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iSouthern Protocol

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We use this protocol and it's working

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Abstract

We present a protocol for detecting DNA modifications in genomic DNA by combining conventional Southern blotting with antibody-based immunological detection. In this immuno-Southern (iSouthern) approach, DNA is digested with methylation-sensitive and -insensitive restriction enzymes and subsequently probed with antibodies that specifically recognise modified bases such as 5-methylcytosine (5mC) and N6-methyladenine (6mA). By integrating enzymatic discrimination with direct antibody recognition, the method offers two complementary strategies in a single experiment, providing both sensitivity and confidence in the analysis of DNA modifications.

Guidelines

Tips on Restriction Digest:

1) Ensure your DNA sample is of good quality before starting the protocol. The DNA should be high-molecular weight with minimal degradation, at a concentration of 50–500 ng/μl, with an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio of 2.0–2.2.

2) Removal of RNA:

When analysing 6mA in DNA, it is essential to ensure that RNA is completely removed during sample preparation. Residual RNA fragments can migrate into the gel and become immobilized on the nylon membrane, leading to false-positive signals. Because 6mA is a common RNA modification, any antibody-based detection must be interpreted only from DNA, not contaminating RNA.

3) Incomplete restriction digest:

The protocol recommends setting up restriction digests in a small reaction volume to ensure that the entire sample can be loaded into a single small agarose gel well. However, very small volumes (e.g., 10 μL) can sometimes result in incomplete digestion. This is evident from the banding pattern on the gel when using a restriction enzyme that is insensitive to DNA modifications at its recognition site. For instance, digestion with *Sau3A* should completely eliminate high-molecular-weight genomic DNA. The presence of residual high-molecular-weight bands indicates incomplete digestion. To achieve complete digestion, it may be necessary to purify the DNA prior to digestion or to increase the reaction volume (e.g., up to 50 μL). If the volume is increased, not all of the reaction can typically be loaded onto a gel; therefore, adjustments to DNA concentration or sample loading may be required.

Rationale for using a master mix:

To assess the genome-wide level of a given DNA modification, equal amounts of DNA should be loaded into adjacent wells of the agarose gel. To ensure consistency, it is advisable to first prepare a single master mix of DNA and buffer, and then split this into multiple reaction tubes. Ideally, the master mix should be prepared in a buffer that supports complete digestion by all selected enzymes. We have found the **New England Biolabs (NEB) buffer system**, particularly **rCutSmart buffer**, to be a convenient option, as all enzymes we have tested so far retain 100% activity in this buffer.

Typically, four reactions are set up:

- **Tube 1:** DNA only (no restriction enzyme), equivalent volume of H₂O added instead of enzyme.
- **Tube 2:** DNA + a restriction enzyme that digests only if the target base is *modified* (e.g., *DpnI*, which cuts GATC sites when adenine is methylated).
- **Tube 3:** DNA + a restriction enzyme that is *blocked* by modification (e.g., *MboI*, which cuts unmethylated GATC sites but is inhibited if adenine is methylated).
- **Tube 4:** DNA + a restriction enzyme *insensitive* to modification (e.g., *Sau3A*, which cuts GATC sites regardless of methylation).

Including a modification-insensitive enzyme (Tube 4) is critical, as it confirms that the DNA is digestible and not protected by contaminants or impurities. This control ensures that any partial digestion observed with



methylation-sensitive enzymes can be attributed to DNA modification at the recognition sites, rather than incomplete digestion due to suboptimal enzyme conditions.

Tips for Southern Blot procedure:

1) In traditional Southern blot protocols, UV-nicking can be replaced by a depurination step facilitating transfer of large DNA molecules from the gel to the membrane. Depurination is suitable when base modifications occur on cytosines or thymines. This involves incubating the agarose gel for  00:15:00 in  500 mL

 0.25 Mass Percent HCl (1:50 dilution of concentrated HCl) on a slow shaker. However, if modifications are present on adenosines or guanines, partial base loss may occur, leading to apurinic sites, and potentially reducing antibody detection of, for example, 6mA, in subsequent steps.

This step is optional, and it may be worth testing whether it is required for a particular blot.

2) Transfer/Denaturing Solution: first add the required amount of NaCl to water and stir until completely dissolved. Then, slowly add NaOH pellets or concentrated NaOH solution while stirring continuously.

Safety Note:

- **Always wear gloves, a lab coat and safety goggles when handling NaOH**, as it is highly caustic and can cause chemical burns.
- Add NaOH slowly to prevent overheating and splashing.
- Prepare the solution in a well-ventilated area or fume hood.

3) Ensure that no air bubbles remain between the individual layers of the Southern blot stack.

4) Disassembling the blot:

The nylon membrane may be labelled with a pen or pencil, provided the writing does not cover regions where DNA is immobilized. For example, adding the date of preparation can help with cataloging. Properly dried membranes can be stored at room temperature for indefinite periods.

Tips on Immunodetection

This step can vary depending on the antibody used. If you already have an established Western blotting protocol, it is recommended to follow that method. Only if results are unsatisfactory should you consider adjusting conditions, for example by adopting steps from this immuno-Southern protocol.

1. The primary antibody is generally stable and can be reused multiple times. However, the signal may weaken after the first use. In that case, add ~1 μ L of fresh primary antibody to the reused solution to restore signal strength. Store the antibody solution at 4 °C between uses.



Materials

The following restriction endonucleases were used to assess 6mA modification levels in DNA (sourced from New England Biolabs/NEB):

Sau3AI (NEB #R0169S)

DpnI (NEB #R0176S)

MboI (NEB #R0147S)

RNase A used during restriction enzyme digests: (NEB #T3018L).

1× TAE buffer: (see *Preparation of 50× TAE Buffer*, protocols.io: ([dx.doi.org/10.17504/protocols.io.gtvbwn6](https://doi.org/10.17504/protocols.io.gtvbwn6)))

Loading dye used for loading and separating DNA on agarose gels: Blue (NEB #B7021S).

Transfer/Denaturing Solution: [M] 0.5 Mass Percent NaOH, [M] 1.5 Mass Percent NaCl

for example:  20 g NaOH and  87 g NaCl in 1L H₂O

Nylon Membrane (Positively charged): ROCHE NYLM-R, #11209299001.

Whatman Grade 3MM Chr Blotting Paper

40mM NaPi (sodium phosphate) buffer / pH 7.2 solution: Use 8 mL NaPi stock solution + 92 mL water to prepare 100 mL of 40 mM NaPi (pH 7.2).

To prepare a **0.5 M NaPi (pH 7.2) stock solution:** dissolve 89 g Na₂HPO₄·2H₂O (disodium hydrogen phosphate dihydrate) in distilled water ~950 mL; adjust the pH to 7.2 by slowly adding 3-4 mL concentrated H₃PO₄ and then add more distilled water to final volume of 1 L.

standard plastic cling film (i.e. Saran wrap), UV transparency required.

1× TBS-T (Tris-Buffered Saline and Tween 20): (see *Preparation of 10× Tris-Buffered Saline and Tween 20*), protocols.io: ([dx.doi.org/10.17504/protocols.io.cvjlw4m](https://doi.org/10.17504/protocols.io.cvjlw4m))

Transfer tray: Any plastic tray with a footprint larger than the agarose gel and side walls approximately 5 cm high can be used.

Immunodetection:

6mA detection (primary antibody) Rabbit anti-m6A (polyclonal) antibody from Synaptic Systems (Cat. No.: 202 003 / <https://sysy.com/product/202003>)

Secondary antibody: polyclonal HRP-conjugated secondary Goat anti-rabbit (ab6721 / <https://www.abcam.com/>)

Skim Milk Powder: Millipore (Cat. No.: 70166) for Biotechnology and Microbiology



ECL Western Blotting Substrate: Thermo Scientific™ Pierce™ ECL Western Blotting Substrate (Cat. No.: 32109)

Safety warnings

⚠ Exercise caution when heating agarose solutions, as they can easily boil over and cause burns.

Handle ethidium bromide with care when preparing and working with stained gels. Users must review and follow all relevant safety guidelines for ethidium bromide prior to use.

Before start

Ensure your DNA sample is of good quality before starting the protocol. The DNA should be high-molecular weight with minimal degradation.

Ensure you have a sufficient amount of paper towels for the blot. You need a sufficient quantity to draw transfer buffer through the gel and membrane overnight. If the stack becomes fully saturated then DNA transfer to the membrane could be affected.

Methylation-Sensitive Restriction Digest

1h

- 1 The DNA should be high-molecular weight with minimal degradation, at a concentration of 50–500 ng/μL, with an A260/A280 ratio of 1.8–2.0 and an A260/A230 ratio of 2.0–2.2.
- 2 Set up a 10-μL restriction digest as follows:
 1 μL - 8.5 μL DNA (0.5 μg - 2 μg), 1 μL 10× restriction buffer, 0.5 μL RNase A (NEB #T3018L), and nuclease-free water to a final volume of 9.5 μL, then add 0.5 μL of restriction enzyme.
Note: Since the same DNA should be tested with both methylation-sensitive and -insensitive restriction enzymes, it is recommended to prepare a **master mix** containing all reagents except the restriction enzyme. Dispense 9.5 μL of the master mix into each reaction tube, then add 0.5 μL of the appropriate restriction enzyme to each tube, or 0.5 μL H₂O for the control/undigested DNA). (see also the *Guidelines & Warnings* section).
- 3 Incubate the samples at 37 °C for 1 - 12 hours

1h



Gel Electrophoresis

30m

- 4 Prepare a 0.8–1% agarose gel in 1× TAE buffer, incorporating a non-toxic nucleic acid stain (e.g., SYBRTM Safe or GelRed[®]) according to the manufacturer's instructions.
Note: A small gel size (e.g., 6 cm × 7 cm) is sufficient for this assay.
Alternative: If a non-toxic dye is not available, 0.5 μg/mL ethidium bromide can be added to the gel instead.
- 5 For each well, mix 10 μL of digested or undigested DNA with 2 μL of 6× Gel Loading Dye (e.g., NEB #B7021S), and load the mixture onto the gel.
Alternative: Other standard loading dyes containing tracking dyes such as bromophenol blue and xylene cyanol may also be used.
- 6 Perform electrophoresis in 1× TAE buffer at 100 V for approximately 30 minutes, or until the DNA fragments are adequately separated by size.
Alternative: 1× TBE buffer may be used instead of TAE if sharper resolution of small DNA fragments is desired.

30m



- 7 Following electrophoresis, visualise and document DNA bands using UV transillumination at 254 nm, or a blue-light transilluminator (with compatible dyes such as SYBRTM Safe or GelRed[®]).

Southern Blot - Transfer of DNA to Nylon Membrane

51m

- 8 After gel electrophoresis, cover the gel with UV-transparent plastic wrap and expose to UV light for 120 seconds to induce nicks in high molecular weight DNA fragments, thereby reducing their size and facilitating transfer to the blotting nylon membrane. 2m
- 9 Place the gel in  50 mL Transfer/Denaturing solution on a slow shaking platform for 30 minutes. Repeat one more time (altogether 2 x 30 minutes) 30m
- 10 Prepare Whatman Grade 3MM blotting paper for the DNA transfer by cutting:
- Two pieces of 3MM paper, cut larger than the gel, to serve as wicks that draw Transfer/Denaturing solution from the tray (see setp 12.2)
 - Two pieces of 3MM paper, cut to the same dimensions as the agarose gel (e.g., 6 cm x 7 cm).
- 11 Cut 1 sheet of positively-charged nylon membrane (ROCHE Nylon Membrane, #11209299001) to the same dimensions of the agarose gel.
- 12 **Assembly of Southern blotting stack:**
- 12.1 Pour about  500 mL of Transfer/Denaturing solution into the transfer tray.
- 12.2 Place a glass plate over the transfer tray. Layer two large sheets of 3MM paper on top, ensuring their ends extend into the reservoir of transfer/denaturing solution. Thoroughly moisten the 3MM sheets with the solution. Use a 20 mL glass pipette to gently roll over the surface and remove any air bubbles.  Caution: trapped air bubbles will block DNA transfer.
- 12.3 Place the agarose gel face down, with the wells against the two large 3MM sheets soaked in transfer/denaturing solution. Use a 20 mL glass pipette to gently roll over the surface and remove any air bubbles between the gel and the 3MM paper.
- 12.4 Moisten the nylon membrane in transfer/denaturing solution, then place it on top of the agarose gel.



- 12.5 Moisten two pieces of pre-cut 3MM paper (see step 10) in transfer/denaturing solution, then place them on top of the nylon membrane. Remove any air bubbles between the nylon membrane and the 3MM paper.
- 12.6 Apply plastic wrap around the stack to prevent direct contact between the stack and the paper towels added in the next step (13).
Note: The transfer/denaturing solution must be absorbed by the paper towels only after passing through the stack.
- 12.7 Place a stack of absorbent paper towels (3–4 cm high) on top of the two pre-cut 3MM papers covering the stack. Cut the towels to the size of the agarose gel or slightly larger.
- 12.8 Add a flat plate (for example plexiglass, glass plate or a tip box) on top of the paper towels to apply gentle weight.
Note: The weight can be increased by placing a water-filled plastic bottle (e.g., an empty solution bottle from a DNA isolation kit) on top. Avoid excessive weight, as this may squash the agarose gel and impair DNA transfer to the nylon membrane.
- 13 Allow the DNA to transfer from the gel to the membrane overnight.
Note: The stack may slant if the weight is not centered; reposition the weight if needed. If the paper towels become saturated after 2–3 hours, replace them with a fresh set.
- 14 **Disassembly of Southern blotting stack**
Remove the weight, glass plate, and paper towels from the top of the stack. Then carefully lift the remaining stack (agarose gel/nylon membrane/3MM paper) and invert it so that the agarose gel faces upward again, in the same orientation as when the DNA samples were originally loaded in the gel tank.
- 15 Before removing the agarose gel, mark the orientation of the nylon membrane: Use a needle to prick one small hole at the position corresponding to the leftmost well of the gel, and two holes at the rightmost well. Alternatively, cut the bottom-right corner of the membrane for easy orientation.
After removing the gel, label the membrane with a pen or pencil, ensuring that writing does not cover areas where DNA is immobilized (e.g., add the date of preparation for cataloging).
- 16 Rinse the membrane in  50 mL 40mM NaPi buffer, pH 7 for 1 minute.
- 17 Cover the nylon membrane with UV-transparent plastic wrap and expose to UV light for 2 minutes, immobilising the DNA.
- 18 With the transparent wrap covering the blot, this membrane can now be stored at room temperature until you are ready to proceed with antibody staining.

1m

1m

2m

II



Note: Properly dried nylon membranes can be stored at room temperature for indefinite periods.

Immunodetection

1d 2h 22m

- 19 **Note:** The following steps describe a procedure to detect 6mA in DNA. Block the nylon membrane in 5% skim milk powder 1x TBST buffer at room temperature for 1 hour.
- 20 Wash the nylon membrane with 1x TBST buffer for 10 minutes. Repeat once (two washes total). 20m
- 21 Incubate the nylon membrane overnight at 4 °C in 10 mL primary antibody solution (6mA antibody, 1:5000 dilution), prepared by adding (2 µL 6mA antibody + 10 mL 5% skim milk powder 1x TBST buffer) at 4 °C . 1d
- 22 Wash the nylon membrane three times with 1x TBST buffer, 10 minutes each wash, on a rocking platform 30m
- 23 Incubate the nylon membrane for 1 hour at room temperature in 5 mL secondary antibody solution (goat anti-rabbit HRP-conjugated antibody, 1:2500 dilution) prepared by adding (2 µL antibody to 5 mL 5% skim milk powder 1x TBST buffer. 1h
- 24 Wash the nylon membrane with 1x TBST buffer for 10 minutes. Repeat twice (three washes total). 30m
- 25 Incubate the nylon membrane with enhanced chemiluminescence (ECL) solution (Pierce **™** ECL Western Blotting Substrate). Prepare the solution by mixing Substrate A and Substrate B in a 1:1 ratio immediately before use and spreading evenly over the membrane. Incubate for 2 minutes, then proceed to imaging. 2m



Protocol references

Adrian P. Bird, Edwin M. Southern,

Use of restriction enzymes to study eukaryotic DNA methylation: I. The methylation pattern in ribosomal DNA from *Xenopus laevis*,

Journal of Molecular Biology,

Volume 118, Issue 1,

1978,

Pages 27-47,

ISSN 0022-2836,

[https://doi.org/10.1016/0022-2836\(78\)90242-5](https://doi.org/10.1016/0022-2836(78)90242-5).

Ruth Sager, Anthony Anisowicz, Neil Howell,

Genomic rearrangements in a mouse cell line containing integrated SV40 DNA,
Cell,

Volume 23, Issue 1,

1981,

Pages 41-50,

ISSN 0092-8674,

[https://doi.org/10.1016/0092-8674\(81\)90268-3](https://doi.org/10.1016/0092-8674(81)90268-3).