

Certificate of Analysis

Beadlyte[®] 8-Plex Multi-Pathway Signaling Kit – Phosphoprotein
(Erk/MAP Kinase 1/2, STAT3, JNK, p70 S6 kinase, I κ B- α , STAT5A/B, CREB, and p38/SAPK)
Catalog # 48-680
Lot # 31371

Kit Components

Beadlyte[®] 8-plex Multi-Pathway-Phosphoprotein, beads, Catalog # 42-680, Lot # 30498. One vial containing **125 μ l** of anti-Erk/MAP Kinase 1/2, STAT3, JNK, p70 S6 kinase, I κ B- α , STAT5A/B, CREB, and p38 Luminex[®] Beads at 4000/ μ l (20x) #:

Protein	Bead #
Erk/MAP Kinase 1/2	9
STAT3	14
JNK	16
p70 S6 kinase	19
I κ B- α	33
STAT5A/B	35
CREB	37
p38	54

Beadlyte[®] 8-plex Multi-Pathway-Phosphoprotein, biotin, Catalog # 44-680, Lot # 30359. One vial containing **125 μ l** of (20X) biotin-conjugated anti-Phospho-Erk/MAP Kinase 1/2 (Thr185/Tyr187), STAT3 (Ser727), JNK (Thr183/Tyr185), p70 S6 kinase (Thr412), I κ B- α (Ser 32), STAT5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) IgG in a proprietary formulation of Tris-buffered salts and animal protein containing 0.05% sodium azide as a preservative.

Beadlyte[®] Cell Signaling Universal Lysis Buffer, Catalog # 43-040, Lot # 30355. One vial containing **50ml** of a proprietary formulation of Tris buffered salts, detergents, and phosphatase inhibitors including 1mM sodium orthovanadate.

Beadlyte[®] Cell Signaling Universal Assay Buffer, Catalog # 43-041, Lot # 30356. One vial containing **50ml** of a proprietary formulation of Tris buffered salts and animal protein containing 0.05% sodium azide as a preservative.

Multi-screen plate

Empty mixing vials

Beadlyte[®] Streptavidin-Phycoerythrin, Catalog # 45-001B, Lot # 30281. One vial containing **100 μ l** of a 1mg/ml solution of Streptavidin-Phycoerythrin in PBS, pH 7.5, containing 0.02% sodium azide. **Do not freeze. Store in the dark.**

Beadlyte[®] Cell Signal Amplification Buffer, Catalog # 43-024A, Lot # 30832. One vial containing **3ml** of a proprietary formulation of Tris buffered salts and animal protein containing 0.05% sodium azide as a preservative.

Beadlyte[®] HeLa Cell lysate: Multi-stim, Catalog # 47-202, Lot # 30383. One vial containing lyophilized cell lysate made from a mixture of EGF-stimulated HeLa cells, TNF α -stimulated HeLa cells, and heat shock/arsenite treated HeLa cells lysed in Beadlyte[®] Cell Signaling Universal Lysis Buffer and protease inhibitors. Make fresh for each assay. Reconstitute in 200 μ l of Beadlyte[®] Cell Signaling Universal Assay Buffer.

Protein	Lysate *Units/vial
Erk/MAP Kinase 1/2	2400
STAT3	600
JNK	1600
p70 S6 kinase	800
I κ B- α	800
STAT5A/B	400
CREB	2400
p38	1120

*Lysate units are values based on MFI generated from freshly prepared cell lysates over a range of lysate concentrations.

Storage and Stability: Stable for 1 year at 4°C from date of shipment. **Store in the dark.**

FOR RESEARCH USE ONLY
DO NOT USE FOR DIAGNOSIS OF DISEASE IN HUMANS OR ANIMALS
DO NOT USE IN HUMANS OR IN ANIMALS

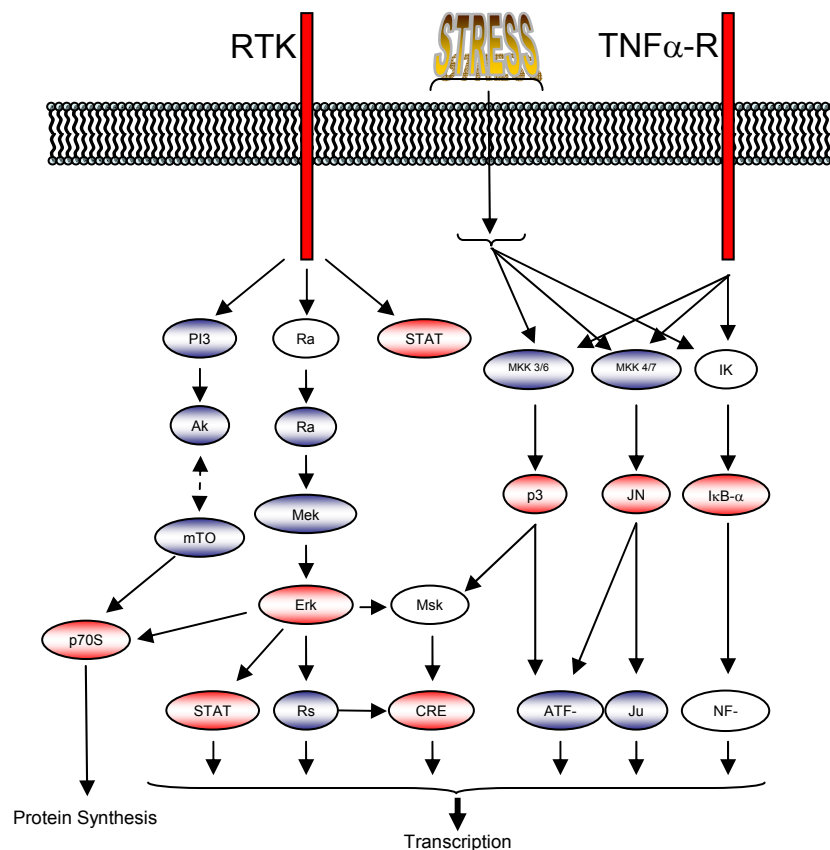
Other components required but not included as part of kit are:

- Cell lysates or cell extracts harboring protein(s) of interest
- Vortex mixer
- Plate shaker
- Timer
- Variable volume (5-200µl) pipette + tips
- Sonication Bath (Catalog # 40-002)
- Protease inhibitors (recommend *Complete protease inhibitor cocktail tablets*, Cat. 1836153, Roche Molecular Biochemicals, Inc., www.biochem.roche.com)
- Millipore multiscreen vacuum manifold (Catalog # MAVM0960R)
- Luminex¹⁰⁰ LabMAP™ System

8-plex Multi-Pathway Signaling kit, phosphoprotein

The Beadlyte® 8-plex Multi-Pathway Signaling kit, phosphoprotein, is used to detect changes in phosphorylated Erk/MAP kinase 1/2 (Thr185/Tyr187), STAT3 (Ser727), JNK (Thr183/Tyr185), p70 S6 kinase (Thr412), IκB-α (Ser32), STAT5A/B (Tyr694/699), CREB (Ser133), and p38 (Thr180/Tyr182) in cell lysates using the Luminex™ x-MAP™ system. The detection assay is a rapid, convenient alternative to Western Blotting and immunoprecipitation procedures. Each kit has sufficient reagents for 100 individual assays.

Summary of Signaling Pathways



Cells respond to their environment in many different ways through intracellular signaling. Signaling through RTKs (Receptor Tyrosine kinases) often promote increased metabolism and cell growth. Activation of Erk/MAP kinase is one of the key Ser/Thr kinases activated via RTK signaling, promoting increased activity of p70 S6 kinase, Msk1, STAT3 (Ser727), CREB, and many other signaling intermediates. Other signaling pathways induced via stress (ex. heat shock/arsenite treatment) or death receptors (ex. TNFα) promote p38, JNK, and IκB signaling pathways.

Materials and Methods

A. Preparation of cell lysates

Beadlyte[®] Cell Signaling Universal Lysis Buffer is supplied as **1X** stock solution. The lysis buffer contains phosphatase inhibitors *including* 1mM sodium orthovanadate (Na₃VO₄) but does **NOT** contain protease inhibitors. It is recommended that *Complete protease inhibitors* (Catalog # 1836153) from Roche Molecular Biochemicals, Inc. be added immediately before use.

Suggested cell lysis protocol for cells

- a. Pellet the cells by centrifugation (500 – 1000 x g) in a tabletop centrifuge for 5 minutes.
- b. Wash the cells in ice cold TBS.
- c. Add ice cold **1X** Beadlyte[®] Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors to cells (1ml per 1 x 10⁷ cells).
- d. Gently rock the lysate for 10-15 minutes at 4°C.
- e. Remove particulate matter by filtration.
Suggested Millipore filters:
 1. 2ml or greater, Millipore Catalog # SLPBDZ5NZ
 2. 0.5 - 2ml, Millipore Catalog # UFC 0DV 25
 3. Less than 0.5ml, Millipore Catalog # UFC30DV00
- f. Aliquot and store the lysate at –70°C. The lysate should be stable for several months.
- g. 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.

- a. Wash the cells by placing the 96 well filter plate containing cells over a vacuum manifold to remove liquid.
- b. Add 100µl of ice cold TBS then remove via vacuum.
- c. To lyse the cells, add 200µl/well of ice cold **1X** Beadlyte[®] Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors.
- d. Place the plate on an orbital shaker (600 – 800rpm) for 10-15 minutes at 4°C.
- e. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
- f. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
- g. Store the filtered lysate at –70°C until ready for use.
- h. 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.

- a. Wash the cells by centrifugation in a microplate carrier 2 min at 500 x g.
- b. Remove the supernatant via aspiration and add 100µl of ice cold TBS.
- c. Centrifuge and remove supernatant via aspiration.
- d. Add 200µl/well of ice cold **1X** Beadlyte[®] Cell Signaling Universal Lysis Buffer containing freshly prepared protease inhibitors.
- e. Place the plate on an orbital shaker (600 – 800 rpm) for 10-15 minutes at 4°C.
- f. Transfer the lysate to a 96-well filter plate that has been pre-wetted with **1X** lysis buffer.
- g. Place a low protein binding, 96-well round bottom or V-bottom plate underneath the filter plate.
- h. Centrifuge the plates in a microplate carrier for 5 minutes at 500 x g.
- i. Store the filtered lysate at –70°C until ready for use.
- j. 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 capture beads

Beadlyte[®] 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 0.125ml of beads with 2.475ml of Beadlyte[®] Cell Signaling Universal Assay Buffer. Use the empty bead-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

Beadlyte[®] 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 0.125ml of reporter antibody with 2.475ml of Beadlyte[®] Cell Signaling Universal Assay Buffer. Use the empty bead-mixing vial provided.
- Dilute Beadlyte[®] Streptavidin-Phycoerythrin 1:25 by adding 0.1ml of Streptavidin-Phycoerythrin with 2.5ml of Beadlyte[®] Cell Signaling Universal Assay Buffer. Use the empty mixing vial provided.
- The Beadlyte[®] Cell Signal Amplification Buffer is provided as a **1X** stock, and does not need to be diluted.

D. Multiplexing additional Beadmates™ with 8-plex Multi-Pathway Signaling Kit, phosphoprotein.

Additional Cell Signaling Phospho-Beadmates™ can be combined with this kit. *Please note that Total Beadmate™ pairs should not be multiplexed with the 8-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 Human T Cell Receptor Signaling Kit 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 Human T Cell Receptor Signaling Kit reporter.

Bead/Reporter Dilution Table for Multiplexing Additional Beadmates™

Number of Additional Beadmate™ Pairs added		Amount of additional 20X Beads or 20X Reporter (ml)		Amount of 8-plex Multi-Pathway 20X Beads or 20X Reporter (ml)		Total Bead or Reporter (ml)		Cell Signaling Universal Assay Buffer (ml)		Total 1X volume (ml)
0	X	0	+	0.125	=	0.125	+	2.475	=	2.6
1	X	0.125	+	0.125	=	0.250	+	2.350	=	2.6
2	X	0.125	+	0.125	=	0.375	+	2.225	=	2.6
3	X	0.125	+	0.125	=	0.500	+	2.100	=	2.6
4	X	0.125	+	0.125	=	0.625	+	1.975	=	2.6
5	X	0.125	+	0.125	=	0.750	+	1.875	=	2.6
6	X	0.125	+	0.125	=	0.875	+	1.725	=	2.6
7	X	0.125	+	0.125	=	1.000	+	1.600	=	2.6
8	X	0.125	+	0.125	=	1.125	+	1.475	=	2.6
9	X	0.125	+	0.125	=	1.250	+	1.350	=	2.6

Luminex[®] 100™ Instrument Settings

- Make sure lines are free of air bubbles (an alcohol flush, followed by a wash will often help).
- Check to make sure the lids of the sheath fluid containers are tightly fastened.
- Choose the 100 region bead map by going through options from the main page of the Luminex™ Data Collector.
- Select **Bead #s** for the protein detection assay. (See page 1 of each Beadmate™ COA).
- Read 50-100 events per bead.
- Set sample size = 75µl. Set gate to 8,000-13,500.
- Adjust needle height using 2 disks and the same plate type as the plate used in assay performed.
- Bio-Plex Instruments (Bio-Rad) should be calibrated with "Low" assay values on the calibration 2 bottle.

E. Preparation of lyophilized Beadlyte® HeLa Cell Lysate: Multi-stim (Catalog # 47-202).

Beadlyte® HeLa Cell Lysate: Multi-stim is provided as a lyophilized stock of mixed cell lysates prepared from HeLa cells stimulated with 50 ng/ml of EGF (5 min), HeLa cells stimulated with 25µM TNF-α (7 min), and HeLa cells heat shocked for 30 min at 42°C then 16 hrs at 37°C followed by 400uM arsenite for 30 min. The cells were lysed in Beadlyte® Cell Signaling Universal Lysis Buffer containing protease inhibitors, mixed together, and lyophilized for stability. The lysate can be used as a positive control sample or alternatively, to create standard curves for relative quantification of different phosphoprotein analytes.

- a. Beadlyte® HeLa Cell Lysate: Multi-stim as a **positive control**
 - (a) Reconstitute the lyophilized cell lysate in 200µl of Beadlyte® Cell Signaling Universal Assay Buffer (**UAB**).
 - (b) Gently vortex and incubate the reconstituted lysate for 5 min at RT (store on ice).
 - (c) 25µl/well of the reconstituted Beadlyte® HeLa Cell Lysate: Multi-stim mixed with 25µl of 8-plex Multi-Pathway Signaling phosphoprotein beads (steps 3 and 4 of main Assay protocol) will provide MFI values comparable to a freshly made mixture of HeLa cell lysates at 400 µg/ml.
- b. Beadlyte® HeLa Cell Lysate: Multi-stim as a **lysate standard (Lot # 30383)**

Protein	*Lysate Units/vial	Overnight Incubation (Lysate Units)	
		Sensitivity	High Range
Erk/MAP Kinase 1/2	2400	0.24	100
STAT3	600	0.90	75
JNK	1600	0.55	100
p70 S6 kinase	800	0.51	100
IκB-α	800	0.99	100
STAT5A/B	400	0.32	50
CREB	2400	2.57	100
p38	1120	0.69	140

Sensitivity: Defined as two standard deviations above the mean MFI (Median Fluorescence Intensity) of 20 replicates of the zero standard.

*Lysate units: These are values based on MFI generated from freshly prepared cell lysates over a range of lysate concentrations. All subsequent lots of 47-202 will be calibrated accordingly to provide consistency.

- (a) Reconstitute the lyophilized cell lysate in 200µl of UAB.
- (b) Gently vortex and incubate the reconstituted lysate for 5 min at RT (store on ice).
- (c) Standard Dilutions: It is recommended to use at least an 8-point curve including a zero, run in duplicate. A sample serial dilution follows:

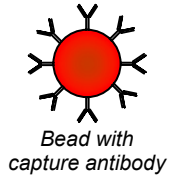
To make Standard	Standard (µl)	UAB (µl)	Erk 1/2 Units/wel	STAT3 Units/wel	JNK Units/wel	p70 S6 Units/wel	IκB-α Units/wel	STAT5 Units/wel	CREB Units/wel	p38 Units/wel
A	initial	0	300.0	75.0	200.0	100.0	100.0	50.0	300.0	140.0
B	100 STD A	200	100.0	25.0	66.7	33.3	33.3	16.7	100.0	46.7
C	100 STD B	200	33.3	8.3	22.2	11.1	11.1	5.6	33.3	15.6
D	100 STD C	200	11.1	2.7	7.4	3.7	3.7	1.9	11.1	5.2
E	100 STD D	200	3.7	0.9	2.5	1.2	1.2	0.6	3.7	1.7
F	100 STD E	200	1.2	0.3	0.8	0.4	0.4	0.2	1.2	0.6
G	100 STD F	200	0.4	0.1	0.27	0.14	0.14	0.07	0.4	0.2
H	-	200	0.0	0.0	0.0	0	0	0.0	0.0	0.0

F. Considerations for Cell Stimulation.

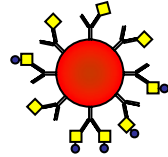
- a. Treating cells with growth factors (ex. EGF), cytokines (ex. TNFα), or other compounds (ex. Arsenite) induce a multitude of signaling cascades. The duration of stimulation in addition to the concentration of the respective factor/compound should be considered since these influence the degree of phosphorylation of any given analyte.
- b. Cellular responses to growth factors are typically improved when cells have been serum starved prior to treatment.
- c. Cell lines will differ in the robustness of their signaling response for any given stimulation.

Detection Protocol Summary

Pre-wet filter plate and add 25µl of diluted cell lysate to each well containing 25µl of 1X beads



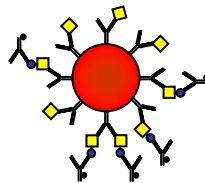
Overnight; dark
(4°C shaking)



Capture antibody binds target proteins

Wash 2X with 100 µl of Beadlyte® Cell Signaling Universal Assay Buffer. Add 25µl of 1X reporter

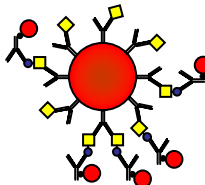
1 hour; dark
(RT shaking)



Biotinylated reporter binds phosphorylated proteins

Remove reporter and add 25µl of diluted Streptavidin-PE

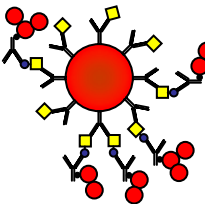
15 min; dark
(RT shaking)



Streptavidin-PE binds Biotinylated reporter

DO NOT WASH and add 25µl of Beadlyte Cell Signal Amplification Buffer

15 min; dark
(RT shaking)



Additional Streptavidin-PE Binds to reporter

Remove Streptavidin-PE/Amplification buffer and resuspend beads in 100µl of Beadlyte® Cell Signaling Universal Assay Buffer. Read results using Luminex 100™.

The Beadlyte® Cell Signaling assay is a simple fluorescence bead-based antibody sandwich immunoassay that is sensitive and easy to perform. A cell lysate or other test sample is incubated with beads coupled to a capture antibody that binds the target protein. After an overnight incubation, the beads are washed and mixed with a biotinylated reporter antibody which binds to an epitope distinct from the capture antibody. The beads are then incubated with streptavidin-phycoerythrin and amplification buffer, washed, and the target protein is quantified using the Luminex¹⁰⁰ xMAP™ system.

Assay Protocol

1. Dilute filtered lysates at least 1:1 in Beadlyte[®] Cell Signaling Universal Assay Buffer. The suggested working range of protein concentration for the assay is 1 to 40 µg of total protein/well (25µl/well at 40 to 1,000µg/ml).
2. Pre-wet filter plate with 25µl/well of Beadlyte[®] Cell Signaling Universal 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. Gently mix the **1X** bead suspension with a pipette and sonicate for 10 seconds. Add 25µl of 1X bead suspension to each well.
4. Add 25µl of diluted cell lysate (or reconstituted HeLa Cell Lysate: Multi-stim standard) to each well and incubate overnight¹ 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 100µl/well of Beadlyte[®] Cell Signaling Universal 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 25µl/well of **1X** Beadlyte[®] 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 25µl of diluted (1:25) Beadlyte[®] Streptavidin-Phycoerythrin (Strept-PE).
12. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
13. **DO NOT REMOVE** Strept-PE. Add 25µl of Beadlyte[®] Cell Signal Amplification Buffer to each well.
14. Incubate on a plate shaker for 15 minutes at room temperature, protected from light.
15. Remove Beadlyte[®] Strept-PE/Amplification buffer by vacuum filtration and gently blot the bottom of the filter plate on a paper towel.
16. Resuspend beads in 100µl of Beadlyte[®] Cell Signaling Universal Assay Buffer.
17. Analyze using the Luminex[®] 100™ system.

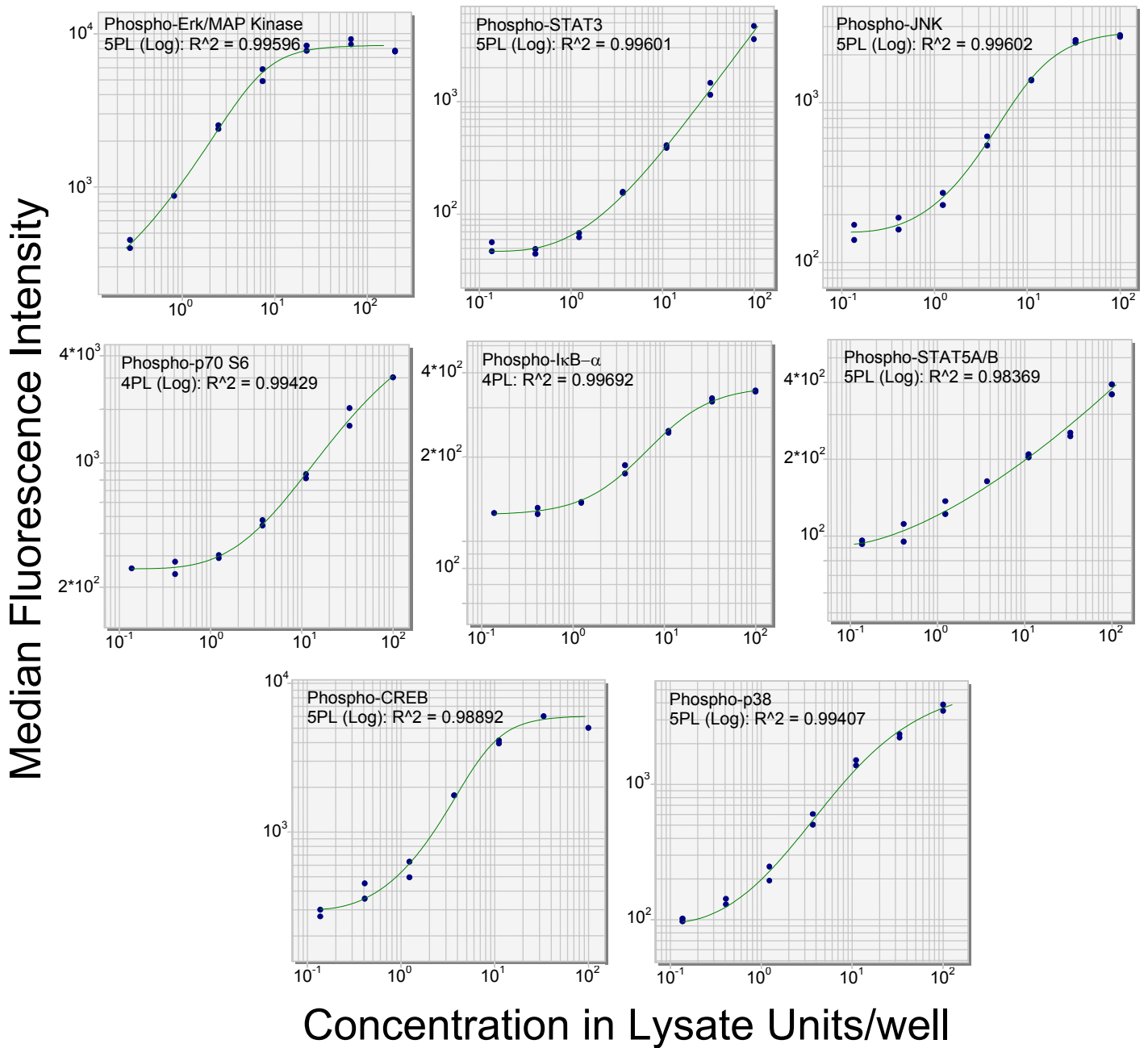
Step	Description
1. Prepare cell lysate samples	Dilute cell lysates in Beadlyte [®] Cell Signaling Universal Assay Buffer
2. Pre-wet filter plate	Add 25µl/well of Beadlyte [®] Cell Signaling Universal Assay Buffer, vacuum
3. Capture beads	Add 25µl/well of 1X Beads
4. Cell lysate/incubation	Add 25µl/well of cell lysate and incubate overnight ¹ at 4°C (or 2 hrs RT) shaking, protected from light
5. Remove lysate	Remove lysate via vacuum
6. Wash	Wash wells with 100µl Beadlyte [®] Cell Signaling Universal Assay Buffer
7. Second wash	Repeat step 6 a second time
8. Biotin reporter antibody	Add 25µl/well of 1X Biotin-labeled Reporter
9. One hour incubation	Incubate 1 hr RT
10. Remove Reporter	Remove reporter via vacuum
11. Streptavidin-PE	Add 25µl/well of Streptavidin-PE (diluted 1:25)
12. 15 minute incubation	Incubate 15 minutes at room temperature, shaking and protected from light
13. Amplification buffer	DO NOT REMOVE STREPTAVIDIN-PE. Add 25µl of Cell Signal Amplification Buffer to each well containing 25µl of Streptavidin-PE
14. 15 min incubation	Incubate 15 min at room temperature, shaking and protected from light
15. Remove streptavidin-PE/Amplification buffer	Remove Streptavidin-PE/Amplification buffer from wells via vacuum
16. Resuspension in Beadlyte [®] Cell Signaling Universal Assay Buffer	Add 100µl/well of Beadlyte [®] Cell Signaling Universal Assay Buffer
17. Analysis	Analyze using the Luminex [®] 100™ system

¹ Beadmates™ can be incubated with cell lysate for 2 hrs at room temperature. However, overnight incubations provide better sensitivity.

Data analysis:

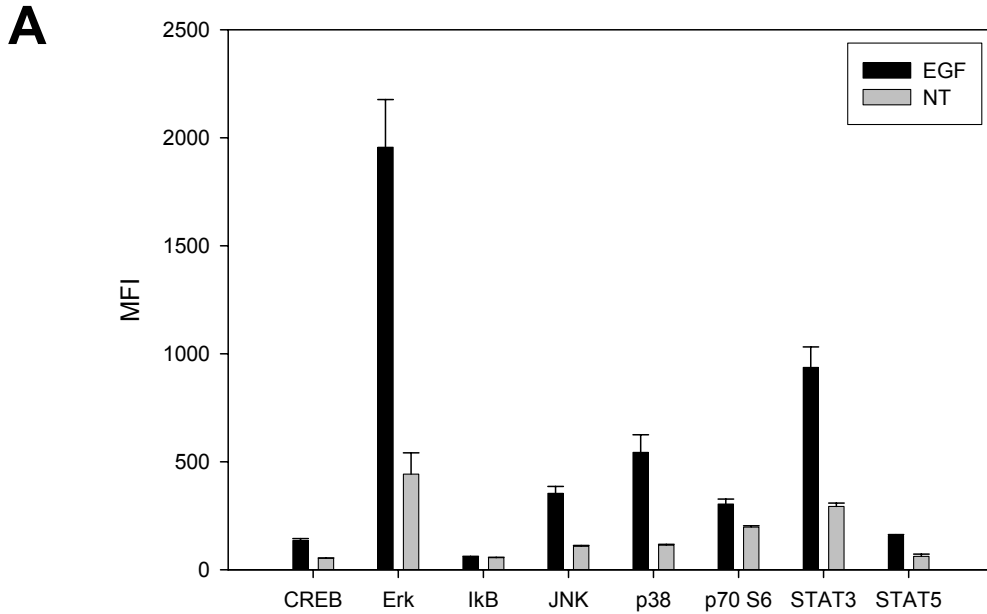
Non-linear curve fitting using lysate standards

Lyophilized Beadlyte[®] HeLa Cell Lysate: Multi-stim was resuspended in 200 μ l of Cell Signaling Universal Assay Buffer, as described on page 5. A tripling dilution series was made from the rehydrated lysate and analyzed with the Beadlyte[®] 8-plex Multi-Pathway Signaling kit, phosphoprotein. The Median Fluorescence Intensity measured with the Luminex¹⁰⁰ System was used to create the following 4 or 5 parameter curves using Beadview[™] software (cat# 40-250).



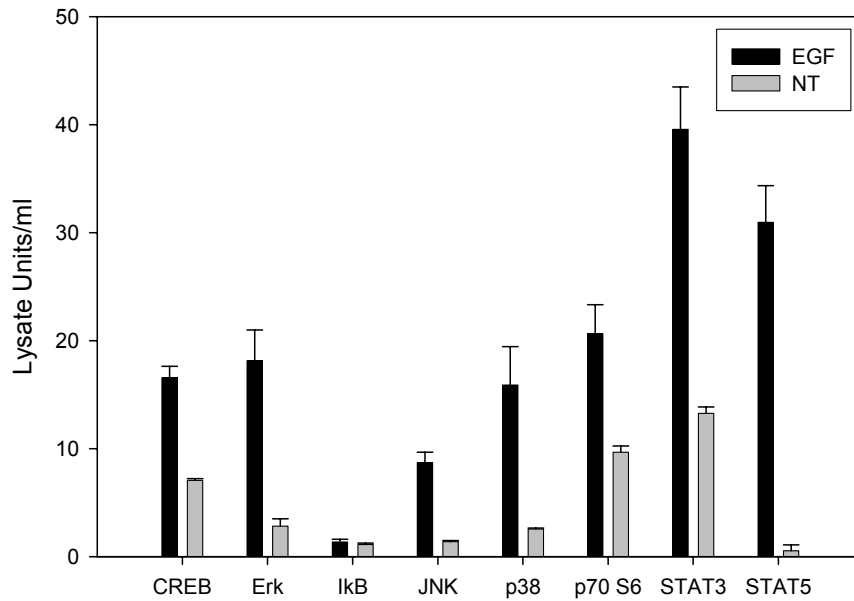
HeLa Cells were serum starved and treated with or without 50ng/ml of EGF for 5 min, washed with TBS, and lysed with Beadlyte[®] Cell Signaling Universal Lysis Buffer and diluted to 200 μ g/ml with Cell Signaling Universal Assay Buffer prior to following the Assay Protocol (lysate incubation at 4°C). The standard curves on page 8 were used to convert (A) Median Fluorescence Intensity (MFI) of each analyte into (B) Lysate Units.

8-plex Multi-Pathway analysis of HeLa cells



B

8-plex Multi-Pathway analysis of HeLa cells



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.

- a. Add 25 μ l/assay point of 1X beads to a 500 μ l centrifuge tube.
- b. Next, add lysate diluted in Beadlyte[®] Cell Signaling Universal Assay Buffer to a final volume of 100 μ l or higher.
- c. Vortex the mixture at high speed for 15 seconds then sonicate for an additional 15 seconds.
- d. Rotate the mixture overnight at 4°C, protected from light.
- e. Centrifuge the beads for 1 min at 2,000 x g and carefully remove the supernatant to minimize bead loss.
- f. Resuspend the pelleted beads in 25 μ l/assay point of Beadlyte[®] Cell Signaling Universal Assay Buffer.
- g. Transfer 25 μ l of the bead mixture to pre-wet filter plate wells and proceed to step 5 of the Main Assay 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 (Millipore Catalog # MAHAS4510 or comparable products). Placing the cells over a gentle vacuum can simplify washing of cells, adding and removing cell treatments, and filtering lysed cells.

- a. Growing cells overnight in 96 well filter plates
 - (a) Wash cells with sterile TBS or culture medium.
 - (b) Gently resuspend the cell pellet in culture medium to give 1 x 10⁵ cells per ml for adherent cells (such as HeLa or A431) or 2 x 10⁵ cells per ml for non-adherent cells (e.g. Jurkat T cells).
 - (c) Pre-wet the filter plate by adding 50 μ l of sterile TBS or culture medium per well of 96 well filter plate.
 - (d) 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.
 - (e) Add 100 μ l of cell suspension to each well.
 - (f) Grow/treat cells under desired conditions.
 - (g) Proceed to "Cell lysis protocol for cells in **96-well filter plates**" on pg. 3, Materials and Method section.
- b. Seeding cells (short durations) in 96 well filter plates
 - (a) Wash cells with sterile TBS or culture medium.
 - (b) Gently resuspend the cell pellet in TBS or culture medium to give 1 x 10⁶ cells per ml for non-adherent cells or adherent cells.
 - (c) Pre-wet the filter plate by adding 50 μ l of sterile TBS or culture medium per well of 96 well filter plate.
 - (d) 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.
 - (e) Add 100 μ l of cell suspension to each well.
 - (f) Treat cells under desired conditions.
 - (g) Proceed to "Cell lysis protocol for cells in **96-well filter plates**" on pg. 3, Materials and Method section.

Technical Considerations

1. The suggested working range of protein concentration for the assay is 1 to 10 μ g of total protein/well (25 μ l/well at 40 to 1000 μ g/ml).
2. Please note that multiplexing phospho-specific and total Beadmate[™] pairs is not recommended due to reporter cross reactivity.
3. 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.

End-User License Agreement

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation, you, the customer, acquire the right under Luminex Corporation'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 Luminex[®] 100[™]. One or more of the following US patents covers this product and the use thereof: #6,046,807, #5,981,180.

Representative Data

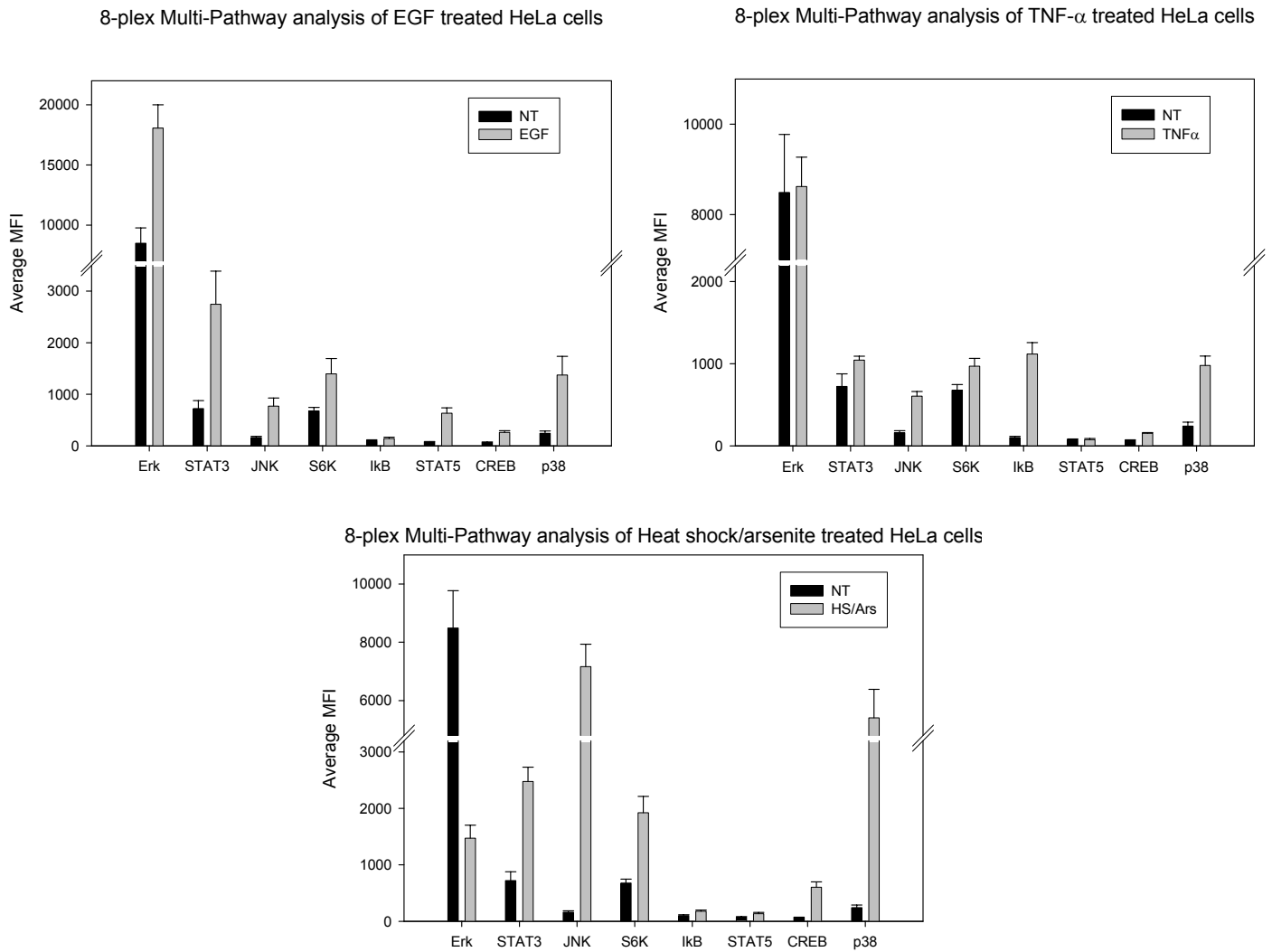


Figure 1. Multiplex analysis of HeLa cells treated with EGF, TNF- α , or heat shock/arsenite. HeLa cells were stimulated with 50ng/ml of EGF (5 min), HeLa cells stimulated with 25 μ M TNF- α (7 min), or HeLa cells heat shocked for 30 min. at 42 $^{\circ}$ C then 16 hrs at 37 $^{\circ}$ C followed by 400 μ M arsenite for 30 min. The cells were lysed in Beadlyte[®] Cell Signaling Universal Lysis Buffer containing protease inhibitors. 200 μ g/ml of each lysate diluted in Beadlyte[®] Cell Signaling Universal Assay Buffer were analyzed according the Assay protocol (lysate incubation at 4 $^{\circ}$ C). The median Fluorescence Intensity (MFI) was measured with the Luminex¹⁰⁰ system. The figures represent the average and standard deviation of three replicate wells.

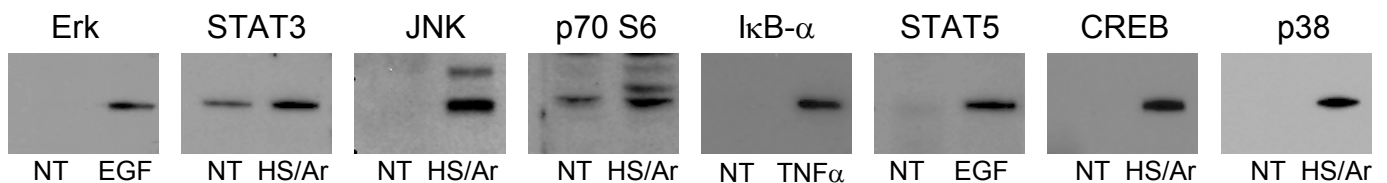


Figure 2. Immunoprecipitation/Western Blot analysis of multiplexed analytes in HeLa cells. 10 μ g of lysates (described in Figure 1) were mixed with capture antibodies to immunoprecipitate each respective protein. The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose, and probed with Biotin labeled phospho-specific reporter antibodies. The proteins were imaged using Streptavidin-HRP and chemiluminescence.