

Detection of Micro-organisms with the Fluoroskan Ascent[®] using Molecular Beacons, Taqman[®], and SYBR[®] Green assays

Key Words

- Fluorescence
- Endpoint Reading
- PCR

Thermo Electron Corporation - Microplate Instrumentation SOLUTION NOTE

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Introduction

Fluorescent labeled probe utilisation has increased in diagnostic laboratories in recent years; uses include medical, agricultural and forestry micro-organism detection and identification. However, several operational diagnostic laboratories do not have access to high-end equipment used in research labs, such as real-time PCR. Here we describe the use of fluorescence detection in endpoint to allow detection and identification of micro-organisms using molecular beacons (3, 4, 5, 6, 7), Taqman (5), or SYBR Green (4).

In this assay, we used specific primers to amplify *Phytophthora ramorum*, the causal agent of Sudden Oak Death (2,8), combined with fluorescent probes (molecular beacons and Taqman) or non-specific label (SYBR Green). The reactions were analysed in endpoint with Thermo Fluoroskan Ascent using endpoint measurement (Figure 1).

Materials and Methods

PCR Amplification

AS-PCR (Allelic Specific-Polymerase Chain Reaction) was performed with a MJ Research thermal cycler PTC-200[®] or a Techne Genius Thermal Cycler.

Locked Nucleic Acids (LNA) (1) primers were used to increase the specificity of the primers. Fluorescent molecules (SYBR Green, dual-labeled probe (Taqman), or molecular beacons) were included in the PCR master mix. All reactions were performed in 25 µl volumes. Negative controls consisted of reactions with all reagents minus the template DNA. Fluorescence values from control reactions were subtracted to account for background fluorescence.

SYBR Green

The PCR reaction contained 0.3 µM of each LNA primer, 1X of QuantiTect[™] SYBRGreen PCR Master Mix (Qiagen), and template DNA. PCR cycling conditions were set at 95°C for 15 min, 36 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Taqman probe

The PCR reaction contained 0.2 µM of each LNA primer, 0.2 µM of Taqman (dual-labeled probe) probe, 1X of QuantiTect Probe PCR Master Mix (Qiagen), and template DNA. PCR cycling conditions were set at 95°C for 15 min, 36 cycles at 94°C for 15 s, and 60°C for 60 s.

Molecular beacons

The PCR reaction contained 0.4 µM of each LNA primer, 0.2 µM of molecular beacon probe, 1X of QuantiTect Probe PCR Master Mix (Qiagen), and template DNA. PCR cycling conditions were set at 95°C for 15 min, 36 cycles at 94°C for 15 s, 60°C for 30 s, and 72°C for 30 s. This was followed by denaturation at 95°C for 1 min and annealing at 60°C for 45 s prior to result readings in the Fluoroskan Ascent. This allows the molecular beacon to open and

anneal with the target sequence.

Plate reading

To detect the fluorescence of the molecular beacons, the Fluoroskan Ascent was used to read in endpoint, the integration time was 20 ms and the measurement type single. The samples were read in a PCR tube/plate (0.2 ml PCR tubes-Flat cap, Promega) with Adapter for PCR tube (Thermo) at 485 and 527 nm of wavelengths (FAM, SYBR Green). The approximate temperature was 23.5°C (ambient temp).



Figure 1: Fluoroskan Ascent

Results and Discussion

SYBR Green

SYBR Green was used to successfully differentiate *Phytophthora ramorum* from all other species in endpoint following PCR amplification (Figure 2a). Fluorescence of *P. ramorum* was significantly higher than that of the negative control and non-targeted species. No non-specific amplification was observed on gel. This is significant since the emission of fluorescence occurs when the SYBR Green inserts in double stranded DNA in a non-specific manner.

Taqman

Taqman probes are widely used in real-time PCR. However, we show here that they can also be used in endpoint in a lower cost high

throughput application (Figure 2b). During amplification, the fluorochrome on the Taqman probe is cleaved and released by the polymerase during extension, thereby separating the fluorochrome from the quencher. The accumulation of fluorescence can be easily detected in endpoint with the Fluoroskan Ascent. A 6.38 or 3.48 RFUs higher fluorescence can be seen in the *P. ramorum* samples compared with the negative control. Some low levels of fluorescence can be detected with the other *Phytophthora* spp.

Molecular Beacons

Molecular beacons work on the same basic principle as Taqman, except that instead of being accumulated, fluorescence is 'read' in real time once every cycle. When the probe hybridizes with its complement, it opens and distances the quencher from the fluorochrome, thereby emitting fluorescence. It is possible to detect the molecular beacon pairing with the target in endpoint (Figure 2c) following PCR. However, it is necessary to denature and hybridize the molecule prior to reading the fluorescence. The fluorescence emission of the molecular beacon with *P. ramorum* was several times higher than that of the control and non-targets yielding a highly specific assay.

Conclusion

We have shown that it is possible to have a present/absent PCR diagnostic operating at a lower cost by combining the use of fluorescence detection with endpoint plate reading. SYBR

Green assays are easy to set up but can yield to unspecific detection of double-stranded DNA, such as primer dimers. Internal reporter probes, such as molecular beacons and Taqman, give a double redundancy in the assay by generating a signal only when the target DNA has been amplified.

These two techniques showed a high signal-noise ratio. Because the Fluoroskan Ascent can read several plates in minutes, a number of thermal cyclers could be combined for more affordable high throughput assays. These methods can be useful in operational settings in diagnostic labs where high throughput and lower costs are important aspects.

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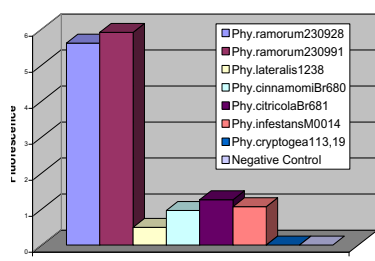


Figure 2a. SYBR Green detection in endpoint reading

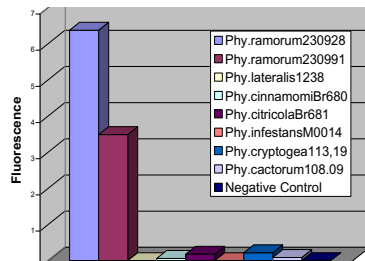


Figure 2b: Taqman PCR reaction read in endpoint

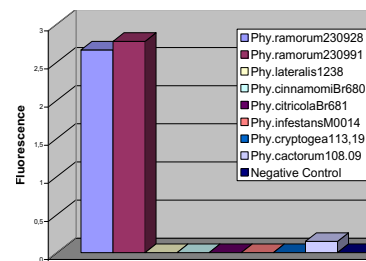


Figure 2c: Molecular beacons read in endpoint