

## Product Information

### Panorama<sup>®</sup> Antibody Microarray - Cell Signaling Kit

Catalog Number **CSAA1**  
Storage Temperature 2–8 °C

## TECHNICAL BULLETIN

### Product Description

The Panorama<sup>®</sup> Antibody Microarray - Cell Signaling 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 fundamental cell biology research as well as for disease diagnosis and treatment. 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<sup>1</sup> so a method that can assay proteins directly is required for analyzing biologically-relevant events. Antibody arrays provide an effective solution to rapidly profile the expression of multiple target proteins in samples. Examples where Panorama Antibody Microarrays have been used for such applications include the differentiation of F9 cells<sup>2</sup> and breast cancer samples,<sup>3</sup> and for monitoring human embryonic stem cell pluripotency and viability.<sup>4</sup>

The Cell Signaling array contains 224 different antibodies each spotted in duplicate on nitrocellulose-coated glass slides. These antibodies represent biological pathways including apoptosis, cell cycle, neurobiology, cytoskeleton, signal transduction, and nuclear proteins.

The expression of a protein in a cell extract is detected when it binds to its corresponding antibody on the array. This binding is visualized by a sensitive fluorescent signal created by directly labeling the proteins in the cell extracts with a fluorescent dye. Each antibody used on the array 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 Cy<sup>™</sup> dye (Cy3 or Cy5) and the two samples are applied simultaneously at equal protein concentrations onto 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 - Cell Signaling Kit include:

- The profiling assay is fast and is completed in less than 2 hours.
- The antibodies are spotted in high density to ensure strong signals.
- Proprietary treatment of the slide coating minimizes background staining.

Important general aspects of the Panorama Antibody Microarray - Cell Signaling Kit:

- The Panorama Antibody Microarray-Cell Signaling 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, Specificity, & Position" on the accompanying diskette. More information on each antibody can be obtained in the antibody specific datasheet found on the Sigma-Aldrich Web site at [sigma.com/antibody](http://sigma.com/antibody). 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 only be performed on corresponding antibody/antigen systems and not between different antibodies.

- Each slide contains 224 antibodies spotted in 32 sub-arrays each containing duplicate spots of 7 antibodies, as well as a single positive control spot for Cy3 and Cy5, and a single negative control. Information regarding specific positioning of each antibody can be found in the file “Antibody List, Specificity, & Position” on the accompanying diskette or at [sigma.com/arrays](http://sigma.com/arrays).
- For best results, it is recommended that freshly prepared extracts be labeled and that only labeled preparations with high dye/protein ratio (>2) be used.
- The results obtained using the array are semi-quantitative 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 – Cell Signaling Catalog Number A9852	2 each
quadriPERM <sup>®</sup> 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	300 µl
Benzonase <sup>®</sup> , ultrapure Catalog Number B8309	1,000 units
Array Incubation Buffer Catalog Number A9602	20 ml
Phosphate Buffered Saline, pH 7.4, with TWEEN <sup>®</sup> 20 (Washing Buffer) Catalog Number P3563	1 each

SigmaSpin<sup>™</sup> Post-Reaction Clean-Up Columns 8 each  
Catalog Number S0185

Collection Tubes, Polypropylene, 2 ml 16 each  
Catalog Number T7813

Panorama Antibody List – Cell Signaling 1 each  
Catalog Number A9727

### 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)
- Microarray Scanner

### Precautions and Disclaimer

The 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 double-distilled water to the vial (Catalog Number P4495). Store the reconstituted solution at –20 °C.

Benzonase Stock Solution – Benzonase, ultrapure (Catalog Number B8309) is supplied as a 50 units/µl solution. For immediate use, prepare a stock solution of 5 units/µl in Extraction/Labeling Buffer (Catalog Number E0655) by adding 2 µl of Benzonase solution to 18 µl of Extraction/Labeling Buffer. Upon dilution store the stock 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 Phosphatase Inhibitor Cocktail II, (Catalog Number P 5726), and 1.2 µl of the diluted Benzonase Stock 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  $\mu$ m filter.

### Storage/Stability

The kit ships on wet ice and should be stored at 2–8 °C. All components can be stored at 2–8 °C for 12 months without loss of activity; however, it is recommended that upon arrival, immediately transfer the Benzonase (Catalog Number B 8309) and Protease Inhibitor Cocktail (Catalog Number P4495) to –20 °C storage.

### Procedure

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

#### 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 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 solution is still viscous.

Cell and tissue extracts can be prepared using any suitable protocol. However, it is important that the final concentration of the sample be high (around 10 mg/ml) so that it could 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 test tube. Spin cells at  $1,500 \times g$  for 5 minutes.
2. Wash the cells twice with cold 0.01 M PBS, pH 7.4, by spinning.
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 method.
6. Dilute the extract 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 protocol 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 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 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.

## II. Sample Labeling and Processing

Use of freshly prepared biological samples for protein labeling is highly recommended. Using extracts from frozen tissues, old protein extracts, or cell lines with low viability 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 removed using SigmaSpin columns (supplied with kit). Other methods for eliminating the excess of dyes, such as PD-10 columns (Catalog Number 54805) or dialysis, may also be used.

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

### IIA. Protein Labeling

1. Use 1 ml of extract (1 mg/ml) for labeling with Cy3 or Cy5. Add the extract solution (1 ml) 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 dye from the labeled sample by applying on a SigmaSpin column as follows:
  - a. Loosen the cap 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 x 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 rest of the labeled protein should be kept in case the column procedure has failed).
  - f. Centrifuge for 4 minutes at 750 x 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 the 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. Use Buffer A as the blank.
2. Calculate the molar concentration of Cy3 and Cy5 taking into account the following:
  - For a non-homogenous sample that contains a mixture of proteins (cell and tissue extracts), the protein molecular weight should be taken as 60 kDa.
  - The µmolar extinction coefficient ( $\epsilon$ ) of Cy3 and Cy5 are:

$$\text{Cy3: } \epsilon_{552} = 0.15 \mu\text{M}^{-1} \text{ cm}^{-1}$$

$$\text{Cy5: } \epsilon_{650} = 0.25 \mu\text{M}^{-1} \text{ cm}^{-1}$$

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 that 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; however, 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. 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.

**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 a marker may affect the final results by introducing extraneous background fluorescence.
2. Wash each slide briefly by dipping in PBS.
3. In a tube add the Cy3 and Cy5 labeled samples at equal protein concentrations (1-10  $\mu\text{g/ml}$  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 (quadriPERM Cell Culture Vessel, Catalog Number Q3756) supplied with 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  $\sim 30$  rpm.
7. Add 5 ml of Washing Buffer to wells 2, 3, and 4.
8. Carefully remove the slide from well 1 and place in the Washing Buffer contained 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 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 microfuge to facilitate drying as this may cause a "comet" effect of the antibodies.

### IV. Scanning the Antibody Array

Scanning can be performed using any microarray scanner. The optimal laser power and PMT should be determined for each one individually.

Each sub-array contains a spot with a mixture of Cy3 and Cy5 labeled BSA. These points can be used as internal references for positioning of the sub-arrays. Furthermore, non-labeled BSA is spotted in each sub-array in order to serve as a control for non-specific signals. Refer to file "Antibody List, Specificity, & Position" on the diskette accompanying the kit or at [www.sigma.com/arrays](http://www.sigma.com/arrays).

The slide 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.

1. Scan the slides using an appropriate scanner and record the numerical value of the signal obtained. Use the attached Worksheet Excel file found on the diskette for compiling the results.

## 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 array 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, so 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. Here we briefly discuss the problem of data normalization. An excellent review of this important subject has been published.<sup>2</sup>

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 3 of the simplest methods (see review article<sup>2</sup> for details):

### 1. Normalization by Reference (Housekeeping) Proteins

In many cases, there is reason to believe that 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 taking 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 so obtained may not be meaningful.

## Troubleshooting Guide

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

## References

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