

cDNA preparation from dsRNA

1. Remove the samples, dNTPs, RT buffers, and primers and RNase A from the freezer and thaw. The dNTPs must be thawed on ice. The rest can be thawed at room temp, but cannot be allowed to warm up, place them on ice immediately after they are thawed.
2. Prepare a mix for the RT reaction. For 8 samples use:
 - 32 μ l 5X superscript buffer (We have used SS I, II, and III with similar results)
 - 16 μ l DTT
 - 8 μ l dNTPs (10 mM)
 - 8 μ l SuperScript III (add this just before use, while the samples are boiling in step 4.)
3. In a boil-proof 1.5 ml tube, mix:
 - 1 μ l of sample
 - 1 μ l Tris EDTA (10 mM of each)
 - 2 μ l primer (20 μ M)
 - 8 μ l H₂O
4. Place in boiling water for 2 min..
5. Remove from boiling water and immediately place on ice until chilled (about 2 minutes).
6. Add 8 μ l of the mix prepared in step 2.
7. Incubate on ice for 15 minutes.
8. Transfer tubes to 50°C heat block.
9. Allow the reaction to go for about 1 hour or a little longer.
10. Remove the tubes from the heat block to room temp and add 1 μ l (10 mg/ml) Boiled RNase A. Incubate at room temp for 15 minutes.
11. Place tubes in the 85 C heat block for 2 minutes.
12. Remove the tubes one at a time, open them and immediately add 100 μ l PBI from the Qiagen PCR purification kit. Remove all of the liquid from the tube to a Qiagen column.
13. Spin the columns in the microfuge for 1 minute at top speed.
14. Wash the column with 750 μ l Guanidine HCL (35% in water).
15. Pour off the liquid that has spun through the column.

16. Wash the column by adding 750 μ l PE from the Qiagen kit and spinning for 1 minute at top speed.

17. Pour off the liquid that has spun through the column, place it back on the tube and spin it for 1 additional minute.

18. Remove the column to a clean 1.5 ml microfuge tube.

19. Add 30 μ l 0.1 X EB (dilute this from the Qiagen kit).

20. Spin for 1 minute at top speed.

21. Label the tube with the date and sample number after spinning and reserve for the PCR reaction.

22. Make a mix for the PCR reaction. For 8 reactions use:

80 μ l H₂O

12 μ l primer

12 μ l 10 X IT Medium buffer

2 μ l dNTPs (10 mM)

2 μ l Taq polymerase

23. Add to a 0.5 ml tube:

13.5 μ l of reaction mix

1.5 μ l template

24. Pipette into an IT* cuvette and cap, then centrifuge for a few seconds using the adaptors to get the liquid to the bottom of the cuvette

OR place a 10 μ l capillary tube into the mixture and draw it up by capillary action. Try to center the liquid in the tube, and seal both ends.

25. Run the PCR reactions on the IT machine. Use the following parameters:

94 C 1 min

65 C 0 sec

72 C 45 sec

Slope = 9

1 cycle

94 C 0 sec

45 C 0 sec

72 C 30 sec

Slope = 5

40 cycles

Hold 72 C 5 min, Hold 37 C 5 min

26. Run about 4 μ l of each sample on a gel to check.

27. Clone by TA cloning or by restriction sites that are built into the primers.

* We use an Idaho Technologies machine and capillary reaction tubes, but this protocol could be adapted to any PCR machine.