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| Step2  Ribominus depletion of rRNA  Time: 2~3h  Note on 2):  I am using 2 times of hybridization buffer and RiboMinus probe for each sample compared to the standard low-input protocol as the RNA volume is 6 times the standard low-input volume, by doing this, the concentration of the probe is the same as the standard low-input protocol (Standard 1/33=3%  Customized 2/74=2.7%)  Note on 9):  I am using 2x beads compared to standard low-input protocol for each sample as the volume of the sample is way bigger than standard low-input protocol, but the beads are suspended to the same concentration as the standard low-input protocol.  (Standard 20/55=36%  Customized 40/114=35%)  Note on 11):  Make sure not to transfer beads into new tube.  200µL magnetic rack tends to have less magnetic force for certain beads. If this is the case, do the transfer twice or use another magnetic device if available. | Ribominus depletion of rRNA  **1 Hybridize Probe and Sample**  1) Set heat block to 75°C  2) Prepare hybridize mix as follows   |  |  |  | | --- | --- | --- | |  | 1000 cells |  | | Component | Vol. (µL) | 8.5x | | Hybridization Buffer | 60 | 510 | | RiboMinusTM Probe (15 pmol/µL) | 2 | 17 | | Total RNA | 12 | 527/62 each |   3) Mix by gentle vortexing, and incubate the tube at 70–75°C for 5 minutes to denature the RNA.  4) Immediately transfer the tubes to a 37°C water bath/heat block, and allow the sample to cool to 37°C over a period of 30 minutes. Slow cooling promotes sequence-specific hybridization. Do not cool samples quickly by placing the tubes in cold water.  While the sample is cooling, proceed to Prepare Beads.  **2 Prepare Beads**  Calculate how much beads are needed for the whole process, which includes  Beads volume   |  |  |  | | --- | --- | --- | | Beads | Vol. (µL) 1x | 8x | |  | 150 | 1200 |   1) Resuspend RiboMinusTM Magnetic Beads in its bottle by thorough vortexing.  2) Pipet calculated required volume (150µL per sample) of bead suspension required for your sample into a sterile, RNase-free, 1.5-mL microcentrifuge tube.  3) Place the tube with the bead suspension on a magnetic separator for 1 minute. The beads settle against the side of the tube facing the magnet. Gently aspirate and discard the supernatant.  4) Add same amount (150µL per sample) of sterile, nuclease-free water to the beads and resuspend beads by slow vortexing.  5) Place tube on a magnetic separator for 1 minute. Aspirate and discard the supernatant.  6) Repeat Steps 4–5 once.  7) Resuspend beads in 75\*N µL (N, number of samples)Hybridization Buffer (75µL per sample). Transfer 25\*N µLof the beads to a new tube **(Tube B)** and maintain the tube at 37°C for use at a later step.  8) Aliquot 50 µL of beads per sample into a new tube (**Tube A)** and put it on a magnetic separator for 1 minute. Aspirate and discard the supernatant.  9) Resuspend beads in 40 µL per sample Hybridization Buffer and keep the beads at 37°C for later use.  **3 Removal of rRNA**  1) After the RNA/ RiboMinusTM Probe mixture has cooled to 37°C for 30 minutes, briefly centrifuge the tube to collect the sample to the bottom of the tube.  2) Transfer the sample (~73 µL) to the prepared RiboMinusTM Magnetic beads from Step 9 (**Prepare Beads**). Mix well by pipetting up and down or low speed vortexing.  3) Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally.  4) Briefly centrifuge the tube to collect the sample to the bottom of the tube.  5) Place the tube on a magnetic separator for 1 minute to pellet the rRNA-probe complex. **Do not discard the supernatant.** The supernatant contains RiboMinusTM RNA.  6) Aliquot Tube B (from **Prepare Beads**, step 7) to 25 µL of beads per sample.Put tubes on magnetic separator for 1 minute. Aspirate and discard the supernatant.  Place the tube (**Tube B**) with 25 µL of beads per sample from **Prepare Beads**, step 7 on a magnetic separator for 1 minute. Aspirate and discard the supernatant.  7) Add the supernatant containing RiboMinusTM RNA from step 5, above (~114 µL) to the new tube of beads. Mix well by pipetting up and down or low speed vortexing.  8) Incubate the tube at 37°C for 15 minutes. During incubation, gently mix the contents occasionally.  9) Briefly centrifuge the tube to collect the sample to the bottom of the tube.  10) Place the tube on a magnetic separator for 1 minute to pellet the rRNA-probe complex. **Do not discard the supernatant.** The supernatant contains RiboMinusTM RNA.  11) Transfer the supernatant (~114 µL) containing RiboMinusTM RNA to a new tube.  **4 Concentrate RiboMinusTM RNA (Standard Protocol)**  1) Transfer RiboMinusTM RNA sample to a clean, RNase-free 1.5-mL microcentrifuge tube.  2) Add the following components to the RiboMinusTM RNA:  1 µL LPA ( µg/µL)  0.1X volume (of eluted RNA sample) of 3 M sodium acetate  2.5X volume of 100% ethanol  3) Mix well and incubate at –80°C for ≥30 minutes.  4) Centrifuge the tube for 15 minutes at ≥12,000 × *g* at 4°C. Carefully discard the supernatant without disturbing the pellet.  5) Add 500 µL of cold 70% ethanol.  6) Centrifuge the tube for 5 minutes at ≥12,000 × *g* at 4°C. Carefully discard the supernatant without disturbing the pellet.  7) Repeat steps 5–6 once.   8) Air-dry the pellet for ~5 minutes. Resuspend the RiboMinusTM RNA pellet in 10–30 µL of nuclease-free water (12µL for the next step in this protocol).  These are ribosome RNA depleted RNA ready for reverse transcription. |