LIMS / SOPs / measurements / microarray

mRNA quantitation using microarrays

Author(s):	Matthew Rolfe
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Abstract

Global transcriptional profiling of chemostat samples is carried out using DNA microarrays. This procedure encompasses the fluorescent-labelling of RNA, the fluorescent-labelling of DNA (if required), blocking of microarray slides, hybridisation to microarrays and scanning and data analysis.

Explanation of experimental procedure

Microarray experiments traditionally have compared two RNA samples by competitive hybridisation, producing direct gene-expression ratios between the two original samples. An alternative method compares the two samples independently on separate microarrays to a common reference sample consisting of either another single RNA sample, pooled RNA or genomic DNA (DeRisi *et al*. (1997) *Science* **270**, 680-686). Here, we use fluorescently-labelled bacterial genomic DNA as a reference channel, which hybridises to every spot on the microarray. The use of a common reference allows a reduction in the number of microarrays needed for analysing a large number of conditions, removes the need for dye-swap controls and facilitates the comparison of datasets between different experiments (Yang & Speed (2002) *Nat Rev Biol* **3**, 579-588).

Content

Preparation

When working with RNA and microarrays the following precautionary procedures are standard:

- Powder-free gloves are worn whenever carrying out procedures and handling equipment or reagents
- Pipette tips are certified as DNase- and RNase-free
- All disposable plasticware (i.e. 1.5 ml microcentrifuge tubes) are baked overnight at 120 ºC

Fluorescent-labelling of RNA

Reagents

Superscript III reverse transcriptase (Invitrogen, 18080-044)
Cyanine 3-dCTP (PerkinElmer, NEL576001EA)
Random hexamer primers (Invitrogen, 48190-011)
dNTPs (Promega, U1420)
Molecular biology water, RNase and DNase free (Sigma, W4502)
QiaQuick PCR cleanup kit (Qiagen, 28104)

1. Combine 16 µg of RNA and 5 µg of random primers (1.7 µl) in a final volume of 14 µl

in Sigma ultra-pure water. If concentration of RNA is too low then concentrate RNA in a speed vac dessicator at lowest temperature setting.
2. Heat at 72 °C for 5 minutes, then place on ice for 5 minutes. Spin briefly to collect at bottom of tube.
3. To each reaction add:

6.0 µl	5x reaction buffer
3.0 µl	0.1 M DTT
0.6 µl	50x dNTP mix (25 mM dATP, dTTP. dGTP; 10 mM dCTP)
1.4 µl	Sigma ultra-pure water

4. Add 3µl Cy3-dCTP and 2 µl of Superscript III. Mix tubes by gently pipetting up & down. Wrap tubes up in foil to protect vulnerable dyes from the light.
5. Incubate in dark at 25 °C for 5 minutes.
6. Incubate in dark at 50 °C overnight.
7. To degrade the RNA (leaving fluorescently-labelled cDNA) add 15 µl 0.1 M NaOH and hydrolyse the RNA at 72 °C for 10 minutes. Add 15 µl 0.1 M HCl to neutralise the alkali.
8. Clean up the reaction using QiaQuick PCR cleanup kit, using buffer PB instead of buffer PBI, washing twice with buffer PE and eluting into 100 µl of DNase-free water.
9. Store reactions in dark on ice until needed.

Fluorescent-labelling of DNA (enough for 4 hybridisations)

Reagents
Invitrogen BioPrime Labelling Kit (Invitrogen, 18094-011)
Cyanine 5-dCTP (PerkinElmer, 577001EA)
dNTPs (Promega, U1420)
Molecular biology water, RNase and DNase free (Sigma, W4502)
QiaQuick PCR cleanup kit (Qiagen, 28104)

1. Combine 2 µg of genomic DNA in a 1.5 ml tube and make up to 21 µl with DNase-free water. Add 20 µl of 2.5x Reaction buffer/primer mix from the BioPrime labelling kit.
2. Heat at 100 ºC for 5 minutes, then place on ice for 5 minutes.
3. Briefly centrifuge to collect at bottom of tube.
4. On ice, add 5 µl of 10x dNTP mix (1.2 mM dATP, dTTP. dGTP; 0.6 mM dCTP; 10 mM Tris pH 8.0; 1 mM EDTA).
5. Add 3 µl 1mM Cy5-dCTP.
6. Add 1 µl Klenow from the kit.
7. Incubate overnight at 37 ºC.
8. The next day, stop the reaction by adding 5 µl of 0.5 M EDTA pH 8.0.
9. Clean up the reaction using QiaQuick PCR cleanup kit, using buffer PB instead of buffer PBI, washing twice with buffer PE and eluting into 100 µl of DNase-free water.
10. Store reactions in the dark on ice until needed, use 25 µl of cleaned up DNA labelling reaction per hybridisation.

Blocking of microarrays

This decreases non-specific background hybridisation by inactivating the surface of the microarray slide. Block slides on the day of use. Slides are obtained from Ocimum Biosolutions (*E. coli* K12 V2 Ocichip, Cat no. 2140-000000).

1. In a glass microscope staining jar dissolve the following at 42 ºC using a small magnetic stirrer.

4 g	Bovine Serum Albumin (fraction V)
300 ml	MilliQ water

80 ml	20x SSC
20 ml	10% SDS

2. Once dissolved, place the microarray slides in a slide-staining tray, which is then placed in the blocking solution. Incubate for 45 minutes at 42 ºC with the stirrer still present.
3. Wash with MilliQ water, by transferring the slide rack to a fresh jar and stirring for 5 minutes. At all times, minimise the length of time the slides are exposed between wash solutions to prevent irregular drying.
4. Wash a further 5 times in MilliQ water for 5 minutes.
5. Dry down in a 50 ml centrifuge tube in a centrifuge. Prepare the tubes in advance with a small amount of paper roll in the bottom. Add microarray slides to tubes with label nearest the bottom. Spin at room temperature at 800 x g for 10 minutes.
6. Store in the dark in a sealed contained to prevent dust accumulation.

Slide hybridisation

1. The entire RNA labelling reaction (100 µl) and ¼ of the DNA labelling reaction (25 µl) is combined in a 1.5 ml tube and dried down in a speed vacuum desiccator (low heat setting) until a volume less than 5 µl is reached.
2. 140 µl of salt-based hybridisation buffer (supplied with the microarray slides) is added to each hybridisation mixture.
3. cDNA is denatured by heating at 100 ºC for 3 minutes.
4. Leave to cool at room temperature in the dark for 5 minutes (do not place on ice as the buffer components may precipitate).
5. Spin down at 13000 rpm, room temperature for 10 minutes to remove any debris from the hybridisation mixture.
6. While the reaction mixture is being spun, prepare the microarray slide. Using the microarray location finder, carefully place a gene frame around the microarray.
7. Add the entire 140 µl+ of hybridisation mixture to the gene frame and carefully lower the plastic cover to seal the array.
8. Seal microarray in a slide chamber and incubate overnight at 42 ºC in the dark.
9. The next morning remove the gene frame and wash the microarrays in the following washes. Once the gene frame is removed, minimise the time slides are not in wash buffer. Twice in 2x SSC, 0.1% SDS at 42 ºC for 5 minutes
   Twice in 1x SSC at room temperature for 5 minutes
   Twice in 0.2x SSC at room temperature for 5 minutes

1. Spin slides dry in 50 ml centrifuge tubes with a small amount of paper towel in the bottom. Centrifuge is set to 800 x g, room temperature, 10 minutes. Microarray bar-code is at bottom of tube.
2. Once dry, store in dark in a dust-free container awaiting scanning.
3. Scan using a microarray scanner at 10 mm resolution and a 2 line average. Use the highest PMT gain possible that gives minimal saturation of features. For the Affymetrix 428 scanner in Sheffield, these are PMT gains of between 53-58 mV.
4. Grid using data acquisition software (e.g.Imagene) using the gene-lists supplied by Ocimum Biosolutions.
5. Normalise the data using a block-by-block normalisation (either LOWESS or by median-shifting). This can be carried out within microarray data analysis software (e.g. GeneSpring or GeneSight).
6. Determine the number of differentially-changing genes (2-fold cutoff, $p<0.05$)

Acceptable Variations

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Parts List

id	item	description	source
1	...	...	...

Changes to Previous Versions

old version	new version	changes	link to old version
...	...	...	...

no previous versions exist

Discussion and Comments