HiLite™ Histone H3 Methyl-Lys9 / Lys27 Binding Assay

(version A1)

Catalog No. 57001

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Overview

DNA is organized by its incorporation into chromatin; 147 base pairs of DNA are coiled around an octamer of core histone proteins to form the basic subunit of chromatin, the nucleosome. A large body of evidence has accumulated to indicate that post-translational modifications of histone proteins are crucial to all genome-based activity. Specific functional groups (phospho-, methyl-, acetyl-, ubiquityl-) are added or subtracted from histone proteins, and these dynamic addition and subtraction events have profound affects on the function of chromatin.

Recent biochemical findings indicate that modified histones recruit specialized chromatin-interacting proteins that facilitate the defined function conferred by the histone modification. It is believed that these histone-effector protein interactions give rise to downstream protein recruitment, as well as enzyme and substrate interactions. Several classes of histone modification-binding modules have been identified, such as the bromo domain (binds to acetyl-histones), chromo domain (methyl-histones) and the MBT domain (methyl-histones), to name a few.

Identifying proteins that interact with modified histones is of great interest to the research community, as such proteins are often important regulators of genome function, transducing the histone modification to generate a specific cellular outcome. For example, HP1 and Polycomb proteins have each been shown to bind to histone H3 that has been methylated at either lysine 9 or lysine 27. Methylation of these residues is involved in specifying regions of the genome that are heterochromatic and transcriptionally silent, and both HP1 and Polycomb are known to be involved in maintaining heterochromatic regions.

With Active Motif's HiLite™ Histone H3 Methyl-Lys9 / Lys27 Binding Assay, you can use fluorescence polarization to determine if your specific protein of interest binds histone H3 that is mono-, di- or trimethylated at either lysine 9 or 27. You can also measure the affinity of binding interactions of your protein to these modifications.

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Introduction to Fluorescence Polarization Assays

Fluorescence Polarization (FP) is based on the observation that when fluorescent molecules in solution are excited with polarized light, they will emit light back as polarized light as long as they remain stationary during the excitation of the fluorophore. Small molecules such as peptides, however, rotate and tumble rapidly in solutions, and the plane of polarization of the emitted light can be very different from the plane used for the initial excitation. The polarization of a molecule is proportional to the molecule's rotational relaxation time, or basically how fast the molecule is spinning in solution. Rotational relaxation time is related to viscosity, temperature and molecular volume. If a molecule is very large, little movement occurs during excitation and the emitted light remains highly polarized in the initial plane of excitation. If a molecule is small, rotation and tumbling is faster and the emitted light is depolarized relative to the excitation plane.

Fluorescence polarization readers excite fluorescent molecules with polarized light and measure the emitted light in both a parallel and a perpendicular polarization plane. Large fluorescent molecules, which move comparatively slowly, emit a greater percentage of light in a direction that is generally parallel to the excitation source. Small molecules rotate quickly during the excited state, and upon emission, have low polarization values. A small molecule, such as a peptide, can be "converted" into a larger molecule through the binding of a larger protein. When the larger molecule binds to a smaller molecule there is a reduction in the amount of rotation during the excited state. This results in higher polarization values. Because these assays are homogenous, there is no need to remove unbound fluorophore. Consequently, fluorescence polarization can be used to differentiate bound and unbound fluorophore. This change in polarization can be measured and used to calculate the amount of binding that has occurred. In the case of this assay, the amount of binding can be used to determine the specificity of a particular protein for methylated histone tail peptides.

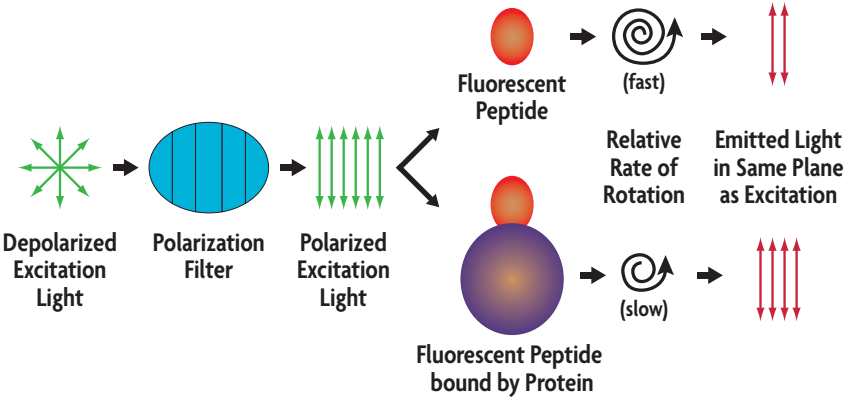


Figure 1. Overview of the theory of fluorescence polarization. See text for details.

The polarization value depends on the size difference between the protein and the peptide. The smaller the peptide and the bigger the binding protein, the larger the change in the P value (Polarization) measured. Based on the size of the peptides in this kit, it should be possible to determine binding equilibria for proteins that range from 10 kDa to 100 kDa. The upper limit is due to the particular properties of the dye and fluorescence, while the lower limit is due to the need for a difference in size between the free ligand and the receptor-ligand complex. The method detects changes in molecular volume, not specifically changes in molecular weight.

By titrating the amount of the larger molecule (*i.e.* protein) in relation to the smaller molecule (peptide), a binding curve can be generated (the amount of polarization observed is proportional to the amount of protein complex formed, which is proportional to the concentration of the binding partners in solution). Curve fitting can be used to determine the K_d and mathematical models can be applied to this binding curve to determine the binding constant of the protein:peptide interaction.

$$A = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \qquad P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

A = anisotropy (the property of being directionally dependent, as opposed to being homogeneous in all directions)

P = polarization

mP = P/1000 (milliP)

$\mathbf{I}_{\parallel}$ = fluorescence intensity parallel to the excitation plane

$\mathbf{I}_{\perp}$ = fluorescence intensity perpendicular to the excitation plane

The theoretical **P** for assays is-0.33-0.5.

The typical range for **P** in biological systems is 0.01 to 0.3 P (or 10 to 300 mP).

The measurement range is small but modern instruments have a high degree of precision, so it is possible to measure **P** +/- 0.002 or +/- 2 mP.

Converting **P** values to **A**:

A = 2 x **P**/3 – **P**

HiLite™ Histone H3 Methyl-Lys9 / Lys27 Binding Assay Advantages

- All the reagents and a detailed protocol have been developed to help you perform methyl-histone binding assays to examine proteins that bind to either methyl-Lys9 or methyl-Lys27 of histone H3.
- A positive control protein (HP1) is included to get you started doing FP assays.
- FP is a homogeneous technology with no washing steps. This increases speed and precision.
- Reactions are very rapid, generally taking only seconds to minutes to reach equilibrium.
- Using this kit, you can measure the dissociation constant (K_d) of your protein binding to methyl-Lys9 or methyl-Lys27 of histone H3.
- The reagents are stable and may be prepared at one time, resulting in high reproducibility.
- Other homogeneous technologies based on fluorescence intensity have been developed such as energy transfer, quenching, and enhancement assays. FP offers several advantages over these including the fact that only one tracer is required and the fluorophore does not have to respond to binding events by undergoing a change in intensity.
- FP experiments are done in solution without solid support, allowing true equilibrium analysis.
- FP is independent of intensity and so works in colored solutions and cloudy suspensions.
- FP experiments are non-perturbing, so the influence on binding of multiple factors can be analyzed on the same samples (*e.g.* changes in pH, temperature, salt concentration, *etc.*).
- FP is a fundamental property of the molecule, and the reagents are stable, so little or no standardization is required.
- FP is relatively insensitive to instrument changes such as drift, gain settings, lamp changes, *etc.*
- Fluorescence intensity may be obtained in addition to polarization, if desired. FP can be used to quantify interactions over a wide range of dissociation constants.

Kit Performance

In order to test the binding of your protein of interest, the kit contains methylated and unmethylated peptides corresponding to regions of histone H3 around lysine 9 and lysine 27. It also contains a positive control protein to ensure that you are performing the assay correctly before testing your protein of interest.

The kit contains five 96-well half area black polystyrene plates. One of the plates is for checking the detection of the fluorescence signal on the microplate reader and for performing a binding curve for the positive control protein; the remaining four plates are for the assay that will be performed with your protein of interest.

The kit requires the use of an fluorescence polarization plate reader, such as the Tecan Infinite 200. Please ensure that your machine is in good working order and that you are well versed in the operation and theory of your particular machine before performing this assay.

This kit requires a data analysis and graphing software package to perform curve-fitting following the acquisition of binding data. Kaleidagraph (Synergy Software) is such a program and was used in the development of this kit. Other such software packages include SigmaPlot, GraphPad Prism and OriginPro.

References

Heyduk, T. *et al.* (1996) *Meth Enz* 274: 492-503.
Fischle, W. *et al.* (2005) *Nature* 438: 1116-1122.
Jacobs, S.A. *et al.* (2004 *Meth Enz* 376: 131.
Lundblad, J.R. *et al.* (1996) *Molec Endo* 10: 607-612.

Kit Components and Storage

Please store each component at the temperature indicated in the table below. All of the fluorescent peptides are conjugated to fluorescein and the concentration of the stock solutions will be 20 µM after reconstitution.

Reagents	Quantity	Storage / Stability
H3 Lys9 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 monomethyl Lys9 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 dimethyl Lys9 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 trimethyl Lys9 peptide, fluorescent conjugate	60 µl	-20°C for 6 months
H3 Lys27 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 monomethyl Lys27 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 dimethyl Lys27 peptide, fluorescent conjugate	40 µl	-20°C for 6 months
H3 trimethyl Lys27 peptide, fluorescent conjugate	60 µl	-20°C for 6 months
Binding Buffer AM10	2 x 20 ml	-20°C for 6 months
1 M DTT	100 µl	-20°C for 6 months
Calibration dye, 50 µM	30 µl	4°C for 6 months
HP1 protein, 1 mM	80 µl	4°C for 6 months
Black half area 96-well plates	5	Room Temperature

HP1 protein is supplied as a frozen solution. If the kit is not to be used upon delivery, HP1 should be stored at -20°C. **Once the HP1 protein has thawed it must not be frozen again, and should thereafter be stored at 4°C.** The protein is stable for up to six months at 4°C.

Peptides are supplied lyophilized and should be stored at -20°C until needed. Peptides should then be re-constituted according to the instructions in the manual. The quantity shown above for each peptide reflects the volume after reconstitution. Once they have been re-suspended, the peptides should be stored at 4°C in the dark until use. Long-term storage at 4°C is not recommended. Stocks should be stored at -20°C if the kit is not used within a day of re-suspending the peptides. Repeated freeze-thaw steps are not recommended.

Additional materials required

- Distilled water
- 1.5 ml Eppendorf tubes
- 50 ml conical tube
- 1 M NaOH
- Ice
- Aluminum foil
- Pipettes capable of dispensing 1 to 25 µl volumes with accuracy
- Microplate reader equipped with 485 (+/-20) nm excitation filters, 535 (+/-25) nm emission filters, and polarizers
- A suitable data analysis and graphing and curve-fitting program (*e.g.* Kaleidagraph)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

The first section of this protocol describes how to reconstitute the lyophilized peptides and determine the optimal final concentration of peptide that will be needed. All peptides should be used at the same final concentration.

For most commercially available fluorescence polarization scanners that have a sensitivity of 2 nM fluorescein, a final peptide concentration of 200 nM per well will be sufficient. This is the peptide concentration that was used to develop this assay.

If the sensitivity of the instrument is not known, or if the G factor of the instrument has not previously been determined, it may be necessary to perform the preliminary calibration step outlined in Protocol A on page 10.

In addition to calculating the G factor, the calibration step is useful in determining whether the instrument being used is sensitive enough to read low peptide concentrations (100-200 nM) or whether a higher concentration should be used (400-500 nM).

Sequence of steps in the assay:

1. Calibrate instrument with calibration dye (optional).
2. Peptide reconstitution.
3. Final dilution of peptides (working stock).
4. Preparation of the binding buffer.
5. Generate binding curves with positive control protein.
6. Preliminary dilution series of the protein of interest (optional).
7. Perform binding assay with protein of interest and all peptides.

Points to consider:

- This kit should only be used with recombinant or purified proteins where the concentration of the protein of interest is known. The kit should not be used with crude extracts or other samples where the concentration of the protein is not known, or where there may be contaminating proteins.
- Ideally, the larger macromolecule (*e.g.* HP1 protein) should be serially diluted to cover a concentration range of 100-fold below to 100-fold above the K_d of the interaction being studied. The peptide concentration remains the same throughout the assay.
- It is essential to keep the fluorescently labeled ligand (*e.g.* the fluorescent histone tail peptide) at least 10-fold (preferably 100-fold) lower in concentration than the K_d; failure to do so will result in inaccurate affinity calculations.

- If you get unexpected values, it may be worth checking that the protein of interest being assayed does not fluoresce, as this will influence the assay. You can perform such a trouble-shooting experiment using any of the unused rows on Plates 3-5. Anisotropy values in such "buffer + protein only" wells (any wells without fluorophore) will be very high due to scatter.
- Protect the fluorescently labeled peptides from light at all times by covering the microplate with aluminum foil.
- Binding Buffer AM10 is optimal for the peptides and positive control protein provided in this assay and can be used with almost any protein, though some proteins of interest may not be stable in this buffer. It is possible that you will need to alter buffer AM10 to suit the needs of your experiments. All protein:protein interactions are sensitive to a variety of factors, including salt concentration and pH, so consistency across your experiments is required.
- Fluorescence polarization is generally independent of concentration within the detectable range of the instrument. Thus, a plot of mP as a function of fluorophore concentration is expected to result in a straight line with a slope of 0.
- Fluorescein has a mP value of about 22-30 depending on the temperature and the buffer used.
- Binding curves are plotted using anisotropy values rather than polarization because anisotropy is an additive, molecular parameter, while polarization is not.
- The quality of results obtained is directly linked to the quality of the instrument. The performance of FP readers may vary and as optical components age, the detection efficiency can diminish and measured polarization may not be optimal.
- About microplate readers: In order for the assay to perform to a high standard, it is important that the reader being used to measure fluorescence polarization meet certain sensitivity criteria and have been properly calibrated using fluorescein to determine the G factor. A sample of fluorescein is provided with this kit for the purpose of calibrating the instrument if this has not been done previously. Instrument requirements for this kit include filters to excite your sample at 485 nm and to read the emission at 535 nm. It is essential to have the correct polarizers in place together with these filters. Please contact your instrument sales representative prior to using this kit if you are not sure that your machine has the correct setup. This kit was developed on a Tecan Infinite 200 with the following filters and polarizers:

  Excitation 1: 485 (+/- 10) nm – parallel

  Excitation 2: 485 (+/- 10) nm – parallel

  Emission 1: 535 (+/- 12.5) nm – parallel

  Emission 2: 535 (+/- 12.5) nm – perpendicular

A. Instrument Calibration (Optional)

This step is optional, but recommended. If the sensitivity and specifications of the instrument to be used are known then it is possible to proceed with the assay protocol and omit this instrument calibration step. If you are following our Suggested Microplate Layouts, this step is performed as Plate 1, rows A and B, which is detailed in Figure 2 on page 20.

Calibration dye is included in this kit so that the G factor for an instrument may be calculated. It is also used to determine the ability of the microplate reader to detect the signal from the fluorescent peptides used in the assay. It is suggested that a 1 nM solution of calibration dye is prepared and used to fill the first two rows on one of the black plates. This will allow one to see any well to well variability that may occur. Alternatively, a serial dilution of calibration dye may be made to test the sensitivity of the instrument at low concentrations.

Note: Do not discard Plate 1 after performing the peptide dilution in rows A and B. This plate is needed to perform the subsequent binding curves with positive control protein in Protocol D. Mark the plate so that it does not get confused with the other unused plates.

The following protocol is used to perform a scan with 1 nM calibration dye in the first two rows of the plate:

1. Reconstitute the calibration dye by adding 30 µl distilled water. Pipette the solution up and down several times to ensure that all the material has dissolved. Protect the dye solution from light.
2. Prepare a 0.01 M NaOH solution by adding 500 µl of 1 M NaOH to 49.5 ml distilled water.
3. Add 1 µl of the calibration dye solution to the 0.01 M NaOH. **Store the remaining calibration dye in the brown vial at 4° for future use.**
4. Mix the solution well by inverting the tube several times.
5. Pipette 25 µl of the solution into all of the wells on rows A and B of Plate 1.
6. Read the plate on the plate reader using "optimal gain" or similar setting. (The instrument should sample the fluorescence from all wells and then determine the gain before reading the plate again.)
7. Ideally for 1 nM calibration dye, the gain should fall between 80 and 120 and the fluorescence intensity measured should be between 10,000 and 100,000.

If desired, the calibration dye can also be used to prepare a serial dilution in a black 96-well plate (provided by the customer, not part of the kit). The concentration of calibration dye stock solution is 50 µM after resuspension.

B. Peptide Reconstitution

Note: It is very important to closely follow the instructions for reconstituting and diluting the peptides. Please be aware that there are two steps to diluting the peptides. The first, which is performed now, is to reconstitute each lyophilized peptide, which will result in stock solutions of 20 µM each. The second step, making working stocks, will be performed in step 4 below.

1. Resuspend the lyophilized peptide samples by adding 40 µl distilled water to the tubes labeled H3 Lys9, H3 monomethyl Lys9, H3 dimethyl Lys9, H3 Lys27, H3 monomethyl Lys27 and H3 dimethyl Lys27. Pipette the solution up an down several times to ensure that all the material has been resuspended.
2. Add 60 µl distilled water to the tubes labeled H3 trimethyl Lys9 and H3 trimethyl Lys27. Pipette the solution up an down several times to ensure that all the material has been resuspended. All the peptides will now be at a stock concentration of 20 µM.
3. Keep peptides in the dark at 4°C until ready to use. Use a table-top centrifuge to spin the samples briefly before use to ensure that the solution is not stuck in the cap or on the walls of the tube.

   Note: If the assay will not be performed on the same day that the peptides are reconstituted, they should be stored at -20°C.

Table 1

Peptides to Reconstitute with 40 µl H_2O	Peptides to Reconstitute with 60 µl H_2O
H3 Lys9 (unmodified)	H3 trimethyl Lys9
H3 monomethyl Lys9	H3 trimethyl Lys27
H3 dimethyl Lys9	
H3 Lys27 (unmodified)	
H3 monomethyl Lys27	
H3 dimethyl Lys27	

4. See Appendix Section A for preparing working stocks of the peptides. Working stocks should only be stored for a few hours, so should be made when you are ready to perform the assay.

C. Preparation of the Binding Buffer

Note: Do not prepare the Binding Buffer until you are ready to dilute the proteins and begin the assay.

Binding Buffer AM10 (pH 8.0) must be supplemented with DTT and then used to dilute the positive control protein and other protein(s) that will be assayed. Store the Binding Buffer AM10 and vial of DTT at -20°C. Add the DTT just before you dilute the proteins, which should be done just before you are ready to begin the assay.

The concentration of DTT stock solution is 1 M and a final concentration of 2 mM will be used. When you are ready, thaw Binding Buffer AM10, then prepare the amount of binding buffer needed for the assay by adding 2 µl of the 1 M DTT per ml of Binding Buffer AM10. (If you are using the entire kit at one time, add 40 µl of the DTT solution to 20 ml of thawed Binding Buffer AM10.)

Binding Buffer AM10 plus DTT should be kept on ice and used within the time needed to perform the assay. Discard any remaining Binding Buffer AM10 plus DTT as it cannot be used later. If only part of the kit is to be used at one time, then the appropriate amount of DTT should be added to a portion of Binding Buffer AM10; the remaining Binding Buffer AM10 should be stored at 4°C until needed. Any unused DTT should be stored at -20°C until needed.

D. Performing the Binding Curves with Positive Control Protein

The chromo domain from the *Drosophila* HP1 protein provided can be used as a positive control for binding to histone H3 peptides methylated at lysine 9 and a negative control for histone H3 peptides methylated at lysine 27. If you are following our Suggested Microplate Layouts, this step is performed as Plate 1, rows C-E and rows F-H, which is detailed in Figure 2 on page 20.

Note: At this stage, the working peptide solutions (2.5, 5, 7.5, 10 or 12.5 µM) should have been prepared according to the instructions in Appendix Section A. When 1 µl of peptide working stock is added to 24 µl of protein sample, the final concentration of peptide in each well will be 100, 200, 300, 400 or 500 nM, respectively.

1. After adding DTT to Binding Buffer AM10, place the Binding Buffer AM10 plus DTT on ice.
2. Label 12 Eppendorf tubes (1.5 ml) from 1-12. They will end up with the following concentrations of HP1 protein: 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39, 0.19 and 0.09 µM.
3. Place the tubes on ice.
4. Add 160 µl Binding Buffer AM10 plus DTT to the tubes labeled 2 to 12 and return to the ice.
5. Add 256 µl of Binding Buffer AM10 plus DTT to tube 1.
6. Add 64 µl of the positive control protein to this tube. Mix well by vortexing, then spin down briefly to collect all the material in the cap and on the sides of the tube.
7. Perform a 1:2 serial dilution of protein by transferring 160 µl of the solution tube 1 to tube 2. Return tube 1 to the ice. Mix tube 2 well, as before, then transfer 160 µl from tube 2 to tube 3. Continue to do this until the dilution series is complete. The last tube (tube 12) will contain 320 µl of 0.09 µM protein.
8. Place the 96-well black plastic plate used previously for the plate reader calibration on ice.
9. Pipette 24 µl of the protein solution from tube 1 into the first column of Plate 1, wells C1 to H1. Continue to pipette 24 µl of each protein dilution into the appropriate column. That is, pipette 24 µl from tube 2 into each of wells C2 to H2, *etc*.
10. Pipette 1 µl of the working solution of H3 trimethyl Lys9 peptide into all wells in rows C to E, and pipette 1 µl of the working solution of H3 trimethyl Lys27 into all wells in rows F to H.

11. Read the plate on the microplate reader using the optimal gain setting. Make a note of the gain used, as this value should be used for subsequent readings.

12. Plot the binding curves for the 2 peptides. Keep in mind that the HP1 positive control protein is not expected to bind to the H3 trimethyl Lys27 peptide. It is done as a negative control.

Note: An alternative way to carry out the protein dilution series is to start with 48 µl of a 200 µM solution in column 1 and then use a multi-channel pipettor to make 1:2 dilutions across the plate, transferring 24 µl of protein into wells containing 24 µl of protein dilution buffer. This method will be quicker, but the quality of the results will depend on the accuracy of the equipment used. If your multi-channel pipettor has not calibrated recently, it is not recommended to use it.

E. Preparing a Dilution Series for the Protein of Interest (Optional)

Ideally, in an FP assay the larger macromolecule (*e.g.* HP1 protein) should be serially diluted to cover a concentration range of 100-fold below to 100-fold above the K_d of the interaction being studied. However, the affinity of the protein being used in this assay for the Lys9 and Lys27 peptides may not be known. In this case, it is important to perform a preliminary dilution series of the protein covering a wide range of concentrations before beginning the main assay. For this concentration test, 12 dilutions of the protein should be made and tested with the two trimethylated peptides in the kit. If you are following our Suggested Microplate Layouts, this step is performed as Plate 2, rows A and B, which is detailed in Figure 3 on page 20.

Most protein-histone tail interactions observed to date occur with an affinity of approximately 10^{-6}. Therefore, protein samples should range from approximately 0.1 µM to 1 mM for initial studies. It is important to keep the concentration of the fluorescently labeled peptide at least 10-fold and preferably 100-fold, less than the K_d. Failure to do so can result in inaccurate affinity calculations. For protein-histone tail interactions, a concentration of approximately 100 µM has been found to be a good starting point. Concentrations can then be adjusted in the appropriate range during the main part of the assay.

1. At this point, the peptides should have already been reconstituted (Protocol A) and working solutions prepared as per Appendix Section A.
2. Label 12 Eppendorf tubes (1.5 ml) from 1 to 12. Place the tubes on ice.
3. Add 50 µl of Binding Buffer AM10 plus DTT to the tubes labeled 2 to 12 and return to the ice.
4. Calculate the amount of protein in Binding Buffer AM10 plus DTT needed to make 100 µl of the highest concentration in the dilution series, then make this up in tube 1. (This volume is necessary to fill two wells and to continue the 1:2 dilution series, *i.e.* 25 µl will be put in both wells A1 + B1, and 50 µl will be transferred to tube 2).
5. Vortex the 100 µl protein solution in tube 1 to mix, then briefly centrifuge the tube to remove any solution from the cap or sides of the tube.

6. Perform a 1:2 serial dilution of the protein by transferring 50 µl from tube 1 to tube 2. Mix well, then transfer 50 µl from tube 2 to tube 3. Continue until the dilution series is complete. (Tube number 12 will have twice the volume of the other 11 tubes when the dilution series has been made.)

   Note: You may want to dilute your protein 1:3 or 1:4 in which case the volumes should be adjusted accordingly.

7. Place one of the 96-well black plastic plates on ice.
8. Pipette 24 µl of the protein solution from tube 1 into wells A1 and B1. Continue to pipette 24 µl of each protein dilution into the appropriate column. That is, pipette 24 µl from tube 2 into each of wells A2 and B2, *etc*.
9. Add 1 µl of the H3 trimethyl Lys9 solution to every well in row A of the 96-well plate containing the protein dilution series. Use a fresh tip for the different concentrations.
10. Add 1 µl of the H3 trimethyl Lys27 solution to every well in row B of the 96-well plate containing the protein dilution series. Use a fresh tip for the different concentrations.
11. Place the plate in the dark at room temperature and wait 20 minutes.
12. Read the plate on the microplate reader using optimal gain settings. **Do not discard this plate after reading as the unused wells will be required for the next step of the assay.**
13. Plot the binding curves and determine the optimal protein concentration to use in the assay.

F. Performing the Binding Curves for the Protein of Interest

In this part of the assay, a dilution series of the protein of interest will be made and the binding affinity of the protein with the four H3 Lys9 and four H3 Lys27 peptides will be determined. If you are following our Suggested Microplate Layouts, this step is performed as Plates 2-5, which are detailed in Figures 3-6 on pages 20-21.

Note: It is important that you **use the same incubation time be for all plates**. As you will be reading 4 different plates, you must plan the experiment to stagger the addition of peptides to each plate so that the plate reader is available when each plate's incubation is complete. You should take into account how long it takes you to transfer the plate to the reader, how long the read takes, *etc*.

1. Label 12 Eppendorf tubes (1.5 ml) from 1 to 12.
2. Add 600 µl Binding Buffer AM10 plus DTT to tubes 2 through 12, then place all tubes on ice.
3. Dilute your protein of interest with Binding Buffer AM10 plus DTT. The range of the dilution series should be broad enough to ensure that binding of the protein to each peptide can be accurately measured. Based upon the optimal protein concentration that was determined in the Protocol E, make 1200 µl of the highest protein concentration in your binding curve in tube 1; 600 µl will be transferred to tube 2 for the 1:2 dilution, while approximately 600 µl will be used to assay each of the 8 peptides in triplicate (24 µl per well x 8 peptides x 3 = 576 µl).

5. Vortex the 1200 µl protein solution in tube 1 to mix, then briefly centrifuge the tube to remove any solution from the cap or sides of the tube.

6. Perform a 1:2 serial dilution of the protein by transferring 600 µl from tube 1 to tube 2, then put tube 1 back on ice. Mix tube 2 well as before, then transfer 600 µl from tube 2 to tube 3. Continue until the dilution series is complete. All tubes should be on ice.

7. Place Plate 2, which was used in the previous step to determine the optimal protein concentration, on ice. (The layout of this plate is Figure 3 on page 20.)

8. Mix tube 1 well, then pipette 24 µl of the protein solution into column 1, rows C through H. Continue to pipette 24 µl of each protein dilution from tubes 2-12 into the appropriate column. That is, pipette 24 µl from tube 2 into column 2, rows C-H, tube 3 into column 3, *etc*.

9. Pipette 1 µl working stock of H3 trimethyl Lys9 peptide into each of the 36 wells that make up a protein dilution series in triplicate (*e.g.* rows C to E). Pipette the second peptide, the working stock of H3 dimethyl Lys9, into the lower 36 wells on the same plate (rows F to H).

10. Read the plate immediately on the microplate reader using optimal gain, then incubate for 20-30 minutes and read again. To reduce the amount of noise in the data, the plate can be read 3-4 times and the results averaged.

    Note: The plate should be placed in the dark at room temperature during incubation. If the protein of interest is particularly sensitive, it may be preferable to keep the plate on ice during the incubation. Some proteins may require up to 60 minutes of incubation time. Also, after reading each plate, it is advised to place each back in the dark and on ice until all plates are read. As detailed in the Troubleshooting, bubbles in wells can make data noisy and some proteins may require more reads/well. By storing them on ice, you may be able to re-read all plates if needed.

11. Continue with the assay by pipetting the protein dilutions and peptides into Plate 3 as before, but using the working stocks of the H3 monomethyl Lys9 and unmodified H3 Lys9 peptides. (The layout of this plate is Figure 4 on page 21.) Read immediately, incubate and read again as before, using the same gain and other settings that were used for Plate 2.

12. Proceed by pipetting the protein dilutions and peptides into Plate 4 as before, this time with the working stocks of the H3 trimethyl Lys27 and H3 dimethyl Lys27 peptides. (The layout of this plate is Figure 5 on page 21.) Read immediately, incubate and read again as before.

13. Complete the assay by pipetting the protein dilutions and peptides into Plate 5 as before using the working stocks of the H3 monomethyl Lys27 and unmodified H3 Lys27. (The layout of this plate is Figure 6 on page 21.) Read immediately, incubate and read again as before.

    Note: An alternative way to carry out the protein dilution is to add 48 µl of the most concentrated protein sample to column 1 of the plate and then use a multi-channel pipettor to make 1:2 dilutions across the plate transferring 24 µl into wells containing 24 µl of the protein dilution buffer This method may be quicker than pipetting into wells individually, but the quality of the results will depend on the accuracy of the equipment used. If your multi-channel pipettor has not calibrated recently, it is not recommended to use it.

Appendix

Section A. Prepare the Peptide Working Stocks

- Decide which final concentration is needed (100-500 nM). This can be done based on the known specifications of the instrument, or by performing the optional calibration dye intensity test (Protocol A on page 10).
- Make **ONE** of the following working stocks (2.5, 5, 7.5, 10 or 12.5 µM) to achieve the desired final concentration of peptide of either 100, 200, 300, 400 or 500 nM.
- For most instruments, 200 nM final peptide concentration should be fine, but extra material has been provided in case it is necessary to use a higher final concentration.
- It is important to keep the concentration of the fluorescently labeled peptide at least 10-fold, and preferably 100-fold, less than the K_d. Failure to do so can result in inaccurate affinity calculations.

1. Using the calibration data, or data previously acquired on the plate reader using 488 nm excitation and 535 nm emission, determine the final concentration of peptide that will be most suitable for the plate reader (100, 200, 300, 400 or 500 nM). For example, if the gain was high (100-120) during the calibration dye scan, one might chose to make a 400-500 nM peptide dilution. Conversely, if the plate reader only required a gain of 80-100 to obtain decent fluorescent values (10,000-100,000) one might select 100-200 nM for the peptide.
2. Make the appropriate working stock solution according to the chart below. The aim is to make a peptide solution that will result in a final concentration of 100, 200, 300, 400 or 500 nM when 1 µl is added to 24 µl of protein in a well of a 96-well plate.

Table 2. Working concentration of peptide needed to generate various final peptide concentrations in each well of the binding assay.

Working conc. (µM)	Volume peptide to add to each well (µl)	Total volume per well (µl)	Final conc. of peptide (nM)
2.5	1	25	100
5	1	25	200
7.5	1	25	300
10	1	25	400
12.5	1	25	500

3. Use one of the charts below to determine how to make a working stock solution of 2.5 µM (for final concentration of 100 nM), 5 µM (for 200 nM), 7.5 µM (for 300 nM), 10 µM (for 400 nM) or 12.5 µM (for 500 nM) from the 20 µM peptide stocks.

4. **PROTECT THE WORKING STOCKS FROM LIGHT.** Clear Eppendorf tubes should be wrapped in aluminum foil and stored at 4°C if the peptide dilutions are made in advance of performing the assay. Working stock solutions can be stored for several hours this way.

5. Prepare the working stocks by combining and appropriate volume of water and peptide in clean Eppendorf tubes. Do not add the water directly to the amber vial because the vial contains an excess of peptide, which will cause an incorrect final concentration.

   To make a 2.5 µM working stock of peptide, which will yield a final concentration of 100 nM for the assay, use the following table:

Peptide	Number of wells (1 µl per well)	Volume of 20 µM stock to use (µl)	Volume of water added to peptide (µl)
H3 Lys9 (unmodified)	36	5.5	38.5
H3 monomethyl Lys9	36	5.5	38.5
H3 dimethyl Lys9	36	5.5	38.5
H3 trimethyl Lys9	84	11.2	78.8
H3 Lys27 (unmodified)	36	5.5	38.5
H3 monomethyl Lys27	36	5.5	38.5
H3 dimethyl Lys27	36	5.5	38.5
H3 trimethyl Lys27	84	11.2	78.8

   To make a 5 µM working stock of peptide, which will yield a final concentration of 200 nM for the assay, use the following table:

Peptide	Number of wells (1 µl per well)	Volume of 20 µM stock to use (µl)	Volume of water added to peptide (µl)
H3 Lys9 (unmodified)	36	11	33
H3 monomethyl Lys9	36	11	33
H3 dimethyl Lys9	36	11	33
H3 trimethyl Lys9	84	22.5	67.5
H3 Lys27 (unmodified)	36	11	33
H3 monomethyl Lys27	36	11	33
H3 dimethyl Lys27	36	11	33
H3 trimethyl Lys27	84	22.5	67.5

To make a 7.5 µM working stock of peptide, which will yield a final concentration of 300 nM for the assay, use the following table:

Peptide	Number of wells (1 µl per well)	Volume of 20 µM stock to use (µl)	Volume of water added to peptide (µl)
H3 Lys9 (unmodified)	36	15.4	28.6
H3 monomethyl Lys9	36	15.4	28.6
H3 dimethyl Lys9	36	15.4	28.6
H3 trimethyl Lys9	84	31.5	58.5
H3 Lys27 (unmodified)	36	15.4	28.6
H3 monomethyl Lys27	36	15.4	28.6
H3 dimethyl Lys27	36	15.4	28.6
H3 trimethyl Lys27	84	31.5	58.5

To make a 10 µM working stock of peptide, which will yield a final concentration of 400 nM for the assay, use the following table:

Peptide	Number of wells (1 µl per well)	Volume of 20 µM stock to use (µl)	Volume of water added to peptide (µl)
H3 Lys9 (unmodified)	36	22	22
H3 monomethyl Lys9	36	22	22
H3 dimethyl Lys9	36	22	22
H3 trimethyl Lys9	84	45	45
H3 Lys27 (unmodified)	36	22	22
H3 monomethyl Lys27	36	22	22
H3 dimethyl Lys27	36	22	22
H3 trimethyl Lys27	84	45	45

To make a 12.5 µM working stock of peptide, which will yield a final concentration of 500 nM for the assay, use the following table:

Peptide	Number of wells (1 µl per well)	Volume of 20 µM stock to use (µl)	Volume of water added to peptide (µl)
H3 Lys9 (unmodified)	36	26.5	17.5
H3 monomethyl Lys9	36	26.5	17.5
H3 dimethyl Lys9	36	26.5	17.5
H3 trimethyl Lys9	84	54	36
H3 Lys27 (unmodified)	36	26.5	17.5
H3 monomethyl Lys27	36	26.5	17.5
H3 dimethyl Lys27	36	26.5	17.5
H3 trimethyl Lys27	84	54	36

Section B. Summary of Each Plate's Contents

Plate 1: FP reader calibration (rows A-B); Positive Control Protein Binding Curve with H3K9me3 peptide (in triplicate, rows C-E) and H3K27me3 peptide (in triplicate, rows F-H)

Plate 2: Preliminary Protein of Interest dilution series with H3K9me3 peptide (row A) and H3K27me3 peptide (row B); Protein of Interest dilution series with H3K9me3 peptide (in triplicate, rows C-E) and H3K9me2 peptide (in triplicate, rows F-H)

Plate 3: Protein of Interest dilution series with H3K9me1 peptide (in triplicate, rows A-C) and unmodified H3K9 peptide (in triplicate, rows D-F)

Plate 4: Protein of Interest dilution series with H3K27me3 peptide (in triplicate, rows A-C) and H3K27me2 peptide (in triplicate, rows D-F)

Plate 5: Protein of Interest dilution series with H3K27me1 peptide (in triplicate, rows A-C) and unmodified H3K27 peptide (in triplicate, rows D-F)

Section C. Suggested Microplate Layouts

Below are suggested layouts for setting up the assays in the provided microplates.

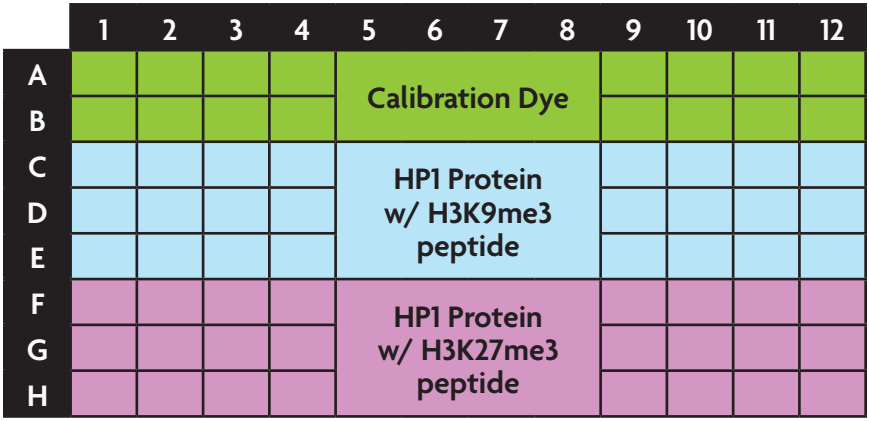


Figure 2. Layout for Plate 1, used for the FP reader calibration and the Positive Control Protein Binding Curves.

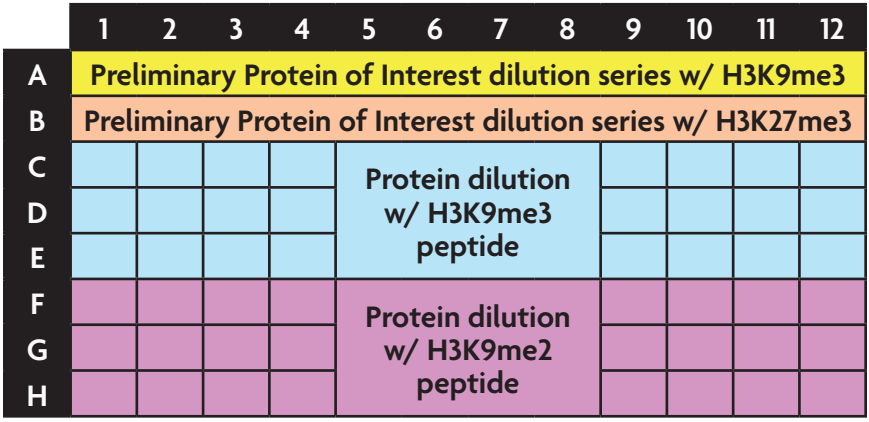


Figure 3. Layout for Plate 2, used to analyze binding of the protein of interest to H3K9me3 and H3K9me2.

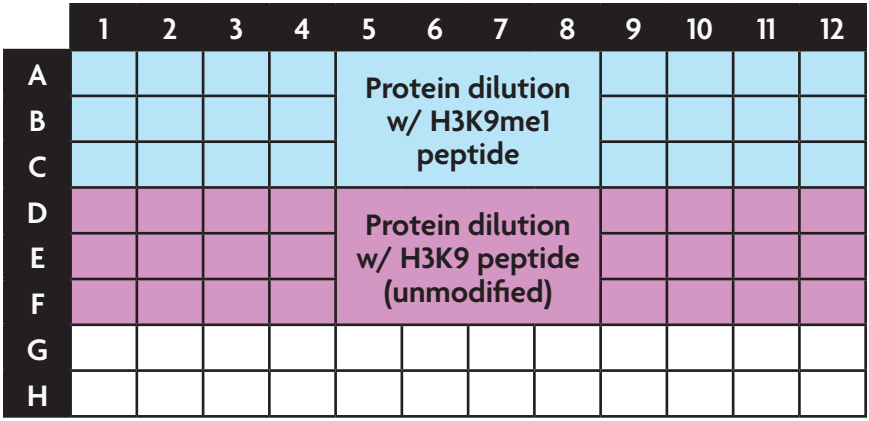


Figure 4. Layout for Plate 3, used to analyze binding of the protein of interest to H3K9me1 and unmodified H3K9.

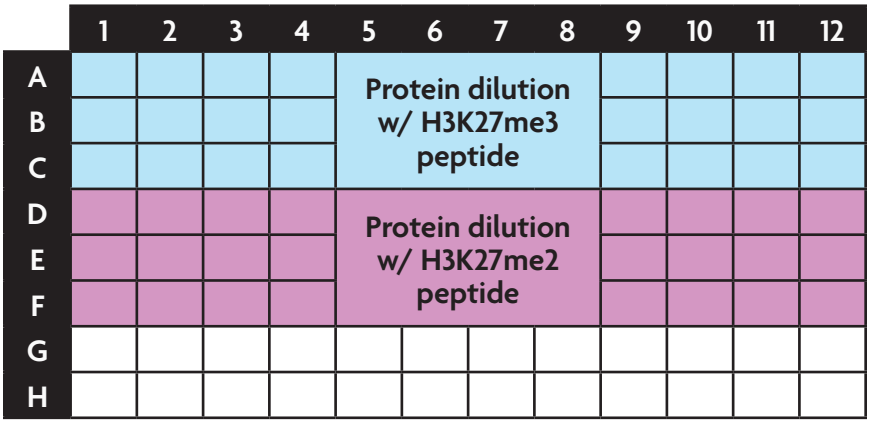


Figure 5. Layout for Plate 4, used to analyze binding of the protein of interest to H3K27me3 and H3K27me2.

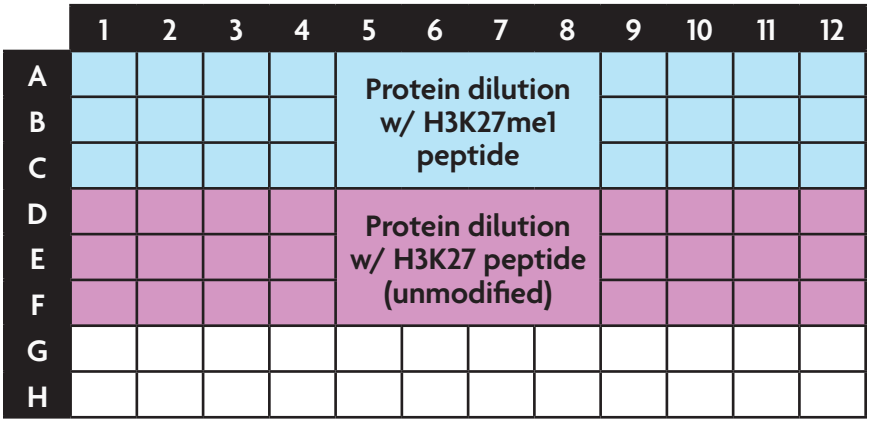


Figure 6. Layout for Plate 5, used to analyze binding of the protein of interest to H3K27me1 and unmodified H3K27.

Section D. Tips on Fluorescence Polarization Plate Scanning

When scanning plates, it may be advantageous to obtain values for polarization, anisotropy and fluorescence intensity. Fluorescence intensity values can indicate the cause of a problem, such as not adding peptide to one of the wells. It is recommended that binding curves be plotted using anisotropy because anisotropy is an additive, molecular parameter, while polarization is not. If the plate reader does not convert polarization values into anisotropy, this can be done using the equation A = (2*P)/(3-P).

Sometimes, data can be quite noisy. In such cases, one should allow for extra time after setting up the assay to give the reaction time to reach equilibrium. This may be as much as 60 minutes. The plate should be protected from light and stored at room temperature (or on ice if the protein is sensitive to degradation) during this time. To reduce noise in the data it may help to increase the number of flashes, or reads, per well. Additionally, you might decide to increase the settle time during the assay (this is the time between reads of adjacent wells). Finally, it can also help to read the plate 3-4 times and then average the resulting values.

Section E. Data Analysis

Note: Your calculations will only be accurate if your peptide concentration is 10-100 fold higher than the K_d.

If the microplate reader reports polarization values only, convert these to anisotropy using the following equation: **A = (2*P)/(3-P)**

Transfer the anisotropy data into a graphing software program. Kaleidagraph is an example of software that works well for this analysis. The data are plotted as log protein concentration versus anisotropy and fit to the equation below by non-linear least squares analysis:

Anisotropy = m1+(m2-m1)*M0/(m3+M0); m1=50; m2=100; m3=15.4

M0 is the dependent variable (protein concentration).

m1 equals the anisotropy of the non-bound state (graphically the left end of the sigmoid curve parallel to the x-axis.

m2 equals the anisotropy of the bound state (graphically the right end of the sigmoid curve parallel to the x-axis. m1 and m2 correspond to the tangents to the curve (in sigmoid representation).

m3 is the inflection point of the sigmoid curve. In some circumstances it may be necessary to reduce the value of m3 in the equation to obtain a good fit for the data.

Kaleidagraph will generate a table giving the R value for the curve and a value for m3, which corresponds to the dissociation constant (K_d). Check the R value as this is a parameter that describes the quality of the curve fit.

Advanced normalization can be performed to determine the degree of binding that has occurred. As the curves from different reads of a plate and especially from different dilution series of pipetting might vary in the m1 and m2 values, these are not really additive (not suitable for averaging). This can be attributed to slight variability in the positions of the mirrors and photo multipliers of the plate readers. To directly compare different readings and especially with different peptides, it is advisable to normalize the binding data (*i.e.* the value 0 is assigned to no binding and the value 1 is assigned to full binding). This can be achieved by converting the data sets in the following way:

$$y = (x-m1)/(m2-m1)$$

This operation can be performed in Excel or in Kaleidagraph. In the latter, go to "window" and "formula entry". A separate mathematical window will appear. There it is possible to enter different mathematical operations. Each button (F1-F8) is assigned a different operation. Select one of the buttons and enter the following formula:

$$c0 = (c0-m1)/(m2-m1)$$

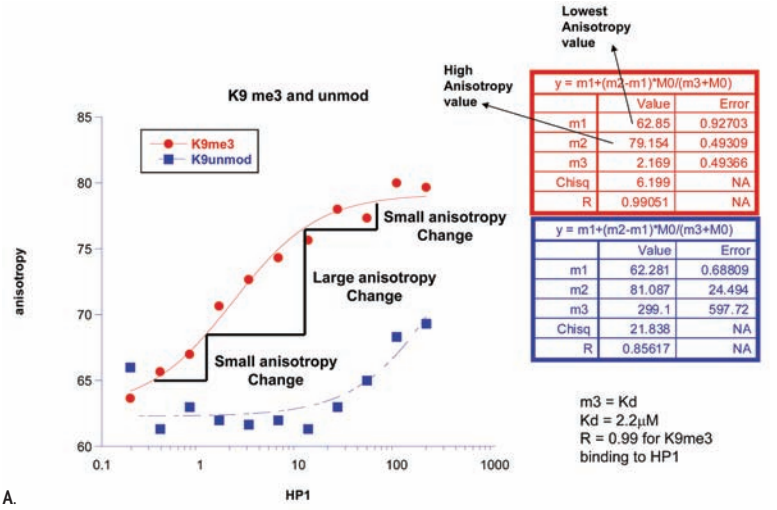
In this equation, m1 and m2 are the corresponding values for a given data set (*i.e.* a binding curve with a peptide) that were obtained from the curve fitting. Select the data column corresponding to the data set in the spread sheet. This marks the values that the operator will use as c0. Go back to the operator and hit the run button. This will normalize the data in the selected column for no binding/full binding.

The normalized values (which are sometimes referred to as fraction bound) are additive. This means that it is possible to build averages from different readings of the same plate as well as from different measurements. This may help to smoothen noise in the data.

Section F. Examples of Acceptable and Unacceptable Data

The graph below is an example of good data for H3 trimethyl Lys9 binding to HP1. Anisotropy values from the Tecan (Graph A) have been converted into normalized, fraction-bound values (Graph B) using the equation y = (x-m1)/(m2-m1). This equation normalizes data by assigning a value of 0 for no binding and a value of 1 for full binding. The closer the results in the graph get to 1, the better the degree of binding to the protein, HP1. Data for H3 trimethyl Lys9 fits the curve well with an R value of 0.99. The K_d for the binding reaction is the inflection point of the curve, or m3. The top table in Graph B shows that the binding of H3 trimethyl Lys9 to HP1 has a K_d of 2.17 µM, whereas the K_d for the binding of unmodified K9 peptide is 296 µM. The change in fraction bound from the lowest to the highest concentration of HP1 is 0.65.

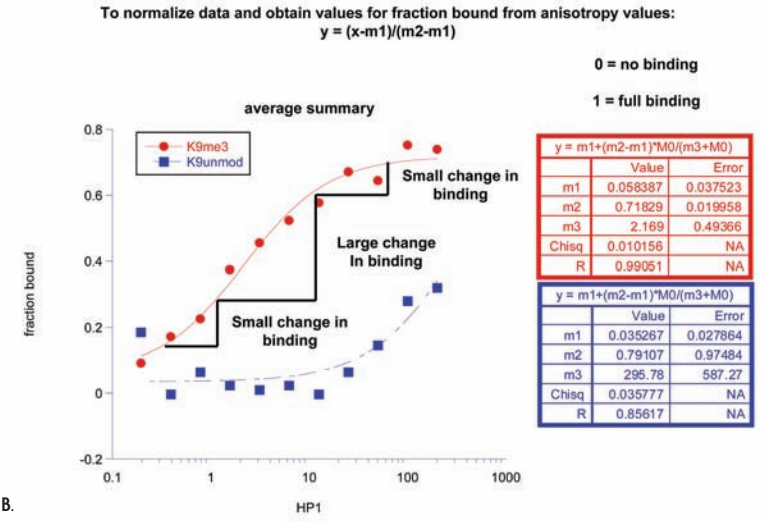
Reasonable results from the interaction of HP1 with the H3 Lys9 peptides are as follows.



y = m1+(m2-m1)*M0/(m3+M0)	Value	Error
m1	62.85	0.92703
m2	79.154	0.49309
m3	2.169	0.49366
Chisq	6.199	NA
R	0.99051	NA

y = m1+(m2-m1)*M0/(m3+M0)	Value	Error
m1	62.281	0.68809
m2	81.087	24.494
m3	299.1	597.72
Chisq	21.838	NA
R	0.85617	NA

Graph A.

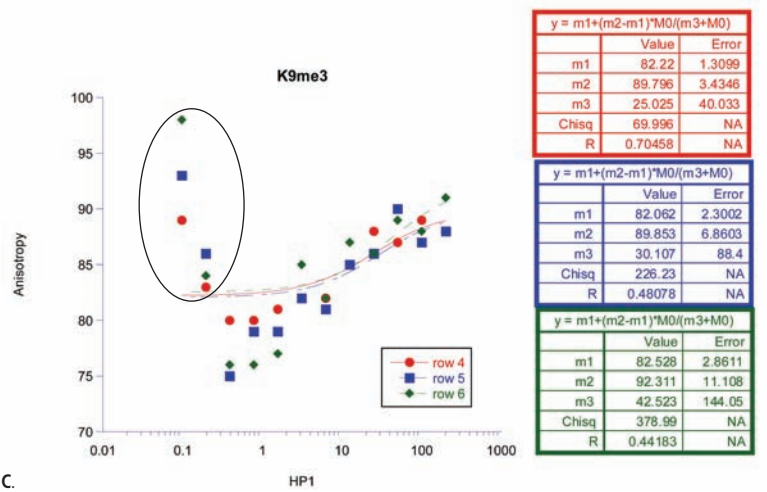


Graph B.

An example of unacceptable anisotropy data is below (Graph C).

- There are artifacts at low protein concentration, possibly caused by light scatter
- The data are noisy and the curve fitting is bad (R values are less than 0.9 for H3K9me3)
- The K_d values are high
- The change in anisotropy is small

Note: Curve fitting for this data set could be improved significantly by removing the outliers at the 2 lowest protein concentrations (circled).



Graph C.

Section G. Troubleshooting Guide

Problem/question	Recommendation
Data seem noisy, or there are outliers	1. Check the fluorescence intensity readings to verify that fluorescent peptide was added to the well(s) in question. Absence of peptide will lead to anomalous FP or anisotropy readings. 2. Rock the plate back and forth gently and then gently tap it on the surface of a bench or desk. This will help to mix the contents of the well and to eliminate bubbles. 3. If bubbles can be seen within a well, use a needle or fine pipette to burst them, then read the plate again. 4. Increase number of flashes / reads per well. 5. Increase the time between reads (also called the "settle time") 6. Try reading the plate several times to see if fluctuations disappear (bubbles burst, well contents properly mixed, *etc.*). 7. Check that pipettors are calibrated. Try using a different set. 8. Increase the concentration of fluorescent peptide used. 9. Allow for additional time before reading the plate by incubating on ice and covered with foil for several minutes (up to 60 minutes).
Fluorescence intensity values are too low (less than 10,000)	1. Increase the gain of the instrument. 2. Add a higher concentration of peptide.
Very high anisotropy values are seen	This may be an artifact caused by the low concentration of protein in solution. The artifact is caused by low protein altering the viscosity of the solution in the well compared to higher concentrations and light scatter. Polarization values for blank wells (buffer + protein with no fluorophore) will be very high as a result of light scatter and this effect will also be seen with low concentrations of protein. Light scatter may also arise from the presence of precipitant material in the well. This can lead to false positive data. Make sure that protein solutions are centrifuged prior to making the dilution series.
The unmodified peptide is binding to the protein	Some proteins might bind more strongly to the unmodified peptides than others so some degree of binding might be observed. At very high protein concentrations the solution might get very viscous. In this case one might see a change in anisotropy but this does not reflect true binding and is due to a decrease in the motion of the peptide. **However, it is possible that this binding event is a true indication of the nature of the particular protein with which you are working and should not be discounted but tested further to determine the authenticity of the interaction.**

Section H. Related Products

Histone ELISAs	Format	Catalog No.
Total Histone H3 ELISA	1 x 96 rxns	53110
Histone H3 dimethyl Lys9 ELISA	1 x 96 rxns	53108
Histone H3 trimethyl Lys9 ELISA	1 x 96 rxns	53109
Histone H3 trimethyl Lys27 ELISA	1 x 96 rxns	53106

Histone Acetyltransferase and Deacetylase Activity	Format	Catalog No.
HAT Assay Kit (Fluorescent)	1 x 96 rxns	56100
Recombinant p300 protein, catalytic domain	5 µg	31205
Recombinant GCN5 protein, active	5 µg	31204
HDAC Assay Kit (Fluorescent)	1 x 96 rxns	56200
HDAC Assay Kit (Colorimetric)	1 x 96 rxns	56210

Histone Purification & Chromatin Assembly	Format	Catalog No.
Histone Purification Kit	10 rxns	40025
Histone Purification Mini Kit	10 rxns	40026
Chromatin Assembly Kit	10 rxns	53500
HeLa Core Histones	36 µg	53501

Methylated Histone Proteins	Format	Catalog No.
Recombinant Histone H3 (C110A)	50 µg	31207
Recombinant Histone H3 monomethyl Lys4	50 µg	31208
Recombinant Histone H3 dimethyl Lys4	50 µg	31209
Recombinant Histone H3 trimethyl Lys4	50 µg	31210
Recombinant Histone H3 monomethyl Lys9	50 µg	31211
Recombinant Histone H3 dimethyl Lys9	50 µg	31212
Recombinant Histone H3 trimethyl Lys9	50 µg	31213
Recombinant Histone H3 monomethyl Lys27	50 µg	31214
Recombinant Histone H3 dimethyl Lys27	50 µg	31215
Recombinant Histone H3 trimethyl Lys27	50 µg	31216
Recombinant Histone H3 monomethyl Lys36	50 µg	31217
Recombinant Histone H3 dimethyl Lys36	50 µg	31218
Recombinant Histone H3 trimethyl Lys36	50 µg	31219
Recombinant Histone H3 monomethyl Lys79	50 µg	31220
Recombinant Histone H3 dimethyl Lys79	50 µg	31221
Recombinant Histone H3 trimethyl Lys79	50 µg	31222
Recombinant Histone H4	50 µg	31223
Recombinant Histone H4 monomethyl Lys20	50 µg	31224
Recombinant Histone H4 dimethyl Lys20	50 µg	31225
Recombinant Histone H4 trimethyl Lys20	50 µg	31226

Methyltransferase Proteins	Format	Catalog No.
Recombinant G9a protein, active	10 µg	31327
Recombinant G9a H904K protein	10 µg	31328
Recombinant PRMT1 protein, active	10 µg	31325
Recombinant PRMT1 E143Q protein	10 µg	31326
Recombinant Set8 protein, active	10 µg	31321
Recombinant Set8 D338A protein	10 µg	31322
Recombinant Set9 protein, active	10 µg	31319
Recombinant Set9 H297A protein	10 µg	31320
Recombinant Smyd2 protein, active	10 µg	31323
Recombinant Smyd2 Y204F protein	10 µg	31324

ChIP-IT™ Kits	Format	Catalog No.
ChIP-IT™ Express	25 rxns	53008
ChIP-IT™ Express Enzymatic	25 rxns	53009
ChIP-IT™ Express HT	96 rxns	53018
Re-ChIP-IT™	25 rxns	53016
ChIP-IT™	25 rxns	53001
ChIP-IT™ Enzymatic	25 rxns	53006
ChIP-IT™ Shearing Kit	10 rxns	53002
Enzymatic Shearing Kit	10 rxns	53005
ChIP-IT™ Protein G Magnetic Beads	25 rxns	53014
Salmon Sperm DNA/Protein G agarose	25 rxns	53003
ChIP-IT™ Control Kit – Human	5 rxns	53010
ChIP-IT™ Control Kit – Mouse	5 rxns	53011
ChIP-IT™ Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021

ChIP-validated Antibodies	Application	Format	Catalog No.
AP-2 pAb	ChIP, EMSA	17 rxns	39304
c-Jun pAb	ChIP, EMSA, IF	100 µg	39309
C/EBPα pAb	ChIP, EMSA, IF, WB	100 µg	39306
CTCF mAb	ChIP, WB	200 µg	39621
DNMT1 mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT3A mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mAb	ChIP, IF, IP, WB	100 µg	39207
E2F-1 pAb	ChIP, EMSA	17 rxns	39313
E2F-6 mAb	ChIP, WB	100 µl	39509
EZH2 pAb	ChIP, IF, IP, WB	200 µl	39103
HBP-1 mAb	ChIP, IF, WB	100 µl	39511
HDAC1 mAb (Clone 10E2)	ChIP, IF, IHC, IP, WB	200 µl	39531
HDAC2 mAb (Clone 3F3)	ChIP, IF, IHC, IP, WB	200 µl	39533
HDAC3 pAb	ChIP, WB	100 µg	40968
HDAC4 pAb	ChIP, WB	100 µg	40969
HDAC5 pAb	ChIP, WB	100 µg	40970
HDAC6 pAb	ChIP, WB	100 µg	40971
Histone H2A pAb	ChIP, WB	200 µl	39235
Histone H2A phospho Ser129 pAb	ChIP, IF, IP, WB	200 µl	39271
Histone H2A.Z pAb	ChIP, WB	200 µl	39113
Histone H2B pAb	ChIP, WB	200 µl	39237
Histone H2B acetyl Lys5 pAb	ChIP, WB	200 µl	39123
Histone H2B acetyl Lys16 pAb	ChIP, WB	200 µl	39121
Histone H2B acetyl Lys46 pAb	ChIP, WB	200 µl	39571
Histone H3, C-terminal pAb	ChIP, WB	200 µl	39163
Histone H3 acetyl Lys4 pAb	ChIP, IF, WB	200 µl	39381
Histone H3 monomethyl Lys4 mAb	ChIP, WB	100 µg	39635
Histone H3 dimethyl Lys4 pAb	ChIP, WB	200 µl	39141
Histone H3 trimethyl Lys4 pAb	ChIP, WB	200 µl	39159
Histone H3 dimethyl Lys9 pAb	ChIP, IF, WB	200 µl	39239
Histone H3 trimethyl Lys9 pAb	ChIP, WB	200 µl	39161
Histone H3 acetyl Lys18 pAb	ChIP, IF, WB	200 µl	39587
Histone H3 acetyl Lys27 pAb	ChIP, IF, WB	200 µg	39133
Histone H3 acetyl Lys27 pAb	ChIP, WB	200 µl	39135
Histone H3 dimethyl Lys27 pAb	ChIP, IF, WB	200 µl	39245

ChIP-validated Antibodies (cont.)	Application	Format	Catalog No.
Histone H3 trimethyl Lys27 mAb	ChIP, WB	200 µl	39535
Histone H3 trimethyl Lys27 pAb	ChIP, IF, WB	200 µg	39155
Histone H3 trimethyl Lys27 pAb	ChIP, WB	200 µl	39156
Histone H3 acetyl Lys36 pAb	ChIP, IF, WB	200 µl	39379
Histone H3 acetyl Lys56 pAb	ChIP, WB	200 µl	39281
Histone H3 acetyl Lys64 pAb	ChIP, IF, WB	200 µl	39545
Histone H3 acetyl Lys79 pAb	ChIP, WB	200 µl	39565
Histone H4 pan-acetyl pAb	ChIP, IF, WB	200 µl	39243
Histone H4 tetra-acetyl pAb	ChIP, WB	50 µl	39179
Histone H4 acetyl Lys5 pAb	ChIP, IF, WB	200 µl	39169
Histone H4 acetyl Lys5 pAb	ChIP, IF, WB	200 µl	39583
Histone H4 acetyl Lys12 pAb	ChIP, IF, WB	200 µl	39165
Histone H4 acetyl Lys16 pAb	ChIP, WB	200 µl	39167
Histone H4 monomethyl Lys20 pAb	ChIP, IF, WB	200 µl	39175
Histone H4 trimethyl Lys20 pAb	ChIP, IF, WB	200 µl	39180
IRF-3 pAb	ChIP, WB	100 µl	39033
JunB pAb	ChIP, EMSA	17 rxns	39326
JunD pAb	ChIP, EMSA	100 µl	39328
L3MBTL1 pAb	ChIP, IP, WB	200 µl	39182
p53 pAb	ChIP, EMSA	17 rxns	39334
PP2A pAb	ChIP, IP, WB	200 µl	39192
RbAp46/48 pAb	ChIP, WB	200 µl	39198
RNA pol II mAb	ChIP, ELISA, IF, IP, WB	200 µl	39097
SNF2h mAb	ChIP, IF, IP, WB	200 µl	39543
Sp1 pAb	ChIP, WB	100 µl	39058
TRF2 Goat pAb	ChIP, IP, WB	100 µg	39223

For an up-to-date list of ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**

Application Key: ChIP = Chromatin Immunoprecipitation; **EMSA** = Electrophoretic Mobility Shift Assay; **IF** = Immunofluorescence; **IHC** = Immunohistochemistry; **IP** = Immunoprecipitation; **WB** = Western blot

Fluorescent Dyes	Excitation / Emission	Format	Catalog No.
Chromeo™ 488 Carboxylic Acid	488 nm / 517 nm	1 mg	15510
Chromeo™ 488 NHS-Ester	488 nm / 517 nm	1 mg	15511
Chromeo™ 488 Antibody Labeling Kit	488 nm / 517 nm	1 kit	15090
Chromeo™ 494 Carboxylic Acid	494 nm / 628 nm	1 mg	15110
Chromeo™ 494 NHS-Ester	494 nm / 628 nm	1 mg	15111
Chromeo™ 494 Antibody Labeling Kit	494 nm / 628 nm	1 kit	15091
Chromeo™ 505 Carboxylic Acid	505 nm / 526 nm	1 mg	15610
Chromeo™ 505 NHS-Ester	505 nm / 526 nm	1 mg	15611
Chromeo™ 546 Carboxylic Acid	545 nm / 561 nm	1 mg	15210
Chromeo™ 546 NHS-Ester	545 nm / 561 nm	1 mg	15211
Chromeo™ 546 Antibody Labeling Kit	545 nm / 561 nm	1 kit	15092
Chromeo™ 642 Carboxylic Acid	642 nm / 660 nm	1 mg	15310
Chromeo™ 642 NHS-Ester	642 nm / 660 nm	1 mg	15311
Chromeo™ 642 Antibody Labeling Kit	642 nm / 660 nm	1 kit	15093

Fluorescent Protein Labeling	Format	Catalog No.
LigandLink™ pLL-1 Kit	1 kit	34001
LigandLink™ pLL-1-NFκB p65 Kit	1 kit	34004
LigandLink™ pLL-1-p53 Kit	1 kit	34005
LigandLink™ pLL-1-STAT1 Kit	1 kit	34006
LigandLink™ Fluorescein Label	300 rxns	34101
LigandLink™ Hexachlorofluorescein Label	300 rxns	34104

Transcription Factor ELISAs	Format	Catalog No.
TransAM™ AML-1/Runx1	1 x 96-well plate	47396
TransAM™ AML-3/Runx2	1 x 96-well plate	44496
TransAM™ AP-1 Family	2 x 96-well plates	44296
TransAM™ AP-1 c-Fos	1 x 96-well plate	44096
TransAM™ AP-1 c-Jun	1 x 96-well plate	46096
TransAM™ AP-1 FosB	1 x 96-well plate	45096
TransAM™ AP-1 JunD	1 x 96-well plate	43496
TransAM™ ATF-2	1 x 96-well plate	42396
TransAM™ c-Myc	1 x 96-well plate	43396
TransAM™ C/EBP α/β	1 x 96-well plate	44196
TransAM™ CREB	1 x 96-well plate	42096
TransAM™ pCREB	1 x 96-well plate	43096
TransAM™ Elk-1	1 x 96-well plate	44396
TransAM™ ER	1 x 96-well plate	41396
TransAM™ FKHR (FOXO1/4)	1 x 96-well plate	46396
TransAM™ GATA Family	2 x 96-well plates	48296
TransAM™ GATA-4	1 x 96-well plate	46496
TransAM™ GR	1 x 96-well plate	45496
TransAM™ HIF-1	1 x 96-well plate	47096
TransAM™ HNF Family	2 x 96-well plates	46296
TransAM™ HNF-1	1 x 96-well plate	46196
TransAM™ IRF-3 (Human)	1 x 96-well plate	48396
TransAM™ IRF-3 (Mouse)	1 x 96-well plate	48496
TransAM™ IRF-7	1 x 96-well plate	50196
TransAM™ MAPK Family	2 x 96-well plates	47296
TransAM™ MEF2	1 x 96-well plate	43196
TransAM™ MyoD	1 x 96-well plate	47196
TransAM™ NF-YA	1 x 96-well plate	40396
TransAM™ NFATc1	1 x 96-well plate	40296
TransAM™ NFκB Family	2 x 96-well plates	43296
TransAM™ Flexi NFκB Family	2 x 96-well plates	43298
TransAM™ NFκB p50	1 x 96-well plate	41096
TransAM™ NFκB p50 Chemi	1 x 96-well plate	41097
TransAM™ Flexi NFκB p50	1 x 96-well plate	41098
TransAM™ NFκB p52	1 x 96-well plate	48196
TransAM™ NFκB p52 Chemi	1 x 96-well plate	48197
TransAM™ NFκB p65	1 x 96-well plate	40096
TransAM™ NFκB p65 Chemi	1 x 96-well plate	40097
TransAM™ Flexi NFκB p65	1 x 96-well plate	40098
TransAM™ Nrf2	1 x 96-well plate	50296
TransAM™ Oct-4	1 x 96-well plate	42496
TransAM™ p53	1 x 96-well plate	41196
TransAM™ PPARγ	1 x 96-well plate	40196
TransAM™ Sp1	1 x 96-well plate	41296
TransAM™ Sp1/Sp3	1 x 96-well plate	40496
TransAM™ STAT Family	2 x 96-well plates	42296
TransAM™ STAT3	1 x 96-well plate	45196
TransAM™ T-bet	1 x 96-well plate	51396

For a complete, up-to-date list of available TransAM™ Kits, please visit **www.activemotif.com/transam**

Co-Immunoprecipitation	Format	Catalog No.
Universal Magnetic Co-IP Kit	25 rxns	54002
Nuclear Complex Co-IP Kit	50 rxns	54001

SUMOylation	Format	Catalog No.
SUMOlink™ SUMO-1 Kit	20 rxns	40120
SUMOlink™ SUMO-2/3 Kit	20 rxns	40220

In-cell Phospho-specific ELISAs	Format	Colorimetric Kit Catalog No.	Chemiluminescent Kit Catalog No.
FACE™ AKT	1 x 96 rxns	48120	48220
FACE™ ATF-2	1 x 96 rxns	48115	48215
FACE™ Bad	1 x 96 rxns	48165	48265
FACE™ c-Jun (S63)	1 x 96 rxns	48125	48225
FACE™ c-Jun (S73)	1 x 96 rxns	48135	48235
FACE™ c-Src	1 x 96 rxns	48155	48255
FACE™ EGFR (Y845)	1 x 96 rxns	48340	48440
FACE™ EGFR (Y992)	1 x 96 rxns	48150	48250
FACE™ EGFR (Y1173)	1 x 96 rxns	48190	48290
FACE™ ErbB-2 (Y877)	1 x 96 rxns	48130	48230
FACE™ ErbB-2 (Y1248)	1 x 96 rxns	48105	48205
FACE™ ERK1/2	1 x 96 rxns	48140	48240
FACE™ FAK	1 x 96 rxns	48145	48245
FACE™ FKHR (FOXO1)	1 x 96 rxns	48160	48260
FACE™ HSP27	1 x 96 rxns	48350	48450
FACE™ JAK1	1 x 96 rxns	48185	48285
FACE™ JNK	1 x 96 rxns	48110	48210
FACE™ MEK1/2	1 x 96 rxns	48180	48280
FACE™ NFκB p65 Profiler	3 x 96 rxns	48300	48400
FACE™ p38	1 x 96 rxns	48100	48200
FACE™ PI3 Kinase p85	1 x 96 rxns	48175	48275
FACE™ STAT2	1 x 96 rxns	48310	48410
FACE™ STAT4	1 x 96 rxns	48320	48420
FACE™ STAT6	1 x 96 rxns	48330	48430
FACE™ Maker	1 x 96 rxns	48000	48050
Suspension Cell FACE™	2 x 96 rxns	48305	48405

For a complete, up-to-date list of available FACE™ Kits, please visit **www.activemotif.com/face**

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