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 NaBH₄-induced reduction of ac4C and conversion to thymidine followed by sequencing (RedaC:T-seq). Total RNA is ribodepleted and then treated with NaBH₄ 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.
Protocol for base resolution mapping of ac4C using RedaC:T-seq

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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 NaBH4-induced reduction of ac4C and conversion to thymidine followed by sequencing (RedaC:T-seq). Total RNA is ribodepleted and then treated with NaBH4 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 (NaBH4) reduction of ac4C. NaBH4 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<sup>−/−</sup> 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<sup>6</sup> cells in 10-cm dishes in complete DMEM.
   c. Incubate at 37°C and 5% CO<sub>2</sub> 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<sub>2</sub>O.

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<sub>2</sub>O. 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<sub>2</sub>O. 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 RNeasy 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    |
| NaBH₄                                      | Sigma-Aldrich                  | Cat#: 452882   |
| Nuclease-free H₂O                          | 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#: E770S    |
| NEBNext® Multiplex Oligos for Illumina® (Index Primers Set 1 and Set 2) | New England Biolabs | Cat#: E7335S, 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.¹                | N/A            |
| **Software and algorithms**                |                                 |                |
| Cutadapt v.1.6                             | Martin⁷                        | RRID: SCR_011841|
| Samtools v.1.11                            | Li et al.⁸                     | 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             | 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 ES-2680v4) | N/A                            | N/A            |
| 8-core, 64 GB RAM, 2 TB storage, OSX or CentOS Linux 7 | N/A                            | N/A            |
**MATERIALS AND EQUIPMENT**

200 mM NaBH₄ solution

△ CRITICAL: Prepare NaBH₄ fresh immediately before use. NaBH₄ should be handled in the fume hood.

| Reagent          | Final concentration | Amount   |
|------------------|---------------------|----------|
| NaBH₄            | 200 mM              | 7.57 mg  |
| Nuclease-free H₂O| N/A                 | 1 mL     |
| **Total**        |                     | 1 mL     |

Dissolve 7.57 mg of NaBH₄ in 1 mL of nuclease-free H₂O.

**Storage:** Do not store. Prepare NaBH₄ fresh immediately before use. NaBH₄ 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 H₂O        | N/A                 | 98.8 mL|
| **Total**                |                     | 100 mL |

**Storage:** Buffer can be stored at 2°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 H₂O| N/A                 | 9 mL   |
| **Total**        |                     | 10 mL  |

**Storage:** Buffer can be stored at 2°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 H₂O. 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.
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: RNA 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 NaBH₄.
   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 NaBH₄. Mix tubes by finger-flicking.
   c. Incubate for 1 h at 55°C in the dark.

![Figure 2. Reduction of ac4C](image)

NaBH₄ reduces ac4C to tetrahydro-ac4C (top). Reduced ac4C less efficiently base pairs with guanosine and can interact with adenosine instead (bottom).
**STAR Protocols**

**Protocol**

**Agarose electrophoresis after NaBH₄**

| NaBH₄ | 37°C | 55°C |
|-------|------|------|
| 28S   | 0    | 0    |
| 18S   | 10   | 10   |
| Small RNAs | 50 | 50 |
|       | 100  | 100  |

**Bioanalyzer of NaBH₄-treated RNA**

(100 mM, 55 °C, 1 hr)

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**CRITICAL:** NaBH₄ 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:** NaBH₄ is an alkaline solution and induces RNA fragmentation to ~100 nt at 55 °C (Figure 3).

**Optional:** Reduction of ac4C with NaBH₄ 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 H₂O.
   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.
   g. **Pause point:** samples can be stored at −80 °C for 16–20 h.
   h. Centrifuge at 16,100 × g for 15 min at 4 °C in a refrigerated microcentrifuge.
   i. Discard the supernatant.
   j. Add 500 μL of 70% ethanol. Mix vigorously.
   k. Centrifuge at 16,100 × g for 5 min at 4 °C in a refrigerated microcentrifuge.
   l. Discard the supernatant.
      i. Remove all traces of alcohol.
   m. Air dry the pellet for 5 min at 22 °C–25 °C with the lid opened.
   n. Resuspend pellets in 6 μL of nuclease-free H₂O.

**Note:** To avoid further freeze and thaw of the NaBH₄-treated RNA, we recommend moving directly to the next step.

**Library preparation**

© Timing: 4 h
This step performs cDNA synthesis from NaBH₄-treated RNA, followed by adapter ligation and PCR amplification. We used the NEBNext Ultrall™ Directional RNA Library Prep Kit for library preparation.

8. First-strand cDNA synthesis.

**Note:** After ribodepletion, NaBH₄ 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).

| 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 |

a. To each sample of 5 µL NaBH₄-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 |
|----------------------------------|--------|
| 5X 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 NaBH₄ treatment. See the “troubleshooting” section for additional comments.

**Alternatives:** Several reverse transcriptases can be used, including TGIRT, Superscript III, and AMV. 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 NaBH₄-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 (10X) | 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 x) 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 x 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   |

   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.
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 \( \mu \)L 0.1TE buffer.
i. Incubate for 2 min at 22°C–25°C.
j. Without disturbing the bead pellet, transfer 21 \( \mu \)L 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 \( \mu \)L 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 NaBH4-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 \( \mu \)L of libraries, add the following components and mix by gentle pipetting:

| Reagent                                      | Amount |
|----------------------------------------------|--------|
| NEBNext Ultra II Q5 Master Mix               | 12.5 \( \mu \)L |
| 18S rRNA helix 45 Primer F (10 \( \mu \)M) – 5’ CGCTACTACCGATGGATGG 3’ | 2.5 \( \mu \)L |
| 18S rRNA helix 45 Primer R (10 \( \mu \)M) – 5’ TAATGATCCCTCCGAGGTTCACC 3’ | 2.5 \( \mu \)L |
| Library DNA                                  | 0.5 \( \mu \)L |
| Nuclease-free Water                          | 7 \( \mu \)L |
| Total volume                                 | 25 \( \mu \)L |
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).

   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 -0 1 \
   -quality-cutoff 6 -m 18 -a GATCGGAAGAGCACA -g ACGCTCTTCCGATCT \
   -A AGATCGGAAGAGC -G GTGCTCTTCCGATC -o Sample1_R1_cutadapt.fastq.gz \
   -p Sample1_R2_cutadapt.fastq.gz Sample1_R1_001.fastq.gz \
   Sample1_R2_001.fastq > 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).

   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 \n   –readFilesIn Sample1_R1_cutadapt.fastq.gz Sample1_R2_cutadapt.fastq.gz \
   –outFilterMultimapNmax 10 –clip3pNbases 6 –clip5pNbases 6 \n   –outFilterMultimapScoreRange 1 –outFileNamePrefix Sample1_genome.bam \
   ```
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](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) NaBH4 treated, KO (NAT10^-/-) NaBH4 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

```
taxmapped <- 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 NaBH₄ 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
```
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. 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 x coverage). In the HeLa transcriptome, we used this approach to estimate total ac4C:C at 0.016%.

**LIMITATIONS**

NaBH4 can react with other nucleobases, including 7-methylguanosine, dihydrouridine, 3-methylcytidine, and wybutosine, 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.

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 NaBH₄).

Cause: Impure NaBH₄, old NaBH₄, NaBH₄ prepared at the wrong concentration.

**Potential solution**
Make sure to use newly prepared NaBH₄.

**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 NaBH₄ 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.

**Problem 3**
Low library yield.

Cause: Low starting RNA material, RNA degradation during ribodepletion, excessive RNA fragmentation during NaBH₄ treatment.

**Potential solution**
Check RNA integrity and concentration before you begin, after ribodepletion, and after NaBH₄. 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 100x for low input procedures. If the DNA library yield is high but shows adapter contamination, perform another purification round using 0.9x 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.

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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.

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