

# RNA isolation and reverse transcription protocol: Cells in culture

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Procedure for the isolation, DNase treatment and reverse transcription of RNA from cell culture.

## RNA isolation procedure for cells

1. Using at least  $10^6$  cells, aspirate the media and wash once with ice cold PBS (1–2 ml).
2. Aspirate the PBS (remove as much as possible) and add 1 ml TRIzol.
3. Scrape the plate briefly, then remove the TRIzol with a pipette and deposit the TRIzol/cell lysate into a 1.5 ml Eppendorf tube.
4. Leave at room temperature for 5 min.
5. Add 250  $\mu$ l chloroform and shake the tube vigorously for about 15 sec.
6. Leave at room temperature for 5 min.
7. Centrifuge at 10,000 rpm for 5 min.
8. At this point, there will be three layers in each tube:

**Top layer:** clear, aqueous

**Middle layer/interphase:** white precipitated DNA

**Bottom layer:** pink organic phase

9. Carefully remove the aqueous phase using a pipette. With a larger pipette, it is harder to control the rate and force of fluid withdrawal and this increases the likelihood of drawing some of the organic or DNA phase. Leave behind some of the aqueous phase (about 1 mm above DNA layer to prevent DNA contamination). Place in another 1.5 ml Eppendorf tube.
10. Add 550  $\mu$ l isopropanol to the aqueous phase and mix gently. Leave at room temperature for 5 min.
11. Centrifuge at 14,000 rpm for 20 min. If a low yield is expected, centrifuge for 30 min.
12. Place samples on ice. There should be a pellet barely visible at the base of each tube. Pour off the isopropanol and add 1 ml 75% ethanol in DEPC treated  $H_2O$ . Mix gently. Recentrifuge at 9,500 rpm for 5 min.
13. Pour off the ethanol and let the pellets air-dry. This is a critical step; if the pellets dry out too much, the RNA crystallizes and is very difficult to resolubilize. If not enough of the ethanol evaporates, this also prevents the RNA from going into solution. After pouring off the bulk of the ethanol wash, there will be approximately 30–40  $\mu$ l left in the bottom of the Eppendorf tube. To quicken the evaporation, centrifuge the tubes briefly to force remaining fluid on the side of the tube to the bottom, then pipette off as much of the ethanol as is feasible. The best time to add DEPC treated water to the RNA pellet is when there is only a tiny meniscus of solution left around the pellet itself.
14. Add approximately 15–25  $\mu$ l (depending on yield) of either DEPC treated TE buffer or water to the RNA pellet. To a small Eppendorf tube, dilute the RNA 1/40 (1.2  $\mu$ l in 48.8  $\mu$ l of TE buffer) and add to a microcuvette (path length = 1 cm). Then measure the absorbance at 260 nm. The 260/280 ratio should be greater than 1.8. If less than 1.5–1.6 or so, the RNA is likely to be at least partially degraded. Lower ratios also suggest DNA or thiocyanate contamination. The concentration is essentially the equivalent of the OD at 260 nm (in  $\mu$ g/ $\mu$ l).

## RNA isolation procedure for tissue

The only difference from the procedure in cells is the first step. Add 1 ml TRIzol to a sterile culture tube (preferably 12x75 mm). To this tube, add the frozen tissue (try not to add more than approximately 20 mg). On ice, pulverize the tissue with a homogenizer at a setting of 25 out of 30 for a total of 2 x 10 sec. Then pour the TRIzol solution into a 1.5 ml Eppendorf tube and proceed as above (from Step 4).

## DNase treatment

1. The DNase cocktail consists of the following (per sample):

RQ1 RNase free DNase: 1  $\mu$ l  
DNase 10X reaction buffer: 2  $\mu$ l  
DEPC-treated H<sub>2</sub>O: 6  $\mu$ l  
RNase Out: 0.5  $\mu$ l

2. Make a master mix of the above based on the number of RNA samples being treated.
3. Prepare the RNA in the following way:

Add 2  $\mu$ g of RNA (calculated by 2  $\mu$ g/the concentration in  $\mu$ g/ $\mu$ l) to a small Eppendorf tube. Bring the total volume of the RNA to 11  $\mu$ l by adding additional DEPC treated water. For example, if your RNA concentration is 1  $\mu$ g/ $\mu$ l, add 2  $\mu$ l of the RNA to 9  $\mu$ l of DEPC water.

Add 9  $\mu$ l of the DNase master mix to the RNA bringing the total volume to 20  $\mu$ l.

4. This step should be done in a thermal cycler. Incubate the samples at 37 °C for 15 min, followed by 65°C for 20 min, then place on ice. Briefly centrifuge each sample to assure all of the volume lies in the bottom of the tube. The DNase treated RNA can then be used immediately for the reverse transcription reaction.

## Reverse transcription of DNase treated RNA

1. Under most circumstances, each sample of RNA (1  $\mu$ g, or 10  $\mu$ l from the DNase treatment reaction) will be run with reverse transcriptase with the second 1  $\mu$ g aliquot being used for a no-RT control.
2. For each sample, mix together the following:

13  $\mu$ l DEPC treated water  
16  $\mu$ l 5X first strand buffer  
7  $\mu$ l DTT (0.1 M)  
8  $\mu$ l random primers (concentration = 0.1  $\mu$ g/ $\mu$ l, or a 1/30 dilution of the 3  $\mu$ g/ $\mu$ l)  
8  $\mu$ l BSA  
3  $\mu$ l dNTPs  
1  $\mu$ l RNase Out

The total from the above reagents is 56  $\mu$ l. Mix by vortexing.

3. Aliquot 28  $\mu$ l each into two separate 0.5 ml Eppendorf tubes. One tube will serve as the RT reaction (to which 2  $\mu$ l of the MMLV reverse transcriptase is added) and the other, the no-RT control, to which 2  $\mu$ l of H<sub>2</sub>O is added.
4. To each tube, add 10  $\mu$ l of the DNase treated RNA from above.
5. Mix well by pipetting.
6. Incubate all samples at 37°C for 1 hr, then 95°C for 5 min. The latter step fully inactivates remaining enzymes (i.e. MMLV RT and any remaining DNase).
7. Use immediately for PCR or store at -20°C first thing the next day.
8. An alternative approach to the above might include the following:

One needs a total 20  $\mu$ l of the non-RT containing mix per sample. Randomly select two or three samples

from a group and prepare no-RT controls by aliquoting out 28  $\mu$ l per sample into 0.5 ml tubes and adding  $\mu$ g of RNA to each along with an additional 2  $\mu$ l of DEPC treated H<sub>2</sub>O (this is optional).

To the remaining RT master mix, add 2  $\mu$ l of MMLV RT/sample being prepared, mix by vortexing, and then aliquot 30  $\mu$ l per tube for each sample. For example, if you have a total of 10 samples, you will reverse transcribe all 10, but want 2 or 3 samples additionally for no RT controls to assure that the DNase treatment step worked. Thus, you would multiply the above master mix by 7 or 8. 1X the master mix is for two samples.

Thus, if you'll need a total of 12 reactions (for both RT and no-RT reactions) then you will need 6X the master mix cocktail. Once made, aliquot off the two no-RT samples (28  $\mu$ l each), add 20  $\mu$ l of the MMLV RT (2  $\mu$ l/sample) to the master mix. Vortex the mix, then aliquot 30  $\mu$ l per tube for 10 tubes. To each, add the corresponding 1  $\mu$ g (10  $\mu$ l) of RNA and incubate as above.