Erenna®

SMC™ Human Phospho AKT1 Immunoassay Kit Instructions

Microparticle Assay

Catalog # 03-0100-01

Immunoassay kit for the quantitative determination of **Human Phospho AKT1** in cell lysates

FOR RESEARCH USE ONLY

NOT FOR USE IN DIAGNOSTIC PROCEDURES

Manufactured & Distributed by:

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INTRODUCTION

The SMC™ Human Phospho AKT1 Immunoassay uses a quantitative fluorescent sandwich immunoassay technique to measure Phospho AKT1 in cell lysate samples. A capture antibody specific for human Phospho AKT1 has been pre-coated onto paramagnetic microparticles (beads). The user pipettes beads, standards, and samples into uncoated microplate wells. During incubation, the Phospho AKT1 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 Phospho AKT1 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 Phospho AKT1 present in the sample when captured. The amount of Phospho AKT1 in unknown samples is interpolated from a standard curve.

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REAGENTS

The SMC™ Human Phospho AKT1 Immunoassay 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	Phospho AKT1 Coated Beads	With cold pack	2-8°C	02-0659-00	1 x 500 µL
2	Sample Diluent	With cold pack	2-8°C	02-0666-00	2 x 10 mL
3	Phospho AKT1 Detection Antibody	With cold pack	2-8°C	02-0661-00	1 x 20 µL
4	Assay Buffer	With cold pack	2-8°C	02-0665-00	1 x 25 mL
5	AKT1 Standard	On dry ice	≤ -70°C	02-0654-00	1 x 20 µL
6	AKT1 Detection Antibody Buffer	With cold pack	2-8°C	02-0663-00	1 x 6 mL
7	10X Wash Buffer Note: Contains 0.5% Proclin	With cold pack	2 - 8°C	02-0001-06	1 x 50 mL
8	Buffer D	With cold pack	2 - 8°C	02-0359-00	1 x 3 mL
9	Elution Buffer B	With cold pack	2 - 8°C	02-0211-02	1 x 5 mL

Storage Instructions

- The SMC™ Human Phospho AKT1 Immunoassay Kit should be stored at 2 - 8°C. The Standard analyte should be stored at ≤ -70°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 System/Wash Buffer w/ Proclin (1 L bottle) (EMD Millipore PN 02-0111-03)
2. Erenna® 10X Wash Buffer (1 L bottle) (EMD Millipore PN 02-0111-00) if using an automated plate washer
3. Elution Buffer (EMD Millipore PN 02-0002-04) for maintenance
4. 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.)

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.)

SAMPLE INFORMATION

- The SMC™ Human Phospho AKT1 Immunoassay validation data have been compiled using cell lystate samples.
- Ensure sample is clear of precipitants and other visible particulate matter before testing with the SMC™ Human Phospho AKT1 Immunoassay.

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	
02-0001-06	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.

ASSAY PREPARATION

Reagent Preparation

1. Warm the following reagents to room temperature prior to use: Sample Diluent, Assay Buffer, Coated Beads, Elution Buffer B, Buffer D, Detection Antibody and 10X Wash Buffer.
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 50 mL of the 10X Wash Buffer into a container capable of holding at least 500 mL.
    b. Add 450 mL of deionized water.
    c. Mix thoroughly by gentle inversion or with a clean, sterile stir bar.
4. Mix Phopho AKT1 Coated Beads (coated microparticles) on a rotisserie spin rotator, or manually by repeat inversion, for 10 - 20 minutes until all beads are completely resuspended.

Sample Preparation

1. Thaw cell lysates on ice
2. Dilution will need to be experimentally determined based on cell line. Use the **Sample Diluent** for dilution (e.g., for Jurkat a 1:3200 dilution was used). Mix thoroughly before transferring to assay plate.

    Note: During data analysis, the interpolated value of the diluted sample needs to be multiplied by x in order to calculate the correct concentration of the analyte in the sample; the dilution factor can be adjusted in Sgx link to do this automatically.

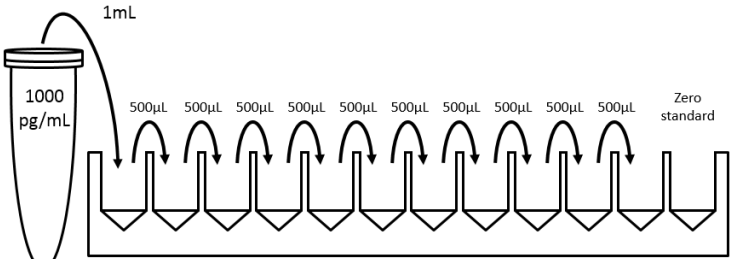
Initial Standard Stock Preparation

1. Quick spin the AKT1 Standard vial in a microcentrifuge and pipette mix prior to preparing standards. Use care when opening the stock 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 AKT1 Standard in the vial.
3. To make your Analyte Working Stock, perform the necessary serial dilutions, in Standard Diluent, to achieve the final working concentration of 1,000 pg/mL in a 1 mL final volume. Ensure that all pipetting steps transfer ≥10 µL of liquid to achieve the best precision.
4. Discard standard after one use.

HUMAN PHOSPHO AKT1 ASSAY PROCEDURE

Standard Curve

Prepare the standard curve dilutions in a 12-channel reservoir. Perform 1:2 serial dilutions of the Analyte Working Stock for standards 2 through 11 to achieve a curve from 1,000 pg/mL to 1.0 pg/mL. Run the standards in triplicate.



1	2	3	4	5	6	7	8	9	10	11	12
1000	500	250	125	62.5	31.3	15.6	7.8	3.9	2.0	1.0	0

1. Add 500 µL Standard Diluent to wells 2 through 12 of a 12-channel reservoir.
2. Add 1,000 µL of the 1,000 pg/mL Analyte Working Stock from standard preparation into well 1.
3. Transfer 500 µL from well 1 into well 2, mixing thoroughly. Continue serial dilutions from well 2, stopping at well 11, mixing thoroughly each time. Use a fresh tip with each transfer.

Target Capture

1. Pipette 100 µL per well of Standards or diluted Samples to Plate 1 (96-well polypropylene).
2. Mix microparticles (coated beads) by gentle inversion until all beads are completely resuspended.
3. Immediately before adding to the assay plate, add the vial of Coated Beads to 10.5 mL of the supplied Assay Buffer. Mix by gentle inversion. Ensure that all beads have been transferred.
4. Pipette 100 µL per well of the Coated Beads into Plate 1.
5. Cover Plate 1 with a plate sealing film.
6. Incubate for 2 hours at 25°C on microplate incubator / shaker, **no shaking**.

7. Approximately 10 minutes prior to the end of Target Capture incubation, prepare the Detection Antibody. Add 15 µL of Detection Antibody to 2,985 µL of Assay Buffer to make a 1:200 dilution. Filter the diluted detection antibody using the syringe with a 0.2 µm filter into a clean tube.
8. When target capture incubation is complete, carefully remove temporary plate cover to avoid splashing.

Post-Capture Wash

The plate can be washed with a plate washer or a manual washer.

1. **Plate Washer**
    a. BioTek; Post Capture Wash (POSTCAP)
    b. HydroFlex; Post Capture Wash (PCW)
2. **Manual Post-Capture Wash Protocol**
    a. Place Plate 1 onto magnet (EMD Millipore PN 90-0003-02).
    b. Wait 2 minutes for beads to settle (ensure all beads are amassed as a pellet near magnet).
    c. Aspirate the supernatant (beads remain visible).
    d. Add 200 µL of Wash Buffer.
    e. Wait ≥ 2 minutes. To ensure that the beads remain amassed, do not suspend or remove beads from the magnet during this time.
    f. Aspirate buffer.

Detection

1. Immediately remove Plate 1 from the magnet and add 20 µL per well of Detection Antibody.
2. Cover Plate 1 with plate sealing film.
3. Incubate for 1 hour at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting # 5).
4. Carefully remove plate sealing film to avoid splashing.

HUMAN PHOSPHO AKT1 ASSAY PROCEDURE (continued)

Pre-Transfer Wash

The plate can be washed with a plate washer or a manual washer.

1. **Plate Washer**
    a. BioTek; 4 cycle Pre-Transfer (4CYCPRE)
    b. HydroFlex; 4 cycle Pre-Transfer (*4cyPrTra)*
2. **Manual Pre-Transfer Wash Protocol**
    a. Place Plate 1 onto magnet (EMD Millipore PN 90-0003-02).
    b. Add 100 µL of Wash Buffer to each well of Plate 1.
    c. Wait 2 minutes.
    d. Aspirate the supernatant and discard into waste, change tips.
    e. Add 200 µL of Wash Buffer to each well.
    f. Wait ≥ 2 minutes. To ensure that the beads remain amassed, do not suspend or remove beads from the magnet during this time.
    g. Aspirate buffer from each well, discard into waste and change tips.
    h. Repeat steps e - g three more times for a total of four washes.
    i. Add 200 µL of Wash Buffer to each well of Plate 1.
    j. Remove Plate 1 from magnet.

Manual Plate Transfer

1. **If using Sphere Mag Plate with SBS Footprint (EMD Millipore PN 90-0003-02 or DynaMag™-96 Side Skirted Magnet (Thermo Fisher PN 12027))**
    a. Prepare manual transfer station:
        i. Set manual 12-channel pipette to 100 µL.
        ii. Fill a reservoir with Wash Buffer.
        iii. Place Plate 2 on a magnet.
    b. In the first row of Plate 1, pipette up and down seven to ten times to resuspend beads gently to minimize bubbles.
    c. Transfer 200 µL (100 µL x 2) of suspended beads from Row A of Plate 1 to Row A of Plate 2.
    d. Change tips.
    e. Aspirate 100 µL of Wash Buffer from reservoir and dispense into Row A of Plate 1.
    f. Pipette up and down seven to ten times to resuspend any remaining beads, and then transfer 100 µL of suspended beads to Row A of Plate 2.
    g. Change tips.
    h. Repeat steps c - h for remaining 7 rows.

i. Inspect Plate 1 for any remaining beads.
    i. Yes, beads are present in Plate 1:
        - Add 100 µL of Wash Buffer to wells containing beads.
        - Gently mix by pipetting to resuspend the bead pellet.
        - Transfer the contents of each well containing beads to Plate 2 on magnet.
    ii. No, beads are not present in Plate 1.
j. Discard Plate 1.
k. Magnetized bead pellet should be visible in Plate 2.

Final Aspiration

1. Plate Washer
    a. BioTek; Final Aspirate (FINASP)
    b. HydroFlex; Final Aspirate (FA_V1)
2. Manual Final Wash Aspirate
    a. While Plate 2 is on the magnet, wait 2 minutes.
    b. Aspirate the supernatant and discard into waste.

Elution

1. Immediately remove Plate 2 from the magnet.
2. Add 10 µL Elution Buffer B per well.
3. Cover Plate 2 with a plate sealing film.
4. Incubate plate for 10 minutes at 25°C on microplate incubator / shaker (if using EMD Millipore PN 70-0009-00, use setting # 5).
5. Add 10 µL per well of Buffer D to Plate 3 (384-well polypropylene plate (Nunc PN 264573 or equivalent)) using a 12-channel manual P20.
6. Place Plate 2 on sphere magnet bed (EMD Millipore PN 90-0003-02), remove plate sealing film, and allow beads to form a tight pellet for 2 minutes.
7. Set manual 8-channel pipette to 15 µL and transfer eluate to Plate 3 by rows, avoiding the pelleted beads.
8. Cover Plate 3 with a universal plate cover and spin plate for 5 minutes at RT, approximately 1,100 x g.
9. Cover Plate 3 with heat sealing foil (EMD Millipore PN 02-01-0216-00 or equivalent), according to manufacturer's instructions for the heat sealer.

Run on Erenna® Immunoassay System

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

APPENDIX A: SMC™ Quick Assay Guide

1. Prepare all reagents, standard curve, and samples as instructed.
2. Add 100 µL of Standard/Samples and 100 µL of **Coated Beads** to **Plate 1**.
3. Cover and incubate for 2 hours at 25°C on microplate incubator/shaker (no shaking).

2 hours 25°C

4. Perform Post-Capture Wash (**Plate 1**).
5. Remove from magnet and add 20 µL of **Detection Antibody** per well.
6. Cover and incubate for 1 hour at 25°C on microplate incubator/shaker.

1 hour 25°C

7. Perform Pre-Transfer Post-Detection Wash (**Plate 1**).
8. Perform Manual Plate Transfer (**Plate 2**).
9. Perform Final Aspiration (**Plate 2**).
10. Remove from magnet and add 10 µL of **Elution Buffer B** to each well.
11. Cover and incubate for 10 minutes at 25°C on microplate incubator/shaker.

10 minutes 25°C

12. Add 10 µL of **Buffer D** per well to **Plate 3**.
13. Transfer contents of **Plate 2** to **Plate 3**.
14. Cover and centrifuge for 5 minutes at 1,100 x g.
15. Cover **Plate 3** with pierceable plate seal cover.

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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