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Product Information

Panorama® Antibody Array – Neurobiology

Catalog Number **NBAA5**
Storage Temperature 2–8 °C

TECHNICAL BULLETIN

Product Description

The Panorama® Antibody Array – Neurobiology kit is designed for studying protein expression in cell or tissue extracts. The ability to identify multiple proteins simultaneously allows global molecular characterization of biological samples with applications in basic biological research as well as in disease diagnosis and treatment.[1] Specifically, the neuroproteome can be profiled using chip-based technologies and genetic-based methods to obtain large-scale protein-protein interaction data.[2] 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. A poor correlation between mRNA and protein expression is often noticed,[3] thus a method that assays proteins directly should be utilized for analyzing biologically-relevant events. Antibody arrays provide an effective solution to rapidly profile expression of multiple proteins in samples. Examples where Panorama Antibody arrays have been used for various applications include the differentiation of F9 cells,[4] breast cancer and lung cancer samples,[5,6] human embryonic stem cell,[7,8] differential protein expression in colorectal and gastric cancer.[9,10]
(for a complete list go to:
http://www.sigmaaldrich.com/life-science/cell-biology/protein-arrays/protein-arrays-applications/literature-references.htmL)

The array contains 224 different antibodies each spotted in duplicate on nitrocellulose coated glass slides. These antibodies represent families of proteins and biological pathways known to be involved in neurobiology in health and disease.

The expression of a protein in an extract is detected when it binds to its corresponding antibody. 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 spotted antibody has been tested for its ability to bind proteins using several 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 CyDye™ (Cy™3 or Cy5) and the two samples are applied simultaneously on the array. An expression profile of each sample (Cy3 and Cy5) is recorded individually and compared. Alternatively, all samples are labeled with the same color (e.g., Cy3), and each sample is applied on a different slide. The advantage of the first approach is that the same antibody evaluates each protein in different samples, while the advantage of the second approach is that the same color is used and thus differences in the intensity and background of the different colors are eliminated.

Important advantages of the Panorama Antibody Array – Neurobiology kit include:

- The profiling assay is fast and can be completed in less than 2 hours.
- Antibodies are spotted in high density to ensure strong signals.
- Proprietary treatment of the slides ensures low background staining.

Important general aspects of the Panorama Antibody Array - Neurobiology kit:

- Panorama Antibody Array - Neurobiology kit is not species specific. The antibodies spotted recognize mainly human, mouse and rat proteins. Information on species specificity of each antibody can be found in the file “Antibody List” on the accompanying disc-on-key. More information on each antibody can be obtained in the antibody specific datasheet found on the Sigma-Aldrich web site (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Antibody_Explorer.htmL). All antibodies spotted can be purchased individually from Sigma-Aldrich.

- Antibody affinity to its target varies significantly between antibodies. The fluorescence 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.

- The 224 antibodies are spotted in 32 sub-arrays each containing duplicate spots of the antibodies, as well as a single positive control spot for Cy3 and Cy5 (a monoclonal antibody that recognizes Cy3 and Cy5), and a single negative control. Information on specific positioning of each antibody can be found in the file "Antibody Position" on the accompanying USB or at sigma.com/arrays.

- To obtain good results, it is recommended freshly prepared extracts be labeled, and that only labeled preparations with high dye/protein ratio (>2) be used.

- Results obtained using the array are semi-quantitative and should be further evaluated by other methods such as immunoblotting assays or ELISA.

- The slides are sensitive, do not touch the surface of the slides and handle all your buffers with latex free gloves.

- The slides are for a single use only.

Components
Sufficient material is provided for performing 2 array reactions. Some of the buffers are provided in excess.

Panorama Antibody Slides–Neurobiology Catalog Number S0452	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 II 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, Polyproplyene, 2 mL Catalog Number T 7813	16 each
Panorama Antibody List – Neurobiology Catalog Number L8420	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 (Amersham Biosciences PA23001)
- Cy5 Monofunctional Reactive dye (Amersham Biosciences PA25001)
- CelLytic™ NuCLEAR™ Extraction Kit (Catalog Number NXTRACT) (optional)
- Carbonate-Bicarbonate buffer (Catalog Number C3041).
- Microarray Scanner

Precautions and Disclaimer
This product 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 at 50 units/μL. For immediate use, prepare a working solution of 5 units/μL in Extraction/Labeling Buffer (Catalog Number E0655) by adding 2 μL 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 μL of the reconstituted Protease Inhibitor Cocktail, 100 μL of the Phosphatase Inhibitor Cocktail II (Catalog Number P5726), and 1.2 μL 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 μm filter.

Storage/Stability
The kit should be stored at 2–8 °C. All components can be stored at 2–8 °C; however, it is recommended that upon arrival immediately transfer the Benzoase (Catalog Number B8309) and Protease Inhibitor Cocktail vial (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.

When using a different method of sample preparation, it is recommended to dialyze the sample against Carbonate-Bicarbonate Buffer, pH 9.5–9.6 (Catalog Number C3041), before the labeling procedure (Step II).

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. This 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, and to eliminate small particles by a rapid centrifugation just before the labeling procedure. An additional amount (0.6 units/mL) of Benzonase can be added in cases where the extract is still viscous.

Cell and tissue extracts can be prepared using any suitable procedure. However, it is important the final concentration of the sample be high (5–10 mg/mL) so that 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% confluence. (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.

From Non-adherent Cells:
1. Grow cells in culture. Collect 10^7 cells into a test tube. Centrifuge the cells at 300 × *g* for 5 minutes.
2. Wash the cells twice with cold 0.01 M PBS, pH 7.4, and collect by centrifugation at 300 × *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 9,500 × *g* (in a microcentrifuge). Transfer the supernatant to a new microcentrifuge tube.
5. Determine the protein concentration in the supernatant by the Bradford method.
6. Dilute the sample to 1 mg/mL of protein in Buffer A.
7. Use 1 mL of extract (1 mg/mL) for labeling with Cy3 or Cy5 (Procedure II).

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 tissue into 2.5 mL).
4. Homogenize the tissue on ice using a homogenizer.
5. Centrifuge the sample for 10 seconds at 9,500 × *g* in a microcentrifuge.
6. Transfer the supernatant into a clean tube and determine the protein concentration by the Bradford method.
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 (Procedure III).

IC. Nuclear Protein Extraction Without the Use of a Detergent.

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

The CelLytic NuCLEAR Extraction Kit (Catalog Number NXTRACT) may be used for preparation of nuclear protein. 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 µL 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 µL 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 sterile 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 $CaCl_2$, 0.3 M Sucrose.
3. To 1,400 µL of Lysis Buffer (hypotonic or isotonic), add 14 µL of the 0.1 M DTT solution and 14 µL 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% confluency.
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 and collect into an appropriate conical centrifuge tube.
5. Centrifuge for 5 minutes at 450 × *g*.
6. Decant and discard the supernatant.
7. Estimate the packed cell volume (PCV).
8. Proceed to step 7 of the procedure for Non-adherent cells.

From Non-adherent Cells

1. Collect the cells into an appropriate centrifuge conical tube.
2. Centrifuge for 5 minutes at 450 × *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 × *g*.
5. Decant and discard supernatant.
6. Estimate the packed cell volume (PCV).
7. Add 1 mL (5× PCV) of Lysis Buffer (including DTT and Protease Inhibitor Cocktail) to 200 µL 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 × *g*. Decant supernatant and resuspend the pellet of packed cells in 400 µL (2× PCV) of the Lysis Buffer.

11. 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.
12. Centrifuge the disrupted cells in suspension for 20 minutes at 10,000–11,000 × *g*.
13. Transfer the supernatant to a fresh tube. This fraction is the cytoplasmic fraction.
14. Add 1.5 µL of the 0.1 M DTT solution and 1.5 µL of the Protease Inhibitor Cocktail to 147µl of the Extraction Buffer.
15. Resuspend the crude nuclei pellet in ~140 µL (2/3× PCV) of Extraction Buffer containing DTT and Protease Inhibitor Cocktail. If the procedure is being performed with a tissue homogenizer, it is recommended to give 10 more strokes at this point.
16. Shake gently for 30 minutes.
17. Centrifuge for 5 minutes at 20,000–21,000 × *g*.
18. Transfer the supernatant to a clean, chilled tube.
19. Continue with dialysis or store at –70 °C.

IC-b Extraction from tissue

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

1. Prepare a fresh solution of 0.1 M DTT with ultrapure sterile water.
2. 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.
3. Prepare Extraction Buffer as described in Section IC-a, step 4. Add 1.5 µL of the prepared 0.1 M DTT solution and 1.5 µL of the Protease Inhibitor Cocktail to 147 µL of the Extraction Buffer.
4. Rinse the tissue twice with PBS buffer. Discard the PBS.
5. Resuspend the tissue gently in 1 mL (5× PCV) of the Lysis Buffer containing DTT and Protease Inhibitor Cocktail.
6. Homogenize the tissue (using the tissue homogenizer) until more than 90% of the cells are broken and nuclei are visualized under the microscope.
7. Centrifuge the disrupted cells for 20 minutes at 10,000–11,000 × *g*.
8. Transfer the supernatant to a fresh tube. This fraction is the cytoplasmic fraction.
9. Resuspend the crude nuclei pellet in ~140 µL (2/3× PCV) of Extraction Buffer containing DTT and Protease Inhibitor Cocktail. At this stage a short homogenization can be performed to facilitate nuclear extraction.
10. Shake gently for 30 minutes.
11. Centrifuge for 5 minutes at 20,000–21,000 × *g*.
12. Transfer the supernatant to a clean, chilled tube.
13. Continue with dialysis or store at –70 °C.

ID. Dialysis

Before performing the labeling procedure (Procedure II) it is important to bring the extract to the required pH by dialysis.

1. Prepare 0.1 M, pH 9.5–9.6, Carbonate-Bicarbonate Buffer (dissolve 2 capsules of Catalog Number C3041 into 100 mL water).
2. Dialyze at 4 °C for 2 hours in a dialysis buffer volume 1000× the volume of the nuclear protein extract.
3. Replace the dialysis buffer with freshly prepared carbonate buffer and dialyze for an additional 2 hours at 4 °C.
4. Determined protein concentration according to the Bradford method. Continue to Step II.

II. Sample Labeling and Processing

Use of freshly prepared extracts for protein labeling is highly recommended. Use of 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 of Cy3/Cy5 dye is eliminated by a rapid and easy method using SigmaSpin columns (Catalog Number S0185). Other methods for eliminating the excess of dyes, such as PD10 columns or dialysis can be used.

The dye to protein ratio (D/P ratio) should be >2. **If this ratio is not achieved, a new sample should be labeled.**

In cases where 1 mg of nuclear 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 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 (Catalog Number S0185) 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 method.
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.

IIB. 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 wavelengths 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 weight should be taken as 60 kDa.
   - The µmolar extinction coefficients (ε^{mM}) of Cy3 and Cy5 are:

     Cy3: $\varepsilon^{mM}_{(552\ nm)} = 0.15\ \mu M^{-1}cm^{-1}$
     Cy5: $\varepsilon^{mM}_{(650\ nm)} = 0.25\ \mu M^{-1}cm^{-1}$

     Calculations:

     Cy3 concentration (µM) = $A_{552}/0.15$

     Cy5 concentration (µM) = $A_{650}/0.25$

     Y (mg/mL) = protein concentration after labeling (see Step IIA-4)

     Protein (µM) concentration = $[Y\ (mg/mL)/60{,}000\ ] \times 10^6$

     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, it may cause background problems.

The incubation time for the assay is short. When the signal is low, it is recommended to prolong the incubation time, but 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. The results can then be normalized according to the D/P ratio of each sample (control and test). To verify results, each control and test sample should be 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.

Note: **Absolutely** do not touch the surface of the array during the whole procedure. Forceps are recommended.

The incubation procedure is performed at room temperature.

1. Mark each slide using only a pencil. A pen or a marker may affect the final results.
2. Wash each slide briefly by dipping in PBS.
3. In a tube add the Cy3 and Cy5 labeled samples at equal protein concentrations (~20 µg/mL each) to 5 mL of Array Incubation Buffer (Catalog Number A9602). Mix well by inverting the tube. Do not vortex.
4. Add the mixture to well 1 of the incubation tray (quadriPREM Cell Culture Vessel) supplied in the kit.
5. Dip the slide into well 1 that already contains the labeled samples. Cover with the lid. Protect the plate from exposure to light by covering with aluminum foil.
6. Incubate for 35–45 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 dip it 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 the slide from the incubation tray and allow to air dry completely for at least 20 minutes (protected 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 two spots with a monoclonal antibody specific for Cy3/Cy5, which serve as positive controls. 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 controls for non-specific signals. Refer to file "Antibody List, Specificity & Position" on the accompanying USB memory stick 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 (**P**hoto**m**ultiplier **T**ube) 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 USB memory stick 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 desirable 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 that 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.[3]

Data Normalization:
A brief discussion on the problem of data normalization is presented. An excellent review of this important subject has been published.[7]

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 article[3] 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 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 intensity 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.

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Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Weak signal	Poor labeling	• 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	• Pass labeled sample again through a spin column.
	Excess of labeled protein	• Decrease the concentration of labeled protein applied on slide.
	Non-specific binding	• 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	• 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 mm)].

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 localizes 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 (GAPDH, or others) serve as internal controls for each dye, for example in Table 1, B2-B3 for Cy3 and C2-C3 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 2.0 (Table 1, B15).

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 (Neurofilament 200, rows 6 and 7) or lower than 0.5 (DVL, rows 10 and 11).

Table 1.
Example Of Sample Calculations

	A	B	C	D	E	
1	**Name**	**Mean F550 -Background**	**Mean F650- Background**	**Mean F550 Normalized**	**F650/F550**	
2	GAPDH	2,000	4,400	4,000	0.90	
3	GAPDH	2,200	4,000	4,400	1.10	
4	LRP1	3,000	6,000	6,000	1.00	
5	LRP1	3,000	6,200	6,000	0.96	
6	Neurofilament 200	4,000	2,000	8,000	4.00	up regulated
7	Neurofilament 200	4,150	1,850	8,300	4.49	up regulated
8	β-Amyloid	6,000	7,000	12,000	1.72	
9	β-Amyloid	6,000	7,150	12,000	1.68	
10	DVL	1,000	8,000	2,000	0.25	down regulated
11	DVL	1,150	8,500	2,300	0.27	down regulated
	GAPDH Average	2,100	4,200			
	F650 /F550	2.0				