

Luminex[®] Performance Assay

Human Cytokine Premixed Kit A

Catalog Number FCST03

For the simultaneous quantitative determination of multiple human cytokine concentrations in cell culture supernates, 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

Cytokines are intercellular signaling proteins released from a wide variety of cells and tissues. They play an integral role in regulating growth and cellular proliferation as well as modulating host response to infection, injury, and inflammation. Cytokines also influence reproduction and bone remodeling. A large number of cytokines are pleiotropic and share similar functions. In addition, many cytokines influence the production of other cytokines. Analysis and quantification of cytokines in biological fluids and cell culture supernates has thus become increasingly important. Methods such as bioassay, enzyme-linked immunosorbent assay (ELISA), intracellular staining, ribonuclease protection assay (RPA) and polymerase chain reaction (PCR) have all been used for quantifying cytokines; however, each of these techniques has limitations associated with it. These techniques are not capable of measuring multiple cytokines simultaneously in a limited sample volume.

This kit can be used to simultaneously assess the levels of multiple cytokines in a single sample. For ease of use, the microparticles and the biotinylated detection antibodies are premixed in respective vials.

Analyte	Microparticle Region	Performance Data Online (www.RnDSystems.com/pdf/...)
CXCL5/ENA-78	37	LUH254.pdf
FGF basic	54	LUH233.pdf
G-CSF	58	LUH214.pdf
GM-CSF	11	LUH215C.pdf
IFN- γ	75	LUH285.pdf
IL-1 α /IL-1F1	05	LUH200B.pdf
IL-1 β /IL-1F2	06	LUH201.pdf
IL-1ra/IL-1F3	16	LUH280.pdf
IL-2	17	LUH202.pdf
IL-4	21	LUH204.pdf
IL-5	09	LUH205.pdf
IL-6	32	LUH206.pdf
CXCL8/IL-8	36	LUH208.pdf
IL-10	50	LUH217.pdf
IL-17	20	LUH317.pdf
CCL2/MCP-1	78	LUH279.pdf
CCL3/MIP-1 α	59	LUH270.pdf
CCL4/MIP-1 β	74	LUH271.pdf
CCL5/RANTES	80	LUH278.pdf
TNF- α	77	LUH210.pdf
Tpo	34	LUH288.pdf
VEGF	52	LUH293.pdf

PRINCIPLE OF THE ASSAY

Magnetic Luminex® Performance Assay multiplex kits are designed for use with the Luminex® 100/200™, Luminex® FLEXMAP 3D® or Bio-Rad® Bio-Plex®, dual laser, flow-based sorting and detection platforms.

Analyte-specific antibodies are pre-coated onto magnetic microparticles embedded with fluorophores at set ratios for each unique bead region. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. Final washes remove unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex® 100/200™, Luminex® FLEXMAP 3D® or Bio-Rad Bio-Plex Analyzer. These analyzers use one laser to excite the dyes inside each bead to identifying the bead region and a second laser to excite the PE to measure the amount of analyte bound to the bead. All excitation emitted as each bead passes through the flow cell is then analyzed to differentiate excitation levels using a Photomultiplier Tube (PMT) and an Avalanche Photodiode.

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 the appropriate 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. However, until these proteins have been tested in Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Luminex® Performance Assays afford the user the benefit of multianalyte analysis of cytokines in a single complex sample. For each sample type, a single multipurpose diluent is used to optimize recovery, linearity, and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only the analytes listed on the Standard Value cards can be measured with this kit. Refer to the enclosed certificate of analysis for specific analytes included in this kit.**

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, DILUTED, OR RECONSTITUTED MATERIAL
Standard Cocktail 1	895531	2 vials of recombinant human cytokines in a buffered protein base with preservatives; lyophilized.	Discard after use. Use fresh standards for each assay.
Standard Cocktail 2	895546	2 vials of recombinant human cytokines in a buffered protein base with preservatives; lyophilized.	
Microparticle Cocktail; Human Cytokine Premixed Kit A	894098	1.2 mL of a concentrated microparticle cocktail with preservative.	May be stored for up to 1 month at 2-8 °C.* <i>Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.</i>
Biotin Antibody Cocktail; Human Cytokine Premixed Kit A	894097	1.2 mL of a concentrated biotinylated antibody cocktail with preservative.	
Streptavidin-PE	892525	0.07 mL of a concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Microparticle Diluent	895529	6 mL of a buffered protein base with blue dye and preservative.	May be stored for up to 1 month at 2-8 °C.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	
Calibrator Diluent RD5K	895119	21 mL of a 2-fold concentrated solution of a buffered protein base with preservatives. <i>For cell culture supernate samples. Use diluted 1:2 in this assay.</i>	
Calibrator Diluent RD6-40	895817	21 mL of a buffered animal serum with preservatives. <i>For serum/plasma samples.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Microplate	640763	1 filter-bottomed 96-well microplate used as a vessel for the assay.	
Mixing Bottles	895505	2 empty 8 mL bottles used for mixing microparticles with Microparticle Diluent.	
Plate Sealers	640445	4 adhesive foil strips.	
Standard Value Card 1	750215	1 card listing the Standard Cocktail 1 reconstitution volume and working standard concentrations for this kit lot.	
Standard Value Card 2	750618	1 card listing the Standard Cocktail 2 reconstitution volume and working standard concentrations for this kit lot.	

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

**Additional reagents supplied if CCL5/RANTES is ordered (See page 4 for details).

MATERIALS PROVIDED & STORAGE CONDITIONS *CONTINUED*

Note: Additional reagents supplied if CCL5/RANTES is ordered.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
Human RANTES Microparticle Concentrate	891045	0.075 mL a concentrated microparticle with preservatives.	May be stored for up to 1 month at 2-8 °C.* <i>Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.</i>

OTHER SUPPLIES REQUIRED

- Luminex® 100/200™, or Bio-Rad Bio-Plex analyzer with X-Y platform.
- Microplate vacuum manifold (Millipore Multiscreen™ Vacuum Manifold Catalog # MAVM096 or equivalent).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

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

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

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.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.
- For best results, adjust the vacuum strength on the plate washer to between 15 and 40 cm of mercury.

SAMPLE COLLECTION AND 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 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.*

SAMPLE PREPARATION

Use polypropylene tubes.

Note: *On the day of the assay, ALL fresh and previously frozen serum and plasma samples require centrifugation at 16,000 x g for 4 minutes immediately prior to use or dilution.*

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 30 μ L of sample + 90 μ L of Calibrator Diluent RD6-40. Mix thoroughly.

When assaying CCL5/RANTES, serum and plasma samples must be further diluted 25-fold to a final 100-fold dilution. A suggested 100-fold dilution is 10 μ L of the 4-fold diluted sample + 240 μ L of Calibrator Diluent RD6-40. Mix thoroughly.

REAGENT PREPARATION

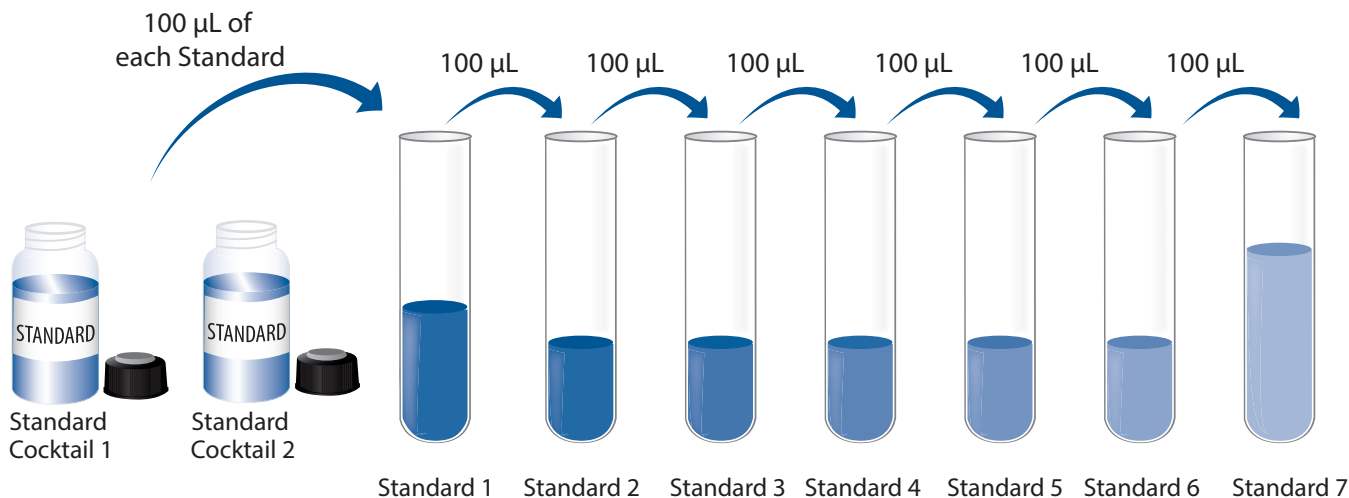
Bring all reagents to room temperature before use.

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 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5K (diluted 1:2) - **For cell culture supernate samples only.** Add 20 mL of Calibrator Diluent RD5K to 20 mL of deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5K (diluted 1:2).

Standards - **Refer to the Standard Value Cards for the reconstitution volumes and the assigned values.** Reconstitute one each of Standard Cocktails 1 and 2 with Calibrator Diluent RD5K (diluted 1:2) (*for cell culture supernate samples*) or Calibrator Diluent RD6-40 (*for serum/plasma samples*). Allow the standards to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. This produces a 5X stock of each Standard Cocktail.

Use polypropylene tubes. Pipette 300 μ L of Calibrator Diluent RD5K (diluted 1:2) (*for cell culture supernate samples*) or Calibrator Diluent RD6-40 (*for serum/plasma samples*) into a tube labeled working standard 1. Pipette 200 μ L of the appropriate calibrator diluent into the remaining tubes. Pipette 100 μ L of each of the 5X reconstituted Standard Cocktail vials 1 and 2 into the working standard 1 tube. Use working standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Working standard 1 serves as the high standard. The appropriate calibrator diluent serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Dilute the Microparticle Cocktail in the mixing bottle provided.

Number of Wells Used	Microparticle Cocktail	+	Microparticle Diluent
96	1000 μ L	+	4.5 mL
72	750 μ L	+	3.375 mL
48	500 μ L	+	2.25 mL
24	250 μ L	+	1.125 mL

2. To prepare the RANTES microparticles, add the RANTES microparticle concentrate into previously diluted microparticles from Step 1 following the table below.

Number of Wells Used	Diluted Microparticle Cocktail	+	RANTES Microparticles
96	5.5 mL	+	55 μ L
72	4.125 mL	+	41.25 μ L
48	2.75 mL	+	27.5 μ L
24	1.375 mL	+	13.75 μ L

Note: Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.

DILUTED BIOTIN-ANTIBODY COCKTAIL PREPARATION

1. Centrifuge the Biotin-Antibody Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Biotin-Antibody Cocktail in Biotin Antibody Diluent 2. Mix gently.

Number of Wells Used	Biotin-Antibody Cocktail	+	Biotin Antibody Diluent 2
96	1000 μ L	+	4.5 mL
72	750 μ L	+	3.375 mL
48	500 μ L	+	2.25 mL
24	250 μ L	+	1.125 mL

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 μ L of Streptavidin-PE to 5.5 mL of Wash Buffer.

Number of Wells Used	Streptavidin-PE Concentrate	+	Wash Buffer
96	55.0 μ L	+	5.50 mL
72	42.0 μ L	+	4.10 mL
48	28.0 μ L	+	2.75 mL
24	14.0 μ L	+	1.35 mL

INSTRUMENT SETTINGS

Note: *Adjust the probe height setting on the analyzer to avoid puncturing the plate. Calibrate the analyzer using the proper reagents for polystyrene microparticles (refer to instrument manual).*

Luminex® 100/200™, Luminex® FLEXMAP 3D® and Bio-Rad Bio-Plex analyzers:

Note: *Ensure that the instrument flow rate is set to the default of 60 µL/minute (fast) for all flow based analyzers.*

- a) Sample volume: 50 µL
- b) Bead Type:
 - i. Luminex® 100/200™ and FLEXMAP 3D® select MicroPlex
 - ii. Bio-Rad Bio-Plex Manager use Bio-Plex Assay (Non-Magnetic)
- c) Doublet Discriminator gates:
 - i. Luminex® 100/200™ and FLEXMAP 3D® set at 7500 and 15,500
 - ii. Bio-Rad Bio-Plex Manager set at 4300 and 10,000
- d) Reporter Gain Setting:
 - i. Luminex® 100/200™ use Default setting
 - ii. Luminex® FLEXMAP 3D® use Standard PMT setting
 - iii. Bio-Rad Bio-Plex Manager use the low RP1 target value for the CAL2 setting
- e) Assign the microparticle region for each analyte being measured (see page 1)
- f) 50 count/region
- g) Collect MFI

ASSAY PROCEDURE

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

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 μ L of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.
Note: *After each final wash cycle and subsequent reagent addition, blot the bottom of the microplate with a paper towel to prevent wicking.*
3. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 μ L of the microparticle mixture to each well of the pre-wet filter-bottomed microplate.
4. Add 50 μ L of Standard or sample* per well. Pipette the assay within 15 minutes. Securely cover with a foil plate sealer. Incubate for 3 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. Using a vacuum manifold designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 μ L), and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the microplate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
6. Add 50 μ L of diluted Biotin Antibody Cocktail to each well. Securely cover with a new foil plate sealer, and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. Add 50 μ L of diluted Streptavidin-PE to each well. Securely cover with a new foil plate sealer, and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. Resuspend the microparticles by adding 100 μ L of Wash Buffer to each well. Incubate for 2 minutes at room temperature on the shaker set at 500 ± 50 rpm.
11. Read within 90 minutes using the Luminex® or Bio-Rad analyzer.
Note: *Resuspend microparticles immediately prior to reading by shaking the plate for 2 minutes on the plate shaker at 500 ± 50 rpm.*

*Samples may require dilution. See the Sample Preparation section.

ASSAY PROCEDURE SUMMARY

Note: Protect microparticles and Streptavidin-PE from light at all times.

- 1 Prepare all reagents as instructed.
- 2 Add 50 μL of diluted Microparticle Cocktail to each well.
- 3 Add 50 μL of standard, control, or sample* to each well.
Incubate for 3 hours at RT on a shaker at 500 rpm.
- 4 Wash by removing the liquid from each well, filling with 100 μL Wash Buffer, and removing the liquid again.
Perform the wash 3 times.
- 5 Add 50 μL of diluted Biotin-Antibody Cocktail to each well.
Cover and incubate for 1 hour at RT on the shaker at 500 rpm.
- 6 Repeat the wash as in step 4.
- 7 Add 50 μL of diluted Streptavidin-PE to each well.
Incubate for 30 minutes at RT on the shaker at 500 rpm.
- 8 Repeat the wash as in step 4.
- 9 Add 100 μL of Wash Buffer to each well.
Incubate for 2 minutes at RT on the shaker at 500 rpm.
- 10 Read within 90 minutes using a Luminex® or Bio-Rad analyzer
Note: Resuspend microparticles immediately prior to reading.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

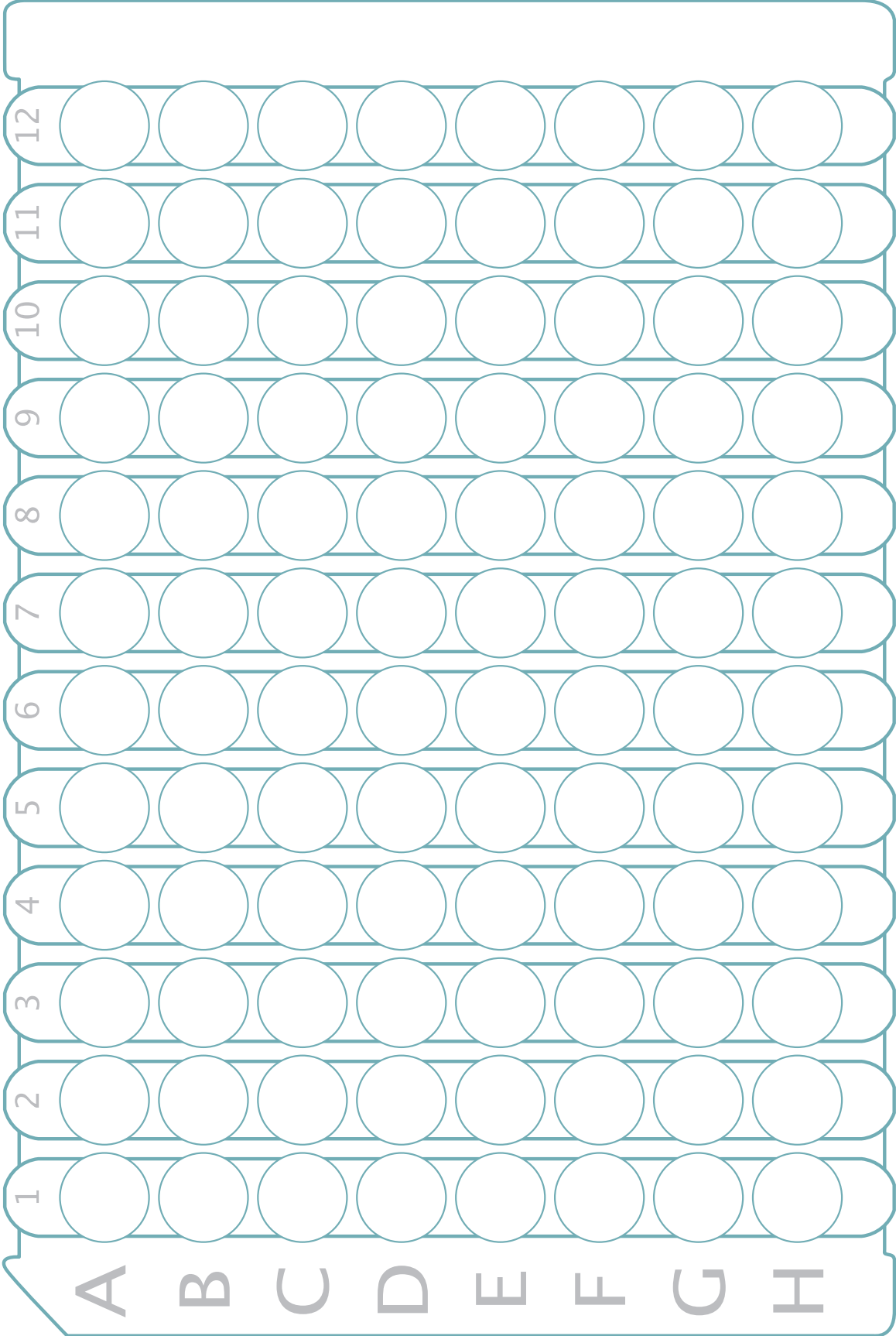
Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

CALIBRATION

This assay is calibrated against highly purified recombinant human cytokines produced at R&D Systems®.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



NOTES

NOTES

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