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Introduction

This application guide has been written to provide users of multiplex bead technology a brief understanding of how BioSource™ products from Invitrogen can be utilized with the Luminex® platform to obtain multiple, accurate results from a small volume of sample.

Communication between cells is mediated by a network of signaling molecules. Cell-to-cell communication influences gene expression patterns, differentiation, division, and survival. Regulation of signaling events is apparent in embryogenesis and development, as well as repair processes and inflammation in response to pathogens. The dysregulation of signaling events has been found to underlie numerous disease states, including diabetes, autoimmune diseases and cancer.

Historically, signaling molecules have been placed into two classes: (1) signaling molecules that act between cells (i.e., growth factors, cytokines, peptide hormones, steroid hormones, and phospholipids), and (2) cell-associated signaling molecules that transduce signals within the responding cells (i.e., receptors and downstream signaling components). Within these two classes of signaling molecules, individual analytes can be assessed as biomarkers for specific diseases.

In many disease states, marked local inflammatory responses induce systematic changes, and cause cytokines to spill over into the general circulation, resulting in detectable levels of these proteins in biological fluids, such as serum and plasma. Among these extracellular proteins, biomarkers for specific diseases include elevated IL-13, which correlates with asthma and allergy; elevated TNF-α, which correlates with Th1-mediated diseases, such as rheumatoid arthritis and Crohn's disease; and elevated VEGF, which correlates with vascularization and tumor metastasis. Among the intracellular signaling molecules, elevated HSP27 and survivin, both of which render cells resistant to apoptosis, or elevated levels of the epidermal growth factor receptor family member Her-2, can serve as biomarkers for cancer, while mutations of receptors such as c-kit and c-Met or protein kinases such as c-Abl have been found to underlie specific disease states.

With the discovery of networks of extracellular and intracellular molecules, interest in profiling multiple analytes has arisen. Examples of cytokine networks are abundant. In rheumatoid arthritis, elevated levels of IL-1α, IL-1β, IL-6, IL-8, GM-CSF are detected; however, administration of neutralizing antibodies to TNF-α results in the reduction of the concentrations of IL-6, IL-8, and IL-1RA, implicating TNF-α in the progression of the disease. Additionally, IL-10, TGF-β, IL-4, and IL-13 are found to inhibit the production of proinflammatory cytokines by macrophages. Cytokine profiling is also of interest when considering members of cytokine families, which share overlapping functions. Examples of cytokine families include the IL-6 family (IL-6, oncostatin M, LIF, and IL-11) and the TNF family (TNF-α, CD40L, CD95L, CD27L, and others).

Among the intracellular analytes, mutations or over-expression of receptors have been linked to alterations in downstream signaling events which correlate with malignant phenotypes. In addition, recalcitrance to insulin in type II diabetes can be detected in alterations in signaling arising from the insulin receptor through the Akt pathway (PI3K, Akt, and PRAS40).

Understanding of the importance of extracellular and intracellular markers as indicators of disease can be difficult when analytes are considered in isolation. Suspension protein arrays have been developed to permit the profiling of multiple markers within individual samples. Suspension protein arrays are sandwich immunoassays that use spectrally encoded beads with a diameter of 5 µm as the solid support. During an assay, capture antibodies covalently bound to the surface of the beads immobilize analytes of interest. After a washing step to remove unbound materials, detector antibodies are reacted with the beads, followed by addition of an R-phycoerythrin (RPE) conjugate that labels the immune complexes on the beads. The spectral properties of the beads are then monitored with the Luminex® xMAP® instrument. This assay method requires low sample volume (50 µl or less). Results are determined by interpolation from standard curves that extend over several orders of magnitudes of analyte concentration. At the present time, 100 different spectrally distinct bead types are available, affording this assay system with the capability of simultaneously monitoring the concentrations of up to 100 different analytes in a single sample. Assay kits are currently available for quantifying the concentrations of cytokines, chemokines, growth factors, neurotrophic factors and neuropeptides, either singly, or in multiplexed assays designed by the researcher. A second line of kits enables the quantification of phosphorylation state of intracellular analytes along with total levels of analytes independent of phosphorylation state. Finally, a third line of kits enables the semiquantification of activated transcription factors. These assay methods are becoming popular as a cost-effective, sample-saving alternative to measurement of individual analytes.

Chapter 1 will review sample preparation for a variety of sample types. Robust methods for sample preparation are critical to accurate and reproducible data. Special attention should be paid to the details in this section. Chapter 2 will provide detailed protocols for successful use of BioSource™ multiplex assays. Chapter 3 discusses the use of several popular acquisition/analysis software programs. Finally, the Appendix provides a handy troubleshooting guide, as well as a list of current references and a list of recommended supplies and suppliers.

Chapter 1—Sample Handling

This chapter reviews specific details for preparing extracellular proteins, intracellular proteins and nuclear proteins for use with BioSource™ multiplex kits for Luminex® from Invitrogen. In our development of assays, we have found a key component to success is the proper and consistent preparation of sample, regardless of the source.

Preparation of Sample for Extracellular Assays

It is clear that to accurately measure cytokines, chemokines, growth factors and other serum proteins in biological fluids, there are special requirements. In this chapter, several biological fluids are discussed, including serum, plasma and culture supernatant, which are validated in BioSource™ extracellular antibody bead kits for Luminex®. Additional sample types discussed include bronchial lavage, synovial fluid, cerebrospinal fluid, cervical secretions and oral mucosa transudates. Additionally, optimized protocols for preparing cell extracts and nuclear extracts are provided and are compatible with BioSource™ multiplex kits for measuring kinases and transcription factors.

General Rules

1. Serum, plasma, and tissue culture medium samples have been evaluated with BioSource™ Multiplex Bead Assays. Suitable sample types are defined on the Information Sheet included with each kit.
2. Samples should be analyzed shortly after collection or frozen in aliquots. Avoid multiple "freeze-thaw" cycles of frozen samples. Thaw completely and gently mix well prior to analysis.
3. Samples with concentrations that exceed the standard curve should be diluted and reanalyzed. Serum or plasma samples should be diluted in Assay Diluent. Tissue culture supernatants should be diluted in the corresponding tissue culture medium.

Serum

Samples should be collected in pyrogen/endotoxin-free tubes. Whole blood should be allowed to sit at room temperature for 15–30 minutes to clot. Spin at 1,000–2,000 x g for 10 minutes in a 4°C refrigerated centrifuge to separate the cells. Transfer the supernatant to a clean, chilled polypropylene tube with a sterile Pasteur pipette. Maintain the samples at 2–8°C while handling. If serum is to be analyzed at a later date, apportion the serum into 0.5 ml aliquot and store at –80°C. Avoid multiple freeze-thaw cycles. When possible, avoid the use of hemolyzed or lipemic sera. Upon thawing, it is recommended that the samples be clarified by centrifugation (14,000 rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates and/or probe. Follow assay procedure provided with kit for appropriate dilutions.

Plasma

Remove the cells from the samples by centrifugation at 2,000 x g for 10 minutes in a refrigerated centrifuge. Centrifugation at this force is necessary to deplete platelets from the sample. Transfer the supernatant to a clean, chilled polypropylene tube with a sterile Pasteur pipette. Maintain the samples at 2–8°C while handling. If the plasma is to be analyzed at a later date, apportion into

aliquots in polypropylene microcentrifuge tubes and store at –80°C. Avoid multiple freeze-thaw cycles. When ready to analyze (allow the samples to thaw on ice). All plasma samples should be clarified by centrifugation at 14,000 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge immediately prior to analysis. Follow assay procedure provided with kit for appropriate dilutions.

Culture Supernatant

Cells should be in log phase growth. Stimulate cells as desired in appropriate cell culture flasks. Using sterile technique, remove the desired volume of conditioned cell culture medium with a pipette and transfer the medium to clean polypropylene microcentrifuge tubes. Centrifuge the medium at 14,000 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge to remove any cells or cellular debris. Aliquot the clarified medium into clean polypropylene microcentrifuge tubes. These samples are ready for the assay. Alternatively, clarified medium samples can be aliquoted and stored at –80°C for future analysis. Avoid multiple freeze-thaw cycles. Frozen samples should be allowed to thaw on ice just prior to running the assay. Thawed samples should be clarified by centrifuging at 14,000 rpm for 10 minutes at 4°C in a refrigerated microcentrifuge prior to analysis to prevent clogging of the Luminex® probe and/or filter plate. Follow assay protocol provided with kit for appropriate dilutions.

Tissue Homogenate Samples

This protocol was developed using the BioSource™ Tissue Extraction Reagent I, (Cat. no. FNN0071), and shows good correlation between ELISA vs. Luminex® as illustrated in Figure 1. This procedure has been applied to multiple tissue types. However, it is recommended that you optimize for each tissue sample type used.

1. Add protease inhibitors to the Tissue Extraction Reagent I just before use.
2. Weigh tissue sample.
3. Add 10 ml of the Tissue Extraction Reagent I per 1 gram of tissue.
4. Homogenize the tissue.
5. Centrifuge the sample at 10,000 rpm for 5 minutes to pellet the tissue debris.
6. Collect the supernatant. Follow assay protocol provided with kit for appropriate dilutions.

If the samples are to be stored, aliquot and freeze at –80°C. Avoid multiple freeze-thaw cycles.

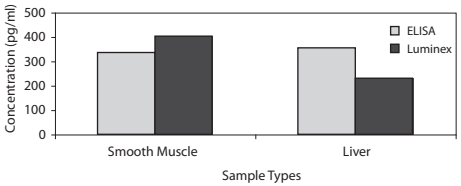


Figure 1—Correlation between VEGF ELISA and Luminex®. Quantitation of mouse tissue extracts using BioSource™ (Cat. no. FNN0071) and multiplex cytokine assay for Mouse VEGF (black bar) (Cat. no. LMG0111). Results indicate a strong correlation to ELISA (gray bar) (Cat. no. KMG0111).

Urine

Use only freshly collected samples. Dilute 2-fold with the Assay Diluent provided in the kit. The final dilution of the sample will be 4-fold and all results should be then multiplied by 4. As needed, clarify samples by centrifugation (14,000 rpm for 10 minutes) and/or filter prior to analysis to prevent clogging of the filter plates and/or probe. This procedure was used to generate the data in Figure 2.

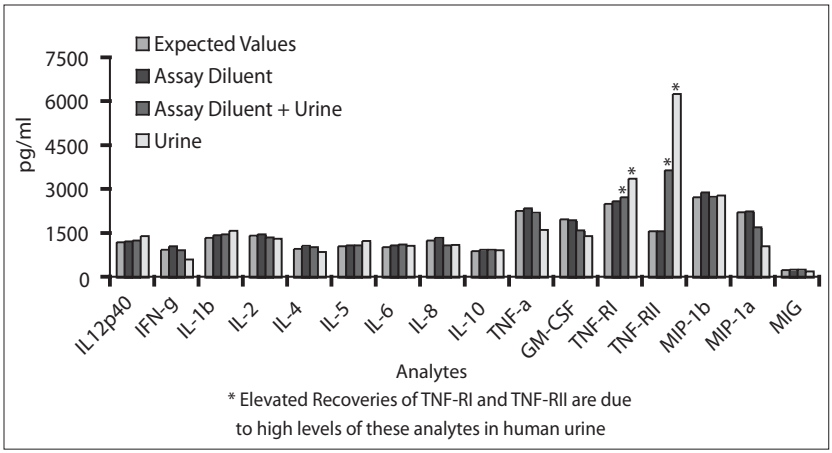


Figure 2—Quantification of cytokines using urine as a sample type. A panel of 16 recombinant protein standards was reconstituted and spiked into Assay Diluent, a 1:1 mixture of Assay Diluent + urine, or 100% urine. The data presented here show that when the urine was diluted with Assay Diluent prior to analysis, the recovery of each protein from urine was comparable to that observed with Assay Diluent.

Synovial Fluid

Collect into nonheparinized tubes and spin at 1,000 x g for 10 minutes within 30 minutes of sample collection. The acellular portion of synovial fluid should be stored at –80°C before subsequent analysis. All samples need to be clarified by centrifugation (14,000 rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates. Dilute samples 1:1 with Assay Diluent prior to addition to the assay. This procedure was used to generate the data in Figure 3 taken from a publication in *Arthritis Research and Therapy.*

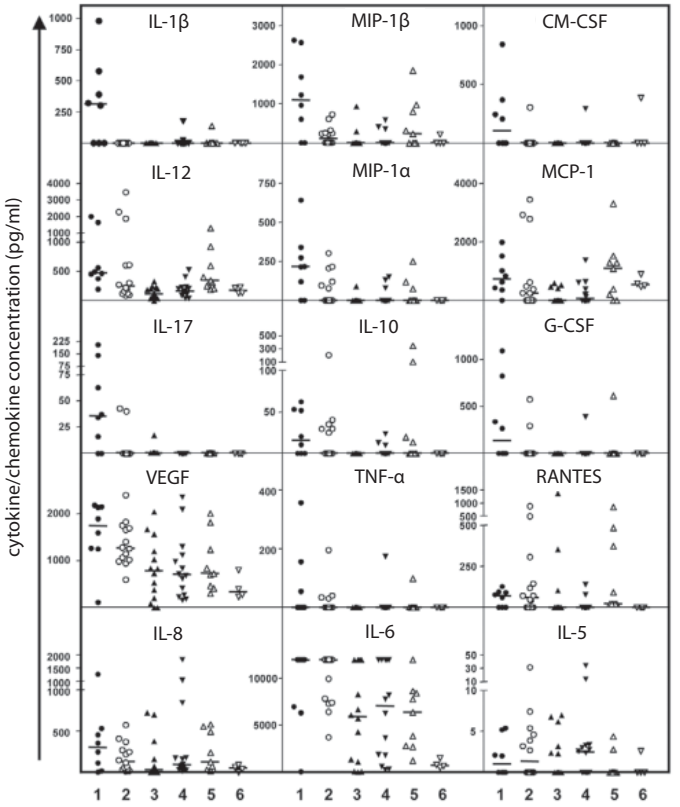


Figure 3— Measuring cytokines in synovial fluid. Synovial fluid cytokines in early and established arthritis. Shown are synovial fluid concentrations (pg/ml) of IL-1β, (MIP)-β, GM-CSF, IL-12, (MCP)-1, IL-17, IL-10, G-CSF, VEGF, TNF-α, RANTES, IL-8, IL-6, and IL-5. Patient groups: 1, early synovitis that develops into rheumatoid arthritis (RA); 2, early synovitis that develops into non-rheumatoid persistent synovitis; 3, early non-crystal related resolving synovitis; 4, crystal-related resolving synovitis; 5, established RA; and 6, osteoarthritis. Figure used with permission from Raza, et al., Arthiritis Res. Ther., Apr 2005.

Cerebrospinal Fluid

All samples need to be clarified by centrifugation (14,000 rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates. However, CSF has relatively low viscosity and unless there is presence of an infected state (abundance of WBCs), it should not require clarification and can be directly applied to the assay. Dilute 2-fold with Assay Diluent provided in the Neuroscience Buffer kit, (Cat. no. LNB0001.) This preparation procedure was initiated in the Luminex® assays and correlated to ELISA (Figure 4).

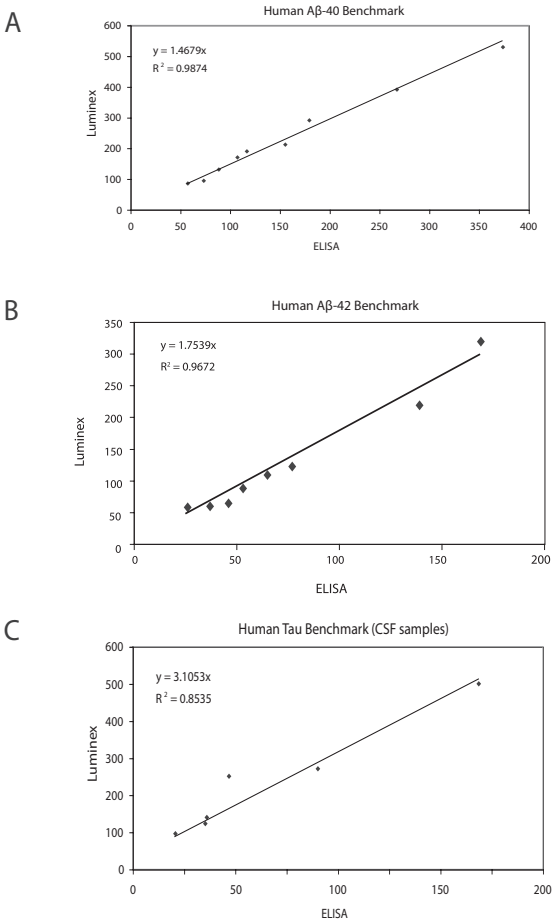


Figure 4— Correlation of Luminex® assays and ELISA. CSF Samples demonstrate excellent correlation of measurement with Luminex® Assays and BioSource™ ELISAs for A) Aβ-40 (Cat. no. LHB3481, KHB3481), B) Aβ-42 (Cat. no. LHB3441, KHB3482), and C) Total Tau (Cat. no. LHB0041, KHB0041).

Bronchial Lavage

The bronchoalveolar lavage (BAL) should be collected in a sterile syringe and kept on ice until ready to analyze. Alternatively, BAL can be aliquoted and frozen in usable sample sizes (such that exposure to freeze-thaw is limited to one). All samples need to be clarified by centrifugation (14,000 rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates. Dilute 2-fold with Assay Diluent before applying to the plate.

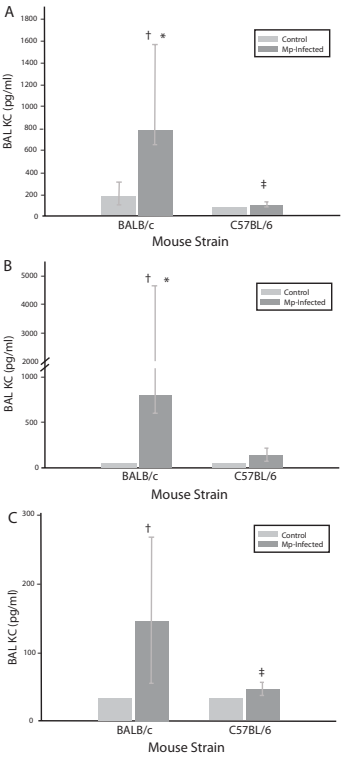


Figure 5— Measurement of chemokines in bronchial lavage. Chemokine concentrations of KC (A), MIP-1α (B), and MCP-1 (C), in BAL specimens from mice inoculated with ***Mycoplasma pneumoniae*** (Mp) or sterile SP4 broth (controls) measured 1 day after inoculation. Values represent results from two independent experiments, each including four to five mice per time point in each group. Values shown are the medians and 25–75 percentiles (error bars). †$P < 0.05$ between the values for BALB/c mice inoculated with Mp and controls. ‡$P < 0.05$ between the values for C57BL/6 mice inoculated with Mp and controls. *$P < 0.05$ between the values for BALB/c mice infected with Mp and C57BL/6 mice infected with Mp. Multiple comparisons were made with Bonferroni correction. Used with permission from Fonseca-Aten, et al. (2005) Am J Respir Cell Mol Biol.

Cervical Secretions

Cervical sponges should be placed on ice immediately upon collection. Samples should be stored at –20°C for up to one week and then stored at –80°C until ready for assay. After thawing, sponges should be weighed and placed into Eppendorf tubes, using forceps cleaned with ethanol after each transfer. Add 200 µl of ice-cold extraction buffer (recipe below) to each tube and incubate overnight at 4°C. The sponges and extraction buffer can then be transferred to microcentrifuge tubes with 0.2 µm cellulose acetate filters and centrifuged at 13,000 rpm for 10 minutes at 4°C. The eluate can then be tested for cytokine expression.

Extraction Buffer	
• 50 mM HEPES, pH 7.5	• 1 mM Na_3VO_4
• 150 mM NaCl	• 1mM NaF
• 1 mM EDTA	• 0.1% Tween 20
• 25 mM EGTA	• 10% Glycerol

Oral Mucosal Transudate

Isolate the site around the tooth and insert a piece of periodontal filter paper into gum pocket around the tooth for 30 seconds. Remove the filter paper and extract 4 times with 50 µl PBS for 5 minutes each at room temperature. The individual extractions can be combined and analyzed. Dilute 2-fold with Assay Diluent before applying to the assay.

Preparation of Sample for Intracellular Assays

Extraction of Cellular Proteins

This protocol has been applied to several human and mouse cell lines. Researchers should optimize the cell extraction procedures for their own application.

Recommended Cell Lysis Buffer	
• NP40 Cell Lysis Buffer (Cat. no. FNN0021)	
Or	
• 50 mM Tris, pH 7.4	• 50 mM NaF
• 250 mM NaCl	• 1 mM Na_3VO_4
• 5 mM EDTA	• 0.02% NaN_3
• 1% Nonidet P40 (Roche Applied Science, Cat. no. 1754599)	

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 Buffer just before use:

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

Alternate Cell Extraction Buffer

Lysates prepared with Cell Extraction Buffer must be diluted at least 10-fold prior to running the assay.

Alternate Cell Extraction Buffer	
• Cell Extraction Buffer (Cat. no. FNN0011)	
Or	
• 10 mM Tris, pH 7.4	• 2 mM Na_3VO_4
• 100 mM NaCl	• 1% Triton X-100
• 1 mM EDTA	• 10% Glycerol
• 1 mM EGTA	• 0.1% SDS
• 1 mM NaF	• 0.5% Deoxycholate
• 20 mM $Na_4P_2O_7$	

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 Cell Extraction Buffer just before use:

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

1. 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 2–5 mg/ml). Incubate 15 minutes on ice with occasional vortexing. Transfer the lysate to a microcentrifuge tube and centrifuge at 14,000 rpm for 10 minutes at 2–8°C. Aliquot the cleared lysate into clean microcentrifuge 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 µg/ml), and incubate 15 minutes on ice. Collect the cell lysate and transfer to a microcentrifuge tube. Centrifuge at 14,000 rpm for 10 minutes at 2–8°C. Aliquot the cleared lysate into clean microcentrifuge tubes and determine total protein concentration.

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.

Sample treatment prior to analysis Some BioSource™ protein immunoassays from Invitrogen receive sample pretreatment for robust antigen recognition. It is important to follow the protocol for accurate data.

For assays not requiring Sample Treatment Buffer: the lysate must be diluted at least 2-fold in Assay Diluent prior to analysis. Suggested final lysate concentration 200–400 µg/ml; however the exact amount should be determined by the individual user.

For assays requiring Sample Treatment Buffer: (see individual INFORMATION SHEET for each analyte) dilute the lysate 2-fold with Sample Treatment Buffer and incubate 20 minutes on ice. Immediately after treatment, the lysate must be diluted at least 4-fold in Assay Diluent. Suggested final lysate concentration 200–400 µg/ml; however the exact amount should be determined by the individual user.

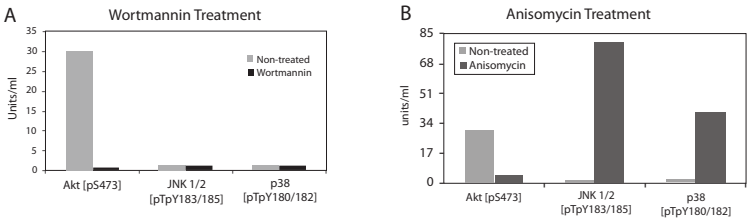


Figure 6—Phospho AKT, JNK1/2, p38 MAPK 3-plex. Jurkat cells were untreated or treated with wortmannin (A), or anisomycin (B), to demonstrate relative expression of phospho-Akt, phospho-JNK 1/2, and phospho-p38 MAPK. (Cat. no. LHO0061)

Preparation of Nuclear Fraction

Invitrogen offers an easy-to-use kit for nuclear extraction, (Cat. no. FNN0031). The kit includes all necessary reagents and the detailed protocol outlined below. Please visit www.invitrogen.com to learn more.

Procedure for Suspension Cells

1. Determine cell density in liquid media.
2. Aliquot 5 x 10^6 cells (or up to 2 x 10^7 cells) per tube into 15 ml conical tubes.
3. Collect the cells by centrifugation at 800 x g for 6 minutes at 4°C. Remove the supernatant and discard.
4. Add 10 ml cold PBS, resuspend cells and pellet by centrifugation at 800 x g for 6 minutes at 4°C. Repeat for a total of 2 PBS washes.
5. During the second centrifugation step required in step 4, prepare the Complete Hypotonic Cell Lysis Buffer according to the following table. Space is provided to allow calculation of volume for each experiment. Each 5 x 10^6 cells contained in the sample requires 0.5 ml Complete Hypotonic Cell Lysis Buffer. Mix the Complete Hypotonic Cell Lysis Buffer by pipeting up and down several times. It is important to note that this buffer must be prepared within 10 minutes of use.

Complete Hypotonic Cell Lysis Buffer	Volume per Extraction of 5 x 10^6 Cells	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Hypotonic Cell Lysis Buffer	0.5 ml		
Phosphatase Inhibitor Cocktail	5 µl		
Protease Inhibitor Cocktail	5 µl		
DTT solution	5 µl		
PMSF	0.5 µl		

*included in the kit

6. At the completion of the centrifugation step, remove the supernatant from the cells by aspiration and discard.
7. Add Complete Hypotonic Cell Lysis Buffer (0.5 ml per 5 x 10^6 cells) to each cell pellet. Resuspend each pellet by pipetting up and down five times. Transfer each mixture into clean 1.5 ml microcentrifuge tubes.
8. Incubate for 10 minutes on ice.
9. Add 25 µl Detergent Solution to each sample for each 0.5 ml of Complete Hypotonic Cell Lysis Buffer used in step 7.
10. Vortex for 5 seconds.

Complete Nuclear Wash Buffer	Volume per Extraction of 5 x 10^6 Cells	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Nuclear Wash Buffer	1.0 ml		
Phosphatase Inhibitor Cocktail	10 µl		
Protease Inhibitor Cocktail	10 µl		
DTT solution	10 µl		
PMSF	1 µl		

*Included in the kit

11. Centrifuge at 800 x g for 6 minutes at 4°C. During this centrifugation step, prepare the Complete Nuclear Wash Buffer according to the table presented above. Space is provided to allow calculation of volume for each experiment. Each sample requires 1.0 ml of Complete Nuclear Wash Buffer. Mix the Complete Nuclear Wash Buffer by pipetting up and down several times.
12. Carefully aspirate the supernatant from the lysed cells and transfer to a clean 1.5 ml microcentrifuge tube. Store this fraction at –80°C for future analysis, if desired. This supernatant contains the cytoplasmic fraction. The pellet contains the cell nuclei.
13. To each nuclear pellet, add 0.5 ml cold Complete Nuclear Wash Buffer. Wash the pellet gently by pipetting up and down several times. It is important to note that this pellet may remain intact through this washing step. Collect the nuclear pellet by centrifuging the mixture at 800 x g for 6 minutes at 4°C. Repeat this washing step for a total of two washes. During the centrifugation step, prepare Complete Extraction Buffer 1 and Complete Extraction Buffer 2 according to the tables presented below. Space is provided to allow calculation of volume for each experiment. A nuclear pellet made from 5 x 10^6 cells has a volume of approximately 25 µl, and requires 12.5 µl (1/2 pellet volume) Complete Extraction Buffer 1 and 12.5 µl (1/2 pellet volume) Complete Extraction Buffer 2. These volumes should be adjusted according to the nuclear pellet size. Mix Complete Extraction Buffer 1 by pipetting up and down several times. Mix Complete Extraction Buffer 2 by pipetting up and down several times.

Complete Extraction Buffer 1	Volume per Extraction (25 uL pellet)	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Extraction Buffer 1	12.5 uL		
Phosphatase Inhibitor Cocktail	0.125 µl		
Protease Inhibitor Cocktail	0.125 µl		
PMSF	0.0125 µl		

Complete Extraction Buffer 2	Volume per Extraction (25 uL pellet)	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Extraction Buffer 1	12.5 uL		
Phosphatase Inhibitor Cocktail	0.125 µl		
Protease Inhibitor Cocktail	0.125 µl		
PMSF	0.0125 µl		

*Included in the kit

14. Remove the supernatant from the nuclear pellet by aspiration.
15. To the nuclear pellet, add Complete Extraction Buffer 1 (1/2 pellet volume), followed immediately by Complete Extraction Buffer 2 (1/2 pellet volume), and vortex the mixture vigorously for 2 seconds.
16. Incubate the mixture on ice for 30 minutes. Vortex the tube briefly at 10 minute intervals.
17. Clarify the nuclear extract by centrifugation at 14,000 x g in a microcentrifuge for 30 minutes at 4°C.
18. Carefully transfer the supernatant to a clean chilled microcentrifuge tube. Discard the pellet. The supernatant contains the nuclear extract. Quantitate protein concentration using the Bradford assay. Nuclear extracts prepared from 5 x 10^6 cells will yield approximately 25-100 µg protein.
19. Aliquot and store the extract at –80°C until ready for analysis. Avoid repeated freeze-thawing.
20. Return all Nuclear Extraction Kit reagents to storage at 2–8°C.

Procedure for Adherent Cells

1. Remove cell culture media by aspiration and wash cells twice with an equal volume of cold PBS (containing no calcium or magnesium).
2. Add an equal volume of ice cold 5 mM EDTA in PBS (containing no calcium or magnesium) to the cells and incubate on ice for 10 minutes.
3. Lift the cells from the culture vessel by gentle scraping. Determine the cell density.
4. Aliquot 5 x 10^6 cells (or up to 2 x 10^7 cells) per tube into 15 ml conical tubes.
5. Collect the cells by centrifugation at 800 x g for 6 minutes at 4°C. Remove the supernatant.
6. Add 10 ml cold PBS, resuspend cells and pellet by centrifugation at 800 x g for 6 minutes at 4°C. Repeat for a total of 2 PBS washes.
7. During the second centrifugation step required in step 6, prepare the Complete Hypotonic Cell Lysis Buffer according to the table on the next page. Each 5 x 10^6 cells contained in the sample requires 0.5 ml Complete Hypotonic Cell Lysis Buffer. Mix the Complete Hypotonic Cell Lysis Buffer by pipetting up and down several times. It is important to note that this buffer must be prepared within 10 minutes of use.

Complete Hypotonic Cell Lysis Buffer	Volume per Extraction of 5 x 10^6 Cells	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Hypotonic Cell Lysis Buffer	0.5 ml		
Phosphatase Inhibitor Cocktail	5 µl		
Protease Inhibitor Cocktail	5 µl		
DTT solution	5 µl		
PMSF	0.5 µl		

***Included in the kit**

8. At the completion of the centrifugation step, remove the supernatant from the cells by aspiration.
9. Add Complete Hypotonic Cell Lysis Buffer (0.5 ml per 5 x 10^6 cells) to each cell pellet. Resuspend each pellet by pipetting up and down five times. Transfer each mixture into clean 1.5 ml microcentrifuge tubes.
10. Incubate for 10 minutes on ice.
11. Add 25 µl Detergent Solution to each sample for each 0.5 ml of Complete Hypotonic Cell Lysis Buffer used in step 9.

12. Vortex for 5 seconds.
13. Centrifuge at 800 x g for 6 minutes at 4°C. During this centrifugation step, prepare the Complete Nuclear Wash Buffer according to the table presented below. Space is provided to allow calculation of volume for each experiment. Each sample requires 1.0 ml of Complete Nuclear Wash Buffer. Mix the Complete Nuclear Wash Buffer by pipetting up and down several times.

Complete Nuclear Wash Buffer	Volume per Extraction of 5 x 10^6 Cells	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Nuclear Wash Buffer	1.0 ml		
Phosphatase Inhibitor Cocktail	10 µl		
Protease Inhibitor Cocktail	10 µl		
DTT solution	10 µl		
PMSF	1 µl		

*** Included in the kit**

14. Carefully aspirate the supernatant from the lysed cells and transfer to a clean 1.5 ml microcentrifuge tube. Store this fraction at –80°C for future analysis, if desired. This supernatant contains the cytoplasmic fraction. The pellet contains the cell nuclei.
15. To each nuclear pellet, add 0.5 ml cold Complete Nuclear Wash Buffer. Wash the pellet gently by pipetting up and down several times. It is important to note that this pellet may remain intact through this washing step. Collect the nuclear pellet by centrifuging the mixture at 800 x g for 6 minutes at 4°C. Repeat this washing step for a total of two washes. During the centrifugation step, prepare Complete Extraction Buffer 1 and Complete Extraction Buffer 2 according to the tables presented below. Space is provided to allow calculation of volume for each experiment. A nuclear pellet made from 5 x 10^6 cells has a volume of approximately 25 µl, and requires 12.5 µl (1/2 pellet volume) Complete Extraction Buffer 1 and 12.5 µl (1/2 pellet volume) Complete Extraction Buffer 2. These volumes should be adjusted according to the nuclear pellet size. Mix Complete Extraction Buffer 1 by pipetting up and down several times. Mix Complete Extraction Buffer 2 by pipetting up and down several times.

Complete Extraction Buffer 1	Volume per Extraction (25 ul pellet)	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Extraction Buffer 1	12.5 µl		
Phosphatase Inhibitor Cocktail	0.125 µl		
Protease Inhibitor Cocktail	0.125 µl		
PMSF	0.125 µl		

***Included in the kit**

Complete Extraction Buffer 2	Volume per Extraction (25 ul pellet)	Number of Reactions + 2	Volume x (Number of Reactions +2)
*Extraction Buffer 2	12.5 µl		
Phosphatase Inhibitor Cocktail	0.125 µl		
Protease Inhibitor Cocktail	0.125 µl		
PMSF	0.125 µl		

***Included in the kit**

16. Remove the supernatant from the nuclear pellet by aspiration.
17. To the nuclear pellet, add Complete Extraction Buffer 1 (1/2 pellet volume), followed immediately by Complete Extraction Buffer 2 (1/2 pellet volume), and vortex the mixture vigorously for 2 seconds.
18. Incubate the mixture on ice for 30 minutes. Vortex the tube briefly at 10 minute intervals.
19. Clarify the nuclear extract by centrifugation at 14,000 x g in a microcentrifuge for 30 minutes 4ºC.
20. Carefully transfer the supernatant to a clean chilled microcentrifuge tube. Discard the pellet. The supernatant contains the nuclear extract. Quantitate protein concentration using the Bradford assay. Nuclear extracts prepared from 5 x 10^6 cells will yield approximately 25–100 µg protein.
21. Aliquot and store the extract at –80ºC until ready for analysis. Avoid repeated freeze-thawing.
22. Return all nuclear extraction kit reagents to storage at 2–8ºC.

Chapter 2—Testing the Samples

Extracellular Multiplex Assay

Invitrogen provides kits as either a singleplex that can be combined with other singleplexes or pre-mixed multiplex panels. The 10-Plex assay example that is used herein is designed for the *in vitro* quantitative determination of the designated ten analytes. The 10-Plex may also be combined with other extracellular bead reagents, allowing higher-level multiplexing of the assay. Samples validated by BioSource include serum, plasma, and tissue culture supernatant.

Principles of the Method

Beads of defined spectral properties conjugated to analyte specific capture antibodies and samples (including standards of known analyte concentration, control specimens, and unknowns) are pipetted into the wells of a filter bottom microplate and incubated for 2 hours. During this first incubation, analytes bind to the capture antibodies on the beads. After washing the beads, analyte-specific biotinylated detector antibodies are added and incubated with the beads for 1 hour. During this second incubation, the analyte-specific biotinylated detector antibodies recognize their epitopes and bind to the appropriate immobilized analytes. After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-phycoerythrin (Streptavidin-RPE), is added and incubated for 30 minutes. During this final incubation, the Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich. After washing to remove unbound Streptavidin-RPE, the beads are analyzed with the Luminex® xMAP® instrument. By monitoring the spectral properties of the beads and the amount of associated R-phycoerythrin (RPE) fluorescence, the concentration of one or more analytes can be determined.

Reagents Provided

Note: Store all reagents at 2–8°C.

Reagents Provided	100 Test Kit
Bead Concentrate (10X). Contains 7.5 mM sodium azide; 0.25 ml per vial.	1 vial
Biotinylated Antibody Concentrate (10X). Contains 15 mM sodium azide; 1 ml per vial.	1 vial
Hu Cytokine Plex Standard.	2 vials
Wash Solution Concentrate (20X); 15 ml per bottle.	1 bottle
Assay Diluent. Contains 15 mM sodium azide; 15 ml per bottle.	1 bottle
Incubation Buffer. Contains 15 mM sodium azide; 12 ml per bottle.	1 bottle
Biotin Diluent. Contains 3.3 mM thymol; 12 ml per bottle.	1 bottle
Streptavidin-RPE Concentrate (10X). Contains 15 mM sodium azide; 1 ml per vial.	1 vial
Streptavidin-RPE Diluent. Contains 3.3 mM thymol; 12 ml per bottle.	1 bottle
96 well Filter Plate (Extra plates can be purchased from Millpore/Fisher Cat. no. MSBVN-1250.)	1 plate

All BioSource™ kits have been configured for research use only and are not to be used in diagnostic procedures.

Disposal Note: 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.

Supplies Required But Not Provided (see page 29)

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Procedural Notes/Lab Quality Control

1. Do not freeze any component of this kit. When not in use, kit components should be stored at 2–8°C. All reagents should be brought to room temperature before use.
2. The fluorescent beads are light-sensitive. Protect the beads from light to avoid photobleaching of the embedded dye. Aluminum foil should be used to cover test tubes used in the assay. Filter plates containing beads should be shielded with an aluminum foil–wrapped plate cover. The amber vial does not provide full protection, and should therefore be kept covered in the box when not in use.
3. Do not expose beads to organic solvents.
4. Do not use reagents after kit expiration date.
5. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
6. 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 inches Hg)** and emptied from the bottom.
7. When pipetting reagents, maintain a consistent order of addition from well-to-well. This method ensures equal incubation times for all wells.
8. Avoid touching the filter plate membrane with pipette tips to prevent tearing.
9. Do not place filter plates on absorbent paper towels during loading or incubations, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.

Preparation of Wash Solution

Upon storage at 2–8°C, a precipitate may form in the 20X Wash Solution Concentrate. If this occurs, warm the 20X Wash Solution Concentrate to 37°C and vortex until the precipitate is dissolved. The Wash Solution Concentrate is provided as a 20X concentrate. To prepare the Working Wash Solution for use with a 96-well plate, transfer the entire contents of bottle to a 500 ml container and add 285 ml of deionized water. If not using an entire 96-well plate, smaller volumes of 1X Working Wash Solution can be made by mixing 1 part of 20X concentrate with 19 parts deionized water. The Working Wash Solution is stable for up to two weeks when stored at 2–8°C.

Directions for Washing

Incomplete washing will adversely affect assay outcome. All washing must be performed with the Wash Solution provided. All phases of the assay, including incubation steps, washing steps, and loading the beads into the Luminex® xMAP® instrument, are performed in the filter bottom plate provided. Unused wells may be left dry during the assay and used at a later time.

1. To wash the beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum. The vacuum setting should be adjusted so 3 seconds are required to empty 0.2 ml solution from the wells. (DO NOT EXCEED 5 inches Hg). Excessive vacuum can cause the membrane to tear, resulting in antibody-bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface.
2. 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.
3. Following the final aspiration step, gently blot the bottom of the filter plate on clean paper towels to remove residual liquid.

Reagent Preparation and Assay Procedure

Prior to starting the assay, prepare standard and antibody conjugated beads in accordance with instructions below, then proceed to Assay Procedure, Step 1 (page 24).

Preparation of Standard

Reconstitute the protein standard within one hour of performing the assay. All standards are calibrated to NIBSC preparations, when available. Additional standards are available from BioSource™. The standard included in this kit is provided as a premixed set of related markers. The concentrations of the protein components of the standard are indicated on the **Information Sheet** included in the kit. Standard dilutions may be performed in glass or plastic tubes. When using serum or plasma samples, reconstitute the standard with Assay Diluent provided. If using other sample types (e.g., tissue culture supernatant), reconstitute the standard with a mixture composed of 50% Assay Diluent and 50% of the matrix which most closely matches the sample type (50%/50% mixture). For example: When the sample type is RPMI medium containing 5% FBS, the standards should be reconstituted in a mixture composed of 50% Assay Diluent and 50% RPMI containing 5% FBS. Pro-

tein standards may be analyzed alone, or may be combined with other protein standards for higher levels of multiplexing. **Do not combine more than 4 vials.**

	Preparation of Standard
One vial of standard	Reconstitute the standard vial in the suggested reconstitution volume, usually 1 ml, of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes. Gently invert 2–3 times.
Two vials of standards	Reconstitute each vial with 0.5 ml of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes. Combine equal volumes from each vial and mix gently.
Three vials of standards	Reconstitute each vial with 0.333 ml of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes. Combine equal volumes from each vial and mix gently.
Four vials of standards	Reconstitute each vial with 0.250 ml of appropriate diluent (see above). Allow the proteins to rehydrate for 10 minutes. Combine equal volumes from each vial and mix gently.

Preparation of the Standard Curve

The standard curve is made by serially diluting the reconstituted standard in Assay Diluent (serum and plasma samples) or 50% Assay Diluent/50% culture medium (tissue culture supernatant) (see Figure 7). Discard all remaining reconstituted and diluted standards after completing assay. Return the Assay Diluent to the refrigerator.

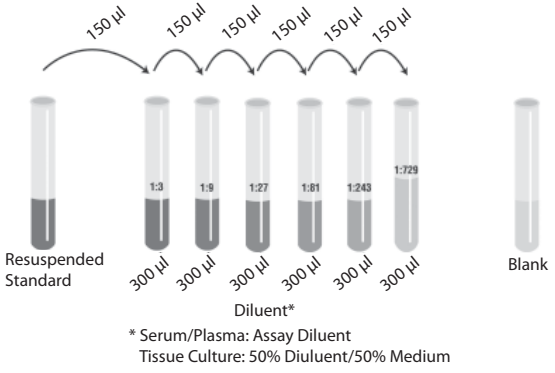


Figure 7— Illustration of standard serial dilution.

Preparation of Antibody Conjugated Beads

Determine the number of wells required for the assay. Standard curves and samples may be run singly or in replicates, as desired. The beads are provided as a 10X concentrate and must be diluted prior to use. Immediately before dispensing, vortex the 10X bead concentrate for 30 seconds followed by sonication in a sonicating water bath for 30 seconds. To make a 1X stock, dilute 2.5 µl 10X beads in 25 µl Working Wash Solution per assay well. Each well requires 25 µl of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Volume 10X Beads	Volume Working Wash Solution
24	0.06 ml	0.60 ml
32	0.08 ml	0.80 ml
40	0.10 ml	1.00 ml
48	0.12 ml	1.20 ml
56	0.14 ml	1.40 ml
64	0.16 ml	1.60 ml
72	0.18 ml	1.80 ml
80	0.20 ml	2.00 ml
88	0.22 ml	2.20 ml
96	0.24 ml	2.40 ml

Extracellular Assay Procedure Flow Chart

A general summary of the assay is shown below in figure 8.

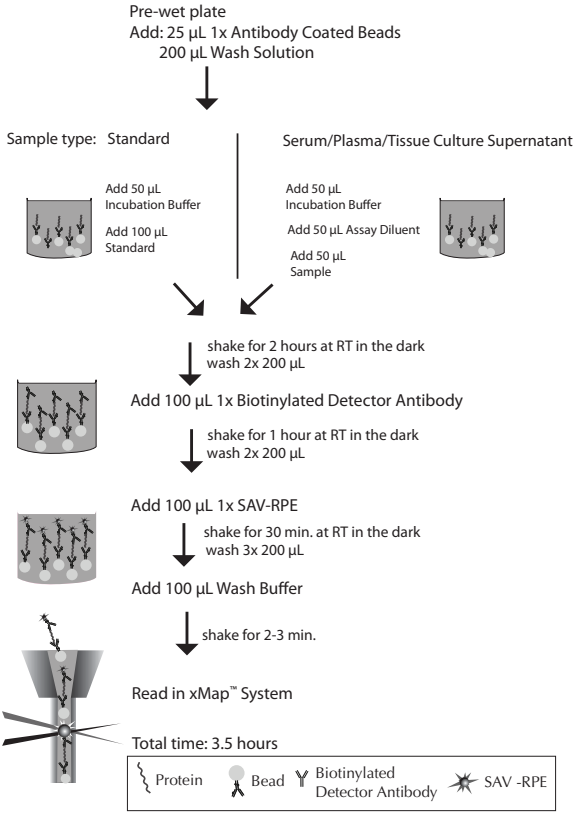


Figure 8—Summary of extracellular assay procedure.

Extracellular Assay Procedure

1. Pre-wet the wells designated for the assay. Pipette 200 µl of Working Wash Solution into designated wells. Wait 15–30 seconds then aspirate the Working Wash Solution from the wells using the vacuum manifold. Wells not used during the assay should be kept dry for future use. An adhesive plate cover may be used to seal the unused wells.
2. Vortex the diluted bead solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.
3. Pipette 25 µl of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then remove the Working Wash Solution from the wells by aspiration with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
5. Pipette 50 µl Incubation Buffer into each well.
6. To the wells designated for the standard curve, pipette 100 µl of appropriate standard dilution.
7. To the wells designated for the sample, pipette 50 µl Assay Diluent followed by 50 µl sample.
8. Incubate the plate for 2 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500-600 rpm).
9. 10–15 minutes prior to the end of this incubation, prepare the biotinylated detector antibody in accordance with instructions below, then proceed to Assay Procedure, Step 10.
10. After the 2 hour capture bead incubation, remove the liquid from the wells by aspiration with the vacuum manifold. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
11. Add 100 µl of 1X stock, diluted Biotinylated Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500–600 rpm).
12. 10–15 minutes prior to the end of the detector incubation step, prepare the Streptavidin-RPE in accordance with instructions below, then proceed to Assay Procedure, Step 13.

Preparation of Detector Antibody

The Biotinylated Detector Antibody is provided as a 10X concentrate and must be diluted prior to use. To make a 1X, dilute 10 µl 10X Biotinylated Detector Antibody in 100 µl Biotin Diluent per assay well. Each well requires 100 µl of the diluted Biotinylated Detector Antibody. See table below for examples of volumes to combine.

Number of Wells	Volume 10X Detector Antibody	Volume Detector Antibody Diluent
24	0.240 ml	2.4 ml
32	0.320 ml	3.2 ml
40	0.400 ml	4.0 ml
48	0.480 ml	4.8 ml
56	0.560 ml	5.6 ml
64	0.640 ml	6.4 ml
72	0.720 ml	7.2 ml
80	0.800 ml	8.0 ml
88	0.880 ml	8.8 ml
96	0.960 ml	9.6 ml

Preparation of Streptavidin-RPE

The Streptavidin-RPE is provided as a 10X concentrate and must be diluted prior to use. To make a 1X, dilute 10 µl 10X Streptavidin-RPE in 100 µl Steptavidin-RPE Diluent per assay well. Each well requires 100 µl of the diluted Streptavidin-RPE. See table below for examples of volumes to combine.

Number of Wells	Volume 10X SAV-RPE	Volume SAV-RPE Diluent
24	0.240 ml	2.4 ml
32	0.320 ml	3.2 ml
40	0.400 ml	4.0 ml
48	0.480 ml	4.8 ml
56	0.560 ml	5.6 ml
64	0.640 ml	6.4 ml
72	0.720 ml	7.2 ml
80	0.800 ml	8.0 ml
88	0.880 ml	8.8 ml
96	0.960 ml	9.6 ml

13. Remove the liquid from the wells by aspiration with the vacuum manifold. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then aspirate with the vacuum manifold Repeat this washing step. Blot residual liquid from the bottom of the plate on clean paper towels.
14. Add 100 µl of 1X, diluted Streptavidin-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).
15. Prepare the Luminex® xMAP® instrument during this incubation step.
16. Remove the liquid from the wells by aspiration with the vacuum manifold. Wash the 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 two additional times for a total of 3 washes.

17. Add 100 µl of Working Wash Solution to each well. Shake the plates on an orbital shaker (500–600 rpm) for 2–3 minutes to resuspend the beads. If the plates cannot be read on the day of the assay, they may be covered and stored in a dark location overnight at 2–8°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 2–3 minutes prior to analysis.
18. Uncover the plate; insert the plate into the XY platform of the Luminex® xMAP® instrument, and analyze the samples.
19. Determine the concentration of samples from the standard curve using curve fitting software. The five-parameter algorithm usually provides the best fit.

IMPORTANT: In addition to dilutions performed on the sample prior to running the assay, the sample concentration calculated from the standard curve must be multiplied by an additional factor of 2 to correct for the 1:2 dilution in Step 7.

Multiplex Assay Combining Plexes

Before mixing plexes it is important to check that each analyte is represented by a unique bead region. Up to 10 bead concentrates (singleplexes or premixed multiplexes) can be combined to increase the number of analytes being monitored.

Preparation of Capture Bead

Volume from each vial of Bead Concentrate to combine:

0.025 ml x _____ assay wells ÷ 10 = _____ ml per vial

Volume of working Wash Solution:

[0.0275 ml x _____ assay wells] –
[_____ ml Bead Concentrate x _____ vials of Bead Concentrate] = _____ ml

Final volume of diluted multiplexed Capture Beads:

[_____ ml working Wash Solution] +
[_____ ml Bead Concentrate x _____ vials of Bead Concentrate] = _____ ml

Sample calculation—Combining 5 vials of Bead Concentrate for 48 assay wells

Volume from each vial of Bead Concentrate to combine:

[0.025 ml x 48 assay wells] ÷ 10 = 0.120 ml per vial

Volume of working Wash Solution:

[0.0275 ml x 48 assay wells] –
[0.120 ml Bead Concentrate x 5 vials] = 0.720 ml

Final volume of diluted multiplexed Capture Beads:
[0.720 ml working Wash Solution] +
[0.120 ml Bead Concentrate x 5 vials] = 1.32 ml

If desired, premixed beads can be stored at 2–8°C until the expiration date printed on the kit box.

Preparation of Biotinylated Antibody

Volume from each vial of Biotinylated Antibody Concentrate to combine:
0.100 ml x _____ assay wells ÷ 10 = _____ ml per vial

Volume of Biotin Diluent:
[0.110 ml x _____ assay wells] –
[_____ ml Biotinylated Antibody Concentrate x _____ vials of Biotinylated Antibody Concentrate] = _____ ml

Final volume of diluted Biotinylated Antibody:
_____ ml Biotin Diluent +
[_____ ml Biotinylated Antibody Concentrate x _____ vials of Biotinylated Antibody Concentrate] = _____ ml

Sample calculation—Combining 5 vials of Biotinylated Antibody Concentrate for 48 assay wells

Volume from each vial of Biotinylated Antibody Concentrate to combine:
0.100 ml x 48 assay wells ÷ 10 = 0.480 ml

Volume of Biotin Diluent:
[0.110 ml x 48 assay wells] –
[0.480 ml Biotinylated Antibody Concentrate x 5 vials] = 2.88 ml

Final volume of diluted Biotinylated Antibody:
2.88 ml Biotin Diluent +
[0.480 ml Biotinylated Antibody Concentrate x 5 vials] = 5.28 ml

If desired, premixed Biotinylated Detector Antibody can be stored at 2–8°C until the expiration date printed on the kit box. Sample data using BioSource™ Multiplexed Luminex® assays is shown below in Figures 9, 10, and 11.

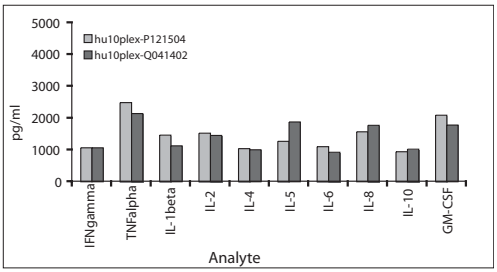


Figure 9— Cytokine 10-plex lot comparison. A spike recombinant sample analyzed using the BioSource™ Human Cytokines 10-Plex from Invitrogen. (Cat. no. LHC0001), lots P121504 and Q041402. Quantitative values obtained for each lot were comparable, indicating good lot-to-lot consistency.

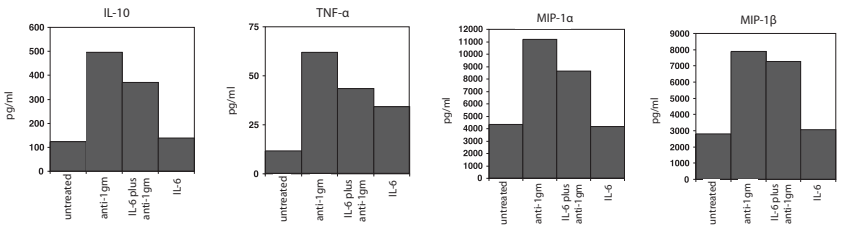


Figure 10— BioSource™ 19-plex bead immunoassay. Conditioned cell culture supernatants (approximately 1 ml per treatment) from untreated cells, cells treated with Goat (polyclonal) $F(ab')_2$ anti-human IgM to engage the BCR, cells pretreated with IL-6 then treated with Goat (polyclonal) $F(ab')_2$ anti-human IgM, or cells treated with IL-6 alone, were analyzed with a BioSource™ 19-Plex bead immunoassay. IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, GM-CSF, MCP-1, eotaxin, RANTES, EGF, VEGF, FGFb, and G-CSF were unaffected by these treatments.

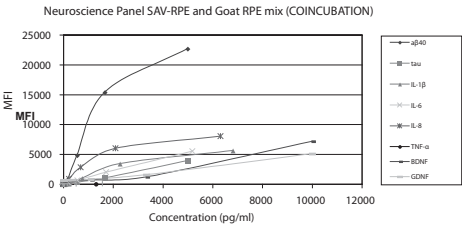


Figure 11— Neuroscience multiplex panel. A BioSource™ multiplex assay that combines single-plexes for the measurement of Aβ40, Tau, IL-1β, IL-6, IL-8, TNF-α, BDNF, and GDNF from cell culture supernatant.

Intracellular Multiplex Assay

Principles of Method

BioSource's Intracellular Multiplex assays from Invitrogen follow the same principles of method as outlined for the Extracellular Assay Method (see page 18 for details).

Reagents Provided

Note: Store all reagents at 2–8°C.

Reagents Provided	100 Test Kit
Wash Solution Concentrate (20X); 15 ml per bottle.	1 bottle
Sample Treatment Buffer. Contains 15 mM sodium azide; 10 ml per bottle.	1 bottle
Assay Diluent. Contains 15 mM sodium azide; 15 ml per bottle.	2 bottle
RPE Diluent. Contains 15 mM sodium azide; 12 ml per bottle.	1 bottle
Goat Anti-Rabbit IgG-RPE Concentrate (10X). Contains 15 mM sodium azide; 1 ml per vial.	1 vial
Detector Antibody Diluent. Contains 3.3 mM thymol; 12 ml per bottle.	1 bottle
Filter Plate, 96 wells per plate.	1 plate

Disposal Note: 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.

Supplies Required But Not Provided

1. Appropriate Cell Biology Protein Bead Kit(s). See www.invitrogen.com for kits available.
2. Luminex® xMap® system. Please contact Invitrogen for instrument and software placement services.
3. Filtration manifold for bead washing (e.g., Millipore, Cat. no. MAVM 096 0R; Qiagen, Cat. no. 9014579).
4. Data analysis and graphing software program (e.g., ACS STarStation™, MiraiBio MasterPlex®, Luminex® IS, MS Excel, or SoftMax).
5. Sonicating water bath (e.g., Cole-Parmer, Cat. no. 08849-00).
6. Orbital shaker (e.g., Fisher, Cat. no. 14-271-9).
7. Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips. (A manifold multichannel pipette is desirable.)
8. Distilled or deionized water.
9. Glass or plastic tubes.
10. Beakers and graduated cylinders in various sizes.
11. Aluminum foil.
12. Extra filter plate covers.

Safety

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Procedural Notes/Lab Quality Control

1. This Buffer Reagent Kit contains a set of common reagents which are intended for use with BioSource™' Cell Biology and Neurobiology Antibody Bead Kits for the Luminex® xMAP® instrument. This assay system allows the design of single analyte or multiplexed assays. To ensure that several Antibody Bead Kits are compatible in a multiplexed assay, verify that the bead region for each analyte (stated on the INFORMATION SHEETS in the Antibody Bead Kits used) is unique.
2. Do not freeze any component of this kit. When not in use, kit components should be stored at 2–8°C. All reagents should be brought to room temperature before use.
3. The fluorescent beads are light-sensitive. Protect the beads from light to avoid photobleaching of the embedded dye. Aluminum foil should be used to cover test tubes used in the assay. Filter plates containing beads should be shielded with an aluminum foil–wrapped plate cover. The amber vial does not provide full protection, and should therefore be kept covered in the box when not in use.
4. Do not expose beads to organic solvents.
5. Do not use reagents after kit expiration date.
6. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
7. 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 inches Hg)** and emptied from the bottom.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This method ensures equal incubation times for all wells.
9. Avoid touching the filter plate membrane with pipette tips to prevent tearing.
10. Do not place filter plates on absorbent paper towels during loading or incubations, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.

Preparation of Wash Solution

The Wash Solution Concentrate is provided as a 20X concentrate. Upon storage at 2–8°C, a precipitate may form. If this occurs, warm the 20X Wash Solution Concentrate to 37°C and vortex until the precipitate is dissolved. To prepare the Working Wash Solution for use with a 96 well plate, transfer the entire contents of bottle to a 500 ml container and add 285 ml of deionized water. If not using an entire 96 well plate, smaller volumes of 1X Working Wash Solution can be made by mixing 1 part of 20X concentrate with 19 parts deionized water. The Working Wash Solution is stable for up to two weeks when stored at 2–8°C.

Directions for Washing

Incomplete washing will adversely affect assay outcome. All washing must be performed with the Wash Solution provided. All phases of the assay, including incubation steps, washing steps, and loading the beads into the Luminex® xMAP® instrument, are performed in the filter bottom plate

provided in this Buffer Reagent Kit. Unused wells may be left dry during the assay and used at a later time.

1. To wash the beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum. The vacuum setting should be adjusted so 3 seconds are required to empty 0.2 ml solution from the wells **(Do not exceed 5 inches Hg)**. Excessive vacuum can cause the membrane to tear, resulting in antibody bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface.
2. 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.
3. Following the final aspiration step, blot the bottom of the filter plate on clean paper towels to remove residual droplets of liquid.
4. **Sample treatment prior to analysis**
   A. Assays not requiring Sample Treatment Buffer: the lysates must be diluted at least 2-fold in Assay Diluent prior to analysis. Suggested final lysate concentration 200–400 µg/ml; however the exact amount should be determined by the individual user.
   B. Assays requiring Sample Treatment Buffer (see the individual INFORMATION SHEET for each analyte) to improve recovery: dilute the lysate 2-fold with Sample Treatment Buffer and incubate 20 minutes on ice. Immediately after treatment, the lysate must be diluted at least 4-fold in Assay Diluent. Suggested final lysate concentration 200–400 µg/ml; however, the exact amount should be determined by the individual user.

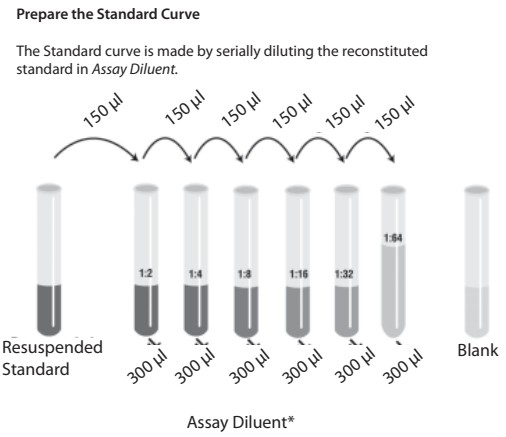


Figure 12—Illustration of standard serial dilution.

Preparation of Standard

Standard dilutions may be performed in glass or plastic tubes. Reconstitute the Protein Standard in Assay Diluent within one hour of performing the assay. The concentrations of the protein components of the standards and the resuspension volumes are indicated on the lot-specific **Information Sheets** which are provided with the Antibody Bead Kits. Additional standards are available for purchase from BioSource™. Allow the standard to rehydrate for 10 minutes before use.

Preparation of Standard Curve

The standard curve is made by serially diluting the reconstituted standard in Assay Diluent. Discard all remaining reconstituted and diluted standards after completing assay. Return the Assay Diluent to the refrigerator.

Preparation of Antibody Conjugated Beads

Determine the number of wells required for the assay. Standard curves and samples may be run singly or in replicates, as desired. The beads are provided as a 10X concentrate and must be diluted prior to use. Immediately before dispensing, vortex the 10X bead concentrate for 30 seconds followed by sonication in a sonicating water bath for 30 seconds. To make a 1X stock, dilute 2.5 µl 10X beads in 25 µl Working Wash Solution per assay well. Each well requires 25 µl of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Volume 10X Beads	Volume Working Wash Solution
24	0.06 ml	0.60 ml
32	0.08 ml	0.80 ml
40	0.10 ml	1.00 ml
48	0.12 ml	1.20 ml
56	0.14 ml	1.40 ml
64	0.16 ml	1.60 ml
72	0.18 ml	1.80 ml
80	0.20 ml	2.00 ml
88	0.22 ml	2.20 ml
96	0.24 ml	2.40 ml

Intracellular Assay Procedure

1. Pre-wet the wells designated for the assay. Pipette 200 µl of Working Wash Solution into designated wells. Wait 15–30 seconds then aspirate the Working Wash Solution from the wells using the vacuum manifold. Wells not used during the assay should be kept dry for future use. An adhesive plate cover may be used to seal the unused wells.
2. Vortex the diluted bead solution for 30 seconds, then sonicate for at least 30 seconds immediately prior to use in the assay.
3. Pipette 25 µl of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then remove the Working Wash Solution from the wells by aspiration with the vacuum manifold. Repeat this washing step. Blot the bottom of the plate on clean paper towels to completely remove all residual droplets of liquid.
5. To the wells designated for the standard curve, pipette 100 µl of appropriate diluted standard.
6. To the wells designated for the sample, pipette 100 µl of sample (Please see Preparation of samples on page 10 for recommended sample preparation). Suggested total protein per well: 20–40 µg. However the exact amount should be determined by the individual user.
7. Incubate the plate for 2 hours at room temperature on an orbital shaker. Shaking should be sufficient to keep beads suspended during the incubation (500–600 rpm).
8. Ten minutes prior to the end of this incubation step, prepare the Detector Antibody, then proceed to Assay Procedure, step 9.

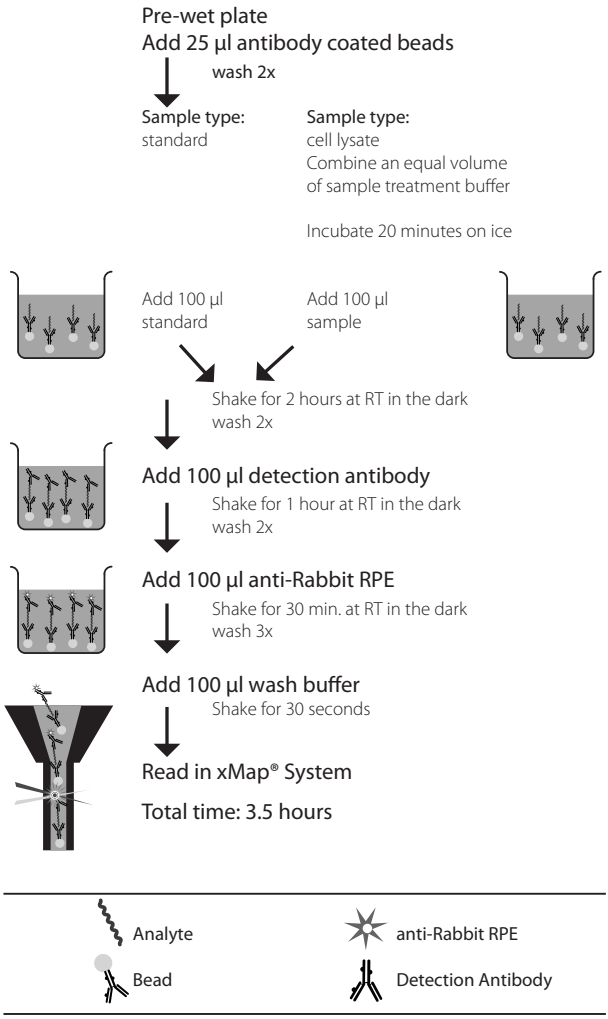


Figure 13—Summary of intracellular assay procedure.

Preparation of the Detector Antibody

The Detector Antibody is provided as a 10X concentrate and must be diluted prior to use. To make a 1X stock, dilute 10 µl 10X Detector Antibody in 100 µl Detector Antibody Diluent per assay well. Each well requires 100 µl of the diluted Detector Antibody. See table below for examples of volumes to combine.

Number of Wells	Volume 10X Detector Antibody	Volume Detector Antibody Diluent
24	0.240 ml	2.4 ml
32	0.320 ml	3.2 ml
40	0.400 ml	4.0 ml
48	0.480 ml	4.8 ml
56	0.560 ml	5.6 ml
64	0.640 ml	6.4 ml
72	0.720 ml	7.2 ml
80	0.800 ml	8.0 ml
88	0.880 ml	8.8 ml
96	0.960 ml	9.6 ml

9. After the 2 hour capture bead incubation, remove the liquid from the wells by aspiration with the vacuum manifold. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the plate on clean paper towels to completely remove all residual droplets of liquid.
10. Add 100 µl of diluted Detector Antibody to each well and incubate the plate for 1 hour at room temperature on an orbital shaker. Shaking should be sufficient to keep the beads suspended during incubation (500–600 rpm).
11. Ten to fifteen minutes prior to the end of this incubation step, prepare the Goat Anti-Rabbit IgG-RPE in accordance to instructions below, then proceed to Assay Procedure, step 12.
12. Remove the liquid from the wells by aspiration with the vacuum manifold. Add 200 µl Working Wash Solution to the wells. Allow the beads to soak for 15–30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the plate on clean paper towels to completely remove all residual droplets of liquid.
13. Add 100 µl of diluted 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 (500–600rpm).
14. Prepare the Luminex® xMAP® instrument during this incubation step.
15. Remove the liquid from the wells by aspiration with the vacuum manifold **(vacuum must not exceed 5 Inches Hg)**. Wash the 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 two additional times for a total of 3 washes. Blot the bottom of the plate on clean paper towels to completely remove all residual droplets of liquid.

Number of Wells	Volume 10X Goat Anti-Rabbit IgG-RPE	Volume RPE Diluent
24	0.240 ml	2.4 ml
32	0.320 ml	3.2 ml
40	0.400 ml	4.0 ml
48	0.480 ml	4.8 ml
56	0.560 ml	5.6 ml
64	0.640 ml	6.4 ml
72	0.720 ml	7.2 ml
80	0.800 ml	8.0 ml
88	0.880 ml	8.8 ml
96	0.960 ml	9.6 ml

16. Add 100 µl of Working Wash Solution to each well. Shake the plates on an orbital shaker (500–600 rpm) for 2–3 minutes to resuspend the beads. If the plates cannot be read on the day of the assay, they may be covered and stored in a dark location overnight at 2–8°C for reading the following day without significant loss of fluorescent intensity. Aspirate Working Wash Solution from stored plates and add 100 ml fresh Working Wash Solution. Place the plate on an orbital shaker 2–3 minutes prior to analysis.
17. Uncover the plate, insert the plate into the XY platform of the Luminex® xMAP® instrument, and analyze the samples.
18. Determine the concentration of samples from the standard curve using curve fitting software. Refer to page 58 for selection of a curve-fitting algorithm that provides the best fit for the data collected.

Important: In addition to dilutions performed on the sample prior to running the assay, the following dilution factors need to be accounted for when determining final concentrations.

A. Lysates not requiring preincubation in Sample Treatment Buffer: The sample concentration calculated from the standard curve must be multiplied by an additional factor of 2 or greater. The exact dilution factor will be determined by the individual user.
B. Lysates REQUIRING pre-incubation in Sample Treatment Buffer: The sample concentration calculated from the standard curve must be multiplied by an additional factor of 8 or greater. The exact dilution factor will be determined by the individual user.

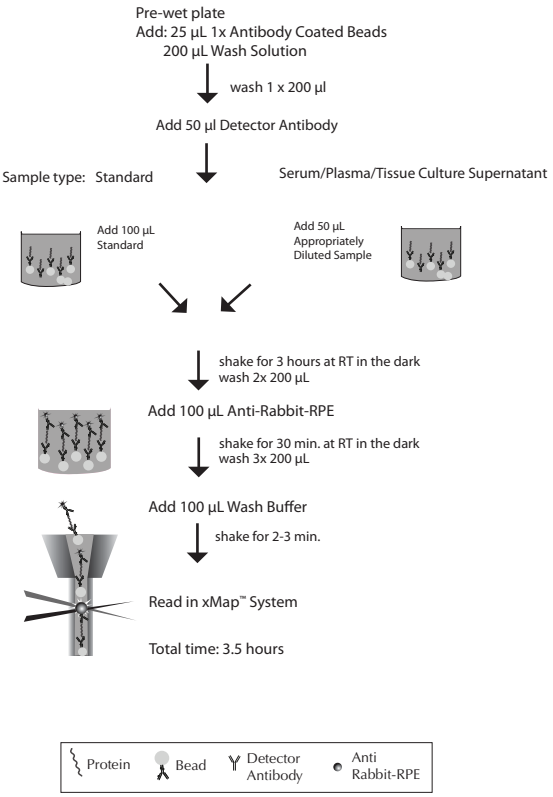


Figure 14—Summary of intracellular assay procedure requiring a co-incubation step.

Multiplexed Assays

Before mixing plexes, it is important to check that each analyte is represented by a unique bead region. Up to 10 bead concentrates (singleplexes or pre-mixed multiplexes) can be combined to increase the number of analytes being monitored.

Preparation of Capture Bead

Volume from each vial of Bead Concentrate to combine:
0.025 ml x _____ assay wells ÷ 10 = _____ ml per vial

Volume of working Wash Solution:
[0.0275 ml x _____ assay wells] – [_____ ml Bead Concentrate x _____ vials of Bead Concentrate] = _____ ml

Final volume of diluted multiplexed Capture Beads:
[_____ ml working Wash Solution] + [_____ ml Bead Concentrate x _____ vials of Bead Concentrate] = _____ ml

Sample calculation: Combining 5 vials of Bead Concentrate for 48 assay wells

Volume from each vial of Bead Concentrate to combine:
[0.025 ml x 48 assay wells] ÷ 10 = 0.120 ml per vial

Volume of working Wash Solution:
[0.0275 ml x 48 assay wells] – [0.120 ml Bead Concentrate x 5 vials] = 0.720 ml

Final volume of diluted multiplexed Capture Beads:
[0.720 ml working Wash Solution] + [0.120 ml Bead Concentrate x 5 vials] = 1.32 ml

If desired, premixed beads can be stored at 2–8°C until the expiration date printed on the kit box.

Preparation of Detector Antibody

Volume from each vial of Detector Antibody Concentrate to combine:
0.100 ml x _____ assay wells ÷ 10 = _____ ml per vial

Volume of Detector Antibody Diluent:
[0.110 ml x _____ assay wells] – [_____ ml Detector Antibody Concentrate x _____ vials of Detector Antibody Concentrate] = _____ ml

Final volume of diluted Detector Antibody:
_____ ml Detector Antibody Diluent + [_____ ml Detector Antibody Concentrate x _____ vials of Detector Antibody Concentrate] = _____ ml

Sample calculation: Combining 5 vials of Detector Antibody Concentrate for 48 assay wells

Volume from each vial of Detector Antibody Concentrate to combine:
0.100 ml x 48 assay wells ÷ 10 = 0.480 ml

Volume of Detector Antibody Diluent:
[0.110 ml x 48 assay wells] – [0.480 ml Detector Antibody Concentrate x 5 vials] = 2.88 ml

Final volume of diluted Detector Antibody:
2.88 ml Detector Antibody Diluent + [0.480 ml Detector Antibody Concentrate x 5 vials] = 5.28 ml

If desired, premixed diluted Detector Antibody can be stored at 2-8°C until the expiration date printed on the kit box.

Co-incubation Calculation

Volume of Detector Antibody Diluent:
[0.100 ml x _____ assay wells] – [_____ ml Detector Antibody Concentrate x _____ vials of Detector Antibody Concentrate] = _____ ml

Final volume of diluted Detector Antibody:
_____ ml Detector Antibody Diluent + [_____ ml Detector Antibody Concentrate x _____ vials of Detector Antibody Concentrate] = _____ ml

If desired, premixed Detector Antibody can be stored at 2 to 8°C until the expiration date printed on the kit box.

Sample data using Biosource™ Akt, p38, and JNK 3-plex assay are shown in Figure 15.

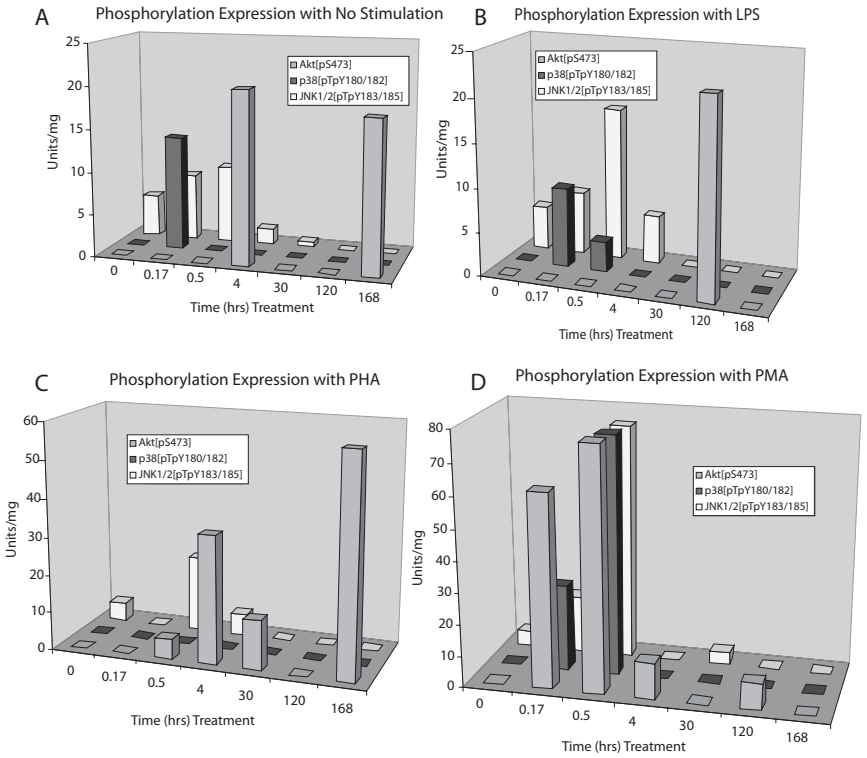


Figure 15— Measurement of phosphorylation of Akt, JNK1/2, p38 MAPK 3-plex. Isolated lymphocytes either left untreated (A) or treated with LPS (B). Isolated lymphocytes were also treated with either PHA (C) or PMA (D). The levels of Akt [pS473], p38MAPK [pTpY180/182], and JNK1/2 [pTpY183/185] in cell lysates were measured using the 3-plex Luminex® Assay (BioSource™ Cat. no. LHO0051 [Total] and Cat. no. LHO0061 (phospho)). All values were normalized to total protein levels.

Nuclear Transcription Factor Assay

Principle of the Method

BioSource™ Transcription Factor (TF) Assays are multiplexed bead assays designed for simultaneously quantifying the levels of multiple transcription factors within nuclear extracts. To perform an assay, cells are first stimulated as desired, and nuclear extracts are prepared using BioSource™ Nuclear Extraction Kit. These extracts can be prepared in advance and stored at –80°C until the Transcription Factor Assay is performed.

In the first phase of a Transcription Factor Assay, biotin labeled DNA probes, provided in a separate Multiplex Transcription Factor Bead Set, are pipetted into the wells of a 96-well PCR thermal cycler–compatible microplate. To these wells, controls or nuclear extract samples are added. Controls include the Positive Reagent Control, Negative Reagent Control, a protein control, nuclear extract from unstimulated cells, and nuclear extract from stimulated cells. Samples are nuclear extracts from the cells under investigation. All controls and samples are incubated with the DNA probes for 20 minutes at 25°C in a PCR thermal cycler. During this incubation, transcription factors contained in the nuclear extract bind to the DNA probes.

In the second phase of the assay, the Positive Reagent Control wells receive Digestion Buffer, while other wells receive Digestion Buffer plus Digestion Reagent (nuclease). The DNA digestion step is allowed to proceed for 20 minutes at 37°C in a PCR thermal cycler. During this phase of the assay, transcription factors bound to their DNA probes protect the probes from nuclease. The amount of biotin that remains incorporated in the probe permits quantitation of the protection afforded by transcription factor in the samples.

In the third phase of the assay, fluorescently encoded microspheres are added to the digestion mixture and incubated for 45 minutes at room temperature. These beads are conjugated to capture DNA sequences designed to bind to the probes through sequence complementarity. In the fourth phase of the assay, the individual mixtures are transferred to the wells of a filter plate. The beads are then washed, and incubated with Streptavidin-RPE. The Streptavidin-RPE binds to the biotin associated with beads. Excess Streptavidin-RPE is removed in a final washing step, and the beads are analyzed in the Luminex® xMAP® instrument.

The efficiency of digestion of the DNA probes in the absence of transcription factors is calculated from the Positive Reagent Control and the Negative Reagent Control. The amount of specific transcription factors bound to the DNA probes is then calculated by subtracting the MFI of the Negative Reagent Control from the MFI of the sample.

Transcription Factor Buffer Reagent Kit

(Cat. no. LNB0004)

Note: Store all reagents at –20°C.

Common Reagent Kit	100 Test Kit
Sample Diluent; 0.75 ml per vial.	1 vial
Binding Mix 1; 1.6 ml per vial.	2 vials
Digestion Reagent (Nuclease); 30 µl per vial.	1 vial
Digestion Buffer. 3.2 ml per vial.	1 vial
Hybridization Buffer; 1.8 ml per vial.	1 vial
Hybridization Accelerator; 120 µl per vial.	1 vial
Detection Reagent (SAV-RPE) in glycerol; 55 µl per vial.	1 vial
10X Assay Wash Buffer.* Contains 0.05% sodium azide; 6 ml per bottle.	1 bottle

*10X Assay Wash Buffer should be stored at 2–8°C following initial thawing.

Disposal Note: 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.

Supplies Required But Not Provided

1. Luminex® xMAP® with the XY platform. Please contact Invitrogen for instrument and placement services.
2. We recommend the use of STarStation™ software from Applied Cytometry Systems for data acquisition and analysis with the Luminex® xMAP® instrument.
3. Multiplex Transcription Factor Bead Set. This kit contains the spectrally encoded fluorescent beads, binding mix (DNA probes in buffered solution), controls, and digestion buffer.
4. 96-well PCR plate.
5. Adhesive PCR plate covers (e.g., Axygen Cat. no. PCR-AS200).
6. Millipore Multiscreen® Filter Plate (Millipore Cat. no. MSBVN1250).
7. Extra filter plate covers.
8. Microcentrifuge.
9. 1.5 ml microcentrifuge tubes.
10. Glass or plastic tubes.
11. Distilled or deionized water.
12. Beakers and graduated cylinders in various sizes.
13. Calibrated, adjustable precision pipettes, preferably with disposable plastic tips.
14. A manifold multichannel pipette.
15. Filtration manifold for bead washing (e.g., Pall, Cat. no. 5017 or Millipore, Cat. no. MAVM 096 OR).
16. 96-well PCR thermal cycler.
17. 37°C water bath.
18. Sonicating water bath (e.g., Cole-Parmer, Cat. no. 08849-00).

19. Vortex mixer.
20. Orbital shaker.
21. Aluminum foil.
22. Absorbent paper towels.

Safety

All biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

Procedural Notes/Lab Quality Control

- → Do not mix or interchange different reagent lots from various kit lots.
- → Do not use reagents after the kit expiration date.
- → The beads provided in the Multiplex Transcription Factor Bead Sets are light-sensitive. Protect the beads from light to avoid photobleaching the embedded dye. Aluminum foil should be used to cover tubes containing beads during the assay. Filter plates containing beads should be shielded with an aluminum foil–wrapped plate cover.
- → Do not place filter plates on absorbent paper towels during loading, as liquid will be lost due to wicking. An extra plate cover serves as a good surface upon which to rest the filter plate. Following plate washing, excess liquid may be blotted from the bottom of the plate by pressing the plate on clean paper towels.
- → In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect. We recommend that with the initial use of this kit, samples and controls be analyzed in triplicate. Experienced users of this kit may consider decreasing the number of replicates with subsequent analyses. Unstimulated Cell Nuclear Extracts, Stimulated Cell Nuclear Extracts, and Protein Controls are recommended in the assay.

Sample Preparation

Invitrogen's BioSource™ Transcription Factor Assays are intended for use with nuclear extracts generated with the Nuclear Extraction Kit. Please see the Nuclear Extraction Kit protocol for complete details. Each assay requires 1–3 µl of nuclear extract at a protein concentration ranging from 1–10 µg/µl. If assays with different volumes of cell extract will be compared, it is important to keep the total reaction volume of the assay constant by adding extract dilution buffer to the samples with the lower volume.

Directions for Washing Beads

This assay includes bead washing steps, which are performed in a filter bottom plate. The filter plate is designed to retain the beads while permitting the flow of liquids which are removed through the bottom of the plate by gentle aspiration using a vacuum manifold. During the assay, the plate must be kept in the upright position to prevent bead loss. When preparing a vacuum manifold for use with these reagents, the vacuum setting should be adjusted so that 5–10 seconds are required to empty solution from the wells. Vacuum pressure should not exceed 5 inches Hg. Excessive vacuum

can cause the membrane to tear, resulting in bead loss. Vacuum surge should be prevented by opening and adjusting the vacuum on the manifold before placing the plate on the surface. To wash the beads, place the filter plate on the vacuum manifold and aspirate the liquid with gentle vacuum. Following each aspiration step, gently blot the bottom of the filter plate on absorbent paper towels to remove residual droplets of liquid. Remove the plate from the vacuum manifold and add 1X Assay Wash Solution to the wells, then empty the wells by aspiration with the vacuum manifold and blot. Do not let the wells completely dry. Repeat as directed under ASSAY PROCEDURE. If solution remains in the wells during vacuum aspiration, remove the plate from the vacuum manifold and firmly press the bottom of the casing up against the filter bottom to improve the vacuum. Covering unused wells with an adhesive plate sealer also improves the vacuum. Unused wells may be left dry during the assay and used at a later time.

Preparation

- → Initiate the Luminex® xMAP® warm up cycle.
- → Prepare reagents.
- → Allow all reagents to warm to room temperature for 30 minutes.
- → Place the Hybridization Buffer in a 37°C water bath to dissolve precipitate.
- → Briefly microcentrifuge the Detection Reagent vial and the Digestion Reagent vial (contained in the Multiplex Transcription Factor Bead Set) to bring their contents to the bottom of the vials.
- → Allow the Protein Controls, Nuclear Extract Controls, and nuclear extract samples to thaw on ice.
- → Determine the number of wells required for the assay. Each assay requires a Bead Control well, Negative Reagent Control wells, Positive Reagent Control wells, plus wells for the samples under investigation. The number of replicates should be determined by the user. We recommend that with the initial use of this kit, the Negative Reagent Control wells, Positive Reagent Control wells, Protein Control, Unstimulated Cell Nuclear Extract Control, Stimulated Cell Nuclear Extract Control, and samples under investigation be analyzed in triplicate. With subsequent analyses the number of sample replicates may be decreased, and the Protein Control, the Unstimulated Cell Nuclear Extract Control, and the Stimulated Cell Nuclear Extract Control may be omitted. A suggested plate plan is presented in Figure 15. Please note that well A1 will be left dry until the assay is analyzed. Well A1 is designated for the beads to permit setting of the gates on the Luminex® xMAP® instrument.

	A	B	C	D	E	F	G	H
1	Bead Control Well*	Protein Control	Sample 1	Sample 3				
2	Positive Reagent Control Well	Protein Control	Sample 1	Sample 4				
3	Positive Reagent Control Well	Unstimulated Cell Extract	Sample 1	Sample 4				
4	Positive Reagent Control Well	Unstimulated Cell Extract	Sample 2	Sample 4				
5	Negative Reagent Control Well	Unstimulated Cell Extract	Sample 2	Sample 5				
6	Negative Reagent Control Well	Stimulated Cell Extract	Sample 2	Sample 5				
7	Negative Reagent Control Well	Stimulated Cell Extract	Sample 3	Sample 5				
8	Protein Control	Stimulated Cell Extract	Sample 3					

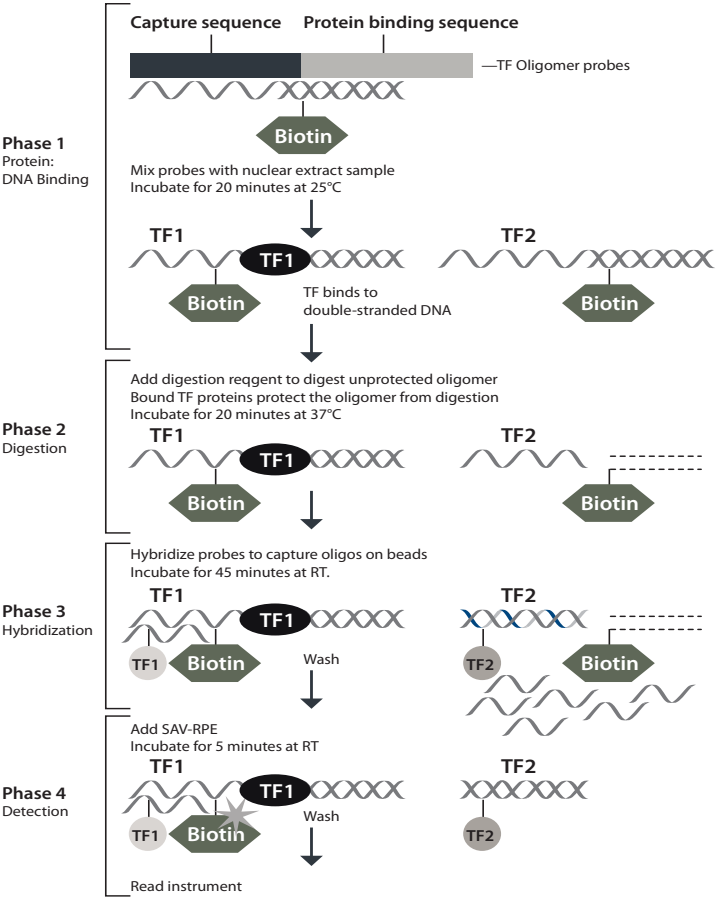


Figure 16—Schematic of transcription factor assay.

Transcription Factor Assay Procedure

* This well is left empty until the analysis on the Luminex® xMAP® instrument. This well is intended for setting the gates on the instrument and for estimating the bead background.

Phase 1—Protein: DNA Binding Step.

1. Mix the Binding Mix by inverting the vial two times.
2. Pipette 25 µl of the Binding Mix into each well, except the Bead Control well, of a 96 well PCR plate required for the assay.
3. Add 1 µl Protein Control, Unstimulated Control Cell Nuclear Extract, and Stimulated Control Cell Nuclear Extract to designated wells. Mix by gently pipetting up and down two times.
4. Add 1–3 µl of sample nuclear extract, diluted as indicated in **Sample Preparation**, to designated wells. Mix by gently pipetting up and down two times.
5. Seal plate with an adhesive plate cover and incubate at 25°C for 20 minutes in a PCR thermocycler.
6. During this incubation step, prepare the Complete Digestion Mix according to the table presented on the next page.

Complete Digestion Mix	Volume per 1 Reaction	Number of Reactions + 2	Volume x (Number of Reactions +2)
Digestion Buffer	24.75 µl		
Digestion Reagent (nuclease)	0.25 µl		
Total Volume	25 µl		

All wells require the Complete Digestion Mix except for the Bead Control well and Positive Reagent Control wells. It is suggested sufficient volume be made for at least two extra wells. Space is provided in the table above for performing calculations. Mix the Complete Digestion Mix by gently pipetting up and down ten times.

Phase 2—Digestion Step

1. At the end of the 20 minutes, protein: DNA binding incubation step, remove the 96-well plate from the PCR thermal cycler. Adjust the thermal cycler temperature to 37°C.
2. Add 25 µl of Complete Digestion Mix to all wells except the Bead Control well and the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
3. Add 25 µl of Digestion Buffer to the Positive Reagent Control wells. Mix by gently pipetting up and down two times.
4. Seal the plate with an adhesive plate cover.
5. Incubate wells for 20 minutes at 37°C in the PCR thermal cycler.
6. During this incubation step, prepare for the next phase of the assay.
   - Mix Hybridization Buffer by inversion and check for precipitate. If precipitate is present, return the Hybridization Buffer to the 37°C water bath until the solution is clear.
   - Mix the Hybridization Buffer again by inversion. Mix the Hybridization Accelerator by brief, gentle vortexing and briefly microcentrifuge the vial to bring the contents to the bottom.

• Resuspend the Bead Mix (provided in the Multiplex Transcription Factor Bead Set) by vortexing for 10 seconds and then incubating for 2 minutes in a sonicating water bath.

• Prepare the Complete Hybridization Mix according to the table presented below. Space is provided to perform calculations of volumes for each experiment. All wells require the Complete Hybridization Mix, including the Bead Control well. It is suggested that sufficient volume be made for at least two extra wells. Vortex the Complete Hybridization Mix for 10 seconds, then incubate the Complete Hybridization Mix for 2 minutes in a sonicating water bath. Protect this mixture from light by wrapping the tube in aluminum foil, as the beads are light-sensitive.

Phase 3—Hybridization Step

1. At the end of the 20 minutes digestion incubation step, remove the 96-well plate from the PCR thermal cycler. All subsequent procedures will be performed at room temperature.
2. Add 10 µl of the Complete Hybridization Mix to each well except the Bead Control well. Mix by pipetting up and down two times. Save the extra Complete Hybridization Mix for use as the Bead Control for analyzing the assay with the Luminex® xMAP® instrument. This Complete Hybridization Mix must be protected from light.
3. Seal the plate with an adhesive plate cover. An aluminum foil–wrapped adhesive plate cover is recommended at this step to shield the beads from light.
4. Incubate wells for 45 minutes at room temperature. Protect the wells from light during this incubation.
5. During this incubation step, prepare the 1X Assay Wash Buffer and Complete Detection Mix.
   • Prepare the 1X Assay Wash Buffer, according to the table presented below. Space is provided to allow calculations of volumes for each experiment. Mix the 1X Assay Wash Buffer by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

1X Assay Wash Buffer	Volume per 1 Reaction	Number of Reactions + 2	Volume x (Number of Reactions +2)
10X Assay Wash Buffer	60 µl		
Deionized Water	540 µl		
Total Volume	600 ml		

• Prepare the Complete Detection Mix, according to the table presented below. Space is provided to perform calculations of volumes for each experiment. Mix the Complete Detection Mix by inverting several times. It is suggested sufficient volume of this reagent be made for at least two extra wells.

Complete Detection Mix	Volume per 1 Reaction	Number of Reactions+ 2	Volume x (Number of Reactions+2)
1X Assay Wash Buffer	49.5 uL		
Detection Reagent	0.50 uL		
Total Volume	50 uL		

Phase 4—Detection Step.

1. Pre-wet the 96-well filter plate by adding 50 µl 1X Assay Wash Buffer to each well designated for the assay, including the Bead Control well. Blot residual liquid from the bottom of the plate with absorbent paper towels.
2. Remove the 1X Assay Wash Buffer from each well by gentle aspiration with the vacuum manifold. Do not allow the filter plate to dry.
3. Carefully transfer the contents of the wells of the PCR plate to the corresponding wells of the pre-wetted filter plate. A multichannel pipette is desirable for this transfer. Care must be taken not to puncture the membranes of the filter plate or cross-contaminate the samples.
4. To the Bead Control well (well A1 in our example), add 50 µl 1X Assay Wash Buffer followed by 10 µl Complete Hybridization Mix.
5. Remove the liquid from the wells of the filter plate by gentle aspiration with the vacuum manifold. Do not allow the membrane to dry. Blot residual liquid from the bottom of the plate with absorbent paper towels.
6. Wash each well by adding 100 µl 1X Assay Wash Buffer. Remove the 1X Assay Wash Buffer by gentle aspiration with the vacuum manifold. Repeat this washing step two more times for a total of three washings. Blot residual liquid from the bottom of the plate with absorbent paper towels.
7. Add 50 µl of Complete Detection Mix to each well.
8. Incubate for 5 minutes at room temperature. Shield the plate from light during this incubation with an aluminum foil–wrapped plate cover.
9. Remove the liquid from the wells of the filter plate by gentle aspiration with the vacuum manifold.
10. Wash each well by adding 100 µl 1X Assay Wash Buffer. Remove the 1X Assay Wash Buffer by gentle aspiration with the vacuum manifold. Blot residual liquid from the bottom of the plate with absorbent paper towels.
11. Carefully resuspend the beads by adding 100 µl 1X Assay Wash Buffer to each well, and pipetting up and down two times. A multichannel pipette is desirable for resuspending the beads. Care must be taken not to puncture the membranes of the filter plate.
12. Analyze the assay on the Luminex® xMAP® instrument.
    - Set bead events to 75.
    - Set minimum number of events to 20.
    - Enter the number of samples.
    - Set Sample Size to 65 µl.
    - Set Flow Rate to Fast. Under the Settings Tab, click Bead.
    - Enter the bead region numbers as indicated on the Multiplex Transcription Factor Bead Set.

Product Information Sheet

- Check the probe height to accommodate Multiscreen® MSBVN1250 filter plates.
- Perform one alcohol flush and one sheath fluid wash.
- Insert the plate into the instrument and initiate the analysis.
- Set the gate for the analysis using the Bead Control well.

13. Representative data for each specific Multiplex Transcription Factor Bead Set and data analysis method are provided on the lot-specific **Product Information Sheet**.
14. Upon initial thawing and use in the assay, the Assay Wash Buffer should be stored at 2–8°C. All other reagents included in this kit (Digestion Reagent, Hybridization Buffer, Hybridization Accelerator, and Detection Reagent) should be returned to storage at –20°C after use.

Using this procedure the following Figure was generated with unstimulated and stimulated THP-1 cells.

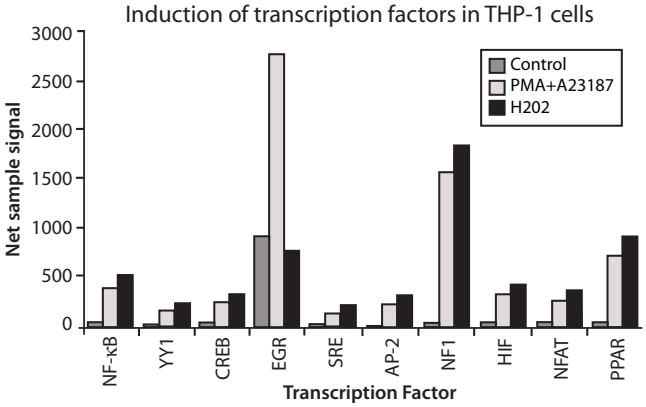


Figure 17— Transcription factor 10-plex panel. THP-1 cells unstimulated (control), or stimulated with PMA+A23187 or hydrogen peroxide, and run on the BioSource™ Transcription Factor Multiplex Kit (Cat. no. LHF1001) f or Luminex®. The data show differential regulation of transcription factors.

Chapter 3—Acquiring and Analyzing the Data

Regardless of the data analysis program selected some basic parameters should be checked during the acquisition phase before proceeding forward with the analysis. It is important that the values for the double discriminator gate are appropriately set and the appropriate bead regions for each analyte assigned. The beads for each analyte should appear as a cloud in the designated region on the classifier plot and the correct number of bead events should observed for each analyte, we recommend 100 events per analyte. For analyzing the data a four– or five–parameter logistic curve fit is recommended. The advantages of choosing the five– versus four–parameter fit are explained in the end of section. In addition, the user can improve the curve fit at the lower concentrations of the standard curve by selecting certain curve weighting functions. Extrapolating unknowns that lie outside the range of the standard curve is not recommended. In addition, unknowns with values that approach the top end of the standard curve should be diluted and re-analyzed. We will discuss four acquisition and analysis programs.

ACS STarStation™ 2.0 Software

The data is initially acquired in the acquisition panel and the results saved to the individual user's Samples Folder. This file can be open either the Analysis window or the Reports window.

Analysis Window

Data displayed in this window include the singlet gate, the bead region maps for each well and analyte, a histogram representing the population of fluorescence intensity observed for each analyte, and the raw mean fluorescence values (MFI). See Figure 18. The Analysis window is unique to STarStation™ Software and allows the end user, depending upon their assigned user level, to manipulate both the singlet gate and the bead region gates (Figure 19).

This function is important for several reasons:

1. Allows the user to re-assign the singlet gate when the wrong singlet gate region was initially assigned
2. Allows the user to re-assign the singlet gate when the beads are shifted outside the assigned singlet gate
3. Allows the user to re-assign the bead regions on the classifier plot when due to an instrument malfunction or bead bleaching the beads fall outside their assigned regions.

After the gates have been adjusted the complete assay or individual wells can be analyzed using the new settings. It is recommended that the altered file be saved seperately so the original data is accessible. In the Analysis window the individual panels can be copied and pasted into other applications to produce reports.

Reports Window

A series of tabs representing a Summary Report (Figure 19), Instrument Status (Figure 20), Plate Layout (Figure 21), and the data for the individual analytes (Figure 22) are displayed in the Results window. Data displayed in the individual analyte tabs (Figure 22) include the standard curve fitted with the selected default regression analysis (a four– or five–parameter fit is recommended), a review grid with the individual well values, and a summary grid containing the median data. Within the Individual Analyte panels the regression fit can be changed and curve weighting assigned to improve the regression fit. A finalized report can be generated in STarStation™ Software or the report file imported into an Excel spreadsheet.

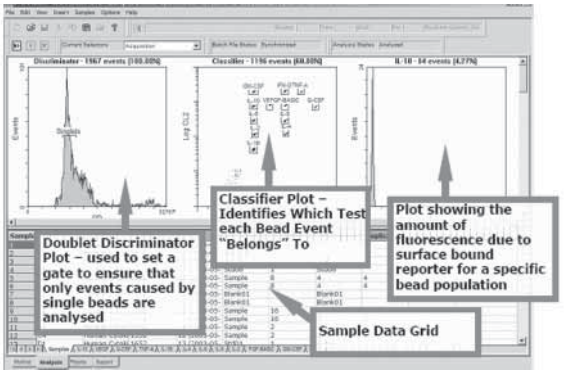


Figure 18—Analysis window.

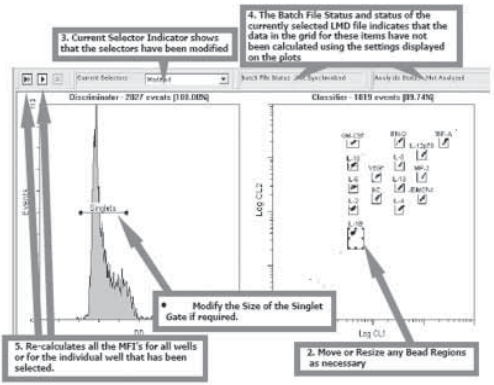


Figure 19—Gate adjustment.

Bio-Plex Manager™ Software

The Bio-Plex Manager™ software is associated with the Bio-Plex® instrument. Two versions of the software are available with or without elevated security, but the following information applies to all versions.

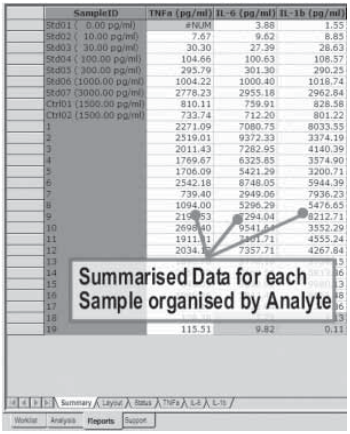
Bio-Plex Manager™ Adjustments for Reading BioSource™ Antibody Bead Assays

1. Creating a new analyte panel: Follow the Bio-Plex Manager™ instructions for setting up a new analyte panel. Briefly:
   a. Under **Protocol Settings** choose 2. Select **Analytes** and click **Add Panel**.
   b. Enter a panel name and click **Add**.
   c. Enter the bead region and name in the appropriate fields then click **Add Continue** panel. Add the next bead region and name and repeat until all the analytes are entered. To finish click **Add** and then click **OK**.
   d. To select all the analytes in the panel click **Add All** or to select a subset of analytes select the desired individual analytes and click **Add**.
2. Calibrating the Instrument: For running the BioSource™ Antibody Bead Kits, we recommend calibrating the Bio-Plex® instrument with the Low RP1 target value (CL2 target, CAL2 calibration microspheres) as stated in the Bio-Plex Manager™ User Guide.
3. Entering Standard Concentrations: Since the standard concentrations of the BioSource™ Antibody Bead Kits are carefully calibrated and thus are intentionally not fixed, the user must enter the concentration for each analyte into the Bio-Plex Manager™ Software.
   a. Uncheck the box that says **All Standards The Same.**
   b. In the Bio-Plex Manager™ Step 4, **Enter Standard Info** click **Standard Info**. For each analyte select **Enter Automatically**.
   c. In the field **Concentration S1,** type the concentration of your highest standard for your first analyte (make sure you selected S1 as the most concentrated standard or else use the reverse).
   d. In the field **Dilution Factor** type 2, then click **Calculate**. The list on the left will show the stan dard concentrations for this analyte.
   e. **Repeat Steps 2 and 3** for each of your analytes.
   f. For PhosphoAssays enter units/ml and for Total-Assays enter ng/ml or pg/ml in the **Concentration Units** field and check the **Same Units For All Analytes** box.

Assessing the data as the plate is being run, or after run completion

1. Data are generated and displayed in real time as a protocol is running. Data are also reviewable after run completion.
2. The data window shows a histogram of the events collected on the left side of the window, a display of the beads collected (and their regions) on the right side of the window, and the individual well results at the bottom of the window (Figure 23).
3. Use the bead region display to assure that all the beads are in the proper gated regions.

A



B

Parameter	Value
Date of Last Classifier Calibration	08/06/2002 12:06:05
Date of Last Reporter Calibration	09/06/2002 12:07:06
Time Taken (CAL1)	43090
Time Taken (CAL2)	57136
Target CL1 Value	3570.00
Target CL2 Value	3805.00
Target DD Value	10000.00
Target RP1 Value	3885.00
CAL1 Lot Number	A3630
CAL2 Lot Number	A3598
Delta Temperature	0.22C
CL1 Voltage	91.23V
CL1 Temperature	26.35C
CL2 Voltage	74.48V
CL2 Temperature	25.48C
DD Voltage	37.42V
DD Temperature	25.91C
RP1 Voltage	674.00V
Model	LX100
Serial Number	LX10000244001

Figures 20—A) Summary Report and B) Instrument Status.

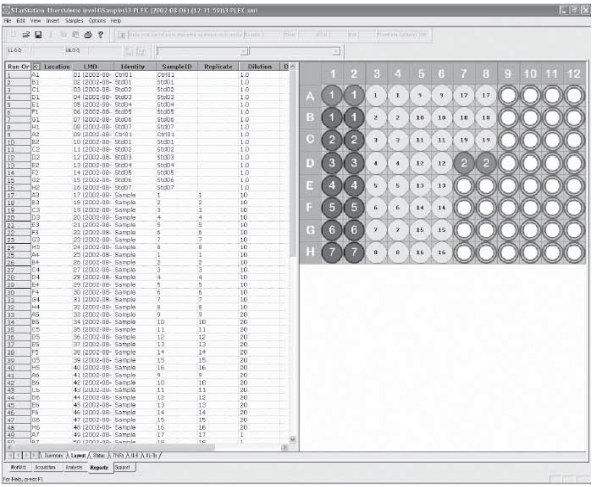


Figure 21—Plate tab.

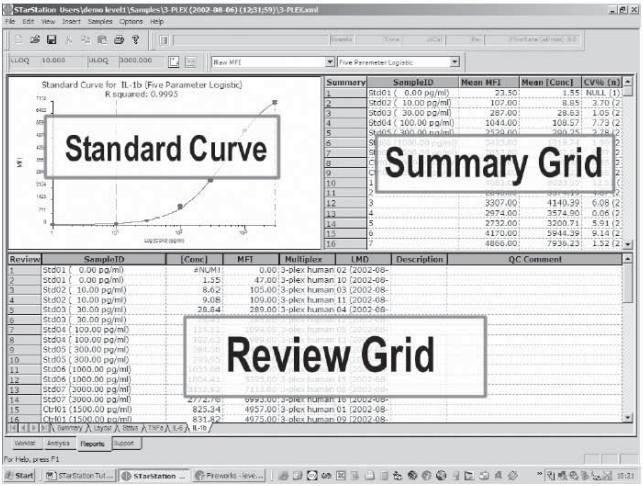


Figure 22—Individual Analyte tab.

4. Use the events histogram to assess singlets from doublets and to adjust (version 4.0 or higher) the gating as needed (based on the histogram and the appearance of beads in step 3 above). This should only be performed after the plate reading has been completed and all data are saved.
5. If any adjustments are made, create a **New Results** file by rerunning the protocol and using **New Results from Protocol.**

Analyzing results

At the conclusion of data acquisition, a results file will be generated and displayed in a new window. Note that a protocol can be modified and the plate rerun a second time to create a new results file. Therefore, it is important to rename your results files when rerunning the plate or the original results file will be overwritten.

Raw Data

This window contains all the information included from the protocol run, but it also includes additional information and functions (Figure 23). Use this button to review the raw data and print or export as needed.

The Report Table

This window (Figure 24) displays an individual analyte's results including any calculations or errors. The Report table can be adjusted to display only the columns of interest to the user and allows for the identification of outliers which can be excluded from the analysis.

a. Click Report Table.
b. Choose the analyte of interest in the drop-down box at the top of the window.
c. Click Report Table Options icon (spreadsheet with a red check mark).

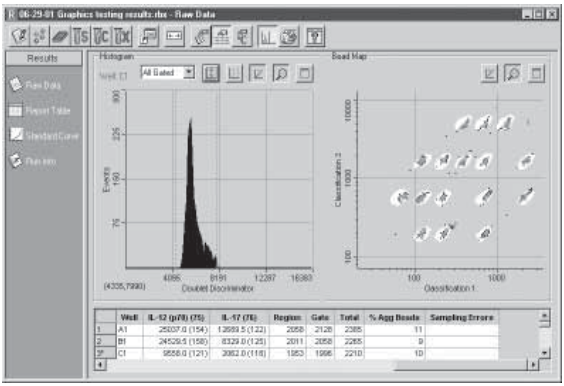


Figure 23—Data window in Bio-Plex™ Manager 3.0.

d. Choose the options of interest. The available options are: Type (mandatory), Well (manda tory), Outlier, Description, FI (fluorescent intensity), FI-bkgd, Std Dev, Std Err, % CV, Obs Conc, Exp Conc, (Obs/Exp)*100, Conc in Range, Group, Ratio, Dilution, and Sampling Errors. Refer to the Bio-Plex Manager™ Operator's Manual for additional descriptions of the available options (Figure 26).
e. Check the appropriate Organize Samples By (as necessary).
f. Check Expand Replicates Info to show results of replicate wells (if desired).
g. Check Exclude table error codes (if desired).
h. Click OK.
i. A Report Table will be displayed based on the options selected.
j. If outliers are selected, review results for eligible outliers and check all relevant outliers to be excluded from analysis.
k. Resave the Report file.
l. Optional, export the report table information. Click on the Export Icon (spreadsheet with green X and an arrow in the lower left corner). Select the relevant options and click OK.
m. Optional, print an individual analyte report or all analytes reports. Click File, Print and choose either a single analyte or all analytes print.

The Standard Curve

This window generates standard curves based on the serial dilutions of the Standards run for each individual analyte. The Bio-Plex Manager™ software allows for several different curve fits based on the number of standard data points available and the desired appearance. The four– or five–parameter logistic curve is recommended.

a. Click **Standard Curve.**
b. Use the drop-down boxes to choose the **Analyte**, **Regresssion Type**, and **Axis Translocation**. The Standard Curve will be refreshed automatically as changes are made (Figure 25).
c. Choose the relevant Label and Error Bars using the drop-down boxes at the top of the window.
d. Optional, print one analyte or all analytes and determine print layout to print multiple curves on a single page.

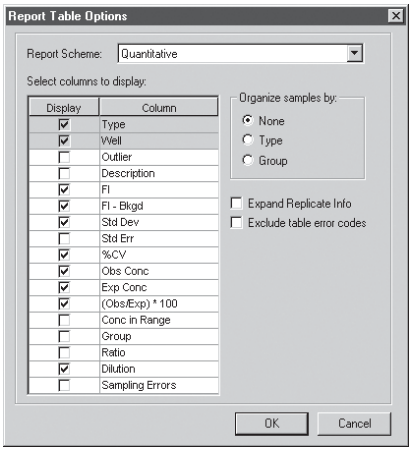


Figure 24—Available options for Report Table display.

Run Info

This window displays the user with all the relevant information about the run including instrument settings used for the run (Figure 26).

MasterPlex® QT 2.0

The Luminex® xMAP® system saves each run results file as a *.csv to a separate directory. The Luminex® default directory is called Output. To begin MasterPlex® QT select File > New Plate. In the Select Data File dialog box, navigate to the appropriate directory containing the *.csv file of interest and open the file. The data will be displayed in the plate view where by using the drop down menu median fluorescence intensity (MFI), bead counts, dilution factors, computed concentrations, and standard values can be displayed.

In the Plate view window, standard, unknown, control, and background wells are assigned and grouped. While in this view enter the appropriate standard concentrations for each of the analytes and any dilution factor for the unknown samples or controls. By default, the first standard curve defined is linked to the background, control, and unknown well groups. If there is more then one standard curve on the plate each curve can be manually linked to its appropriate control and unknown well groups.

A number of model equations are available to create a regression analysis for calculating the concentration of the unknown samples. A four or five parameter curve fit is recommended. In addition, there are weighing functions available to help improve the curve fit.

MasterPlex® QT 2.0 displays MFI, count or concentration data in several formats in the Data Chart window, and a variety of different report types can be generated using the Reports Generator.

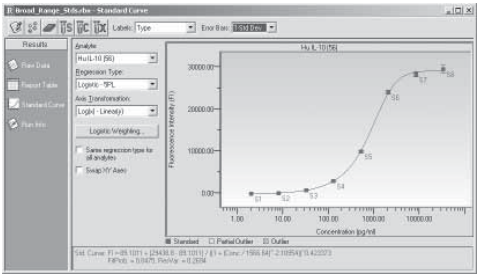


Figure 25—Standard Curve display.

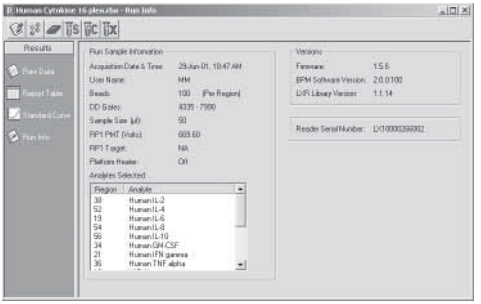


Figure 26—Run Information display.

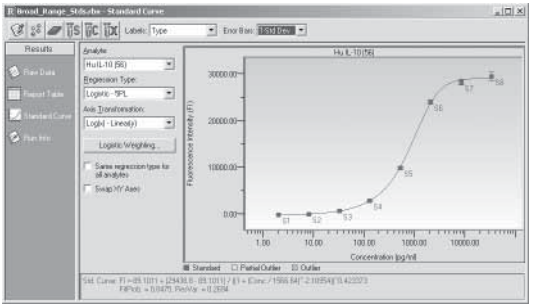


Figure 27—Standard Curve display.

Analysis

Choice of Curve Fit

Selection of a curve-fitting algorithm is an important component of data interpretation. Depending on the software package used, several curve-fitting methods may be available including both linear and non-linear fitting options. More comprehensive description of fitting methods can be found in Bates and Watts, 1988.

The simplest method for quantification of unknowns using a standard curve is the use of a linear regression to model the assay response. Immunoassays typically do not display linear response over a wide range of analyte concentration, and for this reason the use of a linear data model can severely limits an assay's quantifiable range. However, linear regression models are ubiquitous and simple to fit, and have been traditionally used for quantitative ELISA and other immunoassays. Linear models can be fit with reasonable accuracy even with relatively few data points (e.g., 4–6 standard curve points), making them a reasonable choice for assays requiring quantification within a narrow concentration range.

Linear regressions are of the form:

$$y = mx + b$$

Where: m = slope of the line
b = y-axis intercept

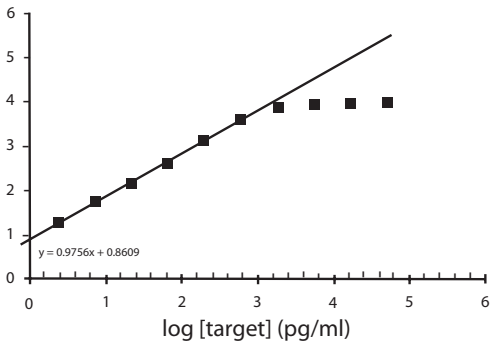


Figure 28—Example of a Linear Fitting model. Results obtained from a multiplex bead-based immunoassay were log-transformed and graphed as a function of analyte concentration (log). The linear region of the assay response is fit using a linear model to generate a standard curve for quantification. Note that the concentration range is limited only to the linear assay response, and does not encompass the entire range of concentration.

This method would be recommended when using fewer than 6 standard curve points and the calibration range of the assay is linear. Data may be log-transformed prior to regression, extending the data that can be modeled using a linear fit (Figure 28).

For greatest utility, Invitrogen recommends a nonlinear curve fitting method to maximize the effective working range of the assay. Nonlinear models typically require more data points for an accurate fit, but are able to provide quantification over a much wider range of analyte concentration. Nonlinear fitting algorithms may use a "weighted-fit" that favors data points with less variance thereby providing a more robust description of assay response. Common nonlinear fitting models include the **four–parameter** (4PL) and **five–parameter logistic** (5PL). Both describe a sigmoidal response when plotted as the log-linear relationship between fluorescence (ordinate) and analyte concentration (abscissa), and while the 4PL model is a symmetric response, the 5PL provides a degree of asymmetry that can compensate for instrumentation and assay effects (Figure 29).

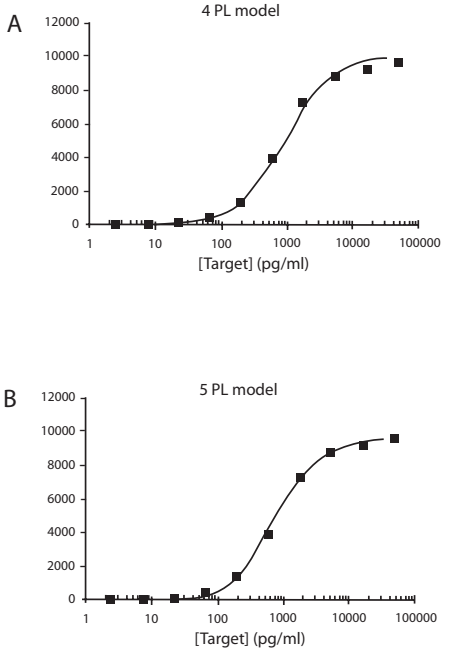


Figure 29—Comparison of 4PL and 5PL fitting models. Identical results from Figure 28 fit to either a 4PL (A) or 5PL (B) model. While both models fit the entire range of data, the 5PL algorithm is better able to describe the assay response, particularly in the higher range of analyte concentration.

Four-parameter logistic (4PL)

Data are fit to a sigmoidal, four-parameter function. 4PL functions are of the form:

$$y = b + \frac{A-B}{[1+(x/c)^D]}$$

Where:

A. estimated response at infinite analyte concentration
B. estimated response at zero analyte concentration
C. concentration at the sigmoidal inflection point
D. slope factor

Select this method to maximize the quantifiable range of the assay and there are 6 or more data points used for the standard curve.

Five-parameter logistic (5PL)

Data are fit to a sigmoidal, five-parameter function. 5PL functions differ from 4PL in that they can accommodate asymmetry of the sigmoidal dose response. The 5PL model is identical to a 4PL model when D=1. 5PL functions are of the form:

$$y = b + \frac{A-B}{[1+(x/c)^D]^E}$$

Where:

A. estimated response at infinite analyte concentration
B. estimated response at zero analyte concentration
C. concentration at the sigmoidal inflection point
D. slope factor
E. asymmetry factor

Select this method to maximize the quantifiable range of the assay when there are 6 or more data points used for the standard curve, and the assay response is asymmetrical over the range of data.

Reference:

Bates, D.M. and Watts, D.G. (1988) Nonlinear Regression Analysis and its Applications. *New York: Wiley*

Troubleshooting Guide

Problem: **During washing steps, the vacuum manifold does not aspirate the liquid from the wells of the filter plate.**

Cause: The filter plate is clogged.

Solution: If the filter plate is clogged, the clog may be dislodged under some circumstances 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. To prevent filter plate clogging, clarify samples by centrifugation at 14,000 x g for 30 seconds prior to analysis. Some samples may also require filtration prior to analysis.

Cause: Lack of a tight seal.

Solution: 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.

Problem: **During loading and incubation steps, the volume of liquid in the wells is observed to increase or decrease.**

Cause: The wells are porous, and are designed to permit flow. If the plate is placed on a wet surface, liquid can potentially enter the wells, altering the volume of liquid in the wells along with the concentrations of assay constituents. If the plate is placed on an absorbent surface, liquid will be lost from the wells due to wicking action.

Solution: Make sure that the plates are placed only a clean, dry, non-absorbent surfaces during loading and incubations. After washing, make sure to blot residual liquid droplets from the bottom of the plate with clean paper towels.

Problem: **During data analysis, beads do not appear in the region gated.**

Cause: Incorrect buffer was used for final step.

Solution: The Wash Solution provided in the kit must be used when washing the beads and for resuspending the beads before loading them into the Luminex® xMap® System instrument. The osmolarity of the solution will impact the size of the bead and any change in bead size will alter detection by the instrument.

Problem: **During data analysis, the beads fall below or to the lower left of their bead region on the bead map.**

Cause: This usually indicates that the beads have been photobleached. This problem can also be caused by exposing the beads to organic solvents.

Solution: Unfortunately, the assay will have to be repeated because the color of the beads cannot be restored. The beads must be protected from light and organic solvents during all steps of the procedure. In the STarStation™ software, bead region can be adjusted to a certain extent and beads regated.

Cause: Instrument out of measurement or calibration issue.

Solution: Call manufacturer for service appointment.

Problem: During data analysis, the beads appear as a diagonal line on the bead map, rather than appearing in the appropriate bead map region.

Cause: A diagonal line on the bead map indicates that the beads have aggregated.

Solution: Unfortunately, the aggregates cannot be broken apart at this stage and therefore the assay will need to be repeated. Make sure to vortex the beads for 30 seconds and then sonicate the beads for at least 30 seconds prior to beginning the assay.

Problem: During data analysis, insufficient and/or erratic bead count is observed.

Cause: Bead aggregation is one potential cause of this problem.

Solution: It is important that the beads be vortexed for 30 seconds and then sonicated for at least 30 seconds prior to beginning the assay to break up the aggregates.

Cause: Bead loss during the assay can also cause this problem. Beads will be lost if the contents of the plate are emptied from the top, rather than from the bottom of the filter plate.

Solution: Make sure to keep the plate in the upright position at all times.

Cause: Beads can also be lost if the filter plate membrane tears.

Solution: 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.

Cause: Care should be exercised with the vacuum manifold, as strong vacuum force can tear the membrane.

Solution: 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.

Cause: Insufficient and/or erratic bead count can also occur if air enters the lines of the Luminex® xMap® System instrument. Air can enter the lines when the volume of the bead suspension is insufficient for the height of the probe of the instrument.

Solution: To avoid this problem, make sure the beads are suspended in 100 µl of solution prior to analysis, as directed in the Assay Procedure. If necessary, probe height may be adjusted to prevent the entry of air into the instrument. Air can also enter the lines when mechanical blockages prevent the flow of liquid through the instrument. One source of this mechanical blockage are the crystals which precipitate from the sheath fluid onto the instrument's probe. To prevent crystal formation on the probe, always make sure the instrument is cleaned and flushed when an assay is completed. A clogged probe may be cleaned by removing it from the instrument and sonicating it for 1 minute using the sonicating water bath required for preparing the beads.

Cause: Another source of mechanical blockage is vacuum formation along the liquid path.

Solution: To relieve vacuum within the lines of the instrument, briefly open the junction of the line with the waste receptacle, then flush the instrument. Additional details on instrument care are provided in the instrument operation manual.

Problem: **During the data analysis, the standard curve Median Fluorescence Intensity is lower than the values presented on the lot-specific Antibody Bead Kit technical data sheet.**

Cause: Depressed standard curves may indicate that one or more reagents in the kit is not performing optimally.

Solution: Make sure that all reagents are at room temperature before use. Check the expiration date of the kit. The Streptavidin-RPE loses activity when stored in the diluted form. It is important that the Streptavidin-RPE be diluted for use just prior to performing the assay. The preparation of the standard dilutions can also be double-checked.

Problem: **In-house controls perform differently in subsequent assays.**

Cause: Incorrect concentration entered in data analysis software.

Solution: The standards included in BioSource'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 lot of standard. Therefore, it is important to check the concentration of the standard listed on the Antibody Bead Kit Information Sheet, and to verify all concentration values entered in data analysis software.

Cause: Improper reconstitution or dilution of the standard

Solution: Check standard reconstitution and dilution.

Recommended Supplies and Suppliers

1. Luminex® xMap® System. Please contact BioSource for instrument and software placement services
2. Filtration manifold for bead washing (e.g., Millipore, Cat. no. MAVM 096 0R; PALL, Cat. no. 5017)
3. Data analysis and graphing software program (e.g., ACS STarStation, MiraiBio MasterPlex®, Luminex® IS, MS Excel, or SoftMax)
4. Sonicating water bath (e.g., Cole-Parmer, Cat. no. 08849-00 or Fisher Scientific, Cat. no. NC9587550)
5. Orbital shaker (e.g., Fisher, Cat. no. 14-271-9)
6. Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips (a manifold multi-channel pipette is desirable)
   a. Brinkmann-Eppendorf Repeator Plus pipetter, Fisher Scientific, Cat. no.21-380-2E
   b. Pipet-Lite LTS 20–200 µl, Rainin, Cat. no. L-200
   c. Pipet-Lite LTS 100–1,000 µl, Rainin, Cat. no. L-1000
   d. Pipet-Lite LTS 2–20 µl, Rainin, Cat. no. L-20
   e. Pipet-Lite LTS 20–300 µl, 8-channel, Rainin, Cat. no. L8300
7. Distilled or deionized water
8. Glass or plastic tubes
9. Beakers
10. Graduated cylinders in various sizes
11. Filter plate covers, BioSource™ Cat. no. PC10
12. Vortex mixer, Fisher Scientific, Cat. no. 02-215-365
13. Vacuum Pressure Pump, Fisher Scientific, Cat. no. 13-875-220

Luminex® References Summary

The references below demonstrate the success our customers achieve when using BioSource™ Multiplex Assays for Luminex® technologies from Invitrogen.

Plasma, Human

P. Szodoray, et al. (2004) Circulating Cytokines in Primary Sjorens Syndrome Determined by a Multiplex Cytokine System. *Scandinavian J Immunol* 59; 592–599. (25-plex)

David H. Chang, et al. (2005) Sustained Expansion of NKT Cells and Antigen-Specific T Cells After Injection of a-Galactosyl-Ceramide Loaded Mature Dendritic Cells in Cancer Patients. *J Exp Med* 201: 1503–1517. (IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 p40, IL-13, IL-15, IL-17, IFN-γ, TNF-α, GM-CSF, MIP-1α, IP-10, MCP-1, Eotaxin)

Ulrike Wille-Reece, et al. (2005) Immunization with HIV-1 Gag Protein Conjugated to a TLR7/8 Agonist Results in the Generation of HIV-1 Gag-Specific Th1 and CD8+ T Cell Responses. *J Immunol* 174: 7676–7683. (IL-6, IL-12p70, IL-10, and TNF-α)

PBMCs, Human

Kinter, Audrey L. et al. (2004) CD25+CD4+ Regulatory T Cells from the Peripheral Blood of Asymptomatic HIV-infected Individuals Regulate CD4+ and CD8+ HIV-specific T Cell Immune Responses *In Vitro* and Are Associated with Favorable Clinical Markers of Disease Status *J Exp Med* 200: 331–343. (IFN-γ, IL-4, IL-10, RANTES, TNF-α)

Serum, Human

Pickering, Alison K. et al. (2004) Cytokine Response to Infection with *Bacillus Anthracis* Spores. *Infect Immun* 72: 6382–6389. (10-plex, IL-12)

Cell Supernatants, Human

Elena Efimova et al. (2003) IG20, a MADD Splice Variant, Increases Cell Susceptibility to γ-Irradiation and Induces Soluble Mediators That Suppress Tumor Cell Growth. *Cancer Res* 63: 8768–8776. (HeLa cells: FGFb, GM-CSF, G-CSF, HGF, IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-1α, MIP-1β, RANTES, TNF-α, VEGF)

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Serum, Mouse

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BAL, Rat

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