Quantikine® ELISA

Human/Cynomolgus Monkey B7-H1/PD-L1 Immunoassay

Catalog Number DB7H10

For the quantitative determination of human and cynomolgus monkey B7 Homolog 1 (B7-H1) concentrations in cell culture supernates, cell lysates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

TABLE OF CONTENTS

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INTRODUCTION

B7 Homolog 1 (B7-H1), also known as PD-L1 and CD274, is an approximately 65 kDa transmembrane glycoprotein in the B7 family of immune regulatory molecules (1). Mature human B7-H1 consists of a 220 amino acid (aa) extracellular domain (ECD) with two immunoglobulin-like domains, a 21 aa transmembrane segment, and a 31 aa cytoplasmic domain (2). Within the ECD, human B7-H1 shares 96%, 73%, and 74% aa sequence identity with cynomolgus monkey, mouse, and rat B7-H1, respectively. Alternative splicing generates additional isoforms that either lack the first Ig-like domain or are truncated within the second Ig-like domain (3). B7-H1 is expressed on inflammatory-activated immune cells including macrophages, T cells, and B cells (4-7), keratinocytes (8, 9), endothelial and intestinal epithelial cells (8, 10), as well as a variety of carcinomas and melanoma (11, 12). B7-H1 binds to T cell B7-1/CD80 and PD-1 (7, 8, 12-15). It suppresses T cell activation and proliferation (5, 8, 14, 16) and induces the apoptosis of activated T cells (11). It plays a role in the development of immune tolerance by promoting T cell anergy (7, 14) and enhancing regulatory T cell development (16). B7-H1 favors the development of anti-inflammatory IL-10 and IL-22 producing dendritic cells (5, 10) and inhibits the development of Th17 cells (16). In cancer, B7-H1 provides resistance to T cell mediated lysis, enhances epithelial to mesenchymal transition, and enhances the tumorigenic function of Th22 cells (6, 9, 12, 15). A soluble form of B7-H1 is elevated in the plasma of cancer patients and in the cerebrospinal fluid in glioma (17-22). It can be released by mature dendritic cells and retains the ability to bind PD-1 and induce T cell apoptosis (21, 23, 24).

The Quantikine® Human/Cynomolgus Monkey B7-H1/PD-L1 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human/cynomolgus monkey B7-H1 in cell culture supernates, cell lysates, serum, plasma, and urine. It contains HEK293-expressed recombinant human B7-H1 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human/cynomolgus monkey B7-H1 showed linear curves that were parallel to the standard curves obtained using the Quantikine® kit standards. These results indicate that this kit can be used to determine relative mass values for naturally occurring human/cynomolgus monkey B7-H1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human B7-H1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any B7-H1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human B7-H1 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of B7-H1 bound in the initial step. The color development is stopped and the intensity of the color is measured.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine® Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human/Cynomolgus Monkey B7-H1 Microplate	894883	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human B7-H1.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human/Cynomolgus Monkey B7-H1 Standard	894885	2 vials of recombinant human B7-H1 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new Standard for each assay. Discard after use.
Human/Cynomolgus Monkey B7-H1 Conjugate	894884	21 mL of a polyclonal antibody specific for human B7-H1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-41	895514	12 mL of a buffered protein base with preservatives.	
Assay Diluent RD1-54	895321	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-33	895813	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:3 in this assay.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution of standards and samples.
- Human/Cynomolgus Monkey B7-H1 Controls (optional; R&D Systems, Catalog # QC226).

If using cell lysate samples, the following is also recommended:

- Sample Diluent Concentrate 2 (2X) (R&D Systems, Catalog # DYC002).

PRECAUTIONS

The protein used in the standard is purified from human cells. As no processing or testing can offer complete assurance of freedom from infectious agents, these reagents should be handled as if capable of transmitting infection.

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

Human 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.

Human Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed and lipemic samples are not suitable for use in this assay.

Human Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cynomolgus Monkey Serum & EDTA Plasma - Whole blood samples were collected from non-medicated and non-immunized cynomolgus monkeys.

SAMPLE PREPARATION

For cell lysate samples, quantitation of sample protein concentration using a total protein assay is recommended. The suggested range for total cell lysate protein added is ≤ 10 µg/well.

If using DYC002 (1X) as a lysis buffer, refer to the DYC002 insert for the preparation of Lysis Buffer 9 (DYC002 (1X) plus the addition of protease inhibitors). Prepare the cell lysate using Lysis Buffer 9 then dilute at least 1:10 prior to assay.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

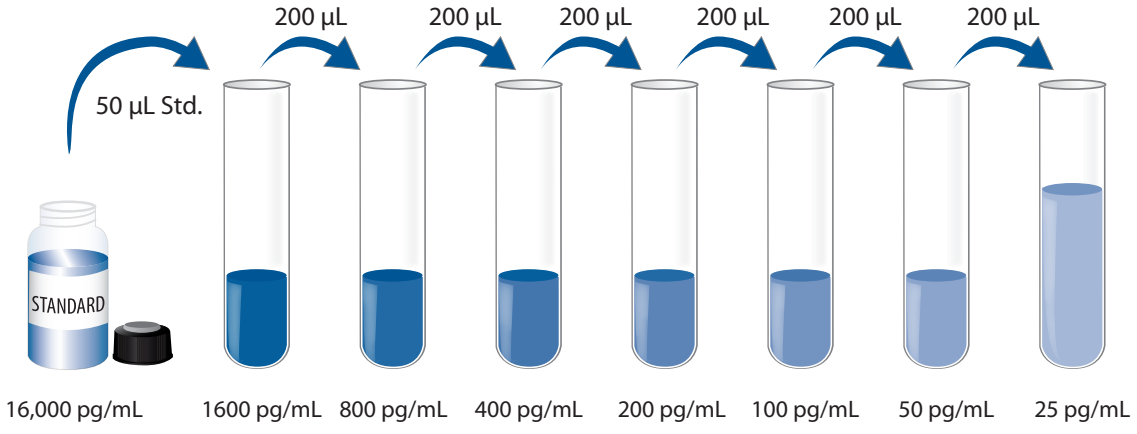
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-33 (diluted 1:3) - Add 10 mL of Calibrator Diluent RD5-33 to 20 mL of deionized or distilled water to prepare 30 mL of Calibrator Diluent RD5-33 (diluted 1:3).

Human/Cynomolgus Monkey B7-H1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human/Cynomolgus Monkey B7-H1 Standard with deionized or distilled water. This reconstitution produces a stock solution of 16,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5-33 (diluted 1:3) into the 1600 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 1600 pg/mL standard serves as the high standard. Calibrator Diluent RD5-33 (diluted 1:3) serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-41 (*for cell culture supernate/cell lysate/serum/plasma samples*) or Assay Diluent RD1-54 (*for urine samples*) to each well.
4. Add 100 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided for a record of standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of Human/Cynomolgus Monkey B7-H1 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature **on the benchtop. Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

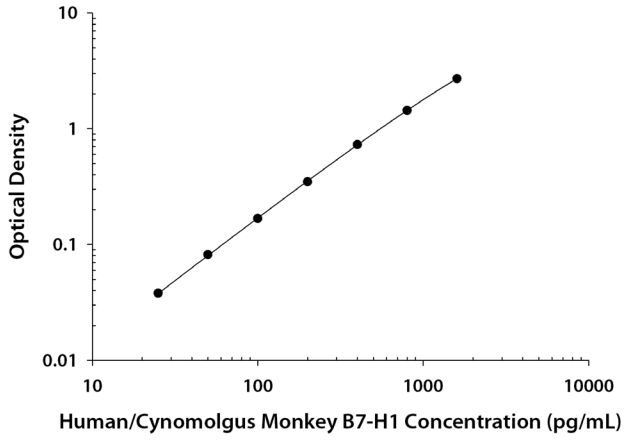
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human/cynomoglus monkey B7-H1concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted prior to assay, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

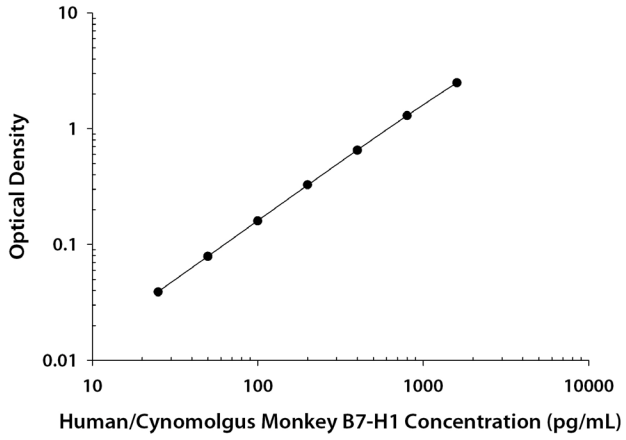
This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/CELL LYSATE/SERUM/PLASMA ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.011 0.012	0.012	—
25	0.050 0.050	0.050	0.038
50	0.093 0.095	0.094	0.082
100	0.179 0.181	0.180	0.168
200	0.351 0.370	0.361	0.349
400	0.738 0.743	0.741	0.729
800	1.442 1.458	1.450	1.438
1600	2.715 2.726	2.721	2.709

URINE ASSAY



(pg/mL)	O.D.	Average	Corrected
0	0.023 0.023	0.023	—
25	0.062 0.062	0.062	0.039
50	0.101 0.103	0.102	0.079
100	0.181 0.185	0.183	0.160
200	0.347 0.354	0.351	0.328
400	0.674 0.675	0.675	0.652
800	1.316 1.325	1.321	1.298
1600	2.511 2.522	2.517	2.494

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.

CELL CULTURE SUPERNATE/CELL LYSATE/SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	260	458	884	244	466	878
Standard deviation	4.25	6.83	14.7	15.9	25.8	66.4
CV (%)	1.6	1.5	1.7	6.5	5.5	7.6

URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	257	490	934	249	469	898
Standard deviation	3.75	7.01	12.7	11.1	21.7	37.7
CV (%)	1.5	1.4	1.4	4.5	4.6	4.2

RECOVERY

The recovery of human/cynomolgus monkey B7-H1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	97-112%
DYC002 (1X)* (n=1)	96	90-100%
Human serum (n=4)	89	81-100%
Cynomolgus monkey serum (n=3)	98	72-123%
Human EDTA plasma (n=4)	90	79-100%
Cynomolgus monkey EDTA plasma (n=4)	98	84-113%
Human heparin plasma (n=4)	85	76-95%
Human urine (n=4)	109	100-116%

*Sample was diluted 1:10 prior to assay.

LINEARITY

To assess the linearity of the assay, samples spiked with high concentrations of human B7-H1 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

Human Samples		Cell culture media (n=4)	DYC002 (1X)* (n=1)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	102	101	100	100	100	101
	Range (%)	98-104	———	96-103	97-107	93-106	100-103
1:4	Average % of Expected	100	104	104	104	105	101
	Range (%)	95-105	———	98-111	101-112	99-111	100-102
1:8	Average % of Expected	100	106	107	108	110	101
	Range (%)	94-106	———	103-114	106-115	102-118	98-103
1:16	Average % of Expected	96	104	108	109	114	100
	Range (%)	88-104	———	101-114	103-116	108-121	96-103

*Sample was diluted 1:10 prior to assay.

To assess the linearity of the assay, samples containing high concentrations of cynomolgus monkey B7-H1 were pooled and diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

Cynomolgus Monkey Samples		Serum (n=2)	EDTA plasma (n=2)
1:2	Average % of Expected	111	107
	Range (%)	110-112	106-107
1:4	Average % of Expected	119	108
	Range (%)	115-122	104-111
1:8	Average % of Expected	122	105
	Range (%)	115-129	102-108
1:16	Average % of Expected	112	96
	Range (%)	101-123	———

SENSITIVITY

Forty-five assays were evaluated and the minimum detectable dose (MDD) of human/cynomolgus monkey B7-H1 ranged from 0.369-4.52 pg/mL. The mean MDD using Assay Diluent RD1-41 was 1.51 pg/mL; the mean MDD using Assay Diluent RD1-54 was 1.81 pg/mL

The MDD was determined by adding two standard deviations to the mean O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified HEK293-expressed recombinant human B7-H1 manufactured at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human/cynomolgus monkey B7-H1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Human serum (n=36)	62.5	44.5-106	13.7
Cynomolgus monkey serum (n=10)	461	248-953	254
Human EDTA plasma (n=36)	56.2	39.1-108	14.2
Cynomolgus monkey EDTA plasma (n=10)	202	89.1-497	135
Human heparin plasma (n=36)	52.2	33.4-110	15.4

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Human urine (n=10)	34.5	10	ND-34.5

ND=Non-detectable

Cell Culture Supernates:
Human peripheral blood mononuclear cells were cultured in RPMI supplemented with 10% fetal bovine serum. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA for 6 days. Aliquots of the cell culture supernates were removed and assayed for human B7-H1.

Condition	(pg/mL)
Unstimulated	109
Stimulated	68.3

ND=Non-detectable

JEG-3 human epithelial choriocarcinoma cells (5 x 10^5) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Media was changed after 4 days and then collected on day 6. An aliquot of the cell culture supernate was removed, assayed for human B7-H1, and measured 142 pg/mL.

Cell Lysates - Human peripheral blood mononuclear cells were cultured in RPMI supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were washed and lysed with Lysis Buffer 9 the next day. 5 µg of the cell lysate was removed and assayed for human B7-H1, and measured 653 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human B7-H1. This assay also recognizes natural cynomolgus monkey B7-H1.

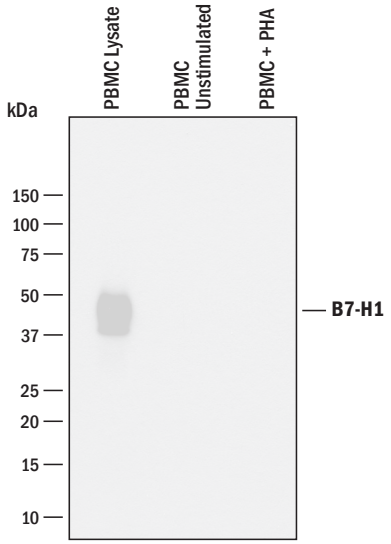
The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human B7-H1 control were assayed for interference. No significant cross-reactivity or interference was observed.

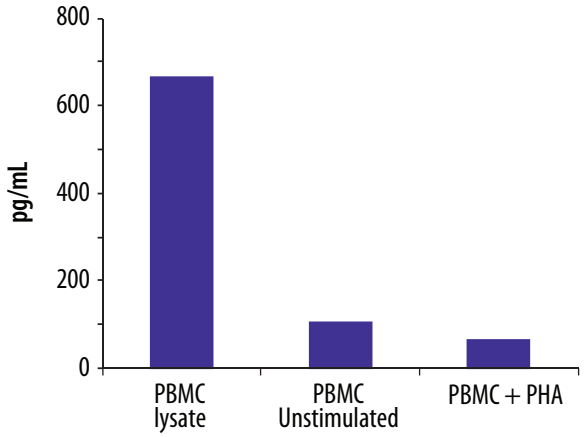
Recombinant human:

B7-1
B7-2
B7-H2
B7-H3
B7-H4
B7-H6
B7-H7
PD-1
PD-L2

Recombinant mouse:

B7-H1





Lysates from human PBMCs cultured overnight in RPMI-10% FBS, and conditioned media from human PBMCs unstimulated or stimulated with PHA for 6 days were analyzed by Western Blot and Quantikine® ELISA. Samples were resolved under reducing SDS-PAGE conditions, transferred to PVDF membrane, and immunoblotted with the detection antibody used in this kit. The Western blot band intensity shows a direct correlation with ELISA sample values. The conditioned media samples are below the level of detection in the Western blot.

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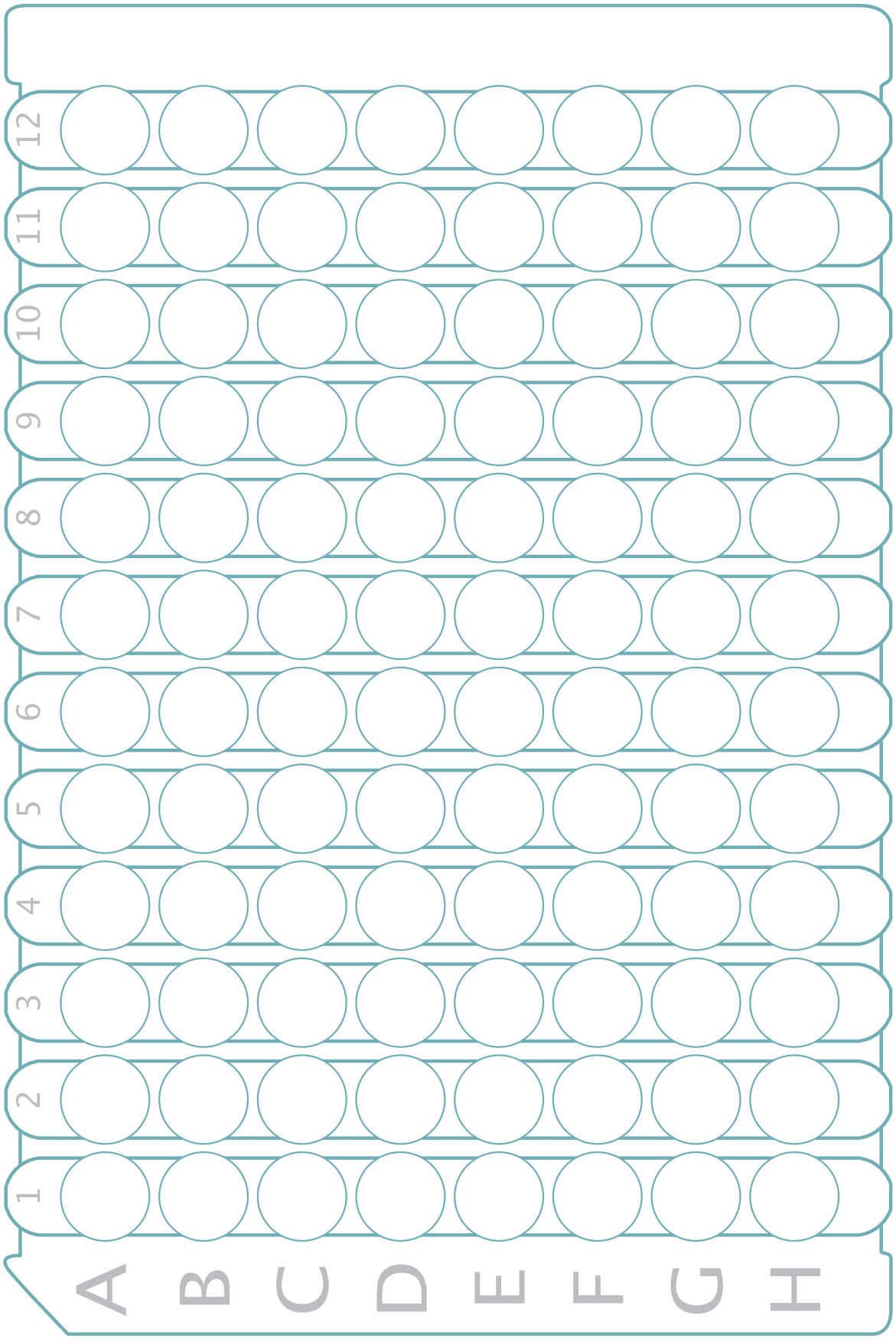
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES