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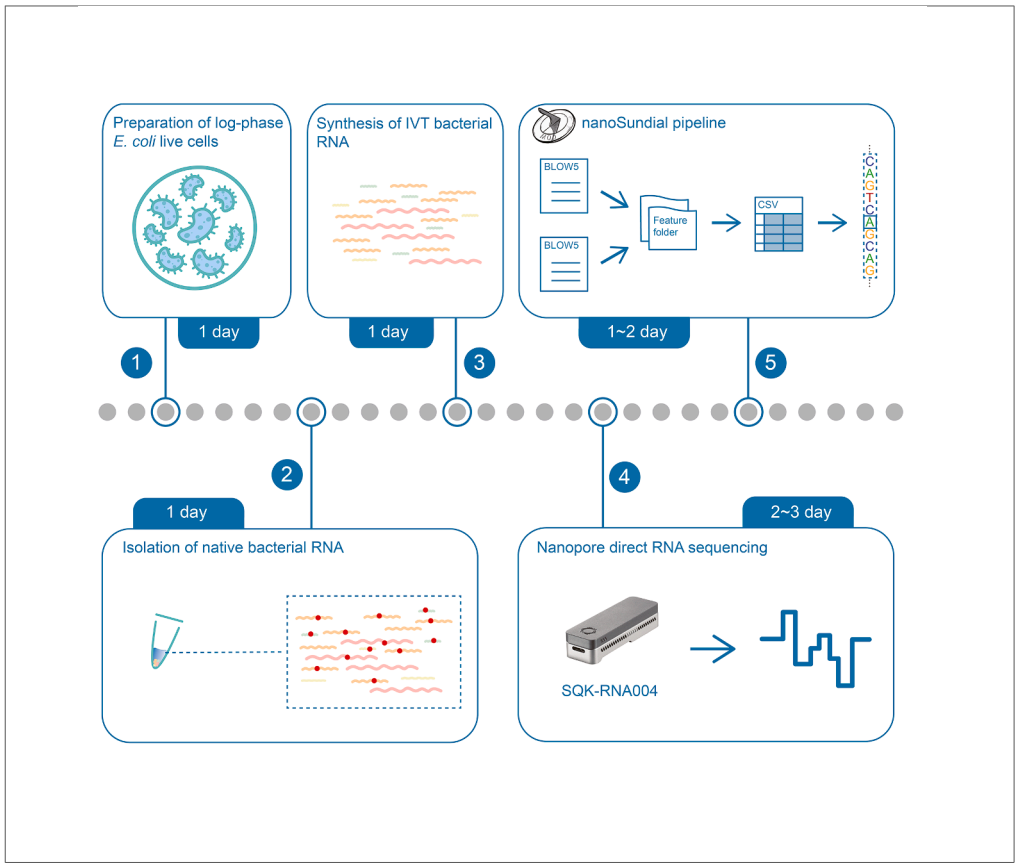
Protocol for generating native and unmodified bacterial RNA for nanopore direct RNA sequencing and downstream analysis



Nanopore direct RNA sequencing (DRS) relies on adapter ligation to poly(A) tails, posing challenges for bacterial mRNAs, which naturally lack these structures. Here, we present a protocol for generating native and unmodified bacterial RNA for nanopore DRS and downstream analysis. We describe steps for preparing log-phase *E. coli* live cells, isolating native bacterial RNA, and synthesizing *in vitro*-transcribed (IVT) bacterial RNA. We then detail procedures for nanosphere DRS and a downstream data analysis pipeline for RNA modification detection.

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

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Highlights

Protocol for nanopore direct RNA sequencing of bacterial RNA

Steps for bacterial RNA extraction and *in vitro* synthesis of unmodified RNA

Details on optimization of the protocol, limitations, and possible solutions

Guidance on data analysis of bacterial RNA modification detection

Guo et al., STAR Protocols 7, 104279
March 20, 2026
https://doi.org/10.1016/j.xpro.2025.104279

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Protocol for generating native and unmodified bacterial RNA for nanopore direct RNA sequencing and downstream analysis

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https://doi.org/10.1016/j.xpro.2025.104279

SUMMARY

Nanopore direct RNA sequencing (DRS) relies on adapter ligation to poly(A) tails, posing challenges for bacterial mRNAs, which naturally lack these structures. Here, we present a protocol for generating native and unmodified bacterial RNA for nanopore DRS and downstream analysis. We describe steps for preparing log-phase *E. coli* live cells, isolating native bacterial RNA, and synthesizing *in vitro*-transcribed (IVT) bacterial RNA. We then detail procedures for nanosphere DRS and a downstream data analysis pipeline for RNA modification detection.

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

BEFORE YOU BEGIN

Nanopore direct RNA sequencing (DRS) enables characterization of bacterial transcript structures and RNA modifications in their native forms. In particular, the modification landscape of bacterial mRNA remains largely unknown, and DRS is a promising tool to identify candidate sites for further validation. However, bacterial DRS typically yields limited mRNA data due to two major challenges. First, small fragments in bacterial RNA libraries form complex secondary structures that rapidly clog nanopores. To overcome this, we employ Solid Phase Reversible Immobilization (SPRI) bead-based size selection to displace short fragments, reducing pore blockage and stabilizing data generation. The second challenge is that bacterial mRNA represents only 2%–5% of total RNA, while the majority is ribosomal RNA (rRNA). Because bacterial mRNA lacks poly(A) tails, the standard nanopore DRS workflow cannot directly ligate sequencing adapters to mRNA molecules or distinguish mRNAs from rRNAs. An additional polyadenylation step is therefore required, preceded by rRNA depletion to enhance mRNA recovery. Although commercial rRNA depletion kits are available, they are often inefficient or prohibitively expensive. In this protocol, we outline the application of a cost-effective kit that achieves an mRNA ratio of ~50%.

This protocol details a complete workflow for extracting total RNA from Gram-negative bacteria, followed by size selection (ss) and ribosomal RNA depletion (rd), with technical considerations provided for each step (Figure 1A). In addition, to enable comparative detection of bacterial RNA

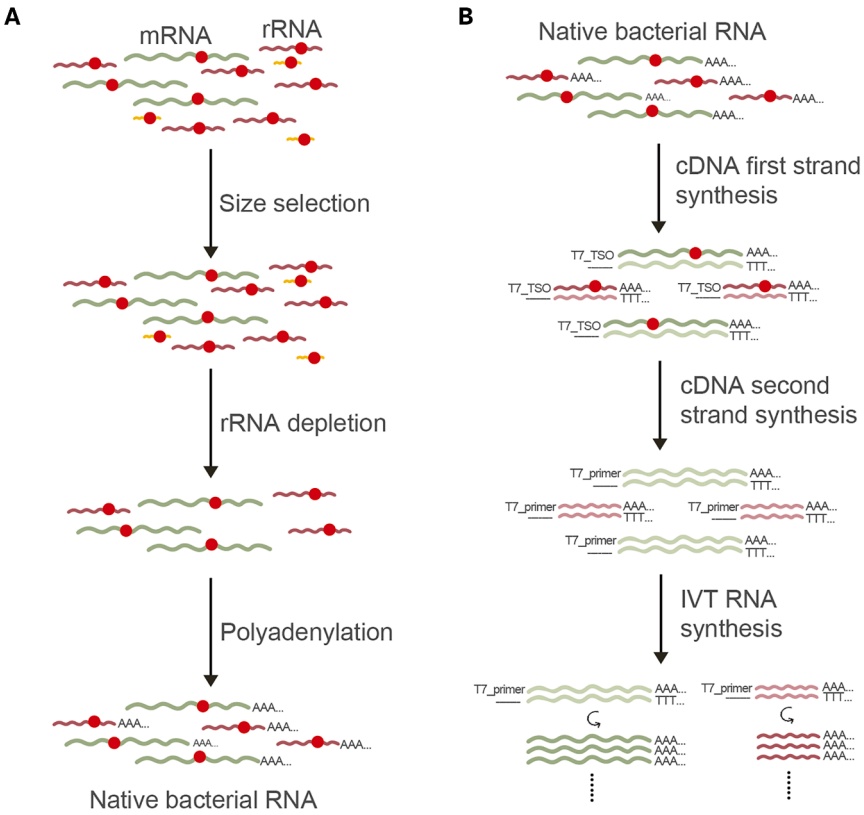

Figure 1. *E. coli* strain K-12 total RNA was isolated from cells grown to logarithmic phase

Prior to Oxford Nanopore Technologies (ONT) Direct RNA Sequencing (DRS) library preparation, (A) the total RNA underwent size selection followed by rRNA depletion and polyadenylation (ss&rd_RNA). mRNA, messenger RNA; rRNA, ribosomal RNA.

(B) The ss&rd_RNA was subsequently reverse-transcribed to synthesize first-strand cDNA, followed by second-strand synthesis to generate double-stranded cDNA. The resulting double-stranded cDNA served as the template for in vitro transcription (IVT) using T7 RNA polymerase.

modifications, our protocol also describes the preparation of unmodified RNA via in vitro transcription (Figure 1B), as well as the downstream pipeline for transcriptome analysis and modification detection. Although *E. coli* was used as the primary model in this protocol, we have successfully applied the workflow to several other Gram-negative bacterial species.

Innovation

Nanopore Direct RNA Sequencing (DRS) offers a robust platform for thorough analysis of RNA modifications. However, its application to bacterial RNA is hindered by low yields and insufficient mRNA reads, limiting transcriptome-wide modification mapping. Moreover, existing official tools for detecting bacterial RNA modifications suffer from high false-positive. Here, we demonstrate that an optimized protocol involving size selection, ribosomal RNA depletion, and polyadenylation markedly improves sequencing data throughput and significantly enriches mRNA read abundance. By using unmodified in vitro-transcribed (IVT) RNA libraries as a negative control, we employed the nano-Sunidal to compare raw current signal feature, achieving more precise detection of bacterial RNA modifications.

Revival of frozen *E. coli* cells

Timing: 15 min, plus overnight incubation

1. Prepare the LB broth 2.5% medium.
   a. Weigh 5 g LB broth and add to a 500 mL glass bottle.
   b. Add 200 mL Milli-Q water.
   c. Sterilize the solution using an autoclave and allow it to cool down to room temperature (18°C–22°C) before proceeding.
2. Revival of frozen *E. coli* cells (BSL1).
   a. Using a sterile inoculation loop, scrape a small amount of frozen stock and directly transfer ice fragments into 2 mL LB broth 2.5% medium in a 14 mL culture tube. The frozen stock tube does not need to be thawed.
   b. Cultivate the sample overnight at 37°C with agitation at 250 rpm.

Pause point: Cultures can be left shaking overnight (~12–16 h).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli strain K-12	American Type Culture Collection, VA, USA	BW25113
Chemicals, peptides, and recombinant proteins		
LB broth	hopebiol	HB0128
Invitrogen TRIzol reagent	Thermo Fisher Scientific	Cat#15596018
Maxima H Minus Reverse Transcriptase	Thermo Fisher Scientific	Cat#EP0752
Deoxynucleotide (dNTP) Solution Mix	New England Biolabs	NEB#N0447S
Thermostable RNase H	New England Biolabs	NEB#M0523S
Poly(A) polymerase	New England Biolabs	NEB#M0276
Q5 Hot Start High Fidelity Master Mix	New England Biolabs	NEB#M0494
RNaseOut	Thermo Fisher Scientific	Cat#10777019
SuperScript III Reverse Transcriptase	Thermo Fisher Scientific	Cat#18080044
Critical commercial assays		
RiboMinus Transcriptome Isolation Kit, bacteria	Thermo Fisher Scientific	K155004
MEGAscript Kit	Ambion, MA, USA	Ambion#AM1334
SQK-RNA004 Kit	Oxford Nanopore Technology, Oxford, UK	version DRS_9195_v4_revB_20Sep2023
Oligonucleotides		
Template switching oligo (TSO) T7 primer: 5′-ACTCTAATACGACTCACTATAGGGA GAGGGCrGrGrG-3′	BGI Genomics Co., Ltd.	N/A
T7 extension primer: 5′-GCTCTAATAC GACTCACTATAGG-3′	BGI Genomics Co., Ltd.	N/A
Software and algorithms		
nanoSundial v.0.0.1	GitHub	https://github.com/lrslab/nanoSundial
Other		
Agilent TapeStation System	Agilent Technologies	RNA ScreenTape 5067-5576
AMPure XP beads	Beckman Coulter, IN, USA	Product No: A63881
VAHTS RNA Clean Beads	Vazyme Biotech Co., Ltd.	N412-02
FLO-MIN004RA Flow Cell	Oxford Nanopore Technology, Oxford, UK	FLO-MIN004RA
SPRIselect Beads	Beckman Coulter, IN, USA	Product No: B23317

MATERIALS AND EQUIPMENT

LB broth 2.5% medium		
Reagent	Final concentration	Amount
LB broth	2.5%	5 g
Milli-Q water	N/A	200 mL
Unless otherwise specified, all steps at room temperature are performed at 18°C–22°C.		

STEP-BY-STEP METHOD DETAILS

Preparation of log-phase *E. coli* live cells

Timing: 1 day

In this part, we describe the procedure for isolating total RNA from E. coli live cells in the logarithmic growth phase, as outlined in steps 1–2.

1. Expansion and harvesting of *E. coli* cells for RNA extraction (20 min hands-on, plus ~1.5 h incubation).
   a. Inoculate overnight revival culture to fresh LB broth at a 1:100 dilution. For example, add 60 μL of the overnight (16–18 hours) suspension into two culture tubes containing 6 mL LB broth each.
   b. Grow at 37°C with shaking at 250 rpm until OD600 reaches ~0.4 (not exceeding 0.6). Measure OD600 using 3 mL aliquots to ensure accuracy. This typically requires ~1 h 30 min.

⚠ CRITICAL: Do not exceed OD600 of 0.6, as cells may have entered the stationary phase and affect RNA integrity.

Note: If OD600 exceeds 0.6, restarting the culture is recommended. Alternatively, additional TRIzol may be added during the lysis step, though the success rate is not guaranteed.

   c. Combine the expanded cultures (6 mL in total) into one 15 mL tube and centrifuge at 4°C, 6000 g for 5 min. Discard the supernatant.
   d. Resuspend the pellet thoroughly in 3 mL TRIzol reagent.
2. RNA extraction from *E. coli* cells (1 h hands-on, overnight incubation, plus ~1 h the following day).
   a. Incubate the TRIzol-treated cells at 65°C for 10 min, then cool to 4°C.

Note: Because bacteria possess a cell wall, direct RNA extraction using TRIzol alone is insufficient to achieve cell lysis. We found that this step not only disrupts the cell wall and releases RNA but also ensures good RNA quality.

   b. Add chloroform (1/5 volume of TRIzol used), vortex for 1 min, incubate on ice for 5 min, and centrifuge at 25,830 g for 10 min. Repeat once.

⚠ CRITICAL: Ensure complete phase separation; avoid disturbing the interphase when collecting the aqueous layer.

Note: After the first chloroform extraction, it is recommended to remove as much of the aqueous phase as possible, even if the interphase is slightly disturbed. The second extraction will yield a much clearer separation, at which point the interphase must not be disturbed.

   c. Insert an equal volume of isopropanol to the aqueous phase. Incubate at −20°C overnight, then centrifuge at 20,817 g for 15 min.

Pause point: Samples can be kept at −20°C overnight before centrifugation.

Note: In this step, using 1.5 mL tubes is recommended.

   d. Wash the RNA pellet once with 400 μL 70% ethanol.
      i. Centrifuge at 20,817 g for 5 min.

ii. Carefully remove the ethanol to avoid aspirating the precipitate.

Note: If there is still liquid, spin down and use a 10 μL pipette to displace the remaining liquid.

iii. Open the lid and evaporate for 1 minute.

e. Dissolve the pellet in 60 μL nuclease-free water.

f. Perform quality control:

i. Measure 1 μL of total RNA to determine concentration and purity using a NanoDrop spectrophotometer.

Note: A260/A280 ratio should be ≥ 2.0; in this step, A260/A230 ratio may be slightly lower than 2 but is acceptable (Table S1).

ii. Assess RNA integrity using a TapeStation or an equivalent platform.

Note: RIN values should be ≥ 8 (Figures 2A and 2B).

Note: More than 30 μg of total RNA can be obtained from cells cultured in 2 mL of LB broth.

Preprocessing of native bacterial RNA for nanopore DRS

Timing: 1 day

In this part, we describe the process of size selection (step 3), ribosomal RNA depletion (step 4), and poly(A) tailing (step 5) of total RNA to obtain native RNA suitable for DRS library preparation.

3. Size selection to remove small RNA fragments using SPRIselect beads or AMPure XP beads.
   a. If using AMPure XP beads, remove the AMPure XP beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. SPRIselect beads are normally stored at room temperature. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.
   b. Dilute 25 μg of total RNA to a final concentration of 200 ng/μL.
   c. Add SPRIselect beads or AMPure XP beads (0.8 volume of RNA solution) to RNA. Flick the tube to ensure thorough mixing. Incubate for 8 mins at room temperature.
   d. Briefly centrifuge the sample and place it on a magnetic stand to pellet. Allow the tube to remain on the magnet for 3 minutes, then carefully remove the supernatant once it appears clear and colorless.
   e. Wash with 200 μL 85% ethanol twice.

⚠ CRITICAL: Do not disturb the beads during the wash steps.

   f. Briefly centrifuge the sample and return the tube to the magnetic stand until the eluate becomes clear and colorless. While keeping the tube on the magnet, carefully remove any residual ethanol by pipetting.

⚠ CRITICAL: Ensure that any residual ethanol is completely removed after washing.

   g. Air dry with lid open for 1 min.

⚠ CRITICAL: Do not overdry the beads to the point of cracking.

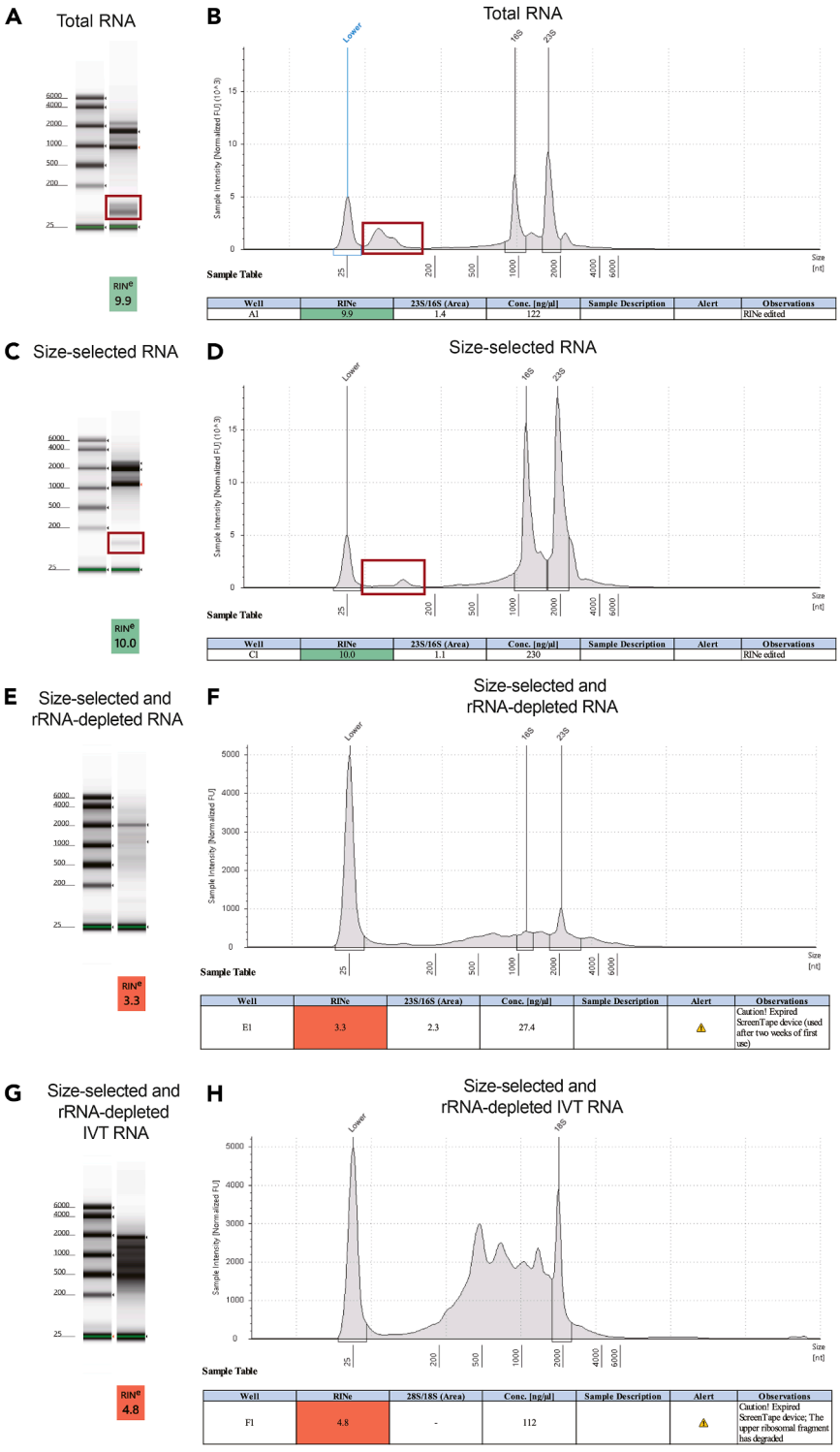

Figure 2. The TapeStation gel plots of the K12 RNA samples

(A and B) are total RNA, (C and D) are size selection RNA, (E and F) are size selection and ribosome depletion RNA (ss&rd_RNA), and (G and H) are IVT RNA. IVT RNA was generated based on ss&rd_RNA.

h. Take the tube off the magnetic stand and dissolve the pellet in 20 μL of nuclease-free water. Allow the sample to stand at ambient temperature for 10 minutes.
i. Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.
j. Perform quality control:
   i. Measure 1 μL of size-selected RNA (ss_RNA) to determine concentration and purity using a NanoDrop spectrophotometer.

Note: A260/A280 ratio should be ≥2.0; A260/A230 ratio should be around 2.0 (Table S1).

   ii. Assess RNA length distribution using a TapeStation or an equivalent platform.

Note: RNA whose length is less than 200 bp should be removed in ss_RNA (Figures 2C and 2D).

4. Perform the ribosome depletion following the instructions of RiboMinus Transcriptome Isolation Kit (manual: https://documents.thermofisher.com/TFS-Assets/LSG/manuals/ribominus_yeast_bacteria_man.pdf).

Note: Resuspend the RiboMinus Magnetic Beads, RiboMinus Probe (100 pmol/uL), Hybridization Buffer (B10) and nuclease-free water were provided by the kit.

a. Resuspend the RiboMinus Magnetic Beads in the bottle by thorough vortexing. Pipette 250 μL of the bead suspension into a 1.5-mL tube.
b. Position the tube containing the bead suspension on a magnetic stand for 1 minute, then carefully aspirate and discard the supernatant.
c. Remove the tube from the magnetic stand. Add 250 μL nuclease-free water to the tube and resuspend the beads. Position the tube on a magnetic stand for 1 minute, then carefully aspirate and discard the supernatant. Repeat this step once.
d. Resuspend beads in 250 μL Hybridization Buffer (B10). Position the tube on a magnetic stand for 1 minute, then carefully aspirate and discard the supernatant.
e. Resuspend beads in 100 μL Hybridization Buffer (B10) and store them at ambient temperature until needed.
f. Add the following reagents to a new 1.5 mL tube:

Component	Volume
Total RNA (20 μg)	<20 μL
RiboMinus Probe (100 pmol/uL)	4 μL
Hybridization Buffer (B10)	100 μL
Total volume	124 μL

Note: Although the official protocol of this kit specifies an input RNA of 10 μg, we have tested up to 20 μg without issues; however, do not exceed 20 μg.

g. Incubate the tube at 37°C for 5 minutes to denature RNA. Immediately place the sample on ice for at least 30 seconds.
h. Transfer ~124 μL of the cooled hybridized sample to the prepared RiboMinus Magnetic beads, and mix well by vortex. Incubate the tube at 37°C for 15 minutes. Gently mix the tube occasionally.

⚠ CRITICAL: It is essential to gently mix the contents every 3 min to greatly improve the efficiency of rRNA removal.

i. Place the tube on a magnetic stand for 1 minute to pellet the rRNA-probe complex. The supernatant contains the rRNA-depleted RNA fraction.
j. Transfer the supernatant (~224 μL) to a new tube.
k. Concentrate and purify using Vazyme RNA clean beads.
    i. Remove the VAHTS RNA Clean Beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.
    ii. Add equal volume of VAHTS RNA Clean Beads to the collected supernatant. Pipette up and down or flick the tube to ensure thorough mixing.
    iii. Incubate at room temperature for 8 minutes to allow RNA to bind to the beads.
    iv. Place the sample on a magnetic stand for 5 minutes. Once the solution becomes clear, carefully remove the supernatant.
    v. Keep the sample on the magnetic stand and add 200 μL freshly prepared 80% ethanol. Do not disturb the beads; gently rotate the tube 180° to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
    vi. Repeat the previous wash step once more (for a total of two washes).
    vii. Spin down the sample briefly and return the tube to the magnetic stand until the eluate becomes clear and colorless. Maintain the tubes on the magnetic stand and carefully pipette off any residual ethanol.
    viii. Air-dry the beads with the lid open for 1 min.
    ix. Take the tube off the magnetic stand and dissolve the pellet in 17 μL of nuclease-free water. Incubate the sample at room temperature for 10 min.
    x. Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.
l. Perform quality control:
    i. Measure 1 μL of size-selected and ribosome-depleted RNA (ss&rd_RNA) to determine concentration and purity using a NanoDrop spectrophotometer.

Note: If rRNA has been effectively depleted, the total amount of ss&rd_RNA should be approximately 600 ng and preferably not exceed 1 μg. The A260/A280 ratio should be ≥ 2.0, and the A260/A230 ratio may be markedly reduced (around 0.1) at this stage (Table S1). This step may not completely remove the salts or organic contaminants in the reaction mixture. However, the ratio generally returns to acceptable levels (>2.0) after the poly(A) tailing step, well before initiating library construction.

    ii. Use 1 μL of ss&rd_RNA to assess length distribution using a TapeStation or an equivalent platform.

Note: Compared with ss_RNA, most rRNA should be removed, as evidenced by a marked reduction of the 16S and 23S peaks (Figures 2E and 2F).

5. Poly(A) tailing using poly(A) polymerase.
    a. Use 15 μL ss&rd_RNA and prepare the following system.

Component	Volume
ss&rd_RNA	15 μL
10X *E. coli* Poly(A) Polymerase Reaction Buffer	2 μL
ATP (10 mM)	2 μL
RNaseOUT	0.5 μL
E. coli Poly(A) Polymerase	1 uL
Total volume	20.5 uL

    b. Incubate the reaction at 37°C for 20 minutes.

c. Cleanup using RNA clean beads.
   i. Remove the VAHTS RNA Clean Beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.
   ii. Add 36 μL VAHTS RNA Clean Beads (bead volume = 1.8 × the original RNA solution volume). Pipette up and down or flick the tube to ensure thorough mixing.
   iii. Incubate at room temperature for 8 minutes to allow RNA to bind to the beads.
   iv. Place the sample on a magnetic stand for 5 minutes. Once the solution becomes clear, carefully remove the supernatant.
   v. Keep the sample on the magnetic stand and add 200 μL freshly prepared 80% ethanol. Do not disturb the beads; gently rotate the tube 180° to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
   vi. Repeat the previous wash step once more (for a total of two washes).
   vii. Spin down the sample briefly and return the tube to the magnetic stand until the eluate becomes clear and colorless. Retain the tubes on the magnetic stand and carefully remove any residual ethanol by pipetting.
   viii. Air-dry the beads with the lid open for 1 min.
   ix. Take the tube off the magnetic stand and dissolve the pellet in 17 μL of nuclease-free water. Incubate the sample at room temperature for 10 min.
   x. Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.
d. Perform quality control: Measure 1 μL of RNA to determine concentration and purity using a NanoDrop spectrophotometer.

Note: The concentration is expected to be higher than that of ss&rd_RNA; the A260/A280 ratio should be $\geq$ 2.0, and the A260/A230 ratio should recover to a higher value above 2.0 compared to that of ss&rd_RNA (Table S1).

Note: To reduce costs, the TapeStation analysis of RNA length distribution could be omitted at this step, as it typically shows minimal differences compared to ss&rd_RNA.

Note: At this stage, the native bacterial RNA (ss&rd_RNA with poly(A) tail) is ready for DRS library preparation. Approximately 300 ng will be used for this purpose. The remaining RNA will be used for subsequent *in vitro* transcription (IVT) of bacterial RNA.

⚠ CRITICAL: For DRS, the prepared native RNA must be used for library construction on the same day. The ss&rd_RNA can be kept on ice temporarily.

Synthesis of IVT bacterial RNA for nanopore DRS

Timing: 1 day

In this part, we describe how to generate IVT bacterial RNA based on ss&rd_RNA. Briefly, ss&rd_RNA is first reverse-transcribed to synthesize the first cDNA strand (step 6), followed by second-strand synthesis (step 7), and finally in vitro transcription to obtain IVT bacterial RNA (step 8).

6. cDNA synthesis and strand switching.
   a. Prepare the primer annealing reaction on ice. Mix thoroughly by pipetting.

Reagent	Volume
Bacteria RNA (size selected, rRNA depleted and poly(A) added)	8.6 μL (300 ng)
Oligo(dT)23VN (10 μM)	1 μL
dNTP (10 mM)	1 μL
Total volume	11.6 μL

b. Incubate the reaction mix in a thermocycler with the following steps, with the lid temperature set at ≥ 85°C, then hold at 4°C until next step.

Temperature	Time (minutes)
72°C	3
4°C	10
25°C	1
4°C	Hold

c. Prepare the reverse transcription (RT) reaction mix. Mix thoroughly by pipetting.

Reagent	Volume
Nuclease-free water	3 µL
5 x RT Buffer	4.4 µL
RNaseOUT	1 µL
T7 TSO (75 µM)	1 µL
Maxima H Minus Reverse Transcriptase	1 µL
Total Volume	10.4 µl

Note: T7 TSO sequence: 5′-ACTCTAATACGACTCACTATAGGGAGAGGGCrGrGrG-3′.

d. Combine 10.4 µL RT reaction mix with 11.6 µL annealed mix, mix well by pipetting. Incubate the combined reaction mix in a thermocycler following the SSIV RT protocol.[2]

Temperature (°C)	Time	Cycles
50	10 min	1
55	30 sec	10
50	30 sec	
60	30 sec	5
55	30 sec	
50	30 sec	1
65	30 sec	5
60	30 sec	
50	30 sec	1
70	30 sec	5
65	30 sec	
50	30 sec	1
75	30 sec	5
70	30 sec	
50	1 min	1
80	10 min	1
4	Hold	1

Pause point: If not proceeding to the second-strand cDNA synthesis step immediately, samples can be stored at 4°C overnight or at −20°C for up to 1 week.

7. Second-strand cDNA synthesis.
   a. Assemble the following reaction mix to hydrolyze the RNA template.

Component	Volume
Template switching cDNA product (from the last step)	10 µL
RNase H Reaction Buffer (10X)	10 µL
Thermostable RNase H	1 µL
Nuclease-free H_2O	79 µL
Total volume	100 uL

b. Incubate at 50°C for 20 minutes.
c. Purify the template switching cDNA product using VAHTS RNA Clean Beads.
    i. Remove the VAHTS RNA Clean Beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.
    ii. Add 180 μl VAHTS RNA Clean Beads (bead volume = 1.8 × the original RNA solution volume). Pipette up and down or flick the tube to ensure thorough mixing.
    iii. Incubate at room temperature for 8 minutes to allow RNA to bind to the beads.
    iv. Place the sample on a magnetic stand for 5 minutes. Once the solution becomes clear, carefully remove the supernatant.
    v. Keep the sample on the magnetic stand and add 200 μL freshly prepared 80% ethanol. Do not disturb the beads; gently rotate the tube 180° to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
    vi. Repeat the previous wash step once more (for a total of two washes).
    vii. Spin down the sample briefly and place the tube back on the magnet until the eluate is clear and colorless. Keep the tubes on the magnet and pipette off any residual ethanol.
    viii. Air-dry the beads with the lid open for 1 min.
    ix. Remove the tube from the magnetic rack and resuspend the pellet in 17 μL nuclease-free water. Incubate the sample at room temperature for 10 min.
    x. Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.
d. Use 1 μL to measure the first-strand cDNA concentration using a NanoDrop spectrophotometer.

Note: The total amount should approximate the initial RNA input, although minor losses are expected and acceptable.

e. Assemble the second strand cDNA synthesis reaction mix on ice. Mix gently.

Component	Volume
Purified template switching cDNA product (from the last step)	20 μL
Q5 Hot Start High Fidelity Master Mix	25 μL
T7 primer (50 μM)	3.75 μL
H_2O	1.25 μL
Total Volume	50 μL

Note: T7 primer sequence: 5′-ACTCTAATACGACTCACTATAGGGA-3′

f. Incubate in a thermocycler, with lid temperature set at ≥ 100°C.

Step	Temperature	Time (minutes)
Initial denaturation	95°C	1
Primer extension	65°C	10
Hold	4°C	∞

Pause point: If not proceeding to the next step immediately, samples can be stored at 4°C overnight or at −20°C for up to 1 week.

g. Purify the double-stranded cDNA product using AMPure XP beads.
    i. Remove the AMPure XP beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.
    ii. Add 50 μL AMPure XP beads (1 volume of cDNA solution) to RNA. Flick the tube to ensure thorough mixing. Incubate for 10 mins at room temperature.

iii .Spin down the sample briefly and pellet on a magnet stand. Keep the tube on the magnet for 3 min and pipette off the supernatant when clear and colourless.

iv. Wash with 200 μL 85% ethanol twice.

⚠ CRITICAL: Do not disturb the beads during the wash steps.

v. Spin down the sample briefly and place the tube back on the magnet until the eluate is clear and colourless. Keep the tubes on the magnet and pipette off any residual ethanol.

⚠ CRITICAL: Ensure that any residual ethanol is completely removed after washing.

vi. Air dry with lid open for 1 min.

⚠ CRITICAL: Do not overdry the beads to the point of cracking.

vii. Remove the tube from the magnetic rack and resuspend the pellet in 11 μL nuclease-free water. Incubate the sample at room temperature for 10 min.

viii Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.

h. Use 1 μL to measure the double-stranded cDNA concentration using a NanoDrop spectrophotometer.

Note: Theoretically, double-stranded cDNA contains twice the total nucleotide content of single-stranded cDNA, since each molecule gains a complementary strand. However, when measured by Nanodrop, we observed that the concentration may not reflect a full two-fold increase. We proposed that this discrepancy may arise from the hypochromic effect of base stacking in double-stranded DNA, incomplete synthesis efficiency, and purification losses, resulting in yields that are only slightly higher than, or sometimes even lower than, those of single-stranded cDNA.

i. Use around 100 ng double-stranded cDNA for *in vitro* transcription. The remaining cDNA could be stored in the −20°C or −80°C refrigerator.

8. *In vitro* transcription.

a. Assemble the transcription reaction at room temperature using MEGAscript Kit. Mix gently by pipetting.

Component	Volume
ATP/CTP/GTP/UTP	2 μL each
10X Reaction Buffer	2 μL
Purified double-stranded cDNA	8 μL (100 ng)
Enzyme Mix	2 μL
Total volume	20 uL

b. Incubate at 37°C for 4 hr.

c. Add 1 μL of TURBO DNase to the transcription reaction mix and mix well.

d. Incubate at 37°C for 15 min.

e. Purify IVT RNA using VAHTS RNA Clean Beads.

i. Remove the VAHTS RNA Clean Beads from 2°C–8°C and equilibrate them to room temperature for 30 minutes. Mix thoroughly by gentle inversion or vortex to fully resuspend the beads.

ii. Add 36 μL VAHTS RNA Clean Beads (bead volume = 1.8 × the original RNA solution volume). Pipette up and down or flick the tube to ensure thorough mixing.

iii. Incubate at room temperature for 8 minutes to allow RNA to bind to the beads.
iv. Place the sample on a magnetic stand for 5 minutes. Once the solution becomes clear, carefully remove the supernatant.
v. Keep the sample on the magnetic stand and add 200 μL freshly prepared 80% ethanol. Do not disturb the beads; gently rotate the tube 180° to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
vi. Repeat the previous wash step once more (for a total of two washes).
vii. Spin down the sample briefly and return the tube to the magnetic stand and allow it to sit until the eluate is clear and colorless. While keeping the tube on the magnet, carefully pipette off any residual ethanol.
viii. Air-dry the beads with the lid open for 1 min.
ix. Take the tube off the magnetic stand and dissolve the pellet in 17 μL of nuclease-free water. Allow the sample to stand at ambient temperature for 10 minutes.
x. Carefully transfer the supernatant to a new nuclease-free low-binding microcentrifuge tube.

f. Measure 1 μL of IVT RNA to determine concentration using a NanoDrop spectrophotometer.

Note: The total amount should be around 20 μg; the A260/A280 ratio should be ≥ 2.0 (Table S1). Check the RNA length distribution with TapeStation; the gel plot should show a similar distribution compared to ss&rd_RNA (Figures 2G and 2H).

g. Take 300–500 ng IVT RNA to prepare the nanopore DRS library. The remaining RNA could be stored in the −80°C refrigerator.

Nanopore DRS

Timing: 2–3 days

In this section, we briefly describe the technical details of DRS in our experiment. For comprehensive instructions, please refer to the official manual available on the Oxford Nanopore Technologies website.

9. The nanopore DRS libraries were prepared in accordance with Oxford Nanopore's official RNA004 protocol, using 300–500 ng of native bacterial RNA and IVT RNA, respectively, as input.

Note: When using the same batch of native RNA to generate IVT RNA and perform sequencing, special attention to timing is required. Because the native bacterial RNA was prepared earlier than the IVT RNA and must be used for library construction and sequencing on the same day, time management is essential. After synthesizing the first-strand cDNA, the remaining steps may be performed on the following day.

10. For the RT step, SuperScript III Reverse Transcriptase was used in the previous version of this protocol, which consistently yielded high library preparation efficiencies. The newer enzyme specified in the updated protocol has not yet been tested.
11. Upon completion of library construction, RNA library concentrations were measured using a NanoDrop spectrophotometer. Although substantial sample loss may occur during this process, sequencing can proceed as long as a measurable RNA concentration is detected.

Installation of the nanoSundial

Timing: 30 min

We developed nanoSundial,[1] an innovative method that utilizes raw current signals from native sample and negative control sample to identify RNA modifications. The pipeline is available as a Python script workflow, compatible with Linux systems (developed and tested on Linux, Ubuntu 24.04.2 LTS).

> ***Note:*** nanoSundial is a comparative method requiring two samples for analysis. We recommend using a wild-type sample(ss&rd_RNA) and a negative control. IVT samples serve as the negative control in this protocol. Alternatively, knockdown or knockout samples are also viable options.

In this part, we detail the installation of a virtual environment for nanoSundial and provide the necessary download commands. nanoSundial offers two installation environments, Conda and Docker, allowing users to choose based on their familiarity and preference. Both environments contain the required software listed below.

Software	Version
SAMtools[3]	v1.16
Minimap2[4]	v2.17
f5c[5]	v1.4
slow5tools[6]	v1.3.0
Python	>=3.9, <=3.11

12. Installation with Conda.
    a. Install the Conda. For comprehensive instructions on installing Conda, refer to the official documentation. (https://docs.conda.io/projects/conda/en/latest/user-guide/install/index.html).
    b. Create a new conda environment named nanosundial_env.

```
>conda create -n nanosundial_env
```

    c. Activate the Environment.

```
>conda activate nanosundial_env
```

    d. Install requirements.

```
> conda install samtools=1.16 minimap2 f5c=1.4 slow5tools python=3.9 git -c conda-forge -c bioconda

> git clone https://github.com/lrslab/nanoSundial

> cd nanoSundial/

> pip install -r requirement.txt
```

13. Installation with Docker.
    The docker documentation page is (https://docs.docker.com/desktop/setup/install/linux/ubuntu/).
    a. If you choose Docker, first pull the docker image from Docker Hub to the local system.

```
>docker pull zhihaguo/nanocem_env
```

    b. Run the Docker container. Start a container named nanosundial_env from the pulled image in detached mode with an interactive terminal.

```
>docker run --name nanosundial_env -idt zhihaguo/nanocem_env
```

    c. Access the Container.

```
>docker exec -it nanosundial_env /bin/bash
```

d. Download nanoSundial from its GitHub repository.

```
>git clone https://github.com/lrslab/nanoSundial

>cd nanoSundial/
```

Note: You only need to choose either Conda or Docker to complete the installation. If you are well-versed in bioinformatics tools, you can directly install nanoSundial in your environment. It has been thoroughly tested and is compatible with Python versions ≥3.9 and ≤3.11.

Data preparation for nanoSundial

Timing: 1 day, 25 min/10 GB raw sequencing data

This section outlines the data preparation requirements for the nanoSundial pipeline, including raw data formats, basecalling, and reference sequence selection.

14. Raw Sequencing Data: BLOW5 Files[6]

Nanopore sequencing generates raw current data in multiple storage formats, such as BLOW5, FAST5, and POD5. The nanoSundial pipeline exclusively supports BLOW5 files. To convert other formats (e.g., FAST5 or POD5) to BLOW5, follow the detailed instructions provided in the data preparation guide (https://nanocem.readthedocs.io/en/latest/preparation/).

15. Basecalled FASTQ Files.

Basecalled fastq files are required for the nanoSundial pipeline. We recommend using Dorado, a high-performance basecaller developed by Oxford Nanopore Technologies (ONT), for converting raw nanopore data into FASTQ format. The current latest model version is 5.2.0. Below is an example of a dorado basecalling command and converting the results to a FASTQ file via SAMtools[3]:

```
>dorado basecaller rna004_130bps_sup@v5.2.0 <raw sequence data> -r > basecall.bam

>samtools bam2fq basecall.bam>basecall.fastq
```

Note: ONT frequently updates Dorado and its basecalling models to improve accuracy and performance. To access the latest version of Dorado and the most current models, consult the official document (https://github.com/nanoporetech/dorado).

16. Reference Sequence: FASTA file.

Note: For the nanoSundial pipeline, a reference sequence in FASTA format is required for alignment, with the choice of reference depending on the organism and analysis type. For prokaryotic analysis, a reference genome is recommended, while for eukaryotic analysis, a reference transcriptome is advised.

Note: To obtain a reference sequence for the nanoSundial pipeline, you can download sequences for various species from the NCBI database in FASTA format. Alternatively, for DNA sequencing projects, a reference sequence can be generated through de novo assembly using suitable assembly tools.

Note: Since raw sequencing data files can be very large (exceeding 100 GB) and involve significant disk read/write operations, nanoSundial recommends running on an SSD for optimal performance.

Upon completion of preparation, the data directory structure should closely align with the following sample:

```
sample/
└── wt.blow5 # Raw signal data, BLOW5 file
└── wt.fastq # Basecalled reads from basecaller, FASTQ file
└── ivt.blow5 # Raw signal data, BLOW5 file
└── ivt.fastq # Basecalled reads from basecaller, FASTQ file
└── reference.fasta # Reference sequence, FASTA file
```

nanoSundial workflow

Timing: 1 day, 30 min/10 GB raw sequencing data

We provide 3 functional python scripts: extract_feature_block.py, sundial_comp.py, and merge_positive_region.py. For the feature extraction part, it should be executed separately for each sample, with consistent parameters applied across both runs (Figure 3). For detailed usage instructions, refer to the nanoSundial documentation at https://github.com/lrslab/nanoSundial.

17. Feature extraction.

We have offered a python script for feature extraction named extract_feature_block.py. nanoSundial performs signal alignment by leveraging the f5c eventalign module, enabling the alignment of raw nanopore signals (electric current measurements) to a reference genomic or transcriptomic sequence. Run it separately for the wild-type (ss&rd_RNA) and negative control (IVT) samples, outputting the results to distinct directories.

```
>python extract_feature_block.py --fastq wt.fastq --blow5 wt.blow5 --ref reference.fasta
--output WT_feature --rna --pore rna004 --cpu 64

>python extract_feature_block.py --fastq ivt.fastq --blow5 ivt.blow5 --ref reference.fasta
--output IVT_feature --rna --pore rna004 --cpu 64
```

Note: Since feature extraction and storage are not compressed, 100 GB of sequencing data may result in a 120 GB feature table. Please ensure the hard drive has sufficient space.

Note: During the signal mapping refinement process in f5c eventalign, there is approximately a 5% failure rate, which may result from issues such as unsuccessful signal mapping or problems with data conversion.

18. Statistical analysis.

Following feature extraction from the two samples, the sundial_comp.py script is used to compare them. This protocol supports three algorithms, which include Multivariate Analysis of Variance (MANOVA), Logistic Regression (LR), and the Kolmogorov-Smirnov test (KS), with MANOVA designated as the default. The script outputs all positions meeting the coverage threshold (default: 50x).

```
>python sundial_comp.py --input WT_feature/ --control IVT_feature/ --cpu 64 --output nanosun-
dial_result.csv --balance --method manova --minimum_coverage 50
```

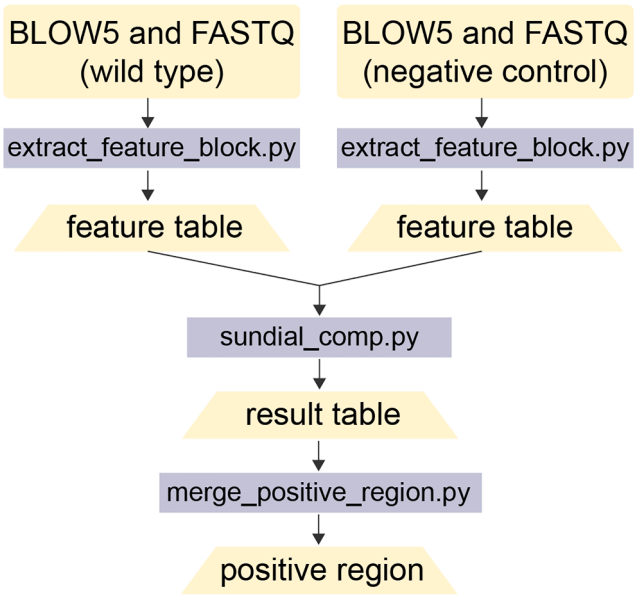

Figure 3. Workflow of the nanoSundial script

The native sample (ss&rd_RNA) and negative control (IVT) are processed separately using extract_feature_block.py. The resulting feature files are then used as input for sundial_comp.py to generate a result table. Finally, merge_positive_region.py is applied to extract high-confidence positive regions.

Note: Handling large datasets with pandas can require substantial memory. Despite our optimization efforts, for sequencing data exceeding 80 GB, we recommend executing the sundial_comp.py script on a server with at least 200 GB of memory to ensure optimal performance.

Note: To ensure statistical results are minimally affected by imbalanced data samples, we provide the –balance option to subsample larger datasets. Additionally, if the coverage is below 50, the minimum_coverage parameter can be adjusted to lower this threshold.

19. Merging strategy.

Following the application of the recommended thresholds (|mean difference|: 0.18, |dwell time difference|: 1, -log10(adjusted p-value): 3), all eligible sites located within a distance less than shift_size(default: 4) are combined into a single region using the merge_positive_region.py script.

```
>python merge_positive_region.py --input nanosundial_result.csv --output positive.bed
--mean_cutoff 0.18 --dwell_cutoff 1 --pvalue_cutoff 3
```

EXPECTED OUTCOMES

The sundial_comp.py script generates a CSV file containing the current differences for all points (Table S2), allowing visualization of the differences at each position. The merge_positive_region.py script produces a BED file that records the highest-confidence positive regions.

LIMITATIONS

The sundial_comp.py script relies on pandas for data processing, which has limitations when handling large datasets exceeding 10 GB, leading to significant time and memory consumption.

Additionally, as nanoSundial is designed primarily to detect high-level modifications, its ability to identify low-level modifications is limited.

TROUBLESHOOTING

Problem 1

Severe RNA degradation was observed when extracting RNA using TRIzol in the step 2.

Potential solution

To minimize RNA degradation, it is advisable to avoid collecting bacterial cells in 50 mL tubes and then adding TRIzol. The use of large tubes may lead to difficulty in mixing the cells well in TRIzol, thereby exacerbating RNA degradation. Instead, using 15 mL tubes can improve the RNA extraction and reduce degradation.

Problem 2

Failed to convert to BLOW5 data format in the step 14.

Potential solution

- The fast5 file is compressed with VBZ but the required plugin is not loaded. Please read the instructions here: https://hasindu2008.github.io/slow5tools/faq.html.
- Convert single format FAST5 to multi format using ont-fast5-api: https://github.com/nanoporetech/ont_fast5_api.
- blue-crab[7] only supports converting POD5 files with the same group ID into a single BLOW5 file. To merge multiple group IDs, first convert them to a BLOW5 folder format and then combine them into a single BLOW5 file like below.

```
>blue-crab p2s <POD5 folder> --out-dir <BLOW5 folder>

>slow5tools merge < BLOW5 folder> -o < BLOW5 file >
```

Problem 3

The current feature file is empty in the step 17.

Potential solution

- Verify whether the intermediate BAM file contains reads aligned to the reference genome.
- The intermediate temporary files encountered issues during previous generation and may not have been regenerated; remove them using the following command and re-run your script.

```
> rm */*aligned*
```

- Verify the software environment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Runsheng Li (runsheng.li@cityu.edu.hk).

Technical contact

For any technical inquiries regarding the execution of this protocol, please contact Zhihao Guo (zhihaguo-c@my.cityu.edu.hk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The nanoSundial code has been deposited at GitHub repository (https://github.com/lrslab/nanoSundial and Zenodo (https://doi.org/10.5281/zenodo.15904129).

ACKNOWLEDGMENTS

Open access was made possible with partial support from the Open Access Publishing Fund of the City University of Hong Kong.

Funding for this research was provided to R.L. through the New Research Initiatives support from City University of Hong Kong (project no. 9610497), the Hong Kong Health and Medical Research Fund (project no. 08194126), as well as the Early Career Scheme (CityU 21100521) and the General Research Fund (11105524) from the Research Grants Council of the Hong Kong Special Administrative Region, China.

AUTHOR CONTRIBUTIONS

Conceptualization, Z.G., Y.S., and R.L.; methodology, Z.G. and Y.S.; investigation, Z.G., Y.S., and R.L.; software, Z.G.; experiments, Y.S. and L.T.; writing – original draft, Z.G. and Y.S.; writing – review and editing, Z.G., Y.S., L.T., and R.L.; supervision, R.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the development of this manuscript, the authors employed ChatGPT and Grok to aid in correcting grammatical errors. The content was thoroughly reviewed and edited by the authors as needed, and they take complete responsibility for the final published work.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2025.104279.

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