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G-quadruplex-generating PCR for visual colorimetric detection of amplicons

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Abstract

We have developed a self-reporting PCR system for visual colorimetric gene detection and distinction of single nucleotide polymorphisms (SNP). Amplification is performed using target-specific primers modified with a 5'-end tail that is complementary to a G-quadruplex deoxyribozyme-forming sequence. At end-point G-quadruplexes are forced to fold from PCR-generated duplex DNA and then used to colorimetrically report the successful occurrence of PCR by assaying their peroxidase activity using a chromogenic substrate. Furthermore, primer design considerations for the G-quadruplex-generating PCR system have allowed us to visually distinguish SNPs associated with *Mycobacterium tuberculosis* drug resistance alleles.

Keywords

G-quadruplex; single nucleotide polymorphism; *Mycobacterium tuberculosis*; polymerase chain reaction; colorimetric detection

Reliable, fast and accessible molecular diagnostics is crucial for management of infectious and metabolic diseases. Polymerase chain reaction (PCR) has become the gold standard for several diagnostic applications particularly due to its specificity and sensitivity of detection [1]. PCR is usually detected either by staining double-stranded DNA with dyes such as SYBR Green, or by using sequence-specific probes, such as TaqManTM probes. In both instances, fluorescent signals are typically read using a real-time PCR machine. While low-cost and field-ready PCR devices are being built to allow widespread application of PCR-based diagnostics, the use of fluorescent dyes and probes remains cost prohibitive in resource poor nations [2].

We have developed a variant of PCR (termed G-quadruplex-generating PCR) that produces as a result of target-specific amplification a short G-quadruplex deoxyribozyme (DNAzyme) with peroxidase activity in the presence of hemin that turns over a chromogenic substrate 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and H₂O₂ to produce a colorimetric signal [3–5]. G-quadruplex/hemin complexes can also oxidize luminescent,

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electrochemical and fluorogenic substrates allowing integration into chemiluminescent, electrochemical and fluorimetric detection platforms.

DNAzymes have been widely used in biosensors, nucleic acid circuits as well as in nucleic acid detection and amplification assays [6–17]. Previous efforts had shown that G-quadruplexes or their complements placed at the termini of PCR primers could be used to follow the progression of amplification reactions [9–12; 18]. In these schemes, the G-quadruplex was either incomplete and was formed upon the addition of several deoxyguanosine residues [10–12], or required displacement or partial degradation of the complementary strand in order to function as a peroxidase [9; 18]. However, each of these schemes had drawbacks. For example, some G-quadruplex completion methods limited where primer-binding sites could be located and primers containing G-quadruplexes also had relatively high background [10], while methods dependent on partial degradation of completed PCR products required column purification of PCR products and relied on exonucleases that were not thermostable, and thus had to be added at the conclusion of PCR [9]. The colorimetric detection schemes that relied on releasing a G-quadruplex sequence sequestered within a hairpin primer by extension-dependent displacement of the complementary strand were especially interesting [18]. However, because the synthesis of these primers required DNA polymerase-blocking ‘Spacer’ moieties between the G-quadruplex and its complement groups may have incurred greater expense compared to unmodified oligonucleotides, while decreases in the efficiency of synthesis likely require purification prior to use. Furthermore, it is possible that displaced primers might generate significant background signal even in the absence of target amplification due to spontaneous folding of G-quadruplex DNAzymes, as has previously been observed during isothermal quadruplex priming nucleic acid amplification reactions [10]. In consequence, while the examples of strand displacement primers that have been assayed have shown excellent signal-to-noise ratios, it is nonetheless a concern that more generalized primer design may not be possible with this approach [18].

To avoid background and ensure G-quadruplex formation only during amplification, we have developed a method that relies on using primers containing sequences complementary to the G-quadruplex at their 5'-end (Figure 1a). Only upon extension is a G-quadruplex sequence formed at each end of the amplicon; since there is not even a partial G-quadruplex in the reaction mixture prior to extension, potential background should be greatly reduced for virtually any primer set. Following the amplification reaction heat denaturation and quick cooling in the presence of hemin and endogenous potassium ions leads to the folding of the G-quadruplex even in the presence of its complement, without the need for additional enzymes or buffer modifications.

We designed allele-specific, G-quadruplex-generating primers for several SNPs associated with drug-resistant *M. tuberculosis* (Supplementary Table S1) [19–21]. The relevant SNP was encoded at the 3'-most position of the primer and a second mismatched base was introduced at the third nucleotide in from the 3'-end (termed 2m primers) (Supplementary Table S2). We also tested 2m primers bisected by a bubble of 5 inosine nucleotides (dual-priming DPO oligonucleotides). Although such general mismatch strategies reduce the amplification efficiency they have been previously shown to improve allelic distinction [22; 23]. The template-complementary regions of these primers were between 20 to 24 nt long and had a fairly wide range of GC contents (between 55% to 73%) and melting temperatures (T_m) (from 61 °C to 72 °C). A 17 nt sequence complementary to a previously reported G-quadruplex DNAzyme was added to the 5'-end of the G-quadruplex-generating PCR primers [24]. In the case of nested PCR reactions, gene-specific outer primers for nested PCR were designed to be between 26 to 30 nt in length with melting temperatures ranging between 72 °C to 74 °C.

Allelic distinction of *rpoBS531L*, *rpoBQ513L* and *katGS315T* by the various G-quadruplex-generating PCR primers was quantitatively compared with an established TaqMan™ real-time PCR assay (Supplementary Table S3). Primers containing a single 3'-end mismatch yielded ΔC_t of amplification between wild-type and SNP-containing alleles in the range of 5 to 11, values consistent with real-time PCR literature [25]. 2m primers with secondary mismatches improved the ΔC_t by 3, 8 and 13 cycles, respectively. The differences between the primers are likely because 3'-end C:A and G:T mismatches are harder to distinguish than purine:purine and C:C mismatches [26–28]. While DPO primers further improved allelic distinction (Supplementary Table S3) they had unacceptably low sensitivities of detection. Therefore, 2m primers were used for further assay development.

Primers complementary to the *M. tuberculosis embB* gene were initially used to assess the performance of G-quadruplex-generating PCR (Supplementary Tables S1 and S2). Plasmid DNA containing segments of either the wild-type or the M306V allelic variant of *embB* were amplified by *Taq* polymerase-driven 30 cycles of 2-step amplification reactions (see Supplementary Materials and Methods).

The amplification products were subsequently denatured at 95 °C for 2 min in the presence of hemin and quickly cooled to 4 °C (fastest ramp rate (2 °C/sec) for the Bio-Rad DNA Engine Peltier thermocycler (Bio-Rad, Hercules, CA, USA)). Cooling by incubation of denatured amplicons on ice also suffices for G-quadruplex folding. Following 30 min incubation at 4 °C the peroxidase activity of the folded G-quadruplex DNazymes was assayed at room temperature by incubating an aliquot of the amplicons with ABTS and H₂O₂. Color development due to G-quadruplex catalytic activity was measured in real-time using a microtitre plate reader (TECAN Safire) (Figure 1b), but could also be observed by eye and / or imaged at reaction end-point using a smartphone camera (Figure 1c).

G-quadruplex peroxidase activity above background could only be detected following amplification of cognate templates by 2m primers. SNP-containing template allelic variants failed to generate a signal above noise. PCR products generated using 2m primers lacking the G-quadruplex complementary tails did not yield any peroxidase activity above background (data not shown). To demonstrate the robustness of our method, we tested additional 2m primer pairs for *M. tuberculosis* alleles *rpoBS531L*, *rpoBH526Y*, *rpoBQ513L*, *katGS315T*, and *gyrAD94G* (Supplementary Tables S1 and S2). In all cases, unambiguous colorimetric allele distinction of cloned plasmid templates was achieved (data not shown).

To ensure that individual genes and alleles could be amplified in the presence of excess non-cognate DNA, we attempted to detect specific amplicons from *M. tuberculosis* genomic DNA in a milieu of human genomic DNA. Genomic DNA from the virulent *M. tuberculosis* strain H37Rv was obtained from PATH (Program for Appropriate Technology in Health, Seattle, WA, USA) at high (H: 26850 genomes/μl), medium (M: 268.5 genomes/μl) and low (L: 1.38 genomes/μl) concentrations each with a background of 5.7 ng/μl of human genomic DNA. Allele-specific colorimetric identification of several genes was then performed by two-step nested PCR in which the first stage of the PCR was performed using gene-specific primers, while the second stage was carried out with allele-specific G-quadruplex-generating 2m primers. Subsequent G-quadruplex folding and peroxidase assays yielded definitive colorimetric detection of the *M. tuberculosis* wild-type alleles for *rpoB*, *embB* and *katG* genes down to 268 copies of genomic DNA (Figure 2). Even following nested G-quadruplex-PCR the observed amplification with 2m primers that were instead specific for the drug resistance alleles (*rpoBQ513L*, *rpoBH526Y*, *embBM306V* and *katGS315T*) was undetectable (Figure 2 and data not shown).

These results demonstrate that G-quadruplex-PCR can be incorporated into a nested PCR scheme resulting in sensitive, yet visual colorimetric gene detection and SNP distinction that can be scored without complicated electronic devices. Our method is more robust than previous methods using G-quadruplex peroxidase activity for detection because the background signal in the absence of a correct template is minimized by eliminating the inclusion of partial G-quadruplex sequences in the primers. Furthermore, post-PCR sample processing prior to colorimetric detection is minimized and simplified by relying only on thermal refolding for G-quadruplex generation and does not require additional enzymes or complicated user manipulations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. G-quadruplex-generating PCR for direct colorimetric gene detection. (a) Schematic of G-quadruplex-generating PCR. (b and c) Validation of G-quadruplex-generating PCR for SNP distinction using artificial templates. Plasmids containing segments of wildtype or SNP M306V of the *M. tuberculosis* gene *embB* were used as templates for colorimetric distinction by G-quadruplex-generating PCR. Peroxidase activity of the accumulated G-quadruplexes was assayed colorimetrically by measuring absorbance in real-time using TECAN Safire (b) and by visual examination at end-point (c).

Figure 2. Colorimetric allele detection in *M. tuberculosis* genomic DNA. (a) Agarose gel analysis of nested PCR amplification of *M. tuberculosis* H37Rv genomic DNA using rpoB-WT or rpoB-Q513L SNP-specific primers. H: 26850 genomes, M: 268.5 genomes, L: 1.38 genomes. (b) Peroxidase activities of the G-quadruplexes accumulated upon target amplification were assayed colorimetrically by measuring absorbance in real-time using TECAN Safire and also by visual examination at end-point (inset).