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In Vitro Histone Methyltransferase Assay

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INTRODUCTION

Histone methyltransferases catalyze the addition of one or more methyl groups to a specific lysine or arginine residue within histones. Currently, there is a great deal of interest in histone methyltransferases, because mutations and misregulation of the genes encoding these proteins have been linked to various cancers and other diseases. Many genes encoding putative histone methyltransferases have been identified in eukaryotes, but the proteins they encode have not been functionally characterized. This protocol describes an in vitro assay for histone methyltransferase activity that uses bacterial cell extracts in which expression of a methyltransferase of interest is induced. In many cases, purification of the enzyme is unnecessary, making this experiment ideal for pilot studies. Bacterial cell extract containing the methyltransferase of interest is incubated with S-adenosyl-L-[methyl-³H]-methionine and various histone substrates, many of which are commercially available. Incorporation of the methyl-³H can be measured easily by scintillation counting. The labeled substrate is visualized by SDS-polyacrylamide gel electrophoresis (PAGE) followed by fluorography. This allows the substrate specificity and activity of a histone methyltransferase of interest to be readily characterized.

RELATED INFORMATION

This protocol was adapted from Strahl et al. (2001). Protocols for histone and nucleosome core particle isolation can be found in Schnitzler (2000).

MATERIALS

Reagents

Bacteria containing an expression plasmid encoding the recombinant methyltransferase of interest

Use common bacterial expression vectors such as pET28 or pGEX 2T for generating His₆ or GST-tagged proteins. His₆ and GST epitopes will allow for detection of protein by Western blot and will serve as a means for purification if necessary.

Bacterial lysis buffer

Coomassie Blue stain

Coomassie destain solution

ENLIGHTNING autoradiography enhancer (PerkinElmer, NEF974G001L)

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Glycerol

2X Histone methyltransferase buffer (50 mM Tris-Cl at pH 8.0, 20% glycerol)

Histone substrates (see Step 24)

Core histones (1 mg/mL)

*Core histones from 293T cells can be isolated as described (Strahl et al. 2001).
Core histones can also be purchased (chicken erythrocytes, Millipore; calf thymus, Sigma).*

Histone peptides (1 mg/mL)

Histone peptides can be custom synthesized or purchased from Millipore.

Nucleosomes from chicken erythrocytes (purified, 4 mg/mL)

Nucleosomes can be purified as described (Mizzen et al. 1999).

Recombinant histones (purified, 1 mg/mL)

Recombinant histones can be generated and purified as described (Luger et al. 1999) or purchased from Millipore.

Isopropyl- β -thiogalactoside (IPTG) (100 mM stock solution)

LB (Luria-Bertani) liquid medium

Add the appropriate antibiotics for selection.

NaHCO₃ (1 M, pH 9.0)

S-adenosyl-L-[methyl-³H]-methionine (GE Healthcare TRK581)

S-adenosyl-methionine is toxic and requires care in handling, in addition to the care needed for radioactive substances.

SDS-polyacrylamide gels (12% and 15%; see
SDS-Polyacrylamide Gel Electrophoresis of Proteins)

2X SDS sample buffer

5X SDS sample buffer

Equipment

Autoradiography cassette

Beaker (1 L)

Boiling water bath

Electrophoresis unit and power supply suitable for SDS-PAGE

Gel dryer

Ice

Ice water bath

Incubator preset to 37°C

Microcentrifuge

Microcentrifuge tubes

Paper towels

Pencil

Plastic wrap

Plate (glass)

Radioactive waste receptacle

Scintillation counter, vials, and scintillation fluid suitable for reading ^3H

Shaker, temperature-controlled (e.g., Eppendorf Thermomixer R, a programmable mixing-heating/cooling block) (optional; see Step 27)

Shaker (platform)

Sonicator (e.g., Misonix) with microtip attachment

Spectrophotometer

Staining tray

Tubes (14-mL sterile disposable polypropylene; e.g., BD Falcon)

Whatman paper (3MM Chr)

Whatman paper (P-81) (2 cm $\times$ 2 cm squares)

X-ray film

METHOD

Expression of Recombinant Methyltransferases

1. In a 14-mL sterile polypropylene tube, inoculate 4 mL of LB plus antibiotic with bacteria containing an expression plasmid encoding the methyltransferase of interest.
Cultures can be started from frozen glycerol stocks or from single colonies on an agar plate.
2. Incubate culture overnight with shaking at 37°C.
3. Inoculate 4 mL of LB liquid medium containing antibiotic with 0.2 mL of the overnight culture, and incubate with shaking at 37°C until OD₆₀₀ measures between 0.6 and 0.8.
4. Split the culture into two 2-mL aliquots in new 14-mL culture tubes.

5. To one tube, add IPTG to a final concentration of 0.4 mM. Do not add IPTG to the second tube. This serves as an uninduced control.
6. Incubate both cultures with shaking for 4 h at room temperature.
7. Transfer the cultures to microcentrifuge tubes and harvest cells by centrifugation in a microcentrifuge at 14,000 rpm for 1 min.
8. Remove and discard the supernatant. At this point, the collected cells can be frozen and stored at -80°C until processed.

The freezing step is not essential and the protocol can be continued if desired.

Preparation and Analysis of Bacterial Cell Extracts

9. If cells have been previously frozen, thaw them on ice. Resuspend the cells in 0.2 mL of bacterial lysis buffer.
10. Sonicate the cells as follows, while suspending the tube in an ice water bath:
 - i. Using a Misonix programmable sonicator, set the program for 10 sec total sonication time, with a 2-sec pulse followed by a 1-sec pause, and an output of level 3 (9 W).
 - ii. Allow the cells to rest for 2 minute on ice.
11. Collect the cells by centrifugation in a microcentrifuge at 14,000 rpm for 5 min.
12. Repeat Steps 10 and 11.
13. Transfer the supernatant to a new microcentrifuge tube; this is the soluble cell extract. The remaining pellet is the insoluble fraction.
14. Add glycerol to the soluble fraction to a final concentration of 10%.
Samples can be aliquoted and stored at -80°C at this point.
15. Combine a 10- μL aliquot of the soluble fraction with 10 μL of 2 $\times$ SDS sample buffer.
16. Resuspend the insoluble fraction in 200 μL of 2 $\times$ SDS sample buffer.
If the insoluble sample becomes too viscous for SDS-PAGE, sonicate it using the conditions in Step 10, but without using an ice bath.
17. Boil all of the samples for 5 min prior to gel electrophoresis.
18. Analyze both soluble induced and uninduced (10 μL) and insoluble induced and uninduced (10 μL) fractions by SDS-PAGE on a 12% SDS polyacrylamide gel (see SDS-Polyacrylamide Gel Electrophoresis of Proteins) followed by staining with Coomassie Blue.
19. If expressed protein is detectable in the soluble fraction by Coomassie Blue staining, assay the sample for histone methyltransferase activity as in Steps 21–39.
If the protein is not detectable in the soluble fraction after staining with Coomassie Blue, analyze these samples by Western blotting using antibodies that detect your protein of interest. If the expressed protein can be visualized by Western blot analysis, perform the histone methyltransferase assay.
See Troubleshooting.
20. Store the soluble protein extracts at -80°C .

In Vitro Histone Methyltransferase Assay

21. Set up a 1.5-mL microcentrifuge tube on ice for each assay.
22. To each tube, add 10 μ L of 2 $\times$ histone methyltransferase buffer.
23. Add 1 μ L of S-adenosyl-L-[methyl- 3 H]-methionine.
From this point onward, reagents are radioactive and must be handled with appropriate caution.
24. Add desired histone substrates.
Substrates can be core histones, purified recombinant histones, histone peptides, or purified nucleosomes. The amount of substrate used in each assay varies based on concentration. Typically, 1 μ g of each recombinant histone to be tested, 1–5 μ g of histone peptide, 4–8 μ g of core histones, or 4–8 μ g of nucleosomes is sufficient. Use enough substrate to allow detection by SDS-PAGE followed by Coomassie Blue staining. If the methyltransferase of interest is uncharacterized, test it against all of the mentioned substrates to determine specificity.
25. Add 1–2 μ L of prepared bacterial extracts from Step 14.
*Using more than 2 μ L of bacterial extract is not recommended. Set up two negative control reactions: one using bacterial extract from uninduced cells and one without bacterial extract. Add bacterial lysis buffer in place of extract in the latter control. Set up a positive control reaction as well using a known methyltransferase, such as *Saccharomyces cerevisiae* Dot1 or Set2 for nucleosome substrates or human Set7/9 for histone or peptide substrates.*
26. Add H₂O to a final volume of 20 μ L.
27. Incubate the reactions in a temperature-controlled shaker at 1000 rpm for 30 min at 20°C.
Alternatively, carry out the reaction at room temperature with occasional mixing by hand.
28. Dilute a 1 M NaHCO₃ (pH 9.0) stock solution to make a 50 mM NaHCO₃ (pH 9.0) working solution. Make this stop/wash solution fresh, just before use in the next step.
29. Stop the reactions as follows:
 - i. Spot 10 μ L of each reaction onto Whatman P-81 paper squares (2 cm $\times$ 2 cm) which have been labeled with a pencil and arranged on a glass plate.
 - ii. Immediately add 2 μ L of 5 $\times$ SDS sample buffer to the remaining half of each reaction for gel analysis.
Store these samples at –20°C until Step 31. Samples can be stored at –20°C indefinitely, and SDS-PAGE analysis can be performed at your convenience.
 - iii. Place the filters into a 1-L beaker.
 - iv. Add 250 mL of 50 mM NaHCO₃ (pH 9.0) to the filters. Place them on a room temperature platform shaker and wash for 5 min.

The shaking speed should be fast enough to keep the filters suspended off the bottom of the beaker.

- v. Carefully pour off the wash buffer into a radioactive waste receptacle.
 - vi. Repeat Steps 29.iv and 29.v three more times for a total of four washes.
 - vii. After the final wash, spread the wet filters on paper towels to dry.
- 30.** Quantify the activity of each reaction by liquid scintillation counting.
- Save these samples and count them again the next day, because the counts will increase over the first several hours.*
- See Troubleshooting.*
- 31.** For gel analysis, run the samples from Step 29.ii on a 15% SDS-PAGE gel (see SDS-Polyacrylamide Gel Electrophoresis of Proteins).
- For peptide substrates, do not let the dye front run off the gel; take into consideration the molecular weight of the substrates and run gels accordingly. Include in the gel prestained molecular weight protein standards in the 6–10 kD range. For optimal resolution of the histone proteins run the gel as far as possible without running the samples off. Caution! After the gel has been run, the running buffer in the gel apparatus will be contaminated with free S-adenosyl-L-[methyl-³H]-methionine.*
- 32.** Stain the gel in Coomassie Blue stain for 1 h or overnight.
- 33.** Destain the gel with Coomassie destain solution until the substrate bands of interest are visible.
- After destaining, the gel can be photographed or scanned for documentation.*
- 34.** Remove the gel from the Coomassie destain solution and place it into a clean dry staining tray.
- 35.** Add ~10 mL of ENLIGHTNING solution which has been mixed well just before use. Use just enough ENLIGHTNING solution to completely cover the gel.
- 36.** Incubate the gel at room temperature for 30 min with gentle shaking.
- The ENLIGHTNING solution will destain the gel further.*
- 37.** Place the gel onto 3MM Chr Whatman paper and lay a sheet of plastic wrap over the top.
- 38.** Dry the gel using a gel dryer for 1 h at 80°C.
- 39.** Remove the plastic wrap from the dried gel. In the dark, insert the gel and a piece of X-ray film in an autoradiography cassette and place it at –80°C.
- X-ray film in an autoradiography cassette and place it at –80°C.*
- Expose the gel from several hours to several days or even longer, depending on how much activity you see from the filter counts (Step 30). Sometimes exposures may have to go for several weeks for a signal to be detected.*

TROUBLESHOOTING

Problem: Expressed protein is insoluble

[Step 19]—Solution: Consider the following:

- The most common solutions are to adjust the time and temperature of induction (see Step 6). An overnight induction at room temperature or at 18°C will sometimes be sufficient to allow the expression of some soluble protein.
- Lower the amount of IPTG used for induction from 0.4 mM to 0.1 mM; this may increase the amount of soluble protein.
- The size of the protein being expressed in bacteria also influences solubility. In the case of some histone methyltransferases, full-length protein is not needed for activity. Expressing the methyltransferase domain with some additional flanking sequence may be more soluble than a full-length protein and sufficient for histone methyltransferase activity.
- If no soluble protein is produced, try an alternative expression system such as baculovirus. However, the protein of interest will have to be purified to eliminate contaminating endogenous histone methyltransferase activity.

Problem: Histone methyltransferase activity is weak or not detected by the filter assay followed by scintillation counting

[Step 30]—Solution: Consider the following:

- Even if no histone methyltransferase activity is detected by scintillation counting, the samples should still be processed by SDS-PAGE and fluorography. Weak activity can sometimes be detected on the autoradiograph after several weeks of incubation at −80°C.
- Using too much bacterial cell extract seems to have an inhibitory effect on methyltransferase activity, possibly due to the contribution of additional salt from the bacterial lysis buffer. Limit the amount of bacterial extract to no more than 2 µL.
- Usually, enough of the methyltransferase of interest is present in the bacterial cell extract to generate detectable activity. If the methyltransferase activity is weak, however, the protein may have to be purified. For example, if the protein is only detectable by Western blot (and not by Coomassie Blue staining), purifying the protein may be required to obtain robust activity. Microgram quantities of the purified protein of interest can be used in this assay. However, if the protein is in high salt buffer, dialysis or buffer exchange may be necessary prior to performing the assay.
- A given histone methyltransferase may prefer some substrates to others. For example, if no activity is detected when using peptides or recombinant histones as a substrate in this assay, try nucleosomes or a mix of core histones.
- Weak methyltransferases may have very little detectable activity if the S-adenosyl-L-[methyl-³H]-methionine is not fresh. If this reagent is more than 6 mo old, its specific activity will decrease and the overall activity of the methyltransferase will appear to be much lower than it actually is. In this case, repeat the assay with fresh S-adenosyl-L-[methyl-³H]-methionine.

DISCUSSION

This protocol can identify and measure histone methyltransferase activity. It can also provide some limited information regarding specificity. While this assay can identify activity in the presence of a particular histone or peptide, identification of the target amino acid requires further investigation. If the histone methyltransferase is active on peptide

substrates, Edman degradation and microsequencing can be performed to identify the labeled amino acid (Strahl et al. 2001). Alternatively, if it is active on recombinant histones, mutations at candidate amino acids within the recombinant histones can be generated and tested as substrates. Additionally, histone methyltransferase assays can be performed using non-radioactive S-adenosyl-L-methionine and the histones can be analyzed by mass spectrometry to look for the addition of mass contributed by the methylated residue. Identifying the site specificity of a methyltransferase that is nucleosome specific is more complicated. It can be analyzed by mass spectrometry, but pre-existing modifications (if the substrates have been isolated from chicken erythrocytes) make this approach problematic. Radioactively labeled nucleosomes can also be separated into purified histones using reverse-phase high performance liquid chromatography (HPLC) and assayed by Edman degradation to look for incorporation of the label. Alternatively, it is also possible to generate recombinant nucleosomes, using recombinant histones and specific DNA sequences. These recombinant nucleosomes can be constituted with histones containing specific mutations. Loss of activity with a particular mutation might suggest it is a target residue. However, the existence of *trans*-tail histone interactions, where activity on one histone is dependent upon a sequence or modification on another histone, can complicate this analysis (Briggs et al. 2002; Fingerman et al. 2007).

This method of analysis may not be suitable for all histone methyltransferases. For example, the Set1 histone methyltransferase, from *S. cerevisiae*, is only active when it is present in a multiprotein complex (Briggs et al. 2001; Krogan et al. 2002; Nagy et al. 2002; Roguev et al. 2001). Reconstitution of these protein complexes in vitro or purification of the complex from cells may be necessary for observing histone methyltransferase activity. Alternatively, this protocol can be adapted for use with other types of modifying enzymes. For example, by using radioactively labeled acetyl-CoA or ATP, it may be possible to assay histone acetyltransferase or kinase activity. However, the histone acetyltransferase or kinase of interest may have to be purified since there could be interfering activities found in the bacterial extracts. Finally, non-histone proteins may also be used as substrates in these assays, because several histone methyltransferases have been shown to be active on non-histone substrates. Overall, this method serves as a starting point for the initial identification and characterization of potentially novel histone methyltransferases and, with various modifications, can be used to examine other histone and non-histone specific enzymatic activities.

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