Magnetic Luminex® Screening Assay

Human RTK Kit B

Catalog Number VMAPMAGB

For the simultaneous detection of multiple phosphorylated human receptor tyrosine kinases (RTKs) in cell lysates.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Receptor tyrosine kinases (RTKs) are a family of widely expressed transmembrane proteins with an extracellular ligand binding domain and an intracellular tyrosine kinase domain. This family includes receptors for growth factors, neurotrophic factors, insulin, and other extracellular signaling molecules. Upon ligand binding, the cytoplasmic domains of RTKs are autophosphorylated on multiple tyrosine residues either as a result of receptor dimerization or due to allosteric interactions between the two halves of the same receptor. These phosphorylated tyrosine residues on RTKs serve as high affinity docking sites for intracellular proteins that promote downstream signal transduction cascades. The signaling pathways initiated by RTK activation are required for normal developmental processes including proliferation, differentiation, and motility.

Mutations in RTKs can cause constitutitive activation of downstream signaling pathways, which have been implicated in the pathogenesis of different forms of cancer [Christensen, J. *et al.* (2005) Cancer Letters **225**(1):1]. Due to the physiological and pathological importance of RTK activation, analysis and quantification of RTK phosphorylation has become increasingly important. Assays that allow several RTKs to be monitored simultaneously simplify the screening processes required to identify pathways involved in establishing specific cellular phenotypes. Having this capability allows proteins of interest to be rapidly identified and targeted for further study.

This kit contains the basic components required to measure relative differences of natural and recombinant human phosphorylated RTKs in a multiplex sandwich ELISA. Magnetic Luminex® Screening Assays can be used to assess the relative levels of tyrosine phosphorylation of up to twenty-five RTKs of your choosing in a single cell lysate sample. For ease of use, the microparticles are pre-mixed.

PRINCIPLE OF THE ASSAY

The Magnetic Luminex Screening Assay is a homogeneous, no wash assay. Briefly, a reaction mixture containing capture antibodies immobilized on superparamagnetic microparticles, biotinylated detection antibody, streptavidin-phycoerythrin (streptavidin-PE), and sample is added to each well of a microplate. Both unphosphorylated and phosphorylated forms of the target RTKs present in the samples are bound by the immobilized capture antibodies. Phosphorylated RTKs on activated receptors are detected by the use of a biotinylated anti-phospho-tyrosine detection antibody and streptavidin-PE. Signals proportional to the relative amounts of phosphorylated RTKs bound are generated and read using a Luminex MAGPIX® analyzer. This analyzer utilizes a magnet to hold the superparamagnetic microparticles in a monolayer while Light Emitting Diodes (LEDs) illuminate and a CCD camera images each well. Alternatively, kits can be used with a Luminex 100™, Luminex 200™, or a Bio-Rad® Bio-Plex® analyzer, dual laser, flow-based sorting and detection platforms.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in sample handling, diluents, operator, pipetting technique, instrumentation, incubation time or temperature, and kit age can cause variation in binding.
- Discrepancies may exist in relative values obtained for the same analyte utilizing different technologies.
- This assay is designed to eliminate interference by proteins present in biological samples. Until all factors have been tested in the Magnetic Luminex Screening Assay, the possibility of interference cannot be excluded.
- The Control supplied in this kit is not intended to be utilized for quantification of RTK levels in samples. It is intended to be utilized as a positive control to ensure all reagents were added to the wells.
- **Only the analytes listed on the enclosed Certificate of Analysis can be measured with this kit.**

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each control level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.

PRECAUTIONS

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

This kit contains sufficient materials to run multiplex assays on one 96 well plate.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
Control RTK B	894173	1 vial of recombinant human RTKs in a buffered protein base with preservatives; lyophilized.	May be stored for up to 1 month at ≤ -20 °C.*
RTK B Microparticle Custom Premix	894176	0.35 mL of a 10X concentrated human RTK microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, these solutions must be discarded. Prepare fresh for each assay.*
RTK Biotin Antibody, 100X	894175	0.04 mL of a 100X concentrated biotinylated anti-phospho-tyrosine antibody with preservatives.	
Streptavidin-PE High Sensitivity, 100X	894177	0.04 mL of a 100X concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Diluent RD2-3	895972	2 vials (21 mL/vial) of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Diluent RD2-4	895973	11 mL of a buffered protein base with preservatives.	
Lysis Buffer 15	895567	21 mL of a cell lysing buffer with phosphatase inhibitors and preservatives	
Microplate	641182	1 round-bottomed plate, used as a vessel for the assay.	
Certificate of Analysis	752296	1 sheet listing the analytes, microparticle regions, and Control reconstitution volume.	
Mixing Bottle	895505	1 (8 mL) empty bottle used for preparing Reaction Mixture.	
Plate Sealers	640445	2 adhesive foil strips.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Luminex MAGPIX, Luminex 100/200, or Bio-Rad Bio-Plex Analyzer with X-Y platform.
- Pipettes and pipette tips.
- **Polypropylene** test tubes for dilution.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Microcentrifuge.

LYSATE PREPARATION AND DILUTION

Since the Magnetic Luminex Screening Assay RTK Kit B detects relative phosphorylation levels of individual analytes, it is important to include appropriate controls (including unstimulated cells and wells with Diluent RD2-3 only).

Cell Lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding Lysis Buffer 15. Solubilize the cells at 3-5 x 10^7 cells/mL in Lysis Buffer 15. Pipette up and down to resuspend and rock the lysates gently at 2-8 °C for 30 minutes. Microcentrifuge at 14,000 x g for 5 minutes, and transfer the supernate into a clean test tube. It is recommended that sample protein concentrations be determined using a total protein assay. Use 1-5 µg of lysate diluted in Diluent RD2-3. A recommended dilution is 1:10. Do not allow the Lysis Buffer to exceed 10% of the sample's final volume. For example, add 10 µL of cell lysate to 90 µL of Diluent RD2-3. The amount of lysate should be optimized for each particular cell type. Lysates should be used immediately or aliquoted and stored at ≤ -70 °C. Thawed lysates should be kept on ice until immediately prior to use. Avoid repeated freeze-thaw cycles.

Lysates Directly from 96-well Microplates - Cells can be seeded and grown directly in uncoated 96-well microplates. Results may be affected by confluency of cells. Treat cells as desired. Rinse the cells with PBS, making sure to remove any remaining PBS before adding Lysis Buffer 15. Add 25 µL of Lysis Buffer 15 per well. Cover the microplate with a plate sealer and incubate on an orbital shaker for 30 minutes at 2-8 °C. Add 225 µL of Diluent RD2-3. Mix and transfer 25 µL to one well of the reaction microplate. Not all cell lines will provide enough lysate for this protocol. This protocol needs to be tested for each cell type.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Control - Reconstitute the Control provided in the kit using Diluent RD2-3. Refer to the Certificate of Analysis for the reconstitution volume. Allow the Control to sit for 15 minutes with gentle agitation prior to use.

Diluted Microparticle Cocktail:

1. Centrifuge the RTK B Microparticle Custom Premix vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. To assay a full plate, dilute the Microparticle Custom Premix using Diluent RD2-4 in the Mixing Bottle provided by adding 300 µL of RTK B Microparticle Custom Premix cocktail to 2.7 mL of Diluent RD2-4. If assaying a partial plate, prepare only as much Diluted Microparticle Cocktail as needed.

Note: *Protect microparticles from light during handling.*

Reaction Mixture (Diluted Microparticle Cocktail, RTK Biotin Antibody, and Streptavidin-PE):

1. Centrifuge the RTK Biotin Antibody vial for 30 seconds at 1000 x g prior to removing the cap. Repeat this for the Streptavidin-PE vial.
2. Gently vortex each vial, taking precautions not to invert them.
3. To assay a full plate, add 30 µL of the RTK Biotin Antibody and 30 µL of the Streptavidin-PE to the Diluted Microparticle Cocktail. If assaying a partial plate, prepare only as much Reaction Mixture as needed.
4. Mix by gentle vortexing or inversion.

ASSAY PROCEDURE

Bring all reagents to room temperature before use. It is recommended that all samples and the Control be assayed in duplicate.

Note: *Protect Reaction Mixture from light at all times.*

1. Prepare all reagents, Control, and samples as directed in the previous sections.
2. Ensure microparticles are in suspension in the Reaction Mix by gentle vortexing or inversion.
3. Add 25 µL of Control or sample to the appropriate wells of the microplate.
4. Add 25 µL of the Reaction Mixture per well. Securely cover with a foil plate sealer. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Read within 60 minutes using a Luminex or Bio-Rad analyzer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the analyzer as directed using the plate provided in this kit. Refer to the instrument manual.

For MAGPIX:

a) Assign the bead region for each analyte being measured (refer to the Certificate of Analysis).
b) 50 events/bead
c) Pre-wash well selected in the protocol.
d) Sample size: 25 µL
e) Collect Median Fluorescence Intensity (MFI)

For Luminex 100/200 and Bio-Plex:

Note: *Calibrate the instrument using the proper reagents and instructions for superparamagnetic microparticles.*

a) Assign the microparticle region for each analyte being measured (refer to the Certificate of Analysis).
b) 50 events/region
c) Minimum events: 0
d) Flow rate: 60 µL/Min (fast)
e) Sample size: 25 µL
f) Doublet Discriminator gates at approximately 8000 and 16,500
g) Collect Median Fluorescence Intensity (MFI)

Note: *For the Bio-Rad Bio-Plex analyzer, set the gates at 4300 and 15,000.*

DATA ANALYSIS

Average duplicate readings. Users should establish their own methods for data interpretation to determine relative changes of tyrosine phosphorylation between samples.