5-Plex Heat Shock Protein Panel Magnetic Bead Kit 96-Well Plate

Cat. # 48-615MAG

MILLIPLEX® MAP

5-Plex Heat Shock Protein Magnetic Bead Kit

Cat # 48-615MAG

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

Heat shock proteins (HSPs) are a family of proteins that function as molecular chaperones within the cell, and have many important roles including folding nascent proteins and maintaining proteins in a folded, active state. They can be induced in response to a variety of stresses including heat shock, radiation, and cytotoxic drug exposure, and have increased expression in cancer cells relative to normal cells. A number of these proteins are being investigated as potential targets for cancer therapy, as they can have effects on proliferation, apoptosis, metastasis, and chemotherapy resistance in certain cancers. The levels of some of the family members are coordinately regulated by the transcription factor Heat Shock Factor 1 (HSF1), in response to a variety of cellular stresses. Interestingly, HSP90 inhibitors can disrupt the interaction of HSP90 with HSF1, which can lead to increased expression of HSP family members such as HSP70 and influence the efficacy of treatment. To better understand the roles and regulation of heat shock proteins and the heat shock response in cancer and other physiological processes, there is increased interest in simultaneous analysis of key heat shock proteins in a panel or multiplex format, rather than studying each protein in isolation.

The MILLIPLEX™ MAP 5-plex Human Heat Shock Protein Magnetic Bead Kit is used to detect changes in HSP27 (Total), HSP27 (pS78/pS82), HSP60, HSP70 (HSP72), and HSP90α 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.

This kit is for research purposes only.

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.
- Once the control lysates have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSITUTED CONTROLS IN LYOPHILIZATION VIALS**. For long-term storage, freeze reconstituted 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
MILLIPLEX MAP 5-plex HSP Panel Magnetic Beads (20X)	42-615K	180 µL	1 tube
MILLIPLEX MAP 5-plex HSP Panel, Biotin (20X) (Detection Antibody)	44-615K	180 µL	1 tube
MILLIPLEX MAP Lysis Buffer	43-040	55 mL	1 bottle
MILLIPLEX MAP Assay Buffer 1	43-010	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 Streptavidin-Phycoerythrin	45-001D	115 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
HSP27 (Total)	19
HSP27 (pS78/pS82)	25
HSP60	52
HSP70 (HSP72)	57
HSP90α	63

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Protease inhibitors (recommend EMD Chemicals Catalog #535140 or similar product)
- Coomassie or BCA-based total protein assay (EMD Chemicals Catalog #71285 or similar product) or an assay normalization control, such as the GAPDH MAPmates (Millipore Catalog #46-667)
- Luminex Sheath Fluid (Luminex Catalog #40-5000) or Luminex Drive Fluid (Luminex Catalog # MPXDF-4PK)

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 (Millipore Catalog # MX-STAND, if using filter plate)
- Filter devices for clearing lysates
  - 2 mL or greater, Millipore Catalog # SLHVX13NL
  - 0.5 – 2 mL, Millipore Catalog # UFC40DV25
  - Less than 0.5 mL, Millipore Catalog # UFC30DV25
  - For 96-well plates, Millipore Catalog # MSBVN1210
- A Hand-held Magnetic Separation Block (Millipore catalog # 40-285 or equivalent) may be used. Contact Technical Service for use of an automatic plate washer.
- If using the filter plate, a Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with 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 15 to 150 ng of total protein/well (25 µL/well at 600 to 6,000 ng/mL). A total protein amount of 100 ng/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 Chemicals 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 off PBS.
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 Millipore filters:
      (i) 2 mL or greater, Millipore Catalog # SLPBDZ5NZ
      (ii) 0.5 – 2 mL, Millipore Catalog # UFC 0DV 25
      (iii) Less than 0.5 mL, 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.

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 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.

B. Preparation of cell lysates (continued)

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 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.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of antibody HSP Panel magnetic beads

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

1. Sonicate **20X** stock capture 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 1. Use one of the empty Mixing Bottles provided.
3. Vortex the **1X** capture beads for 15 seconds.
4. For use, transfer 1X capture beads with a pipette, do not pour from Mixing Bottle.

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.

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

2. Dilute the Detection Antibody to 1X by combining 0.150 mL of Detection Antibody with 2.85 mL of MILLIPLEX MAP Assay Buffer 1. Use one of the empty Mixing Bottles provided.
3. Vortex the MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE) for 10 seconds.
4. Dilute SAPE by combining 30 µL of Streptavidin-Phycoerythrin with 2.97 mL of MILLIPLEX MAP Cell Signaling Assay Buffer 1. Use one of the empty Mixing Bottles provided.
5. Transfer 1X biotinylated detection antibody and diluted SAPE with a pipette; do not pour from Mixing Bottle.

C. Preparation of lyophilized MILLIPLEX MAP Cell Lysates (Millipore Catalog # 47-205, 47-211).

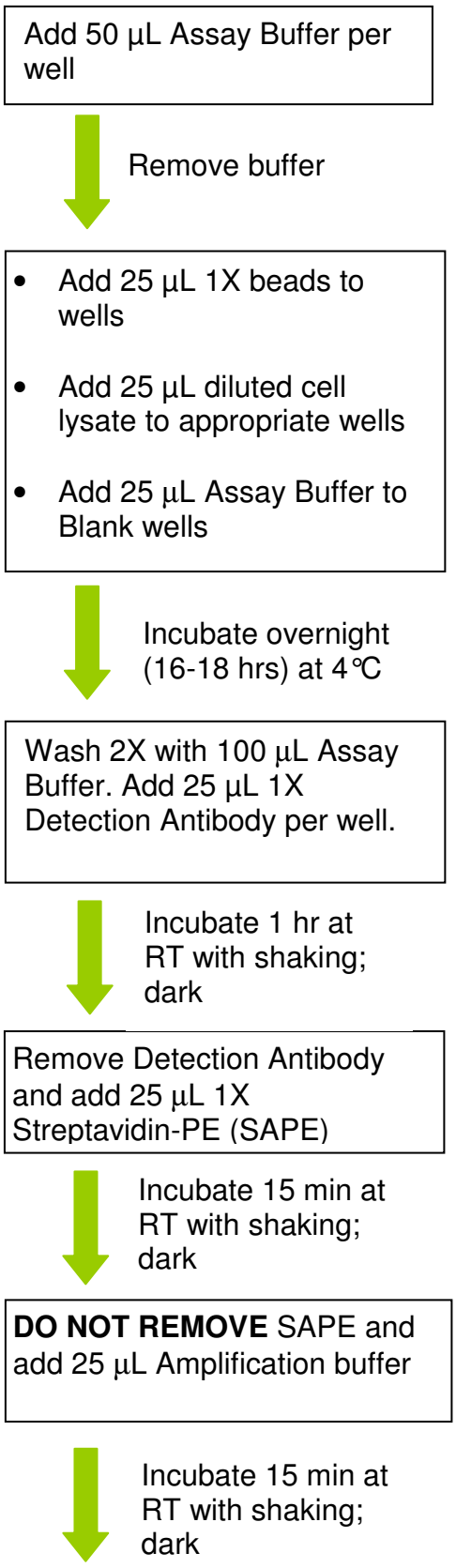
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated (Millipore Catalog #47-205) is provided as a lyophilized stock of cell lysate prepared from unstimulated HeLa cells and is used as a negative control for the HSP27(pS78/pS82) assay. MILLIPLEX MAP HeLa Cell Lysate: HS/Ars (Millipore Catalog #47-211) is provided as a lyophilized stock of cell lysate prepared from HeLa cells heat shocked (42^0C for 30 min.), grown for 16 hrs at 37^0C, then treated with 400 µM arsenite for 30 min; it is used as a positive control for all five assays. 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 positive and negative control samples or alternatively, to create calibration curves for relative quantification of different protein analytes.

MILLIPLEX MAP Cell Lysates as a positive and negative control

1. Reconstitute each of the lyophilized cell lysates in 100 µL of ultrapure water, for each vial this will yield 100 µL of concentrated lysate at a total protein concentration of 2000 µg/mL.
2. Gently vortex and incubate the reconstituted lysates for 5 min at RT (store on ice).
3. Combine 10 µL of reconstituted lysate with 40 µL of MILLIPLEX MAP Assay Buffer 1 in a pre-chilled microcentrifuge tube, and pipette to mix. This will yield diluted lysate stock at 400 µg/mL.
4. To prepare working stocks of cell lysate controls, combine 10 µl of diluted cell lysate stock (prepared in Step 3 above) with 490 µL of MILLIPLEX MAP Assay Buffer 1 and pipette thoroughly to mix. The cell lysate is now prepared for use in the MILLIPLEX MAP 5-plex Heat Shock Protein Magnetic Bead Kit.
5. If desired, unused concentrated lysate stock may be stored in its original container at ≤ -70°C for up to one month.

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

1. Dilute filtered lysates at least 1:1 in MILLIPLEX MAP Assay Buffer 1. The suggested working range of protein concentration for the assay is 15 to 150 ng of total protein/well (25 µL/well at 600 to 6,000 ng/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 diluted working stocks of Control cell lysates, prepared test lysate, or Assay Buffer to appropriate wells and incubate overnight (16-18 hours) at 4°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, allow 60 seconds for beads to settle and decant Detection Antibody.
11. Add 25 µL of diluted 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, allow 60 seconds for beads to settle and decant SAPE /Amplification buffer.
16. Resuspend beads in 150 µL of MILLIPLEX MAP Assay Buffer 1, and mix on plate shaker for 5 minutes, Analyze using the Luminex® system.

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

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 (Millipore Cat.# 40-275) and performance verified with the Performance Verification Kit (Millipore Cat. # 40-276). The Luminex FLEXMAP 3D™ instrument must be calibrated with the FLEXMAP 3D™ Calibrator Kit (Millipore cat#40-028) and performance verified with the FLEXMAP 3D™ Performance Verification Kit (Millipore cat#40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (Millipore cat# 40-049) and performance verified with the MAGPIX® Performance Verification Kit (Millipore cat# 40-050).

NOTE: These assays cannot be run on any instruments using 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 Millipore Cat.# 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:	HSP27 (Total)	19
	HSP27 (pS78/pS82)	25
	HSP60	52
	HSP70 (HSP72)	57
	HSP90α	63

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 1 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 1.
- 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 (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)

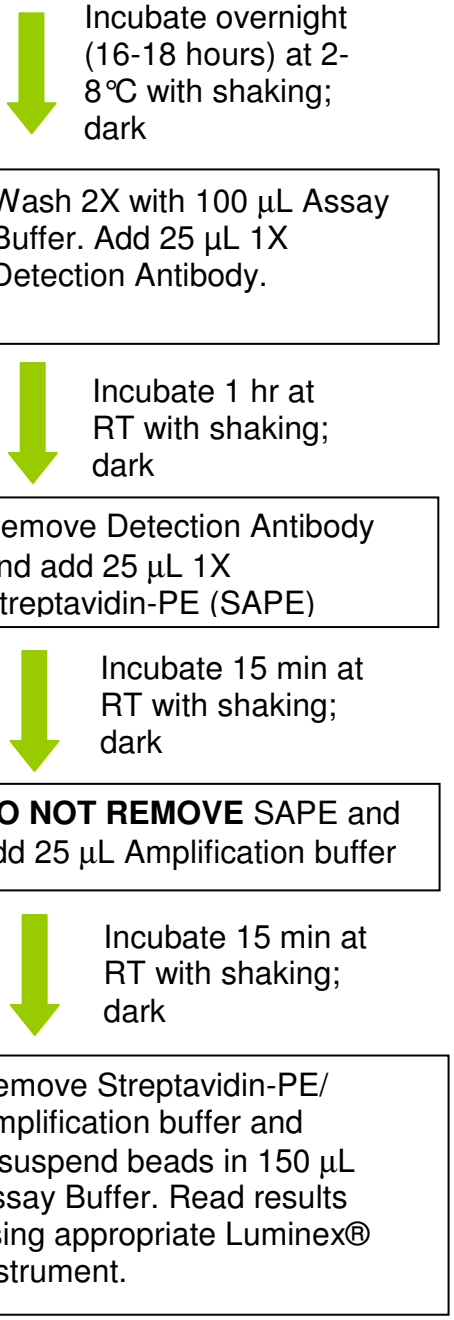
1. Dilute filtered lysates at least 1:1 in MILLIPLEX MAP Assay Buffer 1. The suggested working range of protein concentration for the assay is 15 to 150 ng of total protein/well (25 µL/well at 600 to 6,000 ng/mL).
2. Pre-wet filter plate with 25 µL/well of MILLIPLEX MAP Assay Buffer 1. Remove by vacuum 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.

Add 25 µL Assay Buffer per well

↓ Remove buffer by vacuum.

- Add 25 µL 1X beads to wells
- Add 25 µL diluted cell lysate to appropriate wells
- Add 25 µL Assay Buffer to Blank wells

4. Add 25 µL of diluted working stocks of Control cell lysates, prepared test lysate, or Assay Buffer to appropriate wells and incubate overnight (16-18 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 1. 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 filtration 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 mins 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 mins 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 1, and mix on plate shaker for 5 minutes.
16. Analyze using the Luminex® system.



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.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex 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°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	Incorrect or no Detection	Add appropriate Detection Antibody and

plate is same as background	Antibody was added	continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	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.
	Plate incubation was too long with standard curve and 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 tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve.	See above.
High Variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	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 a vertical 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 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	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum Pressure too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.

		May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly Sample too viscous	Adjust probe to 3 alignment discs in well H6. May need to dilute sample.

REPLACEMENT REAGENTS	**Catalog #**
MILLIPLEX MAP Lysis Buffer	43-040
MILLIPLEX MAP Assay Buffer 1	43-010
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated	47-205
MILLIPLEX MAP HeLa Cell Lysate: HS/Ars	47-211
MILLIPLEX MAP Streptavidin-Phycoerythrin	45-001D
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

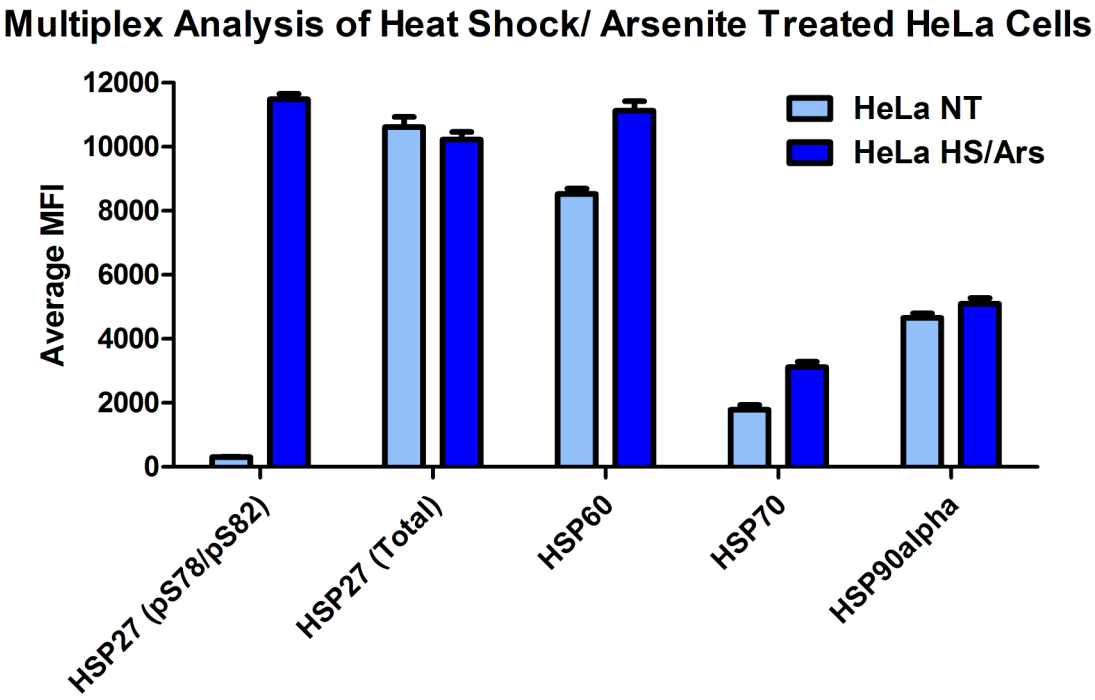


Figure 1. Multiplex analysis of HeLa cells treated with heat shock and arsenite. HeLa cells untreated (NT), and HeLa cells heat shocked (42^{0}C for 30 min.), grown for 16 hrs at 37^{0}C, then treated with 400 µM arsenite for 30 min (HS/Ars) were lysed in MILLIPLEX MAP Lysis Buffer containing protease inhibitors. 200 ng total protein per well of each lysate was analyzed according to the kit instructions. The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The results presented are the average and standard deviation of four replicate wells.

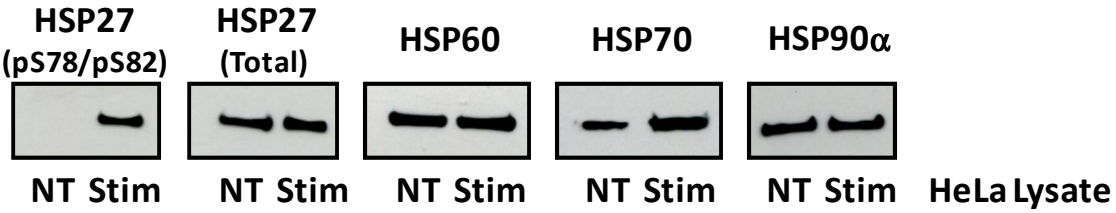


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes in HeLa cells. Cell lysates (20 µg) from HeLa cells that were untreated (NT) or treated with heat shock and arsenite (HS/Ars) as described in Figure 1 were mixed with bead-immobilized capture antibodies specific to each target in order to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin-labeled detection antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescence.
NT: HeLa Cell Lysate Unstimulated; Stim: HeLa Cell Lysate Heat Shock and Arsenite Stimulated

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample Blank (Assay Buffer)	Sample2										
B	Sample Blank (Assay Buffer)	Sample2										
C	HeLa: Unstim Control	Etc.										
D	HeLa: Unstim Control											
E	HeLa: HS/Ars Control											
F	HeLa: HS/Ars Control											
G	Sample1											
H	Sample1											