RES36-EN.01

resDetect™ Human 4-1BB Ligand ELISA Kit (Residue Testing)
(Enzyme-Linked Immunosorbent Assay)

Catalog Number：RES-A036

Pack Size: 96 tests

IMPORTANT: Please carefully read this manual before performing your experiment.

For Research Use Only. Not For Use in Diagnostic or Therapeutic Procedure

INTENDED USE

The kit is developed for the detection and quantitative determination of 4-1BB Ligand in human serum and cellular supernatant. It is intended for research use only (RUO).

BACKGROUND

Tumor necrosis factor ligand superfamily member 9 (4-1BBL) is also known as 4-1BB ligand, CD137L or TNFSF9, which is a cytokine that binds to TNFRSF9. 4-1BBL is the high affinity ligand of 4-1BB. 4-1BBL induces the proliferation of activated peripheral blood T-cells. Also, 4-1BBL may have a role in activation-induced cell death (AICD). Furthermore, 4-1BBL may play a role in cognate interactions between T-cells and B-cells/macrophages. As for diseases, 4-1BBL is involved in cancers, infectious diseases and autoimmune diseases.

To support the development of CAR-T drugs, ACROBiosystems independently developed Human 4-1BB Ligand ELISA Kit via rigorous methodological validation, which is used for detection of GMP human 4-1BB Ligand in samples from CAR-T product preparation processing for evaluation the quality of CAR-T products in drug development and CMC quality control stages.

PRINCIPLE OF THE ASSAY

This assay kit is used to measure the levels of human 4-1BB Ligand by employing a standard sandwich-ELISA format. The micro-plate in the kit has been pre-coated with Anti-4-1BB Ligand Antibody. Firstly, add the standard samples provided in kit and your samples to the plate, incubate and wash the wells. Then add the Biotin-Anti-4-1BB Ligand Antibody to the plate and form Antibody-antigen-biotinylated antibody complex, incubate and wash the wells. Next add Streptavidin-HRP to the plate, incubate and wash the wells. At last, load the substrate into the wells and monitor solution color from blue to yellow. The reaction is stopped by the addition of a stop solution and the intensity of the absorbance can be measured at 450 nm and 630 nm. The OD Value reflects the amount of human 4-1BB Ligand bound.

PRECAUTIONS

1. This kit is for research use only and is not for use in diagnostic or therapeutic applications.

US and Canada: Tel: +1 800-810-0816 | Web: http://www.acrobiosystems.com
Asia and Pacific: Tel: +86 400-682-2521 | E-mail: order@acrobiosystems.com

2. The kit is suitable for cell supernatant, serum and plasma samples.
3. Do not use reagents past their expiration date.
4. Do not mix or substitute reagents with those from other kits or other lot number kits.
5. If samples generate values higher than the highest standard, dilute the samples with the appropriate calibrator diluent and repeat the assay. If cell supernatant samples need step dilution, except for the final dilution with diluent, other intermediate dilutions can be in cell culture medium.
6. Differences in test results can be caused by a variety of factors, including laboratory operator, pipette usage, plate washing technique, reaction time or temperature, and kit storage.
7. This kit is designed to remove or reduce some endogenous interference factors in biological samples, and not all possible influencing factors have been removed.

MATERIALS PROVIDED

Table1. Materials provided

Catalog	Components	Size (96 tests)	Format	Storage	
				Unopened	Opened
RES036-C01	Pre-coated Anti-4-1BB Ligand Antibody Microplate	1 plate	Solid	2-8°C	2-8°C
RES036-C02	Human 4-1BB Ligand Standard	20 μg	Powder	2-8°C	-70°C
RES036-C03	Biotin-Anti-4-1BB Ligand Antibody	20 μg	Powder	2-8°C	-70°C
RES036-C04	Streptavidin-HRP	50 μL	Liquid	2-8°C, avoid light	2-8°C, avoid light
RES036-C05	10×Washing Buffer	50 mL	Liquid	2-8°C	2-8°C
RES036-C06	2×Dilution Buffer	50 mL	Liquid	2-8°C	2-8°C
RES036-C07	Substrate Solution	12 mL	Liquid	2-8°C, avoid light	2-8°C, avoid light
RES036-C08	Stop Solution	7 mL	Liquid	2-8°C	2-8°C

SRORAGE

1. Unopened kit should be stored at 2°C -8°C upon receiving.

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

2. The opened kit should be stored per Table 1. The shelf life is 30 days from the date of opening.

Note: *a. Do not use reagents past their expiration date.*

b. Find the expiration date on the outside packaging.

REAGENTS/EQUIPMENT NEEDED BUT NOT SUPPLIED

Single or multi-channel micropipettes and pipette tips: need to meet 10 µL, 300 µL, 1000 µL injection requirements;

37ºC Incubator;

Single or dual wavelength microplate reader with 450 nm and 630 nm filter;

Tubes: 1.5mL,10mL;

Timer;

Reagent bottle;

Deionized or distilled water.

REAGENT PREPARATION

Bring all reagents and samples to room temperature (20℃-25℃) before use. If crystals have formed in buffer solution, place the sample in an 37℃ incubator until the crystals have completely dissolved and bring the solution back to room temperature before use.

According to Table 2, prepare the provided lyophilized product into a storage solution with ultrapure water, dissolve at room temperature for 15 to 30 minutes, and mix by gently pipetting, avoiding vigorous shaking or vertexing. The reconstituted storage solution should be stored at -70°C. It is recommended that the number of freezing and thawing should not exceed 1 time, the size of the aliquot should not be less than 5 µg.

Note: *Considering inevitable minor quantitation variations between protein batches, it is also reasonable to generate the standard curve with specific lot of proteins used for current production for even better accuracy.*

Table 2. Preparation method

ID	Components	Size (96 T)	Storage solution concentration.	Reconstituted water Vol.
RES036-C02	Human 4-1BB Ligand Standard	20 µg	100 µg/mL	200 µL

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

RES036-C03	Biotin-Anti-4-1BB Ligand Antibody	20 μg	100 μg/mL	200 μL

Note: It is recommended that Streptavidin-HRP be centrifuged briefly before use to deposit liquid from the tube wall or cap to the bottom of the tube.

RECOMMENDED SAMPLE PREPARATION

1. Working Solution Preparation

1.1 Preparation of 1×Washing Buffer:

Dilute 50 mL 10×Washing Buffer with ultrapure water/deionized water to 500 mL.

1.2 Preparation of 1×Dilution Buffer:

Dilute 50 mL 2×Dilution Buffer with 1×Washing Buffer to 100 mL.

1.3 Preparation of Biotin-Anti-4-1BB Ligand Antibody working fluid:

Dilute Biotin-Anti-4-1BB Ligand Antibody to 0.2 μg/mL with 1×Dilution Buffer. Please prepare it for one-time use only.

1.4 Preparation of Streptavidin-HRP working fluid:

Dilute Streptavidin-HRP at 1:2000 with 1×Dilution Buffer. The prepared working fluid should avoid light. Please prepare it for one-time use only.

1.5 Sample preparation

a. If the sample to be tested is the serum, dilute test sample at 1:20 with 1×Dilution Buffer. The volume ratio of sample to diluent is 1:19.

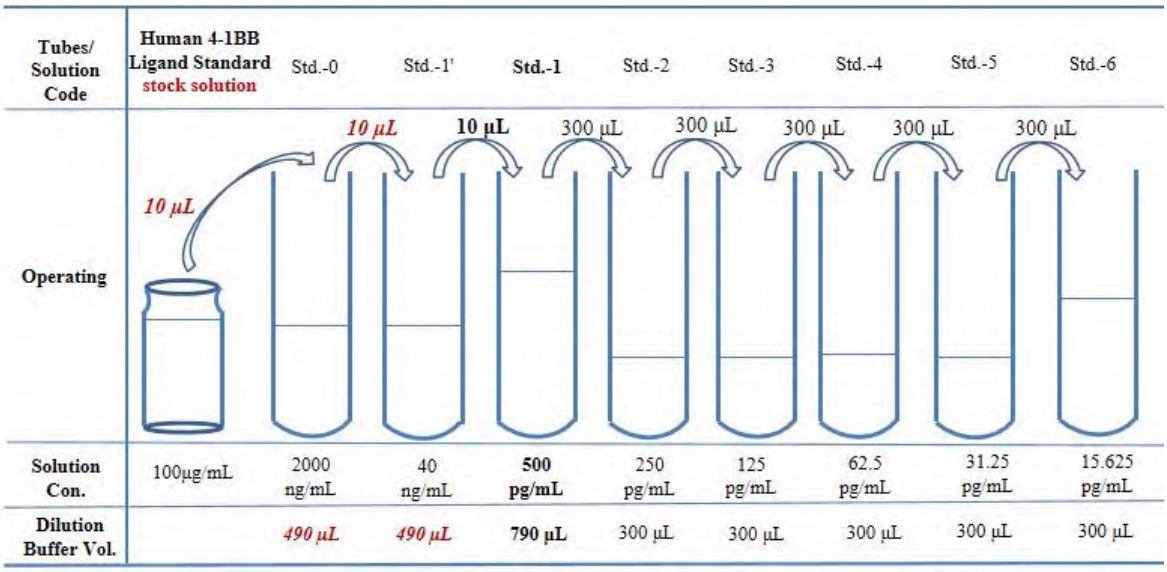
b. If the sample to be tested is the cell supernatant or plasma, dilute test sample at 1:2 with 1×Dilution Buffer. The volume ratio of sample to diluent is 1:1.

2. Preparation of Standard curve

The concentration of the reconstituted human 4-1BB Ligand Calibrator (RES036-C02) is 100 μg/mL，prepare (Std.-0) by diluting 10 μL the reconstituted human 4-1BB Ligand Calibrator into 490 μL Sample Dilution Buffer, mix gently well. Then prepare Std.- 1' by diluting 10 μL Std.-0 into 490 μL Sample Dilution Buffer. At last, prepare the highest concentration of standard curve, Std.-1 (500 pg/mL), by diluting 10 μL Std.- 1' into 790 μL Sample Dilution Buffer. Prepare 1:1 serial dilution for the standard

US and Canada: Tel: +1 800-810-0816 | Web: http://www.acrobiosystems.com
Asia and Pacific: Tel: +86 400-682-2521 | E-mail: order@acrobiosystems.com

curve as follows: Pipette 300 µL of Sample Dilution Buffer into each tube. Make sure to mix well every time. Sample Dilution Buffer serves as blank.



3. Add Samples

Add 100 µL Calibrator and samples to each well. For blank Control wells, please add 100 µL Dilution Buffer.

***Note**: It is recommended to set double holes for samples and standard curves to be tested.*

4. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 1.0 hour.

5. Washing

Remove the remaining solution by aspiration, add 300 µL of 1×Washing Buffer to each well, soak for 10 s, remove any remaining 1×Washing Buffer: by aspirating or decanting, invert the plate and blot it against paper towels. Repeat the wash step above for three times.

6. Add Biotin-Anti-4-1BB Ligand Antibody

For all wells, add 100 µL Biotin-Anti-4-1BB Ligand Antibody (dilute to 0.2 µg/mL) working solution. Please prepare it for one-time use only.

7. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 1.0 hour.

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

8. Washing

Repeat step 5.

9. Add Streptavidin-HRP

For all wells, add 100 µL Streptavidin-HRP (dilute at 1:2000) working solution. Please prepare it for one-time use only, avoid light.

10. Incubation

Seal the plate with microplate sealing film and incubate at room temperature for 30 min.

11. Washing

Repeat step 5.

12. Substrate Reaction

Add 100 µL Substrate Solution to each well. Seal the plate with microplate sealing film and incubate at room temperature for 20 min, avoid light.

13. Termination

Add 50 µL Stop Solution to each well and tap the plate gently to allow thorough mixing.

Note: *The color in the wells should change from blue to yellow.*

14. Data Recording

Read the absorbance at 450 nm and 630 nm using UV/Vis microplate spectrophotometer within 10 minutes.

Note*: To reduce the background noise, subtract the value read at* OD_{450nm} *with the value read at* $OD_{630\ nm}$.

CALCULATION OF RESULTS

1. Calculate the mean absorbance for each standard, control and sample and subtract average zero standard optical density (OD).
2. The standard curve is plotted with the standard concentration as x-axis and the calibrated absorbance value as y-axis. Four parameters logistic are used to draw the standard curve and calculate the sample concentration.
3. Normal range of Standard curve: $R^2 \geq 0.9900$.
4. Detection range: 15.625 pg/mL-500 pg/mL. If the OD value of the sample to be tested is higher than

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

500 pg/mL, the sample shall be diluted with dilution buffer and assay repeated. If the OD value of the sample to be tested is lower than 15.625 pg/mL, the sample should be reported.

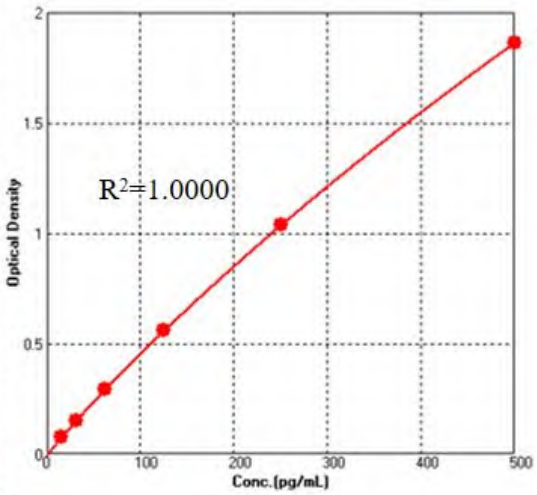
QUICK GUILD

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

TYPICAL DATA

For each experiment, a standard curve needs to be set for each micro-plate, and the specific OD value may vary depending on different laboratories, testers, or equipments. The following example data is for reference only. The sample concentration was calculated based on the results of the standard curve.

Standard (pg/mL)	O.D.-1	O.D.-2	Average	Corrected
500	1.933	1.911	1.922	1.862
250	1.132	1.058	1.095	1.035
125	0.631	0.612	0.622	0.561
62.5	0.361	0.343	0.352	0.292
31.25	0.211	0.213	0.212	0.152
15.625	0.138	0.137	0.138	0.077
0	0.059	0.062	0.061	/



SENSITIVITY

The minimum detectable concentration of 4-1BB Ligand is 1.881 pg/mL. The minimum detectable concentration was determined by adding twice standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

PRECISION

1. Intra-assay Precision

Three samples of known concentration were tested ten times on one plate to assess intra-assay precision.

2. Inter-assay Precision

Three samples of known concentration were tested in three separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	10	10	10	3	3	3

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

Mean (pg/mL)	358.765	93.877	37.003	360.948	92.080	36.437
SD	15.908	2.463	2.146	3.783	1.883	0.491
CV (%)	4.4	2.6	5.8	1.0	2.0	1.3

***Note**: The example data is for reference only.*

RECOVERY

Five parts of blank T cell culture supernatant were added with different concentrations of human 4-1BB Ligand, and the T cell culture supernatant without human 4-1BB Ligand was used as background to calculate the recovery rate. The range of the recovery rate is 88.3-107.2%, and the average recovery is 96.6%.

Sample Type	Average % Recovery	Range
T cell culture supernatant (n=5)	96.6%	88.3-107.2%

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of 4-1BB Ligand were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture medium (DMEM)	Cell culture medium (1640)	Serum
1:2	Average Recovery (%)	91.7	92.1	93.7
	Range (%)	86.8-94.2	87.2-96.7	85.6-99.4
1:4	Average Recovery (%)	95.2	91.2	94.4
	Range (%)	92.7-96.2	86.5-97.5	93.0-95.6
1:8	Average Recovery (%)	100.4	91.3	96.2
	Range (%)	98.7-102.9	86.6-95.5	92.8-100.1
1:16	Average Recovery (%)	104.0	102.2	91.1
	Range (%)	99.5-108.1	94.1-108.6	84.7-95.9

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

***Note**: The example data is for reference only.*

SPECIFICITY

This assay recognizes natural and recombinant human 4-1BB Ligand. No cross-reactivity was observed when this kit was used to analyze the following recombinant cytokines.

Human			
IL-2	IL-10	GM-CSF	Anti-CD3
IL-3	IL-11	G-CSF	Anti-CD28
IL-4	IL-12B	M-CSF	TNF-alpha
IL-5	IL-15	IFN-alpha 1	FGF basic
IL-6	IL-17A	IFN-gamma	Thrombopoietin-TPO
IL-7	IL-18	TGF-beta 1	L1R
IL-8	VEGF165	SCF	BMP-2

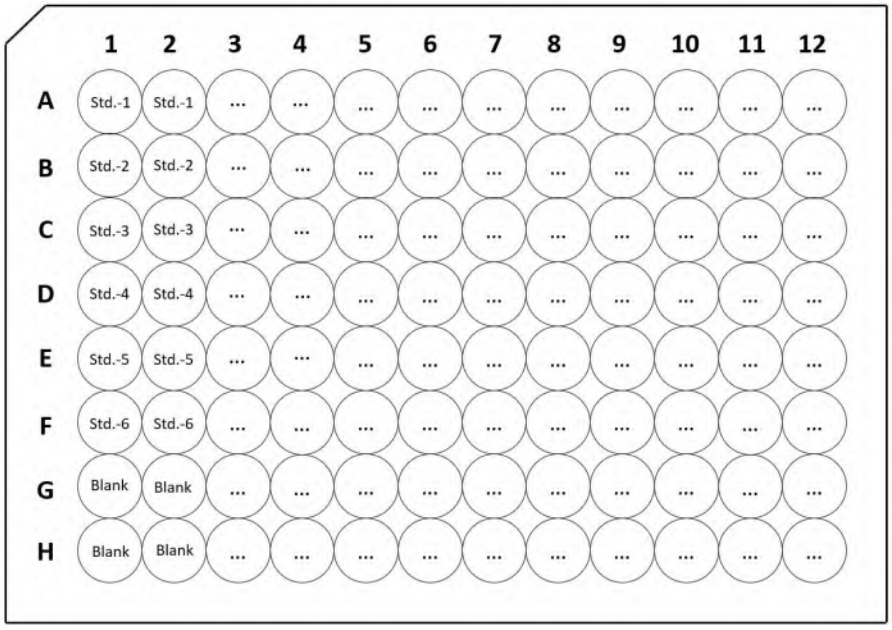
INTERFERING SUBSTANCES

Verify potential matrix effects by adding different levels of DMSO and HSA to the diluted buffer.

Additive	Tolerated concentration
DMSO	10%
HSA	5%

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

PLATE LAYOUT



Note: *Blank is a Blank Dilution Buffer hole.*

TROUBLESHOOTING GUIDE

Problem	Cause	Solution
Poor standard curve	* Inaccurate pipetting	* Check pipettes
Large CV	* Inaccurate pipetting * Air bubbles in wells	* Check pipettes * Remove bubbles in wells
High background	* Plate is insufficiently washed * Contaminated wash buffer	* Review the manual for proper wash. * Make fresh wash buffer
Very low readings across the plate	* Incorrect wavelengths * Insufficient development time	* Check filters/reader * Increase development time
Samples are reading too high, but standard curve looks fine	* Samples contain cytokine levels above assay range	* Dilute samples and run again

US and Canada: Tel: +1 800-810-0816
Asia and Pacific: Tel: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com

Drift	* Interrupted assay set-up * Reagents not at room temperature	* Assay set-up should be continuous - have all standards and samples prepared appropriately before commencement of theassay * Ensure that all reagents are at room temperature before pipetting into the wells unless otherwise instructed in the antibody inserts

US and Canada: **Tel**: +1 800-810-0816
Asia and Pacific: **Tel**: +86 400-682-2521
Web: http://www.acrobiosystems.com
E-mail: order@acrobiosystems.com