Cell-Based Methylation Assays

A technique based on cellular metabolism of methionine has been developed that allows methylated cellular proteins to be labeled *in vivo* using tritiated methionine (Coppard et al., 1983; Liu and Dreyfuss, 1995). In the cell, the universal methyl donor S-adenosylmethionine is generated from free methionine (Freitag and Clarke, 1981). Thus, cells are labeled with [methyl-^{3}H]-L-methionine in the presence of protein-synthesis inhibitors, which prevents the incorporation of the radioisotope into nascently synthesized proteins, while allowing the labeling of methylated proteins. For this experiment to work well, protein synthesis must be effectively blocked, otherwise proteins become labeled due to the incorporation of tritiated methionine. It should be noted that there is no evidence for direct AdoMet transport across the plasma membrane of mammalian cells (Clarke and Tamanoi, 2006; McMillan et al., 2005). Thus, tritiated AdoMet should not be used to label methylated proteins in cultured cells.

1. Seed exponentially growing HeLa cells at 1-2 X 10^6 cells in a 10-cm tissue culture dish in 10 mL of the appropriate complete growth medium until 80% confluence is reached.
2. Wash the cells with 1X PBS and add 10 mL of growth medium A [Dulbecco's Modified Eagle's Medium (DMEM), 10% Fetal Bovine Serum (FBS), cycloheximide (100 μg/mL in ethanol), and chloroamphenicol (40 μg/mL)].
3. Incubate for 30 min at 37 ºC in a tissue culture incubator.
4. Wash the cells once with 5 mL of growth medium B [Dulbecco's Modified Eagle's Medium without methionine (DMEM/-Met), 10% FBS (dialyzed), cycloheximide (100 μg/mL in ethanol), and chloramphenicol (40 μg/mL)].
5. Add 50 μL of L-[methyl-^{3}H]methionine (1mCi/mL) to the plate that contains 5 mL of medium B.
6. Incubate for 3 h at 37 ºC in a tissue culture incubator.
7. Wash the cells twice with cold 1X PBS.
8. Add 600 μL of cold RIPA lysis buffer (50 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, and 0.1% SDS).
9. Collect the cells from the culture plate with a plastic scraper.
10. Transfer the solution into a 1.5-mL microcentrifuge tube and rock for 10 min at 4 ºC.
11. Sonicate 8 s with pulses of 0.5 s on and 0.5 s off (amplitude 30%) to break the cells.
12. Centrifuge at 15,000 *g* for 15 min. Keep the supernatant.
13. While the incubation at step 6 is in progress, wash 25 μL of protein A/G agarose beads with cold RIPA lysis buffer. Then add 2 μg of antibody, against your substrate of interest, to the protein A/G agarose beads in 300 μL of cold RIPA lysis buffer.
14. Incubate for 2-3 h with rocking at 4 ºC to allow binding of the antibody to the protein A/G agarose beads.
15. Centrifuge at 1,000 *g* for 30 s and discard the supernatant.
16. Add cell supernatant from **step 12** to the beads and rock for 2 h at 4 ºC.

17. Centrifuge at 1,000 *g* for 30 s and wash the beads three times with cold RIPA lysis buffer.
18. Carefully remove all of the buffer and add 25 μL of 2X protein sample loading buffer and heat at 95 ºC for 5 min.
19. Run IPed sample by SDS-PAGE at 100 volts for 1-2 h using Tris/Glycine running buffer.
20. Transfer the separated samples from the gel to a PVDF membrane using a semidry electroblotter.
21. Spray the PVDF membrane harboring the immobilized protein samples with EN^{3}HANCE two times, with a 10-min delay between each application.
22. Wait 30 min for the PVDF membrane to completely dry and then expose to X-ray film for 2-5 days.

An example of this experiment is shown in **Figure 1**. CA150 is a well-characterized CARM1 substrate (Cheng et al., 2007), and Sam68, is a well-described substrate for PRMT1 (Cote et al., 2003). Both these proteins can be used as positive control for cell-based methylation assays. Importantly, after fluorography has been performed, the same membrane can be washed and reanalyzed by Western with αCA150 and αSam68 antibodies, respectively, to ensure equal loading.

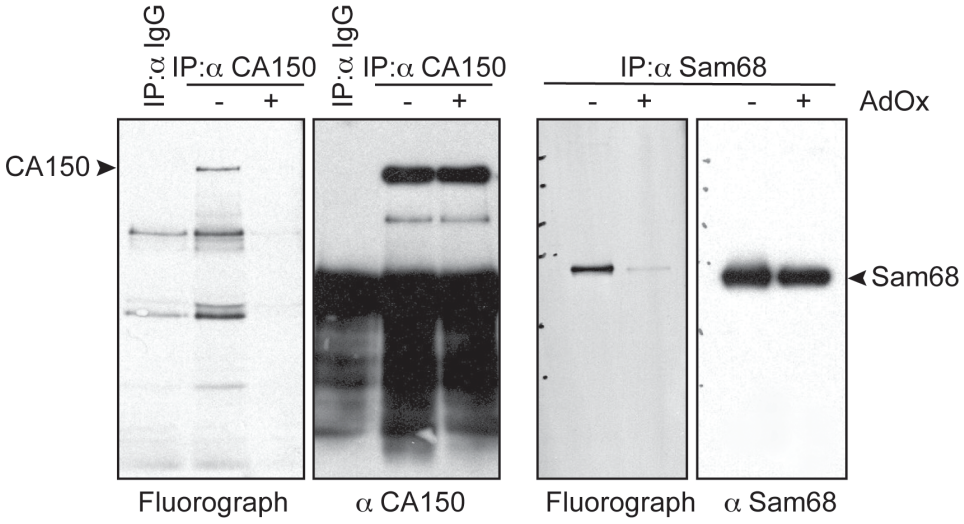

Figure 1. The methyltransferase inhibitor, AdOx, prevents CA150 and Sam68 methylation in cells. HeLa cells were grown for 24 h in the presence of 20 μM AdOx. These cells were then subjected to an *in vivo* methylation assay by tritium labeling, in the presence of 20 μM AdOx. IPs were performed with αCA150 or αSam68 antibodies. The ^{3}H-labeled proteins were visualized by fluorography. After fluorography, the same membrane was washed in TBST and immunoblotted with αCA150 or αSam68 antibodies. We clearly see equal amounts of the proteins in cells that are treated and untreated with AdOx. But methylated bands are only seen in the untreated samples.

References

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