User Guide

MILLIPLEX® Total β-Tubulin Magnetic Bead MAPmates™

46-713MAG

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Introduction

The MILLIPLEX® Total β-Tubulin Magnetic Bead MAPmates™ pair is used in conjunction with the MILLIPLEX® Cell Signaling Buffer and Detection Kit (Cat. No. 48-602) to detect the presence of total β-Tubulin 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® 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® HeLa Cell Lysate: Unstimulated included in the MILLIPLEX® Cell Signaling Buffer and Detection Kit may be utilized as a unstimulated control for this target.

Important note: For a detailed protocol on Cell Signaling Detection Procedures please see the instruction booklet for the MILLIPLEX® Cell Signaling Buffer and Detection Kit (No. 48-602).

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.

Reagents Supplied

Reagents	Luminex® Bead No.	Volume	Quantity	Cat. No.
MILLIPLEX® Anti-β-Tubulin Magnetic Beads (20X)	26	180 µL	1 tube	42-713MAG
MILLIPLEX® Anti-total β-Tubulin, Biotin (20X)	n/a	180 µL	1 tube	44-713

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 standards and controls at ≤ -70 °C. Aliquot if needed. Avoid freeze/thaw cycles.
- **DO NOT FREEZE** Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.

Materials Required (not included)

Reagents

- MILLIPLEX® Cell Signaling Buffer and Detection Kit (Cat. No. 48-602)
- Protease inhibitors (Cat. No. 535140 or similar product)
- BCA-based total protein assay (Cat. No. 71285 or similar product)
- 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)

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
- A Hand-held Magnetic Separation Block (Cat. No. 40-285 or equivalent), or an Automatic Plate washer for magnetic beads (Bio-Tek® ELx405, Cat. No. 40-015 or equivalent) may be used. Contact Technical Service for use of an automatic plate washer.
- 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.

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.
- Do not mix magnetic and non-magnetic MAPmates™
- 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 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 solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, 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 the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, 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 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.
- Do not lysis cells at greater than 5 mg/mL total protein concentration. Sufficient volume of cell lysis buffer is required to solubilize some proteins while other proteins are not as affected. For example, β-Tubulin signal decreases with increasing total protein concentration (signal decrease occurs at approximately 5 mg/mL for Jurkat and PBMC cell lysates).
- 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:
    - phospho-specific and total MAPmates™ pairs (same target)
    - pTyr and site-specific phospho MAPmates™ (the pTyr detect may generate false positives on the site-specific MAPmates™)
    - Phospho MAPmates™ for a single target (Akt, STAT3, p53)

Preparation of lyophilized MILLIPLEX® Cell Lysate

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 2 to each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the MILLIPLEX® Magnetic Bead assay.
4. If desired, unused lysate may be transferred into polypropylene vials and stored at -80 °C for up to one month.

Single and Multi-Plex Analysis

The recommended lysis and assay buffers for single or multi-plex analysis of Total β-Tubulin Magnetic Bead MAPmates™ are MILLIPLEX® Lysis Buffer (Cat. No. 43-040) and MILLIPLEX® Assay Buffer 2 (Cat. No. 43-041). Both buffers are included in the MILLIPLEX® Cell Signaling Buffer and Detection Kit (Cat. No. 48-602). For complete cell signaling assay and cell lysis protocols refer to the MILLIPLEX Cell Signaling Buffer and Detection Kit instructions.

MAPmates™ which are listed in the MILLIPLEX® 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.

Immunoassay Protocol

(96-Well Plate and Handheld Magnetic Separation Block)

1. Dilute filtered lysates at least 1:1 in MILLIPLEX® 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 1,000 µg/mL).
2. Add 50 µL of Assay Buffer 2 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 2, reconstituted control cell lysates or prepared 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 2 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.

Add 50 µL Assay Buffer per well

Shake 10 min, RT

Decant

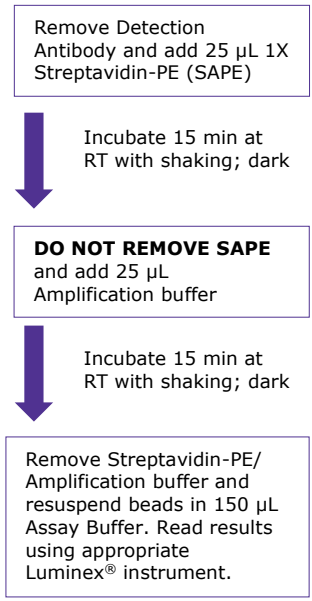
- Add 25 µL 1X beads to wells
- Add 25 µL Assay Buffer to the blank well
- Add 25 µL control and sample lysates to appropriate wells

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 hr at RT with shaking; dark

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 2 and mix on plate shaker for 5 minutes. Analyze using the Luminex® system.



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.

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.

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	26

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 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 1,000 µg/mL).
2. Pre-wet filter plate with 25 µL/well of MILLIPLEX® 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. 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 2, reconstituted control cell lysates or 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 2. 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).

Add 200 µL Assay Buffer per well

↓ Remove buffer by vacuum

- 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 hr at RT with shaking; dark

Remove Detection Antibody and add 25 µL 1X Streptavidin-PE (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 2 and mix on plate shaker for 5 minutes.
16. Analyze using the Luminex® system.

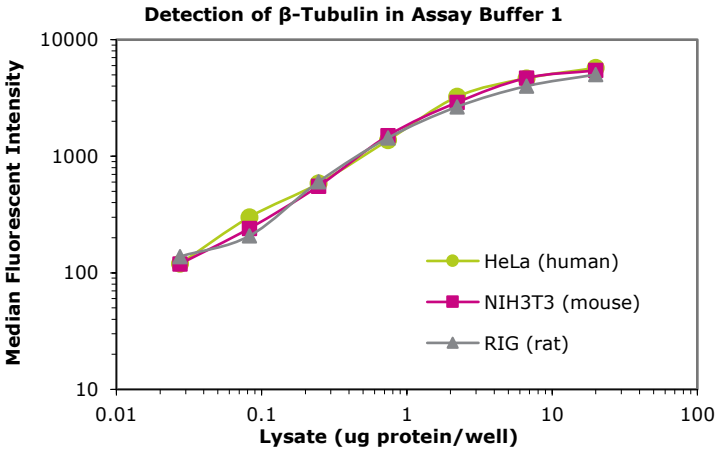
Incubate 15 min at RT with shaking; dark

DO NOT REMOVE SAPE and add 25 µL Amplification buffer

Incubate 15 min at RT with shaking; dark

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

Representative Data



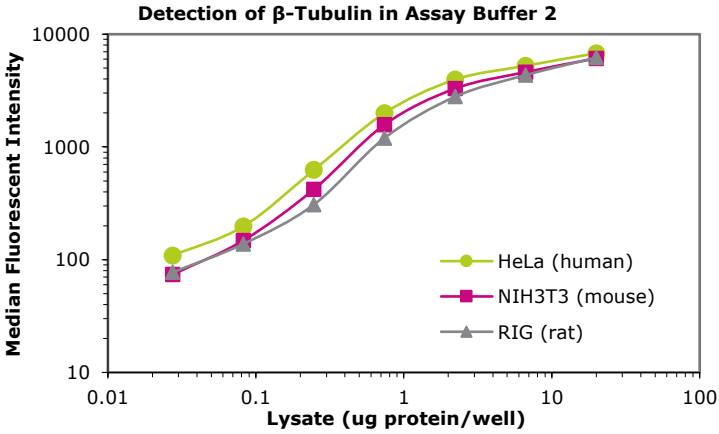
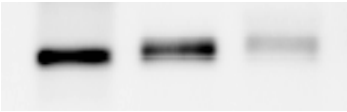


Figure 1. Comparison of β-Tubulin in Assay Buffer 1 vs 2 using Luminex® system. HeLa (human), NIH3T3 (mouse) or RIG (rat) cells were lysed in MILLIPLEX® Lysis Buffer containing protease inhibitors. 1:1 dilutions of HeLa, NIH3T3 or RIG cell lysates were diluted in either MILLIPLEX® Assay Buffer 1 or 2 and assayed 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 of triplicate wells.

β-Tubulin



HeLa NIH3T3 RIG

Figure 2. Immunoprecipitation/Western Blot analysis of β-Tubulin in human, mouse and rat cells. 10 µg HeLa, NIH3T3 or RIG cell lysates (lysed in MILLIPLEX® Lysis Buffer with protease inhibitors) were mixed with capture antibodies to immunoprecipitate β-Tubulin. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with biotin labeled β-Tubulin detection antibody. The proteins were imaged using Streptavidin-HRP and chemiluminescent substrate.

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.

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Contact Information

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

Technical Assistance

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

Standard Warranty

The applicable warranty for the products listed in this publication may be found at SigmaAldrich.com/terms.

Safety Data Sheets (SDS)

Safety Data Sheets are available on the product page at SigmaAldrich.com.

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