Supplementary Protocol

Materials

K562 cells

HyClone RPMI 1640 Media

Fetal bovine serum (FBS), certified

Penicillin-Streptomycin, 100X solution

Phosphate buffered saline (PBS), 1X

4-thiouridine

DEPC-treated water

Qiagen RNeasy Mini kit

2-mercaptoethanol (BME)

Ethanol, 200 proof, molecular biology grade

3 M sodium acetate, pH 5.2

2,2,2-trifluoroethylamine (TFEA)

0.5 M EDTA, pH 8

Sodium periodate ($NaIO_4$)

0.2 mL PCR tube strips

1.5 mL microcentrifuge tubes, PCR clean

15 mL conical centrifuge tubes

1 M dithiothreitol (DTT)

22-gauge disposable needle

Luer slip syringe

Agencocurt RNAClean XP beads

Magnetic PCR tube separation rack

SMARTer Stranded Total RNA-Seq Kit - Pico Input Mammalian

PCR Thermocycler

Nanodrop spectrophotometer

Mini centrifuge for PCR strips

Benchtop microcentrifuge

1. Culture K562 cells at 37°C to approximately 50% confluence in RPMI media supplemented with 10% FBS and 1% P/S.

2. Dissolve 4-thiouridine in a minimal volume of water and add to 5 mL cultured cells at 100 mM final concentration. Agitate cells gently to evenly distribute the metabolic label. Incubate 4 hours at 37°C.

Note: Take steps to protect 4-thiouridine treated cells and RNA in all subsequent steps from light exposure.

Note: 4-thiouridine treatment time and concentration will vary by cell type and desired application. We provide an example above for K562 cells to examine relatively long RNA half-lives.

3. Transfer cells to 15 mL conical Eppendorf tube and place on ice for 1 min.

4. Pellet cells for at 4°C for 3 min at 300xg in a pre-chilled centrifuge. Aspirate cell culture medium from cell pellet.

5. Rinse pellet with ice-cold 1X PBS then pellet and aspirate as in step 4. Proceed to modified RNeasy isolation.

Modified RNeasy isolation

6. Resuspend cell pellet in 350 µl RLT buffer supplemented with 1% BME. Pass cell suspension through a 22-gauge needle 5 times to lyse the cells and transfer the mix to a 1.5 mL Eppendorf tube.

7. Add 350 µl freshly prepared 70% ethanol to the lysis mixture and mix well by inversion. Transfer 700 µl of mixture to an RNeasy spin column, and centrifuge >10,000 RPM for 15 sec. Discard flow through.

8. Add 700 µl RW1 to column and centrifuge >10,000 RPM for 15 sec. Discard flow through.

9. Add 500 µl RPE supplemented with 1% BME to column and centrifuge >10,000 RPM for 15 sec. Discard flow through.

10. Add 700 µl freshly prepared 80% ethanol to column and centrifuge at maximum speed for 2 min. Transfer column to fresh collection tube and centrifuge at maximum speed for an additional 5 min.

11. Transfer column to 1.5 ml Eppendorf tube and add 30 µl DEPC-treated water directly to column membrane. Let column stand 1 min, then centrifuge >10,000 RPM for 1 min. Assess RNA concentration by nanodrop.

TimeLapse chemistry

12. Create a master mix by combining the following on ice (15 µl total per sample, +10% to account for pipetting errors): 0.84 µl 3M sodium acetate, pH 5.2; 12.7 µl DEPC-treated water; 0.2 µl 0.5M EDTA, pH 8; 1.3 µl TFEA. Combine well by vortexing.

Note: TFEA is volatile. Pipette up and down several times prior to dispensing the reagent to ensure vapor pressure equilibration and accurate volumes.

13. Add 15 µl above master mix to 8.7 µl RNA sample (e.g. 2 µg RNA in DEPC-treated water) in a 0.2 ml PCR tube strip. Combine well by flicking PCR tubes, and briefly spin to collect sample at bottom of tube.

14. Add 1.3 µl of a 192 mM solution of $NaIO_4$ in DEPC-treated water. Combine well by flicking PCR tubes, and briefly spin to collect sample at bottom of tube.

15. Incubate samples at 45°C for 1 h in a pre-heated PCR thermocycler with a heated lid. Cool sample to 4°C after incubation.

16. Add an equal volume (25 µl) of RNAClean beads to each sample and gently vortex to combine. Incubate at room temperature 10 min.

Note: An aliquot of RNAClean beads should be brought to room temperature for 30 min prior to use.

17. Briefly spin to collect sample at bottom of tube, and place on a magnetic isolation rack until solution is clear (~5 min).

18. Carefully remove supernatant without disturbing bead pellet, and perform 2 washes with 200 µl of freshly prepared 80% ethanol.

19. After removing second ethanol wash, spin to collect bead pellet at bottom of tube, and recapture beads on magnetic isolation rack for 1 min. Remove residual ethanol with a pipette and allow bead pellet to dry until just cracked (2-4 min).

20. Add 18 µl of DEPC-treated water to dried beads. Flick samples until beads are resuspended, and allow to rehydrate for 2 min.

21. Briefly spin to collect sample at bottom of tube, and place on magnetic isolation rack until solution is clear (~2 min).

22. Carefully collect the supernatant and transfer to a fresh PCR tube strip.

23. Add 2 µl of a freshly made 10X reducing master mix (100 µl recipe): 10 µl 1 M Tris-HCl, pH 7.4; 10 µl 1 M DTT; 20 µl 5 M NaCl; 2 µl 0.5 M EDTA, pH 8; 58 µl DEPC-treated water. Incubate samples at 37°C for 30 min.

24. Repeat steps 16-22 with 20 µl of RNAClean beads, eluted into 12 µl DEPC-treated water. Assess RNA concentration and integrity by bioanalyzer.

25. Proceed to RNA-seq library preparation using the SMARTer Stranded Total RNA-Seq Kit-Pico Input Mammalian.