Luminex® Performance Assay

Human Angiogenesis Premixed Kit A

Catalog Number FCST02

For the simultaneous quantitative determination of multiple human angiogenesis biomarker concentrations in cell culture supernates, serum, 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.

TABLE OF CONTENTS

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

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-A, 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 (www.RnDSystems.com/pdf/...)
Angiogenin	05	LAN265.pdf
Angiopoietin-1	22	LAN923.pdf
Endostatin	18	LAN1098.pdf
FGF acidic	33	LAN232.pdf
FGF basic	54	LUH233.pdf
PlGF	34	LAN264.pdf
PDGF-AA	51	LAN221.pdf
PDGF-BB	37	LAN220.pdf
Thrombospondin-2	59	LAN1635.pdf
VEGF	52	LUH293.pdf
VEGF-D	66	LAN622.pdf

PRINCIPLE OF THE ASSAY

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

Analyte-specific antibodies are pre-coated onto color-coded microparticles. 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 detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE. The microparticles are resuspended in buffer and read using the Luminex or Bio-Plex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

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 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 multianalyte analysis of biomarkers in a single complex sample. 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.**

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.

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 Microparticle Cocktail	894121	1.2 mL of a concentrated microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, any unused microparticle cocktail must be discarded.*
Human Angiogenesis Premixed Kit A Biotin-Ab Cocktail	894120	1.2 mL of a concentrated biotin antibody cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Microparticle Diluent 5	895575	6 mL of a buffered protein base with preservative.	
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. ***Warm to room temperature prior to use.***	
Streptavidin-PE	892525	0.07 mL of a 100-fold concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
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	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 100, Luminex 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

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 ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

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

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 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.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 2-8 °C at 1000 x g within 30 minutes of collection. An additional centrifugation step of the separated plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. 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 and Laboratory 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

Serum and 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 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.*

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 (1.5X). Mix thoroughly.

REAGENT PREPARATION

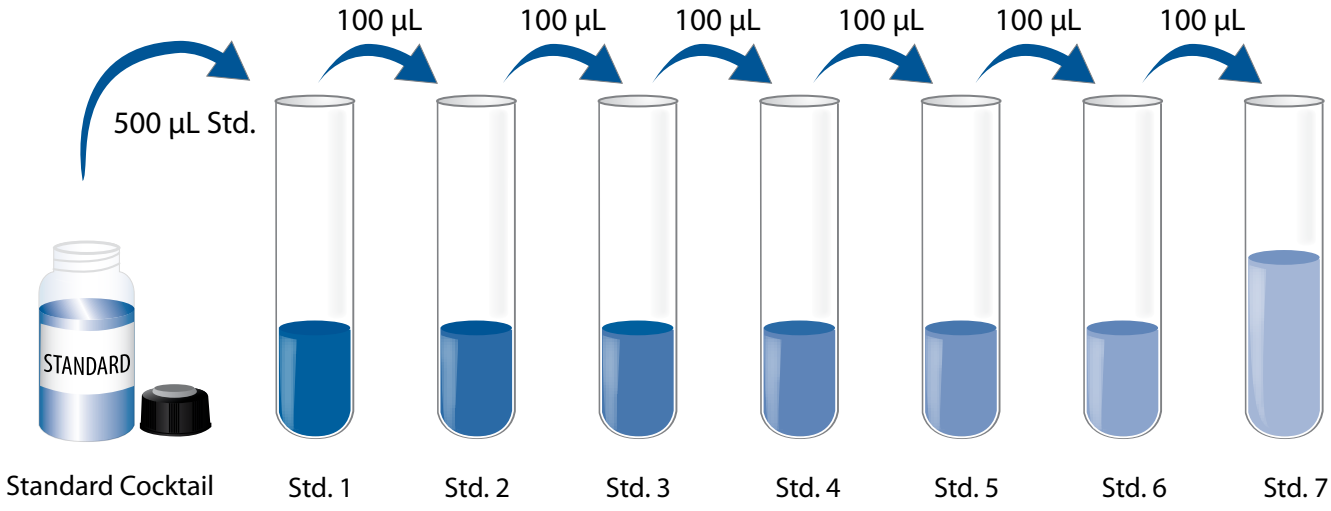
Bring all reagents to room temperature before use.

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

Calibrator Diluent RD6-49 (1.5X) - 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 (1.5X). *For use with cell culture supernates and urine samples only.*

Standard - Reconstitute the Standard Cocktail with Calibrator Diluent RD6-49 (*for serum, plasma, and human milk samples*) or Calibrator Diluent RD6-49 (1.5X) (*for cell culture supernate and urine samples*). Refer to the Standard Value Card for the reconstitution volume and assigned values. 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 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). 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 100X Streptavidin-PE to a 1X concentration by adding 55 µL of Streptavidin-PE to 5.5 mL of Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex analyzer to avoid puncturing the membrane. Refer to the instrument manual.

a) Assign the bead region for each analyte being measured

b) 50 events/bead

c) Minimum events: 0

d) Flow rate: 60 µL/minute (fast)

e) Sample size: 50 µL

f) Doublet Discriminator gates at approximately 7500 and 15,500

g) Collect Median Fluorescence Intensity (MFI)

Note: *For the Bio-Rad Bio-Plex analyzer, set the gates at 4300 and 10,000. The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.*

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards 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 µL of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accomodate 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. Add 100 µL of Standard or sample* per well to the pre-wet filter-bottomed microplate. A plate layout is provided to record standards and samples assayed.
4. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 µL of the microparticle mixture 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 500 ± 50 rpm.
5. Using a vacuum manifold device designed to accomodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 µL), 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.

   Note: *To ease microplate filtration, it may be necessary to soak the bottom of the plate in deionized or distilled water for up to 1 minute.*
6. 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 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. 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 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. 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 500 ± 50 rpm.
11. Read within 90 minutes using the Luminex or Bio-Rad analyzer.

*Samples require dilution. See the 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.