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Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications

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ABSTRACT

A Hot Start Polymerase Chain Reaction (PCR) entails the withholding of at least one reagent from the reaction mixture until the reaction tube temperature has reached 60–80°C. Hot Start amplification with an AmpliWax vapor barrier uses a layer of solid wax to separate the retained reagent(s) and the test sample from the bulk of the reagents until the first heating step of automated thermal cycling melts the wax and convectively mixes the two aqueous layers. Wax-mediated Hot Start PCR greatly increases the specificity, yield, and precision of amplifying low copy numbers of three HIV targets. In the presence of 1 μg of human placental DNA (1.6×10^5 diploid genomes) the specificity improvement entails considerable to complete reduction in the amplification of mis-primed sequences and putative primer oligomers. When mis-priming is negligible, the procedural improvement still suppresses putative primer oligomerization. Hot Start PCR with an AmpliWax vapor barrier permits routine amplification of a single target molecule with detection by ethidium stained gel electrophoresis; nonisotopically visualized probing suffices for confirmation. The improved amplification performance is evident for target copy numbers below approximately 10^3.

INTRODUCTION

The Polymerase Chain Reaction (PCR) can amplify single molecules of a target nucleic acid sequence sufficiently to permit isotopic (1–5) or, if the test sample contains little background DNA, nonisotopic (5–11) detection. However, PCR amplification of low-copy-number targets is vulnerable to interference by the amplified extension of primer pairs annealed to non-target nucleic acid sequences in the test sample ('mis-priming') and by the amplified extension of two primers across one another's sequence without significant intervening sequence ('primer dimerization') (10). Primer dimers may experience amplified oligomerization during PCR to create a complex mixture of primer artifacts (12), the quantity of which often varies inversely with the yield of specific PCR product in low-copy-number amplifications.

Such nonspecificity has several negative consequences for low-copy-number analytical PCR.

(a) Often the ethidium-stained gel electrophoresis pattern for amplified DNA is so complex and the target band is so weak that analyte presence can be determined confidently only after nucleic acid probing (1,5,10,11).

(b) Yields of specific PCR product are more variable than expected for random sampling (or precise sampling when single cells are isolated by micromanipulation) and depend strongly on the amount of background nucleic acid in the test sample (2,4,5,8,11). Failure to run replicate amplifications for each test sample increases the risk of false negative findings in diagnostic settings; replication is impractical in single-cell genotyping applications (4,7–10,12).

(c) This imprecision and sensitivity to background DNA jeopardize target quantitation by PCR.

(d) Reduced confidence in PCR quantitation contributes to demand for an internal amplification standard (13–16), which adds another competing reaction to complicate the analysis.

The analytical context confronting these issues most dramatically is the detection and quantitation of low-copy-number blood-borne infectious agents, usually in the presence of high-copy-number host nucleic acid. PCR detection of HIV-1 is typical of such an analysis, where amplification from 20 μL of blood, containing about 1.6×10^5 diploid human genomes in 1 μg of DNA, often generates an uninterpretable ethidium-stained electrophoretic pattern. Here we use PCR amplification of HIV-1 targets to show that most of the observed mis-priming and primer dimerization arises during the customary and poorly controlled interval (time scale of minutes) that reactants are mixed at room temperature before starting an amplification. In Hot Start PCR, complete mixture of all reactants is delayed until they have been heated to a temperature which prevents primer annealing to non-target sequences. This method increases amplification efficiency and specificity to the point that non-probed and nonisotopically probed detection become practical and routine. The manually

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performed Hot Start process requires the opening and re-closing of all reaction tubes as they sit in the thermal cycler at 60–80°C, in order to add the missing reactant (normally enzyme); it originally was used for unstated reasons (17, 18). A prediction (19) that Hot Start PCR would show improved specificity was later verified by applying the manual procedure to low-copy-number HIV-1 amplification (20). Replacement of the conventional mineral oil vapor barrier with an AmpliWax layer provides a simple mechanism for synchronizing Hot Start amplifications without the need for manual intervention, increasing precision even more.

MATERIALS AND METHODS

Pooled human placental DNA (Sigma Chemical Co.) was repurified by phenol-chloroform extraction, chloroform extraction, and ethanol precipitation (without drying) as described by Sambrook *et al.* (21). It was stored at 4°C at a concentration of about 1 mg/mL in 10 *mM* TrisCl, 0.1 *mM* NaEDTA, pH 8.0. HIV-1 target for most of these experiments was provided by a carefully quantitated stock solution of a plasmid containing the rearranged viral genome, pSYC1857 (22), sold by Perkin-Elmer Cetus Instruments as part of a kit: GeneAmplimer HIV-1 Control Reagents.

Table 1 summarizes the primers and amplification conditions used. The *tat* region primers, QC42 and QC43, are variants of T1 and T2, respectively, reported by Meyerhans *et al.* (24), differing primarily in that introduced 5-terminal restriction sites were replaced with coding sequences of approximately the same length. All amplifications, performed in a DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instruments), employed two temperatures for each cycle (combining annealing and extension segments) and started with two cycles in which DNA denaturation occurred at 98°C for 1 min and annealing-extension occurred for 2 min at a temperature optimized for each target sequence. Thirty-eight subsequent cycles employed 94°C denaturation for 1 min and 1 min annealing-extension at the optimal temperature (**Table 1**). The last cycle was ended with a 10 min incubation at 72°C. Primers SK38 and SK39 were obtained from Perkin-Elmer Cetus Instruments. Primers QC42 and QC43 and all probes were synthesized and purified by polyacrylamide gel electrophoresis by the Cetus Nucleic Acid Chemistry Department; the probes were biotinylated at their 5 ends by use of the reagents, N-TFA-C_6-AminoModifier (Clontech Laboratories) and sulfosuccinimidyl-6-(biotinamido) hexanoate (Pierce).

After mixing of all components, reaction mixtures had the following composition: 10–15 *mM* TrisCl, pH 8.3, 50 *mM* KCl, 2.5 *mM* $MgCl_2$, 0.2 *mM* of each dNTP, 0.5 μM of each primer, and 40 U/mL recombinant *Taq* DNA polymerase. $MgCl_2$, dNTP's, and enzyme (AmpliTaq DNA polymerase) were supplied by the GeneAmp PCR Core Reagents (Perkin-Elmer Cetus Instruments), as were TrisCl buffer and KCl when the two were used in strict 1:5 molar ratio. Total reaction volumes were 100 μL unless otherwise stated. The PCR vapor barrier consisted of either 100 μL of mineral oil (Sigma Chemical Co.), delivered from a Gilson Pipetman, or one AmpliWax™ PCR Gem (Perkin-Elmer Cetus Instruments), a pellet of specially formulated paraffin optimized in mass for the reaction volume (16–17 mg for 100 μL).

For Hot Start PCR, reactants were segregated so that dNTP's, $MgCl_2$, and primers were formulated in 10*mM* TrisCl, pH 8.3 below the vapor barrier. DNA polymerase was formulated in TrisCl and KCl for addition on top of the vapor barrier; test sample, generally a mixture of human placental DNA and HIV-1 plasmid, was formulated in the same concentration of TrisCl and KCl used for the enzyme and was added last to the reaction tube. Equivalent performance was obtained if DNA polymerase and test sample were mixed together (with TrisCl and KCl) before adding above the wax layer, although this mixing sequence is less convenient for multiple test samples than is the separate addition of enzyme and DNA. The exact formulated concentration of each component was set to achieve the desired final concentration after mixing, taking into account the final volume and the added volume of the formulation. The DNA polymerase was added in a small volume, 5–10 μL, to allow the greatest possible volume of test sample to be used. The total volume above the wax layer was always adjusted to be at least 1/2 and sometimes as much as 3/4 of the total reaction volume. For example, if equal volumes were used above and below the wax layer and the total volume was 100 μL, dNTP's, $MgCl_2$, and primers were formulated at 2×final concentration in 50 μL of 10 *mM* TrisCl; DNA polymerase was formulated at 20×final concentration in 5 μL of 10 *mM* TrisCl, 100 *mM* KCl; and test sample was diluted to 45 μL 10 *mM* TrisCl, 100 *mM* KCl. Hot Start performance with AmpliWax vapor barrier is robust if (a) primers and $MgCl_2$ reside below the wax layer, (b) DNA polymerase, KCl, and DNA reside above the wax layer, (c) the KCl concentration is approximately the same in the enzyme and DNA preparations before mixing above the wax layer, and (d) both layers are buffered with TrisCl.

Table 1. Primers, probes, and amplification conditions

HIV-1 Gene	Primer/Probe Name	Sequence Positions (nt, HIV-1 genome)	Target Length(nt)	Optimal Anneal Extend T (°C)	Primary Source
tat	QC42[1]	1127–1151	365	64	this paper
	QC43[2]	1465–1491			
	probe:QC44[3]	1257–1300 (HIVZ6)			
gag	SK38	1551–1578	115	60	23
	SK39	1638–1665			
	probe:SK19	1595–1635 (HIVSF2CG)			

1 QC42: 5GAATTGGGTGTCAACATAGCAGAAT
2 QC43: 5AATACTATGGTCCACACAACTATTGCT
3 QC44: 5ATTGTAAAAAGTGTTGCTATCATTGCCAAGTTTGCTTCATAACG

To prepare reaction tubes for Hot Start PCR with an AmpliWax vapor barrier, the bottom reagent solution and an AmpliWax PCR Gem were added to a reaction tube which then was capped, heated to 80°C for 5 min in a dry bath containing a spare thermal cycler aluminum sample block (Perkin-Elmer Cetus Instruments), and cooled to room temperature over several minutes. The tube was opened long enough to add, in sequence, the enzyme formulation and the test sample. Amplification then was performed in standard fashion. After amplification, the center of the wax layer was easily penetrated by a standard air-displacement sampler to withdraw reaction mixture for electrophoretic analysis. Tubes could be heated to 80°C to re-seal the wax layer for long-term storage of PCR product at 4°C. Chloroform extraction was not done, because negligible wax was drawn into the sampler tip and any wax clinging to the outside of the tip could be rubbed off against the rim of intact wax or the inner surface of the reaction tube.

To minimize potential for reaction back-contamination and cross-contamination, reaction tube preparation was performed on a clean bench in a different room from that where test sample was added (in a laminar flow hood). A third room was used for amplification and PCR product analysis. Sampler tips containing a hydrophobic aerosol filter (250 μL Tipgard tips, DBM Scientific Corp.) and dedicated samplers were used for all transfers. Reaction tube racks were incubated for at least 30 min in 10% bleach before removal from the room used for amplification and product analysis. A disposable lab gown, cap, and mask were worn while preparing reaction tubes, were saved for use while loading the electrophoresis gel with the resulting PCR product, and then were discarded.

Electrophoretic analysis was performed on 8 μL aliquots of PCR mixture diluted with 2 μL of 0.1 *M* Na_2EDTA, 1% sodium lauryl sulfate, 0.25% bromophenol blue, 20% Ficoll 400. Molecular weight standards consisted of 10 μL aliquots of a 10 μg/mL dilution of biotinylated *Hinf* I digest of ΦX174 DNA (Life Technologies Incorporated); the fragment sizes are (726, 713), 533, 500, (427, 417, 413), 311, 249, 200, 151, 140, 118, 100, 82, 66, 48, 40, 24. Horizontal 24×20×0.6 cm tandem gels of 3% NuSieve 1% SeaKem GTG agarose (FMC BioProducts) were run for about 3 hr at 130 V in 89 *mM* Tris-borate, 2.5 *mM* NaEDTA, pH 8.3. Gels were stained for 15 min at room temperature with 5 μg/mL of ethidium bromide in electrophoresis buffer and destained for 15 min with deionized water. After Polaroid photography on a 300 nm 7500 $\mu W/cm^2$ transilluminator (Fotodyne Model 3−300), Southern transfer to Hybond N+ cationic nylon membrane (0.45 μm; Amersham) was performed with the general physical design and operational scheme of Ausubel *et al.* (25), except that (a) the blotting membrane contacted the bottom of the gel and (b) gel pre-treatment and DNA transfer were done in a single alkaline solvent, 0.4 *M* NaOH, 1.5 *M* NaCl, without acidic depurination or neutralization following base treatment. After 15 to 17 hr of transfer, the blotting membrane was washed briefly in 360 *mM* NaCl, 20 *mM* $NaPO_4$, 2 *mM* NaEDTA, pH7.4. This simplified alkaline transfer, a slight modification of the original protocol (26), gave stronger probe signals than traditional blotting in neutral solvent (25) or with recently published alkaline conditions (27). Washed membrane was incubated for 5−60 min with gentle shaking at 52°C in 20 mL (per 10×19 cm blot) of 36 *mM* NaCl, 2 *mM* $NaPO_4$, 0.2 *mM* NaEDTA, 5×Denhardt's solution, 1% sodium lauryl sulfate, pH 7.4 and then for 1 hr in the same solvent containing 1.6 *nM* biotinylated oligonucleotide probe. Probed membrane was rinsed with 36 *mM* NaCl, 2 *mM* $NaPO_4$, 0.2 *mM* NaEDTA, 0.1% Triton X-100, pH 7.4 and then agitated for about 15 min at 58°C in the same buffer. Washed probed membrane was incubated for 10 min at room temperature in 50 mL of 237 *mM* NaCl, 2.7 *mM* KCl, 1.5 *mM* KH_2PO_4, 8.0 *mM* Na_2HPO_4, 5% Triton X-100, pH 7.4 containing 0.16 μg/mL horseradish peroxidase-streptavidin conjugate (Cetus AmpliType® HLA DQα Forensic Kit, PE Xpress).

Visualization of the peroxidase-tagged immobilized probe was completed with the following five incubations at room temperature, each with gentle agitation: (a) 5 min in about 50 mL of the conjugate incubation buffer also containing 1 *M* urea and 1% dextran sulfate; (b) 5 min in about 50 mL of 100 *mM* Na citrate, pH 5.0; (c) 10 min in 60 mL of the buffer from (b) also containing 0.1 mg/mL 3,3′,5,5′-tetramethylbenzidine (TMB), shielded from room light; (d) 10 min in 60 mL of the solution from (c) also containing 1.5×10^{-3}% hydrogen peroxide, shielded from room light; (e) at least twice for 10 min in 50−100 mL deionized water, shielded from room light. Prolonged water washing is desirable to remove all unreacted TMB from the membrane and will not fade the stained pattern.

RESULTS AND DISCUSSION

Figure 1 displays an ethidium-stained electrophoretic gel and its Southern blot for four conditions of PCR amplification of 5 copies of a 365 bp sequence from the HIV-1 *tat* region. Conventional oil-overlayered amplification, in which all reactants were mixed

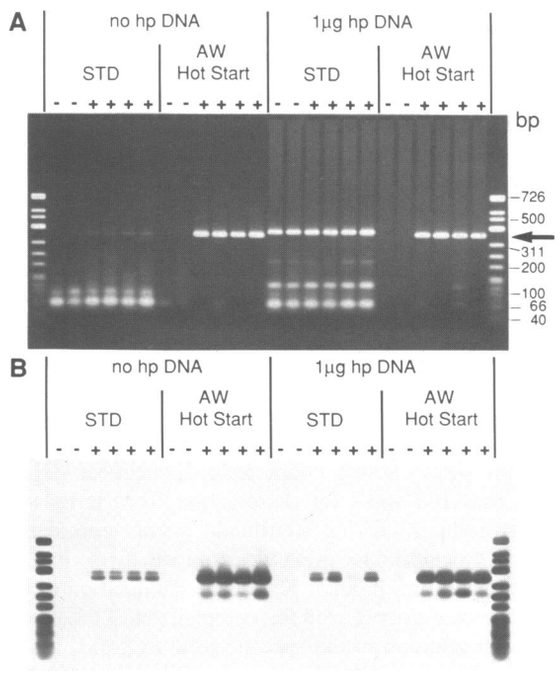

Figure 1. Effect of the Hot Start process with an AmpliWax vapor barrier on PCR amplification of a 365 bp sequence from the HIV-1 *tat* region. Two negative controls and four replicate test samples containing an average of 5 copies of HIV-1 plasmid pSYC 1857 were amplified for 40 cycles under each of four test conditions, gel electrophoresed, and subjected to peroxidase-visualized Southern blotting as described under Materials and Methods. Primers, biotin-labeled probe, and anneal-extend temperature were those in **Table 1** for the *tat* target. The four test conditions were conventional oil-layered and wax-mediated Hot Start amplification, each with and without 1 μg human placental DNA. A: ethidium-stained agarose gel. B: HRP/TMB-stained Southern blots of the gel in A.

at room temperature for an uncontrolled interval of up to 10 min before thermal cycling, is compared to Hot Start PCR with an AmpliWax layer. The wax vapor barrier prevented mixing of DNA polymerase and DNA-containing test sample with the other reactants until the first heating step reached 70–80°C. Each procedure was run with and without the presence of 1 μg of human placental DNA in the 100 μL reaction mixture, intended to model the abundant host nucleic acid usually present in test samples for the PCR detection of blood-borne infectious agents. Ethidium-stained gel electrophoresis of conventional amplifications showed no consistent differences between positive samples and negative controls. The wax-mediated Hot Start process gave high and precise yields of the expected 365 bp band, completely absent in negative controls; the identity of this band was confirmed by Southern blotting visualized by peroxidase oxidation of the TMB chromogen. Probed detection showed that conventional PCR created the specific product, though with greatly reduced average yield; in one of the eight reactions, the yield was so low that a false negative outcome might have been reported. Ethidium staining revealed traces of this specific product in the absence, but not the presence, of 1 μg of background DNA.

Equally dramatic as these effects on specific product yield and its precision was the specificity improvement by wax-mediated Hot Start PCR, which completely suppressed bands at approximately 105 and 75 bp, tentatively assigned to primer oligomers. In the presence of 1 μg of background DNA, it also eliminated strong mis-primed bands at approximately 405 and 135 bp as well as less prominent products elsewhere in the gel lanes. [Low-molecular-weight bands from negative controls lacking background DNA are assigned to primer oligomers; the remaining bands from negative controls containing background DNA represent mis-primed side products. Primer oligomerization is considered putative in the absence of sequence data.] The 405 bp mis-primed product easily could be mistaken for the 365 bp specific product unless the ethidium-stained pattern is closely examined or probing is done, especially with shorter electrophoretic runs or in denser gels. The Southern blots provide two other interesting details: (1) a 410 bp satellite band from conventional amplifications, not seen with AmpliWax-mediated Hot Start, and (2) a probe-complementary product at about 270 bp in wax-mediated PCR, not evident with oil overlayering or in the ethidium-stained pattern from the Hot Start reactions. Such multiplicity often is seen in PCR (see **Figures 2** and **3**) and has been attributed (28) to the truncation of extended primer caused by the relatively low processivity of *Taq* polymerase (28, 29). This enzyme shows poorly characterized, sequence-dependent, hot spots and cold spots for dissociation from template (29); truncation products lacking significant primer-complementary sequence will accumulate in single-stranded form, hard to see by ethidium staining. Indeed, Southern visualization of the Hot Start reaction of **Figure 1** with the complement of the probe used here gave, in addition to the specific product band, a different ethidium-invisible band at approximately 170 bp (data not shown). This outcome is consistent with the truncation hypothesis, as the strength of the enzyme-substrate interaction controlling processivity should be different at a particular locus for a given template and its complement.

Figure 2 explores the mechanism of specificity improvement by Hot Start PCR with an AmpliWax vapor barrier. Three of the four reaction conditions entailed complete mixing of reagents and test sample for 30 min, 10 min, or less than 2 min before starting the thermal cycler; a single AmpliWax pellet was added to each incubation but was not melted to form a vapor barrier before thermal cycling. The fourth condition was a wax-mediated Hot Start reaction as in **Figure 1**. Using the same target, probe, and target copy number as in **Figure 1**, the distinctions between Hot Start and non-Hot Start wax-containing reactions were the same as those in **Figure 1** between wax-mediated Hot Start and oil-overlayered conventional PCR with one conspicuous exception: the nature of the putative truncation product formed when pre-PCR mixing of reactants was permitted. Comparison of the **Figure 2** and **Figure 1** Southern blots suggests that specific product multiplicity is controlled by vapor barrier chemistry, not the Hot Start method. The 270 bp satellite band is generated under wax and not under oil, whereas oil but not wax creates the 410 bp satellite. Apparently, a significant amount of amplification occurs at and is perturbed by the water-vapor barrier interface. The most important message from **Figure 2** is that less than 2 minutes incubation of complete reaction mixture at room temperature suffices to generate abundant side products, to suppress specific amplification, and to reduce the precision of specific amplification. The significant increase in ethidium-stained nonspecific product yield and reduction in probed specific

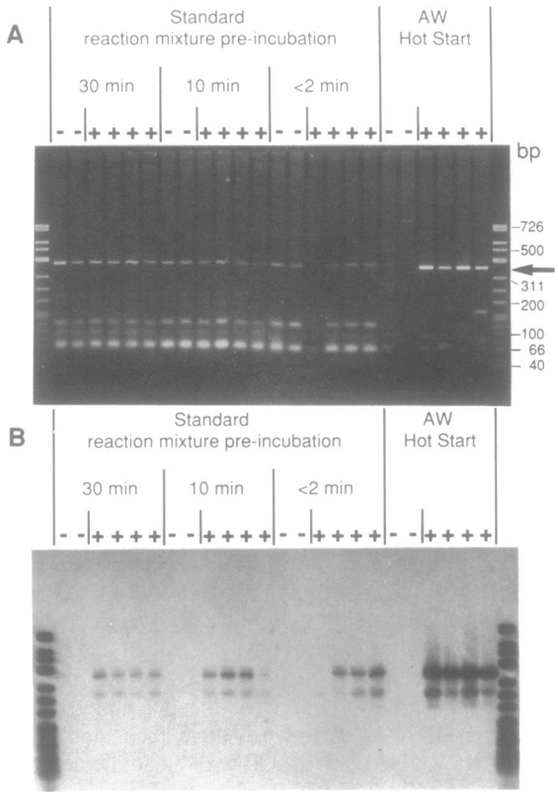

Figure 2. Kinetics of degradation of PCR performance by pre-PCR mis-priming and primer oligomerization. Two negative controls and four replicate test samples containing an average of 5 copies of HIV-1 plasmid pSYC 1857 were amplified for 40 cycles under each of four test conditions, gel electrophoresed, and Southern blotted as in **Figure 1**. All reactions contained 1 μg human placental DNA. The four test conditions were as follows: a) Hot Start amplification with an AmpliWax vapor barrier; b) non-Hot Start amplification with an AmpliWax vapor barrier (one PCR Gem added to each tube after all reactants were mixed), in which reactant mixture occurred less than 2 minutes before start of thermal cycling; c) same as (b), except that reactants were mixed 10 min before thermal cycling started; d) same as (b), except that reactants were mixed 30 min before thermal cycling started. A: ethidium-stained agarose gel. B: HRP/TMB-stained Southern blot of the gel in A.

amplification with increased incubation time confirm that practically all of the nonspecificity for this PCR target is initiated by pre-PCR side reactions, not by events during thermal cycling. Although wax greatly increases the convenience and synchronization of Hot Start amplification, the wax chemistry is not directly responsible for the improvements in specificity, yield and precision. The Hot Start process is essential for improved amplification performance. An experiment like that of **Figure 2** but omitting background DNA gave similar results except for the absence of the mis-primed bands; and analogous Hot Start performance improvement has been seen, with and without background DNA, for every low-copy-number target examined so far: three from HIV-1 and one from human papilloma virus (data not shown).

How does the Hot Start process achieve these benefits? PCR entails a three-way competition among the targeted amplification and the two major side reactions, primer oligomerization and mis-priming. Low target copy number favors both side reactions, and high background DNA promotes mis-priming. Mis-priming, much more than primer oligomerization, complicates the evaluation of specific amplification by physical methods like electrophoresis and HPLC. However, the fact that the specific yield and precision improvements are independent of the presence of background DNA suggests that primer oligomerization threatens specific amplification more seriously than does mis-priming. There has been a chicken-and-egg ambiguity over whether strong side reactions suppress target amplification, or whether low specific amplification simply allows increased side reaction. This virtual identity of the negative control and target-positive gel patterns in non-Hot Start reactions argues that side reactions, not target abundance, control the outcome of low-copy-number amplifications. Eliminating specific amplification does not enhance side reaction; instead, Hot Start reduction of the opportunity for side reaction boosts specific amplification.

Perhaps the greatest surprise in the present work is the dominance of side reactions initiated before thermal cycling has started. This phenomenon implies that *Taq* DNA polymerase may be more active at room temperature under PCR conditions than would be predicted from the conventionally determined temperature dependence of enzyme activity (29). Hot Start suppression of primer oligomerization suggests that this side reaction actually is favored by lowering the temperature. Hot Start reduction of pre-PCR mis-priming requires that the test sample contain substantial amounts of single-stranded DNA. Although this condition might reflect the natural occurrence of single-stranded regions in the eukaryotic genome (30, 31), it probably derives from the background DNA purification method. DNA drying, occasionally performed after ethanol precipitation, causes measurable denaturation (32); and we have observed increased mis-priming after re-extraction and precipitation of commercial human placental DNA, even when drying was carefully avoided (data not shown). Furthermore, the fractional strand separation of the repurified DNA still was quite small, as it showed normal hyperchromism upon heating (data not shown). Insofar as mis-priming depends on poorly controlled denaturation of background DNA, the degree of mis-priming reduction shown here to result from Hot Start PCR with AmpliWax vapor barrier is the arbitrary consequence of our DNA work-up procedure. However multiple solvent extraction and ethanol precipitation, with or without drying, are common PCR sample preparation procedures. Furthermore, even more strongly denaturing pre-PCR processes are common: deliberate pre-PCR strand separation by boiling and cold-quenching (33), cell lysis by boiling in hypotonic medium (3,5), alkalinization and subsequent neutralization to release viral targets (34), and proteinase K digestion followed by incubation above 90°C to inactivate the proteinase (4,5). Therefore the ability of wax-mediated Hot Start to reduce mis-primed nonspecificity may be even more evident in many people's hands than was demonstrated here.

Low-copy-number amplification is best validated for an average of 5 to 10 target molecules per test sample to avoid statistically

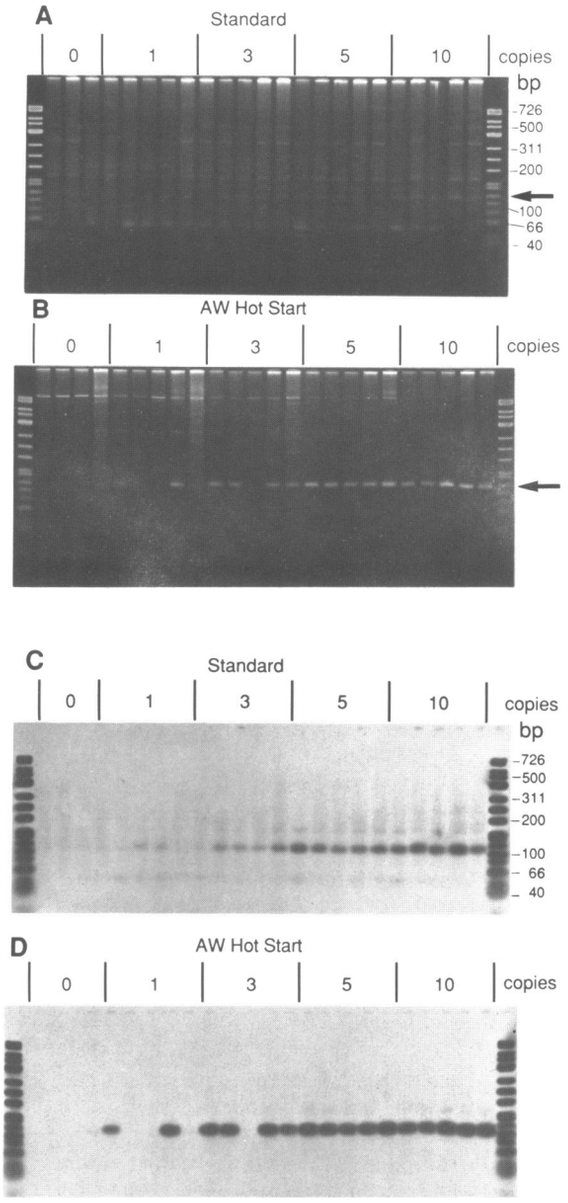

Figure 3. Effect of the wax-mediated Hot Start process on PCR amplification of very low target copy numbers. Three to five replicate amplifications, using each of the indicated average copy numbers of the HIV-1 plasmid pSYC 1857, were performed and detected as in **Figure 1** except that primers, biotin-labeled probe, and anneal-extend temperature were as specified in **Table 1** for the 115 bp HIV-1 *gag* region target. All reactions contained 1 μg human placental DNA. A: ethidium-stained agarose gel, conventional oil-overlayered PCR. B: ethidium-stained agarose gel, wax-mediated Hot Start PCR. C: HRP/TMB-stained Southern blot of the gel in A. D: HRP/TMB-stained Southern blot of the gel in B.

arising drop-outs 'false negatives'. However, the 5-copy Hot Start signals of **Figures 1** and **2** are so strong that the Hot Start method should allow routine single-copy detection. **Figure 3** titrates HIV-1 target copy number in the range of 1−10 genomes (average) per reaction, all reactions containing 1 μg human placental DNA. Conventional oil-overlayered PCR allowed no confident ethidium-stained identification of positive samples, because of the heavy yield of a wide range of mis-primed products and the essential identity of positive samples and negative controls, distinguishable only by probing; the Southern blots indicate a graded increase in product yield with initial target copy number, as well as trace satellite products which annealed to target-specific probe and increased in yield with initial target copy number. Several of the single-copy reactions show marginal signs of probe-specific product. In contrast, Hot Start PCR with an AmpliWax vapor barrier reduced putative primer dimerization, almost completely erased rampant mis-priming, greatly increased specific amplification efficiency, and yielded unmistakable ethidium-stained specific product in every reaction where the Southern blot revealed a specific band. One of the negative controls and one of the single-copy drop-outs show a greater-than-normal yield of a range of mis-primed products (though still much lower than is seen conventionally), including a nonspecific band which migrates slightly faster than the specific product. The Hot Start drop-outs at very low copy number presumably represent the statistically predicted occurrence of samples lacking target molecules. The Poisson distribution predicts that populations with an average of 1, 3, 5, and 10 molecules per sample should yield 37%, 5%, 0.7%, and 0.005% incidences of samples with no target molecules, respectively. The sampling of this experiment was too small to test these predictions sharply, but the respective 60% and 20% drop-out rates for average target densities of 1 and 3 copies per sample are acceptably close to statistical expectations. Over 100 replicate 10-copy reactions gave no drop-outs under the reaction conditions of **Figure 3** (data not shown). A final detail of **Figure 3** is the apparently much reduced relative yield of and different size distribution of sequence-specific satellite product for Hot Start as compared to conventional amplifications. As in **Figures 1** and **2**, the nature and yield of the anomalous (truncated single-stranded?) product were affected by vapor barrier chemistry.

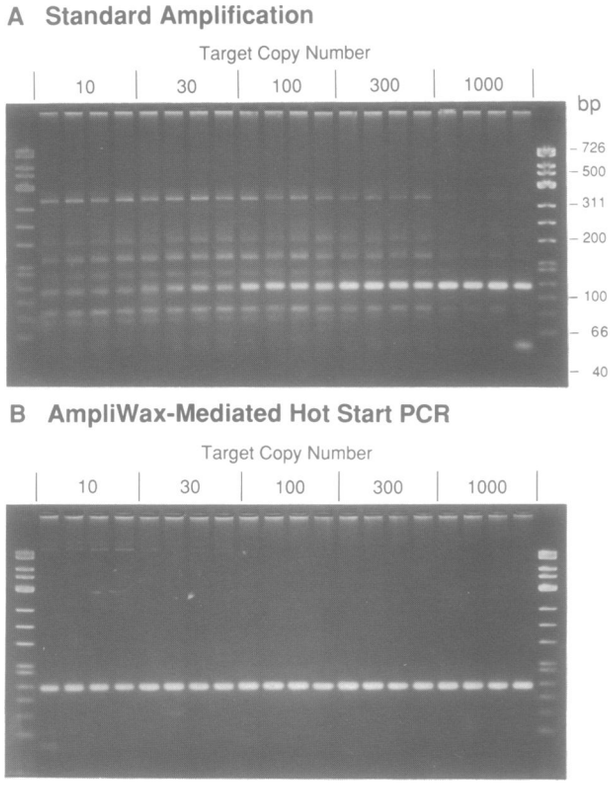

Figure 4. Titration of the upper bound on 'low-copy-number'. Four replicate amplifications of the 115 bp HIV-1 *gag* target were performed under the conditions of **Figure 3** except that mean copy number of the HIV-1 plasmid pSYC 1857 ranged from 10 to 1000 per reaction as indicated, and were detected by ethidium-stained agarose gel electrophoresis. All samples contained 1 μg human placental DNA. A: conventional oil-overlayered PCR. B: Hot Start PCR with an AmpliWax vapor barrier.

If Hot Start PCR with an AmpliWax vapor barrier renders nonisotopically detected amplification of single target molecules routine and reliable, what is the upper bound on the 'low-copy-number' range where specificity is improved? **Figure 4** compares oil- and Hot Start wax-overlayered amplifications for a series of higher target concentrations, all in the presence of 1 μg of human placental DNA. Even at 300 target copies, wax-mediated Hot Start can be a great improvement over conventional amplifications with respect to background reduction, improving confidence that a band of approximately the predicted size is not a mis-primed side product. This 100 to 1000 molecule low-copy-number threshold is suggestive rather than definitive. The vulnerability of individual amplifications to mis-priming and primer oligomerization will depend in a complex manner on primer sequences, primer concentration, target concentration, background DNA concentration, and the chemical procedure used to isolate test sample DNA. **Figure 4** also shows that the wax-mediated Hot Start process increases specific amplification efficiency so much that 40-cycle PCR of 10 to 1000 target molecules has entered the quantitative plateau where product yield no longer is proportional to initial target copy number.

Figure 3 has modeled the detection of infectious agents, residual disease, or potentially transforming nucleic acid in such contexts as clinical diagnostics and prognostics, blood banking, and biotherapeutic quality assurance, where single-copy sensitivity has vital implications but previously has been hard to guarantee even with isotopically tagged DNA probing. Genotype analysis of single sperm (4,10), ova (7,9), or blastomeres (8,12,34) is another area where single-copy-number amplification capability is needed. Currently target re-amplification or nested priming often is used to assure adequate single-copy sensitivity, especially when relying on nonisotopic, non-probed detection (8−10,35). Wax-mediated Hot Start amplification should give equivalent performance in a single reaction. However, confident exploitation of this sensitivity improvement requires scrupulous attention to laboratory hygiene in order to avoid an unacceptable false positive rate. Experience in our laboratory echoes that of Kitchin *et al.* (36); it appears that individual PCR operators can become chronic carriers and shedders of PCR product if they do not adopt clean-room standards of laboratory dress while handling amplified DNA. The common practice of preparing reaction tubes in laminar flow hoods, motivated by a desire to isolate reactions from sources of back-contamination, may actually exacerbate the problem because they draw air past the operator into the work area. In fact, 'clean benches', which blow filtered air from the work area

toward the operator, may be more useful for avoiding false-positive low-copy-number amplifications when wax-mediated Hot Start PCR is applied in routine high-volume assays.

The wax-mediated Hot Start method also should benefit PCR applications where low copy number is not an explicit concern: quantitative PCR and situations which are particularly vulnerable to side reactions. PCR product quantitation should gain in two ways. Improved sensitivity and precision have obvious analytical advantages, but additionally the specificity improvement increases the attractiveness of physical methods for PCR product detection and quantitation, such as electrophoresis and HPLC (37). HPLC has much better precision and dynamic range than the isotopic or enzyme-linked DNA probe approaches currently favored for quantitative PCR (38–41). The conventional arguments against physical (as opposed to probed) identification of amplified DNA lose force when single-copy sensitivity protects against false negatives and increased specificity reduces the chance of false positives. Multiplex (10, 42) and degenerately primed (43,44) PCR applications, which require each reaction to contain more than two distinct primer sequences, are especially prone to both primer oligomerization and mis-priming. Hot Start methods block the pre-PCR component of such nonspecificity. Single-sided PCR (45) and the amplification of long (> 1 kb) targets also may benefit from side reaction suppression because of their special vulnerability to mis-priming. We hope that the present work, showing how a wax-mediated Hot Start procedure improves the specificity, sensitivity, and precision of a particularly demanding PCR application, will stimulate the research community to push back other conventional limits to first-generation PCR performance.

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