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Title: Dot Blot Assay for Detecting Global N6-Methyladenosine RNA Modification Levels

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? Yes

All done

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes. All done**

NOTE: The SC files are of low-resolution

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials: **NO**

Current Protocol Length

Number of Steps: 33

Number of Shots: 58

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Xingyuan Sun** :Our protocol enables semi-quantitative assessment of global m⁶A levels using dot blot.
 - 1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Xingyuan Sun** : This protocol can be applied to routine screening and preliminary functional verification of m⁶A.
 - 1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Xingyuan Sun**: The most important challenges include membrane selection, precise RNA dilution, and uniform spot size.
 - 1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Xingyuan Sun**: Perform sequencing for precise site-specific identification and apply mass spectrometry to achieve comprehensive, global quantitative analysis.
 - 1.4.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Xingyuan Sun:** Future studies can investigate m⁶A mechanisms in disease development and progression.

1.5.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. RNA Extraction for Downstream Analysis

Demonstrator: Suxia Guo

- 2.1. To begin, remove cell culture dishes containing 143B cells with stable SNHG21 knockdown, negative control cells, and cells treated with 3-deazaadenosine and meclofenamic acid from the incubator set to 37 degrees Celsius [1].
 - 2.1.1. WIDE: Talent removing culture dishes containing the indicated 143B cell groups.
Videographer: Please pan to the labeled culture dishes to ensure a long enough capture
- 2.2. Verify that cell confluence is approximately 80 to 90 percent under a microscope [1-TXT].
 - 2.2.1. Talent placing the culture dish on the microscope stage.
AND
LAB MEDIA: [69860-screenshot-2.2.1.png](#).
Video Editor: Please play both shots side by side in a split screen
- 2.3. Rinse the cells twice with 1 milliliter of PBS after removing the culture medium [1]. Replace the PBS with 1 milliliter of trypsin per 6 centimeter dish [2].
 - 2.3.1. Talent adding 1 mL phosphate-buffered saline to rinse the cells.
 - 2.3.2. Talent adding 1 mL trypsin to the dish.
- 2.4. Gently shake the dish to distribute the trypsin evenly and incubate at room temperature for 1 minute [1]. Next, replace the trypsin solution with 1 milliliter of medium containing 10 percent FBS to terminate digestion [2]. Pipette the cell suspension several times to disperse the cells [3].
 - 2.4.1. Talent gently rocking the culture dish by hand.
 - 2.4.2. Talent adding serum-containing medium to the dish.
 - 2.4.3. Talent pipetting the cell suspension up and down in the dish.
- 2.5. Transfer the cell suspension to a 1.5-milliliter ribonuclease-free microcentrifuge tube [1]. Centrifuge the tube at 1000 g for 3 minutes [2-TXT].
 - 2.5.1. Talent transferring the cell suspension into a labeled microcentrifuge tube.
 - 2.5.2. Talent placing the tube into the centrifuge. **TEXT: Pipette out the supernatant after centrifugation**

- 2.6. For RNA extraction, add 600 microliters of lysis buffer from the RNA purification kit to the cell pellet [1]. Vortex thoroughly to lyse the cells [2-TXT].
 - 2.6.1. Talent adding lysis buffer to the pellet.
 - 2.6.2. Talent vortexing the tube . **TXT: Allow the solution to stand for 5 min**
- 2.7. After 5 minutes, add an equal volume of 100 percent ethanol to the lysate and vortex thoroughly [1].
 - 2.7.1. Talent adding ethanol to the tube and vortexing the solution.
- 2.8. Now pipette 600 microliters of the mixture onto a spin column assembled with a collection tube [1].
 - 2.8.1. Talent pipetting the mixture into the spin column placed in a labeled collection tube.
- 2.9. Centrifuge the column at 12,000 *g* for 1 minute [1]. Discard the liquid from the collection tube [2].
 - 2.9.1. Talent placing the column into the centrifuge.
 - 2.9.2. Talent discarding the flow-through from the collection tube.
- 2.10. After spinning the remaining mixture, add 500 microliters of wash buffer from the RNA purification kit to the spin column [1-TXT].
 - 2.10.1. Talent adding wash buffer to the column. **TXT: Incubate at RT for 1 min**
- 2.11. Then centrifuge the column at 12,000 *g* for 1 minute [1]. Discard the liquid from the collection tube before centrifuging the empty column again [2].
 - 2.11.1. Talent centrifuging the column.
 - 2.11.2. Talent discarding the wash solution.
- 2.12. Now transfer the spin column to a new 1.5-milliliter ribonuclease-free tube [1]. Open the lid to air dry for 2 minutes [2].
 - 2.12.1. Talent transferring the column to a new labeled tube.
 - 2.12.2. Talent opening the column lid.
- 2.13. Add 25 to 50 microliters of enzyme-free water to the center of the spin column [1]. After a 2-minute incubation at room temperature, centrifuge the column at 12,000 *g* for 1 minute to elute RNA [2].
 - 2.13.1. Talent pipetting elution buffer into the column.
 - 2.13.2. Talent placing the column in a centrifuge.
- 2.14. Discard the spin column [1] and place the eluted RNA on ice [2].
 - 2.14.1. Talent discarding the column.

2.14.2. Talent placing the RNA tube on ice.

~~2.15. Next, measure RNA concentration of control and knockdown samples using a spectrophotometer [1].~~

~~2.15.1. SCREEN: Shot of the spectrophotometer interface showing RNA concentration measurements.~~

2.16. After measuring the RNA concentration, prepare 1 microgram of total RNA from each cell line [1]. Then serially dilute RNA to 400 nanograms, 200 nanograms, and 100 nanograms using diethyl pyrocarbonate-treated water [2].

2.16.1. Shot of labeled tubes with 1 µg RNA.

2.16.2. Talent preparing serial dilutions in labeled tubes.

2.17. Heat the diluted RNA samples at 95 degrees Celsius for 3 minutes [1]. Immediately transfer the tubes to ice to cool [2].

2.17.1. Talent placing RNA tubes into a heating block.

2.17.2. Talent moving the tubes from the heating block onto ice.

3. Dot Blot Analysis for Detection and Quantification of N6-Methyladenosine RNA Modification

3.1. To perform dot blotting, first draw reference lines on the positively charged nylon membrane [1]. Label the membrane with sample names [2].

3.1.1. Talent marking reference lines on the membrane.

3.1.2. Talent writing sample labels on the membrane.

3.2. Now, mix each RNA sample thoroughly by gentle vortexing or pipetting [1]. Then spot 1 to 2 microliters of each RNA sample onto designated positions on the membrane [2].

3.2.1. Talent gently vortexing RNA tubes.

3.2.2. Talent spotting RNA droplets onto the membrane.

3.3. Irradiate the membrane in a ultraviolet crosslinker at 254 nanometers and 120 millijoules per square centimeter to perform RNA cross-linking [1]. Then transfer the membrane into a dish containing 10 milliliters Tris-buffered saline with Tween 20 [2] and incubate it on a rocking platform for 5 minutes [3].

3.3.1. Talent placing the membrane into the ultraviolet crosslinker.

3.3.2. Talent placing the membrane in 1x TBST.

3.3.3. Talent transferring the membrane in 1x TBST onto a rocking platform.

3.4. Next, prepare a 5 percent bovine serum albumin blocking solution [1]. Incubate the

- membrane in blocking solution at room temperature for 2 hours [2].
- 3.4.1. Talent preparing the blocking solution in a container.
 - 3.4.2. Talent placing the membrane into blocking solution on a rocker.
- 3.5. Then incubate the membrane with anti-m⁶A (*Anti-M-Six-A*) antibody at 4 degrees Celsius overnight on a rocking platform set to 50 revolutions per minute [1].
- 3.5.1. Talent placing the membrane into primary antibody solution in a cold environment.
- 3.6. The next day, wash the membrane three times with 10 milliliters of Tris-buffered saline with Tween 20 for 10 minutes each on a rocking platform [1]. Then incubate the membrane with horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature [2].
- 3.6.1. Talent transferring the membrane to a dish with 10 mL 1_x TBST.
 - 3.6.2. Talent placing the membrane in secondary antibody solution.
- 3.7. After washing the membrane three times again, evenly apply chemiluminescent horseradish peroxidase substrate dropwise onto the membrane [1-TXT].
- 3.7.1. Talent applying substrate evenly across the membrane. **TXT: Incubate for 1 min with gentle rocking**
- 3.8. Detect the signal using a chemiluminescence imaging system [1]. Perform an initial pre-scan using the auto-exposure function [2].
- 3.8.1. SCREEN: [69860-screenshot-3.8.1.png](#).
 - 3.8.2. SCREEN: [69860-screenshot-3.8.2-1.png](#).
- 3.9. For methylene blue staining, incubate the membrane in methylene blue staining solution for 1 minute [1].
- 3.9.1. Talent immersing the membrane in methylene blue solution.
- 3.10. Wash the membrane three times with 10 milliliters of Tris-buffered saline with Tween 20 for 5 minutes each [1]. Image as a loading control [2].
- 3.10.1. Talent washing the membrane on a rocking platform.
 - 3.10.2. SCREEN: 69860-screenshot-3.10.2-(1).png.
- 3.11. Destain the membrane using a solution containing 1 percent sodium dodecyl sulfate on a rocking platform for 15 minutes [1].
- 3.11.1. Talent placing the membrane in destaining solution on a rocker.
- 3.12. Next, import the acquired image into the ImageJ software [1], and adjust the format [2].

3.12.1. SCREEN: 69680-screenshot-3.12-3.16.mp4. 00:00-00:03

3.12.2. SCREEN: 69680-screenshot-3.12-3.16.mp4. 00:04-00:05

3.13. Subtract background by selecting **Process** and choosing **Subtract Background [1]**. Set the radius to 50.0 pixels and enable **light background [2]**.

3.13.1. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:06-00:08

3.13.2. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:09-00:12.

3.14. Use the **Rectangle** tool to select the analysis area **[1]**. Then define lanes sequentially by selecting **Analyze, Gels**, and choosing **Select First Lane [2]**.

3.14.1. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:13-00:22.

3.14.2. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:23-00:26.

3.15. Then sequentially click on **Analyze, Gels, Plot Lanes** and then measure peaks to generate intensity profiles **[1]**. Measure the grayscale values of N6-methyladenosine and methylene blue signals **[2]**.

3.15.1. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:27-00:52.

3.15.2. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:53-00:58.

3.16. Copy the quantified data into a table for analysis **[1]**. Compare signal intensities to assess global N6-methyladenosine modification levels **[2]**.

3.16.1. SCREEN: 69680-screenshot-3.12-3.16.mp4 00:59-01:08.

3.16.2. SCREEN: 69680-screenshot-3.12-3.16.mp4 01:09-01:16.

Results

4. Results

- 4.1. Treatment with the methyltransferase inhibitor 3-deazaadenosine markedly decreased the global N6-methyladenosine signal in 143B (*One-Forty-three-B*) cells [1].
 - 4.1.1. LAB MEDIA: Figure 2A and 2C. *Video Editor: Please highlight columns 1–2 in Figure 2A (shCtrl vs. shCtrl+3-DAA) and corresponding line chart in Figure 2C (shCtrl vs. shCtrl+3-DAA).*
- 4.2. Knockdown of SNHG21 (*S-N-H-G-Twenty-One*) produced a comparable reduction in baseline global N6-methyladenosine levels [1].
 - 4.2.1. LAB MEDIA: Figure 2A and 2C. *Video editor: Highlight 1st and 3rd columns in Figure 2A (shCtrl vs. shSNHG21) and corresponding line chart in Figure 2C (shCtrl vs. shSNHG21).*
- 4.3. Additional treatment with 3-deazaadenosine further decreased the global N6-methyladenosine signal in shSNHG21 cells [1].
 - 4.3.1. LAB MEDIA: Figure 2A. *Video editor: Highlight columns 3–4 in Figure 2A (shSNHG21 vs. shSNHG21+3-DAA) and Figure 2C line chart (shSNHG21 vs. shSNHG21+3-DAA).*
- 4.4. Treatment with the demethylase inhibitor meclofenamic acid significantly increased global N6-methyladenosine levels in 143B cells [1].
 - 4.4.1. LAB MEDIA: Figure 3A and 3C. *Video editor: Please highlighted columns 1–2 in Figure 3A (shCtrl vs. shCtrl+MA) and corresponding line chart in Figure 3C (shCtrl vs. shCtrl+MA).*
- 4.5. Despite lower basal N6-methyladenosine levels, shSNHG21 (*S-H-S-N-G-H-Twenty-One*) cells exhibited a robust increase in N6-methyladenosine following meclofenamic acid treatment [1].
 - 4.5.1. LAB MEDIA: Figure 3A and 3 C. *Video editor: Highlight columns 3–4 in Figure 3A (shSNHG21 vs. shSNHG21+MA) and Figure 3C line chart (shSNHG21 vs. shSNHG21+MA).*

Pronunciation Guide:

Assay

Pronunciation link: <https://www.merriam-webster.com/dictionary/assay>

IPA: /'æ,seɪ/

Phonetic Spelling: a·say

Confluence

Pronunciation link: <https://www.merriam-webster.com/dictionary/confluence>

IPA: /'kɒnfluəns/

Phonetic Spelling: kon·floo·uhns

Trypsin

Pronunciation link: <https://www.merriam-webster.com/dictionary/trypsin>

IPA: /'trɪpsɪn/

Phonetic Spelling: trip·sin

Ribonuclease

Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonuclease>

IPA: /,raɪboʊ'nu:kli:ɛs/

Phonetic Spelling: rye·boh·noo·klee·ayz

Microcentrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/microcentrifuge>

IPA: /,maɪkroʊ'sentrɪfju:dʒ/

Phonetic Spelling: my·kroh·sen·truh·fyooj

Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /'sentɹɪfjuːdʒ/

Phonetic Spelling: sen·truh·fyooj

Spectrophotometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>

IPA: /ˌspek.troʊ.fəˈtɑː.mə.tə/

Phonetic Spelling: spek·troh·fuh·taa·muh·ter

Ethanol

Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>

IPA: /'eθəˌnɔːl/

Phonetic Spelling: eth·uh·nawl

Lysate

Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>

IPA: /'laɪsɪt/

Phonetic Spelling: ly·sayt

Ultraviolet

Pronunciation link: <https://www.merriam-webster.com/dictionary/ultraviolet>

IPA: /ˌʌltrəˈvaɪələt/

Phonetic Spelling: ul·truh·vy·uh·luht

Antibody

Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>

IPA: /'æn.tɪ.baɪ.di/

Phonetic Spelling: an·tee·baa·dee

Bovine

Pronunciation link: <https://www.merriam-webster.com/dictionary/bovine>

IPA: /'boʊvaɪn/

Phonetic Spelling: boh·vyne

Albumin

Pronunciation link: <https://www.merriam-webster.com/dictionary/albumin>

IPA: /æl'bjʊːmɪn/

Phonetic Spelling: al·byoo·min

Chemiluminescent

Pronunciation link: <https://www.merriam-webster.com/dictionary/chemiluminescence>

IPA: /ˌkiːmi.luːmə'nesənt/

Phonetic Spelling: kee·mee·loo·muh·neh·suhnt

Chemiluminescence

Pronunciation link: <https://www.merriam-webster.com/dictionary/chemiluminescence>

IPA: /ˌkiːmi.luːmə'nesəns/

Phonetic Spelling: kee·mee·loo·muh·neh·suhns

Methylene

Pronunciation link: <https://www.merriam-webster.com/dictionary/methylene>

IPA: /'meθəˌliːn/

Phonetic Spelling: meth·uh·leen

Substrate

Pronunciation link: <https://www.merriam-webster.com/dictionary/substrate>

IPA: /'sʌbˌstreɪt/

Phonetic Spelling: sub·strayt

Sequencing

Pronunciation link: <https://www.merriam-webster.com/dictionary/sequencing>

IPA: /'siːkwənsɪŋ/

Phonetic Spelling: see·kwuhn·sing

Spectrometry

Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrometry>

IPA: /spek'trɑːmətri/

Phonetic Spelling: spek·trah·muh·tree

Sulfate

Pronunciation link: <https://www.merriam-webster.com/dictionary/sulfate>

IPA: /'sʌlfet/

Phonetic Spelling: sul·fayt

Methyltransferase

Pronunciation link: No confirmed link found

IPA: /,mɛθəl'trænsfə'reɪs/

Phonetic Spelling: meth·uhl·trans·fuh·rays

Demethylase

Pronunciation link: No confirmed link found

IPA: /di:'mɛθə'leɪs/

Phonetic Spelling: dee·meth·uh·lays

Methyladenosine

Pronunciation link: No confirmed link found

IPA: /,mɛθɪl'ædə'nouʒi:n/

Phonetic Spelling: meth·il·ad·uh·noh·seen

Meclofenamic

Pronunciation link: No confirmed link found

IPA: /,mekloʊfə'næmɪk/

Phonetic Spelling: mek·loh·fuh·nam·ik

Deazaadenosine

Pronunciation link: No confirmed link found

IPA: /di:ˌæzə'ædə'nouʒi:n/

Phonetic Spelling: dee·az·uh·ad·uh·noh·seen