Preparation of Labeled cDNA

2nd strand labeling

This protocol is a modification of the one originally developed by Michael B. Eisen and Patrick O. Brown. 1999. DNA Arrays for Analysis of Gene Expression. In Methods in Enzymology (Weissman SM, ed.) 303: pp179-205. Academic Press, San Diego, CA. The following protocol is prepared to generate labeled cDNA from 1 μg polyA$^+$ RNA. The labeled cDNA will be enough for 1 to 2 20x40 mm microarray hybridization.

◎*Materials and Reagents*

Oligo dT-V (2 μg/μl)

5X Superscript II reaction buffer – Invitrogen Cat. 10864014

0.1M DTT – Invitrogen Cat. 10864014

Superscript II reverse transcriptase (200 U/μl) – Invitrogen Cat. 10864014

10 mM dNTP (dilute from 100mM stock) - Amersham Cat. 27-2035-03

RNaseH - Amersham Cat. E70054Y

TE pH 8.0

Centrifugal filter device Microcon YM-30 - Millipore Cat. 42410

PCR Clean up kit – Qiagen Cat. 28106

10X Klenow reaction buffer – Invitrogen Cat. 18012-021

Klenow - Invitrogen Cat. 18012-021

Random primer (3 mg/ml) - Invitrogen Cat. 48190-011

10X dNTP (0.25mM each except for dTTP at 0.09 mM) - Amersham Cat. 27-2035-01

Cy3 dUTP (25 nmole) – Amersham Cat. PA53022

Cy5 dUTP (25 nmole) – Amersham Cat. PA55022

10X SSC, dilute from 20X SSC - Invitrogen Cat. 15557-036

DEPC-Water

◎***First Strand cDNA Synthesis***

1. Mix the following in 0.2 ml PCR tubes

RNA sample (~1 μg of PolyA RNA)	X μl
DEPC-Water	24 - X μl
Oligo-dT-V	0.5 μl
Total Volume	24.5 μl

2. Incubate for 10 min at 70℃ in a PCR machine. Immediately transfer to ice. Briefly after, add:

5X Superscript Buffer	8 μl
0.1 M DTT	4 μl
10 mM dNTPs	2 μl
Superscript II RT	1.5 μl
Total Volume	40 μl

3. Mix thoroughly upon adding each reagent. Incubate for 1 hr at 42℃
4. Add: 0.25 μl RNase H, mix thoroughly. Incubate at 37℃ for 30 min

◎***First Strand cDNA Clean up:***

1. Label Microcon columns. Insert into receiving tubes. Label 1.5 ml tubes for the elutants and set aside.

2. Add 160 µl TE to first strand synthesis reaction. Mix thoroughly. Transfer to column.
3. Spin at 14,000 g for 5 min and discard flow-thru.
4. Add 200 µl TE to column. Spin again at 14,000 g for 5 min. Discard receiving tube.
5. **Invert and insert the column into a new 1.5 ml tube** for recovery of first strand cDNA product.
6. Spin at 960 x g for 1 min. (Recovery volume of about 2 µl).
7. Add 40 µl ultra pure water to column. Invert and insert the column into original 1.5 ml recovery tube.
8. Spin at 960 x g for 1 min. Adjust recovered volume to 56 µl with ultra pure water. Take out 28 µl into new 0.2 ml tube for 2nd Strand cDNA reaction.
9. Label the original 1.5 ml tube (which has the remaining 28 µl). **Save as backup** by storing at - 20℃.

◎***Second Strand cDNA Synthesis and Labeling :***

1. Mix:

   First-strand cDNA product 28 µl

   Klenow buffer 4 µl

   Random primer 1 µl
2. Incubate at 100℃ for 2 min. Leave at room temperature for 5 min.
3. Add:

   10X dNTPs 4 µl

Cy3-dUTP or Cy5-dUTP	1 μl
Klenow	2 μl
Final volume	40 μl

4. Incubate at 37℃ for 3 hrs

◎***Second Strand cDNA Clean up:***

The two second strand cDNA samples could be combined in the same tube before clean up. Removal of unincorporated nucleotides is carried out using Qiagen PCR clean up Kit, as follows:

1. Insert QiaQuick columns into the receiving tubes. Label lid of the column. (optional: Combine the two cDNA samples for each slide and adjust volume to 100 μl by adding TE).
2. Add 500 μl PB Qiagen buffer. Mix thoroughly. Pipet the solution into the column and spin at maximum speed for 1 min.
3. Discard flow-through and reinsert column into same receiving tube.
4. Wash: Add 750 μl PE Qiagen buffer to column and spin at maximum speed for 1 min.
5. Repeat wash and spin as in 4. again.
6. Spin additional 1 min at maximum speed after removing flow-through wash buffer from receiving tube.
7. Discard receiving tube and insert the column into a new 1.5 ml microcentrifuge tube.
8. Apply 50 μl EB Qiagen buffer to **center** of column. Let sit for 1 min.
9. Spin at maximum speed for 1 min to elute labeled cDNA.

10. Dry eluate in a SpeedVac then adjust final combined volume to 44.28 µl with ultra pure water. This is the labeled cDNA to be used for hybridization.