

ab193662 – Human RTK Phosphorylation Antibody Array - Membrane (71 Targets)

Instructions for Use

For the simultaneous detection of 71 phosphorylated Human receptor tyrosine kinases (RTKs) in cell and tissue lysates

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

Table of Contents

INTRODUCTION

- 1. BACKGROUND** 2
- 2. ASSAY SUMMARY** 3

GENERAL INFORMATION

- 3. PRECAUTIONS** 4
- 4. STORAGE AND STABILITY** 4
- 5. MATERIALS SUPPLIED** 5
- 6. MATERIALS REQUIRED, NOT SUPPLIED** 6
- 7. LIMITATIONS** 6
- 8. TECHNICAL HINTS** 7

ASSAY PREPARATION

- 9. REAGENT PREPARATION** 9
- 10. SAMPLE PREPARATION AND STORAGE** 10
- 11. ARRAY MAP** 12

ASSAY PROCEDURE

- 12. ASSAY PROCEDURE** 13

DATA ANALYSIS

- 13. CALCULATIONS** 16
- 14. TYPICAL DATA** 18

RESOURCES

- 16. TROUBLESHOOTING** 19
- 17. NOTES** 21

1. BACKGROUND

Abcam's Human RTK Phosphorylation Antibody Array - Membrane (71 Targets) ab193662 can be used for the simultaneous detection of 71 phosphorylated Human receptor tyrosine kinases (RTKs) in cell and tissue lysates.

Targets: ALB1, ACK1, ALK, Axl, Btk, BMX, Btk, Csk, Dtk, EGFR, EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphB1, EphB2, EphB3, EphB4, EphB6, ErbB2, ErbB3, ErbB4, FAK, FER, FGFR1, FGFR2, FGFR2 (α isoform), Fgr, FRK, Fyn, Hck, HGFR, IGF-I R, Insulin R (CD220), Itk, JAK1, JAK2, JAK3, LCK, LTK, Lyn, MATK, M-CSFR, MUSK, NGFR (TNFRSF16), PDGFR- α , PDGFR- β , PYK2, RET, ROR1, ROR2, ROS, RYK, SCF R (CD117/c-kit), SRMS, SYK, Tec, Tie-1, Tie-2, TNK1, TRKB, TXK, Tyk2, TYRO10 (DDR2/TKT), VEGFR2, VEGFR3, ZAP70

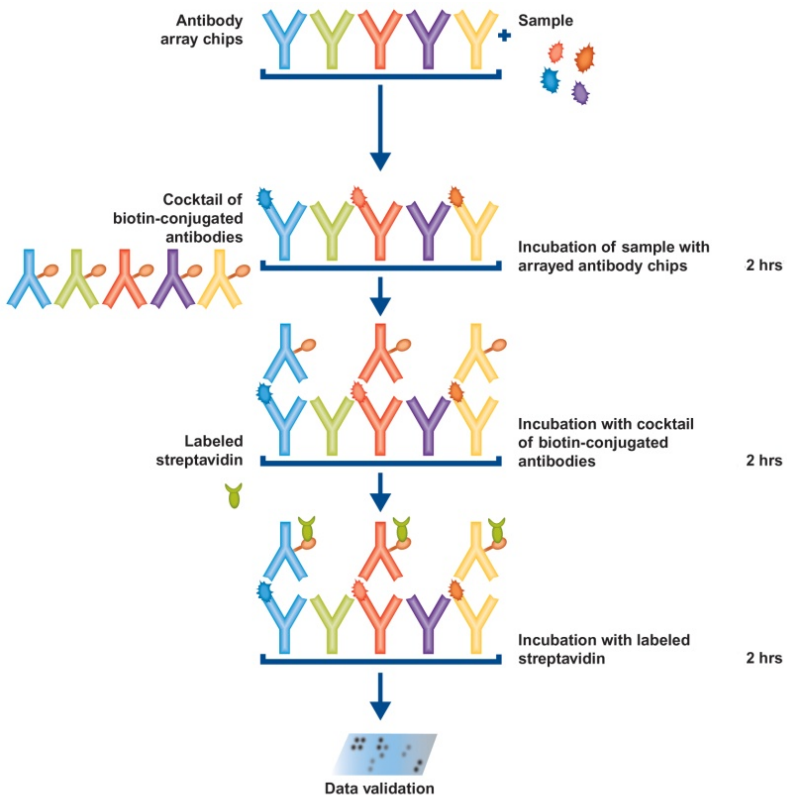
Protein phosphorylation plays an unusually prominent role in cell signaling, development and growth. Abcam's Human RTK Phosphorylation Antibody Array – Membrane ab193662 is a very rapid, convenient and sensitive assay to simultaneously detect multiple protein phosphorylations and can be used to monitor activation or function of important biological pathways.

Abcam's Human RTK Phosphorylation Antibody Array - Membrane (71 Targets) ab193662, is specifically designed for simultaneously identifying the relative levels of phosphorylation of 71 different human receptor tyrosine kinases (RTKs) in cell lysates. By monitoring the changes in protein tyrosine phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in performing an analysis of immunoprecipitation and/or Western Blot.

By using Abcam's Human RTK Phosphorylation Antibody Array - Membrane (71 Targets) ab193662, treated or untreated cell lysate is added into antibody array membranes. The antibody array membranes are washed and biotinylated anti-phosphotyrosine antibody is used to detect phosphorylated tyrosines on activated

receptors. After incubation with HRP-streptavidin, the signals are visualized by chemiluminescence.

2. ASSAY SUMMARY



3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20°C immediately upon receipt.

Once thawed, for short-term storage, store array membranes, 1X Blocking Buffer, Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail II at $\leq -20^{\circ}\text{C}$, and all other component at 2-8°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Reagent Preparation section.

5. MATERIALS SUPPLIED

Item	Quantity			Storage Condition (Before Preparation)
	2X Membranes	4X Membranes	8X Membranes	
Human RTK Antibody Array Membranes (C1)	1X 2 Units	1X 4 Units	2X 4 Units	-20°C
Biotinylated Anti-Phosphotyrosine Antibody (C1)	1X 1 Vial	2X 1 Vial	4X 1 Vial	-20°C
1000X HRP-Conjugated Streptavidin	1X 50 µL	1X 50 µL	1X 50 µL	-20°C
1X Blocking Buffer	1X 25 mL	1X 25 mL	2X 25 mL	-20°C
20X Wash Buffer I	1X 30 mL	1X 30 mL	2X 30 mL	-20°C
20X Wash Buffer II	1X 30 mL	1X 30 mL	2X 30 mL	-20°C
2X Cell Lysis Buffer Concentrate	1X 10 mL	1X 10 mL	1X 16 mL	-20°C
Detection Buffer C	1X 1.5 mL	1X 1.5 mL	1X 2.5 mL	-20°C
Detection Buffer D	1X 1.5 mL	1X 1.5 mL	1X 2.5 mL	-20°C
8-Well Incubation Tray with Lid	1 Unit	1 Unit	1 Unit	-20°C
Protease Inhibitor Cocktail	1 Unit	1 Unit	2X 1 Unit	-20°C
Phosphatase Inhibitor Cocktail II	1 vial	1 vial	2 vials	-20°C
Plastic Sheets	1 Unit	1 Unit	1 Unit	-20°C

The kit also includes a booklet, an array template and a packing list.

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 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

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

8. TECHNICAL HINTS

Handling Array Membranes

- The antibody printed side of each membrane is denoted 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.

Incubation 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 using the 8-Well Incubation Tray provided in the kit.
- Cover the 8-Well Incubation Tray with the lid 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

Biotinylated Antibody Cocktail Incubation

HRP-Conjugated Streptavidin Incubation

- *NOTE: Overnight incubations should be performed at 2-8°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.*

Chemiluminescence Detection

- Beginning with adding the detection buffers and ending with exposing the membranes should take no more than 10-15 minutes as the chemiluminescent signals may start to fade at this point.
- 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.

9. REAGENT PREPARATION

Thaw all reagents to room temperature immediately before use. Reagents should only be used in their 1X working concentration.

9.1. 1X Cell Lysis Buffer

Dilute 2-fold with distilled or deionized water (e.g. 10 mL of 2X concentrate + 10 mL of water = 20 mL of 1X working solution).

9.2. 1X Wash Buffer I

Dilute 20X Wash Buffer I 20-fold with distilled or deionized water to prepare the 1X Wash Buffer I (e.g. 10 mL of 20X concentrate + 190 mL of water = 200 mL of 1X working solution).

9.3. 1X Wash Buffer II

Dilute 20X Wash Buffer II 20-fold with distilled or deionized water to prepare the 1X Wash Buffer II (e.g. 10 mL of 20X concentrate + 190 mL of water = 200 mL of 1X working solution).

9.4. 1X Biotinylated Antibody Cocktail

Briefly centrifuge each vial (1 vial is enough to test 2 membranes) and reconstitute by adding 2 mL of 1X Blocking Buffer. Mix thoroughly and gently.

9.5. 1X HRP-Conjugated Streptavidin

Briefly centrifuge the vial. Dilute 1000X HRP-Conjugated Streptavidin 1000-fold with 1X Blocking Buffer to prepare the 1X working concentration (e.g. 10 μ L of 1,000X concentrate + 9990 μ L of 1X Blocking Buffer = 10 mL of 1X working solution).

9.6. Protease Inhibitor Cocktail

Pipette 60 μ L of 1X Cell Lysis Buffer into the vial to prepare 100X Protease Inhibitor Cocktail concentrate.

9.7. Phosphatase Inhibitor Cocktail II

Add 180 μL of 1X Cell Lysis Buffer into the vial to prepare 25X Phosphatase Inhibitor Cocktail II Concentrate. Dissolve the powder thoroughly by gentle mixing.

NOTE: Prior to preparing cell or tissue lysates: Add 20 μL Protease Inhibitor Cocktail Concentrate (100X) and 80 μL Phosphatase Inhibitor Cocktail Set II Concentrate (25X) into 1.9 mL 1X Lysis Buffer immediately before use. Mix well.

- The Blocking buffer and Detection Buffers C and D are supplied at working concentrations.

10. SAMPLE PREPARATION AND STORAGE

10.1. General Considerations

NOTE: Optimal methods will need to be determined by each experimenter 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 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.

10.2. Recommended Sample Volumes and Dilution Factors

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. 1X Blocking Buffer 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.

10.3. Preparing Cell Lysates

- 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 minutes) 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 Set II (see Reagent preparation note). 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 minutes.
- It is recommended that sample protein concentrations be determined using a total protein assay. For incubation with the Phosphorylation Antibody Array I, use at a protein concentration of 50-1000 $\mu\text{g}/\text{mL}$ 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. If signals are too weak, the cell lysates can be pretreated by immunoprecipitations before incubation with array membranes. Immunoprecipitations can be done using anti-phosphotyrosine and protein A.

11. ARRAY MAP

POS – Positive Control, NEG – Negative Control, BLANK – No Antibody

Array Map for Human RTK Phosphorylation Antibody Array - Membrane C1 (71 Targets) ab193662

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS 1	POS 1	POS 2	POS 2	POS 3	POS 3	ABL1	ABL1	ACK 1	ACK 1	ALK	ALK
2	NEG	NEG	NEG	NEG	Axl	Axl	Blk	Blk	BMX	BMX	Btk	Btk
3	Csk	Csk	Dtk	Dtk	EGFR	EGFR	EphA 1	EphA 1	EphA 2	EphA 2	EphA 3	EphA 3
4	EphA 4	EphA 4	EphA 5	EphA 5	EphA 6	EphA 6	EphA 7	EphA 7	EphA 8	EphA 8	EphB 1	EphB 1
5	EphB 2	EphB 2	EphB 3	EphB 3	EphB 4	EphB 4	EphB 6	EphB 6	EerB 2	EerB 2	EerB 3	EerB 3
6	EerB 4	EerB 4	FAK	FAK	FER	FER	FGFR1	FGFR1	FGFR2	FGFR2	FGFR2 (α isoform)	FGFR2 (α isoform)
7	Fgr	Fgr	FRK	FRK	Fyn	Fyn	Hck	Hck	HGFR	HGFR	IGF-IR	IGF-IR
8	InsulinR	InsulinR	Itk	Itk	JAK 1	JAK 1	JAK2	JAK2	JAK3	JAK3	LCK	LCK
9	LTK	LTK	Lyn	Lyn	MATK	MATK	M-CSFR	M-CSFR	MUSK	MUSK	NGFR	NGFR
10	PDGFR-α	PDGFR-α	PDGFR-β	PDGFR-β	PYK 2	PYK 2	RET	RET	ROR 1	ROR 1	ROR 2	ROR 2
11	ROS	ROS	RYK	RYK	SCFR	SCFR	SRMS	SRMS	SYK	SYK	Tec	Tec
12	Tie-1	Tie-1	Tie-2	Tie-2	TNK 1	TNK 1	TRKB	TRKB	TXK	TXK	NEG	NEG
13	Tyk2	Tyk2	TYRO10	TYRO10	VEGFR2	VEGFR2	VEGFR3	VEGFR3	ZAP70	ZAP70	POS 4	POS 4

12. ASSAY PROCEDURE

Please prepare all reagents immediately prior to use. All incubations and washes must be performed under gentle rotation/rocking.

- 12.1. Place each membrane printed side up into the 8-Well Incubation Tray provided in the kit.
- 12.2. Block membranes by incubating with 2 mL 1X Blocking Buffer at room temperature (RT) for 30 minutes.
- 12.3. Aspirate 1X Blocking Buffer from each well.
- 12.4. Pipette 1 mL of diluted or undiluted sample into each well and incubate for 1.5 - 5 hours at room temperature OR overnight at 2-8°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.

- 12.5. Aspirate samples from each well with a pipette.
- 12.6. Pipette 2 mL of 1X Wash Buffer I into each well and incubate for 5 minutes at RT. Repeat this 2 more times for a total of 3 washes using fresh buffer and aspirating each time.
- 12.7. Pipette 2 mL of 1X Wash Buffer II into each well and incubate for 5 minutes at RT. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating each time.
- 12.8. Pipette 1 mL of 1X Biotinylated Anti-Phosphotyrosine Antibody into each well and incubate for 1.5 - 2 hours at RT or overnight at 2-8°C.
- 12.9. Aspirate 1X Biotinylated Anti-Phosphotyrosine Antibody from each well.
- 12.10. Wash membranes as directed in Steps 12.6 and 12.7.

- 12.11. Pipette 2 mL of 1X HRP-Conjugated Streptavidin into each well and incubate for 2 hours at room temperature or overnight at 2-8°C.
- 12.12. Aspirate 1X HRP-Conjugated Streptavidin from each well.
- 12.13. Wash membranes as directed in Steps 12.6 and 12.7.
- 12.14. Transfer and place each membrane printed side up onto a sheet of chromatography paper, tissue paper, or blotting paper lying on a flat surface, such as a benchtop.
- 12.15. Remove any excess wash buffer by blotting the membrane edges with another piece of chromatography, blotting, or tissue paper.
- 12.16. Transfer and place the membranes, printed side up, onto a plastic sheet (provided) lying on a flat surface.
- 12.17. Into a single, clean tube, pipette equal volumes (1:1) of Detection Buffer C and Detection Buffer D. For 1 membrane add 250 μ L of Detection Buffer C and 250 μ L of Detection Buffer D into tube. Mix well.
- 12.18. Gently pipette 500 μ L of the Detection Buffers mixture onto each membrane (e.g., 500 μ L = 250 μ L of Detection Buffer C and 250 μ L of Detection Buffer D) and incubate for 2 minutes at room temperature (**Do not rock or shake**). Immediately afterwards, proceed to Step 12.19.

NOTE: Exposure should ideally start within 5 minutes after finishing Step 12.18 and completed within 10 – 15 minutes as chemiluminescence signals will fade over time. If necessary, the signals can usually be restored by repeating washing, HRP-Conjugated Streptavidin and Detection Buffers incubations (Steps 12.11. -12.18.).
- 12.19. Place another plastic sheet on top by starting at one end and gently “rolling” the flexible plastic 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 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.

- 12.20. 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.

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

13. CALCULATIONS

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 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 the 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)

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

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) and/or Blank Spots (BLANK) should be similar and are accurate for this purpose.

Positive Control Normalization:

The amount of biotinylated 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 "Reference Array" to which the other arrays are normalized to. The choice of the Reference Array is arbitrary.

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 reference array

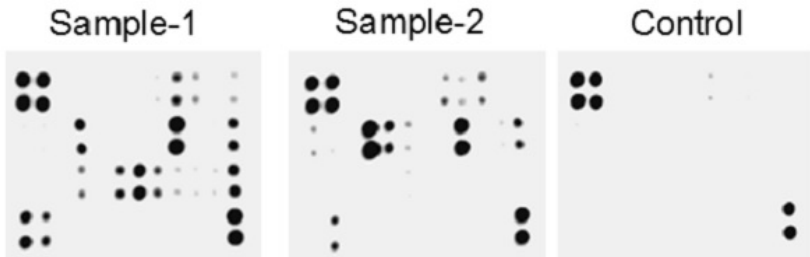
P(y) = mean signal density of Positive Control spots on Array "y"

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

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

14. TYPICAL DATA

Typical results obtained with Abcam Antibody Arrays:



The preceding figure presents typical images obtained with Abcam's Cytokine Antibody Membrane Array. These membranes were probed with conditioned media from two different cell lines. Membranes were exposed to film at room temperature for 1 minute.

Note the strong signals of the Positive Control spots in the upper-left and lower-right corners.

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.

15. TROUBLESHOOTING

Problem	Cause	Recommendation
No signals (not even the positive controls 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-Conjugated Streptavidin	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 controls spots signals visible but no 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).

RESOURCES

Problem	Cause	Recommendation
Uneven signal 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-Conjugated Streptavidin and Biotin 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	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.

16. NOTES



For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)