Chapter 7

Molecular techniques for detection of foodborne Pathogens

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The public health concerns in recent time demands easy and accurate methods for detection of pathogens. The development of molecular techniques, new detection tools and the combination of existing approaches have increased the abilities of pathogenic bacteria monitoring and surveillance by exploring new biomarkers, increasing the sensitivity and accuracy of detection, quantification, and analyzing various genes such as functional genes and antimicrobial resistance genes (ARG). Molecular methods are gradually emerging as the most popular detection approach for pathogens, in addition to the conventional culture-based plate enumeration methods.

The field of molecular biology has a profound impact in life science investigation. Major advances in molecular biology over the last four decades have stimulated research and progress in almost all the disciplines of life science. The application of molecular technology in medicine is almost endless, some of the applications of molecular methods are:

1. Classification of organism by genotyping
2. Identification and confirmation of isolates
3. Early detection of pathogens in clinical specimen
4. Rapid detection of antibiotic resistance
5. Detection of mutations
6. Differentiation of toxigenic from non-toxigenic strains
7. Detection of microorganisms that lose viability
8. Identifying abnormalities in human and forensic medicine.

Polymerase chain reaction (PCR):

Polymerase chain reaction (PCR), a technique used to make numerous copies of a specific segment of DNA quickly and accurately. The polymerase chain reaction enables investigators to obtain the large quantities of DNA that are required for various experiments and procedures in molecular biology, forensic analysis, evolutionary biology, and medical diagnostics.

Polymerase chain reaction (PCR) is a molecular method developed more than 30 years ago (Mullins et al. 1986) to rapidly increase copies of all or part of a DNA sequence specific to a

particular pathogen that allows further analysis of that genetic sequence. As PCR can only detect a particular DNA sequence at a time, and there are often many pathogens in a contaminated food sample, many PCR methods were developed, including multiplex PCR (mPCR), nested PCR, reverse transcription PCR, and real-time fluorescent quantitative PCR (RT-qPCR). Our paper summarizes mPCR and RT-qPCR, as they are the most widely used for the pathogen detection and identification of foodborne pathogens.

Different types of PCR:

1. Multiplex PCR
2. Nested PCR
3. Hot start PCR
4. Semi-quantitative PCR
5. Real time PCR
6. qPCR

Multiplex PCR

The principle of this technology is that multiple pairs of primers present in the reaction mixture amplify different target gene fragments in parallel. Multiplex PCR is mainly used for gene knockout, mutation analysis, and RNA detection. Thereby, this technology can improve the health and safety of food. mPCR can therefore identify many different species of pathogens that commonly contaminate food and that cause similar poisoning symptoms in humans. However, since the design of primers is the key factor in developing mPCR determination, there may be some interactions between multiple primer sets, resulting in low amplification efficiency. Therefore, primer sets should be designed with similar annealing temperature, and provide a method to distinguish amplicons after a thermal cycle .Additionally, it can lead to a false positive result as living and dead bacteria cannot be distinguished . Therefore, mPCR can often lead to unsatisfactory results.

Nested PCR

Nested PCR usually involves two sequential amplification reactions, each of which uses a different pair of primers. The product of the first amplification reaction is used as the template for the second PCR, which is primed by oligonucleotides that are placed internal to the first primer pair. In this type, increases the specificity of the amplified product for a second PCR with new primers that hybridize within the amplified fragment in the first PCR.

The increased sensitivity arises from the high total cycle number. The increased specificity arises from the annealing of the second primer set to sequences found only in the first-round

products, verifying the identity of the first-round product. In nested PCR, one of the primers in the second PCR is identical to the first.

Hot start PCR

Hot Start PCR is a technique that reduces non-specific amplification and offers the convenience of reaction set up at room temperature. The polymerases used in Hot Start PCR are unreactive at ambient temperatures. Polymerase activity can be inhibited at these temperatures through different mechanisms, including antibody interaction, chemical modification and aptamer technology. At permissive reaction temperatures reached during PCR cycling, the polymerase dissociates from its inhibitor and commences polymerization. Use of hot start DNA polymerases is most often recommended for high-throughput applications, experiments requiring a high degree of specificity, or even routine PCR where the added security offered by a hot start enzyme is desired.

Semi quantitative PCR

Fluorescent dyes like SYER Green master mix are used for the identification of samples and probes are used to measure the amount of amplified product in real- time2. cDNA is obtained by RT-PCR for a RNA sample. ApoA1/Bactin are used as markers followed by gel electrophoresis process with ethidium bromide dye staining procedure. Here the main disadvantage is the generation of non-specific hybridisation. All the reactions were performed in quadruplicates using the DNA stocks. This technique allows an approximation to the relative amount of nucleic acids present in a sample quantitative PCR.

RT-PCR

RT-PCR includes chemicals that fluoresce in the PCR reaction system. The presence of pathogenic DNA causes the mixture to fluoresce, thereby enabling pathogen presence to be monitored in real time. RT-qPCR is highly specific and sensitive. Amplified products are detected in real time without the need for post-PCR DNA analysis. For those reasons, RT-qPCR became one of the most preferred methods for detecting and identifying foodborne pathogens. The most commonly used RT-qPCR methods utilize TaqMan™ and LightCycler™ probes.

qPCR

Quantitative PCR (qPCR) is used to detect, characterize and quantify nucleic acids for numerous applications. Commonly, in RT-qPCR, RNA transcripts are quantified by reverse transcribing them into cDNA first, as described above and then qPCR is subsequently carried out. As in standard PCR, DNA is amplified by 3 repeating steps: denaturation, annealing and

elongation. However, in qPCR, fluorescent labelling enables the collection of data as PCR progresses. This technique has many benefits due to a range of methods and chemistries available.

In dye-based qPCR (typically green), fluorescent labelling allows the quantification of the amplified DNA molecules by employing the use of a dsDNA binding dye. During each cycle, the fluorescence is measured. The fluorescence signal increases proportionally to the amount of replicated DNA and hence the DNA is quantified in "real time". The disadvantages to dye-based qPCR are that only one target can be examined at a time and that the dye will bind to any ds-DNA present in the sample.

In probe-based qPCR, many targets can be detected simultaneously in each sample but this requires optimization and design of a target specific probe(s), used in addition to primers. There are several types of probe designs available, but the most common type is a hydrolysis probe, which incorporates the use of a fluorophore and quencher. Fluorescence resonance energy transfer (FRET) prevents the emission of the fluorophore via the quencher while the probe is intact. However, during the PCR reaction, the probe is hydrolysed during primer extension and amplification of the specific sequence it is bound to. The cleavage of the probe separates the fluorophore from the quencher and results in an amplification-dependent increase in fluorescence. Thus, the fluorescence signal from a probe-based qPCR reaction is proportional to the amount of the probe target sequence present in the sample. Because probe-based qPCR is more specific than dye-based qPCR, it is often the technology used in qPCR diagnostic assay. Both the Detection and Quantification of a signal emitted by the amplified product by using the continuous measurement of a fluorescent label. It is also denoted as quantitative PCR—qPCR; usage of RT-PCR is inappropriate (Reverse Transcription PCR). Fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. Both the Detection and Quantification of a signal emitted by the amplified product by using the continuous measurement of a fluorescent label. Fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time.

APPLICATIONS OF PCR

1. Detecting pathogens using genome-specific primer pairs in food and clinical samples.
2. Detection of viral pathogens and other microorganisms persist in low levels in infected cells and are difficult to be identified by routine methods.

4. Quantitative Real-Time can be used to detect viral genomes such as HIV or HPV.

3. Diagnosis of genetic disorders such as phenylketonuria, haemophilia, sickle cell anaemia, thalassemia. Identification of genetic mutations like deletions, insertions and point mutations.

5. Screening specific genes for unknown mutations.

6. Identification and analysis of mutations in eukaryotic DNA.

7. Gene polymorphisms and Gene expression.

Advantages of PCR:

PCR is a powerful tool to amplify minute amounts of nucleic acids. Due to its unprecedented sensitivity, the method has become an essential diagnostic and research tool for infectious dermatology. Additionally, PCR can be used on all tissues or samples (fresh tissues, paraffin-embedded tissues, blood, faeces). It is also possible to analyse samples of poor conditions because only relatively short intact sequences of DNA are required. Archival materials can consequently be used for retrospective studies. In this latter case, however, amplification depends on the conservation of the target DNA. It has, for example, been demonstrated that DNA could be spoiled by long-stay in formalin and that amplification subsequently failed.

Components for PCR reaction:

The success of PCR depends on a number of factors, with its reaction components playing critical roles in amplification.

1. Template DNA
2. DNA polymerase
3. Primers
4. Deoxynucleoside triphosphate
5. MgCl2
6. Buffer

Template DNA

A PCR template for replication can be of any DNA source, such as genomic DNA (gDNA), complementary DNA (cDNA), and plasmid DNA. Nevertheless, the composition or complexity of the DNA contributes to optimal input amounts for PCR amplification. For example, 0.1–1 ng of plasmid DNA is sufficient, while 5–50 ng of gDNA may be required as a starting amount in a 50 µL PCR. Optimal template amounts can also vary based on the type of DNA polymerase used; a DNA polymerase engineered to have higher sensitivity due to

affinity to the template would require less input DNA. Optimization of DNA input is important because higher amounts increase the risk of nonspecific amplification whereas lower amounts reduce yields.

DNA polymerase

DNA polymerases are critical players in replicating the target DNA. Taq DNA polymerase is arguably the best-known enzyme used for PCR—its discovery revolutionized PCR. Taq DNA polymerase has relatively high thermostability, with a half-life of approximately 40 min at 94°C -95°C . It incorporates nucleotides at a rate of about 60 bases per second at 70°C and can amplify lengths of about 5 kb, so it is suitable for standard PCR without special requirements. Nowadays, new generations of DNA polymerases have been engineered for greatly improved PCR performance.

For more specialized applications such as PCR cloning, long amplification, and GC-rich PCR, DNA polymerases with higher performance are preferred. These enzymes are capable of generating lower-error PCR products from long templates in a shorter time with better yields and higher resistance to inhibitors (learn more about DNA polymerase characteristics).

Primers

PCR primers are synthetic DNA oligonucleotides of approximately 15–30 bases. PCR primers are designed to bind (via sequence complementarity) to sequences that flank the region of interest in the template DNA. During PCR, DNA polymerase extends the primers from their 3′ ends. As such, the primers' binding sites must be unique to the vicinity of the target with minimal homology to other sequences of the input DNA to ensure specific amplification of the intended target.

In addition to sequence homology, primers must be designed carefully in other ways for specificity of PCR amplification. First, primer sequences should possess melting temperatures (Tm) in the range of 55–70°C, with the Tm of the two primers within 5°C of each other. Equally important, the primers should be designed without complementarity between the primers (especially at their 3' ends) that promotes their annealing (i.e., primer-dimers), self-complementarity that can cause self-priming (i.e., secondary structures), or direct repeats that can create imperfect alignment with the target area of the template.

Furthermore, the GC content of the primer should ideally be 40–60%, with uniform distribution of C and G bases to avoid mistakes. Similarly, no more than three G or C bases should be present at the 3′-ends of the primers, to minimize nonspecific priming. On the other hand, one C or G nucleotide at the 3′ end of a primer can promote beneficial primer anchoring and

extension. For convenience and simplicity, a number of online tools are available to bioinformatically design and select optimal primer sequences with defined parameters.

Higher primer concentrations often contribute to mispriming and nonspecific amplification. On the other hand, low primer concentrations can result in low or no amplification of the desired target.

Deoxynucleoside triphosphates (dNTPs):

dNTPs consist of four basic nucleotides—dATP, dCTP, dGTP, and dTTP—as building blocks of new DNA strands. These four nucleotides are typically added to the PCR reaction in equimolar amounts for optimal base incorporation. However, in certain situations such as random mutagenesis by PCR, unbalanced dNTP concentrations are intentionally supplied to promote a higher degree of misincorporation by a non-proofreading DNA polymerase.

In common PCR applications, the recommended final concentration of each dNTP is generally 0.2 mM. Higher concentrations may help in some cases, especially in the presence of high levels of Mg2+, since Mg2+ binds to dNTPs and reduces their availability for incorporation. However, dNTPs exceeding optimal concentrations can inhibit PCR. For efficient incorporation by DNA polymerase, free dNTPs should be present in the reaction at a concentration of no less than 0.010–0.015 mM (estimated Km). When using non-proof reading DNA polymerases, fidelity can be improved by lowering dNTP concentrations (0.01–0.05 mM), as well as proportionally reducing Mg2+.

Magnesium ion (Mg2+):

Magnesium ion (Mg2+) functions as a cofactor for activity of DNA polymerases by enabling incorporation of dNTPs during polymerization. The magnesium ions at the enzyme's active site catalyse phosphodiester bond formation between the 3′-OH of a primer and the phosphate group of a dNTP. In addition, Mg2+ facilitates formation of the complex between the primers and DNA templates by stabilizing negative charges on their phosphate backbones.

Mg2+ ions are commonly delivered as a MgCl2 solution to the PCR mixture. However, some polymerases such as Pfu DNA polymerase prefer MgSO4, since sulfate helps ensure more robust and reproducible performance under certain circumstances. The magnesium concentration often needs optimization to maximize PCR yield while maintaining specificity due to its binding to dNTPs, primers, DNA templates, and EDTA (if present).

Buffer:

PCR is carried out in a buffer that provides a suitable chemical environment for activity of DNA polymerase. The buffer pH is usually between 8.0 and 9.5 and is often stabilized by Tris-

HCl. For Taq DNA polymerase, a common component in the buffer is potassium ion (K^+) from KCl, which promotes primer annealing. At times, ammonium sulphate $(NH_4)_2SO_4$ may replace KCl in the buffer. The ammonium ion (NH_4^+) has a destabilizing effect, especially on weak hydrogen bonds between mismatched primer-template base-pairing, thereby enhancing specificity (Figure 8). Note that DNA polymerases often come with PCR buffers that have been optimized for robust enzyme activity; therefore, it is recommended to use the provided buffer to achieve optimal PCR results.

Since Mg^{2+} has a stabilizing effect similar to K^+, the recommended $MgCl_2$ concentrations are generally lower when using a KCl buffer (1.5 ± 0.25 mM) but higher with an $(NH_4)_2SO_4$ buffer (2.0 ± 0.5 mM). Due to antagonistic effects of NH_4^+ and Mg^{2+}, buffers with $(NH_4)_2SO_4$ offer higher primer specificity over a broad range of Mg^{2+} concentrations. It is important to follow buffer recommendations by the enzyme's supplier, since the optimal PCR buffer is dependent upon the DNA polymerase used.

VISUALIZATION OF PCR TEST RESULTS:

Amplicons visualization in post PCR process, the template DNA is usually done by following-

Agarose Gel Electrophoresis-

After PCR is performed, the result can be analysed by agarose gel electrophoresis. This technique will allow a "visualization "of the PCR amplification reaction regarding the number of amplified regions (one or more PCR products) and the size of the PCR products (the size is the expected one based on the predicted amplicon flanked by the primers). Agarose gel electrophoresis separates DNA molecules according to size.

First, a board of agarose gel is assembled with wells (depressions) on top. The gel is submerged in electrophoresis buffer inside the electrophoresis tank. The PCR products (DNA) are then deposited on the wells and an electrical current is passed through the gel, conducted by the salts in the electrophoresis buffer (e.g. TAE - Tris-acetate-EDTA electrophoresis buffer).

Because DNA has a negative charge the current drives the DNA across the gel into the positive electrode. The agarose gel is a porous matrix where DNA molecules move according to size; smaller molecules take less time to move towards the end of the gel because shorter DNA fragments are not retained by the pores of the gel. Therefore, when we have a mixture of different DNA molecules, shorter molecules will migrate faster and will be deposited as bands at the bottom of the gel. Longer DNA molecules will be deposited as bands at the top of the gel because they have been retained by the pores in the gel matrix.

Before applying the DNA into the wells of the gel, the DNA is mixed with loading buffer This buffer is composed of glycerol (to enable the deposition of the DNA inside the wells) and bromophenol blue dye (to visualize the DNA solution when loading the samples and monitoring the progression of the electrophoresis run). The loading buffer does not interfere with the electrophoresis.

After the current is stopped, the DNA bands can be visualized using a fluorescent stain that intercalates the DNA molecules and a UV light equipment. There are several types of stains that intercalate the DNA molecules, emitting fluorescence when the gel is irradiated under UV light (254nm -365 nm) such as ethidium bromide (EtBr), SybrGreen or GelRed. These agents are added to the agarose when assembling the gel

Contaminants and inhibition

Sample quality is critical and therefore it must be verified before the samples are used in qPCR assays. It has been clearly demonstrated that analysis of degraded RNA samples can result in poor quality. In addition, it has been shown that the presence of inhibitors differentially affects qPCR assays. Therefore it is important to determine the integrity and purity of nucleic acid samples. contamination refers to the presence of an unwanted component in a sample. Classic examples of contamination in biological research experiments include the presence of mycoplasma in cell culture or unwanted proteases during serological analysis. Within the context of qPCR assays, the significant forms of contamination refer to nucleic acid template present in the sample which may be detected along with the specific target, thus generating a false positive, or material that may inhibit downstream reactions resulting in a reduced or failed reaction (false negative). Amplicon (target sequence) contamination PCR assays are especially vulnerable to contamination with the specific target sequence of interest for two reasons: PCR is a process that generates billions of copies of the amplicon, the specific target of interest. However, amplified product from one PCR is potentially a source of contamination for future PCRs. In a molecular biology laboratory, it is therefore essential to separate the PCR set up area (the designated space in which the reaction is prepared) from the post-PCR analysis area (the site the product will be subsequently analysed and possibly manipulated). This should be achieved by using separate rooms with dedicated equipment and laboratory coats such that nothing from the post-PCR analysis space is brought into contact with the clean (pre-PCR) space. Many laboratories also introduce a one way policy such that if an individual has entered a post-PCR room they are forbidden from subsequently entering a prePCR room on the same day, and qPCR assays are particularly vulnerable to amplicon contamination given the scope

of detecting a single template molecule; hence minuscule amounts of contamination are sufficient to cause a problem

In addition to PCR generation of the specific amplicon, care is needed in the laboratory setup to ensure that template material is not transferred from one sample to another (i.e. cross contamination). In the case of analysis of human samples, there must be precautions to ensure that material is not introduced from the analyst. It is important to identify and avoid contamination of all samples, and the use of controls for this purpose is highly recommended

CONCLUSION

The invention of PCR and real-time PCR has led to many major scientific advances. Though both methods are still regularly used in laboratories, real-time PCR is gaining popularity and quickly becoming the most cost- and time-effective method for analysing DNA products.

The use of real-time PCR expands to many areas of the clinical laboratory including genetics, virology, and microbiology. With more real-time PCR platforms and practices being created, the growth and potential of this technology is just beginning. However, the concept and process will stay the same and it is important for laboratory professionals to understand and learn about this technology.