7-Plex T Cell Receptor Signaling Kit – Phosphoprotein

Cat. # 48-690

MILLIPLEX® MAP

7-Plex T Cell Receptor Signaling Kit – Phosphoprotein
(CD3ε, Lck, ZAP-70, LAT, Erk/MAP Kinase 1/2, CREB, and Syk)

#48-690

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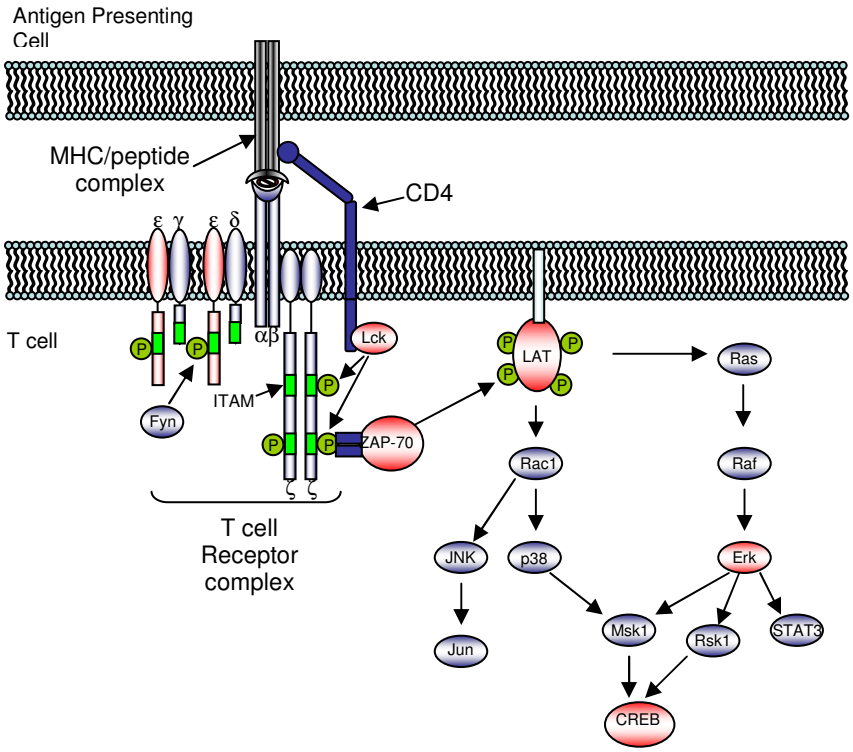
FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

CD4 expressing T cells recognize antigenic peptides presented in the context of MHC class II on the surface of antigen presenting cells via the T cell receptor complex. The T cell receptor complex consists of an α/β heterodimer and the multimeric CD3 subunits, γ, δ, ε, and ζ chains. The binding and recognition of the peptide/MHC class II complexes initiates signaling cascades in the T cell. Early signaling event includes the tyrosine phosphorylation and activation of cytosolic tyrosine kinases such as Fyn and Lck. These phosphorylate tyrosine residues residing in ITAM (Immunoreceptor-Tyrosine based Activation Motifs) regions on CD3 subunits. Phosphorylated ITAMs create binding sites
for the protein tyrosine kinase, ZAP-70, leading to its activation. ZAP-70 phosphorylates the transmembrane adaptor protein, Linker for Activation of T cells (or LAT), which recruits a broad range of signaling molecules in the T cell. The result of these signaling cascades in conjunction with co-stimulatory signals from the antigen presenting cell lead to T cell proliferation and cytokine production..



The MILLIPLEX™ MAP 7-plex T Cell Receptor Signaling kit - phosphoprotein is used to detect changes in phosphorylated CREB (Ser133), Erk/MAP kinase 1/2 (Thr185/Tyr187), and phosphorylated tyrosine residues on CD3 epsilon chain, Lck, ZAP-70, LAT, and Syk in T cell lysates using the Luminex® System. Although the protein kinase Syk is *not* part of the T Cell Receptor pathway, it does play a significant role in B Cell Receptor signaling and acts as a negative control for the analysis of T Cell Receptor signaling. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for 100 individual assays.

PRINCIPLE

MILLIPLEX MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as 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 allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser 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.

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.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- Once the controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED CONTROLS IN GLASS VIALS**. For long-term storage, freeze reconstituted controls at ≤ -80°C.
  Avoid multiple (>2) freeze thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
MILLIPLEX™ MAP 7-plex TCR Signaling Pathway-Phosphoprotein, beads (20x)	42-690	131 µL	1 tube
MILLIPLEX™ MAP 7-plex TCR Signaling Pathway-Phosphoprotein, biotin (20x)	44-690	131 µL	1 tube
MILLIPLEX™ MAP Lysis Buffer	43-040	55 mL	1 bottle
MILLIPLEX™ MAP Assay Buffer 2	43-041	55 mL	1 bottle
MILLIPLEX™ MAP Jurkat Cell lysate: unstimulated	47-206	----------	1 vial
MILLIPLEX™ MAP Jurkat Cell lysate: C305	47-201	----------	1 vial
MILLIPLEX™ MAP Streptavidin-Phycoerythrin	45-001D	115 µL	1 tube
MILLIPLEX™ MAP Amplification buffer	43-024A	3 mL	1 bottle
MILLIPLEX™ MAP 96-well Filter Plate	MX-PLATE	----------	1 plate
Empty mixing vials	----------	----------	3 vials

Target Protein	Bead Region
Erk MAP Kinase 1/2	9
LAT	18
Lck	20
CREB	37
ZAP-70	51
CD3ε	53
Syk**	57

**Syk is not part of the T Cell Receptor Signaling pathway but is included in this kit as a negative control for T Cell Receptor Stimulation.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Cell lysates or cell extracts harboring protein(s) of interest
- Protease inhibitors (Millipore Catalog # 20-201 or similar product)
- 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

MATERIALS REQUIRED BUT NOT PROVIDED (continued)

- 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)
- Filter devices for clearing lysates
  - 2 mL or greater, Millipore Catalog # SLHVX13NL
  - 0.5 – 2 mL, Millipore Catalog # UFC40DV25
  - Less than 0.5 mL, Millipore Catalog # UFC30DV25
  - For 96 well plates, Millipore Catalog # MSBVN1210

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 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 an 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.

- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a plate stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the appropriate Assay Buffer provided.
- After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µL of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one month.
- 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.
- 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. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND PREPARATION OF REAGENTS

A. Preparation of cell lysates

MILLIPLEX™ MAP 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 (Millipore catalog #20-201 or a similar product) be added immediately before use.

Suggested cell lysis protocol for cells

- Pellet the cells by centrifugation (500 – 1000 x g) in a tabletop centrifuge for 5 minutes.
- Wash the cells in ice cold TBS.
- Add ice cold **1X** MILLIPLEX™ MAP Lysis Buffer containing freshly prepared protease inhibitors to cells (1 mL per 1 x 10^7 cells).
- Gently rock the lysate for 10-15 minutes at 4ºC.

- Remove particulate matter by filtration.
- Aliquot and store the lysate at –70°C. The lysate should be stable for several months.
- It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

Cell lysis protocol for cells in **96-well filter plates**

Adherent or non-adherent cells seeded or grown in sterile 96-well filter plates (See supplemental protocols) can be washed, treated, lysed and filtered in the same plate.

- Wash the cells by placing the 96 well filter plate containing cells over a vacuum manifold to remove liquid.
- Add 100 µL of ice cold TBS then remove via vacuum.
- To lyse the cells, add 200 µl/well of ice cold **1X** MILLIPLEX™ MAP Lysis Buffer containing freshly prepared protease inhibitors.
- Place the plate on an orbital shaker (600 – 800rpm) for 10-15 minutes at 4ºC.
- Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
- Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
- Store the filtered lysate at –70°C until ready for use.
- It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

Cell lysis protocol for cells in sterile **96-well tissue culture plates**

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

- Wash the cells by centrifugation in a microplate carrier 2 min at 500 x g.
- Remove the supernatant via aspiration and add 100µl of ice cold TBS.
- Centrifuge and remove supernatant via aspiration.
- Add 200 µL/well of ice cold 1X MILLIPLEX™ MAP Lysis Buffer containing freshly prepared protease inhibitors.
- Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4ºC.
- Transfer the lysate to a 96-well filter plate that has been pre-wetted with 1X lysis buffer.
- Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
- Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
- Store the filtered lysate at –70°C until ready for use.
- It is recommended that the lysate be diluted at least 1:10 in PBS for determining the protein concentration with Coomassie-based assays.

B. Preparation of antibody Multi-pathway phosphoprotein beads

MILLIPLEX MAP capture beads are provided as a **20X** stock solution and should be protected from light.

- Gently resuspend the **20X** stock capture beads with a pipette, then sonicate for 15 seconds.
- Dilute the beads to **1X** by adding 0.125 mL of beads with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty mixing vial provided.
- Gently mix the **1X** stock capture beads with a pipette and sonicate for 15 seconds.

C. Preparation of biotin-labeled reporters and Streptavidin-PE

MILLIPLEX MAP reporter antibodies are provided as a **20X** stock solution.

- Gently vortex and centrifuge the **20X** reporter stock for 1 minute at 1000 x g.
- Dilute the reporter antibody to **1X** by adding 0.125 mL of reporter antibody with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty bead-mixing vial provided.
- Dilute MILLIPLEX MAP Streptavidin-Phycoerythrin 1:25 by adding 0.1ml of Streptavidin-Phycoerythrin with 2.4 mL of MILLIPLEX™ MAP Assay Buffer 2. Use the empty mixing vial provided.
- The MILLIPLEX MAP Cell Signal Amplification Buffer is provided as a **1X** stock, and does not need to be diluted.

D. Multiplexing additional MILLIPLEX MAP Cell Signaling MAPmates with the 7-plex Multi-Pathway Signaling Kit, phosphoprotein.

Additional Cell Signaling Phospho-MAPmates can be combined with this kit. *Please note that Total Beadmate™ pairs should not be multiplexed with the 7-plex Multi-Pathway Signaling Kit, phosphoprotein.*

- Gently resuspend the **20X** stock capture beads for each additional Beadmate with a pipettor, then sonicate for 15 seconds.
- Refer to the Bead/Reporter Dilution Table below for details on adding the **20X** stock beads to the 7-plex TCR Signaling beads.
- Gently vortex and centrifuge the **20X** reporter stock for each additional Beadmate for 1 minute at 1000 x g.
- Refer to the Bead/Reporter Dilution Table below for details on adding **20X** reporter stock to the 7-plex TCR Signaling biotin.

Bead/Reporter Dilution Table for Multiplexing Additional MAPmates™

Number of Additional MAPmates™ added		Amount of additional **20X** Beads or **20X** Reporter (mL)		Amount of 7-plex TCR Pathway **20X** Beads or **20X** Reporter (mL)		Total Bead or Reporter (mL)		Assay Buffer 2 (mL)		Total **1X** volume (mL)
0	X	0	+	0.125	=	0.125	+	2.375	=	2.5
1	X	0.125	+	0.125	=	0.250	+	2.250	=	2.5
2	X	0.125	+	0.125	=	0.375	+	2.125	=	2.5
3	X	0.125	+	0.125	=	0.500	+	2.000	=	2.5
4	X	0.125	+	0.125	=	0.625	+	1.875	=	2.5
5	X	0.125	+	0.125	=	0.750	+	1.750	=	2.5
6	X	0.125	+	0.125	=	0.875	+	1.625	=	2.5
7	X	0.125	+	0.125	=	1.000	+	1.500	=	2.5
8	X	0.125	+	0.125	=	1.125	+	1.375	=	2.5

E. Preparation of lyophilized MILLIPLEX™ MAP Cell Lysates (Catalog # 47-206, 47-201).

MILLIPLEX™ MAP Jurkat Cell Lysate: unstimulated (#47-206) is provided as a lyophilized stock of cell lysate prepared from unstimulated Jurkat cells and is used as a negative control. MILLIPLEX™ MAP Jurkat Cell Lysate: C305 (#47-201) is provided as a lyophilized stock of cell lysate prepared from Jurkat cells treated with C305 (mouse anti-TCR β chain). Each of the cell lysates were prepared in MILLIPLEX™ MAP Lysis Buffer containing protease inhibitors and lyophilized for stability. The lysates can be used as positive and negative control samples or alternatively, to create calibration curves for relative quantification of different phosphoprotein analytes.

MILLIPLEX™ MAP Cell Lysates as a positive and negative control

- Reconstitute each of the lyophilized cell lysates in 100 µL of ultrapure water, for each vial this will yield 100 µL of lysate at 1 mg/mL total protein.
- Gently vortex and incubate the reconstituted lysate for 5 min at RT (store on ice).
- Pipette 150 µL of MILLIPLEX™ MAP Assay Buffer 2 to each cell lysate vial. The cell lysate is now prepared for use in the MILLIPLEX™ MAP 7-plex TCR Signaling Assay.
- Combine prepared lysates (25 µL/well) with 25 µL of 7-plex TCR Signaling beads (steps 3 and 4 of Cell Signaling Assay protocol) and proceed with assay.

F. Considerations for Cell Stimulation.

- 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.
- Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
- Cell lines will differ in the robustness of their signaling response for any given stimulation.

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 1,000 µg/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 (or reconstituted HeLa or A431 Cell Lysate Control) 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 Cell Signal Amplification Buffer to each well.

Add 25 µL Assay Buffer 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. Add 25 µL 1X reporter biotin.

↓ Incubate 1 hr at RT with shaking; dark

Remove reporter and add 25 µL diluted Streptavidin-PE (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. 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		
	Erk MAP Kinase 1/2	9
	LAT	18
	Lck	20
	CREB	37
	ZAP-70	51
	CD3ε	53
	Syk	57

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 MILLIPLEX™ MAP Assay Buffer 2 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 4°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™ MAP Assay Buffer 2.
- Transfer 25 µL of the bead mixture to pre-wet filter plate wells and proceed to step 4 of the Immunoassay protocol.

B. Growing or seeding cells in 96-well filter plates

Adherent or Non-adherent cells can be grown in sterile 96 well filter plates. Placing the cells over a gentle vacuum can simplify washing of cells, adding and removing cell treatments, and filtering lysed cells.

Growing cells overnight in 96 well filter plates

- Wash cells with sterile TBS or culture medium.
- Gently resuspend the cell pellet in culture medium to give 1 x 10^5 cells per mL for adherent cells (such as HeLa or A431) or 2 x 10^5 cells per mL for non-adherent cells (e.g. Jurkat T cells).
- Pre-wet the filter plate by adding 50 µL of sterile TBS or culture medium per well of 96 well filter plate.
- Remove liquid by placing the plate on a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.
- Add 100 µL of cell suspension to each well.
- Grow/treat cells under desired conditions.
- Proceed to "Cell lysis protocol for cells in **96-well filter plates"**.

Seeding cells (short durations) in 96 well filter plates

- Wash cells with sterile TBS or culture medium.
- Gently resuspend the cell pellet in TBS or culture medium to give 1 x 10^6 cells per mL for non-adherent cells or adherent cells.
- Pre-wet the filter plate by adding 50 µL of sterile TBS or culture medium per well of 96 well filter plate.
- Remove buffer by placing the plate on a vacuum manifold and gently applying vacuum. Gently blot the bottom of the filter plate on a paper towel to remove excess liquid.

- Add 100 µL of cell suspension to each well.
- Treat cells under desired conditions.
- Proceed to "Cell lysis protocol for cells in **96-well filter plates"**.

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 10 µg/ well is generally a good starting point for lysates for which target protein expression levels are unknown.
- Please note that multiplexing phospho-specific and total Beadmate™ pairs is not recommended due to reporter cross reactivity.
- To view the dot plot, contact Luminex® Corporation to inquire how to enable the software to view all selected bead events in the dot plot (white oval target). Note that some beads may miss the white oval target; this is a result of variations in the calibration process. This will not affect results as only beads hitting the white target will be counted and read.

TROUBLESHOOTING GUIDE

Situation	Possible Problem	Solution
Data acquisition time exceeds 30 seconds per well and/or "Sample Empty" occurs	**Mechanical** 1. Needle height is not correct (common problem due to variation in well depth between plate types). 2. Sample needle is clogged. 3. Air in the system. 4. Low pressure in the system. **Sample Related** 1. Lysate concentration too high. 2. Particulate matter in lysate. 3. No buffer in well.	**Mechanical** 1. Adjust needle height using 2 disks using "options; XY setup function". 2. Remove needle (see user's manual) and sonicate it to remove obstruction. 3. Perform alcohol flush and then wash with alcohol; followed by a wash with sheath fluid and a prime. 4. Tighten tops on the ***sheath fluid*** container. 5. Loosen the top on the ***waste*** container. **Sample Related** 1. Refer to lysate preparation protocol. 2. Centrifuge or filter lysate to remove particulate matter.
Readings are lower than expected	1. Gate is not set properly. 2. Calibration is not correct. 3. Cells responded poorly to stimulation.	1. Right click within the "doublet discriminator" and create gate. Move lines to 8,000 and 13,500 2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Check stimulation conditions.
Bead pattern is diffuse and missing the bead target (white oval)	1. Precipitate buildup in system.	1. Drain the system, followed by a backflush, proceed with solution for air in the system.

Bead pattern is diffuse and missing the bead target (white oval)	2. Calibration is not correct. 3. Incompatible buffer used to resuspend beads for Luminex® analysis.	2. Run a machine calibration with calibration beads available from Luminex® Corp. 3. Vacuum plate and resuspend beads in MILLIPLEX™ MAP Assay Buffer 2.
Applying vacuum to filter plate does not remove liquid from wells	1. Wells not in use are empty. 2. Particulate matter in lysate.	1. Place tape over the top of empty wells that are not in use. 2. Pre-filter lysate before use.
Aggregation of beads during analysis	1. Particulate matter in lysate. 2. Incompatible lysis buffer. 3. Beads not sonicated for step 3 of main protocol.	1. Pre-filter the lysate. 2. Use recommended lysis buffer. 3. Sonicate ***resuspended*** 1X beads prior to adding to filter plate.
Liquid wicking out from 96-well filter plate	1. Plate was not blotted on paper towel prior to adding next reagent. 2. The bottom of the plate is in contact with absorbent material such as paper towel or bench paper.	1. After vacuum step, gently blot the bottom of the plate using a paper towel to remove excess liquid. 2. Place the plate on a flat, non-absorbent surface during loading steps.

REPRESENTATIVE DATA

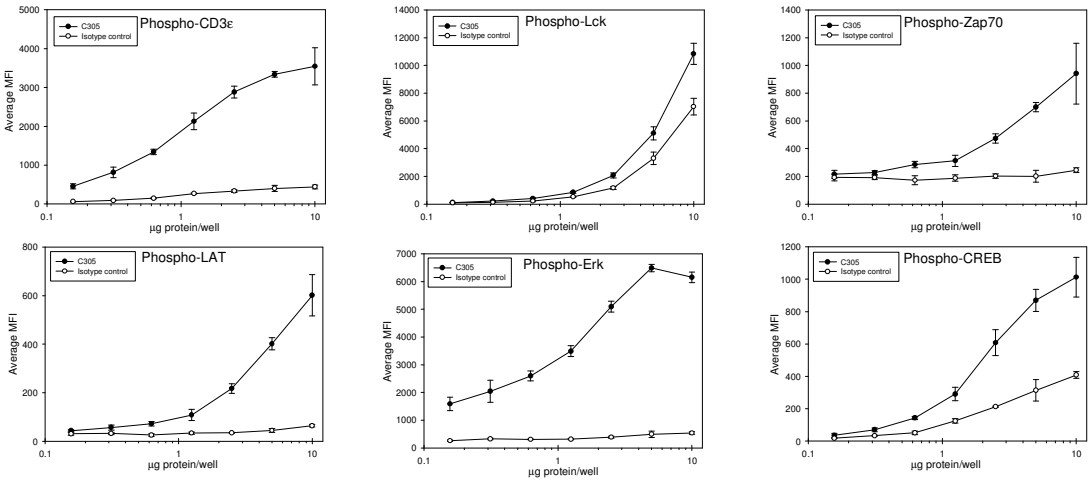


Figure 1. Multiplex analysis of anti-TCR treated Jurkat T cells. Jurkat cells were stimulated with 0.5µg/ml anti-TCRβ antibody (C305) or isotype control antibody for 2 min, washed with TBS, and lysed with Milliplex™ MAP Lysis Buffer. Increasing amounts of lysates were diluted in Milliplex™ MAP Assay Buffer 2 prior to following the Assay Protocol (lysate incubation at 4ºC). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system. The figures represent the average and standard deviation of three replicate wells.

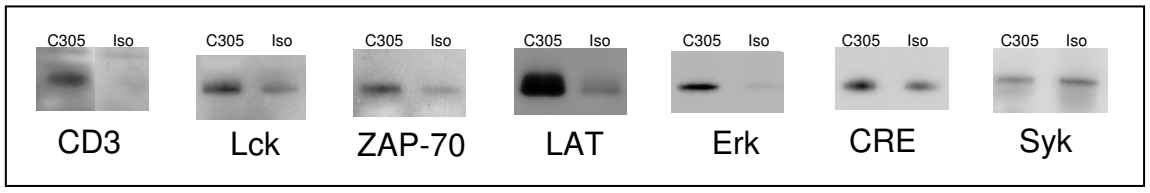


Figure 2. Immunoprecipitation/Western analysis of C305 treated Jurkat T cells. 10 µg of lysates from C305 treated Jurkat cells (Figure 1) were mixed with the appropriate 7-plex Human T cell Receptor phosphoprotein capture antibodies and protein-G agarose to immunoprecipitate signaling proteins. The proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with the appropriate biotinylated 7-plex Human T cell Receptor phosphoprotein reporter antibody followed by streptavidin-HRP. The blots were imaged using a chemiluminescence substrate and film.

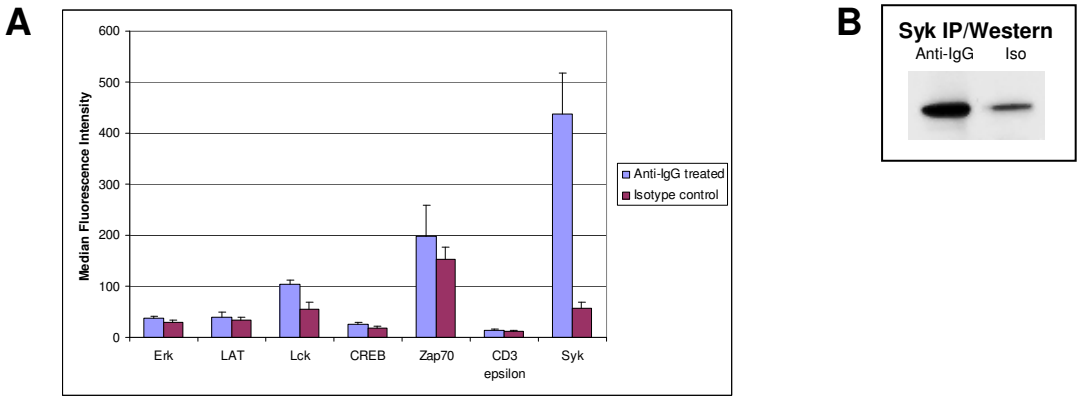


Figure 3. Analysis of surface IgG mediated signaling pathways in Daudi B cells. Human Daudi B cells were treated with 20µg/ml of F(ab)'$_2$ Goat anti-Human IgG/M or F(ab)'$_2$ Goat IgG for 10 min to induce sIgG mediated signaling. Cells were washed in TBS and lysed in MILLIPLEX™ MAP Lysis Buffer. The lysates were diluted in Assay Buffer 2 prior to following the Assay Protocol (lysate incubation at 4ºC). The Median Fluorescence Intensity (MFI) was measured with the Luminex® system (Panel A). The Syk capture antibody was used to immunoprecipitate Syk from 10 µg of F(ab)'$_2$ Goat anti-Human IgG/M or F(ab)'$_2$ Goat IgG isotype control lysates. The proteins were separated with SDS-PAGE, transferred to nitrocellulose, and probed with biotinylated anti-phospho-Syk reporter antibody. The blots were imaged using a chemiluminescence substrate and film (Panel B).

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Jurkat unstim negative control											
B	Jurkat unstim negative control											
C	Jurkat: C305 positive control											
D	Jurkat: C305 positive control											
E												
F												
G												
H												