APPLICATION NOTE

Improved Function with Double-Quenched BHQ® Probes for qPCR

Signaling Mechanisms of qPCR Probes

The predominant probe-type for qPCR applications is the linear hydrolysis probe, as used in the 5′ nuclease assay. This probe is comprised of two dyes at either terminus of the oligonucleotide, typically a 5′ fluorescent reporter and 3′ dark quencher. Within this system the 3′ modification of the probe is actually dual purpose:

1. Prohibit polymerase extension from the probe upon hybridization.
2. Quench signal from the fluorophore before the probe hybridizes to its target.

The terminal quencher modification is responsible for extinguishing the signal of the fluorescent reporter. Both reporter dye and quencher may interact through either Förster Resonance Energy Transfer (FRET) or else static quenching, wherein the chromophores contact one another to form a ground-state complex that is non-fluorescent (Johansson, 2006; Marras et al., 2002). Both FRET and static quenching mechanisms suppress the signal when the probe is free in solution. Upon oligo hybridization, the duplex formed is quite rigid and so increases the effective length compared to single-stranded oligo, which is much more flexible. Binding of the probe to the target sequence is therefore sufficient to disrupt the interaction between the two dyes positioned at opposite ends of an oligo, and thus allows the release of fluorescent signal (Parkhurst and Parkhurst, 1995).

The signal release upon hybridization can be made permanent by hydrolyzing the oligo probe between the fluorophore and the quencher. In fact, cleavage is accomplished when *Taq* polymerase extends from a primer and encounters the probe in its path (Figure 1). The nuclease activity of the polymerase cleaves the probe one or several bases from its 5′ end until the probe loses binding stability and is displaced from the strand. This signaling mechanism is the basis for hydrolysis probes.

Measures of probe performance

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. The initial fluorescence represents the quenching efficiency, and so to double this background signal reduces the S:N by a factor of two. Quenching efficiency depends, in part, upon the oligo length. This is because FRET quenching diminishes quite rapidly with increasing separation between the dyes according to a relationship of $(1/r)^6$, where r is the distance through space (Cardullo et al., 1988). Single-stranded oligos are thought to behave as a random coil and so the effective distance is the average of many conformations in space, but the principle remains the same: increasing sequence length diminishes the quenching efficiency, resulting in elevated baseline fluorescence and poor signal-to-noise values from certain probe designs. For this reason, probe sequences are typically limited to 30 bases or shorter to achieve sufficient quenching efficiency in the final application.

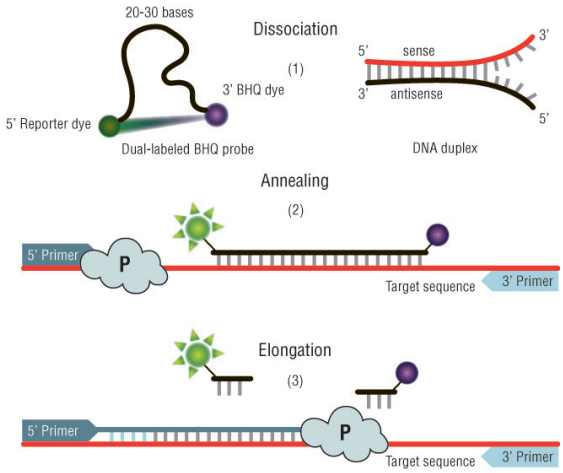

Fig. 1 – Signaling Mechanism of Hydrolysis Probes. *After denaturation, the probe and primers anneal to the dissociated strands. During elongation the polymerase extends from the primer, encounters the probe, and hydrolyzes nucleotide fragments that then become displaced from the strand. Cleavage of the probe untethers the fluorophore and quencher to make signal release permanent.*

The length of an oligo probe is selected with consideration not only to quenching efficiency but also binding stability. A common design guideline is to select probe sequences with a melt temperature (Tm) of 70 °C, elevated above the Tm of the primers. Depending on base composition, probes must typically be longer than 20 bases to accomplish that Tm criterion, in the absence of special modifications to promote hybridization. Sequence design is thus a careful compromise between binding stability and quenching efficiency, but a 20-30 base window is inadequate for many difficult targets. For example, SNP genotyping requires probes shorter than 20 bases in order to achieve the enhanced specificity needed for mismatch discrimination.

Conversely, it is occasionally necessary to extend the probe sequence beyond 30 bases. Many target genes are particularly AT-rich and require longer sequences to obtain the proper Tm. This upper limit on sequence length can be relaxed by positioning the quencher at an internal location closer to the fluorescent reporter, rather than at the 3′ terminus. In fact, some of the earliest hydrolysis probe designs had the quencher at an internal location for this reason—to improve their quenching efficiency (Lee et. al., 1993). However, the 3′ position now vacated must still be modified with a blocker such as a terminal phosphate or aliphatic carbon spacer, to prevent the probe from behaving as a primer and triggering extension.

A sensible approach to improve quenching efficiency is to preserve the terminal quencher for its blocking function while incorporating a second quencher at an internal position. That is the basis of novel double-quenched probe-type from LGC Biosearch Technologies, called BHQnova™ probes:

5’ - [FAM]- Oligo sequence[1] - [Nova] - Oligo sequence[2] - [BHQ-1] - 3’

The internal quencher is positioned between residues 9 & 10 for optimal performance, and this location is paramount. The precise position and combination of modifications improve both quenching efficiency and signal release across a range of oligo lengths.

Performance Results

A panel of probes were designed with increasing sequence lengths, as follows:

21-base probe (end-labeled):	5’-[FAM]-TCCTTTGGGCTCCTGCCATCT-[BHQ-1]-3’
21-base dual-quenched probe:	5’-[FAM]-TCCTTTGGG-[Nova]-CCCTGCCATCT-[BHQ-1]-3’
28-base probe (end-labeled):	5’-[FAM]-AAACCACTTTATGAAAATCTAACTGGACA-[BHQ-1]-3’
28-base dual-quenched probe:	5’-[FAM]-AAACCACTT-[Nova]-ATGAAAATCTAACTGGACA-[BHQ-1]-3’
35-base probe (end-labeled):	5’-[FAM]-AAGAGTAGTAGCCTAAGAGTGTCAGTTGTACATCA-[BHQ-1]-3’
35-base dual-quenched probe:	5’-[FAM]-AAGAGTAGT-[Nova]-AGCCAAGAGTGTCAGTTGTACATCA-[BHQ-1]-3’

These probes are used to signal amplification across a 7-point dilution series of template DNA. The resulting traces are presented before baseline subtraction to better gauge the quenching efficiency, as well as following normalization by the analysis algorithms of the Rotor-Gene Q. These results reveal a significantly reduced baseline signal and improved signal response for each assay, particularly those with longer sequence designs.

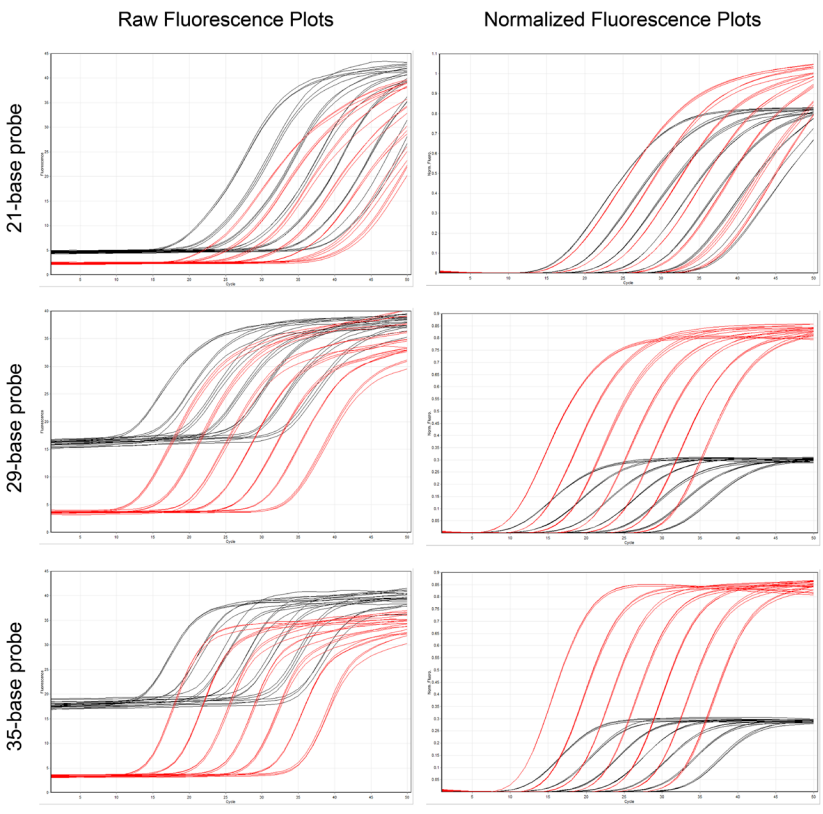

Figure 2 (on left) – Comparison of standard probes and BHQnova probes. *Amplification traces signaled with traditional end-labeled probes in **Black**. Amplification traces signaled with double-quenched BHQnova probes in **Red**. The panels at left present the raw data while the panels at right present the same data following baseline subtraction and normalization.*

Conclusion

A BHQnova probe represents a double-quenched probe-type that overcomes the upper limit of probe length previously restricted by quenching efficiency. This new format incorporates a novel internal modification, the Nova quencher, in conjunction with a BHQ-1 modification at the 3’ terminus. Positioning the Nova quencher between nucleotides 9 and 10 avoids the base-dependency that governs those quenchers formulated onto a thymidine linkage for internal modification. This new probe format is suitable for demanding qPCR applications that require greater flexibility in sequence selection without sacrificing the sensitivity of the highest performing probe designs. For more information about BHQnova probes, please contact techsupport@biosearchtech.com.

References

1. Choosing reporter-quencher pairs for efficient quenching through formation of intramolecular dimers. Johansson MK. *Methods Mol Biol*. **2006**;335:17-29. Review. PMID: 16785617
2. Efficiencies of fluorescence resonance energy transfer and contact-mediated quenching in oligonucleotide probes. Marras SA, Kramer FR, Tyagi S. *Nucleic Acids Res*. **2002** Nov 1;30(21):e122. PMID:12409481
3. Kinetic studies by fluorescence resonance energy transfer employing a double-labeled oligonucleotide: hybridization to the oligonucleotide complement and to single-stranded DNA. Parkhurst KM, Parkhurst LJ. *Biochemistry*. **1995** Jan 10;34(1):285-92. PMID:7819209
4. Detection of nucleic acid hybridization by nonradiative fluorescence resonance energy transfer. Cardullo RA, Agrawal S, Flores C, Zamecnik PC, Wolf DE. Proc *Natl Acad Sci U S A*. **1988** Dec;85(23):8790-4. PMID:3194390
5. Allelic discrimination by nick-translation PCR with fluorogenic probes. Lee LG, Connell CR, Bloch W. *Nucleic Acids Res*. **1993** Aug 11;21(16):3761-6. PMID:8367293

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