# ELISA Development Guide

## A guide for the development of microplate and Luminex ELISA assays using OriGene's ELISA and Luminex-validated antibodies



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# ELISA Plate Coating

## Materials

| Description | Recommended Source |
|---|---|
| ELISA Capture Antibody | OriGene Technologies, Inc. |
| Coating Buffer | Prepared by user. See below and Buffer and Reagent Preparation. |
| ELISA Plate | Corning Costar #2592 (or equivalent) |
| Plate Cover | Corning Costar #3930 (or equivalent) |
| Post-Coat/Blocking Buffer | Prepared by user. See below and Buffer and Reagent Preparation. |
| 5.5" x 7.0" Ziplock Mylar Bag | Sorbent Systems #55MFS07OZE or 6-mil Ziplock™ bag, VWR # 82027-710 |
| Indicating Dessicant | Sigma Cat. No. Z163562-100EA |
| Absorbent Paper Towel | WYPALL X60 towels from Kimberly Clark |

Coating Buffer

Each capture antibody has been tested with three different coating buffers. The optimal coating buffer for each antibody is listed on the antibody data sheet. The three coating buffers tested are listed below. Formulations and instructions for preparing them can be found in the last section of this document, or by following the hyperlinks.

100 mM Carbonate, pH 9.5
100 mM Borate, pH 8.5
100 mM Phosphate, pH 7.5

ELISA Plates

OriGene ELISA-validated capture antibodies are tested on Corning-Costar High-Binding stripwell plates, #2592. Other Corning-Costar high-binding plates should give similar results. Similar plates from Nunc and Greiner may also give acceptable results, but should be tested for each application. Each ELISA assay will need to be optimized for a specific type and vendor of microplate.

Post-Coat/Blocking Buffer

After antibody coating, plates are coated with a buffer that blocks the plate from further binding and preserves the coated antibody. For some applications, it may be beneficial to add additional blocking proteins to this buffer such as BSA, animal IgG, gelatin, casein, or non-fat dry milk. To prepare, see: Post-Coat/Blocking Buffer.

Storage of antibody-coated microplates

Although we have not done extensive stability studies, antibody-coated plates are usually quite stable when stored dessicated in sealed mylar bags at 2-8ºC. If Mylar bags are not available, a 6-mil polyethylene Ziplock™ bag can also be used, but the desiccant will not last as long and will need to be replaced periodically.

## Antibody Plate Coating Procedure

1. Immediately before use, dilute antibody to recommended dilution in Coating Buffer. Antibody coating concentrations of 2-8 μg/ml are typical, but may vary significantly for some antibodies. Do not store diluted antibody.
2. Add 70-100 μl diluted antibody to each well.
3. Cover and incubate plates overnight at room temperature. Shaking is not usually necessary.
4. Aspirate liquid from wells so that no more than 5 μl of diluted antibody remains.
5. If more than 5 μl of diluted antibody remains, then tap plates sharply on absorbent paper towel to remove remaining liquid.
6. Add 250-300 μl Post-Coat solution to each well.
7. Cover and incubate plates for 3-4 hours (or overnight at 4ºC).
8. Aspirate liquid from wells. If more than 5 μl of Post Coat solution remains in the wells, then tap plates sharply on absorbent paper towel to remove remaining liquid.
9. Plates are then ready for immediate or same-day use. For later use, plates should be dried for 3-24 hours at room temperature and then stored in a sealed Mylar bag containing a pack of indicating desiccant. Antibody-coated plates can generally be stored desiccated at 2-8ºC for up to 6 months.

# Basic ELISA Protocol

## Materials

| Description | Recommended Source |
|---|---|
| Antibody coated microplate | See previous section. |
| Assay Buffer<br><br>For dilution of detection antibody, secondary antibodies, and streptavidin conjugates. | OriGene has four different Assay Buffers, each containing a different optimized mixture of buffer, salts, detergent and proteins. We recommend starting with Assay Buffer D. Different buffers may perform better than others depending upon the species origin of the capture and detection antibodies. |
| Assay Buffer A - contains mouse and goat IgG — Part No.: AR100012<br>Assay Buffer B- contains BSA — Part No.: AR100013<br>Assay Buffer C- contains BSA and rabbit IgG — Part No.: AR100014<br>Assay Buffer D- contains BSA, mouse and goat IgG — Part No.: AR100015 | |
| Assay Diluent<br><br>Serum-based diluent for dilution of standards and samples. | OriGene, Part No.: AR100016<br><br>Contains heat-activated animal serum, detergent and a preservative. |
| Biotinylated Detection Antibody | OriGene biotinylated detection antibodies are supplied at 0.5 mg/ml. |
| ELISA Wash Buffer (10X) | OriGene Part No.: AR100010 (100 ml), AR100011 (1L) |
| Streptavidin-HRP Conjugate | OriGene Part No.: AR100017 |
| TMB Substrate, soluble, 1-part | OriGene Part No.: AR100018 (100 ml), AR100019 (1L) |
| Stop Solution | OriGene Part No.: AR100020 (100 ml), AR100021 (1L) |
| Absorbent paper towels | WYPALL X60 towels Kimberly Clark |

## Recommended Plate Layout

Before starting, it is recommended that a plate plan be designed. Such a plan will assist in assay workflow and data analysis. A suggested plate plan is shown below. A plate plan template is provided on page 41 (link here).

| | 1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Std 1 | Std 1 | Std 1 | | | | | | | | |
| **B** | Std2 | Std2 | Std2 | | | | | | | | |
| **C** | Std 3 | Std 3 | Std 3 | | | | | | | | |
| **D** | Std 4 | Std 4 | Std 4 | | | | | | | | |
| **E** | Std 5 | Std 5 | Std 5 | | | | | | | | |
| **F** | Std 6 | Std 6 | Std 6 | | | | | | | | |
| **G** | Std 7 | Std 7 | Std 7 | | | | | | | | |
| **H** | Neg | Neg | Neg | | | | | | | | |

Sample Wells

Std 1 is the highest concentration of the protein standard and Std 7 is the lowest concentration of the protein standard. The Negative wells contain the buffer or mixture that is used to dilute the standards. Running all standards, samples, and controls in duplicate or triplicate is recommended.

## Preparation of Materials and Buffers

1. Prepare Wash Buffer by diluting 10X Wash Buffer to 1X with deionized water. Store diluted wash buffer at 2-8ºC for up to one month.

2. For accurate quantitation, it is important to prepare protein standards in a matrix that mimics the composition of the samples. For example, if the samples are cell culture supernatants, then the protein standards should be prepared in the cell culture medium. For serum or plasma samples, dilute protein standards in OriGene Assay Diluent (AR100016) or similar serum- or plasma-based matrix.

3. The performance of an ELISA assay can vary dramatically depending upon the composition of the samples, particularly with serum, plasma and other samples of clinical origin. Samples of clinical origin may contain substances that inhibit the assay causing falsely low readings. Dilution of samples with an appropriate matrix may reduce some or all of the inhibition or interference.

Example:

a. Protein stock concentration is 385 µg/ml.
b. Samples to be tested are serum samples
c. Sample volume per well is 50 µl.
d. Total volume added per well is 100 µl.

➡ To prepare a 1 ug/ml stock, add 1.3 µl of protein stock to 498.7 µl of Assay Buffer. To prepare Standard 1 at 100 ng/ml, add 100 µl of the 1 µg/ml stock to 900 µl of Assay Diluent.

| To Make | Add | To | Conc. (ng/ml) |
|---|---|---|---|
| Standard 2 | 200 µl of Standard 1 | 400 µl of Assay Diluent | 33.33 |
| Standard 3 | 200 µl of Standard 2 | 400 µl of Assay Diluent | 11.11 |
| Standard 4 | 200 µl of Standard 3 | 400 µl of Assay Diluent | 3.70 |
| Standard 5 | 200 µl of Standard 4 | 400 µl of Assay Diluent | 1.23 |
| Standard 6 | 200 µl of Standard 5 | 400 µl of Assay Diluent | 0.41 |
| Standard 7 | 200 µl of Standard 6 | 400 µl of Assay Diluent | 0.14 |

## Detailed ELISA Assay Protocol

1. Dilute samples into Assay Diluent or an appropriate matrix so that the signals will fall within the linear range of the assay.
2. Add 100 µl prepared Standard to designated standard wells.
3. Add 100 µl Assay Diluent to Negative wells.
4. Add 100 µl diluted sample to designated sample wells.
5. Cover and incubate the plate for 1-2 hours at room temperature on an orbital plate shaker (500-700 rpm).
6. Ten to fifteen minutes before the end of this incubation, prepare working stock of Detection Antibody. Biotinylated detection antibody from OriGene is typically provided at 0.5 mg/ml. Check antibody data sheet for recommended working concentration. Typical working concentrations for colorimetric assays range from 0.5 to 2.0 µg/ml. Lower working concentrations may be suitable for chemiluminescent assays.

   Example:

   a. Biotinylated detection antibody stock is 0.5 mg/ml.
   b. Working biotinylated detection antibody concentration is 1 µg/ml.
   c. Prepare 100 µl per well plus 10% extra.

d. Assay uses 48 wells.
e. Volume Assay Diluent required = 48*100*1.1 = 5280 µl
f. Volume detection antibody required = 5280 * 1/500 = 10.56 µl

➡ Add 10.56 µl biotinylated detection antibody to 5280 µl of Assay Buffer.

7. Aspirate solution from wells.
8. Wash wells 3-5 times with 200-300 µl of 1X Wash Buffer. If using an automated plate washer, fill wells completely before aspiration. After final wash, tap plate thoroughly on absorbent paper to remove all traces of wash buffer from the wells.
9. Add 100 µl working stock of Detection Antibody to each well.
10. Cover and incubate the plate for 1 hour at room temperature on an orbital plate shaker at 500-700 rpm.
11. Ten to fifteen minutes before the end of this incubation, prepare the working solution of streptavidin-HRP (SA-HRP). A typical working concentration range for colorimetric assays is from 0.1 to 2.0 µg/ml. Lower working concentrations may be suitable for chemiluminescent assays.

    Example:
    a. Streptavidin HRP stock is 1.0 mg/ml.
    b. Working streptavidin-HRP concentration is 0.5 µg/ml.
    c. Prepare 100 µl per well plus 10% extra.
    d. Assay uses 48 wells.
    e. Volume Assay Diluent required = 48*100*1.1 = 5280 µl
    f. Volume SA-HRP stock required = (0.5/1000) * 5280 = 2.64 µl

    ➡ Add 2.64 µl of streptavidin-HRP to 5280 µl of Assay Diluent.

12. Wash wells 3-5 times with 200-300 µl of 1X Wash Buffer. If using an automated plate washer, fill wells completely before aspiration. After final wash, tap plate thoroughly on absorbent paper to remove all traces of wash buffer from the wells.
13. Add 100 µl of diluted streptavidin-HRP to each well.
14. Incubate plate at room temperature for 30 minutes on an orbital plate shaker at 500-700 rpm.
15. Wash wells five times with 200-300 µl of 1X Wash Buffer. If using an automated plate washer, fill wells completely before aspiration.
16. After final wash, tap plate sharply on clean absorbent paper to remove all traces of wash buffer from the wells.
17. Add 100 µl soluble TMB substrate to each well.
18. Cover the plate and incubate plate at room temperature for 15-30 minutes.

19. Add 100 µl of Stop Solution to each well. Incubate for 1-2 minutes.
20. Read the absorbance at 450 nm.

## Data Analysis

1. Use commercial data analysis software to analyze the data as analysis of ELISA data with Excel can be quite difficult. Many plate readers are sold with a software package that can be used to perform the analysis. Stand-alone analysis software is also available. ReaderFit from MiraiBio (Hitachi), Prism from GraphPad, and SigmaPlot from Systat are all suitable. A free (but limited) web-based analysis package is available at www.readerfit.com.
2. Generate a standard curve by fitting the results of the protein standard data to a model equation.
3. For microplate ELISA data, generally a 4-parameter logistic (4-PL) model gives a good fit. For some assays, a 5-parameter logistic (5-PL) model may give a better fit than the 4-PL model.
4. To account for greater variance at the low end of the curve, it is recommended to consider $1/Y$ or $1/Y^2$ weighting in conjunction with the 4-PL or 5-PL models.
5. Use the standard curve to calculate the concentration of the analytes in the samples (unknowns). Be sure to adjust for any dilution of the samples relative to the standards.
6. The following on-line articles provide additional in-depth discussion and analysis of the various factors and parameters to consider for ELISA data analysis.

    - Tips for ELISA Data Analysis
    - The 4 Parameter Logistic Regression Model
    - The 5 Parameter Logistic Regression Model
    - The Importance of Weighting with 4PL and 5PL Regression Models

## ELISA Protocol Summary

1. Prepare standards and samples.
2. Add 100 µl standards and samples to designated wells of capture plate.
3. Cover and incubate the capture plate for 1-2 hours at room temperature on an orbital plate shaker (500-700 rpm). Prepare detection antibody.
4. Wash wells 3-5 times with 200-300 µl of 1X Wash Buffer.
5. Add 100 µl Detection Antibody to each well.
6. Cover and incubate the plate for 1 hour at room temperature on an orbital plate shaker at 500-700 rpm. Prepare streptavidin-HRP solution at end of incubation.
7. Wash wells 3-5 times with 200-300 µl of 1X Wash Buffer.
8. Add 100 µl of diluted streptavidin-HRP to each well.
9. Incubate plate at room temperature for 30 minutes on an orbital plate shaker at 500-700 rpm.
10. Wash wells five times with 200-300 µl of 1X Wash Buffer.
11. Add 100 µl soluble TMB substrate to each well.
12. Incubate plate at room temperature for 15-30 minutes.
13. Add 100 µl of Stop Solution to each well.
14. Read absorbance at 450 nm.

## ELISA Assay Optimization

Assays can be optimized for many different purposes such as maximum sensitivity, widest dynamic range, maximum specificity, improved linearity or recovery, or shortest time to results. Some common assay parameters that can be modified to achieve an optimal assay are listed in the table below.

| Parameter | Modification | Possible Effects |
|---|---|---|
| Antibody Coating Concentration | Increased | Higher signal<br>Higher background<br>Lower limit of detection<br>Faster capture |
| Antibody Coating Concentration | Decreased | Lower signal<br>Lower background<br>Wider dynamic range<br>Longer capture time required |
| Post-Coat/Blocking Buffer | Addition of a protein such as BSA, IgG, gelatin or casein at a concentration of 0.1 – 10 mg/ml<br><br>Alternate Buffer and salt concentration<br><br>Alternate detergent and/or detergent concentration. | Lower background<br>Lower limit of detection<br>Improved recovery<br>Improved stability |
| Plate Type (Binding) | High-binding vs Low-binding | Difficult to predict but may significantly affect assay performance |
| Plate Type (Color) | Use of White or Black plates instead of clear with fluorescent or chemiluminescent detection | Higher signal<br>Higher sensitivity<br>Wider dynamic range |
| Plate Vendor | Use ELISA plates from different vendors | Difficult to predict. Plates from different vendors may give different results under identical assay conditions. |
| Sample and Standard Diluent | Samples and standards can be diluted into a different assay matrix. | Increased recovery<br>Increased linearity<br>Increased sensitivity |
| Standard Concentrations | Standard can be diluted over different ranges | Wider dynamic range<br>Different dynamic range |

| Parameter | Modification | Possible Effects |
|---|---|---|
| Sample concentrations | Samples can be used undiluted or can be diluted to different concentrations in an assay matrix | Increased recovery<br>Optimal linearity<br>Increased sensitivity |
| Composition of Assay Matrix | Addition of blocking agents such as proteins, polymers, or additional detergents<br><br>Increase or decrease the salt (NaCl) concentration in the buffers<br><br>Increase or decrease in the pH of the buffer<br><br>Alternate buffers: Tris, borate, MES | Increased recovery<br><br>Optimal linearity<br><br>Increased sensitivity<br><br>Reduced sample interference |
| Capture time | Increase or decrease | Increased or decreased sensitivity.<br>Wider dynamic range |
| Number of Washes: Post-Capture | Increase or decrease | Increased or decreased signal and/or background.<br><br>Increased or decreased assay time. |
| Composition of Wash Buffer | Increased or decreased salt concentration<br><br>Increased or decreased pH<br><br>Increased or decreased detergent concentration<br><br>Different buffer (Tris, etc.)<br><br>Different detergent | Increased or decreased signal and/or background.<br><br>Increased sensitivity<br><br>Alternate dynamic range<br><br>Reduced sample interference<br><br>Improved recovery |
| Biotinylated detection antibody concentration | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Wider or smaller dynamic range |

| Parameter | Modification | Possible Effects |
|---|---|---|
| Composition of the biotinylated detection antibody diluent | Addition of blocking agents such as proteins, polymers, or additional detergents.<br><br>Increase or decrease in the salt (NaCl) concentration<br><br>Increase or decrease in the buffer pH<br><br>Alternate buffers: Tris, borate, MES<br><br>Alternate buffer concentrations | Increased recovery<br><br>Increased linearity<br><br>Increased sensitivity<br><br>Reduced sample interference |
| Detection antibody incubation time | Increase or decrease. | Increased or decreased signal<br>Increased or decreased sensitivity<br>Altered dynamic range |
| Composition of the streptavidin-HRP conjugate diluent | Addition of blocking agents such as proteins, polymers, or additional detergents<br><br>Increase or decrease in the salt (NaCl) concentration.<br><br>Increase or decrease in the buffer pH<br><br>Alternate buffers: Tris, borate, MES<br><br>Alternate buffer concentrations | Increased or decreased signal<br><br>Increased or decreased sensitivity<br><br>Increased or decreased dynamic range<br><br>Increased or decreased detection time |
| Streptavidin-HRP conjugate concentration | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Alternate dynamic range |
| Streptavidin-HRP incubation time | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity |
| Number of washes after Streptavidin-HRP incubation | Increase or decrease | Increased or decreased signal and/or background. |

# ELISA Assay Troubleshooting

| Problem | Possible Causes | Solutions |
|---|---|---|
| **High Background** | Insufficient washing | Increase number of washes.<br>Add 1-5 minute soak step between washes.<br>Increase the volume of wash buffer per well.<br>If using a plate washer, check to ensure that all ports are clean and that the target volume is delivered to all wells. Check that the residual volume after aspiration is less than 5 µl per well. |
| | Detection antibody concentration is too high | Check dilution. Titrate to find the optimal concentration.<br><br>Use a medium-binding plate from a reliable source (Costar, Nunc, Greiner). |
| | Streptavidin-HRP conjugate concentration is too high | Check dilution. Titrate to find the optimal concentration. |
| | Non-specific interactions | Add additional protein to assay buffers and wash buffer. |
| | Contaminated buffers | Remake buffers. |
| | | |
| **No Signal or Low Signal** | Non-binding (eg, tissue culture) microplate used for antibody coating | Use a high-binding plate from a reliable source (Costar, Nunc, Greiner) |
| | Diluted capture antibody stored in coating buffer for extended period before coating. | Dilute antibody into coating buffer immediately before use. Do not store diluted capture antibody. |
| | Reagents omitted or added in incorrect order | Check calculations and protocol. Re-make solutions. Repeat assay. |
| | Incorrect standard was used or standard dilutions were made incorrectly | Verify that the correct standard was used. Check calculations for standard dilutions. Re-dilute standards. |
| | Inhibition of HRP by azide | Make sure that the SA-HRP dilution buffer or final wash buffer does not contain azide. |
| | Concentration of Detection Antibody or Streptavidin-HRP is too low. | Check dilution. Titrate to find the optimal concentration. |

| Problem | Possible Causes | Solutions |
|---|---|---|
| **No Signal or Low Signal- continued** | Capture antibody coated at too low a concentration | Titrate coating antibody concentration to determine optimal coating level. |
| | Incorrect wavelength used in plate reader | Read plate at 450 nm for yellow color (with stop solution).<br>Read plate at 650 nm for blue color (without stop solution). |
| | Insufficient color development time | Increase color development time until background is visible in the Negative Standard. |
| **Too much signal – plate is uniformly blue or yellow.** | Final washing step skipped or insufficient – unbound HRP remains in the wells | Wash plates thoroughly according to the protocol. Increase the number of washes. Increase the volume of wash buffer used per well. |
| | Substrate contaminated | Use a fresh bottle of substrate.<br>Pour required amount of substrate into a new reagent reservoir. Do not return unused substrate to the bottle. |
| | Detection Antibody Concentration is too high | Check dilution. Titrate to find the optimal concentration. |
| | Streptavidin-HRP Conjugate Concentration is too high. | Check dilution. Titrate to find the optimal concentration. |
| | Plate sealers or reagent reservoirs contaminated by residual buffer or reagents | Use a fresh plate sealer and reagent reservoir for each step. |
| | Insufficient washing | Increase number of washes.<br>Add 1-5 minute soak steps between washes.<br>Increase the volume of wash buffer per well.<br><br>If using a plate washer, check to ensure that all ports are clean and that the target volume is delivered to all wells. Check that the residual volume after aspiration is less than 5 µl per well. |
| | Pipettors or plate washer are contaminated | Clean pipettors and plate washer thoroughly. Test for residual HRP activity. |

| Problem | Possible Causes | Solutions |
|---|---|---|
| **Poor reproducibility of duplicates** | Uneven concentration of antibody across the plate | Check coating and blocking volumes.<br>Check dispensing equipment.<br>Incubate plate in a uniform environment.<br>Use a high-binding microplate from a different vendor.<br>Increase coating concentration.<br>Extend blocking time to overnight. |
| | Insufficient washing | Increase number of washes.<br>Add a 1-5 minute soak steps with each wash.<br>Increase the volume of wash buffer per well.<br><br>If using a plate washer, check to ensure that all ports are clean and that the target volume is delivered to all wells. Check that the residual volume after aspiration is less than 5 µl per well. |
| | Buffers contaminated | Make fresh buffers.<br>Use new reagent reservoirs for each reagent.<br>If using a plate washer, clean, decontaminate, and use fresh wash buffer. |
| | Well-to-well carryover (from standards or positive samples) | Adjust shaker speed to minimize carryover.<br><br>Completely seal wells with a plate sealer between steps or use a plate cover with individual rings that separate wells.<br><br>Use a fresh plate sealer for each incubation. |
| Poor assay-to-assay reproductibility | Insufficient or inconsistent washing | Increase number of washes.<br>Add a 1-5 minute soak steps with each wash.<br>Increase the volume of wash buffer per well.<br><br>If using a plate washer, check to ensure that all ports are clean and that the target volume is delivered to all wells. Check that the residual volume after aspiration is less than 5 µl per well. |

| Problem | Possible Causes | Solutions |
|---|---|---|
| Poor assay-to-assay reproductibility-continued | Insufficient or inconsistent washing | For manual washing, decanting technique may affect assay results. Develop a consistent procedure. |
| | Variations in incubation times or temperatures | Run assay in a consistent environment. All assay incubation times must be consistent from assay to assay. |
| | Incorrect dilution of protein standards | Check dilutions. Re-dilute and re-run assay. |
| | Variation in equipment used | Use the same equipment from assay to assay including pipettors, shaker, plate washer, plate reader. |
| | Difference in the standard curve calculation | Use the same equation to fit the standard curve. |
| Samples are all above the linear range of the standard curve | Analyte concentration in samples is too high | Dilute samples are re-run. |
| | Assay sensitivity is not optimized for the natural range of the analyte | Reduce detection antibody concentration.<br>Reduce concentration of streptavidin-HRP.<br>Reduce capture and detection incubation times.<br>Re-dilute the protein standards to cover the appropriate range of the analyte. |
| Standard curve is fine, but all samples are very low or negative | No analyte in sample or analyte is present at a level below the limit of detection. | Increase the amount of sample used per well.<br>Decrease the dilution factor of the samples. |
| | Samples may contain an inhibitory factor | Spike a negative sample with protein standard and measure the recovery.<br><br>Dilute samples 1:2 or further. |

# Preparing Antibody-Coupled Luminex Beads

## Materials and Equipment Required

| Description | Recommended Source |
|---|---|
| Filter Plate (for Bead Coupling) | OriGene Part No. AR100012 contains 5 filter plates and 15 plate sealers. |
| Aluminum Plate Sealer | OriGene Part No. AR100012 contains 5 filter plates and 15 plate sealers. |
| EDC | Pierce (Thermo Fisher) # 22980 (store dessicated at -20ºC) |
| Sulfo-NHS | Pierce (Thermo Fisher) # 24510 (store dessicated at -20ºC) |
| Luminex MicroPlex® or MagPlex® Microspheres | Luminex Corp (www.luminexcorp.com), MiraiBio (www.miraibio.com), or Bio-Rad (www.bio-rad.com) |
| ELISA Capture Antibody | OriGene Technologies. Capture antibodies are supplied at 0.5 mg/ml. |
| NaH2PO4 (Sodium phosphate monobasic, anhydrous) | Sigma # S3139 |
| Water, Ultra-pure | Invitrogen # 10977 or equivalent |
| MES Buffer | Sigma # M2933 |
| 5N NaOH | ThermoFisher# SS256-500 |
| Tween-20 | Sigma # P9416 |
| BSA | Sigma # A7888 |
| 10X PBS | EMD Chemicals # 6506 or equivalent |
| Sodium Azide | Sigma # S8032 |
| ELISA Wash Buffer (10X) | OriGene Part No.: AR100010 (100 ml), AR100011 (1L) |
| Activation Buffer | 100 mM Phosphate, pH 6.2 (see Buffer and Reagent Preparation) |
| Coupling Buffer | 50 mM MES, pH 5.0 (see Buffer and Reagent Preparation) |
| Blocking Buffer | (see Buffer and Reagent Preparation) |
| Microplate vacuum manifold | Pall #5017 Multi-well plate vacuum manifold (or equivalent) |
| Vacuum pump or vacuum source | Millipore WP6111560 Chemical Duty Pump, 115 V/60 Hz or equivalent |
| Dessicant containers | Plastic or glass containers containing dessicant. |
| Absorbent paper towel | WYPALL X60 towels from Kimberly Clark |

## Set-Up

Determine the wells to be used for coupling. Cover remaining wells with an aluminum Plate Sealer. It is most convenient to use wells in groups of 8 (columns) and to use an 8-channel pipettor for addition of reagents.

The following protocol is designed for coupling of 0.5 - 8 μg of antibody to 100-150 μl (1.25-1.9 x $10^6$ ) of activated beads in a total volume of 150 μl. An example plate layout is shown below. In this example, 4 different antibodies are being coupled at four different coupling ratios: 1.0, 2.0, 3.0, and 4.0 μg of antibody per well. Couple each antibody to a different bead region (0-100) so that all coupled beads can be assayed simultaneously.

| | 1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Ab1-1 | Ab3-1 | | | | | | | | | |
| **B** | Ab1-2 | Ab3-2 | | | | | | | | | |
| **C** | Ab1-3 | Ab3-3 | | | | | | | | | |
| **D** | Ab1-4 | Ab3-4 | | | | | | | | | |
| **E** | Ab2-1 | Ab4-1 | | | | | | | | | |
| **F** | Ab2-2 | Ab4-2 | | | | | | | | | |
| **G** | Ab2-3 | Ab4-3 | | | | | | | | | |
| **H** | Ab2-4 | Ab4-4 | | | | | | | | | |

Wells covered by Aluminum Plate Sealer

## Procedure Notes

1. The vacuum pressure on the vacuum manifold should not exceed 5 mm Hg. Optimal filtration occurs between 1 and 4 mm Hg.

2. The fluorescent beads are light sensitive and are especially sensitive to direct sunlight and incandescent light. Use aluminum foil to cover plates and tubes. Store plates in a dark location during incubations.

3. After vacuum filtration, blot the bottom of the filter plate on clean absorbent material such as WYPALL X60 towels from Kimberly Clark.

4. During loading or incubations do not place the filter plate on absorbent material as liquid will be drawn through the plate by wicking.

## Coupling Procedure

| | |
|---|---|
| Step 1 | Add 100 µl 1X Wash Buffer to each well of the filter plate. Vacuum gently and blot. |
| Step 2 | Vortex bead stock for 10 seconds at high speed |
| Step 3 | Add 100-125 µl of Bead Stock to each well of the filter plate |
| Step 4 | Vacuum gently and blot. |
| Step 5 | Wash beads 2 Times with 200 µl Activation Buffer. |
| Step 6 | Vacuum gently and blot. |
| Step 7 | Add 80 µl Activation Buffer to each well. |
| Step 8 | Shake plate at 1000 rpm for at least 5 minutes. |
| Step 9 | Prepare 50 mg/ml Sulfo-NHS in water. |
| Step 10 | Add 10 µl of the 50 mg/ml Sulfo-NHS to each well. |
| Step 11 | Prepare a 50 mg/ml solution of EDC in water. Work quickly as EDC is not stable in water. |
| Step 12 | Add 10 µl of the 50 mg/ml EDC to each well. Work quickly as EDC is not stable in water. |
| Step 13 | Mix wells by pipetting up and down 5 times. |
| Step 14 | Shake plate for 20-30 minutes @ 600-1000 rpm in the dark or covered with a dark cover. |
| Step 15 | Mix wells by pipetting up and down 5 times. |
| Step 16 | Shake plate for 20-30 minutes @ 600-1000 rpm in the dark or covered with a dark cover. |
| Step 17 | Wash Beads 3 Times with 200 µl Coupling Buffer. |
| Step 18 | Vacuum gently and blot. |
| Step 19 | Add 146 µl Coupling Buffer to each well. |
| Step 20 | Add 2.0 µl of antibody solution to the 1.0 µg antibody wells.<br>Add 4.0 µl of antibody solution to the 2.0 µg antibody wells.<br>Add 6.0 µl of antibody solution to the 3.0 µg antibody wells.<br>Add 8.0 µl of antibody solution to the 4.0 µg antibody wells. |
| Step 21 | Mix wells by pipetting up and down 5 times. |
| Step 22 | Mix for 1 hour with shaking at 600 RPM. |
| Step 23 | Mix wells by pipetting up and down 5 times. |
| Step 24 | Mix for 1-3 hours with shaking at 600 RPM. |
| Step 25 | Vacuum gently. |
| Step 26 | Wash 3 Times with 250 µl Blocking Buffer. |
| Step 27 | Vacuum gently and blot. |

| | |
|---|---|
| Step 29 | Add 100 µl Blocking Buffer to each well |
| Step 30 | One well at a time, mix wells by pipetting up and down 5 times and then remove the solution containing suspended beads from the well. |
| Step 31 | Transfer the solution (~ 100 µl) to a new labeled tube. |
| Step 32 | Store beads @ 4ºC in the dark. |
| Step 33 | (Optional). Count the beads to determine the bead concentration using a hemocytometer or a particle counting instrument such as a Cellometer. Alternatively, assume the beads are at a concentration of 10,000 beads per µl and adjust the concentration after initial testing. |

# Luminex Assay Protocol

## Required or Recommended Reagents and Supplies

| Description | Recommended Source |
|---|---|
| Filter Plate | OriGene Part No. AR100012 contains 5 filter plates and 15 plate sealers. |
| Aluminum Plate Sealer | OriGene Part No. AR100012 contains 5 filter plates and 15 plate sealers. |
| Luminex beads coupled with an OriGene capture antibody | Prepared as described in the coupling procedure. |
| Biotinylated Detection Antibody | OriGene Detection antibodies are supplied at 0.5 mg/ml. |
| Recombinant Protein for use as a protein standard | OriGene proteins purified from HEK293 cells.<br>OriGene over-expression HEK293 Cell Lysates. |
| Assay Buffer<br><br>For dilution of detection antibody, secondary antibodies, and streptavidin conjugates. | OriGene has four different Assay Buffers, each containing a different optimized mixture of buffer, salts, detergent and proteins. We recommend starting with Assay Buffer D. Different buffers may perform better than others depending upon the species origin of the capture and detection antibodies.<br><br>Assay Buffer A- contains mouse and goat IgG — Part No.: AR100012<br>Assay Buffer B- contains BSA — Part No.: AR100013<br>Assay Buffer C- contains BSA and rabbit IgG — Part No.: AR100014<br>Assay Buffer D- contains BSA, mouse and goat IgG — Part No.: AR100015 |
| Assay Diluent<br><br>Serum-based diluent for dilution of standards and samples. | OriGene, Part No.: AR100016<br><br>Contains heat-activated animal serum, detergent and a preservative. |
| Streptavidin-Phycoerythrin Conjugate | Moss PN: SAPE-001 1 mg/ml stock<br><br>or<br><br>OriGene PN: AM100109<br>2 vials of 10X (30 µg/ml) solution of streptavidin-PE in stabilizing buffer for use with Luminex immunoassays. Makes a 3 µg/ml solution when diluted to 1X. Sufficient quantity for 192 assays. |

## Equipment and software required:

| *Description* | *Recommended Supplier & Catalog No.* |
|---|---|
| Luminex 100™, 200™ or equivalent | Luminex, Millipore, Hitachi, Bio-Rad |
| Luminex Data Acquisition Software | xPONENT 3.1<br>Luminex IS 2.3<br>Luminex LDS 1.7<br>BioPlex Manager |
| Vortex Mixer | VWR Analog Vortex Mixer<br>58816-121 (120V)<br>58816-123 (230V) |
| Mini centrifuge | VWR Minifuge<br>93000-196 (120V)<br>93000-198 (230V) |
| Orbital Shaker | Eppendorf Mix Mate<br>022674200 (120V/60Hz)<br>022674226 (230V/50Hz) |
| Water Bath Sonicator | Bransonic Model B3/B5<br>000-951-005 (Model B3, 115V)<br>000-951-103 (Model B5, 230V) |
| Microplate vacuum manifold with pressure gauge | Pall Multi-well plate vacuum manifold (PN 5017). |
| Data Analysis Software | MasterPlex QT from Hitachi Software or equivalent. A free trial download is available. |

**Note :** To achieve optimal results, the use of the "High Gain" or "High PMT" setting on the Luminex 100 or 200 is recommended. Some instruments may use this setting as the default. Check with your instrument supplier for more information.

## Setting the Luminex 100 or 200 for "High Gain" or "High PMT" Reading

1. Create a new lot number for Cal 2 and enter it into the Luminex software as a new Cal 2 lot number (use the actual lot number with an HG at the end to designate High Gain).
2. Record the Cal 2 target value, which is usually around 3800.
3. Multiply the Cal 2 target value by 4.55 to get a new Target value of approximately 17,290.
4. Enter the new Target Value as the value for your "New" Cal 2 lot.
5. Run the Cal 2 Calibration.

## Procedure Notes

1. The vacuum pressure on the vacuum manifold should not exceed 5 mm Hg. Optimal filtration occurs between 1 and 4 mm Hg.
2. The fluorescent beads are light sensitive and are especially sensitive to direct sunlight and incandescent light. Use aluminum foil to cover plates and tubes. Store plates in a dark location during incubations.
3. After vacuum filtration, blot plates on clean absorbent material such as WYPALL X60 towels from Kimberly Clark.
4. During loading or incubations do not place the filter plate on absorbent material as liquid will be drawn through the plate by wicking.

## Recommended Plate Layout

Before starting, it is recommended that a plate plan be designed. Such a plan will assist in assay workflow and data analysis. A suggested plate plan is shown below. A plate plan template is provided in the appendix.

| | 1 | 2 | 3 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | Std 1 | Std 1 | Std 1 | | | | | | | | |
| **B** | Std2 | Std2 | Std2 | | | | | | | | |
| **C** | Std 3 | Std 3 | Std 3 | | | | | | | | |
| **D** | Std 4 | Std 4 | Std 4 | | | | | | | | |
| **E** | Std 5 | Std 5 | Std 5 | | | | | | | | |
| **F** | Std 6 | Std 6 | Std 6 | | | | | | | | |
| **G** | Std 7 | Std 7 | Std 7 | | | | | | | | |
| **H** | Neg | Neg | Neg | | | | | | | | |

Sample Wells

Std 1 is the highest concentration and Std 7 is the lowest concentration.
Running all standards, samples, and controls in duplicate or triplicate is recommended.

## Sample Collection and Preparation

1. Serum, plasma and tissue culture supernatants are common sample types and will generally be suitable for testing with this protocol. Protocols for additional sample types will need to be tested and validated.
2. Avoid the use of lipemic or hemolyzed samples.
3. Collect serum and plasma samples according to standard protocols. Immediately mix and separate, and then aliquot the samples into polypropylene tubes. If not tested immediately, store samples at -80ºC.
4. Analyze fresh samples as quickly as possible after collection. Allow frozen samples to thaw on ice, then mix well before testing. Avoid multiple freeze-thaw cycles of frozen samples.
5. Turbid samples should be clarified by centrifugation before use.
6. If the measured analyte concentrations exceed the value of the upper range of the standard curve, dilute samples appropriately and reanalyze. Dilute serum and plasma samples in Assay Diluent or Assay Buffer. Dilute tissue culture supernatants in tissue culture medium or Assay Buffer.

7. For accurate quantitation, it is important that the composition of the standards be exactly the same as the composition of the samples. Dilute the standards in a buffer that reflects the final composition of the samples after dilution.

## Dilution of Samples

It is important to dilute the samples so that the analyte levels are within the linear range of the assay and to minimize the effect of the sample matrix on the sample assay.

## Reagent Preparation

1. Prepare Wash Buffer by diluting 10X Wash Buffer to 1X with deionized water. Store diluted wash buffer at 2-8ºC for up to one month.

2. For accurate quantitation, it is important to prepare protein standards in a matrix that mimics the composition of the samples. For example, if the samples are cell culture supernatants, then the protein standards should be prepared in the cell culture medium. For serum or plasma samples, dilute protein standards in OriGene Assay Diluent (AR100016) or similar serum- or plasma-based matrix.

3. The performance of a Luminex ELISA assay can vary dramatically depending upon the composition of the samples, particularly with serum, plasma and other samples of clinical origin. Samples of clinical origin may contain substances that inhibit the assay causing falsely low readings. Dilution of samples with an appropriate matrix may reduce some or all of inhibition or interference.

   Example – Serum Samples:
   a. Protein stock concentration is 385 µg/ml.
   b. Samples to be tested are serum samples.
   c. Sample volume per well is 50 µl.
   d. Total volume added per well is 100 µl.

   ➡ Assay matrix is a mixture of 50% cell Assay Diluent and 50% Assay Buffer B.

   ➡ To prepare a 1 ug/ml stock, add 1.3 µl of protein stock to 498.7 µl of Assay Buffer B. To prepare Standard 1 at 100 ng/ml, add 100 µl of the 1 µg/ml stock to 900 µl of Assay Matrix.

| To Make | Add | To | Conc. (ng/ml) |
|---|---|---|---|
| Standard 2 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 33.33 |
| Standard 3 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 11.11 |
| Standard 4 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 3.70 |

| | | | |
|---|---|---|---|
| Standard 5 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 1.23 |
| Standard 6 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 0.41 |
| Standard 7 | 200 µl of Standard 1 | 400 µl of Assay Matrix | 0.14 |

Filter Plate

Before starting the assay, cover the wells that will not be used in the assay with an aluminum plate sealer. Press sealer down so that all wells are tightly sealed. Trim edges with a razor blade.

Bead Mix

Prepare Bead Mix in Assay Buffer or 1X Wash Buffer containing 1 mg/ml BSA. Dilute beads to a final concentration of 25 beads per µl.

Example:

a. Bead stocks are at a concentration of 10,000 per µl.
b. Prepare 100 µl per well plus 10% extra.
c. Assay uses 48 wells.
d. Volume Assay Buffer B required = 48*100*1.1 = 5280 µl
e. Volume bead stock required = 5280 * 25/10,000 = 13.2 µl

➡ Add 13.2 µl of each bead stock to 5280 µl of Assay Buffer B.

f. When all beads have been added, vortex thoroughly for 10 seconds and then sonicate for 2 minutes.

## Assay Protocol

Analyte Capture

1. Briefly vortex the 1X Bead Mix. Add 100 µl of 1X Bead Mix to each assay well (this is approximately 2500 beads of each type per well).
2. Remove liquid from the plate by gentle vacuum. Blot plate thoroughly on absorbent paper.
3. Add 100 µl diluted standards into designated standard wells (including negative).
4. Add 50 µl serum samples into designated sample wells.
5. Add 50 ul Assay Buffer B into the designated sample wells.
6. Cover and incubate the plate for 1-2 hours at room temperature on an orbital plate shaker (500-700 rpm).

7. Ten to fifteen minutes before the end of this incubation, prepare 1X Detection Antibody at 0.5-4 µg/ml.

   Example:
   a. Biotinylated detection antibody stock is 0.5 mg/ml.
   b. Working biotinylated detection antibody concentration is 2 µg/ml.
   c. Prepare 100 µl per well plus 10% extra.
   d. Assay uses 48 wells.
   e. Volume Assay Buffer B required = 48*100*1.1 = 5280 µl
   f. Volume detection antibody required = 5280 * 2/500 = 21.12 µl

   ➡ Add 21.12 µl biotinylated detection antibody to 5280 µl of Assay Buffer D.

Analyte Detection

8. Remove the liquid from the wells by gentle vacuum.
9. Wash beads three time by adding 100 µL 1X Wash Buffer to the wells and removing liquid with gentle vacuum.
10. After washing, blot plate thoroughly on absorbent material.
11. Add 100 µl Detection Antibody into each well.
12. Cover and incubate the plate for 1-2 hours at room temperature on an orbital plate shaker at 500-700 rpm.
13. During this incubation, prepare the Luminex instrument for reading (see below).
14. During the last 10 minutes of this incubation, prepare the Detection Reagent (Streptavidin-Phycoerythrin).

Preparation of Detection Reagent (Streptavidin-Phycoerythrin Conjugate

Method 1 – Dilution of Moss 1 mg/ml SAPE concentrate:
   a. Streptavidin-Phycoerythrin (SAPE) stock is 1.0 mg/ml.
   b. Working SAPE concentration is 2 µg/ml.
   c. Prepare 100 µl per well plus 10% extra.
   d. Assay uses 48 wells.
   e. Volume Assay Diluent required = 48*100*1.1 = 5280 µl
   f. Volume SAPE stock required = (2/1000) * 5280 = 10.56 µl

   ➡ Add 10.56 µl of SAPE to 5280 µl of Assay Buffer D.

Method 2 – Dilution of TruePlex™ 10X Detection Reagent Concentrate:
   a. Prepare 100 µl per well plus 10% extra.

b. Assay uses 48 wells.
c. Total Volume Required = 48 * 100 * 1.1 = 5280 µl
d. Volume Assay Diluent required = 0.9 * 5280 = 4754 µl
e. Volume 10X Detection Reagent required = 0.1 * 5280 = 528 µl

➡ Add 528 µl of 10X Detection Reagent to 4754 µl of Assay Buffer D.

Prepare the Luminex instrument for reading

15. Set up the instrument as described in the user's manual. Instruments from different vendors may have different set-up procedures.
16. Warm up the instrument. This may take up to 30 minutes.
17. General Parameter settings:
    - The XY platform heater should be off.
    - The minimum events setting should be 30.
    - The sample size should be set to 50 µL.
    - The flow rate should be set to Fast.
18. Enter the analyte names and bead numbers.
19. Check the probe height and adjust it, if necessary to accommodate the filter plate.
20. Perform one prime cycle with sheath fluid, one alcohol flush, and two sheath fluid washes.
21. Calibrate to "High Gain" setting if necessary (see page 25).

Detection with Streptavidin-Phycoerythrin

22. Remove the liquid in the wells by gentle vacuum.
23. Wash beads three times by adding 100 µl 1X Wash Buffer to all wells and removing liquid with gentle vacuum.
24. Blot plate thoroughly on absorbent material.
25. Add 100 µl Detection Reagent (Streptavidin-PE conjugate) into each well.
26. Cover and incubate the plate for 30 minutes at room temperature on an orbital plate shaker at 500-700 rpm.
27. Wash beads three times by adding 100 µl 1X Wash Buffer to all wells and then removing liquid with gentle vacuum.
28. Blot plate thoroughly on absorbent material.
29. Add 100 µL 1X Wash Buffer to each well.

30. Optional: shake plate for 2 minutes on an orbital plate shaker at 500-700 rpm (this optional step may increase the bead count during Luminex reading.
31. Read the plate in the Luminex instrument.

## Data Analysis

1. Use commercial data analysis software to analyze the data as analysis of ELISA data with Excel can be quite difficult. Some Luminex instruments may come with analysis software. MasterPlex ReaderFit or MasterPlex QT from Hitachi software (www.miraibio.com) are suitable third-party software packages for quantitative analysis of Luminex ELISA data. Time-limited trial versions can be downloaded from www.miraibio.com . Follow the instructions for installing the software and licenses.
2. Tutorials in PDF format, PowerPoint presentations, FAQs and other training materials are also available on the MiraiBio website.
3. A free web-based data analysis tool is available at www.readerfit.com. The free version is limited to analyzing a total of 32 data points (standards and samples), but a paid version allows broader use.
7. Enter the data for your standards and samples and generate a standard curve by fitting the results of the protein standard data to a model equation.
8. For Luminex assays, a 5-parameter logistic regression with $1/Y^2$ weighting usually gives the best fit.
9. Use the standard curve to calculate the concentration of the analytes in the samples (unknowns). Be sure to adjust for any dilution of the samples relative to the standards.
10. The following on-line articles provide additional in-depth discussion and analysis of the various factors and parameters to consider for ELISA data analysis.

    - Tips for ELISA Data Analysis
    - The 4 Parameter Logistic Regression Model
    - The 5 Parameter Logistic Regression Model
    - The Importance of Weighting with 4PL and 5PL Regression Models

## Luminex ELISA Protocol Summary

1. Prepare standards and samples.
2. Dilute beads to 25 beads/µl in Assay Buffer.
3. Add 100 µl beads to each well of a filter plate.
4. Vacuum gently to remove liquid and blot thoroughly.
5. Add 100 µl standards and samples to the appropriate wells.
6. Cover and incubate the plate for 1-2 hours at room temperature on an orbital plate shaker at 500-700 rpm. Prepare Detection Antibody.
7. Warm up the Luminex instrument.
8. Wash wells three times with 100 µl of 1X Wash Buffer Vacuum gently and blot.
9. Add 100 µl Detection Antibody to each well.
10. Cover and incubate the plate for 1-2 hours at room temperature on an orbital plate shaker at 500-700 rpm. Prepare SAPE solution at 2-4 µg/ml.
11. Wash wells three times with 100 µl of 1X Wash Buffer. Vacuum gently and blot.
12. Add 100 µl of diluted SAPE to each well.
13. Incubate plate at room temperature for 30 minutes on an orbital plate shaker at 500-700 rpm.
14. Prepare the Luminex instrument for reading.
15. Wash wells three times with 100 µl of 1X Wash Buffer. Vacuum gently and blot.
16. Add 100 µl 1X Wash Buffer to each well.
17. Read plate in Luminex instrument.

## Luminex Assay Optimization

Assays can be optimized for many different purposes such as maximum sensitivity, widest dynamic range, maximum specificity, improved linearity or recovery, or shortest time to results, to give a few examples. Some common assay parameters that can be modified to achieve an optimal assay are listed in the table below.

| Parameter | Modification | Possible Effects |
|---|---|---|
| **Antibody Coupling Concentration** | Increase or Decrease the amount of antibody used in the coupling reaction. | Higher signal<br>Higher background<br>Lower limit of detection<br>Faster capture |
| **Standard Concentrations** | Standard can be diluted over different ranges. | Wider dynamic range<br>Different dynamic range |
| **Sample concentrations** | Samples can be used undiluted or can be diluted to different concentrations in an assay matrix. | Increased recovery<br>Increased linearity<br>Increased sensitivity |
| **Composition of Assay Matrix** | Addition of blocking agents such as proteins, polymers, or additional detergents.<br><br>Increase or decrease in the salt (NaCl) concentration.<br>Increase or decrease the pH of the buffer.<br><br>Alternate buffers: Tris, borate, MES<br><br>Alternate buffer concentrations | Increased recovery<br>Increased linearity<br>Increased sensitivity<br>Reduced sample interference |
| **Capture time** | Increase or decrease | Increased or decreased sensitivity<br>Wider dynamic range |
| **Number of Washes – Post Capture** | Increase or decrease | Increased or decreased signal and/or background<br><br>Increased or decreased assay time |

| Parameter | Modification | Possible Effects |
|---|---|---|
| **Composition of Wash Buffer** | Increased or decreased salt concentration<br><br>Increased or decreased pH<br><br>Increased or decreased detergent concentration<br><br>Different buffer (Tris, etc.)<br><br>Different detergent | Increased or decreased signal and/or background<br><br>Increased sensitivity<br><br>Alternate dynamic range<br><br>Reduced sample interference<br><br>Improved recovery |
| **Biotinylated detection antibody concentration** | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Alternate dynamic range |
| **Composition of the biotinylated detection antibody diluent** | Addition of blocking agents such as proteins, polymers, or additional detergents.<br><br>Increase or decrease in the salt (NaCl) concentration.<br><br>Increase or decrease in the buffer pH.<br><br>Alternate buffers: Tris, borate, MES | Increased recovery<br>Increased linearity<br>Increased sensitivity<br>Reduced sample interference |
| **Detection antibody incubation time** | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Altered dynamic range |
| **Composition of the streptavidin-PE conjugate diluent** | Addition of blocking agents such as proteins, polymers, or additional detergents.<br><br>Increase or decrease in the salt (NaCl) concentration.<br><br>Increase or decrease in the buffer pH. | Increased or decreased signal<br>Increased or decreased sensitivity<br>Altered dynamic range<br>Increased or decreased detection time |

| Parameter | Modification | Possible Effects |
|---|---|---|
| **Streptavidin-PEconjugate concentration** | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Alternate dynamic range |
| **Streptavidin-PE incubation time** | Increase or decrease | Increased or decreased signal<br>Increased or decreased sensitivity<br>Altered dynamic range |
| **Number of Washes after Streptavidin-PE binding** | Increase or decrease | Increased or decreased signal and/or background<br><br>Increased or decreased assay time |
| **Composition of Wash Buffer** | Increased or decreased salt concentration<br><br>Increased or decreased pH<br><br>Increased or decreased detergent concentration<br><br>Different buffer (Tris, etc.)<br><br>Different detergent | Increased or decreased signal and/or background<br><br>Increased sensitivity<br><br>Altered dynamic range<br><br>Reduced sample interference<br><br>Improved recovery |

## Luminex ELISA Troubleshooting

To troubleshoot problems with the Luminex instrument, consult the appropriate Luminex manuals, contact Luminex technical support (http://www.luminexcorp.com/support), or contact your instrument supplier.

To troubleshoot problems with the using MasterPlex QT or ReaderFit for data analysis, contact Hitachi Software (http://www.miraibio.com/support).

| Problem | Cause | Solution |
|---|---|---|
| Insufficient bead count | Bead mix not prepared correctly | Sonicate and vortex beads before dilution. Check bead concentration. |
| | Vacuum pressure too high | Adjust vacuum pressure to 1-4 mm Hg during filtration. |
| | Plate leaked in the Luminex instrument | See section on leaking plate. |
| | Clogged sample probe | Clean sample probe. Remove and sonicate, if necessary. |
| | Clogged flow cell | Not correctable by user. Contact instrument technical support. |
| Wells in filter plate will not vacuum | Clogged wells | Spin samples @ 14,000 x g for 2 minutes before removing aliquot for testing. |
| | Plate has not made a tight seal with the vacuum manifold | Replace gasket on vacuum manifold. |
| Leaking plate | Probe height not adjusted correctly | Adjust probe height with three alignment disks using the recommended filter plate. |
| | Insufficient blotting of filter plate | Blot filter plate thoroughly onto paper towels after each washing cycle. Make sure that the bottom of the filter plate is completely dry. |
| | Plate placed on absorbent surface | If there is liquid in the wells of the filter plate, do not place the plate on any absorbent materials. The wicking action of the material will draw liquid through the bottom of the filter plate. |

| Problem | Cause | Solution |
|---|---|---|
| | Vacuum pressure too high | Ensure that the vacuum pressure is less than 5 mm Hg. |
| High Background | Cross-contamination of wells | Pipette carefully to ensure that no material from the standard or sample wells reaches the background (Neg) wells. |
| | Ineffective or omitted wash steps | Follow wash instructions carefully. Increase number of washes if necessary. |
| | Ineffective blocking of non-specific interactions | Add additional blocking protein to Assay Buffer. |
| Low signal across the plate | Luminex instrument gain setting is incorrect | Make sure that the reporter channel is calibrated to the “High Gain” or “High PMT” setting. |
| | Instrument is out of calibration | Recalibrate instrument. |
| | 1X Detection Antibody prepared incorrectly | Prepare new 1X Detection Antibody. Increase concentration of Detection Antibody. |
| | Incubations were too short, or shaking was insufficient | Increase incubation times. Verify and/or increase shaking speed. |
| Beads not in region | Instrument is out of calibration | Recalibrate instrument. |
| Sample readings are out of range | Beads are photo-bleached | Protect beads from light at all times. Use amber colored tubes. Cover filter plate with a black cover or aluminum foil. |
| | Incorrect bead regions entered | Check protocol template. Correct if necessary. |
| | Analyte level is below the detection limit of the assay | Prepare an additional low standard by diluting Standard 7 1:3 and re-run the samples and the standard curve. |
| | Analyte level is above of the quantifiable range of the assay | Dilute samples and re-run. |

## Buffer and Reagent Preparation

100 mM Carbonate, pH 9.5

To prepare 1000 mL, add the following to 500-800 ml of deionized water:

| | |
|---|---|
| $Na_2CO_3$ (FW=105.99) | 3.18 g |
| $NaHCO_3$ (FW=84.01) | 5.88 g |

Mix thoroughly, then bring volume to 1000 ml with deionized water.
Verify that pH is 9.3-9.7.
Filter sterilize through a 0.2 μm filter unit.
Store at room temperature.
Check pH monthly or before use.

100 mM Borate, pH 8.5

To prepare 1000 mL, add the following to 900 ml of deionized water:

| | |
|---|---|
| 20X Borate Buffer (Pierce PN 28341) | 100 mL |

The final borate concentration is 2X or 100 mM.

Verify that the pH is 8.3-8.7
Filter sterilize through a 0.2 μm filter unit.
Store at room temperature.
Check pH monthly or before use.

100 mM Phosphate, pH 7.4

To prepare 1000 mL, add the following to 500-800 ml deionized water

| | |
|---|---|
| $NaH_2PO_4 \cdot H2O$ (FW = 156.01) | 2.50 g |
| $Na_2HPO_4 \cdot 7\ H2O$ (FW = 268.07) | 22.52 g |

Mix thoroughly, then bring volume to 1000 ml with deionized water.
Verify that pH is 7.3-7.7.
Filter sterilize through a 0.2 μm filter unit.
Store at room temperature.
Check pH monthly or before use.

Post-Coat/Blocking Buffer

To prepare 1000 mL, add the following to 800 ml of deionized water:

| | |
|---|---|
| 10X Wash Buffer<br>(Part No. AR100010 – 100 ml)<br>(Part No. AR100011 – 1000 ml) | 100 mL |
| Sucrose | 50 g |
| Sodium Azide (optional) | 0.5 g |
| Protein (optional)<br>BSA, animal IgG, casein, gelatin, or non-fat dry milk. | 1-10 g |

Mix thoroughly, and then bring volume to 1000 ml with deionized water.
Verify that the pH is 7.3-7.7.
Filter sterilize through a 0.2 μm filter unit.
Store at room temperature.

Activation Buffer (Luminex)
(100 mM Phosphate, pH 6.2)

To prepare 1000 mL, add the following to 900 ml of deionized water:

| | |
|---|---|
| NaH2PO4 (Sodium phosphate monobasic, anhydrous) | 12.0 g |

Mix thoroughly, then add 5N NaOH to bring the pH to 6.2.
Bring the volume to 1000 ml with deionized water.
Verify that pH is 6.1-6.3.
Filter sterilize through a 0.2 μm filter unit.
Store at room temperature.
Check pH monthly or before use.

Coupling Buffer (Luminex)
(50mM MES, pH 5.0)

To prepare 1000 mL, add the following to 900 ml of deionized water:

| | |
|---|---|
| MES (2[N-Morpholino] ethanesulfonic acid) | 9.76 g |

Mix thoroughly, then add 5N NaOH to bring the pH to 5.0.
Bring the volume to 1000 ml with deionized water.
Verify that pH is 5.0-5.1.
Filter sterilize through a 0.2 µm filter unit.
Store at 4ºC.
Check pH monthly or before use.

Blocking Buffer (Luminex)

To prepare 1000 mL, add the following to 900 ml of deionized water:

| | |
|---|---|
| 10X Wash Buffer<br>(Part No. AR100010 – 100 ml)<br>(Part No. AR100011 – 1000 ml) | 100 mL |
| BSA | 1 g |
| Sodium Azide | 0.5 g |

Mix thoroughly until the solution is homogeneous. Filter sterilize through a 0.2 µm filter unit.
Store at 4ºC.

## Plate Plan Template

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