Nucleic Acids Research, 1993, Vol. 21, No. 16 3917–3918

A novel method employing UNG to avoid carry-over contamination in RNA-PCR

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Received March 9, 1993; Revised and Accepted July 6, 1993

The uracil-N-glycosylase (UNG) – dUTP approach has been shown to be effective in controlling the problem of carry-over contamination, a potent source of error (1), in the case of DNA-PCR (2, 3). This approach, however, is not useful in reactions involving the amplification of RNA templates, since substituting dUTP for dTTP in the reverse transcription (RT) reaction makes the nascent cDNA a substrate for UNG, if the latter is also present in the reaction mixture (4 and see below). Conventionally, RNA may be reverse transcribed with dTTP and a part of the RT mixture used as template for amplification in the presence of UNG. Although this strategy should reduce the incidence of false positivity, carry-over contamination could still occur. In order to minimize the chances of carry-over contamination in RNA-PCR it is essential to eliminate the transfer step between RT and PCR and perform both reactions in the presence of UNG. The end product of such a reaction must be UNG-sensitive.

We have developed a simple and novel technique to reverse transcribe RNA and subsequently to amplify the cDNA, both reactions performed in the presence of UNG. As depicted in Figure 1, both reactions are performed sequentially in the same vial, without opening the vial once the reagents have been assembled. We refer to this method as the in vial RNA-PCR (IVRP). During the course of reverse transcription the RT-mix and the Taq-mix are physically separated from each other by means of a wax-barrier. since the cDNA generated in this reaction contains thymidine and not uracil, it is resistant to degradation by UNG also present in the reaction mixture. As reverse transcription is followed by PCR, the elevated temperatures cause the wax-barrier to melt, thereby bringing the two reaction mixtures together. The PCR reaction mixture comprises of both dUTP and dTTP which are randomly incorporated into the amplified fragments. The random incorporation of uracil across the length of DNA fragments is sufficient to incapacitate such fragments from contaminating a subsequent PCR, by employing UNG.

Figure 2 shows the UNG-sensitivity of the end products of RT-PCR where total RNA was amplified under three different conditions, all performed using the IVRP format. After amplification the reaction mixtures were incubated with freshly added UNG for various periods. The products of RT and PCR, both performed in the presence of dTTP, were UNG resistant. Extensive incubation of the PCR product for 15 hrs with UNG did not affect the signal intensity (panel A). Amplicons generated in this reaction can, therefore, be a potential source of carry-over contamination. There was a remarkable loss in signal intensity when dUTP was substituted for dTTP in RT and PCR reactions (compare the 0 hr lanes of panels A and B). As pointed out before, this result indicated that cDNA containing uracil was destroyed by UNG also present in the reaction mixture. In contrast, products of reactions where RNA was reverse

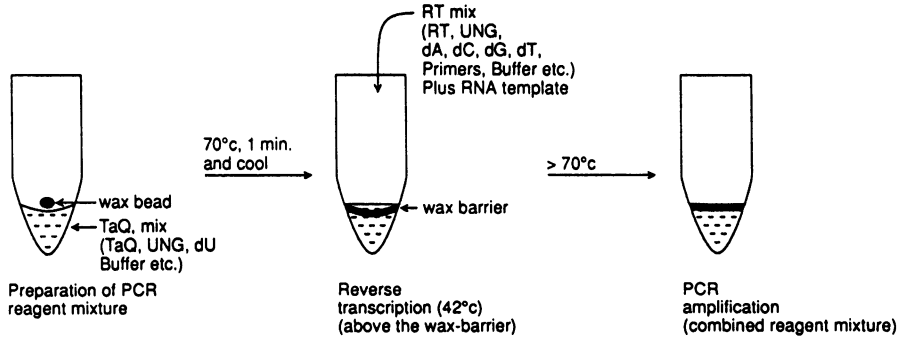

Figure 1. Schematic representation of the in vial RNA-PCR.

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transcribed in the presence of dTTP and the cDNA subsequently amplified in the presence of dTTP plus dUTP had signals of undiminished intensity (compare the 0 hr lanes of A and C). More importantly, amplicons generated in this reaction were readily digested by UNG. No signal was visible after one hour of digestion with UNG (panel C). These results demonstrated that the transfer step between RT and PCR can be eliminated, that UNG can be added to RT reaction in the presence of dTTP and that the final product of such an amplification can be UNG-sensitive provided dUTP is added during amplification. In summary, we have attempted to extend the range of the UNG-DUTP approach to RNA-PCR, in an effort to reduce the problem of carry-over contamination arising during the amplification process. To our knowledge, we have described the first attempt that is applicable to any primer pair used to amplify RNA.

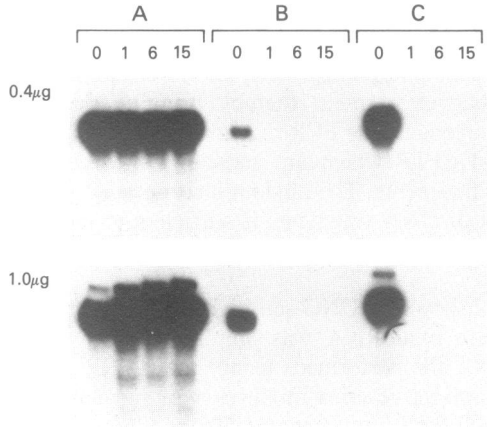

Figure 2. Amplification of HIV-1 RNA by the IVRP technique. RNA isolated (5) from H9 cells infected with the IIIB strain of HIV-1 was used at two different concentrations (0.4 or 1.0 μg/reaction). A primer pair derived from a well conserved envelope sequence of HIV-1 was used to amplify a fragment of 329 bp length (unpublished). The RNA-PCR was performed using the IVRP format under three different conditions: (**A**) both RT reaction and PCR in the presence of dTTP; (**B**) both reactions with dUTP and (C) reverse transcription and PCR in the presence of dTTP and a mixture of dTTP and dUTP, respectively. The concentrations of the reagents used for reaction C have been described below. Similar concentrations of the reagents were used for other reactions. The RT-mix of a reaction volume of 50 μl, consisted of DEPC treated sterile distilled water, 10 U of AMV-RT (Boehringer Mannheim), 0.1 U UNG (Gibco BRL), 2.0 mm DTT, 10 U RNAsin (Promega), 250 nM of each primer, 200 μM each of dATP, dCTP, dGTP and dTTP, 1.5 mM Mg^{2+}, 50 mM KCl, 10 mM Tris−HCl, pH 8.3, 100 μg/ml gelatin. The Taq-mix of a volume of 50 μl consisted of 2.0 U of Taq polymerase (Perkin Elmer Cetus), 0.1 U UNG, 200 μM dUTP, 1.5 mm Mg^{2+}, 50 mM KCl, 10 mM Tris−HCl, pH 8.3 and 100 μg/ml gelatin. The individual reaction volumes of RT and Taq mixes might vary depending on the assay conditions. Concentrations of the individual components in either reaction might as well vary depending on the primer pair used. The RT reaction was set up as depicted in Figure 1. Reverse transcription was performed for 30 min at 42°C following which the vials were incubated at 94°C for three mins to inactivate RT. PCR was performed for 33 cycles using the following profile, melting at 94°C, annealing at 55°C and extension at 65°C, each step for one min. Following amplification, to check UNG-sensitivity of the individual reaction products, the contents of each sample were divided into four equal fractions, two units of fresh UNG were added to each fraction and the vials were incubated at room temperature, for different periods as shown at the top of the lanes. At the end of the specified incubation period, two or three drops of chloroform were added to the samples to inactivate UNG. The amplified product was detected by means of solution hybridization to an end labelled oligo probe that is internal to the amplified sequence. 5 μl of ^{32}P end-labeled probe (100,000 CPM) were added to 20 μl of the UNG-treated samples. The mixture was heated to 100°C for 5 min and incubated at 56°C for one hr. The amplified DNA was separated using a 6% polyacrylamide gel and the gel was directly exposed to the X-ray film at −70°C for five or six hrs.

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