10-Plex MAPK/SAPK Signaling Magnetic Bead Panel

96-well Plate Assay

Cat. # 48-660MAG

MILLIPLEX® MAP

10-Plex MAPK/SAPK Signaling Magnetic Bead Kit
96-well Plate Assay

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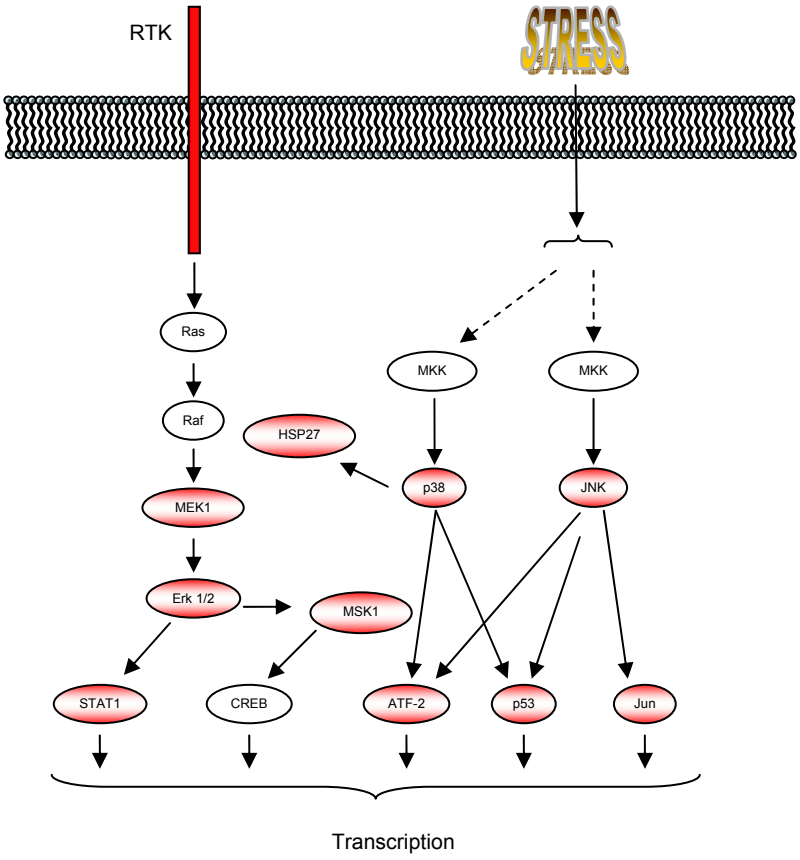
INTRODUCTION

Cells respond to their environment in various ways through intracellular signaling. Signaling through RTKs (Receptor Tyrosine kinases) often promotes increased metabolism and cell growth. Activation of ERK/MAP kinase is one of the key Ser/Thr kinases activated via RTK signaling, promoting increased activity of p70 S6 kinase, MSK1, STATs, CREB, and many other signaling intermediates. Other signaling pathways induced via stress (ex. heat shock/arsenite treatment) or death receptors (ex. TNFα) promote p38, JNK, and p53 signaling pathways.

Summary of Signaling Pathways



The MILLIPLEX® MAP 10-Plex Human MAPK/SAPK Signaling Magnetic Bead Kit, is used to detect changes in phosphorylated ERK/MAP kinase 1/2 (Thr185/Tyr187), STAT1 (Tyr701), JNK (Thr183/Tyr185), MEK1 (Ser217/221), MSK1 (Ser212), ATF2 (Thr71), p53 (Ser15), HSP27 (Ser78), c-Jun (Ser73) and p38 (Thr180/Tyr182) 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.

PRINCIPLE

MILLIPLEX® MAP is 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 beads known as Magplex™ microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets 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.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
MILLIPLEX® MAP 10-plex MAPK/SAPK, Magnetic Beads (20X)	42-660MAG	180 µL	1 tube
MILLIPLEX® MAP 10-plex MAPK/SAPK, Biotin (20X) (Detection Antibody)	44-660KMG	180 µL	1 tube
MILLIPLEX® MAP Lysis Buffer	43-040	55 mL	1 bottle
MILLIPLEX® MAP Assay Buffer 2	43-041	55 mL	1 bottle
MILLIPLEX® MAP HeLa Cell Lysate: unstimulated	47-205	----------	1 vial
MILLIPLEX® MAP Hela Cell Lysate: HS/Ars	47-211	----------	1 vial
MILLIPLEX® MAP NIH/3T3 Cell Lysate: anisomycin	47-219	----------	1 vial
MILLIPLEX® MAP A431 Cell Lysate: EGF	47-210	----------	1 vial
MILLIPLEX® MAP Streptavidin-Phycoerythrin	45-001H	150 µL	1 tube
MILLIPLEX® MAP Amplification Buffer (1X)	43-024A	3 mL	1 bottle
Set of one 96-well Filter Plate and 2 sealers	----------	----------	1 plate, 2 sealers
Set of one 96-well Plate and 2 sealers	----------	----------	1 plate, 2 sealers
Empty mixing bottles	----------	----------	3 bottles

Analyte	Magnetic Bead Region
ATF2	15
JNK	18
HSP27	27
p38	38
Erk/MAP Kinase 1/2	42
p53	53
MEK1	55
MSK1	56
STAT1	61
c-Jun	73

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Protease inhibitors (EMD Millipore Catalog #535140 or similar product)
- Coomassie or BCA-based total protein assay (EMD Millipore Catalog #71285 or similar product) or an assay normalization control, such as the GAPDH MAPmate (EMD Millipore Catalog #46-667MAG)
- Luminex Sheath Fluid (Luminex Catalog #40-5000) or Luminex Drive Fluid (Luminex Catalog # MPXDF-4PK)
- 10X Assay Buffer 1 (EMD Millipore Catalog # 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 #B200 or equivalent)
- Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
- Luminex 200™, HTS, FLEXMAP 3D® , or MAGPIX® with xPONENT software by Luminex Corporation
- Plate Stand (EMD Millipore Catalog # MX-STAND, if using filter plate)
- Filter devices for clearing lysates
  - 2 mL or greater, EMD Millipore Catalog # SLHVX13NL
  - 0.5 – 2 mL, EMD Millipore Catalog # UFC40DV25
  - Less than 0.5 mL, EMD Millipore Catalog # UFC30DV25
  - For 96 well plates, EMD Millipore Catalog # MSBVN1210
- Use of a hand-held Magnetic Separation Block (EMD Millipore Catalog # 40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (Bio-Tek ELx405, EMD Millipore Catalog #40-015 or equivalent), consult Supplemental Protocol C.
- If using the filter plate, a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent). Consult Supplemental Protocols Section for Filter Plate protocol use.

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.

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.
- 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 Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 4 alignment discs. When reading the assay on FLEXMAP 3D® , adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 2 alignment discs.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Considerations for Cell Stimulation.

1. Treating cells with growth factors (ex. EGF), cytokines (ex. TNFα), or other compounds (ex. 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.
2. Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
3. Cell lines will differ in the robustness of their signaling response for any given stimulation.
4. 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.

B. Preparation of cell lysates

MILLIPLEX® MAP 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 (EMD Millipore catalog #535140 or a similar product) be added immediately before use.

<u>Suggested cell lysis protocol for adherent cells</u>

1. After treatments, wash cells with ice cold Buffered Saline (PBS or TBS) and drain.
2. Add ice-cold **1X** MILLIPLEX® MAP 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.
   a. Suggested EMD Millipore filters:
      (i) 2 mL or greater, EMD Millipore Catalog # SLPBDZ5NZ
      (ii) 0.5 – 2 mL, EMD Millipore Catalog # UFC 0DV 25
      (iii) Less than 0.5 mL, EMD Millipore Catalog # UFC30DV00
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. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmate (EMD Millipore Catalog #46-667MAG), is used.

<u>Suggested cell lysis protocol for non-adherent cells</u>

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.

SAMPLE COLLECTION AND STORAGE (continued)

3. Add ice-cold **1X** MILLIPLEX® MAP Lysis Buffer containing freshly prepared protease inhibitors to cells (1 mL per 1 x 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. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmate (EMD Millipore Catalog #46-667MAG), is used.

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 µL of ice-cold PBS or TBS.
2. Centrifuge and remove supernatant via aspiration.
3. Add 30-50 µL/well of ice-cold 1X MILLIPLEX® MAP Lysis Buffer containing freshly prepared protease inhibitors.
4. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C.
5. Transfer the lysate to a 96-well filter plate that has been pre-wetted with 1X Lysis Buffer.
6. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
7. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
8. Store the filtered lysate at -70°C until ready for use.
9. 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. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH MAPmate (EMD Millipore Catalog #46-667MAG), is used.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of MAPK/SAPK magnetic beads

MILLIPLEX® MAP 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. Dilute the beads to **1X** by combining 0.150 mL beads with 2.85 mL of MILLIPLEX® MAP Assay Buffer 2. Use one of the Mixing Bottles provided.
3. Vortex the **1X** capture beads for 15 seconds.
4. For use, transfer 1X beads with a pipette into a reservoir, do not pour from Mixing Bottle.
5. Please note that multiplexing phospho-specific and total or panTyr MAPmate pairs is not recommended due to cross-reactivity.

B. Preparation of Biotin-Labeled Detection Antibody and Streptavidin-PE

MILLIPLEX® MAP 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 MILLIPLEX® MAP Assay Buffer 2. Use one of the Mixing Bottles provided.
3. Vortex the MILLIPLEX® MAP Streptavidin-Phycoerythrin 1:25 (SAPE) for 10 seconds.
4. Dilute SAPE by combining 0.120 mL of Streptavidin-Phycoerythrin with 2.88 mL of MILLIPLEX® MAP Cell Signaling Assay Buffer 2. Use one of the Mixing Bottles provided.
5. Transfer 1X biotinylated detection antibody and SAPE with a pipette to separate reservoirs. Do not pour from Mixing Bottles.

C. Multiplexing additional MILLIPLEX® MAP Cell Signaling Magnetic MAPmates with the 10-Plex MAPK/SAPK Signaling Magnetic Bead Kit, phosphoprotein.

Additional Cell Signaling Phospho-MAPmates may be combined with this kit, up to a maximum of 9 additional MAPmates.

Please note that Total or PanTyr MAPmate pairs should not be multiplexed with the 10-plex MAPK/SAPK Kit, phosphoprotein.

1. For each additional Magnetic Bead MAPmate, sonicate **20X** stock capture beads for 15 seconds, then vortex for 30 seconds.
2. Add 0.150 mL 10-plex MAPK/SAPK Magnetic beads to the mixing vial
3. For each additional MAPmate, add 0.150 mL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer 2. Vortex the mixed beads well.
4. Use the same preparation volumes for the Detection Antibody

Example 1: When using 2 additional MAPmates, add 0.150 mL 10-plex MAPK/SAPK Beads/ Detection Antibody and 0.150 mL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 2.55 mL Assay Buffer 2, for a final volume of 3.0 mL.

Example 2: When using 5 additional MAPmates, add 0.150 mL 10-plex MAPK/SAPK Beads/ Detection Antibody and 0.150 mL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 2.1 mL Assay Buffer 2, for a final volume of 3.0 mL.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

D. Preparation of lyophilized MILLIPLEX® MAP Cell Lysates (Catalog # 47-205, 47-210, 47-211 and 47-219).

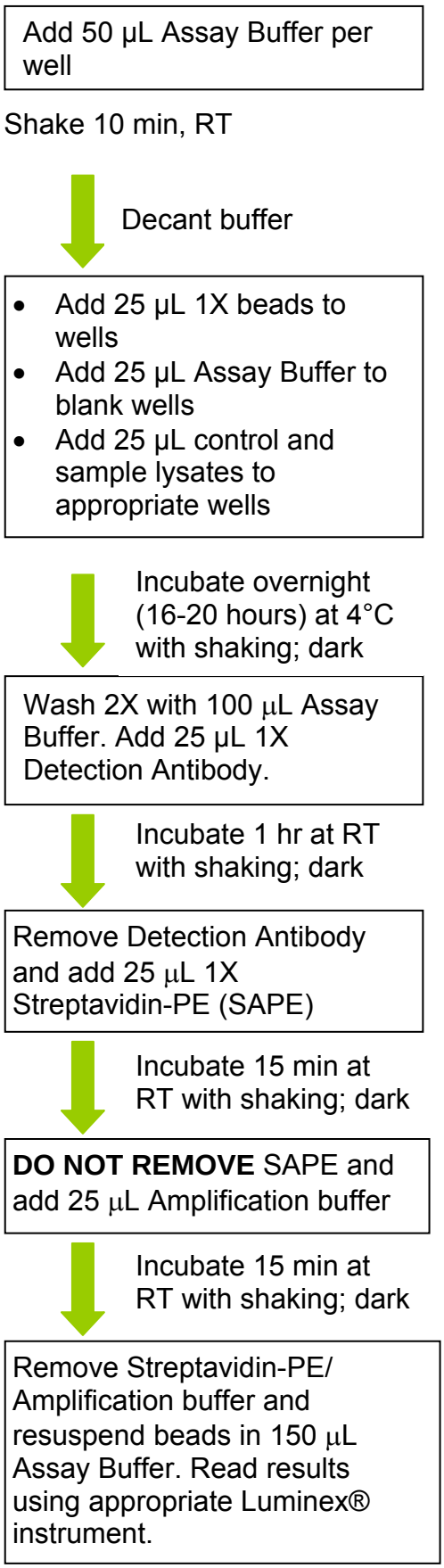
MILLIPLEX® MAP HeLa Cell Lysate: unstimulated (#47-205) is provided as a lyophilized stock of cell lysate and used as a negative control. MILLIPLEX® MAP Hela Cell Lysate: HS/Ars (#47-211) is provided as a lyophilized stock of cell lysate prepared from Hela cells treated with Heat shock (42°C 30 min., 37°C 16 hours) and 400µM Arisenite for 30min.. MILLIPLEX® MAP NIH/3T3 Cell Lysate: anisomycin (#47-219) is provided as a lyophilized stock of cell lysate prepared from anisomycin treated NIH/3T3 cells (10µM 30mins.). MILLIPLEX® MAP A431 Cell Lysate: EGF (#47-210) is provided as a lyophilized stock of cell lysate prepared from EGF treated A431 cells (100ng/mL for 5 min). Each of the cell lysates were prepared in MILLIPLEX® MAP 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® MAP Cell Lysates as an unstimulated and stimulated control

1. Reconstitute each of the lyophilized cell lysates in 100 µL of ultrapure water, for each vial this will yield 100 µL of lysate at a total protein concentration of 2 mg/mL.
2. Gently vortex and incubate the reconstituted lysates for 5 min at RT (store on ice).
3. Pipette 150 µL of MILLIPLEX® MAP Assay Buffer 2 into each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the MILLIPLEX® MAP 10-plex MAPK/SAPK Magnetic Bead Kit.
4. If desired, unused lysate may be stored in its original container at -80°C for up to one month. For long-term storage, freeze reconstituted standards and controls at ≤ -70°C. Aliquot if needed. Avoid freeze/thaw cycles.

IMMUNOASSAY PROTOCOL (96-well Plate and Hand-held Magnetic Separation Block)

1. Dilute filtered lysates at least 1:1 in MILLIPLEX MAP 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 to blank wells, 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** MILLIPLEX MAP 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 MILLIPLEX MAP 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).
13. **DO NOT REMOVE SAPE**. Add 25 µL of MILLIPLEX MAP 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°C).
15. Attach Magnetic Separation Block, wait for 60 seconds and decant SAPE /Amplification buffer.
16. Suspend beads in 150 µL of MILLIPLEX MAP Assay Buffer, and mix on plate shaker for 5 minutes, Analyze using the Luminex® system.



WASHING NOTE: For hand-held 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 µL 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.

INSTRUMENT 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, LABScan100) 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: 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 EMD Millipore Catalog #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:	ATF2	15
	JNK	18
	HSP27	27
	p38	38
	Erk/MAP Kinase 1/2	42
	p53	53
	MEK1	55
	MSK1	56
	STAT1	61
	c-Jun	73

SUPPLEMENTAL PROTOCOLS

A. Analysis of viscous cell lysates

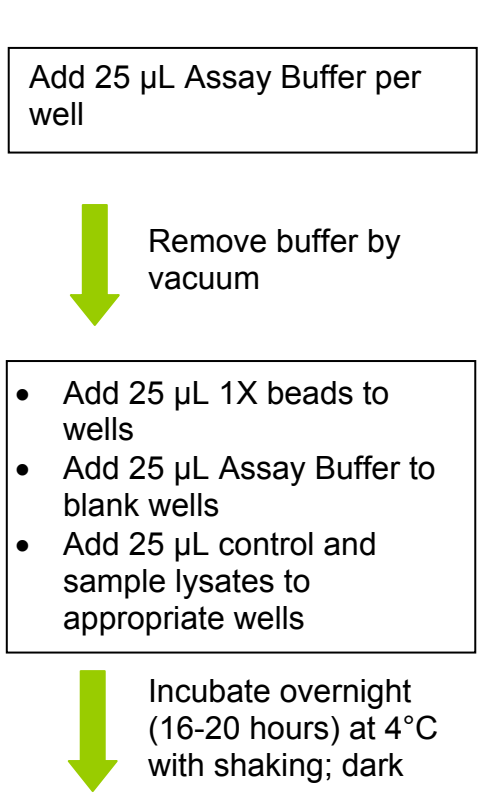
Some cell lysates may not flow through the filter plate efficiently due to high viscosity or the formation of particulate matter from long-term storage. For these samples, the initial capture and wash steps can be done in microcentrifuge tubes. The beads are then transferred into 96-well filter plates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

- Add 25 µL/assay point of 1X beads to a 500 µL centrifuge tube.
- Next, add lysate diluted in MILLIPLEX® MAP Assay Buffer 2 to a final volume of 100 µL or higher.
- Vortex the mixture at high speed for 15 seconds then sonicate for an additional 15 seconds.
- Rotate the mixture overnight at 2-8°C, protected from light.
- Centrifuge the beads for 1 min at 2,000 x g and carefully remove the supernatant to minimize bead loss.
- Resuspend the pelleted beads in 25 µL/assay point of MILLIPLEX® MAP Assay Buffer 2.
- Transfer 25 µL of the bead mixture to pre-wet filter plate wells and proceed to step 4 of the Immunoassay protocol.

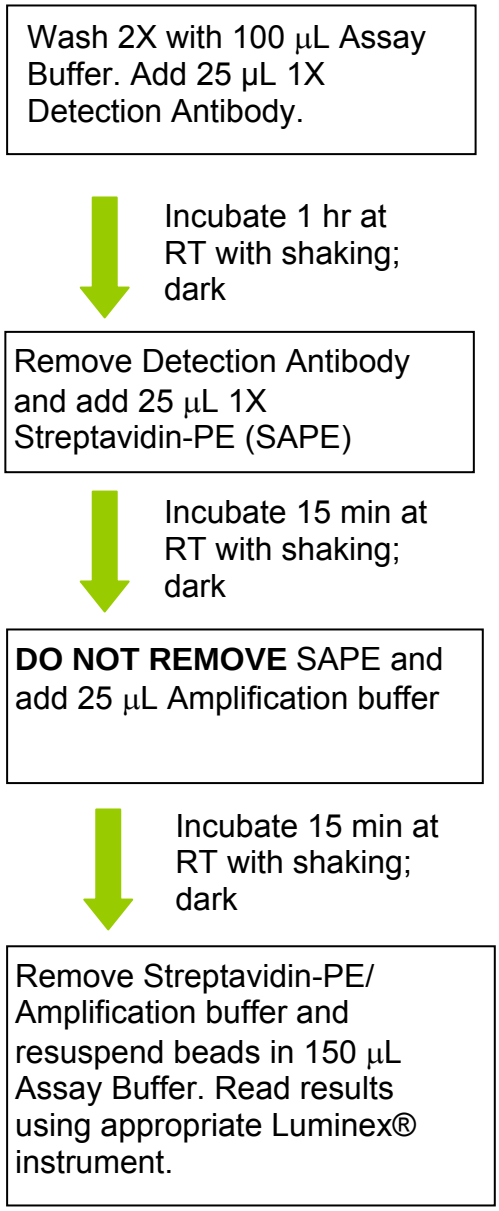
B. Filter Plate Immunoassay Protocol

NOTE: This protocol requires the use of the included 96-well Filter plate and a Vacuum Manifold (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560).

1. Dilute filtered lysates at least 1:1 in MILLIPLEX® MAP 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. Pre-wet filter plate with 25 µL/well of MILLIPLEX® MAP Assay Buffer. Remove by vacuum filtration by placing the filter plate over a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
3. Vortex the **1X** bead suspension for 10 seconds. Add 25 µL of 1X bead suspension to each well.
4. Add 25 µL of Assay Buffer to blank wells, 25 µL reconstituted control cell lysates and sample lysates to appropriate wells and incubate overnight (16-20 hours) at 2-8°C. Seal, cover with lid and incubate with agitation on a plate shaker at 600-800 rpm.
5. Remove the lysate by vacuum filtration.



6. Add 100 µL/well of MILLIPLEX® MAP Assay Buffer. Remove buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel. Repeat this step again for a total of two washes.
7. Add 25 µL/well of **1X** MILLIPLEX® MAP Detection Antibody.
8. Seal, cover with lid and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C).
9. Remove Detection Antibody by vacuum and gently blot the bottom of the filter plate on a paper towel.
10. Add 25 µL of 1X MILLIPLEX® MAP Streptavidin-Phycoerythrin (SAPE).
11. Seal, cover with lid and incubate with agitation on a plate shaker for 15 min at room temperature (20-25°C).
12. **DO NOT REMOVE** SAPE. Add 25 µL of MILLIPLEX® MAP Amplification Buffer to each well.
13. Seal, cover with lid and incubate with agitation on a plate shaker for 15 min at room temperature (20-25°C).
14. Remove MILLIPLEX® MAP SAPE /Amplification buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
15. Resuspend beads in 150 µL of MILLIPLEX® MAP Assay Buffer, and mix on plate shaker for 5 minutes.
16. Analyze using the Luminex® system.



C. 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 EMD Millipore Tech Service if additional instructions are required.

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient Bead Count	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.
	Probe height not adjusted correctly	When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 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. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipeting with Multichannel pipets without touching reagent in plate.
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex not calibrated correctly or recently	Calibrate Luminex based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by >3^{o}C.
	Gate Settings not adjusted correctly	Some Luminex instruments (e.g. Bioplex) 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 4 times to rid 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.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added Streptavidin-Phycoerythrin was not added	Add appropriate Detection Antibody and continue. 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 Plate incubation was too long with samples	With some Luminex Instrument (e.g. Bio-plex) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate. 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 tech support for appropriate protocol modifications.
High Variation in samples	Multichannel pipet may not be calibrated Plate washing was not uniform Samples may have high particulate matter or other interfering substances Plate agitation was insufficient Cross well contamination	Calibrate pipets. Confirm all reagents are removed completely in all wash steps. See above. Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing. Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.
	FOR FILTER PLATES ONLY	
Filter plate will not vacuum	Vacuum pressure is insufficient Samples have insoluble particles High lipid concentration	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Centrifuge samples just prior to assay setup and use supernatant. After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum Pressure too high Plate set directly on table or absorbent towels during incubations or reagent additions	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. 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 Sample too viscous	Adjust probe to 3 alignment discs in well H6. May need to dilute sample.

REPLACEMENT REAGENTS

MILLIPLEX® MAP 10-plex MAPK/SAPK - Magnetic Beads (20X)	42-660MAG
MILLIPLEX® MAP 10-plex MAPK/SAPK, Biotin (20X) (Detection Antibody)	44-660KMG
MILLIPLEX® MAP Lysis Buffer	43-040
MILLIPLEX® MAP Assay Buffer 2	43-041
MILLIPLEX® MAP HeLa Cell Lysate: Untsimulated	47-205
MILLIPLEX® MAP HelaCell Lysate: HS/Ars	47-211
MILLIPLEX® MAP NIH/3T3 Cell Lysate: anisomycin	47-219
MILLIPLEX® MAP A431Cell Lysate: EGF	47-210
MILLIPLEX® MAP Streptavidin-Phycoerythrin	45-001H
MILLIPLEX® MAP Amplification Buffer (1X)	43-024A
Set of two MILLIPLEX® MAP 96-well Plates with sealers	MAG-PLATE
Set of two MILLIPLEX® MAP 96-well Filter Plates with sealers	MX-PLATE

REPRESENTATIVE DATA

10-Plex MAPK/SAPK Magnetic Bead Kit Analysis of Treated Cells

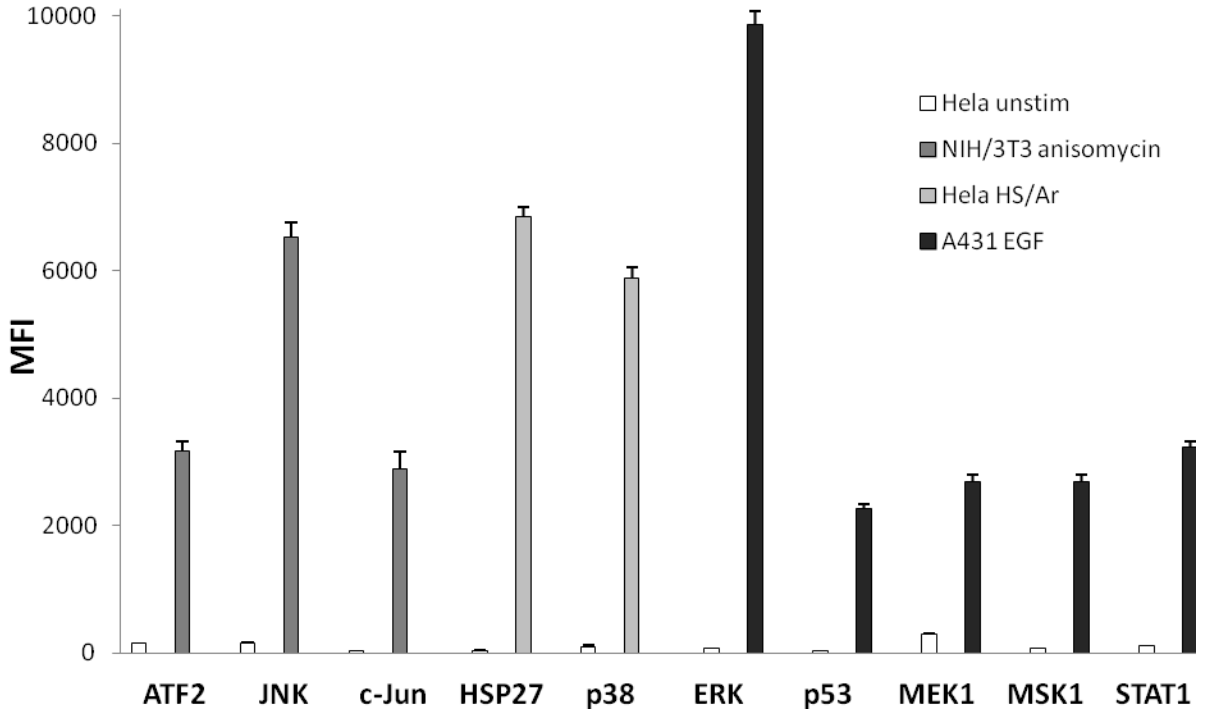


Figure 1. Multiplex analysis of Positive Control Samples 47-210, 47-211, 47-219. Hela: unstimulated negative control (47-201), A431: EGF stimulated (47-210), Hela: Heat Shock/ Arsenite stimulated (47-211) and NIH/3T3: anisomycin stimulated (47-219) positive controls were assayed according to instructions using the the MILLIPLEX® MAP 10-plex MAPK/SAPK kit. The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate wells.

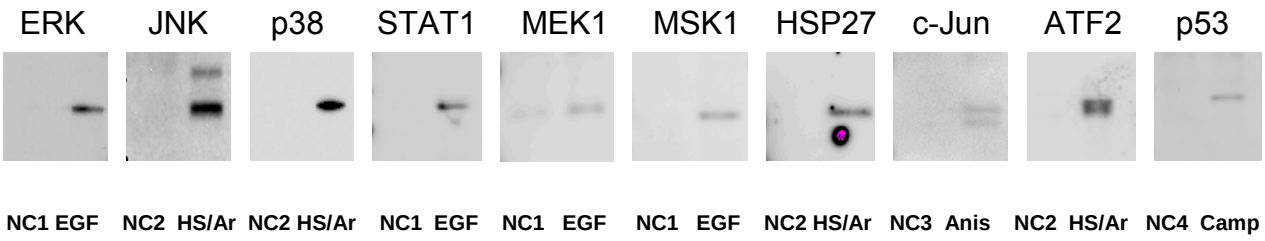


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes. Control lysates (described in Figure 1) were mixed with capture antibodies to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled phospho-specific reporter antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescence. NC1 = Negative Control A431:unstim.; NC2 = HeLa: unstim.; NC3 = NIH/3T3: anisomycin; NC4 = A549: unstim.; EGF = A431:EGF treated; HS/Ar = HeLa: Heat shock/ Arsenite treated; Anis = NIH/3T3: anisomycin treated; Camp = A549: camptothecin treated. NOTE: p53 is present constitutively phosphorylated in the A431 control.

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Buffer 2 Blank	A431: EGF positive control										
B	Assay Buffer 2 Blank	A431: EGF positive control										
C	HeLa: unstimulated negative control	Sample 1										
D	HeLa: unstimulated negative control	Sample 1										
E	Hela: HS/Ars positive control	Sample 2										
F	Hela: HS/Ars positive control	Sample 2										
G	NIH/3T3: Anis positive control	Etc.										
H	NIH/3T3: Anis positive control	Etc.										