Laboratory Operating Procedure

Conversion of RNA to double-stranded cDNA for next-generation sequencing

Date

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LOP-Version: 1

Attachments:

Introduction

Whole Genome Sequencing of RNA and DNA samples now possible with the advent of next generation sequencing technologies. This is achieved by determining the DNA sequence of short fragments of genomic DNA and then computationally reassembling them in the correct order.
In order for RNA samples to be analysed using this approach it must first be converted to double-stranded complimentary DNA (ds-cDNA). RNA is single stranded and a complimentary DNA strand is synthesized by a process called reverse transcription. The second DNA strand can then be generated using DNA polymerase.

Sample Material

High quality RNA extracted from clinical material or tissue culture supernatent

Equipment and Reagents

(general remarks and list of equipment and material needed, bullet points)

Equipment

Single & multichannel pipettes suitable for volumes of 0.1 ul – 1000 ul	Various
Pipette tips (sterile with and without filters) suitable for volumes of 0.1 ul – 1000 ul	Alpha/Starlab
96-well microtitre plates	Alpha/Starlab
Plate sealing film	Alpha/Starlab
Centrifuge (capable of spinning 96-well plates between 100 x g and 1100 x g, room temperature)	Various
Thermocyler for 96-well plates	Various
Vortex mixer	Various
SPRIPlate Super Magnet Plate	Beckman Coulter
1.7 ml microcentrifuge tubes	Sigma
Reagent reservoirs	Various
Magnetic tube stand	Various

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Reagents

Agencourt Ampure XP	Beckman Coulter
Molecular Biology Grade Ethanol	Sigma
10mM Tris-Cl pH 7.5	Sigma
cDNA Synthesis System	Roche
Molecular Biology Grade Water	Various

Random hexamer primer mix	Roche
0.2M EDTA	Sigma

General remarks

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Literature

- *(e.g. Kit handbooks, publications)*

Before getting started

All reagents, apart from Random Primer Mix, 0.2 M EDTA, Ampure XP, Ethanol and 10 mM Tris, are part of the Roche cDNA synthesis kit

Procedure

1. Make up a stock of 400µM Random Primer Mix by diluting 200µl of 1 mM Roche Primer "random" with 300µl 10 mM Tris HCl, pH 7.5. This stock can be stored at -20°C for up to 2 years. If sufficient mix exists go straight to next step
2. Retrieve RNA sample from -80°C storage and thaw.
3. Retrieve Random Primer Mix, 5× RT-buffer AMV, DTT (0.1 M) and dNTPs (10 mM) mix from -20°C storage and thaw.
4. Pipette 2 µl Random Primer Mix (400µM) into individual PCR tubes or wells of a 96 well plate according to how many samples are to be processed in parallel.
5. Add 8.5 µl of RNA sample to the Random Primer Mix. Pipette up and down to mix.
6. Transfer the tubes or plate to a thermal cycler and incubate at 70°C for 10 minutes, followed by 4°C for at least 2 minutes.
7. During this incubation Master Mix 1 with sufficient for all the samples can be prepared. For each sample add the following amounts in a 1.7ml tube:
   4 µl 5× RT-buffer AMV
   2 µl DTT, 0.1 M
   2 µl dNTPs, 10 mM
   0.5 µl Protector RNase Inhibitor, 25 U/µl
   1 µl AMV RT, 25 U/µl
8. The enzymes can be used straight from the freezer so do not need thawing before use. Return all reagents (except dNTPs as these are required for a subsequent step) to the freezer and retrieve 5× 2nd strand synthesis buffer and redistilled water from -20°C storage and thaw.
9. Add 9.5 µl of Master Mix 1 prepared in step 7 to each of the samples from 6 and mix by vortexing
10. Transfer the tubes or plate back to the thermal cycler and run the following programme:
    25°C for 10 minutes
    42°C for 60 minutes
    4°C Hold
11. Toward the end of the incubation in step 10 Master Mix 2 for the next stage can be prepared. For each sample add the following amounts in a 1.7ml tube:
    15 µl 5x 2nd strand synthesis buffer
    38 µl Redistilled water
    0.75 µl dNTPs (10 mM)
    2.5 µl 2nd strand enzyme
12. The enzyme can be used straight from the freezer so do not need thawing before use.
13. When step 10 is complete add 55 µl of Master Mix 2 to each sample and mix by pipetting.

14. Incubate the plate or tubes in a thermal cycler at 16°C for 2 hours
15. After 2 hours add 8.5 µl of T4 DNA Polymerase to each sample and incubate at 16°C for a further 5 minutes.
16. Add 8.5 µl 0.2 M EDTA to each sample to stop the reaction.
17. For each sample label a fresh 1.7 µl microcentrifuge tube and pipette 150 µl Ampure XP into it.
18. Transfer the all the sample (~92 µl) from step 16 into the correctly labelled 1.7 ml microcentrifuge tube and vortex to mix. Incubate at room temperature for 5 minutes
19. Briefly (2-3 s) pulse the tubes in a microcentrifuge to collect all the sample at the bottom of the tube. Place the tube(s) into the magnet tube stand and wait until brown magnetic beads have collected at the side of the tube (~3-5 minutes).
20. Keeping the tubes in the magnetic stand, remove and discard the supernatant with a pipette without disturbing the magnetic beads. Care should be taken to remove as much supernatant as possible
21. Keeping the tubes in the magnetic stand, carefully add 500 µl 70% Ethanol to each tube with a pipette, without disturbing the beads.
22. Keeping the tubes in the magnetic stand, carefully remove the 70% Ethanol to each tube with a pipette, without disturbing the beads.
23. Repeat steps 21 and 22 twice more (a total of three washes)
24. Briefly (2-3 s) pulse the tubes in a microcentrifuge to collect all the sample at the bottom of the tube. Place the tube(s) into the magnet tube stand and remove any excess liquid.
25. Keep the lids of the tubes open and allow the beads to dry for 5-10 minutes
26. Add 10 µl of 10 mM Tris (pH 7.5) to each tube and vortex. Incubate at room temperature for 5 minutes.
27. Briefly (2-3 s) pulse the tubes in a microcentrifuge to collect all the sample at the bottom of the tube. Place the tube(s) into the magnet tube stand.
28. There is no requirement for the supernatant to be transferred to a new tube. Samples can be store in a refrigerator until required (up to one week) or taken immediately for further processing as indicated in section 5.1. If samples are store for any length of time then section 27 should be repeated before proceeding to quantification and library preparation