

Maximizing sensitivity and specificity of PCR by pre-amplification heating

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We have found that assembling the reaction mixture at a temperature greater than the annealing temperature improved both product yield and specificity of PCR. When reactions were maintained at 70°C in a dry heating block during addition of denatured samples to aliquotted reagent master mix, a reproducible increase in product yield was observed compared to duplicates maintained at room temperature (Table 1). Greater specificity was also seen with heating in a gel electrophoretic analysis (not shown). In addition, improved sensitivity and specificity was seen with pre-amplification heating using templates of double stranded plasmid DNA that had not been previously denatured (Figure 1), both with (Figure 1) and without (not shown) the addition of tetramethylammonium chloride (TMAC) (Fisher Scientific) (3) to the reaction master mix. This effect was observed with 3 different primer-template pairs (Table I and Figure 1). It is of note that pre-amplification heating did not improve product yield with primers of the same sequence as those used in the experiment in Table I that had been documented to lack failure sequences by HPLC. We speculate that pre-amplification heating may promote stringent primer annealing and subsequent extension, thereby increasing effective primer length. Minimization of 'primer-dimer' or primer self-annealing may also contribute. Pre-amplification heating has allowed the use of primer pairs which were inadequate for amplification of low copy number templates when reactions were assembled at room temperature.

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Table 1. Effect of sample addition at 70°C on PCR product yield from previously denatured cell lysate samples.

Sample	OD after Sample addition at: Room Temp. or	70°C	% Increase at 70°C
Uninfected cells	0.023	0.019	–
HIV-1 infected cells # 1	0.128	0.654	410%
HIV-1 infected cells- # 2	0.838	1.980	136%
HIV-1 infected cells- # 3	1.923	2.270	18%

PCR amplification of a 323 bp fragment of the HIV-1 pol gene was performed using 1 × standard PCR reaction buffer (GeneAmp®, Perkin Elmer Cetus), 200 μM dNTPs, 1.25 units Taq polymerase (AmpliTaq®, Perkin Elmer Cetus), and 20 pMol of each 20-mer primer in a 50 μl reaction (5' primer: sense strand of HIV-1 BRU – GenBank accession # KO2013 – base pair # 1889–1908; 3' primer: antisense strand of HIV-1 BRU base pair # 2211–2192) and cell lysate (1.2 × 10⁵ cell equivalents of DNA) (1). After one cycle of 94°C for 2 minutes, 45°C for 2 minutes, 72°C for 2 minutes, 39 additional cycles of 1 minute were performed at each of these temperatures (Perkin Elmer DNA Thermal Cycler). PCR products were hybridized to both HRP-linked and biotinylated probes, captured in a streptavidin-coated titerplate well, and optical density (OD) read at 450 nM (2).

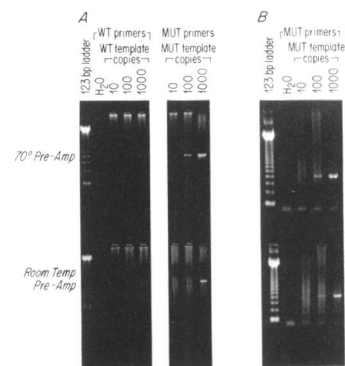


Figure 1. Effect of sample addition at 70°C on sensitivity and specificity of PCR from non-denatured, double stranded plasmid DNA templates. **A.** Amplification of a 480 bp fragment of the HIV-1 RT gene was performed using 5mM TMAC and 20 pM each of either a wild type specific (WT) or mutation-specific (MUT) primer pair (both pairs include the sense strand of HIV-1 BRU bp # 2318–2330 and the antisense strand of HIV-1 BRU bp # 2797–2774, but differ at codons that confer AZT resistance (4)). Recombinant RT plasmid DNAs (WT or MUT) diluted in 0.1 μg/μl salmon sperm DNA were added to individual reaction tubes containing matching primers at either 70°C or at room temperature. After one, cycle at 94°C for 2 minutes, 55°C for 2 minutes, and 72°C for 3 minutes, 49 additional cycles of 2 minutes at each of those temperatures were performed and 15 μl of each 50 μl reaction were electrophoresed (1.5% agarose gel). **B.** The experiment was repeated with the MUT primer and templates without TMAC. Results were identical with 0.5 mM TMAC (not shown).