

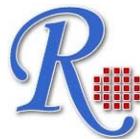
RayBio[®] Human Apoptosis Antibody Array G Series

User Manual

RayBio[®] Human Apoptosis Antibody Array

Cat# AAH- APO-G1-4

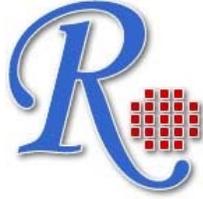
Cat# AAH- APO-G1-8



RayBiotech, Inc.

**We Provide You with Excellent
Protein Array Systems and Service**

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RayBiotech, Inc.

RayBio® Human Apoptosis Array G series Protocol

TABLE OF CONTENTS

I.	Introduction.....	2
	How It Works.....	5
II.	Materials Provided.....	6
	Additional Materials Required.....	6
III.	Overview and General Considerations.....	7
	A. Preparation of Samples.....	7
	B. Handling Glass Chips	8
	C. Incubation.....	8
IV.	Protocol.....	8
	A. Blocking and Incubation.....	8
	B. Detection.....	11
V.	Interpretation of Results.....	12
VI.	RayBio Analysis Tool.....	14
VII.	Array Map.....	15
VIII.	Troubleshooting Guide.....	16
IX.	Reference List.....	17

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I. Introduction

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

Apoptotic studies 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 an 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-X1* 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 a group of caspases, which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen 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*.

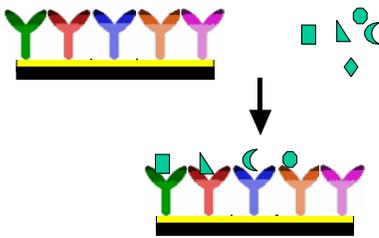
The tumor-suppressor protein p53 also plays critical role in apoptosis. p53 accumulates in response to DNA damage via interferon-alpha and interferon-beta pathways, which induce transcription of the *p53* gene and result in the increase of p53 protein level and enhancement of cancer cell apoptosis. p53 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.

Here's how it works

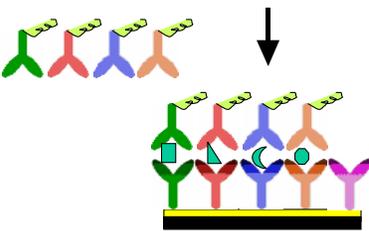
Array support



Samples

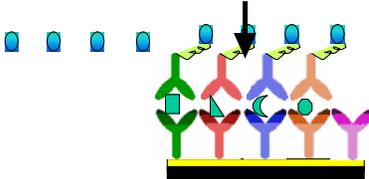
Incubation of Sample With arrayed antibody } Overnight Supports

Cocktail of Biotin-Ab



Incubation with Biotinylated Ab } 2 hrs

Labeled – streptavidin

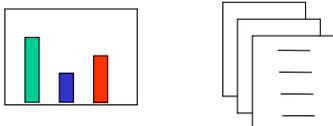


Incubation with Labeled- streptavidin } 2 hrs

Detection of signals



Data analysis and graph



II. Materials Provided

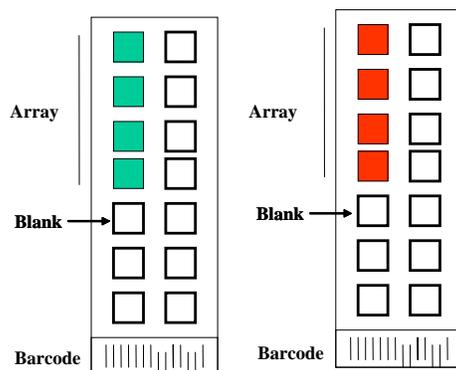
Upon receipt, all components of the RayBio[®] Human Apoptosis Antibody Array kit should be stored at -20⁰C. At -20⁰C the kit will retain full activity for up to 6 months. Once thawed, the glass chips, Alexa-Flour 555-streptavidin, Internal Control, Proteinase Inhibitor and 2X Blocking Buffer should be kept at -20⁰C and all other component should be stored at 4⁰C. Use within three months after reagents have been thawed. Please use within six months after purchase.

1. RayBio[®] Human Apoptosis Antibody Microarray slides (4 subarray wells in one slide)
2. Biotin-Conjugated Anti-Apoptosis Antibody cocktail mix (1 vial for 4 wells of each slide)
3. 1,500X Alexa Flour 555-Conjugated Streptavidin (1 vial)
4. 2X Blocking Buffer (10 ml)
5. 20X Wash Buffer I (30ml)
6. 20X Wash Buffer II (30ml)
7. 2X Cell Lysis Buffer (10 ml)
8. Proteinase Inhibitor (1 vial)
9. RayBio[®] G series antibody array accessory (including slide incubation chamber, Gasket, Protective cover, Snap-on sides and adhesive film)
10. Manual

Additional Materials Required

- Orbital shaker
- Laser scanner for fluorescence detection
- Aluminum foil
- Distilled water
- Plastic box

Layout of RayBio[®] Human Apoptosis Antibody Array



4 arrays in one glass chip

III. Overview and General Considerations

A. Preparation of Samples

- For cell lysates and tissue lysates, we recommend using 1X Cell Lysis Buffer to extract proteins from cell or tissue (e.g. using homogenizer). After extraction, spin the sample and save supernatant for experiment. Determine protein concentration. Dilute 2X Cell Lysis Buffer with H₂O (we recommend adding proteinase inhibitors to Cell Lysis Buffer before use). Prepare relative concentrated lysate since we recommend diluting lysate at least 10 folds with Blocking Buffer for array assay.
- *Protease Inhibitor Cocktail: Briefly spin down the Protease Inhibitor Cocktail tube before use. Add 60 μ l of 1X Lysis Buffer into the vial to prepare a 100X Protease Inhibitor Cocktail Concentrate.*
- *2X Cell Lysis Buffer: Cell lysis buffer should be diluted 2-fold with deionized or distilled water before use. Add 20 μ l of prepared 100X Protease Inhibitor Cocktail Concentrate (bring the tube to room temperature to thaw the solution before use) into 1.98 ml 1X Lysis Buffer before use. Mix well.*
- We recommend using 500 to 600 μ g of protein for cell lysates and tissue lysates.

- The kit can also be used for serum and plasma samples, as well as conditioned medium.
If you experience high background, you may further dilute your sample.

B. Handling glass chips

- The microarray slides are sensitive, do not touch the surface. Hold the slides by the edges only.
- Handle all buffers and slides with latex free gloves.
- Avoid breaking glass slide.
- Handle glass chip in clean environment.

C. Incubation

- Completely cover array area with sample or buffer during incubation, and cover the incubation chamber with adhesive film or plastic sheet protector to avoid drying.
- Avoid foaming during incubation steps.
- Perform all incubation and wash steps under gentle rotation.
- Cover the incubation chamber with adhesive film during incubation, particularly when incubation is more than 2 hours or 50 μ l of sample or reagent is used.
- Avoid cross-contamination from overflowing solution to neighboring wells.
- Several incubation steps such as step 3 (blocking), step 4 (sample incubation), step 8 (biotin-Ab incubation) or step 11 (Alexa Flour 555-streptavidin incubation) may be done at 4⁰C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

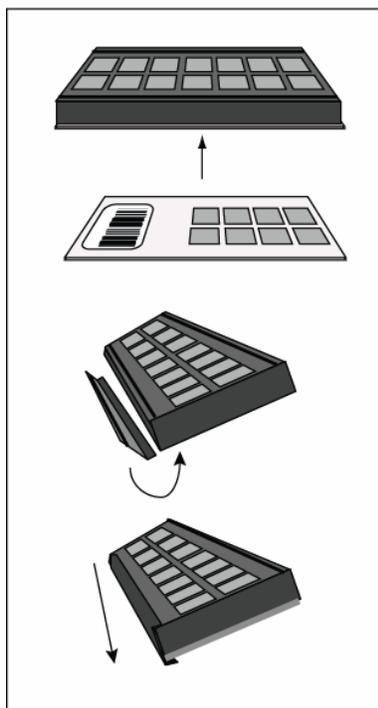
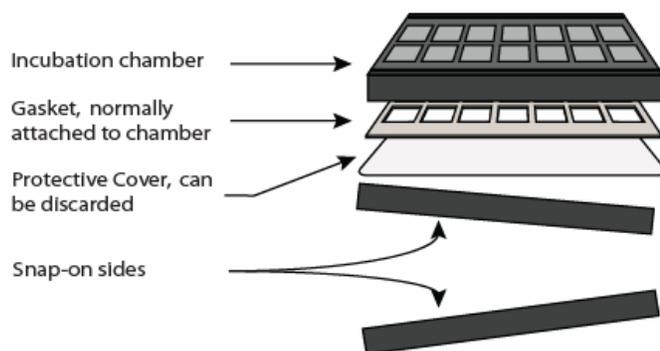
IV. Protocol

A. Blocking and Incubation

1. Take the glass chip out from the box. Let air dry for 60 minutes.

2. Assemble the glass chip into incubation chamber and incubation frame as shown below. (Note: if you slide has been pre- assembled, you can go to step 3 directly).

Instructions for incubation chamber assembly ***G Series and Q series arrays***



1 Carefully place slide at bottom of the chamber as shown. The slide will adhere somewhat to the bottom. Warning: the slide is fragile, so do not apply more than gentle force to the apparatus.

2 While gently holding chamber and slide, place side on chamber as shown, beginning with bottom flap first.

3 Then, press the top of the side into groove on chamber, and then apply even, gentle pressure from one end to the other. Repeat this procedure with the other side.

3. Add 100 μ l 1 X Blocking Buffer into each well and incubate at room temperature for 30 min to block slides. Dilute 2X Blocking Buffer with H₂O. Make sure no bubbles are in the well.

Note: only add reagents to wells printed with antibodies.

4. Decant Blocking Buffer from each well. Add 50 to 100 μ l of each sample to array wells. Incubate arrays with sample at 4^oC overnight. Dilute sample using 1X Blocking Buffer if necessary. Add 100 μ l of Blocking Buffer to IC tube, mix well and transfer 1 μ l of IC to each well (50 to 100 μ l of sample).
 - *Note: Dilute the lysate at least 10 folds with 1 X blocking buffer to make a total volume of 50 to 100 μ l. Make sure there is no bubble in the wells.*
 - *Note: The amount of sample used depends on the abundance of Apoptotic molecules. More sample can be used if signals are too weak. If signals are too strong, the sample needs further dilution.*
 - *Note: Incubation may be done for 2 hours at room temperature.*
 - *Note: when transfer IC, please use 0.1 μ l to 2 μ l pipettor*
5. Decant the samples from each well, and wash 5 times with 150 μ l of 1X Wash Buffer I at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer I with H₂O. Completely remove Wash Buffer I in each wash step.
 - *Note: avoid solution flowing into neighboring wells.*
6. Wash 2 times with 150 μ l of 1X Wash Buffer II at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer II with H₂O. Completely remove wash buffer II in each wash step.
7. Prepare working solution for biotin-conjugated antibodies. After brief spinning, Add 300 μ l of 1x blocking buffer to the Biotin-Conjugated Antibody mix vial. Mix gently.
 - *Note: the diluted biotin-conjugated antibodies can be stored at 4^oC for 2-3 days.*

8. Add 70 μ l of diluted biotin-conjugated antibodies to each corresponding well. Incubate at room temperature for 2 hours.

➤ *Note: incubation may be done at 4⁰C for overnight.*

9. Wash as directed in steps 5 and 6.

10. Add 70 μ l of 1,500 fold diluted Alexa Flour 555-conjugated streptavidin (after brief spinning, add 1.5 ml of Blocking Buffer to Alexa Flour 555-conjugated streptavidin tube) to each subarray. Cover the incubation chamber with Adhesive film. Cover the plate with aluminum foil to avoid exposure to light or incubate in dark room.

11. Incubate at room temperature for 1 to 2 hours.

➤ *Note: incubation may be done at 4⁰C for overnight.*

12. Wash with Wash Buffer I **twice** as directed in steps 5.

B. Fluorescence Detection

1. Decant excess Wash Buffer from wells.

2. Disassemble the slide out of the incubation frame and chamber.

3. Place the whole slide in 50 ml centrifuge tube, add enough Wash Buffer I (about 30 ml) to cover the whole slide and gently shake at room temperature for 10 minutes. Decant Wash Buffer I. Repeat Wash Buffer I once. Wash with Wash Buffer II (about 30 ml) with gentle shake at room temperature for 10 minutes. Or wash using slide chamber. Rinse the slide with distilled H₂O.

4. Remove water droplets by centrifuge at 1,000 rpm for 3 minutes and then let slide dry completely in air at least 20 minutes

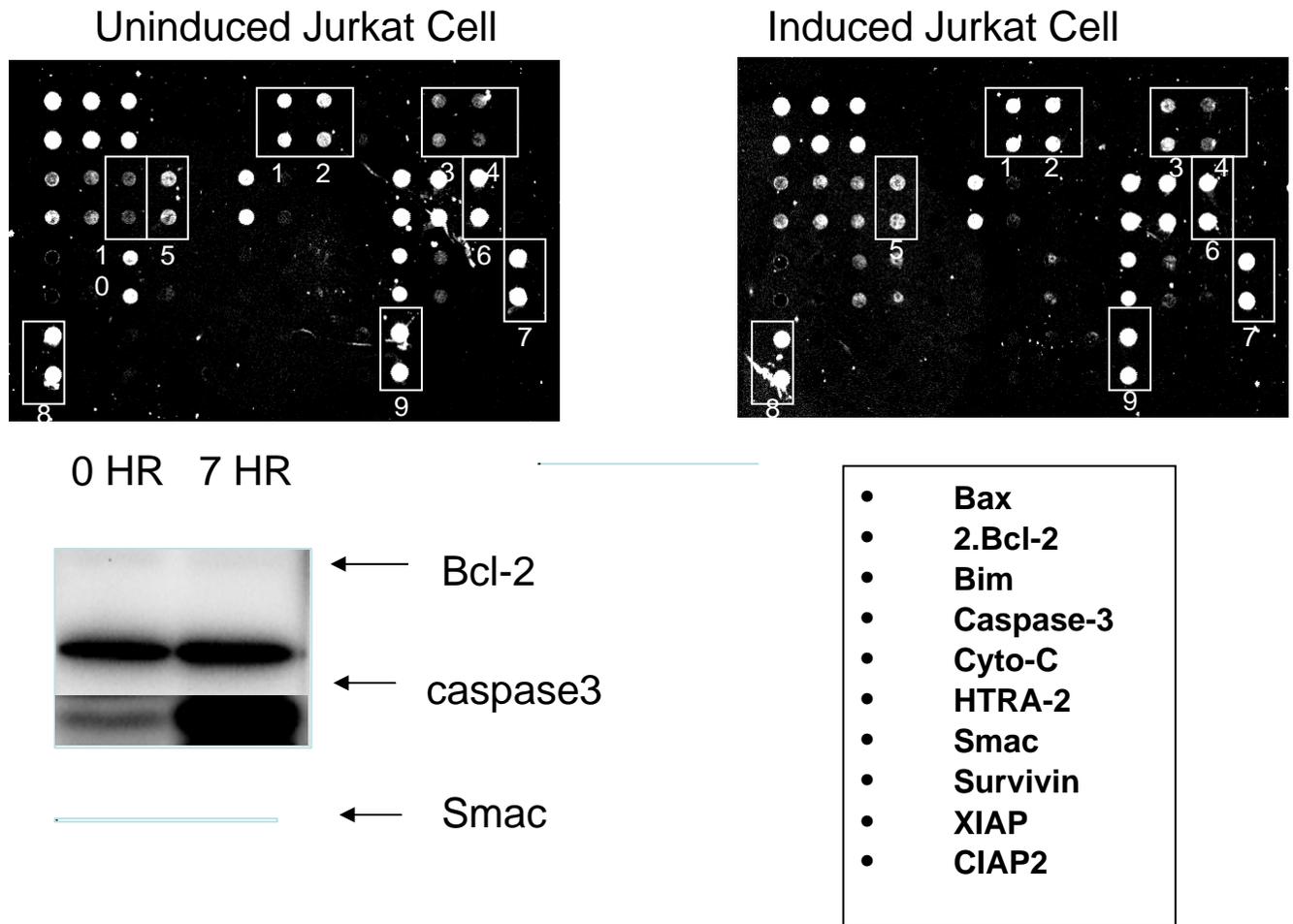
(protect from light). Make sure the slides are absolutely dry before the scanning procedure.

5. Image the signals using laser scanner such Axon GenePix using cy3 channel.

Note: we recommend scanning slides right after experiment. You also can store the slide at -20°C in dark for several days. If you do not have a laser scanner, we can provide service for you. Just simply send your slide to us and we will take care of it.

V. Interpretation of Results

The following figure shows RayBio[®] Human Apoptosis Antibody Array using cell lysates from induced and uninduced Jurkat cell line. The images were captured using laser scanner. The biotin-conjugated protein produces positive signals, which can be used to identify the orientation and to compare the relative expression levels among the different wells. The internal control (IC) can also be used to normalize the signal intensities among array membranes in different experiments.



C. Western Results

Fig 1. Apoptotic protein profiling in Induced and uninduced Jurkat cell lines

Jurkat cells were treated with apoptosis inducer set for 7 hours (10mM Actinomycin D, 2mM Camptothecin, 100mM Cycloheximide, 10mM Dexamethasone, and 100mM Etoposide). 50 µg of cell lysate from both induced and uninduced Jurkat cells were incubated overnight with RayBio Human Apoptosis Antibody Array slide. The antibody array slides were then washed and cocktail of biotinylated antibody mix was used to detect apoptosis-related proteins. After incubation with fluorescence dye, the signals were scanned with fluorescence scanner. Array results are shown in Figure A for uninduced Jurkat cells and Figure B for induced Jurkat cells. Representative markers are highlighted in the numbered rectangular boxes. Figure C shows Western Blotting of apoptotic markers (Bcl-2, Caspase-3, HTRA-2) using both induced (0 Hr) and induced (7 Hr) Jurkat cell lysates.

VI. RayBio® Analysis Tool

How to use RayBio[®] Analysis Tool

The signal intensities obtained from laser scanner can simply be imported into our analysis tool. The analysis tool will help you:

- Locate your signal intensities to antibody array map
- Link the protein to website for more detailed information on the particular protein
- Protein list sorting
- Average signal intensities
- Subtract background
- Normalize the data from different samples
- Obtain protein level comparison charts among different samples

This analysis tool is very simple and affordable, which will not only assist in compiling and organizing your data, but also reduces your calculations to a “copy and paste” step.

If you do not use our RayBio[®] Analysis Tool, you can locate the Apoptosis by referring to RayBio[®] Human Apoptosis Antibody Array.

Normalization and comparison

For biomarker discovery or for analysis of large number of arrays, great attention must be paid to the normalization. Our apoptosis antibody array design includes controls for normalization and comparison of arrays performing in different membranes and different experiments.

Positive control. Positive control is biotinylated protein. It can be used to normalize the streptavidin incubation step. If the positive signals from different array membranes are similar, positive control is a simple and effective way for normalization.

Negative control. Negative control is BSA. Normally, it should only give a background reading.

V. Array Map

RayBio[®] Human Apoptosis Antibody Array G series

	A	B	B	D	E	F	G	H	I	J	K	L	M
1	Pos 1	Pos 2	Pos 3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
2	Pos 1	Pos 2	Pos 3	Neg	Neg	bad	bax	bcl-2	bcl-w	BID	BIM	Caspase3	caspase8
3	CD40	CD40L	clAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-I
4	CD40	CD40L	clAP-2	cytoC	DR6	Fas	FasL	neg	HSP27	HSP60	HSP70	HTRA	IGF-I
5	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
6	IGF-II	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-5	IGFBP-6	IGF-1sR	livin	p21	p27	p53	SMAC
7	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg
8	Survivin	sTNF-R1	sTNF-R2	TNF-alpha	TNF-beta	TRAILR-1	TRAILR-2	TRAILR-3	TRAILR-4	XIAP	Neg	Neg	Neg

We also offer sample testing service and Custom Human Apoptosis Antibody Arrays at an affordable price. For more information, please visit our website, www.raybiotech.com.

VI. Troubleshooting guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power Check PMT parameters
	Inadequate reagent volume Improper dilution	Check pipettor Ensure correct preparation
	Inadequate incubation times	Ensure sufficient incubation Time Incubate sample overnight
	Low protein concentration in sample	Don't make too low dilution Concentrate sample
	Improper storage of kit	Store kit at recommended temperature
High Background	Excessive biotinylated antibodies	Make sure correct amount of antibodies
	Excessive streptavidin	Make sure correct amount of streptavidin
	Inadequate detection	Check laser power Check PMT parameters
	Unclean environment such as dust	Work in clean environment
	Insufficient wash	Increase wash time Use more wash buffer
Uneven Signal	Bubbles formed during incubation	Avoid bubble formation during incubation
	Arrays are not completed Covered by reagent	Completely cover arrays with solution

VII. Reference List

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Note:

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Apoptosis antibody arrays are RayBiotech patent-pending technology.

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