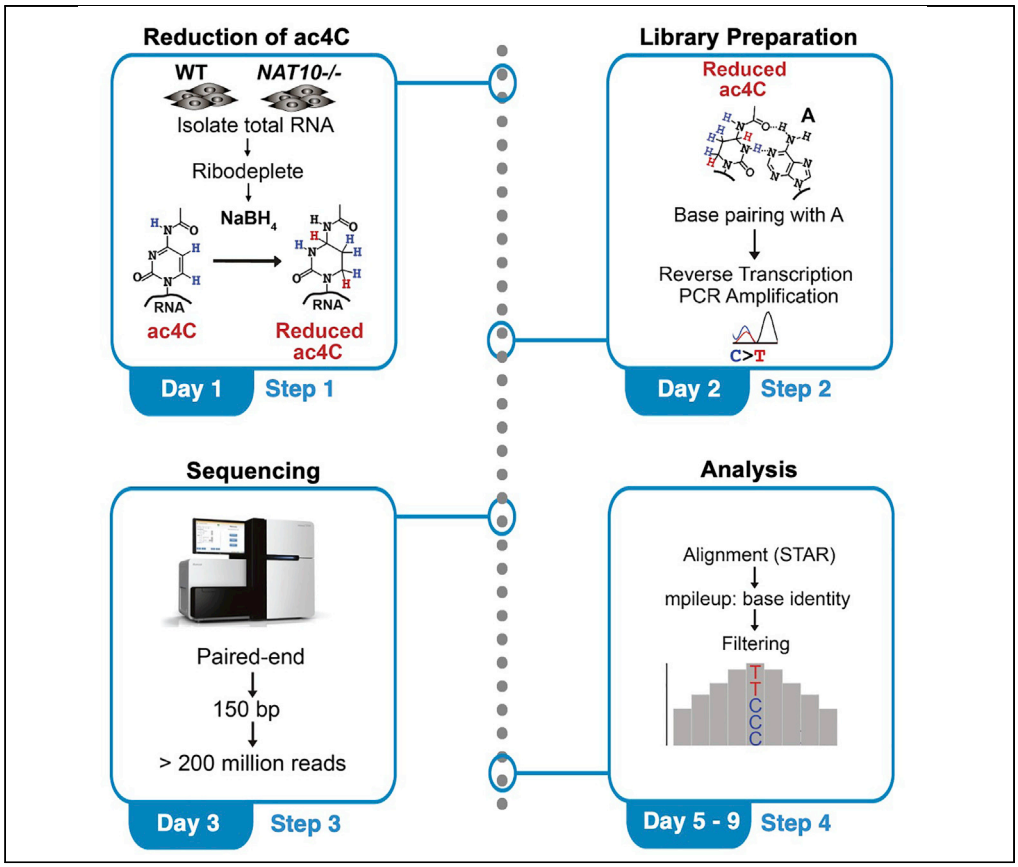
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for base resolution mapping of ac4C using RedaC:T-seq



N4-acetylcytidine (ac4C) is an mRNA modification catalyzed by the enzyme N-acetyltransferase 10 (NAT10), with position-dependent effects on mRNA translation. This protocol details a procedure to map ac4C at base resolution using $NaBH_4$-induced edruction of ac4C and conversion to thymidine followed by sequencing (RedaC:T-seq). Total RNA is ribodepleted and then treated with $NaBH_4$ to reduce ac4C to tetrahydro-ac4C, which specifically alters base pairing during cDNA synthesis, allowing the detection of ac4C at positions called as thymidine following Illumina sequencing.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

David Sturgill,
Daniel Arango,
Shalini Oberdoerffer

davidsturgill@nih.gov (D.S.)
daniel.arangotamayo@northwestern.edu (D.A.)
shalini.oberdoerffer@nih.gov (S.O.)

Highlights

Steps for RNA sample preparation and ribosomal RNA depletion

Specific reduction of ac4C to tetrahydro-ac4C using sodium borohydride ($NaBH_4$)

Quality control and library preparation for Illumina sequencing

Computational pipeline for analysis and mapping of ac4C sites

Sturgill et al., STAR Protocols *3*, 101858
December 16, 2022
https://doi.org/10.1016/j.xpro.2022.101858

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for base resolution mapping of ac4C using RedaC:T-seq

David Sturgill,[1,3,4,*] Daniel Arango,[1,2,3,4,*] and Shalini Oberdoerffer[1,5,*]

[1]Laboratory of Receptor Biology and Gene Expression, Center for Cancer Research, National Cancer Institute, NIH, Bethesda, MD 20892, USA

[2]Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611, USA

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: davidsturgill@nih.gov (D.S.), daniel.arangotamayo@northwestern.edu (D.A.), shalini.oberdoerffer@nih.gov (S.O.)
https://doi.org/10.1016/j.xpro.2022.101858

SUMMARY

N4-acetylcytidine (ac4C) is an mRNA modification catalyzed by the enzyme N-acetyltransferase 10 (NAT10), with position-dependent effects on mRNA translation. This protocol details a procedure to map ac4C at base resolution using $NaBH_4$-induced reduction of ac4C and conversion to thymidine followed by sequencing (RedaC:T-seq). Total RNA is ribodepleted and then treated with $NaBH_4$ to reduce ac4C to tetrahydro-ac4C, which specifically alters base pairing during cDNA synthesis, allowing the detection of ac4C at positions called as thymidine following Illumina sequencing.

For complete details on the use and execution of this protocol, please refer to Arango et al. (2022).[1]

BEFORE YOU BEGIN

To achieve base-resolution mapping of ac4C, we employed sodium borohydride ($NaBH_4$) reduction of ac4C. $NaBH_4$ specifically reduces ac4C to tetrahydroacetylcytidine (tetrahydro-ac4C) without affecting unmodified cytidine.[2] Reduced ac4C cannot efficiently base pair with guanosine and interacts with adenosine. Thus, adenosine is incorporated opposite of reduced ac4C during reverse transcription, culminating in detection as thymidine during second strand cDNA synthesis.

The RedaC:T-seq protocol begins with purified total RNA. The procedures described below were performed in total RNA from wildtype and *NAT10*$^{-/-}$ HeLa cells. However, it can be performed in any cell type, tissue, or organism. HeLa cells were purchased from ATCC (Cat. #: CCL-2). *NAT10*$^{-/-}$ HeLa cells were generated by CRISPR/cas9-mediated ablation of NAT10.[3]

⚠ CRITICAL: Genetic ablation or knockdown of NAT10 is essential to reduce the incidence of false positives and identify specific ac4C sites in downstream analysis of RedaC:T-seq.

Institutional permissions

All procedures can be performed in a Biosafety Level 2 (BSL2) laboratory.

Preparation of RNA samples

Timing: 2 days

1. Culture of wildtype and *NAT10*$^{-/-}$ HeLa cells.
   a. Cells are maintained in 10-cm tissue culture dishes in Dulbecco's Modified Eagle Medium (DMEM) containing 25 mM glucose, 1 mM sodium pyruvate and supplemented with 2 mM L-glutamine and 10% bovine calf serum in the absence of antibiotics.
   b. Seed 2 × 10^6 cells in 10-cm dishes in complete DMEM.
   c. Incubate at 37°C and 5% CO_2 for 48 h until cells reach 70%–80% confluency.
2. Harvest cells.
   a. Discard DMEM using a vacuum aspirator.
   b. Add 5 mL of pre-warmed phosphate-buffered saline (PBS) to each plate and distribute homogenously across culture dishes.
   c. Discard PBS using a vacuum aspirator. Make sure to completely aspirate all traces of medium.
   d. Add 1 mL of TriZol to each plate and distribute homogenously across culture dishes.
   e. Scrape out the cellular monolayer using a cell scraper.
   f. Collect lysed cells into 1.5 mL RNase-free microcentrifuge tubes.
3. Isolate total RNA.
   a. Add 200 μL of Chloroform to each tube. Mix vigorously. Incubate at 22°C–25°C for 3 min.
   b. Centrifuge at 16,100 × *g* for 15 min at 4°C in a refrigerated microcentrifuge.
   c. Transfer ~500 μL of the upper clear layer to new 1.5 mL RNase-free microcentrifuge tubes.
   d. Add 500 μL of isopropanol. Mix vigorously.
   e. Incubate at 22°C–25°C for 10 min.
   f. Centrifuge at 16,100 × *g* for 10 min at 4°C in a refrigerated microcentrifuge.
   g. Discard the supernatant.
   h. Add 500 μL of 70% ethanol. Mix vigorously.
   i. Centrifuge at 16,100 × *g* for 5 min at 4°C in a refrigerated microcentrifuge.
   j. Discard the supernatant. Remove all traces of alcohol.
   k. Air dry the pellet for 5 min at 22°C–25°C with the lid open.
   l. Resuspend pellets in 87 μL of nuclease-free H_2O.
4. DNase treatment.
   a. Prepare the following mix per sample:
   10× Turbo DNase Buffer 10 μL.
   Turbo DNase I (2 U/μL) 2 μL.
   Murine RNase Inhibitor (40 U/μL) 1 μL.
   b. Add 13 μL of mix to each tube. Mix well by pipetting up and down.
   c. Incubate at 37°C for 20 min.
   d. Add 300 μL of nuclease-free H_2O. Mix well.
   e. Add 400 μL acid-phenol:chloroform, pH 4.5. Mix vigorously.
   f. Incubate at 22°C–25°C for 3 min.
   g. Centrifuge at 16,100 × *g* for 15 min at 4°C in a refrigerated microcentrifuge.
   h. Transfer ~360 μL of the upper clear layer to new 1.5 mL RNAse-free microcentrifuge tubes.
   i. Add 40 μL of 3 M Sodium Acetate pH 5.5. Mix well.
   j. Add 1.1 mL of 100% ethanol. Mix well.
   k. Incubate at −80°C for at least 1 h.
   l. Centrifuge at 16,100 × *g* for 15 min at 4°C in a refrigerated microcentrifuge.
   m. Discard the supernatant.
   n. Add 500 μL of 70% ethanol. Mix vigorously.
   o. Centrifuge at 16,100 × *g* for 5 min at 4°C in a refrigerated microcentrifuge.
   p. Discard the supernatant. Remove all traces of alcohol.
   q. With the lid opened, air dry the pellet for 5 min at 22°C–25°C.
   r. Resuspend pellets in 100 μL of nuclease-free H_2O. Total RNA can be stored at −80°C until further use.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Total RNA from wildtype HeLa cells	Arango et al.[1]	N/A
Total RNA from *NAT10*$^{-/-}$ HeLa cells	Arango et al.[1]	N/A
Chemicals, peptides, and recombinant proteins		
Acid-Phenol:Chloroform, pH 4.5	Thermo Fisher Scientific	Cat#:AM9722
Agencourt AMPure XP beads	Beckam Coulter	Cat#:A63881
Agencourt RNAclean XP beads	Beckam Coulter	Cat#:A63987
Agilent RNA 6000 kit	Agilent	Cat#:5067-1511
Agilent High Sensitivity DNA Kit	Agilent	Cat#:5067-4626
Bovine Calf Serum (BCS)	HyClone	Cat#:SH30073.03
Chloroform	Fisher Scientific	Cat#:BP1145-1
DMEM	Thermo Fisher Scientific	Cat#:11995073
EDTA (0.5 M), pH 8.0, RNAse-free	Thermo Fisher Scientific	Cat#:AM9260G
Ethanol	Fisher Scientific	Cat#:BP2818100
HCl	Sigma-Aldrich	Cat#:320331
Isopropanol	Sigma-Aldrich	Cat#:I9516
L-glutamine	Thermo Fisher Scientific	Cat#:25030149
Linear acrylamide	Thermo Fisher Scientific	Cat#:AM9520
Murine RNase inhibitor	New England Biolabs	Cat#:M0314
$NaBH_4$	Sigma-Aldrich	Cat#:452882
Nuclease-free H_2O	Thermo Fisher Scientific	Cat#:AM9932
PBS	Thermo Fisher Scientific	Cat#:10010023
RNase-away	Thermo Fisher Scientific	Cat#:10328011
Sodium Acetate Solution pH5.5	Thermo Fisher Scientific	Cat#:AM9740
Tris (1 M), pH 8.0, RNAse-free	Thermo Fisher Scientific	Cat#:AM9856
TRIzol Reagent	Thermo Fisher Scientific	Cat#:15596026
Turbo™ DNase I	Thermo Fisher Scientific	Cat#:AM2239
Critical commercial assays		
NEBNext® Magnesium RNA Fragmentation buffer	New England Biolabs	Cat#:E6150
NEBNext® rRNA Depletion Kit	New England Biolabs	Cat#:E6310L
NEBNext® UltraII™ Directional RNA Library Prep Kit	New England Biolabs	Cat#:E7770S
NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1 and Set 2)	New England Biolabs	Cat#:E7335S Cat#:E7500S
Deposited data		
Raw and processed RedaC:T-seq data	Arango et al.[1]	GEO: GSE162043
Experimental models: Cell lines		
Human: HeLa (Human cervical carcinoma, female)	ATCC	Cat#:CCL-2; RRID: CVCL_0030
Human: *NAT10*$^{-/-}$ HeLa (Human cervical carcinoma, female)	Arango et al.[3]	N/A
Software and algorithms		
Cutadapt v.1.6	Martin[4]	RRID: SCR_011841
Samtools v.1.11	Li et al.[5]	RRID: SCR_002105
Rstudio v.3.6	N/A	RRID: SCR_000432
STAR v.2.5.4.a	Dobin et al.[6]	RRID: SCR_019993
mpileup2readcounts	IARC bioinformatics platform (Lyon, France)	https://github.com/IARCbioinfo/mpileup2readcounts
Genomation v.1.24.0	Akalin et al.[7]	RRID: SCR_003435
GenomicFeatures v.1.44.2	Lawrence et al.[8]	RRID: SCR_016960
Rtracklayer v.1.52.1	Lawrence et al.[9]	RRID: SCR_021325
Other		
2100 Bioanalyzer or TapeStation	Agilent	RRID: SCR_019715 or RRID: SCR_019394
Workstation or cluster with 2.4 GHz CPU (Intel E5-2680v4) 8-core, 64 GB RAM, 2 TB storage, OSX or CentOS Linux 7	N/A	N/A

MATERIALS AND EQUIPMENT

200 mM $NaBH_4$ solution

⚠ CRITICAL: Prepare $NaBH_4$ fresh immediately before use. $NaBH_4$ should be handled in the fume hood.

Reagent	Final concentration	Amount
$NaBH_4$	200 mM	7.57 mg
Nuclease-free H_2O	N/A	1 mL
Total		1 mL

Dissolve 7.57 mg of $NaBH_4$ in 1 mL of nuclease-free H_2O.

Storage: Do not store. Prepare $NaBH_4$ fresh immediately before use. $NaBH_4$ should be handled in the fume hood.

1× TE buffer

Reagent	Final concentration	Amount
1 M Tris-HCl (pH 8.0)	10 mM	1 mL
0.5 M EDTA (pH 8.0)	1 mM	0.2 mL
Nuclease-free H_2O	N/A	98.8 mL
Total		100 mL

Storage: Buffer can be stored at 22°C–25°C for up to 3 months.

0.1× TE buffer

Reagent	Final concentration	Amount
1× TE Buffer	0.1× TE	1 mL
Nuclease-free H_2O	N/A	9 mL
Total		10 mL

Storage: Buffer can be stored at 22°C–25°C for up to 3 months.

Computer equipment

High-performance computational resources are required for downstream analysis. Working within a large cluster environment is recommended. Minimum requirements for a standalone computing environment are provided in the key resources table.

Note: Smaller transcriptomes or targeted approaches may be analyzed with more moderate resources. The requirements specified are for data from whole human transcriptomes.

STEP-BY-STEP METHOD DETAILS

Ribodepletion

Timing: 2 h

Note: It is necessary to reduce the excess of ribosomal reads in sequencing experiments. The step described below removes ribosomal RNA (rRNA) from total RNA preparations. We used the rRNA NEBNext® rRNA Depletion Kit from New England Biolabs.

⚠ CRITICAL: To avoid unintended degradation of RNA samples, perform all procedures in a clean area, using RNase-free reagents, RNase-free tubes, and spraying all pipettes with RNase-way reagent.

1. Anneal rRNA depletion probes.
   a. Prepare 1 μg of total RNA in 12 μL of nuclease-free H_2O. Add the following components:

Reagent	Amount
NEBNext rRNA Depletion Solution	1 μL
NEBNext Probe Hybridization Buffer	2 μL

   b. Place samples in a Thermocycler and run the following program with the lid set at 105°C:

Steps	Temperature	Time
Denaturation	95°C	2 min
Anneal	95°C–22°C	ramp 0.1°C/s
Hold	22°C	5 min

   c. Spin down the samples and place them on ice.
2. Prepare the RNase H master mix.
   a. Mix the following reagents:

Reagent	Amount
NEBNext RNase H	2 μL
NEBNext RNase H Reaction Buffer	2 μL
Nuclease-free Water	1 μL

   b. Add 5 μL of the above mix to the RNA samples. Mix by pipetting up and down.
   c. Place samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 min.
   d. Spin down the samples in a tabletop centrifuge and place them on ice.
3. Prepare the DNAse I master mix:
   a. Mix the following reagents:

Reagent	Amount
NEBNext DNase I reaction Buffer	5 μL
NEBNext DNase I (RNase-free)	2.5 μL
Nuclease-free Water	22.5 μL

   b. Add 30 μL of the above and mix by pipetting up and down.
   c. Place the samples in a thermocycler (with lid at 40°C) and incubate at 37°C for 30 min.
   d. Spin down samples in a tabletop centrifuge and place on ice.
4. Purify ribodepleted RNA.
   a. Add 110 μL (2.2×) of resuspended Agencourt RNAclean XP beads to the RNA samples.
      i. Mix well by pipetting up and down.
      ii. Incubate for 15 min on ice.
   b. Spin, place on a magnetic rack for 5 min.
      i. Remove supernatant.
   c. Add 200 μL 80% ethanol.
      i. Incubate at 22°C–25°C for 30 s and remove supernatant.
      ii. Repeat for a total of 2 washing steps.
   d. With lid opened, air dry the beads for 5 min at 22°C–25°C.
   e. Elute RNA into 10 μL nuclease-free water.

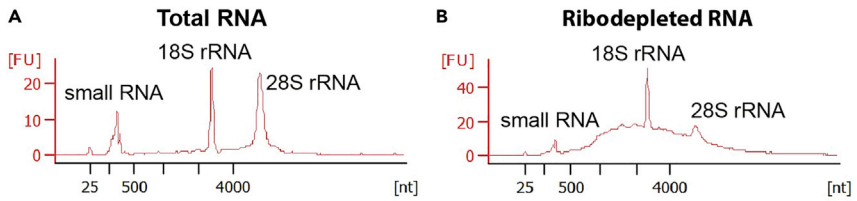


Figure 1. RNA integrity and efficiency of ribodepletion
(A and B) Bioanalyzer profile of total RNA pre- and post-ribodepletion.

i. Incubate for 2 min at 22°C–25°C.

f. Place tubes on a magnetic rack to separate.
   i. Remove 10 μL of the supernatant.
   ii. Transfer it to a clean nuclease-free PCR tube.

Pause point: Ribodepleted RNA can be stored at −80°C for prolonged periods of time. However, we moved to the next step immediately.

Note: rRNA ribodepletion efficiency can be verified through RT-qPCR or by running 1 μL of sample in a bioanalyzer using the Agilent RNA 6000 kit (Figure 1). Approximately 100 ng ribodepleted RNA is expected.

Critical: Sodium borohydride treatment

Timing: 2 h

Note: This step reduces ac4C to tetrahydro-ac4C without affecting unmodified cytidine (Figure 2). It is the most critical step of the procedure.

5. Treat RNA with $NaBH_4$.
   a. Place 10 μL of ribodepleted RNA (~50–100 ng) in a 0.2 mL RNase-free tube.
   b. Add 10 μL of 200 mM $NaBH_4$. Mix tubes by finger-flicking.
   c. Incubate for 1 h at 55°C in the dark.

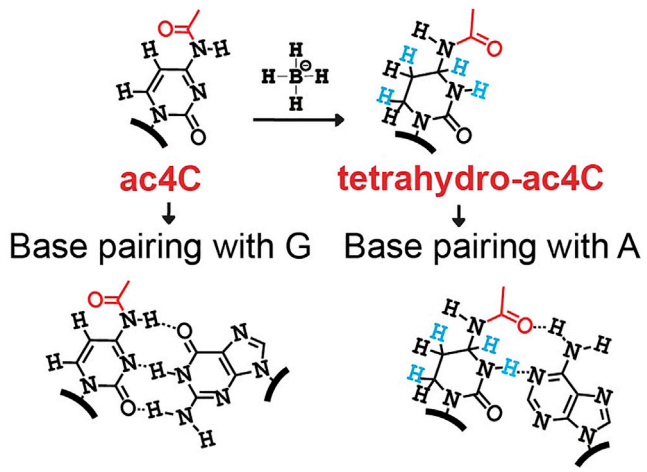


Figure 2. Reduction of ac4C
$NaBH_4$ reduces ac4C to tetrahydro-ac4C *(top)*. Reduced ac4C less efficiently base pairs with guanosine and can interact with adenosine instead *(bottom)*.

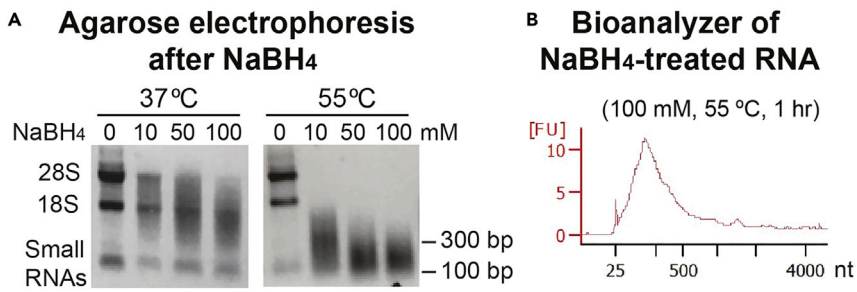


Figure 3. RNA integrity after $NaBH_4$ treatment

(A) Agarose gel electrophoresis of total RNA samples treated with $NaBH_4$ or left untreated.

(B) Bioanalyzer profile of total RNA treated with 100 mM $NaBH_4$ for 1 h at 55°C.

⚠ CRITICAL: $NaBH_4$ releases heat and gas, generating bubbles within the solution. Since bubbles are generated, to avoid increased pressure inside the tubes, open the lids every 10 min and quickly spin down the tubes.

Note: $NaBH_4$ is an alkaline solution and induces RNA fragmentation to ~100 nt at 55°C (Figure 3).

Optional: Reduction of ac4C with $NaBH_4$ may also be performed at 37°C. However, an extra step of RNA fragmentation needs to be included when using temperatures lower than 55°C. While not performed in this protocol, in case further RNA fragmentation is needed, we use the NEBNext Magnesium RNA Fragmentation Module following the manufacturer's instructions.

6. Neutralize the reaction by adding 10 µL of 200 mM HCl.
7. Purify RNA.
   a. Transfer the solutions to new 1.5 mL RNase-free Tubes.
   b. Add 370 µL of nuclease-free H_2O.
   c. Add 3 µL of 5 mg/mL linear acrylamide.
      i. Mix well by pipetting up and down.
   d. Add 40 µL of 3 M Sodium Acetate pH 5.5.
      i. Mix well by inverting the tube several times.
   e. Add 1.1 mL of 100% ethanol. Mix well.
   f. Incubate at −80°C for at least 1 h.

Pause point: samples can be stored at −80°C for 16–20 h.

   g. Centrifuge at 16,100 × *g* for 15 min at 4°C in a refrigerated microcentrifuge.
   h. Discard the supernatant.
   i. Add 500 µL of 70% ethanol. Mix vigorously.
   j. Centrifuge at 16,100 × *g* for 5 min at 4°C in a refrigerated microcentrifuge.
   k. Discard the supernatant.
      i. Remove all traces of alcohol.
   l. Air dry the pellet for 5 min at 22°C–25°C with the lid opened.
   m. Resuspend pellets in 6 µL of nuclease-free H_2O.

Note: To avoid further freeze and thaw of the $NaBH_4$-treated RNA, we recommend moving directly to the next step.

Library preparation

Timing: 4 h

Table 1. Samples description

	Amount of NaBH4-treated RNA	Final library yield	Final volume	Barcode	NEBNext® multiplex oligos
HeLa WT Rep 1	10 ng	24.2 ng/μL	20 μL	ACTGAT	NEBNext Index 25
NAT10$^{-/-}$ Rep 1	10 ng	42.3 ng/μL	20 μL	ATTCCT	NEBNext Index 27
HeLa WT Rep 2	10 ng	14.2 ng/μL	20 μL	GGCTAC	NEBNext Index 11
NAT10$^{-/-}$ Rep 2	10 ng	14.4 ng/μL	20 μL	CTTGTA	NEBNext Index 12

Note: This step performs cDNA synthesis from $NaBH_4$-treated RNA, followed by adapter ligation and PCR amplification. We used the NEBNext® UltraII™ Directional RNA Library Prep Kit for library preparation.

8. First-strand cDNA synthesis.

Note: After ribodepletion, $NaBH_4$ treatment and all isolation steps, we obtained ~10–40 ng of RNA material. NEB recommends using 1 ng–100 ng of ribodepleted RNA for library preparation. We used 10 ng of RNA (Table 1).

a. To each sample of 5 μL $NaBH_4$-treated RNA, add 1 μL of 50 μM NEBNext Random primers. (Provided with Kit).
b. Incubate the sample at 65°C for 5 min, with a heated lid set at 105°C.
   i. Hold at 4°C.
c. To each sample (6 μL), add the following components and mix by gentle pipetting:

Reagent	Amount
5× Buffer	4 μL
NEBNext Strand Specificity Reagent	8 μL
NEBNext First Strand Synthesis Enzyme Mix	2 μL

d. Incubate samples (20 μL total volume) in a preheated thermal cycler (with the heated lid set at 105°C) as follows:

Steps	Temperature	Time
Anneal	25°C	10 min
Reverse transcription	48°C	10 min
Heat inactivation	70°C	15 min

Note: The temperature is elevated compared to the recommended by NEB. We have observed that elevating the temperature of reverse transcriptases increases C>T conversion upon $NaBH_4$ treatment. See the "troubleshooting" section for additional comments.

Alternatives: Several reverse transcriptases can be used, including TGIRT, Superscript III, and AMV.[2] An adapter ligation to the 3′ end of RNAs can be performed, followed by reverse transcription using a cDNA primer specific to the adapter. However, we have observed that adapter ligation is inefficient in $NaBH_4$-treated RNA resulting in very poor library yield.

9. Second strand cDNA synthesis.
   a. Add the following reagents to the first strand synthesis reactions:

Reagent	Amount
Nuclease-free water	48 μL
NEBNext Second Strand Synthesis Reaction Buffer (10×)	8 μL
NEBNext Second Strand Synthesis Enzyme Mix	4 μL

b. Mix thoroughly by gentle pipetting.
c. Incubate samples (60 μL total volume) in a thermal cycler for 1 h at 16°C, with a heated lid set at ≤ 40°C.

10. Purify cDNA.
    a. Add 144 μL (1.8×) of resuspended AMPure XP Beads to the second strand synthesis reaction.
        i. Mix well by pipetting up and down.
        ii. Incubate for 5 min at 22°C–25°C.
    b. Spin and place the tube on a magnetic rack for 5 min.
        i. Remove supernatant.
    c. Add 200 μL of 80% ethanol to the tube.
        i. Incubate at 22°C–25°C for 30 s, and then remove supernatant.
        ii. Repeat for a total of 2 washing steps.
    d. Air dry the beads for 5 min with lids open.
        i. Remove the tube from the magnet.
    e. Elute the DNA target from the beads into 53 μL 0.1× TE buffer.
        i. Mix well by pipetting up and down.
        ii. Incubate for 2 min at 22°C–25°C.
    f. Place the tube in the magnetic rack until the solution is clear.
    g. Remove 50 μL of the supernatant.
    h. Transfer it to a clean nuclease-free PCR tube.
11. End Prep of cDNA Library.
    a. Mix the following components in a sterile nuclease-free tube:

Reagent	Amount
Purified double-stranded cDNA	50 μL
NEBNext Ultra II End Prep Reaction Buffer	7 μL
NEBNext Ultra II End Prep Enzyme Mix	3 μL

    b. Mix by pipetting up and down.
    c. Incubate samples (60 μL total volume) in a thermal cycler (with the heated lid set at 75°C) as follows:

30 min at 20°C.
30 min at 65°C.
Hold at 4°C.

12. Perform Adaptor Ligation.

Note: Dilute the NEBNext Adaptor for Illumina by mixing 1 μL of stock adapter (provided with Kit) with 9 μL with adaptor dilution buffer (provided with Kit)).

    a. Add the following components directly to the End Prep Reaction. **Caution:** Do not pre-mix the components to prevent adaptor-dimer formation.

Reagent	Amount
End Prep Reaction	60 μL
Diluted NEBNext Adaptor* (1:10 dilution)	2.5 μL
NEBNext ligation enhancer	1 μL
NEBNext Ultra II Ligation Master Mix	30 μL

    b. Mix samples (93.5 μL total volume) by pipetting up and down.
        i. Quick spin to collect all liquid from the sides of the tube.

c. Incubate 15 min at 20°C in a Thermomixer.
d. Add 3 μL USER Enzyme (provided with the kit) to the ligation mixture resulting in a total volume of 96.5 μL.
   i. Mix well by pipetting up and down.
e. Incubate at 37°C for 15 min with the lid set to higher than 45°C.

13. Purify adapter-ligated cDNA.
    a. Add 96.3 μL (1×) AMPure XP Beads.
       i. Mix well by pipetting up and down.
    b. Incubate for 10 min at 22°C–25°C.
    c. Quickly spin, and place on a magnetic rack for 5 min.
       i. Discard the supernatant.
    d. Add 200 μL of freshly prepared 80% ethanol to the tube.
    e. Incubate at 22°C–25°C for 30 s.
       i. Remove supernatant.
       ii. Repeat for a total of 2 washing steps.
    f. Spin the tube and put the tube back in the magnetic rack.
    g. Completely remove the residual ethanol and air dry beads for 5 min.
    h. Elute DNA with 17 μL 0.1× TE buffer.
    i. Incubate for 2 min at 22°C–25°C.
    j. Without disturbing the bead pellet, transfer 15 μL of the supernatant to a clean PCR tube.
14. PCR enrichment.
    a. To the adapter-ligated cDNA (15 μL total volume), add the following components and mix by gentle pipetting:

Reagent	Amount
NEBNext Ultra II Q5 Master Mix	25 μL
Barcoded Primer – Use a different barcode per sample	5 μL
Universal PCR Primer	5 μL
Total volume	50 μL

    b. Set the thermocycler with the following steps:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	13 cycles
Annealing/Extension	65°C	75 s	
Final extension	65°C	5 min	1
Hold	4°C	forever	

15. Purify libraries.
    a. Take samples out of the thermocycler and add 50 μL (1×) of resuspended AMPure XP Beads.
       i. Mix well by pipetting up and down.
    b. Incubate for 5 min at 22°C–25°C.
    c. Spin and place the tube on a magnetic rack for 5 min.
       i. Discard the supernatant.
    d. Add 200 μL of freshly prepared 80% ethanol.
       i. Incubate at 22°C–25°C for 30 s.
       ii. Remove supernatant.
    e. Repeat for a total of 2 washing steps.

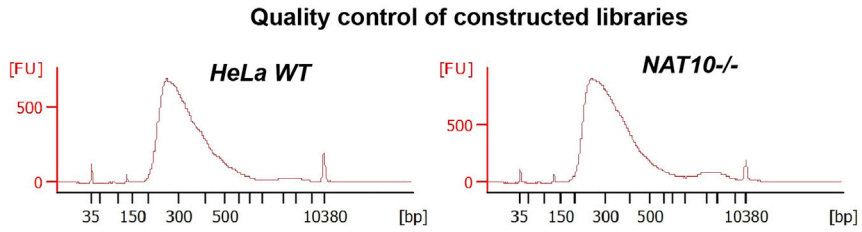


Figure 4. DNA library integrity
Bioanalyzer profiles of DNA libraries from HeLa WT and *NAT10*$^{-/-}$ cells.

f. Spin the tubes and put back into the magnetic rack.
g. Completely remove the residual ethanol and air dry beads for 5 min.
h. Elute DNA target from the beads with 23 μL 0.1 TE buffer.
i. Incubate for 2 min at 22°C–25°C.
j. Without disturbing the bead pellet, transfer 21 μL of the supernatant to a clean PCR tube.

Pause point: Libraries can be stored at −20°C indefinitely.

Quality control step: Bioanalyzer of constructed libraries

Timing: 2 h

Note: The integrity, purity, and size distribution of DNA libraries should be checked in a Bioanalyzer or TapeStation.

16. Usually, 1 μL of sample is used for quality control using the Bioanalyzer and Agilent High Sensitivity DNA Kit.
17. Follow the manufacturer's suggestions. Representative results for libraries made from $NaBH_4$-treated RNA used in our study are provided in Figure 4.

Quality control step: Checking the efficiency of C>T conversion by PCR

Timing: 2 days

Note: This step is required to estimate the efficiency of C>T in a conserved ac4C site in 18S rRNA. A pair of primers surrounding position 1842 in 18S rRNA is used to amplify a region that contains an ac4C site at 100% stoichiometry. The residual amount of rRNA in the libraries is enough to perform this quality control. Following PCR, amplicons are analyzed by Sanger sequencing. We typically observed ~50% C>T efficiency in position 1842 (Figure 5). While the PCR and amplicon purification takes ~2 h, sending the samples for Sanger sequencing and analyzing the data can take up to two days.

18. To 0.5 μL of libraries, add the following components and mix by gentle pipetting:

Reagent	Amount
NEBNext Ultra II Q5 Master Mix	12.5 μL
18S rRNA helix 45 Primer F (10 μM) – 5′ CGCTACTACCGATTGGATGG 3′	2.5 μL
18S rRNA helix 45 Primer R (10 μM) – 5′ TAATGATCCTTCCGCAGGTTCACC 3′	2.5 μL
Library DNA	0.5 μL
Nuclease-free Water	7 μL
Total volume	25 μL

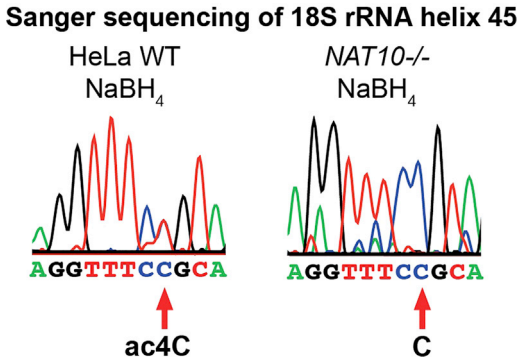


Figure 5. Sanger sequencing of 18S rRNA helix 45 after $NaBH_4$ treatment

19. Set the thermocycler with the following steps:

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	30 s	1
Denaturation	98°C	10 s	35 cycles
Annealing/Extension	68°C	60 s	
Final extension	68°C	5 min	1
Hold	4°C	forever	

20. Purify 18S rRNA amplicons.
    a. Take samples out of the thermocycler and add 45 μL (1.8×) of resuspended AMPure XP Beads.
        i. Mix well by pipetting up and down.
    b. Incubate for 5 min at 22°C–25°C.
    c. Spin and place the tube on a magnetic rack for 5 min.
        i. Discard the supernatant.
    d. Add 200 μL of freshly prepared 80% ethanol.
        i. Incubate at 22°C–25°C for 30 s.
        ii. Remove supernatant.
        iii. Repeat for a total of 2 washing steps.
    e. Spin the tubes and put back into the magnetic rack.
    f. Completely remove the residual ethanol and air dry beads for 5 min.
    g. Elute DNA target from the beads with 12 μL 0.1 TE buffer.
    h. Incubate for 2 min at 22°C–25°C.
    i. Without disturbing the bead pellet, transfer 11 μL of the supernatant to a clean PCR tube and store at −20°C.
    j. Send samples for Sanger sequencing using the 18S rRNA helix 45 Primer F (10 μM) – 5′ CGCTACTACCGATTGGATGG 3′
    k. Align and verify C>T conversion at the ac4C site in position 1842 (Figure 5).

Library sequencing

Timing: 2–6 days

Instrument run time (HiSeq 2500): 40 h (rapid run mode) to 6 days (high output mode).

This step generates sequencing reads for the constructed library and should be performed by a sequencing facility. General guidance on run parameters includes:

21. Sequence on an Illumina HiSeq 2500 or comparable four-channel instrument (see Note) for 126–150 cycles in paired-end mode (For 126–150 bp paired-end reads).

Note: Illumina's two-channel instruments have different baseline error profiles than their four-channel instruments. This protocol describes data produced with four-channel chemistry. It is critical that data that are compared are produced on the same instrument to ensure mismatch differences detected are not due to differences in chemistry.

Read processing and alignments

Timing: 1.5 days

22. Obtain raw reads from the sequencing facility.
23. For each sample, perform adapter trimming with cutadapt (v1.16).[4]
    a. Consult the documentation (https://cutadapt.readthedocs.io/en/stable/) to customize parameters for your dataset. For example:

```
cutadapt -f fastq –match-read-wildcards –times 1 -e 0.1 -O 1 \
–quality-cutoff 6 -m 18 -a GATCGGAAGAGCACA -g ACGCTCTTCCGATCT \
-A AGATCGGAAGAGCGT -G GTGCTCTTCCGATC -o Sample1_R1_cutadapt.fastq.gz \
-p Sample1_R2_cutadapt.fastq.gz Sample1_R1_001.fastq.gz \
Sample1_R2_001.fastq.gz > Sample1.adapterTrim.metrics
```

24. Prepare a genomic reference for mapping.
    a. You can obtain genomic sequence and gene annotation from sources such as UCSC (http://hgdownload.soe.ucsc.edu/downloads.html#human) or igenomes (https://support.illumina.com/sequencing/sequencing_software/igenome.html).
    b. Format the reference for mapping as in this example:

```
STAR –runMode genomeGenerate –runThreadN 8 –genomeDir indexes/hg19 \
  –genomeFastaFiles ref.fa –sjdbGTFfile genes.gtf –sjdbOverhang 100
```

25. Perform the alignment using STAR (v 2.5.4.a).[6]
    a. Consult the documentation (https://github.com/alexdobin/STAR) to customize parameters for your dataset. For example:

```
STAR –runMode alignReads –runThreadN 8 –genomeDir /data/indexes/STAR/hg19 \
  –genomeLoad LoadAndRemove \
  –readFilesIn Sample1_R1_cutadapt.fastq.gz Sample1_R2_cutadapt.fastq.gz \
  –outFilterMultimapNmax 10 –clip3pNbases 6 –clip5pNbases 6 \
  –outFilterMultimapScoreRange 1 –outFileNamePrefix Sample1_genome.bam \
```

```
  –outSAMattributes All –readFilesCommand zcat –outStd BAM_Unsorted \
  –outSAMtype BAM Unsorted –outFilterMismatchNmax 5 –outFilterType BySJout \
  –outReadsUnmapped Fastx –outFilterScoreMin 10 –outSAMattrRGline ID:foo \
  –alignEndsType Local
```

26. Sort and index the alignments, merge alignments as desired.

```
samtools sort -T sort_scratch -o Sample1_genome.sorted.bam Sample1_genome.bam \
  && samtools index Sample1_genome.sorted.bam
samtools merge -f WT_NaBH4.bam Sample1_genome.sorted.bam Sample2_genome.sorted.bam \
  && samtools sort -T sort_scratch -o WT_NaBH4.sorted.bam WT_NaBH4.bam \
  && samtools index WT_NaBH4.sorted.bam
```

Variant analysis

Timing: 2.5 days

Note: This step will produce a pileup, or summary of coverage and base calls by position, across samples. Conversion to interpretable read counts is performed by the mpileup2readcounts script, which enforces additional quality criteria on alignments. Before beginning, install this script by following the instructions at (https://github.com/IARCbioinfo/mpileup2readcounts). Place the executable in your working directory or a directory in your $PATH.

27. Run the mpileup command and pipe to the mpileup2readcounts script.
    a. This example runs this on three samples, called – wildtype (WT) $NaBH_4$ treated, KO (*NAT10*$^{-/-}$) $NaBH_4$ treated, and WT Untreated:

```
samtools mpileup -A -R -Q20 -C0 -d 100000 –ff UNMAP,SECONDARY,QCFAIL,DUP \
  -f /data/indexes/STAR/hg19/ref.fa WT.BH4.bam KO.BH4.bam WT.Ctrl.bam \
  | sed 's/ / * */g' | \
  mpileup2readcounts 0 –5 true 0 0 > mpileup_output.txt;
```

Optional: To reduce downstream compute time, you may restrict output to positions with a minimum depth, with an additional pipe, as this enforces a depth of 10 in each sample:

```
samtools mpileup -A -R -Q20 -C0 -d 100000 –ff UNMAP,SECONDARY,QCFAIL,DUP \
  -f /data/indexes/STAR/hg19/ref.fa WT.BH4.bam KO.BH4.bam WT.Ctrl.bam \
  | sed 's/ / * */g' | \
  mpileup2readcounts 0 –5 true 0 0 | \
  awk '$4 >= 10 && $15 >= 10 && $26 >= 10' > mpileup_output.txt;
```

28. Parse the output to produce tidier results for comparing mismatch rates.
    a. This parsing script is available at Github: https://github.com/dsturg/RedaCT-Seq.
    b. Usage: redact_parse_script.pl [starting file] [number of samples].

```
redact_parse_script.pl mpileup_output.txt 3 > mpileup_output_parsed.txt
```

Quantification and statistical analysis

Timing: 2 h

Note: Following the generation of base calling summaries via pileup, the next step is to load and process these data in the R environment. An example workflow with sample data is provided at Github: https://github.com/dsturg/RedaCT-Seq. The timing estimate above reflects computational run time along with consideration of diagnostic plots within the workflow. The workflow consists of 4 major steps, described below:

1. Calculation of mismatch rates at each queried position, for each sample. For each mismatch relative to the reference genome, the mismatch rate is calculated as:

```
MismatchRate = MismatchCounts / depth
```

2. Projection of genomic coordinates into transcript coordinates. Candidate converted sites are projected onto reference transcripts using functions in the Genomation, GenomicFeatures, and Rtracklayer packages.[7–9]

```
txmapped <- mapToTranscripts(bed, exon_by_tx,ignore.strand=FALSE)
```

3. QC and determination of candidate modified sites.
    a. Before statistical testing, screening of sites is performed to ensure specificity of transcript assignment, absence of polymorphism, and mismatch rate above sequencing error:
        i. Mismatch rate in untreated control < 1%.
        ii. Mapping to a single reference transcript.
        iii. Absence of multiple mismatch types at the same position.
        iv. Mismatch rate elevated relative to untreated sample.
4. Statistical testing, thresholding, and exploratory plots.

Note: Statistical testing is performed on mismatch and reference base calls between $NaBH_4$ treated WT and *NAT10−/−* samples. To perform this test, 2 × 2 matrices are constructed for each relevant site, using the data: Mismatched base counts (WT), Reference base counts (WT), Mismatched base counts (*NAT10−/−*), Reference base counts (*NAT10−/−*). Fisher's Exact Tests are performed in R as:

```
pvalue <- fisher.test(matrix)$p.value
```

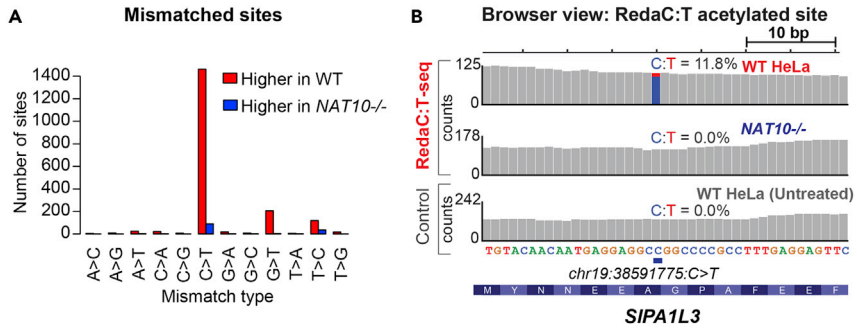


Figure 6. Mismatches observed in RedaC:T-seq data

(A) Bar plot of frequency of each mismatch type. Sample data from chromosome 19, sites selected at $p \leq 0.01$ and ≥ 5 fold mismatch rate difference.

(B) Browser view of a site in the *SIPA1L3* gene. $NaBH_4$ treated samples in WT and NAT10$^{-/-}$ are shown, along with untreated control.

Final selection of sites uses criteria on magnitude of difference of mismatch rate (as measured by fold change), in addition to the p-value.

EXPECTED OUTCOMES

Following the procedure described above, where non-C>T mismatches are included in the analysis as quality control, we expect C>T mismatches to be most highly represented, and mismatch rates to be elevated in the WT sample (as in Figure 6A).

Mismatch rates at individual mRNA sites will cover a range of values reflecting differences in stoichiometry (Figure 6B), with a maximum that reflects the conversion efficiency of the experiment. This can be assessed by observing mismatch rates in a positive control. In the HeLa transcriptome, mismatch rates at acetylated sites covered a broad range, but generally plateaued at 25%. This maximum reflects the conversion rate we observed at the 100% acetylated 18S rRNA site at position 1842.

An example acetylated site is shown in Figure 6B. The total number of acetylated locations is dependent on the sample, conversion efficiency, and sequencing depth. With the depth and conditions we describe here, we detected 7,851 acetylated locations.[1] The total ratio of ac4C to C in the transcriptome can be estimated by comparing the total C>T mismatches to the sequencing depth at reference cytidines, after applying a minimum depth threshold (for example, 10× coverage). In the HeLa transcriptome, we used this approach to estimate total ac4C:C at 0.016%.[1]

LIMITATIONS

$NaBH_4$ can react with other nucleobases, including 7-methylguanosine, dihydrouridine, 3-methylcytidine, and wybutosine,[10–12] potentially producing mismatches unrelated to ac4C. To accurately call ac4C sites, a *NAT10*$^{-/-}$ sample must be used. Using the analysis routine described above, RedaC:T-seq analysis filters out non-specific mismatches and detects only NAT10-mediated sites.

One limitation thus relates to obtaining *NAT10*$^{-/-}$ samples, especially when working with primary cells or tissues. In such cases, chemical deacetylation of RNA in mild alkaline conditions may be used.[13]

While not included in this study, we recommend spiking in samples with acetylated RNA probes containing ac4C at known positions and stoichiometries. Probes will aid in the absolute quantitation of ac4C and help control for reduction variability across different samples. We also recommend using

unique molecular identifiers (UMIs) to filter duplicated reads and reduce artifacts related to sequencing errors.

With the non-targeted approach that we describe, we avoid potential selection bias arising from the targeting/enrichment technique. However, this creates a limitation with regards to depth requirements. In an RNA pool with heterogeneous representation, acetylated sites with low stoichiometry on low expressed transcripts will be under-detected. For this reason, efforts should be directed toward maximizing sequencing depth to achieve the best detection. In our HeLa whole transcriptome experiment, we obtained greater than 200 million reads (100 million mate pairs) per replicate, for greater than 400 million reads (200 million mate pairs) per sample type. Additionally, high depth in a control untreated sample is important for evaluating the relevance of low mismatch rates.

Successful nucleotide conversion and completed reverse-transcription are critical for the success of our approach. Adoption of this protocol for another modification or condition that induces RT stops would fail to identify modified locations. We found no evidence of induction of RT-stops at ac4C locations in our data, via searching for "coverage cliffs" or biases in read offset positions.

TROUBLESHOOTING

Problem 1

Low C>T conversion rate in positive control (Related to $NaBH_4$).

Cause: Impure $NaBH_4$, old $NaBH_4$, $NaBH_4$ prepared at the wrong concentration.

Potential solution

Make sure to use newly prepared $NaBH_4$.

Problem 2

Low C>T conversion rate in positive control (Related to reverse transcription).

Cause: Improper reverse transcription conditions.

Potential solution

We have observed that elevating the temperature of reverse transcriptases increases C>T conversion upon $NaBH_4$ treatment. Thus, we recommend optimizing the reverse transcription temperature. This is particularly important when using a new reverse transcriptase. In addition, decreasing the concentration of GTP in the reaction increases the efficiency of C>T conversion.[2]

Problem 3

Low library yield.

Cause: Low starting RNA material, RNA degradation during ribodepletion, excessive RNA fragmentation during $NaBH_4$ treatment.

Potential solution

Check RNA integrity and concentration before you begin, after ribodepletion, and after $NaBH_4$. If excessive loss of RNA is observed at any step, use more starting material. NEB recommends using 1 ng–100 ng of ribodepleted RNA for library preparation. We used 10 ng of RNA.

Problem 4

Adapter contamination in final libraries.

Cause: Excessive concentration of adapter in the Adapter Ligation step or poor clean up.

Potential solution

Optimize the concentration of adapter. NEB recommends dilutions as low as 100× for low input procedures. If the DNA library yield is high but shows adapter contamination, perform another purification round using 0.9× AMPure beads.

Problem 5

Insufficient memory to process data.

Cause: Analyzing a whole transcriptomic dataset, including mismatch types that are not the expected nucleotides of interest, is valuable for troubleshooting. However, this may involve more data than can be processed in R, leading to memory errors or inability to execute code.

Potential solution

Data can be pre-filtered before loading into R, such as by minimum depth, mismatch type, or mismatch frequency. Moderate filtering will enable you to evaluate the data on this reduced dataset. The vector memory limit in R may also be increased, for example with the R_MAX_VSIZE command.

Problem 6

Lack of enrichment of C>T mismatches compared to G>A.

Cause: Misassignment of the strand for the mismatch.

Potential solution

Ensure that the transcript assignment section in the code workflow has been run. An effective way to diagnose potential alignment or strand issues is to visualize the alignments in the IGV genome browser. In the browser, reads can be color coded by strand, and mismatch rates presented in barplots (as in Figure 6B). Note that for mismatches to be highlighted in a barplot, you may need to adjust the allele frequency threshold for it to be visible, as this is commonly set to a high default value of 20%.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shalini Oberdoerffer (shalini.oberdoerffer@nih.gov).

Materials availability

This protocol did not generate new unique reagents.

Data and code availability

The accession number for the RedaC:T-seq data is GEO: GSE162043

Analysis code is provided at Github: https://github.com/dsturg/RedaCT-Seq and has been deposited at Zenodo: https://doi.org/10.5281/zenodo.7186739.

ACKNOWLEDGMENTS

This protocol utilized the Center for Cancer Research (CCR) Sequencing Facility for the National Cancer Institute (NCI, Frederick, MD) for Illumina sequencing services. This protocol used the Biowulf Linux cluster at the National Institutes of Health, Bethesda, MD (https://hpc.nih.gov) to analyze sequencing data. This work is supported by the Intramural Research Program of NIH, Center for Cancer Research, National Cancer Institute. D.A. was supported by the NCI K99/R00 grant R00CA245035.

AUTHOR CONTRIBUTIONS

Conceptualization, D.A., D.S., and S.O.; methodology, D.A., D.S., and S.O.; data analysis and curation, D.A. and D.S.; investigation and validation, D.A. and D.S.; writing – original draft, D.A. and D.S.; writing – review & editing, D.A., D.S., and S.O.; supervision, S.O.; funding acquisition, S.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Arango, D., Sturgill, D., Yang, R., Kanai, T., Bauer, P., Roy, J., Wang, Z., Hosogane, M., Schiffers, S., and Oberdoerffer, S. (2022). Direct epitranscriptomic regulation of mammalian translation initiation through N4-acetylcytidine. Mol. Cell *82*, 2797–2814.e11. https://doi.org/10.1016/j.molcel.2022.05.016.

2. Thomas, J.M., Briney, C.A., Nance, K.D., Lopez, J.E., Thorpe, A.L., Fox, S.D., Bortolin-Cavaille, M.L., Sas-Chen, A., Arango, D., Oberdoerffer, S., et al. (2018). A chemical signature for cytidine acetylation in RNA. J. Am. Chem. Soc. *140*, 12667–12670. https://doi.org/10.1021/jacs.8b06636.

3. Arango, D., Sturgill, D., Alhusaini, N., Dillman, A.A., Sweet, T.J., Hanson, G., Hosogane, M., Sinclair, W.R., Nanan, K.K., Mandler, M.D., et al. (2018). Acetylation of cytidine in mRNA promotes translation efficiency. Cell *175*, 1872–1886.e24. https://doi.org/10.1016/j.cell.2018.10.030.

4. Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet. J. *17*, 10–12. https://doi.org/10.14806/ej.17.1.200.

5. Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G., and Durbin, R.; 1000 Genome Project Data Processing Subgroup (2009). The sequence alignment/map format and SAMtools. Bioinformatics *25*, 2078–2079. https://doi.org/10.1093/bioinformatics/btp352.

6. Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., Batut, P., Chaisson, M., and Gingeras, T.R. (2013). STAR: ultrafast universal RNA-seq aligner. Bioinformatics *29*, 15–21. https://doi.org/10.1093/bioinformatics/bts635.

7. Akalin, A., Franke, V., Vlahoviček, K., Mason, C.E., and Schübeler, D. (2015). Genomation: a toolkit to summarize, annotate and visualize genomic intervals. Bioinformatics *31*, 1127–1129. https://doi.org/10.1093/bioinformatics/btu775.

8. Lawrence, M., Huber, W., Pagès, H., Aboyoun, P., Carlson, M., Gentleman, R., Morgan, M.T., and Carey, V.J. (2013). Software for computing and annotating genomic ranges. PLoS Comput. Biol. *9*, e1003118. https://doi.org/10.1371/journal.pcbi.1003118.

9. Lawrence, M., Gentleman, R., and Carey, V. (2009). rtracklayer: an R package for interfacing with genome browsers. Bioinformatics *25*, 1841–1842. https://doi.org/10.1093/bioinformatics/btp328.

10. Cerutti, P., Kondo, Y., Landis, W.R., and Witkop, B. (1968). Photoreduction of uridine and reduction of dihydrouridine with sodium borohydride. J. Am. Chem. Soc. *90*, 771–775. https://doi.org/10.1021/ja01005a039.

11. Enroth, C., Poulsen, L.D., Iversen, S., Kirpekar, F., Albrechtsen, A., and Vinther, J. (2019). Detection of internal N7-methylguanosine (m7G) RNA modifications by mutational profiling sequencing. Nucleic Acids Res. *47*, e126. https://doi.org/10.1093/nar/gkz736.

12. Macon, J.B., and Wolfenden, R. (1968). 1-Methyladenosine. Dimroth rearrangement and reversible reduction. Biochemistry *7*, 3453–3458. https://doi.org/10.1021/bi00850a021.

13. Sas-Chen, A., Thomas, J.M., Matzov, D., Taoka, M., Nance, K.D., Nir, R., Bryson, K.M., Shachar, R., Liman, G.L.S., Burkhart, B.W., et al. (2020). Dynamic RNA acetylation revealed by quantitative cross-evolutionary mapping. Nature *583*, 638–643. https://doi.org/10.1038/s41586-020-2418-2.