Technical Manual

Human EGFR Phosphorylation Array (16 targets)

- Catalogue Code: SARB0062
- Sandwich Principle
- Research Use Only

Contents

1. Key features and Sample Types

Size:

2, 4 or 8

Number of Targets Detected:

16

Gene Symbols:

EGFR, ERBB2, ERBB3, ERBB4

Detection principle:

Sandwich (Semi-Quantitative)

Sample Types:

Cell Culture Supernatants / Cell Lysates / Plasma / Serum / Tissue Lysates

Species Detected:

Human

Method of Detection:

Chemiluminescence

Pathway:

HER/ErbB Signaling

Solid Support:

Membrane

Expiry:

See Kit Label

2. Introduction

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

The conventional approach to analyzing multiple protein expression levels has been to use 2-D SDS-PAGE coupled with mass spectrometry. However, these methods are slow, expensive, labor-intensive and require specialized equipment. Thus, effective study of multiple protein expression levels can be complicated, costly and time-consuming. Moreover, these traditional methods of proteomics are not sensitive enough to detect most cytokines (typically at pg/ml concentrations).

Cytokines, broadly defined as secreted cell-cell signaling proteins distinct from classic hormones or neurotransmitters, play important roles in inflammation, innate immunity, apoptosis, angiogenesis, cell growth and differentiation. They are involved in most disease processes, including cancer, obesity and inflammatory and cardiac diseases.

Simultaneous detection of multiple cytokines undoubtedly provides a powerful tool to study cell Signaling pathways. Regulation of cellular processes by cytokines is a complex, dynamic process, often involving multiple proteins. Positive and negative feedback loops, pleiotrophic effects and redundant functions, spatial and temporal expression of or synergistic interactions between multiple cytokines, even regulation via release of soluble forms of membrane-bound receptors, all are common mechanisms modulating the effects of cytokine Signaling. As such, unravelling the role of individual cytokines in physiologic or pathologic processes generally requires consideration and detection of multiple cytokines rather than of a single cytokine.

1. More Data, Same or Less Sample: Antibody arrays provide high-content screening using about the same sample volume as traditional ELISA.

2. Global View of Cytokine Expression: Antibody array screening improves the chances for discovering key factors, disease mechanisms, or biomarkers related to cytokine signalling.

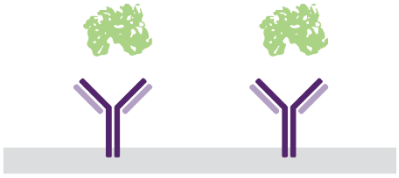
3. Similar Sensitivity (occasionally better): As little as 4 pg/ml of MCP-1 can be detected using the Assay Genie array format. In contrast, our similar MCP-1 ELISA assay has a sensitivity of 40 pg/ml of MCP-1.

4. Increased Detection Range: ELISA assays typically detect a concentration range of 100- to 1000-fold. However, Assay Genie arrays can, for example, detect IL-2 at concentrations of 25 to 250,000 pg/ml, a range of 10,000-fold.

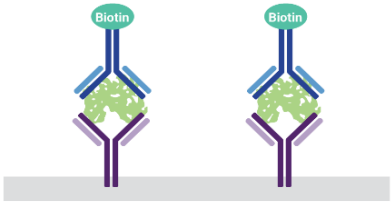
5. Better Precision: As determined by densitometry, the inter-array Coefficient of Variation (CV) of spot signal intensities is 5-10%, comparing favourably with ELISA testing (CV = 10-15%).

3. Workflow

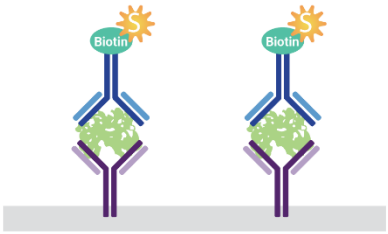
Step 1: Capture antibodies on a nitrocellulose membrane



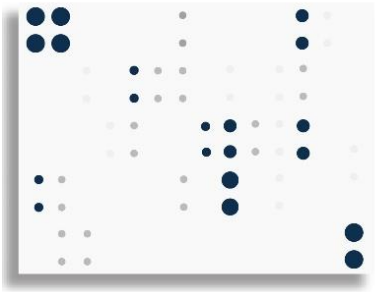
Step 2: Addition of target sample.



Step 3: incubation with biotin-labeled detection antibodies.



Step 4: detection of target analyte with labeled-streptavidin.



Step 5: Data analysis and imaging

4. Kit Components and Storage

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

Components	2 Arrays	4 Arrays	8 Arrays	Storage**
Antibody Arrays	2 membranes	4 membranes	8 membranes	-20°C
Blocking Buffer	1 vial (25ml)		2 vials (25ml/ea)	
Detection Antibody Cocktail	1 vial	2 vials	4 vials	2-8°C (for up to 3 days after dilution)
1000x HRP-Anti-Rabbit IgG Concentrate	1 vial (50µl)			2-8°C
20X Wash Buffer I Concentrate	1 vial (10ml)		1 vial (20ml)	
20X Wash Buffer II Concentrate	1 vial (10ml)		1 vial (20ml)	
2X Cell Lysis Buffer Concentrate	1 vial (10ml)		1 vial (16ml)	
Detection Buffer C	1 vial (1.5ml)		1 vial (2.5ml)	
Detection Buffer D	1 vial (1.5ml)		1 vial (2.5ml)	
Protease Inhibitor Cocktail	1 vial		2 vials	-20°C
100x Phosphatase Inhibitor Cocktail I	1 vial		2 vials	
Phosphatase Inhibitor Cocktail II	1 vial		2 vials	
8-Well Incubation Tray and Lid	1 tray			RT

*Each package contains 2 or 4 membranes

**Storage AFTER THAWING: up to 3 months (unless stated otherwise) or until expiration date

5. Additional Materials Required

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

6. Sample Suggestions and General Tips

1. Sample Collection, Preparation and Storage

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

- If not using fresh samples, freeze samples as soon as possible after collection.
- Avoid multiple freeze-thaw cycles. If possible, sub-aliquot samples prior to initial storage.
- It is strongly recommended to add a protease inhibitor cocktail to cell and tissue lysate samples.
- Avoid using EDTA as an anti-coagulant for collecting plasma if testing MMPs or other metal-binding proteins.
- 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.

- **Cell Lysate can be prepared as follows:**
  For attached cells, remove supernatant from cell culture, wash cells twice with cold 1X PBS (for suspension cells, pellet the cells by spinning down the cells at 1500 rpm for 10 min) making sure to remove any remaining PBS before adding Lysis Buffer. Solubilize the cells at $2x10^7$ cells/ml in 1X Lysis Buffer containing Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktail (see preparation note shown on page 8 under Component Preparation Section). Pipette up and down to resuspend cells and rock the lysates gently at 2–8 °C for 30 minutes. Transfer extracts to microfuge tubes and centrifuge at 14,000 x g for 10 min.
  It is recommended that sample protein concentrations should be determined using a total protein assay. For sample incubation with the Phosphorylation Antibody Array, use at a protein concentration of 50-1000 µg/ml for cell lysates.
  Lysates should be used immediately or aliquot and stored at -70 °C. Thawed lysates should be kept on ice prior to use.
  If you experience high background, you may further dilute your samples.

- **General tips for preparing serum, plasma, cell culture media, urine, and lysate samples can be viewed on the online at https://www.assaygenie.com/elisa-sample-preparation-and-collection**

2. Sample Type and Recommended Dilutions/Amounts

NOTE: *Optimal sample dilutions and amounts will need to be determined by each researcher empirically, but the below recommendations may be used as a starting point. Blocking Buffer should be used to dilute samples. Equal amount of protein should be used per sample.*

- **Cell and Tissue Lysates:** Load 50 to 1000 µg of total protein (after a 5-fold to 10- fold dilution to minimize the effects of any detergent(s)). Therefore the original lysate concentration should be 250 µg to 5 mg/ml.

3. Handling Membranes

- The antibody printed side of each membrane is marked by a dash (-) or number (#) in the upper left corner.
- Do not allow membranes to dry out during the experiment or they may become fragile and break OR high and/or uneven background may occur.
- Grasp membranes by the corners or edges only using forceps. DO NOT touch printed antibody spots.

4. Incubation and Washing

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

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

7. Chemiluminescence Detection Guidelines

1. 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.
2. Trying multiple exposure times is recommended to obtain optimum results.
3. A few seconds to a few minutes is the recommended exposure time range, with 30 seconds to 1 minute being suitable for most samples.

8. Component Preparation

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

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

Detection Antibody Cocktail*

Pipette 2 ml of Blocking Buffer into each vial. Mix gently with a pipette.

*1 vial is enough to test 2 membranes

1000X HRP-Anti-Rabbit IgG Concentrate

Dilute 1,000-fold with Blocking Buffer. Mix gently with a pipette.

Example: 10 µl of 1,000X concentrate + 9990 µl of Blocking Buffer = 10 ml of 1X working solution

Wash Buffer Concentrate (I + II)

Dilute each 20-fold with distilled or deionized water.

Example: 10 ml of 20X concentrate + 190 ml of water = 200 ml of 1X working solution

2X Cell Lysis Buffer Concentrate**

Dilute 2-fold with distilled or deionized water.

Example: 10 ml of 2X concentrate + 10 ml of water = 20 ml of 1X working solution

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

Protease Inhibitor Cocktail

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

Phosphatase Inhibitor Cocktail II

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

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

The antibody arrays, blocking buffer, detection buffer C and D and 100x Phosphatase Inhibitor Cocktail I require no preparation.

9. Array Protocol

NOTE: *Prepare all reagents and samples immediately prior to use. See Sections 6 + 7 'Sample Tips and General Considerations' and 'Component Preparation'. ALL incubations and washes must be performed under gentle rotation/rocking (~0.5-1 cycle/sec). Make sure no bubbles appear on or between the membranes to ensure even incubations.*

1. Remove the kit from storage and allow the components to equilibrate to room temperature.

2. Carefully remove the Antibody Arrays from the plastic packaging and place each membrane (printed side up) into a well of the Incubation Tray. One membrane per well.

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

A. Blocking

3. Pipette 2 ml of Blocking Buffer into each well and incubate for 30 minutes at room temperature.
4. Aspirate blocking buffer from each well with a pipette.

B. Sample Incubation

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

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

6. Aspirate samples from each well with a pipette.

C. First Wash

NOTE: *The 20x Wash Buffer Concentrates I and II must be diluted 20-fold before use. See component preparations for details.*

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

8. *Wash Buffer II Wash*: Pipette 2 ml of 1X Wash Buffer II into each well and incubate for 5 minutes at room temperature. Repeat this 1 more time for a total of 2 washes using fresh buffer and aspirating out the buffer completely each time.

D. Detection Antibody Cocktail Incubation

NOTE: *The Detection Antibody Cocktail must be prepared before use. See Component Preparation for details*

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

10. Aspirate Detection Antibody Cocktail from each well.

E. Second Wash

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

F. HRP-Anti-Rabbit IgG Incubation

NOTE: *The 1000X HRP-Anti-Rabbit IgG Concentrate must be diluted before use. See Component Preparation for detail.*

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

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

G. Third Wash

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

H. Chemiluminescence Detection

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

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

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

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

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

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

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

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

NOTE: *Exposure should ideally start within 5 minutes after finishing Step 19 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-Anti-Rabbit IgG and Detection Buffers incubations (Steps 11-19).*

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

NOTE: *Avoid "sliding" the top plastic sheet along the membranes' printed surface.*

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

NOTE: *Optimal exposure times will vary so performing multiple exposure times is strongly recommended. See Component Preparation for additional details.*

I. Storage

22. To store, without direct pressure, gently sandwich the membranes between 2 plastic sheets (if not already), tape the sheets together or use plastic wrap to secure them, and store at < -20°C for future reference.

10. Typical Data and Interpretation of Results

The following figure shows Assay Genie Human EGFR Phosphorylation Antibody Array I membranes probed with different cell lines. The signals were detected by using a chemiluminescence imaging device. Alternatively, membranes may also be exposed to Kodak X-Omat film at room temperature. A biotinylated protein provides positive signals (indicated “Pos” on the array map, page 15) which can be used to orient the membrane and to normalize the results from different arrays being compared. The signals of pan EGFR, ErbB2, ErbB3, and ErbB4 can also be used to normalize the results of their corresponding phospho-proteins if the pan proteins are detectable.

One important parameter is the background signal. To obtain the best results, we suggest that several exposures be attempted. We also strongly recommend using a negative control in which the sample is replaced with an appropriate mock buffer according to the array protocol, particularly during your first experiment.

By comparing the signal intensities, relative expression levels of target proteins can be made. The intensities of the signals can be quantified by densitometry. Positive controls may be used to normalize the results from different membranes. If the pan (total) EGFR, ErbB2, ErbB3, or ErbB4 signals are detectable, then they may also be used to normalize the signals of their corresponding phospho-proteins. If desired, the phospho-protein signals may be normalized to both the positive controls and the pan EGFR signals.

Normalization of Signals:

One array should defined as the "reference" to which the signal intensities of the other arrays should be compared. It is up to the researcher to define which array should be the reference. The normalization of the array signals to the positive controls may be calculated as follows:

Pos(1) = average signal intensity of positive controls on the reference array

Pos(2) = average signal intensity of positive controls on Array 2

X(2) = signal intensity for a particular spot on Array 2

X(N2)= the normalized value for that particular spot on Array 2

X(N2) = X(2) * Pos(1)/Pos(2)

This calculation may be repeated for the remaining arrays 3, 4, 5, etc. for a particular experiment.

After positive control normalization, normalization of phospho EGFR signals to the pan EGFR signals may be calculated according to the following example:

EGFR(1) = average signal intensity of pan EGFR on the reference array
EGFR(2) = average signal intensity of pan EGFR in array 2
Y845(2) = average signal intensity of EGFR (Tyr845) in array 2
Y845(N2) = normalized signal of EGFR (Tyr845) in array 2

Y845(N2) = Y845(2) * EGFR(1) / EGFR(2)

Antibody affinity to its target varies significantly between antibodies. The intensity detected on the array with each antibody depends on this affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.

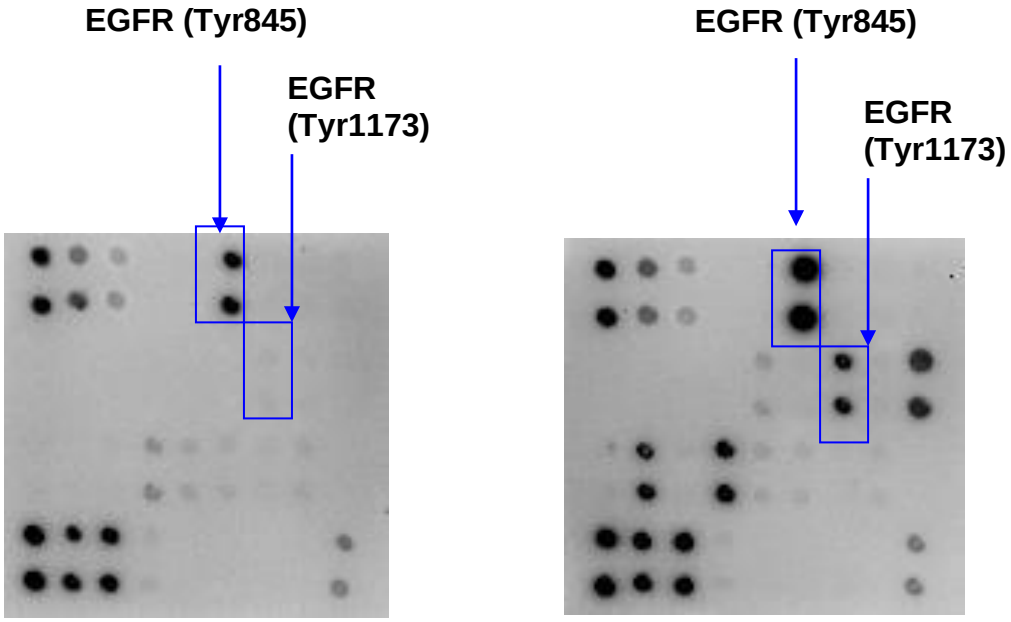


Figure 1: Human epidermoid carcinoma cell line, A431 cells that were 80- 90% confluent were serum starved overnight, then exposed to 100 ng/ml EGF for 20 minutes at 37 °C. Control cells were serum starved without the subsequent stimulation with EGF. Cell lysates were prepared following the "Preparation of Sample" portion of our protocol V. To use the Assay Genie Phosphorylation Antibody Array 1, treated or untreated cell lysate was added into antibody array membrane. The antibody array membranes were washed and cocktail of biotinylated anti-EGFR was used to detect phosphorylated proteins on activated receptors. After incubation with HRP-Conjugated Streptavidin, the signals were visualized by chemiluminescence.

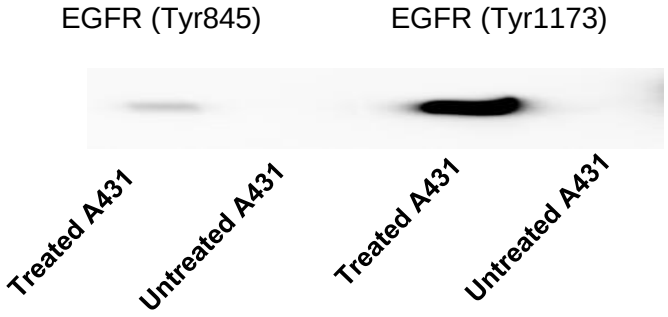


Figure 2: Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells or untreated A431 cells. Phospho-EGFR (Tyr845) or Phospho- EGFR (Tyr1173) antibodies was used in this assay.

Each antibody is spotted in duplicate vertically

	A	B	C	D	E	F	G	H	I
1 2	POS 1	POS 2	POS 3	BLANK	NEG	EGFR (P-Tyr845)	EGFR (P-Tyr922)	EGFR (P-Tyr1045)	EGFR (P-Tyr1068)
3 4	BLANK	BLANK	BLANK	BLANK	EGFR (P-TYR1086)	EGFR (P-TYR1148)	EGFR (P-TYR1173)	EGFR (SER1046/1047)	BLANK
5 6	ErbB2 (P-Tyr877)	ErbB2 (P-Tyr1112)	ErbB2 (P-Tyr1221/1222)	ErbB2 (P-TYR1248)	ErbB2 (P-Thr686)	ErbB2 (P-Ser1113)	ErbB3 (P-Tyr1289)	ErbB4 (P-Tyr1284)	BLANK
7 8	EGFR	ErbB2	ErbB3	ErbB4	BLANK	BLANK	NEG	BLANK	POS4

POS = positive
NEG = negative

12. Troubleshooting

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

Notes

Notes

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Contact Details

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