TECHNICAL ADVANCE

Cooperative Primers

2.5 Million—Fold Improvement in the Reduction of Nonspecific Amplification

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The increasing need to multiplex nucleic acid reactions presses test designers to the limits of amplification specificity in PCR. Although more than a dozen hot starts have been developed for PCR to reduce primer-dimer formation, none can stop the propagation of primer-dimers once formed. Even a small number of primer-dimers can result in false-negatives and/or false-positives. Herein, we demonstrate a new class of primer technology that greatly reduces primer-dimer propagation, showing successful amplification of 60 template copies with no signal dampening in a background of 150,000,000 primer-dimers. In contrast, normal primers, with or without a hot start, experienced signal dampening with as few as 60 primer-dimers and false-negatives with only 600 primer-dimers. This represents more than a 2.5 million—fold improvement in reduction of nonspecific amplification. We also show how a probe can be incorporated into the cooperative primer, with 2.5 times more signal than conventional fluorescent probes. (J Mol Diagn 2013, 15;1–11; http://dx.doi.org/10.1016/j.jmoldx.2013.10.004)

Q2 In the short time since its inception, PCR has become an almost indispensable part of medical and diagnostic sciences. In PCR, a short DNA segment, called a primer, anneals to a target nucleic acid segment and is extended by the polymerase, amplifying the DNA present. Although rules have been described for the selection of primers, primers often anneal to and amplify each other in an unpredictable manner, forming primer-dimers. This problem is compounded in multiplexed tests, where the large number of primers increases the probability of primer-dimer formation exponentially. Primer-dimers compete with the target for the primers and can result in signal dampening, false-negatives, or even false-positives if the nonspecific amplification product also interacts with a probe.

To try to prevent formation of primer-dimers, more than a dozen hot start methods have been invented, each of which limits the activity of the polymerase before an initial denaturing step. However, none of these can stop 100% of primer-dimer formation. Furthermore, the hot start protection is lost after the initial denaturing step, allowing more primer-dimers to be formed and propagated during amplification. Because the number of possible primer-dimer products increases exponentially with the number of primers in the reaction according to the function

\[ \frac{n^2 + n}{2} \]  

where \( n \) is the number of primers, highly multiplexed tests can be difficult to design. With the increasing demand for highly multiplexed assays to diagnose cancers, drug resistance, and other disease, a solution must be found to the primer-dimer problem.

Herein, we report on cooperative primers, the first technology to prevent primer-dimer formation and propagation during the actual amplification rounds (Figure 1). The primers consist of short sequences that would ordinarily not amplify the template. Because of the low primer melting temperature

Disclosure: DNA Logix Inc. is a commercial enterprise that owns the rights to the cooperative primer technologies described herein. B.C.S. is the owner of DNA Logix Inc.
(Tm), unless the capture sequence binds holding the primer in close proximity to the template, the primers will not amplify. The polyethylene glycol linker connecting the primer and the capture sequence prevents the polymerase from extending through the capture sequence, retaining the primer specificity in each round of amplification. Nonspecific amplicons that do not have a complementary region to the capture sequence, such as primer-dimers, are not propagated. Because this process is repeated in every round of amplification, nonspecific amplification products that do not contain sequences complementary to the capture sequence, such as primer-dimers, do not propagate. Because capture sequence hybridization is required during every round of amplification for the primer to bind, it results in an exponential reduction of nonspecific amplification in contrast to traditional hot starts that are effective only before starting amplification. The polymerase extension of the cooperative primer linked to the 5' end of the capture sequence (A) favors displacement of the capture sequence, whereas extension of the cooperative primer linked to the 3' end of the capture sequence (B) favors 5'-3' exonuclease cleavage of the capture sequence. Cleavage of the capture sequence allows it to double as a sequence-specific probe, releasing a fluorophore on nucleic acid cleavage. Because the capture sequence must bind for the primer to extend, the efficiency of probe hybridization and the resultant cleavage are theoretically very high.

**Materials and Methods**

**Oligonucleotide Design and Synthesis**

Adapting math previously derived for cooperative probes and neglecting entropic/enthalpic penalties due to restricting mobility from the linker, the effective equilibrium constant for cooperative primers can be expressed as follows:

\[
K_{\text{eff}} = K_{\text{primer}} + K_{\text{cap}} + P_t K_{\text{primer}} K_{\text{cap}} = \frac{C_{\text{cap}} + C_{\text{primer}} + C_{\text{both}}}{PT}
\]

where \( K \) is the equilibrium constant, with the added subscripts \( \text{cap} \), \( \text{primer} \), \( \text{both} \), and \( \text{eff} \) referring to capture sequence, primer, both capture sequence and primer, and effective, respectively.

\[
P = P_o - C_{\text{cap}} - C_{\text{primer}} - C_{\text{both}} \quad T = T_o - C_{\text{cap}} - C_{\text{primer}} - C_{\text{both}}
\]

where \( P_o \) and \( T_o \) are initial primer and target concentrations, respectively. \( P_t \) refers to the local primer concentration \([P_t = 1 \text{ molecule/(volume swept out by linker length} \times \text{Avogadro's number)}]\), and \( C \) is the concentration of the sequence indicated by the subscript that is hybridized to the template. From this equation, the effective primer binding efficiency \((E_{\text{ff}})\) in the initial rounds of amplification (eg, \( P_o >> T_o \)) can be calculated as follows:

\[
E_{\text{ff}} = \frac{C_{\text{primer}} + C_{\text{both}}}{T_o} = \frac{(K_{\text{primer}} + P_t K_{\text{primer}} K_{\text{cap}}) P_o}{1 + K_{\text{eff}} P_o}
\]

Exploration of predicted enthalpy and entropy values to evaluate the equilibrium constants in equation (2) reveals that primers with predicted Tms 7°C to 12°C below the reaction temperature can still amplify with up to 99% efficiency when coupled with capture sequences with an equal or greater Tm and when separated by three or fewer hexaethylene glycol sequences (HEGs). When six HEG linkers separate the primer and the capture sequence, 99% efficiencies can still be obtained with predicted primer Tms 4°C to 7°C below the reaction temperature.

For proof of concept, cooperative primers were designed to the human beta-actin gene, the mitochondrial sequence of Plasmodium spp., and the rpoB D516V mutation in Mycobacterium tuberculosis (Table 1). The primer sequence was
designed with a Tm 3°C to 9°C below the reaction temperature. The capture sequence was designed with a Tm 3°C to 11°C below the reaction temperature or 5°C and 7°C above the reaction temperature. The capture sequence was also labeled with a FAM/Dabcyyl FRET pair to determine an optimal method of incorporating a probe into the cooperative primer. One of the primers, PfcF inv 62HP, had hairpin secondary structure intentionally designed into the capture sequence to improve quenching of the fluorophore. Sufficient HEGs were used as linkers for the primer and the capture sequence to bind in a rigid double helix. Three or six HEGs (for cooperative primers with the linker attached to the 5′ or 3′ end of the capture sequence, respectively) were used to link the capture sequence to the primer.

For the cooperative primers with the linker attached to the 5′ end of the capture sequence, a blocking carbon chain was placed at the 3′ end of the capture sequence to prevent it from being used as a primer. To attach the linker to the 5′ end of the capture sequence (eg, with the 5′ end of the primer linked to the 5′ end of the capture sequence), the primer had to be synthesized with an inverted linkage (Table 1). Reverse amidites were used to switch the strand polarity at this linkage using otherwise standard solid-phase synthesis for oligonucleotide manufacture. A reverse amide comprises a dimethoxyltrityl or similar protecting group at the 3′-hydroxyl of a deoxynucleoside and a phosphate group at the 5′-hydroxyl of a deoxynucleoside such that the 5′ region of the reverse amide is amenable for linkage to a 5′-OH or 3′-OH of another nucleoside. Sequences made from inverted bases function like normal primers. The orientation affects only the direction of binding (Figure 1A).

Control primers for determining normal primer amplification efficiency and susceptibility to primer-dimers were designed with a Tm 5°C to 7°C above the reaction temperature. This gave the primers just enough affinity to maximize binding to the template for good amplification efficiency without unduly increasing the likelihood of spurious product formation. An additional set of control primers was identical to the short primer sequence in the cooperative primer and was designed to verify that cooperative primers cannot amplify properly without the capture sequence. Primer-dimers, by definition, were designed as the perfect complement of each primer set, with 3′ ends touching each other. Synthesis of cooperative primers was performed by Biosearch Technologies (Petaluma, CA). Dual high-performance liquid chromatography purification was used to purify the cooperative primers. Template was synthesized by either Biosearch Technologies or Integrated DNA Technologies (Coralville, IA) with no purification.

### Cooperative Primer PCR Efficiency

Human beta-actin real-time PCR was run by making a master mix with a 250-nmol/L final concentration of each primer/probe (b-act P, b-act cF, b-act cR), a 5-mmol/L final concentration of MgCl₂, and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp., Madison, WI). Dilutions of template were made using 600,000, 6,000, and 0 copies. The reaction was run on the StepOne system (Applied Biosystems, Foster City, CA) and included a 20-second denaturing step at 95°C followed by 45 cycles at 95°C for 1 second and 55°C for 20 seconds. Reactions were run in duplicate. A log plot of the concentration versus the CT was used to find the slope from which to calculate PCR efficiency.

### Impact of Primer-Dimers

*Plasmodium falciparum* PCR was run by making a master mix with a 250-nmol/L final concentration of each primer for cooperative primers (PfcF inv62, PfcR inv) or normal primers (PfnF, PfnR, PIP), a 5-mmol/L final concentration of MgCl₂, and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp.). Sixty copies of template were added to each reaction and 0, 60, 600, 6000, 60,000, 600,000, 6,000,000, 60,000,000, 150,000,000, or 150,000,000,000 primer-dimers were placed in each. Each reaction was run in duplicate. The reaction with normal primers was repeated using GoTaq hot start and GoTaq hot start colorless master mix. The reactions were run on the StepOne system (Applied Biosystems) and included a 20-second denaturing step at 95°C followed by 50 cycles at 95°C for 1 second and 55°C for 20 seconds. After amplification, the PCR products were run on a 2.2% FlashGel system (Lonza Inc., Walkersville, MD) and were imaged.

As a follow-up to verify that the results from the cooperative primers were due to cooperativity, the cooperative primers were made without a capture sequence (Pf Low Tm F and Pf Low Tm R). Combined with the probe (PIP) and using the same master mix and thermocycling conditions as noted previously herein, 0, 60, 600, 600,000, or 6,000,000 copies of template were added to the reaction. Reactions were run in duplicate.

### Effect of Complex Samples

To analyze performance in complex samples, 5000 copies of *P. falciparum* DNA were spiked into 0, 0.6, 1.2, or 3 μg of human gDNA (BioChain Institute Inc., Newark, CA). A cooperative primer with a long capture sequence (PfcF inv62) and a cooperative primer with a short capture sequence (PfcF) were compared with normal primers (PfnF and PfnR). Each master mix had a 500-nmol/L final concentration of primers, a 5-mmol/L final concentration of MgCl₂, and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp.). Each reaction was run in duplicate. The reactions were run using the ABI 7500 system (Applied Biosystems) and included a 20-second denaturing step at 95°C followed by 50 cycles at 95°C for 3 seconds and 55°C for 32 seconds.
<table>
<thead>
<tr>
<th>Name</th>
<th>Sequences*</th>
<th>Tm</th>
<th>Tm</th>
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<tbody>
<tr>
<td><strong>M. tuberculosis</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Normal primers/probes</td>
<td></td>
<td></td>
<td></td>
</tr>
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<td>b-act P</td>
<td>5’- [FAM] TGTGGCCGAGGACTTTGAGCG[PHQ1] -3’</td>
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<td>Templates</td>
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<td>Normal primers/probes</td>
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<td>PfnR</td>
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<td>PfcF inv</td>
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<td>52.8</td>
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<td>46.6</td>
<td>62.0</td>
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<td>PfcF inv62HP</td>
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<td>46.6</td>
<td>62.0</td>
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<tr>
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<td>5’- GAAAGCACCACTGCGGAGTAA-3’</td>
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<tr>
<td>Templates</td>
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<tr>
<td>Normal primer-dimer</td>
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<td>475</td>
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<td><strong>M. tuberculosis</strong></td>
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<tr>
<td>Normal primers/probes</td>
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<tr>
<td>MTb P</td>
<td>5’- [FAM] CGCCCGAGTCAAGGACTTTGCG [PHQ1] -3’</td>
<td>483</td>
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<td>Cooperative primers/probes</td>
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<tr>
<td>MTb cF</td>
<td>3’- AACCTGCGAGGAG-5’ [Sp18] [Sp18] 5’- CGCAGAGCTGTGAT [DABC] -3’</td>
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<tr>
<td>MTb cR1</td>
<td>5’- [CF 560] TGGAGACTGATGGTC [PHQ1] [Sp18] [Sp18] [Sp18] 5’- CGCAAGGCTTCCGCATTCCATTTTCCTACATCTTCAATTGCAGTGGTACGACTTTGTCAGTCAAAACATGGAA-3’ [DABC] -3’</td>
<td>50.9</td>
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<tr>
<td>MTb cR2</td>
<td>3’- [CF 560] TGGGAGACTGATGGTC [PHQ1] [Sp18] [Sp18] [Sp18] 5’- CGCAAGGCTTCCGCATTCCATTTTCCTACATCTTCAATTGCAGTGGTACGACTTTGTCAGTCAAAACATGGAA-3’ [DABC] -3’</td>
<td>50.9</td>
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<td>MTb cR3</td>
<td>3’- [CF 560] TGGGAGACTGATGGTC [PHQ1] [Sp18] [Sp18] [Sp18] 5’- CGCAAGGCTTCCGCATTCCATTTTCCTACATCTTCAATTGCAGTGGTACGACTTTGTCAGTCAAAACATGGAA-3’ [DABC] -3’</td>
<td>50.9</td>
<td></td>
</tr>
<tr>
<td>MTb cR4</td>
<td>3’- [CF 560] TGGGAGACTGATGGTC [PHQ1] [Sp18] [Sp18] [Sp18] 5’- CGCAAGGCTTCCGCATTCCATTTTCCTACATCTTCAATTGCAGTGGTACGACTTTGTCAGTCAAAACATGGAA-3’ [DABC] -3’</td>
<td>50.9</td>
<td></td>
</tr>
</tbody>
</table>

* (table continues)
After amplification, the PCR products were run on a 2.2% FlashGel system (Lonza Inc.) and were imaged.

**Incorporating a Probe into Cooperative Primers**

*Plasmodium falciparum* real-time PCR was run by making a master mix with a 250-nmol/L final concentration of each primer (PfcF inv, PfcF inv62, PfcF inv62HP, or PfcF with PfcR inv), a 5-mmol/L final concentration of MgCl₂, and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp.). Five million, 600,000, 50,000, 500, or 0 copies of template were added to each reaction. The reaction was run on the StepOne system (Applied Biosystems) and included a 20-second denaturing step at 95°C followed by 45 cycles at 95°C for 1 second and 55°C for 20 seconds. Each reaction was run in duplicate.

Because of higher fluorescent signals, the capture sequence end attached to the linker was selected as the method for making the probe in the remaining experiments.

The cooperative primers PfcF and PfcR inv, selected previously herein, were then used with the same cycling conditions to analyze 5 μL of 1:1000 dilutions (approximately 50 pg of purified genetic material, frozen for 4 years) from *Plasmodium* samples obtained from BEI Resources Repository (Manassas, VA), including *P. falciparum* parent type strain in Rhesus, *Plasmodium knowlesi* H strain, and *P. falciparum* FCR-3/Gambia clone D-4, knobless. Negative controls were included, and all the tests were run in duplicate.

**Single Nucleotide Polymorphism Differentiation**

*M. tuberculosis* real-time PCR for the D516V mutation in the rpoB gene conferring rifampicin resistance was run by making a master mix with a 250-nmol/L final concentration of each primer/probe (MTb cF, MTb P, and one of MTb cR1, MTb cR2, MTb cR3, MTb cR4, MTb cR5, MTb cR6, MTb cR7, MTb cR8, or MTb cR9), a 5-nmol/L final concentration of MgCl₂, and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp.). Fifty thousand copies of template (MTb WT or MTb D516V) were added to each reaction. Each reaction was run in duplicate.

The reaction was run using the ABI 7500 system (Applied Biosystems) and included a 20-second denaturing step at 95°C followed by 45 cycles at 95°C for 3 seconds and 55°C for 32 seconds. The Ct₅ was automatically determined and an additional 0.275 U per reaction of GoTaq polymerase in GoTaq colorless master mix (Promega Corp.). Fifty thousand copies of template (MTb WT or MTb D516V) were added to each reaction. Each reaction was run in duplicate.

The reaction was run using the ABI 7500 system (Applied Biosystems) and included a 20-second denaturing step at 95°C followed by 45 cycles at 95°C for 3 seconds and 55°C for 32 seconds. The Ct₅ was automatically determined by the machine with a threshold of 10,000, and the ΔRn was taken from cycle 45 of the exported data.

**Results**

**Cooperative Primer PCR Efficiency**

To evaluate the concept for cooperative primers, the first step was to see whether they would even amplify and, if so, with what efficiency (Figure 2). The beta-actin forward and reverse primers were selected to be Cooperative Primers.
amplification of just 600 P-Ds in the reaction with normal primers eclipsed the normal primers and cooperative primers. Each was run in duplicate. The DNA (the causative agent of malaria) with and without spiked-in P-Ds for dimers (P-Ds). Gels of PCR products amplifying 60 copies of 

Impact of Primer-Dimers

Once the amplification efficiency was shown to be sufficient, we tested the central hypothesis behind cooperative primers: the ability to eliminate propagation of primer-dimers. We spiked up to 600,000 primer-dimers (100 fmol/L final concentration) into a malaria reaction with only 60 copies of *P. falciparum* template (Figure 3). There was no visible amplification of spiked-in primer-dimers or dampening of the amplification product when cooperative primers were used. In contrast, the addition of only 600 primer-dimers to a reaction with normal primers resulted in false-negatives. Next, we determined whether a traditional antibody-mediated hot start could stop primer-dimer propagation with regular primers (Figure 4). However, the results were similar with or without hot start. Primer-dimers were already competing with the template with only 60 primer-dimers and completely eclipsed the template amplification with 600 primer-dimers. Hot starts are capable only of preventing primer-dimer formation during reaction setup. Once primer-dimers form, they cannot stop primer-dimer propagation.

In a subsequent experiment, we attempted to find the limit of specificity for cooperative primers (Figure 5). Only once the primer-dimer concentration was within an order of magnitude of the primer concentration with 150,000,000,000 primer-dimers spiked into the 10-μL reaction were primer-dimers finally formed and the product eclipsed. In contrast, it took only 600 primer-dimers to eclipse the *P. falciparum* amplification product using normal primers with or without a hot start. This is consistent with our experience that a 10-fold excess of a competitive reaction product is sufficient to result in false-negatives when using normal primers. Cooperative primers were spiked with up to 150,000,000 primer-dimers,
Incorporating a Probe into Cooperative Primers

Because probes incorporated into primers have shown relatively high signal to noise in the past, we attempted to incorporate a probe into the cooperative primer. This was done by labeling the capture sequence. First, inverted primers were attached to the 5' end of capture sequences. To characterize the effectiveness of labeling the capture sequence, three different capture sequences were used. The first had a Tm below the reaction temperature (PfcF inv), the second had a Tm above the reaction temperature (PfcF inv2), and the third formed a hairpin to encourage greater quenching (PfcF inv 62HP). However, very little signal was observed from any of these primers (data not shown), and electrophoretic gels showed that very few of the primers were cleaving the capture sequence [Figure 3 (the barely visible bands below the amplicon of the cooperative primers)].

We hypothesized that conformational strain from the linker was lifting the 5' end of the capture sequence and causing the polymerase to displace the sequence rather than cleave it (Figure 1). Consequently, if we moved the strain from the 5' end to the 3' end (eg, by changing where the linker was attached), the polymerase might cleave the capture sequence with greater efficiency. On testing this hypothesis (with PfcF), the fluorescent signal rose dramatically (Figure 6). Although the labeled capture sequence had a Tm below the reaction temperature, the signal was still 2.5 times higher than the signal from normal hybridization probes.

The cooperative primers with an incorporated probe were then run on a series of Plasmodium samples obtained from BEI Resources Repository, including P. falciparum St. Lucia, P. vivax Panama in Aotus, P. brasilianum Peruvian III in Saimiri, P. cynomolgi bastianelli in Rhesus, P. cynomolgi Smithsonian in Rhesus, P. fragile—type strain in Rhesus, P. simium Howler in Saimiri, P. knowlesi H strain, and P. falciparum FCR-3/Gambia clone D-4, knobless (Figure 7). The pan-plasmodium primers were able to successfully detect DNA from each species.

Single Nucleotide Polymorphism Differentiation

Finally, we analyzed the ability of these efficient, primer-dimer resistant, cooperative primers to differentiate single nucleotide polymorphisms (SNPs). Cooperative primers

Table 2  Summary of Amplification Results in gDNA

<table>
<thead>
<tr>
<th>gDNA (µg)</th>
<th>52.8°C capture</th>
<th>62°C capture</th>
<th>Normal primers</th>
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</thead>
<tbody>
<tr>
<td>0</td>
<td>Amplified</td>
<td>Amplified</td>
<td>Amplified</td>
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<tr>
<td>0.6</td>
<td>Amplified</td>
<td>Amplified</td>
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<tr>
<td>1.2</td>
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<td>Failed</td>
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<tr>
<td>3.0</td>
<td>Failed</td>
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</table>

Cooperative primers and normal primers amplify the target in a background of 0.6 µg of gDNA but not in a background of 3.0 µg of gDNA. The cooperative primer with a longer capture sequence (Tm of 62°C) was not as effective as the cooperative primer with a shorter capture sequence (Tm of 52.8°C) at amplifying the target in a background of gDNA.

2.5 million times more primer-dimers than template, with no signs of dampening of the amplification product.

To verify that the result was truly due to cooperativity, the cooperative primers were created without a capture sequence. These isolated, low-Tm primers did not amplify primer-dimers but were also incapable of detectably amplifying even up to 600,000 copies of P. falciparum DNA (data not shown). In contrast, cooperative primers exhibited efficient amplification and detection of low copy numbers, demonstrating the importance of the cooperative link to the capture sequence.

Effect of Complex Samples

To evaluate cooperative primers in complex samples, two different cooperative primers, one with a long capture sequence (PfcF inv62) and the other with a short capture sequence (PfcF), were compared with normal primers PfnF and PfnR (Table 2). Five thousand copies of Plasmodium DNA were spiked into 0.6, 1.2, or 3 µg of human gDNA. All the primers were able to successfully amplify the Plasmodium sequence in a background of 0.6 µg of gDNA, but the cooperative primer with the 62°C-Tm capture sequence did not amplify template in a background of 1.2 µg of gDNA. The short capture sequence cooperative primer and the normal primers did not amplify the template in a background of 3 µg of gDNA.

Figure 6 Cooperative primers with Integrated DNA Technologies probes. Labeled cooperative primers (blue) or normal hybridization probes (red) were used for real-time detection of 5,000,000, 50,000, 500, or 0 copies of P. falciparum template. Each was run in duplicate. Labeled capture sequence in cooperative primers had a fluorescent signal 2.5 times higher than that of normal hybridization probes, although the capture sequence had a Tm below the reaction temperature.
were designed to the rpoB gene D516V mutation, which is present in up to 7.4% of rifampicin-resistant M. tuberculosis isolates in India. Two different strategies were used: the amplification-refractory mutation system (ARMS) method and labeled capture sequence differentiation. Both methods resulted in the ability to differentiate SNPs similar to standard primers and probes (Figure 8 and data summary in Table 3).

For the probe-based method, cooperative primer MTb CR6 gave the best ratio of fluorescent signals between the mutant and wild-type strains. For the ARMS-based method, MTb CR8 gave the best difference in CT values. Both are shown in Figure 8.

**Figure 7** Cooperative primers with multiple Plasmodium species. Panplasmodium cooperative primers were able to successfully detect multiple species of Plasmodium parasites, including P. falciparum St. Lucia, P. vivax Panama in Aotus, P. brasilianum Peruvian III in Saimiri, P. cynomolgi bastianelli in Rhesus, P. cynomolgi Smithsonian in Rhesus, P. fragile—type strain in Rhesus, P. simium Howler in Saimiri, P. knowlesi H strain, and P. falciparum FCR-3/Gambia clone D-4, knobless. Each was run in duplicate, and only the negative control was negative.

**Figure 8** SNP differentiation with cooperative primers. Cooperative primers differentiate between tuberculosis complex with the rpoB D516V SNP causing rifampicin resistance (blue) and without the SNP (red) using probe-based differentiation with the SNP under the capture sequence (left panel) and the ARMS-based method with the SNP under the 3’ end of the primer (right panel).

**Discussion**

Despite the development of more than a dozen hot starts, no currently existing method has the ability to stop the propagation of primer-dimers once formed (an example of antibody-mediated hot start is shown in Figure 4). We have created low-Tm primers that ordinarily would not amplify the target or primer-dimers. However, on linking the primers to a capture sequence, they are capable of amplifying the target with up to 100% efficiency (Figure 2). Spurious amplification products, such as primer-dimers, which do not have a complementary sequence to the capture sequence, are not propagated (Figures 3 and 5).

The principle of cooperativity is what enables the cooperative primers to function. The capture sequence binds to the target, holding the primer in close proximity to the template. This increases the effective concentration by several orders of magnitude, which increases the number of collisions between the primer and the target DNA, shifting its effective Tm above that of the reaction temperature and allowing extension to occur. In this manner, the target DNA is amplified, but primer-dimers and other artifacts not possessing a region complementary to the capture sequence are not propagated (Figure 3).

Cooperative primers are not the first real-time PCR technology to make use of cooperativity. We previously reported on Tentacle Probes (Arcxis Biotechnologies, Pleasanton, CA), which were built on cooperativity and led to a 100- to 200-fold improvement in kinetics and up to a 10,000-fold improvement in concentration-independent specificity. However, Tentacle Probes are nonextendable and do not solve the problem of primer-dimers. Note that herein we used cooperativity to make another multiple order of magnitude improvement in PCR, only this time with respect to resistance to amplification of primer-dimers. In fact, cooperative primers reduced propagation of primer-dimers and corresponding false-negatives by up to 250 million—fold compared with normal primers, with or without a hot start. This is such a strong reduction in sensitivity to primer-dimers that it would take nearly every primer in the reaction forming a primer-dimer during reaction setup to cause...
Cooperative Primers

Table 3 Summary of SNP Differentiation Methods

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Method</th>
<th>ΔTm (°C)</th>
<th>Probe ΔTm (°C)</th>
<th>ΔCt</th>
<th>ΔRnVar/ΔRnWT</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTb CR1</td>
<td>Probe</td>
<td>4.1</td>
<td>4.5</td>
<td>1.68</td>
<td>3.01</td>
</tr>
<tr>
<td>MTb CR2</td>
<td>Probe</td>
<td>4.1</td>
<td>2.5</td>
<td>4.79</td>
<td>3.35</td>
</tr>
<tr>
<td>MTb CR5</td>
<td>Probe</td>
<td>6.6</td>
<td>2.5</td>
<td>4.83</td>
<td>1.89</td>
</tr>
<tr>
<td>MTb CR6</td>
<td>Probe</td>
<td>4.1</td>
<td>7.1</td>
<td>3.83</td>
<td>3.67</td>
</tr>
<tr>
<td>MTb CR7</td>
<td>Probe</td>
<td>4.2</td>
<td>11.7</td>
<td>5.30</td>
<td>3.62</td>
</tr>
<tr>
<td>MTb CR3</td>
<td>ARMS</td>
<td>6.3</td>
<td>4.3</td>
<td>4.43</td>
<td>NA</td>
</tr>
<tr>
<td>MTb CR4</td>
<td>ARMS</td>
<td>10.2</td>
<td>4.3</td>
<td>5.99</td>
<td>NA</td>
</tr>
<tr>
<td>MTb CR8</td>
<td>ARMS</td>
<td>20.2</td>
<td>4.3</td>
<td>7.57</td>
<td>NA</td>
</tr>
<tr>
<td>MTb CR9</td>
<td>ARMS</td>
<td>25.5</td>
<td>4.3</td>
<td>7.13</td>
<td>NA</td>
</tr>
</tbody>
</table>

Each primer is listed together with whether it uses ARMS- or probe (labeled capture sequence)-based differentiation, the number of degrees the predicted Tm for the primer or probe is above or below the reaction temperature (values below the reaction temperature are shown in parenthesis), the difference between mutant and wild-type Ct values, and the ratio of the mutant and wild-type fluorescence.

NA, not available.

mispriming event.

Table 2 shows that something about the inverted cooperative primer caused

mispriming. This is because a large capture sequence is more likely to nonspecifically bind to the genomic DNA, providing sufficient stability for the short priming region to amplify the nontarget gDNA sequence after the initial mispriming event.

Even when the capture sequence is designed optimally, such as the 52.8°C capture sequence in Table 2, cooperative primers still do not solve all the problems of normal primers. Despite reducing primer-dimer propagation up to 250 million—fold more effectively than normal primers, the cooperative primers were still no more effective than normal primers in reducing mispriming of gDNA.

In the present study, we used a single annealing temperature of 55°C with just one type of master mix. We find this temperature convenient because it is compatible with single-tube reactions for RT-PCR, where both the reverse transcriptase step and the annealing step during polymerization are performed at the same temperature. The principal behind cooperative primers suggests that they should be compatible with any reaction temperature and in a variety of different salt or primer concentrations. However, slight tweaking of the general design parameters (eg, the ideal Tm for the primer and the capture sequence) will likely be necessary. Further work will be needed to fully explore the range of conditions possible and the optimum design parameters for each in real-time PCRs.

Scorpion primers may be somewhat similar in appearance to cooperative primers, but they are not cooperative. Scorpion primers bind to themselves after the primer is extended rather than cooperatively binding to the template. Because there is no cooperative capture sequence, a low-Tm primer would not work in a Scorpion primer.

Dual-priming oligonucleotides are the only example of a primer that could be considered cooperative in function. A short primer of 6 to 12 bases is linked to a high-Tm capture sequence connected via a series of deoxynucleosines. The difference between the function of the dual-priming oligonucleotide and the present cooperative primer is that the capture sequence in the former binds upstream from the primer rather than downstream. Because the linker is composed of deoxynucleosines, the polymerase extends through the entire primer, including the capture sequence, enabling high-efficiency PCR. However, this same principle prevents it from being able to stop propagation of primer-dimers once formed. If a nonextendable linker were to be used in the dual-priming oligonucleotide, then the first-round amplification product would not include a complementary region to the capture sequence, preventing further amplification. In contrast, the cooperative primers in the present work maintain affinity and specificity during every round of amplification owing to the unique mechanism of the capture sequence binding downstream from the primer.

Given that the capture sequence in the cooperative primer must bind for the primer to extend, it seemed convenient to label the capture sequence and use it as a probe. This could eliminate the need to identify a third conserved region in a given target or the need to add a separate probe to the reaction mix. Furthermore, the potential to design the capture sequence with a Tm below the reaction temperature could make it impervious to false-positives from spurious amplification products, seeing as the capture sequence cannot light up unless the primer binds to a region of the target immediately upstream from it. All these ideas lent themselves toward incorporating a probe directly into the cooperative primer.

However, when we labeled the capture sequence attached to the inverted primer, there was very little fluorescence. This did not change even for high-Tm capture sequences or capture sequences with hairpin structures. We noticed that the electrophoretic gels, like the one shown in Figure 3, had a faint band underneath the amplicon for cooperative primers. This band was consistently the length of the amplicon minus the capture sequence(s). Thus, it seemed that something about the inverted cooperative primer caused...
the polymerase to favor displacing the capture sequence rather than cleaving it. This, in turn, would allow amplification but would prevent generation of a signal.

We hypothesized that the strain of the linker on the 5' end of the capture sequence was what caused the polymerase to favor displacement. Consequently, a cooperative primer that was attached to the capture sequence at the 3' end might allow for efficient 5'-3' nuclease activity by removing the strain from the 5' end. As it turned out, multiple primers with the linker attached to the 3' end of the capture sequence generated large fluorescent signal, even when the capture sequence was as much as 11.7°C below the reaction temperature (Figures 6 and 8). In fact, the fluorescent signal was so high that it was more than 2.5 times the signal of normal hybridization probes. It could be thought that the signal was higher purely because of better quenching efficiency on the short capture sequence versus the relatively long hybridization probe. However, the hybridization probe in these experiments used secondary structure, placing the quencher within seven bases of the fluorophore, theoretically giving it more efficient quenching than the short capture sequence in the cooperative primer. Although the short distance between the quencher and the fluorophore on the capture sequence certainly contributes to the signal, it does not seem to be the only factor. It is possible that some of the increased fluorescence is because the primer does not have to compete with the synthesized template for hybridization to the target sequence the way that a hybridization probe does.

Differentiating SNPs using ARMS- and probe-based differentiation seemed to yield similar results for cooperative primers as for normal primers and probes. The 3' mismatch on the primer delayed the C_t by approximately four cycles. An intentional mismatch added to the third base from the 3' end in addition to the SNP under the 3' end delayed the C_t by approximately six to seven cycles (Table 3). These are typical results for ARMS-based methods using normal primers.21-23 Probe-based discrimination showed strong signal for an exact match, with a weak signal for a mismatch (Figure 5). This is typical of results for dual-labeled hybridization probes.24-26

Although we were able to obtain signal and amplification from all the designs that were tried, some seemed to be more efficient than others. This has led to a few general observations for reasonably effective design for reaction temperatures at 55°C. For best results with cooperative primers containing three HEG linkers (eg, inverted primers attached to the 5' end of the capture sequence), a Tm 5°C to 7°C below the reaction temperature should be used. For best results with cooperative primers containing six HEG linkers (eg, primers attached to the 3' end of the capture sequence), a Tm for both oligonucleotides 4°C to 6°C below the reaction temperature is desirable. Although results can be obtained with lower Tms, the risk of losing either signal or amplification efficiency grows dramatically. Higher Tm designs can also be used, but, in theory, the lower the Tm of the primer, the more resistant it will be to amplification of spurious products. Also, for SNP differentiation, the lower the Tm in the target range, the better. High-Tm capture sequences could potentially be used to provide additional stability for highly polymorphic targets, but additional work needs to be done to explore this application.

Conclusion

More than a dozen hot starts have been developed to reduce primer-dimer formation during PCR setup; however, none of these can prevent primer-dimer formation after the initial hot start or the propagation of primer-dimers once formed. With increasing multiplexing demands, the need for a real solution to primer-dimers is only increasing. Cooperative primers are the first instance of a technology that prevents the propagation of primer-dimers. The implications to the field of life sciences, which relies on the use of PCR, are tremendous. We believe that this technology will provide researchers with the ability to rapidly and easily create multiplexed tests almost without hindrance from primer-dimers.

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