User Guide

MILLIPLEX® 7-Plex DNA Damage/Genotoxicity Magnetic Bead Kit

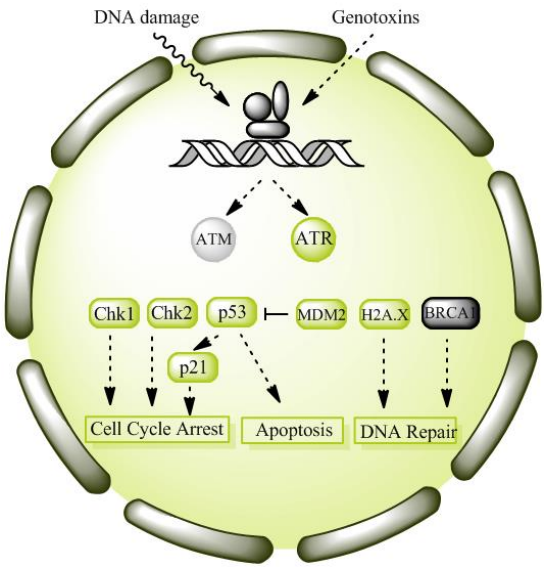
96-Well Plate

48-621MAG

Introduction

7-DNA damage and repair is constantly occurring in all living cells. This damage occurs naturally as a result of cellular respiration and metabolism (both normal and aberrant), but may also be elicited by exposure to genotoxic xenobiotics. Repair of this damage is necessary to retain cell function and viability, as well as to maintain the accurate transference of genetic data to daughter cells. There are numerous proteins involved in the detection and repair of DNA damage, these proteins can be broadly classified as Sensors, Mediators, Transducers and Effectors. Sensor proteins like Rad9, Rad1and Hus1 accumulate at the site of DNA damage and facilitate phosphorylation of checkpoint proteins, this phosphorylation is affected by the transducers ATM and ATR. Activation of mediators like H2AX, BRCA1 and SMC1 lead to sustained multi-protein interactions which facilitate ATM signaling and in turn activate checkpoint kinases. Checkpoint kinases like Chk1 and Chk2 are essential for cell-cycle arrest before mitosis in response to DNA damage. Effector molecules include p53 and MDM2. p53 is a tumor suppressor protein which plays a critical role in the decision of a cell to undergo cell-cycle arrest or apoptosis after diverse stresses, including radiation or chemical induced DNA damage, hypoxia and the activation of oncogenes. Traditional genotoxicity assays detect gain or loss of function mutations, but provide little or no mechanistic data as to how this DNA damage may have occurred. Protein expression and phosphorylation detection of multiple proteins involved in DNA damage or genotoxicity provides a faster and more accurate assessment of the status of the cell for researchers exposing cells to potentially genotoxic compounds or researching DNA damage and repair mechanisms.

Summary of DNA Damage/Genotoxicity Pathway



The MILLIPLEX® 7-plex DNA Damage/Genotoxicity Magnetic Bead kit is used to detect changes in phosphorylated Chk1 (Ser345), Chk2 (Thr68), H2A.X (Ser139) and p53 (Ser15) as well as total protein levels of ATR, MDM2 and p21 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

The MILLIPLEX® portfolio 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®-C microspheres.

- Luminex® products use 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. 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	Volume	Quantity	Cat. No.
MILLIPLEX® 7-plex DNA Damage/Genotoxicity, Magnetic Beads (20X)	180 µL	1 tube	42-621MAG
MILLIPLEX® 7-plex DNA Damage/Genotoxicity, Biotin (20X) (Detection Antibody)	180 µL	1 tube	44-621KMG
MILLIPLEX® Lysis Buffer (1X)	55 mL	1 bottle	43-040
MILLIPLEX® Assay Buffer 1 (1X)	55 mL	1 bottle	43-010
MILLIPLEX® HeLa Cell Lysate: Lambda Phosphatase	-	1 vial	47-229
MILLIPLEX® Jurkat Cell Lysate: Anisomycin	-	1 vial	47-207
MILLIPLEX® A549 Cell Lysate: Camptothecin	-	1 vial	47-218
MILLIPLEX® Streptavidin-Phycoerythrin (25X)	150 µL	1 tube	45-001H
MILLIPLEX® Amplification Buffer (1X)	3 mL	1 bottle	43-024A
Set of one 96-well Filter Plate and 2 sealers	-	1 set	-
Set of one 96-well Plate and 2 sealers	-	1 set	-
Empty Mixing Bottles	-	3 Bottles	-

Analyte	Magnetic Bead Region
ATR	21
Chk1	28
Chk2	35
H2A.X	39
p53	53
MDM2	67
p21	75

Materials Required (not included)

Reagents

- Protease inhibitors (Cat. No. 535140 or similar product)
- Coomassie or BCA-based total protein assay (Cat. No. 71285 or similar product) or an assay normalization control, such as the GAPDH (Cat. No. 46-667MAG) or β-Tubulin (Cat. No. 46-713MAG) MAPmate™
- MAGPIX® Drive Fluid PLUS (Cat. No. 40-50030), xMAP® Sheath Fluid PLUS (Cat. No. 40-50021), or xMAP® Sheath Concentrate PLUS (Cat. No. 40-50023)
- 10X Assay Buffer 1 (Cat. No. MPEQ-AB) if using a magnetic plate washer (see Supplemental Protocol C)

Instrumentation/Materials

- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL
- Multichannel pipettes capable of delivering 25 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Rubber bands
- Aluminum foil
- Absorbent pads
- Laboratory vortex mixer

- Sonicator (Branson Ultrasonic Cleaner Model No. B200 or equivalent)
- Titer plate shaker (Lab-Line Instruments Model No. 4625 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software, or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Plate stand (Cat. No. MX-STAND, if using filter plate)
- Filter devices for clearing lysates
    - 2 mL or greater, Cat. No. SLHVX13NL
    - 0.5 – 2 mL, Cat. No. UFC40DV25
    - Less than 0.5 mL, Cat. No. UFC30DV25
    - For 96 well plates, Cat. No. MSBVN1210
- Use of a handheld Magnetic Separation Block (Cat. No. 40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (BioTek® ELx405, Cat. No.40-015 or equivalent), consult Supplemental Protocol C.
- If using the filter plate, a Vacuum Filtration Unit (Vacuum Manifold, Cat. No. MSVMHTS00 or equivalent with Vacuum Pump Cat. No. 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.

Symbol Definitions

Ingredient	Cat. No.	Full Label	
A549 Cell Lysate: Camptothecin treated	47-218		**Danger**. Harmful if swallowed Causes skin irritation. Causes serious eye damage. Very toxic to aquatic life. Toxic to aquatic life with long lasting effects. Avoid release to the environment. IF SWALLOWED: Call a POISON CENTER/ doctor if you feel unwell. Collect spillage. Dispose of contents/ container to an approved waste disposal plant.
Jurkat T Cell Lysate: Anisomycin	47-207		

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 the Luminex® 200™ instrument, adjust probe height according to the protocols recommended by Luminex® to the kit filter plate using 3 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit filter plate using 1 alignment disc. When reading the assay on the MAGPIX® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit filter plate using 2 alignment discs.
- For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final suspension should be in 150 µL and 75 µL should be aspirated.
- For the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.

- Vortex all reagents well before adding to plate.

Sample Collection and Storage

Considerations for Cell Stimulation.

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.

Preparation of cell lysates

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

Suggested cell lysis protocol for adherent cells

1. After treatments, wash cells with ice cold Buffered Saline (PBS or TBS) and drain.

2. Add ice-cold **1X** MILLIPLEX® Lysis Buffer with freshly added protease inhibitors to cells (0.6 mL per 150 mm dish, 0.3 mL per 100 mm dish, or 0.1 mL per well of 24-well plate).

3. Scrape adherent cells off the dish with a cell scraper. Transfer the cell suspension into a centrifuge tube and gently rock for 10-15 minutes at 4 °C.

4. Remove particulate matter by filtration.

   Suggested filters:

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

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 (Cat. No. 46-667MAG) or β-Tubulin (Cat. No. 46-713MAG) 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 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 (Cat. No. 46-667MAG) or β-Tubulin (Cat. No. 46-713MAG) MAPmate™, is used.

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

Adherent or non-adherent cells grown in sterile 96-well tissue culture grade plates can be treated, washed and lysed in the same plate, but need to be filtered in a separate 96-well filter plate.

1. For non-adherent cells, centrifuge tissue culture plate 2 minutes at 500 x g to pellet cells, if using adherent cells skip to next step.
2. Remove the media via aspiration and add 100 µL ice-cold PBS or TBS.
3. For non-adherent cells, repeat step 1.
4. Remove wash via aspiration.
5. Add 35 µL/well ice-cold 1X MILLIPLEX® Lysis Buffer containing freshly prepared protease inhibitors.
6. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4 °C.
7. Pipette samples up and down without making bubbles.
8. Transfer the lysate to a 96-well filter plate that has been pre-wetted with 5 µL Lysis Buffer/well.
9. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
10. Centrifuge the stacked plates for 5 minutes at 500 x g.
11. Store the filtered lysate at -70 °C until ready for use.
12. 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 (Cat. No. 46-667MAG) or β-Tubulin (Cat. No. 46-713MAG) MAPmate™, is used.

Preparation of Reagents for Immunoassay

Preparation of 7-plex DNA Damage/Genotoxicity 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 MILLIPLEX® Assay Buffer 1. Use one of the Mixing Bottles provided.
3. Vortex the **1X** capture beads for 15 seconds.
4. For use, transfer 1X beads with a pipette into a reservoir, do not pour from Mixing Bottle.
5. Please note that multiplexing phospho-specific and total or panTyr MAPmate™ pairs is not recommended due to cross-reactivity.

Preparation of Biotin-Labeled Detection Antibody and Streptavidin-PE

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

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

Multiplexing additional MILLIPLEX® Cell Signaling Magnetic MAPmates™ with the 7-plex DNA Damage/Genotoxicity Magnetic Bead Kit.

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

Please note that Total or Pan Tyr MAPmate™ pairs should not be multiplexed with the 7-plex DNA Damage 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 7-plex DNA Damage/Genotoxicity Magnetic beads to the mixing vial.

3. For each additional MAPmate™, add 0.150 mL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer 1. 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 7-plex DNA Damage/Genotoxicity 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 1, for a final volume of 3.0 mL.

Example 2: When using 5 additional MAPmates™, add 0.150 mL 7-plex DNA Damage/Genotoxicity Beads/ Detection Antibody and 0.150 mL of each additional MAPmate™ Beads/Detection Antibody to the mixing vial. Then add 2.1 mL Assay Buffer 1, for a final volume of 3.0 mL.

Preparation of lyophilized MILLIPLEX® Cell Lysates (Cat. No. 47-229, 47-207 and 47-218).

MILLIPLEX® HeLa Cell Lysate: Phosphatase (Cat. No. 47-229) is provided as a lyophilized stock of cell lysate prepared from unstimulated HeLa cells treated with lambda phosphatase and is used as an unstimulated control. Jurkat Cell Lysate: Anisomycin (Cat. No. 47-207) is provided as a lyophilized stock of cell lysate prepared from Jurkat cells treated with 25 µM anisomycin (4 hours); it is used as a stimulated control. A549 Cell Lysate: Camptothecin (Cat. No. 47-218) is provided as a lyophilized stock of cell lysate prepared from A549 cells stimulated with 5 µM camptothecin (overnight); it is also used as a stimulated control. 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.

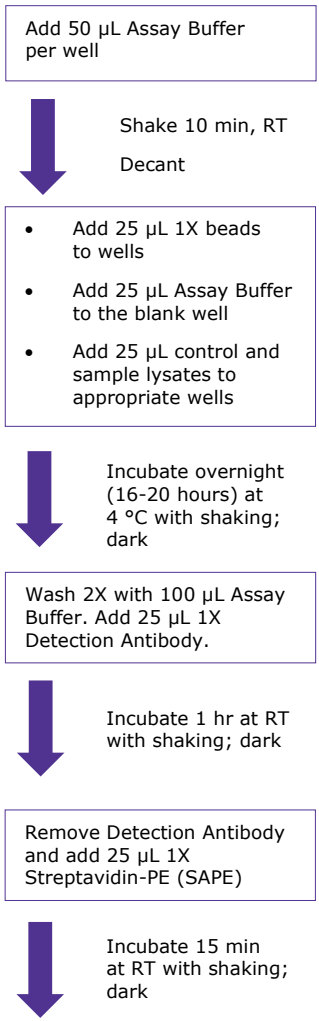
2. Gently vortex and incubate the reconstituted lysates for 5 min at RT (store on ice).

3. Pipette 150 µL of MILLIPLEX® Assay Buffer 1 to each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the MILLIPLEX® 7-plex DNA Damage/Genotoxicity Magnetic Bead Kit.

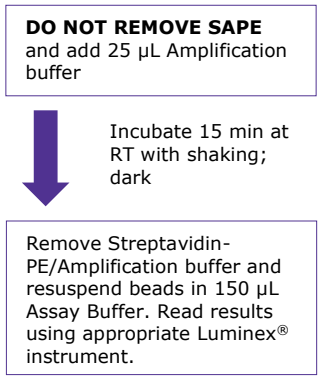
4. If desired, unused lysate may be transferred into polypropylene vials. For long-term storage, freeze reconstituted controls at ≤ -70 °C. Aliquot if needed. Avoid freeze/thaw cycles.

Immunoassay Protocol (96-well Solid 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** MILLIPLEX® Detection Antibody.
9. Seal, cover with lid and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25 °C).
10. Attach Magnetic Separation Block, wait for 60 seconds and decant Detection Antibody.
11. Add 25 µL of 1X MILLIPLEX® 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® Amplification Buffer to each well.
14. Seal, cover with lid and incubate with agitation on a plate shaker for 15 minutes at room temperature (20-25 °C).
15. Attach Magnetic Separation Block, wait for 60 seconds and decant SAPE/Amplification buffer.
16. Suspend beads in 150 µL of MILLIPLEX® 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.

Equipment Settings

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

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

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

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (Cat. No. LX2R-CAL-K25)	Performance Verification Kit (Cat. No. LX2R-PVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (Cat. No. F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (Cat. No. F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (Cat. No. IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (Cat. No. IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (Cat. No. MPX-CAL-K25)	MAGPIX® Performance Verification Kit (Cat. No. MPX-PVER-K25)

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

NOTE: When setting up for a magnetic bead assay using the xPONENT® software, you must select [MagPlex®] as the Bead Type in the Acquisition settings. The default setting is [MicroPlex] for polystyrene beads.

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

Events	50 per bead	
Sample Size	100 µL	
Gate Settings	8,000 to 15,000	
Reporter Gain	Default (Low PMT)	
Time Out	60 seconds	
Bead Region	ATR	21
	Chk1	28
	Chk2	35
	H2A.X	39
	p53	53
	MDM2	67
	p21	75

Supplemental Protocols

Analysis of viscous cell lysates

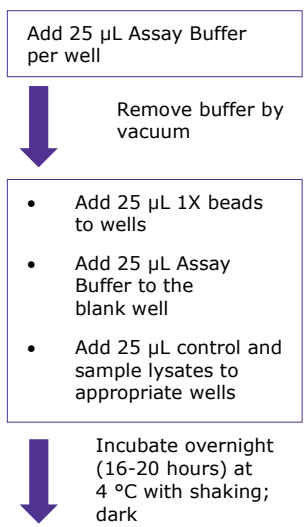
Some cell lysates may not flow through the filter plate efficiently due to high viscosity or the formation of particulate matter from long-term storage. For these samples, the initial capture and wash steps can be done in microcentrifuge tubes. The beads are then transferred into 96-well filter plates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

- Add 25 µL/assay point of 1X beads to a 500 µL centrifuge tube.
- Next, add lysate diluted in MILLIPLEX® 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® 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.

Filter Plate Immunoassay Protocol

NOTE: This protocol requires the use of the included 96-well Filter plate and a Vacuum Manifold (Vacuum Manifold, Cat. No. MSVMHTS00 or equivalent with Vacuum Pump, Cat. No. WP6111560).

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



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

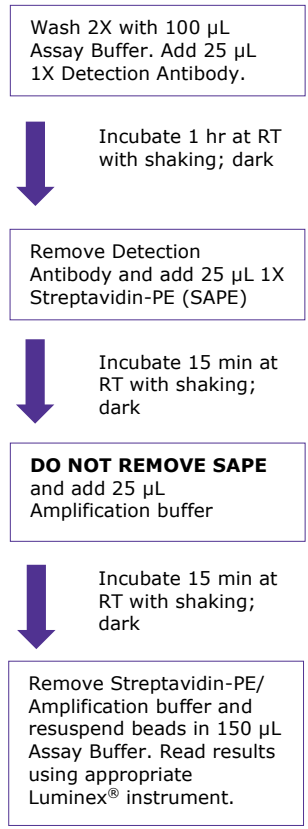


Plate Washer Use

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 Tech Service if additional instructions are required.

Troubleshooting

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 the Luminex® 200™ instrument, adjust probe height to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, 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 the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with Multichannel pipettes without touching reagent in plate.
	Insufficient washes	Increase number of washes.

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 of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	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® Instrument (for example, Bio-Plex®) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.

Problem	Probable Cause	Solution
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 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 pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

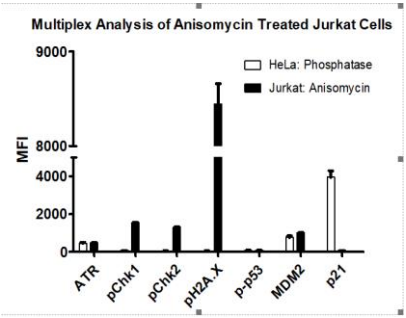
FOR FILTER PLATES ONLY

Problem	Probable Cause	Solution
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	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

Product Ordering

Replacement Reagents	Cat. No.
MILLIPLEX® 7-plex DNA Damage, Magnetic Beads (20X)	42-621MAG
MILLIPLEX® 7-plex DNA Damage, Biotin (20X) (Detection Antibody)	44-621KMG
MILLIPLEX® Lysis Buffer (1X)	43-040
MILLIPLEX® Assay Buffer 1 (1X)	43-010
MILLIPLEX® HeLa Cell Lysate: Lambda Phosphatase	47-229
MILLIPLEX® Jurkat Cell Lysate: Anisomycin	47-207
MILLIPLEX® A549 Cell Lysate: Camptothecin	47-218
MILLIPLEX® Streptavidin-Phycoerythrin (25X)	45-001H
MILLIPLEX® Amplification Buffer (1X)	43-024A
Set of two MILLIPLEX® 96-well Plates with sealers	MAG-PLATE
Set of two MILLIPLEX® 96-well Filter Plates with sealers	MX-PLATE

Representative Data



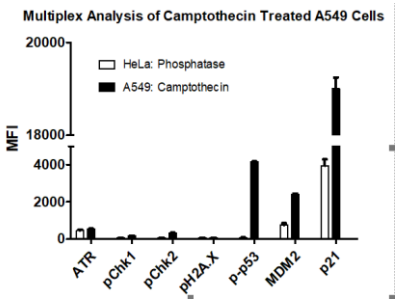


Figure 1. Multiplex analysis of Jurkat and A549 cells treated with anisomycin or camptothecin. HeLa cells treated with lambda phosphatase (negative control), Jurkat cells stimulated with 25 µM anisomycin (4 hours) and A549 cells stimulated with 5 µM camptothecin (overnight) were assayed. The cells were lysed in MILLIPLEX® Lysis Buffer containing protease inhibitors. 20 µg total protein of each lysate diluted in MILLIPLEX® Assay Buffer 1 were analyzed according 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 three replicate wells.

Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes in various cells. Cell lysates (100 µg, described in Figure 1) were mixed with capture antibodies to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled phospho-specific or total detection antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescent substrate. Non-treated (NT), Anisomycin-treated (A) or Camptothecin-treated (C) lysate controls.

Well Map

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Buffer 1 Blank	Sample 1										
B	Assay Buffer 1 Blank	Sample 1										
C	HeLa: Ppase Control	Sample 2										
D	HeLa: Ppase Control	Sample 2										
E	Jurkat: Anisomycin Control	Sample 3										
F	Jurkat: Anisomycin Control	Sample 3										
G	A549: Camptothecin Control	Etc.										
H	A549: Camptothecin Control	Etc.										

Notice

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