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David L. Hacker *Editor*

Recombinant Protein Expression in Mammalian Cells

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Edited by

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Preface

One can consider recombinant protein production in mammalian cells to be a mature technology. After all, the first commercial therapeutic recombinant proteins produced with mammalian cells were available to patients in the mid-1980s. Large-scale transient transfection of mammalian cells in suspension was developed in the mid-1990s, first with calcium phosphate as the DNA delivery vehicle followed by the cationic polymer polyethyleneimine, today's standard reagent for transfection. The protein yields in these early days were very modest compared to the levels achieved now. The improvements have resulted from advances in vector design, DNA delivery, cell cultivation, host cell engineering, and bioprocess optimization. For this volume, I have organized the protocols around these topics. Almost all of the protocols presented here use either Chinese hamster ovary cells (CHO) or human embryonic kidney 293 cells (HEK293), the workhorses of the field, as the production host. However, the methods can be adapted to other mammalian hosts under the appropriate cell-specific conditions.

Although the book is not formally organized into sections, the first three chapters describe methods for transient protein production. Chapters 4–7 concern methods for the generation of recombinant cell lines for either constitutive or induced recombinant gene expression. The subsequent section (Chapters 8–11) is focused on cell cultivation methods for WAVE, stirred-tank (batch and perfusion modes), and orbitally shaken bioreactors. This is followed by three chapters on high-throughput technologies for protein production and for RNAi-based host cell screening. Chapters 15 and 16 describe the engineering of CHO cells for improved protein production and quality using CRISPR/Cas9 gene editing. Finally, the volume closes with three special topics: codon optimization for gene expression in mammalian cells, transient recombinant adeno-associated virus vector production in HEK293 cells, and cell-free protein synthesis in CHO cell lysates.

Having worked in this field for over 15 years, I have seen the technology change dramatically toward more high-yielding, cost-efficient processes, and I am encouraged to see that more and more academic and industrial researchers now have access to recombinant proteins generated from mammalian cells. When I began to operate a protein production service facility at the EPFL in 2006, there were few such facilities in the world that were using mammalian cells in suspension as production hosts. Now there is a large and growing network of facilities and individual laboratories using the technology as it has become more reliable and affordable. Its continued development promises to build on our knowledge of protein structure and function and to speed the discovery of new therapeutic proteins.

In closing, I wish to thank Professor John Walker for advising me on the organization and editing of this volume. I also want to express my appreciation to all of the authors who took time from their busy schedules to contribute protocols. Lastly, I thank Professor Florian Wurm who many years ago gave me the opportunity to join his research group at the EPFL to help develop large-scale protein production technology with suspension-adapted mammalian cells.

Lausanne, Switzerland ***David L. Hacker***

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Chapter 1

Transient Gene Expression in Suspension HEK293-EBNA1 Cells

Denis L'Abbé, Louis Bisson, Christian Gervais, Eric Grazzini, and Yves Durocher

Abstract

Transient gene expression in human embryo kidney 293 (HEK293) cells is an established approach for the rapid production of large amounts of recombinant proteins (r-proteins). Milligram to gram quantities of r-proteins can be typically obtained within less than 10 days following transfection. In this chapter, we describe a simple and robust transfection process of suspension-growing human embryo kidney 293 cells using two commercially available serum-free media and polyethylenimine as the transfection reagent. This chapter provides examples for the production and purification of a his-tagged recombinant protein and two monoclonal antibodies.

Key words Transfection, 293-6E, Polyethylenimine, Deacylated PEI, Recombinant protein, Suspension culture, IMAC, Protein-A, Antibody, Serum-free medium

1 Introduction

Fast and efficient production of recombinant proteins (r-proteins) remains a major challenge for the academic and biopharmaceutical communities. Pure r-proteins are often required in large amounts (hundreds of milligrams to gram quantities) when being developed as biotherapeutics, or in smaller quantities (milligrams) for high-throughput screening campaigns, structural or functional studies. Mammalian cells are often preferred over prokaryotic systems when expressing cDNAs of mammalian origin due to their superior capability to conduct elaborate posttranslational modifications [1, 2]. The conventional way to produce large quantities of r-proteins in mammalian cells is to transfect a small volume of cells and then select for clones that have stably integrated one or multiple copies of the gene of interest into their chromosomes. This process is tedious and generally requires many months to isolate stable clones that express adequate levels of the r-protein of interest. Transfection of mammalian cells is a well-known

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technique that has been widely used at small-scale for many decades [3–5]. This technology has gained significant awareness following key developments in transfection-compatible culture media, highly efficient expression vectors, robust serum-free and suspension-growing cell lines, and cost-effective transfection reagents [6]. Large-scale transfection of mammalian cells is now an established technology that allows the production of milligram to gram quantities of recombinant proteins within a week or two after cDNA cloning into an expression vector. This technology is mostly used at large-scale with suspension-growing human embryonic kidney (HEK) 293 and Chinese hamster ovary (CHO) cells, but other cell lines including insect cell lines (Sf9 and others), HKB11 and CAP-T cells have also been used [7–18].

Many gene carriers can be used for the large-scale transfection of mammalian cells, including commercially available cationic lipid mixtures, but so far only one carry out the process in a cost-effective way: polyethylenimine (PEI). PEI was first shown to efficiently bind and precipitate DNA [19] and these DNA–PEI complexes were later exploited to efficiently transfect mammalian cells [20]. PEI-mediated transfection involves the formation of nanoparticles (polyplexes) which are obtained by its interaction with plasmid DNA. A short incubation period that allows polyplexes to reach their optimal size for transfection is typically performed prior to their addition to the culture.

This chapter describes all the steps needed for a successful protein expression following PEI-mediated transfection of HEK293 cells grown in serum-free media in suspension cultures.

2 Materials

2.1 Cell Thawing and Freezing

The 293 cell line stably expressing the Epstein Barr Virus (EBV) Nuclear Antigen-1 (293-EBNA1 or 293E) is widely used for large-scale transfection. When using expression vectors bearing the EBV origin of replication oriP (such as the pTT™ vectors), a threefold improvement in r-protein yield is generally obtained compared to EBNA1-negative 293 cells [21]. The protocols described in this chapter use the 293-6E cells that do express a truncated version of EBNA1 and the pTT5™ expression vector. Other 293 cell lines (e.g., 293E, 293T, 293S, 293SF, 293SFE or 293F), expression vectors and culture medium can also be used, but reoptimization of the transfection conditions (cell density at transfection, DNA concentration, PEI:DNA ratio) would be needed. The protocols can be done in any cell culture laboratory with access to typical equipment such as inverted microscope with fluorescence detection capability, autoclave, centrifuges (1–1000 ml capacities), high-purity water system (e.g., Milli-Q®), freezers (−20 °C and −80 °C) and a liquid nitrogen freezer. It is important to note that cells growing in serum-free media are very sensitive to chemical contaminations; thorough rinsing of all non-single-use culture vessels that contact the cells with high-purity water prior to sterilization is highly recommended.

1. 293-6E vials from a research cell bank (stored in liquid nitrogen vapor phase).
2. Pluronic® F68: 10% (w/v) stock solution (Invitrogen) (*see* **Note 1**).
3. L-Glutamine: 200 mM stock solution in water.
4. Geneticin® (G418 sulfate): 50 mg/ml stock solution (Invitrogen).
5. FreeStyle™-17 (F17) medium (Invitrogen; *see* **Note 2**) or BalanCD® HEK293(BCD; Irvine Scientific; *see* **Note 3**).
6. Erythrosin B (Sigma): 25 mg/ml in PBS.
7. Dimethylsulfoxide (DMSO), cell culture grade (Sigma).
8. Disposable Erlenmeyer plastic shake flasks (Corning) or reusable Erlenmeyer glass shake flasks (*see* **Note 4**).
9. Humidified incubator controlled at 37 °C with 5% CO_2.
10. Orbital shaker set at 120 rpm, 19–25 mm throw (*see* **Note 5**).
11. 2 ml cryovials (Corning).
12. Styrofoam box to contain cryovial boxes.

2.2 Plasmid DNA Preparation (MAXIPREP)

1. LB agarose plate with proper antibiotic (e.g., 50 μg/ml ampicillin).
2. Maxiprep DNA kit (*see* **Note 6**).
3. CircleGrow™ (Q-Biogen) or Terrific Broth media (*see* **Note** 7).
4. 250 ml Erlenmeyer shaker flask (*see* **Note 8**).
5. Orbital shaker plate in a nonhumidified incubator controlled at 37 °C.
6. Centrifuge for 50 ml polypropylene tubes (running at 10,000 × *g*).
7. Competent *E. coli* (DH5alpha strain).
8. Equipment for agarose gel electrophoresis.
9. UV-spectrophotometer.

2.3 Polyethylenimine MAX Solution

1. PEI MAX powder (Polysciences Inc.) (*see* **Note 9**).
2. Milli-Q® water.
3. 1 N sodium hydroxide solution.
4. Magnetic stir plate.
5. Graduated cylinder.

6. pH meter.
7. 0.22 μm vacuum filtration unit.
8. Sterile polypropylene tubes (15 ml or 50 ml).

2.4 Transfection of 293-6E Cells with PEI MAX

1. 293-6E maintenance cells in shake flask.
2. 1 mg/ml PEI MAX solution.
3. Freestyle™ F17 or BalanCD® HEK293 medium.
4. 20% (w/v) TN1, sterile stock solution (*see* **Note 10**) or BalanCD® HEK293 Feed solution.
5. Purified plasmid DNA of interest.
6. Sterile disposable or glass cell culture vessel (e.g., 6-well plates, Erlenmeyer flask, glass bottle) (*see* **Note 11**).

2.5 Batch Purification of His-Tagged Proteins

1. Nickel Sepharose® excel resin (GE Healthcare) or Fractogel® EMD chelate (M) resin (EMD Biosciences) charged with cobalt chloride (*see* **Note 12**).
2. Milli-Q® water.
3. Wash Buffer 1: 50 mM sodium phosphate, 300 mM NaCl, pH 7.4 (*see* **Note 13**).
4. Wash Buffer 2: 50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 7.4.
5. Elution Buffer: 50 mM sodium phosphate, 300 mM NaCl, 500 mM imidazole, pH 7.4.
6. Phosphate Buffered Saline (PBS).
7. Rotator for 1.5 ml to 50 ml polypropylene tubes.
8. UV spectrophotometer.

2.6 Column Purification of Monoclonal Antibody or Fc-Fusion Proteins

1. HiTrap™ MabSelect SuRe™ 5 ml column (GE Healthcare).
2. 100 mM citrate buffer pH 3.6 (68.5 mM citric acid monohydrate, 31.5 mM trisodium citrate dihydrate). Check pH and adjust if necessary.
3. 1 M Tris-base pH 11 solution (should not need pH adjustment).
4. 0.45 μm bottle-top vacuum filter system (Corning Inc.).
5. Stericup® filter units (Millipore) with a 500 ml filtrate capacity.
6. Centrifugation bottles with conical bottom (Corning Inc.).
7. HiPrep™ 26/10 desalting columns (GE Healthcare).
8. SDS-PAGE gel electrophoresis system.

3 Methods

3.1 Cell Thawing and Maintenance (see Note 14)

1. Dispense 16 ml of complete F17 medium (without G418) in a disposable 125 ml plastic shaker flask.
2. Place the flask on an orbital shaker plate in a humidified incubator controlled at 37 °C with 5% CO_2 and wait for a minimum of 1 h prior to thawing the cells.
3. Return the vessel to the incubator.
4. Rapidly thaw cells by dipping the cryovial (containing 5×10^6 cells in 1 ml) in a 37 °C water bath and immediately transfer cells to the culture vessel containing the conditioned medium.
5. Remove a 100 μl aliquot and dilute with 100 μl Erythrosin B to determine cell density and viability.
6. Return the vessel to the incubator.
7. At 72 h postthawing, count cells and dilute to 0.25×10^6 cells/ml using medium without G418.
8. Subculture every 2 or 3 days to maintain cell densities between 0.25×10^6 and 2.0×10^6 cells/ml. Only supplement culture medium with 25 μg/ml G418 when cell doubling time is around 24 h.
9. Dilute cultures to 0.125×10^6 for long weekends (4 days).
10. Freeze a large working cell bank as soon as the cells exhibit good growth rate.
11. Do not allow the maintenance cells to exceed 2.2×10^6 cells/ml.
12. Thaw a new vial every 10 weeks.

3.2 Cell Freezing

1. Prepare freezing mixture by adding DMSO to fresh medium (10:90, v/v).
2. Label cryogenic vials as needed.
3. Count cells and determine volume needed for cryopreservation. Cells must be in log growth phase (between 0.8 and 1.2×10^6 cells/ml) (*see* **Note 15**).
4. Centrifuge cells at 200 × *g* for 5 min, decant supernatant and dissociate pellet by gently tapping the tube.
5. Add ice-cold freezing medium to cells while swirling the tube to obtain a density of 5×10^6 cells/ml.
6. Quickly aliquot into vials (1 ml per vial) and immediately transfer to a − 80 °C freezer. Do not open freezer door for at least 2 h.
7. Transfer vials to liquid nitrogen freezer storage (vapor phase) the following day.

3.3 Plasmid DNA Preparation

1. Pick a fresh colony to inoculate 50 ml of growth medium with appropriate antibiotic (e.g., 50 μg/ml ampicillin) in a 250 ml glass or polypropylene shake flask.
2. Incubate for 16–20 h under vigorous shaking (~250 rpm).
3. Extract plasmid DNA as recommended by the kit manufacturer (*see* **Note 16**).
4. Dissolve DNA in 1 ml of sterile TE (10 mM Tris, 1 mM EDTA, pH 8.0).
5. Measure absorbance at 260 and 280 nm. DNA should be diluted in TE or 50 mM Tris–HCl pH 8.0 to obtain an accurate measurement. The A_{260}/A_{280} ratio of purified plasmid DNA should be between 1.85 and 1.95.
6. Verify DNA integrity on 0.8% agarose gel electrophoresis.

3.4 Linear PEI-MAX (1 mg/ml Solution)

1. Pour ~450 ml Milli-Q® water into a 500 ml glass beaker (*see* **Note 17**).
2. Weigh 500 mg PEI and add to beaker with stirring.
3. Stir until dissolved (5–15 min).
4. Adjust pH to 6.9–7.1 using 1 N NaOH dropwise (*see* **Note 18**).
5. Pour solution into a 500 ml cylinder; adjust final volume to 500 ml with Milli-Q® water.
6. Filter-sterilize solution through a 0.22 μm membrane.
7. Aliquot to desired volumes and store at 4 °C.

3.5 Transfection of HEK293 Cells

The following transfection protocols were developed in our laboratory with our 293-6E cell line (Figs. 1 and 2). The procedures describe scalable protocols to transfect cultures grown in Freestyle™ F17 (F17) or BalanCD® 293 medium (Table 1). The optimal DNA to PEI ratio for transfection differs from one medium to another (Fig. 3 and Table 2), so if using another PEI-transfection compatible culture medium, the ratio should be reoptimized. This is most simply performed using a statistical Design of Experiment (DoE) approach to evaluate a number of parameters simultaneously [22, 23]. Secreted protein production is enhanced by supplementation with the casein hydrolysate TN1 or BalanCD® HEK293 Feed at 24 hpt (hours posttransfection). Supplementation is not necessary for intracellular or membrane proteins production since maximal expression (and thus harvest time) typically peaks much sooner (48–72 hpt).

The cultures are typically seeded 1 or 2 days before transfection so that the optimal cell density is reached on the day of transfection. This avoids needing to centrifuge the cells which may have a detrimental effect on transfection efficiency if performed shortly prior to transfection. For small-scale transfection (e.g., transfection

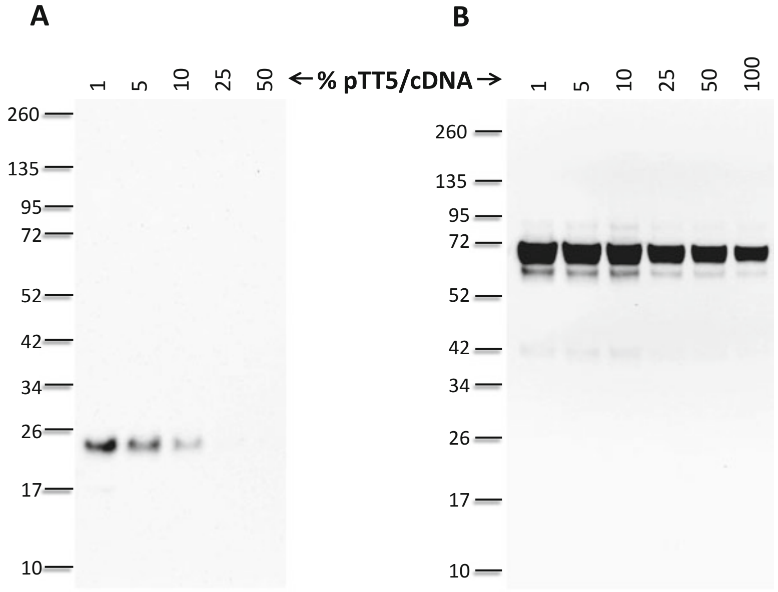

Fig. 1 pTT plasmid titration for optimal expression. To ensure optimal protein expression, pTT5™ plasmid encoding the cDNA of interest (pTT5/cDNA) can be titrated (100% down to 1%) with noncoding stuffing (filler) DNA (in this case, sonicated salmon testis DNA [sstDNA]) while maintaining the absolute amount of DNA constant. In Panel (**a**), the pTT5 plasmid encoding the Prolactin-Inducible Protein (PIP—P12273; aa 1-146 with a C-terminal His_{10}Gly tag) cDNA was used to transfect 293-6E cells grown in F17 medium (2 ml final) in an agitated 6-well plate. The pTT5™ plasmid was mixed at various ratios (w:w) with sstDNA (1:99, 5:95, 10:90, 25:75, and 50:50). A total of two microgram of DNA was used per well to transfect the cells. Culture supernatants at day 5 posttransfection were analyzed by western blot using anti-his antibody. The blot shows that highest PIP expression (~2 mg/l) was observed when only 1% of pTT5/PIP-H10G plasmid was used. In Panel (**b**), increasing amounts of pTT5™ plasmid encoding the Proprotein Convertase Subtilisin/Kexin type 9 (PCSK9—Q8NBP7; aa 1-692 with C-terminal FLAG tag) cDNA were mixed with decreasing amounts of sstDNA and the mixtures were used to transfect 293-6E cells as described in Panel (**a**). The blot shows that highest PCSK9 expression (6 mg/l) was obtained when only 1% of pTT5/PCSK9-FLAG plasmid was present in the DNA solution

in 96- or 6-well plates), a seed culture grown in a shaker flask can be made and the cells, at the appropriate density, transferred into the plates just prior to transfection.

1. Warm culture medium to 25–37 °C, take out DNA from −20 °C freezer to thaw.
2. From a previously seeded flask, determine cell density and viability. Cells should be at 1.5 to 2.2 × 10^6/ml at the time of transfection, with >99% viability (*see* **Note 19**).

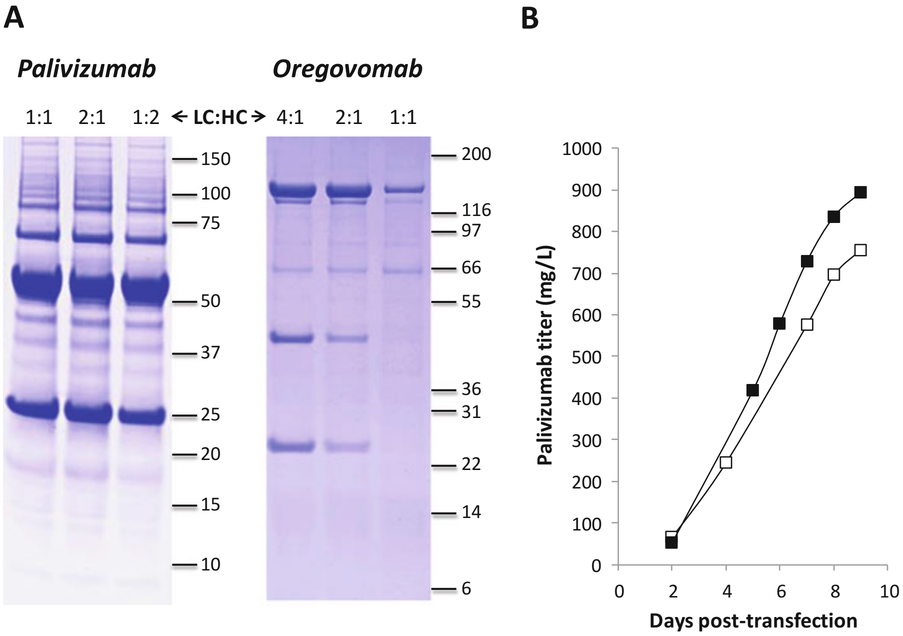

Fig. 2 Expression of monoclonal antibodies in 293-6E cells. Panel (**a**): the pTT5™ plasmids encoding light (pTT5/LC) and heavy (pTT5/HC) chains were mixed together at various ratios (w:w). For the Palivizumab (MEDI-493) IgG1 antibody, pTT5/LC and pTT5/HC were mixed at ratios of 1:1, 2:1, and 1:2. For the Oregovomab (B43) IgG3 antibody, ratios were of 4:1, 2:1, and 1:1. The plasmid mixtures were used to transfect cells grown in a 6-well plate (2 ml final). Antibody secreted in the culture medium was measured 6 days posttransfection by protein-A HPLC (Palivizumab) or estimated by integration of the Coomassie-stained bands relative to an IgG1 control (Oregovomab, not shown). Palivizumab SDS-PAGE gel was run under reducing conditions while Oregovomab samples were run under nonreducing conditions. Palivizumab titers were of 502 (1:1), 308 (2:1), and 442 (1:2) mg/l, while Oregovomab titers were estimated at 300 (4:1), 270 (2:1), and 80 (1:1) mg/l. Panel (**b**): 293-6E cells (360 ml) were grown in a 2 l Corning Erlenmeyer flask in F17 (solid squares) or BalanCD® HEK293 (BCD; empty squares) media. Cells were transfected at 1.5×10^6 (F17) or 1.9×10^6 (BCD) cells/ml and fed as described in the Subheading 3. Antibody titers were determined by protein-A HPLC using a calibration curve made with purified Palivizumab

3. Add appropriate cell volume to the culture vessel to transfect according to Table 1 and transfer the vessel to the incubator.
4. Add culture medium (5% of final transfected culture volume) to an appropriate tube according to Table 1.
5. Add plasmid DNA to the tube (final DNA concentration should be 20 ng/µl) and vortex/mix gently (*see* **Note 20**).
6. Add culture medium (5% of final transfected culture volume) to a second tube according to Table 1.
7. Add PEI MAX to each tube and vortex. Make sure to add the appropriate amount of PEI depending on the culture medium used (*see* Table 1).

Table 1
Culture vessels commonly used for transfection of suspension-growing cells

	6-well plates	250 ml flasks	2 l flasks	5 l flasks (*see* Note 27)
Culture volume (prior to transfection)	1.8 ml/well	45 ml	450 ml	1.8 l
DNA solution (20 ng/µl)	100 µl in a 1.5 ml PP tube (for each well to transfect)	2.5 ml in a 15 ml PP tube	25 ml in a 50 ml PP centrifuge tube (e.g., Corning)	100 ml in a 250 ml PP centrifuge tube (e.g., Corning)
PEI solution (50 ng/µl for F17; 80 ng/µl for BCD)	100 µl in a 1.5 ml PP tube (for each well to transfect)	2.5 ml in a 15 ml PP tube	25 ml in a 250 ml PP centrifuge tube (e.g., Corning)	100 ml in a 500 ml PP centrifuge tube (e.g., Corning)
Agitation speed	120 rpm	120 rpm	120 rpm	120 rpm
Agitation orbit	19 mm	19 mm	25 mm	25 mm
TN1 feed (F17)	50 µl	1.25 ml	12.5 ml	50 ml
BalanCD® HEK293 feed (BCD)	100 µl at 1 dpt100 µl at 2 dpt 100 µl at 3 dpt 200 µl at 4 dpt	2.5 ml at 1 dpt2.5 ml at 2 dpt 2.5 ml at 3 dpt 5.0 ml at 4 dpt	25 ml at 1 dpt25 ml at 2 dpt 25 ml at 3 dpt 50 ml at 4 dpt	100 ml at 1 dpt100 ml at 2 dpt 100 ml at 3 dpt 200 ml at 4 dpt

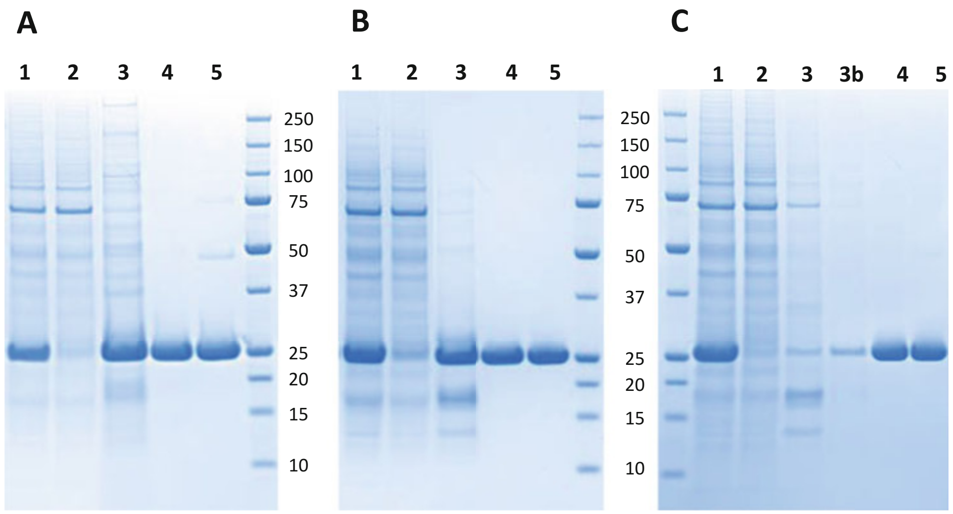

Fig. 3. Expression and purification of a his-tagged secreted protein. The mouse perlecan fragment LG3 (aa 3514-3707 with a C-terminal His$_8$Gly tag and N-terminal signal peptide [24]) cDNA was cloned into pTT5™ plasmid. 293-6E cells were inoculated 3 days prior to transfection in a 5 l Corning glass bottle (**a** and **b**) or in a 2 l Corning Erlenmeyer flask (**c**) and transfected at 1.8 × 10^6/ml (**a** and **b**) or 2.0 × 10^6/ml (**c**) with 95% (w:w) pTT5/mLG3 and 5% (w:w) pTT/GFP (to monitor for transfection efficiencies). Culture were collected at the indicated time (*see* Table 2) and purified by IMAC on Fractogel®-Cobalt (**a** and **b**) or Ni Sepharose™ excel (**c**) resins. Samples were analyzed by SDS-PAGE (reducing conditions) and stained with Coomassie Blue (lane 1, Load; lane 2, Flow-Through; lane 3, Wash 1; lane 3b, Wash 2; lane 4, Elution; lane 5, Elution—nonreduced)

Table 2
Production and purification of mouse LG3 (*see* Fig. 3)

Flask	A	B	C
Culture medium	Freestyle™-17	BalanCD® HEK293	BalanCD® HEK293
Culture volume	1012 ml	1086 ml	468 ml
Transfection efficiency (% GFP + at 24 hpt)	29%	37%	22%
Harvest time (viability)	4 dpt (69%)	7 dpt (69%)	7 dpt (76%)
Cell density at harvest	2.2 × 10^6	5.1 × 10^6	7.4 × 10^6
IMAC resin (column volume)	Fractogel®-cobalt (5 ml)	Fractogel®-cobalt (2 × 5 ml)	Ni Sepharose™ excel (5 ml)
Yield (post-IMAC)	91 mg	201 mg	114 mg
Volumetric yield	90 mg/l	186 mg/l	244 mg/l
Purity (SEC-HPLC)	96% monomers	97% monomers	94% monomers
Endotoxins (*see* **Note 28**)	<0.005 EU/mg	<0.003 EU/mg	<0.013 EU/mg

8. Add PEI solution to DNA solution and immediately vortex/mix thoroughly (3–5 s).
9. Incubate mixture for 5 min at room temperature for polyplexes to form.
10. Remove culture vessel (**step 3**) from incubator, add the DNA–-PEI mixture and swirl.
11. Return culture vessel to incubator.
12. For secreted protein production in F17 medium, add pre-warmed 20% (w/v) TN1, 4 to 48 hpt (Table 1) and return culture to incubator. The final TN1 concentration in the culture(s) is 0.5% (w/v). For production in BalanCD® HEK293 medium, add BalanCD® HEK293 Feed according to Table 1.
13. If intracellular protein, harvest cells at 48–96 hpt. If secreted protein, harvest supernatant between 96 and 240 hpt, as long as culture viability is high (>70%) and/or product titer is increasing (*see* **Note 21**).

3.6 Batch Purification of Secreted His-Tagged Proteins

Recombinant proteins often need to be as pure as possible and exempt of endotoxin prior to their characterization or to begin animal study. The following protocol describes an immobilized metal affinity chromatography (IMAC) purification technique for polyhistidine-tagged proteins. Small-scale IMAC purification (e.g., <500 ml of culture medium) can be easily achieved using a batch capture, wash and elution with gravity column chromatography approach. Larger volumes can be processed by gravity flow but are more conveniently purified using column chromatography with the aid of automated chromatography systems such as the AKTA™ system. We recommend using the Ni Sepharose™ excel resin (GE Healthcare) since it is less sensitive to metal leaching resulting from the presence of chelating components that are present in many serum-free medium formulations. Other resins such as cobalt-charged Fractogel® EMD Chelate (EMD Millipore) can also be used with this protocol. If a large volume of culture medium is to be purified, the clarified culture supernatant can be concentrated 10 to 20-fold by tangential flow filtration prior to r-protein capture on the IMAC column. When using column chromatography, 1 to 5 ml gravity columns can be packed with a resin bed height of at least 1 cm.

Below is a protocol for the batch purification of his-tagged protein from small volume of culture medium (~1.5 ml). This is typically performed to assess if the protein of interest can be efficiently captured and to estimate its expression level. This protocol can be scaled up in 50 ml polypropylene tubes or 500 ml polypropylene centrifuge bottles.

1. Gently suspend the nickel resin (provided as slurry in 20% ethanol).

2. Transfer 60 μl in a 1.5 ml polypropylene tube (*see* **Note 22**).
3. Add 400 μl of Milli-Q® water, rotate for 5 min.
4. Centrifuge for 3 min at 500 × *g* and remove supernatant.
5. Add 300 μl of PBS, rotate 5 min.
6. Centrifuge for 3 min at 500 × *g* and remove supernatant.
7. Add clarified sample ("Load") to bead, rotate 30 min (*see* **Note 23**).
8. Centrifuge for 3 min at 500 × *g* and collect supernatant ("Flow-Through") (*see* **Note 24**).
9. Add 300 μl of Wash 1 Buffer, rotate 5 min.
10. Centrifuge for 3 min at 500 × *g* and collect supernatant ("Wash 1").
11. Repeat **steps 9** and **10**.
12. Add 300 μl of Wash 2 buffer, rotate 5 min.
13. Centrifuge for 3 min at 500 × *g* and collect supernatant ("Wash 2").
14. Add 120 μl of Elution Buffer, rotate 5 min.
15. Centrifuge for 3 min at 500 × *g* and collect supernatant ("Elution") (*see* **Note 25**).
16. Repeat **steps 14** and **15** to generate "Elution 2".
17. Prepare samples for SDS-PAGE and/or Western blot (18 μl sample + 6 μl 4 × loading buffer with or w/o DTT).
18. Heat at 70 °C for 10 min.
19. Load samples on SDS-PAGE gel for analysis (Coomassie Blue staining and/or western blotting).

3.7 Purification of Monoclonal Antibody or Fc-Fusion Protein

This section describes mAb or Fc-fusion protein purification by gravity-flow using premade protein A column (HiTrap™ MabSelect SuRe™). However, this can also be easily achieved in batch mode using bulk resin following a process similar to that described in Subheading 3.6. Note that MabSelect SuRe™ has a protein binding capacity of about 30 mg/ml of resin, so make sure not to overload the resin for optimal recovery.

1. Centrifuge the harvested culture at 4000 × *g* for 30 min.
2. Filter the clarified culture medium through a 0.45 μm filter unit under vacuum.
3. Load the filtered medium at 5 ml/min onto a HiTrap™ MabSelect SuRe™ Protein-A column preequilibrated with 5 column volumes (CV) of PBS. Save the flow-through for further analysis.

4. Wash the column with 5 CV of PBS at 5 ml/min. Save the wash fraction for further analysis.
5. Elute the protein of interest with 5 CV of citrate buffer, pH 3.6 (*see* **Note 26**). Collect fractions of 1 ml each and pool those containing the protein.
6. The concentration of the purified protein may be determined from the absorbance at 280 nm using its molar extinction coefficient (https://web.expasy.org/protparam/).
7. Load the pooled eluted protein fractions on a HiPrep™ desalting column equilibrated with 2 CV of PBS at 10 ml/min. Collect the fractions containing the protein based on UV absorbance at 280 nm.
8. Filter the desalted protein using a 0.22 μm syringe filter. Aliquot the purified protein in sterile screw-cap 2 ml polypropylene tubes and store them at −80 °C.
9. Evaluate the production and purity of protein by SDS-PAGE and Coomassie staining of collected samples including the clarified culture medium supernatant, flow-through, wash and the eluate from the protein-A column.

4 Notes

1. Pluronic® F68 can be replaced by a 0.2 μm filtered 10% Kolliphor® P 188 (BASF) stock solution.
2. Add 10 ml Pluronic® F68 stock solution per liter of culture medium (0.1% w/v, final) and 20 ml of 200 mM L-glutamine stock solution per liter of culture medium.
3. Add 20 ml of 200 mM L-glutamine stock solution per liter of culture medium (4 mM final). There is no need to add Pluronic® F68, as it is already present at 0.1% w/v in this medium.
4. Rinse glassware 3 times using Milli-Q® water (or equivalent) followed by steam sterilization.
5. Orbital shaker must be supplied with a platform that possesses holders for shake flask sizes ranging from 50 to 2000 ml. If no holders are supplied, the platform should contain an anti-slip adhesive mat. Check with the manufacturer that orbital shaker can operate continuously under humidified conditions.
6. We recommend using “endotoxin-free” commercial kits based on anion-exchange chromatography, especially if the protein to be produced will be used in vivo or in cell-based assays.
7. These rich media should generate >1 mg of plasmid DNA per 50 ml culture when using high-copy-number plasmids such as the pTT5™ vector.

8. The use of baffled flasks such as Ultra Yield flasks (Thomson Instrument Co.) will result in higher biomass and plasmid yields.
9. Polyethylenimines are available as linear or branched polymers. The two most efficient ones for HEK293 transfection in suspension culture are PEIpro® (deacylated linear PEI from Polyplus-transfection® SA, Illkirch, France) and PEI MAX (deacylated linear PEI from Polysciences Inc., Warrington, PA, USA). Both PEIpro® and PEI MAX are available as a 1 mg/ml sterile solution while PEI MAX is also available as a powder (hydrochloride salt). We recommend using PEI MAX powder for cost-effectiveness.
10. Tryptone N1 (OrganoTechnie) can be dissolved in Freestyle medium, supplemented with Pluronic® F68 at 0.1% (w/v) and filter-sterilized.
11. If using 6-well plates, we recommend placing the plates in a plastic container (e.g., Tupperware) lined with a wet paper towel (to minimize evaporation), making sure not to close the lid too tightly to allow for gas exchange.
12. Please see http://www.cshprotocols.org/cgi/content/abstract/2008/4/pdb.prot4980 for charging the resin with cobalt.
13. All solutions must be filtered (0.45 μm).
14. Conduct all operations aseptically in a laminar flow hood; all media and reagents added to cultures must be sterile.
15. We recommend freezing the cells at 5×10^6 cells/ml/vial. Place the vials in a small 20-position styrofoam box and place this box in a larger styrofoam box in a −80 °C freezer.
16. The final step of plasmid DNA preparation (e.g., 70% ethanol wash and dissolution in TE) should be performed in a laminar hood to ensure sterility.
17. Rinse all glassware 3 times with high-purity water.
18. If pH is over 7.1, add 1 N HCL dropwise to lower it.
19. Cells can be diluted 24–48 h prior to transfection. The presence of G418 does not interfere with transfection.
20. When producing secreted proteins, we highly recommend monitoring cell transfection by using a plasmid encoding a fluorescent protein (e.g., GFP). Adding 5% of a GFP-plasmid in the transfection mixture does not significantly alter expression of the gene of interest while allowing a visual confirmation of transfection efficiency using a fluorescence microscope (or quantitative, if using flow cytometry). Good transfection efficiency happens when more than 25% of cells express GFP 48 hpt using 5% of GFP plasmid in the transfection mixture. A

separate transfection with GFP plasmid must however be done when expressing intracellular proteins.

21. Establish best harvesting time by performing an expression kinetic study.
22. The manufacturer's binding capacity specification is >10 mg/ml of his-tagged protein, but we have found that it could be higher in many instances. It is important to use an adequate volume of resin for optimal recovery of the protein of interest.
23. Cells and debris should be removed by centrifugation. If performing column chromatography, the supernatant must be filtered (0.45 μm) before loading onto the column to prevent clogging.
24. Do not discard flow-through or Wash fractions until it is determined that the r-protein has been captured and eluted from the resin.
25. We recommend exchanging the buffer of the eluted protein with PBS using commercial prepacked NAP ™—25 columns (GE Healthcare). These columns can be sanitized with 0.1 N NaOH prior to use for low-endotoxin requirements.
26. Some antibodies bind strongly to pA resin and may require more acidic pH (e.g., 3.2 or 3.0). We recommend however always eluting at pH 3.6 first since some antibodies will precipitate at lower pH.
27. Optimum Growth™ flasks from Thomson Instrument or Erlenmeyer flasks from Corning.
28. Endotoxins were measured using the Endosafe—PTS™ system (Charles River Laboratories).

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Chapter 2

Transient Expression of Recombinant Membrane-eGFP Fusion Proteins in HEK293 Cells

Joanna Pieprzyk, Samuel Pazicky, and Christian Löw

Abstract

Membrane proteins play important roles in many biological processes and are a major drug target. However, only a limited number of structures of eukaryotic membrane proteins have been determined so far. Besides the challenges in crystallizing these proteins, one of the main bottlenecks in structure determination is their recombinant expression. The mammalian HEK293 cell line provides a natural environment for expression of eukaryotic membrane proteins but optimization of transfection and cultivation time is often necessary to yield amounts of protein suitable for structural studies.

Here we describe a detailed protocol for expression and purification of membrane proteins from HEK293 cells with an example of the human peptide transporter, PepT2. In the first part, we focus on the expression optimization by changing transfection protocol and cultivation time. In the second part, we describe a robust protocol for large-scale expression and purification of membrane proteins based on affinity chromatography and gel filtration.

Key words Membrane protein, Transient transfection, HEK293 cell suspension culture, Protein expression, Protein purification

1 Introduction

Over 22% of the human genome encodes for integral membrane proteins (IMPs) and more than half of FDA-approved drugs target proteins on the cell surface [1, 2]. However, the expression of eukaryotic IMPs is a major bottleneck for their functional and structural characterization. For human membrane proteins in particular, the most widely used bacterial expression systems did not prove to be efficient, with only nine unique protein structures solved so far (IMPs with more than one transmembrane segment). For most of the eukaryotic IMP structures, the proteins were expressed using the baculovirus infected insect cell system [3]. However, this system is different in the lipid composition of the membrane, lacks human molecular chaperones and results in a different glycosylation pattern, characteristics which are crucial for

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the expression of many eukaryotic proteins [4]. Expression of soluble proteins in HEK293 (human embryonic kidney) cells became popular in recent years [4, 5]; however, the expression of IMPs remains challenging due to the low expression yields. In such cases, simple optimization of basic transfection steps can dramatically enhance protein expression, hence the yield after purification [6]. Here, we describe a detailed protocol for optimization of IMP expression in HEK293 cells, its large-scale expression and purification based on an example of the human peptide transporter, PepT2.

The predicted structure of PepT2 follows the typical topology of major facilitator superfamily (MFS) transporters with 12 transmembrane α helices, and this protein contains an additional large extracellular domain located between the eighth and ninth transmembrane helix. We expressed the protein with an N-terminal (*see* **Note 1**), TEV-cleavable His/GFP fusion tag from the mammalian expression vector pXLG_PepT2 (Fig. 1).

For protein expression, we chose the HEK293F cell line cultured in suspension to increase the biomass. We transfect the cells at high density with linear polyethylenimine (LPEI) and harvest the cells after optimized time points and purify the target

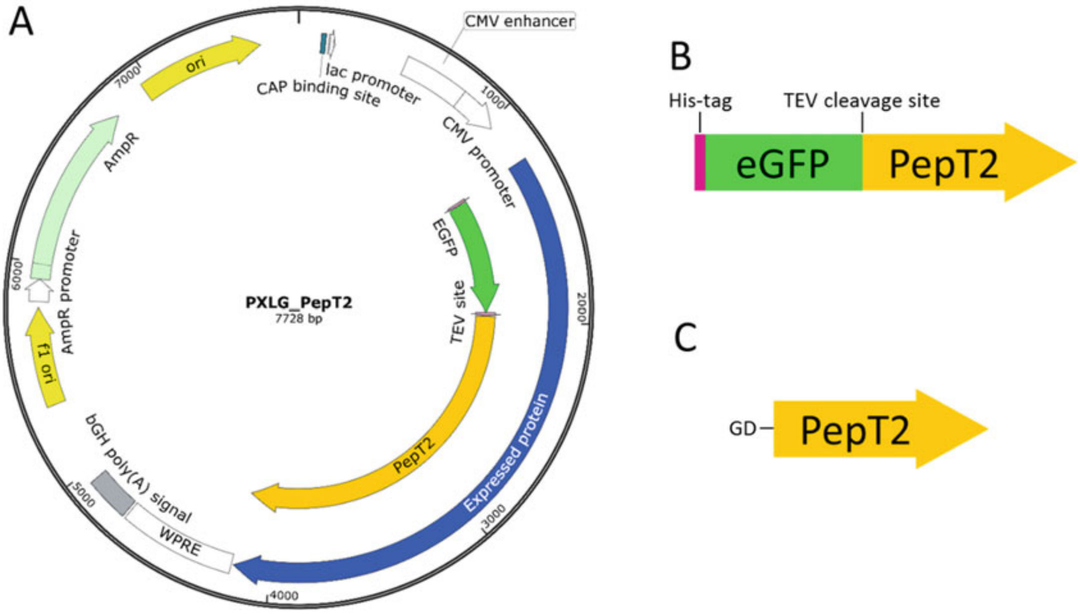

Fig. 1 Mammalian expression vector pXLG with cloned human PepT2 cDNA sequence. (**a**) Vector expression cassette of pXLG_PepT2 is flanked by two inverted terminal repeats and contains a strong CMV promoter followed by an artificial intron, a Kozak consensus sequence upstream of the cloning site and the GFP encoding sequence, two stop codons, a woodchuck post regulatory element (WPRE), and a bovine growth hormone polyadenylation signal downstream of the cloning site. (**b**) Expressed protein with the N-terminal TEV-cleavable His/GFP fusion tag. (**c**) N-terminal glycine and aspartate residues remain at the N-terminus of the protein after tag cleavage

protein using affinity chromatography. The optimization procedure for overexpression described in the first part of the protocol depends on the localization of the expressed protein using fluorescence microscopy and monitoring the amount of overexpressed material by western blot. We consider the optimization of the transfection procedure and the time of protein expression after infection as the most critical parameters. We recommend screening a combination of several conditions of three variables: (1) transfection incubation time (0.5 h, 1.5 h, 3 h), (2) DNA:LPEI ratio (w/w) (1:1, 1:2, 1:3), and (3) expression time (8 h, 16 h, 24 h, 48 h and up to 5 days). The optimized transfection protocol should yield high protein expression with minor impact on cell viability. In the second part of the protocol a detailed description for large-scale transfection and expression of the human peptide transporter PepT2 in suspension-cultured HEK293F cells followed by purification is given. Several additional factors like cultivation temperature [7], cultivation media [8], stirring speed and type of bioreactor [9], and passage number [6] can influence the protein yield upon large-scale expression. Another strategy to increase protein expression level in mammalian cell cultures is the addition of histone deacetylase inhibitors [10] or plasmids encoding proteins regulating the cell cycle [11, 12]. However, in all cases, these studies were conducted with systems expressing soluble proteins.

Here, we describe the conditions that proved most efficient for the membrane proteins we expressed in our laboratory.

2 Materials

2.1 Transfection and Protein Expression

1. FreeStyle™ 293-F cells (*see* **Note 2**).
2. pXLG_PepT2 (or other mammalian expression vector with gene of your protein of interest; *see* **Note 3**).
3. RPMI 1640 medium with 0.1% Pluronic F-68 (*see* **Note 4**).
4. Pluronic® F-68 nonionic surfactant.
5. FreeStyle™ 293 Expression Medium (*see* **Note 5**).
6. Linear 25 kDa polyethyleneimine (LPEI mg/ml in water; *see* **Note 6**).
7. 0.4% solution of trypan blue to determine viable and total cell counts.
8. LYSIS buffer: 20 mM Na-Phosphate (pH 7.5), 300 mM NaCl, 5% glycerol, 5 U/ml DNase, mixture of protease inhibitors.
9. SDS Sample buffer: 100 μl of NuPAGE® LDS Sample buffer, 20 μl of water, 20 μl of 0.5 M TCEP (tris(2-carboxyethyl) phosphine).
10. Pipets, filter tips, syringe, and 0.22 μm filters for syringe.

11. Orbital shaker in 37 °C incubator with humidified atmosphere at 8% CO_2 (*see* **Note 7**).
12. Benchtop centrifuge.
13. Water bath (37 °C).
14. Cell counter.
15. 50 ml TubeSpin® Bioreactors.
16. 600 ml TubeSpin® Bioreactors.
17. Cell counting slides.
18. Microcentrifuge tubes.

2.2 Cell Lysis and Protein Purification

1. LYSIS buffer: 20 mM Na-Phosphate (pH 7.5), 300 mM NaCl, 5% glycerol, 0.5 mM TCEP, 5 U/ml DNase, mixture of protease inhibitors.
2. Solubilization buffer: 20 mM Na-Phosphate (pH 7.5), 300 mM NaCl, 10 mM imidazole, 1% DDM (Dodecyl Maltoside), mixture of protease inhibitors.
3. WASH buffer: 20 mM Na-Phosphate (pH 7.5), 300 mM NaCl, 10 mM imidazole, 5% glycerol, 0.5 mM TCEP, 0.03% DDM.
4. ELUTION buffer: 20 mM Na-Phosphate (pH 7.5), 150 mM NaCl, 250 mM imidazole, 5% glycerol, 0.5 mM TCEP, 0.03% DDM.
5. SEC buffer: 20 mM Na-Phosphate (pH 7.5), 150 mM NaCl, 5% glycerol, 0.5 mM TCEP, 0.03% DDM.
6. FPLC purification system.
7. Superose 6 Increase 10/300 GL column (or any SEC column suitable for the protein of interest).
8. Potter-Elvehjem pestle.
9. IMAC gravity purification column.
10. Ni-NTA affinity resin.
11. Protein concentrator (100 kDa cutoff).

3 Methods

3.1 Cell Cultivation

1. All solutions and the equipment used to work with cell cultures must be sterile. Use proper sterile technique and work in a laminar flow hood.
2. Before starting the experiment, make sure that the cells are well established: the cells should divide with a constant rate and the viability should not drop below 95% (this usually takes at least five passages). It is generally recommended to use early-passage cells for experiments (<30 passages).

3. Keep the culture at a density between 0.3 and 3.0 × 10^6 cells/ml. The cell viability should not drop below 95%.
4. Always use prewarmed media at 37 °C.
5. Determine the amount of cell clumping by using an automated cell counter or count the cells manually. To determine the viability, transfer a small aliquot of the cell suspension to a microcentrifuge tube, combine the cell suspension with 0.4% trypan blue in a volume ratio of 1:1. Gently mix the cells and the dye by pipetting (*see* **Note 8**).

3.2 Small-Scale Optimization of Transfection

1. On Day 1 seed the cells in a 600 ml TubeSpin® Bioreactor in 100 ml prewarmed FreeStyle™ 293 Expression Medium at a density of 0.3 × 10^6 cells/ml. Incubate at 37 °C, 8% CO_2, 175–225 RPM in a humidified atmosphere.
2. Grow the cells until they reach a density of 2.5–3 × 10^6 cells/ml. This usually takes 3–4 days as the cells divide approximately once per 24 h (Fig. 2).
3. On Day 4 count the cells (*see* Subheading 3.1 point 5) and centrifuge at 100 × *g* for 5 min at room temperature (RT).
4. Aspirate the medium and resuspend the cells in prewarmed FreeStyle™ 293 Expression Medium to reach the density of 2 × 10^6 cells/ml (125–150 ml of media).
5. Incubate at 37 °C, 8% CO_2, 175–225 RPM in a humidified atmosphere for 24 h (Fig. 3).
6. On Day 5 label sterile TubeSpin® Bioreactors:
   - (a) 1 × 600 ml TubeSpin® Bioreactor labeled ‘transfection mix’.
   - (b) 3 × 50 ml TubeSpin® Bioreactor labeled 1:1, 1:2, 1:3.
   - (c) 9 × 50 ml TubeSpin® Bioreactor labeled: 1:1–0.5 h, 1:1–1.5 h, 1:1–3 h, 1:2–0.5 h, 1:2–1.5 h, 1:2–3 h, 1:3–0.5 h, 1:3–1.5 h, 1:3–3 h.

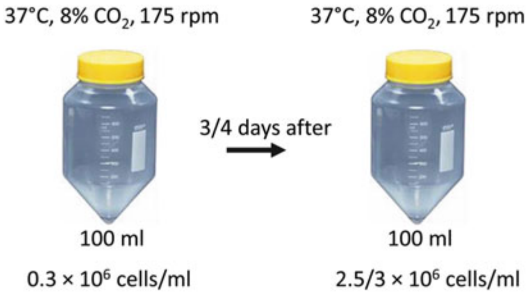

Fig. 2 Cone-shaped growth vessels for cell growth of HEK293F cells in 100 ml of medium

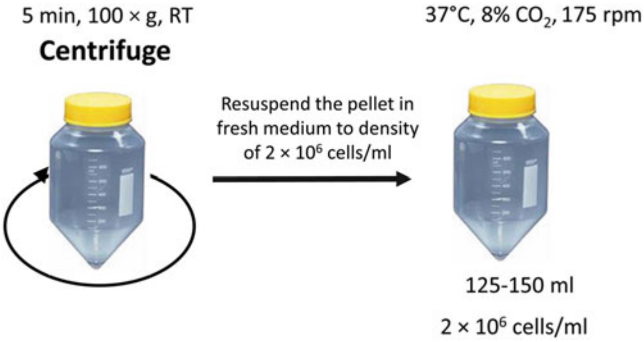

Fig. 3 Resuspend cells into fresh medium at a cell density of 2 × 10^6 cells/ml

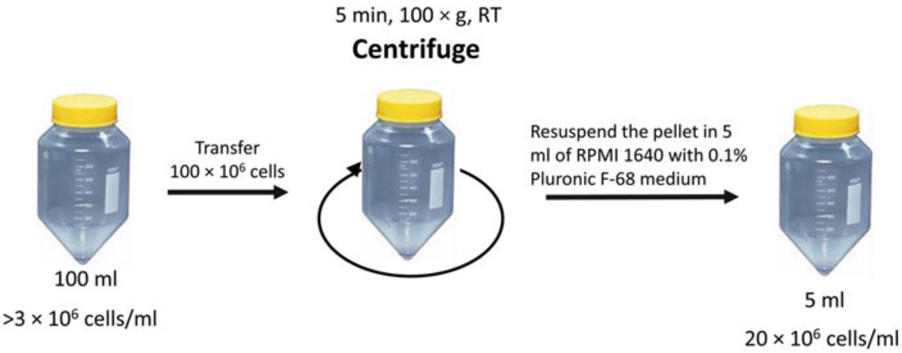

Fig. 4 Transfer 100 × 10^6 cells to a 600 ml TubeSpin® Bioreactor, centrifuge and resuspend them in 5 ml of prewarmed RPMI 1640 with 0.1% Pluronic F-68 medium

7. Count the cells (the cell density should be above 3 × 10^6 cells/ml), determine the cell viability, and the amount of cell clumping using the trypan blue dye (*see* Subheading 3.1 point 5). The viability must not drop below 95%.
8. Transfer 100 × 10^6 cells to a 600 ml TubeSpin® Bioreactor ('transfection mix') and centrifuge at 100 × *g*, 5 min, RT.
9. Carefully remove the medium by decanting.
10. Add 5 ml of prewarmed RPMI 1640 with 0.1% Pluronic F-68 medium to the cell pellet (Fig. 4). Gently mix to resuspend the cells (*see* **Note 9**).
11. Transfer 1.5 ml of the dense suspension culture into three 50 ml TubeSpin® Bioreactors (1:1, 1:2, 1:3; 1.5 ml to each; Fig. 5).
12. Add 45 μg of DNA directly into each tube containing the resuspended cells and mix gently.
13. Immediately add 45, 90 and 135 μl of LPEI (1 mg/ml) into the tubes containing the resuspended cells labeled 1:1, 1:2 and 1:3, respectively. Mix gently.

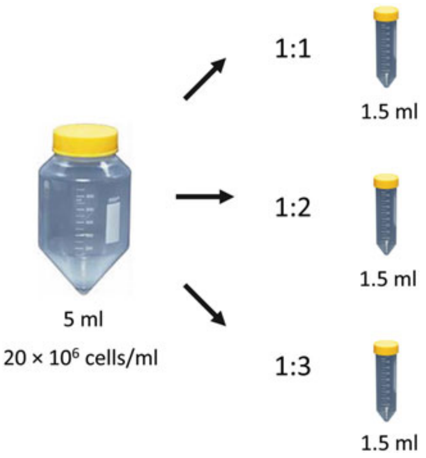

Fig. 5 Split the suspension into three 50 ml-TubeSpin® Bioreactors (1.5 ml each)

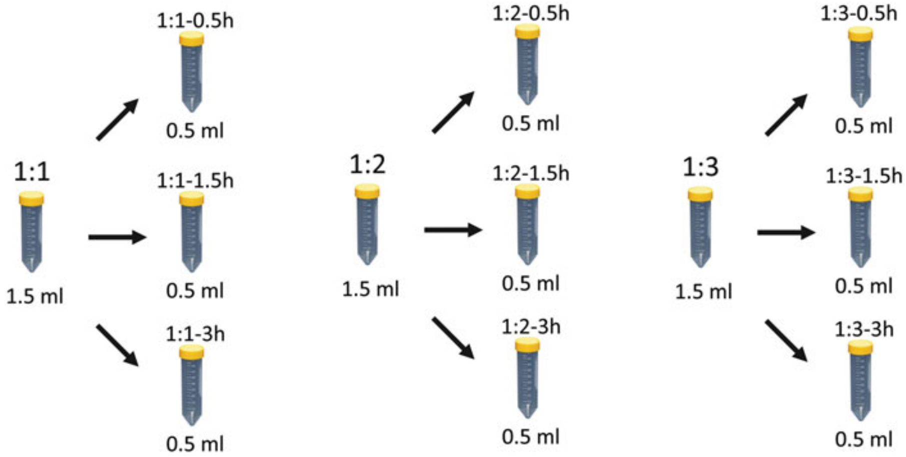

Fig. 6 Further split the suspensions into nine labeled 50-ml TubeSpin® Bioreactors (0.5 ml each)

14. From each tube (labeled 1:1, 1:2 and 1:3), transfer 0.5 ml of the resuspended cells into nine 50-ml TubeSpin® Bioreactor labeled: 1:1–0.5 h, 1:1–1.5 h, 1:1–3 h, 1:2–0.5 h, 1:2–1.5 h, 1:2–3 h, 1:3–0.5 h, 1:3–1.5 h, 1:3–3 h (Fig. 6).
15. Incubate the tubes in the orbital shaker at 37 °C, 8% CO_2, 175–225 RPM in a humidified atmosphere.
16. After the incubation time indicated on the tubes (0.5 h, 1.5 h, 3 h), transfer 9.5 ml of FreeStyle™ 293 Expression Medium directly in each culture tube to obtain a 10 ml culture in each tube.

17. Incubate the tubes in the orbital shaker at 37 °C, 8% CO_2, 175–225 RPM in a humidified atmosphere.
18. After transfection, take a sample from each tube after 8 h, 16 h and every day up to 5 days.
19. Estimate the cell density and collect 1 ml from each tube.
20. Centrifuge 0.5 ml of each sample (16,000 × *g*, 5 min, 4 °C), remove the supernatant and store the pellet at −20 °C for subsequent analysis by western blotting.
21. Use the remaining 0.5 ml of each sample for fluorescence analysis.

3.3 Sample Analysis

3.3.1 Western Blot

1. Thaw the cell pellets on ice.
2. Add LYSIS buffer to pellets to reach a density of 2×10^6 cells/ml. Resuspend the pellets by pipetting (*see* **Note 10**).
3. Transfer 30 μl of each sample into a fresh Eppendorf tube and add 15 μl of SDS sample buffer directly to each sample.
4. Incubate and shake at room temperature for a minimum of 10 min to shear the DNA and reduce the sample viscosity.
5. Load the SDS-PAGE gel with 15 μl of each sample (approx. 1×10^5 cells/well). Run the gel and proceed with western blot according to standard protocols (*see* **Note 11**).

3.3.2 Fluorescence Microscopy

1. Observe the membrane-associated eGFP-specific fluorescence of each cell culture sample using the FITC filter.
2. Note the localization of the expressed protein (membrane association versus location in the cytoplasm).

3.3.3 Evaluation of Small-Scale Expression Test

Several factors can limit the overexpression of membrane proteins. Besides space limitation in the membrane, often the insertion machineries can be overloaded leading to aggregation of misfolded proteins and toxic effects [13]. Localization studies by fluorescence microscopy are typically a good indicator for the correct folding state of an overexpressed protein. Western blot analysis, however yields information on the total amount of overexpressed protein only but not on the folding state. Typically, one selects the condition with the highest protein expression level while retaining membrane localization for later scale-up experiments.

In the case of PepT2, the fluorescence signal indicates that the majority of the protein is integrated into the cell membrane one and 2 days after the transfection. However, the signal starts to accumulate in the cytoplasm from the third day of expression (Fig. 7a). The blotted samples were divided into three groups differing by the transfection time. In all cases, the strongest signal was derived from the samples corresponding to an expression time of 2 days

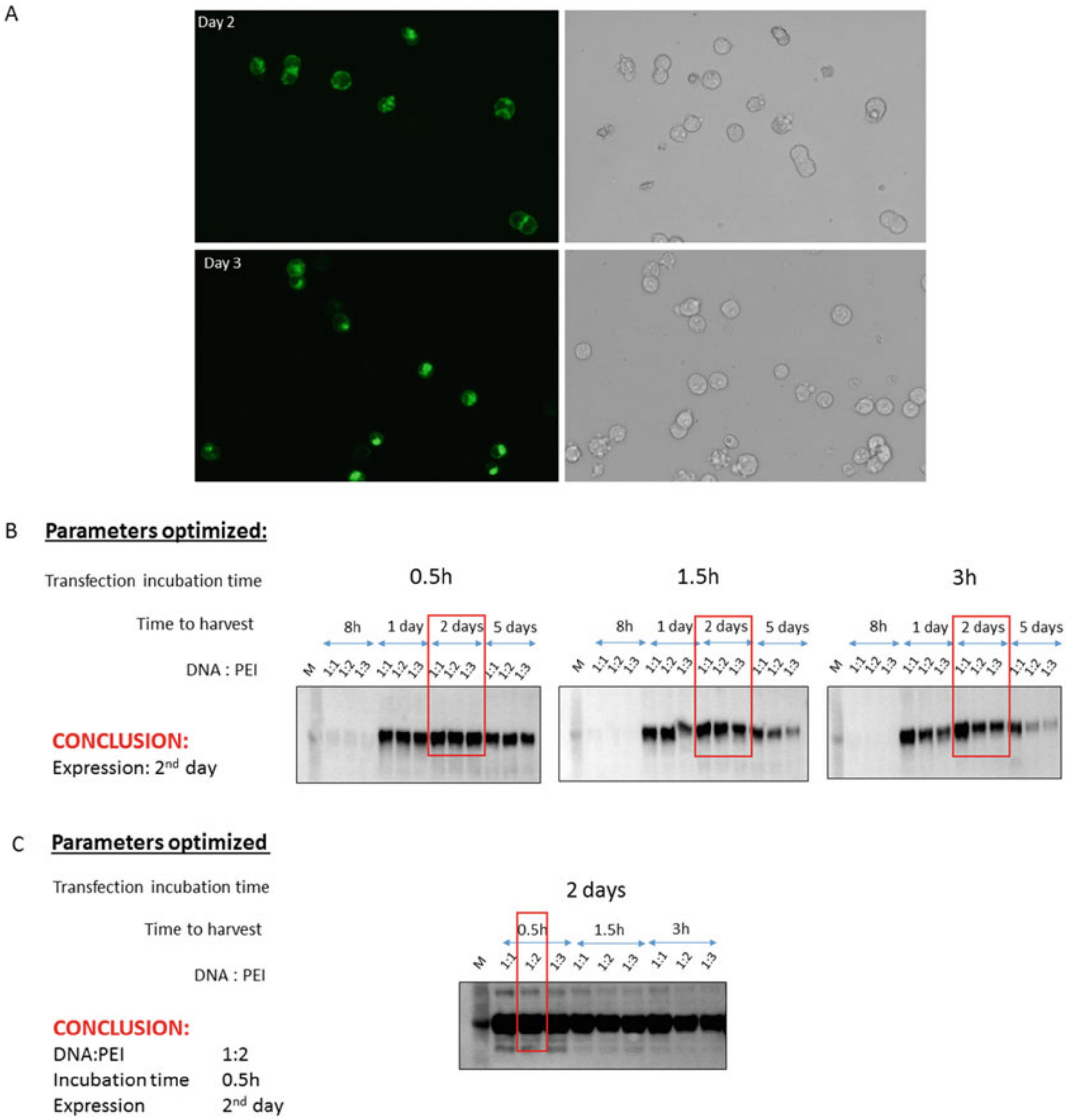

Fig. 7 Transfection optimization analysis. (**a**) Representative fluorescence image (left) and corresponding bright field image (right) of GFP-fused membrane protein expressed in HEK293F cells at day 2 (top image) and day 3 (bottom image) post-transfection. (**b**) The representative Western blot analysis of PepT2 after three different transfection incubation times; DNA:LPEI ratios are indicated on the top of the gel, time of the harvest is marked by blue arrows on top of the gel. Red frames indicate the best conditions. (**c**) Western blot analysis of PepT2 expression at day 2. Red frames indicate the best conditions

(Fig. 7b). Further parameter analysis revealed that 0.5 h transfection incubation time and a DNA:LPEI ratio of 2:1 (Fig. 7c) resulted in the highest expression levels of PepT2.

Hence, the optimized conditions for the transfection and expression of PepT2 are a DNA:LPEI ratio of 1:2 (w/w), transfection incubation time of 0.5 h and expression for 2 days.

3.4 Transfection and Expression in Large Scale (1 L in Total)

1. Seed five 600 ml TubeSpin® Bioreactors with 200 ml of cell culture each at a density of 0.4 × 10^6 cells/ml).
2. Grow the cells until the cell culture reaches a density of 2.5–3.0 × 10^6 cells/ml. This usually takes 3–4 days, as the cells divide approximately once per 24 h.
3. Count the cells separately for each bioreactor (*see* Subheading 3.1, point 5) and centrifuge at 100 × *g*, 5 min, RT.
4. Aspirate the medium and resuspend the cells in each bioreactor in prewarmed FreeStyle™ 293 Expression Medium to reach a density of 2 × 10^6 cells/ml (125–150 ml of medium).
5. Incubate cultures in an orbital shaker at 37 °C, 8% CO_2, 175–225 RPM in a humidified atmosphere for 24 h.
6. Determine the cell density, viability and the amount of cell clumping using trypan blue. Viability of the cells must be over 95%.
7. Transfer the volume of cell culture corresponding to 1000 × 10^6 cells into a new 600 ml TubeSpin® Bioreactor and centrifuge (100 × *g*, 5 min).
8. Carefully remove the medium by decanting. Do not disturb the cell pellet.
9. Add 50 ml of prewarmed RPMI 1640 with 0.1% Pluronic F-68 medium to the cell pellet. Gently mix to resuspend the cells (*see* **Note 9**).
10. Add 1500 μg of DNA directly to the cell culture and mix gently.
11. Immediately add 3 ml of a 1 mg/ml solution of LPEI directly to the culture and mix gently.
12. Incubate cultures in an orbital shaker at 37 °C, 8% CO_2, 175 RPM in humidified atmosphere for 45 min.
13. Split the transfection mix in five new 600 ml TubeSpin® Bioreactors by transferring 10 ml of transfection mix into one bioreactor.
14. Transfer 190 ml of FreeStyle™ 293 Expression Medium directly into each bioreactor with the transfection mix to yield 200 ml of the cell culture in total.
15. Incubate in the orbital shaker at 37 °C, 8% CO_2, 175–225 RPM in humidified atmosphere.
16. Harvest the cells 48 h after transfection: centrifuge at 3500 × *g*, 10 min, 4 °C. The pellet can be stored at −20 °C. The wet weight of the pellet is typically 6–8 g per liter of cell culture.

3.5 Purification of Membrane Protein (PepT2)

Perform all steps at 4 °C. Take a small sample at each step and add SDS loading dye for subsequent analysis.

3.5.1 Membrane Preparation

1. Resuspend the cell pellet in LYSIS buffer (5 ml of LYSIS buffer per 1 g of the pellet) and incubate for 30 min.
2. Disrupt the cells by passing the suspension three times through the emulsifier, applying pressure of 10,000–15,000 psi.
3. Remove the cell debris by centrifugation at 10,000 × *g*, 12 min, 4 °C.
4. Harvest the cell membranes by ultracentrifugation at 140,000 × *g*, 1 h, 4 °C.
5. Resuspend the pellet (=membrane fraction) in the solubilization buffer with a Potter-Elvehjem pestle. Use the same volume of solubilization buffer as the LYSIS buffer used in **step 1**.
6. Add 1% DDM and stir for 1 h at 4 °C (*See* **Note 12**).
7. Remove nonsolubilized material by ultracentrifugation at 105,000 × *g*, 30 min, 4 °C (take a sample from the supernatant).
8. Directly proceed with the next steps with the supernatant.

3.5.2 Affinity Chromatography—IMAC (See **Note 13**)

1. Transfer 2 ml of Ni-NTA resin into the IMAC gravity column.
2. Equilibrate the column with five column volumes of WASH buffer.
3. Transfer 2 ml of IMAC Ni-NTA resin into a falcon tube and incubate with solubilized material for 1 h on a rotating wheel.
4. Transfer the IMAC resin into a closed plastic column and let the beads settle at the bottom of the column.
5. Open the column and collect the flow-through (take a sample). Reapply the flow-through once the solution has passed through the column.
6. Wash the resin with 2 × 10 ml of WASH buffer (*see* **Note 14**). Do not let the beads dry out completely. After the last step of washing, close the column.
7. Add 10 ml of WASH buffer with 0.5 mg of TEV protease.
8. Incubate the beads with the bound protein and TEV protease on the rotating wheel overnight.

3.5.3 Affinity Chromatography—Negative IMAC

1. Let the beads settle at the bottom of the column.
2. Open the column and collect the flow-through. The flow-through contains the cleaved target protein. Proceed directly to the gel filtration step and simultaneously continue with the following steps.
3. Wash the beads with 2 × 3 ml of WASH buffer.

4. Elute the residual protein from the beads with 2 × 5 ml of ELUTION buffer.
5. Run the SDS-PAGE gel with all collected samples (*see* **Note 15**).

3.5.4 Gel Filtration

1. Equilibrate the Superose 6 Increase 10/300 GL column with 1.5–2 column volumes of GF buffer.
2. Concentrate the target protein using a concentrator with 100 kDa cutoff to 0.5 ml (*see* **Note 16**).
3. Wash the injection loop with water and GF buffer, and carefully load the loop with the concentrated sample and inject the sample on the column. Run size exclusion chromatography with the recommended flow rate and maximum pressure settings (Fig. 8).
4. Collect 0.5–1 ml fractions.
5. Analyze the collected fractions by SDS-PAGE (Fig. 8, inset) and pool the fractions containing the target protein.
6. Concentrate the protein to 1–5 mg/ml, aliquot in 50-μl fractions, snap freeze the aliquots in liquid nitrogen and store at −80 °C until further use.

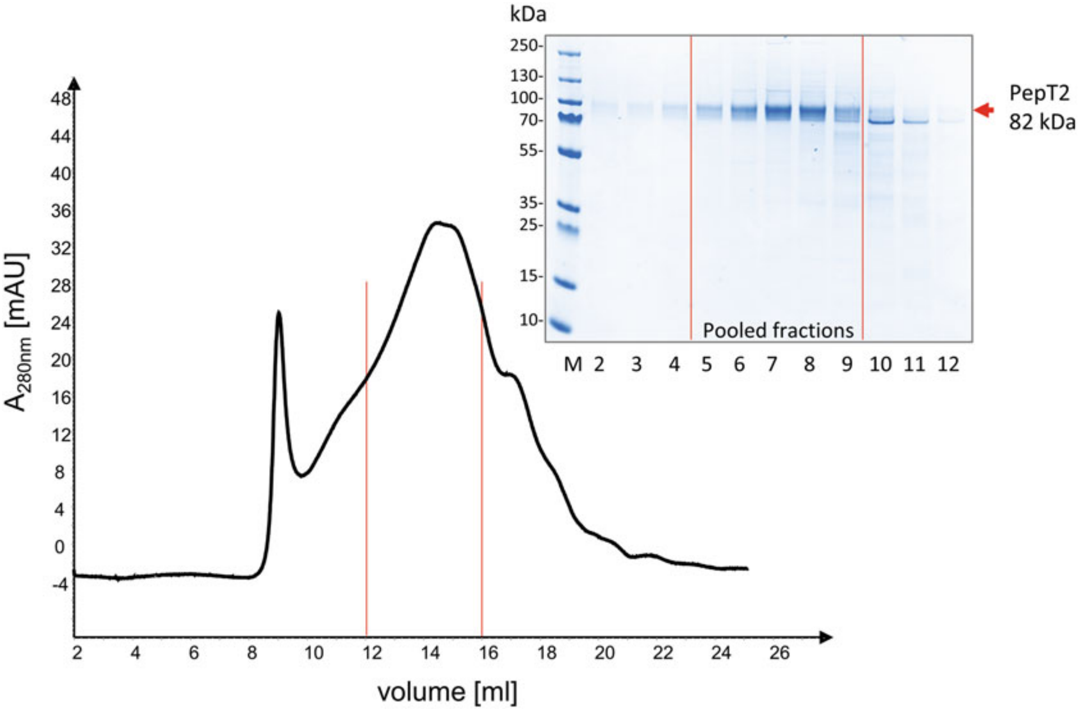

Fig. 8 Purification of PepT2. Representative gel-filtration chromatogram of PepT2. Inset: Instant blue-stained SDS-PAGE analysis of the size exclusion chromatography run. Lane 1: protein molecular weight marker (M). Lanes 2–12: fractions eluted from the Superose6 column. The arrow indicates the band corresponding to PepT2. Red lines indicate the pooled fractions used for further analysis

4 Notes

1. Although the construct used in this particular case contains an N-terminal His-GFP tag, often membrane proteins are tagged C-terminally, as this ensures that only the fully translated protein is purified. We recommend testing the expression of both N-terminally and C-terminally tagged proteins in small scale.
2. The transfection protocol provided here is transferable to most other HEK293 subclones. Of particular interest might be the HEK293S GnTI−/− subclone, which expresses proteins with a more homogenous glycosylation pattern, suitable for structural studies.
3. For plasmid preparation, use purification kits suited for transfection-grade DNA isolation. The quality of the plasmid DNA can influence the transfection efficiency [8].
4. RPMI 1640 medium (Sigma) already contains L-glutamine at 0.3 g/l and does not require further addition of L-glutamine. On the day of transfection, add Pluronic F-68 to a final concentration of 0.1%.
5. FreeStyle™ 293 Expression Medium is a ready-to-use medium, supplemented with GlutaMAX™-I and is animal-origin free. This medium supports the growth and transfection of FreeStyle™ 293-F cells in suspension. The medium needs to be prewarmed in a 37 °C water bath prior to use. Store at 4 °C with minimize exposure to light.
6. Polyethylenimine, Linear, MW 25000, Transfection Grade (LPEI 25 K) is a cost-effective transient transfection reagent. To prepare 100 ml of 1 mg/ml stock solution, dissolve 100 mg of powder in 60 ml of water and adjust the pH to 3.0 with 1 M HCl (solution becomes clear). Once the powder completely dissolves, adjust the pH to 7.0 with 1 M NaOH. Adjust the volume to 100 ml. Filter through a 0.22 μm membrane (this step is necessary to sterilize the solution and to remove undissolved LPEI particles). Prepare 0.5–1 ml aliquots and store at −20 °C. Once thawed, keep the solution at 4 °C. At this condition, the LPEI is stable for at least 2 months. Avoid multiple freezing and thawing cycles.
7. Incubator should be adapted for growing suspension cells requiring gentle agitation, humidified atmosphere of 8% CO_2 in air, proper gas exchange and minimal exposure of cultures to light.
8. Trypan blue is toxic for the cells and prolonged exposure can introduce errors when determining the live/dead cell ratio. Always count the cells 1–2 min after mixing with the dye.

9. For optimal transfection results, make sure that the cells do not aggregate in suspension. Gently mix the culture by pipetting with a P1000 pipette.
10. In some cases, it is necessary to isolate the membrane fraction and remove soluble cytoplasmic proteins to be able to detect the low expression of membrane proteins. After lysis and low speed centrifugation, harvest the membrane fraction by an ultracentrifugation step (105,000 × *g*, 1 h, 4 °C), resuspend the pellet and solubilize the membranes with WASH buffer in the presence of strong detergents such as 1% SDS or FC12.
11. Membrane proteins are likely to bind more SDS molecules and stay partially folded even after treatment with SDS. Due to these effects, membrane proteins often exhibit different electrophoretic mobility than expected. Moreover, different protein modifications, such as glycosylations, can result in "smeared" bands or in the appearance of multiple bands.
12. We recommend screening multiple detergents before proceeding with the large-scale production and purification. It is essential to identify a suitable detergent, which is able to solubilize the target protein efficiently and keep it stable in solution. The solution typically clears up upon membrane solubilization.
13. Affinity chromatography can be adjusted to specific proteins. Amount of the Ni-NTA resin can be changed according to resin binding capacity and the amount of the expressed protein. In some cases, the His tag is not cleavable with the protein bound on the beads or the protein might precipitate upon prolonged incubation with the beads. In such cases, after the washing step, elute the protein with 2 × 10 ml of ELUTION buffer, transfer the protein into a dialysis bag, add TEV protease, dialyze against the GF buffer and transfer into an equilibrated gravity column with washed 1 ml of Ni-NTA resin on the next day. Amount of the TEV protease required depends on the protein expression level.
14. Resuspend the beads with WASH buffer with the 5 ml pipette when you apply the buffer the first time. Do not repeat it in second washing step.
15. Analyze samples from all purification steps on a SDS-PAGE gel before proceeding with the next step. Make sure that the target protein was cleaved by the TEV protease.
16. Surrounding detergent increases the molecular weight of a detergent solubilized membrane protein. It is common to use concentrators with molecular weight cut off membranes greater than the molecular weight of the membrane protein to minimize the concentration of free detergent micelles.

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Chapter 3

PEI-Mediated Transient Gene Expression in CHO Cells

Yashas Rajendra

Abstract

We describe a method for polyethyleneimine (PEI) mediated transient transfection of suspension-adapted Chinese hamster ovary (CHO-DG44) cells for protein expression applicable at scales from 2 mL to 2 L. The method involves transfection at a high cell density (5×10^6 cells/mL) by direct addition of plasmid DNA (pDNA) and PEI to the culture and subsequent incubation at 31 °C with agitation by orbital shaking. This method requires 0.3 mg/L of coding pDNA, 2.7 mg/L of nonspecific (filler) DNA and 15 mg/L of PEI. The production phase is performed at 31 °C in the presence of 0.25% *N*,*N*-dimethylacetamide (DMA). We also provide information on culture vessel options, recommended working volumes, and recommended shaking speeds for transfections at scales from 2 mL to 2 L.

Key words CHO cells, Transfection, Polyethyleneimine, Orbital shaking, Recombinant protein

1 Introduction

Rapid production of recombinant proteins by transient gene expression (TGE) in mammalian cells is not only an important tool used during the early phases of biologic drug development but also a very widely used tool in academic settings for generation of recombinant proteins for research purposes. In the recent years, transient expression in CHO cells has significantly improved with publications reporting titers of up to 3 g/L [1–3]. However, TGE methods described for CHO cells in the literature often require the use of engineered host cells, TGE-specific expression vectors, and proprietary cell culture media [1, 2], which means they are often not directly reproducible. We describe a TGE method here for which all of the materials necessary are commercially available and hence should be easier to reproduce with a minimum amount of optimization [4].

Even with all the advancements, pDNA remains a relatively high cost component of PEI-mediated transfection processes. Hence, it is important to minimize pDNA requirements while

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maintaining high protein expression levels. We and others have previously reported that the amount of transgene-bearing pDNA can be reduced by replacing some of it with nonspecific DNA with only a moderate loss in volumetric productivity [5, 6]. When using reduced pDNA amounts, the transient protein yield from transfected CHO cells can be enhanced by use of polar solvents such as *N*,*N*-dimethylacetamide (DMA) [7, 8].

Here, we describe the procedure for PEI mediated transfection of CHO-DG44 cells applicable to scales from 2 mL to 2 L that, depending on the scale, can be performed in different types of culture vessels including 24 deep-well plates, disposable TubeSpin® bioreactor 50 (TPP, Trasadingen, Switzerland), glass bottles and Erlenmeyer flasks [2, 4, 9–11]. The production of human monoclonal antibody from a bicistronic plasmid carrying the full-length cDNAs of the IgG light and heavy chain genes is described, but the method is suitable for the production of most other mammalian proteins for research use. The transfection method described here requires considerably less pDNA (300 μg/L) than most reported levels.

2 Materials

2.1 Cell Culture

1. CHO-DG44 cells adapted to cultivation in serum-free suspension.
2. Cylindrical and square-shaped glass bottles with nominal volumes of 100 mL to 5 L (Schott Glass, Mainz, Germany).
3. ProCHO5 medium (Lonza AG, Verviers, Belgium) without L-glutamine, hypoxanthine, thymidine, and phenol red.
4. 50× L-glutamine and phenol red solution: 200 mM glutamine and 250 μg/mL phenol red. Dissolve 29.23 g glutamine and 250 mg phenol red in 800 mL water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration through a 0.2 μm Steritop bottle-top filter with a neck size of 45 mm (Merck & Cie, Schaffhausen, Switzerland). The solution is transferred into sterile 50 mL centrifuge tubes and kept frozen at −20 °C. For each liter of ProCHO5 medium, 20 mL of the stock solution is added.
5. 50× HT solution: 5 mM hypoxanthine and 0.8 mM thymidine. Dissolve 680 mg of hypoxanthine and 194 mg of thymidine in 800 mL of water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration and frozen as aliquots as explained in the previous step. For each liter of $ProCHO_5$ medium, 20 mL of the stock solution is added.

6. Trypan blue solution (0.4%).
7. Neubauer hemocytometer.
8. Inverted phase contrast microscope (100× magnification, Telaval 31, Carl Zeiss AG, Feldbach, Switzerland).
9. Tabletop centrifuge (Labofuge 200, Heraeus AG).
10. Incubator shaker (model ISF-4-W with a rotational diameter of 50 mm; Kühner AG, Birsfelden, Switzerland).
11. Floor model centrifuge (Cryofuge 6000i, Heraeus AG).

2.2 Plasmids

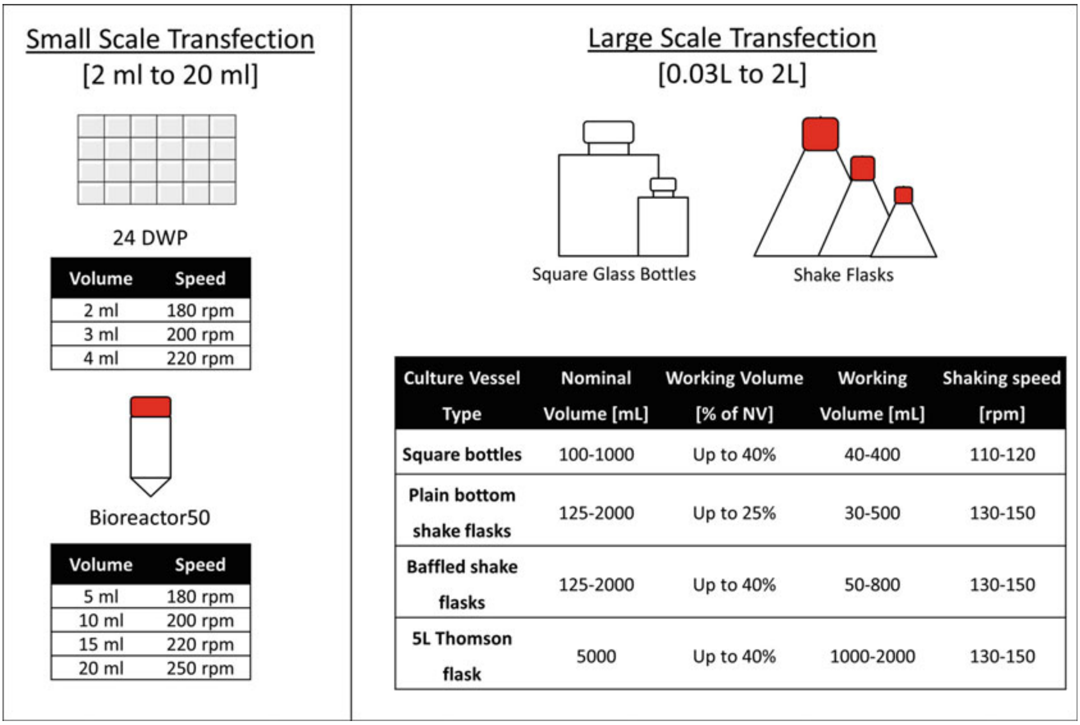
1. pXLG$^{\text{CHO}}$-A3 expressing the anti-Rhesus D IgG1 with the heavy and light chain cDNAs cloned in separate expression cassettes in a head-to-head orientation [4] (*see* **Notes 1** and **2**).
2. Based on our experience, any one of the following commercial pDNA purification kits can be used for generating transfection grade pDNA.
    - (a) NucleoBond (Macherey-Nagel, Düren, Germany)
    - (b) Qiagen Plasmid Kits (Qiagen, USA)
    - (c) Purelink Expi Plasmid Purification Kits (ThermoFisher Scientific, USA).

2.3 Transfection

1. Linear 25 kDa polyethyleneimine (PEI) (Polysciences, Eppenheim, Germany) solution: 1 mg/mL PEI (pH 7). PEI (1 g)is added to 800 mL deionized water. When stirring the solution, lower the pH to 3 with 1 N HCl. When the PEI is in solution, the pH is increased to 7.0 with 1 N NaOH and the volume is increased to 1 L. The solution is filter-sterilized, aliquoted into sterile 50 mL tubes, and stored at −20 °C. It can be stored frozen for years as long as repeated freeze–thaw cycles are avoided (*see* **Notes 3** and **4**).
2. ProCHO5 medium without L-glutamine, hypoxanthine, thymidine, and phenol red (*see* Subheading 2.1).
3. 50× L-glutamine–phenol red solution (*see* Subheading 2.1).
4. 50× HT solution (*see* Subheading 2.1).
5. Sheared herring sperm DNA to be used as filler DNA (*see* **Note 5**).
6. *N, N*-Dimethyl acetamide (DMA).

2.4 Culture Vessel

1. *See* Fig. 1 for a noncomprehensive list of culture vessel options and recommended culture volumes and shaking speeds. All shaking speed recommendations are for a shaking platform with a shaking diameter of 50 mm.



Volume	Speed
2 ml	180 rpm
3 ml	200 rpm
4 ml	220 rpm

Volume	Speed
5 ml	180 rpm
10 ml	200 rpm
15 ml	220 rpm
20 ml	250 rpm

Culture Vessel Type	Nominal Volume [mL]	Working Volume [% of NV]	Working Volume [mL]	Shaking speed [rpm]
Square bottles	100-1000	Up to 40%	40-400	110-120
Plain bottom shake flasks	125-2000	Up to 25%	30-500	130-150
Baffled shake flasks	125-2000	Up to 40%	50-800	130-150
5L Thomson flask	5000	Up to 40%	1000-2000	130-150

Fig. 1 List of small-scale and large-scale vessel options and recommended shaking speeds

3 Methods

3.1 Plasmid Purification

1. *E. coli* strain DH5α is transformed with pXLG^{CHO}-A3 by the standard $CaCl_2$ method and spread onto LB agar plates with 100 μg/mL ampicillin (*see* **Note 6**).
2. Incubate the plates overnight (16 h) at 37 °C.
3. With a sterile loop or a pipette tip, transfer a single colony from the transformed plate to a sterile round-bottom, polypropylene 14-mL culture tube containing 3 mL LB broth with 100 μg/mL ampicillin.
4. Incubate at 37 °C for 4–6 h with agitation at 220 rpm.
5. Use this starter culture to inoculate the appropriate volume of LB depending on the scale of plasmid preparation as per kit manufacturer's protocol.
6. Incubate the culture for 12–16 h at 37 °C with agitation at 220 rpm.
7. Follow manufacturer's protocol for purification of pDNA from this culture.

3.2 Routine Cell Culture

1. CHO-DG44 cells are subcultivated every 3–4 days (*see* **Note 7**) by inoculation in 100 mL ProCHO5 medium (when used for cell culture, the medium contains L-glutamine, hypoxanthine, thymidine, and phenol red as indicated in Subheading 2.1) (*see* **Note 8**) in a 250 mL square-shaped glass bottle at an initial cell density of 0.3×10^6 cells/mL.
2. Determine the cell density and viability by Trypan blue staining using a Neubauer hemocytometer and an inverted phase contrast microscope or any other suitable method.
3. After cell counting, transfer 3×10^7 cells into a 50 mL centrifuge tube and centrifuge at $500 \times g$ for 3 min in a standard tabletop centrifuge.
4. Remove medium by aspiration or decanting. The cell pellet is resuspended in 10 mL of ProCHO$_5$ medium and transferred to a 250 mL square-shaped bottle containing 90 mL of prewarmed ProCHO$_5$ medium. This will lead to a culture at 0.3×10^6 cells/mL.
5. Attach the bottle to a platform mounted on an orbital shaker using double-sided adhesive transfer tape and agitate at 110 rpm at 37 °C in a 5% CO$_2$ atmosphere without humidity. Preferably, vented caps should be used. Alternatively, keep the cap of the bottle opened about one quarter of a turn.

3.3 Cell Expansion for Transfection

1. Count the cells prepared as in Subheading 3.2 after reaching a density of about 5×10^6 cells/mL.
2. Culture should be scaled up based on the transfection volumes. Below is an example of scale up for a transfection volume of 1 L, but applicable to any desired transfection volume.
3. Transfer 100 mL of CHO-DG44 cells from the 250 mL bottle into the 2 L bottle with 400 mL of prewarmed ProCHO$_5$ medium to reach a cell density of about 1×10^6 cells/mL.
4. Incubate the culture at 37 °C with agitation as described in Subheading 3.2 (**step 5**) until reaching a cell density of about 5×10^6 cells/mL (approximately 2 days).
5. On the day before transfection, transfer the culture into two sterile 500 mL centrifuge bottles (*see* **Note 9**).
6. Centrifuge for 5 min at $500 \times g$ at room temperature in a floor model centrifuge.
7. Remove the medium by aspiration and gently resuspend each cell pellet in 50 mL of prewarmed ProCHO$_5$ medium with a 25 mL pipette (*see* **Note 8**).
8. Transfer the cells into a 5 L cylindrical glass bottle with 900 mL of prewarmed ProCHO$_5$ medium. Alternatively, use a 5 L

Thomson flask. The starting cell density of the culture is 2.0–2.5 × 10^6 cells/mL.

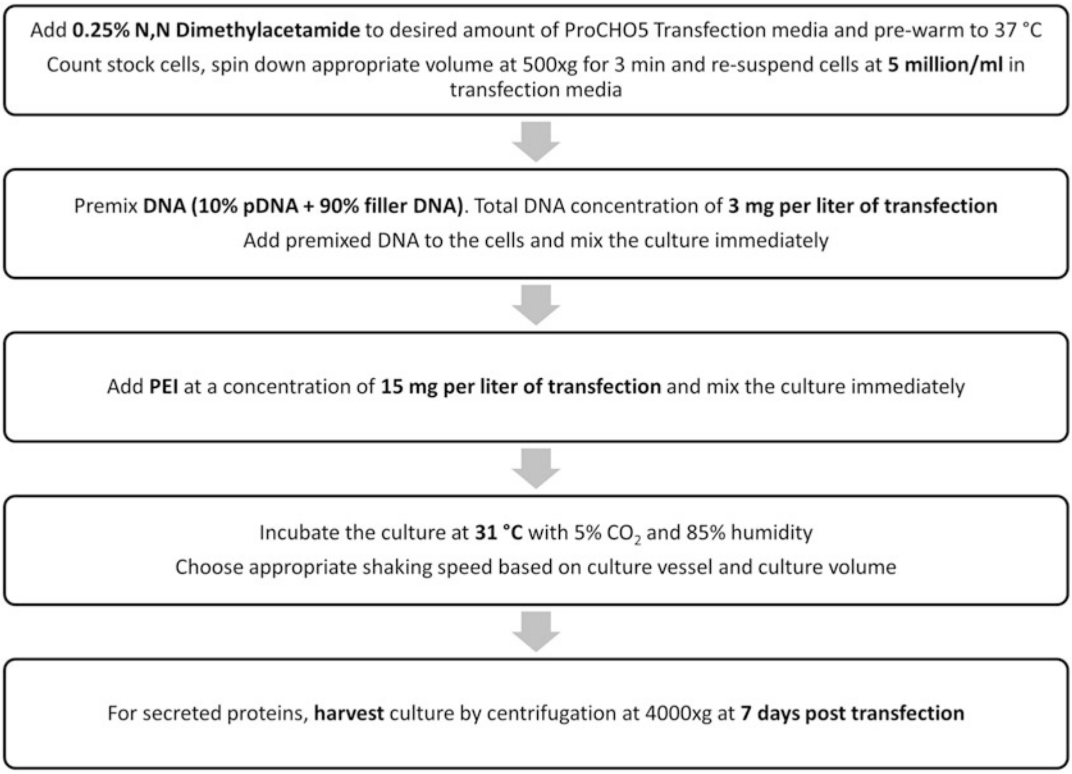
9. Transfer the 5 L bottle onto an orbital shaker and incubate at 37 °C overnight (at least 16 h) with agitation as described in Subheading 3.2 (**step 6**).

3.4 Transfection

1. On the day of the transfection, the cells are counted as described in Subheading 3.2.
2. Below is an example of a transfection volume of 1 L, but this is also applicable to any desired transfection volume.
3. Spin down appropriate amount of cells at 500 × *g* for 5 min and resuspend cells at 5 × 10^6 cells/mL in 1000 mL prewarmed ProCHO$_5$ medium. Make sure to completely aspirate out the old media prior to resuspension in fresh medium. Transfer the cells into an appropriately sized culture vessel.
4. Mix 0.3 mg/L of pDNA and 2.7 mg/L of herring sperm DNA (*see* **Note 10**). Add this to the culture and swirl immediately. Total DNA for this transfection method is fixed at 3 mg/L of the transfection volume.
5. Subsequently add PEI to a final concentration of 15 mg/L directly in the culture and immediately mix the culture by swirling after addition of PEI (*see* **Note 4**).
6. Add 2.5 mL of DMA to the culture and mix immediately by swirling the bottle (*see* **Notes 11** and **12**). DMA can also be added to medium prior to transfection and is stable for up to 1 month.
7. Place the culture on an orbital shaker as described in Subheading 3.2 (**step 5**) and incubate at 31 °C in 5% CO_2 and 85% humidity with appropriate agitation (*see* **Note 13**).
8. For secreted proteins, at day 7 post-transfection, harvest the culture by centrifuging at 4000 × *g* for 15 min (*see* **Note 14**).
9. A flow chart representation of the transfection method is shown in Fig. 2.

3.5 Analysis of Antibody Production

1. To measure recombinant protein accumulation over time, 100 μL aliquots of the culture can be taken daily during the production phase. After centrifugation to remove cells, the antibody concentration in each sample is measured by sandwich ELISA.
2. Detailed protocol for antibody detection by ELISA has been previously published [12].



Add **0.25% N,N Dimethylacetamide** to desired amount of ProCHO5 Transfection media and pre-warm to 37 °C
Count stock cells, spin down appropriate volume at 500xg for 3 min and re-suspend cells at **5 million/ml** in transfection media

Premix **DNA (10% pDNA + 90% filler DNA)**. Total DNA concentration of **3 mg per liter of transfection**
Add premixed DNA to the cells and mix the culture immediately

Add **PEI** at a concentration of **15 mg per liter of transfection** and mix the culture immediately

Incubate the culture at **31 °C** with 5% CO_2 and 85% humidity
Choose appropriate shaking speed based on culture vessel and culture volume

For secreted proteins, **harvest** culture by centrifugation at 4000xg at **7 days post transfection**

Fig. 2 Flowchart representation of the transfection method

4 Notes

1. Typically, for antibodies, both the IgG light and heavy chain genes are expressed from the human cytomegalovirus (HCMV) major immediate early promoter/enhancer. This is generally the most favorable promoter for TGE in either CHO or HEK-293 cells.
2. For antibodies, IgG light and heavy chain genes can either be expressed using a dual vector or by cotransfecting individual vectors carrying light and heavy chain. If cotransfecting, it is recommended to test cotransfection ratios of 1:1, 1:2, and 1:3 of HC–LC.
3. Once thawed, the PEI solution in 50 mL tubes can be aliquoted into 15 mL tubes and either used for transfection that day or stored at 4 °C for up to 4 weeks.
4. Alternatively, PEI HCl Max (Polysciences Inc.) can also be used. PEI-Max dissolves in water without any need for pH adjustment. Note that reoptimization of PEI-Max amounts

for transfection would be necessary if replacing the traditional 25 kDa PEI as the optimal amount needed may be different.

5. Sheared herring sperm DNA can be diluted to a desirable concentration in either sterile deionized water or TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 7.5).
6. It is recommended to use expression vectors with a high copy number origin of replication. This is an important point because a significant amount of pDNA is necessary for TGE at large scale. The LB culture volume (2 L) is sufficient for a Giga Prep yielding 6–10 mg of pDNA. If a low copy number plasmid is used, its propagation may require a larger culture volume to obtain a sufficient pDNA yield.
7. To maintain the transfectability of the cells, it is best to keep cells in culture for no longer than 2–3 months (20–25 passages). We highly recommend maintaining the cells in exponential growth phase at all times.
8. ProCHO$_5$ medium contains plant-derived peptone hydrolysates that may be a source of lot-to-lot variation of the medium. We have observed that different lots of ProCHO5 medium can result in differences in the percentage of transfected cells following PEI-mediated gene delivery. Therefore, each new lot of the medium should be tested for its support of cell cultivation and transfection before purchase, if feasible. The CHO cells should be adapted to any new medium lot for at least 4 passages (about 2 weeks) prior to testing cell growth and transfection.
9. It is recommended to passage the cells into fresh medium on the day prior to transfection to ensure exponential growth as this is optimal for PEI-mediated transfection.
10. The method described here does not involve precomplex formation with DNA and PEI prior to addition to the culture. It is very important to minimize the time delay between addition of pDNA and PEI and to mix the culture well after each component is added.
11. The method described here used 10% pDNA and 90% filler DNA with the addition of 0.25% DMA. It is recommended to use higher pDNA amounts (while keeping total amount of DNA constant) if low expression is observed with the method described here. It may also be necessary to optimize the amount of DMA added to the culture.
12. It is important to note that DMA is a hazardous chemical and it is very important to follow appropriate usage and disposal practices. Alternatively, acetonitrile may be used to replace DMA; however, the concentration must be optimized (test range 0.1% to 2%)

13. The method described here was found to be optimal when performed at 31 °C. However, it may be beneficial to test temperatures between 30 °C and 33 °C to obtain the best yields for the production of any given protein.
14. Although the supernatant was harvested on day 7 post-transfection, the culture can be extended further if its viability is high (>80%) at that time. Preferably, the viability of the cells at harvest should be at least 50%.
15. If two or more plasmids are being cotransfected, for multi-protein complex formation as an example, then the optimal plasmid ratio needs to be empirically determined.
16. The efficiency of transfection is typically 60–70% for the method described here as determined by the percentage of fluorescent cells following transfection with a vector expressing a fluorescent protein.
17. While, the TGE method described here was originally developed for the combination of CHO-DG44 cells and ProCHO5 medium, it is also applicable to suspension adapted CHO-K1 and CHO-S. For CHO-S and CHO-K1 grown and transfected in ProCHO5, the optimal amounts of PEI and DNA remained unchanged; however, we observed 30–40% lower titer.
18. If using a different CHO strain and/or a different medium, it is necessary to optimize the amounts of DNA, PEI, and DMA added as well as the cell density at the time of transfection. Some commercially available media inhibit PEI-mediated transfection due to the presence of known components such as dextran sulfate, heparin sulfate, ferric ammonium citrate and certain hydrolysates or other unknown components. Hence, it is essential to choose a medium which supports PEI-mediated transfection.
19. It is recommended to confirm efficiency of transfection by performing a transfection with 100% EGFP-coding pDNA. If transfection efficiency at 24 h is less than 50%, optimization of PEI will be necessary.

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Chapter 4

Stable Expression by Lentiviral Transduction of Cells

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Abstract

Lentiviral gene transfer represents a versatile and powerful method for genetic transduction of many cell lines and primary cells including "hard-to-transfect" cells. As a consequence of the integration of the recombinant lentiviral vector into the cellular genome the transgene is stably maintained and long term producing cells are established. Here, we describe the current state of the art and give details for lab scale production of lentiviral vectors as well as for infection and titration of the viral vectors.

Key words Lentiviral vectors, Gene transfer, Stable expression, Primary cells, Cell lines

1 Introduction

Lentiviral gene transfer emerged as a highly efficient and widely applicable method for stable expression of transgenes in a broad spectrum of primary cells as well as cell lines. Lentiviruses belong to family of retroviridae and carry two copies of the positive sense strand viral RNA genome. Upon infection, the viral RNA is reverse-transcribed and one copy of double stranded DNA is stably integrated into the host's genome. In this way, the viral genome is replicated together with the cellular genome. In contrast to γ-retroviruses, which crucially rely on cell division to access the nucleus as a prerequisite for integration into the cellular genome, lentiviruses rely on active nuclear transport and can infect both dividing and nondividing cells.

To exploit the viral transfer principles for transduction of genetic material of choice different vector systems have been developed (for a recent review *see* [1–3]). Mostly, these transduction systems rely on the human immune deficiency virus HIV-1. The vector developments have resulted in protocols that facilitate the specific transfer of the lentiviral vector with the gene of interest but avoid packaging and transduction of the viral genes, so that only the transfected cells but not the infected cells have the ability to produce viral particles. Efforts have been undertaken to reduce viral

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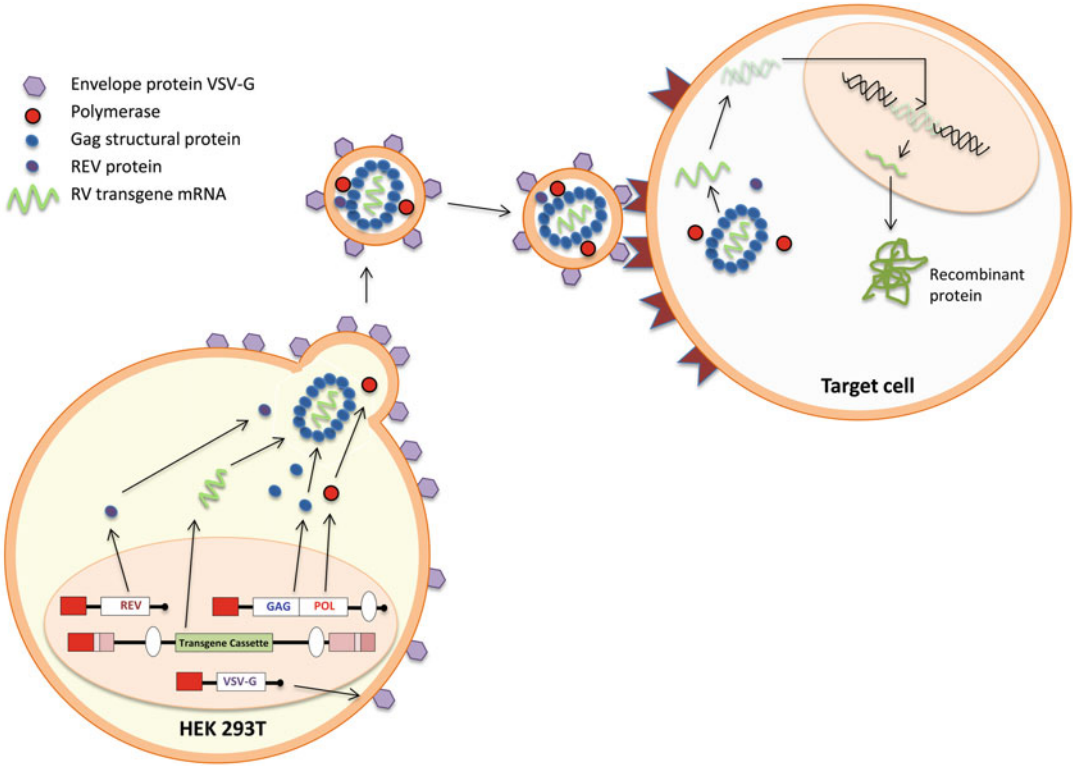

Fig. 1 Production of recombinant lentiviral particles and their infection. Left: HEK 293T helper cells are transfected with plasmids encoding helper functions and the LV vector. Upon expression of the encoded helper proteins and the LV RNA from the vector, LV particles are formed, enveloped and released from the helper cells. Right: Mediated by the expression of the specific receptor on the surface of the target cells, LV particles infect these cells. The vector RNA is converted into DNA and becomes integrated into the host cell genome allowing expression of the LV encoded gene(s)

sequences and homologies to minimize the risk for recombination that might result in rearranged viruses with novel properties. The technological improvements finally resulted in so-called third-generation packaging systems that allow both efficient and safe transduction of genes of interest [4]. Since they represent the state of the art and are used most frequently the current protocol focuses on one of these systems.

Production of recombinant lentiviral particles is usually accomplished by transfection of HEK293T cells with a set of three helper plasmids and additionally a lentiviral vector that encodes the gene(s) of interest as well as optional selection genes. The helper plasmids encode the viral gagpol, REV and envelope proteins (Fig. 1). To facilitate infection of a broad range of cells from various organisms, the G protein from vesicular stomatitis virus (VSV-G) is mostly used as viral envelope protein. By employing envelope genes from other viruses or fusion

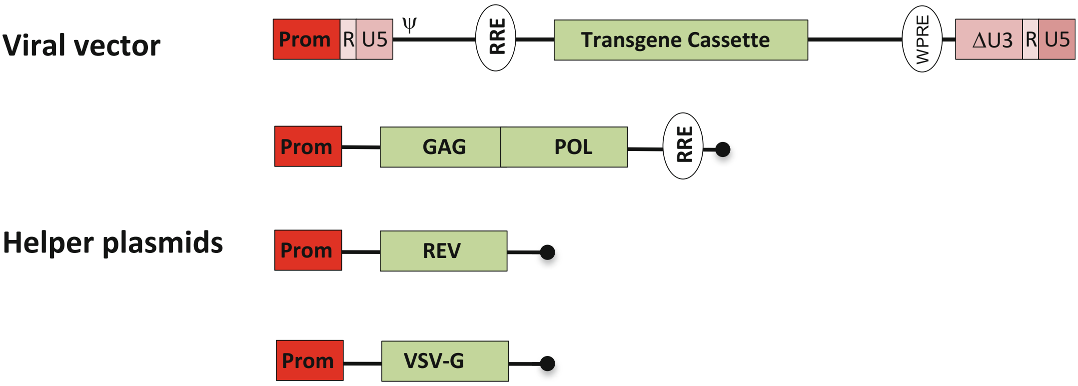

Fig. 2 Schematic of plasmids for transfection of helper cells. Symbols and abbreviations; *Ψ* packaging signal, *RRE* REV responsive element facilitating the transport of GAG-POL and vector mRNAs to the cytoplasm, *WPRE* woodchuck hepatitis virus post-transcriptional regulatory element, • poly(A) signal, *VSV-G* vesicular stomatitis virus G protein

proteins, infectious particles can be produced that provide a more restricted or even cell type specific host range [5]. The plasmids with helper functions of the third-generation systems are devoid of any viral regulatory elements (Fig. 2). As a consequence, they can only be expressed but their genes cannot become packaged by the recombinant virus. The lentiviral vectors are constructed in a way that the transcribed RNA comprises a packaging sequence that facilitates specific incorporation into virions. Cotransfection of the three helper plasmids together with the lentiviral vector ensures that the released infectious viral particles have exclusively incorporated the RNA of the vector (Fig. 1). Accordingly, only the viral vector RNA with the encoded transgene(s) of interest is packaged and transduced to the target cells. The infectious particles comprise the gagpol polyprotein encoding the enzymatic functions and allowing reverse transcription of the imported vector RNA and integration of the proviral DNA into the cellular genome. The infected cells cannot release viral particles. The genetic information for the helper functions is restricted to the HEK293T producer cells.

Various lentiviral vector systems are available that allow convenient cloning of the transgene (*see* **Note 1**). This includes vectors with a broad range of internal promoters (constitutive and inducible polymerase II and polymerase III promoters) and multicistronic vectors (IRES or 2A peptide based) that facilitate (co)expression of several genes encoding for example cDNAs, shRNAs, and/or gRNAs. Frequently, a selection marker (fluorophore or resistance marker) is encoded that facilitates enrichment of infected cells (Fig. 3, for review, *see* [1–3]).

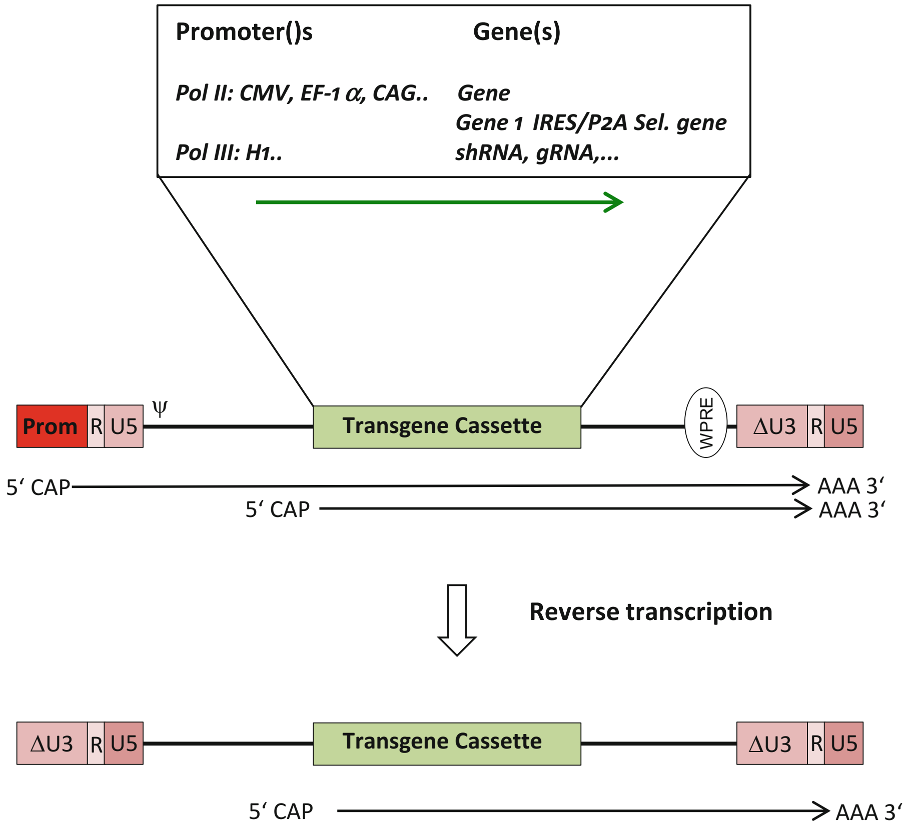

Fig. 3 Scheme of a third generation lentiviral vector and its conversion to the proviral gene. Upper: Lentiviral vector as it is transfected into helper cells. The upper size limit of the transcribed RNA is not much more than 10 kb. The transgene cassette can be varied as exemplified in the upper box. Note that transcription of this vector is driven by a nonlentiviral promoter (red). The ΔU3 element from the right LTR is not able to promote transcription (SIN). Lower: A schematic of the proviral gene is shown. Upon reverse transcription and integration into the host cell genome the defective left LTR is not able to drive transcription of the viral RNA. Instead, an internal promoter can create diverse types of RNA (see upper panel for examples). The green arrow represents the orientation of the transgene cassette. The black arrows represent proviral and transgene derived RNAs. Other symbols—*Ψ* packaging signal, *WPRE* signal sequence for strong transgene expression

Lentiviral gene transfer represents a prominent method for transduction of cells and shows a number of advantages over physical transduction methods. These include the following:

- Transduction of hard-to-transfect cells, including nonproliferating cells.
- Possibility to influence the transgene copy number and/or the percentage of infected cells by adjusting the ratio of virus/cell (multiplicity of infection, MOI, see below).
- Integration of transgene into transcriptionally open chromatin resulting in high expression levels.

The drawbacks of this method concern the elaborate production of the virus particles including the need for BSL2 conditions for production of most pseudotypes and a limited capacity of around 10 kb of packaged RNA.

2 Materials

All solutions are prepared with purified H_2O and cell culture grade chemicals.

1. DMEM: 13.63 g/L DMEM powder (containing sodium pyruvate and high glucose), 3.67 g/L $NaHCO_3$, 2.6 g/L HEPES, pH 7.2, 10% of heat-inactivated fetal calf serum (FCS), 100 μM ß-mercaptoethanol (1:500 dilution of a 50 mM stock solution), 1:100 dilution of 100x nonessential amino acids (GIBCO), 1:100 dilution of 100x GlutaMAX (Thermo Fisher), and (optionally) 1:100 dilution of a 100x stock solution of ampicillin and streptomycin (6.06 mg/mL ampicillin; 10 mg/mL streptomycin, pH 7.2) (*see* **Note 2**).
2. 1.25 M $CaCl_2$: 18.38 g $CaCl_2$ dihydrate are dissolved in 70 mL H_2O, adjusted to 100 mL with H_2O. The solution is sterile filtered through a 0.2 μm filter and stored in aliquots at −20 °C.
3. 2xHBS buffer (HEPES buffered salt solution): 280 mM NaCl (16.4 g/L), 50 mM HEPES (23.8 g/L), and 1.5 mM Na_2HPO_4 (0.4 g $Na_2HPO_4 \times 7H_2O$/L) are dissolved in H_2O. The pH is adjusted to 7.1. The solution is sterile filtered through a 0.2 μm filter and stored in aliquots at −20 °C. (*see* **Note 3**).
4. 50 mL centrifugation tubes (NUNC).
5. 125× Polybrene: 1 mg hexadimethrine-bromide (Sigma) is dissolved in 1 mL of sterile PBS.
6. 1000× chloroquine (25 mM): 0.129 g of chloroquine diphosphate salt (Sigma) is dissolved in 10 mL sterile H_2O.
7. PBS: 80 g NaCl, 2.2 g KCl, 14.3 g Na_2HPO_4, and 2 g KH_2PO_4 are dissolved in 10 L H_2O and the pH is adjusted to 7.0.
8. TEP: 6 mM EDTA, 0.1% trypsin (Sigma) in PBS.

3 Methods

The protocol described here gives rise to lentiviruses that efficiently infect human cells. This imposes certain safety issues that might be further affected by the nature of the transduced gene(s). Accordingly, appropriate measures have to be taken. Usually, BSL2 containment is required for the production of recombinant lentiviruses and infection. Infected cells that are free from viral components can be frequently classified as BSL1 organisms. For details, we wish to refer the reader to the local regulatory authorities. For a detailed discussion of the biosafety features of lentiviral vectors please refer to [4, 6, 7].

3.1 Virus Production in Helper Cells (See Note 4)

1. Use low passage number HEK293T cells to achieve optimal transfection efficiency. For expansion, cultivate HEK293T cells in a cell culture incubator at 37 °C and 5% CO_2 (*see* **Note 5**). Passage them every three to 4 days in DMEM. Take care that the cells never grow to confluence. Freeze aliquots of 10^7 cells in 5% DMSO/95% FCS per vial.
2. The frozen cells (10^7 cells) are thawed, mixed with 10 mL of DMEM, centrifuged, and resuspended in 100 mL DMEM. 10 mL of the cell suspension is plated on each of 10 dishes (10 cm).
3. After 3 days the cells are washed with 10 mL warm PBS and trypsinized with 5 mL trypsin in PBS for 2 to 5 min at 37 °C. The cell suspension is transferred in 50 mL centrifugation tubes with 10 mL of prewarmed DMEM. Residual cells are collected by washing each plate with 10 mL DMEM which is added to the 50 mL tube. Centrifuge the tubes for 10 min at 150 × *g* at room temperature.
4. The medium is removed and the cell pellets are suspended in DMEM and counted. Cells are adjusted to a density of 4×10^6 cells in 9 mL DMEM. This cell suspension is transferred into 10 cm dishes resulting in about 66,000 cells per cm^2 (*see* **Note 6**).
5. The cells are cultured in the incubator for at least 20 h allowing them to undergo at least one cell division. To achieve optimal transfection rates the cell density should be close to confluence (*see* **Note 7**).
6. Thaw an aliquot of the validated 2× HBS buffer (*see* **Note 3**).
7. At the day of transfection confirm cell density and homogenous distribution of cells. 1 h prior to transfection renew the medium (9 mL per plate) with chloroquine-containing medium. Prepare a 250 μM chloroquine solution in prewarmed DMEM. Add 1 mL of the 250 μM chloroquine solution to each cell plate (with 9 mL DMEM) to reach a chloroquine concentration of 25 μM in the cell culture plate and mix gently.
8. Transfer the plates to the incubator and incubate cells again at 37 °C and 5% CO_2 until transfection.
9. Prepare the transfection cocktail (here described for one 10 cm cell culture plate) in a 15 mL centrifugation tube. Add

the following plasmid DNAs to sterile water (Millipore quality) and adjust to a final volume of 1.6 mL: 10 μg of lentiviral vector, 10 μg gagpol encoding plasmid, 5 μg Rev. encoding plasmid, and 3 μg VSV-G encoding plasmid (*see* **Notes 8** and **9**).

10. Add 0.4 mL of 1.25 M $CaCl_2$ to the DNA mixture, mix by inverting the tube several times and incubate at room temperature for 2 min.
11. 2 mL of prewarmed 2× HBS buffer is added to this DNA solution. Without delay this mixture is repeatedly and heavily mixed by pipetting (6–7 times) followed by vortexing for 6–10 s. Coprecipitates are formed, comprising $Ca_3(PO_4)_2$ and DNA. The size of the precipitates is checked by light microscopy (*see* **Note 10**).
12. The suspension is dropwise pipetted to the 10 cm dishes and gently mixed (*see* **Note 11**).
13. 4 h after adding the DNA/$Ca_3(PO_4)_2$ coprecipitate suspension, the culture plates are checked by light microscopy for sedimentation of the precipitates onto the cell layer.
14. 4–6 h after transfection, the medium is exchanged for 10 mL of fresh DMEM (*see* **Note 12**) containing 10–15% heat-inactivated FCS (*see* **Note 13**), and the cells are cultured under standard conditions. During this incubation time, viral particles are produced.
15. 48–72 h after transfection, the particles are harvested by collecting the supernatant by centrifugation (*see* **Note 14**).

3.2 Concentration of Virus by Centrifugation

1. Transfer the virus-containing cell supernatant to a 50 mL centrifugation tube (maximally loaded with 30 mL of supernatant) and remove dead cells and cell debris by centrifugation at 150 × *g* for 10 min at room temperature.
2. The supernatant is filtered using membrane filters with 0.45 μm pores.
3. The filtrate is transferred into centrifugation tubes and centrifuged for 6 h at 16,000 × *g* at 4 °C (*see* **Note 15**).
4. After removal of the supernatant the virus pellet is resuspended in PBS or the appropriate medium (around 150 μL per 50 mL tube) and stored in aliquots at −80 °C. An aliquot (about 25 μL) is used to determine the titer (*see* **Notes 16** and **17**).

3.3 Determination of the Virus Titer by Flow Cytometry (for Lentivirus Preparations that Transfer a Fluorophore Gene)

1. Plate target cells (*see* **Note 18**) in 12-well plates at a density of 30,000–100,000 cells/well (depending on their proliferation activity) and incubate them for 24 h.
2. Determine the specific cell count per well at the time point of infection after trypsinizing the cells of control wells. Replate these cells to use them later as a negative control in flow cytometry analysis.
3. Infection is performed in the presence of polybrene to enhance the efficiency of infection (*see* **Note 19**). To this end, dilutions of viral stocks (*see* **Note 20**) are prepared in 250 μL prewarmed medium aliquots supplemented with polybrene at a concentration of 8 μg/mL.
4. Wash the cells on the 12-well plates with warm PBS and replenish the cells with 250–500 μL of prepared viral suspensions and incubate cells.
5. 8–24 h after infection, wash cells twice with warm PBS and add 1 mL of fresh medium.
6. At day 2 after infection trypsinize the cells from the wells, wash them with PBS/2% FCS to inactivate the trypsin, and transfer the cell suspensions into flow cytometry tubes.
7. Equilibrate the flow cytometer with the uninfected/mock control and determine the percentage of fluorescence positive cells in the virus infected cell samples (*see* **Note 21**). This reflects the percentage of primarily infected cells and allows calculation of the number of infectious virus particles in a given dilution.
8. For calculation of the titer (transduction units, TU) use the dilutions in which you find between 5% and 60% positive cells. Calculate the titer according to the formula

$$\mathrm{TU/mL} = \frac{\%\ \text{positive cells} \times \text{cell count at day of infection} \times \text{dilution factor of the virus}}{\text{volume of virus stock (mL)}}$$

The resulting virus titer indicates the TU number per mL of the virus stock. It is specific for the particular cell type (compare to **Note 17**).

3.4 Determination of the Virus Titer by Colony Forming Assay (for Lentivirus Preparations that Confer Antibiotic Selection)

1. Plate target cells in 6-well plates at a density of 2×10^5 cells per well (depending on their proliferation activity) and incubate them for 24 h before infection. Include 2 control wells (*see* **Note 21**).
2. Infection is performed in the presence of polybrene to enhance the efficiency of infection (*see* **Note 19**). To this end, dilutions of viral stocks (*see* **Note 20**) are prepared in 1 mL prewarmed medium supplemented with polybrene at a concentration of 8 μg/mL.

3. Wash the cells with warm PBS, replenish with the 1 mL virus dilutions and incubate overnight.
4. For calculation of the titer, determine the cell count per well at the time point of infection by trypsinization of the first control well. The second control well is incubated with DMEM and treated as the infected cells.
5. 8–24 h after transfection the medium is exchanged.
6. 2 days after infection, cells are trypsinized, suspended in medium supplemented with the appropriate selection drug (s) (*see* **Note 22**), and incubated on appropriate plates for 7–14 days. Every 3–4 days, gently aspirate the media and replace it with fresh DMEM containing the respective antibiotic.
7. After 7–14 days resistant cell clones are visible and can be counted (*see* **Note 23**) while in the mock control no living cells should be visible.
8. For calculation of the titer count the colonies from at least 2 of the dilutions. Calculate the transduction units per mL (TU/mL):

$$\frac{\text{Average of number of colonies per plate} \times \text{dilution factor of the virus}}{\text{total volume in the well (mL)}}$$

3.5 Transduction of Cells by Lentiviral Vectors

1. For transduction, seed the cells of interest in a plate or flask so that they will become confluent on day 2. On the day after seeding, add virus supernatant (or dilutions in media) and supplement with polybrene (*see* **Note 19**).
2. 8–24 h later, virus supernatant is removed and fresh medium is added and the cell population is further propagated. At this stage, the cell population will comprise infected cells with stably integrated the lentiviral vectors and also a certain fraction of noninfected cells. To enrich infected cells and eliminate noninfected cells, the cell population can be subjected to drug selection or sorting, depending on the lentiviral vector used.
3. For transduction, the multiplicity of infection (MOI) can be adjusted. The multiplicity of infection is defined as the number of infectious virus particles that is applied per target cell at the time point of infection. Depending on this ratio, the probability of infection and the average number of infection events per cell can be calculated. This allows for example to establish cell cultures in which literally all cells are infected, or cell cultures in which infected cells have a high probability to have single copy integrations.

4. The number of infected cells can be calculated by the following formula:

$$P(k) = \frac{e^{-m} \times m^k}{k!}$$

where m is the MOI and $P(k)$ is the fraction of cells infected by k virus particles.

Calculation example for the number of uninfected cells infected with an MOI 10:

$P(0) = e^{-m}$, $P(0) = e^{-10} = 4.5 \times 10^{-5}$.

This means if 10^6 cells were infected with an MOI of 10 ($10^6 \times 4.5 \times 10^{-5}$), 45 cells are not infected.

Calculation example for the number of cells infected with an MOI 10 that will have a single copy:

$P(1) = me^{-m}$, $P(1) = 10 \times e^{-10} = 4.5 \times 10^{-4}$.

This means if 10^6 cells were infected with an MOI of 10 ($10^6 \times 4.5 \times 10^{-4} = 450$), 450 cells will show single copy integration.

Calculation example for the number of multiple infected cells upon infection with an MOI 10.

$P(>1) = 1 - e^{-m}(m + 1)$, $P(>1) = 1 - e^{-10}(10 + 1) = 0.9995$

This means if 10^6 cells were infected with an MOI of 10 ($10^6 \times 0.9995 = 999{,}500$ cells) the vast majority of cells will have more than one viral copy.

Depending on the following application, infected cells can be pooled or enriched by sorting and further cultivated as bulk populations. Alternatively, clonal populations can be established by picking individual resistant colonies or sorting individual cells.

4 Notes

1. Various lentiviral vector systems as well as packaging plasmids are available (*see* Fig. 3 for examples). Please consult for example
   http://www.addgene.org/viral-vectors/lentivirus/?f=c&cmd=browse&browse=vector&v.vectype=Lentiviral&gclid=$EAIaIQobChMI2su00siH1wIVwpPtCh145w9VEAMYAyAAEgI3EPD_BwE
   http://www.clontech.com/US/Products/Viral_Transduction/$ Lentiviral_Vector_Systems/Constitutive_Promoter
   https://www.systembio.com/products/gene-expression-systems/lentiviral-vectors/
2. Depending on the laboratory standards the medium can be supplemented with antibiotics.
3. Accurate preparation of the HBS is crucial for optimal transfection efficiency, consequently, for high titers. Minor variations in

the buffer composition may have drastic effects on transfection performance. One important factor for the preparation of the HBS buffer is the exact molarity of the Na_2HPO_4 and its accurate neutralization to the pH. In particular, higher salt concentrations as a consequence of overtitration during pH adjustment should be avoided. It is highly recommended to pretest individual HBS buffer preparations with respect to the transfection efficiency. To do so, cells are seeded on a 6 well plate in 80–90% density. The next day the cells are transfected using the buffer preparations, using a fluorescence reporter plasmid that allows estimation of the amount of transfected cells 2 days after transfection. Aliquots of buffer preparations that give highest transfection efficiencies should be stored in aliquots at −20 °C.

4. This protocol describes the laboratory scale production of lentiviruses. Details concerning the upscaling are described in [3].
5. Some protocols describe the transfection at 3% CO_2 to improve $Ca_3(PO_4)_2$ precipitation resulting in higher transfection efficiencies (*see* ref. 8).
6. A homogenous distribution of cells on the plate is important. Cell patches reduce the transfection efficiency and thereby the efficiency of virus production.
7. A near-confluency cell layer before transfection is important to achieve highest transfection rates and optimal virus titers.
8. The ratio of a lentiviral vector DNA and helper packaging plasmids is important for efficient virus titer production.
9. For transformation of lentiviral plasmids bacterial strains should be used with reduced recombination activity to avoid recombination of repetitive vector elements (e.g., the long terminal repeats). Recombination can be frequently reduced by expanding the transformed bacteria at 30 °C. To enhance transfection efficiency, the plasmid DNA should be free of endotoxins. To this end, appropriate kits should be used (e.g., the endotoxin-free maxiprep (Qiagen, cat no. 12632)).
10. The size of the formed precipitation complexes should be small since they will be taken up more efficiently than the bigger aggregates.
11. Take care that the 2× HBS buffer is immediately mixed with the $CaCl_2$/DNA solutions, in particular in case you prepare a series of transfection mixes.
12. If 5–8 mL medium is used the final virus titer will be increased.
13. FCS at a concentration of 15% is recommended if virus production is maintained for 72 h instead of 48 h.

14. Usually, the virus is harvested 48 h posttransfection. However, a second harvest can be performed at 72 h. Later, due to the toxicity of the VSV-G protein, cells are lysed.
15. Note that the centrifugation time can be reduced by increasing the speed. For example ultracentrifugation at 70,000 × *g* reduces the time to 2 h (*see* ref. 8).
16. Freezing aliquots of virus preparations is recommended to avoid any loss in titer associated with repeated freeze–thaw of virus stocks.
17. Titers of lentiviral vectors depend on the lentiviral vector backbone, the transfection efficiency, the nature of the transduced genes, and the target cells. At optimal conditions, titers up to 10^7 IU/mL can be achieved, higher titers require concentration of virus supernatants.
18. Titrations need to be performed on the target cells of choice using the appropriate media.
19. Polybrene is a polycation that is added to neutralize the negative charges of cell membranes and viral particles. Alternatively, protamine sulfate (8–10 μg/mL) can be used. Not all cells tolerate the suggested concentrations. In this case, the optimal concentration of these compounds should be predetermined.
20. The dilutions of the virus should be defined according to the needs. E.g., to achieve high probabilities of cells with single-copy transduction, low MOI ($\text{MOI} < 0.01$) must be used. Higher MOI will increase the number of cells with elevated copy numbers which may increase the expression level per cell. Typical viral dilutions are in the range of 1:10–1:10,000.
21. Since polybrene or protamine sulfate treatment can influence the cells and their morphology it is important to treat the control cells as well with these compounds, in particular for flow cytometry titrations.
22. Before beginning a colony formation assay, the dose of antibiotic required to kill the nontransduced cells needs to be empirically determined for each cell line/type.
23. To visualize the colonies, culture plates are washed with PBS and incubated with 1 mL of a 0.1% crystal violet solution for 10 min at room temperature. After the incubation time the cells are extensively washed with H_2O.

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Chapter 5

Inducible Protein Production in 293 Cells Using the *piggyBac* Transposon System

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Abstract

Recombinant proteins are widely used to study various pathophysiological processes. Nevertheless, the establishment of the desired protein-producing stable mammalian cell lines using traditional methods is hampered by multiple laborious steps. In this chapter, we describe a simple and robust system that allows for the derivation of stable transgenic cell lines in 293 cells, yielding high protein expression levels, in a short time period. This methodology is based on the *piggyBac* transposon system and, notably, it allows for inducible production of the protein of interest. Moreover, it can easily be used in conventional laboratory cell culture settings and does not require any specialized devices. Herein, we outline all the steps of this procedure in detail and point out specific considerations.

Key words Protein production, Antibodies, Biologics, *piggyBac*, Tetracycline-inducible, 293 cells

1 Introduction

Production of recombinant proteins and biologics, such as antibodies, can facilitate our efforts to understand the role of proteins in human physiology and in various diseases. Depending on the nature of the protein, different heterologous expression systems could be utilized for protein production, including bacteria (*E. coli*), yeast (*S. cerevisiae* and *P. pastoris*), insects (*S. frugiperda*), and mammalian cells [1, 2]. The latter are favored for mammalian proteins, since they are produced in their native environment, and therefore, maintain correct folding, post-translational modifications, and function [3]. Over the years, different mammalian cell lines, such as mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK) 293, and Chinese hamster ovary (CHO) cells have been utilized to produce mammalian proteins, with the latter two being the most widely used.

Traditional methods for the establishment of mammalian cell lines expressing a high yield of recombinant proteins present some major drawbacks. After transfection of the cell line of choice with

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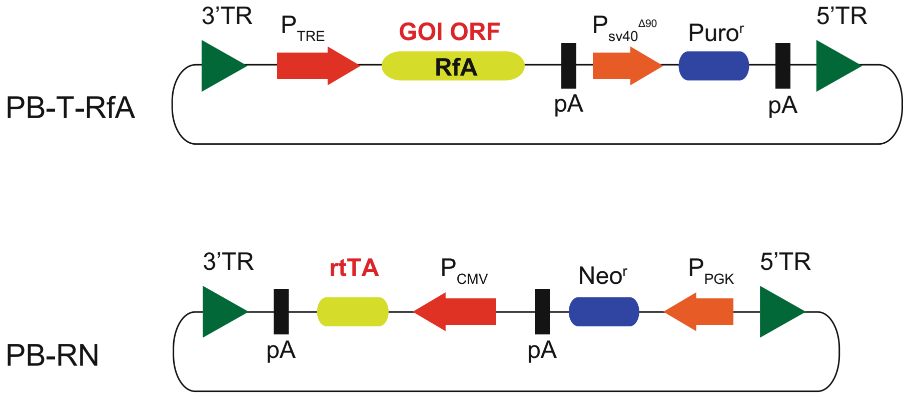

Fig. 1 *piggyBac* transposon vectors. (**a**) PB-T-RfA transposon allows for cloning of the gene of interest (GOI) under the control of the tetracycline promoter P_{TRE}. The gene encoding for the puromycin resistance (Puro^r) marker under the control of attenuated SV40 promoter allows for selection of stable cell lines. The plasmid contains an ampicillin resistance marker. (**b**) PB-RN transposon encodes for the reverse tetracycline transactivator (rTA) under the control of CMV promoter. The gene encoding for the neomycin resistance marker (Neo^r) under the control of PGK promoter allows for selection of stable cell lines. The plasmid contains an ampicillin resistance marker

the plasmid encoding for the gene of interest (GOI) and initial selection of stable cell lines, additional steps, such as gene amplification and screening of single clones for identification of those with high expression, results in a laborious process, lasting up to 12 months [1].

To circumvent the limitations mentioned above, we and others have utilized various transposon systems that allow for faster and simplified establishment of mammalian cell lines for protein production [4, 5]. DNA transposons (Class II) are composed of a transposase gene flanked by two terminal repeats (TRs) (Fig. 1). The transposase has the ability to recognize the TRs, "cut" the DNA sequence between them, and then "paste" it into a new genomic location. For protein production, the transposase and transposon are used in trans, meaning that they are located on two different plasmids (Fig. 1). In this arrangement, the sequence in the transposon between the TRs encodes the GOI, and once it is "cut" from the transposon, it is inserted ("pasted") in the genome of the desired cell line used. This "cut and paste" mechanism makes the process of GOI integration in mammalian cells very efficient and enhances the derivation of stable cells lines compared to traditional methods using plasmids. Furthermore, the integration of single copies of the transposon into various locations of the genome increases the possibility of integration in a genomic location that permits gene expression, and more importantly, that is not subject to "repeat induced gene silencing", a common problem that arises during plasmid transfection methods whereby genes inserted as concatamers are prone to silencing.

Three transposon systems, *Tol2*, *Sleeping Beauty* (SB), and *piggyBac* (PB), have been utilized for protein production in mammalian cells. The PB transposon is more efficient than the *Tol2* and SB [6, 7], and it can accommodate larger cargo, up to 14 kb, without significantly affecting its efficiency [8]. In addition, with the PB there is no overproduction inhibition due to high transposase production, a phenomenon that can limit SB efficiency [9]. For the aforementioned reasons, we use the PB transposon system for establishment of stable cell lines for protein production [4].

Finally, conventional promoters utilized for production in mammalian cells allow for constitutive expression of the GOI and are therefore problematic since clones with high protein expression tend to proliferate slower compared to those with low expression and are, as a result, selected against those with low expression. To avoid the expression of the protein during the establishment and expansion of the stable cell lines, we use the tetracycline inducible expression system, which allows for very high expression [10].

The method described here has been previously used to produce various recombinant proteins, such as rhodopsin [11], as well as biologics, such as VEGF-trap and anti-HER2 Ab [4, 12]. Herein, as an example, we describe the production of the biosimilar antibody B20S, which neutralizes the angiogenic activity of VEGF [13, 14].

2 Materials

2.1 DNA Cloning

1. Plasmid pDONR221 (ThermoFisher).
2. *piggyBac* plasmid PB-T-RfA [4].
3. Takara PrimeSTAR™ HS DNA Polymerase (Clontech).
4. SyberSafe.
5. Agarose.
6. TAE buffer.
7. TE buffer, pH 8.0.
8. Gateway BP Clonase II Enzyme Mix (ThermoFisher).
9. Gateway LR Clonase II Enzyme Mix (ThermoFisher).
10. Subcloning Efficiency DH5α Competent Cells.
11. One Shot TOP10 Chemically Competent *E. coli* (ThermoFisher).
12. One Shot ccdB Survival 2 T1^R Competent Cells (ThermoFisher).
13. Plasmid isolation Miniprep and Maxiprep kits.

2.2 Establishment of Stable Cell Lines

1. Plasmid PBase (pCyL43; *see* **Note 1**).
2. *piggyBac* plasmid PB-RN [4].
3. *piggyBac* plasmid(s) PB-T-GOI [4].
4. Freestyle 293 F cells (ThermoFisher).
5. Freestyle medium (ThermoFisher).
6. OptiPRO SFM (ThermoFisher).
7. Freestyle MAX reagent (ThermoFisher).
8. Geneticin.
9. Puromycin.
10. Disposable Polycarbonate Erlenmeyer Flasks with vent cap; 125 mL.
11. Disposable Polycarbonate Erlenmeyer Flasks with vent cap; 1000 mL.
12. Falcon tubes (50 mL).
13. Conventional cell culture incubator allowing for 8% CO_2.
14. MAXQ2000 CO_2 resistant shaker (ThermoFisher).

2.3 Protein production

1. Doxycycline hyclate (powder). Doxycycline is solubilized in H_2O at final stock concentration of 10 mg/mL. It is sterilized through filtration by a 0.2 nm filter.
2. Aprotinin from bovine lung (lyophilized powder 3-8TIU/mg solid). Aprotinin is solubilized in H_2O at final stock concentration of 10 mg/mL. It is sterilized through filtration by a 0.2 nm filter.

3 Methods

3.1 DNA Cloning

3.1.1 cDNA Cloning in the pDONR221 Vector

In order to clone the cDNA encoding for the desired protein in the pDONR221 vector, the attB1 and attB2 sites should be included in the forward and reverse primer, respectively. In addition, a Kozak sequence is required before the start codon.

Primer Design

1. Fw primer: 5'–GGGGACAAGTTTGTACAAAAAAGCAGGCT**GCCACC***ATG*+~18bp–3'
   The AttB1 site is underlined, and the Kozak sequence is highlighted with bold font. The start codon, ATG, is shown in *italics*. Around 18 bp from the 5′ of the cDNA are added at the end of the primer. The annealing temperature of this fragment should be between 55–60 °C.
2. Rev. primer: 5′ – GGGGACCACTTTGTACAAGAAAGCTGGGT*TCA*+~18bp – 3'
   The AttB2 site is underlined. The reverse of the stop codon, TGA, is shown in *italics*. Around 18 bp from the 3′ of the

cDNA are added at the end of the primer in a reverse complementary orientation. The annealing temperature of this fragment should be between 55–60 °C.

PCR Amplification

1. It is recommended to set up a 50 μL reaction and use 2-step PCR cycling conditions for gene amplification. The conditions below apply to the Takara Prime Star DNA Polymerase.
    - 5× Buffer: 10.0 μL.
    - Fwd Primer: 1.0 μL (final concentration 0.2 μM).
    - Rev Primer: 1.0 μL (final concentration 0.2 μM).
    - dNTPs: 4.0 μL (final concentration 0.2 mM).
    - Template: 1.0 μL (~1 ng for plasmid).
    - Polymerase: 0.5 μL.
    - H_2O: Up to 50 μL.
2. Cycling conditions are listed below:
    - Step 1: Denaturation: 95 °C 60 s.
    - Annealing: Tm-5 °C 5 s.
    - Extension: 72 °C 1 min/Kb.
    - Repeat 4 additional cycles for a total of 5 cycles.
    - Step 2: Den./Anneal.: 68 °C 5 s.
    - Extension: 72 °C 1 min/Kb.
    - Repeat 29 more cycles for a total of 30 cycles.
    - Step 3: Go to: 4 °C.

Gel Purification

1. Prepare 1% agarose gel with SyberSafe.
2. Add 10 μL of 6×DNA loading dye in each PCR reaction and load 50 μL.
3. Run the gel in fresh TAE buffer at 100 V for 30 min. A sharp single band is expected corresponding to the length of the amplicon.
4. Cut the band using a clean scalpel under a long-wave UV.
5. Extract the DNA, and elute in 30 μL of TE buffer, pH 8.0 (*see* **Note 2**).
6. Using a spectrophotometer, measure the absorbance at 260 and 280 nm to determine the purity and concentration of the DNA. The 260/280 ratio should be above 1.8, and the concentration at least 10 ng/μL.

BP Cloning

1. Set up the reaction below in a 1.5 mL Eppendorf tube, and incubate at room temperature for 2 h.
    - pDONR221: 150 ng.

- purified PCR product: 100–150 ng.
- BP clonase: 2 μL.
- TE buffer: Up to 10 μL.

2. At the end of the incubation terminate the reaction with 1 μL of Proteinase K (*see* **Note 3**).
3. Use 2–4 μL of the reaction for transformation in DH5a cells, and plate them in LB agar plates with 50 μg/mL kanamycin.
4. The next day pick and inoculate 4–8 resistant colonies in 4 mL of LB medium with 50 μg/mL kanamycin (overnight: up to 16 h, at 37 °C).
5. Use 3 mL of the culture to isolate the plasmid, pENTRY-GOI, using a plasmid isolation kit, and store the remainder of the solution at 4 °C for preparation of glycerol stocks.
6. Proceed with screening of the colonies with enzyme digestion. BsrGI could be used to screen the colonies, since each of the generated attL recombination sites contains a recognition site for it.
7. Sequencing of the correct plasmids to verify the insert is highly recommended.

3.1.2 Subcloning in PB-T-RfA Vectors: LR Cloning

1. Set up the reaction below and let it incubate at room temperature for at least 2 h.
   - PB-T-RfA: 150 ng.
   - pENTRY-GOI: 100–150 ng.
   - LR clonase: 2 μL.
   - TE buffer: Up to 10 μL.
2. Terminate the reaction with 1 μL of Proteinase K.
3. Use 2–4 μL of the reaction for transformation in TOP10 cells, and plate them in LB agar plates with 100 μg/mL ampicillin.
4. The following day pick and inoculate 6–12 resistant colonies in 4 mL of LB medium with 100 μg/mL ampicillin.
5. The following day use 3 mL of the culture for isolation of plasmid using a plasmid isolation kit.
6. Store the remaining 1 mL at 4 °C for preparation of glycerol stocks and to set up maxiprep.
7. Proceed with screening the colonies using enzyme digestion.
8. Use 0.5 mL from the correct clones to set up bacteria cultures for plasmid maxipreps (*see* **Note 4**).

3.2 Establishment of Stable Cell Lines

3.2.1 Thawing 293-F Cells

1. Thaw one vial of 293-F cells in a 125 mL flask with 35 mL of medium (*see* **Note 5**) (Fig. 2a).
2. Culture the cells on a shaker (125 rpm) in a regular cell culture incubator (37 °C, 8% CO_2).
3. The following day change the medium. Transfer the cells in a 50 mL falcon tube, and centrifuge at 110 × *g* for 5 min.
4. Discard the medium and resuspend the cells in fresh medium. Passage the cells 2–3 times before transfection (*see* **Note 6**).

3.2.2 Transfection

1. Split the cells the day before the transfection (*see* **Note 6**). The recommended cell density is 0.8–1 × 10^6 cells/mL.
2. The day of the transfection, the cell density should be ~ 1–1.2 × 10^6 cells/mL, and cell viability above 95%.
3. Prepare the DNA plasmid according to the quantities shown in Table 1 (*see* **Notes** 7 and **8**).

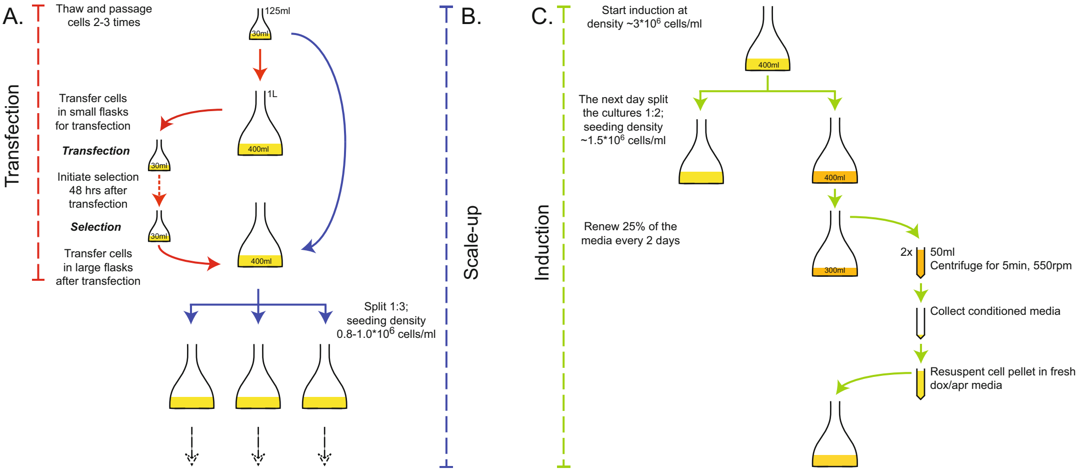

Fig. 2 Outline of the three main steps of the protocol: (**a**) transfection of 293 cells for establishment of stable cell lines, (**b**) scale-up of stable cell-lines, and (**c**) induction of protein production

Table 1
DNA quantities for transfection

	One transgene	Two transgenes	Antibodies (HC-LC: 1–2)
PBase	5 μg	5 μg	5 μg
PB-RN	5 μg	3 μg	3 μg
PB-T-RfA-A (transgene A)	25 μg	16 μg	12 μg (heavy chain)
PB-T-RfA-B (transgene B)	/	16 μg	24 μg (light chain)
Total DNA	35 μg	40 μg	44 μg

4. Dilute the plasmid DNA in an Eppendorf tube with OptiPRO SFM medium to a final volume of 0.6 mL, and mix gently.
5. In a separate Eppendorf tube, dilute the equivalent amount of MAX reagent (e.g., 35–44 μL) in OptiPRO SFM medium to a final volume of 0.6 mL, and mix gently.
6. Transfer the components of the latter Eppendorf tube (step 5) into the former (step 4), and mix by inverting the tube 10–12 times.
7. Incubate at room temperate for 15–20 min (*see* **Note 9**).
8. Add 1.2 mL of mix to the cell culture flask dropwise while swirling.

3.2.3 Selection for Stable Transfectants

1. Selection can be initiated 48 h after the transfection (Fig. 2a). Centrifuge the cells and resuspend in fresh medium containing 500 ng/mL G418/Geneticin.
2. The selection period lasts 2 to 3 weeks. During this period, the cells are centrifuged at 60 × *g* for 5 min, 2–3 times per week and resuspended in fresh medium (*see* **Note 10**).
3. The selection is considered successful once the number of viable cells is above 2 × 10^6 cells/mL, and cell viability is above 80%. Once the selection with G418 is complete, transfer the 35 mL of cells to a large 1 L flask, and add fresh medium without Geneticin to a final volume of 400 mL.
4. The speed of the shaker for 1 L flasks should be ~110 rpm.

   Change the medium every 2–3 days, until the cells reach a density of 2–3 × 10^6 cells/mL (*see* **Note 11**).
5. To ensure the presence of PB-T-RfA, selection for puromycin (1 ng/mL) can be initiated when the cells reach 1 × 10^6 cells/mL, and viability ~95%.
6. The selection is maintained for 7 days (*see* **Note 12**).

3.2.4 Cell Freezing

1. It is recommended to freeze 10 × 10^6 cells per cryovial in 1 mL of freezing medium (regular medium containing 10% DMSO). To freeze the cells, remove the required number of cells and transfer them to 50 mL falcon tubes.
2. Centrifuge the cells at 110 × *g* for 5 min, and subsequently resuspend them with the required amount of freezing medium.
3. Transfer 1 mL of the mixture per cryovial, and place in a styrofoam box (or similar container).
4. Store the box in the −80 °C freezer overnight.
5. The next day transfer the vials to liquid N_2 (*see* **Note 13**).

3.3 Scale-Up and Protein Production

3.3.1 Scale-Up (~Days 8–15)

1. Thaw the cells as described in Subheading 3.2.1, and culture until the cells reach a density of 2–2.5 × 10^6 cells/mL (Fig. 2b).
2. Transfer the cells from the small flask to a large 1 L flask, and add 370 mL of medium to reach a final volume of 400 mL.
3. Culture the cells on a shaker (110 rpm) in a regular cell culture incubator (37 °C, 8% CO_2).
4. Change half of the medium every 2–3 days, until the cells reach a density of 3 × 10^6 cells/mL. Once the cells reach density of 3 × 10^6 cells/mL, split the cells 1:3 and transfer to 1 L flasks. This process is to be continued until half the number of desired flasks for protein production is reached (*see* **Note 14**).

3.3.2 Induction (~Days 15–22)

1. On the last day during the scale-up period, add DOXY (2 μg/mL) and aprotinin (1 μg/mL) to the cells (Fig. 2c).
2. The following day, split the cells 1:2 using medium containing DOXY/aprotinin. Thus, ~1.5 × 10^6 cells/mL would be the starting concentration for protein production. From this point, for optimal protein production, it is recommended to change one quarter of the medium every other day (e.g., days 2, 4, and 6 postsplitting).
3. Remove 100 mL of cells from each 1 L-flask, and transfer in two 50 mL falcon tubes. Centrifuge the cells at 60 × *g* for 5 min. If the protein is secreted, such as in the case of antibodies, collect the supernatant and store it at 4 °C.
4. Resuspend the pellet of cells in 100 mL of media containing DOXY (2 μg/mL) and aprotinin (1 μg/mL). Return the cells to the original flask.
5. The density and survival of cells should be monitored during this period each time the cells are split (*see* **Note 15**).
6. At the end of induction, collect the supernatant or the cell pellet by centrifuging at 110 × *g* for 5 min.

3.4 Assessment of Protein Production Levels

1. Supernatant from either the initial small flasks or during the scale-up (1 L flasks) could be used in order to assess the levels of recombinant protein production using SDS-PAGE. In the case of Abs, using a HC:LC ratio of 1:2 leads to higher production than with a 1:1 ratio (Fig. 3a). Furthermore, there is very small variation in protein production between flasks during the scale-up phase (Fig. 3b). In the case of the biosimilar antibody B20S, the average yield after purification is ~300 mg/L.

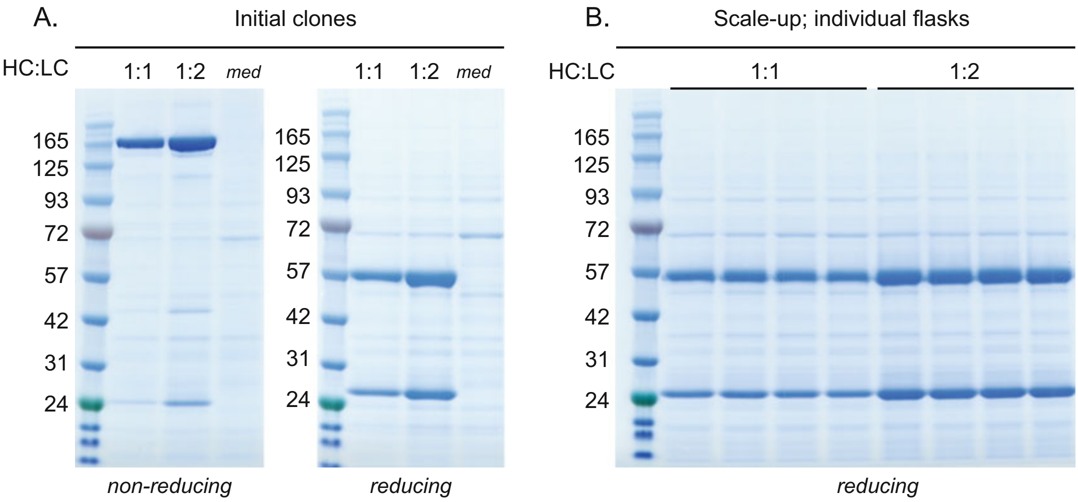

Fig. 3 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of conditioned medium from production of B20S Ab. (**a**) Samples were collected from small flasks induced for 48 h with doxycycline: 20 μL were loaded in each lane. Two separate conditions using different ratios of heavy to light chain genes are shown. *med* indicates conditioned medium from nontransfected 293 cells. (**b**) Medium samples were collected from 4 large individual flasks induced for 48 h with doxycycline: 20 μL were loaded in each lane

4 Notes

1. The plasmid pCyL43 encoding for the transposase can be obtained from the Welcome Trust Sanger Institute (http://www.sanger.ac.uk/form/Sanger_CloneRequests).
2. It is critical to use TE buffer. Eluting with H_2O will dramatically reduce the efficiency of the BP reaction.
3. For very large inserts, remove 5 μL from the BP reaction and use 0.5 μL of Proteinase K to stop the reaction. Use this part of the BP reaction to proceed with bacterial transformation, and leave the rest of the reaction overnight at RT. The latter part of the BP reaction can be used in case of failure of the initial transformation.
4. For very large inserts, it is recommended to incubate the bacteria at 30 °C, to avoid any possible recombination.
5. It is not necessary to centrifuge the cells to remove the DMSO. Quickly thaw the cryovial in a 37 °C water bath, spray with 70% EtOH, and thaw the cells by adding 1 mL of medium to the cryovial using a Pasteur pipette. Then transfer the cells to a small flask containing medium. Always prewarm the medium to room temperature.
6. The maximum cell density should not be above a density of 2–2.5 × 10^6 cells/mL, and the viability should remain above 95%. Passage the cells by splitting them 1:3. This can be done

by transferring 10 mL of cells into a new flask and adding 25 mL of fresh medium. In general, when less than 30% of the cultured cells are used to achieve the desired cell density, it is not necessary to centrifuge the cells and completely change the medium.

7. A high quality of plasmid is required, thus it is recommended that the plasmid is prepared using a MAXI prep or a similar method.
8. The 1:5 ratio between PB-RN and PB-T-RfA is absolutely necessary. This increases the possibility of double, i.e., PB-RN and PB-T-RfA, (or triple) transfectants after selection with Geneticin.
9. Longer incubations are not required and will not increase the transfection efficiency.
10. Using low speed for centrifugation allows for the removal of the dead cells and debris and enrichment of the stable/transfected cells. This process is slow, and the enrichment for stably transfected cells becomes apparent after 10–12 days. To make sure that the stably transfected cells are not discarded during this process, it is better to check for viable cells in the supernatant after the centrifugation. If viable cells are present, then the centrifugation speed must be increased. It is unavoidable to have some dead cells and debris remaining in the pellet after centrifugation.
11. During this phase, it is necessary to change only 50% of medium every 2–3 days. For this purpose, remove 200 mL of supernatant from the flask, and use four 50 mL falcon tubes to centrifuge the cells at 110 × *g* for 5 min. Resuspend the cells in fresh medium, and return to the same flask. The cell viability should be ~95%.
12. In our experience, very few cells die during the selection with puromycin, which is indicative of the fact that the cells that are stably transfected with PB-RN are also stably transfected with PB-T-RfA.
13. The cryovials can be kept in the −80 °C freezer for up to 1 month without affecting cell survival upon thawing. However, it is recommended to transfer them to liquid N_2 as soon as possible.
14. For example, if 18 large flasks are required for protein production, it is recommended to twice split the cells 1:3, which will leave nine flasks.
15. The cells could reach a density of 4–4.5 × 10^6 cells/mL. The viability should remain above 70% for secreted proteins. In the case of production of intracellular proteins, higher viability is desired (e.g., above 85%).

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Chapter 6

Recombinant CHO Cell Pool Generation Using piggyBac Transposon System

Sowmya Balasubramanian

Abstract

CHO cell pools with desirable characteristics of high titer and consistent product quality are useful for rapid production of recombinant proteins. Here we describe the generation of CHO cell pools using the piggyBac transposon system for mediating gene integration. The method describes the co-transfection of cells with the donor plasmid (coding for the gene of interest) and the helper plasmid (coding for the transposase) using polyethyleneimine (PEI). This is followed by a genetic selection for the generation of a cell pool. The resulting cell pool can be used to start a batch or fed-batch culture. Alternatively it can be used for generation of clonal cell lines or generation of cell banks for future use.

Key words CHO cells, Transposon system, Transfection, Orbital shaking, Recombinant protein

1 Introduction

Clonally derived Chinese hamster ovary (CHO) cell lines, generated by stable gene expression, are predominantly used for manufacturing of recombinant therapeutic proteins [1–3]. It is typically a time consuming process requiring approximately 6 months for the generation, characterization, and selection of a CHO cell line. Alternatively, CHO pools, the population of cells resulting from transfection and genetic selection, can be generated in 2–3 weeks. Due to the heterogeneous nature of a CHO cell pool, both genetically and phenotypically, they have historically only been used for rapid generation of material for nonclinical research [4, 5]. Due to the rapid nature of the process, CHO cell pools are an attractive approach to recombinant protein production for good laboratory practice toxicology studies or, potentially, first-in-human studies, if they can exhibit the desired attributes of high titers and consistent and representative product quality [6–10]. Targeted or semitargeted gene integration strategies have been used to obtain such high-expressing CHO pools with representative product quality [8–10].

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Transposon systems have been shown to be reliable tools for generating recombinant cells yielding high protein titers in a short time period [11–13]. Several class II transposons have been modified for catalyzing gene integration in mammalian cells [12]. The transposase enzyme recognizes a unique pair of inverted terminal repeats (ITRs) flanking the expression cassette, excising it from the plasmid DNA and inserting it into the genomic DNA of the cells. The transposases favor integration in or near actively transcribed genes [14]. The most widely used transposon for recombinant protein production is piggyBac (PB) [4, 5, 11, 13, 15]. PB-mediated gene integration has been reported to generate highly productive CHO pools with consistent product quality attributes when scaled up in bioreactors [13]. Other transposon systems that have been used for recombinant antibody production in CHO cell lines include Sleeping Beauty and Tol2 [12].

Here, I describe a method for the generation of CHO cell pools for recombinant protein production using the PB transposon system to catalyze gene integration. I describe the process of PEI-mediated transfection, genetic selection using puromycin, and a fed-batch production process. In my experience PB-mediated CHO pool generation is a very robust process allowing a simple “plug and play” application of the technology with an existing CHO platform (i.e., cell line, transfection method, and media package) with a few modifications. I also discuss the optimizations that are recommended when adapting the protocol to a different CHO platform.

2 Materials

2.1 Cell Culture

1. CHO-DG44 cells adapted to cultivation in serum-free suspension.
2. Cylindrical and square-shaped glass bottles with nominal volumes of 100 mL to 5 L (Schott Glass, Mainz, Germany).
3. TubeSpin bioreactor 50 tubes (TPP, Trasadingen, Switzerland).
4. ProCHO5 medium (Lonza AG, Verviers, Belgium) without L-glutamine, hypoxanthine, thymidine, and phenol red.
5. 50× L-glutamine and phenol red solution: 200 mM glutamine and 250 μg/mL phenol red in water. Dissolve 29.23 g glutamine and 250 mg phenol red in 800 mL water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration through a 0.2 μm bottle-top filter with a neck size of 45 mm. The solution is transferred into sterile 50 mL centrifuge tubes and kept frozen at −20 °C. For each liter of ProCHO5 medium, 20 mL of the stock solution is added.

6. 50× HT solution: 5 mM hypoxanthine and 0.8 mM thymidine in water. Dissolve 680 mg of hypoxanthine and 194 mg of thymidine in 800 mL of water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration and frozen as aliquots as explained in the previous step. For each liter of ProCHO5 medium, 20 mL of the stock solution is added.
7. Inverted phase contrast microscope (100× magnifications, Telaval 31, Carl Zeiss AG, Feldbach, Switzerland).
8. Standard tabletop centrifuge (Labofuge 200, Heraeus AG).
9. Incubator shaker (model ISF-4-W with a rotational diameter of 50 mm; Kühner AG, Birsfelden, Switzerland).
10. 0.4% trypan blue solution.

2.2 Plasmids

1. The donor vector (pMP-PB-TNFR:Fc) carrying two PB ITRs flanking two expression cassettes, one coding for the ectodomain of the human tumor necrosis factor α receptor 2 fused to a human Fc domain (TNFR-Fc) under the control of the mouse cytomegalovirus major immediate early promoter (mCMV) and the puromycin resistance gene (puromycin *N*-acetyl-transferase—*pac*) under the control of the herpes simplex virus thymidine kinase promoter (HSV-tk) (*see* **Notes 1–3**) (Fig. 1a).
2. The helper vector (pmPBase) coding for a codon-optimized variant of the PB transposase gene under the control of the human CMV (hCMV) immediate early promoter (*see* **Note 4**) (Fig. 1b).
3. Chemically competent *E. coli* DH5α cells.
4. LB medium with 100 μg/mL ampicillin.
5. LB agar plates with 100 μg/mL ampicillin.
6. Based on experience, any one of the following commercial plasmid DNA purification kits can be used for generating transfection grade plasmid DNA.
    (a) NucleoBond Kits (Macherey-Nagel, Düren, Germany).
    (b) Qiagen Plasmid Kits (Qiagen, USA).
    (c) PureYield™ Kits (Promega, USA).

2.3 Transfection and Genetic Selection

1. Linear 25 kDa polyethyleneimine (PEI) (Polysciences, Eppenheim, Germany) solution: 1 mg/mL PEI, pH 7. Dissolve 1 g of PEI in 800 mL water. As the PEI dissolves, lower the pH with 1 N HCl so that the solution is acidic. When the entire PEI is in solution, the pH is increased to 7 with 1 N NaOH. The volume is then adjusted to 1 L by further addition of water. The solution is filter sterilized, aliquoted into sterile 50 mL tubes,

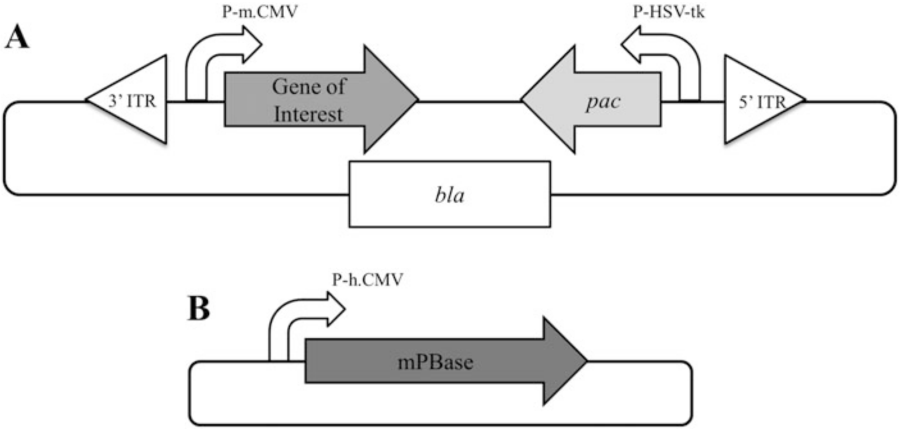

Fig. 1 Representation of the maps of donor and helper vectors. (**a**) The gene of interest was placed under the control of the mCMV promoter and the selection marker, puromycin resistance gene, was placed under the control of the HSV-tk promoter. The cassette expressing the selection marker was cloned in the opposite orientation of that of the gene of interest to avoid influence of the mCMV promotor on the selection marker gene. (**b**) The gene coding for the PB transposase enzyme (mPBase) was driven by the human CMV promoter. *P-mCMV* mouse cytomegalovirus major immediate early promoter, *P-HSV-tk* herpes simplex virus thymidine kinase promoter, *ITR* inverted terminal repeat, *bla* beta-lactamase gene, *pac* puromycin N-acetyl-transferase

and stored at −20 °C. It can be stored frozen for years as long as repeated freeze–thaw cycles are avoided (*see* **Notes 5** and **6**).

2. ProCHO5 medium without L-glutamine, hypoxanthine, thymidine, and phenol red (*see* Subheading 2.1).
3. 50× L-glutamine–phenol red solution (*see* Subheading 2.1).
4. 50× HT solution (*see* Subheading 2.1).
5. Puromycin solution: 10 mg/mL puromycin in water. Dissolve 100 mg puromycin in 10 mL water and sterilize by filtration using a 0.22 μM filter. Store at −20 °C until ready for use.
6. 50× Glucose solution: 150 g/L D-(+)-glucose in water. Dissolve 150 g D-(+)-glucose in 1 L water and sterilize by filtration using a 0.22 μM filter and store as aliquots in 50 mL centrifuge tubes.
7. MEM amino acids solution (50×) (Sigma-Aldrich) and MEM nonessential amino acids solution (100×) (Sigma-Aldrich).

3 Methods

3.1 Plasmid Purification

1. *E. coli* strain DH5α is transformed with pDNA by the standard $CaCl_2$ method and spread onto LB agar plates with 100 μg/mL ampicillin (*see* **Note 7**).
2. Incubate the plates overnight (16 h) at 37 °C.

3. With a sterile loop or a pipette tip, transfer a single colony from the transformed plate to a sterile round-bottom, polypropylene 14 mL culture tube containing 3 mL LB broth with 100 μg/mL ampicillin.
4. Incubate at 37 °C for 4–6 h with agitation at 220 rpm.
5. Use this starter culture to inoculate appropriate volume of LB depending on the scale of plasmid preparation as per kit manufacturer's protocol.
6. Incubate the culture for 12–16 h at 37 °C with agitation at 220 rpm.
7. Follow manufacturer's protocol for purification of plasmid DNA from this culture.

3.2 Routine Cell Culture

1. CHO-DG44 cells are subcultivated every 3–4 days (*see* **Note 8**) by inoculation in 100 mL ProCHO5 medium containing L-glutamine, hypoxanthine, thymidine, and phenol red (*see* **Note 9**) in a 250 mL square-shaped glass bottle at an initial cell density of 0.3×10^6 cells/mL.
2. Determine the cell density and viability by trypan blue staining using a Neubauer hemocytometer or any other suitable method.
3. After cell counting, transfer 3×10^7 cells into a 50 mL centrifuge tube and centrifuge at 500 × *g* for 3 min in a standard tabletop centrifuge.
4. Remove medium by aspirating or decanting. The cell pellet is resuspended in 10 mL of ProCHO5 medium with additives (*see* **step 1**) and transferred to a 250 mL square-shaped bottle containing 90 mL of prewarmed ProCHO5 medium with additives. This will result in a culture at 0.3×10^6 cells/mL.
5. Attach the bottle to a platform mounted on an orbital shaker platform using double-sided adhesive transfer tape and agitate at 110 rpm at 37 °C in a 5% CO_2 atmosphere without humidity. Preferably, vented bottle caps should be used. Alternatively, keep the cap of the bottle opened about one quarter of a turn.

3.3 Transfection

1. One day prior to transfection, the cells are inoculated in ProCHO5 with additives at a density of 2×10^6 cells/mL and grown overnight with agitation by orbital shaking as described (*see* **Note 10**).
2. On the day of the transfection, the cells are counted as described in Subheading 3.2.
3. Below is an example of one transfection at a volume of 5 mL, but the procedure is applicable to any desired small-scale transfection volume (5–20 mL).

4. Centrifuge the appropriate amount of cells at 500 × *g* for 5 min and completely aspirate the spent medium. Resuspend the cell pellet at a density of 3×10^6 cells/mL in 5 mL of prewarmed ProCHO5 medium with additives and transfer into a TubeSpin bioreactor 50 tube.
5. The cells are transfected with donor–helper plasmid ratio of 9:1 (w/w) (*see* **Note 11**). Premix 20.25 μg (1.35 μg/10^6 cells) of donor pDNA and 2.25 μg (0.15 μg/10^6 cells) of helper pDNA. Add the mix to the culture and mix immediately by swirling the tube.
6. Subsequently add 45 μg (3 μg/10^6 cells) PEI directly in the culture and immediately mix by swirling the tube (*see* **Note 12**).
7. Place the culture on an orbital shaker with a rack for TubeSpin bioreactor 50 tubes and incubate at 37 °C in 5% CO_2 and 85% humidity with agitation at 180 rpm at a shaking diameter of 50 mm.

3.4 Genetic Selection

1. At 2 days posttransfection, the cells are counted and centrifuged as above and resuspended in 5–20 mL of prewarmed ProCHO5 medium with additives and 10 μg/mL puromycin (*see* **Note 2**), at a density of 0.5×10^6 cells/mL in a TubeSpin bioreactor 50 tube.
2. Every 3–4 days, the cells are counted as described in Subheading 3.2.
3. Then centrifuge the appropriate amount of cells at 500 × *g* for 5 min and resuspend cells at a density of 0.5×10^6 cells/mL in 5–20 mL of prewarmed ProCHO5 medium with additives and 10 μg/mL puromycin into a TubeSpin bioreactor 50 tube.
4. Continue passing the cells in the presence of the selection agent until the cell viability is >98% (*see* **Note 13**).
5. Once the cells have recovered (>98% viability) the CHO cell pool can be used for inoculating a batch or fed-batch culture. Alternatively, they may be used for single-cell cloning for the generation of clonally derived cell lines.

3.5 Fed-Batch Culture

Below is an example of a 2-week fed-batch culture at a volume of 5 mL, but the protocol can be applied to any desired culture volume.

1. Cells are counted as described in Subheading 3.2
2. The cells are then centrifuged and resuspended in 5 mL of prewarmed ProCHO5 medium with additives but without puromycin at a density of 0.5×10^6 cells/mL.

3. Place the cells in an incubator shaker with a rack for TubeSpin bioreactor 50 tubes and incubate at 37 °C in 5% CO_2 and 85% humidity with agitation at 180 rpm.
4. On day 3 postinoculation, the culture is fed with 3 g/L glucose and 1.5 g/L essential and nonessential amino acids (*see* **Note 14**).
5. After feeding, the culture is transferred to an incubator shaker at 31 °C with 5% CO_2 and 85% humidity with appropriate agitation (*see* **Note 15**).
6. At day 14 postinoculation, harvest the culture by centrifugation at 1000 × *g* for 15 min (*see* **Note 16**).
7. Filter the medium with a 0.22 μm filter and store at 4 °C or at −20 °C until the recombinant protein can be purified.

4 Notes

1. The mCMV major immediate early promoter/enhancer, a strong constitutive promoter, is used for expression of the gene of interest (recombinant protein) in CHO cells. A weaker promotor (HSV-tk) was chosen for expressing the genetic selection marker for effective selection stringency. Additionally, the cassette expressing the selection marker was cloned in the opposite orientation of that of the gene of interest to avoid influence of the mCMV promotor on the selection marker gene.
2. Depending on the CHO strain, other selection agents can be used including blasticidin, hygromycin B, zeocin, methotrexate, and methionine sulfoximine (MSX). However, the optimal concentration of each agent needs to be determined empirically for each CHO strain [5, 16].
3. For expression of more than one gene as is the case for antibodies or multiprotein complexes, a single plasmid DNA with multiple expression cassettes can be used or multiple single vectors (expressing one gene each) can be cotransfected [17–20].
4. Synthetic mRNA coding for the transposase can be used instead of pDNA [21].
5. Once thawed, the PEI solution in 50 mL tubes can be aliquoted into 15 mL tubes and either used for transfection that day or stored at 4 °C for up to 4 weeks.
6. Other methods of transfection, including electroporation, have also been used for gene delivery. The protocol (DNA amount, amount of transfection reagent, e.g., PEI, transfection cell density) must be optimized for the respective cells and gene delivery method.

7. The donor vector consists of the PB inverted terminal repeats (ITRs) which are AT-rich and contain repeat elements. Therefore, other *E. coli* strains designed especially for cloning direct repeats can be used, for example One Shot® Stbl3.
8. To maintain the transfectability of the cells, it is best to keep them in culture for no longer than 2–3 months (20–25 passages). We highly recommend maintaining the cells in exponential growth phase at all times.
9. ProCHO5 medium contains plant-derived peptone hydrolysates which can potentially cause lot-to-lot variation. We have previously observed differences in transfection efficiency (percentage of transfected cells following PEI-mediated gene delivery) using different lots of ProCHO5 medium. Therefore, each new lot of the medium should be tested for its support of cell cultivation and transfection before purchase, if feasible. The CHO cells should be adapted to any new medium lot for at least four passages (about 2 weeks) prior to testing cell growth and transfection.
10. It is recommended to passage the cells into fresh medium on the day prior to transfection to ensure exponential growth as this is optimal for PEI-mediated transfection.
11. The optimal amount of helper vector required for efficient transposition must be tested for each different CHO cell strain [12].
12. The method described here does not involve precomplex formation with DNA and PEI prior to addition to the culture. It is very important to minimize the time delay between addition of pDNA and PEI and to mix the culture well after each component is added.
13. The cells can be scaled up to larger volumes when they start recovering from selection pressure so as to have enough cells to start a fed-batch study on complete recovery (>98%) [5].
14. The feeding strategy must be optimized for different cells and media. The factors to optimize include the nutrients and their amounts and frequency of feeding.
15. The temperature shift of the culture during fed-batch culture causes cell growth arrest and results in an increase in specific productivity (Qp). The temperature shift of the culture is typically from 37 °C to 30–35°, before the cells reach a stationary growth phase (3–4 days postinoculation) [22, 23]
16. Although the supernatant was harvested on day 14 postinoculation, the culture can be extended further if its viability is high (>80%) at that time. Preferably, the viability of the cells at harvest should be at least 50%.

17. The method described here was developed for the combination of CHO-DG44 cells and ProCHO5 medium. CHO pools have also been successfully generated using suspension adapted CHO-K1, CHO-S, and CHO-GS cells [16, 24].
18. If using a different CHO strain and/or a different medium, it is necessary to optimize the amounts of DNA and PEI, as well as the cell density at the time of transfection. Some commercially available media inhibit PEI-mediated transfection due to presence of known components such as dextran sulfate, heparin sulfate, ferric ammonium citrate, and certain hydrolysates or other unknown components. Hence, it is essential to choose a medium which supports PEI-mediated transfection.
19. It is recommended to confirm the efficiency of transfection by performing a transfection with a plasmid expressing the enhanced green fluorescent protein (EGFP) or another fluorescent protein. If the transfection efficiency at 24 h posttransfection is less than 50%, optimization may be necessary.

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Chapter 7

Genome Engineering of Hybridomas to Generate Stable Cell Lines for Antibody Expression

Cristina Parola, Derek M. Mason, Andreas Zingg, Daniel Neumeier, and Sai T. Reddy

Abstract

From the perspective of academic and small research laboratories, the most common and practical strategy for recombinant expression of full-length monoclonal antibodies is to perform transient plasmid transfection of mammalian cells, resulting in small-scale and limited protein production. The generation of stable antibody producing mammalian cell lines enables larger-scale and consistent expression, however this approach is rarely pursued due to the time-consuming and expensive process of single colony screening and characterization. In order to bridge the gap between the simplicity of transient transfection and consistent production by stable cell lines, we describe a method to stably integrate antibody genes into the endogenous immunogenomic loci of hybridoma cells using CRISPR/Cas9 genome editing. Initially, the antibody variable light (VL) chain is deleted by multiplexed Cas9 cleavage; subsequently, the variable heavy (VH) chain is replaced by a fluorescent reporter gene (mRuby) by Cas9-assisted homology-directed repair (HDR). This cell line is customized by replacing mRuby with a synthetic antibody (consisting of a VL, light constant region and VH) by once again using Cas9-assisted HDR. Due to hybridomas' inherent ability to surface display and secrete antibodies, they provide a suitable host for both the selection and the production process, substantially streamlining the process for stable cell line generation, and thus we refer to this platform as *plug-and-(dis)play* (PnP) hybridomas.

Key words Antibody, Protein expression, Hybridoma, Genome editing, Stable cell lines, CRISPR/Cas9, Mammalian cells, B cells

1 Introduction

Mammalian cells are the host of choice for the production of full-length monoclonal antibodies (e.g., IgG), due to their ability to accommodate correct antibody folding, assembly, and posttranslational modifications (glycosylation) [1, 2]. Recombinant antibody production in mammalian cells is typically performed by transient transfection of plasmids, typically of human embryonic kidney (HEK293) cells [3, 4], or by generating stable cell lines by random transgene integration [5], with Chinese hamster ovary (CHO) cells being the most prominently used host [6, 7]. Intuitively, the

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advantages of one method are the drawbacks of the other: transient transfection is simple, rapid, and inexpensive and allows an immediate, albeit temporary, supply of the desired antibody; while in contrast, stable cell line generation is laborious, slow, and expensive, but results in a consistent (and long-term) supply of antibody. Overall, due to time and resource limitations, transient transfection is often the preferred choice for small-scale production, whereas stable cell lines are used most exclusively for large-scale production (e.g., therapeutic monoclonal antibodies).

Another commonly used cell line for antibody production is hybridomas. Hybridomas are created by fusing primary B cells from an immunized mouse with a myeloma tumor cell partner [8, 9], they are a well-established technology and represent one of the most common methods for discovering and expressing monoclonal antibodies. While hybridomas are most notable for their role in the discovery of therapeutic drug molecules, they are also used for the production of antibody laboratory reagents, which are used extensively in a plethora of biological assays such as western blots, enzyme-linked immunoabsorbent assays (ELISA), and flow cytometry. Because of the central role antibodies play in experiments, it has been suggested that the quality of antibody reagents largely influences the reproducibility and consistency of research [10]. Additionally, the validation process of future monoclonal antibody drug candidates begins at the bench, where small scale but solid expression systems are crucial for the preliminary investigation steps.

A real revolution in genetic engineering was started by the introduction of technologies for targeted genome editing [11], in particular the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas)9 system [12]. This technology relies on the programmable specificity of the nuclease Cas9, which is recruited by two short RNA molecules: a scaffold trans-activating CRISPR RNA (tracrRNA), and a target-dependent CRISPR RNA (crRNA). These RNAs associate with each other and with Cas9 to direct the nuclease toward the target genomic site by complementary base pairing with the crRNA; notably, the two RNAs are often combined in a synthetic (single) guide RNA (sgRNA, or gRNA) molecule, playing the same role as the duplex. After target recognition, Cas9 performs a double-stranded break to which the cell's DNA damage repair machinery can respond with two main alternative pathways: (1) nonhomologous end-joining (NHEJ) involves nontemplated, random nucleotide insertions and deletions, with consecutive gene knockout; (2) or alternatively, in the presence of a donor DNA template with homology arms, homology-directed repair (HDR) is promoted leading to targeted insertion of the donor [13]. A major advantage of the system is its great versatility, since virtually, the only requirement for a specific genomic sequence to be a CRISPR target is the presence of a short recognition sequences known as the protospacer adjacent motif (PAM) at its 3′ end; the most commonly used Cas9 variant from *Streptococcus pyogenes* has a PAM sequence of 5′-NGG-3′ [14, 15].

Here, we describe a method that utilizes hybridomas as a platform for stable cell line generation for full-length antibody production. What makes hybridomas ideal hosts for both the selection and the production process of stable cell lines is the potential for alternative splicing in B cells: based on their maturation stage, B cells and terminally differentiated plasma cells have an inclination toward surface expressing and/or secreting their antibody [16]. Hybridomas are a heterogeneous class of cell lines, and some clones retain the capability to express both the secreted and the membrane-bound form simultaneously. Our method relies on performing CRISPR/Cas9 genome editing on the endogenous antibody loci of hybridomas, leading to fast, easy, and inexpensive generation of stable cell lines for antibody production [17].

To develop our plug-and-(dis)play (PnP) antibody expression platform, we started by selecting a hybridoma clone (Wen1.3) that maintains alternative splicing of membrane-bound and secretory antibodies. We proceeded stepwise and applied CRISPR/Cas9 genome editing on both the antibody variable heavy (VH) and the variable light (VL) chain loci. Initially, we deleted the VL gene by multiplexed targeting of Cas9; next, by targeting Cas9 to the VH genomic locus, we integrated a donor DNA template bearing 5′ and 3′ homology arms and encoding a red fluorescent reporter gene (mRuby). As a result, a platform cell line devoid of antibody expression but positive for red fluorescence was obtained. Antibody expression is subsequently restored by targeting mRuby with Cas9 to promote HDR with a synthetic antibody (sAb) donor construct. The sAb construct contains a full-length light chain (VL and constant light chain), a self-cleaving 2A peptide, and a variable heavy (VH) chain, and is flanked by homology arms corresponding to the VH locus. Antibody expression is dependent on correct splicing with the constant heavy chain region (CH), therefore the sAb donor must be integrated in the former VH locus in order for cells to restore antibody expression. Successfully edited cells are isolated through phenotypic screening via fluorescence-activated cell sorting (FACS). This PnP platform allows isolation of a >90% antibody-positive population with one single round of FACS, substantially shortening and simplifying the conventional workflow for the generation of stable cell lines [17].

We describe here a detailed protocol for our PnP platform comprising several procedures, which we grouped in four main sequential steps (*see* Subheading 3): (1) CRISPR/Cas9 target selection, crRNA cloning and testing; (2) HDR donor DNA design and cloning; (3) generation of PnP-mRuby cell line; (4) generation of PnP-sAb cell lines and antibody characterization. While our efforts were exclusively focused on using the Wen1.3 hybridoma as a starting cell line, one of the strengths of the method lies in the possibility to repeat the workflow with virtually any murine hybridoma of IgG, κ isotype. Prior to starting the engineering process, some preliminary knowledge is required concerning: ability to surface express IgG; ability to secrete IgG; and sequence information of the antibody variable regions in the selected hybridoma clone. For variable gene sequencing, we recommend RNA isolation and RT-PCR with universal primer mixes (as previously described in Reddy et al. [18] and Haessler and Reddy [19]), followed by bacterial cloning and Sanger sequencing. A thorough protocol on hybridoma variable gene sequencing was described in Fields et al. [20]. Phenotypic characterization of surface expression and secretion should be performed by flow cytometry and ELISA, respectively.

2 Materials

2.1 Mammalian Cell Culture, Transfection, and Flow Cytometry

1. Mouse hybridoma, previously characterized (*see* Subheading 1).
2. Hybridoma growth medium: Dulbecco's Modified Eagle's Medium with high glucose (DMEM—GlutaMAX™), 10% fetal bovine serum (FBS), 10 mM HEPES buffer, 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol (all from Thermo Fisher Scientific).
3. Hybridoma freezing medium: DMEM—GlutaMAX™, 40% FBS, 10 mM HEPES buffer, 100 U/ml penicillin/streptomycin, 50 μM β-mercaptoethanol, 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich).
4. Trypan Blue.
5. PBS, pH 7.4, without calcium and magnesium (Thermo Fisher Scientific).
6. Flow cytometry staining antibodies: Brilliant Violet (BV) 421™ anti-mouse Ig light chain κ antibody (BioLegend) and suitable labeling antibody for the specific IgG subclass expressed by the hybridoma. Recommended options for IgG2C: Alexa Fluor® 488 AffiniPure Goat Anti-Mouse IgG, Fcγ Subclass 2c Specific (Jackson Immuno) and Allophycocyanin (APC) AffiniPure Goat Anti-Mouse IgG, Fcγ Subclass 2c Specific (Jackson Immuno).
7. If available: purified or recombinant protein antigen, fluorescently labeled with Alexa Fluor™ 647 Protein Labeling Kit (Thermo Fisher Scientific) or analogous product.
8. SF Cell Line 4D-Nucleofector™ X Kit (Lonza).
9. Opti-MEM™ I Reduced-Serum Medium (Thermo Fisher Scientific).

2.2 Bacterial Cell Culture and Plasmid Generation

1. Electrocompetent *E. coli* cells (suggested: MegaX DH10B™ $T1^R$ Electrocomp™ Cells, Thermo Fisher Scientific; TransforMax™ EC100™ Electrocompetent Cells, Lucigen).
2. LB medium: Luria Broth Base powder in MilliQ H_2O, autoclaved.
3. Kanamycin sulfate: 50 mg/ml in H_2O, working dilution 1:1000.
4. Ampicillin sodium salt: 100 mg/ml in H_2O, working dilution 1:1000.
5. Ampicillin and kanamycin agar plates: LB Agar (Thermo Fisher Scientific) in MilliQ H_2O, autoclaved and poured in petri dishes.
6. Plasmids: pcDNA3-mRuby2 (Addgene #40260); pUC57-Kan (GenScript); and pSpCas9(BB)-2A-GFP (pX458) (Addgene #48138). Equivalent variants of the latter include pSpCas9 (BB)-2A-BFP (as in Pogson et al. [17], obtained replacing GFP with BFP by Gibson cloning) and pSpCas9(BB)-2A-Puromycin (pX459) (Addgene #62988).
7. Nuclease-free H_2O.
8. KAPA HiFi HotStart 2× ReadyMix (KAPA Biosystems).
9. KAPA2G Fast Genotyping 2× Mix (KAPA Biosystems).
10. Restriction enzyme BbsI (NEB).
11. T4 DNA ligase (NEB).
12. Gibson Assembly® 2× Master Mix (NEB).
13. Restriction enzyme DpnI (NEB).
14. DNA gel reagents: 6× Loading Dye, 100 bp and 1 kb GeneRuler ladders, SYBR Safe DNA gel stain (all from Thermo Fisher Scientific), TAE buffer.
15. Agarose 1–2% (g/ml) in 1× TAE buffer.
16. PCR purification and gel extraction kits.
17. Plasmid DNA isolation miniprep and midiprep kits.
18. DNA precipitation solutions: Precipitation buffer (3 M Na-Acetate, pH 5.2 in H_2O), 100% isopropanol, 70% ethanol.
19. Glycerol anhydrous (50%, diluted in MilliQ H_2O and autoclaved).

2.3 Genotyping Analysis

1. QuickExtract™ DNA Extraction Solution (Epicentre).
2. Surveyor® Mutation Detection Kit (Integrated DNA Technologies).
3. 5× Phusion® HF Buffer (NEB).
4. TRIzol™ reagent (Thermo Fisher Scientific).

5. PureLink® RNA Mini Kit (Thermo Fisher Scientific).
6. Reverse transcription materials: Maxima Reverse Transcriptase, Olgo(dT)$_{20}$ Primer, dNTP mix (10 mM each), Ribolock RNase Inhibitor (all from Thermo Fisher Scientific).

2.4 Supernatant ELISA

1. Washing buffer: PBS with 0.05% (v/v) Tween® 20.
2. Blocking buffer: PBS with 0.05% (v/v) Tween® 20 and 2% (w/v) nonfat dried milk powder.
3. Suitable coating and detection antibodies. Recommended options: AffiniPure Goat Anti-Mouse IgG, Light Chain Specific (Jackson Immuno), Anti-Mouse IgG (Fc specific)–HRP (Sigma), and Rat monoclonal 187.1 Anti-Mouse kappa light chain (HRP) (Abcam).
4. If available: purified or recombinant protein antigen, resuspended at 1–10 mg/ml in PBS (e.g., Hen egg lysozyme).
5. 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Fisher Scientific).
6. Stopping solution: 1 M sulfuric acid (H_2SO_4) in H_2O.

2.5 Software

1. Geneious (Biomatters) or equivalent (*see* **Note 1**).
2. FlowJo or equivalent platform for flow-cytometric analysis.
3. GraphPad Prism, Microsoft Excel, or equivalent software for the analysis of ELISA data.

2.6 Disposables

1. Filter tips, serological pipettes, and Pasteur pipettes.
2. DNA LoBind tubes (Eppendorf).
3. 0.2 ml PCR tubes.
4. EconoSpin® All-In-One Mini Spin Columns (Epoch).
5. Bacterial cell culture tubes.
6. MF-Millipore™ membrane filters (Merck Millipore).
7. 1 mm cuvettes compatible with electroporator (*see* Subheading 2.7).
8. Autoclaved CellTrics® 30 μm disposable filters (Sysmex).
9. Nunc™ Cell-Culture Treated Multidishes (Thermo Fisher Scientific).
10. Nunc™ Cell Culture Treated EasYFlasks™, filter cap (Thermo Fisher Scientific).
11. ELISA plates.
12. 15 and 50 ml centrifugation polypropylene tubes.
13. Cryovials, System 100™, Nalgene®.
14. 0.22 μm syringe filters and compatible syringes.

15. Stericup-GP and Steriflip-GP filtration systems (Merck Millipore).
16. Razor blades.

2.7 Equipment

1. Pipettor [e.g., Pipetboy (Integra)] and micropipettes (e.g., Gilson, Rainin).
2. Mammalian cell culture basic equipment: tissue culture hood; CO_2 incubator kept at 37 °C and 5% CO_2; vacuum aspirator; refrigerable centrifuge; microscope; cell counting equipment (manual or automated); water bath.
3. 4D-Nucleofector™ System (Lonza); alternatively, transfection system of choice (chemical or electrical).
4. Flow cytometry analyzer (e.g., BD LSR Fortessa^{TM}) and sorter (e.g., BD FACS Aria^{TM}).
5. Bacterial cultivation and gene cloning basic equipment: 37 °C incubator; temperature-controlled shaker (e.g., Kühner); electroporator [e.g., MicroPulser™ (Bio-Rad)]; Bunsen burner.
6. PCR Thermal Cycler with temperature gradient and ramp functions [e.g., Veriti^{TM} cyclers (Applied Biosystems)].
7. DNA electrophoresis and purification equipment: NanoDrop™ (Thermo Fisher Scientific) (or equivalent spectrophotometer); tabletop centrifuge; horizontal electrophoresis chamber with power supply; Gel Doc™ EZ System with Blue Sample Tray (Bio-Rad), or equivalent; transilluminator with compatible goggles; water bath or heating block for microcentrifuge tubes; microwave oven; vacuum source with compatible tube; vacuum concentrator.
8. Microplate reader [e.g., Infinite® 200 PRO (Tecan)].

3 Methods

Unless specified otherwise, carry out all procedures at room temperature (RT), and perform all cell centrifugation steps at $250 \times g$ for 5 min. Water in all cloning, PCR and purification procedures should be PCR-grade nuclease-free H_2O. Prepare all agarose gels at 1% (g/ml), unless otherwise specified.

3.1 CRISPR/Cas9 Target Selection, crRNA Cloning and Testing

1. Start culturing the hybridoma cell line in normal growth medium (*see* Subheading 2). Ideally, the cells should be monitored to make sure that they are growing and healthy for at least 1 week before the beginning of any experiment (*see* **Note 2**).
2. The VH and VL gene sequences of the selected hybridoma clone should be known before starting all procedures. Submit the nucleotide sequence on the Immunogenetics database

(IMGT V-Quest) (https://www.imgt.org/IMGT_vquest/vquest) to infer the germ line V and J genes.

3. On IMGT, search the sequence of the signal peptide (SP) of the retrieved VL and VH genes (http://www.imgt.org/IMGTrepertoire/). This step is important to be able to map the germ line genes in the chromosomal loci, and sequence the surrounding regions, which is helpful for designing the Cas9 gRNA targets and homology arms. An exemplary schematic can be found in Fig. 1.

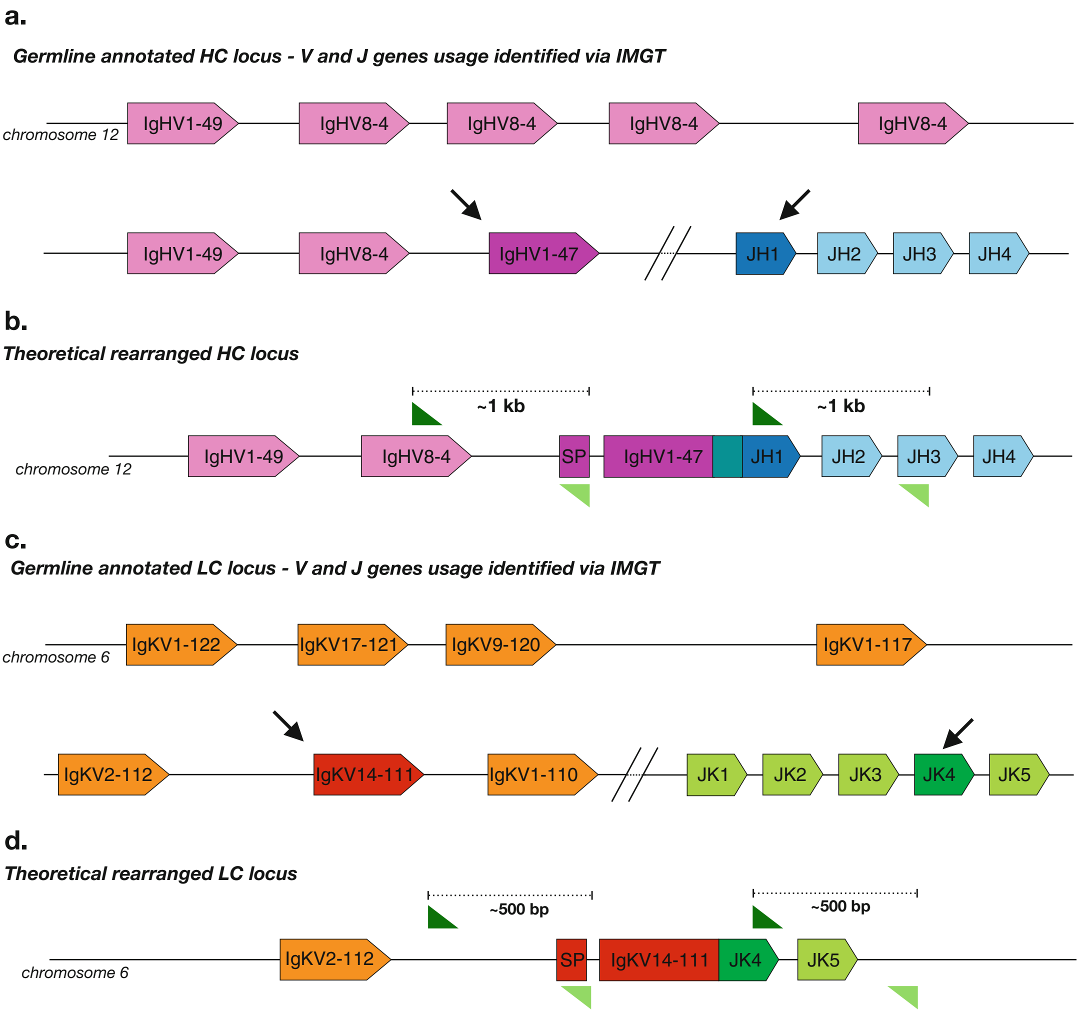

Fig. 1 Sequencing strategy for the VH and VL loci. (**a** and **c**) Starting from the known VH and VL sequences, the germ line VH, JH, VK, and JK genes need to be inferred with IMGT. (**b** and **d**) Primers are designed approximately 1 kb upstream of the VH and downstream of the JH, and 500 bp upstream of VK and downstream of JK. In each case, the complementary primer is ideally designed in the 5′ or 3′ terminal part of the coding region. The figure shows the loci structure for a hypothetical antibody obtained by rearrangement of IgHV1-47, any D region, and JH1 (heavy chain) and IgKV14-111 and IgKJ4 (light chain) (n.b.: distances not in scale)

4. Oligonucleotide primers should be ordered that cover approximately 1 kilobase (kb) upstream of the VH signal peptide and downstream of the JH region, and at least ~500 base pairs (bp) upstream of the VK signal peptide and downstream of the JK region.
5. Extract the genomic DNA (gDNA) by first recovering 10^6 cells by centrifugation.
6. Remove the supernatant, wash the cell pellet with 1 ml PBS.
7. Repeat the centrifugation step.
8. Resuspend the cells in 100 μl QuickExtract solution and incubate in a thermal cycler with the following parameters:
   - 68 °C for 15 min.
   - 95 °C for 8 min.
   - 4 °C until removal.

   The gDNA can be stored at 4 °C for up to 1 week and −20 °C for long term storage.
9. PCR amplify the genomic regions of interest. Mix the components as follows (*see* **Note 3**):
   - 25 μl KAPA HiFi HotStart 2× ReadyMix.
   - 1.5 μl forward primer (10 μM).
   - 1.5 μl reverse primer (10 μM).
   - 2 μl gDNA.
   - 20 μl H_2O.
10. Incubate in a thermal cycler with the following parameters (*see* **Note 4**):
    - 95 °C for 3 min.
    - 35 cycles:
      - 98 °C for 20 s.
      - 60–75 °C for 15 s.
      - 72 °C for 15–60 s/kb.
      - 72 °C for 1 min/kb.
    - 4 °C until removal.
11. As an alternative method of PCR amplification:
    - 15 μl KAPA2G Fast Genotyping 2× Mix.
    - 1 μl forward primer (10 μM).
    - 1 μl reverse primer (10 μM).
    - 2 μl gDNA.
    - 11 μl H_2O.

12. Incubate in a thermal cycler with the following parameters (*see* **Note 4**):
    - 95 °C for 3 min.
    - 35 cycles:
        - 95 °C for 15 s.
        - 55–75 °C for 15 s.
        - 72 °C for 15 s/kb.
    - 72 °C for 1 min/kb.
    - 4 °C until removal (*see* **Note 5**).
13. Check the DNA purity by running ~5 μl on an agarose gel. If the product is sufficiently clean, proceed with PCR purification; otherwise, run the remaining product on a new gel and isolate the DNA by gel extraction.
14. After purification, measure the DNA concentration with a spectrophotometer (e.g., NanoDrop).
15. Good quality DNA can then be submitted for Sanger sequencing (*see* **Note 6**).
16. Once the sequences of the regions upstream of VH and downstream of JH are obtained, design homology arms of 700–1000 bp. Homology regions should start immediately before the signal peptide and immediately after the J region (*see* **Note 7**).
17. Search for Cas9 target sites: the VL has to be deleted by NHEJ, and multiplexed cleavage should be induced, ideally, 5′ of the SP sequence or within the SP sequence and 3′ of the J region.
18. The VH has to be exchanged with another gene, and cleavage can be induced anywhere along the sequence (*see* Fig. 2a and **Note 8**).
19. While multiple online tools are available for the interrogation of a specific sequence for CRISPR/Cas9 target sites, we suggest the comprehensive online tools CRISPOR (http://crispor.tefor.net/) and GPP Web Portal (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design), which classify targets according to multiple existing algorithms. Even though these online tools are often reliable, it is recommended to select 3–4 possible gRNA target sites for each region for testing Cas9 cleavage.
20. gRNA oligonucleotides should be designed and ordered according to the protocols from the Zhang group (Ran et al. [21], http://www.genome-engineering.org). For each gRNA target, a forward and a reverse oligonucleotide should be ordered with the configuration depicted in Fig. 2b, inclusive of BbsI cloning extensions. Order the oligonucleotides with 5′ phosphorylation (*see* **Note 9**).

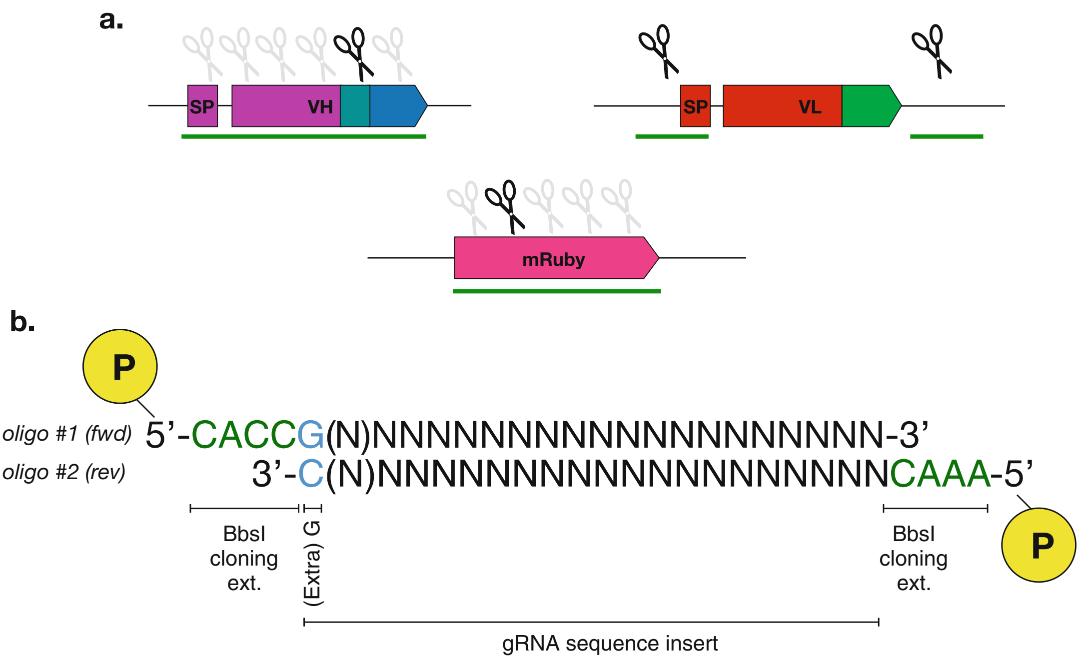

Fig. 2 Cloning strategy for CRISPR/Cas9 gRNAs. (**a**) At least one functional gRNA should be available for the VH gene, the 5′ and 3′ regions flanking the VL gene, and the mRuby gene. (**b**) Structure of the 5′-phosphorylated oligos that need to be designed for each Cas9 target to be tested. As explained in Ran et al. [21], the U6 promoter, which drives gRNA transcription in pX458, requires a G as a first transcript base. For this reason, when the gRNA starts with any other nucleotide, an extra G is added upstream of the gRNA’s 5′ end (n.b.: in the latter case, the original first nucleotide can be omitted or preserved, resulting in a total gRNA length of 20 or 21 nucleotides, respectively)

21. Restriction digest 1 μg of pX458 with BbsI and gel-purify it.
22. In parallel, anneal the forward and reverse oligos by mixing:
    - 1 μl fwd oligo (100 μM).
    - 1 μl rev oligo (100 μM).
    - 8 μl H_2O.
23. Anneal in a thermal cycler with the following parameters:
    - 37 °C for 30 min.
    - 95 °C for 5 min.
    - Ramp down to 25 °C at 5 °C/min.
24. Dilute the annealed oligos 1:200 in H_2O and set up the ligation procedure as follows (*see* **Note 10**):
    - 50 ng restriction digested pX458.
    - 1 μl diluted annealed oligos.
    - 1 μl 10× T4 Ligase buffer.
    - 0.5 μl T4 Ligase.
    - H_2O to 10 μl.

Table 1
Primers used for amplification and sequencing procedures

Primer ID	Sequence 5′ → 3′	Description
hU6seq	ACTATCATATGCTTACCGTAAC	Sequencing cloned CRISPR gRNA from pX458 (or equivalent)
HDR-linear-for	AGCGGATAACAATTTCACACAG	Linearizing pUC57-Kan-HDR plasmid prior to transfection
HDR-linear-rev	AGGGTTTTCCCAGTCACG	Linearizing pUC57-Kan-HDR plasmid prior to transfection

25. Incubate overnight (O/N) at 16 °C.
26. De-salt 5–7 μl of ligation product on a membrane filter, and transform 3 μl in 30 μl electrocompetent *E. coli* cells.
27. Recover with 300 μl of LB medium (without antibiotic) for 45 min at 37 °C in a shaking incubator and plate on ampicillin agar plate.
28. Isolate DNA from clones by miniprep and submit them for Sanger sequencing with the primer hU6seq (*see* Table 1).
29. Prepare a midiprep and a frozen stock (*see* **Note 11**) from the selected positive clones (ideally, one per gRNA) and perform an additional cleaning step by ethanol precipitation:
    - Mix with 1/10 volume of Precipitation buffer.
    - Mix with 1 volume of 100% isopropanol.
    - Incubate for at least 1 h at −20 °C.
    - Centrifuge for 15 min at maximum speed (e.g., 17,000 × *g*).
    - Decant supernatant and wash pellet with 1 ml 70% ethanol.
    - Repeat centrifugation step.
    - Decant supernatant and completely dry the pellet.
    - Resuspend in H_2O, typically half of the starting volume.
30. The plasmid DNA can now be transfected (by nucleofection electroporation) into hybridomas to assess Cas9 cleavage. Approximately 24 h before transfection, seed the cells at a low density (e.g., 2×10^5/ml) which enables the cells to divide approximately once before reaching a density of about 5×10^5 cells/ml, the optimal density for nucleofection. The total volume and cell count should be calculated to have a sufficient amount the following day.
31. On the day of transfection, start by prewarming a 24-well plate with 1 ml growth medium for each sample.

32. Isolate 10^6 cells/sample by centrifugation at 90 × *g* for 10 min.
33. Remove the supernatant and resuspend the cells in 1 ml Opti-MEM reduced serum medium, and repeat the centrifugation step. During centrifugations, prepare the Nucleofection mix (10% excess):
    - 5.5 μg (×μl) pX458-gRNA.
    - (110-×) μl Lonza SF buffer.
34. After the Opti-MEM washing step, remove the supernatant, resuspend the cells in Nucleofection mix (100 μl), and then transfer in a Nucleofection cuvette.
35. Nucleofect the cells with the program CQ-104.
36. After electroporation, transfer the cells the prewarmed plate (*see* **Note 12**).
37. 24 h after transfection, isolate a small aliquot of cells (~100–200 μl) by centrifugation, resuspend them in PBS and analyze them by flow cytometry for the detection of GFP signal.
38. 48 h after transfection, centrifuge the full samples, resuspend the cells in PBS and sort the GFP^+ population by flow cytometry sorting (FACS). Culture the sorted cells until recovery (*see* **Notes 13** and **14**).
39. After recovery, extract gDNA from 10^6 cells as previously explained. Cleavage performance is measured by Surveyor assay [21]. DNA amplicons need to be obtained containing the gRNA target site, and having a length of approximately 1 kb, with one end more proximal to the cleavage site (300–400 bp) (*see* **Note 15**). PCR amplify the region of interest from all the samples (*see* **Note 16**). Isolate the amplified DNA by PCR purification (cleanup).
40. If all samples are concentrated >34 ng/μl (i.e., volume for 400 ng ≤ 12 μl), assemble the denaturation and hybridization reaction as it follows:
    - 400 ng (×μl) DNA.
    - (12-×) μl H_2O.
    - 3 μl 5× Phusion HF buffer.
41. If some samples have a lower concentration, calculate the amount of DNA in 12 μl for the least concentrated sample, and use the same amount for the other samples as well, adjusting the volume to 12 μl (total 15 μl including buffer) with H_2O.
42. Incubate in a thermal cycler with the following parameters:
    - 95 °C for 10 min.

- Ramp down to 85 °C at 2 °C/s.
- 85 °C for 1 min.
- ramp down to 75 °C at 0.3 °C/s.
- Repeat **previous 2 steps** until 25 °C.
- 4 °C until removal.

43. Add 1 μl of Surveyor Enhancer S and 2 μl of Surveyor Nuclease S, and incubate at 42 °C for 40 min.
44. Run product on a 2% agarose gel (*see* **Notes 17–19**). For examples of Cas9 targets validation via Surveyor analysis, *see* Figs. S2 and S6 in Pogson et al. [17].
45. At this point, functional Cas9 targets should have been found for:
    - 5′ VL region.
    - 3′ VL region.
    - VH coding region.

    (*See* Subheading 3.1, **steps 17–18** and Fig. 2a)

3.2 HDR Donor DNA Design and Cloning

1. Clone the hybridoma's VH genomic locus (inclusive of homology arms, total length ~2–2.5 kb) in pUC57-Kan with Gibson cloning [22] to obtain a basic HDR donor plasmid, subsequently customizable with different cassettes.
2. Purify the pUC57-Kan and pcDNA3-mRuby2 plasmids from a miniprep culture.
3. Design primers to amplify:
    - The VH genomic locus with overhangs for pUC57-Kan.
    - pUC57-Kan to be linearized for VH locus insertion.
4. *See* Fig. 3 for a schematic of the strategy to clone a basic pUC57-Kan-HDR vector and subsequent derivatives.
5. Set up insert and donor PCRs as follows:
    - 25 μl KAPAHiFi HotStart 2× ReadyMix.
    - 1.5 μl forward primer (10 μM).
    - 1.5 μl reverse primer (10 μM).
    - 1 ng plasmid/2 μl gDNA.
    - H_2O to 50 μl.
6. Incubate in a thermal cycler with the following parameters:
    - 95 °C for 3 min.
    - 25 cycles (plasmid DNA/backbone) or 35 cycles (gDNA/insert).
        - 98 °C for 20 s.
        - 60–75 °C for 15 s.
        - 72 °C for 15–60 s/kb.

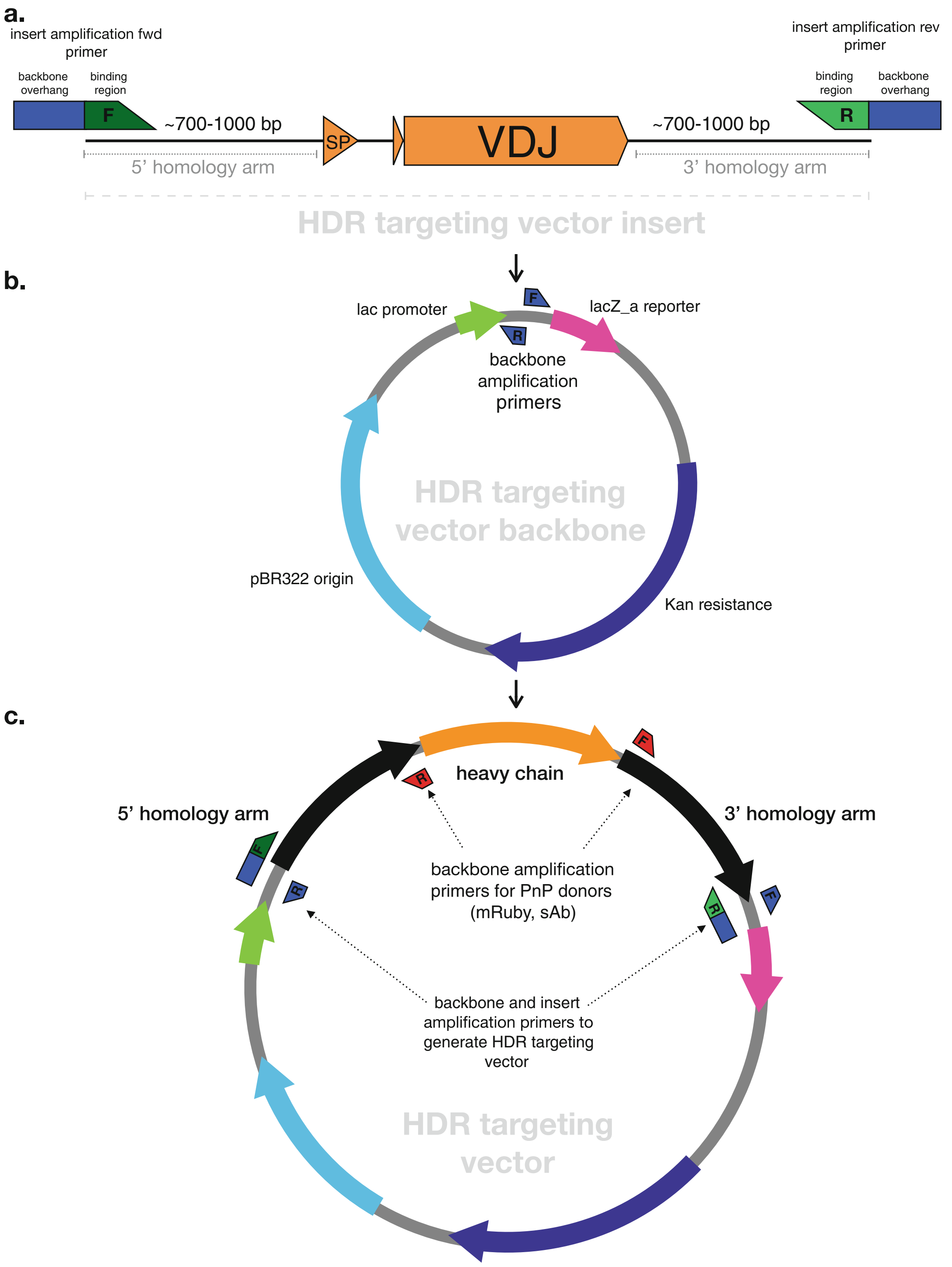

Fig. 3 Strategy to clone the HDR donor cassette in pUC57-Kan. (**a**) Primers to amplify the VH locus from the genome are designed approximately 700–1000 bp upstream and downstream of the recombined VDJ of the hybridoma, having Gibson overhangs overlapping with the plasmid backbone in the indicated area. (**b**) pUC57-Kan is amplified with primers binding in the regions matching to the overhangs of the primers for the insert (blue primers). (**c**) The plasmid after successful Gibson cloning of the VH region inclusive of the homology arms is shown. The cloning procedure to replace the original VDJ region with mRuby or sAb donors is analogous, but in this case the homology arms are kept as part of the backbone (red primers)

- 72 °C for 1 min/kb.
- 4 °C until removal.

7. Add 1 μl DpnI for the digestion of methylated DNA (*see* **Note 20**) and incubate in a thermal cycler as follows:
   - 37 °C for 60 min.
   - 80 °C for 20 min.
8. Run on an agarose gel and purify amplified and digested DNA.
9. Set up the Gibson Assembly reaction as follows (*see* **Notes 10** and **21**):
   - 5 μl Gibson Assembly 2× Master Mix.
   - 0.025 pmol backbone DNA.
   - 0.075 pmol insert DNA.
   - H_2O to 10 μl.
10. Assemble in a thermal cycler or heating block at 50 °C for 1 h.
11. De-salt 5–7 μl of assembled product and transform 3 μl in 30 μl electrocompetent *E. coli* cells.
12. Recover with 300 μl of LB medium (without antibiotic) for 45 min at 37 °C in a shaking incubator and plate on a kanamycin agar plate.
13. As a preliminary screen for successful clones, a colony PCR is recommended (*see* **Note 22**).
14. Then, clones that successfully integrated the insert should be sequenced with as many reactions as necessary to cover the whole insert.
15. The pUC57-Kan-HDR vector can be used in all downstream procedures to target the VH locus and exchange the VDJ region with mRuby and, subsequently, mRuby with synthetic antibodies.
16. To clone the PnP-mRuby-HDR donor, a Gibson cloning strategy analogous to the previous one needs to be designed, but including the homology arms in the backbone structure (*see* Fig. 3c and **Note 23**). The gene for mRuby, including the bGH polyA sequence, should be amplified from the commercial vector pcDNA3-mRuby2.
17. To clone the PnP-sAb-HDR donors, synthetic antibody cassettes must be designed as follows: VL signal peptide (exon 1—intron—exon 2)—VL gene—CK gene—Furin cleavage site and 2A peptide—VH signal peptide (exon 1—intron—exon 2)—VH gene (*see* Fig. 4) and ordered as synthetic gene fragments (e.g., gBlocks from IDT). *See* **Note 24** for more specifications and tips about the sAb construct design.

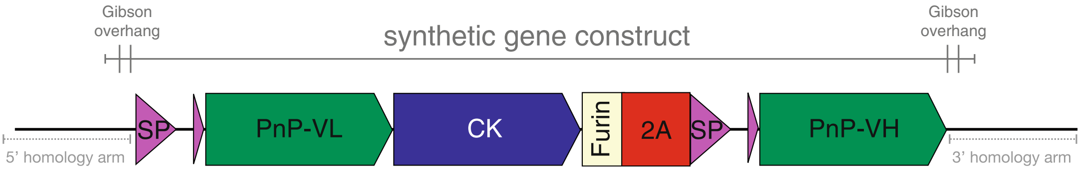

Fig. 4 Schematic representation of the sAb donors for the PnP system. The sAb construct(s) is designed to have Gibson overhangs for the homology arms (already in the HDR vector, *see* Fig. 3)

3.3 Generation of PnP-mRuby Cell Line

1. Make sure to have pX458 versions containing all the required VL- and VH-targeting gRNAs, and the pUC57-Kan-mRuby targeting vector, purified by midiprep and ethanol precipitation.
2. The first step is the VL knockout by multiplexed NHEJ. Prepare the hybridoma cells for transfection as previously described, and set up the Nucleofection as follows:
   - 5.5 μg (X μl) pX458-VL5′-gRNA.
   - 5.5 μg (Y μl) pX458-VL3′-gRNA.
   - (110-X–Y) μl Lonza SF buffer.
3. 48 h after transfection, sort the cells for GFP expression and recover them as previously explained (*see* **Note 25**).
4. To check the cells for VL knockout, perform flow cytometry staining by preparing cells as follows:
   - Isolate 10^6 cells/sample by centrifugation.
   - Resuspend in 500 μl PBS and repeat centrifugation.
   - Resuspend the cells in 100 μl labeling mix, containing staining antibody diluted at proper working concentration in PBS (*see* **Note 26**). For the anti-mouse-IgK-BV421 (BioLegend, *see* Subheading 2), in our hands, it is 1:80.
   - Incubate for 30 min on ice, protected from light.
   - Centrifuge the cells and resuspend in 500 μl PBS.
   - Centrifuge again; repeat this washing step 1–2 times.
   - After the final wash, resuspend the cells in 500 μl PBS and analyze by flow cytometry. Figure S5a (middle panel) from Pogson et al. [17] shows an example of light chain knockout prior to isolation of single-cell clones.
5. If knockout is successful, proceed to single-cell sorting of cells that have lost IgK expression. Label the cells as previously described, and sort in a round-bottom 96-well plate containing 100 μl medium per well (*see* **Note 27**).
6. Extract the gDNA from the recovered clones as previously described, and PCR amplify the whole VL locus, making sure to use primers that bind upstream of the 5′ and downstream of the 3′ Cas9 site (the outmost primers used for sequencing the locus can be used as well, as long as they are compatible with each other).

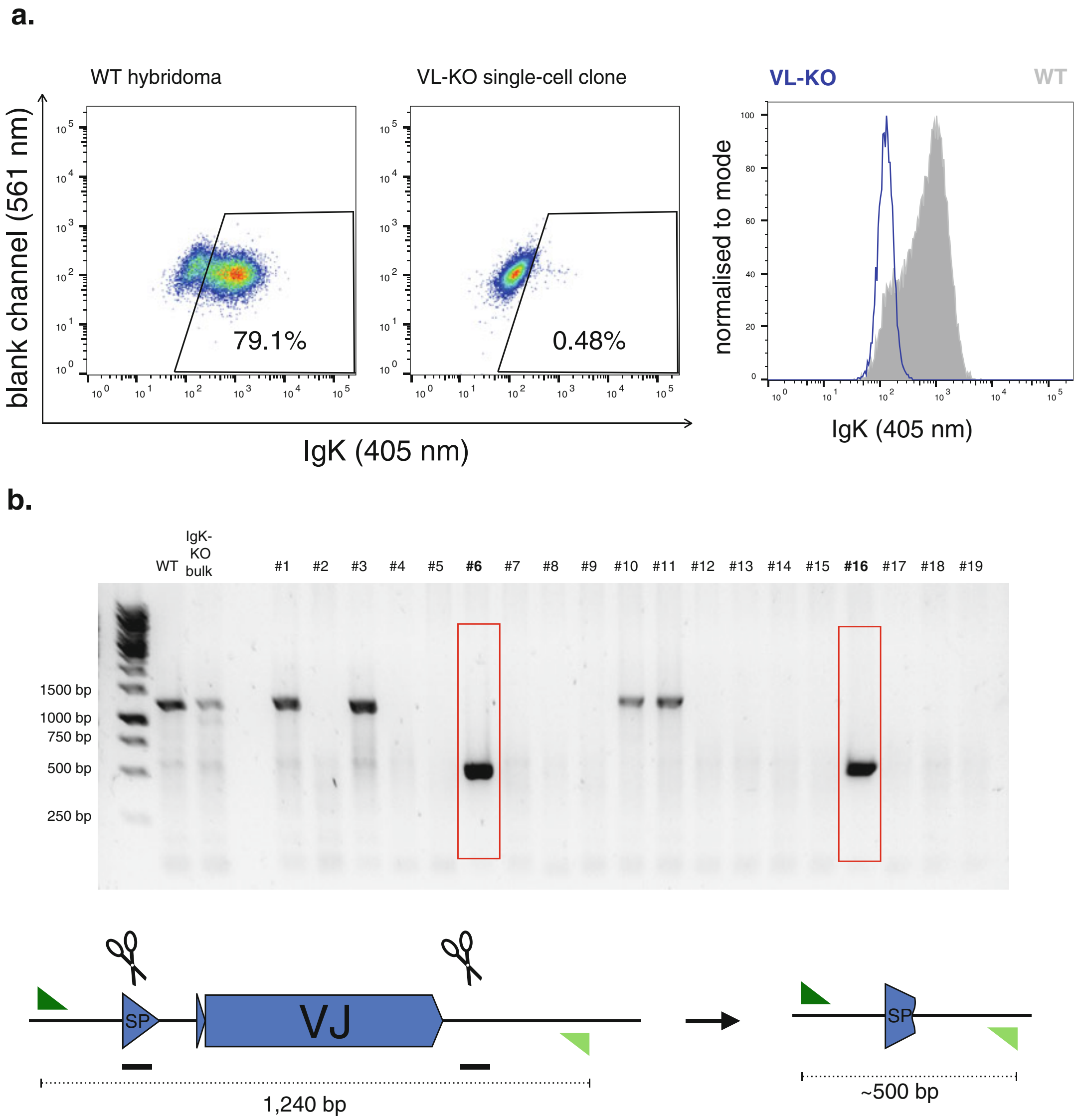

Fig. 5 Light chain knockout in the parental hybridoma cell line via multiplexed Cas9 targeting. (**a**) Flow cytometry data shows that the original hybridoma (Wen1.3), albeit the heterogeneity of the population, surface expresses IgK, opposite of the selected knocked-out single-cell clone, which is negative. (**b**) Genotypically, deletion of the region between the two Cas9 gRNA target sites causes a reduction of the PCR amplicon from 1240 to approximately 500 bp. Two of the analyzed clones (named, for reference, #6 and #16) display the deletion band, and either of them can be chosen for downstream characterization and, most importantly, to proceed to the next step

7. Analyze the clones by running the PCR product on an agarose gel. For an X bp distance between the two Cas9 sites, the successfully edited ones will display a band X bp smaller than the wild-type (WT) amplicon (*see* Fig. 5b and **Note 28**).
8. Select the clones displaying the right band and repeat the flow cytometry staining to verify that the whole population is IgK

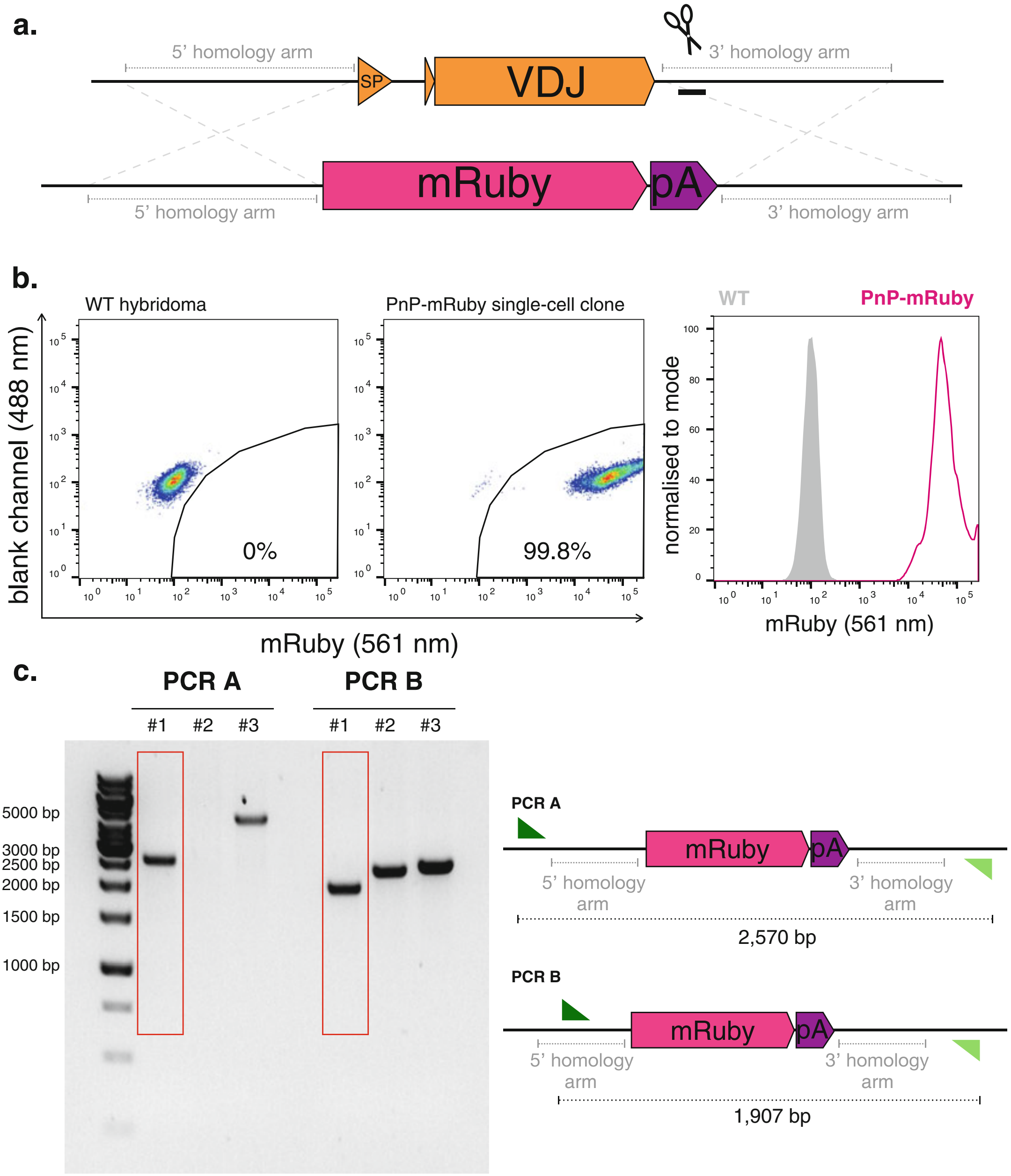

Fig. 6 Reporter mRuby knockin in the VH locus. (**a**) HDR-driven cassette exchange to replace the signal peptide + VDJ region with mRuby+polyA. In this specific case, the gRNA used induces cleavage within the 3′ homology arm, but it is generally advisable to target the region to be exchanged (i.e., VH region). (**b**) Flow-cytometric analysis of the single-cell clone selected for having correctly integrated mRuby. (**c**) Genotyping strategy: two possibilities to genotype for correct integration are shown. In the case of PCR A, both primers bind outside the homology arms; for PCR B, the forward primer binds inside the 5′ homology arm. The result of the genotyping analysis is shown for three representative clones. Notably, for only one of them (clone #1) the result of both PCRs is correct, while in the case of the other clones, while still expressing mRuby, those are likely duplications or aberrant integration events. This example highlights the importance of combining multiple gDNA PCRs together when characterizing the HDR outcome

negative. Figure 5a shows an exemplary outcome after successful light chain knockout (KO).

9. Choose the best performing clone, create frozen stocks (*see* **Note 29**) and proceed with the next step (*see* Fig. 6a).
10. Linearize the pUC57-Kan-mRuby HDR donor by PCR (*see* **Note 30**). Mix the following:
    - 525 μl KAPA HiFi HotStart 2× ReadyMix.
    - 31.5 μl HDR-linear-for (10 μM).
    - 31.5 μl HDR-linear-rev (10 μM).
    - 105 ng plasmid.
    - H_2O to 1050 μl.

    (*See* Table 1 for the primer sequences) Split reaction mix in single PCR tubes (e.g., 50 μl/tube for 20 reactions or 100 μl/tube for 10 reactions) (*see* **Note 31**).

    Incubate in a thermal cycler at the following conditions:
    - 95 °C for 3 min.
    - 20 cycles:
      - 98 °C for 20 s.
      - 67.5 °C for 15 s.
      - 72 °C for 2 min.
    - 72 °C for 3 min.
    - 4 °C until removal.
11. Check the DNA purity by running ~5 μl on an agarose gel. If the product is sufficiently clean, proceed with PCR purification (*see* **Note 32**).
12. If the DNA concentration is significantly lower than 500–1000 ng/μl, concentrate it to bring it within that range. Consider amplifying more DNA if there is not enough material available.
13. Set up a transfection with the following nucleofection mix:
    - 5.5 μg (X μl) pX458-VH-gRNA.
    - 5.5 μg (Y μl) linearized pUC57-Kan-mRuby HDR donor.
    - (110-X–Y) μl Lonza SF buffer.
14. 48 h after, sort the cells for GFP expression and recover them as previously explained.
15. After recovery, check the cells by flow cytometry for gained mRuby expression; mRuby fluoresces with the same spectrum as mCherry (561 nm laser, 610/20 filter or equivalent). A population in the 1–2% range is to be expected (*see* Fig. S4 from Pogson et al. [17]).

16. If successful, proceed to single-cell sorting of mRuby-positive cells.
17. Genotype the recovered single-cell clones by gDNA PCR. An amplicon obtained with both primers binding outside of the homology arms is an option: in this case, size differences with the WT hybridoma will be observed in the positive clones, since mRuby is bigger than the average VDJ region.
18. Alternatively, one primer can be designed to bind inside one homology arm. As a third alternative, one primer can bind inside mRuby's coding sequence, and the cognate primer outside of one of the homology regions. In the latter two cases, two complementary PCRs should be designed in order to cover the whole locus (mRuby + homology arms). Whichever strategy is preferred, include a Sanger sequencing step (*see* Fig. 6c and **Note 33**).
19. Once a clone has been selected, perform a final flow cytometry characterization, including light and heavy chain staining (perform the light chain labeling as previously described; for the heavy chain, make sure to have an antibody compatible with the hybridoma's isotype and titrate it before use) (*see* **Note 34**).
20. Ideally, the successful clone will be positive for mRuby and negative for both antibody chains (*see* Figs. 6b and 1 from Pogson et al. [17]). This cell line should from now on be referred to as PnP-mRuby (*see* **Note 35**).

3.4 Generation of PnP-sAb Cell Lines and Antibody Characterization

1. To target mRuby (*see* Fig. 7a), use one of the Cas9 gRNAs indicated in Table 2, after cloning in pX458 as previously explained (*see* **Note 36** and **37**), or use custom-designed ones, as long as they are appropriately validated to cleave within mRuby's coding sequence (*see* Fig. 2a).
2. Linearize the sAb donor with the same procedure performed for mRuby, taking care to always verify the PCR purity.
3. Set up a transfection of the PnP-mRuby cells, with the following Nucleofection mix:
   - 5.5 μg (X μl) pX458-mRuby-gRNA.
   - 5.5 μg (Y μl) linearized pUC57-Kan-sAb HDR donor.
   - (110-X–Y) μl Lonza SF buffer.
4. 48 h after, sort the cells for GFP expression and recover them as previously explained.
5. After recovery, perform a flow cytometry staining by labeling heavy and/or light chain (one is sufficient, *see* **Note 38**) and plotting either of them against mRuby. If cleavage was successful, a significant proportion of the cells will have lost mRuby fluorescence; antibody expression is regained by (a) HDR and

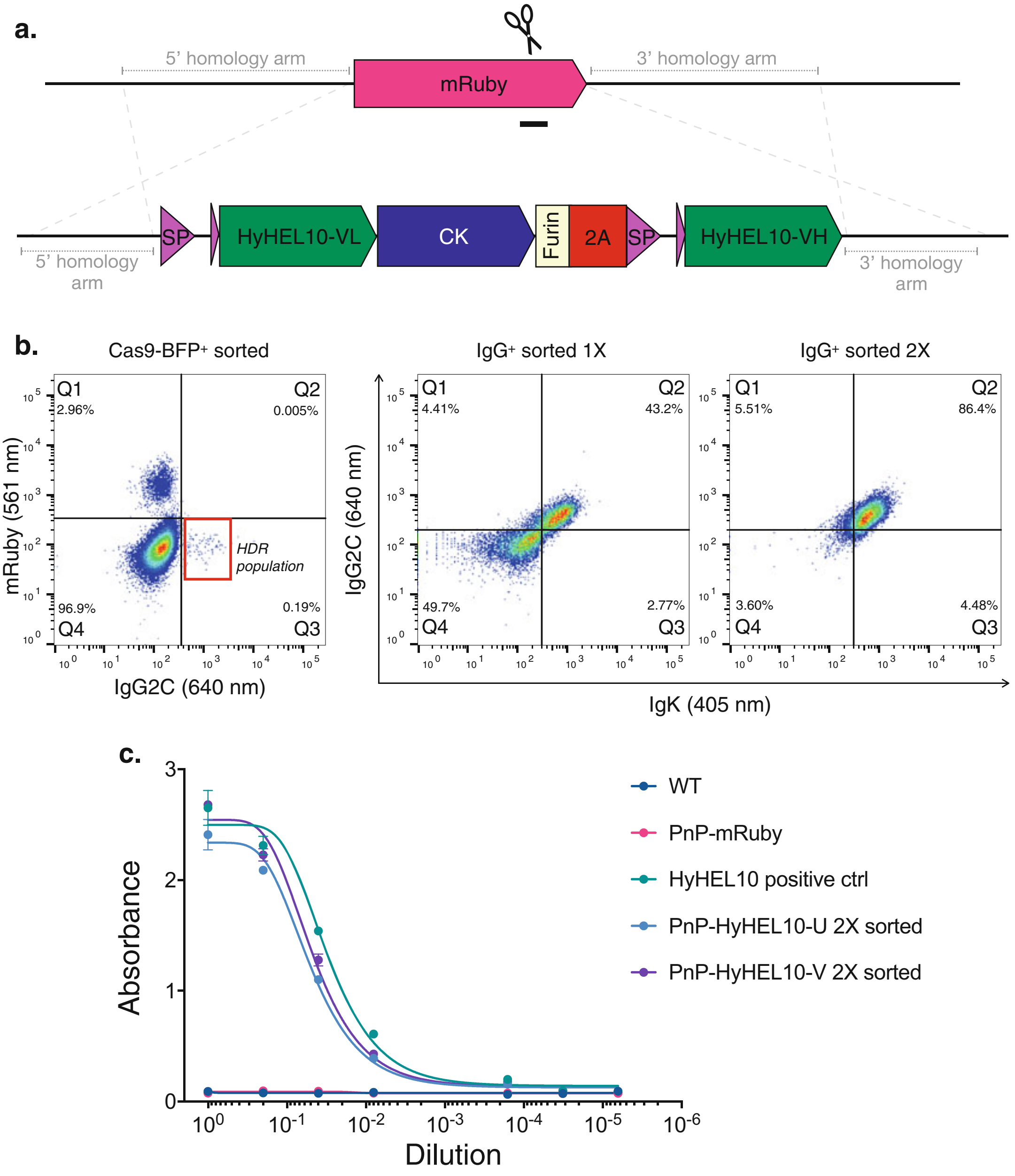

Fig. 7 Antibody PnP: exchange of mRuby with a sAb encoding an antibody specific for hen-egg lysozyme (HyHEL10). (**a**) Schematic of the experiment. A gRNA targeting the terminal region of mRuby (gRNA-K) was used, together with a PCR-linearized version of the plasmid donor. In contrast to what is advised in this protocol, the experiment illustrated in this figure was performed with a platform cell line lacking a polyA tail after mRuby. (**b**) Flow cytometry analysis of the cells after Cas9 enrichment (via BFP$^+$), and after the first and second IgG$^+$ enrichment steps. (**c**) Antigen (hen egg lysozyme) ELISA on the supernatant of the PnP-HyHEL10 samples V (gRNA-K, *see* (**a**) and (**b**)), and U (obtained with the same workflow but targeting mRuby with gRNA-J, binding 5′ of gRNA-K). A HyHEL10 hybridoma was used as the positive control, and the parental hybridoma and the PnP-mRuby cells as the negative controls (supernatant serial dilution 1:5)

Table 2
gRNA target sequences for mRuby

gRNA ID	*Target sequence* 5′ → 3′ including PAM	Description
mRuby-J	*GTCATGGAAGGTTCGGTCAACGG*	mRuby targeting for sAb HDR donor incorporation
mRuby-K (*see* **Note 37**)	*CATGCCGTTGATCACCGCCTGG*	mRuby targeting for sAb HDR donor incorporation

(b) splicing with IgG Fc region, and if both occurred, a very small population (0.5–1%) will display concurrent loss of mRuby signal and gained antibody staining (*see* Fig. 7b).

6. Isolate the RNA and synthetize the complementary DNA (cDNA) from sample(s) and controls.

   Mix:

   - 500 ng RNA.
   - 1 µl dNTP.
   - 0.5 µl oligo(dT).
   - H_2O to 14.5 µl.

7. Heat in a thermal cycler at 65 °C for 5 min, then add:
   - 4 µl 5× RT buffer.
   - 0.5 µl Ribolock RI.
   - 1 µl Maxima RT.
8. Incubate in a thermal cycler at the following conditions:
   - 50 °C for 30 min.
   - 85 °C for 5 min.
   - 4 °C until removal.
9. PCR amplify the spliced product by using a forward primer binding in the light chain signal peptide, and a reverse primer within the IgG constant region. Set up the PCR as follows:
   - 7.5 µl KAPA HiFi HotStart 2× ReadyMix.
   - 0.45 µl forward primer (10 µM).
   - 0.45 µl reverse primer (10 µM).
   - 1 µl cDNA.
   - 5.6 µl H_2O.
10. Incubate in a thermal cycler at the following conditions:
    - 95 °C for 3 min.
    - 35 cycles:
      - 98 °C for 20 s.

    - 60–75 °C for 15 s.
    - 72 °C for 15–60 s/kb.
  - 72 °C for 1 min/kb.
  - 4 °C until removal.

  Check the splicing outcome by running on an agarose gel (*see* Fig. 8a). Verify correct splicing by Sanger sequencing after PCR cleanup or gel extraction, depending on PCR purity (*see* **Note 39**).

11. Perform FACS to isolate antibody positive cells and allow them to recover; while the method was validated to only require a bulk sorting step, single-cell sorting can also be performed if desired.
12. After expansion, the sorted cells should be checked by flow cytometry for positive expression of heavy and light chain; ideally, even in case of bulk sorting, a population with a purity of at least 90% should be obtained. If desired, an additional sorting step can be included (*see* Figs. 7b and 8b).
13. If available, the protein antigen can be fluorescently labeled and used to check for antibody specificity (*see* **Note 40**).
14. After the antibody expression-sorting step, the samples should be genotyped for genomic integration as well (*see* Fig. 8b). Like in the case of mRuby, primers can be both external to the antibody cassette, which allows comparison with the WT hybridoma and with the PnP-mRuby cell line, or one of them can bind inside the antibody construct: in this case, a band will only be present in the antibody-expressing samples (*see* **Note 41**).
15. Collect the supernatant to check for antibody secretion by ELISA (*see* Fig. 2 from Pogson et al. [17]). Isolate 10^6 cells/sample; normalize the volume of all samples according to the least concentrated by adding growth medium (*see* **Note 42**). WT hybridoma and PnP-mRuby cells should always be included as controls.
16. Pellet the cells by centrifugation, isolate the supernatant and syringe-filter it to remove any remaining cells or debris (*see* **Note 43**).
17. Perform all ELISA incubations either at room temperature (RT) for 1 h or at 4 °C O/N.
18. Proceed as follows:
    - Coat an ELISA plate with 50 μl anti-mouse-IgK antibody in PBS, concentrated 4 μg/ml.
    - Block plate with 360 μl Blocking buffer, and wash 3× with Washing buffer.

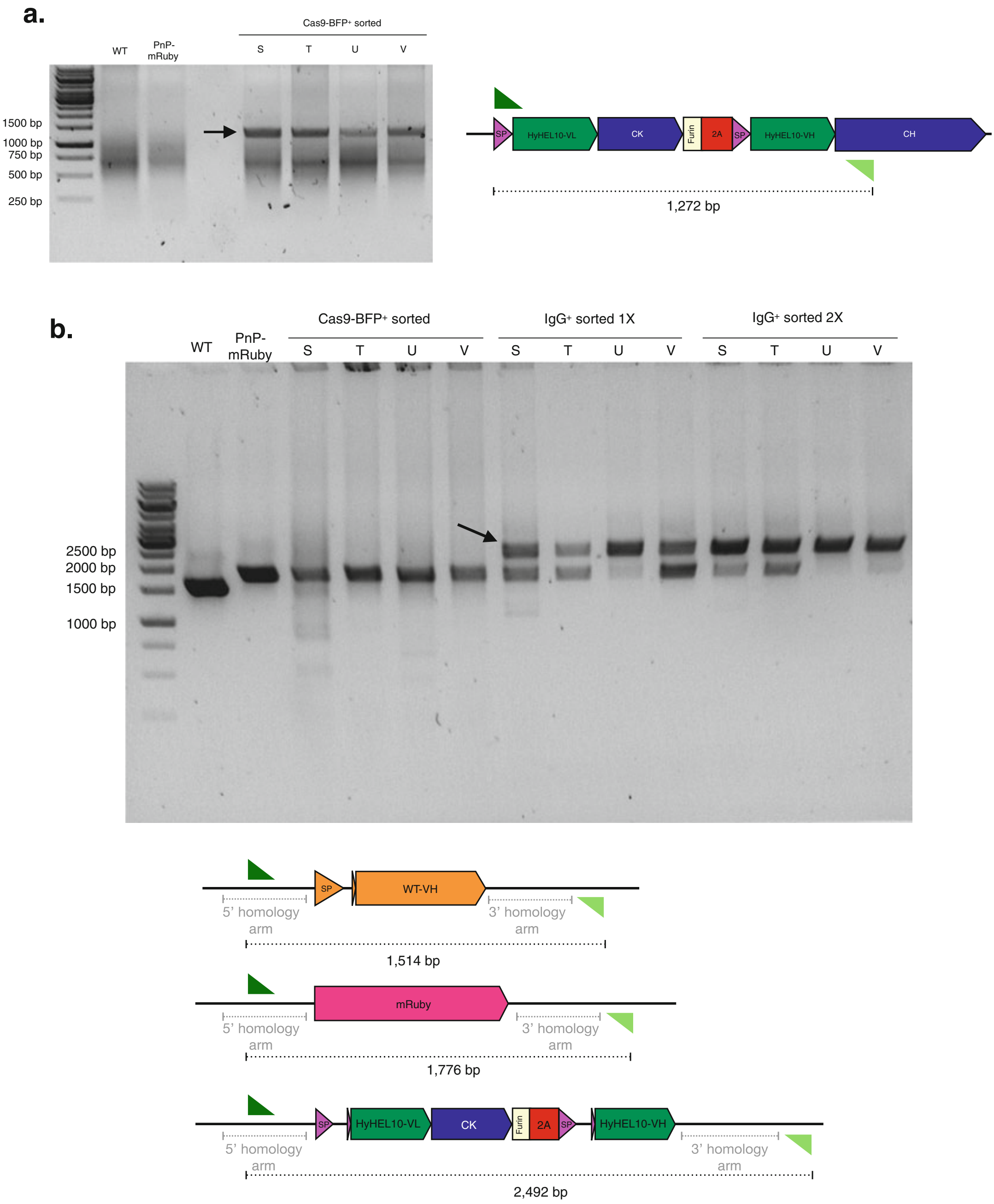

Fig. 8 Genotyping analysis of the PnP-HyHEL10 samples; genotyping analysis is shown for samples obtained using HyHEL10-sAb in its plasmid form (S, T) and linear (U, V, *see* Fig. 7). (**a**) cDNA amplification shows splicing with the IgG2C constant region (CH); the correct band, present only in the PnP samples, is indicated by the black arrow. (**b**) gDNA amplification with primers external to the fragment to be exchanged, but the forward primer inside the 5′ homology arm shows progressive enrichment of the HyHEL10-sAb band (black arrow) along with the subsequent sorting steps. This genotyping PCR is performed with the same primers used to verify integration of mRuby, as shwon in Fig. 6c (PCR 'B')

- Add supernatant: start with undiluted sample (75 μl) and perform a 1:3–1:5 serial dilution (*see* **Note 44**) in Blocking buffer; after supernatant incubation, wash 3× with Washing buffer.
- Add 50 μl anti-mouse-IgH-HRP antibody concentrated 1.7 μg/ml (*see* **Note 45**) in Blocking buffer; wash 3× with Washing buffer.
- Add 50 μl peroxidase substrate (TMB), and when the blue color develops, block the reaction with 50 μl Stopping solution.
- Measure absorbance at 450 nm.

19. The ELISA procedure can be performed also to test antigen specificity (*see* Figs. 7c and 3 from Pogson et al. [17]). In this case, the plate would be coated with the protein antigen at the same concentration, and all the steps repeated as described (possibly varying the supernatant dilution factor). An anti-IgK- or IgH-HRP can both be used as secondary reagents (*see* **Note 45**).
20. The antibody can now be purified with the desired method and used for downstream applications (e.g., ELISA).

4 Notes

1. All the cloning procedures included in this protocol, as well as recombinant constructs and genotyping experiments were assisted by using the commercial software Geneious (version R9).
2. We typically culture our hybridoma cells in 25 cm^2 cell culture flasks and split them every 2–3 days at a 1:10–1:20 dilution. A digital cell counter (e.g., Bio-Rad TC10), measuring cell viability, is advisable; alternatively, the cells can be counted with a Neubauer chamber. In our experience, healthy hybridomas usually reach a viability higher than 90% up to a density of $1–2 \times 10^6$ cells/ml. However, we recommend to make sure that the density is at least one order of magnitude lower ($1–5 \times 10^5$/ml) whenever a transfection is to be performed because a higher fraction of the cells will likely be in their dividing phase, increasing the likelihood of HDR. When carrying out a flow cytometry staining, low density cells will have a significantly lower level of unspecific, background labeling.
3. The gDNA extraction reaction can be scaled down or up, as long as the ratio 10^6 cells/100 μl is kept. gDNA in QuickExtract solution is extremely viscous and a precipitate can form over time. This is not problematic as long as care is taken not to

touch it when pipetting the DNA. The presence of the precipitate would in fact likely inhibit the PCR.

4. The annealing temperature of the PCRs should ideally be optimized using a thermal cycler with the gradient function. We suggest to run the optimization PCRs in a smaller volume than the one indicated, and once the optimal temperature has been established, perform the reaction in 50 μl (KAPA HiFi) or 30 μl (KAPA2G) as described.
5. In our hands, both KAPA HiFi and KAPA2G mixes have been successful for genotyping purposes, and the better performance of one over the other should usually be established empirically. Therefore, when optimizing a particular genotyping PCR, we suggest to try both.
6. Sanger sequencing reads will typically lose quality after approximately 1 kb. When the PCR amplicon to be sequenced exceeds this size, make sure to submit multiple sequencing reactions with primers able to cover the whole region.
7. Care should be taken that the 3′ (downstream) homology arm starts immediately after the last in-frame amino acid of the JH region. The presence of extra bases before the splice site should be carefully avoided as this will ensure that the sAb construct used in the PnP system will keep the correct reading frame.
8. In Pogson et al. [17], the Cas9 target site used to exchange the VH with mRuby was downstream of the JH region, inside the 3′ homology arm. When functional sites are not found inside the VDJ region, this option is also acceptable, always taking into account that the more distal the cleavage site is from the cassette to be exchanged, the less likely it is that successful HDR will occur.
9. To speed up the cloning procedure, make sure to have the pX458 (or analogous) plasmid purified by the time the oligonucleotides are received. For cloning purposes, a miniprep is satisfactory as a source of plasmid DNA.
10. In restriction and Gibson cloning procedures, always include a negative control without the insert; for both ligation and Gibson Assembly, NEB recommends a 20 μl reaction, but 10 μl can be used to save material, without losses in efficiency.
11. Prepare bacterial frozen stocks after cloning every new vector. Mix 500 μl of an O/N culture with an equal volume of 50% (v/v) glycerol and store the frozen stocks at −80 °C.
12. The transfection protocol was optimized for nucleofection. However, other transfection methods, including both electrotransfection and chemical transfection, can be used as well, with consideration for high transfection efficiency and cell viability. Due to the presence of a fluorescent reporter protein

in pX458, transfection efficiency can be easily assessed prior to any gene editing experiment (even without having cloned any guide RNA in the plasmid). When performing nucleofection, a 5 min incubation at RT after transfection (prior to moving the cells to the culture vessel) can be included if this improves cell viability.

13. In order to avoid cell clumping, it is advised to filter the cells prior to sorting using a 30 μm disposable filter (*see* Subheading 2) in a tissue culture hood. This step should be performed before any flow cytometry sorting experiment included in this protocol.
14. The fraction of cells that will be GFP$^+$ (Cas9$^+$) after transfection depends on a number of factors (cell cycle stage at transfection, transfection efficiency, etc.). We typically obtain results that can vary between 10 and 50%. Always keep track of the number of cells sorted as a number higher than 10^5 can be easily recovered in a fresh 24-well plate. 48-well plates should be preferred for immediate recovery of smaller numbers, to be able to transfer them to larger wells in the following days after they settle down and start growing.
15. If the gRNA is functional, the Surveyor assay will result in two bands corresponding to the two fragments in which the amplicon is cleaved by the Surveyor nuclease, the "breaking point" corresponding to the gRNA target site. For an ideal amplicon of 1 kb, expected fragments of approximately 300 and 700 bp size each are optimal to ensure a good resolution of the uncleaved product as well as the two cleavage bands on an agarose gel. However, smaller amplicons are also suited for Surveyor, and 1 kb should generally be taken as an upper size limit.
16. It is important to meticulously optimize this PCR so that no unspecific bands are present. Besides facilitating the interpretation of the Surveyor outcome, this optimization step allows DNA recovery by PCR cleanup instead of gel purification, which typically ensures a better yield, required for the Surveyor assay.
17. A negative control is important to validate the Surveyor result. Possibilities include: (1) cells not transfected or transfected with pX458 without a gRNA and incubated in presence of the Surveyor nuclease; and (2) cells transfected with pX458 with the gRNA of interest but without the Surveyor nuclease (in this case, the hybridized pool can be split in two and the nuclease and enhancer are added only to one half, adjusting the volumes accordingly).
18. Other products following the same principle as Surveyor (e.g., T7 Endonuclease I, NEB) can be used as well.

19. Optionally, the mutational activity of a specific gRNA can be evaluated by checking the presence of indels by next-generation sequencing (NGS) or decomposition (https://tide.nki.nl). However, a positive Surveyor outcome is sufficient to select a successful gRNA for the subsequent steps.
20. The elimination of methylated (parental) DNA prevents its propagation along the cloning procedure and reduces the occurrence of background (negative) colonies.
21. The amount of DNA indicated was empirically observed to be functional, but can be modified. However, an insert: backbone molar ratio of 3:1 should be preserved. The manufacturer's recommendations for the total amount of DNA are 0.02–0.5 pmol/20 μl reaction.
22. The PCR previously designed to amplify the insert can be used for colony PCR. Instead of adding the plasmid template, pick a clone from the agar plate and repeatedly pipet it into the PCR mix. Mark the position of the colonies on the plate so that the ones that yield a successful PCR can be subsequently grown and sequenced.
23. In our experience, Gibson cloning for HDR template construction is very efficient. However, it is based on PCR and, therefore, polymerase errors should be taken into consideration. Whenever cloning a new insert between the homology arms, successful colonies should be sequenced making sure to cover the whole insert as well as the homology arms, in order to confirm that the sequence was preserved during the backbone amplification step.
24. We have preserved the intronic regions between signal peptide and start of the V(D)J region for VL and VH to keep the native antibody configuration found in the B cell genome. In case of space constraints, introns can be removed. Such an approach was observed to work also with already spliced sAb. Concerning the 2A peptide choice [23], we have been able to obtain successful antibody expression both with F2A (published [17]) and with T2A.
25. Make sure that at least 3–5 × 10^6 cells recover before proceeding since there have to be enough cells for a preliminary staining and the subsequent sorting step.
26. The staining antibody should initially be titrated using WT hybridoma cells. In every labeling experiment, always include transfected and labeled cells (test samples) as well as nontransfected, labeled cells and nontransfected, unlabeled cells as negative controls.
27. The recovery of hybridoma cells from single-cell sorting should be estimated to take at least 1 week (until the colonies can be

moved from a 96-well plate to a higher volume vessel), during which the cells should be monitored every 2–3 days to check for growth. A recovery rate of ~30% is considered successful, and for this reason sorting more than one plate per experiment (e.g., 2–3) increases the odds of success.

28. The clones are sorted based on lost IgK expression, which does not necessarily result from multiplexed cleavage. For this reason, only a small fraction of the recovered clones are expected to show the correct band.

29. Create hybridoma frozen stocks by isolating 10^6 cells by centrifugation and resuspending them in 1 ml of Freezing medium (*see* Subheading 2). Store short-term at −80 °C, and move to a liquid nitrogen (LN2) tank for long-term storage. Whenever a new cell line is created, we recommend to create at least 4–5 vials of the frozen stock.

30. Linearized DNA is more efficient than plasmid for HDR knockin. However, we have observed that plasmid DNA can as well be integrated in our workflow. Whenever the linearization PCR results in a low yield and trouble-shooting becomes too demanding, we suggest to attempt plasmid transfection as well. A small percentage of cells incorporating mRuby is sufficient, since they will be single-cell sorted anyway.

31. 1 ml PCR mix (reported with 5% excess) should likely allow to purify enough linear DNA for transfection. If this is not the case, further scale up the reaction.

32. We reported the primers used by us for the linearization process (*see* Table 1), which we suggest to try with the described cycling conditions. However, especially when using a custom antibody donor (*see* Subheading 3.4), PCR purity can be suboptimal. In this case, a further temperature optimization should be performed until the product is clean and no other bands are observed.

33. Sequencing not only mRuby, but also the homology arms is important to make sure that the homology arms in the platform cell line are preserved for the integration of antibody constructs.

34. Choose light chain and heavy chain labeling antibodies compatible with each other and with mRuby for multi-color staining.

35. We hereby report an optimized workflow, which we consider more suitable and convenient to carry out by the user. When performing the experiments reported in Pogson et al. [17], the procedure and materials used were slightly different, albeit leading to the same final result. (1) mRuby knockin via HDR was performed before deleting the light chain, opposite to

what is reported in this protocol. The order of the two steps is not so critical, as long as both steps are correctly executed and the outcome thoroughly characterized. (2) The published version of the PnP-mRuby cell line does not have a polyA terminator after mRuby's coding sequence (as shown in Figs. 7 and 8), but we strongly encourage its incorporation, as described in this protocol. This feature will substantially boost cellular mRuby fluorescence due to higher protein expression levels, and render the signal less sensitive to cell density.

36. The experiments published in Pogson et al. [17] and shown in Figs. 7 and 8 to generate PnP-sAb cell lines were performed with a version of pX458 where GFP had been replaced by BFP prior to cloning mRuby-targeting gRNAs. The strategy is exactly the same whichever version is chosen, as long as the flow cytometry scanning and sorting steps after transfection are adjusted with the appropriate laser and filter.
37. The targeting sequence of gRNA mRuby-K is 19 bp long because, when designing the oligonucleotides to clone the gRNA in the px458-BFP plasmid, the 5′ C in the 20 bp target site was replaced with a G as explained in Ran et al. [21]. As we observed empirically in other contexts, however, the G can be added when not naturally present also by preserving the whole 20 bp target site (therefore having a 21-nucleotide (nt) long gRNA).
38. In order to avoid interference with possible residual pX458, it is better at this stage to choose a labeling antibody not overlapping with the GFP (or BFP) spectrum.
39. Splicing can be assessed by the presence of the correct band; however, the sequencing results should be carefully analyzed to check for the correct frame of both variable and constant regions. While this step can also be performed at a later point (i.e., after sorting the antibody positive cells), including it at this stage will help to verify the authenticity of the positive population observed by flow cytometry. While the instructions in this protocol recommend the use of the Kapa HiFi HotStart 2X ReadyMix, successful cDNA amplification at this stage was obtained also with Taq DNA Polymerase (NEB), as in the case of the gel shown in Fig. 8a as well as the results published in Pogson et al. [17].
40. In our experience, antigen labeling typically displays a lot of unspecific background staining of negative cells. For this reason, we do not recommend to add this step before sorting antibody positive cells. When labeling the sorted cells with the fluorescent antigen, make sure to include anti-heavy and/or light chain antibody as costaining agent(s) to positively correlate antigen signal with antibody expression.

41. Unlike cDNA amplification, where a small percentage of positive cells is sufficient since they produce a large amount of RNA, a significant level of enrichment is necessary for the PCR on gDNA to show evidence of integration. For this reason, it is suggested to wait until the cells have been sorted for antibody expression. Even in this case, when amplifying with external primers, the mRuby band can still be visible and can actually be quite prominent when the cells are mostly antibody-positive (and mRuby-negative) when analyzed by flow cytometry (*see* Figs. 7 and 8). The reason is the PCR bias toward smaller products when the template originates from a heterogeneous pool of cells.
42. Because of normalization, cell number and volume will be equal for all the samples. This way, differences in ELISA signal will be granted to reflect actual differences in antibody productivity. As an example: sample A has a concentration of 1×10^6 cells/ml, and the necessary volume for 1×10^6 cells is 1 ml; sample B is half as concentrated (5×10^5 cells/ml) and the necessary volume is therefore doubled (2 ml). To normalize the samples, 1 ml of growth medium needs to be added to sample A in order to render them equal in terms of volume and concentration (1×10^6 cells/2 ml).
43. Samples for ELISA should ideally all be cultured under comparable conditions (i.e., seeded at the same time).
44. In both antibody secretion and antigen binding ELISA, the serial dilution factor should be determined empirically. We suggest to try 1:5 or 1:3 as starting options.
45. We provide information about the antibodies we use to test the supernatants by ELISA. However, any equivalent setup can be chosen instead as long as full-length IgG is detected. For example, antibodies can be inverted (coating with anti-IgH, secondary anti-IgK-HRP). When doing antigen ELISA with hen egg lysozyme as shown in Fig. 7c, we use an anti-IgK-HRP as the secondary antibody (*see* Subheading 2).

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Chapter 8

Protein Expression via Transient Transfection of Mammalian Cells in a WAVE Bioreactor

David I. Fisher

Abstract

Large culture volumes are often required when expression constructs are particularly low-yielding or when end-uses require significant amounts of material. In these cases, a single homogenous culture is usually more convenient, in terms of both consistency of expression and labour/resource requirements, than multiple parallel cultures. Using a WAVE Bioreactor culture volumes as high as 500 L may be achieved in a single vessel. Here we describe the transfection of 293-6E cells in a disposable 50 L Cellbag on a WAVE Bioreactor platform to produce recombinant protein. The methods described herein may be adapted, with suitable optimizations, for other suspension-adapted mammalian cell lines.

Key words Transient transfection, Recombinant protein, Polyethylenimine, WAVE Bioreactor, Mammalian, Cell culture

1 Introduction

Delivery of recombinant genes into mammalian cells is commonly achieved via transfection with plasmid DNA, either through association with a suitable transfection reagent or electroporation. For large-scale, multiliter expression, polyethylenimine (PEI) is an extremely cost-effective option [1]. Traditional transient transfection techniques involve the formation of polyplexes with a transfection reagent prior to addition to a cell culture. More recently, however, direct transfection techniques, whereby DNA and transfection reagents are added sequentially to the culture, have become more common [2, 3].

In order to carry out large, single vessel transfections, we have invested in WAVE Bioreactors. A WAVE Bioreactor consists of a rocking platform with integrated heater and surface temperature sensors suitable for use with disposable, single-use Cellbags. The rocking platform induces a wave action within the culture to facilitate mixing and gas transfer [4, 5]. This removes the need for an impeller or similar as used in traditional stirred-tank bioreactors.

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The Cellbag is a presterilized, inflated bag that provides a robust, disposable cell culture vessel. While reusable versions of the technology have recently been described [6], this chapter focusses on the original single-use Cellbag.

The 293-6E cells used in this chapter were developed at NRC-BRI in Montreal, Canada. Based on a suspension-adapted HEK293 cell line they constitutively express a truncated form of EBNA1 to enable episomal replication of *oriP*-based plasmids [3].

2 Materials

Prepare all solutions using ultrapure water (18 MΩ cm at 25 °C) and analytical grade reagents. Store all solutions at 4 °C, unless otherwise stated.

2.1 Transient Transfection

1. Complete cell culture medium: FreeStyle™ F17 Expression Medium (Thermo Fisher Scientific) supplemented with 0.004 M L-glutamine, 0.1% (v/v) Pluronic® F-68.
2. Linear polyethylenimine hydrochloride (PEI Max 40 K; Polysciences): 5 g/L in ultrapure water. Filter-sterilize through a 0.22 μm filter. Leave one aliquot at 4 °C for current use and store any remaining aliquots at −20 °C.
3. Expression plasmid DNA: Transfection grade at 1 mg/mL in TE (0.01 M Tris, 0.001 M EDTA, pH 8). For best results with EBNA1 expressing cell lines, use a plasmid with an *oriP* origin of replication (*see* **Note 1**).
4. 10% HyPEP™ 1510 (w/v) in ultrapure water. Filter sterilize through a 0.22 μm filter.
5. Cellbag 50 L, manifold and transfer bottles (*see* Fig. 1). The manifold and transfer bottles should be presterilized by autoclave.
6. Sterile Luer lock syringes (20 mL & 5 mL).
7. 293-6E cells (NRC-BRI).

2.2 SDS-PAGE Analysis

1. MES SDS Running Buffer: 0.05 M MES, 0.05 mM Tris, 0.1% (w/v) SDS, 0.01 M EDTA.
2. LDS (lithium dodecyl sulfate) Sample Buffer (4×): 0.424 M Tris–HCl, 0.564 M Tris, 8% (w/v) LDS, 40% (w/v) glycerol, 0.002 M EDTA, 0.0009 M SERVA Blue G250, 0.0007 M Phenol Red. Store at room temperature.
3. NuPAGE™ 10% Bis-Tris protein gels, 1.0 mm, 10-well (Thermo Fisher Scientific).
4. SeeBlue™ Plus2 Pre-stained standard (Thermo Fisher Scientific).
5. InstantBlue™ Protein Stain (Expedeon).

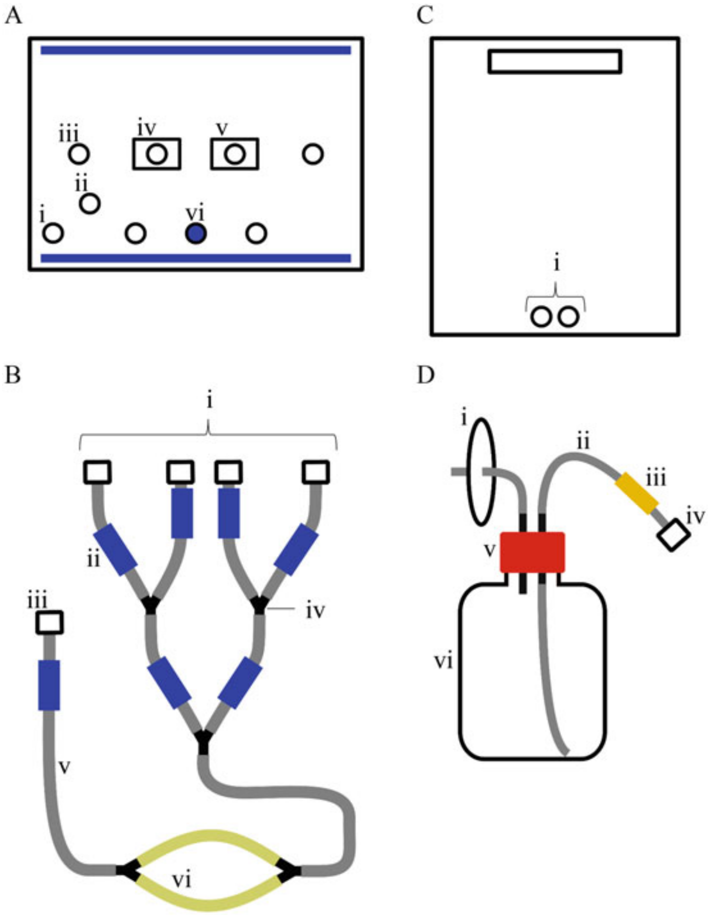

Fig. 1 Schematics of Cellbag 50 L, manifold, media bag and transfer bottle. (**a**) (Cellbag 50 L. (i) main inlet (1/4 in. × 7/16 in. × 39 in.) female MPC; (ii) addition port (3/16 in. × 3/8 in. × 2 in.) female Luer; (iii) addition port (3/16 in. × 3/8 in. × 2 in.) female Luer; (iv) air outlet and filter; (v) air inlet and filter; (vi) one-way, needless sample port (3/16 in. × 3/8 in. × 2 in.) female Luer. The remaining ports are not required for this process. (**b**) Manifold. (i) secondary hose connections, female MPC (×4); (ii) tubing clamps (×7) (e.g., Keck KT); (iii) primary hose connection, male MPC; (iv) barbed Y fitting (×5); (v) hose (eg. Masterflex HV-96410-18); (vi) pump hose (e.g., Masterflex HV-06508-18). Manifold designed for dual pump head. For single pump head, omit double tubing at vi. (**c**) Media bag 20 L (Thermo Fisher Scientific Universal Bag B). (i) outlet hoses (male MPC × 1, female MPC × 1). (**d**) Transfer bottle(s). (i) sterile 0.2 μm filter; (ii) hose (e.g., Masterflex HV-96410-25); (iii) tubing clamp (e.g., Keck KT); iv) outlet connection, male MPC; (v) Duran® stainless steel 2-Port connector cap GL 45, (vi) Duran® 2 L bottle. When assembling a manifold and transfer bottles, all MPC fittings should be secured to hoses with cable ties to prevent leakage due to pressure build-up

3 Methods

Here we describe the transfection of 293-6E cells, though this process is generally applicable (with optimization) for culture and transfection of many other suspension adapted mammalian cell lines. *See* **Note 2** for optimized PEI-based transfection parameters for some other cell lines.

Values are given for a 50 L Cellbag on a WAVE Bioreactor 20/50 platform (final volume ~23 L). This method can be scaled pro rata as required for different Cellbag sizes (*see* **Note 3**).

Stock 293-6E cells should be routinely cultured in vented shake flasks or vented 850 cm^2 roller bottles. Incubate at 37 °C, 5% CO_2, 140 RPM (in an orbital incubator with 25 mm orbit). Stock cells should be cultured in the presence of 25 mg/L G418, but this should be omitted prior to scaling for transfection.

3.1 WAVE Bioreactor Preparation

1. In a Class II Microbiological Safety Cabinet (MSC), remove the Cellbag 50 L from its external plastic packaging. Ensure that all ports are capped and close clamps on all lines except for those on the air inlet and outlet.
2. Aseptically attach the primary manifold hose to the Cellbag 50 L, via the female MPC coupling on the main inlet (*see* **Note 4**). Then use one of the four secondary hose connections to attach the media bag.
3. Transfer the Cellbag 50 L to the WAVE Bioreactor 20/50 platform and clamp the bag in place.
4. Attach the filter heater to the air outlet filter to prevent condensation and attach the air inlet to your 5% CO_2 gas supply.
5. Inflate the Cellbag 50 L at 0.5 mL/min. The Cellbag has a pressure valve built into the air outlet that will keep the bag inflated whilst preventing excess pressure build-up.
6. Once inflated, reduce the airflow to 0.25 mL/min.
7. Open all clamps that lead from the Cellbag 50 L to your medium bag. Using a calibrated peristaltic pump, transfer 5 L of complete cell culture medium to the Cellbag (*see* **Note 5**). Once the medium has been transferred, reclose the clamps.
8. To prewarm the medium, set the WAVE Bioreactor 20/50 platform to 37 °C, 22 RPM, 7° rocking angle (*see* **Note 6**).

3.2 Transient Transfection of 293-6E Cells

1. Count stock 293-6E cells and calculate the volume of cells require to seed 7 L at 4×10^5 cells/mL. Ideally stock cells will be at ~1.5×10^6 cells/mL and therefore volume of cells required will be <2 L.
2. In a Class II MSC, aseptically decant the appropriate volume of stock 293-6E cells into a 2 L transfer bottle.

3. Aseptically attach the transfer vessel to one of the remaining three secondary manifold hose connections (*see* **Note 7**).
4. Open all clamps leading from the Cellbag to the transfer bottle. Using your gas supply, or a hand pump, transfer the cell culture to the Cellbag (*see* **Note 8**). Once the cells suspension has been transferred, reclose the clamps.
5. As described above, transfer sufficient complete cell culture medium to bring the final volume of cell suspension to 7 L. Once the media has been transferred, reclose the clamps.
6. Incubate the cell suspension for 48 h at 37 °C, 22 RPM, 7° rocking angle, 5% CO_2.
7. Using a 5 mL Luer lock syringe, remove an aliquot of culture via the one-way sample port and perform a cell count (*see* **Note 9**).
8. As described above, transfer sufficient complete cell culture medium from the media bag to dilute the culture to 8×10^5 cells/mL. It is important that the total volume not exceed 14 L at this stage. If necessary, cells can be removed back to a transfer bottle prior to dilution. Once the media has been transferred, reclose the clamps.
9. Incubate the cells as previously (37 °C, 22 RPM, 7° rocking angle, 5% CO_2) for 24 h.
10. As described in **step 7**, perform a cell count and ensure the culture is at an appropriate density for transfection ($1.6–1.8 \times 10^6$ cells/mL). Cell viability should be >95%. If parameters are met, proceed with transfection. If cells are at a lower density, continue to incubate and monitor until acceptable density is reached.
11. In a Class II MSC, aseptically fill a 20 mL Luer lock syringe with sufficient DNA to yield 0.75 mg/L [e.g., 10.5 mg for 14 L; 10.5 mL at 1 mg/mL] and cap. Similarly fill a 20 mL Luer lock syringe with sufficient PEI Max 40 K to yield 2.8 mg/L [e.g., 39.2 mg for 14 L; 7.84 mL at 5 mg/mL] and cap.
12. Briefly stop the WAVE platform and aseptically attach the DNA-containing syringe to one of the available addition ports on the Cellbag (*see* Fig. 1). Restart the WAVE platform, open the appropriate clamp and add the DNA to the culture. Reclamp prior to removing the syringe.
13. Repeat **step 12** for the PEI Max 40 K-containing syringe.
14. Continue to incubate the cells as previously (37 °C, 22 RPM, 7° rocking angle, 5% CO_2 (0.25 L/min)) for 24 h.
15. As described in **step 7**, perform a cell count. At this stage you should observe significant growth arrest (the cells will not have

doubled in density overnight) though a high viability should be maintained.

16. Determine the volume of HyPep™ 1510, based on the transfection and feed volumes. The culture should be fed with 60% of the transfection volume [e.g., 8.4 L of feed for 14 L transfection]. This feed should consist of complete cell culture medium supplemented with sufficient HyPep 1510 to yield a final concentration of 0.3% (w/v) [e.g., 0.672 L for final volume of 22.4 L].
17. As described above, transfer sufficient complete cell culture medium from the medium bag to bring the final culture volume to the values calculate in **step 16** [e.g., 8.4–0.672 ≈ 7.7 L for the 14 L transfection]. Once the medium has been transferred, reclose the clamps.
18. Aseptically attach the HyPEP™ 1510 transfer vessel to one of the two remaining secondary manifold hose connections (*see* **Note 7**).
19. Open all clamps leading from the Cellbag to the transfer bottle. Using your gas supply, or peristaltic pump, transfer the HyPep™ 1510 to the Cellbag. Once the feed has been transferred, reclose the clamps.
20. Due to the increased volume it is necessary to increase the rocking angle to ensure that the wave motion is maintained (*see* **Note 6**). Thus, incubate the culture at 37 °C, 22 RPM, 7.5° rocking angle, 5% CO_2 (0.25 L/min) for a further 6 days (*see* **Note 10**).
21. Once the expression phase has been completed, perform a cell count as described in **step 7**. At this stage a high viability should still be maintained and the cells should reach ~1×10^7 cells/mL.
22. Harvest the cell suspension by removing the manifold from the Cellbag and draining the culture into 1 L or 2 L centrifuge pots (*see* **Note 11**). Leaving the bag inflated will aid the removal of cell suspension from the Cellbag.
23. Clarify the culture medium by centrifugation @ 10,000 × *g*, 30 min, 4 °C. Decant supernatant into appropriate storage bottles (*see* **Note 12**).

3.3 SDS-PAGE Analysis

For secreted proteins, we recommend SDS-PAGE analysis of crude culture supernatant using standard practices (*see* **Note 13**).

1. Add 10 μL LDS Sample Buffer (4×) to 30 μL of clarified culture supernatant (*see* **Note 14**).
2. Set up a NuPAGE™ 10% Bis-Tris protein gel (1.0 mm, 10-well) according to manufacturer's instructions.

3. Load 20 μL of sample along with appropriate molecular weight markers, such as SeeBlue™ Plus2 Pre-stained Protein Standard.
4. Run gel for 35 min at constant 100 V.
5. Remove gel and stain in 10 mL InstantBlue Protein Stain with continuous mixing for >1 h (*see* **Note 15**).
6. Once stained, wash the gel with water and take an appropriate image.

4 Notes

1. Due to the fact the 293-6E cells express a truncated form of Epstein-Barr nuclear antigen 1 (EBNA1) [3], expression levels are improved by use of a plasmid containing the *oriP* origin of replication from Epstein-Barr. Use of an *oriP*-based plasmid enables episomal replication and therefore semi-stable maintenance of the gene of interest. Plasmids recommended for use with the 293-6E system included the pTT [7] series and pDEST12.2-*OriP* [8].
2. We have optimized PEI Max-mediated transfection conditions for CHO-EBNA-GS cells [8] and Expi293F cells. As with 293-6E cells, CHO-EBNA-GS can be successfully transfected by direct addition of DNA (0.5 mg/L) and PEI Max (7 mg/L) to cells at 1.0–1.2 × 10^6 cells/mL. We have been unable to optimize transfection of Expi293F cells via direct addition, as such we recommend a priori complex formation using DNA (1.5 mg/L) and PEI Max (6 mg/L) incubated at room temperature in OptiMEM at 10% of the transfection volume for 15 min before adding to cells at 3.5–4.2 × 10^6 cells/L.
3. Some groups routinely monitor glucose and/or dissolved oxygen during their WAVE cultivations. This allows for more precise optimization and control of conditions that may give rise to improved product titers. However, we do not routinely monitor any parameters besides cell number and viability, as such the protocol described does not include any additional monitoring. The original publication of the WAVE Bioreactor technology indicates that by controlling appropriate parameters (rocking and CO_2 supply) dissolved oxygen control is unnecessary [4].
4. When making any connections, whether within the confines of a sterile MSC or on the open bench, be sure to spray the connections with a suitable disinfectant (i.e., 70% ethanol) prior to opening. Before making connections on the open bench it is a good idea to practice within a MSC to ensure

you can make the connections as quickly as possible to reduce the potential for contamination.

5. Alternatively, an integrated load cell can be used to determine the weight of culture within the bag and can thus be used as a surrogate for volume.
6. It is critical that the motion of the rocking platform be appropriately set to ensure robust oxygen transfer and mixing [4]. The rocking rate should be set at the highest value that avoids excessive foaming. The rocking rate or angle should be adjusted to ensure a wave is visible across the culture; with the culture "overlapping" at the bottom of the motion. In general, for a WAVE Bioreactor 20/50 and a 50 L Cellbag, a rocking angle of 6–8 degrees is sufficient along with a rocking rate of 15–25 RPM.
7. If your WAVE platform is sufficiently adjacent to a MSC, it is worth ensuring your manifold is long enough that you can continue to attach transfer bottles within the confines of the cabinet. Otherwise you will need to make these attachments aseptically on the open bench (*see* **Note 4**).
8. It is possible to use the peristaltic pump to pump cells from the transfer bottles, but we do not recommend this due to the increased shear stress. The use of compressed gas (air or 5% CO_2) or a hand pump attached to the transfer bottle results in a more gentle transfer. If your cells are sufficiently robust you may not see a difference in viability between these methods.
9. Try to keep the culture in motion as much as possible during sampling and only stop the rocking motion briefly to avoid cell settling. As with all connections, even though this is a one-way port, we recommend wiping with disinfectant (i.e., 70% ethanol) prior to attachment. It is important to realize that the blue sample port itself screws onto the Cellbag tubing in the same orientation as the Luer lock. For this reason it is imperative that you ensure that the port is tightly attached to the tubing and hold it in place as you unscrew the cap or syringe. Failure to do so can cause the port to unscrew from the Cellbag and your culture to be forced out of the sample tubing, in addition to exposing your culture to the open air. We recommend drawing a small sample through the tubing to discard directly prior to sampling to ensure your actual sample is taken from the Cellbag culture, and not liquid trapped in the tubing.
10. For secreted targets in 293-6E cells using the process outlined, we find 5–7 days post-transfection gives maximal expression before observing negative viability effects. For intracellular targets we recommend harvesting 2–4 days post-transfection.
11. For secreted targets it is also possible to harvest the culture medium using tangential flow filtration (TFF) if you have that

option available. To perform TFF you can connect your TFF device to your Cellbag via the MPC coupling and pump the contents through the device.

12. For intracellular targets you will need to keep the pellets rather than supernatant. We recommend using harvest bags/liners in your centrifuge pots for this purpose.
13. For particularly low expressing targets, you may need to perform a purification step, such as affinity purification, to increase the concentration sufficient to see by Coomassie-stained gel. Alternatively, you can perform a western blot if you have an appropriate antibody for your target protein. For intracellular proteins we recommend standard lysis and purification prior to visualization on a gel.
14. For secreted targets we do not find it necessary to use reductant or heating prior to loading and visualization. For intracellular targets we recommend both reductant and heating to >95 °C.
15. For convenience we routinely stain overnight to ensure maximal, even staining, though 1 h is usually sufficient to see appropriate bands.

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Chapter 9

CHO and HEK293 Cultivation and Transfection in Single-Use Orbitally Shaken Bioreactors

David L. Hacker, Laurence Durrer, and Soraya Quinche

Abstract

Chinese hamster ovary (CHO) and human embryonic kidney 293 (HEK293) cells are the two most popular mammalian hosts for the production of recombinant proteins. In this chapter the suspension cultivation and transfection of these cells in small-scale disposable bioreactors is described. The TubeSpin bioreactor 50 and TubeSpin bioreactor 600 are designed for the cultivation of suspension cells by orbital shaking and have maximum working volumes of about 15 mL and 400 mL, respectively.

Key words CHO cells, HEK293 cells, Disposable bioreactors, Suspension cells, Transfection

1 Introduction

When using orbital shaking as a mixing principle for the suspension cultivation of mammalian cells there are several types of vessels to choose from including Erlenmeyer and Thomson flasks and square-shaped and cylindrical glass bottles [1, 2]. For the production of recombinant proteins in mammalian cells it may be sufficient to use small volumetric scales (less than 500 mL) to generate the amount of protein needed for the intended application. Recently, conical centrifuge tubes with nominal volumes of 50 mL and 600 mL have been developed for the suspension cultivation of animal cells [3–5]. Commercially available TubeSpin bioreactor 50 (OSR50) and TubeSpin bioreactor 600 (OSR600) tubes are single-use tubes with a conical bottom and a filter cap for gas exchange. These tubes have been shown to have a low mixing time, low shear stress, and a high gas transfer rate, making them excellent choices for the small-scale cultivation of animal cells at high cell densities [6, 7].

Here, we describe methods for the small-scale cultivation and transfection of suspension-adapted Chinese hamster ovary (CHO) and human embryonic kidney 293 (HEK293) cells in OSR50 and OSR600 tubes. The transfections are based on DNA transfer in the

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presence of polyethyleneimine as previously described for large-scale transfections of these same two cell lines [8, 9].

2 Materials

2.1 Cell Culture

1. CHO-DG44 and HEK293E cells adapted to cultivation in serum-free suspension (*see* **Note 1**).
2. TubeSpin bioreactor 50 (OSR50) and TubeSpin bioreactor 600 tubes (OSR600) (TPP, Trasadingen, Switzerland) (*see* **Note 2**).
3. ProCHO5 medium without L-glutamine, hypoxanthine, thymidine, and phenol red (Lonza AG, Verviers, Belgium).
4. Ex-cell293 serum-free medium without L-glutamine and phenol red (Sigma-Aldrich, Buchs, Switzerland).
5. 50× L-glutamine and phenol red: dissolve 29.23 g glutamine and 250 mg phenol red in 1 L water. Sterilize by filtration, transfer into sterile 50-mL tubes and store frozen at −20 °C.
6. 50× HT: dissolve 680 mg of hypoxanthine and 194 mg of thymidine in 1 L water, filter and store at −20 °C in sterile 50-mL tubes.
7. 0.4% Trypan Blue solution.
8. Phosphate-buffered saline (PBS).
9. Neubauer hemocytometer.

2.2 Transfection

1. Linear 25 kDa polyethyleneimine (PEI) at 1 mg/mL: dissolve 1 g of PEI (Polysciences, Eppenheim, Germany) in 800 mL water. Adjust the pH to 3 with 1 N HCl. When the PEI is in solution, adjust the pH to 7 with 1 N NaOH. Adjust the volume to 1 L, filter and store at −20 °C in sterile 50-mL tubes (*see* **Note 3**).
2. RPMI 1640 medium containing 25 mM HEPES and 4 mM L-glutamine.
3. 10% Pluronic F-68 (Sigma-Aldrich): dissolve 100 g Pluronic F-68 in 1 L water, filter and store at −20 °C in sterile 50-mL tubes.
4. Plasmid DNA in water or TE (10 mM Tris–HCl and 1 mM EDTA, pH 7.5) at a concentration of 1–2 mg/mL (*see* **Note 4**).
5. 0.5 M valproic acid (VPA): dissolve 72.1 g of VPA in 1 L water, filter and store at −20 °C in sterile 50-mL tubes.

2.3 Equipment

1. Laminar flow hood.
2. Two incubator shakers maintained at 37 °C and 31 °C (Climo-Shaker ISF-1 XC; Kühner AG, Birsfelden, Switzerland).

3. Racks for holding OSR50 and OSR600 tubes (Kühner AG) (*see* **Note 5**).
4. Floor-model centrifuge.
5. Tabletop centrifuge.
6. Inverted phase contrast microscope.

3 Methods

3.1 Cultivation of CHO Cells in an OSR50

1. Prepare 1 L of ProCHO5 by addition of 20 mL of 50× HT and 20 mL of 50× L-glutamine and phenol red (referred to as ProCHO5 in the protocol).
2. CHO-DG44 cells are subcultivated every 3–4 days (*see* **Note 6**) by inoculation in 10 mL ProCHO5 in an OSR50 at an initial cell density of 0.3×10^6 cells/mL.
3. Determine the cell density and viability of the culture by Trypan Blue staining using a Neubauer hemocytometer and an inverted phase contrast microscope.
4. After cell counting, transfer 3×10^6 cells into an OSR50 and centrifuge at $500 \times g$ for 4 min in a tabletop centrifuge.
5. Remove the medium from the tube by aspiration. Resuspend the cell pellet in 10 mL of prewarmed ProCHO5.
6. Place the OSR50 in a rack in the incubator shaker set at 37 °C with 5% CO_2 and 85% humidity. If the shaking diameter is 5 cm, agitate at 180 rpm (*see* **Note 7**).
7. Repeat cell passing after 3–4 days.

3.2 Transfection of CHO Cells in an OSR50

1. On the day before transfection, determine the cell density and viability of the OSR50 culture.
2. Transfer 4×10^7 cells into an OSR50.
3. Centrifuge for 5 min at $500 \times g$.
4. Remove the medium by aspiration and gently resuspend the cell pellet in 20 mL of prewarmed ProCHO5. The starting cell density of the culture is 2.0×10^6 cells/mL.
5. Transfer 10 mL of the culture into a second OSR50 (*see* **Note 8**).
6. Place the two tubes in a rack within the incubator shaker and agitate overnight at 37 °C as above.
7. On the following day, determine the cell density and viability of the two cultures.
8. For each 10-mL transfection, transfer 5×10^7 cells into a clean OSR50 and centrifuge as before.

9. Aspirate the medium from the tube and resuspend the cell pellet in 10 mL of prewarmed ProCHO5.
10. Add 30 μg of plasmid DNA to the culture and mix gently by hand.
11. Add 150 μL of 25 kDa PEI (1 mg/mL) to the tube and mix gently by hand (*see* **Note 9**).
12. Place the tube in an incubator shaker at 31 °C and agitate at 180 rpm. The duration of the production phase is protein-dependent (*see* **Note 10**).
13. Following the production phase, centrifuge the culture at 1000 × *g* for 10 min (*see* **Note 11**).
14. If an intracellular protein is being produced, the medium is removed by aspiration and the cell pellet is washed once with cold PBS. After centrifugation the cell pellet is flash frozen in liquid nitrogen and stored at −80 °C as necessary.
15. If a secreted protein is being produced, the medium is removed by pipetting following centrifugation and transferred to a tube. The medium can be stored at −20 °C until needed for protein purification or analysis.

3.3 Cultivation of CHO Cells in an OSR600

1. Determine the cell density and viability of the starting culture in an OSR600.
2. Transfer 9×10^7 cells into another OSR600 and centrifuge at 500 × *g* for 4 min.
3. Remove the medium by aspiration.
4. Resuspend the cell pellet in 25 mL of prewarmed ProCHO5 by pipetting the culture up and down several times with a 25-mL pipette to ensure that cell aggregates are dispersed.
5. Add prewarmed ProCHO5 to a total volume of 300 mL.
6. Place the OSR600 in a rack in the incubator shaker at 37 °C. Agitate at 180 rpm in the presence of 5% CO_2 and 85% humidity.
7. Cells can be maintained in culture for 3–4 days before passing.

3.4 Transfection of CHO Cells in an OSR600

1. On the day before transfection, determine the cell density and viability of the culture in an OSR600 as before.
2. Transfer 8×10^8 cells into an OSR600.
3. Centrifuge for 5 min at 500 × *g*.
4. Remove the medium by aspiration and gently resuspend the cell pellet in 25 mL prewarmed ProCHO5.
5. Add prewarmed ProCHO5 to 400 mL to achieve a cell density of 2×10^6 cells/mL.

6. Place the OSR600 in a rack within an incubator shaker at 37 °C and agitate overnight as before.
7. On the day of transfection, determine the cell density and viability of the culture.
8. Transfer 15×10^8 cells to an OSR600 and centrifuge as before.
9. Aspirate the medium from the OSR600 and resuspend the cell pellet in 25 mL of prewarmed ProCHO5 using a pipette.
10. Add prewarmed ProCHO5 to 300 mL.
11. Add 900 μg of plasmid DNA to the culture and mix gently by hand.
12. Add 4.5 mL of 25 kDa PEI (1 mg/mL solution) to the culture and mix gently by hand.
13. Place the culture in a rack within the incubator shaker at 31 °C and agitate at 180 rpm. The duration of production phase is protein-dependent as indicated in Subheading 3.2.

3.5 Cultivation of HEK293E Cells in an OSR50

1. Prepare 1 L of Ex-Cell293 medium by addition of 20 mL of 50× L-glutamine and phenol red (referred to as Excell293 in the protocol).
2. HEK293E cells are subcultivated every 3–4 days (*see* **Note 6**) by inoculation in 10 mL prewarmed Ex-Cell293 in an OSR50 at an initial cell density of 0.3×10^6 cells/mL.
3. Determine the cell density and viability.
4. Transfer 3×10^6 cells into an OSR50 and centrifuge at $500 \times g$ for 4 min.
5. Remove the medium by aspiration. Resuspend the cell pellet in 10 mL of prewarmed Ex-Cell293.
6. Place the OSR50 in a rack in an incubator shaker at 37 °C and agitate at 180 rpm in the presence of 5% CO_2 and 85% humidity.

3.6 Transfection of HEK293E Cells in an OSR50

1. On the day before transfection, count the cells in the OSR50 as before.
2. Transfer 2×10^7 cells into an OSR50.
3. Centrifuge for 5 min at $500 \times g$.
4. Remove the medium by aspiration and resuspend the cell pellet in 10 mL of prewarmed Ex-cell293. The starting cell density is 2.0×10^6 cells/mL.
5. Place the tube in a rack within the incubator shaker and agitate overnight.
6. On the day of transfection, determine the cell density and viability of the culture.

7. For each 10-mL transfection, transfer 2 × 10^7 cells into an OSR50 and centrifuge as before.
8. Aspirate the medium from the tube and resuspend the cell pellet in 0.5 mL prewarmed RPMI1640 containing 0.1% Pluronic F-68.
9. Add 15 μg of plasmid DNA to the culture and mix gently by hand.
10. Add 30 μL of 25 kDa PEI (1 mg/mL solution) to the culture and mix gently by hand.
11. Place the tube in a rack within an incubator shaker at 37 °C and agitate at 180 rpm for 1 h.
12. Add 9.5 mL of prewarmed Ex-cell293 to each tube (*see* **Note 12**).
13. Add 75 μL of 0.5 M VPA, if necessary.
14. Return the tube to the incubator shaker at 37 °C and agitate as before.
15. The duration of the production phase is protein-dependent (*see* **Note 11**).
16. After the production phase, retain the cell pellet or the cell culture medium as described in Subheading 3.2.

3.7 Cultivation of HEK293E Cells in an OSR600

1. Determine the cell density and viability of the culture in an OSR600.
2. Transfer 9 × 10^7 cells into an OSR600 and centrifuge at 500 × *g* for 4 min.
3. Remove the medium by aspiration.
4. Resuspend the cell pellet in 25 mL of prewarmed Ex-cell293 using a pipette.
5. Add prewarmed Ex-cell293 to 300 mL.
6. Place the OSR600 in a rack in the incubator shaker at 37 °C and agitate at 180 rpm in the presence of 5% CO_2 and 85% humidity.
7. The culture is maintained for 3–4 days before passing.

3.8 Transfection of HEK293E Cells in an OSR600

1. On the day before transfection, determine the cell density and viability.
2. Transfer 4 × 10^8 cells into an OSR600.
3. Centrifuge for 5 min at 500 × *g*.
4. Remove the medium by aspiration and gently resuspend the cell pellet in 25 mL Ex-Cell293 using a pipette.
5. Add prewarmed Ex-cell293 to 200 mL to achieve a cell density of 2 × 10^6 cells/mL.

6. Place the OSR600 in a rack within an incubator shaker at 37 °C and agitate overnight at 180 rpm.
7. On the day of transfection, determine the cell density and viability of the culture.
8. Transfer 6 × 10^8 cells to an OSR600 and centrifuge as described above
9. Aspirate the medium from the tube and resuspend the cell pellet in 15 mL of RPMI1640 containing 0.1% Pluronic F-68 using a pipette.
10. Add 450 μg of plasmid DNA to the culture and mix gently by hand.
11. Add 900 μL of 25 kDa PEI (1 mg/mL solution) to the culture and mix gently by hand.
12. Place the tube in a rack within the incubator shaker at 37 °C and agitate at 180 rpm for 1 h.
13. Add 285 mL of prewarmed Ex-cell293.
14. Add 225 μL of 0.5 M VPA, if necessary.
15. Return the culture to the rack in the incubator shaker at 37 °C and agitate as before.
16. The duration of production phase is protein-dependent.
17. After the production phase, retain the cell pellet or the cell culture medium as described in Subheading 3.2.

4 Notes

1. The CHO-DG44 and HEK293E cells described here were adapted to serum-free suspension culture in ProCHO5 and Ex-cell293 over several months before they were used for the optimization of the transfection methods described here. The transfection methods can be applied to other suspension-adapted strains of CHO and HEK293 cells grown in various serum-free media. However, it will be necessary to reoptimize the amounts of DNA and PEI as well as the cell density at the time of transfection.
2. The TubeSpin bioreactor 450 is also available (TPP). It is closer in size to a 500-mL centrifuge tube. It should be noted that the TubeSpin Bioreactor 600 does not fit into every floor-model centrifuge as it is somewhat taller than the standard 500-mL centrifuge tube.
3. Once thawed, the PEI solution in a 50-mL tube can be maintained at 4 °C for up to 1 week or refrozen after being further aliquoted into 15-mL tubes. One should avoid repetitive

freeze–thaw cycles. The tubes can be maintained frozen for several years.

4. The DNA must be sterile since no antibiotics are used in the cell cultivation system described here. The DNA can be precipitated in the presence of 95% ethanol and washed in 70% ethanol. The centrifuge tube containing the DNA in these steps is only opened inside a laminar flow hood. After the ethanol wash step, the DNA pellet is air-dried in the open tube in a laminar flow hood. The DNA is then resuspended in sterile water or TE.
5. It is important that the rack holds the OSR50 or OSR600 tightly so that it does not rotate while being agitated.
6. To maintain the transfectability of the cells, it is best to keep cells in culture for no longer than 3 months (20–25 passages). We also recommend maintaining the cells in exponential growth phase at all times. The transfection efficiency of CHO-DG44 and HEK293E cells in the methods described here are about 60% and 85%, respectively.
7. The agitation conditions described here depend on a shaking diameter of 5 cm for the shaker platform. For incubator shakers with a lower shaking diameter, the shaking speed needs to be increased. For a shaking diameter of 2.5 cm, for example, the OSR50 and OSR600 may be agitated at 220 rpm.
8. If multiple transfections in OSR50s need to be performed, then the cells are prepared in an OSR600 as described in Subheading 3.4.
9. The method described here does not involve precomplex formation with DNA and PEI prior to addition to the culture. It is very important to minimize the time delay between addition of DNA and PEI and to mix the culture well after each component is added.
10. The method described here is optimal when performed at 31 °C. However, it may be beneficial to test other temperatures between 30 °C and 33 °C to obtain the best yields for the production of any given protein.
11. The duration of the production phase needs to be determined empirically for each recombinant protein. For intracellular and membrane proteins the maximum yield is usually reached between days 2 and 3 posttransfection. For secreted proteins, the maximum yield is usually reached between days 5 and 9 posttransfection.
12. Nickel-affinity chromatography of a secreted histidine-tagged protein from Ex-cell293 medium may be difficult due to stripping of the nickel by a component of the medium. This can be avoided by producing a secreted his-tagged protein in another

medium that supports nickel-affinity chromatography such Freestyle293 (Life Technologies) or Pro293s (Lonza). Alternatively, it is possible to purify the protein from Ex-cell293 with Ni Sepharose Excel Fast Flow (GE Healthcare).

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Chapter 10

Bench-Scale Stirred-Tank Bioreactor for Recombinant Protein Production in Chinese Hamster Ovary (CHO) Cells in Suspension

Dominique T. Monteil and Jeffrey Kuan

Abstract

Most pharmaceutical biotechnology companies use stirred-tank bioreactors (STR) for recombinant protein manufacturing. These bioreactors are used at a variety of different scales ranging from bench to production scales, with working volumes from 10 mL to 25,000 L. Bench-scale STRs are commonly used to culture mammalian cells for process development, to troubleshoot production scale bioreactors using scale-down models (SDM), or to conduct fundamental research. In this chapter, we describe the operations of a bench-scale STR for the production of recombinant proteins with suspension-adapted Chinese hamster ovary (CHO-DG44) cells. These operations include bioreactor setup and configuration, batching media, inoculation of the seed cell culture, production phase, and harvest of cell-free fluids.

Key words CHO cells, Stirred-tank bioreactor, Bench scale, Recombinant protein, Production

1 Introduction

For the production of recombinant protein in microbial and mammalian cell cultures at large scale, stirred-tank bioreactors (STR) are the standard. The biotechnology industry has invested heavily in large facilities of multiple large-scale stainless steel STRs ranging from 2000 to 25,000 L in order to supply the market with the required therapeutic protein demands [1–3]. To develop bioprocesses for the production of these therapeutic proteins, development of scaled-down versions of STRs were necessary to conduct multifactor experiments. These 1–20 L scale-down models (SDM) have been proven to show reliable cell culture performance at the bench-scale when compared to large-scale bioreactors [4, 5]. Different versions of these STRs have since been developed for more cost-efficient solutions, such as ambr® bioreactors, which are milliliter-scale STRs with automated functions. However, most labs still use glass autoclavable bench-scale bioreactors [6, 7]. These glass

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bioreactors, typically made by Applikon or Sartorius [4, 8], range in volume from 1 to 20 L.

The smaller bench scales of glass STRs allow scientists to complete multifactor experiments while mimicking production-scale STRs, increasing the speed and efficiency of process development. In this chapter, we describe the operations involved in the bench-scale STR production of recombinant proteins with suspension-adapted Chinese hamster ovary (CHO-DG44) cells. The materials required for completing these processes are listed. Next, the methods to operate these STRs are described. This operation includes bioreactor setup and configuration, batching media, inoculation of the seed cell culture, production phase, and harvest of cell-free fluids.

2 Materials

2.1 Cell Culture

1. CHO-DG44 cells adapted to cultivation in serum-free suspension.
2. TubeSpin® bioreactors 50 (TS50) and TubeSpin® bioreactors 600 (TS600) with nominal volumes of 50–600 mL (TPP, Trasadingen, Switzerland).
3. Cell cultures are maintained in an orbitally shaken incubator at 37 °C with 5% CO_2 saturation and 85% humidity set at 5.0 cm shaking diameter (ISF1-XC, Kuhner AG, Birsfelden, Switzerland) [9].
4. Erlenmeyer shake flask with vented caps with nominal volumes of 125, 500, and 1000.
5. The cultivation vessels are attached to the shaken platform using a rack (Kuhner AG) or by using double-sided adhesive tape.
6. ProCHO5 medium (Lonza AG, Verviers, Belgium) without L-glutamine, hypoxanthine, thymidine, and phenol red.
7. 50× L-glutamine and phenol red solution: A stock solution with 200 mM glutamine and 250 μg/mL phenol red is made by dissolving 29.23 g glutamine and 250 mg phenol red in 800 mL water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration through a 0.2-μm Steritop bottle-top filter with a neck size of 45 mm. The solution is transferred into sterile 50-mL centrifuge tubes and kept frozen at −20 °C. For each liter of ProCHO5 medium, 20 mL of the stock solution is added to yield a final glutamine concentration of 4 mM.
8. 50× HT solution: A stock solution is made by dissolving 680 mg of hypoxanthine and 194 mg of thymidine in

800 mL of water. After complete dissolution, the volume is adjusted to 1 L by further addition of water. The solution is sterilized by filtration and frozen as aliquots as explained in the previous step. For each liter of ProCHO5 medium, 20 mL of the stock solution is added to yield a final concentration of 13.6 mg/L hypoxanthine and 3.9 mg/L thymidine.

9. 0.4% Trypan blue solution.
10. 300 g/L D-glucose in water. The solution is sterilized by filtration through a 0.2-μm Steritop bottle-top filter.
11. 30% Antifoam C emulsion (Sigma-Aldrich). This stock solution is diluted using deionized water until 1% volume by volume. Sterilize this solution by autoclaving.
12. 1 M sodium bicarbonate solution. The solution is sterilized by filtration through a 0.2-μm Steritop bottle-top filter.
13. Centrifuge tubes at different sizes: 1550,500 mL.

2.2 3-L Glass Bioreactor and Controller

1. 3-L single-wall glass bioreactor (Applikon Biotechnology, Schieda, Netherlands).
2. 3-L bioreactor head plate accessories including: Sample port, triport for additions, septum, inoculation port, overlay port, exhaust port, harvest port, gas outlet with condenser, pH/DO ports, temperature probe well, impeller shaft, blank ports, and two vertical baffles (Applikon Biotechnology).
3. An L-type fluted 7-hole sparger with 1.0-mm holes and sparge cap (Applikon Biotechnology) (*see* **Note 1**).
4. One 4.5 cm 6-blade Rushton impeller (bottom) and one 4.5 cm 3-blade Marine-type propeller (top) (*see* **Note 2**). Fix the impellers such that the distance between the Rushton impeller and the bottom of the shaft is equal to the distance between the Marine-type impeller and the liquid surface.
5. Acro filters for sparger and exhaust (Pall, Basel, Switzerland).
6. 3.2 mm size tubing with 1.6 mm wall thickness used for welding on of attachments to the bioreactor (Pureweld or Marprene, Watson-Marlow, Zollikon, Switzerland).
7. Prepare multiple size cylindrical bottles (250,1000, and 2000 mL) with dip tubes and vents for the sterile transfer of cells, media and feeds. Sterilize by autoclaving (Schott Glass, Mainz, Germany) (Fig. 1).
8. 10-mL, 20-mL, and 60-mL syringes with Luer-Lok. 18-gauge by 1.5″ needles are used for bolus feed additions.
9. Welder or aseptic connectors.
10. Tubing clamps for bioreactor feed and sample lines (Nalgene, Rochester, NY, USA)

Fig. 1 Image of a 500-mL cylindrical glass bottle assembly with dip tube, vent, and tubing

11. Peristaltic pump for sterile transfer of liquid (100 and 500 series, Watson-Marlow)
12. Controller for monitoring and controlling the agitation, temperature, pH and dissolved oxygen concentration (ez-Control, Applikon Biotechnology) (*see* **Note 3**) (Fig. 2).
13. Mass flow controller to control gas flow and composition (FlowCon4, Kuhner AG) or (ez-Control, Applikon Biotechnology).
14. Temperature probe, PT100 (Applikon Biotechnology).
15. 235 mm dissolved oxygen and pH sensors (AppliSens, Applikon Biotechnology).
16. Heat blanket (Applikon Biotechnology) (*see* **Note 4**).

2.3 Cell Analysis

1. The viable cell density (VCD) and cell viability are determined by the trypan blue exclusion method using a hemocytometer.
2. The cell biomass is determined using packed cell volume (PCV) tubes (TPP).
3. The air saturation, CO_2 saturation, and pH are analyzed offline from fresh samples using a blood gas analyzer (BGA), the NOVA BioProfile pHOx analyzer (NOVA Biomedical, Waltham, MA).
4. The glucose, lactate, glutamine, glutamate, and ammonium ion concentrations are determined using a NOVA BioProfile 400 analyzer (NOVA Biomedical).

Fig. 2 Images of different available bench-scale bioreactors and controllers. (**a**) ez-Control, Applikon Biotechnology; (**b**) G3lab Universal, Finesse; (**c**) BioSTAT B, Sartorius; (**d**) BioFlo 120, Eppendorf

3 Methods

3.1 Cell Culture and Seed Train Expansion

1. CHO-DG44 cells are subcultivated every 3–4 days starting at an initial cell density of 0.3 × 10^6 cells/mL in ProCHO5 medium (when used for cell culture, the medium contains L-glutamine, hypoxanthine, thymidine, and phenol red as indicated in Subheading 2.1).
2. Cells are cultivated in 5–20 mL in TS50 at 180 rpm or alternatively at 20 mL in 125-mL Erlenmeyer shake flasks, with a vented cap, orbitally shaken at 120 rpm.

3. After 3–4 days of cultivation at the smaller scale, expand the cell culture into a larger volume using a TS600 at 100–500 mL shaken at 180 rpm or a 1-L Erlenmeyer shake flask, with a vented cap, at 200 mL shaken at 120 rpm.
4. Determine the viable cell density and cell viability by Trypan blue staining using a Neubauer hemocytometer and an inverted phase contrast microscope.
5. Ensure that each subcultivation starts at an initial cell density of 0.3×10^6 cells/mL by diluting the existing culture with fresh ProCHO5 medium.
6. Repeat **step 5**–7 using multiple vessels to further expand the volume of cell culture. For example, one TS600 at 100 mL working volume expanded into two TS600 with a working volume of 400 mL.

3.2 Bioreactor Preparation

3.2.1 Assembly and Sterilization

1. Assemble the bioreactor head plate with the necessary components. Typical head plate components include a sample port, harvest port, inoculation port, septum port, pH/DO probe ports, overlay port, gas exhaust port, blank ports, triport for additions, 7-hole sparger, impeller shaft, and a temperature well. Install gaskets between each component and the head plate (*see* **Note 5**) (Fig. 3).
2. Install 2 baffles on the head plate to promote efficient mixing during operation. Ensure the baffles are installed with the faces perpendicular to the direction of flow in the bioreactor.
3. Install a sparge cap on the 7-hole sparger (*see* **Note 6**).

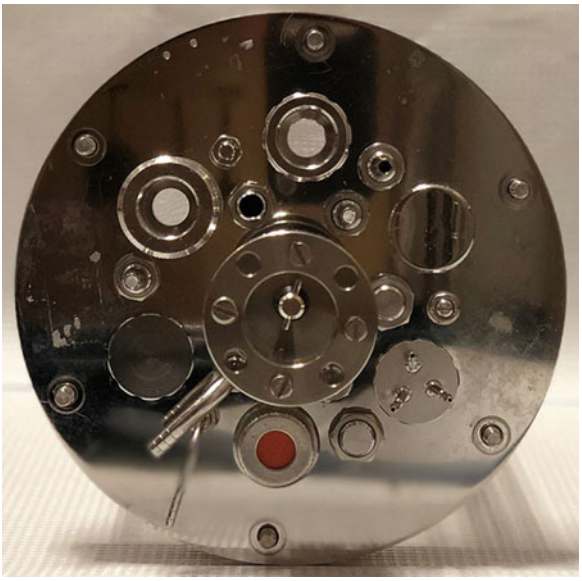

Fig. 3 Image of bioreactor head plate

4. Install one 4.5 cm 3-blade Marine-type impeller approximately 11 cm from the bottom of the impeller shaft, then install one 4.5 cm Rushton impeller approximately 1 cm from the bottom of the impeller shaft.
5. Attach tubing to the bioreactor: attach tubing with Acro filters for the overlay and sparge lines. Attach a Luer-Lok syringe injection connector on the tubing attached to the sample port. For the harvest port, inoculation port, and triport addition lines, include Marprene tubing with Acro filters such that attachments or feeds can later be sterilely attached. If using aseptic connector, attach in place of the filter at the end of the tubing before autoclaving.
6. On the exhaust port of the bioreactor, attach a glass bottle with tubing leading to an Acro filter. This allows condensate to collect in the bottle, keeping the Acro filter dry.
7. Attach silicone tubing to the bottom of the harvest tube and cut flutes. This creates a fluted dip-tube that allows for the full bioreactor volume to be harvested (Fig. 4).
8. Fasten the head plate to a 3 L glass vessel using millinuts (*see* **Note** 7).

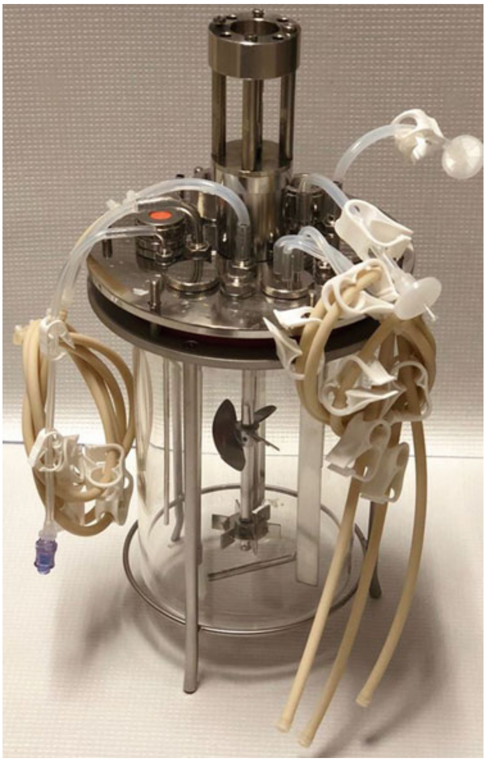

Fig. 4 Image of assembled bioreactor with tubing

9. Calibrate a pH probe with the bioreactor controller using pH 4 and pH 7 buffers (*see* **Note 8**).
10. Calibrate a DO probe with the bioreactor controller using nitrogen (0% air saturation) and air (100% air saturation).
11. Place approximately 5 mL of DI water in the vessel (*see* **Note 9**) and then install the pH and DO probes.
12. Autoclave the bioreactor. Ensure all installed clamps are open during the autoclave cycle. Close these clamps immediately after taking the bioreactor out of the autoclave.
13. Retighten all millinuts and ports postautoclave. The head plate and its components may come loose during the autoclave cycle.
14. Attach gas lines and agitator motor head to the bioreactor. Connect the probes to the controller.
15. Initiate an air overlay of 0.1 L/min (*see* **Note 10**) and start agitation at 150 rpm (*see* **Note 11**).
16. Configure the bioreactor controls to prepare for the cell culture. Set a pH setpoint of 7.00 with a deadband of 0.25. Configure the CO_2 gas mass flow controller (MFC) to be used when the pH goes above the upper deadband. Configure a pump, attached and primed with the 1 M sodium bicarbonate solution, to be used when the pH goes below the lower deadband [10].
17. If only using a constant sparge rate, set the air MFC to 0.77 L/min containing 5% CO_2 (*see* **Note 12**).
18. If using a DO controller, set the DO setpoint to 30% air saturation, set the air MFC flow rate to 0.02 L/min and supply O_2 on demand.

3.2.2 Medium Batching

1. Obtain ProCHO5 media in a bag with tubing that can be welded onto the inoculation line on the reactor or transfer medium into a 2-L cylindrical glass bottle with a dip tube (from Subheading 2.2).
2. Place the medium bag on a scale and weld the media bag onto the inoculation line on the reactor (*see* **Note 13**).
3. Place the tubing in a peristaltic pump and prime the line with fluid.
4. Using the scale as reference, gently pump 1.50 L batch volume of medium into the bioreactor (*see* **Note 14**).
5. When batching is complete, reverse the pump direction and clear the line of fluid.
6. Aseptically disconnect the medium bag from the inoculation line. This can be completed using a tube sealer or by folding the tubing and zip tying twice, then cutting the tube on the nonsterile side.

7. Ensure that the agitation is on and mixing, then activate temperature control and allow the bioreactor to reach the temperature set-point of 37 °C. Once the bioreactor has reached temperature, pull a 5-mL sample from the reactor and run it with the BGA.
8. Compare online and offline DO and pH values. Make corrections to the calibrations if the difference is greater than 10% saturation or pH 0.01 units (*see* **Notes 15** and **16**).
9. Once DO and pH corrections have been completed, activate DO and pH control for the bioreactor to allow air, oxygen, carbon dioxide, and base to be added to the bioreactor as needed.

3.3 Operation of Bioreactor

3.3.1 Inoculation

1. Once the batched medium has equilibrated for more than 4 h and less than 24 h, sample the bioreactor again. Purge 5 mL of sample to clear the sample line, and then sample 5 mL for the BGA.
2. Confirm that the online and offline DO and pH values are similar. Make 1-point corrections to the calibrations if the difference is greater than 10% air saturation or pH 0.01.
3. Count the cells in the seed train culture and determine the VCD as prepared in Subheading 3.1.
4. Calculate the amount of seed train cell culture required to inoculate the batched 3-L STR at a starting cell density of 0.3×10^6 cells/mL. Use the below equation:

$$\begin{aligned}&\text{Seed train vol required (L)}\\&\quad=\frac{\text{Media vol batched (L)} \times 0.3 \times 10^6\text{cells/mL}}{\text{Seed train VCD}\left(10^6\text{cells/mL}\right) - 0.3 \times 10^6\text{cells/mL}}\end{aligned} \tag{1}$$

5. Using a prepared 250-mL cylindrical glass bottle assembly with dip tubes (from Subheading 2.2), sterilely transfer the required volume of seed culture into the transfer bottle. This is typically completed in a laminar flow hood.
6. Weld or aseptically attach the transfer bottle containing seed culture to the inoculation port on the 3-L STR. Transfer the cell culture into the STR using a pump or by gravity, ensuring that the seed culture is well mixed throughout the transfer (*see* **Note 17**).
7. After the transfer is completed, the transfer bottle may be left attached to the STR as a secondary exhaust vent bottle (*see* **Note 18**).
8. Ensure that all of the control loops are functioning (i.e., DO, pH, and temperature).

3.3.2 Daily Sampling

1. Take daily samples to monitor cell culture performance. Using a 10-mL Luer-Lok syringe, pull 10 mL of cell culture to purge the sample port line and discard. Immediately after, sample 10 mL of cell culture with a fresh 10-mL syringe.
2. Immediately after pulling a sample, run a BGA sample to determine the pH, DO saturation and CO_2 saturation. Compare the offline values from the BGA with the online values of the probes. If the online and offline values differ by over pH 0.05, complete a 1-point correction.
3. Determine the VCD and viability of the cell culture as described in Subheading 3.1.
4. Run the daily sample on the NOVA BioProfile 400 analyzer. This will determine the glucose, lactate, glutamine, glutamate, and ammonium ion concentrations. If the glucose concentration is ≤ 2.0 g/L, then addition of glucose is needed.
5. Bring the glucose concentration up to 4.0 g/L using the 300 g/L glucose stock solution. Calculate the amount of stock solution to add using the equation below:

$$\text{Gluc vol required (L)} = \frac{(4.0\text{g/L} - \text{Current Gluc conc.(g/L)}) \times \text{Bioreactor current vol (L)}}{0.3} \tag{2}$$

6. To add glucose, sterilely transfer the target feed amount into a syringe with a needle. Using the septum port in the head plate, inject the glucose solution with the syringe. Keep the septum port saturated with isopropanol during this operation to maintain bioreactor sterility.
7. Inspect the foam level at the top of the liquid in the bioreactor. If the foam has accumulated to a height of more than 3 cm, then an antifoam addition is needed. Sterilely transfer 0.5 mL of the antifoam stock solution into a syringe with a needle. Using the septum port in the head plate, inject the target amount with the syringe.
8. Take the remaining sample and centrifuge at 1050 × *g* in a 15-mL conical centrifuge tube for 5 min. Collect the supernatant, label the centrifuge tube, and then freeze at −20 °C. This retain can be used later to determine the protein concentration or to reanalyze metabolites.
9. Ensure that all of the control parameters are within the process conditions.
10. Repeat **steps 1** through **9** of this subsection daily, until the cell viability drops below 50%. Then proceed to harvest the bioreactor.

3.4 Harvest of Bioreactor

1. Once the cell culture is ready to harvest, typically around 7–8 days, attach a 2-L bottle assembly with a dip tube to the harvest port on the bioreactor, sterility is not important for this step.
2. Disable the temperature and pH control loops to avoid overheating or unwanted additions of base during the harvest.
3. Pump the cell culture out of the bioreactor, transferring all of the culture into the 2-L glass bottle.
4. Transfer the cell culture into four 500-mL conical centrifuge tubes.
5. Centrifuge at 5000 × *g* for 20 min at room temperature.
6. Using a 0.2-μm Steritop filter, sterile filter the supernatant.
7. Sterilely aliquot the harvested cell culture into two 1-L Nalgene bottles and store at −20 °C.

4 Notes

1. Different spargers can be installed in a bioreactor. This operation uses a drilled hole sparger, which generates small bubbles with a large surface for oxygen transfer. Alternatively, a fritted or sintered sparger can be used, which generates even smaller bubbles with a larger surface area for oxygen transfer, but causes increased foaming. An open-hole sparger can also be used, which produces large bubbles that cause less foam.
2. Different combinations of Rushton and Marine-type impellers can be used depending on the needs of a given cell line. These different combinations will have various effects in mixing time, shearing, oxygen transfer efficiency and foaming.
3. Different controllers can be used (refer to Fig. 1).
4. Some glass bioreactors have double-walled water jackets which can be used as an alternative to heating blankets.
5. Gaskets are to be checked for integrity prior to each use of the bioreactor. Loose or warped gaskets can compromise sterility.
6. Sparge cap needs to be flush with the sparger and should be checked pre and post-autoclaving. A slightly loose sparge cap will fall off a sparger during operation.
7. Fasten bioreactor head plate by tightening millinuts on opposite ends simultaneously. This will prevent the head plate from warping asymmetrically.
8. pH probes will eventually dry out if they are not submerged in liquid. For this reason, probes should be left in buffer or electrolyte solution until they need to be removed for autoclaving.

9. Placing water in the bioreactor during autoclave will increase the effectiveness of sterilization. Post-autoclave, the increased moisture content within the vessel will prolong the lifespan of the pH probes, which can dry out otherwise.
10. After autoclaving, immediately initiate air overlay to maintain positive pressure within the bioreactor. Start agitation to ensure the impeller is securely attached.
11. Starting agitation immediately after autoclaving ensures that the impeller is attached securely. It is important to verify this before batching or inoculating a bioreactor. If the impeller falls off, disassemble, refasten the impeller and reautoclave.
12. If using a constant sparge rate, the upper pH deadband control with CO2 should be disabled.
13. When welding attachment bottles onto the bioreactor, place clamps on either side of the tubing to be welded. This will keep lines sterile in the event of a bad weld.
14. If one requires a larger culture starting volume, the batch volume will have to be adjusted. The required batch volume could also change based on the seeding bioreactor final seed density.
15. The BGA provides the DO saturation in mm Hg units. To convert from partial pressure of O_2 to percent saturation use the following conversion factor: 1.59 mm Hg O_2 = 1% saturation O_2.
16. Perform one-point standardization of DO only before inoculation. Doing so after inoculation is not recommended and is not typically needed.
17. If inoculum is being transferred via a peristaltic pump, operate the pump at no more than 180 rpm for 3.2 mm size tubing with 1.6 mm wall thickness. High pump speeds will induce cell shear and lower viability. The controller should be working at the time of inoculation.
18. Leave inoculation transfer bottle welded to the bioreactor. Avoid disconnecting attachments to the bioreactor, as these are potential sources of contamination.

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Chapter 11

Continuous and Integrated Expression and Purification of Recombinant Antibodies

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Abstract

This chapter introduces the necessary concepts to design continuous expression and purification processes for monoclonal antibodies. The operation of a perfusion bioreactor is discussed containing the preparation procedures, the seeding train and the preparation and control of a long-term production run. The downstream processes exploit the benefits of countercurrent chromatography. Their design from batch experiments is presented. The CaptureSMB process is introduced for continuous capturing while for polishing applications the design of the two-column MCSGP process is described. The chapter also puts these processes together in the context of their integration to an end-to-end production process.

Key words Expression, Purification, Production, Perfusion bioreactor, CaptureSMB, MCSGP, Integrated biomanufacturing, Continuous chromatography, Countercurrent chromatography, Monoclonal antibody

1 Introduction

1.1 Processing of mAbs

Therapeutic proteins have been one of the promising drug families targeting a large range of diseases for 30 years [1]. The variety of therapeutic proteins is dominated by monoclonal antibodies (mAbs) whose worldwide sales are expected to rise up to nearly $125 billion by 2020 [2, 3]. Chinese hamster ovary (CHO) cells have been and remain the most common expression system for the production of therapeutic proteins [4]. They are able to perform human-like posttranslational protein modifications like N-linked glycosylation affecting efficacy, stability, and immunogenicity of the proteins [5–7]. This provides the basis for the wide approval of CHO-derived proteins by regulatory authorities [8]. The industrial state-of-the-art mAb production is performed in well-established batch platform operations, strictly divided into upstream and downstream processing [9]. While the upstream

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part is typically performed in large scale fed-batch bioreactor systems, the downstream purification train consists of a protein A capture step, at least two polishing steps as well as virus inactivation and filtration steps. However, recent technology advances and growing interest point toward the transition from batch to continuous processing in a number of instances of industrial relevance [10]. A variety of industries has already achieved significant process intensification through the conversion from batch to continuous manufacturing. Besides, continuous manufacturing offers a set of advantages including steady state operation, more homogeneous product quality and improved controllability, reduced equipment size, high volumetric productivity, low cycle times, and reduced cost economics [10–13]. In addition, the US Food and Drug Administration (FDA) has encouraged the efforts in continuous bioprocessing and underlined its great potential to address issues of agility, flexibility, and robustness [14]. The transition from batch to continuous processing requires both the development and implementation of alternative continuous unit operations of the different production steps, and the robust connection and control of the different process parts.

1.2 Upstream Processing

Upstream processing offers two alternatives for the continuous production of therapeutic proteins, both well known in biotechnology for many years: the chemostat and the perfusion bioreactor [15–17]. The two setups are schematically presented in Fig. 1. Both processes share the continuous mode of operation in terms of constant medium exchange. However, in the chemostat, the outlet stream is the same as the bioreactor content and includes cells, target protein, and metabolites [18]. On the other hand, the perfusion setup uses an additional cell retention device in order to remove only by-products (target protein, metabolites) from the reactor and keep cells inside, thus enabling process intensification due to much higher viable cell densities. The chemostat is commonly applied when using microbial production systems which typically have a much higher cell division rate compared to mammalian cells. Applying a chemostat CHO culture results in much lower viable cell densities compared to a perfusion operation due to the slow division rate of mammalian cells.

State-of-the-art cell retention devices use separation either based on cell size or on cell density. They range from filter devices, centrifuges, gravity settlers to acoustic wave separators [10, 19, 20]. However, today's most promising devices employ tangential flow, in particular the alternating tangential flow (ATF) filtration and tangential flow filtration (TFF) systems both using hollow fiber filter modules for cell retention. Comparing these two devices, Clincke et al. showed that by using the TFF device, a higher maximum viable cell density could be achieved compared to the ATF [21]. However, the alternating flow operation induces a self-

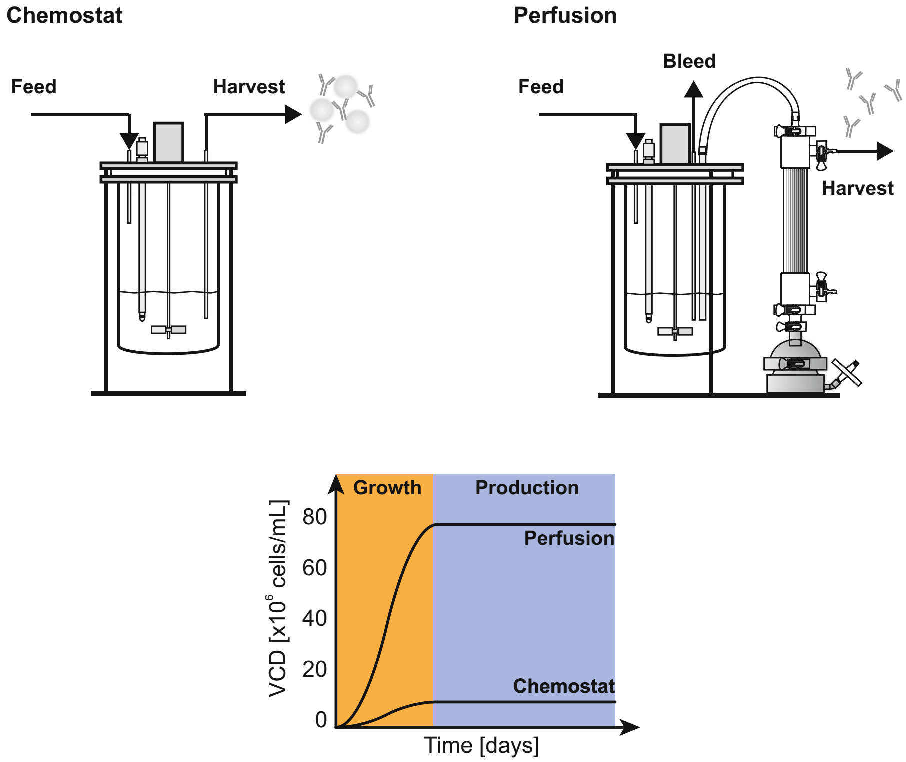

Fig. 1 Schematic setup of chemostat and perfusion bioreactors (top) and the resulting viable cell density profiles for CHO cultures (bottom)

cleaning of the hollow fibers reducing the probability of filter-fouling [22]. In a comparative study, Karst et al. reported, on the one hand, significantly lower maximum shear stress, and on the other hand, almost no protein retention in the ATF device compared to the TFF [23].

A stable and robust perfusion process targets steady state operation at a fixed viable cell density set point. In order to maintain this set point for a long process duration, cells need to be kept in a growing state and have to be fed with sufficient nutrients to prevent nutritional limitations potentially inducing cell death. The cellular growth must be balanced by the removal of cells from the reactor. Therefore, a second outlet stream, the bleed stream, is necessary to fulfill both a constant cell density set point and a viable cell culture. Nevertheless, the bleed stream contains not only cells, but also the protein of interest. Therefore, this stream has to be regarded as waste since further processing of this cell containing stream is challenging. Reducing the bleed stream to a minimum (5–10% of the overall medium exchange rate), while maintaining a viable cell culture will allow improvement of the overall process yield [24].

In order to evaluate and compare the performance of perfusion processes it is necessary to introduce specific parameters. The overall medium exchange rate, often called perfusion rate P, equals the sum of both outlet streams, bleed B and harvest H rate.

$$P = H + B \left[\frac{\mathrm{RV}}{\mathrm{day}}\right] \tag{1}$$

The cell specific perfusion rate (CSPR) describes the amount of medium fed to a single cell per day. More generally, it is defined by the perfusion rate normalized to cell density and is often reported in $\mathrm{pL}_{\mathrm{medium}}/\mathrm{cell}/\mathrm{day}$:

$$\mathrm{CSPR} = \frac{P}{\mathrm{VCD}} \left[\frac{\mathrm{pL}}{\mathrm{cell\ day}}\right] \tag{2}$$

which eventually indicates the performance of the used medium, the so-called medium depth. In order to operate at a process specific optimum in terms of cell density, medium consumption, and productivity it is important to assess the potential of the given expression system and medium, the so called minimum CSPR, $\mathrm{CSPR}_{\mathrm{min}}$. At the $\mathrm{CSPR}_{\mathrm{min}}$, the ratio of perfusion rate and viable cell density cannot be further decreased without influencing cellular viability or productivity [25]. Over the last two decades, perfusion performances, cell line and medium formulations have been certainly improved as indicated by accomplished cell specific perfusion rates, which have been reduced from around 100 pL/cell/day down to 14 pL/cell/day [16, 23, 26–28] as summarized in Table 1.

The proper design and development of mammalian cell perfusion processes requires a careful analysis of suitable cell specific perfusion rates. Defining suitable operating set points (flow rates, viable cell density, and medium composition) guarantees a robust and stable process operation. Robust and stable conditions are the key requirement for a further integration with continuous downstream processing for the development of an end-to-end integrated production stream.

Table 1
Cell specific perfusion rates for steady state CHO perfusion operation set points reported in literature

Reference	Product	Cell density (10^6 cells/mL)	Perfusion rate (RV/day)	CSPR (pL/cell/day)
Konstantinov et al. [16]	Therapeutic protein	~ 35	3.6	102
Dowd et al. [26]	t-PA	~ 5	0.25–2	50–400
Warikoo et al. [27]	mAb	50–60	2–3	40–50
Karst et al. [23]	IgG	60	1.05	17.5
Xu et al. [28]	mAb	68	1	14.7

1.3 Downstream Processing

Apart from virus inactivation and filtration steps, downstream processing typically consists of three chromatographic steps [29]: capture followed by two polishing units. The capture is used to concentrate the antibody from the harvest employing Protein A affinity chromatography using pH step elution with the positive side effect of significantly decreasing process-related impurity levels (e.g., DNA). The polishing steps specifically target the removal of process- and product-related impurities (e.g., aggregates) applying two orthogonal chromatography modes such as cation (CEX) and anion exchange (AEX). For an introduction to batch methods, the reader is referred to the article of Müller-Späth et al. [30].

In the frame of batch technology, continuous operation can be enabled by time-shifting multiple identical columns within the same processing step, so-called tandem batch operation [31]. This, however, does not improve the performance potential of the unit beyond that of traditional batch technology. For this, various continuous countercurrent processes for each application have been developed and many of them patented [32, 33].

1.3.1 Capture

The capture is a loading or "saturation" adsorption [33, 34] process operated under conditions with yields close to 100%, i.e., no breakthrough of mAb [35]. Due to the low diffusivity of mAbs, the loading of bead-based stationary phases occurs under mass transfer limitation, which becomes more pronounced with increasing flow rates resulting in shallower breakthrough curves (Fig. 2). In batch mode, high productivity originating from high loading flow rates leads to earlier breakthrough and therefore occurs at the expense of decreased eluate concentration and capacity utilization (CU), which is a parameter of relevance due to the high cost and limited lifetime associated with Protein A resins. This could be improved by

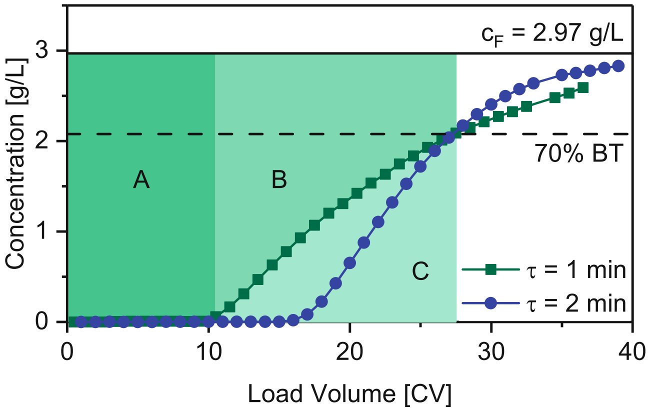

Fig. 2 Breakthrough curves of an IgG1 type antibody from prepacked Eshmuno A ($d_C = 0.8$ cm, $L_C = 5$ cm, $V_C = 2.5$ mL) at two different flow rates. The higher flow rate shows earlier breakthrough. Shaded areas correspond to $\tau = 1$ min and are named in accordance with Fig. 3

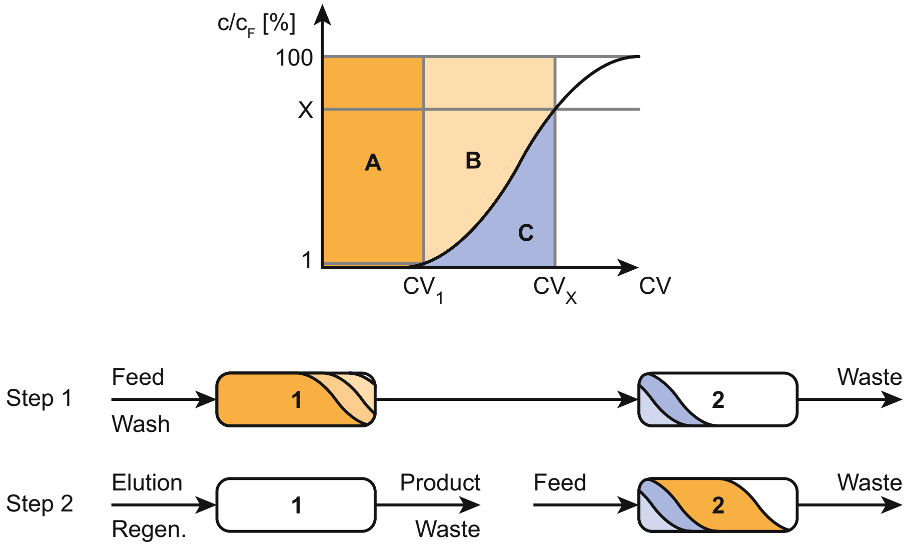

Fig. 3 Top: Schematic breakthrough curve highlighting the amount of antibody bound to one column in batch (A) and countercurrent processing (A + B). Bottom: Scheme of one switch of the CaptureSMB process. Columns switch position after step 2 and the sequence is repeated

lowering the loading flow rate, which also decreases productivity. This trade-off between productivity and capacity utilization is sought to be alleviated using processing schemes exploiting countercurrent loading and internal recycling (Fig. 3): At least two columns are interconnected, allowing the antibody to break through from the first column as it is captured on the downstream column(s). The first column can therefore be loaded beyond its dynamic binding capacity (DBC) resulting in high load, capacity utilization and product concentration even at elevated flow rates. Various processing schemes with at least two interconnected columns during feeding and first wash step have been proposed for this and were reviewed by Steinebach et al. [33]. These include sequential multicolumn chromatography (SMCC, e.g., BioSC using four columns [36]), which however does not allow feeding during the interconnected wash; periodic countercurrent processes (PCC) with at least three columns and continuous loading capability [35, 37]; stepSMB using 8 columns assigned to different zones for parallel regeneration [38]; BioSMB using a single-use flow path cassette with up to 16 columns [39]; CaptureSMB using only two columns, but not allowing loading during the interconnected wash at maximum flow rate [40]. Several process variations of each process exist, e.g., with additional columns in regeneration, which becomes more beneficial at very high-titer regimes as regeneration starts to limit productivity [41]. Due to its simplicity and comparably low equipment demand, the design and operation of a CaptureSMB process will be considered in this chapter.

1.3.2 Polishing

In contrast to capture steps, polishing in bind-elute mode can be referred to as "elution separation" [34], where the majority of the column (length) is used to resolve different solutes by step or linear gradients. In case of antibody purification, the typical objective is the removal of fragments and aggregates, although the separation of charge isoforms could also be of interest. For these separations, impurities elute both before and after the antibody, referred to as weakly and strongly adsorbing, yielding a center-cut three-component separation problem. Due to similar adsorptive behavior of the impurities and the mAb, achievable resolution remains low even on modern high-performance materials unless loadings are chosen low and gradients very flat, which reduces productivity and final product concentration. In batch processing, this leads to a trade-off between purity and yield at a given productivity value as exemplified in Fig. 4 for a charge variant separation; either a small central section of the chromatogram is taken as product resulting in high purity but low yield, or a larger portion is collected increasing yield while sacrificing purity. As for the capture step, the trade-off can be alleviated by countercurrent chromatography, where the unresolved portions of the elution stream are recycled internally. The first processes to exploit this were true (TMB) [34, 42] and simulated moving bed (SMB) [43] processes; however, they are only rarely applied in biopharmaceutical polishing steps because only binary separations and no linear gradients are possible. To resolve these limitations, the multicolumn countercurrent solvent

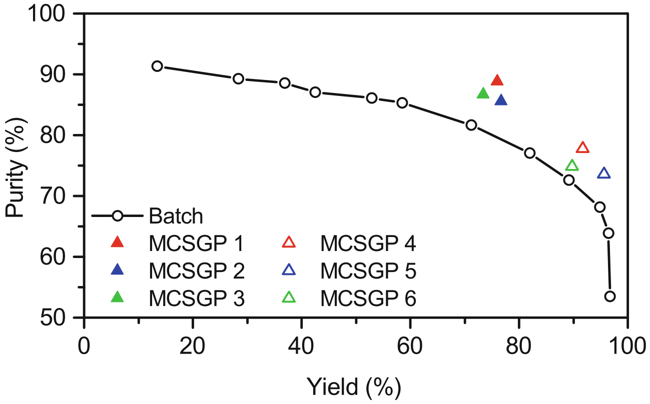

Fig. 4 Purity and yield of a charge isoform separation by gradient elution in batch and MCSGP processing. Different setpoints of the MCSGP were evaluated, all of which exceed the performance of the corresponding batch separation. Setpoint MCSGP 1 roughly doubles the yield at a purity of 90% and corresponds to the discussed example. The other setpoints use 70% (MCSGP 2, 3, 4) or 50% (MCSGP 5, 6) as purity threshold for t_C (2, 5), t_D (3, 6) or both (4). For MCSGP 5 and 6, the duration of the recycle windows was kept constant with respect to MCSGP 4

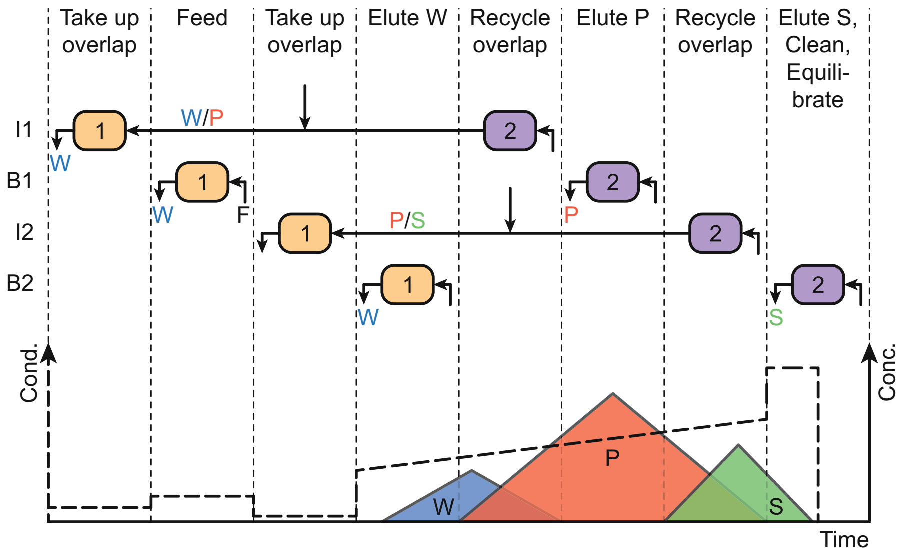

Fig. 5 Scheme of one switch of the two-column MCSGP process. The zones of the schematic batch chromatogram divide the process in tasks (dashed vertical lines). Phases I1 to B2 are carried out sequentially

gradient purification (MCSGP) process was developed [44, 45]. Different versions with two to six columns exist allowing cyclic [46, 47] or continuous [44, 48] feeding and elution as well as multiproduct separation [49] and different cleaning-in-place (CIP) steps [50]. The process can be applied to any bind-elute chromatographic mode (CEX, AEX, hydrophobic interaction etc.), although for mAbs, CEX is the most commonly applied one. As working principle of the MCSGP (Fig. 5), sufficiently pure portions of the chromatogram are collected, while those unresolved are recycled to a second column, thus allowing to achieve both high yield and purity (Fig. 4). This is enabled by alternating the columns between interconnected and batch states: In the first interconnected phase (I1), product (P) eluting with weakly adsorbing impurities (W) from column 2 is recycled onto column 1. Inline dilution is applied to bring the solutes into a binding state. Once the outlet product stream is sufficiently pure, the first batch phase (B1) starts during which product is collected from column 2 and feed is applied to column 1. As soon as strongly adsorbing impurities (S) contaminate the product to a certain degree, the columns are again interconnected (I2) for recycling including inline dilution. After the elution of all product from column 2, the second batch phase starts consisting of regeneration (strip, CIP, equilibration) of column 2 and begin of the gradient on column 1, where only W elutes. These four steps constitute a switch, after which the positions of the columns are exchanged and the phases are repeated. The four switching times provide the key to control yield and purity more independently than possible in traditional batch technology, therefore alleviating the encountered trade-off. In the following, the development of the two-column MCSGP process will be discussed as it provides the simplest realization of this process. In addition, it is capable of receiving the cyclically occurring elution from the capture step in order to enable integration of the two unit operations [51].

1.4 Integration of USP and DSP

The technological advances in upstream and downstream processes mentioned above facilitated the implementation of continuous integrated end-to-end platforms. An exemplary scheme of such a platform is presented in Fig. 6. The integration of a perfusion bioreactor connected with a continuous countercurrent capture step has been reported by a few groups. Warikoo et al. showed stable uninterrupted performance for 30 days [27]. Karst et al. implemented successfully an additional at-line HPLC combined with a mechanistic model of the capture unit in order to adapt operating conditions of the capture process to changing product concentrations in the harvest as a consequence of varying process parameters in the perfusion bioreactor [52]. Furthermore, Steinebach et al. and Godawat et al. presented case studies on end-to-end fully continuous production platforms [51, 53]. Godawat et al. also proved that continuous processing enables steady state product quality, process train simplification, and high volumetric throughput over long process durations. In particular, from an economic point of view, continuous biomanufacturing could lead to a significant decrease in average costs and additional cost of goods, and operational robustness benefits combined with improved and more homogeneous product quality [12, 13, 54].

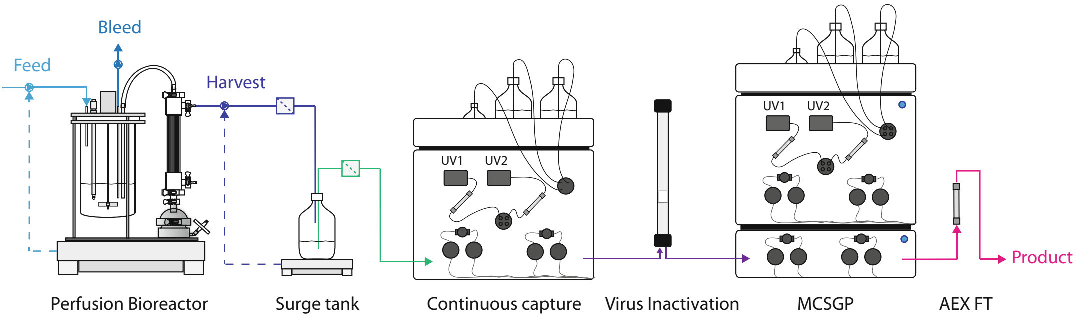

Fig. 6 Scheme of the integrated setup as used by Steinebach et al. [51]. Besides the unit operations discussed in the text, a surge tank to decouple flow rate variations and sterilization requirement in upstream and downstream, virus inactivation, and anion exchange flow-through polishing are included

2 Materials

2.1 Upstream Processing

2.1.1 Equipment

1. A benchtop bioreactor system (e.g., Vaudaux Eppendorf AG, Switzerland) consisting of a set of glass vessels (1.0–2.0 L working volume), a reactor control system with implemented modules for monitoring and control of pH and DO, heating and stirring, gas flow rate and mixing as well as additional peristaltic pump modules is used for the realization of a lab scale perfusion system. Additionally, the process control computer needs to allow the implementation of a biocapacitance probe (e.g., ABER Instruments Ltd., UK) and the connection of balances (e.g., Mettler Toledo, USA) for online cell density and reactor weight control, respectively.
2. A cell retention device is essential to achieve and maintain high cell densities and to continuously harvest cell free supernatant. Currently, the integration of cross flow filtration devices using PES hollow fiber modules (e.g., 25 cm length, pore size 0.5 μm, filtration area of 1570 cm^2, 1 mm fiber diameter) is standard coupled with either an alternating tangential flow system or a tangential flow filtration device. For lab scale, an ATF2 device (Repligen Cooperation, USA) or a bearingless centrifugal pump for the TFF operation (PuraLev MU200, Levitronix GmbH, Switzerland) are suitable systems.

2.1.2 Cell Line and Medium

1. A cryopreserved CHO cell line (5–10 × 10^6 cells/mL) producing either an IgG monoclonal antibody or an unstable protein stored in culture medium including 10% DMSO in liquid nitrogen are typically used. For the cultivation, a chemically defined medium for CHO cell culture is necessary, preferably a commercially available or supplied medium formulation.

2.1.3 Additional Equipment

1. Humidified shaking incubator (e.g., Adolf Kuhner AG, Switzerland).
2. Different types of containers (e.g., 50 mL TubeSpin Bioreactors, 600 mL Tube Spin Bioreactor, TPP, Switzerland or roller bottles, Corning®, USA) are needed for the cell expansion.
3. For in process control (IPC) a mammalian cell counting system is necessary, e.g., the Cedex HiRes (Roche Diagnostics, Switzerland).
4. An analyzer for bioprocesses needs to be used for daily measurements of key metabolites (glucose, lactate, ammonia), e.g., Cedex Bio (Roche Diagnostics).
5. For preparation of cell culture medium in large amounts, suitable bags equipped with connections to the bioreactor system are recommended (e.g., BPC, 100 L, Life Technologies).

6. The sterile connection of different system can be performed by a sterile tubing welder (Terumo, Japan) in combination with appropriate tubing (e.g., PharMed® BPT tubing, Saint-Gobain, France.)

2.2 Downstream Processing

2.2.1 Equipment

1. For continuous downstream processes, as discussed above, a preparative LC system with at least two column positions and the possibility to interconnect them by valve switching is needed (e.g., GE Healthcare, ChromaCon, or Pall Life Sciences). The devices feature accurate pump, monitoring (UV, conductivity, pH) and fractionation systems, and are provided with the supplier's control software. They also allow regular batch chromatography operation.
2. For the two-column MCSGP process, a gradient pump is required in addition to two isocratic pumps.
3. For the processes described below, all needed calculations can be carried out automatically by the ChromIQ software (ChromaCon, CUBE hardware required).

2.2.2 Resins and Columns

1. Typical preparative stationary phases for the capture and purification of monoclonal antibodies are summarized in Table 2. Equilibrium binding capacities range between 60 g/L$_{col}$ and 70 g/L$_{col}$ for Protein A resins, and exceed 100 g/L$_{col}$ for ion exchange materials. The resin can be obtained in bulk or prepacked in columns of various sizes.
2. For small amounts of antibodies to be purified, inner diameters of 0.5–1.0 cm and lengths of 5–15 cm are usually chosen. For the multicolumn processes, each column needs to be packed with the same material; furthermore for the MCSGP process,

Table 2
Stationary phases for the capture and polishing of mAbs

Chromatography type	Resin name	Supplier	Mean particle diameter [μm]
Protein A affinity	MAbSelect SuRe	GE Healthcare	85
Protein A affinity	Eshmuno A	Merck KGaA	50
Protein A affinity	Toyopearl AF-rProtein A HC-650F	Tosoh	45
CEX (strong)	Poros 50 HS	ThermoFisher	50
CEX (strong)	Eshmuno CPX	Merck KGaA	50
CEX (strong)	BioPro SmartSep S10	YMC	10
AEX (strong)	Eshmuno Q	Merck KGaA	85
HIC	Toyopearl Phenyl-650 M	Tosoh	65

packing needs to be reproducible as it impacts the separation performance (*see* **Note 1**).

3. The capture method was successfully run with MAb Select SuRe (GE Healthcare) and Eshmuno A (Merck KGaA), but can be expected to work with any preparative Protein A resin, provided the ligand is stable in 0.1 M NaOH.
4. The MCSGP process was implemented with Eshmuno CPX (Merck KGaA) for aggregate and fragment removal, and with BioPro SmartSep S10 (YMC) for charge isoform purification including slight buffer and gradient adjustments (buffers A-C: pH 6.1; buffer B:150 mM NaCl; gradient: 30 CV from 35% to 60% B).

2.2.3 Buffers and Feed

1. The buffer recipes for the Protein A capture step as well as for the CEX polishing step are summarized in Tables 3 and 4, respectively. pH is adjusted using 1 M base (NaOH) or acid (HCl). The measured conductivity can be an indicator for the correctness of the buffer. Alternatives for the Protein A elution buffer are 50 mM glycine or acetate. It has to be noted that an adaptation of CEX elution buffer B in terms of NaCl concentration might be necessary if protein elution occurs early or late during the gradient or in a very narrow region (*see* **Note 2**). Buffer A is also used for inline compensation during recycling in MCSGP processing.
2. For antibody capture, the feed needs to be clarified of insoluble components (e.g., cells and cell debris) to prevent column fouling. When obtaining it from a perfusion cultivation with

Table 3
Buffer recipes for Protein A capture

Substance [g]	Buffer A (Equil.)	Buffer B (Wash)	Buffer C (Elution)	Buffer D (CIP)
Composition	20 mM phosphate, 150 mM NaCl, pH 7.5	20 mM phosphate, 1 M NaCl, pH 7.5	50 mM citric acid, pH 3.0	0.1 M NaOH, 1 M NaCl
NaH_2PO_4	0.39	0.36		
Na_2HPO_4	2.37	2.41		
NaCl	8.77	58.44		58.44
Citric acid monohydrate			10.51	
NaOH (solid)				4.0
DI water	1000	1000	1000	1000
Adjust pH to	7.5	7.5	3.0	n/a
Typ. cond. [mS/cm]	18	82	2	n/a

Table 4
Buffer recipes for CEX polishing

Substance [g]	Buffer A (Equil./Comp.)	Buffer B (Elution)	Buffer C (Strip)	Buffer D (CIP)
Composition	25 mM phosphate, pH 6.0	25 mM phosphate, 0.5 M NaCl, pH 6.0	25 mM phosphate, 1 M NaCl, pH 6.0	1 M NaOH, 1 M NaCl
NaH_2PO_4	2.72	2.52	2.54	
Na_2HPO_4	0.33	0.56	0.54	
NaCl		29.22	58.44	58.44
NaOH (solid)				40.0
DI water	1000	1000	1000	1000
Adjust pH to	6.0	6.0	6.0	n/a
Typ. cond. [mS/cm]	2	46	82	n/a

cell retention by hollow fiber, it can be used directly; otherwise, centrifugation and filtration are strongly advised. For connection to the LC system, *see* **Note 3**.

3. For polishing applications using CEX, binding of the antibody to the stationary phase is sufficient if the feed solution pH is at least 1 unit below the pI of the mAb and its conductivity does not exceed about 5 mS/cm. Reduction of ionic strength can be accomplished by diafiltration against an appropriate buffer or dilution with DI water or buffer; in the latter case however, mAb concentration should not become smaller than about 5 g/L in order to avoid feeding limitation in the MCSGP operation.

3 Methods

3.1 Upstream Processing

3.1.1 Cell Expansion

1. Cells stored in a working cell bank in liquid nitrogen need to be thawed and expanded in expansion medium.
2. The cultivation is performed in shake tubes at 36.5 °C, 5% CO_2 and 320 rpm in a humidified incubator for 1 week, passaging every second day.
3. At each passage state, cells are diluted to 0.3×10^6 cells/mL in incrementally larger containers (spin tubes or roller bottles) to accumulate a sufficiently high cell number for the inoculation of the seed perfusion bioreactor.

3.1.2 Medium Preparation

1. Medium preparation has to be performed according to the supplier's protocol. Typically, a perfusion run at a working volume of 1.5 L operated at a perfusion rate of 1 reactor volume per day during 30 days, consumes 45 L medium. It is recommended to prepare at least 20–25 L of medium at a time. After preparation and pH adjustment (*see* **Note 4**), the medium can be sterile filtered into the corresponding medium bag.

3.1.3 Reactor Preparation

1. Both seed and production bioreactors need to be assembled as shown in Fig. 7. Additionally, polarization of the DO probes and calibration of the pH probes have to be performed. For the pH calibration, technical pH buffers (pH = 7 for offset calibration, pH = 4.01 for slope calibration) are used. A more detailed description of the calibration procedures is given in [55].
2. The tubing for the different inlet and outlet streams has to be prepared, together with a condenser in combination with an off-gas filter. Typically, a small bottle is used in between to prevent filter blocking in case of excessive foaming. The hollow fiber has to be connected to the bioreactor.
3. All probes (pH, DO, biomass capacitance probe), the condenser, the sparger, the stirrer (e.g., equipped with Rushton or Pitch Blade turbine), the different tubing (harvest, feed, bleed, inoculation/antifoam), and the connection to the cell retention device have to be carefully assembled as shown in Fig. 7 and a small amount of water has to be added to the assembled setup before autoclaving.
4. The bioreactor setup is sterilized at 121 °C for 45 min.
5. The bottles for the collection of harvest and bleed, as well as for antifoam addition can be prepared independently. A calibration of the different pumps is necessary in order to ensure high precision of the targeted flow rates.
6. After sterilization, all lids have to be closed tightly and tube connections have to be checked carefully. Additionally, all clamps have to be closed.
7. After the connection of all probes to the corresponding cables, the air supply has to be attached to the air filter, followed by the insertion of the temperature probe.
8. The DO calibration can be performed after cooling down the setup. As for the pH calibration, the slope (100% atmospheric air, 21% oxygen) and the offset (pure nitrogen) have to be calibrated.
9. All tubes can be connected to the corresponding bags and bottles (*see* **Note 5**).

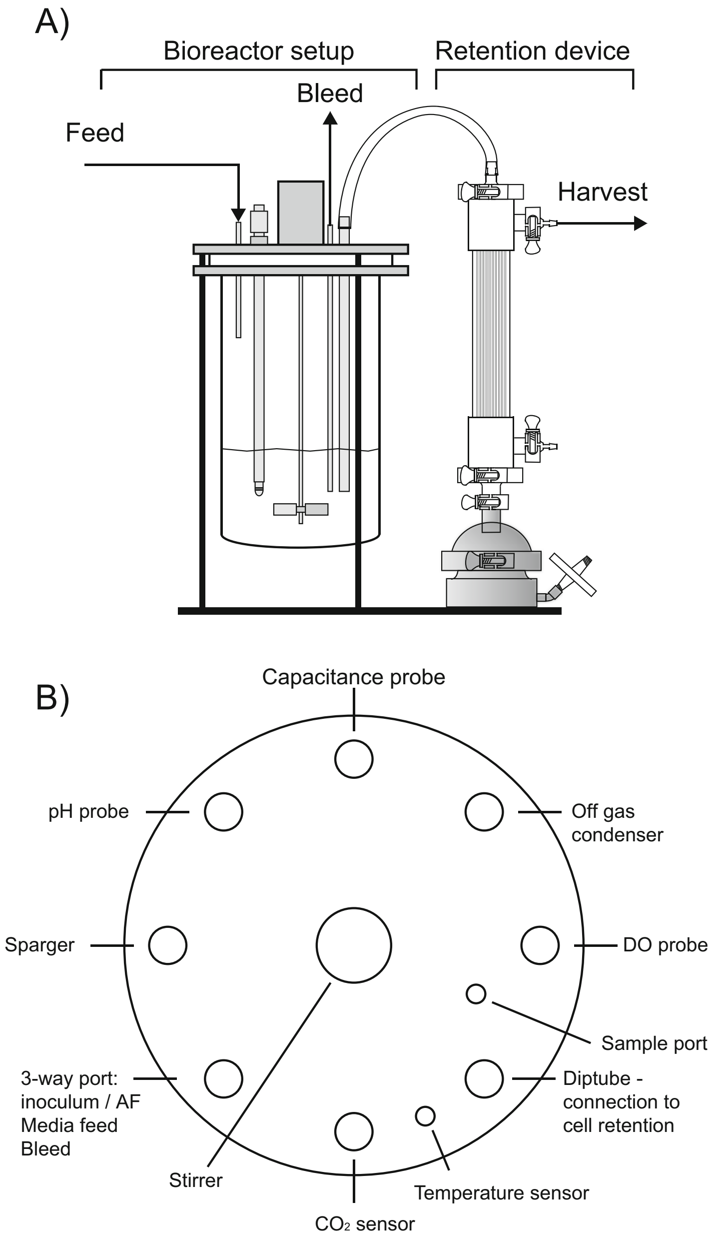

Fig. 7 Overview of bioreactor assembly: (**a**) Side view and (**b**) Head plate configuration

3.1.4 Preparation of Inoculation

1. The reactor has to be filled with 750–950 mL of fresh medium (in case of 1.5 working volume). The external loop comprising the cell retention device has to be initiated at the desired flow rate (1.0–1.5 L/min).
2. The heating mantle can be installed and the medium can be heated to the process temperature.

3. Stirring and gassing (stirrer speed: 100 rpm, gas flow rate: 0.22 vessel volumes per minute (vvm), X_{O2}: 21%, X_{CO2}: 10%) can be started.
4. After equilibration, the DO slope calibration has to be repeated. The process set points (50% dissolved oxygen tension, pH 7.1, gas flow rate of 0.22 vvm, and agitation of 250–400 rpm) can be initialized and the baseline capacitance of the biomass sensor can be set in the plain medium after equilibration of all process variables at the respective set point.

3.1.5 Seed Bioreactor/N-1 Perfusion

1. Targeting a perfusion process, the inoculation viable cell density (VCD) of the production bioreactor is aimed to be close to the targeted VCD set point. Consequently, an N-1 seed perfusion bioreactor can be used to shorten the time to reach the production phase. Besides, the split of growth and production phase into two processes is intended to not impact the life time of the hollow fiber in the retention device of the production vessel.
2. Cells from expansion are directly inoculated into the seed bioreactor after 1 week, targeting an inoculation cell density between 0.3–0.5 × 10^6 cells/mL.
3. After inoculation, the harvest pump is started and a gravimetric feedback control of the feed pump is implemented. The seed reactor targets maximizing the cell growth and preventing nutrient limitation.
4. During the first 4 days a perfusion rate of 0.5–1.0 RV/day is recommended, followed by a stepwise increase aligned with the increasing cell density to maintain the cell specific perfusion rate above a critical value (2–3 times higher than the $CSPR_{min}$).
5. After 1 week, cell densities between 40 and 60 × 10^6 cells/mL can be achieved as shown in Fig. 8.

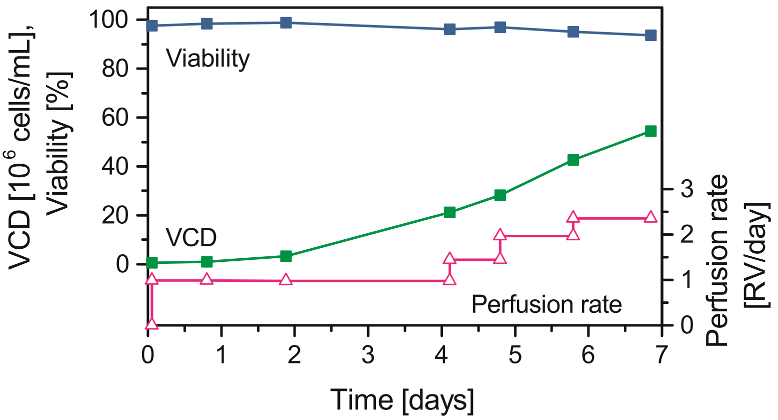

Fig. 8 Exemplary time profiles of viable cell density, viability, and perfusion rate in an N-1 seed bioreactor

3.1.6 Production Bioreactor

1. The production bioreactor is inoculated from the seed production bioreactor after 7–10 days, depending on the growth properties of the cell line. Cells can be transferred by connecting the two vessels directly (e.g., sterile welding). One possibility to enable the transfer of the cells is to apply a slight overpressure in the seed reactor not exceeding 1.5–2 bar.
2. Upon inoculation, the essential control loops have to be initiated: (1) constant harvest rate (2) gravimetric feedback loop of the feed pump, (3) cell density control of the bleed pump (*see* **Note 6**).
3. The cell density set point should be selected carefully based on preliminary studies on the medium depth/$\mathrm{CSPR}_{\mathrm{min}}$. As mentioned before, the cell density can be monitored by a biocapacitance probe. The online biomass value should be compared and adjusted according to the offline VCD measurements in order to account for changes in cell diameter at the beginning of the culture. The combination of inoculation close to the cell density set point and immediate start of perfusion operation facilitates a fast transition to a steady state bioreactor operation. The operation can be maintained for the desired process duration (usually 20–60 days).
4. At the end of the culture, all control loops can be stopped including disconnecting the probes, turning off cooling of the condenser, clamping off and disconnecting the different tubes and a careful cleaning of all probes and all the parts of the reactor vessel.

3.1.7 Bioreactor Monitoring

1. Daily in process control (IPC) is performed including measuring all cell related characteristics (viable cell density, viability, diameter, and aggregation rate) preferably using the trypan blue exclusion method.
2. Samples for metabolite analysis are withdrawn from the reactor and the harvest to analyze main metabolites (glucose, lactate, potentially ammonia, and IgG titer) using a proper cell culture analyzer. Samples for further analysis of product quality can be kept frozen. For analytical methods, *see* **Note 7**.
3. Additionally, in process control involves a daily check of the different flow rates and the corresponding pumps. Bleed and harvest rate can be calculated and compared based on the weight change of the different hold up tanks capturing the outlets flows. In case of a constant harvest rate, the pump rate has to be adjusted accordingly. With illustrative purposes the time profiles of the daily offline measurements are shown in Figs. 9 and 10. In this case, the targeted viable cell density set point was 30×10^6 cells/mL and the harvest rate was set to 0.8 RV/day to operate at an overall perfusion rate of 1.0 RV/day.

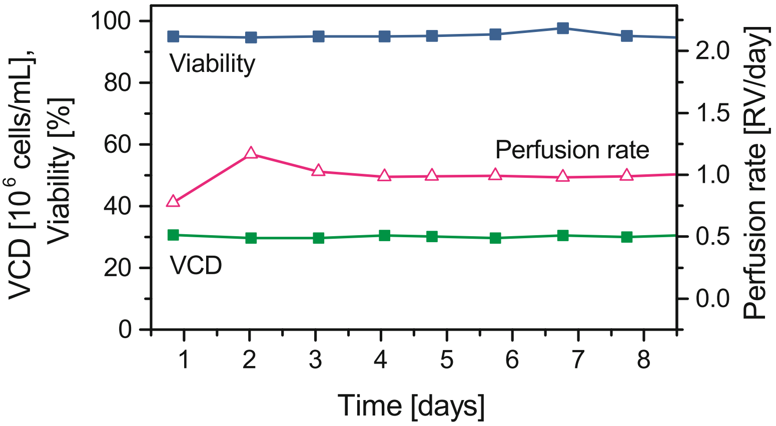

Fig. 9 Exemplary time profiles of viable cell density, viability and perfusion rate in a production bioreactor

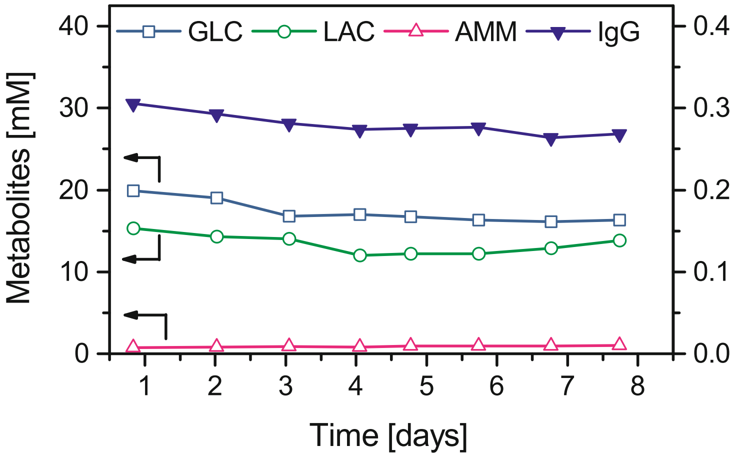

Fig. 10 Time evolution of different metabolites (GLC, LAC, AMM, IgG) in a perfused production bioreactor after high cell density inoculation at day zero

In case of strong deviations between online biomass reading and offline viable cell density count, the probe's biomass conversion factor has to be corrected (*see* Subheading 3.1.6).

4. In a last step of the IPC, online monitored values (reactor weight, pump flow rates, gas flow rate and gas fractions, pH, DO, temperature) shall be checked as they are indicative for the current status of the operation (stable operation, steady state, pump status) as shown in Fig. 11.

3.1.8 Integration to Downstream Purification Train

1. As presented in [52], the connection of the perfusion bioreactor to the subsequent downstream process can be realized by placing a small surge tank (5% of reactor volume) between the

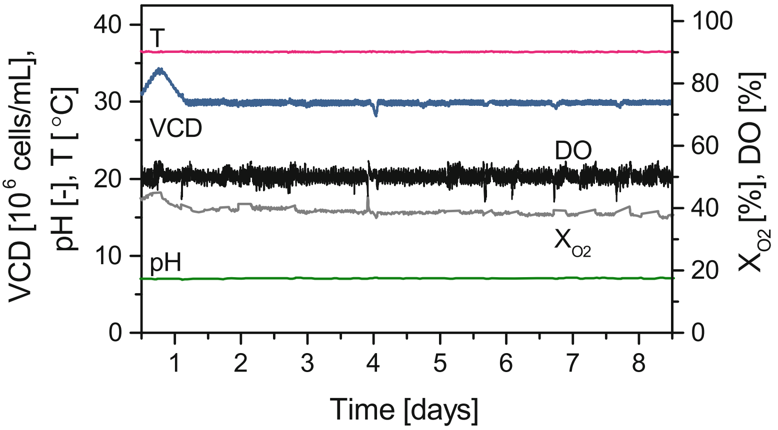

Fig. 11 Time profiles of monitored and controlled parameters (temperature, VCD, dissolved oxygen (DO), oxygen fraction in the gas inlet stream (X_{O2}), and pH

two corresponding units. This replaces the holdup tank for the harvest mentioned above (*see* Subheading 3.1.3).

2. Sterility can be assured by installing sterile barriers before and after the surge tank.

3.2 Downstream Processing

Volumes of processing steps are typically given in terms of column volumes (CV), i.e., the total volume of the empty column. For example, a column with an inner diameter of 0.8 cm and length of 5 cm has a volume V_C of 1 CV = 2.5 mL. Flow rates are reported as linear velocities u defined as the ratio between volumetric flow rate Q and cross-sectional area A of the column. As an example, applying a flow rate of 2.5 mL/min to the above column results in a linear velocity of $u = 300$ cm/h, which corresponds to 1 CV/min. In all procedures described below, buffers are named according to Tables 3 and 4. Before starting any procedure, the chromatographic system and columns should be cleaned and equilibrated with buffer A.

3.2.1 CaptureSMB: Breakthrough Curves

1. For the experimental design of the CaptureSMB process, a breakthrough curve needs to be obtained (Fig. 2) at a given feed concentration and the desired flow rate. For this, a single column is loaded to almost reach its static binding capacity (SBC), which is typically achieved after feeding the amount of mAb equivalent to twice the SBC. As an example, for a resin with 70 g/L_{col} SBC and a feed of 3 g/L_{feed}, the static binding capacity is equivalent to 23 CV of feed. Hence, to obtain the breakthrough curve about 46 CV should be loaded. As can be seen in Fig. 2, this is a rather conservative estimation.
2. The column is initially equilibrated (5 CV, buffer A) and then the UV signal is set to 0 (autozero).

3. Feeding is carried out at the desired flow rate until the required volume is loaded.
4. The flow-through is fractionated and subsequently analyzed using analytical Protein A chromatography yielding the breakthrough curve schematically shown in Fig. 2.
5. The column can be regenerated and product collected as described in the next section.

3.2.2 CaptureSMB: Batch Regeneration Protocol

1. Regeneration in practically all continuous capture processes occurs in batch mode. A typical, nonoptimized protocol starting after loading is given as follows:

Wash 1	4 CV	100% buffer A	Interconnected in CaptureSMB
Wash 2	5 CV	100% buffer B	Reduces HCP content
Wash 3	4 CV	100% buffer A	Reduces cond. At start of elution
Elution	5 CV	100% buffer C	Collect as pool or in fractions
CIP	8 min	100% buffer D	Time-based, flow rate can be halved
Reequilibration 1	2 CV	100% buffer B	Reduces pH faster
Reequilibration 2	4 CV	100% buffer A	

2. In the CaptureSMB, wash 1 is carried out in the interconnected mode so to adsorb unbound mAb to the second column thus increasing the process yield. If continuous feeding is desired, the loading flow rate must be chosen below the maximum tolerable flow rate in order to enable merging of the streams of interconnected wash and batch loading without harming the second column. Adaptations to subsequent calculations are therefore also necessary.

3.2.3 CaptureSMB: Design

1. There are two process-related degrees of freedom in the CaptureSMB: The loading volume until 1% breakthrough (CV_1) in batch mode and the volume loaded in the interconnected phase ($CV_{IC} = CV_X - CV_1$). As a first guess, a good choice for CV_X is the loaded volume at 70% breakthrough (CV_{70}). This is because capacity utilization is already significantly increased while the breakthrough curve starts to flatten out resulting in less CU gain with further feeding.
2. The amount loaded to the second column during the interconnected phase needs to be estimated because it reduces the loadable amount before breakthrough in the batch phase. This corresponds to area C in Fig. 2 and can be obtained by integration of the concentrations c_{mAb} over the fractionation

interval ΔCV. To find the corresponding feed volume $\mathrm{CV_{BT}}$, this value is divided by the feed concentration c_{feed}:

$$CV_{\mathrm{BT}} = \frac{1}{c_{\mathrm{feed}}} \int_{CV_1}^{CV_X} c_{mAb} \mathrm{d}CV = \frac{\Delta CV}{c_{\mathrm{feed}}} \sum_{CV_1}^{CV_X} c_{mAb}[\mathrm{CV}] \tag{3}$$

3. At this point, several parameters are determined allowing the design of the capture process: Loading volume until 1% ($\mathrm{CV_1}$, area A) and 70% breakthrough ($\mathrm{CV_{70}}$, B + C), preloaded amount ($\mathrm{CV_{BT}}$, C) and time required for batch regeneration t_{RR}, which are 10.5 CV, 27.5 CV, 7.1 CV and 28 min in this example, respectively. As mentioned above, the loading volume in the batch phase $\mathrm{CV_B}$ is reduced by the amount that has already been preloaded, corresponding to $\mathrm{CV_1} - \mathrm{CV_{BT}}$ (A–C, 3.4 CV).
4. Parameters of the two phases of the process can now be calculated: The batch loading flow rate Q_{B} is the ratio of the net batch load amount $\mathrm{CV_B}$ and the regeneration duration t_{RR}, in this example 0.12 CV/min or 36 cm/h. Due to the steeper breakthrough curve associated with smaller Q_{B}, the process has an intrinsic safety margin. The length of the interconnected phase t_{IC} is calculated from $\mathrm{CV_{IC}}$ and the design flow rate: In the above example 17 CV are equivalent to a duration of 17 min.
5. For very low mAb concentrations, the batch loading flow rate might be higher than the design flow rate. In this case, the duration until 1% breakthrough is calculated with the design flow rate and the difference to the regeneration duration added to the length of the interconnected phase. Therefore, the columns are interconnected already before 1% breakthrough occurs.

3.2.4 CaptureSMB: Operation

1. Having both phase durations (t_{RR}, t_{IC}) as well as all flow rates (Q_{B}, Q_{IC}, Q_{RR}), the method can be implemented in the controller software. For the CaptureSMB, a cycle conventionally starts with the interconnected phase. Therefore, valves are set to put the columns in series and feed is pumped for the found t_{IC}.
2. The interconnected wash is carried out, followed by the batch regeneration protocol for column 1, while column 2 is loaded at the found batch feed flow rate Q_{B}. This constitutes a switch.
3. The valves have to position column 2 upstream to column 1 and the steps are repeated finishing a cycle.
4. The same method can be used in a procedure to run several cycles. The UV signal of four consecutive cycles with Eshmuno

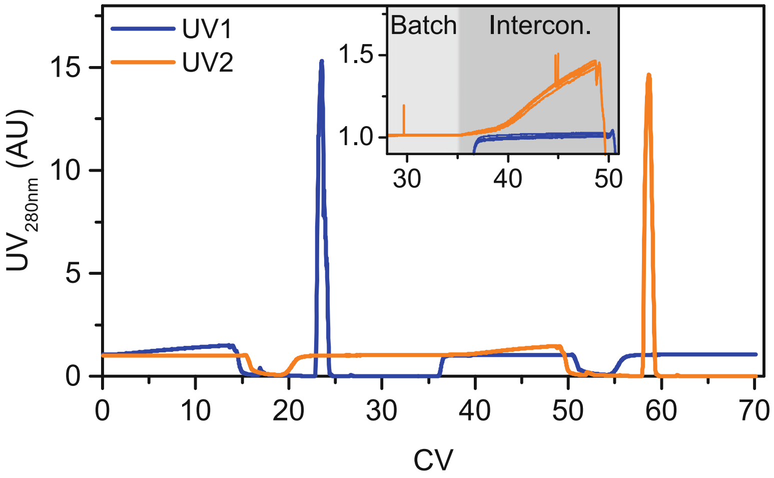

Fig. 12 UV profiles of four consecutive cycles of the CaptureSMB process run on Eshmuno A ($d_C = 0.8$ cm, $L_C = 5$ cm, $V_C = 2.5$ mL). All cycles show consistent behavior. Peaks before 25 and 60 CV correspond to the product elution, while the columns are loaded when the UV is around 1 AU (unbound impurities). Breakthrough from the upstream column is well visible; it occurs only after interconnection is established and is captured by the downstream column (overlay)

A resin and a modified regeneration protocol are presented in Fig. 12 showing consistent behavior. Breakthrough occurring from the upstream column during the interconnected phase is clearly visible as well as the corresponding capturing by the second column (constant UV).

5. In order to achieve cyclic steady state already during the first switches, a startup method can be implemented, where the first column is loaded to a degree close to the state at the end of the batch phase, i.e., an amount equivalent to area A is loaded. This can be done either in batch or interconnected mode (preferred).
6. At the end of the process, a shutdown without feeding needs to be performed. As each cycle ends with the batch loading of column 1, this column can be regenerated applying the above protocol without interconnection of the first wash step to column 2.

3.2.5 MCSGP: Design Batch

1. The following procedure is based on the method described by Steinebach et al. [56]. For the experimental design of the MCSGP, a convenient batch with linear gradient elution first needs to be identified at the desired loading, e.g. 10 g/L_{col}. A nonoptimized, typical protocol consists of the following steps.

Equilibration	3 CV	100% buffer A	UV autozero afterward
Loading		100% feed	Amount depends on target loading
Wash	3 CV	10% buffer B	% B same as the gradient start
Linear gradient	20 CV	10–90% buffer B	Elution, at least 20 fractions
Strip	3 CV	100% buffer C	Removes most remaining proteins
CIP	3 CV	100% buffer D	Cleaning
Reequilibration 1	2 CV	100% buffer B	Reduces pH faster
Reequilibration 2	3 CV	100% buffer A	

2. The same gradient is used in the MCSGP. Therefore, this should not start below 10% buffer B as the inline dilution might not be sufficient, and should not end above 90% to ensure reliable flow rates of the gradient pumps.
3. The gradient (and buffer B) might need some adjustments in order to set the elution in the gradient center with only little margins at the beginning and the end (*see* **Note 2**). For the example in Fig. 13, which refers to the case where the main charge variant is separated from the others, buffer B contained 150 mM instead of 0.5 M NaCl and the gradient ranged from 35% B to 60% B over 30 CV.

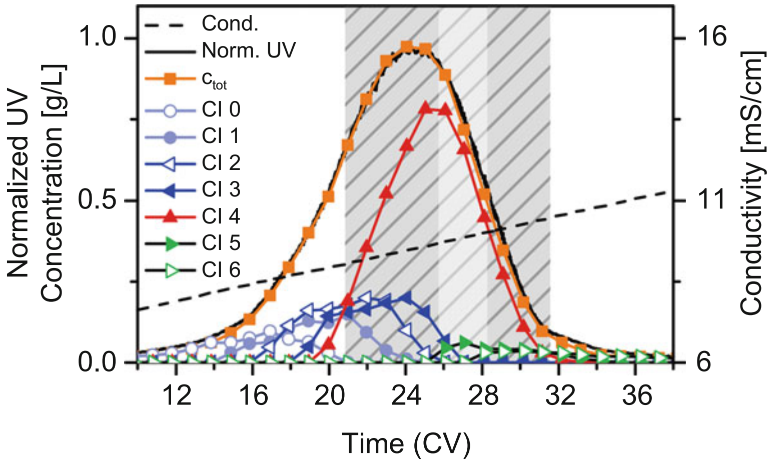

Fig. 13 Batch separation of charge isoforms of a mAb on BioPro SmartSep S10 ($d_C = 0.5$ cm, $L_C = 5$ cm, $V_C = 1.0$ mL) using a linear salt gradient. The hatched areas correspond to the phases of the MCSGP process (W/P recycling, P collection, P/S recycling)

4. The collected fractions need to be analyzed toward the impurity to be removed, e.g., weak cation exchange analytics for charge isoforms (*see also* **Note 7**). The obtained concentration and purity profile can then be used to design the MCSGP process.

3.2.6 MCSGP: Characteristic Times

1. In contrast to batch polishing, which includes only two process design parameters, that is start (t_C) and end (t_D) of product collection, the MCSGP process includes two additional parameters: The start of the W/P recycle window t_B and the end of the P/S recycle window t_E. These four times need to be selected to finalize the design of the MCSGP.
2. For the product window, the purity profile is used: Collection is done in the range of sufficient purity, e.g., ≥85% in the considered example, i.e., from $t_C = 25.7$ min to $t_D = 28.2$ min. A broader window typically yields a more robust process, but it might not be feasible for a specific system.
3. For the recycle windows, the concentration profile of the target isoform (e.g., main charge variant) is used: W/P recycling starts around the onset, while P/S recycling is stopped at the end of target elution.
4. Beginning and stop of product collection determines the end of W/P and start of P/S recycling, respectively. However, there are two aspects to be considered to obtain a stable process: First, smaller recycle windows typically make the purification more robust. Second, due to accumulation occurring in the recycle windows, the amount of impurities increases until cyclic steady state and might – depending on isotherm type – contaminate the collected product.
5. In cases of rather low initial purity, it is advised to use smaller recycle windows and sacrifice some yield. In the example, a purity threshold of 25% was used for the recycle windows ($t_B = 20.9$ min, $t_E = 31.4$ min).

3.2.7 MCSGP: Design

1. For the MCSGP, flow rates and durations of each step as well as the amount of fresh feed to be loaded need to be determined based on the characteristic times. Therefore, the starting and end time of the gradient (t_A and t_F) and the modifier (salt) concentration of the inline dilution c_{P2} need to be known. In the example, they are 8.0 min, 38.0 min, and 0 mM (buffer A, 0% B), respectively. Each of the times t_A to t_F is associated with a corresponding modifier concentration (c_A to c_F), which will be expressed as percent of buffer B. The gradient slope m can be obtained from the known c_A, c_F, t_A, and t_F as follows:

$$m = \frac{c_F - c_A}{t_F - t_A} \left[\frac{\%B}{\text{min}}\right] \quad (4)$$

It is used to calculate the unknown modifier concentrations ($i = \text{B, C, D, E}$)

$$c_{\text{i}} = m(t_{\text{i}} - t_{\text{A}}) + c_{\text{A}} \; [\%\text{B}] \tag{5}$$

The fraction of recycled target protein y can be estimated by

$$y = \frac{\int_{t_{\text{B}}}^{t_{\text{C}}} c_P \text{d}t + \int_{t_{\text{D}}}^{t_{\text{E}}} c_P \text{d}t}{\int_{t_{\text{A}}}^{t_{\text{E}}} c_P \text{d}t} \tag{6}$$

where the denominator corresponds to the batch loading volume $V_{\text{feed,B}}$ multiplied by the feed concentration and purity.

2. The numerator can be obtained by numerical integration of the product concentration c_{P} in the fractions over the fractionation interval for both recycle windows. In order to keep the **target product** loading constant, the amount of fresh feed $V_{\text{feed,M}}$ supplied at each switch is given by:

$$V_{\text{feed,M}} = (1 - y) V_{\text{feed,B}} \; [\text{CV}] \tag{7}$$

3. For the following design, the flow rate used for the batch separation is considered the maximum tolerable for the column ($Q_{P1,\text{batch}} = Q_{\text{max}}$). If this is not the case, the flow rates calculated below may be scaled proportionally and durations shortened accordingly. Be aware, that resolution becomes worse if the elution flow rate is increased. Therefore, it is not advised to increase any flow rate of the gradient pump ($Q_{P1,i}$) above the elution flow rate of the design batch.
4. Furthermore, a critical modifier concentration c_{crit} needs to be determined, which is defined as the maximum concentration of the modifier for which the target product does not move along the column. This is typically set equal to the modifier concentration in the feed or during the wash step. In the above example, it corresponds to 35% B. With these parameters, the MCSGP is fully designed.
5. By convention, an MCSGP cycle starts with phase I1 (W/P recycling). Parameters to be determined are the flow rates for the gradient pump $Q_{P1,I1}$ and the inline compensation $Q_{P2,I1}$ as well as the duration t_{I1}.

$$Q_{P1,I1} \le Q_{\text{max}} \frac{c_{\text{B}} - c_{P2}}{c_{\text{C}} - c_{P2}} \left[\frac{\text{mL}}{\text{min}}\right] \tag{8}$$

$$Q_{P2,I1} = Q_{\text{max}} - Q_{P1,I1} \left[\frac{\text{mL}}{\text{min}}\right] \tag{9}$$

$$t_{I1} = \frac{(t_{\text{C}} - t_{\text{B}}) Q_{P1,\text{batch}}}{Q_{P1,I1}} \; [\text{min}] \tag{10}$$

6. The recycle is compensated in a way such that the modifier concentration at t_{C} is diluted to c_{B}. In case $Q_{P1,I1}$ is very close to $Q_{\max}$, the resulting $Q_{P2,I1}$ becomes very small leading to unreliable pump behavior. Therefore, $Q_{P1,I1}$ should be decreased such that $Q_{P2,I1}$ is sufficiently high (at least 1% of the maximum pump flow rate). t_{I1} has to be adapted accordingly.
7. During the subsequent step B1 (P collection), one column elutes the product ($Q_{P1,B1}$), which is typically collected as pool, while the other is loaded with fresh feed ($Q_{P3,B1}$). The process with the higher volume is run at $Q_{\max}$ and determines the step duration t_{B1}. The flow rates are obtained as follows:

$$Q_{P1,B1} = \frac{(t_{\mathrm{D}} - t_{\mathrm{C}})Q_{P1,\mathrm{batch}}}{t_{B1}} \left[\frac{\mathrm{mL}}{\mathrm{min}}\right] \tag{11}$$

$$Q_{P3,B1} = \frac{V_{\mathrm{feed,M}}}{t_{\mathrm{B1}}} \left[\frac{\mathrm{mL}}{\mathrm{min}}\right] \tag{12}$$

8. In the second interconnected phase I2 (P/S recycling), the same constraints are valid as for I1 with the modification that inline compensation should be carried out to reach c_{crit}. Therefore, the following equations need to be applied:

$$Q_{P1,I2} \le Q_{\max} \frac{c_{\mathrm{crit}} - c_{P2}}{c_{\mathrm{E}} - c_{P2}} \left[\frac{\mathrm{mL}}{\mathrm{min}}\right] \tag{13}$$

$$Q_{P2,I2} = Q_{\max} - Q_{P1,I2} \left[\frac{\mathrm{mL}}{\mathrm{min}}\right] \tag{14}$$

$$t_{I2} = \frac{(t_{\mathrm{E}} - t_{\mathrm{D}})Q_{P1,\mathrm{batch}}}{Q_{P1,I2}} [\mathrm{min}] \tag{15}$$

9. Gradient elution is stopped after P/S recycling as the remaining bound protein would not be collected anyway and can be stripped off. Therefore, the eluted column is regenerated with the same Strip/CIP/Reequilibration procedure as in the batch protocol (i.e., same flow rate), while the gradient is started on the now fully loaded column ($Q_{P1,B2}$) in the second batch step B2. The longer step, which is typically the regeneration, defines the step duration t_{B2}.

$$Q_{P1,B2} = \frac{(t_{\mathrm{B}} - t_{\mathrm{A}})Q_{P1,\mathrm{batch}}}{t_{B2}} \left[\frac{\mathrm{mL}}{\mathrm{min}}\right] \tag{16}$$

3.2.8 MCSGP: Operation

1. With the knowledge of flow rates ($Q_{P1,i}$, $Q_{P2,i}$, $Q_{P3,i}$) and duration (t_i) of each step i as well as starting and end modifier concentrations (c_j, $j = A,B,C,D,E$), the process can be implemented in the controller software.

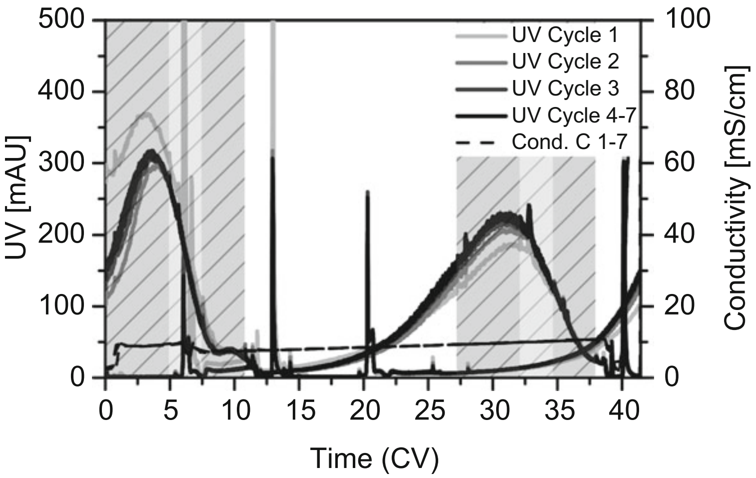

Fig. 14 Overlay of seven cycles of an MCSGP run for the purification of charge isoforms based on the batch design chromatogram shown in Fig. 13. The hatched areas in each switch correspond to W/P recycling, P collection and P/S recycling, respectively. Cyclic steady state in the UV signal is reached after cycle 3

2. A cycle starts with phase I1. Valves are therefore set to interconnect column 2 to column 1 (or vice versa). The gradient pump runs at a flow rate of $Q_{P1,I1}$ from c_B to c_C for the duration of t_{I1}.
3. The eluate is compensated by pump 2 with the flow rate $Q_{P2,I1}$. Proceeding in the same way, all the following processing steps are implemented.
4. The four phases constituting a switch are repeated for the reversed column order so as to complete a cycle. The result of this procedure is shown in Fig. 14 for the charge variant separation process under examination.
5. Cyclic steady state can be deduced by overlaying UV profiles at different cycles. This can be validated by offline analytics and performance parameters such as purity and yield as shown in Fig. 15. The overall performance is compared to that of the corresponding batch process as shown in Fig. 4.
6. In order to accelerate the transient period to reach steady state, a proper startup procedure has been developed. This requires to load the upstream column with the first steps of the batch protocol: Equilibration, loading (as in batch) and gradient from c_A to c_B. For the shutdown, a single switch without recycling and feeding is typically performed.

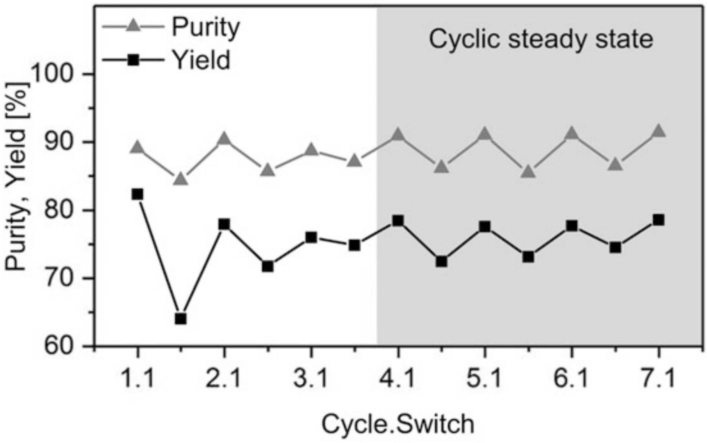

Fig. 15 Purity and yield values in the product stream leaving the MCSGP as a function of the switch numbers. Cyclic steady state is reached after cycle 3

4 Notes

1. Packing quality of chromatographic columns can be assessed with little product demand by loading it only to about 1 g/L_{col}. Subsequently, the gradient of choice is run. Overlay of the obtained UV profiles allows the selection of the most similarly packed columns. Generally, prepacked columns show less variability.
2. The gradient can also be optimized using low loadings of 1–3 g/L_{col}. Usual parameters are length and initial and final modifier concentrations. As mAbs typically adsorb on CEX materials under a Langmuir-type isotherm, attention has to be paid to the effect of increasing the load: Broadening tends to occur only in the direction of earlier elution. Therefore, the initial portion of the gradient should not result in elution when optimizing the gradient at low loadings.
3. Harvest remains a good medium for microbial growth. To reduce contamination potential and therefore column blocking, harvest should be contained in sterilized bottles with the possibility to withdraw liquid from the bottom. Therefore, unsterile tubing from the chromatographic system can be directly connected to this port. Furthermore, the possibility to compensate the volume loss with air through a sterile filter should be implemented. Regular cleaning of the feed tubing and pump with 1 M NaOH is advised. Do not apply 1 M NaOH to Protein A columns.
4. In order to avoid excessive addition of base during the perfusion process, a slightly basic medium is prepared (pH adjustment to pH 7.2). During the process, pH is

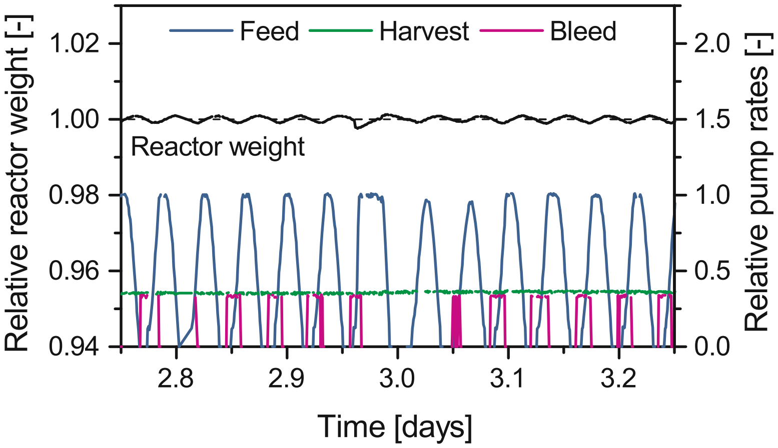

Fig. 16 Flow rates of the perfusion bioreactor streams (feed, harvest, bleed) and reactor weight as a function of time

maintained by balancing the CO_2 fraction in the inlet gas and the produced lactate in the culture.

5. For sterile connection of the reactor to the medium bag, as well as to the hold-up tanks for bleed and harvest, sterile connectors or welding tube can be used. The bottle volume should range between 3.5–5.0 L.
6. The implemented control loops result in different pump flow rates. The harvest pump is set to a constant value and consequently the pump profile shows a constant pattern over time. The bleed pump is activated when the online monitored viable cell density exceeds the VCD set point. As a result, the pump is switched on and off to a certain pump flow rate. The gravimetric feedback control loop of the feed pump results in an oscillating pump profile of the feed pump. The reactor weight also fluctuates around the set point due to the implemented control structure. The different time profiles are shown in Fig. 16.
7. Critical quality attributes can be monitored by various techniques. For aggregates, size exclusion chromatography is typically applied; for fragments and charge isoforms, weak cation exchange analytics or microfluidic devices are available; for glycosylation, a standard method is capillary gel electrophoresis with laser-induced fluorescence detection. Process-related impurities can be quantified with UV fluorescence tagging (DNA) and immunoassays (HCP, leached Protein A).

Acknowledgments

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Chapter 12

High Throughput Transfection of HEK293 Cells for Transient Protein Production

Tia A. Arena, Peter D. Harms, and Athena W. Wong

Abstract

Transient transfection of mammalian cells is used in the biotechnology industry to quickly supply recombinant protein for research and large molecule drug development. Here, we describe a method for high throughput transient transfection of Human Embryonic Kidney 293 (HEK293) cells in 30 mL tubespins using polyethylenimine (PEI) as a transfection reagent. An automated liquid handler can be used to perform pipetting steps for transfecting batches of 96 tubespins, and septa in the tubespin caps allow for rapid processing without decapping. The addition of valproic acid (VPA) to transfection cultures enhances recombinant protein production. The thawing and passaging operations for HEK293 cultures to source the transient transfections are also described.

Key words Transient transfection, HEK293 cells, Polyethylenimine, High throughput, Recombinant protein production, Automation

1 Introduction

Popularized in the 1990s, transient transfection of mammalian cells is an efficient tool to express recombinant proteins for research and preclinical studies [1, 2]. Transient transfections for protein production are not only quicker than the generation of stably transfected cell lines—days compared to months—but the transient transfection process is also less labor intensive and highly automatable by liquid handling robotics [3–5]. This enables high throughput screening of candidate proteins to accelerate the identification of lead molecules for use in clinical trials.

In particular, HEK293 cells are used for early stage pipeline development because these cells are highly transfectable. HEK293 cells contain the early region 1 (E1) of the adenoviral genome and express the adenoviral E1A and E1B proteins [6]. The cells used in this protocol, 293T, are derived from HEK293—stably transfected with the SV40 large T-antigen and suspension adapted (*see* **Note 1**). While it is recognized that proteins produced from HEK293

David L. Hacker (ed.), *Recombinant Protein Expression in Mammalian Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 1850, https://doi.org/10.1007/978-1-4939-8730-6_12, © Springer Science+Business Media, LLC, part of Springer Nature 2018

cells may exhibit different product quality than those produced from Chinese hamster ovary (CHO) cells, HEK293 cells are frequently chosen for recombinant protein expression during research because they have comparable or higher titers with faster timelines than CHO (typically 5–7 days for HEK293 vs. 10–14 days for CHO) [7, 8].

Here, we describe a method for high throughput transient transfection of 293T cells using PEI as a transfection reagent at the 30 mL scale in tubespins [9]. PEI is a cationic polymer that interacts with negatively charged plasmid DNA to form a DNA–-PEI complex. The DNA–PEI complex enters the host cell by endocytosis. The plasmid is then transcribed and translated by the cell's protein expression machinery to produce protein encoded by the plasmid. Various parameters of the PEI transfection process can be modulated to optimize protein production including the amount of PEI, the amount of DNA, the time the DNA–PEI complex is allowed to form, the media in which the DNA–PEI complex is formed, and the cell culture media [10]. As these parameters influence the formation and uptake of the complex into the cell [11], we suggest that all of these factors be optimized using a few representative plasmids prior to using the process as a platform for expression of diverse proteins.

A typical PEI transient transfection protocol is conducive to high throughput transfections as the steps are straightforward and thus easily automatable by liquid handling robotics. Timing and duration of the steps can influence transfection productivity. Our previous publications have detailed execution of 1 mL and 30 mL transient transfections by liquid handling robotics [3, 12]. Here, we suggest using a liquid handler (e.g., Biomek FX^{P}) capable of pipetting at least 1 mL to perform the liquid addition steps of a 30 mL transient transfection protocol and using tubespins with septa to avoid decapping for high throughput processing of many different transfections. During the automated transfection of 96 tubespins, cultures may be held at room temperature in the absence of agitation for up to 1 h without negatively impacting production titers.

Histone deacetylase inhibitors such as VPA are commonly added to mammalian cell culture to increase recombinant protein production [13, 14]. Chromatin accessibility for transcription factors can be regulated by acetylation and deacetylation of histones. By inhibiting histone deacetylation, chromatin remains in a more open configuration for transcription. VPA has been reported to increase mRNA levels of a recombinant protein during transient transfection of HEK293 and CHO cells [15]. We have found that VPA added during the seeding of production cultures (as described here) or 24 h after transfection similarly improve protein production (data not shown).

We have used the described method for 30 mL 293T transient transfections to successfully generate thousands of proteins to support various discovery efforts and pipeline projects across a biopharmaceutical organization. This high throughput process uses PEI as a transfection reagent, VPA to enhance titers, and an automated liquid handler to efficiently transfect batches of 96 tubespins.

2 Materials

2.1 Thawing and Passaging 293T Seed Train Cultures

1. Preestablished 1 mL vial of ~3×10^7 suspension adapted 293T cells (HEK293 cells stably transfected with SV40 large T-antigen) stored in liquid nitrogen.
2. Seed train medium (Freestyle 293 expression medium (ThermoFisher) supplemented with 1% low IgG fetal bovine serum).
3. 125 mL, 250 mL, 500 mL, 1 L, or 2 L baffled shake flasks with vent cap (Corning or equivalent).
4. Clean glass 500 mL beaker.
5. Sterile water.
6. 70% ethanol or isopropanol.
7. Cell counter (e.g., Beckman Coulter Vi-CELL or Roche Cedex).
8. Orbital shaker at 150 rpm with orbital diameter of 25 mm (e.g., Kuhner or Innova) with adhesive matting (Infors HT, Sticky Stuff).
9. Incubator at 37 °C, 5% CO_2, and 80% relative humidity (RH).

2.2 Seeding and Transfecting 293T Production Cultures

1. 293T seed train culture (*see* Subheadings 3.1 and 3.2 on thawing and passaging 293T seed train cultures).
2. Sterile 3 L bottle.
3. Production medium (Freestyle 293 expression media (ThermoFisher) supplemented with 1% low IgG fetal bovine serum and 0.1 g/L gentamicin).
4. 1 M valproic acid (VPA) (Sigma) in deionized water.
5. 50 mL conical tubespin with filters and septa (TPP or Optimum Processing).
6. Cell counter (e.g., Beckman Coulter Vi-CELL or Roche Cedex).
7. Incubator shaker at 37 °C, 5% CO_2, 80% RH, and 225 rpm with orbital diameter of 50 mm (Kuhner).
8. EPFL table for incubator shaker (Kuhner).

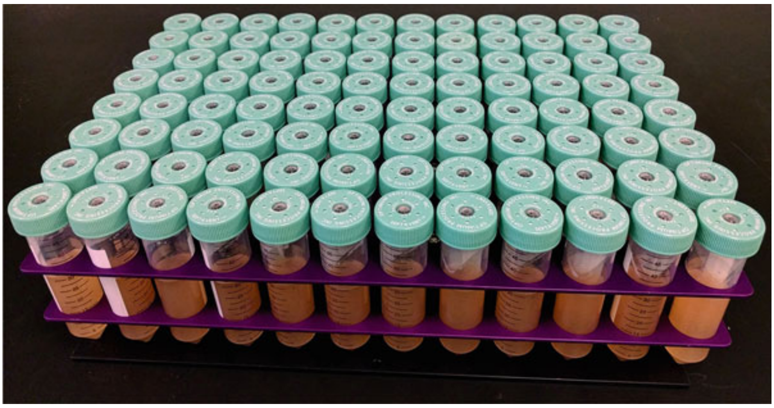

Fig. 1 Example of an 8 × 12 tubespin holder rack for 96 × 50 mL conical tubespins. This rack is 300 × 440 mm

9. Tubespin holder for 96 × 50 mL conical tubes (8 × 12 holder, *see* Fig. 1).
10. Pipeline (Essen Bioscience).
11. Peripette (Essen Bioscience).
12. Automated liquid handler capable of pipetting at least 1 mL per pass (e.g., Biomek FX^{P}), inside a Class II biosafety cabinet.
13. 48-deep-well plates.
14. Serum free medium. (e.g., Opti-MEM I Reduced-Serum medium (ThermoFisher)).
15. 30 μg plasmid DNA at >0.2 g/L in Tris–EDTA buffer for each 30 mL transfection (*see* **Note 2**).
16. 60 μL PEI (linear 25 kDa at 7.5 mM; Polyplus-Transfection) for each 30 mL transfection (*see* **Note 2**).

3 Methods

All cell culture operations should be completed inside a sterile biosafety cabinet using aseptic techniques. *See* Fig. 2 for a flow chart depicting these methods.

3.1 Thawing 293T Seed Train Cultures

1. Fill a 125 mL shake flask, 4–24 h before thaw, with 49 mL of seed train medium and place the flask on the orbital shaker inside the incubator.
2. Warm a bottle of sterile water to 37 °C.
3. On the day of thaw, thoroughly clean the beaker with 70% ethanol or isopropanol; place the beaker inside a biosafety cabinet and fill the beaker with 37 °C sterile water.

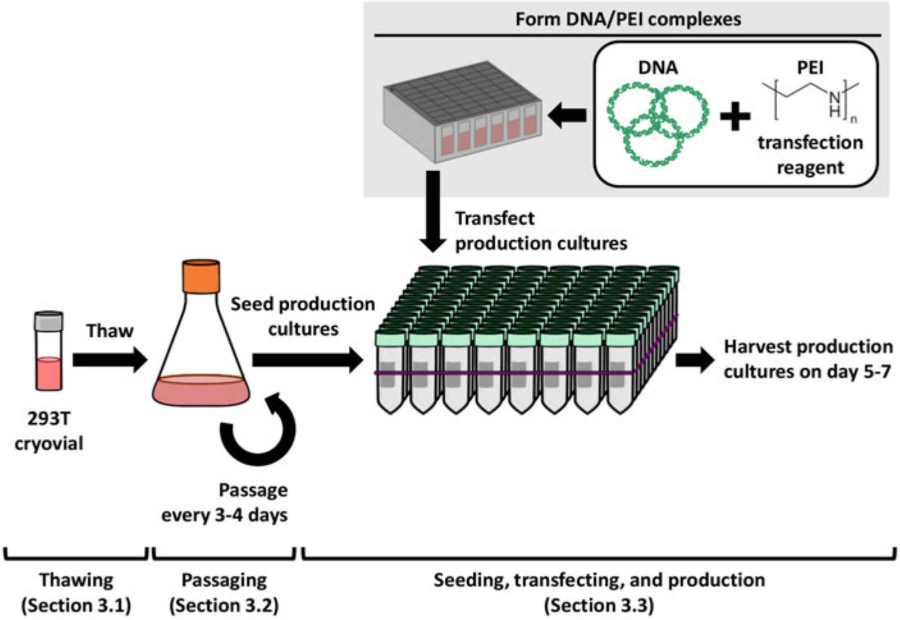

Fig. 2 Flow chart showing the entire process from thaw to transfection. Diagrams represent thawing 293T cells, passaging 293T cells, and seeding and transiently transfecting 293T cells in 30 mL tubespins for recombinant protein production

4. Remove the preestablished vial of 293T cells from liquid nitrogen storage.
5. Swirl the vial of 293T cells in the sterile water in the biosafety cabinet; do not submerge the entire vial.
6. When the contents of the vial are almost thawed, remove the vial from the water and thoroughly clean the vial with 70% ethanol or isopropanol.
7. Open the vial and transfer the contents to the baffled shake flask containing prewarmed medium.
8. Measure cell density and viability using the cell counter; the cells should be ≥80% viability. If viability is <80%, *see* **step 11** for instructions.
9. Place the flask on the orbital shaker inside the incubator.
10. On the 3rd day after thaw, measure cell density and viability using the cell counter; cells should be $\geq 3 \times 10^6$ cells/mL and ≥ 90% viability. If viability is <90%, *see* **step 11** for instructions. Passage this 293T seed train per Subheading 3.2 on passaging 293T seed train cultures.
11. If cells are <80% viability after thaw, <90% viability 3 days after thaw, or < 95% viability during passaging, try performing a

media exchange (*see* **step 12**) or thawing another preestablished vial of 293T cells (*see* **step 13**).

12. To perform a media exchange, centrifuge the culture at 228 × *g*, aspirate the spent media from the cell pellet, and resuspend the cells in 50 mL of fresh medium. Fill a 125 mL shake flask with the cell suspension and place the flask on the orbital shaker inside the incubator.
13. If the low viability problem persists, try creating another bank of vials of 293T cells.

3.2 Passaging 293T Seed Train Cultures

1. Passage every 3–4 days. Measure cell density and viability of day 3 or day 4 seed train cultures using the cell counter; cells should grow to $\geq 4 \times 10^6$ cells/mL and $\geq$ 95% viability after each passage.
2. To passage, combine day 3 or day 4 seed train culture and fresh seed train medium in a new shake flask so that the final viable cell density is 0.6×10^6 cells for a 3 day passage or 0.3×10^6 cells/mL for a 4 day passage. Use the following formulas to calculate the volume of day 3 or day 4 seed train culture and the volume of fresh seed train medium needed.

$$V_c = \frac{X_P V_P}{X_C}$$

$$V_M = V_P - V_C$$

Where:

V_C = volume of day 3 or day 4 seed train culture needed (mL)
X_P = viable cell density desired for next passage (0.6 or 0.3×10^6 cells/mL)
V_P = volume desired for next passage (mL; *see* **Note 3**)
X_C = viable cell density of day 3 or day 4 seed train culture (cells/mL)
V_M = volume of seed train medium needed for next passage (mL)

3. Place the newly passaged flask on the orbital shaker inside the incubator.

3.3 Seeding and Transfecting 293T Production Cultures

3.3.1 Seeding

1. Measure cell density and viability of day 3 or day 4 seed train cultures using the cell counter; cells should grow to $\geq 4 \times 10^6$ cells/mL and $\geq$ 95% viability after each passage. It is important that cells have $\geq$95% viability for transfections.
2. In a sterile bottle, combine 293T seed train and fresh production media so that the final viable cell density is 1.2×10^6 cells/mL. Use the following formulas to calculate the volume of day 3 or day 4 seed train culture and the volume of fresh production medium needed.

$$V_c = \frac{X_F V_F}{X_C}$$

$$V_M = V_F - V_C$$

Where:

V_C = volume of day 3 or day 4 seed train culture needed (mL)
X_F = final viable cell density desired for production cultures (1.2×10^6 cells/mL)
V_F = final volume desired for production cultures (3000 mL for 96 × 30 mL cultures)
X_C = viable cell density of day 3 or day 4 seed train culture (cells/mL)
V_M = volume of production medium needed (mL)

3. Supplement the production culture with 2.25 mL of 1 M VPA solution (final concentration of VPA in the final production culture will be 0.75 mM).
4. Aliquot 27 mL of production culture per tubespin. Use a pipeline dispenser and peripette or an automated liquid handler. Ensure that tubespin cultures sit out for $\leq$60 min during seeding.
5. Place tubespins in holder and clamp the latter onto the EPFL table inside the incubator shaker. Allow the production cultures to equilibrate for at least 2 h.

3.3.2 Transfecting

1. For each 30 mL transfection, pipette 30 μg of DNA to the bottom of a well in the 48-deep-well plate; fill 2 × 48-deep-well plates for 96 tubespin transfections.
2. Add 3 mL of serum-free medium to each well containing DNA in the 48-deep-well plates and mix the DNA–media gently.
3. Remove the tubespins (in the tubespin holder) from the incubator.
4. If using an automated liquid handler, place the tubespins (in the tubespin holder), and the 48-deep-well plates containing DNA and serum-free medium on the deck of the robot.
5. Form DNA–PEI complex; this may be done with an automated liquid handler. Add 60 μL of PEI to the DNA/medium for each transfection and mix the PEI/DNA/medium gently. If using an automated liquid handler, add the PEI to the DNA/media one column (8 wells) at a time. Stagger the addition of PEI to each column to allow sufficient time for completion of **step 6**.
6. Incubate PEI/DNA/media at 2–8 °C for 8–10 min (*see* **Note 2**). Use timers to ensure that this incubation time is controlled for each column.

7. Transfect the equilibrated production cultures by adding the entire complex volume (PEI/DNA/medium) to the culture and mixing gently. If using an automated liquid handler, aspirate DNA–PEI complexes one column at a time and dispense them into the production cultures through the tubespin septa. Wash fixed tips or load new disposable tips between columns to prevent cross contamination.
8. Ensure that tubespin cultures sit out for ≤60 min during transfections.
9. Clamp the tubespins (in the tubespin holder) onto the EPFL table inside the incubator shaker.
10. Harvest cultures 5–7 days after transfection.

4 Notes

1. The 293T used in this protocol were generated at Genentech. An adherent 293T cell line is available from ATCC.
2. The amount of DNA, the amount of PEI, and the incubation time to form the DNA–PEI complex have been optimized for the described cell line and media. Additional optimization may be needed if different HEK293 cells or media are used.
3. We recommend a working volume of ~50% the nominal volume of the shake flask (e.g., a 250 mL working volume for a 500 mL shake flask).

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Chapter 13

Microfluidic Transfection for High-Throughput Mammalian Protein Expression

Kristina Woodruff and Sebastian J. Maerkl

Abstract

Mammalian synthetic biology and cell biology would greatly benefit from improved methods for highly parallel transfection, culturing, and interrogation of mammalian cells. Transfection is routinely performed on high-throughput microarrays, but this setup requires manual cell culturing and precludes precise control over the cell environment. As an alternative, microfluidic transfection devices streamline cell loading and culturing. Up to 280 transfections can be implemented on the chip at high efficiency. The culturing environment is tightly regulated and chambers physically separate the transfection reactions, preventing cross-contamination. Unlike typical biological assays that rely on end-point measurements, the microfluidic chip can be integrated with high-content imaging, enabling the evaluation of cellular behavior and protein expression dynamics over time.

Key words Microfluidics, Transfection, Microarrays, DNA array, Protein array, Surface chemistry, Protein expression, Mammalian cells, High-throughput screening, Automated microscopy

1 Introduction

Cell-based research and therapeutic endeavors frequently entail expressing and studying specific proteins. Transfection is the process of introducing foreign genetic material into mammalian cells [1]. In contrast to those produced in prokaryotic systems, the expressed proteins undergo proper folding and posttranslational modifications. This technology is thus pertinent to protein production, functional assays, and therapeutic gene delivery.

Chemical transfection can be achieved with widely used reagents such as cationic lipids, which neutralize the negative charge of DNA and facilitate entry into the cell by endocytosis. The integration of lipid-based transfection with contact spotting has enabled high-throughput transfection. In this technique, called reverse transfection, purified cDNA samples are mixed with transfection reagent and spotted onto glass slides [2]. The arrays are next seeded with cells, which undergo transfection in situ, resulting in

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exogenous gene expression. Unlike protein microarrays [3–5], this method does not require individual purification of each sample and proteins can be analyzed in the natural cellular context. More than 5000 samples can be printed on a single glass microscope slide using standard techniques [2, 6, 7]. This throughput enables massively parallel characterization of complex synthetic networks and the screening of genome-wide RNAi and cDNA libraries [8, 9].

Although reverse transfection has been optimized for a variety of genetic materials [10, 11] and cell types [12], methods for cell manipulation on the arrays still stand to be improved. Cell seeding and culturing is performed manually, and cross-contamination is a concern because spots on the array are not physically separated from one another. Standard reverse transfection offers poor control over the cell microenvironment and cannot support sophisticated downstream experiments.

Microfluidics could compensate for these shortcomings by providing a means to enclose each position on the array in a cell culture chamber. Microfluidic devices are readily fabricated using standard photolithography and soft lithography techniques [13] and contain micromechanical components such as valves that execute complex fluidic manipulations. These devices are characterized by high throughput, automation, small sample requirements, and compatibility with other analytical techniques. Standard microfluidic chips designed for mammalian cells are capable of a variety of functions, including long-term perfusion culture and the ability to individually address cell chambers [14].

This protocol describes the high-throughput transfection of mammalian cells on a microfluidic chip [15]. The device consists of two components: a glass slide patterned with transfection reagent, and a microfluidic device with chambers that support cell culturing (Fig. 1). The 280 microfluidic chambers have a capacity of ~600 cells each. The chambers of the chip are aligned to the transfection array so that each chamber contains a unique transfection mixture. The arraying of DNA-containing transfection mixtures is coupled to the arraying of poly-L-lysine (PLL), which serves to anchor the DNA to the glass slide. This process has been optimized to enable highly efficient transfection with low cross-contamination. The chip is operated and cultured in a microscopy setup, facilitating the interrogation of protein expression dynamics and synthetic gene network performance.

2 Materials

2.1 Mask and Wafer Fabrication

1. Computer-aided design (CAD) software.
2. Heidelberg VPG200 laser lithography system (Heidelberg Instruments Mikrotechnik GmbH).

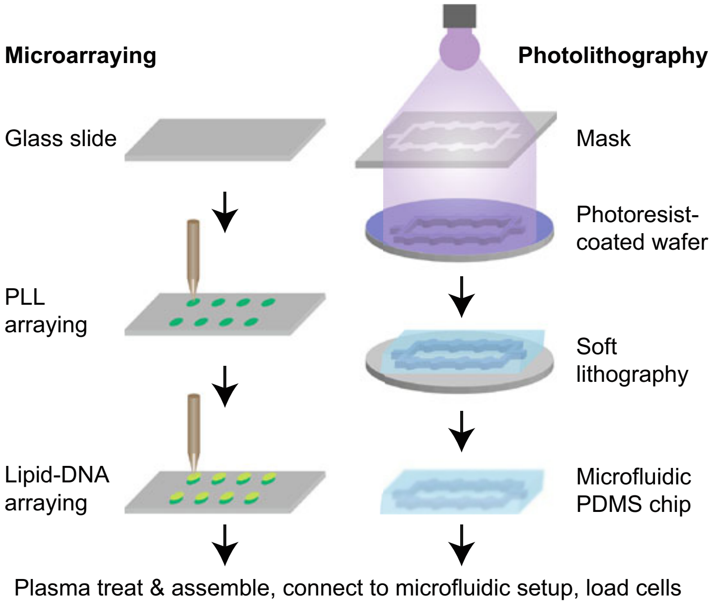

Fig. 1 Workflow to generate a microfluidic transfection array. The transfection device consists of two components: a glass slide patterned with transfection reagent (left) and a microfluidic device that supports cell culturing (right). The lipid-DNA transfection mixtures are arrayed into distinct spots on the glass slide. The microfluidic component is fabricated using standard photolithography and soft lithography techniques. The two components are assembled together so that one lipid-DNA spot is enclosed inside each microfluidic cell culturing chamber. The assembly is connected to a microfluidic setup, and cells loaded into the device undergo transfection

3. DV10 developer (Süss MicroTec AG).
4. Tepla300 dry etcher, oxygen plasma (PVA Tepla AG).
5. MA6 mask aligner (Süss MicroTec AG).
6. Sawatec LSM250 programmable coater for SU-8 (Sawatec AG).
7. EVG150 coater and developer system for positive resist (EV Group).
8. Dektak XT surface profilometer (Bruker).
9. Silicon wafers, diameter: 100 ± 0.5 mm, thickness: 525 ± 25 μm, conductivity type: N or P, dopant: Boron or Phosphorous, resistivity range: 0.1–100 Ωcm (Okmetic).
10. Chrome-coated mask plates, 5 × 5 inches (Nanofilm).
11. Developer MP 351 (Merck).
12. Chromium etch CR7 MOS: $(NH_4)_2Ce(NO_3)_6$; $HClO_4$.
13. TechniStrip P1316 (Microchemicals).

14. AZ9260 photoresist (Clariant GmbH).
15. Developer AZ 400 K (Merck).
16. GM1070 (Gersteltec).
17. Propylene glycol methyl ether acetate (PGMEA).
18. Isopropanol.

2.2 PDMS Chip Fabrication

1. Thinky mixer ARE-250 with adaptor for 100 ml PP beakers (C3 Prozess- und Analysentechnik GmbH).
2. Vacuum desiccator.
3. SCS G3P-8 spin coater (Specialty Coating Systems).
4. Manual hole punch machine and pin vises, 21 gauge, 0.04” OD (Technical Innovations, Inc.).
5. Stereomicroscope SMZ1500 (Nikon AG).
6. Femto plasma cleaner (Diener electronic).
7. 80 °C oven.
8. Trimethylchlorosilane (TMCS).
9. Polydimethylsiloxane (PDMS), Sylgard 184 (Dow Corning).
10. 100 ml polypropylene (PP) beakers.
11. 10-cm diameter glass petri dishes.
12. Tweezers, scalpel.
13. Scotch tape.
14. Aluminum foil.

2.3 Microarray Fabrication

1. QArray2 microarrayer (Genetix GmbH).
2. Conical-well, poly(propylene) 384-well plates (Arrayit).
3. Arraying pin with a 300 μm spot diameter and 3.3 nl delivery volume (Arrayit).
4. Standard glass microscope slides, 76 × 26 × 1 mm.
5. Glass slide rack and dish.
6. Dish soap.

2.4 PLL and Transfection Mixture Preparation

1. NaOH and ethanol solution: 57% ethanol and 10% NaOH in water.
2. Milli-Q water.
3. 0.1% poly-L-lysine (PLL) solution.
4. Boric acid solution: 0.225 M boric acid, pH 8.4, in water.
5. Gelatin, Type B, 225 g Bloom (Sigma).
6. 0.1% fibronectin solution (Sigma) or bovine plasma fibronectin dissolved in water at 1 mg/ml.

7. Supercoiled plasmid DNA (*see* **Note 1**); eGFP and tdTomato genes expressed from the human cytomegalovirus (hCMV) immediate early promoter/enhancer.
8. Effectene (Qiagen).
9. 20 ml syringe with Luer-Lok tip.
10. 25 mm syringe filter with 0.45 μm cellulose acetate membrane.
11. Vortex.
12. Small benchtop centrifuge.

2.5 Cell Culture

1. HEK 293-T cells.
2. Autoclave.
3. Dulbecco's Modified Eagle Medium (DMEM).
4. Fetal bovine serum (FBS) (*see* **Note 2**).
5. Antibiotic–antimycotic solution.
6. TrypleE express (Life Technologies).
7. CO_2-independent medium (Life Technologies).
8. GlutaMAX solution (Life Technologies).
9. T-75 flasks, serological pipets, 0.2 μm filter, other standard cell culture materials.
10. 25-ml glass laboratory bottles with open-top cap (Schott) and silicon septa.

2.6 Microfluidic Setup

1. Precision pressure regulator, BelloFram Type 10, 2–25 psi, 1/8″ port size (Bachofen SA).
2. Bourdon tube pressure gauges, 0–30 psi, G 1/4 male connection (Kobold Instruments AG).
3. Custom-designed manual manifolds (rectangular metal casing: 14.5 × 1 × 1″) with toggles and barbs for 1/16" ID tubing, 1/4 NPT connection (Pneumadyne.).
4. Fittings to connect regulators to gauges and to luer manifolds: tee union and male adaptor union (Serto AG).
5. Polycarbonate luer fittings (ThermoFisher Scientific), multiport luer manifolds for flow inlet regulation (Cole Parmer); male luer to luer connector (Cole Parmer).
6. Tygon tubing for pneumatic setup, 1/4" OD x 1/8" ID (Fisher Scientific AG).
7. Disposable stainless steel dispensing needles to connect to syringe, 23 gauge, 1/2″ long, 0.33 mm ID (Gonano).
8. Flexible plastic tubing for fluidic connections, Tygon S54HL, 0.51 mm ID (ThermoFisher Scientific).

9. PTFE tubing for fluidic connections, 0.022“ ID x 0.042” OD (Cole Parmer).
10. Steel pins for chip-to-tube interface, Tube AISI 304 OD/ID x L 0.65/0.30 × 8 mm, cut, deburred, passivated (Unimed SA).

2.7 Microscope Setup

1. Nikon Ti-E Eclipse automated epi-fluorescence microscope.
2. Filter cubes: TexasRed (HC 562/40, HC 624/40, BS 593) and FITC (HC 482/35, HC 536/40, BS 506) (AHF Analysentechnik AG).
3. Cube air heater and incubation chamber to enclose the microscope (Life Imaging Services).
4. Ixon DU-888 camera (Andor Technology).
5. NIS Elements, Fiji, and MATLAB imaging and image processing software.

2.8 Lipid–DNA Microarraying

1. 0.5% gelatin solution: 0.15 g of gelatin is added to 30 ml of prewarmed Milli-Q water at 60 °C. The mixture is incubated in a 60 °C water bath and swirled gently eight times during a 15 min period. The solution is removed from the bath and left at room temperature until it has cooled to 37–40 °C. The mixture is then loaded into a syringe, filtered through a 0.45 μm membrane, and stored in 700 μl aliquots at 4 °C (*see* **Note 3**).
2. 0.2 M Sucrose-EC solution: sucrose is dissolved in EC buffer (Effectene transfection kit) to a final concentration of 0.2 M, and the solution is sterilized by filtration.

3 Methods

High-throughput microfluidic transfection incorporates several techniques including photolithography, soft lithography, microarraying, operation of a microfluidic setup, and cell culture (Fig. 1) [16]. The 280-chamber transfection device consists of 2 layers: a flow layer (containing cells, media, etc.) and a superimposed control layer (valves to manipulate the flow layer). Both layers are written on chrome masks in a clean room facility. The masks transfer the design to photoresist-coated wafers, which serve as molds for PDMS casting. Soft lithography techniques are employed to fabricate the PDMS chips.

The PDMS chip is aligned and bonded to a transfection microarray to generate the final device. The microarray is similar to those used for reverse transfection [2, 17–19] and consists of 280 spots containing DNA mixed with a lipid-based transfection reagent. The surface of the microarray is patterned with PLL (a hydrophilic, DNA-retaining substance) in the positions of the chambers prior to lipid–DNA microarraying (Fig. 1). This protocol ensures robust DNA attachment, even under the high flow rates experienced on the microfluidic chip. Unlike traditional approaches that use glass slides entirely covered in PLL, this method is compatible with hydrophobic PDMS chips since the chip is not directly in contact with the positions coated with PLL.

The assembled device is connected to a microfluidic setup. A suspension of cells is loaded into the channels, and valves are actuated to segregate individual chambers. Medium is perfused through channels running parallel to the cell chambers, eliminating sheer stress during culturing. Diffusion from the channels introduces nutrients into and eliminates waste from the chambers. The cell chip is cultured directly on a microscope stage encased in an incubation chamber, enabling manipulation and monitoring of transfection over several days.

3.1 Mask and Wafer Fabrication

All mask and wafer fabrication steps are performed in a class 100 clean room. For researchers without access to a clean room or PDMS fabrication facilities, many commercial mold and device fabrication services are available. The design files of the transfection device are available for download from zenodo.org (https://zenodo.org/record/820777#.WVSrbhPfrOQ).

3.1.1 Chip Design

1. The microfluidic device is designed using CAD software. The chip measures 1.6 × 5.8 cm and contains 280 culturing and transfection chambers (Fig. 2a). Two molds should be designed: one for the control layer (30 μm height), which contains the valves, and another for the flow layer (30 μm height), which contains the channels and chambers necessary for reagent introduction and cell culturing (Fig. 2b,c, *see* **Note 4**).
2. The flow layer mold contains two patterns (SU-8 and AZ9260) on the same wafer, so alignment marks are added to the design to enable alignment of the layers during the exposure step.
3. The control layer is scaled by 101.5% to account for PDMS shrinkage during curing.

3.1.2 Mask Fabrication

1. A Heidelberg VPG200 laser lithography system with a 20 mm writing head and a UV-light source (i-line $\lambda = 355$ nm) is used to write each layer of photoresist on a separate mask (2 masks for the flow layer, 1 for the control layer).
2. Masks are developed using the DV10 instrument. The development dispenser is purged before the mask is placed inside the chamber and developer (a 1:5 mixture of MP 351 and Milli-Q water) is applied twice (15 s application followed by 45 s agitation), followed by rinsing and drying.

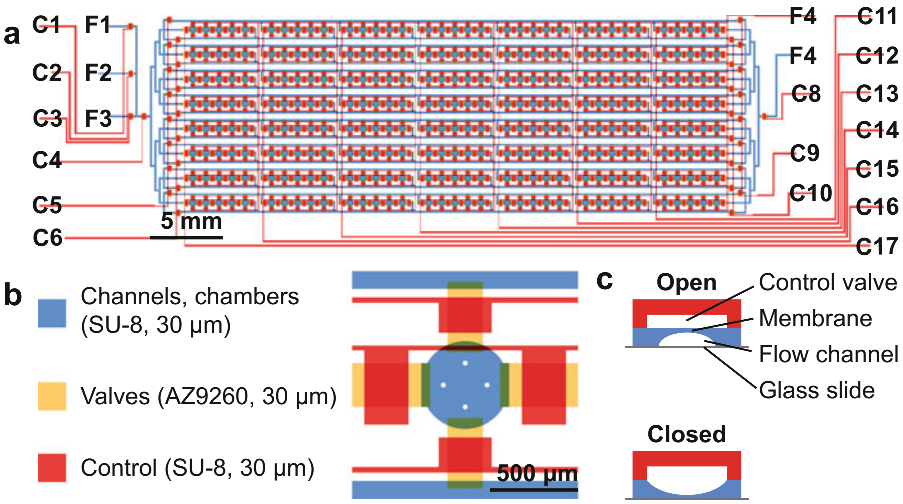

Fig. 2 Design of the high-throughput transfection chip. (**a**) Design of the 280-chamber transfection and cell culturing chip. Red, control lines for valve actuation, blue/yellow: flow channels and cell culturing chambers. F1–F3 are flow inputs, F4 is the flow outlet. C1–C3 control inputs F1–F3. C4 and C8 control access to the body of the chip. C7 controls access of cell chambers to medium channels. C5 and C9 control flow in the upper medium channel. C6 and C10 control flow in the lower medium channel. C11-C17 segregate the chambers in individual columns. (**b**) Close-up of one chamber. The features are created by patterning different layers of photoresist. (**c**) Schematic of valve opening and closing. When the control line is actuated, the resulting pressure depresses the PDMS membrane separating the control valve and the flow channel, closing the flow channel

3. The chrome layer of the mask is etched in a perchloric acid solution for 120 s, then washed with water (quick dump rinse followed by an ultrapure water bath) and dried. The remaining photoresist is removed using TechniStrip 1316 (manual application to mask followed by complete immersion in bath for 10 min). The mask is washed with water (quick dump rinse followed by an ultrapure water bath) and air-dried.

3.1.3 Control Layer Wafer Fabrication

1. Wafers are cleaned with oxygen plasma for 10 min at 500 W power, 2.45 GHz plasma frequency, Gas: O_2 and CF_4, max. Flow 500 ml/min.
2. Immediately after, the Sawatec LSM250 is used to spin coat a 30 μm thick layer of SU-8 (GM1070): 5 s ramp up, 5 s at 500 rpm, 20.3 s ramp up, 40 s at 2527 rpm, 1 s at 3527 rpm, 1 s ramp down to 2527 rpm, 5 s at 2527 rpm, and 25.3 s ramp down to 0 rpm.
3. The wafers are relaxed for 20–45 min at room temperature.
4. The wafers are baked using the hot plate of the Sawatec LSM250 for 5 min at 130 °C, with ramp up and ramp down times of 50 min each.

5. The MA6 mask aligner is used to expose the baked wafers to light for 8 s in soft contact mode with an alignment gap of 30 μm using a lamp intensity of 20 mW/cm^2 (WEC type: cont; N2 purge: no; WEC-offset: off).
6. The wafers are baked on the Sawatec LSM250 hot plate as follows: 25 min ramp up, 15 min at 70 °C, 15 min ramp up, 40 min at 100 °C, 60 min ramp down to room temperature.
7. The wafers are placed in a storage box overnight to encourage rehydration under ambient conditions.
8. The wafers are developed in 2 consecutive 5 min PGMEA baths, followed by 1 min in isopropanol and air drying. If white residues appear during the isopropanol bath, the wafer is returned to the PGMEA bath for further development.
9. The wafers are inspected by microscopy, and if cracks are visible in the features, a hard bake (120 min at 135 °C, with ramp up and down times of 40 min each) is performed.
10. The features are measured using a Dektak XT surface profilometer.

3.1.4 Flow Layer Wafer Fabrication

1. The flow layer is patterned with SU-8 (for the channel features) as described above. For the valve features, AZ9260 is next patterned on the same wafers as described below.
2. An automated wafer coating, baking, and development machine (EVG150) is used. The wafers are dehydrated for 4 min 30 s at 160 °C (*see* **Note 5**). AZ9260 is then spin coated as follows: dispensing of resist at 100 rpm, increase to 500 rpm, 3 s ramp up, 45 s at 1700 rpm, 60 s ramp down to 0 rpm. Edge bead removal (15 s) and backside removal (20 s) are performed at 1000 rpm. The coated wafers are baked for 1 min 35 s at 110 °C. AZ9260 resist is deposited a second time (using the same recipe) to yield a final height of 30 μm. The wafers are baked for 3 min 15 s at 110 °C.
3. The coated wafers are rehydrated overnight.
4. The MA6 mask aligner is used to expose the wafers to light for 2 sessions of 28 s, separated by a 10 s wait. The mask used with the exposure tool is aligned to the existing SU-8 pattern on the wafer. The exposure tool is operated in hard contact mode with an alignment gap of 30 μm and a lamp intensity of 20 mW/cm^2 (WEC type: cont; N2 purge: no; WEC-offset: off).
5. The EVG150 is used to dispense developer AZ 400 K onto the wafers (9 min at 250 rpm), rinse them with Milli-Q water (30 s at 250 rpm), perform backside removal (10 s at 250 rpm), and drying (30 s at 3000 rpm).

6. The wafers are washed with water (quick dump rinse followed by a water bath in ultrapure water) to remove any remaining developer residues.
7. The features are annealed (to create a rounded profile) by placing the wafers on a Sawatec LSM250 hot plate for 300–600 s at 140 °C.
8. The features are measured with a Dektak XT surface profilometer. If the profile of the valve regions is not completely rounded, the annealing bake should be repeated for a longer amount of time.

3.2 PDMS Chip Fabrication

1. Flow and control wafers are incubated for 10 min in a sealable wafer holder containing a plastic cap filled with 1 ml TMCS. This process is repeated prior to each chip fabrication to prevent PDMS from lifting photoresist features off the wafer.
2. Both wafers are placed into glass petri dishes lined with aluminum foil (*see* **Note 6**).
3. For the control layer, 60 g of a 5:1 PDMS mixture (50 g Part A: 10 g Part B) is placed in a disposable polypropylene (PP) cup, mixed for 1 min at 2000 rpm (~400 × *g*), and degassed for 2 min at 2200 rpm (~440 × *g*) in a centrifugal mixer.
4. The control wafer is cleaned with a nitrogen air gun to remove any debris or small dust particles (*see* **Note 7**). The PDMS mixture is immediately poured onto the control layer wafer and the assembly is degassed in a vacuum chamber for 20 min. Upon removal of the PDMS-coated wafer from the chamber, any remaining bubbles are pushed to the edges of the petri dish using a pipet tip.
5. For the flow layer, 20 g of a 20:1 PDMS mixture (20 g Part A: 1 g Part B) is placed in a disposable PP cup and mixed and degassed as described above.
6. The flow wafer is placed inside the spin coater and cleaned with a nitrogen air gun, then coated with PDMS using a ramp of 15 s and a spin of 35 s at 650 rpm.
7. Both the control and flow molds are baked in an oven for 30 min at 80 °C (*see* **Note 8**).
8. The molds are removed from the oven and the PDMS of the control layer is cut with a scalpel to remove each chip design from the wafer. To enable attachment of tubing to the control channels, holes are punched at the control line inlets. The holes are punched with the patterned side of the chip facing up, and displaced PDMS stubs are removed with tweezers.
9. Scotch tape is placed in contact with the edges and the patterned side of the chip and subsequently removed to clean debris from the surface.

10. The control layer chips are aligned to the PDMS-coated flow wafer using a stereomicroscope. Slight pressure is applied throughout the chip to ensure that all regions of the control layer are in contact with the flow layer.
11. The assembly is baked for 90 min at 80 °C.
12. A scalpel is traced around the outline of each device to cut the PDMS layer coating the flow wafer (*see* **Note 9**).
13. The bonded control/flow devices are removed from the wafer, and holes for the flow inlets and outlets are punched with the patterned side of the chip facing up.
14. The edges and patterned side of the chip are cleaned with Scotch tape before bonding to the transfection arrays.
15. For the flow wafer, the remaining thin PDMS layer is removed by pouring a 10:1 PDMS mixture (mixed and degassed) onto the wafer and baking for 30 min at 80 °C. Once cured, the thick PDMS layer can be easily removed from the entire wafer.
16. For the control wafer, excess PDMS is cut away from the body of the wafer and a frame is left near the edges to reduce the quantity of PDMS required for subsequent batches of chips.

3.3 PLL Microarraying

1. Glass slides are arranged in a rack and submerged in a covered dish containing 57% Ethanol and 10% NaOH. The slides are incubated for 2 h with gentle shaking, then rinsed five times with Milli-Q water and air dried.
2. The arraying pin is placed in a 15-ml conical tube containing a soap–water mixture (~0.5 ml dish soap diluted in 15 ml water) and placed in a sonication bath for 10 min (*see* **Note 10**). The soap–water mixture is then replaced with 70% ethanol and the pin is sonicated for another 10 min. The pin is dried with a nitrogen air gun and placed in the microarrayer head.
3. The PLL sample is prepared by adding 25 μl of a 0.1% PLL solution to 50 μl of 0.225 M boric acid, pH 8.4. The sample is loaded into a well on a 384-well plate. One sample can be used to prepare up to 2 glass slides. To simultaneously prepare more glass slides, multiple PLL samples should be used.
4. The glass slides and the well plate are loaded into the microarrayer (*see* **Note 11**). PLL is stamped four times per spot in a cyclic fashion, with approximately 8 min between cycles (modifying the wait times to 6–14 min has no detrimental effect on transfection efficiency). The pitch of the spots matches that of the chambers on the microfluidic chip: 8 rows, 41 columns (7 sets of 5, with one blank position between each set; x-pitch 900 μm, y-pitch 1700 μm). Spotting parameters are 1 s inking time, 500 ms printing time, 4 stamps/ink. Every 40 inks, the

pin is washed with water for 500 ms and dried for 500 ms. Humidity in the spotter is set to 50%.

5. Orientation marks are placed on the slides (*see* **Note 12**) and 2 h after arraying is complete, PLL arrays are washed 5× 30 s with Milli-Q water, dried using a nitrogen air gun, and stored in a desiccator. PLL slides should be used between 2 and 8 weeks post-coating, since the quality of the PLL has been shown to deteriorate beyond this time period [20].

3.4 Lipid–DNA Microarraying

1. 1.5 μg of supercoiled plasmid DNA is diluted in 15 μl of the 0.2 M sucrose-EC buffer (*see* **Note 13**). In the case of cotransfection the total DNA of all plasmids should add up to 1.5 μg and the mixture should be vortexed for 10 s, then incubated for 15 min. The tubes are quickly spun down after each vortexing step. For a modification of this method (gelatin-DNA method), *see* **Note 14**.
2. 1.5 μl Enhancer (Effectene transfection kit) is added. The mixture is vortexed for 1 s and incubated for 5 min.
3. 5 μl Effectene is added. The mixture is gently vortexed for 10 s and incubated for 10 min.
4. 12.7 μl of the 0.5% gelatin solution and 12.7 μl of a 0.1% fibronectin solution [12] are added and mixed by pipetting up and down. Samples are transferred to a 384-well plate for microarraying. Each sample can be used to array up to 140 spots.
5. The microarray pin is cleaned as previously described and placed in the microarray head. The PLL-patterned slides are oriented in the holders and the spotter is programmed to spot in the same positions as previously used for PLL.
6. The lipid–DNA is stamped once per spot (*see* **Note 15**). Spotting parameters are 1 s inking time, 500 ms printing time, 4 stamps/ink. Every 40 inks, the pin is washed with water for 500 ms and dried for 500 ms. Humidity in the spotter is set to 65%.
7. Printed slides are stored in a desiccator and used within a few weeks.

3.5 Device Assembly

1. The spotted glass slides are cleaned with a nitrogen air gun and placed into a plasma chamber with the spots facing up.
2. The PDMS chips are cleaned with Scotch tape and placed into the plasma chamber with the features facing up.
3. The slides and chips are simultaneously plasma treated (settings: 23 ml oxygen, power setting of 5, 1 min vacuum pump, 1 min gas, 7 s plasma). Immediately afterward, the functionalized surfaces are aligned (one-shot) using a stereomicroscope

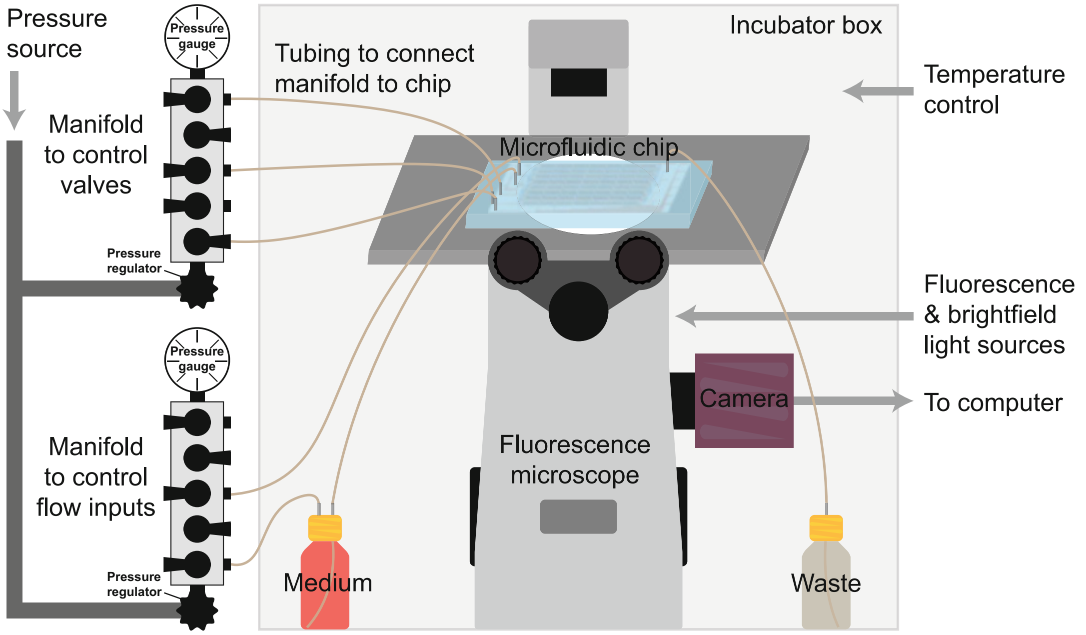

Fig. 3 Schematic of the microfluidic setup. A pressure source is connected to manifolds that enable manipulation of specific control and flow lines on chip. The ports of the manifold are individually controllable and attached to the chip with flexible tubing. The chip is cultured on the stage of a fluorescence microscope contained inside an incubation chamber

(*see* **Note 16**). Slight pressure can be applied to ensure that all parts of the chip are in contact with the glass slide (*see* **Note 17**). The assembly is baked for 1 h at 80 °C.

4. The transfection devices are stored in a desiccator.

3.6 Microfluidic Setup and Operation

The microfluidic setup comprises pressurized manifolds for flow and control layer manipulation, a fluorescence microscope, an incubation chamber, and a camera (Fig. 3).

3.6.1 Microfluidic Setup: Control Layer

1. A pressure source is attached to manifolds controlled by pressure regulators, with gauges connected to the ends of the manifolds. The toggle switches on the manifolds control the on/off state of individual ports. Each port is connected to a flexible Tygon tube that is fitted with a steel pin at the other end, enabling connection to the holes punched on the chip. The pneumatic setup can be custom built to allow for different pressure ranges and to accommodate larger numbers of control and flow lines.
2. A syringe is used to aspirate and transfer filtered water to a control line tube (a short piece of Tygon tubing serves as an adaptor between the syringe needle and the pin). The control line is placed in the corresponding hole of the chip and

pressurized at 5 psi (*see* **Note 18**). This process is repeated for each control line (Fig. 1a, C1-C17). Once the air in the chip's control lines has been replaced by water, the pressure is increased to 22 psi.

3.6.2 Microfluidic Setup: Flow Layer

1. For flow connections, small samples (such as PBS for washing or cells for loading) are contained in Tygon tubing attached to a manifold at one end and the chip at the other end. The Tygon tubes are loaded and connected as described above for the control lines.
2. For large volume or long-term flow connections, PTFE tubing is used (*see* **Note 19**). A bottle containing filtered CO_2-independent medium supplemented with 10% FBS, antibiotic–antimycotic solution, and GlutaMax is attached to the microfluidic setup (*see* **Note 20**). A piece of tubing is placed inside the bottle, running from top to bottom. The tube is connected to a pin that punctures the septa of the cap (this entire assembly is autoclaved prior to use). On the other side of the septa, the pin is connected to a longer piece of tubing that connects to the chip. Finally, a second piece of tubing is attached to a manifold at one end and punctures the septa on the other end. This setup pressurizes the bottle to drive flow into the chip (Fig. 3).

3.6.3 Microfluidic Operation: Flow Inputs

1. To flow samples on chip, all valves are initially closed (control lines C1-C17 pressurized).
2. The tube containing the sample is connected to the chip (for example to position F1) and pressurized at ~2–4 psi.
3. The corresponding valve (C1) controlling the flow of F1 is opened.
4. A second valve (such as C3) controlling an additional flow input (such as F3) is opened, serving as a purge to remove any air introduced at the end of the sample.
5. To terminate the purge and begin flowing on chip, C3 is activated and the master valves (C4, C8) controlling access to the body of the chip are opened. The outlet, F4, is connected to a waste bottle with Tygon tubing.
6. All valves controlling channel and chamber features (C5-C7, C9-C17) are opened to facilitate filling of the entire chip.
7. To disperse any air bubbles trapped in the chip, the outlet valve (C8) is activated while the sample is flowed into the chip. The pressure can be increased to accelerate this step. Once all air has been removed, C8 is opened to permit continuous flow.

3.6.4 Microfluidic Operation: Cell Loading

1. A T-75 flask of HEK 293-T cells at 70–80% confluence is harvested. 800,000 cells are centrifuged and resuspended in 20 μl PBS (*see* **Note 21**).
2. As quickly as possible, the sample is loaded into a Tygon tube, attached to the manifold and chip, and purged as described above.
3. The cells are loaded into the chip at a rate of 27 μl/min (*see* **Note 22**), with columns loaded in sets sequentially (one column of 5-chamber segments at a time, Fig. 4a). When a sufficient number of cells (~70% confluent) is obtained in the chamber segment (*see* **Note 23**), chamber-separating valves (C11-C17) are immediately actuated to capture cells in the chambers (*see* **Note 24**).

3.6.5 Microfluidic Operation: Cell Culturing

1. The chip is cultured on the stage of an automated microscope contained in an incubation chamber maintained at 37 °C.
2. For the first hour after loading, medium is pulse perfused [21] to promote attachment and prevent cells from being washed out of the chambers (Fig. 4b, **Note 25**). Medium is flowed for

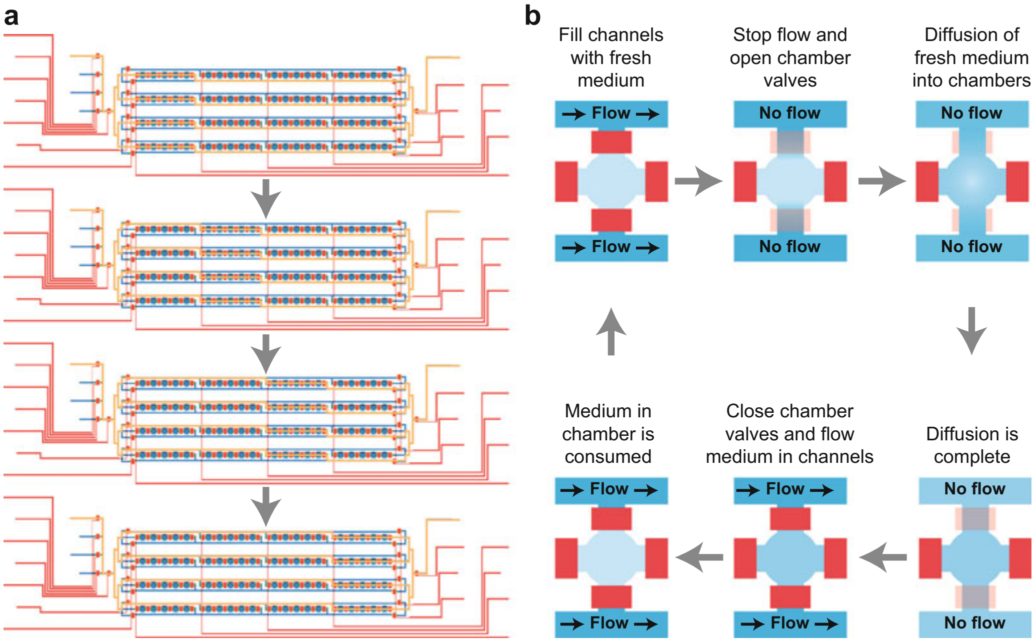

Fig. 4 Schematic of cell loading and medium perfusion. (**a**) Cell loading is performed by sequentially loading each segment of 5 chambers. The yellow lines designate the flow trajectory through the chip. A low-throughput chip (4 columns) is shown for clarity, but the procedure is the same for the 7-segment high-throughput chip. (**b**) Medium is pulse perfused for the first hour after cell loading to promote attachment to the surface. The device is switched between two states: (1) flow of medium through the channels with access to the cell chambers closed, and (2) no flow, with exposure of chambers to the channels for medium diffusion

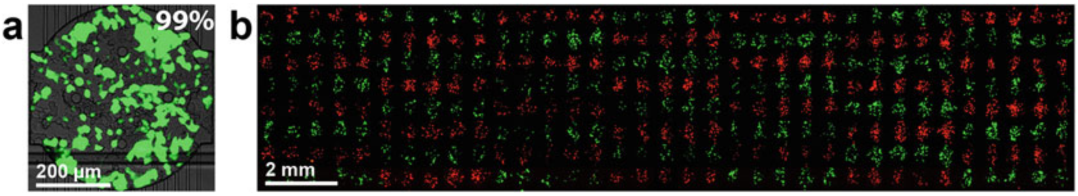

Fig. 5 Transfection on the high-throughput chip. (**a**) Composite fluorescence image showing high efficiency eGFP transfection on the device. (**b**) Fluorescence micrograph of an entire 280-chamber chip, with each 5-chamber segment transfected with a plasmid for expression of eGFP (green) or tdTomato (red)

5 min through the channels with access to the chambers closed. For the next 5 min, the flow of medium is stopped and the valve joining the chambers and medium channels are opened.

3. After 1 h of pulse perfusion, the top/bottom chamber valves are opened and medium is continuously flowed at a rate of ~1 ml/h (*see* **Note 26**).

3.7 Imaging

1. To measure transfection efficiency, images are acquired 48 h (*see* **Note 27**) after introducing cells onto the chip using 20× magnification to capture an entire chamber in the field of view (Fig. 5a). Images can also be acquired continuously after cell loading to monitor the progression of protein expression. Chambers are imaged in fluorescence and brightfield modes to count both the number of fluorescent cells and the number of total cells.
2. Images can be stitched together (for example using the Grid/Collection Stitching plugin in Fiji [22]) to reconstruct the entire array (Fig. 5b). Cells can be counted using programs such as CellProfiler or Matlab.
3. Transfection efficiency is calculated as the area occupied by fluorescent cells in the entire chamber (500 μm diameter) divided by the area occupied by cells within the 300 μm diameter spot where the lipid–DNA was deposited (*see* **Note 28**).

4 Notes

1. DNA samples should be supercoiled plasmids obtained using standard plasmid preparation kits, with a concentration of 200–500 ng/μl to avoid significant dilution of the transfection mixture.
2. The FBS should be of high quality and subjected to limited freeze–thaw cycles to ensure high transfection efficiency.
3. Proper gelatin preparation is important for transfection efficiency, and aliquots should not be stored longer than 1 month.

4. Designs intended for use with positive photoresist should be inverted before mask writing to ensure proper polarity, and a frame should be added around the features to provide sufficient empty space around the design.
5. If problems with AZ9260 adhesion to the wafer are experienced, an HMDS bake may be performed prior to dehydration and coating.
6. Multiple layers of foil may be necessary to prevent holes. The foil prevents direct contact and bonding between the PDMS poured on the wafer and the glass dish.
7. To fabricate functional microfluidic chips, it is essential to work quickly, protect the exposed flow layer, and keep wafers and chips free of dust. Chip fabrication can alternatively be performed in a clean room environment. Wafers should never be cleaned with water or organic solvents.
8. Wafer-containing glass petri dishes should not be stacked in the oven, since this results in suboptimal heat distribution.
9. Space allowing a strip of PDMS can be removed along each of the 4 edges of the chip to ensure a clean cut of both the flow and control layers. Small diagonal cuts made at each corner will also discourage delamination during chip operation.
10. Care should be exercised when handling microarraying pins. The tips of the pins are easily damaged, which results in printing or alignment problems.
11. Glass slides should be carefully aligned to the bottom right corner of the microarray holder positions, and vacuum should be applied to prevent the slides from moving. Proper alignment is critical since a second round of spotting (lipidDNA) will be performed on the same slides and the lipidDNA must be deposited precisely on top of the PLL spots.
12. PLL spots are no longer visible after the slides have been washed to remove excess PLL. To enable proper orientation of the slide in the holder for subsequent lipid–DNA spotting, the bottom right corner of the PLL slide is marked with a permanent marker prior to washing.
13. In the case of limited DNA samples, the lipid–DNA recipe can also be halved.
14. For the gelatin-DNA technique, DNA mixtures lacking transfection reagent are spotted and the Effectene is flowed on chip prior to cell loading. Samples are prepared similarly to the lipid–DNA mixtures and contain 1.5 μg DNA and buffer EC (total of 25.4 μl), 12.7 μl gelatin, and 12.7 μl fibronectin. A mixture of 16 μl Enhancer, 150 μl EC buffer, and 25 μl Effectene is flowed on chip for 30 min using the same conditions

used for medium perfusion. Medium is flowed on the chip for 20 min prior to cell loading.

15. Depositing lipid–DNA multiple times per spot does not increase transfection efficiency, and as has been previously reported [17] results in imprecisely localized spots due to spreading of excess DNA.
16. A maximum of two chips should be plasma treated at a time since alignment is time sensitive and must be performed immediately after plasma treatment.
17. Application of high pressure to the chip immediately after plasma treatment should be avoided since valves or chambers may depress, resulting in permanently closed valves or collapsed chambers.
18. When filling control lines and flow samples, the end of the tube fitted with the pin should be positioned downward and the free end (for connection to the manifold) should be elevated. In addition to careful aspiration with the syringe, this configuration discourages the sample from sliding further back into the tube, minimizing bubbles introduced on chip.
19. PTFE tubing is used to supply long-term culturing medium because cell toxicity may be observed when using Tygon tubing, as has been previously reported [23].
20. The medium bottle should be carefully handled since any frothing may introduce bubbles onto the chip.
21. As with traditional transfection, efficiency is increased when using low passage number cells. Transfection of different cell types (e.g., CHO) is possible, but the composition of the lipid–DNA mixture may need to be optimized accordingly.
22. Flow rate is determined by measuring the volume of liquid exiting the chip over a period of time.
23. In the case of clogging during cell loading, additional pressure may be applied to disperse the clog. To ensure homogeneity, it is recommended to purge a large amount of the sample (discard 10–50%) before running the cells through the chip for loading.
24. Example of chip loading (left to right) for a cell sample connected to F1. Cells will enter the first column through the middle channel and exit through the bottom right (all valves closed except C1, C4, C8, C10, and C17). For columns 2–6, cells enter through the top left and exit through the bottom right (all valves closed except C1, C4, C5, C8, C10, and C12–C16, depending on the column being loaded). For the last column, cells enter through the top left and exit through the middle channel (all valves closed except C1, C4, C5, C8, and C11).

25. Example of pulse perfusion from F2, medium flow stage: all valves closed except C2, C4–C6, C8–C10. Medium diffusion stage: all valves closed except C2, C5, C6, C7, C9, C10. To automate pulse perfusion, solenoid pneumatic valves arranged on a manifold (Pneumadyne) and controlled by a custom written LabVIEW VI program can be used.
26. Example of continuous medium flow from F2: all valves closed except C2, C4-C10). Long-term (longer than 48 h) culturing on the chip may result in cell outgrowth from the chamber and migration into the medium perfusion channels, causing clogging and preventing proper medium flow. To discourage this, the C7 valve can be periodically opened and closed to crush and disperse outgrown cells. Another concern is the presence of bubbles in the chip during culturing, which may be mitigated by closing the outlet (C8) valve while the chip is pressurized with medium. In the case of frequent problems with bubbles, an automated lab program such as LabVIEW may be used to periodically close C8.
27. For co-transfection experiments, imaging at time points beyond 48 h may be necessary (e.g., for long maturation times, or for co-transfections in which one protein must first be expressed before activating the expression of a second protein).
28. Normalization when calculating transfection efficiency is necessary because only some of the cells loaded into the chamber have access to the transfection mixture (a 300 μm spot within a 500 μm chamber). Some cells that initially settle in the lipid–DNA area migrate to different parts of the chamber after 48 h (when images are captured), resulting in the dispersed pattern visible in the images.

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Chapter 14

Genome-Wide High-Throughput RNAi Screening for Identification of Genes Involved in Protein Production

Sarah Inwood, Michael J. Betenbaugh, Madhu Lal, and Joseph Shiloach

Abstract

With an increasing number of blockbuster drugs being recombinant mammalian proteins, protein production platforms that focus on mammalian proteins have had a profound impact in many areas of basic and applied research. Many groups, both academic and industrial, have been focusing on developing cost-effective methods to improve the production of mammalian proteins that would support potential therapeutic applications. As it stands, while a wide range of platforms have been successfully developed for laboratory use, the majority of biologicals are still produced in mammalian cell lines due to the requirement for posttranslational modification and the biosynthetic complexity of target proteins. An unbiased high-throughput RNAi screening approach can be an efficient tool to identify target genes involved in recombinant protein production. Here we describe the process of optimizing the transfection conditions, performing the genome-wide siRNA screen, the activity and cell viability assays and the validation transfection to identify genes involved with protein expression.

Key words siRNA, Protein production, HEK 293, Screen

1 Introduction

Recombinant proteins are produced for purposes such as biotechnology research and medicine, the protein products include antibodies, growth factors, membrane products, and vaccines, among others [1–3]. Common mammalian hosts for recombinant protein expression include Chinese hamster ovary cells (CHO) and human embryonic kidney (HEK) 293 cells. The former are more commonly used industrially due to their ability to produce high quality protein with post-translational modifications that are similar to those of human proteins. CHO cells also grow in chemically defined media in suspension and are resistant to viral infection [4]. Sometimes HEK 293 cells are preferred when CHO cells are not able to produce the required proteins, for example, in the case of some growth factors where proper glycosylation and protein

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folding is required [5]. The impressive success associated with using CHO cell lines to produce recombinant protein has to do with their unparalleled adaptability that allows these cells to grow uniformly in suspension cultures and adapt to serum-free conditions. However, this adaptability has its drawbacks. Phenotypic drift between CHO production clones is not uncommon, making it a challenge to produce recombinant proteins in a reproducible manner. While CHO cell lines were the workhouse of recombinant protein production, especially antibodies, the HEK 293 cell line has come to the forefront of recombinant protein production because proteins produced in HEK cells are a much closer match to naturally occurring proteins in terms of function and posttranslational modifications. The use of human cell lines also allows for the use of tools such as RNAi, which can target the whole human genome [6, 7].

The 2006 Nobel Prize in Physiology or Medicine went to Andrew Fire and Craig Mello for their discovery of RNA interference (RNAi), which was first identified in *Caenorhabditis elegans* and is found in almost all eukaryotes [8, 9]. It regulates gene expression at the mRNA level by suppressing transcription or triggering RNA degradation [10]. Small interfering RNAs (siRNAs) are 21- and 22- nucleotide sequence specific mediators of RNA inference [11]. As noncoding RNA, both endogenous and synthetic exogenous siRNA have the potential to be manipulated for use in biomedical research, drug development, and treatment [12, 13].

RNAi screening has proved useful for identifying genes and gene networks that are involved in various biological processes, diseases, and responses of host cells to pathogens and drugs [14]. Multiple types of RNAi screening are available including siRNA, enzymatically generated siRNA (esiRNA), small hairpin RNA (shRNA), and microRNA (miRNA) screenings. These are arranged as pools or individual arrays looking for a positive or negative phenotype. Each screen type has its own advantages and disadvantages [7, 15].

Using a high-throughput genome-wide siRNA screen, our laboratory identified antizyme 1 (OAZ1) as a target for improving luciferase expression in HEK 293 cells without affecting transcription. From the human genome screen, 56 genes were identified for a validation screen with three additional genes. Then, ten genes were identified for follow-up using three additional reporter proteins. OAZ1 was found to consistently improve the expression of a cytosolic, a secreted and a membrane protein in HEK 293 cells with minimal effect on cell growth [16].

Here, we present a method for performing a genome-wide siRNA screen for identifying genes involved in recombinant protein production using the *Photinus pyralis* (firefly) luciferase as a reporter protein. An initial assay development is followed by the primary screen, in which 21,585 genes are individually knocked

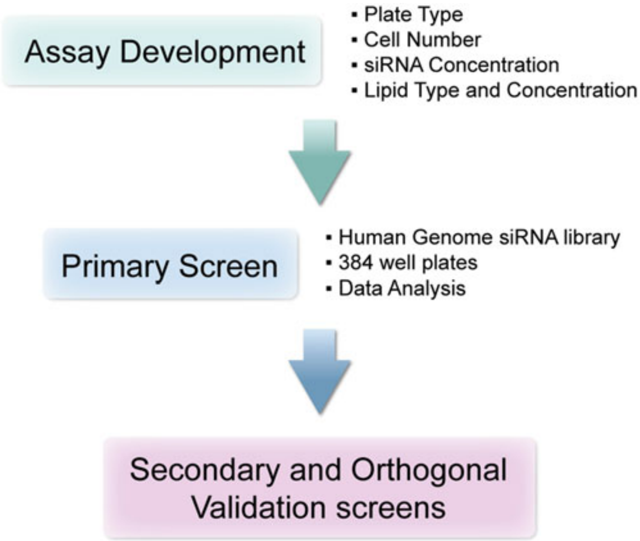


Fig. 1 Work flow for genome-wide RNAi screening

down with three unique siRNAs per target in a high-throughput format. This is followed by a validation screen to confirm the top genes as shown in Fig. 1. We have expanded the section on assay optimization and development to include many parameters that should be taken into consideration when designing a high-throughput RNAi screen to identify genes that may have been outside the scope of this luciferase based readout. We hope that the methods presented here serve as a platform for anyone designing a high-throughput genome-wide, functional genomics screen to identify genes involved in production of proteins.

2 Materials

2.1 Cells and Media

1. HEK-CMV-Luc2-Hygro cell line constitutively expressing *P. pyralis* luciferase pGL4.50 (luc2/CMV/Hygro vector) (Promega) [16] (*see* **Note 1**).
2. HEK-GPC3-hFc cell line constitutively secreting glypican-3 hFc-fusion protein [17], inducible T-Rex-SERT-GFP cell line [18], and T-Rex-NTSR1-GFP cell line [19] (nonscreening cell lines for orthogonal validation) [16] (*see* **Note 2**).
3. Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, pyruvate.
4. Fetal Bovine Serum (FBS).

2.2 Transfection Reagents and Instrumentation

1. Silencer select negative control #2 (Ambion® Silencer® Select, Thermo Fisher Scientific, Waltham, MA) and siPLK control (Ambion® Silencer® Select, Thermo Fisher Scientific).
2. Silencer select Human Genome siRNA library (Thermo Fisher Scientific).
3. 384-well white solid bottom tissue culture plates (Corning, Corning, NY).
4. Lipofectamine TM RNAiMAX (Thermo Fisher Scientific).
5. Agilent robotic system.
6. Silencer1 siRNAs (Thermo Fisher Scientific).
7. Humidified sterile incubator maintained at 37 °C, 5% CO_2.
8. Hoechst dye (Thermo Fisher Scientific).
9. Image Xpress, (Molecular Devices, San Jose, CA).
10. Pipettes.
11. Eppendorf tubes.

2.3 Luciferase Assay

1. OneGlo™ Reagent Luminescent Cell Luciferase Assay (Promega, Madison, WI).
2. Cell Titer Glo™ (CTG; Promega).
3. EnVision Multilabel plate reader (PerkinElmer, Waltham, MA).

2.4 Data Visualization

1. R computational environment (https://www.R-project.org/) [20].
2. "hexbin" [21] and "ggplot2" [22] packages.
3. Spotfire (Perkin Elmer) (*see* **Note 3**).

3 Methods

3.1 Assay Optimization and Development

Assay development includes experimental design, optimization, miniaturization, validation, and, if necessary, small-scale pilot screens to assess the assay performance and to identify lead candidates and genes/controls. The plate type, cell number, siRNA concentration, and lipid reagent and concentration optimization are all part of assay development as shown in Fig. 2.

3.1.1 Cell Number Optimization and Plate Selection

1. Seed HEK 293 cells in DMEM with 10% FBS in a 384-well plate at densities ranging from 250 cells/well to 5000 cells/well to assess growth for 72–96 h, using 3–4 columns of wells per cell density. The goal is to have cells that are no more than 80–85% confluent at the time of endpoint measurement.

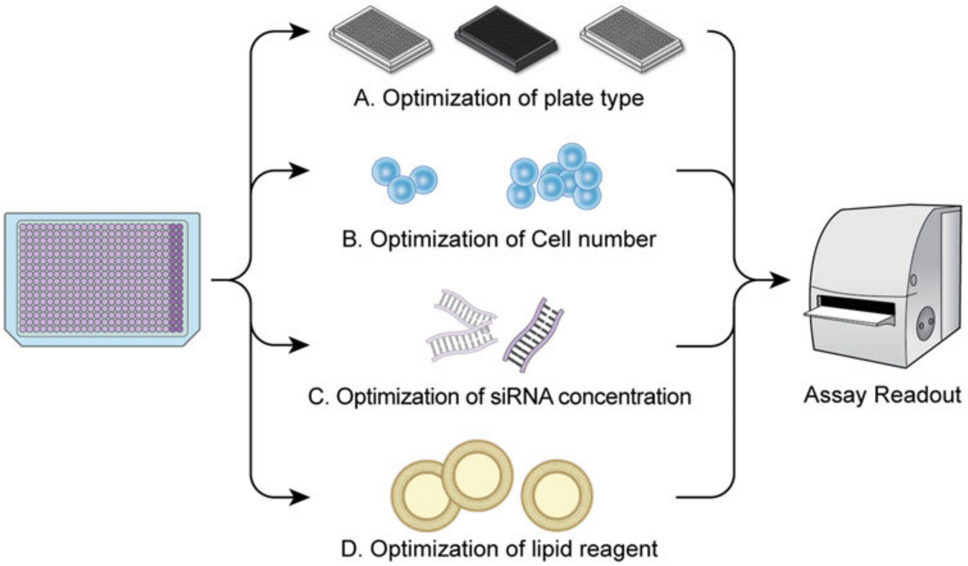
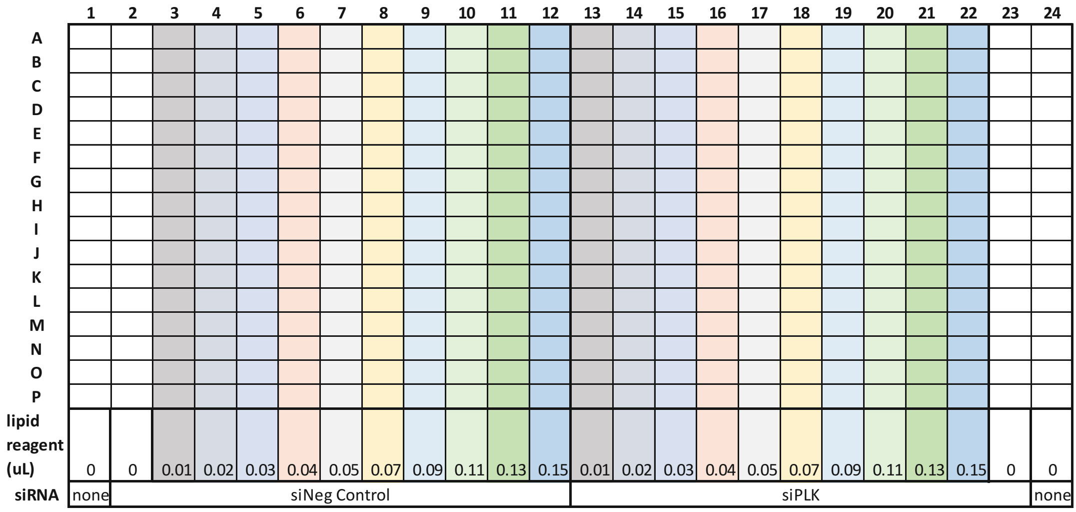


Fig. 2 Assay development strategies

2. Growth is usually assessed by either staining with Hoechst dye for nuclear number (ImageXpress) or by using a luminescence based readout (EnVision) for cell viability. This is done to negate the effects on viability and cell number that might come into play due to cell overcrowding, contact inhibition, and other factors.
3. While determining cell number, if necessary, assess different multiwell plates with different growth surfaces (for finicky cells) in order to optimize cell growth and assay read out (*see* **Note 4**).
4. Once the optimum number of cells has been determined, the next step is to access the transfection efficiency. There are two components for assessing the transfection efficiency: (1) choice and concentration of transfection reagent; and (2) concentration and suitability of transfection controls. The assay is experimentally designed for calculating the transfection efficiency between positive and negative siRNA control using a concentration of lipid reagent that causes the least amount of transfection-mediated cytotoxicity. Figure 3 shows a sample plate layout for the transfection efficiency assessment (*see* **Note 5**).

3.1.2 Transfection Efficiency Assessment

1. Transfer 2 μL of 400 nM stocks, (0.8 pmol) of 1) the non-targeting control (Silencer® Select Negative Control No. 2 siRNA, siNC) and 2) the positive control (siPLK1) into a 384-well plate with a multichannel pipette (*see* **Note 6**).
2. Dilute different amounts of RNAiMAX (0–0.15 μL per well) in screening media (20 μL per well of DMEM with no FBS or



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A																								
B																								
C																								
D																								
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J																								
K																								
L																								
M																								
N																								
O																								
P																								
lipid reagent (uL)	0	0	0.01	0.02	0.03	0.04	0.05	0.07	0.09	0.11	0.13	0.15	0.01	0.02	0.03	0.04	0.05	0.07	0.09	0.11	0.13	0.15	0	0
siRNA	none	siNeg Control											siPLK											none

Fig. 3 Sample plate layout for transfection efficiency assessment

penicillin/streptomycin), add to the wells with a multichannel pipette, and incubate for at least 30 min at room temperature (RT) (*see* **Note 7**).

3. While the plates are incubating, centrifuge HEK 293 cells at 200 × *g* for 8 min and resuspend in DMEM containing 20% FBS to the concentration required to achieve the previously determined cell number in 20 μL of cell suspension.
4. Seed 20 μL of the cell suspension in the wells already containing the siRNAs plus the lipofection reagent with a multichannel pipette. These experiments are performed in replicates.
5. Incubate the plates at 37 °C, 5% CO_2 and humidified air.
6. After 72 h of incubation, add 20 μL of OneGlo™ with a multichannel pipette to the wells of one replicate set to get an overall luciferase yield.
7. To the wells in the second replicate set, add 30 μL of CTG with a multichannel pipette to get a read for total cell viability.
8. Incubate the plates for 20 min at RT to stabilize the luminescent signal and then collect the luminescence readouts with the EnVision multilabel reader.
9. Assess transfection efficiency with the fold change in the viability and the luciferase yield, separately, between the positive and negative transfection controls.
10. The lipid reagent concentration, siRNA concentration and cell number that give the best transfection efficiency with the least associated cytotoxicity are then chosen for the primary screening (*see* Subheading 3.2) (*see* **Note 8**).

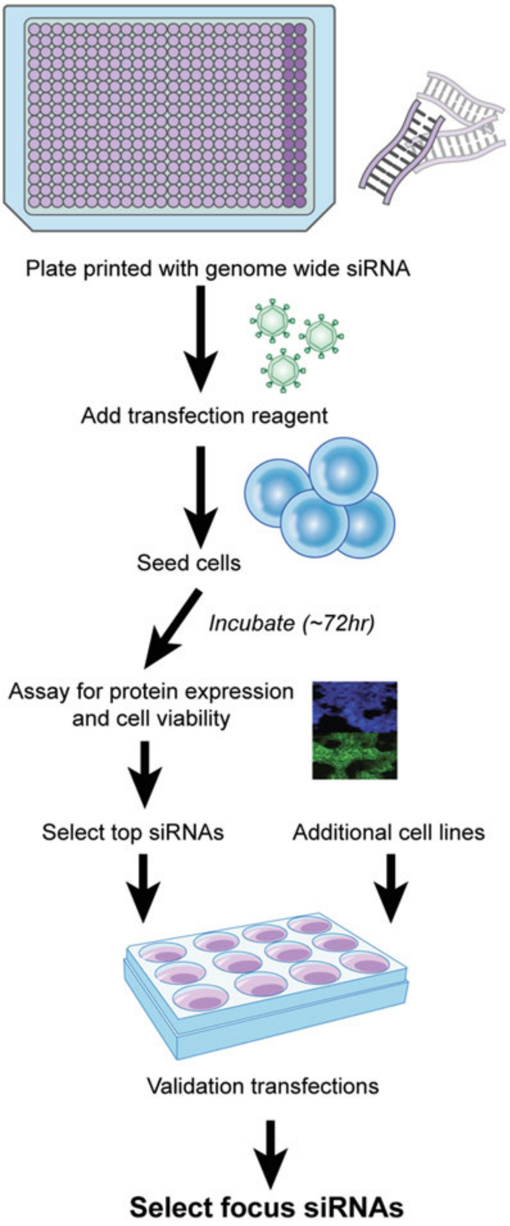

Fig. 4 Work flow for primary and secondary validation screen. Note, the additional cell lines step is to ensure the selected genes focus on recombinant protein production and are not specific to one protein

3.2 Primary Screen

The Silencer Select Human genome siRNA library, which targets 21,585 human genes with three siRNAs per gene, is used for primary screening. A workflow for the primary and secondary screens can be seen in Fig. 4 (*see* **Note 9**).

1. Each siRNA is arrayed in an individual well. All plates have a full column (16 wells) of Silencer Select Negative Control #2 for data normalization, and a full column of siPLK1 used as on-plate reference for transfection efficiency.
2. Each transfection is done in duplicate. Using the Agilent Robotic system, 0.8 pmol of each siRNA is spotted to a different well of a 384-well plate, and 20 μL of serum-free DMEM containing 0.07 μL of RNAiMAX is then added to each well (*see* **Note 10**).
3. This lipid-siRNA mixture is incubated at ambient temperature for 30 min prior to addition of 4000 cells in 20 μL of DMEM containing 20% FBS (*see* **Note 11**).
4. After incubating the transfected cells at 37 °C in 5% CO_2 for 72 h, 20 μL of OneGlo™ is added to one set of replicates for quantification of "overall luciferase yield" and 30 μL of CTG is added to the second set of replicates for measurement of "viable cell density" with the Agilent Robotic system.
5. All plates are incubated at room temperature for 20 min to stabilize the luminescent signal and then measured with the EnVision plate reader.
6. Both controls, Silencer Select Negative Control #2 and siPLK1, are used in all validation transfections. The genes that are targeted by at least two independent siRNAs (out of three) resulting in enhanced luciferase production with median absolute deviation (MAD)-based z-score > 3 from the primary screen are then subjected to validation screens using three additional Silencer1 siRNAs with different sequences from those used in the primary screen.
7. Gene candidates for further downstream orthogonal follow-up are selected based on the criteria that three out of the six siRNAs displayed a MAD-based z-score > 3. The transfection and assay processes are the same as in the primary genome-wide screen.
8. Data visualization is performed in R computational environment. The screen generates end-point data for "overall luciferase yield" and "viable cell density" in each well. For each plate, the median value of the negative control wells is set as 100% and is used to normalize corresponding sample wells. The "overall luciferase yield" and "viable cell density" are exported as the percentage of the negative control, and the MAD-based z-score was calculated for each sample [23].

3.3 Secondary and Orthogonal Validation Screens

1. Genes with at least two siRNAs that are in the range of >3 MAD for enhanced luciferase expression are selected for validation transfections.

2. Three additional Silencer1 siRNAs with different sequences from those used in the primary screen are used in the secondary screen with the same assay conditions as the primary screen.
3. The data are analyzed together with the primary screen and candidates with MAD-based z-score > 3 for at least 4 out of 6 siRNA sequences (combining the primary and secondary screen data) are selected.
4. The selected SiRNA are then funneled through orthogonal lower throughput assays or performed for different cell lines in the same assay to focus on genes that are important for the production of recombinant proteins.

4 Notes

1. Other cell lines can be used but should have an assay that is measurable on a high-throughput scale such as a GFP-based reporter protein.
2. Other cell lines can be used for orthogonal assays. These additional cell lines ensure the selected genes are focused to recombinant protein production and are not specific to one protein.
3. Use “hexabin” and “ggplot2” or Spotfire.
4. Typically, for imaging-based screens, black clear bottom TC-treated 384-well plates are used. White opaque bottom plates are used for assays that have a luminescence-based readout while black opaque bottom plates are used in cases where the readout is total fluorescence. During the assay optimization steps, white and black clear bottom plates are used to assess cell health and morphology while determining optimum assay readouts.
5. The sample plate layout is only for reference; many plate layouts are possible depending on individual experimental needs.
6. The initial stages of transfection efficiency are assessed with a final concentration of 20 nM siRNA per well. However, if this siRNA concentration is not sufficient to suitably transfect the cells, a concentration response of siRNA is performed to determine the siRNA concentration required for reliable knockdown of the target genes (controls). Knockdown efficiency is also assessed by measuring mRNA transcript levels post knockdown in addition to the phenotypic effect.
7. Typically, the first reagent tested for transfection efficiency is a Lipofectamine derivative, RNAiMAX. In the event that RNAiMaX does not work to suitably transfect the cell line of choice, other lipid reagents are used in the assay optimization. These

include, but are not limited to, Dharmafect (1–4), DNAIn, CRISPR Max, and Transit.

8. For assays that employ the use of GFP or other reporters, an siRNA to that reporter gene can also be used to assess transfection efficiency. Furthermore, known biological controls for the phenotype of interest are also tested to serve as good biological assay controls. Depending on the physiological complexity of the assay, anything from a tenfold difference (viability from an Adenosine TriPhosphate (ATP) luciferase-based read out) to a robust, reproducible twofold difference with Z factors above 0.5 in more complex image-based or HTRF assays is considered a screenable assay. The process of assay development usually involves running a small pilot screen, to assess data quality and robustness of the assay. This pilot screen is typically done in duplicate and if the correlation between pilot screens is good, the primary screen is then embarked upon.
9. The conditions listed in this section are based on the results of our assay development for the HEK-CMV-Luc2-Hygro cell line.
10. Prepare enough Lipofectamine RNAiMax/serum-free media for all wells plus some extra.
11. Prepare enough cells in DMEM with 20% FBS for all wells plus some extra. Cell concentration = (4000 cells/well)/(20 μL/well)*(1000 μL/mL) = 2×10^5 cell/mL.

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Chapter 15

Targeting miRNAs with CRISPR/Cas9 to Improve Recombinant Protein Production of CHO Cells

Kevin Kellner, Ankur Solanki, Thomas Amann, Nga Lao, and Niall Barron

Abstract

MicroRNAs with their unique ability to target hundreds of genes have been highlighted as powerful tools to improve bioprocess behavior of cells. The common approaches to stably deplete miRNAs are the use of sponge decoy transcripts or shRNA inhibitors, which requires the introduction and expression of extra genetic material. As an alternative, we implemented the CRISPR/Cas9 system in our laboratory to generate Chinese hamster ovary (CHO) cells which lack the expression of a specific miRNA for the purpose of functional studies. To implement the system, miR-27a/b was chosen as it has been shown to be upregulated during hypothermic conditions and therefore may be involved in controlling CHO cell growth and recombinant protein productivity. In this chapter, we present a protocol for targeting miRNAs in CHO cells using CRISPR/Cas9 and the analysis of the resulting phenotype, using miR-27 as an example. We showed that it is possible to target miRNAs in CHO cells and achieved ≥80% targeting efficiency. Indel analysis and TOPO-TA cloning combined with Sanger sequencing showed a range of different indels. Furthermore, it was possible to identify clones with no detectable expression of mature miR-27b. Depletion of miR-27b led to improved viability in late stages of batch and fed-batch cultures making it a potentially interesting target to improve bioprocess performance of CHO cells.

Key words CRISPR/Cas9, MicroRNA depletion, Chinese hamster ovary cells, Cell line engineering, Productivity

1 Introduction

Chinese Hamster Ovary (CHO) cells are the most prominent cell line used in biopharmaceutical production for therapeutic applications in treatment of various diseases such as hemophilia, cancer, and inflammatory disorders. However, compared to other expression systems like bacteria or yeast, CHO cells generally yield considerably lower amounts of protein product. Optimization approaches like the improvement of media formulations and bioprocess regimes have achieved great benefits, however, genetic engineering strategies to generate cell lines with improved bioprocess phenotypes are also being considered. Targeting of protein-coding genes for engineering of apoptosis [1], secretion [2],

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protein folding [3], metabolism [4], or glycosylation [5] have been shown to be very successful strategies to create tailored CHO producer cell lines.

MicroRNAs (miRNAs) have been demonstrated to be useful tools for cell line engineering to improve the bioprocess characteristics such as growth, productivity, longevity, expression of difficult-to-express proteins, and many more potentially critical processes [6, 7]. MiRNAs are small (~22 nts) noncoding RNAs that are involved in the regulation of hundreds of different mRNAs. Two mechanisms of miRNA target interaction are well understood, involving the block of translation due to a physical hindrance or the degradation of the target mRNA catalyzed by the RNA-induced silencing complex (RISC). Besides being able to regulate entire pathways, miRNAs are processed from a noncoding precursor and therefore do not impose a translational burden if used as an engineering tool. The noncoding primary transcript is processed into an immature pre-miR consisting of three regions: the mature sequence, the star sequence, and the stem loop [8]. The mature sequence is the more commonly selected strand of the duplex and is more likely to be incorporated into the RISC, whist the star sequence is degraded [9]. Depending on the location in the pre-miR, the miRNA is also referred to as -5p and -3p (e.g., miR-24-5p or miR-24-3p).

Differential expression of miR-27 was first identified as part of a temperature shift study by Gammel and colleagues [10] where it was significantly reduced during hypothermic conditions, suggesting a role in proliferation as well as increased productivity, and therefore represented an interesting target in cell line engineering. As part of a microRNA-cluster (miR-23-24-27 cluster), miR-27 consists of two genomic paralogs: miR-27a and miR-27b which only differ in one nucleotide. Several studies showed that miR-27a/b play a role in apoptosis by either targeting proapoptotic or antiapoptotic proteins possibly dependent on the specific circumstances of the cell environment. Agrawal and colleagues showed that miR-27 targets APAF1 which inhibits apoptosis during hypoxia [11]. Further studies also showed that miR-27 negatively regulates FADD which interacts with caspase-8 in the apoptotic signaling pathway and thereby prevents apoptosis in several human cell lines [12]. In addition, miR-27 is involved in cell survival by targeting Myt-1 which has been shown to inhibit G_2-M phase [13] presumably leading to a higher percentage of cells in S phase supporting our hypothesis that it is involved in proliferation.

A common method for the stable depletion of miRNAs is the use of sponge decoys. The generation of miRNA sponges is straightforward and has been shown to be very reliant and effective [14]. Several studies in CHO showed that stable inhibition of miRNAs can increase recombinant protein production and enhances longevity [15, 16]. Whilst miRNA sponges are a valuable tool for stable miRNA inhibition, the necessity to introduce a reporter gene (e.g., GFP or luciferase) is not desired for the generation of industrial cell lines, and a complete knockout of a miRNA could potentially be more beneficial than a knockdown. To address this, we proposed to utilize the recently developed CRISPR/Cas9 system. This system consists of two main components: the single guide RNA (sgRNA) and a CRISPR-associated endonuclease (Cas9) which induces double strand breaks (DSBs). These DSBs can result in the insertion or deletion (indels) of base pairs which can disrupt gene function. Several studies have already proved the successful application of CRISPR/Cas9 in CHO cells with knockouts or insertion of genes, aiming to influence product quality of monoclonal antibodies, for example [17, 18]. Besides targeting protein-coding genes, it has been also shown that miRNAs can be targeted using CRISPR/Cas9 [19, 20]. However, targeting noncoding RNA represents a special challenge as deletion of one or two base pairs may not impair the function of the RNA. It was proposed that indels in the stem loop of pre-miRNAs could inhibit the recognition by Dicer or Drosha and therefore lead to lower levels of mature miRNA, making this system a suitable tool for miRNA loss-of-function studies. In this protocol, we show that CRISPR/Cas9 can be successfully used to target miRNAs in CHO cells and that this system is a valuable alternative to sponge decoy overexpression, negating the requirement for introducing transgenes.

2 Materials

2.1 Expression Plasmid Construction

1. Guide RNA design tool such as GPP Web Portal (Broad Institute).
2. Genomic DNA sequence of miRNA target.
3. Ampicillin.
4. PX459 sgRNA expression plasmid (Addgene).
5. LB broth media and LB-Agar.
6. Petri dishes 90 mm.
7. Subcloning Efficiency *E. coli* DH5-α.
8. 250 mL Erlenmeyer flasks (glass).
9. DNA/RNA quantification device (e.g., Nanodrop1000; Thermo Fisher).
10. Sterile ultrahigh purity water.
11. 1.7 mL reaction tubes.
12. PCR thermo cycler.
13. BbsI FastDigest restriction enzyme (Thermo Fisher Scientific).

14. Agarose.
15. 50× Tris-Acetate-EDTA buffer (Sigma-Aldrich).
16. Electrophoresis gel chamber and power supply.
17. Mini/midi prep kits for plasmid DNA extraction.
18. Table top centrifuge.
19. Sterile pipette tips.
20. Sterile spatula.
21. T4 polynucleotide kinase.
22. Alkaline phosphatase.

2.2 Transfection of CHO Cell Lines

1. TransIT-X2 Dynamic Delivery System (LLC Bio).
2. CHO-mAb cell line, IgG producing [21].
3. Puromycin.
4. Flat bottom 6-well plates.
5. DMEM/F12 medium.
6. Foetal Bovine Serum.
7. BalanCD® CHO Growth A (Irvine Scientific).
8. T75 flasks vented lid or 50 mL bioreactor tubes (TPP).
9. Incubator ISF1-X Climo shaker (Kuhner Shaker).
10. Static incubator 37 °C, humidified.

2.3 Indel Analysis

1. Agarose gel extraction kit.
2. Surveyor® Mutation Detection Kit (IDT).
3. PCR thermo cycler.
4. Agarose.
5. Platinum® High fidelity Polymerase kit (Invitrogen).
6. Genomic DNA isolation kit (Qiagen).
7. Phosphate buffered saline (PBS).
8. TOPO®TA cloning kit (Invitrogen).
9. Plasmid DNA Mini prep kit.
10. Oligonucleotide primers.
11. SafeView™ Nucleic Acid Visualization Kit (abm Inc.).

2.4 Analysis of Mature miRNA Expression

1. TaqMan™ assays (Applied Biosystems).
2. Real-time PCR cycler (Applied Biosystems 7500).
3. TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems).
4. MicroAmpTM Optical 96-well reaction plates (Applied Biosystems).

5. TRIzol reagent (Ambion).
6. Nuclease-free H_2O.
7. Isopropanol.
8. Ethanol.

2.5 Analysis of Phenotype in Batch and Fed-Batch Cultures

1. 50 mL TubeSpin bioreactor tubes.
2. 250 mL Erlenmeyer flasks.
3. ISF1-X Climo orbital shaker.
4. BalanCD® CHO Growth A.
5. BalanCD® CHO Feed 1 (Irvine Scientific).
6. **Optional:** anti clumping agent, e.g., Polyvinyl alcohol (PVA).
7. Guava easyCyte benchtop flow cytometer (Merck Millipore).
8. ViaCount reagent (Merck Millipore).
9. Human IgG ELISA Quantitation Kit (Bethyl Laboratories).

3 Methods

The following describes a general procedure for targeting miRNAs in CHO cells as established in our laboratory. Figure 1 describes the general workflow and approach of targeting a miRNA in CHO as well as screening for clones with complete knockouts. As there is no tool available for the design of gRNAs targeting noncoding RNAs in CHO we used the published and publicly available genomic information (https://www.ncbi.nlm.nih.gov/) and standard design tools as provided by the Broad Institute for spCas9 (https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).

3.1 Guide RNA Design Considerations for Noncoding RNAs

1. Find the genomic sequence of the miRNA target by using publicly available sources (e.g., (https://www.ncbi.nlm.nih.gov/).
2. Identify important regions of the miRNA hairpin (i.e., seed sequence, mature miRNA, and passenger strand).
3. Copy your sequence into a gRNA design tool (e.g., provided by https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design).
4. Select gRNAs ideally proximal to the seed region of the mature miRNA. Targeting the seed region however, can be limited by the presence of a Protospacer Adjacent Motif (PAM) used by Cas9 for recognition. SpCas9 uses the trinucleotide repeat NGG which statistically occurs very frequently in the human genome. If no PAM site is located close to the seed region another Cas nuclease (e.g., *Staphylococcus aureus* [NNGRRT with R = purine] or Cpf1 [YTN with Y = pyrimidine] can be selected).

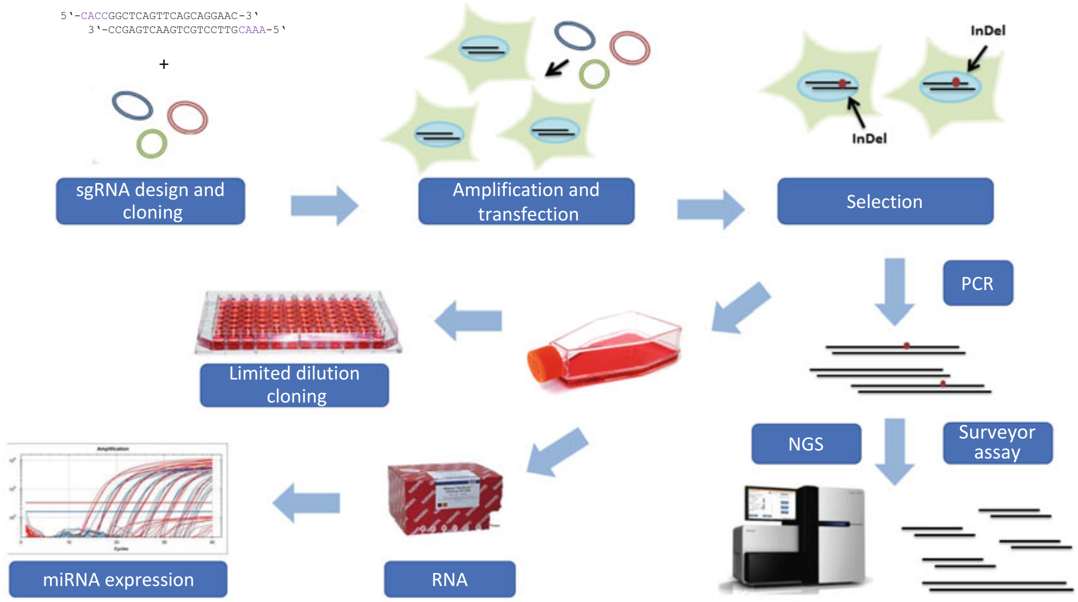


Fig. 1 General overview of steps involved in the generation of cells with knockdown of miRNAs. The first steps involve the design and cloning of sgRNAs before the transfection of cells. Depending on the approach, transfections can be performed transiently or mixed stable pools can be generated using a selection marker, e.g., puromycin resistance gene. High-fidelity PCR followed by TOPO-TA cloning as well as the Surveyor® assay can be used to assess targeting, or alternatively, indels can be analyzed using next-generation sequencing. Furthermore, miRNA expression after targeting can be analyzed using qPCR assays for stable mixed populations and for screening single cell clones

5. Design your gRNA oligos with additional overhangs for BbsI restriction enzyme recognition.
6. Nonphosphorylated oligos can be synthesized by various providers
7. Once the gRNA is cloned into the expression plasmid it will be expressed as a single guide RNA (sgRNA) together with the scaffold which is located in the PX459 plasmid.

3.2 Generation and Preparation of sgRNA Expression Plasmid

1. Purchase plasmid PX459 which is delivered as an agar stab. It encodes a human codon optimized spCas9 gene as well as U6 promoter-driven scaffold for expression of sgRNAs.
2. Prepare glycerol stocks according to the supplier's protocol.
3. Inoculate 50 mL LB-broth cultures containing 100 μg/mL ampicillin using 100 μL sterile pipette tips.
4. Incubate cultures overnight on a shaker at 37 °C at 250 rpm in sterile 250 mL Erlenmeyer flasks.
5. Isolate plasmid DNA using a midi prep plasmid isolation kit according to the manufacturer's protocol.
6. Measure the plasmid concentration.

3.2.1 Annealing, Ligation, and Amplification of sgRNA Expression Vector

1. Before the gRNA can be ligated into the vector, it is necessary to digest and purify the linear plasmid first.
2. Digest 1 μg of plasmid DNA using 1 U of BbsI in a total volume of 20 μL for 1 h at 37 °C.
3. Treat digested plasmid additionally with alkaline phosphatase (AP) at 37 °C for 1 h.
4. To remove enzymes and buffer which could inhibit the ligation reaction purify plasmid using a PCR purification kit with nuclease-free H_2O for elution.
5. **Optional:** Oligos are usually provided dephosphorylated and must be phosphorylated for a successful ligation using PNK. Annealing and PNK treatment can be performed in one step as follows:

Component	Amount (μL)
Sense oligo (100 μM)	1
Antisense oligo (100 μM)	1
PNK buffer (10×)	1
PNK	1
H_2O	6
Total	10

6. Incubate at 37 °C for 1 h and inactivate PNK at 95 °C for 20 min.
7. Anneal phosphorylated oligos into a duplex using a stepwise reduction of the temperature (5 °C per min) until the reaction mix reaches 20 °C.
8. Dilute duplex 1:200 for ligation and store at −20 °C.
9. Set up ligation mix as follows:

Component	Amount (μL)
BbsI digested and AP treated PX459	100 ng (× μL)
Diluted annealed duplex gRNA	2
10× ligation buffer	2
T4 DNA ligase (1 U/μL)	1
H_2O	Top up to 20
Total	20

10. Ligate overnight at 16 °C and store at −20 °C until ready for transformation.

3.2.2 Transformation

1. Transform ligated plasmid using competent *E. coli* DH5α.
2. Add 2 μL of the ligation mix into 20 μL chemically competent *E. coli* and incubate for 30 min on ice.
3. Heat-shock at 42 °C for 40 s.
4. Incubate on ice for 2 min.
5. Add 700 μL of prewarmed SOC medium.
6. Incubate at 37 °C for 1 h at 250 rpm in a shaker.
7. Plate on LB-Agar plates containing 100 μg/mL ampicillin and incubate overnight at 37 °C.
8. Pick colonies the next day and cultivate overnight at 37 °C at 250 rpm in 5 mL LB-Broth containing 100 μg/mL ampicillin.
9. Isolate plasmid DNA using a miniprep kit according to the manufacturer's protocol.
10. Analyze positive ligation of gRNA using Sanger sequencing with specific primers targeting the regions upstream and downstream the scaffold sequence.
11. Expand positive clones into 50 mL LB-Broth cultures containing 100 μg/mL ampicillin.
12. Isolate plasmid DNA using a midi prep kit according to the manufacturer's protocol and measure DNA concentration.

3.3 Transfection and Generation of Stable Cell Lines

For the transfection, a CHO-mAb cell line expressing an IgG was used. Transfection was performed in healthy cell cultures with viability above 95%. Transfections can be performed in suspension as well as using adherent conditions when medium is supplemented with FBS (5–10%). For our transfections, we used serum-free conditions. Cell culture conditions for all steps were at 37 °C, 170 rpm and 5% CO_2 with a humidity of 80%.

1. Exchange medium 24 h prior to transfection to ensure cell growth and removal of dead cells.
2. Exchange medium on day of transfection using 5 mL prewarmed DMEM/F12 (*see* **Note 1**).
3. **Optional:** Trypsinize cells if attached conditions are used.
4. Count cells and transfer 1×10^6 cells into a final volume of 1 mL into 50 mL TubeSpin bioreactor tubes. **Optional:** For attached cultures transfer 1×10^6 cells into a well of a 6-well plate.
5. Perform DNA-PEI complex formation according to the manufacturer's protocol. For plasmid DNA a ratio of 1:1 (w/w) plasmid DNA:TransIT-X2 is common. Allow 30 min for complex formation.
6. Add complex to cell suspension and incubate for 3 h.

7. Spin down and exchange medium to BalanCD® CHO Growth A or another culture medium (*see* **Note 2**).
8. Exchange medium after 24 h and apply selection pressure using 10 μg/mL puromycin.
9. Maintain selection pressure for 2–3 weeks with routine passaging of cells using a seeding density of 2×10^5 cells/mL (*see* **Notes 3** and **4**).
10. Sample for RNA and DNA extraction after selection for further analysis of indels and for mature miRNA expression.

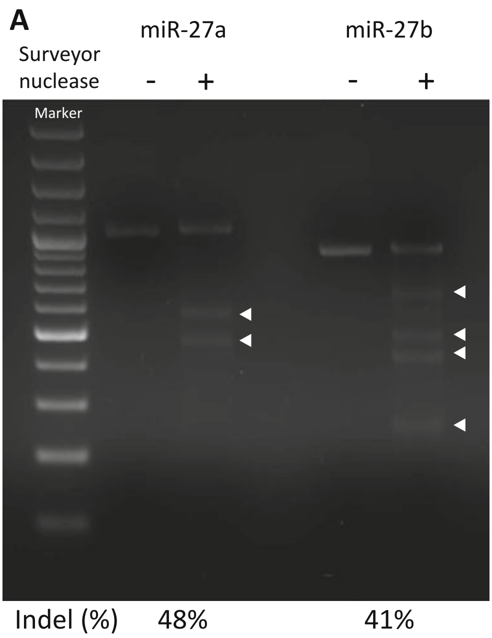
3.4 Analysis of Indels Generated by CRISPR/Cas9 Using TOPO® TA Cloning and Surveyor® Assay

For the analysis of Indels generated, cellular DNA can be isolated and then amplified by PCR with forward and reverse primers specific to the targeted region (± 400 bp) of the miRNA coding sequence. PCR products can be ligated into TOPO®TA cloning vectors and analyzed by Sanger sequencing. For the use of TOPO®TA in combination with Sanger sequencing as well as Surveyor® assays it is recommended that only PCR products which show a single specific band on an agarose gel are used. Therefore, the optimization of annealing temperatures for PCR reactions for each primer is essential to avoid ligation of nonspecific amplicons into TOPO®TA plasmids or the cleavage of unspecific amplicons by the Surveyor® assay. Furthermore, high fidelity enzymes for PCR amplification are required to exclude amplification errors.

1. Isolate genomic DNA using 1×10^6 cells following the manufacturer's protocol.
2. Measure DNA concentration.
3. Perform high-fidelity PCR according to the manufacturer's protocol using optimized annealing temperatures, 100 ng of genomic DNA and 10 nM final concentration of forward and reverse primers.
4. Purify the PCR product using a PCR purification kit according to the manufacturer's protocol.
5. Run an analytical agarose gel to ensure specificity of PCR. If nonspecific bands are detectable perform gel extraction of the correct amplicon (*see* **Note 5**).

3.4.1 TOPO® TA Cloning

1. PCR products can be directly ligated according to the manufacturer's protocol into TOPO®TA plasmids without purifying the PCR product (*see* **Note 6**).
2. Spread 40 μL of a 20 mg/mL X-Gal solution in DMF on a kanamycin (50 μg/mL) containing LB-agar plate and incubate for 30 min as 37 °C.
3. Transformation according to Subheading 3.2.2.



B

miR-27a	Sequence	Indel
WT	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	--
1	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
2	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
3	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
4	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
5	CCGCAGCAAGTCGTGTTC---CAGTGGCTAAGTT	-1
6	CCGCAGCAAGTCGTGTTCCCCAGTGGCTAAGTT	+2
7	CCGCAGCAAGTCGTGTTCA----GT-AGCTAAGTT	-2
8	CCGCAGCAAGTCGTGTTCACAGTGGCTAAGTT	0
9	--CAGTGGCTAAGTT	-62
10	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
11	CCGCAGCAAGTCGTGTTCAACAGTGGCTAAGTT	+1
12	CCGCAGCAAGTCGTGTTCACCAGTGGCTAAGTT	+1

miR-27b	Sequence	Indel
WT	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	--
1	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
2	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
3	TTGGTTTCCGCTTT---------CAGTGGCTAAGTT	-5
4	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
5	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
6	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
7	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
8	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
9	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0
10	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
11	TTGGTTTCCGCTTTGTTCAACAGTGGCTAAGTT	+1
12	TTGGTTTCCGCTTTGTTCACAGTGGCTAAGTT	0

miRNA seed

C

sgRNA	% (wt)	% (indel)
miR-27a	16.29%	83.71%
miR-27b	11.16%	88.84%

Fig. 2 Indel analysis using TOPO-TA cloning combined with Sanger sequencing, Surveyor® assay and next-generation sequencing. (**a**) Analysis of Surveyor® Mutation Detection Assay shows digestion of the amplified genomic region due to mismatch formation for both targeted miRNAs (arrows). (**b**) PCR products cloned into TOPO®TA and analyzed. Individual clones show a variety of indels proximal to the targeted region. C) Next-generation sequencing shows high efficacy of targeting of both miRNAs up to 88%

4. Recover positive colonies the next day and expand them in 5 mL LB-broth cultures in the presence of 50 μg/mL kanamycin.
5. Incubate overnight at 37 °C.
6. Perform plasmid isolation using miniprep kits.
7. Sequence inserts using Sanger sequencing for comparison to wild-type genomic sequence (*see* Fig. 2).

3.4.2 Surveyor® Assay for Detection of Indels

1. Purify PCR product and anneal wild-type DNA with mutated DNA.
2. Denature double stranded DNA at 95 °C for 10 min.
3. A stepwise reduction of the temperature is recommended and described in the manufacturer’s protocol.
4. Treat wild-type and reannealed PCR amplicon with Surveyor® nuclease.
5. Analysis of treated PCR products on 2% agarose gel (*see* Fig. 2).

3.4.3 Next-Generation Sequencing (NGS) for Analysis of Indels

For higher resolution information of the frequency of indels as well as the efficacy of targeting we recommend the use of NGS (*see* Fig. 2). It will give more information whether both alleles are targeted efficiently. First, the targeted region is amplified using high-fidelity PCR (*see* Subheading 3.4). Primers are designed to amplify approximately 200–300 base pairs around the targeted region. For library generation linkers are added to the primers. Linker sequence will depend on the NGS provider used.

3.5 Analysis of Mature miR-27 Expression After Targeting Using CRISPR/Cas9

For the analysis of mature miRNA expression, RNA was isolated and reverse transcribed using specific stem loop primers for miR-27b. Expression was analyzed using TaqMan™ assays for miR-27b and miR-27b* on a qPCR cycler and normalized to U6 snRNA expression (*see* Fig. 3).

1. Count cells using Trypan Blue stain and a hemocytometer.
2. Transfer 1 × 10^6 cells into a centrifuge tube.
3. Centrifuge at 800 × *g* for 5 min and discard supernatant.
4. Wash pellet three times with PBS.
5. Resuspend cell pellet in 1 mL TriZOL and isolate RNA following the manufacturer's protocol. Store at −80 °C
6. Measure RNA concentration and determine RNA quality (*see* **Note 7**)

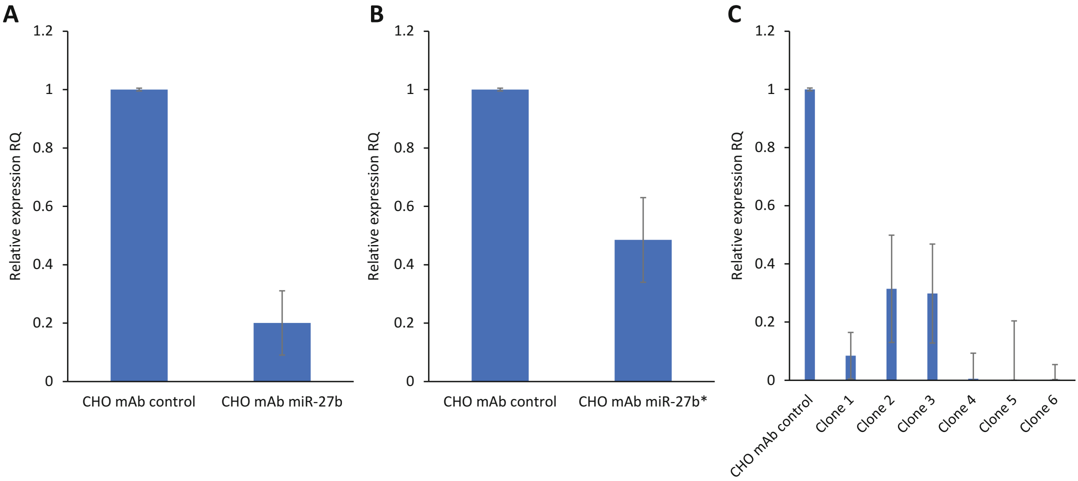


Fig. 3 Expression of miR-27b and miR-27b*. (**a**) and (**b**) Targeting miR-27b using CRISPR/Cas9 led to reduced levels of mature miRNAs in mixed cell populations with a knockdown of up to 80% of the control. Furthermore, mature levels of the passenger strand (miR-27b*) were reduced as well. (**c**) Analysis of mature miR-27b expression after limited dilution cloning and isolation of single cell clones showed cell clones with a significantly reduced level or no miR-27b expression compared to the control

7. Use a total of 100 ng RNA for reverse transcription according to the manufacturer's protocol using specific stem loop primers for U6 snRNA, miR-27b and miR-27b*.
8. **Optional:** To avoid the risk of contaminating genomic DNA, the RNA can be treated with DNase I. However, the final concentration must be adjusted for use in RT-qPCR.
9. Analyze mature miRNA expression on a qPCR cycler using the corresponding assays and normalize to U6 snRNA expression. Use the ddCt method for analysis of differential expression.
10. The same principle can be applied for a larger clone screen to find clones which show an appropriate knockdown of miR-27b or any other miRNA.

3.6 Analysis of Phenotype in Batch and Fed-Batch Cultures After Knockdown of miR-27b Using CRISPR/Cas9

For the analysis of the phenotype, the mixed miR-27b depleted population was compared to a control population expressing Cas9 as well as a nontargeting scaffold, in batch cultures of 5 mL as well as fed-batch cultures of 30 mL (*see* Fig. 4).

1. Count cells using Guava and ViaCount reagent (*see* **Note 8**).
2. For batch cultures seed cells with a density of 2×10^5 cells/mL in 50 mL TubeSpin bioreactor tubes with a total volume of 5 mL. For fed-batch cultures use 250 mL Erlenmeyer flasks with a total volume of 25 mL at day 0. Cultures are seeded in triplicate and assayed for viability every day or every second day using ViaCount reagent on a Guava benchtop flow cytometer.
3. Take conditioned medium samples every second day for IgG quantification using ELISA.
4. Addition of feed corresponds to the medium used and can be optimized according to the medium provider. For cultures using BalanCD CHO Growth A, we added 10% BalanCD CHO Feed 1 on days 1, 5, and 7 (*see* **Note 9**).
5. Centrifuge conditioned medium samples for 5 min at 800 × *g* and store at −80 °C until needed.
6. Use ELISA IgG quantification kit to assess titer according to the manufacturer's instructions. Calculate the specific productivity (*see* **Note 10** and ref. 22).

4 Notes

1. BalanCD® CHO Growth A can limit transfection efficacy and it is recommended to exchange the medium before transfection.
2. If the phenotype will be assessed, transfer cells into the appropriate medium and adapt cells for 3–4 passages prior to assessment.

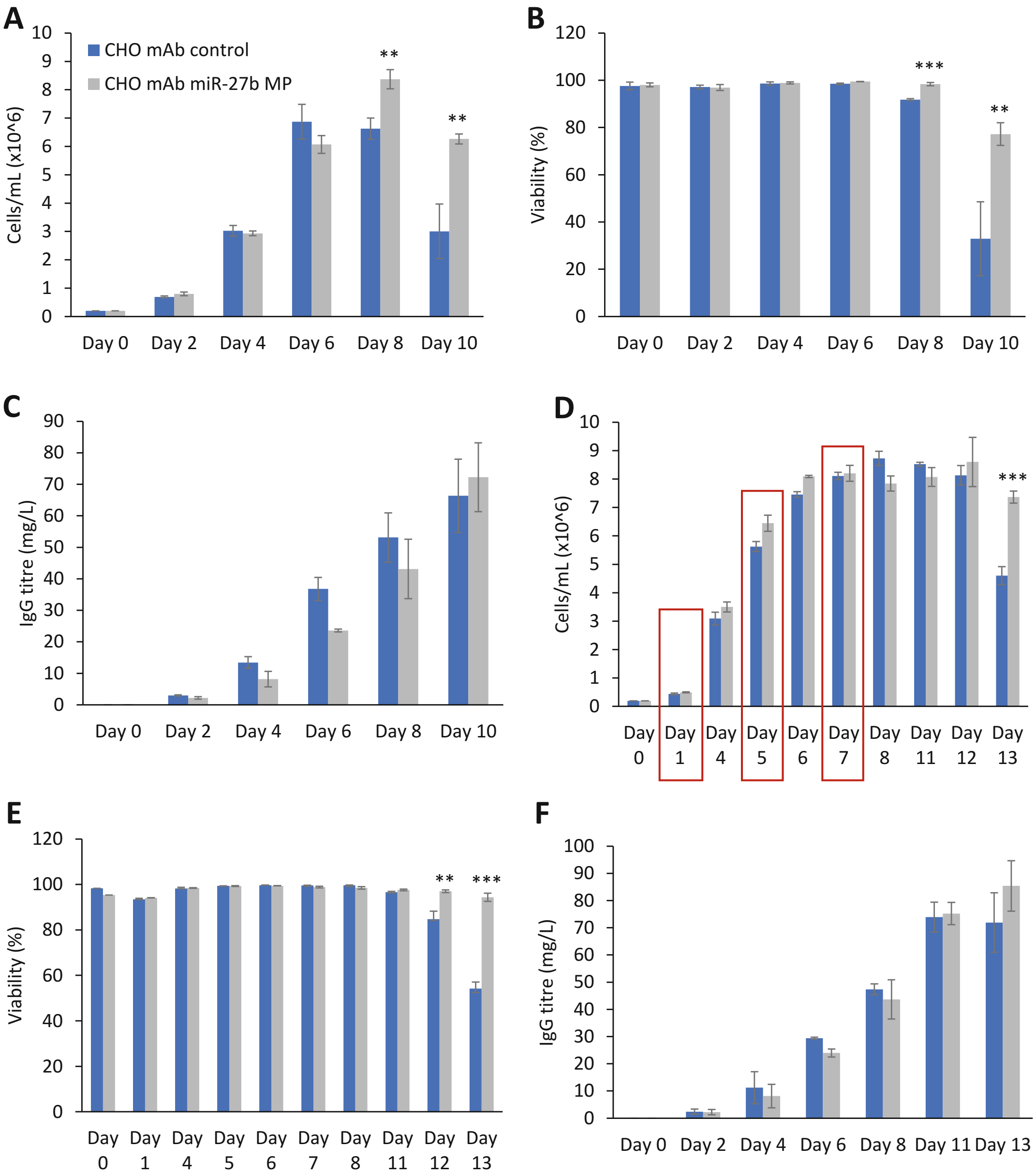


Fig. 4 Phenotype after depletion of miR-27b in batch and fed-batch cultures. (**a**) and (**b**) Batch cultures showed increased viable cell densities and increased viability on day 8 and 10 of the cultures. (**c**) IgG titer was increased but not significantly due to increased longevity. (**d**) and (**e**) Fed-batch cultures showed increased viable cell density on day 13 and increased viability on day 12 and day 13 compared to the control. Feed was added on day 1, day 5 and day 7 (red boxes). (**f**) IgG titer was not significantly affected in fed-batch. For analysis of statistical significance, an unpaired two tailed student's *t*-test was applied (*$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$) on biological triplicates between two independent sets of samples

3. After selection limited dilution cloning or FACS for generation of single cell clones can be performed, if desired. Cells overexpressing Cas9 fused to GFP can be effectively sorted using FACS.
4. The usual concentration of puromycin varies between 1 and 10 μg/mL for mammalian cell lines. For each cell line, determination of a kill curve is recommended.
5. The same purified PCR amplicon can be used for the TOPO®TA and Surveyor® assays.
6. Many high-fidelity PCR kits will give a mix of blunt- and sticky-end amplicons which can be used for TOPO®TA cloning. If high-fidelity polymerases are used (e.g., Pfu-polymerase which will result in a blunt end PCR amplicon), a TOPO® kit with corresponding vector can be purchased or the addition of an A-overhang using Taq-polymerase is recommended.
7. Only high quality RNA is suitable for use in downstream qPCR applications. The quality can be determined using the OD 260/280 as well as 260/230 ratio. Both values should be >1.8 to ensure good quality.
8. Use only cultures with viability >95%.
9. Growth curves can take up to 10–12 days depending on medium used for batch cultures and are usually stopped once viability falls below 80%.
10. Cell specific productivity (pg protein/cell/day) (Qp) can be calculated using data obtained by ELISA for product titer, viable cell density, and growth rate.

Acknowledgments

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Chapter 16

Application of the CRISPR/Cas9 Gene Editing Method for Modulating Antibody Fucosylation in CHO Cells

Qiong Wang, Cheng-Yu Chung, Julian N. Rosenberg, Geng Yu, and Michael J. Betenbaugh

Abstract

Genetic engineering plays an essential role in the development of cell lines for biopharmaceutical manufacturing. Advanced gene editing tools can improve both the productivity of recombinant cell lines as well as the quality of therapeutic antibodies. Antibody glycosylation is a critical quality attribute for therapeutic biologics because the glycan patterns on the antibody fragment crystallizable (Fc) region can alter its clinical efficacy and safety as a therapeutic drug. As an example, recombinant antibodies derived from Chinese hamster ovary (CHO) cells are generally highly fucosylated; the absence of fucose significantly enhances antibody dependent cell-mediated cytotoxicity (ADCC) against cancer cells. This chapter describes a protocol applying clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) to disrupt the α-1,6-fucosyltranferase (FUT8) gene and subsequently inhibit α-1,6-fucosylation on antibodies expressed in CHO cells.

Key words Chinese hamster ovary cells, CRISPR/Cas9, Gene editing, Fucosylation, Knockout, ADCC

1 Introduction

Targeted gene knockout has been extensively studied and widely used in mammalian cell line development. Traditionally, targeted gene editing is achieved by homologous recombination [1], but due to inherently low recombination rates, homologous recombination can be less efficient and more time-consuming [2]. The emergence of advanced genetic engineering technologies, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats (CRISPR) with CRISPR-associated protein 9 (Cas9), has dramatically accelerated the gene editing process and significantly improved gene knockout efficiency [3]. These advanced gene editing tools are based on the use of engineered nucleases comprised of programmable and sequence-specific

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DNA-binding domains fused to a nonspecific DNA cleavage module [1]. Specifically, ZFNs and TALENs employ a protein–DNA binding domain fused to a FokI endonuclease and require a pair of ZFNs or TALENs to generate a DNA double-strand break (DSB). More recently, the development of the CRISPR/Cas9 system, utilizing a guided RNA complementary to the target DNA sequence fused to a Cas9 nuclease, exhibits higher target binding specificity than ZFNs or TALENs and only requires RNA-binding to a single strand of DNA in order to generate a nick in the sequence [1]. The evolutionary origin of CRISPR stems from an adaptive immunological response in some bacteria and archaea to protect the host against foreign DNA [1]. Among the three identified CRISPR systems, the Type II CRISPR/Cas9 system of *Streptococcus pyogenes* (SpCas9) has been studied most extensively (Fig. 1a). In this system, short segments of foreign DNA (approx. 20 nucleotides) known as "protospacers" are integrated within the bacterial CRISPR genomic loci; individual protospacers are separated by short palindromic repeats [1]. To generate short CRISPR-targeting RNA (crRNA), protospacers are transcribed as pre-crRNA, which anneals to trans-acting crRNA (tracrRNA) via a conserved dinucleotide protospacer adjacent motif (PAM) upstream of the target region [1]. The CRISPR/Cas9 system can thereby direct sequence-specific cleavage to excise foreign DNA by Cas9 proteins. By redesigning the crRNA sequence, CRISPR can be engineered to target any DNA sequence of interest in the host genome. For the CRISPR/Cas9 gene editing system, there are two prerequisites: the targeted sequence must be unique in the host genome and the target sequence must be present immediately upstream of a protospacer adjacent motif (PAM).

Monoclonal antibody (mAb) therapeutics comprise a multibillion dollar market [4]. The wide-ranging applications of mAb biologics not only treat major diseases such as cancer, autoimmune diseases, inflammation and Alzheimer's disease [4], but also provide diverse types of therapies, including radioimmunotherapy [5], antibody-directed enzyme prodrug therapy [6], antibody–drug conjugates [7], immunoliposome therapy [8], and checkpoint inhibitor therapy [9]. Except for neutralization with pathogens, antibodies (especially IgGs) oftentimes execute their functions by phagocytosis of antibody-bound pathogens. In particular, antibody-dependent cell-mediated cytotoxicity (ADCC) is triggered when pathogen-bound antibodies interact with Fc receptors of lymphocytes (mainly natural killer cells) [10]. The conserved N-glycan on the IgG Fc domain greatly affects this binding affinity. The core-fucose residue of the heavy chain constant region (CH)-associated N-glycans plays a critical role in regulating antibody effector function in ADCC [2, 11–17]. Compared to the fucosylated antibodies from wild-type CHO cells, defucosylated antibodies exhibited 100-fold higher ADCC in vitro [18, 19] as a result of

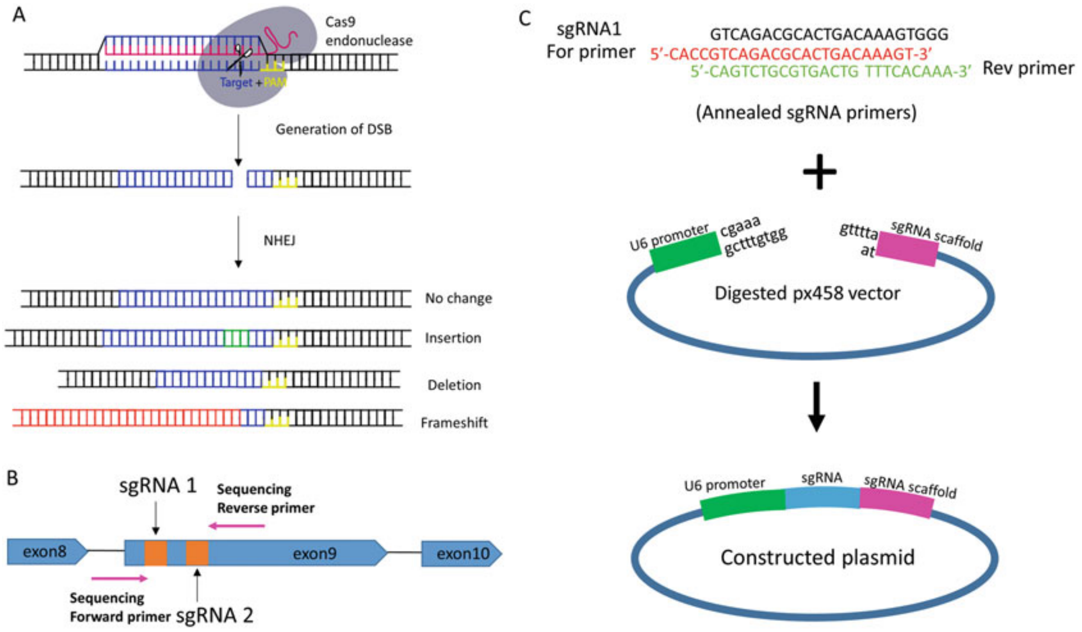

Fig. 1 (**a**) Schematic of CRISPR/Cas9-mediated gene mutation. (**b**) The illustration of sgRNA binding sites in the FUT8 gene. (**c**) The workflow of px458 plasmid construction

a subtle conformational change [12, 17, 20]. Inactivation of the α-1,6-fucosyltransferase, encoded by the FUT8 gene in mammals, represents the most efficient single modification to inhibit the addition of fucose to the core of the complex N-glycan [21, 22].

In this chapter, we describe a robust method for generating a single-gene knockout CHO cell line with an engineered glycosylation pathway using the CRISPR/Cas9 system (Fig. 2). The biological significance of the FUT8 gene makes it an ideal target for our proof-of-concept study. Importantly, we employed the lectin selection methodology to isolate specific glycan types in antibody-expressing clones, which is a unique step in the knockout cell line development process. The choice of stable antibody-expressing CHO cells as our parental cells makes our protocol practical and useful for industrial purposes.

2 Materials

2.1 Reagents and Kits

1. Plasmid Miniprep kit.
2. 1.5 mL Eppendorf microcentrifuge tubes.
3. 12 × 75 mm tubes with cell strainer cap (FACS tubes).
4. Nuclease-free water.
5. BbsI restriction enzyme (New England Biolabs).

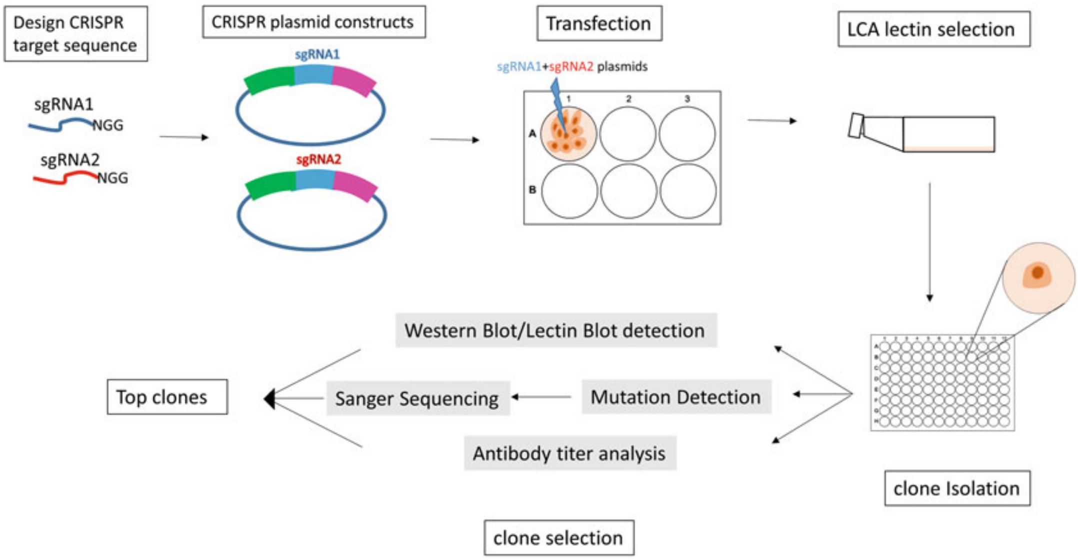

Fig. 2 Workflow of the knockout cell line generation and analysis using the CRISPR/Cas9 method described in this protocol

6. NEBuffer 2.1. (New England Biolabs).
7. QIAquick Gel Extraction Kit (Qiagen).
8. QIAquick PCR Purification Kit (Qiagen).
9. S.O.C. Medium.
10. LB Broth (Miller).
11. LB Agar powder (Lennox).
12. NEBuffer 4 (New England Biolabs).
13. T4 DNA ligase buffer.
14. Ampicillin.
15. T4 DNA ligase.
16. Taq DNA polymerase with standard Taq buffer.
17. PCR tubes.
18. NEB 5-alpha component *E. coli* (New England Biolabs).
19. Opti-MEM™ I Reduced Serum Medium (ThermoFisher Scientific).
20. Lipofectamine™ 3000 Transfection Reagent. (ThermoFisher Scientific).
21. Lens Culinaris Agglutinin (LCA) (Vector Laboratories).
22. Biotinylated Lens Culinaris Agglutinin (LCA) (Vector Laboratories).
23. Fetal Bovine Serum (FBS).
24. L-Glutamine (200 mM).

25. T25 flask.
26. T75 flask.
27. Dimethyl sulfoxide (DMSO).
28. 6-well cell culture plate.
29. 12-well cell culture plate.
30. 96-well cell culture plate.
31. Ham's F-12 K (Kaighn's) Medium (ThermoFisher Scientific).
32. Trypan Blue Solution.
33. UltraPure™ Agarose.
34. 50× TAE buffer.
35. Phusion High-Fidelity DNA Polymerase (New England Biolabs).
36. Cryovial tubes 1.2 mL.
37. Surveyor mutation detection kit (Integrated DNA technologies (IDT)).
38. Phosphate buffered saline (PBS) solution.
39. Ethylenediaminetetraacetic acid (EDTA).
40. HEPES Buffer.
41. Zero Blunt™ TOPO™ PCR Cloning Kit (ThermoFisher Scientific).
42. 17 mm × 100 mm round-bottom snap-capped tubes for *E. coli* culture.
43. Petri dishes.
44. Qiagen DNeasy Blood & Tissue Kit (Qiagen).
45. GeneRuler 1 kb DNA ladder (ThermoFisher Scientific).

2.2 Formulas

1. CHOK1 cell culture: F12-K medium with 10% FBS and 2 mM L-glutamine.
2. FACS sorting buffer: 1× PBS with 1 mM EDTA, 25 mM HEPES, and 1% FBS
3. 1% agarose gel: 1 g agarose powder in 100 mL 1× TAE buffer, boil the solution to dissolve the agarose
4. 1000× ampicillin stock: 1 g ampicillin powder in 10 mL sterile water
5. LB-agar (ampicillin): 35 g LB agar powder in 1 L deionized water, then autoclave it. After LB-agar solution cools to 50 °C, add ampicillin stock in 1000× dilution ratio.
6. 1× TAE buffer for DNA gel electrophoresis: dilute 50× TAE buffer with deionized water.

2.3 Equipment

1. Forma™ Series II 3110 Water-Jacketed CO_2 Incubators (ThermoFisher Scientific).
2. Microcentrifuge.
3. Laboratory water bath.
4. Heat block.
5. NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific).
6. Fluorescence-activated cell sorter (FACS).
7. Hemocytometer.
8. Microscope.
9. PCR thermocycler.
10. ChemiDoc™ XRS+ System (Bio-Rad).
11. Horizontal DNA Electrophoresis System (Bio-Rad).
12. Protein blot Electrophoresis System (Bio-Rad).
13. Microwave oven.

3 Methods

3.1 Design sgRNAs Against Targeted Gene

The sequence specificity of the CRISPR/Cas9 system is determined by choosing 20 nucleotides (nts) from the target gene to serve as the single guide RNA (sgRNA) sequence [23]. In order to express the small chimeric sgRNA, an RNA pol III promoter, such as U6, is required [24, 25]. Since the U6 promoter initiates transcription at guanine (G), this base must be present in the 5′ end of the gene target site. Thus, the 20 nts from target DNA site located immediately at 5′ of a protospacer-adjacent motif (PAM, 5’-NGG-3′) are in the form of 5’-GNNNNNNNNNNNNNN$ NNNNN-NGG-3′ (N can be A,G, C,or T) [23, 26].

1. Search your gene of interest using the CHO Cas9 target Finder http://staff.biosustain.dtu.dk/laeb/crispy [27] (*see* **Note 1**). Be aware that your gene of interest may have multiple variants in CHO cells. For example, FUT8 gene (Gene ID: 100751648) has two variants in CHO: one variant (protein ID: XP 003501783.1) containing 11 exons as a full-size α-1,6-fucosyltransferase, the other variant (protein ID: XP 007640580.1) containing 9 exons as a truncated α-1,6-fucosyltransferase (Fig. 1b).
2. A list of target sequences will be presented. We choose the target sequence from Exon 9 because Exon 9 exists in both CHO α-1,6-fucosyltransferase variants and its amino acid sequence covers the active region of the FUT8 enzyme [28, 29] (*see* **Note 2**).

Table 1
sgRNAs and their primer sequences

guided RNA sequence	Primers for cloning into Px458 (5′ to 3′)		
sgRNA 1	GTCAGACGCACTGACAAAGTGGG	Forward	CACCGTCAGACGCACTGACAAAGT
		Reverse	AAACACTTTGTCAGTGCGTCTGAC
sgRNA 2	GGATAAAAAAAGAGTGTATCTGG	Forward	CACCGGATAAAAAAAGAGTGTATC
		Reverse	AAACGATACACTCTTTTTTTATCC

3. From the CHO Cas9 target Finder search result, two target sequences in Exon 9, each followed by a PAM sequence (5’-NGG-3′) was chosen, as shown in Table 1 (*see* **Note 3**).
4. Design sgRNA primers

Based on the target sgRNA sequences from Subheading 3.1, **step 3**, we designed the sgRNA cloning primers according to the px458 CRISPR protocol from Dr. Feng Zhang’s lab at Addgene http://www.addgene.org/crispr/zhang/ [27]. The target sequence minus the “5’-NGG-3′” PAM sequence is the varying region that primers overlap with each other, as described in Table 1 and illustrated in Fig. 1c.

3.2 Construction of px458 Plasmid with Designed sgRNAs

3.2.1 Preparation of px458 Vector

1. The construct plasmid px458 was kindly provided by Dr. Feng Zhang (MIT) [30]. Subclone this plasmid into *E. coli* to make a bacterial glycol stock.
2. Inoculate a small amount of *E. coli* from the frozen bacterial glycol stock in LB medium supplemented with 100 μg/mL ampicillin.
3. Culture overnight at 37 °C with shaking at 250 rpm.
4. Extract plasmid DNA from *E. coli* culture using a plasmid purification kit (depending on the culture volume to choose mini, midi, or maxi plasmid purification kit and follow the manufacturer’s protocol). Elute the empty px458 plasmid in nuclease-free water (NFW) and measure the DNA concentration using the NanoDrop 2000 (*see* **Note 4**).

3.2.2 Digestion of px458 Plasmid

1. Based on the px458 empty plasmid concentration, digest an appropriate amount of px458 plasmid with BbsI enzyme and 1× NEBuffer 2.1 digestion buffer as shown in Table 2.
2. Analyze digested product by DNA electrophoresis on 1% agarose gel and check the size and linearization of px458 plasmid.

Table 2
px458 digestion reaction

Component	Volume
BbsI enzyme	3 μL
px458 plasmid	3 μg
1× NEBuffer 2.1	5 μL
Total reaction volume	50 μL
Reaction conditions	
Incubation time	2 h
Incubation temperature	37 °C

Table 3
reagents for sgRNA construct

Component	Volume
10× NEBuffer 4	10 μL
sgRNA forward primer (100 μM)	10 μL
sgRNA reverse primer (100 μM)	10 μL
NFW	70 μL
Total	100 μL

3. Cut the DNA band (containing linearized px458 plasmid) with appropriate size (~9.3 kb) to remove primer dimers and isolate the linearized px458 plasmid by using a gel extraction kit following the manufacturer's protocol.
4. Elute the linearized px458 plasmid in nuclease-free water (NFW).
5. Measure the DNA concentration using a NanoDrop 2000.

3.2.3 Anneal sgRNA Primers for the sgRNA Construct (See **Note 5***)*

1. The primers are synthesized by a custom primer service and dissolved in NFW to a concentration of 100 μM.
2. Mix the sense and antisense primers (100 μM) with following components in a tube as shown in Table 3.
3. Boil the primer mixture at 95 °C for 5 min using a heating block or thermocycler and allow the oligo duplex to slowly cool to room temperature. Freshly annealed oligo duplex is recommended for the sgRNA plasmid construction.

Table 4
Reagents for sgRNA plasmid ligation

Component	Ligation (20 μL reaction)
T4 DNA ligase buffer (10×)	2 μL
px458 linearized plasmid DNA	100 ng
Annealed sgRNA insert DNA	1 μL of oligo duplex from Subheading 3.2, **step 3** (1:20 dilution)
T4 DNA ligase	1 μL
NFW	To 20 μL
Reaction conditions	
Incubation time	2 h
Incubation temperature	Room temperature

3.2.4 The sgRNA Plasmid Construction (Ligation)

Mix components in a tube according to Table 4. If not immediately used, store at −20 °C.

3.2.5 Transformation of sgRNA Plasmid in E. coli

1. Take the DH5-alpha component *E. coli* vials out from −80 °C freezer and thaw them on ice.
2. After *E. coli* thaws, add 1–3 μL ligation product into one vial of competent cells.
3. Mix gently by flicking the bottom of the tube with your finger twice.
4. Prepare another vial of competent cells without adding ligation product as a negative control.
5. Incubate the competent *E. coli* cells on ice for 30 min
6. Heat-shock the vial in a 42 °C water bath for 30 s
7. Place the vial in ice for 5 min.
8. Add 950 μL room temperature SOC medium to each vial and incubate in a shaker at 250 rpm at 37 °C for 1 h.
9. Centrifuge at 5000 × *g* for 5 min and remove the supernatant.
10. Add 150 μL fresh SOC media to each vial, pipette up and down to resuspend the *E. coli* pellet.
11. Spread two densities (high and low) of transformed *E. coli* onto 10 cm LB agar plates containing 100 μg/mL ampicillin.
12. Incubate the agar plates overnight at 37 °C. Very few or none colonies should be expected on the negative control agar plate.

3.2.6 Analyze Transformation Colony

1. Pick a few colonies using a pipette tip and individually transfer to 4 mL LB medium supplemented with 100 μg/mL ampicillin in an *E. coli* culture snap-capped tube.
2. Place tubes in shaking incubator at 250 rpm at 37 °C overnight.
3. Purify the sgRNA plasmid using a plasmid purification kit following the manufacturer's protocol and measure the DNA concentration using the NanoDrop 2000.
4. Sequence the constructed plasmid using Sanger sequencing.
5. After confirming the sequence of the constructed sgRNA plasmid, store sgRNA plasmid at −20 °C for further transfection (*see* **Note 6**).

3.3 Transfection of sgRNA Plasmids into an IgG-Expressing CHO Cell Line

3.3.1 Day 0: Seed Cells for Transfection

1. Use healthy IgG expressing CHO cell line (viability >97%) at early passage, for higher transfection efficiency. The IgG expressing CHO cell line was previously described [31].
2. Count cells using a cell counter or hemocytometer.
3. Plate cells at a density 7×10^5 cells per well in a 6-well plate, add culture medium to 2 mL per well. At least two wells are needed: one well for transfection and another well for control.
4. Incubate cell cultures at 37 °C in a humidified incubator with 5% CO_2 for 24 h.

3.3.2 Day 1: Transfection with Lipofectamine 3000

1. Check cell confluency. Cells should be 80–90% confluent at the time of transfection. Replace the old culture medium with 2 mL reduced serum Opti-MEM medium in each well.
2. Use Lipofectamine 3000 for transfection following the manufacturer's protocol. Warm Lipofectamine 3000 reagent to room temperature and thaw sgRNA plasmids from −20 °C freezer. Flick the tube a couple times and quick spin before using.
3. Place two sterile tubes, each containing 125 μL of Opti-MEM medium, and label them Tube 1 and Tube 2
4. Lipofectamine 3000–Opti-MEM mixture: Add 5 μL Lipofectamine 3000 reagent into Tube 1
5. Diluted DNA mixture: Add 2.5 μg sgRNA plasmids (sgRNA1 plasmid: sgRNA2 plasmid = 1:1 (w/w)) into Tube 2. And then add 5 μL P3000 reagent. Gently pipet up and down to mix them well.
6. Add Lipofectamine 3000–Opti-MEM mixture to the plasmid-P3000 reagent mixture.
7. Incubate for 15 min at room temperature

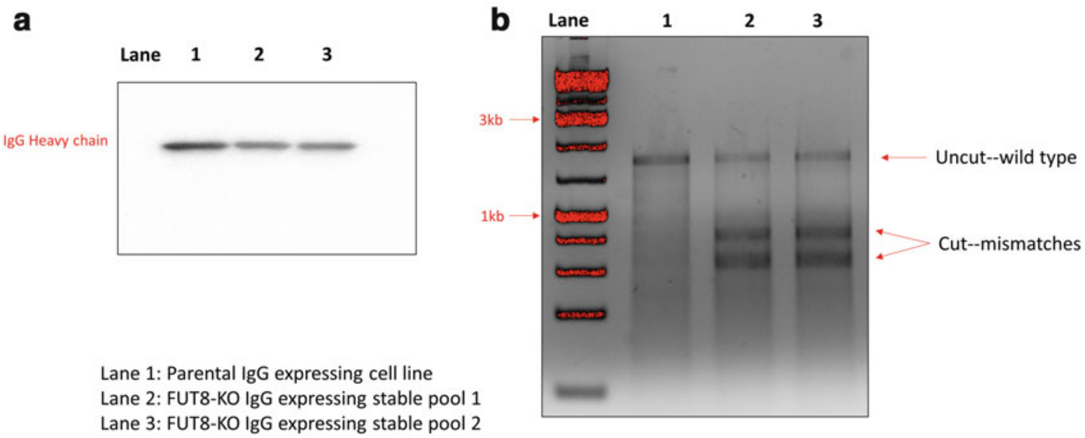

Fig. 3 (**a**) LCA lectin blot analysis of α-1,6-fucosylation level on antibody secreted from FUT8-KO stable pools. (**b**) The mutation detection assay of FUT8 exon 9 knockout efficiency in stable pools. In the mutation detection analysis, equal amounts of knockout stable pool DNA and wild-type DNA were mixed together to serve as mutant/WT cross hybridization (Lane 2 and 3). At the same time, equal amounts of wild-type DNA was also prepared to serve as the self- hybridization control (Lane 1). After annealing, equal amounts of self-hybridization and cross hybridization mixtures were treated with Surveyor Nuclease individually and analyzed by gel electrophoresis. The mutant/WT cross hybridization results in mismatched heteroduplexes. Surveryor Nuclease can digest the mismatched DNA and produce cleavage products as shown as an uncut wild-type fragment and multiple cleavage fragments in the cross hybridization mixtures (lanes 2 and 3). While no mismatch results from the wild-type self-hybridization as the Surveryor Nuclease does not cleave this homoduplex (lane 1)

8. Add the transfection mixture into a well in the 6-well plate.
9. Gently shake the 6-well plate to evenly distribute the transfection mixture.
10. Incubate cells for 2 days at 37 °C in 5% CO_2 with humidity.

3.4 LCA Lectin Selection of the Transfected Cells

1. Two days posttransfection, the supernatant from transfected cells is collected and subjected to LCA lectin blot analysis to detect fucosylation of the recombinant antibody. An example of LCA lectin result is shown in Fig. 3a.
2. For transfected cells, selection of FUT8 knockout cells was performed by supplementing the culture medium with 50 μg/mL *lens culinaris agglutinin* (LCA). The transfected pool in one well of 6-well plate was passed into a T25 flask with F12-K culture medium containing 50 μg/mL unconjugated LCA for a week to select the transfected cells with reduced fucosylation. The same amount of untransfected parental cells supplemented with equal concentrations of LCA is also performed as a negative control (*see* **Note 7**).

3.5 (Optional) Fluorescence Activated Cell Sorting to Enrich for sgRNA Plasmid Expression (See Note 8)

1. Prior to LCA selection, the transfected pool is seeded at 1×10^6 cells per 100 mm culture dish for overnight incubation.
2. When the confluency is above 90%, cells are collected by trypsinization and centrifuged at 500 × *g* for 15 min to obtain a cell pellet.
3. Wash the cells twice using sterile PBS and resuspend the cell pellet in sorting buffer using a 12 × 75 mm tube with cell strainer cap.
4. Cells are then subjected to FACS.
5. Cells expressing GFP within the top 5% intensity of signal are sorted and collected in culture medium.
6. Sorted cells are transferred to a 50 mL tube with culture medium up to 40 mL.
7. Centrifuge 2100 × *g* for 5 min and remove sorting buffer and culture medium.
8. Resuspend cells in a T75 flask with 50 μg/mL LCA for selection.
9. Incubate the sorted cells for 3 days at 37 °C in 5% CO_2 with humidity.
10. When the sorted cells grow, this indicates a successful sorting process. Passage the cells and allow them to grow to >80% confluency for the following analysis or cryopreservation.

3.6 Surveyor Mutation Assay to Check the Knockout Efficiency

The Surveyor enzyme can cleave the mismatch in the DNA duplex where a mutated sequence is hybridized with the wild-type sequence. The PCR product after cleavage will produce bands of different size that are separable on an agarose gel (*see* **Note 9**).

1. After 2–3 passages, harvest 1×10^6 cells from the LCA selected stable pool.
2. Centrifuge at 1000 × *g* and remove the supernatant.
3. Extract the genomic DNA from the cell pellet using DNeasy blood & Tissue kit following the manufacturer's protocol. Also extract the genomic DNA from untransfected parental cells for use as a negative control.
4. Design sequencing forward and reverse primers: Design your primers using ApE software (http://biologylabs.utah.edu/jorgensen/wayned/ape/) or NCBI primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Please *see* **Note 10** for primer design instructions. The sequencing primers can be used for Surveyor mutation detection as described here or for Sanger sequencing as described in Subheading 3.8.
5. Mix the following components (Table 5) in a 1.5 mL Eppendorf tube (master mix) and add 25 μL from the master mix to

Table 5
Reagents for FUT8 sequencing PCR reaction

Component	FUT8-KO (4.5 reactions)	Control (4.5 reactions)
5× HF buffer	22.5 μL	22.5 μL
NFW	71.55 μL	71.55 μL
10 mM dNTPs	2.7 μL	2.7 μL
10 μM forward primer	5.625 μL	5.625 μL
10 μM reverse primer	5.625 μL	5.625 μL
Genomic DNA (from Subheading 3.6, **step 3**)	FUT8-KO genomic DNA 100 ng	Control genomic DNA 100 ng
Phusion polymerase	1.35 μL	1.35 μL
Total reaction volume	112.5 μL	112.5 μL

Table 6
PCR program to amplify FUT8 knockout sequence

Step		Temperature	Time
Initial denaturation		98 °C	3 min
30 cycles	Denaturation Annealing Extension	98 °C 62 °C 72 °C	10 s 20 s 22 s
Final extension		72 °C	7 min
Hold		4 °C	∞

each PCR tube. Prepare four tubes for the knockout sample and four tubes for the control sample.

6. Place the PCR tubes in a thermocycler, and run the program outlined in Table 6 (*see* **Note 11**):
7. After PCR amplification, pool four PCR repeats together and take 5 μL of the PCR product from knockout and control samples and run them individually on 1% agarose gel. There should be only one clear band from your control sample (if not, redo your PCR or troubleshoot your primer design).
8. Check the PCR product on the gel, if not much primer dimer is present, the PCR product can be purified by a PCR purification kit following the manufacturer's protocol; if primer dimers are abundant, the PCR product has to be processed through a gel extraction step to remove primer dimers before proceeding to the next step.
9. Combine purified knockout PCR product with control wild-type PCR product in a new PCR tube as shown in Table 7.

10. Place the combined PCR mixture in a thermocycler and run the DNA hybridization protocol as shown in Table 8. When the PCR amplification is done, put the sample on ice immediately.
11. Mix the components for the following two reactions as outlined in Table 9.
12. Incubate the PCR tubes for 60 min at 42 °C.

Table 7
Reagents for DNA hybridization

Component	Volume
Knockout PCR	400 ng
Control wild-type PCR	400 ng
10× Taq buffer	2 μL
NFW	Up to 20 μL
Total	20 μL

Table 8
PCR program for DNA hybridization

95 °C	10 min
95 °C–85 °C	–2.0 °C/s
85 °C	1 min
85–75 °C	–0.3 °C/s
75 °C	1 min
75–65 °C	–0.3 °C/s
65 °C	1 min
65–55 °C	–0.3 °C/s
55 °C	1 min
55–45 °C	–0.3 °C/s
45 °C	1 min
45–35 °C	–0.3 °C/s
35 °C	1 min
35–25 °C	–0.3 °C/s
25 °C	1 min
4 °C	Hold ∞

Table 9
Reagents for surveyor enzyme mutation detection

Component	Control WT PCR product	KO-WT hybridized PCR mixture
Hybridized DNA sample	10 μL	10 μL
$MgCl_2$ solution	1 μL	1 μL
Enhancer S	1 μL	1 μL
Nuclease S	1 μL	1 μL
Total	13 μL	13 μL

13. Then add 1.3 μL Stop solution to each PCR tube to terminate the surveyor endonuclease reaction. Put the samples on ice.
14. Load the products on a 2% agarose gel and run for 30 min. Your results should be similar to what is shown in Fig. 3b.

3.7 Limited Dilution Cloning (Clone Isolation) (See Note 12)

1. Trypsinize the cells from the stable knockout pool and count the cell density.
2. Perform a 1:10 serial dilution with fresh medium to a final concentration of ~ 1000 viable cells/mL.
3. Transfer 160 μL of 1000 cell/mL solution to 39.8 mL fresh medium to bring the concentration to about 4 cell/mL. Low LCA lectin concentration (10–20 μg/mL) in the medium is preferred in this step.
4. Add 200 μL of the 4 cells/mL solution into each well of the 96-well plate, allowing each well to be seeded at an average density of 0.8 cells/well.
5. Leave the cells undisturbed in an incubator until Day 7 or 8, then start to check cell growth to identify single colonies in the 96-well plates. Wells with more than one colony should not be marked.
6. After 11 or 12 days, check the growth of single-cell clones daily.
7. Before the cultures become over-confluent, trypsinize the clonal cells from each well and expand them to 12-well plates individually.

3.8 Clonal Cell Line Selection

After the isolated clones grow, make three repeats of each. One culture serves a maintenance purpose. One culture is subjected to screen the clonal cells for FUT8 knockout efficiency by using surveyor mutation detection assay (Fig. 4a) and Sanger sequencing

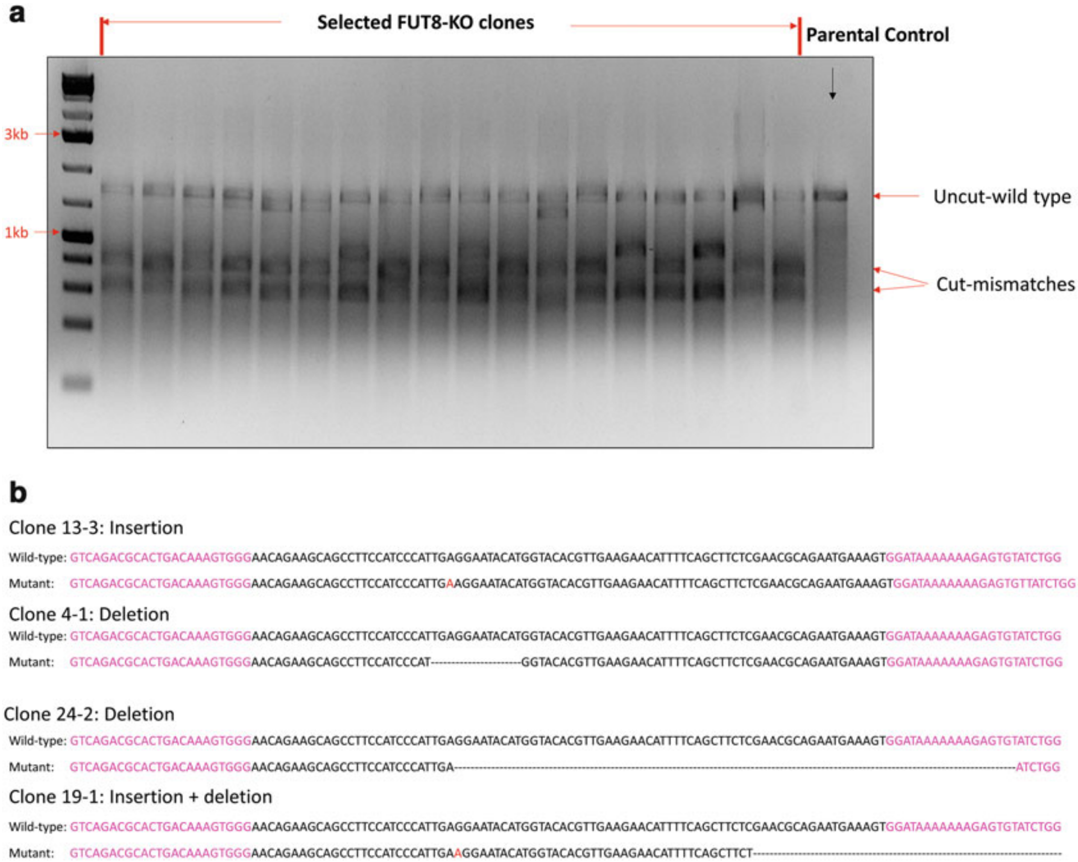

Fig. 4 (**a**) Mutation detection analysis of knockout efficiency in FUT8-knockout clones. (**b**) The DNA sequences of selected FUT8-knockout cell clones

(Fig. 4b). The other culture is subjected to LCA lectin blot and western blot analysis against α-1,6-fucosylation and antibody titer analysis.

3.8.1 Sanger Sequencing to Analyze Gene Mutation

1. After the clones in 6-well plate become confluent, the genomic DNA from each clone is extracted using a Qiagen DNeasy Blood & Tissue Kit DNA extraction kit following the manufacturer's protocol.
2. Measure the extracted genomic DNA concentration and use the designed sequencing primers in Subheading 3.6 to perform the PCR amplification and PCR product purification.
3. Check the PCR product quality by DNA gel electrophoresis and measure the purified PCR product concentration using a Nanodrop 2000.
4. Perform a Surveyor mutation analysis to detect FUT8-knockout efficiency in each clone.

5. The mutant clones from the analysis are chosen and processed as in the next step.
6. For sequencing the mutated region in each clone, TOPO cloning of the PCR product is performed following the manufacturer's protocol. Alternative methods are also provided in **Note 13.**
7. The TOPO plasmids constructed with the mutated region from each clone are purified and mixed with the forward and reverse sequencing primers as in Subheading 3.6, individually based on the Sanger sequencing service company's instructions.
8. Analyze Sanger sequencing results using ApE software.

3.8.2 LCA Lectin Blot to Check FUT8 Knockout Efficiency

A LCA lectin blot against potential FUT8 knockout clone cell lysates can also be used to verify the FUT8 knockout efficiency. (Please *see* Fig. 5 for an example of LCA lectin blot result.)

Fig. 5 LCA lectin blot of CHO cell lysates

3.8.3 Antibody Titer Analysis

The antibody productivity in each clone is analyzed by enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography (HPLC).

4 Notes

1. Other CRISPR sgRNA design software are also available [32], such as CRISPR MultiTargeter (http://www.multicrispr.net/), E-CRISP (http://www.e-crisp.org/E-CRISP/), and CRISPR-seek (http://bioconductor.org/packages/release/bioc/html/CRISPRseek.html). We chose CRISPY because this Cas9 target finding software is built on the CHO-K1 genome.
2. For improving gene disruption efficiency, we chose two sgRNAs targeting two close sites in one exon in the targeted gene (as shown in Fig. 1b). The exon or exons covering the activity domain of the targeted protein are recommended. This protocol is also applicable for one target site using one sgRNA.
3. sgRNA selection criteria are as follows:
   (a) If the targeted protein's activity domain has been defined, design the sgRNA within the activity domain coding sequence. For example, Exon 9 covers the active site of both variants of FUT8 in CHO cells. If the targeted protein's activity domain has not yet been determined, it is preferable to design multiple sgRNAs targeting different exons and test the sgRNA knockout efficacy of each one.
   (b) The sgRNA should be unique in the CHO genome with minimum off-target effects. Given that Cas9 digests between 3 and 4 nts upstream of the PAM sequence of the targeted knockout sequence [26], it is important to make sure the first 10 nts in the selected sgRNA has no off-target activity. Many sgRNA software programs also predict the off-target activity for each sgRNA. Alternatively, you can manually align your sgRNA sequence with the CHO genome using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). No or low off-target activity is an important consideration for sgRNA selection.
4. It is recommended to check the px458 vector sequence before BbsI digestion, especially the BbsI digestion region for sgRNA insertion.
5. It is not necessary to phosphorylate the oligo duplex. The oligo duplex without phosphorylation can also work well.
6. For transfection purposes, it is suggested to use sterile NFW to elute the sgRNA plasmid.

7. LCA is a plant lectin which preferentially binds to the α-linked mannose of N-glycans. LCA can cause cytotoxicity in cells expressing core-fucosylated proteins [2].
8. The px458 plasmid contains the GFP marker, which can be used for cell analysis by FACS to enrich for cells with a high plasmid expression level. Given that the CRISPR system is efficient and LCA selection alone is enough to generate FUT8−/− cells, we provide the FACS protocol here as a supplement. FACS should be performed prior to LCA lectin blot selection because after LCA selection, transient px458 plasmid expression may be terminated.
9. Other enzymes can also detect mismatched DNA sequences, such as T7 endonuclease. For an example of a detailed mutation assay methodology, please refer to the Surveyor mutation detection protocol from Integrated DNA Technologies (IDT).
10. Generally, a PCR product between 600 and 1000 bp is a suitable length for PCR amplification. This product should span the knockout binding site in your targeted gene. Primers should be placed ≥50 bp outside the region of interest and have 18–25 bp in length with a GC content around 45–60%.
11. The annealing temperature is dependent on the length and GC content of your primers.
12. For a more detailed CHO limited dilution protocol, please refer to the Freedom™ CHO-S™ protocol. As an alternative to the limited dilution protocol, single cell sorters can also be applied to perform clone isolation.
13. The uracil-specific excision reagent (*USER*) cloning method can also be used as a cloning tool to check for mutated sequences [33].

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Chapter 17

Scalable Production and Purification of Adeno-Associated Viral Vectors (AAV)

Daniel Blessing, Nicole Déglon, and Bernard L. Schneider

Abstract

Here we describe methods for the production of adeno-associated viral (AAV) vectors by transient transfection of HEK293 cells grown in serum-free medium in orbital shaken bioreactors and the subsequent purification of vector particles. The protocol for expression of AAV components is based on polyethyleneimine (PEI) mediated transfection of a 2-plasmid system and is specified for production in milliliter to liter scales. After PEI and plasmid DNA (pDNA) complex formation the diluted cell culture is transfected without a prior concentration step or medium exchange. Following a 3-day batch process, cell cultures are further processed using different methods for lysis and recovery. Methods for the purification of viral particles are described, including iodixanol gradient purification, immunoaffinity chromatography, and ultrafiltration, as well as quantitative PCR to quantify vector titer.

Key words Transient transfection, HEK293, Adeno-associated viral vector, Orbital shaken bioreactors, Suspension cell culture, Affinity chromatography purification

1 Introduction

Adeno-associated viral (AAV) vectors are amongst the most popular viral vectors for gene therapy applications in preclinical and clinical research [1]. Discovered in 1965, AAV are nonpathogenic members of the *Dependovirus* genus of the parvoviruses and are endemic in humans [2, 3]. Besides the numerous clinical trials that have been conducted to date, the recent approval by the FDA of Luxturna™, an AAV-based gene therapy to treat an inherited condition leading to blindness (Spark Therapeutics), demonstrates the popularity and feasibility of AAV-based gene therapy approaches. For many years, optimizing transgene delivery and expression, and the cell or tissue specificity of AAV variants, has been the focus of translational research. It is now that this technology reaches the market when needs and challenges concerning manufacturing and quality become most apparent. As a consequence, the focus of research and development is shifting into process development.

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Today, one commonly used AAV production platform is based on plasmid transfection of adherent HEK293 cells grown in serum-containing media. This production method is difficult to scale-up, labor-intensive, and disadvantageous for manufacturing. Therefore cost-effective and scalable protocols are needed for large-scale AAV vector production. Recent research has validated the use of orbital shaking technology (OSR) for the cultivation of HEK293 cells in suspension, using disposable vessels up to a 1000-L scale [4]. Key features of the OSR technology are high gas transfer rates, low sheer stress for cells, low mixing times, and low specific power consumption [5–7]. These characteristics make the OSR technology one of the most promising disposable bioreactor platforms for animal cell culture for small- and large-scale operations. In parallel, different groups have demonstrated the technical feasibility to scale-up AAV production by transfecting suspension adapted HEK293 cells without prior concentration of cells or exchange of medium [8, 9]. Here, we describe the transient transfection of suspension-adapted HEK293 cells in orbital shaken bioreactors for the production of AAV vectors (Fig. 1).

Next to the upstream processing for vector manufacturing, scalable methods are also required for the downstream processing in order generate AAV vectors that satisfy the requirements for clinical application. In many labs, the purification via gradient centrifugation is commonly used for lab-scale isolation of AAV vectors harvested from cell pellets. This density-based method has the advantage that it allows the removal of empty AAV particles. Nevertheless, as this operation is very laborious to process larger liquid volumes, it would not be suitable at a scale relevant for studies in large animal models or for clinical trials. As an alternative to centrifugation, chromatographic purification procedures can be applied. Various methods have been described for AAV purification in the past including ion exchange chromatography, hydrophobic interaction chromatography, and size exclusion chromatography [10–13]. These have been applied successfully to achieve high purity, but often suffer from low recovery rates. The recent development of affinity ligands and resins offers a solution to this issue. Thanks to their high selectivity, affinity ligands allow the efficient removal of impurities while maximizing vector recovery [14, 15]. The possibility to scale-up and to process large liquid volumes makes affinity chromatography an ideal option for downstream processing of AAV vectors at all scales.

2 Materials

2.1 Cell Culture

1. HEK293 cells (ExcellGene SA, Monthey, Switzerland) adapted to serum-free medium and cultivated in orbital shaken bioreactors.

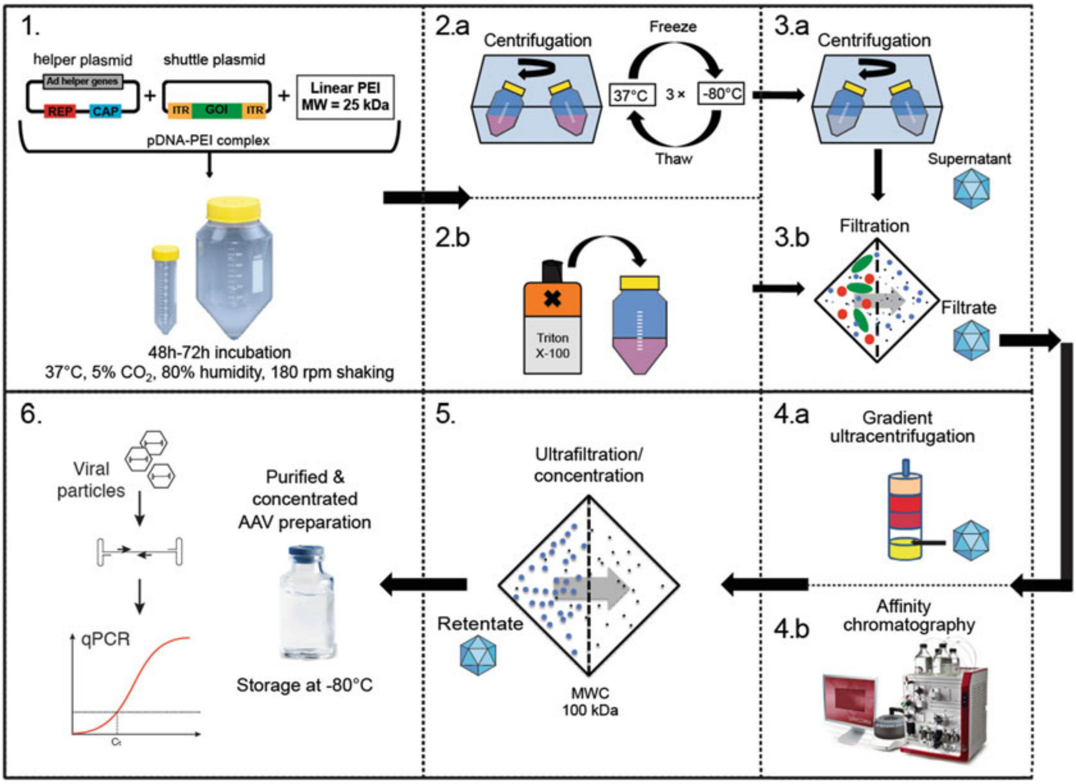

Fig. 1 Process-scheme for the transient production of AAV vectors with HEK293 cells in suspension. (**1**) PEI-mediated transfection of HEK293 cells in suspension using a two-plasmid system for AAV expression. (**2a**) Collection of cells via centrifugation and subsequent cell lysis via repeated freeze–thaw cycles. (**2b**) Cell lysis by direct addition of Triton X-100 to the cell suspension. (**3a**) Removal of cell debris (clarification) via centrifugation (supernatant contains AAV vectors). (**3b**) Clarification by filtration (depending on production scale, use membrane and/or depth filtration) (filtrate contains AAV vectors). (**4a**) Immunoaffinity chromatography purification. (**4b**) Purification via density gradient centrifugation**.** (**5**) Buffer exchange and concentration using ultrafiltration (retentate contains AAV particles). (**6**) Retentate is aliquoted for storage. The number of genome-containing particles in the purified AAV preparation is quantified via qPCR

2. TubeSpin 50 bioreactor and TubeSpin 600 bioreactor tubes (TPP, Trasadingen, Switzerland) (*see* **Note 1**).
3. Serum-free Freestyle F17 Expression medium (Thermo Fisher Scientific AG, Reinach, Switzerland) (F17) is supplemented with L-alanyl-L-glutamine (Gibco Glutamax, Thermo Fisher Scientific) by adding 20 mL of a 200 mM stock solution to 1 L of F17 medium. Additionally Pluronic F-68 (Thermo Fisher Scientific) was added to a final concentration of 0.1% (10 mL of a 10% stock solution in 1 L F17 medium).
4. 0.4% Trypan Blue solution.
5. Phosphate-buffered saline (PBS).

6. ISF1-X or ISF4-X incubator shaker (Adolf Kühner AG, Birsfelden, Switzerland).
7. Phase contrast microscope.

2.2 Transfection

1. Linear 25 kDa polyethyleneimine (PEI) (Polysciences, Eppenheim, Germany): to prepare the 1 mg/mL PEI stock solution, 1 g of PEI is weighed and transferred in 800 mL water. To dissolve the PEI, the pH is lowered to about 3 by adding 1 N HCl. Once PEI is dissolved, the pH is increased to 7.0 with 1 N NaOH and the volume adjusted to 1 L with water. Next, the solution is sterile-filtered using a bottle-top filter (0.2 μm) and aliquoted into sterile 50-mL tubes for storage at −20 °C.
2. Shuttle plasmid containing the sequence encoding the gene of interest flanked by two ITRs, typically derived from AAV serotype 2 (*see* **Note 2**).
3. pDP-helper plasmid encoding the rep (AAV2) and cap genes (serotype dependent), and adenoviral helper genes (VA, E2A, E3, and E4) (*see* **Note 3**).
4. Plasmid DNA (pDNA) obtained from contract manufacturer. Adjusted to concentrations of 1 mg/mL with distilled water for long-term storage (−80 °C).

2.3 Harvest and Lysis

1. Triton X-100 (Merck, Zug, Switzerland).
2. 10× Lysis buffer: 1% Triton X-100, 20 mM $MgCl_2$ in 500 mM Tris buffer pH 7.4.
3. Dry ice.
4. 70% ethanol.
5. 5 M NaCl, sterile filtered.
6. Benzonase 25 kU/mL (Merck).
7. 10% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) (Axon Lab AG, Le Mont-sur-Lausanne, Switzerland).
8. Supracap™ depth filter, 0.4–1.0 μm, Seitz BIO 20 media (Pall, Basel, Switzerland).
9. AcroPak™ 0.8/0.45 μm membrane filter (Pall).
10. Hermle Z 513 K centrifuge and Hermle 220.70 rotor with tube holder for TubeSpin 50 and Tubespin 600 (Hermle Labortechnik GmbH, Wehingen, Germany) (for cell harvest at 1000 × *g*).
11. Sorvall Evolution RC centrifuge and Sorvall SLA-600TC Super-Lite rotor (50 mL centrifugal tubes) (Thermo Fisher Scientific) (for removal of cell debris at 10,000 × *g*).

2.4 Iodixanol Gradient Centrifugation

1. Optiprep (Axon Lab AG, Baden-Dattwil, Switzerland), sterile and endotoxin tested multipurpose medium based on 60% iodixanol in water.
2. 0.5% phenol red solution.
3. PBS-MK buffer: DPBS (500 mL) supplemented with 50 mg $MgCl_2 \times 6H_2O$.
4. PBS-MK 1 M NaCl: obtained by addition of 36 g NaCl to 1 L PBS-MK.
5. Preparation of four gradient solutions, 50 mL final volume:

   15%: 12.5 mL Optiprep, 37.5 mL PBS-MK 1 M NaCl.

   25%: 20.8 mL Optiprep, 29.2 mL PBS-MK and 125 μL Phenol red.

   40%: 33 mL Optiprep and 17 mL PBS-MK.

   60%: 50 mL Optiprep and 125 μL Phenol red.
6. Beckman Coulter ultracentrifuge Optima 90-K (Beckman Coulter, Nyon, Switzerland).
7. Rotor 70Ti (Beckman Coulter).
8. OptiSeal polypropylene tubes (32.4 mL) (Beckman Coulter).
9. Spacer (Adapter for tubes in Rotor 70Ti) (Beckman Coulter).
10. Syringes 10 mL (B.Braun, Sempach, Switzerland).
11. Needle 18 Gauge (Thermo Fisher Scientific).
12. Syringe equipped with spinal needle (1.27 × 89 mm) (Becton Dickinson, Allschwil, Switzerland).

2.5 Immunoaffinity Chromatography

1. POROS™ CaptureSelect™ AAV9, AAV8 and AAVX resin (Thermo Fisher Scientific) (*see* **Note 4**).
2. Omnifit® column (Diba Industries Inc., Danbury, CT, USA) with movable end piece.
3. Equilibration buffer: PBS, pH 7.4.
4. 0.1 M citric acid monohydrate solution.
5. 0.1 M trisodium citrate dehydrate solution.
6. 50 mM citrate elution buffer: citric acid buffer pH 3 is prepared by mixing 82 mL of the 0.1 M citric acid monohydrate solution and 18 mL of the 0.1 M trisodium citrate dihydrate solution. The resulting solution is diluted two fold to obtain 50 mM citrate elution buffer (*see* **Note 5**).
7. Regeneration buffer: 5 M guanidine hydrochloride.
8. Cleaning buffer: 0.1 M phosphoric acid.
9. ÄKTA Pure chromatography controller (GE Healthcare, Glattbrugg, Switzerland).
10. Fraction collector F9-R (GE Healthcare).
11. UNICORN 7 control software for ÄKTA purification systems (GE Healthcare).

2.6 Ultrafiltration

1. DPBS (without calcium and magnesium) (Thermo Fisher Scientific).
2. DPBS (Thermo Fisher Scientific).
3. Amicon® Ultra-15 centrifugal filter units, molecular weight cutoff (MWC) 100 kDa (Merck).
4. Amicon® Ultra-4 centrifugal filter units, MWC 100 kDa (Merck).

2.7 Quantitative Polymerase Chain Reaction (qPCR)

1. Rotor gene probe qPCR kit (Qiagen AG, Hombrechtikon, Switzerland).
2. Forward primer (4 μM) (*see* **Note 6**).
3. Reverse primer (4 μM) (*see* **Note 6**).
4. qPCR probe (2 μM) (*see* **Note 6**).
5. QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific).

3 Methods

3.1 Cell Culture

1. Frozen HEK293 cells are thawed at 37 °C and immediately transferred into a centrifuge tube containing 10 mL prewarmed F17 medium.
2. After quick shaking by hand the tube is centrifuged at 500 × *g* for 3 min.
3. The supernatant is discarded and cells are resuspended with prewarmed 10 mL F17 medium and transferred into a TubeSpin 50 bioreactor tube. Henceforth, the cultures are maintained at 37 °C, 80% humidity, 5% CO_2 and constant shaking at 180 rpm (shaking diameter 5 cm) (*see* **Note 7**).
4. After 3–4 days cells are counted using the Trypan blue staining, a Neubauer hemocytometer and an inverted phase contrast microscope (*see* **Note 8**).
5. Based on the viable cell density, the cell suspension is diluted to a final concentration of 1×10^6 cells/mL by transferring the according cell culture volume into fresh and prewarmed F17 medium (*see* **Note 8**).
6. To maintain the cell culture at high viability, the cells are passaged twice per week.

3.2 Transient Transfection

1. 3 days before transfection, cells are seeded at 1×10^6 cells/mL in a volume corresponding to the desired scale of vector production (TubeSpin 50 or TubeSpin 600 bioreactor tube) (*see* **Note 9**).

Table 1
Parameters for transfections at different scales using TubeSpin bioreactors

Culture vessel	Culture volume [mL]	Cell density transfection	Cells total	Premix volume [mL]	DNA [μg]	PEI [μg]
TubeSpin 50	10	1×10^6	10×10^6	0.5	15	30
TubeSpin 600	100	1×10^6	100×10^6	5	150	300
TubeSpin 600	250	1×10^6	250×10^6	12.5	375	750

2. On the day of transfection, cells are counted as previously described (*see* Subheading 3.1) and diluted to 1×10^6 cells/mL in fresh F17 medium (TubeSpin 50 or TubeSpin 600 bioreactor tube).
3. The helper and shuttle plasmids are premixed at equimolar ratios, with the total amount of DNA and volume depending on the scale of transfection and the total cell number (Table 1).
4. A volume of F17 medium (room temperature) corresponding to 5% of the cell culture is added to a sterile polystyrene tube (depending on scale of transfection use 5 mL or 19 mL tube).
5. A total of 1.5 μg of pDNA per million cells is added to the F17 medium. The tube is then shaken briefly (*see* **Note 10**).
6. 3 μg of PEI per million cells is added to the tube (DNA: PEI = 1:2 [w/w]). (*see* **Note 10**).
7. At this point the tube is gently vortexed.
8. The tube is then incubated for 10 min at room temperature for precomplex formation (*see* **Note 11**).
9. The whole content of the tube is then transferred to the cell suspension for transfection.
10. The cell culture is subsequently incubated at standard conditions described above (Subheading 3.1, **step 3**) for 48–72 h before vector harvest (Fig. 1) (*see* **Note 12**).

3.3 Harvest

If purification via gradient centrifugation is intended as a one-step purification, only the cells are processed to isolate intracellular AAV particles. For immunoaffinity chromatography, it is optional to collect cells and/or culture medium. As it is possible to process larger liquid volumes with this method, cells can also be lysed in the cell culture directly (*see* Subheading 3.4.2).

1. The cells are harvested by centrifuging the TubeSpin 50 bioreactors at 1000 × *g* for 5 min or TubeSpin 600 bioreactor tubes at 1000 × *g* for 10 min. It is recommended to collect and

store at −20 °C a sample of the supernatant at this point to determine the amount of particles released by the packaging cells (e.g., by quantifying the amount of genome-containing particles via qPCR).

2. Discard the supernatant and gently resuspend the pellet in 10 mL PBS.
3. Transfer in a 50 mL polypropylene centrifuge tube and centrifuge at 1000 × *g* for 7 min.
4. After discarding the remaining supernatant, the cell pellet is further processed, continuing with the steps described in Subheading 3.4.1 or 3.4.2. The cell pellet can be stored at −20 °C before further processing.

3.4 Lysis

3.4.1 Freeze–Thaw Lysis (See **Note 13**)

1. Mix dry ice with 70% ethanol.
2. If required, thaw the pellet initially in a water bath at 37 °C.
3. Resuspend the pellet in PBS with a volume corresponding to 2% of the initial cell culture volume.
4. Then freeze (10 min) in dry ice–ethanol mix and thaw (10 min) in 37 °C water bath. After thawing, the suspension is vortexed vigorously for 30 s. This procedure is repeated three times (Fig. 1).
5. Add Benzonase at 50 U/mL and incubate 30 min at 37 °C under agitation (100 rpm).
6. Add 0.5% CHAPS, incubate 30 min at 37 °C and 100 rpm.
7. Add 5 M sodium chloride to a final concentration of 0.1 M and vortex briefly. The volume can be estimated based on the graduation displayed on the 50 mL polypropylene centrifuge tube.
8. Centrifuge for 10 min at 10,000 × *g*, 4 °C.
9. Pass the supernatant through a 0.45 μm filter (*see* **Note 14**). The lysate is ready for iodixanol gradient centrifugation or immunoaffinity chromatography (*see* **Note 15**).

3.4.2 Triton X-100 Lysis

1. Add 10% (v/v) lysis buffer to the cell culture (Fig. 1). If only the cell pellet is processed, resuspend the pellet in PBS with a volume corresponding to 2% of the initial cell culture volume (*see* **Note 16**).
2. Add Benzonase at 50 U/mL.
3. Incubate at 37 °C, 100 rpm shaking for 30 min.
4. Add 5 M sodium chloride to a final concentration of 0.1 M and briefly vortex. The volume can be estimated based on the graduation displayed on the 50 mL centrifuge tube.

5. If only the cell pellet is processed and the volume does not exceed 15 mL, centrifuge for 10 min at 10,000 × *g*, 4 °C (Fig. 1). For larger volumes, proceed with **step 6**.
6. Pass the lysate through a depth filter for clarification and removal of debris.
7. Pass the supernatant through a 0.8 μm prefilter and then through a 0.45 μm filter. It is also possible to use filter capsules with a built-in prefilter (*see* **Note 14**).
8. The lysate is ready for iodixanol gradient centrifugation or immunoaffinity chromatography (*see* **Note 15**).

3.5 Iodixanol Gradient Centrifugation

1. Rinse ultracentrifuge tube with sterile PBS to remove electrostatic charge that may cause air bubbles otherwise.
2. Place sterile OptiSeal tube, washed in PBS, in the assembly rack.
3. Pipette the lysate (6 mL maximal volume) at the bottom of the 30 mL ultracentrifuge tube.
4. Using a needle connected to a syringe, underlay the lysate with 15% (7 mL), 25% (5 mL), 40% (5 mL) and 60% (8 mL) iodixanol solutions (Optiprep). This can be achieved by carefully placing the tip of the needle at the very bottom of the tube for each of the different solutions sequentially added according to increasing iodixanol concentrations.
5. The tube is then sealed before centrifugation.
6. Centrifuge in 70Ti rotor at 350,000 × *g* for 1 h at 4 °C.
7. Fix the tube on holder, remove the rubber lid from top and insert syringe with 18G needle at the border between the 60% and the 40% iodixanol fractions, with the needle facing upward to the lower portion of the 40% iodixanol fraction where the viral particles accumulate.
8. Carefully remove the entire 40% iodixanol layer (transparent), without disturbing the layer of protein contaminants that accumulate at the interphase between the 40% and 25% iodixanol layers. It is typically possible to collect 4–5 mL of suspension.
9. Dilute 1:1 in PBS pH 7.4.
10. Continue with the ultrafiltration described in Subheading 3.7.

3.6 Immunoaffinity Chromatography

This method has been implemented using the Poros™ Capture-Select™ AAV9 and AAV8 resins. The AAVX resin has recently been introduced. Together, these three commercially available resins allow the purification of capsids of the AAV1 through AAV9 serotypes, and possibly a variety of modified and chimeric serotypes (*see* **Note 17**).

1. After gently mixing the bottle containing the resin (resins are supplied as approximately 56% slurry in 18% ethanol), the desired amount of slurry is transferred to an Omnifit® column with a serological pipette.
2. After transferring the slurry, the resin is allowed to settle by gravity. Do not let the resin dry out.
3. Once the resin is not compacting any further, the column is filled with distilled water. A convex meniscus should form at the very top of the column.
4. The removable column end piece is screwed back on the column without introducing any air bubble into the column. The part with the 25 μm PTFE frit is moved on top of the resin leaving 1–2 mm space between resin and frit (watch out for liquid that may spill out of the column!).
5. Once the end piece is in place, the column is connected to the chromatography controller.
6. A flow rate of 10 mL/min (linear velocity ≈ 763 cm/h) is applied to pack the column. Water is used for this step. Using 10 mL of slurry, the volume of the packed column should be approximately 6 mL under these conditions.
7. The column is either equilibrated with 10 column volumes (CVs) at a flow rate of 2 mL/min (linear velocity ≈ 152 cm/h) with equilibration buffer for immediate use, or a 20% ethanol solution for storage at 4–8 °C.
8. The clarified lysate (*see* Subheading 3.4) is loaded with a flow rate of 1 mL/min (linear velocity ≈ 76 cm/h).
9. The resin is then washed with equilibration buffer at a flow rate of 2 mL/min (linear velocity ≈ 152 cm/h). Washing is made with at least 5 CVs or until the 280 nm absorbance reaches baseline value or remains stable.
10. Bound AAV9 particles are recovered by a one-step elution using 50 mM citrate buffer (pH 3) with a flow rate of 2 mL/min (*see* **Note 18**).
11. Peak fractions are collected with a fraction collector and immediately after collection adjusted to a neutral pH by adding 50 μL neutralization buffer per mL elute (1 M Tris, pH 9) (*see* **Note 19**).
12. The peak fractions are pooled based on the recorded 280 nm absorbance (*see* **Note 20**).
13. At this point continue with Subheading 3.7 for further sample processing. The steps below describe the procedure for column regeneration and storage.
14. After the elution step, the column is regenerated with 5 M guanidine hydrochloride buffer.

15. The chromatography system and resin can be cleaned using 0.1 M phosphoric acid (*see* **Note 21**).
16. The column is reequilibrated with equilibration buffer (5 CVs).
17. For storage at 4–8 °C repeat **step 7** using 20% ethanol.

3.7 Ultrafiltration

With this final step the preparations deriving from the gradient centrifugation (Subheading 3.5) or the immunoaffinity chromatography (*see* Subheading 3.6) can be subjected to buffer exchange and concentrated (Fig. 1).

1. Add 12 mL sterile water to the Amicon centrifugal filter unit and centrifuge at 3000 × *g*.
2. Discard the flow-through.
3. Add 12 mL sterile DPBS (without calcium and magnesium) to the Amicon centrifugal filter units and centrifuge at 3,000 × *g* (*see* **Note 22**).
4. Discard the flow-through.
5. Add gradient fraction or the pooled elution fractions to the Amicon Ultra-15 (a maximum of 12 mL)
6. Centrifuge at 3,000 × *g* until volume is reduced to 1 mL (the volume depends on the desired AAV particle concentration).
7. Discard the flow-through.
8. Add DPBS (without calcium and magnesium) to the concentrated particles to a final volume of 15 mL.
9. Repeat **steps 6–8** up to 3 times.
10. Add DPBS to the concentrated particles to a final volume of 15 mL.
11. Centrifuge at 3000 × *g* until volume is narrowed down to 500 μL.
12. The AAV particle suspension can at this point be aliquoted in low retention tubes for storage at −80 °C (*see* **Note 23**).
13. For further concentration, transfer the vector suspension to the Amicon Ultra-4 and add DPBS to a final volume of 4 mL.
14. Centrifuge at 3000 × *g* until the desired volume is reached (the volume depends on the desired AAV particle concentration) and discard the flow-through.
15. Aliquot the concentrated AAV particle suspension in low retention tubes for storage at −80 °C (Fig. 1).

3.8 Determination of the Amount of Genome-Containing AAV Particles

There is a variety of methods that are frequently used to quantify AAV vectors. The most commonly used method is based on qPCR and determines the number of vector particles that contain a genome (Fig. 1). The advantage of this method is that it is fast and inexpensive and it can be applied independently of the vector serotype. However, this method does not provide any measurement for the infectivity of vectors or the amount of empty particles in a preparation.

1. Thaw an aliquot of purified vector at room temperature and pipette twice 2 μL of the purified vector to low retention reaction tubes.
2. Dilute 1:10^4 and 1:10^5 with PBS using serial dilution steps.
3. Prepare pDNA standards containing the same template DNA used to amplify and quantify the vector genomes. Establish a standard curve based on a known number of pDNA copies, ranging from 10^3 to 10^7 copies. Note that each plasmid copy is equivalent to two single stranded AAV vector genomes.
4. Prepare master mix for PCR by combining 5 μL of 2× Rotor-Gene probe PCR master mix, 1 μL forward primer (4 μM), 1 μL reverse primer (4 μM), 1 μL probe (2 μM). Vortex the master mix before further use.
5. Distribute reaction master mix to reaction tubes (8 μL/tube).
6. Prepare triplicates for each sample. 2 μL of the diluted samples or standards are transferred to each of the three tubes containing the reaction master mix (10 μL total reaction volume). The tubes are then quickly centrifuged for 5–10 s.
7. To lyse the AAV capsid and release the vector genome (PCR template) the reaction tubes are heated to 95 °C for 20 min in the PCR cycler before initiating the PCR cycling (40 cycles of 3 s at 95 °C followed by 10 s at 60 °C).
8. Determine the number of genome-containing AAV particles, taking into account the quantification according to the standard curve, sample dilution, and the volume of vector suspension added to the PCR. Of note, a factor 2 correction has to applied when the single-stranded AAV genome is compared to double-stranded plasmid DNA.

4 Notes

1. OSRs are available as single-use bioreactor systems up to a scale of 1000 L [4]. The mixing principle of these reactors is based on horizontal movement and shaking of the entire reactor in contrast to the mixing of stirred tank bioreactors with an impeller. Key features of the OSR technology are high gas transfer rates, low mixing times and low specific power consumption [5–7]. These characteristics make the OSR technology one of the most promising disposable bioreactor platforms for animal cell culture for small- and large-scale operations.

2. The backbone of the shuttle plasmid can be a standard vector containing a bacterial origin of replication and a bacterial selection gene. The sequence flanked by the ITRs will be incorporated into the AAV capsid as the recombinant genome and has to be designed to encode the protein or the small RNA of interest.
3. The pDP helper plasmid is a part of a two-plasmid system typically used to produce rAAV vectors. As an alternative, a three-plasmid system is commercially available. This system encodes the AAV essential genes (rep and cap) and the adenoviral helper genes (VA, E2A, E3, and E4) on two separate plasmids. We have compared both systems and did not observe a significant impact on the overall vector yield between them.
4. The nature of the Poros™ CaptureSelect™ AAV resins and their mechanical resistance to pressures up to 10 MPa allows the application of high linear velocities. Hence, these resins are ideally suited to process large liquid volumes in a manufacturing scale.
5. The buffer pH should be around 3 but should be checked using a pH probe and be adjusted, if necessary.
6. The primers are designed according to the recombinant AAV genome (pPCR template).
7. Using the TubeSpin 50 bioreactor a working volume of 20 mL should not be exceeded. The ideal working volume is 10–15 mL. Above this, the formation of cell clusters can be observed with some cell lines.
8. When HEK293 cells are seeded in F17 medium at 1×10^6 cells/mL, cells have to be passaged after a maximum of 5 days. Seeding at a lower cell density ($0.3–0.5 \times 10^6$ cells/mL) allows prolonging the cultivation time to 6–7 days.
9. Based on the growth characteristics of the HEK293 cells the dilution factor on the day of transfection is approximately 5–6 fold. For a 1-liter transfection, a minimum of 200 mL of cell suspension at 1×10^6 cells/mL will need to be seeded 3 days before the transfection. As the cells are typically maintained in 10 mL cultures, it might be necessary, depending on the scale of production, to expand the cell culture volume already 1 week (two passages) before the planned transfection.
10. The parameters for transient transfection of HEK293 cell suspensions are based on protocols optimized in our lab, which are used to define total pDNA amount per cell and the w/w ratio of pDNA:PEI [16, 17]. In combination with the findings from other groups we adapted protocols toward the transfection of HEK293 cells in conditioned Freestyle-F17 medium [8, 9]. Depending on several parameters including the cell line,

the plasmids used for vector production, and the type of AAV, the amounts of pDNA and PEI have to be optimized on a case-by-case basis.

11. After a short incubation time (30 s), an increased turbidity can be observed. This is an indicator of complex formation. The tube with medium containing pDNA and PEI appears "milky" compared to a tube with medium only.
12. The incubation time depends on the cell line, the medium and the process strategy (e.g., batch, fed-batch or perfusion). Under standard batch conditions we have observed a maximum accumulation of AAV vectors (intracellular) between 48 and 72 h post-transfection. Furthermore, we did not observe any significant increase of the overall vector yield for longer process times. After 72 h, the number of particles in the culture medium may increase due to a loss of cell viability.
13. Freeze–thaw lysis is mostly used when lysates are directly purified via gradient centrifugation. We have observed that detergents in the lysate, in particular sodium deoxycholate, can interfere with gradients and separation.
14. The choice of filter depends on the production scale and the particle burden of the lysate. At a liter-scale of production, syringe filters are not suitable anymore. At this scale, membrane filter capsules connected to a peristaltic pump are best suited. With an increasing scale of production the usage of membrane filters becomes too expensive and inefficient. A solution at this scale is the application of depth filtration to remove most of the insoluble particles followed by a membrane filtration (0.45–0.8 μm).
15. The vectors are usually not purified when transfections are carried out at a small scale (10–15 mL in TubeSpin 50 bioreactors) for screening purposes in order to optimize the transfection efficiency, AAV yields or other process parameters. Indeed, depending on the number of transfections, this would be very laborious. Also, the quantities produced at this scale could not be efficiently recovered. To quantify the vector yield, cells are collected and lysed in 1.5 mL tube following the same protocol as described in Subheading 3.4.1 or 3.4.2. A sample of the supernatant (1 mL) is taken as well. The amount of intracellular vector and the amount of vector in the cell culture supernatant can be quantified using qPCR as described in Subheading 3.8.
16. Triton X-100 addition as compared to the freeze–thaw method is very simple to perform and less time-consuming. The method is applied when lysates are directly purified using immunoaffinity chromatography.
17. The purification of AAV vectors via gradient centrifugation (iodixanol or CsCl) has long been used to produce research

grade vector preparations for preclinical studies [18]. The combination of gradient centrifugation and chromatographic procedures results in a higher purity and can help to decreases possible adverse effects of protein impurities during in vivo studies.

18. The elution conditions have been optimized for the step gradient elution of AAV9. We found that elution was most efficient at a pH of 3. Instead of citrate buffer other acidic buffers can be used for elution.
19. The AAV capsids are in general relatively heat and pH stable. Nevertheless, extended storage under extreme conditions (pH or temperature) will result in the denaturation of AAV capsids and a decreased vector amount. To avoid this, the pH is immediately adjusted to 6.5–7.5 after fraction collection.
20. The peak is not defined very stringently. One fraction before the A280 nm signal starts increasing and one fraction after the signal reaches baseline are included in the pooled fractions, with the aim to increase vector recovery.
21. It is important to clean the system and resin sufficiently in order to avoid residual contaminations.
22. Above vector particle concentrations of 10^{13} vectors/mL, aggregation of AAV particles can occur. Previous studies have demonstrated that by increasing ionic strength of the suspension buffer, it is possible to limit AAV aggregation [19].
23. The aliquot volume is chosen based on the application and the vector amount needed for an experiment. Refreezing a vial should be avoided, as repeated freeze–thaw cycles might cause vector degradation.

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Chapter 18

Considerations in the Use of Codon Optimization for Recombinant Protein Expression

Vincent P. Mauro and Stephen A. Chappell

Abstract

Codon optimization is a gene engineering approach that is commonly used for enhancing recombinant protein expression. This approach is possible because (1) degeneracy of the genetic code enables most amino acids to be encoded by multiple codons and (2) different mRNAs encoding the same protein can vary dramatically in the amount of protein expressed. However, because codon optimization potentially disrupts overlapping information encoded in mRNA coding regions, protein structure and function may be altered. This chapter discusses the use of codon optimization for various applications in mammalian cells as well as potential consequences, so that informed decisions can be made on the appropriateness of using this approach in each case.

Key words Codon, Optimization, Synonymous, mRNA, Translation, Wobble, Recombinant, Bioproduction, Nucleic acid, Therapeutics

1 Introduction

Translation is one of the most conserved and fundamental mechanisms in biology, which enables information encoded in the genome to be decoded into protein. It involves three distinct steps: initiation, elongation, and termination [1, 2]. Although ribosome translocation during the elongation cycle is most relevant for a discussion of codon optimization, initiation is also relevant, as codon optimization can disrupt alternative open reading frames within mRNAs and introduce new ones [3]. Codon-optimized mRNAs often encode novel peptides, which has implications both for bioproduction in cell lines and for in vivo expression, through mRNA or DNA constructs. As such, both initiation and elongation with be briefly reviewed.

Translation initiation involves processes that lead to the formation of a ribosomal complex at the translation start site, which consists of two major processes: ribosome recruitment and start site selection. In eukaryotes, recruitment includes mechanisms that

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enable mRNAs and the small (40S) ribosomal subunit to interact. Recruitment can occur at different locations within mRNAs and may require various accessory factors. A major recruitment site occurs at the 5′ ends of mRNAs, which contain an m7G posttranscriptional modification termed the cap-structure. Recruitment at the cap-structure is mediated by various eukaryotic initiation factors (eIFs). In addition, ribosomal recruitment can occur at other locations within mRNAs by cap-independent mechanisms. In many cases, cap-independent recruitment is facilitated by an internal ribosome entry site (IRES). IRESes encompass a broad category of sequence elements that function by various mechanisms that range from direct binding to 40S subunits to specific structures that interact with various eIFs or other noncanonical initiation factors.

For most mRNAs, recruitment of the translation machinery typically occurs some distance upstream of the initiation codon, usually an AUG codon, which necessitates movement of the 40S ribosomal subunit from the recruitment site to the initiation codon. This mechanism is not yet fully understood. Although linear scanning is generally thought to explain this movement, the scanning hypothesis is poorly supported and lacks direct evidence. There is stronger evidence for nonlinear (shunting) mechanisms of ribosomal movement that include tethering and clustering of ribosomal subunits [4, 5]. In any case, many mRNAs do not initiate translation from the first AUG codon, or necessarily from the first AUG codon residing in a good nucleotide context. Indeed, there is strong evidence that translation often initiates at multiple start sites within mRNAs, including both AUG codons and noncanonical start sites [6–9]. As will be discussed below, the fact that multiple initiation sites can function within mRNAs is significant because codon optimization disrupts many of the alternative open reading frames within mRNAs and introduces new ones that can encode novel peptides [3].

When the 40S subunit reaches the initiation codon, this codon is recognized via the initiator Met-tRNA, which is associated with the subunit as part of a 43S preinitiation complex [10]. After the initiator Met-tRNA base pairs to the initiation codon, the 60S ribosomal subunit joins to form an 80S complex that is capable of mRNA template-driven peptide synthesis in a process termed translation elongation [11]. The 80S ribosomal complex facilitates interactions between mRNA codons and aminoacylated tRNAs, which are tRNAs covalently bound to their cognate amino acids. tRNAs recognize specific codons via 3-nt base pairing interactions. In a translation elongation cycle, the ribosome, which is a peptidyl transferase, catalyzes the transfer of an amino acid from an aminoacylated tRNA to a growing polypeptide chain. Ribosomes then shift downstream to the next codon, and this process is repeated until a termination codon is encountered, at which point the ribosomal complex dissociates and the newly synthesized protein is released.

2 Codon Usage

The genetic code is universal, with minor exceptions observed in some bacteria, protists, mitochondria, and yeast [12]. It consists of 61 3-nt codons that specify 20 amino acids. Three codons do not specify any amino acids but function as termination codons. Eighteen of the amino acids are encoded by multiple (2, 3, 4, or 6) synonymous codons; only methionine and tryptophan are encoded by one codon each. This degeneracy enables many different mRNA coding sequences to encode the same polypeptide [13]. However, synonymous codon usage is not random. Different synonymous codons are used with different relative frequencies. Synonymous codon bias varies between different organisms, and even between different tissues of the same organism [14].

The complexity of codon usage is increased by the fact that organisms generally express only a subset of the 61 possible tRNA genes, for example, human cells only contain tRNA genes corresponding to 48 codons [15]. Nevertheless, all codons are used, even those that cannot be decoded by a cognate tRNA. This flexibility is possible because of tRNA wobble.

For a cognate tRNA–codon interaction, the tRNA base pairs to the codon using three standard (Watson–Crick) base-pairing interactions. For a wobble tRNA–codon interaction, the tRNA uses Watson–Crick pairing for the first two nucleotides and a less-stringent base-pairing interaction for the third nucleotide, e.g., G:U. Interestingly, ribosome profiling studies in yeast, *C. elegans*, and a human cell line (HeLa) indicate that translation elongation at codons decoded by wobble interactions is slower than that at codons decoded by standard base-pairing interactions [16, 17]. Ribosome profiling is a technique that has provided important insights about translation by determining which segments of mRNAs are protected by ribosomes in cells. Deep sequencing is used to identify the ribosome-protected fragments under conditions that block translation to provide a static picture of ribosome positions on mRNAs at a moment frozen in time. Analysis of the relative distribution of protected fragments within an mRNA can provide information about the uniformity of ribosomal movement at specific sites along the mRNA (Fig. 1). For example, in human cells, ribosome occupancy was increased by ≈65–300% at codon positions for which the third base interaction was a wobble G:U base pair compared to a standard G:C base pair [16].

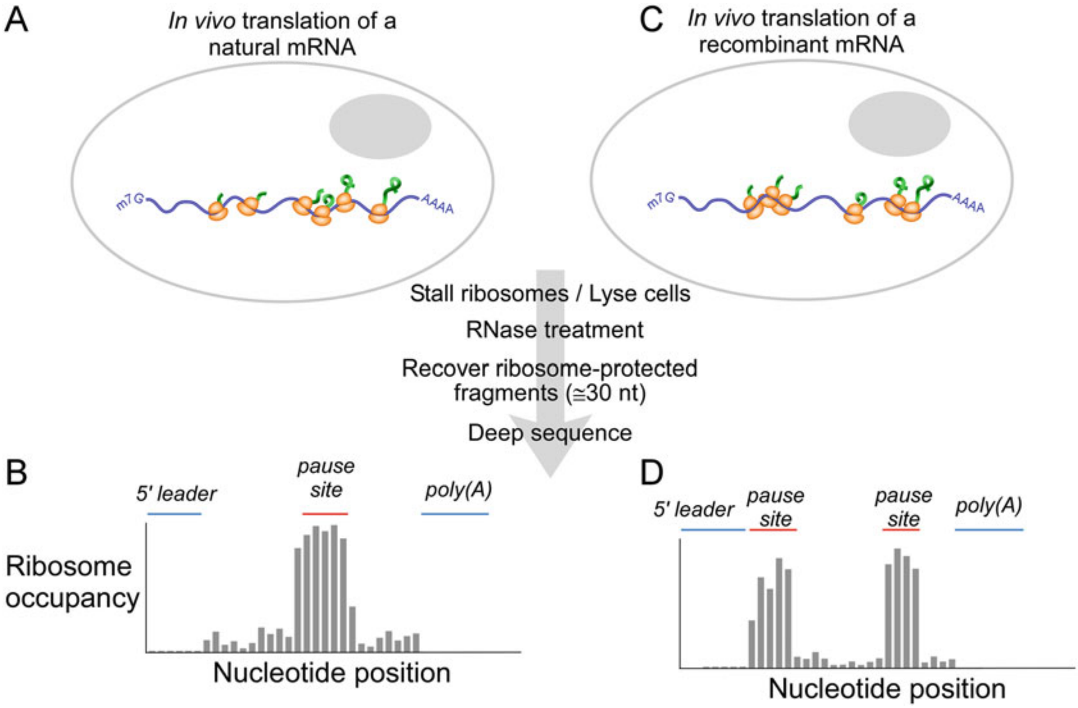

Fig. 1 Ribosome profiling to identify ribosome pause sites in mRNAs. Ribosome profiling can provide a snapshot of ribosomes in the process of peptide synthesis. (**a**) Schematic representation of a cell translating a natural mRNA indicated as a blue line with a m7G cap structure at the 5′ end and a poly(A) tail at the 3′ end. Ribosomes are indicated as orange objects and the nascent peptide is indicated in green. Ribosome profiling involves stalling ribosomes using an antibiotic, such as cycloheximide, to block elongation. After cells are lysed, an RNase treatment is performed to degrade mRNA not protected by ribosomes. Recovery and sequencing of these protected fragments provides information about which specific segments of mRNAs are protected by ribosomes. (**b**) By mapping ribosome occupancy based on sequence data from the protected mRNA fragments in (**a**) along the coding sequence of a given mRNA (Nucleotide position), it is possible to observe segments where translation is slower, such as pause sites. Sequences corresponding to these sites will be overrepresented compared to regions where translation is faster. (**c**) Schematic representation of a cell translating a codon optimized recombinant mRNA corresponding to the one depicted in (**a**). (**d**) The ribosome occupancy map from a recombinant mRNA may be different than that of a natural mRNA if codon optimization disrupts natural pausing signals, which may include codon bias, wobble decoding, and mRNA secondary structure, and/or introduces new pause sites

3 Codon Optimization

Codon optimization refers to approaches using synonymous mutations to increase protein expression from a gene of interest. This approach is routinely used to enhance recombinant protein expression, both for bioproduction applications, as well as for in vivo nucleic acid therapeutic applications [18, 19]. This approach is often used simply to increase expression, particularly when a protein is expressed in a heterologous expression system, to match the expression system [20, 21].

Early assumptions about the origin and effects of codon bias suggested that more frequently used codons are translated at a faster rate than less-frequently used “rare codons.” A variety of codon-optimization algorithms have been developed and used based on the notion that nonoptimal codon usage restricts protein expression by slowing elongation. Different approaches vary in complexity from simply replacing all codons with the most frequently used ones [22, 23] to trying to maintain regions of slow translation that may be important for protein folding [20, 21]. In addition, various approaches use synonymous mutations to address other variables and issues, e.g., to eliminate potential splice sites that may that cause expression problems, to modify restriction sites to facilitate cloning, and to avoid specific sequence elements known to cause problems [3].

The ability to increase recombinant protein expression is often essential, for example, to enable basic research, to perform clinical trials of a therapeutic protein or to ensure the commercial viability of an industrial enzyme. However, the benefits of codon optimization appear to be variable and in general reported results are modest; reviewed in [3, 24].

Codon optimization can provide a benefit of increased expression. However, the effects on expression may result from different underlying mechanisms. For example, increases in protein expression from codon optimized mRNAs have been shown to result from elevated mRNA levels via increased transcription or RNA stability [25–28]. In addition, expression has also been shown to be affected by codon changes altering mRNA secondary structures. In one study, Kelsic and colleagues generated a library of codon mutants in the *E. coli* essential gene infA and correlated predicted 5′ RNA structures in the coding sequences with fitness [29]. This study showed that even if codons were located far from the start of the coding sequence, they could be deleterious if codon changes disrupted the native 5′ RNA conformation.

Without fully understanding the effects of different synonymous mutations, there is potential risk introduced by using this approach. As discussed below, these potential risks include effects that alter protein conformation. These potential risks should be considered upfront, and will vary with different applications. For example, for human therapeutics there is increased potential risk if a protein drug is required at high doses, or is used for treatment of chronic diseases, which require extensive or lifetime use. For some therapeutic applications, there is a lower potential safety risk associated with codon optimization, and for some applications, there is no risk, for example, in nontherapeutic industrial applications. Interestingly, synonymous codon modifications have been used to alter protein conformation and function in various studies, both to modify enzymatic activity as well as to attenuate viruses [30–33].

4 Effects of Codon Optimization on Translation

As indicated in Fig. 1, the elongation rate of ribosomes along mRNA coding regions is not necessarily uniform. The notion that clusters of rare codons can slow elongation and the possibility of using this information to preserve pause sites and protein folding patterns appears to have been overgeneralized. We do not yet fully understand all the variables affecting elongation rates, which includes codon bias in some instances. However, other variables include wobble decoding, effects associated with specific dicodons, and mRNA secondary structures. There is also evidence that codons that are thought to facilitate a fast rate of elongation are important for protein conformation by reducing the possibility of misfolded intermediates [34]. It is also important to consider that alterations in the rhythm of elongation may result from other variables introduced by expressing recombinant mRNAs in heterologous host cells, which may express different sets of tRNAs at different relative levels compared to in vivo.

The effects of specific dicodons on elongation rates have been reported in yeast [35]. Interestingly, these effects were independent of the encoded dipeptides. In most cases, one or both codons were wobble codons, and in many cases, the order of the two codons was important, suggesting that the effects were more complex than simply having two slow codons in a row. These inhibitory dicodons also appear to increase mRNA decay [36]. The potential effects of dicodons have also been suggested in humans based on an analysis of synonymous single nucleotide polymorphisms in numerous different genes for various human genetic diseases or traits [37]. This study identified disruptions that appeared to be caused by dicodon pairs, rather than bias associated with individual codons.

The picture that is emerging from many recent studies suggests that codon bias arises from different factors in various organisms and depending on the organism, the effect of codon bias on translation varies dramatically. There appears to be a fundamental distinction between nonmammalian and mammalian organisms. In many nonmammalian organisms, codon bias has been reported to be correlated with expression and various studies have linked elongation rates with tRNA expression levels [3] and have demonstrated faster elongation rates at frequently used synonymous codons than at less frequently used synonymous codons [38–42]. However, the situation in mammals appears to be different; indeed, correlations between codon bias and translation efficiency in mammals are very weak [3, 43–45]. Moreover, there does not appear to be any strong evidence supporting the notion that codon bias influences protein expression by altering the rate of ribosome translocation through the coding region. Consequently, in mammalian cells, codon bias appears to arise through different mechanisms unrelated to translocation rates. Various other observations fail to support the idea that codon usage is correlated with expression [43–45]. For example, different highly expressed genes in the same cell can vary in their synonymous codon usage. In addition, for many individual genes, codon usage does not match tRNA abundance.

A major factor suggested to affect codon bias in mammals is the GC composition of the genome. Large segments of chromosomal DNA, known as isochores, have a uniform GC composition, and analysis of synonymous codon usage indicates that the GC content of chromosomal regions accounts for ≈70% of codon bias in mammals [43].

One variable associated with assessing codon bias is that codon bias is often based on a tally of gene sequences, and does not consider which mRNAs are expressed, to what extent, and the possible effects of these variables on overall codon bias. However, several recent studies have taken these variables into consideration and the results indicated that in different mammalian cells, the overall codon usage of the transcriptome is correlated with the tRNA pools, i.e., there appears to be a balance between the codon populations and the tRNAs that are necessary for their translation. In an analysis of proliferating and differentiated human cell types, it was observed that codon usage was balanced through the expression of two distinct tRNA pools [46]. A comparable balance of codon usage and tRNA pools was also observed in an analysis of mouse liver and brain tissues at eight different developmental stages [44]. Notably, and in contrast to reports from nonmammalian organisms, no differences in codon usage were seen when highly expressed and poorly expressed genes were compared. Similar findings were reported when tRNA pools and codon usage were compared in human and mouse liver cancer cell lines (in vitro) and quiescent liver cells (in vivo) [45]. The results suggested that the tRNA pools from the different cell types were capable of translating various mRNA transcriptomes with similar efficiencies. As in the previous study, there was no evidence that the codon usage of highly expressed mRNAs was optimized for expression in different cell types; the data were most consistent with the notion that differences in codon usage in different gene sets were caused by chromosomal variations in GC content.

5 Potential Consequences Regarding the Use of Codon Optimization

Caution about the use of codon optimization approaches is justified since many diseases have been linked to synonymous codon changes [47, 48]. Studies that analyze the effects of synonymous mutations reveal that they affect gene expression at multiple levels that contribute to disease progression. These effects include aberrant splicing, reduced ribonucleoprotein complex formation, alterations to mRNA stability or half-life, and alterations to mRNA secondary structure. In addition, some mutations also affect protein folding, altering both conformation and functional properties, including altered substrate specificity and extracellular protein levels. A recent detailed analysis of a synonymous mutation identified in blood factor IX (F9) was shown to decrease expression and alter the conformation of the protein [49]. As noted by the authors, there are at least nine other synonymous mutations in F9 that are associated with hemophilia B.

Additional caution about the use of codon optimization is warranted since coding regions not only specify amino acid sequences, but often also contain overlapping genetic information which includes RNA secondary structures that can affect protein folding [50, 51].

As already mentioned, one of the major assumptions of codon optimization approaches is that “rare codons” are decoded more slowly than more commonly used codons, and are rate-limiting for translation. In mammals, data from a ribosome profiling study failed to support this assumption in an analysis of the kinetics of translation in mouse embryonic stem cells [52]. In this study, cells were treated with the drug harringtonine to stall new initiation events at start codons, and run-off elongation was monitored using ribosome profiling. This study found that translation speed was largely independent of codon usage. In addition, analysis of ribosome protected fragments corresponding to rare codons indicated that there was no evidence of ribosomal pausing at these codons or for a large effect of codon usage on the overall rate of elongation.

Based on the assumption that rare codons are rate limiting for translation, the corollary that replacing rare codons with more frequently used ones increases protein production is also unfounded. Although there are examples showing that substituting rare codons for major codons [53] or vice versa [54] increases or decreases expression, respectively, these examples are anecdotal and other examples do not support a strong correlation between the two variables.

It has been assumed that synonymous codons are interchangeable without affecting protein structure and function. However, numerous examples indicate that this is not necessarily the case, and the following examples detail recent reports of such instances. For example, replacing rare Arginine codons of a Tobacco Etch Virus Protease variant adversely affects both solubility and activity when expressed in bacterial hosts [55]. In another study, Zhou and colleagues noted that nonoptimal codons were preferentially used in intrinsically disordered regions, while optimal codons were found in structural domains [56]. However, targeted codon optimization of the predicted disordered, but not well-structured regions of the *Neurospora* circadian clock gene frequency (*frq*) impaired clock function and altered the structure of the FRQ protein. It has also been reported that synonymous codon variants in the gene encoding gamma-B crystallin resulted in different conformations as determined by 2D NMR spectroscopy leading to altered stability in vivo and protease resistance in vitro. Similarly, the coding sequences of the *Aspergillus tubingensis* raw starch hydrolyzing α-amylase and glucoamylase encoding genes were redesigned using synonymous codons expressed in *S. cerevisiae* [57]. This codon-optimization resulted in decreased extracellular enzymatic activity and altered glycosylation patterns. In addition, these proteins were shown to fold differently as demonstrated using differential scanning fluorimetry. A final example illustrates that codon optimization of vesicular stomatitis virus glycoprotein envelope, a key protein for lentiviral vector function, resulted in an increase in its expression. However, the expressed protein was not appropriately glycosylated, exported to cytosol, or processed, resulting in the functional impairment of this protein [58].

As we have previously noted an implicit assumption associated with codon-optimization approaches is that protein expression is limited to the expression of the full-length protein [3]. However, numerous examples demonstrate that translation can initiate from multiple start sites within mRNAs, resulting in peptides from both in-frame and out-of-frame open reading frames. In addition to the ribosome profiling studies discussed above [52], some of the best examples of alternative initiation comes from the immune system in which cryptic, antigenic peptides are produced and presented on the surface of cells by the Major Histocompatibility Complex 1 to mediate cellular immunity and assist in self-recognition, e.g., [59].

Some studies illustrate specifically how codon optimization can affect the expression of cryptic epitopes which may modulate an immune response. For example, codon optimization of the human papillomavirus E7 oncogene sequence introduced a cryptic epitope from an out-of-frame open reading frame that was not present in the wild type sequence [60]. Expression of this epitope induced a T cell response in a myelogenous leukemia line demonstrating that codon optimization rendered this sequence to be artificially immunogenic. In addition, Li and colleagues showed that a cryptic epitope expressed from an alternative open reading frame in the human coagulation factor F9 coding sequence induced an immune response when expressed in Mice [61]. Of note, a codon optimized variant of the F9 coding sequence did not encode this epitope and did not elicit an immune response.

The unintended generation of novel, cryptic peptides described above represents potential, but nonetheless real safety concerns for in vivo applications such as gene therapy, RNA therapeutics, or nucleic acid vaccines. In contrast to recombinant protein expression in ex vivo systems, these peptides cannot be separated from the full length, desired protein. As such, these peptides, if expressed in the "human bioreactor," can potentially exert their biological effects on the host.

In addition to cryptic peptides with immunogenic properties, other peptides may also be expressed that have unanticipated, undesirable functional activities. For example, one can imagine peptides having hormone or oncogenic activities. While this may be regarded as a theoretical consideration, it cannot be dismissed easily out-of-hand. Support for this notion comes from genetic selection studies in mouse cells to isolate proteins with amino acid sequences that do not exist in nature that possess a specific biological property [62]. Using this approach, the authors selected short proteins that induced oncogenic transformation of mouse cells. Although unlikely, studies such as these highlight the potential to generate peptides from novel out-of-frame coding sequences with functional activities that could arise from the seemingly infinite number of possible codon combinations for a given sequence.

The expression of cryptic proteins may also have implications for recombinant expression in ex vivo systems in that they may interfere with the host cell physiology, or may even have toxic effects that adversely affect protein yields, or prevent the establishment of stable expressing lines. For example, the biological activity of functional Granzyme B expressed in HEK293 cells induced apoptosis of the host cells preventing the establishment of stable expressing cells [63]. While this is an example in which the desired protein itself has deleterious properties, it is possible to envisage that cryptic peptides may also affect cells in other unintended ways, which may include interfering with metabolic pathways or inhibiting cellular proliferation.

Finally, codon optimization may also disrupt posttranscriptional modifications by eliminating sites of modification and creating new ones by affecting adenosine-to-inosine (A-to-I) editing. A-to-I editing is an important mechanism used by cells to generate multiple transcript isoforms by affecting splicing and microRNA binding, and may be used to optimize protein function in response to varying conditions [64]. Since inosine is recognized as guanosine by the translational machinery A-to-I editing within coding sequences can lead to amino acid substitutions. Consequently, the resulting protein will differ from that intended, and may affect its expression and functional activity.

A-to-I modifications are introduced by enzymes that bind double stranded regions and while efforts have been made to predict editing sites, they are largely insufficient due to the inability to accurately predict higher order structures in vivo, e.g., [65]. Consequently, altering codon use may also result in the disruption of existing sites and/or the generation of new sites which may affect amino acid sequence, posttranscriptional RNA processing, or microRNA binding sites that may have unanticipated consequences. Although this issue may affect the quality of a therapeutic expressed both in ex vivo and in vivo contexts, in vivo applications are particularly concerning inasmuch as various tissues have different levels of editing and there is less opportunity to fully characterize the expressed therapeutic protein.

In addition to the abovementioned potential effects of codon optimization on recombinant protein expression, there are other considerations associated with the expression of natural mRNAs in heterologous host cell lines, typically Chinese hamster ovary (CHO) or human embryonic kidney 293 (HEK293). Both cell lines are heteroploid and are likely to differ significantly in their tRNA pools from those in the tissues from which they were derived. These tRNA pools are also likely to differ from those of the cell types in which the recombinant protein is naturally expressed. Moreover, it is expected that additional variation will arise in individual clonal cell lines and may be further altered by culture conditions.

6 Closing Notes

Codon optimization has proven to be a powerful tool for the enhancement of recombinant protein synthesis and is based on the notion that protein synthesis is limited at the level of translation elongation. This idea is based on findings in various nonmammalian organisms but does not seem to apply to mammalian cells, where codon optimization can nevertheless sometimes still benefit expression by inadvertently affecting other mechanisms. However, the potential costs of increased expression in any system include altered protein conformation and function. In addition, for in vivo applications such as gene therapy, RNA therapeutics, or nucleic acid vaccines, there are additional potential safety risks, which include the production of novel cryptic peptides. As such, the use of codon optimization for recombinant protein expression in cultured cells or in vivo warrants careful consideration with respect to the potential risks compared to the potential benefit of this approach to increase expression, which is many cases is rather modest. However, for some applications, the potential risks may be less of an issue if the potential benefits clearly outweigh these risks, or if the protein products are not for human use, for example industrial enzymes.

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Chapter 19

Versatile Cell-Free Protein Synthesis Systems Based on Chinese Hamster Ovary Cells

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Abstract

We present an alternative production platform for the synthesis of complex proteins. Apart from conventionally applied protein production using engineered mammalian cell lines, this protocol describes the preparation and principle of cell-free protein synthesis systems based on CHO cell lysates. The CHO cell-free system contains endogenous microsomes derived from the endoplasmic reticulum, which enables a direct integration of membrane proteins into a nature like milieu and the introduction of posttranslational modifications. Different steps of system development are described including the cultivation of CHO cells, cell harvesting and cell disruption to prepare translationally active CHO cell lysates. The requirements for DNA templates and the generation of linear DNA templates suitable for the CHO cell-free reaction is further depicted to underline the opportunity to produce different protein variants in a short period. This experimental setup provides a basis for high-throughput applications. The productivity of the CHO cell-free systems is further increased by using a non-canonical translation initiation due to the attachment of an internal ribosomal entry site of the Cricket paralysis virus (CRPV IRES) to the 5′ UTR of the desired gene. In this way, a direct interaction of the IRES structure with the ribosome facilitates a translation factor independent initiation of translation. Cell-free reactions were performed in fast and efficient batch reactions leading to protein yields up to 40 μg/mL. The reaction format was further adjusted to a continuous exchange CHO cell-free reaction (CHO CECF) to prolong reaction time and thereby increase the productivity of the cell-free systems. Finally, protein yields up to 1 g/L were obtained. The CHO CECF system represents a sophisticated resource to address structural and functional aspects of difficult-to-express proteins in fundamental and applied research.

Key words Cell-free protein synthesis, CHO cells, Continuous exchange cell-free systems, Difficult-to-express proteins, In vitro protein production, Internal ribosomal entry sites, Linear expression templates

1 Introduction

Nowadays, protein synthesis plays a pivotal role in the pharmaceutical industry for the development and screening of novel therapeutical treatments and the production of drugs. There is an increasing demand of fast and efficient protein production platforms, while different cellular host organisms are conventionally

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used for the production of a target protein [1, 2]. One of the currently most relevant production hosts are Chinese hamster ovary (CHO) cells, a well-established, optimized, safe, and reliable production host for more than three decades [3, 4]. The development of appropriate cell hosts comprises a laborious and time-consuming developmental process and is limited for proteins, which show cytotoxic effects and folding issues during cellular overexpression. Membrane proteins and proteinogenic toxins belong to this class of so-called "difficult-to-express" proteins. Despite their challenging production process, these proteins harbor a high potential for pharmaceutical applications [5, 6]. In this context, novel protein productions platforms so called cell-free protein synthesis systems are continuously developed to provide a value-added technology. Cell-free protein synthesis system are typically based on cell lysates harboring the entire translational machinery in an active state [7, 8]. Until now, a broad range of cell-free protein synthesis systems has been developed differing in their cellular origin and their performance [9]. In general, the systems can be subdivided into prokaryotic and eukaryotic cell-free systems, while the selection of the production platform depends on the complexity of the desired target protein [10]. Eukaryotic cell-free protein synthesis systems are generally used for the production of pharmaceutically relevant and complex mammalian proteins. Special eukaryotic cell-free systems contain endogenous microsomes derived from the endoplasmic reticulum due to a mild cell disruption procedure during lysate preparation. Microsomes enable an integration of membrane embedded and associated proteins into a nature-like milieu, a cotranslational translocation of proteins as reported in cellular protein expression processes and they harbor a fully ER based set of enzymes for folding and posttranslational modifications including relevant glycosyltransferases for attachment of glycan moieties, chaperones and essential molecules for the introduction of disulfide bridges [11–14]. In order to combine a mammalian host cell often used for the production of versatile, mammalian proteins and a cell-free platform, we have recently developed novel systems based on the prominent industrial protein production host CHO cells [15, 16]. This platform provides a user-friendly system completing the classical industrial protein production pipeline. The fast, efficient, and high-throughput compatible protein synthesis tool promotes DNA template pre-evaluation as well as production of difficult-to-express proteins [17]. To realize these demands a coupled transcription/translation system is developed containing endogenous microsomal structures for posttranslational modifications and cotranslational membrane protein integration. To address individual applications, the system is adapted to different reaction modes including a fast batch based [18] and a highly productive continuous exchange cell-free system (CECF) leading to protein yields up to 1 g/L [19]. A concept is proposed for the application of linear DNA templates apart from circular DNA to expand the platform by a fast and cloning-free DNA template generation process.

This chapter includes an overview covering the entire process pipeline for the development of CHO cell-free systems. In this protocol, we present the preparation of cell-free systems containing the cultivation of CHO cells, the preparation of translationally active lysate, the generation of linear expression templates and finally the cell-free production of proteins using batch and CECF formatted systems.

2 Materials

2.1 Cell Cultivation

1. CHO-K1 cells are purchased from a cell bank (DSZM, Germany) and adapted to serum-free suspension cultivation (*see* **Note 1**).
2. For the initial cultivation of the CHO cells, shake flasks (Corning) of different sizes (from 125 mL up to 1 L) are used.
3. Cell density and viability are evaluated using a Thoma cell counting chamber and trypan blue staining.
4. CO_2-Incubator Multitron (Infors) for cultivation of CHO cells in shake flasks.
5. Conventional stirred tank bioreactors in a scale of 5 L (Biostat B-DCUII, Sartorius Stedim Biotech GmbH) up to 30 L (Biostat D-DCU) including an aeration basket for bubble-free aeration (*see* **Note 2**). An internal spin filter is implemented to enable perfusion based fermentation.
6. Diverse sensor units for online monitoring of cell cultivation process: pH probe, pO_2 probe, level sensor (Hamilton and Sartorius Stedim Biotech GmbH).
7. MFCS SCADA software (bioprocess control) (Sartorius Stedim Biotech GmbH) (*see* **Note 3**).
8. 1 M Sodium carbonate solution (Sigma-Aldrich) for the adjustment of pH during fermentation.
9. Chemically defined PowerCHO2CD medium (Lonza) supplemented with 6 mM stabilized glutamine (Sigma-Aldrich).
10. Wash and resuspension buffer: 40 mM HEPES-KOH (pH 7.5), 100 mM NaOAC and 4 mM DTT. Store at 4 °C.
11. Light microscope BX40 and Cell D Software (Olympus) (*see* **Note 4**).

2.2 Cell Disruption and CHO Lysate Preparation

1. Wash buffer (*see* Subheading 2.1, **item 9**).
2. Syringe with 20-gauge needle or high-pressure homogenizer.
3. Nanodrop 2000c (Thermo Scientific).
4. Sephadex G-25 columns (NAP 25 or Äkta Prime (GE-Healthcare)). Stored at 4 °C.
5. S7 Nuclease (Roche) (200 U/μL). Stored at −20 °C.
6. 80 mM $CaCl_2$ and 80 mM EGTA dissolved in water and stored at 4 °C.
7. Creatine kinase dissolved in water (1 mg/mL) and stored at −20 °C.
8. Baker's yeast tRNA (17.5 μg/mL) dissolved in water and stored at −80 °C.
9. High speed centrifuge (Beckmann Coulter) for cell harvesting.

2.3 Generation of Suitable Linear and Circular DNA Expression Templates

1. Sequence of the gene of interest (GOI).
2. Regulatory sequence donor vector containing the 5′-terminal regulatory sequences necessary for CHO cell-free synthesis (*see* **Note 5**).
3. T7 RNA polymerase promotor (5′ UTR) and terminator (3′ UTR) sequence.
4. Sequence of the Cricket paralysis virus (CRPV) IGR IRES (Genbank accession no. AF218039, nucleotides 6025–6216).
5. Gene specific primer pairs for the amplification of the gene of interest and the attachment of regulatory overlap sequences (*see* **Note 6**).
6. Regulatory adapter primers (Reg-F, Reg-R, CRPV-R) for the introduction of required regulatory sequences.
7. PCR thermocycler (Bio-Rad).
8. HotStar HiFidelity DNA Polymerase and appropriate buffer including dNTPs (Qiagen) (*see* **Note 7**).
9. Perfect Blue Gel System and universal Agarose (Peqlab).
10. Rotiphorese 10× TBE-buffer (Carl Roth).
11. DNA Stain Clear G (SERVA).
12. 2-log DNA ladder 0.1–10 kb (NEB).
13. EasyXpress pIX3.0 cell-free expression vector backbone for direct cloning of generated PCR products into a circular plasmid applicable for cell-free protein synthesis (Biotechrabbit) (*see* **Note 8**).
14. Plasmids including the gene of interest suitable for cell-free protein synthesis (Geneart Gene Synthesis service (Thermo Fisher Scientific) or BioCat).

15. Electroporation device and cuvettes (Biorad).
16. E.coli JM109 strain (New England Biolabs).
17. LB media (Carl Roth).
18. Antibiotics (Sigma-Aldrich).
19. PureLink HiPure Plasmid Prep Kit (Thermo Fisher Scientific).
20. Restriction enzymes for the verification of target gene integration into a plasmid.
21. Nanodrop C for the estimation of plasmid DNA concentration.
22. Primers for DNA sequencing: M13-F5′ GTA AAA CGA CGG CCA GTG3′, M13-R 5′ CAG GAA ACA GCT ATG AC 3′.

2.4 Batch Formatted CHO Cell-Free Synthesis of Proteins

1. 1.5 mL reaction tubes.
2. Thermomixer comfort (Eppendorf).
3. Previously prepared CHO lysate. Store at −80 °C.
4. Translation buffer (10×): 300 mM HEPES-KOH (pH 7.6), 2250 mM KOAc, 2.5 mM spermidine, 25 mM of DTT, 1 mM of each of the 20 canonical amino acids (Merck) and 39 mM $Mg(OAc)_2$. Store at −80 °C (*see* **Note 9**).
5. Energy buffer (5×): 100 mM creatine phosphate, 8.75 mM ATP, 1.5 mM CTP, 1.5 mM UTP, 1.5 mM GTP (Roche), and 0.5 mM $m^7G(ppp)G$ cap analogue. Store at −80 °C (*see* **Note 9**).
6. T7 RNA polymerase (3 U/μL) for performing the transcription reaction in the transcription-translation coupled system. Store at −20 °C.
7. ^{14}C Leucine (PerkinElmer) (final concentration of 66.67 dpm/pmol) for radiolabeling of proteins. Store at −20 °C.
8. Linear expression template or plasmid DNA containing the GOI. Store at −20 °C.

2.5 Continuous Exchange Cell-Free Synthesis Based on CHO Lysate

1. Two chamber dialysis device (Membrane cut-off: 10 kDa, 50 μL reaction chamber, 1000 μL feeding chamber) (Biotech Rabbit).
2. Thermomixer C (Eppendorf).
3. Translationally active CHO lysate. Store at −80 °C (*see* **Note 9**).
4. Translation buffer (10×) (*see* Subheading 2.5, **item 4**).
5. Energy buffer: Energy buffer (5×) containing the following components: 92.5 mM creatine phosphate, 8.75 mM ATP, 1.5 mM CTP, 1.5 mM UTP, 1.5 mM GTP (Roche). Store at −20 °C.
6. T7 RNA polymerase (1 U/μL) for performing the transcription reaction in the transcription-translation coupled system. Store at −20 °C.

7. Caspase inhibitor AC-DEVD CMK (Santa Cruz Biotechnology) diluted in water (*see* **Note 10**).
8. 2500 mM $Mg(OAc)_2$ in water. Store at −20 °C.
9. ^{14}C Leucine (PerkinElmer) (final concentration of 66.67 dpm/pmol) for radiolabeling of proteins. Store at −20 °C.
10. Linear expression template or plasmid DNA containing the GOI. Store at −20 °C.

3 Methods

3.1 Biomass Production

1. CHO-K1 cells are initially cultured in shake flask starting with a density between 0.5 and 0.7 × 10^6 cells/mL. Cultivation conditions are set to a temperature of 37 °C, a CO_2 concentration of 5% and a shaking speed of 100 RPM.
2. For biomass production the stirred tank bioreactor is inoculated with CHO-K1 cells precultured in shake flasks at an initial density of around 1.0 × 10^6 cells/mL.
3. The fermentation process can be performed in repeated batch or perfusion mode. The repeated batch process included a cell growth phase of approximately 2 days to obtain cell densities between 4 and 5 × 10^6 cells/mL grown in the exponential growth phase, which is followed by the cell harvest.
4. A defined volume of cell suspension remains in the cultivation vessel to start a second cycle of batch cultivation. The repeated batch is performed for 3 cycles.
5. To increase the produced biomass, a perfusion fermentation process can be applied to the CHO cell lysate preparation protocol. For this, CHO cells are inoculated at a density of 1 × 10^6 cells/mL, a batch based process is performed until a density between 3–4 × 10^6 cells/mL is reached and perfusion mode is started using a perfusion rate of 0.5 RV/day (RV: reactor volume).
6. Exponentially grown CHO cells can be harvested at different cell densities during the perfusion process without the loss of translational activity of CHO cell lysate (Fig. 1).

3.2 Cell Harvest, Disruption and Preparation of Translationally Active CHO Cell Lysates

1. Exponentially grown CHO cells are harvested by centrifugation using a fixed angle rotor or a continuous flow rotor depending on the volume of cell suspension.
2. After harvesting cells are washed with HEPES based wash buffer (*see* Subheading 2.1, **item 9**) to remove culture medium components.
3. Before cell disruption, CHO cells are suspended in wash buffer to obtain a final density of approximately 1.0 × 10^8 cells/mL.

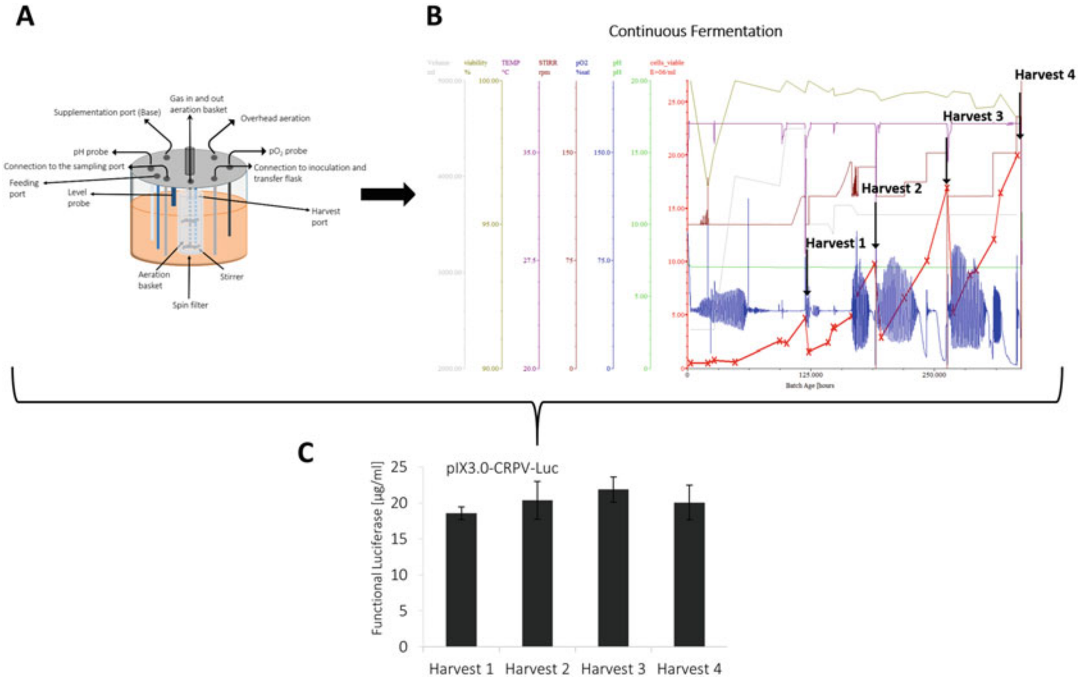

Fig. 1 Application of continuous fermentation for CHO biomass production used for the preparation of CHO cell-lysates. CHO cells are cultured in a 5 L cultivation vessel connected to a Biostat B-DCU II regulation unit (Sartorius Stedim). Different regulation and monitoring systems are connected to the fermentation vessel including a level probe, pH sensor, and pO_2 sensor as illustrated in (**a**). Continuous cultivation is realized by the introduction of a spin filter for cell retention and implementation of several pumps to enable feeding with fresh culture medium and removal of inhibitory byproducts. The perfusion process is started at a cell density of around 4×10^6 cells/mL and a perfusion rate of 0.5 reactor volumes/day. Fermentation parameters are monitored using MFCS SCADA software (**b**). CHO cell densities and viabilities are estimated by a Thoma counting chamber and trypan blue staining. Cells are harvested at different cell densities to evaluate the possibility to use high cell-density cultures for production of translationally active CHO cell lysate. Times of harvest are indicated by black arrows. (**c**) shows the quality of the produced CHO cell lysate analyzed using batch formatted cell-free protein synthesis. Quality control was based on the cell-free protein synthesis of model protein luciferase (DNA template: pIX3.0-CRPV-Luc) and analysis of luciferase activity using a standard luciferase assay (Promega) and luminescence detection using a multimode microplate reader (Berthold). Protein yield was calculated by application of a previously prepared calibration curve. Error bars indicate one standard deviation ($n = 6$, harvests 1 and 4; $n = 12$, harvests 2 and 3). No significant differences in luciferase activity were detected using CHO cell lysates harvested at different cell densities showing the possibility of using high cell-density cultures for lysate production

4. For preparation of translationally active CHO lysate, which contains endogenous microsomes, a mild disruption procedure is required. Therefore, CHO cells are disrupted mechanically by passing the suspended cell pellet through a 20-gauge needle or disruption using high-pressure homogenization (*see* **Note 11**).

5. A raw CHO lysate is further prepared by differential centrifugation at 10,000 × *g*, 4 °C, for 10 min. This step is required for

the removal of cell debris, cell nuclei and cellular organelles (*see* **Note 12**). Raw lysate can be flash frozen using liquid nitrogen and stored at −80 °C prior to further recondition steps.

6. Raw lysate is applied to a preequilibrated Sephadex G-25 column and eluted using wash buffer (*see* Subheading 2.1, **item 9**). Fractions of 1 mL are collected and RNA concentration is quantified by absorbance estimation using the NanoDrop 2000C. Fractions displaying an absorbance above 1 at 260 nm are pooled.
7. Pooled fractions are treated with micrococcal S7 nuclease (10 U/mL) to digest and remove endogenous mRNA. The S7 nuclease has a calcium dependent digestion activity, therefore 1 mM of $CaCl_2$ is added to the enzyme containing eluate and incubated for 2 min at room temperature.
8. The enzymatic reaction is inactivated using the Ca^{2+} chelating regent EGTA (6.7 mM final concentration).
9. The energy regenerating enzyme creatine kinase (100 μg/mL final concentration) is added to the lysate to enable the regeneration of ATP using creatine phosphate as a phosphate donor.
10. The final translationally active CHO lysate is flash frozen and stored at −80 °C (*see* **Note 13**).

3.3 Generation of Linear Expression Templates

The application of linear DNA templates to the cell-free reaction offers a fast, flexible and cost-efficient approach to produce diverse proteins with different modifications, signal peptides and protein tags. To obtain a linear DNA template suitable for CHO cell-free protein synthesis, a two-step PCR procedure needs to be performed to add the required regulatory sequences to a target gene. A general overview of the procedure is illustrated in Fig. 2.

1. The gene of interest is amplified using gene specific adapter primers harboring an overlap to the 5′- and 3′- regulatory sequences.
2. The 5′- regulatory sequences, which contains a stem loop for stabilization, the T7 RNA promotor and the CRPV IGR IRES, are amplified using a regulatory sequence donor vector, Reg-F and CRPV-R primer.
3. Subsequently, the presence of both PCR products is verified on agarose gel prior to their fusion and the generation of the final linear expression template (*see* **Note 14**).
4. Fusion PCR is performed by application of both previously generated DNA fragments (gene fragment and 5′- regulatory fragment) in an equimolar amount to the reaction and supplementation of regulatory primers (Reg-F and Reg-R). A high-fidelity DNA dependent polymerase is used for DNA

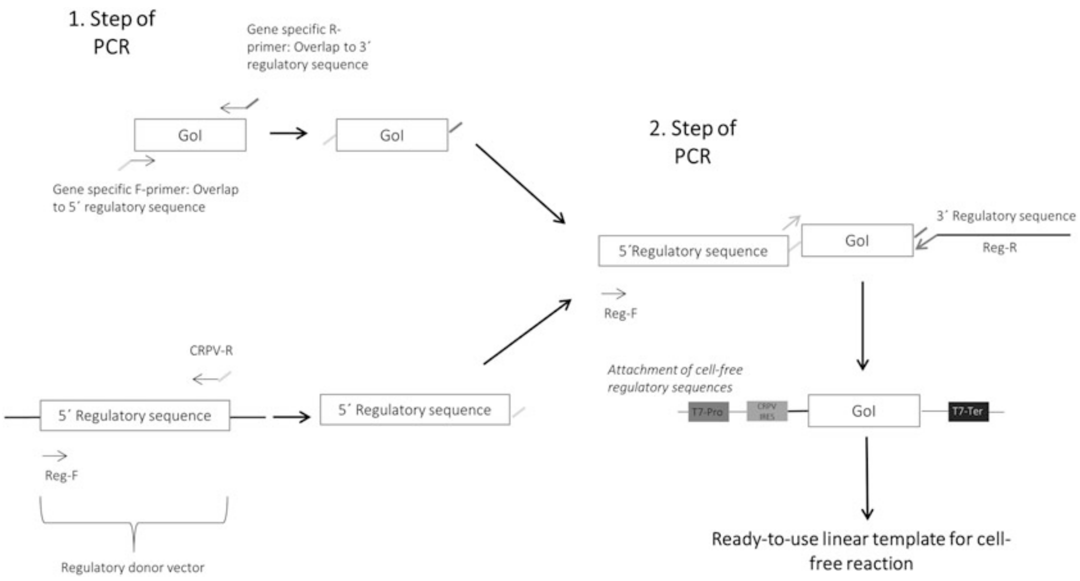

Fig. 2 Schematic overview of linear expression template generation for CHO cell-free synthesis. A two-step procedure is used for the generation of cell-free compatible linear DNA templates. The first step includes the amplification of the gene of interest (GOI) using gene-specific primer pairs. This step includes the fusion of overlap sequences to 5′- and 3′- regulatory sequences. Additionally, the 5′-regulatory sequence including stem loops for stabilization, the T7 RNA polymerase promotor for initiation of transcription and the Cricket paralysis virus IGR IRES for translation initiation factor independent translation initiation, is amplified using a regulatory donor vector and the Reg-F and CRPV-R primers. Both PCR products are fused during the second step of PCR using the Reg-F primer, and 3′-terminal regulatory sequences are added to the GOI by application of the Reg-R primer. After the second step, a ready-to-use linear DNA template for the CHO cell-free reaction is prepared

amplification to enable a high correctness of produced DNA sequence by low misincorporation rates and proof reading activities.

5. The produced linear PCR fragment is analyzed on an agarose gel prior to the application to the cell-free reaction (*see* **Note 15**).
6. Different signal peptides, purification tags and fluorescence tags can be integrated into the DNA sequences by the previously described procedure. For this, overlap sequences and regulatory primer pairs need be adapted to the individual requirements.

3.4 Preparation of Circular Plasmid Templates

1. Transformation of the desired plasmid template into *E. coli* JM109 cells is performed by electroporation using a 1 mM cuvette and a Biorad electroporation system.
2. Transformed cells are plated on agar-antibiotic plats for *E. coli* clone growth. Transformed *E. coli* cell stocks are stored in glycerol at −80 °C.

3. *E. coli* cell clone is picked and inoculated into a shake flask containing LB medium and the appropriate antibiotics and cultured overnight at 37 °C and 120 RPM.
4. Amplified plasmid DNA is isolated from *E. coli* overnight culture using the PureLink HiPure Plasmid Prep Kit (*see* **Note 16**).
5. Concentration of isolated plasmid DNA was analyzed at an absorbance of 260 nm using the Nanodrop 2000 C. A 260/280 purity value around 1.8 is required for further applicability of the plasmid DNA to a cell-free protein synthesis reaction.
6. Gene integration is evaluated by treatment of isolated plasmid DNA with restriction enzymes and separation of obtained DNA fragments by agarose gel electrophoresis. DNA fragment size is estimated using Clone Manager Software.
7. The DNA sequence is further verified by gene sequencing using M13-F and M13-R primers.

3.5 Cell-Free Synthesis in a Batch-Formatted Reaction

1. All required components for cell-free protein synthesis are thawed on ice prior to their use in the reaction (*see* **Note 17**).
2. Batch formatted CHO cell-free reactions are performed in Eppendorf tubes containing reaction volumes from 20 μL up to 500 μL. Reports of prokaryotic cell-free systems show the possibility for process scale up to 100 L reaction volume [20].
3. To prevent RNase contamination, CHO cell-free reactions are pipetted using filter tips.
4. The preparation of the cell-free CHO batch reaction is performed on ice. The general principle of the CHO lysate based cell-free protein synthesis is illustrated in Fig. 3a.
5. The transcription/translation coupled CHO cell-free batch reaction is composed of 40% CHO cell lysate, 10% translation buffer and 20% energy buffer.
6. Typically, 20 nM of plasmid DNA is added to the reaction but the optimal concentration depends on the applied vector backbone (*see* **Note 18**).
7. To enable transcription of mRNA, 1 U/μL T7 RNA polymerase are supplemented to the CHO cell-free reaction.
8. For further analysis of produced proteins, ^{14}C leucine (specific radioactivity: 66.67 dpm/pmol) is added to the reaction and cotranslationally incorporated into the synthesized proteins.
9. Reaction volume is adjusted by supplementation with Millipore water to the final volume.

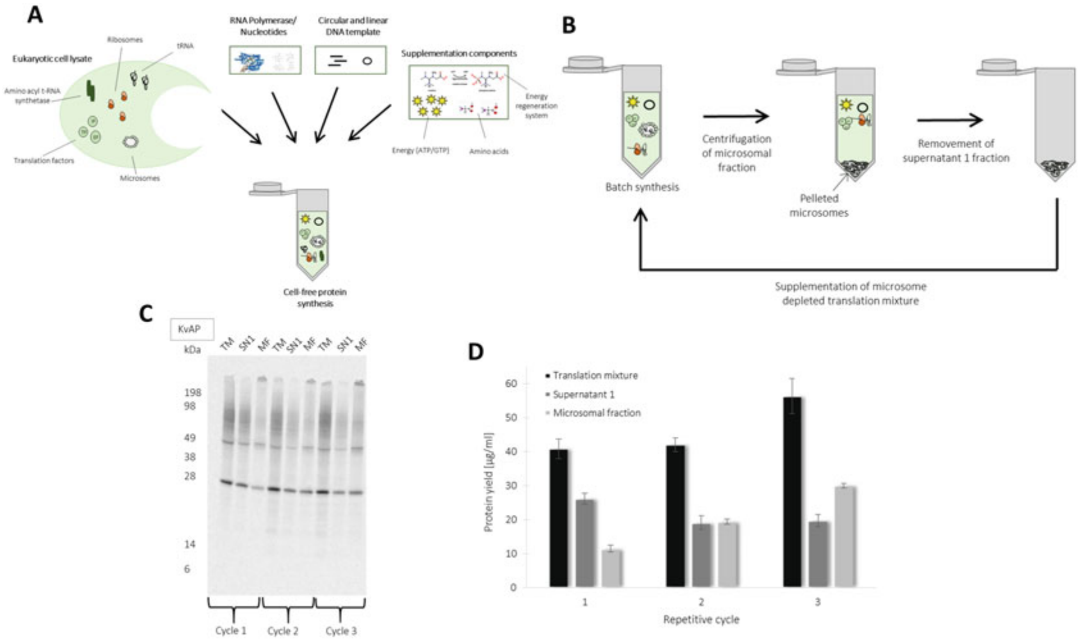

Fig. 3 Batch formatted cell-free synthesis based on CHO cell lysates. (**a**) Schematic overview of CHO cell-free protein synthesis using batch mode. The batch formatted cell-free protein synthesis system consists of the CHO cell lysate, T7 RNA polymerase, nucleotides, a DNA template containing the GOI, and supplementation components. The exact protocol is described in Subheading 3.5. The translation mixture can be separated into ER derived microsomes and a supernatant 1 fraction by centrifugation (16,000 × *g*, 4 °C, 15 min). The microsomal fraction contains proteins, which are cotranslationally translocated into the microsomes by signal peptide SRP-complex interaction. The microsomes enable direct integration of membrane proteins into a natural milieu having ER based posttranslational modifications. (**b**) describes a repetitive translation of proteins into the microsomes for enrichment of secretory and membrane embedded proteins. For this, a general cell-free reaction is performed. After finishing the reaction, microsomes are separated by centrifugation and supernatant 1 is removed. Previously addressed microsomes are supplemented with a freshly prepared translation mixture depleted of microsomes. An example for an enrichment of a membrane protein is depicted in (**c**) and (**d**). For this, the ion channel KvAP is synthesized into the microsomes using three repetitive protein production cycles in the batch formatted CHO cell-free system. (**c**) shows an autoradiograph of translations mixture (TM), supernatant 1 (SN1), and microsomal fraction (MF) of the different repetitive cycles. Appropriate monomer (30 kDa) and dimer (60 kDa) bands of KvAP were detected. The quantification of protein yield by TCA precipitation followed by scintillation measurement showed an increase of protein yield in the microsomal fraction from around 11 μg/mL to approximately 30 μg/mL (**d**)

10. Optimization experiments included a further increase of T7 RNA polymerase to 3 U/μL and the supplementation of molecular crowding reagents (PEG, Ficoll) (*see* **Note 19**).
11. All components were pipetted directly to the reaction while adjusting the volume by adding ddH20. Standard conditions present in a batch-formatted CHO cell-free reaction are summarized in Table 1.
12. Ready prepared cell-free translation mixture is incubated for 3 h at 30 °C and 600 RPM using a thermomixer.

Table 1
List of standard reaction conditions present in a batch-formatted CHO cell-free system: optimized conditions are indicated in bold letters

Component/Parameter	CHO batch reaction conditions
Cell lysate	40% (vol/vol)
HEPES KOH, pH 7.6	30 mM
KoAc	150 mM
$Mg(OAc)_2$	3.9 mM
ATP	1.75 mM
CTP,GTP,UTP	0.3 mM
$m^7G(ppp)G$ cap (cap I)	0.1 mM
Amino acids	100 μM
^{14}C Leucine	66.67 dpm/pmol
Plasmid concentration	20 nM
T7 RNA polymerase	1 U/μL (**3 U/μL**)
DTT	2.5 mM
PEG 1500	**2%**
Temperature	30 °C
Agitation	600 RPM
Reaction time	3 h

13. The CHO cell-free translation mixture can be directly used for further analysis of the produced protein. Cell-free synthesized proteins can be applied to various assays. Radio labeled proteins can be quantified using TCA precipitation followed by scintillation measurement.
14. Molecular weight and protein modifications can be analyzed by separation of acetone-precipitated proteins on SDS-PAGE followed by autoradiography.
15. Figure 3 shows exemplary CHO cell-free synthesis of membrane protein and ion channel KvAP. Functional membrane proteins are integrated into the ER derived microsomes of CHO lysate. Microsomes can be separated by centrifugation (16,000 × *g*, 4 °C, 15 min). Proteins integrated into the microsomes can be enriched by a repetitive addressing procedure. For this, a batch based cell-free reaction is initially performed followed by the separation of microsomal fraction. The separated microsomes are dissolved in a freshly prepared cell-free reaction depleted from microsomes, which enables the

enrichment of translocated proteins into microsomes. This procedure is performed for three repetitive cycles. A schematic overview is illustrated in Fig. 3b. The produced membrane protein KvAP shows the formation of monomeric and multimeric structures in the autoradiograph (Fig. 3c). An increase in produced membrane protein is detected in the microsomal fraction (Fig. 3d) (*see* **Note 20**).

3.6 High-Yield Production of Proteins Using a Continuous Exchange Cell-Free System

1. As reported for the batch formatted cell-free reaction, all components are thawed on ice prior to their use in the reaction (*see* **Note 17**).
2. The preparation of CECF reaction is performed on ice.
3. The CHO continuous exchange cell-free reaction is performed in a two chamber device consisting of a reaction chamber (50 μL) and a feeding chamber (1000 μL). A general scheme of CHO CECF reaction is depicted in Fig. 4a**.** The reaction chamber contains a general cell-free reaction, the feeding chamber includes additional energy components. Both chambers are separated by a semipermeable membrane and due to diffusion gradients energy components are continuously delivered to the reaction chamber and inhibitory byproducts are removed from the reaction chamber. Thereby, protein production time is prolonged resulting in an increased protein yield. Analysis of reactor membrane cut-off by CECF synthesis of membrane protein EGFR fused to a C-terminal eYFP, results in an optimal protein yield using a membrane cut-off between 10 and 14 kDa in comparison to smaller cut-offs (3–8 kDa) (Fig. 4b) (*see* **Note 21**).
4. Feeding and reaction mix are prepared separately.
5. The feeding mix consists of 1× translation buffer and 1× energy buffer harboring the conditions described in Table 2. Additionally, 0.02% sodium azide (*see* **Note 22**), caspase inhibitor Ac-DEVD-CMK (30 μM), ^{14}C leucine (9.9 dpm/pmol), and ddH_2O (filling up to 1000 μL) are added to complete the feeding mixture.
6. The reaction mix is then prepared. The optimized conditions of the reaction mix differ from the standard CHO cell-free batch reaction and are illustrated in Table 2. Essential parameters which differ from the standard conditions are the concentration of Mg(OAC) (22 mM), creatine phosphate (18.5 mM), caspase inhibitor Ac-DEVD-CMK (30 μM), plasmid concentration (30 nM),and ^{14}C leucine (9.9 dpm/pmol).
7. The reaction mixture is transferred into the dialysis device followed by the feeding mix.

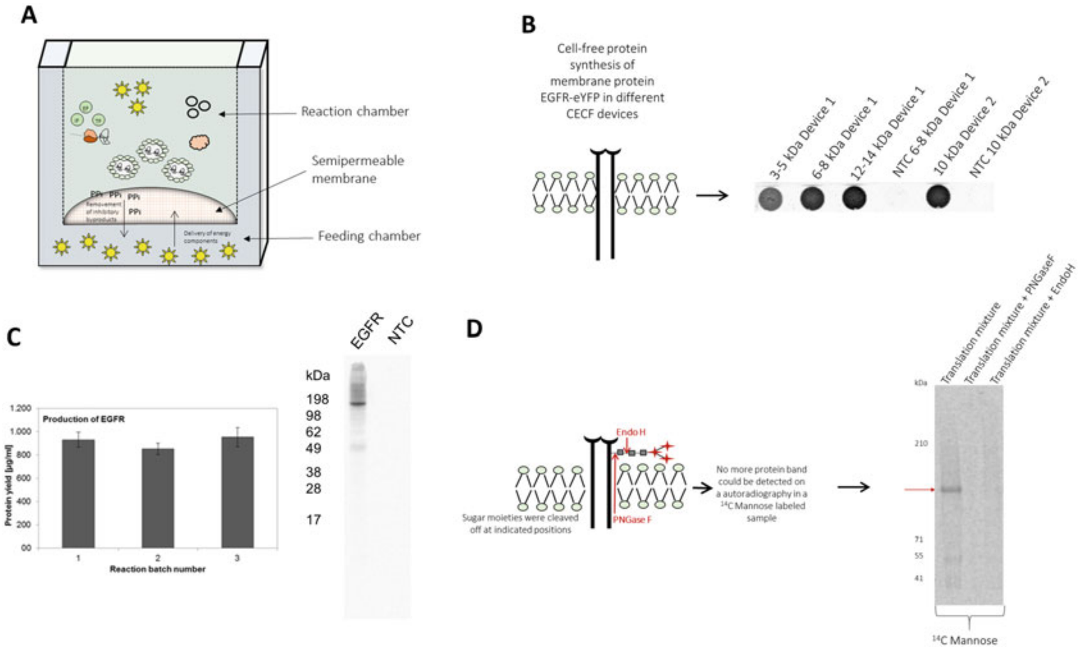

Fig. 4 Continuous exchange cell-free synthesis based on CHO cell lysates. (**a**) Schematic overview of the general principle of the CHO CECF reaction. The reaction vessel is divided into a reaction chamber and a feeding chamber, separated by a semipermeable membrane. The membrane protein EGFR fused to a C-terminal eYFP is used as a model protein for CHO CECF synthesis. The cut-off of the dialysis membrane is analyzed using different dialysis devices (device 1 (Scienova); device 2 (RiNA GmbH)). Fluorescence intensity of the produced EGFR-eYFP fusion protein was analyzed on µ-Ibidi-Slides using a multimode imager (Typhoon Trio Plus, GE Healthcare). An increase in protein fluorescence was achieved using a cut-off between 10 and 14 kDa compare to a smaller cut-off (3–8 kDa) (**b**). For further experiments, device 2 was used. After optimizing the reaction conditions (Table 2), protein yields of approximately 1000 µg/mL of "difficult-to-express" protein EGFR were obtained in different reaction batches (**c**). The quantification of protein yield was performed by TCA precipitation followed by scintillation measurement. An appropriate protein band is detected on the autoradiograph, while no band is detected in the sample containing no DNA template (NTC). The glycosylation of the protein was verified by the supplementation of ^{14}C mannose during the CHO CECF reaction and further analysis by autoradiography (**d**). Glyco residues were removed by EndoH and PNGaseF treatment to underline the specificity of the glycosylation band

8. The device is sealed with plastic foil and placed into a thermomixer C for incubation at 30 °C, 600 RPM and 48 h.
9. After finishing the CECF synthesis, the sealing is removed and reaction mixture is transferred into an Eppendorf tube for further analysis.
10. Like reported for the batch based cell-free reaction, reaction mixture can be directly used for the analysis of the produced proteins. Different batches of CECF synthesized membrane protein EGFR were quantified and analyzed using autoradiography (Fig. 4c).

Table 2
List of standard reaction conditions present in a CHO CECF system

Component/Parameter	CHO CECF reaction conditions	Reaction mix	Feeding mix
Cell lysate	40% (Vol/Vol)	×	
HEPES KOH, pH 7.6	30 mM	×	×
KoAc	150 mM	×	×
$Mg(OAc)_2$	3.9 mM		×
$Mg(OAc)_2$	22 mM	×	
ATP	1.75 mM	×	×
CTP,GTP,UTP	0.3 mM	×	×
Amino acids	100 μM	×	×
^{14}C Leucine	9.9 dpm/pmol	×	×
Sodium azide	0.02%	×	
Plasmid	30 nM	×	×
T7 RNA polymerase	1 U/μL	×	
DTT	2.5 mM	×	×
Caspase inhibitor Ac-DEVD-CMK	30 μM	×	×
Temperature	30 °C	×	×
Agitation	600 RPM	×	×
Reaction time	48 h	×	×

11. The quantification was performed using 3 μL of reaction mixture for hot TCA precipitation and further analysis by scintillation measurement. Typically, protein yields of approximately 1000 μg/mL are obtained. An autoradiograph of EGFR shows the expected molecular weight at around 170 kDa.
12. Protein glycosylation can be monitored by adding 3 nM ^{14}C mannose to the CHO CECF reaction.
13. After finishing cell-free protein synthesis, ^{14}C mannose labeled sample is acetone precipitated and can be directly applied to SDS-PAGE.
14. The dried gel is analyzed by autoradiography.
15. For the purpose of verifying the specificity of protein glycosylation, a treatment of CHO CECF sample using glycosidases PNGaseF (NEB) and EndoH (NEB) is performed according to the manufacturer's protocol. The results show the specificity of EGFR glycosylation performed in the CHO CECF system (Fig. 4d).

3.7 Application of Linear Expression Templates to CHO Cell-Free Systems

The application of linear expression templates to a CHO cell-free system offers new possibilities for fast and easy template generation and modification. This procedure is mandatory for cloning-free high-throughput applications and the development of novel screening technologies for the production and analysis of difficult-to-express proteins.

1. For the application of PCR products to the CHO cell-free reactions, standard batch formatted as well as CECF reactions are performed. Plasmid DNA is replaced by previously prepared linear expression template.
2. The productivity of batch cell-free systems based on linear expression templates are strongly dependent on the concentration of applied PCR-product. Figure 5a shows the dependency of PCR product concentration on the received protein yield in a batch formatted CHO cell-free reaction for the production of secreted protein EPO. A maximum protein yield is obtained by the application of 12.5 ng/μL linear expression template to the batch formatted CHO cell-free protein synthesis system.

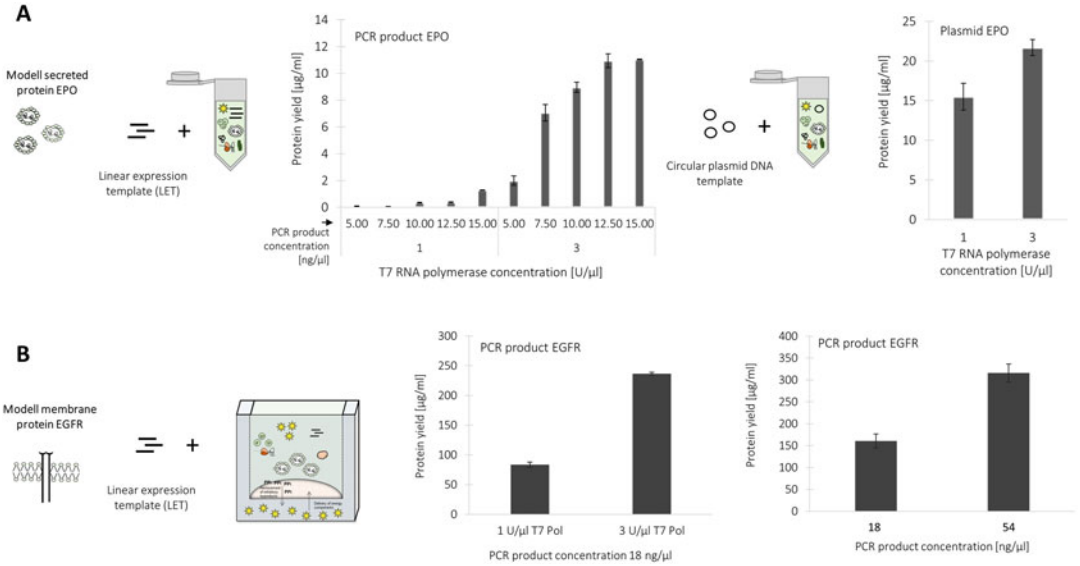

Fig. 5 Application of linear expression DNA templates to CHO cell-free protein synthesis systems. (**a**) Linear expression templates (LET) of secreted protein EPO were applied to a batch formatted CHO cell-free protein synthesis system. An increase of protein yield was detected by increasing the PCR product and T7 RNA polymerase concentrations. Protein yields up to approximately 12 μg/mL were obtained as compared to 22 μg/mL using a plasmid DNA template. (**b**) Linear expression templates of membrane protein EGFR were applied to a CHO CECF system. An influence of T7 RNA polymerase and PCR product concentrations on the productivity of the protein was detected. An increase of T7 RNA polymerase and PCR production lead to protein yields up 350 μg/mL of EGFR using a PCR product as a DNA expression template. Protein yields were derived by TCA precipitation of radio labeled proteins followed by scintillation measurement

3. An increase in T7 RNA polymerase concentration from 1 U/μL to 3 U/μL results in a significant increase in protein yield in the batch system. Protein yields up to approximately 12 μg/mL of secretory EPO were obtained by a combined increase of T7 RNA polymerase and PCR product concentration (Fig. 5a) (*see* **Note 23**).
4. The application of linear DNA templates to the CHO CECF system requires an increase of T7 RNA polymerase and PCR product concentration as reported for the batch formatted system. A maximum protein yield of 350 μg/mL of membrane protein EGFR was obtained using 3 U/μL and 54 ng/μL of linear PCR product.

4 Notes

1. Serum-free and chemically defined media are used for the cultivation of CHO cells prior to their utilization in lysate production procedure. This enables low batch-to-batch variation and therefore a high reproducibility in the cultivation step for the subsequent production of translationally active CHO cell lysates.
2. Aeration and stirring conditions in the bioreactor for the production of CHO cell biomass are adapted to obtain a stress reduced cultivation procedure. Induction of cell stress response can lead to inactivation of translation factors due to kinase dependent phosphorylation reactions and activation of the cell death response including the overexpression of caspases, a class of cysteinyl-aspartate specific proteases that reportedly cleave translation factors thereby reducing the productivity of the protein translation machinery.
3. All cultivation relevant parameters were directly implemented into MFCS SCADA bioprocess software. Thereby growth relevant values can be directly monitored to estimate the point of harvest of the biomass for lysate production. CHO cells harvested in the late log phase resulted in the highest translational activity of produced cell lysates.
4. Cultured CHO cells were microscopically analyzed during cell cultivation, after harvesting and washing procedures, and after cell disruption to verify cell stress response by the morphology of cells and the degree of cell disruption.
5. The regulatory sequence donor vector contains the 5′ regulatory sequences required for transcription and translation reaction including the Cricket Paralysis Virus IGR IRES and the T7 RNA polymerase promotor.

6. Regulatory overlap sequences consist of 10–15 base pairs complementary to the 3′ end of the CRPV IGR IRES for the N-terminal gene specific primer and a 10 base pair overlap to the T7 terminator for the C-terminal gene specific primer.
7. The HotStar HiFidelity DNA polymerase is a hot-start proofreading enzyme, which enables the amplification of PCR-products with a minimized rate of mutation incorporation. Factor SB in the PCR buffer prevents degradation of primers and DNA-template during PCR.
8. The EasyXpress pIX3.0 cell-free expression vector backbone contains regulatory N- and C-terminal sequences for the application in various prokaryotic and eukaryotic cell-free protein synthesis systems.
9. Repeated freeze/thaw cycle (up to 5 times) does not affect the quality of the translation and energy buffers. Ready prepared CHO lysate shows no loss of activity by three repeated freeze/thaw cycles.
10. The caspase inhibitor is initially dissolved in DMSO to increase its stability during freezing and storage. For the application to the cell-free synthesis reaction, the caspase inhibitor is further diluted in water.
11. The degree of cell disruption is monitored by microscopic imaging. If a high degree of intact cells is present after initial disruption, a repetition of the cell disruption step is performed.
12. If intact cells are available after cell disruption, these cells will be removed by the centrifugation step due to their sedimentation coefficient.
13. The ready prepared raw lysate can be stored for more than 5 years at −80 °C without significant loss of activity.
14. 1 μL of the PCR sample is diluted in 2 μL of sample buffer and 9 μL of Millipore water and loaded onto a 1% agarose gel. The agarose gel is run for 60 min at 100 V for the separation of DNA samples. Detection of DNA was accomplished using DNA intercalating dye DNA safe stain and an UV detection unit.
15. For application of a linear expression template to a cell-free reaction no further purification of the PCR product is required.
16. The preparation of plasmid DNA is performed according to the manufacture's protocol.
17. Thawing of cell-free components on ice enables a mild and slow thaw procedure which prevents the degradation and inactivation of enzymatic and translation related components.

18. Apart from the standardized pIX3.0 cell-free expression vector, various vector backbones can be applied to the CHO cell-free synthesis reaction. Requirements for the application to the CHO cell-free reaction are the presence of a T7 RNA polymerase promotor/terminator and an IRES sequence. Optimal concentrations of the plasmid depend on the type of plasmid applied. It is mandatory to evaluate each individual vector backbone to obtain increased protein yields.
19. Supplementation of PEG and Ficoll induce molecular crowding effects, which lead to an increased activity of T7 RNA polymerase.
20. Autoradiographs are prepared after separation of protein samples on SDS-PAGE. The SDS-PAGE is dried on an unigel dryer and exposed on a phosphor-screen for 3 days. The autoradiography is completed using a multimode imager (Typhoon Trio Plus, GE-Healthcare).
21. The evaluation of fluorescent proteins was performed using a multimode imager. For this, 5 μL of cell-free produced protein sample is diluted with 20 μL PBS and directly applied to an μ-Ibidi slide. The Ibidi slide can be placed in the multimode imager for fluorescence imaging.
22. Sodium azide is a supplement for the CHO CECF reaction to prevent microbial contamination.
23. The application of linear expression templates to a cell-free system generally leads to decreased protein yields compared to a circular DNA expression template. This fact is due to the decreased stability of linear DNA templates in cell-free systems.

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