MOLab Standard Protocol for Preparing RT-PCR cDNA Stocks for qPCR

(Lan Zhou 11/02/00, Yang Bi 02/24/08, updated by Yi Shu @ 03/30/2025; commented by TCH)

NOTE:

A) Yang has recently compared the RT enzyme from NEB (**Cat# M0253S**) vs. Invitrogen's Superscript II, and found no significant difference between those enzymes. Thus, **NEB RT enzyme is the recommended choice** in our lab. However, we do not use any manufacturer's protocol!

B) After numerous pre-testing runs by former/current lab members, we decide that the RT enzyme in each reaction can be reduced to as low as **0.25µl** (the original suggested volume was 1.25µl).

C) These conditions work well for most regular RT-PCR cDNAs prepared for real-time PCR assays, especially if your RNA is isolated from cultured cells and/or fresh tissue samples. However, if you are concerned about the yield and quality of your RNA samples, you are always recommended to check a couple microliters of your RNA samples on RNA gel (see **Protocol C12**).

D) Make sure you use enough total RNA for each RT reaction. Typically, a subconfluent T-25 flask yields about 5-10µg total RNA, and a subconfluent 100mm dish yields about 10-20µg total RNA. Please keep in mind that the RNA yield will be much lower if the cells are cultured in low serum medium 8-12h prior to RNA isolation.

E) In our TRIzol or Nucleozol RNA isolation protocol, we do not routinely use RNase-free DNase to digest away potential genomic DNA. Thus **-RT tube** is set up in some cases.

RT-PCR PROTOCOL

1. Turn on two heating blocks: one at 70°C and one at 42°C.
2. Make Hexamer mix-RNA [prepare half of the volume if **-RT tube** is not set up]:

Hexamer (0.5 µg/µL)	2 µl
Total RNA	10 µl
Total volume	12 µl

3. Incubate @ 70°C for 5 minutes.
4. Prepare RT mix [prepare half of the volume if **-RT tube** is not set up]:

10 X M-MuLV buffer	2.5 µl
5mM dNTPs	2 µl
M-MuLV	0.2µl
ddH2O	3.5 µl
Total volume	8.2 µl (~8µl /RT Rx)

5. Prepare RT Reactions:

	+RT tube
Hexamer mix-RNA	12.0 µl
RT mix	8.0 µl
Total volume	**20µl**

7. The cycling program of RT-PCR:

   42^{o}C X 60 minutes
   95^{o}C X 1 minute (for killing RT; RT interferes with Taq DNA Pol)
   4 ^{o}C Hold or kept at -80°C freezers

8. Add 80µl ddH_2O to get 100µl original cDNA, and then take 10µl out, add 40µl ddH_2O to dilute into 5 times (**total volume = 100µl, aka., RT-PCR cDNA stock**), and then use the diluted cDNA for real-time PCR. *It's a good practice to aliquot the initial 100µl into multiple aliquots. Keep the aliquots at -80°C.*

9. For most real-time PCR reactions, the cDNA mix needed to be further diluted 5 to 100 times, depending on transcript abundance of the gene of your interest. *The ideal Ct or Cq values for internal reference genes should be between 15~20 cycles.*