# TGFβ 1,2,3 Magnetic Bead Kit

## 96-Well Plate Assay

## Cat. # TGFBMAG-64K-03

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**MILLIPLEX® MAP**

**TGFβ1,2,3 MAGNETIC BEAD KIT**
**96-Well Plate Assay**

**# TGFBMAG-64K-03**

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By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex® Corporation ("Luminex®"), you, the customer, acquire the right under Luminex®'s patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex®'s laser based fluorescent analytical test instrumentation marketed under the name of Luminex® 100™ IS, 200™, HTS, FLEXMAP 3D®, MAGPIX®.

## INTRODUCTION

The transforming growth factor beta (TGFβ) system, a superfamily of cytokines as well as signaling pathways, is highly conserved throughout the animal kingdom. TGFβ functions in angiogenesis, wound healing and embryonic development, and plays a critical role in immunity, heart disease, and cancer. In its normal state TGFβ is one of the few classes of proteins able to inhibit cell growth by halting mitosis at the G1 state, inducing cell differentiation or apoptosis. However, during oncogenesis mutations in the TGFβ signaling pathway result in tumor cell resistance to the effects of normally functioning TGFβ, causing proliferation without regulation. Initial research suggests that VE-cahedrin may enhance the mutated TGFβ signaling pathway, while other research indicates that DNA methylation plays a role in pathway mutation.

The secreted TGFβ cytokine exists in three isoforms: TGFβ1, TGFβ2 and TGFβ3. Secreted by most immune cells, TGFβ1 plays a critical role in controlling the immune system, acting on cells differently depending on cell type as well as stage of differentiation. TGFβ2, also known as glioblastoma-derived T-cell suppressor factor (G-TSF), plays a role in embryonic development and has the ability to suppress the effects of interleukin-dependent T-cell tumors. TGFβ3 regulates cellular adhesion molecules and extracellular matrix formation, as well as lung and palate development. TGFβ3 deficiency during mammalian development results in the cleft palate deformity. In addition TGFβ3 controls wound healing by regulating epidermal and dermal cell movement in injured skin.

TGFβ3 may not be detected in normal plasma and serum samples. Due to the involvement of all three isotypes in neonate development and lactation regulation, literature suggests detectable levels in milk.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® MAP TGFβ 1, 2 and 3 Magnetic Bead Panel thus enables you to focus on the therapeutic potential of Immunology/Immune Response. Coupled with the Luminex® xMAP® platform in a magnetic bead format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

EMD Millipore's MILLIPLEX® MAP TGFβ 1, 2 and 3 Magnetic Bead Panel is part of the most versatile system available for Immunology/Immune Response research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation. MILLIPLEX® MAP offers you a convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP TGFβ 1, 2 and 3 Magnetic Bead Panel is a 3-plex kit to be used for the simultaneous quantification of the following analytes in serum, plasma and cell/tissue culture samples: TGFβ1, TGFβ2, TGFβ3

***For Research Use Only. Not for Use in Diagnostic Procedures.***

***Please read entire protocol before use.***

***It is important to use same assay incubation conditions throughout your study.***

## PRINCIPLE

MILLIPLEX® MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex®-C microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex® instruments to acquire and analyze data using two detection methods:
    - The Luminex® analyzers Luminex® 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

## STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

## REAGENTS SUPPLIED

**Note: Store all reagents at 2 – 8°C**

| Reagents Supplied | Catalog Number | Volume | Quantity |
|---|---|---|---|
| TGFβ1,2,3 Standard | TGFB-8064-3 | Lyophilized | 1 vial |
| TGFβ1,2,3 Quality Controls 1 and 2 | TGFB-6064-3 | Lyophilized | 2 vials |
| TGFβ Detection Antibody | LTGF-1064 | 3.2 mL | 1 bottle |
| Streptavidin-Phycoerythrin | L-SAPE9 | 3.2 mL | 1 bottle |
| Serum Matrix<br>Note: Contains 0.08% Sodium Azide | LMC-SD | Lyophilized | 1 vial (required for serum and plasma samples only) |
| Sample Diluent<br>Note: Contains 0.08% Sodium Azide | LTGF-SD | 5.0 mL | 1 bottle |
| 1.0 N Hydrochloric Acid (HCl) | L-HCL | 1.0 mL | 1 bottle |
| 1.0 N Sodium Hydroxide (NaOH) | L-NAOH | 1.0 mL | 1 bottle |
| Assay Buffer | L-AB1 | 30 mL | 2 bottles |
| 10X Wash Buffer<br>Note: Contains 0.05% Proclin | L-WB | 60 mL | 1 bottle |
| Set of one 96-Well Microtiter Plate with 2 Sealers | ----------- | ----------- | 1 plate<br>2 sealers |

**TGFβ1,2,3 Premixed Antibody-Immobilized Magnetic Beads:**

| Bead/Analyte Name | Luminex® Magnetic Bead Region | (1X concentration, 3.5 mL) | |
|---|---|---|---|
| | | Available | Cat. # |
| Anti-TGFβ1 Bead | **18** | ✓ | **TGFBPMX3-MAG** |
| Anti-TGFβ2 Bead | **54** | ✓ | |
| Anti-TGFβ3 Bead | **36** | ✓ | |

## MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex® Sheath Fluid (EMD Millipore Catalog # SHEATHFLUID) or Luminex® Drive Fluid (EMD Millipore Catalog # MPXDF-4PK)

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
10. Titer Plate Shaker (VWR® Microplate Shaker Cat # 12620-926 or equivalent)
11. Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
12. Automatic Plate washer for magnetic beads (BioTek® 405 LS and 405 TS, EMD Millipore Catalog # 40-094, # 40-095, # 40-096, # 40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog # 40-285 or equivalent).

Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog # MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog # MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog # WP6111560 or equivalent).

## SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

**Note: See Full Labels of Hazardous components on next page.**

**Full Hazardous labels for components in this kit:**

| Ingredient, Cat # | | Full Label | |
|---|---|---|---|
| 1.0N NaOH | L-NAOH | | **Danger.** May be corrosive to metals. Causes severe skin burns and eye damage. Wear protective gloves/ protective clothing/ eye protection/ face protection. IF SWALLOWED: Rinse mouth. Do NOT induce vomiting. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. IF exposed or concerned: immediately call a POISON CENTER or doctor/ physician. |
| Serum Matrix | LMC-SD | No Symbol Required | Harmful to aquatic life with long lasting effects. Avoid release to the environment. |
| Streptavidin-Phycoerythrin | L-SAPE9 | | **Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| Wash Buffer | L-WB | | **Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water. |
| Assay Buffer | L-AB1 | | **Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| TGFβ1,2,3 Standard | TGFB-8064-3 | | Harmful to aquatic life with long lasting effects. Avoid release to the environment. |
| TGFβ1,2,3 Quality Controls | TGFB-6064-3 | | Harmful to aquatic life with long lasting effects. Avoid release to the environment. |
| 1.0 N Hydrochloric Acid (HCl) | L-HCL | | **Warning.** May be corrosive to metals. |

## TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final suspension should be in 150 µL and 75 µL should be aspirated.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.
- For serum/plasma samples that require further dilution beyond 1:30, use the Serum Matrix provided in the kit.

## TECHNICAL GUIDELINES (continued)

- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

## SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:
   - Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
   - Avoid multiple (<2) freeze/thaw cycles.
   - When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
   - See Preparation of Reagents Step E for detailed sample treatment information before setting up the assay.

B. Preparation of Plasma Samples:
   - Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20°C.
   - Avoid multiple (>2) freeze/thaw cycles.
   - When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
   - See Preparation of Reagents Step E for detailed sample treatment information before setting up the assay.

C. Preparation of Tissue Culture Supernatant:
   - Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
   - Avoid multiple (>2) freeze/thaw cycles.
   - Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.
   - See Preparation of Reagents Step E for detailed sample treatment information before setting up the assay.

## SAMPLE COLLECTION AND STORAGE (continued)

**NOTE:**

- A maximum of 25 µL per well of diluted serum or plasma can be used. Tissue culture or other media may also be used.
- To measure circulating TGFβ1 in plasma it is necessary to remove platelets from the samples. Platelet granules contain TGFβ1 which are released upon activation.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

## PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the antibody-bead bottle 30 seconds and then vortex for 1 minute before use. Unused portion may be stored at 2-8°C for up to six months.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL **Assay Buffer**. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Unused portion may be stored at ≤ 20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

**This step is required for serum or plasma samples only.**
Add 1.0 mL of deionized water and 4.0 mL of Assay Buffer to the bottle containing the lyophilized Serum Matrix, mix and let it set for at least 10 min at room temperature to allow complete reconstitution. Mix well. In a separate tube, add 0.1 mL of the reconstituted Serum Matrix to 0.5 mL Assay Buffer for a final dilution of 1:30. Mix well. This is the working Serum Matrix to be used for the Standards and Controls when measuring serum /plasma samples.

E. Treatment of Samples

For measuring active TGFβ, it is necessary to treat serum/plasma samples and tissue/cell culture samples prior to the assay. **Note: Tissue/cell culture samples may or may not require dilution, depending on the sample concentration of TGFβ.**

Serum/Plasma Samples:

1. Centrifuge samples to remove debris and excess lipids.
2. Dilute serum/plasma samples by adding 1 part of serum/plasma to 4 parts of Sample Diluent (e.g. 20 µl serum and 80 µl Cat. # LTGF-SD).
3. Add 2.0 µl of 1.0 N HCl to each 25 µl of the 1:5 diluted samples. Make sure sample pH drops below 3.0. After mixing, acidified samples should be moderately shaken at room temperature for 15 min or incubated with no shaking for 1 hour at room temperature.
4. Acid-treated serum/plasma samples should be further diluted 1:6 using Assay Buffer (Cat. # L-AB1) as the diluent for a final sample dilution of 1:30 immediately prior to addition to sample wells.

Tissue/Cell Culture Samples Requiring Dilution:

1. Centrifuge samples to remove debris and excess lipids.
2. Dilute tissue/cell culture samples with Sample Diluent. The dilution factor should be determined by the user. For example, adding 1 part of sample to 4 parts of Sample Diluent (e.g. 20 µl sample and 80 µl Cat. # LTGF-SD) for 1:5 dilution.
3. Add 2.0 µl of 1.0 N HCl to each 25 µl of the 1:5 diluted culture medium samples. Make sure sample pH drops below 3.0. After mixing, acidified samples should be moderately shaken at room temperature for 15 min or incubated with no shaking for 1 hour at room temperature.
4. The acid-treated tissue/cell culture samples should be neutralized by 1.0 N NaOH prior to addition to sample wells. The volume of 1.0 N NaOH required is approximately similar to the volume of 1.0 N HCl used. But actual volume of 1.0 N NaOH required to achieve neutral pH may vary, depending on initial sample pH and buffering capacity of the samples.

Tissue/Cell Culture Samples Requiring **NO DILUTION**:

1. Centrifuge samples to remove debris and excess lipids.
2. Add 2.0 µl of 1.0 N HCl to each 25 µl of the culture medium samples. Make sure sample pH drops below 3.0. After mixing, acidified samples should be moderately shaken at room temperature for 15 min or incubated with no shaking for 1 hour at room temperature.
3. The acid-treated tissue/cell culture samples should be neutralized by 1.0 N NaOH prior to addition to sample wells. The volume of 1.0 N NaOH required is approximately similar to the volume of 1.0 N HCl used. But actual volume of 1.0 N NaOH required to achieve neutral pH may vary, depending on initial sample pH and buffering capacity of the samples.

F. Preparation of TGFβ1,2,3 Standard

1.) Prior to use, reconstitute the TGFβ1,2,3 Standard with 250µL **Assay Buffer** to give a 10,000 pg/mL concentration of standard. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as the 10,000 pg/mL standard; the unused portion may be stored at ≤ -20°C for up to one month.

## PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

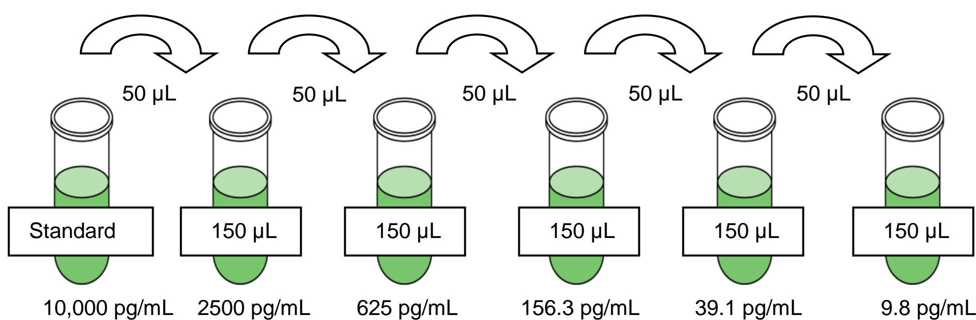
2). Preparation of Working Standards

Label five polypropylene microfuge tubes 2500, 625, 156.3, 39.1, and 9.8 pg/mL. Add 150 µL of the Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2500 pg/mL tube, mix well and transfer 50 µL of the 2500 pg/mL standard to the 625 pg/mL tube, mix well and transfer 50 µL of the 625 pg/mL standard to the 156.3 pg/mL tube, mix well and transfer 50 µL of the 156.3 pg/mL standard to 39.1 pg/mL tube, mix well and transfer 50 µL of the 39.1 pg/mL standard to the 9.8 pg/mL tube and mix well. The 0 pg/mL standard (Background) will be the Assay Buffer.

| Standard Concentration (pg/mL) | Volume of **Assay Buffer** to Add | Volume of Standard to Add |
|---|---|---|
| 10,000 | 250 µL | 0 |

| Standard Concentration (pg/mL) | Volume of Assay Buffer to Add | Volume of Standard to Add |
|---|---|---|
| 2500 | 150 µL | 50 µL of 10,000 pg/mL |
| 625 | 150 µL | 50 µL of 2500 pg/mL |
| 156.3 | 150 µL | 50 µL of 625 pg/mL |
| 39.1 | 150 µL | 50 µL of 156.3 pg/mL |
| 9.8 | 150 µL | 50 µL of 39.1 pg/mL |

### Preparation of Standards



## IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), 9.8, 39.1, 156.3, 625, 2500, 10,000 pg/mL], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying tissue culture samples, use identical control medium (provided by users) for Background, Standard and Quality Control wells. For serum/plasma samples, use the Serum Matrix (see Preparation of Reagents Step D.) for Background, Standard, and Quality Control wells.
6. Add 25 µL of treated sample into the appropriate wells (see Preparation of Reagents Section Step E.).
7. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker for 2 hours at room temperature (20-25°C) or overnight (16-18 hours) at 4°C.

Add 200 µL Assay Buffer per well

Shake 10 min, RT

Decant

- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background and sample wells
- Add 25 µL appropriate matrix solution to background, standards, and control wells
- Add 25 µL treated Samples to sample wells
- Add 25 µL Beads to each well

Incubate 2 hours at RT or overnight at 4°C with shaking

9. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section.

10. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**

12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

14. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section.

15. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 100 µL Sheath Fluid or Drive Fluid per well

Read on Luminex® (50 µL, 50 beads per bead set)

## PLATE WASHING

**1.) Solid Plate**

**If using a solid plate, use either a handheld magnet or magnetic plate washer.**

A.) Handheld magnet (EMD Millipore Catalog # 40-285) - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

B.) Magnetic plate washer (EMD Millipore Catalog # 40-094, # 40-095, # 40-096 and # 40-097) - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 µL of residual wash buffer in each well. This is expected when using the BioTek® plate washer and this volume does not need to be aspirated from the plate.

**If using an automatic plate washer other than BioTek® 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.**

**2.) Filter Plate (EMD Millipore Catalog # MX-PLATE)**

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

## EQUIPMENT SETTINGS

Luminex® 200™, HTS, FLEXMAP 3D® and MAGPIX® with xPONENT® software:

These specifications are for the Luminex® 200™, Luminex® HTS, Luminex® FLEXMAP 3D® and Luminex® MAGPIX® with xPONENT® software. Luminex® instruments with other software (e.g. MasterPlex®, StarStation, LiquiChip, Bio-Plex Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® Magnetic Beads.

For magnetic bead assays, the Luminex® 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog # 40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog # 40-276). The Luminex® FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog # 40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog # 40-029). The Luminex® MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog # 40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog # 40-050).

**NOTE: When setting up a Protocol using the xPONENT® software, you must select MagPlex as the Bead Type in the Acquisition settings.**

**NOTE: These assays cannot be run on any instruments using Luminex® IS 2.3 or Luminex® 1.7 software.**

## EQUIPMENT SETTINGS (continued)

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Catalog # MAG-PLATE, if additional plates are required for this purpose.

| Events: | 50, per bead | |
|---|---|---|
| Sample Size: | 50 µL | |
| Gate Settings: | 8,000 to 15,000 | |
| Reporter Gain: | Default (low PMT) | |
| Time Out: | 60 seconds | |
| Bead Set: | TGFβ1 | 18 |
| | TGFβ2 | 54 |
| | TGFβ3 | 36 |

## QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website emdmillipore.com using the catalog number as the keyword.

## ASSAY CHARACTERISTICS

### Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies for an analyte and any of the other analytes in this panel.

1. In addition to human human TGFβ1, the assay can detect TGFβ1 in serum of the following: horse, rabbit, mouse, rat, mini pig, canine, hamster, guinea pig, rhesus monkey and cynomolgous monkey. However, the exact amount of cross reactivity has not been determined.

2. In addition to human TGFβ2, the assay can detect TGFβ2 in serum of the following: horse, rabbit, mouse, rat, mini pig, canine, hamster, guinea pig, rhesus monkey and cynomolgous monkey. However, the exact amount of cross reactivity has not been determined.

3. In addition to human TGFβ3, the assay can detect TGFβ3 in serum of the following: mouse, rat, mini pig, hamster, guinea pig, rhesus monkey and cynomolgous monkey samples. The exact amount of cross reactivity has not been determined.

## ASSAY CHARACTERISTICS (continued)

### Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

| Analyte | Overnight Protocol (n=5 Assays) | | 2 Hour Protocol (n = 2 Assays) | |
|---|---|---|---|---|
| | MinDC (pg/mL) | MinDC+2SD (pg/mL) | MinDC (pg/mL) | MinDC+2SD (pg/mL) |
| TGFβ1 | 6.0 | 11.4 | 9.3 | 11.9 |
| TGFβ2 | 6.6 | 13.4 | 1.7 | 2.9 |
| TGFβ3 | 2.2 | 7.4 | 2.2 | 7.5 |

### Precision

Intra-assay precision is generated from the mean of the %CV's from **8** reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 5 different assays.

| Analyte | Overnight Protocol | |
|---|---|---|
| | Intra-assay %CV | Inter-assay %CV |
| TGFβ1 | 7.2 | 4.6 |
| TGFβ2 | 7.0 | 4.4 |
| TGFβ3 | 6.3 | 8.1 |

### Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrices (n=5).

| Analyte | Overnight Protocol |
|---|---|
| | % Recovery in Serum Matrix |
| TGFβ1 | 99.5 |
| TGFβ2 | 128.2 |
| TGFβ3 | 62.1 |

## TROUBLESHOOTING GUIDE

| Problem | Probable Cause | Solution |
|---|---|---|
| Insufficient bead count | Plate washer aspirate height set too low | Adjust aspiration height according to manufacturers' instructions. |
| | Bead mix prepared inappropriately | Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate. |
| | Samples cause interference due to particulate matter or viscosity | See above. Also sample probe may need to be cleaned with alcohol flushes, back flushes and washes; or, if needed, probe should be removed and sonicated. |
| | Probe height not adjusted correctly | When reading the assay on Luminex® 200™, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc.<br>For FLEXMAP 3D® when using the solid plate in the kit, the final suspension should be in 150 µL and 75 µL should be aspirated. |
| Background is too high | Background wells were contaminated | Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate. |
| | Matrix used has endogenous analyte or interference | Check matrix ingredients for cross-reacting components (e.g. interleukin modified tissue culture medium). |
| | Insufficient washes | Increase number of washes. |
| Beads not in region or gate | Luminex® instrument not calibrated correctly or recently | Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3°C. |
| | Gate settings not adjusted correctly | Some Luminex® instruments (e.g. Bio-Plex®) require different gate settings than those described in the kit protocol. Use instrument default settings. |
| | Wrong bead regions in protocol template | Check kit protocol for correct bead regions or analyte selection. |
| | Incorrect sample type used | Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required. |
| | Instrument not washed or primed | Prime the Luminex® instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid. |
| | Beads were exposed to light | Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps. |

| Problem | Probable Cause | Solution |
|---|---|---|
| Signal for whole plate is same as background | Incorrect or no Detection Antibody was added<br><br>Streptavidin-Phycoerythrin was not added | Add appropriate Detection Antibody and continue.<br><br>Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low. |
| Low signal for standard curve | Detection Antibody may have been removed prior to adding Streptavidin-Phycoerythrin<br><br>Incubations done at inappropriate temperatures, timings or agitation | May need to repeat assay if desired sensitivity not achieved.<br><br>Assay conditions need to be checked. |
| Signals too high, standard curves are saturated | Calibration target value set too high<br><br>Plate incubation was too long with standard curve and samples | With some Luminex® instruments (e.g. Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.<br><br>Use shorter incubation time. |
| Sample readings are out of range | Samples contain no or below detectable levels of analyte<br><br>Samples contain analyte concentrations higher than highest standard point<br><br>Standard curve was saturated at higher end of curve | If below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.<br><br>Samples may require dilution and reanalysis for just that particular analyte.<br><br>See above. |
| High variation in samples and/or standards | Multichannel pipette may not be calibrated<br><br>Plate washing was not uniform<br><br>Samples may have high particulate matter or other interfering substances<br><br>Plate agitation was insufficient<br><br>Cross-well contamination | Calibrate pipettes.<br><br>Confirm all reagents are removed completely in all wash steps.<br><br>See above.<br><br>Plate should be agitated during all incubation steps using an orbital plate shaker at a speed where beads are in constant motion without causing splashing.<br><br>Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate. |

| FOR FILTER PLATES ONLY | | |
|---|---|---|
| **Problem** | **Probable Cause** | **Solution** |
| Filter plate will not vacuum | Vacuum pressure is insufficient | Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. |
| | Samples have insoluble particles | Centrifuge samples just prior to assay set-up and use supernatant. |
| | High lipid concentration | After centrifugation, remove lipid layer and use supernatant. |
| Plate leaked | Vacuum pressure too high | Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue. |
| | Plate set directly on table or absorbent towels during incubations or reagent additions | Set plate on plate holder or raised edge so bottom of filter is not touching any surface. |
| | Insufficient blotting of filter plate bottom causing wicking | Blot the bottom of the filter plate well with absorbent towels after each wash step. |
| | Pipette touching plate filter during additions | Pipette to the side of plate. |
| | Probe height not adjusted correctly | Adjust probe to 3 alignment discs in well H6. |
| | Sample too viscous | May need to dilute sample. |

| **REPLACEMENT REAGENTS** | **Catalog #** |
|---|---|
| TGFβ1,2,3 Standard | TGFB-8064-3 |
| TGFβ1,2,3 Quality Controls 1 and 2 | TGFB-6064-3 |
| TGFβ Detection Antibodies | LTGF-1064 |
| Sample Diluent | LTGF-SD |
| Steptavidin-Phycoerythrin | L-SAPE9 |
| Serum Matrix | LMC-SD |
| 1.0 N HCl | L-HCL |
| 1.0 N NaOH | L-NAOH |
| Assay Buffer | L-AB1 |
| Wash Buffer | L-WB |
| Set of two 96-Well Filter Plates with Sealers | MAG-PLATE |

## Antibody-Immobilized Magnetic Beads

| *Analyte* | *Bead #* | *Cat. #* |
|---|---|---|
| TGFβ1,2,3 Premixed Beads | 18, 54, 36 | TGFBPMX3-MAG |

## ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

### Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

### Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be downloaded through our website at emdmillipore.com/msds.

## WELL MAP

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 0 pg/mL Standard (Background) | 625 pg/mL Standard | QC 2 Control | | | | | | | | | |
| B | 0 pg/mL Standard (Background) | 625 pg/mL Standard | QC 2 Control | | | | | | | | | |
| C | 9.8 pg/mL Standard | 2500 pg/mL Standard | Sample 1 | | | | | | | | | |
| D | 9.8 pg/mL Standard | 2500 pg/mL Standard | Sample 1 | | | | | | | | | |
| E | 39.1 pg/mL Standard | 10000 pg/mL Standard | Sample 2 | | | | | | | | | |
| F | 39.1 pg/mL Standard | 10000 pg/mL Standard | Sample 2 | | | | | | | | | |
| G | 156.3 pg/mL Standard | QC 1 Control | Etc. | | | | | | | | | |
| H | 156.3 pg/mL Standard | QC 1 Control | | | | | | | | | | |