Magnetic Luminex® Performance Assay

Human Angiogenesis Base Kit A

Catalog Number LANM000

For the simultaneous quantitative determination of multiple human protein biomarker concentrations in cell culture supernates, serum, platelet-poor plasma, 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

Angiogenesis, involving the sprouting and branching of new blood vessels from pre-existing vessels, plays a critical role in wound healing and tumor growth. The typically quiescent adult vasculature does not require ongoing angiogenesis, except in female reproductive organs or in response to injured tissue. Pathologic angiogenesis occurs in tumor development, since the generation of a tumor blood supply is a rate-limiting step in tumor progression and metastasis, and in other vasculature disorders (1). In addition, angiogenesis also represents an excellent therapeutic target for the treatment of cardiovascular disease (2).

The emergence and maturation of new vessels are complex and highly-regulated processes that require multiple signaling cascades and affect endothelial cell proliferation and migration (3). Activators and inhibitors of angiogenesis coordinate the "angiogenic balance", which dictates whether an endothelial cell will be quiescent or angiogenic. Positive regulators of angiogenesis include FGFs, VEGFs, PDGF-BB, and EGF. Negative regulators include thrombospondin-1, angiostatin, and endostatin. Factors such as VEGF, placenta growth factor (PlGF), and Angiopoietin-1 stimulate angiogenesis as well as the de novo incorporation of marrow-derived endothelial cells into the walls of growing vessels (1, 3).

When combined with separately available analyte-specific bead sets, this kit is an excellent tool for simultaneously assessing the levels of multiple pro- and anti-angiogenic molecules in a single sample.

Any combination of the following bead sets are suitable for use with this base kit.

Analyte	Catalog Number	Microparticle Region
Angiogenin	LANM265	12
Angiopoietin-1	LANM923	25
Endostatin	LANM1098	14
FGF acidic	LANM232	15
FGF basic	LUHM233	13
PlGF	LANM264	20
PDGF-AA	LANM221	18
PDGF-BB	LANM220	19
Thrombospondin-2	LANM1635	21
VEGF	LUHM293	39
VEGF-D	LANM622	22

PRINCIPLE OF THE ASSAY

Magnetic Luminex® Performance Assay multiplex kits are designed for use with the Luminex® MAGPIX® CCD Imager. Alternatively, kits can be used 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 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 Luminex® MAGPIX® Analyzer. 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 filter to differentiate excitation levels.

Analysis with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad Bio-Plex uses one laser to excite the dyes inside each microparticle to identifying 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 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. Until these factors have been tested in the Magnetic Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Magnetic 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 base 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	893605	2 vials of recombinant human protein biomarkers in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Microparticle Diluent 5	895575	6 mL of a buffered protein base with preservative.	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.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	
Streptavidin-PE	892525	0.07 mL of a concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Calibrator Diluent RD6-49	895580	2 vials (21 mL/vial) of a buffered protein base with preservatives. *Use undiluted for serum/plasma/human milk samples. Use diluted 2:1 for cell culture supernate/urine samples.* ***Must be warmed to room temperature prior to use.***	May be stored for up to 1 month at 2-8 °C.*
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	749101	1 card listing the Standard reconstitution volume and working standard concentrations for this lot of base kit.	

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

OTHER SUPPLIES REQUIRED

- **Magnetic Luminex® Performance Assay analyte-specific kit(s) (see page 1)**.
- Luminex® MAGPIX®, Luminex® 100/200™, Luminex® FLEXMAP 3D®, 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®, Catalog # QC06).

PRECAUTIONS

Calibrator Diluent RD6-49 contains sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

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 at room temperature 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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. For more complete platelet removal, an additional centrifugation step of the separated plasma at 1500 x g for 10 minutes at 2-8 °C is recommended. 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.*

Angiopoietin-1, PDGF-AA, and PDGF-BB are present in platelet granules and are released upon platelet activation. Therefore, to measure circulating levels of these factors, platelet-free plasma should be collected for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and Standards Institute (CLSI), result in incomplete removal of platelets from blood. This will cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

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

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.*

Cell culture supernate and urine samples require a 5-fold dilution. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD6-49 (diluted 2:1). Mix thoroughly.

Serum and platelet-poor plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD6-49. Mix thoroughly.

Note: *Angiogenin serum and platelet-poor plasma samples require an additional 10-fold dilution. A suggested 10-fold dilution is 50 µL of diluted sample + 450 µL of Calibrator Diluent RD6-49 to complete the 50-fold dilution. Mix thoroughly.*

Human milk samples require a 5-fold dilution. A suggested 5-fold dilution is 50 µL of sample + 200 µL of Calibrator Diluent RD6-49.

Note: *Angiogenin milk samples require an additional 4-fold dilution. A suggested 4-fold dilution is 100 µL of diluted sample + 300 µL of Calibrator Diluent RD6-49 to complete the 20-fold dilution. Mix thoroughly.*

REAGENT PREPARATION

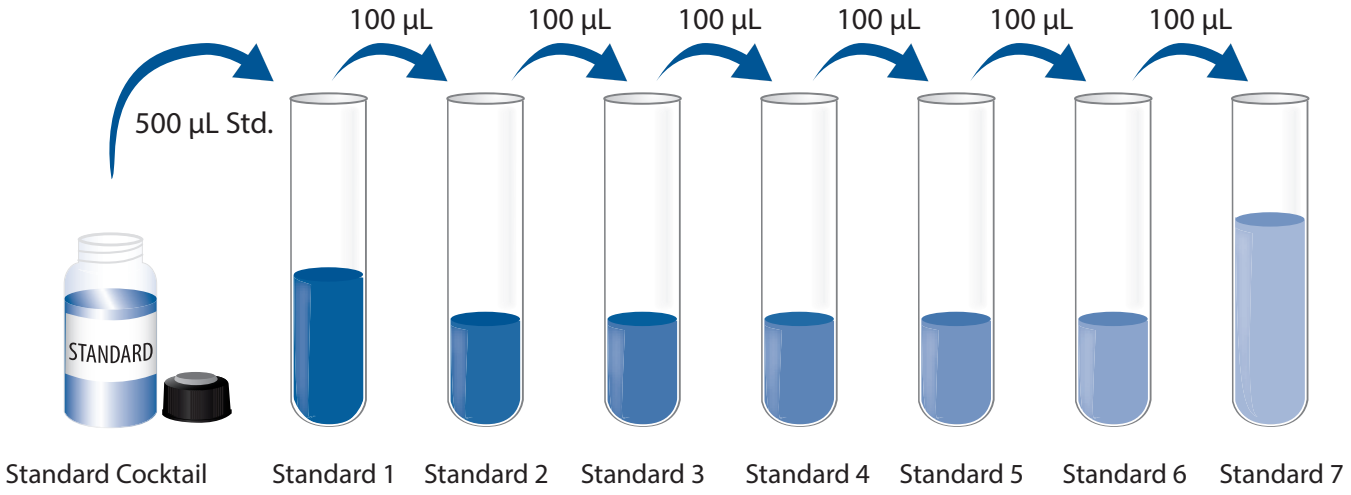
Bring all reagents to room temperature before use.

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 RD6-49 (diluted 2:1) - **For cell culture supernate and urine samples.** Add 20 mL of Calibrator Diluent RD6-49 to 10 mL of deionized or distilled water to prepare 30 mL of Calibrator Diluent RD6-49 (diluted 2:1).

Standard - **Refer to the Standard Value Card for the reconstitution volume and assigned values.** Reconstitute the Standard Cocktail with Calibrator Diluent RD6-49 (*for serum, plasma, and human milk samples*) or Calibrator Diluent RD6-49 (diluted 2:1) (*for cell culture supernate and urine samples*). Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions. **Use reconstituted standards within 4 hours.**

Use polypropylene tubes. Pipette 500 µL of the reconstituted Standard into a tube labeled Standard 1. Pipette 300 µL of the appropriate calibrator diluent into the remaining tubes. Use Standard 1 to produce a 4-fold dilution series (below) *Refer to analyte specific data sheets for details*. Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. The appropriate calibrator diluent serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge each Microparticle Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials to resuspend the microparticles, taking precautions not to invert the vials.
3. Dilute the Microparticle Concentrates in the mixing bottle provided. The volume of the Microparticle Concentrate listed in the table below is for each analyte (*e.g.* if measuring a full plate of FGF basic and VEGF, add 50 µL of FGF basic Microparticle Concentrate and 50 µL of VEGF Microparticle Concentrate to 5 mL of Microparticle Diluent).

Number of Wells Used	Microparticle Concentrate	+	Microparticle Diluent
96	50.0 µL	+	5.00 mL
72	37.5 µL	+	3.75 mL
48	25.0 µL	+	2.50 mL
24	12.5 µL	+	1.25 mL

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 each Biotin-Antibody Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials, taking precautions not to invert the vials.
3. Dilute the Biotin-Antibody Concentrates in Biotin Antibody Diluent 2. The volume of the Biotin-Antibody listed in the table below is for each analyte (e.g. if measuring a full plate, add 50 µL of each Biotin-Antibody to 5 mL of Biotin Antibody Diluent 2). Mix gently.

Number of Wells Used	Biotin-Antibody Concentrate	+	Biotin Antibody Diluent
96	50.0 µL	+	5.00 mL
72	37.5 µL	+	3.75 mL
48	25.0 µL	+	2.50 mL
24	12.5 µL	+	1.25 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	55.0 µL	+	5.50 mL
72	42.0 µL	+	4.10 mL
48	28.0 µL	+	2.75 mL
24	14.0 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).*

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

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 stanadrds, 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 100 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 cocktail to each well of the microplate. Securely cover with a foil plate sealer. Incubate for 2 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. Complete 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.

   Note: *Refer to the magnetic device user manual for proper wash technique using a round bottom microplate.*
5. Add 50 µL of diluted Biotin-Antibody Cocktail to each well. Securely cover with a 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 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 on the shaker set at 800 ± 50 rpm.
10. Read within 90 minutes using a 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 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.

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❷ Add 100 µL of standard, control, or sample* to each well.

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❸ Add 50 µL of diluted Microparticle Cocktail to each well.
Incubate for 2 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: *Incubate for 2 minutes at RT on the shaker at 800 rpm immediately prior to reading.*

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 4-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 (human FGF basic utilizes a 4-PL curve fit).

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

CALIBRATION

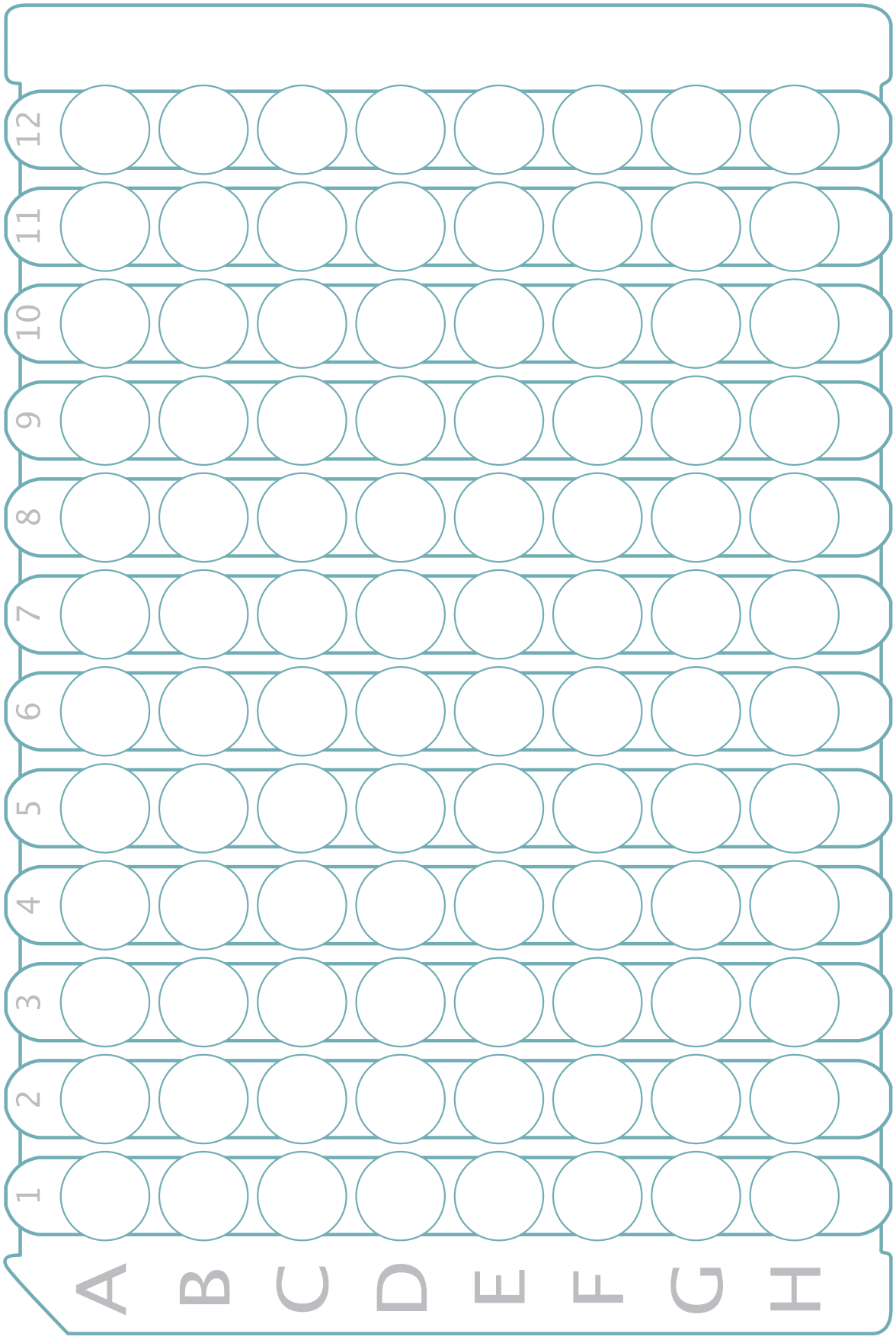
This assay is calibrated against highly purified recombinant human protein biomarkers produced at R&D Systems®.

REFERENCES

1. Bergers, G. and L.E. Benjamin (2003) Nat. Rev. Cancer **3**:401.
2. Atluri, P. and Y.J. Woo (2008) BioDrugs **22**:209.
3. Karamysheva, A.F. (2008) Biochemistry (Mosc) **73**:751.

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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