[Letter to the Editor]

Many commercial hot-start polymerases demonstrate activity prior to thermal activation

The polymerase chain reaction (PCR) is an enzymatic technique for the *in vitro* synthesis of targeted nucleic acid regions (1), and it is an integral method in most aspects of molecular biology and genetics. PCR relies on thermally stable DNA polymerase to synthesize DNA by adding nucleotides complementary to those on a single-stranded template. By repeated cycling through 3 key temperatures (usually 95°C, ~60°C, and 72°C), exponential amplification of a target template is obtained (2).

Many molecular applications, including forensics and clinical diagnostics, are highly reliant on PCR, requiring consistent, accurate, and repeatable assays (3,4). In such instances, the generation of misleading or false results may have profound implications and, therefore, a high level of analytical accuracy is required.

The development of hot-start DNA polymerases was a significant PCR innovation. Hot-start polymerases remain inactive until the reaction is

heated to a temperature at which oligonucleotide primers can no longer anneal to the DNA template (5). This prevents non-specific amplification and primer-dimer formation, and allows reaction mixes to be prepared at room temperature without the negative impact of spurious polymerase activity (6–10).

During establishment of an assay for measuring telomere length, we observed that several commercial hot-start polymerases mediated formation of primer-dimers (11). Here,

Figure 1. Assay for polymerase activity prior to thermal activation. Polymerase activity assay performed using (A) Primer Set 1 and (B) Primer Set 2. Lanes 1–17 represent analysis of 17 different hot-start polymerase enzymes; M denotes 25-bp DNA ladder (Invitrogen, Carlsbad, CA). (C) Positive amplification control for activity of the hot-start polymerase enzymes used above; M denotes Universal DNA 150-bp ladder (KAPABIOSYSTEMS, Wilmington, MA).

we expand these observations and describe a simple method for detecting polymerase activity prior to the initial heat denaturation step. In this assay, 2 different primer pairs (Table 1) are used, and each primer pair can form a 3-bp overlap capable of annealing at room temperature. If a DNA polymerase extends either pair of primers prior to thermal activation, an ~79-bp primer-dimer will be formed which acts as the template for amplification in subsequent cycles (Figure 1). However, an effective hot-start enzyme will not produce a primer-dimer, as the enzyme activation and subsequent PCR cycling temperatures are higher than the annealing temperature of the 3-bp overlap. No genomic DNA template is required in this simple assay format.

PCR was carried out in a Mastercycler pro thermal cycler (Eppendorf, Stevenage, UK). A total reaction volume of 30 μ l containing 1 $\times$ buffer, $MgCl_2$, dNTPs, hot-start polymerase, and primers (IDT, Singapore) was used, at the concentrations recommended by the manufacturers. A robust amplicon was used as a positive control for polymerase activity (Table 1) in a reaction containing ~30 ng of human genomic DNA (Figure 1C). The reactions were prepared at room temperature, and hot-start polymerase activity was assayed using touchdown PCR (12). The initial denaturation time and extension temperature were adjusted according to the enzyme suppliers' specifications and are specified in Table 2. Cycling conditions consisted of denaturation at 95°C for 15 s, annealing for 15 s, and extension for 45 s. The initial annealing temperature was 65°C, and this was decreased by 1°C per cycle for 10 cycles, followed by 25 cycles at 55°C. A final extension was performed for 5 min.

In total, 17 hot-start DNA polymerase enzymes (Table 2) were assayed for activity prior to thermal denaturation using 2 primer pairs (Primer Sets 1 and 2), in addition to a positive control primer pair to confirm the overall polymerase activity in each preparation. The presence of a primer-dimer at ~79 bp indicates activity of the hot-start enzyme prior to thermal activation. The absence of a primer-dimer indicates that the enzyme was only active after thermal activation, as would be expected for a

Table 1. Oligonucleotide sequences used.

Primer name	Primer sequence: 5' to 3'	T _m ***
Primer Set 1F*	ATTATCGTAGATGCTAGCTGGTATGGTACGTAATGAATCTA	69.49°C
Primer Set 1R*	TTATACATCGGACCAGCATATTCATGTCATCTAGGAAGATAG	71.73°C
Primer Set 2F (telg)	ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT	76.80°C
Primer Set 2R (telc)	TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACAA	73.05°C
Positive control PF**	GTGCCCCGCTAAACTTCAGA	61.09°C
Positive control PR**	TGTGGGACAGTTTCAGTAACCT	57.15°C

*Primer Set 1 was generated from a random sequence and contains no homology to human genomic DNA (13). The sequences of Primer Set 2 were previously published by Cawthon et al. (14). The 3' complementary bases of the primer pairs are underlined. A primer-dimer formed by Primer Set 1 will contain a central *EcoRI* site (GAATTC).
 **Positive control primers span exon 13 of *ATP12A*.
 *** Primer T_m was calculated using Applied Biosystems' T_m calculator (<http://www6.appliedbiosystems.com/support/techtools/calc/>).

Table 2. Hot-start polymerases assayed.

Enzyme name*	Company	Method of enzyme inactivation	PCR extension temperature	PCR initial denaturation time	Batch number
HotMaster Taq DNA Polymerase	ThermoFisher Scientific	Derivatized natural affinity ligand to DNA polymerases	72°C	3 min	PG177P
TAQ-Ti Heat Activated DNA Polymerase	Fisher Biotec	Antibody-mediated	72°C	2 min	211031
KAPA LongRange HotStart DNA polymerase	Kapa Biosystems	Antibody-mediated	68°C	3 min	KE3502
KAPA SYBR FAST qPCR Master Mix	Kapa Biosystems	Antibody-mediated	72°C	3 min	KM4108K
Syberfast (for use with the Roche LightCycler 480)	Kapa Biosystems	Antibody-mediated	72°C	5 min	KM4111
MyFi Mix	Bioline	Antibody-mediated	72°C	1 min	MFx-111E
KAPA HiFi HotStart Uracil+ ReadyMix	Kapa Biosystems	Antibody-mediated	72°C	1 min	KM2800
Platinum Green Hot Start PCR 2 $\times$ Master Mix	Invitrogen	Antibody-mediated	72°C	2 min	00319539
AccuPrime Pfx DNA Polymerase	Invitrogen	Antibody-mediated	68°C	2 min	465063
AccuPrime Taq DNA Polymerase System	Invitrogen	Antibody-mediated	68°C	2 min	1196356
Platinum Taq DNA Polymerase	Invitrogen	Antibody-mediated	68°C	2 min	385786
Pfx50 DNA Polymerase	Invitrogen	Intrinsic hot-start capability	68°C	2 min	878488
Platinum PCR SuperMix High Fidelity	Invitrogen	Antibody-mediated	68°C	2 min	876413
Hot FIREPol DNA Polymerase	Solis BioDyne	Chemically modified	72°C	15 min	HF0730
EpiTaq HS	TaKaRa	Antibody-mediated	72°C	30 s	A201-1
PrimeSTAR HS DNA Polymerase	TaKaRa	Antibody-mediated	72°C	30 s	N2001EA
Ex Taq Hot-Start	TaKaRa	Antibody-mediated	72°C	30 s	N6501BA

*The order enzymes are listed in this table does not reflect the order presented in Figure 1.

hot-start enzyme. Analysis using Primer Set 1 revealed a primer-dimer for 7 of the polymerases tested, and analysis using Primer Set 2 revealed a primer-dimer for 12 of the polymerases tested (Figure 1). Polymerases that produced a primer-dimer with Primer Set 1 also produced primer-dimers using Primer

Set 2, suggesting that Primer Set 2 was more sensitive. The difference in detection sensitivity between primer pairs likely reflects the relative stability of the 3-bp overlap or the T_m of each primer, which may have relevance to amplification efficiency during the subsequent PCR cycles. These results

Figure 2. Validation of primer-dimer. Lane 1: “Manual hot-start” PCR; Lane 2: Standard PCR protocol; Lane 3: EcoRI digestion of the remaining PCR product shown in Lane 2. M denotes 25-bp DNA ladder (Invitrogen).

were consistent over multiple assays (data not shown). We have not disclosed the performance of individual enzymes as our findings are based on testing of individual batches of the hot-start DNA polymerases, and we do not know if there is batch-to-batch variation for each manufacturer’s hot-start DNA polymerase.

To ensure that the observed amplicon was indeed a primer-dimer, two experiments were performed. First, a manual hot-start PCR was carried out where *Taq* polymerase was added to aliquots from a PCR master mix at either 95°C or room temperature, prior to thermal cycling. The primer-dimer was only apparent when the polymerase was added at room temperature, which was indicative of polymerase activity prior to thermal activation (Figure 2, Lanes 1 and 2). Second, the origin of this band was verified by restriction digestion. The design of Primer Set 1 was such that formation of a true primer-dimer would generate a recognition site for *Eco*RI (Table 1). Digestion of the PCR product with *Eco*RI did indeed lead to cleavage (Figure 2, Lane 3), confirming formation of a true primer-dimer.

We found that many commercial enzyme preparations marketed as “hot-start” exhibit polymerase activity prior to thermal activation. PCR applications, particularly in forensic or molecular diagnostic settings, rely on accurate and consistent assays. The failure of many hot-start enzymes to perform as expected could, therefore, have quite profound implications.

Although enzyme suppliers provide evidence of tests for polymerase activity in documents accompanying their products, there is no evidence that they test for polymerase activity prior to thermal activation. We provide here a simple and effective assay that can be used to screen hot-start polymerases for this undesirable property.

Author contributions

A.S. carried out much of the experimental work and drafted the manuscript. S.A. made initial observations, contributed to experimental work and editing of the manuscript. M.K. contributed to design of the study and editing of the manuscript.

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Competing interests

The authors declare no competing interests.

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