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R&D SYSTEMS

Luminex® Performance Assay

Human Cytokine Premixed Kit A

Catalog Number FCSTM03

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
CXCL5/ENA-78	12
FGF basic	13
G-CSF	14
GM-CSF	15
IFN-γ	18
IL-1α/IL-1F1	19
IL-1β/IL-1F2	20
IL-1ra/IL-1F3	21
IL-2	22
IL-4	25
IL-5	26
IL-6	27
CXCL8/IL-8	28
IL-10	29
IL-17	30
CCL2/MCP-1	33
CCL3/MIP-1α	34
CCL4/MIP-1β	35
CCL5/RANTES	36
TNF-α	37
Tpo	38
VEGF	39

PRINCIPLE OF THE ASSAY

Luminex® Performance Assay multiplex kits are designed for use with any Luminex® analyzer including the MAGPIX®, Luminex® 100/200™, FLEXMAP 3D®, xMAP INTELLIFLEX®, 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 microparticle 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 MAGPIX®. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles. One LED excites the dyes inside each microparticle to identify the region and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a CCD camera with a set of filters to differentiate excitation levels.

Analysis with the Luminex® 100/200™, FLEXMAP 3D®, xMAP INTELLIFLEX®, or Bio-Rad® Bio-Plex® uses one laser to excite the dyes inside each microparticle to identify the microparticle region and the second laser to excite the PE to measure the amount of analyte bound to the microparticle. All excitation emitted as each microparticle 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 fall outside the dynamic range of the assay, 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 these factors have been tested in the Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Luminex® Performance Assays afford the user the benefit of multi-analyte analysis of biomarkers 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 Card 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.	
Human Cytokine Premixed Kit A Microparticle Cocktail**	894537	1.2 mL of a concentrated microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.*
Human Cytokine Premixed Kit A Biotin-Ab Cocktail	894097	1.2 mL of a concentrated biotinylated antibody cocktail with preservatives.	
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. *For cell culture supernate samples. Use diluted 1:2 in this assay.*	
Calibrator Diluent RD6-40	895817	21 mL of a buffered animal serum with preservatives. *For serum/plasma samples. May contain a precipitate. Mix well before and during use.*	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Microplate	641385	1 flat-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).**

Additional wash buffer (#WA126) and plates (#LYX010) are available for purchase.

MATERIALS PROVIDED & STORAGE CONDITIONS *CONTINUED*

Note: *Additional reagents supplied if CCL5/RANTESis ordered.*

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
Human RANTES Microparticle Concentrate	894448	0.075 mL a concentrated microparticle with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.*

OTHER SUPPLIES REQUIRED

- **Luminex® Performance Assay analyte-specific kit(s) (see page 1)**
- MAGPIX®, Luminex® 100/200™, FLEXMAP 3D®, xMAP INTELLIFLEX®, or Bio-Rad® Bio-Plex® analyzer with X-Y platform
- Hand-held microplate magnet or platewasher with a magnetic platform
- 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 800 ± 50 rpm
- Microcentrifuge
- **Polypropylene** test tubes for dilution of standards and samples
- Luminex® Performance Assay Controls (optional; R&D Systems®, # QC02)

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.

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 centrifuging 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 µL of sample + 90 µL 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 µL of the 4-fold diluted sample + 240 µL of Calibrator Diluent RD6-40. Mix thoroughly.

REAGENT PREPARATION

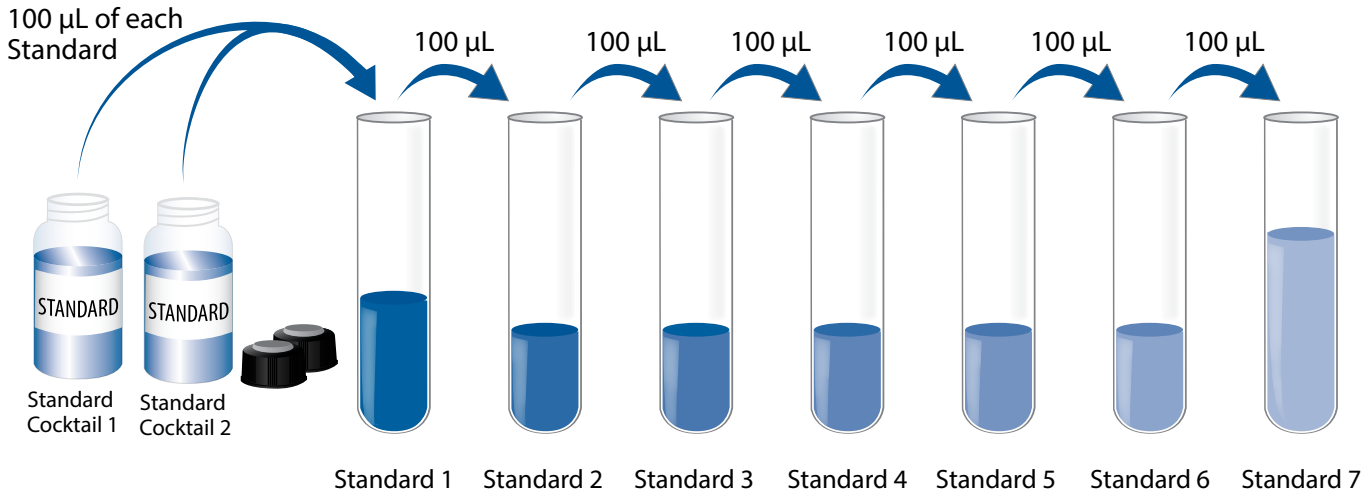
Bring all reagents to room temperature before use.

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 concentrate 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.
Note: *Do NOT vortex standard cocktail. Gentle agitation should be initiated only after the 15-minute reconstitution step is complete.*

Use polypropylene tubes. Pipette 300 µL of the appropriate calibrator diluent into a tube labeled working standard 1. Pipette 200 µL of the appropriate calibrator diluent into the remaining tubes. Pipette 100 µL 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). *Refer to the analyte specific datasheet for details.* 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. Centrifuge the Microparticle Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Cocktail using Microparticle Diluent in the mixing bottle provided.

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

4. To prepare the CCL5/RANTES microparticles, add the CCL5/RANTES microparticle concentrate into previously diluted microparticles from Step 3 following the table below. **Note:** *When measuring CCL5/RANTES as a 1-plex, dilute microparticles in Microparticle Diluent using the same volumes as outlined in the table below.*

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

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 µL	+	4.5 mL
72	750 µL	+	3.375 mL
48	500 µL	+	2.25 mL
24	250 µL	+	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 Streptavidin-PE concentrate in Wash Buffer.

Number of Wells Used	Streptavidin-PE Concentrate	+	Wash Buffer
96	55 µL	+	5.50 mL
72	42 µL	+	4.10 mL
48	28 µL	+	2.75 mL
24	14 µL	+	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 superparamagnetic microparticles (refer to instrument manual).*

MAGPIX® analyzer:

a) Sample volume: 50 µL

b) Assign the microparticle region for each analyte being measured (see page 1)

c) 50 count/region

d) Collect Median Fluorescence Intensity (MFI)

Luminex® 100/200™, FLEXMAP 3D®, xMAP INTELLIFLEX®, 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™ , FLEXMAP 3D®, and xMAP INTELLIFLEX select MagPlex

ii. Bio-Rad® Bio-Plex® Manager use Bio-Plex® MagPlex Beads (Magnetic)

c) Doublet Discriminator gates:

i. Luminex® 100/200™ and FLEXMAP 3D®, set at 8000 and 16,500

ii. xMAP INTELLIFLEX® set at 7000 and 17,000

iii. Bio-Rad® Bio-Plex® Manager set at 8000 and 23,000

d) Reporter Gain Setting:

i. Luminex® 100/200™ use Default setting

ii. FLEXMAP 3D® use Standard PMT setting

iii. xMAP INTELLIFLEX® use Luminex® 200™ Operating Mode on Low PMT setting

iv. 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, controls, 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. Add 50 µL of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
3. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 µL of the microparticle mixture to each well of the microplate. 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 800 ± 50 rpm.
4. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, allow 1 minute before removing the liquid, filling each well with Wash Buffer (100 µL) and allow 1 minute before removing the liquid again. Uniform removal of liquid is essential for good performance. **Note:** ***Do NOT blot; this may cause a loss of microparticles.*** Perform the wash procedure three times.
5. Add 50 µL 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 800 ± 50 rpm.
6. Repeat the wash as in step 4.
7. Add 50 µL 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 800 ± 50 rpm.
8. Repeat the wash as in step 4.
9. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 2 minutes at room temperature on the shaker set at 800 ± 50 rpm.
10. 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 800 ± 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.*

❶ Prepare all reagents as instructed.

❷ Add 50 µL of standard, control, or sample* to each well.

❸ Add 50 µL of diluted Microparticle Cocktail to each well.
Incubate for 3 hours at RT on a shaker at 800 rpm.

❹ Wash by removing the liquid from each well, filling with 100 µL Wash Buffer, and removing the liquid again.
Perform the wash 3 times.

❺ Add 50 µL of diluted Biotin-Antibody Cocktail to each well.
Cover and incubate for 1 hour at RT on the shaker at 800 rpm.

❻ Repeat the wash as in step 4.

❼ Add 50 µL of diluted Streptavidin-PE to each well.
Incubate for 30 minutes at RT on the shaker at 800 rpm.

❽ Repeat the wash as in step 4.

❾ Add 100 µL of Wash Buffer to each well.
Incubate for 2 minutes at RT on the shaker at 800 rpm.

❿ 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, such as Bio-Techne® Quantist™, capable of generating a five parameter logistic (5-PL) curve-fit.

If 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®.

SPECIFICITY

The assay was tested for cross-reactivity and interference with the following factors. Less than 0.5% cross-reactivity and interference was observed unless otherwise noted on the analyte specific datasheet.

Recombinant human:

6Ckine
CNTF
β-ECGF
FGF acidic
FGF-4
FGF-5
FGF-6
FGF-9
FGF-10
FGF-18
GCP-2
GROα
GROβ
GROγ
I-309
IGF-I
IGF-II
IL-1 RI
IL-1 RII
IL-2 Rα
IL-2 Rβ
IL-2 Rγ
IL-3 Rα
IL-4 R
IL-5 Rα
IL-6 R
IL-10 R
IL-3
IL-7
IL-9
IL-11
IL-12 p40
IL-12 p70
IL-13
IL-15
IL-16
IL-17
IL-18
LIF
LIF R
MIP-1α
MIP-3α
MIP-3β
MCP-2
MCP-3
MCP-4
M-CSF
TNF RI
$VEGF_{121}$
$VEGF_{165}$
VEGF-D

Recombinant mouse:

G-CSF
GM-CSF
IFN-γ
IL-1α
IL-1ra
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-17
MIP-1α
MIP-1β
RANTES
Tpo
TNF-α
VEGF

Recombinant rat:

GM-CSF
IFN-γ
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-10
TNF-α

Recombinant porcine:

GM-CSF
IL-1α
IL-1β
IL-2
IL-4
IL-6
IL-8
IL-10
Leptin
TNF-α

Recombinant human multiplex partners:

ENA-78
FGF basic
GM-CSF
IFN-γ
IL-1α
IL-1β
IL-1ra
IL-2
IL-4
IL-5
IL-6
IL-8
IL-10
IL-17
MCP-1
MIP-1α
MIP-1β
RANTES
Tpo
TNF-α
VEGF

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.