Phospho-IR (Tyr1162/Tyr1163) MAPmates™

Cat. # 46-688

MILLIPLEX® MAP
Phospho IR (Tyr1162/Tyr1163) MAPmates™
(100 Assay Points)

#46-688

INTRODUCTION

The MILLIPLEX MAP Phospho IR (Tyr1162/Tyr1163) MAPmates pair is used in conjunction with the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Catalog # 48-602) to detect the presence of phosphorylated IR (Tyr1162/Tyr1163) in cell lysates using the Luminex® 100™ IS, 200™, or HTS system. Each MAPmates pair is ordered individually and may be combined for simultaneous multiplex analysis of cellular events. The MILLIPLEX MAP Cell Signaling Buffer and Detection Kit is ordered separately and consists of a common set of reagents needed for performing MAPmates assays. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit contains sufficient reagents for 100 individual assays. The MILLIPLEX MAP HeLa Cell Lysate: Unstimulated included in the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit may be utilized as a negative control for this target, while the HepG2 Cell Lysate: Insulin Stimulated included with this MAPmates pair may be utilized as a positive control.

Important note: For a detailed protocol on Cell Signaling Detection Procedures please see the instruction booklet for the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (#48-602).

REAGENTS SUPPLIED	CATALOG NUMBER	LUMINEX BEAD #	VOLUME	QUANTITY
MILLIPLEX MAP Anti-IR Beads (20X)	42-688	65	131 µL	1 tube
MILLIPLEX MAP Anti-phospho IR (Tyr1162/Tyr1163), biotin (20X)	44-688	n/a	131 µL	1 tube
MILLIPLEX MAP HepG2 Cell Lysate: Insulin Stimulated	47-227	n/a	--------	1 vial

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- DO NOT FREEZE Streptavidin-Phycoerythrin.

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.

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation ("Luminex"), you, the customer, acquire the right under Luminex's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex's laser based fluorescent analytical test instrumentation marketed under the name of Luminex100, 200, HTS.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Catalog # 48-602)
- Cell lysates or cell extracts harboring protein(s) of interest prepared using the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Catalog # 48-602)
- Protease inhibitors (Millipore Catalog #20-201 or equivalent)
- Luminex Sheath Fluid (Luminex Catalog #40-50000)

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
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator
- Titer Plate Shaker
- Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
- Luminex 100™ IS, 200™, or HTS by Luminex Corporation
- Plate Stand (Millipore Catalog # MX-STAND)

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 and Proclin may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

TECHNICAL CONSIDERATIONS

- 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 20 µg/ well is generally a good starting point for lysates for which target protein expression levels are unknown.

- The following MAPmates should not be multiplexed:
  a. phospho-specific and total MAPmates pairs
  b. pTyr and site-specific phospho MAPmates (the pTyr detect may generate false positives on the site-specific MAPmates)
  c. Phospho MAPmates for a single target (Akt, STAT3, p53)

PREPARATION OF LYOPHILIZED MILLIPLEX MAP CELL LYSATE

MILLIPLEX MAP HepG2 Cell Lysate: Insulin stimulated as a positive control

- Reconstitute the lyophilized cell lysate in 100 µL of ultrapure water, this will yield 100 µL of lysate at 2 mg/mL total protein.
- Gently vortex and incubate the reconstituted lysate for 5 min at RT (store on ice).
- Pipette 150 µL MILLIPLEX MAP Assay Buffer 2 to the reconstituted cell lysate vial. The cell lysate is now prepared for use in the MILLIPLEX MAP Cell Signaling Assays.
- Combine prepared lysate (25 µL/well) with 25 µL Cell Signaling beads (step 4 of Immunoassay Protocol) and proceed with assay.

IMMUNOASSAY PROTOCOL

1. Dilute filtered lysates at least 1:1 in MILLIPLEX MAP Assay Buffer 2. 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 1,000µg/ml).
2. Pre-wet filter plate with 25 µL/well of MILLIPLEX MAP Assay Buffer 2. 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. Gently mix the **1X** bead suspension with a pipette and sonicate for 10 seconds. Add 25 µL of 1X bead suspension to each well.
4. Add 25 µL of diluted cell lysate to each well and incubate overnight at 4°C (or 2 hours RT) on a plate shaker (600-800rpm) protected from light.
5. Remove the lysate by vacuum filtration.
6. Add 100 µL/well of MILLIPLEX MAP Assay Buffer 2. Remove buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
7. Wash the beads a second time by repeating step 6.
8. Add 25 µL/well of **1X** MILLIPLEX MAP Biotinylated Reporter.
9. Incubate on a plate shaker for 1 hour at room temperature, protected from light.
10. Remove reporter by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
11. Add 25 µL of diluted (1:25) MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE).
12. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
13. **DO NOT REMOVE** SAPE. Add 25 µL of MILLIPLEX MAP Amplification Buffer to each well.

Add 25 µL Assay Buffer 2 per well

↓ Remove buffer by vacuum.

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

↓ Incubate overnight at 4°C or 2 hour at RT with shaking; dark

Wash 2X with 100 µL Assay Buffer 2. Add 25 µL 1X reporter biotin.

↓ Incubate 1 hr at RT with shaking; dark

Remove reporter and add 25 µL diluted SAPE.

↓ Incubate 15 min at RT with shaking; dark

DO NOT REMOVE SAPE and add 25 µL Amplification buffer.

14. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
15. Remove MILLIPLEX MAP SAPE/Amplification buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
16. Resuspend beads in 150 µL of MILLIPLEX MAP Assay Buffer 2.
17. Analyze using the Luminex® system.

Incubate 15 min at RT with shaking; dark

Remove Streptavidin-PE/ Amplification buffer and resuspend beads in 150 µL Assay Buffer 2. Read results using appropriate Luminex® instrument.

EQUIPMENT SETTINGS

These specifications are for the Luminex100 v.1.7 or Luminex100IS v2.1/2.2, Luminex200 v2.3, xPONENT, and Luminex HTS. 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.

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 Set	65

SINGLE AND MULTI-PLEX ANALYSIS

The recommended lysis and assay buffers for single or multi-plex analysis of Phospho IR (Tyr1162/Tyr1163) MAPmates are MILLIPLEX MAP Lysis Buffer (Catalog # 43-040) and MILLIPLEX MAP Assay Buffer 2 (Catalog # 43-041). Both buffers are included in the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit (Catalog # 48-602). For complete cell signaling assay and cell lysis protocols refer to the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit instructions.

MAPmates which are listed in the MILLIPLEX MAP Cell Signaling Buffer and Detection Kit buffer selection chart as “not recommended” for the above buffer conditions must be assayed separately using appropriate buffer conditions.

Note: Phospho and Total MAPmates should not be multiplexed together.

REPRESENTATIVE DATA:

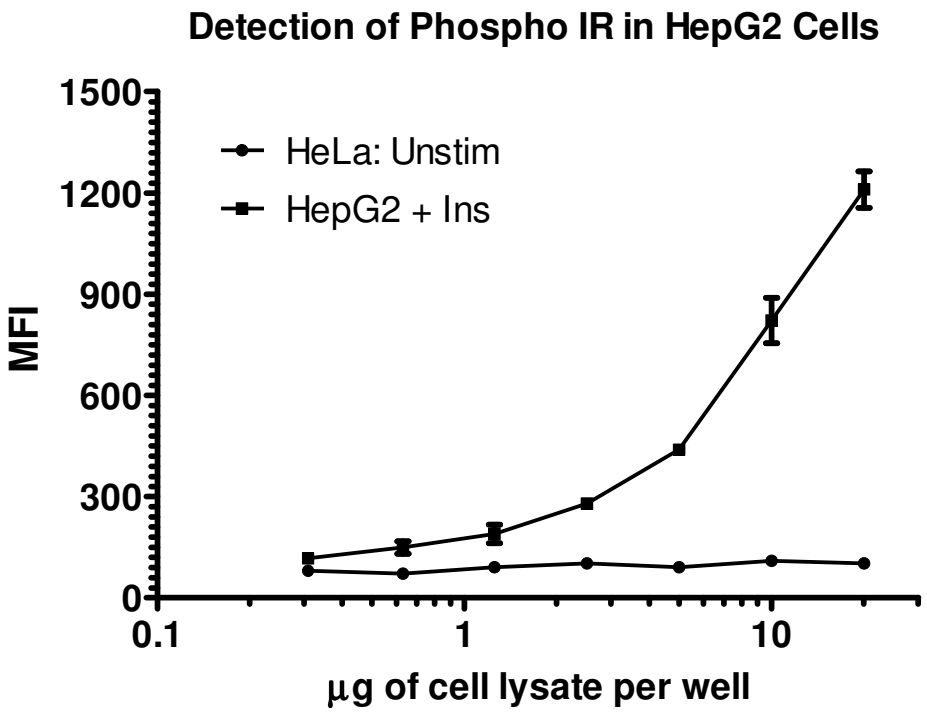

Figure 1. **MILLIPLEX MAP detection of phosphorylated IR in HepG2 cell lysate**. HepG2:Insulin stimulated (#47-227) and HeLa: Unstimulated (#47-205) were prepared according to instructions. Increasing amounts of cell lysate were incubated overnight at 4°C with anti-IR capture beads. The beads were washed and probed with biotin labeled anti-phospho IR, followed by incubation with streptavidin-PE and amplification buffer. The Median Fluorescent Intensity (MFI) in triplicate wells was measured using the Luminex® Instrument. This graph displays the MFI values obtained utilizing the Phospho IR MAPmates™.

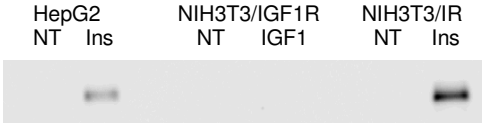

Figure 2. Immunoprecipitation/Western blot detection of phosphorylated IR in HepG2 and NIH3T3 overexpressing IGF1R or IR cell lysate. HepG2 cells cells were grown to confluence and stimulated with or without 1µM insulin for 5 minutes. NIH3T3 cells overexpressing IGF1R were grown to confluence and stimulated with or without 50ng/ml of IGF for 5 minutes. NIH3T3 cells overexpressing IR were grown to confluence and stimulated with or without 1uM of insulin for 5 minutes. 100 µg of HepG2, 20 µg of NIH3T3/IGF1R or NIH3T3/IR cell lysates (lysed in MILLIPLEX MAP Lysis Buffer with protease inhibitors) were mixed with capture antibody beads to immunoprecipitate phospho IR protein from non-treated (NT), IGF1-treated (IGF1) or insulin-treated (Ins) cell lysates. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled phospho IR (Tyr1162/Tyr1163) reporter antibody. The proteins were imaged using Streptavidin-HRP and chemiluminescence

ORDERING INFORMATION

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Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits

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