



Catalog #LMB0002

*Multispecies*  
**Growth Factor  
Buffer Reagent  
Kit**

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## TABLE OF CONTENTS

Introduction .....	4
Principle of the Method.....	6
Reagents Provided.....	7
Supplies Required But Not Provided .....	8
Safety .....	8
Procedural Notes/Lab Quality Control.....	9
Preparation of Wash Solution.....	10
Directions for Washing .....	11
Preparation of Sample .....	12
Reagent Preparation and Assay Procedure.....	13
Preparation of Standard.....	13
Preparation of Antibody Conjugated Beads.....	16
Assay Procedure.....	17
Preparation of Detector Antibody.....	18
Preparation of Streptavidin-RPE .....	20
Limitations of the Procedure.....	22
Performance Characteristics .....	22
Appendices .....	23
References .....	29
Protocol Summary .....	32

## INTRODUCTION

Advances in the field of cell biology have defined a complex and interdependent set of extracellular and intracellular signaling molecules that control normal cell function. Perturbations in signaling pathways may be important indicators, and possibly the root cause, of many diseases. Therefore, there is growing interest among clinicians as well as drug discovery groups in monitoring multiple components of signaling pathways simultaneously. Solid phase multiplex protein assays have become the primary tools of choice in these studies as they maximize efficiency by permitting several analytes to be profiled within individual samples.

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

This Buffer Reagent Kit contains a set of common reagents that are intended for use with Invitrogen's Multispecies Growth Factor Antibody Bead Kits. This kit should also be used when multiplexing Multispecies Growth Factor Antibody Bead Kits with other Cytokine Antibody Bead Kits. Invitrogen's Multiplex Bead Immunoassay Kits have been developed to maximize flexibility in experimental design, permitting the measurement of one or multiple analytes in panels designed by the researcher.

Invitrogen's Buffer Reagent Kits and Antibody Bead Kits are intended for the *in vitro* quantitative determination of analytes in serum, plasma, and tissue culture medium. Other Buffer Reagent Kits are available from Invitrogen which are specifically formulated for use with Mouse and Rat Cytokine Antibody Bead Kits, Human Extracellular Antibody Bead Kits, Human Transcription Factor Kits, or Human Neuroscience Antibody Bead Kits. For a current listing of available Invitrogen multiplex bead immunoassays and reagents, please visit the Invitrogen web site at [www.invitrogen.com](http://www.invitrogen.com).

**This kit has been configured for research use only and is not to be used in diagnostic procedures.**

**Read entire protocol before use.**

## PRINCIPLE OF THE METHOD

Invitrogen's Multiplex Bead Immunoassays are solid phase sandwich immunoassays, which are designed to be analyzed with a Luminex 100™ instrument. The spectral properties of 100 distinct bead regions can be monitored with the Luminex 100™ instrument, a capability that affords this assay system the potential for measuring up to 100 different analytes in a single sample. 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 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 100™ 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 to 8°C.

<i>Buffer Reagent Kit</i>	<i>100 Test Kit</i>
<i>Wash Solution Concentrate</i> (20x); 15 mL per bottle.	1 bottle
<i>Assay Diluent.</i> Contains 15 mM sodium azide; 15 mL per bottle.	1 bottle
<i>Incubation Buffer.</i> Contains 15 mM sodium azide; 12 mL per bottle.	1 bottle
<i>Biotin Diluent.</i> Contains 3.3 mM thymol; 12 mL per bottle.	1 bottle
<i>Streptavidin-RPE Concentrate</i> (10x). Contains 15 mM sodium azide; 1 mL per vial.	1 vial
<i>Streptavidin-RPE Diluent.</i> Contains 3.3 mM thymol; 12 mL per bottle.	1 bottle
<i>96 well Filter Plate</i> (Extra plates can be purchased from Millipore/Fisher Cat. # MSBVN-1250 or Pall Cat. # S5039.)	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 Multispecies Growth Factor Antibody Bead Kit(s). See [www.invitrogen.com](http://www.invitrogen.com) for kits available from Invitrogen.
2. Luminex xMAP™ system. Please contact Invitrogen for instrument and software placement services.
3. Filtration manifold for bead washing (e.g., Pall, Cat. # 5017 or Millipore, Cat. # MAVM 096 0R).
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. # 08849-00).
6. Orbital shaker (e.g., Fisher, Cat. # 14-271-9).
7. Calibrated, adjustable, precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel 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 (e.g., Invitrogen, Cat. # PC10).

## **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 Invitrogen's Multispecies Growth Factor Antibody Bead Kits and the Luminex 100™ instrument. This assay system allows the design of single analyte or multiplexed assays. See **APPENDIX I** for further information. 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 to 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 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.
6. Do not use reagents after kit expiration date.

7. In-house controls should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
8. 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.
9. When pipetting reagents, maintain a consistent order of addition from well-to-well. This method ensures equal incubation times for all wells.
10. Avoid touching the filter plate membrane with pipette tips to prevent tearing.

### **PREPARATION OF WASH SOLUTION**

Upon storage at 2 to 8°C, a precipitate may form in the 20x *Wash Solution Concentrate*. If this occurs, warm the 20x *Wash Solution Concentrate* to 37°C and 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 to 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 100™ 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 wash solution 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.

## PREPARATION OF SAMPLE

1. Serum, plasma, and tissue culture medium samples have been evaluated with Invitrogen's Multiplex Bead Assays. When possible, avoid the use of hemolyzed or lipemic sera. Suitable sample types are defined on the **INFORMATION SHEET** included in the Antibody Bead Kit(s) used.
2. Samples should be collected in pyrogen/endotoxin-free polypropylene tubes.
3. Samples should be analyzed shortly after collection or frozen. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well (DO NOT VORTEX) prior to analysis.
4. All samples need to be clarified by centrifugation (14,000 x rpm for 10 minutes) and/or filtered prior to analysis to prevent clogging of the filter plates.
5. 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.

## REAGENT PREPARATION AND ASSAY PROCEDURE

This Buffer Reagent Kit is intended for use with Invitrogen's Multispecies Growth Factor Antibody Bead Kits. Prepare components of the bead kits according to instructions below.

Prior to starting the assay, prepare standard and antibody conjugated beads, then proceed to **ASSAY PROCEDURE, Step 1** (page 17).

### 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 Invitrogen. The concentrations of the protein components of the standard are indicated on the **INFORMATION SHEET** included in the Antibody Bead Kit(s) used. 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.

## Reconstitution of Lyophilized Standards

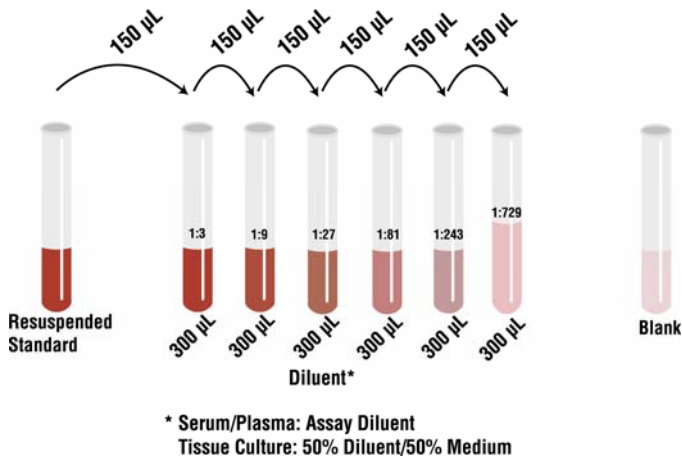
To the standard vial(s) add the suggested reconstitution volume listed below of the appropriate diluent (see above). Do not mix. Replace the vial stopper and allow the vial to stand undisturbed for 10 minutes. Gently swirl and invert the vial 2-3 times to ensure complete reconstitution and allow the vial to sit for an additional 5 minutes.

Protein 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.**

One vial of standard	Reconstitute the standard vial in the suggested reconstitution volume, usually 1 mL, of appropriate diluent (see above).
Two vials of standards	Reconstitute each vial with 0.5 mL of appropriate diluent (see above). Combine 300 $\mu$ L from each vial and mix by pipetting up and down 5-10 times.
Three vials of standards	Reconstitute each vial with 0.333 mL of appropriate diluent (see above). Combine 200 $\mu$ L from each vial and mix by pipetting up and down 5-10 times.
Four vials of standards	Reconstitute each vial with 0.250 mL of appropriate diluent (see above). Combine 150 $\mu$ L from each vial and mix by pipetting up and down 5-10 times.

## Prepare the Standard Curve

The standard curve is made by serially diluting the reconstituted standard in *Assay Diluent* (serum and plasma samples) or a mixture of 50% *Assay Diluent* plus 50% culture medium. See diagram below. DO NOT VORTEX. Mix by pipetting up and down 5-10 times.



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  $\mu$ L 10x beads in 25  $\mu$ L Working Wash Solution per assay well. Each well requires 25  $\mu$ L of the diluted beads. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Beads	Vol. 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



## Assay Procedure

1. Pre-wet the wells designated for the assay. Pipette 0.2 mL of Working Wash Solution into designated wells. Wait 15 to 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  $\mu$ L of the diluted bead solution into each well. Once the beads are added to the plate, keep the plate shielded from light.
4. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 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 filter plate on clean paper towels to remove residual liquid.
5. Pipette 50  $\mu$ L *Incubation Buffer* into each well.
6. To the wells designated for the standard curve, pipette 100  $\mu$ L of appropriate standard dilution.
7. To the wells designated for the sample, pipette 50  $\mu$ L *Assay Diluent* followed by 50  $\mu$ L 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. Ten to fifteen 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** (page 19).

### 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 stock, dilute 10  $\mu$ L 10x *Biotinylated Detector Antibody* in 100  $\mu$ L *Biotin Diluent* per assay well. Each well requires 100  $\mu$ L of the diluted *Biotinylated Detector Antibody*. See table below for examples of volumes to combine.

Number of Wells	Vol. 10x Biotinylated Detector Antibody	Vol. Biotin 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

10. After the 2 hour capture bead incubation, remove the liquid from the wells by aspiration with the vacuum manifold. Add 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
11. Add 100  $\mu$ L of prepared 1x *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. Ten to fifteen 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** (page 21).

### Preparation of Streptavidin-RPE

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

Number of Wells	Vol. 10x Streptavidin-RPE	Vol. Streptavidin-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 0.2 mL Working Wash Solution to the wells. Allow the beads to soak for 15 to 30 seconds, then aspirate with the vacuum manifold. Repeat this washing step. Blot the bottom of the filter plate on clean paper towels to remove residual liquid.
14. Add 100  $\mu$ L of prepared 1x *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 100™ instrument during this incubation step. Please see **APPENDIX II Section A** for Luminex 100™ and **Section B** for Bio-Plex instrument setup.
16. Remove the liquid from the wells by aspiration with the vacuum manifold. Wash the beads by adding 0.2 mL 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 filter plate on clean paper towels to remove residual liquid.
17. Add 100  $\mu$ L 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 to 8°C for reading the following day without significant loss of fluorescent intensity. Aspirate Working Wash Solution from stored plates and add 100  $\mu$ L 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 100™ instrument, and analyze the samples.

19. Determine the concentration of samples from the standard curve using curve fitting software. The four 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.

## LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the highest standard point; the dose-response is non-linear in this region and inaccurate. Dilute samples that are greater than the highest standard with *Assay Diluent* or appropriate matrix diluent; reanalyze these samples and multiply results by the appropriate dilution factor. The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum/plasma and tissue culture medium samples have not been thoroughly investigated. The rate of degradation of analytes in various matrices may not have been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

**This kit is for research use only.**

**Not for human therapeutic or diagnostic use.**

## PERFORMANCE CHARACTERISTICS

Please refer to analyte specific **INFORMATION SHEET** for performance claims.

## APPENDIX I:

### MULTIPLEXING 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.

### MULTIPLEXING MULTISPECIES GROWTH FACTORS WITH OTHER ANALYTES

For multispecies growth factor kits (Cat. # LHC7071, LHG0041, LMC2031, LMG0041) use the reagent buffers supplied with Multispecies Growth Factor Buffer Reagent Kit LMB0002 for all dilutions. For the pre-mixed growth factor 4-Plex (Cat. # LMC0004) use the reagent buffers supplied with the kit for all dilutions.

### CAPTURE BEAD PREPARATION

**Volume from each vial of 10x Bead Concentrate to combine:**

$$\frac{0.025 \text{ mL} \times \text{Assay Wells}}{10} = \text{___ mL per vial}$$

**Volume Working Wash Solution:**

( 0.0275 mL x \_\_\_ Assay Wells ) –

( \_\_\_ mL 10x Capture Bead per vial x \_\_\_ vials ) = \_\_\_ mL

**Final Volume of diluted multiplexed Capture Beads:**

( \_\_\_\_ mL Working Wash Solution ) +

( \_\_\_\_ mL 10x Capture Bead per vial x \_\_\_\_ vials ) = \_\_\_\_ mL

**Example Calculation:** Combining 5 vials of 10x Capture Bead Concentrate for 48 assay wells.

**Volume from each vial of each 10x Capture Bead Concentrate to combine:**

$$\frac{0.025 \text{ mL} \times 48 \text{ Assay Wells}}{10} = \underline{0.120} \text{ mL per vial}$$

**Volume Working Wash Solution:**

( 0.0275 mL x 48 Assay Wells ) –

( 0.120 mL 10x Capture Beads per vial x 5 vials ) = 0.720 mL

**Final Volume of diluted multiplex Capture Beads:**

( 0.720 mL Working Wash Solution ) +

( 0.120 mL 10x Capture Beads per vial x 5 vials ) = 1.32 mL

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



## BIOTINYLATED ANTIBODY PREPARATION

**Volume from each vial of each 10x Biotinylated Antibody to combine:**

$$\frac{0.100 \text{ mL} \times \text{Assay Wells}}{10} = \text{___ mL per vial}$$

**Volume Biotin Diluent:**

( 0.110 mL x \_\_\_ Assay Wells ) –

( \_\_\_ mL 10x Biotinylated Ab. per vial x \_\_\_ vials ) = \_\_\_ mL

**Final Volume diluted Biotinylated Antibody:**

( \_\_\_ mL Biotin Diluent ) +

( \_\_\_ mL 10x Biotinylated Ab. per vial x \_\_\_ 10x vials ) = \_\_\_ mL

**Example Calculation:** Combining 5 vials of 10x Biotinylated Antibody Concentrate for 48 assay wells.

**Volume of each 10x Biotinylated Antibody to combine:**

$$\frac{0.100 \text{ mL} \times 48 \text{ Assay Wells}}{10} = \underline{0.480} \text{ mL per vial}$$

### Volume Biotin Diluent:

( 0.110 mL x 48 Assay Wells ) –

( 0.480 mL 10x Biotinylated Ab. per vial x 5 vials ) = 2.88 mL

### Final Volume diluted multiplexed Biotinylated Antibody:

( 2.88 mL Biotin Diluent ) +

( 0.480 mL 10x Biotinylated Ab. per vial x 5 vials ) = 5.28 mL

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

## APPENDIX II:

### INSTRUMENT SETUP

#### A. HELPFUL GUIDES FOR LUMINEX 100™ USERS

1. Assign the appropriate **Bead Region** (refer to the kit-specific technical data sheet) to each analyte.
2. We recommend that the user count **100 events/bead region**.
3. Set **Minimum Events** to 0.
4. Set the **Sample Size** to 50 µL.
5. Set **Flow Rate** to 60 µL/minute.
6. For Invitrogen kits we recommend an initial **Double Discriminator (DD)** gate setting of 7800-15200. (This setting may vary among instruments and must be determined by the user.)
7. Collect **Median RFU**.

## **B. BIO-PLEX MANAGER ADJUSTMENTS FOR READING Invitrogen 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 on the **Add Panel** tab.
  - b. Enter a panel name and click the **Add** tab.
  - c. Enter the bead region and name in the appropriate fields then click on the **Add Continue** panel. Add the next bead region and name and repeat until all the analytes are entered. To finish click on the **Add** tab and then click the **OK** tab.
  - d. To select all the analytes in the panel click the **Add All** tab or to select a subset of analytes select the desired individual analytes and click the **Add** tab.
2. **Calibrating the Instrument:** For running the Invitrogen Antibody Bead Kits, we recommend to calibrate 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 Invitrogen Antibody Bead Kits are intentionally not fixed, the user has to enter the concentration for each analyte into the Bio-Plex Manager.
  - a. Uncheck the box that says **All standards the same**.
  - b. In the Bio-Plex Manager Step 4, **Enter Standard Info** click on the **Standard Info** tab. 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 3., then click the **Calculate** button. The list on the left will show the standard concentrations for this analyte.
  - e. Repeat Steps c and d for each of your analytes.
  - f. Type in pg/mL in the **Concentration Units** field and check the **Same Units For All Analytes** box.
4. **Setting the DD Gates:** In the Advanced Settings in Step 7 **Run Protocol**, you can choose the Doublet Discriminator (DD) gates. Check the box **Override DD Gates** and change the box labeled High to 10,000. The Low box is fine as the default.

## REFERENCES

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# Protocol Summary

Pre-wet plate  
Add: 25  $\mu$ L 1x Antibody Coated Beads  
200  $\mu$ L Wash Solution

↓ wash 1x 200  $\mu$ L

*Sample type:* **Standard**

Add 50  $\mu$ L  
Incubation Buffer  
Add 100  $\mu$ L  
Standard



**Serum/Plasma/Tissue Culture Supernatant**

Add 50  $\mu$ L  
Incubation Buffer  
Add 50  $\mu$ L Assay Diluent  
Add 50  $\mu$ L  
Sample



↓ shake for 2 hours at RT in the dark  
wash 2x 200  $\mu$ L



Add 100  $\mu$ L 1x Biotinylated Detector Antibody

↓ shake for 1 hour at RT in the dark  
wash 2x 200  $\mu$ L



Add 100  $\mu$ L 1x SAV-RPE

↓ shake for 30 min. at RT in the dark  
wash 3x 200  $\mu$ L

Add 100  $\mu$ L Wash Buffer

↓ shake for 2-3 min.



Read in xMap™ System

Total time: 3.5 hours

Protein Bead Biotinylated  
Detector Antibody SAV-RPE