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Citation for published version:

Jacob, Y & Voigt, P 2017, In Vitro Assays to Measure Histone Methyltransferase Activity Using Different Chromatin Substrates. in M Bemer & C Baroux (eds), *Plant Chromatin Dynamics*. 1st ed. 2018 edition edn, vol. 1675, Methods in Molecular Biology, vol. 1675, HUMANA PRESS INC, pp. 345-360.
https://doi.org/10.1007/978-1-4939-7318-7_20

Digital Object Identifier (DOI):

[10.1007/978-1-4939-7318-7_20](https://doi.org/10.1007/978-1-4939-7318-7_20)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Plant Chromatin Dynamics

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Chapter 20

***In vitro* assays to measure histone methyltransferase activity using different chromatin substrates**

Yannick Jacob^{1*} and Philipp Voigt^{2*}

* These authors contributed equally to this work.

Correspondence: yannick.jacob@yale.edu and philipp.voigt@ed.ac.uk

¹Department of Molecular, Cellular & Developmental Biology, Yale University, 165 Prospect St
New Haven, Connecticut, 06511-2106, USA.

²Wellcome Trust Centre for Cell Biology, The University of Edinburgh, Michael Swann
Building, Max Born Crescent, Edinburgh (UK), EH9 3BF

Running Head: *In vitro* histone methylation assays

Summary/Abstract:

In vitro histone modification (HM) assays are used to characterize the activity of chromatin-modifying enzymes. These assays provide information regarding the modification sites on histones, the product specificity, and the impact of other histone or nucleotide modifications on enzyme activity. In particular, histone methyltransferase (HMT) assays have been instrumental in elucidating the activity and site specificity of many plant HMT enzymes. In this chapter, we describe a general protocol that can be used to perform HMT assays using different chromatin substrates, detection methods, and enzymes directly purified from plant material or heterologous sources.

Key Words

Histone modifications, histone variants, histone lysine methylation, histone peptides, histone octamers, nucleosomes.

1. Introduction

The study of epigenetics is largely focused on understanding how different chemical modifications on DNA and histones affect chromatin-based biological processes. Identifying the specific activity of a chromatin-modifying enzyme is often the first step in elucidating the molecular function of a particular epigenetic mark. *In vitro* histone modification (HM) assays have long been the method of choice to precisely identify the substrate of histone methyltransferases, acetyltransferases, and

kinases, as well as their antagonizing counterparts, the “erasers” of histone modifications [1]. These assays have significantly facilitated the study of plant chromatin since the discovery and characterization of the first plant histone methyltransferase (HMT) enzymes [2,3]. Even though many chromatin-modifying enzymes have now been functionally characterized in terms of their substrate preferences, HM assays still remain a very useful method in chromatin biology. For example, HM assays can be used to determine product specificity (e.g. mono-, di-, or trimethylation) and the impact of histone variants and neighboring histone/DNA modifications on the enzymatic activity of histone-modifying enzymes (Fig. 1). Recent work in plant epigenetics on the histone H3 lysine 27 (H3K27) methyltransferases ATXR5 and ATXR6 underscores the usefulness of *in vitro* HM assays for understanding the functions of chromatin-modifying enzymes. HMT assays were used to show 1) that ATXR5 and ATXR6 do not methylate H3K4 (as predicted by sequence comparison) but rather H3K27 and 2) that the enzymatic activity of ATXR5 and ATXR6 is specific for replication-dependent H3.1 variants [4,5].

In vitro HM assays rely on setting up a chemical reaction between a histone-modifying enzyme and a chromatin substrate. The chromatin substrate used in these assays can be peptides, histone monomers, histone dimers/tetramers/octamers, or even complete nucleosomes. Many of these substrates are now commercially available. In addition, new techniques have made it possible to design and synthesize complex nucleosome substrates (e.g. including different histone/DNA modifications and/or histone variants) to answer precise questions about the interplay between different epigenetic marks [6,7]. In this chapter, we describe a general protocol that can be used to perform HMT assays using different chromatin substrates. The addition of methyl groups can either be detected with radioactive assays, or by using antibodies against a specific histone mark.

All these detection methods are described here in different sections of the protocol (Fig. 2). We seek to provide sufficient experimental details to help scientists adapt this assay to their specific scientific needs.

2. Materials

2.1 HMT reactions

1. 5x methylation buffer: 250 mM Tris-HCl pH 8.5 (pH measured at 21°C), 25 mM MgCl₂ (*see Note 1*).
2. ³H-labeled S-adenosylmethionine (³H-SAM, available from Perkin Elmer, e.g. NET155H250UC, or in Europe also from Hartmann Analytic, e.g. ART0288), with the ³H label present in the methyl group. Aliquot and store at -20°C, avoid repeated freeze-thaw cycles (Radioactive assay only, *see Note 2*).
3. 0.2 M DTT (dithiothreitol) stock solution. Aliquot and freeze at -20°C.
4. Non-radioactive SAM stock as a 32 mM stock solution. Alternatively, a 2.5 mM SAM stock solution can be created by dissolving 1 mg of SAM in 788 µl of 5 mM sulfuric acid, pH 2, 10% ethanol. Aliquot and freeze at -20°C.
5. TE buffer: 10 mM Tris-HCl pH 8.0, 1 mM EDTA.
6. BC100 buffer: 20 mM Tris-HCl pH 8.0, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT. Add DTT fresh before using.
7. 4X SDS-PAGE loading buffer: 200 mM Tris-HCl (pH 6.8), 400 mM DTT, 8% SDS, 0.4% bromophenol blue and 40% glycerol. Aliquot and store at -20°C.
8. Histone methyltransferase preparation in suitable buffer: enzymes for these assays are typically obtained from recombinant expression in *E. coli* or Sf9 insect cells followed by purification using affinity tags grafted onto the methyltransferase (*see Note 3*).
9. Methylation substrate: suitable substrates include histone peptides (*see Note 4*), recombinant or native histones (*see Note 5*), and mono- or oligonucleosomes (*see Note 6*). The choice of substrate depends on the specificity of the enzyme and experimental

needs, however, the unavailability of more complex substrates may impose limitations in selecting the optimal substrate.

10. Heat block (with heated lid to prevent evaporation) or incubator set to 30°C or reaction temperature of choice (*see Note 7*).
11. Laboratory space equipped and certified for the use of ^3H radioactive material (Radioactive assay only, *see Note 8*).

2.2 Detection of methylated histones by SDS-PAGE and radioactivity

1. SDS-PAGE reagents and equipment for standard mini gels. Use of prestained protein marker is recommended to monitor transfer.
2. Equipment and reagents for wet or semi-dry transfer.
3. PVDF membrane.
4. Laboratory platform rocker.
5. Coomassie stain solution: 45% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) coomassie brilliant blue R. For 500 ml, dissolve 1.25 g of coomassie brilliant blue R in 225 ml of methanol. Add distilled water up to a final volume of 450 ml. Add 50 ml glacial acetic acid. Filter to remove residual undissolved coomassie dye.
6. Destain solution: 45% (v/v) methanol, 10% (v/v) acetic acid.
7. Camera or imaging system capable of taking white-light monochrome or color images. Alternatively, a scanner can be used.
8. Autoradiography enhancer (e.g. EN³HANCE spray, Perkin Elmer).
9. Autoradiography film with high sensitivity for ^3H .

10. Autoradiography cassette and bag.
11. -80 °C freezer.
12. Darkroom with red safelight.
13. Automated X-ray developer or manual film development setup.
14. Hair dryer or laboratory heat gun capable of running in cool mode (recommended but not essential; *see* **Notes 12 and 13**).

If performing scintillation counting (Subheading 3.2, steps 11–13):

15. Scalpel or razorblade.
16. Liquid scintillation cocktail suitable for ^3H
17. Scintillation vials.
18. Scintillation counter suitable for ^3H detection.

2.3 Detection of methylated peptides by SDS-PAGE and radioactivity

1. Same materials as under 2.2 with the exception of blotting reagents and equipment, coomassie stain solution, EN³HANCE spray, and items for scintillation counting.
2. Autoradiography Enhancer (e.g. ENLIGHTNING Rapid, Perkin Elmer, or Amplify Fluorographic Reagent, Amersham).
3. Filter paper (e.g. Whatman 3MM Chr).
4. Thin clear wrap such as saran wrap.
5. Gel drying apparatus.

2.4 Detection of methylated histones or peptides by SDS-PAGE and antibodies

1. SDS-PAGE reagents and equipment for standard mini gels. Use of prestained protein ladder is recommended.
2. Equipment and reagents for semi-dry transfer.
3. Nitrocellulose or PVDF membrane.
4. Laboratory platform rocker.
5. Primary antibody against a specific histone mark. Follow the manufacturer's recommendation regarding the dilution to be used in a Western Blot.
6. Secondary antibody coupled to horseradish peroxidase. Make sure that the secondary antibody used has specificity for the antibody species and isotype of the primary antibody. Refer to manufacturer's protocol for determining which dilution to use.
7. TBS: 20 mM Tris pH 7.5, 100 mM NaCl.
8. TBS-T: 20 mM Tris pH 7.5, 100 mM NaCl, 0.1 % (v/v) Tween.
9. Blocking solution: 5 % Nonfat Dry Milk in TBS-T solution.
10. Antibody solution: 2 % Nonfat Dry Milk in TBS-T solution or follow the antibody manufacturer's recommendation.
11. ECL Western Blotting Detection Reagents.
12. X-ray film for Western blot.
13. Standard film cassette.
14. Automated X-ray developer or manual film development setup.

2.5 Detection of methylated peptides and histones by using radioactivity and filter assays

1. Whatman P-81 filter papers (cellulose phosphate paper which is also a strong cation exchanger).
2. BD solution: 50 mM NaHCO₃ at pH 9.0. Prepare a fresh solution before use.
3. 1 L beaker.
4. Rocking shaker.
5. Liquid scintillation cocktail suitable for ³H (e.g. Gold Star by Meridian) and scintillation vials.
6. Liquid scintillation counter suitable for ³H detection.

3. Methods

3.1 HMT reaction

This protocol can be adapted to a broad range of methyltransferases and substrates. Depending on the choice of substrate and detection method, subsequent steps differ and are outlined separately in the following sections (see Subheadings 3.2-3.5 and Fig. 2). If no activity is detectable, pointers for troubleshooting are provided at the end (see Subheading 3.6). If performing multiple assays with different substrates or enzyme preparations, it is advisable to prepare a master mix containing all common components.

1. On ice, combine distilled water (to make up a total reaction volume of 25 μ l including substrate), 5 μ l 5x methylation buffer, 0.5 μ l 0.2 M DTT (4 mM final), 10 μ M unlabeled SAM (e.g. 0.25 μ l of a 1 mM pre-dilution in water of the stock solution) or 25–75 kBq of 3 H-SAM (if relying on radioactivity-based detection methods), and methyltransferase preparation (in BC100, *see Note 3*). 0.5-1 pmol (25–50 ng for a 50 kDa protein) of purified enzyme is usually sufficient to detect activity, but higher amounts might be necessary for some methyltransferases. If higher reaction volumes are required, for instance due to diluted enzyme or substrate preparations, scale up 5x methylation buffer, DTT, and SAM accordingly to maintain their final concentrations.
2. Add substrate to start the reaction. If using peptides (*see Note 4*), add peptide to a final concentration of 0.1–1 mM (2.5–25 nmol). If using core histones (*see Note 5*), add 0.5–4 μ g of histones (0.18–1.47 μ M or 4.6–36.7 pmol for recombinant *Xenopus laevis* histones). If using nucleosomal substrates (*see Note 6*), use the same amounts as indicated for core histones.
3. Mix well by pipetting up and down. Centrifuge briefly if needed (*see Note 8*).

4. Incubate at 30°C (*see Note 7*) for 1 h if doing end-point assays or for shorter time points if analyzing reaction kinetics to obtain enzymatic parameters (*see Note 9*).
5. Stop reactions by adding 8.3 µl of 4x SDS sample buffer (to reach 1x final) and boiling at 95°C for 5 min. Centrifuge for 10 sec at full speed. Different detection procedures can be followed after this step. For detection of methylated histones or methylated peptides using SDS-PAGE and ³H fluorography, go to subheading 3.2 or 3.3, respectively. For detection of methylated histones or peptides using antibodies, go to subheading 3.4. If using filter assays to detect incorporation of radioactivity into histones or peptides, it is not necessary to stop the reaction here. Skip this step entirely and go directly to Subheading 3.5. Please refer to Fig. 2 for an overview of the different detection methods available and the sections of this protocol covering each method.

3.2 Detection of methylated histones by SDS-PAGE and ³H fluorography

This protocol outlines the steps required to detect methylation of histone proteins if radioactive ³H-SAM has been used for the HMT reaction described in Subheading 3.1. It is applicable to all reactions performed on histone proteins, irrespective of the nature of the methyltransferase studied. ³H-SAM incorporation is visualized by exposing an autoradiography film (steps 1–10). Quantitative data can be obtained subsequent to film exposure by liquid scintillation counting (optional steps 11–13).

1. Resolve samples on a 15% SDS polyacrylamide gel (*see Note 10*). Dispose of radioactive waste from this and subsequent steps following the relevant regulations in your jurisdiction (*see Note 8*).

2. Transfer polypeptides from the gel onto a PVDF membrane (*see Note 11*). Most standard semi-dry or tank blot setups and protocols will be suitable (e.g. perform semi-dry transfer for 75 min at 100 V in SDS running buffer containing 20 % methanol).
3. Stain PVDF membrane with coomassie stain solution for 2–5 min with agitation on a platform rocker. Remove the coomassie stain solution.
4. Wash the membrane with destain solution 2–3 times for 5 min each with agitation.
5. Air-dry membrane until completely dry (*see Note 12*).
6. Document membrane with a camera, imaging system, or scanner. The image of the membrane obtained at this step should be similar to the bottom panel of Fig. 1, clearly showing the characteristic four core histone bands around 15 kDa (three in the case of *Xenopus* histones due to virtually identical size of H2A and H2B).
7. In a fume cabinet, spray the membrane with EN³HANCE spray (Perkin Elmer). Make sure to coat the membrane evenly with the solution. Let sit for 5 min, and then repeat two times.
8. Air-dry membrane until completely dry (*see Note 13*).
9. Expose to autoradiography film for 12–96 h in an autoradiography cassette in a -80°C freezer (*see Note 14*). Bend one corner of the film to mark its orientation relative to the membrane.
10. Develop film in an X-ray developer.
11. Optional: To obtain quantitative information, cut out the coomassie-stained bands of interest from the membrane with a scalpel or razorblade.
12. Place each individual band into a scintillation vial and add liquid scintillation cocktail.
13. Perform scintillation counting of all samples as well as vials containing only scintillation cocktail (for background count determination) in a scintillation counter suitable for ³H.

3.3 Detection of methylated peptides by SDS-PAGE and ³H fluorography

This protocol outlines the steps required to detect methylation of histone peptides if radioactive ³H-SAM has been used for the HMT reaction described in Subheading 3.1. It is applicable to all reactions performed using peptide substrates. To avoid loss of peptide in blotting, the SDS polyacrylamide gel used to resolve samples, rather than a membrane, is treated with autoradiography enhancer solution and exposed to an autoradiography film for detection of ³H-SAM incorporation.

1. Resolve samples on a 10 % SDS polyacrylamide gel. Run the gel until the dye front migrated about halfway into the resolving gel. This should result in a sharp band for the peptides migrating close to the dye front. Dispose of radioactive waste from this and subsequent steps following the relevant regulations in your jurisdiction (*see Note 8*).
2. Stain the gel in coomassie staining solution for 30–60 min with agitation.
3. Destain gel with several washes of destain solution (15–30 min each) until the bands of interest are clearly visible and background is nearly clear.
4. Document the gel with a camera, imaging system or scanner.
5. Incubate the gel in ENLIGHTNING solution for 30 min. It is sufficient to use just enough to cover the gel.
6. Lift gel onto Whatman paper that has been presoaked in water.
7. Cover with clear wrap.
8. Dry on a gel dryer set to 50-55 °C until dry (1-1.5 h). Higher temperatures may increase the risk of cracks in the gel.

9. Remove clear wrap for optimal sensitivity.
10. Expose to autoradiography film for 12–96 h in an autoradiography cassette in a -80 °C freezer (*see Note 14*). Bend one corner of the film to mark its orientation relative to the membrane.
11. Develop film in an X-ray developer.

3.4 Detection of methylated peptides or histones using antibodies

Using antibodies as a detection method is advantageous over radioactivity for a few reasons. First, albeit not as sensitive as radioactive detection, it is safer and easier to perform than radioactive HMT assays. Secondly, antibodies can be used to reveal the product specificity (mono-, di- or trimethylation) of a methyltransferase [8,5]. A few caveats of using antibodies for HMT assays is that they are costly and there is a need to know which lysine of the histone is modified by a specific methyltransferase (otherwise, many antibodies would have to be used to “screen” for the modified lysine). Also, antibodies are less suitable when using native histones as substrates, as many modifications are already present and can mask the signal generated in the HMT assay.

1. Resolve samples by SDS-PAGE as described in subheading 3.2 Step 1 (for histones) or Subheading 3.3 Step 1 (for peptides).
2. Transfer to nitrocellulose or PVDF membrane by wet or semi-dry transfer (*see Note 15*).
3. Quickly wash membrane by covering it in TBS-T solution. Let it sit for a few minutes. Remove the TBS-T.

4. Proceed to soak the membrane into the blocking solution. Gently shake the membrane in the blocking solution (add enough to cover the membrane) for 30-60 min at room temperature using a laboratory platform rocker. Wash briefly with TBS-T solution.
5. Dilute the primary antibody to a working concentration using the antibody solution. The minimum volume needed of antibody solution (containing the primary antibody at the appropriate concentration) is just enough to cover the membrane. Gently shake the membrane for 1 hour at room temperature or at 4 °C overnight using a laboratory platform rocker. Remove the solution.
6. Perform three washes of the membrane in TBS-T solution (10 min each) using the platform rocker.
7. Dilute the secondary antibody to its working concentration in antibody solution and add to the membrane. Gently shake the membrane for 1 hour at room temperature using a laboratory platform rocker. Remove the solution.
8. Perform two washes of the membrane in TBS-T solution (10 min each) using the platform rocker. Perform a third and final wash of the membrane in TBS solution (10 min). Remove the TBS solution at the end of the third wash.
9. Prepare the ECL substrate as recommended by the manufacturer. Add the ECL substrate directly to the membrane making sure the substrate is present on the whole membrane. Let it sit for 1–5 minutes.
10. Cover the membrane with clear wrap.
11. Expose to X-ray film (*see Note 16*).
12. Develop the film in an X-ray developer.

3.5 Detection of methylated peptides and histones by using radioactivity and filter assays

This section describes the use of a rapid detection method for measuring the enzymatic activity based on incorporation of radioactive ^3H -SAM. Contrary to other detection methods described in this chapter, this method does not rely on separating the product(s) of the HMT reaction on a gel. However, it cannot resolve whether multiple histones or other proteins are being methylated in the same HMT reaction, as all ^3H -SAM incorporated into peptides or proteins will be detected.

1. The reactions are stopped by spotting the reactions onto Whatman P-81 filter papers (~1.5 cm² each). The filter papers are dried for ~15min at room temperature before proceeding to the next step. Each filter paper should be labeled, as they will be combined in the next step.
2. Unincorporated ^3H -SAM is removed from the filters by washing them in 250 ml (using a 1 L beaker) of BD solution in a 1 L beaker. A maximum of 20 filter papers per 250 ml of BD solution should be used. Wash three times for 30 min each. Gently rock the beaker using a rocking shaker. Do not use a magnetic bar when washing, as the filters will get damaged.
3. Dry the filter papers at room temperature.
4. Each filter paper is quantified separately by liquid scintillation counting.

3.6 Troubleshooting

Fig. 1 shows an example of a successful HMT assay using radioactive detection. If no methyltransferase activity is detectable with the protocol described in Subheading 3.1 followed by any of the appropriate detection methods, several options for optimization are available. Radioactivity-based detection methods are usually most sensitive and may be required to detect

activity of certain methyltransferases. Although the amounts stated should be sufficient in most cases, the amount of ^3H -SAM can be increased to enhance the signal. Exposure times for fluorography on autoradiography film can be extended to several days or even weeks.

The methylation reaction itself can be enhanced by 1) increasing incubation times up to overnight, 2) altering the reaction temperature (both above and below 30°C), 3) increasing reaction pH, and/or by 4) increasing concentration of SAM (both unlabeled and ^3H labeled). If the SAM stocks have been stored for prolonged times, consider replacing them with fresh ones. In addition, as the final molar concentration of ^3H -SAM is often in the submicromolar range especially for ^3H -SAM preparations with high labeling density, supplementing the reactions with additional $10\text{--}20\ \mu\text{M}$ unlabeled SAM may increase sensitivity in cases where the increase in enzymatic activity due to elevation of SAM concentrations closer to its K_m for the enzyme is greater than the competition effect observed by adding an excess of unlabeled SAM.

The amount of methyltransferase can be increased as well, however, care should be taken to prevent introduction of significant amounts of salt, which might decrease activity especially on nucleosomes [9]. Moreover, potential inhibitory factors from bacteria or cell lysates may be introduced with increasing amounts of methyltransferase preparations as well. It is also possible that further factors might be required for activity; for instance the catalytic subunits of PRC2 do not exhibit activity without the other complex members [10,1]. Furthermore, while increasing the amount of substrate might enhance activity, assaying different types of substrates should also be considered since the activity of a methyltransferase can vary depending on the substrate [11,10]. Also, enzymatic activity might only be detected with specific histone isoforms or in the presence of other modifications [4].

4. Notes

1. Both lysine and arginine methyltransferases have been shown to be optimally active in alkaline buffers, with pH optima often around 9-10 [12,13]. We therefore recommend

slightly alkaline buffer conditions with pH 8.5 usually providing a good starting point for optimization.

2. For optimum sensitivity of the assay, ^3H -SAM with a high specific activity should be used (~ 3 TBq/mmol, if available). Both radiolabeled and unlabeled SAM are relatively prone to decay and stock solutions may lose significant activity within 6 months or less of storage. Stock solutions of SAM must be kept in a strongly acidic buffer (e.g. 5 mM sulfuric acid pH 2, 10 % ethanol), as it is unstable at neutral or alkaline pH. Storage at -80 °C is not recommended, as this accelerates decay compared to storage at -20 °C.
3. Providing detailed instructions for the purification of specific enzymes is beyond the scope of this protocol. However, the protocol described here should allow for the detection of methyltransferase activity from a variety of sources, ranging from highly purified recombinant or native enzymes and enzyme complexes to crude or fractionated cellular extracts. Both *E. coli* and insect cell (Sf9) expression hosts have been successfully used to prepare active methyltransferases using protein affinity tags such as His, GST, or FLAG. As a general recommendation, it is likely easier to use purified recombinant proteins expressed in *E. coli* (or insect cells if there are issues with expression/folding in *E. coli*) than native enzymes extracted from plant tissues. Insect cell systems also allow for the expression of individual subunits and purification of reconstituted protein complexes. Regardless of source, it is usually advisable to keep the volume of enzyme preparation per reaction to a minimum in order to minimize carry-over of potential inhibitors of activity. Activity of many methyltransferases, especially when using nucleosomes as substrates, is also inhibited by salt [9]. Enzyme preparations should therefore ideally be provided in a low-salt buffer, for instance by dialyzing the final

purification product into BC100 buffer (see Subheading 2.1). Dialysis will also remove elution agents such as FLAG peptide, even though the latter commonly does not interfere with activity. Enzyme preparations should be aliquotted and stored at -80 °C to preserve activity.

4. Although not always representative of the native conformation of histones in chromatin, peptides derived from histone sequences such as the N-terminal tail of histone H3 are commonly used as substrates in HMT assays. Histone peptides containing several well-characterized modifications are readily available from several commercial sources or can be custom-made by peptide synthesis services. Peptides should be centered around the residue to be methylated and of sufficient length (>20 residues) to include neighboring residues that might be required for substrate recognition. Unless containing native N- or C- termini, cap the peptides with acetyl (N terminus) and amide (C terminus) groups to maintain electrostatic properties of peptides. As the mass of lyophilized material is a poor measure for peptide amounts due to varying amounts of residual salts, it is highly advisable to add a C-terminal tyrosine residue to allow for quantification by UV spectroscopy. Alternatively, a biotin moiety can be introduced in the form of a C-terminal biotinylated lysine residue to enable quantification by western blotting with anti-biotin antibodies. When comparing activities between differentially modified peptides, accurate quantification and matching of peptide amounts is crucial to obtain meaningful results. Peptides should be resuspended to a final concentration of 10 mM in water (minimal amounts of TFA or ammonium hydroxide can be added to help solubilize the peptides if needed). Aliquot and store at -20°C. Use protein low-bind tubes to avoid loss of peptide due to adhesion to tube walls. Consider filtering through a 0.22 µm low protein binding

filter to prevent degradation due to potential bacterial contamination. If TFA or ammonium hydroxide is present in peptide stock solutions, ensure that the pH of the HMT reaction is unchanged by spotting 0.5–1 μ l of the reaction onto each field of a suitable pH strip and determine pH based on color change.

5. Histone preparations from various sources can also be used as substrates. Recombinant, unmodified histones are available from commercial sources or can be expressed and purified from *E. coli* using well established protocols [14,15]. Recombinant histones containing lysine/arginine-to-alanine point mutations are commonly used to elucidate the site specificity of methyltransferases with unknown substrate specificity. Recombinant histones with defined, site-specific modifications can further be generated by cysteine alkylation approaches or by native chemical ligation [16]. A growing selection of modified histones is also available commercially. They can also be isolated from native sources such as chicken erythrocytes [17] or HeLa cells (also available from vendors). Histones can either be provided as histone monomers or as complexes, such as H3-H4 tetramers or complete histone octamers. Histones are often provided in buffers containing 2 M NaCl or KCl, which is required to maintain solubility and integrity of the highly charged histones in absence of DNA. Histone preparations can therefore be a significant source of salt in HMT reactions, potentially inhibiting activity of the enzyme to be tested [9]. It is important to keep in mind that, when using histone octamers, the low concentration of salt in HMT reactions promotes dissociation into H2A–H2B dimers and H3–H4 tetramers [18], which will therefore represent the major histone species in the reaction.

6. Histones from recombinant or native sources can be assembled into nucleosomal substrates using well-established salt dialysis-based protocols [15]. DNA templates for assembly are commonly plasmids containing repeats of the 601 nucleosome position sequence [19] interspaced with linkers of varying lengths. These plasmids can be used directly for assembly. If exact positioning is crucial, the stretch of repeats can be excised and separated from the plasmid backbone, for which uneven spacing is expected due to the absence of positioning sequences. For mononucleosome particles, 601 DNA can either be amplified by PCR or excised from plasmids. The degree of saturation of DNA templates with histones should be monitored and oversaturation avoided. When simply seeking to detect activity, undersaturation is usually acceptable as methyltransferases often do not require complete occupancy of DNA with histone octamers to display activity, although specific modes of regulation might be missed [20]. Reconstituted chromatin is usually stored in TE buffer, which is ideally suited for use in HMT assays due to its low salt content. Of the options presented here for substrates in these assays, reconstituted chromatin most accurately reflects the native substrate of most methyltransferases that act on chromatin, however, others may not be active on such reconstituted templates at all.
7. The optimal reaction temperature depends on the methyltransferase and needs to be determined experimentally. For example, *Neurospora crassa* Dim-5 is optimally active at 10 °C, remains 50 % active at 30 °C, but is essentially inactive at 37 °C [13]. 30 °C should yield activity for a wide range of enzymes, however, the growth temperature of plants may provide a useful starting point for plant-derived histone methyltransferases or other plant enzymes.

8. When using radioactive detection methods, it is advisable to collect samples at the bottom of tubes by brief centrifugation in a table-top centrifuge (10 sec at full speed) before opening the tubes after all mixing and incubation steps in order to minimize the risk of radioactive contamination. Moreover, be cautious to properly handle and dispose of all tubes, pipette tips, blotting paper, and other consumables that have been in contact with ^3H -SAM. Buffers from SDS-PAGE and blotting transfer likewise need to be handled with appropriate care and disposed of properly. Equipment such as gel tanks and blotting setups should be checked for contamination. All procedures need to be carried out in accordance with local rules for the use of ^3H in laboratories.
9. If the goal of the assay is primarily to detect activity, incubation times as long as overnight can be employed. Usually, 30 min – 4 h should be sufficient to observe activity. However, when aiming to determine kinetic parameters of the enzyme, shorter time points within the linear range of the reaction are required to accurately determine initial velocities.
10. 15 % gels are recommended as they provide good resolution of all four core histones while still allowing adequate transfer of higher molecular weight methyltransferases in subsequent blotting steps. Both 18 % and 12.5 % gels may be used as well.
11. It is imperative to use PVDF membranes as nitrocellulose membranes are incompatible with the subsequent staining and scintillation cocktail spraying steps. Remember to pre-soak PVDF in methanol before use. Methanol for this purpose can be stored and reused.
12. Importantly, letting the PVDF membrane dry completely increases the signal-to-background ratio of the coomassie-stained bands and should be done before taking a picture. To air dry takes ~30 min, but the process can be sped up with a hair dryer or

laboratory heat gun, as long as these can be run at low temperature or cool mode to not heat up the membrane. Carefully hold the membrane with forceps and use a low fan speed to keep the membrane from flying away and becoming damaged.

13. A hair dryer can be used to speed up the drying process.
14. Exposing the film at -80 °C increases the sensitivity of detection, but it can be performed at ambient temperature as well.
15. For peptides, make sure to use a 0.2 µm or similar pore size membrane along with shorter transfer times to prevent transfer of peptides through the membrane. Staining the membrane by Ponceau can be helpful to ensure successful transfer of the peptide. This step may need to be optimized depending on the peptides used.
16. The strength of the signal obtained can vary based on a few parameters (e.g. efficiency of the HMT reaction, primary antibody concentration used). For these reasons, the time of exposure should be extended if no signal is observed on the film after a 1-2 min exposition.

Acknowledgements

We want to thank Jean-François Couture (University of Ottawa) for his advice on some of the assays described and Chantal LeBlanc for her helpful comments on this chapter. Work in the

Voigt lab is supported by the Wellcome Trust and the Royal Society (joint Grant Ref. 104175/Z/14/Z, Sir Henry Dale Fellowship to PV) and has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (ERC-STG grant agreement No 639253). The Wellcome Trust Centre for Cell Biology is supported by core funding from the Wellcome Trust (Grant Ref. 092076).

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Figure legends

Fig. 1. HMT assay using the Arabidopsis histone H3 lysine 27 (H3K27) methyltransferase ATXR5. The enzyme with an N-terminal GST tag was expressed in E.coli and purified by affinity chromatography. The substrates used in the assay were plasmid-based nucleosomes containing different histone H3 variants: plant H3.1, mammalian H3.3, or plant H3.3. ³H-labeled SAM was used to detect the methylated histones. The result of the assay demonstrates the specific role of threonine 31 (T31) of plant H3.3 variants in inhibiting the activity of ATXR5.

Fig. 2. Schematic overview of the different methods described in this Chapter to perform HMT assays. LSC = Liquid scintillation counter.

Figure 1

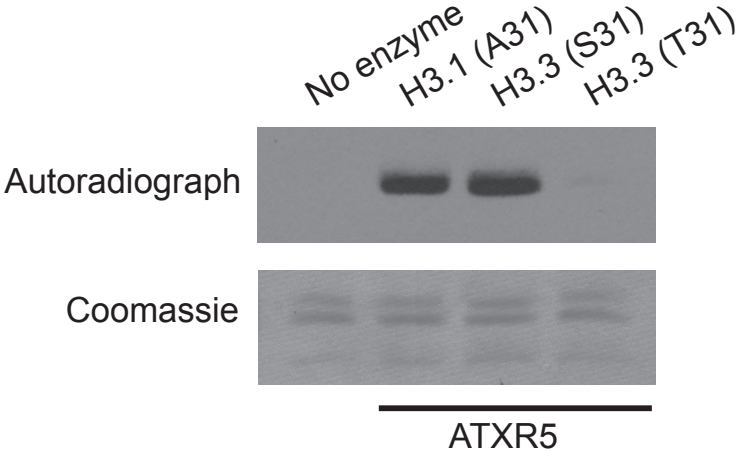


Figure 2

