RNA isolation-purification and first-strand cDNA synthesis Entered by Kevin Janes
Janes Lab Protocols 12/28/16

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I. RNA isolation and purification

1. Aspirate the cell culture medium and lyse cells directly in 1 ml RNA STAT-60 (Tel-Test #CS-110) per ~10^6 cultured cells.
   - *This roughly corresponds to 1 ml per well for a 6-well plate or 0.5 ml for a 12-well plate (samples smaller than 12-well are not recommended)*
   - *Chamber slides containing 3D structures can be lysed in 0.5 ml RNA STAT-60 per chamber*
   - *Washing with PBS is not necessary and the delay can degrade unstable mRNAs*
   - *Note the expiration date on RNA STAT-60; the phenol will oxidize over time*
2. In a chemical fume hood, shear the denatured genomic DNA in each sample by pipetting the lysate up and down several times with a P200 micropipet.
   - *For 3D structures, use a P1000 to help dissolve the matrigel thoroughly*
3. After the lysate has become less viscous, transfer to a microcentrifuge tube and incubate at room temperature for 5 min to complete the dissociation of mRNAs from ribonuclear proteins.
   - *Lysates can be stored at this point for several weeks at –80°C*
   - *Make sure that the lysate does not touch the cap of the tube or else it can contaminate the later steps in the procedure; if the cap is contaminated, transfer to a fresh microcentrifuge tube*
4. Add 200 μl chloroform per ml of RNA STAT-60 and shake the samples vigorously for 15 sec.
   - *Many samples can be shaken in parallel by stacking two microcentrifuge racks on top of one another with the tubes in between*
   - *Chloroform causes the lysate to separate into aqueous and organic phases with denatured proteins at the interface*
   - *Chloroform degrades with light and with air over time, so small quantities should be purchased*
5. Centrifuge the samples for 15 min at max speed on a benchtop centrifuge in the cold room.
6. In a chemical fume hood, transfer the upper aqueous phase of the samples to a fresh microcentrifuge tube.
   - *~40–60% of the volume of the RNA STAT-60 used for the lysis will be present in the aqueous phase; the volume of the aqueous phase depends upon the sample that was lysed*
   - *Be very careful not to pick up any of the organic phase or the protein interface at this step; do not touch anywhere on the microcentrifuge tube when extracting the very top of the aqueous phase*
7. Add 0.5 ml isopropanol per ml RNA STAT-60, mix well by vortexing, and incubate for 30 min at 4°C.
   - *Use molecular biology-grade isopropanol that is reasonably fresh (we have run into problems with yellow precipitates when "old" isopropanol is used here)*
8. Centrifuge the samples for 10 min at max speed on a benchtop centrifuge in the cold room.
   - *A white glassy pellet may be visible after this step, but for small starting numbers of cells, the pellet may be invisible*
   - *Orient the tubes in the centrifuge so that you can anticipate where the pellet will be formed*
9. Carefully aspirate the supernatant and wash the pellets with 1 ml 75% EtOH per ml RNA STAT-60
   - *It is not critical to remove all of the isopropanol and risk aspirating the pellet; simply aspirate so that less than 100 μl isopropanol remains*
10. Mix gently by inverting 5–6 times and centrifuge at 7500 rcf for 5 min at 4°C.
    - *Do not vortex vigorously or the pellet will break up and then not pellet well at the next step*
11. Carefully aspirate the supernatant and allow the pellet to air dry for 5-10 min at room temperature.
    - *Aspirate so that less than 100 μl EtOH remains and then pull off the remaining liquid with a micropipet and gel-loading tip; tip the microcentrifuge tube sideways and then draw off the liquid far away from the base of the tube where the pellet is located*
    - *If the pellet is drawn up by the micropipet, redispense the liquid into the tube and spin at max speed on a benchtop centrifuge for 15 sec to resediment the pellet*

- *Wet pellets can be warmed for a few seconds at 37°C to increase evaporation*
- *Smaller or broken pellets will need to have the remaining liquid evaporated off at 42ºC or, if 42ºC is ineffective, 55ºC*

12. Resuspend in 10 μl nuclease-free H_2O and incubate for 10 min at 37°C
    - *Resuspend each pellet as soon as the white precipitate has dried to a brownish hazy film at the base of the microcentrifuge tube*
    - *Do not overdry the pellet, because this will make it much more difficult to redissolve*
    - *When resuspending, make sure that the pipet tip does not have any residual liquid in it; if it does, that is a chunk of RNA that has not completely resuspended, and the sample must be pipetted up and down thoroughly to wash off this chunk*
13. Quantify the RNA concentration on a Nanodrop and store at –80°C.
    - *After mixing the sample, pull 2 μl carefully off the top to avoid particulate material that will cause unstable absorbance readings*
    - *To confirm that the RNA has fully dissolved, measure each sample two separate times, removing the sample, pipetting it up and down in the tube extensively, and then re-adding it to the Nanodrop for the second reading—concentrations between separate measurements should be ±20% (if not, repeat until measurements stabilize)*
    - *The A260/A280 ratio will only be 1.6–1.7 because the sample was dissolved in H_2O rather than elution buffer*
    - *The A260/A230 ratio will be low because of residual guanidinium contamination from the RNA STAT-60; however, we have not seen this adversely affect the downstream first-strand synthesis for qRT-PCR*
    - *This procedure does not yield clean enough DNA for sequencing, microarrays, etc.*

II. TURBO DNAse treatment

1. Remove residual genomic DNA with the TURBO DNA-free kit (Ambion # AM1907). Assemble the following reactions in PCR tubes:
   2, 1, or 0.4 μg total RNA (use a constant amount across all samples)
   2 μl 10× TURBO DNAse buffer
   1 μl TURBO DNAse
   Volume to 20 μl with nuclease-free H_2O
   - *Add as much RNA as you can based on the yield from the different samples that will be analyzed simultaneously*
2. Incubate for 30 min at 37°C on a PCR thermocycler ("DNAse treatment" protocol on MyCycler)
3. Add 2 μl of DNAse Inactivation Reagent and incubate for 5 min at room temperature, flicking the tubes 2-3 times during the inactivation to keep the Inactivation Reagent resuspended.
4. Spin in a PCR centrifuge for 2 min and then transfer 5 μl of the supernatant to a fresh PCR tube for first-strand cDNA synthesis
   - *This will correspond to 500, 250, or 100 ng DNAse-treated RNA per first-strand reaction depending upon what starting amount was used in Step #1*
   - *Take an additional 2.5 μl of DNAse-treated RNA from two samples you expect to be different; mix these together to provide a "no RT" control sample that checks for contaminating genomic DNA in RT-qPCR experiments*
   - *Also, put 5 μl of nuclease-free H_2O in a fresh PCR tube to serve as a "Blank" control sample for first-strand cDNA synthesis and RT-qPCR*

III. First-strand cDNA synthesis

1. To 5 μl DNAse-treated RNA, add the following as a master mix:
   0.5 μl 50 μM oligo$(dT)_{24}$ primer
   0.5 μl 10 mM dNTPs
   0.5 μl nuclease-free H_2O
   6.5 μl total volume

- *We run the reverse transcription reaction at half the manufacturer's recommended volume, which is sufficient for 100 RT-qPCR experiments (see Janes_RTqPCR.pdf)*

2. Heat denature for 5 min at 65°C on a PCR thermocycler ("RNA denaturation" protocol on MyCycler) and then place on ice.
3. Prepare the following as a master mix:
   2 μl 5× first-strand buffer
   0.5 μl 0.1 M DTT
   0.5 μl RNAsin Plus RNAse inhibitor (Promega #N2615)
   0.5 μl Superscript III RT
   3.5 μl total volume
   - *Remember not to add this master mix to the no RT control. This sample must get a separate mix lacking Superscript III.*
4. Mix thoroughly by pipetting and then incubate RT reactions at 50°C for 60 min, followed by heat denaturation at 70°C for 15 min on a PCR thermocycler ("SSIII rt" protocol on MyCycler).
5. Transfer cDNA samples to a fresh microcentrifuge tube and store at –20°C