Benchmarks

Use of Elevated Reverse Transcription Reaction Temperatures in RT-PCR

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The use of reverse transcriptase coupled with the polymerase chain reaction (RT-PCR) has been proven to be a valuable tool in the synthesis and isolation of cDNA clones as well as the quantification of rare messenger RNA species. While a number of different approaches can be used to permit gene-specific cDNA synthesis and amplification, a recurring problem is one of high background signal (i.e., mispriming). A primary contributor to this problem may be that the RT reaction is traditionally performed at the optimal temperature for reverse transcriptase, 42°C. At this temperature and routine salt concentrations (50 to 100 mM), the initial primer annealing step is normally far below the melting temperature ($T_m$) of the 3′ primer. This can produce illegitimate (unwanted) hybridization and the subsequent amplification of unexpected PCR products. We report here that a potential solution to this problem is to increase the stringency of the RT step by using elevated reaction temperatures. In fact, the use of elevated RT reaction temperatures (50–55°C) has been utilized to overcome problems of mRNA secondary structure (1,3,5).

As a model system, a synthetic RNA for the dopamine D2 receptor (4) was amplified in an RT-PCR using sequence-specific primers. Briefly, 10 pg (121 fmol) of in vitro-synthesized D2 RNA were converted to a first-strand cDNA using a 3′-specific primer (5′-TCTGCGGCTCATCGTCTTAG - 3′; $T_m = 64$°C at 2°C per A/T and 4°C per G/C). The RT reaction was performed at a number of temperatures between 42°C and 60°C for 60 min. A portion of the RT reaction (2.5 µL) was then amplified by PCR. The PCR step was conducted in a total volume of 25 µL containing the following components (exclusive of those constituents contributed by the RT reaction): 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton® X-100, 2.5 mM MgCl₂, 100 µCi/mL ($[\alpha-32P]dCTP$, 50 units/mL Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT, USA), 1 µM of the 3′-primer described above and 1 µM of a gene-specific 5′-primer (5′-GCAGTCGAGCTTTTCAGGCC-3′, $T_m = 64$°C). The amplification was performed through 20 PCR cycles (94°C for 1.5 min, 65°C for 2 min and 72°C for 2 min) following initiation using a “hot-start” addition of the polymerase. The “hot start” entailed 5 min at 94°C during which the polymerase was added through the mineral oil layer. The products were resolved on a non-denaturing 10% polyacrylamide gel (2). The gel was then dried and the products visualized by autoradiography. A Betascope™ 603 Blot Analyzer (Betagen, Waltham, MA, USA) was used to directly quantify the radiolabeled products.

Figure 1. Dependence of RT-PCR efficiency on RT reaction temperature. RT-PCR amplifications of a synthetic D2 dopamine receptor RNA were performed at increasing RT reaction temperatures as detailed in the text. The reaction products were labeled by the inclusion of α-labeled [32P]dCTP within the PCR amplification. The products (404 bp) were resolved by polyacrylamide gel electrophoresis, quantified by using a Betascope blot analyzer (direct measurement of radioactivity) and visualized by autoradiography. The inset shows a typical autoradiograph observed at five different RT temperatures. The quantitative results from four independent amplifications are presented in the graph. The data have been normalized such that the signal from the 42°C RT reaction is assigned a value of 100%. Each point represents the mean ± SEM of four determinations.
Figure 1 illustrates the results from RT-PCR analysis of the same RNA at increasing RT temperatures. Notably, under these reaction conditions, there is no decrease in amplification when the RT step is performed at temperatures up to 50°C. This is not true of greater temperatures. When performed at 60°C, no amplification products could be detected. There was considerable variability in the amplification observed when the RT step was performed at 55°C (coefficient of variance = 1.18). This is probably due to the fact that this temperature is very near the upper temperature limit for active reverse transcriptase, and therefore minor discrepancies in the temperature will produce marked differences in the amount of cDNA product. These results are seemingly at odds with the work of Mallet and co-workers who have reported possible reverse transcriptase activity between 55°C and 65°C during RNA denaturation (1). Since we have never observed amplification when the RT reaction is conducted at 60°C or above, this discrepancy points to the possibility of two separate thermoproperties of reverse transcriptase: one being the thermoactivity (the temperatures at which the enzyme is active) and the second being thermostability (temperature at which the enzyme is not active but is not denatured). From the work of Mallet et al. (1), it appears that reverse transcriptase can survive short periods at 65°C and regain activity when cooled to 55°C and lower.

These results establish guidelines for varying the RT temperature in a specific RT-PCR application. The ability to raise the initial reaction temperature will increase specificity since the 42°C reaction temperature is generally 20 to 25 degrees below the Tm of most primers. In fact, an increase of only 3°C in RT reaction temperature (42°C vs. 45°C) dramatically decreased background signal when amplifying growth hormone receptor sequences from 1 µg total rat mRNA (X. Xu and W. Sonntag, personal communications). In this laboratory, the amplification of the D4 dopamine receptor cDNA from total striatal RNA was facilitated by performing the RT reaction at 50°C, because the high number of cycles required to visualize this rare mRNA product generates considerable unwanted PCR products (data not shown). RT reaction temperatures can therefore be tailored for best results in a specific experimental application. It must be emphasized, however, that optimal RT reaction temperatures are sensitive to the specific application (primer Tm’s, source of reverse transcriptase [AMV vs. MMLV (Moloney murine leukemia virus)], source and quality of RNA, number of cycles, etc.), and that specific conditions should be determined empirically.

REFERENCES

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