

Klenow labelling of double stranded DNA derived from 3 to 5 µg total RNA

Outline

Klenow labelling of double stranded DNA can be used when the amount of biological material is limiting. With this method, amplification by *in vitro* transcription of the sample can be avoided. The RNA samples are reverse transcribed to cDNA and second strand synthesis is then performed to obtain double stranded DNA (dsDNA). Fluorescent dyes are incorporated using Klenow fragment and the labelled samples are then subjected to paired competitive hybridisations.

Equipment and reagents

- Oligo(dT)23 anchored primer (Sigma; Cat. No. 04387)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080-044)
- RNAsin (Promega; Cat. No. 18064-014)
- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Second strand buffer (Invitrogen; Cat. No. 10812-014)
- DNA Polymerase I (Invitrogen; Cat. No. 1801-025)
- RNaseH (New England Biolabs; Cat. No. M0297S)
- *E. coli* DNA ligase (GE Healthcare Bio-Sciences AB; Cat. No. E70020Z)
- QIAquick PCR Purification Kit (Qiagen; Cat. No. 28104)
- AutoSeq G-50 column (GE Healthcare Bio-Sciences AB, Cat. No. 27-5340-01)
- DEPC - Diethyl pyrocarbonate (Sigma; Cat. No. D 5758)
- DEPC-treated MilliQ water
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632-011)
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- GeneVac miVac vacuum concentrator
- Dyad thermal cycler (PCR block)

Removal of RNase

All materials should be autoclaved and only handled using gloves. Glassware should be baked at 180 °C overnight. Water and solutions should be treated with DEPC. The work area can be cleaned using RNase Zap to further limit the risk of RNase contamination. If at all possible, it is also a good precaution to use a separate set of pipettes for RNA work.

Procedure

Preparing the dNTP mixes

Making the 10mM dNTP mix:

1. Make a large 10 mM dNTP mix for the RT-reaction and second strand synthesis
 - ◆ 100 µl 100mM dNTA
 - ◆ 100 µl 100mM dNTT

- ◆ 100 µl 100mM dNTG
 - ◆ 100 µl 100mM dNTC
2. Then make up to 1 ml using DEPC-water
 3. Aliquot the dNTP mix and then store at -20 °C

Making the 10 X low-C dNTP mix:

1. Make a large 10 X low-C dNTP mix for the labelling reaction (5 mM A-,G-,T-dNTPs and 3mM C-dNTP)
 - ◆ 25 µl 100mM dNTA
 - ◆ 25 µl 100mM dNTT
 - ◆ 25 µl 100mM dNTG
 - ◆ 15 µl 100mM dNTC
2. Then make up to 500 µl using DEPC-water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

Reverse Transcription reaction:

The following steps are performed in 200 µl PCR tubes and the PCR block

1. Take between 3 to 5 µg of the extracted RNA (small scale RNA extraction protocol), add 1 µl of Oligo(dT)23 anchored primer (500 ng/µl) and then make up to a total volume of 5 µl with DEPC-water
2. Incubate at 65 °C for 10 min
3. Snap freeze on ice
4. Make up a premix for the RT reaction:
 - ◆ 4 µl 5x First Strand Buffer (from Superscript Kit)
 - ◆ 2 µl 0.1M DTT (from Superscript Kit)
 - ◆ 1 µl 10 mM dNTP mix
 - ◆ 0.25 µl 40 U/µl RNasin
 - ◆ 0.75 µl 200 U/µl Superscript III
5. Add 8 µl to each sample
6. Incubate at 46 °C for 2 hours
7. Incubate at 65 °C for 15 minutes
8. Snap freeze on ice

Second strand synthesis:

The following steps are performed in 200 µl PCR tubes and the PCR block

9. Make up a premix for the second strand synthesis:
 - ◆ 9.15 µl DEPC-water
 - ◆ 7.5 µl Second Strand Buffer
 - ◆ 0.75 µl 10 mM dNTP mix
 - ◆ 2 µl 10 U/µl DNA Polymerase I
 - ◆ 0.1 µl 5 U/µl RNaseH
 - ◆ 0.5 µl 10 U/µl *E. coli* Ligase
10. Add 20 µl to each sample
11. Incubate at 16 °C for 2 hours

Purification of dsDNA using QIAquick PCR Purification Kit:

The following steps are performed in 1.5 ml microfuge tubes. Make sure that ethanol has been added to Buffer PE.

Making the 10mM dNTP mix:

12. Add 5 volumes (200 μ l) of Buffer PB to 1 volume (40 μ l) of the reaction and mix
13. Apply sample to QIAquick column and spin at 13,000 rpm for 1 minute. Discard flow-through and place column back into same collection tube
14. Wash column with 750 μ l Buffer PE, spin at 13,000 rpm for 1 minute. Discard flow-through and place column back into same collection tube
15. Centrifuge column at 13,000 rpm for 1 minute
16. Place column into a clean 1.5 ml microcentrifuge tube
17. Add 30 μ l water to the center of the membrane, incubate for 1 minute, then spin at 13,000 rpm for 1 minute
18. Reduce the volume of the sample to 12-15 μ l in the speed vac
19. Measure concentration on Nanodrop

Klenow labelling:

The following steps are performed in 200 μ l PCR tubes and the PCR block

20. Take up to 1 μ g double stranded DNA and make up to a total volume of 25 μ l with DEPC-water
21. Add 20 μ l 2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
22. Incubate at 100 °C for 5 minutes
23. Snap freeze on ice
24. Mix together the following to make a master mix:
 - ◆ 1 μ l 10 X low-C dNTP mix
 - ◆ 2 μ l Cy3 or Cy5 dCTP
 - ◆ 1 μ l 40U/ μ l Klenow
25. Add 4 μ l to each sample and mix by pipetting up and down
26. Incubate at 37°C for 2 to 3 hours
27. Stop the reaction by adding 5 μ l Stop Buffer (supplied in the Bioprime Labelling System Kit)
28. Combine the Cy3 and Cy5 pairs
29. Reduce the volume to ~60 μ l in a speed vac with medium heat

Probe clean-up:

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides. AutoSeq G-50 columns are quick and easy to use. Microcon 30 columns (Millipore) or Qiaquick PCR purification columns (Qiagen) work equally well.

Purify probe using an AutoSeq G-50 column as follows:

30. Prepare the G50 columns (need 2 columns per combined sample):
 - ◆ Resuspend the resin in the G-50 column by vortexing gently
 - ◆ Loosen the cap a quarter turn and snap off the bottom closure
 - ◆ Place the column in a 1.5 ml tube
 - ◆ Pre-spin column at 5,000 rpm (2000 x g) for 1 min to remove the buffer
 - ◆ Remove the top cap and place column in a new 1.5 ml tube
31. Pipette half the sample to the G50 columns onto the centre of the angled surface of the compacted resin bed being careful not to disturb the resin. Do not allow any of the sample to flow around the sides of the bed.
32. Centrifuge for 1 minute at 5000 rpm
33. Place a second column into the same 1.5 ml microfuge tube and then add the second half of the sample. Spin for 1 minute and 5,000 rpm.
34. Reduce volume of sample to between 2 to 5 μ l by placing in a speed vac with medium heat
35. Add 2 μ l of sonicated salmon sperm DNA

The samples have now been labelled and combined together for hybridisation to a microarray with the

blocking agent, sonicated salmon sperm DNA. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.

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