2-Plex Phospho/Total p38 Magnetic Bead Kit

96-well Plate Assay

Cat. # 48-624MAG

MILLIPLEX®

2-Plex Phospho/Total p38 Magnetic Bead Kit
96-well Plate Assay

Cat # 48-624MAG

TABLE OF CONTENTS **PAGE**

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

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

p38 is a member of the MAPK (Mitogen Activated Protein Kinase) family that mediates cellular stress induced by inflammatory cytokines, UV, osmotic shock, heat shock, etc. Activation of p38 pathway can lead to cell differentiation, proliferation, survival, apoptosis and autophagy. Dysfunction of p38 pathway has been implicated in various diseases such as inflammatory diseases, neurodegenerative diseases and cancer.

Four p38 MAPKs, p38α (MAPK14), β (MAPK11), γ (SAPK3 /MAPK12), and δ (SAPK4/MAPK13), have been identified. Each p38 MAPK is encoded by different genes and have different tissue expression patterns, with p38α being ubiquitously expressed at significant levels in most cell types, whereas the others are expressed in a more tissue-specific manner with p38β expressed mainly in brain, p38γ in skeletal muscle and p38δ in endocrine glands.

In response to appropriate stimuli, dual-specificity MAPK kinases (MKKs/MAP2Ks) can phosphorylate p38 on threonine and tyrosine residues on the activation loop. MKK6 can phosphorylate all four p38 MAPK family members. MKK3 can phosphorylate p38α, p38γ and p38δ, but not p38β. Both MKK3 and MKK6 are highly specific for p38 MAPKs. In addition, MKK4, an activator of the JNK (c-Jun N-terminal kinase) pathway can phosphorylate p38α.

It has been estimated that p38 MAPKs can phosphorylate approximately 200–300 substrates, both *in vitro* and *in vivo.* These substrates include transcription factors (p53, CHOP, ATF, etc.), kinases (MSK1/2, MNK1/2, MK2/3, etc.), and many other nuclear and cytoplasmic proteins. In response to appropriate stimuli, activation of these p38 substrates can regulate cellular processes such as protein synthesis and degradation, mRNA stability, cytoskeleton dynamics, vesicle trafficking and cell migration.

Protein phosphorylation represents the major mechanism used in regulating cellular functions in all eukaryotic cells. Aberrant phosphorylation has been implicated in the onset and development of many diseases including metabolic disorders, inflammatory disease, cancer, etc. Changes in protein phosphorylation can be attributed to both changes in phosphorylation events as well as changes due to total protein levels. In order to distinguish the changes in phosphorylation from changes in protein expression, it is important to normalize the signal from phosphorylation over the signal from total protein. For this need, the MILLIPLEX® 2-plex Phospho/Total p38 kit has been developed for the simultaneous detection of phosphorylated p38 (Thr180/Tyr182) and total p38 in a single well using the Luminex® system. According to the original vendor's antibody information sheets, phospho-p38 detects p38α, p38β and p38γ but not p38-δ, while total p38 only detect p38α. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures for the analysis of cell lysate samples. Each kit has sufficient reagents for one 96 well plate assay.

It is possible to multiplex this kit together with other MILLIPLEX® 2-plex Phospho/Total Magnetic Bead kits (refer to protocol on page 9). For more information, please contact Technical Services (see the last page of this protocol for contact information).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.

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® 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) and non-magnetic bead (MicroPlex®) microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with multiple fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm non-magnetic or 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 SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
2-plex Phospho/Total p38, Magnetic Beads (20X)	42-624MAG	180 µL	1 tube
2-plex Phospho/Total p38, Biotin (20X) (Detection Antibody)	44-624KMG	180 µL	1 tube
Lysis Buffer	43-040	55 mL	1 bottle
Assay Buffer 2	43-041	55 mL	1 bottle
HeLa Cell Lysate: Unstimulated	47-205	----------	1 vial
HeLa Cell Lysate: TNFα/Calyculin A	47-230	----------	1 vial
Streptavidin-Phycoerythrin	45-001H	150 µL	1 tube
Amplification Buffer (1X)	43-024A	3 mL	1 bottle
Set of one 96-well Plate and 2 sealers	----------	----------	1 plate, 2 sealers
Empty mixing vials	----------	----------	3 vials

Analyte	Magnetic Bead Region
Phospho p38 (Thr180/Tyr182)	35
Total p38	38

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 (Catalog # 46-667MAG) MAPmate™ or β-Tubulin (Catalog # 46-713MAG) MAPmate™
- Luminex® Sheath Fluid (20 L, EMD Millipore Catalog # 40-50015) or Luminex® Drive Fluid (EMD Millipore 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 (VWR® Microplate Shaker Cat # 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
- 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

  NOTE: If using a filter plate and Vacuum Filtration Unit, a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog # MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog # WP6111560 or equivalent) may be ordered.
- Use of a handheld Magnetic Separation Block (EMD Millipore Catalog # 40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (BioTek® ELx405, EMD Millipore Catalog # 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.

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.
- 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.
- 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® 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.

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.
   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

   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. Alternatively, protein quantification may be omitted if an assay normalization control, such as the GAPDH (Catalog # 46-667MAG) MAPmate™ or β-Tubulin (Catalog # 46-713MAG) MAPmate™, is used.

Suggested cell lysis protocol for non-adherent cells

1. Pellet the cells by centrifugation (500 – 1000xg) 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 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 (Catalog # 46-667MAG) MAPmate™ or β-Tubulin (Catalog # 46-713MAG) MAPmate™, 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 500xg.

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® 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 500xg.
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 (Catalog # 46-667MAG) MAPmate™ or β-Tubulin (Catalog # 46-713MAG) MAPmate™, is used.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Phospho/Total p38 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. Dilute the beads to **1X** by combining 0.150 mL beads with 2.85 mL of 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.

B. 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 2. 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 2. 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.

C. Multiplexing an assay normalization control, such as the GAPDH (Catalog # 46-667MAG) MAPmate™ or β-Tubulin (Catalog # 46-713MAG) MAPmate™ with the 6-plex TGFβ Signaling Magnetic Bead Kit.

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 2-plex Phospho/Total p38 magnetic beads to the mixing vial.
3. For each additional MAPmate™, add 0.150 mL from each antibody bead vial to the mixing vial 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 2-plex Phospho/Total p38 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.

D. Preparation of lyophilized MILLIPLEX® Cell Lysates (Catalog # 47-205 and 47-230).

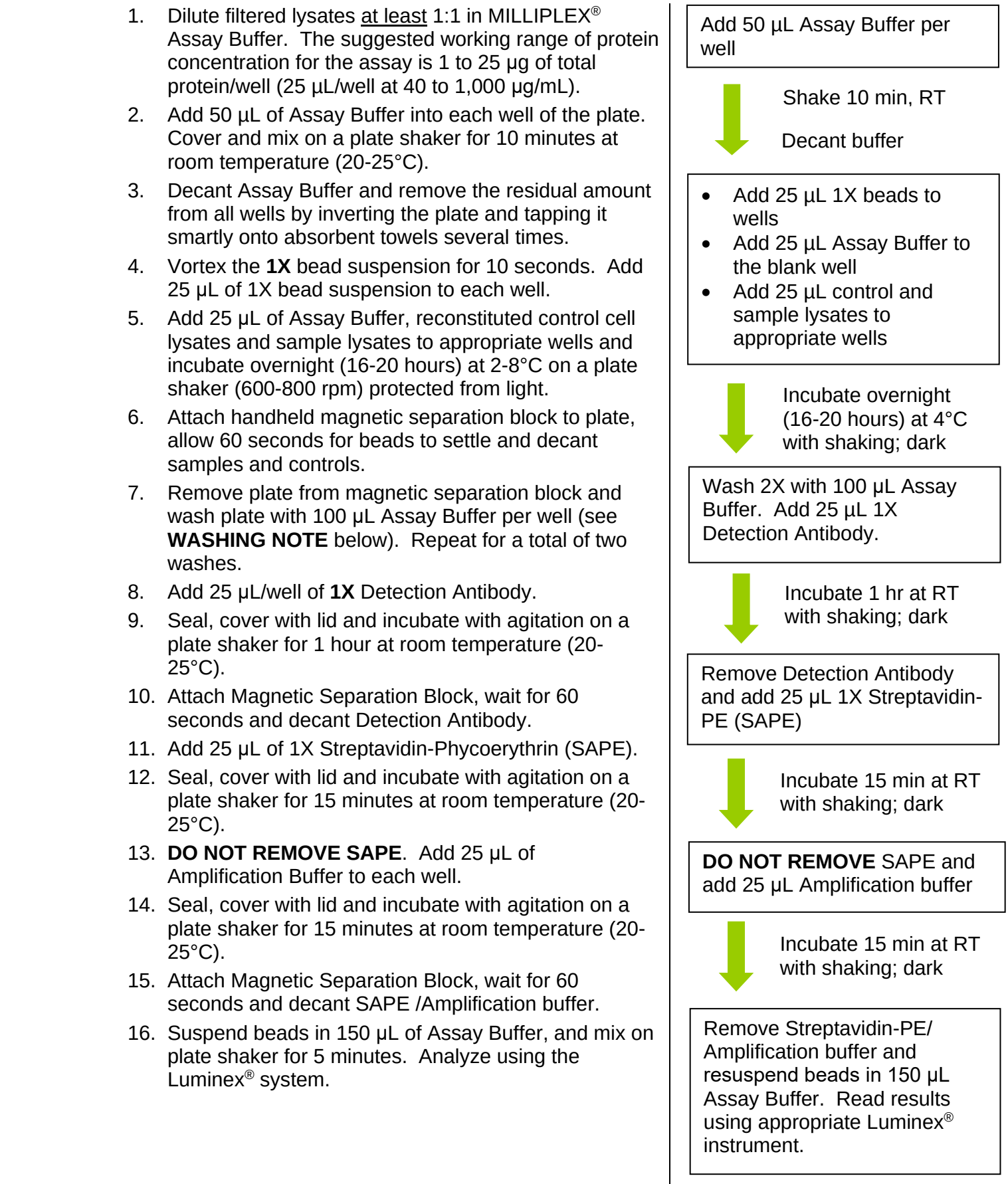
MILLIPLEX® HeLa Cell Lysate: Unstimulated (Catalog #47-205) is provided as a lyophilized stock of cell lysate prepared from HeLa cells and is used as an unstimulated control. MILLIPLEX® HeLa Cell Lysate: TNFα/Calyculin A (Catalog # 47-230) is provided as a lyophilized stock of cell lysate prepared from HeLa cells treated with calyculin A (50nM) and TNFα (50 ng/mL for 15 min). 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 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 minutes at RT (store on ice).
3. Pipette 150 µL of Assay Buffer 2 to each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the 2-plex Phospho/Total p38 Magnetic Bead Kit.
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).
13. **DO NOT REMOVE SAPE**. Add 25 µL 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°C).
15. Attach Magnetic Separation Block, wait for 60 seconds and decant SAPE /Amplification buffer.
16. Suspend beads in 150 µL 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 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® 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 # LX2R-CAL-K25) and performance verified with the Performance Verification Kit (EMD Millipore Catalog # LX2R-PVER-K25). The Luminex® FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D Calibrator Kit (EMD Millipore Catalog # F3D-CAL-K25) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog # F3D-PVER-K25). The Luminex® MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog # MPX-CAL-K25) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog # 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 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:	Phospho p38	35
	Total p38	38

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 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 2000xg and carefully remove the supernatant to minimize bead loss.
- Resuspend the pelleted beads in 25 µL/assay point of 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

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 (Catalog # MX-PLATE includes a set of two MILLIPLEX® 96-well Filter Plates with sealers). Contact EMD Millipore Technical Support if additional instructions are required.

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 Technical Service if additional instructions are required.

REPRESENTATIVE DATA

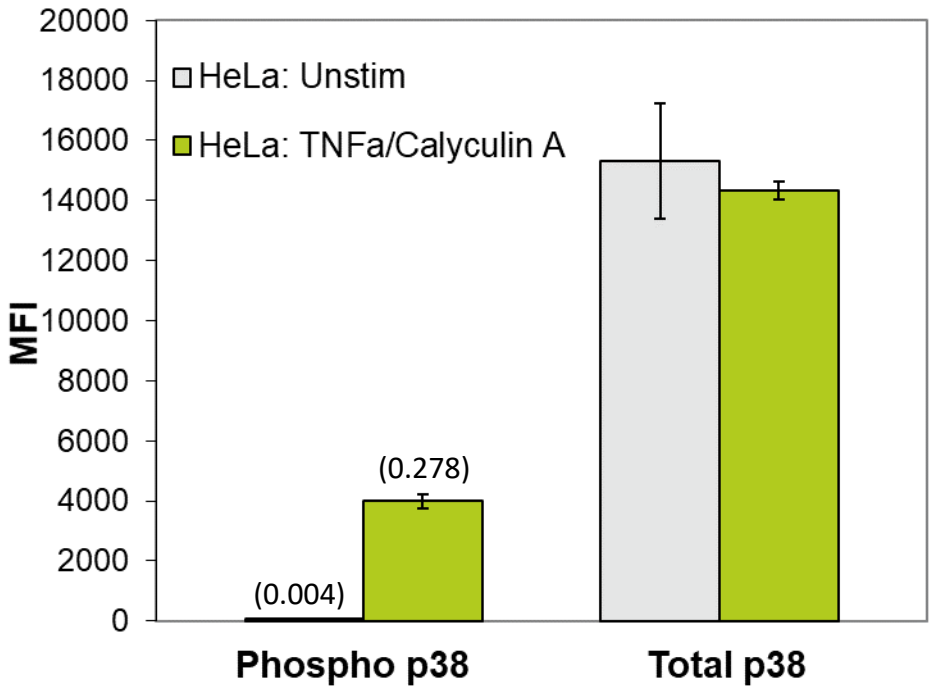


Figure 1. Multiplex analysis of Phospho and Total p38 in HeLa cells treated with TNFα + calyculin A. HeLa cells untreated or HeLa cells were treated with calyculin A (50nM) and TNFα (50 ng/mL for 15 min) were assayed. The cells were lysed in MILLIPLEX® Lysis Buffer containing protease inhibitors. Each lysate (20 μg total protein) was diluted in MILLIPLEX® Assay Buffer 2 and analyzed according the assay protocol (lysate incubation at 4ºC overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate wells. The ratio of Phospho-p38 over Total p38 signal is given in the parenthesis.

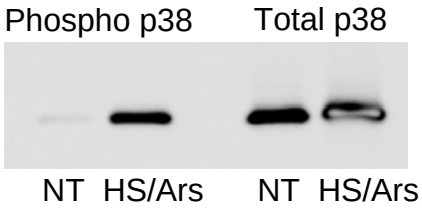


Figure 2. Immunoprecipitation/Western Blot analysis of Phospho and Total p38 in HeLa cells. Non-treated (NT) and heat shock/arsenite-treated (HS/Ars) HeLa cell lysates (100 μg) were mixed with capture antibodies to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled total p38 detection antibody. The proteins were imaged using Streptavidin-HRP and chemiluminescent substrate.

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 to the kit solid plate using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height 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.
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.
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	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 (e.g. 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 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.

REPLACEMENT REAGENTS

2-plex Phospho/Total p38 - Magnetic Beads (20X)	42-624MAG
2-plex Phospho/Total p38, Biotin (20X) (Detection Antibody)	44-624KMG
Lysis Buffer	43-040
Assay Buffer 2	43-041
HeLa Cell Lysate: Unstimulated	47-205
HeLa Cell Lysate: TNFα + calyculin A	47-230
Streptavidin-Phycoerythrin	45-001H
Amplification Buffer (1X)	43-024A
Set of two MILLIPLEX® 96-well Plates with sealers	MAG-PLATE

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	Assay Buffer 2 Blank	Sample 2										
B	Assay Buffer 2 Blank	Sample 2										
C	HeLa: Unstim negative control	Sample 3										
D	HeLa: Unstim negative control	Sample 3										
E	HeLa: TNFα/ Calyculin A positive control	Sample 4										
F	HeLa: TNFα/ Calyculin A positive control	Sample 4										
G	Sample 1	Etc.										
H	Sample 1	Etc.										