



AbSurance™Pro **Histone Peptide Microarray**

Catalog No. 16-671

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Not for use in diagnostic procedures

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Introduction

In eukaryotic cells, genetic information is organized into chromatin that is composed of repeating units called nucleosomes. Each nucleosome consists of an octamer of histone proteins. These core histone proteins, H2A, H2B, H3, H4 and their variants, are post-translationally modified at specific residues by nuclear enzymes. The placement of these chemical marks (acetylation, phosphorylation, methylation and others) at specific locations in a given histone protein create an elaborate code that can be “read” in a position and context specific manner. These modifications therefore reflect what is known as the “histone code”, which can be used to infer “chromatin states” (1, 2). The histone code influences cellular state and can be correlated with gene activation or repression, DNA damage, cell cycle status, etc.

Within chromatin, the accessible regions of histones represent substrates for chromatin writer and eraser enzymes, as well as recognition domains for chromatin reader proteins that facilitate higher order structure important in chromatin, gene and nuclear regulation. An understanding of how these various proteins interact with and modify the histone code to regulate gene expression is useful for understanding not only understanding how gene expression is regulated, but also how it can be biochemically and chemically modulated.

Product Overview

The AbSurance™ Pro Histone Peptide Microarray is a microarray containing peptides corresponding to sequences of all four core histone subunits (H2A, H2B, H3, H4) plus select variants). Over 260 histone peptides representing various post-translational modifications are present on the array as unmodified, singly modified as well as multiply modified sequences with up to six modifications per peptide. In addition, multiple peptides representing common protein epitope tags are also printed for use as controls for anti-epitope tag detection scenarios. With over 270 different peptide sequences, AbSurance Pro Microarrays are ideal for protein interaction analysis or histone antibody specificity screening using scanning densitometry or chemiluminescent detection (see figure 1).

The AbSurance Pro Histone Peptide Microarray is printed as two subarrays (see figure 2) with each peptide represented six times per subarray to ensure confident binding analysis and quantitation. This configuration enables analysis of up to two binding experiments per slide when using the provided two chamber adhesive gasket (see figure 3). Using this convenient format, side by side analysis of chromatin readers, writers, and erasers in the absence and presence of small molecules or other protein complex members can be assayed to elucidate substrate specificity and binding characteristics (see Figure 1). Alternatively, this array can be used for comprehensive analysis of histone antibody specificity. For a macroarray analysis of histone antibody specificity using single modification sites and a simple western blot like detection approach, the PVDF-FL membrane—based AbSurance™ Complete macroarray (cat# 16-668) is recommended.

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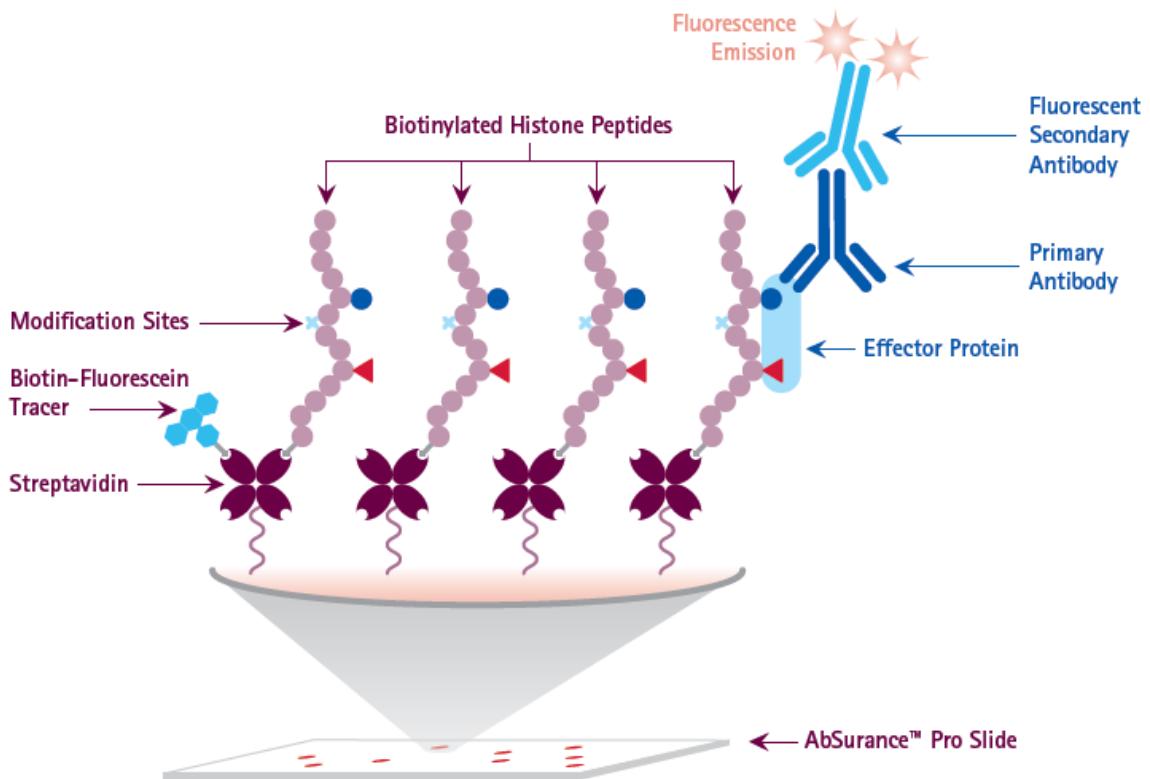


Figure 1. AbSurance Pro Histone Peptide Microarray slide design

Interaction of an effector protein with peptides on the array is detected by a primary antibody to the protein (or to an affinity tag) and a fluorescently-labeled secondary antibody directed against the primary antibody. Alternatively, to assess specificity of a histone antibody, you need a primary antibody to a histone modification and a fluorescently-labeled secondary antibody recognizing the primary antibody. The entire procedure is similar to the one employed in immunofluorescence microscopy.

Peptide Array Layout and Features

Each AbSurance Pro Histone Peptide Microarray contains 270 different biotinylated peptides (approximately 20 amino acids in length) immobilized on a streptavidin-coated glass microscope slide. To ensure consistent printing and to allow normalization of interaction signals, all AbSurance Pro peptides are produced and spotted with a tracer dye (see figure 1). Details about these peptides, including their sequences can be found in the spreadsheet “AbSurance Pro Peptides.xlsx”. This file and other templates that facilitate data analysis can be downloaded from the AbSurance™ Pro product page on our company website.

Peptides are spotted on two identical sub-arrays (labeled A and B on Figure 2). Each peptide is spotted twice in groups of 3 within each sub-array (red dots on Figure 2). Thus each peptide is featured 12 times in defined positions across a single array slide that carries over 4,000 features. This format also mitigates spotting variability, local binding differences across the slide surface, and allows the user to statistically evaluate the quality of individual peptide: protein interactions.

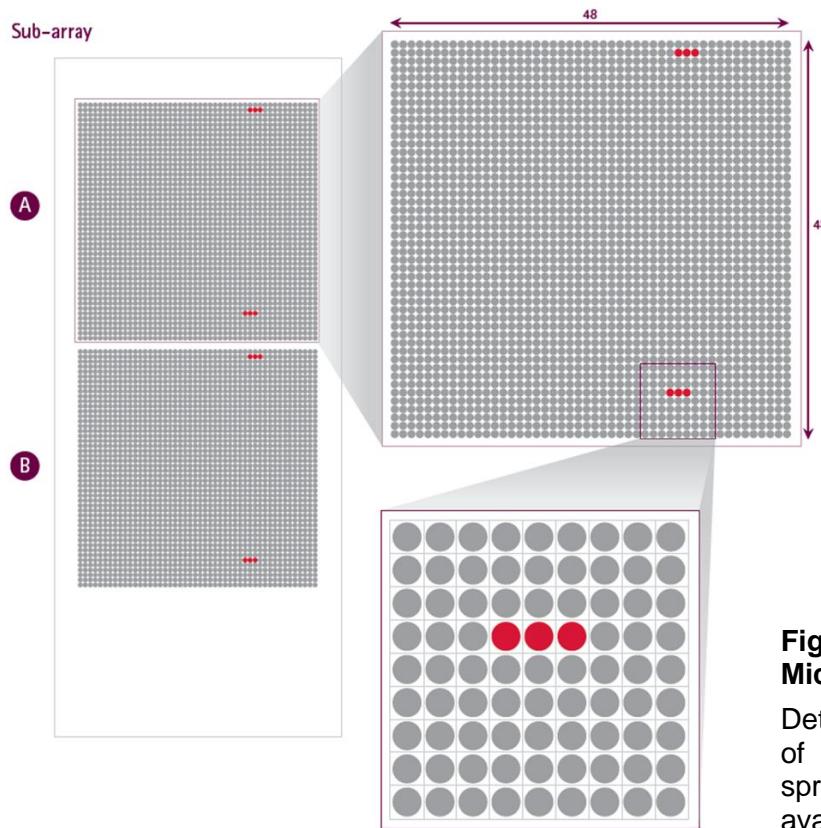


Figure 2. AbSurance Pro Histone Peptide Microarray glass slide design.

Detailed information about the exact position of each peptide can be found in the Excel spreadsheet “AbSurance Pro Peptides.xlsx” available on our website.

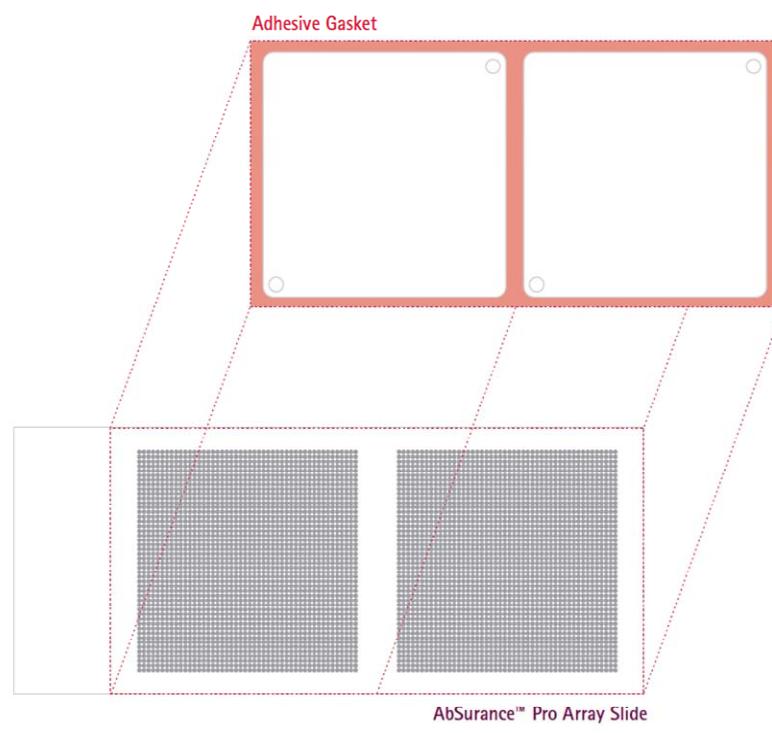


Figure 3. Gasket Placement on AbSurance Pro Histone Peptide Microarray

Placement of the gasket on a glass slide is shown to scale relative to spotted peptides. See Step 2 of the Protocol for instructions on how to apply the chamber gasket. However, if the entire array is to be used for a single hybridization then cover it with a 24 x 60 mm No. 2 glass coverslip. Place the coverslip to the far right edge of the array, relative to the logo on the slide.

AbSurance Pro Histone Peptide Microarray Features and Advantages

- Peptide layout and the provided adhesive gasket accommodates two separate experiments at a time to enable testing of multiple targets, positive control samples, and side-by-side comparisons.
- Arrays spotted with multiple epitope tag and IgG controls to facilitate array orientation and simplify data analysis.
- Guaranteed full length peptides –all peptides purified by HPLC and analyzed by mass spectrometry prior to spotting.
- Each peptide spotted 6 times on each of two subarrays allowing generation of statistically relevant data.
- Fluorescent spotting tracer controls for printing efficiency, enables normalization and eliminates false negative results.
- Readily visualize and collect data using fluorescence scanning or standard ECL techniques.

Materials Provided

1. AbSurance Pro Histone Peptide Microarray (Part #CS210586). Store at 4°C in the dark.
2. Adhesive gasket and sheet of 20 adhesive sealing tabs (Part #CR208893 and CR208894). Store at room temperature.

Materials Required But Not Supplied

Reagents

- Cold, 1x phosphate-buffered saline (PBS) filtered
- Powdered bovine serum albumin (BSA)
- Tween®-20
- Far-red fluorescently-labeled secondary antibody, such as AlexaFluor® 647, Cy5™ or DyLight™ 650
- X-ray film and high sensitivity ECL detection reagents

Equipment

- Microarray scanner capable of scanning at least 25 µm resolution. For details, see section III “Guidelines for Fluorescent Scanning Detection”
- Microscope slide dish, Coplin jar or glass array tube for washing slides
- If only a single hybridization is performed per array: 24x60mm glass coverslip

Warnings and Precautions

- When applying the gasket avoid touching the surface of the slide by handling the glass side from the edges with fingers placed by engraved end of the slide.
- When possible, wear nitrile gloves when handling slides. Latex gloves should be avoided. The powder present in latex gloves (even those sold as powder-free) will contaminate the surface of the slide resulting in autofluorescence.
- Avoid touching the surface of the slide with pipette tips when dispensing liquids, as this can dislodge peptides from the surface of the slide.
- Particle-free buffers should be used for experiments with AbSurance Pro Histone Peptide Microarrays. To eliminate particles, pass all buffer solutions through a 0.2 µm filter.

Storage and Stability

Store AbSurance Pro Histone Peptide Microarrays in dry environment at 4°C and protect from light and dust. Performance is guaranteed for 3 months from date of receipt when reagents are stored properly and handled as indicated.

Important Notes before Starting

- Read the entire protocol before starting your experiments with AbSurance Pro Microarrays.
- The top surface of each slide is marked with either a “+” or the glass manufacturer logo). The top surface of the glass slide should always be facing up.
- Make sure that the glass slide is dry when attaching the adhesive gasket
- Spotting tracer and fluorescent antibodies are light sensitive. Protect slides from light and perform experiments under reduced light if possible.
- To avoid compromising experimental data, do not allow slides to dry out during processing.
- All incubation and washing steps should be performed in the dark at 4°C and can be facilitated by gentle rotation

AbSurance Pro Histone Peptide Microarray Protocols

PLEASE READ: The Histone Interacting Protein and Histone Antibody Specificity Screening protocols below describe procedures for two independent hybridizations carried out on a single array slide with the adhesive gasket attached. If you are planning to perform only a single hybridization on the entire array, use a 24x60 mm glass coverslip, and place it to the far right edge of the array, relative to the logo on the slide. It is important to maintain humidity during incubations. If not using the adhesive gasket, place the glass slide on damp Whatman® filter paper inside a chamber with a tight fitting lid to prevent slide from drying out.

I. Hybridization Procedure –Interacting Protein

IMPORTANT: If performing antibody specificity screening please go to Section II “Hybridization Procedure –Histone Antibody Specificity Screening.”

This procedure has been optimized based on extensive testing of domains ranging from 25-60 kDa. Hybridization concentration for proteins outside of this size range may require further optimization. This procedure was optimized for the detection of GST- and His-tagged proteins. Detection of FLAG-tagged proteins is not advised with the M2 monoclonal. A polyclonal antibody with higher avidity is recommended. See Related Products section for additional suggested reagents.

1. Prepare Hybridization Buffer (1x PBS, 5% BSA (w/v), 0.1% Tween-20), and pass the solutions through a 0.2 µm filter.
2. Place an array slide on a clean surface with the slide logo facing up. If using the adhesive gasket, remove the backing from the gasket with forceps. Carefully align the edges of the gasket and glass slide, so both subarrays are separated by the chambers as shown in Figure 3. Please note that the two subarrays may not be visible. Careful placement of the gasket to the far right edge of the array, relative to the logo on the slide will ensure that both arrays are accessible to your solutions. To ensure a tight seal, press

the edges of the gasket firmly against the glass making sure that the gasket adheres along the entire perimeter of the slide.

Note: Perform the following steps for each chamber independently.

3. Use a pipette with 1 mL tip pressed directly against one of the access ports to fill the chamber with 400 μ L of Hybridization Buffer. Maintain a slow and steady flow while filling the chamber to help avoid air bubbles. Seal both access ports by covering with the provided adhesive sealing tabs. Incubate the array with hybridization buffer for 30 minutes at 4°C in the dark to equilibrate the array.
4. Prepare 400 μ L of a 2 μ M solution of your protein of interest in Hybridization Buffer.
5. Remove the sealing tabs from both access ports. Use a pipette with 1 mL tip to remove liquid from hybridization chamber (holding the glass slide at an angle helps to collect the liquid at one corner where it can be removed through the access port). Carefully fill the chamber with 400 μ L of the protein solution prepared in Step 4. Avoid introducing air bubbles by maintaining a slow and steady flow. Seal ports with adhesive sealing tabs.
6. Incubate at 4°C overnight in the dark.
7. Empty the chambers as described in Step 5. Perform five sequential washes by filling the chambers with ice cold 1x PBS and emptying chambers as described in step 5. Incubating the chamber filled with 1x PBS for 5 min at 4°C at each wash may improve the stringency of the washes.
8. The bound protein is detected by a series of antibody incubation steps. Prepare primary antibody dilution (specific to protein affinity tag or to the protein itself) in Hybridization Buffer. Transfer solution into the chamber as previously described, seal the ports and incubate for several hours at 4°C in the dark. The incubation time will vary. Although two hours is generally sufficient, this step may need optimization depending on antibody chosen and protein used. GST-fusion proteins are typically readily detected and a GST positive control peptides are spotted on the array. The use of GST fusion protein as binding partners is recommended when possible. Several other commonly used protein tags (FLAG, His, Myc and V5) are also printed on the array as positive controls for their respective epitopes. See Related Products section for suggested reagents and usage.
9. Wash each chamber 3-5 times as described in Step 7.

Note: To detect antibody binding using the ECL method, go to Data Analysis section “ECL Detection Procedure”.

10. For each chamber prepare 400 μ L of a fluorescent secondary antibody (1:2,000 to 1:5,000 dilution) in Hybridization Buffer. Pipette in the chamber, seal the ports and incubate for 30 minutes in the dark at 4°C.
11. Wash each chamber 3-5 times as described in Step 7.
12. Remove the gasket using forceps and wash the entire array by submerging it in 1x PBS for 5 min at 4°C with rotation (using a Coplin jar or glass array tube). Repeat.
13. To remove excess salt, dip the array several times in 0.1x PBS.

14. Dry the slide using either **filtered** forced air or centrifugation in a 50 mL conical tube at 800 x g for 2 minutes.
15. Proceed to the detection step (Section III). Fluorescent antibody signals may remain for 24 hours or longer. However, it is recommended to scan the array slide within a few hours of incubation for best results.

II. Hybridization Procedure – Histone Antibody Specificity Screening

IMPORTANT: This section is for screening specificity of a histone antibody. If performing a protein interaction assay please go to Section I “Hybridization Procedure –Interacting Protein”

1. Prepare Hybridization Buffer (1x PBS, 5% BSA (w/v), 0.1% Tween-20), and pass the solutions through a 0.2 μ m filter.
2. Place an array slide on a clean surface with the slide logo facing up. If using the adhesive gasket, remove the backing from the gasket with forceps. Carefully align the edges of the gasket and glass slide, so both subarrays are separated by the chambers as shown in Figure 3. The two subarrays may not be visible. However, careful placement of the gasket to the far right edge of the array, relative to the logo on the slide will ensure that both arrays are accessible to your solutions. To ensure a tight seal, press the edges of the gasket firmly against the glass making sure that the gasket adheres along the entire perimeter of the slide.

Note: The steps below should be performed for each chamber independently.

3. Use a pipette with 1 mL tip pressed directly against one of the access ports to fill the chamber with 400 μ L of Hybridization Buffer. Maintain a slow and steady flow while filling the chamber to help avoid air bubbles. Seal both access ports by covering with the provided adhesive sealing tabs. Incubate the array with hybridization buffer for 30 minutes at 4°C in the dark to equilibrate the array.
4. Prepare 400 μ L of your antibody of interest in Hybridization Buffer. The dilution used should be comparable to that used for Western blotting.
5. Remove sealing tabs from both access ports. Use a pipette with 1 mL tip to remove liquid from hybridization chamber (holding the glass slide at an angle helps to collect the liquid at one corner where it can be removed through the access port). Carefully fill the chamber with 400 μ L of the antibody solution prepared in Step 4. Avoid introducing air bubbles by maintaining a slow and steady flow. Seal ports with adhesive sealing tabs.
6. Incubate at 4°C for 2-3 hours in the dark.
7. Empty the chambers as described in step 5. Perform five sequential washes by filling the chambers with ice cold PBS and emptying chambers as described in step 5. Incubating the chamber filled with PBS for 5 min at 4°C at each wash may improve stringency of the washes.

Note: The procedure below is for detection with a fluorescent secondary antibody. For detection using the ECL method, go to “ECL Detection Procedure” on page 12.

8. For each chamber prepare 400 μ L of a fluorescent secondary antibody (1:2,000 to 1:5,000 dilution) in Hybridization Buffer. Pipette in the chamber, seal the ports and incubate for 30 minutes in the dark at 4°C.
9. Wash each chamber 3-5 times as described in Step 7.
10. Remove the gasket using forceps and wash the entire array by submerging it in 1x ice cold PBS for 5 min at 4°C with rotation (using a Coplin jar or glass array tube). Repeat.
11. To remove excess salt dip the array several times in 0.1x PBS.
12. Dry the slide using either **filtered** forced air or centrifugation in a 50 mL conical tube at 800 x g for 2 minutes.
13. Proceed to the detection step below. Fluorescent antibody signals may remain for 24 hours or longer. However, it is recommended to scan the array slide within a few hours of incubation for best results.

III. Guidelines for Fluorescent Scanning Detection

1. Detection of features on an AbSurance Pro Microarray requires an array scanner with a resolution of at least 25 μ m. Higher resolution data can be collected at 10 μ m if desired. A variety of array scanners are appropriate. AbSurance Pro arrays have been scanned and analyzed successfully on Agilent G2505C, Typhoon™ 9400 (GE), Typhoon™ Trio+ (GE), GenePix® 4000B (Molecular Devices/Axon Instruments). See the AbSurance Pro product page on our company website to download the GAL file called “AbSurance Pro.gal”. This GenePix® Array List (GAL) file describes the size and position of blocks and features on the array, as well as IDs of the printed peptides.
2. Set the scanner acquisition mode to fluorescence and select the 526 nm and 670 nm emission filter sets for the spotting tracer and secondary antibody, respectively. It is important to scan both channels as the green channel contains the data on the spotting tracer and provides information about peptide spotting efficiency, which factors into the data analysis.
3. See Product Performance Section for an example of actual array data.

Data Analysis

Note: There are a variety of ways to analyze AbSurance Pro microarray data. Two approaches are described below. Depending on your experiment, you have the flexibility to perform a simple qualitative approach to quickly identify positive interactions or a more rigorous statistical analysis.

A. Simple and Rapid Qualitative Analysis Procedure

For interpretation of peptide array-protein or antibody interactions a statistical analysis is the preferred approach. However, in some cases it is desirable to quickly assess positive peptide interactions with a given protein or antibody of interest. In such cases, we recommend using the provided ‘peptide grid’ in the file named “AbSurance Pro Array Grid.pptx”. This file can be found on the AbSurance Pro Histone Peptide Microarray product page on our company website. A quick qualitative analysis can be performed by overlaying the array grid on the raw image of the scanned array slide. The grid numbers correspond to specific peptides found on the second tab of the spreadsheet.

Paste the image of a single subarray as a picture on a PowerPoint slide or any other program that allows manipulation of images (e.g. Microsoft® Word, Adobe® Photoshop®, Adobe® Illustrator).

Copy the subarray grid from the “AbSurance Pro Array Grid.pptx” file and paste it as a picture on the same slide.

Adjust the grid picture size and position to fit the image of the array. See Figure 4 for an example of the grid positioned over an array. You can adjust brightness of the grid picture to get the best visual effect by right clicking on the image and selecting ‘Format Picture’. Each cell on the grid should cover three peptide spots on the array.

Positives appear as bright red over the green spotting tracer background. Refer to the peptide list in the “AbSurance Pro Peptides.xlsx” spreadsheet to identify which peptides appear as positive.

Peptides are spotted as a series of three adjacent spots. True positive results will be represented by all three peptide spots lighting up in the same rectangle. A single spot in a set of three should be considered a false positive result and should be ignored.

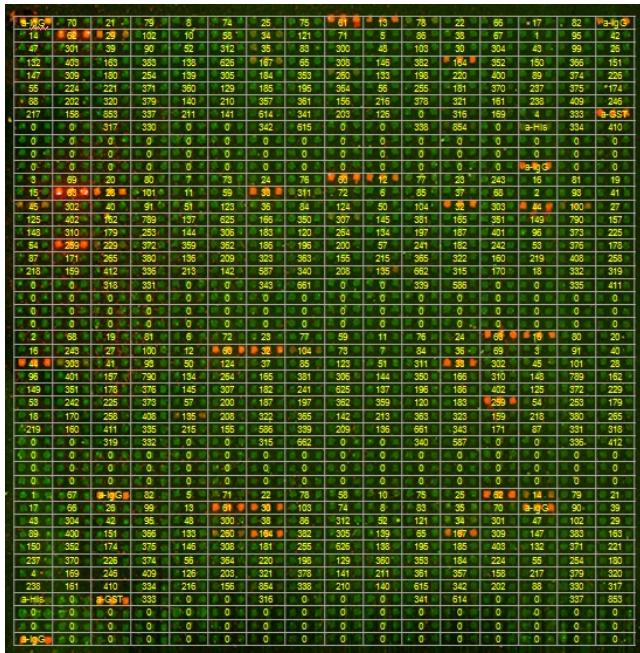


Figure 4. AbSuriance Pro Histone Peptide Microarray with Grid Overlay. Scanned data (both red and green channels) from a single sub-array with peptide grid on top of the array data. This grid overlay file “AbSuriance Pro Array Grid.pptx” is available on the AbSuriance Pro product page of our website to allow you to download the array grid for your own use.

B. Quantitative Analysis of AbSuriance Pro Results

Detailed analysis of histone peptide microarray results can be performed by densitometry analysis using analysis software such as ImageQuant™ TL. These software programs allow determination of raw signal intensities for each spot on the scanned array. An overview of how to use the ImageQuant TL software package is presented below.

1. Open ImageQuant TL and click on “Array Analysis.”
2. Open your scanned array file.
3. Adjust the contrast on Channel 1 (green) as needed to make all spots clearly visible.
4. For visual inspection, adjust the contrast on Channel 2 (red).

Note: Contrast adjustments have no effect on the quantification of data as described below. However, the quantification procedure presented here does require both Channel 1 and Channel 2 to be toggled on (you can toggle Channels on/off by clicking the Channel 1/Channel 2 icons above the array image).

5. On the left frame, click on “Background Correction” and select “Spot Edge Average.”
6. On the left frame, click on “Spot Definition”. Create a 48 x 24 square grid (15 x 15 radius).
7. Beginning with the cursor in the upper left spot on sub-array A (see figure 2), overlay the grid on the sub-array. Please note that subarray A is 48 x 48, so this grid covers only ½ of the features. The other half of subarray A will be addressed in subsequent steps.

Note: Once the grid is created, it may be necessary to align the grid over the 48 x 24 series of peptides in sub-array A section 1 by dragging the corners of the grid.

8. Open the “Measurements” window in the lower left corner and select the “All Spots” tab.
9. Click “Edit” > “Export to Excel”. This will copy the measurements window into Microsoft Excel. Save this file.

10. Return to ImageQuant TL and click “Image Window” in the lower left corner.
11. Drag the 48 x 24 grid down the array to cover the other half of subarray A. If necessary, further align the grid as described in Step 7.
12. Repeat Steps 8-9.
13. This will open your original Excel File created in Step 9 as well as a new workbook with the second set of data from the new measurements window. Copy the entire second dataset. Click on Cell A1156 and paste the entire second dataset into your original Excel File created in Step 9.

Note: If Step 13 is performed correctly, you should now have two datasets in the original file separated by a single row (Row 1155). It is crucial that there only be one row separating the datasets for the data to be analyzed correctly once you transfer it to the analysis spreadsheet.

14. Repeat Steps 11-13 for peptides in sub-array B (see Figure 2).
15. You should now have four datasets merged into a single Excel file, each separated by a single row. Copy columns D (Channel 1; green) and E (Channel 2; red) from the compiled spreadsheet and paste into columns E and F of the provided Excel “AbSurance Pro Analysis Template.xlsx” available at the AbSurance Pro product page of our company website.

Note: The “AbSurance Pro Analysis Template.xlsx” automatically applies an initial filter to remove individual spots whose green intensity is less than 5% of the average intensity for all spots on the peptide array. This eliminates false negative points that may result from inefficient spotting or damage to the array during handling. If a spot passes this initial filter, the corresponding cell in column I will turn green with the word ‘OK.’ Filtered spots that do not meet pass criteria turn red with the word “NA” and are not included into subsequent analysis. The red channel intensity of the three spots for each peptide are then averaged and treated as a single value (Spot Avg x). The eight averaged values for each peptide triplicate (columns P-W) are then used to calculate a mean, standard deviation, and standard error of the mean (columns Z-AB) for each peptide interaction. The value in “Peptide” columns refers to the peptide number in the “AbSurance Pro Peptides.xlsx”.

16. Copy columns Y-AB in the “AbSurance Pro Analysis Template.xlsx” and select “Paste Special” option and paste only the values into a new set of columns (AD-AG). This data is now paired with the peptide name and sequence from “AbSurance Pro Peptides.xlsx” information in columns AH-AJ. To determine the best interacting peptides, select columns AD-AJ and use Excel’s “Sort Data” feature to sort by the “AVGsum” value in descending order. Make sure your selection include the peptide name and sequence columns so that these will sort with the peptide number and the intensity value. The peptides at the top of this list are the strongest interactors with your protein or antibody of interest.

C. ECL Detection Procedure

Detection of antibody or protein binding to the AbSurance Pro Histone Peptide Microarray by ECL detection is similar to developing a Western blot. At this point, there is a primary antibody on the array or a histone binding protein with an antibody to the protein or the protein's epitope tag on the array. This protocol assumes use of X-ray film to detect ECL signal, although high resolution CCD-based gel documentation systems can be employed as well.

1. Prepare an appropriate dilution of HRP-conjugated secondary antibody in Hybridization Buffer. Pipette the diluted secondary antibody into the chamber, seal the ports and incubate for 30 minutes at 4°C in the dark.
2. Wash the chambers 3-5 times as described in step 7 of the Hybridization Protocol.
3. Remove the gasket using forceps and wash the entire AbSurance Pro microarray slide by submerging it in 1x PBS for 5 min at 4°C with rotation (using a Coplin jar or glass array tube). Repeat.
4. Detect the HRP-conjugated secondary using an ultrasensitive ECL reagent. Less sensitive ECL substrates can be used, but will require longer exposure times.
5. Mix 100 µl of each of the two substrate components (1:1) and transfer the mixture to the top surface of the AbSurance Pro Microarray slide. Cover the array with a cover slip (or a 25mm x 60mm piece of Parafilm®).
6. Incubate at room temperature for 5 minutes.
7. Place the AbSurance Pro Microarray slide between a clear plastic sheet protector.
8. Expose the microarray to an X-ray film in a darkroom in an X-ray film cassette.
9. Develop X-ray film to reveal binding of antibody or protein to the histone peptide array.

Note: For X-ray film exposure it is important to ensure that the AbSurance Pro Microarray slide remains stationary during the exposure to prevent blurring of the image. To secure the slide inside the exposure cassette, place the array in the top right corner of the plastic sheet protector and tape the sheet protector containing the array to the lid of an 8" x 10' X-ray film cassette. To prevent attenuation of the exposure, be sure to orient the top surface of the array towards the film.

By securing the slide to the swing lid of the cassette, it is possible to expose the film by opening and closing the lid. This also decreases the chance that the slide may move during handling. To achieve up to four different exposures per sheet of film, rotate the film 90 degrees between exposures. Exposure times vary depending on type of film and the intensity of the signal. Multiple exposures between 5 seconds and 5 minutes per exposure are suggested.

D. Data Analysis with ImageJ Software

For basic analysis of antibody or protein binding data using a scanned image (fluorescent or ECL) using the freely available image analysis software package ImageJ, an array analysis template that allows comparison of relative binding to the peptides on the AbSurance Pro Histone Peptide Microarray is available from our website. If you plan to use ImageJ software, it is recommended that you familiarize yourself with this program before starting your analysis. In addition to the ImageJ software, you will need an image manipulation program (e.g. Photoshop) to resize and crop scanned histone peptide array images.

Getting Started with ImageJ

1. Download ImageJ package specific for your computer's operating system.
ImageJ requires the usage of Java platform which is bundled with the package for your specific OS. Visit <http://rsb.info.nih.gov/ij/download.html>
2. Download MicroArray Profile plugin for ImageJ from OptiNav.
This is a Java resource (called a "jar") that you download and place in the Plugins folder in ImageJ. This allows you to measure spots on scanned images from the AbSurance Pro Histone Peptide Microarray. Familiarize yourself with the interface and the controls for the plugin prior to using it. Visit http://www.optinav.com/MicroArray_Profile.htm
3. Obtain AbSurance Pro ImageJ Analysis Template.xls. This is an Excel spreadsheet for use in analyzing data gathered using ImageJ. To download, visit the AbSurance Pro product page on our company website.
4. AbSurance Pro 48x48 grid is a custom grid for use with ImageJ and the MicroArray Profile plugin. To download go the AbSurance Pro product page on our company website.

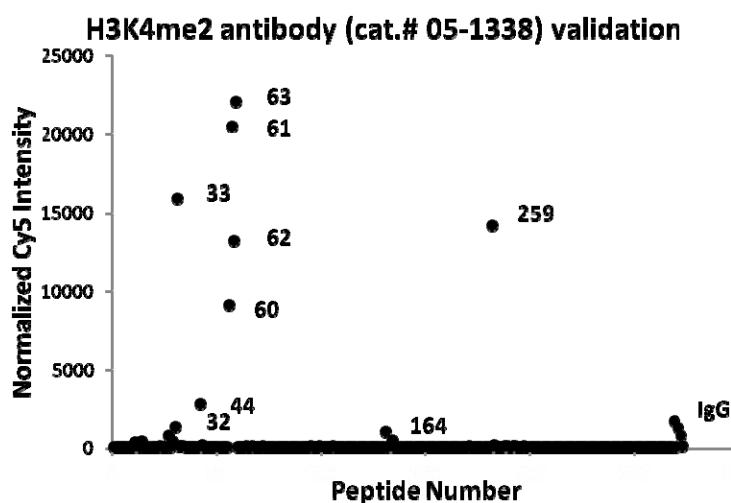
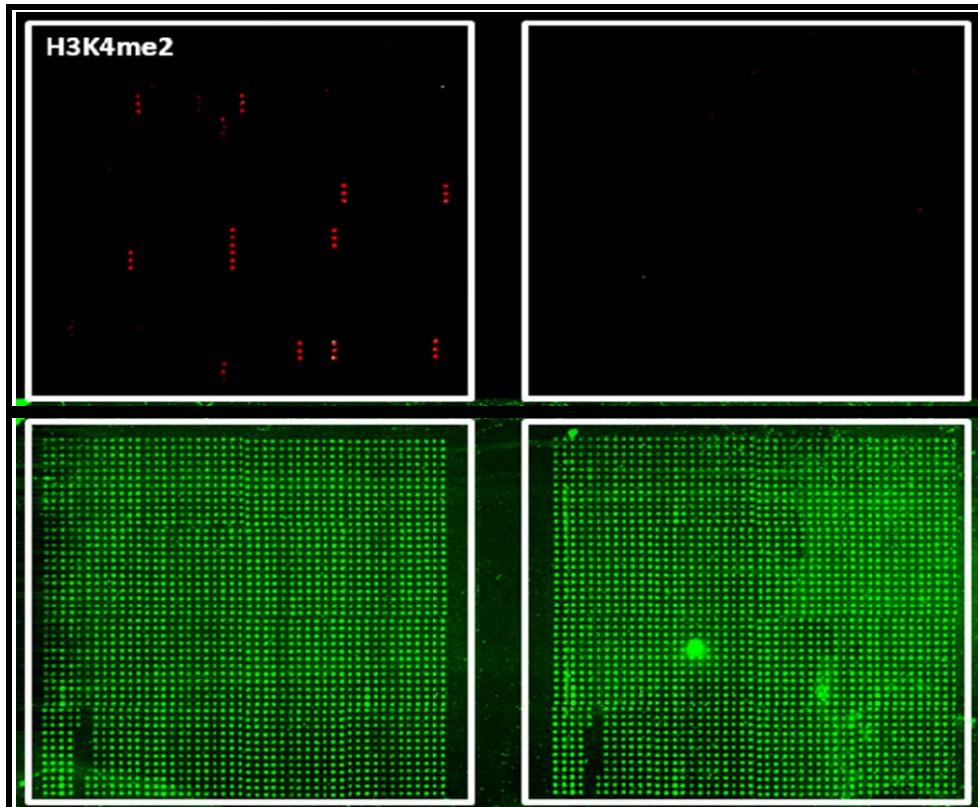
E. Instructions for Analysis with ImageJ

1. Starting with your scanned peptide array image (file format should be jpg), the IgG control spots will appear (in groups of three) in the top right and left corners and the bottom left corner, allowing you to orient the image properly. You will need to orient the image so that the peptide spots are perpendicular. This will make the peptide spots parallel to each other. You might need to rotate the image slightly clockwise or counterclockwise to achieve this. You can determine this by drawing a parallel line next to your peptide spots and rotating the image until the line is parallel to the spots. This is crucial as the **MicroArray Profile** plugin selects specific parts of the peptide array for measurement of signal and requires that the image to be perpendicular.
2. Once you have the image perpendicular, you will need to adjust the image size so that the top row corner to corner (center of spot to center of spot) is 820 pixels and the left column corner to corner is 820 pixels (center to center). You can resize the MicroArray Profile grid but it is easier to resize the image.
3. Launch ImageJ and open your newly resized peptide array image. Zoom in so that a single sub-array is visible and fills as much of your screen as possible.
4. Navigate to "Plugins" toolbar and scroll down to "jars" and open "MicroArray Profile" plugin. This launches the microarrays plugin. You will see yellow circles appear over your image.

5. On the MicroArray Profile pulldown menu, click “Read Grid.” This will allow you to load the custom grid specific for the AbSurance Pro array. The filename is “AbSurance Pro 48x48 grid”. This file can be downloaded from the AbSurance Pro product page on our company website. Select the grid file from where you saved it and it will load and you will see a different grid of yellow circles.
6. You will need to reposition the grid to match the features on the AbSurance Pro array. Use Alt-Click to select (and hold the mouse button down) any part of the grid to move the entire grid. Drag a corner so that it aligns with the corresponding corner spot in the image.
7. Shift-click and hold/drag an adjacent corner so that it is positioned properly. Now the whole edge connecting the two corners should be lined up with the spots.
8. Shift-click the other adjacent corner so that it is positioned properly. Now the whole edge connecting the two corners should be lined up with the spots.
9. Shift-click a spot in the interior of the opposite edge until that edge is lined up with the spots. Unless the pattern of spots is distorted, all of the grid circle should now be aligned with the spots on the array image. If at any point the grid array becomes too far out of alignment, just reload the AbSurance Pro 48x48 grid template using the “Read Grid” feature of the plugin.
10. On the MicroArray Profile plugin menu, click on “Measure RT”. This will create a new window to appear with the measurement data named “Results”. Click the toolbar in the “Results” window to select it and then click “Edit” and select “Copy”. You should see the message “2304 lines copied to clipboard” in the ImageJ toolbar menu.
11. Open the Excel file downloaded from our company website titled “AbSurance Pro ImageJ Analysis Template.xls”. Make sure the first tab of the file “Analysis Template 1” is open. Select cell D2 and select “Edit->Paste” to past the data from ImageJ into the Excel worksheet. You should see data fill columns D through J. The signal data is automatically copied from column J to column C.
12. Carefully select cells A2 through C2305 and only these cells and copy the data to the clipboard. You can do this easily by selecting cell A2, then scrolling down and hitting the shift key before selecting cell C2305 and then hitting ctrl-C.
13. Switch to the second tab in the worksheet, “Sheet 2”, select cell A2. in the “Edit” pulldown menu, select “Paste Special” and click the radio box next to “Values and Number Formats” in the “Paste” section of the window that pops up and then click “OK”. The data from the first worksheet, including the peptide names and the signal data, is now copied into “Sheet 2”. A calculation is made automatically to subtract the background from the signal, and appears in the “Bkd-Sig” column.
14. Select columns A-D and copy them to the clipboard. Navigate to “Sheet 3”, select column A and “Paste Special..Values and Number Formats” as before.
15. Sort the data in columns A-D by Column A data “Pep #” sorting smallest to largest. Make sure the box “My list has headers” is selected before sorting.
16. You should now see the data sorted by peptide number, 0 being the lowest number. This represents peptide spots with no peptide present and should occupy Rows 2 through 667.

17. Select Rows 2-667 and delete them. Data for peptides 1-835 should now be visible. There are not 835 different peptides on the array, these numbers represent an identifying number used for internal reference for each peptide.
18. Select columns A-D and copy them to the clipboard. Navigate to “Sheet 4”, select column A and “Paste Special..Values and Number Formats” as before. Make sure not to overwrite the formulas in Columns E and F.
19. Select columns A-F and copy them to the clipboard. Navigate to “Sheet 4”, select column A and “Paste Special..Values and Number Formats” as before.
20. Sort the data in columns A-F by Column E data “Average” sorting largest to smallest. Make sure the box “My list has headers” is selected before sorting. Delete Columns C and D and Rows 272 and higher. These data now represent the averages for all the individual peptides on the AbSurance Pro Histone Peptide Array.
21. The same procedure can be used for the analysis of sub-array B.

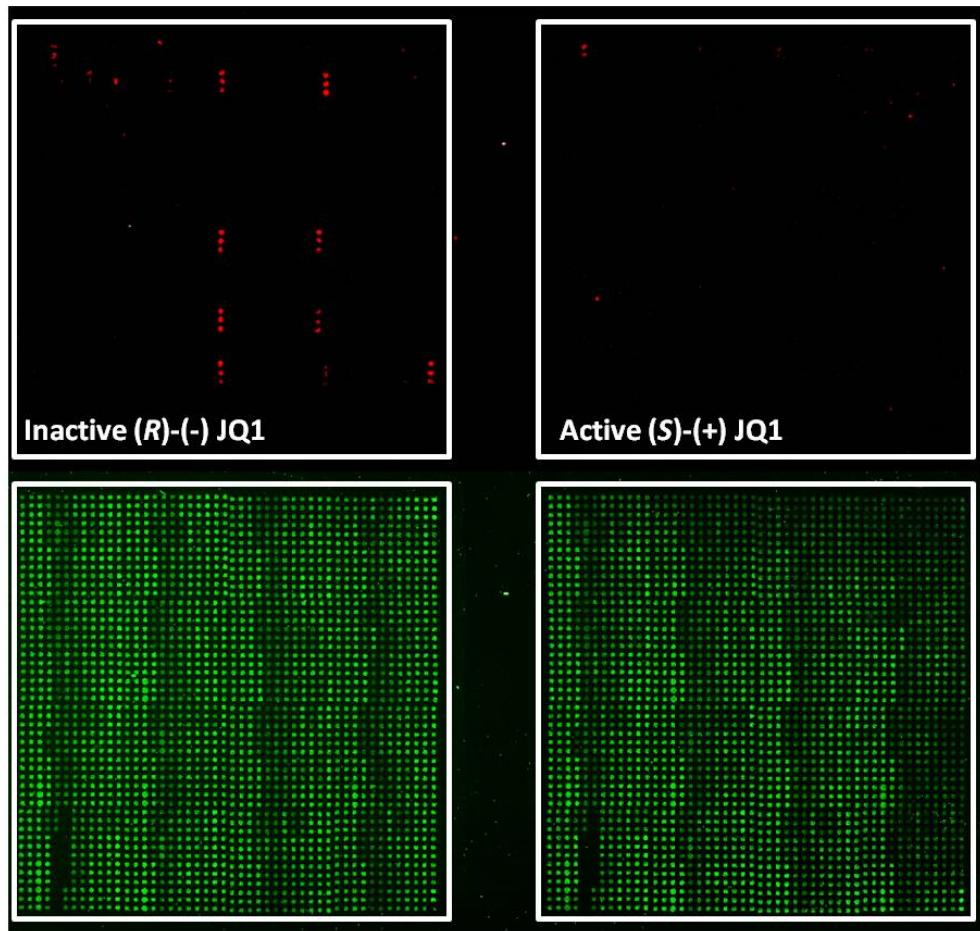
AbSuriance Pro Histone Peptide Microarray Product Performance



| ## | Peptide ID | Cy-5 signal |
|---------------------------------|---------------------------------------|-------------|
| 63 | H3Cit2 + K4me2 | 22'054 |
| 61 | H3R2me2s + K4me2 | 20'438 |
| 33 | H3K4me2 + K9ac + K14ac + K18ac | 15'935 |
| 259 | H3K4me2 + K9me2 | 14'195 |
| 62 | H3R2me1 + K4me2 | 13'263 |
| 60 | H3R2me2a + K4me2 | 9'150 |
| 44 | H3K4me2 + K9ac + K18ac | 2'836 |
| 32 | H3K4me2 | 1'346 |
| 164 | H3K4me2 + T6p | 1'040 |
| No peptide (background reading) | | 72 |

Figure 5. Binding Specificity Analysis of Anti-dimethyl-Histone H3 (Lys4)

The left chamber of an AbSuriance Pro Histone Peptide Microarray slide was incubated overnight with anti-H3K4me2 antibody (Cat. # 05-1338, 1:10,000 dilution), followed by a 30 min incubation with anti-mouse IgG antibody conjugated to Cy5 (cat. #192S, 1:1,000 dilution). The right chamber was mock incubated as a control. Data was collected on an Agilent G2505C scanner at 25 μ m resolution. Upper panel (red spots): H3K4Me2 antibody binding. Lower panel (green spots): detection of peptide spotting tracer. Authentic binding events appear as three adjacent red dots, indicating antibody binding to all three peptides of in a set. Graph and tables show quantified normalized data indicating antibody specificity.



| Peptide # | Peptide ID | Cy5 signal | |
|--|--|------------|---------|
| | | (R)-(-) | (S)-(+) |
| 81 | H4R3me1 + K5ac + K8ac + K12ac + K16ac + K20ac | 3542 | 1246 |
| 59 | H4K5ac + K8ac + K12ac + K16ac | 3296 | 1416 |
| 72 | H4K5ac + K8ac + K12ac | 2788 | 1453 |
| 80 | H4R3me2s + K5ac + K8ac + K12ac + K16ac + K20ac | 2703 | 480 |
| 79 | H4R3me2a + K5ac + K8ac + K12ac + K16ac + K20ac | 1994 | 774 |
| No peptide (averaged background reading) | | 276 | 348 |

Figure 6. Analysis of BRD4 binding to H4 peptides in presence of JQ1. Human recombinant BRD4 protein (a.a. 44-459) was pre-incubated for 30 min with 250 nM of active (S)-(+) or 250 nM of inactive (R)-(-) JQ1 enantiomers (cat.# 500586, reconstituted in ethanol). Treated BRD4 protein was pipetted into separate chambers of AbSurance Pro peptide microarray, and incubated at 4°C overnight. Detection was performed with an Anti-His6 antibody (cat. # 05-949) at a 1:2,000 dilution for 3 hrs) followed by a 30 min incubation with anti-mouse IgG antibody conjugated to Cy5 (cat. #192S) at a 1:5,000 dilution). The array was scanned at 25 μ m resolution with Agilent G2505C scanner. Upper panel (red spots): Left side shows treatment with inactive enantiomer. Right side shows treatment with active JQ1 enantiomer. Lower panel (green spots) shows detection of peptide spotting tracer. Table shows tracer-normalized Cy5-signal intensity is shown for top 5 strongest interactions.

Related and Suggested Products

These reagents have worked well in combination with AbSurance Pro Histone Peptide Microarrays. Dilutions provided are intended to be used as starting points and may need to be adjusted for your experiments.

Anti-GST: antibody for detection of GST-tagged proteins

Antibody: Sigma anti-GST, rabbit polyclonal (Cat. No. G7781)

Dilution: 1:1,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 2hr at 4°C (in dark)

Washes: 3 x 5' in PBS.

Anti-His tag antibody for detection of His-tagged proteins

Antibody: EMD Millipore Anti-His Tag, clone HIS.H8 mouse monoclonal (Cat. # 05-949)

Dilution: 1:2,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 2hr at 4°C (in dark)

Washes: 3 x 5' in PBS.

Anti-Mouse fluorescent secondary antibody

Antibody: EMD Millipore Donkey Anti-mouse IgG antibody, Cy5 conjugate (Cat. #. AP192S)

Dilution: 1:5,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 30' at 4°C (in dark)

Washes: 3 x 5' in PBS.

Anti-Rabbit fluorescent secondary antibody

Antibody: EMD Millipore Donkey Anti-rabbit IgG antibody, Cy5 conjugate (Cat. # AP182SA6)

Dilution: 1:5,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 30' at 4°C (in dark)

Washes: 3 x 5' in PBS.

Anti-Rabbit HRP Conjugate for ECL detection

Antibody: EMD Millipore Donkey anti-Rabbit IgG antibody, HRP conjugate (Cat. # AP182P)

Anti-Mouse HRP Conjugate for ECL detection

Antibody: EMD Millipore Goat anti-Mouse IgG antibody, HRP conjugate (Cat. # AP124P)

Anti-Rat HRP Conjugate for ECL detection

Antibody: EMD Millipore Goat anti-Rat IgG antibody, HRP conjugate (Cat. # AP136P)

References

1. Tarakhovsky A. 2010. *Nature Immunology*. Tools and landscapes of epigenetics.
2. Helin K and Dhanak D. 2013. *Nature*. Chromatin proteins and modifications as drug targets.

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