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Genetic Amplification: Quantitative Polymerase Chain Reaction and Its Problems and Uses

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Introduction

Quantitative polymerase chain reaction (qPCR), also called real-time PCR, has become a cornerstone of DNA analysis, enabling detection of minute amounts of nucleic acids (Whittwer et. al, 1997). In 1983, Kary Mullis developed a new method of genetic amplification—the polymerase chain reaction [PCR] (Bartlett & Stirling, 2003). A little over 20 years later, PCR now is a common and often crucial technique used in medical and biological research laboratories for a variety of applications. Some of these applications include DNA cloning for sequencing, DNA-based phylogeny, the diagnosis of hereditary diseases, the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing), and the detection and diagnosis of infectious diseases. qPCR is a modification of the classic PCR method which, due to the presence of a fluorescent-labeled probe, allows for the quantification of DNA. Precise DNA quantification is a valuable insight that qPCR provides over other diagnostic techniques and can affect treatment options or preventative measures. This is especially the case in the detection of infectious diseases, where pathogens may be harmless in insignificant amounts but cause disease once the infectious dose is reached. With the prevalence of this technique and its many uses, it is important to research qPCR and its successes as well as its potential issues.

Polymerase Chain Reaction (PCR)

PCR involves the genetic amplification of a target DNA fragment from a template. PCR requires four main reagents: (1) sample DNA [which contains the DNA sequence to be amplified], (2) forward and reverse primers, (3) a heat-stable DNA Polymerase, and (4) deoxynucleoside triphosphates [dNTPs]. The PCR reaction occurs in three main stages: the denaturation stage, the annealing stage, and the

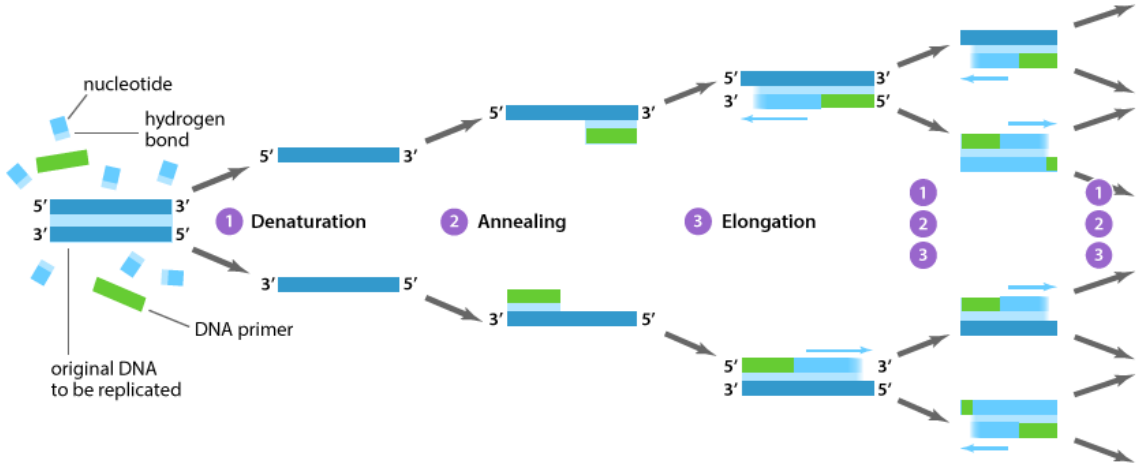

Figure 1. PCR Reaction Sequence (source: https://www.abmgood.com/marketing/knowledge_base/polymerase_chain_reaction_introduction.php)

extension stage. These stages are illustrated in Figure 1. In the denaturation stage, the sample DNA is heated to 94° C for one minute. At this temperature denaturation occurs and the double-stranded helix breaks apart. The reaction is then cooled to 54° C for 45 seconds, allowing the forward and reverse primers to anneal to the DNA [the annealing stage]. The sample is heated again, this time to 72° C for 2 minutes, allowing the heat-stable DNA polymerase to form the complementary strand of DNA via the dNTPs during the elongation stage. When the process is complete, there is double the amount of DNA compared to when the cycle started.

One PCR reaction usually repeats these three steps ~36 times, exponentially amplifying the sample DNA at each cycle.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative PCR employs the same PCR reaction, but it involves a fifth ingredient: a fluorescent-labeled probe. Two important discoveries led to the development of real time PCR: first, that the *Taq* polymerase possesses 5' → 3' exonuclease activity (Holland et al., 1991); second, the construction of dual-labeled oligonucleotide probes based on the fluorescence resonance energy transfer (FRET) principle (Stryer, 1978). The Taqman assay, a commonly used assay for laboratories employing the qPCR method, combines these findings. Figure 2 highlights the use of the probe in qPCR cycle. In a qPCR reaction, the internal labeled nonextendable probe (the "Taqman" probe) will anneal to the sample DNA at a spot downstream from the forward primer during the annealing stage. The probe has a fluorophore on one end (e.g., FAM: 6-carboxyfluorescein) and a quencher (e.g., TAMRA: 6-carboxytetramethylrhodamine) on the other (Giulietti et al., 2001). While the probe is intact, fluorescence energy transfer occurs through which the fluorescence emission of the reporter dye is absorbed by the quenching dye (Giulietti et al., 2001). As the DNA is extended, the probe will be cleaved. When the fluorophore and quencher are no longer near each other, the reporter dye emission is no longer transferred to the quenching dye (no more FRET), resulting in an increase in fluorescence emission. This process occurs in every cycle and does not interfere

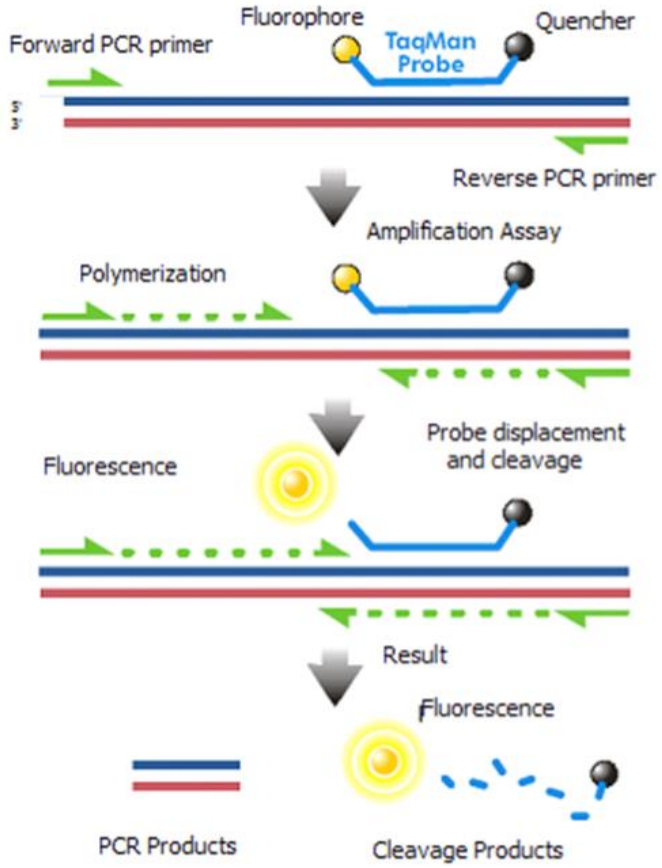

Figure 2. Use of the Taqman Probe in a qPCR reaction. (source: https://en.wikipedia.org/ wiki/Reverse_transcription_polymerase_chain_reaction) wiki/Reverse_transcription_polymerase_chain_reaction)

with the accumulation of PCR product (Giulietti et al., 2001). The qPCR instrument will take a "snapshot" of the fluorescence in each well for each qPCR cycle, producing a visualization of how much target DNA is present as the qPCR reaction progresses in real-time. It is for this reason that quantitative PCR is sometimes called "real-time" PCR.

qPCR Applications

qPCR has many practical applications. In 2015, Sik-Wing Yeung et al. at the Chinese University of Hong Kong presented a study employing the qPCR method as a way to detect group B streptococcus (GBS) colonization in pregnant women. GBS is one of the most common pathogens that cause neonatal sepsis (McKenna & Iams, 1998). The main source of this infection occurs at the time of vaginal delivery: colonization of GBS in the maternal genital tract results in vertical transmission (Schuchat A, 1998). Intrapartum antibiotics (IPA) administration is currently the most effective way to reduce the colonization at delivery, and also works well to reduce early onset GBS neonatal infection. However, the bacterial population can change so rapidly that the colonization of GBS between 35-37 weeks of gestation may not reflect exactly the same status during labor (Yeung et al., 2015). Unnecessary use of high-dose IPA can also cause various potential adverse effects for mother and baby, most notably potentially fatal anaphylaxis (Weiss & Adkinson, 1988). On the other hand, the GBS carriage diagnosed by the standard culture procedure would usually take 2-5 days for results, sometimes rendering it too late for IPA prophylaxis (Yeung et al., 2015). qPCR offers the quickest and most accurate method for intrapartum testing, with a sensitivity of 94-100% and a specificity of 90-100% (Honest et al., 2006). For intrapartum testing, the advantage lies with the speed and accuracy of a qPCR test. This method yields results in a timely manner and helps greatly in avoiding the unnecessary use of antibiotics in uncolonized women.

qPCR also is often used for agricultural applications. In 2014, Marios-Créhan et al. presented a study in France on diagnosing *Actinobacillus pleuropneumoniae* in swine populations. *A. pleuropneumoniae* is the causative agent of porcine pleuropneumonia, a respiratory disease responsible for significant worldwide economic losses to the swine industry (Gottschalk, 2012). Serological tests are usually performed for diagnosis, and it is relatively easy to isolate and characterize *A. pleuropneumoniae* from pneumonic lesions in acute cases (Marios-Créhan et al., 2014). However, bacterial detection tends to be more difficult in chronic infections or in healthy carrier pigs. In these silent infections, outbreaks may occur abruptly and explosively and result in the decimation of the affected herd. It is therefore crucial to diagnose carrier pigs early to prevent transmission between herds (Gottschalk, 2012). Unfortunately, serotyping can be tedious and requires the use of several different serological techniques for each strain (Mittal et al., 1992). qPCR, on the other hand, offers a better choice for diagnosis: it presents a specific and sensitive test, allowing for the detection of as few as 5 copies of the target sequence. Additionally, qPCR is extremely fast and does not require post-amplification manipulations. As these advantages are proving themselves increasingly worthwhile, more and more industries are employing qPCR testing as the standard.

Verstraete et al. present another example of this via a 2014 study conducted in Belgium. This study developed a qPCR assay to detect and quantify Shiga toxin-producing *E. coli* (STEC) in cattle and on farms. STEC are prominent food-borne pathogens capable of causing severe diseases such as hemorrhagic colitis, hemolytic uremic syndrome, and kidney failure (Karmali, 2009). Cattle are the main reservoir

for STEC pathogens (Blanco et al., 2004). The phage-encoded Shiga toxins 1 or 2 (*stx1* or *stx2* genes) are the important virulence factor for this strain, and were the targets of this study's qPCR assay. Due to the lack of common biochemical properties, STEC are often difficult to distinguish from other *E. coli* (Verstaete et al., 2014). Current culture methods tend to isolate merely a subset of serogroups, leaving other serogroups undetected (Verstaete et al., 2014). By developing this molecular approach, screening for STEC (or at least *stx1* and *stx2*) is more reliable. However, during development of this study, the authors noticed a big difference in results based on the efficiency of the primer. Primer base-matching is one of the few drawbacks of the PCR reaction, but one that can be compensated for to maximize the production of reliable data.

As previously stated, the primers in a PCR reaction anneal to the complementary sites of the target gene. 100% matching ensures optimal efficiency and excludes quantitative underestimation of the target (Werbrouck et al., 2007). During the method development of Verstaete et al.'s study, they observed that a single base mismatch in the primer resulted in a log 3 reduction in the gene copy number. This illustrates the importance of primer matching, and brings to light one of the drawbacks of using qPCR. Another more common disadvantage of qPCR is inhibition. Inhibitors can be divided into two main groups depending on how they disturb the qPCR: amplification inhibitors and detection inhibitors (Sidstedt et al., 2015). Amplification inhibitors, as the name implies, interfere with the amplification of the target gene directly by inhibiting the DNA polymerase, by changing the buffer composition, or by binding to nucleic acids (Sidstedt et al., 2015). Many molecular-

weight compounds in the source water (e.g. complex carbohydrates) have the ability to combine with metal ions and sequester nucleic acids from polymerases, preventing amplification (Watson & Blackwell, 2000). Detection inhibitors are a more recent discovery, but it is suggested that some molecules have the ability to quench the fluorescence signal from dyes or probes or may alter background fluorescence (Sidstedt et al., 2015). Environmental samples including soils and aqueous sediment are known to contain PCR inhibitors (Tebbe & Vahjen, 1993). Due to the prevalence of environmental studies' use of qPCR method, more advanced DNA purification methods have been developed. Pressure cycling technology (PCT) subjects the extracts to high pressure (Marshall et al., 2013). Synchronous coefficient of drag alteration (SCODA) focuses the DNA into a small area on a gel through altering voltage (Schmedes et al., 2013). However, extensive sample treatment and DNA purification inevitably leads to DNA loss and, depending on the chosen methods, recovery rates can be anywhere from approximately 10 to 80% (Miller et al., 1999). To avoid this DNA loss, researchers typically attempt to dilute the sample or add adjuvants such as bovine serum albumin to deal with inhibition in analysis of water samples (Kreader, 1996). Despite these disadvantages, qPCR continues to be an effective and efficient tool in DNA quantification.

Future Public Health Applications of qPCR

An increasingly widespread use of qPCR in public health is in the area of beach monitoring. Because of the great diversity of pathogenic microorganisms transmitted by contaminated water and the difficulty and cost of directly measuring

all possible microbial pathogens, indicator organisms are often used for monitoring and regulation of recreational and drinking waters. These organisms are common inhabitants of the intestinal tract of warm-blooded animals and indicate the presence of sewage and fecal contamination in water samples. While indicator organisms themselves do not cause illness under normal conditions, they represent a measure of fecal contamination (Wade et al., 2006). In beach monitoring, common indicator organisms used for analysis are *Escherichia coli* and *Enterococcus* spp.

Traditional beach monitoring enumeration methods include EPA method 1600 and Enterolert/Colilert-18 (Noble et al., 2010). The former method is a membrane filtration approach based on passing water through a filter, and then plating the filter on a medium selective for the bacterial group of interest. The Enterolert/Colilert-18 method uses defined substrate technology and relies on quantification through a most-probable number (MPN) calculation. These culture methods are widely accepted and practiced due to their low cost, relative ease of use, and demonstrated relationship to health risk. However, recent advances in technology allow new opportunities to measure bacterial water quality more rapidly. Boehm et al. (2002) demonstrated that fecal indicator bacteria concentrations change substantially on a time scale of hours, so rapid detection was crucial to protect public health. Speedy detection allows public health officials to post warnings or close beaches on the same day that samples are collected, rather than keep contaminated beaches open while waiting on overnight lab results. The response time of qPCR also can reduce the number of unnecessary beach closings. In 2004, Kim and Grant estimated that up to 40% of beach closures were in error.

Molecular techniques have a large advantage over culture methods due to their quicker response times and specificity.

While current standard methods rely on bacterial growth and metabolic activity, new methods such as qPCR have the capability to directly measure DNA or RNA. As a result, this direct approach is in theory capable of more accurate quantification of fecal indicator bacteria. However, since qPCR measures genetic material rather than the viable cells quantified culture-based methods, it may overestimate bacterial concentrations due to the inclusion of target DNA from dead or dying cells in the sample (Noble et al., 2010). This is especially a problem at inland lake beaches. Inland water bodies are popular swimming and boating destinations and differ in terms of hydrology and water quality than ocean or Great Lakes beaches (Francy et al., 2013). Due to their size, inland beaches are more susceptible to build-up of dead cells and weather changes, which can skew qPCR results. Nonpoint source runoff dominated by animal associated fecal contamination is also attributed to interfering with bacterial counts at beaches (Colford et al., 2007). Enteric viruses and bacterial viruses such as coliphage are promising indicators of fecal contamination, but have been inadequately studied as predictors of health effects on swimmers (Bosch, 1998).

Conclusions

Since its development, qPCR has been an indispensable tool for molecular testing. While inhibition of the qPCR reaction remains one of the most prominent drawbacks, a range of techniques are available to combat inhibitors and ensure

accurate results. qPCR is often used in time-sensitive situations—be it beach monitoring or intrapartum GBS testing—and thus the speed of the reaction proves itself to be the method's greatest advantage. Today qPCR is widely used in clinical diagnosis and agricultural environments, and more assays for different organisms continue to emerge. As researchers develop this method further, qPCR could become the standard practice in clinical and environmental situations.

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