USER GUIDE

novex® by life technologies™

Akt Phospho Magnetic 7-Plex Panel

For simultaneous quantitative determination of Akt [pS473], GSK-3ß [pS9], IGF-1R [pYpY1135/1136], IR [pYpY1162/1163], IRS-1 [pS312], p70S6K [pTpS421/424], PRAS40 [pT246] in human, mouse, or rat cell lysates and tissue homogenates

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life technologies™

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Kit Contents and Storage

Storage

All components of the Akt Phospho Magnetic 7-Plex Panel are shipped at 2–8°C. Upon receipt, store all kit components at 2–8°C. **Do not freeze**.

Contents

The components and amounts included in the Akt Phospho Magnetic 7-Plex Panel are listed below.

Reagents Provided	100 Test Kit
AKT Pathway Phopho Magnetic 7-Plex Antibody Bead Concentrate (10X) (contains 0.05% sodium azide)	0.25 mL × 1 vial
AKT Pathway Phopho Magnetic 7-Plex Standard (contains 0.1% sodium azide)	2 vials
AKT Pathway Phopho Magnetic 7-Plex Detection Antibody Concentrate (10X) (contains 0.1% sodium azide)	0.50 mL × 1 vial
Wash Solution Concentrate (20X) (contains 0.1% sodium azide)	15 mL × 3 bottles
Assay Diluent (contains 0.1% sodium azide)	15 mL × 1 bottle
RPE Diluent (contains 0.1% sodium azide)	12 mL × 1 bottle
Goat Anti-Rabbit IgG RPE Concentrate (10X) (contains 0.1% sodium azide)	1 mL × 1 vial
Detector Antibody Diluent (contains 0.1% sodium azide)	12 mL × 1 bottle
96-well Filter Plate	1 × 96-well plate
96-well Flat Bottom Plate	1 × 96-well plate

Caution

This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin, and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

Intended use

For Research Use Only. Not for use in diagnostic procedures.

Introduction

Overview

Purpose

Multiplex Bead Immunoassay Kits from Life Technologies are developed to maximize flexibility in experimental design, permitting the measurement of one or multiple proteins in panels designed by the researcher. The Akt Phopho Magnetic 7-Plex Panel contains all the reagents that are intended for use with the Luminex® 100™, 200™, FLEXMAP 3D®, or MAGPIX® system with xPONENT software. These instruments are manufactured by Luminex Corporation and sold by Life Technologies and other vendors.

Background information

Advances in the field of cell biology have defined a complex and interdependent set of extracellular and intracellular signaling molecules that control normal cell function. There is growing interest among researchers as well as drug discovery groups in simultaneously monitoring multiple components of signaling pathways. Solid phase multiplex protein assays are the tools of choice in these studies as they maximize efficiency by simultaneously profiling several proteins within individual samples.

Multiplex Bead Immunoassays from Life Technologies are solid phase protein immunoassays that use spectrally encoded antibody-conjugated beads as the solid support. The spectral beads are suitable for use in singleplex assays or may be mixed for multiplex assays according to the researcher's requirements. Each assay is carefully designed and tested to assure that sensitivity, range, and correlation are maximized. The assay is performed in a 96-well plate format and analyzed with a Luminex® 100™, 200™, FLEXMAP 3D®, or MAGPIX® instrument which monitors the spectral properties of the capture beads while simultaneously measuring the quantity of associated fluorophore. Standard curves generated with this assay system extend over several orders of magnitude of concentrations, while the sensitivity and quantitation of the assays are comparable to ELISAs (Enzyme Linked-Immuno-Sorbent Assays). Assay standards are calibrated to National Institute for Biological Standards and Controls (NIBSC) reference preparations, when available, to assure accurate and reliable results.

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Background information, continued

The Akt Phopho Magnetic 7-Plex Panel is designed for the quantitative determination of For simultaneous quantitative determination of Akt [pS473], GSK-3β [pS9], IGF-1R [pYpY1135/1136], IR [pYpY1162/1163], IRS-1 [pS312], p70S6K [pTpS421/424], PRAS40 [pT246] in human, mouse, or rat cell lysates and tissue homogenates. The antibodies used in this assay are human, mouse and rat cross-reactive. This kit has not been tested for multiplexing with other markers. Should user elect to multiplex this kit with other Luminex kits, the assay conditions should be determined empirically for each specific application.

Visit **www.lifetechnologies.com/luminex** for a current listing of available multiplex bead immunoassays and reagents.

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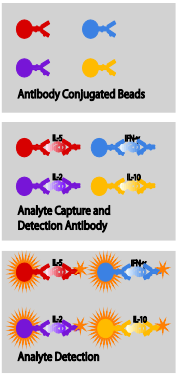
Overview, Continued

Assay overview



The xMAP® technology combines the efficiencies of multiplexing up to 100 different proteins for simultaneous analysis, with reproducibility similar to ELISA. This assay uses 6.5 µm polystyrene beads which contain magnetite.

Assays performed with these beads may be washed using a filter plate, washed manually with the aid of a magnetic separator, or washed with the aid of automated magnetic bead washing equipment.

The beads are internally dyed with red and infrared fluorophores of differing intensities. Each bead is given a unique number, or bead region, allowing differentiation of one bead from another.

Beads of defined spectral properties are conjugated to protein-specific capture antibodies and added along with detector antibody, samples (including standards of known protein concentration, control samples, and test samples), into the wells of a filter-bottom microplate and where proteins bind to the capture antibodies and the protein-specific detector antibodies bind to the appropriate immobilized proteins over the course of a 3 hour incubation.

After washing the beads, R-Phycoerythrin (RPE) conjugate, is added and allowed to incubate for 30 minutes. The RPE conjugate binds to the detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich.

After washing to remove unbound RPE conjugate, the beads are analyzed with the Luminex detection system. By monitoring the spectral properties of the beads and the amount of associated RPE fluorescence, the concentration of one or more proteins can be determined.

Experimental Overview

Experimental outline

Experimental outline for using the Akt Phopho Magnetic 7-Plex Panel is shown below.

Note: Pre-wet step required only with the filter bottom plate.

Methods

Before Starting

Materials required but not provided

- Luminex® xMAP® system with data acquisition and analysis software:
  - Luminex® 200™ system (Cat. no. MAP0200)
  - FLEXMAP 3D® system (Cat. no. FM3D000)
  - MAGPIX® system (Cat. no. MPX0001)

  Contact Life Technologies for instrument and software placement services, see page 27
- Washing equipment:
  - EveryPrep™ Universal Vacuum Manifold (Cat. no. K2111-01), or equivalent for use with 96-well Filter Plates
  - Magnetic 96-Well Separator (Cat. no. A14179) for use with 96-well Flat-Bottom Plates
  - Automated magnetic bead washing equipment for use with 96-well Flat-Bottom Plates
- Sonicating water bath
- Vortex mixer
- Orbital shaker (small diameter rotation recommended)
- Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips (A manifold multi-channel pipette is desirable)
- Distilled or deionized water
- Glass or polypropylene tubes
- Aluminum foil

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Procedural notes

Review the procedural notes below before starting the protocol.

- All phases of the assay are performed using the filter plate or flat-bottom plate provided with the kit. **Do not invert the plates during the assay**.
- A filter plate is provided for use when washing steps are performed with a vacuum manifold. **Do not exceed 5 mm Hg**. With the filter-bottom plate, contents are emptied from the bottom of the plate during washing.
- A flat-bottom plate is provided for use when washing steps are performed with a magnetic separator. With the flat-bottom plate, contents are removed from the top of the plate during washing. Washing with the flat-bottom plate may be performed manually or with the aid of automated washing equipment.
- **Do not freeze any component of this kit**. Store kit components at 2–8°C when not in use. Allow all reagents to warm to room temperature before use (air-warm all reagents at room temperature for at least 30 minutes, or alternatively, in a room-temperature water bath for 20 minutes [except plate and vials of standard]).
- **The fluorescent beads are light-sensitive**. Avoid light exposure to prevent photobleaching of the embedded dye. Use aluminum foil to cover test tubes used in the assay. Cover microplates containing beads with an opaque or aluminum foil-wrapped plate cover. Since the amber vial does not provide full protection, keep the vial covered in the box or drawer when not in use.
- Do not expose beads to organic solvents.
- Do not place filter plates on top of absorbent paper towels during loading or incubations, as liquid may be lost due to contact wicking. We recommend placing the filter plate on an extra plate. Following plate washing, remove excess liquid and blot the bottom of the plate by pressing the plate on clean paper towels.
- When pipetting reagents, maintain a consistent order of addition from well-to-well to ensure equal incubation times for all wells.
- To prevent filter tearing, avoid touching the filter plate membrane with pipette tips.

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Before Starting, Continued

Procedural notes, continued

- Do not use reagents after kit expiration date.
- It is recommended that in-house controls be included with every assay. If control values fall outside pre-established ranges, the assay may be suspect. Contact Technical Support for product and technical assistance.
- Do not mix or substitute reagents with those from other lots or sources.
- Handle all blood components and biological materials as potentially hazardous. Follow standard precautions as established by the Centers for Disease Control and Prevention and by the local Occupational Safety and Health Administration when handling and disposing of infectious agents.

Recommended plate plan

We recommend designing a plate plan before starting the assay. A plate plan template is provided on page **Error! Bookmark not defined.**. The following is a suggested plate plan:

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B										
B	Std 7	Std 7										
C	Std 6	Std 6										
D	Std 5	Std 5										
E	Std 4	Std 4										
F	Std 3	Std 3										
G	Std 2	Std 2										
H	Std 1	Std 1										

B= blank (Assay Diluent), Std 7 = Standard 7 (lowest concentration) through Std 1 = Standard 1 (highest concentration).

The remainder of the plate is available for controls and samples which may be run as a singlet or in duplicate, as desired.

Note: we recommend running all standards, samples, and controls in duplicate.

Preparing Reagents

Review the information in this section before starting. The Akt Phopho Magnetic 7-Plex Panel includes both antibody bead reagents and buffer reagents. Prepare components of the Akt Phopho Magnetic 7-Plex Panel according to the instructions below.

Note: Bring all reagents and samples to room temperature before use.

Preparing 1X Wash Solution

Upon storage at 2–8°C, a precipitate may form in the Wash Solution Concentrate (20X). If this occurs, warm the Wash Solution Concentrate (20X) to 37°C and mix until the precipitate is dissolved.

1. Prepare a 1X Wash Solution for use with a 96-well plate by transferring the entire contents of the Wash Solution Concentrate (20X) bottle to a 500 mL container (or equivalent) and then add 285 mL of deionized water. Mix well.
2. The 1X Wash Solution is stable for up to 2 weeks when stored at 2–8°C.

Note: To prepare smaller volumes of 1X Wash Solution, mix 1 part of 20X concentrate with 19 parts of deionized water. Mix well.

Guidelines for standard curve preparation

- Each Kit comes with two vials of standard, so that two runs on the plate can be made with freshly prepared standards.
- Reconstitute the protein standard within 1 hour of performing the assay. All standards are calibrated to NIBSC preparations, when available. Additional standards are available from Life Technologies custom services.
- Before performing standard mixing and serial dilutions confirm reconstitution volumes on the Technical Data Sheet, included with the Akt Phopho Magnetic 7-Plex Panel.
- The concentrations of the protein components of the standard are indicated on the Technical Data Sheet.
- Perform dilutions in glass or polypropylene tubes.

Important: The impact of adding additional standards to this assay has not been evaluated.

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Preparing Reagents, Continued

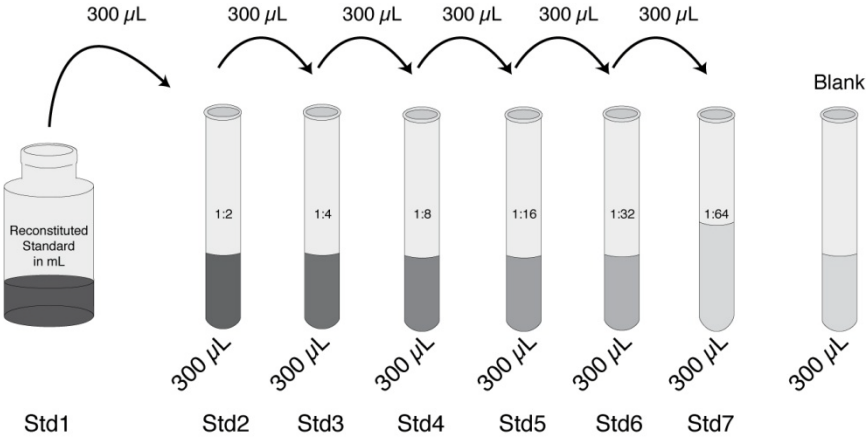
Reconstituting lyophilized standards

Reconstitute the Akt Phopho Magnetic 7-Plex Standard as described below. One vial of standard is sufficient for making a standard curve in duplicate

1. Add the suggested reconstitution volume of Assay Diluent, indicated on the Technical Data Sheet to the vial of standard. **Do not vortex.** Reconstitute protein solutions gently to avoid foaming**.**
2. Replace the vial stopper and allow the vial to stand undisturbed for 10 minutes.
3. Gently swirl and invert the vial 2–3 times to ensure complete reconstitution and allow the vial to sit at room temperature for an additional 5 minutes.

Preparing standard curve

The standard curve is made by serially diluting the reconstituted standard in Assay Diluent. See below. **Do not vortex.** Mix by gently pipetting up and down 5–10 times.



Discard all remaining reconstituted and diluted standards after completing assay. Return the Assay Diluent to the kit.

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Preparing 1X antibody beads

Determine the number of wells required for the assay.

The Antibody Bead Concentrate is supplied as a **10X concentrate and must be diluted prior to use.** The fluorescent beads are light-sensitive. **Protect antibody conjugated beads from light during handling.**

1. Immediately before dispensing, vortex the 10X Antibody Bead Concentrate for 30 seconds followed by sonication in a sonicating water bath for 30 seconds.
2. Prepare 1X Antibody Bead stock by diluting 2.5 µL of 10X beads in 25 µL of 1X Wash Solution (page 8) per assay well. Each well requires 25 µL of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Vol. 10X Antibody Bead Concentrate	Vol. 1X Wash Solution
24	0.06 mL	0.6 mL
32	0.08 mL	0.8 mL
40	0.10 mL	1.0 mL
48	0.12 mL	1.2 mL
56	0.14 mL	1.4 mL
64	0.16 mL	1.6 mL
72	0.18 mL	1.8 mL
80	0.20 mL	2.0 mL
88	0.22 mL	2.2 mL
96	0.24 mL	2.4 mL

Note: Dilution factor is 1:11 for extra pipetting volume.

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Preparing Reagents, Continued

Preparing 1X detector antibody

The Detector Antibody is supplied as a **10X concentrate and must be diluted prior to use.**

To prepare a 1X Detector Antibody stock, dilute 5 µL of 10X Detector Antibody in 50 µL of Detector Antibody Diluent per assay well. Each well requires 50 µL of the diluted Detector Antibody. See table below for examples of volumes to combine.

Number of Wells	Vol. 10X Detector Antibody Concentrate	Vol. Detector Antibody Diluent
24	0.12 mL	1.2 mL
32	0.16 mL	1.6 mL
40	0.20 mL	2.0 mL
48	0.24 mL	2.4 mL
56	0.28 mL	2.8 mL
64	0.32 mL	3.2 mL
72	0.36 mL	3.6 mL
80	0.40 mL	4.0 mL
88	0.44 mL	4.4 mL
96	0.48 mL	4.8 mL

Note: Dilution factor is 1:11 for extra pipetting volume.

Preparing Samples

This protocol has been applied to several human, mouse and rat cell lines. Researchers should optimize the cell/tissue extraction buffers and procedures for their own applications.

Cell extraction buffer preparation

Recommended Cell Extraction Buffer		
Cell Extraction Buffer (Cat. no. FNN0011)		
	or	
10 mM Tris, pH 7.4	2 mM Na_3VO_4	1 mM EDTA
100 mM NaCl	1% Triton X-100	1 mM EGTA
20 mM Na_4P2O_7	10% glycerol	1 mM NaF
0.5% deoxycholate	0.1% SDS	

Buffer without protease inhibitor cocktail and PMSF is stable for 2–3 weeks at 2–8°C or 6 months when stored in aliquots at –20°C. Add FRESH to the NP40 Lysis Bufer just before use:

- 1 mM PMSF (stock 0.3 M in DMSO)
- Protease inhibitor cocktail (Sigma, Cat. no. P-2714)

An alternative cell extraction buffer is listed below.

Alternative Cell Lysis Buffer		
NP40 Lysis Buffer (Cat. no. FNN0021)		
	or	
50 mM Tris, pH 7.4	1% Nonidet P40	250 mM NaCl
5 mM EDTA	1 mM Na_3VO_4	50 mM NaF

Buffer without protease inhibitor cocktail and PMSF is stable for 2–3 weeks at 2–8°C or 6 months when stored in aliquots at –20°C. Add FRESH to the NP40 Lysis Bufer just before use:

- 1 mM PMSF (stock 0.3 M in DMSO
- Protease inhibitor cocktail (Sigma, Cat. no. P-2714)

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Preparing Samples, Continued

Cell lysis procedure

1. Prepare your cell lysate at a recommended concentration of 2–5 mg/mL according to the table below:

Cell Type	Action
Non-adherent	1. Pellet cells by low speed centrifugation. 2. Remove medium from the pellet, and wash twice with ice-cold PBS. 3. Remove the PBS, and resuspend the cell pellet in cell lysis buffer by gently pipetting. 4. Incubate 15 minutes on ice with occasional vortexing.
Adherent	1. Remove tissue culture medium from the cells, and wash twice with ice-cold PBS. 2. Remove the PBS, and add cell lysis buffer 3. Incubate 15 minutes on ice.

2. Collect the cell lysate
3. Transfer the lysate to a microfuge tube and centrifuge at 14,000 rpm for 10 minutes at 2–8°C.
4. Aliquot the cleared lysate into clean microfuge tubes and determine total protein concentration.

Storage: Lysates should be frozen and stored at –80°C or analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely, mix well and clarify by centrifugation (14,000 rpm for 5 minutes) prior to analysis to prevent clogging of the filter plates.

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Preparing Samples, Continued

Sample treatment procedure

- Dilute lysate prepared using Cell Extraction Buffer (Cat. no. FNN0011) at least 10-fold in Assay Diluent prior to analysis.
- Dilute lysate prepared using NP40 Lysis Buffer (Cat. no. FNN0021) at least 5-fold in Assay Diluent prior to analysis.
- For samples with concentrations that fall ouside of the standard curve, dilute the sample in Assay Diluent so that it falls within the range of the standard curve and reanalyze.

Co-Incubation Assay Procedure

Washing methods

This assay may be washed using a vacuum manifold (requires the filter bottom plate provided), or by aid of a magnetic separator (requires the flat bottom plate provided).

Incomplete washing adversely affects assay results. Perform all wash steps with 1X Wash Solution.

Filter plate method

1. To wash beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum (**do not exceed 5 mm Hg**). Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Prevent any vacuum surge by opening and adjusting the vacuum on the manifold before placing the plate on the manifold surface.
2. Stop the vacuum pressure as soon as the wells are empty. Do not attempt to pull the plate off the vacuum manifold while the vacuum is still on or filter plate damage may occur. Release the vacuum prior to removing the plate.
3. If solution remains in the wells during vacuum aspiration, **do not detach the bottom of the 96-well filter plate.** In some cases, minor clogs in the filter plate may be dislodged by carefully pressing the bottom of the plate under the clogged well with the pointed end of a 15-mL plastic conical tube. Place the filter plate on a clean paper towel and use a gloved thumb or a 1-mL Pasteur pipette bulb to plunge the top of the clogged well. Empty all clogged wells entirely before continuing the washes. **Note**: Do not attempt to repetitively pull vacuum on plates with clogged wells. This can compromise the unclogged wells and bead loss may occur.
4. After all wells are empty, lightly tap or press the filter plate onto clean paper towels (hold the plate in the center for tapping) to remove excess fluid from the bottom of the filter plate. **Do not invert plate.**
5. Following the last aspiration and plate taps, use a clean absorbent towel to blot the bottom of the filter plate before addition of next liquid phase or data acquisition step.
6. **Do not leave plate on top of absorbent surfaces when adding reagents**.

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Co-Incubation Assay Procedure, Continued

Washing methods, continued

Magnetic separator method

1. Set the plate on a magnetic separator for 90 seconds to allow immobilization of the magnetic beads. Next, aspirate the liquid from the wells using a multichannel pipette.
2. Refill the wells with washing solution, remove the plate from the magnet and then allow the well contents to soak for 60 seconds. Again, prepare to remove the liquid from the wells by setting the plate on the magnetic separator for 90 seconds and then aspirate the liquid contents of the wells with a multichannel pipette.

Guidelines for automated plate washers

1. Some optimization of the automated plate washer set up may be required. As with manual washing with a magnetic separator, the program used for automated washing should include a 90 second period in which the beads are immobilized onto a magnetic separator. After the beads are immobilized, liquid may be aspirated using automated washing equipment. A suggested probe height of 4.8 mm is recommended.
2. As with the manual washing method, the wells should then be refilled with washing solution, the plate removed from the magnet, and the contents of the wells allowed to soak for 60 seconds. Again, prepare to remove the liquid from the wells by setting the plate on the magnetic separator for 90 seconds, then aspirate the well contents with the automated plate washing equipment.

Co-Incubation Assay Procedure, Continued

Reverse pipetting instructions

To reduce bubbles and loss of reagents due to residual fluid left in pipette tips, use the recommended reverse pipetting technique.

1. To reverse pipette, set the pipette to the appropriate volume needed. **Note:** Do not reverse pipette volumes <20 µL.
2. Press the push-button slowly to the first stop and then press on past it. **Note**: the amount past the first stop will depend on the volume of liquid available for aspiration.
3. Immerse the tip into the liquid, just below the meniscus.
4. Release the push-button slowly and smoothly to the top resting position to aspirate the set volume of liquid. Drag the tip up the side of the tube or reservoir to remove excess volume from the outside of the tips
5. Place the end of the tip against the inside wall of the recipient vessel at an angle above the fluid level.
6. Press the push button slowly and smoothly to the first stop. Some liquid will remain in the tip, this should not be dispensed.
7. Remove the tip, keeping the pipette pressed to the first stop and return to step 3 (above) if reusing tips and contamination is not an issue.

Note: Bring all reagents and samples to room temperature before use.

Co-Incubation Assay Procedure, Continued

Analyte capture and detection

1. Choose the filter bottom plate when washing with a vacuum manifold. Choose the flat bottom plate when washing manually with a magnetic separator or with automated magnetic bead washing equipment. An adhesive plate cover may be used to seal any unused wells; this will keep the wells dry for future use.
2. **The filter bottom plate requires pre-wetting before use in the assay**. Pre-wet the designated wells of the filter bottom plate by adding 200 µL of 1X Wash Solution. Incubate the plate 30 seconds at room temperature. Aspirate the 1X Wash Solution from the wells using the vacuum manifold.

   The flat-bottom plate may be used without this pre-wetting step.
3. Vortex the 1X Antibody Beads solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay. **The magnetic beads settle rapidly. It is therefore important that the 1X Antibody Beads solution is well-mixed immediately prior to use**.
4. Pipette 25 µL of the 1X Antibody Beads solution into each well. Once the beads are added to the plate, **keep the plate protected from light**.
5. Add 200 µL 1X Wash Solution to the wells. Allow the beads to soak for 15–30 seconds.
6. Wash the wells two times, aspirating the 1X Wash Solution at the end of each washing step (when using the filter-bottom plate, blot the bottom of the plate on clean paper towels to remove any residual liquid).

   Note: Place the filter plate on a plate cover or non-absorbent surface before all incubations.
7. Pipette 50 µL of prepared 1X Detector Antibody into each well.
8. Pipet 50 µL of appropriate standard dilution (page 9) to each well designated for the standard curve.
9. Pipet 50 µL of sample or in-house controls (if used) to the appropriate wells designated for samples.

   Note: Suggested total protein per well is 10–40 µg. However, the exact amount should be determined by the individual user.

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Analyte capture and detection, continued

10. Cover the microplate containing the beads with an aluminum foil-wrapped plate cover. Incubate the plate for 3 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500–600 rpm). Larger radius shakers will need a lower speed and smaller radius shakers will typically handle higher speeds without splashing.
11. Ten to fifteen minutes prior to the end of the incubation step, prepare the Goat Anti-Rabbit IgG-RPE, and then proceed with **Analyte detection, step 1**.

Preparing 1X Goat Anti-Rabbit IgG-RPE

The Goat Anti-Rabbit IgG-RPE is supplied as a **10X concentrate and must be diluted prior to use. Protect Goat Anti-Rabbit IgG-RPE from light during handling.**

To prepare a 1X Goat Anti-Rabbit IgG-RPE stock, dilute 10 µL of 10X Goat Anti-Rabbit IgG-RPE in 100 µL of RPE Diluent per assay well. Each well requires 100 µL of the diluted Goat Anti-Rabbit IgG-RPE. See table below for examples of volumes to combine.

Number of Wells	Vol. 10X Goat Anti-Rabbit IgG-RPE Concentrate	Vol. RPE Diluent
24	0.24 mL	2.4 mL
32	0.32 mL	3.2 mL
40	0.40 mL	4.0 mL
48	0.48 mL	4.8 mL
56	0.56 mL	5.6 mL
64	0.64 mL	6.4 mL
72	0.72 mL	7.2 mL
80	0.80 mL	8.0 mL
88	0.88 mL	8.8 mL
96	0.96 mL	9.6 mL

Note: Dilution factor is 1:11 for extra pipetting volume.

Co-Incubation Assay Procedure, Continued

Analyte detection

1. After the 3 hour capture bead incubation, remove the liquid from wells with the vacuum manifold (filter bottom plate), or with magnetic washing equipment (flat bottom plate).
2. Wash the plate by adding 200 µL of 1X Wash Solution to the wells. Allow the beads to soak for 30 seconds. Remove the liquid with the vacuum manifold, or with magnetic washing equipment. Repeat this washing step for a total of 2 washes. (the bottom of the filter plate should be blotted on clean paper towels to remove residual liquid after the second wash).
3. Add 100 µL of prepared 1X Goat Anti Rabbit IgG RPE (page 19) to each well and incubate the plate for 30 minutes at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500–600 rpm).
4. Prepare the Luminex® 100™, 200™, FLEXMAP 3D®, or MAGPIX® instrument during this incubation step. Refer to the Technical Data Sheet for all bead regions and standard concentration values.

Co-Incubation Assay Procedure, Continued

Assay reading

1. Remove the liquid from the wells using the vacuum manifold (**filter bottom plate**), or with magnetic washing equipment (**flat bottom plate**).
2. Wash the plate by adding 200 µL 1X Wash Solution to the wells. Allow the beads to soak for 30 seconds. Remove the liquid with the vacuum manifold, or with magnetic washing equipment. Repeat the wash step two more times for a total of 3 washes (blot the bottom of the filter plate on clean paper towels to remove residual liquid after the second wash).
3. Add 100 µL of 1X Wash Solution to each well. Shake the plate on an orbital shaker (500–600 rpm) for 2–3 minutes to resuspend the beads.

   Note: If the plate cannot be read on the day of the assay, cover and store the plate in the dark overnight at 2–8°C for reading the following day without significant loss of fluorescent intensity. Aspirate 1X Wash Solution from stored plates and add 100 µL fresh 1X Wash Solution. Place the plate on an orbital shaker for 2–3 minutes at 500–600 rpm prior to analysis.
4. Uncover the plate and insert the plate into the XY platform of the Luminex® 100™, 200™, FLEXMAP 3D®, or MAGPIX® instrument, and analyze the samples.
5. Determine the concentration of samples from the standard curve using curve fitting software. The five parameter logistic algorithm with a weighted function ($1/y^2$)is recommended.

Instrument Setup

Luminex® 100™ and 200™ instruments

Helpful guides for Luminex® 100™ and 200™ instrument users with xPONENT® software follows:

- Assign the appropriate **Bead Region** (refer to the kit-specific Technical Data Sheet) to each analyte.
- We recommend that the user **count 100 events/bead regions**.
- Set **Sample Size** to 75 µL.
- For kits using MagPlex® beads, we recommend starting with an initial **Double Discriminator (DD)** gate setting of 7800–20,000. Adjustment of this setting may be required for individual instruments, and must be determined by the user.
- Collect **Median Fluorescent Intensity (MFI)**.

Note: Use the default setting **low PMT** for the Luminex® 100™ and 200™ instruments.

Luminex® FLEXMAP 3D® instrument

Helpful guides for Luminex® FLEXMAP 3D® instrument follows:

- Assign the appropriate **Bead Region** (refer to the kit-specific Technical Data Sheet) to each analyte.
- We recommend that the user **count 100 events/bead regions**.
- Set **Sample Size** to 75 µL.
- For kits using MagPlex® beads, we recommend starting with an initial **Double Discriminator (DD)** gate setting of 7800–20,000. Adjustment of this setting may be required for individual instruments, and must be determined by the user.
- Collect **Median Fluorescent Intensity (MFI)**.

Note: Use the default setting **high PMT** for the Luminex® FLEXMAP 3D® instrument.

Performance Characteristics and Limitations of the Procedure

Performance characteristics

Refer to analyte specific Technical Data Sheet for performance claims.

Procedure limitations

- Do not extrapolate the standard curve beyond the highest or lowest standard point; the dose-response and data collected in these regions may be non-linear and should be considered inaccurate. Note: In some cases, further dilution of the standard beyond 7 points may be possible to extend the low end of the standard curve.
- Dilute samples that are greater than the highest standard with Assay Diluent or appropriate matrix diluent; reanalyze these samples and multiply results by the appropriate dilution factor.
- **Samples are diluted in the assay 1:2 (50 µL of sample and 50 µL of diluent) relative to the standards. Be sure to account for this dilution factor during sample calculations.**
- The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum, plasma and tissue culture supernatant samples have not been thoroughly investigated. The rate of degradation of analytes in various matrices may not have been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

Troubleshooting

Refer to the following tables to troubleshoot problems encountered with the use of Multiplex Bead Kits on the Luminex® platform.

To troubleshoot problems with the Luminex® instrument, refer to the manual supplied with the instrument.

For more troubleshooting solutions, visit **www.lifetechnologies.com/luminex**.

Observation	Cause	Solution
In-house controls perform differently in subsequent assays	Incorrect concentration entered in data analysis software	The standard proteins included in Bead Kits from Life Technologies are calibrated to NIBSC preparations, whenever possible. This calibration assures lot-to-lot consistency in performance. However, the concentration of the reconstituted standards may vary with each new lot of standard. Therefore, it is important to check the concentration of the standard listed on the Technical Data Sheet, and to verify all concentration values entered into the data analysis software.
	Improper reconstitution or dilution of the standard	Check standard reconstitution and dilution as described on page 9.

Continued on next page

Troubleshooting, Continued

Observation	Cause	Solution
During washing steps, the vacuum manifold does not aspirate the liquid from wells of the filter plate	The filter plate is clogged	Dislodge the clog by gently pushing the pointed end of a 15-mL plastic conical tube into the bottom of the plate under the clogged well. This procedure clears the small opening in the plastic casing.
		Dislodge by placing the filter plate on a clean paper towel and use a gloved thumb or a 1-mL Pasteur pipette bulb to plunge the top of the clogged well.
		To prevent filter plate clogging, clarify samples by centrifugation at 1000 × *g* for 10 minutes prior to analysis. Some samples may also require filtration prior to analysis.
	Lack of a tight seal	Hold the plate firmly against the vacuum manifold to form a tight seal. If only a partial plate is being analyzed, cover the empty wells with a self-adhesive plate seal.
Leaky filter plate	Solution remains on the bottom of the wells after vacuum aspiration, causing wicking and leakage of well contents during next incubation	After final wash step and plate taps, use a clean absorbent towel to blot the bottom of the filter plate before addition of next liquid phase or data acquisition step.
	Filter plate membrane tearing	Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Prevent any vacuum surge by opening and adjusting the vacuum on the manifold before placing the plate on the manifold surface.

Continued on next page

Troubleshooting, Continued

Observation	Cause	Solution
During data analysis, insufficient and/or erratic bead count is observed	Magnetic bead settling	Make sure that the rate of plate shaking is sufficient to keep the beads suspended during incubations and prior to analysis.
	Bead aggregation	Make sure to vortex the beads for 30 seconds and then sonicate the beads for at least 30 seconds prior to beginning the assay, to break up any bead aggregates.
		Empty wells and add fresh wash buffer. Shake for 2–3 minutes to resuspend the beads.
	Loss of beads due to the filter plate membrane tearing	To prevent membrane tearing, place pipette tips on the side of the well, rather than straight down onto the membrane when dispensing liquid into the wells.
		Turn the vacuum manifold on before placing the filter plate on the top to prevent vacuum surge. When evaluating a new vacuum manifold, adjust the vacuum force so that 3 seconds are required to empty 0.2 mL from the wells of a plate.
	Clog in instrument or probe	Remove probe, sonicate for 5 minutes, rinse the probe and reinstall. Run an unclog protocol. See instrument manual.
	Probe height set incorrectly	Readjust the instrument probe height. If it is too low, it could puncture the well membrane. If it is too high, air could be pulled up with the liquid which may appear as bead fragments to the instrument.

Appendix

Technical Support

Obtaining support

For the latest services and support information for all locations, go to **www.lifetechnologies.com**

At the website, you can:

- Access worldwide telephone and fax numbers to contact Technical Support and Sales facilities
- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support (**techsupport@lifetech.com**)
- Search for user documents, SDSs, vector maps and sequences, application notes, formulations, handbooks, certificates of analysis, citations, and other product support documents
- Obtain information about customer training
- Download software updates and patches

7305 Executive Way
Frederick, MD 21704
Tel (Toll Free): 1 800 955 6288
E-mail: **techsupport@lifetech.com**

Safety data sheets (SDS)

Safety Data Sheets (SDSs) are available at **www.lifetechnologies.com/support**

Certificate of analysis

The Certificate of Analysis provides detailed quality control and product qualification information for each product. Certificates of Analysis are available on our website. Go to **www.lifetechnologies.com/support** and search for the Certificate of Analysis by product lot number, which is printed on the box.

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References

The references below demonstrate the success customers achieve when using Multiplex Assays from Life Technologies. For a complete list, visit **www.lifetechnologies.com/luminex**.

1. Chang, D. H., et al. (2005) Sustained expansion of NKT cells and antigen-specific T cells after injection of α-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J. Exp. Med.* 201:1503–1517.
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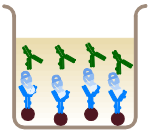
Co-Incubation Protocol Summary

Pre-wet plate
Add 25 µL 1X antibody-coated beads
and 200 µL Wash Solution

Wash 1 × 200 µL

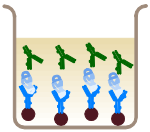
Add 50 µL 1X detector antibody

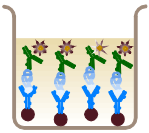
Sample type: **Standard** **Cell lysate**



Add 50 µL
standard

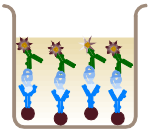
Add 50 µL
appropriately
diluted sample





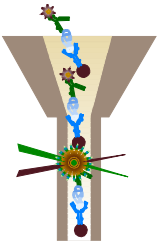
Shake for 3 hr at RT in the dark
Wash 2 × 200 µL

Add 100 µL 1X anti-Rabbit-RPE



Shake for 30 min at RT in the dark
Wash 3x 200 µL

Add 100 µL wash buffer



Shake for 2-3 min

Read in Luminex® Detection System

Total time: 3.5 hr

Plate Plan Template

Kit atalog number: _____ Lot Number: _____

Plate I : _____ ate: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

Explanation of Symbols

Symbol	Description	Symbol	Description
	Manufacturer		Catalog number
	Use by		Temperature limitation
	Consult instructions for use		Caution, consult accompanying documents
	Batch code		

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Notes

Notes

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