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RNA amplification and labeling of RNA probes

This protocol generates dye-labeled antisense RNA probes for microarray hybridizations. In a first step polyA-RNA is amplified by *in vitro* transcription (according to Eberwine and collaborators). During *in vitro* transcription aminoallyl-UTP is incorporated into the newly synthesized RNA. NHS-ester dyes are then directly coupled to the modified bases in a simple chemical reaction.

METHOD:

Protect all dye-containing solutions from light to prevent photo bleaching. We are using total RNA purified on Qiagen RNeasy columns as starting material but other RNA isolation methods may work as well.

I: cDNA synthesis

1: Mix:

Total RNA	3-10 µg
T7dT primer (0.5µg/µl)	1 µl
Control RNA (optional)	x µl
RNase-free H_2O	to 12 µl

2: Incubate at 70°C for 10 min. Quick-chill on ice. Collect the contents of the tube by quick centrifugation.

3: Add:

5X First Strand Buffer	4 µl
0.1 M DTT	2 µl
dNTP-mix (10 mM each)	1 µl

4: Incubate at 37°C for 2 min to equilibrate the temperature.

5: Add 1 µl of Superscript II and mix gently.
Final volume of the 1st strand reaction: 20 µl

6: Incubate at 37°C for 1 hour. Put on ice.

7: Second strand synthesis.

Add:

H_2O	92 µl
5X Second Strand Buffer	30 µl
dNTP-mix (10 mM each)	3 µl
E.coli DNA Polymerase I	4 µl
E.coli RNase H	1 µl

Final volume of the 2nd strand reaction: 150 µl

8: Incubate at 16°C for 2 hours. Do not let the temperature rise above 16°C.

9: Add 10 µl of 0.5 M EDTA to stop the reaction.

II: Purification of cDNA

1: Transfer reaction mix into a phase-lock gel tube (spin tubes briefly before use to collect the gel on the bottom).

2: Add 160 µl of phenol:chloroform:isoamyl alcohol (25:24:1). Mix thoroughly, but do not vortex. Spin 5 min at 14k rpm (12,000-16,000 x *g*).

3: Pipet off aqueous layer and place in a fresh tube.

4: Add an equal volume (~ 160 µl) of 5M $NH_4OAcetate$.

5: Add 2.5x volumes of 100% EtOH (~ 800 µl). Add 1 µl of Linear Polyacrylamide. Mix and spin for 5 min at 14k rpm, RT.

6: Remove supernatant carefully and wash pellet with 500 µl of 80% EtOH. Do not vortex.

7: Spin for 5 min at 14k rpm.

8: Repeat the wash step.

9: Dry pellet in a speed-vac.

10: Resuspend pellet in 10 µl of RNase-free H_2O.

III: *In vitro* transcription

Use Ambion's Megascript T7 kit and aminoallyl-UTP. Set up reaction at room temperature.

1: Mix the following:

RNase-free H_2O	2.5 µl
ATP solution (75 mM)	2 µl
GTP solution (75 mM)	2 µl
CTP solution (75 mM)	2 µl
UTP solution (75 mM)	1 µl
aa-UTP solution (50 mM)	1.5 µl
10X reaction buffer	2 µl
cDNA from previous step	5 µl
T7 Enzyme	2 µl
FINAL VOLUME:	20µl

2: Incubate the reaction at 37°C for 6 hours to overnight.

3: Clean up the RNA on a Qiagen RNeasy column:

- Add 80 µl of RNase-free water

- Add 350 µl of buffer RLT to the sample (add β-ME to buffer RLT before use; 10 µl β-ME / 1 ml buffer).
- Add 250 µl 100% EtOH to the sample. Mix well by pipetting.
- Transfer sample (700 µl) to an RNeasy mini spin column.
- Centrifuge for 15 sec at full speed.
- Reload column with flow-through and spin for 15 sec at full speed. This step may increase the RNA yield.
- Transfer RNeasy column to a new 2-ml collection tube (supplied in kit)
- Add 500 µl of phosphate wash buffer. (Don't use Qiagen's buffer RPE since it contains Tris that might interfere with the labeling reaction). Centrifuge for 15 sec at 14 k (>8,000 x g), and discard flow-through
- Pipet 500 µl of phosphate wash buffer onto the column. Centrifuge for 2 min at maximum speed. Discard flow-through.
- Centrifuge at full speed for 1 min.
- Transfer RNeasy column into a 1.5 ml collection tube.
- Add 30 µl of RNase-free water directly onto the RNeasy membrane.
- Centrifuge for 1 min at full speed.
- Repeat elution step with another 30 µl of water, into the same collection tube. Final volume: 50-60 µl.

4: Run 2 µl of the RNA on a 1% agarose gel or run an aliquot on a Bioanalyzer. Determine RNA concentration and calculate total yield. The transcription reaction should result in at least 30-50 µg of RNA. Store RNA at –80^{o}C until use.

IV. RNA labeling

Important: Make sure, to never over-dry the RNA in the following steps. Precipitated RNA will appear as colored speckles on the membrane of the RNeasy column after elution.

1: Dry down 5-10 µg of RNA in a speed-vac to 3 µl.

2: Add 1 µl of 0.4 M Na_2CO_3 pH 8.5. Vortex vigorously to resuspend any precipitated RNA.

3: Add 4 µl of dye solution, mix by vortexing and incubate for 1 h in the dark.

4: Add 92 µl of RNase-free water and purify RNA on an RNeasy column as described above. Use buffer RPE for the wash steps. Elute twice with 30 µl of RNase-free water. Check the membrane for colored speckles (see above).

5: Measure dye incorporation and RNA recovery by spectrophotometry: Dilute 4 µl of the eluate into 46 µl of water and analyze the sample in a spectrophotometer using a micro-cuvette. For Cy3-containing samples measure the absorbance at 260 and 550 nm and for Cy5 at 260 and 650 nm. Calculate the dye incorporation as follows:

dye molecules per 1000 nt= $(A_{dye}/A_{260})\bullet(9010\ cm^{-1}M^{-1}/\varepsilon_{dye})\bullet 1000$

with ε_{Cy3}= 150,000 $cm^{-1}M^{-1}$ and ε_{Cy5}= 250,000 $cm^{-1}M^{-1}$.

The labeling reaction normally incorporates 25-50 dye molecules per 1000 nt.

V. Probe hydrolysis

1: Combine the labeled RNAs of a sample pair (~110 μl). Dry down RNA solution to 9 μl in a speed-vac. To avoid over-drying, take the tubes out of the speed-vac a few time during the drying procedure and vortex them thoroughly.
Add 1 μl of 10X fragmentation buffer (Ambion) and mix by vortexing. Incubate at 70°C for exactly 10 min. Vortex the tubes for a few seconds. Put tubes on ice and add 1 μl of stop buffer.
Note: This method generates RNA fragments <200 nt (peak at ~85 nt).

2: Add 20 μl of RNase-free water to the sample.

- Pre-spin a Spin-50 column in a microcentrifuge at 1000 *g* for 3 min. Empty collection tube.
- Add 500 μl of RNase-free water to the column and spin column in a microcentrifuge at 1000 *g* for 3 min.
- Discard collection tube and transfer column to an amber tube.
- Load RNA sample onto the center of the column. Spin column in a microcentrifuge at 1000 *g* for 3 min.

3: Continue with the hybridization protocol for RNA probes.

MATERIALS AND REAGENTS:

Invitrogen:
E.coli Ribonuclease H (Cat. No. 18021071); 120 units, enough for 60 rxns
E.coli DNA Polymerase I (Cat. No.18010025); 1000 units, enough for 25 rxns
Superscript II (Cat. No.18064071); 4x10,000 units, enough for 200 rxns
dNTP set (100 mM) (Cat. No. 10297018)

Ambion:
Megascript T7 kit (Cat. No. 1334), enough for 40 rxns
Fragmentation reagents (Cat. No. 8740), enough for 200 rxns
Aminoallyl-UTP (Cat. No. 8437), enough for 33 rxns

Amersham:
Cy3 Mono-Reactive Dye Pack (Cat. No. PA23001)
Cy5 Mono-Reactive Dye Pack (Cat. No. PA25001)

Eppendorf:
Phase Lock Tubes, Gel Light (200), 1.5 ml tubes (Cat. No. 0032 007.961)

Qiagen:
RNeasy Mini Kit (250) (Cat. No. 74106)

Sigma:
Linear Polyacrylamide (Cat. No. 5-6575)

USA Scientific:
Spin-50 Mini-Column (Cat. No. 1415-1602)

Various sources:

T7dT primer (PAGE purified):

5′- TCT AGT CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AGG GCG TTT TTT TTT TTT TTT TTT TTN N-3′

Phenol:chloroform:isoamyl alcohol (25:24:1).

SOLUTIONS:

5x Second Strand Buffer (store at -20°C):

100 mM Tris-HCl pH 6.9
450 mM KCl
23 mM $MgCl_2$
50 mM $(NH_4)SO_4$
0.75 mM β-NAD^+

0.1 M Na_2CO_3 pH 8.5:

Prepare fresh buffer every 3-4 weeks. Make up with RNase-free water.

Phosphate Wash Buffer:

Prepare: 1 M K_2HPO_4 and 1 M KH_2PO_4 solutions using RNase-free water.
Mix 9.5 ml of 1 M K_2HPO_4 and 0.5 ml of 1 M KH_2PO_4 to generate 1 M KPO_4 pH 8.5 buffer.
For 100 ml of wash buffer mix:

0.5 ml 1 M KPO_4 pH 8.5
80 ml 100% ethanol
19.5 ml RNase-free water

Cy-dye solutions (store at -20°C protected from light):

Resuspend the dried dye of one vial in 73 μl of DMSO. Avoid repeated thawing of the dye solutions.