Quantikine™ HS ELISA

Human CTLA-4 Immunoassay

Catalog Number HSCT40

For the quantitative determination of human Cytotoxic T-Lymphocyte-Associated molecule-4 (CTLA-4) 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.

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INTRODUCTION

Cytotoxic T-Lymphocyte-Associated molecule-4 (CTLA-4), also known as cluster of differentiation 152 (CD152) is located on band q33-q34 on chromosome 2 in humans, and band C of chromosome 1 in mice (1). CTLA-4 has over 100 single nucleotide polymorphisms (SNPs) associated with autoimmunity and cancer. SNPs in the CTLA-4 promoter have been correlated with a variety of cancers including breast cancer, cervical cancer, colorectal cancer, head and neck cancer, hepatocellular cancer, melanoma, non-small-cell lung carcinoma (NSCLC), renal cancer and more. The 223 amino acid (aa) protein product consists of several domains including: a 36 aa leader peptide, a 116 aa extracellular Ig V-like ligand binding domain, a 37 aa transmembrane domain and a 34 aa cytoplasmic domain. The cytoplasmic domain contains tyrosine motifs, at Y201 VKM and Y218 FIP. Several intracellular proteins bind Y201 VKM including: phosphatidyl-inositol 3 kinase (PI3-K), Src homology 2 domain containing protein tyrosine phosphatase (SHP-2), the serine threonine phosphatase PP2a and clathrin adaptor proteins activator proteins (AP1, AP2).The mature type 1 transmembrane glycoprotein has a native molecular weight of 33-37 kDa (2,3).

CTLA-4 plays a role in immune self-tolerance, which is important for avoiding autoimmune diseases. Unfortunately, this mechanism can be co-opted in order to suppress immune responses in the context of cancer. CTLA-4's highly studied role in the T cell response has been well described (4). CTLA-4 was originally identified as a gene that was specifically expressed by cytotoxic T lymphocytes. However, CTLA-4 transcripts have since been found in both T helper type 1 and 2 (Th1 and Th2) cells, and $CD4^+$ and $CD8^+$ T cell clones. CTLA-4 is a negative costimulatory molecule that is upregulated over the course of 2-3 days in T cells in response to T cell receptor (TCR) engagement with peptide major histocompatibility complex (pMHC) on antigen presenting cells. CTLA-4 recruitment to the immunological synapse is positively correlated with TCR signal strength. CTLA-4 outcompetes CD28, a positive costimulatory molecule, for the ligands B7-1 (CD80) and B7-2 (CD86) in order to attenuate T cell activation. CTLA-4 has a much higher affinity and avidity for B7-1 and B7-2 than CD28. CTLA-4 and CD28 are structurally homologous molecules that are members of the immunoglobulin (Ig) gene superfamily. CTLA-4 and CD28 are both expressed on the cell surface as disulfide-linked homodimers or as monomers. Unlike CTLA-4, CD28 expression is constitutive on the surfaces of 95% of $CD4^+$ T cells and 50% of $CD8^+$ T cells and is down regulated upon T cell activation. CTLA-4 negative co-stimulation suppresses T cell activation, thereby facilitating immune evasion. CTLA-4's effects occur via cell intrinsic and cell extrinsic mechanisms. Cell intrinsic mechanisms include inhibition of protein translation, recruitment of phosphatases, activation of ubiquitin ligases, inhibition of cytokine receptor signaling, and inhibition of lipid domain formation at membrane of T cells. Cell extrinsic mechanisms include stimulating the release of the suppressive indoleamine (2,3) deoxygenase (IDO), modulation of regulatory T cell function and the aforementioned competitive inhibition of CD28 costimulatory signaling (5).

CTLA-4 is a well-known immune checkpoint target. The human monoclonal CTLA-4 antibody Ipilimumab was approved by the food and drug administration (FDA) to treat melanoma in 2011 (4). Since then, it has been approved for colorectal cancer in children 12 and older, hepatocellular carcinoma, NSCLC, and renal cell carcinoma. The Ipilimumab binding epitope overlaps with the B7 binding domain and blocks the interaction via steric hinderance of B7(4). Ipilimumab allows for B7-CD28 positive costimulatory signaling, which is an important signal for T cell activation (5).

The Quantikine™ HS Human CTLA-4 Immunoassay is a 4.0 hour solid-phase ELISA designed to measure human CTLA-4 in cell culture supernates, cell lysates, serum, plasma, and urine. It contains CHO cell-expressed recombinant human CTLA-4 and antibodies raised against the recombinant protein. Results obtained using natural human CTLA-4 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 natural human CTLA-4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human CTLA-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any CTLA-4 present is bound by the immobilized antibody. After washing away any unbound substances, a biotinylated monoclonal antibody specific for human CTLA-4 is added to the wells. Following a wash to remove any unbound antibody-biotin reagent, an enzyme-linked streptavidin polymer is added to the wells. After washing away any unbound streptavidin polymer-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of CTLA-4 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 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 CTLA-4 HS Microplate	899185	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human CTLA-4.	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 CTLA-4 HS Standard	899187	2 vials of recombinant human CTLA-4 in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Use a new standard for each assay. Discard after use.
Human CTLA-4 HS Conjugate	899186	21 mL of a monoclonal antibody specific for human CTLA-4 conjugated to biotin with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-40	895513	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-17	895512	21 mL of a buffered protein base with preservatives.	
Streptavidin Polymer-HRP Diluent	898387	21 mL of a solution with preservatives.	
Streptavidin Polymer-HRP (100X)	898350	0.3 mL of Streptavidin Polymer-HRP in a buffer with preservatives.	
Wash Buffer Concentrate	895003	2 vials (21 mL/vial) 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	8 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
- 1000 mL graduated cylinder
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm
- **Polypropylene** test tubes for dilution of standards and samples
- Human CTLA-4 HS Controls (optional; R&D Systems®, Catalog # QC283)

PRECAUTIONS

CTLA-4 is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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 SDS 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 were lysed prior to assay as described in the Sample Values section.

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.

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.*

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

SAMPLE PREPARATION

Use polypropylene tubes.

Cell culture supernates and urine samples may require dilution due to high endogenous levels.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Concentrations of CTLA-4 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 40 mL of Wash Buffer Concentrate to 960 mL of deionized or distilled water to prepare 1000 mL of Wash Buffer.

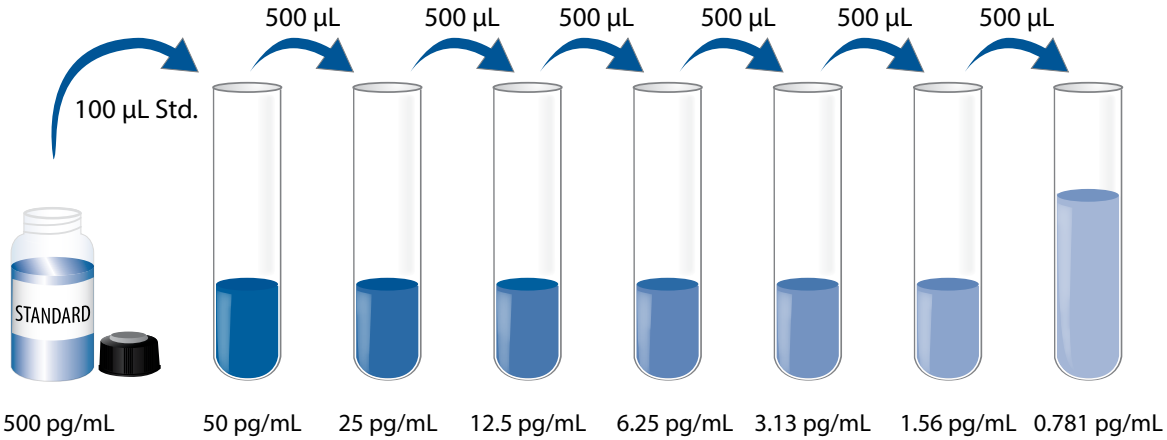
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Streptavidin Polymer-HRP (1X) - Add 0.215 mL of Streptavidin Polymer-HRP (100X) directly to the Streptavidin Polymer-HRP Diluent. Mix well.

Human CTLA-4 HS Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human CTLA-4 Standard with deionized or distilled water. This reconstitution produces a stock solution of 500 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Note: *Do not vortex. Use standard stock within 60 minutes.*

Use polypropylene tubes. Pipette 900 µL of Calibrator Diluent RD5-17 into the 50 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 50 pg/mL standard serves as the high standard. Calibrator Diluent RD5-17 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 standards, controls, and samples be assayed in duplicate.

Note: *Concentrations of CTLA-4 are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 100 µL of Assay Diluent RD1-40 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 to record 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 CTLA-4 HS Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour** at room temperature on the shaker.
7. Repeat the wash as in step 5.
8. Add 200 µL of Streptavidin Polymer-HRP (1X) to each well. Cover with a new adhesive strip. Incubate for **30 minutes** at room temperature on the shaker.
9. Repeat the wash as in step 5.
10. Add 200 µL of Substrate Solution to each well. Incubate for **30 minutes** at room temperature **on the benchtop. Protect from light.**
11. 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.
12. 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.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

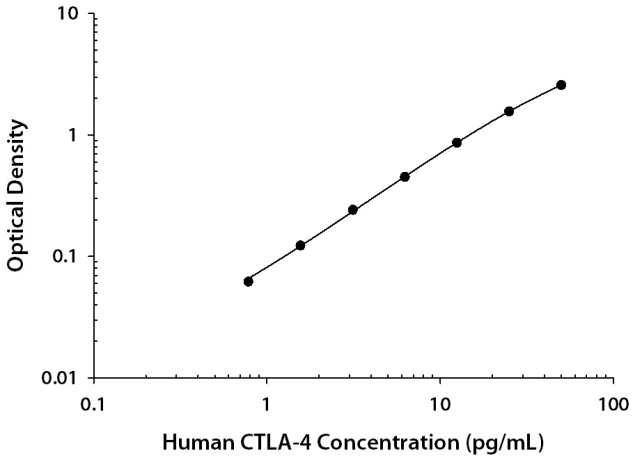
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 CTLA-4 concentrations 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, 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.



(pg/mL)	O.D.	Average	Corrected
0	0.046 0.046	0.046	—
0.781	0.105 0.110	0.108	0.062
1.56	0.161 0.176	0.169	0.123
3.13	0.272 0.304	0.288	0.242
6.25	0.466 0.529	0.498	0.452
12.5	0.894 0.926	0.910	0.864
25	1.536 1.686	1.611	1.565
50	2.555 2.684	2.620	2.574

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	5.53	15.6	32.1	5.32	14.7	31.2
Standard deviation	0.191	0.409	0.528	0.331	0.851	1.98
CV (%)	3.5	2.6	1.6	6.2	5.8	6.3

RECOVERY

The recovery of human CTLA-4 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	105	102-108%
Lysis buffer (n=1)	76	75-78%
Serum (n=4)	94	85-102%
EDTA plasma (n=4)	90	79-97%
Heparin plasma (n=4)	93	84-100%
Urine (n=4)	103	100-107%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human CTLA-4 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates* (n=4)	Lysates* (n=4)	Serum (n=4)	EDTA plamsa (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	95	98	103	101	103	99
	Range (%)	88-100	95-102	102-105	98-104	101-105	96-103
1:4	Average % of Expected	96	95	111	108	108	102
	Range (%)	86-102	91-98	108-117	105-112	104-112	98-109
1:8	Average % of Expected	97	90	112	111	111	101
	Range (%)	87-109	88-92	109-116	108-117	106-117	97-109
1:16	Average % of Expected	95	90	111	110	110	102
	Range (%)	85-102	89-92	105-117	108-112	106-114	98-107

*Samples were diluted prior to assay.

SENSITIVITY

Twenty-three assays were evaluated and the minimum detectable dose (MDD) of human CTLA-4 ranged from 0.029-0.130 pg/mL. The mean MDD was 0.071 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 CHO cell-expressed recombinant human CTLA-4 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human CTLA-4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (pg/mL)	% Detectable	Range (pg/mL)
Serum (n=30)	1.94	90	ND-13.2
EDTA plasma (n=30)	1.87	93	ND-13.1
Heparin plasma (n=30)	1.87	97	ND-13.8

ND=Non-detectable

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Urine (n=10)	22.8	10.5-74.4	19.2

Cell Culture Supernates:
Unstimulated $CD4^+$ T cells were isolated from human peripheral blood mononuclear cells (0.5×10^6 cells/mL) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 6-7 days. Cells were left unstimulated. Aliquots of the cell culture supernates and lysates were removed and assayed for levels of human CTLA-4.

Stimulated $CD4^+$ T cells were isolated from human peripheral blood mononuclear cells (0.5×10^6 cells/mL) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 20 ng/mL recombinant human IL-2, 20 ng/mL recombinant human IL-23, 40 ng/mL recombinant human IL-6, and 10 ng/mL recombinant human IL-1β for 6 days on a flask coated with 1 µg/mL mouse anti-human CD3ε. After the 6-7 day culture, $CD4^+$ Th17 cells were treated with 10 ng/mL PMA and 500 ng/mL Ionomycin overnight. Aliquots of the culture supernates and lysates were removed and assayed for levels of human CTLA-4.

Stimulated $CD4^+$ $CD25^+$ T Cells were isolated from Human peripheral blood mononuclear cells (0.5×10^6 cells/mL) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and 1 µg/mL mouse anti-human CD28 for 6-7 days on a flask coated with 2 µg/mL goat anti-mouse IgG and 1 µg/mL mouse anti-human CD3ε. Cells were treated with 10 ng/mL PMA and 500 ng/mL Ionomycin overnight. Aliquots of the cell culture supernates and lysates were removed and assayed for levels of human CTLA-4.

Condition	Cell culture supernates (pg/mL)	Lysates (pg/mL)
Unstimulated $CD4^+$ T cells	ND	ND
Stimulated $CD4^+$ TH17 cells	45.6	33.3
Stimulated $CD4^+$ $CD25^+$ Treg cells	283	24

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human CTLA-4.

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 CTLA-4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

B7-1
B7-2
CD28
IL-17
NKp30

Recombinant mouse:

CTLA-4

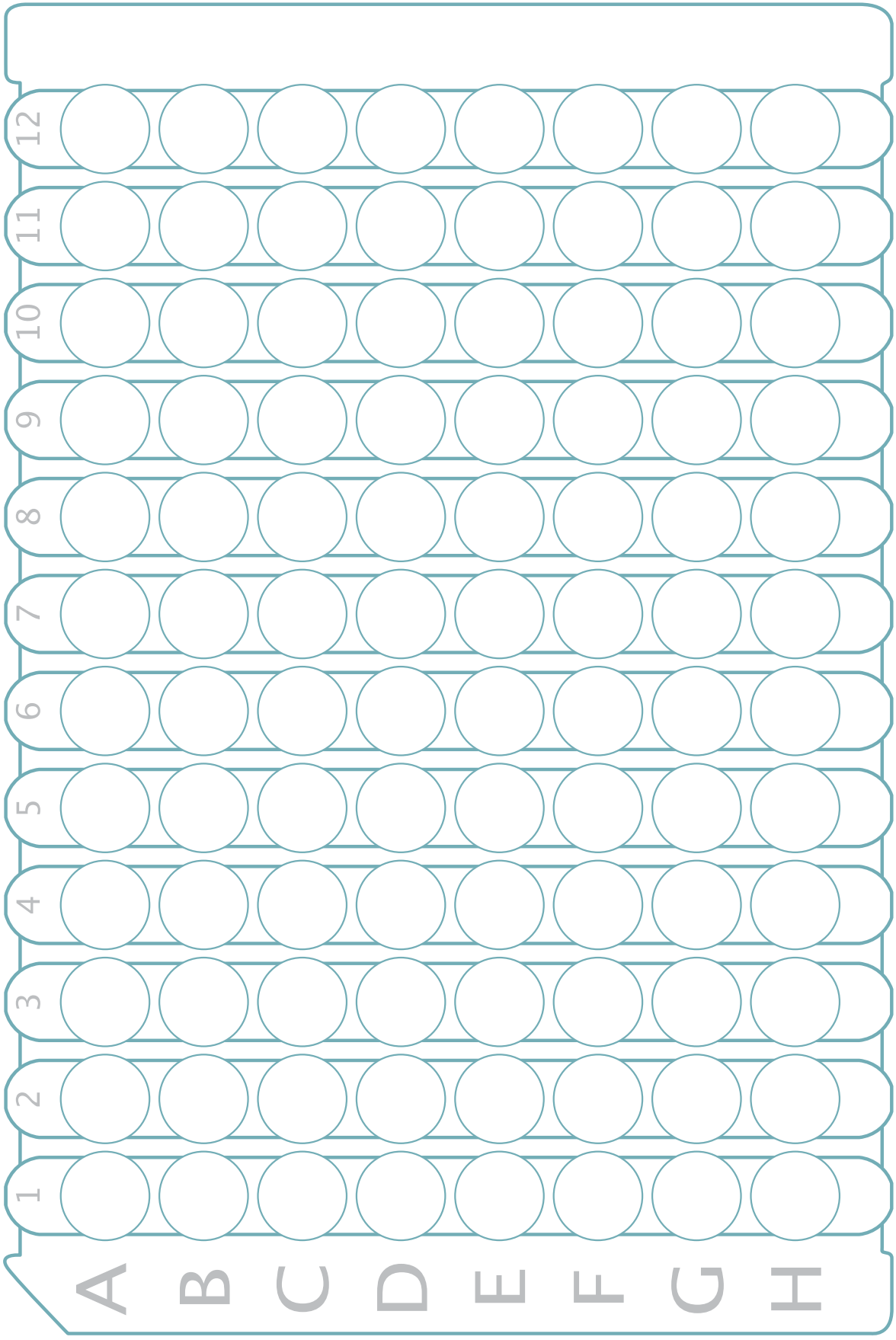
Ipilimumab Antibody (R&D Systems, Catalog # MAB9928), does not cross-react in this assay but does interfere at concentrations > 100 ng/mL.

REFERENCES

1. Zhao, Y. *et al.* (2018) Cell Physiol Biochem. **47**:721.
2. Brunet, J-F. *et al.* (1987) Nature **328**:267.
3. Walunas, T.L. *et al.* (1994) Immunity **1**:405.
4. Wei, S.C. *et al.* (2018) Cancer Discovery **9**:1609.
5. Brunner-Weinzierl, M. C and C. E. Rudd (2018) Front. Immunol. **9**:1.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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