

Affymetrix GeneChip Resource

Total RNA to Double-stranded cDNA Protocol

Please do not make copies of or distribute this protocol.

A. Required reagents:

One-Cycle cDNA Synthesis Kit (Affymetrix, P/N 900431)

B. Equipment and supplies:

Microcentrifuge with 1.5 ml rotor

Strip Tube microcentrifuge

Pipettors

Aerosol-barrier tips

Vortex mixer

Thermocycler

Powder-free gloves

1.5 ml RNase-free microcentrifuge tubes

0.2 ml RNase-free PCR 8-strip tubes

PROCEDURE GUIDELINES

- Starting material: High quality total RNA in DEPC-treated water. RNA should be isolated by using RNeasy kit (Qiagen). If RNA is isolated by any other method, perform cleanup using RNeasy kit.
- Assess the quality of total RNA by gel electrophoresis. The intact RNA indicates that there is no contamination of RNase, which could affect cRNA synthesis in the next step. If the RNA does not appear to be intact, do not proceed with cDNA reaction.
- The concentration of total RNA should be at least 1 $\mu\text{g}/\mu\text{l}$. A dilute solution of RNA can be concentrated by using a lyophilizer or speedvac.
- Pipet up and down 10 times when removing and adding RNA sample and enzymes to ensure complete transfer.
- All vortexing is done with the vortex set at 8. Press tubes down on vortex and release 5 times to ensure complete mixture of components.
- Vortex and spin down all enzymes for 30 sec. at top speed before use.

I. FIRST STRAND cDNA SYNTHESIS

1. Add 5 µg of RNA in a volume of 9 µl to a 0.2 ml 8-strip tube.
2. Add 2 µl of T7-(dT)₂₄ primer.
3. Cap, vortex, and spin down.
4. Run '1st Strand' method in thermocycler.
5. Incubate at 70°C for 10 min. Cool at 4°C for at least 2 min.
6. Remove samples, spin down, and transfer to ice.
7. Allow '1st Strand' method to heat block to 42°C and PAUSE thermocycler at 42°C.
8. Prepare First-Strand Master Mix for n+1 samples, where n = # of RNA samples.

5X First Strand Buffer	4 µl
0.1M DTT	2 µl
10 mM dNTP mix	1 µl
9. Vortex and spin down.
10. Let samples stand at room temperature for 30 sec.
11. Carefully remove caps from tubes. Add 7 µl of master mix to each sample.
 - DTT precipitates at cold temperatures. Ensure sample is at room temperature before adding First Strand reaction mix.
12. Cap, vortex, and spin down.
13. RESUME '1st Strand' method for the incubation at 42°C for 2 min.
14. Hit PAUSE after 2 min. Carefully remove caps from tubes and discard caps.
15. Add 1 µl of SSII RT to each sample. Cover samples with a new cap.
16. Remove samples from thermocycler, mix samples by gently flicking the tubes a few times, and spin down.
17. Return to thermocycler, hit RESUME and incubate at 42°C for 1 hour.

II. SECOND STRAND cDNA SYNTHESIS:

1. Spin down samples and place on ice.
2. Prepare Second-Strand Master Mix for n+1 samples, where n = # of RNA samples.

DEPC-treated water	91 ul
5X Second Strand Reaction Buffer	30 ul
10mM dNTPs	3 ul
10 U/ μ l DNA Ligase	1 ul
10 U/ μ l DNA Polymerase I	4 ul
2 U/ μ l RNase H	1 ul

3. Vortex and spin down.
4. Carefully remove and discard sample caps.
5. Add 130 μ l of master mix to each sample.
6. Vortex and spin down.
7. Run '2nd Strand' method on the thermocycler.
7. Place samples in the thermocycler and incubate at 16^oC for 2 h.
8. Hit PAUSE after the 2h incubation.
9. Carefully remove and discard the sample caps.
10. Add 2 μ l (10 U) T4 Polymerase to each sample.
11. Cap samples, vortex, spin down, and return the samples to thermocycler.
12. Hit RESUME and incubate for 5' at 16 ^oC.
13. Remove samples from thermocycler.
14. Carefully remove the caps.
10. Add 10 μ l of EDTA per sample and recap samples.
11. Vortex and spin down.
12. Proceed to cDNA cleanup protocol or store at -20 ^oC.