ab134001 – Human Apoptosis Antibody Array – Membrane

Instructions for Use

For the simultaneous detection of 43 Human Apoptosis marker concentrations in cell and tissue lysates

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

Apoptosis is the process of programmed cell death that involves a series of biochemical events leading to characteristic cell morphology and death. These events include blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation.

Studies on apoptosis have increased substantially since the early 1990s. In addition to its importance as a biological phenomenon such as cell termination, homeostasis, development and lymphocyte interactions, deregulation of apoptosis has been implicated in many diseases. Excessive apoptosis causes hypotrophy, such as in ischemic damage, whereas insufficient apoptosis results in uncontrolled cell proliferation, such as HIV progression and cancer development.

Apoptosis is mediated by a diverse range of cell signals, both extracellular and intracellular. Extracellular signals may include toxins, hormones, growth factors, nitric oxide or cytokines. Intracellular apoptotic signaling may be induced in response to stress via, heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration or the binding of nuclear receptors by glucocorticoids. These signals may positively or negatively induce apoptosis.

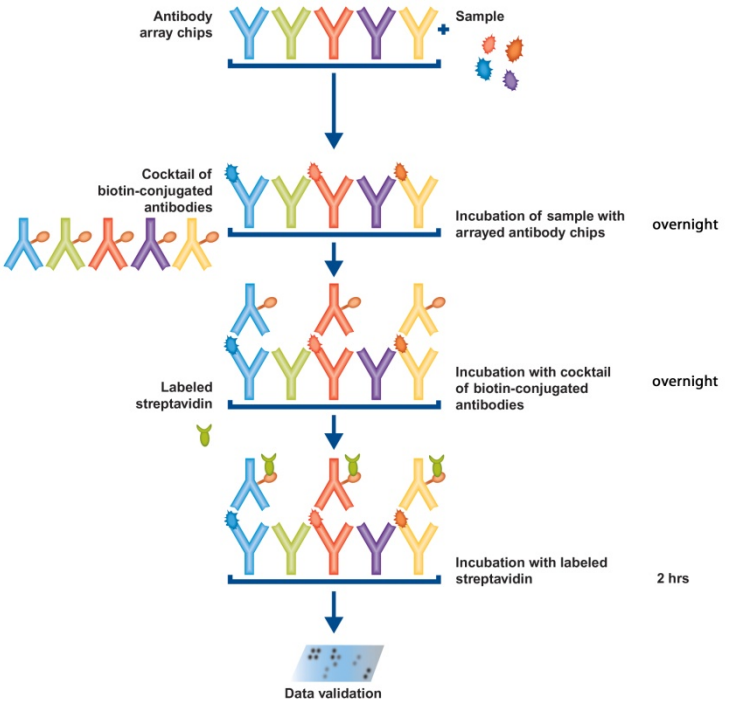
Two apoptotic signal transduction pathways in mammals have been reported: the TNF-induced model and the Fas-Fas ligand-mediated model. TNF is the major extrinsic mediator of apoptosis. Most cells in the human body have two receptors for TNF: TNF-R1 and TNF-R2. The binding of TNF to TNF-R1 has been shown to initiate the pathway that leads to caspase activation via the intermediate membrane proteins TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD). Binding of this receptor can also indirectly lead to the activation of transcription factors involved in cell survival and inflammatory responses. The Fas receptor (also known as Apo-1 or CD95) binds the Fas ligand. The interaction between Fas and FasL results in the formation of the death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10. Following TNF-R1 and Fas activation in mammalian cells a balance between pro-apoptotic (BAX, BID, BAK, or BAD) and anti-apoptotic (Bcl-Xl and Bcl-2) members of the Bcl-2 family is established. This balance is the proportion of pro-apoptotic homodimers that form in the outer-membrane of mitochondrion. The pro-apoptotic homodimers are required to make the mitochondrial membrane permeable for the release of caspase activators such as cytochrome c and SMAC. Control of pro-apoptotic proteins under normal cell conditions of non-apoptotic cells is incompletely understood.

Mitochondria are an important site for apoptosis. Mitochondrial proteins known as SMACs (second mitochondria-derived activator of caspases) are released into the cytosol following an increase in

permeability. SMAC binds to inhibitor of apoptosis proteins (IAPs) and deactivates them, preventing the IAPs from arresting the apoptotic process and therefore allowing apoptosis to proceed. IAP also normally suppresses the activity of caspases (cysteine aspartic acid proteases), which carry out the degradation of the cell. Therefore, the degradative activity of caspases seems to be indirectly regulated by mitochondrial permeability. Cytochrome c is also released from mitochondria due to formation of a channel, MAC, in the outer mitochondrial membrane, and serves a regulatory function as it precedes morphological change associated with apoptosis. Once cytochrome c is released it binds with Apaf-1 and ATP, which then bind to pro-caspase-9 to create a protein complex known as an apoptosome. The apoptosome cleaves the pro-caspase to its active form of caspase-9, which in turn activates the effector caspase-3.

The tumor-suppressor protein p53 also plays critical role in apoptosis. p53 accumulates in response to DNA damage via interferon-alpha and interferon-beta pathways, which induce transcription of the p53 gene and result in the increase of p53 protein level and enhancement of cancer cell apoptosis. p53 prevents the cell from replicating by stopping the cell cycle at G1, or interphase, to give the cell time to repair, however it will induce apoptosis if damage is extensive and repair efforts fail. Any disruption to the regulation of the p53 or interferon genes will result in impaired apoptosis and the possible formation of tumors.

A recent report has shown the involvement of IGFBPs (insulin-like growth factor-binding protein) in apoptosis. IGFBP1 protein localizes to mitochondria where it binds to the BAK and hinders BAK activation and apoptosis induction. When IGFBP1 is in a complex with BAK, formation of a proapoptotic p53/BAK complex and apoptosis induction is impaired, both in cultured cells and in liver. In contrast, livers of IGFBP1-deficient mice exhibit spontaneous apoptosis that is accompanied by p53 mitochondrial accumulation and BAK oligomerization. These results identify IGFBP1 as a negative regulator of the BAK-dependent pathway of apoptosis, whose expression integrates the transcriptional and mitochondrial functions of the p53 tumor suppressor protein.


Antibody array chips
Sample
Cocktail of biotin-conjugated antibodies
Incubation of sample with arrayed antibody chips
overnight
Labeled streptavidin
Incubation with cocktail of biotin-conjugated antibodies
overnight
Incubation with labeled streptavidin
2 hrs
Data validation

2. Storage and Components

A. Storage

For best results, store the entire kit at –20°C or –80°C upon arrival.

Once thawed, store array membranes and 1X Blocking Buffer at –20°C or –80°C, and all other components at 4°C. Array kits are robust and will retain full activity even if stored for up to 24 hours at room temperature.

B. Components

Item	Quantity		
Apoptosis Antibody Array Membranes	2 membranes	4 membranes	8 membranes
Biotin-Conjugated Anti-Cytokines	1 vial	2 vials	4 vials
1000X HRP-Conjugated Streptavidin	50 µl	50 µl	50 µl
1X Blocking Buffer	25 ml	25 ml	50 ml
20X Wash Buffer I	10 ml	10 ml	20 ml
20X Wash Buffer II	10 ml	10 ml	20 ml
2X Cell Lysis Buffer	10 ml	10 ml	16 ml
Detection Buffer C	1.5 ml	1.5 ml	2.5 ml
Detection Buffer D	1.5 ml	1.5 ml	2.5 ml
Protease Inhibitor Cocktail	1 vial	1 vial	2 vials
8-Well Plastic Tray	1 unit	1 unit	1 unit

*Plastic Sheets also provided.

C. Additional Materials Required

- 1X PBS (for cell culture sample preparation)
- Small plastic boxes or containers
- Pipettors, pipette tips and other common lab consumables
- Orbital shaker or oscillating rocker
- Saran Wrap or similar plastic film
- A chemiluminescent blot documentation system
- X-ray Film and a suitable film processor, or other chemiluminescent detection system.

3. Preparation and Storage of Samples

A. General Considerations

- Freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot your samples prior to initial storage.

- Spin samples hard (5-10 minutes at 10K to 15K RPM) immediately prior to incubation of samples with array.

- Optimal sample concentrations may need to be determined empirically based on the signal intensities of spots and background signals obtained with each sample.

- If spot intensities are weak, increase sample concentration in subsequent experiments.

- If background or spot intensities are too strong, decrease sample concentration in subsequent experiments.

B. Recommended Sample Volumes and Dilution Factors

NOTE: All sample dilutions should be made using the 1X Blocking Buffer provided in this kit. For all sample types, final sample volume = 1.0-1.2 ml per membrane

- Cell and Tissue Lysates: At least 10 fold dilution to minimize the effect of any detergent(s), to equal concentrations of total protein in lysate.

  IMPORTANT: Lysate sample volumes required must be determined empirically and will depend upon the total protein concentration of each lysate and the intensity of background signals for each sample.

- You must determine the total protein concentration of each lysate/homogenate. We recommend using the BCA method, as it is insensitive to detergents commonly found in lysis buffers.

- Minimum Recommended Total Lysate Protein Concentration (prior to sample dilution) = 2.5 µg/µl

- Minimum Recommended Dilution of Lysates (prior to sample incubation): at least 10 fold dilution with 1X Blocking Buffer to minimize the effect of any detergent(s). Dilute all lysate samples to the same final concentration of total lysate protein in 1X Blocking Buffer to 1.2 ml final volume.

- For your first experiment, we recommend using 400-600 µg of total protein in 1.2 ml of 1X Blocking Buffer (final volume) for each array membrane.

- Optimal amounts of total lysate protein may range from 200-1000 µg per array membrane. Based upon the signal intensities of background and spots obtained with each sample, you may need to increase or decrease the volume of lysate used in subsequent experiments.

NOTE: Sample types other than cell or tissue lysates are not recommended for use with Abcam's Human Apoptosis Antibody Array - Membrane.

C. Preparing Samples

- We recommend preparing cell or tissue lysates using 2X Cell Lysis Buffer, which is provided in this kit. Be sure to properly dilute 2X Cell Lysis Buffer (1:1 with deionized H_2O) and add Protease Inhibitor Cocktail before use.

- Other lysis buffers can be used if they are non-denaturing, non-reducing, total salt concentration ≤700 mM), contain ≤2% total detergent (v/v) and are free of sodium azide (NaN_3). Be sure to add Protease Inhibitor Cocktail before use.

- The volume of 1X Cell Lysis required should be determined empirically to produce lysates with ≥2.5 µg/µl

D. Preparation of Cell Lysates

- For adherent cell culture, wash cells 2X with cold 1X PBS before adding lysis buffer.

- For suspension culture, spin cells down (10 min @ 1500 rpm).

- Remove remaining liquid from cell pellet.

- Add 1X Lysis Buffer to cell pellet (approx. $2x10^7$ cells/ml). Pipette cells up and down to resuspend cells.

- Incubate at 2–8°C for 30 minutes, with gentle rocking or shaking (0.5 to 1 cycle/sec).

- Spin down lysates at 14000 x *g* for 10 min.

- Transfer supernatants to fresh microfuge tubes. Preparing sub-aliquots is strongly recommended.

E. Preparation of Tissue Lysates

- Wash tissue 2X with cold 1X PBS before adding lysis buffer. Remove remaining liquid from tissue sample.

- Add 1X Lysis Buffer to tissue sample.

- Use appropriate mechanical disruption technique to solubilize tissue samples. **See Notes below.**

- Incubate at 2–8°C for 30 minutes, with gentle rocking or shaking (0.5 to 1 cycle/sec).

- Spin down lysates at 14000 x *g* for 10 min.

- Transfer supernatants to fresh microfuge tubes. Preparing sub-aliquots is strongly recommended.

NOTES:

a) Optimal protocols for mechanical disruption vary for different tissue types. Please consult the scientific

literature for examples of successful detection of proteins using ELISA or multiplex ELISA techniques in lysates made from cell or tissue samples similar to yours.

b) Sonication can quickly heat volumes of 1 ml or less and denature proteins in your samples!

4. Handling Array Membranes

- Array membranes are fragile when dry. Handle with care.
- Wet or dry, grasp membranes by the edges using forceps.
- Do not allow membranes to dry out during experiments.
- The printed side of each membrane is denoted by a dash mark (-) or array number in the upper left corner.

5. Incubations and Washes

- All washes and incubations in the standard protocol can be performed using the 8-well tray provided in the kit.
- Place the cover on 8-well trays with lid to avoid drying, particularly during extended incubation or wash steps.

- Be sure to completely cover the membranes with sample or reagent during each incubation

- During incubation steps, avoid foaming and be sure to remove all bubbles from the membrane surface.

- Perform all incubation and wash steps under gentle rotation or rocking motion (~0.5 to 1 cycle/s).

- Incubations with sample and Biotin-conjugated Anti-Cytokines should be performed overnight at 4°C.

- Wash steps in Wash Buffer II and all other incubation steps may be performed overnight at 4°C.

- Overnight Blocking and Wash steps are useful for reducing background signal intensities even with completed membranes. Wash with Wash Buffer II followed by repeating incubation with Streptavidin-HRP and chemiluminescent detection may greatly improve signal-to-noise ratios in your developed array images.

6. Chemiluminescence Detection

- We strongly recommend using multiple exposures to obtain optimum images. Begin by exposing the membranes for 40 seconds. Then re-expose the film accordingly.

- If the signals are too strong (or background is too high), reduce exposure time (e.g., 5-30 seconds).

- If the signals are weak, increase exposure time (up to overnight).

- Blot documentation systems that use CCD cameras to detect chemiluminescence are ideal for imaging Abcam array membranes. They can easily be programmed to take multiple exposures, and the dynamic range of these detectors tends to be 2-3 orders of magnitude greater than that of X-ray film or and much more sensitive to chemiluminescence than phospho-imaging systems.

7. Protocol

A. Preparation and Storage of Reagents

NOTE: During this protocol, prepare reagents immediately prior to use and keep working dilutions of all reagents on ice at all times.

1. Protease Inhibitor Cocktail:

   a) Briefly spin down the Protease Inhibitor Cocktail tube before use.

b) Add 60 µl of 1X Lysis Buffer into the vial to prepare a 100X Protease Inhibitor Cocktail Concentrate.

2. Cell Lysis Buffer is supplied at 2X concentration.

   a) Dilute 1 ml of 2X Cell Lysis Buffer with 980 µl of deionized water (~2-fold dilution).

   b) Add 20 µl of 100X Protease Inhibitor Cocktail Concentrate to 1X Cell Lysis Buffer immediately prior to use.

3. Blocking Buffer is supplied at 1X concentration, no reconstitution or dilution is required. Store at –20°C or –80°C when not in use.

4. Wash Buffers I and II are supplied at 20X concentration.

   a) For each membrane to be used in the experiment, dilute 1 ml of Wash Buffer I to a final volume of 20 ml deionized water.

   b) For each membrane to be used in the experiment, dilute 1 ml of Wash Buffer II to a final volume of 20 ml deionized water.

   c) Wash Buffer reagents at working dilution (1X) can be stored at 4°C for up to 1 month. Stock solutions at 20X can be stored 4°C for up to 3 months.

5. Biotin-Conjugated Anti-Cytokines preparation.

    a) Briefly centrifuge the Biotin-Conjugated Anti-Cytokines vial before opening, to ensure maximum recovery.

    b) Mix well as precipitates may form during storage.

    c) Pipette 150 µL of 1x Blocking Buffer into the vial and mix gently.

    d) Transfer the contents of the vial into a tube containing 1.8 mL of Blocking Buffer.

    e) The 1X Biotin-Conjugated Anti-Cytokines may be stored for 2-3 days at 4°C.

6. Streptavidin-HRP is supplied at 1000x concentration.

    a) Mix the tube containing 1000X Streptavidin-HRP well before use, as precipitants may form during storage.

    b) Add 2 µl of 1000X Streptavidin-HRP to 1998 µl of 1X Blocking Buffer.

    c) This working dilution can be stored for 3-5 days at 4°C.

7. Detection Buffers C & D are supplied as 1X solutions that are intended to be mixed in a 1:1 ratio *immediately prior use*. Detection Buffers C & D may be stored at 4°C for up to 3 months.

B. Blocking and Incubations

NOTE: Please prepare all reagents immediately prior to use as described above and carefully read Sample Preparation and Incubations and Washes sections before proceeding.

1. Place each membrane printed side up (see Section 4) into the 8-well tray provided in the kit.

2. Add 2 ml 1X Blocking Buffer to each membrane. Be sure to remove all bubbles above and below the membrane. Incubate at room temperature (RT) for 30 min with gentle rocking or shaking to block the membrane surface.

3. Decant Blocking Buffer and incubate membranes with 1.2 mL of sample overnight at 4°C with gentle rocking or shaking.

   NOTES:

   a) To prevent evaporation, be sure to place the lid on the 8-well tray during overnight incubation.

   b) Incubation with sample may be done at RT for 4 hours, but with a significant loss of sensitivity.

4. Aspirate samples from membranes and wash 3 x 5 min with 2 ml Wash Buffer I at RT. Use fresh buffer for each wash.

OPTIONAL Large Volume Wash to Reduce Background: After Step 4 and before Step 5, place membranes into clean container(s). Add 20-30 ml of Wash Buffer I per membrane, and wash at RT with gentle shaking or rocking for 30-45 min. Return membranes to the 8-well tray.

5. Wash 2 x 5 min with 2 ml of 1X Wash Buffer II each at RT. Decant and use fresh wash buffer each time.

6. Carefully remove remaining wash buffer using aspiration or pipette. Add 1 ml of 1X Biotin-conjugated Anti-Cytokines to each membrane. Incubate overnight at 4°C with gentle rocking or shaking.

   NOTE: To prevent evaporation, be sure to place the lid on the 8-well tray during overnight incubation.

7. Decant or aspirate Anti-Cytokine reagent and repeat washes as described in steps 4 and 5 above.

8. Pipette 2 ml of 1X HRP-Streptavidin into each well and incubate for 2 hours at RT or overnight at 4°C.

9. Wash membranes as directed in steps 4 and 5.

10. Proceed with Chemiluminescent Detection protocol below or store membrane for detection at a later date as directed in Section 4 - Handling Array Membranes.

C. Chemiluminescence Detection

NOTE: Do not allow membranes to dry out during detection. Detection of chemiluminescence should be started within 5 minutes after removing Detection Buffers and must be completed within 20 minutes.

11. Place a plastic sheet (provided in the kit) on your benchtop.

12. Place one or more array membranes protein side up (see Section 4) on the plastic sheet. Drain excess liquid by touching one edge to blotting paper or tissue paper.

13. Into a single, clean tube, add equal volumes of Detection Buffer C and Detection Buffer D immediately prior to detection. Mix well. Add 250 µl of each buffer per membrane to be detected, e.g., for 4 membranes, combine 1 ml of each detection buffer.

14. Pipette the mixed Detection Buffers on to each membrane. Place another plastic sheet on top, starting at one end and "rolling" the flexible plastic across the surface to the opposite end. During this process, ensure that the detection mixture completely covers each membrane, and gently smooth out any air bubbles. Avoid sliding the plastic sheet along the membranes' printed surfaces.

15. Incubate at room temperature for 2 minutes.

16. Remove top plastic sheet and remove excess liquid (see Step 12).

17. Gently replace the membranes (protein side up) on the bottom plastic sheet and replace the top plastic sheet (see Step 14). Gently smooth out any air bubbles on the membrane surfaces.

18. Detect signals using a chemiluminescence imaging system or expose the array membranes to X-ray film and detect the signal using a film developer (See tips for obtaining array images in Section 6).

19. For each array, try multiple exposures to obtain an image with low background and strong Positive Control signals that do not bleed into one another. Typical exposure times are 10 seconds to 2 minutes.

20. When you finish your last exposure, remove the top plastic sheet. Gently rinse membranes and plastic sheets with Wash Buffer II. Remove excess wash buffer as described in Step 14 and replace the membranes between the plastic sheets.

21. Wrap the sheets in plastic wrap and store the membranes at –20°C to –80°C. (Alternatively, you may store membranes for up to 5 days at 4°C in Wash Buffer II. Be sure to cover the container to avoid evaporation).

8. Interpretation of Results

Typical results obtained with Abcam Apoptosis Antibody Arrays:

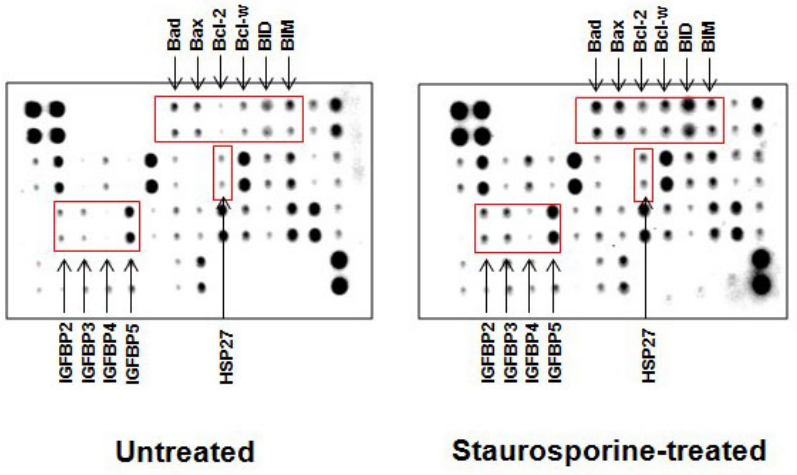


Figure 1. Apoptotic protein profiling in Staurosporine-treated and untreated Jurkat cell lines

10^7 Jurkat cells were untreated or treated with staurosporine (0.5 µM) for 3 hours. Equal total lysate protein was loaded on each Abcam Apoptosis Antibody Array membrane. Antibody spots exhibiting signal differences are indicated in red boxes.

The preceding figure presents typical images obtained with Human Apoptosis Antibody Array. Note the strong signals of the Positive Control spots, printed directly onto the array membrane in the upper-left and lower-right corners. These Positive Control spots are useful

for proper orientation of the array image and for normalization of results from array to array (see Normalization of Array Data, below).

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.

Obtaining Densitometry Data:

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.

To obtain densitometry data from an X-ray film, one must first scan the film to obtain a digitized image using an ordinary office scanner with resolution of 300 dpi or greater. 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 (for more info, visit http://rsbweb.nih.gov/ij/).

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

- For each array membrane, identify a single exposure that the exhibits low background signal intensity and strong Positive Control signals that do not "bleed" into one another. Exposure times do not need to be identical for each array, but Positive Control signals on each 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 circle (area 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)

Once you have obtained the raw densitometry data, you should subtract the background and normalize to the Positive Control signals before proceeding to analysis.

Background Subtraction:

On each array, several "Negative Control" and/or "Blank" spots will be included. Blank spots are literally blank; nothing has been printed there. Negative Control spots are printed with the same buffer used to dilute antibodies printed on the array. Thus, the signal intensities

of the Negative Controls represent the background plus non-specific binding to the printed spots. We recommend subtracting the mean of 4 or more Negative Control spots for background correction.

Normalization of Array Data:

The amount of biotin-conjugated IgG protein 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 "reference" to which the other arrays are normalized. This choice can be arbitrary. You can calculate the normalized values as follows:

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

Where:

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

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

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

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

After normalization to Positive Control signal intensities, you can compare the relative expression levels, analyte-by-analyte, among or between your samples or groups. By comparing these signal intensities, one can determine relative differences in cytokine expression in each sample.

9. Array Maps

Detects 43 Apoptosis markers in one experiment.

	A	B	B	D	E	F	G
1	Pos	Pos	Neg	Neg	BLANK	BLANK	Bad
2	Pos	Pos	Neg	Neg	BLANK	BLANK	Bad
3	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL
4	CD40	CD40L	cIAP-2	cytoC	DR6	Fas	FasL
5	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR
6	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR
7	sTNF-R2	TNF-α	TNF-β	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4
8	sTNF-R2	TNF-α	TNF-β	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4

	H	I	J	K	L	M	N
1	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
2	Bax	Bcl-2	Bcl-w	BID	BIM	Caspase 3	Caspase 8
3	BLANK	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
4	BLANK	HSP27	HSP60	HSP70	HTRA	IGF-I	IGF-II
5	Livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
6	Livin	p21	p27	p53	SMAC	Survivin	sTNF-R1
7	XIAP	BLANK	BLANK	Neg	Neg	Neg	Pos
8	XIAP	BLANK	BLANK	Neg	Neg	Neg	Pos

10. Troubleshooting Guide

Problem	Cause	Recommendation
No signal for any spots, including Positive Controls	Global detection failure	Repeat incubation with HRP-Streptavidin and Detection Buffers.
Weak or no signals antigen-specific spots	Sample is too dilute	Repeat experiment using higher sample concentration.
	Improper dilution of HRP-Streptavidin	Tube may contain precipitants. Repeat detection; mix 1000X HRP-Streptavidin well before diluting reagent.
	Waiting too long to detect chemiluminescent signals	Repeat detection, making sure to complete this process within 20 min.

Weak or no signals antigen-specific spots	Other Tips	Incubate with sample O/N at 4°C.
		Increase concentration of HRP-Streptavidin.
		Increase concentration of Biotin-conjugated Anti-Cytokine.
		Extend exposure time (may go overnight).
Uneven signal or background	Bubbles present on membrane during incubations	Be sure to completely remove all bubbles from membrane surface.
	Membranes were not evenly covered during washes/incubations or allowed to dry out	Completely cover membranes with solution, use a rocker or shaker during washes and incubations.
High background signals	Overexposure	Decrease exposure time.
	Sample is too concentrated	Repeat experiment using more dilute sample.
	NOTE: To reduce background on completed membrane, wash O/N @ 4°C in Wash Buffer II, then re-incubate with HRP-Streptavidin and repeat detection.	

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