PCR: design delivers success

Polymerase Chain Reaction (PCR) has gained wide acceptance as a pathogen detection tool in the food industry and the popularity of the method continues to grow. Two choices in PCR technology are available for the industry to choose from: end-point and real-time PCR (RT-PCR).

In end-point PCR, primers or short oligonucleotides are designed to flank the target sequence which is amplified during the PCR reaction. The amount of PCR product (amplicon) produced during the PCR is detected using a non-specific double strand DNA (dsDNA) binding dye.

Traditionally, the amplicon was visualised using agarose gel electrophoresis. But fluorescent dyes such as SYBR Green are now used to measure the accumulated PCR product at the end of PCR without the need to run a gel and thus keeping the system closed. Typically, this is achieved by performing a heating step during which the double stranded (ds)DNA comes apart releasing the fluorescent dye and consequently resulting in a decrease in fluorescent signal. The amount and temperature at which the fluorescent signal changes corresponds to the strength and type of PCR product generated during the PCR event.

In comparison to end-point PCR, real-time PCR (RT-PCR) detects the increasing amount of amplicon in real-time after each PCR cycle by measuring the fluorescence signal. The changes in fluorescence intensity with each PCR cycle are monitored in an amplification plot, from which the cycle threshold (Ct) is calculated. The Ct value, also referred to as the crossing point (Cp), is inversely correlated to the amount of DNA present in the sample, and is used to measure the amount of amplicon produced in real-time. This measurement can be used for absolute quantification through a standard curve or relative quantification by comparison with a known amount of a reference target.

The fluorescent signal in RT-PCR is generated by a fluorescently labelled, target-specific DNA probe which is included in addition to the PCR primers. A quencher present in the probe suppresses the fluorescence in the absence of the target sequence, but is released when the target is present. RT-PCR assays provide an option of multiplexing to include many targets in a single assay. Probes for different targets are designed with reporter dyes that emit fluorescence at different emission wavelengths. Together with a unique set of primers, amplicons generated for each target emit a unique fluorescent signal.

The advantages of RT-PCR are added specificity due to the probe and the ability to multiplex. However, these advantages should be weighed against the complexity of probe design and the cost of the probe, which is expensive due to the fluorescent reporter dye and quencher. On the other hand, end-point PCR affords a cheaper PCR option and simpler design with more reliance on the primers for specificity.

Regardless of the PCR technology selected, the key to the success of a PCR assay in food testing lies in its design and its validation. For an end-point assay, the primers should be carefully designed to be selective for the target and to meet inclusivity/exclusivity criteria. For an RT-PCR assay, in addition to the primers, the design of the probe is critical to the selectivity of the assay. For both assays, initial primers/probe design is done by using bioinformatics, followed by wet laboratory testing with bacterial strains to confirm specificity and selectivity.

Furthermore, to achieve robustness, it is imperative to optimise amounts of other critical PCR components including DNA polymerase, nucleotides and salts. The addition of an internal positive control as an indicator of assay performance provides increased reliability and confidence in results. Finally, validating the performance of the PCR chemistry on a wide range of appropriate matrices is essential to ensure the success of the assay and monitoring food safety!

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Designing robustness into PCR tests

One of the most decisive factors for the commercial success of any PCR assay for microbial screening in the food safety market is the accuracy and reliability of the results. This involves much more than designing the specificity of the detection system. Precision is achieved by performing inclusivity and exclusivity studies that detect the desired target and exclude the rest without compromising on sensitivity of detection.

The testing should be validated using multiple manufacturing lots of assay components to ensure reproducibility with the supply of materials. Once the target PCR chemistry has been achieved, it is further developed to meet the practical requirements of the industry.

The most challenging aspect for detection of microbial pathogens in food is the food matrix itself where the requirement is to detect 1 viable organism per analytical unit (e.g. from 25g to 375g).

No analytical method is currently capable of this without an enrichment procedure during which the target organism grows to a detectable level. It imperative to develop an accurate detection method that must perform consistently on a varied range of food matrices some of which may have specific enrichment requirements.

Subsequent procedures are then required to transfer and prepare the enriched sample for analysis that achieves the limit of detection (LOD) for the overall assay.

A procedure to lysis of target cells in the enriched sample is required to release DNA which serves as the template for amplification in the PCR reaction. These procedures need to be simple and easy to use to facilitate the high testing volumes in food testing laboratories.

The complexity and number of potential variables in food testing requires meticulous and stringent testing of the PCR chemistry during development and beyond. The desirable LOD in food matrices is verified through post enrichment spike studies with known levels of target cells.

Field trial studies with collaborative laboratories and customers provide the opportunity to determine 'real-world' performance and ease of use of the overall assay to the end consumer. It is also critical to incorporate as many known failure modes into the development of a product as possible to ensure that the overall performance is minimally compromised in the event of any deviation from recommended procedure.

The deviations range between studies examining failures during manufacturing processes to common customer errors. These studies are important in determining the effects of innate variables such as supply of test components that could affect the overall product. Data from development activities are used to develop and validate the final output.

An essential requirement for the food testing market is that results should be easy to interpret and permit quick decisions to be made with confidence. This is achieved by the development of analysis algorithms to generate a definitive output. A well designed screening method must incorporate data interpretation into the design process.

Test methods based on molecular biology have great specificity but many other variables need to be built into each stage of the procedure to deliver a robust and reliable assay.

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PCR: instrumentation & data interpretation

Ascertaining the presence of a micro-organism in a sample by analysing the data output of a polymerase chain reaction (PCR) is a highly complex mathematical and computational task. Nevertheless, the analysis must be robust, reliable, fast, and provide the user with simple and clear results.

Current PCR technologies, both end-point and real-time, rely on fluorescent dyes to detect the amplification of targeted DNA. PCR instruments use an array of filters, lenses, prisms, and charge-coupled devices (CCD) to measure the luminescence of these dyes.

End-point and real-time PCR require different data analysis methods. In end-point PCR, fluorescence is emitted when dye binds to the many copies of double-stranded DNA produced during the reaction. The objective of the algorithm in end-point PCR is to detect rapid reductions in luminescence levels during a 'melt' stage at the 'end' of PCR.

As the temperature of the amplified PCR product is increased, the double-stranded DNA chain comes apart, resulting in a decrease of the fluorescent signal. Each amplification product in an end-point assay has a characteristic melt curve, allowing differentiation of multiple products within a single assay.

On the other hand, in real-time PCR, the amplification of DNA is tracked throughout the PCR cycles by measuring the increasing fluorescent signal as the reaction progresses. In this instance, the algorithm attempts to fit an amplification curve with a known model. The resulting parameters of the fit are used to determine the presence of targeted micro-organisms in the sample and to calculate metrics such as the threshold cycle (Ct), which identifies the cycle number at which fluorescent signal reaches the detection threshold.

Another complexity of real-time PCR is the presence of multiple signals in a single reaction due to multiplexing of several dyes, each one with its own characteristic feature. The algorithm must be robust enough to draw insights from multiple dye signals.

The profiles of the melt curves (reduction in fluorescence in end-point PCR) and the amplification curves (increase in fluorescence in real-time PCR) are extremely sensitive to factors such as the organism being detected, dye concentrations used, food matrices, and the data collection process. Due to this sensitivity, the signal processing methods and data analysis algorithms must be thoroughly tested to ensure the reliable interpretation of the raw data is made. As new data is generated, the analysis algorithms are refined to consider unexpected and previously untested scenarios in future software releases. It is also important to test and calibrate the data analysis algorithms using several PCR devices to account for any instrumental variability. Throughout the refinement process, the internal positive control (IPC) reaction that exhibits a good degree of consistency under all conditions serves as a standard to evaluate assay performance and to ensure conformity.

Regardless of the complexity of the data analysis algorithms, the software should make data interpretation simple for the user. The software should also provide a user-friendly interface that allows for the selection of PCR parameters appropriate for the assay such as target species, concentration thresholds, etc. The algorithms should be designed to run in the background and to provide the user with straightforward results.

Software that provides the user with a simple symbolic qualitative result for each sample would facilitate data interpretation with minimal training. The results could be simple positive (+) or negative (-) icons that indicate the presence or absence, respectively, of the target organism.

The results could also be quantitative, such as plots of the melt and amplification curves, and if applicable, display values of the organism concentrations in the sample (in CFU/ml) and of the threshold cycle (Ct).

The composition of the sample matrix has a significant effect on the result and data interpretation.

Different foods and ingredients as well as increasing sample size cause continuing challenges for PCR detection methods.

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Challenges in detecting foodborne pathogens

Foodborne illness is primarily caused by pathogenic micro-organisms that inadvertently enter the food supply. The United States Centers for Disease Control and Prevention (CDC) estimates an average of 48 million people get sick every year from consuming contaminated foods or beverages. Therefore, the safety and quality of food relies on the ability to detect these contaminating pathogens before product is released into commerce.

Rapid pathogen detection technologies such as the polymerase chain reaction (PCR) are available to reduce this disease burden; however, food analysis has many inherent difficulties.

Pathogens are often present at low levels

Regulatory testing of foods requires test kit manufacturers of pathogen detection methods to achieve a limit of detection (LOD) close to 1 cell per analytical test portion since pathogens are often found at low levels. To detect such a low contamination level, the food matrix being tested is added to a specialised media and incubated for several hours. During this enrichment step, the bacterial population will multiply in the medium leading to an increase in the number of cells to a detectable level. The enrichment procedure significantly increases the chance of detecting the pathogen in subsequent steps allowing PCR based methods to achieve a high sensitivity. Additionally, the enrichment process enables cells that have been injured during food processing a chance to recover and be detected.

Complex food matrices

The complexity of food matrices can present major challenges during analysis especially when very small volumes of the enrichment is needed for detection. The various physical and chemical compositions of different foods can interfere with the performance of many rapid detection methods in a number of ways. When preparing a food sample for analysis, the intrinsic properties of the food matrix must be considered. This can include pH, fat content, moisture, and levels of competing background flora. Additionally, food matrices often contain inhibitory compounds such as proteinases, polyphenols, calcium and others. If these components and inhibitors are not abated from the sample, even a highly sensitive assay can be inefficient. Sample preparation techniques must be able to prepare the pathogen from the food enrichment for detection.

Differentiation of viable cells

The accurate detection of viable cells is a key factor when investigating a foodborne disease outbreak. Many food processers rely on rapid methods to quickly screen food samples. Food samples can contain cells that have lost viability during processing steps therefore many food processers also rely on traditional culture-based methods which allow for the isolation of the pathogen. Generally, this is not an issue for rapid methods since the enrichment promotes the growth of live bacterial cells rather than dead cells. If high levels of dead cells are a concern, selective chemicals and reagents have been successfully evaluated to remove DNA from non-viable cells.

Technological advancements

Although there are considerable challenges to overcome, it is imperative to analyse food for the presence of contaminating pathogens. There has been much progress made to advance detection methodologies in food and as a result the quality and safety of food has improved.

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Validation and verification of food testing methods are critical success factors in food safety

Most consumers do not realise the number of recalls issued on food products across the globe every day. Often, only those resulting in hospitalisations and death are reported. Recalls can involve foods that are contaminated with a pathogen, allergen, foreign material or have been mislabelled or mishandled.

Recalls are issued by regulatory agencies such as the USDA and FDA in the US, and EFSA and RASFF in the European Union.

Recalls can irreparably damage a food manufacturer's reputation and profitability, opening the door to expensive litigation. To avoid recalls, manufacturers must proactively identify and control potential hazards to ensure the safety and integrity of the food being introduced into commerce. Doing so requires risk assessment, process monitoring, validation testing, documentation and more.

Manufacturers perform food pathogen testing either on site or at a third-party laboratory. They commonly employ a test-and-hold method for high-risk (and often highly perishable) foods. This is a method where food is tested and held in containers ready to be shipped, while waiting for laboratory results to clear the products for distribution to retailers. Every hour these products are held, inventory cost increases while product shelf life decreases. This underscores the need for rapid and accurate food pathogen testing methods.

Deciding on the method that best fits a company's food safety program is not an easy assessment. Certification agencies such as AOAC (US), Health Canada, AFNOR (France) and NORDVAL (Denmark) provide guidance in this decision and allows food manufacturers to sift through various approved food pathogen testing platforms. These agencies require test kit manufacturers to compare their testing methods to known reference methods such as ISO, USDA, FDA and Health Canada Compendium to determine equivalent performance for the food product being tested. The performance of the assay is also assessed for sensitivity, inclusion of relevant bacterial strains, cross reactivity to non-specific strains, stability and ruggedness.

Another question food manufacturers must ask themselves when determining a food pathogen testing method is; which one is the right fit for their specific products? The most common testing methods include traditional culture-based detection methods, EIA (enzyme immunoassay) or ELISA (enzyme linked immuno-sorbent assay) and PCR (polymerase chain reaction). Because each method has its own set of advantages and limitations, which testing method is right for a food manufacturer might not be clear. For companies selling perishable goods, the need for a rapid testing method such as PCR might be the only way to maintain the level of speed, specificity and accuracy needed to verify the safety of their food products. On the other hand, processed, shelf stable foods, may be able to sustain a more time-consuming traditional culture method.

In most cases, verification of a company's specific food products might be the only way to evaluate the testing method that best fits their needs.

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