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Using Real-Time PCR for Pathogen Detection

Technical Note: Bioinformatics for Use in the Design of TaqMan-based Assays

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Traditionally, scientists and clinicians have used assays that involve growing cultures to screen samples for the presence of pathogenic microorganisms. Today, culture-based methods for pathogen detection are rapidly being replaced by faster and more specific Realtime PCR assays that discriminate between microorganisms based on a signal from specific nucleic acid sequences.

Applied Biosystems (Part of **Life Technologies**, Carlsbad, CA) developed a program for designing these same kinds of assays for the detection of pathogenic microbial sequences. These pathogen detection assays can be designed using the same technology that is currently being used for the company's commercially available gene expression assays, which include predesigned expression assays for human, mouse, rat, *Arabidopsis*, and *Drosophila* genes.

The Applied Biosystems' bioinformatics assay design program for producing TaqMan®-based real-time PCR assays for pathogen detection is described here, as well as applications of these assays for biosecurity programs.

In collaboration with **Tetracore**, a biotechnology company based in Gaithersburg, MD, Applied Biosystems used this software tool to produce realtime PCR assays for U.S. Department of Defense biodefense applications.

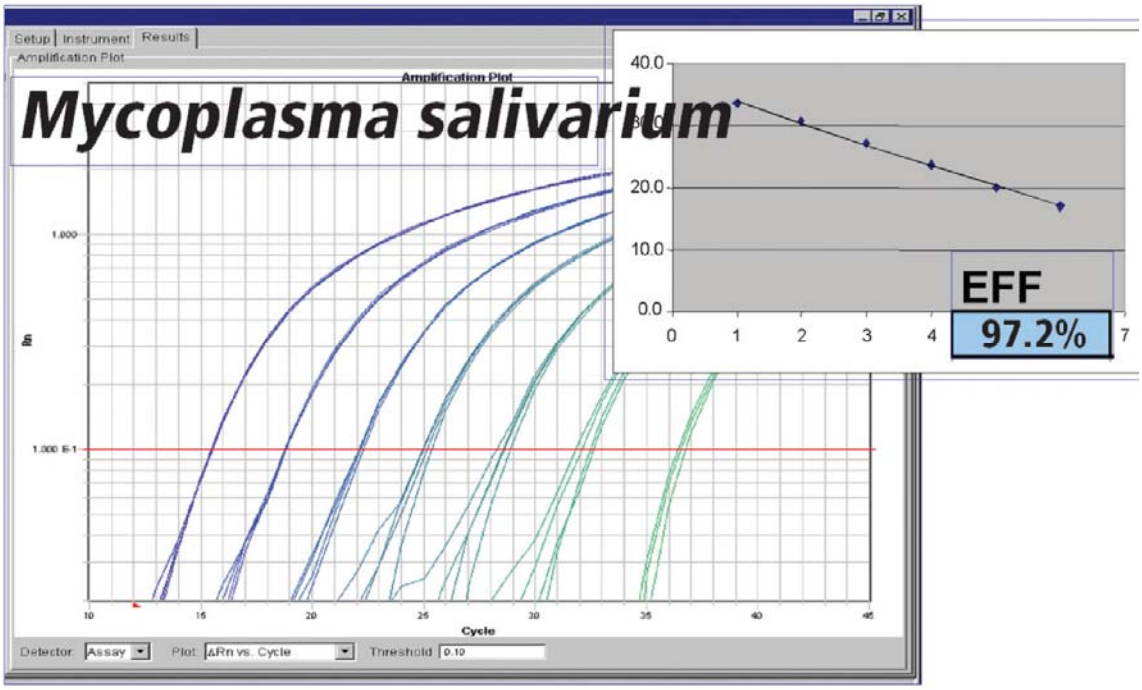

Figure 1. The graph shows an efficiency test for Mycoplasma salivarium. Efficiency is close to 100%.

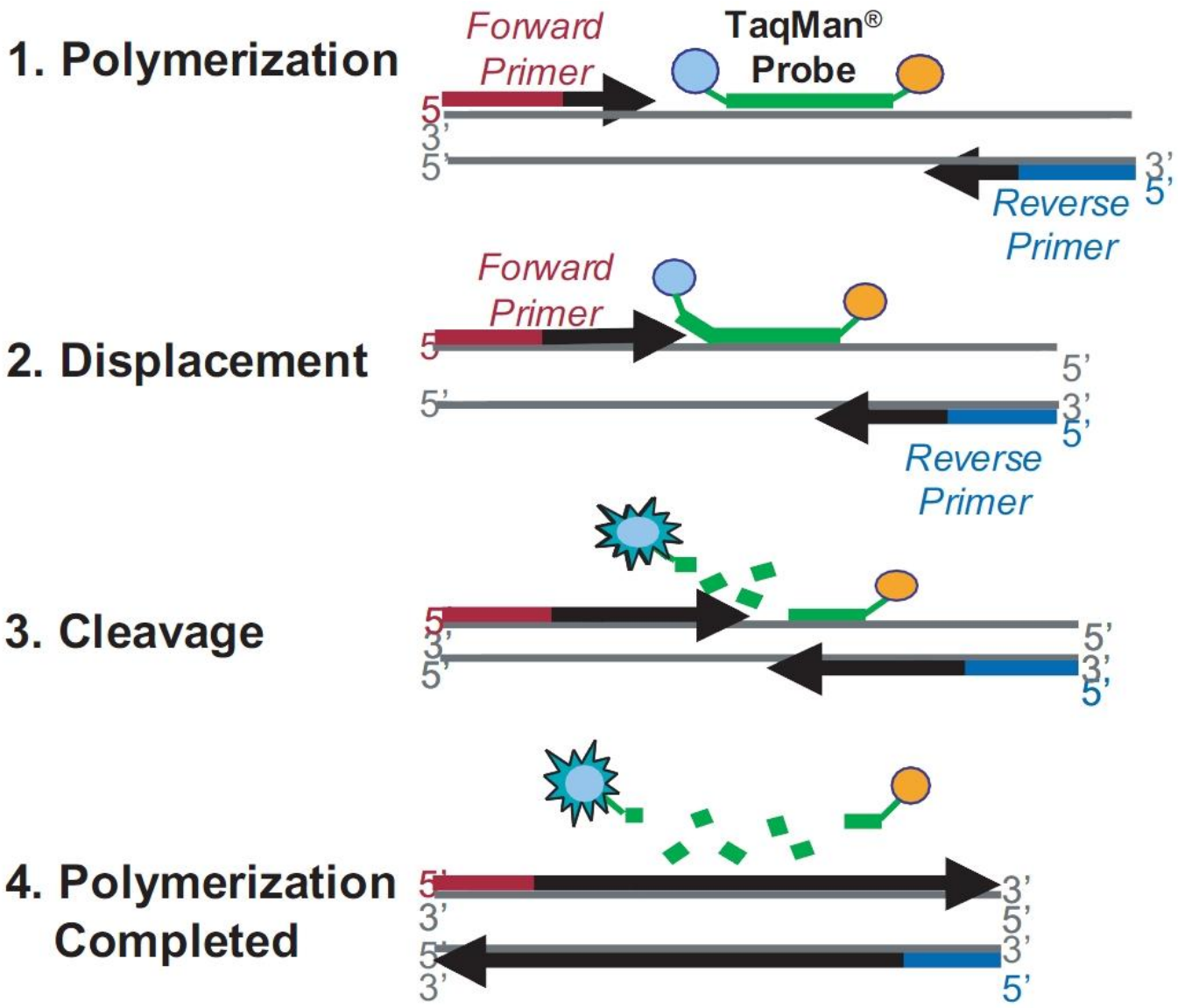

Figure 2. TaqMan® Probe-based Assay Chemistry. [1]The assay probe carries a fluorogenic reporter dye at its 5' end, and a quencher at its 3' end. The quencher absorbs the fluorescence emission of the reporter while the two are joined in close proximity to each other as part of the probe. [2] [3] During each cycle of PCR, the probe hybridizes to the PCR products to which it is targeted, and the 5' terminal reporter dye is then cleaved through the 5'-to-3' nuclease activity of Taq DNA polymerase as it copies the complementary strand. [4] The physical separation of the reporter and quencher dyes results in an increase in fluorescent signal that is proportional to the amount of amplification product that is generated in the reaction mixture As the 5' nuclease activity of Taq DNA polymerase is double-strand specific, all unbound probe remains intact. Consequently, if non-specific PCR product is amplified during the reaction, the unbound TaqMan probe remains uncleaved, resulting in an absence of reporter fluorescence.[2]

Applied Biosystems also developed and manufactured reagents for real-time PCR assays aimed at detecting the presence of potentially harmful pathogens in mail sorting facilities.

Assay Design Software Program

Applied Biosystems developed a software program to assist scientists in the design of primer and probe combinations for real-time PCR pathogen detection assays. A proprietary algorithm applies a set of rules for design of the assay, and recommends candidate primer and probe sequences that target a signature nucleic acid sequence in a microbe of interest.

The algorithm evaluates a set of optimal assays, considering criteria of melting temperature and nucleotide composition of primer-probe combinations. The algorithm then selects the assay with the highest specificity, based on nucleic acid sequence comparisons of assay primers and probes with genomic sequences from other closely related bacterial species.

Based on this sequence comparison, a proprietary TaqMan assay scoring algorithm selects an assay with the highest mismatch scores in other bacteria. This minimizes the possibility that an assay will be selected that generates a false-positive signal. The design algorithm consistently generates assays that have near 100% efficiency.[1]

The Applied Biosystems assay design software searches primers for all known target microbial sequences and optimizes selection of primer pairs based on hybridization patterns of the two primers to the intended target sequence.

Unlike many assay design software programs that produce a list of numerous candidate assays, the Applied Biosystems program eliminates many of the potential primer-probe combinations, resulting in a lower number of candidate primers and probes that need to be evaluated for effectiveness.

As an example, we have demonstrated a design of highly sensitive and specific real-time PCR assays against closely related species: *Streptococcus pneumoniae, Mycoplasma pneumoniae, M. orale* y *M. salivarium* (Figure 1).[1]

TaqMan Real-Time PCR Pathogen Detection Assays

TaqMan real-time PCR pathogen detection assays amplify target nucleic acid sequences from select microbes present in samples collected from complex biological environments. Specific amplification of target sequences is directed by custom designed primers and probes.

The first degree of specificity is achieved by the combination of amplification primer sequences. An additional degree of specificity results from a probe that hybridizes to a region of nucleic acid sequence that identifies the microbe of interest (Figure 2).

The real-time PCR pathogen detection assays are automated by the Applied Biosystems family of real-time PCR systems, all of which are capable of simultaneously analyzing at least 96 and as many as 384 sealed samples.

As PCR amplification proceeds, fluorescence is excited by laser or halogen light and is detected by a charge-coupled device (CCD) camera. Because the probe does not inhibit the PCR reaction, these systems give a linear response to template concentration over at least five orders of magnitude.

Specificity and Sensitivity

Target sequences that confer specificity to pathogenic microorganisms are found within the portion of the microbe's genome that encodes their virulent agents. The genes or a portion of the genes that contribute to the disease phenotype of the pathogen distinguish a target microbe from its nearest neighbors.

For instance, for *Bacillus anthracis*, the genetic components responsible for the Anthrax disease phenotype distinguish its genome from that of closely related *Bacillus* species.

In addition to specificity, the assay limit of detection (LOD) greatly impacts its ability to detect a target sequence for a microbe of interest. In many cases, there is only a small amount of target sequence present in a sample.

Traditionally, microbiologists have used colony forming units (CFUs) expressed as CFUs per milliliter to quantify the sensitivity of a culture-based pathogen detection assay. For real-time PCR assays, sensitivity refers to the number of copies of a target sequence that must be present in a sample in order for the assay to positively identify the presence of the pathogenic microbe.

TaqMan real-time PCR assays routinely detect as few as 10 copies of target sequence present in a sample, and, in some cases, have been known to detect one copy of target sequence.

Real-time PCR assays generate results in much less time than culture-based assays. While culture-based detection methods often require days to obtain a result, the TaqMan-based real-time PCR assays generate accurate results in a matter of hours.

Although the TaqMan real-time PCR assays are more specific and more sensitive than culture-based assays, they cannot be used to determine the viability of a pathogenic microorganism. If a microbe is detected using culture-based assays, it is viable.

Alternatively, TaqMan-based assays can only be used to determine if a particular pathogenic microbe is present or absent from a sample. Because the assay identifies the presence of microbe based on detection of a signature nucleic acid sequence, that sequence may be intact, but the organism containing the sequence may be dead.

However, usually applications of pathogen detection assays are used to investigate if a pathogenic organism is present in a sample or not. Culture-based techniques remain the best method to assess viability.

Biosecurity Applications

TaqMan-based real-time PCR pathogen detection assays are currently being used in several different biosecurity applications. For instance, assays have been designed for detection of non-pathogenic surrogates in dispersion analysis of biothreat organisms.

In dispersion analysis, researchers release surrogates of biothreat microbes or benign microorganisms and model how the environment influences the distribution of released biological agents. Strategically placed collection devices are used to evaluate how a cloud filled with surrogate microbes disperses in that area.

The key to successful dispersion analysis is the reliable detection of non-pathogenic surrogate microorganisms. Surrogate organisms are non-pathogenic species selected to reflect characteristics of disease causing agents.

For example, *B. atrophaeus*, which is a gram-positive spore former, acts in a manner very similar to *B. anthracis*, environmentally, so it provides a model for how the virulent version of the microbe that causes Anthrax would behave.

Highly sensitive assays capable of accurate quantification that are also highly specific for the target of interest are necessary to correctly model an actual release event.

Applied Biosystems developed highly specific real-time PCR assays for detection of three nonpathogenic surrogate organisms: *B. atrophaeus* (var. globigii), *Pantoea agglomerans* (formerly *Erwinia herbicola*), and the RNA coliphage MS2 are employed as nonpathogenic surrogates for *B. anthracis* (anthrax), *Yersinia pestis* (plague), and *Variola major* (smallpox), respectively.

Real-time PCR pathogen detection assays are also being applied to the field of microbial forensics, where pathogens detected at one location, such as crime scene, can be traced to a source, such as the location where the germ was first released.

Detecting Microbial Pathogens in Complex Environments

For pathogen detection applications, such as those for biosecurity programs, scientists often need to identify a single microbial species in samples collected from complex biological environments such as air, soil, or water.

Unlike assays that detect specific human DNA sequences present in samples, where the template available for the PCR reaction is human DNA, real-time PCR pathogen detection assays must account for the presence of genetic material from multiple microbial species in a single sample.

To identify a single target microbe from among a spectra of microbial species present in a sample requires an assay capable of discriminating between nucleic acid sequences from the species of interest and those from the species that represent the nearest evolutionary neighbors of the target species.

The key to detecting a target microbial pathogen amidst closely related neighbor species is selecting the optimum target sequence within the pathogen of interest. This target sequence forms the template from which to design primers and a probe of complementary sequence.

While scientists generally select target sequences within genes that are widely represented in all microorganisms, this decision is complicated in that most microbial sequence is unknown, or has not yet been deciphered. In target selection, assay designers need to identify a highly specific target sequence in a gene or portion of a gene that uniquely identifies the species of interest.

For primer and probe design, specificity can be defined as a measure of how well an assay will be able to distinguish a single species from its nearest evolutionary neighbors.

In this definition, specificity implies two properties: inclusion, which implies the assay will detect all strains of the target species; and exclusion, which implies the assay will not detect near neighbor species.

Summary

Applied Biosystems has developed a bioinformatics design program for generating highly efficient and specific Taq-Man-based real-time PCR assays. These assays provide fast and accurate detection of potentially pathogenic microorganisms. The program includes a software tool for designing optimum primer-probe combinations that target signature nucleic acid sequences in microbes of interest.

Real-time PCR pathogen detection assays are currently being used for biosecurity applications that include dispersion analysis of surrogate pathogens and detecting the presence of potentially harmful pathogens in highrisk environments.

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