



Phospho AKT1 Immunoassay Kit Instructions

Microparticle Assay

Catalog #03-0100-01

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

FOR RESEARCH USE ONLY

NOT INTENDED FOR DIAGNOSTIC OR THERAPEUTIC PROCEDURES

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INTRODUCTION

The Erenna[®] 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 (MPs). The user pipettes MPs, standards, and samples into uncoated microplate wells. During incubation, the Phospho AKT1 present in the sample binds to the capture antibody on the coated MPs. Unbound molecules are washed away during the subsequent buffer exchange and 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 MPs. During the following wash step the MPs are transferred to a clean plate. Elution buffer is then added and incubated. The elution buffer dissociates the bound protein sandwich from the MP 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.

FOR RESEARCH USE ONLY

MATERIALS

The Erenna[®] Phospho AKT1 Immunoassay kit includes all reagents listed in Table 1: Reagents Provided. Additional reagents and supplies may be required to run this immunoassay, as listed in APPENDIX B: Additional Supplies Required and below 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	2x 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	With cold pack	2-8°C	02-0001-06	1 x 30 mL
8	Elution Buffer B	With cold pack	2-8°C	02-0211-02	1 x 5 mL
9	Buffer D	With cold pack	2-8°C	02-0359-00	1 x 3 mL
10	Erenna [®] PHOSPHO AKT1 Immunoassay Kit Instructions	N/A	Ambient	05-0468-02	1

Table 1: Reagents Provided

Storage Instructions

The **Erenna® Phospho AKT1** Immunoassay Reagent Kit should be stored at 2–8°C. The Standard analyte should be stored at $\leq -70^{\circ}\text{C}$. Proper kit performance can only be guaranteed if the materials are stored properly.

General Supplies Required but Not Provided

- De-ionized or distilled water
- 12-channel pipettes capable of transferring 20 μL - 250 μL
- 8-channel pipette capable of transferring 10 μL
- Micro-centrifuge tubes
- Micro-centrifuge
- Container capable of holding 300 mL
- 500 mL graduated cylinder
- Rotisserie rotator for microparticle resuspension
- If using an automated plate washer additional **10x Wash Buffer** may be needed.
- Two 96-well polypropylene plates labeled **Plate 1** and **Plate 2** (Axygen P-96-450V-C)
- One 384-well polypropylene plate labeled **Plate 3** (Nunc 264573)

TECHNICAL HINTS DUE TO HIGH SENSITIVITY

- Wipe down bench and pipettes with 70% isopropanol before use.
- Quickly spin concentrated standard before opening vials.
- Use sterile filter pipette tips and reagent trays to avoid contamination.
- Use filter tips while transferring concentrated standard.
- Use a 12-channel reservoir for preparing standards.
- Pre-wet tips (aspirate and dispense within well) twice before each transfer.

ADDITIONAL SAMPLE INFORMATION

- The Erenna® Phospho AKT1 Immunoassay data has been compiled using cell lysate samples
- Ensure sample is clear of precipitants and other visible particulate matter before testing with the Erenna® Phospho AKT1 Immunoassay.

PRECAUTIONS

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

ASSAY PREPARATION

Reagent Preparation

1. Warm the following reagents to room temperature prior to use: **Sample Diluent**, **Assay Buffer**, **AKT1 Detection Antibody 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 the contents of the 30 mL bottle of 10X Wash Buffer into a container capable of holding at least 300 mL
 - b. Add 270 mL of deionized water
 - c. Mix thoroughly by gentle inversion or with a clean, sterile stir bar
4. Mix **Coated Beads** (coated microparticles) on a rotisserie spin rotator, or manually by repeat inversion, for 10-20 minutes until all MPs 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.

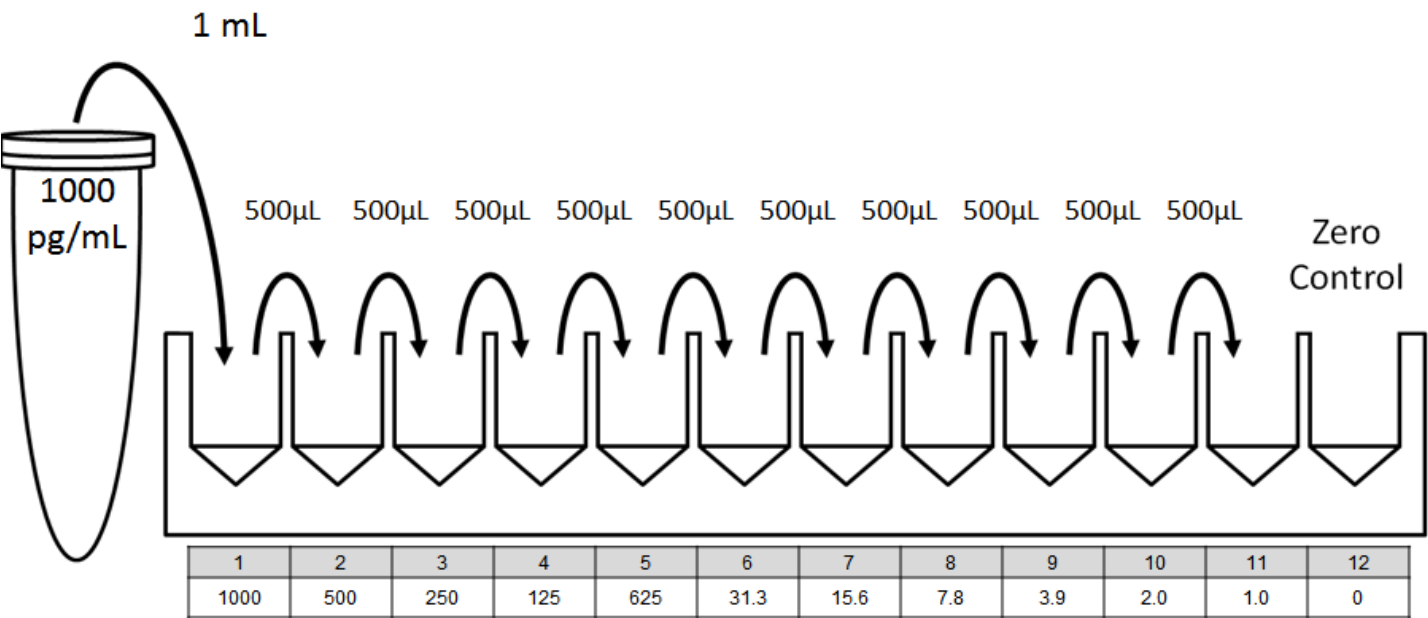
Initial Standard Stock Preparation

1. Quick spin and pipette mix the **Standard Analyte** vial in a mini-centrifuge prior to opening. 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 for the starting concentration of the **Standard Analyte** in the vial.
3. To make your **Analyte Working Stock**, perform the necessary serial dilutions to achieve the final working concentration of 1000 pg/mL in a 1 mL final volume. Ensure that all pipetting steps transfer ≥ 10 μ L of liquid to achieve the best precision.

PHOSPHO AKT1 ASSAY PROCEDURE

Standard Curve

Prepare the standard curve in a 12-channel reservoir dilution plate. Perform 1:2 serial dilutions of the **Analyte Working Stock** for dilutions 2 through 11 to achieve a curve from 1000 pg/mL to 1.0 pg/mL. Well 12 will not contain any standard analyte and will be run as your zero standard. Run the standards in triplicate.



1. Add 500 µL **Standard Diluent** to wells 2 through 12.
2. Add 1000 µL of the 1000 pg/mL **Analyte Working Stock** from standard preparation into well 1.
3. Mix well 1 thoroughly then transfer 500 µL from well 1 into well 2. Change tips. Continue serial dilutions from well 2 stopping at well 11. **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 (MPs) by gentle inversion until all MPs 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 an Axyseal plate cover.
6. Incubate for 2 hour at 25°C on Boekel Scientific, The Jitterbug™, **no shaking**.
7. Approximately 10 minutes prior to the end of Target Capture incubation, prepare the **Detection Antibody**. Add 15 µL of the **Detection Antibody** to 2985 µL **Detection Antibody Buffer**. Filter the diluted detection antibody using the syringe with a 0.2 µm filter into a clean tube.
8. When incubation is complete, carefully remove temporary plate cover to avoid splashing.

Post-Capture Wash

1. Manual Post-Capture Wash Protocol

- a. Place **Plate 1** onto magnet (DynaL MPC® - 96S)
- b. Wait 2 minutes for MPs to settle (ensure all MPs are amassed as a pellet by magnet)
- c. Aspirate the supernatant (MPs remain visible)
- d. Add 200 µL of Wash Buffer
- e. Wait ≥ 1 minute, to be sure that the MPs remain amassed
- f. Aspirate buffer

2. If using automation please contact your technical service representative, techsupport@singulex.com, for the appropriate automation procedure.

Detection

1. Immediately remove **Plate 1** from the magnet and add 20 µL per well of **Detection Antibody**.
2. Cover **Plate 1** with an Axyseal plate cover.
3. Incubate for 60 mins at 25°C on Jitterbug setting 5
4. Carefully remove Axyseal plate cover to avoid splashing.

Pre-Transfer Wash

1. Manual Pre-Transfer Wash Protocol

- a) Place **Plate 1** onto magnet
- 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 ≥ 1 minute, to be sure that the MPs remain amassed, do not suspend or remove MP 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

2. If using automation please contact your technical service representative, techsupport@singulex.com, for the appropriate automation procedure.

Plate Transfer

1. Manual Plate transfer Protocol

- a) Prepare manual transfer station:
 - a. Set manual 12 channel pipette to 100 μ L
 - b. Fill reservoir with **Wash Buffer**
 - c. Plate **Plate 2** on a magnet
- b) Put clean tips onto 12 channel manual pipette.
- c) In the first row of **Plate 1**, pipette up and down 10x to resuspend MPs gently to minimize bubbles.
- d) Transfer 200 μ L (100 μ L x 2) of suspended MPs from Row A of **Plate 1** to Row A of **Plate 2**.
- e) Change tips.
- f) Aspirate 100 μ L of **Wash Buffer** from reservoir and dispense into Row A of **Plate 1**.
- g) Pipette up and down 10x to resuspend any remaining MPs, then transfer 100 μ L of suspended MPs to Row A of **Plate 2**.
- h) Change Tips.
- i) Repeat steps c-h for remaining 7 rows.
- j) Inspect **Plate 1** for any remaining MPs
 - a. Yes, MPs are present in **Plate 1**:
 - Add 100 μ L of **Wash Buffer** to wells containing MPs
 - Gently mix by pipetting to re-suspend the MP pellet.
 - Transfer the contents of each well containing MPs to **Plate 2** on magnet.
 - b. No, MPs are not present in **Plate 1**.
- k) Discard Plate 1
- l) Magnetized MP pellet should be visible in **Plate 2**.

2. If using automation please contact your technical service representative, techsupport@singulex.com, for the appropriate automation procedure

Final Aspiration

1. While **Plate 2** is on the magnet, wait 2 minutes
2. 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 an AxySeal plate cover.
4. Incubate plate for 10 minutes at 25°C on Jitterbug setting 5.
5. Add 10 µL per well of **Buffer D** to assay **Plate 3** (384-well polypropylene plate, Nunc, PN 264573) using a 12-channel manual P20.
6. Place **Plate 2** on bar magnet bed, remove AxySeal plate cover, and allow MPs to form a tight pellet for 2 min.
7. Set manual 8 channel pipette to 15 µL and put 8 tips onto the pipettor. Transfer eluate to **Plate 3** by columns, avoiding the pelleted MPs.
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, according to manufacturer instructions for the heat sealer.

Run on Erenna Immunoassay System

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

APPENDIX A: ERENNA® Quick Assay Guide

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

2 Hour
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 60 minutes at 25°C on Jitterbug (setting 5).

60 minutes
25°C

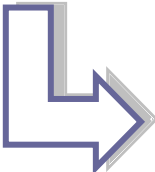


10 Minutes
25°C



- 1. Perform Pre-Transfer/Post-Detection Wash (**Plate 1**).
- 2. Perform Manual Plate Transfer (**Plate 2**).
- 3. Perform Final Aspiration (**Plate 2**).
- 4. Remove from magnet and add 10 µL of **Elution Buffer** to each well.
- 5. Cover and incubate at 25°C for 10 minutes on Jitterbug (setting 5).

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



**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	Singulex	02-0111-00	Systems (Analysis) Buffer, fluid used to run Erenna System	1L (10L mixed)
Erenna® 10X Wash Buffer* Contains Proclin	Singulex	02-0514-00	Wash buffer used for manual and automation wash protocols	1L (10L mixed)
Reservoirs for 12-Channel Pipettors	VWR	80092-466	Standard Curve	10/pkg
96-Well V-Bottom PolyPropylene Plate, 480 µL	Axygen	P-96-450V-C or P-96-450V-C-S	Assay Plate 1 and Plate 2, Receptacle plates	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	Assay Pate 3, 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
AcroPrep™ 96-well Filter Plate (Supor Membrane)	Pall	5041	Alternate sample preparation	10/pkg
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
Dynal MPC® - 96S	Dynal™	120.27	Rare Earth Magnet to capture MP during wash	1 plate
Sphere Mag Plate -SBS Foot Print	Singulex	45-0036-00	Sphere magnet used to capture MPs during automated washes	1 plate
Centrifuge w/ Plate Rotor	---	---	Remove MP via filter plate ~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	---
Heat Sealer with adjustable temperature and time	FluidX	XTS-384 or equivalent	Sealing plate for analysis on Erenna	1
Universal Adapter for SBS format plates	FluidX	42-1001 or equivalent	Required for proper sealing on XTS-384	1

CONTACT INFORMATION

To reach Singulex, Inc. reagent technical support, call **(510) 995-9000 ext.3**, or in the U.S. you may call us toll-free at (888) 603-3033.

You can also send us an e-mail at techsupport@singulex.com

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