

16 RNA Microarray Labelling Procedure UFIR

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Transcriptomics

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We used the NuGO Standard Operating Procedure (SOP) number 16 produced by the University of Florence Details of the SOP are available via the web link:

<http://www.nugo.org/frames.asp?actionID=28250&action=loginFromPP>

For more details please contact Department of Pharmacology, University of Florence

Backgrounds

--to be edited--

Materials

Amount	Name	Supplier	Catalogue Number	Further information
	DEPC Water	Ambion, Inc	9922	
2µl to each tube	Primer Solution			Poly dT _(12-18mers) 5µg/µl with Random Hexamers 5µg/µl
6µl 0.8µl 3µl	Reaction solution: 5 x Buffer 50 x Amino Allyl-dUTP/dNTP 0.1 M DTT			
2µl to each tube then 1µl to each tube	Superscript II Reverse Transcriptase Kit	Invitrogen	18064-022	Contains enzyme, 5x buffer (6µl), 0.1M DTT solution (3µl)
50 x 0.8 µl	Amino Allyl-dUTP/dNTP solution	Invitrogen Sigma	10297-018 A-0410	10µl dATP, 10µl dCTP, 10µl dGTP, 6µl dTTP, 4µl amino allyl dUTP (dissolved to 100mM with DEPC water).
10µl to each tube	Sodium Hydroxide			1N
10µl to each tube	EDTA			0.5M
25µl to each tube	HEPES			1M, pH 7.5
1µl to each sample	Sodium Bicarbonate			1M, pH 9.0
	CyDye post-labelling reactive dye packs	Amersham	RPN5661	12xCy3 12xCy5
	PCR purification kit	Qiagen	28104	PB Buffer Qiaquick columns
	Microcon YM-30	Millipore	42409	

	concentrating units			
20µl to each tube	Sodium acetate (NaOAc)			3M, pH5.5
	20 x SSC			
	Microfilters	Milkipore	UFC30HVOS	0.45µm
	SDS solution			10%

Main Procedures

- Probe cDNA Synthesis - Reverse Transcription Reaction
- Hydrolysis, Initial Purification, and Labelling
- Final Probe Purification

Sub Procedures

Probe cDNA Synthesis - Reverse Transcription Reaction

1) Calculate the volume of sample needed

For each reaction calculate the volume of sample needed to process 20µg of total RNA. If this volume exceeds 16.5µl, the RNA source sample will require concentrating.

2) Add samples to PCR tubes

Bring volumes to 16.5µl with DEPC water. Incubate tubes at 4°C.

3) Add primer solution

Add 2µl of the Primer Solution (oligo(DT) and random examers 1:1) to each tube.

4) Place tubes in thermocycler and prepare Reaction Solution - 15 minutes

Place tubes in thermocycler and incubate at 70°C for 10 minutes (put on ice for at least 5 minutes). Meanwhile prepare the following Reaction Solution (x1):

5x Buffer	6µl
50x Amino Allyl-dUTP/dNTP	0.8µl
0.1M DTT	3µl

Once the incubation is complete add 9.8µl of the Reaction Solution to each tube.

5) Add Reverse Transcriptase and incubate - 2 hours

Add 2µl of Superscript II Reverse Transcriptase to each tube. Place the tubes into a thermocycler, and incubate at 42°C for 2 hours. After 1 hour add 1µl of Superscript II Reverse Transcriptase to each tube.

Hydrolysis, Initial Purification, and Labelling

1) Add NaOH and EDTA

After the 2 hours incubation, remove the tubes from the thermocycler and add 10µl of 1N NaOH and 10µl of 0.5M EDTA to each.

2) Incubate - 15 minutes

Incubate for 15 min using the thermocycler at 65°C

3) Add HEPES

Add 25µl of 1M HEPES to each tube.

4) Weigh and label a Microcon YM-30 filter

For each sample, weigh and label a Microcon YM-30 filter, and assemble with its collection tube.

5) Transfer samples to the appropriately labelled YM-30 filters units

Add 450ul of DEPC water to each tube, and transfer samples to the appropriately labelled YM-30 filters units.

6) Centrifuge - 8 minutes

Centrifuge these units at 10000 x g for 8 mins.

7) Remove effluent and add DEPC water

Remove the effluent from the collection tubes, and add 470ul of DEPC water to the filter units.

8) Centrifuge - 8 minutes

Centrifuge for 10000 x g for 8 minutes.

9) Determine the retained volume

Remove the YM-30 filter units from their collection tubes and weigh to determine the retained liquid volume. 1mg = 1ul.

10) Centrifuge

Continue centrifuging these units at 10000 x g until the liquid volume is between 20ul and 18ul. Avoid centrifuging to dryness, as it will be impossible to recover the sample.

11) Centrifuge - 5 minutes

Invert the filter into a clean collection tube, and centrifuge at 1900 x g for 5 minutes.

12) Add Sodium Bicarbonate

Adjust the volume to 10ul. Add 1ul of the Sodium Bicarbonate solution to each collected sample.

13) Centrifuge

Pipette each of these samples into an appropriate Cy-dye tube and centrifuge to ensure that the liquid is at the bottom of the tube.

14) Incubate - 1.5 hours

Incubate this dye-probe coupling reaction for 1½ hours at room temperature in total darkness.

Final Probe Purification

1) Add DEPC water and sodium acetate

Add 50ul of DEPC water and 20ul of NaOAc 3M pH 5.5 to each reaction tube.

2) Add PB Buffer

Add 500ul of PB Buffer (from the PCR Purification Kit) to each tube.

3) Add solutions to Qiaquick columns

Add these solutions to appropriately labelled Qiaquick columns (from the PCR Purification Kit).

4) Centrifuge

-1 Minute

Centrifuge at 18300 x g for 1 min.

5) Observe the columns' glass-fibre filters

Decant the effluent from the collection tubes, and observe the columns' glass-fibre filters. Successful probe synthesis will be indicated by obvious blue and pink tints to these filters.

6) Add PE Buffer

Add 700ul of PE Buffer to each column, and centrifuge at 18300 x g for 1 min. Decant effluent.

7) Add PE Buffer

Repeat step #6.

8) Centrifuge

Centrifuge the columns again, empty to ensure dryness.

9) Place the columns in fresh collection tubes

Place the columns into fresh 1.7ml collection tubes.

10) Add EB Buffer and centrifuge

-<10

minutes

Add 30ul of EB Buffer to the columns' glass-fibre surface, incubate at room temperature for 5 mins, and centrifuge at 18300 x g for 1 min.

11) Add EB Buffer and centrifuge

-<10

minutes

Repeat step #10.

12) Assemble YM-30 filter units

Assemble one **YM-30 filter** unit for each dual-hybridisation sample. Into each unit combine the appropriate 60ul of collected Cy3 and Cy5 labelled probes purified above.

13) Centrifuge - 1.5 hours

Centrifuge the filter units at 10000 x g for 1hr 30mins.

14) Centrifuge - 5 minutes

Invert the filter into a fresh collection tube, and centrifuge at 1900 x g for 5 mins.

15) Adjust the collected probe solution

Adjust the collected probe solution to 80ul (70ul; 90ul) with DEPC water.

16) Add SSC and HEPES

Add the following reagents to each probe:

20x SSC	9.6 ul	8.4ul	10.8ul
1M HEPES, pH 7.5	1.53 ul	1.34ul	1.72ul

17) Add DEPC water and centrifuge

For each combined probe, add 10ul of DEPC water to a 0.45 micro filter unit, and centrifuge it at 18300 x g for 1min.

18) Transfer to new collection tubes

Discard the effluent and collection tubes, and add each filter unit to new 1.7ml collection tubes.

19) Centrifuge

Add a probe sample to each of these tubes, and centrifuge at 18300 x g for 1min.

20) Add SDS

To each effluent-probe add 1.44ul of 10% SDS (1.26ul; 1.62ul).

21) Transfer to PCR tubes, vortex and centrifuge

Transfer each probe sample to individually labelled PCR tubes, vortex, and centrifuge briefly to ensure sample is at the bottom of these tubes.

22) Place tubes in thermocycler - 2 minutes

Denature at 100°C for 2 min.

Safety

Users must comply with COSHH and local safety regulations.

Definitions

dUTP – 2'Deoxyuridine 5'-Triphosphate

DEPC - diethylpyrocarbonate

EDTA - ethylenediaminetetraacetic acid

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

PCR – polymerase chain reaction

SDS – sodium dodecyl sulphate

SSC – solution containing sodium chloride and sodium citrate

References

DeRisi J. Amino-allyl dye coupling protocol.

<http://www.microarrays.org/pdfs/amino-allyl-protocol.pdf>.