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

(Lan Zhou 11/02/00, updated by Yang Bi 02/24/08, 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, the **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. However, we have found that the -RT tube is not necessary in most cases; so **it is optional**.

1. Turn on two heating blocks: one at 70^{o}C and one at 37^{o}C.
2. Make Hexamer mix [prepare half of the volume if **-RT tube** is not set up]:

	1X
Hexamer(random primer,1µg/µl)	4 µl
SSB (0.5µg/µl) (**optional**)	2 µl
RNase-free H2O	3 µl
Total volume	9 µl (or 4.5µl per RT)

3. Make Hexamer mix-RNA [prepare half of the volume if **-RT tube** is not set up]:

Hexamer mix	9 µl
Total RNA (10µg) or mRNA (0.5µg)	x µl
RNase-free H2O	xx µl
Total volume	25 µl (or 12.5µl per RT)

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

5 X First strand buffer	10 µl
0.1M DTT	4 µl
10mM dNTPs	2 µl
RNasin (**optional**)	0.4 µl
Total volume	16.4 µl (or ~8µl per RT)

6. Prepare +/- RT Reactions:

	+RT tube	-RT tube (**optional**)
Hexamer mix-RNA	12.0 µl	12.0 µl
RT mix	7.8 µl	7.8 µl
RT enzyme (NEB)	0.25 µl	0.0 µl
RNase-free H2O	0.0 µl	0.25 µl
Total volume	**20µl**	20µl

7. The cycling program of RT-PCR:

   37^{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 Ct or Cq values for internal reference genes should be ideally between 15~20 cycles.