OmniKine™

Human 4-1BB-R
Colorimetric Sandwich ELISA Kit

Catalog #: OK-0100

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

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

Store entire kit at 4°C until use.
Kit expiration is 6 months from date of shipment

Manual Version: 89.741.254

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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.

ASSAY PRINCIPLES

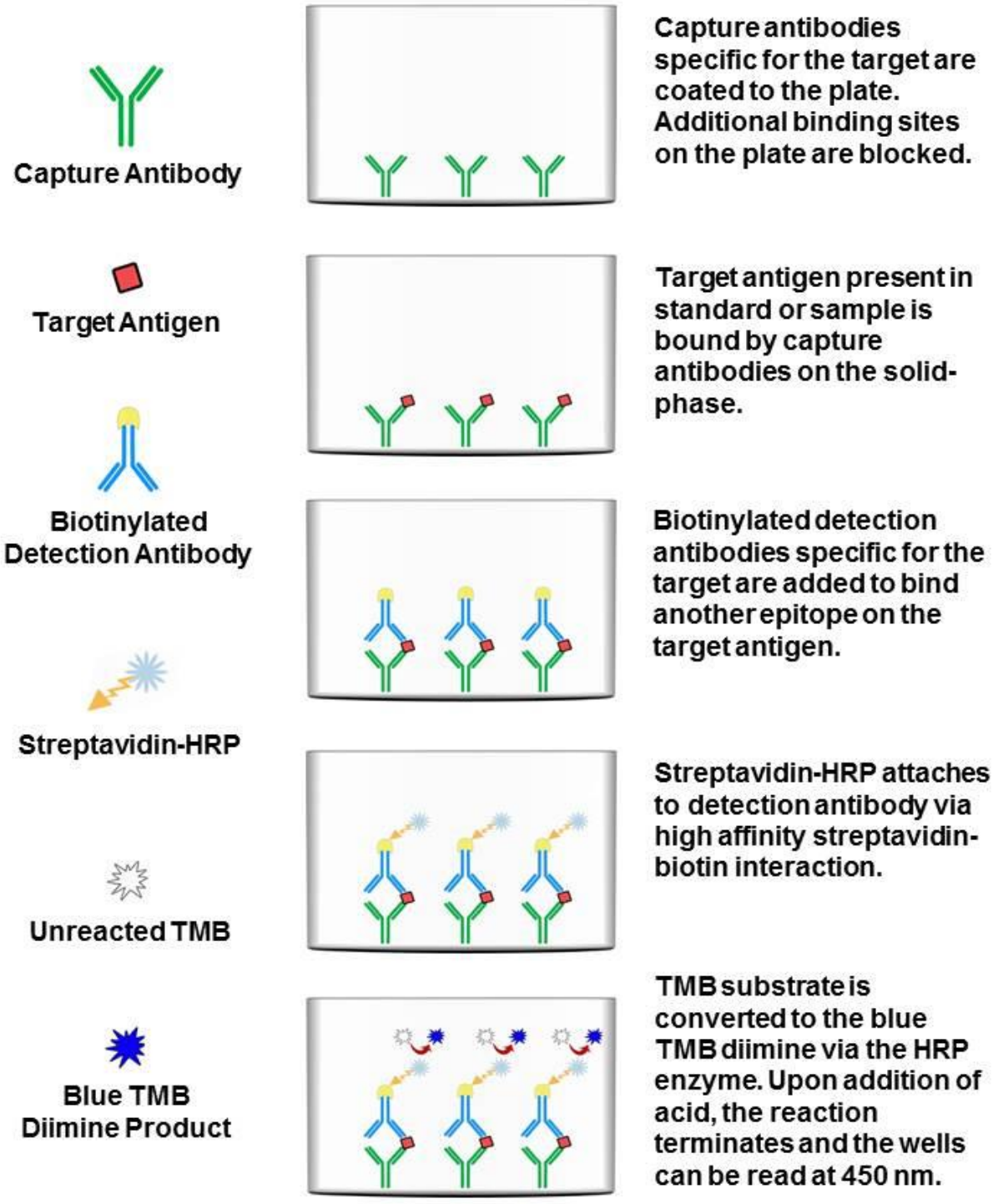
The OmniKine™ Human 4-1BB-R ELISA Kit contains the components necessary for quantitative determination of natural or recombinant Human 4-1BB-R concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” 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 colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3’, 5, 5’-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration.

Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

ASSAY FORMAT



MATERIALS INCLUDED

Component	Quantity Per Kit	Storage/Stability after first use
Microstrips Coated w/ Capture Antibody	12 x 8-Well Microstrips	1 month at 4°C
Protein Standard	Lyophilized	
Biotinylated Detection Antibody	Lyophilized	
400x Streptavidin-HRP A	30 µl	
Wash Buffer (15x)	60 ml	
Assay Diluent 45HB	15 ml	
Assay Diluent 1TD	50 ml	
Ready-to-Use Substrate	12 ml	
Stop Solution	12 ml	
Adhesive Plate Sealers	2 Sheets	-
Technical Manual	1 Manual	-

Any unused strips should be rewrapped with plate sealer and placed back into pouch with zipper closed until next use**

ADDITIONAL MATERIALS REQUIRED

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

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm)
- Micropipettes ranging from 1 µl to 1 ml
- Distilled, deionized, and or purified water (recommended TOC 1-50 ppb, MΩ-cm 18.0)
- 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)

SAMPLE PREPARATION AND STORAGE

Sample Type	Recommended Dilution Factor
Plasma	1 to 4
Serum	1 to 4
Cell Culture Supernatant	1 to 4

Levels of Human 4-1BB-R may vary between samples. Optimal dilution factors for every sample must be determined by the investigator.

Cell Supernatants

Remove large cell components via centrifugation and perform the assay. Dilute cell supernatants at a minimum of 1 to 4 using Assay Diluent 1TD and assay.

Serum

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. Dilute serum samples 1 to 4 using Assay Diluent 45HB and incubate 20 mins room temperature (RT) prior to continuing with the assay.

Plasma

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. Dilute plasma samples 1 to 4 using Assay Diluent 45HB and incubate 20 mins prior to RT prior to continuing with the assay.

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.

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

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

REAGENT PREPARATION

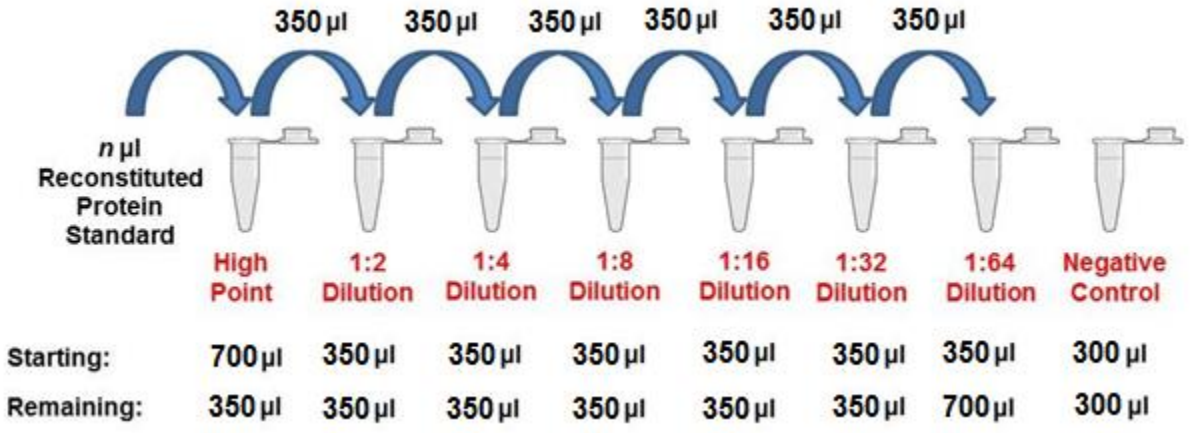
All provided solutions should be at ambient temperature prior to use. **We recommend performing the assay in duplicate**. Reagents provided are enough to assay 96 wells and it is recommended to only prepare as much needed on the day of the experiment. All incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells.

1. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH2O and 1 volume of 15x Wash Buffer.

2. Reconstitute Detection Antibody with 100 µl of ddH2O for a concentration of 100x. Mix gently and dilute to 1x prior to use with Assay Diluent 1TD.

3. Reconstitute Protein Standard with 83 µl of ddH2O for a concentration of 250 ng/mL Mix gently and dilute to the working range of the kit, 32-2000 pg/mL. To obtain a 7 point 2 fold dilution for the standard curve 5.6 µl of standard into 694.4 µl of diluent.

    a. Dilute Protein Standard with Assay Diluent 45HB when experimenting with Serum/Plasma samples. **OR**.
    b. Dilute Protein Standard with Assay Diluent 1TD when experimenting with Cell Supernatant samples.



4. Mix the 400x Streptavidin-HRP A (SAV-HRP) gently. Dilute to 1x using Assay Diluent 1TD.

5. Stop Solution and Ready to Use Substrate are ready to use and do not need dilution.

ASSAY PROCEDURE

1. Prepare all reagents to working concentrations, standards to desired range, and samples to appropriate dilution factors.

2. Remove desired number of capture antibody coated strips for experiment and place remaining strips back into dry pouch with desiccant for 4°C storage.

3. Add 100 µl of Standards/ Samples to each well and incubate on orbital shaker at room temperature (RT) for 2 hrs.

4. Aspirate the solution and add 300 µl of 1x Wash Buffer to each well being used and gently shake for 2-3 mins on an orbital shaker. Repeat this process 3 times. After the last wash ensure no liquid remains by inverting the plate and tapping it against clean paper towels.

5. Add 100 µl of 1x Detection Antibody to each well and incubate for 2 hrs. on an orbital shaker at RT.

6. Repeat step 4.

7. Add 100 µl of 1x SAV-HRP A to each well and incubate for 30 mins on an orbital shaker at RT

8. Repeat step 4.

9. Add 100 µl of Ready to use Substrate to each well, cover plate from light, and incubate for 10 mins on an orbital shaker at RT.

10. Add 100 µl of Stop Solution to each well and read at 450 nm.

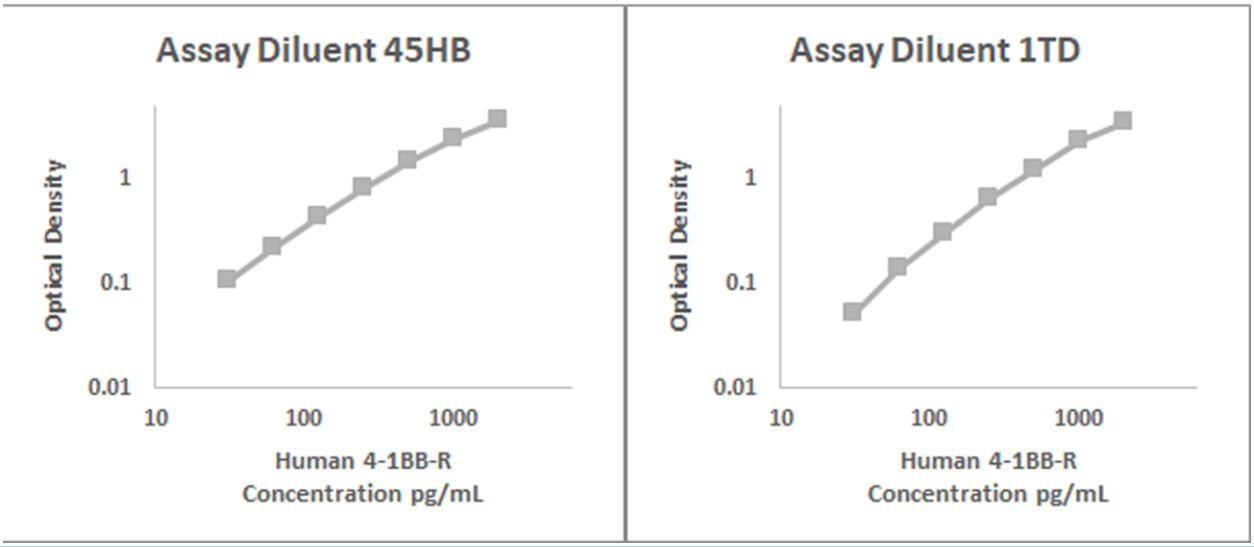
DATA ANALYSIS

Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard optical density.

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).

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.

TYPICAL DATA



Concentration pg/ml	2000.00	1000.00	500.00	250.00	125.00	62.50	31.25
Average Optical Density 45HB	3.66	2.47	1.48	0.80	0.43	0.21	0.11
Average Optical Density 1TD	3.48	2.32	1.23	0.64	0.30	0.14	0.05

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

The standard curves shown are for demonstration. A new curve must be generated for each experiment.

RECOVERY

Three concentrations of recombinant Human 4-1BB-R was spiked into various samples to measure the recovery percentage of recombinant protein in duplicate.

Sample Type	Average Recovery %	Range %
Plasma (25%)	95	92-97
Serum (25%)	96	93-101
RPMI 10% FBS (25%)	99	86-102

LINEARITY

Human 4-1BB-R recombinant protein was spiked into various biological samples and serial diluted with Assay Diluent 45HB for serum samples and Assay Diluent 1TD for RPMI 10% FBS.

		EDTA Plasma	Serum	RPMI 10% FBS
Neat	pg/ml	969.74	1019.27	1015.13
	Expected %	97.00	102.00	102.00
1 to 2	pg/ml	461.68	193.99	491.02
	Expected %	99.00	92.00	98.00
1 to 4	pg/ml	232.85	235.52	256.24
	Expected %	94.00	94.00	102.00
1 to 8	pg/ml	118.46	119.99	131.41
	Expected %	96.00	96.00	105.00

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 within the range of 32-2000 pg/ml.

CROSS REACTIVITY AND SPECIFICITY

The OmniKine™ 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

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 Ready-to-Use Substrate to each well and incubate at room temperature for color development.

Add 100ul of Stop Solution and read plate at 450nm.

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.

- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

ASSAY RESTRICTIONS

- This 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.

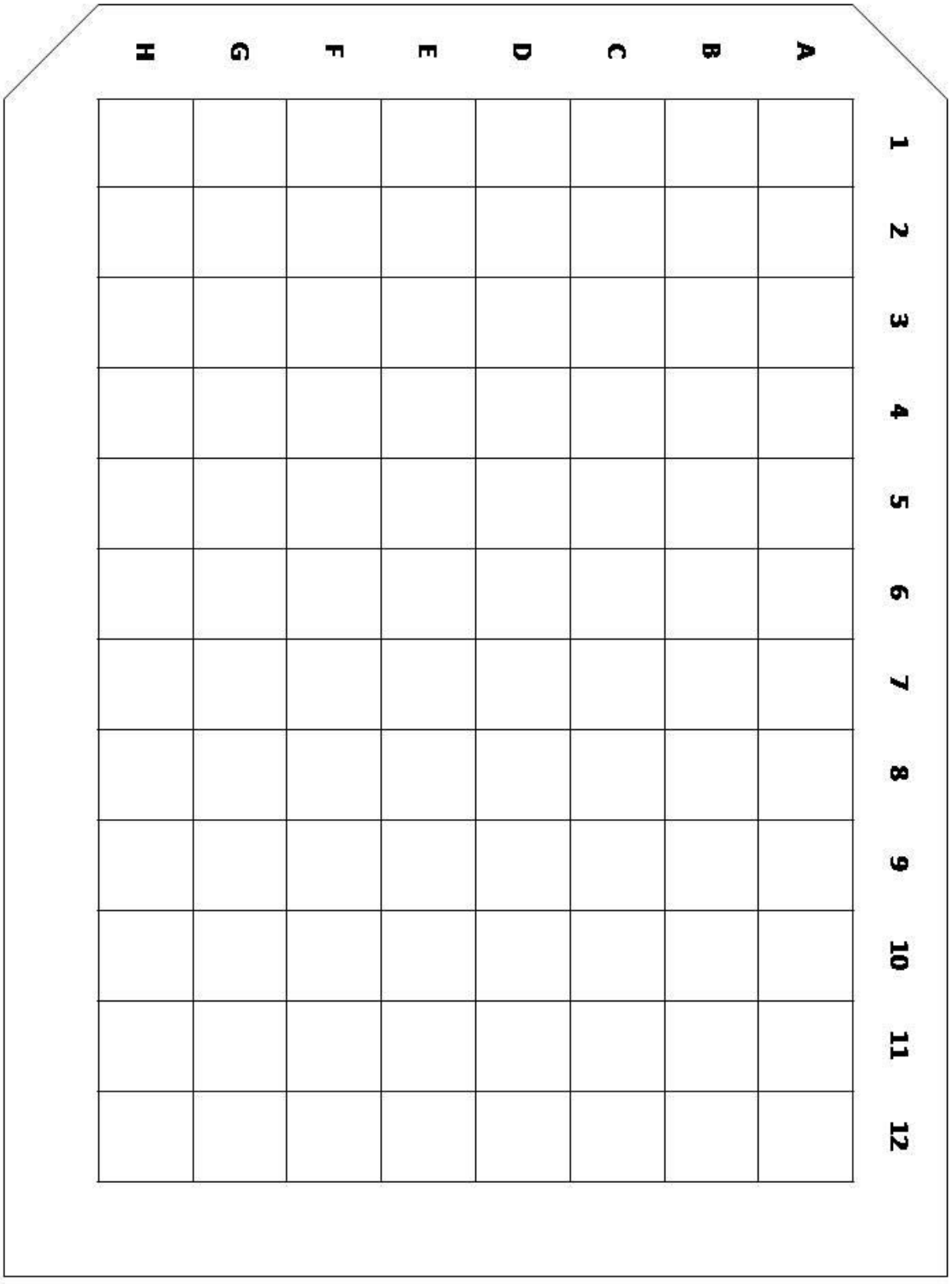
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.

- 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.

- Individual results may vary due to differences in technique, plasticware and water sources.

ELISA PLATE TEMPLATE



TECHNICAL SUPPORT

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