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Targeted rRNA degradation

Modified from a protocol by Greg Smaldone (Mitch Singer lab) by Kaisa Kajala (Siobhan Brady lab), UC Davis.

Citation

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Purpose and Background

Degrade rRNA from a total RNA sample where poly-A purification of mRNA cannot be done; e.g., INTACT nuclei.

Materials

- Sample tissue / Total RNA / RNeasy Micro kit (Qiagen)
- Turbo DNase kit (Thermo Fisher AM2238)
- Agencourt RNAClean XP beads
- Probes designed against rRNA sequences (for nuclei; these are 5S, 5.8S, 18S and 25S rRNAs, probes at 60 bp length, reverse complements the rRNA sequences, ordered as a mix at 10 nmol in 1000 ul of water). Prepare a working mix with each probe at 1uM.
- Hybridase Thermostable RNase H (Epicenter # H39500)
- RNAse free PCR strips
- Magnetic rack for PCR strips
- PCR machine

Buffers and solutions

- 70% ethanol
- RNase-free water
- 5x Hybridization buffer H1 (0.5 Tris-HCl pH 7.0, 1M NaCl; RNase-free; filter sterilized and frozen)
- 10x Hybridase buffer H2 (0.5 Tris-HCl pH 7.4, 1M NaCl, 200mM MgCl2; RNase-free; filter sterilized and frozen)

Procedure

1. RNA isolation with Qiagen RNeasy Micro kit

For nuclei from INTACT:

- Purify the nuclear RNA using the Qiagen RNeasy Micro kit. Start by adding 350ul of lysis buffer RLT (with 10ul B-ME/1ml added) to the 20ul of purified nuclei. Vortex vigorously for 2 min.
- Centrifuge the lysate at 1,000g (3,100rpm) for 2min at RT to pellet the beads. Use a magnet to help transfer the supernatant to a new 1.5ml tube, add 350ul of 70% ethanol and vortex several times to mix.
- Pipette lysate/ethanol mixture into a RNeasy MinElute spin column resting in a 2ml collection tube and centrifuge at 10,000g (9,700rpm) for 1min at RT. Discard flowthrough.
- Add 350ul of buffer RW1 to the column. Centrifuge at 10,000g (9,700rpm) for 1min at RT. Discard flowthrough and move the column to a new 2ml collection tube.
- Add 500ul of buffer RPE to the column. Centrifuge at 10,000g (9,700rpm) for 1min at RT. Discard flowthrough.
- Add 500ul of 80% ethanol to the column. Centrifuge at 10,000g (9,700rpm) for 1min at RT. Discard flowthrough and move the column to a new 2ml collection tube.

- Open the column lid and centrifuge at top speed (16,000g) for 5min at RT. Discard the flowthrough and place the column into a new 1.5ml tube.
- Add 20ul RNase-free water onto the column membrane and allow to stand for 1mnin. Centrifuge at 16,000g for 1min at RT.
- Store RNA at -80C.
- Quantification can be done using RiboGreen RNA quantitation kit, expected yield is 100-500ng.

2. DNaseI treatment (eliminate genomic DNA contamination)

- Use DNaseI protocol for Turbo DNaseI
- Add to 20ul of RNA:
  2ul of 10x DNaseI reaction buffer
  1uL DNaseI
- Incubate 30min at 37°C
- Add 2uL DNaseI inactivation reagent (vortex well before adding) and incubate 5min at RT (vortex every 1min)
- Spin down (2000g for 5min) and recover 20uL into a new tube.

3. Agencourt RNAClean XP bead cleanup.

- Add 1.8 volume (e.g. 18ul for 10ul of RNA) of RNAClean XP beads to rxn
- Split each reaction into up to 140uL aliquots
- Incubate at RT for 10min
- Onto magnet for 5min
- Remove most of the supernatant (leave 5ul behind to avoid pulling up beads)
- Leave tubes on magnet, add 200uL 70% EtOH, let stand for 30s
- Remove all the supernatant
- Repeat 70% wash as above once more (two washes total)
- Remove as much of the EtOH as possible
- Air dry beads on magnet for 10min (until appear light in colour)
- Add 15uL RNase-free water to the first aliquot dried bead and mix
- If you have aliquoted samples, pool back together.
- Incubate at RT for 5min
- Onto magnet for 5min
- Recover 15uL eluate

4. **NanoDrop for [RNA] and 260/280 if you want to crush your dreams.**

5. rRNA probe hybridization (bind DNA probes to rRNA)

- Start with 3ug total RNA (rxn volume must be ~6uL, 3x 1ug rxns is okay). Or start with whatever you have.
- Add following together:
  1.2ul 5x Hybridization buffer H1 (0.5M Tris-HCl (pH 7.0), 1M NaCl, RNase-free)
  1.0ul probe mix – choose your concentration as follows:
    If your RNA amount is 1ug, use 1µM/oligo (working stock of probe mix)
    If your RNA amount is 0.1ug, dilute probes to 0.1 µM/oligo
    If your RNA amount is 0.01ug, dilute probes to 0.01 µM/oligo
    If your RNA amount is under Nanodrop range, use 0.01 µM/oligo
  3.8ul RNA (make up with RNase-free water)
- Incubate at 95°C for 2min, ramp down to 45°C at 0.1°C/s, 45°C for 5min, hold at 45°C

6. Hybridase® (thermostable RNaseH) reaction (digest RNA of an RNA:DNA hybrid)

- Prepare a master mix for your samples that you preheat to 45°C (in a hotblock):
  1ul 10x Hybridase buffer H2 (500mM Tris-HCl pH 7.4, 1M NaCl, 200mM MgCl2)
  1ul Hybridase (5U/uL)
  2ul RNase-free H2O

- Add 4ul of the MM to hybridization reaction still at 45°C.
- Incubate at 45°C for 30min.
- Remove to ice

7. DNaseI treatment (digest DNA oligos from mRNA pool)

- Use DNaseI protocol for Turbo DNaseI
- Add to 40ul of RNA:
  4ul of 10x DNaseI reaction buffer
  2uL DNaseI
- Incubate 30min at 37°C
- Add 2uL DNaseI inactivation reagent (vortex well before adding) and incubate 5min at RT (vortex every 1min)
- Spin down (2000g for 5min) and recover 43uL into a new tube.

8. Agencourt RNAClean XP bead cleanup.

- Add 1.8 volume (e.g. 72ul for 40ul of RNA) of RNAClean XP beads to rxn
- Split each reaction into up to 140uL aliquots
- Incubate at RT for 10min
- Onto magnet for 5min
- Remove most of the supernatant (leave 5ul behind to avoid pulling up beads)
- Leave tubes on magnet, add 200uL 70% EtOH, let stand for 30s
- Remove all the supernatant
- Repeat 70% wash as above once more (two washes total)
- Remove as much of the EtOH as possible
- Air dry beads on magnet for 10min (until appear light in colour)
- Add **10uL** RNase-free water to the first aliquot dried bead and mix
- If you have aliquoted samples, pool back together
- Incubate at RT for 5min
- Onto magnet for 5min
- Recover 10uL eluate

→ Use RNA for RNA-seq library prep.