

Improved efficiency for single-sided PCR by creating a reusable pool of first-strand cDNA coupled to a solid phase

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The cloning of a defined cDNA by single-sided PCR (1) can be a cumbersome procedure leading to a variety of differently sized amplified fragments that have to be re-cloned and re-analysed. One of the reasons for this problem is the unequal requirement of specific 5′ and common 3′ oligo-dT primers by this reaction. We thought that an improvement of specificity could be achieved when PCR is performed exclusively with the cDNA strand synthesized from the specific 5′ primer. Figure 1A shows schematically the protocol used.

The mRNA is captured by the Dynabeads® Oligo $(dT)_{25}$ (10 µl beads/10^5 cells) either directly from cell lysates or from total RNA preparations as specified by the supplier. The beads are washed three times in washing buffer (10 mM Tris HCl pH 7.5, 0.15 M LiCl, 1 mM EDTA, 0.1% SDS) and two final washes are done in RT buffer containing 50 mM Tris HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$. Reverse transcription reaction is performed in a volume of 20 µl RT buffer containing 10 mM DTT, 0.7 mM dNTP, 20 U RNAse inhibitor and 200 U of MoMLV reverse transcriptase for 1 h at 42°C. Beads are kept in suspension by shaking in a waterbath. After the first strand cDNA synthesis is completed, the mRNA/cDNA complex is heated to 95°C for 1 min in 2 mM EDTA pH 7.5 and the supernatant containing the melted mRNA is removed and discarded. This heat treatment is repeated once. Then, the beads are washed in 1×TB buffer (50 mM KCl, 20 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$ and 0.1 mg/ml BSA). For the synthesis of the second strand a specific 5′ primer (0.5 pmoles) and 1 U Taq polymerase in 100 µl 1×TB buffer containing 200 µM dNTP each are added and the following PCR cycle is performed once: 20 sec 94°C, 2 min 55–60°C and 10 min at 72°C. After a melting step at 94°C for 2 min, the vial is placed in the magnet holder and the supernatant containing the second strand DNA is recovered. Beads containing the cDNA library may be reused. Aliquots of the supernatant are then taken for synthesis of a new complementary strand (1°′ strand) using an oligo dT_{21}-anchor primer (0.5 pmoles) for the 3′ end. The reaction mix is subjected to a primer extension reaction consisting of 94°C for 20 sec, 50–55°C for 5 min and 72°C for 5 min. After completion of this reaction a new primer pair (20 pmoles each) is added and a PCR is started for 40–50 cycles. Figure 1B shows the results of the amplification of murine IL-3 and human N-*ras* mRNA/cDNA by this method.

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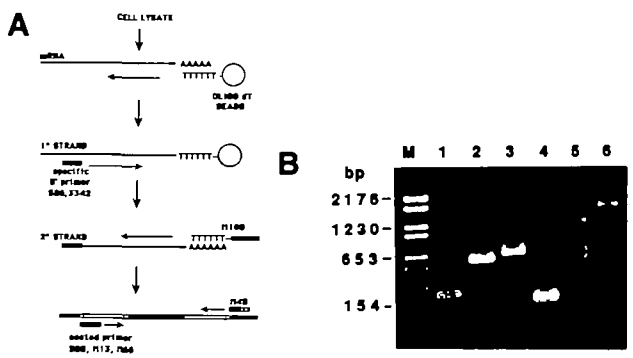

Figure 1A. Schematic protocol for single-sided PCR. The indicated oligonucleotides are as follows: 3342: 5′-dCTGCTCCTGATGCTCTTCCA-3′ for IL-3 exon 1; M13: 5′-GAGCTTTCGGAGAGTAAACC-3′ for IL-3 exon 3; 586: 5′-dATGACTGAGTACAAACTGGTGGTGGTTGGA-3′ for N-*ras* exon 1; 588: 5′-dAGAAAACAAGTGGTTATAGATGGTGAAACC-3′ for N-*ras* exon 2; M66: 5′-dGTTGTATGGGATTGCCATGTG-3′ for N-*ras* exon 4; M108: 5′-dGTTGACAGGTGACAGAGACAGT$_{21}$-3′ as oligo-dT anchor and M45: 5′-dGACAGGTGACAGAGACAGTT-3′ as anchor primer The sequences are taken from Miyatake *et al.* (2) for the IL-3 and from Hall and Brown (3) for the N-*ras* gene. **B** Agarose gel electrophoresis and ethidium bromide staining of the amplification products. Lanes 1–3 amplification of IL-3 cDNA. The nested primers in lane 1 (control) are M13–M20 (M20: 5′-dAGGTTAGCACTGTCT-CCAGA-3′); in lane 2 (control): M13–M77 (M77: 5′-dCATGGCCCCAGT-CTTCCTTG-3′); in lane 3: M13–M45. Lanes 4–6 represent the amplification products of N-*ras* cDNA. The nested primers in lane 4 (control) are 588–589 (589: 5′-dATTGATGGCAAATACACAGAGGAAGCCTTC-3′); in lane 5: M66–M45 and in lane 6: 588–M45. Lane M shows the size standard (mixture of pBR328/BglI and pBR328/HinfI). The expected correctly sized fragments are 227 bp (lane 1), 573 bp (lane 2) and 655 bp (lane 3) for IL-3 and 134 bp (lane 4), 1205 bp (lane 5) and 1623 bp (lane 6) for N-*ras*. The identity of the fragments are confirmed by Southern blotting and hybridization analysis using the corresponding oligonucleotide probes (data not shown).

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