

User Guide

MILLIPLEX[®] RTK Phosphoprotein Magnetic Bead Kit

96-Well Plate

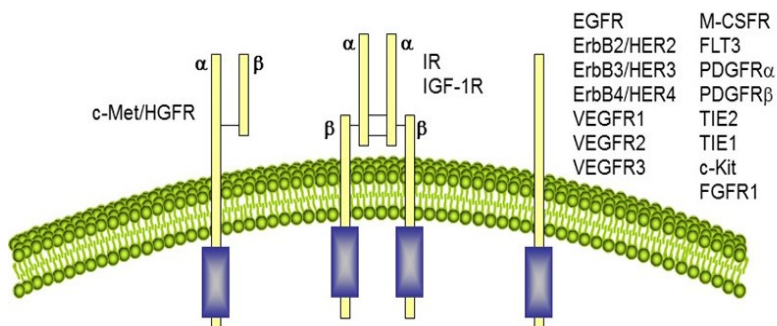
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Introduction

RTKs (Receptor Tyrosine Kinases) are transmembrane proteins that act as receptors for growth factors, neurotrophic factors and other extracellular signaling molecules. Upon binding extracellular ligands, RTKs can undergo receptor dimerization and tyrosine phosphorylation on specific residues in the cytoplasmic tails which then lead to RTK-mediated signal transduction. Of the greater than 500 known protein kinases in human genome, approximately 60 are members of the RTK family. They are central components of cell signaling networks and play crucial roles in normal physiological processes and disease processes ranging from diabetes to cancer.



The MILLIPLEX[®] portfolio offers the broadest selection of analytes across a wide range of pathways. Once the panel of interest has been identified, you can rely on the quality we build into each kit to produce results you can trust. Performance criteria evaluated during the verification process include: cross-reactivity, assay CVs, kit stability, and sample behavior. In addition, each kit meets stringent Quality Control criteria to ensure lot-to-lot reproducibility.

The MILLIPLEX[®] RTK Phosphoprotein Magnetic Bead Panel includes:

- Stimulated and unstimulated cell lysates provided to qualify assay performance
- Configurable magnetic beads to capture analytes of interest
- Optimized detection antibody cocktails designed to yield consistent analyte profiles within a panel

The customizable MILLIPLEX[®] RTK Phosphoprotein Magnetic Bead Panel is used to detect changes in phosphorylated (panTyr) c-Kit, c-Met/HGFR, EGFR, ErbB2/HER2, ErbB3/HER3, ErbB4/HER4, FGFR1, FLT3, IGF-1R, M-CSFR, PDGFR α , PDGFR β , TIE1, TIE2, VEGFR1, VEGFR2, and VEGFR3 in cell lysates using the Luminex[®] system. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for one 96-well plate assay.

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Please read entire protocol before use.

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

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Principle

MILLIPLEX® assays are based on the Luminex® xMAP® technology - one of the most respected multiplex technologies available. This technology finds applications throughout the life sciences and enables a variety of bioassays, including immunoassays, on the surface of fluorescent-coded magnetic bead (MagPlex®-C) microspheres.

- Luminex® products use proprietary techniques to internally color-code microspheres with multiple fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 80-6.45 µm magnetic polystyrene 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.
- The microspheres are illuminated, and the internal dyes fluoresce, marking the microsphere set(s) used in a particular assay. A second illumination source excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

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.
- Once the control lysates have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED CONTROLS IN LYOPHILIZATION VIALS.** For long-term storage, freeze reconstituted standards and controls at ≤ -70 °C. Aliquot if needed. Avoid freeze/thaw cycles.
- **DO NOT FREEZE** Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

Reagents Supplied

Reagents	Volume	Quantity	Cat. No.
RTK Phosphoprotein Biotin (20X)	180 µL	1 tube	PRTK-1001
Lysis Buffer	55 mL	1 bottle	43-040
Assay Buffer 1	55 mL	1 bottle	43-010
HeLa Cell Lysate: Unstimulated	-	1 vial	47-205
HEK293 Cell Lysate: Serum	-	1 vial	47-233
HUVEC Cell Lysate: Serum	-	1 vial	47-238
THP-1 Cell Lysate: Serum	-	1 vial	47-246
HFF-1 Cell Lysate: Serum	-	1 vial	47-247
Streptavidin-Phycoerythrin (25X)	150 µL	1 tube	45-001H
Amplification Buffer (1X)	3 mL	1 bottle	43-024A
Set of one 96-well Plate and 2 sealers	-	1 set	-
Mixing Bottles	-	3 bottles	-

Included lysates are dependent on selection of analytes within the panel:

Analyte	HeLa: Unstim (47-205)	HEK293: Serum (47-233)	HUVEC: Serum (47-238)	THP-1 :Serum (47-246)	HFF-1 :Serum (47-247)
VEGFR1	✓		✓		
c-Kit	✓	✓			
VEGFR2	✓		✓		
c-Met/HGFR	✓	✓			
VEGFR3	✓			✓	
EGFR	✓	✓			
ErbB2/HER2	✓	✓			
M-CSFR	✓			✓	
IGF-1R	✓	✓			
TIE2	✓		✓		
FGFR1	✓	✓			
TIE1	✓		✓		
PDGFRβ	✓				✓
FLT3	✓	✓			
PDGFRα	✓				✓
ErbB3/HER3	✓	✓			
ErbB4/HER4	✓	✓			

Included RTK Phosphoprotein Panel Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

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RTK Phosphoprotein Panel Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Magnetic Bead Region	Customizable 18 Analytes (20X concentration, 180 µL)	
		Available	Cat. No.
Anti-VEGFR1, Magnetic Beads (20X)	14	✓	VEGFR1-MAG
Anti-c-Kit, Magnetic Beads (20X)	19	✓	SCKIT-MAG
Anti-VEGFR2, Magnetic Beads (20X)	21	✓	VEGFR2-MAG
Anti-c-Met/HGFR, Magnetic Beads (20X)	29	✓	HGFR-MAG
Anti-VEGFR3, Magnetic Beads (20X)	33	✓	VEGFR3-MAG
Anti-EGFR, Magnetic Beads (20X)	34	✓	EGFR-MAG
Anti-ErbB2/HER2, Magnetic Beads (20X)	35	✓	ERBB2-MAG
Anti-MCSFR, Magnetic Beads (20X)	39	✓	MCSFR-MAG
Anti-IGF1R, Magnetic Beads (20X)	43	✓	IGF1R-MAG
Anti-TIE2, Magnetic Beads (20X)	44	✓	TIE2-MAG
Anti-FGFR1, Magnetic Beads (20X)	47	✓	FGFR1-MAG
Anti-TIE1, Magnetic Beads (20X)	52	✓	TIE1-MAG
Anti-PDGFR β , Magnetic Beads (20X)	64	✓	PDGFRB- MAG
Anti-FLT3, Magnetic Beads (20X)	66	✓	FLT3-MAG
Anti-PDGFR α , Magnetic Beads (20X)	67	✓	PDGFRA- MAG
Anti-ErbB3/HER3, Magnetic Beads (20X)	74	✓	ERBB3-MAG
Anti-ErbB4/HER4, Magnetic Beads (20X)	78	✓	ERBB4-MAG

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Materials Required (not included)

Reagents

- Protease inhibitors (Cat. No. 535140 or similar product)
- Coomassie or BCA-based total protein assay (Cat. No. 71285 or similar product)
- MAGPIX® Drive Fluid PLUS (Cat. No. 40-50030), xMAP® Sheath Fluid PLUS (Cat. No. 40-50021), or xMAP® Sheath Concentrate PLUS (Cat. No. 40-50023)
- 10X Assay Buffer 1 (Cat. No. MPEQ-AB) if using a magnetic plate washer (see supplemental protocol C)

Instrumentation/Materials

- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL
- Multichannel pipettes capable of delivering 25 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Rubber bands
- Aluminum foil
- Absorbent pads
- Laboratory vortex mixer
- Sonicator (Branson Ultrasonic Cleaner Model No. B200 or equivalent)
- Titer plate shaker (VWR® Microplate Shaker Cat. No. 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software, or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Filter devices for clearing lysates
 - 2 mL or greater, Cat. No. SLHVX13NL
 - 0.5-2 mL, Cat. No. UFC40DV25
 - Less than 0.5 mL, Cat. No. UFC30DV25
 - For 96-well plates, Cat. No. MSBVN1210


NOTE: If using a filter plate and vacuum filtration unit, Vacuum Manifold, Cat. No. MSVMHTS00 or equivalent with Vacuum Pump, Cat. No. WP6111560 or equivalent, may be ordered.

- Use of a handheld Magnetic Separation Block (Cat. No. 40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (BioTek® ELx 405, Cat. No. 40-015 or equivalent), consult Supplemental Protocol C.

Safety Precautions

- All tissue 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 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.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
HeLa Cell Lysate: Unstimulated	47-205		Danger. Harmful if swallowed Causes skin irritation. Causes serious eye damage. Very toxic to aquatic life. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Collect spillage. Dispose of contents/ container to an approved waste disposal plant.
THP-1 Cell Lysate: Serum	47-246		
HEK293 Cell Lysate: serum	47-233		
HFF-1 Cell Lysate: Serum	47-247		
HUVEC Cell Lysate: Serum	47-238		

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 Assay Buffer provided.

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- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8 °C for up to one week.
- 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 the Luminex® 200™ instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 2 alignment discs.
- When reading the assay on the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
- For the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Considerations for Cell Stimulation

- Treating cells with growth factors (for example, EGF), cytokines (for example, TNF α), or other compounds (for example, Arsenite) induce a multitude of signaling cascades. The duration of stimulation in addition to the concentration of the respective factor/compound should be considered since they influence the degree of phosphorylation of any given analyte.
- Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
- Cell lines will differ in the robustness of their signaling response for any given stimulation.
- The suggested working range of protein concentration for the assay is 1 to 25 µg of total protein/well (25 µL/well at 40 to 1000 µg/mL). A total protein amount of 10 µg/well is generally a good starting point for lysates for which target protein expression levels are unknown.

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Preparation of Cell Lysates

MILLIPLEX® Lysis Buffer is supplied as **1X** stock solution. The Lysis Buffer contains phosphatase inhibitors *including* 1 mM sodium orthovanadate (Na_3VO_4) but does **NOT** contain protease inhibitors. It is recommended that protease inhibitors (Cat. No. 535140 or a similar product) be added immediately before use.

Suggested cell lysis protocol for adherent cells

1. After treatments, wash cells with ice cold Buffered Saline (PBS or TBS) and drain.
2. Add ice-cold **1X** MILLIPLEX® Lysis Buffer with freshly added protease inhibitors to cells (0.6 mL per 150 mm dish, 0.3 mL per 100 mm dish, or 0.1 mL per well of 24-well plate).
3. Scrape adherent cells off the dish with a cell scraper. Transfer the cell suspension into a centrifuge tube and gently rock for 10-15 minutes at 4 °C.
4. Remove particulate matter by filtration.

Suggested filters:

- 2 mL or greater (Cat. No. SLPBDZ5NZ)
- 0.5–2 mL (Cat. No. UFC 0DV 25)
- Less than 0.5 mL (Cat. No. UFC30DV00)

Alternative method: remove particulate matter by centrifugation at 12,000 rpm for 10 minutes at 4 °C. Transfer supernatant, without disturbing pellet, into a clean new tube.

5. Aliquot and store the lysate at –70 °C. The lysate should be stable for several months.
6. It is recommended that the lysate be diluted at least 1:10 with PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays.

Suggested cell lysis protocol for non-adherent cells

1. Pellet the cells by centrifugation (500 – 1000 x g) in a tabletop centrifuge for 5 minutes.
2. Wash the cells in ice-cold PBS or TBS.
3. Add ice-cold **1X** MILLIPLEX® Lysis Buffer containing freshly prepared protease inhibitors to cells (1 mL per 1×10^7 cells).
4. Gently rock the lysate for 10-15 minutes at 4 °C.
5. Remove particulate matter by filtration (See above). Aliquot and store the lysate at –70 °C. The lysate should be stable for several months.
6. It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays.

Cell lysis protocol for cells in sterile 96-well tissue culture plates

Adherent or non-adherent cells seeded or grown in sterile 96-well tissue culture grade plates (see supplemental protocols) can be washed, treated, and lysed in the same plate, but need to be filtered in a separate 96-well filter plate. Wash the cells by centrifugation in a microplate carrier 2 minutes at 500 x *g*.

1. Remove the supernatant via aspiration and add 100 μ L of ice-cold PBS or TBS.
1. Centrifuge and remove supernatant via aspiration.
2. Add 30-50 μ L/well of ice-cold 1X MILLIPLEX® Lysis Buffer containing freshly prepared protease inhibitors.
3. Place the plate on an orbital shaker (600-800 rpm) for 10-15 minutes at 4 °C.
4. Transfer the lysate to a 96-well filter plate that has been pre-wetted with 1X Lysis Buffer.
5. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
6. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x *g*.
7. Store the filtered lysate at -70 °C until ready for use.
8. It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays or 1:4 for BCA assays.

Preparation of Reagents for Immunoassay

Preparation of RTK Phosphoprotein Panel Antibody-Immobilized magnetic beads

MILLIPLEX® magnetic beads are provided as a **20X** stock solution and should be protected from light.

1. Sonicate 20X stock magnetic beads for 15 seconds, then vortex for 30 seconds.
2. Add 0.150 mL of each bead to the Mixing Bottle provided.
3. Bring final volume to 3.0 mL with Assay Buffer 1.
4. Vortex the 1X capture beads for 15 seconds.
5. For use, transfer 1X beads with a pipette into a reservoir, do not pour from Mixing Bottle.

Example 1: When using 6 antibody-immobilized beads, add 150 μ L from each of the 6 bead vials to the Mixing Bottle. Then add 2.1 mL Assay Buffer 1.

Example 2: When using 15 antibody-immobilized beads, add 150 μ L from each of the 15 bead vials to the Mixing Bottle. Then add 0.75 mL Assay Buffer 1.

Preparation of Biotin-Labeled Detection Antibody and Streptavidin-PE

Detection Antibody is provided as a **20X** stock solution.

1. Vortex the 20X Detection Antibody stock for 10 seconds, it may be necessary to centrifuge briefly after vortexing for complete recovery of contents.
2. Dilute the Detection Antibody to 1X by combining 0.150 mL of Detection Antibody with 2.85 mL of Assay Buffer 1. Use one of the Mixing Bottles provided.
3. Vortex the Streptavidin-Phycoerythrin 1:25 (SAPE) for 10 seconds.
4. Dilute SAPE by combining 0.120 mL of Streptavidin-Phycoerythrin with 2.88 mL of Assay Buffer 1. Use one of the mixing vials provided.
5. Transfer 1X biotinylated detection antibody and SAPE with a pipette to separate reservoirs. Do not pour from mixing vials.

Multiplexing additional MILLIPLEX® Cell Signaling Magnetic MAPmates™ with the RTK Phosphoprotein Magnetic Bead Panel

Additional Cell Signaling MAPmates™ cannot be combined with this kit. Most analytes contain tyrosine phosphorylation sites which can mask detection of specific serine or threonine phosphorylation sites using Phospho-MAPmates™.

Please note that Total or PanTyr MAPmate pairs should not be multiplexed with the RTK Phosphoprotein Magnetic Bead Panel.

Preparation of Lyophilized MILLIPLEX® Cell Lysates (Cat. No. 47-205, 47-233, 47-238, 47-246, 47-247)

- MILLIPLEX® HeLa Cell Lysate: Unstimulated (Cat. No. 47-205) is provided as a lyophilized stock of cell lysate prepared from untreated HeLa cells and is used as a negative control.
- MILLIPLEX® HEK293 Cell Lysate: Serum (Cat. No. 47-233) is provided as a lyophilized stock of cell lysate prepared from HEK293 cells treated with fetal calf serum (15 minutes).
- MILLIPLEX® HUVEC Cell Lysate: Serum (Cat. No. 47-238) is provided as a lyophilized stock of cell lysate prepared from HUVEC cells treated with 10% fetal bovine serum and 100 μ M pervanadate (30 minutes).
- THP-1 Cell Lysate: Serum (Cat. No. 47-246) is provided as a lyophilized stock of cell lysate prepared from THP-1 cells stimulated with 10% serum and 100 μ M pervanadate (30 minutes).
- HFF-1 Cell Lysate: Serum (Cat. No. 47-247) is provided as a lyophilized stock of cell lysate prepared from HFF-1 cells stimulated with 10% serum and 100 μ M pervanadate (30 minutes).

Each of the cell lysates were prepared in MILLIPLEX® Lysis Buffer containing protease inhibitors and lyophilized for stability. The lysates can be used as unstimulated and stimulated control samples or alternatively, to create calibration curves for relative quantification of different phosphoprotein analytes.

MILLIPLEX® Cell Lysates as an unstimulated and stimulated control

1. Reconstitute each of the lyophilized cell lysates in 100 μ L of ultrapure water. For each vial this will yield 100 μ L of lysate at a total protein concentration of 2 mg/mL.
2. Gently vortex and incubate the reconstituted lysates for 5 minutes at RT (store on ice).
3. Pipette 150 μ L of Assay Buffer 1 to each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the MILLIPLEX® RTK Phosphoprotein Magnetic Bead Panel.
4. If desired, unused lysate may be stored in its original container at -80 °C for up to one month.

Immunoassay Protocol

(96-well Plate and Handheld Magnetic Separation Block)

1. Dilute filtered lysates at least 1:1 in MILLIPLEX[®] Assay Buffer. The suggested working range of protein concentration for the assay is 1 to 25 µg of total protein/well (25 µL/well at 40 to 1,000 µg/mL).
2. Add 50 µL of Assay Buffer into each well of the plate. Cover and mix on a plate shaker for 10 minutes at room temperature (20-25 °C).
3. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
4. Vortex the 1X bead suspension for 10 seconds. Add 25 µL of 1X bead suspension to each well.
5. Add 25 µL of Assay Buffer, reconstituted control cell lysates and sample lysates to appropriate wells and incubate overnight (16-20 hours) at 2-8 °C on a plate shaker (600-800 rpm) protected from light.
6. Attach handheld magnetic separation block to plate, allow 60 seconds for beads to settle and decant samples and controls.
7. Remove plate from magnetic separation block and wash plate with 100 µL Assay Buffer per well (see Washing Note below). Repeat for a total of two washes.
8. Add 25 µL/well of 1X Detection Antibody.
9. Seal, cover with lid and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C).
10. Attach Magnetic Separation Block, wait for 60 seconds and decant Detection Antibody.
11. Add 25 µL of 1X Streptavidin-Phycoerythrin (SAPE).
12. Seal, cover with lid and incubate with agitation on a plate shaker for 15 minutes at room temperature (20-25 °C).

Add 50 µL Assay Buffer per well

↓ Shake 10 min, RT
Decant buffer

- Add 25 µL 1X beads to wells
- Add 25 µL Assay Buffer to the blank well
- Add 25 µL control and sample lysates to appropriate wells

↓ Incubate overnight (16-20 hours) at 4 °C with shaking; dark

Wash 2X with 100 µL Assay Buffer. Add 25 µL 1X Detection Antibody.

↓ Incubate 1 hr at RT with shaking; dark

Remove Detection Antibody and add 25 µL 1X Streptavidin-PE (SAPE)

↓ Incubate 15 min at RT with shaking; dark

DO NOT REMOVE SAPE and add 25 µL Amplification buffer

↓ Incubate 15 min at RT with shaking; dark

Remove Streptavidin-PE/Amplification buffer and resuspend beads in 150 µL Assay Buffer. Read results using appropriate Luminex[®] instrument.

13. DO NOT REMOVE SAPE. Add 25 μ L of Amplification Buffer to each well.
14. Seal, cover with lid and incubate with agitation on a plate shaker for 15 minutes at room temperature (20-25 $^{\circ}$ C).
15. Attach Magnetic Separation Block, wait for 60 seconds and decant SAPE/Amplification buffer.
16. Suspend beads in 150 μ L of Assay Buffer and mix on plate shaker for 5 minutes. Analyze using the Luminex[®] system.

Washing Note

For handheld magnet, 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 100 μ L of Assay Buffer by removing plate from magnet, adding Assay 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.

Equipment Settings

Luminex[®] 200™, HTS, FLEXMAP 3D[®], MAGPIX[®] instruments with xPONENT[®] software and xMAP[®] INTELLIFLEX instrument with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex[®] instruments with other software (for example, 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, each instrument must be calibrated and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex [®] 200™ and HTS	xPONENT [®] 3.1 compatible Calibration Kit (Cat. No. LX2R-CAL-K25)	Performance Verification Kit (Cat. No. LX2R-PVER-K25)
FLEXMAP 3D [®]	FLEXMAP 3D [®] Calibrator Kit (Cat. No. F3D-CAL-K25)	FLEXMAP 3D [®] Performance Verification Kit (Cat. No. F3D-PVER-K25)

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Instrument	Calibration Kit	Verification Kit
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (Cat. No. IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (Cat. No. IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (Cat. No. MPX-CAL-K25)	MAGPIX® Performance Verification Kit (Cat. No. MPX-PVER-K25)

NOTE: These assays cannot be performed on any instruments running Luminex® IS 2.3 or Luminex® 1.7 software.

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Cat. No. MAG-PLATE, if additional plates are required for this purpose.

Events	50 per bead
Sample Size	100 µL
Gate Settings	8,000 to 15,000
Reporter Gain	Default (Low PMT)
Time Out	60 seconds
Bead Region	VEGFR1 14
	c-Kit 19
	VEGFR2 21
	c-Met/HGFR 29
	VEGFR3 33
	EGFR 34
	ErbB2/HER2 35
	M-CSFR 39
	IGF-1R 43
	TIE2 44
	FGFR1 47
	TIE1 52
	PDGFRβ 64
	FLT3 66
	PDGFRα 67
	ErbB3/HER3 74
	ErbB4/HER4 78

Supplemental Protocols

Analysis of viscous cell lysates

Some cell lysates may not flow through the filter plate efficiently due to high viscosity or the formation of particulate matter from long-term storage. For these samples, the initial capture and wash steps can be done in microcentrifuge tubes. The beads are then transferred into 96-well filter plates for the rest of the assay.

1. Add 25 μL /assay point of 1X beads to a 500 μL centrifuge tube.
2. Next, add lysate diluted in Assay Buffer 1 to a final volume of 100 μL or higher.
3. Vortex the mixture at high speed for 15 seconds then sonicate for an additional 15 seconds.
4. Rotate the mixture overnight at 2-8 $^{\circ}\text{C}$, protected from light.
5. Centrifuge the beads for 1 min at 2000 $\times g$ and carefully remove the supernatant to minimize bead loss.
6. Resuspend the pelleted beads in 25 μL /assay point of Assay Buffer 1.
7. Transfer 25 μL of the bead mixture to pre-wet filter plate wells and proceed to step 4 of the immunoassay protocol.

Filter Plate Immunoassay Protocol

The use of a filter plates is not a recommended method of running magnetic bead MILLIPLEX[®] cell signaling immunoassays. If desired, filter plates may be purchased separately (Cat. No. MX-PLATE includes a set of two MILLIPLEX[®] 96-well Filter Plates with sealers). Contact Technical Support if additional instructions are required.

Plate Washer Use

The use of a plate washer is not a recommended method of washing for cell signaling assays. Deterioration of assay performance and well-to-well variability have been noted when using plate washers. If desired, MPEQ-AB may be purchased and used as a general wash buffer with plate washers. MPEQ-AB should be diluted to 1X for use in plate washers. Follow standard protocol wash instructions when using a plate washer (2 washes after sample incubation). Contact Technical Service if additional instructions are required.

Representative Data

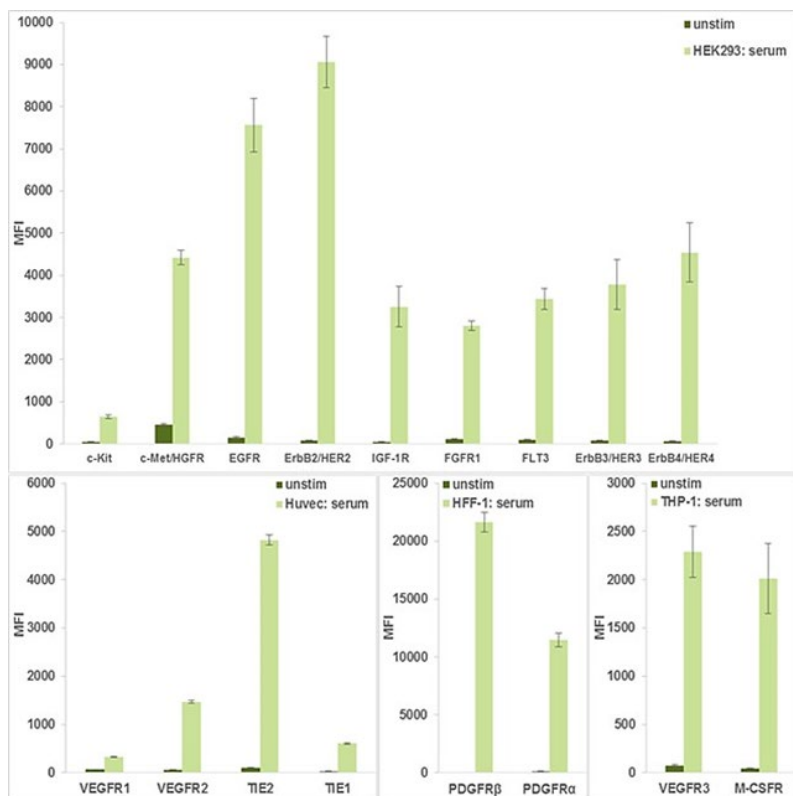


Figure 1. Multiplex analysis of HEK293, HUVEC, THP-1, and HFF-1 cells treated with serum or serum and pervanadate. HEK293 cells stimulated with fetal calf serum (15 minutes), HUVEC cells stimulated with 10% fetal bovine serum and 100 μ M pervanadate (30 minutes), THP-1 cells stimulated with 10% fetal calf serum and 100 μ M pervanadate (30 minutes), and HFF-1 cells stimulated with 10% fetal calf serum were assayed. The cells were lysed in MILLIPLEX[®] Lysis Buffer containing protease inhibitors. A sample of 20 μ g total protein of each lysate diluted in MILLIPLEX[®] Assay Buffer 1 was analyzed according to the Assay protocol (lysate incubation at 4 $^{\circ}$ C overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex[®] system. The figures represent the average and standard deviation of three replicate wells.

	VEGFR1	c-KIT	VEGFR2	c-MET	VEGFR3	EGFR	HER2	M-C SFR	IGF-1R	TIE2	FGFR1	TIE1	PDGFRβ	FL T3	PDGFRα	HER3	HER4
VEGFR1 [†]	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
c-KIT [†]	0%	100%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
VEGFR2 [†]	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
c-MET [†]	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
VEGFR3 [†]	0%	0%	0%	0%	100%	0%	0%	0%	0%	10%	0%	0%	0%	0%	0%	0%	0%
EGFR [†]	0%	0%	1%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
HER2 [†]	0%	0%	1%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
M-C SFR [†]	0%	0%	1%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%	0%
IGF-1R [†]	0%	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%
TIE2 [†]	0%	0%	1%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%	0%	0%
FGFR1 [†]	0%	0%	2%	0%	0%	0%	0%	0%	1%	0%	100%	0%	0%	2%	3%	0%	0%
TIE1 [†]	0%	0%	0%	0%	0%	0%	0%	0%	2%	0%	0%	100%	0%	0%	5%	0%	0%
PDGFRβ [†]	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%	0%
FL T3 [†]	0%	0%	1%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%	0%	0%	0%
PDGFRα [†]	0%	0%	0%	0%	2%	0%	0%	0%	1%	2%	0%	0%	1%	0%	100%	0%	0%
HER3 [†]	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%	0%
HER4 [†]	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	100%

Figure 2. Cross-reactivity. Biotinylated recombinant standards at concentrations of 5 ng/mL*, 10 ng/mL[†], or 20 ng/mL[‡] were analyzed according to the Assay protocol (lysate incubation at 4 °C overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex[®] system.

Troubleshooting

Problem	Probable Cause	Solution
	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 flush, back flush and washes; or if needed probe should be removed and sonicated.
Insufficient bead count	Probe height not adjusted correctly	When reading the assay on the Luminex [®] 200™ instrument, adjust probe height to the kit solid plate using 3 alignment discs. When reading the assay on the MAGPIX [®] instrument, adjust probe height to the kit solid plate using 2 alignment discs. When reading the assay on the FLEXMAP 3D [®] instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D [®] instrument, when using the

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Problem	Probable Cause	Solution
		solid plate in the kit, the final resuspension should be with 150 μ L Sheath Fluid PLUS in each well and 75 μ L should be aspirated. When reading the assay on the xMAP [®] INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex [®] .
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.
	Insufficient washes	Increase number of washes.
	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 (for example, Bio-Plex [®]) require different gate settings than those described in the kit protocol. Use instrument default settings.
Beads not in region or gate	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 PLUS or water if there is any remnant alcohol or sanitizing liquid.

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Problem	Probable Cause	Solution
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Signals too high	Calibration target value set too high	With some Luminex [®] instruments (for example, Bio-Plex [®]) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with technical support for appropriate protocol modifications.
High variation in samples	Multichannel pipette may not be calibrated	Calibrate pipettes.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using an orbital plate

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Problem	Probable Cause	Solution
		shaker at a speed where beads are in constant motion without causing splashing.
	Cross-well contamination	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.

Product Ordering

Replacement Reagents	Cat. No.
RTK Phosphoprotein Biotin (20X)	PRTK-1001
Lysis Buffer	43-040
Assay Buffer 1	43-010
HeLa Cell Lysate: Unstimulated	47-205
HEK293 Cell Lysate: Serum	47-233
HUVEC Cell Lysate: Serum	47-238
THP-1 Cell Lysate: Serum	47-246
HFF-1 Cell Lysate: Serum	47-247
Streptavidin-Phycoerythrin	45-001H
Amplification Buffer (1X)	43-024A
Set of two MILLIPLEX® 96-well Plates with sealers	MAG-PLATE

Antibody-Immobilized Magnetic Beads

Analyte	Bead No.	Cat. No.
VEGFR1	14	VEGFR1-MAG
c-Kit	19	SCKIT-MAG
VEGFR2	21	VEGFR2-MAG
c-Met/HGFR	29	HGFR-MAG
VEGFR3	33	VEGFR3-MAG
EGFR	34	EGFR-MAG
ErbB2/HER2	35	ERBB2-MAG
M-CSFR	39	MCSFR-MAG
IGF-1R	43	IGF1R-MAG
TIE2	44	TIE2-MAG
FGFR1	47	FGFR1-MAG
TIE1	52	TIE1-MAG
PDGFR β	64	PDGFRB-MAG
FLT3	66	FLT3-MAG
PDGFR α	67	PDGFRA-MAG
ErbB3/HER3	74	ERBB3-MAG
ErbB4/HER4	78	ERBB4-MAG

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Well Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Buffer 1 Blank	THP-1: Serum positive control	Sample 3									
B	Assay Buffer 1 Blank	THP-1: Serum positive control	Sample 3									
C	HeLa: Unstim negative control	HFF-1: Serum positive control	Etc.									
D	HeLa: Unstim negative control	HFF-1: Serum positive control	Etc.									
E	HEK293: Serum positive control	Sample 1										
F	HEK293: Serum positive control	Sample 1										
G	HUVEC: Serum positive control	Sample 2										
H	HUVEC: Serum positive control	Sample 2										

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