

RayBio[®] C-Series Human and Mouse AKT Pathway Phosphorylation Array C1

For the semi-quantitative detection of 18 phosphorylated human and mouse proteins in cell and tissue lysates

Patent Pending Technology

User Manual

Cat# AAH-AKT-1-2 (2 Sample Kit)

Cat# AAH-AKT-1-4 (4 Sample Kit)

Cat# AAH-AKT-1-8 (8 Sample Kit)

Please read manual carefully
before starting experiment





C-Series Antibody Arrays

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I. INTRODUCTION

New techniques such as cDNA microarrays have enabled us to analyze global gene expression¹⁻³. However, almost all cell functions are executed by proteins, which cannot be studied simply through DNA and RNA techniques. Experimental analysis clearly shows disparity can exist between the relative expression levels of mRNA and their corresponding proteins⁴. Therefore, analysis of the proteomic profile is critical.

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry^{5,6}. However, these methods are slow, expensive, labor-intensive and require specialized equipment⁷. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming.

The AKT pathway, or PI3K-Akt Pathway is one of the most important signaling pathways that link extracellular signals to the machinery that controls fundamental cellular processes such as survival, growth, cell migration and apoptosis.

The RayBio® Human and Mouse AKT Pathway Phosphorylation Array Kit is a rapid, sensitive, and economical tool for the simultaneous detection of the relative levels of phosphorylation of 18 AKT pathway proteins. Each antibody was carefully validated using appropriate cell lysates. With the RayBio® Human and Mouse AKT Pathway Phosphorylation Array Kit, you can verify multiple activated targets in your system without spending excess time and effort in performing multiple ELISAs and/or Western blots. The RayBio® Human and Mouse AKT Pathway Phosphorylation Array Kit has several advantages over the detection of AKT pathway markers using single-target ELISA kits or Western blots:

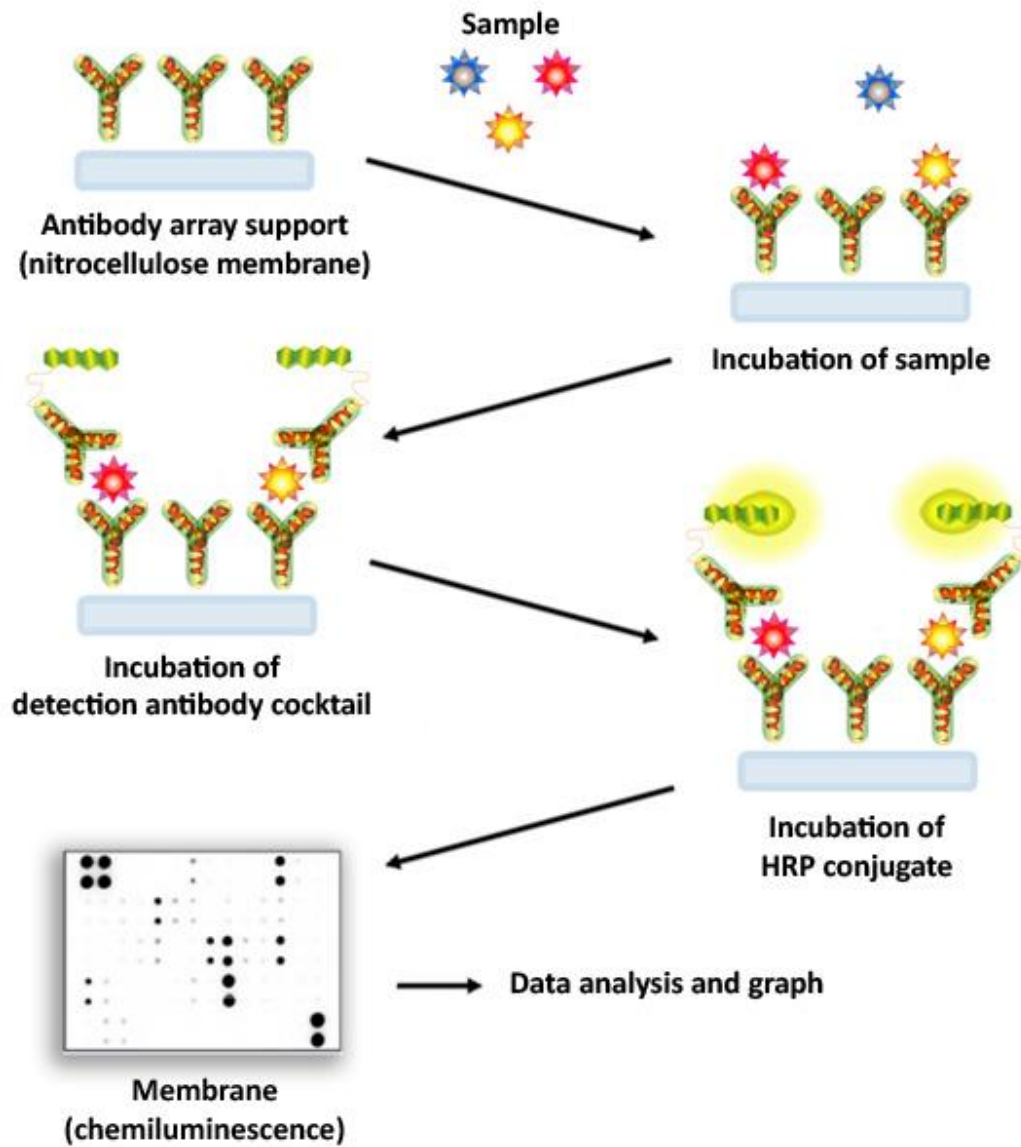
1. More Data, Same or Less Sample: Antibody arrays provide high-content screening using about the same sample volume as traditional ELISA.
2. Global View of Protein Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cell signaling.
3. Similar (sometimes better) Sensitivity: As little as 4 pg/ml of MCP-1 can be detected using the C-Series array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.
4. Wider Detection Range: ELISA typically detects a concentration range of 100 to 1000-fold, while RayBiotech arrays can detect concentration ranges up to 10,000-fold.
5. Better Precision: As determined by densitometry, the inter-array Coefficient of Variation (CV) of spot signal intensities is 5-10%, comparing favorably with ELISA testing (CV = 10-15%).

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II. HOW IT WORKS



III. COMPONENTS AND STORAGE

ITEM	COMPONENT	AAH-AKT-1-2	AAH-AKT-1-4	AAH-AKT-1-8	STORAGE TEMPERATURE AFTER THAWING**
1	Antibody Arrays*	2 membranes	4 membranes	8 membranes	≤ -20 °C
2	Blocking Buffer	1 vial (25 ml)		2 vials (25 ml/ea)	
3	Detection Antibody Cocktail	1 vial	2 vials	4 vials	2-8 °C (for up to 3 days after dilution)
4	1,000X HRP-Anti-Rabbit IgG Concentrate	1 vial (20 µl)			2-8 °C
5	20X Wash Buffer I Concentrate	1 vial (10 ml)		1 vial (20 ml)	
6	20X Wash Buffer II Concentrate	1 vial (10 ml)		1 vial (20 ml)	
7	2X Cell Lysis Buffer Concentrate	1 vial (10 ml)		1 vial (16 ml)	
8	Detection Buffer C	1 vial (1.5 ml)		1 vial (2.5 ml)	
9	Detection Buffer D	1 vial (1.5 ml)		1 vial (2.5 ml)	
10	8-Well Incubation Tray w/ Lid	1 tray			Room Temperature
11	Protease Inhibitor Cocktail	1 vial		2 vials	≤ -20 °C
12	100x Phosphatase Inhibitor Cocktail I	1 vial		2 vials	
13	Phosphatase Inhibitor Cocktail II	1 vial		2 vials	
Other Kit Components: Plastic Sheets, Array Map Template, User Manual					

Store kit at ≤ -20 °C immediately upon arrival. Kit must be used before the 6 month expiration date.

*Each package contains 2 or 4 membranes

**For up to 3 months (unless stated otherwise) or until expiration date

IV. ADDITIONAL MATERIALS REQUIRED

- Pipettors, pipet tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Tissue paper, blotting paper or chromatography paper
- Adhesive tape or plastic wrap
- Distilled or de-ionized water
- A chemiluminescent blot documentation system:
 - CCD Camera
 - X-Ray Film and a suitable film processor
 - Gel documentation system
 - Or another chemiluminescent detection system capable of imaging a western blot

V. SAMPLE TIPS AND GENERAL CONSIDERATIONS

A. Sample Collection, Preparation, and Storage

NOTE: Optimal methods will need to be determined by each researcher empirically based on researched literature and knowledge of the samples.

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease and phosphatase inhibitor cocktail to cell and tissue lysate samples.
- Avoid sonication of 1 ml or less as this can quickly heat and denature proteins.
- Most samples will not need to be concentrated. If concentration is required, a spin column concentrator with a chilled centrifuge is recommended.
- Always centrifuge the samples hard after thawing (~10,000 RPM for 2-5 minutes) in order to remove any particulates that could interfere with detection.

- **The Cell Lysate can be prepared as follows:**

- For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1500 rpm for 10 min) making sure to remove any remaining PBS before adding Lysis Buffer. Solubilize the cells at 2×10^7 cells/ml in 1X Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (see preparation note shown on page 8 under Component Preparation Section). Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 min.

It is recommended that sample protein concentrations should be determined using a total protein assay. For sample incubation with the Phosphorylation Antibody Array, use at a protein concentration of 50-1000 µg/ml for cell lysates.

Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.

If you experience high background, you may further dilute your samples.

General tips for preparing lysate samples can be viewed on the online Resources page of the website.

B. Sample Types and Recommended Dilutions/Amounts

NOTE: Optimal sample dilutions and amounts will need to be determined by each experimenter empirically but the below recommendations may be used as a starting point. Blocking Buffer (ITEM 2) should be used to dilute samples. Normalize by loading equal amounts of protein per sample.

- **Cell and Tissue Lysates:** load **50 to 1000 µg** of total protein (after at least a 5-fold dilution to minimize the effect of any detergent(s)). Therefore the original lysate concentration should be **250 µg to 5 mg/ml**.

C. Handling Membranes

- The antibody printed side of each membrane is marked by a dash (-) or number (#) in the upper left corner.

- Do not allow membranes to dry out during the experiment or they may become fragile and break OR high and/or uneven background may occur.
- Grasp membranes by the corners or edges only using forceps. DO NOT touch printed antibody spots.

D. Incubations and Washes

- Perform **ALL** incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/sec) using an orbital shaker or oscillating rocker to ensure complete and even reagent/sample coverage. Rocking/rotating too vigorously may cause foaming or bubbles to appear on the membrane surface which should be avoided.
- All washes and incubations should be performed in the Incubation Tray (ITEM 10) provided in the kit.
- Cover the Incubation Tray with the lid provided during all incubation steps to avoid evaporation and outside debris contamination.
- Ensure the membranes are completely covered with sufficient sample or reagent volume during each incubation.
- Avoid forceful pipetting directly onto the membrane; instead, gently pipette samples and reagents into a corner of each well.
- Aspirate samples and reagents completely after each step by suctioning off excess liquid with a pipette. Tilting the tray so the liquid moves to a corner and then pipetting is an effective method.
- Optional overnight incubations may be performed for the following steps to increase overall spot signal intensities:
 - Sample Incubation
 - Detection Antibody Cocktail Incubation
 - HRP-Anti-Rabbit IgG Incubation

NOTE: *Overnight incubations should be performed at 4 °C (also with gentle rocking/shaking). Be aware that longer incubations can also increase the background response so complete liquid removal and washing is critical.*

VI. CHEMILUMINESCENCE DETECTION TIPS

- The time between adding the detection buffers and exposing the membranes should be no more than 10-15 minutes as the chemiluminescent signals may start to fade after this time.
- Trying multiple exposure times is recommended to obtain optimum results.
- A few seconds to a few minutes is the recommended exposure time range, with 30 seconds to 1 minute being suitable for most samples.

Don't have time or the equipment to image your membranes? Let the experts at RayBiotech image and analyze your membranes. Contact us for pricing details.

VII. COMPONENT PREPARATION

NOTE: Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.

NOTE: The Detection Antibody Cocktail (ITEM 3) and the HRP-Anti-Rabbit IgG Concentrate (ITEM 4) vials should be briefly centrifuged (~1000 xg) before opening to ensure maximum recovery and mixed well as precipitates may form during storage.

*1 vial is enough to test 2 membranes

**Only for use for preparation of cell or tissue lysates. General tips for preparing lysates and other common sample types can be found on the online Resources Page

ITEM	COMPONENT	PREPARATION	EXAMPLE
1	Antibody Arrays	No Preparation	N/A
2	Blocking Buffer		
3	Detection Antibody Cocktail*	Pipette 2 ml of Blocking Buffer into each vial. Mix gently with a pipette.	N/A
4	1,000X HRP-Anti-Rabbit IgG Concentrate	Dilute 1,000-fold with Blocking Buffer. Mix gently with a pipette.	10 µl of 1,000X concentrate + 9990 µl of Blocking Buffer = 10 ml of 1X working solution
5	20X Wash Buffer I Concentrate	Dilute each 20-fold with distilled or deionized water.	10 ml of 20X concentrate + 190 ml of water = 200 ml of 1X working solution
6	20X Wash Buffer II Concentrate		
7	2X Cell Lysis Buffer Concentrate**	Dilute 2-fold with distilled or deionized water.	10 ml of 2X concentrate + 10 ml of water = 20 ml of 1X working solution
8	Detection Buffer C	No Preparation	N/A
9	Detection Buffer D		
10	8-Well Incubation Tray w/ Lid		
11	Protease Inhibitor Cocktail	Pipette 60 µl of 1X Cell Lysis Buffer into the vial to prepare 100X Protease Inhibitor Cocktail concentrate.	N/A
12	100x Phosphatase Inhibitor Cocktail I	No Preparation	N/A
13	Phosphatase Inhibitor Cocktail II	Add 180 µl of 1X Lysis Buffer into the vial to prepare 25X Phosphatase Inhibitor Cocktail Set II Concentrate. Dissolve the powder thoroughly by gentle mixing.	N/A

Note: Prior to preparing cell or tissue lysates: Add 20 µl Protease Inhibitor Cocktail Concentrate (100X), 20 µl Phosphatase Inhibitor Cocktail Set I (100x) and 80 µl Phosphatase Inhibitor Cocktail Set II Concentrate (25X) into 1.9 ml 1X Lysis Buffer immediately before use. Mix well.

VIII. PROTOCOL

NOTE: Prepare all reagents and samples immediately prior to use. See Sections V and VII. **ALL** incubations and washes must be performed under gentle rotation/rocking (~0.5-1 cycle/sec)

- 1) Remove the kit from storage and allow the components to equilibrate to room temperature (RT).
- 2) Carefully remove the Antibody Arrays (ITEM 1) from the plastic packaging and place each membrane (printed side up) into a well of the Incubation Tray (ITEM 10). One membrane per well.

NOTE: The antibody printed side is marked by a dash (-) or number (#) in the upper left corner.

A. Blocking

- 3) Pipette 2 ml of Blocking Buffer (ITEM 2) into each well and incubate for 30 minutes at RT.
- 4) Aspirate blocking buffer from each well with a pipette.

B. Sample Incubation

- 5) Pipette 1 ml of diluted or undiluted sample into each well and incubate for 1.5 to 5 hours at RT OR overnight at 4 °C.

NOTE: Longer incubations can help maximize the spot signal intensities. However, doing so can also increase the background response so complete liquid removal and washing is critical.

- 6) Aspirate samples from each well with a pipette.

C. First Wash

NOTE: The 20X Wash Buffer Concentrates I and II (ITEM 5 and 6) must be diluted 20-fold before use. See Section VII for details.

- 7) Wash Buffer I Wash: Pipette 2 ml of **1X** Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times using fresh buffer for a total of 3 washes, aspirating out the buffer completely each time.
- 8) Wash Buffer II Wash: Pipette 2 ml of **1X** Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time using fresh buffer for a total of 2 washes, aspirating out the buffer completely each time.

D. Detection Antibody Cocktail Incubation

NOTE: The Detection Antibody Cocktail (ITEM 3) must be prepared before use. See Section VII for details.

- 9) Pipette 1 ml of the **prepared** Detection Antibody Cocktail into each well and incubate for 1.5 to 2 hours at RT OR overnight at 4 °C.

10) Aspirate Detection Antibody Cocktail from each well.

E. Second Wash

11) Wash membranes as directed in Steps 7 and 8.

F. HRP-Anti-Rabbit IgG Incubation

NOTE: *The 1,000X HRP-Anti-Rabbit IgG Concentrate (ITEM 4) must be diluted before use. See Section VII for details.*

12) Pipette 2 ml of **1X** HRP-Anti-Rabbit IgG into each well and incubate for 2 hours at RT OR overnight at 4 °C.

13) Aspirate HRP-Anti-Rabbit IgG from each well.

G. Third Wash

14) Wash membranes as directed in Steps 7 and 8.

H. Chemiluminescence Detection

NOTE: *Do not allow membranes to dry out during detection.*

15) Transfer the membranes, printed side up, onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface (such as a benchtop).

16) Remove any excess wash buffer by blotting the membrane edges with another piece of paper.

17) Transfer and place the membranes, printed side up, onto a plastic sheet (provided) lying on a flat surface.

NOTE: *Multiple membranes can be placed next to each other and fit onto a single plastic sheet. Use additional plastics sheets if necessary.*

18) Into a single clean tube, pipette equal volumes (1:1) of Detection Buffer C (ITEM 8) and Detection Buffer D (ITEM 9). Mix well with a pipette.

EXAMPLE: *250 µl of Detection Buffer C + 250 µl of Detection Buffer D = 500 µl (enough for 1 membrane)*

19) Gently pipette 500 µl of the Detection Buffer mixture onto each membrane and incubate for 2 minutes at RT (DO NOT ROCK OR SHAKE). Immediately afterwards, proceed to Step 20.

NOTE: *Exposure should ideally start within 5 minutes after finishing Step 19 and be completed within 10-15 minutes as chemiluminescence signals will fade over time. If necessary, the signals can usually be restored by repeating washing, HRP-Anti-Rabbit IgG and Detection Buffers incubations (Steps 11-19)*

20) Place another plastic sheet on top of the membranes by starting at one end and gently “rolling” the flexible plastic sheet across the surface to the opposite end to smooth out any air bubbles. The membranes should now be “sandwiched” between two plastic sheets.

NOTE: Avoid “sliding” the top plastic sheet along the membranes’ printed surface. If using X-ray film, do not use a top plastic sheet so that the membranes can be directly exposed to the film.

21) Transfer the sandwiched membranes to the chemiluminescence imaging system such as a CCD camera (recommended) and expose.

NOTE: Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Section VI for additional details.

I. Storage

22) To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or use plastic wrap to secure them, and store at $\leq -20^{\circ}\text{C}$ for future reference.

IX. TYPICAL RESULTS

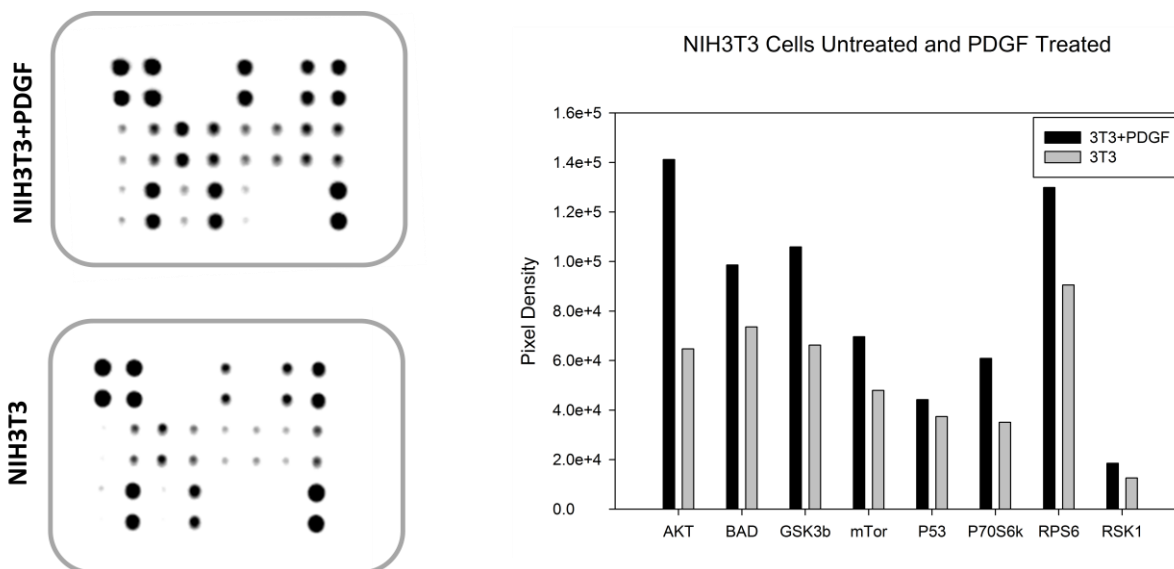


Figure 1. NIH3T3 cells were grown to 80% confluency and then serum starved overnight. Cells were either untreated (bottom panel) or treated (top panel) with PDGF β for 10 minutes. Data shown are from a 20 second exposure using a chemiluminescence imaging system.

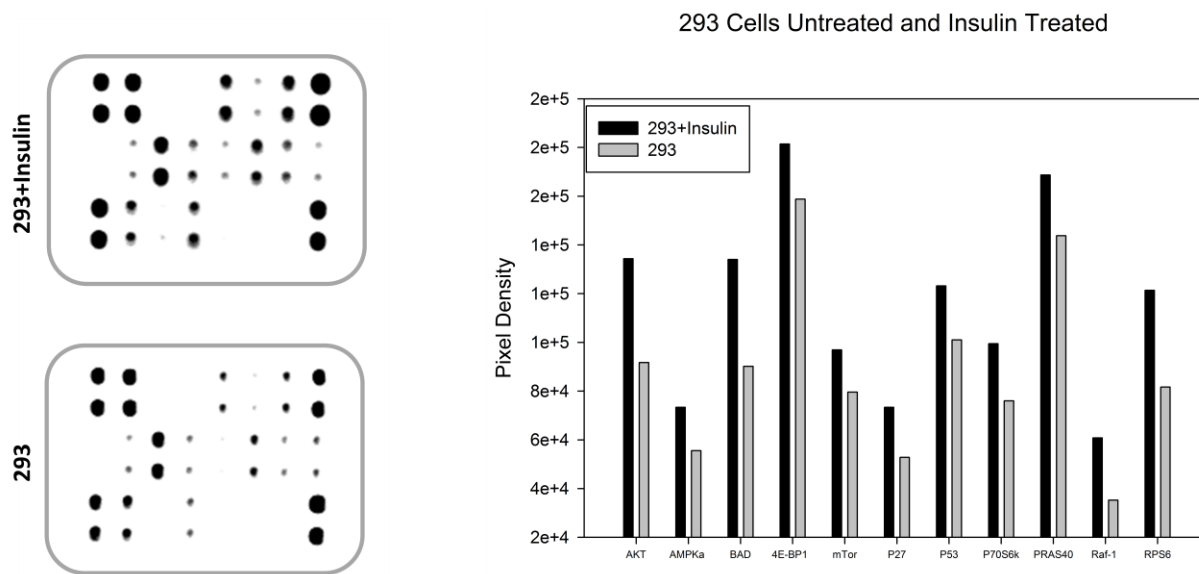


Figure 2. 293 cells were grown to 80% confluency and then serum starved 24 hours. Cells were either untreated (bottom panel) or treated (top panel) with Insulin for 30 minutes. Data shown are from a 20 second exposure using a chemiluminescence imaging system.

Note the strong signals of the Positive Control spots in the upper left and lower right corners. (See below for further details on the control spots.)

The signal intensity for each antigen-specific antibody spot is proportional to the relative concentration of the antigen in that sample. Comparison of signal intensities for individual antigen-specific antibody spots between and among array images can be used to determine relative differences in expression levels of each analyte sample-to-sample or group-to-group.

X. INTERPRETING THE RESULTS

A. Control Spots

Positive Control Spots (POS) – controlled amount of Detection antibody printed onto the array. Used for normalization and to orientate the arrays.

Negative Control Spots (NEG) – buffer printed (no antibodies) used to measure the baseline responses. Used for determining the level of non-specific binding of the samples.

B. Data Extraction

Visual comparison of array images may be sufficient to see differences in relative protein expression. However, most researchers will want to perform numerical comparisons of the signal intensities (or more precisely, signal *densities*), using 2-D densitometry. Gel/Blot documentation systems and other chemiluminescent or phosphorescent detection systems are usually sold as a package with compatible densitometry software.

Any densitometry software should be sufficient to obtain spot signal densities from your scanned images. One such software program, ImageJ, is available for free from the NIH website along with an array plug-in.

We suggest using the following guidelines when extracting densitometry data from our array images:

- For each array membrane, identify a single exposure that exhibits a high signal to noise ratio (strong spot signals and low background response). Strong Positive Control Spot signals but not too strong that they are “bleeding” into one another is ideal. The exposure time does not need to be identical for each array, but Positive Control signals on each array image should have similar intensities.
- Measure the density of each spot using a circle that is roughly the size of one of the largest spots. Be sure to use the same extraction circle dimensions (area, size, and shape) for measuring the signal densities on every array for which you wish to compare the results.
- For each spot, use the summed signal density across the entire circle (i.e., total signal density per unit area)

C. Data Analysis

NOTE: RayBiotech offers Microsoft® Excel-based Analysis Software Tools for each array kit for automatic analysis. Please visit the website at www.raybiotech.com or contact us for ordering information.

Once the raw numerical densitometry data is extracted, the background must be subtracted and the data need to be normalized to the Positive Control signals.

Background Subtraction: Select values which you believe best represent the background. If the background is fairly even throughout the membrane, the Negative Control Spots (NEG) should be similar and are accurate for this purpose.

Positive Control Normalization: The amount of Detection antibody printed for each Positive Control Spot is consistent from array to array. As such, the intensity of these Positive Control signals can be used to

normalize signal responses for comparison of results across multiple arrays, much like housekeeping genes and proteins are used to normalize results of PCR gels and Western Blots, respectively.

To normalize array data, one array is defined as the "Reference Array" to which the other arrays are normalized to. The choice of the Reference Array is arbitrary.

NOTE: The RayBio® Analysis Software Tools always designate Array 1/Sample 1 as the Reference Array.

Next, the simple algorithm below can be used to calculate and determine the signal fold expression between like analytes.

$$X(Ny) = X(y) * P1/P(y)$$

Where:

P1 = mean signal density of Positive Control spots on the reference array

P(y) = mean signal density of Positive Control spots on Array "y"

X(y) = mean signal density for spot "X" on Array "y"

X(Ny)= normalized signal intensity for spot "X" on Array "y"

For example:

Let's determine the relative expression of AKT on two different arrays (Arrays 1 and 2). Let's assume that the duplicate signals for the AKT spots on each array are identical (or that the signal intensity used in the following calculation is the mean of the two duplicate spots). Also assume the following:

P1 = 2500

P2 = 2700

AKT (1) = 300

AKT (2) = 455

Then AKT(N2) = 455 * 2500/2700 = 421.30

The fold increase of AKT(N2) vs AKT(1) = 421.3/300 = 1.40-fold increase or a 40% increase in the signal intensity of AKT in Array 2 vs. Array 1.

XI. ARRAY MAP

Each antibody is spotted in duplicate vertically	1	POS	POS	NEG	NEG	Akt (P-Ser473)	AMPKa (P-Thr172)	BAD (P-Ser112)	4E-BP1 (P-Thr36)
	2								
	3	ERK1 (P-T202/Y204)	GSK3a	GSK3b	mTOR	p27	P53	P70S6K	PDK1
	4	ERK2 (P-Y185/Y187)	(P-Ser21)	(P-Ser9)	(P-Ser2448)	(P-Thr198)	(P-Ser15)	(P-Thr421/Ser424)	(P-Ser241)
	5	PRAS40	PTEN	Raf-1	RPS6	RSK1	RSK2	NEG	POS
	6	(P-Thr246)	(P-Ser380)	(Ser301)	(P-Ser235/236)	(P-Ser380)	(P-Ser386)		

POS = Positive Control Spot

NEG = Negative Control Spot

NOTE: Protein alternative names, accession numbers, and official symbols can be accessed on www.raybiotech.com via the Resources Page.

XII. TROUBLESHOOTING GUIDE

PROBLEM	CAUSE	RECOMMENDATION
No signals (not even the positive control spots)	Chemiluminescent imager is not working properly	Contact image manufacturer
	Too short exposure	Expose the membranes longer
	Degradation of components due to improper storage	Store entire kit at $\leq -20^{\circ}\text{C}$. Do not use kit after expiration date. See storage guidelines
	Improper preparation or dilution of the HRP-Anti-Rabbit IgG	Centrifuge vial briefly before use, mix well, and do not dilute more than 1000-fold
	Waiting too long before exposing	The entire detection process should be completed in 10-15 minutes
Positive control spot signals are visible but not other spots	Low sample protein levels	Decrease sample dilution, concentrate samples, or load more protein initially
	Skipped sample incubation step	Samples must be loaded after the blocking step
	Too short of incubations	Ensure the incubations are performed for the appropriate time or try the optional overnight incubation(s)
Uneven signals and/or background	Bubbles present on or below membrane	Don't rock/rotate the tray too vigorously or pipette the sample or reagent with excessive force
	Insufficient sample or reagent volume	Load enough sample and reagent to completely cover the membrane
	Insufficient mixing of reagents	Gently mix all reagents before loading onto the membrane, especially the HRP-Anti-Rabbit IgG and Detection Antibody Cocktail
	Rocking/Rotating on an uneven surface while incubating	Rock/rotate on a flat surface or the sample or reagent can "pool" to one side
High background signals or all spots visible	Too much HRP-Anti-Rabbit IgG or Detection Antibody Cocktail	Prepare these signal enhancing components precisely as instructed
	Membranes dried out	Do not let the membranes dry out during the experiment. Cover the incubation tray with the lid to minimize evaporation
	Too high of sample protein concentration	Increase dilution of the sample or load less protein
	Exposed too long	Decrease exposure time
	Insufficient washing	Ensure all the wash steps are carried out and the wash buffer is removed completely after each wash step
	Non-specific binding	Ensure the blocking buffer is stored and used properly

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