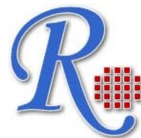
RayBio® Human Apoptosis Antibody Array G Series

User Manual

RayBio® Human Apoptosis Antibody Array
Cat# AAH- APO-G1-1
Cat# AAH- APO-G1-2
Cat# AAH- APO-G1-4



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

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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-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 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*.

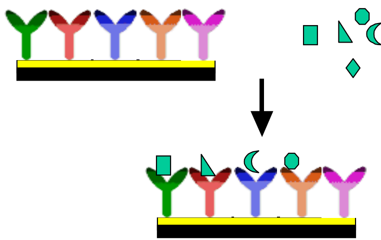
The tumor-suppressor protein p53 also plays critical role in apoptosis. p53 accumulates in response to DNA damage via interferon-alpha and interferon-beta pathways, which induce transcription of the *p53* gene and result in the increase of p53 protein level and enhancement of cancer cell apoptosis. p53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

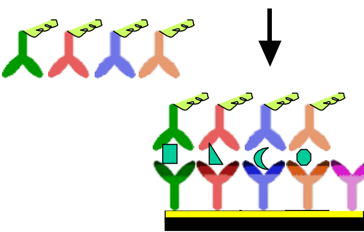
A recent report has shown the involvement of IGFBPs (insulin-like growth factor-binding protein) in apoptosis. IGFBP1 protein localizes to mitochondria where it binds to the BAK and hinders BAK activation and apoptosis induction. When IGFBP1 is in a complex with BAK, formation of a proapoptotic p53/BAK 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

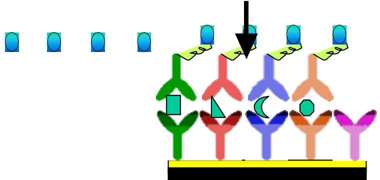


Samples



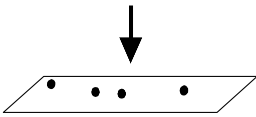
Incubation of Sample With arrayed antibody Supports — **Overnight**

Cocktail of Biotin-Ab



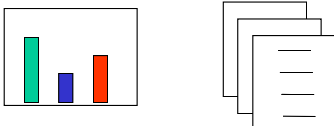
Incubation with Biotinylated Ab — **2 hrs**

Labeled – streptavidin



Incubation with Labeled- streptavidin — **2 hrs**

Detection of signals



Data analysis and graph

II. Materials Provided

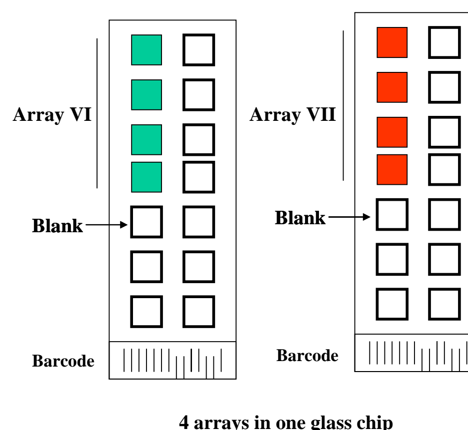
Upon receipt, all components of the RayBio® Human Apoptosis Antibody Array kit should be stored at -20^{0}C. At -20^{0}C the kit will retain complete activity for up to 6 months. Once thawed, the glass chips, Alexa-Flour 555-streptavidin, Internal Control and 2X Blocking Buffer should be kept at -20^{0}C and all other component should be stored at 4^{0}C. Use within three months after reagents have been thawed. Please use within six months of purchase.

- RayBio® Human Apoptosis Antibody Microarray slides (4 subarray wells in one slide)
- Biotin-Conjugated Anti-Apoptosis Antibody cocktail mix (1 tube for 4 wells of each slide)
- 1,500X Alexa Flour 555-Conjugated Streptavidin (1 tube)
- 2X Blocking Buffer (4 ml/8ml)
- 20X Wash Buffer I (30ml/60ml)
- 20X Wash Buffer II (20ml/40ml)
- Internal control (powder, 1 tube)
- 2X Cell Lysis Buffer (10 ml/ 16 ml)
- RayBio® G series antibody array accessory (including slide incubation chamber, Gasket, Protective cover, Snap-on sides and adhesive film)
- Manual

Additional Materials Required

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

Layout of human antibody array G series



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 H_2O (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.
- We recommend using 400 to 600 µg 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 μl 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 4^0C for overnight. Please make sure to cover the incubation chamber tightly to prevent evaporation.

IV. Protocol

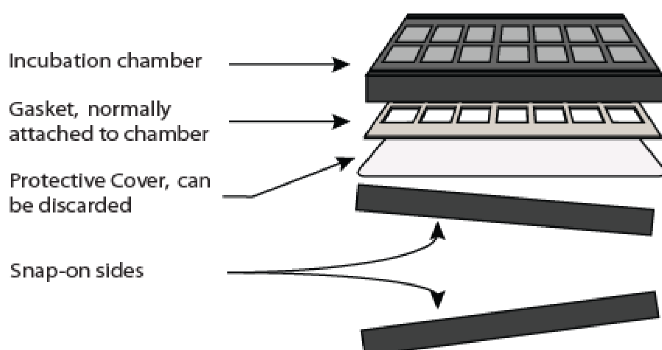
A. Blocking and Incubation

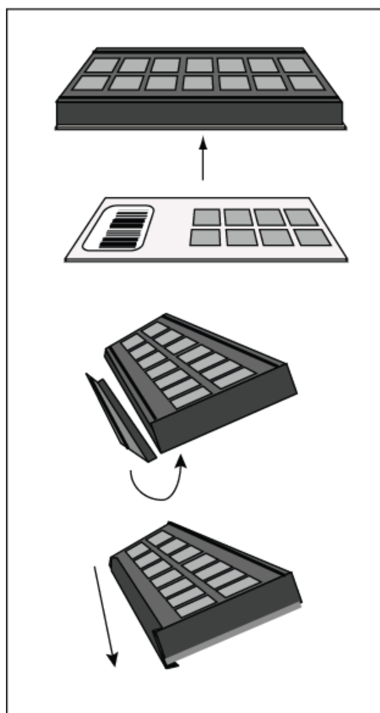
1. Take the glass chip out from the box. Let air dry for 60 minutes.

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 grove on chamber, and then apply even, gentle pressure from one end to the other. Repeat this procedure with the other side.

3. Add 100 µl 1 X Blocking Buffer into each well and incubate at room temperature for 30 min to block slides. Dilute 2X Blocking Buffer with H_2O. 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 µl of each sample to array wells. Incubate arrays with sample at room temperature for 1 to 2 hours. Dilute sample using 1X Blocking Buffer if necessary. We strongly recommend including Internal Control (IC) in your assay. Add 100 µl of Blocking Buffer to IC

tube, mix well and transfer 1 µl of IC to each well (50 to 100 µl of sample).

Note: We recommend using 50 to 100 µl of conditioned media or 50 to 100 µl of original or 2-5 fold diluted serum or plasma or 10-200 ug of protein for cell lysates and tissue lysates. ***Dilute the lysate at least 10 folds with 1 X blocking buffer to make a total volume of 50 to 100 µl. 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 can be diluted further.
Note: Incubation may be done at 4^0C for overnight.
Note: when transfer IC, please use 0.1 µl to 2 µl pipettor

5. Decant the samples from each well, and wash 5 times with 150 µl of 1X Wash Buffer I at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer I with H_2O. Completely remove Wash Buffer I in each wash step.

Note: avoid solution flowing into neighboring wells.

6. Wash 2 times with 150 µl of 1X Wash Buffer II at room temperature with gentle shaking. 2 min per wash. Dilute 20X Wash Buffer II with H_2O. Completely remove wash buffer II in each wash step.

7. Prepare working solution for biotin-conjugated antibodies. After brief spinning,

   Add 300 µl of 1x blocking buffer to the Biotin-Conjugated Antibody VI tube. Mix gently.

   Add 300 µl of 1x blocking buffer to the Biotin-Conjugated Antibody VII tube. Mix gently.

Note: the diluted biotin-conjugated antibodies can be stored at 4^0C for 2-3 days.

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

Note: incubation may be done at 4^0C for overnight.

9. Wash as directed in steps 5 and 6.

10. Add 70 µl 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 4^0C 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 H_2O.

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^{0}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 G series 1000 probed with different cell culture supernatant. 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.

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 G series 1000. Please keep in mind that G series 1000 consists of two individual arrays; human Apoptosis antibody array 6 and human Apoptosis antibody array 7. Refer to corresponding map.

Normalization and comparison

For biomarker discovery or for analysis of large number of arrays, great attention must be paid to the normalization. Our antibody array design includes several controls for normalization and comparison of arrays performing in different membranes and different experiments (for more information please read the reference 17).

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.
Internal control. RayBio® antibody arrays also include spiking-in protein serving as internal control (IC). The spiking-in proteins do not have cross-reactivity with protein in the array. It can be used to normalize the entire process.
Negative control. Negative control is BSA. Normally, it should only give a background reading.

RayBio® Human Apoptosis Antibody Array G series

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

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

RayBiotech, Inc., the protein array pioneer company, strives to research and develop new products to meet demands of the biomedical community. RayBio's patent-pending technology allows detection of over 180 Apoptosis, chemokines and other proteins in a single experiment. Our format is simple, sensitive, reliable and cost effective. Products include: Apoptosis Arrays, Chemokine Arrays, ELISA kits, EIA kits, Phosphotyrosine kits, Recombinant Proteins, Antibodies, and custom services.

1. Antibody arrays

   Apoptosis antibody array
   - Human Apoptosis antibody arrays
   - Mouse Apoptosis antibody arrays
   - Rat Apoptosis antibody arrays

   Pathway- or disease-focused antibody arrays
   - Inflammation antibody array
   - Angiogensis antibody array
   - Chemokine antibody array
   - Growth factor antibody array
   - MMP antibody array
   - Atherosclerosis antibody array

   Quantibody arrays for quantitative measurement of Apoptosis and other protein concentration

   Phosphorylation antibody arrays

   Biotin label-based antibody arrays for high density antibody arrays

Antibody analysis tool, software

2. ELISA
3. EIA
4. Cell-based phosphorylation assay
5. Custom antibody arrays
6. Antibody
7. Recombinant protein
8. Peptide
9. Protein arrays

RayBiotech also provides excellent custom service:

1. Antibody arrays
2. Protein arrays
3. Peptide synthesis
4. Production of recombinant protein and antibody
5. Peptide arrays
6. Phosphorylation arrays
7. ELISA
8. EIA

Just simply send your samples and we will do the assay for you.

Technology transfer program
Have you developed technologies or reagents interested to the scientific and research community? RayBiotech can help you commercialize your technologies, reagents and dream.

VI. Troubleshooting guide

Problem	Cause	Recommendation
Weak signal	Inadequate detection	Check laser power and PMT parameters
	Inadequate reagent volumes or improper dilution	Check pipetters and ensure correct preparation
	Short incubation times	Ensure sufficient incubation Time and change sample incubation step to overnight
	Too low protein concentration in sample	Don't make too low dilution Or concentrate sample
	Improper storage of kit	Store kit at suggested temperature
High background	Excess of biotinylated antibodies	Make sure correct amount of antibodies
	Excess of streptavidin	Make sure correct amount of streptavidin
	Inadequate detection	Check laser power And PMT parameters
	dust	Work in clean environment
	Insufficient wash	Increase wash time and 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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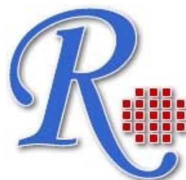

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