MILLIPLEX® Human Cell Health Magnetic Bead Panel

96-Well Plate Assay

48-691MAG

Introduction

Cell health is affected by the careful balance of various physiological processes and signaling pathways in response to diverse stimuli including drug treatment, disease, culture conditions, and stress. For example, cells respond to different stressors by expressing stress-related proteins that may lead to the activation of survival pathways or initiation of cell death. Identifying markers of specific stressors (for example oxidative, heat shock, genotoxicity, ER stress, inflammation, et cetera), activated repair or death pathways (for example, apoptosis, autophagy, DNA repair/damage, et cetera), and affected cellular functions (mRNA synthesis, chromatin condensation, proliferation, OXPHOS, et cetera) may elucidate further details about ongoing pathological processes or pharmacological response mechanisms. Comprehensive, simultaneous analysis of cell health in response to various stimuli through a single, multiplexed detection platform provides researchers with valuable insights into diverse cell signaling pathways and provides a fast and accurate assessment of the overall cell health status. Further, measurement of specific protein expression and phosphorylation levels can provide mechanistic insights to researchers performing drug tox screens, studying changes in cell health due to disease, or researching various cell death and survival pathways.

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

Each MILLIPLEX® cell signaling kit includes:

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

The MILLIPLEX® 16-plex Human Cell Health Panel is used to detect changes in total CHOP, Cyclin B1, Complex IV, Ki67, LC3B, HIF-1a, Polyubiquitin K48-linkage, HSP70, cleaved PARP, and GRP78 and phosphorylated eIF4B (Ser422), NF-κB (Ser536), Histone H2A.x (Ser139), Histone H3 (Ser10), p53 (Ser15), and RNA Pol II (Ser2) 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.

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 two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500, 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
- The Luminex® analyzers Luminex® 200, FLEXMAP 3D®, and xMAP® INTELLIFLEX are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
- The Luminex® analyzer, MAGPIX®, is a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified, and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the streamlined data acquisition power of Luminex® xPONENT acquisition software with sophisticated analysis capabilities of MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.
- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user to freely select which data points to include and to reduce the time to analysis.

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

Storage Conditions Upon Receipt

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

Reagents Supplied

Reagents	Volume	Quantity	Catalogue No.
16-plex Human Cell Health Panel Magnetic Beads (20X)	180 µL	1 tube	42-691MAG
16-plex Human Cell Health Panel Detection (20X)	180 µL	1 tube	44-691KMG
Blocker Mix	150 µL	1 tube	43-055
Lysis Buffer	55 mL	1 bottle	43-040
Cell Signaling Assay Buffer 2	55 mL	2 bottles	43-041
Streptavidin-Phycoerythrin	150 µL	1 tube	45-001H
Cell Signaling Amplification Buffer	3 mL	1 bottle	43-024A
HeLa Cell Lysate: Lambda Phosphatase	-	1 vial	47-229
Hela Cell Lysate: TNFα + CalyculinA	-	1 vial	47-230
A549 Cell Lysate: Camptothecin	-	1 vial	47-218
MCF7 Cell Lysate: MG132	-	1 vial	47-319
Jurkat Cell Lysate: Anisomycin	-	1 vial	47-207
Set of one 96-well Plate and 2 sealers	-	1 plate, 2 sealers	-
Mixing Bottle	-	3 Bottles	-

Analyte	Magnetic Bead Region
phopspho-eIF4B (Ser422)	19
CHOP	22
Cyclin B1	25
Complex IV	26
Ki67	29
LC3B	33
phospho-NFkB (Ser536)	36
phospho-H2A.X (Ser139)	39
HIF-1a	45
Polyubiquitin K48-linkage	48
phospho-H3 (Ser10)	52
phospho-p53 (Ser15)	53
HSP70	61
Cleaved PARP	72
phospho-RNA Pol II (Ser2)	77
GRP78	78

Materials Required (Not Provided)

Reagents

- Protease inhibitors (535140 or similar product)
- Coomassie or BCA-based total protein assay (71285 or similar product) or an assay normalization control, such as the GAPDH (46-667MAG) MAPmate™ or β-Tubulin (46-713MAG) MAPmate™.
- MAGPIX® Drive Fluid PLUS (40-50030), xMAP® Sheath Fluid PLUS (40-50021), or xMAP® Sheath Concentrate PLUS (40-50023).
- Wash Buffer (L-WB) if using a magnetic plate washer. (see Supplemental Protocols).

Instrumentation/Materials

- Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
- Multichannel Pipettes capable of delivering 25 µL to 200 µL
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Rubber Bands
- Aluminum Foil
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator (Branson Ultrasonic Cleaner Model No. B200 or equivalent)
- Titer Plate Shaker (VWR® Microplate Shaker, 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® with xPONENT® software or xMAP® INTELLIFLEX with INTELLIFLEX software by Luminex® Corporation
- Filter devices for clearing lysates
  - 2 mL or greater (SLHVX13NL)
  - 0.5-2 mL, (UFC40DV25)
  - Less than 0.5 mL, (UFC30DV25)
  - For 96-well plates, (MSBVN1210)

  Note: If using a filter plate and Vacuum Filtration Unit, a Vacuum Filtration Unit (Vacuum Manifold, MSVMHTS00 or equivalent with Vacuum Pump, WP6111560 or equivalent) may be ordered.
- Use of a handheld Magnetic Separation Block (40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (BioTek® Agilent 405TS, 40-015 or equivalent), consult Supplemental Protocols.

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 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.

Hazard Labels

Ingredient	Catalogue No.	Label	
HeLa Cell Lysate: Lambda Phosphatase Hela Cell Lysate: TNFα + CalyculinA A549 Cell Lysate: Camptothecin MCF7 Cell Lysate: MG132 Jurkat Cell Lysate: Anisomycin	47-229 47-230 47-218 47-319 47-207		**Danger.** Harmful if swallowed. Causes skin irritation. Causes serious eye damage. Very toxic to aquatic life. Toxic to aquatic life with long lasting effects. Wash skin thoroughly after handling. Do not eat, drink or smoke when using this product. Avoid release to the environment. Wear protective gloves/ eye protection/ face protection. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Rinse mouth. IF ON SKIN: Wash with plenty of water. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. If skin irritation occurs: Get medical advice/ attention. Take off contaminated clothing and wash it before reuse. Collect spillage. Dispose of contents/ container to an approved waste disposal plant.
Blocker Mix	43-055	No Label Required	Harmful to aquatic life with long lasting effects. Avoid release to the environment. Dispose of contents/ container to an approved waste disposal plant.
Lysis Buffer	43-040		**Warning.** Causes serious eye irritation. Toxic to aquatic life. Harmful to aquatic life with long lasting effects. Wash skin thoroughly after handling. If eye irritation persists: Get medical advice/ attention. Dispose of contents/ container to an approved waste disposal plant.

For research use only. Not for use in diagnostic procedures.

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.
- Unused 1X mixed Antibody-Immobilized Beads cannot be stored. **Beads must be used immediately.**
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8 °C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm. Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
- For xMAP® INTELLIFLEX, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Considerations for Cell Stimulation

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

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 (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.
5. Suggested filters:
    - 2 mL or greater, (SLPBDZ5NZ)
    - 0.5-2 mL, (UFC0DV25)
    - Less than 0.5 mL, (UFC30DV00)
6. 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.
7. Aliquot and store the lysate at -70 °C. The lysate should be stable for several months.

8. 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 (46-667MAG) MAPmate™, is used.

Suggested cell lysis protocol for non-adherent cells

1. Pellet the cells by centrifugation (500-1000 x g) in a tabletop centrifuge f or 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 (46-667MAG) 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 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® 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 (46-667MAG) MAPmate™ or, is used.

Preparation of Reagents for Immunoassay

Preparation of Human Cell Health Panel 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. This must be completed immediately prior to assay set up.
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.

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 and 25X Blocker Mix 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 and 0.120 mL of Blocker Mix with 2.73 mL of Assay Buffer 2. Use one of the Mixing Bottles provided.
3. Vortex the 25X Streptavidin-Phycoerythrin (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.

Multiplexing an assay normalization control (such as the GAPDH (46-667MAG) MAPmate™ with the 16-plex Human Cell Health Panel 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 16-plex Human Cell Health Panel 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 MAPmate™, add 0.150 mL 16-plex Cell Health 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.

Preparation of lyophilized MILLIPLEX® Cell Lysates

(47-229, 47-230, 47-218, 47-319, 47-207)

- MILLIPLEX® HeLa Cell Lysate: Lambda Phosphatase (47-229) is provided as a lyophilized stock of cell lysate prepared from unstimulated HeLa cells treated with lambda phosphatase and is used as a negative control.
- MILLIPLEX® HeLa Cell Lysate: TNFα + Calyculin A (47-230) is provided as a lyophilized stock of cell lysate prepared from HeLa cells treated with 15 ng/mL TNFα +Calyculin A (15 minutes).
- MILLIPLEX® A549 Cell Lysate: Camptothecin (47-218) is provided as a lyophilized stock of cell lysate prepared from A549 cells stimulated with 5 µM Camptothecin (overnight).
- MILLIPLEX® MCF7 Cell Lysate: MG132 (47-319) is provided as a lyophilized stock of cell lysate prepared from MCF7 cells stimulated with 10 µM MG132 (24 hours).
- MILLIPLEX® Jurkat Cell Lysate: Anisomycin (47-207) is provided as a lyophilized stock of cell lysate prepared from Jurkat cells stimulated with 25 µM Anisomycin (4 hours).
- 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 protein 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 then store lysate on ice.
3. Pipette 150 µL of Assay Buffer 2 to A549 Cell Lysate: Camptothecin (47-218) MCF7 Cell Lysate: MG132 (47-319) vial and vortex mix for a final concentration of 20 µg/well.
4. Pipette 900 µL of Assay Buffer 2 to HeLa Cell Lysate: Lambda Phosphatase (47-229) and HeLa Cell Lysate: TNFα + Calyculin A (47-230) vial and vortex mix for a final concentration of 5 µg/well.
5. Pipette 900 µL of Assay Buffer 2 to Jurkat Cell Lysate: Anisomycin (47-207) vial, then transfer 1 mL of reconstituted lysate to a vial with 4 mL of Assay Buffer 2 and vortex mix for a final concentration of 1µg/well.
6. If desired, unused lysate may be stored in a polypropylene tube at –20 °C for up to one month. Avoid multiple freeze/thaws.

Immunoassay Protocol (96-well Plate and Handheld Magnetic Separation Block)

1. Dilute filtered lysates at least 1:1 in 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 and 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

Add 50 µL Assay Buffer per well.

Shake 10 min, RT

Decant

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

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

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

Incubate 1 hour at RT with shaking; dark.

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

Incubate 15 min at RT with shaking; dark.

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 100 µL of Assay Buffer and mix on plate shaker for 5 minutes. Analyze using the Luminex® system.

DO NOT REMOVE SAPE
Add 25 µL Amplification buffer

Incubate 15 minutes at RT with shaking; dark

Remove Streptavidin-PE/ Amplification buffer and resuspend beads in 100 µL Assay Buffer. Read results using appropriate Luminex® instrument.

Equipment Settings

Luminex® 200™, HTS, FLEXMAP 3D® MAGPIX® with xPONENT® software and xMAP® INTELLIFLEX with INTELLIFLEX Software.

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example. MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® Magnetic Beads.

For magnetic bead assays, each instrument must be calibrated, and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (LX2RCAL-K25)	Performance Verification Kit (LX2RPVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (F3DCAL-K25)	FLEXMAP 3D® Performance Verification Kit (F3DPVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (MPXCAL-K25)	MAGPIX® Performance Verification Kit (MPXPVER-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 (MAG-PLATE), if additional plates are required for this purpose.

Events	50, per bead
Sample Size	50 µL
Gate Settings	8,000 to 15,000
Reporter Gain	Default (low PMT)
Time Out	60 seconds

Bead Region	
phopspho-eIF4B (Ser422)	19
CHOP	22
Cyclin B1	25
Complex IV	26
Ki67	29
LC3B	33
phospho-NFkB (Ser536)	36
phospho-H2A.X (Ser139)	39
HIF-1a	45
Polyubiquitin K48-linkage	48
phospho-H3 (Ser10)	52
phospho-p53 (ser15)	53
HSP70	61
Cleaved PARP	72
phospho-RNA Pol II (Ser2)	77
GRP78	78

Supplemental Protocols

Plate Washer Use

Handwashing is recommended for cell signaling assays; however, this kit has been evaluated for plate washer use. If desired, 10X L-WB may be purchased and used as a general wash buffer with plate washers. 10X L-WB should be diluted to 1X for use in plate washers. Follow standard protocol wash instructions when using a plate washer (2 washes after sample incubation). Contact Technical Service if additional instructions are required.

Representative Data

16-plex Cell Health Signaling Panel Analysis of Stimulated Cell Lines

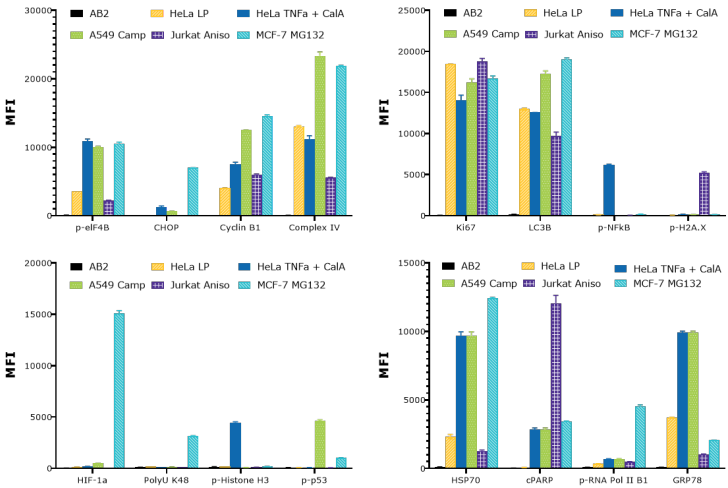

Figure 1. Multiplex analysis of HeLa, A549, Jurkat, and MCF7 cells treated with TNFa/CalyculinA, Camptothecin, Anisomycin, or MG132. HeLa cells treated with lambda phosphatase (LP), HeLa pretreated with 50 nM Calyculin A (15 minutes) prior to stimulation with 50 ng/mL TNF-α (15 minutes), A549 cells stimulated with 5 µM Camptothecin (overnight), Jurkat cells treated with 25 µM Anisomycin (4 hours), and MCF-7 cells treated with 10 µM MG132 (24 hrs) were assayed. 20 µg total protein of A549 Camptothecin and MCF-7 MG132, 5 µg of HeLa LP and TNFa/CalA, and 1 µg of Jurkat Anisomycin lysates diluted in MILLIPLEX® Assay Buffer 2 were analyzed according to the Assay protocol (lysate incubation at 4 °C overnight). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of duplicate wells.

Figure 2. Immunoprecipitation/Western Blot analysis in various cell lines. 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 phospho-specific or total detection antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescent substrate. Non-treated (HNT or MNT), HeLa Lambda Phosphatase-treated (HLP), HeLa TNFα/Calyculin A-treated (HT), A549 Camptothecin-treated (AC), Jurkat Anisomycin-treated (JA), and MCF-7 MG132 (MM) lysate controls.

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.
Background is too high	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. When reading the assay on xMAP® INTELLIFLEX, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
	Background 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.

Problem	Probable Cause	Solution
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 (for example. 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 PLUS 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.	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added.	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Signals too high	Calibration target value set too high.	With some Luminex® instruments (for example, Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with samples.	Use shorter incubation time.

Problem	Probable Cause	Solution
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.

Product Ordering

Products are available for online ordering at SigmaAldrich.com.

Description	Catalogue Number
16-plex Human Cell Health Panel Magnetic Beads (20X)	42-691MAG
16-plex Human Cell Health Panel Detection (20x)	44-691KMG
Detection Blocker Mix (25x)	43-055
Lysis Buffer	43-040
Cell Signaling Assay Buffer 2	43-041
HeLa Cell Lysate: Lambda Phosphatase	47-229
HeLa Cell Lysate: TNFα + CalyculinA	47-230
A549 Cell Lysate: Camptothecin	47-218
Jurkat Cell Lysate: Anisomycin	47-207
MCF7 Cell Lysate: MG132	47-319
Cell Signaling Streptavidin-Phycoerythrin (25x)	45-001H
Cell Signaling Amplification Buffer (1X)	43-024A
Set of two MILLIPLEX® 96-well Plates with sealers	MAG-PLATE

Notice

We provide information and advice to our customers on application technologies and regulatory matters to the best of our knowledge and ability, but without obligation or liability. Existing laws and regulations are to be observed in all cases by our customers. This also applies in respect to any rights of third parties. Our information and advice do not relieve our customers of their own responsibility for checking the suitability of our products for the envisaged purpose.

The information in this document is subject to change without notice and should not be construed as a commitment by the manufacturing or selling entity, or an affiliate. We assume no responsibility for any errors that may appear in this document.

Technical Assistance

Visit the tech service page at SigmaAldrich.com/techservice.

Terms and Conditions of Sale

Warranty, use restrictions, and other conditions of sale may be found at SigmaAldrich.com/terms.

Contact Information

For the location of the office nearest you, go to SigmaAldrich.com/offices.