

Measurement of molecular beacons in the SPECTRAmax[®] GEMINI spectrofluorometer[†]

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Abstract: This article discusses the use of a dual monochromator based microplate spectrofluorometer in the analysis of molecular beacons used to monitor PCR reactions. The SPECTRAmax GEMINI spectrofluorometer is the first such instrument that incorporates Excitation and Emission dual scanning monochromators in its design. This feature gives the sensitivity that is required to analyse PCR products and provides the platform to excite and collect emission light from any wavelength pair in the region 250 to 850 nm, directly from the PCR plate. Up to 4 wavelength pairs can be selected to analyse up to four probes in a single mixture. In this article we show data from typical targets, and dyes such as TET, FAM and TAMRA.

The use of this analytical technology with a 96 or 384 well PCR plate gives the scientist a true high throughput method of monitoring the PCR reaction in their laboratory without the need for more dedicated expensive equipment.

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INTRODUCTION

Molecular beacons are fluorogenic oligonucleotide probes developed by Kramer and associates to detect specific nucleic acids in homogeneous solutions.^{1–4} They are single-stranded oligonucleotides that exist in a hairpin shape because of the complementary ‘arms’ at the ends which bind together to form a ‘stem’. The strained loop portion contains the nucleotide that is complementary to the intended target. A fluorophore is covalently bound to one end of the oligonucleotide and a quencher to the other end. In the absence of target, the fluorophore and quencher are held in close proximity by the arms and the fluorescence is internally quenched. When the probe hybridises to the target molecule, it undergoes a conformational change, resulting in separation of the fluorophore and quencher, and restoration of fluorescence. Molecular beacons are appealing because they can be used in homogeneous solutions and obviate the need to isolate probe/target hybrids from an excess of unbound hybridisation probes. They have been used to monitor polymerase chain reactions.³ Many combinations of fluorophores and quenchers are possible. Kramer’s group has found that 4-(4′-dimethylaminophenylazo) benzoic acid (DABCYL) can serve as a ‘universal quencher’ for a number of fluorophores. Even if the fluorophore emission spectrum does not overlap with the DABCYL absorption spectrum, quenching (with

>95% efficiency) occurs by direct energy transfer from fluorophore to quencher because of their close proximity.² This paper describes measurement of probe/target complexes with the SPECTRAmax GEMINI microplate spectrofluorometer.

MATERIALS AND METHODS

Samples of molecular beacons and complementary targets were obtained from Research Genetics, Huntsville, Alabama. The oligonucleotide sequences are not important to this discussion. The microplates used were, black 384-well microplates (Nunc), surface-treated to minimise binding (cell culture-treated). All samples were prepared in 10 m mol dm⁻³ Tris buffer, pH 8, containing 1 m mol dm⁻³ MgCl₂, and the final concentrations of beacon and target were 0.3 μmol dm⁻³ and 1.6 μmol dm⁻³ respectively, in a total volume of 54 μl³. The reaction mixtures were incubated for at least 30 min at ambient temperature before measurement in a SPECTRAmax GEMINI microplate spectrofluorometer.

RESULTS AND DISCUSSION

Figure 1 shows the fluorescence spectrum of a tetrachlorofluorescein (TET)-labelled probe in the presence and absence of complementary target. At the

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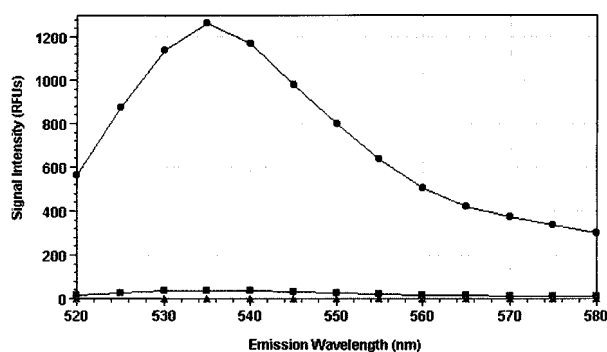


Figure 1. Emission spectra of TET probe with target (●) and without target (■). The bottom plot (▲) is buffer only. The excitation wavelength was 490nm and a 515nm emission cut-off filter was used.

peak maximum (approximately 535nm), the probe/target complex had approximately 35 times higher signal than did the probe alone.

The optimal wavelength settings for TET and several other molecular beacons were determined by examination of signal/blank ratios as previously described.^{5,6} The combination of excitation/emission wavelengths and emission cut-off filters giving the highest ratios are summarised in Table 1.

There is considerable interest in measuring multiple targets ('multiplexing') in a single sample. This can be done by using a different fluorophore for each probe, assuming the probes do not interfere with each other. If their emission spectra do not overlap, detection of one in the presence of the other is straightforward. Such is the case for fluorescein (FAM)- and tetramethylrhodamine (TAMRA)-labelled probes. Using the instrument settings intended for FAM, the TAMRA probe/target complex does not interfere with the FAM signal (Fig 2).

Conversely, when TAMRA settings are used, the FAM probe/target complex does not interfere with the TAMRA signal (Fig 3). There are many other two-probe combinations that would be compatible. If two probes have partially overlapping emission spectra, they may be resolved by adjusting their excitation and/or emission wavelengths to minimise interference. The SPECTRAMax GEMINI system's dual monochromators facilitate the optimisation process by making it easy to select different wavelengths.

There are also a number of three-probe combina-

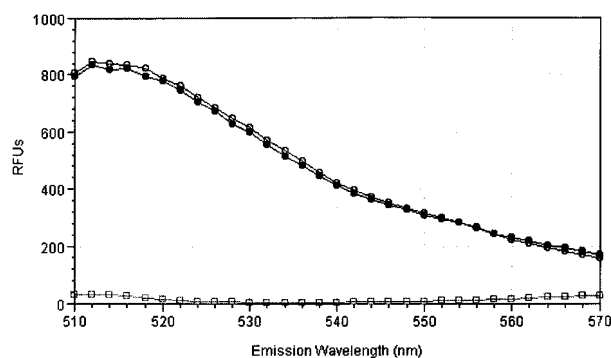


Figure 2. Fluorescence of an equimolar mixture of FAM and TAMRA probe/target complexes (●), FAM complex alone (○), and TAMRA complex alone (□). Scans were obtained with an excitation wavelength of 484nm and a 515nm emission cut-off filter.

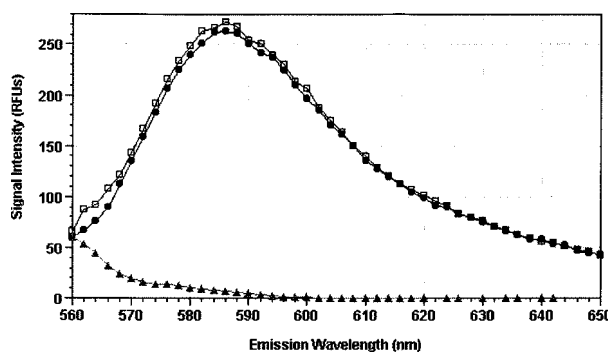


Figure 3. Fluorescence of an equimolar mixture of TAMRA and FAM probe/target complexes (●), TAMRA complex alone (□), and FAM complex alone (▲). Scans were obtained with an excitation wavelength of 550nm and a 570nm emission cut-off filter.

tions that should be suitable for use in a multiplex assay, for example, FAM and CY5 with HEX, CR 6G, CY3, or TAMRA (see Table 1). It is also possible that a four-probe combination such as FAM, HEX, TAMRA, and CY5 could be resolved.

CAUTIONS

Molecular beacons, like other oligonucleotides, will readily adhere to untreated plastic. Therefore, it is important to use microplates which have been surface treated to minimise binding. We have found that plates

Table 1. Recommended SPECTRAMax GEMINI instrument settings for selected individual beacons

Fluorophore	Excitation (nm)	Emission (nm)	Emission cut-off filter (nm)
FAM (fluorescein)	484	525	515
TET (tetrachlorofluorescein)	490	535	515
HEX (hexachloro-fluorescein)	525	554	550
CR (6-carboxyrhodamine 6G) 6G	505	570	530
CY [®] 3 ^a	550	605	590
TAMRA (tetramethylrhodamine)	555	605	590
CY [®] 5 ^a	640	675	665

^a Amersham Pharmacia Biotech.

treated for cell culture exhibit less binding than do untreated plates.

Molecular beacons, like other analytical tools, have potential pitfalls. Depending on the temperature, they will react with targets containing one or more nucleotide mismatches.²⁻⁴ Temperature control is especially important for discrimination between perfectly complementary targets and targets containing a single-nucleotide mismatch.^{3,4} Tyagi et al state that fluorescence increases as much as 900-fold when probes bind to their target.² In practice, the ratios are typically much lower. A high ratio is obviously desirable because it affords high sensitivity. A low ratio can be due to a number of factors, including contamination with free fluorophore and contamination with oligonucleotides that are missing the quencher. At least three commercial suppliers guarantee a minimum ratio of 25 (Research Genetics; SyntheGen, Houston, Texas; and Pacific Oligos, Toowong, Australia).

Summary

The reaction of molecular beacons with their targets can be measured in the SPECTRAMax GEMINI

microplate spectrofluorometer. With its dual scanning monochromators, it is easy to optimise excitation and emission wavelengths for specific fluorophores and to customise the settings for combinations of probes. Mixtures of two, three and (probably) four probes in a single mixture can be resolved.

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