

Product Information

Panorama® Antibody Microarray – p53 Pathways Kit

Catalog Number **PPAA4**

Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The Panorama® Antibody Microarray – p53 Pathways Kit is designed for studying protein expression in cell or tissue extracts, and blood plasma or serum samples. The ability to identify multiple proteins simultaneously allows global molecular characterization of biological samples with applications in fundamental cell biology research.¹ The use of DNA arrays for profiling mRNA expression in cells has accelerated research for understanding biological systems from a genomic perspective. However, mRNA undergoes a number of alternative processing steps prior to and following translation. There is often poor correlation between mRNA and protein expression,² so a method that can assay proteins directly is required for analyzing biologically-relevant events. Antibody arrays provide an effective solution to rapidly profile expression of multiple proteins in samples. Examples where Panorama Antibody Microarrays have been used for such applications include the differentiation of F9 cells,³ breast cancer samples,⁴ maintenance of human embryonic stem cell pluripotency and viability,⁵ and for differential protein expression in colorectal cancer.⁶

The array contains 112 different antibodies each spotted in duplicate on nitrocellulose-coated glass slides. These antibodies represent families of proteins known to be involved in p53 pathways.

The expression of a protein in a cell extract is detected when it binds to its corresponding antibody spotted on the slide. This binding is visualized by a sensitive fluorescent signal created by directly labeling the proteins in the cell or nuclear extract with a fluorescent dye. Each antibody has been tested for its ability to bind proteins using at least two biological samples.

The array can be used for comparing protein expression profiles of two samples (test versus reference samples). Each sample is labeled with a different Cy™ dye (Cy™3 or Cy5) and the two samples are applied simultaneously at equal protein concentrations on the array. Fluorescent signal intensity for each sample is then recorded individually at the wavelength corresponding to the dye label of the sample and compared.

Important advantages of the Panorama Antibody Microarray – p53 Pathways Kit include:

- Accurate and robust protein expression profiling in half a day.
- Antibodies are spotted in high density to ensure strong signals.
- Proprietary treatment of the slide coating minimizes background staining, thereby, maximizing the signal-to-noise ratio.

Important general aspects of the Panorama Antibody Microarray – p53 Pathways Kit:

- The Panorama Antibody Microarray – p53 Pathways Kit is not species specific. The antibodies spotted recognize primarily human, mouse, and rat proteins. Information on species specificity of each antibody can be found in the file “Antibody List” on the accompanying small CD. More information on each antibody can be obtained in the antibody specific datasheet found on the Sigma-Aldrich Web site at sigma.com/arrays. All antibodies spotted can be purchased individually from Sigma-Aldrich.
- Binding and dissociation constants of an antibody with its target can vary significantly between antibodies. The fluorescence intensity detected on an array with each antibody depends on this binding affinity; therefore, signal intensity comparison can be performed only within the same antibody/antigen system and not between different antibodies.

- Each slide contains 112 antibodies spotted in 16 sub-arrays each containing duplicate spots of the antibodies, as well as spots for positive (a monoclonal antibody that recognizes Cy3 and Cy5) and negative controls. Information regarding specific positioning of each antibody can be found in the file "Antibody Position" on the accompanying small CD or at sigma.com/arrays.
- For best results, it is recommended that freshly prepared extracts be labeled and that only labeled preparations with dye/protein ratio >2 be used.
- Results obtained using the array are qualitative and should be further evaluated by other methods such as immunoblot assays or ELISA.
- The slides are sensitive. Do not touch the surface of the slides and handle all buffers with latex free gloves.
- The slides are for a single use only.

Components

Sufficient material is provided for performing 2 array reactions. Please be aware that some of the buffers are provided in excess.

Panorama Antibody Slides – p53 Pathways Catalog Number P2624	2 each
quadriPERM [®] Cell Culture Vessels Catalog Number Q3756	2 each
Extraction/Labeling Buffer Catalog Number E0655	30 ml
Protease Inhibitor Cocktail Catalog Number P4495	1 vial
Phosphatase Inhibitor Cocktail 2 Catalog Number P5726	0.3 ml
Benzonase [®] , Ultrapure Catalog Number B8309	1,000 units
Array Incubation Buffer Catalog Number A9602	20 ml
Phosphate Buffered Saline, pH 7.4, with TWEEN [®] 20 (Washing Buffer) Catalog Number P3563	1 each

SigmaSpin [™] Post-Reaction Clean-Up Columns Catalog Number S0185	8 each
Collection Tubes, Polypropylene, 2 ml Catalog Number T7813	16 each
Panorama Antibody List – p53 Pathways Catalog Number P2749	1 each

Reagents and Equipment Required But Not Provided

- Bradford Reagent (Catalog Number B6916)
- 0.01 M Phosphate Buffered Saline (PBS), pH 7.4 (Catalog Number P3813)
- Microcentrifuge
- Microcentrifuge tubes
- Rocking shaker
- Rubber policeman (for adherent cells)
- Homogenizer (for tissues)
- Cy3 Monofunctional Reactive dye (GE Healthcare)
- Cy5 Monofunctional Reactive dye (GE Healthcare)
- CellLytic[™] NuCLEAR[™] Extraction Kit (Catalog Number NXTRACT) (optional)
- Carbonate-Bicarbonate buffer (Catalog Number C3041)
- Microarray Scanner

Precautions and Disclaimer

This kit is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

—Protease Inhibitor Cocktail - Add 0.3 ml of ultrapure water to the vial (Catalog Number P4495). Store the reconstituted solution at –20 °C.

Benzonase Working Solution – Benzonase, Ultrapure (Catalog Number B8309) is supplied as a 50 units/μl solution. For immediate use, prepare a working solution of 5 units/μl in Extraction/Labeling Buffer (Catalog Number E0655) by adding 2 μl of Benzonase, Ultrapure to 18 μl of Extraction/Labeling Buffer. Upon dilution store the Benzonase Working Solution on ice.

Buffer A - To each 10 ml of Extraction/Labeling Buffer add 50 μ l of the reconstituted Protease Inhibitor Cocktail, 100 μ l of Phosphatase Inhibitor Cocktail II, (Catalog Number P 5726), and 1.2 μ l of the Benzonase Working Solution (final concentration in Buffer A of 0.6 units/ml). Keep Buffer A on ice. Use it immediately; do not store unused buffer.

Washing Buffer - Open the foil pouch of Phosphate Buffered Saline, pH 7.4, with TWEEN 20, and dissolve in 1 liter of water. Filter through a 0.45 μ m filter.

Storage/Stability

The kit ships on wet ice and should be stored at 2–8 °C. It has a shelf life of 2 years when stored at 2–8 °C; however, it is recommended that upon arrival, immediately transfer the Benzonase (Catalog Number B8309) and Protease Inhibitor Cocktail (Catalog Number P4495) to –20 °C storage.

Procedure

Note: Wear disposable gloves (non-latex) while performing all procedures.

The use of total cell extract with the array may be sufficient for some cells or tissues (Procedure I, Protein Extraction from Cell Lines or Tissues). For examination of the nuclear protein content, enrichment of the nuclear protein fraction in the sample is suggested. For these assays, using the CelLytic NuCLEAR Extraction Kit (Catalog Number NXTRACT) is recommended. A nuclear protein extract prepared with this kit requires dialysis to adjust the pH prior to labeling with the Cy dyes.

Detergents can interfere with the labeling efficiency of the extracted proteins. Therefore, any procedure used for the preparation of nuclear proteins should not include the use of detergents. If the CelLytic NuCLEAR Extraction Kit is used, be certain to use the procedure that omits the use of a detergent.

I. Protein Extraction from Cell Lines or Tissues

A unique extraction/labeling buffer has been developed that can be used for extraction of proteins from cells and tissues, and is suitable for labeling of proteins. With this buffer a high ratio of dye to protein (D/P molar ratio) can be achieved, which is very important for the success of the experiment.

The extract should be clear and not viscous. Therefore, it is important to add the Benzonase, which is a potent DNase, to Buffer A to ensure DNA degradation. An additional amount (0.6 units/ml) of Benzonase can be added in cases where the extract is still viscous. Small particles should be eliminated by a rapid centrifugation just before the labeling procedure.

Cell and tissue extracts can be prepared using any suitable procedure. However, it is important the final concentration of the sample be high (~10 mg/ml) so it can be diluted at least 10-fold into Buffer A to enable adequate protein labeling.

Note: All protein extract preparations should be performed in a cold room or kept on ice.

IA. Extraction from Cells

From adherent cells:

1. Grow cells to 70–80% confluency (2–3 Petri dishes of 10 cm will give enough material for labeling).
2. Wash the cells twice with cold 0.01 M PBS, pH 7.4.
3. Add 1 ml of Buffer A directly onto each plate. Incubate for 5 minutes on ice. Scrape the plate with a rubber policeman and collect the sample into a microcentrifuge tube.
4. Proceed to step 4 for non-adherent cells.

For non-adherent cells:

1. Grow cells in culture. Collect $\sim 10^7$ cells into a tube. Centrifuge cells at 1,500 $\times g$ for 5 minutes.
2. Wash the cells twice with cold 0.01 M PBS, pH 7.4, and collect by centrifugation at 1,500 $\times g$.
3. Transfer the cells to a microcentrifuge tube, add 1 ml of Buffer A, and vortex. Incubate for 5 minutes on ice.
4. Centrifuge the sample for 10 seconds at 10,000 $\times g$ in a microcentrifuge. Transfer the supernatant to a new tube.
5. Determine the protein concentration in the supernatant by the Bradford protein assay.
6. Dilute the sample to 1 mg/ml in Buffer A.
7. Use 1 ml of extract (1 mg/ml) for labeling with Cy3 or Cy5.

IB. Extraction from Tissues

Note: The following procedure is used for extracting proteins from mouse brain and may be suitable for use with other soft tissues.

1. Rapidly remove the tissue from the animal.
2. Weigh the tissue and record wet tissue weight. Cut the tissue into small pieces with a scalpel or a tissue slice blade.
3. Transfer the tissue pieces into 5 volumes (w/v) of Buffer A (0.5 g of tissue into 2.5 ml).
4. Homogenize the tissue on ice using a homogenizer.
5. Centrifuge the sample for 10 seconds at $10,000 \times g$ in a microcentrifuge.
6. Transfer the supernatant into a clean tube and determine the protein concentration by the Bradford protein assay.
7. Dilute the extract to 1 mg/ml in Buffer A.
8. Use 1 ml of extract (1 mg/ml) for labeling with Cy3 or Cy5 dyes.

IC. Nuclear Protein Extraction Without the Use of a Detergent

Detergents can interfere with the labeling efficiency of extracted nuclear proteins. Therefore, a procedure that does not include the use of detergents should be used for the preparation of nuclear proteins.

The CellLytic NuCLEAR Extraction Kit (Catalog Number NXTRACT) may be used for preparation of nuclear proteins. Be certain to use the procedure that omits the use of a detergent. A nuclear protein extract prepared with this kit requires dialysis to adjust the pH prior to labeling with the Cy dyes.

The following procedure is for protein extraction from 200 μ l of packed cell volume (PCV) and represents the non-detergent procedure in the kit. For different packed cell volumes, calculate accordingly.

Note: The following procedure describes the preparation of crude nuclear extracts using a syringe or a glass tissue homogenizer. The procedure requires at least 100 μ l of PCV. Use of a syringe is recommended for small-scale preparations (0.1–1 ml). Passage of more than 1 ml through a syringe may cause difficulties due to the needle gauge size.

IC-a. Extraction from Cells

1. Prepare a fresh solution of 0.1 M DTT with ultrapure water.
2. Prepare Lysis Buffer:
 - Hypotonic: 10 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$ and 10 mM KCl.
 - Isotonic (or protein extraction from fragile cells): 10 mM Tris HCl, pH 7.5, with 2 mM $MgCl_2$, 3 mM $CaCl_2$, and 0.3 M Sucrose
3. To 1,400 μ l of Lysis Buffer (hypotonic or isotonic), add 14 μ l of the 0.1 M DTT solution and 14 μ l of the Protease Inhibitor Cocktail.
4. Prepare Extraction Buffer: 20 mM HEPES, pH 7.9, with 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) Glycerol.

From adherent cells

1. Grow cells to 70–80% confluence.
2. Remove the growth medium from the cells.
3. Rinse the cells twice with PBS, being careful not to dislodge any cells.
4. Discard the PBS. Scrape the cells using fresh PBS into an appropriate conical centrifuge tube.
5. Centrifuge for 5 minutes at $450 \times g$.
6. Decant and discard the supernatant.
7. Estimate the packed cell volume (PCV).
8. Proceed to step 7 for non-adherent cells.

For non-adherent cells

1. Collect the cells into an appropriate centrifuge conical tube.
2. Centrifuge for 5 minutes at $450 \times g$.
3. Decant and discard the supernatant.
4. Wash cells twice by resuspending the cell pellets in PBS and centrifuge for 5 minutes at $450 \times g$.
5. Decant and discard supernatant.
6. Estimate the packed cell volume (PCV).
7. Add 1 ml ($5 \times$ PCV) of Lysis Buffer (including DTT and protease inhibitors) to 200 μ l of PCV.
8. Resuspend the cell pellet gently. Avoid foam formation. If working with small volumes, the suspended cells may be transferred to a microcentrifuge tube.
9. Incubate the packed cells in Lysis Buffer for 15 minutes, allowing cells to swell.
10. Centrifuge the suspended cells for 5 minutes at $420 \times g$. Decant supernatant and resuspend the pellet of packed cells in 400 μ l ($2 \times$ PCV) of the Lysis Buffer.

- Using a glass tissue homogenizer, transfer the cells into a glass tissue grind tube. Grind on ice slowly with five up-and-down strokes using a type B pestle. Avoid foam formation.

Or

Using a syringe with a narrow-gauge (No. 27) hypodermic needle, fill the syringe with Lysis Buffer. The syringe plunger is used to displace the buffer as fully as possible. This removes all the air from the syringe and prevents excess air being pumped into the cell suspension during lysis. Draw the cell suspension slowly into the syringe and then eject with a single rapid stroke. Repeat five times.

Notes:

- The number of strokes needed (using the tissue homogenizer or the syringe) varies between cell lines. Start with 5 strokes and then check lysis under the microscope. Lysis should be 80–90 %. If the lysis is not sufficient, perform several more strokes until lysis is complete.
 - Lysis can be observed by the addition of a Trypan Blue solution to an aliquot of cells. The dye is excluded from the intact cells, but stains the nuclei of lysed cells. If nuclear lysis or clumps of nuclei are visualized, or if a gelatinous mass is observed, the cell disruption may have been too vigorous or too many strokes were performed.
- Centrifuge the disrupted cells in suspension for 20 minutes at $10,000 \times g$.
 - Transfer the supernatant to a fresh tube. This fraction is the cytoplasmic fraction.
 - Add $1.5 \mu\text{l}$ of the 0.1 M DTT solution and $1.5 \mu\text{l}$ of the Protease Inhibitor Cocktail to $147 \mu\text{l}$ of the Extraction Buffer.
 - Resuspend the crude nuclei pellet in $\sim 140 \mu\text{l}$ ($2/3 \times \text{PCV}$) of Extraction Buffer containing DTT and Protease Inhibitors. If the procedure is being performed with a tissue homogenizer, it is recommended to give 10 more strokes at this point.
 - Shake gently for 30 minutes.
 - Centrifuge for 5 minutes at $20,000\text{--}21,000 \times g$.
 - Transfer the supernatant to a clean, chilled tube.
 - Continue with dialysis or store at -70°C .

IC-b. Extraction from Tissues

The following procedure is for extraction of nuclear proteins from 100 mg of tissue. For different tissue weight, calculate accordingly.

- Prepare a fresh solution of 0.1 M DTT with ultrapure water.

- Prepare Lysis Buffer as described in Section IC-a, steps 1-3.
Note: For tissues tested by Sigma the hypotonic buffer worked better than the isotonic. If the tissue is found to be too fragile, one can use the isotonic lysis buffer.
- Prepare Extraction Buffer as described in Section IC-a, step 4. Add $1.5 \mu\text{l}$ of the prepared 0.1 M DTT solution and $1.5 \mu\text{l}$ of the Protease Inhibitor Cocktail to $147 \mu\text{l}$ of the Extraction Buffer.
- Rinse the tissue twice with PBS buffer. Discard the PBS.
- Resuspend the tissue gently in 1 ml ($5 \times \text{PCV}$) of the Lysis Buffer containing DTT and protease inhibitors.
- Homogenize the tissue (using the tissue homogenizer) until more than 90% of the cells are broken and nuclei are visualized under the microscope.
- Centrifuge the disrupted cells for 20 minutes at $10,000 \times g$.
- Transfer the supernatant to a fresh tube. This fraction is the cytoplasmic fraction.
- Resuspend the crude nuclei pellet in $\sim 140 \mu\text{l}$ ($2/3 \times \text{PCV}$) of Extraction Buffer containing DTT and protease inhibitors. At this stage a short homogenization can be performed to facilitate nuclear extraction.
- Shake gently for 30 minutes.
- Centrifuge for 5 minutes at $20,000 \times g$.
- Transfer the supernatant to a clean, chilled tube.
- Continue with dialysis or store at -70°C .

ID. Dialysis

When protein samples are prepared using a procedure without Buffer A or the Extraction/Labeling Buffer, it is recommended to dialyze the sample against Carbonate-Bicarbonate Buffer, pH 9.5–9.6 (Catalog Number C3041) before the labeling procedure.

- Prepare 0.1 M, pH 9.5–9.6, Carbonate-Bicarbonate Buffer (Catalog Number C3041) by dissolving 2 capsules in 100 ml of ultrapure water.
- Dialyze at 4°C for 2 hours in a dialysis buffer volume $1,000 \times$ the volume of the protein extract.
- Replace the dialysis buffer with freshly prepared carbonate buffer and dialyze for an additional 2 hours at 4°C .
- Determined protein concentration according to the Bradford protein assay. Continue to Procedure II.

IE. Blood Plasma or Serum Samples

Suggested guidelines for the use of the arrays with blood plasma or serum samples are presented; however, this procedure may require further optimization.

1. Deplete the highly abundant proteins from the sample. This can be done using the ProteoPrep® 20 Plasma Immunodepletion technology (Catalog Numbers PROT20, PROT20S, and PROT20LC) for 20 of the high abundance proteins or by using the ProteoPrep Immunoaffinity Albumin and IgG depletion kit (Catalog Number PROTIA) to deplete the top two most abundant proteins.
2. Concentrate sample to the original volume. For example, if the starting volume of plasma or serum used was 100 µl, reduce the final volume to 100 µl.
3. It is not necessary to determine protein concentration at this time. Protein concentration will only need to be considered during Procedure II. Samples should be compared at equal volumes when analyzed on the slide. Continue to Procedure II.

II. Sample Labeling and Processing

Use of freshly prepared biological samples for protein labeling is highly recommended. Using extracts from frozen tissues or cell lines with low viability, or old protein extracts may give inadequate results.

For successful labeling, the extract should be clear. If small particles are observed, it is recommended to perform a rapid centrifugation just before the labeling procedure.

Excess Cy3/Cy5 dye is eliminated by a rapid and easy method using SigmaSpin columns. Other methods for eliminating the excess dyes, such as PD10 columns (Catalog Number 54805) or dialysis, can be used.

For best results, the dye to protein ratio (D/P ratio) should be >2. If this ratio is not achieved, either perform the labeling step again on the same sample or a new sample should be labeled.

In cases where 1 mg of the protein extract is not obtained, lower amounts can be labeled. However, the ratio of dye to protein in the labeling mix should be kept the same. The Cy3/Cy5 dyes can be dissolved in 50–100 µl of Carbonate-Bicarbonate Buffer, pH 9.5–9.6, and used for the labeling procedure. For example, if 400 µg of nuclear extract is obtained, then 20 µl of Cy3 or Cy5 can be used (assuming the dyes were dissolved in a total volume of 50 µl of buffer).

IIA. Protein Labeling

1. Use 1 ml of extract (1 mg/ml) for labeling with Cy3 or Cy5. Add 1 ml of extract solution to the dye vial. Cap the vial and mix thoroughly. Care should be taken to prevent foaming of the protein solution.
2. Incubate the reaction at room temperature for 30 minutes, mixing the solution every 10 minutes.
3. Remove the free Cy3/Cy5 from the labeled sample by applying on SigmaSpin columns as follows:
 - a. Loosen the cap of the column by half a turn and then snap off the bottom closure.
 - b. Place the column in a microcentrifuge tube and centrifuge for 2 minutes at 750 × g.
 - c. Discard the eluate.
 - d. Place the column in a new collection tube.
 - e. Pipette 150 µl of the labeled protein sample solution directly onto the center of the SigmaSpin column. The remaining labeled extract can be stored at –70 °C.
 - f. Centrifuge for 4 minutes at 750 × g.
 - g. Discard the column and retain the eluate. This is the labeled protein sample. Protect it from prolonged exposure to light.
4. Determine the protein concentration by Bradford protein assay.
5. Store the labeled protein at 2–8 °C. The sample may be frozen in case it is not possible to proceed immediately to the next step.

II.B. Determination of Dye to Protein Molar Ratio (D/P ratio)

1. Measure the Cy3 and Cy5 absorbance at 552 nm and 650 nm, respectively. Read the absorbance of the dyes at their exact absorbance wavelength maxima. Use Buffer A as the blank.
2. Calculate the molar concentration of Cy3 and Cy5 taking into account the following:
 - For a non-homogenous extract that contains a mixture of proteins, the protein molecular mass should be taken as 60 kDa.
 - The µmolar extinction coefficient ($\epsilon^{\mu M}$) of Cy3 and Cy5 are:

$$\text{Cy3: } \epsilon^{\mu M}_{(552 \text{ nm})} = 0.15 \mu\text{M}^{-1} \text{ cm}^{-1}$$

$$\text{Cy5: } \epsilon^{\mu M}_{(650 \text{ nm})} = 0.25 \mu\text{M}^{-1} \text{ cm}^{-1}$$

Calculations:

$$\text{Cy3 concentration } (\mu\text{M}) = A_{552}/0.15$$

$$\text{Cy5 concentration } (\mu\text{M}) = A_{650}/0.25$$

Y (mg/ml) = protein concentration after labeling
(see step IIA-4)

$$\text{Protein } (\mu\text{M}) \text{ concentration} = \\ [Y \text{ (mg/ml)}/60,000] \times 10^6$$

$$\text{D/P} = \frac{\text{Cy3 or Cy5 concentration}}{\text{Protein concentration of sample}}$$

III. Sample Incubation on the Array

Before starting the array assay, make sure the dye to protein molar ratio (D/P) is >2. A lower ratio may work; however, a higher background may be observed.

The incubation time for the assay is short. When the signal is low, it is recommended to prolong the incubation time, but for not longer than 45 minutes.

The control and test samples are labeled with different dyes and are mixed before applying on the array. These samples may have different D/P values. It is recommended to mix equal amounts of protein from the two different samples rather than equal dye concentration. With blood serum or plasma samples, it is recommended to mix equal volumes. The results can then be normalized according to the D/P ratio of each sample (control and test). To verify results, a dye swap should be performed, in which each control and test sample is labeled by both Cy3 and Cy5 and mixed with its counterpart labeled with the other dye. Thus the experiment is fully controlled and doubly tested.

Important: Do not touch the surface of the array. The use of forceps is recommended.

The incubation procedure is performed at room temperature.

1. Mark each slide using a pencil only. A pen or marker may affect the final results by introducing extraneous background fluorescence.
2. Wash each slide briefly by dipping in PBS.

3. In a tube, mix 10–50 μg each of the Cy3 and Cy5 labeled samples at equal protein concentrations (2–10 $\mu\text{g/ml}$ each) with 5 ml of Array Incubation Buffer (Catalog Number A9602). Mix well by inverting the tube. Do not vortex.
Note: For labeled samples with low D/P ratios (<3), use of the higher protein concentration is recommended.
4. Add the mixture to well 1 of the incubation tray (quadriPREM Cell Culture Vessel) supplied in the kit.
5. Immerse the slide into well 1 containing the labeled samples then cover with the lid.
Important: Protect the plate from exposure to light by covering with aluminum foil.
6. Incubate for 30 minutes at room temperature on a rocking shaker at a moderate shaking frequency of ~30 rpm.
7. Add 5 ml of Washing Buffer to wells 2, 3, and 4.
8. Carefully take the slide out of well 1 and place in the Washing Buffer in well 2. Incubate for 5 minutes while shaking on a rocking shaker.
9. Repeat steps 7–8 twice by transferring the slide to well 3 and then to well 4.
10. Decant all the liquid from well 4 and add 5 ml of water.
11. Incubate for 2 minutes.
12. Carefully remove slide from incubation tray and allow to air dry completely for at least 20 minutes (protect from light). Do not use a centrifuge to facilitate drying as this may cause a “comet” effect of the antibodies.
Note: The slide should be absolutely dry before the scanning procedure. Water may cause background problems.

IV. Scanning the Antibody Array

Each sub-array contains a spot with a monoclonal antibody specific for Cy3/Cy5, which serves as a positive control. These points can be used as internal references for positioning the sub-arrays. Furthermore, the array is spotted with buffer at several spots in order to serve as control for non-specific signals. Refer to file “Antibody List, Specificity & Position” on the accompanying small CD or at www.sigma.com/arrays.

The slides should be absolutely dry before the scanning procedure. Water may cause background problems.

Due to the short half-life of the dyes it is recommended that the array be scanned as soon as the experiment is completed and no more than 24–48 hours later.

The arrays can be scanned with most commercially available 'overhead' light source DNA array scanners that accommodate standard microscope slides. Scanners with bottom-lit light sources (e.g., Agilent) cannot be used, as the nitrocellulose coating employed with antibody arrays will interfere with the instrument's signal detection system. The optimal laser power and PMT should be determined for each scanner individually.

Instrument Compatibility:

- Accommodation of a standard microscope glass slide (25 mm × 75.6 mm × 1 mm)
- Light filter system reading within the near-red spectrum of cyanine dyes:

Cy3
excitation 550 nm
emission 570 nm

Cy5
excitation 659 nm
emission 670 nm

Typical Scanner Settings:

The optimal laser power and PMT (Photomultiplier Tube) parameters should be determined for each scanner individually. The following parameters are a recommendation in order to obtain a signal-noise ratio greater than 10:1.

Laser power: 40–80%
PMT: 40–80%

- It is advised to use the lower settings for the initial scan. Depending on the abundance of protein and thus, the corresponding spot signal intensity, the highest settings may accentuate the nitrocellulose background, thereby, "washing-out" any positive bound-protein signals.
- Confocal plane focus should be adjusted for arrays from different batches. Nitrocellulose coating thickness may vary between 7–10 μm.

Use the attached Excel worksheet file found on the small CD for compiling these results.

Note: Occasionally, positive control spots may vary in intensity, resulting in low to high responses.

V. Data Analysis

While the two-color antibody microarray is a relatively new technology, the basic data analysis principles are the same as for DNA microarrays. For both types of arrays, it is highly recommended to perform appropriate control experiments, average observations over as many replicates as possible, and confirm results with an alternative technique. Just as DNA microarray results are routinely confirmed by quantitative RT-PCR, antibody microarray results should be confirmed by immunoblotting.

The mechanical collection of microarray data does not guarantee significant results will be obtained. Appropriate attention must be given to experimental design, data normalization, data visualization, and statistical rules for identifying differentially expressed proteins. The relative merits of various alternative approaches have been treated at length in numerous articles and books, and are beyond the scope of this Technical Bulletin. An excellent review of this important subject has been published.²

Data Normalization:

A brief discussion on the problem of data normalization is presented. An excellent review of this important subject has been published.⁵

Because the two Cy dyes differ in fluorescence intensity and labeling efficiency, fluorescence intensities derived from two-color microarray experiments must be normalized. There are many ways to do this ranging from simple to complex. Here are three of the simplest methods (See review article² for details):

1. Normalization by Reference (housekeeping) Proteins

In many cases, there is reason to believe certain proteins do not change their expression levels for the two different samples in a microarray experiment. The fluorescence intensity obtained for each element in the array is then divided by the fluorescence intensity of the signal obtained for a highly expressed reference protein. Better results may be obtained by normalizing with an appropriate average of several reference proteins. The obvious drawback of this approach is that the reference protein expression level may not be constant.

2. Normalization by Summed Fluorescence Intensities

One can easily derive a normalization factor by separately summing the intensities of the Cy3 and Cy5 channels over all elements of the array and then dividing them (Cy3/Cy5) to obtain the ratio. This approach has a solid theoretical basis for large arrays where the two samples have roughly equivalent numbers of up and down-regulated proteins. However, this assumption may not hold for small arrays.

3. Normalization by Dye Swapping

A popular method for DNA microarrays is to perform one experiment labeling each sample with a different dye and then perform a second experiment with the dyes reversed. The normalized intensity for each element of each sample is calculated as the geometric average of the Cy3 and Cy5 intensities in the two experiments. This method is attractive for antibody microarrays, because it takes into account any label-specific differences in antigen-antibody interactions. However, for big differences in Cy3 and Cy5 fluorescence intensity, the average ratios obtained may not be meaningful.

References

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quadriPERM is a registered trademark of Heraeus Instrument GmbH.

TWEEN is a registered trademark of Croda International PLC

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Weak signal	Poor labeling	<ul style="list-style-type: none"> • Check D/P ratio. • Repeat the labeling procedure. • Increase concentration of labeled protein. • Increase the incubation time up to 45 minutes (Step III-6). • Check laser power and PMT parameters.
High background	Excess of free Cy3/Cy5	<ul style="list-style-type: none"> • Pass labeled sample again through a spin column.
	Excess of labeled protein	<ul style="list-style-type: none"> • Decrease the concentration of labeled protein applied on slide.
	Non-specific binding	<ul style="list-style-type: none"> • Add BSA to the Washing Buffer at 100 µg/ml or add 0.4 M NaCl (final concentration).
No signal from a specific antibody	Low level of protein in sample	<ul style="list-style-type: none"> • Check by Western blotting whether the protein of interest is expressed. • Increase the concentration of the labeled protein extract applied on the slide. • Label the sample again, in order to achieve a higher D/P ratio.
	Recognition of the antigen by the antibody is lost after labeling the protein.	

Appendix

Example of calculations for numerical results obtained from the arrays.

1. Confirm the assay was performed with extract samples having dye to protein molar ratios >2.
2. Scan the Panorama slide with parameters set to optimize the signal-to-noise ratio [PMT, laser power, laser focus, and resolution (no less than 10 μm)].
3. Make sure that the background is low and equally distributed before starting the calculations. Save the files as TIFF files.
4. Use the gal file in order to obtain the position of each spot in the array. Carefully examine each circle and hand localize it exactly in its correct position (the use of the automatic position matching feature in the analysis software is not recommended).
5. Use the Mean minus Background results of the Cy3 channel (Table 1, column B) and the Cy5 channel (Table 1, column C) for the calculations. Do not use the positive control spots in the calculations. They are used only for slide orientation.
6. The housekeeping proteins (actin, tubulin, GAPDH, or others) serve as internal controls for each dye, for example in Table 1, B2-B5 for Cy3 and C2-C5 for Cy5. Average these values and divide the average of Cy5 values for the housekeeping proteins by that of Cy3 housekeeping proteins. In our example the ratio is 2.02 (Table 1, B16).
7. Normalize the numerical values of all Cy3 spots by multiplying them by the ratio obtained in step 6 (Table 1, column D).
8. After the normalization, divide the Cy5 results by the Cy3 results for each individual protein (Table 1, column E).
9. Proteins of interest are those with a Cy5/Cy3 ratio higher than 2 (MDMX, rows 6 and 7) or lower than 0.5 (cJun, rows 11 and 12).

Table 1.
Sample Calculations

	A	B	C	D	E	
1	Name	Mean F550 - Background	Mean F650- Background	Mean F550 Normalized	F650/F550	
2	Actin	2000	4400	4020	0.91	
3	Actin	2200	4000	4422	1.11	
4	β Actin	3000	6000	6030	1.01	
5	β Actin	3000	6200	6030	0.97	
6	MDMX	4000	2000	8040	4.02	up regulated
7	MDMX	4150	1850	8341.5	4.51	up regulated
8	P53	6000	7000	12060	1.72	
9	P53	6000	7150	12060	1.69	
10	cJun	1000	8000	2010	0.25	down regulated
11	cJun	1150	8500	2311.5	0.27	down regulated
	Actin Average	2550	5150			
	F650 /F550	2.02				

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