

EpiTitan™ Histone Peptide Array

Array Version 3.0

Catalog No. 11-2001

Store in Darkness at 4°C Upon Receipt

User's Manual ver 5.0

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Description

EpiCypher EpiTitan™ Histone Peptide Arrays are designed for semi-quantitative and high-throughput analysis of the binding specificity of antibodies and histone binding ‘effector’ proteins. They can also be used to determine the substrate specificity of histone-modifying enzymes. A typical workflow for ‘effector’ protein analysis is depicted in Figure 1. A standard hybridization and detection protocol for antibodies and ‘effector’ proteins and antibodies is described in the “Experimental Procedure” section starting on page 7. The standard procedure for antibodies can be completed in half a day, and ‘effector’ protein analysis can be completed in less than two days.

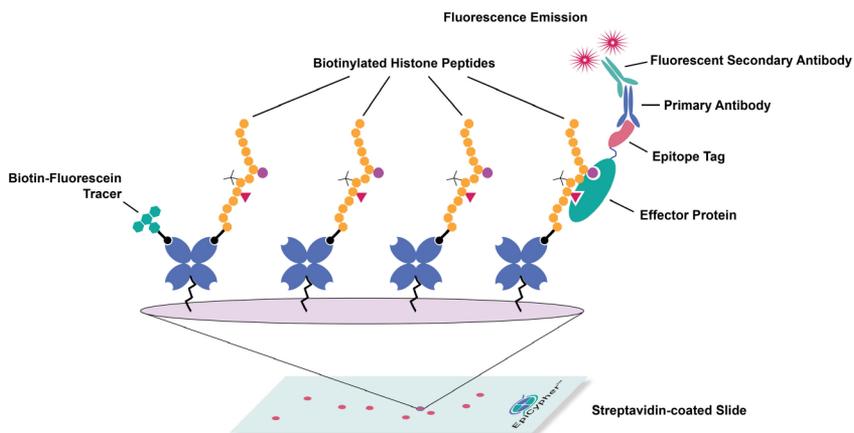


Figure 1. EpiTitan™ Histone Peptide Array slide design.

For detection of the interaction of an effector protein with peptides on the array as shown above, you need a primary antibody to the protein (or to an affinity tag) and a fluorescently-labeled secondary antibody to the primary. This is much like the detection procedure employed in immunofluorescence microscopy. For analysis of histone antibody specificity, you need the primary antibody to a histone modification to be studied and a fluorescently-labeled secondary antibody recognizing the primary antibody.

Storage and Handling

- EpiCypher EpiTitan™ Histone Peptide Arrays should be stored in a dry environment at 4°C and must be protected from light and dust.
- Proper storage will maintain the integrity of the array slide for at least 4 months from the date of shipping.
- Arrays should be handled with care.
- Avoid touching the surface of the slide with hands by handling from the edges towards the engraved end of the slide.
- When possible, wear nitrile gloves when handling slides. The powder present in latex gloves (even those sold as powder-free) will autofluoresce and should be avoided.
- Avoid touching the surface of the slide with pipette tips when dispensing liquids, as this can remove the peptides from the surface of the slide.
- High-quality particulate-free buffers should be used throughout slide processing. Pass all solutions through a 0.2 µM filter.
- Incubation of array slides with solutions other than those described in this manual may compromise the integrity of the array's biological surface.

Materials Needed but not Supplied

Note: See **Appendix 1** for suggestions on reagents to use with EpiTitan™ Histone Peptide Arrays.

- Cold, 1x phosphate-buffered saline (PBS) filtered.
- Powdered bovine serum albumin (BSA).
- Tween-20.
- 24 x 60 mm No. 2 coverslips.
- Humidified slide incubation box (e.g. Simport M918 StainTray™). This can also be achieved using a plastic container, such as a large square plastic petri dish with a lid (Greiner 688 102 or Corning CLS431111), and wet blotter paper or ScotchBrite. Place something on the padding to elevate the slides so they are not sitting directly on the pad.
- Microscope slide chamber dish (e.g. Thermo-Nunc 267061 4-well dish with lid) for washing slides.
- Far-red fluorescently-labeled secondary antibody, such as AlexaFluor™ 647, Cy5™ or DyLight™ 650.
- Microarray scanner capable of scanning with at least 25 μM resolution (e.g., Typhoon Trio+ from GE, Axon GenePix 4000).
- X-ray film and ECL detection reagents.
- Densitometry analysis software (e.g., ImageQuant TL from GE, ImageJ). A protocol for using ImageJ for data analysis is located in Appendix 4 on page 20.
- For detection of epitope tagged proteins on the array, an anti-tag antibody and a corresponding fluorescent secondary antibody are necessary.

Background

Histone post-translational modifications (PTMs) regulate the diverse biological processes associated with chromatin. The study of histone PTMs such as lysine and arginine methylation, lysine acetylation, serine and threonine phosphorylation has been at the forefront of chromatin biology research over the past decade. Recent studies demonstrate that histone modifications function in the form of a combinatorial 'histone code' to recruit 'effector' proteins to defined genomic loci where they facilitate distinct biological functions. However, progress deciphering the 'histone code' has been limited due to a lack of quality-controlled technologies to address this complex hypothesis.

EpiCypher's EpiTitan™ Histone Peptide Array platform is designed for rapid and high-throughput screening of effector protein, antibody and enzyme interactions with a comprehensive library of combinatorially-modified histone peptides encompassing more than 95 unique modifications (acetylation, arginine methylation, lysine methylation and phosphorylation) on the four core histones (H3, H4, H2A, and H2B) and several histone variants. There are also several additional amino acids, including norleucine and citrulline. There are up to six modifications on each peptide. Each modified peptide also has an unmodified version as a control. A variety of epitope tag peptides are also included on the arrays for use as a control in experiments to detect protein binding.

Extensive validation and purification of peptides is performed prior to array printing to ensure the highest quality product for your studies. Additionally, all peptides are co-spotted with a fluorescent tracer to control for printing efficiency and eliminate the potential for false negative results. This meticulous attention to detail translates to a superior array platform to help rapidly progress your scientific discovery.

EpiTitan™ Histone Peptide Array Advantages

- EpiTitan™ arrays can be used for two separate experiments per slide, allowing the inclusion of a positive control or incubation of a protein or antibody at two different dilutions.
- The EpiTitan™ array includes a number of epitope tag and IgG controls to help with the successful completion of your experiments and makes data analysis easier.
- All peptides are purified by HPLC and analyzed by mass spectrometry prior to spotting, and guaranteed to be full length.
- Each peptide is spotted 12 times on each array to allow for high quality analysis of protein or antibody binding.
- The patent-pending fluorescent spotting tracer controls for printing efficiency and eliminates false negative results.
- The EpiTitan™ Histone Peptide Array is the only histone peptide array that can be visualized using fluorescence scanning or standard ECL techniques.

Important Notes Before Beginning

- Read the protocol completely before starting your experiments with EpiTitan™ Histone Peptide Arrays.
- Make sure that you have all the necessary additional reagents (see **Appendix 1** for suggestions on additional reagents).
- The slides are marked with either the logo of the company that made the slide precursors (PolyAn). This is the top of slide and they should always be oriented face up.
- During the course of the experiment, it is critical to keep the slides in the dark as much as possible. The fluorescent spotting tracer is light sensitive, as is the fluorescent secondary antibody used to detect protein or antibody binding to the array.
- It is very important that the slides not be allowed to dry out during processing, or your data will be compromised.
- Download additional files for use with the EpiTitan arrays at http://www.epicypher.com/epititan_array.aspx

Peptide Array Layout

Each EpiCypher EpiTitan™ Histone Peptide Array contains several hundred biotinylated histone peptides (approximately 20 amino acids in length) immobilized on a streptavidin-coated glass microscope slide. The complete listing of peptides is available in the spreadsheet **EpiCypher Annotated Peptide List v3.xls**, on the product page at www.epicypher.com. Peptides are spotted as two identical sub-arrays, labeled A and B in Figure 2. Within each sub-array, each peptide is spotted twice in groups of 3 (see Figure 2, red dots). This format results in each peptide being featured 12 times in defined positions across the array, and allows the user to examine interactions with over 4,000 features on a single slide. This format also accounts for spotting variability, local hybridization differences across the slide surface, and allows the user to statistically evaluate the quality of individual peptide interactions.

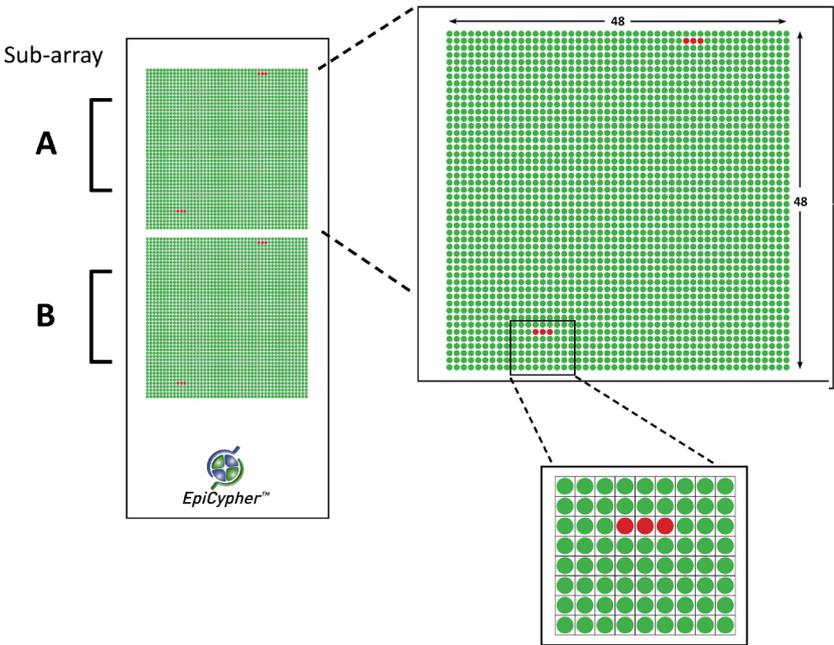


Figure 2. EpiTitan™ Histone Peptide Array slide design.

Please refer to the Excel spreadsheet “EpiCypher Annotated Peptide List v3.xls” available on our website for details on the exact layout of each peptide. This figure does not represent the exact placement of each of the sub-arrays on the slide: see Figure 3 on page 8.

Experimental Protocol

Hybridization Procedure - Antibody

Note: For use of antibodies with EpiCypher EpiTitan™ Histone Peptide Arrays, the dilution range used for Western blotting should serve as a starting point.

Note: For use of the multi-well gasket with the EpiTitan Histone Peptide Array, see instructions and modifications to the protocol in Appendix 5 on page 21.

1. Prepare Peptide Array Hybridization Buffer (1x PBS, 5% BSA (w/v), 0.1% Tween-20).
2. Carefully place a array slide face up (“+” or logo facing up and visible) in a clean non-treated microscope slide chamber dish (e.g. Thermo-Nunc 267061 untreated 4-well dish).
3. Incubate the array with hybridization buffer (enough to submerge the slide completely) in a covered microscope slide chamber dish for 30 minutes at 4°C with gentle rotation to equilibrate the array.
4. Prepare 200 µl* of your antibody of interest of your protein of interest in hybridization buffer. The dilution used should be comparable to that used for Western blotting.

Note: If you are using the multi well gasket, the volume for each sub-array chamber is **450 µl**.

5. Place the array in a humidified slide incubation box to prevent the slide from drying out.
6. Pipet 200 µl of the antibody solution prepared in Step 4 directly over the printed peptides (see Figure 3).
7. Carefully overlay the slide with a coverslip, avoiding the introduction of air bubbles (see Figure 3).
8. Cover the box and incubate at 4°C for 2-3 hours in the dark without rotation in the humidified microscope slide incubation box with water in the chamber. Alternatively, slides can be incubated in a large petri dish or a plastic container with a tight-fitting lid. To maintain humidity, place a damp piece of Whatman paper

in the container with the array.

- Carefully remove coverslip (should slide off without touching).
- Wash the array in a microscope slide chamber dish three times for 5 minutes each (with rotation) with 1x PBS at 4°C. Use enough PBS to submerge the slide completely.

Note: To detect antibody binding using ECL techniques, go to page 16. "Detection Procedure - ECL."

- Prepare a diluted solution of fluorescent secondary antibody (we recommend AlexaFluor™ 647) in hybridization buffer and incubate with the array for 30 minutes in the dark at 4°C with gentle rotation in the microscope slide chamber dish.
- Wash the slide 3 x 5 minutes with 1x PBS in the dark at 4°C in the microscope slide chamber dish (with rotation).
- Holding the array with forceps from the **end without the logo**, dip the array several times (using a Coplin jar or small beaker) in 0.1x PBS to remove excess salt.
- Dry the slide using either **filtered** forced air or centrifugation in a 50 ml conical tube at 800 xG for 2 minutes.
- Proceed to the detection step. 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.

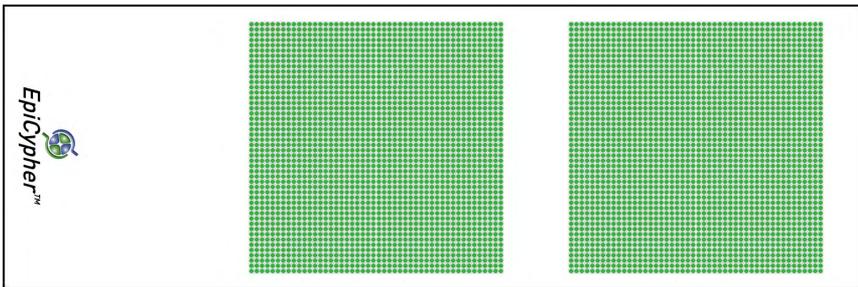


Figure 3. Peptide Placement on EpiTitan™ Histone Peptide Array.

Placement of peptides on glass slide shown to scale. Use only a 24 x 60mm No. 2 glass coverslip to cover array during incubations. Place the coverslip to the far left edge of the array, relative to the logo on the slide.

Hybridization Procedure - Binding Protein

Note: 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 has also been optimized for the detection of GST-tagged proteins. Detection of His-tagged proteins has also been successful. Detection of FLAG-tagged proteins is not advised with the M2 monoclonal. A polyclonal antibody with higher avidity is recommended. See **Appendix 1** for additional suggested reagents.

Note: A key to a successful experiment using the EpiTitan Histone Peptide Array is that the tested protein has to be very concentrated ($\sim 50 \mu\text{M}$). It always works better by adding $2 \sim 3 \mu\text{l}$ of protein in $200 \mu\text{l}^*$ of array hybridization buffer to reach at least 500 nM final protein concentration.

1. Prepare Peptide Array Hybridization Buffer (1x PBS, 5% BSA (w/v), 0.1% Tween-20).
2. Carefully place a array slide face up (“+” or logo facing up and visible) in a clean non-treated microscope slide chamber dish (e.g. Thermo-Nunc 267061 untreated 4-well dish).
3. Incubate the array with hybridization buffer (enough to submerge the slide completely) in a covered microscope slide chamber dish for 30 minutes at 4°C with gentle rotation to equilibrate the array.
4. Prepare $200 \mu\text{l}$ of a $2 \mu\text{M}$ solution of your protein of interest in hybridization buffer.
5. Place the array in a humidified slide incubation box to prevent the slide from drying out.
6. Pipet $200 \mu\text{l}$ of protein antibody solution prepared in Step 4 directly over the printed peptides (see Figure 3).
7. Carefully overlay the slide with a coverslip, avoiding the introduction of air bubbles.
8. Cover the box and incubate at 4°C overnight in the dark without rotation in the humidified microscope slide incubation box

with water in the chamber. Alternatively, slides can be incubated in a large petri dish or a plastic container with a tight-fitting lid. To maintain humidity, place a damp piece of Whatman paper in the container with the array.

9. Remove the coverslip (should easily slide off).

10. Wash the array in a microscope slide chamber dish three times for 5 minutes each (with rotation) with 1x PBS at 4°C. Use enough PBS to submerge the slide completely.

11. 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 and incubate on the array for several hours at 4°C with gentle rotation. The incubation time will vary depending on antibody chosen, and this step may need optimization. Generally, two hours is sufficient. GST-fusion proteins are recommended. Several other commonly used protein tags (FLAG, His, Myc and V5) are printed on the array as positive controls for these epitopes. See **Appendix 1** for suggested reagents and usage.

12. Wash the array 3 x 5 minutes with 1x PBS at 4°C in the microscope slide chamber dish.

Note: To detect antibody binding using ECL techniques, go to page 16. "Detection Procedure - ECL."

13. Prepare a diluted solution of fluorescent secondary antibody (we recommend AlexaFluor™ 647) in hybridization buffer and incubate with the array for 30 minutes in the dark at 4°C with gentle rotation in the microscope slide chamber dish.

14. Wash the array in the dark in a microscope slide chamber dish three times for 5 minutes each (with rotation) with 1x PBS at 4°C. Use enough PBS to submerge the slide completely.

15. Holding the array with forceps from the **end without the logo**, dip the array several times (using a Coplin jar or small beaker) in 0.1x PBS to remove excess salt.

16. Dry the slide by either filtered forced air or centrifugation in a 50 ml conical tube at 800 xG for 2 minutes.

Appendix 1 - Suggested Additional Reagents

A list of suggested reagents that have worked well in combination with EpiTitan™ Histone Peptide Arrays.

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: Bethyl 6-His-Antibody rabbit polyclonal (Cat. No. A190-114A)

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-DYKDDDDK Tag Antibody for detection of FLAG-tagged proteins.

Antibody: CST Anti-DYKDDDDK Tag Antibody rabbit polyclonal (Cat. No. 2368)

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-Mouse fluorescent secondary antibody

Antibody: Invitrogen Alexa Fluor® 647 Anti-Mouse IgG (Cat. No. A-21235)

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

Incubation: 30' at 4°C (in dark) with rotation

Washes: 3 x 5' in PBS.

Anti-Rabbit fluorescent secondary antibody

Antibody: Invitrogen Alexa Fluor® 647 Anti-Rabbit IgG (Cat. No. A-21244)

Dilution: 1:10,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 30' at 4°C (in dark) with rotation

Washes: 3 x 5' in PBS.

Anti-Rabbit HRP Conjugate (for ECL detection)

Antibody: GE Life Sciences Donkey anti-Rabbit IgG, HRP-linked (Cat. No. NA934V)

Dilution: 1:4,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 30' at 4°C (in dark) with rotation

Washes: 3 x 5' in PBS.

Anti-Mouse HRP Conjugate (for ECL detection)

Antibody: GE Life Sciences Sheep anti-Rabbit IgG, HRP-linked (Cat. No. NA934V)

Dilution: 1:10,000 in PBS + 0.1% Tween and 5% BSA

Incubation: 30' at 4°C (in dark) with rotation

Washes: 3 x 5' in PBS.

ECL Substrate

Substrate: Thermo-Fisher Pierce SuperSignal West Femto (Cat No. 34095)

Exposure: 5 seconds to 2 minutes

Appendix 2 - Fluorescent Scanning Detection Procedure

The process requires a array scanner capable of at least 25 μM resolution. The following detection protocol is for use of a Typhoon Trio+ and ImageQuant TL software from GE, but other scanners can easily be adapted. The arrays have also been scanned and analyzed successfully on a Typhoon 9400 (GE), a GenePix 4000B (Molecular Devices/Axon Instruments) as well as the SureScan microarray scanner from Agilent. See the product page on the website to download the GAL file for use with Axon GenePix scanners.

1. Place the array slide face down in a microarray slide holder such as the GE #00-3759-30 AA. The slide holder allows for scanning of up to two arrays and places the array at a uniform height (+3 mm) above the glass for accurate data collection. Position the slide holder in the lower left corner of the scanner. It is not advisable to place the slide directly on the scanner glass.
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. Set the focal plane to + 3 mm, check the “Press Sample” box, and set the orientation as “Я”.
4. The scanning resolution should be set at 25 μM . It takes roughly 6 minutes per array to scan at this resolution. Higher resolution data can be collected at 10 μM if desired. See Figure 4 for an example of actual array data.

Analysis Procedure

There are numerous ways to analyze array data obtained. A simple qualitative approach and a more detailed statistical analysis are provided below.

A. Rapid qualitative analysis of results

Although statistical analysis is required for best interpretation of any peptide array-protein interaction, it is sometimes desired 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 "EpiTitan™ Array Grid v3.doc" found on the website to overlay on the raw image of the scanned array slide. The grid numbers correspond to specific peptides found on the second tab of the spreadsheet.

1. Paste a single array as a picture on a PowerPoint slide or any other program that allows manipulation of images (e.g. MS Word, Photoshop, Illustrator).
2. Copy the array grid (just the grid from with the peptide numbers, not the array image under it) from the "EpiTitan™ Array Grid v3.doc" file from the website and paste it as a picture on the same slide. See Figure 5 for an example of this.
3. Change the grid picture size and position to fit the array picture. You can also adjust brightness of the grid picture to get the best visual effect (right click and select 'Format Picture'). Each cell on the grid should cover three peptide spots on the array.
4. Positives will appear as bright red over the green spotting tracer background. Refer to the peptide list in the "EpiTitan Annotated Peptide List v3.xls" spreadsheet to identify which peptides appear as positive.
5. Peptides are spotted in series of three adjacent spots. A true positive result will be represented by all three peptide spots lighting up in the same rectangle. A single spot in a set of three is not a true positive result and should be discounted.

B. Quantitative analysis of results

For detailed analysis of your peptide array results, the raw signal intensities for each spot on a scanned array can be determined by densitometry using microarray analysis software such as GE's ImageQuant TL, as described below.

1. Open ImageQuant TL and click on "Array Analysis."
2. Open your scanned array file.
3. If necessary, adjust the contrast on Channel 1 (green) so all peptide spots are clearly visible.
4. For visual inspection, adjust the contrast on Channel 2 (red).

Note: Contrast adjustments have no effect on the quantification of data described below. However, the following quantification procedure 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" and create a 48 x 24 square grid (15 x 15 radius).
7. For analysis purposes, the array slide is divided into 4 sections (see Figure 2). Beginning with the cursor in the upper left spot on sub-array A, overlay the grid on Section 1 of the sub-array as depicted in Figure 3.

Note: Once the grid is created, it may be necessary to further align the grid over the 48 x 24 series of peptides in sub-array A section 1 by dragging each of 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 Section 2 (Figure 3). 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 sections 1 and 2 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 “EpiTitan Typhoon Analysis Template v3.xlsx.”

Note: The “EpiTitan Typhoon Analysis Template v3.xlsx” 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 removes false negative points in cases where peptides were not efficiently spotted. 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 the 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 peptide number in **EpiTitan Annotated Peptide List v3.xls**.

16. Copy columns Y-AB in the “EpiTitan Typhoon Analysis Template v3.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 “EpiTitan Annotated Peptide List v3.xls” 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 “AVG-sum” 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.

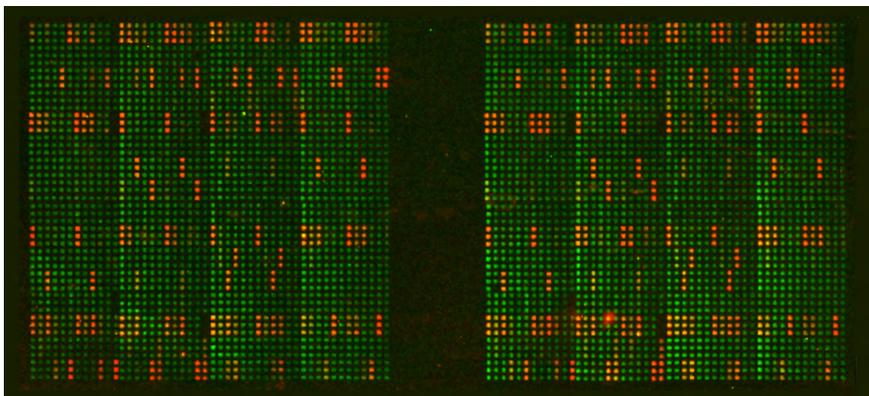


Figure 5. EpiTitan™ Histone Peptide Array Data.

Detection of histone modification binding of Histone H3 K4Me3 Antibody (Catalog No. 13-0004) using the EpiTitan™ Histone Peptide Array, scanned at 25 μ M resolution with Typhoon 9400 scanner (GE). The peptide spotting tracer appears green, and the H3 K4Me3 Antibody protein is shown in red. Authentic binding events appear as three adjacent red dots, indicating that the antibody has bound all three of the same peptide in the set.

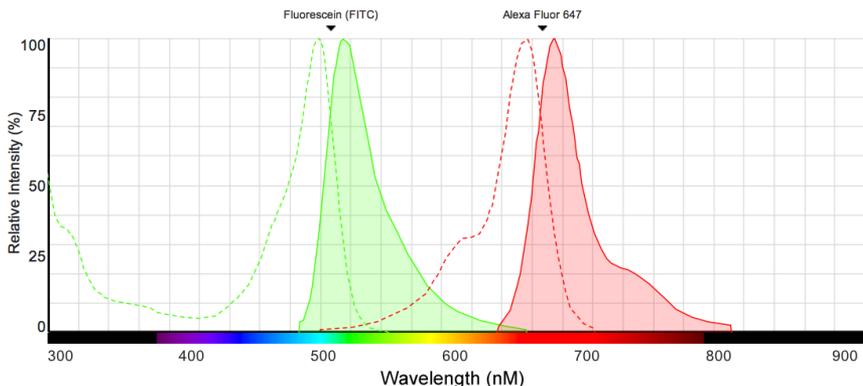


Figure 6. Fluorescent Spectra Comparison.

Fluorescent spectra of fluorescein (green) and AlexaFluor™ 647 (red). The absorption spectrum for each fluorophore is shown in dotted outline, while the emission spectrum is shaded solid. The separation of the emission spectrum of AlexaFluor™ 647 from that of the fluorescein-conjugated spotting tracer makes it ideal for detection of proteins and antibodies on the EpiTitan™ Histone Peptide Array.

Appendix 3 - ECL Detection Procedure

Detection of antibody or protein binding to the EpiTitan histone peptide array by ECL detection is very similar to developing a Western blot. At this point, you will have a primary antibody on the array, or have a binding protein with an antibody to the protein's epitope tag on the array. This protocol assumes usage of X-ray film to detect the ECL signal, but CCD camera gel documentation systems can be employed as well, provided that the camera resolution is sufficiently high.

1. Prepare a diluted solution of your HRP-conjugated secondary antibody in hybridization buffer and incubate with the array for 30 minutes in the dark at 4°C with gentle rotation in the microscope slide chamber dish.
2. Wash the array in the dark in a microscope slide chamber dish three times for 5 minutes each (with rotation) with 1x PBS at 4°C. Use enough PBS to submerge the slide completely.
3. Detect the HRP-conjugated secondary using SuperSignal West

Femto Chemiluminescent Substrate (Pierce / Thermo-Fisher Scientific) to do standard ECL Western Blot procedure. The use of other ECL substrates is possible, but WestFemto gives the most sensitive detection possible. Less sensitive ECL substrates will require longer exposure times.

4. Mix 100 μ l of each of the two substrate components (1:1) and put the mix on the array and then cover the array with a cover slip (or a 25mm x 60mm piece of parafilm).
5. Incubate at room temperature for 5 minutes.
6. Place the array between a clear plastic sheet protector.
7. Expose the array to an X-ray film in a darkroom using an X-ray film cassette.
8. Develop X-ray film to reveal binding of antibody or protein to the histone peptide array

Note: For X-ray film exposure, the method that works best is to place the array in the top right corner of the plastic sheet protector and tape the sheet protector containing the array (array facing down) to the lid of an 8" x 10" X-ray film cassette. You then put the film in the cassette so that you can expose the film just by opening and closing the lid. This reduces the chance that there will be any slippage during the exposure that will blur your film. Rotate the film 90 degrees between exposures to achieve four different exposures per sheet of film. Exposure time will vary depending on type of film and the strength of the signal, so use multiple exposures, between 5 seconds and 5 minutes per exposure.

Appendix 4 - Data Analysis with ImageJ Software

For basic analysis of antibody or protein binding data using a scanned image (fluorescent or ECL), we have created an array analysis template that allows comparison of relative binding to the peptides on the EpiTitan Histone Peptide Array using the freely available image analysis software package ImageJ. It is recommended that you fully familiarize yourself with this program before undertaking. You will also need an image manipulation program (such as Photoshop) that will allow you to resize and crop your scanned histone peptide array image.

To get started, **you will need to first download the following:**

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>

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. It allows you to measure spots on scanned images from the EpiTitan histone peptide array.

Familiarize yourself with the interface and the controls for the plugin prior to using it.

Visit http://www.optinav.com/MicroArray_Profile.htm

EpiCypher EpiTitan Analysis Template ver3.xls

This is an Excel spreadsheet for use in analyzing data gathered using ImageJ.

Visit <http://www.epicypher.com/epititan-array.aspx>

EpiCypher EpiTitan 48x48 grid ver2

This is a custom grid for use with ImageJ and the MicroArray Profile plugin.

See http://www.epicypher.com/epititan_array.aspx

Note: There are also tutorial videos specific for use with ImageJ, the MicroArray Profile plugin and EpiTitan array images.

Visit <http://www.epicypher.com/youtube.aspx>.

Also, there are additional tips for working with ImageJ at <http://www.epicypher.com/ImageJ.aspx>.

ImageJ Analysis Instructions

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 counter-clockwise 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 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 its 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, scroll down to “jars” and open “**MicroArray Profile**” plugin. This will launch the microarrays plugin and 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 EpiTitan array. The filename is **EpiTitan 48x48 grid ver2** and can be downloaded from the arrays file page on the EpiCypher website (http://www.epicypher.com/epititan_array.aspx). 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 EpiTitan Histone Peptide 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 correspond-

ing 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 **EpiTitan 48x48 grid ver2** 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 the EpiCypher website titled “**EpiCypher EpiTitan Analysis Template ver3.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. 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.
17. 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.
18. 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.
19. 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 EpiTitan Histone Peptide Array.
20. The same procedure can be used for the analysis of the second sub-array.

Appendix 5 - Use of the Multiwell Gasket

An improvement of the EpiTitan arrays over the previous histone peptide array platform includes the ability to perform multiple experiments per array using the multiwell gasket. The gasket facilitates the independent interrogation of each sub-array, to test different proteins, multiple dilutions of the same antibody, or to include a positive control with your experiment. It is important that the slide be dry before attempting to apply the gasket.

1. Remove the backing from the gasket with forceps. This reveals the adhesive that allows the gasket to adhere to the glass slide.
2. Align gasket with the top of the array to the edge of the slide **with the etched logo**. Gently press the gasket to the slide.
3. Turn the slide upside and place gasket side down on a flat surface and gently press down on the slide to seal the gasket completely.
4. With both ports open, you may begin the hybridization procedure. When performing any steps using the multi-well gasket, the volume per chamber is 450 μ l.
5. When you are finished with the primary hybridization, you can remove the gasket and perform the secondary incubation without the gasket in place.

Note: For more information on the use of the multi-well gasket, there is a video on its use on EpiCypher's Youtube page: <http://www.epicypher.com/youtube>

