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Exquisite allele discrimination by toehold hairpin primers

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ABSTRACT

The ability to detect and monitor single nucleotide polymorphisms (SNPs) in biological samples is an enabling research and clinical tool. We have developed a surprising, inexpensive primer design method that provides exquisite discrimination between SNPs. The field of DNA computation is largely reliant on using so-called toeholds to initiate strand displacement reactions, leading to the execution of kinetically trapped circuits. We have now similarly found that the short toehold sequence to a target of interest can initiate both strand displacement within the hairpin and extension of the primer by a polymerase, both of which will further stabilize the primer:template complex. However, if the short toehold does not bind, neither of these events can readily occur and thus amplification should not occur. Toehold hairpin primers were used to detect drug resistance alleles in two genes, *rpoB* and *katG*, in the *Mycobacterium tuberculosis* genome, and ten alleles in the *Escherichia coli* genome. During real-time PCR, the primers discriminate between mismatched templates with Cq delays that are frequently so large that the presence or absence of mismatches is essentially a 'yes/no' answer.

INTRODUCTION

Real-time polymerase chain reaction (PCR) is the gold standard for the detection of nucleic acids, especially in a diagnostic context (1,2). An important problem for both research applications and molecular diagnostics is discrimination between closely related alleles of genes (3–5). Unfortunately, most real-time assays rely heavily on extensive sample preparation and detailed analysis in machines that detect the number of cycles required for amplification (6). The presence of impurities or contaminants in samples can lead to non-specific amplification and increasing difficulties in discriminating between alleles (7). In order to better adapt PCR methods, including real-time PCR, for point-of-care applications, it would be desirable to be able to robustly discriminate between alleles, irrespective of sample provenance, condition, preparation or purity. Allele discrimination via PCR commonly relies upon the use of allele-specific specific primers (8). General primers can also be used for amplification, and amplicons then probed by single base extension with unlabeled or fluorescently tagged dideoxynucleotides, ultimately leading to products that are distinguished based on mass (in matrix-assisted laser desorption/ionization time-of-flight) (9) or fluorescence (10).

Allele-specific primers typically contain mismatches at their 3′ ends (so-called ARMS, amplification refractory mutation SNP primers) (11). In real-time PCR with allele-specific primers, there is a delay in amplification of the mismatched target, typically of 5 to 10 PCR cycles, often detected via a so-called TaqMan probe in which a fluor:quencher pair is separated by the exonuclease activity of the polymerase (12). However, certain mismatches are efficiently extended, leading to inaccurate genotyping (13,14). Improved discrimination against mismatches (a delay in amplification of 5 or 6 cycles) has been reported using locked nucleic acid nucleotides (LNAs) at the 3′ end of a primer, overlapping the mismatch. It has been postulated that the increased melting temperature of LNAs correctly paired with a DNA target resulted in a greater differential in melting temperatures (15). SNP-specific hairpin primers have also been designed (16). In many cases the hairpin is also a molecular beacon that is triggered when the single-stranded loop sequence hybridizes to the primer-binding site. Scorpion SNP primers are specialized hairpin primers engineered with a full-length linear ARMS primer appended to the 3′ end of a hairpin probe. These primers can be used for the real-time amplification and detection of specific targets via end-point fluorescence rather than quantification cycle (Cq). Five nanograms of human genomic DNA amplified with Scorpion primers through 40 cycles was sufficient for detection and genotyping of a BRCA2 SNP (17).

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Non-fluorescent hairpin primers with a single-stranded targeting loop sequence and a SNP-specific nucleotide at the 3′ position in the stem also improved the mean cycle difference between matched and unmatched templates in SYBR Green qPCR assays from 7.6 for linear primers to 11.2 for hairpin primers (18), presumably because of the competition between correct inter- and intramolecular pairing. The field of nucleic acid computation frequently relies on programming DNA molecules as kinetic traps, which undergo conformational rearrangements upon interactions with input molecules, leading to the execution of algorithms (19,20). One of the chief features of the kinetically trapped nucleic acid substrates is the presence of a short so-called toehold sequence that can initiate strand displacement reactions (21,22). We have now applied these principles to the design of hairpin primers that have an initiating toehold sequence that is exquisitely sensitive to mismatches. In the presence of the correct toehold, both strand displacement and elongation can lead to productive amplification of particular SNPs, and discriminate with high fidelity against single mismatches.

MATERIALS AND METHODS

Oligonucleotides and plasmid construction

All oligonucleotides used in this study were obtained from Integrated DNA Technologies (IDT; Coralville, IA, USA). *Mycobacterium tuberculosis* gene segments were PCR amplified using Phusion DNA polymerase (New England Biolabs, NEB; Ipswich, MA, USA) from commercially available genomic DNA of the virulent strain H37Rv (ATCC; Manassas, VA, USA) and gene-specific primers:

katG forward: TGGGCGGACCTGATTGTTTTCGCC GGC

katG reverse: GCTCTTAAGGCTGGCAATCTCGGC TTCGCC

rpoB forward: TGCGATCGACGCTGGAGAAGGA CAA CACCG

rpoB reverse: TGTAGTCGGCCGA CACCTCCTCG ATGACGC

The PCR products were purified from agarose gels using the Wizard SV gel and PCR purification system (Promega; Madison, WI, USA). SNP-containing alleles were then built by overlap PCR amplification of the wild-type (WT) gene segments using site-specific mutagenic primers. Following A-tailing using *Taq* DNA polymerase (NEB), the PCR products were TA cloned into a pCR2.1 TOPO vector (Life Technologies; Grand Island, NY, USA) and verified by sequencing at the Institute of Cellular and Molecular Biology Core DNA sequencing facility (University of Texas at Austin; Austin, TX, USA). Plasmid templates for PCR were prepared using the Qiaprep spin mini-prep kit (Qiagen; Valencia, CA, USA), and DNA concentrations were determined using a Nanodrop 2000 (Thermo Scientific; Waltham, MA, USA).

End-point PCR

End-point PCR assays were performed using 200 μM deoxynucleotides (Thermo Scientific), 50 pg/μl (2×10^8 total targets) of cloned plasmid template, and 5 units of *Taq* polymerase in a 20 μl reaction on an MJ Research PTC-200 Thermal Cycler. 1X PCR buffer consisted of 50 mM KCl, 10 mM Tris-Cl, pH 8.3 and 1.5 mM $MgCl_2$. Five microliters of each PCR reaction were electrophoresed on a 4% SeaKem LE Agarose gel (Lonza; Rockland, ME, USA) with 0.2 μg/ml ethidium bromide and were visualized with a UV lamp. To determine optimal annealing temperatures, gradient temperatures between 55 and 68°C (55, 56.1, 58.7, 62.8, 66 and 67.8°C) were tested and analyzed in the following protocol: *katG* WT linear primers and THPs with toehold lengths of 0, 3, 4, 5, 6, 7, 8 and 9 nucleotides (nt) were used at a concentration of 200 nM in a three-step PCR reaction with the following conditions: 95°C for 2 min, followed by 30 cycles of 95°C for 30 s, annealing at the gradient temperatures listed above for 30 s, and extension at 68°C for 30 s. Preliminary results favored an annealing temperature of 60°C in a three-step PCR, with no improvement of amplification for toeholds longer than 6 nt (data not shown). Thus, further optimization of 0, 3 and 6 nt toehold (i.e. T0, T3 and T6) primers was performed with varying annealing times, $MgCl_2$ concentrations and steps in the PCR reaction. Reaction conditions were: 95°C for 2 min, followed by 20 cycles of 95°C for 30 s, annealing at 60°C for 30 or 20 s, and extension at 68°C for 30 s. Separate reactions with $MgCl_2$ concentrations of 1.5 and 2.5 mM were run. Testing of two-step PCR was initiated with these conditions: 95°C for 2 min, followed by 20 cycles of 95°C for 30 s and combined annealing/extension at 68°C for 30 or 45 s. Separate reactions with $MgCl_2$ concentrations of 1.5 and 2.5 mM were run.

Real-time PCR of *Mycobacterium tuberculosis* alleles

All real-time PCR assays were performed on the LightCycler 96 System (Roche Diagnostics; Indianapolis, IN, USA) in 96-well format with three technical replicates per sample using Fast Universal Probe Master (ROX) (Roche) and FAM-labeled hydrolysis probes with an Iowa Black quencher (IDT). Toehold-dependent hairpin primers with 3, 4, 5 and 6 nt toeholds were tested for each allele. LightCycler 96 software was used to determine the Cq and analyze primer efficiencies. For all assays, unless explicitly stated, template concentration was 50 pg/μl of plasmid, primer concentration was 200 nM, and probe concentration was 55 nM. Ramp times were 1.1°C/s for cooling and 2.2°C/s for heating. The default parameters of the LightCycler SW 1.1 software were adopted for all analyses. For *katG* THPs, conditions were as follows: 95°C for 10 min, followed by 45 cycles of 95°C for 10 s and annealing/extension at 68°C for 30 s. For assays with *rpo*B Q513 alleles, we performed a three-step PCR gradient on the LightCycler to determine optimal conditions for amplification with SNP discrimination. This initial amplification reaction was performed with 500 pg/μl of template with reaction conditions of 95°C for 10 min, followed by 60 cycles of 95°C for 15 s, annealing between 65 and 72°C for 20 s, and extension at 72°C for 20 s. We determined that a two-step PCR with 95°C for 10 min followed by at least 45 cycles of 95°C for 15 s and combined annealing/extension at 72°C for 30 s was optimal for amplification and discrimination. To quantify primer efficiency and establish a limit of detection, at least three real-

time assays each for the *katG* WT- and *rpo*B WT-specific primers were run with template concentrations (in triplicate) of 50 pg/μl (2×10^8 molecules), 5 pg/μl (2×10^7 molecules), 0.5 pg/μl (2×10^6 molecules) and 50 fg/μl, (2×10^5 molecules). Efficiencies (E) were calculated as $E = 10^{(-1/\text{slope of the standard curve})}$.

Real-time PCR with multiple *E. coli* alleles

Colonies from *Escherichia coli* strains REL606 and CZB154 were grown in LB media for several hours. DNA was isolated from cell pellets using the PureLink Genomic DNA Mini Kit (Life Technologies) and DNA concentrations determined using the Nanodrop 2000 (Thermo Scientific). Primers (400 nM) were assayed in real-time PCRs on a Light Cycler 96 using 2.5 ng/μl (2×10^7 molecules) of genomic target DNA and the FastStart PCR Master Mix (Roche). Real-time amplification was monitored by including Evagreen (Biotium), an intercalating dye, in the reaction mixture. Amplification conditions were 95°C for 10 min, followed by either a two-step or three-step PCR. The two-step protocol is described above. The three-step protocol included a 10 min pre-incubation at 95°C followed by 60 cycles of 95°C for 20 s, 65°C for 20 s and 72°C for 20 s. Limit of detection assays for *yaaH* N174 alleles were performed in a two-step PCR using the conditions described above. We generated a dilution series from 10^7 copies/μl to 10 copies/μl of target genome by decades and added 1 μl from each dilution into a well containing 10^7 copies of the mismatched genome (e.g. for N174T SNP-detecting primers we diluted CZB154 genomes into wells containing 10^7 REL606 genomes).

RESULTS

Design of toehold hairpin primers

In attempting to identify SNPs during real-time PCR amplification, the ideal result would be that a SNP-specific primer would perfectly bind its matched template and not react at all with the mismatched template. While this is energetically impossible, it may nonetheless be possible to create situations where the initial discrimination between matched and mismatched primers leads to much more productive amplification of only the matched sets. By manipulating the DNA toehold strand displacement designs originally described in the field of DNA computing, we have come up with a model for mismatch discrimination that relies on equilibration of a very small sequence 'seed', rather than equilibration of a much larger primer. In this model, the initial binding of the seed leads to two processes, which may occur in parallel: first, strand displacement that leads to additional primer-binding and second, strand extension (Figure 1).

In designing our so-called THPs, it was clear that there were several variables that would likely impact their performance, including the length and sequence of the toehold, the length of the hairpin and the placement of mismatches within either the toehold or the hairpin. For example, in a previous study, toehold length was shown to play an important role in toehold-mediated strand-displacement reactions. Changes in the length of the toehold from 5 to 6 nt led to changes in branch migration rates of upwards of 10-fold (23).

We initially attempted to ensure maximum qPCR discrimination of two common SNPs conferring drug resistance in *M. tuberculosis*: *katG* S315T and *rpo*B Q513L (Table 1). Isoniazid susceptibility in *M. tuberculosis* is mediated by the product of the *katG* gene that encodes a heme-containing catalase. A single nucleotide mutation that changes amino acid 315 from serine to threonine is sufficient to confer isoniazid resistance and is a commonly observed mutation in drug resistant *M. tuberculosis* infections (24–27). The antibiotic rifampin inhibits *M. tuberculosis* RNA polymerase and resistance frequently arises from mutations in codon 513 of the beta subunit of the polymerase, the *rpo*B gene (more than 50% of rifampin resistant isolates in some studies (28,29)).

In order to promote maximum discrimination between these alleles and their WT counterparts, we chose to put the mismatch within the short toehold region. Our rationale was that any equilibration that occurred between the short toehold and the target sequence would be greatly affected by the mismatch, preventing either subsequent strand displacement and/or strand elongation by a thermostable polymerase. Further, by using the ARMS strategy of placing the allele-specific nucleotide at the 3′ end of the toehold, we could use the discriminating properties of *Taq* polymerase, which binds but does not efficiently extend a 3′ mismatched primer (Figure 1) (11,14).

Two important considerations for determining the stem length were that (i) the sequence of the extended primer was long enough to be specific for the target and that (ii) the hairpin structure remained stable at annealing and elongation temperatures typical of real-time assays (between 60 and 72°C). A stem length of 18 base pairs (bp) was chosen for the *katG* target and 19 bp for the *rpo*B target. The loop sequence for both targets was a stretch of six thymidines. There are no previous studies of the kinetics of toehold-mediated strand-displacement at a high temperature and we therefore initially assessed toehold lengths from 3 to 9 nt (i.e. T3 to T9 primers) for single mismatch discrimination. All allele-specific primers shared a common linear reverse primer (Table 1).

Optimization of end-point PCR with toehold hairpin primers

Because it was unclear whether and how the THPs would work in qPCR as well as what background and side reactions they might produce, we first evaluated their ability to generate PCR products of the correct size. PCR conditions were initially optimized as described in the 'Materials and Methods' section. The THPs were predicted to have melting temperatures of 62.5°C for *katG* and 69.8°C for *rpo*B (calculated based on a 2.5 mM $MgCl_2$ concentration and assuming complete strand displacement). The common second primers for the PCRs were therefore designed to have T_m values of 62.9 and 73.2°C, respectively. Thermal cycles were designed around these predicted melting temperatures.

The linear positive controls for these assays were primers that had previously yielded efficient amplification and allele discrimination in our hands, and that contained the same target-binding sequence as the THP (Table 1) but without

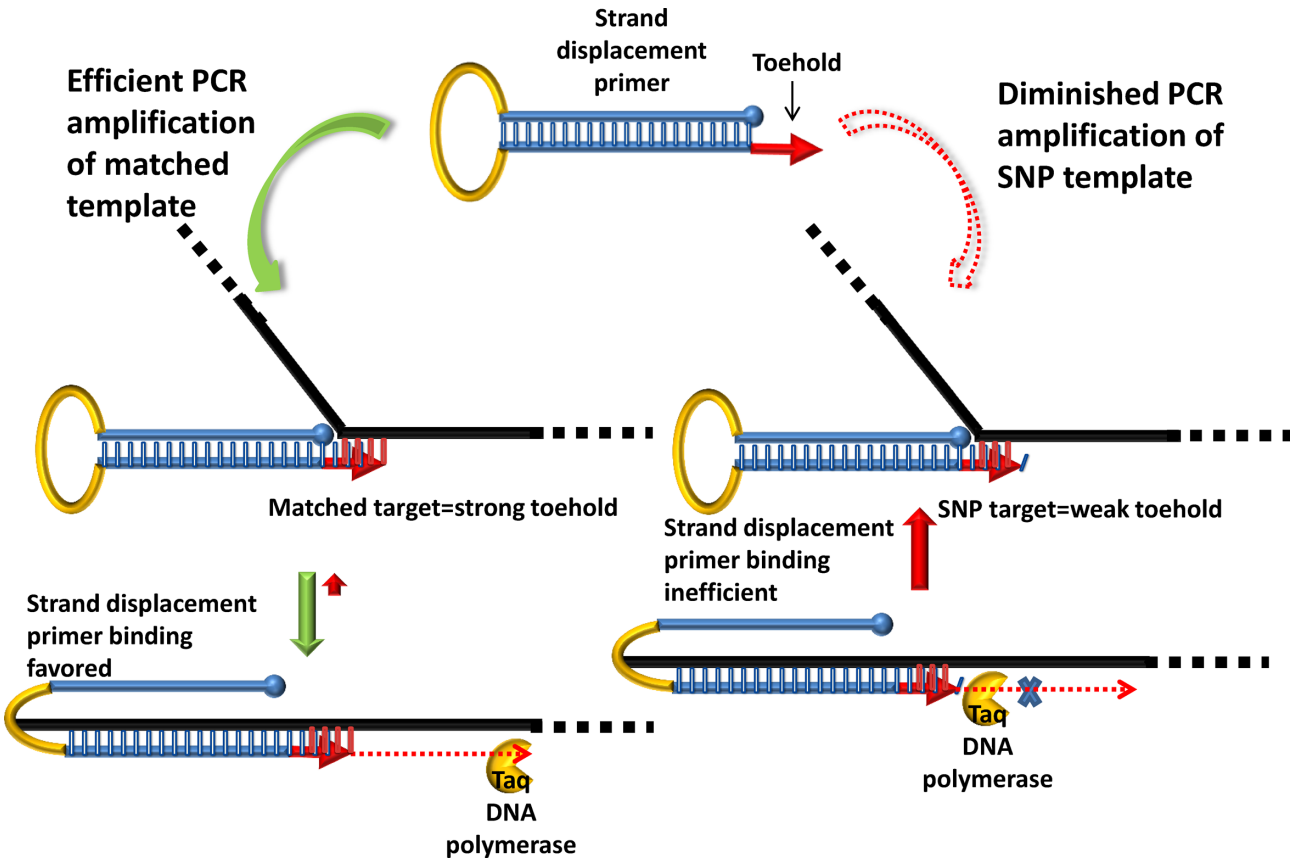


Figure 1. Schematic of toehold-dependent hairpin (THP) primers for enhanced SNP distinction. Most of the target-binding sequence of the hairpin primer is sequestered by hybridization to the complementary sequence in the stem (shown in blue) leaving a short target-specific single stranded 'toehold' (red arrow) at the 3′-end. Binding of the toehold to its complementary target sequence initiates strand displacement leading to primer unfolding and stabilization of the primer:template duplex by extended hybridization. Concomitant initiation of polymerization from the 3′-end by the DNA polymerase leads to template amplification. The toehold hybridization efficiency (and the ensuing primer strand displacement) is designed to be significantly impaired by even a single mismatched nucleotide resulting in primer destabilization and significantly diminished amplification.

a competing complement. As negative controls, we carried out amplifications in the absence of target as well as amplifications with a primer that contained a complementary sequence extension that completely covered the toehold (i.e. a T0 primer) (Table 1).

Reactions were assessed by gel electrophoresis to ensure that an amplicon of the correct size was being produced. Initial experiments revealed no difference between T6 and T9 primers (data not shown). We then attempted to identify conditions that would yield efficient amplification by either T3 or T6 primers yet no amplification in the absence of template or with a T0 primer. We assessed several different buffer conditions and both three-step and two-step PCR cycles.

A simple protocol that produced visible bands for the T6 primer and no bands with the T0 primer at 20 cycles with 50 pg/μl (2.3×10^8 molecules) of template was a two-step PCR with a 2 min denaturing step at 95°C and 20 cycles with a 30 s 95°C denaturing step followed by a 30 s annealing/extension incubation at 68°C (Supplementary Figure S1). These conditions were also amenable to real-time PCR and were therefore used in further analyses.

Optimization of real-time PCR with toehold hairpin primers

Having shown that THPs could produce bands of the correct size, primer designs and reaction conditions were then further optimized in a real-time PCR assay. It might be anticipated that shorter toeholds would produce greater discrimination between alleles. However, since the T3 primer gave weak or no bands in end-point PCR, we tested toehold lengths of 4, 5 and 6 nt for amplification and SNP discrimination. Assays were performed using two-step, real-time PCR and conditions similar to those described above but with the inclusion of a 10 min 95°C incubation to activate the real-time *Taq* 'HotStart' polymerase. To ensure reproducibility and translation to clinical use, we used a commercial master mix (Fast Universal Probe Master, Rox), a qPCR machine designed for clinical applications (LightCycler 96) and FAM-labeled hydrolysis probes. We designated Cq delay, the difference in Cq (i.e. the number of cycles required to achieve a basal signal above background), as a measure of how well our primers discriminate between alleles.

The linear primers (Lin) demonstrated relatively small Cq differences between matched and mismatched targets (Cq delay = 6.2). The THPs showed greater discrimination: the

Table 1. Primers for discrimination of cloned multiple drug resistance alleles of *M. tuberculosis*

M. tuberculosis S315T (AGC>ACG)	Gene *katG* catalase peroxidase	Antiobiotic resistance Rifampin
WT Lin[a]	CCGGTAAGGACGCGATCACCAG	
SNP Lin[a]	CCGGTAAGGACGCGATCACCAC	
WT T0[b]	CTGGTGATCGCGTCCTTACCGGTTTTTTCCGGTAAGGACGCGATCACCAG	
SNP T0[b]	CTGGTGATCGCGTCCTTACCGGTTTTTTCCGGTAAGGACGCGATCACCAC	
WT T3[c]	GTGATCGCGTCCTTACCGTTTTTTCGGTAAGGACGCGATCACCAG	
SNP T3[c]	GTGATCGCGTCCTTACCGTTTTTTCGGTAAGGACGCGATCACCAC	
WT T4	TGATCGCGTCCTTACCGGTTTTTTCCGGTAAGGACGCGATCACCAG	
SNP T4	TGATCGCGTCCTTACCGGTTTTTTCCGGTAAGGACGCGATCACCAC	
WT T4 scrambled[d]	TACGGTTCCGGCGTTACCTTTTTTGGTAACGCCGGAACCGTACCAG	
SNP T4 scrambled[d]	TACGGTTCCGGCGTTACCTTTTTTGGTAACGCCGGAACCGTACCAC	
WT T5	GATCGCGTCCTTACCGGTTTTTTTACCGGTAAGGACGCGATCACCAG	
SNP T5	GATCGCGTCCTTACCGGTTTTTTTACCGGTAAGGACGCGATCACCAC	
WT T6	ATCGCGTCCTTACCGGTTTTTTTTAACCGGTAAGGACGCGATCACCAG	
SNP T6	ATCGCGTCCTTACCGGTTTTTTTTAACCGGTAAGGACGCGATCACCAC	
WT T7	TCGCGTCCTTACCGGTTCTTTTTTGAACCGGTAAGGACGCGATCACCAG	
SNP T7	TCGCGTCCTTACCGGTTCTTTTTTGAACCGGTAAGGACGCGATCACCAC	
WT T8	CGCGTCCTTACCGGTTCCTTTTTTGGAACCGGTAAGGACGCGATCACCAG	
WT T9	GCGTCCTTACCGGTTCCGTTTTTTCGGAACCGGTAAGGACGCGATCACCAG	
Reverse (Lin)[e]	CAGCAGGGCTCTTCGTCAGCTC	
Hydrolysis probe[f]	5′FAM/TGTTGTCCCATTTCGTCGGGGTGTTCGTCC 3′Iowa Black	
Q513L (CAA>CTA)	***rpoB* RNA polymerase beta subunit**	**Isoniazid**
WT Lin[a]	GGCACCAGCCAGCTGAGCCA	
SNP Lin[a]	GGCACCAGCCAGCTGAGCCT	
WT T0[b]	TGGCTCAGCTGGCTGGTGCTTTTTTGCACCAGCCAGCTGAGCCA	
SNP T0[b]	TGGCTCAGCTGGCTGGTGCTTTTTTGCACCAGCCAGCTGAGCCT	
WT T3[c]	CTCAGCTGGCTGGTGCTTTTTTGCACCAGCCAGCTGAGCCA	
SNP T3[c]	CTCAGCTGGCTGGTGCTTTTTTGCACCAGCCAGCTGAGCCT	
WT T4	TCAGCTGGCTGGTGCCTTTTTTGGCACCAGCCAGCTGAGCCA	
SNP T4	TCAGCTGGCTGGTGCCTTTTTTGGCACCAGCCAGCTGAGCCT	
WT T4 scrambled[d]	CGGTGGCCGCTATCGTTTTTTTACGATAGCGGCCACCGGCCA	
SNP T4 scrambled[d]	CGGTGGCCGCTATCGTTTTTTTACGATAGCGGCCACCGGCCT	
WT T5	CAGCTGGCTGGTGCCGTTTTTTCGGCACCAGCCAGCTGAGCCA	
SNP T5	CAGCTGGCTGGTGCCGTTTTTTCGGCACCAGCCAGCTGAGCCT	
WT T6	AGCTGGCTGGTGCCGATTTTTTTCGGCACCAGCCAGCTGAGCCA	
SNP T6	AGCTGGCTGGTGCCGATTTTTTTCGGCACCAGCCAGCTGAGCCT	
Reverse (Lin)[e]	GCCCGGCACGCTCACGTGACAG	
Hydrolysis probe[f]	5′FAM CCGACTGTTGGCGCTGG 3′ Iowa black	

[a]Lin whose 3′-end nucleotide is complementary to the wild-type (WT) or the mutant (SNP) allele.
[b]WT or SNP-specific hairpin primers without a 3′-end toehold.
[c]WT or SNP specific toehold hairpin primers with the 3′-end toeholds denoted as 'T' followed by the number of nucleotides in the toehold.
[d]Only the primer toehold displays target complementarity. The stem sequence is scrambled to prevent target recognition.
[e]Common reverse primer.
[f]Template-specific fluorescent probe for real-time amplicon detection.

T6 primer gave a Cq delay of 8.7, the T5 primer gave a Cq delay of 15, while the T4 primer did not amplify the mismatched target (Figure 2A). The T4 primers reproducibly gave an average Cq of 32.5 for the WT template and showed no amplification through 45 cycles with the mutant template (Figure 2B**)**. These results were in general concordance with the notion that mismatch discrimination by THPs was highly dependent upon the initial contact of the toehold with the template. It should also be noted that these results show much greater Cq delay values than previously published hairpin primers without allele-specific toeholds (16). For example, a hairpin primer with the toehold in the loop of the hairpin yielded a maximum difference in cycle number between a matched template and a single mismatch of 11.2 cycles, as opposed to the 15 or greater cycle differences that we routinely observe.

In order to demonstrate that both strand extension and strand displacement were important for the function of THPs, we created a primer that was similar to T4, but in which the complementary sequence beyond the toehold was scrambled (T4Scr). The T4Scr primer showed no amplification of either the WT or mutant targets.

Having shown that WT THPs could discriminate against mutant alleles, we attempted to show that primers specific for the mutant could be readily generated and would in turn similarly discriminate against the WT gene. To this end, we modified the 3′ nucleotide on the *katG* WT T4 primer from a C to a G (*katG* S315T T4). The linear version of the primer gave a Cq delay between mutant and WT templates of 9 cycles, while the *katG* S315T T4 primer once again did not yield amplification of the mismatched (in this case WT) template (Figure 2C).

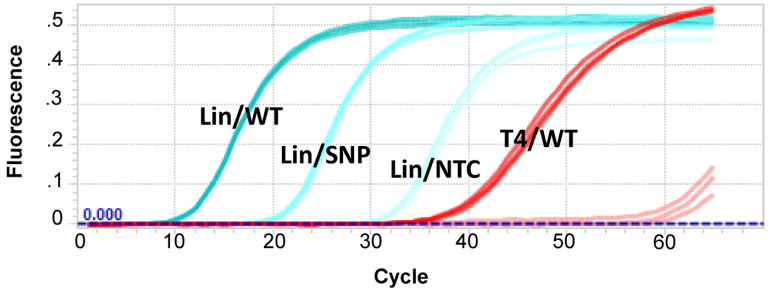
A. **KatG WT THPs Magnify Discrimination Against Mismatches**

Toehold/Allele	Cq Mean	Delay in Cq
T4/WT	31.4	28.1
T4/SNP	60	
T5/WT	12	15
T5/SNP	26.9	
T6/WT	11.7	8.7
T6/SNP	20.4	
Linear/WT	11	6.2
Linear/SNP	17.2	

C. **Cq of KatG WT and SNP T4 Primers on Matched and Mismatched Templates**

	KatG Target Plasmid		
Toehold/Allele	S315 WT	S315T SNP	No template
T4 WT	32.5		
T4 SNP		22.2	
Linear WT	11.4	14.1	37.1
Linear SNP	17.6	13.1	36.8
T0 WT			
T0 SNP		37.2	
T4Scr WT			
T4Scr SNP			

B. **KatG WT Amplification Curves**



D. **Cq of RpoB WT and SNP T4 Primers on Matched and Mismatched Templates**

	RpoB Target Plasmid		
Toehold/Allele	Q513 WT	Q513L SNP	No template
T4 WT	28		
T4 SNP		31.2	
Linear WT	17.6	33.3	
Linear SNP	41.1	12.1	36.0
T0 WT			
T0 SNP			
T4Scr WT			
T4Scr SNP			

Figure 2. Effect of toehold length on SNP distinction by THP primers. Hairpin primers specific for the *Mycobacterium tuberculosis* wild-type (WT) *katG* allele were designed with 4, 5 or 6 nucleotide long toeholds (T4, T5 and T6, respectively) at their 3′-ends. The amplification efficiency of linear (Lin) and THP primers and their ability to discriminate between the WT and single nucleotide mismatch (SNP)-containing alleles of *katG* was compared by real-time qPCR analysis of cloned gene variants. The average Cq of amplification from triplicate experiments, (**A**) a representative set of qPCR amplification curves, (**B**) and Cqs of amplification from triplicate experiments for T4 WT and SNP primers for *katG* (**C**) and *rpo*B alleles (**D**) are depicted.

Generalization to other genes

We then tested a similar THP design with the *rpo*B WT gene and its Q513L allele. The previous results with the *katG* gene suggested that a T4 primer yielded exquisite discrimination. Therefore we designed primers for *rpo*B that had only a 4 nt toehold and a 19 bp stem-obscured sequence complementary to the *rpo*B WT target (Table 1). Gradient PCR analysis revealed that the T4 primer performed well in a two-step PCR, with annealing and extension at 72°C (Supplementary Figure S2). We then verified allele discrimination with 50 pg/μl of template (2.3×10^8 targets in a 20 μl volume). The linear primer amplified the WT allele at a Cq of 17.6 and the Q513L SNP template at a Cq of 41.1, while the *rpo*B WT T4 primer amplified the WT template at an average Cq of 28, but showed no amplification of the mismatched SNP target, even through 60 cycles (Figure 2D).

We also changed the 3′ nucleotide on these primers to be specific for the Q513L SNP (Table 1). The resultant linear primer amplified the Q513L template at a Cq of 12.1 and the WT at a Cq of 33.3 while, again, the *rpo*B Q513L T4 primer amplified the Q513L template with an average Cq of 31.2 but showed no amplification of the WT, even through 60 cycles (Figure 2D). This 'digital discrimination' of different alleles should prove useful for diagnostics.

In order to more fully assess THP designs against multiple targets we attempted to identify individual SNPs within the genomes of two fully sequenced *E. coli* B strains, REL606 and CZB154. CZB154 is a strain that evolved the ability to aerobically utilize citrate during the course of a long-term evolution experiment, and REL606 is its parent (30,31). CZB154 harbors 79 known mutations that are not present in the genome of the parent (32) and thus was particularly suitable for the development of genotyping assays. We designed THP primers for mutations specific for ten SNPs within CZB154. The primers should preferentially recognize a variety of mutational changes relative to the parental strain, including five transversions and five transitions, T>G, T>C, A>C, A>G, A>T, C>T, C>A, G>C, G>A and G>T (Table 2). To further show the generality of our new method, qPCR experiments were carried out with Evagreen, a double-stranded DNA-binding dye (Evagreen, Biotium; Hayward, CA.), rather than with a TaqMan probe.

We attempted to specifically identify 10^7 targets (2.5 ng/μl in a 20 μl reaction) 20× fewer than the 2×10^8 targets

Table 2. Primers for detection of genomic mutations in *E. coli*

CZBa 154 mutation	Gene	CZB 154 mutation	Gene
N174T (AAC>ACC)	***yaaH inner membrane protein (acetate transport)***	**T792K (ACG>AAG)**	***topA DNA topoisomerase I***
WT Lin5	GGTGCGACCAAACTGCTCGT	WT Lin5	CTTCCACCAGTGGCGCACGCG
SNP Lin5	GGTGCGACCAAACTGCTCGG	SNP Lin5	CTTCCACCAGTGGCGCACGCT
WT T5	CAGTTTGGTCGCACCTTTTTTGGTGCGACCAAACTG CTCGT	WT T5	GCGCCACTGGTGGAAGTTTTTTCTTCCACCAGTGGC GCACGCG
SNP T5	CAGTTTGGTCGCACCTTTTTTGGTGCGACCAAACTG CTCGG	SNP T5	GCGCCACTGGTGGAAGTTTTTTCTTCCACCAGTGGC GCACGCT
Rev 181	GCGCACGCGTTCTGCAATTC	Rev 220	CATGGCCTGCACCAACGAAGAGTG
E245A (GAG>GCG)	***setA broad specificity sugar efflux system***	**T46A (ACC>GCC)**	***malT transcriptional regulator MalT***
WT Lin4	CGTTGTGGATCAGTAGCGA	WT Lin4	CCACTGGGAAATGAGGGTGGT
SNP Lin4	CGTTGTGGATCAGTAGCGC	SNP Lin4	CCACTGGGAAATGAGGGTGGC
WT T4	TACTGATCCACAACGTTTTTTCGTTGTGGATCAGTA GCGA	WT T4	CCCTCATTTCCCAGTGGTTTTTTCCACTGGGAAATG AGGGTGGT
SNP T4	TACTGATCCACAACGTTTTTTCGTTGTGGATCAGTA GCGC	SNP T4	CCCTCATTTCCCAGTGGTTTTTTCCACTGGGAAATG AGGGTGGC
Rev 164	CAGTACTCCTGCCGCCAC	Rev 129	CCGGTTCGACTCGACCATACC
S27G (AGT>GGT)	***fadE acyl CoA dehydrogenase***	**L100Q (CTG>CAG)**	***rplS 50S ribosomal protein L19***
WT Lin4	CAGGCGAGCAAAATCAGACT	WT Lin4	CTTACCAGTACGCTCACGCA
SNP Lin4	CAGGCGAGCAAAATCAGACC	SNP Lin4	CTTACCAGTACGCTCACGCT
WT T4	TGATTTTGCTCGCCTGTTTTTTCAGGCGAGCAAAAT CAGACT	WT T4	TGAGCGTACTGGTAAGTTTTTTCTTACCAGTACGCT CACGCA
SNP T4	TGATTTTGCTCGCCTGTTTTTTCAGGCGAGCAAAAT CAGACC	SNP T4	TGAGCGTACTGGTAAGTTTTTTCTTACCAGTACGCT CACGCT
Rev 246	ACTTTTTGACCTGAAGTGCGG	Rev 193	ATTCGAGGGCGTGGTTATCG
G45A (GGC>GCC)	***secF protein export***	**Q79L (CAA>CTA)**	***arcB hyb sensory his kinase in 2comp reg sys with ArcA***
WT Lin4	GTTATTATGGGCGTGCGCGG	WT Lin4	CTACTTTCTATCGGTGGTCGTCGAGCA
SNP Lin4	GTTATTATGGGCGTGCGCGC	SNP Lin5	CTACTTTCTATCGGTGGTCGTCGAGCT
WT T4	GCACGCCCATAATAACTTTTTTGTTATTATGGGCGT GCGCGG	WT T4	CGACGACCACCGATAGAAAGTAGTTTTTTCTACTTT CTATCGGTGGTCGTCGAGCA
SNP T4	GCACGCCCATAATAACTTTTTTGTTATTATGGGCGT GCGCGC	SNP T5	GACGACCACCGATAGAAAGTAGTTTTTTCTACTTTC TATCGGTGGTCGTCGAGCT
Rev 153	ACCAAAGTTTTGCAGCATCGG	Rev 187	GGTTTCCTGCAGTTCTGCTTCCG
A258T (GCT>ACT)	***gltA citrate synthase***	**S33R (AGC>AGA)**	***mepA penicillin insensitive murein endopeptidase***
WT Lin4	CAGGTCCCCACAGTGAAGC	WT Lin5	GAAAAACTGCCTATCGATTGTGCG
SNP Lin4	CAGGTCCCCACAGTGAAGT	SNP Lin5	GAAAAACTGCCTATCGATTGTGCT
WT T4	CACTGTGGGGACCTGTTTTTTCAGGTCCCCACAGTG AAGC	WT T5	AATCGATAGGCAGTTTTTCTTTTTTGAAAAACTGCC TATCGATTGTGCG
SNP T4	CACTGTGGGGACCTGTTTTTTCAGGTCCCCACAGTG AAGT	SNP T5	AATCGATAGGCAGTTTTTCTTTTTTGAAAAACTGCC TATCGATTGTGCT
Rev 144	TCTGGAACGTGCTATGGACC	Rev 115	GATTGCGCTGCTGGCTCTGC

used in the *M. tuberculosis* SNP allele assays. The potential background in these experiments was also much more extensive, as we went from detecting targets on a 4 kb plasmid to detecting the SNPs in a bacterial genome (4600 kb.) This also provided us with an opportunity to better understand what parameters could be optimized for THPs to ensure successful amplification and allelic discrimination.

In greater detail, we began by using NCBI primer blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) with the *E. coli* REL606 NC_012967.1 FASTA sequence as a reference to design the qPCR assays. Our design strategy was to first designate a forward linear primer that ended in a 3′ allele-specific residue and that had a predicted melting temperature between 60°–65°C in 50 mM Na^+, 2 mM Mg^{++} and 200 nM primer. For the SNP-detecting primers, we then changed the 3′ base to pair with the SNP-specific nucleotide. Amplicon lengths were kept between 100 and 200 nucleotides to remain in the range for efficient qPCR.

Based on the linear primer designs, THPs were designed using the same principles that had worked with allele detection in the *M. tuberculosis* genome: a single-stranded, four residue toehold that contained the allele discriminating base at its 3′ end was juxtaposed with a hairpin stem that encompassed the remaining bases in the primer, followed by a six thymidine loop and a complementary stem.

Initially, we screened all linear primers in a two-step PCR under the previously described conditions to ensure that amplicons would arise. We then began to test and optimize THPs. We first assayed the *yaaH* N174 SNP site using THPs with 4 nt toeholds and a two-step PCR with 200 nM primer. However, amplification was inefficient and the LightCycler 96 machine calls were negative. In moving from a 4-kb plasmid to a 4600-kb genome, we had increased the amount of potentially interfering DNA by three orders of magnitude. To reduce competition for toehold hybridization, we extended the THP toehold to 5 nt. This moved the Cq values to 51 and 46 for THPs specific for SNP and WT alleles with their respective target genomes, and resulted in positive calls by the machine. To improve amplification efficiency, primer concentrations were doubled to 400 nM, which improved the Cq values to 29 and 23 for SNP and WT alleles, again with no amplification of the cross-pairs (WT-detecting THP primer on SNP-containing genome; SNP-detecting THP primer on WT genome) (Table 3).

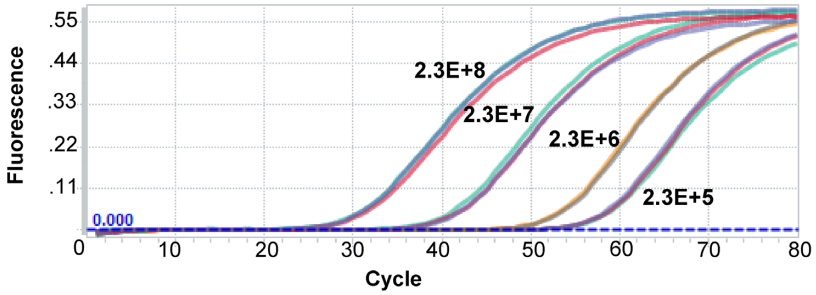
Further optimization was also required for THPs that targeted other mutation sites, as Cq values were frequently beyond 50 cycles. To further improve the initial annealing

Table 3. Distinction of *E. coli* strain-specific genomic SNPs by real-time qPCR using toehold hairpin primers

Gene	Primer[a]	Mutation	Mismatch	Strength of mismatch[b]	Cq matched target[c]	Cq mismatched target[d]	Cq delay[e]
yaaH	N174 WT T5	T>G	T:C	Maximum	23.1	No amplification detected	
	N174 WT Lin		T:C		10.1	31.1	21.0
	N174T SNP T5		G:A	Maximum	29.2	No amplification detected	
	N174T SNP Lin		G:A		15.8	21.7	5.9
setA	E245A WT T4	A>C	A:G	Maximum	28.7	No amplification detected	
	E245A WT Lin		A:G		11.3	32.6	21.3
	E245A SNP T4		C:T	Maximum	26.7	46.5	19.8
	E245A Lin SNP		C:T		14.8	27.0	12.2
fadE	S27 WT T4	T>C	T:G	Weak	28.2	No amplification detected	
	S27 WT Lin		T:G		10.1	24.6	14.5
	S27G SNP T4		G:A	Maximum	35.6	51.1	15.5
	S27G Lin SNP		G:A		15.5	18.8	3.3
secf	G45 WT T4	G>C	G:G	Medium	33.8	No amplification detected	
	G45 WT Lin		G:G		11.7	32.0	20.3
	G45A SNP T4		C:C	Strong	37.9	No amplification detected	
	G45A Lin SNP		C:C		15.6	28.3	12.7
gltA	A258 WT T4	C>T	C:A	Weak	24.3	No amplification detected	
	A258 WT Lin		C:A		9.9	31.2	21.3
	A258T SNP T4		T:G	Weak	40.7	53.9	13.2
	A258T SNP Lin		T:G		14.6	19.6	5.0
topA	T792 WT T5	C>A	G:A	Maximum	24.7	No amplification detected	
	T792 WT Lin		G:A		11.1	28.8	17.7
	T792K SNP T5		T:C	Maximum	33.1	54.8	21.7
	T792K Lin SNP		T:C		17.0	27.0	10.0
malT	T46 WT T4	A>G	T:G	Weak	23.7	48.4	24.7
	T46 WT Lin		T:G		10.5	19.0	8.4
	T46A SNP T4		C:A	Weak	23.7	42.3	16.3
	T46A SNP Lin		C:A		14.6	20.7	6.1
rplS	L100 WT T4	A>T	A:A	Medium	26.1	No amplification detected	
	L100 WT Lin		A:A		10.0	34.1	24.1
	L100Q SNP T4		T:T	Maximum	40.0	No amplification detected	
	L100Q SNP Lin		T:T		17.6	31.4	13.8
arcB	Q79 WT T4	T>A	A:A	Medium	22.0	49.3	27.3
	Q79 WT Lin		A:A		10.5	29.2	18.7
	Q79Ls SNP T5		T:T	Maximum	28.2	44.1	15.9
	Q79L SNP Lin		T:T		14.6	19.3	4.7
mepA	S33 WT T5	G>T	G:A	Maximum	28.4	No amplification detected	
	S33 WT Lin		G:A		12.4	30.6	18.2
	S33R SNP T5		T:C	Maximum	41.6	No amplification detected	
	S33R SNP Lin		T:C		17.8	23.9	6.1

[a]'WT' primers are specific for the parental *E. coli* strain REL606 while the 'SNP' primers are specific to genomic alleles in the mutant strain CZB154.
[b]mismatch strength was predicted based on previous reports (33).
[c]Average Cq of allele detection using complementary primers.
[d]Average Cq of allele detection using primers with a 3′-end mismatch.
[e]'Cq delay' measures the average delay in amplification of mismatched genomic alleles by THP and linear control primers.

A. *M. tuberculosis* KatG WT T4 THP Limit of Detection Amplification Curves



B. KatG and RpoB WT Primers Standard Curves

	THP (T4) *KatG* WT	Linear *KatG* WT	THP (T4) *RpoB* WT	Linear *RpoB* WT
Efficiency	1.3	1.9	1.3	1.6
Slope	-9.3	-3.5	-8.2	-4.6
Error	1.6	0.4	2.8	0.8
R^2	1.0	1.0	0.9	1.0
Y intercept	110.0	-24.8	97.6	53.9

C. KatG and RpoB WT T4 THP Reproducibility

Standard Concentration	Average Detected Concentration *KatG* T4 WT	Interassay Standard Deviation	Average Detected Concentration *KatG* WT Linear	Interassay Standard Deviation	Average Detected Concentration *RpoB* T4 WT	Interassay Standard Deviation	Average Detected Concentration *RpoB* WT Linear	Interassay Standard Deviation
2.3E+08	2.5E+08	12%	2.2E+08	17%	2.0E+08	11%	2.2E+08	29%
2.3E+07	2.6E+07	12%	2.9E+07	5%	2.1E+07	14%	3.3E+07	2%
2.3E+06	1.9E+06	26%	1.9E+06	4%	5.8E+05	56%	1.2E+06	42%
2.3E+05	3.3E+05	20%	2.6E+05	10%	4.5E+05		3.8E+05	21%

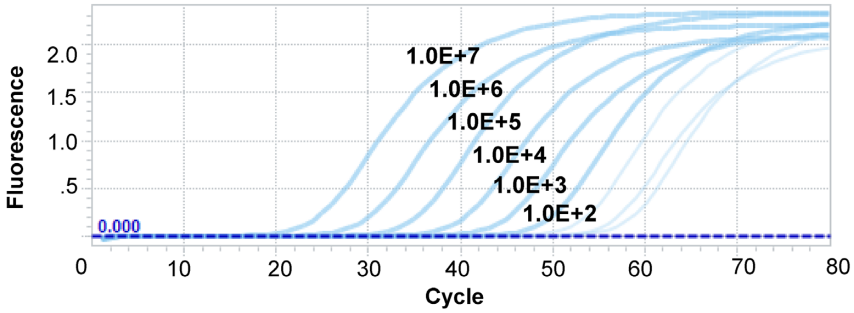
Figure 3. Comparison of amplification efficiency, reproducibility and limit of detecting cloned *Mycobacterium tuberculosis katG* and *rpo*B genes by qPCR using specific linear versus THP primers. (**A**) A representative set of qPCR amplification curves generated by amplification of 2.3×10^8 to 2.3×10^5 copies of cloned *kat*G WT genes using the *katG* WT T4 primer. (**B**) Comparison of the amplification efficiencies of linear versus T4 THP primers specific for *kat*G and *rpo*B WT alleles. Standard curves were generated from triplicate qPCR amplifications of 2.3×10^8 to 2.3×10^5 copies of cloned templates. (**C**) Comparison of linear versus THP primer-based qPCR assay reproducibility. Absolute quantification of cloned WT *kat*G and *rpo*B genes from replicate assays using specific linear versus T4 THP primers is depicted. The lowest reproducibly detected template concentration is reported as the limit of detection for each primer.

of the toehold, we moved from two-step to three-step PCR, again with 400 nM primer concentrations. In the three-step PCR an annealing temperature of 65°C was chosen, three degrees below the 68°C annealing and extension temperature, and included an extension step at 72°C for 20 s. This improved parameter set resulted in working assays for five mutation sites, including *gltA* A258, *secF* G45, setA E245, *fadE* S27 and *rplS* L100 (Table 3).

For the remaining assays, we noted that at least one primer in each set might have more weakly annealed to the template than its partner. For example, in the *mepA* S33 assay we noted that while the common reverse linear primer had 50% GC content and a T_m of 63.9°C, its 3′ end contained two adenosines. The efficiency of both the *mepA* S33 SNP and WT primers was improved by developing a new common reverse primer. This primer was identified by allowing a 5°C difference between the forward and reverse primers, rather than the 3°C default difference set by the NCBI Primer Blast program. A reverse primer (Table 2) predicted to have a T_m of 68.3°C under our assay conditions and which yielded a shorter product length (115 versus 190 bp) was identified. In a three-step PCR, the new primer yielded Cq values of 44 and 32 for the SNP- and WT-detecting primers on their respective target genomes (Table 3). This primer could also be extended to a 5 nt toehold, terminating in a 3′ G, which further resulted in Cq values of 42 for the SNP and 28 for the WT primers. No amplification was detected for either toehold length on mismatched target sequences. In contrast, the linear ARMs primer with a 3′ SNP-specific base had a Cq delay of only 6 cycles between matched and unmatched targets (Table 3, Figure 5).

The remaining alleles (*topA* T792, *malT* T46 and *arcB* Q79) did not show amplification even with higher primer concentrations, longer toeholds and three-step PCR. We noted that each had at least one allele-specific primer with a low GC content (0–25%). We therefore designed different

A. *E. coli* yaaH N174 WT T5 Limit of Detection Amplification Curve



B. *E. coli* yaaH N174 Primers Standard Curves

	THP (T5) N174 WT	Linear N174 WT	THP (T5) N174T SNP	Linear N174T SNP
Efficiency	1.6	1.9	1.6	2.0
Slope	-5.2	-3.5	-4.9	-3.3
Error	1.6	1.6	1.7	1.3
R^2	1.0	0.9	0.9	0.9
Y intercept	58.0	61.0	71.0	52.0

C. *E. coli* yaaH N174 Primers Reproducibility Between Assays

Standard Concentration	Average Detected Concentration N174 T5 WT	Interassay Standard Deviation	Average Detected Concentration N174 Linear WT	Interassay Standard Deviation	Average Detected Concentration N174T T5 SNP	Interassay Standard Deviation	Average Detected Concentration Linear N174T SNP	Interassay Standard Deviation
1.00E+07	1.17E+07	5%	1.29E+07	15%	6.92E+06	87%	4.78E+08	78%
1.00E+06	9.70E+05	7%	1.23E+06	15%	1.21E+06	104%	8.28E+05	53%
1.00E+05	9.33E+04	0%	7.51E+04	26%	1.21E+05	42%	3.35E+03	125%
1.00E+04	7.10E+03	39%	8.61E+03	35%	1.15E+04	77%	1.06E+04	139%
1.00E+03	1.42E+03	28%	1.31E+03	33%				
1.00E+02			8.61E+01	11%				

Figure 4. Comparison of amplification efficiency, reproducibility and limit of detecting *Escherichia coli yaaH* WT (N174) and SNP (N174T) genomic alleles by qPCR using allele-specific linear or THP primers. (**A**) A representative set of qPCR amplification curves generated by amplification of 10^7 to 10^2 wild-type *E. coli* genomes using the *yaa*H N174 WT-specific T5 THP primers. (**B**) Comparison of the amplification efficiencies of linear versus T5 THP primers specific for *E. coli yaa*H N174 WT or the N174T SNP genomic alleles. Standard curves were generated from triplicate qPCR amplifications of 10^7 to 10^2 copies of target genomes mixed with 10^7 copies of non-specific genomes. (**C**) Comparison of linear versus THP primer-based qPCR assay reproducibility. Absolute quantification of *E. coli yaa*H WT and N174T SNP genomic alleles from replicate assays using specific linear versus T5 THP primers is depicted. The lowest reproducibly detected template concentration is reported as the limit of detection for each primer.

primers using the template sequences rather than the coding sequences of these genes. The new primer sets had toeholds with 50–80% GC content; we tried toehold lengths of both 4 and 5 nt. In testing the 4 nt toeholds, we found that the primer for the WT allele of *arcB* Q79 produced good amplification, while the other alleles required 5 nt toeholds. The THPs for these three sites now gave robust discrimination, with the lowest delay in Cq between matched and mismatched templates of 16 cycles and no amplification detection of mismatched template in the T792 WT allele-detecting primer. The linear versions of the primers on these three alleles had an average Cq delay between matched and mismatched templates of 11 cycles compared with an average Cq delay of 23 cycles for THPs (Table 3, Figure 5). Interestingly, in THP designs the canonical strength of the terminal mismatch had only a mild effect, with weak G:T and A:C mismatches enabling robust discrimination in several THPs (33).

Overall, the designed primers could specifically amplify all 10 of the new SNPs, in most cases with the same extraordinary specificity as the THPs directed against drug resistance markers. For many alleles, no amplification of mismatched (parental) templates was detected after 60 cycles; the smallest delay in Cq between matched and mismatched targets observed was 13 (Table 3, Figure 5). The empirical rule set for primer design involves adjusting primer concentrations and positions, extending toeholds, choosing optimal GC contents in toeholds and/or lowering annealing temperatures. We anticipate that THPs can be engineered

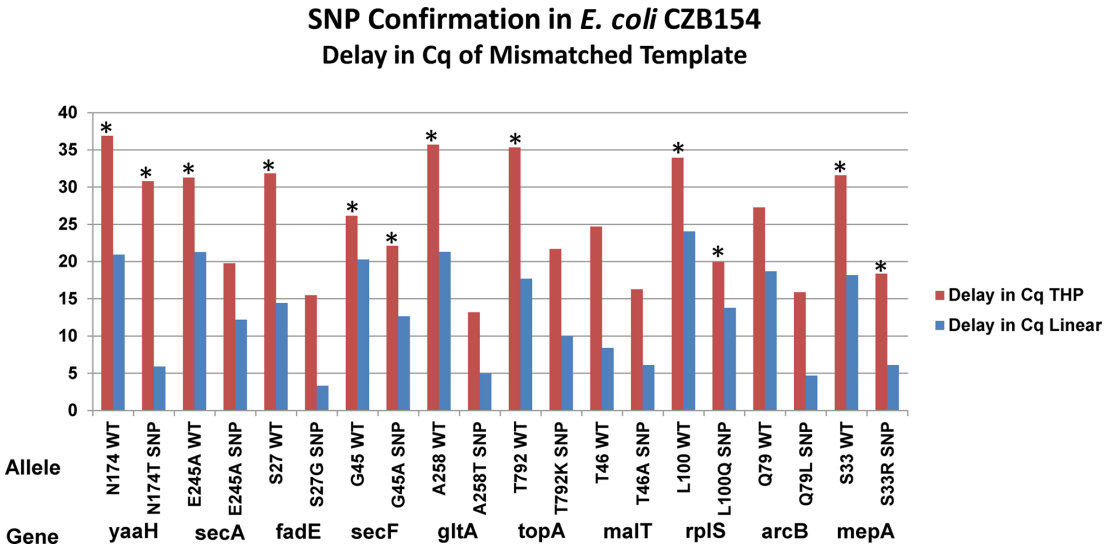


Figure 5. Comparison of the Cq delay in qPCR amplification of mismatched *Escherichia coli* genomic alleles using THP versus linear primers. Triplicate qPCR assays were used to determine the Cq delay in amplification of crosspaired *E. coli* genomic targets using linear (blue bars) or THP (red bars) primers specific for WT (REL606) or SNP (CZB154) alleles. Primers that did not generate significant fluorescent signals from their mismatched target genome at the end of 60 amplification cycles are tagged with an asterisk.

for most genomic targets, including those in the human genome, by adjusting these parameters.

Efficiencies, reproducibility and limits of detection for Toehold Hairpin Primers

While the THPs showed excellent discrimination between alleles, they were less efficient than their linear counterparts. This could potentially limit their applicability for the detection of small amounts of template. We therefore carried out real-time PCR assays with the *katG* and *rpo*B THPs at different template concentrations (between 50 pg/μl (2.3×10^8 targets) and 50 fg/μl (2.3×10^5 targets) to better establish their efficiencies and limits of detection (Figure 3A and C). Perfectly optimized real-time PCR primers should exhibit an efficiency of 2, indicating a doubling of the target sequence at each cycle. *KatG* linear primer efficiencies averaged 1.9, while comparable T4 primer efficiencies were 1.3. efficiencies for the *rpo*B linear primers averaged 1.6, while the THPs averaged 1.4 (Figure 3B). Efficiencies for the *E. coli yaaH* N174 T5 THPs were 1.6 for both WT- and SNP-detecting primers, while their linear counterparts were 1.9 and 2, respectively. Even so, the THPs could detect down to 1000 copies of relevant *E. coli* genomes relative to no template controls (Figure 4B and C). It is possible that even smaller amounts of template would not be amplified by THPs, but this could be readily overcome by using nested PCR amplification with linear primers specific for extensions embedded within the THPs.

The limits of detection (LOD) were assessed for the primer sets that specifically amplified *E. coli yaaH* N174 SNP and WT alleles. Decreasing amounts of target genome (10^7 to 10 copies) were spiked into a background of 10^7 non-target genomes; i.e. for SNP-detecting primers, 10^7 to 10 CZB154 genomes were spiked into wells containing 10^7 REL606 parental genomes and vice versa for WT-detecting primers. We found that THPs demonstrated limits of detection similar to those observed for linear primers. The THPs specific for the WT allele could reproducibly detect 1000 molecules of cognate genome in the presence of 10^7 non-cognate genomes, while the linear primers could detect 100 molecules; conversely, the LOD for both linear and toehold hairpin SNP-specific primers was 10^4 SNP alleles in a background of 10^7 WT genomes (Figure 4A and C).

Reproducibility was assessed by determining the LODs with different dilutions of primers and targets on different days. Standard curves were generated for each new experiment (Figures 3B and 4B). The THPs for *M. tuberculosis katG* and *rpo*B alleles and *E. coli yaaH* N174 alleles were similar to linear primers in terms of reproducibility, while the N174 THP primer set generally demonstrated a lower standard deviation between runs for detecting absolute concentrations of the standard curve than the linear primer set (Figures 3C and 4C).

One caveat when determining both the Cq delay and the limits of detection of our THPS is the dependence on the concentration and quality of the polymerase used in the assay. While we used commercial master mixes to make the technology transferable, variations in the Cq delay and LODs should be monitored with an allele-specific standard curve for each new master mix.

While THPs are not as efficient as linear primers, they are far more efficient than previously described hairpin primers. The T0 primer specific for the *katG* S315T SNP did not show amplification until an average of 37.3 cycles while the T4 primer for the same SNP had an average Cq value of 22.2. This result is very consistent with our proposed mechanism for toehold binding followed by both extension via *Taq* polymerase and strand displacement.

DISCUSSION

In summary, a simple primer design method adapted from the field of DNA computation allows synthetic DNA

oligonucleotides to be generated that can yield exquisitely high discrimination between even single nucleotide mismatches during real-time PCR. Our results suggest that mismatch discrimination by toehold hairpin primers was highly dependent upon the initial contact of the toehold with the template, and that the stability of this contact determined whether strand displacement and extension by the polymerase could subsequently occur. Toehold hairpin primers show much greater Cq delay values for SNPs than previously published linear primers. The differentiation between mismatches is typically on the order of 10 000-fold. While more qPCR cycles must be carried out, the diminution in the efficiency of detection is likely to be minimal, especially because of the exquisitely low background amplification exhibited by toehold hairpin primers. We anticipate that these reagents will see broad use for allele detection, especially in Point of Care settings where yes/no answers are most valued.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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