Using a “loop-out” Primer for Identification and Prevention of MEN1 Genotyping Errors Caused by G-quadruplex- and i-motif-like Sequences

Kelli Sumner1, Jeffrey Swensen1,3, Mohammed Jama1, Benjamin Brulotte2, Rebecca Godfrey2, Genevieve Pont-Kingdon1,3, D. Hunter Best1,3
1ARUP Institute for Clinical & Experimental Pathology®, Salt Lake City, UT; 2ARUP Laboratories®, Salt Lake City, UT; 3Dept. of Pathology, University of Utah School of Medicine, Salt Lake City, UT
kelli.sumner@aruplab.com

Previous reports have shown that fluorescence energy transfer (FRET) probe and unlabeled probe (oligonucleotide sequence that is block on the 3' end to prevent extension) designs that exclude up to 80 nucleotides surrounding at least 11 nucleotides bind to template DNA and can be used for genotyping or haplotyping. We designed a primer that would loop-out a portion of the template and exclude from amplification.

DNA secondary structure results from tetrad of hydrogen bonded guanine residues (1A) held together by loops of interacting mixed-sequence nucleotides (G-quadruplex; figure 1B). A motif is the opposite strand of a G-quadruplex sequence. The G-quadruplex sequence follows a basic formula: \( G\_2\_7X1\_7G\_2\_7X1\_7G\_2\_7X1\_7G\_2\_7; \) where X can be any base. The bases surrounding the motif were revealed by loop-out primer set one; the rs509606 polymorphism was present and the 4 base sequence, c.249_252delGTCT, was clearly discernible. Primer set 1 forward primer will amplify through the polymorphism, rs509606, and appear homozygous. However, when this sample was amplified using the forward primer that was designed to loop-out the 10 nucleotide region including the polymorphism, the c.249_252delGTCT could be discerned.

The loop-out primer was validated for accuracy, intra- and inter-run reproducibility. Primer set 1 was accurate and reproducible using the same protocol for amplification of the entire MEN1 gene allowing us to keep consistent conditions throughout the assay, which is desirable for use in a clinical/ laboratory setting.

Results

Accuracy results: All samples amplified with both primer sets. Eight of the 19 samples characterized had the rs509606 polymorphism, one of which also had a known polymorphism mutation, c.213C>T, on exon 1. (Chandrasekharappa et al. 1997; figure 3). Of the remaining samples, 10 had normal wild-type sequence, and one had a polymorphism, c.435C>T. Results are summarized in table 3.

When amplifying sample 3 with primer set 1 the results showed preferential amplification of the allele containing the polymorphism, rs509606, and it was homozygous (figure 4); no other sequence variants were found (figure 5A). When amplifying sample 3 with primer set 2 (loop-out forward primer) the c.249_252delGTCT was discernible (figure 5B).

The sample containing the c.435C>T polymorphism was clearly heterozygous when amplified by either primer set, because it did not contain the rs509606 polymorphism (not shown). Reverse sequences show the 10 base deletion in the primer area (figure 6).

Accuracy results: All samples amplified with both primer sets. After amplification, the 20 samples were characterized by Sanger sequencing using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Life Technologies, Grand Island, NY) on the SeqCap® 9700 PCR system (Applied Biosystems, Carlsbad, CA). PCR cycling conditions were as follows: 95°C for 5 min; 10 cycles of: 94°C for 45 sec. with a decrease in temperature by 0.5°C per cycle, and an initial extension at 72°C for 1 min. Sanger sequencing was performed using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA) and an Applied Biosystems 3730 DNA Analyzer. Sequences were aligned using Mutation Surveyor® software (SoftGenetics, State College, PA) using NG_008929.1 as the reference sequence. Loop-out primers were validated for accuracy with 19 samples that were also sequenced (figure 6). Primer set 1 forward primer was designed to amplify through the rs509606 polymorphism and are indicated in blue. Primer set 1 reverse primer was designed to exclude a 10 nucleotide region including the rs509606 polymorphism.

Future works will reveal that fluorescence energy transfer (FRET) probe and unlabeled probe (oligonucleotide sequence that is block on the 3' end to prevent extension) designs that exclude up to 80 nucleotides surrounding at least 11 nucleotides bind to template DNA and can be used for genotyping or haplotyping. We designed a primer that would loop-out a portion of the template and exclude from amplification.

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Allele dropout resulting from the common MEN1 gene polymorphism, rs509606, can be prevented by using a noncontiguous primer that loops out a 10 nucleotide portion including this polymorphism. Additionally, the use of this design principle for primers may simplify the analysis of other exons that are flanked by sequences that complicate amplification or sequencing.

Conclusions

Previous reports have shown that fluorescence energy transfer (FRET) probe and unlabeled probe (oligonucleotide sequence that is block on the 3' end to prevent extension) designs that exclude up to 80 nucleotides surrounding at least 11 nucleotides bind to template DNA and can be used for genotyping or haplotyping. We designed a primer that would loop-out a portion of the template and exclude from amplification.

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