SMC™ Plate Based Immunoassay Development Kit Instructions

Assay Development Kit

Catalog # 03-0128-00

Kit for the development of

SMC™ Plate Based Immunoassays

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Manufactured & Distributed by:

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INTRODUCTION

The SMC™ Plate-Based Immunoassay uses a quantitative fluorescent sandwich immunoassay technique to measure analyte in a matrix. A capture antibody specific for an analyte has been pre-coated onto paramagnetic microparticles (beads). The user pipettes beads, standards, and samples into uncoated microplate wells. During incubation, the analyte present in the sample binds to the capture antibody on the coated beads. Unbound molecules are washed away during the wash steps. Fluor-labeled detection antibody is added to each well and incubated. This detection antibody recognizes and binds to the analyte that has been captured onto the beads. Following a stringent wash step to remove unbound detection, the beads are transferred to a clean plate. After a final aspirate, elution buffer is then added and incubated. The elution buffer dissociates the bound protein sandwich from the bead surface releasing the labeled antibodies. These antibodies are separated during transfer to a final microplate. The plate is loaded into the Erenna® System where the labeled molecules are detected and counted. The number of fluor-labeled detection antibodies counted is directly proportional to the amount of analyte present in the sample when captured. The amount of analyte in unknown samples is interpolated from a standard curve.

The SMC™ Plate Based Immunoassay Development Kit is intended to be used with the SMC™ derivatization services or antibody labeling kits and contains the necessary buffers and protocols to build a SMC™ plate-based immunoassay.

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REAGENTS

The SMC™ Plate Based Immunoassay Development Kit includes all reagents listed in Reagents Provided. Additional reagents and supplies may be required to run this immunoassay, as listed in the section titled General Supplies Required But Not Provided. All reagents supplied are for Research Use Only.

Reagents Provided

Item #	Description	Shipping Conditions	Storage Conditions	Component Part No.	Packaging Details
1	Assay Buffer	With cold pack	2-8°C	02-0865-00	1 x 100 mL
2	Blocking Buffer	With cold pack	2-8°C	02-0866-00	1 x 250 mL
3	Plate Standard Diluent	With cold pack	2-8°C	02-0867-00	1 x 125 mL
4	10X System/Wash Buffer w/Proclin	With cold pack	2-8°C	02-0111-03	1 x 1 L
5	Elution Buffer B	With cold pack	2-8°C	02-0297-00	1 x 100 mL
6	Buffer C	With cold pack	2-8°C	02-0868-00	1 x 30 mL
7	10X PBS	With cold pack	2-8°C	02-0869-00	1 x 10 mL

Storage Instructions

- The SMC™ Plate Based Immunoassay Development Kit should be stored at 2 - 8°C.
- Discard standards after one use.
- Proper kit performance can only be guaranteed if the materials are stored properly.

REAGENTS (continued)

General Supplies Required But Not Provided

Reagents

1. Erenna® 10X Wash Buffer (1 L bottle) (EMD Millipore PN 02-0111-00) if using an automated plate washer
2. Elution Buffer (EMD Millipore PN 02-0002-04) for maintenance
3. De-ionized or distilled water

Washing Options

Automated

a. Bio-Tek ELx405™ Microplate Washer (EMD Millipore PN 95-0004-05) *or*
b. Tecan HydroFlex™ microplate washer (EMD Millipore PN 95-0005-02)

Manual

a. Sphere Mag Plate SBS Footprint (EMD Millipore PN 90-0003-02) *or*
b. DynaMag™-96 Side Skirted Magnet (Thermo Fisher PN 12027)

Instrumentation / Materials

1. Jitterbug™ Microplate incubator / shaker (EMD Millipore PN 70-0009-00 or equivalent)
2. ALPS™ 50V microplate heat sealer (Thermo Fisher PN AB1443A or equivalent)
3. Centrifuge with plate rotor capable of reaching a speed of 1,100 xg
4. 12-channel pipettes capable of transferring 20 µL - 250 µL
5. 8- or 12-channel pipette capable of transferring 15 µL
6. Rotisserie rotator
7. Microcentrifuge
8. MultiScreen$_{HTS}$ BV 96-Well Filter Plate (EMD Millipore PN MSBVN1210 or equivalent)
9. 96-well V-bottom polypropylene plate, 500 µL (Axygen PN P-96-450V-C)
10. 384-well round bottom polypropylene plate, 120 µL (Thermo Fisher PN 264573)
11. 0.2 µm syringe filter (EMD Millipore PN SLGPR33RS or equivalent)
12. Universal plate cover (Thermo Fisher PN 253623 or equivalent)
13. Sealing tape (Thermo Fisher PN 236366 or equivalent)
14. Heat sealing plate foil (EMD Millipore PN 02-01-0216-00 or equivalent)
15. 12-channel reagent resevoirs for preparing standards
16. 5 mL syringe
17. Microcentrifuge tubes
18. Container capable of holding 300 mL
19. 500 mL graduated cylinder

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

WHEN TO USE THIS KIT

This kit should be used in conjunction with the SMC™ Detection Antibody Labeling Kit (03-0076-XX). Follow instructions in the indicated kit to label detection antibody.

TECHNICAL HINTS DUE TO HIGH SENSITIVITY

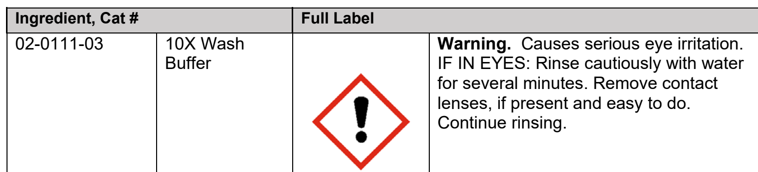
To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set-up.

- Wipe down bench and pipettes with 70% isopropanol before use. It is important to allow all reagents to warm to room temperature (20 - 25°C).
- Use sterile filter pipette tips and reagent trays to avoid contamination.
- Use filter tips while transferring standard.
- Pre-wet tips (aspirate and dispense within well) twice before each transfer.
- The standards prepared by serial dilution must be used within 10 minutes of preparation. It is recommended that the standards are prepared as the last step prior to plate setup.
- The detection antibody is light sensitive and must be protected from light at all times.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the wash buffer provided.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- The plates should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate with the pierceable foil seal and store the plate at 2-8°C for up to 48 hrs. Bring to room temperature then centrifuge the plate at 1,100 x g for 5 minutes prior to reading on the Erenna.
- The plate shaker should be set at a speed to provide maximum orbital mixing without splashing liquid on the sealer or outside the wells. For the recommended plate shaker, this would be a setting of 3 - 5.
- For optimal instrument performance, complete a cycle routine (10,000 µL at 1,000 µL/min) followed by a bubble test, and an instrument calibration prior to reading the plate.
- If a clean routine is required, run using three wells of elution buffer (EMD Millipore PN 02-0002-04), one well of 10% bleach and five wells of elution buffer (EMD Millipore PN 02-0002-04). (Note: This elution buffer is not provided and should be ordered separately.)

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 Proclin 950 at 5% have not been thoroughly investigated. At this concentration, this biocidal preservative is irritating to eyes and skin, and may be detrimental if enough is ingested (quantities above those found in the kit). ProClin 950 is a potential sensitizer by skin contact; prolonged or repeated exposure may cause allergic reaction 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 0.1% 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 Hazardous Label:



Ingredient, Cat #		Full Label	
02-0111-03	10X Wash Buffer		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

PREPARATION OF COATED ASSAY PLATE

1. Prepare 20 mL of 1X PBS by diluting 2 mL of 10X PBS into 18 mL deionized water.
2. Prepare four concentrations of capture antibody; for example, 4 µg/mL, 2 µg/mL, 1 µg/mL, and 0.5 µg/mL. Prepare and make all dilutions in 1X PBS.
    a. Prepare 5 mL of the 4 µg/mL stock.
    b. Prepare the lower concentrations by serial dilution.
    c. Make at least 2 mL of each concentration to ensure sufficient volume to use a multichannel pipettor.
    d. Ensure that all pipetting steps transfer ≥10 µL of liquid to achieve the best precision.

PREPARATION OF COATED ASSAY PLATE (continued)

3. Pipette 50 µL of diluted capture antibody into each well of the Assay Plate. Ensure that the solution has evenly coated the bottom of each well.

	1	2	3	4	5	6	7	8	9	10	11	12
A	4 µg/mL capture antibody											
B												
C	2 µg/mL capture antibody											
D												
E	1 µg/mL capture antibody											
F												
G	0.5 µg/mL capture antibody											
H												

4. Seal the Assay Plate with an AxySeal plate cover and incubate without shaking overnight at 4°C.

BLOCK THE ASSAY PLATE

1. Warm the following reagents to room temperature prior to use: Coated Assay Plate, Plate Standard Diluent, Assay Buffer, Blocking Buffer, Elution Buffer B, Buffer C, 10X Wash Buffer, Detection Antibody.
2. Store the Detection Antibody away from light until ready to use.
3. Prepare 1X Wash Buffer (from 10X Wash Buffer) as follows:
    a. Pour 100 mL of the 10X Wash Buffer into a container capable of holding at least 1L mL.
    b. Add 450 mL of deionized water.
    c. Mix thoroughly by gentle inversion or with a clean, sterile stir bar.
4. Wash the plate 1 time with 250 µL 1X Wash Buffer. This can be done manually or with an automated plate washer. Ensure that the well is free of residual volume.
5. Block the plate with 200 µL/well of Blocking Buffer. Cover with an universal plate cover (Thermo Fisher PN 253623 or equivalent) and incubate with shaking for 1 hour at 25°C on Boekel Scientific, The Jitterbug™ (EMD Millipore PN 70-0009-00 or equivalent) setting # 5. The plate can also be blocked overnight at 4-8°C.

ANALYTE INCUBATION

1. Quick spin the Standard analyte vial in a mini-centrifuge prior to opening, and pipette mix. Use care when opening this concentrated standard vial to prevent loss of materials and contamination of specimens or plates with aerosols.
2. Refer to the standard value assignment on the Certificate of Analysis for the starting concentration of the Standard in the vial.
3. To make your Analyte Working Stock, perform the necessary serial dilution in Plate Standard Diluent to achieve a working analyte concentration. Choose a concentration appropriate for your analyte, plus a zero (for example 50 pg/mL, and 0 pg/mL). Prepare 4 mL of each concentration to ensure sufficient volume to use a multichannel pipettor. Ensure that all pipetting steps transfer ≥10 µL of liquid to achieve the best precision.
4. After blocking for 1+ hour, wash the plate 1 time with 250 µL 1X Wash Buffer. This can be performed manually or with an automated plate washer. Ensure that the well is free of residual volume.
5. Add 50 µL of analyte at the appropriate concentration, according to the example plate map below:

	Capture Ab:	1	2	3	4	5	6	7	8	9	10	11	12
A	4 µg/mL	50 pg/mL Analyte											
B		0 pg/mL Analyte											
C	2 µg/mL	50 pg/mL Analyte											
D		0 pg/mL Analyte											
E	1 µg/mL	50 pg/mL Analyte											
F		0 pg/mL Analyte											
G	0.5 µg/mL	50 pg/mL Analyte											
H		0 pg/mL Analyte											

5. Cover with an universal plate cover and incubate for 2 hours at 25°C on the Jitterbug, setting # 5.

DETECTION ANTIBODY INCUBATION

1. Approximately 10 minutes prior to the end of analyte incubation, prepare the **Detection Antibody**. Prepare and make all dilutions in **Assay Buffer**.
2. Prepare four concentrations of detection antibody; for example, 400 ng/mL, 200 ng/mL, 100 ng/mL and 50 ng/mL.
    a. Prepare 7 mL of the 400 ng/mL stock.
    b. Filter the detection antibody using a syringe with a 0.2 µm filter into a clean tube.
    c. Prepare the lower concentrations by serial dilution.
    d. Make at least 3 mL of each concentration to ensure sufficient volume to use a multichannel pipettor.
    e. Ensure that all pipetting steps transfer ≥10 µL of liquid to achieve the best precision.
3. When incubation is complete, remove universal plate cover carefully to avoid splashing.
4. Wash the plate 3 times with 250 µL 1X Wash Buffer. This can be performed manually or with an automated plate washer. Ensure that the well is free of residual volume.
5. Add detection antibody (50 µL/well) in the appropriate concentration, according to the plate map below:

<table>
<tr><th></th><th>Capture Ab:</th><th>Analyte [pg/mL]</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="2">4 µg/mL</td><td>50</td><td colspan="3" rowspan="8">50 ng/mL</td><td colspan="3" rowspan="8">100 ng/mL</td><td colspan="3" rowspan="8">200 ng/mL</td><td colspan="3" rowspan="8">400 ng/mL</td></tr>
<tr><td>B</td><td>0</td></tr>
<tr><td>C</td><td rowspan="2">2 µg/mL</td><td>50</td></tr>
<tr><td>D</td><td>0</td></tr>
<tr><td>E</td><td rowspan="2">1 µg/mL</td><td>50</td></tr>
<tr><td>F</td><td>0</td></tr>
<tr><td>G</td><td rowspan="2">0.5 µg/mL</td><td>50</td></tr>
<tr><td>H</td><td>0</td></tr>
</table>

6. Cover with an universal plate cover and incubate for 1 hour at 25°C on Jitterbug setting # 5.

ELUTION AND PLATE TRANSFER

1. Remove universal plate cover carefully to avoid splashing.
2. Wash the plate 6 times with 250 µL 1X Wash Buffer. This can be performed manually or with an automated plate washer. Ensure that the well is free of residual volume.
3. Add 50 µL Elution Buffer B per well.
4. Cover Assay Plate with an universal plate cover.
5. Incubate plate for 10 minutes at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting # 5).
6. While the Assay Plate is incubating, add 10 µL per well of Buffer C to Read Plate (384-well polypropylene plate, Nunc, PN 264573) with a multi-channel manual P20.
7. Set manual pipette to 30 µL, and transfer 30 µL eluate to Read Plate by <u>rows</u>.
8. Cover Read Plate with heat sealing foil (EMD Millipore PN 02-01-0216-00 or equivalent) according to heat sealer manufacturer instructions, and spin plate for 5 minutes at room temperature at 1,100 *x g*.

Run on Erenna® Immunoassay System

1. Load completed assay Plate 3 onto the Erenna® Immunoassay System.

INTERPRETATION OF RESULTS

Following the above protocol will yield a set of results consistent with the following plate map: 16 conditions run in triplicate, against one analyte concentration and a 0 pg/mL point.

A typical set of results may appear as in Table 5 below. Detected Events (DE) may be used to estimate the sensitivity of the conditions. Event Photons (EP) should be used if the analyte condition is near saturating DE values (approximately 12,000 DE). The best condition balanced a low background, high slope, and good signal to noise ratio.

<table>
<tr><th></th><th>Capt. Ab. [µg/mL]</th><th>Analyte [pg/mL]</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td rowspan="2">4</td><td>50</td><td colspan="3">7186</td><td colspan="3">5808</td><td colspan="3">3221</td><td colspan="3">2347</td></tr>
<tr><td>B</td><td>0</td><td colspan="3">160</td><td colspan="3">91</td><td colspan="3">65</td><td colspan="3">23</td></tr>
<tr><td>C</td><td rowspan="2">2</td><td>50</td><td colspan="3">8295</td><td colspan="3">5393</td><td colspan="3">3233</td><td colspan="3">2066</td></tr>
<tr><td>D</td><td>0</td><td colspan="3">171</td><td colspan="3">105</td><td colspan="3">55</td><td colspan="3">30</td></tr>
<tr><td>E</td><td rowspan="2">1</td><td>50</td><td colspan="3">7820</td><td colspan="3">5351</td><td colspan="3">4920</td><td colspan="3">1897</td></tr>
<tr><td>F</td><td>0</td><td colspan="3">133</td><td colspan="3">94</td><td colspan="3">61</td><td colspan="3">36</td></tr>
<tr><td>G</td><td rowspan="2">0.5</td><td>50</td><td colspan="3">6934</td><td colspan="3">5882</td><td colspan="3">4636</td><td colspan="3">1672</td></tr>
<tr><td>H</td><td>0</td><td colspan="3">172</td><td colspan="3">111</td><td colspan="3">78</td><td colspan="3">33</td></tr>
<tr><td></td><td colspan="2">Det. Ab. [ng/mL]</td><td colspan="3">400</td><td colspan="3">200</td><td colspan="3">100</td><td colspan="3">50</td></tr>
</table>

INTERPRETATION OF RESULTS (continued)

Assay Development Considerations

- **Very low DE** at 0 pg/mL analyte (<50 counts) may be less reproducible. Consider the precision (%CV) of the 0 pg/mL replicates before choosing assay conditions.
- **High DE** at 0 pg/mL analyte (>300 counts) suggest there is opportunity for further optimization. Achieving an assay with a very low background will also be limited by the choice/availability of antibodies, and the target itself.
- Determine slope between the 0 pg/mL and the analyte. Higher slopes suggest that the assay will be quantifiable at a lower value.
- Determine signal to noise between the 0 pg/mL and the analyte. Higher signal:noise suggests that the assay will be more robust at the lower end of the curve.
- DE will plateau and hook slightly at or near 12,000. In that instance, EP may be a better indication for assay sensitivity.
- Observe patterns between common concentrations.
- Proceed with assay optimization with 2 best conditions to ensure the results are repeatable. Consider fit-for-purpose (i.e., is the assay sensitivity appropriate for the target and sample matrix) before proceeding with further optimization.

APPENDIX A: SMC™ Quick Assay Guide

1. Add 50 µL of diluted capture antibody to each well.
2. Coat sealed plate overnight at 4°C.

overnight 4°C

3. Wash Assay Plate 3 times with 250 µL of Wash Buffer.
4. Block plate with 200 µL /well of Blocking Buffer at 25°C for 1 hour on microplate incubator/shaker.
5. Prepare all reagents, standard curve and samples as instructed.

1 hour 25°C

6. Wash Assay Plate 3 times with 250 µL Wash Buffer.
7. Add 50 µL of Standard/Samples to Assay Plate.
8. Cover and incubate for at 25°C for 2 hours on microplate incubator/shaker.

2 hours 25°C

9. Wash Assay Plate 3 times with 250 µL Wash Buffer.
10. Add 50 µL of Detection Antibody per well..
11. Cover and incubate for at 25°C for 1 hour on microplate incubator/shaker.

1 hour 25°C

12. Wash Assay Plate 6 times with 250 of Wash Buffer.
13. Add 50 L of Elution Buffer B to the Assay Plate.
14. Cover and incubate at 25°C for 10 minutes on microplate incubator/shaker.

10 minutes 25°C

15. Add 10 µL of **Buffer C** per well to **Read Plate**.
16. Transfer 30 µL from **Assay Plate** to **Read Plate**.
17. Cover **Read Plate** with pierceable plate seal cover.
18. Cover and centrifuge for 5 minutes at 1,100 x g.

LOAD ON ERENNA® SYSTEM

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipeting 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.
		Change tips for each dilution of the standard curve.
		Insufficient washes—washer may need to be cleaned or reprogrammed.
	Instrument needs cleaning	See Technical Guidelines for appropriate Erenna® cleaning protocol.
	Plate was over-incubated	Confirm correct incubation times were followed.
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 PI and lab SOPs. 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. Should splashing occur on the plate sealer pulse spin plate to remove excess material prior to removing the seal. A new plate seal should be used every time the plate is sealed.

TROUBLESHOOTING GUIDE (continued)

Problem	Probable Cause	Solution
Sample variability is high (continued)	Cross-well contamination (continued)	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.
	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.
Published LLoQ was not achieved	Improper dilution/reconsititution of the standard reference material	Confirm appropriate kit protocol was followed when preparing standard curve.
		Check plate washer to confirm no beads were lost during washes and that plate contains <2 uL following the post-capture and final aspiration protocols.
		Ensure time from thawing the standard to starting the capture incubation is ≤10 minutes
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/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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