SMC™ Immunogenicity Bead Based Assay Development Kit

Microparticle Assay

Catalog # 03-0175-00

Guidelines for Bridging Immunogenicity Bead Based Assay Development

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Manufactured & Distributed by:

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INTRODUCTION

The Single Molecule Counting (SMC™) technology is a fluorescent based immunoassay technique offering superior sensitivity, dynamic range and assay development capabilities. With up to 5-logs of dynamic range SMC™ is ideal for reducing the need for multiple dilutions of sample. SMC™ uses digital counting for low level protein detection and offers a number of advantages with a unique platform design, in addition to specialized chemistry for enhanced specificity. Digital counting affords advantages such as ease of use, low volume sample requirement, greater sensitivity and excellent inter- and intra-assay precision.

SMC™ enables the detection of anti-drug antibodies (ADA) for multiple therapeutics (e.g. biologics), using a homogenous species-independent assay format that is simple, easy to design and validate. This assay format is often referred to as a "bridging assay" since the ADA acts as a bridge between the capture and detection labeled drug Figure 1, which is captured onto streptavidin beads. With the reduced number of wash steps, this aids in the detection of low affinity antibodies and decreases overall assay time. Elution buffer is then added to dissociate the complex, and the eluate containing the labeled Alexa Fluor drug and ADA (in sample) is transferred to the final reading microplate. The plate is loaded into the Erenna® or SMCxPRO™ System where labeled molecules are detected and counted. The number of fluor-labeled detection antibodies counted is directly proportional to the amount of ADA captured in the sample.

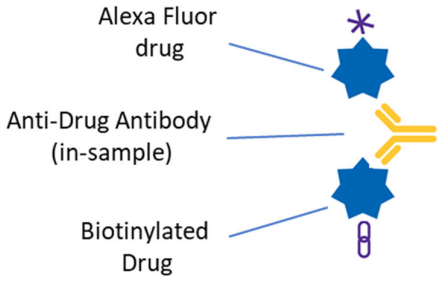

Figure 1. Bridging Assay format on Beads

By using a 642nm laser focused 250 μm from the base of an Aurora plate, a rotating objective scans through the eluate, exciting fluorochromes as they pass through the interrogation space. The focused interrogation space of acquisition reduces cross talk from well to well, flare from meniscus diffusion of light, as well as inherent interference from turbid solutions. A low noise Avalanche Photodiode (APD) then counts individual photons as they are emitted. The xPro software interprets the digital read-out as fluoresence response.

The SMC™ immunogencity assay kit includes capture and labeling reagents as well as buffers for the development of ADA assays on the SMC™ technology. Considerations such as those listed below can be easily studied with the SMC technology platform:

- Sensitivity/Dynamic range of the assay
- Cut point/Matrix Tolerance
- Drug tolerance
- Acid Dissociation
- Reproducibility

This guide is an aid to the development of an immunogenicity assay on the SMC technology. *Please note further optimization is required to fully validate for the system being studied.

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SUPPLIES

The SMC™ Immunogenicity package includes all reagents listed in *Table 1: Reagents Provided*. Additional reagents and supplies are required to run this immunoassay and are listed in *Table 2: Additional Supplies Required (not provided)*. All reagents supplied are for Research Use Only.

Table 1: Reagents Provided

Item #	Description	Shipping Conditions	Storage Conditions	Component Part No.	Packaging Details
1	Capture Label	On dry ice	≤ -70ºC	02-0557-00	1 x 20 µL
2	Detection label	On dry ice	≤ -70ºC	02-0574-00	1 x 20 µL
3	Buffer 1	With cold pack	2 - 8ºC	02-0552-00	2 x 25 mL
4	Buffer 2	With cold pack	2 - 8ºC	02-0553-00	1 x 500 µL
5	Buffer 3 (10x)	With cold pack	2 - 8ºC	02-0554-00	1 x 5 mL
6	Filter Tube Ultra 4	Cold Pack	2 – 8 °C	02-0556-00	4 x 2 Pack
7	10X Wash Buffer	With cold pack	2 - 8ºC	02-0001-03	1 x 5 mL
8	Coated Bead Buffer	With cold pack	2 - 8ºC	02-0558-00	1 x 5 mL
9	Uncoated SMC Beads	With cold pack	2 - 8ºC	02-0559-00	1 x 4 mL
10	Discovery Standard Diluent	With cold pack	2 - 8ºC	02-0560-00	1 x 500 mL
11	Elution Buffer B	With cold pack	2 - 8ºC	02-0297-00	1 x 100 mL
12	Buffer D	With cold pack	2 - 8ºC	02-0368-00	1 x 100 mL
13	10X System/Wash Buffer w/Proclin	With cold pack	2 - 8ºC	02-0111-03	1 x 1 Liter

Storage Instructions

The SMC™ immunogenicity Assay Development Immunoassay Kit should be stored at 2 - 8°C.

Supplied 10X Wash Buffer does not contain preservative. After dilution, the 1X Wash Buffer may be filter sterilized with Stericup® filter, EMD Millipore PN SCGPU11RE 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)

Materials

Item #	Product Description	Supplier	Product Number	Product Uses
1	Manual Pipettes 1 - 5000 µL	--	--	Transferring reagents
2	12-Channel Manual Pipette 10 – 20 µL	--	--	Transferring 10 µL
3	12-Channel Manual Pipette 20 – 250 µL	--	--	Transferring 20 µL, 100 µL
4	Tube Rotator	--	--	Microparticle resuspension
5	15 mL and 50 mL Conical tubes	--	--	Reagent preparation
6	Magentic Bead separator	EMD Millipore	LSKMAGS08	Magentic bead separation
7	Sodium Azide (NaN_3) (optional)	--	--	Preservative
8	Sphere Mag Plate	EMD Millipore	90-0003-02	Capturing/pelleting microparticles
9	Jitterbug™ Microplate Incubator/Shaker	EMD Millipore	70-0009-00	Incubating/Shaking at 25°C
10	VWR® Microplate Shaker	VWR International	12620-926	Plate shaking for overnight incubation, if recommended
11	Bio-Tek ELx™ 405 Microplate Washer	EMD Millipore	95-0004-05	Automated plate washing option
12	Tecan Hydroflex™ Microplate Washer	EMD Millipore	95-0005-02	Automated plate washing option
13	Centrifuge able to reach speed of 1,100 x g and 3,000 x g	--	--	Centrifuging samples, plates
14	Spectrophotometer or Nanodrop capable of reading A280 and A650	--	--	Measuring absorbance/protein concentration and Fluor absorbance.
15	Micro-Centrifuge	--	--	Centrifuge reagents
16	12-Channel Reagent Reservoir (sterile)	Fisher Scientific	13-681-800	Standard curve dilution
17	VistaLab™ 25 mL Reservoirs	Fisher Scientific	21-381-27C	Addition of Reagents
18	MultiScreenHTS BV 96-Well Filter Plate	EMD Millipore	MSBVN1210	Sample filtration
19	96-well V-bottom plate	Fisher Scientific	14-222-241	Assay plate

Additional Supplies Required (not provided) continued

Materials

Item #	Product Description	Supplier	Product Number	Product Uses
20	5 mL Luer-Lok™ Syringe	Fisher Scientific	14-829-45	Alexa Fluor Drug filtration
21	0.2 µm Syringe Filter	EMD Millipore	SLGPR33RS	Alexa Fluor Drug filtration
22	Nunc™ Clear Adhesive Plate Seal	Fisher Scientific	236366	Sealing assay plate
23	384-well round bottom plates	Fisher Scientific	12-565-384	Erenna® reading plate
24	Heat sealing foil	Fisher Scientific	NC0276513	Sealing plates for Erenna® reading
25	1L Stericup® Filter; 0.22 µm	EMD Millipore	SCGPU11RE	Filter sterilizing Erenna® system buffer
26	SMCxPRO™ 384-well plate, 1 plate with adhesive seal	EMD Millipore	02-1008-00	SMCxPRO™ reading plate, seal
27	SMCxPRO™ 384-well plate, case of 32	EMD Millipore	ABB2-00160A	SMCxPRO™ reading plate
28	SMCxPRO™ aluminum adhesive plate seals	Fisher Scientific	276014	SMCxPRO™ reading plate seals
29	ALPS™ 50V Microplate Heat Sealer	EMD Millipore	70-0018-00	Heat sealing 384-well plates before Erenna Reading
30	Universal plate cover	Fisher Scientific	253623	Covers assay plate
31	500 mL Container	--	--	Wash Buffer Dilution
32	Micro-centrifuge tubes	--	--	Sample storage, standard preparation
33	Elution Buffer (5 mL)	EMD Millipore	02-0002-04	Required for Erenna® maintenance
34	SMC™ 10X Wash Buffer (1 L)	EMD Millipore	02-0111-00	Automated plate washing
35	De-ionized or Distilled water	--	--	Dilution of 10X Wash or System Buffer

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 assay. The following notes should be reviewed 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 recommended 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 plate should be read immediately.
    a. For Erenna® Immunoassay System, use heat sealing plate foil.
    b. For SMCxPRO™ Immunoassay System – use adhesive seal.
9. The plates may be stored at 2-8°C for up to 48 hours away from light if same day reading is not possible.
    a. After the assay is complete, seal the plate before storing at 2 - 8°C
        i. For Erenna® Immunoassay System, use heat sealing plate foil
        ii. For SMCxPRO™ Immunoassay System – use aluminum adhesive plate seal
    b. Bring to RT then centrifuge the plate 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-well plate and 20 µL/well:

i. *3 wells of elution buffer*
ii. *1 well of 10% bleach*
iii. *5 wells of elution buffer*

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

PRECAUTIONS

1. Use caution when handling biological samples. Wear protective clothing and gloves.
2. 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.

Ingredient, Cat #		Full Label	
Capture Label	02-0557-00	No symbol Required	**Warning.** Combustible liquid.
Detection label	02-0574-00	No symbol Required	**Warning.** Combustible liquid.
10X Wash Buffer	02-0001-03		**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.

SECTION I

LABELING OF DRUG WITH BIOTIN AND ALEXA FLUOR

The labeling protocol recommended is intended for the labeling of **0.3-1.0 mg** of a 150KDa antibody-based drug with Alexa Fluor and Biotin to capture onto paramagnetic microparticle (MP) beads. The labeling procedure suggested is to be used in conjunction with a bridging assay format to be developed for the SMC™ Immunoassay system.

Note: For different molecular weight drugs please contact EMD Millipore Tech Support.

Preparation of drug for labeling

Drugs need to be unlabeled and free of carrier proteins (e.g. BSA) and supplemental reagents such as gelatin and glycerol to ensure success.

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

1. Bring all labeling reagents to room temperature.
2. Dilute drug to approximately 1 mg/mL in Buffer 1.
3. Dilute 10X Buffer 3 by diluting the 5 mL of the 10X Buffer 3 into 45 mL of DI water.
4. Confirm the drug concentration by reading the absorbance at A280, using Buffer 1 as blank: Using Beer's Law A=Ɛ*cl*, determine the drug concentration:

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

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

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

5. Using the calculated drug concentration, determine the volume required to label 0.3-1 mg drug.

E.g.:To label 300 µg of drug: 0.30 mg/0.87 mg/mL= 345 µL drug.

6. Label two 15 mL Ultra 430K filter tubes, one with Alexa Fluor drug and the other with Biotin drug:
    a. Pre-wet the Ultra 4 30K filter tubes by adding 4 mL Buffer 1.
    b. Centrifuge for 5 minutes at 3900 x g (3900 RCF). Discard the flow through.
7. Add the drug volume calculated in step 5 to the respective filter tubes. Bring the volume up to 4 mL with Buffer 1. Centrifuge for 10 minutes at 3900 x g (3900 RCF). Save the flow through in the conical tube.

E.g.:0.345 mL drug + 3.65 mL Buffer 1 = 4 mL total volume

8. Transfer the filters containing the drug into a two new 15 mL conical tube and bring the volume up to 4 mL with Buffer 1. Centrifuge for 10 minutes at 3900 x g (3900 RCF). Save the flow through in conical tube.
9. Mix the concentrated drug in the filter tube and transfer to Eppendorf tubes. Determine the approximate volume recovered.

10. Verify the drug concentration by reading the absorbance at A280 (using Buffer 1 as the blank). *If required adjust the drug volume to the approximate starting concentration using Buffer 1.* After the concentration of drug is confirmed discard the flow through tubes. If drug yield is low, check the flow through tubes to extract the drug by using new filters

Tip: Use Buffer 1 to rinse the membrane to recover more drug

Drug conjugation

1. Calculate the volume of Biotin and Alexa fluor label required to conjugate to the drug.

E.g.:To label 300 µg of biotin drug requires (6 x 0.30) = 1.8 µL biotin label

Biotin labeling: volume of biotin label is 6 µL per 1 mg drug

$$\text{Biotin label (uL)} = 6 \text{ x drug (mg)}$$

Alexa Fluor labeling: volume of Alexa fluor label is 15 µL per 1 mg drug

$$\text{Alexa Fluor label (uL)} = 15 \text{ x drug (mg)}$$

2. Add the calculated volume of label to the respective tubes. Vortex and incubate for **1 hour** at room temperature. For Alexa Fluor Drug conjugation store in the dark at RT.

3. Calculate the volume of Buffer 2 required to the respective labeled drug to quench the reaction.

Biotin Drug: the volume of Buffer 2 is 5x the volume of biotin Label. Mix by vortex.

E.g.:Buffer 2: 1.8 µL x 5 µL= 9 µL to quench Biotin reaction.

$$\text{Buffer 2 (uL)} = \text{Biotin label (uL)} \text{ x } 5$$

Alexa Fluor Drug: the volume of Buffer 2 is 3.75x the volume of Alexa Label. Mix by vortex.

Tip:Excess can be added to round up (e.g. 10 µL instead of 9 µL)

$$\text{Buffer 2 (uL)} = \text{Alexa Fluor label (uL)} \text{ x } 3.75$$

4. Label 2 Ultra 4 30K filter tubes, W1:

   a. Pre-wet the Ultra 4 30K filter tubes by adding 4 mL Buffer 3.

   b. Centrifuge for 5 minutes at 3900 x g (3900 R.C.F). Discard the flow through.

5. Transfer each of the labeled drug volume to the Ultra 4 30K filter tubes, W1. Bring the volume up to 4 mL with Buffer 3. Centrifuge for 10 minutes at 3900 x g (3900 RCF). Save the flow through in conical tubes W1.

6. Label 3 additional 15 mL conical tubes W2, W3 and W4 for both the biotinylated and Alexa Fluor labeled drug. Repeat the buffer exchange with Buffer 3 three more times and save the flow through for each of the tubes.

7. Mix and transfer each of the labeled drug volumes from the filter tubes (W4) into eppendorf tubes. Determine the volume recovered. Verify the drug concentration by reading the absorbance at A280 (use Buffer 3 as the blank). *If required adjust the drug volume to the approximate starting concentration using Buffer 3.* If drug yield is low, check the flow through tubes to extract the drug by using new filters. If the concentration is acceptable, discard W1-W4 flow through.

E.g.: If A280 = 1.22 and A650 = 2.89 the Alexa Fluor labeling ratio is 2.4

8. Read the A650 to determine Alexa Fluor labeling ratio.

$$\text{Ratio} = \text{A650}/\text{A280}$$

9. Filter the labeled drug using the Ultrafree filter in a minicentrifuge for two minutes at maximum speed. Label and store the drug at 4°C in the dark. If desired, add NaN_3 to 0.1% as a preservative.

SECTION II

SMC™ BRIDGING IMMUNOASSAY DEVELOPMENT

1. Testing Feasibility

To determine feasibility of the labeled drug it is recommended that equal concentrations of capture (Biotin drug) and detection (Alexa Fluor drug) is tested against a serial dilution of ADA and MP Beads. A suggested plate map is below.

	ADA ng/mL	1	2	3	4	5	6	7	8	9	10	11	12
		Biotin + Alexa Fluor											
		0.25 μg/mL + 0.25 μg/mL			0.5 μg/mL + 0.5 μg/mL			1 μg/mL + 1 μg/mL			2 μg/mL + 2 μg/mL		
A	10,000	10 μg Beads per well											
B	1000												
C	100												
D	0												
E	10,000	20 μg Beads per well											
F	1000												
G	100												
H	0												

Figure 2: Sample plate map for Biotinylated Drug and AlexaFluor Drug titration

Preparation of Biotinylated and Alexa Fluor Drug MasterMix

1. Prepare a MasterMix of equal concentrations of Biotinylated drug and Alexa Fluor drug. Suggested starting concentrations are 2.0, 1.0, 0.5, and 0.25 μg/mL.

    a. Prepare 10 mL of biotinylated drug and Alexa Fluor drug at a final concentration of 2 μg/mL in standard diluent.

    b. Serially dilute to 2-fold in standard diluent to make 1.0, 0.5 and 0.25 μg/mL respectively.

Preparation of ADA

2. Prepare a 10x (100,000 ng/mL) intermediate stock of the ADA. Dilute the ADA accordingly to prepare 5 mL of each concentration.

ADA ng/mL	Dilution Scheme
10000	Add 500 μL of 100,000 ng/mL to 4500 μL of Standard diluent
1000	Add 500 μL of 10,000 ng/mL to 4500 μL of Standard diluent
100	Add 500 μL of 1,000 ng/mL to 4500 μL of Standard diluent
0	Add 5 mL of Standard diluent

3. In a 96 deep well block mix 300 μL of the MasterMix according to the plate map (Fig. 2).

4. Add 150 μL of the ADA to each of the wells according to the plate map.

5. Incubate either 2hrs RT or overnight shaking at 4°C.

Preparation of Microparticle Beads for Blocking

1. Rotate the uncoated MP bead vial at room temperature for 20-30 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. Wash the MP beads as follows:
    a. Add 300 μL (3 mg) of MP beads into an Eppendorf tube. Place the tube in a bench top magnetic bead separator and allow beads to sit in magnet for ≥ 2 minutes. Visually confirm that all beads have been pulled toward the magnet.
    b. Aspirate the supernatant with a pipette and discard. Add 1 mL of Wash Buffer to the tube. Turn the tube in the magnet holder several times to wash the beads. Allow beads to sit in magnet for ≥ 2 minutes, remove and discard the Wash Buffer.

Note: Additional MP beads can be blocked for multiple experiments. Blocked MP beads can be stored at 4°C

c. Repeat the wash step two more times using fresh Wash Buffer. Aspirate and discard the final Wash Buffer.

4. Add equal volume of Coated Bead Buffer to block the beads at a 10 mg/mL. Rotate either for 1hr at RT or overnight at 4°C rotating.

E.g. If 3 mg of MP beads is used then block with 300 μL of coated bead buffer.

Preparation of MP Beads

5. Prepare blocked MP beads to deliver 100 μL of 20 μg/well and 10 μg/well:

   a. 20 μg/well: transfer 180 μL of 10 mg/mL MP beads to 8,820 μL of standard diluent. Mix by inversion.
   b. 10 μg/well: transfer 3 mL of the to 20 μg/well to 3 mL of standard diluent. Mix by inversion.

6. Post offline incubation, transfer 100 μL per well of blocked MP beads into a 96 well Axygen plate (Fig. 2) Transfer 100 μL per well of the bridged complex to the respective wells (Fig 2)

7. Seal plate with plate sealing film. Incubate for 1 hr. at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting #5).

Refer to Appendix I to complete the assay depending on which SMC™ platform, Erenna™ of SMCxPro™ is being used.

2. Factorial Titration of Biotin and Alexa Fluor Drug

Optimal response in the bridging assays should occur when the concentrations of capture (Biotin-drug) and detection (Alexa Fluor drug) are at equimolar levels in solution to provide equal competition for the ADA. However, in a homogenous assay the relative antibody affinity may differ and can compete, it is imperative to titrate these two reagents to determine optimal concentrations for use in the assay. Once the MP bead concentration is determined, titrate the Biotinylated drug and Alexa Fluor drug. A factorial approach is the most efficient route to determine the best concentrations of Biotinylated drug and Alexa Fluor drug to use. Suggested starting concentrations to evaluate are 0.25, 0.5, 1, and 2 μg/mL (Fig. 3).

	20 μg Beads per well											
	Biotin μg/mL	1	2	3	4	5	6	7	8	9	10	11	12
		Alexa Fluor μg/mL											
		0.25 μg/mL			0.5 μg/mL			1 μg/mL			2 μg/mL		
A	0.25	100 ng/mL			100 ng/mL			100 ng/mL			100 ng/mL		
B		0 ng/mL			0 ng/mL			0 ng/mL			0 ng/mL		
C	0.50	100 ng/mL			100 ng/mL			100 ng/mL			100 ng/mL		
D		0 ng/mL			0 ng/mL			0 ng/mL			0 ng/mL		
E	1.00	100 ng/mL			100 ng/mL			100 ng/mL			100 ng/mL		
F		0 ng/mL			0 ng/mL			0 ng/mL			0 ng/mL		
G	2.00	100 ng/mL			100 ng/mL			100 ng/mL			100 ng/mL		
H		0 ng/mL			0 ng/mL			0 ng/mL			0 ng/mL		

Figure 3: Sample plate layout for factorial experiment spiked at 250 ng/mL ADA into standard diluent and a zero spike (standard diluent) using 20 μg MP beads per well.

Preparation of Biotinylated Drug and Alexa Fluor Drug MasterMix

1. Prepare 8 mL each of biotin drug and Alexa Fluor Drug:

Example below:

Biotin and Alexa-Fluor Drug	Dilution Scheme
2 μg/mL	Prepare 8 mL of 2 μg/mL in Standard Diluent
1 μg/mL	Transfer 4 μL of 2 μg/mL to 4 mL Standard Diluent
0.5 μg/mL	Transfer 4 μL of 1 μg/mL to 4 mL Standard Diluent
0.25 μg/mL	Transfer 4 μL of 0.5 μg/mL to 4 mL Standard Diluent

Preparation of ADA

2. Prepare 8 mL of ADA sample at 250 ng/mL in Standard Diluent and 8 mL of Standard for the zero.

3. In a 96 deep well block transfer 150 μL/well of Biotinylated drug and 150 μL/well of Alexa drug at each of the concentration to each well according to the suggested plate map (Fig 3.). Transfer 150 μL of 250 ng/mL ADA to each well.

4. Incubate either for 2hrs RT or overnight shaking at 4°C .

Preparation of MP Beads

1. Each 96 well plate will require 11 mL of blocked MP beads at the desired concentration:

    a. *For example, for 20 μg MP bead per plate, transfer 220 μL of blocked MP beads into 10,780 μL of Standard Diluent*

2. Post incubation, transfer 100 μL per well of MP beads into a 96 well Axygen plate (Fig. 3) Transfer 100 μL per well of the bridged complex to the respective wells (Fig. 3)

3. Seal plate with plate sealing film. Incubate for 1 hr. at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting #5).

Refer to Appendix I to complete the assay depending on which SMC™ platform, Erenna™ of SMCxPro™ is being used.

3. Testing Sensitivity and Dynamic Range in Matrix

Once the optimal Biotin Drug and Alexa Fluor Drug well has been determined assay sensitivity and dynamic range can be evaluated in matrix to determine minimum required dilution (MRD),. Fig.4. provides an example playout of testing MRD, the ADA concentration range will have to be empirically determined.

	1	2	3	4	5	6	7	8	9	10	11	12
	ADA ng/mL											
	0% matrix		1% matrix		5% matrix		10%% matrix		25% matrix		100% matrix	
A	5000		5000		5000		5000		5000		5000	
B	1250		1250		1250		1250		1250		1250	
C	312.5		312.5		312.5		312.5		312.5		312.5	
D	78.13		78.13		78.13		78.13		78.13		78.13	
E	19.53		19.53		19.53		19.53		19.53		19.53	
F	4.88		4.88		4.88		4.88		4.88		4.88	
G	1.22		1.22		1.22		1.22		1.22		1.22	
H	0		0		0		0		0		0	

Figure 4: Sample of plate layout to test dynamic range and sensitivity in matrix

Preparation of MasterMix

Prepare 3 mL each of Biotin drug and Alexa Fluor drug in each test matrix at the optimal determined concentration. Each well will receive 100 μL of the MasterMix.

Preparation of Matrix

Prepare a series of diluted sample matrix; Suggested dilutions are 100%, 25%, 5%, 1% an 0% matrix. Dilute matrix in standard diluent.

Preparation of MP Beads

Each 96 well plate will require 11 mL of blocked MP beads at the desired concentration as described above.

Preparation of ADA

Prepare a 10x intermediate stock of the ADA. *For example, if 5000 ng/mL is the upper limit of the assay then prepare 50,000 ng/mL of the ADA. Dilute 4-fold accordingly*.

ADA ng/mL	Dilution Scheme
5000.00	Add 50 µL of 50,000 ng/mL to 450 µL of matrix
1250.00	Add 125 µL of 5,000 ng/mL to 375 µL of matrix
312.50	Add 125 µL of 1250 ng/mL to 375 µL of matrix
78.13	Add 125 µL of 312.50 ng/mL to 375 µL of matrix
19.53	Add 125 µL of 78.13 ng/mL to 375 µL of matrix
4.88	Add 125 µL of 19.53 ng/mL to 375 µL of matrix
1.22	Add 125 µL of 4.88 ng/mL to 375 µL of matrix
0	Add 500 µL matrix

1. In a 96 well deep well block mix 100 µL of Biotin drug and 100 µL of Alexa Fluor drug of each of the MasterMix and 100 µL of ADA/sample to each well.

2. Incubate either for 2hrs RT or overnight shaking at 4°C .

3. Post incubation, transfer 100 µL per well of MP beads into a 96 well Axygen plate Transfer 100 µL per well of the bridged complex to the respective wells (Fig. 4).

4. Seal plate with plate sealing film. Incubate for 1 hr. at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting #5).

Refer to Appendix I to complete the assay depending on which SMC™ platform, Erenna™ of SMCxPro™ is being used.

APPENDIX I
Post-Detection Wash

1. Wash assay plate 4 times with a plate washer (Wash Buffer PN 02-0111-03).

   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)

Elution

1. Dispense 11 µL Elution Buffer B per well using reverse pipetting without disturbing the bead pellet.
2. Seal assay plate with a clear adhesive plate seal.
3. Incubate plate for 10 minutes at 25°C on microplate incubator/shaker (Jitterbug setting #5).

ASSAY READING

To read on the Erenna® Immunoassay System:

1. Add 10 µL per well of Buffer D using reverse pipetting to Erenna® reading plate (Thermo Fisher PN 264573) using a 12-channel manual P20.
2. Place assay plate onto sphere magnet bed and allow beads to form a tight pellet for ≥ 2 minutes.
3. While keeping assay plate on the magnet bed, gently remove plate sealing film 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.
4. Seal reading plate with plate sealing film. Centrifuge plate for 1 minute at RT, approximately 1,100 *x g*.
5. Seal reading plate with heat sealing foil (EMD Millipore PN 02-01-0216-00) according to manufacturer's instructions for the heat sealer.
6. Load completed reading plate onto the Erenna® Immunoassay System.

To read on the SMCxPRO™ Immunoassay System:

1. Add 10 µL per well of Buffer D using reverse pipetting to the SMCxPRO™ reading plate using a 12-channel manual P20.
   NOTE: This plate comes individually wrapped with a lid; handle by touching only the sides of the plate. Leave the plate on the plate holder at all times except when loading into SMCxPRO™ for analysis.
2. Place Plate 1 onto sphere magnet bed (EMD Millipore PN 90-003-02), remove plate sealing film, and allow beads to form a tight pellet for 2 minutes.
3. Set manual 12-channel pipette to 10 µL and transfer eluate to reading plate by aspirating directly from the v-bottom of the plate, avoiding the pelleted beads, and changing tips with each dispensed row.
4. Cover reading plate with a universal plate cover and spin plate for 2 minutes at RT, approximately 1,100 *x g*.
5. Seal reading plate with adhesive plate sealer (Thermo PN 276014). If need to read on Erenna® after SMCxPRO™ then seal with piercable heat sealer (EMD Millipore PN 02-01-0216-00 or equivalent).
6. Remove the plate holder and load sealed reading plate onto the SMCxPRO™ Immunoassay System.
7. There is a warm up period of 30 minutes to bring the plate to the appropriate temperature prior to the start of a new plate read.
8. After 30 minutes, hit 'Start' to start the plate read.

For both platforms, if the assay plate cannot be read on the same day, the sealed reading plate can be stored at 4°C overnight (plate can be stored for up to 48 hours at 4°C). Note: For Aurora reading plates, ensure plate remains on the plate holder.

The following day remove the reading plate from 4 °C and bring to RT for 15 min, spin for 2 minutes at RT, approximately 1,100 x g; for the SMCxPRO™ platform, place in instrument, wait for 30 minutes and click 'start' to start plate reading.

SECTION III

Further Recommendations for Assay Optimization

Further optimization of different variables can take place to produce the most sensitive assay. These include:

- Drug concentration (capture and detection reagent)
- Assay Diluents (to mitigate HAMA or other interfering factors)
- Sample volume
- Incubation time
- Standard / sample diluent
- Determination of minimum required dilution (MRD)
- Evaluation of Drug interference / tolerance
- Acid Dissociation to Improve ADA quantification

To effectively address these issues, a number of different evaluations need to be considered:

i. Incubation time can have a large effect on immunogenicity assays due to the low affinities of the antibodies under investigation. Overnight incubations are standard but shorter times may be used once the assay has been optimized and guard bands established.
ii. In bridging assays of this type, it is important to minimize the amount of free (unlabelled capture or detection reagent) drug.
iii. The sensitivity of the SMC™ system allows for dilution of samples which can mitigate matrix effect & improve specificity. Undiluted samples should be serially diluted to determine the dilution at which linearity is observed.
iv. The use of acid dissociation of immune complexes has been proven to increase the drug tolerance of immunogenicity assays. This technique can be employed to increase the sensitivity of the assay under conditions where drug interference is a factor.

WORKSHEET FOR LABELING OF DRUG

See preceding labeling protocol for more detailed information for each step.

Biotinylated Drug	Alexa Fluor-Drug
Initial Drug Concentration	
A280: _____ Conc.: (C) _____ mg/mL	A280: _____ Conc.: (C) _____ mg/mL
Pre-label concentration	
A280: _____ Conc.: (C) _____ mg/mL	A280: _____ Conc.: (C) _____ mg/mL
Amount of drug labeled	
Ab Amount (A): _____mg (up to 1 mg) Volume.: (A/C) _____ mL	Ab Amount (A): _____mg (up to 1 mg) Volume.: (A/C) _____ mL
Post-label concentration	
A280: _____ Conc.: (C) _____mg/mL	A280: _____ Conc.: (C) _____mg/mL
	A650: _____ A650/A280 ratio: _____

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using seal appropriately. Pipette with multichannel pipets without touching reagent in plate. Change tips when adding reagents if cross contamination is expected.
		Ensure reagents (including wash and system buffers) are not contaminated.
		Insufficient washes—washer may need to be cleaned or reprogrammed.
	Instrument needs cleaning	See Technical Hints for appropriate Erenna® cleaning protocol.
Sample variability is high	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm that there is no residual left in the wells following post-capture wash step and Final Aspirate. Ensure that you have < 2 µL or residual remaining in the well.
	Samples may have high particulate matter or other interfering substances	Samples should be centrifuged or filtered according to the Assay Preparation section. Unprocessed samples could lead to higher imprecision.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing (~650 - 1000 RPM).
	Cross-well contamination	Ensure that the plate is sealed well at each incubation step. If splashing occurs on plate seal, centrifuge plate at 1,100 x g for 1 minute to remove material prior to removing the seal. A new plate seal should be used every time the plate is sealed.
		Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.
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.

TROUBLESHOOTING GUIDE (continued)

Problem	Probable Cause	Solution
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	Labeled 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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