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GENOTYPING WITH BHQ*plus*™

FORTIFIED PROBES ACHIEVE POWERFUL DISCRIMINATION

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ABSTRACT:

We introduce a novel probe for qPCR that is embedded with a duplex stabilizing technology and terminated with the Black Hole Quencher®. These BHQ*plus* probes permit the design of shorter oligonucleotides to detect targets that do not easily accommodate traditional probes. We demonstrate this capability against an AT-rich gene, conserved across several species of closely related bacteria. Fluorescent melting curves reveal the added stabilization of the BHQ*plus* chemistry and also the consequences of hybridizing to a mismatch, such as the opposite allele of a single-nucleotide polymorphism. Our free PCR application, RealTimeDesign™, is being upgraded to incorporate the thermodynamic parameters specific to this chemistry and support probe-based genotyping. This software program will design numerous SNP assays in a high-throughput fashion, and also accommodate multi-nucleotide polymorphisms. Here, we demonstrate the amplification performance of several SNP assays proposed by RealTimeDesign, with scatter plots resolving the genotypes.

COMPACT PROBES FOR QPCR:

Oligonucleotide sequences for fluorescence-quenched probes are selected to anneal at a precise temperature, usually elevated above that of the primers. BHQ*plus* probes are synthesized with a duplex-stabilizing chemistry so that truncated oligos continue to anneal at the proper temperature. This feature allows probe design into difficult AT-rich regions.

Amplification of an AT-rich target, detected with BHQ*plus* and traditional probes:

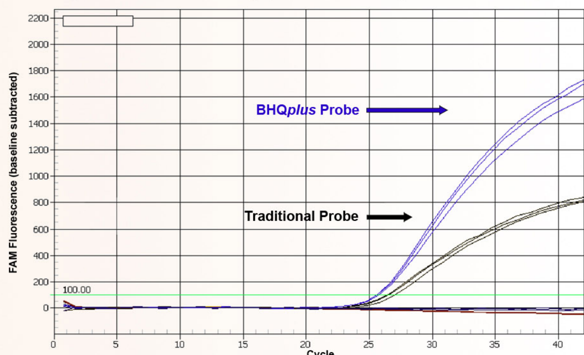
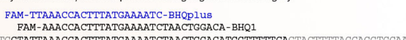


FIGURE 1. Replicate PCR reactions, all amplified using the same primer-set, but detected using either a traditional FAM-BHQ1 probe (black) or a BHQ*plus* probe (blue). The amplification product is a region within the AT-rich hemolysin III gene of *Bacillus anthracis* (TIGR locus #: BA_2725). The assay is as follows, with the primer-binding regions shown in grey:

```
FAM-TTAAACCACTTTATGAAAATC-BHQplus
FAM-AAACCACTTTATGAAAATCTAACTGGACA-BHQ1
```



At 29 bases in length, the traditional probe remains sufficiently short for FRET quenching, but is predicted to melt from its target below the ideal temperature of 70 ˚C. In contrast, the BHQ*plus* probe is only 21 bases in length and yet shows an improved fluorescent response, possibly due to its elevated melting temperature.

DUPLEX STABILIZATION

provided by the BHQ*plus* chemistry is revealed by ramping the temperature of a mixture containing the probe and its complement, and recording fluorescence as the strands melt apart. Melting curves measured upon mismatched templates reveal single-base discrimination, making BHQ*plus* probes well-suited for SNP genotyping.

Melting curves of FAM- and TET-labeled BHQ*plus* probes:

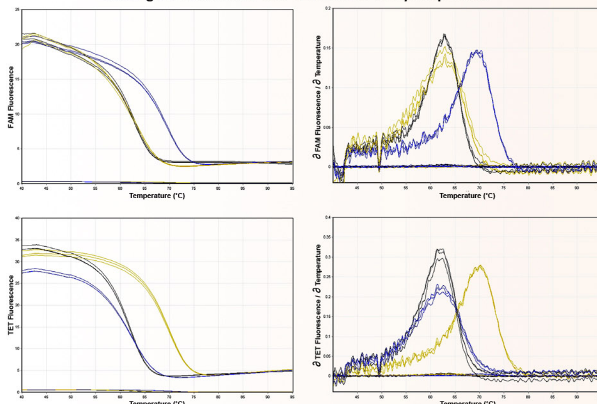


FIGURE 2: BHQ*plus* probes that target a SNP within the gene UGT2B15 were melted in the presence of templates representing each allele. The FAM-labeled probe targets the "C" allele (blue) while the TET-labeled probe targets the "T" allele (gold). The melting temperature of each probe, recorded upon their mismatched template is 6 - 8 ˚C lower than that of the matched template.

Identical probe sequences were also synthesized without the BHQ*plus* chemistry, and melted in the presence of their matched templates (black). These melting curves are also shifted toward lower temperatures, confirming the binding stability of BHQ*plus* probes.

REALTIMEDESIGN

www.qPCRDesign.com

RealTimeDesign is a free software program for qPCR assay design and can be run entirely through a web browser. To assist in the design of genotyping assays, we are developing a module to discriminate SNPs and multi-nucleotide polymorphisms by incorporating the thermodynamic parameters specific to the BHQ*plus* chemistry. Each allele-specific probe can be labeled with a variety of spectrally-distinct fluorophores so that separate optic channels will record the amplifications of each genotype. Because both probes are present within the PCR reaction, they will compete to bind the amplified template and further enhance the mismatch discrimination.

To confirm the performance of RealTimeDesign and the BHQ*plus* technology, four distinct SNPs were submitted to its scrutiny, all within genes thought to be involved in drug metabolism. The default assay proposed by the software was then synthesized at Biosearch, with each probe-set labeled with FAM and TET.

UGT2B15 G/A

Target	Parameter Set		Assays
UGT2B15 G/A-R-201	Most Restrictive	Diagnostics	1
Assay 0031	59.45		

Oligo	Tm	GC%	Length	5' Pos	3' Pos	5'-Sequence-3'
Forward	59.2	53	17	235	219	GAGCTACTGGCTGAACT
Reverse	59.2	50	18	149	166	TCCTCCACCATTCTTCTC
Allele 1	71.4	41	17	214	198	AACATACCCTTTCCGTA BHQ*plus*
Allele 2	70.8	32	19	216	198	TTAACATACCCTTTCTGTA BHQ*plus*

Identified Mis-Alignments

Self Align	Dimer	3'Align	Hairpin	Total ΔG°
Forward	4	2	3	-10.83
Reverse	2	1	0	-10.83
Allele 1	3	3	3	-5.14
Allele 2	4	3	3	-4.36

Pair Align	Dimer	3' Align	Bi-ΔG°
Forward/Reverse	3	3	.00
Allele1/Allele2	3	3	.00
Allele1/Reverse	2	2	.00
Allele2/Forward	3	3	.00
Allele2/Reverse	2	2	.00
Allele1/Forward	3	3	.00

Amplicon Detail

Amplicon	Rank	AT Run	GC Run	GC%	Length	Variation
Allele 1	86.65	3	2	44	87	C
GAGCTACTGG CTGAACTATT TAACATACCC TTTCCGTACA GTCTTCGATT CTCTGTTGGC TACACATTTG AGAAGAATGG TGGAGGA						
Allele 2	84.93	3	2	43	87	T
GAGCTACTGG CTGAACTATT TAACATACCC TTTCTGTACA GTCTTCGATT CTCTGTTGGC TACACATTTG AGAAGAATGG TGGAGGA						

ePCR Submission

Reverse-Forward

FIGURE 3: Screenshot from RealTimeDesign, displaying the oligo sequences of the genotyping assay targeting UGT2B15, a gene involved in the metabolism of certain drugs such as tamoxifen. Properties of the oligos and amplicons are also presented.

Primerset 325 Maximum Pair-Alignments Display

```
Duplex: 3
   5' GAGCTACTGGCTGAACT
                   |||
   3'              CTCTTCTTACCACCTCCT

Match Count: 7
   5' GAGCTACTGGCTGAACT
      |||        | || |
   3' CTCTTCTTACCACCTCCT

3' Duplex: 3
   5' GAGCTACTGGCTGAACT
                   |||
   3'              CTCTTCTTACCACCTCCT

3' Match Count: 3
   5' GAGCTACTGGCTGAACT
                   |||
   3'              CTCTTCTTACCACCTCCT

3' GC Duplex: 2
   5' GAGCTACTGGCTGAACT
      |||
   3' CTCTTCTTACCACCTCCT
```

UGT2B15 G/A-R-201-Assay 31 Hybridization Display

```
  1 AAAACTGGTC CCACTTCTTC AGATCATAAA TTTGAAACCA AAAGTCAAAA TAAAGCATAT
    TTTTGACCAG GGTGAAGAAG TCTAGTATTT AAACTTTGGT TTTCAGTTTT ATTTCGTATA

 61 GTATCATATT TTTTATCCTC TCCATGAAAA TCATTTGATC ACTTAATTCT GACATAACAA
    CATAGTATAA AAAATAGGAG AGGTACTTTT AGTAAACTAG TGAATTAAGA CTGTATTGTT

                                TC CTCCACCATT CTTCTC
121 CAGGTACATA GGAAGGAGGG AACAGAAATC CTCCACCATT CTTCTCAAAT GTGTAGCCAA
    GTCCATGTAT CCTTCCTCCC TTGTCTTTAG GAGGTGGTAA GAAGAGTTTA CACATCGGTT

181 CAGAGAATCG AAGACTGTAC RGAAAGGGTA TGTTAAATAG TTCAGCCAGT AGCTCACCAC
    GTCTCTTAGC TTCTGACATG YCTTTCCCAT ACAATTTATC AAGTCGGTCA TCGAGTGGTG
                                                  TC AAGTCGGTCA TCGAG
                      ATG CCTTTCCCAT ACAA
                      ATG TCTTTCCCAT ACAATT

241 AGGGATTAAG GGCATCTGCC AGAATGACAT CAAACTTTGA CTCTTGTAGT TTCATCATAA
    TCCCTAATTC CCGTAGACGG TCTTACTGTA GTTTGAAACT GAGAACATCA AAGTAGTATT

301 GTTTCTTATT CAAAACTGCA TCTTTACAGA GCTTGTTACT GTAGTCATAA TATTCCCAAC
    CAAAGAATAA GTTTTGACGT AGAAATGTCT CGAACAATGA CATCAGTATT ATAAGGGTTG

361 ACAATTCTTG TAATTGTGAA AAATATGACC AAAATGTATT T
    TGTTAAGAAC ATTAACACTT TTTATACTGG TTTTACATAA A
```

Oligo	Tm	5' Pos	Length	Sequence
Forward Primer	59.2	235	17	GAGCTACTGGCTGAACT
Reverse Primer	59.2	149	18	TCCTCCACCATTCTTCTC
Allele 1 Probe	71.4	214	17	AACATACCCTTTCCGTA
Allele 2 Probe	70.8	216	19	TTAACATACCCTTTCTGTA

Ambiguity Code	SNP Sequence
R	[A/G]
Y	[C/T]

FIGURE 4: LEFT: The software avoids certain misalignments between the primers, but presents those that remain. RIGHT: The alignment of the oligos upon the target sequence. Both the sense and antisense strands are considered for optimal placement of the probes.

GENOTYPING

Scatter plots for the four SNP assays record the final fluorescence values from amplification of each allele. All assays were amplified across a seven-point dilution series of the homozygous and heterozygous genotypes, spanning 5,000,000 copies down to 5 copies. The "FAM" allele is presented in blue, the "TET" allele is presented in gold, and the heterozygote presented in green.

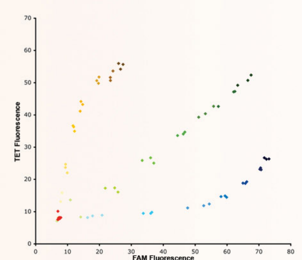


FIGURE 5: Scatterplot of a SNP assay targeting UGT2B15
allele1 probe: [FAM]-AACATACCCTTTCCGTA-[BHQ*plus*]
allele2 probe: [TET]-TTAACATACCCTTTCTGTA-[BHQ*plus*]

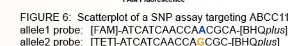


FIGURE 6: Scatterplot of a SNP assay targeting ABCC11
allele1 probe: [FAM]-ATCATCAACCAACGCA-[BHQ*plus*]
allele2 probe: [TET]-ATCATCAACCAGCGC-[BHQ*plus*]

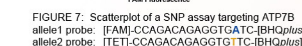


FIGURE 7: Scatterplot of a SNP assay targeting ATP7B
allele1 probe: [FAM]-CCAGACAGAGGTGATC-[BHQ*plus*]
allele2 probe: [TET]-CCAGACAGAGGTGTTC-[BHQ*plus*]

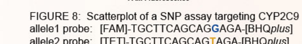


FIGURE 8: Scatterplot of a SNP assay targeting CYP2C9
allele1 probe: [FAM]-TGCTTCAGCAGGAGA-[BHQ*plus*]
allele2 probe: [TET]-TGCTTCAGCAGTAGA-[BHQ*plus*]

METHODS:

All oligo sequences were designed using RealTimeDesign. Synthetic oligonucleotides representing the amplicon sequences were used as templates for each allele. Template dilutions were prepared in water containing yeast tRNA, Roche Molecular systems, with triplicate reactions prepared for all dilution points. All melting curves and PCR amplifications were performed upon a Rotor-Gene 6000, kindly provided by Corbett Research. Melting curve thermal profile: 95 ˚ C for five min. followed by rapid cooling to 40 ˚C. Temperature was slowly ramped back to 95 ˚C over the course of three hours with fluorescence recorded every 0.1 ˚C. PCR thermal cycling profile: 95 ˚C for five min. followed by 40 cycles of 95 ˚C for 20 s, 60 ˚C for 60 s.

Melting curve reaction components:

Component	Volume
• nuclease-free water	8.00 µL
•10X PCR buffer	2.00 µL
• MgCl2 (50mM)	2.00 µL
• Probe (100 µM)	4.00 µL
• Template (100 µM)	4.00 µL
Total	20.0 µL

PCR reaction components:

Component	Volume
• nuclease-free water	0.40 µL
• 2X PCR master mix	10.0 µL
• DNA template	5.00 µL
• forward primer (10 µM)	1.80 µL
• reverse primer (10 µM)	1.80 µL
• allele 1 probe (10 µM)	0.50 µL
• allele 2 probe (10 µM)	0.50 µL
Total	20.0 µL

CONCLUSIONS:

The boosted binding stability provided by the BHQ*plus* chemistry allows these probes to be accommodated into difficult targets and new applications where traditional probes are not well-suited. We demonstrate this capability upon four SNP targets and successfully resolve the genotypes from one another. We have upgraded RealTimeDesign to target single- and multi-nucleotide polymorphisms so that assay design is rapidly automated. BHQ*plus* may also be suitable for adapting preexisting probe sequences that rely upon other binding stabilizers. We envision other appropriate uses of this technology, including the discrimination of closely-related species and the detection of small targets such as microRNA.

ACKNOWLEDGEMENTS:

We would like to gratefully acknowledge the contributions of all individuals who developed and refined the BHQ*plus* technology, including Matt Lyttle who manufactured the starting material, Daren Dick and Joe Knecht who have optimized the synthesis of this probe type, and Mary Katherine Johansson who provided excellent suggestions and advice. Raymond Peterson, Dean Fiala, and the rest of the team at Celadon Laboratories are responsible for the sophisticated code that powers RealTimeDesign.
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