Quantikine® ELISA

Human CX_3CL1/Fractalkine Immunoassay

Catalog Number DCX310

For the quantitative determination of human Fractalkine concentrations in cell culture supernates, serum, plasma, saliva, urine, and human milk.

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

Fractalkine, also known as CX_3CL1 or neurotactin, is the only member of the CX_3C or delta chemokine subfamily (1-4). The name Fractalkine derives from the "fractal" nature of its discovery and placement within the chemokine family (4). Fractalkine is produced as a transmembrane protein, unlike all other chemokines except CXCL16 (1, 4). The 95-100 kDa type I transmembrane form of Fractalkine can be proteolytically processed to generate a secreted 60-80 kDa soluble form (4). Soluble Fractalkine has been identified in serum, urine, cerebrospinal, amniotic and synovial fluids (1, 5). The metalloproteinase ADAM10 cleaves Fractalkine constitutively, while TACE/ADAM17 does so inducibly (6, 7). Fractalkine contains an extracellular 76 amino acid (aa) chemokine domain that includes binding and chemotactic determinants, which is held away from the membrane by a 226 aa mucin-like stalk region (4, 8-10). Human Fractalkine shares 60% or 63% aa sequence identity within the entire extracellular domain, and 78% or 85% aa sequence identity within the chemokine domain only, with mouse or rat Fractalkine, respectively. Fractalkine is predominantly expressed by vascular endothelium and smooth muscle, neurons, dendritic cells, and the epithelial linings of the intestine, bronchi, renal proximal tubules, endometrium, fallopian tube, and bile duct (11-23). Most Fractalkine expression is induced by inflammatory cytokines such as TNF-α and IFN-γ, but expression on forebrain neurons as well as Leydig and Sertoli testicular cells is also constitutive (11-24). Expression by astrocytes, osteoblasts, stratum basal keratinocytes, and fibroblasts has also been reported (13, 17, 25, 26).

CX_3CR1, a 40 kDa 7-transmembrane non-glycosylated G-protein coupled receptor, is the only known endogenous receptor for Fractalkine (1-3). It is expressed by cytotoxic effector cells and cytokine producers, including type I helper and cytotoxic T cells, γδ T cells, $CD16^+$ NK cells, monocytes and microglia (3, 13, 27, 28). In humans, three isoforms of the receptor that vary at the N-terminus differ in binding kinetics and chemotactic responses to the Fractalkine ligand (29, 30). Cytomegalovirus produces a protein called US28 that is also a receptor for human Fractalkine (8).

The functions of Fractalkine are mainly dependent on its adhesion properties and chemoattractant activity and include some differences between soluble and transmembrane forms (1-3). Transmembrane Fractalkine, with cooperation from platelet P-Selectin under arterial high shear, mediates firm adhesion to circulating CX_3CR1-expressing cells (6, 12, 31). Cleavage of the membrane-bound Fractalkine may then free adhering leukocytes to allow diapedesis (6, 10, 12, 32). Both soluble and transmembrane forms are chemoattractants that bring cytotoxic effector and cytokine producing cells to areas of inflammation (1-3, 23, 26, 27). Fractalkine, especially in the soluble form, enhances the cytotoxic response of CX_3CR1-expressing NK cells, which may be responsible for its antitumor activity (33, 34). Fractalkine is thought to mediate interactions between neurons and microglia in the brain, and to protect the brain against microglial neurotoxicity during neuroinflammation (35, 36). Production of Fractalkine by the endometrium is postulated to promote trophoblast migration for implantation, and its abundance in endometrium and placenta implies presence of Fractalkine in the plasma during pregnancy (37). While Fractalkine is important in resolving normal inflammatory processes such as wound healing, it may also contribute to the pathogenesis of some conditions (3, 38). For example, both CX_3CR1 and soluble Fractalkine are often over-expressed in coronary artery disease and may contribute to arterial plaque destabilization (39). Fractalkine may also be upregulated in systemic lupus erythematosis and in rheumatoid arthritis synovial fluid (1, 40).

The Quantikine® Human CX_3CL1/Fractalkine Immunoassay is a 4.5 hour solid phase ELISA designed to measure human Fractalkine in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains NS0-expressed recombinant human Fractalkine and has been shown to accurately quantitate the recombinant factor. Results obtained using natural Fractalkine 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 Fractalkine.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Fractalkine has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Fractalkine present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Fractalkine 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 Fractalkine 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, further dilute the samples with the appropriate 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 Fractalkine Microplate	893583	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Fractalkine.	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 Fractalkine Conjugate	893584	21 mL of a monoclonal antibody specific for human Fractalkine conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Fractalkine Standard	893585	Recombinant human Fractalkine in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-88	895880	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5K	895119	21 mL of a buffered protein base with preservatives. *For cell culture supernate/ saliva samples.*	
Calibrator Diluent RD6-11	895489	21 mL of a buffered protein base with preservatives. *For serum/plasma/urine/ human milk samples.*	
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.
- 2-8 °C refrigerator for assay incubation steps.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Human Fractalkine Controls (optional; R&D Systems®, Catalog # QC93).

PRECAUTIONS

Calibrator Diluent RD6-11 contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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.

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.

Note: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

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.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Saliva samples require a 2-fold dilution. A suggested 2-fold dilution is 200 µL of sample + 200 µL of Calibrator Diluent RD5K.

Urine samples require a 2-fold dilution. A suggested 2-fold dilution is 200 µL of sample + 200 µL of Calibrator Diluent RD6-11.

Human milk samples may require dilution in Calibrator Diluent RD6-11.

REAGENT PREPARATION

The conjugate must remain at 2-8 °C. Bring all remaining reagents to room temperature before use.

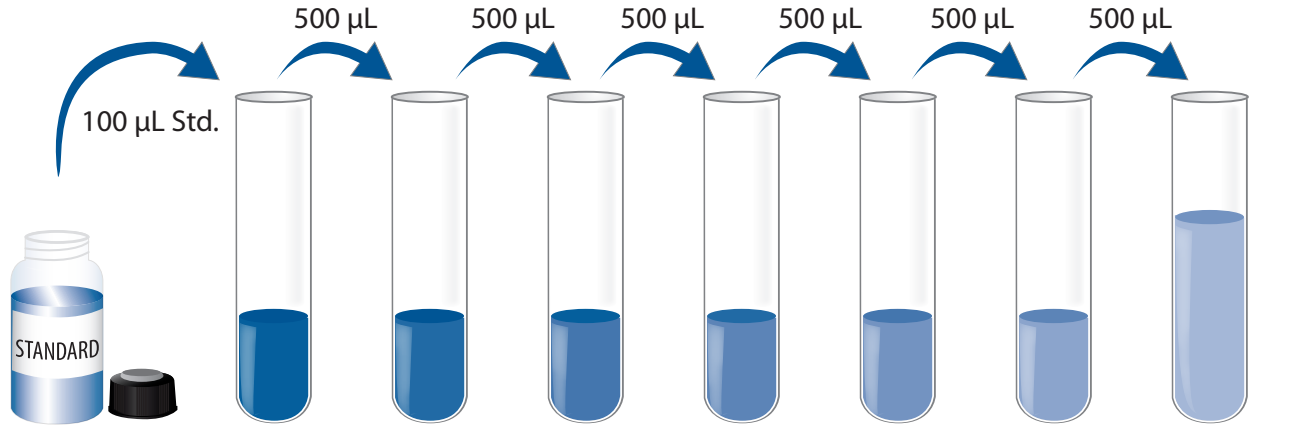
Note: *High concentrations of Fractalkine are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 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.

Human Fractalkine Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Fractalkine Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5K (*for cell culture supernate/saliva samples*) or Calibrator Diluent RD6-11 (*for serum/plasma/urine/human milk samples*) into the 10 ng/mL tube. Pipette 500 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10.0 ng/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

The conjugate must remain at 2-8 °C. Bring all remaining reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *High concentrations of Fractalkine 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 RD1-88 to each well.
4. Add 100 µL of standard, control, or sample* per well **within 15 minutes.** Cover with the adhesive strip provided. **Incubate for 3 hours at 2-8 °C.** 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 toweling.
6. Add 200 µL of **cold** Human Fractalkine Conjugate to each well. Cover with a new adhesive strip. **Incubate for 1 hour at 2-8 °C.**
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. **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.

*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.).

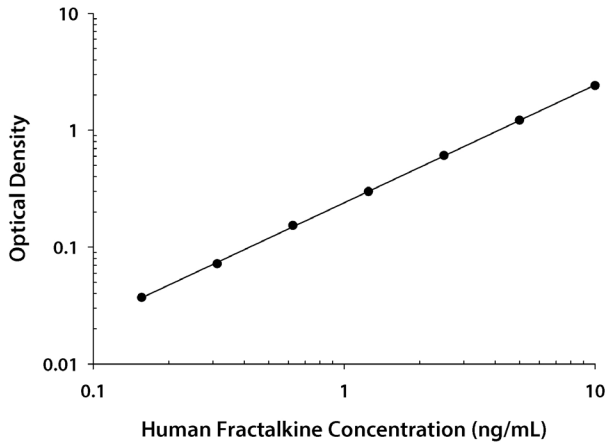
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the human Fractalkine concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

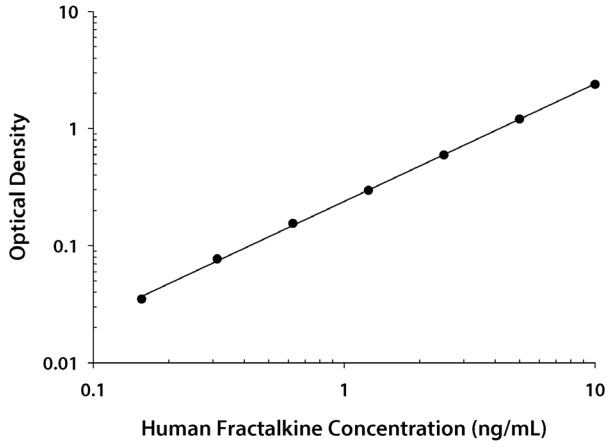
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SALIVA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.007 0.010	0.009	—
0.156	0.045 0.046	0.046	0.037
0.313	0.080 0.082	0.081	0.072
0.625	0.158 0.165	0.162	0.153
1.25	0.301 0.314	0.308	0.299
2.50	0.599 0.635	0.617	0.608
5.00	1.194 1.254	1.224	1.215
10.0	2.381 2.462	2.422	2.413

SERUM/PLASMA/URINE/HUMAN MILK ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.008 0.009	0.009	—
0.156	0.043 0.044	0.044	0.035
0.313	0.084 0.087	0.086	0.077
0.625	0.162 0.165	0.164	0.155
1.25	0.304 0.308	0.306	0.297
2.50	0.586 0.619	0.603	0.594
5.00	1.189 1.233	1.211	1.202
10.0	2.338 2.451	2.395	2.386

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 components.

CELL CULTURE SUPERNATE/SALIVA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.18	3.33	6.43	1.19	3.53	6.85
Standard deviation	0.03	0.11	0.24	0.07	0.15	0.31
CV (%)	2.5	3.4	3.7	6.1	4.3	4.5

SERUM/PLASMA/URINE/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.24	3.80	7.12	1.23	3.59	6.88
Standard deviation	0.04	0.08	0.17	0.11	0.20	0.37
CV (%)	3.2	2.1	2.4	8.9	5.6	5.4

SENSITIVITY

One hundred four assays were evaluated and the minimum detectable dose (MDD) of human Fractalkine ranged from 0.006-0.072 ng/mL. The mean MDD was 0.018 ng/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 human Fractalkine (full length) produced at R&D Systems®.

RECOVERY

The recovery of human Fractalkine spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	98	93-102%
Serum (n=4)	99	88-111%
Heparin plasma (n=4)	97	85-108%
EDTA plasma (n=4)	101	88-113%
Urine* (n=4)	96	86-109%

*Samples were diluted prior to assay as described in the Sample Preparation section.

LINEARITY

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

		Cell culture supernates (n=3)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva* (n=4)	Urine* (n=4)	Human milk* (n=4)
1:2	Average % of Expected	101	101	99	100	99	108	100
	Range (%)	94-107	98-103	93-107	98-104	96-102	98-115	96-102
1:4	Average % of Expected	104	99	100	104	101	105	104
	Range (%)	102-107	94-107	94-110	98-113	96-105	97-115	98-112
1:8	Average % of Expected	105	100	98	101	100	103	102
	Range (%)	104-107	95-110	92-111	94-112	96-107	97-113	95-110
1:16	Average % of Expected	103	102	96	101	96	99	102
	Range (%)	103-103	93-113	87-108	92-109	93-102	98-101	97-111

*Samples were diluted prior to assay as directed in the Sample Preparation section.

SAMPLE VALUES

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

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	0.836	0.456-2.23	0.291
Heparin plasma (n=36)	0.741	0.404-2.32	0.313
EDTA plasma (n=36)	0.738	0.377-2.06	0.282
Saliva (n=12)	6.67	1.74-23.6	5.72
Urine (n=10)	3.55	0.520-6.82	2.01

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Human milk (n=12)	38.6	92	ND-148

ND=Non-detectable

Cell Culture Supernates:

Human peripheral blood leukocytes (PBL) were cultured in RPMI supplemented with 10% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for human Fractalkine. All samples measured below the lowest standard, 0.156 ng/mL.

HUVEC human umbilical vein endothelial cells were cultured in EGM®-2 media and grown to 80% confluency. The cells were cultured unstimulated or stimulated with 40.0 ng/mL of recombinant human TNF-α and 50.0 ng/mL of recombinant human IFN-γ for 24 hours. Aliquots of the cell culture supernates were removed and assayed for levels of human Fractalkine.

Condition	(ng/mL)
Unstimulated	ND
Stimulated	57.5

HMVEC human microvascular endothelial cells were cultured in EGM-2MV media for one week. The cells were cultured unstimulated or stimulated with 40.0 ng/mL of recombinant human TNF-α and 50.0 ng/mL of recombinant human IFN-γ for 24 hours. Aliquots of the cell culture supernates were removed and assayed for levels of human Fractalkine.

Condition	(ng/mL)
Unstimulated	ND
Stimulated	2.46 ng/mL

ND=Non-detectable

SPECIFICITY

This assay recognizes natural and recombinant human Fractalkine.

The factors listed below were prepared at 100 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 100 ng/mL in a mid-range recombinant human Fractalkine control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

CX_3CR1
CXCL6/GCP-2/LIX

Recombinant mouse:

CXCL6/GCP-2/LIX
Fractalkine (chemokine domain; aa 22-105)
Fractalkine (chemokine domain; aa 25-105)
Fractalkine (full length; aa 25-337)
Fractalkine (N-truncated; aa 29-102)

Recombinant rat:

Fractalkine (chemokine domain; aa 22-100)
Fractalkine (chemokine domain; aa 25-100)
Fractalkine (full length; aa 25-334)

This assay detects all forms of human Fractalkine containing the chemokine domain.

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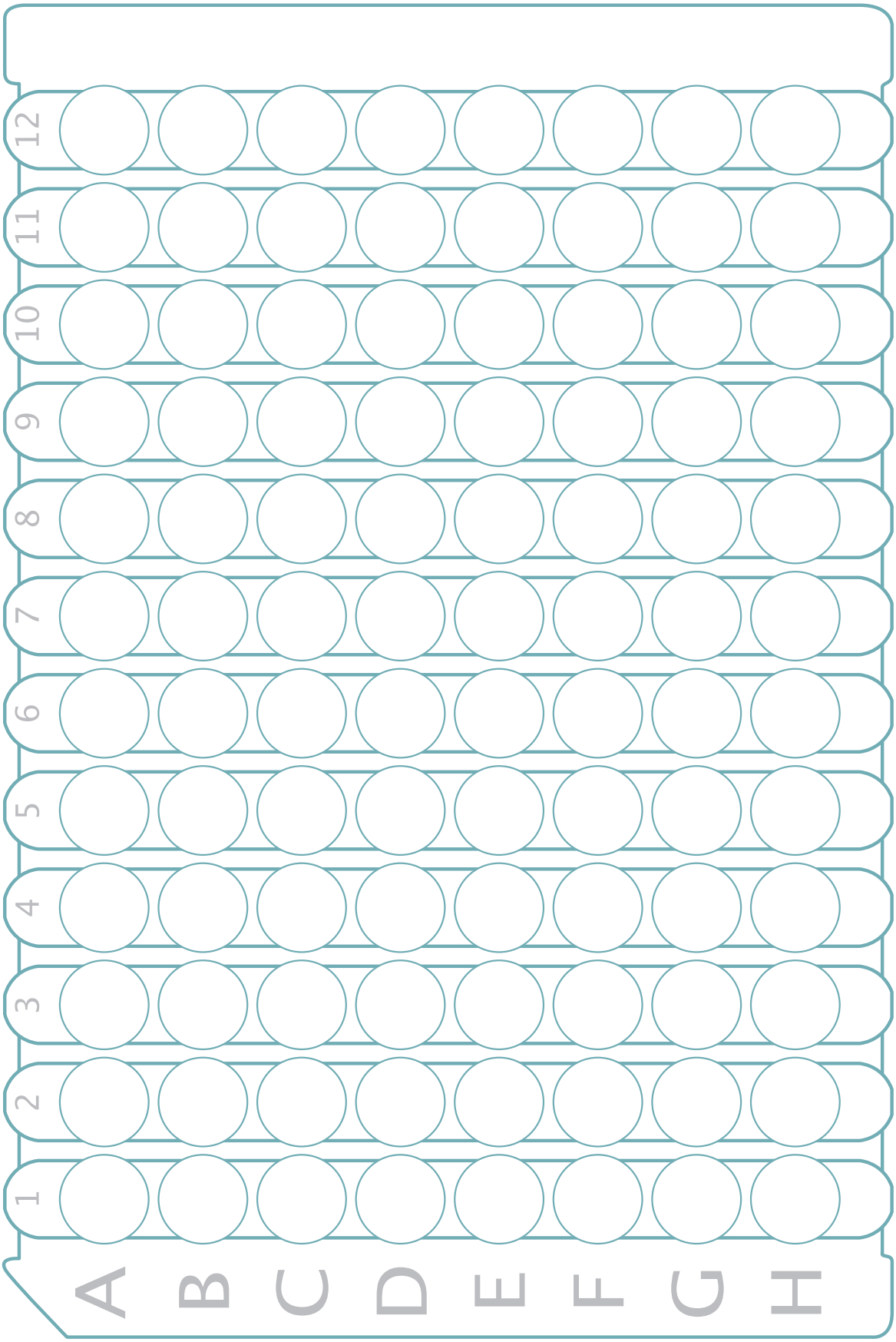
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES