Molecular Beacon Probes: Micro-Volume Fluorescence Measurement of HPLC Isolated Probes

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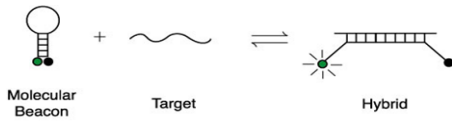
Micro-Volume Fluorescence Technology

The Thermo Scientific NanoDrop 3300 Fluorospectrometer uses a patented "cuvette-less" sample retention system for routine fluorescent measurements with as little as one micoliter. The system uses surface tension to hold micro-volume samples in place, allowing for instant, 'at-strength' readings without dilution. Cleaning involves simply wiping or blotting the instrument's optical surfaces with a common lab wipe. These capabilities, combined with the intuitive, user-friendly software, provides quick and accurate fluorescence measurements with minimal consumption of sample.

Molecular Beacon Technology

Molecular Beacons are single-stranded oligonucleotide hybridization probes in a stem-and-loop configuration. The loop contains a probe sequence complementary to a target nucleic acid sequence while the stem is formed by annealing of complementary arm sequences located on either side of the probe sequence. A fluorophore (donor) is covalently linked to the end of one arm and a quencher is covalently linked to the end of the other arm (Figure 1). Due to the close proximity of the donor and quencher when molecular beacon probes are free in solution, they transiently share electrons eliminating the donor's ability to fluoresce. In the presence of a target nucleic acid sequence, the probe hybridizes to the target and undergoes a conformational change, displacing the quencher and allowing the fluorophore to fluoresce brightly.

Figure 1: General Molecular Beacon Technology



Non-hybridized molecular beacons are fluorescence-quenched and ideally suited to homogeneous assay design, making it unnecessary to isolate the probe-target hybrids to determine the presence of a nucleic acid target. In this study, conducted at the Public Health Research Institute (Newark, New Jersey), a series of molecular beacon probes were used to evaluate the NanoDrop™ 3300 performance against a reference Photon Technology International (PTI) QuantaMaster I spectrofluorometer.

Observations and Conclusions

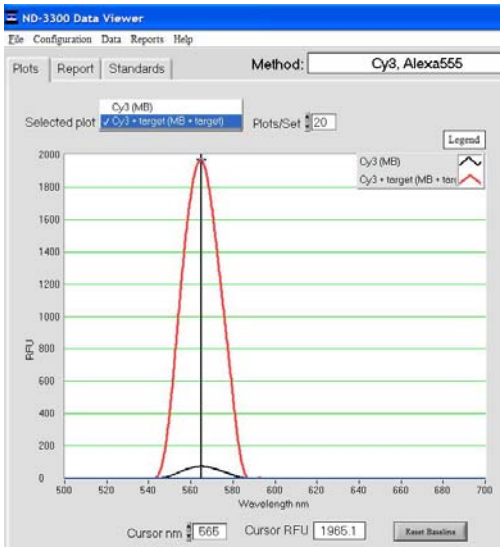
In a fraction of the time required for the reference instrument, the NanoDrop 3300 exhibited comparable signal-to-background without the need for instrument warm-up, sample dilution, cuvettes, or filter changes.

The NanoDrop 3300 software is pre-programmed with methods for measuring a variety of common fluorophores. The signal-to-background (S/B) for eight different fluorescent-tagged molecular beacon probes was determined at three (3) concentrations on the NanoDrop 3300 (0.1uM, 1uM and 3uM) and compared with the reference PTI spectrofluorometer at a single concentration (0.1uM). Three of the study fluorophores (FAM, Cy3, and Cy5), were tested using the pre-configured methods directly. Methods to monitor emission of the fluorophores for the remaining molecular beacon probes were initially created and saved using the NanoDrop 3300 software (Method Editor).

Fluorophore linked to probe	**Emission wavelength** (nm) ± DI*	**ND-3300 MEAN S/B** (# concentrations tested)	**PTI - S/B** (# concentrations tested)
FAM	515	57 (3)	100 (1)
TET	537 ± 30	28 (3)	50 (1)
HEX	554 ± 30	30 (3)	60 (1)
TexasRed	615 ± 30	22(3)	---
Cy3	565 ± 20	18 (3)	50 (1)
Cy5	665 ± 30	68 (3)	100 (1)
Alexa 546	570 ± 20	88 (1)	75 (1)
Alexa 594	612 ± 20	161 (1)	200 (1)

For each unique molecular beacon probe, the NanoDrop 3300 was first initialized (or blanked) with 2 ul of PCR buffer. The fluorescence emission (RFU) of the molecular beacon probe, without target, was measured using a 1ul sample mixed with 1ul of PCR buffer. The fluorescence of the molecular beacon probe (1ul) in the presence of its respective oligonucleotide target (1ul) was then measured in like manner (Figure 2).

Figure 2: Example of fluorescence emission (RFU) of a molecular beacon probe with and without target.



The signal-to-background is defined as the ratio of the molecular beacon probe's fluorescence in the presence of a target to the fluorescence of the probe in the absence of a target.

nm = Emission wavelength (setting within the NanoDrop 3300 Method Editor software).

$\Delta\lambda$* = the respective wavelength range over which the residual signal (with scaled background compensation removal) is displayed and calculated. (Based on the inherent "off-angle" light rejection properties of the optical fiber, the reduction in scattering from directly wetted optical surfaces, and the spectral stability of the LED, the sample fluorescence can be determined by subtracting a scaled representation of white LED source from the total fluorescence.)

Fluorophore = Reporter fluorophore at the 5' end of molecular beacon probe.

Quencher = Molecular beacon probes labeled with 5' FAM, TET and HEX are labeled with a 3' dabcyl quencher moiety and molecular beacon probes labeled with 5' TexasRed, Cy3, Cy5, Alexa 546 and Alexa 594 are labeled with a 3' BHQ-2 quencher moiety.

PTI's QuantaMaster I spectrofluorometer utilizes a xenon short arc lamp as a light source and monochromators for both excitation wavelengths and emission wavelengths. The QuantaMaster I contains a lens-based optic sample compartment with a 150ul mini-cell cuvette option and utilizes a PMT photon counting detection system. According to the manufacturer's specifications, it requires a 10 minute warm-up period before the first measurement can be taken. In contrast, the NanoDrop 3300 LED excitation sources do not require any warm-up time.

Lower overall costs result from reduced operator time, no disposables, and very small sample requirement. The entire cycle time, which includes dispensing one microliter of sample onto the optical surface, measuring its fluorescence, and blotting the optical surface for the next sample, is commonly completed within 30 seconds.

One option is to add one microliter of probe directly onto the NanoDrop 3300 optical surface and one microliter of the target oligo, followed by an immediate measurement. This option can be accomplished provided binding is not time dependent with such small volumes.

Increased confidence in operator-generated data can be an unrecognized by-product of 'smart-design' instruments. With the NanoDrop 3300, the probability of error from sample dilution steps is greatly reduced since a single 1ul sample volume can be used across a wide concentration range encountered in HPLC-purified probes. This simplification contrasts with conventional mini-sample compartment-fitted spectrofluorometers and fluorescent plate readers requiring reaction volumes in the hundreds of microliters. For such instruments, variable sample volumes are commonly used to prevent erroneous fluorescent values resulting from probes that are too concentrated for accurate fluorescent measurement.

The NanoDrop 3300 small footprint and portability are always welcome when allocating laboratory bench space. The instrument is smaller than a common tissue box and is powered through a USB connection to a computer. The instrument is easily transported and ready for use in minutes.

Based on the PHRI study, the NanoDrop 3300 has proven to be a valuable, cost-effective tool for Molecular Beacon quality assurance protocols.