Erenna®

Plate Based Immunoassay Development Kit Instructions

Assay Development Kit

Catalog # 03-0128-00

Kit for the Development of

Singulex Plate-Based Immunoassays

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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05-0583-00

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INTRODUCTION

The Erenna® SMC™ Plate-Based Immunoassay uses a quantitative fluorescent sandwich immunoassay technique to measure analyte in a matrix. An analyte-specific capture antibody is coated onto a plate overnight. The operator blocks the plate, followed by the addition of standards or samples into the plate wells. During incubation, the analyte present in the sample binds to the capture antibody on the plate. Unbound molecules are washed away during the subsequent wash steps. Fluor-labeled detection antibody is added to each well and incubated. The detection antibody recognizes and binds to the captured analyte. The plate is washed and elution buffer is then added and incubated. The elution buffer dissociates the bound protein sandwich from the plate, releasing the labeled antibodies. These antibodies are separated during transfer to a final plate. 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 captured analyte present in the sample. The amount of analyte in unknown samples is interpolated from a standard curve. This kit is intended to be used with the Singulex derivatization services or antibody labeling kits and contains the necessary buffers and protocols to build a Singulex plate-based immunoassay.

FOR RESEARCH USE ONLY

MATERIALS

The Erenna Plate Based Immunoassay Development kit includes all reagents listed in Table 1. Additional reagents and supplies may be required to run an immunoassay, as listed in APPENDIX B: Additional Supplies Required. 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	100 mL
2	**Blocking Buffer**	With cold pack	2-8°C	02-0866-00	250 mL
3	**Plate Standard Diluent**	With cold pack	2-8°C	02-0867-00	125 mL
4	**10X Wash Buffer**	With cold pack	2-8°C	02-0514-00	1 L
5	**Elution Buffer B**	With cold pack	2-8°C	02-0297-00	100 mL
6	**Buffer C**	With cold pack	2-8°C	02-0868-00	30 mL
7	**10X PBS**	With cold pack	2-8°C	02-0869-00	10 mL
8	**Erenna Plate Based Immunoassay Development Kit Instructions**	N/A	Ambient	05-0583-00	1

Table 1: Reagents Provided

Storage Instructions

The Erenna Plate Based Immunoassay Development Kit should be stored at 2–8°C. Proper kit performance can only be guaranteed if the materials are stored properly.

General Supplies Required but Not Provided

- One 96-well polystyrene plate (**Assay Plate**, Nunc™ MaxiSorp™, 449824)
- De-ionized or distilled water
- 8- or 12-channel pipettes capable of transferring 10 µL – 250 µL
- Micro-centrifuge tubes
- Micro-centrifuge
- Container capable of holding 300 mL
- 500 mL graduated cylinder
- If using an automated plate washer additional **10X Wash Buffer** may be needed (02-0514-00).
- One 384-well polypropylene plate (**Read Plate**, Nunc, 264573)

WHEN TO USE THIS KIT

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

TECHNICAL HINTS DUE TO HIGH SENSITIVITY

- Wipe down bench and pipettes with 70% isopropanol before use.
- Use sterile filter pipette tips and reagent trays to avoid contamination.
- Pre-wet tips (aspirate and dispense within well) twice before each transfer.

PRECAUTIONS

- Components of this 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.
- Use caution when handling biological samples; wear protective clothing and gloves.

REAGENT PREPARATION

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.

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												

Table 2: Capture Antibody Plate Map

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 bottle of 10X Wash Buffer into a container capable of holding at least 1 L
   b. Add 900 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 AxySeal plate cover and incubate with shaking for 1 hour at 25°C on Boekel Scientific, The Jitterbug™ setting #5. The plate can also be blocked overnight at 4°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. To make your **Analyte Working Stock**, perform the necessary 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.

3. 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.
4. 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											

Table 3: Analyte Addition Plate Map

5. Cover with an AxySeal 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 AxySeal 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 rowspan="8" colspan="3">50 ng/mL</td><td rowspan="8" colspan="3">100 ng/mL</td><td rowspan="8" colspan="3">200 ng/mL</td><td rowspan="8" colspan="3">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>

Table 4: Detection Antibody Addition Plate Map

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

ELUTION AND PLATE TRANSFER

1. Remove AxySeal 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 AxySeal plate cover.
5. Incubate plate for 10 minutes at 25°C on Jitterbug 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 **rows**.
8. Cover **Read Plate** with heat sealing foil, 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

Load completed assay **Read Plate** 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.

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

Table 5: Representative Assay Development Data (DE Values)

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.

CONTACT INFORMATION

To reach Singulex, Inc. reagent technical support, call **(510) 995-3870**, or in the U.S. you may call us toll-free at (888) 603-3033.

You can also send us an e-mail at:

North America: LSTechSupportNA@singulex.com

Europe: LSTechSupportEU@singulex.com

APPENDIX A: **ERENNA** 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** 1 time with 250 µL/well Wash Buffer.
4. Block plate with 200 µL/well of Blocking Buffer for 1 hour with shaking.
5. Prepare analyte at various concentrations as instructed.

1 Hour
25°C

6. Wash **Assay Plate** 1 time with 250 µL/well Wash Buffer.
7. Add 50 µL/well of Analyte to **Assay Plate**.
8. Cover and incubate for 2 hours at 25°C on Jitterbug (setting 5).

2 Hours
25°C

9. Wash **Assay Plate** 3 times with 250 µL/well Wash Buffer.
10. Add 50 µL/well of **Detection Antibody** per well at various concentrations as instructed.
11. Cover and incubate for 1 hour at 25°C on Jitterbug (setting 5).

1 Hour
25°C

12. Wash **Assay Plate** 6 times with 250 µL/well Wash Buffer.
13. Add 50 µL/well of **Elution Buffer B** to Assay Plate.
14. Cover and incubate at 25°C for 10 minutes on Jitterbug (setting 5).

10 min
25°C

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

LOAD ON ERENNA SYSTEM

APPENDIX B: Additional Supplies Required (not provided)

Description	Mfr Supplier	Component Part Numbers	Product Uses	Packaging Detail
Erenna 10X Systems Buffer Contains Proclin	Singulex	02-0111-03	Systems (Analysis) Buffer, fluid used to run Erenna System	1 L (10 L mixed)
Erenna 10X Wash Buffer Contains Proclin	Singulex	02-0514-00	Wash buffer used for manual and automation wash protocols	1 L (10 L mixed)
Erenna Detection Reagent Labeling Kit	Singulex	03-0076-XX	Label detection antibody	1
Reservoirs for 12-Channel Pipettors	VWR	80092-466	Standard Curve	10/pkg
Nunc™ MaxiSorp™ Immunoassay Plate	Fisher Scientific	449824	Assay Plate	10 plates/unit 5 units/case
8-Well Low Profile Reservoir	VWR	12000-732	Transfer of Reagents	Variable
384-Well Round Bottom Polypropylene Plate, 120 µL	Nunc	264573	Read Plate, analysis plate	20/pk or 120/cs
Syringe (5 mL)	VWR	66064-772 (or equivalent)	To filter diluted detection antibody	100 units/pk
0.2 µm Syringe Filter	Pall	4187	To filter diluted detection antibody	50/pk
Universal Plate Cover	Nunc	253623	Cover the plate	25 units/pk
AxySeal—PCRSP Plate sealing film series	Axygen	PCR-SP	Sealing plates during incubation/ mix/store	100 films/ case
Centrifuge w/ Plate Rotor	- - -	- - -	Spin down Plate contents ~1,100 xg	1
Vacuum Pump	Welch	2511B-01	Degassing systems buffer	1
Microplate Incubator / Shaker	Boekel Scientific	130000 The Jitterbug™	Incubating plate	1
Heat Sealing Plate Foil	Singulex	01-0216-00 or equivalent	Sealing plate for analysis on Erenna System	- - -
ALPS™ 50V Microplate Heat Sealer	Fisher Scientific	AB1443A	Sealing plate for analysis on Erenna System	1

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