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A Method to Study the Expression of DNA Methyltransferases in Aging Systems In Vitro

Joel B. Berletch, Sharla M. O. Phipps, Sabrina L. Walthall, Lucy G. Andrews, and Trygve O. Tollefsbol*

Summary

The methylation of CpG dinucleotides located in key protein binding sites within gene regulatory regions often leads to gene silencing. A mechanism of aging is proposed whereby an accumulation of methylation at gene regulatory sites contributes to cellular senescence. DNA methyltransferases (DNMTs) are enzymes that catalyze the transfer of a methyl moiety from *S*-adenosyl-L-methionine (SAM) to the cytosine of a CpG dinucleotide and are responsible for establishing and maintaining methylation patterns in the genome. It is important to study not only transcription of the DNMTs, but also their protein expression because studies illustrate that it is possible for the enzymes to undergo posttranslational physical changes in response to stimulation even though gene transcription remains unchanged. Here, we discuss an in vitro method to study protein expression of DNMTs in aging systems.

Keywords

SAM; DNMTs; posttranslational; CpG dinucleotides; aging

1. Introduction

Immunoblot analysis is a common tool used to provide a semiquantitative measurement of both protein expression and stability. Proteins have been known to undergo many posttranslational modifications which include acetylation, ubiquitination and phosphorylation, and indeed many studies have used this technique to study these protein alterations (1-4). DNA methyltransferases (DNMTs) are important enzymes involved in transferring methyl moieties to the 5-position of cytosine in CpG dinucleotides and thereby regulating gene activity. In addition to changes in transcription of DNMTs, protein levels have also been shown to fluctuate as cells age and enter senescence (5,6). Moreover, it has also been revealed that DNMTs can undergo posttranslational modifications (7), which indicates the importance of studying DNMTs at the protein level. The following describes a technique used in our laboratory to study DNMT protein expression in aging systems. In brief, total cellular protein is isolated and quantified, followed by denaturation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The separated protein fraction is then transferred to a membrane and incubated with antibodies specific for the protein or proteins of interest. The probed membrane is subjected to a technique for visualization (as described under **Subheading 3.4.**).

*Joel Berletch and Sharla Phipps contributed equally to this work.

⁵Secondary antibodies are usually commercially made and come with a suggested dilution. It is advised to first try what the company recommends before changing dilution. Secondary antibodies should be chosen based on the primary antibody used. If probing the membrane for more than one primary antibody, note that the background will be extremely high. It is recommended that you probe for one antibody at a time or use a stripping agent after each detection.

2. Materials

2.1. Sample Preparation

1. Phosphate-buffered saline.
2. Trypsin-EDTA: 0.25% w/v trypsin/0.2% w/v EDTA in sterile Hank's balanced salt solution.
3. Protease inhibitors (*see* Table 1).
4. NE-PER kit (Pierce, Rockford, IL).
5. Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA).

2.2. SDS-Polyacrylamide Gel Electrophoresis

1. 2X SDS loading buffer: 250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β -mercaptoethanol.
2. Prestained protein molecular weight marker.
3. 5% precast SDS polyacrylamide gel.
4. 1X Running Buffer: 3.03 g Tris, 14.41 g Glycine, 1.5 g SDS in 1 L water.

2.3. Transfer and Western Blot

1. 1X transfer buffer: 3.03 g Tris, 14.41 g Glycine, 100 mL methanol in 1 L water.
2. Extra thick precut Whatman filter paper (Bio-Rad, Hercules, CA).
3. Sponge pads.
4. Hybond-ECL nitrocellulose membrane.
5. Heat-sealable plastic bags.
6. Nonfat dehydrated milk.
7. TBST: 10 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.1% Triton X-100.
8. Primary antibodies (*see* Table 2).
9. Horseradish peroxidase (HRP)-conjugated anti-immunoglobulin (Ig)G secondary antibodies.

2.4. Detection

1. ECL kit (Amersham, Pharmacia Biotech, Piscataway, NJ).
2. Hyperfilm™ ECL (Amersham, Pharmacia Biotech, Piscataway, NJ).

3. Methods

3.1. Sample Preparation

The majority of aging cells are grown in monolayer and can easily be harvested for DNMT protein level analysis with trypsin digest. The use of protease inhibitors (*see* Table 1) during the process of harvesting cells and extracting protein is customary. While there are many methods to extract cellular protein (i.e., RIPA, NP-40), the NE-PER kit (Pierce, Rockford, IL) allows for rapid isolation of nuclear and cytoplasmic extracts and is recommended in this case to extract nuclear DNMTs.

1. Grow cells to be examined to approx 70–80% confluence. Wash cells with cold PBS. Harvest monolayer cells with trypsin-EDTA and pellet cells at $300\text{--}350 \times g$ (1500 rpm in a swinging bucket or 45° fixed-angle rotor) for 5 min at 4°C .
2. Wash pellet with cold PBS containing protease inhibitors (*see* Table 1).
3. Lyse cells and extract protein according to NE-PER kit protocol.
4. Protein can be flash-frozen in liquid nitrogen for future use.
5. Perform protein determination with the Bradford Assay (Bio-Rad, Hercules, CA).

3.2. SDS-Polyacrylamide Gel Electrophoresis

Because DNMTs range in molecular weight from 98 to 195 kDa, there is no need to separate these peptides using the Tris-Tricine buffer system. The following method describes the traditional Laemmli discontinuous gel system which utilizes 0.1% SDS to denature the proteins. With pore size in the polyacrylamide gel matrix decreasing with higher acrylamide concentrations, the optimal gel concentration for DNMTs to migrate near the midpoint of the gel is 5% (*see* Note 1).

1. Boil $100\ \mu\text{g}$ of protein in an equal amount (1:1 v/v dilution) of 2X SDS loading buffer for 10 min.
2. Load prestained marker and samples into the wells of a 5% SDS polyacrylamide gel.
3. Electrophorese 0.75-mm thick gel at 135 V of constant current until the bromophenol blue tracking dye reaches the bottom of the separating gel (*see* Note 1).

3.3. Transfer and Western Blot

DNMTs can be electrophoretically transferred in a tank for wet transfer (*see* Fig. 1) or semi-dry apparatus to a nitrocellulose, polyvinyl difluoride (PVDF), or nylon membrane. Hybond-ECL membranes (Amersham Pharmacia Biotech, Piscataway, NJ) perform well for this assay. Incubations are best performed with low volumes (2–5 mL) in heat-sealable plastic bags and on orbital shakers. If plastic trays are used, increase volumes to 20–50 mL to ensure that membranes are submerged and always handle membrane with clean, gloved hands.

1. Set up transfer apparatus (*see* Fig. 1), taking care to equilibrate gel, transfer components, and activated membrane (*see* Note 2) in transfer buffer at room temperature for 30 min and rolling out any air bubbles between filter paper, gel, and membrane with a glass rod or test tube.
2. Transfer at 100 V for 30–80 min at 4°C (*see* Note 3).
3. Turn off power supply, disassemble transfer apparatus, remove membrane and block in a solution of TBST for 30 min to 1 h at room temperature with gentle agitation or 4°C overnight. Alternatively, membrane can be blocked in 5% milk or 5% milk plus .01 mg/mL bovine serum albumin (BSA) for 2 h at room temperature or overnight at 4°C .

¹If interested in two or more proteins with different molecular weights, use of a gradient gel is more efficient. Run gels at 150 V for 30 min or more depending on power pack. If gels are run at 200 V it does not yield a gel suitable for immunoblotting. In general, gel concentrations for proteins 60–600 kDa should be approx 5%, 16–70 kDa approx 10% and 12–45 kDa approx 15% (8).

²Equilibrate the gel and nitrocellulose in transfer buffer that has 10% methanol for 15–30 min at room temperature. This allows the gel time to swell before it is transferred to a membrane. If this step is not done, the gel will swell during transfer and protein will not properly transfer to the membrane.

³It is highly recommended that transfer occur for less than 80 min. Longer exposure to transfer buffer may lead to increased amounts of protein leaching out of the gel. Also, if using two complete transfer apparatuses, make sure that the power pack is able to produce the correct amount of voltage. Some of the older power supplies can not handle two setups and will take longer to complete the transfer. It has been noted that such immunoblots are usually of poor quality.

4. Dilute primary antibody in blocking buffer (determine dilution empirically; *see* Note 4). Probe membrane with primary antibody for at least 1 h at room temperature or overnight at 4°C. Place membrane in plastic tray and wash (with agitation) three times with TBST, 15 min per wash. Dilute secondary antibody in blocking buffer. Probe membrane with diluted secondary antibody in a new heat-sealable bag for 30 min to 1 h at room temperature with agitation.
5. Wash membrane 3X with TBST as in **step 4**.

3.4. Detection

Bound antigens can be visualized with chromogenic (i.e., BCIP/NBT) or chemiluminescent substrates. It is preferable to use HRP-conjugated secondary antibodies for the increased sensitivity of enhanced chemiluminescence. The Amersham ECL (Enhanced Chemiluminescence) system works well for this assay. Steps outlined in this section should be performed in a darkroom.

1. Remove traces of final wash by holding membrane vertically and dabbing edge of membrane to Whatman/absorbent paper.
2. Mix two substrate components (1:1 ratio) in a small plastic tray and lay membrane target-side down in this mixture for 1 min.
3. Place membrane target-side down in the center of a piece of plastic wrap and fold plastic wrap around membrane to create a flat, liquid tight seal.
4. Expose membrane to autoradiogram (X-ray) film for the appropriate time (5 s to 30 min, depending on the signal strength).
5. Develop autoradiogram, rinse membrane in PBS and air-dry.

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⁴Primary antibodies are either polyclonal, monoclonal, ascites, or antiserum bleeds. Monoclonal antibodies are usually used at a starting dilution of 1:1000 but can go up to 1:10,000 or more. Polyclonal antibodies can range from a dilution as concentrated as 1:1 to a dilution of 1:500. Ascites antibodies are more concentrated than the monoclonal, so suggested starting concentration is 1:2000. Antiserum is rarely used for immunoblotting unless it has been purified by peptide/column or nitrocellulose. It generally gives high background and should only be used if other options are not readily accessible. It is recommended that different dilutions should be tested in the beginning for all antibodies to find the exact dilution needed.

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Fig. 1. Immunoblot transfer apparatus. Diagram shows the set up of the transfer “sandwich”, 3D representation on the right and 2D detailed schematic of layering on the left.

Table 1

Suggested Protease Inhibitors for Use During Sample Preparation Procedure

Inhibitor	Working concentrations	Inhibition
Benzamidine	1 mM	Trypsin-like serine proteases (thrombin, plasmin) (9)
Leupeptin	1–10 μ M	Serine protease, plasmin and cystine proteases (10)
Aprotinin	10 mg/mL	Serine protease, and plasmin (11)
Phenylmethylsulfonyl flouride (PMSF)	1–2 mM	Serine protease with a trypsin-like specificity (12)

Table 2

Primary Antibodies Used for Expression Analysis of the Primary DNA Methyltransferases (DNMTs)

DNA methyltransferase	Target protein size	Specificity
DNMT1	170–195 kDa	Goat polyclonal IgG
DNMT 3a	101–120 kDa	Goat polyclonal IgG
DNMT 3b	98–110 kDa	Goat polyclonal IgG