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. 27-2035-03

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/dNTPs (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°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. 27-2035-03

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