

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: 2 μ g RNA and DEPC water to 22 μ l
3 μ l 10 μ M primer (gene specific 3' to 5' or oligo d(T)₁₅)

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

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

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

3. Hydrolyse template RNA by adding 4 μ l 2M NaOH and boiling for 5 min.
4. Neutralise with 4.6 μ l 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:
4 μ l cDNA
2 μ l 10x RNA ligase buffer
10 μ l 40% PEG 8000
2 μ l 10mM hexamine cobalt chloride

1 μ l T4 RNA ligase
1 μ l 25 μ M adaptor (LA-PCR-ANCHOR)

Leave overnight in dark at room temperature.

PCR reaction

1. Set up PCR reaction as follows:

2 μ l	cDNA template (from above)
5 μ l	10 X PCR buffer
4 μ l	25 mM MgCl ₂
1 μ l	10 mM dNTP's
1 μ l	Primer 1 (25 μ M) (LA-PCR-T3)
1 μ l	Primer 2 (25 μ M) (can be same as use in 1)
0.5 μ l	Taq DNA Polymerase
35.5 μ l	dH ₂ O
50 μ l	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 μ l 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 μ l 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 phosphorylated at the 5' end and blocked with a ddNTP at the 3' end)

5' TTTAGTGAGGGTTAATAAGCGGCCGCGTCGTGACTGGGAGCGC 3'