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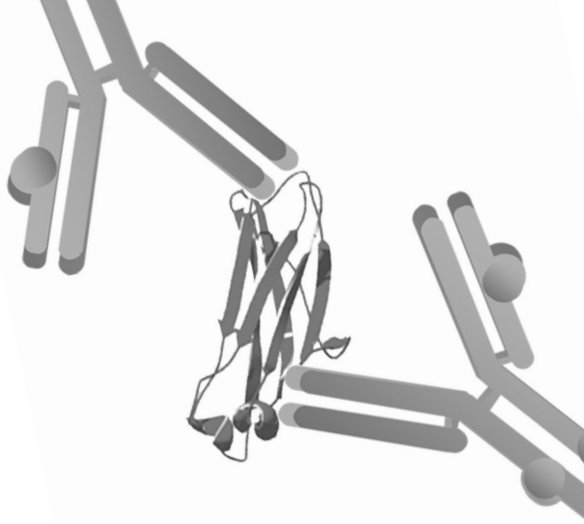


Akt Phospho 7-Plex Panel

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

Catalog no. LHO0001

Rev. 0.0
19 December 2008
PRLHO0001

User Manual

Table of Contents

Kit Contents and Storage

Storage

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

Contents

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

Reagents Provided	100 Test Kit
Akt Phospho 7-Plex Antibody Bead Concentrate (10X) (contains 7.5 mM sodium azide)	0.25 mL × 1 vial
Akt Phospho 7-Plex Standard	2 vial
Akt Phospho 7-Plex Detector Antibody Concentrate (10X) (contains 15 mM sodium azide)	0.50 mL × 1 vial
Wash Solution Concentrate (20X) (contains 15 mM sodium azide)	15 mL × 1 bottle
Assay Diluent (contains 15 mM sodium azide)	15 mL × 1 bottle
RPE Diluent (contains 15 mM sodium azide)	12 mL × 1 bottle
Goat Anti-Rabbit IgG-RPE Concentrate (10X) (contains 15 mM sodium azide)	1 mL × 1 vial
Detector Antibody Diluent (contains 3.3 mM thymol)	12 mL × 1 bottle
96-well Filter Plate	1 × 96-well plate

Introduction

Overview

Intended Use

Invitrogen's Multiplex Bead Immunoassay Kits are developed to maximize flexibility in experimental design, permitting the measurement of one or multiple proteins in panels designed by the researcher. The Akt Phospho 7-Plex Panel contains a set of common reagents that are intended for use with the Luminex® 100™ or 200™ dual laser detection system manufactured by Luminex Corporation and sold by Invitrogen and other vendors. For research use only. Not intended for any animal or human therapeutic or diagnostic use.

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. Perturbations in signaling pathways may be important indicators, and possibly the root cause, of many diseases. Therefore, there is growing interest among clinicians 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.

Invitrogen's Multiplex Bead Immunoassays 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™ or 200™ 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 NIBSC (National Institute for Biological Standards and Controls) reference preparations, when available, to assure accurate and reliable results.

Continued on next page

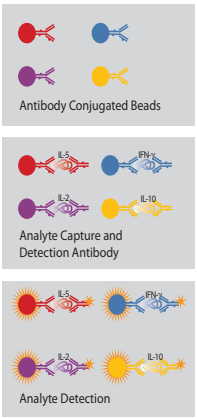
Overview, Continued

Background Information, Continued

Invitrogen's Akt Phospho 7-Plex Panel is designed for the *in vitro* quantitative determination of Akt [pS473], GSK-3β [pS9], IGF-1R [pYpY1135/1136], IR [pYpY1162/1163], IRS-1 [pS312], p70S6K [pTpS421/424], PRAS40 [pT246] in 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 the Invitrogen web site for a current listing of available Invitrogen multiplex bead immunoassays and reagents, at www.invitrogen.com/luminex.

Assay Overview



The xMAP® technology combines the efficiencies of multiplexing up to 100 different proteins for simultaneous analysis, with reproducibility similar to ELISA. The technology uses 5.6 µm polystyrene beads which 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.

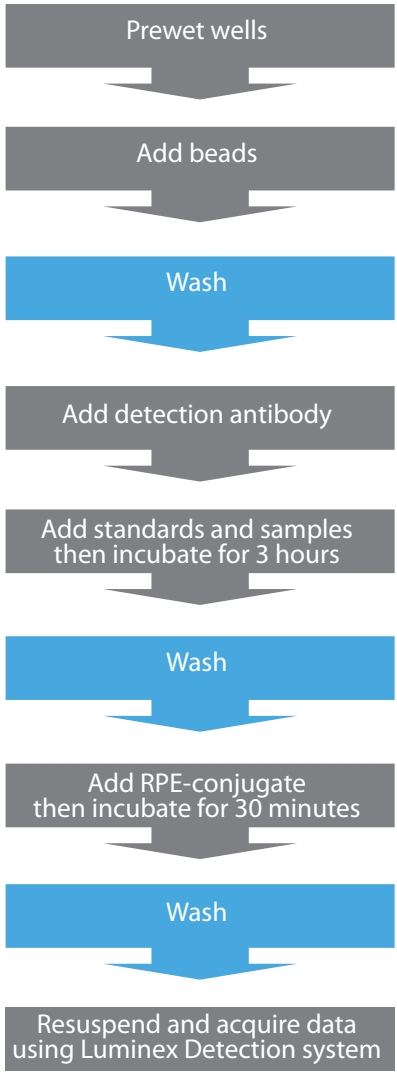
After washing the beads, R-Phycoerythrin (RPE) conjugate, is added and allowed to incubate for 30 minutes. The RPE conjugate binds to the detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich.

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

Co-Incubation Assay Procedure

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



Methods

Before Starting

Materials Required but Not Provided

- Luminex® xMAP® system with data acquisition and analysis software (Invitrogen, Cat. no. MAP0200), contact Invitrogen for instrument and software placement services, see page 22
- Filtration vacuum manifold for bead washing (Pall, Cat. no. 5017 is recommended)
- 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 or opaque 96-well plate cover (Invitrogen, Cat. no. PC10)

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Before Starting, Continued

Note

Review the procedural notes below before starting the protocol.

- **This kit has not been tested for multiplexing with other markers. Should the user elect to multiplex this kit with other Luminex kit, the assay conditions should be determined empirically for each specific application.**
- **Do not invert the filter plates during the assay**. The filter plates are designed to be used in conjunction with a vacuum manifold (**do not exceed** 5 mm Hg) and emptied from the bottom.
- Do not freeze any component of this kit. Store kit components at 2 to 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 standard vials).
- **The fluorescent beads are light-sensitive**. Protect the beads from light to avoid photobleaching of the embedded dye. Use aluminum foil to cover test tubes used in the assay. Cover filter plates 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 absorbent paper towels during loading or incubations, as liquid may be lost due to contact wicking. An extra plate cover is a recommended surface to rest the filter plate. Following plate washing, remove excess liquid and blot from 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.
- 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 Invitrogen Technical Support for product and technical assistance.
- Do not mix or substitute reagents with those from other lots or sources.

Continued on next page

Before Starting, Continued

- 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.
- 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.

Recommended Plate Plan

It is recommended that a plate plan be designed before starting the assay. A plate plan template is provided on page 26 for a fill in template. 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), Standards 7 through 1, lowest concentration to highest.
The remainder of the plate is available for controls and samples which may be run as a singlet or in duplicate, as desired.

Note

Running all standards, samples and controls in duplicate is recommended.

Preparing Reagents

Introduction

Review the information in this section before starting. Prepare components of the Akt Phospho 7-Plex Panel according to instructions below. Note: bring all reagents and samples to room temperature before use.

Preparing Wash Solution

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

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

Note: To prepare smaller volumes of 1X Working 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 2 complete sets of standard vials, so that 2 runs on the plate can be made with freshly prepared standards.
- Reconstitute the protein standard within 1 hour of performing the assay. Additional standards are available from Invitrogen custom services.
- Before performing serial dilutions confirm reconstitution volumes on the INFORMATION SHEET, included in the Antibody Bead Kit(s).
- The concentrations of the protein components of the standard are indicated on the INFORMATION SHEET.
- Perform standard dilutions in glass or polypropylene tubes.

Continued on next page

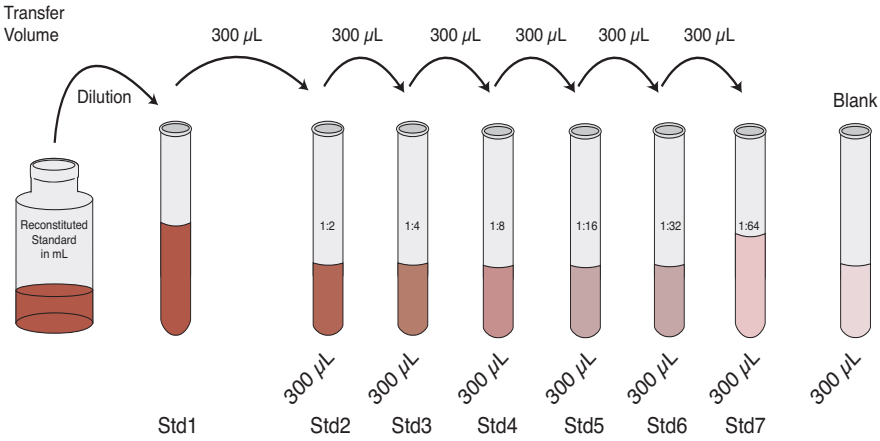
Preparing Reagents, Continued

Reconstituting Lyophilized Standards

1. To the standard vial, add the suggested reconstitution volume of Assay Diluent, indicated on the INFORMATION SHEET. **Do not vortex. When reconstituting protein solutions, always 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 to 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 to 10 times.



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

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Preparing Reagents, Continued

Go to http://www.invitrogen.com/luminex under Multiplex Solution Tools, click Luminex® Calculation Worksheet for auto calculation of all assay dilutions.

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 Working Wash Solution (page 7) per assay well. Each well requires 25 µL of the diluted beads. See table below for examples of volumes to combine.

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

Number of Wells	Vol. 10X Antibody Bead Concentrate	Vol. Working 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

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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.

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

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

Preparing Samples

Introduction

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 (Invitrogen 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° 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 (Invitrogen 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° 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

Non-adherent cells: Pellet cells by low speed centrifugation. Remove medium from the pellet, and wash twice with ice-cold PBS. Remove the PBS, and resuspend the cell pellet in cell lysis buffer (recommended cell lysate concentration is 2 to 5 mg/mL) by gently pipetting. Incubate 15 minutes on ice with occasional vortexing. Transfer the lysate to a microfuge tube and centrifuge at 14,000 rpm for 10 minutes at 2 to 8°C. Aliquot the cleared lysate into clean microfuge tubes and determine total protein concentration.

Adherent cells: Remove tissue culture medium from the cells, and wash twice with ice-cold PBS. Remove the PBS, add cell lysis buffer (recommended cell lysate concentration is 2 to 5 mg/mL), and incubate 15 minutes on ice. Collect the cell lysate and transfer to a microfuge tube and centrifuge at 14,000 rpm for 10 minutes at 2 to 8°C. 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.

Important

Certain samples may require incubation with Sample Treatment Buffer prior to analysis, to improve recovery. See analyte specific INFORMATION SHEET included with the Antibody Bead Kit.

Sample Treatment Procedure

1. Assays not requiring Sample Treatment Buffer: Lysate prepared using Cell Extraction Buffer (Cat. no. FNN0011) must be diluted at least 10-fold in Assay Diluent prior to analysis. Lysate prepared using NP40 Lysis Buffer (Cat. no. FNN0021) must be diluted at least 5-fold in Assay Diluent prior to analysis.
2. Assays requiring Sample Treatment Buffer: Lysate prepared using Cell Extraction Buffer (Cat. no. FNN0011) must be diluted 2-fold with Sample Treatment Buffer and incubated for 20 minutes on ice. Immediately after treatment, the lysate must be diluted at least 5-fold in Assay Diluent with a net dilution of 10-fold. Lysate prepared using NP40 Lysis Buffer (Cat. no. FNN0021) must be diluted 2-fold with Sample Treatment Buffer and incubated for 20 minutes on ice. Immediately after treatment, the lysate must be diluted at least 4-fold in Assay Diluent, with a net dilution of 8-fold.
3. Samples with concentrations that exceed the standard curve should be diluted in Assay Diluent and reanalyzed.

Method of Washing

Method of Washing

Incomplete washing adversely affects assay results. Perform all wash steps with the Wash Solution supplied with the kit. All phases of the assay, including incubations, wash steps, and loading beads, are performed in the filter bottom plate supplied with the kit. For a demonstration of proper washing techniques, download Luminex video from www.invitrogen.com/luminexinstrument .

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 absorbent surface when adding reagents**.

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Method of Washing, Continued

Reverse pipetting recommendation:

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 to aspirate from.
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.
5. Place the end of the tip against the inside wall of the recipient vessel at an angle.
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.

Bring all reagents and samples to room temperature before use.

Co-Incubation Assay Procedure

Analyte Capture and Detection

1. Use an adhesive plate cover to seal any unused wells. This will keep the wells dry for future use.
2. Pre-wet the designated assay wells by adding 200 µL of Working Wash Solution into designated wells. Incubate plate 15 to 30 seconds at room temperature.
3. Aspirate the Working Wash Solution from the wells using the vacuum manifold.
4. Vortex the diluted bead solution (prepared on page 9) for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.
5. Pipette 25 µL of the diluted bead solution into each well. Once the beads are added to the plate, **keep the plate protected from light**.
6. Add 200 µL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds.
7. Aspirate the Working Wash Solution from the wells with the vacuum manifold. Repeat this washing step.
8. Blot the bottom of the filter 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.

9. Add 50 µL of prepared 1X Detector Antibody (page 10) to the wells.
10. To wells designated for the standard curve, pipette 50 µL of appropriate standard dilution.
11. To the wells designated for the sample, pipette 50 µL of sample. Suggested total protein per well, 10 to 40 µg. However, the exact amount should be determined by the individual user.
12. Cover filter plate containing 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.
13. Ten to fifteen minutes prior to the end of the detector incubation step, prepare the Goat Anti-Rabbit IgG-RPE, and then proceed with Assay Reading, Step 1.

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Co-Incubation Assay Procedure, Continued

Preparing 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 of µL 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.

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

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

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Co-Incubation Assay Procedure, Continued

Assay Reading

1. Remove the liquid from wells by aspiration with the vacuum manifold.
2. Add 200 µL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step.
3. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
4. Add 100 µL of prepared 1X Goat Anti-Rabbit IgG-RPE 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).
5. Prepare the Luminex® 100™ or 200™ instrument during this incubation step. Refer to the Luminex Instrument Quick Reference card provided in kit. Refer to the INFORMATION SHEET for all bead regions and standard concentration values.
6. Remove the liquid from wells by aspiration with the vacuum manifold.
7. Wash beads by adding 200 µL Working Wash Solution to the wells; allow the beads to soak for 10 seconds, then aspirate with the vacuum manifold. Repeat this washing step 2 additional times for a total of 3 washes.
8. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
9. Add 100 µL of Working Wash Solution to each well. Shake the plate on an orbital shaker (500-600 rpm) for 2 to 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 to 8°C for reading the following day without significant loss of fluorescent intensity. Aspirate Working Wash Solution from stored plates and add 100 µL fresh Working Wash Solution. Place the plate on an orbital shaker for 2 to 3 minutes at 500-600 rpm prior to analysis.
10. Uncover the plate and insert the plate into the XY platform of the Luminex® 100™ or 200™ instrument, and analyze the samples.
11. Determine the concentration of samples from the standard curve using curve fitting software. It is recommended to use the five parameter algorithm with a weighted function ($1/y^2$), depending on the software package used.

Performance Characteristics and Limitations of the Procedure

Performance Characteristics

Refer to analyte specific INFORMATION 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, 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

Introduction

Refer to the table below to troubleshoot problems encountered with the use of Invitrogen's 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.invitrogen.com/luminex.

Problem	Cause	Solution
During data analysis, insufficient and/or erratic bead count is observed	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 to 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.

Continued on next page

Troubleshooting, Continued

Problem	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 1,000 × 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.
In-house controls perform differently in subsequent assays	Incorrect concentration entered in data analysis software	The standard proteins included in Invitrogen's Antibody Bead Kits 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 INFORMATION 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 8.

Continued on next page

Troubleshooting, Continued

Problem	Cause	Solution
Leaky 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.

Appendix

Technical Support

World Wide Web

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, INFORMATION SHEET, quick calculation worksheet, application notes, MSDSs, FAQs, formulations, citations, handbooks, and more
- Complete Technical Support contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

Contact Us

For more information or technical assistance, call, write, fax, or email. Additional international offices are listed on our website (www.invitrogen.com).

Corporate Headquarters:
Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008 USA
Tel: 1 760 603 7200
Tel (Toll Free): 1 800 955 6288
Fax: 1 760 602 6500
E-mail:
techsupport@invitrogen.com

Japanese Headquarters:
Invitrogen Japan
LOOP-X Bldg. 6F
3-9-15, Kaigan
Minato-ku, Tokyo 108-0022
Tel: 81 3 5730 6509
Fax: 81 3 5730 6519
E-mail:
jpinfo@invitrogen.com

European Headquarters:
Invitrogen Ltd
Inchinnan Business Park
3 Fountain Drive
Paisley PA4 9RF, UK
Tel: +44 (0) 141 814 6100
Tech Fax: +44 (0) 141 814 6117
E-mail:
eurotech@invitrogen.com

Manufacturing Site

Invitrogen Corporation
542 Flynn Road
Camarillo, CA 93012 USA
Tel (Toll Free): 1 800 955 6288
E-mail: techsupport@invitrogen.com

MSDS Requests

Material Safety Data Sheets (MSDSs) are available at www.invitrogen.com/msds.

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References

The references below demonstrate the success customers achieve when using Invitrogen Multiplex Assays. For complete list visit, www.invitrogen.com/luminex.

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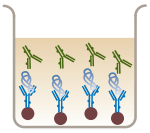
Co-Incubation Protocol Summary

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

Wash 1 × 200 µL

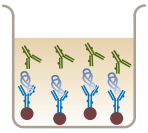
Add 50 µL 1X detector antibody

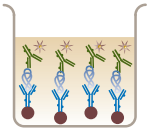
Sample type: **Standard** **Cell lysate**



Add 50 µL
standard

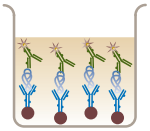
Add 50 µL
appropriately
diluted sample





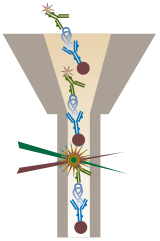
Shake for 3 hr at RT in the dark
Wash 2 × 200 µL

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 Catalog number: _____

Lot Number: _____

Plate ID: _____

Date: _____

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

invitrogen™

Corporate Headquarters
Invitrogen Corporation
5791 Van Allen Way
Carlsbad, CA 92008
T: 1 760 603 7200
F: 1 760 602 6500
E: tech_support@invitrogen.com

For country-specific contact information visit our web site at **www.invitrogen.com**