Quantikine® ELISA

Mouse CD40 Immunoassay

Catalog Number MCCD40

For the quantitative determination of mouse CD40 concentrations in cell culture supernates, cell lysates, serum, and plasma.

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

CD40, also known as TNFRSF5, is a 45-50 kDa variably glycosylated type I transmembrane protein belonging to the TNF receptor (TNFR) superfamily (1, 2). It exhibits diverse activities in normal immune system development and function and participates in the control or progression of a variety of diseases. Mature mouse CD40 consists of a 174 amino acid (aa) extracellular domain (ECD) with four TNFR repeats, a 22 aa transmembrane segment, and a 74 aa cytoplasmic domain (3). Within the ECD, mouse CD40 shares 58% and 83% aa sequence identity with human and rat CD40, respectively. Additional splice forms show substitutions and/or deletions within and following the fourth TNFR repeat (4). Soluble CD40 (27-28 kDa), which functions as an inhibitor of membrane bound CD40 bioactivity, can be generated by alternate splicing or by proteolytic cleavage of transmembrane CD40 by TACE (4-6).

CD40 is expressed on B cells, dendritic cells, monocytes, macrophages, T cells, neutrophils, platelets, fibroblasts, smooth muscle cells, epithelial cells, endothelial cells, neurons, and many hematopoietic and epithelial cancers (1, 7, 8). It is upregulated on these cell types during inflammation and notably on $CD4^+$ T cells in autoimmune mice (9-11). CD40 homotrimerization is important for its ability to initiate signaling through TRAF family proteins (12). This is induced by the binding of CD40 to the 39 kDa transmembrane glycoprotein CD40 Ligand/CD154 (CD40L). CD40L itself is upregulated on T cells, B cells, dendritic cells, neutrophils, platelets, vascular endothelial cells, and smooth muscle cells during activation or inflammation (1, 7, 13, 14). CD40L is expressed on the cell surface in heteromultimers of different isoforms (15). Soluble CD40L is generated by intracellular proteolytic cleavage and is secreted as a homotrimer of 15-18 kDa subunits (15, 16). Both the membrane bound and soluble forms of CD40L induce signaling through CD40 (17, 18).

Interactions between CD40 and CD40L are involved in multiple aspects of humoral immunity, cellular immunity, and inflammation. CD40L on activated $CD4^+$ T cells provides a costimulatory signal that augments B cell proliferation, germinal center formation, immunoglobulin class switching, and antibody secretion (1, 11, 18). CD40 ligation likewise promotes the activation of the many other cell types which express it and additionally promotes the development of medullary thymic epithelial, Th17, and $FoxP3^+$ Treg cells (14, 19-23). CD40-CD40L interactions regulate lymphocyte development and the balance between immune self-tolerance and autoimmunity (11, 24). CD40 signaling reinforces inflammatory responses but can also fulfill a tissue protective role by enhancing epithelial cell survival during oxidative stress (7, 23, 25-28). CD40-CD40L interactions in disease can be beneficial (contributing to the immune response to cancer) or detrimental (contributing to the progression of atherosclerosis and graft versus host disease) (1, 7, 8). CD40-mediated responses are modulated by TLR, RANK, and TNF receptor activity through intracellular signaling crosstalk or direct interaction of CD40 with those receptors (2, 20, 21).

The Quantikine® Mouse CD40 Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse CD40 in cell culture supernates, cell lysates, serum, and plasma. It contains NS0-expressed recombinant mouse CD40 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural CD40 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 mouse CD40.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse CD40 has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any CD40 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse CD40 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 CD40 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.
- For best results, pipette reagents and samples into the center of each well.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- 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.

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
Mouse CD40 Microplate	893825	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse CD40.	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.*
Mouse CD40 Conjugate	893826	12 mL of a polyclonal antibody specific for mouse CD40 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse CD40 Standard	893827	Recombinant mouse CD40 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse CD40 Control	893828	Recombinant mouse CD40 in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Assay Diluent RD1-21	895215	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-3	895436	21 mL of a buffered protein base with preservatives.	
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	895174	23 mL of diluted hydrochloric 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.
- **Polypropylene** test tubes for dilution of standards.

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

- Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347).

PRECAUTIONS

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 20 minutes at 2000 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.*

REAGENT PREPARATION

Bring all reagents to room temperature before use.

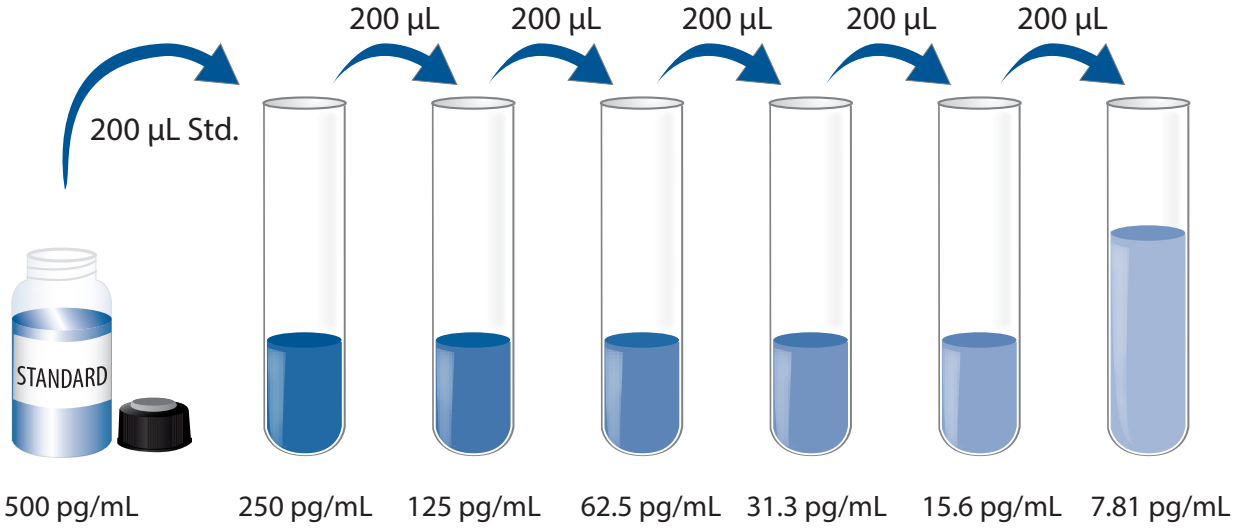
Mouse CD40 Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

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. 100 µL of the resultant mixture is required per well.

Mouse CD40 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse CD40 Standard with Calibrator Diluent RD5-3. This reconstitution produces a stock solution of 500 pg/mL. Mix the standard to ensure complete reconstitution, and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-3 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse CD40 Standard (500 pg/mL) serves as the high standard. Calibrator Diluent RD5-3 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 standards, control, and samples be assayed in duplicate.

1. Prepare all reagents, working standards, control, 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 RD1-21 to each well.
4. Add 50 µL of standard, control, or sample per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 100 µL of Mouse CD40 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. 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

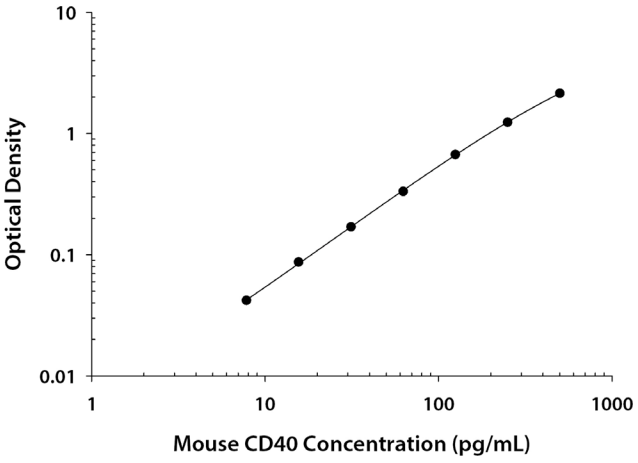
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse CD40 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	0.036 0.041	0.039	—
7.81	0.079 0.082	0.081	0.042
15.6	0.122 0.129	0.126	0.087
31.3	0.205 0.213	0.209	0.170
62.5	0.367 0.376	0.372	0.333
125	0.703 0.719	0.711	0.672
250	1.257 1.298	1.278	1.239
500	2.145 2.225	2.185	2.146

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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	21.0	67.2	159	22.7	70.9	169
Standard deviation	1.5	3.3	5.2	1.7	2.9	7.8
CV (%)	7.1	4.9	3.3	7.5	4.1	4.6

RECOVERY

The recovery of mouse CD40 spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	102	86-119%
Serum (n=4)	92	81-106%
EDTA plasma (n=4)	96	82-109%
Heparin plasma (n=4)	92	83-109%

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of mouse CD40 were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture samples (n=4)	Cell lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)
1:2	Average % of Expected	103	98	109	111	109
	Range (%)	101-108	94-102	99-115	109-115	106-112
1:4	Average % of Expected	104	100	112	115	111
	Range (%)	93-111	91-109	101-119	111-120	111-112
1:8	Average % of Expected	109	102	111	114	110
	Range (%)	102-117	95-116	98-120	110-119	107-114
1:16	Average % of Expected	104	93	106	107	107
	Range (%)	87-119	86-101	95-119	102-118	105-109

SENSITIVITY

Fifty-nine assays were evaluated and the minimum detectable dose (MDD) of mouse CD40 ranged from 0.31-3.70 pg/mL. The mean MDD was 1.49 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 NS0-expressed recombinant mouse CD40 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples were evaluated for the presence of mouse CD40 in this assay.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=20)	89.3	49.5-153	26.7
EDTA plasma (n=20)	74.2	47.5-142	23.2
Heparin plasma (n=20)	68.8	38.9-91	12.4

Cell Culture Supernates:

Organs from 2-4 mice were homogenized, seeded in RPMI, and supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 1.0 µg/mL LPS or 10 µg/mL concanavalin A (Con A). Aliquots of the cell culture supernates were removed and assayed for levels of mouse CD40.

Tissue Type	Incubation Time	Observed Levels (pg/mL)
Kidney, Unstimulated	18 hours	210
Kidney, Stimulated with LPS	18 hours	210
Kidney, Stimulated with Con A	18 hours	152
Liver, Unstimulated	3 days	27.0
Liver, Stimulated with LPS	3 days	36.3
Lung, Unstimulated	3 days	59.6
Lung, Stimulated with LPS	3 days	55.4
Spleen, Stimulated with LPS	1 day	586

Bone marrow cells (1 x 10^6 cells/mL) in DMEM were supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. An aliquot of the cell culture supernate was removed, assayed for mouse CD40, and measured 21.2 pg/mL.

SAMPLE VALUES *CONTINUED*

J774A.1 mouse reticulum cell sarcoma macrophage cells (3.5 x 10^6 cells/mL) were cultured in DMEM supplemented with 10% fetal bovine serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 2.5 µg/mL of LPS and 100 ng/mL recombinant mouse (rm) GM-CSF or rmIL-10 for 3-6 days. Aliquots of the cell culture supernates were removed and assayed for levels of mouse CD40.

Condition	Incubation Time	Observed Levels (pg/mL)
Unstimulated	3 days	17.4
Stimulated with LPS and rmGM-CSF	6 days	258
Stimulated with LPS and rmIL-10	6 days	274

Cell Lysates - Organs from 2-3 mice were rinsed with PBS to remove excess blood, chopped into 1-2 mm pieces, homogenized with a tissue homogenizer, and 1.0 mL of Cell Lysis Buffer 2 was added (2.0 mL of Cell Lysis Buffer 2 was added to liver tissue). Organs were lysed at room temperature for 30 minutes with gentle agitation and centrifuged to remove debris. Aliquots of the cell lysates were removed and assayed for levels of mouse CD40.

Tissue Type	Observed Levels (pg/mL)
Brain	1202
Heart	1800
Kidney	14,780
Liver	11,060
Lung	33,890
Spleen	10,693

SPECIFICITY

This assay recognizes natural and recombinant mouse CD40.

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 mouse CD40 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

4-1BB
BCMA
CD27
CD30
CD40 Ligand
DR3
Fas
GITR
OPG
OX40
RANK
TACE
TACI

Recombinant human:

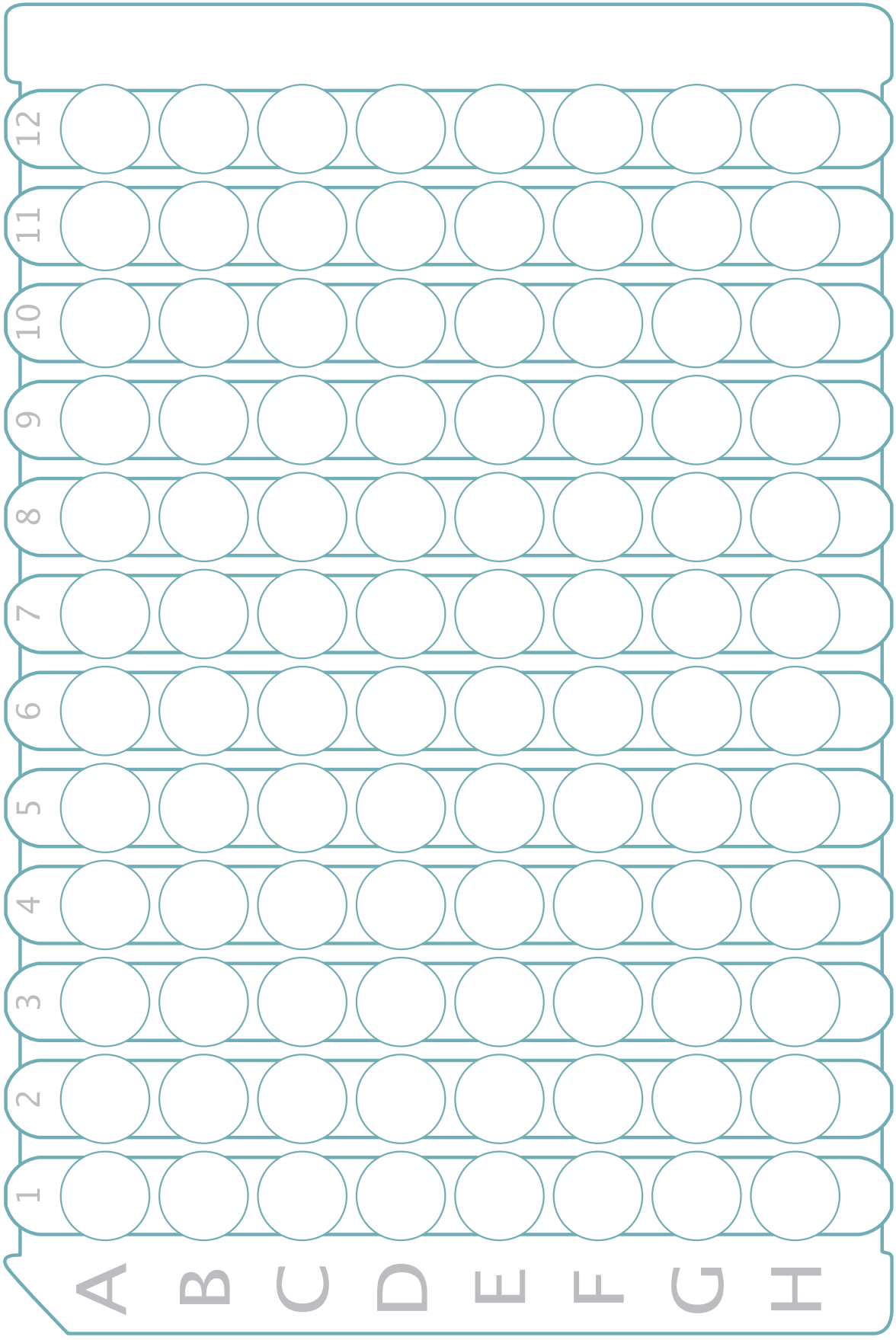
CD40

REFERENCES

1. Elgueta, R. *et al.* (2009) Immunol. Rev. **229**:152.
2. Vaitaitis, G.M. and D.H. Wagner, Jr. (2010) Mol. Immunol. **47**:2303.
3. Torres, R.M. and E.A. Clark (1992) J. Immunol. **148**:620.
4. Tone, M. *et al.* (2001) Proc. Natl. Acad. Sci. USA **98**:1751.
5. Eshel, D. *et al.* (2008) Mol. Immunol. **46**:250.
6. Contin, C. *et al.* (2003) J. Biol. Chem. **278**:32801.
7. Lievens, D. *et al.* (2009) Thromb. Haemost. **102**:206.
8. Loskog, A.S.I. and A.G. Eliopoulos (2009) Sem. Immunol. **21**:301.
9. Cagnoni, F. *et al.* (2004) J. Immunol. **172**:3205.
10. Baker, R.L. *et al.* (2008) J. Autoimmun. **31**:385.
11. Munroe, M.E. (2009) Sem. Immunol. **21**:283.
12. Pullen, S.S. *et al.* (1999) Biochemistry **38**:10168.
13. Armitage, R.J. *et al.* (1992) Nature **357**:80.
14. Ma, D.Y. and E.A. Clark (2009) Sem. Immunol. **21**:265.
15. Hsu, Y.M. *et al.* (1997) J. Biol. Chem. **272**:911.
16. Pietravalle, F. *et al.* (1996) J. Biol. Chem. **271**:5965.
17. Mazzei, G.J. *et al.* (1995) J. Biol. Chem. **270**:7025.
18. Hollenbaugh, D. *et al.* (1992) EMBO J. **11**:4313.
19. Fanslow, W.C. *et al.* (1994) J. Immunol. **152**:4262.
20. Iezzi, G. *et al.* (2009) Proc. Natl. Acad. Sci. USA **106**:876.
21. Akiyama, T. *et al.* (2008) Immunity **29**:423.
22. Spence, P. *et al.* (2008) Proc. Natl. Acad. Sci. USA **105**:973.
23. Suttles, J. and R.D. Stout (2009) Sem. Immunol. **21**:257.
24. Peters, A.L. *et al.* (2009) Sem. Immunol. **21**:293.
25. Vanichakarn, P. *et al.* (2008) Thromb. Res. **122**:346.
26. Dugger, K. *et al.* (2009) Sem. Immunol. **21**:289.
27. Merendino, A.M. *et al.* (2006) Am. J. Respir. Cell Mol. Biol. **35**:155.
28. Laxmanan, S. *et al.* (2005) J. Am. Soc. Nephrol. **16**:2714.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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