SMC™ Plate-based Immunoassay Development Kit (5 plates)

Assay Development Kit

Catalog # 03-0179-00

SMC™ Plate-based Immunoassay Kit for the Development of 5 Plate-based Immunoassays

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Manufactured & Distributed by:

EMD Millipore Corporation
3050 Spruce Street
St. Louis, Missouri 63103
United States of America
emdmillipore.com

This page intentionally left blank.

TABLE OF CONTENTS

INTRODUCTION

The Single Molecule Counting (SMC™) Plate-based Immunoassay Development kit provides necessary reagents to 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 feasibility, prototype, and an optimization experiment.

An analyte-specific capture antibody is coated onto an assay plate and an analyte-specific detection antibody is fluorescently labeled. The user pipettes standards and samples into coated microplate wells. During incubation, the analyte in the sample binds to the capture antibody coated assay plate. 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 specifically captured onto the plate, 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 eluate (containing labeled detection antibody and analyte) is transferred to the final reading plate containing neutralizing buffer. The reading 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™ Plate-based Immunoassay Development kit contains detection labeling reagents, buffers for assay development feasibility and a set of Optimization Standard Diluents: Optimization Standard Diluent A (a proprietary mixture of blockers to decrease non-specific antibody binding), Optimization Standard Diluent B (high salt standard diluent), and Optimization Standard Diluent C (a high detergent diluent). The optimization of salt and detergent concentrations will minimize background, reduce non-specific binding, and improve immunoassay stringency.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

SUPPLIES

The SMC™ Plate-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	Detection Label	Dry Ice	< -70°C	02-0574-00	1 x 20 µL
2	Buffer 1	Cold Pack	2 – 8 °C	02-0552-00	2 x 25 mL
3	Buffer 2	Cold Pack	2 – 8 °C	02-0553-00	1 x 500 µL
4	Buffer 3 (10X)	Cold Pack	2 – 8 °C	02-0554-00	2 x 5 mL
5	Filter Tube Ultra 4	Cold Pack	2 – 8 °C	02-0556-00	2 x 2 Pack
6	Assay Buffer	Cold Pack	2 – 8 °C	02-0865-00	1 x 100 mL
7	Blocking Buffer	Cold Pack	2 – 8 °C	02-9980-00	3 x 50 mL
8	Plate Standard Diluent	Cold Pack	2 – 8 °C	02-0867-00	1 x 125 mL
9	Elution Buffer B	Cold Pack	2 – 8 °C	02-0297-00	1 X 100 mL
10	Buffer C	Cold Pack	2 – 8 °C	02-9988-00	1 x 15 mL
11	10x PBS	Cold Pack	2 – 8 °C	02-0869-00	1 x 10 mL
12	10x Wash Buffer	Cold Pack	2 – 8 °C	02-0111-00	1 x 1,000 mL
13	10x System Buffer w/ 0.5% Proclin	Cold Pack	2 – 8 °C	02-9983-00	1 x 100 mL
14	Optimization Standard Diluent A	Cold Pack	2 – 8 °C	02-9989-00	1 x 50 mL
15	Optimization Standard Diluent B	Cold Pack	2 – 8 °C	02-9986-00	1 x 50 mL
16	Optimization Standard Diluent C	Cold Pack	2 – 8 °C	02-9982-00	1 x 10 mL

Storage Instructions

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

The Detection Label should be stored at ≤ -70°C. Discard unused label.

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	Jitterbug™ Microplate Incubator/Shaker	EMD Millipore	70-0009-00	Incubating/Shaking at 25°C
4	Bio-Tek™ 405 TS Microplate Washer	EMD Millipore	95-0004-05	Automated Plate Washing
5	Centrifuge, with bucket rotors, capable of 3,900 x g	--	--	Centrifuging Samples and Filter Tubes
6	Micro-Centrifuge	--	--	Centrifuging Samples & Antibody
7	ALPS™ 50V Microplate Heat Sealer	EMD Millipore	70-0018-00	Heat sealing 384-well plates (Erenna)
8	Nanodrop™ or Spectrophotometer capable of measuring A280 and A650	--	--	Measuring Ab Conc. and Labeling

Materials

Item #	Product Description	Supplier	Product Number	Product Use(s)
9	12-Channel Reagent Reservoir (sterile)	Argos/Cole Parmer	04395-33	Standard Curve Dilution
10	VistaLab™ 25 mL Reagent Reservoirs	Fisher Scientific	21-381-27C	Addition of Reagents
11	MultiscreenHTS BV 96-well Filter Plate	EMD Millipore	MSBVN1210	Sample Filtration
12	Nunc™MaxiSorp™ 96-well round bottom polystyrene plate	ThermoFisher Scientific	449824	Assay Plate
13	96-well Deep-well Plate	--	--	Assay Buffer Optimization Screen
14	5 mL Luer-Lok™ Syringe	Fisher Scientific	14-829-45	Detection Antibody Filtration
15	0.2 µM Syringe Filter	EMD Millipore	SLGP033RS	Detection Antibody Filtration
16	Nunc™ Clear Adhesive Plate Seal	Fisher Scientific	236366	Sealing Assay Plate
17	384-well Round Bottom Plates	Fisher Scientific	12-565-384	Erenna® Reading Plate
18	Heat Sealing Foil	Fisher Scientific	NC0276513	Sealing plates for Erenna® Reading
19	1L Stericup® Filter; 0.22µM Filter	EMD Millipore	S2GPU11RE	Filter sterilizing System Buffer
20	SMCxPRO™ 384-well plate, 1 adhesive sealer	EMD Millipore	02-1008-00	SMCxPRO™ Reading plate, seal
21	SMCxPRO™ 384-well plates, case of 32	EMD Millipore	ABB2-00160A	SMCxPRO™ Reading Plates
22	SMCxPRO™ Adhesive Plate Seals	Fisher Scientific	276014	SMCxPRO™ Reading Plate
23	Universal Plate Cover	Fisher Scientific	253623	Assay Plate Cover
24	500 mL Container	--	--	Wash Buffer Dilution
25	Micro-centrifuge Tubes	--	--	Sample storage, standard

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 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 reservoirs 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

1. 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)

2. 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

03-0179-00

Detection Label	02-0574-00		**Warning.** Combustible liquid.

K-03-0179-00

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 Standard Diluent C	02-9982-00		**Warning.** Causes serious eye irritation. Very toxic to aquatic life. Toxic 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 – Detection Antibody Labeling and Plate Coating

A. Preparation of Detection Antibody for Labeling

Antibodies need to be unlabeled and free of carrier proteins such as BSA as well as supplemental reagents such as gelatin and glycerol to ensure success.

Some antibody stabilizers (including sodium azide) and amine-containing buffers (including Tris) will significantly reduce detection labeling efficacy. Testing both orientations of an antibody pair will require at least 0.5 mg of each.

1. Bring all reagents to room temperature. Leave the detection label vial in the dark.

2. Dilute antibody to approximately 1.0 mg/mL in Buffer 1. Confirm the antibody concentration 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=Ɛ*cl*, determine the antibody concentration:

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

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 concentration to calculate the volume of antibody solution needed to label 0.2-0.5 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. Label two 15 mL conical tubes: W1 and W2.

6. Add the provided Ultra 4 30K filter tube to W1 and pre-wet by adding 4.0 mL Buffer 1. 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*

7. Add the antibody volume calculated in step 4 to the Ultra 4 30K filter tube 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.*

8. Centrifuge for 10 minutes at 3900 x g (3900 RCF), retaining flow-through in conical tube W1 and transferring the Ultra 4 30K filter tube to W2.

9. After centrifugation, bring the volume in the Ultra 4 30K filter back up to 4.0 mL with Buffer 1 and centrifuge for another 10 minutes at 3900 x g (3900 RCF), saving the flow through in conical tube W2.

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

10. Mix the concentrated antibody in the filter by pipette and transfer to an Eppendorf tube. Determine the approximate volume remaining.

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 antibody is confirmed, discard the flow through tubes*

B. Detection Antibody Labeling

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

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

Equation 2: Volume of Detection Label

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

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

4. After incubation, calculate the volume of Buffer 2 required before adding to the labeled antibody to quench the reaction and mix by vortex.

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

Equation 3: Volume of Buffer 2

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

5. Label four 15 mL conical tubes W1, W2, W3, and W4.

6. Add the provided Ultra 4 30K filter tube to conical tube W1 and pre-wet by adding 4.0 mL Buffer 3. Centrifuge for 5 minutes at 3900 x g (3900 RCF), discarding flow through.

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

7. Add the antibody volume to the Ultra 4 30K filter tube and bring the volume up to 4.0 mL with Buffer 3.

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

8. Centrifuge for 10 minutes at 3900 x g (3900 RCF) retaining flow-through in conical tube W1 and transferring the Ultra 4 30K filter tube to W2.

9. Repeat the buffer exchange with 1X Buffer 3 three more times using conical tubes W2, W3, and W4.

   Note: *Flow-through should become progressively less blue as buffer exchange proceeds.*

10. Mix the concentrated antibody in the filter by pipette and transfer to an Eppendorf tube. Determine the approximate volume remaining.

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

11. Verify the antibody concentration by reading the absorbance at A280 (use 1X Buffer 3 as the blank.). If required, adjust the antibody volume to the approximate starting concentration using Buffer 3.

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

12. Read the A280 and A650 to determine the detection labeling ratio.

    Note*: Detection Labeling Ratio is (A650/A280).*

13. Store the antibody at 4°C in the dark. If desired, add NaN_3 to 0.1% as a preservative.

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 capture antibody and detection antibody concentrations is tested against the analyte 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™).

A suggested plate map is below:

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

	1	2	3	4	5	6	7	8	9	10	11	12	[Analyte] pg/mL	[Cap Ab]
A													50	4.0 μg/mL
B													0	
C													50	2.0 μg/mL
D													0	
E													50	1.0 μg/mL
F													0	
G													50	0.5 μg/mL
H													0	
	1,000			500			100			50			[Det Ab] ng/mL	

Figure 1: Sample Plate Layout for Feasibility Testing

A. Coating Plate with Capture Antibody

1. Prepare 20 mL of 1X PBS by diluting 2 mL of 10X PBS into 18 mL deionized water.

2. Prepare 4 concentrations of capture antibody in 1X PBS accordingly. Prepare 4 mL of the 4 μg/mL stock, and serially dilute to generate at least 2 mL of each concentration.

Tip: *It is recommended to prepare a minimum 10x stock concentration to make the top concentration (40 μg/mL)*

[Cap Ab]	Volume (μL)	1X PBS or Sodium Bicarbonate (μL)	Total volume (μL)	Dilution Factor
4.0 μg/mL	400 μL of Stock	3,600	4,000	-
2.0 μg/mL	2000 μL of 4 μg/mL	2,000	4,000	2
1.0 μg/mL	2000 μL of 2 μg/mL	2,000	4,000	2
0.5 μg/mL	2000 μL of 1 μg/mL	2,000	4,000	2

Figure 2: Dilution Scheme for Capture Antibody

Tip: *Sodium Bicarbonate buffer can also be used in place of 1X PBS*

3. Pipette 50 µL of each capture antibody dilution into each well of the assay plate, ensuring that the solution has evenly coated the bottom of each well. Seal an adhesive plate sealer and incubate without shaking overnight at 4ºC.

B. Assay Plate Blocking

4. Prepare 1x Wash Buffer (from 10x Wash Buffer) as follows:

    a. Transfer 50 mL of the 10x Wash Buffer to 450 mL of DI water. Mix thoroughly by gentle inversion or with a clean, sterile stir bar.

5. Following capture antibody coating, wash assay plate 3 times with 250 µL 1x Wash Buffer. Refer to **Appendix A**.

6. Transfer 200 µL/well of Blocking Buffer. Seal with an adhesive plate seal and incubate with shaking for 1 hour at 25ºC (Jitterbug Setting #3). The plate can also be blocked overnight at 4-8ºC without shaking.

C. Preparation of Analyte

7. Prepare 8.0 mL of the desired analyte concentration spike in Plate Standard Diluent.

8. After blocking of plate, wash the coated assay plate 3 times with 250 µL 1x Wash Buffer. Refer to **Appendix A**

9. Transfer 50 µL per well of the analyte or the Plate Standard Diluent (as blank, 0 pg/mL) according to the plate layout **(Fig. 1)**. Seal assay plate with an adhesive plate seal. Incubate for 2 hours at 25°C on microplate incubate / shaker (Jitterbug Setting #5).

D. Preparation of Detection Antibody

10. Prepare Detection antibody in Assay Buffer. Dilute labeled detection antibody to generate an intermediate stock at 10,000 ng/mL, then filter using a 0.2 µM syringe filter. Dilute further to prepare each detection antibody concentration below.

Tip: *It is recommended to prepare a minimum 10x stock concentration to make the top concentration (10,000 ng/mL)*

[Detection Ab]	Volume (µL)	Assay Buffer (µL)	Total volume (µL)	Dilution Factor
1,000 ng/mL	400 µL of 10,000 ng/mL	3,600	4,000	-
500 ng/mL	2,000 µL of 1,000 ng/mL	2,000	4,000	2
100 ng/mL	800 µL of 500 ng/mL	3,200	4,000	5
50 ng/mL	2,000 µL of 100 ng/mL	2,000	4,000	2

Figure 3: Detection Antibody Dilution Scheme

11. When capture incubation is complete, wash the plate 3 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A.**

12. Transfer 50 µL of detection antibody per well for each detection concentration according to the plate layout (**Fig. 1**.). Seal assay plate with an adhesive plate seal. Incubate for 1 hour at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

13. When detection antibody incubation is complete, wash the plate 6 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A**.

13. Elute and then neutralize in the 384 well reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems. Refer to **Appendix B.**

2. Interpretation of Results (Feasibility Testing)

After reading plate(s), export the assay data to a spreadsheet program. The following section describes how to interpret the feasibility testing.

Tip: *SMCxPRO™ can be set to export data as tab-delimited .txt files prior to reading the plate or .csv file*

	1	2	3	4	5	6	7	8	9	10	11	12	[Analyte] pg/mL	[Cap Ab]
A	359			***290***			161			117			50	**4 µg/mL**
B	8			***5***			3			1			0	
C	415			270			162			103			50	**2 µg/mL**
D	9			5			3			2			0	
E	391			268			246			95			50	**1 µg/mL**
F	7			5			3			2			0	
G	347			294			232			84			50	**0.5 µg/mL**
H	9			6			4			2			0	
	1,000			**500**			**100**			**50**			**[Det Ab] ng/mL**	

Slope [pg/mL]/DE	S:B	Slope [pg/mL]/DE	S:B	Slope [pg/mL]/DE	S:B	Slope [pg/mL]/DE	S:B	[Capture]
141	45	***114***	***64***	63	50	46	102	**4 µg/mL**
162	49	106	51	64	59	41	69	**2 µg/mL**
154	59	105	57	97	81	37	53	**1 µg/mL**
135	40	115	53	91	59	33	51	**0.5 µg/mL**
1,000		**500**		**100**		**50**		**[Det Ab] ng/mL**

Figure 4: Sample SMCxPRO™ Feasibility Testing Data.

Recommendations on Determining Optimal Conditions:

a. Consider conditions that demonstrate low background, a good test-point signal, with a good slope.
b. Very low signals may not be reproducible. Consider the precision (%CV) of blank replicates
    a. **Erenna® Immunoassay System:** < 50 DE Count may be too low. Select a range between 50-200 DE
    b. **SMCxPRO™ Immunoassay System:** < 2 Response may be too low. Select a range between 2 and 10 RE.

SECTION III – Immunoassay Prototype Evaluation

1. Evaluating Sensitivity and Dynamic Range

Once the optimal antibody orientation and 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:

	Standards pg/mL											
	1	2	3	4	5	6	7	8	9	10	11	12
A	STD01	STD02	STD03	STD04	STD05	STD06	STD07	STD08	STD09	STD10	STD11	STD12
B	STD01	STD02	STD03	STD04	STD05	STD06	STD07	STD08	STD09	STD10	STD11	STD12
C	STD01	STD02	STD03	STD04	STD05	STD06	STD07	STD08	STD09	STD10	STD11	STD12
D	*Sample 1*											
E	*Sample 2*											
F	*Sample 3*											
G	*Sample 4*											
H	*Sample 5*											

Figure 5: Sample Plate Layout for Prototype Evaluation

A. Coating plate with capture antibody and blocking of plate

1. Prepare 7 mL of capture antibody in 1x PBS. Pipette 50 µL of the capture antibody into each well of the assay plate, ensuring that the solution has evenly coated the bottom of each well. Seal the plate with an adhesive plate seal and incubate without shaking overnight at 4ºC.

2. Following capture antibody coating, wash the plate 3 times with 250 µL 1x Wash Buffer. Refer to **Appendix A**.

3. Transfer 200 µL/well of Blocking Buffer. Seal with an adhesive plate seal. Incubate with shaking for 1 hour at 25ºC (Jitterbug Setting #3). The plate can also be blocked overnight at 4-8ºC, no shaking.

B. Preparation of Standard Curve

4. Prepare the standard curve in plate standard diluent 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.

Recommendations:

a. Generate a 12-point curve to measure the analytical range of the assay to provide an estimated LLOQ
b. Curve should be performed as 2-fold dilutions from ULOQ to the LLOQ and 2 points below, including a blank
c. Analytical dynamic range of curve can be adjusted accordingly to fit assay purpose in future experiments.

5. After blocking the plate, wash the coated assay plate 3 time with 250 µL 1x Wash Buffer. Refer to **Appendix A**.

6. Transfer 50 µL per well of Standards or Samples to assay plate (Fig. 5). Seal plate with an adhesive plate seal. Incubate for 2 hours at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

C. Preparation of Detection Antibody

7. Approximately 10 minutes prior to end of the analyte incubation, prepare 7 mL of Detection Antibody at the desired concentration determined in **Section II.**

8. When capture incubation is complete, wash the plate 3 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A**

9. Transfer 50 µL of detection antibody per well for each detection concentration according to the plate layout (**Fig. 5**.). Seal assay plate with an adhesive plate seal. Incubate for 1.0 hour at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

10. When detection antibody incubation is complete, wash the plate 6 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A**.

11. Elute and then neutralize in the 384 well reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems. Refer to **Appendix B.**

2. Interpretation of Results (Prototype Evaluation)

After reading plate(s), export the assay data to a spreadsheet program.
The following section describes how to interpret the prototype evaluation.

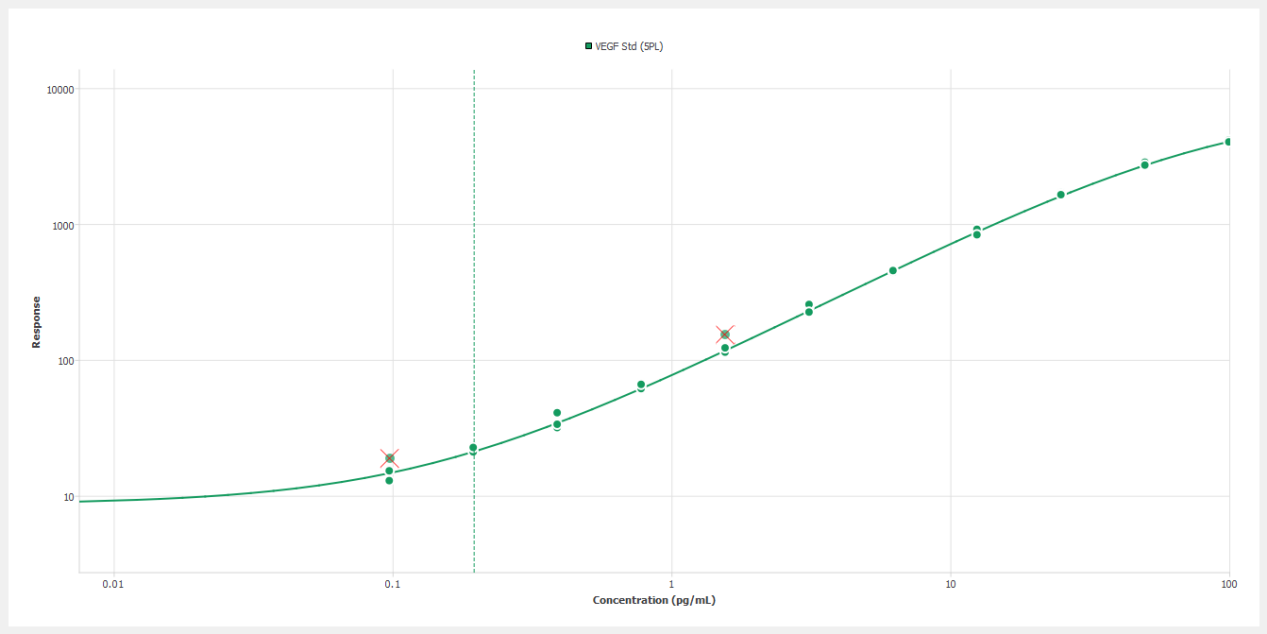

Figure 6: Sample SMCxPRO™ Graph

	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 7: Example Prototype Standard Curve (SMCxPRO™)

A. Sensitivity

1. The **LLoQ** is typically defined as the lowest standard point concentration at which the coefficient of variance (CV) is < 20%, the recovery is between 80% and 120%.

B. Dynamic Range

1. The **Dynamic Range** is determined by the LLoQ and the Upper Limit of Quantification (ULoQ).
    a. Signals above the ULoQ are considered saturated (SAT) on the Erenna®. On the SMCxPRO™, the software will extrapolate linearly beyond the top of the curve until % recovery and precision fails based on 20%. Consider the appropriate ULoQ for your assay.
    b. Signals below the LoD are considered not detectable (ND).

SECTION IV – Standard Diluent Optimization Screening

1. Standard Diluent Optimization

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

A suggested plate layout is below:

	1	2	3	4	5	6	7	8	9	10	11	12	[Analyte] pg/mL
A	Diluent 1			Diluent 5			Diluent 9			Diluent 13			50
B													0
C	Diluent 2			Diluent 6			Diluent 10			Diluent 14			50
D													0
E	Diluent 3			Diluent 7			Diluent 11			Diluent 15			50
F													0
G	Diluent 4			Diluent 8			Diluent 12			Diluent 16			50
H													0

Figure 8: Sample Plate Layout for Standard Diluent Optimization

A. Capture Antibody Coating and blocking of plate

1. Prepare 7 mL of the desired capture antibody concentration in 1x PBS. Pipette 50 µL of the capture antibody into each well of the assay plate, ensuring that the solution has evenly coated the bottom of each well. Seal with adhesive plate seal and incubate without shaking overnight at 4ºC.

2. Following capture antibody coating, wash the plate 3 times with 250 µL 1x Wash Buffer. Refer to **Appendix A**.

3. Transfer 200 µL/well of Blocking Buffer. Seal with an adhesive plate seal and incubate with shaking for 1 hour at 25ºC (Jitterbug Setting #3). The plate can also be blocked overnight at 4-8ºC.

B. Preparation of Optimization Standard Diluent

4. During blocking, bring all optimization Standard Diluents to room temperature. Label sixteen 15.0 mL conical tubes for Diluent #1 through Diluent #16. Prepare each optimized standard according to the table below:

Buffer #	Standard Diluent A (mL)	Standard Diluent B (mL)	Standard Diluent 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 #	Standard Diluent A (mL)	Standard Diluent B (mL)	Standard Diluent 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 9: Optimization Standard Diluents Preparation

5. Transfer the 16 diluents to a 96 deep well block per the suggested plate map **(Fig. 10)**. The top 450 µL aliquot will be used for positive analyte spike; the bottom 500 µL aliquot will be used for background, or 0 pg/mL analyte.

	1	2	3	4	5	6	7	8	9	10	11	12
A	**Diluent 1**	450 µL		**Diluent 5**	450 µL		**Diluent 9**	450 µL		**Diluent 13**	450 µL	
B		500 µL			500 µL			500 µL			500 µL	
C	**Diluent 2**	450 µL		**Diluent 6**	450 µL		**Diluent 10**	450 µL		**Diluent 14**	450 µL	
D		500 µL			500 µL			500 µL			500 µL	
E	**Diluent 3**	450 µL		**Diluent 7**	450 µL		**Diluent 11**	450 µL		**Diluent 15**	450 µL	
F		500 µL			500 µL			500 µL			500 µL	
G	**Diluent 4**	450 µL		**Diluent 8**	450 µL		**Diluent 12**	450 µL		**Diluent 16**	450 µL	
H		500 µL			500 µL			500 µL			500 µL	

Figure 10: Suggested Deep-well Plate Layout – Analyte and Background

C. Preparation of Analyte

6. Prepare 10x intermediate desired analyte concentration in Plate Standard Diluent. Spike in the desired analyte concentration into each of the diluents 1-16.

7. Cover prepared block with either a Universal Plate Cover or a plate seal until ready for use.

E.g. *If the desired spike concentration is 15 pg/mL, and stock is 150 pg/mL, then add 50 µL of the analyte into 450 µL of each of the diluents.*

8. After blocking of plate, wash the coated assay plate 3 times with 250 µL 1x Wash Buffer. Refer to **Appendix A**.

9. Transfer 50 µL per well of the analyte or the plate standard diluent blank according to the plate layout **(Fig. 8)**.

10. 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 #5).

D. Preparation of Detection Antibody

11. Approximately 10 minutes prior to end of the analyte incubation, prepare the Detection Antibody. Based on results from **Section II,** prepare 7 mL of detection antibody in Assay Buffer. Filter the intermediate diluted antibody into a clean tube using a 0.2 µM syringe filter.

12. When capture incubation is complete, wash the plate 3 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A**.

13. Transfer 50 µL of detection antibody to each well. Seal assay plate with an adhesive plate seal. Incubate for 1.0 hour at 25°C on microplate incubator / shaker (Jitterbug Setting #5).

14. When the detection incubation is complete, wash the plate 6 times with 250 µL of 1X Wash Buffer. Refer to **Appendix A.**

15. Elute and then neutralize in the 384 well reading plate for reading on either the Erenna® or SMCxPRO™ Immunoassay Systems. Refer to **Appendix B.**

2. Interpretation of Results (Assay Optimization)

After reading plate(s), export the assay data to a spreadsheet program. The following section describes how to interpret the Assay Optimization.

Tip: *SMCxPRO™ can be set to export data as tab-delimited .txt files prior to reading the plate or .csv file*

A. Example Standard Diluent 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, Standard Diluent #7 was determined to be the optimal condition as it provided the highest S:B ratio.

Standard Diluent	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 12: Example Optimization Screening Data (SMCxPRO™)

B. Assay Buffer Optimization Considerations

1. **Very Low Signals** may be less reproducible. Consider the precision (%CV) of the blank 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

Below are instructions of using the Biotek Washer

Post- Antibody Coating, Post-Blocking and Post-Capture Wash

Wash plate 3 times with a plate washer.

Plate Washer

a. BioTek; W-Nunc_Round_96 program
b. Ensure wells are free of residual volume

Post-Detection Wash

Wash assay plate 6 times with a plate washer.

Plate Washer

a. BioTek; W-Nunc_Round_96 program

***Note**: default can be set to 3 on washer, wash twice if necessary)*

b. Ensure wells are free of residual volume

Below are instructions for washing the plate manually

Post- Antibody Coating, Post-Blocking and Post-Capture Wash

Wash plate 3 times manually

a. Wash by filling each well with Wash Buffer (200 µL) 3 times using a multichannel pipette.
b. Aspirate or decant by inverting the plate and blot it against clean paper towels. Ensure wells are free of residual volume

Post-Detection Wash

Wash assay plate 6 times manually

a. Wash by filling each well with Wash Buffer (200 µL) 6 times using a multichannel pipette.
b. Aspirate or decant by inverting the plate and blot it against clean paper towels. Ensure wells are free of residual volume

APPENDIX B – SMC™ Immunoassay Reading Guide

To Read on the Erenna® Immunoassay System

1. Dispense 50 µL Elution Buffer B per well using reverse pipetting.
2. Seal assay plate with a clear adhesive plate seal. Incubate plate for 10 minutes at 25°C on microplate incubator/shaker (Jitterbug setting #5).
3. Add 10 µL per well of Buffer C using reverse pipetting to Erenna® reading plate (Fisher Scientific PN 12-565-384) using a 12-channel manual P20.
4. Transfer 30 µL of eluate from assay plate to reading plate, changing tips with each dispensed row.
5. Cover plate with universal plate cover, and 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 reading plate onto the Erenna® Immunoassay System.

To Elute and Read on the SMCxPRO™ Immunoassay System:

1. Dispense 50 µL Elution Buffer B per well using reverse pipetting.
2. Seal assay plate with a clear adhesive plate seal. Incubate plate for 10 minutes at 25°C on microplate incubator/shaker (Jitterbug setting #5).
3. During incubation, add 10 µL per well of Buffer C using reverse pipetting to the SMCxPRO™ reading plate.
4. After incubation, transfer 30 µL of eluate from assay plate to the reading plate, changing tips with each dispensed row.
5. Place reading plate on plate holder and either cover with plate lid or seal with clear adhesive plate seal.
6. Place reading plate 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.

7. Seal reading plate with SMCxPRO™ aluminum adhesive plate seal.
8. Remove the plate holder and load the reading plate onto the SMCxPRO™ Immunoassay System.

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 – Detection Antibody Labeling Worksheet

Pre-Label

Step	Result
Dilute Antibody to ~1 mg/mL in Buffer 1	Volume Buffer 1 added: _____ mL
Read A280 of antibody	A280: _____ Conc.: (C) _____ mg/mL
Determine amount and volume of antibody to label	Ab Amount (A): _____mg (up to 1 mg) 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 + _____mL Buffer = 4 mL
Wash #2 with Buffer 1 (10 min.)	Up to 4 mL Buffer 1
Recovered Ab volume	Recovered Vol: _____ mL Buffer 1 added: _____ mL
Read A280 of antibody	A280: _____ 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: _____ 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 + _____ 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 Buffer 3 added: _____ mL
Read A280 of antibody	A280: _____ Conc: (C) _____ mg/mL
Read A650 of antibody	A650: _____ A650/A280 ratio: _____

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

This page intentionally left blank.

EMD Millipore Corporation
3050 Spruce Street
St. Louis, Missouri 63103
United States of America

Toll-Free US: (800) 645-5476
Fax: (800) 645-5439
emdmillipore.com