Preparation of DNA Microarray Printing Solution

The DNA microarray printing solution consists of PCR products at a final concentration of greater than 100 ng/μl (referably greater than 200 ng/μl). For quality control, all PCR reactions are evaluated on gels for size and number of bands. The concentration of the PCR products is determined using the PicoGreen Assay.

PCR Amplification of DNA

◎*Materials and Reagents*

96-Well polypropylene, V-bottom Microplate

5 mM dNTPs

25 mM $MgCl_2$

Taq DNA Polymerase

Pfu DNA Polymerase

Appropriate DNA Primers (e.g. universal primers if clones from a library are used as templates)

10X PCR buffer (300 mM Tricine pH 8.4, 500 mM KCl)

TE pH 7.5 (filtered)

◎*Cycles Temp / time*

94℃ 2min

35 cycles 94℃ 30sec; 65℃ 30sec; 72 ℃ 2min

1 cycle 72 ℃ 10min

1 cycle 4 ℃ hold to end

Note: PCR condition is optimized for GeneAmp PCR System 9700, PE Applied Biosystem

Annealing temp and extension time should be optimized according to the nature of primers and the DNA sequence to be amplified.

PCR Products Clean up

Ⅰ*-Ethanol Precipitation Method*

◎*Materials and Reagents*

3M sodium acetate pH 5.2

95% ethanol

80% ethanol

3X SSC, dilute from 20X SSC - Invitrogen Cat. 15557-036

3M Scotch aluminum tape

Plastic wrap

Multichannel pipet

1. Dry down 100 μl the PCR products to approximately 50 μl in a SpeedVac at low temperature setting (approximately 1 hour and 20 minutes).

2. Add 5 μl 3M sodium acetate pH 5.2 and 150 μl of 95% ethanol per well (or 155 μl of a mixture of both).

3. Precipitate DNA at –20℃ overnight.

4. Centrifuge at 2,750 x g at 4℃ for 60 min.

5. Remove supernatant by rapidly inverting plates, then blotting on paper towel. Be careful otherwise pellets may be lost.

6. Wash pellet with 100 µl 80% ethanol.
7. Centrifuge at 4℃, 2,750 x g for 45 min.
8. Remove supernatant by carefully inverting plates. Tap plates gently on paper towel to remove ethanol.
9. Let plate sit open in a laminar flow hood to allow remaining ethanol to evaporate. This takes anywhere from 30min to 2 hrs. Use speedvac if needed, but **do not overdry!**
10. Add 10-30 µl of filtered 3X SSC or other appropriate printing buffer. Seal plate with 3M Scotch aluminum tape.
11. Wrap plate in wet paper towels, then plastic wrap. Store at 4℃ for 48 hrs to allow DNA to go into solution.
12. Store plates with DNA solutions at –20℃ in a non-defrosting freezer.

Ⅱ-*Millipore Multiscreen Method*

◎*Materials and Reagents*

Multiscreen Vacuum Manifold – Millipore Cat. MAVM 096 OR

Multiscreen PCR plates – Millipore Cat. MANU 030

3X SSC, dilute from 20X SSC - Invitrogen Cat. 15557-036

TE pH 7.5

Multichannel pipet

96-Well polypropylene, V-bottom Microplate

3M Scotch aluminum tape

Plastic wrap

Ziploc plastic bags

1. At the conclusion of the PCR reaction, pipet the reaction products (100 μl) into the 96 well Millipore Multiscreen PCR plate.

2. Place the Multiscreen plate on top of the Multiscreen Vacuum Manifold and apply vacuum at 20 inches of Hg for 10 min until no liquid remains in the wells.

3. Wash by adding 120 μl water to each well and apply vacuum again for 13 min.

4. To resuspend PCR products, add 35 μl of 3X SSC to each well. Cover plate with cover provided and shake vigorously on a plate shaker for 10 min.

5. Remove as much as possible of the resuspended products (recovery about 30 μl) from each well using a multichannel pipet and place in a new, 96 well plate for storage. Seal plate with 3 M Scotch aluminum tape, wrap in moist paper towels and plastic wrap, then place it in a Ziploc plastic bag. Store at -20℃ (non-defrosting freezer).

Quality Control (QC) of PCR Products

Quality Control (QC) analysis of the PCR products is carried out to assure the quality of the DNA microarray spots.

QC involves two parts:

1. Agarose gel analysis is used to examine the quality of each PCR. Failed PCR or PCR with multiple products will be recorded for flagging purpose.

2. The PicoGreen assay is used to determine the concentration of the DNA printing solution (Picogreen; Molecular Probes, Oregon, USA)

Preparation of DNA Microarray Slides

DNA Microarray Slides Preparation

For commercial resources for pre-coated slides, please refer to (http://ihome.cuhk.edu.hk/~b400559/array.html)

For more information on home-made poly-lysine, see (http://cmgm.stanford.edu/pbrown/protocols/1_slides.html)

More information regarding microarray printing, see Hegde, P.;Qi, R.;Abernathy, K.;Gay, C.;Dharap, S.;Gaspard, R.; Hughes, J.E.;Snesrud, E.;Lee, N.;Quackenbush, J.(2000) A Concise Guide to cDNA Microarray Analysis.BioFeature, BioTechniques 29:548-56.

DNA Microarray Slides Printing

Printing of the DNA microarray slides should be carried out according to the recommendations of the manufacturer of choice. If non-barcoded slides are used, mark the slides with a diamond pen.

Post-processing of DNA Microarray Slides

Slide Processing Protocols

Prehybridiztion

Immobilization

1. Rehydrate arrays by holding slides (array slide down) over a bath of hot double distilled H_2O (90°C) for approximately 3 sec until a light vapor film is observed across the slide.
2. Snap-dry each array (array side up) on a 100°C hot plate for approximately 5 sec.
3. UV cross-link DNA to the slide by using a UV crosslinker (150 to 300 mJoules) or by baking the array at 80℃ for 2 to 4 hours.

Slide pre-hybridization

1. Bake the slides at 65℃, 10 min right before prehybridization.
2. Incubate slides in 3XSSC, 0.1 % SDS, 0.1 mg/ml BSA in a Coplin jar for 30 minutes to 1 hour at 50°C.
3. Wash the slides by immersing in water followed by isopropanol.
4. Dry slides using a centrifuge or blow-dry using compressed N_2.

Store slides in the slide container in a desiccator at room temp. For use at a later date.

Staining DNA microarray

◎*Materials and Reagents*

1 mM ToTo-3 iodide in dimethylformamide (DMSO) (Molecular Probes, Cat. P-3604) or

1 mM PoPo-3 iodide in dimethylformamide (DMSO) (Molecular Probes, Cat. T-3604)

10X PBS (Invitrogen, Cat. 70013-032)

Plastic cover slip (e.g. Hybrislip from Research Products)

Slide rack (Wheaton, Cat. 900234)

glass jar

SpeedVac

Microarray scanner

Wash solution

1%SDS, 10 mM Tris, 1mM EDTA, pH 7.5

1. Thaw the frozen dye (ToTo-3 iodide or PoPo-3 iodide) and then dilute the dye to final concentration of 1μM in1X PBS at room temperature (ToTo-3 staining appears to yield lower background fluorescence).
2. Apply 50 μl of the diluted dye to a plastic lip. Place a slide (array faces down) to the plastic lip. Make sure that the diluted dye immediately covers the whole slide. Alternatively, add appropriate volume of the dye to the center of the array (face up) and immediately cover the array with plastic cover slip. Note: it is critical to quickly apply diluted dye on the array.
3. Incubate the array with the diluted dye for 30 minutes at room temperature in the dark.
4. After the completion of 30 minutes incubation, plunge slide in a rack up and down for two minutes in a glass jar filled with 1X PBS.
5. Dry the slides with SpeedVac for 5 minutes.
6. If the DNA is stained by ToTo-3 iodide, scan the array by using the red laser and Cy5 filter. (ToTo-3 exhibits absorption/emission maximum of 642/660 nm when bound to double stranded DNA.) If the DNA is stained by PoPo-3 iodide, scan the slide by using

the green laser and Cy3 filter. (PoPo-3 iodide exhibits absorption/emission maximum of 534/570 nm when bound to double stranded DNA.)

RNA Isolation and Purification

TRIzol: a fast and easy method that will give good quality and yield of RNA for samples from various types of tissues.

The Pine Tree method: a very effective for isolation of RNA from tissue containing a high amount of polysaccharides and/or phenolic compounds.

Ⅰ-*TRIzol Method*

This is a modification of the procedure originally described by Chomczynski P and Sacchi N. 1987.Signal-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate –Phenol -Chloroform Extraction. *Analytical Biochemistry* 162: 156-159

◎*Materials and Reagents*

TRIzol Reagent (Commercially available from many venders)

Home-made recipe for 1 L:

Reagents	Final Concentration
Acid Phenol 380 ml	38 %
Guanidine thiocyanate 94.53 g	0.8 M
Ammonium thiocyanate 30.45 g	0.4 M
Sodium acetate, pH 5.0 33.4 ml of 3 M stock	0.1 M
Glycerol 50 ml	5 %

DEPC-Water Adjust the final volume to 1 L

0.8 M sodium citrate / 1.2 M NaCl

Isopropanol (2-Propanol)

Chloroform

DEPC-Water

75% ethanol prepared with DEPC-Water

RNase Inhibitor (e.g. aseERASE TM – BIO 101 – Cat. 2601-104)

50 ml sterile plastic screw-cap centrifuge tubes

1. Grind 1g tissue in liquid nitrogen in a mortar and pestle.
2. Transfer powdered tissue to a 50 ml sterile plastic screw-cap centrifuge tube containing 15 ml TRIzol reagent. Incubate samples at room temperature or at 60℃ for 5 min.
3. Homogenize tissue with homogenizer for 15 seconds. Repeat once.
4. Centrifuge samples at 12,000 x g at 4 ℃ for 10 min.
5. Transfer supernatant into new sterile 50 ml sterile plastic screw-cap centrifuge tube. Discard pellet.
6. Add 3 ml chloroform to each tube in hood. Shake tubes vigorously with vortex for 15 sec.
7. Let tubes sit at room temp 2-3 min. Centrifuge tubes at 10,000 x g at 4℃ for 15 min.
8. Carefully pipet aqueous phase into a clean screw-cap centrifuge tube; discard interphase and lower phase into waste.

9. Precipitate RNA by adding Isopropanol and 0.8 M sodium citrate/1.2 M NaCl, half volume of the aqueous phase each. Cover tube and mix by gentle inversion. Let sit at room temperature for 10 min.

10. Centrifuge tubes at 10,000 x g at 4℃ for 10 min. Discard supernatant.

11. Wash pellet with 20 ml of 75% ethanol. Vortex briefly.

12. Centrifuge at 10,000 x g at 4℃ for 10 min. Discard supernatant; briefly dry pellet on kimwipe.

13. Add 100-250 µl DEPC-Water, to pellet. Resuspend RNA by pipetting up and down a few times.

14. Add 1 µl RNase inhibitor aseERASE to a 250 µl RNA sample. If having problems resuspending the RNA pellet, we suggest incubation at 55 - 60℃ for 10 min.

15. Transfer sample to microcentrifuge tube at room temperature.

16. Spin samples at high speed in microcentrifuge tube for 5 min at room temperature (to pellet the material that would not resuspend).

17. Transfer RNA solution (supernatant) to a new tube. Determine RNA concentration and quality by spectrophotometry.

Note: For optimal spectrophotometric measurements, RNA aliquots should be diluted with water or buffer with a basic pH. Water with pH< 7.5 falsely decreases the 260/280 ratio.

Ⅱ-*Pine Tree Method*

This method was originally described by Chang S., Puryear J., Cairney J. (1993) A Simple and Efficient Method for Isolating RNA from Pine Trees. Plant Molecular Biology Reporter 11: 113-116.

◎***Materials and Reagents****

Extraction buffer (For RNA extraction Pine Tree Method)

2% CTAB (hexadecyltrimethylammonium bromide)

2% PVP (polyvinylpyrrolidone K 30)

100 mM Tris-HCl pH 8.0

25 mM EDTA

2.0 M NaCl

0.5 g/L spermidine

Mix and autoclave.

2% beta-mercaptoethanol **(add just before use)**

Chloroform:isoamyl alcohol (24:1)

10 M Lithium chloride

1. Warm 5 ml extraction buffer to 65℃ in a water bath, quickly add 1g ground tissue and mix by inverting the tube and vortexing.

2. Extract two times with an equal volume of chloroform:isoamyl alcohol, separating phases at room temperature by centrifugation for 10 min at 12,000 x g. Centrifuge longer if phases are not well separated.

3. Add 1/4 volume 10 M LiCl to the supernatant and mix. The RNA is precipitated overnight at 4℃ and harvested by centrifugation at 12,000 x g for 20 min. Shorter precipitations time may also be used with lower yield.

4. Optional: wash pellet with 20 ml of 75% ethanol. Vortex briefly. Centrifuge at 10,000 x g at 4℃ for 10 min. Discard supernatant; briefly dry pellet on kimwipe.

5. Dissolve pellet into 100-250 µl DEPC-H_2O and proceed with polyA^+ RNA selection directly.

Poly (A)+ RNA Purification

Poly $(A)^+$ RNA can be purified with the Oligotex mRNA Kit (Qiagen)

Quality Control of Poly $(A)^+$ RNA

The quality of the poly $(A)^+$ RNA is done by evaluation of the quality of the 1^{st} strand cDNA. That is done by gel electrophoresis analysis of labeled 1^{st} strand cDNAs.

◎*Materials and Reagents*

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

Klenow enzyme – Invitrogen Cat.18012-021

Cy3 dUTP or Cy5-dUTP (25 nmole) – Amersham Cat. PA53022 / PA55022

Oligo dT-V (A/C/G) (2 µg/µl)

5X Superscript II reaction buffer -Invitrogen Cat. 10864014

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

50X dNTPs (25mM each except for dTTP at 10 mM) - Amersham Cat. 27-2035-01

10X dNTPs (0.25 mM each except dTTP at 0.09 mM – Amersham Cat. 27-2035-01

0.1M DTT - Invitrogen Cat. 10864014

PCR clean up kit – Qiagen Cat. 28106

TE pH 8.0

2X loading buffer (0.08% bromophenol blue, 14% (w/v) sucrose in ultra pure water)

1X TAE buffer

Lambda *Hi*nd III DNA marker

◎Labeling 1st strand cDNA with Cy-dye

1. Mix 2 μl RNA (80 ng mRNA) with 0.5 μl oligo dT-V (2 μg/μl)
2. Heat 10 min at 70℃ and quickly chill on ice.
3. Add

5X Superscript II buffer	1.0 μl
0.1 M DTT	0.5 μl
50X dNTPs (25 mM dNTPs except dTTP at 10 mM)	0.1 μl
Cy3- or Cy5-dUTP	0.5 μl
Superscript II reverse transcriptase (200 U/μl)	0.4 μl

4. Incubate at 42℃ for 2 hrs.
5. Clean up reaction and elute with 50 μl EB buffer from Qiagen PCR clean up kit.
6. Dry the eluted DNA down and resuspend in 3 μl TE pH 8.0.
7. Mix 3 μl of labeled 1st strand cDNA (labeled 1st strand cDNA from 80 ng mRNA) with 3 μl of 2X loading buffer.

8. Load 6 μl into each well.

9. Run a 1.2% agarose gel in 1X TAE buffer at 100mA, 30 min. A labeled DNA molecular weight marker is used for comparison. DNA molecular weight marker is labeled as follows:

◎***Labeling Lambda Hind III with Cy-dye***

1. Mix the following components and incubate at 37℃ for 1hr

Klenow 10X buffer	4 μl
10X dNTP (0.25 mM each except dTTP at 0.09 mM)	4 μl
λ/*Hind* III (0.5 μg /μl)	4 μl
Cy3-dUTP/or Cy5-dUTP	1 μl
Klenow enzyme	2 μl
Ultra pure water	25 μl

2. Clean up reaction with Qiagen PCR clean up kit and elute with 50 μl EB buffer.

3. Dry the eluted DNA down and resuspend in 10 μl TE.

4. Mix 3 μl of the labeled DNA with 3 μl of 2X loading buffer.

5. Load 6 μl into each well. Run together with your labeled sample on a 1.2% agarose gel in 1X TAE buffer at 100mA, 30 min.

◎***QC gel analysis***

Scan gel using a Fluorescence Imager (e.g., Typhoon, Variable Mode Imager, Molecular Dynamics, NJ).Generally we look for a fairly bright smear between 500-2000 bp in size.

Preparation of Labeled cDNA

Ⅰ *-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. 27-2035-01

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 2^{nd} 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.

* From this eluate an aliquot can be taken to run Quality Control of the cDNA labeling, as described below.

Ⅱ -Aminoallyl Labeling of cDNA for Array Hybridization

◎Material and reagents

Oligo dT-V (2 µg/µl) desalted

5X Superscript II reaction buffer – Invitrogen Cat. 10864014

0.1 M DTT– Invitrogen Cat. 10864014

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

100 mM dNTPs – Amersham Cat. 27-2035-01

aminoallyl-dUTP - Sigma Cat. A0410

Monofunctional NHS-ester Cy3 - Amersham Cat. PA23001

Monofunctional NHS-ester Cy5 - Amersham Cat. PA25001

Sodium bicarbonate – Sigma Cat. S5761

Hydroxylamine hydrochloride– Sigma Cat. H9876

Microcon YM 30 – Millipore Cat. 42410

PCR clean up kit – Qiagen Cat. 28106

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

Step-1 Annealing primer to mRNA template

In two 0.2 ml RNase free PCR tubes add the following

	Cy5 mRNA	Cy3 mRNA
Oligo dTV primer	0.5 µl (1.25 µl)	0.5 µl (1.25 µl)
Random Primer	0.33 µl (1.2 µl)	0.33 µl (1.2 µl)
mRNA 1 µg (2.5~3 µg)	X µl	X µl
RNase free water	To a final volume of 15 µl	
( ) for 2.5~3 µg mRNA use		

Mix and Spin

Note: When using total RNA do not use random primer. Use only Oligo-dTV primer Incubate at 70℃ for 10 min. Spin tube and chill on ice for 10 min.

Step-2 Reverse transcription (first strand cDNA synthesis)

In a separate tube add. (Make cocktail for the desired number of reactions, except SuperScript II RTase)

Reagent	Amount
Water	3.4 µl
5X superscript buffer (Invitrogen)	6.0 µl
50X aa dUTP/dTTPs (4:1)	0.6 µl
0.1 M DTT	3.0 µl

1. Aliquot 13 µl of the cocktail in each tube containing the primer template mix.
2. Mix well and spin.
3. Incubate tubes at 65℃ for 2 min only.
4. Immediately transfer tubes to 42℃. Incubate 3 min.
5. Add 1 µl Superscript II (2 µl when use 2.5~3 µg mRNA) in each tube. Mix by flicking the tube. Spin. (Do not vortex)

6. Incubate at 42℃ for 1 hour.

7. Add 1 µl of Superscript II.

8. Mix and Spin.

9. Incubate another 1 hour at 42℃.

Note:

50X dNTP stock solution using a 4:1 ratio aminoallyl-dUTP to dTTP***.

5 µl each 100 mM dATP, dGTP, dCTP (Amersham)

4 µl 100 mM aminoallyl-dUTP** (Sigma, #A0410)

1 µl 100 mM dTTP

** Dissolve 1 mg aminoallyl-dUTP in 18 µl DEPC water, add approximately 0.68 µl 1 N NaOH, Final pH is roughly 7.0 (using pH paper).

***Altering the ratio of aminoallyl-dUTP to dTTP will affect the incorporation of Cy dye.

Step-3 Hydrolysis of RNA

Add

Reagent	Amount
0.5 M EDTA	10 µl
1.0 N NaOH	10 µl

Mix, Spin.

Incubate: 15 min. at 65℃.

Neutralize with 25 µl 1 M HEPES pH 7.4.

Step-4 Purification of Aminoallyl labeled cDNA

To continue with the amino-allyl dye coupling procedure all Tris must be removed from the reaction to prevent the monofunctional NHS-ester Cy dyes from coupling to free amine groups in solution.

1. Fill one Microcon-30 concentrator with 375 µl of ddH_2O.
2. Add neutralized reaction (75 µl) from step 3.
3. Spin at 12,000 x g for 10 min – Wash I.
4. Discard flow through.
5. Add 450 µl water to the column- Wash II.
6. Spin at 12,000 x g for 10 min.
7. Discard flow through.
8. Wash again adding 450 µl of water to the column – Wash III.
9. Spin at 12,000 x g for 10 min.
10. Discard flow through.
11. For elution, invert and insert column into a clean tube.
12. Spin for 3 min at 1000 x g.
13. Collect solution.
14. Concentrate to 9 µl in SpeedVac (Samples may be transferred to -20℃ for storage here).

Step-5 Coupling reaction

1. Add 1 µl 0.5 M sodium bicarbonate, pH 9.0 to Aminoallyl-cDNA solution to make 50 mM final concentration.

2. Add DNA- buffer solution to microfuge tube containing dried down dye (see below for instructions to prepare the monofunctional NHS-ester Cy dyes).

3. Pipet up and down to mix completely.

4. Incubate 1 hour at RT in the dark. Mix and Spin every 15 minutes.

Note: Check 0.5 M stock solution periodically for fluctuations in pH. Carbonate buffer changes composition over time; make it fresh every couple of week or keep at -20℃.

Monofunctional NHS-ester Cy3 (PA23001) and Cy5 (PA25001, Amersham) is supplied as a dry pellet. Each tube is sufficient to label 10 reactions under normal conditions. Dissolve dry pellet in 10 μl (20 μl) DMSO. Aliquot 1 μl (2 μl) into 10 single use tubes that are then dried in SpeedVac without heating. Dried dye are stored desiccated in the dark at 4℃. NHS-ester conjugated Cy dye is rapidly hydrolyzed in water. Don't store in DMSO or water. Decreasing the number of aliquots/dye tube may increase your signal.

Step-6 Quenching and cleanup

Before combining Cy3 and Cy5 samples for hybridization, unreacted NHS-ester Cy dye must be quenched to prevent cross coupling.

1. Add 4.5 μl 4 M hydroxylamine.

2. Let reaction incubate for 15 minutes in the dark.

3. To remove unincorporated/quenched Cy dyes, proceed with the use of PCR clean up kit (QIAGEN).

4. Combine Cy3 and Cy5 reactions.

5. Add 70 μl water.

6. Add 500 μl Buffer PB.

7. Apply to QIAquick column and spin at 13 K for 1 minute. (optional: reapply flow-through for optimal binding).

8. Decant flow-through.

9. Add 750 µl Buffer PE and spin 1 minute.

10. Decant flow-through.

11. Repeat washing again (for 3 times).

12. Decant flow-through.

13. Spin at 13 K rpm for 1 minute to dry the column.

14. Transfer spin unit to fresh microfuge tube.

15. Add 50 µl Buffer EB to the center of the membrane. Let sit for 1 minute.

16. Spin and collect eluatant.

17. Dry eluatant in a SpeedVac to concentrate the volume and continue with cDNA

Step-7 Hybridization reaction

To 44.28 µl probe (for 48 pins)

yeast tRNA (2 µg/µl)	6.23 µl
20X SSC	12.60 µl
2% SDS	11.90 µl

Mix well, 90 sec, 100^{o}C; 13.2 K, 2min

Ⅲ .Protocol for mRNA amplification and target preparation

Isolate total RNA using Pine Tree extraction protocol. Resuspend total RNA in DEPC water at 1 µg/µl concentration.

◎***Materials and Reagents:***

RNeasy Mini Kit (Qiagen, Cat. 74104)

Phenol: Chloroform: Isoamyl alcohol = 25: 24: 1 (Phenol: pH 7.9)

RNAsin (Promega, Cat. N2111)

SuperScript II (200 U/μl) (Invitrogen, Cat. 18064-071)

RNase H (5 U/μl) (USB, Cat. 70054Y)

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

Advantage Polymerase (Clontech, Cat. 8417-1)

Standard Stocks of 1 M NaOH contain 2 mM EDTA

T7 Transcription Kit (Ambion, T7 Megascript Kit. 1334)

QIAquick PCR Purification Kit (Qiagen, Cat. 28104)

Microcon YM-30 (Millipore, Cat. 42410)

Primer sequences (Invitrogen):

Oligo dT(15)-T7 primer:

5’ AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG CGC T(15) [57-mer] TS (template switch) oligo primer:

5’ AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG [30-mer]

All incubations are done in a thermal cycler. For starting amounts of less than or equal to 1 μg of total RNA a second round of amplification may be required to generate enough aRNA for a microarray hybridization (1 μg aRNA/ hybridization) 20X stock 10 mM each of dATP, dGTP, dCTP and 4 mM of dTTP. For a total volume of 250 μl: 25 μl dATP, dGTP, dCTP and 10 μl of dTTP + 165 μl H_2O

◎*Protocol:*

- **First strand cDNA synthesis:**

In PCR reaction tube, mix

Reagent	Amount
DEPC water (Total RNA: 0.5-3 μg)	7 μl
Oligo dT(15)-T7 primer (1 μg/μl)	1 μl

70℃ for 3 min, snap cool on ice.

To each reaction, make 1st Strand Master Mix:

Reagent	Amount
5 X First strand buffer	4 μl
TS (template switch) oligo primer (1 μg/μl)	1 μl
RNAsin	1 μl
0.1M DTT	2 μl
10mM dNTP	2 μl
Superscript II	2 μl
Total volume	12 μl
Final volume	20 μl

Incubate at 42℃ for 90 min.

- **Second strand synthesis:**

Make 2nd Strand Master Mix:

Reagent	Amount
DEPC H_2O	108 μl
Advantage PCR buffer	15 μl
10 mM dNTP mix	3 μl
Advantage Polymerase	3 μl
RNase H (5 U/μl)	1 μl

Add total of 130 μl to each tube from above (20 μl).

Incubate at 37℃ for 5 min to digest mRNA, 94℃ for 2 min to denature, 65℃ for 3 min for specific priming and 75℃ for 30 min for extension.

Stop reaction by adding 7.5 μl 1M NaOH solution containing 2 mM EDTA and incubate at 65℃ for 10min to inactivate enzyme.

- **Double strand cDNA cleanup:**

Reagent	Amount
ds cDNA sample	157.5 μl
Phenol: Chloroform: Isoamyl alcohol (25:24:1)	157.5 μl

1. Mix well by pipeting (be careful not to spill or contaminate).
2. Spin at 14,000 rpm for 5 min. at room temperature.
3. Transfer the aqueous phase to RNase/DNase-free Microcon column (YM30).
4. Label Microcon columns (YM30). Insert into receiving tubes.
5. Label 1.5 μl tubes for the elutants and set aside.
6. Add 42.5 μl DEPC water to first strand synthesis reaction. Mix thoroughly. Transfer to column.
7. Spin at maximum speed for 5 min and discard flow-thru.
8. Add 200 μl to column. Spin again at maximum speed for 5 min.
9. Discard receiving tube. **(twice)**
10. **Invert and insert the column into a new 1.5 ml tube** for recovery of first strand cDNA product.

11. Spin at 960 x g for 1 min. (Recovery volume of about 2 µl).

12. Add 20 µl DEPC water to column. Invert and insert the column into original 1.5 ml recovery tube.

13. Spin at 960 x g for 1 min,

14. Dry sample by speedvac and resuspend in 8 µl DEPC water.

- **In vitro transcription (Ambion; T7 Megascript Kit. 1334)**

Reagent	Amount
ds cDNA	8 µl
18.75 mM NTP(A, G, C and UTP)	8 µl
Reaction buffer	2 µl
Enzyme mix(RNase inhibitor and T7 phage polymerase)	2 µl
Total volume	20 µl

Incubate at 37℃ for 5-6 hr.

- **a-RNA purification using Qiagen RNeasy cloumn**

Make up RLT w/β-ME and H_2O Master Mix:

Per sample:

3.5 µl β-ME

80 µl DEPC H_2O

350 µl RLT buffer

1. Pre-aliquot 430 µl RLT w/β-ME and H_2O to 1.5 ml RNase/DNase-free tubes.

2. Transfer contents of in vitro transcription mix to the tube. Mix well.

3. Add 250 µl ethanol (95%) and mix well by pipetting. (Do not centrifuge!)

4. Apply sample (700 μl) to RNeasy mini spin column sitting in a collection tube.

5. Centrifuge 15 sec. at >= 8000 x g. Discard flow through.

6. Transfer RNeasy column to a new 2-ml collection tube (supplied). Add 500 μl Buffer RPE (which must contain ethanol) and centrifuge 15 sec. at >=8000 x g.(Discard flow-through but re-use tube.)

7. Remove flow through and pipet another 500 μl Buffer RPE onto column. Centrifuge for 2 min. at maximum speed.

8. Place RNeasy spin column into a new 2-ml collection tube and spin at full speed for 1 min. To completely dry column.

9. Transfer RNeasy column into a new 1.5-ml collection tube (supplied) and add 30 μl RNase-free water directly onto membrane. Centrifuge for 1 min. at >=8000 x g to elute. Repeat if expected yield is >= 30 μg Check RNA concentration and quality by measuring $OD_{260/280/320}$.

◎*Flourescent probe preparation:*

A. Aminoallyl labeling (aminoallyl-dUTP)

◎Material and reagents:

5X Superscript II reaction buffer – Invitrogen Cat. 10864014

0.1 M DTT– Invitrogen Cat. 10864014

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

100 mM dNTPs – Amersham Cat. 27-2035-01

aminoallyl-dUTP - Sigma Cat. A0410

Monofunctional NHS-ester Cy3 - Amersham Cat. PA23001

Monofunctional NHS-ester Cy5 - Amersham Cat. PA25001

Sodium bicarbonate – Sigma Cat. S5761

Hydroxylamine hydrochloride– Sigma Cat. H9876

Microcon YM 30 – Millipore Cat.42410

PCR clean up kit – Qiagen Cat. 28106

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

- **Protocol:**

Annealing primer to aRNA template

In two 0.2 ml RNase free PCR tubes add the following

	Cy5 mRNA	Cy3 mRNA
Random Primer (3mg/ml)	1.25 µl	1.25 µl
aRNA (1 µg)	X µl	X µl
RNase free water	To a final volume of 15 µl	

Mix and Spin

Incubate at 70℃ for 10 min.

Spin tube and chill on ice for 10 min

Reverse transcription (first strand cDNA synthesis)

In a separate tube add. (Make cocktail for the desired number of reactions, except SuperScript II RTase)

Reagent	Amount
Water	3.4 µl
5X superscript buffer (Invitrogen)	6.0 µl
50X aa dUTP/dTTPs (4:1)	0.6 µl
0.1 M DTT	3.0 µl

1. Aliquot 13 µl of the cocktail in each tube containing the primer template mix.
2. Mix well and spin
3. Incubate tubes at 65℃ for 2 min only
4. Immediately transfer tubes to 42℃. Incubate 3 min.
5. Add 1 µl Superscript II in each tube. Mix by flicking the tube. Spin. (Do not vortex)
6. Incubate at 42℃ for 1 hour
7. Add 1 µl of Superscript II
8. Mix and Spin
9. Incubate another 1 hour at 42℃.

Note:

50X dNTP stock solution using a 4:1 ratio aminoallyl-dUTP to dTTP***.

5 µl each 100 mM dATP, dGTP, dCTP (Amersham)

4 µl 100 mM aminoallyl-dUTP** (Sigma, #A0410)

1 µl 100 mM dTTP

** Dissolve 1 mg aminoallyl-dUTP in 18 µl DEPC water, add approximately 0.68 µl 1 N NaOH, Final pH is roughly 7.0 (using pH paper).

***Altering the ratio of aminoallyl-dUTP to dTTP will affect the incorporation of Cy dye.

Hydrolysis of RNA

Add

Reagent	Amount

0.5 M EDTA	10 µl
1.0 N NaOH	10 µl

Mix, Spin

Incubate: 15 min. at 65℃.

Neutralize with 25 µl 1 M HEPES pH 7.4.

Purification of Aminoallyl labeled cDNA

To continue with the amino-allyl dye coupling procedure all Tris must be removed from the reaction to prevent the monofunctional NHS-ester Cy dyes from coupling to free amine groups in solution.

1. Fill one Microcon-30 concentrator with of 375 µl of ddH_2O
2. Add neutralized reaction (75 µl)
3. Spin at 12,000 x g for 10 min – Wash I
4. Discard flow through
5. Add 450 µl water to the column- Wash II
6. Spin at 12,000 x g for 10 min
7. Discard flow through
8. Wash again adding 450 µl of water to the column – Wash III
9. Spin at 12,000 x g for 10 min
10. Discard flow through
11. For elution, invert and insert column into a clean tube

12. Spin for 3 min at 1000 x g

13. Collect solution.

14. Concentrate to 9 µl in speedvac (Samples may be transferred to -20℃ for storage here)

Coupling reaction

1. Add 1 µl 0.5 M sodium bicarbonate, pH 9.0 to Aminoallyl-cDNA solution to make 50 mM final concentration.
2. Add DNA- buffer solution to microfuge tube containing dried down dye (see below for instructions to prepare the monofunctional NHS-ester Cy dyes)..
3. Pipet up and down to mix completely.
4. Incubate 1 hour at RT in the dark. Mix and Spin every 15 minutes.

Note:*Check 0.5 M stock solution periodically for fluctuations in pH. Carbonate buffer changes composition over time; make it fresh every couple of week or keep at -20℃.

*Monofunctional NHS-ester Cy3 (PA23001) and Cy5 (PA25001, Amersham) is supplied as a dry pellet. Each tube is sufficient to label 10 reactions under normal conditions. Dissolve dry pellet in 10 µl (20µl) DMSO. Aliquot 1 µl (2 µl) into 10 single use tubes that are then dried in speed-vac without heating. Dried dyes are stored desiccated in the dark at 4℃. NHS-ester conjugated Cy dye is rapidly hydrolyzed in water. Don't store in DMSO or water. Decreasing the number of aliquots/dye tube may increase your signal.

Quenching and cleanup

Before combining Cy3 and Cy5 samples for hybridization, unreacted NHS-ester Cy dye must be quenched to prevent cross coupling.

1. Add 4.5 µl 4 M hydroxylamine.
2. Let reaction incubate for 15 minutes in the dark.
3. To remove unincorporated/quenched Cy dyes, proceed with the use of PCR clean up kit.
4. Combine Cy3 and Cy5 reactions.
5. Add 70 µl water.
6. Add 500 µl Buffer PB.
7. Apply to Qiagene column and spin at 13 K for 1 minute. (optional: reapply flow-through for optimal binding).
8. Decant flow-through.
9. Add 750 µl Buffer PE and spin 1 minute.
10. Decant flow-through.
11. Repeat washing again (for 3 times).
12. Decant flow-through.
13. Spin at 13 K rpm for 1 minute to dry the column.
14. Transfer spin unit to fresh microfuge tube.
15. Add 50 µl Buffer EB to the center of the membrane. Let sit for 1 minute.
16. Spin and collect eluatant.
17. Dry eluatant in a speed-vac to concentrate the volume and continue with cDNA

Hybridization Protocol

Hybridization reaction

To 44.28 µl probe (for 48 pins)

yeast tRNA (2 µg/µl)	6.23 µl
20X SSC	12.60 µl
2% SDS	11.90 µl

Mix well, 90 sec, 100^{o}C; 13.2 K, 2min

B. Second strand labeling (Cy dye-dUTP)

◎Materials and Reagents

Oligo dT-V (2 µg/µl) – Sigma custom ordered

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. 27-2035-01

RNaseH - Amersham Cat. E70054Y

TE pH 8.0 - Invitrogen Cat. 15568-025

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

PCR Clean up kit – Qiagen Cat. 28106

Klenow - Invitrogen Cat. 18012-021

Random primer (3mg/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

- **Protocol:**

First strand cDNA synthesis:

Mix the following in 0.2 ml PCR tubes

Reagent	Amount
aRNA sample (1 μg) +DEPC water	23.5 μl
TS primer (1 μg/μl)	1 μl
Total volume	24.5 μl

Incubate for 10 min at 70℃ in a PCR machine. Immediately transfer to ice.

Briefly after, add:

Reagent	Amount
5X superscript buffer	8 μl
0.1M DTT	4 μl
10mM dNTPs	2 μl
Superscript II RT	1.5 μl
Total volume	40 μl

Mix thoroughly upon adding each reagent.

Incubate for 1 hr at 42℃ (in a PCR machine).

Add: 0.25 μl RNase H, mix thoroughly.

Incubate at 37℃ for 30 min (in the PCR machine).

First strand cDNA clean up:

1. Label Microcon columns. Insert into receiving tubes.
2. Label 1.5 ml tubes for the elutants and set aside.
3. Add 160 µl TE to first strand synthesis reaction. Mix thoroughly. Transfer to column.
4. Spin at 14000 g for 5 min and discard flow-thru.
5. Add 200 µl TE to column. Spin again at 14000 g for 5 min. Discard receiving tube.
6. **Invert and insert the column into a new 1.5 ml tube** for recovery of first strand cDNA product.
7. Spin at 960 x g for 1 min. (Recovery volume of about 2 µl).
8. Add 40 µl ultra pure water to column. Invert and insert the column into original 1.5 ml recovery tube.
9. Spin at 960 x g for 1 min. Adjust recovered volume to 28 ul with ultra pure water.
10. Take out 28 µl into new 0.2 ml tube for 2nd Strand cDNA reaction.
11. 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

Mix:

Reagent	Amount
First-strand cDNA product	28 µl

Buffer2 (Klenow buffer)	4 µl
Random primer	1 µl

Incubate at 100℃ for 2 min. Leave at room temperature for 5 min.

Add:

Reagent	Amount
10X dNTPs	4 µl
Cy3-dUTP or Cy5-dUTP	1 µl
Klenow	2 µl
Final volume	40 µl

Incubate at 37℃ for 3 hrs in the PCR machine.

Note: The two different dyes are used to label different samples for comparison.

Second strand cDNA clean up:

1. The two second strand cDNA samples are combined in the same tube before clean up.
2. Removal of unincorporated nucleotides is carried out using PCR clean up Kit, as follows:
3. Insert QiaQuick columns into the receiving tubes.
4. Label lid of the column (**not** the side of the columns!).
5. Combine the two cDNA samples for each slide and adjust volume to 100 µl by adding 20 µl TE.
6. Add 500 µl PB Qiagen buffer. Mix thoroughly.
7. Pipet the solution into the column.
8. Spin at maximum speed for 1 min.
9. Discard flow-through and reinsert column into same receiving tube.

10. Wash: Add 750 µl PE Qiagen buffer to column.

11. Spin at maximum speed for 1 min.

12. Discard flow-through and reinsert column into same receiving tube.

13. Repeat wash and spin.

14. Spin additional 1 min at maximum speed.

15. Discard receiving tube and insert the column into a new 1.5 ml microcentrifuge tube.

16. Apply 50 µl EB Qiagen buffer to **center** of column. Let sit for 1 min.

17. Spin at maximum speed for 1 min to elute labeled cDNA.

18. Dry eluate in a speed-vac for 15 min.

19. Adjust final combined volume to 44.28 µl with ultra pure water. This is the labeled probe to be used for hybridization.

Hybridization reaction

To 44.28 µl probe

yeast tRNA (2 µg/µl)	6.23 µl
20X SSC	12.60 µl
2% SDS	11.90 µl

Mix well, 90 sec, 100℃; 13.2 K, 2min

DNA Microarray Hybridization and Washing

Ⅰ-*DNA Microarray Hybridization*：

◎***Materials and Reagents***

2% SDS - dilute from 10% SDS stock - Invitrogen Cat. 15553-027

yeast tRNA (2 μg/μl)

Printed and processed array slides

Lifterslip

3X SSC, dilute from 20X SSC - Invitrogen Cat. 15557-036

Hybridization chambers

Wheaton slide rack – Wheaton Cat. 900234

Wheaton staining dish – Wheaton Cat. 900204

Wash solutions (listed in the protocol below)

Water

1. Add to the 44.28 μl labeled cDNAs

yeast tRNA (2 μg/μl)	6.23 μl
20X SSC	12.60 μl
2% SDS	11.90 μl

2. Set slide in hybridization chamber.

3. Clean a Lifterslip with EtOH and Kimwipes. Place slip on array using either fingers or forceps.

4. Heat denature for 90 sec at 100℃.

5. Spin at maximum speed for 2 min to get rid of any dust particles.

6. Slowly inject the probe without capturing air bubbles under one corner of the Lifterslip until the array surface is covered.

7. Pipet 3 drops of 15 μL 3X SSC each onto the lower edge of the slide.

8. Tightly screw down chamber lid and carefully place chamber in a 65°C water bath. Let hybridize for 16 hrs.

Ⅱ-*Washing Conditions*

◎***Prepare beforehand:***

Turn Scanner on to let it warm up while the slides are being washed. Wipe off dust from the SpeedVac for 96-well plates, use ethanol and Kimwipe. Prepare a balance with metal slide-rack and blank glass slides.

Get wash solutions:

I 2X SSC, 0.03% SDS

II 1X SSC

III 0.05X SSC

1. Retrieve the hybridization chamber from water bath and disassemble quickly. Be sure to wipe off excess water from the chamber before opening it up.
2. Carefully place the slides into the slide holder and into the washing solution in Dish I as fast as possible.
3. Let the Lifterslip fall away by themselves. Remove the Lifterslip from the solution and discard. Make sure corners of the slips do not scratch the surface of the slides.

4. Plunge the slides up and down in a continuous motion for 2 min.

5. Transfer **just the slides** to Dish II. Wash for 2 min.

6. Transfer **slides and slide rack** to Dish III. Wash for 2 min.

7. Transfer the slide rack with the slides to the metal spin-rack. Place rack in SpeedVac. Spin (with vacuum, no heat) for 5 min.

*Retrieve slides and put into a light-proof slide box. Scan slides as soon as possible. Otherwise, slides may be stored at– 80℃ for 1-2 weeks.