Protocol for 4sU-2P-seq and DIM-2P-seq

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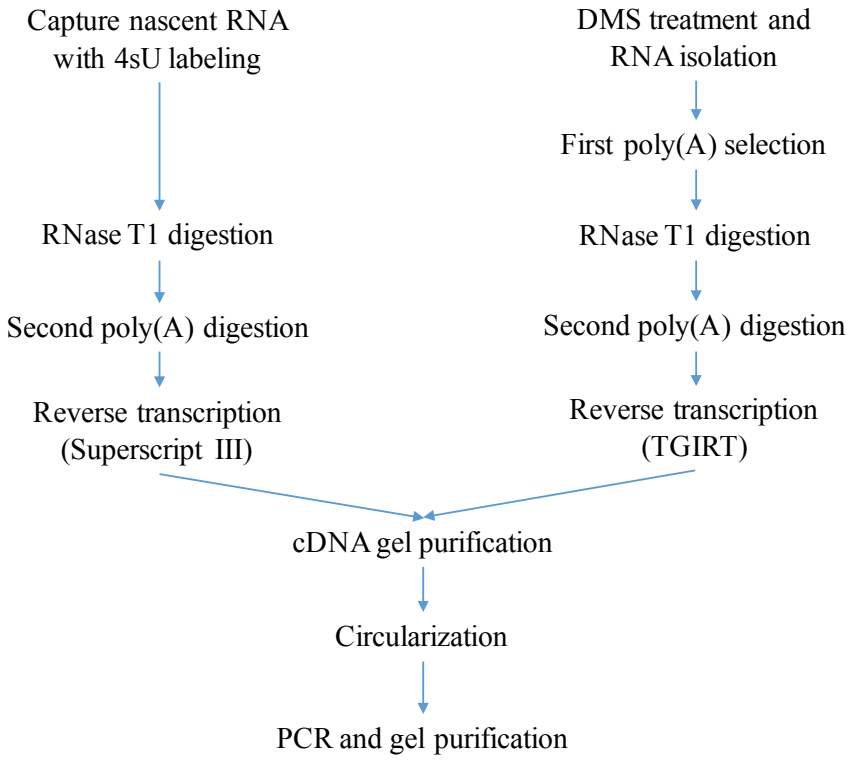
Modified from Noah Spies's protocol for poly(A)-primed sequencing (2P-seq).

Overview



Capture nascent RNA with 4sU labeling

Please follow the detailed protocol described in the following paper:

Rädle B, Rutkowski AJ, Ruzsics Z, Friedel CC, Koszinowski UH, Dölken L. **Metabolic labeling of newly transcribed RNA for high resolution gene expression profiling of RNA synthesis, processing and decay in cell culture.** J Vis Exp. 2013 Aug 8;(78). doi: 10.3791/50195.

With video: http://www.jove.com/video/50195/metabolic-labeling-newly-transcribed-rna-for-high-resolution-gene

DMS treatment and RNA isolation

Modified from Silvi Rouskin's protocol for DMS-seq.

1. grow adherent cells to 80% confluency in 15 cm plates
2. carefully move cells/plates to a chemical hood
3. transfer 1 mL DMS to a 50 mL tube per 15 cm plate (~3.3% final DMS concentration)
4. carefully transfer ~30 mL media from the plate to the tube with DMS, mix well
5. carefully transfer the DMS-containing media back to the plate
6. incubate at 37°C for 5 min at room temperature
7. prepare stop solution in the hood: 30% BME in 1x PBS
8. carefully remove the DMS-containing media
9. add the 40 mL stop solution to each plate, resuspend cells and transfer to 50 mL tube
10. spin at 1000 rpm for 4 min and add 10 mL trizol + 0.1 mL BME to cell pellet
11. follow trizol protocol to prep the RNA, resuspend in 50 µL water
12. DNase treatment: add 6 µL 10x Turbo DNase buffer and 3 µL DNase, 37°C for 20 min
13. add 6 µL inactivation reagent, mix and sit for 5 min room temp, shake occasionally
14. add 340 µL water to 400 µL, add 400 µL acid phenol/chcl3, mix and transfer to phase lock gel tube
15. spin at max speed for 5 min at 4°C
16. transfer top layer to a new tube, precipitate with 40 µL 3M Sodium Acetate, pH 5.2, 880 µL 100% EtOH.
17. resuspend in 100 µL water

First poly(A) selection

Omit if starting with nascent RNA captured by 4sU

1. setup 65°C heat block or water bath
2. per sample magnetize **160 µL** Dynabeads Oligo(dT)^25 for 1 min and remove supernatant
3. wash beads 1x with 100 µL binding buffer per sample
4. resuspend beads in 100 µL binding buffer
5. dilute **60 µg** total RNA in 100 µL water, add 100 µL binding buffer
6. incubate RNA at 65°C for 2 min, then place on ice for 2 min to denature
7. Change heat block to 75°C
8. mix 200 µL RNA with 100 µL washed beads, rotate at room temp for 5 min
9. wash 2x with 200 µL buffer B, each with 1 min rotation at room temp
10. resuspend in 50 µL 1x sequence buffer (RNase T1 kit, Ambion #AM2283)
11. heat at 75°C for 2 min, then magnetize
12. transfer supernatant to PCR tubes

RNase T1 digestion

1. To each sample in PCR tubes, add 1 µL glycoblue
2. 50°C for 5 min, then hold at 22°C

3. at 22°C, add 1 µL RNase T1 (1 U/µL, RNase T1 kit, Ambion #AM2283) , mix and incubate at 22°C for 20 min
4. add 108 µL stop solution, mix and incubate at –20°C for 30 min or longer
5. spin 30 min max speed at 4°C
6. wash 1x with 80% EtOH
7. resuspend in 100 µL water

Second poly(A) selection

1. repeat poly(A) selection once, elute in 13 µL water in PCR tubes after 75°C for 2 min

Reverse transcription

1. prepare template mix
    - 12.2 µL RNA
    - 1 µL 10 mM dNTP
    - 1 µL IW-RT1p primer, 5 µM
2. denature 5 min at 65°C, chill on ice 1 min
3. add
    - 4 µL 5x Superscript III first strand synthesis buffer
    - 1 µL 0.1 M DTT
    - 0.2 µL SuperaseIn
    - 1 µL Superscript III RT (4sU-2P-seq) or TGIRT (DIM-2P-seq)
4. RT incubation: 48°C for 30 min for Superscript III RT, or 48°C for 15 min, then 57°C for 2 hrs for TGIRT (DIM-2P-seq)
5. Add 2.3 µL 1M NaOH
6. 98°C for 15 min
7. add 22.5 µL 2x denaturing loading dye

cDNA gel purification

8. run a 6% TBE-Urea gel
    - 24 µL sample per well, two wells per sample
    - also asymmetrically load 1 µL century plus ladder per well, 0.5 µL RT primer (5 µM) per well
    - run until bottom dye reaches the bottom of the gel
9. stain gel with SYBR gold for 5-10 min
10. cut between 200 bp to 400 bp
11. crush the gel and elute in 500 µL 0.3M NaCl at 65°C for 2 hrs, 1000 rpm shaking
12. precipitation: 1200 µL 100% etoh, 2 µL glycoblue, –80°C for 30 min or overnight

Circularization

1. resuspend in 15 µL water total, then add
    - 2 µL 10x circligase I buffer
    - 1 µL 1 mM ATP

    - 1 µL 50 mM MnCl2
    - 1 µL Circligase I
2. 60°C for 4 hrs
3. 80°C 10 min

PCR

1. use 10 µL (half) circularized cDNA in 167 µL total PCR reaction (10 reactions)
    - 10 µL cDNA
    - 33.4 5x HF buffer
    - 3.34 10 mM dNTP
    - 0.84 µL PCR index primer, 100 µM
    - 0.84 µL PCR forward primer
    - 1.67 Phusion HF
    - 117 µL water
2. PCR
    - initial denaturation 30s at 98°C
    - 10 cycles of
        - 10s denaturation at 98°C
        - 10s annealing at 60°C
        - 20s extension at 72°C
3. column purify PCR reaction (QIAquick PCR Purification Kit, Qiagen), elute in 30 µL water at 56°C,
4. add 6 µL 6x dye and run on 2% agarose gel
5. cut between 200–600 bp, and purify DNA using QIAquick Gel Extraction Kit, Qiagen
6. repeat 5–10 PCR cycles depending on band intensity
7. Send samples for Pippin sizing (200–600), QC, and Illumina sequencing