Published in final edited form as:

Curr Protoc Chem Biol. ; 8(4): 234–250. doi:10.1002/cpch.12.

Enriching s⁴U-RNA using methane thiosulfonate (MTS) chemistry

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Abstract

Metabolic labeling of cellular RNA is a useful approach to study RNA biology. 4-thiouridine (s⁴U) is a convenient nucleoside for metabolic labeling because it is cell-permeable, incorporated into newly transcribed RNA, and the sulfur moiety provides a handle for biochemical purification. However, a critical step in the purification of s⁴U-RNA is the efficiency of the chemistry used to enrich s⁴U-RNA. Here, we present a protocol for s⁴U-RNA enrichment that includes efficient and reversible covalent chemistry to biotinylate s⁴U-RNA using the activated disulfide methane thiosulfonate conjugated to biotin (MTS-biotin), followed by enrichment on streptavidin beads. The efficiency of this chemistry reduces enrichment bias and requires less starting material, thereby expanding the utility of s⁴U to study RNA biology.

Keywords

RNA metabolism; metabolic labeling; 4-thiouridine; methane thiosulfonate

INTRODUCTION

Metabolic labeling is a useful tool to understand RNA dynamics without perturbing RNA transcription or processing. This protocol describes how to enrich 4-thiouridine-labeled RNA from HEK293T cells following metabolic labeling. First, cells are cultured using standard techniques, followed by the addition of 4-thiouridine nucleoside to the cell culture media. Labeling is stopped by removing culture media and lysing the cells in TRIzol reagent, which also prevents cellular RNases from degrading cellular RNA. The RNA is then be purified away from genomic DNA and proteins. DNA contamination may interfere with downstream analyses, and proteins contain cysteine residues whose thiols can react with the activated disulfide reagent). We have included an optional RNA shearing step at the end of the RNA purification that decreases nonspecific background in the enrichment. Reducing agents are present throughout the RNA purification protocol in order to prevent s⁴U-RNA oxidation and potential disulfide formation.

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Conflicts of Interest

The authors declare no competing financial interests.

- The s^4U -RNA is then reacted with an activated disulfide conjugated to biotin, which specifically biotinylates s^4U . The biotinylated RNA is then enriched on streptavidin coated magnetic beads, nonspecific background is washed away, and s^4U -RNA can be specifically eluted from beads by reducing the disulfide bond between biotin and s^4U . In this way, biotin remains bound to the beads and the RNA eluent is collected. Finally, s^4U -RNA enrichment is quantified relative to any nonspecific background and the enriched samples are prepared for downstream analysis (e.g., RT-qPCR or RNASeq). In addition, protocols for positive control *in vitro* transcribed RNAs that contain s^4U are included to help researchers gauge the efficiency of s^4U -RNA enrichment steps independently from the success of a metabolic labeling experiment. In summary, here we present protocols for enrichment of metabolically labeled s^4U -RNA and *in vitro* transcribed (IVT) s^4U -RNA. This protocol has been successful at enriching s^4U -RNA from cells that have been treated with media containing s^4U at concentrations ranging from 100 μM to 1 mM and at times ranging from minutes to days.

Strategic planning

- Extent of s^4U incorporation: When enriching s^4U -RNAs from a population, there are two related considerations: what fraction of the RNAs has any s^4U , and of the RNA that contains s^4U , what fraction of the uridine nucleotides has been substituted with s^4U .
- Amount of starting RNA: This protocol is designed for quantities of total RNA ranging from 1-50 μg of metabolically labeled RNA.
- Length of input RNAs: This protocol is focused on the purification of long RNAs (>200 nt), but can be adapted for RNAs <200 nt (for example, miRNAs). For long RNAs, pre-shearing the RNA is not necessary but can improve enrichment levels.
- Handling of reagents and RNA: The s^4U and s^4U -RNA is light sensitive and can also oxidize upon handling. Without precautions, we have found that these properties can lead to low enrichment and poor reproducibility. This protocol provides handling suggestions to increase the integrity of the s^4U -RNA prior to enrichment. Similarly, some of the chemicals used in this protocol can hydrolyze or oxidize, and so care should be taken to use fresh reagents and to store the stocks under appropriate conditions as described.

BASIC PROTOCOL

ISOLATION OF METABOLICALLY LABELED 4-THIOURIDINE-CONTAINING RNA USING ACTIVATED DISULFIDE METHANE THIOSULFONATE BIOTIN (MTSBIOTIN) FOLLOWED BY ENRICHMENT ON STREPTAVIDIN BEADS

There are a variety of approaches to chemically enrich different sub-populations of RNA. These techniques are particularly powerful for examining RNA turnover (Tani and Akimitsu, 2012), acute changes in transcription (Fuchs *et al.* 2014, Schwalb *et al.* 2016), and tissue specific expression (TU-tagging) (Miller *et al.* 2009). Critical to the success of these approaches is the choice of chemical label and the chemistry used to enrich the labeled RNA. While there are multiple nucleosides that can be metabolically incorporated into cellular RNA, the non-canonical nucleoside 4-thiouridine (s^4U) has several advantages compared to alternative chemistries such as 5-bromouridine and 5-ethynyluridine. For example, s^4U is structurally very similar to uridine, displays low toxicity at concentrations necessary for s^4U -RNA enrichment (even after prolonged treatments), and can be chemically modified using sulfur chemistry that is orthogonal to the canonical RNA nucleotides. Chemical approaches that have been used to enrich s^4U RNA include mercury columns, chemical alkylation of the sulfur, and disulfide chemistry. Disulfide chemistry has the advantage that disulfide bonds formed with s^4U can be easily reversed through reduction with relatively gentle reducing reagents. While the majority of reports use a pyridylthio-activated disulfide to retrieve s^4U RNA, we have found these reagents to be inefficient (Duffy *et al.* 2015). As an alternative, methanethiosulfonate (MTS)-activated disulfides have also been found to modify s^4U (Qin *et al.* 2003, Wunnicke *et al.* 2011) and we have developed conditions in which these reagents are efficient at modifying s^4U -RNA for the purposes of enriching subpopulations of RNA (Duffy *et al.* 2015). In summary, MTS chemistry is efficient, reversible, and provides a facile means of enriching s^4U -RNA.

This protocol describes steps to isolate cellular RNAs that have been metabolically labeled with the 4-thiouridine (s^4U). First, cellular RNA is metabolically labeled by culturing cells in the presence of 4-thiouridine nucleoside for the desired amount of time. Total cellular RNA is isolated, and the 4-thiouridine-containing subpopulation of RNA is biotinylated using the activated disulfide MTS conjugated to biotin. The biotinylated RNAs are enriched on streptavidin resin and eluted from resin via reduction of the disulfide bond. This protocol yields enriched s^4U RNA samples that are suitable for various analyses including RT-qPCR and RNA-Seq. Appropriate controls for this experiment include RNA from cells that have not been exposed to s^4U , and input RNA prior to enrichment. The protocol also includes a description of how to make s^4U -RNAs using in vitro transcription (IVT), and the use of these IVT s^4U -RNAs as positive controls for enrichment. These protocols are similar to ones previously described (Duffy *et al.* 2015) with added tips and improvements for enriching long cellular RNAs from microgram amounts of starting total RNA. As many applications exist for this chemistry beyond the metabolic labeling described here, we conclude with a discussion of considerations and limitations when adapting this protocol for other uses.

Equipment and Materials

Cultured cells (e.g., HEK293T cells; at least 1×10^6)

DMEM high glucose (Invitrogen, cat. no. 11965-092)

Fetal bovine serum (Invitrogen, cat. no. 16000-044)

Penicillin/streptomycin (Millipore, cat. no. TMS-AB2-C)

4-thiouridine (MP Biomedicals, cat. no. 0215213405)

TRIzol reagent (Life Technologies, cat. no. 15596-026)

Chloroform (AmericanBio, cat. no. AB00350-00500)

Kimwipes (Kimberly Clark, cat. no. 730830)

RNeasy Mini Kit (Qiagen, cat. no. Q74106)

100% ethanol (AmericanBio, cat. no. AB04010-00500)

10x TURBO DNase reaction buffer

TURBO DNase (Life Technologies, cat. no. AM2238)

DTT (VWR International, cat. no. EM-3860)

DEPC-Treated Water (Life Technologies, cat. no. AM9906)

HEPES free acid (DOT Scientific, cat. no. DSH75030-250)

EDTA disodium salt (Sigma Aldrich, cat. no. E5134-500G)

Dimethyl formamide (J.T. Baker, cat. no. 9344-13)

MTSEA-biotin-XX (Biotium, cat. no. 89139-636)

Chloroform: Isoamyl alcohol (Fisher Scientific, cat. no. 3160-450ML)

Phase-lock gel tube, heavy 1.5 mL (5-Prime, cat. no. FP2302820)

Glycogen (AmericanBio, cat. no. AB00670-00020)

Dynabeads MyOne Streptavidin C1 magnetic beads (Life Technologies, cat. no. 65001)

Tris•HCl (Fisher Scientific, cat. no. T5941-500G)

Sodium chloride (Sigma Aldrich, cat. no. S9888-2.5KG)

Tween-20 (Mp Biomedicals Inc., cat. no. ICN19484180)

β-mercaptoethanol (Sigma Aldrich, cat. no. M3148-25ML)

Qubit RNA HS assay kit (Thermo Fisher Scientific, cat. no. Q32855)

Ambion century plus RNA marker (Thermo Fisher Scientific, cat. no. AM7145)

MAXIscript T7 transcription kit (Thermo Fisher Scientific, cat. no. AM1312)

Cy5-CTP (GE Healthcare, cat. no. 25-8010-87)

4-thiouridine-5'-triphosphate (s⁴UTP) (Trilink Bio, cat. no. N-1025)

SequaGel UreaGel 29:1 Denaturing Gel System (National Diagnostics, cat. no. EC-829)

Gel cassettes 1.0 mm (Life Sciences, cat. no. NC2010)

Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, cat. no. ND-2000c)

MagRack 6 magnetic stand (GE Healthcare, cat. no. 28948964)

DynaMag 96 side (Life Technologies, cat. no. 12331D)

Qubit 3.0 Fluorometer (Thermo Fisher Scientific, cat. no. Q33216)

Rotator (Glas-Col, cat. no. 099A MR1512)

Typhoon FLA 9500 (GE Healthcare)

Metabolic labeling of cells and isolation of total cellular RNA: In this section we describe the metabolic labeling of HEK293T RNA with s^4U . The protocol describes the labeling of cells in a 6-well plate, and includes two time points and one control, with each sample in duplicate (six total, Figure 1A). This protocol can be adapted to enrich s^4U -RNA from several different types of input RNAs from various sources. Labeling experiments with s^4U have been described in several organisms and cellular systems including bacterial (e.g., Favre et al. 1986), yeast (e.g., Miller et al. 2011, Sun et al. 2012), insect (e.g., Burow et al. 2015), and mammalian (e.g., Dolken et al. 2008, Rabani, et al. 2011) cells. The experimental design can also be adapted to different time points or treatment conditions. The details of the metabolic labeling described here offer a guide to one successful labeling regime that provides strong enrichment.

1. Plate HEK293T cells in a 6-well plate at 3×10^5 cells/well and allow the cells to recover in RPE1 media (without s^4U) overnight.

We generally plate cells at a low enough density so that they will not reach confluence during the experiment. In this protocol, we expect the cells to reach ~80% confluence by the end of the experiment. While we generally do not split cells during treatment, s^4U treatments have been successful through splitting and re-plating cells (including trypsin treatment).

2. Freshly dissolve solid s^4U in water to 1 M (260 mg/mL). Dilute 14 μ L of this 1 M s^4U stock into 20 mL of RPE1 media (to 700 μ M s^4U final) and mix by vortexing for 10 seconds. Aspirate media from cells in all six wells. For wells 1-2, add 3 mL/well of s^4U containing media. For the samples in wells 3-6, add 3 mL/well of media without s^4U .

While we recommend freshly dissolving s^4U solid prior to the experiment, we have observed enrichment when using stocks that were stored at -20°C for more than a week.

Successful experiments have been performed using a range of concentration of s^4U in the media. Because of the efficiency of MTS chemistry, we have found lower concentrations (as low as 100 μ M) of s^4U provide sufficient enrichment. In other

cases, s^4U can be used up to 1 mM---these higher concentrations may be advantageous for especially short treatments (Fuchs et al. 2014, Schwalb et al. 2016). One concern that has been raised about s^4U is its potential for toxicity (Burger et al. 2013). We have found that at low concentrations of s^4U (100 μM), the total RNA levels in cells are very similar between treated and untreated cells, even after 22 days of treatment (Duffy et al. 2015), consistent with others' findings (Gregersen et al. 2014, Hafner et al. 2010), suggesting that any perturbations to the steady state RNA levels caused by these s^4U treatment conditions are minimal.

3. After 1.5h of s^4U labeling, remove media from cells in wells 3-4 and add 3 mL of s^4U -containing media (described in step 2).
4. After the samples in wells 1-2 have been exposed to s^4U for 2h total (and the samples in wells 3-4 have been exposed for 30 min), remove the media from all wells and immediately add 1 mL TRIzol. Pipet up and down five times and transfer the TRIzol samples to labeled 1.7 mL microfuge tubes.

Ideally the RNA purification will be performed immediately, but we have observed successful enrichment for RNA samples that have been frozen at $-80^{\circ}C$ for up to three months.

RNA isolation

5. Add 200 μL chloroform to each of the 1 mL TRIzol samples from step 4. Shake the tubes vigorously for 15 s and let sit for 2 min.
6. Centrifuge the tubes for 5 min at $12,000 \times g$, $4^{\circ}C$. Transfer aqueous phase ($\sim 500 \mu L$) to new labeled tubes.
7. To each aqueous phase from step 6, add 0.5 mL of 100% isopropanol, 1 μL 100 mM DTT (final concentration: 0.1 mM DTT) and 1 μL RNase-free glycogen (5-10 μg). Incubate samples at room temperature for 10 min.

The s^4U -RNA is light sensitive and prone to oxidation. While these steps can be performed under standard laboratory lighting, try to minimize the time of light exposure. The DTT is included to help minimize oxidation of the s^4U .

8. Centrifuge samples at $12,000 \times g$ for 20 min at $4^{\circ}C$. Carefully remove the isopropanol from the RNA/glycogen pellet.
9. Add 1mL of 75% ethanol to the pellet, shake for 10 s and centrifuge at $12,000 \times g$ for 10 min at $4^{\circ}C$.
10. Remove the ethanol completely from the RNA/glycogen pellet. To do so, first remove the majority of the ethanol with a P1000, then spin the tubes again on a counter top centrifuge. Use a gel-loading tip to remove the remaining ethanol. Let the pellet air-dry for 2 min by leaving the tube open under a Kimwipe cover.
11. Resuspend each pellet in 20 μL of RNase-free water. Measure the RNA concentrations using a Nanodrop spectrophotometer. Adjust the volume to bring RNA concentration to 200 ng/ μl with water.

Generally we retrieve 10-20 μg of material from 10^6 HEK293T cells after this step, which includes RNA as well as any contamination from DNA and free nucleotides that were carried through the TRIzol extraction and precipitation.

12. Add 10x Turbo DNase buffer to a final 1x concentration. Add 1 μL Turbo DNase (2U) per 10 μg of RNA. Invert the tube several times and concentrate the liquid in the bottom by centrifugation for ~ 5 s. Incubate the reaction at 37°C for 30 min.
13. Prepare and label six (one per sample) phase lock gel tubes by centrifugation at $10,000 \times g$ for 30 sec according to the manufacturer's directions.
14. Adjust the volume of the RNA samples from step 12 to 315 μL with water. Add 35 μL of sodium acetate (3M stock, pH 5.5) and add samples to phase lock tubes. Then add 350 μL phenol:chloroform:isoamyl alcohol (ratio 25:24:1 and pH 7.7-8.3) to each phase lock tube. Shake the tubes vigorously for 15 s and let incubate at room temperature for 2 min. Centrifuge at $12,000 \times g$ for 10 min at 4°C .
15. Transfer the aqueous phase into in a new tube. Repeat steps 7-10 to precipitate the RNA with isopropanol.
16. Resuspend the RNA pellet in 30 μL of RNase-free water.
17. Assay RNA quality by Nanodrop to calculate RNA concentration and also perform a UV-Vis scan for $s^4\text{U}$ concentration.

Generally this protocol yields 10-12 μg of RNA from 10^6 HEK293T cells. $s^4\text{U}$ absorbs light at 334 nm. In principle this absorbance can be used to calculate the amount of $s^4\text{U}$ in the sample, but we have found that conventional Nanodrop is not sufficiently sensitive to allow accurate quantitation. However, this scan does provide a qualitative check to see that there is a local maximum at 334 nm.

RNA shearing (optional)

While this protocol successfully enriches $s^4\text{U}$ -RNA with unsheared RNA samples, we have found that RNA shearing can increase yields and decrease background. As most short-read RNA-seq protocols require shearing of the RNA samples as part of a library preparation, it is convenient to perform the shearing prior to enrichment if it does not conflict with experimental design.

18. Adjust the RNA samples from step 16 to 40 μL with water. Add 40 μL 2x fragmentation buffer and place sample at 94°C for exactly 4 min.
19. Quickly spin the RNA sample on a countertop centrifuge and immediately place on ice.
20. Add 20 μL of 250 mM EDTA (final concentration: 50 mM EDTA) to each sample, mix by vortexing and incubate on ice for 2 min.

Modified RNeasy MinElute Cleanup

21. Add 350 μ L buffer RLT and 250 μ L 100% EtOH to each RNA sample from step 16 or 20, mix well by pipetting. Apply these samples to RNeasy columns.
22. Centrifuge the columns 15 s at $12,000 \times g$, 4°C. Discard the flow through.
23. To each column, add 500 μ L RPE buffer supplemented with 35 μ L of 1% β -me (final concentration: 10 mM β -me).

Note that the addition of a reducing agent in this step is important to reduce any disulfides that have formed with the s^4U . [See figure 2]

24. Centrifuge the samples 15 s at $12,000 \times g$, 4°C. Discard the flow through.
25. Add 500 μ L freshly prepared 80% EtOH and centrifuge the samples for 2 min at $12,000 \times g$, 4°C. Discard the flow through.
26. Switch the columns to new 2 mL collection tubes. Centrifuge the samples 5 min at maximum speed, 4°C.
27. Transfer column to a 1.7 mL microfuge tube, and add 14 μ L RNase-free water. Centrifuge the samples 1 min at $12,000 \times g$, 4°C.

Biotinylation of s^4U -RNA with the activated disulfide methane thiosulfonate (MTS) bitoin

28. Dilute solid MTSEA-biotin-XX (MW 607.7 g/mol) in dry DMF to 1 mg/mL (1.64 mM) to make a concentrated stock.

The MTSEA-biotin-XX stocks are stable at -20°C for at least 3 months.

29. In order to biotinylate the RNA samples, mix the following reagents in a 1.7 mL microfuge tube:
 - 2 to 5 μ g of RNA from step 27
 - 1 μ L 1 M HEPES, pH 7.4 (final concentration: 20 mM HEPES, pH 7.4)
 - 1 μ L 0.5 M EDTA (final concentration: 1 mM EDTA)
 - Nuclease-free water to 40 μ L total volume
30. Dilute 4 μ L of the 1 mg/mL MTSEA-biotin-XX stock into 76 μ L DMF (50 μ g/mL, 82 μ M final concentration) and mix by vortexing.
31. Add 10 μ L of the MTSEA-biotin-XX solution from step 30 into each reaction from step 29 (results in the addition of 500 ng MTSEA-biotin-XX to each reaction, final concentrations 16.4 μ M MTSEA-biotin-XX, and 20% DMF).

These conditions include excess MTSEA-biotin-XX and should be sufficient for up to 5 μ g of RNA. For larger scale reactions with more RNA, we recommend increasing the volume of the reaction but retaining the concentrations of each component.

32. Cover the reactions with foil and incubate these reactions at room temperature in the dark for 30 min with rotation.

Remove unreacted MTS-biotin from RNA samples

33. Adjust the volume of each sample to 100 μL by adding 50 μL of water. Add the solutions to labeled phase lock tubes and add 100 μL chloroform:isoamyl alcohol to each tube.
34. Shake the samples vigorously for 15 s and let sit 2 min.
35. Centrifuge the samples for 5 min at $12,000 \times g$, 4°C . For each sample, transfer the aqueous phase (100 μL) to a new labeled 1.7 mL microfuge tube.
36. To each aqueous phase from step 35, add 350 μL buffer RLT and 250 μL 100% EtOH, mix well by pipetting, and apply to an RNeasy column.
37. Centrifuge the columns for 15 s at $12,000 \times g$, 4°C . Discard the flow through.
38. Add 500 μL buffer RPE to each column and centrifuge the columns for 15 s at $12,000 \times g$, 4°C . Discard the flow through.
39. Add 500 μL freshly prepared 80% EtOH to each column and centrifuge the columns for 2 min at $12,000 \times g$, 4°C . Discard the flow through.
40. Switch the columns to new 2 mL collection tubes. Centrifuge samples 1 min at maximum speed, 4°C .
41. Transfer the columns to new microfuge tubes. Add 50 μL RNase-free water to the center of each column. Elute the RNA by centrifuging these samples for 1 min at $12,000 \times g$, 4°C . Proceed immediately to isolation of the biotinylated $s^4\text{U}$ -RNA.

Block streptavidin beads

This portion of the protocol can be carried out in parallel with the RNA biotinylation step, as steps 28-41 require ~1h to complete.

42. For six samples, aliquot 66 μL Dynabeads MyOne Streptavidin C1 beads into one 1.7 mL microfuge tube. Place the tube in a magnetic rack for 2 min and remove supernatant with a pipet.

Prepare 10 μL of beads per sample and include 10% extra volume.

43. Wash the beads twice by resuspending them in 500 μL nuclease-free water and mixing by pipetting up and down five times. Place the tubes in a magnetic rack for 2 min and remove supernatant with a pipet after each rinse.
44. Wash beads twice with 500 μL high salt wash buffer, mixing and capturing the beads as described in step 43.
45. Add 330 μL freshly made bead blocking buffer, resuspend the bead mixture completely by pipet, and incubate for 1h at room temperature. Place the tubes in a magnetic rack for 2 min and remove the supernatant with a pipet.

46. Wash the beads twice with 500 μ L high salt wash buffer. Place the tubes in a magnetic rack for 2 min and remove the supernatant with a pipet.
47. Resuspend the beads in 660 μ L of high salt wash buffer. Pipet up and down and immediately aliquot 100 μ L of bead suspension into each of six PCR tubes in a PCR-strip.
48. Immediately before applying the RNA samples (step 49), capture the beads in a 96-well magnetic rack and remove the buffer by pipet.

Isolate s⁴U-containing transcripts with streptavidin beads

49. Add 5 μ L high salt wash buffer to the 50 μ L RNA solutions from step 34 and mix well. Add each sample to beads from step 48. Cover with foil and incubate the samples at room temperature for 15 min on a rotator at 30 rpm (or similar) to ensure the beads are mixing during the incubation.
50. Place the tubes in a 96-well magnetic rack for 2 min and remove supernatant with a pipet. Save the supernatant on ice as “Flow through”.
51. Wash the beads three times by resuspending them in 100 μ L high salt wash buffer and mix by inverting the tubes five times rapidly. Quickly centrifuge the tubes. Place the tubes in a magnetic rack for 2 min and remove the supernatant with a pipet after each rinse.
52. Add 25 μ L freshly made elution buffer to the beads, mix the beads by inversion as in step 51, wrap the tubes in foil, and incubate them at room temperature in the dark for 15 min with rotation as in step 49.

This elution buffer will reduce the disulfide bond that formed between biotin and 4-thiouridine, thereby eluting the s⁴U-RNA and leaving biotin bound to the streptavidin beads.

53. Quickly centrifuge the tubes and capture the beads in a 96-well magnetic rack for 2 min. Carefully retrieve the supernatant with a pipet and save this sample as “elution”.
54. Add another 25 μ L elution buffer and immediately place tubes in a 96-well magnetic rack for 2 min. Remove the supernatant with a pipet and combine this elution for each sample with the corresponding elution from step 53. Place samples on ice.

Quantify s⁴U-RNA enrichment

Successful enrichment can be tested immediately after step 54 by examining the total amount of s⁴U-RNA retrieved in s⁴U treated samples in comparison to the untreated controls. We have found this enrichment is easily observed for long s⁴U treatments using a Qubit HS assay kit according to the manufactures directions. This analysis is presented to provide assay conditions and expected yields from the protocol above.

55. Prepare a 10% Input sample by diluting 200-500 ng RNA from step 29 (10% of RNA used in biotinylation) into 50 μ L of elution buffer. Vortex and place on ice with samples from steps 50 (Flow through) and 54 (Elution).
56. Make a master mix of 3582 μ L of Qubit buffer and 18 μ L dye (199 μ L buffer and 1 μ L dye per sample, 18 samples total) and vortex for 10 s to mix.
57. Aliquot 190 μ L of master mix into two Qubit tubes. Vortex both Qubit RNA HS standards for 10 s, then add 10 μ L of standard 1 and standard 2 to each tube, respectively.
58. Aliquot 194 μ L of master mix into another Qubit tube. Add 5 μ L of elution buffer to the tube.
59. Aliquot 194 μ L of master mix into the remaining 15 tubes. Add 5 μ L of the appropriate sample (Input, Flow through, or Elution) to each tube.
60. Vortex all Qubit tubes for 3 sec, incubate at room temperature for 2 min, and measure the fluorescence in each tube by Qubit, starting with standards 1 and 2.
61. To estimate the yield, first subtract RFU of elution buffer sample from all sample RFU values. Then calculate percent input using the equation $(\text{RFU}_{\text{Sample}}/\text{RFU}_{\text{Input}})*10$.

Calculate the yield of the experiment by percent input rather than Qubit concentration because the elution buffer gives slightly different readings than RNA in water. Expected yields from this experiment are 3-5% Input.

Prepare RNA samples for RNA-Seq or RT-qPCR

62. Adjust the RNA samples from steps 50 (Flow through), 51 (Elution), and 55 (10% Input) to 100 μ L with water.
63. Purify and concentrate the RNA samples using the RNeasy Minelute protocol described in steps 21-27.

The RNeasy Minelute cleanup kit removes excess salts and DTT that could complicate downstream analysis. Following cleanup, samples can be assayed via qPCR or RNASeq, using standard procedures.

Enrich an in vitro-transcribed RNA ladder

This in vitro transcribed ladder is a useful positive and negative control for MTS-biotin enrichment independent of metabolic labeling in cells. We suggest including this control in any MTS-biotin enrichment experiment to monitor the efficiency of enrichment. Under these conditions we expect nearly quantitative retrieval of the positive control and retrieval of undetectable amounts of the negative control.

RNA ladder synthesis

This protocol follows the MAXIscript T7 transcription kit instructions, with the addition of modified nucleoside triphosphates to incorporate a fluorescent label (Cy5) and 4-thiouridine.

64. Label two 0.2 mL PCR tubes “+s⁴U” and “-s⁴U”. To the “+s⁴U” tube, add the following:
 - 1 µL Ambion century plus RNA marker template (1 µg/µL)
 - 2 µL 10x transcription buffer
 - 1 µL 10 mM GTP, ATP, and Cy5-CTP
 - 0.5 µL 10 mM UTP
 - 0.5 µL 10 mM s⁴UTP
 - 9 µL nuclease-free water
 - 2 µL T7 enzyme mix
65. To the “-s⁴U” tube, add the following:
 - 1 µL Ambion century plus RNA marker template (1 µg/µL)
 - 2 µL 10x transcription buffer
 - 1 µL 10 mM GTP, ATP, UTP, and Cy5-CTP
 - 9 µL nuclease-free water
 - 2 µL T7 enzyme mix
66. Invert both tubes three times vigorously and quickly centrifuge. Repeat once more.
67. Cover the samples in foil and incubate at 37°C for 1h.
68. Add 1 µL of TURBO DNase to samples and mix as in step 66.
69. Cover the samples in foil and incubate at 37°C for 15 min.
70. Prepare and label two phase lock gel tubes by centrifugation at 10,000 × g for 30 s according to the manufactures directions.
71. Using material from step 69, adjust the volume of the RNA samples to 150 µL with water and add samples to phase lock tubes. Then add 200 µL phenol:chloroform:isoamyl alcohol (ratio 25:24:1 and pH 7.7-8.3) to each phase lock tube. Shake the tubes vigorously for 15 s and let incubate at room temperature for 2 min. Centrifuge at 12,000 × g for 10 min at 4°C.
72. Transfer the aqueous phase into in a new tube. Adjust the volume to 200 µL with nuclease-free water.

73. Add 10 μ L of sodium acetate (3M stock, pH 5.5; final concentration: 150 mM sodium acetate), 1 μ L 100 mM DTT (final concentration: 0.1 mM DTT) and 1 μ L RNase-free glycogen (5-10 μ g). Mix well by vortexing.
74. Add 600 μ L of ice cold 100% ethanol, mix well by vortexing, and incubate at -80°C for 20 min.
75. Centrifuge samples at $12,000 \times g$ for 30 min at 4°C . Carefully remove the ethanol from the RNA/glycogen pellet.
76. Add 1 mL of 75% ethanol to the pellet, shake for 10 s and centrifuge at $12,000 \times g$ for 10 min at 4°C .
77. Remove the ethanol completely from the RNA/glycogen pellet. To do so, first remove the majority of the ethanol with a P1000, then spin the tubes again on a counter top centrifuge. Use a gel loading tip to remove the remaining ethanol. Let the pellet air-dry for 2 min by leaving the tube open under a Kimwipe cover.
78. Resuspend each pellet in 40 μ L of RNase-free water.
79. Assay RNA quality by Nanodrop to calculate RNA concentration and also perform a UV-Vis scan to qualitatively analyze the $s^4\text{U}$ concentration using the absorbance at 334 nm.

RNA ladder enrichment

80. Biotinylate and enrich 500 ng RNA ladder using steps 28-54 of the main text protocol.
81. Adjust the RNA ladder from steps 50 (Flow through), 51 (Elution), and 55 (10% Input) to 100 μ L with water.
82. Purify and concentrate RNA ladder samples using the RNeasy Minelute protocol in steps 21-27.

Visualize RNA products by urea-PAGE

83. Using the SequaGel UreaGel 29:1 Denaturing Gel System, make a 5% urea gel by combining the following in a 15 mL falcon tube:
- 14 mL system diluent
 - 4 mL system concentrate
 - 2 mL system buffer
 - 80 μ L 10% (w/v) ammonium persulfate
 - 4 μ L TEMED
84. Mix well by inverting the tube and quickly pour into a 1.0 mM gel cassette and insert a 10-well comb. Let the gel polymerize for 30-60 min.

85. Load the gel into a gel electrophoresis apparatus containing 1x TBE buffer and pre-run the gel for at least 15 min at 200V.
86. While pre-running the gel, add 7 μ L of each RNA ladder sample to 7 μ L of urea gel loading buffer and mix well by pipetting.
87. Heat samples to 80°C for 5 min, then load 14 μ L sample into each well using a gel loading tip. Wash the wells with a syringe prior to loading.
88. Run the gel for 45 min at 200V. Visualize RNA ladder on a Typhoon FLA 9500 instrument using Cy5 fluorescence filters (PMT: 1000).

REAGENTS AND SOLUTIONS

Use nuclease-free water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2

RPE1 medium

- 500 mL DMEM high glucose
- 50 mL fetal bovine serum
- 5 mL penicillin/streptomycin

RNA fragmentation buffer, 2x

- 150 mM Tris pH 8.3
- 225 mM KCl
- 9 mM MgCl₂

High salt wash buffer

- 10x stock solution contains:
 - 100 mM Tris, pH 7.4
 - 10 mM EDTA
 - 1 M NaCl
- Add 0.05% Tween-20 fresh to 1x solution each time
- Store at room temperature up to 6 months

Bead blocking buffer

- 1x high salt wash buffer
- 40 ng/ μ L glycogen (20 μ g/ μ L stock solution)

Elution buffer, 1x

- 100 mM DTT
- 20 mM HEPES, pH 7.4

1 mM EDTA
100 mM NaCl
0.05% Tween
Prepare solution fresh each experiment

1x TBE

100 mM Tris Base
100 mM Boric Acid
2 mM EDTA
Store at room temperature up to 6 months

Urea gel loading buffer

990 μ L deionized formamide
10 μ L 500 mM EDTA
Store at room temperature up to 6 months

COMMENTARY

Background Information

Traditional RNA-Seq methods allow the quantification of RNA steady-state levels in cells. In contrast, the study of RNA dynamics is enabled through biochemical enrichment for different sub-populations of RNA prior to RNA-Seq. Metabolic labeling with non-canonical nucleosides is a widely used technique to separate distinct RNA populations. Ideally, the nucleoside should be absent from endogenous RNA, readily incorporated into cellular RNA, and provide a handle for biochemical purification following metabolic labeling.

To this end, numerous studies have employed 5-bromouridine (5-BrU; Tani et al., 2012), 5-ethynyluridine (5-EU; Jao and Salic, 2008), and 4-thiouridine (TU or s⁴U; Cleary et al., 2005 and Miller et al., 2009). 5-BrU-labeled transcripts can be purified by anti-BrdU antibodies, which also recognize BrU, but this approach is limited by the efficiency of antibody enrichment and nucleoside uptake. 5-EU can be used for isolation of nascent transcripts from whole cells, and click chemistry can be used to covalently enrich metabolically labeled RNA. However, 5-EU introduces a greater structural perturbation, and prolonged exposure to 5-EU causes cell toxicity, raising challenges for experiments that require longer treatments.

In contrast, 4-thiouridine can be introduced into cell culture media and is readily incorporated into cellular RNA. Under the conditions listed here, we have not detected significant toxicity even after prolonged exposure, making s⁴U suitable to measure acute changes in transcription and long-lived transcripts in the same experimental set-up. In addition, unlike antibody-based approaches, the biotin enrichment allows for stringent washes to decrease nonspecific background and the disulfide chemistry is reversible,

allowing for elution of RNA from streptavidin beads followed by standard RNA-Seq library preparation methods. While MTS chemistry is specific to s^4U when using isolated RNA, MTS is not specific to s^4U in the context of a cell or cell extract because the activated disulfide reacts with other thiols, including cysteines on proteins. Therefore the MTS chemistry described here is only appropriate for isolated RNA. In order to purify metabolically labeled RNA in a more complex environment, alternative chemistry such as 5-EU, which displays orthogonality to other biomolecules, would be more suitable.

For studying RNA turnover, and other applications where different RNA populations need to be separated from each other (e.g., TU-tagging) MTS-biotin increases labeling yields and decreases enrichment bias. Therefore this MTS chemistry expands the utility of s^4U metabolic labeling by decreasing the number of s^4U 's required to reliably capture a given RNA, the amount of starting material required, and the concentration of s^4U required during cellular labeling.

Critical Parameters

s^4U incorporation—The enrichment will only be successful when the RNA contains sufficient levels of s^4U . In metabolic labeling experiments, incorporation of s^4U into RNA can be controlled by two factors: concentration of s^4U during cell treatment and time of s^4U exposure. Insufficient s^4U incorporation can lead to many transcripts without s^4U , and therefore will lead to low yields and less enrichment over background. Insufficient labeling can also favor enrichment of longer transcripts that have more uridine residues (and therefore a greater probability of s^4U incorporation).

RNA quality—4-thiouridine is light sensitive, so RNA should be kept in the dark as much as possible throughout the protocol. In addition, s^4U oxidation occurs during RNA cleanup, which is why DTT or β -me should be included during these steps of the protocol. Oxidation decreases the amount of s^4U available to react with MTS-biotin, which can decrease yields and introduce biases.

Control samples—RNA from cells that have not been labeled with s^4U serves as a negative control to assess nonspecific background. We routinely reserve 10% of an input sample to quantify RNA yields and calculate fold enrichment of s^4U -RNA relative to the negative control. A useful positive control is an in vitro transcribed RNA with s^4U , which should be captured with nearly quantitative efficiency.

Next-generation RNA sequencing—If s^4U -RNAs are to be analyzed by next-generation RNA sequencing, samples can be depleted of rRNA (preferred) or selected for polyA-RNA. In order to compare RNA enrichment between two samples, we recommend adding an exogenous spike to the enriched samples from step 63, using the same amount of spike per sample. Examples of normalization spikes include RNA from *S. pombe* or synthetic RNA standards. Normalization spikes can be used to quantify fold enrichment of entire samples or individual transcripts relative to input.

Troubleshooting

Suggestions for overcoming or avoiding commonly encountered problems

Step 32

Problem: Inefficient RNA biotinylation

- Possible reason: The MTS biotin has hydrolyzed.
 - Solution: Store MTS-biotin stocks in dry DMF and replace stocks every 3-6 months.
- Possible reason: s^4U has oxidized.
 - Solution: Pre-aliquot s^4U solid and dissolve fresh in water before each experiment.
- Possible reason: The biotin-MTS-DMF stock is not mixed well with the RNA.
 - Solution: Vortex the solution well after adding MTS biotin and make sure that the solution is mixed in the tube rotator. Try changing the mode or speed of rotation until the solution is constantly mixing within the tube.
- Possible reason: s^4U -RNA is oxidized.
 - Solution: Use fresh DTT and β -me stocks for all RNA cleanup procedures in order to pre-reduce oxidized products (e.g., s^4U - s^4U disulfides). Use the IVT ladder in the supplemental protocol as a positive control by cleaning up RNA in parallel to cellular RNA samples in steps 7-16 and 21-27. Run cleaned up IVT ladder samples on a gel and look for slower migrating species that may be indicative of disulfide formation.
- Possible reason: s^4U has been photocrosslinked.
 - Solution: Protect RNA from light during all incubation steps and turn off all unnecessary benchtop lighting while working with RNA. Use the IVT ladder in the supplemental protocol as a positive control. Photocrosslinked IVT ladder may cause an upshifted smear when run on a gel.

Step 61

Problem: Low RNA recovery

- Possible reason: RNA starting amounts are too small.
 - Solution: Measure the concentration by NanoDrop in step 41. The concentration should ideally be equal to the starting concentration in step 29. If the amount of starting material in

step 29 is too low, there may still be RNA enrichment but it is below the limit of detection of the instrument. These samples can still be assayed by more sensitive assays including RNA-Seq.

- Possible reason: Inefficient elution.
 - Solution: Dissolve DTT fresh to make elution buffer. If necessary, repeat elution step with an additional 25 μ L of elution buffer and pool with elutions 1 and 2.
- Possible reason: Inaccurate measurement by Qubit.
 - Solution: Repeat measurement with fresh reagents or use alternative method (e.g., qPCR for expected transcripts).
- Possible reason: RNA has been exposed to RNase.
 - Solution: Clean your work area, pipettes and gloves to remove RNases. Prepare new solutions if needed.

Step 61

Problem: High background in negative control

- Possible reason: Beads were insufficiently blocked in step 45.
 - Solution: Make sure beads are fully resuspended in blocking buffer and make sure that the beads are constantly mixing in a rotator during the blocking step. Try changing the mode or speed of rotation until the solution is constantly mixing within the tube.
- Possible reason: Beads are not washed properly in step 51.
 - Solution: Make sure beads are fully resuspended in between washes and that supernatant is not trapped in tube cap or at the bottom of the tube. Make sure that the beads do not dry out between washes.

Anticipated Results

Before proceeding to downstream analysis, it is important to assay the yield relative to input and fold enrichment over background for each sample. Figure 2 represents the expected yield for sheared and unsheared RNA samples after 2h or 30 min of s^4U treatment as well as a no s^4U control. Sheared samples give slightly better yield and lower background compared to unsheared samples, which is why we prefer shearing if the downstream analysis permits. 2h s^4U treatment is expected to give RNA yields of 4-5% input, whereas 30 min s^4U treatment is expected to give RNA yields of 2-3% input. The yield of the negative control samples is limited by the Qubit assay, so it is difficult to accurately determine the fold enrichment, but greater than 10-fold enrichment is expected.

The in vitro transcribed ladder serves as a useful positive and negative control for s⁴U RNA enrichment. Following our supplemental protocol, we would expect near complete capture of the +s⁴U-RNA ladder, with undetectable levels of the –s⁴U-RNA ladder. These samples can be enriched in parallel to cellular RNA samples to ensure the robustness of the protocol.

Time Considerations

As presented, this protocol requires 2 days of experiments, with 8.5h on day 1 and 3.5h on day 2. Once the cells are plated, the s⁴U treatment requires 2.5 h. Following cell harvest, RNA isolation from cells and DNase treatment and optional shearing takes 6 h. Starting on day 2, RNA biotinylation takes 30 min. Removal of excess biotin takes 30 min and binding to streptavidin beads takes 15 min if bead blocking is performed in parallel. The streptavidin bead wash and RNA elution steps should take no more than an hour, at which point samples can be stored at -20°C or proceed to downstream analysis. Therefore, the total amount of time required for the RNA isolation from the start of the s⁴U treatment is 8.5h, and the enrichment steps from isolated RNA requires 3.5h.

Acknowledgements

This work was supported by the NIH grant DP2 1DP2HD083992 (M.D.S.), the Searle Scholars Program (M.D.S.), NSF Graduate Research Fellowship (E.E.D.). The authors would like to acknowledge Daniele Canzio, Xinyang Bing, and members of the Simon lab for helpful discussions and comments on the manuscript.

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Figure 1.

A) Structure of s^4U and schematic of s^4U -RNA metabolic labeling and enrichment. B) Flow chart of experiments that are generally performed on the first day of the protocol, which includes s^4U metabolic labeling in HEK293T cells and RNA isolation. Dotted lines indicated optional steps for RNA shearing. C) Flow chart of the remainder of the enrichment, which includes s^4U -RNA biotinylation and enrichment on streptavidin beads. Pink and blue boxes indicate fractions that are saved as flow through and elution, respectively.

Figure 2.

Example of yields from the s⁴U-RNA enrichment described in this protocol. RNA from cells treated for 2h or 30 min with s⁴U was enriched using the main text protocol and assayed by Qubit. Percent input was calculated for enriched samples relative to 10% input standards for each sample. Bars indicate the average of two biological replicates, with filled circles indicating the yield from individual experiments.