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

Catalog No: E-UNEL-H0385

Product size: 96T*5/96T*15

Elabscience® Uncoated Human RANκL (Receptor Activator of Nuclear Factor Kappa B Ligand) ELISA Kit

This manual must be read attentively and completely before using this product.

If you have any problems, please contact our Technical Service Center for help.

Tel: 1-832-243-6086
Fax: 1-832-243-6017
Email: techsupport@elabscience.com
Website: www.elabscience.com

Please refer to specific expiry date from label outside of box.

Please kindly provide us with the lot number (on the outside of the box) of the kit for more efficient service.

Intended use

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

Kit components & Storage

Item	Specifications	Dilution	Storage
Human RANκL Micro ELISA pre-Plate	96T*5: 5 plates, 96T 96T*15: 15plates, 96T	/	-20℃, 12 months
Human RANκL Capture Ab	96T*5: 1 vial, 120μL 96T*15: 1 vial, 350μL	1/500	-20℃, 12 months
Human RANκL Biotinylated Detection Ab	96T*5: 1 vial, 120μL 96T*15: 1 vial, 350μL	1/500	-20℃, 12 months
Human RANκL Reference Standard	96T*5: 5 vials 96T*15: 15 vials	5000 pg/vial	-20℃, 12 months(Use a new standard for each assay. Discard after use)
Human RANκL HRP Conjugate	96T*5: 1 vial, 120μL 96T*15: 1 vial, 350μL	1/500	-20℃(Protect from light), 12 months
Product Description	1 copy	/	
Certificate of Analysis	1 copy	/	

Note:

1) All reagent bottle caps must be tightened to prevent evaporation and microbial pollution.
2) The volume of reagents in partial shipments is a little more than the volume marked on the label, please use accurate measuring equipment instead of directly pouring into the vial(s).
3) Do not reuse the reconstituted standard, biotinylated detection Ab working solution, HRP conjugate working solution. The unspent undiluted biotinylated detection Ab and other stock solutions should be stored according to the storage conditions in the above table.

Other required reagents: purchase separately or prepare your own

➢**Ancillary Reagent Kit** (Cat No. E-ELIR-K001): The kit contains a full set of ancillary reagents to complete the 96T*5 ELISA assay.

➢Or if there are other experimental requirements, the following auxiliary reagent products may be purchased separately:

Item	Catalog No.
ELISA Plate Coating Buffer(5×)	E-ELIR-002
ELISA Plate Blocking Buffer	E-ELIR-003
Wash Buffer for Sandwich-ELISA(25×)	E-ELIR-004
Stop Solution(5×)	E-ELIR-012
HRP-conjugate Diluent	E-ELIR-008
Biotinylated Antibody Diluent	E-ELIR-010
Sample Diluent	E-ELIR-011
One-component TMB Substrate	E-IR-R201

➢Or refer to the following formula to prepare each universal reagent.

(Note: The following formula only contains the basic component information of each reagent, which can be optimized according to the experimental requirements and results)

Name	Main Component and Preparation method
PBS(1×)	3.58 g $Na_2HPO_4 \cdot 12H_2O$, 0.21 g $NaH_2PO_4 \cdot 2H_2O$, 8 g NaCl,0.2 g KCl, Make up to 1 L with pure water, pH 7.2-7.4
Coating Buffer	1.59 g Na_2CO_3, 2.94 g $NaHCO_3$, Make up to 1 L with pure water, pH 9.2-9.6
Blocking Buffer	1× PBS, 0.01% PC300, 0.01% Tween® 20, 1% Protective substance, pH 7.0-7.4, 0.2 μm filtered
HRP conjugate Diluent	1× PBS, 0.01% PC300, 0.01% Tween® 20, 1% Protective substance, pH 7.0-7.4, 0.2 μm filtered
Antibody Diluent	1× PBS, 0.01% PC300, 0.01% Tween® 20, 1% Protective substance, pH 7.0-7.4, 0.2 μm filtered
Standard & Sample Diluent	1× PBS, 0.01% PC300, 0.01% Tween® 20, 1% Protective substance, pH 7.0-7.4, 0.2 μm filtered
Wash Buffer	0.05% Tween® 20 in PBS, pH 7.2-7.4.
Stop Solution	5% sulfuric acid

Other supplies required

Microplate reader with 450 nm wavelength filter
High-precision transfer pipette, EP tubes and disposable pipette tips
Incubator capable of maintaining 37℃
Deionized or distilled water
Absorbent paper
Loading slot

Sample collection

Serum

- Collect blood in pyrogen-free, endotoxin-free coagulation tubes or plain tubes.
- Allow clotting, at room temperature, for 2 hours, or overnight, at 2-8 °C.
- Centrifuge at 1,000×g for 20 minutes. For hyperlipidemic samples, centrifuge at 10,000×g at 2-8 °C for 10 minutes to separate serum and remove chylomicrons.
- Aliquot the supernatant (serum) into sterile cryopreservation tubes.

Plasma

- Use EDTA-K2/Na2 (1.5-2 mg/mL) as anticoagulant; sodium citrate can be used for coagulation-related targets.
- Gently invert tubes 5-8 times immediately after collection to prevent air bubbles and hemolysis.
- Centrifuge at 1,000×g at 2-8 °C for 15 minutes, within 30 minutes after collection.
- Collect the plasma fraction after centrifugation.

Tissue Homogenate

- Rinse tissue 2-3 times with pre-cooled PBS (0.01 M, pH 7.2-7.4) to remove residual blood.
- Cut tissue into small pieces (2-3 mm^3) and add to a glass homogenizer with PBS containing protease inhibitor (add phosphatase inhibitor for phosphorylated targets; tissue-to-homogenate ratio 1:9).
- Homogenize on ice 30-50 times. For further lysis, repeat freeze-thaw cycles or use ultrasonic disruption.
- Centrifuge at 5,000 × g at 2-8 °C for 5—10 minutes and collect supernatant.
- Optional: Determine total protein concentration using a BCA Protein Quantification Kit.

Cell Lysate

- Gently rinse adherent cells with cold PBS; digest with trypsin if needed.
- Collect cells by centrifugation at 1,000×g for 5 minutes; suspension cells can be collected directly.
- Wash cells 3 times with cold PBS and resuspend in 150–200 µL PBS per 1.0×10^6 cells (add protease inhibitor if required).
- Lyse cells using repeated freeze-thaw cycles or ultrasonic disruption.
- Centrifuge at 1,500 × g at 2-8 °C for 10 minutes and collect supernatant.

Cell Culture Supernatant

- Collect aseptically and centrifuge at 1,000×g at 2-8 °C for 20 minutes to remove debris.
- Collect the clarified supernatant for ELISA measurement.

Urine

- Centrifuge at 1,500×g at 2-8 °C for 15 minutes to remove particulate matter.
- Use supernatant immediately or aliquot and store at ≤ -20 °C.

Recommended Reagents for Sample Processing

- PMSF Protease Inhibitor (Cat No. E-EL-SR002)
- 0.25% Trypsin Solution (Cat No. E-EL-SR001)
- BCA Protein Quantification Kit (Cat No. E-BC-K318-M)

Note

■Note for kit

1)For research use only. Not for use in diagnostic procedures.
2)ELISA Plate Coating Buffer(5×), Wash Buffer for Sandwich-ELISA(25×) and Stop Solution(5×) should be diluted into working solution according to the instructions before use.
3)Please wear lab coats, eye protection and latex gloves for protection. Please perform the experiment following the national security protocols of biological laboratories, especially when detecting blood samples or other bodily fluids.
4)A freshly opened ELISA plate may appear a water-like substance, which is normal and will not have any impact on the experimental results. Return the unused wells to the foil pouch and store according to the conditions suggested in the above table.
5)The microplate reader should be able to be installed with a filter that can detect the wave length at 450±2 nm. The optical density should be within 0-3.5. Follow the Instructions of the Microplate Reader for set-up and preheat it for 15 min before OD measurement.
6)Do not mix or substitute reagents with those from other lots or sources.
7)Change pipette tips in between adding of each standard level, between sample adding and between reagent adding. Also, use separate reservoirs for each reagent.
8)The kit should not be used beyond the expiration date on the kit label.

■Note for sample

1)Tubes for blood collection should be disposable and be non-endotoxin. Samples with high hemolysis or much lipid are not suitable for ELISA assay.
2)Samples should be assayed within 7 days when stored at 2-8℃, otherwise samples must be divided up and stored at -20℃ (≤1 month) or -80℃ (≤3 months). Avoid repeated freeze-thaw cycles. Prior to assay, the frozen samples should be slowly thawed and centrifuged to remove precipitates.
3)Please predict the concentration before assaying. If the sample concentration is not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
4)If the sample type is not included in the manual, a preliminary experiment is suggested to verify the validity. If a lysis buffer is used to prepare samples, there is a possibility of causing a deviation due to the introduced chemical substance.
5)Some recombinant protein may not be detected due to a mismatching with the coated antibody or detection antibody.

Reagent preparation

1.Bring all reagents to room temperature (18-25℃) before use. If the kit will not be used up in one assay, please only take out the necessary strips and reagents for present experiment, and store the remaining strips and reagents at required condition.

2.Micro ELISA Plate:

a) The capture antibody was diluted to the working concentration using the ELISA Plate Coating Buffer(1×) (1/500 fold dilution is recommended).

b) Take out the Micro ELISA pre-Plate, add 100 µL of capture antibody working solution to each well. Cover the plate with the sealer provided in the kit. Incubate overnight at 2-8℃.

c) Decant the liquid from each well, do not wash. Add 200 µL of ELISA Plate Blocking Buffer to each well. Cover the plate with the sealer. Incubate for 1 hour at 37℃.

d) Decant the liquid from each well, do not wash, and the plate is ready for sample addition. Or the plate was dried at 37℃ for 30 min. The dried plate can be stored at -20℃ for 6 months after sealing with desiccant.

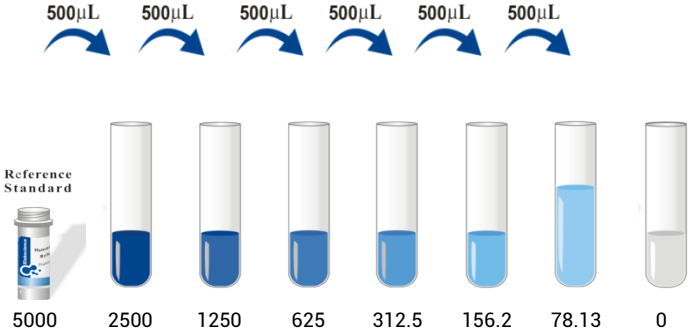
3.Standard working solution:

a) Centrifuge the standard at 10,000×g for 1 min. Add 1 mL of Sample Diluent, let it stand for 10 min and invert it gently several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 5000 pg/mL (or add 1 mL of Sample Diluent, let it stand for 1-2 min and then mix it thoroughly with a vortex meter of low speed. Bubbles generated during vortex could be removed by centrifuging at a relatively low speed). Then make serial dilutions as needed. The recommended dilution gradient is as follows: 5000, 2500, 1250, 625, 312.5, 156.25, 78.13,0 pg/mL.

b) Dilution method: Take 7 EP tubes, add 500 µL of Sample Diluent to each tube. Pipette 500 µL of the 5000 pg/mL working solution to the second tube and mix up to produce a 2500pg/mL working solution. Pipette 500 µL of the solution from the former tube into the latter one according to this step. The illustration on the next page is for reference. Note: the last tube is regarded as a blank. Don't pipette solution into it from the former tube. Gradient diluted standard working solution should be prepared just before use.

4.**Biotinylated Detection Ab working solution:** Calculate the required amount before the experiment (100 µL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the Concentrated Biotinylated Detection Ab at 800×g for 1 min, then dilute the Biotinylated Detection Ab to

working solution with Biotinylated Antibody Diluent (1/500 fold dilution is recommended). The working solution should be prepared just before use.



5. **HRP Conjugate working solution:** HRP Conjugate is HRP conjugated avidin. Calculate the required amount before the experiment (100 μL/well). In preparation, slightly more than calculated should be prepared. Centrifuge the HRP Conjugate at 800×g for 1 min, then dilute the HRP Conjugate to working solution with HRP Conjugate Diluent (1/500 fold dilution is recommended). The working solution should be prepared just before use.
6. **Wash Buffer:** Dilute 30 mL of Concentrated Wash Buffer with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: if crystals have formed in the concentrate, warm it in a 40℃ water bath and mix it gently until the crystals have completely dissolved. For same day use only.

Dilution Method

For 100 fold dilution: One-step dilution. Add 5 μL sample/concentrate to 495 μL diluent to yield 100 fold dilution.

For 1000 fold dilution: Two-step dilution.Add 5 μL sample/concentrate to 95 μL diluent to yield 20 fold dilution,then add 5 μL 20 fold diluted sample/concentrate to 245 μL diluent, after this, the neat sample has been diluted at 1000 fold successfully.

For 100000 fold dilution: Three-step dilution. Add 5 μL sample/concentrate to 195 μL diluent to yield 40 fold dilution,then add 5 μL 40 fold diluted sample/concentrate to 245 μL diluent to yield 50 fold dilution, and finally add 5 μL 2000 fold diluted sample/concentrate to 245 μL diluent, after this, the neat sample has been diluted at 100000 fold successfully.

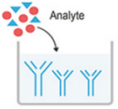
General Operation Procedure

1. Determine wells for diluted standard, blank and sample. Add 100 µL each dilution of standard, blank and sample into the appropriate wells (It is recommended that all samples and standards be assayed in duplicate. It is recommended to determine the dilution ratio of samples through preliminary experiments or technical support recommendations). Cover the plate with the sealer provided in the kit. Incubate for 90 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Decant the liquid from each well, do not wash. Immediately add 100 µL of Biotinylated Detection Ab working solution to each well. Cover the plate with a new sealer. Incubate for 1 hour at 37°C.
3. Decant the solution from each well， add 350 µL of wash buffer to each well. Soak for 1 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps. Make the tested strips in use immediately after the wash step. Do not allow wells to be dry.
4. Add 100 µL of HRP Conjugate working solution to each well. Cover the plate with a new sealer. Incubate for 30 min at 37°C.
5. Decant the solution from each well, repeat the wash process for 5 times as conducted in step 3.
6. Add 90 µL of Substrate Reagent to each well. Cover the plate with a new sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30 min. Preheat the Microplate Reader for about 15 min before OD measurement.
7. Add 50 µL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
8. Determine the optical density (OD value) of each well at once with a microplate reader set to 450 nm.

Assay Procedure Summary



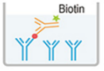
1.1 Take out the Micro ELISA pre-Plate, add 100μL Capture antibody working solution to each well. Incubate overnight at 2-8°C
1.2 Discard the liquid, add 200μL ELISA Plate Blocking Buffer to each well. Incubate for 60 min at 37°C



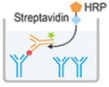
2. Discard the liquid, add 100μL standard or sample to the wells. Incubate for 90 min at 37°C



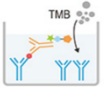
3. Discard the liquid, immediately add 100μL Biotinylated Detection Ab working solution to each well. Incubate for 60 min at 37°C



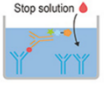
4. Aspirate and wash the plate for 3 times



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



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



7. Add 50μL Stop Solution



8. Read the plate at 450nm immediately.
Calculation of the results

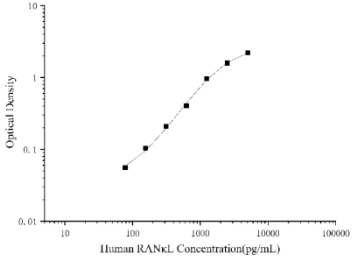
Calculation of results

Average the duplicate readings for each standard and samples, then subtract the average zero standard optical density. Plot a four parameter logistic curve on log-log axis, with standard concentration on the x-axis and OD values on the y-axis.

If the OD of the sample surpasses the upper limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

Typical data

As the OD values of the standard curve may vary according to the conditions of the actual assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), the operator should establish a standard curve for each test. Typical standard curve and data is provided below for reference only.

pg/mL	OD	Corrected OD	Standard Curve
5000	2.290	2.210	
2500	1.668	1.588	
1250	1.046	0.966	
625	0.484	0.404	
312.5	0.288	0.208	
156.25	0.183	0.103	
78.13	0.136	0.056	
0	0.080	-	

Performance

■Specificity

This kit recognizes RANκL in samples.No significant cross-reactivity or interference between RANκL and analogues was observed.

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. Bring substrate to room temperature before use.
	Inadequate reagent volumes	Check pipettes and ensure correct preparation.
	Improper dilution	
	HRP conjugate inactive or TMB failure	Mix HRP conjugate and TMB, rapid coloring.
Deep color but low value	Plate reader setting is not optimal	Verify the wavelength and filter setting on the Microplate reader.
		Open the Microplate Reader ahead to pre-heat.
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.
	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.

Declaration

1. 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. This assay is designed to eliminate interference by factors present in biological samples. Until all factors have been tested in the ELISA immunoassay, the possibility of interference cannot be excluded.
3. The final experimental results will be closely related to the validity of products, operational skills of the operators, the experimental environments and so on. We are only responsible for the kit itself, but not for the samples consumed during the assay. The users should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
4. To get the best results, please only use the reagents supplied by the manufacturer and strictly comply with the instructions.
5. Incorrect results may occur because of incorrect operations during the reagents preparation and loading, as well as incorrect parameter settings of the Micro-plate reader. Please read the instructions carefully and adjust the instrument prior to the experiment.
6. Even the same operator might get different results in two separate experiments. In order to get reproducible results, the operation of every step in the assay should be controlled.
7. Every kit has strictly passed QC test. However, results from end 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.
8. Kits from different manufacturers or other methods for testing the same analyte could bring out inconsistent results, since we haven't compared our products with those from other manufacturers.
9. The kit is designed for research use only, we will not be responsible for any issues if the kit is applied in clinical diagnosis or any other related procedures.