Protocol

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Protocol to analyze and quantify protein-methylated RNA interactions in mammalian cells with a combination of RNA immunoprecipitation and nucleoside mass spectrometry

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SUMMARY
Cellular RNAs are modified by both physiological factors and exogenous agents, such as methyl methanesulfonate (MMS). However, techniques for analyzing how proteins may interact with these modified RNAs are limited. Here, we provide a protocol combining RNA immunoprecipitation (RIP) with mass spectrometry (MS) to analyze the methylation state of the RNAs bound by Flag-tagged proteins in mammalian cells. The approach is highly quantitative and can simultaneously detect several methylated nucleosides in a single experiment.
For complete details on the use and execution of this protocol, please refer to Tsao et al. (2021).

BEFORE YOU BEGIN
Cell culture

- Timing: few days

This protocol describes the specific steps for using suspension adapted HeLa (HeLa-S3) cells. However, we have also successfully used 293T cells.

1. Culturing HeLa-S3 cells to sufficient numbers.

  **Note:** The cell line used (HeLa-S3) was obtained from ATCC.

  **Note:** We used a retroviral infection system to express the desired Flag-tagged proteins in cells. Please refer to Brickner et al. (2017) for details. Otherwise, this protocol can be applied broadly to other Flag-tagged protein expression systems or cell lines.

  **Note:** Typically, one 15-cm dish with $2 \times 10^7$ cells expressing Flag-tagged protein of interest is sufficient for the detection of methylated nucleosides. Since some methylated nucleosides are rare, lowering the number of cells is not recommended. Maintain low passage ($< 15$) for optimal results. Do not grow the cells into a confluent state ($> 3 \times 10^7$) since it will affect protein expression and the efficiency of MMS treatment. Different promoters used will also result in different protein expression levels. Depending on protein expression levels, more cells may need to be used. We plate cells 1–2 days before starting the experiment.
Immunoprecipitation and RNA extraction
Because this protocol combines protein immunoprecipitation and RNA sample preparation, it is important that a specific area is prepared for RNA work. Furthermore, use of RNase free consumables is needed to guarantee RNA samples are not degraded during the procedure. All immunoprecipitation steps need to be performed at 4°C to avoid degradation of the protein samples.

Titration of methylating agent
If there is a desire to induce exogenous methylation, it may be necessary to titrate concentration of the alkylating agent (MMS), depending on the cell line and protein of interest. A dose that induces sufficient methylation adducts is needed, yet not so high that it causes cell death during the experiment. We typically use a dose of 5.0 mM MMS, and recommend using a dose of at least 1.0 mM MMS but not more than 10 mM. The dose used should induce sufficient methylation adducts to be detected and quantified in the linear range, both in the input RNA as well as the immunoprecipitated sample. This does not apply for studying proteins that bind to physiological methylation marks in RNA (e.g., m6A).

MS protocol set-up for analyzing nucleosides

시간: 2 h

This protocol is specifically set up for analyzing N1-methyladenosine (m1A), N6-methyladenosine (m6A), N3-methylcytosine (m3C), and N7-methylguanosine (m7G). The conditions of nucleoside analysis are optimized for the Agilent 1290 Infinity II UHPLC system with the Agilent 6470 Triple Quadrupole MS using a ZORBAX RRHD Eclipse Plus C18 2.1 × 50 mm (1.8 μm) column.

2. Set up sample run conditions in the Agilent MassHunter Workstation Data Acquisition software (Figure 1).
   a. Go to “Method Editor”. Click to create a “New Method” (Figure 1A).
   b. Go to “Multisampler”, set the injection volume to 10 μL (Figure 1B).
   c. Go to “Quat Pump”, and set up with following parameters (Figures 1C and 1D):
      i. “Flow Rate” to 0.5 mL/min.
      ii. “Solvents” with 98% Buffer A and 2% Buffer B.
      iii. “Stoptime” and “Posttime” with 7 min and 4 min, respectively.
      iv. “Timetable” of flow rates and buffers as below:

| Time (min) | A (%) | B (%) | Flow (mL/min) |
|-----------|-------|-------|--------------|
| 0         | 98    | 2     | 0.5          |
| 3         | 92    | 8     | 0.5          |
| 3.01      | 2     | 98    | 0.3          |
| 7         | 2     | 98    | 0.3          |

Buffer A: 0.1% formic acid in water. Buffer B: 0.1% formic acid in methanol.

d. Go to “Column Comp” and set “Temperature” as below (Figure 2A):
   i. Left: 25°C.
   ii. Right: Combined.

e. Go to “DAD” (Figure 2B),
   i. Only check “Signal A” with the following setting:
ii. Go to “Advanced”. Change the “Store” to “All” for all the range of detection.

f. Go to “QQQ” and set up acquisition parameters for nucleosides (Figure 2C):
   i. Ion source: AJS/ESI.
   ii. Delta EMV (+): 200.
   iii. The run settings for each methylated compound are as below:

| Compound name | Precursor ion | Product ion | Dwell | Fragmentor | Collision energy | Cell accelerator voltage | Polarity |
|---------------|---------------|-------------|-------|------------|-----------------|--------------------------|---------|
| m7G           | 298           | 166         | 50    | 75         | 10              | 5                        | Positive|
| m1A           | 282           | 150         | 50    | 75         | 16              | 5                        | Positive|
| m3C           | 258           | 126         | 50    | 75         | 8               | 5                        | Positive|
| m6A           | 282           | 150         | 200   | 107        | 20              | 4                        | Positive|

**Note:** ‘Precursor ion’ and ‘product ion’ refer to the m/z of each compound before and after fragmentation, respectively. ‘Dwell time’ refers to the amount of time (in ms) used to acquire the targeted ion transition. ‘Fragmentor’ refers to voltage used for fragmenting each compound. ‘Collision energy’ refers to the energy (in eV) used in the second quadrupole. ‘Cell accelerator voltage’ relates to the rate at which ions move out of the collision cell. ‘Polarity’ refers to the ion detection mode used in the method.

g. Keep the rest of the settings as default. Save method (Figure 2C).

3. Verify the method settings by running methylated nucleoside standards.
   a. Dilute m1A, m3C and m7G standards to 10 nM.
   b. Run the standards with the above parameters. Open the Agilent Qualitative Navigator analysis software (please see below nucleoside mass spectrometry section for how to run a worklist and analyze data) to analyze the ion intensity signals.

**Note:** At this concentration, an ion intensity of at least $5 \times 10^4$ to $1 \times 10^5$ is expected within a single peak. If peaks are not detected to this magnitude for a given nucleoside, run the MassHunter Optimizer program to determine optimal conditions. Re-run the standards at 10 nM and ensure an ion intensity of at least $5 \times 10^4$ for 10 nM of each standard. Please refer to the Agilent MassHunter Optimizer Quick Start Guide for the details of the settings.

**Note:** If a different MS system is utilized, we recommend running the standards before analyzing the RIP samples to make sure the settings are optimized.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Methyl methanesulfonate (MMS) | Sigma-Aldrich | 129925 |
| ANTI-FLAG® M2 Affinity Gel | Sigma-Aldrich | A2220 |
| Protease inhibitor cocktail | Thermo Fisher Scientific | A32963 |
| Murine RNase inhibitor | New England BioLabs | M0314 |
| β-mercaptoethanol | Sigma-Aldrich | M6250 |
| Dithiothreitol (DTT) | Sigma-Aldrich | D0632 |
| Flag peptide | Sigma-Aldrich | F3290 |
| 1-methyladenosine ribonucleoside | Cayman Chemical Company | 16937 |
| 7-methylguanosine ribonucleoside | Cayman Chemical Company | 15988 |
| 3-methylcytidine ribonucleoside | Cayman Chemical Company | 21064 |

(Continued on next page)
## MATERIALS AND EQUIPMENT

### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE             | IDENTIFIER |
|---------------------|-------------------|------------|
| Nuclease S1         | Sigma-Aldrich     | N5661      |
| FastAP™            | Thermo Fisher Scientific | EF0651    |
| LC-MS grade water  | Fisher scientific | W64        |
| Formic acid (LC-MS grade) | Fisher scientific | A117-50 |
| Methanol (LC-MS grade) | Fisher scientific | A456-500 |

### Critical commercial assays

- miRNAeasy Mini Kit: QIAGEN, 217004

### Experimental models: Cell lines

- HeLa-S3: ATCC, CCL-2.2

### Other (For LC-MS)

- 11 mm plastic crimp/snap top autosampler vials: Thermo Fisher Scientific, C4011-13
- Clear snap-it, 11 mm vial Caps: Thermo Fisher Scientific, C4011-50
- Millex syringe filter (0.22 µm pore size): MilliporeSigma, SLGVR04NK
- Agilent 1290 Infinity II UHPLC system: Agilent Technologies, SKU: 8012-30-0034
- Agilent 6470 Triple Quadrupole MS: Agilent Technologies, G6470BA
- ZORBAX RRHD Eclipse Plus C18 2.1 × 50 mm (1.8 um) column: Agilent Technologies, B21725-902
- Agilent MassHunter Optimizer software (Version B.08.02): Agilent Technologies, N/A
- Agilent MassHunter Workstation Data Acquisition software (Version B.08.02): Agilent Technologies, N/A
- Agilent Qualitative Navigator analysis software (Version B.08.00): Agilent Technologies, N/A

### Buffer A

| Reagents                        | Final concentration | Amount |
|---------------------------------|---------------------|--------|
| Formic acid (LC-MS grade)       | 0.1%                | 1 mL   |
| dH2O                             | n/a                 | 1 L    |
| **Total**                        | n/a                 | ~1 L   |

Store at 25°C for 3 months.

### Buffer B

| Reagents                        | Final concentration | Amount |
|---------------------------------|---------------------|--------|
| Formic acid (LC-MS grade)       | 0.1%                | 1 mL   |
| Methanol (LC-MS grade)          | n/a                 | 1 L    |
| **Total**                        | n/a                 | ~1 L   |

Store at 25°C for 3 months.

### Lysis Buffer

| Reagents                        | Final concentration | Amount |
|---------------------------------|---------------------|--------|
| 0.5 M HEPES-KOH pH7.5           | 10 mM               | 1 mL   |
| 2 M KCl                         | 150 mM              | 3.76 mL|
| 20% NP-40                       | 0.5%                | 1.25 mL|
| 0.5 M EDTA                      | 2 mM                | 0.2 mL |
| 100 mM Zn(Cl)₂ (optional, see Note below) | 0.5 mM | 0.25 mL|
| dH2O                             | n/a                 | 43.54 mL|
| **Total**                        | n/a                 | 50 mL  |

Store at 4°C for 1 month. Aliquot 1.0 mL Lysis Buffer for each sample with freshly added 10 mM β-mercaptoethanol, 1× protease inhibitor cocktail, and 400 U/mL RNase inhibitor, then place on ice.
**Note:** Zinc is specifically used for RING domain-containing proteins or other proteins that require this divalent metal; it is otherwise optional for other types of proteins.

**Alternatives:** 10 mM β-mercaptoethanol can be substituted with 1 mM Dithiothreitol (DTT).

### NT2 Buffer

| Reagents                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| 0.5 M HEPES-KOH pH 7.5                       | 50 mM               | 5 mL    |
| 2 M KCl                                       | 200 mM              | 5 mL    |
| 0.5% NP-40                                    | 20%                 | 0.125 mL|
| 0.5 M EDTA                                    | 2 mM                | 0.2 mL  |
| 100 mM Zn(Cl)\(_2\) (optional, see Note below)| 0.5 mM              | 0.25 mL |
| dH\(_2\)O                                     | n/a                 | 39.425 mL|
| **Total**                                    | n/a                 | 50 mL   |

Store at 4°C for 1 month. Just prior to use, aliquot 10 mL NT2 buffer for each sample with freshly added 10 mM β-mercaptoethanol, 1× protease inhibitor cocktail, and 200 U/mL RNase inhibitor, then place on ice.

**Note:** Prepare a media containing 5 mM MMS from a 100 mM stock described in above materials and equipment.

**Note:** We use this dosage of MMS to generate a sufficient amount of methylated RNAs for detection. This dosage is not recommended to use for more than 2 h since it will cause cell death. Lower concentrations may be used if longer incubations are necessary, although this may compromise methylated RNA signal as such modified RNA may be degraded in the cell.

### 100 mM MMS media

| Reagents                                      | Final concentration | Amount  |
|----------------------------------------------|---------------------|---------|
| MMS (stock: 11.8 M)                          | 100 mM              | 8.7 μL  |
| DMEM supplied with 10% FBS and 100 U/mL Penicillin/Streptomycin | n/a                 | 1 mL    |
| **Total**                                    | n/a                 | ~1 mL   |

Freshly prepare just before use. Make 1.0 mL 100 mM MMS media for each sample, then add into culture dish to make a final concentration of 5 mM.

**△ CRITICAL:** Use “LC-MS” grade reagents to make all the mobile phase solvents for the LC-MS/MS.

**Alternatives:** Other mass spectrometry systems may be used for setting up the protocol for nucleoside analysis.

### STEP-BY-STEP METHOD DETAILS

#### Step 1: MMS treatment, cell collection, and immunoprecipitation

**Timing:** 8 h

The procedure for RNA immunoprecipitation is as previously described (Wang et al., 2014) with minor modifications. Also, to avoid protein degradation, the immunoprecipitation steps (steps 2–14) should be all performed at 4°C or on ice.

1. Treat cells with 5 mM MMS at 37°C for 1 h.

**Note:** Prepare a media containing 5 mM MMS from a 100 mM stock described in above materials and equipment.

**Note:** We use this dosage of MMS to generate a sufficient amount of methylated RNAs for detection. This dosage is not recommended to use for more than 2 h since it will cause cell death. Lower concentrations may be used if longer incubations are necessary, although this may compromise methylated RNA signal as such modified RNA may be degraded in the cell.
Figure 1. Setting an MS protocol for analyzing nucleosides in Agilent MassHunter Workstation Data Acquisition software (steps 2a–c, continues with Figure 2)

(A–D) The sequence of MS protocol set-up for analyzing nucleosides: (A) 1) Go to “Method Editor”. 2) Click “New Method” as indicated. (B) 3) Go to “Multisampler”. 4) Set “injection volume” as indicated. (C) 5) Go to “Quat. Pump”. Set 6) Flow and 7) Solvents as indicated. (D) Stay in “Quat. Pump”. 8) Set “Stoptime” and “Posttime” as indicated. 9) Set “Timetable” as indicated.
Figure 2. Setting an MS protocol for analyzing nucleosides in Agilent MassHunter Workstation Data Acquisition software (steps 2d–g, continued from Figure 1)

(A–C) The sequence of MS protocol set-up for analyzing nucleosides continued from 9) of Figure 1: (A) 10) Go to "Column Pump." 11) Change "Temperature" as shown in the screenshot. (B) 12) Go to "DAD". Set 13) "Signals" and 14) "Store" as indicated. (C) 15) Go to "QQQ". Set 16) "Ion source", 17) "Delta EMV (+)", and 18) "Acquisition" for different methylated compounds as indicated. 19) Save method.
2. Harvest cells as follows:
   a. Remove media from the dish.
   b. Add 6 mL of cold 1× PBS, then use cell lifter to scrape cells and transfer them into a 15 mL tube.
   c. Centrifuge at 500 × g for 2 min to pellet cells.
   d. Wash pellets once with 1 mL of cold 1× PBS then transfer cells into 1.5 mL microfuge tubes.
   e. Centrifuge at 1,000 × g for 2 min to pellet cells again.

   "Pause point: Cell pellet can be store at –80°C for two months.

3. Cell lysis: Resuspend cell pellets with 2 volumes of Lysis Buffer, pipette up and down several times until the lysates become completely homogenous, and then incubate on ice for 5 min for cell lysis.

   Note: Usually, the pellet volume of 2 × 10⁷ HeLa-S3 cells is ~200 µL. So, the cell pellet is lysed with 400 µL Lysis Buffer.

4. Place tubes at −80°C to shock-freeze for 30 min.

   Note: We do not recommend long-term storage of cell lysates at this point since this will potentially dissociate protein-RNA complexes.

5. Thaw cell lysates on ice and centrifuge 15,000 × g for 15 min.

   Note: Cell lysates usually take ~20–30 min to thaw completely.

   Note: Repeat if the lysates are cloudy.

   Note: The user can go to step 8 while waiting.

6. Transfer supernatants to new 1.5 mL microfuge tubes.

7. Save 5% of each clarified lysate as Input samples.

   Note: If a cell pellet is lysed with 400 µL Lysis Buffer, then total supernatant after centrifuge will be ~420–450 µL. Then, use 400 µL of supernatant for immunoprecipitation and save 20 µL as Input.

8. While cells are being centrifuged, prepare ANTI-FLAG® M2 Affinity Gel as follows:
   a. Carefully mix the ANTI-FLAG® M2 Affinity Gel until completely suspended. Immediately aliquot 40 µL of the suspension (20 µL of packed gel volume) into a 1.5 mL microfuge tube for each sample using a wide orifice pipette tip (or cut 1 mm off the end of a regular pipette tip).

   Note: Please refer to the manufacturer’s manual for more details of ANTI-FLAG® M2 Affinity Gel.

   b. Wash the resin by adding 500 µL of NT2 Buffer, mix by vortexing for ~5–10 s or inverting tubes several times.

   Note: While the manufacturer suggests not using reducing agents in the buffer with the ANTI-FLAG® M2 Affinity Gel, we have found that β-mercaptoethanol is needed to prevent oxidation of our protein of interest. We have not observed a significant reduction in the recovery of IP material with up to 10 mM β-mercaptoethanol.
c. Spin down the resin at 700 × g for 1 min and discard the supernatant.
d. Repeat steps b and c for three total washes.
e. Resuspend beads with NT2 Buffer (equal volume of the supernatant, e.g., 400 µL of NT2 Buffer for 400 µL of cell lysate).

9. **Immunoprecipitation:** Add the supernatants from step 6 onto the M2 gel suspension and incubate at 4°C with rotation for 4 h.

10. Spin down the resin at 700 × g for 1 min and remove supernatant.

11. Wash beads eight times with 1 mL of NT2 buffer as follows:
   a. Add 1 mL NT2 Buffer to the resin. Vortex mix for ~5–10 s or invert tubes several times.
   b. Spin down the resin at 700 × g for 1 min and discard the supernatant.
   c. Repeat steps 11a and b for eight total washes.
   d. Remove the remaining liquid completely by using a P200 pipette tip.

12. **Elution:** Elute Flag-tagged proteins from the beads with 5 bead volumes of NT2 Buffer containing 400 µg/mL Flag peptide. Incubate at 4°C with rotation for 2 h.

   *Note:* For 20 µL of the packed gel, we use 100 µL NT2 Buffer containing 400 µg/mL Flag peptide for the elution.

13. Spin down the resin at 700 × g for 1 min. Collect and transfer supernatants into new 1.5 mL microfuge tubes.

14. Wash beads with another 100 µL of NT2 Buffer, pipette several times, spin down the beads as above, then pool the supernatant with the eluate from above.

   *Note:* For 20 µL of beads, a total of 200 µL eluate will be collected.

**Step 2: RNA extraction**

© **Timing:** 2 h

RNA extraction procedure is performed according to the manufacturer’s manual (Qiagen RNasey Mini Kit #217004). We recommend performing these steps with RNase free consumables and in an area prepared for RNA work, it would guarantee a RNA extraction with higher quality.

15. Add 800 µL TRIzol reagent to 200 µL of eluate from step 14. Invert tubes several times to mix completely. For input samples, add NT2 Buffer to 200 µL total volume then mix with 800 µL TRIzol reagent.

   *Pause point:* TRIzol mixtures can be stored at −80°C for future use.

16. Follow the manual for RNA extraction procedure.

   CRITICAL: We recommend using the protocol with DNase I treatment to remove any DNA contamination in the samples.

17. Elute RNAs with 30 µL of RNase-free water. For Input samples elute with 150 µL of water.

   *Pause point:* RNA samples can store at −80°C for future use.

   *Note:* For m1A detection, we recommend running the samples as soon as possible, as this modification may be converted to m6A through Dimroth rearrangement (Macon and Wolfenden 1968).
Step 3: RNA digestion and dephosphorylation

**Timing:** 3–25 h

RNA is broken down to individual nucleosides by treating with S1 nuclease and alkaline phosphatase.

18. Pipette 25 μL of purified RNA from step 17 and add 10 units of S1 nuclease.
   a. Dilute stock S1 nuclease 100 units/μL to 10 units/μL using RNase-free water.
   b. Pipette 1 μL of diluted S1 nuclease into 25 μL of purified RNA.
   c. Vortex and spin down using a microcentrifuge.
   d. Incubate at 37°C for at least 2 h.

**Note:** Step 18d may be left to incubate for 24 h.

**Note:** One unit of S1 nuclease is defined as the amount of need to digest 1 μg of single-stranded nucleic acid to become soluble in perchloric acid per minute at pH 4.6 at 37°C. The RNA concentration of the input samples can be measured by UV spectrophotometry. If desired, the RIP sample RNA concentrations can be measured, but typically requires a high sensitivity method (e.g., Qubit). The amount of S1 nuclease used here is a significantly greater amount than what is necessary to complete digestion. Input RNA concentrations are typically 100–200 ng/μL. By digesting 25 μL, we are digesting 2.5–5.0 μg in total; therefore using 10 units of S1 nuclease for two hours is sufficient. Under the conditions described here, the typical ion intensity of a fully digested input sample is typically $5 \times 10^4$ to $1 \times 10^5$ for m1A.

19. Treat with alkaline phosphatase as follows:
   a. Prepare alkaline phosphatase reagent (50 μL of total volume).

| S1 nuclease-treated RNA | 26 μL |
|-------------------------|-------|
| 10x FastAP buffer       | 5 μL  |
| FastAP alkaline phosphatase | 2 μL |
| RNase-free water        | 17 μL |

**Note:** One unit of phosphatase is the amount of the enzyme required to dephosphorylate 5’-termini of 1 μg of linearized pUC57 DNA in 10 min at 37°C in FastAP buffer. The amount of phosphatase can be adjusted based on the unit definition.

   b. Vortex and spin down briefly using a microcentrifuge to collect the sample at the bottom of the tube.
   c. Incubate at 37°C for 1 h.

20. Filter digested samples with 0.22 μm filter into 11 mm vials. Cap vials and shake tube to move all of sample to the bottom of the vial. Ensure no bubbles are in the sample. Filter at least 150 μL of water into a vial for use as a blank.

21. Prepare 10 nM of m1A, m3C and m7G standards, and filter 50 μL of each standard sample as above.

Step 4: Nucleoside mass spectrometry

**Timing:** Several hours (12 min/sample)

Create and run a worklist for the digested samples.
2) Create a new worklist

3) Edit list

1) Click

Plate 1 (P1)

Plate 2 (P2)

A

B

C

D

E

F

P1-A1 P1-A2 P1-A3 P1-A4 P1-A5

P1-B1 P1-B2 P1-B3

C

Multisampler

Quat. Pump

Column Comp.

DAD

QQQ

5) Run worklist

4) Click “On”, wait until all components become “Idle”.
22. Open the MassHunter acquisition software to create a worklist for the run (Figure 3A).
   a. Type sample names for all the samples including water, nucleoside standards, Input and RIP samples.
   b. Put water sample at the beginning of the list as “Sample” for the purpose of clearing the system (see Note). Then use the same water sample as “Blank” following the first step.
   c. Include script at the end of the program to put the machine on standby.

Note: Water samples should be run at the beginning of every worklist to ensure the system is clear of remnants of any previous samples. Additionally, when first optimizing a method, it can be helpful to run a water sample between every sample to ensure the column is completely clear and no run-off is present that would skew the determined concentration of subsequent samples. If excessive run-off is observed in these water samples, it may be helpful to extend the run time of the solvent for each sample or change the elution methodology.

23. Load vials into the sample rack with the corresponding positions (Figure 3B).
24. Turn on system and allow all the components to come to “Ready” or “Idle” status. Run the worklist (Figure 3C).
25. When the run has ended, open the Agilent Qualitative Navigator analysis software. Select the samples of interest to open the chromatograms (Figure 4A).
26. Under Method Editor, select “MRM” for chromatogram with “All” the transitions, and add it to the method. Then press “Extract Additional Chromatograms” (Figure 4B). This function will integrate the area under the curve of each peak identified by the program.
27. Compare the peaks on the chromatograms of the samples by retention time to the peaks on the standards’ chromatograms to determine which peak is representing the nucleoside of interest (see Note) (Figure 4C). The integrated area under the curve can be compared to determine the amount of methylated nucleoside (Figure 4C). The amount of the RIP sample is then normalized with the amount of its counterpart Input samples.

Note: Some mass transitions can be the same for different compounds, such as m6A and m1A, due to structural similarities. Therefore, it is important to compare the peak of the standard by retention time to the samples to determine which of multiple peaks represents the compound of interest (Figure 5).

EXPECTED OUTCOMES
For RIP, the volume of $2 \times 10^7$ pelleted HeLa-S cells is about 200 μL (step 3). The amount of total RNA extracted from 5% Input sample (~1 $\times$ 10⁶ cells per sample) is about 15–30 μg (step 7). The ion intensity of the methylated standards (10 nM) is about $5 \times 10^5$ with our MS system (step 27). The ion intensity of methylated nucleosides in a 1% Input sample (which is 5x fold diluted from 5% Input, see step 16) is usually at least $10^6$. The intensity of RIP samples is variable, depending on the target protein of interest.

LIMITATIONS
This protocol is set up to analyze three specific RNA modifications from protein-bound RNAs: m1A, m3C, and m7G. For other RNA modifications, new running parameters need to be determined and optimized. This protocol is unable to distinguish m3C and m5C nucleosides due to
Figure 4. Data analysis using the Agilent Qualitative Navigator analysis software (steps 25–27)

(A) Select sample of interest to open.

(B) Process the qualitative analysis as follows: 1) Go to “Method Editor”. Select 2) “Type” as “MRM” and 3) “Transition” as “All”. 4) “Add” this analysis method and select samples. 5) Click “Extract Additional Chromatograms” to obtain the results.

(C) The chromatography results of different samples. Compare the peaks on the chromatograms of the samples to the peaks on the chromatograms of the standards (indicated by red lines). The numbers indicate the total ion intensity (integrated area under the curve).
the overlapping of retention times; further optimization of the LC method is necessary for this purpose. The protocol of RIP is designed specifically for immunoprecipitating Flag-tagged proteins. For other affinity tag systems as well as endogenous proteins, the protocol would need to be further optimized.

**TROUBLESHOOTING**

**Problem 1**
Step 27, ion intensity exceeds $5 \times 10^6$ (most commonly seen with input samples).

**Potential solution**
A linear range of each methylated nucleoside is typically between 1 nM and 200 nM with the ion intensity ranging from approximately $1 \times 10^4$ to $5 \times 10^6$. An ion intensity exceeding $5 \times 10^6$ will likely be out of linear detection range. These samples need to be diluted accordingly and run again.

**Problem 2**
Step 27, ion intensity below $5 \times 10^3$ (most commonly seen with the RIP samples).

**Potential solution**
As mentioned above, sometimes the ion intensity of methylated nucleosides generated from RIP samples are a very small fraction of the input (i.e., 1/1000 to 1/10,000). In such cases it would be helpful to serially dilute the nucleoside standards from 0.05 nM to 10 nM and generate a standard curve in this range to see if the amounts are still within the linear range for detection and quantification. If the RIP sample intensity is still lower than the linear range of the standards, try to increase the amount of cells used for RIP. Varying the dosage and time of drug treatment may also help to generate increased RNA methylation events in cells.

Besides the treatment conditions, the tagged protein expression levels can also affect the amount of methylated nucleoside generated from protein-bound RNA. If needed, using different expression systems may help increase the bait quantity, which in turn would improve methylated nucleoside ion intensity.

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**Figure 5.** The different retention times between m1A and m6A in mass spectrometry analysis

Note that m1A elutes much earlier (< 1 min; middle row) relative to m6A (3.8 min; bottom row).
**Note:** Different MS systems, buffers as well as different types of nucleosides will have significantly different linear range characteristics. We recommend each user to carefully detect the linear range for each nucleoside using their systems.

**Alternatives:** For the purpose of quantitation, generate a standard curve for each methylated nucleoside, from 1 nM to 200 nM. Then, these serially diluted standards can be analyzed with the RIP samples in the same run.

**Problem 3**
Step 27, the input sample has high ion intensity, but the intensity of the RIP sample is near background levels (similar to a water sample).

**Potential solution**
Either the immunoprecipitation has failed, or this protein does not bind to RNA. Check the immunoprecipitation of the protein by Western blotting.

**Problem 4**
Step 14, the amount of immunoprecipitated protein is unequal between different samples.

**Potential solution**
Theoretically, the immunoprecipitation efficiency of a given protein should be equal when comparing between different samples within a given experiment. However, due to various technical reasons, the expression of tagged proteins may vary between samples, thus resulting in a different amount of immunoprecipitated protein, and hence RNA, in the same batch of samples. This makes quantitative comparisons of modified ribonucleosides difficult in a given RIP-MS experiment. In such cases, we suggest performing a quantitative Western blot with immunoprecipitated samples (obtained in step 14). This can then be quantified to determine the quantity of protein isolated within each sample. The ion intensity of a RIP sample can then be normalized with the quantified immunoprecipitated protein. Alternatively, the amount of immunoprecipitated Flag-tagged protein can be first determined by quantitative Western blot prior to MS analysis. Subsequently, a normalized amount of this material can be used for the MS analysis (from step 14 onwards to perform the downstream steps).

**Problem 5**
Step 5, the supernatant is cloudy after centrifugation.

**Potential solution**
Depending on the cell type, the supernatant contains a high concentration of lipids, causing the supernatant to be cloudy. We suggest repeating doing the centrifugation again to solve this problem. Alternatively, the supernatant can be further cleared by filtering through a 0.22 μm membrane syringe as described (Wang et al., 2014).

**RESOURCE AVAILABILITY**

**Lead contact**
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Nima Mosammaparast (nima@wustl.edu)

**Materials availability**
This study did not generate any unique materials.

**Data and code availability**
This study did not generate any unique data sets or codes. Please refer to Tsao et al. (2021) for the experimental data generated using this protocol.
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AUTHOR CONTRIBUTIONS
J.M.S. set up the detection protocols using LC-MS. N.T. optimized the experimental conditions, performed experiments, and organized data. N.T., J.M.S., and N.M. wrote the paper. N.M. supervised the study.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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