MILLIPLEX® MAP
EpiQuant™ Phosphotyrosine Cell Signaling Panel 1 Protocol

#MPEQ-100K

TABLE OF CONTENTS **PAGE**

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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, 200™, HTS and FLEXMAP 3D™.

INTRODUCTION

MILLIPLEX® MAP EpiQuant™ technology uses computational algorithms to identify unique, continuous linear sequences (EpiQuant Sequences) in proteins. EpiQuant antibodies generated against EpiQuant sequences have pre-defined target specificity. Protein abundance and/or protein phosphorylation measurements are made at the peptide level, using EpiQuant antibodies, following proteolytic fragmentation of samples and liberation of EpiQuant-bearing peptides.

A unique feature of the EpiQuant sample processing protocol is fragmentation or digestion of the protein sample. This process has a number of benefits:

- Protein:protein interactions are disrupted
- A synthetic peptide can be used as a quantitative standard
- Specificity of the antibodies can be narrowed to a small linear peptide sequence
- Cross-reactivity with similar peptide sequences in other proteins can be predicted and avoided
- Residual enzymatic activity of the sample (e.g. phosphatases and proteases) is quenched

EpiQuant technology represents a major advance in the design and implementation of quantitative immunoassays.

The MILLIPLEX® MAP EpiQuant™ Phosphotyrosine Cell Signaling Panel 1 is a Luminex® based approach for assessing the functional states of cells by profiling tyrosine phosphorylation changes across signaling pathways and cell phenotypes. Site-specific, quantitative measurements, with picomolar sensitivity, support diverse sample types and applications across the drug discovery/development continuum.

The MILLIPLEX® MAP EpiQuant™ Phosphotyrosine Cell Signaling Panel 1 is used for the assay of cellular lysates prepared utilizing the EpiQuant Sample Preparation Kit (MPEQ-SP, purchased separately). **ONLY SAMPLES PREPARED USING THE MPEQ-SP KIT WILL GENERATE RELIABLE RESULTS IN THIS ASSAY**. EpiQuant beads may be combined in desired configurations for the profiling of prepared lysate samples.

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.

PRINCIPLE

MILLIPLEX 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.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED STANDARDS OR CONTROLS IN LYOPHILIZATION VIALS**. Freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibodies, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Cell Signaling Assay Buffer 1	43-010	55 mL	1 bottle
EpiQuant Dilution Buffer	MPEQ-DB	3 mL	1 bottle
EpiQuant Phosphotyrosine Detection Antibody (25X)	MPEQ-1100	150 µL	1 tube
Streptavidin-Phycoerythrin (SAPE)	45-001D	115 µL	1 tube
EpiQuant Phosphotyrosine Panel 1 Standard	MPEQ-8100	lyophilized	1 vial
EpiQuant Phosphotyrosine Panel 1 Quality Controls 1 and 2	MPEQ-6100	lyophilized	2 vials
Mixing Bottles	----------	--------	3 bottles
Set of one 96-Well filter Plate with 2 sealers	MX-PLATE	----------	1 plate 2 sealers

EpiQuant Cell Signaling Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 41 Analytes (50X concentration, 90µL) Available	Cat. #
EGFR (pY1110/1125)	2	✓	EQEGFR-Y1110
p38MAPKα (pY181)	8	✓	EQP38A-Y181
JNK1/2/3 (pY185/185/223)	10	✓	EQJNK-Y185
Shc (pY349/350)	12	✓	EQSHC-Y349
PDK1 (pY373/376)	14	✓	EQPDK1-Y373
FAK (pY397/407)	16	✓	EQFAK-Y397
Ezrin (pY477)	20	✓	EQEZRIN-Y477
Plakoglobin (γ–catenin) (pY548)	22	✓	EQPLAK-Y548
JAK3 (pY785)	24	✓	EQJAK3-Y785
Raf 1 (pY340/341)	26	✓	EQRAF1-Y340
PLCγ1 (pY1253)	28	✓	EQPLCG1-Y1253
PLCγ2 (pY1197/1217)	30	✓	EQPLCG2-Y1197
Cbl (pY700/731/774)	32	✓	EQCBL-Y700
IRS1 (pY612)	34	✓	EQIRS1-Y612
FAK (pY861)	36	✓	EQFAK-Y861
SPTAN1 (pY1176)	38	✓	EQSPTAN1-Y1176
Crk (pY221/239)	40	✓	EQCRK-Y221
CUB domain containing protein 1 (pY734)	42	✓	EQCDCP1-Y734
SH2 domain containing protein 3A (pY95)	44	✓	EQSDCP3A-Y95
SH2 domain containing protein 3A (pY231)	46	✓	EQSDCP3A-Y231
Interleukin-15 receptor α chain (pY227)	48	✓	EQIL15RA-Y227

Bead/Analyte Name	Luminex Bead Region	Customizable 41 Analytes (50X concentration, 90µL) Available	Cat. #
Growth hormone receptor (pY566)	50	✓	EQGHR-Y566
Tec (pY206)	52	✓	EQTEC-Y206
WASP (pY291)	54	✓	EQWASP-Y291
ITK (pY180)	56	✓	EQITK-Y180
HGFR (met) (pY1003)	58	✓	EQMET-Y1003
TfR (pY20)	60	✓	EQTFR-Y20
SCF38 (pY20)	62	✓	EQSCF38-Y20
HER2 (pY1023)	64	✓	EQHER2-Y1023
PI3Kp110δ (pY485)	66	✓	EQPI3K-Y485
STAT3 (pY705)	68	✓	EQSTAT3-Y705
Gab1 (pY285/307/317)	70	✓	EQGAB1-Y285
LAT (pY255)	72	✓	EQLAT-Y255
Fyb (pY697/651 (iso1/2))	74	✓	EQFYB-Y697
Gab2 (pY266)	76	✓	EQGAB2-Y266
Gab2 (pY584)	78	✓	EQGAB2-Y584
VEGFR1 (pY1169)	82	✓	EQVEGFR1-Y1169
FRS2 (pY436)	84	✓	EQFRS2-Y436
ZAP70 (pY126)	88	✓	EQZAP70-Y126
RapGEF1 (GRF2) (pY504 (522 in iso1))	90	✓	EQGRF2-Y504

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. MILLIPLEX EpiQuant Sample Preparation Kit (MPEQ-SP)

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Absorbent Pads
7. Laboratory Vortex Mixer
8. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
11. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
12. Plate Stand (Millipore Catalog # MX-STAND)

SAFETY PRECAUTIONS

- All 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 opaque plate lid 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 Assay Buffer 1 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).
- After hydration, all Standards and Controls should be used immediately or transferred to polypropylene tubes and stored at $\leq$ -20°C.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with 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 targets.

- 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.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

1. Sonicate each bead vial for 30 seconds and vortex for 1 minute.
2. Add 60 µL from each bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer 1.
3. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month.

Example 1: When using 5 EpiQuant beads, add 60 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.7 mL Assay Buffer 1.

Example 2: When using 15 EpiQuant beads, add 60 µL from each of the 15 bead sets to the Mixing Bottle. Then add 2.1 mL Assay Buffer 1.

B. Preparation of EpiQuant Quality Controls

Reconstitute Quality Control 1 and Quality Control 2 by adding 250 µL Dilution Buffer to each. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Any unused portion may be stored at ≤ -20°C.

C. Preparation of EpiQuant Peptide Standard

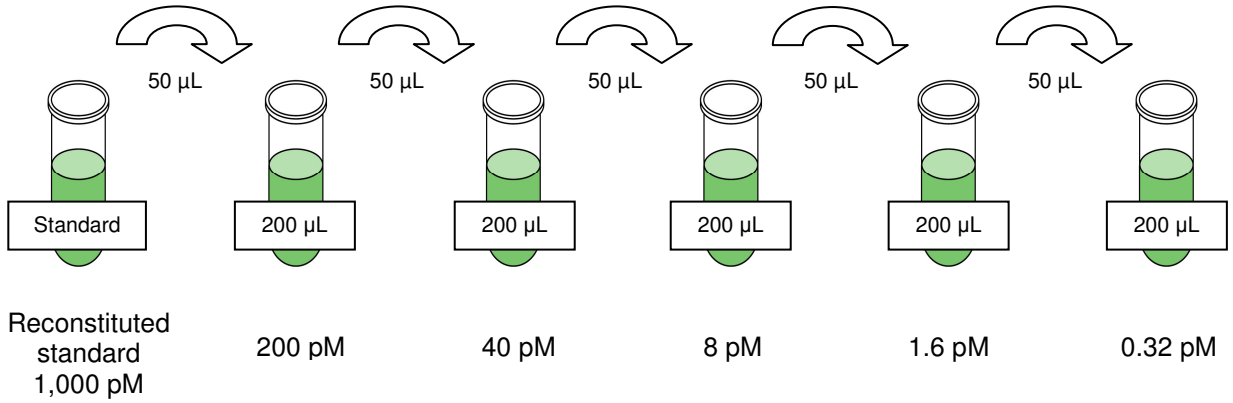
1. Reconstitute the EpiQuant Peptide Standard with 250 µL Dilution Buffer. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the 1000 pM standard; any unused portion may be stored at ≤ -20°C.

   NOTE: The reconstituted Peptide Standard is at a concentration of **1000 pM FOR ALL ANALYTES**.

2. Label five polypropylene microfuge tubes 200, 40, 8, 1.6 and 0.32 pM. Add 200 µL Dilution Buffer to each of the five tubes. Prepare serial dilutions by adding 50 µL of the 1000 pM reconstituted standard to the 200 pM tube; mix well and transfer 50 µL of the 200 pM standard to the 40 pM tube; mix well and transfer 50 µL of the 40 pM standard to the 8 pM tube; mix well and transfer 50 µL of the 8 pM standard to 1.6 pM tube; mix well and transfer 50 µL of the 1.6 pM standard to the 0.32 pM tube and mix well. The 0 pM standard (Background) will be Dilution Buffer only.

Preparation of EpiQuant Peptide Standard

Standard Concentration (pM)	Volume of Dilution Buffer to Add	Volume of Standard to Add
1,000	250 µL	0
200	200 µL	50 µL of 1,000 pM
40	200 µL	50 µL of 200 pM
8	200 µL	50 µL of 40 pM
1.6	200 µL	50 µL of 8 pM
0.32	200 µL	50 µL of 1.6 pM



D. Preparation of Samples

The following protocol allows for the preparation of an appropriate amount of sample for duplicate well measurement. If triplicate or single well measurement is desired scale up or down as appropriate.

1. For each sample, in a clean tube combine 30 µL digested sample (completed cell lysate samples prepared using EpiQuant Sample Preparation Kit (MPEQ-SP) and 30 µLCell Signaling Assay Buffer 1.
2. Repeat for each sample to be assayed.

E. Preparation of Detection Antibody and SAPE

1. Vortex the EpiQuant Phosphotyrosine Detection Antibody (25X) and the SAPE for 10 seconds.
2. In a Mixing Bottle, combine 120 µL pTyr Detection Antibody (25X) and 2.88 mL Assay Buffer 1.
3. In a separate Mixing Bottle, combine 100 µL SAPE (25X) and 2.4 mL Assay Buffer 1.

IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), 0.32, 1.6, 8, 40, 200, and 1,000 pM], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- Set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Prewet the filter plate by pipetting 25 µL of Assay Buffer 1 into each well of the microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Assay Buffer 1 by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Assay Buffer 1 from the bottom of the plate with an absorbent pad or paper towels.
3. Add 25 µL of each Standard or Control into the appropriate wells. Dilution Buffer should be used for the 0 pg/mL standard (Background).
4. Add 25 µL of prepared sample into the appropriate wells.
5. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
6. Seal the plate with a plate sealer, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker for 2 hours at room temperature (20-25°C).
7. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE.)**
8. Wash plate 2 times with 100 µL/well of Assay Buffer 1, removing Assay Buffer 1 by vacuum filtration between each wash. Blot excess Assay Buffer 1 from the bottom the plate with an absorbent pad or paper towels.
9. Add 25 µL of 1X Detection Antibody into each well.

Add 25 µL Assay Buffer 1 per well

Shake 10 min, RT

Vacuum

- Add 25 µL Standard or Control to appropriate wells and 25µL Dilution Buffer to background wells.
- Add 25 µL prepared samples to sample wells
- Add 25 µL Beads to each well

Incubate 2 hours at RT with shaking

Vacuum and wash 2X with 100 µL Assay Buffer 1

Add 25 µL Detection Antibody per well

10. Seal, cover with lid, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). Remove contents by vacuum after agitation. **(NOTE: DO NOT INVERT PLATE.)**
11. Add 25 µL 1X Streptavidin-Phycoerythrin to each well.
12. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C
13. Remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE.)**
14. Add 150 µL of Assay Buffer 1 to all wells. Resuspend the beads on a plate shaker for 5 minutes.
15. Analyze plate on Luminex 100™ IS, 200™, HTS or FLEXMAP 3D™.
16. Save and analyze the Median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or spline curve-fitting method for calculating peptide concentrations in samples.

Incubate 1 hour RT

Vacuum

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Vacuum

Add 150 µL Assay Buffer 1 per well

Read on Luminex (100 µL, 50 beads per bead set)

EQUIPMENT SETTINGS

These specifications are for the Luminex 100™ IS v.1.7 or Luminex 100™ IS v2.1/2.2, Luminex 200™ v2.3, xPONENT v3.0/3.1, 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

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located on the Millipore website www.millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

No significant cross-reactivity was observed between the antibodies and/ or non-specific analytes in this panel.

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of target peptide in one experiment. Inter-assay precision is generated from the mean of the %CV's from two reportable results, each for two different concentrations of target peptide across 6 different experiments. Intra-assay precision for all analytes ranges from **1.8%** to **17.5%** and Inter-assay precision for all analytes ranges from **5.6%** to **19.6%**.

Accuracy

Spike-Recovery values are generated from the mean percent recovery of 3 levels of spiked standards ranging from 8pM to 200pM in sample matrix in 8 experiments. Spike-recovery values for all analytes range from **81.2%** to **119.6%**.

Assay Sensitivity (minimum detectable concentrations, pM)

MinDC: Minimum Detectable Concentration is calculated by STATLIA Immunoassay Analysis Software. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions. MinDC values were determined from 6 different experiments.

MinDC < 0.32	MinDC = 0.33-1.10	MinDC = 1.11-3.0	MinDC > 3.1
EGFR (pY1110/1125)	Shc (pY349/350)	HER2 (pY1023)	Fyb (pY697/651 (iso1/2))
p38MAPKα (pY181)	IRS1 (pY612)	Gab1 (pY285/307/317)	SH2 domain containing protein 3A (pY95)
JNK1/2/3 (pY185/185/223)	FAK (pY861)	IL-15 receptor α chain (pY227)	Tec (pY206)
PDK1 (pY373/376)	SPTAN1 (pY1176)	JAK3 (pY785)	WASP (pY291)
FAK (pY397/407)	Crk (pY221/239)	CUB domain containing protein 1 (pY734)	LAT (pY255)
SH2 domain containing protein 3A (pY231)	Growth hormone receptor (pY566)	RapGEF1 (GRF2) (pY504 (522 in iso1))	Plakoglobin (γ–catenin) (pY548)
Raf 1 (pY340/341)	ITK (pY180)	TfR (pY20)	SCF38 (pY20)
PLCγ1 (pY1253)	HGFR (met) (pY1003)	FRS2 (pY436)	VEGFR1 (pY1169)
PLCγ2 (pY1197/1217)	STAT3 (pY705)		
Cbl (pY700/731/774)	Gab2 (pY266)		
Ezrin (pY477)	Gab2 (pY584)		
Pl3Kp110δ (pY485)	ZAP70 (pY126)		

Natural Sample Response

Prepared cell lysates (MPEQ-LY001/2/3/4/5) were assayed using the MPEQ-100K kit. Results are tabulated below; values are the average of triplicate measurements.

TARGET	TARGET CONCENTRATION IN CONTROL LYSATES (pM)				
	MPEQ-LY001	MPEQ-LY002	MPEQ-LY003	MPEQ-LY004	MPEQ-LY005
EGFR (pY1110/1125)	0.84	<0.04	1.28	88.09	71.63
p38MAPKα (pY181)	0.26	<0.16	0.50	2.70	0.43
JNK1/2/3 (pY185/185/223)	0.07	<0.04	0.36	<0.04	0.05
Shc (pY349/350)	11.37	10.82	6.95	16.87	38.65
PDK1 (pY373/376)	<0.1	<0.1	1.44	2.19	<0.1
FAK (pY397/407)	<0.04	4.20	1.54	13.69	3.97
Ezrin (pY477)	<0.13	<0.13	1.43	4.36	0.62
Plakoglobin (γ–catenin) (pY548)	42.17	29.97	18.67	16.57	31.64
JAK3 (pY785)	9.32	4.75	4.14	8.15	15.45
Raf 1 (pY340/341)	0.20	0.17	0.34	0.61	0.37
PLCγ1 (pY1253)	0.80	0.49	10.67	1.31	4.03
PLCγ2 (pY1197/1217)	1.19	0.82	7.45	3.51	1.41
Cbl (pY700/731/774)	2.79	1.21	14.33	7.51	9.95
IRS1 (pY612)	2.01	1.41	2.06	5.53	3.45
FAK (pY861)	2.00	3.04	3.79	21.08	3.62
SPTAN1 (pY1176)	12.43	10.88	11.32	10.94	11.77
Crk (pY221/239)	<0.42	<0.42	1.56	5.91	17.16
CUB domain containing protein 1 (pY734)	24.30	19.16	13.11	30.59	26.12
SH2 domain containing protein 3A (pY95)	17.37	12.37	23.44	20.96	17.72
SH2 domain containing protein 3A (pY231)	1.54	1.21	0.81	1.36	1.48
Interleukin-15 receptor α chain (pY227)	12.13	1.87	16.63	29.68	13.92
Growth hormone receptor (pY566)	10.90	9.65	7.40	9.30	13.31
Tec (pY206)	26.29	<1.23	5.91	23.72	31.79
WASP (pY291)	13.21	7.50	5.97	13.61	14.19
ITK (pY180)	8.29	6.50	30.39	4.74	6.31
HGFR (met) (pY1003)	<0.04	<0.04	<0.04	10.57	1.85
TfR (pY20)	6.38	4.05	9.91	9.91	6.49
SCF38 (pY20)	24.83	14.03	18.13	21.52	32.86
HER2 (pY1023)	52.67	27.47	16.42	112.85	59.93
Pl3Kp110δ (pY485)	<0.19	0.74	0.54	1.29	1.71
STAT3 (pY705)	<0.27	<0.27	<0.27	2.95	<0.27
Gab1 (pY285/307/317)	6.64	1.73	3.81	2.58	4.60
LAT (pY255)	13.52	2.15	28.09	16.60	12.47
Fyb (pY697/651 (iso1/2))	5.88	<0.15	4.06	12.94	5.35
Gab2 (pY266)	10.08	9.71	7.28	13.35	10.97
Gab2 (pY584)	7.01	6.72	3.15	7.54	8.83
VEGFR1 (pY1169)	14.13	1.20	12.52	23.21	20.81
FRS2 (pY436)	17.38	8.88	6.86	10.72	16.86
ZAP70 (pY126)	2.99	1.25	2.45	5.33	3.75
RapGEF1 (GRF2) (pY504 (522 in iso1))	1.64	<0.28	1.16	2.48	2.47

MPEQ-LY001- EpiQuant Jurkat: Unstimulated Lysate
MPEQ-LY002- EpiQuant A431: Unstimulated Lysate
MPEQ-LY003- EpiQuant Jurkat: H_2O_2 Stimulated Lysate
MPEQ-LY004- EpiQuant A431: H_2O_2 Stimulated Lysate
MPEQ-LY005- EpiQuant A431: EGF Stimulated Lysate

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use supernatant.
	Sample too viscous	May need to dilute sample.
	Plate not sealing on vacuum manifold	Ensure plate and manifold surfaces are clean and aligned properly.
Insufficient bead count	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Bead mix prepared incorrectly	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting 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, backflush and washes; or, if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate stand or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of well.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and by pipeting with multichannel pipets without touching reagent in plate.
	Insufficient washes	Increase number of washes.

Beads not in region or gate	Luminex not calibrated correctly or recently	Calibrate Luminex based on instrument 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.
	Instrument not washed or primed	Prime the Luminex 4 times to eliminate 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	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been vacuumed out, sensitivity may be low.
	Incubations done at incorrect temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	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.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
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.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for that particular analyte.
	Standard curve was saturated at higher end of curve	See above.
High variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are vacuumed out completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.

	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without splashing.
	Cross-well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.

REPLACEMENT REAGENTS	**Catalog #**
Cell Signaling Assay Buffer 1	43-010
EpiQuant Phosphotyrosine Detection Antibody (25X)	MPEQ-1100
Streptavidin-Phycoerythrin (SAPE)	45-001D
EpiQuant Phosphotyrosine Panel 1 Standard	MPEQ-8100
EpiQuant Phosphotyrosine Panel 1 Quality Controls 1 and 2	MPEQ-6100
96 well filter plate with plate sealers	MX-PLATE

EpiQuant Cell Signaling Antibody-Immobilized Beads:

EGFR (pY1110/1125)	EQEGFR-Y1110
p38MAPKα (pY181)	EQP38A-Y181
JNK1, 2, 3 (pY185/185/223)	EQJNK-Y185
Shc (pY349/350)	EQSHC-Y349
PDK1 (pY373/376)	EQPDK1-Y373
FAK (pY397/407)	EQFAK-Y397
Ezrin (pY477)	EQEZRIN-Y477
Plakoglobin (γ-catenin) (pY548)	EQPLAK-Y548
JAK3 (pY785)	EQJAK3-Y785
Raf 1 (pY340/341)	EQRAF1-Y340
PLCγ1 (pY1253)	EQPLCG1-Y1253
PLCγ2 (pY1197/1217)	EQPLCG2-Y1197
Cbl (pY700/731/774)	EQCBL-Y700
IRS1 (pY612)	EQIRS1-Y612
FAK (pY861)	EQFAK-Y861
SPTAN1 (pY1176)	EQSPTAN1-Y1176
Crk (pY221/239)	EQCRK-Y221
CUB domain containing protein 1 (pY734)	EQCDCP1-Y734
SH2 domain containing protein 3A (pY95)	EQSDCP3A-Y95
SH2 domain containing protein 3A (pY231)	EQSDCP3A-Y231
Interleukin 15 receptor α chain (pY227)	EQIL15RA-Y227

Growth hormone receptor (pY566)	EQGHR-Y566
Tyrosine protein kinase Tec (pY206)	EQTEC-Y206
WASP (pY291)	EQWASP-Y291
ITK (pY180)	EQITK-Y180
Hepatocyte growth factor receptor (c-met) (pY1003)	EQMET-Y1003
TfR (pY20)	EQTFR-Y20
SCF38 (pY20)	EQSCF38-Y20
HER2 (pY1023)	EQHER2-Y1023
Pl3Kp110δ (pY485)	EQPI3K-Y485
STAT3 (pY705)	EQSTAT3-Y705
Gab1 (pY285/307/317)	EQGAB1-Y285
LAT (pY255)	EQLAT-Y255
Fyb (pY697/651 (iso1/2))	EQFYB-Y697
Gab2 (pY266)	EQGAB2-Y266
Gab2 (pY584)	EQGAB2-Y584
VEGFR1 (pY1169)	EQVEGFR1-Y1169
FRS2 (pY436)	EQFRS2-Y436
RapGEF1 (GRF2) (pY504 (522 in iso1))	EQGRF2-Y504
ZAP70 (pY126)	EQZAP70-Y126

EpiQuant Prepared Lysates

EpiQuant Jurkat: Unstimulated Lysate	MPEQ-LY001
EpiQuant A431: Unstimulated Lysate	MPEQ-LY002
EpiQuant Jurkat: H_2O_2 Stimulated Lysate	MPEQ-LY003
EpiQuant A431: H_2O_2 Stimulated Lysate	MPEQ-LY004
EpiQuant A431: EGF Stimulated Lysate	MPEQ-LY005

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pM Standard (Background)	40 pM Standard	QC-2 Control									
B	0 pM Standard (Background)	40 pM Standard	QC-2 Control									
C	0.32 pM Standard	200 pM Standard										
D	0.32 pM Standard	200 pM Standard										
E	1.6 pM Standard	1,000 pM Standard										
F	1.6 pM Standard	1,000 pM Standard										
G	8 pM Standard	QC-1 Control										
H	8 pM Standard	QC-1 Control										