WideScreen®

WideScreen® Receptor Tyrosine Kinase Assay Kits

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Overview

Bead-based flow cytometric assays enable sensitive, precise quantification of analytes within a sample. When directed towards protein analytes, such assays are essentially ELISAs on a bead. Samples are combined with labeled microparticles covalently conjugated to a capture antibody. Analytes captured on the beads are identified with detection antibodies and a fluorescent label. A major advantage over traditional protein analyte quantification methods (such as ELISA) is the capacity for multiplexing, as bead-based assays allow simultaneous quantification of multiple analytes in a small sample volume.

Receptor tyrosine kinases (RTKs) are critical regulators of numerous cell signaling pathways and have been implicated in various disease states. Ligand binding to the extracellular domain of transmembrane RTKs triggers receptor dimerization and autophosphorylation of an intracellular kinase domain. This event ultimately triggers activation of downstream pathway proteins via phosphotyrosine-SH2 domain interactions. The WideScreen® RTK Assay Kits allow quantification of a set of key RTKs, including:

- Epidermal Growth Factor Receptor (EGFR)
- Insulin-like Growth Factor 1 Receptor (IGF-1R)
- Hepatocyte Growth Factor Receptor (HGFR)
- Platelet-Derived Growth Factor Receptor beta (PDGFRβ)
- Human Epidermal Growth Factor Receptor 2 (HER-2)
- Vascular Endothelial Growth Factor Receptor 2 (VEGFR2)
- Tyrosine Kinase with Immunoglobulin and EGF Repeats 2 (Tie-2)

The WideScreen RTK Assay workflow is shown in Figure 1 on p 4. If expressed in the cell line of interest, individual RTK proteins may be present in either inactive or active states depending on availability of the RTK ligand. Cellular protein samples are prepared by gentle membrane extraction. Extracts are combined with dye-labeled Luminex xMAP beads covalently conjugated to capture antibodies specific to each RTK. After incubation and several washes, the beads are incubated with biotin-labeled detection antibodies. The resulting bead-immobilized immunosandwich is detected with streptavidin-phycoerythrin and quantified using a Luminex xMAP instrument.

Importantly, detection antibodies differ between the WideScreen Total RTK assay and the WideScreen pTyr RTK assay. In the Total assay, the detection antibody recognizes a second epitope on the RTK, allowing quantification of RTK levels without regard to phosphorylation state. In the pTyr assay, the detection antibody is specific to conserved pTyr on all RTKs. Relative quantification of RTK phosphorylation (expressed in median fluorescence intensity units) is possible using the pTyr kit. Because antibodies would compete for the same analyte, the RTK Total Assays cannot be multiplexed with the RTK pTyr assays.

Applications of the WideScreen RTK Assay Kits include:

- Biomarker quantification
- Expression profiling
- Confirmation of knock-down experiments
- RTK agonist or antagonist profiling
- Pathway analysis
- High-throughput compound library screening

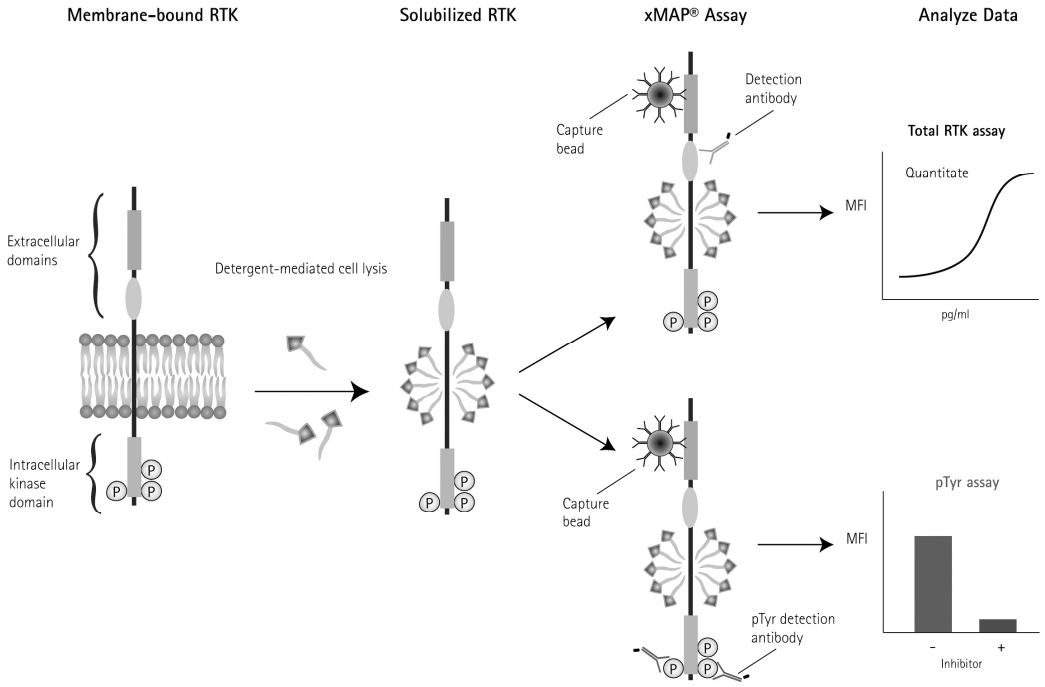


Figure 1. WideScreen® RTK Assays using Luminex® xMAP® Technology. The WideScreen RTK Assays consist of a series of phosphotyrosine-specific RTK assays and companion RTK total protein assays. The phosphotyrosine assays utilize RTK-specific capture antibodies and a broad-spectrum phosphotyrosine detection antibody. The total RTK assays, which include standards, allow the signals from the phosphotyrosine assays to be compared to the total amount of RTK in the sample.

Components

RTK Bead Kits and Standards are used together for multiplex analysis of cell lysates. The WideScreen RTK Total Assay Complete Kit and the WideScreen RTK pTyr Assay Complete Kit contain sufficient reagents to run 96 test wells. For maximum flexibility and user-defined multiplex assay configuration, components of the WideScreen RTK Total Assay Complete Kit and the WideScreen RTK pTyr Assay Complete Kit are available separately. All components are necessary for carrying out the RTK bead-based assays.

The RTK Bead Kits and buffers are not compatible with other bead kits and reagents sold by EMD Chemicals, Inc. or other vendors.

WideScreen RTK Total Assay Complete Kit

The WideScreen RTK Total Assay Complete Kit includes the entire set of reagents to run 96 test wells, including the RTK Total 7-plex, RTK Total Standards Mix, Cell Extraction Kit, and WideScreen Reagent Kit.

WideScreen RTK pTyr Assay Complete Kit

The WideScreen RTK pTyr Assay Complete Kit comprises the entire set of reagents to run 96 test wells, including the RTK pTyr 7-plex, Cell Extraction Kit, and WideScreen Reagent Kit.
No standards are included in the pTyr Assay Complete Kit.

RTK Bead Kits

RTK Bead Kits contain antibody-coated Capture Beads and biotinylated Detection Antibodies used for target detection via immunoassay sandwiches. Bead Kits may be purchased separately, or as pre-mixed panels. Available Bead Kits are listed in Appendix A and at www.novagen.com/WideScreen. Performance specifications for the Bead Kits are detailed in the individual Certificates of Analysis, available online. Each RTK Bead Kit contains sufficient reagents for 100 tests.

The RTK recombinant standards are used to create standard curves when performing quantitative assays. Each individual RTK Total Bead Kit (used to quantify a specific RTK target regardless of phosphorylation state) includes the appropriate individual recombinant standard. Pre-mixed RTK recombinant standards are supplied with the RTK Total Assay Complete Kit and the RTK Total 7-Plex. The concentration of standards in the standard curves can be found in Appendix B and at www.novagen.com/WideScreen. Each recombinant standards mix or individual standard contains reagents sufficient to generate eight singleplex or multiplex standard curves, or four standard curves in duplicate.

Cell Extraction Kit

The Cell Extraction Kit contains a cell extraction reagent that releases soluble and membrane proteins efficiently. Benzonase® Nuclease reduces viscosity due to chromosomal DNA. Phosphatase and protease inhibitor cocktails maintain the phosphorylation state and integrity of target proteins during cell extraction. The kit contains reagents sufficient to make 20 ml of cell lysate or to process 160 wells of cells grown in 96-well plates. Additional extraction reagent is included for preparation of titration buffer.

WideScreen® Reagent Kit

The WideScreen® Reagent Kit contains reagents needed for the bead-based immunoassays, including all buffers, a 96-well filter plate, a plate sealer, and a streptavidin-phycoerythrin solution used in the final detection step. The kit contains sufficient reagents to perform 96 singleplex or multiplex bead-based tests.

Components and Storage

Cell Extraction Kit		**71926-3**
25 ml	Extraction Reagent	Store at -20°C
500 µl	Phosphatase Inhibitor Cocktail Set V (50x)	Store at -20°C
25µl	Protease Inhibitor Cocktail Set III (1000x)	Store at -20°C
10 µl	Benzonase® Nuclease HC, Purity >99% (250 U/µl)	Store at -20°C

WideScreen™ Reagent Kit		**71783-3**
100 µl	Streptavidin-PE Concentrate	Store at 4°C
20 ml	10X Wash Buffer	Store at 4°C
25 ml	5X Assay Diluent	Store at 4°C
1 ea	Polyethylene Plate Sealer	Store at room temperature
1 ea	96-well Filter Plate	Store at room temperature

Components and storage conditions for WideScreen RTK Bead Kits and RTK Assay Complete Kits are described in Appendix A.

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™, 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 TBS) (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 17 for sample protocols for stimulation with growth factors in presence or absence of RTK 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 17 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 5X Assay Diluent (WideScreen® Reagent Kit) 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:

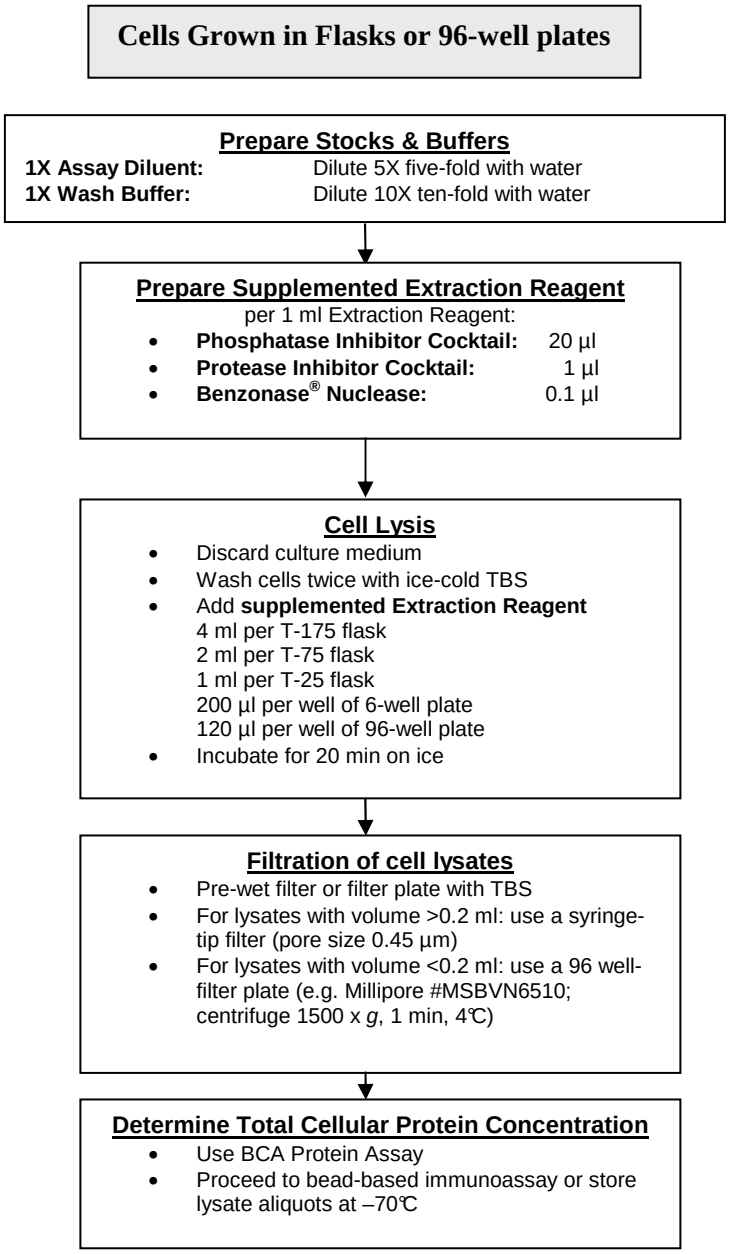
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)
1 µl	Protease Inhibitor Cocktail III (1000X)
0.1 µl	Benzonase® Nuclease

   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 #MSBVN6510, filtration 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 mg/ml to 0.5 mg/ml.

Flowchart for RTK Lysate Preparation



Bead-Based Immunoassay Protocol

Considerations Before You Begin

- Have on hand the 1X Assay Diluent and 1X Wash Buffer that was 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 sec.
  - 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.
- Capture Beads contain fluorescent dyes and are therefore light-sensitive. To avoid photobleaching, keep beads in microcentrifuge tubes covered. Cover filter plates containing beads with aluminum foil during incubation steps. Streptavidin-PE solution is also light-sensitive; protect 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 standards and 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.
- Run standard dilution series and experimental samples using the same multiplex configuration. For instance, if a 7-plex of Bead Kits is used to measure experimental samples, the same 7-plex should be used to create the standard dilution series. Multiplexing causes slight shifts in some standard curves, which will make quantification inaccurate unless experimental samples are measured using the same multiplex.
- 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 quantification. Final dilutions less than 2-fold are not recommended.

Step 1: Prepare Titration Buffer

Quantitative immunoassays are sensitive to buffer composition. Therefore, include the same proportion of Extraction Reagent in all dilutions of standards and samples. 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 2000 µl titration buffer is needed to prepare a duplicate standard curve (see *Step 2: Prepare Standard Dilution Series* below). 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 3: Prepare Sample Dilutions* on p 11).

Sample Calculation:

2	Standard dilution series	= 2000 µl
30	Diluted lysate samples	= 9000 µl (30 X 300 µl)

Make at least **11000 µl** titration buffer

2. Prepare the required volume titration buffer by mixing Extraction Reagent from the Cell Extraction Kit and 1X Assay Diluent prepared from the WideScreen™ Reagent Kit. Use a ratio of 25% Extraction Reagent to 75% 1X Assay Diluent. In the example above, take 2750 µl Extraction Reagent + 8250 µl 1X Assay Diluent = 11000 µl Titration Buffer (allowing for additional buffer to account for pipetting error).

Step 2: Prepare Standard Dilution Series

Notes: Standards are only available for RTK Total Bead Kits. No standards are available for RTK pTyr Bead Kits.

Prepare fresh diluted standards for each assay and use within 1 h.

1. To prepare duplicate 7-point standard curves, label eight microcentrifuge tubes and add 240 µl Titration Buffer to tubes 2-8. See *Step 1: Prepare Titration Buffer* on p 9.
2. Resuspend the appropriate lyophilized RTK Total Standards in 120 µl Titration Buffer for each analyte being tested. These represent 10x Standard solutions. Vortex briefly to ensure all standards are in solution.
3. If conducting a singleplex or user-assembled multiplex assay, add 30 µl of each of the individual RTK Total Standards (10x) being assayed to tube 1. Bring the total volume of tube 1 to 300 µl with Titration Buffer and mix well. This tube is "Dilution 1" of the standard dilution series. If using the RTK Total Standards Mix, it is only necessary to add 30 µl premixed 7-plex standards to 270 µl Titration Buffer.
4. Prepare 4-fold serial dilutions from Dilution 1, as follows:
   - Transfer 80 µl from tube 1 to the 240 µl titration buffer in tube 2; mix well.
   - Change tips. Transfer 80 µl from tube 2 to the 240 µl titration buffer in tube 3; mix well.
   - Proceed in similar manner with the serial dilutions through tube 7.
5. The 8th tube contains 240 µl titration buffer only. This will serve as the blank control.

   Note: Refer to Appendix B for concentrations of the serially-diluted standards.

Table 2. Serial dilution of pre-mixed RTK recombinant standards (7-Plex):

Tube/ Dilution	Volume Standard	Volume Titration Buffer	Final Concentration
1	30 µl RTK Total Standards Mix, 7-Plex (10X)	270 µl	See Appendix B
2	80 µl from tube 1	240 µl	
3	80 µl from tube 2	240 µl	
4	80 µl from tube 3	240 µl	
5	80 µl from tube 4	240 µl	
6	80 µl from tube 5	240 µl	
7	80 µl from tube 6	240 µl	
8/ BLANK	None	240 µl	0

Table 3. Example of serial dilution of five individual RTK Total Standards (user-assembled multiplex):

Tube/ Dilution	Vol. Standard	Volume Titration Buffer	Final Concentration
1	5 x 30 µl of each individual RTK Standard (10X) = 150 µl total volume	150 µl	See Appendix B
2	80 µl from tube 1	240 µl	
3	80 µl from tube 2	240 µl	
4	80 µl from tube 3	240 µl	
5	80 µl from tube 4	240 µl	
6	80 µl from tube 5	240 µl	
7	80 µl from tube 6	240 µl	
8/ BLANK	None	240 µl	0

Step 3: Prepare Sample Dilutions

Notes: Thaw and (if applicable) dilute samples within 1 h 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).*

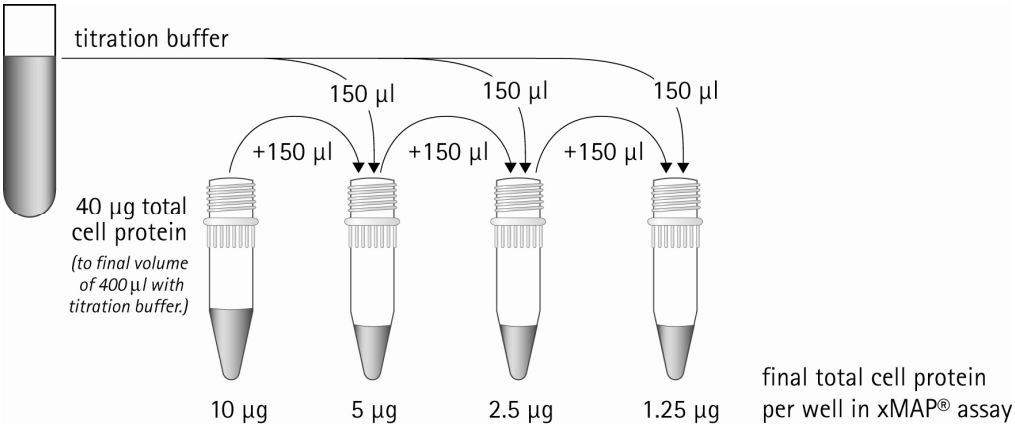
1. Dilute lysate samples four-fold in 1X Assay Diluent (e.g., 100 µl lysate with 300 µl 1X Assay Diluent). Mix well.
2. Calculate the protein concentration of the four-fold diluted lysate samples based on the protein quantification values previously determined using BCA assay. For example, if the original 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 signal quantification. 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 microfuge 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 4: Prepare Capture Beads

Individual RTK Total Bead Kits can be multiplexed in all combinations, and individual RTK pTyr Bead Kits can be multiplexed in all combinations. However, RTK Total Beads and RTK pTyr Beads cannot be multiplexed together because antibodies compete for the same analyte.

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 2000 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
RTK 7-plex (premixed)	1 µl per well

3. Thoroughly resuspend each vial of Capture Beads (50X) by vortexing for 10 sec, sonicating in an ultrasonic bath for 10 sec, and vortexing again for 5 sec.
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 table on the next page for example calculations.
5. Add the calculated volumes of Capture Beads (50X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 sec. Protect from light and store at 4°C until use.

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

	Singleplex, *or* RTK 7-plex (premixed)	**User-assembled multiplex** (e.g., 5-plex)
Test wells	40	40
Volume Capture Beads (50X)	1 µl per well	1 µl each bead per well = 5 µl total
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 beads per well x 40 wells **= 200 µl beads (40µl ea)**
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. Pre-wet 96-well filter plate wells with 50 µl 1X Assay Diluent for 5 min. Leave dry any wells that will not be used. These wells can be used in future assays (use the plate sealer for storage). 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 9 for guidelines on using the filter plate vacuum and manifold.*

2. Vortex (10 sec) the diluted Capture Beads solution prepared as per *Step 4: Prepare Capture Beads* on p 12. Add 50 µl to each well being used.
3. Remove liquid from filter plate by vacuum filtration.
4. To bead-containing wells reserved for the standards, add 100 µl from the standard dilutions (Dilutions 1-7 + blank) prepared as per *Step 2: Preparing Standard Dilution Series* on p 10.
5. To bead-containing wells reserved for analyzing experimental samples, add 100 µl diluted samples prepared as per *Step 3: Prepare Sample Dilutions* on p 11. 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 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.

6. 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 Antibodies

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. Note the volume of 100X Detection Antibody needed per well, based on the assay format (see table on next page):

Assay Format	Volume Detection Antibodies (100X) needed
RTK pTyr singleplex (one target)	1 µl per well
RTK Total singleplex (one target)	1 µl per well
User-assembled RTK pTyr multiplex	1 µl per well
User-assembled RTK Total multiplex	1 µl from each individual Bead Kit per well
RTK pTyr 7plex (premixed)	1 µl per well
RTK Total 7plex (premixed)	1 µl per well

3. Each well receives a total of 100 µl diluted (1X) Detection Antibody solution. Determine the total volume of 100X Detection Antibodies needed per well (refer to the table above) 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.
4. Add the calculated volumes of Detection Antibodies (100X) and 1X Assay Diluent to a microcentrifuge tube. Vortex 3 sec and store at 4°C until use.

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

	Singleplex, *or* RTK 7plex (premixed)	**User-assembled RTK Total multiplex** (e.g., 5-plex)
Test wells	40	40
Volume Detection Antibodies (100X)	1 µl per well	1 µl each Antibody per well = 5 µl total
Volume 1X Assay Diluent	99 µl per well	95 µl per well
Total Volume Detection Antibodies (100X)	1 µl Antibody per well x 40 wells **= 40 µl Detection Antibody**	5 µl Antibodies per well x 40 wells **= 200 µl Detection Antibodies (40µl ea)**
Total Volume 1X Assay Diluent	99 µl per well x 40 wells **= 3960 µl**	95 µl per well x 40 wells **= 3800 µl**

5. Remove liquid from filter plate by vacuum filtration.
6. 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.
7. Immediately add 100 µl 1X Detection Antibody solution to each well.
8. Incubate for 1 h at room temperature on a platform plate shaker (750 rpm). Protect from light.

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

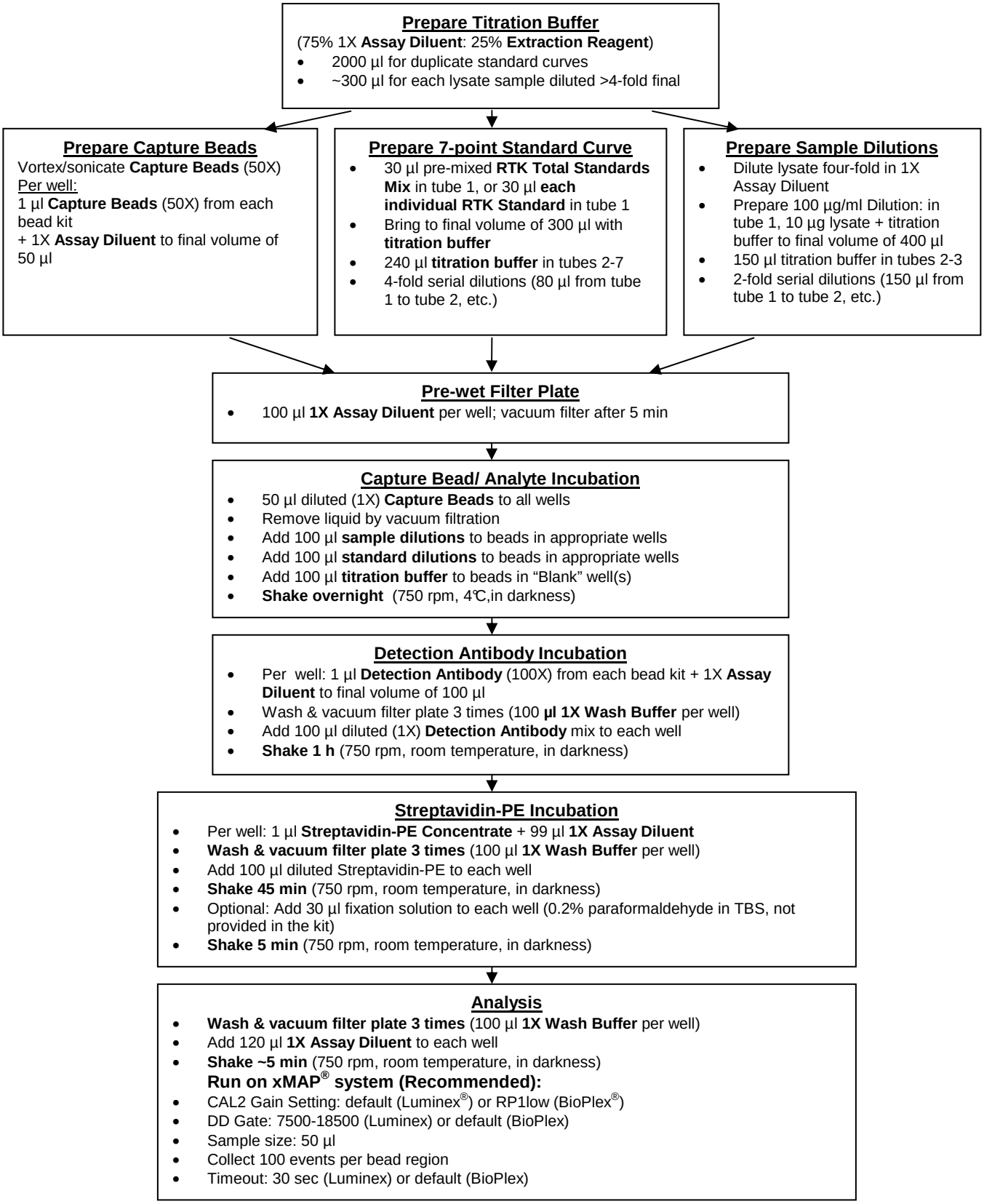
Step 7: Add Streptavidin-Phycoerythrin (PE)

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

1. Calculate the total volume of 1X Streptavidin-PE solution required. 100 µl is needed for each test well.
2. Prepare the calculated volume of 1X Streptavidin-PE solution by diluting Streptavidin-PE Concentrate 1/100 in 1X Assay Diluent. Vortex 3 sec. Protect from light and store at 4°C until use.
3. 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.
4. Immediately add 100 µl 1X Streptavidin-PE solution to each well.
5. Incubate for 45 min at room temperature on a platform plate shaker (750 rpm). Protect from light.
6. Optional: Add 30 µl fixation solution to each well (0.2% paraformaldehyde in TBS, 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.
7. 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.
8. 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. Protect from light.
9. Analyze samples with a Luminex® xMAP® system according to the manufacturer's instructions.

Flowchart for RTK 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 standards, samples, and controls that will be run. The ranges of final concentrations found in the RTK Total Standards Mix 7-plex are shown in Appendix B.
2. Select the bead regions used in the assay. The bead regions used for the RTK Bead Kits are shown in Appendix B.
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 sec	7500-18500	default
ACS StarStation	50 µl	100	30 sec	default	default
Bio-Plex® Manager™	50 µl	100	default	default	RP1 Low

Generation of Standard Curves and Quantitation of Experimental Samples

- Standards are available for all of the RTK Total Bead Kit assays (see Appendix B), allowing accurate quantification. Representative standard curves and assay performance information can be found in the Certificates of Analysis for the individual bead kits.
- The 7-point standard curves are plotted using Median Fluorescent Intensity (MFI) as the signal readout (Y-axis) against concentration of standard dilutions (X-axis). Measurements of the blank are useful for assessing background and lower limits of detection. However, it is not necessary to subtract the MFI value of the blank from other measurements, and the blank is generally not plotted as part of the curve.
- Five-Parameter Logistic (5PL) curve fitting is recommended for modeling data. Most ranges of standard curve concentrations are too wide for accurate linear regression analysis. Four-parameter (4PL) equations will often give a good fit, but are not ideal because they assume the standard curve is symmetrical.
- If the signal from an experimental sample exceeds the highest point of the standard curve, the concentration of the unknown should *not* be extrapolated because the non-linear shape of the standard curve cannot be accurately modeled past the last measured point. In this case, samples should be diluted and tested again.

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 RTK inhibitors, or by treating cell extracts with lambda protein phosphatase. In this last case, 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 a final concentration of 100 ng/ml in tissue culture medium lacking fetal bovine serum (FBS) (refer to table on p 18). This results in a 1X solution. Growth factors can be added separately, with each of the following added to a separate T-75 flask (6 flasks total):
   - EGF
   - IGF
   - HGF
   - PDGF A/B
   - VEGF
   - Vanadate for stimulation of Tie-2

   Alternatively, all six growth factors can be added simultaneously to one T-75 flask. In either case, use 5 ml induction medium per T- 75 flask. For mock inductions, prepare tissue culture medium lacking FBS and growth factors.
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 10 min.

   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 7).

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. 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 all growth factor stocks to a final concentration of 200 ng/ml in tissue culture medium lacking FBS (refer to table on p 18). This results in a 2X solution. Growth factors can be added separately, with each of the following added to a separate T-75 flask (6 flasks total):
   - EGF
   - IGF
   - HGF
   - PDGF A/B
   - VEGF
   - Vanadate for stimulation of Tie-2

   Alternatively, all six growth factors can be added simultaneously to one T-75 flask. In either case, use 5 ml induction medium per T- 75 flask. For mock inductions, prepare tissue culture medium lacking FBS and growth factors.
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 7).

Table 4. Growth factor reconstitution and dilution

Growth factor	Cat. No.	Reconstitution	Amount [µg]	Reconstitution volume [µl]	Stock solution [µg/ml]	Final Concentration in 1X Induction Medium [ng/ml]
EGF	#324831	10 mM acetic acid, 0,1% BSA	200	2000	100	100
HGF	#375228	PBS, 0,1% BSA	5	50	100	100
IGF	#407240	PBS, 0,1% BSA	50	500	100	100
PDGF-AB	#521220	4 mM HCl, 0,1% BSA	10	100	100	100
VEGF	#676472	PBS, 0,1% BSA	10	100	100	100
Vanadate	#567540	water			200 mM	10 mM

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 the wells and add 120 µl/well 1X Assay Diluent.
	Perforation of filter plate membranes	Adjust the vacuum setting to <5 inches (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 inches (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. 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 sec 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 resuspended 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 photobleaching	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	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, don't 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 TBS to covalently fix PE to bead surfaces.
	Lysates assayed at different times show assay-to-assay variability	Generate standard curves carefully (using at least duplicate dilutions series) to increase inter-assay precision. Fully resuspend standards and lysate samples by thawing to room temperature and vortexing carefully.
Sample measurements not falling on the standard curve	Dilution of digested lysate is too low or too high	If values are higher than the standard curve, dilute samples 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.
	Standard curve and background values increased due to multiplexing	The standard curves of some assays shift slightly upon multiplexing. Therefore, for accurate quantitation, the same multiplex of assays must be prepared when comparing standard curves and 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: RTK Bead Kits Ordering and Storage Information

Each Bead Kit contains the following components:

- 100 µl Capture Bead (50X, use 1 µl per test)
- 100 µl Detection Antibody (100X, use 1 µl per test)
- 120 µl RTK Total Standard(s) (only available in Total Bead Kits)

Note: Individual RTK Total Bead Kits can be multiplexed in all combinations and individual RTK pTyr Bead Kits can be multiplexed in all combinations. However, RTK Total Beads and RTK pTyr Beads cannot be multiplexed together, because antibodies would compete for their respective analyte.

RTK Total 7-plex Kits		**Store at 4°C**	**71924-3**
100 tests	EGFR Total, IGF-1R Total, HGFR Total, PDGFRβ Total, HER-2 Total, VEGFR2 Total, Tie-2 Total		

RTK pTyr 7-plex Kits		**Store at 4°C**	**71925-3**
100 tests	EGFR pTyr, IGF-1R pTyr, HGFR pTyr, PDGFRβ pTyr, HER-2 pTyr, VEGFR2 pTyr, Tie-2 pTyr		

Individual RTK Total Bead Kits			
100 tests	EGFR Total Bead Kit	Store at 4°C	71928-3
100 tests	IGF-1R Total Bead Kit	Store at 4°C	71929-3
100 tests	HGFR Total Bead Kit	Store at 4°C	71930-3
100 tests	PDGFRβ Total Bead Kit	Store at 4°C	71931-3
100 tests	HER-2 Total Bead Kit	Store at 4°C	71932-3
100 tests	VEGFR2 Total Bead Kit	Store at 4°C	71933-3
100 tests	Tie-2 Total Bead Kit	Store at 4°C	71934-3

Individual RTK pTyr Bead Kits			
100 tests	EGFR pTyr Bead Kit	Store at 4°C	71935-3
100 tests	IGF-1R pTyr Bead Kit	Store at 4°C	71936-3
100 tests	HGFR pTyr Bead Kit	Store at 4°C	71937-3
100 tests	PDGFRβ pTyr Bead Kit	Store at 4°C	71938-3
100 tests	HER-2 pTyr Bead Kit	Store at 4°C	71939-3
100 tests	VEGFR2 pTyr Bead Kit	Store at 4°C	71940-3
100 tests	Tie-2 pTyr Bead Kit	Store at 4°C	71941-3

WideScreen RTK Total Assay Complete Kit		**71942-3**
1	RTK Total 7-Plex, which includes: • RTK Capture Beads Premix, 7-Plex • RTK Total Detection Antibody Premix, 7-Plex • RTK Total Standards Mix, 7-Plex	Store at 4°C
1	Cell Extraction Kit (see p 5 for components)	Store at –20°C
1	WideScreen Reagent Kit (see p 5 for components)	see p 5 for storage conditions

WideScreen RTK pTyr Assay Complete Kit		71943-3
1	RTK pTyr 7-Plex, which includes: • RTK Capture Beads Premix, 7-Plex • pTyr Detection Antibody	Store at 4°C
1	Cell Extraction Kit (see p 5 for components)	Store at –20°C
1	WideScreen Reagent Kit (see p 5 for components)	see p 5 for storage conditions

Note: The RTK Bead Kits and reagents are not compatible with other bead kits and reagents sold by EMD Chemicals, Inc. or other vendors.

Appendix B: Dilution Series for Generating Standard Curves

The standard curve is used to quantify target proteins found in cell extracts and other analytes. Standards are recombinant proteins representing the extracellular domain of target proteins.

Notes: Standards are supplied with RTK Total Bead Kits only. No standards are available for RTK pTyr Bead Kits.

Standard concentrations are assay-dependent. This is because the linear range and lower limit of each assay depends on assay sensitivity. Values shown are the final concentrations in pg/ml.

Standards supplied with Bead Kits for individual Total targets contain only the standard of interest, but can be mixed for multiplex analysis.

RTK Total Standards Mix: Final concentrations in the 4-fold serial dilution of the standards

	Total-EGFR	Total-IGF-1R	Total-HGFR	Total-PDGFRβ	Total-HER-2	Total-VEGFR2	Total-Tie-2
Molecular Weight [kDa]	**68**	**48**	**129**	**84**	**96**	**160**	**100**
Bead Region	**#21**	**#25**	**#30**	**#43**	**#72**	**#76**	**#80**
	pg/ml	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**	**pg/ml**
Dilution 1	20000	100000	50000	20000	20000	100000	10000
Dilution 2	5000	25000	12500	5000	5000	25000	2500
Dilution 3	1250	6250	3125	1250	1250	6250	625
Dilution 4	313	1563	781	313	313	1563	156
Dilution 5	78	391	195	78	78	391	39
Dilution 6	20	98	49	20	20	98	10
Dilution 7	5	24	12	5	5	24	2
Blank	0	0	0	0	0	0	0