http://www.flychip.org.uk/protocols/gene_expression/direct_labelling.php

Reverse transcription and direct labelling of total RNA for cDNA and oligo arrays

Overview

The samples or samples and controls to be compared are each labelled with a different fluorescent dye and then subjected to paired competitive hybridisations. The described protocol for reverse transcription and direct labelling is based on the method recommended by BioRobotics (http://www.genomicsolutions.com/).

Removal of RNase

All materials should be autoclaved and only handled using gloves to avoid RNase contamination. Glassware should be baked at 180 °C overnight. MilliQ 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 possible, keep a set of pipettes purely for RNA work.

Equipment and reagents

- dATP,dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP−100A)
- Oligo(dT)23 anchored (Sigma; Cat. No. 04387)
- DEPC − Diethyl pyrocarbonate (BDH; Cat. No. 44170 3D)
- DEPC−treated MilliQ water
- Cy3 dCTP (Amersham; Cat. No. PA 53021)
- Cy5 dCTP (Amersham; Cat. No. PA 55021)
- RNAsin (Promega; Cat. No. 18064−014)
- Superscript III Reverse Transcriptase (Invitrogen; Cat. No. 18080−044)
- EDTA (BDH; Cat. No. 100935V)
- NaOH, (BDH; Cat. No. 102525P)
- Tris−HCl solid (BDH; Cat. No. 443864E)
- Sonicated Salmon Sperm DNA (Invitrogen; Cat. No. 15632−011)
- AutoSeq G−50 column (Amersham, Cat. No. 27−5340−01)
- Hettich micro 20 centrifuge
- Grant QBT2 hot−block
- Savant Speed Vac

Procedure

Reverse transcription and direct labelling reaction:

1. Prepare a concentrated stock of low−C dNTP mix:
    - ♦ 25 µl of 100 mM dATP
    - ♦ 25 µl of 100 mM dGTP
    - ♦ 25 µl of 100 mM dTTP
    - ♦ 10 µl of 100 mM dCTP
    - ♦ Make to 500 µl with DEPC−treated MilliQ water
    - ♦ Store in small aliquots at −20 °C
2. Mix together 100 µg total RNA, DEPC MilliQ water and spike mix to a total volume of 28 µl in an RNAse−free 1.5 ml tube. Add 1 µl of 500 ng/µl oligo (dT)23 anchored primer.
3. Incubate at 65 °C for 10 minutes in a hot−block to denature RNA tertiary structure, then place on ice.
4. Mix together the following to make a master mix:

- 8 µl of 5x first strand buffer
- 2 µl of conc. low−C dNTP mix
- 2 µl of 1 mM Cy3 or Cy5 dCTP
- 2 µl of 0.1 M DTT
- 0.5 µl of RNAsin
- 2 µl of Superscript III reverse transcriptase

5. Add 16.5 µl master mix to each tube of RNA/MilliQ water mixing carefully to avoid bubbles. Do not expose samples to light any more than necessary, ie. wrap in foil when possible.
6. Incubate at 42 °C for 1−2 hours.

Hydrolysis and neutralisation:

7. Hydrolyse the remaining RNA by mixing equal volumes of 0.5 M EDTA and 1 M NaOH. Then add 20 µl of this mix to the reaction and incubate at 65 °C for 15 minutes.
8. Bring samples to room temperature and add 25 µl of 1 M Tris−HCl (pH 7.5) to neutralise. If required, the labelled probe can be stored at −20 °C in the dark at this point.

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:

9. Reduce volume of probe to approximately 25 µl, by placing in a speed vac with medium heat. With our machine, this takes about 30 mins. Then combine the Cy3− and Cy5−labelled probe (sample and control) into one 1.5 ml microfuge tube.
10. Resuspend the resin in the G−50 column by vortexing gently.
11. Loosen the cap a quarter turn and snap off the bottom closure.
12. Place the column in a 1.5 ml tube.
13. Pre−spin column at 5,000 rpm for 1 minute to remove the buffer. Blot the tip of the column dry using a clean paper towel.
14. Remove the top cap and place column in a new 1.5 ml tube. Pipette half of the sample 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.
15. Spin for 1 minute at 5,000 rpm. The unincorporated dye and nucleotides should be retained by the column and the purified labelled probe should pass through into the support tube. Discard the column.
16. 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 rmp.
17. Reduce volume of probe to between 2 to 5 µl by placing in a speed vac with medium heat
18. Add 2 µl of 10 mg / ml sonicated salmon sperm DNA

The two samples (i.e. sample and control) have been combined together for hybridisation to a microarray and the blocking agent, sonicated salmon sperm DNA, has been added. This material should now be used immediately to prevent any decay. Please refer to the appropriate hybridisation protocol for the next steps.