# SMC™ Bead-Based Immunoassay Development Kit (5 plates)

**Microparticle Assay Development Kit**

Catalog # 03-0178-00

## SMC™ Bead-Based Immunoassay Kit for the Development of 5 Bead-Based Immunoassays

**FOR RESEARCH USE ONLY**

**NOT FOR USE IN DIAGNOSTIC PROCEDURES**

**Manufactured & Distributed by:**

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## INTRODUCTION

This Single Molecule Counting (SMC™) Bead-Based Immunoassay Development kit provides necessary reagents to label antibodies and evaluate feasibility of an assay using the customer's own antibodies. Enough reagents are provided to run five [5] plates, where the end user can perform a matrix, feasibility, prototype and an optimization experiment.

An analyte-specific capture antibody is labeled and coated onto paramagnetic microparticles (MP) beads and an analyte-specific detection antibody is fluorescently labeled. The user pipettes beads, standards, and samples into microplate wells. During incubation, the analyte in the sample binds to the capture antibody on the coated MP beads. Unbound molecules are washed away during a wash step. The fluor-labeled detection antibody is added to each well and incubated. The detection antibody recognizes and binds to analyte that has been captured onto the beads, thus completing the immunocomplex. Following a wash step, elution buffer is then added and incubated. The elution buffer dissociates the immunocomplex releasing the analyte and labeled detection antibody. The capture antibody remains bound to the MP bead, which is magnetically separated and the eluate (containing labeled detection antibody and analyte) is transferred to the final reading plate. The plate is loaded onto the Erenna® or SMCxPRO™ System where the labeled detection antibodies are detected and counted. The number of fluor-labeled detection antibodies counted is directly proportional to the amount of captured analyte in the sample. The amount of analyte in unknown samples is interpolated from a standard curve.

The SMC™ Bead-Based Immunoassay Development kit contains detection and capture label reagents as well as buffers for assay development feasibility. Additionally, the kit contains base assay buffer (Optimization Assay Buffer A), high salt assay buffer (Optimization Assay Buffer B), and high detergent assay buffer (Optimization Assay Buffer C) that contain a proprietary mixture of blockers to decrease non-specific antibody binding. The optimization of salt and detergent concentrations will minimize background, reduce non-specific bindings, and improve immunoassay stringency. Immunoassay buffer optimization can also improve results for assay validation.

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## SUPPLIES

The SMC™ Bead-Based Immunoassay Development Kit includes all reagents listed in ***Table 1:*** *Reagents Provided.* All reagents supplied are for Research Use Only

Additional reagents and supplies are required to run this immunoassay and are listed in ***Table 2:*** *Additional Supplies Required (not provided)*.

### **Table 1:** Reagents Provided

| Item # | Description | Shipping Conditions | Storage Conditions | Component Part No. | Packaging Details |
|---|---|---|---|---|---|
| **1** | Capture Label | Dry Ice | < -20°C | 02-0557-00 | 1 x 20 µL |
| **2** | Detection Label | Dry Ice | < -20°C | 02-0574-00 | 1 x 20 µL |
| **3** | Buffer 1 | Cold Pack | 2 – 8 °C | 02-0552-00 | 2 x 25 mL |
| **4** | Buffer 2 | Cold Pack | 2 – 8 °C | 02-0553-00 | 1 x 500 µL |
| **5** | Buffer 3 | Cold Pack | 2 – 8 °C | 02-0554-00 | 2 x 5 mL |
| **6** | Filter Tube Ultra 4 | Cold Pack | 2 – 8 °C | 02-0556-00 | 4 x 2 Pack |
| **7** | Uncoated Beads | Cold Pack | 2 – 8 °C | 02-9985-00 | 1 x 2 mL |
| **8** | Coated Bead Buffer | Cold Pack | 2 – 8 °C | 02-0558-00 | 1 x 5 mL |
| **9** | Assay Buffer | Cold Pack | 2 – 8 °C | 02-0865-00 | 2 x 100 mL |
| **10** | Standard Diluent | Cold Pack | 2 – 8 °C | 02-9984-00 | 1 x 100 mL |
| **11** | Elution Buffer B | Cold Pack | 2 – 8 °C | 02-0211-02 | 6 x 5 mL |
| **12** | Buffer D | Cold Pack | 2 – 8 °C | 02-0359-00 | 6 x 3 mL |
| **13** | 10x Wash Buffer | Cold Pack | 2 – 8 °C | 02-9987-00 | 2 x 250 mL |
| **14** | 10x System Buffer w/ 0.5% Proclin | Cold Pack | 2 – 8 °C | 02-9983-00 | 1 x 100 mL |
| **15** | Optimization Assay Buffer A | Cold Pack | 2 – 8 °C | 02-9978-00 | 1 x 50 mL |
| **16** | Optimization Assay Buffer B | Cold Pack | 2 – 8 °C | 02-9977-00 | 1 x 50 mL |
| **17** | Optimization Assay Buffer C | Cold Pack | 2 – 8 °C | 02-9976-00 | 1 x 10 mL |

## Storage Instructions

The SMC™ Bead-Based Immunoassay Development Kit (Catalogue # K-03-0178-00) should be stored at 2 - 8°C.

The Capture and Detection Labels should be stored at ≤ -70°C.

Discard standards after one use.

Supplied 10X Wash Buffer does not contain preservative. After dilution, the 1X Wash Buffer may be filter sterilized with Stericup® filter, EMD Millipore PN S2GPU11RE for storage of up to 1 month at 2 – 8°C. If not filter sterilized, all remaining 1X wash buffer should be discarded upon experiment completion.

Proper kit performance can only be guaranteed if the materials are stored properly.

**Table 2:** Additional Supplies Required (not provided)

**Instrumentation**

| Item # | Product Description | Supplier | Product Number | Product Use(s) |
|---|---|---|---|---|
| 1 | Multichannel Manual Pipette 10 – 20 µL | -- | -- | Transferring 10 µL |
| 2 | Multichannel Manual Pipette 20 – 250 µL | -- | -- | Transferring 20 µL, 100 µL |
| 3 | Tube Rotator | -- | -- | Microparticle Resuspension |
| 4 | Magnetic Bead Separator | EMD Millipore | LSKMAGS08 | Magnetic Bead Separation |
| 5 | Sphere Mag Plate | EMD Millipore | 90-0003-02 | Capturing/Pelleting Microparticle Beads |
| 6 | Jitterbug™ Microplate Incubator/Shaker | EMD Millipore | 70-0009-00 | Incubating/Shaking at 25°C |
| 7 | VWR® Microplate Shaker | VWR International | 12620-926 | Incubating/Shaking overnight |
| 8 | Bio-Tek Elx™ 405 Microplate Washer | EMD Millipore | 95-0004-05 | Automated Plate Washing |
| 9 | Tecan Hydroflex™ Microplate Washer | EMD Millipore | 95-0005-02 | Automated Plate Washing |
| 10 | Centrifuge, with bucket rotors, capable of 3,900 x g | -- | -- | Centrifuing Samples and Filter |
| 11 | Micro-Centrifuge | -- | -- | Centrifuging Samples & |
| 12 | ALPS™ 50V Microplate Heat Sealer | EMD Millipore | 70-0018-00 | Heat sealing 384-well plates (Erenna) |
| 13 | Nanodrop™ or Spectrophotometer capable | -- | -- | Measuring Ab Conc. and Labeling |

**Materials**

| Item # | Product Description | Supplier | Product Number | Product Use(s) |
|---|---|---|---|---|
| **14** | 12-Channel Reagent Reservoir (sterile) | Argos/Cole Parmer | 04395-33 | Standard Curve Dilution |
| **15** | VistaLab™ 25 mL Reagent Reservoirs | Fisher Scientific | 21-381-27C | Addition of Reagents |
| **16** | MultiscreenHTS BV 96-well Filter Plate | EMD Millipore | MSBVN1210 | Sample Filtration |
| **17** | 96-well V-bottom Plate | Fisher Scientific | 14-222-241 | Assay Plate |
| **18** | 96-well Deep-well Plate | -- | -- | Assay Buffer Optimization Screen |
| **19** | 5 mL Luer-Lok™ Syringe | Fisher Scientific | 14-829-45 | Detection Antibody Filtration |
| **20** | 0.2 µM Syringe Filter | EMD Millipore | SLGPR33RS | Detection Antibody Filtration |
| **21** | Nunc™ Clear Adhesive Plate Seal | Fisher Scientific | 236366 | Sealing Assay Plate |
| **22** | 384-well Round Bottom Plates | Fisher Scientific | 12-565-384 | Erenna® Reading Plate |
| **23** | Heat Sealing Foil | Fisher Scientific | NC0276513 | Sealing plates for Erenna® Reading |
| **24** | 1L Stericup® Filter; 0.22µM Filter | EMD Millipore | S2GPU11RE | Filter sterilizing System Buffer |
| **25** | SMCxPRO™ 384-well plate, 1 adhesive sealer | EMD Millipore | 02-1008-00 | SMCxPRO™ Reading plate, seal |
| **26** | SMCxPRO™ 384-well plates, case of 32 | EMD Millipore | ABB2-00160A | SMCxPRO™ Reading Plates |
| **27** | SMCxPRO™ Adhesive Plate Seals | Fisher Scientific | 276014 | SMCxPRO™ Reading Plate Seals |
| **28** | Universal Plate Cover | Fisher Scientific | 253623 | Assay Plate Cover |
| **29** | 500 mL Container | -- | -- | Wash Buffer Dilution |
| **30** | Micro-centrifuge Tubes | -- | -- | Sample storage, standard preparation |

**Reagents**

| Item # | Product Description | Supplier | Product Number | Product Use(s) |
|---|---|---|---|---|
| **31** | Elution Buffer | EMD Millipore | 02-0002-04 | Required for Erenna® Maintenance |
| **32** | SMC™ 10x Wash Buffer (1 L) | EMD Millipore | 02-0111-00 | Addition of Reagents |
| **33** | SMC™ 10x System/Wash Buffer with Proclin (1 L) | EMD Millipore | 02-0111-03 | Use in Erenna® Platform |
| **34** | De-ionized or Distilled Water | -- | -- | Dilution of 10X Wash Buffer |
| **35** | Sodium Azide Solution | EMD Millipore | 08591-1ML-F | Preservative |

*Please contact your technical services representative for additional information or assistance selecting required but not provided supplies.*

## TECHNICAL HINTS

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assays. The following notes should be review and understood before the assay is set up.

### Assay Hints

1. Wipe down bench and pipettes with 70% isopropanol before use.
2. It is important to allow all reagents to warm to room temperature (20 - 25°C).
3. Use sterile filter pipette tips and reagent trays to avoid contamination.
4. Pre-wet tips (aspirate and dispense within well) twice before each transfer.
5. The standards prepared by serial dilution must be used within 10 minutes of preparation.
    a. It is recommended that the standards are prepared as the last step prior to plate setup.
6. All washing must be performed with the wash buffer provided.
7. The recommend plate shaker settings are between #3-#7 to provide maximal orbital mixing without splashing liquid or causing cross-contamination.
8. After the assay is complete, the 384-well reading plate should be sealed and read immediately.
    a. For the Erenna® Immunoassay System – use heat-sealing foil.
    b. For the SMCxPRO™ Immunoassay System – use adhesive aluminum seals.
9. If the 384 reading plate cannot be read immediately, sealed plates may be stored at 2 - 8°C for up to 48 hours away from light.
    a. Bring plate to room temperature and centrifuge at 1,100 x g for 1 minute prior to reading.

### Instrument Hints

10. For optimal Erenna® performance, execute the following prime of the instrument before reading:
    a. Cycle Routine (10,000 µL at 1,000 µL/min)
    b. Bubble Test (200 µL at 1,000 µL/min)
    c. Complete Erenna® calibration prior to reading the plate.

***Note:*** *if carry-over is experienced, perform a clean routine using a 384-plate and 20 µL/well of:*

i. 3 wells of elution buffer (MilliporeSigma PN# 02-0002-03)
ii. 1 well of 10% bleach
iii. 5 wells of elution buffer (MilliporeSigma PN# 02-0002-03)

11. For Optimal SMCxPRO™ performance, perform ASSIST testing on a daily basis (ideally at beginning of the day before assay is prepared).

## PRECAUTIONS

- Use caution when handling biological samples. Wear protective clothing and gloves.
- Proclin-containing solutions and their containers must be disposed of in a safe way and in accordance with local, regional, and national regulations.
- The chemical, physical, and toxicological properties of Proclin 950 at 5% have not been thoroughly investigated. At this concentration, this biocidal preservative is not irritating to eyes and skin and may be detrimental if enough is ingested (in quantities above those found in this kit). Proclin 950 is a potential sensitizer by skin contact; prolonged or repeated exposure may cause allergic reactions in certain sensitive individuals. The potential for these adverse health effects is unknown for the highly diluted, small volume of Proclin in this kit but unlikely if handled appropriately with the requisite good laboratory practices and universal precautions. For full concentration information, please refer to the SDS.
- Components of this reagent kit contain approximately 0.08% sodium azide as a preservative. Sodium azide is a toxic and dangerous compound when combined with acids or metals. Solutions containing sodium azide should be disposed of properly.

**Full Hazard Labels**

**03-0178-00**

| | | | |
|---|---|---|---|
| Capture Label | 02-0557-00 | No Symbol Required | **Warning.** Combustible liquid. |
| Detection Label | 02-0574-00 |  | **Warning.** Combustible liquid. |

**K-03-0178-00**

| | | | |
|---|---|---|---|
| 10x Wash Buffer | 02-9987-00 |  | **Warning**: Causes serious eye irritation. Harmful to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| 10x System Buffer w/ 0.5% Proclin | 02-9983-00 |  | **Warning:** Causes serious eye irritation. Harmful to aquatic life with long lasting effects. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Avoid release to the environment. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |
| Optimization Assay Buffer C | 02-9976-00 |  | **Danger:** Causes serious eye damage. Very toxic to aquatic life. Toxic to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/ attention. |

# SECTION I – Antibody Labeling and Bead Coating

## 1. Capture and Detection Antibody Preparation

### A. Preparation of Antibodies

Antibodies need to be unlabeled and free of protein carrier such as BSA as well as supplemental reagents such as gelatin and glycerol to ensure success. Obtain carrier-free antibodies or purify these materials before proceeding. Testing both orientations of an antibody pair will require at least 1.0 mg of each antibody.

Some antibody stabilizers (including sodium azide) and amine-containing buffers (including Tris) will significantly reduce capture labeling. Follow the steps below to remove these prior to antibody coupling.

1. Bring all reagents to room temperature.

2. Dilute antibodies to approximately 1 mg/mL in Buffer 1. Confirm the capture and detection antibody concentrations by reading the absorbance at A280, using Buffer 1 as blank.

   ***E.g.*** *If the A280 = 1.22, the concentration is (1.22/1.4) = 0.87 mg/mL*

3. Using Beer's Law, $A=\varepsilon cl$, determine the antibody concentrations.

$$\text{Conc.}\,(\text{mg/mL}) = \text{A280}/1.4$$

***Equation 1: Antibody Concentration***

***Note:*** *this example assumes a 1 cm path length and molar extinction coefficient of 1.4*

4. Use the calculated antibody concentrations to calculate the volume of antibody solution needed to label 0.2-1.0 mg of antibody.

   ***E.g.*** *If labeling 200 µg of Antibody, the volume required is (0.20 mg/0.87 mg/mL) = 230 µL.*

5. Prepare two Ultra 4 30K filter tubes:
   a. Pre-wet the Ultra 4 30K filter tubes by adding 4 mL of Buffer 1.
   b. Centrifuge for 5 minutes at 3900 x g (3900 RCF), discarding flow through.

   ***Tip:*** *Five minutes is an approximate time for complete flow-through. If residual remains, extend centrifugation time*

6. Label four 15 mL conical tubes W1 and W2 each. Add the antibody volumes calculated in step 4 to the Ultra 4 30 K filter tubes labeled W1 and bring the volume up to 4.0 mL with Buffer 1.

   ***E.g.*** *If transferring 0.23 mL of Antibody, the volume of Buffer 1 required is (4.00 mL total – 0.23 mL Ab) = 3.77 mL Buffer 1.)*

7. Centrifuge for 10 minutes at 3900 x g (3900 RCF), saving flow through in conical tube W1.

8. Transfer the filter containing the antibody into conical tube W2 and bring the volume up the 4.0 mL with Buffer 1.

9. Centrifuge for 10 minutes at 3900 x g (3900 RCF), saving flow through in conical tube W2.

10. Mix and remove the concentrated antibody from the filter and transfer to an Eppendorf tube. Determine the approximate volume remaining.

***Tip:*** *Rinse the membrane with Buffer 1 to recover more antibody.*

11. Verify the antibody concentration by reading the absorbance at A280 (using Buffer 1 as the blank). If required, adjust the antibody volume to the approximate starting concentration using Buffer 1.

***Note:*** *If antibody yield is low, check the flow through tubes to extract the antibody by using new filters. After the concentration of the antibody is confirmed, discard the flow through tubes.*

## 2. Capture and Detection Antibody labeling

### A. Capture Antibody Labeling

1. Calculate the volume of Capture Label required as follows.

$$\text{Vol. Capture Label } (\mu\text{L}) = 6\ \mu\text{L x mg Antibody}$$

***Equation 2: Volume of Capture Label***

2. Add the calculated volume of Capture Label to the antibody tube. Vortex and incubate for 1 hour at room temperature.

***E.g.*** *labeling 200 µg requires (6 µL x 0.20 mg) = 1.2 µL of Capture Label.*

### B. Detection Antibody Labeling

1. Calculate the volume of the Detection Label required as follows.

$$\text{Vol. Detection Label } (\mu\text{L}) = 15\ \mu\text{L x mg Antibody}$$

***Equation 3: Volume of Detection Label***

2. Add the calculated volume of Detection Label to the antibody tube. Vortex and incubate for 1 hour at room temperature in the dark.

***E.g.*** *labeling 200 µg requires (15 µL x 0.20 mg) = 3.0 µL of Detection Label.*

## 3. Post Labeling Procedure

1. During the incubation, prepare 1X Buffer 3 by diluting the 5 mL of the 10x Buffer 3 into 45 mL of DI water.

*E.g. quenching 1.2 µL requires (1.2 µL x 5) = 6 µL of Buffer 2*

2. After incubation, calculate the volume of Buffer 2 required to the labeled antibodies to quench the reaction and mix by vortexing.

*Tip: Excess can be added to round up (e.g. 10 µL instead of 6 µL*

**Capture Antibody:**

$$\text{Vol. Buffer 2 } (\mu L) = 5 \text{ x Vol. Capture Label } (\mu L)$$

***Equation 4: Volume of Buffer 2 for Capture Ab quench***

**Detection Antibody**

$$\text{Vol. Buffer 2 } (\mu L) = 3.75 \text{ x Vol. Detection Label } (\mu L)$$

***Equation 5: Volume of Buffer 2 for Detection Ab quench***

3. Prepare two Ultra 4 30K filter tubes:
   a. Pre-wet the Ultra 4 30K filter tubes by adding 4.0 mL of Buffer 3.
   b. Centrifuge for 5 minutes at 3900 x g (3900 RCF), discarding flow through.

*E.g. quenching 3.0 µL requires (3.0 µL x 3.75) = 11.25 µL of Buffer 2*

*Tip: Excess can be added to round up (e.g. 15 µL instead of 11.25 µL*

*Tip: Five minutes is an approximate time for complete flow-through. If residual remains, extend centrifugation time*

4. Label eight 15 mL conical tubes W1, W2, W3, and W4.

5. Add the antibody volumes to the Ultra 4 30K filter tubes labeled W1. Bring the volume up the 4.0 mL with Buffer 3.

6. Centrifuge for 10 minutes at 3900 x g (3900 RCF), saving the flow through in conical tube W1.

7. Repeat the buffer exchange with 1X Buffer 3 three more times and save the flow through in tubes W2, W3, and W4.

8. Remove the labeled antibody from the filter (W4) and dilute to approximately 1.0 mg/mL in Buffer 3.

*Tip: Rinse the membrane with Buffer 3 to recover more antibody.*

9. Verify the antibody concentration by reading the absorbance at A280 (use 1X Buffer 3 as the blank).

***Note:** the concentration should be similar to the initial A280 reading. If the concentration is acceptable, discard the W1-W4 flow through.*

10. Transfer the antibodies to a labeled storage container.

11. Store the antibodies at 4°C. If desired, add $NaN_3$ to 0.1% as a preservative.

## 4. Coating of Microparticle (MP) Beads with Capture Antibody

This procedure describes the process of coating magnetic microparticle beads with the labeled capture antibody. The procedures described later in this protocol with require up to 60 µL, 110 µL, and 110 µL of stock coated beads per plate for sections II, III, and IV, respectively.

### A. Preparation and Coating of Microparticle Beads with Capture Antibody

1. Rotate the uncoated MP bead vial at room temperature for 30 minutes to fully resuspend the beads.

2. During the rotation, prepare 1x Wash Buffer by diluting the 5 mL of the 10x Wash Buffer into 45 mL of DI water.

3. Calculate the volume of the labeled capture antibody required to coat at the 12.5 µg IgG/mg beads and 25 µg IgG/mg bead.

$$\text{Vol. Capture Ab } (\mu L) = \frac{\text{Ab. Coating Ratio } (\frac{\mu g \text{ IgG}}{\text{mg MP}})}{\text{Ab. Conc } (\frac{\text{mg}}{\text{mL}})} \text{ X 1 mg of MP}$$

***Equation 4: Volume of Labeled Capture Antibody***

   a. ***Note:*** *the concentration of uncoated MP beads provided is 10 mg/mL, therefore, 100 µL is 1 mg of MP beads.*

***E.g.*** *to coat 1.0 mg of Beads at 12.5 µg IgG/mg Bead with 0.87 mg/mL Antibody, the required volume of Antibody is (12.5 µg IgG/mg bead / 0.87 mg/mL) x 1.0 mg = 14.4 µL*

4. Wash the volume of beads from **Equation 4** as follows:
   a. Label two Eppendorf tubes, 12.5 and 25.0 µg/mg.
   b. Transfer required volume of uncoated beads into labeled tubes.
   c. Place the tubes(s) containing the beads in a bench top magnetic bead separator.
   d. Allow beads to sit in magnet for ≥ 2 minutes. Visually confirm that all beads have been pulled toward the magnet.
   e. Aspirate the supernatant with a pipette and discard.
   f. Add 1.0 mL of 1X Wash Buffer to the tubes. Rotate the tube in the magnet holder 180° several times to wash the beads.

g. Allow the beads to sit in magnet ≥ 2 minutes, then remove and discard the Wash Buffer.
h. Repeat the wash step two more times using fresh 1X Wash Buffer.
i. Aspirate and discard the 1X Wash Buffer.

5. Add the calculated volume of capture antibody to each of the tubes followed by the calculated volume of wash buffer based on the mg of beads being labeled.

$$\text{Total Volume } (\mu L) = \text{Vol. Capture Ab. } (\mu L) + \text{Vol. Wash Buffer } (\mu L)$$

***Equation 5: Volume of Wash Buffer***

***E.g.*** *if coating 1.0 mg (100 µL of Beads) with 14.4 µL of Antibody, the required volume of 1X Wash buffer is (100 µL – 14.4 µL) = 85.6 µL.*

6. Incubate for 60 minutes at room temperature with gentle inversion on a rotator.

7. After incubation, wash the beads by placing in a bead magnetic separator and repeat wash **step 4**, five times.

8. After final wash, resuspend antibody coated MP beads in Coated Bead Buffer as follows:
   a. After final wash in above step, allow beads to sit in magnet for ≥ 2 minutes.
   b. Visually confirm that all MPs have been pulled to the magnet.
   c. Aspirate the supernatant with a pipette and discard.
   d. Add volume of Coated Bead Buffer to resuspend and block the coated beads at 10.0 mg/mL.
   e. Either block the coated beads for 1.0 hour at room temperature or overnight at 4°C rotating.

***E.g.*** *If blocking 2.0 mg of beads, the volume of Coated Bead Buffer required is (2.0 mg / 10.0 mg/mL) = 0.2 mL.*

9. Store the blocked beads at 4°C until use.

## B. Worksheet: Capture Antibody Labeling and Bead Coating

### Pre-Label

| Step | Result |
|---|---|
| Dilute Antibody to ~1 mg/mL in Buffer 1 | Vol. added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc.: (C) _____ mg/mL |
| Determine amount and volume of antibody to label | Ab Amount (A): _____mg (up to 1 mg)<br>Volume.: (A/C) _____ mL |
| Rinse Ultra-4 30K filter tube with 4 mL Buffer 1, 5 minutes at 3900 RCF | |
| Add Antibody to Ultra-4 30K filter tube | |
| Wash #1 with Buffer 1 (10 min.) | Ab. Vol.: _____ mL<br>+ _____mL Buffer = 4 mL |
| Wash #2 with Buffer 1 (10 min.) | Up to 4 mL Buffer 1 |
| Recovered Ab volume | Recovered Vol: _____ mL<br>Buffer 1 added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc.: (C) _____mg/mL |

### Labeling

| Step | Result |
|---|---|
| Add capture Label. Mix by vortexing. | Capture label added = _____µL |
| Incubate 1 hour at RT. | Start time: _____<br>End Time: _____ |
| Add volume of Buffer 2 to Quench Reaction | Volume of Buffer 2: _____µL |

**Post-label**

| Step | Result |
|---|---|
| Rinse new Ultra 4 30K filter tube with 4 mL 1x Buffer 3, 5 minutes at 3900 RCF | |
| Add labeled Ab to Ultra-4 30K filter tube | Ab vol: _____ mL |
| Wash #1 with1X Buffer 3 (10 min.) | Ab vol: _____ mL<br>+ _____ mL Buffer 3 = 4mL |
| Wash #2, #3, #4 with 1X Buffer 3, (10 min.) | Up to 4 mL Buffer 3 |
| Volume of Recovered antibody | Recovered Vol: _____ mL<br>Buffer 3 added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc: (C) _____mg/mL |

**MP Conjugation**

| Step | Result |
|---|---|
| Wash uncoated beads with 1x Wash Buffer 3 times. | |
| Add calculated volume of antibody to coat at 12.5 and 25 µg/IgG/mg bead. | Ab vol. added to coat 12.5 µg: _____µL<br>Ab vol. added to coat 25 µg: _____µL |
| Incubate 1 hour at RT, rotating. | Start time: _____<br>End Time: _____ |
| Wash beads with 1x Wash Buffer 5 times | |
| Add calculated volume of coated bead buffer to block beads at RT for 1hr, or overnight at 4°C rotating. | Start time: _____<br>End Time: _____ |

### C. Worksheet: Detection Antibody Labeling

See preceding protocol for more detailed information on each step.

**Pre-Label**

| Step | Result |
|---|---|
| Dilute Antibody to ~1 mg/mL in Buffer 1 | Volume Buffer 1 added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc.: (C) _____ mg/mL |
| Determine amount and volume of antibody to label | Ab Amount (A): _____mg (up to 1 mg)<br>Volume.: (A/C) _____ mL |
| Rinse Ultra-4 30K filter tube with 4 mL Buffer 1, 5 minutes at 3900 RCF | |
| Add Antibody to Ultra-4 30K filter tube | |
| Wash #1 with Buffer 1 (10 min.) | Ab. Vol.: _____ mL<br>+ _____mL Buffer = 4 mL |
| Wash #2 with Buffer 1 (10 min.) | Up to 4 mL Buffer 1 |
| Recovered Ab volume | Recovered Vol: _____ mL<br>Buffer 1 added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc.: (C) _____ mg/mL |

**Labeling**

| Step | Result |
|---|---|
| Add detection Label. Mix by vortexing. | Detection label added: _____µL |
| Incubate 1.0 hour at RT in the dark. | Start time: _____<br>End Time: _____ |
| Add volume of Buffer 2 to Quench Reaction | Volume of Buffer 2: _____µL |

**Post-label**

| Step | Result |
|---|---|
| Rinse new Ultra 4 30K filter tube with 4 mL 1x Buffer 3, 5 minutes at 3900 RCF | |
| Add labeled Ab to Ultra-4 30K filter tube | Ab vol: _____ mL |
| Wash #1 with1X Buffer 3 (10 min.) | Ab vol: _____ mL<br>+ _____ mL Buffer 3 = 4mL |
| Wash #2, #3, and #4 with 1 X Buffer 3, (10 min.) | Up to 4 mL Buffer 3 |
| Recovered Ab volume | Recovered Vol: _____ mL<br>Buffer 3 added: _____ mL |
| Read A280 of antibody | A280: _____<br>Conc: (C) _____ mg/mL |
| Read A650 of antibody | A650: _____<br>A650/A280 ratio: _____ |

# SECTION II – Immunoassay Feasibility Testing

## 1. Feasibility Testing

When determining feasibility of an assay, a matrix approach is the most efficient route to determine the optimal concentrations. It is recommended that a factorial of coating ratios (12.5 µg IgG/mg Bead and 25.0 µg IgG/mg Bead) and detection antibodies is tested against the analyte and coated MP beads per well to establish a signal:background (S:B) ratio. The starting analyte concentration should be determined based on estimated mid-range signal (e.g. ~10x desired LLoQ for Erenna®, ~25x for SMCxPRO™). On the SMCxPRO™, it is recommended to increase the starting concentration to 100 ng/mL, with the highest concentration used at 2000 ng/mL.

An example plate map is below:

***Note:*** *Plate map below is for one Antibody orientation. A second factorial is required to test reverse orientation.*

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Analyte (pg/mL) | µg IgG/mg MP | µg MP/well |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | | | | | | | | | | | | | 20 | 12.5 | 5 |
| **B** | | | | | | | | | | | | | 0 | | |
| **C** | | | | | | | | | | | | | 20 | | 10 |
| **D** | | | | | | | | | | | | | 0 | | |
| **E** | | | | | | | | | | | | | 20 | 25 | 5 |
| **F** | | | | | | | | | | | | | 0 | | |
| **G** | | | | | | | | | | | | | 20 | | 10 |
| **H** | | | | | | | | | | | | | 0 | | |
| | **50** | | | **100** | | | **500** | | | **1000** | | | **Detection Antibody (ng/mL)** | | |

***Figure 1: Suggested Plate Layout – Feasibility Testing***

**A. Preparation of Microparticle Beads**

1. Rotate MP bead vial at room temperature for 20-30 minutes to fully resuspend the beads.

2. Prepare MP beads in Assay Buffer to deliver 100 µL of 10 µg Bead/well and 5 µg Bead/well at each coating concentration in a 96 well plate.
    a. Label four 15 mL conical tubes: 2 tubes at 10 µg Bead/well (12.5 and 25 µg IgG/mg Bead) and 2 tubes at 5 µg Bead/well (12.5 and 25 µg IgG/mg Bead)

        **i. For 12.5 µg IgG/mg Bead:**
            1. Transfer 60 µL of 12.5 µg IgG/mg Bead stock coated MP beads into 6.0 mL of Assay Buffer to give 10 µg Bead/well. Mix by inversion.
            2. Transfer 2.0 mL of the 10 µg Bead/well beads into 2.0 mL of Assay Buffer to give 5 µg Bead/well beads. Mix by inversion.

        **ii. For 25.0 µg IgG/mg Bead:**
            1. Transfer 60 µL of 25.0 µg IgG/mg Bead stock coated MP beads into 6.0 mL of Assay Buffer to give 10 µg Bead/well. Mix by inversion.
            2. Transfer 2.0 mL of the 10 µg Bead/well beads into 2.0 mL of Assay Buffer to give 5 µg Bead/well beads. Mix by inversion.

3. Transfer 100 µL per well of the appropriate MP bead concentration into a 96-well Axygen plate per Plate Layout **(Fig. 1)**.

**B. Preparation of Analyte**

1. Prepare 6.0 mL of the desired analyte concentration spike. Transfer 100 µL per well of the analyte according to the plate layout **(Fig. 1)**.

2. Seal assay plate with clear adhesive plate seal, apply pressure to seal to prevent leaking and cross-contamination. Incubate for 2 hours at 25°C on microplate incubate / shaker (Jitterbug Setting #4).

3. When capture incubation is complete, wash plate per **Post-Capture Wash** instructions.
    a. Refer to **Appendix A.**

### C. Preparation of Detection Antibody

1. Dilute stock labeled detection antibody to 1,000 ng/mL, then filter using a 0.2 µM syringe filter and dilute further to prepare 600 µL of each detection antibody concentration to deliver 20 µL per well.

   An example dilution scheme shown below:

***Tip:*** *It is recommended to prepare a 10x intermediate stock solution (e.g. 1,000 ng/mL).*

| [Det. Ab] ng/mL | Volume (µL) | Assay Buffer (µL) | Total volume (µL) | Dilution Factor |
|---|---|---|---|---|
| 1000 | 10 µL of Stock | 1190 | 1200 | - |
| 500 | 600 µL of 1000 ng/mL | 600 | 1200 | 2 |
| 100 | 240 µL of 500 ng/mL | 960 | 1200 | 5 |
| 50 | 600 µL of 100 ng/mL | 600 | 1200 | 2 |

***Figure 2: Example Detection Antibody Dilution Scheme***

2. Transfer 20 µL of detection antibody per well for each detection concentration according to the plate layout (**Fig. 1**.). Seal assay plate with clear adhesive plate seal and apply pressure to seal to prevent leaking and cross-contamination. Incubate for 1.0 hour at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

3. When detection antibody incubation is complete, wash plate per **Post-Detection Wash**, **Post-Detection Shake**, and **Final Aspirate** instructions.
   a. Refer to **Appendix A.**

4. Elute, neutralize, and transfer assay plate contents into reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems.
   a. Refer to **Appendix B.**

## 2. Interpretation of Results (Feasibility Testing)

After having read the Feasibility Testing plate(s), export the assay data to a spreadsheet program. The resultant data will be in DE Counts or Response (RE) based on whether the Erenna® Immunoassay System or the SMCxPRO™ Immunoassay System was utilized.

The following section describes how to interpret the feasibility (matrix) test. Sample data provided in **Figure 3** below is based on the suggested plate layout, **Figure 1**.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | Analyte (pg/mL) | µg IgG/mg MP | µg MP/well |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | 931 | | | 1507 | | | 2270 | | | 2292 | | | 20 | 12.5 | 5 |
| **B** | 3 | | | 5 | | | 4 | | | 5 | | | 0 | | |
| **C** | 910 | | | 1417 | | | 2169 | | | 2488 | | | 20 | | 10 |
| **D** | 7 | | | 4 | | | 5 | | | 7 | | | 0 | | |
| **E** | 750 | | | 1204 | | | 1958 | | | 2107 | | | 20 | 25 | 5 |
| **F** | 3 | | | 7 | | | 3 | | | 5 | | | 0 | | |
| **G** | 696 | | | 1101 | | | 1864 | | | 1940 | | | 20 | | 10 |
| **H** | 2 | | | 2 | | | 4 | | | 7 | | | 0 | | |
| | **50** | | | **100** | | | **500** | | | **1000** | | | **Detection Antibody (ng/mL)** | | |

***Figure 3: Example Erenna® Feasibility Testing Data***

### A. Test Point and Background Signal

1. A higher test point signal and a lower background signal are preferable for assay development.

***Tip:*** *For comparable performance from different concentrations of the same components, select the*

# SECTION III – Immunoassay Prototype Evaluation

## 1. Evaluating Sensitivity and Dynamic Range

Once the optimal Antibody orientation and concentration and the optimal bead concentration has been determined, assay sensitivity and dynamic range can be evaluated. Preliminary reference samples can also be tested to determine quantifiability.

A suggested plate layout is shown below:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | STD 01 | STD 02 | STD 03 | STD 04 | STD 05 | STD 06 | STD 07 | STD 08 | STD 09 | STD 10 | STD 11 | STD 12 |
| **B** | STD 01 | STD 02 | STD 03 | STD 04 | STD 05 | STD 06 | STD 07 | STD 08 | STD 09 | STD 10 | STD 11 | STD 12 |
| **C** | STD 01 | STD 02 | STD 03 | STD 04 | STD 05 | STD 06 | STD 07 | STD 08 | STD 09 | STD 10 | STD 11 | STD 12 |
| **D** | *Sample 1* | | | | | | | | | | | |
| **E** | *Sample 2* | | | | | | | | | | | |
| **F** | *Sample 3* | | | | | | | | | | | |
| **G** | *Sample 4* | | | | | | | | | | | |
| **H** | *Sample 5* | | | | | | | | | | | |

***Figure 4: Suggested Plate Layout – Prototype Evaluation***

### A. Preparation of Microparticle Beads

1. Rotate MP bead vial at room temperature for 20-30 minutes to fully resuspend the beads.

2. Prepare ≥ 11.0 mL of blocked coated MP beads for each 96 well plate using the optimal conditions determined in **Section II**.

3. Transfer 100 µL of MP beads per well.

***E.g.*** *if using 10 µg MP/well, transfer 110 µL of 10.0 mg/mL MP beads to 10,890 µL of Assay Buffer. Mix by inversion.*

*If using 5 µg MP/well, transfer 55 µL of 10.0 mg/mL MP beads to 10,945 µL of Assay Buffer. Mix by inversion*

### B. Preparation of Standard Curve

1. Prepare the standard curve by performing 1:2 serial dilutions of the analyte Standard 1 for Standards 2 through Standard 11 in a 12-channel reagent reservoir. Standard 12 is the blank (Standard Diluent only). The starting analyte concentration should be determined based on **Section II** results.

An example standard curve dilution scheme is provided in **Figure 5**.

a. ***Note:*** *Dynamic range of assay will be empirically determined and can be adjusted to fit purpose.*

| Standard Point | [Analyte] pg/mL | Volume (µL) | STD Diluent (µL) | Total volume (µL) | Dilution Factor |
|---|---|---|---|---|---|
| STD01 | 100.00 | 10 µL of Stock | 990 | 1000 | N/A |
| STD02 | 50.00 | 500 µL of STD01 | 500 | 1000 | 2 |
| STD03 | 25.00 | 500 µL of STD02 | 500 | 1000 | 2 |
| STD04 | 12.50 | 500 µL of STD03 | 500 | 1000 | 2 |
| STD05 | 6.25 | 500 µL of STD04 | 500 | 1000 | 2 |
| STD06 | 3.13 | 500 µL of STD05 | 500 | 1000 | 2 |
| STD07 | 1.56 | 500 µL of STD06 | 500 | 1000 | 2 |
| STD08 | 0.78 | 500 µL of STD07 | 500 | 1000 | 2 |
| STD09 | 0.39 | 500 µL of STD08 | 500 | 1000 | 2 |
| STD10 | 0.20 | 500 µL of STD09 | 500 | 1000 | 2 |
| STD11 | 0.10 | 500 µL of STD10 | 500 | 1000 | 2 |
| STD12 | 0.00 | 500 µL of Std. Dil. | 500 | 1000 | N/A |

***Figure 5: Example Standard Dilution Scheme***

2. Transfer 100 µL per well of Standards or Samples to assay plate. Seal plate with clear adhesive plate seal, apply pressure to seal to prevent leaking and cross-contamination. Incubate for 2.0 hours at 25°C on micro incubator / shaker (Jitterbug Setting #4).

3. When incubation is complete, wash plate per **Post-Capture Wash** instructions.
    a. Refer to **Appendix A**

**C. Preparation of Detection Antibody**

1. Approximately 10 minutes prior to end of the analyte incubation, prepare ≥ 3.0 mL of Detection Antibody at the desired concentration determined in **Section II.**

2. When the detection incubation is complete, wash plate per **Post-Detection Wash**, **Post-Detection Shake**, and **Final Aspirate** instructions.
    a. Refer to **Appendix A**

3. Elute, neutralize, and transfer assay plate contents into reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems.
    a. Refer to **Appendix B**

## 2. Interpretation of Results (Prototype Evaluation)

After having read the Assay Buffer Optimization Assay, Export the assay data to a spreadsheet program. The resultant data will be in DE Counts or Response based on whether the Erenna® Immunoassay System or the SMCxPRO™ Immunoassay System was utilized.

The following section describes how to interpret the prototype evaluation.

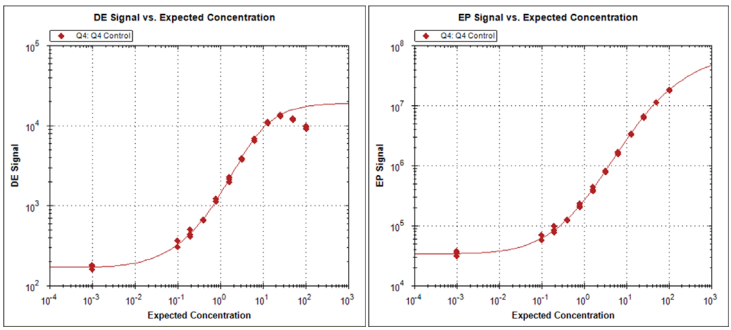


***Figure 6: Example Erenna® DE and EP signal Graphs***

***Erenna® Signals: DE / EP / TP  Interpolated Concentration***

| Std (pg/mL) | DE signal | EP signal | TP signal | Mean | SD | CV | Recovery |
|---|---|---|---|---|---|---|---|
| 100.00 | 8484 | 23358784 | 57788279 | 92.97 | 1.67 | *2%* | *93%* |
| 50.00 | 10924 | 14059955 | 32865905 | 45.70 | 1.74 | *4%* | *91%* |
| 25.00 | 13249 | 8100613 | 22755887 | 25.55 | 1.88 | *7%* | *102%* |
| 12.50 | 11509 | 3999155 | 17504679 | 12.65 | 0.67 | *5%* | *101%* |
| 6.25 | 7728 | 2041953 | 14981380 | 6.36 | 0.43 | *7%* | *102%* |
| 3.13 | 4335 | 960102 | 13604658 | 3.02 | 0.05 | *2%* | *97%* |
| 1.56 | 2354 | 487792 | 13114119 | 1.55 | 0.07 | *4%* | *99%* |
| 0.78 | 1293 | 252798 | 12606187 | 0.80 | 0.05 | *6%* | *102%* |
| 0.39 | 764 | 146593 | 12452015 | 0.42 | 0.04 | *9%* | *106%* |
| 0.20 | 472 | 91079 | 12314012 | 0.20 | 0.02 | *8%* | *102%* |
| 0.10 | 320 | 65032 | 12312233 | 0.09 | 0.01 | *21%* | *89%* |
| 0.00 | 178 | 37886 | 12325289 | - | - | - | - |

***Figure 7: Example Prototype Standard Curve (Erenna®)***

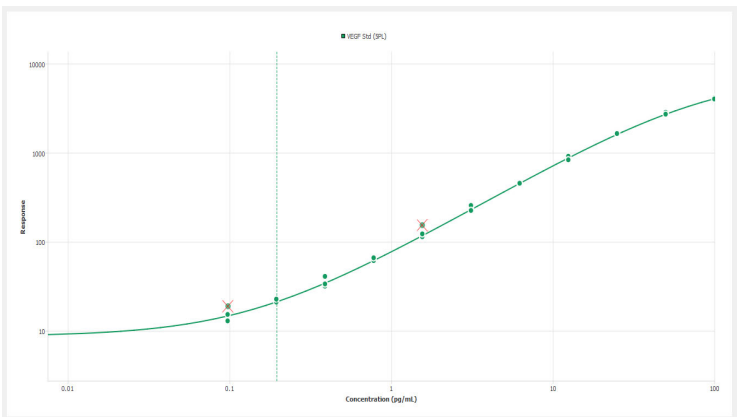


***Figure 8: Example SMCxPRO™ Graph***

| | *xPro Signal: Response* | | | *Interpolated Concentration* | | | |
|---|---|---|---|---|---|---|---|
| **Std (pg/mL)** | **Mean RE** | **SD** | **CV** | **Mean** | **SD** | **CV** | **Recovery** |
| 100.00 | 4062 | 65 | *2%* | 98.28 | 2.30 | *2%* | *98%* |
| 50.00 | 2764 | 69 | *3%* | 50.69 | 1.87 | *4%* | *101%* |
| 25.00 | 1660 | 32 | *2%* | 25.63 | 0.59 | *2%* | *103%* |
| 12.50 | 868 | 40 | *5%* | 12.23 | 0.61 | *5%* | *98%* |
| 6.25 | 447 | 8 | *2%* | 6.09 | 0.11 | *2%* | *98%* |
| 3.13 | 240 | 16 | *7%* | 3.22 | 0.21 | *7%* | *103%* |
| 1.56 | 117 | 6 | *5%* | 1.55 | 0.08 | *5%* | *99%* |
| 0.78 | 64 | 3 | *5%* | 0.81 | 0.04 | *5%* | *104%* |
| 0.39 | 35 | 5 | *14%* | 0.40 | 0.07 | *17%* | *101%* |
| 0.20 | 22 | 1 | *5%* | 0.21 | 0.02 | *8%* | *106%* |
| 0.10 | 14 | 2 | *12%* | 0.09 | 0.03 | *30%* | *87%* |
| 0.00 | 9 | 1 | *9%* | - | - | - | - |

***Figure 9: Example Prototype Standard Curve (SMCxPRO™)***

**A. Sensitivity**

1. The **LLoQ** is defined as the lowest standard point concentration at which the coefficient of variance (CV) is < 20%, the recovery is between 80% and 120%, and contains ≥ 2 replicates.
    a. **For Example:** In Figure 7 and Figure 9 above, the LLoQ is 0.20 pg/mL and shaded in grey.
    b. **Note:** the LLoQ in Figure 7 and Figure 9 is limited by the CV of the 0.10 pg/mL point. This can be improved by removal of an outlier to enhance precision.

**B. Dynamic Range**

1. The **Dynamic Range** is determined by the LLoQ and the Upper Limit of Quantification (ULoQ).
    a. Signals falling above the ULoQ are considered saturated (SAT).
    b. Signals falling below the LoD are considered not detectable (ND).

# SECTION IV – Assay Buffer Optimization Screening

## 1. Assay Buffer Optimization

To optimize the assay, it is recommended to perform a factorial of salt and detergent to determine the most stringent and robust assay conditions to provide optimal signal-to-noise ratio. The optimal analyte, MP, and detection antibody conditions determined in **Section II** and **Section III** should be utilized.

An example Assay Buffer Optimization Screen is below **(Fig 10.)**:

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Buffer 1 | | | Buffer 5 | | | Buffer 9 | | | Buffer 13 | | |
| B | | | | | | | | | | | | |
| C | Buffer 2 | | | Buffer 6 | | | Buffer 10 | | | Buffer 14 | | |
| D | | | | | | | | | | | | |
| E | Buffer 3 | | | Buffer 7 | | | Buffer 11 | | | Buffer 15 | | |
| F | | | | | | | | | | | | |
| G | Buffer 4 | | | Buffer 8 | | | Buffer 12 | | | Buffer 16 | | |
| H | | | | | | | | | | | | |

***Figure 10: Suggested Plate Layout – Assay Buffer Optimization***

**A. Preparation of Optimization Assay Buffers**

1. Bring all reagents to room temperature and label sixteen 15.0 mL conical tubes for Buffer #1 through Buffer # 16.

2. Prepare 5.0 mL of each Optimization Assay Buffer per **Figure 11** below:

| Buffer # | Assay Buffer A (mL) | Assay Buffer B (mL) | Assay Buffer C (mL) |
|---|---|---|---|
| **1** | 4.05 | 0.75 | 0.20 |
| **2** | 3.85 | 0.75 | 0.40 |
| **3** | 3.65 | 0.75 | 0.60 |
| **4** | 3.45 | 0.75 | 0.80 |
| **5** | 2.55 | 2.25 | 0.20 |
| **6** | 2.35 | 2.25 | 0.40 |
| **7** | 2.15 | 2.25 | 0.60 |
| **8** | 1.95 | 2.25 | 0.80 |

| Buffer # | Assay Buffer A (mL) | Assay Buffer B (mL) | Assay Buffer C (mL) |
|---|---|---|---|
| **9** | 1.80 | 3.00 | 0.20 |
| **10** | 1.60 | 3.00 | 0.40 |
| **11** | 1.40 | 3.00 | 0.60 |
| **12** | 1.20 | 3.00 | 0.80 |
| **13** | 1.30 | 3.50 | 0.20 |
| **14** | 1.10 | 3.50 | 0.40 |
| **15** | 0.90 | 3.50 | 0.60 |
| **16** | 0.70 | 3.50 | 0.80 |

***Figure 11: Optimization Assay Buffers Preparation***

3. Transfer a 1.0 mL and a 0.5 mL aliquot of each assay buffer to a 96-well deep well plate as per **Figure 12** below. The top 1000 µL aliquot will be used for test-point analyte spike; the bottom 500 µL aliquot will be used for detection antibody.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **A** | **Buffer 1:** | 1 mL | | **Buffer 5:** | 1 mL | | **Buffer 9:** | 1 mL | | **Buffer 13:** | 1 mL | |
| **B** | | 500 µL | | | 500 µL | | | 500 µL | | | 500 µL | |
| **C** | **Buffer 2:** | 1 mL | | **Buffer 6:** | 1 mL | | **Buffer 10:** | 1 mL | | **Buffer 14:** | 1 mL | |
| **D** | | 500 µL | | | 500 µL | | | 500 µL | | | 500 µL | |
| **E** | **Buffer 3:** | 1 mL | | **Buffer 7:** | 1 mL | | **Buffer 11:** | 1 mL | | **Buffer 15:** | 1 mL | |
| **F** | | 500 µL | | | 500 µL | | | 500 µL | | | 500µL | |
| **G** | **Buffer 4:** | 1 mL | | **Buffer 8:** | 1 mL | | **Buffer 12:** | 1 mL | | **Buffer 16:** | 1 mL | |
| **H** | | 500 µL | | | 500 µL | | | 500 µL | | | 500 µL | |

***Figure 12: Suggested Deep-well Plate Layout – Assay Optimization***

**B. Preparation of Analyte**

1. Prepare 6.0 mL of the desired analyte concentration spike in **Standard Diluent**.

**C. Preparation of Microparticle Beads**

1. Rotate MP bead vial at room temperature for 20-30 minutes to fully resuspend the beads.

2. Transfer MP beads to each 1.0 mL aliquot of optimization assay buffer
    a. Based on the results observed during **Section II and III**, prepare the better concentration of MP beads per well.
        i. To prepare 5 µg MP/well, add 5 µL of beads to 995 µL of each optimization assay buffer. Mix by pipette.
        ii. To prepare 10 µg MP/well, add 10 µL of beads to 990 µL of each optimization assay buffer. Mix by pipette.

3. Transfer MP beads, analyte, and standard diluent to assay plate as per plate layout **(Fig. 13 and Fig. 14**)
    a. Transfer 100 µL per well of coated MP beads per plate layout.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Buffer 1 100 µL coated beads/well | | | Buffer 5 100 µL coated beads/well | | | Buffer 9 100 µL coated beads/well | | | Buffer 13 100 µL coated beads/well | | |
| B | | | | | | | | | | | | |
| C | Buffer 2 100 µL coated beads/well | | | Buffer 6 100 µL coated beads/well | | | Buffer 10 100 µL coated beads/well | | | Buffer 14 100 µL coated beads/well | | |
| D | | | | | | | | | | | | |
| E | Buffer 3 100 µL coated beads/well | | | Buffer 7 100 µL coated beads/well | | | Buffer 11 100 µL coated beads/well | | | Buffer 15 100 µL coated beads/well | | |
| F | | | | | | | | | | | | |
| G | Buffer 4 100 µL coated beads/well | | | Buffer 8 100 µL coated beads/well | | | Buffer 12 100 µL coated beads/well | | | Buffer 16 100 µL coated beads/well | | |
| H | | | | | | | | | | | | |

***Figure 13: Suggest Plate Layout – Coated Beads***

b. Transfer 100 µL of analyte to **Rows A**, **C**, **E**, and **G**.
c. Transfer 100 µL of Standard Diluent to **Rows B**, **D**, **F**, **H**.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Buffer 1 + Analyte | | | Buffer 5 + Analyte | | | Buffer 9 + Analyte | | | Buffer 13 + Analyte | | |
| B | Buffer 1 + Standard Dil. | | | Buffer 5 + Standard Dil. | | | Buffer 9 + Standard Dil. | | | Buffer 13 + Standard Dil. | | |
| C | Buffer 2 + Analyte | | | Buffer 6 + Analyte | | | Buffer 10 + Analyte | | | Buffer 14 + Analyte | | |
| D | Buffer 2 + Standard Dil. | | | Buffer 6 + Standard Dil. | | | Buffer 10 + Standard Dil. | | | Buffer 14 + Standard Dil. | | |
| E | Buffer 3 + Analyte | | | Buffer 7 + Analyte | | | Buffer 11 + Analyte | | | Buffer 15 + Analyte | | |
| F | Buffer 3 + Standard Dil. | | | Buffer 7 + Standard Dil. | | | Buffer 11 + Standard Dil. | | | Buffer 15 + Standard Dil. | | |
| G | Buffer 4 + Analyte | | | Buffer 8 + Analyte | | | Buffer 12 + Analyte | | | Buffer 16 + Analyte | | |
| H | Buffer 4 + Standard Dil. | | | Buffer 8 + Standard Dil. | | | Buffer 12 + Standard Dil. | | | Buffer 16 + Standard Dil. | | |

***Figure 14: Suggested Plate Layout – Analyte and Standard Diluent Wells***

4. Seal assay plate with clear adhesive plate seal, apply pressure to seal to prevent leaking and cross-contamination. Incubate for 2.0 hours at 25°C on microplate incubator / shaker (Jitterbug Setting #4)

5. When capture incubation is complete, wash plate per **Post-Capture Wash** instructions
   a. Refer to **Appendix A**

**D. Preparation of Detection Antibody**

1. Approximately 10 minutes prior to end of the analyte incubation, prepare the Detection Antibody. Based on results from **Section II and III**, prepare 1.0 mL of a 20x intermediate dilution (if feasible based on stock concentration) in Buffer 1.

Filter the intermediate diluted antibody into a clean tube with a 0.2 µM syringe filter.

2. Dilute the intermediate detection antibody stock 20-fold by adding 25 µL of the intermediate stock into the 475 µL aliquot of each optimization assay buffer in the deep-well plate.
    a. Refer to **Figure 12** for suggested plate layout.

3. Seal assay plate with clear adhesive plate seal, apply pressure to seal to prevent leaking and cross-contamination. Incubate for 1.0 hour at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

4. When the detection incubation is complete, wash plate per **Post-Detection Wash**, **Post-Detection Shake**, and **Final Aspirate** instructions.
    a. Refer to **Appendix A.**

5. Elute, neutralize, and transfer assay plate contents into reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems.
    a. Refer to **Appendix B.**

## 2. Interpretation of Results (Assay Optimization)

After having read the Assay Buffer Optimization Assay, Export the assay data to a spreadsheet program. The resultant data will be in DE Counts or Response based on whether the Erenna® Immunoassay System or the SMCxPRO™ Immunoassay System was utilized.

The following section describes how to interpret the optimization assay buffer screen.

### A. Example Assay Buffer Optimization Data

1. **Test Point:** The measured signal for the selected concentration of analyte.
2. **Background:** The measured signal for the background well
3. **Slope:** The average rate of increase in signal per analyte concentration up to the first point with ≥ 1000 DE Count or ≥ 50 Response.
4. **Signal:Background (S:B) Ratio:** the ratio of test point signal to background
    a. **Note:** in the examples below, Assay Buffer #7 was determined to be the optimal condition as it provided the highest S:B ratio.

| Assay Buffer | Test Point (DE Count) | Background (DE Count) | Slope (DE/pg/mL) | S:B Ratio |
|---|---|---|---|---|
| **# 1** | 2412 | 87 | 3874 | 28 |
| **# 2** | 2413 | 70 | 3905 | 34 |
| **# 3** | 2271 | 69 | 3670 | 33 |
| **# 4** | 2347 | 64 | 3804 | 36 |
| **# 5** | 3220 | 64 | 5259 | 50 |
| **# 6** | 3096 | 71 | 5042 | 44 |
| **# 7** | **3046** | **56** | **4984** | **55** |
| **# 8** | 3061 | 72 | 4982 | 43 |
| **# 9** | 3300 | 73 | 5378 | 45 |
| **# 10** | 3245 | 63 | 5304 | 52 |
| **# 11** | 3106 | 72 | 5056 | 43 |
| **# 12** | 3031 | 73 | 4930 | 42 |
| **# 13** | 3276 | 61 | 5358 | 53 |
| **# 14** | 3319 | 73 | 5410 | 45 |
| **# 15** | 3022 | 82 | 4899 | 37 |
| **# 16** | 2904 | 76 | 4714 | 38 |

***Figure 15: Example Optimization Screening Data (Erenna®)***

| Assay Buffer | Test Point (Response) | Background (Response) | Slope (RE/pg/mL) | S:B Ratio |
|---|---|---|---|---|
| **# 1** | 143 | 5 | 230 | 30 |
| **# 2** | 140 | 4 | 227 | 39 |
| **# 3** | 131 | 4 | 210 | 30 |
| **# 4** | 133 | 3 | 216 | 41 |
| **# 5** | 188 | 4 | 307 | 48 |
| **# 6** | 186 | 3 | 304 | 54 |
| **# 7** | **209** | **3** | **343** | **62** |
| **# 8** | 183 | 4 | 298 | 46 |
| **# 9** | 195 | 4 | 317 | 44 |
| **# 10** | 212 | 4 | 347 | 56 |
| **# 11** | 189 | 4 | 308 | 46 |
| **# 12** | 182 | 4 | 296 | 42 |
| **# 13** | 188 | 3 | 308 | 58 |
| **# 14** | 189 | 5 | 307 | 40 |
| **# 15** | 179 | 5 | 291 | 39 |
| **# 16** | 168 | 4 | 273 | 45 |

***Figure 16: Example Optimization Screening Data (SMCxPRO™)***

**B. Assay Buffer Optimization Considerations**

1. **Very Low Signals** may be less reproducible. Consider the precision (%CV) of the 0.0 pg/mL replicates before choosing an optimized buffer condition.
    a. **Erenna® Immunoassay System:** < 50 DE Count may be too low.
    b. **SMCxPRO™ Immunoassay System:** < 2 Response may be too low.

2. **High Background Signal** suggests the opportunity for further optimization. Achieving a very low background will also be limited by choice and/or availability of antibodies and the target. Consider an appropriate sensitivity for the target and sample matrix before further optimization.
    a. **Erenna® Immunoassay System:** > 200 DE Count background signals.
    b. **SMCxPRO™ Immunoassay System:** >10 Response background signals.

3. **Salt and Detergent Concentrations** may impact the biological integrity of critical components when measuring analyte in biological samples. Consider the assay target if choosing very high or very low salt/detergent conditions.

# SECTION V – Appendices

# APPENDIX A – SMC™ Plate-Washing Guide

## Post-Capture Wash

Wash plate once with a plate washer.

**Plate Washer**

a. BioTek; Post Capture Wash (POSTCAP) or
b. HydroFlex; Post Capture Wash (PCW)

If using automation, please contact your technical service representative for the appropriate automation procedure.

## Post-Detection Wash

Wash assay plate 4 times with a plate washer.

**Plate Washer**

a. BioTek; 4 cycle Pre-Transfer (4CYCPRE) or
b. HydroFlex; 4 cycle Pre-Transfer (*4cyPrTra)*

If using automation, please contact your technical service representative for the appropriate automation procedure.

## Post-Detection Shake

1. After 4 cycle Pre-Transfer wash, visually verify that each well contains ~200 µL of wash buffer.
2. Seal assay plate with clear adhesive plate seal and apply pressure to the seal to prevent leaking and cross-contamination.
3. Place plate on microplate/incubator shaker for 2 min (Jitterbug setting #3)
4. Remove the plate from the Jitterbug, carefully remove clear adhesive plate seal to avoid splashing and place it on the plate washer to perform Final Aspiration.

## Final Aspiration

**Plate Washer**

a. BioTek; Final Aspirate (FINASP)
b. HydroFlex; Final Aspirate (FA_V1)

# APPENDIX B – SMC™ Immunoassay Reading Guide

## To Read on the Erenna® Immunoassay System

1. Dispense 10 µL Elution Buffer B per well using reverse pipetting without disturbing the bead pellet. Seal assay plate with a clear adhesive plate seal. Incubate plate for 10 minutes at 25°C on microplate incubator/shaker (Jitterbug setting #5).
2. Add 10 µL per well of Buffer D using reverse pipetting to Erenna® reading plate (Fisher Scientific PN 12-565-384) using a 12-channel manual P20.
3. Place assay plate onto sphere mag plate and allow beads to form a tight pellet for ≥ 2 minutes.
4. While keeping assay plate on the sphere mag plate, gently remove clear adhesive plate seal and transfer 10 µL of eluate from assay plate to reading plate by aspirating directly from the v-bottom of the plate, avoiding the pelleted beads, and changing tips with each dispensed row.
5. Seal reading plate with clear adhesive plate seal. Centrifuge plate for 1 minute at RT, approximately 1,100 *x g*.
6. Seal reading plate with heat sealing foil (Fisher Scientific PN NC0276513) according to manufacturer's instructions for the heat sealer.
7. Load completed reading plate onto the Erenna® Immunoassay System.

## To Read on the SMCxPRO™ Immunoassay System

1. Dispense 10 µL Elution Buffer B per well using reverse pipetting without disturbing the bead pellet. Seal assay plate with a clear adhesive plate seal. Incubate plate for 10 minutes at 25°C on microplate incubator/shaker (Jitterbug setting #5).
2. Secure the plate holder to the bottom of the SMCxPRO™ reading plate (EMD Millipore PN 02-1008-00).
3. Add 10 µL per well of Buffer D using reverse pipetting to the reading plate using a 12-channel manual P20.
4. Place assay plate onto sphere mag plate and allow beads to form a tight pellet for 2 minutes.
5. While keeping assay plate on the sphere mag plate, gently remove clear adhesive plate seal and transfer 10 µL of eluate from assay plate to reading plate by aspirating directly from the v-bottom of the plate, avoiding the pelleted beads, and changing tips with each dispensed row.
6. Place reading plate on plate holder and either cover with plate lid or seal with clear adhesive plate seal.
7. Place reading plate (on plate holder) into Jitterbug and shake for 1 minute at 25°C (Jitterbug setting #7), centrifuge plate for 1 minute at RT, approximately 1,100 *x g*.

   ***Alternative to Shaking option***
   *If operator elects not to shake the plate at the neutralization step, the plate may be stored at room temperature, sealed and light protected, for a minimum of 30 minutes to allow the neutralization process to reach equilibrium by simple diffusion.*

8. Seal reading plate with SMCxPRO™ aluminum adhesive plate seal.
9. Remove the plate holder and load the sealed reading plate onto the SMCxPRO™ Immunoassay System. Start read.

*Note: there is a smart warm up period of up to 30 minutes to wait for the read plate to be close to the internal instrument temperature. Once achieved the read will start automatically.*

## APPENDIX C – SMC™ Trouble-Shooting Guide

| Problem | Probable Cause | Solution |
|---|---|---|
| Beads are lost during the wash | Plate washer needs optimization/cleaning | Contact Tech Support or local BCS to schedule washer programming. Refer to user guide for cleaning procedure. |
| | Insufficiently primed washer | Washer should be primed with wash buffer prior to running the post capture wash protocol. |
| Beads are lost during the wash (continued) | Beads came in contact with water | Washer should be primed with wash buffer sufficiently prior to plate wash. Viscosity of water changes the performance of the magnetic particles. |
| | Proper magnet was not used | Ensure that the mag plate (EMD Millipore PN 90-0003-02) was present on plate wash stage prior to running wash protocol. |
| Microparticles do not resuspend into homogenous solution | Beads were not properly stored and may have been frozen | Labelled microparticles should be stored at 4°C. If microparticles are frozen they will not resuspend properly. |
| | Samples may be causing interference due to excess particulate matter | Samples should be properly processed prior to testing to remove particulate matter or lipids. |

## ORDERING INFORMATION

To place an order or to obtain additional information about SMC™ products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

**Conditions of Sale**

For Research Use Only. Not for Use in Diagnostic Procedures.

**Safety Data Sheets (SDS)**

Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at emdmillipore.com/msds

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