

Micro-cDNA amplification

1. Make volume of RNA up to 5ul (for ~ 100ng total RNA)
2. Heat for 5 mins at 65-70°C (on heat block or PCR machine)
3. In the meantime, prepare beads by washing 10ul of stock oligo-dT beads with 50ul of 1x oligo-dT binding buffer twice
4. After RNA is heated, add equal volume of 2x binding buffer to it
5. Remove last wash from beads in step 3 and add RNA from step 4
6. Incubate beads at room temperature for 20 mins, keeping the beads re-suspended
7. Remove sup (may want to save if RNA sample is valuable)
8. Wash beads with 50ul of 1x wash buffer twice
9. Wash beads once with 50ul of 1x reverse transcriptase (RT) buffer
10. Remove last wash, and add the following to the beads:
 - 4.5ul of water
 - 0.5ul of 10mM dNTP mix
 - 1ul of 10uM 3G-SP6-NotI primer
 - 1ul of 0.1M DTT
 - 2ul of 5x RT buffer
 - 1ul of SuperScript II enzyme (200U)

10ul final reaction volume

11. Incubate at 42°C for 1 hour on PCR machine
12. Remove and discard supernatant
13. Wash beads with 50ul of water twice
14. Wash beads with 50ul of 1x NotI buffer once
15. Remove last wash, and add the following to the beads:
 - 8.5ul of water
 - 1ul of 10x NotI buffer
 - 0.5ul of NotI (~5-10U)

10ul final reaction volume

16. Incubate at 37°C for 20 mins on PCR machine
17. Remove sup and discard
18. Wash beads with 50ul of water thrice
19. Remove last wash, and add the following to the beads:
 - 41 ul of water
 - 1ul of 10mM dNTP mix
 - 1ul of 1uM T7-oligo-dT₍₂₄₎ primer
 - 1ul of 1uM short SP6 primer (SP6-5')
 - 5ul of 10x Advantage Taq buffer
 - 1ul of Advantage Platinum Taq

50ul final reaction volume

20. Carry out PCR using the following parameters:

95°C for 2 mins
95°C for 15 secs
42°C for 15 secs } x 4 cycles
68°C for 6 mins }
68°C for 4 mins
4°C hold

21. Transfer supernatant to a new PCR tube and add the following:

39ul of water
1ul of 10mM dNTP mix
2ul of 10uM T7-oligo-dT₍₂₄₎ primer
2ul of 10uM 3G-SP6-NotI primer
5ul of 10x Advantage Taq buffer
1ul of Advantage Platinum Taq

100ul final reaction volume

22. Carry out PCR using the following parameters:

95°C for 2 mins
95°C for 15 secs
60°C for 15 secs } x 10 cycles
68°C for 6 mins }
68°C for 4 mins
4°C hold

23. Prepare beads for storage:

Wash 3x in 50ul water or TE
Resuspend in 50ul water or TE
Store at 4°C

24. Pass PCR reaction from 21 above through a Sephadex G-50 column equilibrated with water (or cleanup reaction using Qiagen's PCR Purification Kit).

25. Quantify cDNA in eluate, but don't waste too much in the process.

26. Concentrate cDNA to ~30ul. Use 22ul (at least ~200ng) of cDNA in an *in vitro* transcription reaction using kit from Enzo (to make cRNA for Affymetrix) or Ambion (to make mRNA). Reactions can run 6-10 hours at 37°C.

27. Clean transcription reactions using Qiagen's RNeasy Mini Kit to obtain amplified RNA. Then quantify and run ~1ug on gel after denaturing.

28. Fragment labeled RNA:

Bring RNA to 0.5 ug/ul in 1X Fragmentation buffer
Heat to 95°C for 35 min, then put on ice.

2x binding buffer

20mM Tris ph 7.5
1M LiCl
2mM EDTA

1x wash buffer

10mM Tris ph 7.5
0.15M LiCl
1mM EDTA

Superscript II from Invitrogen, Cat. No 18064-014

Not1 enzyme from Invitrogen, Cat. No.15441-025

Advantage 2 PCR kit from Clontech, Cat. No. 639207

Beads available from Dynal (now part of Invitrogen)
Dynabeads Oligo (dT)₂₅ Cat. No. 610.02

T7 transcription kit to make Affymetrix cRNA available from Enzo
BioArray High Yield RNA Transcript Labeling Kit (T7) Cat. No. 42655-10

SP6 transcription kit to make mRNA available from Ambion, Cat. No. 1330

PCR Purification Kit from Qiagen, Cat no. 28106

RNeasy Mini Kit from Qiagen, Cat. No. 74104

5x fragmentation buffer

200mM Tris Acetate pH 8.1
500 mM Potassium acetate
150 mM Mg acetate

Primers

3G-SP6-NotI (PAGE-purified):
GCGGCCGCTATTTAGGTGACACTATAGAAGAGGG

SP6-5':
ATTTAGGTGACACTATAGAA

T7-oligo-dT (PAGE-purified)
GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGGTTTTTTTTTTTTTTT
TTTTTTTTTTVN

For cDNA libraries and microarray protocols see the following :

<http://genome.wustl.edu/GSCGAP/> protocols

Hawkins RD, Bashiardes S, Helms CA, Hu L, Saccone NL, Warchol, W.E. and M. Lovett (2003) Gene expression differences in quiescent versus regenerating hair cells of avian sensory epithelia: implications for human hearing and balance disorders. *Human Molecular Genetics* 12: 1261–1272.

HAWKINS, R. D., C. A. HELMS, J. B. WINSTON, M. E. WARCHOL and M. LOVETT, 2006 Applying genomics to the avian inner ear: development of subtractive cDNA resources for exploring sensory function and hair cell regeneration. *Genomics* **87**: 801-808.

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Sajan, S.A. Warchol, W.E. and M. Lovett. 2007 Towards a Systems Biology of Mouse Inner Ear Organogenesis: Gene Expression Pathways, Patterns and Network Analysis. *Genetics* . In Press September