Research Square

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Nascent RNA 4sU labelling and enrichment

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Abstract

4sU-seq allows for measuring or nascent transcription which can reflect dynamic changes more accurately than traditional RNA-seq. Incorporation of the nucleoside analog 4-thiouridine into RNA allows the separation of nascent transcripts though reversible coupling to biotin and streptavdin bead pull-down. The protocol takes roughly two days to generate a sequencing ready library.

Introduction

4sU-seq allows for measuring or nascent transcription which can reflect dynamic expression changes more accurately than traditional RNA-seq. Incorporation of the nucleoside analog 4-thiouridine into RNA allows the separation of nascent transcripts though reversible coupling to biotin and streptavdin bead pull-down[1-4]. The protocol takes roughly two days to generate a sequencing ready library.

Reagents

- 10^5 cells in suspension

- TRIS, pH 7.5 (ThermoFisher: 15567027)

- EDTA (Invitrogen: AM9760G)

- DEPC-treated water (Ambion: AM9915G)

- NaCl, 5 M (Sigma: S9625)

- Tween 20 (Sigma: P9416)

- 4-thiouridine, 4sU (Sigma: T4509) [500 mM in PCR grade water]

- PBS (Invitrogen: 10010031)

- TRI reagent (Sigma: T9424)

- RNAseZap (Sigma: R2020)

- Chloroform (Sigma: C2432)

- Glycoblue (ThermoFisher: AM9515)

- Isopropanol (Sigma: I9516)

- Ethanol (Sigma: 51976)

- MTSEA-biotin-XX (Biotium: #90066) [0.1 mg/mL in dimethylformamide]

- Dimethylformamide (Sigma: PHR1553)

- µMacs (Miltenyi: 130-074-101)

- Chloroform/isoamylalcohol, 24:1 (Sigma: 25668)

- Phase-lock tube (5Prime: 2900309)

- Dithiothreitol, DTT (Merck: 1114740025) [Make Fresh, 100 mM in PCR grade water]

- RNeasy RNA clean-up kit (Qiagen: 74204)

- QuantiFluor RNA kit (Promega: E3310)

Buffers:

10x Biotinylation buffer (25 µL per sample):

- 100mM Tris, pH 7.4

- 10mM EDTA

- DEPC-treated water

MACS wash buffer (4 mL per sample):

- 100mM Tris, pH 7.5

- 10mm EDTA

- 1M NaCl

- 0.1% Tween 20

- DEPC-treated water

Equipment

- Nanodrop One, or quivalent (ThermoFischer Scientific: ND-ONEC-W)

- Qubit 4 Fluorometer, or quivalent (ThermoFischer Scientific: Q33238)

- MACS MultiStand (Miltenyi: 130-042-303)

- µMACS Separator (Miltenyi: 130-042-602)

Procedure

4sU Incorperation

*** Prepare 10^5 cells per mL of media ***

1. Add 10 µL of 500 mM 4-thiouridine (4sU) per mL of cells for a final concentration of 500 µM.

2. Incubate under standard growth conditions for 45 minutes (e.g. 37°C, 5% CO_2)

3. Pellet the cells by centrifugation at 200 rcf for 5 min.

4. Discard supernatant and resuspend cells in an equal volume of PBS.

5. Pellet the cells by centrifugation at 200 rcf for 5 min.

6. Resuspend pellet in 1 mL of TRI reagent and transfer to microcentrifuge tube.

7. Snap freeze cells using liquid nitrogen OR dry ice and ethanol.

*** Store lysed cells at -80ºC for up to 3 months ***

RNA Extraction

*** Chill centrifuge to 4ºC ***

1. Thaw TRI reagent lysed cells on ice.

2. Add 200 µL chloroform.

3. Add 1 µL glycoblue and close tube securely.

4. Shake the tube vigorously for 15 sec.

5. Incubate at room temperaturefor for 2–3 min.

5. Centrifuge for 30 min at 12,000 rcf (4°C).

6. Transfer the upper aqueous phase to a new collection tube containing 500µL isopropanol.

7. Incubate for 10 min at room temperature.

8. Centrifuge for 10 min at 12,000 rcf (4°C).

9. Discard supernatant and wash the RNA pellet with with 700 µL 70% ethanol in DEPC-treated water.

10. Centrifuge for 10 min at 12,000 rcf (4°C).

11. Discard supernatant and air dry for 10 min.

12. Re-suspend in 20 µL DEPC-treated water.

13. Measure RNA concentration using a Nanodrop.

4sU Enrichment

*** Pre-spin one phase-lock tube per sample ***

*** Heat 2 mL MACS wash buffer to 65°C ***

1. Combine 20-100 µg of total RNA, 50 µL MTSEA-biotin-XX, 25 µL 10x Biotinylation buffer and sufficient DEPC-treated water to generate a 250 µL reaction.

2. Incubate for 30 min at room temperature with rotation.

3. Add 400 µL chloroform/isoamylalcohol (24:1).

4. Add 1 µL glycoblue.

5. Mix and incubate for 3 min.

6. Transfer mixture to a phase-lock tube.

7. Centrifuge for 5 min at 12,000 rcf (room temp).

8. Transfer upper phase (~230 µL) to a new tube.

9. Add 1:10 volume 5 M NaCl and an equal volume of isopropanol and mix.

10. Centrifuge for 20 min at 20,000 rcf (4ºC).

11. Discared supernatant and wash pellet with 500 µL of 75% ethanol in DEPC-treated water.

12. Centrifuge for 20 min at 20,000 rcf (4ºC).

13. Discared supernatant and resuspend pellet in 60µl DEPC-treated water.

14. Denature RNA at 65°C for 10 min followed by rapid cooling on ice for 5 min.

15. Incubate RNA with 60 µL streptavidin magnetic beads for 15 min at room temperature with rotation.

16. Place a µMACS column into a magnetic separator with a 15 mL waste collection tube.

17. Add the biotinylation reaction to the coulmn.

18. Wash the column with 1 mL 65ºC MACS was buffer, repeat for a total of two washes.

19. Wash the column with 1 mL room temp MACS was buffer, repeat for a total of two washes.

20. Replace the waste tube with a collection microcentrifuge tube.

21. Elute RNA from the column by adding 100 µL of freshly prepared 100 mM dithiothreitol (DTT), followed by a second elution with another 100 µL 5 min later.

22. Clean up labelled RNA using a Qiagen RNeasy RNA clean-up kit with on-column DNase digestion.

23. Elute purified RNA in 20 µL of DEPC-treated water.

24. Measure RNA concentration using the QuantiFluor RNA kit.

Further steps:

A. Index RNA using SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian following the manufacturer's instructions with a fragmentation time of 3 min and 14 cycles of PCR amplification.

B. Sequence using 75-cycle paired end reads.

Troubleshooting

- To avoid RNA degradation thoroughly clean bench surfaces and pipettes with RNAseZAP.

- Cell centrifugation speeds are optimised for erythroid progenitor cells and may need adjusting.

Time Taken

Anticipated Results

References

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