cDNA Synthesis by Reverse Transcription

Introduction

This protocol described synthesis of cDNA from polyA mRNA. The protocol is based on Oxford Nanopore´s Direct cDNA Native Barcoding Protocol supplemented with the Native Barcoding Kit.

Materials

- AMPure XP magnetic beads (Agencourt)
- NEBNext End repail/ dA-tailing module (NEB)
- NEB Blunt/TA Ligase Master Mix (NEB)
- 1,5ml Eppendorf DNA LoBind tubes
- 0,2ml PCR tubes
- Pure water
- 70% ethanol
- 10mM dNTP solution
- LongAmp Taq 2x Master Mix
- SuperScript IV reverse transcriptase, 5x RT buffer and 100mM DTT (ThermoFisher)
- RNaseOUT, 40U/ul (Invitrogen)
- RNase Cocktail Enzyme Mix
- Magnetic Rack
- Ice bucket
- Pre-chilled freezer block
- Microfuge
- 250ng PolyA+ RNA
- Direct cDNA sequencing kit

Procedure

Preparing input RNA

1. Transfer 250 ng RNA into a LoBind tube

   CRITICAL RNA needs to be of good quality (2kb fragments, 250 ng, A260:280 = 2.0, A260:230 = 2.2

   CRITICAL As fragments of E.coli mRNA are 1 kb in size, the input needs to be adjusted to 125 ng

2. Adjust the volume up to to 7,5ul with pure water

3. Mix by flicking the tube
4. Spin down briefly in a microfuge
5. Measure the quality of the RNA

Reverse transcription and strand-switching

6. In a 0.2 ml PCR tube, mix the following:

   2.5 µl of VNP primer
   1 µl of 10mM dNTPs
   7.5 µl of mRNA in Nuclease free water

7. Mix by flicking the tube, and spin down
8. Incubate at 65°c for 5 min and then snap cool on a pre-chilled freezer block
9. In a separate tube, mix together the following:

   4 µl Superscript IV buffer
   1 µl RNaseOUT
   1 µl 100mM DTT
   2 µl Strand-Switching Primer (SSP).

10. Mix by flicking the tube, and spin down
11. Add the strand switching buffer to the snap-cooled, annealed mRNA and mix by flicking the tube.
12. Incubate at 42° C for 2 minutes.
13. Add 1 µl of 200 U / µl SuperScript IV Reverse Transcriptase. The total volume is now 20 µl.
14. Mix gently by flicking the tube, and spin down.
15. Incubate as follows:

    Reverse transcription 10 mins @ 50° C
    Strand switching 10 mins @ 42° C
    Heat inactivation 10 mins @ 80° C
    Hold @ 4° C

RNA degradation and second strand synthesis

16. Add 1 µl of RNase Cocktail Enzyme Mix (ThermoFisher, AM2286) to the reverse transcription reaction.
17. Incubate the reaction for 10 minutes at 37° C.
18. Prepare the AMPure XP beads for use; resuspend by vortexing.
19. Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.
20. Add 17 µl of resuspended AMPure XP beads to the reaction and mix by flicking the tube.

21. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

    As Hula mixer was not available, mixing was done manually by rotating the tube.

22. Prepare 500 µl of fresh 70% ethanol in Nuclease-free water.

23. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

24. Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard.

25. Repeat the previous step.

26. Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.

27. Remove the tube from the magnetic rack and resuspend pellet in 20 µl Nuclease-free water.

28. Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.

29. Pellet beads on magnet until the eluate is clear and colourless

30. Remove and retain 20 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.

Second Strand Synthesis

31. Prepare the following reaction in a 0.2 ml thin-walled PCR tube:

    25 µl 2x LongAmp Taq Master Mix
    2 µl PR2 Primer (PR2)
    20 µl Reverse-transcribed sample from above
    3 µl Nuclease-free water

32. Incubate using the following protocol:

    94 °C 1 mins
    50 °C 1 mins
    65 °C 15 mins
    4 °C ∞

33. Prepare the AMPure XP beads for use; resuspend by vortexing.

34. Transfer the sample to a 1.5 ml DNA LoBind Eppendorf tube.

35. Add 40 µl of resuspended AMPure XP beads to the reaction and mix by pipetting.

36. Incubate on a Hula mixer (rotator mixer) for 5 minutes at RT.

37. Spin down the sample and pellet on a magnet. Keep the tube on the magnet, and pipette off the supernatant.

38. Keep on magnet, wash beads with 200 µl of freshly prepared 70% ethanol without disturbing the pellet. Remove the 70% ethanol using a pipette and discard.

39. Repeat the previous step.
40. Spin down and place the tube back on the magnet. Pipette off any residual 70% ethanol. Briefly allow to dry.
41. Remove the tube from the magnetic rack and resuspend pellet in 21 µl Nuclease-free water.
42. Incubate on a Hula mixer (rotator mixer) for 10 minutes at RT.
43. Pellet beads on magnet until the eluate is clear and colourless
44. Remove and retain 21 µl of eluate into a clean 1.5 ml Eppendorf DNA LoBind tube.
45. Analyse 1 µl of the strand-switched DNA for size, quantity and quality.