WideScreen®

WideScreen® EpiTag™ Assay Kits

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About the Kits

Overview

Bead-based flow cytometric assays enable sensitive, precise quantification of analytes within a sample. When directed towards protein analytes, such assays are essentially sandwich immunoassays on a bead. Samples are combined with fluorescently labeled microparticles (beads) covalently conjugated to a capture antibody. Analytes captured on the beads are identified with detection antibodies and a fluorescent label.

WideScreen® EpiTag™ Assays employ Luminex® xMAP® Technology for use on Luminex instruments.
A major hallmark of bead-based assays over traditional protein quantitation methods (such as ELISA) is the capacity for multiplexing, as bead-based assays allow simultaneous quantitation of multiple analytes in a small sample volume. Other advantages of xMAP assays include flexibility, robustness, simple sample handling, and requirement of minimal sample volumes, making them an ideal platform for immunodetection of biomarkers and signaling proteins.

Ideally, bead-based assay methods would enable robust sample handling and absolute quantification of analytes. WideScreen EpiTag Assays combine bead-based assays with the EpiTag™ technology licensed from Millipore Corporation to accommodate both of these needs. In the EpiTag ™ approach (Figure 1, p 4), peptides and phosphopeptides are released from cultured cells or tissues by denaturing and reducing the cell extracts, followed by digestion with a site-specific protease. Unique analyte-specific amino acid sequences (EpiTag™ Sequences) - identified using bioinformatic algorithms - are recognized using anti-peptide capture and detection antibodies. Sample lability is minimal, because denaturation and digestion inactivates endogenous proteases and phosphatases. Analyte quantification is sensitive and highly specific, as antibodies to EpiTag Sequences discern between highly related proteins and between phosphorylated and non-phosphorylated forms of the same protein. The use of synthetic peptide and phosphopeptide standards enables absolute quantification.

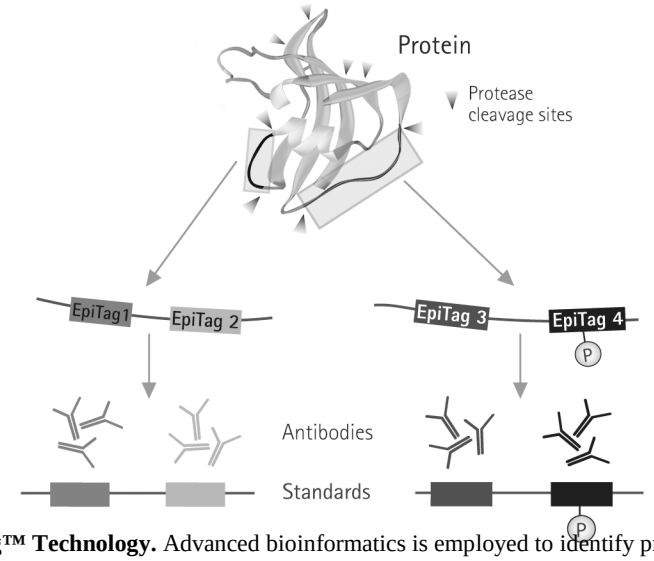


Figure 1. EpiTag™ Technology. Advanced bioinformatics is employed to identify proteolytic fragments of target proteins that contain two or more unique regions, or EpiTag™ Sequences. Antibodies against the EpiTag Sequences are used as capture and detection reagents to form sandwich immunoassays. Synthetic peptides that represent the targeted fragment are used as standards.

WideScreen EpiTag Assays offer the following advantages:

- streamlined sample preparation methods
- denaturation and digestion of sample lysates, eliminating concerns about protease and phosphatase activity
- ability to distinguish between closely-related proteins
- ability to distinguish phosphorylated and non-phosphorylated forms of the same protein
- small sample volumes
- true quantitation with reference to synthetic peptides and phosphopeptide standards

For more information on EpiTag™ technology, visit www.merck4biosciences.com/WideScreen and www.millipore.com.

Description

WideScreen® EpiTag™ kits and standards are used together for quantitative singleplex or multiplex measurements from cell lysates and tissue homogenates. WideScreen EpiTag™ Panel Complete Kits contain all reagents required to run 96 assays. The assays are also available separately in singleplex to allow maximum flexibility and user-defined assay customization. See Figure 2 below for kit configurations.

Due to the unique nature of the EpiTag technology and sample preparation, EpiTag™ Bead Kits and reagents are not compatible with other bead kits and reagents sold by EMD Chemicals, Inc. or other vendors. Product descriptions beginning on p4 apply to all WideScreen EpiTag product lines, regardless of multiplex panel.

For ordering information, storage information, and panel-specific products, see Appendix A.

For quantification standards, see Appendix B.

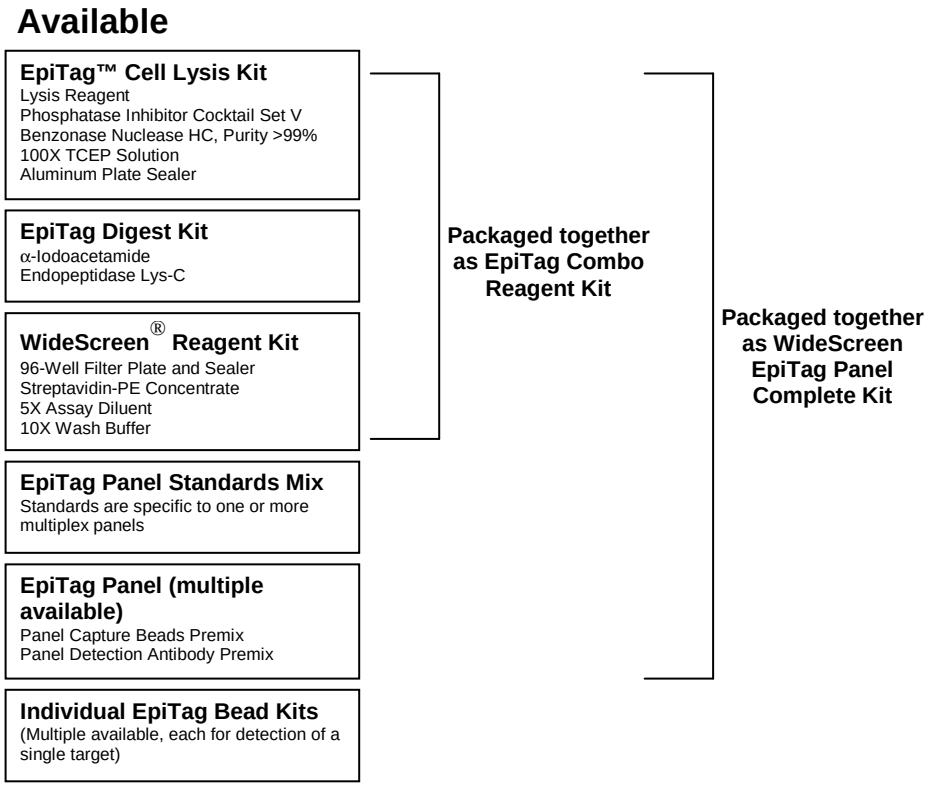


Figure 2. WideScreen® EpiTag™ Kit configurations. See Appendices A and B for panel-specific product information, ordering information, and storage conditions.

WideScreen® EpiTag™ Panel Complete Kits

The WideScreen EpiTag Panel Complete Kits comprise the entire set of reagents to run 96 assays, including an EpiTag Panel (which includes a multiplex panel of Capture Beads and Detection Antibodies), an EpiTag™ Standards Mix, EpiTag™ Cell Lysis Kit, EpiTag™ Digest Kit, and WideScreen Reagent Kit.

EpiTag™ Bead Kits and Panels

EpiTag 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. Lists of available EpiTag Bead Kits can be found in Appendix A and at www.merck4biosciences.com/WideScreen. Performance specifications for bead kits are detailed in the individual Certificates of Analysis, available online. To verify that various individual bead kits can be multiplexed together, consult the EpiTag™ Assay Compatibility Chart in Appendix C. Each EpiTag Bead Kit contains reagents sufficient for 100 tests.

EpiTag™ Standards

EpiTag Standards are used to create standard curves when performing quantitative assays. The standards are a mixture of synthetic peptides and phosphopeptides, formulated so that the same standard mix can be used to perform any user-customized combination of multiplex assays. EpiTag™ Standard Mixes and compositions can be found in Appendix B

and at www.merck4biosciences.com/WideScreen. The standards mix contains reagents sufficient to create six singleplex or multiplex standard curves.

The EpiTag Raf1 Total High Sensitivity Bead Kit contains its own peptide standard (10X solution), to be used if the assay is run in singleplex. See Appendix B for details.

EpiTag™ Cell Lysis Kit

The EpiTag Cell Lysis Kit contains a denaturing and reducing Lysis Reagent that efficiently releases proteins from the cell cytosol, membranes, and nucleus. Benzonase® Nuclease reduces viscosity due to chromosomal DNA, and a cocktail of phosphatase inhibitors maintains the phosphorylation state of target proteins during cell lysis. An aluminum plate sealer is included for use when cells are grown in 96-well plates. The kit contains reagents sufficient to make 5 ml of lysate from cells or tissue, or a total of 160 wells of cells grown in 96-well plates. The kit also contains additional Lysis Reagent for preparing titration buffer.

EpiTag™ Digest Kit

The EpiTag™ Digest Kit contains reagents necessary to alkylate and proteolytically digest cell and tissue lysates. Alkylation with α-iodoacetamide prevents disulfide bond reformation, and endopeptidase Lys-C digests proteins into peptides by cleaving after most lysine residues. The kit contains sufficient reagents to process up to 3.6 mg of total cell protein from cell or tissue lysates, or 120 wells of cells grown in 96-well plates.

Widescreen® Reagent Kit

The WideScreen® Reagent Kit contains reagents needed for the bead-based immunoassay, 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 tests.

EpiTag™ Combo Reagent Kit

The EpiTag Combo Reagent Kit combines the EpiTag™ Cell Lysis Kit, EpiTag™ Digest Kit and Widescreen Reagent Kit for convenience.

Additional Reagents and Equipment Required

- Experimental samples (e.g., stimulated vs. unstimulated cultured cell lines)
- Luminex® xMAP® System (or comparable, such as Bio-Plex® Suspension Array System)
- xMAP data analysis software (Luminex IS, ACS STarStation, Bio-Plex Manager™, or comparable)
- Vacuum manifold for filter plates (Millipore Cat. No. MAVM0960R)
- Plate-shaking platform, such as IKA MTS4
- Heat block (95°C)
- BCA Protein Assay Kit (EMD chemicals, Inc., Cat. No. 71285)
- Screw-top microcentrifuge tubes
- 15 ml and 50 ml polypropylene centrifuge tubes
- Rotator for microcentrifuge tubes
- Microcentrifuge
- Vortexer
- Ultrasonic bath, such as Cole Parmer EW-08849
- Multichannel pipet (optional)
- Plate heater for 96-well plates (optional, for use with cells grown in 96-well plates)
- If additional 96-well filter plates are required, we recommend Millipore Cat. No. MSBVN1210

If preparing lysates from tissue samples:

- Tissue homogenizer, such as Biospec Products Tissue-Tearor™ device
- 5 ml round-bottomed polypropylene tubes
- Bradford protein assay kit

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 19 for sample protocols for stimulation with growth factors and mitogens.

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
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

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

If cells will be stimulated prior to lysis, serum-starve them for 4–16 h before stimulation. See Supplementary Protocols *for sample protocols describing stimulation with growth factors and mitogens.*

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 $0.5–1.0 \times 10^6$ per ml.

Lysate Preparation and Digestion

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. Reducing and denaturing steps serve to extract and denature all cellular proteins. Cysteine alkylation with α–iodoacetamide prevents reformation of disulfide bonds that could lead to cross-reactions, and Lys-C cleaves proteins into peptides recognized by the immunoassay antibodies.
- If it is important to know the lysate protein concentration from cells grown in 96-well plates, additional wells should be plated with cells solely for the purpose of performing a protein assay.

- Protein assay kits are available that are compatible with the levels of anionic detergent and reducing agent used for lysis, such as the Non-Interfering Protein Assay™ Kit (EMD Chemicals, Inc., Cat. No. 488250) or the BCA-Reducing Agent Compatible Kit (Pierce Cat. No. 23250). If such protein assays are used, the 100X TCEP reducing agent may be added to the master mix of supplemented Lysis Reagent before cell lysis.
- If using cells grown in 96-well plates, avoid plating cells in the outermost rows and columns. This minimizes cell growth edge effects and also ensures that lysates are completely denatured if a conventional heating block is used instead of a specialized plate heater.
- Cell lysates are denatured and digested into peptides. They are therefore free of endogenous protease and phosphatase activity. Digested lysate samples subjected to five freeze-thaw cycles show only minor signal decreases compared to digested lysates subjected to only one freeze-thaw cycle.
- Recombinant proteins (including insoluble inclusion bodies expressed in *E. coli*) can be used as positive controls in the EpiTag™ Bead-Based Assays, provided they are denatured and digested according to the Lysate Preparation and Digestion protocol.

Lysis Protocol for Cells Grown in Tissue Culture Dishes

1. Prepare 1X Assay Diluent by adding 25 ml 5X Assay Diluent (WideScreen® Reagent Kit) to 100 ml sterile distilled deionized water. 1X Assay Diluent that will be used within one month can be stored at 4°C. To avoid microbial growth, dispense aliquots of the 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. Prepare a 100 mM α-Iodoacetamide Stock Solution (EpiTag™ Digest Kit) by adding 1.4 ml distilled deionized water to the vial and vortexing until the solid is dissolved. Store aliquots of α–Iodoacetamide stock solution at –20°C. Avoid multiple freeze-thaw cycles.
4. Calculate the total amount of Lysis Reagent needed:

Format	Vol. Lysis Reagent
T-25 flask	350 µl
T-75 flask	1 ml
10 cm culture dish	1 ml
6-well plate	300 µl/ well
24-well plate	100 µl/ well
96-well plate	30 µl/ well: Use protocol on p 8.

Note: For non-adherent cells grown in flasks, use ~1 ml Lysis Reagent per 10^7 cells. For smaller cells such as Jurkat, use 0.5 ml Lysis Reagent per 10^7 cells.

5. Prepare the required volume of Supplemented Lysis Reagent.

   Per 1 ml Lysis Reagent:
   20 µl Phosphatase Inhibitor Cocktail Set V (50X)
   2 µl Benzonase® Nuclease

   Notes: Prepare fresh Supplemented Lysis Reagent each time cell lysates are made.

   Do not add protease inhibitors to the Lysis Reagent. They will inhibit the Lys-C proteolytic digestion step.

6. Preheat a heat block to 95°C.
7. On ice, carefully rinse the cell monolayer twice with ice-cold PBS. Remove all PBS.
   For non-adherent cells: transfer the cells to centrifuge tubes, centrifuge at 500 x *g*, and wash twice with ice-cold PBS.
8. Add room-temperature Supplemented Lysis Reagent to the adherent cells. Incubate for 5 min with gentle agitation (rocking platform or occasional swirling) at room temperature.
   For non-adherent cells: flick pellet to loosen and add Supplemented Lysis Reagent. Incubate for 5 min with occasional vortexing.
9. Dislodge and solubilize all adherent cells using a rubber policeman or repeated pipetting. Lysates should be clear and non-viscous. If not, continue room temperature incubation for an additional 5 min.
10. Transfer lysates to 1.5 ml microcentrifuge tubes. Rock or rotate tubes for an additional 20 min at 4°C.

11. Retain a 50 µl sample of each lysate to perform a BCA Protein Assay (EMD Chemicals, Inc., Cat. No. 71285).

    Note: TCEP is a reducing agent that interferes with the BCA assay. Thus, the sample used for the BCA assay must be TCEP-free.

12. Determine the total cellular protein concentration of each lysate sample by BCA assay. Keep the remainder of the lysates on ice to prevent degradation. Alternatively, perform the BCA protein assay at the same time as the denaturing and alkylating steps described below. Typical total cellular protein concentrations range from 0.4 mg/ml to 2 mg/ml, depending on the particular cell line and confluence.

    Note: For optimum assay performance, lysates with concentrations > 2 mg/ml should be diluted to approximately 1 mg/ml with Lysis Reagent before downstream processing.

13. Transfer a 100 µl aliquot of each cell lysate into screw-top microcentrifuge tubes.

    Notes: Although 100 µl is a convenient size for downstream processing, this volume can be scaled up or down. Adjust volumes of added reagents as necessary. A 100 µl aliquot is sufficient for at least eight immunoassay tests.

    The remaining cell lysate can be stored at –70°C fo r future processing. Avoid multiple freeze-thaw cycles.

14. Add 1 µl 100X TCEP Solution to each 100 µl lysate aliquot (2 mM final). Mix by inverting.
15. Denature lysates by heating at 95°C for 5 min.
16. Clarify the denatured lysate by centrifuging at 12,000 x *g* for 10 min at room temperature. Transfer supernatants to new microcentrifuge tubes.
17. Alkylate samples by adding 11 µl α–Iodoacetamide Stock Solution to each 100 µl lysate aliquot (10 mM final). Mix and incubate for 30 min at room temperature.
18. Digest alkylated samples by adding Endopeptidase Lys-C (5.7 U/ml) at a ratio of 100 mU Lys-C per mg total cellular protein. For example, if lysate concentration is 1 mg/ml (100 µl aliquot = 100 µg), add 1.7 µl (10 mU) Lys-C. Digest samples 3 h to overnight at 37°C.
19. After digestion, add 287 µl 1X Assay Diluent to each sample. The final volume (400 µl) is a 4-fold dilution of the original cellular extract.

    Note: Either proceed directly to the WideScreen® EpiTag™ Bead-Based Immunoassay Protocol, or store digested extracts at –70°C for future analysis.

Lysis Protocol for Cells Grown in 96-well Plates

1. Prepare 1X Assay Diluent by adding 25 ml 5X Assay Diluent (WideScreen® Reagent Kit) to 100 ml sterile distilled deionized water. 1X Assay Diluent that will be used within one month can be stored at 4°C. To avoid microbial growth, dispense aliquots of the 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. Prepare a 100 mM α-Iodoacetamide Stock Solution (EpiTag™ Digest Kit) by adding 1.4 ml distilled deionized water to the vial and vortexing until the solid is dissolved. Store aliquots of α–Iodoacetamide stock solution at -20°C. Avoid multiple freeze-thaw cycles.
4. Calculate the total amount of Lysis Reagent needed (30 µl per well), and increase by 10% to allow for pipetting error.
5. Prepare the required volume of Complete Lysis Reagent:

Per 1 ml	Lysis Reagent:
20 µl	Phosphatase Inhibitor Cocktail Set V (50X)
2 µl	Benzonase® Nuclease
10 µl	100X TCEP Solution

    Notes: Prepare fresh Complete Lysis Reagent each time cell lysates are made.

    If lysate protein quantification is desired, prepare a small amount of TCEP-free Lysis Reagent, and reserve this for wells of cells plated for this purpose. Lysates from these wells can be measured using the BCA Protein Assay (EMD Chemicals, Inc., Cat. No. 71285).Lysate containing TCEP cannot be used for BCA assays.

6. Preheat a heat block or plate heater to 95°C.

7. Keep plate on ice. Use a multichannel pipet to carefully rinse cell monolayers twice with ice-cold PBS. Remove all PBS.
   For non-adherent cells: centrifuge and wash cells in a plate with U- or V-bottom wells.
8. Add room-temperature Complete Lysis Reagent to the adherent cells (30 µl per well). Incubate for 10 min at room temperature on a platform plate shaker (750 rpm).
9. Dislodge and solubilize all adherent cells by pipetting up and down several times. Extracts should be clear and non-viscous. If not, continue room temperature incubation for an additional 5 min.

   Note: If TCEP-free wells have been reserved for BCA protein assays, transfer these lysates to microcentrifuge tubes. Clarify by centrifuging for 10 min at 12,000 x g*. Typical total cellular protein concentrations from 96-well plates range from 0.1 mg/ml to 0.5 mg/ml, depending on the particular cell line and confluence.*
10. Seal plate with the supplied aluminum plate sealer. Denature lysates by placing plate on a 95°C plate heater for 10 min. Alternatively, place sealed plate on a 95°C conventional heat block, with the block oriented flat side up.
11. Centrifuge plate in a centrifuge with plate holders to spin down condensation (30 sec, 500 x *g*).
12. Alkylate samples by adding 3.3 µl α-Iodoacetamide Stock Solution to each well using a multichannel pipet. Agitate plate for 30 sec on a platform plate shaker. Incubate for 30 min at room temperature without shaking.
13. Dilute Endopeptidase Lys-C Stock Solution 10-fold in distilled deionized water (0.57 U/ml final concentration). This dilution facilitates accurate pipetting.
14. Digest alkylated samples by adding diluted Endopeptidease Lys-C Stock Solution (5 µl per well) using a multichannel pipet. Agitate plate for 30 sec on a platform plate shaker. Seal wells with the supplied polyethylene plate sealer (included in WideScreen® Reagent Kit) to prevent evaporation. Incubate plate 3 h to overnight at 37°C.

    Note: Although protein concentration of the lysate is unknown, this amount of Lys-C (2.9 mU/ well) will efficiently digest lysates with up to 1 mg/ml total cellular protein. If a BCA protein assay was performed, the amount of Lys-C can be adjusted to the optimized ratio (100 mU Lys-C per mg total lysate protein).
15. After digestion, add 82 µl 1X Assay Diluent to each well. The final volume (120 µl) is a 4-fold dilution of the original cellular extract.

    Note: Either proceed directly to the WideScreen EpiTag™ Bead-Based Immunoassay Protocol, or seal plate and store lysates at –70°C for future analysis.
16. Immediately before beginning the bead-based assay, centrifuge plate at the maximum allowable speed for 10 min to clarify samples. Pellets may not be visible.

Lysis Protocol for Tissue Samples

Fresh or flash-frozen tissue samples can be used. This protocol has been successfully applied to the tissue types listed below, using a 1:15 (w/v) ratio of tissue to Complete Lysis Reagent. Other tissue types may require ratio optimization. For homogenization, use a tube large enough to accommodate some foaming.

1. Prepare 1X Assay Diluent by adding 25 ml 5X Assay Diluent (WideScreen Reagent Kit) to 100 ml sterile distilled deionized water. 1X Assay Diluent that will be used within one month can be stored at 4°C. To avoid microbial growth, dispense aliquots of the 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. Prepare a 100 mM α-Iodoacetamide Stock Solution (EpiTag™ Digest Kit) by adding 1.4 ml distilled deionized water to the vial and vortexing until the solid is dissolved. Store aliquots of α–Iodoacetamide stock solution at -20°C. Avoid multiple freeze-thaw cycles.
4. Calculate the total amount of Lysis Reagent needed (750 µl per 50 mg tissue sample). Allow 10% extra for pipetting error.

5. Prepare the required volume of Complete Cell Lysis Reagent.

   Per 1 ml Lysis Reagent:
   20 µl 50X Phosphatase Inhibitor Cocktail Set V
   2 µl Benzonase® Nuclease
   10 µl 100X TCEP Solution

   Note: Prepare fresh Complete Lysis Reagent each time lysates are made.

6. Preheat a heat block to 95°C.

7. Determine tissue weight. Transfer aliquots of 50–100 mg tissue to 5 ml round-bottom polypropylene tubes. Keep samples on ice.

8. Add 15 parts (v/w) room temperature Complete Lysis Reagent for each part tissue (e.g., 750 µl Complete Lysis Reagent per 50 mg tissue.) Immediately return sample to ice.

9. Homogenize with a hand-held homogenizer or other suitable device. Homogenization time will vary with tissue type, but ~1 minute is generally required for efficient protein extraction. Return sample to ice.

10. Transfer tissue lysates to screw-top microcentrifuge tubes. Denature lysates by heating at 95°C for 5 min.

11. Clarify denatured lysate by centrifuging at 12,000 x *g* for 10 min at room temperature. Transfer supernatants to new microcentrifuge tubes.

12. Remove 50 µl from each sample and dilute 1:10 with water. Determine protein concentration using the Bradford protein assay.

    Note: The Bradford protein assay is used in this protocol because it is unaffected by the TCEP in the Complete Lysis Reagent, and diluting samples 1:10 avoids detergent interference. Alternatively, detergent- and reducing agent-compatible protein assays may be used.

    Table 2. Example tissue lysate protein concentrations

Mouse Tissue Sample	Protein Concentration
Brain	4–6 mg/ml
Lung	4–6 mg/ml
Liver	7–9 mg/ml
Spleen	6–8 mg/ml

13. In separate tubes, prepare a 100 µl aliquot of each tissue lysate, diluting to 1 mg/ml total protein. Use unsupplemented Lysis Reagent to make the dilution.
    To determine volume of lysate and lysis reagent:

$$\frac{(1\ \text{mg/ml})(0.1\ \text{ml})}{X\ \text{mg/ml}} \times 1000 = Y\ \mu\text{l tissue lysate}$$

$$100\ \mu\text{l} - \mu\text{l tissue lysate} = \mu\text{l Lysis Reagent required}$$

    Notes: Although 100 µl is a convenient size for downstream processing, this volume can be scaled up or down. Adjust volumes of added reagents as necessary. A 100 µl aliquot is sufficient for at least eight immunoassay tests.

    Store remaining tissue lysate at –70°C for future processing and analysis.

    Avoid multiple freeze-thaw cycles.

14. Alkylate samples by adding 11 µl α-Iodoacetamide Stock Solution to each 100 µl lysate aliquot (10 mM final). Mix and incubate for 30 min at room temperature.

15. Digest alkylated samples by adding Endopeptidase Lys-C (5.7 U/ml) at a ratio of 100 mU Lys-C per mg total protein. For 100 µl aliquots diluted to 1 mg/ml, add 1.7 µl (10 mU) Lys-C. Digest samples 3 h to overnight at 37°C.

16. After digestion, add 287 µl 1X Assay Diluent to each sample. The final volume (400 µl) is a 4-fold dilution of the 1 mg/ml tissue extract.

    Note: Either proceed immediately to the WideScreen® EpiTag™ Bead-Based Immunoassay protocol, or store digested lysate samples at –70°C for future analysis.

Flowchart for EpiTag™ Lysate Preparation and Digestion

Cells Grown in Flasks or Dishes

Prepare Stocks & Buffers

- **1X Assay Diluent:** Dilute 5X five-fold with water
- **1X Wash Buffer:** Dilute 10X ten-fold with water
- **100 mM iodoacetamide:** Add 1.4 ml water

Prepare Supplemented Lysis Reagent

per 1 ml Lysis Reagent:

- **Phosphatase Inhibitor Cocktail:** 20 µl
- **Benzonase® Nuclease:** 2 µl

Cell Lysis

- Wash cells twice with ice-cold PBS
- Add **Supplemented Lysis Reagent** (1 ml per dish or T-75 flask)
- Rock for 5 min at room temperature
- Pipet repeatedly and transfer to centrifuge tubes
- Rotate for 20 min at 4°C

Determine Total Cellular Protein

- Use BCA protein assay to determine cell lysate protein concentration
- Store (– 70°C) any lysate aliquots that will not be processed

Denature Lysates

- Transfer 100 µl of each lysate to screw-top tubes
- Add 1 µl **100X TCEP** to each tube
- Heat at 95°C for 5 min
- Centrifuge 10 min (12,000 x *g*, room temperature)
- Transfer supernatants to new tubes

Alkylate and Digest Lysates

- Add 11 µl **α-iodoacetamide** stock per tube
- Incubate 30 min at room temperature
- Add 1.7 µl **Lys-C** stock (5.7 U/ml) per 100 µg lysate protein
- Digest 3 h to overnight at 37°C

Dilute Digested Lysates

- Add 287 µl **1X Assay Diluent** to each digested lysate sample
- Samples are ready for assay or storage (– 70°C)

Cells Grown in 96-well Plates

Prepare Stocks & Buffers

- **1X Assay Diluent:** Dilute 5X five-fold with water
- **1X Wash Buffer:** Dilute 10X ten-fold with water
- **100 mM iodoacetamide:** Add 1.4 ml water
- **0.57 U/ml Lys-C:** Dilute stock 10-fold

Prepare Complete Lysis Reagent

per 1 ml Lysis Reagent:

- **Phosphatase Inhibitor Cocktail:** 20 µl
- **Benzonase Nuclease:** 2 µl
- **100X TCEP** **10 µl**

Cell Lysis

- Wash cells twice with ice-cold PBS
- Add **Complete Lysis Reagent** (30 µl per well)
- Shake for 10 min (750 rpm, room temperature)
- Pipet repeatedly (10 times) to solubilize cells

Denature Lysates

- Seal plate with **Aluminum Plate Sealer**
- Heat at 95°C for 10 min
- Spin down condensation (30 sec, 300 x *g*, room temperature)

Alkylate and Digest Lysates

- Add 3.5 µl **α-iodoacetamide** stock to each well
- Shake 1 min, then incubate 30 min at room temperature
- Add 5 µl **diluted Lys-C** (0.57 U/ml) to each well
- Seal plate & digest 3 h to overnight at 37°C

Dilute Digested Lysate

- Add 82 µl **1X Assay Diluent** to each well
- Samples are ready for assay or storage (– 70°C)
- ***Note: Immediately before immunoassay, centrifuge plate ~3000 rpm to clarify lysates***

Considerations Before You Begin

- Have on hand the 1X Assay Diluent and 1X Wash Buffer that was prepared during the Lysate Preparation and Digestion procedure.
- Important guidelines to follow when using filter plates and the vacuum manifold:
  - Excessive vacuum will perforate the filter plate membrane. Adjust the manifold using a non-filter (ELISA or tissue culture) plate so that the vacuum cannot exceed 5 in (127 mm) Hg.
  - After adjusting the vacuum, place filter plate on the manifold. Apply pressure evenly across the plate using your fingertips. 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 > 1 min before proceeding to the next step and 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 samples or reagents are added. 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.
- 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 and should be protected from light.
- Many of the washing and pipetting steps are done most easily with an 8-channel or 12-channel pipet (manual or automatic). However, for best results, use an accurate single-channel pipet to manipulate standards and experimental samples as described in Steps 2, 3 and 5.
- For efficiency, prepare reagents for the next step during incubation periods. Diluted standards, Detection Antibodies, and Streptavidin-PE should be used within 1 h of preparation.
- When calculating the amount of reagents needed during the various steps, prepare a 10% excess to allow for pipetting errors.
- Run standard dilution series and experimental samples using the same multiplex assay configuration. For instance, if a 6-plex of EpiTag™ Bead Kits is used to measure your unknowns, the same 6-plex should be used to create the standard dilution series. Multiplexing causes slight shifts in some standard curves compared to singleplex assays, which will make quantitation inaccurate unless the experimental samples are measured using the same multiplex of beads and detection antibodies.
- For best overall assay performance, lysates are diluted at least 8-fold final when incubated with Capture Beads. If desired, lysates can be tested at a 4-fold final dilution, although this concentration of Lysis Reagent decreases the sensitivity of some EpiTag Bead Kits. If a 4-fold dilution is used, change the titration buffer composition to 50% Lysis Reagent/ 50% 1X Assay Diluent to ensure accurate quantitation. Final lysates dilutions less than 4-fold are not recommended.

Step 1: Prepare Titration buffer

Quantitative immunoassays are sensitive to buffer composition. Therefore, include the same proportion of Lysis Reagent in all dilutions of standards and samples. The best overall assay performance occurs when lysates are diluted at least 8-fold during incubation with Capture Beads. Titration buffer (25% Lysis Reagent, 75% 1X Assay Diluent) is used for all analyte dilutions and, once added to beads (in Assay Diluent), maintains an 8-fold final dilution of Lysis 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 510 µl titration buffer is needed for each standard dilution series (see *Step 2: Prepare Standard Dilution Series* on page 13). A minimum of 60 µl titration buffer is needed for each lysate sample that is diluted beyond 8-fold final (see *Step 3: Prepare Sample Titrations (optional)* on page 13). If duplicate or triplicate standard dilution series will be run, increase volumes accordingly.

Example Calculation: (1 standard dilution series and 30 experimental sample dilutions)

1 Standard dilution series = 510 µl

30 diluted lysate samples = 1800 µl (30 x 60 µl)

Minimum of 2310 µl titration buffer x 1.10 to accommodate pipet error = **2541 µl**

2. Prepare the required volume of titration buffer by mixing Lysis Reagent from the EpiTag™ Cell Lysis Kit and 1X Assay Diluent prepared from the EpiTag™ Reagent Kit (increase by 10% to allow for pipetting error). Use a final composition of 25% Lysis Reagent to 75% 1X Assay Diluent.

Step 2: Prepare Standard Dilution Series

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

The EpiTag Raf1 Total High Sensitivity Bead Kit requires a modified standard dilution series compared to the EpiTag Raf1 Total Bead Kit. If using the EpiTag Raf1 High Sensitivity Bead Kit, see Appendix B for details.

1. To prepare a 7-point standard dilution series as described below, label 8 microcentrifuge tubes and add 60 µl titration buffer to tubes 2–8. If the standard dilution series will be performed in duplicate or triplicate, double or triple all volumes, respectively. Preparation of titration buffer is described in *Step 1: Prepare Titration buffer* on page 12.
2. Thaw the appropriate EpiTag™ Standards Mix and equilibrate the tube to room temperature for 10 min. Vortex 10 sec to ensure all peptides are in solution.
3. In tube 1, add 10 µl EpiTag Standards Mix (10X solution) to 90 µl Titration buffer. This tube is "Dilution 1" of the standard dilution series.
4. Prepare 3-fold serial dilutions from Dilution 1:
   - Transfer 30 µl from tube 1 to the 60 µl titration buffer in tube 2; mix well.
   - Change tips, and transfer 30 µl from tube 2 to the 60 µl titration buffer in tube 3; mix well.
   - Proceed in similar manner with serial dilutions through tube 7.
5. The 8th tube, containing 60 µl titration buffer only, will serve as the blank control.

   Note: Molar concentrations of standard dilutions series are indicated in Appendix B (page 26).

Table 3. Serial Dilution of EpiTag™ Standards Mix

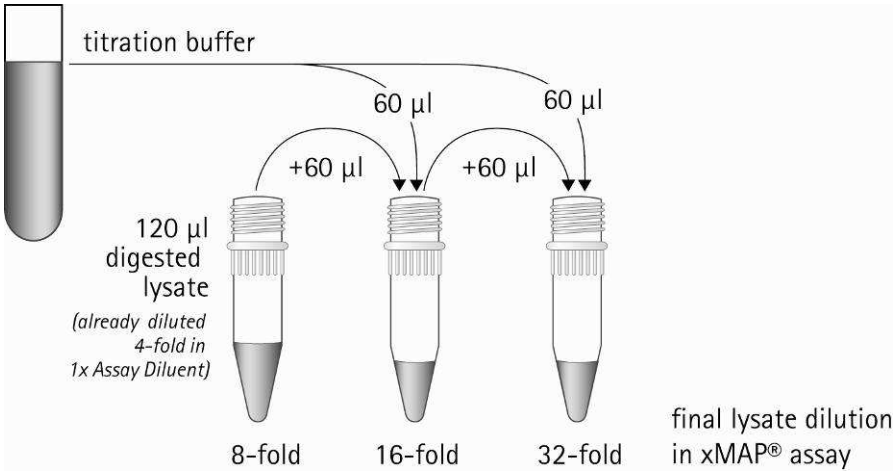
Tube/ Dilution	Vol. Standard	Vol. Titration buffer	Final Conc.
1	10 µl EpiTag Standards Mix	90 µl	See Appendix B
2	30 µl from Tube 1	60 µl	
3	30 µl from Tube 2	60 µl	
4	30 µl from Tube 3	60 µl	
5	30 µl from Tube 4	60 µl	
6	30 µl from Tube 5	60 µl	
7	30 µl from Tube 6	60 µl	
8/ BLANK	None	60 µl	0

Step 3: Prepare Sample Dilutions (optional)

Digested lysates prepared according to the *Lysate Preparation and Digestion* protocols (page 6–9) have already been diluted 4-fold in Assay Diluent. Lysates can be added directly (at 1:1 ratio) to Capture Beads, resulting in an 8-fold final dilution. Follow the procedure below if further dilution of digested lysates is desired.

Note: Thaw and dilute samples within 1 h of use.

1. Label 3 microcentrifuge tubes. Add 60 µl Titration buffer to tubes 2 and 3. If samples will be analyzed in triplicate, triple all volumes.
2. To tube 1, add 120 µl diluted digested lysate from the final step of the *Lysate Preparation and Digestion* protocols.
3. Prepare 2-fold serial dilutions:
   - Transfer 60 µl from tube 1 to the 60 µl titration buffer in tube 2 and mix well.
   - Change tips, and transfer 60 µl from tube 2 to the 60 µl titration buffer in tube 3 and mix well.
   - Once added to Capture Beads, these serial dilutions correspond to 8-, 16-, and 32-fold dilutions of the original cellular lysate.



Step 4: Prepare Capture Beads

Note: Use diluted Capture Beads within 1 h.

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 2000-3000 beads per bead region per well.

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

3. Thoroughly resuspend each vial of Capture Beads (50X) by vortexing for 10–20 sec, sonicating in an ultrasonic bath for 20 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: (37 test wells, therefore calculate for 40 wells to allow ~10% extra)

	Singleplex, *or* EpiTag™ Panel multiplex (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. On the 96-well filter plate, tape off wells that are not going to be used for the assay with lab tape for future use.
2. Pre-wet the 96-well filter plate for at least 5 min by adding 100 µl 1X Assay Diluent to each well that will be used. Using the vacuum manifold, apply gentle vacuum (3 inHg/76 mmHg) to filter plate just until all liquid is aspirated. Tap filter plate on a paper towel to remove any buffer from underside. See *Considerations Before You Begin* on page 12 for guidelines on using the filter plate and vacuum manifold.

   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, do not place filter plate on an absorbent surface during incubations.
3. Vortex (10 sec) the diluted Capture Beads solution prepared as per *Step 4: Prepare Capture Beads* on page 14. Add 50 µl to each well being used.
4. To bead-containing wells designated for the standard dilution series, add 50 µl standard dilutions (Dilutions 1–7 + blank) prepared as per *Step 2: Prepare Standard Dilution Series* on page 13.
5. To bead-containing wells designated for experimental sample analysis, add 50 µl prepared and digested lysates. If additional sample titrations were prepared as per *Step 3: Prepare Sample Dilutions (optional)* on page 13, add 50 µl to bead-containing wells.
6. Incubate for 2 h at room temperature on a platform plate shaker (750 rpm). Use aluminum foil to protect filter plate from light.

   Note: Longer incubations will increase overall signals from targets, but generally do not improve signal-to-background ratios.

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:

Assay Format	**Vol. Detection Antibodies (100X) needed**
Singleplex (one target)	1 µl per well
EpiTag™ Panel multiplex (premixed)	1 µl per well
User-assembled multiplex	1 µl from each individual Bead Kit 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* EpiTag™ Panel multiplex (premixed)	**User-assembled 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 Antibodies per well x 40 wells **= 40 µl Detection Antibodies**	5 µl Antibodies per well x 40 wells **= 200 µl Detection Antibodies (40 µl each)**
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. To each well, add 100 µl 1X Wash Buffer. 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, and add the next solution immediately after tapping the 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 the platform plate shaker (750 rpm). Protect from light.

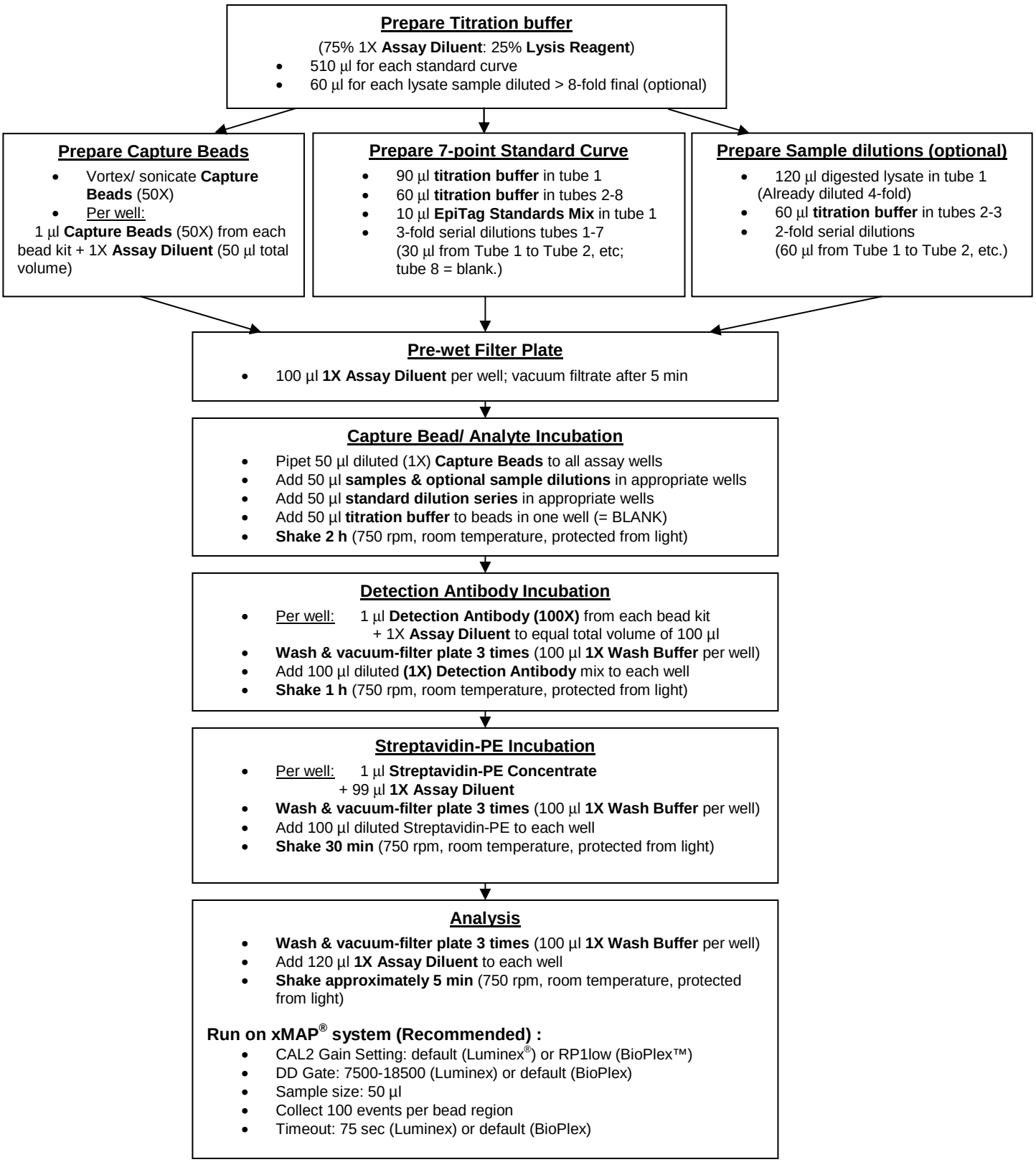
   Note: Turn on the Luminex® xMAP® system. The lasers require a 30 min warm-up period.

Step 7: Add Streptavidin-Phycoerythrin (PE)

Note: Prepare 1X Streptavidin-PE solution within 1 h of use. Dilute only the amount needed based on the number of wells, plus 10% extra to allow for pipetting error. If there is an insufficient volume of Streptavidin-PE Concentrate for your final experiment, making a slightly more dilute working stock will not adversely affect results.

1. Calculate the total volume of 1X Streptavidin-PE solution needed. 100 µl is needed for each test well. Prepare 10% extra to allow for pipetting error.
2. Briefly spin (5 sec) the Streptavidin-PE Concentrate vial to ensure all material is in the bottom of the tube.
3. Prepare the required volume of Streptavidin-PE by diluting Streptavidin-PE Concentrate 1/100 in 1X Assay Diluent. Vortex 3 sec. Protect from light and store at 4°C until use.
4. Wash sample wells three times with 100 µl 1X Wash Buffer as described above. Remove buffer by vacuum filtration between washes. 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 30 min at room temperature on a platform plate shaker (750 rpm). Protect from light.
7. Wash sample wells three times with 1X Wash Buffer as described above. After 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 each assay well. To fully resuspend beads before running samples on the Luminex system, incubate for 5–7 min on a platform plate shaker (750 rpm). Protect from light.

Flowchart for EpiTag™ Bead-Based Immunoassay Protocol



Collecting Data and Data Analysis

Data Acquisition

For detailed instructions on the operation of Luminex® systems, refer to user guides for your specific instrument and software. 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, standards, samples, and controls that will be run. The ranges of final concentrations found in the EpiTag™ Standards Mix are shown in Appendix B.
2. Select the bead regions used in the assay. The bead regions used for the EpiTag™ Bead Kits are shown in Appendix B.
3. Format the assay plate, indicating which wells contain each 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	75 sec	7500-18500	default
ACS STarStation	50 µl	100	75 sec	default	default
Bio-Rad® Bio-Plex® Manager™	default (50 µl)	100	default	default (4335-10000)	RP1 Low

**If the time spent acquiring samples needs to be reduced, collect as low as 50 events per bead region, or adjust the timeout as short as 30 sec.*

Generation of Standard Curves and Quantification of Experimental Samples

- Peptide or phosphopeptide standards are available for all of the EpiTag Bead Kit assays (see Appendix B), allowing accurate quantification. Representative standard curves and assay performance information are available in the Certificates of Analysis for 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 obtained from bead-based immunoassays. 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. The EpiTag standard dilutions generally focus on the lower half of the curve to allow maximum sensitivity in this range.
- 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.
- When determining concentrations of experimental samples by reference to the standard curve, to multiply these values by the lysate dilution factor to obtain target concentration in the original cell lysate. Upon completion of the Lysate Preparation and Digestion protocol (page 6–9), samples have been diluted 4-fold from the original cell extract. An additional 2-fold dilution occurs when samples are added to the Capture Beads (8-fold final dilution).
- EpiTag™ technology uses peptide standards to quantitate target protein proteolytic fragments. Because each target protein (or phosphoprotein) is found in equimolar amounts in comparison to the proteolytic fragment being assayed, the molar concentration of each unknown can be predicted using the standard curve. To convert target protein molar concentrations to the more traditional mass/ volume, use the following equation:

  (Concentration in pM) x (Target molecular weight in kDa) = Conc. in pg/ml

  Example Calculation: (for 15.6 pM phospho-ERK1)
  (15.6 pM pERK1) x (MW 43.3 kDa) = 675 pg/ml pERK1

Supplementary Protocols

Stimulation of Cell Lines

A431 cell line (ATCC CRL-1555): Stimulation with Epidermal Growth Factor (EGF)

1. Maintain early passage cells at a density of 1–5 x 10^6 cells per T-75 flask. After establishing consistent growth rate, plate cells into 100 mm dishes or 6-well plates at a density that will result in ~ 70% confluence by the time of serum starvation initiation. Prepare cells in parallel for induction (stimulated) and mock induction (unstimulated).
2. On the day before induction, replace tissue culture medium with the same type of medium used for cultivation, but lacking FBS. Cells should be ~ 70% confluent. Incubate at 37°C and 5% CO_2 overnight (up to 16 h).

   Note: For adherent cell lines that begin to detach after prolonged (overnight) serum starvation, similar stimulation results can be obtained by serum starving for 4 h prior to induction.
3. Prepare induction medium by diluting rh-EGF (Cat. No. 324831) stock to a final concentration of 100 ng/ml in tissue culture medium lacking FBS. Approximately 5 ml per 100 mm dish, T-75 flask or 6-well plate is sufficient. For mock inductions, prepare tissue culture medium lacking FBS and EGF. Warm to 37°C.

   Notes: If the mitogen (and/or inhibitors being tested) are suspended in DMSO, the mock induction should contain the same amount of DMSO.

   Have all reagents for cell extraction ready before inducing cells.
4. Remove cell medium and replace with warmed induction medium (or mock induction medium). Immediately return cells to incubator.
5. Incubate at 37°C and 5% CO_2 for 5-10 min.

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

Jurkat cell line (ATCC TIB-152): Stimulation with anti-CD3 and anti-CD28 antibodies

1. Maintain early passage cells at a density between 1 x 10^5 and 1 x 10^6 cells/ml.
2. On the day before induction, collect cells by centrifugation. Resuspend pellet at 5 x 10^5 in the same type of medium used for cell cultivation, but lacking fetal bovine serum (FBS). Incubate at 37°C and 5% CO_2 overnight, up to 16 h.
3. On the day of induction, adjust cell density to 1 x 10^6 cells/ml with serum-free medium. Prepare cells for induction and mock induction in parallel.
4. Add anti-CD3 antibody (BD Pharmingen Clone UCHT1, Cat. No. 555329) to a final concentration of 0.25 µg/ml. Add anti-CD28 antibody (BD Pharmingen Clone CD28.2, Cat. No. 555725) to a final concentration of 1.0 µg/ml. Do not add antibodies to mock-induced.
5. Incubate at 37°C and 5% CO_2 for ~1 h.
6. Extract cells according to Lysate Preparation and Digestion protocol (page 6).

Stimulation Conditions for Select Cell Lines (Phosphorylation of ERK Pathway Kinases)

Cell line	Type[a]	Species	Tissue/ Morphology	Stimulation[c] ($37^{o}C$ and 5% CO_2)
A431	adh	H	skin/ epithelial	SFM (4 h); 100 ng/ml EGF (5 min)
HEK 293	adh	H	kidney/ epithelial	SFM (4 h); 100 ng/ml EGF (5 min)
HEK 293	adh	H	kidney/ epithelial	SFM (4 h); 400 ng/ml PMA (30 min)
HeLa	adh	H	cervix/ epithelial	SFM (16 h); 100 ng/ml EGF (5 min)
Raji	susp	H	B-cell/ lymphoma	SFM (4 h); 200 ng/ml PMA (30 min)
Jurkat	susp	H	T-cell/ lymphoblast	SFM (16 h); 0.25 µg/ml α-CD3/ 1 µg/ml α-CD28 (1 h)
MCF-7	adh	H	breast/ epithelial	SFM (16 h); 100 ng/ml EGF (5 min)
NIH 3T3	adh	M	embryo/ fibroblast	SFM (16 h); 75 ng/ml PDGF x (10 min)
RAW 264.7	adh	M	ascites/ macrophage	SFM (16 h); 100 ng/ml LPS (30 min)
SK-Br-3	adh	H	breast/ epithelial	SFM (16 h); 100 ng/ml EGF (5 min)

[a] adh = adherent; susp = suspension

[b] H = human; M= mouse

[c] SFM = serum free medium; EGF = epidermal growth factor; PMA = Phorbol myristate acetate; PDGF = platelet-derived growth factor; LPS = lipopolysaccharide

Troubleshooting

Problem	Probable Cause	Solution
Lysate is viscous	Genomic DNA not digested	Make sure Benzonase® Nuclease was added to Lysis Reagent. Incubate lysate longer at room temperature. If the lysate can be pipetted, proceed to the 95°C incubation, which will eliminate viscosity. For cell lines with recurring viscosity problems, additional Benzonase Nuclease can be added (available separately).
Leaking wells in filter plate	Wicking due to adherant drops	Tap filter plate on paper towel several times 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 samples. Blot underside of the affected wells and add 120 µl/well 1X Assay Diluent).
	Perforation of filter plate membranes	Adjust vacuum setting to < 5 in (127 mm) Hg. Do not touch membranes with pipet tips. Adjust probe height on the Luminex® instrument.
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 on filter plate to ensure a good seal is formed. Use a plate sealer to cover wells not in use.
	Cell debris clogs membranes	Use the non-tip end of a 200 µl pipet tip to flick the center support on the underside of the well, then reapply vacuum. Clarify lysates by centrifugation. Avoid disturbing pellets when removing supernatants. If lysate protein concentration is high, dilute further before assaying. If necessary, filter digested lysates using syringe-tip filters or a filter plate (0.45 µm pore size) before proceeding with immunoassay.
Low bead counts during data acquisition	No beads (or wrong beads) in the wells	See solutions above for leaking wells. Ensure that the appropriate beads were added, 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 instrument user manual (sanitize, alcohol flush, probe sonication probe, etc.). Make sure that the probe height is set correctly. Clarify viscous lysates as described in "Filter plate wells not draining under vacuum" above. Make sure that beads are in suspension by incubating plate for 3–5 min on a 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 (50 µl sample, 100 events per bead, 75 sec timeout, etc.).
Data acquisition is slow	No beads or wrong beads in the wells, or fluidics system clogged	See "Low bead counts during data acquisition" solutions, above.
	Some bead regions being acquired are not in the wells	Make sure that the appropriate 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.
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 the 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 falling outside the bead region gates due to photobleaching	Do not use expired beads. Do not expose 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 SD fluidics module is turned on. Check system calibration using approved calibration beads. Verify correct system pressure. Confirm that system is free of air or particulate buildup. Follow maintenance steps in equipment user manual.

Problem	Probable Cause	Solution
Insufficient volume of an immunoassay reagent	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, TBST + 1% BSA (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.
		If there is insufficient Streptavidin-PE for you final experiment, making a slightly more dilute working stock (e.g. 1:150 instead of 1:100) will not adversely affect results.
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 the outermost wells of plate.
		Plate cells uniformly. Add lysis and digestion reagents accurately.
		Be careful not to dislodge adherent cells during pre-lysis wash steps. If necessary, decant liquid (instead of aspirating) and tap plate on paper towels.
		If cells become less adherent during overnight serum starvation, shorten the serum starvation step to 4 h.
	Lysates assayed at different times show assay-to-assay variability	Generate standard curve using multiple dilution series replicates (e.g., in triplicate) to increase inter-assay precision.
		Fully resuspend standards and digested lysate samples by thawing to room temperature and vortexing 15 sec.
		Values derived from the same digested lysate sample will show less variability than values from lysates samples digested at different times.
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.
		If values are near the bottom of the standard curve, digested lysates may be tested at concentrations higher than the recommended 8-fold dilution. In this case, adjust titration buffer such that all wells receive the same amount of Lysis Reagent.
		Signals may be boosted by increasing the lysate protein concentration, by lysing cells at a higher confluence, or by using less Lysis 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 unknowns.
		Do not multiplex assays that result in significantly higher background values. See the EpiTag™ Assay Compatibility Chart (Appendix C) for details.
	Target concentration is below detection	Ensure that stimulation conditions are optimized.
		Screen additional cell lines or tissue types for target of interest. Expression may be suboptimal in some cell lines and tissues.
		Confirm that antibodies used in the assay recognize the species being tested.

Appendix

A: Product Ordering and Storage Information

Common Kits

The following kits are compatible with all panels and individual bead kits.

EpiTag™ Cell Lysis Kit		**71784-3**
10 ml	Lysis Reagent	Store at Room Temp
100 µl	Phosphatase Inhibitor Cocktail Set V (50X)	Store at –20°C
10 µl	Benzonase® Nuclease HC, Purity > 99%	Store at –20°C
50 µl	100X TCEP Solution	Store at –20°C
1 ea	Aluminum Plate Sealer	Store at Room Temp

EpiTag™ Digest Kit		**71785-3**
26 mg	α-iodoacetamide	Store at –20°C
60 µl	Endopeptidase Lys-C (5.7 U/ml)	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 Temp
1 ea	96-well Filter Plate	Store at Room Temp

EpiTag™ Combo Reagent Kit		**71794-3**
1	EpiTag Cell Lysis Kit	See storage directions above
1	EpiTag Digest Kit	See storage directions above
1	WideScreen Reagent Kit	See storage directions above

Bead Kits

Each EpiTag™ Bead Kit contains the following components:

- 100 µl Capture Beads (50X, use 1 µl per test)
- 100 µl Detection Antibody (100X, use 1 µl per test)

Individual EpiTag Bead Kits			
100 tests	EpiTag ERK 1 Total Bead Kit	Store at 4°C	71829-3
100 tests	EpiTag ERK 2 Total Bead Kit	Store at 4°C	71830-3
100 tests	EpiTag B-Raf (pS446) Bead Kit	Store at 4°C	71831-3
100 tests	EpiTag MEK1/2 (pS217, pS221/ pS222, pS226) Bead Kit	Store at 4°C	71832-3
100 tests	EpiTag ERK1 (pT202, pY204) Bead Kit	Store at 4°C	71833-3
100 tests	EpiTag ERK2 (pT185, pY187) Bead Kit	Store at 4°C	71834-3
100 tests	EpiTag MEK1 Total Bead Kit	Store at 4°C	71835-3
100 tests	EpiTag MEK2 Total Bead Kit	Store at 4°C	71836-3
100 tests	EpiTag STAT1 Total Bead Kit	Store at 4°C	71837-3
100 tests	EpiTag Raf1 Total Bead Kit	Store at 4°C	71838-3
100 tests	EpiTag Raf1 (pS338) Bead Kit	Store at 4°C	71839-3
100 tests	EpiTag Raf1 Total High Sensitivity Bead Kit*	Store at 4°C*	72307-3

* The EpiTag Raf1 Total High Sensitivity Bead Kit contains its own peptide standard (10X solution), to be used if the assay is run in singleplex. See Appendix B for details. Store the Raf1 Total HS Standard at 4°C for one month. For long term storage more than a month, store at –20°C.

EpiTag™ ERK Panel I		**71826-3**
100 tests	Pre-mixed EpiTag ERK Pathway 6-plex Bead Kit: • ERK1 Total • ERK2 Total • B-Raf (pS446) • MEK1/2 (pS217, pS221/ pS222, pS226) • ERK1 (pT202, pY204) • ERK2 (pT185, pY187)	Store at 4°C

EpiTag ERK Panel II		**71890-3**
100 tests	Pre-mixed EpiTag ERK Pathway 6-plex Bead Kit: • MEK1 Total • MEK2 Total • MEK1/2 (pS217, pS221/ pS222, pS226) • Raf1 Total • Raf1 (pS338) • STAT1 Total	Store at 4°C

EpiTag™ ERK Pathway Standards

EpiTag™ ERK Pathway Standards Mix		**71791-3**
The EpiTag ERK Pathway Standards Mix contains reagents sufficient to create six singleplex or multiplex standard curves for all of the EpiTag ERK Pathway Bead Kits*:		
60 µl	Mix of EpiTag™ peptide standards (10X solution)	Store at 4°C (1 mo); –20°C (long term)

Note: The EpiTag Raf1 Total High Sensitivity Bead Kit contains its own peptide standard (10X solution), to be used if the assay is run in singleplex. For special instructions for preparing the Raf1 Total High Sensitivity dilution series, refer to Appendix B.

Complete Kits

EachWideScreen® EpiTag™ Panel Complete Kit contains the entire set of reagents to run 96 assays, including an EpiTag Panel (which includes a multiplex panel of Capture Beads and Detection Antibodies), an EpiTag™ Standards Mix, EpiTag™ Cell Lysis Kit, EpiTag™ Digest Kit, and WideScreen Reagent Kit.

WideScreen® EpiTag ERK Pathway Panel I Complete Kit		**71782-3**
100 tests	Pre-mixed EpiTag ERK Panel I 6-plex Bead Kit: • ERK1 Total • ERK2 Total • B-Raf (pS446) • MEK1/2 (pS217, pS221/ pS222, pS226) • ERK1 (pT202, pY204) • ERK2 (pT185, pY187)	Store at 4°C
1	EpiTag ERK Pathway Standards Mix	See storage information on page 24
1	EpiTag™ Cell Lysis Kit	See storage information on page 23
1	EpiTag™ Digest Kit	See storage information on page 23
1	WideScreen® Reagent Kit	See storage information on page23

WideScreen EpiTag ERK Pathway Panel II Complete Kit		**71891-3**
100 tests	Pre-mixed EpiTag ERK Pathway 6-plex Bead Kit: • MEK1 Total • MEK2 Total • MEK1/2 (pS217, pS221/ pS222, pS226) • Raf1 Total • Raf1 (pS338) • STAT1 Total	Store at 4°C
1	EpiTag ERK Pathway Standards Mix	See storage information on page 24
1	EpiTag Cell Lysis Kit	See storage information on page 23
1	WideScreen Reagent Kit	See storage information on page 23

Note: Due to the unique nature of the EpiTag™ technology and sample preparation, the EpiTag™ Bead Kits and reagents are not compatible with other bead kits and reagents sold by EMD Chemicals, Inc. or other vendors.

Caution: Some kit components contain hazardous chemicals. Refer to MSDS for additional information (MSDS are available on www.emdbiosciences.com).

B: Dilution Series For Generating Standard Curves

The standard curve allows quantification of target proteins and phosphoproteins found in cell lysates. The EpiTag™ Standards Mixes are synthetic peptides and phosphopeptides corresponding to Lys-C-generated target protein proteolytic fragments. Because each target protein (or phosphoprotein) is present in equimolar amounts in comparison to the proteolytic fragment being detected, standard curve concentrations are expressed in molarity.

Note: The range of standard concentrations varies by individual assay, as the linear range and lower limit of each assay depends on assay sensitivity. Values shown are the final concentrations, which accounts for the 2-fold dilution after addition to Capture Beads (50 µl standard + 50 µl Capture Beads)

EpiTag™ ERK Pathway Standards Mix

Table 4: Final concentrations in the 3-fold serial dilution of standards

	Total-ERK2	Total-ERK1	Phos-ERK2	Phos-ERK1	Phos-MEK1/2	Total-MEK1	Total-MEK2	Total-STAT1	Total-Raf1	Phos-Raf1	Phos-B-RAF	Phos-RSK2	Phos-STAT3
Bead Region	**#21**	**#25**	**#72**	**#74**	**#76**	**#30**	**#40**	**#44**	**#43**	**#77**	**#80**	**#75**	**#28**
Conc in:	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**	**pM**
Dilution 1	1111	10000	370	123	1111	10000	3333	10000	3333	10000	10000	123	3333
Dilution 2	370	3333	123	41	370	3333	1111	3333	1111	3333	3333	41	1111
Dilution 3	123	1111	41	14	123	1111	370	1111	370	1111	1111	14	370
Dilution 4	41	370	14	4.6	41	370	123	370	123	370	370	4.6	123
Dilution 5	14	123	4.6	1.5	14	123	41	123	41	123	123	1.5	41
Dilution 6	4.6	41	1.5	0.5	4.6	41	14	41	14	41	41	0.5	14
Dilution 7	1.5	14	0.5	0.17	1.5	14	4.6	14	4.6	14	14	0.17	4.6

Standard Dilution Series Instructions for the EpiTag™ Raf1 Total High Sensitivity Bead Kit

The concentration of Raf1 Total Standard in the EpiTag™ ERK Pathway Standards Mix is too high to generate an optimal standard curve for the EpiTag Raf1 Total High Sensitivity Bead Kit. Therefore, use the following modifications to the EpiTag Bead-Based Immunoassay Protocol (Step 2: Prepare Standard Dilution Series) to generate the standard dilution series for the EpiTag Raf1 Total High Sensitivity Bead Kit.

- If the EpiTag Raf1 Total High Sensitivity Bead Kit (Bead Region #27) will be run alone (singleplex), dilute the Raf1 Total HS Standard (10X, supplied with the bead kit) as described in the chart below (Table 5).
- If the EpiTag Raf1 Total High Sensitivity Bead Kit (Bead Region #27) will be multiplexed with other EpiTag ERK Pathway bead kits, dilute the EpiTag ERK Pathway Standards Mix (10X, available separately) with two additional dilutions, as described in the chart below (Table 6). Use values obtained from Dilutions 3–9 to generate the Raf1 Total HS standard curve, and the values obtained from Dilutions 1–7 to generate standard curves for the other assays in the multiplex.

Table 5: EpiTag™ Raf1 Total High Sensitivity Bead Kit: SINGLEPLEX format

Tube/ Dilution	Volume of Standard	Volume of Titration buffer	Final Conc. Raf1 Total (pM)
1	10 µl **Raf1 Total HS Standard**	90 µl	370
2	30 µl from Tube 1	60 µl	123
3	30 µl from Tube 2	60 µl	41
4	30 µl from Tube 3	60 µl	14
5	30 µl from Tube 4	60 µl	4.6
6	30 µl from Tube 5	60 µl	1.5
7	30 µl from Tube 6	60 µl	0.5

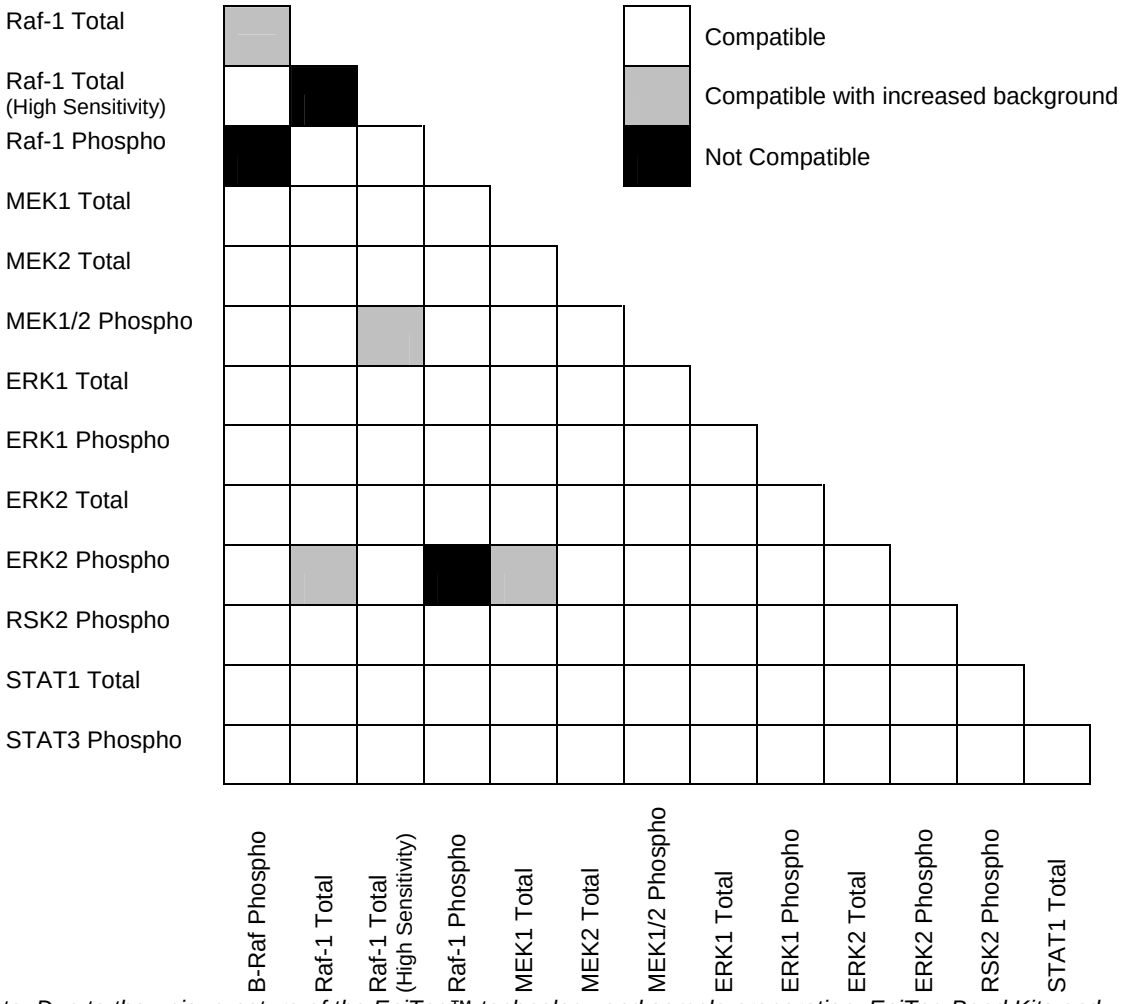
Table 6: EpiTag™ Raf1 Total High Sensitivity Bead Kit: MULTIPLEX format

Tube/ Dilution	Volume of Standard	Volume of Titration buffer	Final Conc. Raf1 Total (pM)
1	10 µl **EpiTag ERK Pathway Standards Mix**	90 µl	3333
2	30 µl from Tube 1	60 µl	1111
3	30 µl from Tube 2	60 µl	370
4	30 µl from Tube 3	60 µl	123
5	30 µl from Tube 4	60 µl	41
6	30 µl from Tube 5	60 µl	14
7	30 µl from Tube 6	60 µl	4.6
8	30 µl from Tube 7	60 µl	1.5
9	30 µl from Tube 8	60 µl	0.5

C: EpiTag™ Assay Compatibility Chart

Some individual bead-based assays should not be multiplexed together, typically because detection antibodies from one assay will bind non-specifically to capture beads from another assay. This matrix indicates compatibility between all paired combinations of the EpiTag™ Bead Kits. "Not Compatible" signifies a combination that should not be used together, typically due to unacceptably high background in one of the assays. "Compatible with increased background" combinations may be used with only a minor loss in sensitivity. To multiplex the EpiTag Raf1 Total High Sensitivity Bead Kit with other ERK Pathway bead kits, see the special instructions in Appendix B for making the standard dilution series.

Note: This Compatibility Chart identifies cases of non-specific interference between assays, and is not meant to represent assay cross-recognition or target specificity. For information on assay specificity, see the Certificates of Analysis for individual EpiTag Bead Kits.



Note: Due to the unique nature of the EpiTag™ technology and sample preparation, EpiTag Bead Kits and reagents are not compatible with other bead kits and reagents sold by EMD Chemicals, Inc. or other vendors.