(FOR RESEARCH USE ONLY. DO NOT USE IT IN CLINICAL DIAGNOSIS!)

Cat No.: EH10238

Human TLR4 (Toll-Like Receptor 4) ELISA Kit Manual

If you have any questions or need further help during experiment, please don't hesitate to contact us through the following methods:

Email (Order) order@enkilife.com

Email (Techsupport) techsupport@enkilife.com

Tel: 0086-27-87002838

Website: www.enkilife.com

Shelf life: Please refer to the label on the outer package.
Techsupport: In order to provide you with better service, please inform us the lot number on the label of the outer package.

Product description

This ELISA kit applies to the in vitro quantitative determination of Human TLR4 concentrations in serum, plasma and other biological fluids.

Key Features

●Sensitivity：3.75pg/ml.
●Detection range：6.25-400pg/ml.
●Specificity：It can detect Human TLR4 in samples without obvious cross-reaction with other analogs.
●Repeatability：The coefficient of variation within and between plates is <10%.

Test Principle

This ELISA kit uses the Sandwich-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with an antibody specific to Human TLR4. Standards or samples are added to the micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for Human TLR4 and Avidin-Horseradish Peroxidase (HRP) conjugate are added successively to each micro plate well and incubated. Free components are washed away. The substrate solution is added to each well. Only those wells that contain Human TLR4, biotinylated detection antibody and Avidin-HRP conjugate will appear blue in color. The enzyme-substrate reaction is terminated by the addition of stop solution and the color turns yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The OD value is proportional to the concentration of Human TLR4. You can calculate the concentration of Human TLR4 in the samples by comparing the OD of the samples to the standard curve.

Components and storage

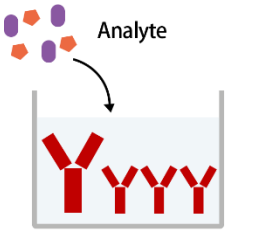
Store the product at 2-8°C before unopened. Upon receipt, unpack promptly and store as recommended in the instructions.

Components	Specifications	Storage and Notes	
Micro Plate	96T: 8 wells×12 strips	**Unopened:** -20℃, 12 months	**Unused:** Put it back in the aluminum foil bag and seal it, store it at -20℃
	48T: 8 wells×6 strips		
Reference Standard	96T: 2 vials	**Undissolved:** -20℃, 12 months	Please use freshly dissolved standards for

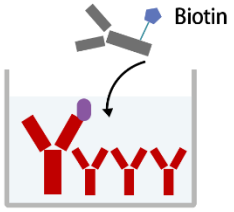
	48T: 1 vial		each experiment. Discard any unused standards after dissolution
Biotinylated Detection Ab Concentrate (100×)	96T: 120μL×1 vial	-20℃, 12 months	**Unused:** Please seal the concentrate and store it at -20℃, and discard the working solution
	48T: 60μL×1 vial		
HRP Conjugate Concentrate (100×)	96T: 120μL×1 vial	-20℃(Protect from light), 12 months	**Unused:** Please seal the concentrate and store it at -20℃, and discard the working solution
	48T: 60μL×1 vial		
Reference Standard & Sample Diluent	20mL×1	2-8℃, 12 months	
Biotinylated Detection Ab Diluent	14mL×1		
HRP Conjugate Diluent	14mL×1		
Wash Buffer Concentrate (25×)	30mL×1		
Substrate Reagent(TMB)	10mL×1	2-8℃(Protect from light),12 months	
Stop Solution	7mL×1	2-8℃/Room temperature	
Plate Sealer	5 pieces		
Product manual	1 copy		
Certificate of Analysis	1 copy		

Note:
Please ensure that the caps of all reagent bottles are tightened to prevent reagent evaporation and avoid microbial contamination.
The actual volume will be slightly larger than the volume indicated on the label. Please use accurate measuring equipment instead of pouring directly.

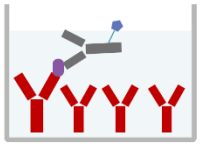
Assay Procedures



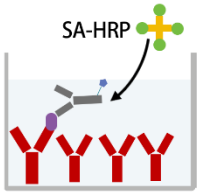
1.Add 100μL standard or sample to each well, incubate at 37°C for 90 min.



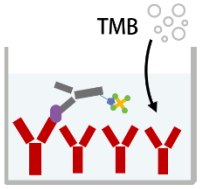
2.Discard the liquid, add 100μL Biotinylated Detection Ab working solution to each well. Incubate at 37°C for 60 min.



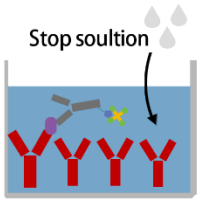
3.Aspirate and wash the plate for 3 times.



4.Add 100μL HRP conjugate working solution. Incubate at 37°C for 30 min. Aspirate and wash the plate for 5 times.



5.Add 90μL Substrate Reagent. Incubate at 37°C for 15 min.



6.Add 50μL Stop Solution.

7.Readings at 450nm. Calculation of results.

Operation method

ⓘ Precautions

1. Please wear protective equipment during the experiment. Please pay attention to biosafety protection according to the Biological Laboratory Safety Protection Regulations when contacting blood samples or other biological samples.
2. Kit components of different batches cannot be mixed (except Stop Solution).
3. The EP tubes and pipette tips used in the experiment are disposable, please do not mix

them.
4. There may be a small amount of water-like substances in the wells of the newly opened ELISA plate. This is a normal phenomenon and will not affect the experimental results. Temporarily unused strips should be placed in a spare aluminum foil bag and stored according to the required storage conditions.
5. Do not reuse the diluted standard, biotinylated antibody working solution, and enzyme conjugate working solution.
6. Unused Biotinylated Detection Ab Concentrate (100×), HRP Conjugate Concentrate (100×) and other original solutions should be stored according to the required storage conditions.
7. The ELISA reader needs to be equipped with a filter that can detect a wavelength of 450±10nm, and the detection range is between 0-3.5.

Materials required but not supplied

1. Microplate reader (450nm wavelength filter), Incubator capable of maintaining 37℃.
2. 1.5mL EP tube, absorbent paper.
3. Pipette and disposable tip: 0.5-10μL, 2-20μL, 20-200μL, 200-1000μL.
4. Double distilled water or deionized water.

Sample processing

Sample collection

Different samples may require different processing methods. Before sample preparation, it's essential to understand the sample's characteristics and avoid reagents that could affect subsequent experiments. Also, prevent samples from being exposed to unsuitable temperatures or durations. The following methods are for reference only. For more details, see the official **ELISA Sample Preparation Guide**.

Sample processing suggestions:
1.Serum: Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Avoid using hemolyzed or hyperlipidemia samples.
2.Plasma: Collect plasma using EDTA-Na2 as an anticoagulant (It is recommended to use tubes containing anticoagulants for blood collection). Centrifuge samples for 15 minutes at 1000×g within 30 minutes after collection. Collect the supernatant to carry out the

assay. For short - term storage, place the sample at 2 - 8°C. Avoid using hemolyzed or hyperlipidemia samples.

3.Tissue homogenization:

3.1 For general information, hemolyzed blood may affect the results, so the tissues should be minced into small pieces and rinsed in ice-cold PBS (0.01M, pH=7.4) to remove excess blood thoroughly. Then weigh for usage.

3.2 Tissue pieces should be weighed and then homogenized in PBS (Tissue weight (g): PBS (mL) volume=1:9. It is recommended to add a protease inhibitor, such as PMSF, to the PBS to inhibit the activity of proteases released during homogenization and prevent the proteolysis of target proteins.) with a glass homogenizer on ice.

3.3 To further break down the cells, you can sonicate the suspension with an ultrasonic cell disrupter (Use an ice bath to cool the sample, and strictly control the ultrasonic power and time to prevent protein denaturation and excessive free radical production). The homogenates are then centrifuged for 5-10 minutes at 5000×g, and collect the supernatant to carry out the assay.

Notes:

A. Some tissue samples (e.g., liver, kidney, and pancreas) contain high levels of endogenous peroxidase, which can react with TMB substrate and cause false positives. In such cases, mince the tissue and soak it in 3% H_2O_2 for 10–15 minutes. Rinse off the residual H_2O_2 with pre-cooled PBS, then homogenize and test as usual.

B. You may also use a mild lysis buffer to facilitate tissue lysis and release of intracellular proteins. However, avoid lysis buffers with Triton X-100, NP-40, or SDS, as they can lyse lysosomes and increase protease release in the absence of protease inhibitors, degrading target proteins and reducing test accuracy. Also, their high content can severely interfere with the test kit reaction. If physical lysis is ineffective, try the EnkiLife new-generation lysis liquid, RC0005 Cell/Tissue Lysis Buffer.

4.Cell lysates:

4.1 Cell collection:

Adherent cells: Gently wash the cells with moderate amount of pre-cooled PBS and dissociate the cells using trypsin (It is recommended to use EnkiLife RC0001 Trypsin Digestion Medium). Collect the cell suspension into a centrifuge tube and centrifuge for 5 min at 1000×g.

Suspended cells: Centrifuge for 5 min at 1500×g.

Semi-adherent cells: Collect the supernatant using the method for suspended cells. Then collect the adherent cells using the method for adherent cells. Resuspend both collected cell fractions in PBS, mix well, and centrifuge at 1000×g for 5 minutes to complete the cell collection.

4.2 Cell lysis:

Wash the cells 3 times with pre-cooled PBS. For each 1×10^6 cells, add 150-250 μL of pre-cooled PBS (If the content is very low, the volume of PBS can be reduced) to keep the cells suspended. Use an ultrasonic cell disrupter until the cells are fully lysed. (Reference settings: Use an ice bath, a 3 - 5mm probe, 150 - 300W power. Work for 1 - 2 seconds each time, with 30 - second intervals, and perform 3 - 5 cycles) Centrifuge for 10 min at 1500×g. Remove the cell fragments, collect the supernatant to carry out the assay.

5.Cell culture supernatant or other biological fluids: Centrifuge samples for 20 min at 1000×g, remove impurities and cell debris. Collect the supernatant to carry out the assay.

ⓘNote for samples

1. Sample storage after collection:

Assay within 1 week: store at 2-8℃.

Assay within 1 month: please divide into single-use amounts and store at -20℃.

Assay within 3 months: please divide into single-use amounts and store at -80℃.

All samples should avoid repeated freezing and thawing.

2.Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve**,** it is recommended to conduct a preliminary experiment to determine the appropriate dilution ratio to ensure the accuracy of the results. (It is recommended to conduct a preliminary experiment after consulting the literature to determine the dilution multiple)

3. If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity

4.If a lysis buffer is used to prepare tissue homogenates or cell lysates, there is a possibility of causing a deviation due to the introduced chemical substance.

5. Please be aware that some recombinant proteins may not be detected due to a mismatching with the coated antibody or detection antibody.

◊Preparation

1. Equilibrate to room temperature: Take the ELISA Kit out of the refrigerator 20 minutes in advance and let it equilibrate to room temperature.

2. Preheat the microplate reader: Please turn on the microplate reader at least 15 minutes in advance to stabilize the light source of the microplate reader during experiment.

3. Washing solution: Dilute the Wash Buffer Concentrate (25×) with double distilled water

(1:24).Tip: The Wash Buffer Concentrate (25×) taken out of the refrigerator may have crystals, which is a normal phenomenon. You can use a 40℃ water bath to slightly heat it to completely dissolve the crystals before preparing the washing solution. Use it on the same day.

4. Standard working solution:

4.1 Centrifuge the standard at 10000×g for 1 minute.

4.2 Add 1.0 mL of Reference Standard & Sample Diluent to the freeze-dried standard. After tightening the tube cap, let it stand for 10 minutes and invert it gently several times.

4.3 After the standard is completely dissolved, gently mix with a pipette to prepare a 400pg/ml standard working solution.

4.4 According to the experimental requirements, the standard is diluted in multiples. The recommended concentration gradient is: 400、200、100、50、25、12.5、6.25、0 pg/ml.

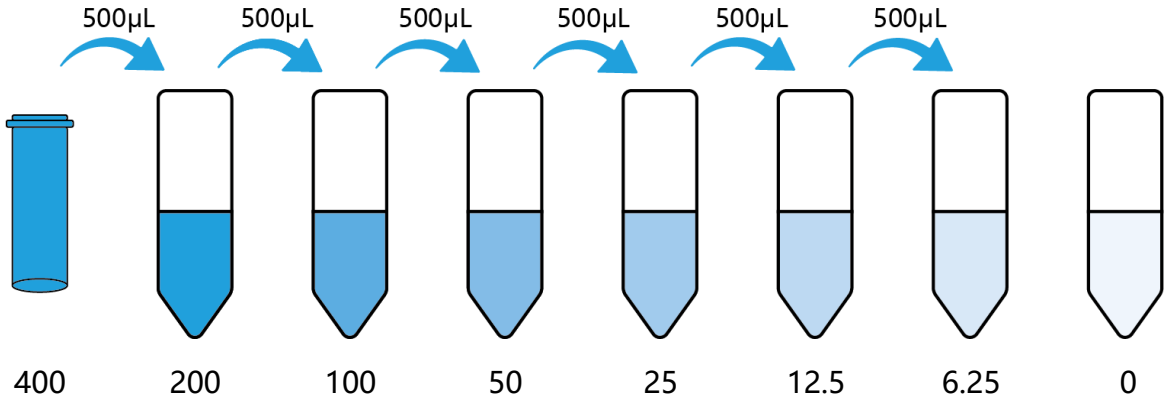
4.5 Multiple dilution method:

Prepare 7 EP tubes and add 500μL of Reference Standard & Sample Diluent to each tube. Pipette 500μL of the 400pg/ml standard working solution and add it to the first EP tube. Mix it with a pipette to prepare a 200 pg/ml standard working solution.

Next, take 500μL of the 200 pg/ml EP tube and add it to the second EP tube. Mix it with a pipette to prepare a 100 pg/ml standard working solution. And so on to obtain a standard working solution with multiple dilutions.

Note: The last tube is regarded as a blank. Just add the Reference **Standard & Sample Diluent.**

The illustration as below is for reference:



5. Biotinylated Detection Ab working solution:

Calculate the required amount before the experiment (100 μL/well), and prepare an additional 100-200μL in case of shortage. Centrifuge the **Biotinylated Detection Ab Concentrate (100×)** at 800×g for 1 min, then dilute the Biotinylated Detection Ab Concentrate

(100×) to 1× working solution with **Biotinylated Detection Ab Diluent.** (Biotinylated Detection Ab Concentrate (100×): Biotinylated Detection Ab Diluent= 1: 99). Prepare the working solution before use, and it should be used on the same day.

6. HRP Conjugate working solution:

Calculate the required amount before the experiment (100 μL/well), and prepare an additional 100-200μL in case of shortage. Centrifuge the **HRP Conjugate Concentrate (100×)** at 800×g for 1 min, then dilute the HRP Conjugate Concentrate (100×) to 1× working solution with **HRP Conjugate Diluent.** (HRP Conjugate Concentrate (100×): HRP Conjugate Diluent = 1: 99). Prepare the working solution before use, and it should be used on the same day.

Operation Steps

1. Add standard and sample:

Add different concentrations of **Standard working solution** to the first two columns of ELISA plate wells from top to bottom, two wells for each concentration, 100μL per well; Add 100μL of the sample to each of remaining wells. High concentration samples need to be diluted first.

During operation, solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall, shake gently to mix, avoid bubbles, and complete the sample addition operation within 10 minutes.

2. Cover the plate and incubate:

Cover the plate with the **Plate Sealer** provided in the kit. Incubate at 37℃ for 90min.

3. Biotinylated Detection Ab working solution:

Tear off the sealer, remove and dry the liquid in the plate wells, no need to wash.

Add 100μL of **Biotinylated Detection Ab working solution** to each well. Shake gently to mix, and cover the plate with a new **Plate Sealer**. Incubate at 37℃ for 60min.

4. Wash plate 1:

Manual Washing:

Aspirate or decant the liquid in the plate wells, add 350μL of **Washing solution** to each well, Soak for 1-2min, aspirate or decant the liquid in the plate wells, pat it dry against clean absorbent paper, and complete one wash. Repeat this wash step 3 times. Complete removal of liquid at each step is essential to good performance.

Microplate Washer:

350μL/well, shake the plate for 3–5 seconds.

5. Add HRP Conjugate working solution:

Add 100μL of **HRP Conjugate working solution** to each well. Gently shake to mix, cover the plate with a new **Plate Sealer**. Incubate at 37℃ for 30min.

6. Wash plate 2:

Wash the plate 5 times, the steps are the same as step 4 (**Wash plate 1**).

7. Add Substrate Reagent (TMB):

Add 90μL of **Substrate Reagent (TMB)** to each well. Cover the plate with a new **Plate Sealer**. Incubate at 37℃ for about 15min, Protect the plate from light.
Tips: Adjust the incubation time according to the color change, but do not exceed 30 minutes. Once the standard wells show a clear gradient, the incubation can be stopped.

8. Stop the reaction:

Add 50μL of **Stop Solution** to each well to stop the reaction.
Tip: The order of adding Stop Solution should be as consistent as possible with the order of adding Substrate Reagent (TMB).

9. Measure the OD value:

Immediately measure the OD value (optical density) of each well of the ELISA plate with a micro-plate reader set to 450nm.

Calculation of results

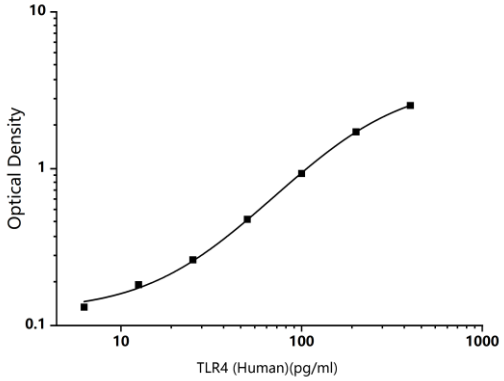
1. Create a standard curve:
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.
Create a four-parameter logistic (4-PL) curve using the curve fitting software offered by the microplate reader by plotting the mean OD value for each standard on the y-axis against the concentration on the x-axis.
2. Calculation of sample concentration:
Substitute the OD value of the sample into the standard curve to get the concentration. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.
3. Processing of high OD value samples:
If the OD of the sample surpasses the upper limit of the standard curve, the sample should be appropriately diluted and re-measured.

Technical Data

Typical Data:

The following data is for reference only. A standard curve must be run with each assay.

Concentration: pg/ml	400	200	100	50	25	12.5	6.25	0
OD	2.439	1.714	0.989	0.492	0.268	0.172	0.121	0.067
Corrected OD	2.372	1.647	0.922	0.425	0.201	0.105	0.054	-



Precision:

Intra-Assay Precision (Precision within an assay): Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays): Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/ml)	21.1	34.03	166.46	21.7	34.99	159.64
Standard deviation	1.43	1.5	7.34	1.37	1.71	6.43
CV (%)	6.8	4.42	4.41	1.37	4.9	4.03

Recovery:

The recovery of Human TLR4 spiked to three different levels in samples throughout the range of the assay in various matrices was evaluated.

Sample Type	Range (%)	Average Recovery (%)

Serum (n=5)	94-106	100
EDTA plasma (n=5)	97-112	102
Cell culture media (n=5)	92-106	100

Linearity:

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of Human TLR4 in various matrices were diluted with the Reference Standard & Sample Diluent to produce samples with values within the dynamic range of the assay.

		Serum (n=5)	Plasma (EDTA)(n=5)	Cell culture media (n=5)
1:2	Range (%)	96-109	87-99	90-105
	Average (%)	101	92	96
1:4	Range (%)	97-109	82-94	86-97
	Average (%)	103	95	92
1:8	Range (%)	90-104	88-101	87-100
	Average (%)	97	95	92
1:16	Range (%)	96-110	85-100	88-102
	Average (%)	102	92	93

ⓘ Troubleshooting

If the results are not good enough, please take pictures and save the experimental data in time. Keep the used plate and remaining reagents. Then contact our technical support to solve the problem. Meanwhile, you could also refer to the following materials:

Problem	**Causes**	**Solutions**
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure briefly spin the vial of standard and dissolve the powder thoroughly by gentle mixing
	Wells are not completely aspirated	Completely aspirate wells in between steps
Low signal	Insufficient incubation time	Ensure sufficient incubation time

	Incorrect assay temperature	Use recommended incubation temperature
	Inadequate reagent volumes	Check pipettes and ensure correct preparation
	Improper dilution	Check dilution steps
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB, rapid coloring
Low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader(450nm)
		Open the Microplate Reader ahead to pre-heat at least 15 min
Large CV	Inaccurate pipetting	Check pipettes.
High background	Concentration of target protein is too high	Use recommended dilution factor
	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed. Ensure to use the wash buffer supplied in the Elisa kit
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	All the reagents should be stored according to the instructions
	Stop solution is not added	Stop solution should be added to each well before measurement.

ⓘStatement:

1. **Quality and Technical Risk Tips**: Limited by current conditions and scientific technology, we can't conduct comprehensive identification and analysis on all the raw material provided. So there might be some qualitative and technical risks for users using the kit.

2. **Factors affecting experimental results:** The experimental results are affected by many factors such as the effectiveness of the reagents, the skills of the operator and the experimental environment. To ensure the accuracy of the results, please prepare sufficient samples to be tested.

3. **Reagent Usage Guide:** To obtain the best experimental results, it is recommended to only use the reagents provided in this kit and operate strictly in accordance with the instructions. Avoid mixing with products from other manufacturers.

4. **Operation Precautions:** Improper reagent preparation or microplate reader parameter settings may lead to abnormal experimental results. Please read the instructions carefully before the experiment and adjust the instrument parameters correctly.

5. **Result Reproducibility:** Even the same operator may get different results in two independent experiments. To ensure the reproducibility of the results, please strictly control every step of the operation during the experiment.

6. **Quality Assurance and Difference Description:** Every kit has strictly passed QC test. However, results from users might be inconsistent with our data due to some variables such as transportation conditions, different lab equipment, and so on. Intra-assay variance among kits from different batches might arise from the above reasons too.

7. This kit is for research use only, do not use it in clinical diagnosis!

ⓘ We are always committed to providing high-quality products and thank you for your understanding and support. If you have any questions, please feel free to contact our technical support team.