

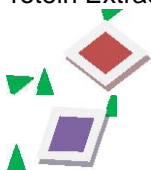
Antibody Microarray User's Guide

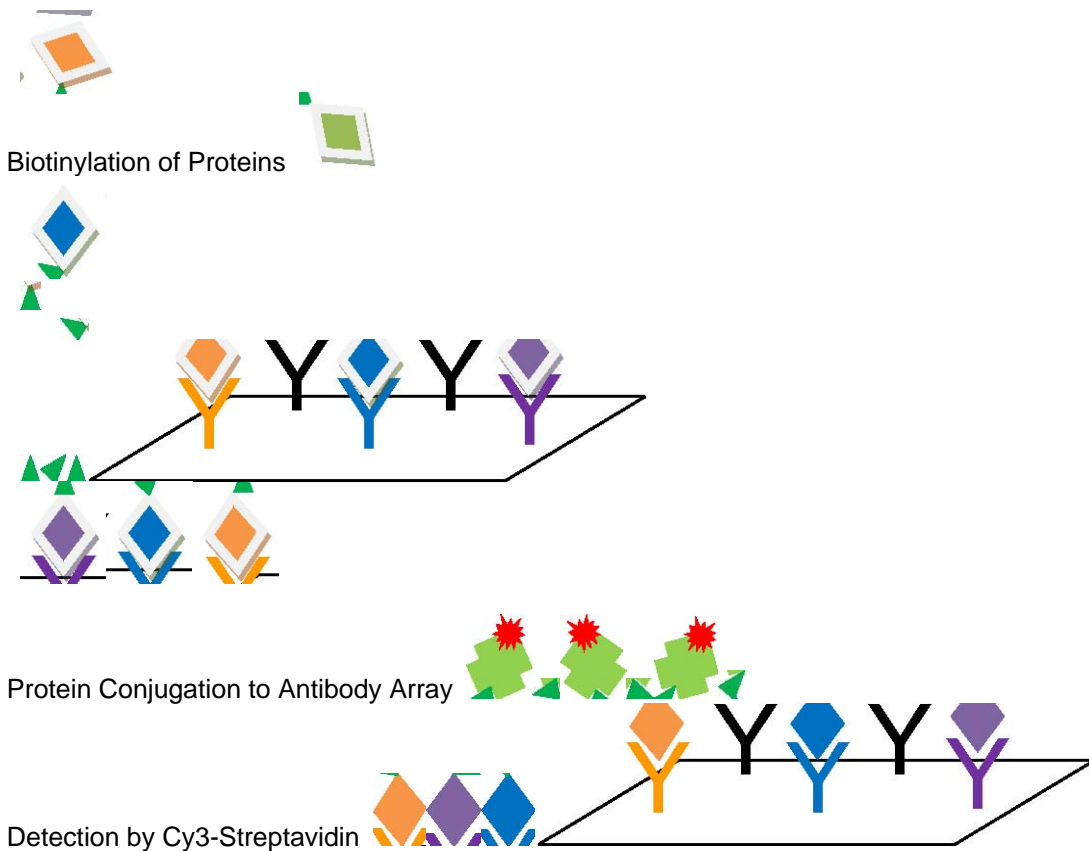
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Antibody Microarrays provide a high-throughput platform for sensitive, efficient and accurate protein expression profiling, screening, and comparison between normal, diseased or treated samples. US Biomax, Inc.'s' antibody arrays allow researchers to detect and analyze hundreds of proteins simultaneously on a single slide without performing numerous immunoprecipitation and/or Western blot analyses, saving precious resources and reducing the number of variables that affect experimental outcome. Our unique collection of antibody arrays includes the comprehensive *Explorer Antibody Array* that allows investigators to examine their samples against 656 antibodies in a single experiment, and the *PathwayFocused Antibody Arrays* and *Phosphorylation Antibody Arrays*, which are designed for researchers to study highly relevant proteins in their specific research fields. Proteins from cell extracts, tissue lysates, or treated samples can be used for analysis. The antibodies are covalently immobilized on high quality glass surface coated with our proprietary 3-D polymer materials to ensure high binding efficiency and specificity. All arrays are printed on standard-size microscope slides. The arrays utilize fluorescent detection and can be scanned on all microarray scanners that are compatible with 76 x 25 x 1 mm (3 inch x 1 inch x 1 mm) slides. For *PathwayFocused Arrays* and *Phosphorylation Antibody Arrays*, each slide consists of an array of well-characterized antibodies with six replicates and multiple positive and negative controls to maximize data reliability. For *Explorer Antibody Array*, each slide is printed with 656 unique antibodies in duplicates. Each set of antibody arrays contains two slides (two identical arrays) – one slide is for control sample, and the other is for treated sample. GAL files are provided for each array and can be downloaded from <http://www.biomax.us/support>. The *Antibody Array Assay Kit* is designed for easy and reliable processing of US Biomax, Inc.'s' antibody arrays. It provides the major reagents required to perform protein extraction, labeling, conjugation and detection. The reagents are convenient, easy to use, and optimized to work with US Biomax, Inc.'s' antibody arrays. Each kit provides sufficient reagents to perform two assays on two slides.



Protein Extraction from cells, tissues, or bodily fluids





All reagents and materials are intended for research use only.
 Always wear gloves before handling any reagents.
 Handle the slides by holding the area with barcode labels. Do not touch the slide surface.
 Use extra care. Any variation in buffers, operator, pipetting technique, washing technique, and incubation time or temperature can alter the performance of the kit.
 Only use reagents and materials recommended by this user's guide. Do not substitute buffers or solutions from other sources.
 Do not allow the arrays dry out between blocking, coupling and washes. It can cause high background.
 Wash the arrays extensively with Wash Solutions and water. It helps remove excess residual reagents from the slide surface.
 The reagents provided in the Antibody Array Assay Kit do not contain protease inhibitors. To prevent protein degradation, once you start the extraction, you should work quickly and proceed diligently towards the array analysis step. Alternatively, you may use inhibitors if you prefer or plan to store the proteins for a long period of time.

Antibody Arrays

Material/Reagent	Quantity	Purpose	Storage Condition
Antibody Microarray	2 slides	Microarray	4°C (6 months)
Gal File	1	Data Analysis	Online download

Antibody Array Assay Kit

Catalog No.	Description	Quantity
KAS02	Antibody Array Assay Kit, 2 Reactions	2 Reactions
KAS20	Antibody Array Assay Kit, 20 Reactions	20 Reactions

Assay Kit Components

Material/Reagent	Quantity	Purpose	Storage Condition	
Biotin Reagent	2-Rxn Kit 1 mg	20-Rxn Kit 5 x 1mg	Labeling	-20 °C
Blocking Reagent	60 mL	600 mL	Blocking	4 °C
Coupling Chamber	1	5	Coupling	RT
Coupling Reagent	12 mL	120 mL	Coupling	-20 °C
Detection Buffer	60 mL	600 mL	Detection	4 °C
DMF	200 uL	1 mL	Labeling	4 °C
Dry Milk	1.8g & 0.36g	18g & 3.6g	Coupling	4 °C
Extraction Buffer	1.5 mL	15 mL	Cell and tissue lysis	4 °C
Labeling Buffer	2 mL	20 mL	Labeling	4 °C
Lysis Beads	2 tubes	20 tubes	Cell and tissue lysis	RT or 4 °C
Spin Columns	2 sets	20 sets	Buffer Exchange	RT
Stop Reagent	100 uL	1 mL	Stop labeling reaction	4 °C
10X Wash Buffer	100 mL	500 mL x 2	Washing	4 °C

50-ml conical tube with cap
1X PBS (pH=7.4)
BCA Protein Assay Kit (Pierce, Cat. #: 23227) or Bradford Protein Assay Kit (Pierce, Cat. #: 23200; BioRad, Cat. #: 500-0201)
Cy3-Streptavidin (GE Healthcare, Cat. #: PA43001)
Centrifuge
Compressed nitrogen/clean air. Do not use canned air duster.
Microarray scanner compatible with 3 x 1 inch (76 x 25 mm) slides
Milli-Q Grade Water or dd H₂O
Orbital shaker
Petri dishes, 100 x 15mm (9 cm in diameter)
Vortexer

ALL REAGENTS MUST BE WARMED BEFORE USE

Blocking Solution Coupling Solution Wash Solution Warm to 25-30°C in a water bath.

All other reagents

Warm to room temperature.

2-RXN Kit (KAS02)

- 1 1X Wash Solution Make 1:10 dilution. In a one-liter reagent bottle, add 100 ml of 10X Wash Buffer to 900 ml of dd H₂O. Shake to mix.
- 2 Blocking Solution Add 1.8 g of Dry Milk to 60 ml of Blocking Reagent. Shake to mix. **Use within one week.**
- 3 Coupling Solution Add 0.36 g of Dry Milk to 12 ml of Coupling Reagent. Shake to mix. **Use within one week.**

20-RXN Kit (KAS20)

- 1 1X Wash Solution Make 1:10 dilution. For example, add 100 ml of 10X Wash Buffer to 900 ml of ddH₂O to make 1L of 1X Wash Solution. Shake to mix.
- 2 Blocking Solution If you plan to perform 20 assays within one week, add 18 g of Dry Milk to 600 ml of Blocking Reagent. For two assays, aliquot 60 ml of Blocking Reagent and add 1.8 g of Dry Milk. Shake to mix. **Use within one week.**
- 3 Coupling Solution If you plan to perform 20 assays within one week, add 3.6 g of Dry Milk to 120 ml of Coupling Reagent. For two assays, aliquot 12 ml of Coupling Reagent and add 0.36 g of Dry Milk. Shake to mix. **Use within one week.**

Detection by Cy3-Streptavidin **IMPORTANT – ALL REAGENTS MUST BE WARMED BEFORE USE** (See Reagent Preparation for detailed instructions)

A. Protein Extraction

Note: It is highly recommended that protein extraction is performed using the Extraction Buffer provided in the Antibody Array Assay Kit. If you used extraction or lysis buffers from other manufacturers, please be sure the buffer is free of Tris or other amine based compounds. The presence of such compound will adversely affect biotinylation of protein samples in the next step. If such buffer was used to extract proteins from cells or tissues, do not omit Step B (Lysate Purification/Buffer Exchange). This step ensures the removal of unwanted buffer from your protein extract and replaces it with the Labeling Buffer provided in the Antibody Array Assay Kit.

Note: The reagents provided in the Antibody Array Assay Kit do not contain protease inhibitors. To prevent protein degradation, once you start the extraction, you should work quickly and proceed diligently towards the array analysis step. Alternatively, you may use inhibitors if you prefer or plan to store the proteins for a long period of time.

a. Extraction from Body Fluid

1. Centrifuge the sample at 10,000 x g (14,000 rpm) for 15 to 20 minutes at 4°C.
2. Transfer the supernatant to a clean tube.
3. Proceed immediately to Step B (Lysate Purification).

b. Extraction from Cells

1. Extraction **with** Lysis Beads

- a. Harvest 1 to 5 million cells. Remove as much culture medium from the cells as possible.
- b. Wash the cells with ice cold 1X PBS and centrifuge at 4°C. Aspirate and discard the supernatant.
- c. Wash the cells two more times with ice cold 1X PBS and centrifuge at 4°C.
- d. Add one tube of Lysis Beads to the cell pellet and add Extraction Buffer. The amount of Extraction Buffer should be determined based on the number of cells harvested. For 1 – 2.5 million cells, add 100 ul of Extraction Buffer; for 2.5 – 5 million cells, add 200 ul of Extraction Buffer. Mix rigorously by vortexing for 30 seconds to 1 minute.

Incubate the mixture on ice for 10 minutes.

- e. Repeat vortexing for 30 seconds to 1 minute at 10-minute intervals for 40 – 60 minutes. Be sure to incubate the mixture on ice between vortexing.
- f. Centrifuge the mixture at 10,000 x g (14000 rpm) for 15 to 20 minutes at 4°C.
- g. Transfer the supernatant to a clean tube. Discard the beads.
- h. Proceed immediately to Step B (Lysate Purification).

2. Extraction **without** Lysis Beads

- a. Harvest 1 to 5 million cells. Remove as much culture medium from the cells as possible.
- b. Wash the cells with 1X PBS and centrifuge at 4°C. Aspirate and discard the supernatant.
- c. Wash the cells two more times with cold 1X PBS and centrifuge at 4°C.
- d. Add Extraction Buffer to the cell pellet. The amount of Extraction Buffer should be determined based on the number of cells harvested. For 1 – 2.5 million cells, add 100 ul of Extraction Buffer; for 2.5 – 5 million cells, add 200 ul of Extraction Buffer. Mix thoroughly by pipetting.
- e. Incubate the mixture on ice for 40 – 60 minutes with occasional mixing.
- f. Centrifuge the mixture at 10,000 x g (14000 rpm) for 15 to 20 minutes at 4°C.
- g. Transfer the supernatant to a clean tube.
- h. Proceed immediately to Step B (Lysate Purification).

c. Extraction from Tissues

1. Extraction **with** Lysis Beads

- a. Add one tube of Lysis Beads to 10 – 40 mg of tissues.
- b. Add Extraction Buffer to the tissues. The amount of Extraction Buffer should be determined

based on the amount of tissues harvested. For 10 – 20 mg of tissues, add 100 ul of Extraction Buffer; for 20 – 40 mg of tissues, add 200 ul of Extraction Buffer.

- c. Mix rigorously by vortexing for 30 seconds to 1 minute. Incubate the mixture on ice for 10 minutes.
- d. Repeat vortexing for 30 seconds to 1 minute at 10-minute intervals for 40 – 60 minutes. Be sure to incubate the mixture on ice between vortexing.
- e. Centrifuge the mixture at 10,000 x g (14000 rpm) for 15 to 20 minutes at 4°C.
- f. Transfer the supernatant to a clean tube. Discard the beads.
- g. Proceed immediately to Step B (Lysate Purification).

2. Extraction **without** Lysis Beads

- a. Place 10 – 40 mg of frozen tissues in a homogenizing tube.
- b. Add Extraction Buffer to the tissues. The amount of Extraction Buffer should be determined based on the amount of tissues harvested. For 10 – 20 mg of tissues, add 100 ul of Extraction Buffer; for 20 – 40 mg of tissues, add 200 ul of Extraction Buffer.

- c. Homogenize the tissues on ice.
- d. Transfer the homogenate to a centrifuge tube.
- e. Centrifuge the homogenate at 10,000 x g (14000 rpm) for 15 to 20 minutes at 4°C.
- f. Transfer the supernatant to a clean tube.
- g. Proceed immediately to Step B (Lysate Purification).

B. Lysate Purification/Buffer Exchange

Note: Do not omit this step if you used extraction buffers containing amine based compounds. This step ensures the removal of unwanted buffer from your protein extract and replaces it with the Labeling Buffer provided in the Antibody Array Assay Kit.

1. The sample volume capacity of each spin column is 100uL.
2. Gently tap the columns to ensure that the dry gel has settled to the bottom of the column. Remove the top column cap and reconstitute the column by adding 650 uL of Labeling Buffer.
3. Replace the column cap and vortex vigorously for about 5 seconds. Remove air bubbles by sharply tapping the bottom of the column. Allow at least 30 to 60 minutes of room temperature hydration time before using the column.
4. After hydration, remove the top column cap and then remove the column end stoppers from the bottom.
5. Spin the column in its wash tubes at 750 x g for two minutes to remove excess fluid.
6. Blot excess drops from the bottom of the column. Discard the wash tubes and the excess fluid. Do not allow the gel material to dry excessively. Process the samples within the next few minutes.
7. Hold the column up to the light. Transfer 30 – 100uL of protein extract, from cells, tissues or bodily fluid, to the top of the gel of each column. Carefully dispense the sample directly onto the center of the gel bed at the top of the column without disturbing the gel surface. Do not touch the sides of the columns with the reaction mixture or the sample pipet tip since this can reduce the purification efficiency.
8. Place the column into a collect tube and place both together into the rotor. Maintain proper column orientation.
9. Spin the column and collection tube at the 750 x g for 2 minutes.
10. The purified protein will collect at the bottom of the collection tube. Discard the spin column.
11. Measure the protein concentration using BCA Protein Assay Kit or Bradford Protein Assay Kit.
12. Proceed immediately to the next step (Protein Labeling) or store the samples at -80°C.

C. Protein Labeling – Biotinylation of Protein Samples

1. Briefly centrifuge Biotin Reagent before use.
2. Add 100 ul of DMF (*N,N*-Dimethylformamide) to 1 mg of Biotin Reagent to give a concentration of 10 ug/ul. Label this solution as Biotin/DMF.

3. Aliquot 10 – 25 ul of the protein sample containing 40 – 100 ug of proteins. **Note:** be sure that the concentration of the proteins is within the range of 2 – 10 ug/ul. If the

concentration is too low, the proteins must be concentrated at 4°C in a vacuum centrifuge, such as SpeedVac, or using YM-10 filters (Millipore Corporation).

4. Add 25 – 40 ul Labeling Buffer to the protein sample to bring the volume to 50 ul.

5. Add 1.5 ul of the Biotin/DMF solution to the protein sample. Store the rest of the Biotin/DMF solution at -20°C for future use.

6. Mix and incubate at room temperature for 2 hours with mixing or shaking.

7. Add 25 ul of Stop Reagent. Incubate for 30 minutes at room temperature with mixing or shaking.

8. Proceed immediately to the next step, or store the sample at -80°C for future use.

D. Blocking

1. Remove the Antibody Microarray from the freezer. **Important:** Allow the slides to warm up to room temperature (30 to 45 minutes) before opening the package.

2. Add 30 ml of Blocking Solution (See "Reagent Preparation") in a 100x15 mm Petri dish. Submerge one slide in the Blocking Solution. Be sure the side with arrays and barcode label faces up.

3. Incubate on an orbital shaker rotating at 55 rpm for 30 to 45 minutes at room temperature.

4. Rinse the slide extensively with Milli-Q grade water as follows:

a. Place the slide into a 50-ml conical tube filled with 45 ml of water. Close the cap.

b. Invert the tube up and down or shake with your hand for 10 seconds. Pour off the water.

c. Filled the tube with fresh water. Shake for 10 seconds and pour off the water.

d. Repeat 5 to 10 times.

Important: It is crucial to rinse the slide extensively to remove any blocking solution residues from the slide surface.

5. Shake off excessive water on the slide surface. Proceed immediately to the next step. **Note:** do not allow the slide to dry out. You can prepare the Protein Coupling Mix (Step E.1 and E.2) in advance so that you can start coupling immediately after this step.

E. Coupling

1. In a tube, add 6 ml of Coupling Solution (See "Reagent Preparation").

2. Add one tube of biotin labeled proteins (40 – 100 ug). Vortex briefly to mix. Label it as "Protein Coupling Mix."

3. Place the slide in Well 1 (or any clean well) of the Coupling Chamber, with the arrays facing up.

4. Slowly pour all 6 ml of Protein Coupling Mix over the slide. **Important:** Make sure the slide is completely submerged. Cover the Coupling Chamber.

5. Incubate on an orbital shaker rotating at 35 rpm for 1 – 2 hours at room temperature.

6. Transfer the slide to a 100x15 mm Petri dish containing 30 ml of 1X Wash Solution (See "Reagent Preparation").

7. Incubate on an orbital shaker rotating at 55 rpm for 10 minutes at room temperature. Discard the wash solution. Repeat the wash step twice.

8. Rinse the slide extensively with Milli-Q grade water as follows:

a. Place the slide into a 50-ml conical tube filled with 45 ml of water. Close the cap.

b. Invert the tube up and down or shake with your hand for 10 seconds. Pour off the water.

c. Filled the tube with fresh water. Shake for 10 seconds and pour off the water.

d. Repeat 5 to 10 times.

9. Shake off excessive water on the slide surface and proceed to the next step immediately. **Note:** do not allow the slide to dry out.

F. Detection

1. Add 60 μ l of Cy3-Streptavidin (0.5 mg/ml) to 60 ml of Detection Buffer.
2. Pour 30 ml of Cy3-Streptavidin Solution into a 100x15 mm Petri dish.
3. Submerge the slide in the Cy3-Streptavidin solution. Incubate on an orbital shaker rotating at 55 rpm for 30 to 45 minutes at room temperature in the dark or covered with aluminum foil.
4. Transfer the slide to a new 100x15 mm Petri dish containing 30 ml of 1X Wash Solution.
5. Incubate on an orbital shaker set at 55 rpm for 10 minutes at room temperature. Discard the wash solution. Repeat the wash step twice.
6. Rinse the slide extensively with Milli-Q grade water as follows:
 - a. Place the slide into a 50-ml conical tube filled with 45 ml of water. Close the cap.
 - b. Invert the tube up and down or shake with your hand for 10 seconds. Pour off the water.
 - c. Filled the tube with fresh water. Shake for 10 seconds and pour off the water.
 - d. Repeat 5 to 10 times.
7. Dry the slide by centrifugation or with compressed nitrogen (less than 30 psi of pressure).
8. The slide is now ready for scanning.