rRNA degradation

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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. profiling pre-mRNA from different stages of processing from INTACT nuclei.

Materials

- Total RNA, or sample tissue + 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, and their internal and external transcribed spacers.
  Probes designed to be 60bp long and to reverse complement the rRNA sequences.
  Probes ordered as a mix at 10nmol in 1000ul 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 (e.g. EdgeBio #57624)
- 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 MgCl; RNase-free; filter sterilized and frozen)

Procedure

1. Total 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 so that you can adjust probe concentration.**

5. **rRNA probe hybridization (bind DNA probes to rRNA)**
    - Reaction size for steps 5+6 needs to be 6ul+4ul (total 10ul) for the buffers to be optimal for each step. You can prepare multiple aliquots of the reaction and pool back before DNaseI treatment. Successful libraries have been made from a single 0.1ug reaction.
    - 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 (if this would be more than 1ug 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 from RNA:DNA hybrids)**
    - Prepare a master mix 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 nuclease-free water
    - Add 4ul of the MM to hybridization reaction still at 45°C (keep in the pcr machine).
    - Incubate at 45°C for 30min.
    - Remove to ice

7. **DNaseI treatment (digest DNA oligos/probes from mRNA pool)**
    - Use DNaseI protocol for Turbo DNaseI
    - Add to 40ul of RNA (for four aliquots pooled):
        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). The minimum volume of inactivation reagent to add is 2ul – so even smaller volumes (e.g. 10ul), add 2ul.
- 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 (due to volume constraints in pcr tubes)
- 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

Go make libraries! (or store at -70C)

References

Morlan JD, Qu K, Sinicropi DV (2012) Selective Depletion of rRNA Enables Whole Transcriptome Profiling of Archival Fixed Tissue. PLoS ONE 7(8): e42882. doi:10.1371/journal.pone.0042882