A reliable method for amplifying cDNA using the anchored-polymerase chain reaction (A-PCR)

Jitra Kriangkum, Inna Vainshtein and John F.Elliott*
Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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A-PCR is a useful method for analyzing RNA transcripts (e.g., T-cell receptor mRNAs) where the sequence of the 5′ portion of the molecule is not known. The procedure involves reverse transcription of mRNA using $(dT)_{18}$ or a specific primer, homopolymer tailing of the first strand cDNA with dGTP and then by amplification by PCR using a non-specific anchor-oligo(dC) oligonucleotide on the 5′ end and a specific primer on the 3′ end (1, 2). Although the procedure appears straightforward, many investigators have found it difficult or impossible to amplify cDNA using the A-PCR method, largely because of technical difficulties. This report identifies the nature of these technical difficulties and provides a method for successful, reliable amplification of cDNA by A-PCR.

Initially, we found empirically that if the oligonucleotide $(dT)_{18}$ used to prime first strand synthesis was not completely removed from the cDNA prior to the homopolymer tailing step, then the A-PCR reaction would fail, even though the same material could be successfully amplified by using specific 5′ and 3′ primers. Presence of the oligonucleotide did not appear to interfere with the homopolymer tailing reaction itself, since under the reaction conditions used all of the cDNA molecules received homopolymer tails (data not shown). We then reasoned that any free $(dT)_{18}$ which remained after first strand synthesis would be dG tailed along with the cDNA, and we hypothesized that the presence of the dG-tailed $(dT)_{18}$ might inhibit the subsequent A-PCR. To test this idea we prepared dG-tailed cDNA which we knew could be successfully amplified by A-PCR, and then did a series of A-PCR reactions containing increasing amounts of either $(dT)_{18}$ or dG-tailed $(dT)_{18}$ along with the 5′ non-specific and 3′ specific primers (Figure 1). The expected A-PCR products (bands A and B) disappeared when 1 ng or more of dG-tailed $(dT)_{18}$ was added to the reaction, whereas 5 ng of $(dT)_{18}$ alone had no effect on DNA amplification. Additional experiments using the same reaction conditions showed that as much as 400 ng $(dT)_{18}$ had no inhibitory effect on A-PCR (data not shown). These results indicate that complete or nearly complete removal of the $(dT)_{18}$ primer prior to the dG tailing step is critical to the success of any subsequent A-PCR reaction.

We examined several different methods to determine which was most effective in removing $(dT)_{18}$ from the first strand cDNA. 10 μg of ^{32}P labelled $(dT)_{18}$ was added to 30 μg of test DNA, and the mixture was subject to two serial precipitations at 4°C using either polyethylene glycol (PEG 8000), ammonium acetate/ethanol, or spermine. These methods removed 84%, 90% and 93% of the $(dT)_{18}$ respectively, while three washes through a centricon-100 membrane (Amicon, each wash 1 ml TE; TE = 10 mM Tris−HCl, 1 mM EDTA, pH 8.0) removed 97% of the oligonucleotide. In contrast, passing the mixture over a Biogel A5M (BioRad) column as described in the legend for Figure 1 removed virtually all of the $(dT)_{18}$ from the early column fractions (i.e. first half of the peak). First strand cDNA was synthesized in a 100 μl reaction containing 10 μg of $(dT)_{18}$, purified by each of these methods, and then dG tailed and subject to A-PCR. Only the early fractions of the Biogel A5M column could be successfully amplified by A-PCR, indicating that none of the other methods of purification removed enough of the $(dT)_{18}$ to prevent the subsequent inhibition of A-PCR by the dG-tailed oligo (generated during the tailing reaction). However, when the amount of $(dT)_{18}$ used to prime cDNA synthesis was decreased to only 1 μg, and the number of washes was increased to five (2 ml TE each time), then centricon-100 purification also resulted in a successful amplification by A-PCR. We also tried using ribo-substituted primers, since we reasoned that they might be tailed less efficiently than $(dT)_{18}$. The primer $(dT)_{17}(rU)$ effectively primed first strand cDNA synthesis, but it was also efficiently tailed (~50% of $(dT)_{18}$), and therefore of little benefit. The oligonucleotide $(rU)_{18}$ could not be tailed, but under the reaction conditions used it was very inefficient in priming first strand cDNA synthesis.

In summary, the presence of homopolymer-tailed oligonucleotide inhibits A-PCR, and complete removal of the first strand primer prior to the tailing step is critical to the success of the subsequent amplification reaction. The larger the amount of primer used, the more stringent the purification method must be in order to obtain A-PCR amplification. Column chromatography over Biogel A5M was shown to be a very effective method for removing the oligonucleotide primer and ensuring a successful A-PCR amplification. Although the results reported above were obtained using $(dT)_{18}$, we have also obtained the same results using a sequence-specific oligonucleotide to prime first strand cDNA synthesis. The exact mechanism by which a homopolymer-tailed oligonucleotide inhibits A-PCR is unknown. For the primers used here it may be related to the depletion of the non-specific 5′ primer in a side reaction which predominates whenever a dG-tailed oligonucleotide is present in the amplification mixture.

* To whom correspondence should be addressed

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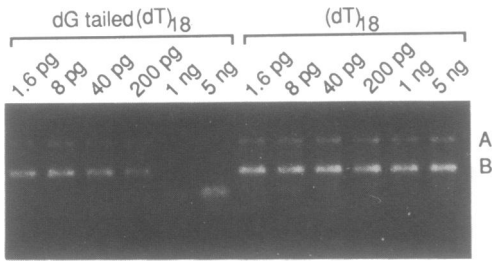

Figure 1. 25 μg total cellular RNA from human peripheral blood mononuclear cells was reverse transcribed in a 100 μl reaction containing 1 μg of $(dT)_{18}$ and 50 μCi (α-^{32}P)dCTP. The reaction was purified by passage over a Biogel A5M column (4×300 mm; BioRad) run in 0.1×TE, and the radiolabelled cDNA was collected in 1 drop (~ 25 μl) fractions. All fractions coming before the peak fraction were pooled and lyophilized. The resuspended cDNA was dG tailed with terminal transferase (Boehringer-Mannheim) and subjected to A-PCR using Taq DNA polymerase and primer, buffer, and nucleotide concentrations recommended by the manufacturer (Perkin-Elmer Cetus). The amplifications were performed in the presence of varying amounts of dG-tailed $(dT)_{18}$ or $(dT)_{18}$. Primers were 5′ CTATCTAGAGAGCTCGCGGCCG$(C)_{14}$ 3′ (anchor-oligo (dC)) and 5′ GACAAGCGACATTTGTTCC 3′ (3′ specific for human T-cell receptor Cδ). The reaction cycle was 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, for a total of 30 cycles. The PCR products were analyzed by agarose gel electrophoresis (1.4% gel) and ethidium bromide staining. A and B indicate the fragments expected in this A-PCR.