Application note

BHQ Probe Master Mix in qPCR applications

Introduction

BHQ™ Probe Master Mix is a highly robust, inhibitor-tolerant PCR master mix that is well established for use in end-point genotyping in conjunction with BHQplus™ Probes from LGC, Biosearch Technologies. Our optimised protocols are particularly relevant to the agrigenomics sector where the starting biological plant material (seed, leaf) can be a challenging sample type. Fluorescence-based end-point genotyping enables the characterisation of single nucleotide polymorphisms (SNPs) and insertion-deletion (InDel) events. Genotypes can be quickly and accurately determined based on the position of a data point on a Cartesian plot, using data captured at the end of the thermal cycling PCR protocol.

Data can, however, be captured in each cycle during the PCR protocol in real-time which, when run alongside the appropriate controls and standards, can generate quantitative data. Real-time data capture during the exponential phase of the PCR protocol, rather than the plateau phase as for end-point PCR protocols, can be used to determine copy number variation (CNV), adventitious presence (AP) and the presence/absence of pathogens. Quantitative techniques can include comparison of an unknown target against an endogenous (or housekeeping) gene, as in the case of CNV, or the generation of a standard curve using serial-diluted standards, as with pathogen detection.

Here we demonstrate the performance of BHQ Probe Master Mix in various qPCR applications using a variety of sample types and a range of BHQ Probe formats, all available from Biosearch Technologies. Highly robust and reproducible data was generated, validating BHQ Probe Master Mix for diploid and polyploidy agrigenomics testing for qPCR-based quantification applications.

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Application note

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Materials, methods and results

a) Plant material and DNA preparation

All plant material was collected using the Biosearch Technologies' BioArk™ Leaf or BioArk Seed collection kits. DNA was purified using Biosearch Technologies' sbeadex™ kits and standard protocols unless otherwise stated.

b) Assay design and reaction set up

Standard BHQ, BHQplus and BHQnova™ assays were designed using Biosearch Technologies' RealTimeDesign software. Competitor A assays were designed using appropriate design software and run using recommended protocols.

Working assay mixes were prepared (40x or 80x) as recommended in the BHQ Probe Master Mix user guide. Final oligonucleotide concentrations of 200 nM probe and 400 nM primer were used unless otherwise stated. A starting DNA concentration of 5-50 ng/µL was used in each reaction, with a total reaction volume of 10 µL in 384-well plates and 20 µL in 96-well plates.

c) Thermal cycling protocols

For each assay, an initial optimisation was performed using 40 cycles as per Table 1. Depending on the dynamics of the reaction, this cycle number was adjusted between 30 and 45 cycles.

Step	Temperature	Time	Number of Cycles
1	95 °C	15 minutes	1
2*	95 °C	15 seconds	40
	60 °C	1 minute	
	Read		

Table 1. Standard thermal cycling protocol for qPCR with BHQ Probe Master Mix. *Step 2 was modified to account for the specific Tm of the oligonucleotides in each assay where necessary.

d) Copy number variation

Seven wheat varieties were tested for the presence of two genes known to show CNV[1] (photoperiod gene Ppd-B1 and vernalisation gene Vm-A1). BHQnova™ Probes were compared with Competitor A probes in 20 µL singleplex reactions on the CFX96 instrument (BIORAD), in conjunction with BHQ Probe Master Mix. The determined CNV values for each probe type were compared with the expected CNV for each target tested.

e) Adventitious presence

Genetically modified organism (GMO) events were tested for cotton (3006-210-23), maize (NK603) and soybean (44406-6) by comparing the C-target value (nuclear DNA content3) against the absolute copy number from a generated calibration curve of Certified Reference Material (CRM). FAM-labelled BHQ Probes were compared to Competitor A probes in 10 µL singleplex reactions on the CFX96 instrument (BIORAD) instrument, in conjunction with BHQ Probe Master Mix.

f) Pathogen detection

Using the detection of two species of potato cyst nematode (*Globodera pallida and Globodera rostochiensis*), sensitivity and specificity of BHQ Probe Master Mix was determined by comparison of BHQplus Probes against Competitor A probes (200 nM) and Competitor A PCR Master Mix using published methods. Samples achieving a Cq value of ≤35 were deemed as positive results. Amplification plots and standard curves were generated for 10 µL reactions with BHQplus Probes to determine reaction efficiency and R^2 values. A total of 52 plant samples were tested, in duplicate, for the presence of both pathogens.

Application note

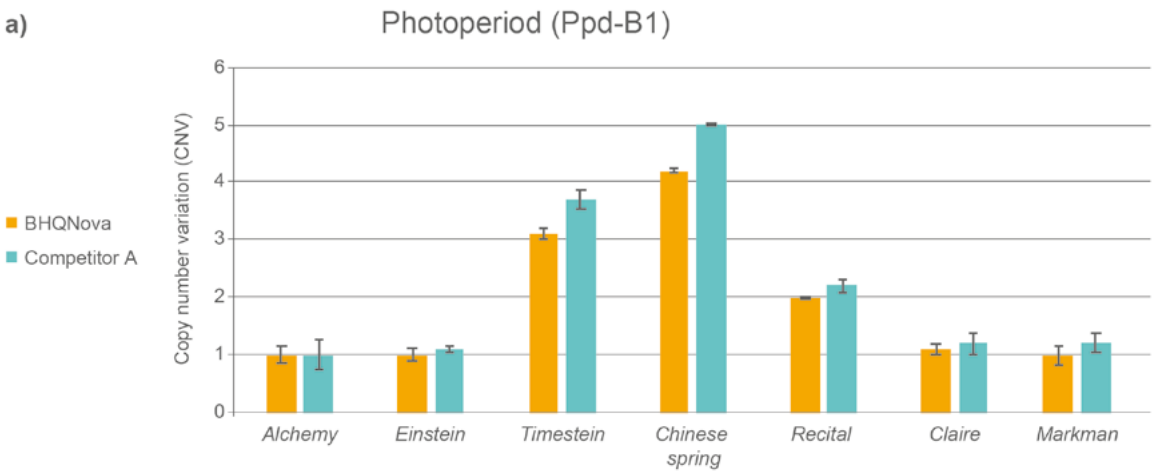
BHQ Probe Master Mix in qPCR applications

Results

a) Copy number variation

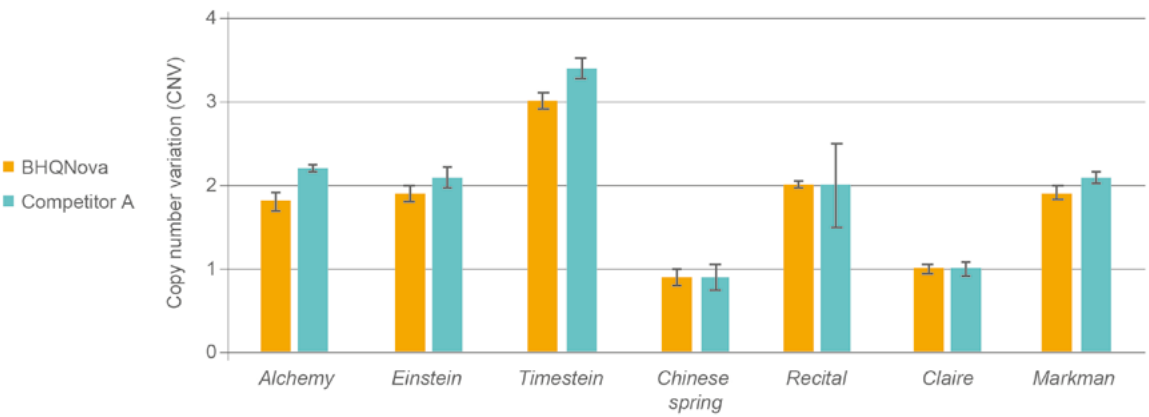
CNV is an important measurement for identifying trait associations. Seven wheat varieties were tested for the presence of the photoperiod-B1 (light-exposure time) and vernalisation-A1 (induction of flowering process) genes, both known to present CNV in the wheat genome[1]. Figure 1 illustrates the improved accuracy of CNV determination achieved with BHQ Probe Master Mix, when used in combination with BHQnova Probes[2], for both targets, down to a single gene-copy event in the wheat genome.

a)



Expected CNV	1	1	3	4	2	1	1
BHQnova	1	1	3.1	4.2	2	1.1	1
Competitor A	1	1.1	3.7	5	2.2	1.2	1.2

b)



Expected CNV	2	2	3	1	2	1	2
BHQnova	1.8	1.9	3	0.9	2	1	1.9
Competitor A	2.2	2.1	3.4	0.9	2	1	2.1

Figure 1: Accurate copy number variation (CNV) values were determined for two wheat genes known to present CNV in the wheat genome. BHQnova probes and BHQ Probe Master Mix were used in a qPCR reaction, and compared to Competitor A probes and BHQ Probe Master Mix. Error bars represent standard deviation across replicates tested.

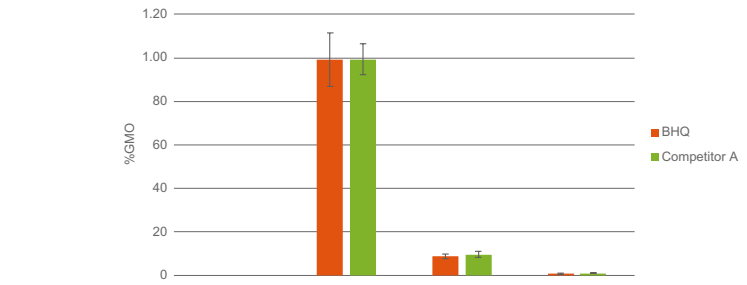
Application note

BHQ Probe Master Mix in qPCR applications

b) Adventitious presence

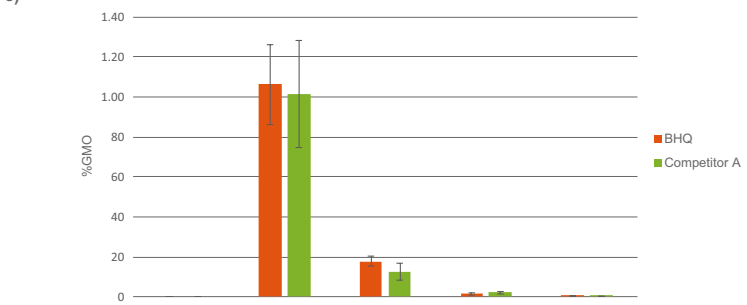
Detection of transgene events is vital for determining the presence of genetically modified organisms (GMO) within plant varieties. Cotton, maize and soybean were tested for AP against certified reference materials for each species from LGC Standards. Figure 2 illustrates the ability of BHQ Probes with BHQ Probe Master Mix to detect the presence of specific GMO events in the three plant species, with the percentage GMO for each sample tested shown in the blue bar. These results demonstrate sensitivity to support AP testing down to a limit-of-detection of <1% GMO event per genome.

a)



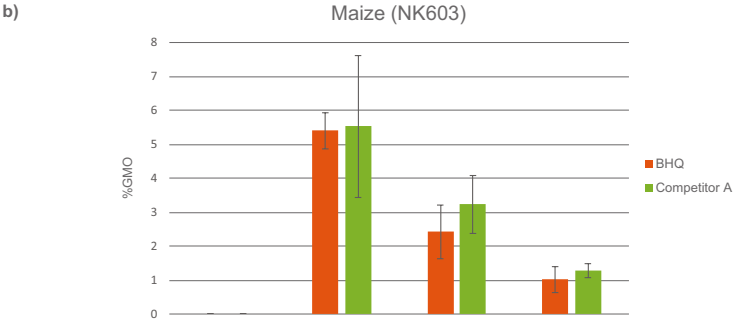
CRM (%GMO)	Negative control	100%	10%	1%
BHQ	0	99.0	8.8	0.8
Competitor A	0	99.2	9.7	1.1

c)



CRM (%GMO)	Negative control	100%	2%	1%	0.1%
BHQ	0	106.3	17.8	1.3	0.3
Competitor A	0	101.6	12.5	1.9	0.2

b)



CRM (%GMO)	Negative control	5%	2%	1%
BHQ	0	5.4	2.4	1.0
Competitor A	0	5.5	3.2	1.3

Figure 2. Unknown target events for (a) cotton (3006-210-23), (b) maize (NK603) and (c) soybean (44406-6) were compared against Certified Reference Material (CRM) amplification from LGC Standards, and quantified down to absolute copy number by determining the appropriate C-value (nuclear DNA content[3]) for each organism. Percentage content for each target is subsequently illustrated. Data generated with BHQ Probes and BHQ Probe Master Mix was comparable with Competitor A probes and BHQ Probe Master Mix. Error bars represent standard deviation across replicates tested.

Application note

BHQ Probe Master Mix in qPCR applications

c) Pathogen detection

The detection and identification of infectious agents of agricultural crops is crucial for infection control. BHQplus probes with BHQ Probe Master Mix were used to detect the presence of two species of the potato cyst nematode, *Globodera pallida* and *Globodera rostochiensis*, known to be infectious agents[4], on extracted plant material. Figure 3 illustrates amplification plots and standard curves for both species. PCR efficiencies of 90-110% and an R^2 value of 0.998 were achieved for both targets.

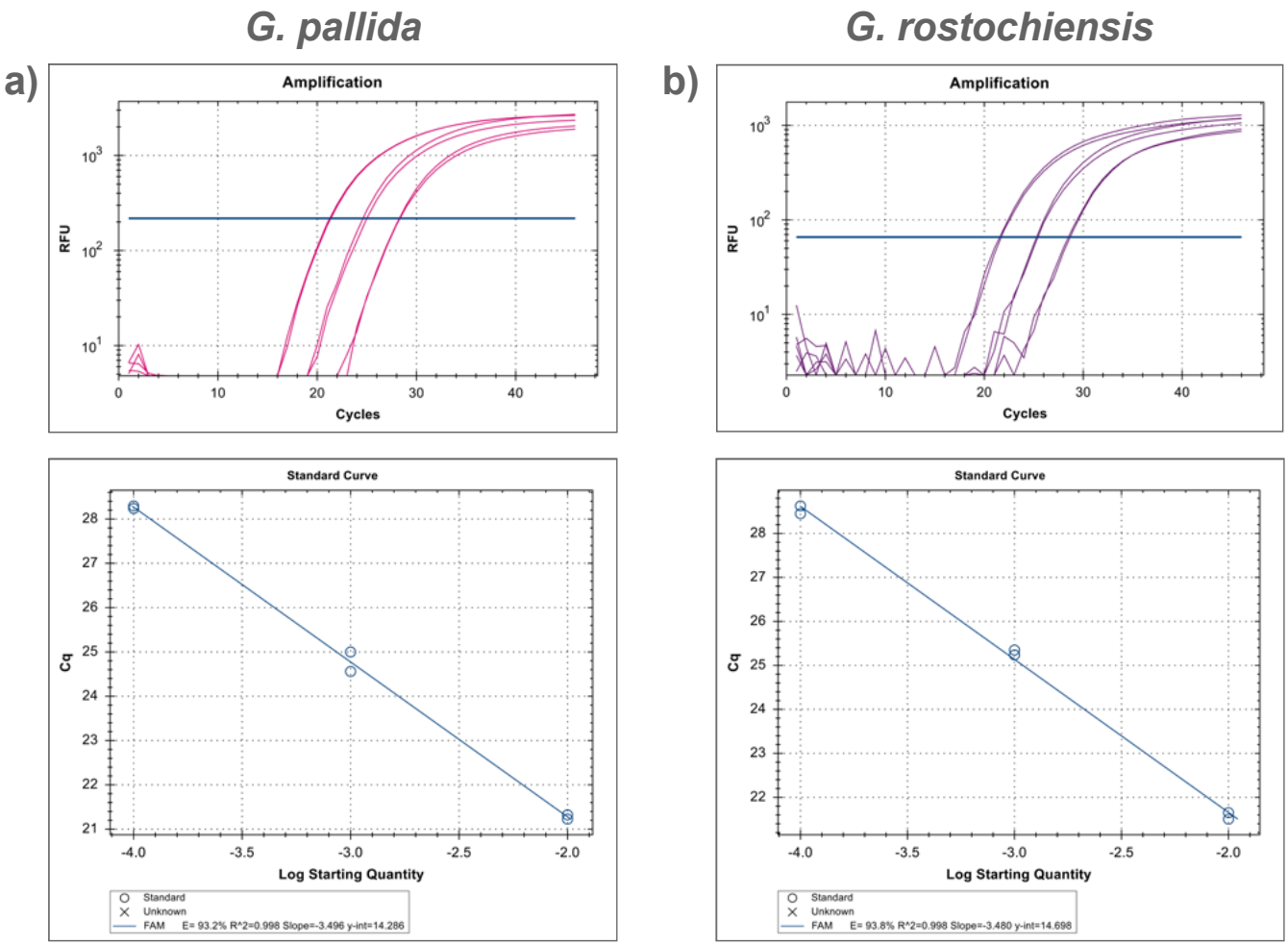


Figure 3. Amplification plots and standard curves for FAM-labelled BHQplus Probes with BHQ Probe Master Mix for (a) *G. pallida* and (b) *G. rostochiensis* targets. For both targets, PCR efficiency was 90-110%, and R^2 = 0.998.

Application note

BHQ Probe Master Mix in qPCR applications

	G. pallida		*G. rostochiensis*	
	BHQplus	**Competitor A**	**BHQplus**	**Competitor A**
Positive samples	25/52 (48%)	20/52 (38%)	19/52 (36%)	18/52 (34%)
Negative samples	27/52 (52%)	32/52 (61%)	33/52 (63%)	34/52 (65%)
Discrepansies	7/52 (13%)		7/52 (13%)	
Sensitivity	95% (19/20)		83% (15/18)	
Specificity	81% (26/32)		88% (30/32)	

Table 1. Sensitivity and specificity of BHQ Probe Master Mix, determined by comparison against Competitor A probes and reagents. Data generated was comparable between the two chemistries.

The results for BHQplus probes with BHQ Probe Master Mix were compared with Competitor A reagents to determine specificity and sensitivity (Table 1). Overall the data generated was comparable between the two chemistries, and specificity and sensitivity of BHQ Probe Master Mix was high.

Conclusion

BHQ Probe Master Mix is a robust master mix that can be used for both end-point and real time qPCR applications. It can be used in agrigenomics qPCR-based quantification applications with challenging diploid and polyploid samples, in conjunction with a range of BHQ Probe formats from the Biosearch Technologies portfolio. BHQ Probe Master Mix has been optimised with a range of nucleic acid concentrations and purities and has a wide range of possible reaction volumes. Its high PCR efficiency, wide dynamic range and multiplexing capabilities make it a reliable choice for the agrigenomics sector.

References

1. Diaz A., Zikhail M., Turner A.S., Isaac P. and Laurie D.A.(2012) PLoS ONE 7(3): e33234.

2. https://biosearchassets.blob.core.windows.net/assets/app-note-bhqnova-cnv.pdf

3. https://cvalues.science.kew.org/

4. Reid A., Evans F., Mulholland V., Cole Y. and Pickup J (2015). Plant Pathology: Techniques and Protocols, Methods in Molecular Biology, vol 1302 (11)

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