WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit

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About the Kit

Assay Overview

Bead-based flow cytometric xMAP® assays enable a sensitive and precise detection of multiple analytes within a single sample. The assays are in essence a multiplex of sandwich immunoassays on beads in solution. Samples are combined with differentially fluorescent-labeled microparticles (beads) covalently conjugated to a capture antibody. Analytes from the prepared samples are captured on these individually specific capture beads. The analytes are then quantified with the use of specific biotinylated detection antibodies and streptavidin-PE. A major hallmark of bead-based assays over traditional protein detection methods (such as ELISA) is that bead-based assays allow for the simultaneous detection of multiple analytes in a small sample volume. Other advantages of xMAP assays also include flexibility, robustness, simplicity of sample handling, and requirement of minimal sample volumes. Together, these characteristics constitute an ideal platform for the immunodetection of biomarkers and signaling proteins.

Target Overview

Receptor Tyrosine Kinases (RTKs) are important regulators of numerous cell signaling pathways and have been implicated in various disease states. Phosphorylation of RTKs play a critical role in the signaling cascades that regulate cell proliferation and development. More specifically, the Epidermal Growth Factor Receptor (EGFR) family of RTKs that includes EGFR (erbB1), HER2 (erbB2), HER3 and HER4 plays a key role in propagating signals regulating cell proliferation, differentiation, motility, and apoptosis. EGFR is a 175 kDa receptor tyrosine kinase that gets activated by ligands like the epidermal growth factor (EGF). EGF binding induces EGFR autophosphorylation at specific tyrosine residues. These phosphorylation events promote the docking of several SH2 domain-containing adaptor proteins to EGFR that, in turn, activate downstream signaling pathways involved in regulating cell growth and proliferation such as the Ras-mediated Mitogen Activated Protein Kinase (MAPK) signaling pathway, the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and the signal transduction and activator of transcription (STAT) pathway. In addition to autophosphorylated tyrosine residues, EGFR contains other phosphorylation sites including phosphothreonine and phosphoserine residues that are involved in cross-talk with other signaling pathways and EGFR down-regulation.

The WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit employs sandwich immunoassay methodology to detect any or all of the following sites on EGFR:

- Phosphorylated Thr654
- Phosphorylated Thr669
- Phosphorylated Tyr845
- Phosphorylated Tyr1045
- Phosphorylated Ser1047
- Phosphorylated Tyr1068
- Phosphorylated Tyr1086
- Phosphorylated Tyr1173
- total EGFR

Note: Depending on the tissue sample, cell type, and/or stimulation conditions, individual EGFR phosphorylation sites may be differentially phosphorylated. In the WideScreen™ BeadPlex™ assay, cellular protein samples are prepared by gentle membrane extraction as described in the solutions and protocol sections of this technical bulletin.

Components and Storage

The WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit contains the entire set of reagents to assay one 96-well plate, including a pre-selected set of WideScreen™ BeadPlex™ Total and Phospho-EGFR Capture Beads, a biotinylated Detection Antibody, a premix of various Control Peptides, Cell Extraction reagents, and a WideScreen™ Reagent Kit. The premix of various control peptides contain enough material to generate control data for 6 or 12 control wells in duplicate. Since these peptides do not mimic the phosphorylation site in the cellular context, these assays are not quantitative, and only allow for the qualitative comparison of site-specific EGFR phosphorylation in response to stimuli or inhibition.

For maximum flexibility, end users may configure their WideScreen™ BeadPlex™ Phospho-EGFR assays according to the following components.

WideScreen™ BeadKit™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit (Cat. No. 72254-3)			
Cat. No.	**Volume**	**Description**	**Storage**
72231-0.1ML	100 µl	Biotinylated-EGFR Detection Antibody	Store at 2-8°C
72220-0.1ML	100 µl	Phospho-EGFR (Thr654) Capture Beads	Store at 2-8°C
72221-0.1ML	100 µl	Phospho-EGFR (Thr669) Capture Beads	Store at 2-8°C
72222-0.1ML	100 µl	Phospho-EGFR (Tyr845) Capture Beads	Store at 2-8°C
72223-0.1ML	100 µl	Phospho-EGFR (Tyr1045) Capture Beads	Store at 2-8°C
72224-0.1ML	100 µl	Phospho-EGFR (Ser1047) Capture Beads	Store at 2-8°C
72225-0.1ML	100 µl	Phospho-EGFR (Tyr1068) Capture Beads	Store at 2-8°C
72226-0.1ML	100 µl	Phospho-EGFR (Tyr1086) Capture Beads	Store at 2-8°C
72228-0.1ML	100 µl	Phospho-EGFR (Tyr1173) Capture Beads	Store at 2-8°C
71905-0.1ML	100 µl	Total EGFR Capture Beads	Store at 2-8°C
72241-1.2ML	1.2 ml	phospho-EGFR Control Peptide Mixture	Store at -20°C
71927-25ML	25 ml	Extraction Reagent	Store at -20°C
524629-500UL	500 µl	Phosphatase Inhibitor Cocktail Set V (50X)	Store at -20°C
539134-25UL	25 µl	Protease Inhibitor Cocktail Set III (100X)	Store at -20°C
71206-2.5KUN	2.5 KUN	Benzonase® Nuclease HC, Purity >99% (250 U/µl)	Store at -20°C
71821-0.1ML	100 µl	Streptavidin-PE Concentrate	Store at 2-8°C
71824-20ML	20 ml	10X Wash Buffer	Store at 2-8°C
71823-25ML	25 ml	5X Assay Diluent	Store at 2-8°C
71871-1PKG	1 PKG	96-well Filter Plate and Polyethylene Plate Sealer	Store at 2-8°C

Additional Reagents and Equipment Required

- Experimental samples, such as cultured cell lines treated with or without stimulant
- Luminex® xMAP™ System (or comparable, such as Bio-Plex® Suspension Array System)
- xMAP data analysis software (e.g., Luminex IS™, xPonent 3.1, ACS StarStation, Bio-Plex Manager™, or comparable)
- Vacuum manifold for filter plates (Millipore Cat. No. MAVM0960R)
- 96-well plate platform shaker, such as IKA MTS4
- BCA protein assay kit (EMD Cat. No. 71285)
- Polypropylene microcentrifuge tubes
- 15 ml and 50 ml polypropylene centrifuge tubes
- Microcentrifuge
- Vortexer
- Ultrasonic bath, such as Cole Parmer EW-08849 (optional)
- Multichannel pipet (optional)
- Fixation solution (0.2% paraformaldehyde in PBS) (optional)
- Syringe-tip filter (0.45 µm) and syringe, or 96-well filter plate (e.g. Millipore #MSBVN6510) and 96-well collector plate
- Tris-buffered saline (TBS) (10 mM Tris, pH 7.5, 150 mM NaCl)

Growth of Cell Lines

Considerations Before You Begin

- Growth rate and requirements for optimal growth vary considerably between cell lines; even the same cell line will grow differently in different laboratories. The following conditions are intended as general guidelines only.
- Cells maintained in culture for long periods of time tend to exhibit slower growth rates and become refractory to stimulation conditions. In general, cell lines passaged <15 times are recommended.
- See *Supplementary Protocols* on p 15 for sample protocols for stimulation with growth factors in presence or absence of inhibitors.

Protocol for Growth of Cell Lines

1. Culture cells in T-75 flasks until steady growth is established. Most cell lines will tolerate a split of 1:10–1:20 without slowing their growth rate.
2. Culture adherent cells until they approach a confluent monolayer, or suspension cells until they approach 10^6 cells per ml. Slower-growing cell lines (such as A431) may initially take up to a week to approach confluency.
3. Plate cells, using the following table as a general guide. Harvest cells for lysate preparation after 2 or 3 days, depending on whether the cells are serum starved overnight before harvesting.

Table 1. Approximate Cell Numbers for Seeding Cell Lines

Cell Line	T-75 Flask or 10 cm Dish	6-well Plate (per well)	96-well Plate (per well)
A431	2.0×10^6	2.8×10^5	4.0×10^4
HeLa	1.2×10^6	1.7×10^5	1.5×10^4
HepG2	4.8×10^6	6.8×10^5	8.0×10^4
HT29	2.4×10^6	3.4×10^5	3.0×10^4
HUVEC	1.5×10^5 *	2.0×10^5	not recommended
NHDF	1.5×10^5 *	1.5×10^5	not recommended
SK-Br-3	2.0×10^6	2.8×10^5	3.0×10^4
Jurkat	1.0×10^6	1.4×10^5	1.5×10^4

* HUVEC and NHDF cells plated with these cell numbers are serum-starved after 6 days and lysed after 7 days.

Note: If cells are grown in 96-well plates, plate extra wells for determining total protein concentration of the lysates.

Note: If cells will be stimulated prior to extraction, serum-starve them for 4–16 h before stimulation. See Supplementary Protocols *on p 15 for sample protocols for growth factor stimulation in presence or absence of inhibitor treatment.*

4. Prepare lysates when cell density is high, but cells are still growing logarithmically. For adherent cells, this is typically a monolayer that is ~ 80% confluent. For suspension cells, this is typically a density of 0.5–1.0 x 10^6 per ml.

Lysate Preparation

Considerations Before You Begin

- Lyse induced and uninduced cells at the same time.
- Do not omit steps from the sample preparation protocol. All steps are necessary for optimum assay performance.
- If it is important to know the lysate protein concentration from cells grown in 96-well plates, prepare additional wells of cells solely for this purpose.
- If using cells grown in 96-well plates, avoid plating cells in the outermost rows and columns. This minimizes cell growth edge effects.

Lysis Protocol for Cell Lines

1. Prepare 1X Assay Diluent by adding 25 ml of the included 5X Assay Diluent to 100 ml sterile distilled deionized water. Store 1X Assay Diluent that will be used within one month at 4°C. To avoid microbial growth, dispense aliquots of any remaining 1X Assay Diluent and store at –20°C.
2. Prepare 1X Wash Buffer by adding 20 ml 10X Wash Buffer (WideScreen™ Reagent Kit) to 180 ml sterile distilled deionized water. Store at 4°C.
3. Calculate the total amount of Extraction Reagent needed. Prepare 10% excess to account for pipetting error.

Format	Extraction Reagent
T-175 flask	4 ml
T-75 flask	2 ml
T-25 flask	1 ml
6-well	200 µl/ well
96-well	120 µl/ well

4. Prepare the required volume of supplemented Extraction Reagent:

   Per ml Extraction Reagent, add:

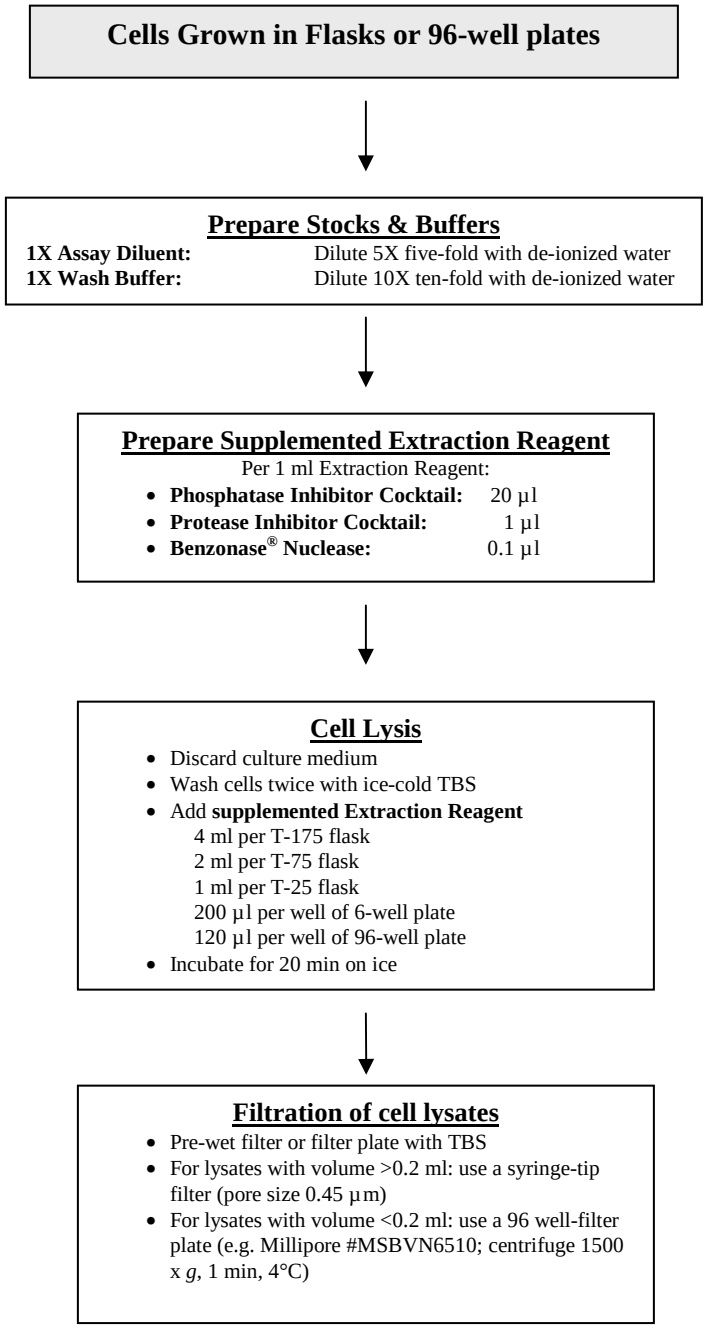
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)
1 µl	Protease Inhibitor Cocktail III (1000X)
0.1 µl	Benzonase® Nuclease

   Note: Prepare fresh supplemented Extraction Reagent each time cell lysates are made.

5. Aspirate and discard culture medium.
6. On ice, rinse cell monolayer twice with cold Tris-buffered saline (TBS). Remove all TBS.
   For non-adherent cells: transfer cells to centrifuge tubes, centrifuge at 500 x *g*, and wash twice with ice-cold TBS.
7. Add cold supplemented Extraction Reagent to adherent cells. Incubate for 20 min at 4°C with gentle agitation (rocking platform or occasional swirling).
   For non-adherent cells: flick pellet to loosen. Add supplemented Extraction Reagent.
   Incubate for 20 min at 4°C with occasional vortexing.
8. Dislodge and solubilize all adherent cells using a rubber policeman or by repeated pipeting. Extracts should be clear and non-viscous.
9. Clear lysates by filtration. Pre-wet filter or filter plate with TBS, then remove all excess buffer. For lysates with volume >0.2 ml, use syringe-tip filter (pore size 0.45 µm). For lysates with volume < 0.2 ml, use a 96 well-filter plate (e.g. Millipore #MSBVN1210). Filter the lysates by centrifugation at 1500 x *g* for 1 min at 4°C. Place a 96-well plate under the filter plate during centrifugation to collect lysates.)
10. Either proceed immediately to the Bead-based Immunoassay Protocol, or store aliquots at –70°C. Avoid multiple freeze-thaw cycles.
11. Remove a 50 µl aliquot of each extract for protein quantification by BCA Protein Assay (Cat. No. 71285). Determine the total protein concentration of each extract.

    Note: Typical total protein concentrations from cells grown in flasks range from 0.4 mg/ml to 2 mg/ml, depending on the cell line and confluence. Typical total protein concentrations from cells grown in 96-well plates range from 0.1–0.5 mg/ml.

Flowchart for Lysate Preparation



WideScreen™ BeadPlex™ Immunoassay Protocol

Considerations Before You Begin

- Have on hand the 1X Assay Diluent and 1X Wash Buffer that were prepared during the Lysate Preparation protocol.
- Important guidelines to follow when using filter plates and the vacuum manifold:
- Excessive vacuum will cause the filter plate membrane to perforate. Adjust the manifold using a non-filter (ELISA or tissue culture) plate, ensuring that the vacuum cannot exceed 5 in (127 mm) Hg.
- After adjusting the vacuum, place filter plate on the manifold. Use fingertips to apply pressure evenly across the plate. The liquid should drain in 2–5 s.
- To avoid drying out the beads, vacuum only long enough to drain all wells. Do not allow drained beads to sit for more than 1 min before rehydrating with buffer.
- It is critical to remove excess buffer from the underside of the filter plate by tapping it on a paper towel several times before adding samples or reagents. This prevents samples from wicking out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations.
- To avoid perforating the filter plate membrane, be sure that the probe height on the xMAP® system is adjusted correctly. Do not touch the membrane with pipet tips. For accurate pipetting, touch tips to the sides of the filter plate wells. Change tips as necessary to prevent cross-contamination.
- The antibody-conjugated Capture Beads contain fluorescent dyes and are therefore light-sensitive. To avoid photo-bleaching, protect the beads from light. Cover filter plates containing beads with aluminum foil during incubation steps. Streptavidin-PE is also light-sensitive must be protected from light.
- To prevent fluorescent dye loss, do not use organic solvents with capture beads. Beads are incompatible with DMSO concentrations >1%.
- Many of the washing and preparation of aliquots steps are done most easily with an 8-channel or 12-channel pipet (manual or automatic). However, for best results, use accurate single-channel pipets for manipulation of experimental samples.
- If using multichannel pipets, ensure that tips fit correctly. Verify volume accuracy and consistency.
- To conduct the protocol efficiently, prepare reagents for the next step during incubation periods.
- When calculating the amount of reagents needed during the various steps, prepare 10% excess to allow for pipetting error.
- For best overall assay performance, lysates are diluted at least 4-fold when incubating with the Capture Beads. If desired, lysates can be tested at a 2-fold final dilution, although this concentration of Lysis Buffer decreases the sensitivity of some Bead Kits. If a 2-fold final dilution is used, change the titration buffer composition to 50% Lysis Buffer/50% 1X Assay Diluent to ensure accurate measurement. Final dilutions less than 2-fold are not recommended.

Step 1: Prepare Titration Buffer

Immunoassays are sensitive to buffer composition. Therefore, include the same proportion of Extraction Reagent in all dilutions of samples and controls. The best overall assay performance occurs when lysates are diluted at least 4-fold when incubated with the Capture Beads. Titration buffer as described here (25% Extraction Reagent, 75% 1X Assay Diluent) maintains a 4-fold final dilution of Extraction Reagent in all assay wells.

Note: Prepare fresh titration buffer for each assay.

1. Calculate the total amount of Titration Buffer needed. A minimum of ~300 µl titration buffer is needed for each lysate sample that is diluted more than 4-fold final (see optional steps in *Step 2: Prepare Sample Dilutions* on p 9).

Sample Calculation: 30 Diluted lysate samples = 9000 µl (30 X 300 µl)
2 Blanks = 200 µl (2 X 100 µl)

Make at least **10,000** µl titration buffer

2. Prepare the required volume titration buffer by mixing Extraction Reagent and 1X Assay Diluent prepared from the buffers included. Use a ratio of 25% Extraction Reagent to 75% 1X Assay Diluent. In the example above, take 2,500 µl Extraction Reagent + 7,500 µl 1X Assay Diluent = 10,000 µl Titration Buffer (preparing additional buffer to account for pipetting error).

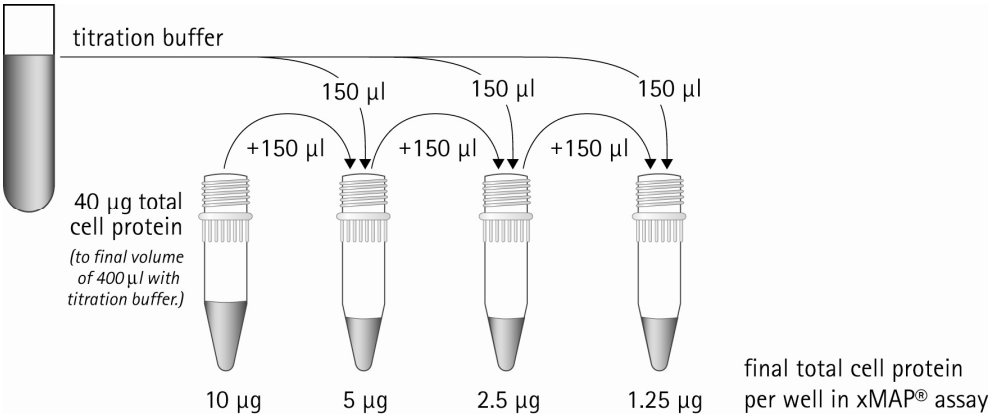
Step 2: Prepare Sample Dilutions

Note: Thaw and (if applicable) dilute samples within 1 hr of use. Avoid multiple freeze/thaw cycles.
96-well samples can be diluted 4-fold with 1X Assay Diluent in the immunoassay-plate later in the protocol (see Step 5: Combine Capture Beads with Analytes *on p 12).*

1. Dilute lysate samples four-fold in 1X Assay Diluent (e.g., 100 µl lysate with 300 µl 1X Assay Diluent). Mix well.
2. Calculate the protein concentration of the four-fold diluted lysate samples based on the protein quantification values previously determined using BCA assay. For example, if the original sample concentration was 1.6 mg/ml, the dilution results in 400 µg/ml.

   Note: If desired, cell extracts can be further diluted to ensure more accurate signals. In this case, follow the optional steps below (Steps 3–5 within this section). A range from 1–10 µg total cell protein per assay well is usually optimal.

3. Label four microcentrifuge tubes. In tube 1, mix the four-fold diluted lysate and titration buffer to a final volume of 400 µl and final protein concentration of 100 µg/ml (10 µg/well later in the assay). For example, if the four-fold diluted extract has a total protein concentration of 400 µg/ml, mix 100 µl diluted extract with 300 µl titration buffer.
4. If additional dilutions of the extract are desired, prepare three additional 2-fold dilutions of the cell extract, as follows:
   - Add 150 µl titration buffer to tubes 2, 3, and 4.
   - Transfer 150 µl from tube 1 to the 150 µl titration buffer in tube 2 and mix well.
   - Change tips. Transfer 150 µl from tube 2 to the 150 µl titration buffer in tube 3. Mix well.
   - Proceed in similar fashion with the serial dilutions through tube 4.
5. These dilutions will result in 10 µg, 5 µg, 2.5 µg,or 1.25 µg total cell protein per assay well, respectively (refer to figure below).



Step 3: Prepare Control-Peptide solution

1. Resuspend the lyophilized control-peptide mixture in 1200 µl deionized water. Mix the vial by vortexing at medium speed for 10–15 s. Store at 4°C until use.
2. 100µl per well of reconstituted control-peptide solution will be used later in the assay.
3. Process the Control Peptide samples similar to the test samples as per *Step 5: Combine Capture Beads with Analytes* on p 11.

Note: Reconstituted control peptide solution can be stored at –80°C.

Note: Since the WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit includes control peptides that do not mimic the phosphorylation site in the cellular context, these assays are not quantitative. These assays allow only the qualitative comparison of site-specific EGFR phosphorylation signal in response to different stimuli or inhibition.

Step 4: Prepare WideScreen™ BeadPlex™ Capture Beads

Note: WideScreen™ BeadPlex™ Capture Beads can be multiplexed in all combinations.

Note: Prepare diluted Capture Beads within 1 h of use.

1. Calculate the number of test wells needed, allowing ~10% extra for pipetting error.
2. Note the volume of 50X Capture Beads needed per well, based on the assay format. In all cases, this results in 3,000 beads per bead region per well.

Assay Format	Vol. Capture Beads (50X) needed
Singleplex (one target)	1 µl per well
User-assembled multiplex	1 µl from each individual Bead Kit per well

3. Thoroughly re-suspend each vial of Capture Beads (50X) by vortexing for 10 s, sonicating in an ultrasonic bath for 10 s, and vortexing again for 5 s.
4. Each well receives a total of 50 µl diluted (1X) Capture Beads. Determine the total volume of 50X Capture Beads needed per well (refer to table above) and the volume of 1X Assay Diluent needed to bring the total volume per well to 50 µl. Multiply these volumes by the number of test wells to determine the total volumes of each component needed. Refer to the following table for example calculations.
5. Add the calculated volumes of Capture Beads (50X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 s. Protect from light and store at 4°C until use.

Example Calculations: **(40 test wells, including 10% extra)**

	Singleplex	User-assembled multiplex (e.g., 5-plex)
Test wells	40	40
Volume Capture Beads (50X)	1 µl per well	1 µl each bead x five different 50X bead types= 5 µl total of five different 50X beads types per well
Volume 1X Assay Diluent	49 µl per well	45 µl per well
Total Volume Capture Beads (50X)	1 µl beads per well x 40 wells **= 40 µl beads**	5 µl of five different 50X bead types per well x 40 wells **= 200 µl total of five different 50X beads**
Total Volume 1X Assay Diluent	49 µl per well x 40 wells **= 1960 µl**	45 µl per well x 40 wells **= 1800 µl**

Step 5: Combine Capture Beads with Analytes

1. On the 96-well filter plate, tape off wells that are not going to be used for the assay with the provided Plate Sealer (cut to size) or lab tape for future use.
2. Pre-wet the 96-well filter plate for at least 5 min by adding 50 µl 1X Assay Diluent to each well that will be used. With the vacuum manifold, apply gentle vacuum (3 in Hg/76 mm Hg) to filter plate just until liquid aspiration is complete. Tap filter plate on a paper towel to remove any buffer on the underside.

   Note: It is critical to remove excess buffer from the underside of the filter plate before adding samples or reagents. Otherwise, samples may wick out of the wells during incubation steps. For the same reason, avoid placing filter plate on an absorbent surface during incubations. See Considerations Before You Begin *on p 8 for guidelines on using the filter plate vacuum and manifold.*
3. Vortex (10 s) the diluted Capture Beads solution prepared as per *Step 4: Prepare Capture Beads* on p 11. Add 50 µl to each well being used.
4. Remove liquid from filter plate by vacuum filtration.
5. To bead-containing wells reserved for the controls, add 100 µl from the peptide premix prepared as per *Step 3: Prepare Control-Peptide solution* on p 11 to each well.
6. To bead-containing wells reserved for analyzing experimental samples, add 100 µl diluted samples prepared as per *Step 2: Prepare Sample Dilutions* on p 10. If additional sample dilutions were prepared (optional), add 100 µl of these dilutions to bead-containing wells.

   Note: If working with samples generated from cells grown in 96-well plates, dilute them directly four-fold with 1x Assay Diluent in the immunoassay plate. Add 75 µl l 1X Assay Diluent and 25 µl cell lysate directly to the appropriate wells of the 96-well filter plate. For convenience, we recommend using a multichannel pipet.
7. Incubate overnight at 4°C on a platform plate shaker (750 rpm). Use aluminum foil to protect filter plate from light. *Note: Shorter incubations are possible, but will decrease overall signal strength.*

Step 6: Add Detection Antibody

Note: Prepare 1X Detection Antibody solution within 1 h of use.

1. Calculate the number of test wells needed, allowing 10% extra for pipetting error.
2. Each well receives a total volume of 100 µl diluted (1X) Detection Antibody solution. Determine the total volume of 100X Detection Antibody needed per well (refer to the table below) and the volume of 1X Assay Diluent needed to bring the total volume per well to 100 µl. Multiply these volumes by the number of test wells to determine the total volumes of each component needed. Refer to the table below for example calculations.
3. Add the calculated volumes of Detection Antibody (100X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 s and store at 4°C until use.

Example Calculations:(40 test wells, including 10% extra)

	Singleplex, *or* User-assembled multiplex (e.g., 5-plex)
Test wells	40
Volume Detection Antibody (100X)	1 µl per well
Volume 1X Assay Diluent	99 µl per well
Total Volume Detection Antibody (100X)	1 µl Antibody per well x 40 wells **= 40 µl Detection Antibody**
Total Volume 1X Assay Diluent	99 µl per well x 40 wells **= 3960 µl Assay Diluent**

4. Remove liquid from filter plate by vacuum filtration.
5. Add 100 µl 1X Wash Buffer to each well. Remove liquid by vacuum filtration. Repeat wash and filtration steps twice more, for a total of three washes. Tap filter plate on a paper towel to remove any buffer on the underside.

Note: Do not allow the beads to dry out. Vacuum only long enough to remove all liquid. Add the next solution immediately after tapping filter plate on a paper towel.

6. Immediately add 100 µl 1X Detection Antibody solution to each well (sample and control) and add 100 µl 1X Assay Diluent to blank wells.
7. Incubate for 1 h at room temperature on a platform plate shaker (750 rpm). Protect from light.

   Note: Turn on the Luminex® system. The lasers require a 30 min warm-up period.

Step 7: Add Streptavidin-Phycoerythrin (PE)

Note: Prepare 1X Streptavidin-PE solution within 30 min of use.

1. Briefly spin the tubes containing the 100X Streptavidin-PE to collect reagent that might be trapped in the tube cap.
2. Calculate the total volume of 1X Streptavidin-PE solution required. 100 µl is needed for each test well.
3. Prepare the calculated volume of 1X Streptavidin-PE solution by diluting Streptavidin-PE Concentrate 1/100 in 1X Assay Diluent. Vortex 3 s. Protect from light and store at 4°C until use. Refer to the table below for example calculations.

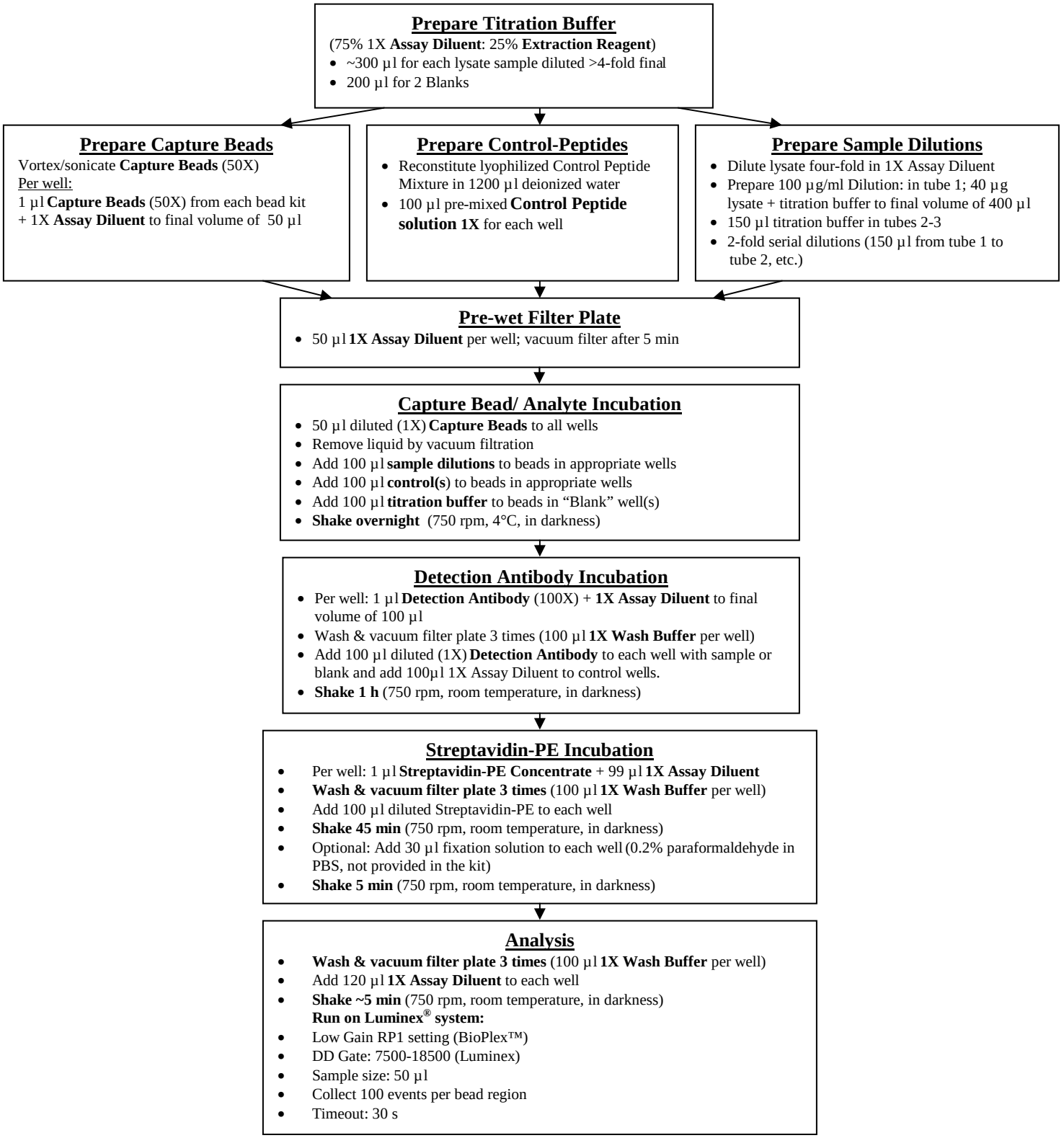
Example Calculations: **(40 test wells, including 10% extra)**

	Singleplex, *or* User-assembled multiplex (e.g., 5-plex)
Test wells	40
Volume Streptavidin-PE (100X)	1 µl per well
Volume 1X Assay Diluent	99 µl per well
Total Volume Streptavidin-PE (100X)	1 µl Antibody per well x 40 wells **= 40 µl Streptavidin-PE**
Total Volume 1X Assay Diluent	99 µl per well x 40 wells **= 3960 µl Assay Diluent**

4. Wash wells three times with 1X Wash Buffer as described above. After the final vacuum filtration, tap filter plate on a paper towel to remove any buffer on the underside.
5. Immediately add 100 µl 1X Streptavidin-PE solution to each well.
6. Incubate for 45 min at room temperature on a platform plate shaker (750 rpm). Protect from light.
7. Optional: Add 30 µl fixation solution to each well (0.2% paraformaldehyde in PBS, not provided in the kit). Incubate for 5 min at room temperature on a platform plate shaker (750 rpm). Protect from light.

   Note: Fixation will improve well-to-well assay reproducibility.
8. Wash wells three times with 1X Wash Buffer as described above. After the final vacuum filtration, tap filter plate on a paper towel to remove any buffer on the underside.
9. Immediately add 120 µl 1X Assay Diluent to the beads in each well. To fully resuspend beads before running samples on the Luminex® system, incubate for 3-5 min on a platform plate shaker (750 rpm). Protect from light.
10. Analyze samples with a Luminex system according to the manufacturer's instructions.

Flowchart for WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel Immunoassay Protocol



Collecting Data and Data Analysis

Data Acquisition

For detailed instructions on the operation of Luminex® systems, refer to the user guide for your specific software and instrument. General recommendations are given below.

1. Using your Luminex system software, prepare a Protocol for the assay you will run. Enter in information for each Bead Kit target, and for the samples and controls that will be run.
2. Select the bead regions used in the assay. The bead regions used for the WideScreen™ BeadPlex™ Phospho-EGFR Bead Kits are shown in Appendix A (see p 30).
3. Format the assay plate, indicating which wells contain which type of analyte.
4. Acquire data using the system settings shown below:

Software	Sample Size	Events per Bead Region	Timeout	Doublet Discriminator	CAL2 Gain Setting
Luminex® 100 IS™	50 µl	100	30 s	7500-18500	default
ACS StarStation	50 µl	100	30 s	default	default
Bio-Plex® Manager™	50 µl	100	default (60 s)	default (4335–10000)	RP1 Low

Data Analyses

- Since the WideScreen™ BeadPlex™ Phospho-EGFR Profiling 9-Plex Complete Assay Kit uses control peptides that do not mimic the phosphorylation site in the cellular context, these assays are not quantitative. These assays allow only the qualitative comparison of site-specific EGFR phosphorylation signal in response to different stimuli or inhibition.
- The signal readout is Median Fluorescent Intensity (MFI). This is a relative value to compare signal between unstimulated cell lysates and stimulated cell lysates.
- Measurements of the blank are useful for assessing background. However, it is not necessary to subtract the MFI value of the blank from other measurements.

Supplementary Protocols

Considerations Before You Begin

Include appropriate positive and negative controls whenever possible. Increases in target protein phosphorylation can be demonstrated by comparison to unstimulated cells, cells treated with growth factors or inhibitors, or by treating cell extracts with lambda protein phosphatase. When treating with lambda protein phosphatase, Phosphatase Inhibitor Cocktail should not be added to the Extraction Reagent.

Alternative 1: Stimulation of Cell Lines (in absence of inhibitor treatment)

Note: Have all reagents for cell extraction ready before inducing cells.

1. Prepare induction medium by diluting all growth factor stocks to each individual final concentration in tissue culture medium lacking fetal bovine serum (FBS) (refer to the table below). This results in a 1X solution.
   Growth factors can be added separately, with each of the following added to a separate T-75 flask.
   Alternatively, all growth factors can be added simultaneously to one T-75 flask. In either case, use 5ml induction medium per T-75 flask. For mock inductions, prepare tissue culture medium lacking FBS and growth factors.

Growth factor reconstitution and dilution 1X

Growth factor	Cat. No.	Reconstitution	Stock solution	Final Concentration in 1X Induction Medium	Incubation time
EGF	#324831	10 mM acetic acid, 0.1% BSA	100 µg/ml	100 ng/ml	10 min
PMA	#524400	DMSO	10 mg/ml	1000 ng/ml	60 min
Vanadate* (Sodium Orthovanadate)	#567540	1) Reconstitute Vanadate in water to a final concentration of 200mM 2) Adjust solution to pH 10 (with NaOH/HCl). The solution becomes coloured. 3) Boil solution for ~10min until it gets transparent 4) Let solution cool down to room temperature and adjust again to pH 10 5) Repeat this cycle for 2-3 times.	200 mM		
Vanadate/ H_2O_2		1) Dilute 30% H_2O_2 1:88 to 100mM 2) Dilute reconstituted Vanadate 1:2 to 100mM 3) Mix diluted Vanadate and diluted H_2O_2 in equal volumes	100 mM	10 mM	10 min

* Vanadate (Natrium-Orthovanadate) can be converted to pervanadate by hydrogen peroxide. Vanadate-Hydrogen-Peroxide-Mix should be used for stimulation to get a higher signal (MFI).

2. Following serum starvation, remove medium. Add 1X induction medium (or mock induction medium) to starved cells. Immediately return cells to incubator.
3. Incubate at 37°C and 5% CO_2 for each individual time (refer to table above)
   Note: Phosphorylation of many signaling pathway proteins peaks at 5–10 min, followed by rapid dephosphorylation.
4. Extract cells immediately according to the Lysate Preparation protocol (p 6).

Alternative 2: Inhibition and Subsequent Stimulation of Cell Lines

Note: Have all reagents for cell extraction ready before inhibiting and inducing cells.

1. Reconstitute inhibitor in DMSO according to the manufacturer's instructions. Prepare inhibition medium by diluting the inhibitor stock to the desired concentration in 5 ml tissue culture medium lacking FBS per T-75 flask. For mock inhibitions, prepare serum-free tissue culture medium lacking inhibitors, but including an equivalent volume DMSO.
2. Following serum starvation, remove medium. Replace with inhibition medium (or mock inhibition medium). Immediately return cells to incubator.
3. Incubate at 37°C and 5% CO_2 for 1 h.
4. Prepare induction medium by diluting growth factor stocks to each individual final concentration in tissue culture medium lacking FBS (refer to table below). This results in a 2X solution.
   Use 5 ml induction medium per T- 75 flask. For mock inductions, prepare tissue culture medium lacking FBS and growth factors.

Growth factor reconstitution and dilution 2X

Growth factor	Cat. No.	Reconstitution	Stock solution	Final Concentration in 2X Induction Medium	Incubation time
EGF	#324831	10 mM acetic acid, 0.1% BSA	100 µg/ml	200 ng/ml	10 min

5. Add 2X induction medium (or mock induction medium) directly to inhibitor-treated cells. Immediately return cells to incubator.
6. Incubate at 37°C and 5% CO_2 for 10 min.

   Note: Phosphorylation of many signaling pathway proteins peaks at 5-10 min, followed by rapid dephosphorylation.
7. Extract cells immediately according to the Lysate Preparation protocol (p 6).

Sample Data and Results

Note: All data and results shown below are representative and not lot specific.

Specificity Performance Data

Figure 1: Inhibitor Profiling of WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit
MDA-MB-231 cells were cultured in 96-well plates, serum-starved overnight, and treated with increasing amounts of the inhibitor PD168393 for 1 h. Following growth factor stimulation with EGF, cell lysates were prepared and assayed using the WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay kit. Data shown are averaged Median Fluorescence Intensity (MFI) signals obtained from triplicate cell culture wells, expressed as % of the DMSO-treated positive control. The data shows that the EGFR kinase inhibitor PD168393 has influence on the phosphorylation of tyrosine sites of EGFR but not on the phosphorylation of threonine nor serine sites of EGFR.

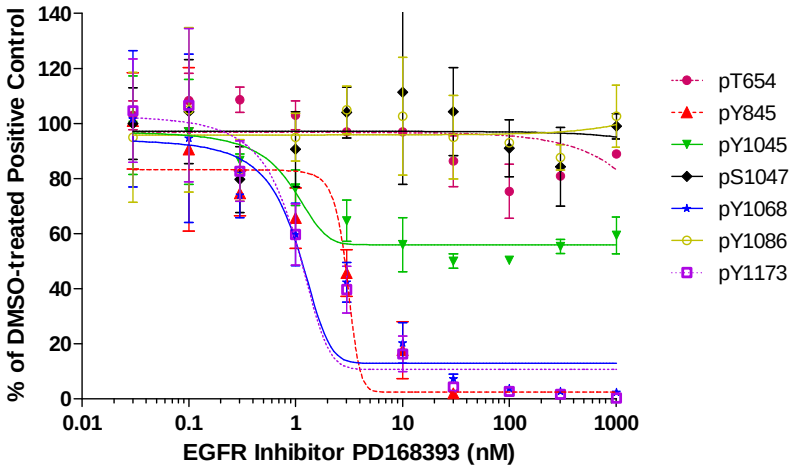
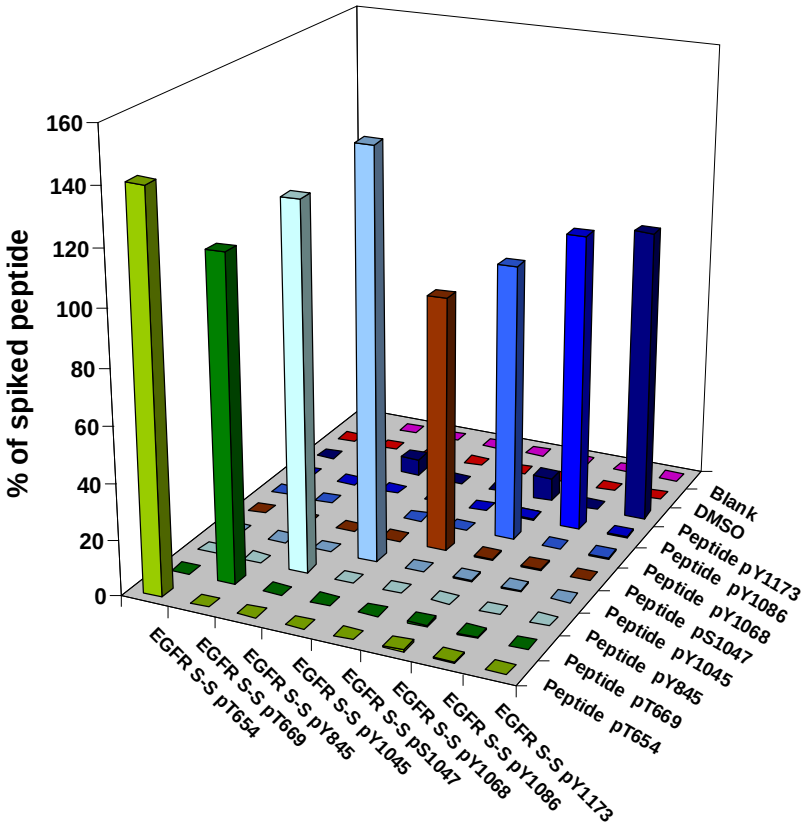


Figure 2: Sequence-Specificity of the WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit

The WideScreen™ BeadPlex™ Phospho-EGFR Profiling Panel 9-Plex Complete Assay Kit was tested for cross-recognition against the peptides from all of the individual phospho-specific Widescreen EGFR Capture Beads. Cross-reactivity was less than 1% in most cases with the exception that the Phospho-EGFR (Tyr845) Capture Bead showed 6% cross-reactivity with the Phospho-EGFR (Tyr1173) peptide and the phospho-EGFR (Tyr1068) Capture Bead showed 8.6% cross-reactivity with the phospho-EGFR (Tyr1173) peptide.



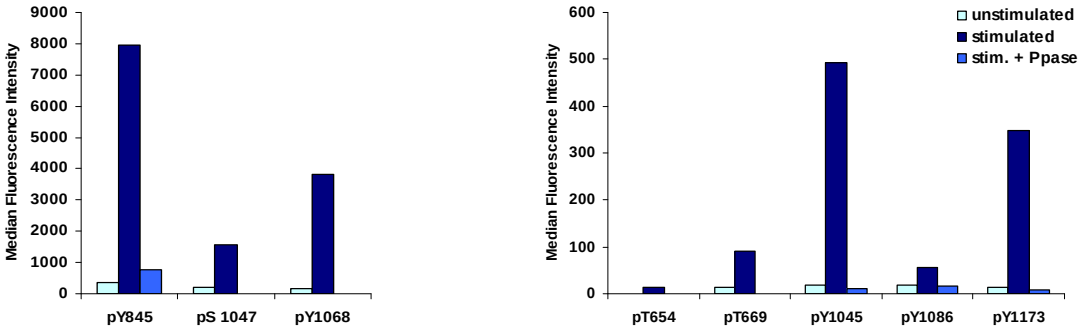
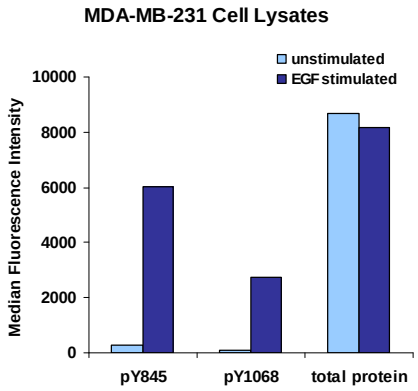
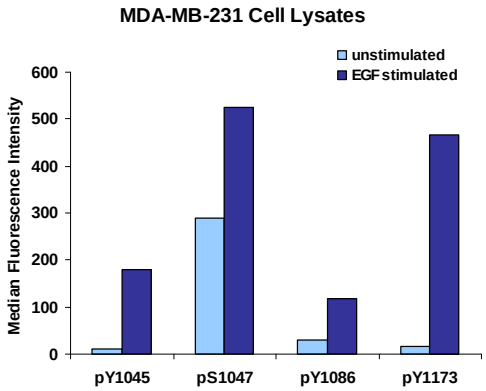
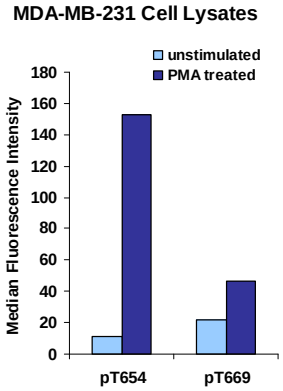


Figure 3: Phospho-Specificity of the Widescreen® BeadPlex™ Phospho-EGFR Profiling 9-Plex Assay Kit

Unstimulated and Vanadate/H_2O_2-stimulated MDA-MB-231 cell lysates (with or without subsequent dephosphorylation) with Lambda Phosphatase (denoted stim.+Ppase in the graph above) were profiled using the Widescreen™ BeadPlex™ Phospho-EGFR Profiling Assay Panel. Treatment of stimulated cell lysates with 1000U of phosphatase resulted in a decrease in signal. The decrease of signal in the phosphatase treated stimulated cell lysate demonstrates the phospho-specificity of the Widescreen™ BeadPlex™ Phospho-EGFR Profiling Assay Panel. Specific detection of low levels of phosphorylated EGFR in unstimulated cells is also shown. Representative data is shown in Median Fluorescence Intensity (MFI) units.







	A431		MDA-MB-231		SK-BR-3		HepG2	
	Unstim. Cell Lysate Signal (MFI)	**EGF stim. Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**EGF stim. Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**EGF stim. Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**EGF stim. Cell Lysate Signal** (MFI)
pY845	5609	9061	293	6008	912	3642	188	1268
pY1068	2639	8330	82	2761	1052	4909	42	350
total protein	11196	10507	8663	8162	8371	7959	3399	3547
pY1045	266	3270	10	179	35	1231	3	24
pS1047	4300	4574	289	526	198	579	182	281
pY1086	126	342	29	117	18	73	3	12
pY1173	333	3502	15	467	53	1094	5	24

	Unstim. Cell Lysate Signal (MFI)	**PMA treated Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**PMA treated Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**PMA treated Cell Lysate Signal** (MFI)	**Unstim. Cell Lysate Signal** (MFI)	**PMA treated Cell Lysate Signal** (MFI)
pT654	168	610	11	153	79	161	1	26
pT669	328	451	22	47	23	25	1	6

Figure 4: EGFR phosphorylation profiling of cell lines and stimulation
Serum-starved A431, MDA-MB-231, SK-BR-3 and HepG2 cell lines were stimulated with EGF or PMA as shown above. Lysates were prepared according to the WideScreen™ BeadPlex™ ImmunoAssay Protocol (p 8). Ten micrograms of total cell protein were analyzed per well. Representative multiplex data are shown in Median Fluorescence Intensity (MFI) units.

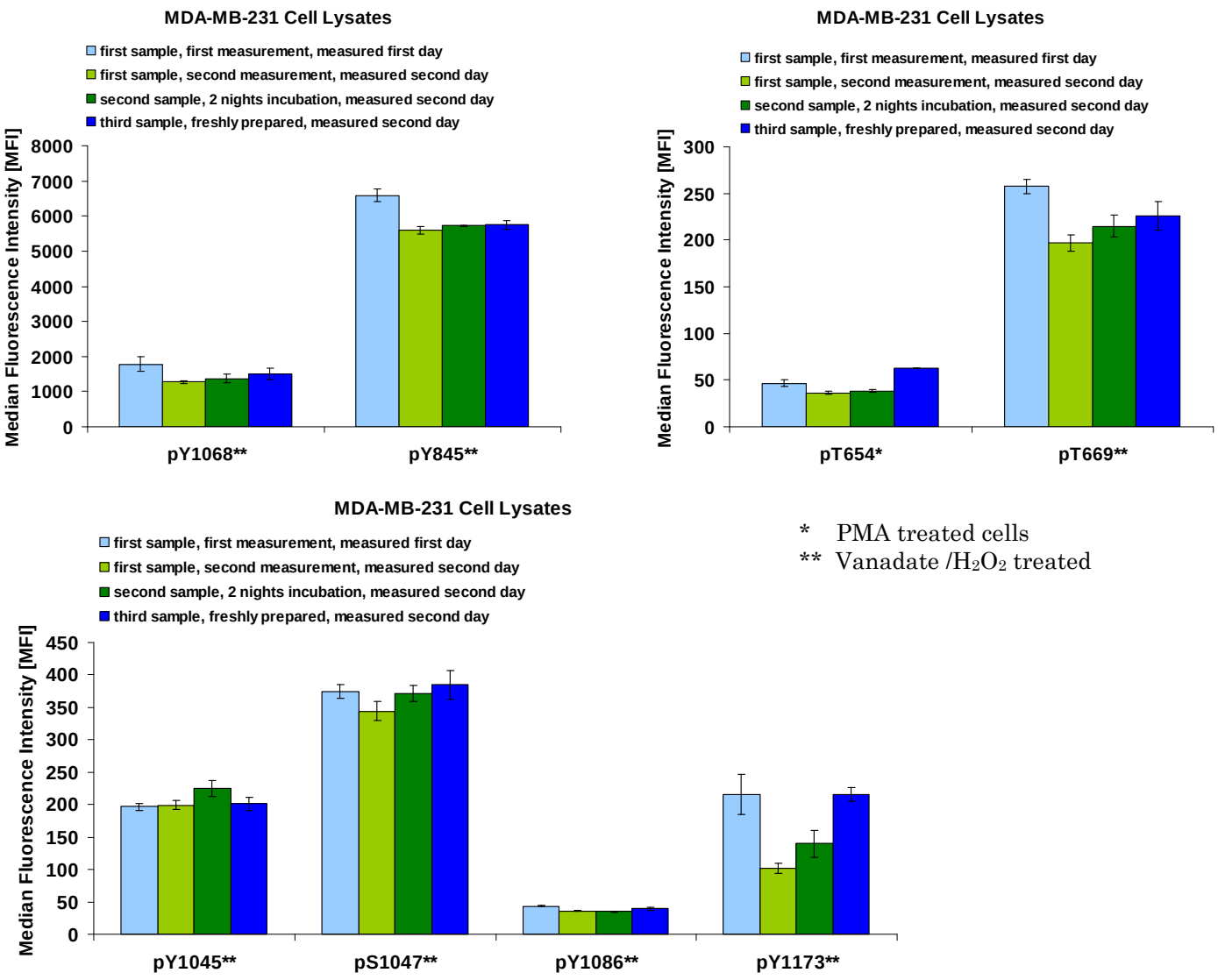


Figure 5: Proof of Principle for Multiplexing the WideScreen™ BeadPlex™ Phospho-EGFR Assay Kit

Serum-starved MDA-MB-231 cells were stimulated with PMA or Vanadate/ H_2O_2 as shown.
To demonstrate the proof of principle that multiple WideScreen™ BeadPlex™ Phospho-EGFR Capture Beads can be used in multiplex, site-specific phosphorylated EGFRs from the same cell lysate were assayed three times in accordance with the WideScreen™ BeadPlex™ ImmunoAssay Protocol (p 8). In the first assay, 10 µg of total cellular protein per well were incubated with a mixture of all eight WideScreen™ BeadPlex™ Phospho-EGFR pairs and the levels of each site-specific phosphorylated EGFR were measured on the same day (first sample, first measurement, measured first day). Levels of each site-specific phoshorylated EGFR are measured in Median Flourescence Intensity (MFI) units using a Luminex® xMAP® system and xMAP® software. In the second assay, an additional 10 µg of total cellular protein per well were incubated overnight with another batch of all eight WideScreen™ BeadPlex™ Phospho-EGFR pairs and MFI levels for each site-specific phosphorylated EGFR were determined the next day (first sample, second measurement, measured second day). In the third assay, 10 µg were incubated overnight without any capture beads as an appropriate control (results not shown above), while another 10 µg were incubated for two nights with all eight WideScreen™ BeadPlex™ Phospho-EGFR pairs to demonstrate that phosphorylation levels do not decrease during the incubation process (second sample, 2 nights incubation, measured second day). As an additional control, a fresh sample was prepared and phosphorylation levels were measured on the second day (third sample, freshly prepared, measured second day). The data shows no significant effects between sequential measurements of the same Phospho-EGFR site indicating that product performance is not affected by the respective assay conditions and therefore, it is possible to multiplex several WideScreen™ BeadPlex™ Phospho-EGFR capture beads.

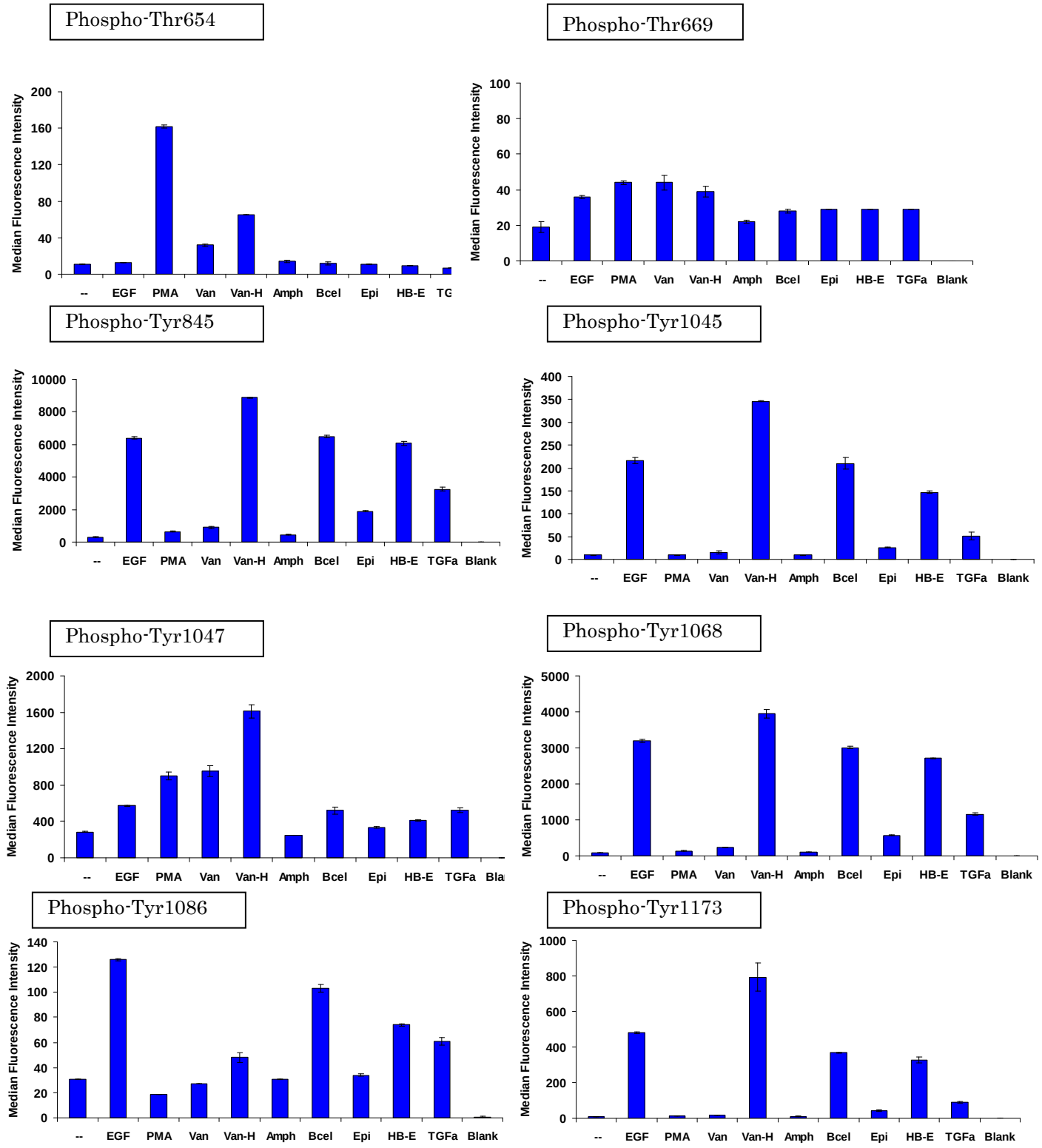


Figure 6: Stimulation dependent Specificity

The WideScreen™ BeadPlex™ Phospho-EGFR Profiling assay was tested with MDA-MB-231 cells stimulated with various growth factors that included Epidermal Growth Factor (EGF), Phorbol 12-myristate 13-acetate (PMA), Vanadate (Van), Vanadate/H_2O_2 (Van-H), Amphiregulin (Amph), Betacellulin (Bcel), Epiregulin (Epi), Heparin Binding EGF (HB-E), or Transforming Growth Factor α (TGFα)].

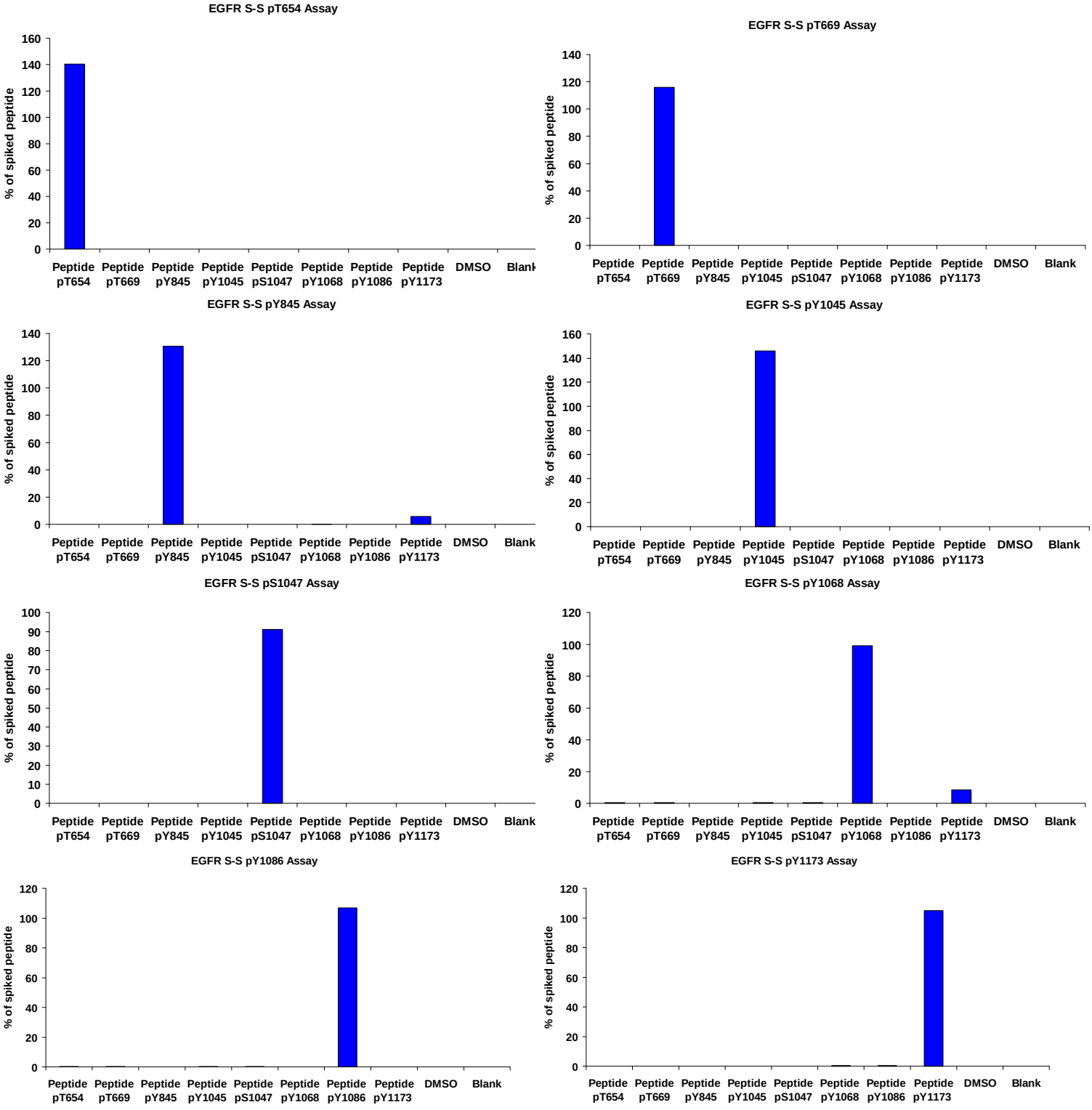


Figure 7: Sequence-Specificity
The Widescreen™ BeadPlex™ Phospho-EGFR Profiling assay kit was tested for cross-recognition against the peptides from all of the Site-Specific Phospho-EGFR bead sets. Cross-reactivity was less than 1% in all cases.

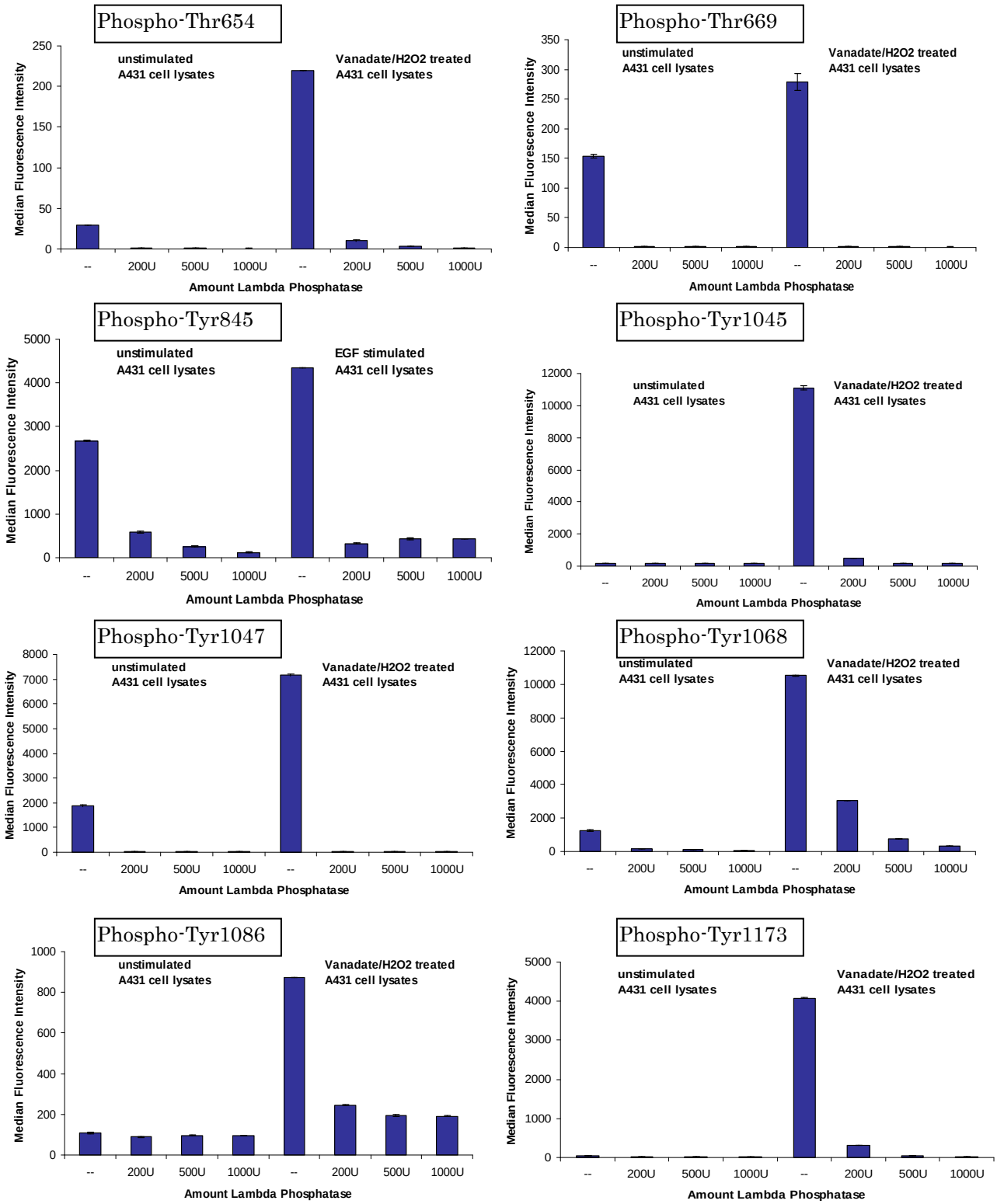


Figure 8: Phospho-Specificity of the WideScreen™ BeadPlex™ Phospho-EGFR Profiling Assay Kit
Vanadate/H_2O_2-stimulated A431 cell lysates were treated with increasing amounts of Lambda Phosphatase to determine the phospho-specificity of the Widescreen™ BeadPlex™ Phospho-EGFR Profiling various capture antibodies. Representative data is shown in Median Fluorescence Intensity (MFI) units. Treatment of stimulated cell lysates with 1000U phosphatase results in a 200-fold decrease in signal. This demonstrates the phospho-specificity of the Widescreen™ BeadPlex™ Phospho-EGFR Profiling Assay Kit.

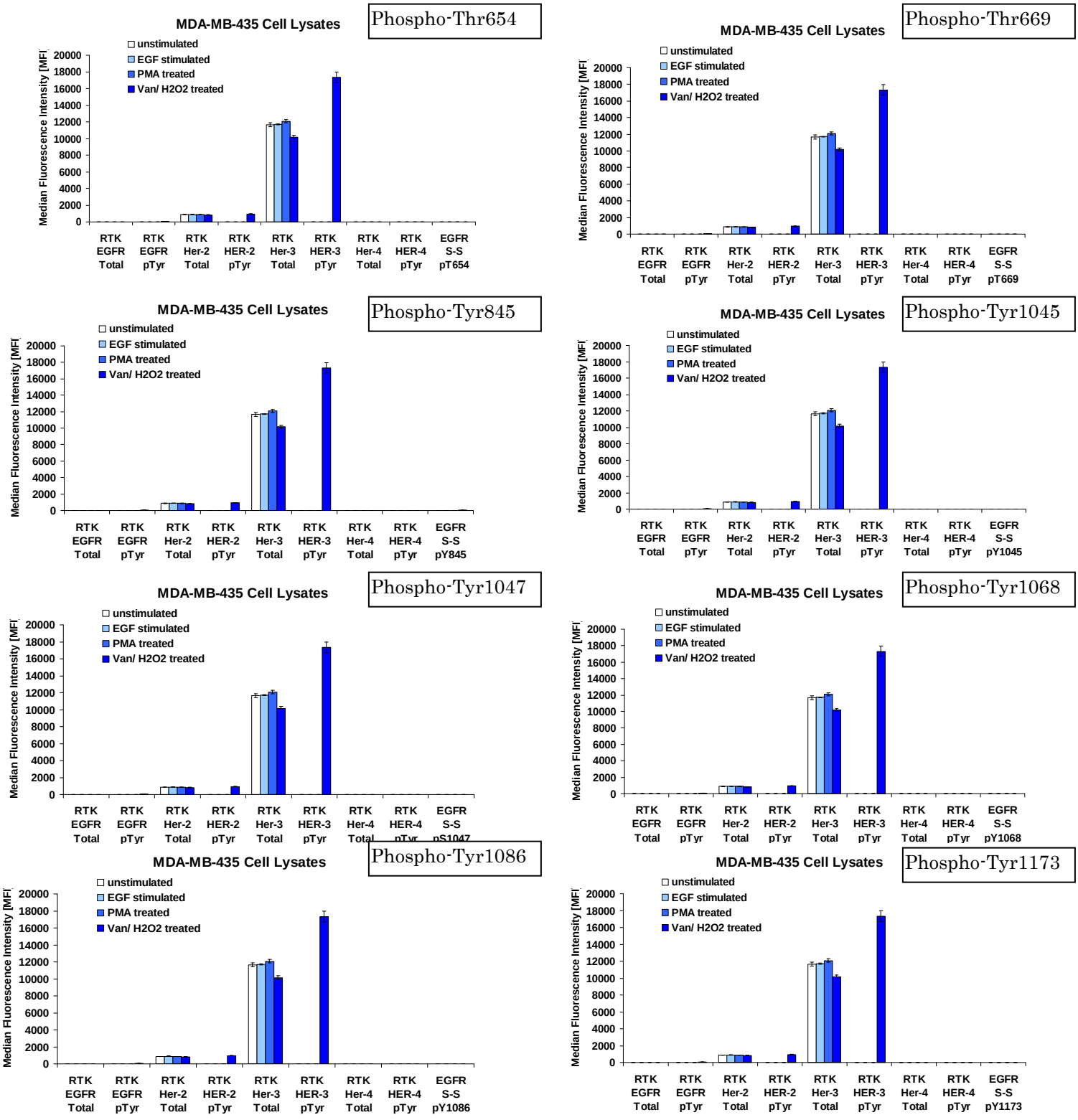


Figure 9: Protein-Specificity

Serum-starved MDA-MB-435 cells (either do not express EGFR or express at extremely low levels) were stimulated with either EGF, PMA or Vanadate/H_2O_2. Lysates were prepared according to the protocol above. 10µg of total cell protein was analyzed with the WideScreen™ RTK (Receptor Tyrosine Kinase) Total and pTyr assays (EGFR, HER-2, HER-3 or HER-4) as well as the WideScreen™ BeadPlex™ Phospho-EGFR Profiling Kit. Representative data are shown in Median Fluorescence Intensity (MFI) units. The data indicates no cross-reactivity for the BeadPlex™ Phospho-EGFR profiling with neither HER-2 (erbB2) nor HER-3.

Intra-Assay and Inter-Assay Variation

Intra-assay Precision

	pT654		pT654		pT654
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	140	78	769	407	1883
St Dev	5	3	25	11	141
% CV	**3.7**	**3.4**	**3.3**	**2.7**	**7.5**

Inter-assay Precision

	pT654		pT654		pT654
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	191	92	868	479	1934
St Dev	12	6	82	37	333
% CV	**6.0**	**6.8**	**9.5**	**7.7**	**17.2**

Intra-assay Precision

	pT669		pT669		pT669
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	42	56	237	310	68
St Dev	2	3	13	14	6
% CV	**5.0**	**5.6**	**5.4**	**4.6**	**9.2**

Inter-assay Precision

	pT669		pT669		pT669
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	41	58	280	364	62
St Dev	4	4	26	36	10
% CV	**8.9**	**7.7**	**9.4**	**9.8**	**16.3**

Intra-assay Precision

	pY845		pY845		pY845
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	7630	10010	9922	13057	2793
St Dev	217	322	251	383	262
% CV	**2.8**	**3.2**	**2.5**	**2.9**	**9.4**

Inter-assay Precision

	pY845		pY845		pY845
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	7262	9891	10139	13317	2245
St Dev	350	531	586	887	534
% CV	**4.8**	**5.4**	**5.8**	**6.7**	**23.8**

Inter-assay Precision

	pY1045		pY1045		pY1045
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	416	604	5165	8479	4909
St Dev	18	23	363	470	704
% CV	**4.2**	**3.8**	**7.0**	**5.5**	**14.3**

Inter-assay Precision

	pS1047		pS1047		pS1047
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	1671	2098	5741	6659	1640
St Dev	88	149	249	363	296
% CV	**5.3**	**7.1**	**4.3**	**5.4**	**18.1**

Intra-assay Precision

	pS1047		pS1047		pS1047
Lysate	**MDA-MB-231 PMA stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 PMA stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	1669	2082	5394	6553	2191
St Dev	40	42	136	131	227
% CV	**2.4**	**2.0**	**2.5**	**2.0**	**10.3**

Intra-assay Precision

	pY1068		pY1068		pY1068
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	3983	4040	8815	10339	1811
St Dev	139	106	348	191	44
% CV	**3.5**	**2.6**	**4.0**	**1.8**	**2.4**

Inter-assay Precision

	pY1068		pY1068		pY1068
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	3818	3957	9273	10829	1719
St Dev	105	204	556	563	239
% CV	**2.7**	**5.2**	**6.0**	**5.2**	**13.9**

Intra-assay Precision

	pY1086		pY1086		pY1086
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	121	83	392	611	1617
St Dev	3	3	20	25	47
% CV	**2.1**	**3.6**	**5.0**	**4.0**	**2.9**

Inter-assay Precision

	pY1086		pY1086		pY1086
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	119	81	404	638	1595
St Dev	6	3	16	34	162
% CV	**5.3**	**3.6**	**4.0**	**5.3**	**10.1**

Intra-assay Precision

	pY1173		pY1173		pY1173
Lysate	**MDA-MB-231 EGF stim**	**MDA-MB-231 Vanadate / H_2O_2**	**A431 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	16	16	16	16	16
MFI	164	139	1066	2227	2225
St Dev	7	6	49	98	171
% CV	**4.1**	**4.0**	**4.6**	**4.4**	**7.7**

Inter-assay Precision

	pY1173		pY1173		pY1173
Lysate	**SKBR3 EGF stim**	**SK-BR-3 Vanadate / H_2O_2**	**HepG2 EGF stim**	**A431 Vanadate / H_2O_2**	**control peptide**
n=	5	5	5	5	5
MFI	452	363	9	180	2150
St Dev	34	41	1	19	507
% CV	**7.5**	**11.2**	**6.3**	**10.6**	**23.6**

Figure 10: Precision
Reproducibility within an assay (intra-assay precision) was tested using four cell lysates and a control peptide 16 times on the same plate. Reproducibility between assays (inter-assay precision) was tested using four cell lysates and a control peptide in at least five separate assays.

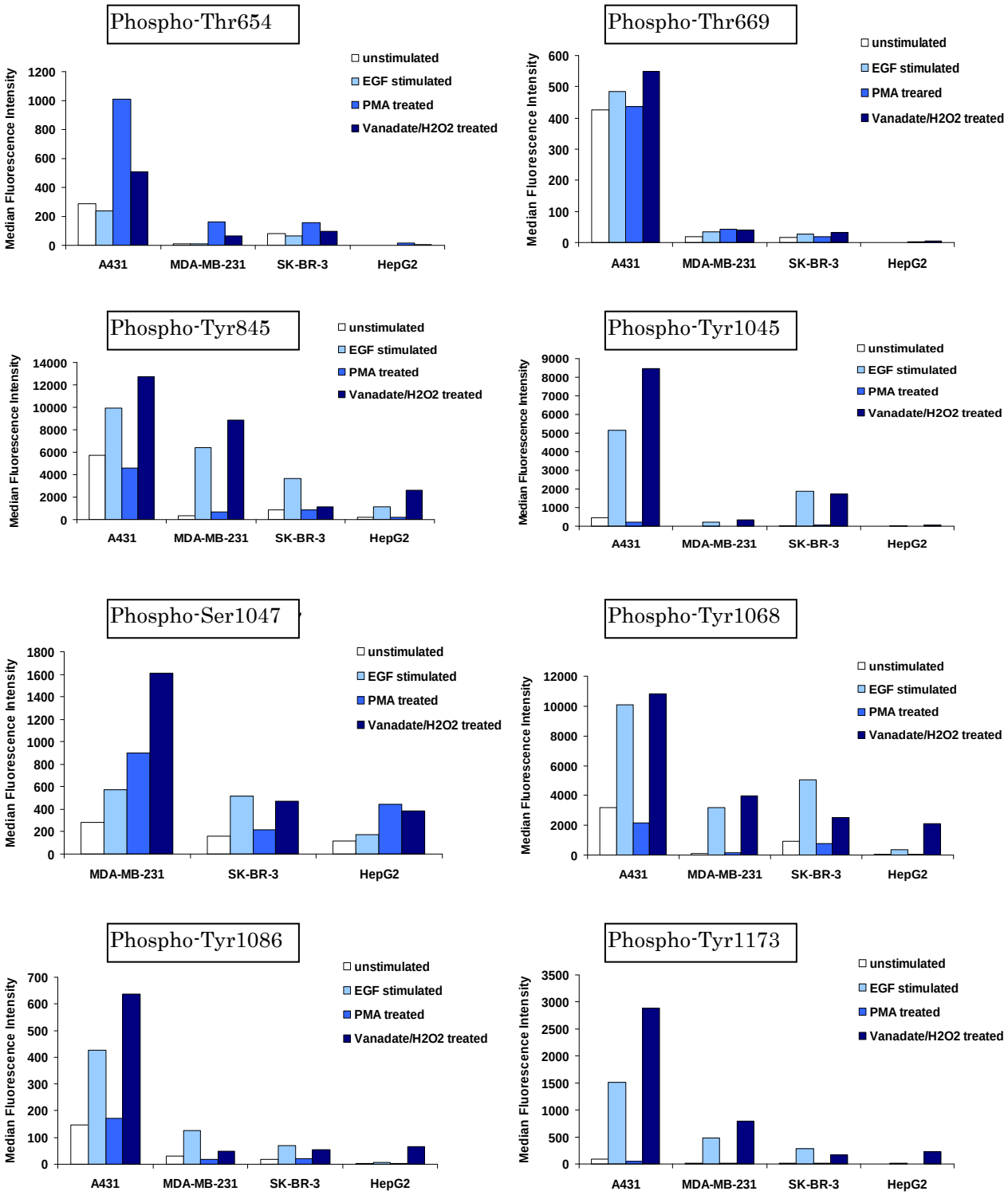


Figure 11: Representative data

Serum-starved cell lines A431, MDA-MB-231, SK-BR-3, and HepG2 were stimulated with either EGF, PMA or Vanadate/H_2O_2. Lysates were prepared according to the protocol as described above. 10µg of total cell protein was analyzed using WideScreen™ BadPlex™ Phospho-EGFR Profiling Assay kit as described above. Representative data are shown in Median Fluorescence Intensity (MFI) units.

Troubleshooting

Problem	Probable Cause	Solution
Lysate is viscous	Genomic DNA is not digested	Make sure Benzonase® Nuclease was added to Extraction Reagent. Incubate lysate longer. For cell lines with recurring viscosity problems, additional Benzonase Nuclease can be added (available separately).
Leaking wells in filter plate	Wicking due to adherent drops	Tap filter plate several times on paper towel before adding samples or reagents. Do not place filter plate on an absorbent surface during incubations. If wells leaked during data acquisition, it may be possible to reacquire these wells. Blot underside of wells and add 120 µl/well 1X Assay Diluent.
	Perforation of filter plate membranes	Adjust the vacuum setting to <5 in. (127 mm) Hg. Do not touch membranes with pipet tips.
Filter plate wells not draining under vacuum	Vacuum is too low	Adjust vacuum setting to 3–5 in. (76–127 mm) Hg. Clean rubber seals. Apply fingertip pressure to filter plate to ensure formation of a good seal. Use a plate sealer to cover wells not in use.
	Cell debris clogs membranes	Clarify lysates by centrifugation. Avoid disturbing pellets when removing supernatant. Use the non-tip end of a fresh 200 µl pipet tip to flick the center support on the underside of the well, then reapply vacuum. If lysate protein concentration is high, dilute further before assaying.
Low bead counts during data acquisition	No beads (or wrong beads) in the wells	See solutions above for leaking wells. Verify that the appropriate beads were added at the correct concentration, and that the correct bead regions and wells were selected during acquisition setup.
	Luminex® fluidics system is clogged	Clear system of clogs or air using maintenance steps described in the instrument user manual (sanitize, alcohol flush, probe sonication, etc.). Make sure that the probe height is set correctly. Make sure that beads are in suspension by incubating plate for 3-5 min on the platform plate shaker (750 rpm) immediately before analysis. Microbial growth in buffers can cause beads to stick to the filter plate membrane. Do not use contaminated reagents.
	Timeout limit is set too low	Use the recommended settings for acquisition setup first (50 µl sample, 100 events per bead, 30 s time out, etc.). However, timeout limit can be set higher, e.g. 75 s.
Data acquisition is slow	No beads in the wells, or fluidics system is clogged	See "Low bead counts during data acquisition" solutions, above.
	Some bead regions being acquired are not in the wells	Make sure that the intended beads were added, and that the correct bead regions and wells were selected during acquisition setup. Attempting to acquire inappropriate bead regions will cause each sample to time out.

Problem	Probable Cause	Solution
Beads are not falling into the gates properly	Beads were not re-suspended in 1X Assay Diluent before analysis	The setting of the Doublet Discriminator (DD) gate is buffer-specific. This gate can be adjusted, but 1X Assay Diluent is the buffer recommended for running samples. Other buffers may also cause bead aggregation.
	Beads were exposed to organic solvents	Do not use organic solvents in the immunoassay, as they will damage beads irreversibly.
	Beads are falling outside the bead region gates due to photo-bleaching	Do not use expired beads. Do not expose the beads to ambient light for >10 min. Avoid intense light.
	Fluidics system is not running properly	Confirm that the waste container is not full, the sheath fluid is not empty, and the SD fluidics module is turned on. Check system calibration using approved calibration beads. Verify correct system pressure. Confirm that the system is free of air or particulate buildup. Follow maintenance steps described in the instrument user manual.
An immunoassay reagent is used up	Solutions were not prepared or used as described in the protocol	Briefly spin tubes to collect reagents that might be trapped in the tube cap. Confirm correct buffer dilutions and use. If additional Wash Buffer is needed, TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20) may be substituted. If additional Assay Diluent is needed, 10 mM Tris pH 7.5, 225 mM NaCl, 0.05% Tween-20, 1% BSA may be substituted. If additional 96-well filter plates are required, we recommend Millipore Cat. No. MSBVN1210.
High coefficients of variance (CVs) between replicates	Cells grown in 96-well plates show well-to-well variability	To avoid edge effects, do not plate cells in outermost wells of plates. Plate cells uniformly. Add lysis reagents accurately. Do not dislodge adherent cells during pre-lysis wash steps. If necessary, decant (instead of aspirating) liquid and tap plate on paper towels. If cells become less adherent during overnight serum starvation, shorten the serum starvation step to 4 h.
	A gradual drop in signal strength as many samples are read on the xMAP® system	Group samples such that those being compared directly (including replicates) are not being read with a long delay in between. Use 0.2% paraformaldehyde in PBS to covalently fix PE to bead surfaces.
	Lysates assayed at different times show assay-to-assay variability	Generate control data carefully (using at least duplicate wells) to increase inter-assay precision. Fully resuspend control peptide solution and lysate samples by thawing to room temperature and vortexing carefully.
Sample measurements not falling on the standard curve	Dilution of lysate is too low or too high	If the MFI signals from the lysates are too high, samples can be diluted further in titration buffer. Signal strength may be boosted by increasing lysate protein concentration, by lysing cells at a higher confluence, or by using less Extraction Reagent.
	Background values increased due to multiplexing	Background levels of some assays shift slightly upon multiplexing. Therefore, the same multiplex of assays must be prepared when comparing experimental samples.
	Target concentration is below detection	Ensure that stimulation conditions are optimal. Screen additional cell lines. Target expression may be suboptimal in some cell lines. Confirm that antibodies used in the assay recognize target in the species being tested.

Appendix A: WideScreen™ BeadPlex™ Phospho-EGFR Bead Regions

	EGFR-Thr654	EGFR-Thr669	EGFR-Tyr845	EGFR-Tyr1045	EGFR-Ser1047	EGFR-Tyr1068	EGFR-Tyr1086	EGFR-Tyr1173	EGFR-Total
Bead Region	**# 47**	**# 10**	**# 16**	**# 40**	**# 81**	**# 27**	**# 77**	**# 28**	**# 21**