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 amplifying target sequence is revealed through a fluorescent-labeled probe. A series of fluorophores, 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 quadruplex 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' exo-sequencing probes, Molecular Beacons™, and Scorpions™. Finally, CAL Fluor™ dyes are compatible with the range of real-time PCR instruments including the ABI 7500™, the Rotor-Gene 3000™, and the Bio-Rad iCycler™, among others.

**Real-Time PCR Performance Across the Spectrum**

Linear 5' exo-sequencing, 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.

**Quadruplex Assay Incorporating CAL Fluor™ BHQ™ Beacons**

A quadruplex assay was designed and optimized by Bio-Rad Laboratories to detect the following human genomic DNA targets: a-tubulin, IL-1B, 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™.

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

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

**Molecular Beacon Amplifications using CAL Fluor™ Orange 560**

**Scorpion™ Amplifications using CAL Fluor™ Orange 560**

**BD QZyme™ Assay Mechanism**

5' Primer containing inactive DNAzyme

The sense strand is synthesized containing active DNAzyme

The DNAzyme sense strand binds substrate & catalyzes cleavage.

**Conclusions:**

- CAL Fluor™ dyes offer superior detection to FAM in singleplex assays.
- These fluorophores can be successfully 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.

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

**CAL Fluor™ Red 610 Amplification Generated using the ABI 7700**

**CAL Fluor™ Orange 560 Amplifications Generated using the ABI 7700**

**CAL Fluor™ Gold 540 and CAL Fluor™ Orange 560 Amplifications Generated using the Cepheid SmartCycler™**

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

**CAL Fluor™ Orange 560 Amplifications Generated using the Cepheid SmartCycler™**

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*Bio-Rad multiplexing protocol outlined in Bio-Rad Tech Note 3079*