CAL Fluor™ Dyes: Multiplexing Reporters for Real-Time PCR Instruments and Probe Designs

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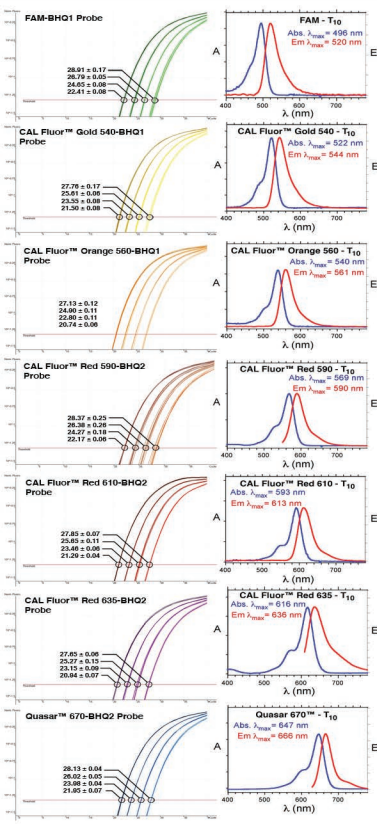
Introduction

The specificity and sensitivity of real-time PCR has revolutionized the fields of haplotyping, pathogen detection, and gene expression quantification. In each of these applications, the ampliflying target sequence is revealed through the use of a fluorescent-labeled probe. A series of fluorescent dyes, collectively known as CAL Fluor™ dyes, is presented for incorporation into these oligonucleotide probes. With emission maxima ranging from 522 nm through 636 nm, these dyes are ideal for multiplexing applications where minimal cross-talk is desired. This capability is demonstrated by simultaneously amplifying four different genomic DNA targets in a quadraplex assay. CAL Fluor™ probes also incorporating Black Hole Quenchers™ exhibit large signal to noise ratios and thus produce amplification traces with early CT values. These dyes function in a variety of probe designs, including dual-labeled 5' exonuclease probes, Molecular Beacons™, and Scorpions™. Finally, CAL Fluor™ dyes are compatible with the range of real-time PCR instruments including the ABI real-time machines, the Rotor-Gene 3000™, and the Bio-Rad iCycler™, among others.



Real-Time PCR Performance Across the Spectrum

Linear 5' exonuclease probes, incorporating either a CAL Fluor™ dye or FAM, target a telomerase reverse-transcriptase gene in a singleplex assay. Quasar 670™, a dye with emission in the far red is also included.

Each reporter type was quenched using a Black Hole Quencher™, and the probe sequence was kept identical.

Amplification traces were generated using a Rotor-Gene 3000™, and document a four-fold dilution series of human genomic DNA. Serial dilutions cross the threshold at an interval of two cycles, with six replicates per dilution.

CAL Fluor™ Dyes perform with superior detection compared to fluorescein (FAM), demonstrated by earlier CT values.

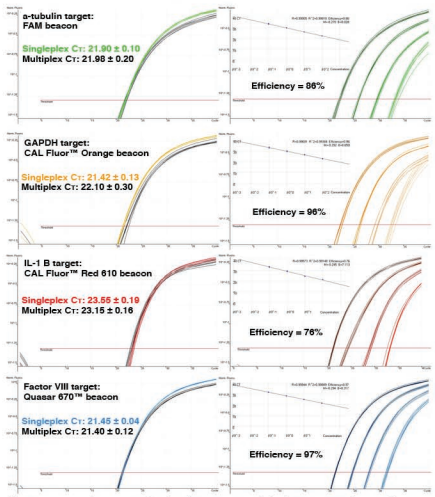
Absorption and emission spectra are shown for each unquenched reporter when linked to a T10 oligonucleotide. Blue traces are the absorption spectra and red traces are the emission spectra.

Probe and Primer Sequences:

Fwd. Primer Sequence:	CAG GTG GAG ACC CTG AGA A
Rev. Primer Sequence:	ACA CCT TTG GTC ACT CCA AAT
Probe Sequences:	[Reporter]-TCC CAG AGC TCC CAG GGT CC-[BHQ]

Quadraplex Assay Incorporating CAL Fluor™ BHQ™ Beacons

A Quadraplex assay was designed and optimized by Bio-Rad Laboratories to detect the following human genomic DNA targets: a-Tubulin, IL-1 B, GAPDH, and Factor VIII.* Here we modify this assay to incorporate CAL Fluor™ reporters as well as Black Hole Quenchers™. All multiplexing results were obtained using a Rotor-Gene 3000™.



Primer and Probe Sequences

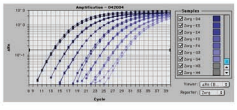
a-tubulin fwd. primer:	CCA AGC TGG AGT TCT CTA
a-tubulin rev. primer:	CAG AGT GCT CCA GG
a-tubulin probe:	[FAM]-CCC AGG TTT CCA CAG CTG TAG TTG ACC TGG G-[BHQ1]
GAPDH fwd. primer:	CAT GTT CCA ATA TGA TTC CAC
GAPDH rev. primer:	CCT GGA AGA TGG TGA TG
GAPDH probe:	[CAL Fluor™ Orange 560]-CAA GGC TGA GAA CGG GAA GCT TGT CCA GCC TTG-[BHQ1]
IL-1 B fwd. primer:	TGC TCC TTC CAG GAC CT
IL-1 B rev. primer:	GTG GTG GTC GGA GAT TC
IL-1 B probe:	[CAL Fluor™ Red 610]-CTC TGC CCT CTG GAT GGC GGC AGA G-[BHQ2]
Factor VIII fwd. primer:	GAC AGT GGA AAT GTT ACC
Factor VIII rev. primer:	CAT CCC AGC ATG TAG ATG
Factor VIII probe:	[Quasar 670™]-AGC TGG AAT TTG GCG GGT GGA ATG TCC AGC T-[BHQ2]

Left: comparisons of single vs. multiplex reactions for each target when amplified from 100 ng of genomic DNA. The CT value for each amplified target in singleplex reactions (colored traces) is nearly equivalent to that target when amplified in multiplex reactions (black traces). Singleplex and multiplex CT values that are statistically distinguishable, such as the GAPDH target, indicate that further fine-tuning of the primer concentrations is necessary. **Right:** To gauge the amplification efficiency for each target, the multiplex assay was repeated using 10-fold serial dilutions of genomic DNA, with six replicates per dilution.

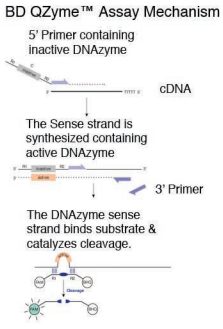
*Bio-Rad multiplexing protocol outlined in Bio-Rad Tech Note 2679

CAL Fluor™ Compatibility with Variety of Real-Time PCR Probe Designs

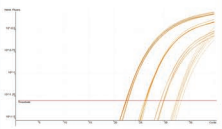
BD QZyme™ Assay showing CAL Fluor™ Orange 560 Amplification Traces



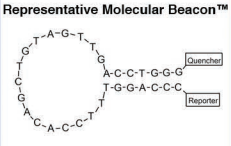
BD QZyme™ Assay Mechanism



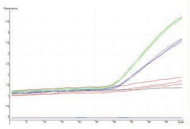
Molecular Beacon Amplifications using CAL Fluor™ Orange 560



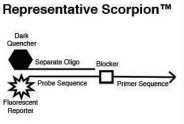
Representative Molecular Beacon™



Scorpion™ Amplifications using CAL Fluor™ Orange 560

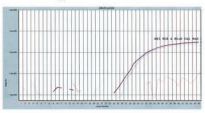


Representative Scorpion™

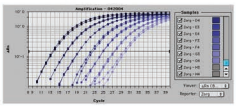


CAL Fluor™ Compatibility with Range of Real-Time PCR Instruments

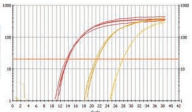
CAL Fluor™ Red 610 Amplification Generated using the ABI 7000



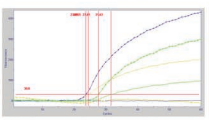
CAL Fluor™ Orange 560 Amplifications Generated using the ABI 7700



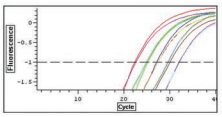
CAL Fluor™ Red 610 Amplifications Generated using the Bio-Rad iCycler™



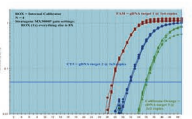
CAL Fluor™ Gold 540 and CAL Fluor™ Orange 560 Amplifications Generated using the Cepheid Smartcycler™.



CAL Fluor™ Gold 540 Amplifications Generated using the MJ Research Opticon 2™



CAL Fluor™ Orange 560 Amplifications Generated using the Stratagene MX 3000P™



Conclusions:

- CAL Fluor™ dyes offer superior detection to FAM in singleplex assay.
- These fluorescent dyes can be succesfully employed as multiplexing reporters.
- As versatile fluorophores, they can be incorporated into a variety of real-time PCR probe designs.
- CAL Fluor™ dyes are compatible with the range of quantitative PCR instruments.

Acknowledgments

We would like to gratefully acknowledge Bio-Rad Laboratories for quadraplex assay design and optimization, Dale Levitzke, Biotage for supplying the Rotor-Gene 3000 necessary for real-time PCR, Bob Larsen, BD Biosciences Clontech for his assistance in performing the QZyme™ assay, and for providing the figure of the QZyme™ mechanism, David Whitcombe, DxS for supplying the Scorpion™ amplification image, Cassandra Kelly, Wadsworth Center, NYSDOH for providing the ABI 7000 amplification image, Minh Le, Bayer for supplying the Stratagene MX 3000P amplification image, and Aaron Sechler, USDA for providing the Cepheid Smartcycler amplification image. These individuals also provided invaluable guidance in the sophisticated field of real-time PCR.