8-Plex Human Src Family Kinase Kit – Phosphoprotein

Cat. # 48-650

MILLIPLEX® MAP

8-Plex Human Src Family Kinase Kit – Phosphoprotein

(Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes)

#48-650

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INTRODUCTION

The Src family of proto-oncogenic kinases (SFK) is the largest nonreceptor protein tyrosine kinase family. SFK consists of Src, Yes, Fyn, Fgr, Lck, Hck, Blk, and Lyn. Expressed either ubiquitously or predominately in hematopoietic immune-competent cells, the Src kinases share a high degree of homology in the catalytic SH1, and SH2 and SH3 domains, whereas the amino-terminal portion gives each of them some degree of specificity. The SFK proteins play a crucial role in embryonic development and cell growth, regulating such functions as proliferation, adhesion, differentiation, and survival that are common to both. Consequently, Src kinases also play a role in the oncogenesis of tumors such as non-small cell lung cancer, squamous cell head and neck and pancreatic cancers. In addition, studies have demonstrated that Src. Lck, Fyn and Hck have roles in trauma signaling pathways such as Akt and STAT3 phosphorylation and Ras activation, as well as the regulation of p38-mediated IL-6 production following hypoxia [(1-6)].

Millipore provides important tools to understand better the role of the Src family kinases in normal embryonic stem cells, development and cell growth, as well as its role in tumor proliferation and metastasis. Based on the Luminex xMAP platform, the MILLIPLEX MAP Human Src Family Kinase Phosphoprotein Panel (pan Tyr) is an ideal addition to our cell signaling portfolio.

The MILLIPLEX™ MAP 8-plex Human Src Family Kinase kit, phosphoprotein (pan Tyr), is used to detect tyrosine phosphorylation in Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, and Yes 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.

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.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
MILLIPLEX MAP 8-plex Human SFK-Phosphoprotein, Beads (20x)	42-650K	131 µL	1 tube
MILLIPLEX MAP 8-plex Human SFK-Phosphoprotein, Biotin (20x) (Detection Antibody)	44-650K	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 Ramos Cell Lysate: Pervanadate	47-224	----------	1 vial
MILLIPLEX MAP HeLa Cell Lysate: Pervanadate	47-223	----------	1 vial
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated	47-205	----------	1 vial
MILLIPLEX MAP Streptavidin-Phycoerythrin	45-001D	115 µL	1 tube
MILLIPLEX MAP Amplification Buffer (1X)	43-024A	3 mL	1 bottle
MILLIPLEX MAP 96-well Filter Plate	MX-PLATE	----------	1 plate
Empty mixing vials	----------	----------	3 vials

Protein	Bead Set
Lck	20
Lyn	26
Src	30
Yes	40
Fgr	44
Fyn	64
Blk	70
Hck	84

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

- Protease inhibitors (recommend Millipore Catalog #20-201 or similar product)
- Coomassie or BCA-based total protein assay for determination of lysate concentrations

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)
- 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 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.
- Mix only the required amount of beads and biotin-labeled detection antibody prior to assay setup. Discard any unused premixed beads and biotin-labeled detection antibodies.
- 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).
- 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 STORAGE

A. 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.
- 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.

B. 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 4°C.
- Remove particulate matter by filtration. Suggested Millipore filters:
  - 2 mL or greater, Millipore Catalog # SLPBDZ5NZ
  - 0.5 – 2 mL, Millipore Catalog # UFC 0DV 25
  - Less than 0.5 mL, Millipore Catalog # UFC30DV00
- 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 and 1:4 when using BCA-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 50 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 (Millipore Catalog # MSNBVN1210) 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 and 1:4 when using BCA-based assays.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of antibody Human SFK phosphoprotein beads

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

- Sonicate **20X** stock capture beads for 15 seconds, then vortex for 30 seconds.
- Dilute the beads to **1X** by combining 0.125 mL beads with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty mixing vial provided.
- Vortex the **1X** capture beads for 15 seconds.
- For use, transfer 1X capture beads with a pipette, do not pour from mixing vial.
- Please note that multiplexing phospho-specific and total MAPmate pairs is not recommended due to cross-reactivity

B. Preparation of Biotin-Labeled Detection Antibody and Streptavidin-PE

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

- Vortex the **20X** Detection Antibody stock for 10 seconds, it may be necessary to centrifuge briefly after vortexing for complete recovery of contents.
- Dilute the Detection Antibody to **1X** by combining 0.125 mL of Detection Antibody with 2.375 mL of MILLIPLEX MAP Assay Buffer 2. Use the empty bead-mixing vial provided.
- Vortex the MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE) for 10 seconds.
- Dilute SAPE by combining 0.1mL of Streptavidin-Phycoerythrin with 2.4 mL of Milliplex MAP Cell Signaling Assay Buffer 2. Use the empty mixing vial provided.
- Transfer 1X biotinylated detection antibody and SAPE with a pipette do not pour from mixing vial.

C. Multiplexing additional MILLIPLEX MAP Cell Signaling MAPmates™ with the 8-plex Human Src Family Kinase, phosphoprotein.

Additional Cell Signaling Phospho-MAPmates may be combined with this kit, up to a maximum of 8 additional MAPmates.

Please note that Total Human SFK MAPmate pairs should not be multiplexed with the 8-plex Human Src Family Kinase Kit, phosphoprotein.

- For each additional MAPmate, sonicate **20X** stock capture beads for 15 seconds, then vortex for 30 seconds.
- Add 125 µL 8-plex Human SFK beads to the mixing vial
- For each additional MAPmate, add 125 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 2.5 mL with Assay Buffer 2. Vortex the mixed beads well.
- Use the same preparation volumes for the Detection Antibody

Example 1: When using 2 additional MAPmates, add 125 µL 8-plex Human SFK Beads/ Detection Antibody and 125 µL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 2.125 mL Assay Buffer 2, for a final volume of 2.5 mL.

Example 2: When using 5 additional MAPmates, add 125 µL 8-plex Human SFK Beads/ Detection Antibody and 125 mL of each additional MAPmate Beads/ Detection Antibody to the mixing vial. Then add 1.75 mL Assay Buffer 2, for a final volume of 2.5 mL.

E. Preparation of lyophilized MILLIPLEX MAP Cell Lysates (Catalog # 47-205, 47-223, 47-224).

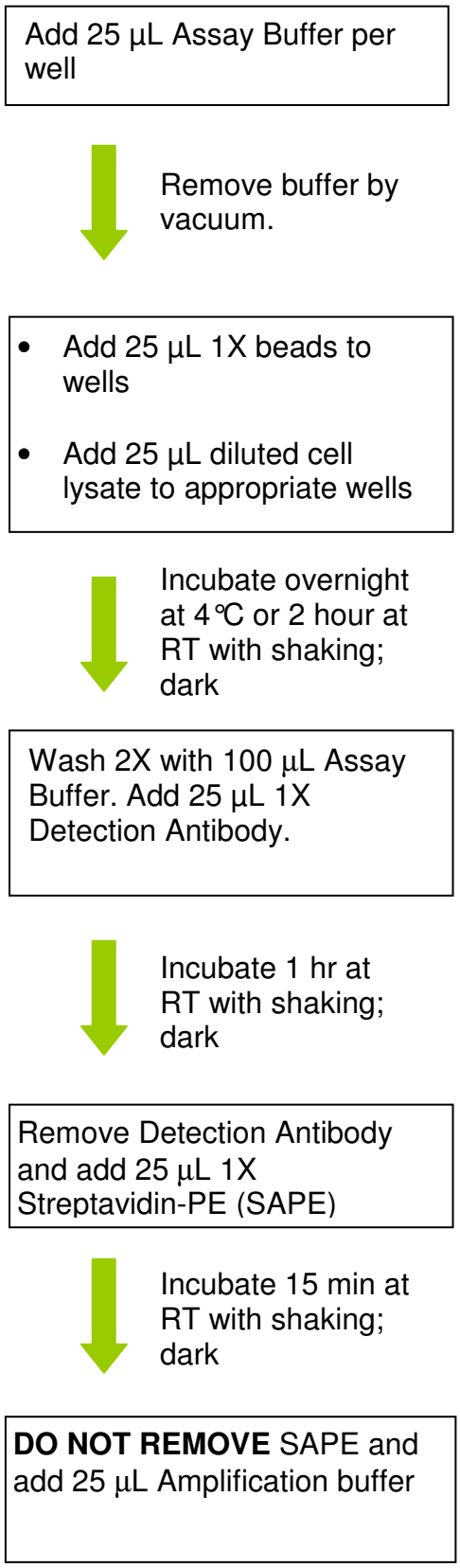
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated (#47-205) is provided as a lyophilized stock of cell lysate prepared from unstimulated HeLa cells and is used as a negative control. MILLIPLEX MAP HeLa Cell Lysate: Pervanadate (#47-223) is provided as a lyophilized stock of cell lysate prepared from HeLa cells treated with pervanadate for 5 min. MILLIPLEX MAP Ramos Cell Lysate: Pervanadate (#47-224) is provided as a lyophilized stock of cell lysate prepared from Ramos cells treated with pervanadate for 5 min. 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 2 mg/mL total protein.
- Gently vortex and incubate the reconstituted lysates 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 8-plex Human SFK Cell Signaling Assay.
- If desired, unused lysate may be stored in its original container at -80°C for up to one month.

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. Vortex the **1X** bead suspension 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 Ramos 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. Repeat this step again for a total of two washes.
7. Add 25 µL/well of **1X** MILLIPLEX MAP Detection Antibody.
8. Incubate on a plate shaker for 1 hour at room temperature, protected from light.
9. Remove Detection Antibody by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
10. Add 25 µL of 1X MILLIPLEX MAP Streptavidin-Phycoerythrin (SAPE).
11. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
12. **DO NOT REMOVE** SAPE. Add 25 µL of MILLIPLEX MAP Amplification Buffer to each well.



13. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
14. Remove MILLIPLEX MAP 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 MAP 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

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

INSTRUMENT SETTINGS

These specifications are for Luminex100/200 instruments. 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		
	Lck	20
	Lyn	26
	Src	30
	Yes	40
	Fgr	44
	Fyn	64
	Blk	70
	Hck	84

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.

REPLACEMENT REAGENTS

MILLIPLEX MAP Lysis Buffer	43-040
MILLIPLEX MAP Assay Buffer 2	43-041
MILLIPLEX MAP HeLa Cell Lysate: Pervanadate	47-223
MILLIPLEX MAP Ramos Cell Lysate: Pervanadate	47-224
MILLIPLEX MAP HeLa Cell Lysate: Unstimulated	47-205
MILLIPLEX MAP Streptavidin-Phycoerythrin	45-001D
MILLIPLEX MAP Amplification Buffer (1X)	43-024A
MILLIPLEX MAP 96-well Filter Plate	MX-PLATE

REPRESENTATIVE DATA

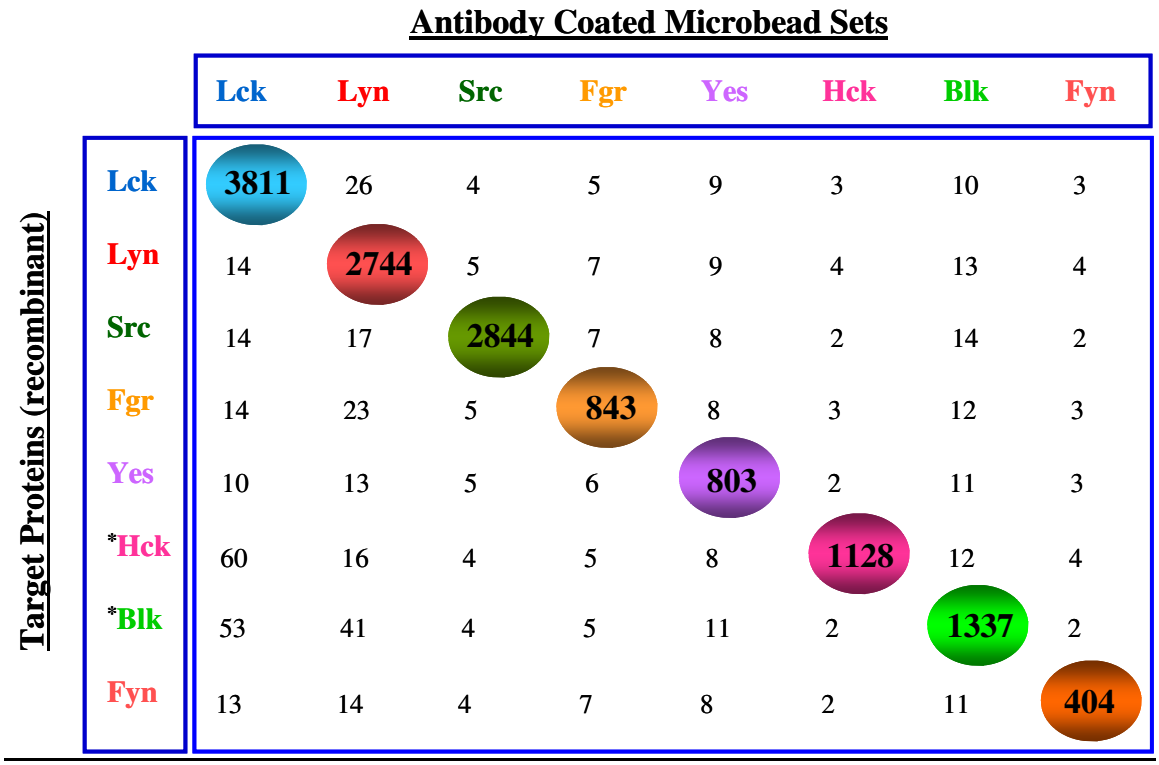


Figure 1 Capture antibody specificity. Selected capture antibodies were coated on Luminex microbeads. Specificity data for each optimized SFK capture antibody is shown. All eight individual microbeads were mixed and incubated with one individual recombinant SFK protein at a time. We took advantage of the basal phosphorylation level of the expressed proteins and detected them with biotinylated antiphosphotyrosine detection antibody. SFK proteins were used at a concentration of 8 ng/well (Hck and Blk were 1.6 ng/well as denoted by asterisk). The results show that the capture antibodies were specific against their respective target SFKs. These multiplex results for antibody specificity were confirmed by immunoblot analysis in which each individual capture antibody was probed for reactivity against all eight purified recombinant SFKs (data not shown).

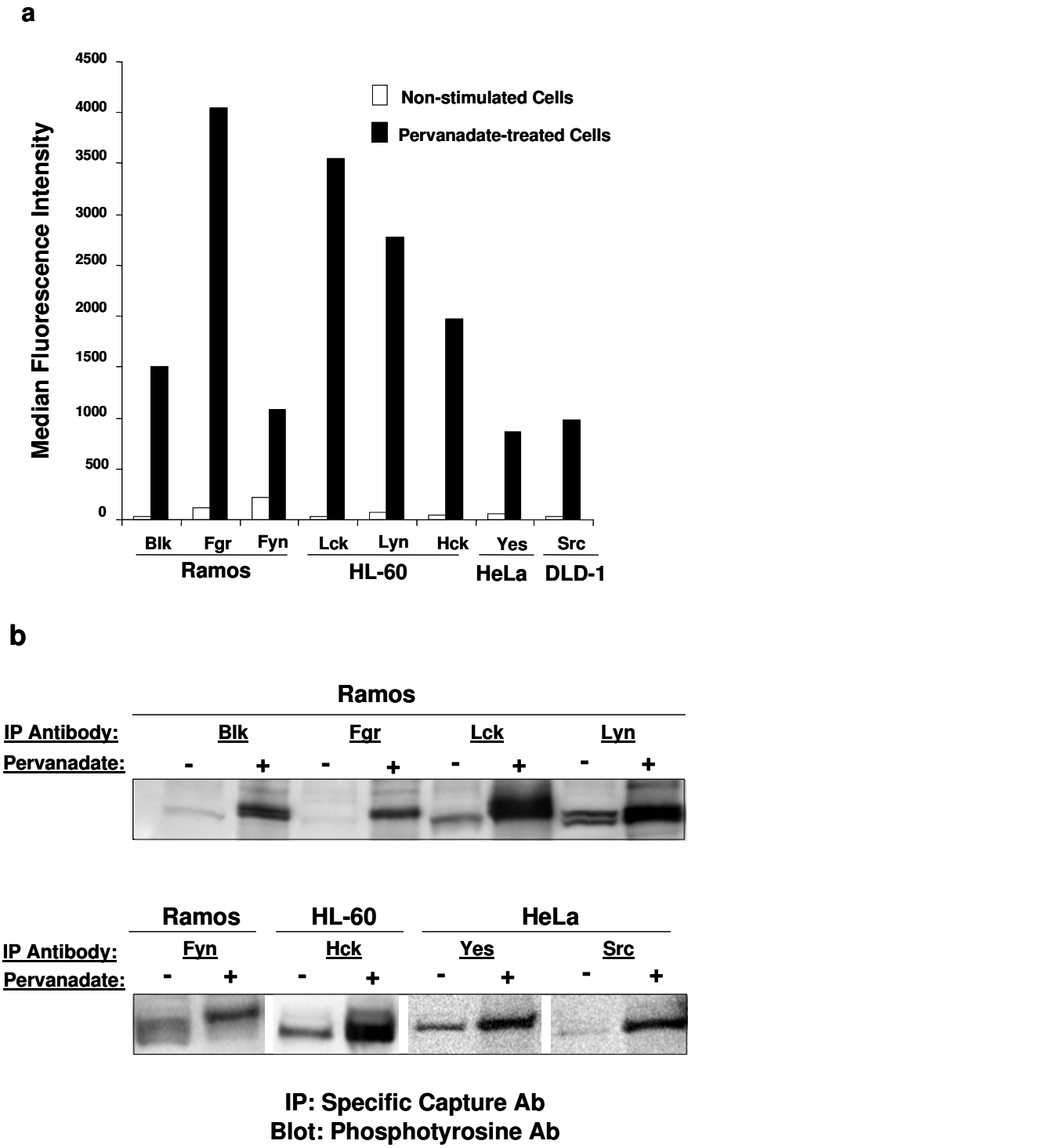


Figure 2. Phospho-SFK detection in cell lysates. Tumor Cells (Ramos, HL-60, HeLa and DLD-1) were treated with pervanadate (5 min) for tyrosine phosphorylation of signaling proteins. Cell lysates prepared from untreated and pervanadate treated cells were incubated with a mixture of all eight antibody coated microbeads. Tyrosine phosphorylation of SFKs was detected with antiphosphotyrosine antibody. The data show that the multiplex assay readily detected tyrosine phosphorylation. Blk, Fgr and Fyn phosphorylation in Ramos cells; Lck, Lyn and Hck phosphorylation in HL-60 cells, and Yes and Src phosphorylation were detected in HeLa and DLD-1 cells, respectively. Representative multiplex bead assay data are shown in Figure 2a. The confirmatory immuoprecipitation/western blots (IP/IB) are shown in Figure 2b.

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. 2. Calibration is not correct. 3. Incompatible buffer used to resuspend beads for Luminex® analysis.	1. Drain the system, followed by a backflush, proceed with solution for air in the system. 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.

REFERENCES

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WELL MAP

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