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MODified Histone Peptide Array

I. Purpose

To screen histone peptide modification interactions and cross reactivity of antibodies used for Chromatin ImmunoPrecipitation (ChIP) protocol by using the MODified Histone Peptide Array by Active Motif.

II. Scope

All procedures are applicable to the BCGSC Library Technology Development and Library Core Groups.

III. Policy

This procedure will be controlled under the policies of the Genome Sciences Centre, as outlined in the Genome Sciences Centre High Throughput Production Quality Manual (QM.0001). Do not copy or alter this document. To obtain a copy see a QA associate.

IV. Responsibility

It is the responsibility of all personnel performing this procedure to follow the current protocol. It is the responsibility of the Group Leader to ensure personnel are trained in all aspects of this protocol. It is the responsibility of Quality Assurance Management to audit this procedure for compliance and maintain control of this procedure.

V. References

Reference Title	Reference Number
MODified Histone Peptide Array Manual	13001 and 13005

VI. Related Documents

Document Title	Document Number
Western Blot	LIBPR.0076

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VII. Safety

All Laboratory Safety procedures will be complied with during this procedure. The required personal protective equipment includes a laboratory coat and gloves. See the material safety data sheet (MSDS) for additional information.

VIII. Materials and Equipment

Name	Supplier	Number	Model or Cat.#	
1M Tris-HCl,pH 8.0	Ambion	AM9856		✓
10% Tween-20	Bio-rad	161-0781		✓
5M NaCl	In-house	n/a		✓
Ultrapure Water	Invitrogen	10977-023		

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Non-fat dried Milk powder	Bio-rad	170-6404		✓
Custom Made Slide Cassettes	In-house	n/a		
5mL serological pipettes	Costar	CS004487		✓
10mL serological pipettes	Costar	CS004488		✓
25mL serological pipettes	Costar	CS004489		✓
50mL serological pipettes	Costar	CS004489	✓	
15mL Conical tubes	BD Falcon	352097		✓
50mL Conical tubes	BD Falcon	352070		✓
Fisher Filter Forceps	Fisher Scientific	09-753-050		✓
Large Kimwipes (Kimberley Clark)	Fisher Scientific	06-666-1A		✓
Small size safetouch nitrile gloves	Ultident	296359683		✓
LabQuake Shaker/Rotator with Clips	Barnstead	415110		✓
Supersignal West Femto Maximum Sensitivity Substrate	Thermo Scientific	34095		✓
Goat Anti-Mouse HRP Conjugated Secondary Antibody	Thermo Scientific	32430		
Goat Anti-Rabbit HRP Conjugated Secondary Antibody	Thermo Scientific	32460	✓	
Portable Pipet Aid,Multispeed XP, Rechargeable	Fisher Scientific	13-681-15E	✓	✓
Black ink permanent marker pen	VWR	52877-310		✓
Wet Ice	In house	n/a		✓
Ice Bucket			n/a	n/a
Gilson P10 pipetman	Fisher	11-675-58		✓
Diamond Filter tips DFL10 (10 Tipacks of 96 racked filter tips)	Mandel	GF-F171203		✓
mall Autoclave waste bags 10"x15"	Fisher Scientific	01-826-4		✓
Galaxy mini-centrifuge	VWR	37000-700		✓
MODified Histone Peptide Array	Active Motif	13005		✓
5M Sodium Chloride	Ambion	AM9760G		✓

IX. Procedure

1. Buffer Preparation

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1.1. Prepare 500mL of TTBS Buffer containing 10mM Tris-HCl, pH 7.4, 0.05% Tween-20, 150mM NaCl, using Table 1 as a guide. Filter sterilizes the buffer once made. The buffer can be stored at room temperature once made.

Table 1: Preparation of TTBS Stock Buffer

Reagent	Stock Concentration	Volume to Add (mL)
Tris-HCl,pH 8.0	1M	5
Tween-20	10%	2.5
NaCl	5M	15
Ultrapure Water	n/a	Up to 500mL

1.2. Prepare Blocking Buffer using Table 2 as a guide. This buffer should be made fresh for the day of use and can be stored for a maximum of 24 hours at 4°C. Keep on ice if using the day of preparation.

Table 2: Preparation of Blocking Buffer

Reagent	Stock Concentration	Amount to Add
Non-fat dried milk powder	n/a	0.5g
TTBS Buffer	n/a	Up to 10mL

2. Blocking the Array

2.1. Add 3mL of blocking buffer directly to the array cassette (constructed by the Engineering Group).

2.2. Retrieve 1 array from the 4°C refrigerator and submerge into the dish containing the blocking buffer. Proceed to step 2.3 **OR** 2.4.

2.3. Incubate the array for **1-4 hours** on an orbital shaker at room temperature. Ensure the shaking is vigorous or gentle enough to ensure thorough mixing of the blocking buffer while the array is still entirely submerged in the buffer. Do not let the slide dry out once placed in the buffer.

2.4. Incubate the array **OVERNIGHT** on an orbital shaker at 4°C. Ensure the shaking is vigorous or gentle enough to ensure thorough mixing of the blocking buffer while the

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array is still entirely submerged in the buffer. Do not let the slide dry out once placed in the buffer.

2.5. Decant the blocking buffer. Perform a quick rinse with TTBS buffer for 30 sec and decant.

2.6. Add 5mL of TTBS buffer to the array dish holding the array.

2.7. Shake on orbital shaker for 5 minutes. Decant.

2.8. Repeat Steps 2.6 and 2.7 two more times for a total of 3 washes. Do not decant on the last wash.

2.9. During the last wash, prepare the primary antibody dilution in 3mL of blocking buffer. Follow the manufacturer's recommendations listed for a western blot dilution.

3. Probing with Primary Antibody

3.1. Ensure the TTBS buffer is completely decanted from the last wash step.

3.2. Add the diluted primary antibody (from Step 2.9) to the array dish holder containing the array.

3.3. Incubate 1 hour at room temperature with shaking on the orbital shaker.

3.4. Decant the diluted primary antibody. Perform a quick rinse with TTBS buffer for 30 sec and decant.

3.5. Add 5mL of TTBS buffer to the array dish holding the array.

3.6. Shake on orbital shaker for 5 minutes. Decant.

3.7. Repeat Steps 3.5 and 3.6 two more times for a total of 3 washes. Do not decant on the last wash.

3.8. During the last wash, prepare the secondary antibody dilution in 3mL of blocking buffer. Follow the manufacturer's recommendations listed for a western blot dilution. Ensure the

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secondary antibody is HRP (horseradish peroxidase conjugated) and the correct species specificity.

4. Probing With Conjugated Secondary Antibody

4.1. Ensure the TTBS buffer is completely decanted from the last wash step.

4.2. Add the diluted conjugated secondary antibody (from Step 3.8) to the array dish holder containing the array.

4.3. Incubate 1 hour at room temperature with shaking on the orbital shaker.

4.4. Decant the diluted secondary antibody. Perform a quick rinse with TTBS buffer for 30 sec and decant.

4.5. Add 5mL of TTBS buffer to the array dish holding the array.

4.6. Shake on orbital shaker for 5 minutes. Decant.

4.7. Repeat Steps 4.5 and 4.6 two more times for a total of 3 washes. Do not decant on the last wash.

4.8. Carefully wrap the dish with saran wrap to prevent spilling during transport to the CRC for imaging.

5. Detection of the Array (Done at CRC Site)

5.1. Bring the following to the CRC for developing:
-Pierce substrate
-SuperSignal West Femto Maximum Sensitivity Substrate
-1mL pipettor and 1ml barrier tips
-15mL falcon tube
-KimWipes
-Timer
-2 Pair of Gloves
    -2 pieces of mylar

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5.2. At the CRC, put on disposable lab coat and gloves. Log into the computer connected to the ChemiDOC System (Figure 1). Double click on the Image Lab icon to initialize the software.

Figure 1 ChemiDoc System

5.3. Prepare Detection solution by mixing equal parts of the Stable Peroxide Solution and the Luminol/Enhancer Solution. 1mL of detection solution is more than sufficient for 1 array. Prepare in a 15mL falcon tube. Ensure the solution is mixed well. The Detection solution is stable up to 8 hours at room temperature. Typical laboratory lighting will not harm the Detection solution.

5.4. Unwrap the cassette holding the array. Pour off the wash buffer slowly. **Add the Detection solution to the array immediately ONLY PRIOR TO SCANNING.** Using a P1000 pipette, gently add the detection solution directly to the slide and only enough to cover the slide. The slide does not have to be saturated or immersed in the solution. With gloved hands, transfer the array to a piece of mylar sheet. **Immediately** proceed to the next step.

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5.5. Follow Steps 8.2.1-8.2.10 of LIPBR.0076 on how to use the ChemiDOC System to image the array.

5.6. Take a screenshot of the array image and save it as a **jpeg file**. Save the image with the name of the primary antibody and date in a pre-labeled folder in R:\\filer05b\geneexplab\Library Core\Epigenomics\Peptide Array\(1° Antibody Name).

5.7. Once the image is saved, exit out of the software and log off of the computer.

5.8. Remove the blot from the imager deck and wipe down the deck with dH_20. Tidy up area and place all supplies and reagents back into transporting bag. Discard gloves and disposable lab coat.

6. **Imaging and Analysis of Array**

6.1. Ensure the array analysis software downloaded on the computer. Software can only be downloaded on a PC.

6.2. Open the Array Analysis program.

6.3. Select Open Slide and upload the file.

6.4. Use the mouse to click and drag a rectangle around the border of the array image, making sure to leave a small amount of space outside of the spots.

      a. Click the Crop button (the original file will not be modified).

Select the option "Dark Spots on Light Background" or "Light Spots on Dark Background" which corresponds to the image type.

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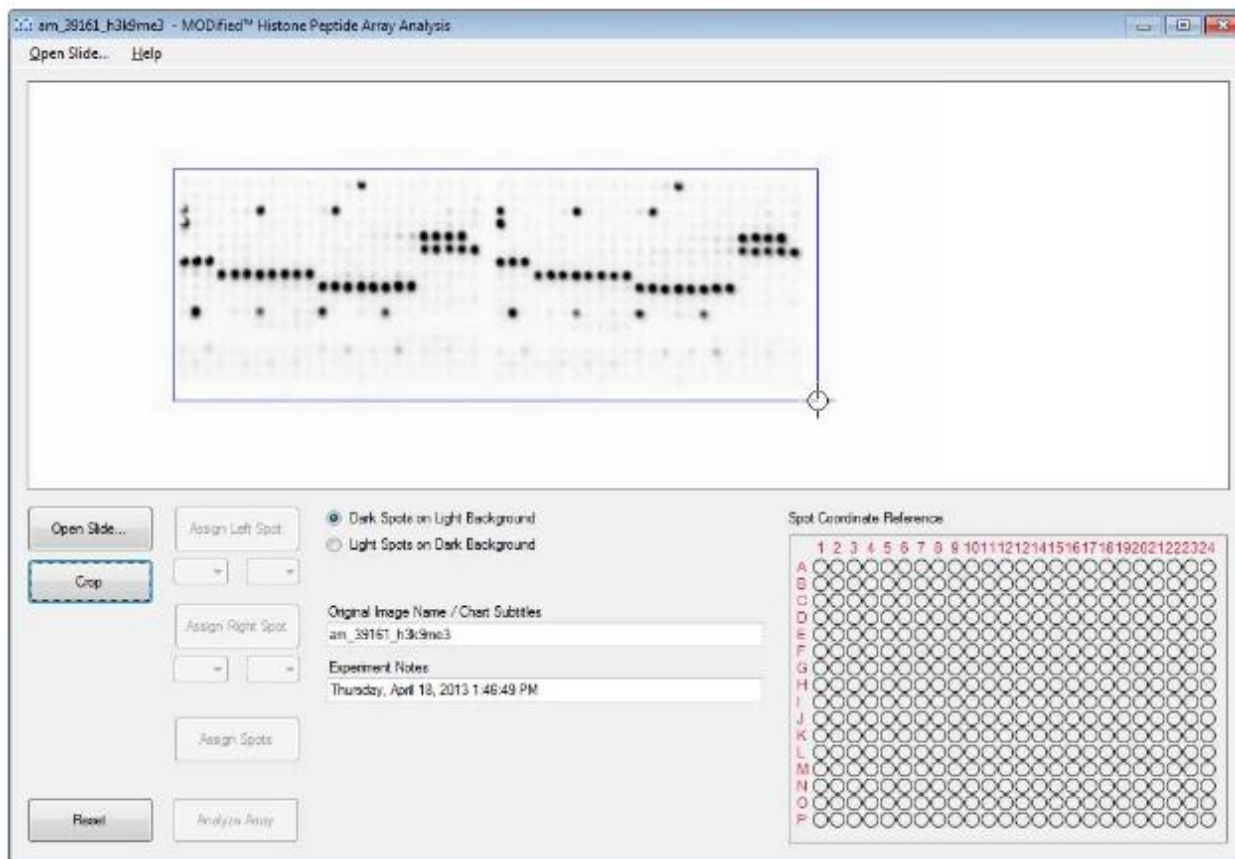**Note: Controlled Versions of this document are subjected to change without notice*

Figure 2. Example of Array To be Analyzed And Cropped

6.5. In order to align the array reference grid with the slide, two spots will need to be identified. It is best if spot 1 is in the upper left corner of the left half of the array and spot 2 is in the lower right corner of the right half of the array. However, in the case that only a few spots show up, take care to correctly identify their location and use those spots as the reference spots.

    a. Align the cursor with the center of a spot on the upper left side of the left array.
    b. Click on the center of the spot.
    c. Use the drop down menu to select the coordinates of the selected spot
    d. Click the **Assign Left Spot** button.
    e. Repeat for the lower right side of the right array and click **Assign Right Spot** button.

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6.6. Once the left and right side of the array have been assigned, press the **Assign Spots** button. The grid image will be overlaid onto the array image. Verify the grid is in alignment with the array spots. If not, click the **Reset** button and repeat spot alignment.
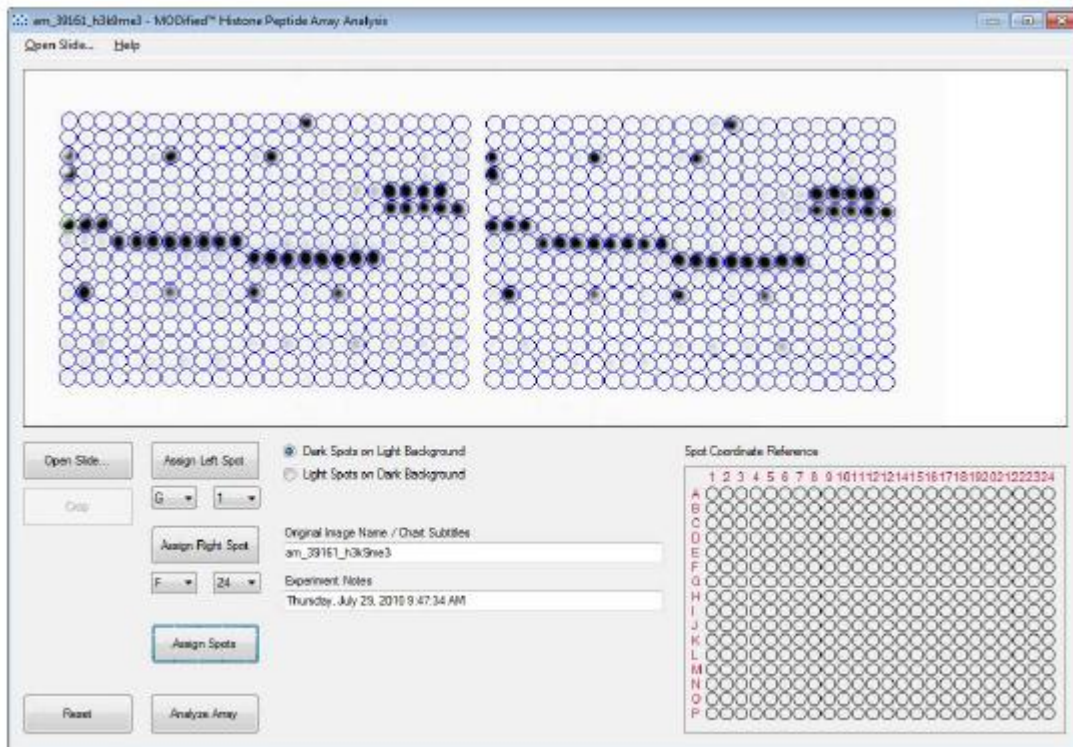

Figure 3. Alignment of Spots on Grid

6.7. If these steps are performed out of sequence, the program will not respond.

6.8. Click the **Analyze Array** button. A new tabbed Window will open containing the analysis results.

6.9. To save any of the results, select one of the Save options from the File menu. "Save All" saves results from all the tabs, or it is possible to save the output displayed on each tab individually. **By default, the results will be saved in the same directory as the original image file.**

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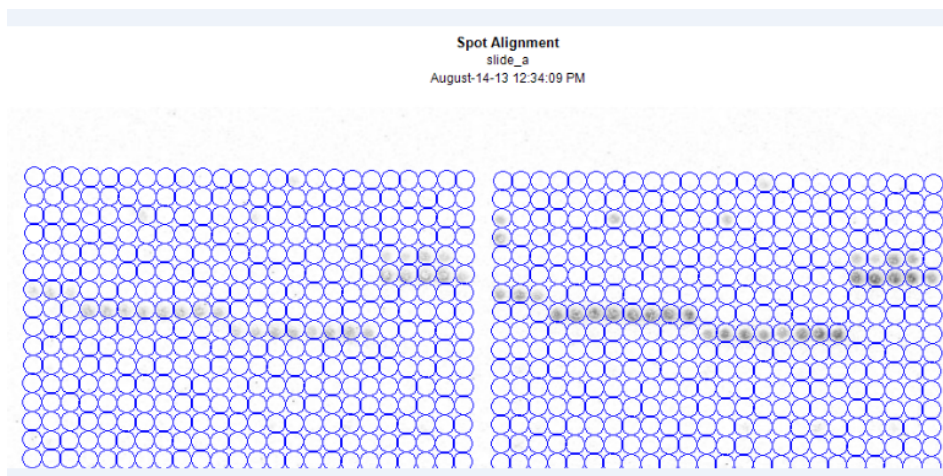6.10. The Spot Alignment tab contains an image of the grid overlay. See Figure 4.

Figure 4. Spots Aligned on Grid

The **Spot statistics** tab gives a comparison of the intensities of each spot in the left and right array (left image). The data points ideally should be on a straight line connecting 0,0 and 1,1 which indicates perfect duplicates. It is ideal to have most of the peptides contain an error range of 0-5% (which means very little variation between the left and right sides), as seen in Figure 5 below.

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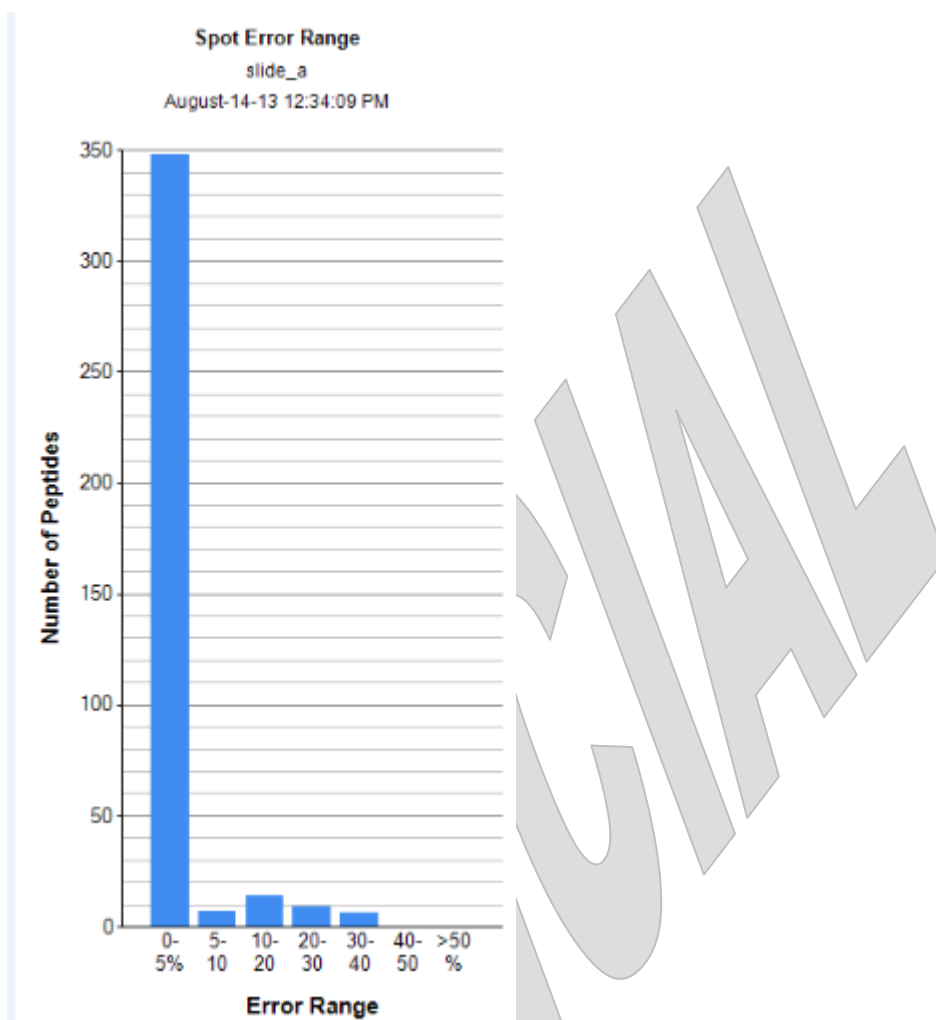

Figure 5. Example of Spot Statistics Graph

6.11. The Background Setting tab defines the background for the calculation of the Specificity, Reactivity and Modification Analysis profiles. **DO NOT** adjust the default parameters of this setting.

6.12. The Specificity tab displays the background subtracted graph of the 10 modifications with the greatest specificity factors. Only the 10 modifications with the highest specificity factor values will be displayed. The **Multiple Peptide Array** calculates the

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ratio of the average intensity of all spots containing the mark divided by the average intensity of all spots not containing the mark. An example of a Multiple Peptide Array can be seen in Figure 6. **The Multiple Peptide Array should be sent to supervisors.**
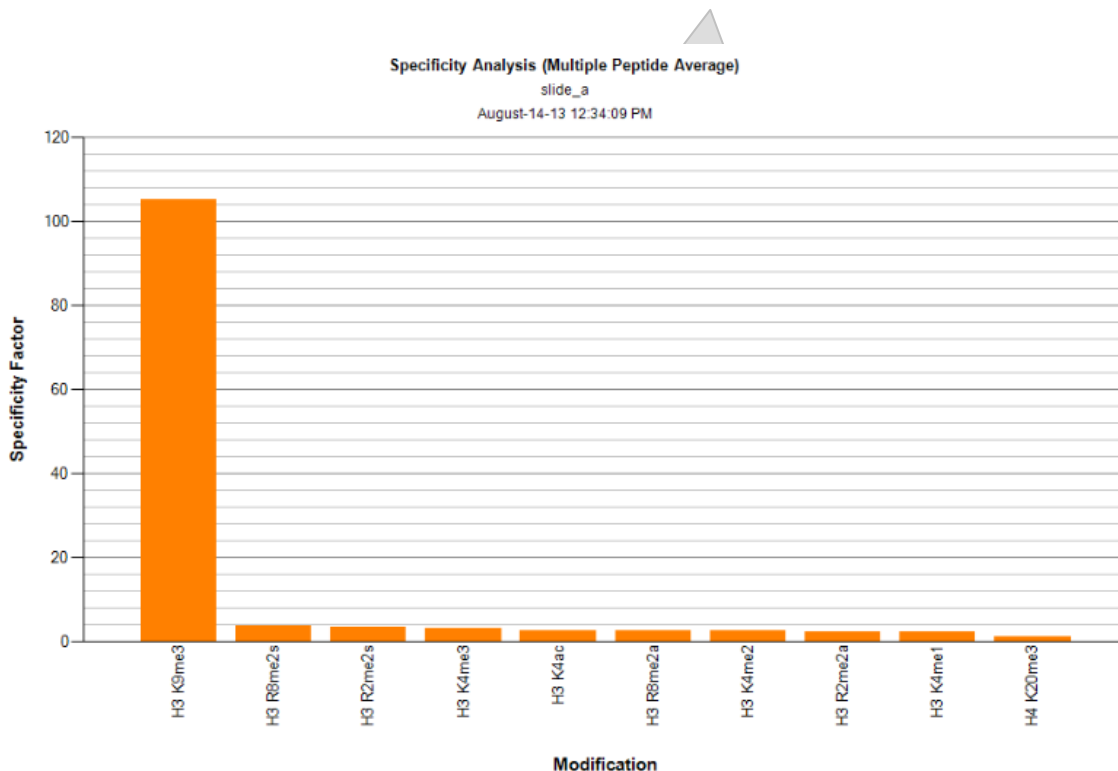

Figure 6. Example of a Multiple Peptide Average Graph

6.13. The **Single Peptide Intensity** option displays only single modified peptides without averaging all spots containing the mark. Whereas the Multiple Peptide Average plot provides a general overview of specificity, the Single Peptide Intensity plot reveals a more precise and exact indication of specificity of the antibody in question. **The Single Peptide Intensity Plot should be sent out to supervisors to determine whether the antibody can be put into production.** See Figure 7 of an example of a Single Peptide Intensity Graph.

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Figure 7. Example of a Single Peptide Intensity Plot for H3K9me3 Antibody

6.14. The **Reactivity tab** displays the background subtracted intensity values for all single modified peptides, as well as for the control spots. See Figure 8 for an example.

Figure 8. Example of a Reactivity Plot

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6.15. The **Modification Analysis** tab displays the background subtracted intensity values for all spots containing the selected.

6.16. The **Annotated Array** tab allows the user to select up to three modifications to be displayed in superposition to the experimental data. The modification selections can be order to see the array grid overlay or to identify the modifications on both sides of the array. Use the colour coding to identify the modifications. The annotated array also helps align the grid to the experimental data. The user can use this tab as a guiding tool for grid alignment. See **Figure 9** below for an example.
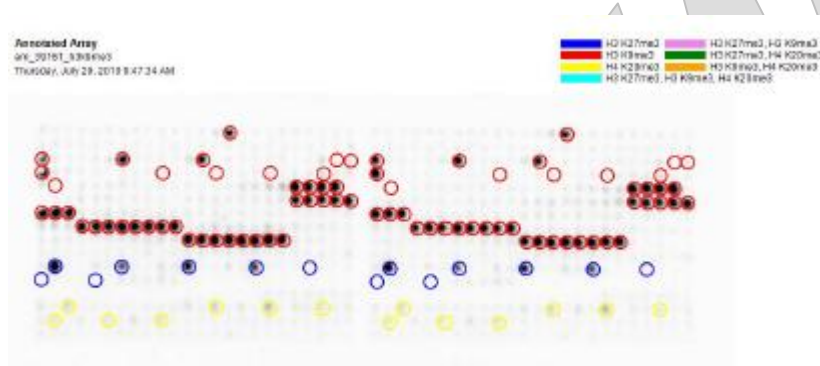

Figure 9. Example of an Annotated Array Overlay

6.17. To start a new analysis or edit the analysis of the current image, select **Open Image** from the file menu.

6.18. To close the program, click the 'X' in the upper right corner of the program.

6.19. Email the results of the top 10 Single Peptide Intensity histone modifications graph and the Multiple Peptide Average Array graph to supervisors. Including all pertinent information such as Antibody name, Manufacturer, Catalogue Number, Lot Number and the dilution factor of the antibody.

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Appendix A. Worksheet

The worksheet can be found by following path: R:\\filer05b\geneexplab\Library Core\Epigenomics\Peptide Array\Worksheets\Peptide_Array_Worksheet_Template. Save As a copy of the template worksheet with the name of the antibody and date.