

**Human Stem Cell
Pluripotency Magnetic
Bead Kit 2**

96-well Plate

Cat. # 48-620MAG

MILLIPLEX[®] MAP

Human Stem Cell Pluripotency Magnetic Bead Kit 2 96-well Plate Assay

Cat # 48-620MAG

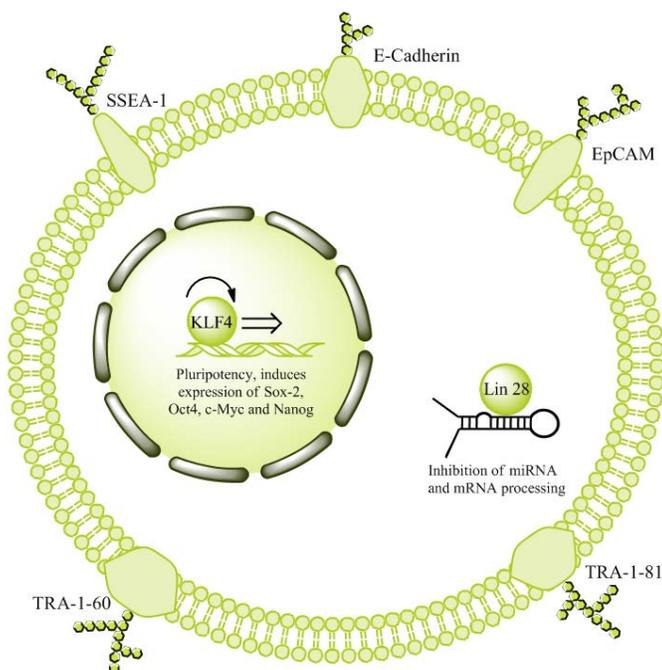
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By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation (“Luminex”), you, the customer, acquire the right under Luminex’s patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex’s laser based fluorescent analytical test instrumentation marketed under the name of Luminex 100[™] IS, Luminex 200[™], Luminex HTS, FLEXMAP 3D[®], MAGPIX[®].

INTRODUCTION

The expression of transcription factors, translational regulators and trans-membrane proteins, as well as cell surface glycostructures, play critical roles in the maintenance of pluripotency and self renewal in stem cells and can serve as markers of cell status. The zinc finger-containing transcription factor KLF4 has regulatory functions in cell growth, proliferation, differentiation, and embryogenesis. It is one of the factors for iPSC reprogramming used by Takahashi and Yamanaka in 2006. The translational enhancer LIN-28 contributes to stem cell pluripotency by possibly blocking miRNA-mediated differentiation. The trans-membrane proteins E-Cadherin and EpCam are adhesion molecules. E-Cadherin is a calcium-dependent trans-membrane protein, it influences pluripotency and self-renewal signaling pathways in ES and iPS cells. EpCam is a homophilic calcium-independent adhesion molecule that is expressed in undifferentiated hESCs being localized to Octamer 4 positive pluripotent cells, its expression is down-regulated upon differentiation. TRA-1-60 and TRA-1-81 are glycan modifications of the membrane protein Podocalyxin, these epitopes are expressed on ES cells. SSEA1 is the stage-specific embryonic antigen-1 (SSEA1), an antigenic epitope defined as a Lewis X carbohydrate structure. It is expressed in mouse ES cells but repressed in human ES cells therefore serving as a negative control in this kit.



The MILLIPLEX[®] MAP Human Stem Cell Pluripotency Magnetic Bead Kit 2 is a 7-plex used to detect total protein levels of E-Cadherin, EpCam, KLF4, LIN-28, and the glycostructures TRA-1-60, TRA-1-81 and SSEA1 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[®] MAP is based on the Luminex xMAP[®] technology — one of the most respected multiplex technologies available. This technology finds applications throughout the life sciences and enables a variety of bioassays, including immunoassays, on the surface of fluorescent-coded beads known as MagPlex[®]-C microspheres.

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are illuminated, and the internal dyes fluoresce, marking the microsphere set(s) used in a particular assay. A second illumination source excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Human Pluripotency Kit 2, Magnetic Beads (20X)	42-620MAG	180 μ L	1 tube
Human Pluripotency Kit 2, Biotin (20X) (Detection Antibody)	44-620KMG	180 μ L	1 tube
Lysis Buffer	43-040	55 mL	1 bottle
Assay Buffer 1	43-010	55 mL	1 bottle
Jurkat T Cell Lysate: Unstimulated	47-206	-----	1 vial
Ntera-2cl.D1 Lysate: Unstimulated	47-241	-----	1 vial
MCF-7 Lysate: Unstimulated	47-242	-----	1 vial
Streptavidin-Phycoerythrin	45-001H	150 μ L	1 tube
Amplification Buffer (1X)	43-024A	3 mL	1 bottle
Set of one 96-well Filter Plate and 2 sealers	-----	-----	1 plate, 2 sealers
Set of one 96-well Plate and 2 sealers	-----	-----	1 plate, 2 sealers
Empty mixing bottles	-----	-----	3 bottles

Analyte	Magnetic Bead Region
TRA-1-60	18
TRA-1-81	20
SSEA1	22
KLF4	36
E-Cadherin	62
EpCam	67
LIN-28	77

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Protease inhibitors (EMD Millipore Catalog #535140 or similar product)
- Coomassie or BCA-based total protein assay (EMD Millipore Catalog #71285 or similar product) or an assay normalization control, such as the GAPDH Magnetic Bead MAPmate™ (EMD Millipore Catalog #46-667MAG) or β -Tubulin (Catalog #46-713MAG) MAPmate™
- Luminex Sheath Fluid (Luminex Catalog #40-5000) or Luminex Drive Fluid (Luminex Catalog #MPXDF-4PK)
- 10X Assay Buffer 1 (EMD Millipore Catalog #MPEQ-AB) if using a magnetic plate washer (see supplemental protocol C)

Instrumentation / Materials

- Adjustable Pipettes with Tips capable of delivering 25 μ L to 1000 μ L
- Multichannel Pipettes capable of delivering 25 μ L to 200 μ L
- Reagent Reservoirs
- Polypropylene Microfuge Tubes
- Rubber Bands
- Aluminum Foil
- Absorbent Pads
- Laboratory Vortex Mixer
- Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
- Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
- Luminex 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex Corporation
- Plate Stand (EMD Millipore Catalog #MX-STAND, if using filter plate)
- Filter devices for clearing lysates
 - 2 mL or greater, EMD Millipore Catalog #SLHVX13NL
 - 0.5 – 2 mL, EMD Millipore Catalog #UFC40DV25
 - Less than 0.5 mL, EMD Millipore Catalog #UFC30DV25
 - For 96 well plates, EMD Millipore Catalog #MSBVN1210
- Use of a Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent) is recommended. If using an Automatic Plate washer for magnetic beads (BioTek® ELx405, EMD Millipore Catalog #40-015 or equivalent), consult Supplemental Protocol C.
- If using the filter plate, a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent). Consult Supplemental Protocols Section for Filter Plate protocol use.

SAFETY PRECAUTIONS

- All tissue components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide may react with lead and copper plumbing to form highly

explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state and local regulations.

TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Assay Buffer provided.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one week.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 1 alignment disc. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 2 alignment discs.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Considerations for Cell Stimulation.

1. Treating cells with growth factors (ex. EGF), cytokines (ex. $\text{TNF}\alpha$), 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 $\mu\text{g}/\text{mL}$). A total protein amount of 10 $\mu\text{g}/\text{well}$ is generally a good starting point for lysates for which target protein expression levels are unknown.

B. Preparation of cell lysates

Lysis Buffer is supplied as **1X** stock solution. The Lysis Buffer contains phosphatase inhibitors *including* 1 mM sodium orthovanadate (Na_3VO_4) but does **NOT** contain protease inhibitors. It is recommended that protease inhibitors (EMD Millipore Catalog #535140 or a similar product) be added immediately before use.

Suggested cell lysis protocol for adherent cells

1. After treatments, wash cells with ice cold Buffered Saline (PBS or TBS) and drain.
2. Add ice-cold **1X** Lysis Buffer with freshly added protease inhibitors to cells (0.6 mL per 150 mm dish, 0.3 mL per 100 mm dish, or 0.1 mL per well of 24-well plate).
3. Scrape adherent cells off the dish with a cell scraper. Transfer the cell suspension into a centrifuge tube and gently rock for 10-15 minutes at 4°C.
4. Remove particulate matter by filtration.
 - a. Suggested EMD Millipore filters:
 - (i) 2 mL or greater, EMD Millipore Catalog #SLPBDZ5NZ
 - (ii) 0.5 – 2 mL, EMD Millipore Catalog #UFC40DV25
 - (iii) Less than 0.5 mL, EMD Millipore Catalog #UFC30DV25
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 (Catalog #46-667MAG) or β -Tubulin (Catalog #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** Lysis Buffer containing freshly prepared protease inhibitors to cells (1 mL per 1×10^7 cells).

SAMPLE COLLECTION AND STORAGE (continued)

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.
7. 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 (Catalog #46-667MAG) or β -Tubulin (Catalog #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 μ L ice-cold PBS or TBS.
3. For non-adherent cells, repeat step 1.
4. Remove wash via aspiration.
5. Add 35 μ L/well ice-cold 1X Lysis Buffer (Catalog #43-040) containing freshly prepared protease inhibitors.
6. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C.
7. Transfer the lysate to a 96-well filter plate that has been pre-wetted with 5 μ L Lysis Buffer/ well.
8. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
9. Centrifuge the stacked plates for 5 minutes at 500 x g.
10. Store the filtered lysate at -70°C until ready for use.
11. 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 (Catalog #46-667MAG) or β -Tubulin (Catalog #46-713MAG) MAPmate™, is used.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Human Stem Cell Pluripotency Kit 2 magnetic beads

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 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 Magnetic Bead MAPmate™ pairs is not recommended due to cross-reactivity.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

B. 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 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 Assay Buffer 1. Use one of the Mixing Bottles provided.
3. Vortex the Streptavidin-Phycoerythrin 1:25 (SAPE) for 10 seconds.
4. Dilute SAPE by combining 0.120 mL of Streptavidin-Phycoerythrin with 2.88 mL of Cell Signaling 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.

C. Multiplexing additional MILLIPLEX® MAP Cell Signaling Magnetic MAPmates™ with the Human Stem Cell Pluripotency Magnetic Bead Kit 2

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

Please note that Phospho- Magnetic Bead MAPmates™ or PanTyr Magnetic Bead MAPmate™ pairs should not be multiplexed with the Human Stem Cell Pluripotency Magnetic Bead Kit 2.

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 Human Stem Cell Pluripotency Magnetic Bead Kit 2 Magnetic beads to the mixing vial
3. For each additional Magnetic Bead 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 Magnetic Bead MAPmates™, add 0.150 mL Human Stem Cell Pluripotency Magnetic Bead Kit 2 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 Magnetic Bead MAPmates™, add 0.150 mL Human Stem Cell Pluripotency Magnetic Bead Kit 2 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 REAGENTS FOR IMMUNOASSAY (continued)

D. Preparation of Lyophilized Cell Lysates (Catalog #47-206, 47-241, 47-242)

Jurkat T Cell Lysate: Unstimulated (#47-206) is provided as a lyophilized stock of cell lysate prepared from unstimulated Jurkat T cells and is used as a negative control for all analytes E-Cadherin, EpCam, KLF4, LIN-28, SSEA1, TRA-1-60, and TRA-1-81.

Ntera-2cl.D1 Lysate: Unstimulated (#47-241) is provided as a lyophilized stock of cell lysate prepared from unstimulated Ntera-2cl.D1 cells. It is used as a positive control for the analytes LIN-28, TRA-1-60 and TRA-1-81.

MCF-7 Lysate: Unstimulated (#47-242) is provided as a lyophilized stock of cell lysate prepared from unstimulated MCF-7 cells. It is used as a positive control for the analytes E-Cadherin, EpCam, KLF4 and SSEA1.

Each of the cell lysates were prepared in 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.

Cell Lysates as an unstimulated and stimulated control

1. Reconstitute each of the lyophilized cell lysates in 100 μ L of ultrapure water, for each vial this will yield 100 μ L 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 μ L of Assay Buffer 1 to each cell lysate vial and vortex mix. The cell lysate is now prepared for use in the Human Stem Cell Pluripotency Magnetic Bead Kit 2.
4. If desired, unused lysate may be stored in its original container at -80°C for up to one month. For long-term storage, freeze reconstituted standards and controls at $\leq -70^{\circ}\text{C}$. Aliquot if needed. Avoid freeze/thaw cycles.

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 $\mu\text{g}/\text{mL}$).
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** 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 / well of 1X 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 / well of Amplification Buffer.
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 Assay Buffer, and mix on plate shaker for 5 minutes, Analyze using the Luminex system.

Add 50 μL Assay Buffer per well



Shake 10 min, RT
Decant buffer

- 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)



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

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.

INSTRUMENT SETTINGS

Luminex 200™, HTS, FLEXMAP 3D® and MAGPIX® with xPONENT® software:

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D® and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex 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, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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 EMD Millipore Catalog #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:	TRA-1-60	18
	TRA-1-81	20
	SSEA1	22
	KLF4	36
	E-Cadherin	62
	EpCam	67
	LIN-28	77

SUPPLEMENTAL PROTOCOLS

A. Analysis of viscous cell lysates

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

- Add 25 μL /assay point of 1X beads to a 500 μL centrifuge tube.
- Next, add lysate diluted in 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 Assay Buffer 1.
- Transfer 25 μL of the bead mixture to pre-wet filter plate wells and proceed to step 4 of the Immunoassay protocol.

B. Filter Plate Immunoassay Protocol

NOTE: This protocol requires the use of the included 96-well Filter plate and a Vacuum Manifold (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560).

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 $\mu\text{g}/\text{mL}$).
2. Pre-wet filter plate with 25 μL /well of 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.

Add 25 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

6. Add 100 μL /well of Assay Buffer. 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** 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. Remove Detection Antibody by vacuum and gently blot the bottom of the filter plate on a paper towel.
11. Add 25 μL of 1X Streptavidin-Phycoerythrin (SAPE).
12. Seal, cover with lid and incubate with agitation on a plate shaker for 15 min at room temperature (20-25°C).
13. **DO NOT REMOVE SAPE.** Add 25 μL / well of Amplification Buffer.
14. Seal, cover with lid and incubate with agitation on a plate shaker for 15 minutes at room temperature (20-25°C).
15. Remove 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 Assay Buffer, and mix on plate shaker for 5 minutes.
17. Analyze using the Luminex system.

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)



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

C. 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 EMD Millipore Technical Service if additional instructions are required.

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient Bead Count	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with alcohol flush, back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 3 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipetting with multichannel pipettes without touching reagent in plate.
	Insufficient washes	Increase number of washes.
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 (e.g. 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	<p>Incorrect or no Detection Antibody was added</p> <p>Streptavidin-Phycoerythrin was not added</p>	<p>Add appropriate Detection Antibody and continue.</p> <p>Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.</p>
Signals too high	<p>Calibration target value set too high</p> <p>Plate incubation was too long with samples</p>	<p>With some Luminex instruments (e.g. Bio-Plex[®]) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.</p> <p>Use shorter incubation time.</p>
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.
High Variation in samples	<p>Multichannel pipette may not be calibrated</p> <p>Plate washing was not uniform</p> <p>Samples may have high particulate matter or other interfering substances</p> <p>Plate agitation was insufficient</p> <p>Cross-well contamination</p>	<p>Calibrate pipettes.</p> <p>Confirm all reagents are removed completely in all wash steps. See above.</p> <p>Plate should be agitated during all incubation steps using a orbital plate shaker at a speed where beads are in constant motion without causing splashing.</p> <p>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.</p>
FOR FILTER PLATES ONLY		
Filter plate will not vacuum	<p>Vacuum pressure is insufficient</p> <p>Samples have insoluble particles</p> <p>High lipid concentration</p>	<p>Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.</p> <p>Centrifuge samples just prior to assay setup and use supernatant.</p> <p>After centrifugation, remove lipid layer and use supernatant.</p>
Plate leaked	<p>Vacuum pressure too high</p> <p>Plate set directly on table or absorbent towels during incubations or reagent additions</p> <p>Insufficient blotting of filter plate bottom causing wicking</p>	<p>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.</p> <p>Set plate on plate holder or raised edge so bottom of filter is not touching any surface.</p> <p>Blot the bottom of the filter plate well with absorbent towels after each wash step.</p>

	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly Sample too viscous	Adjust probe to 3 alignment discs in well H6. May need to dilute sample.

REPLACEMENT REAGENTS

Human Pluripotency Kit 2, Magnetic Beads (20X)	42-620MAG
Human Pluripotency Kit 2, Biotin (20X) (Detection Antibody)	44-620KMG
Lysis Buffer	43-040
Assay Buffer 1	43-010
Jurkat T Cell Lysate: Unstimulated	47-206
Ntera-2cl.D1 Lysate: Unstimulated	47-241
MCF-7 Lysate: Unstimulated	47-242
Streptavidin-Phycoerythrin	45-001H
Amplification Buffer (1X)	43-024A
Set of two 96-well Plates with sealers	MAG-PLATE
Set of two 96-well Filter Plates with sealers	MX-PLATE

REPRESENTATIVE DATA

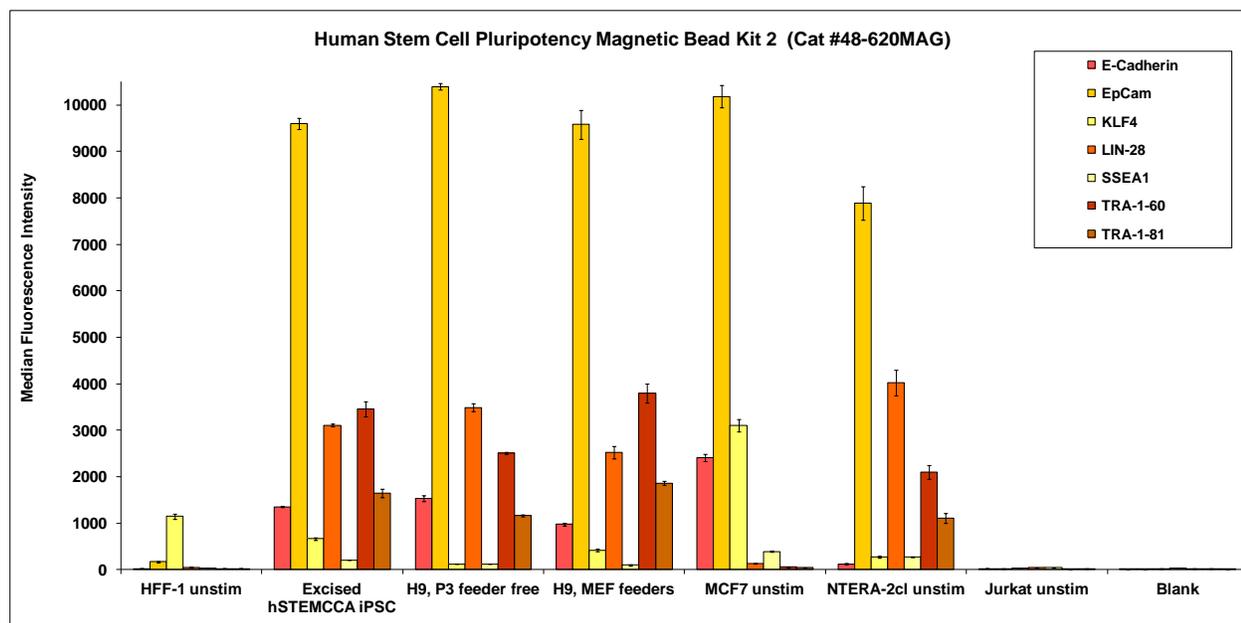


Figure 1. Analysis of HFF, iPS, hES cells and control cell lines MCF-7, Ntera-2cl.D1 and Jurkat.

Differentiated Human Foreskin Fibroblasts (EMD Millipore, Catalog #SCC058) were used for reprogramming using the STEMCCA Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit (EMD Millipore Catalog #SCR511) yielding virus excised hSTEMCCA iPSC. H9 P46 hES cells (WiCell Catalog #WA09) were cultured both in feeder free medium or on a monolayer of mitotically inactivated primary mouse embryonic fibroblast feeder cells. All cells were lysed according to the protocol and assayed with the Human Stem Cell Pluripotency Magnetic Bead Kit 2. Kit control lysates (unstimulated MCF-7, Ntera-2cl.D1 and Jurkat cells) were assayed and results are shown. Lysates were diluted in assay buffer and analyzed according to the protocol. Median Fluorescence Intensity was measured with the Luminex system. The figure represents the average and standard deviation of three replicate wells. The MCF-7 cell line serves as a positive control for E-Cadherin, EpCam, KLF4 and SSEA1, while the Ntera-2cl cell line serves as a positive control for LIN-28, TRA-1-60 and TRA-1-81. Jurkat is used as a negative control for all analytes.

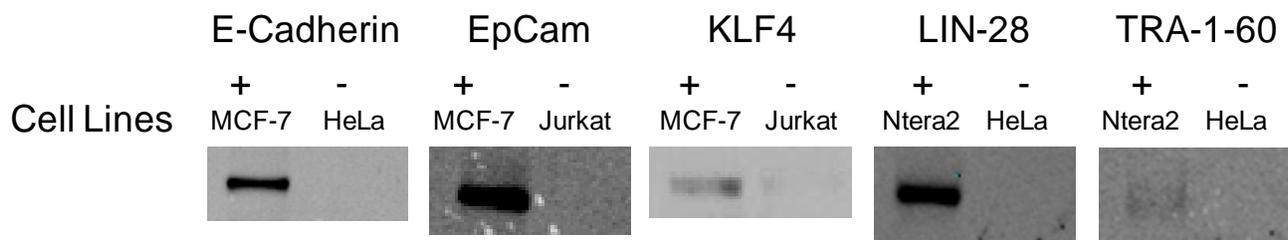


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes in various cells. 100 µg of lysates (Unstimulated Jurkat, Ntera-2cl.D1, MCF-7 or HeLa) were mixed with kit capture beads to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with kit biotin labeled detection antibodies. The proteins were imaged using fluorescently labeled secondary antibodies. NOTE: HeLa and Jurkat expression levels for E-Cadherin, LIN-28, and TRA-1-60 are comparable. Suitable Western Blotting antibodies for TRA-1-81 and SSEA1 could not be obtained.

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

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

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Assay Buffer 1 Blank	Sample 1	Etc.									
B	Assay Buffer 1 Blank	Sample 1	Etc.									
C	Jurkat T: unstim negative control	Sample 2										
D	Jurkat T: unstim negative control	Sample 2										
E	Ntera- 2cl.D1: unstim positive control	Sample 3										
F	Ntera- 2cl.D1: unstim positive control	Sample 3										
G	MCF-7: unstim positive control	Sample 4										
H	MCF-7: unstim positive control	Sample 4										