User-Developed Protocol:

Labeling of cDNA using labeled dCTP and <50 ng RNA with the Sensiscript™ RT Kit (Labeling protocol C-50)

This procedure has been adapted by customers from the Sensiscript™ Protocol for Reverse Transcription and is for use with the Sensiscript RT Kit. **It has not been thoroughly tested or optimized by QIAGEN**.

The protocol has been used successfully for Cy3-, Cy5-, and biotin-labeling of cDNA from <50 ng of total RNA or poly A^+ mRNA.

Please be sure to read the *Sensiscript Reverse Transcriptase Handbook* and the detailed Sensiscript Protocol for Reverse Transcription carefully before beginning this procedure.

Solutions and reagents to be supplied by user

- Sensiscript RT Kit
- QIAquick® PCR Purification Kit
- RNase inhibitor
- Oligo-dT_{15} primer
- RNase A
- dNTPs: dATP, dCTP, dGTP, and dTTP (Amersham Pharmacia cat. nos. 27-2050-01, 27-2060-01, 27-2070-01, and 27-2080-01, respectively)
  or dATP/dCTP/dGTP/dTTP Set (Roche Molecular Biochemicals cat. no. 1 969 064)
- Labeled dCTP: biotin-16-dCTP, cyanine 3-dCTP (NEN Life Science cat. no. NEL576), or cyanine 5-dCTP (NEN Life Science cat. no. NEL577)

Important notes before starting

- A number of different labeling protocols are available from QIAGEN. The choice of protocol depends on the type of labeled nucleotide and the amount of starting RNA. See Table 1 or call QIAGEN Technical Services to determine which protocol is best for you.

Table 1. Labeling protocol selection guide

Amount of RNA (total or poly A^+)	Cy3-, Cy5-, or biotin-labeled		^{32}P-dCTP
	dUTP	dCTP	
<50 ng	Labeling protocol U-50	**Labeling protocol C-50**	
50 ng – 2 µg	Labeling protocol U-2000	Labeling protocol C-2000	Labeling protocol P*
5–50 µg	Labeling protocol U-5000	Labeling protocol C-5000	

** Optimized for use with 1 µg RNA*

- If working with RNA for the first time, please read the recommendations in "Appendix A: General Remarks for Handling RNA" in the *Sensiscript Reverse Transcription Handbook*.
- Thaw dNTPs immediately before use. When using for the first time, divide the stock solution into aliquots, and refreeze unused aliquots immediately. Handle nucleotides carefully, and avoid multiple freeze–thaw cycles.
- Set up all reactions on ice to avoid premature cDNA synthesis and minimize the risk of RNA degradation.
- Separate denaturation and annealing steps are generally not necessary. However, for some RNAs with a high degree of secondary structure, a denaturation step may be desired. If so, denature the RNA in RNase-free water before reaction setup: incubate the RNA for 5 min at 65°C, then place immediately on ice. Do not denature the RNA in the reaction mix.

Procedure

1. **Thaw your template RNA solution on ice. Thaw the primer solutions (not supplied), 10x Buffer RT, dNTP aliquots (labeled and unlabeled), and RNase-free water at room temperature. Store on ice immediately after thawing. Mix each solution by vortexing, and centrifuge briefly to collect residual liquid from the sides of the tubes.**
2. **Prepare the dNTP mix C on ice according to Table 2. Mix thoroughly by vortexing, and centrifuge briefly to collect residual liquid from the sides of the tubes. Refreeze unused dNTP aliquots immediately.**

Table 2. Composition of dNTP mix C

Component	Volume per dNTP mix C (µl)	Final concentration
dATP (100 mM)	5.0	5 mM
dGTP (100 mM)	5.0	5 mM
dTTP (100 mM)	5.0	5 mM
dCTP (100 mM)	2.0	2 mM
RNase-free water	83.0	–
Total volume	**100 µl**	**–**

3. **Dilute RNase inhibitor (not supplied) to a final concentration of 10 units/µl in ice-cold 1x Buffer RT (dilute an aliquot of the 10x Buffer RT accordingly using the RNase-free water supplied). Mix carefully by vortexing for no more than 5 s, and centrifuge briefly to collect residual liquid from the sides of the tubes.**

   Commercially available RNase inhibitor is commonly supplied at 40 units/µl. Dilute to make it easier to pipet small amounts when preparing the master mix in step 4.

   Note: Prepare a fresh dilution of RNase inhibitor. To minimize the amount of RNase inhibitor and Buffer RT used, dilute no more than needed for your current series of reactions.

4. **Prepare on ice a fresh master mix according to Table 3. Mix thoroughly and carefully by vortexing for no more than 5 s. Centrifuge briefly to collect residual liquid from the walls of the tube, and store on ice.**

   The master mix contains all components required for the labeling reaction except the template RNA. If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reverse-transcription reactions to be performed.

   Note: The protocol is optimized for use with <50 ng RNA. With other amounts of RNA, see Table 1 or call QIAGEN Technical Services. Calculate the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA labeled.

Table 3. Master mix using labeled dCTP and <50 ng RNA

Component	Volume/reaction (µl)	Final concentration
Master mix		
10x Buffer RT	2.0	1x
dNTP mix C (see Table 2)	2.0	0.5 mM each dATP, dGTP, dTTP; 0.2 mM dCTP
Labeled dCTP (1 mM)*	1.0	0.05 mM
Oligo-dT_{15} primer (10 µM)	2.0	1 µM
RNase inhibitor†	1.0	10 units (per reaction)
Sensiscript Reverse Transcriptase	1.0	1 µl (per reaction)
RNase-free water	Variable	–
Template RNA		
Template RNA, added at step 6	Variable (<50 ng)	<50 ng‡ (per reaction)
Total volume	**20 µl**	**–**

* *Use Cy3-, Cy5-, or biotin-labeled dCTP.*

† *Not provided. If supplied at >10 units/µl, dilute in 1x Buffer RT (dilute an aliquot of the 10x Buffer RT accordingly).*

‡ *This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA, and carrier RNA present, and regardless of the primers used or cDNA labeled.*

5. **If setting up more than one reverse-transcription reaction, distribute the appropriate volume of master mix into individual reaction tubes. Keep tubes on ice.**
6. **Add template RNA to the individual tubes containing the master mix. Mix thoroughly and carefully by vortexing for no more than 5 s. Centrifuge briefly to collect residual liquid from the walls of the tube.**
7. **Incubate for 2 h at 37°C.**

8. **Add 2 µl RNase A (10mg/ml). Vortex for no more than 5 s. Incubate the reaction mix for 10 min at 65°C.**
9. **Incubate for 5 min at 93°C. Cool immediately on ice.**
10. **Purify the labeled probe using one of the QIAquick PCR Purification Kit Protocols in the *QIAquick Spin Handbook*. Perform an extra 35% guanidine-hydrochloride wash as described in the Troubleshooting Guide of the handbook (under the heading "Eluate contains primer–dimers").**

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