

# **Magnetic Luminex<sup>®</sup> Performance Assay**

## **Human Angiogenesis Premixed Kit A**

Catalog Number FCSTM02

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).

This kit can be used to simultaneously assess the levels of multiple biomarkers 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 ( <a href="http://www.RnDSystems.com/pdf/...">www.RnDSystems.com/pdf/...</a> )
Angiogenin	12	LANM265.pdf
Angiopoietin-1	25	LANM923.pdf
Endostatin	14	LANM1098.pdf
FGF acidic	15	LANM232.pdf
FGF basic	13	LUHM233.pdf
PlGF	20	LANM264.pdf
PDGF-AA	18	LANM221.pdf
PDGF-BB	19	LANM220.pdf
Thrombospondin-2	21	LANM1635.pdf
VEGF	39	LUHM293.pdf

## 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 filters 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 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 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 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	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.
Human Angiogenesis Premixed Kit A Magnetic Microparticle Cocktail	894299	1.2 mL of a concentrated microparticle cocktail 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>
Human Angiogenesis Premixed Kit A Biotin-Ab Cocktail	894120	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 5	895575	6 mL of a buffered protein base with 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 RD6-49	895580	2 vials (21 mL/vial) of buffered protein base with preservatives. <i>Used undiluted for serum/platelet-poor plasma/human milk samples. Used diluted 2:1 for cell culture supernate/urine samples. <b>Must be warmed to room temperature prior to use.</b></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	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 Cocktail reconstitution volume and working standard concentrations for this kit lot.	

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

## OTHER SUPPLIES REQUIRED

- 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  $800 \pm 50$  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  $\leq -20^{\circ}\text{C}$ . 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  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Platelet-poor Plasma** - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at  $2-8^{\circ}\text{C}$  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  $2-8^{\circ}\text{C}$  is recommended. Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . 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  $\leq -20^{\circ}\text{C}$ . Avoid repeated freeze-thaw cycles.

**Human Milk** - Assay immediately or aliquot and store samples at  $\leq -20^{\circ}\text{C}$ . 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.

Serum and platelet-poor plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 50  $\mu\text{L}$  of sample + 200  $\mu\text{L}$  of Calibrator Diluent RD6-49. Mix thoroughly.

**Note:** Angiogenin serum and plasma samples require an additional 10-fold dilution. A suggested 10-fold dilution is 50  $\mu\text{L}$  of diluted sample + 450  $\mu\text{L}$  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  $\mu\text{L}$  of sample + 200  $\mu\text{L}$  of Calibrator Diluent RD6-49.

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

Cell culture supernate and urine samples require a 5-fold dilution. A suggested 5-fold dilution is 50  $\mu\text{L}$  of sample + 200  $\mu\text{L}$  of Calibrator Diluent RD6-49 (diluted 2:1)\*. Mix thoroughly.

\*See Reagent Preparation section.

## REAGENT PREPARATION

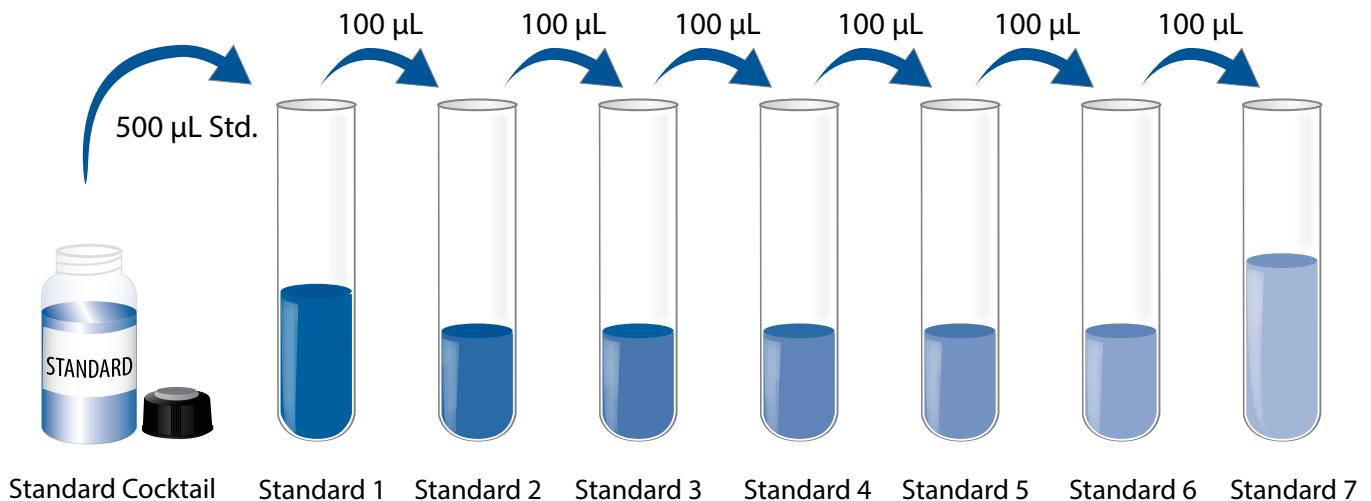
**Bring all reagents to room temperature before use.**

**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 use in cell culture supernates and urine samples only.** 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, platelet-poor 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  $\mu$ L of reconstituted standard into a tube labeled Standard 1. Pipette 300  $\mu$ L of the appropriate calibrator diluent into the remaining tubes. Use Standard 1 to produce a 4-fold dilution series (below). 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 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 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

**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	55.0 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 standards, controls, and samples be assayed in duplicate.**

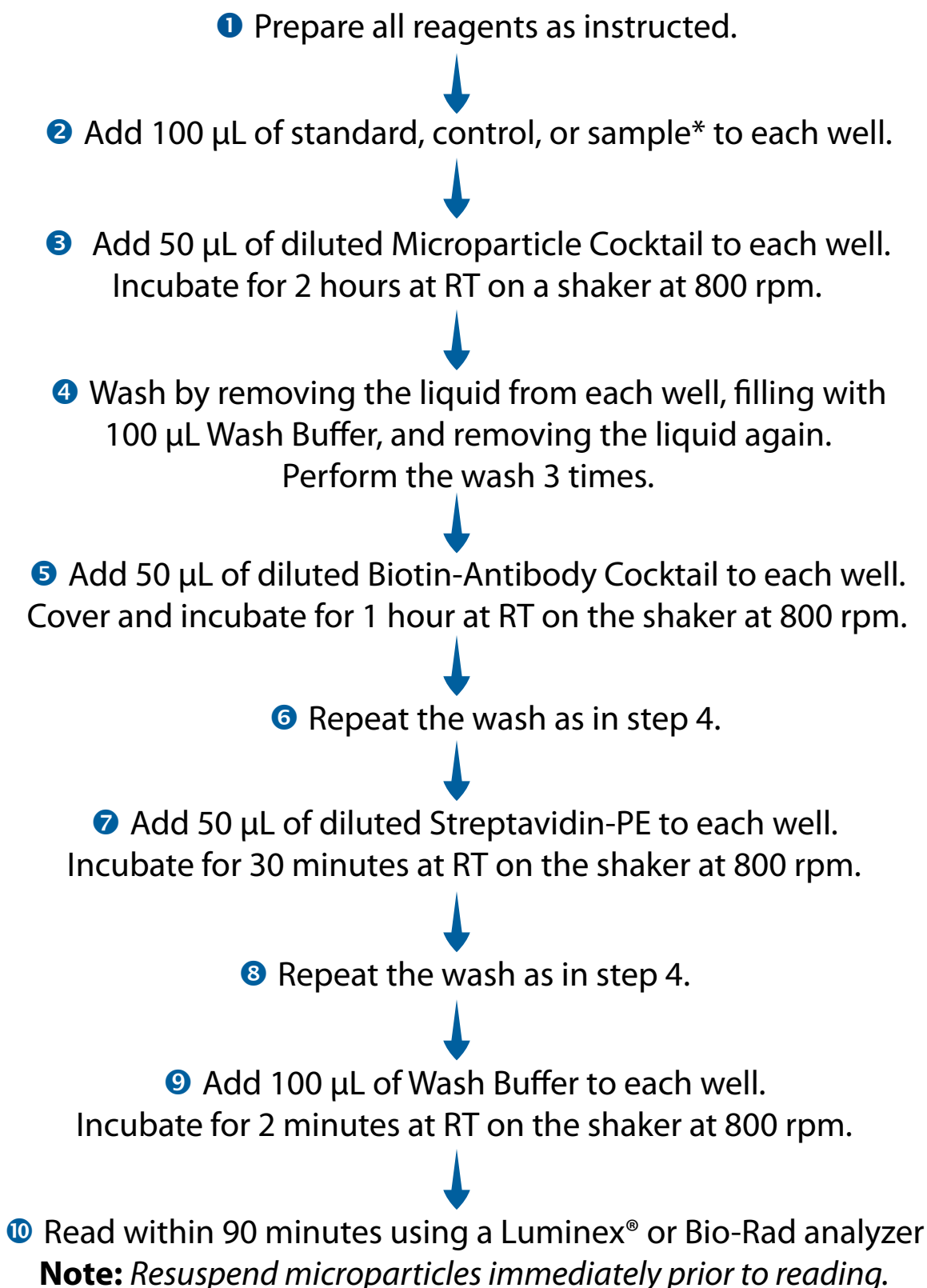
**Note:** *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Add 100  $\mu$ L 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  $\mu$ L of the Microparticle Cocktail to each well. 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  $800 \pm 50$  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  $\mu$ L) 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  $\mu$ 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  $800 \pm 50$  rpm.
6. Repeat the wash as in step 4.
7. Add 50  $\mu$ 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  $800 \pm 50$  rpm.
8. Repeat the wash as in step 4.
9. Resuspend the microparticles by adding 100  $\mu$ L of Wash Buffer to each well. Incubate for 2 minutes at room temperature on the shaker set at  $800 \pm 50$  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  $800 \pm 50$  rpm.*

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

## ASSAY PROCEDURE SUMMARY

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



\*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.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H



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