LumiAb™

Human 4-1BB-R
Chemiluminescent ELISA Kit

Catalog #: Lum-8100

Detection and Quantification of Human 4-1BB-R Concentrations in Cell Lysates, Sera and Plasma.

Please read the provided manual as suggested experimental protocols may have changed.

Research Purposes Only. Not Intended for Diagnostic or Clinical Procedures.

CONTENTS PAGE

INTRODUCTION

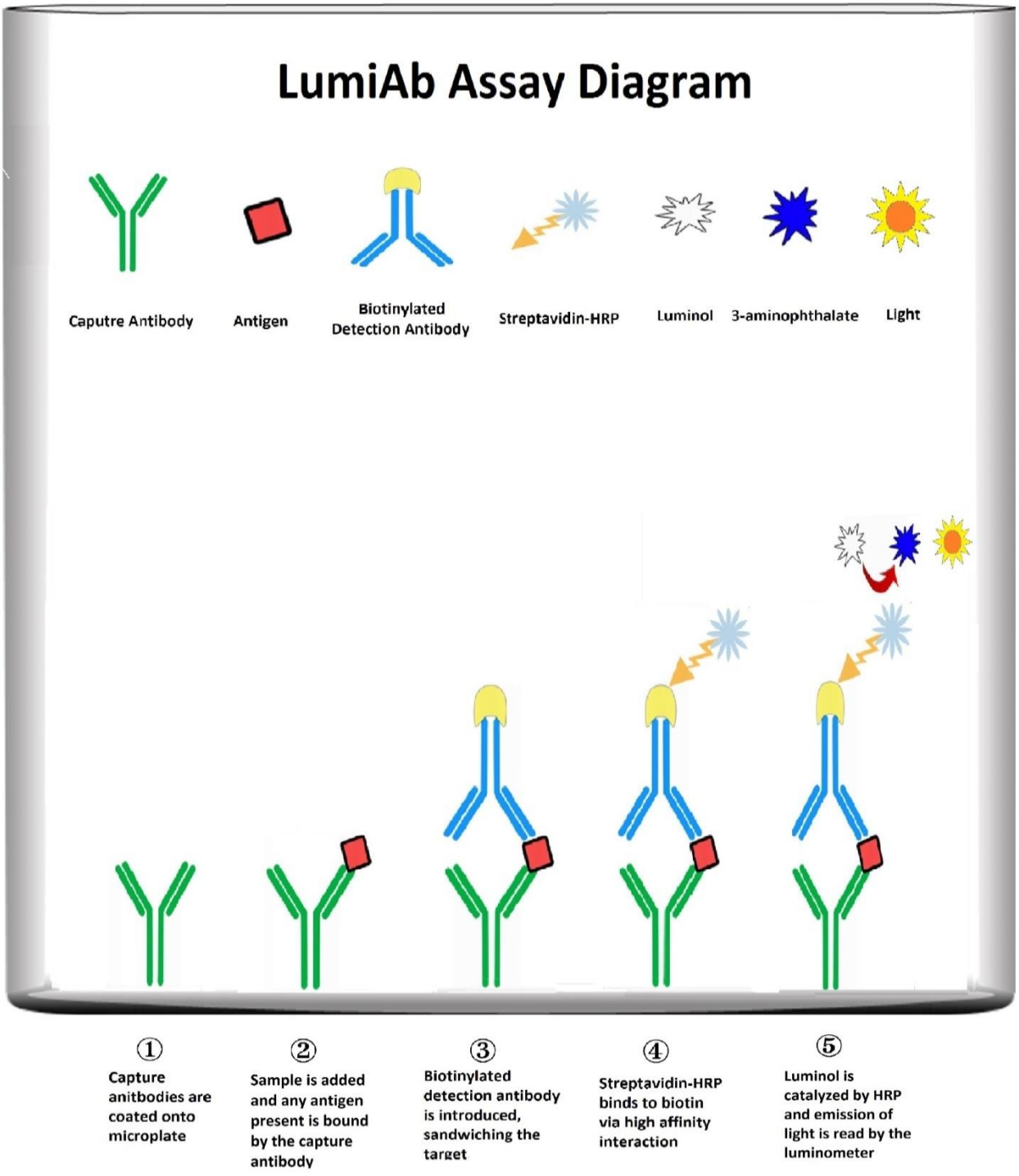
Human 4-1BB Ligand Receptor, also known as the Tumor Necrosis Factor Receptor Superfamily Member 9, is a 255 amino acid cytokine protein implicated in T-cell activation pathways. After initial synthesis of the peptide, the cytokine is proteolytically cleaved by proteases, thus separating the 23 residue signal sequence from the actual mature 232 residue 4-1BB Receptor sequence. The protein is expressed from the TNFRSF9 gene located at locus 1p36 on chromosome 1. The 4-1BB receptor or TNFRSF9 inherently has high affinity binding for the 4-1BB ligand such that upon binding, the receptor contributes to clonal expansion, survival and development of T-cells. It can also induce proliferation in peripheral monocytes, enhance T-cell apoptosis induced by TCR/CD3 triggered activation and regulate CD28 co-stimulation to promote Th1 cell responses. The expression of this receptor is induced by lymphocyte activation. TRAF adaptor proteins such as TRAF1, TRAF2 and TRAF 3 have been shown to bind to this receptor and transducer the signals leading to activation of NF-κB.

Source: Entrez Gene: TNFRSF9 tumor necrosis factor receptor superfamily, member 9 [Homo sapiens]; Swiss-Prot: Q07011

ASSAY PRINCIPLES

The LumiAb™ Human 4-1BB-R Chemiluminescent ELISA Kit contains the components necessary for quantitative determination of natural or recombinant Human 4-1BB-R concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator. The capture antibodies coated to the bottom of each well are specific for a particular epitope on Human 4-1BB-R while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase. After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a sensitive luminescent reaction to ensue upon substrate addition. When the Peroxide Enhancer solution is added, the reaction catalyzed by peroxidase yields light that is representative of the antigen concentration. After a brief incubation, the microplate can be read with a luminometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

ASSAY FORMAT



ASSAY RESTRICTIONS

- This Lum-ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

MATERIALS INCLUDED

Component	Quantity Per Plate	Container
Microstrips Coated w/ Capture Antibody	12 x 8-Well Microstrips	-
Protein Standard	Lyophilized	Red
100^{X} Biotinylated Detection Antibody	Lyophilized	Yellow
400^{X} Streptavidin-HRP	30 µl	Blue
Wash Buffer (15^{X})	50 ml	Clear
Assay Diluent	50 ml	Clear
Enhancer Solution	8 ml	Brown
Peroxide	8 ml	Clear
Adhesive Plate Sealers	2 Sheets	-
Technical Manual	1 Manual	-

ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Luminometer able to measure total light output
- Micropipettes with capability of measuring volumes ranging from 1 µl to 1 ml
- Sterile deionized water
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.

STORAGE INFORMATION

Note: If used frequently, reagents may be stored at 4°C.

Unopened Kits: Store at 4°C for 6 months.

Component	Storage Time	Storage Information
Microstrips Coated w/ Capture Antibody	6 Months	4°C
400^{x} Streptavidin-HRP		
Wash Buffer (15^{x})		
Assay Diluent		
Enhancer Solution		
Peroxide Solution		
Protein Standard	Lyophilized: 6 Months Reconstituted: 1 Month	4°C
Biotinylated Detection Antibody		
Adhesive Plate Sealers	-	-
Technical Manual	-	-

SAMPLE PREPARATION AND STORAGE

If samples are to be used within 24 hours, aliquot and store at 4°C. If samples are to be used over a long period of time, aliquot and store between -20°C and -80°C, depending on the duration of storage.

Note: Samples containing a visible precipitate or pellet must be clarified prior to use in the assay.

Caution: Avoid repeated freeze/thaw cycles to prevent loss of biological activity of proteins in experimental samples.

Cell Lysate and Supernatants

Remove large cell components via centrifugation and perform the assay. Cell lysates and supernatants require a dilution using Assay Diluent. A serial dilution may be performed to determine a suitable dilution factor for the sample.

Serum Preparation

Allow samples to clot in a serum separator tube (SST) for 30 minutes. After sufficient clotting, centrifuge at 1000 x g for 15 minutes and remove serum from SST in preparation for the assay. A serial dilution may be performed to determine a suitable dilution factor for the sample.

Plasma Preparation

Use heparin, citrate or EDTA as an anticoagulant to gather plasma from original biological sample. After collection of the plasma, centrifuge for 15 minutes at 1000 x g. This step must be performed within 30 minutes of plasma collection.

Serum and Plasma Sample Dilution Recommendation

Dilute the plasma or serum samples with 10-50% animal serum in PBS. Do not reconstitute or dilute the detection antibody or Streptavidin-HRP in the buffer with animal serum. However, it is important to use the same diluent for the samples and the standard so it reflects the same environment of the samples being measured.

SAMPLE EXPERIMENT LAYOUT

	1	2	3	4	5	6
A	Standard (High Point)	Standard (High Point)	Standard (High Point)	Sample	Sample	Sample
B	Standard (1:2)	Standard (1:2)	Standard (1:2)	Sample	Sample	Sample
C	Standard (1:4)	Standard (1:4)	Standard (1:4)	Sample	Sample	Sample
D	Standard (1:8)	Standard (1:8)	Standard (1:8)	Sample	Sample	Sample
E	Standard (1:16)	Standard (1:16)	Standard (1:16)	Sample	Sample	Sample
F	Standard (1:32)	Standard (1:32)	Standard (1:32)	Sample	Sample	Sample
G	Standard (1:64)	Standard (1:64)	Standard (1:64)	Sample	Sample	Sample
H	Negative Control	Negative Control	Negative Control	Sample	Sample	Sample

IMMUNOASSAY PROTOCOL

Note: If possible, all incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells. Also, all provided solutions should be at ambient temperature prior to use.

Reconstitution of Provided Materials

1. Reconstitute the lyophilized Biotin-Conjugated Detection Antibody with 100 µl of Assay Diluent to get 100^{x} Biotin-Conjugated Detection Antibody. Store it at 4°C if it not used immediately or completely.

2. Reconstitute the Protein Standard with 500 µl of Assay Diluent for a concentration of 1000 pg/ml. Store it at 4°C if it is not used immediately or completely.

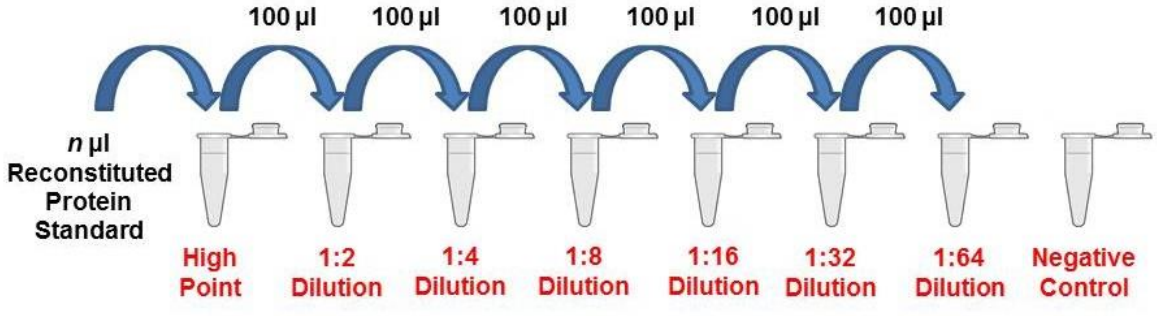
3. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH_2O and 1 volume of 15x Wash Buffer. Use as necessary.

Addition of Known Standard and Unknown Sample to Immunoassay

The LumiAb™ Human 4-1BB-R ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human 4-1BB-R proteins as low as 8 pg/ml or beyond.

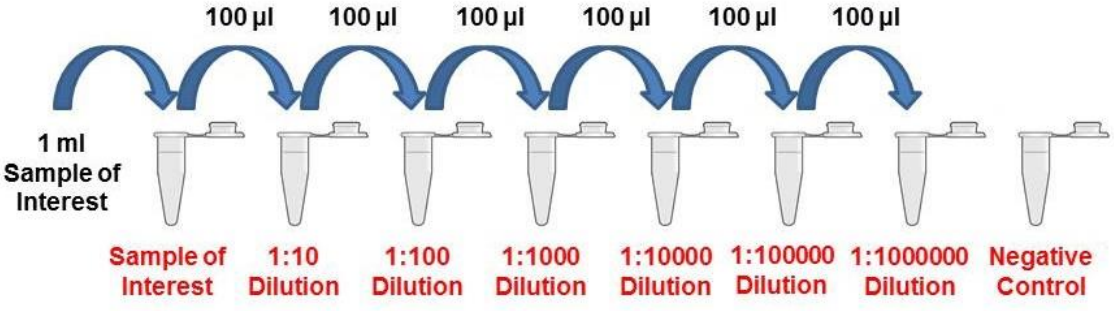
1. Dilute the known standard sample from 500 pg/ml to 0 pg/ml in a series of microfuge tubes using Assay Diluent. Mix each tube thoroughly by inverting several times or by vortexing lightly to ensure proper equilibration. Add 100 µl of each serial dilution step into the wells of a specified row or column of the 96-well microtiter plate in duplicate or triplicate and incubate at room temperature for 2 hours. Unknown samples of interest can be serial diluted with Assay Diluent to concentrations within the detection range of this assay kit and added to the plate at 100 µl per well. Seal the microplate air-tight using one of the microplate adhesive seals provided in this kit or Parafilm if readily available. See the following figure for serial dilution diagram.

To obtain serial dilution high point, dilute reconstituted Protein Standard to the maximum concentration for serial dilution with Assay Diluent. Determine how much volume is needed for the High Point (100ul/well) and dilute as necessary from the given reconstituted concentration (1000pg/ml) to a desired concentration (500pg/ml). Pipette *double* this amount into the High Point tube in order begin a 7 point 2-fold pattern by serial dilution in a similar fashion as illustrated below.



Starting:	200 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl
Remaining:	100 µl	100 µl	100 µl	100 µl	100 µl	100 µl	200 µl	100 µl

For samples of unknown protein concentrations, serial dilute the experimental sample using Assay Diluent to determine range of detection and acceptable dilutions. Shown below is a diagram illustrating a 10-fold serial dilution on a given sample of interest.



Starting:	1000 µl	900 µl	900µl	900 µl	900 µl	900 µl	900 µl	100 µl
Remaining:	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl	1000 µl	100 µl

Addition of Detection Antibody to Capture Antibody-Bound Samples

2. Aspirate the protein standard solution out of the microplate wells. If your lab does not have a vacuum-based aspirator, you may dump the solutions from the microplate into a waste container and blot 3-4 times on a stack of paper towels until most or all of the liquid is removed from the wells. Add 300 µl of 1^x Wash Buffer to each well being used and gently shake for 3-4 minutes on an orbital shaker. Perform this wash step 4 times consecutively.

3. After the 4^{th} wash step, dilute the detection antibody solution 100^x to 1^x with Assay Diluent. Ensure that there is enough detection antibody solution for all wells being used Mix the test tube either by inverting several times or vortexing to ensure proper equilibration. Ensure that there is enough detection antibody solution for all wells being used. Add 100 µl of the diluted detection antibody solution into each well, seal the plate and incubate at room temperature for 2 hours.

Conjugation of Streptavidin-HRP to Biotinylated Detection Antibody

4. Remove the detection antibody solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Perform 4 consecutive wash steps with gentle shaking between each wash.

5. Dilute the 400^x Streptavidin-HRP with Assay Diluent to a 1^x Streptavidin-HRP solution.

6. After the 4^{th} wash step, add 100 µl of 1^x Streptavidin-HRP solution into each well and incubate at room temperature for 30 minutes.

Application of Liquid Substrate for Luminescent Reaction

7. Remove the 1^x Streptavidin-HRP solution out of the microplate wells by either vacuum-based aspirator or paper towel blotting. Perform 4 consecutive wash steps with gentle shaking between each wash. Prepare the Peroxide and Enhancer Solution by bringing it to room temperature without exposure to light as these may degrade the substrate. Mix the Peroxide and Enhancer solution in a 1:1 ratio andstore in room temperature

8. After the 4th wash step, add 100 µl of the Peroxide/Enhancer solutions into each well and incubate at room temperature for light development for 5 minutes. The microplate should be kept out of direct light by either covering with an opaque object or putting it into a dark room. The microplate is now ready to be read by the luminometer.

Generation of Standard Curve and Interpretation of Data

9. Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard light units.

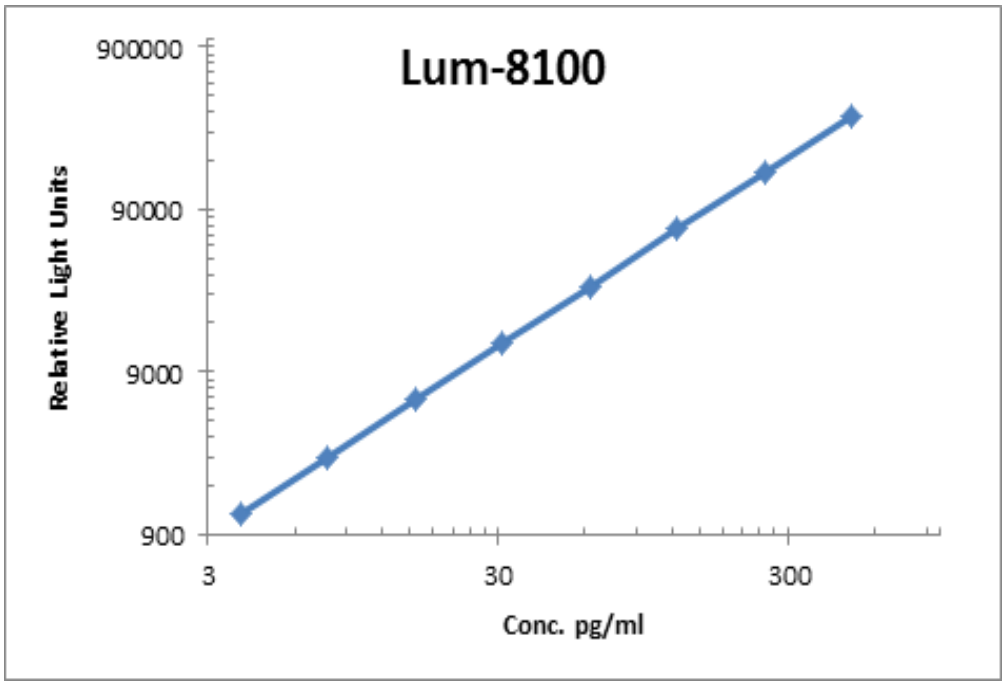
Generate a standard curve by using Microsoft Excel or other computer software capable of establishing a 4-Parameter Logistic (4-PL) curve fit. If using Excel or an alternative graphing tool, plot the average optical density values in absorbance units (y-axis) against the known standard concentrations in pg/ml (x-axis).

Note: Only use the values in which a noticeable gradient can be established. Afterwards, generate a best fit curve or "trend-line" through the plotted points via regression analysis.

Note: Shown on the next page is an example of typical data produced by analysis of the standard sample.

The data and subsequent graph was obtained after performing a cytokine ELISA for Human 4-1BB-R. Each known sample concentration was assayed in triplicate.

Concentration pg/ml	Relative Light Units
500	343664
250	153262
125	68349
62.5	30481
31.25	13593
15.625	6062
7.8125	2703
3.90625	1205



SUMMARIZED PROTOCOL

Reconstitute Biotinylated Detection Antibody and Protein Standard and dilute the 15x Wash Buffer as specified.

Perform serial dilution of Protein Standard and prepare samples as desired. See sample preparation section for instructions to dilute serum and plasma samples.

Add 100ul of Protein Standard, sample or control to each well and incubate for 2 hours at room temperature.

Aspirate Protein Standards, samples or controls out and wash plate 4 times.

Dilute Biotinylated Detection Antibody as specified. Add 100ul to each well and incubate for 2 hours at room temperature.

Aspirate Biotinylated Detection Antibody out and wash plate 4 times.

Dilute 400x Streptavidin-HRP as specified. Add 100ul of 1x Streptavidin-HRP to each well and incubate at room temperature for 30 minutes.

Aspirate 1x Streptavidin-HRP out and wash plate 4 times.

Add 100ul of the Peroxide/Enhancer Solution to each well and shake at room temperature for 5 minutes for light development.

SENSITIVITY

The Human 4-1BB-R ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human 4-1BB-R proteins as low as 8 pg/ml.

CROSS REACTIVITY AND SPECIFICITY

The LumiAb™ Human 4-1BBR ELISA is capable of recognizing both recombinant and naturally produced Human 4-1BBR proteins. The antigens listed below were tested at 50 ng/ml and did not exhibit significant cross reactivity or interference.

• Human: 4-1BBL, AITRL, BAFF, BAFF Receptor, BCMA, CD40 Ligand/TRAP, Fas Receptor, LIGHT, OPG, sOX40L, sRANK (Receptor), sRANKL, TACI, TL-1A, TNF-alpha, TNF-beta, sTNF-Receptor Type I, sTNF-Receptor Type II, sTRAIL/Apo2L, sTRAIL Receptor-1, sTRAIL Receptor-2, TWEAK, TWEAK Receptor

TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.assaybiotechnology.com or contact us at:

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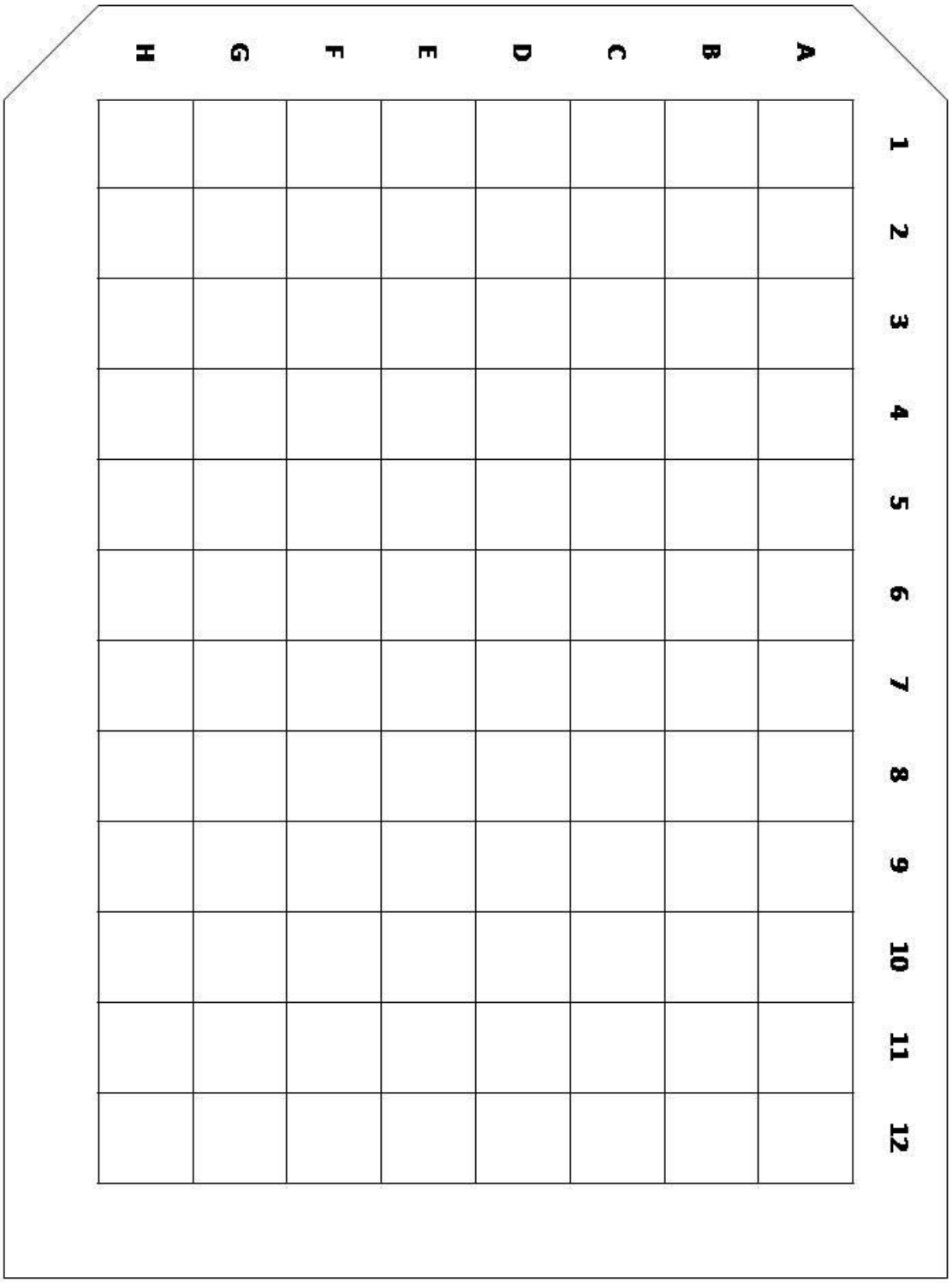
Manual Version 1.3

ELISA PLATE TEMPLATE



NOTES

Over 2,000 Assay Kits including Sandwich, Cell-Based and Transcription Factor ELISA Kits

Visit us at www.AssayBiotechnology.com