LIGATION ANCHORED PCR

This protocol uses either oligo d(T) or a gene specific primer (priming from 3′ to 5′) to make cDNA. After RNA hydrolysis, a single stranded adaptor is ligated to the 5′ end of the single stranded cDNA. Amplification is subsequently performed using primers to the adaptor and a gene specific primer (which can be the same as that used in the first step). PCR should be performed immediately after adapter ligation as the single stranded cDNA is sensitive to degradation. Ref: PNAS **89** 9823-9825 (1992)

First strand synthesis

1. Mix: 2μg RNA and DEPC water to 22μl
   3μl 10μM primer (gene specific 3′ to 5′ or oligo $d(T)_{15}$)

   Heat to 65°C for 2 mins then put on ice.

2. Add:
   5μl 0.1M DTT
   10μl 5x RT buffer
   2.5μl 10mM dNTPs
   2μl enzyme (Superscript II)
   0.5μl RNAsin
   5μl DEPC water (to give 50μl total)

   Incubate at 42°C for up to 2 hrs to reverse transcribe RNA

3. Hydrolyse template RNA by adding 4μl 2M NaOH and boiling for 5 min.

4. Neutralise with 4.6μl 1.7M HCl (check sample is at neutral pH before proceeding by pipetting a little on to pH indicator paper).

Adaptor ligation

1. Ligate cDNA to adaptor:

   4μl cDNA
   2μl 10x RNA ligase buffer
   10μl 40% PEG 8000
   2μl 10mM hexamine colbalt chloride

1μl T4 RNA ligase
1μl 25μM adaptor (LA-PCR-ANCHOR)

Leave overnight in dark at room temperature.

PCR reaction

1. Set up PCR reaction as follows:

2 μl	cDNA template (from above)
5 μl	10 X PCR buffer
4 μl	25 mM $MgCl_2$
1 μl	10 mM dNTP's
1 μl	Primer 1 (25μM) (LA-PCR-T3)
1 μl	Primer 2 (25μM) (can be same as use in 1)
0.5 μl	Taq DNA Polymerase
35.5 μl	dH_2O
50 μl	TOTAL

2. Cycle as follows:

94°C (2')	X 1
94°C (30s), 55°C (30s), 72°C (2')	X 34
72°C (5')	X 1
cool to 4°C.	

25 μl of the final PCR product should be run out on a 1% agarose gel, viewed and blotted for band verification by hybridization. The remaining 25 μl of the PCR reaction can then be used, if positives are forthcoming, to clone and sequence the band of interest.

Primers

LA-PCR-T3

5' GCGGCCGCTTATTAACCCTCACTAAA 3'

LA-PCR-ANCHOR (needs to be phsophorylated at the 5' end and blocked with a ddNTP at the 3' end)

5' TTTAGTGAGGGTTAATAAGCGGCCGCGTCGTGACTGGGAGCGC 3'