APPLICATION NOTE

Improved qPCR Assays for Copy Number Variation Using Double-Quenched BHQ® Probes.

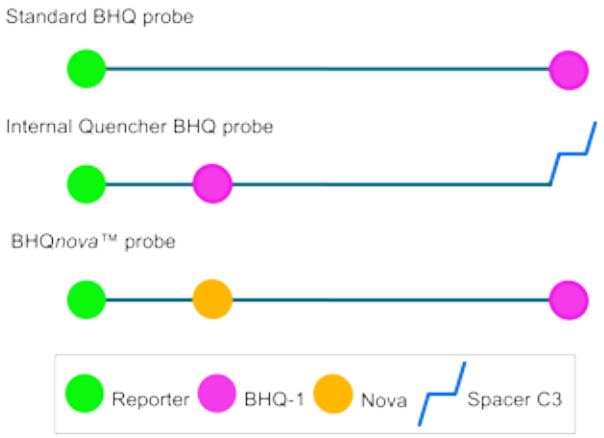
Introduction

An application of quantitative PCR with commercial significance is the measurement of genomic copy number variation (CNV). In this study, we introduce a new probe format for accurate CNV determination in human and plant samples. A BHQnova™ probe is a Black Hole Quencher® probe that is double-quenched for improved functionality. CNV determination requires a robust detection chemistry that is sensitive across a broad dynamic range, and with little technical noise that causes variation in cycle threshold (Ct) values. For this purpose, BHQnova probes have enhanced signal-to-noise ratios upon amplification, while also providing greater flexibility in sequence design.

Traditional probes including standard BHQ probes comprise a 5′ reporter dye and 3′ quencher as end labels upon an oligonucleotide tether. The dye labels interact through both Förster Resonance Energy Transfer (FRET) and static (contact) quenching mechanisms, but their quenching efficiency varies according to the distance dependence of FRET (Johansson, 2006; Marras et al., 2002). Poor quenching typifies those probe sequences that exceed 30 bases in length, unless the quencher is positioned internally so that it is closer to the fluorescent reporter (Fig. 1). The vacated 3′ terminus is then blocked with a Spacer C3 modification to prevent extension of the probe. While there is a long history of probes with internal quenchers, they are typically conjugated to a thymidine base and so the quencher configuration introduces new limitations to the probe sequence.

The novel BHQnova probe maintains the typical 5′ reporter dye and 3′ BHQ quencher, but also introduces a unique Nova quencher between bases 9 and 10 from the 5′ terminus. The Nova quencher's inter-nucleotide linkage simplifies probe design while improving the quenching efficiency of longer sequences. This format provides considerable flexibility toward the base composition while selecting probe sequences.

Figure 1.



In this study, we designed a BHQnova probe to *CYP2D6*, a common human CNV target, using LGC Biosearch Technologies' RealTimeDesign™ software. This region of the human genome is known to harbor sequence rearrangements with clinical

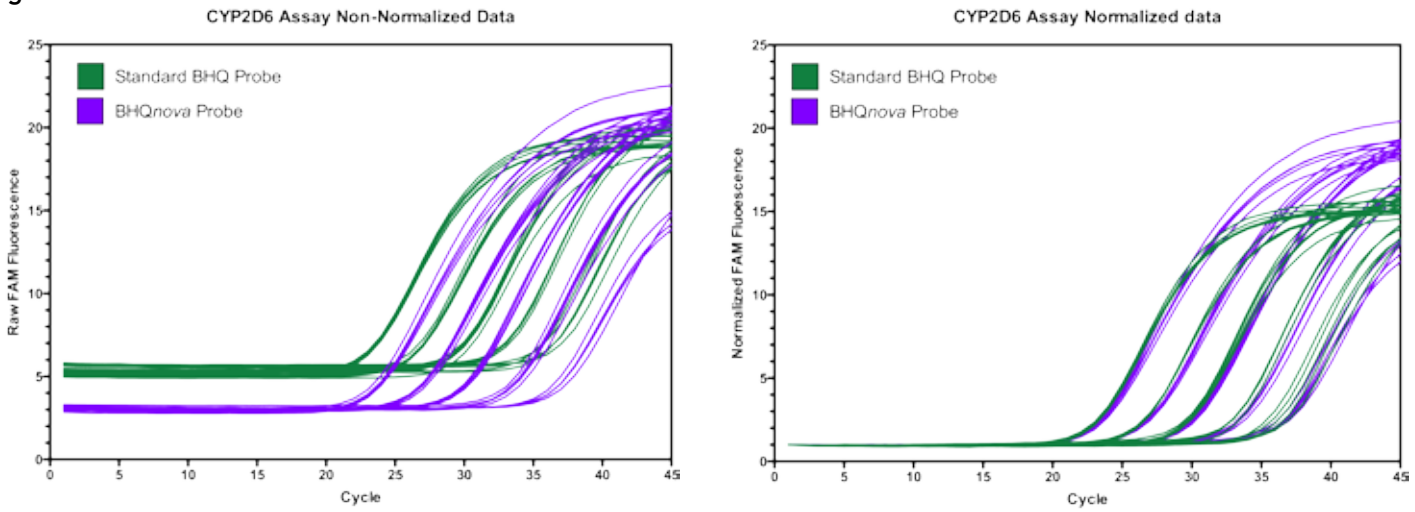
Table 1: Oligo Sequences

CYP2D6	Forward Primer:	CCATGCTCACACCTCCCTAG
	Reverse Primer:	GGGTCGTGGACATGAAACAGG
	Traditional BHQ Probe:	FAM-CAGGTGGTTTCTTGGCCCGCT-BHQ-1
	BHQnova Probe:	FAM-CAGGTGGTT-[Nova]-TCTTGGCCCGCT-BHQ-1
RNase P (reference)	Forward Primer:	GCGGAGGGAAGCTCATCAG
	Reverse Primer:	CTGGCCCTAGTCTCAGACCTT
	Traditional BHQ Probe:	CAL Fluor Orange 560-CCACGAGCTGAGTGCGTCCTGT-BHQ-1
	BHQnova Probe:	CAL Fluor Orange 560-CCACGAGCT-[Nova]-GAGTGCGTCCTGT-BHQ-1

Table 2: Oligo Sequences for Wheat Assay

Ppd-B1	Forward Primer:	GCGTAAGTTACTATCTCTCATGGTGTATC
	Reverse Primer:	TTTGTTTTAGTACCCAGTACCATACCAG
	BHQnova Probe:	FAM- CTGCTGCTT-[Nova]-CAGTTCCTAGTTTCACTTGTGTCC-BHQ-1
Vrn-A1	Forward Primer:	GCAGCCCACTTTTGGTCTCTA
	Reverse Primer:	TCTGCCCTCTCGCCTGTT
	BHQnova Probe:	FAM-TGTGTTCGC-[Nova]-TTTGGTTGTGCAGCA-BHQ-1
TaC02 (reference)	Forward Primer:	TGCTAACCGTGTGGCATCAC
	Reverse Primer:	GGTACATAGTGCTGCTGCATCTG
	BHQnova Probe:	CAL Fluor Orange 560- CATGAGCGT-[Nova]-GTGCGTGTCTGCG-BHQ-1

Figure 2.



significance, through variation in gene copy number. Similarly, we apply BHQnova probes for analysis of wheat samples that contain copy number variations that influence crop characteristics. The results reveal accurate quantification, but also the performance advantages of double-quenched probes.

Materials and Methods

The principal of CNV determination is to amplify the target of interest in a duplexed qPCR reaction alongside a different target known to remain constant, and therefore act as a normalizer for genome copy number. The difference in Ct values between the variable target and fixed reference gene provides a measure of copy number per genome. For detection of *CYP2D6* we designed an assay within intron 2 of the human gene, as well as an assay to the *RNase P* gene as a reference. For oligo sequences see Table 1.

For the wheat samples we utilized two published assays targeting the *Ppd-B1* and *Vrn-A1* genes which are known to present with copy number variations (CNV's) in the wheat genome (Díaz et al., 2012). We also utilized an assay for a single copy gene known as *TaCO2* (also called *TaHd1*), as a reference assay to both of these targets. For oligo sequences see Table 2.

Thermal cycling conditions: 10 minutes at 95 °C enzyme activation, 45 cycles of 95 °C for 20 seconds then 60 °C for 60 seconds with fluorescence recorded in the FAM channel (Ex 465 nm / Em 510 nm) and the CAL Fluor® Orange 560 Channel (Ex 465 nm / Em 510 nm).

These assays were performed in 15 µL duplexed reactions in a 384-well format on a Roche LightCycler 480 instrument. Each assay was amplified using 200 nM concentration of the probe and 600 nM of each primer. Reactions were formulated with 2x KlearKall™ Master Mix (LGC), and amplified from human genomic DNA (Promega) across a 1:10 dilution series, spanning 30,000 copies/µL down to 3 copies/µL. The data was subsequently normalized and Ct values were recorded.

Improved Signal-to-Noise Values in CYP2D6 Assay

A 5-point dilution series of human genomic DNA was utilized for the *CYP2D6* assay (Fig. 2), to test the performance characteristics of the single and double-quenched probes. Examining the raw data for each amplification trace confirms the BHQnova probe (purple) has lower baseline fluorescence and therefore improved quenching efficiency, compared to a standard probe with the same sequence (green) (Fig. 2, left). There is simultaneously an improved signal response, with a plateau phase that exceeds the standard probe (Fig. 2, right). Probe performance can be quantified in a general sense by a signal-to-noise value (S:N): the final fluorescence following amplification divided by the initial fluorescence preceding amplification. By this measure, the BHQnova probe is significantly improved across all dilution points (Fig. 3, N=5, $p < 0.01$).

A semi-log plot of the dilution series indicates that the reaction efficiency and sensitivity are unaffected by the conversion from the standard probe format (Fig. 4, left) to the double-quenched

Figure 3.

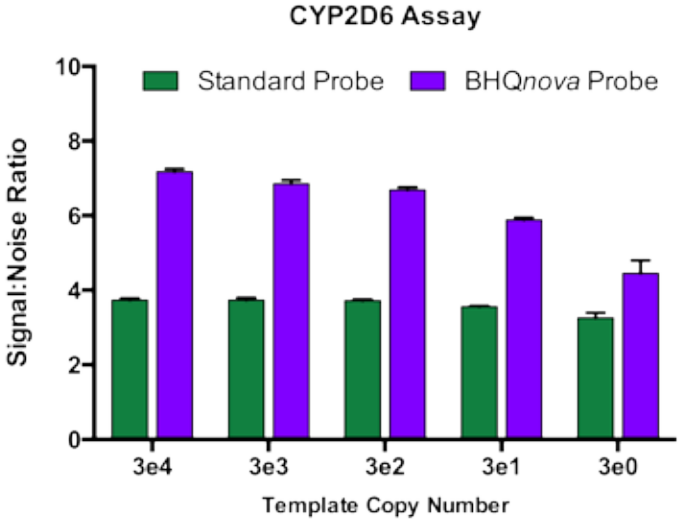
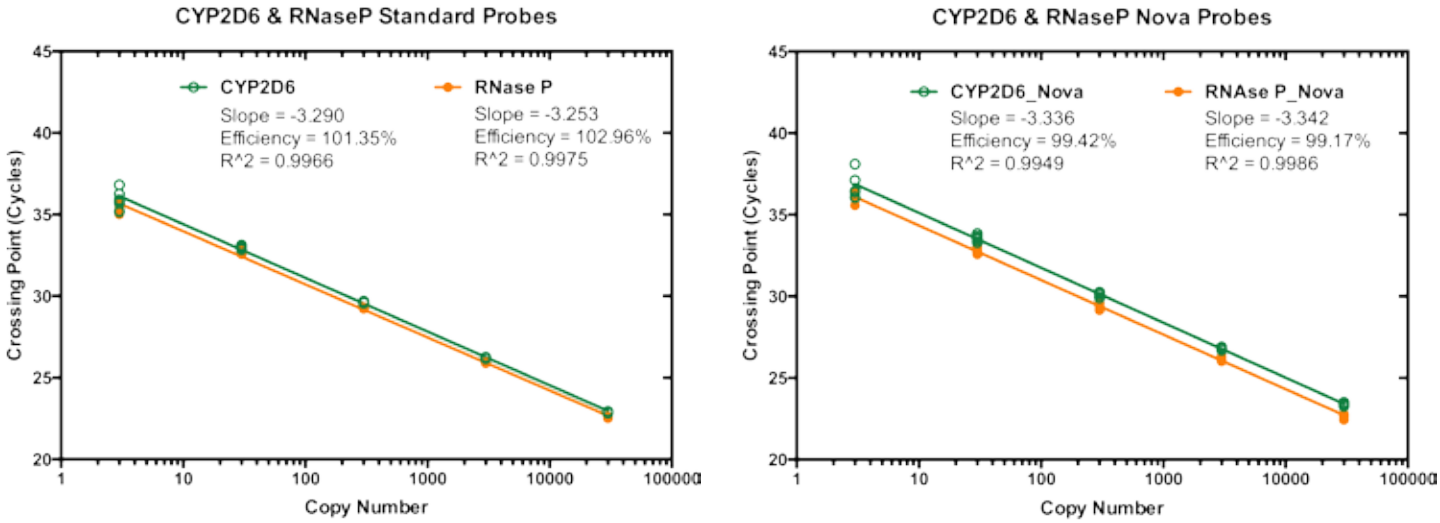


Figure 4



BHQnova probe format (Fig. 4, right). Within each plot the standard curve for *CYP2D6* is colored green while the reference *RNase P* is shown in orange.

Overall, double-quenched probes achieve improved signaling without sacrificing other measures of amplification performance. The data also confirms excellent compatibility between BHQ probes and KlearKall Master Mix for qPCR applications across a wide range of template concentrations.

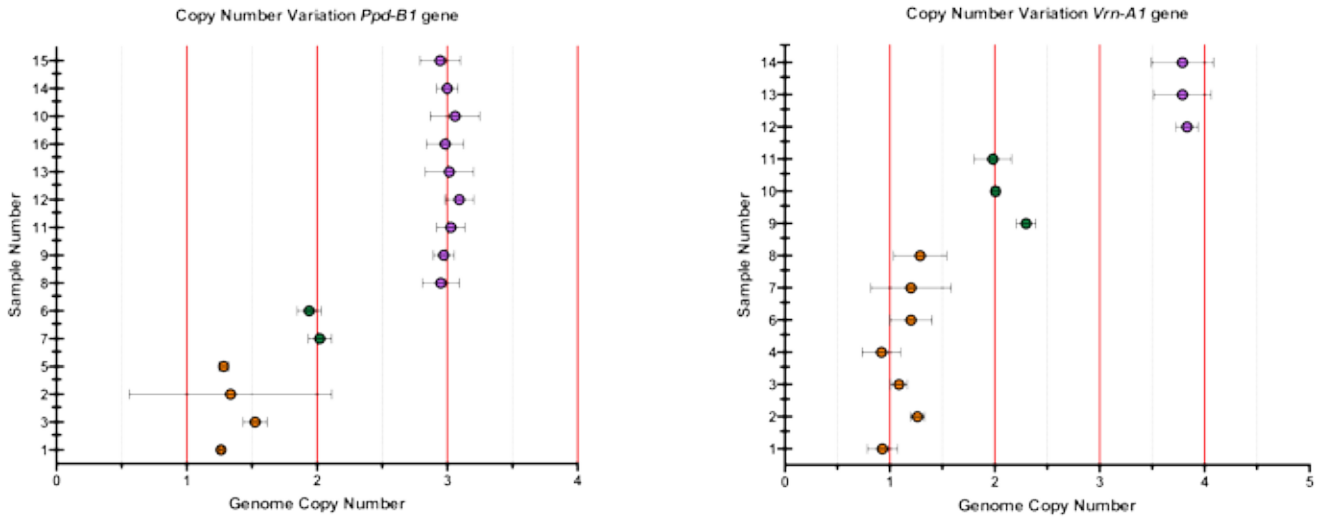
Wheat Assays for CNV Determination

To demonstrate the ability of these probes in a common qPCR application we sought to evaluate their function in a copy number variation assay. This assay consists of a target gene typically designed with a probe in the FAM channel and then duplexed with a second reference assay which is designed to report signal in a second channel on the device. This reference assay is targeted to a gene which is known to have a single haploid copy in the genome of the organism and typically does not display variation in the duplication or deletion of this region.

We utilized a recently published assay that examined two biologically relevant target gene within the wheat (*Triticum aestivum*) genome (Diaz et al., 2012). What is particularly interesting to note is that these gene targets do not contain regional variations, such a SNPs, which would present during the determination of their genotype, the phenotypic variation is fully dependent on genomic copy number. The two targets were the *Ppd-B1* gene which regulates the onset of flowering according to the length of light exposure (photoperiod), and the *Vrn-A1* gene which regulates vernalization in response to winter conditions. Gene duplication of each target is noted to confer desirable phenotypes through adaptation to local growing environments.

The wheat samples represent a variety of strains originating from regions in China. Genomic DNA was extracted using the sbeadex™ high-throughput bead extraction kit (LGC), and incorporated directly into the qPCR reaction at non-normalized concentrations ranging from 30 to 50 ng/µL. Copy number variation was determined via the ΔΔCt calculation between the target and reference assay. The data was then plotted as the

Figure 5.



relative copy number to confirm duplication events in certain samples. Points represent the mean ± standard deviation of triplicate reactions per sample.

For *Ppd-B1* (Fig. 5, left), the majority of samples reveal 3 copies per haploid genome while the remaining samples register two or fewer. For *Vrn-A1* (Fig. 5, right), the majority of the crop samples harbor a single copy of the gene while other samples indicate two or more copies per haploid genome.

Conclusion

BHQnova probes release signal upon amplification that is usually elevated above standard probes, while the background fluorescence (baseline) is consistently lower. This new format provides greater flexibility in probe design by enabling the selection of longer sequences that are otherwise poorly quenched. BHQnova probes are perfectly partnered with technologies from LGC Genomics to complete the qPCR workflow: sbeadex extraction and KlearKall Master Mix. This ensemble of components achieves optimal amplification efficiency and sensitivity by working together in unison. In summary, double-quenched BHQnova probes are a robust tool for commercial applications of qPCR, including *in vitro* diagnostics and crop selection.

References

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