First strand cDNA synthesis

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Equipment and reagents[a]

- 0.2 ml thin-walled MicroAmp (Perkin Elmer) PCR[b] tubes and caps
- The following thermal cyclers have worked successfully: Perkin Elmer GeneAmp 9600; Genomyx CycleLR thermal cycler (GX102) with a heated lid; or MJ Research PTC100 thermal cycler with a heated lid. Other thermal cyclers may require protocol modification.
- SuperScript™ II RNase H^- reverse transcriptase[c]: 200 U/μl supplied with 100 mM DTT and 5 × first strand cDNA synthesis buffer (250 mM Tris–HCl pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) (Invitrogen)
- Two or more total RNA samples,[d] high quality, free from DNA contamination (see *DNase treatment of total RNA samples*), and prepared according to a properly designed experiment
- Genomyx HIEROGLYPH mRNA Profile Kit(s) (Beckman Coulter, Inc.)
- Sterile nuclease-free H_2O (Amresco; Ambion; or DEPC-treated H_2O)

Method

1 Thaw out the reagents (total RNA samples, unlabelled HIEROGLYPH APs, dNTP mix, HeLa RNA, 5 × SuperScript II RT first strand buffer, DTT) on ice, and keep them on ice. Mix each thawed reagent gently but do not vortex; spin the tubes briefly to collect the contents in the bottom of each tube.

2 Dilute the total RNA samples with sterile, nuclease-free water to a working concentration of 0.1 mg/ml. Dilute only as much RNA as needed for the RT rxns; diluted RNAs should not be refrozen for later use. Keep the tubes on ice.

3 Using the 0.2 ml thin-wall PCR tubes, prepare RT tubes for each total RNA sample (one RT rxn for each AP). Prepare a set of positive control reactions using the HeLa kit control RNA, freshly diluted to 0.1 mg/ml in nuclease-free H_2O, with each of the APs that are to be used.[e] Set up a negative control tube (–RNA control) containing nuclease-free H_2O in place of total RNA. For each experimental RNA sample (control and test conditions) prepare another negative control tube (–RT control) containing experimental RNA and no RT enzyme.[f]

4 In the appropriate tubes combine the following:

- Total RNA, freshly diluted to 0.1 mg/ml 2.0 μl
- HIEROGLYPH T7(dT_{12})AP (2 mM) 2.0 μl

5 Mix the samples carefully with a micropipette (do not vortex), and spin the tubes briefly. Cap the tubes securely.

6 Incubate the RNA and AP at 70 °C for 5 min in a thermal cycler with a heated lid. Quickly chill the tubes on ice. Spin the tubes briefly.

7 On ice, set up the RT core mix in sufficient volume for the number of RT rxns. Also prepare a separate –RT core mix, substituting sterile nuclease-free H_2O for the RT enzyme, for use in the –RT control. Add 16 μl of core mix into the appropriate tubes for a final volume of 20 μl per tube. Mix the samples well with gentle pipetting. Cap all of the tubes securely. Spin the tubes briefly.

(a) RT core mix.

Reaction component	**[Stock]**	**1 × vol.**	**[Final]**
Sterile nuclease-free H_2O	–	7.8 μl	–
SuperScript II RT buffer	5 ×	4.0 μl	1 ×
dNTP mix (1:1:1:1)	5 mM each	2.0 μl	0.5 mM each
DTT	100 mM	2.0 μl	10 mM
SuperScript II RT enzyme	200 U/μl	0.2 μl	2 U/μl

(b) –RT core mix for –RT negative control tubes.

Reaction component	**[Stock]**	**1 × vol.**	**[Final]**
Sterile nuclease-free H_2O	–	8.0 μl	–
SuperScript II RT buffer	5 ×	4.0 μl	1 ×
dNTP mix (1:1:1:1)	5 mM each	2.0 μl	0.5 mM each
DTT	100 mM	2.0 μl	10 mM
SuperScript II RT enzyme	200 U/μl	–	–

8 Perform the RT rxns in a thermal cycler with a heated lid.

(c) 42 °C for 5 min.

(d) 50 °C for 50 min.

(e) 70 °C for 15 min.

(f) Hold at 4 °C.

9 Store in a –20 °C constant temperature, nonfrost-free freezer for use in fluoroDD-PCR within one week.

Notes

a All equipment and reagents must be kept RNase-free. Note that the use of RNase inhibitor is less important than taking extreme care to avoid introduction of RNases during RNA sample processing and RT reaction set up. Be aware that the commercially available RNase inhibitors do not inactivate RNase T1 or bacterial or fungal RNases, and that RNases may be released from inhibitor complexes during oxidation or denaturation. Do not use vanadyl RNase inhibitors, which can interfere with subsequent reactions. If RNase inhibitor (Ambion or Promega) is to

be used, it must be added to all tubes: for each reaction add 1 μl of 20 U/μl stock to the RT core mix for a final concentration of 1 U/μl. Adjust the volume of sterile nuclease-free water accordingly.

b The polymerase chain reaction (PCR) is covered by US patents owned by Hoffman-LaRoche.

c Be sure to store this temperature-sensitive enzyme in a constant temperature (not frost-free) –20 °C freezer. Leave the SuperScript II RT in the freezer until just before use and return the enzyme to the freezer immediately after use.

d At least 2.4 μg of each RNA sample will be required for the RT step (200 ng RNA with each of the 12 APs) to provide sufficient cDNA in duplicate DD-PCR reactions with the 12 oligo(dT) 3' APs and the four 5' ARPs, for a complete screening with the 48 primer-pair combinations of one mRNA Profile Kit.

e The HeLa kit control RNA is a positive control to check the performance of the reaction. It is not an appropriate reagent for the –RT negative controls.

f Generally one AP can be used for the negative controls. The purpose of the –RNA control is to test the purity of the RT reaction components (there should be no products). The purpose of the –RT control is to test for genomic DNA contamination in the experimental RNAs that can contribute artefactual DD-PCR bands to the DD pattern.