Faster binding kinetics with BHQ*plus*™ probes

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BHQ*plus*™ *is a new probe chemistry from Biosearch Technologies that increases probe melting temperature and enables more sensitive fluorescence detection. Using a* BHQ*plus* *probe, real-time PCR was performed in 15 min on the Spartan DX*™.

Introduction

TaqMan® probes (Applied Biosystems) are linear oligos that are typically 20 to 30 nucleotides (nt) in length. Probes are usually designed to have a fluorophore reporter molecule at the 5′ end and a quenching dye at the 3′ end. When the fluorophore is excited by light, its excitation energy is transferred to the probe *via* the process of fluorescence resonance energy transfer (FRET) and no fluorescence is emitted. During PCR, the DNA polymerase cleaves the probe and separates the fluorophore and quencher. Now the fluorophore is free of the quencher and emits fluorescence when excited by light of the appropriate wavelength (Ref 1).

The melting temperature (Tm) of the probe is typically designed 10°C higher than the Tm of the PCR primers. The reason is because this increases the probability that the probe will bind before the primers during the annealing step of PCR. This is important because the probe must be bound in order to be cleaved by the action of the DNA polymerase as it extends the primers. If the probe is not bound before the primers extend, then it will not be cleaved, and there will be no increase in fluorescence.

One method of increasing probe Tm is to simply increase the length of the probe. The disadvantages are that longer probes have slower binding kinetics and can be more difficult to synthesize. An alternative method of increasing probe Tm is to add additional chemistries that promote tighter binding with the DNA template. One example is Minor Groove Binder (MGB) probes™ (Ref 2). MGB probes are typically 12-15 nt in length and have chemical compounds attached to their 3′ ends which bind to the minor groove of DNA. The binding of this MGB portion compensates for the lower melting temperature of the probe. An additional advantage is that the shorter probe length enables them to be used for detecting single nucleotide polymorphisms (SNPs). In contrast, conventional TaqMan probes are 20-30 nt in length and are less sensitive at detecting SNPs.

Another weakness of some TaqMan probe chemistries is that there is not enough quenching to decrease the fluorescence background. The consequence of the higher background is that it takes more amplification cycles to increase the signal over background and this results in delayed threshold cycle (Ct) values.

Similar to MGB, BHQ*plus* is a new probe chemistry that increases the fluorescence signal by increasing the binding strength of a probe, while maintaining a shorter length.

The purpose of this study was to compare the reaction kinetics and fluorescence sensitivity of BHQ*plus* probes versus conventional BHQ-1 probes.

Materials and Methods

Real-time PCR

The DNA template was a synthetic oligonucleotide of 82 base pairs in length (Biosearch Technologies). Oligonucleotide primers were designed against the hemolysin III (HlY–III) gene, which is specific to *Bacillus cereus* Group organisms. The forward primer sequence was 5′-gga tgg ctc ata atc gtt g-3′, and the reverse primer was 5′-ttc cac ctg cta aaa gta g-3′. The primers were calculated to have a Tm of 58°C (www.dnastar.com). The expected amplicon size was 82 bp and consisted of the same sequence as the DNA template.

For real-time detection, the BHQ*plus* TaqMan probe consisted of a FAM fluorophore at the 5′ end and a BHQ*plus* quencher at the 3′ end. The BHQ*plus* probe was 21 nt in length and had a sequence of 5′-tta aac cac ttt atgaaa atc-3′. The conventional BHQ-1 TaqMan probe consisted of a FAM fluorophore at the 5′ end and a BHQ-1 quencher at the 3′ end. The BHQ-1 probe was 29 nt in length and had a sequence of 5′-aaa cca ctt tat gaa aat cta act gga ca-3′. Components of the real-time PCR mixtures are listed in Table 1.

Thermal cycling

A two-temperature cycling program was performed by combining the annealing and extension steps. Cycling parameters are listed in Table 2. As previously described (Ref 3), thermocouple measurements showed that this particular set of cycling conditions resulted in average liquid temperatures of 82°C during the denaturation step and 58°C during the annealing/extension step. Reactions were performed in 0.2 ml thin-wall, flat-cap PCR tubes (VWR Cat.

Component	Final amount
10X PCR Reaction Buffer (No $MgCl_2$) (Invitrogen)	1 X
$MgCl_2$ (Invitrogen)	5.75 mM
dNTP mix (Invitrogen)	0.2 mM
Platinum TAQ Polymerase (Invitrogen)	1 U
TaqMan Probes (Biosearch)	0.1 μM
PCR primers (Biosearch)	0.3 μM each
Template DNA	4 x 10^6 copies
Sterile water	
Total reaction volume	**20 μl**

Table 2. Components of real-time PCR mixture.

Step	Temperature	Time	Cycles
Initial denaturation	96°C	7 s	1
Denaturation	96°C	10 s	35
Annealing/extension	45°C	12 s	35

Table 3. Cycling parameters.

No. 53550-106). Reactions were topped with 15 μl of mineral oil (Biotools, Cat. No. 20.032) to prevent evaporation. Real-time PCR was performed using the Spartan DX instrument. Reactions were performed in triplicate.

DNA analysis

Fluorescence values were downloaded from the Spartan DX to a computer and graphed using Microsoft Excel®. PCR results were confirmed by agarose gel electrophoresis using 5 μl of the amplification products.

Results

Samples with BHQ*plus* probes had an average cycle threshold (Ct) value of 18, compared to 22 with BHQ-1 probes. At the end of 35 cycles, the BHQ*plus* probes also yielded a higher fluorescence intensity of 29 (arbitrary units) compared to only 21 for the BHQ-1 probes. Figures 1 and 2 show real-time PCR plots for BHQ*plus* and BHQ-1 reactions, respectively. Gel electrophoresis showed a single amplicon of the correct size for all samples. The total time for 35 cycles of real-time PCR was about 15 min.

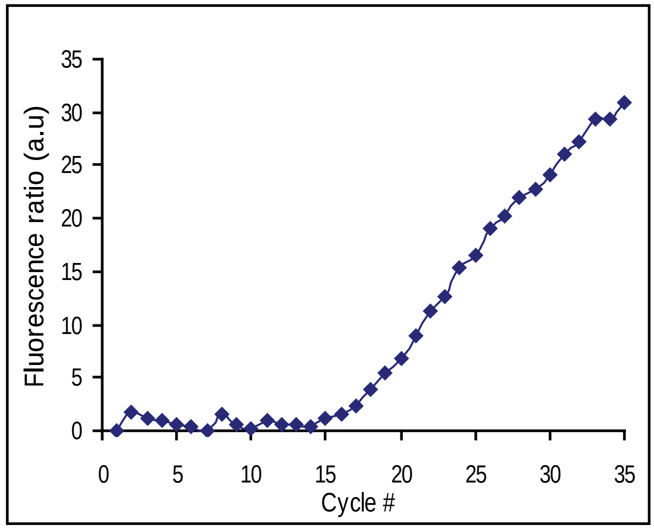

Figure 1. Real-time PCR result with BHQ*plus* probe.

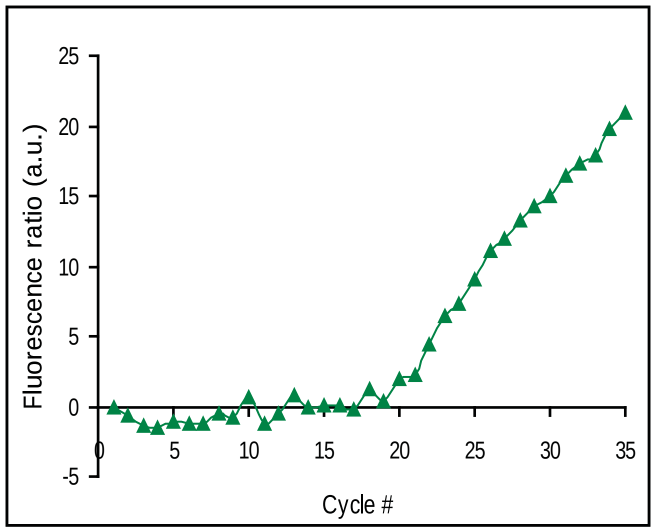

Figure 2. Real-time PCR result with regular BHQ-1 probe.

Discussion and Conclusions

BHQ*plus* chemistry increases the Tm of a probe without increasing its nucleotide length. As expected, BHQ*plus* reactions were detectable at earlier cycles and achieved higher end-point fluorescence intensities compared to regular BHQ-1 reactions where the probe Tm was lower.

Interestingly, it was possible to perform 35 cycles of real-time PCR in only 15 min. One factor in achieving this low run-time was that the amplicon was only 82 bp in length and had a low Tm (68°C). This meant that the liquid temperature in the reactions was able to cycle between 82°C and 58°C and still achieve efficient denaturation, annealing, and extension.

In summary, BHQ*plus* probes enable more sensitive fluorescent detection for real-time PCR and permit faster cycling on the Spartan DX.

References

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