WHITE PAPER

Molecular Technologies Commonly Used in Food Pathogen Detection

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The ability to detect pathogenic microbes accurately and rapidly is one of the key requirements in the production of safe food. Several different technologies are used for pathogen detection, and they are classified based on the biological principles used in each method. Conventional methods utilize the differences between the metabolism and growth conditions of various microbes, and pathogens are identified through the use of specific culture media and biochemical tests. More recently, immunological and molecular methods have been developed for more rapid pathogen detection.

Immunological methods are based on the binding of specific antibody proteins to corresponding antigens. In microbial diagnostics, the antigens are typically proteins residing on the surface of the microbial cells or on viral capsids.

The term "Molecular methods" is generally applied to a group of technologies that are based on the detection and analysis of DNA, or sometimes RNA. In order to detect and study DNA, the genetic material is first amplified, either through Polymerase Chain Reaction (PCR), which is the most commonly used method, or through isothermal amplification methods. In different commercial products developed for food pathogen detection, several variations of the basic DNA amplification principles have been applied. An overview of these methods is presented below.

PCR

The most commonly used application of PCR within the diagnostics field is real-time PCR. As opposed to end-point PCR, the amplification of DNA is monitored during each temperature cycle of the PCR, and a DNA amplification curve is produced at the end of the reaction to verify the presence of DNA of interest in the reaction, without the need to open the PCR tubes for analysis. This reduces the risk of amplified DNA contaminating the testing laboratory.

Fluorescent dyes are used in real-time PCR to detect DNA amplification. The fluorescent dye can either be a DNA-binding dye, such as Applied Biosystems™ SYBR™ Green, which binds to all double-stranded DNA, or the dye can be incorporated into a specific DNA probe. Probe-based detection yields better detection specificity, as the amplification signal is only detected if the specific target DNA is present in the sample. More details about the two detection principles are presented below.

SYBR Green–based detection

SYBR Green is a fluorescent dye that binds to double-stranded DNA. The resulting DNA-dye-complex produces green light when excited by a light source in the real-time PCR instrument. SYBR Green technology is relatively inexpensive to use, but has the downside of being non-specific: all double-stranded DNA present in the sample will contribute to the fluorescence signal, and if the DNA polymerase occasionally amplifies non-target DNA, this unspecific DNA is detected, and the food pathogen analysis yields a false positive result.

To overcome the specificity issue, a DNA melting curve can be produced after the PCR cycles. In this method, the amplified DNA is slowly heated, and the fluorescence produced by SYBR Green is monitored. At a specific melting temperature, the two strands of the DNA separate, the SYBR Green dye is released, and the fluorescence signal decreases. As the melting temperature is dictated by the sequence of the amplified DNA, it can be used to verify if the fluorescence signal seen in the amplification curve was produced by the amplification of the target DNA. This additional melting step increases the level of specificity in SYBR Green detection; however, it has the disadvantage of increasing the time needed for the analysis. The DNA melting step can add up to 30-45 minutes onto the PCR time.

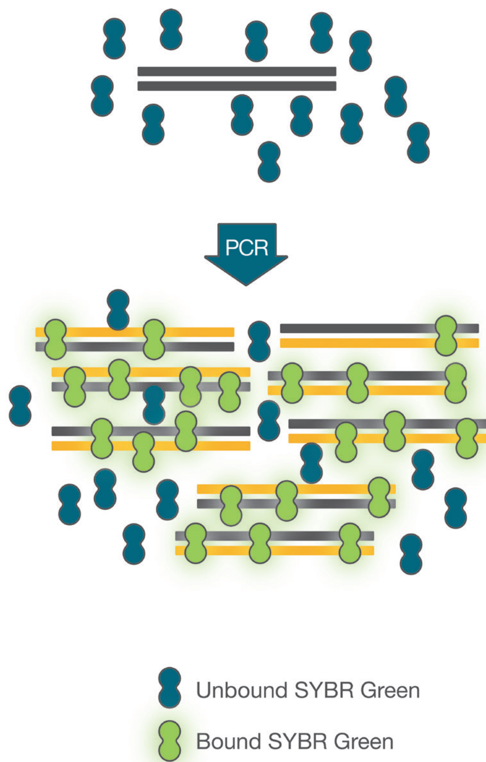


Figure 1. Mechanism for DNA-Binding dye SYBR Green

Probe-based DNA detection: Hydrolysis (TaqMan) probes

Another way of monitoring DNA amplification during PCR is the use of short DNA molecules or probes, that carry a fluorescent reporter molecule. The most widely used probe technology in real-time PCR applications is TaqMan™ chemistry. TaqMan probes contain a fluorogenic molecule or Reporter (fluorophore) at their 5'-end and a Quencher molecule at their 3'-end. When the probe is intact in the solution, the Quencher catches the fluorescence produced by the Reporter and quenches the signal. During the PCR amplification step, however, the probe binds to its specific location on the target DNA, and the DNA polymerase digests the probe. As the Quencher is no longer in close proximity to the Reporter, a fluorescence signal is produced and can be detected[1].

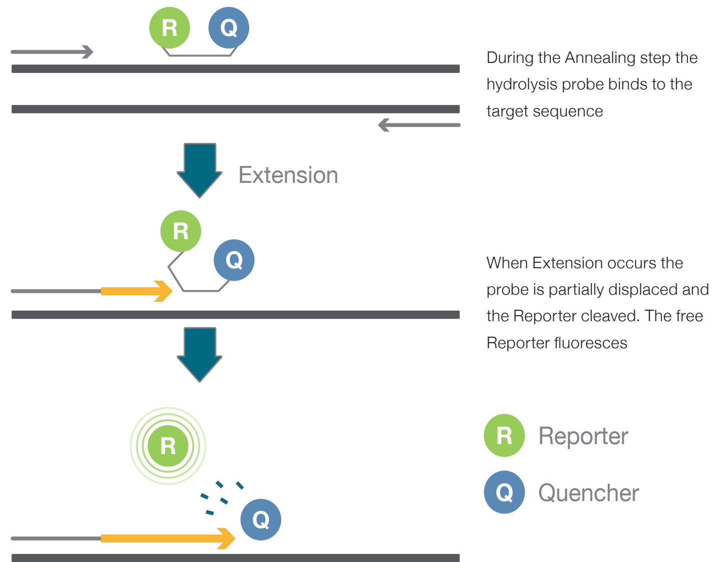


Figure 2. Principle of TaqMan probe–based real-time PCR

TaqMan probes can have excellent detection sensitivity and specificity. The simplicity of the detection mechanism also makes it easy to apply TaqMan probes to multiplex PCR where several DNA targets are detected in one PCR tube if different fluorophores are used. This also facilitates the inclusion of an internal synthetic amplification control into each PCR assay. The Internal Amplification Control (IAC) uses a specific probe labeled with a different fluorophore, and this fluorescence signal verifies that the amplification reaction was successful. Without an IAC present in each reaction, interpretation of negative results in any method based on amplification of DNA is not fully reliable.

Other real-time PCR probe technologies

Examples of other real-time PCR probe chemistries used in food pathogen detection include molecular beacon probes, Solaris™ probes, Scorpions™ probes, and FRET probe pairs.

Molecular beacon assays consist of a fluorescently labelled probe for a target sequence called a molecular beacon. It forms a hairpin structure with a stem and a loop. The 5' and 3' ends of the probe have complementary sequences of 5–6 nucleotides that form the stem structure. The loop portion of the hairpin is designed to specifically hybridize to a 15–30 nucleotide section of the target sequence. A fluorescent Reporter molecule is attached to the 5'-end of

the molecular beacon, and a Quencher is attached to the 3'-end. Formation of the hairpin brings the Reporter and Quencher together, so no fluorescence is emitted.

Molecular beacons are probes with a Reporter and Quencher at either end. In their hairpin structure the Reporter and Quencher are in close proximity

When Annealing occurs during PCR the hairpin structure binds to the target DNA separating by distances the Reporter and Quencher enabling the Reporter to fluoresce

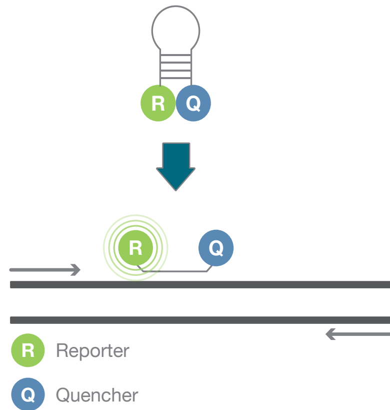


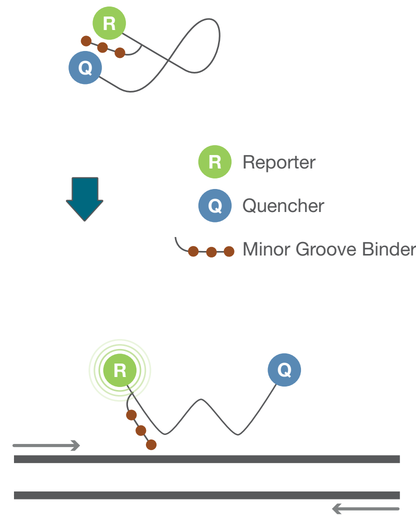
Figure 3. Mechanism of action of molecular beacon probes

During the annealing step of the amplification reaction, the loop portion of the molecular beacon binds to its target sequence, causing the stem to denature separating the Reporter and Quencher so that the quenching is abolished and the Reporter fluorescence is then detectable. As the fluorescence is emitted from the probe only when it is bound to the target, the amount of fluorescence detected is proportional to the amount of target in the reaction.

Molecular beacons have some advantages over other probe chemistries. They are highly specific, can be used for multiplexing, and if the target sequence does not match the beacon sequence exactly, hybridization and fluorescence will not occur. Unlike hydrolysis (TaqMan) assays, molecular beacons are displaced but not degraded during amplification. The main drawback of molecular beacons is that they are difficult to design. The stem of the hairpin must be strong enough that the molecule will not spontaneously fold into a random structure and result in unintended fluorescence. At the same time the stem of the hairpin must not be overly strong or the beacon may not properly hybridize to the target sequence.

Solaris probes like molecular beacon probes, share a very similar design and working mechanism with TaqMan probes; like molecular beacons, Solaris probes are not cleaved by the DNA polymerase during the PCR. The probe emits fluorescence when bound to the target DNA and is very effectively quenched when no target DNA is present.

Solaris probes are based on Pleiades probe chemistry. The unique feature of this probe chemistry is the presence of a Minor Groove Binder (MGB™) molecule adjacent to the fluorophore at the 5'-end of the probe[2]. The MGB increases the binding efficiency of the probe, prevents the degradation of the probe by the DNA polymerase, and enhances the fluorescence quenching in an unbound probe[2]. These features together make Solaris probes very specific.



Solaris probes possess a Reporter and Quencher molecule at either end which are closely positioned in the unbound state meaning fluorescence is quenched. The Minor Groove Binder (MGB) molecule increases the quenching efficiency

During Annealing the MGB increases the binding efficiency of the probe and prevents degradation of the probe during the Extension phase

Figure 4. Mechanism of action of Solaris probes

In contrast to linear TaqMan, molecular beacon probes and Solaris probes, Scorpions probes are designed as DNA hairpins, where the probe sequence is linked to one of the primers, and the molecule forms a stem-loop configuration[3]. This unimolecular probe structure enables very fast reaction kinetics in the PCR, if other components of the reaction mixture and the instrument are also carefully optimized, but at the same time, the hairpin structure complicates assay design and probe synthesis.

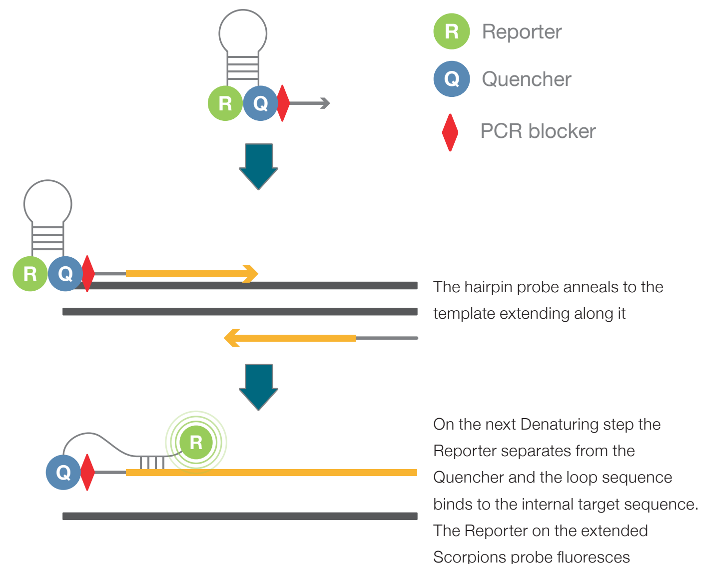


Figure 5. Mechanism of action of Scorpions probes

Dual hybridization probes, often referred to as FRET probe pairs, are always designed as a two-probe system, where both probes carry different fluorescent dyes. The probe pair is designed to bind to target DNA in close proximity, and the fluorescence resonance energy transfer (FRET) between the two dyes on both probes produces a detectable fluorescence signal[4]. The need to design and use two probes with different fluorescent dyes for each target DNA makes assay design more challenging and decreases the possibilities to apply this technology in multiplex PCR. In theory, the possibility to multiplex for at least two targets, in the case of a four channel instrument, should be achievable as the donor probe can have the same dye for several acceptor probes. In practice, if the donor probes and acceptor probes are at opposite ends of the light spectrum the light emitted from the donor probe is not at the correct wavelength to efficiently excite the acceptor probe. For this reason, some methods use a similar melting temperature analysis with FRET probes as was described for SYBR Green above to achieve a multiple target reaction. The specificity is at a similar level to other probe systems, but the fluorescence signal level is often lower with FRET probes than with TaqMan probes which means achieving the same level of sensitivity can be a challenge.

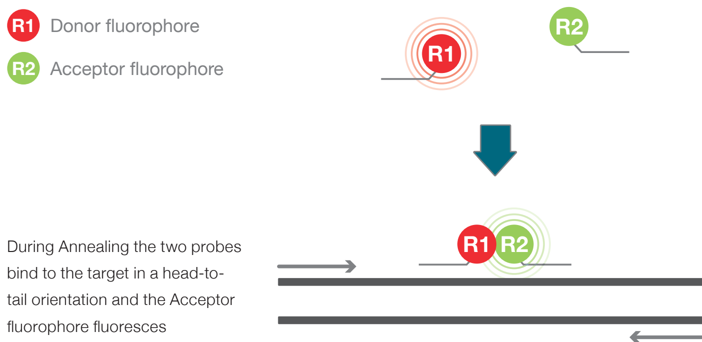


Figure 6. Mechanism of action of dual hybridization probes

Isothermal amplification methods used in food pathogen detection systems

Real-time PCR is the most widely used molecular method in molecular diagnostics. The underlying principle of enzymatic amplification reaction is relatively straightforward in PCR: alternating temperatures and thermostable DNA polymerases are used to amplify DNA. Recently, other methods for amplifying DNA have been developed for diagnostic platforms. These isothermal methods do not use alternating temperatures during the amplification reaction, rather than rely on more complicated enzyme or primer cascade reactions to separate the DNA strands and generate multiple copies of the original target DNA.

The benefit of isothermal reactions is the ability to use relatively simple and fast detection instrumentation with low capital costs, as the instruments do not need to be capable of accurate and precise temperature cycling. On the other hand, the biochemical reactions needed to produce DNA amplification isothermally are much more complicated than those in PCR. Multiplexing is typically very challenging with isothermal systems, hence, IACs can only very rarely be used in the reactions to validate negative results.

Loop-mediated isothermal amplification

Loop-mediated isothermal amplification (L-AMP) was one of the first isothermal amplification methods in commercial use. Target DNA is amplified through the use of multiple primers and a DNA polymerase that has a strong strand displacement activity, i.e. ability to separate two annealed DNA strands. Primers bind onto the DNA and form loop structures to facilitate several rounds of amplification[5]. Detection of the amplification can occur through various chemical reactions, one example being a bioluminescence reaction. It utilizes a two-step enzymatic process to produce light from the molecules released during the DNA amplification. The need to add multiple primers for each target organism, complicated DNA secondary structures formed during the amplification reaction, and the use of several moderately stable enzymes in the amplification and detection increase the complexity of these reaction designs significantly. Therefore, multiplexing is very difficult, and IACs cannot be used. The L-AMP reactions may also often be more prone to unspecific cross-reactions with non-target DNA than PCR due to the presence of multiple primers and low primer binding temperatures.

Summary

	Molecular Technology					
		Probe-based PCR				
	Fluorescent Dye PCR	TaqMan	Molecular Beacon (Solaris)	Scorpions	Dual Hybridization (FRET)	L-AMP
Fast processing & analysis	+ (requires melt-curve)	++	++	++	+/++ (requires melt-curve)	+++
Relative cost + low +++ high	++	+++	+++	+++	+++	+
Design scalability (Multiplexing)	++ (with melt-curve)	+++	+++	+++	++	No
Suitability for inclusion of IAC	Yes (with melt-curve)	Yes	Yes	Yes	Yes (with melt-curve)	No

Table 1. Comparison of molecular detection technologies for practical applications

Conclusion

The last ten to fifteen years have seen considerable up-take of molecular methods in food safety testing laboratories with around 15% of all pathogen tests being carried out using a DNA-based technique today, rising to a projected 18% by 2018[6]. The faster time to result and potential for improved accuracy can mean considerable benefits to a business that is able to release product to market sooner or take faster interventions and corrective actions. Given the significant burden for the laboratory when implementing a change in method it is worth taking time to consider the most appropriate and future-proof system to adopt. Technologies such as L-AMP and SYBR Green PCR may present a cheaper option but could be presenting a false economy when lower accuracy and/or delayed time in getting an actionable result are considered.

Another area for consideration is the instrument that will be used to run the chosen molecular tests. Some systems are closed meaning that only a narrow number of tests may be run on them. Exactly which tests could be determined by either the limitations of the instrument and its software as well as the particular technology or by the supplier of that instrument locking down the system for their own tests. An open instrument, in combination with a technology that allows for multiplexing, inclusion of an IAC, rapid cycling times and the possibility to scale-up test throughput could present the most attractive investment for a laboratory and provides the possibility to run molecular tests for other targets whether that be using assays from the same supplier, an alternative supplier or using in-house developed methods.

References

1. Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. Proc Natl Acad Sci U S A. 1991; 88(16):7276–7280
2. Lukhtanov EA, Lokhov SG, Gorn VV, Podyminogin MA, Mahoney W. Novel DNA probes with low background and high hybridization-triggered fluorescence. Nucleic Acids Research 2007; 35(5):e30.
3. Whitcombe D, Theaker J, Guy SP, Brown T, Little S. Detection of PCR products using self-probing amplicons and fluorescence. Nature Biotechnology 1999; 17:804-807.
4. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 1997; 22:130-139.
5. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research 2000; 28(12):e63.
6. Strategic Consulting, Inc. Food Micro, 8th Edition – Microbiology Testing in the Global Food Industry. Published June 2013

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