Proceedings of the 3rd International Conference on Environmental Geoscience and Earth Ecology
DOI: 10.54254/2753-8818/2025.23394

Analysis of the Advantages and Disadvantages of PCR Technology and Ideas for Improvement

Xiang Tang

Faculty of Mines, Guizhou University, Guiyang, China
TXiang331@outlook.com

Abstract: PCR began in the late 60s and early 70s of the 20th century for the study of gene in vitro isolation technology, in 1953 Watson and Crick proposed the double helix structure of DNA, in 1959 Kornberg reproduced the replication process of DNA in a test tube, in 1969 Taq enzyme was discovered, in 1984 PCR was proven to be feasible, since its inception, through many technical iterations, now in medicine, agriculture, Food safety testing and other fields are widely used as a mature technology, however PCR technology is not perfect, it cannot always provide satisfactory results in experiments, the exploration of PCR technology in improving specificity and accuracy is still a long way, the article introduces the development history of PCR, the mechanism, advantages and limitations of different PCR technologies, summarizes the technical points and precautions of traditional PCR through the analysis of operation methods and materials, and introduces the application of various PCR technologies. The development of PCR technology was also discussed. This article helps readers understand the existing problems of PCR and gives ideas for improvement, which is helpful for updating PCR technology.

Keywords: PCR, qPCR, RT-PCR&RT-qPCR, dPCR

1. Introduction

Semi-preserved replication of DNA increases the stability of heredity and lays the foundation for the evolution and development of organisms. When DNA is replicated in an organism, double-stranded DNA is formed by helicase enzyme, and then two new DNA molecules are generated under the action of DNA polymerase, according to the principle of base complementarity pairing. In the exploration of in vitro synthesis of DNA, it has been found that high temperature can replace helicase to denature DNA into single-stranded molecules, and when the temperature is lowered, it can be re-transformed into double-stranded molecules through cooperation with base pairs. Based on this principle, in vitro DNA amplification can be achieved by controlling DNA denaturation through high and low temperature conversion, selecting the desired replication fragment through primers, using dNTP as raw material and providing energy, adding buffer as adjustment and circulating in a specific instrument, which is the PCR technology.

PCR is characterized by its ability to ramp up small amounts of DNA in large quantities quickly, allowing scientists to make millions or even billions of copies of specific DNA sequences in just a few hours. This makes it extremely widely used in the field of detection, whether it is paleontological fossils, the remains of historical figures, the hair, skin or blood left by the murderer in a homicide case decades ago, or a mutated gene, if a little bit of DNA can be isolated, it can be amplified and

DOI: 10.54254/2753-8818/2025.23394

compared by PCR. This technology has revolutionized fields such as medicine, forensics, and genetic research.

2. Mechanism

2.1. PCR

PCR, which stands for polymerase chain reaction, is the first generation of PCR technology, and its detection mainly relies on gel electrophoresis.

PCR mainly contains three basic reaction steps: denaturation, annealing, and elongation (1) Denaturation of template DNA: After 95 °C, 5-10 minutes of pre-denaturation, even the large mass template has been basically dissociated, and then the cycle can begin, according to the proportion of base pairs in the DNA, the denaturation of 95-98 °C for about 30 seconds makes the DNA molecule a single strand, preparing for the next step of binding to primers. (2) Annealing of template DNA: reduce the temperature to about 55 °C, renaturation of template DNA and binding of primers according to the principle of base complementary pairing. (3) Extension of primers: primers and templates are heated to about 72 °C (the optimal working temperature of high-temperature resistant DNA polymerase) With the help of polymerase, dNTP is used as raw material, the target sequence as the template, according to the principle of base complementary pairing to form a new DNA single strand, and then the PCR machine repeats the cycle process, each cycle takes 2-4 minutes and contains the above three parts of denaturation, annealing, and extension, the number of synthetic DNA single strands will increase exponentially with the number of cycles, so after a few hours, millions of target gene fragments will be obtained [1].

2.2. qPCR

qPCR stands for Quantitative real time polymerase chain reaction, At present, the theoretical basis of all probe qPCR is to use the fluorescence resonance energy transfer phenomenon(FRET), there is a pair of groups on the probe that can produce fluorescence resonance energy transfer, and some processes in the PCR reaction (enzyme digestion, hybridization, etc.) are used to change the distance between the two groups, so that the fluorescence intensity or fluorescence type in the system changes, and this change is directly related to the type and amount of PCR products, and by detecting this change, we can detect the type and amount of products in the PCR reaction system. The mechanism of FRET is briefly introduced below.

There is a phenomenon between fluorescent substances, if the emission spectrum of one fluorophore (Donor) overlaps with the absorption spectrum of another group (Acceptor), when the distance between the two is close to a certain degree (1-10nm), the donor is excited by the excitation light but does not emit the emission light of the substance, but transfers the energy to the acceptor, and the whole energy transfer process is mediated by a pair of dipoles, which does not involve the emission and reabsorption of photons. If the acceptor fluorescence quantum yield is zero, energy transfer fluorescence extinguishing occurs (i.e., the donor fluorescence is quenched by the acceptor); If the receptor is also a fluorescent emitter, it exhibits the fluorescence of the receptor and causes redshift in the secondary fluorescence spectrum.

2.3. RT-PCR&RT-qPCR

RT-PCR stands for Reverse transcription PCR, and its key point is to combine PCR technology with reverse transcription technology, so that PCR can be applied to RNA. Compared with PCR, the first step of RT-PCR is the synthesis of the first strand of cDNA, which is based on enzymatic catalysis to reverse transcribe RNA into the first strand of cDNA. An oligodeoxynucleotide primer is

DOI: 10.54254/2753-8818/2025.23394

hybridized to RNA and then catalyzed by reverse transcriptase to synthesize a corresponding complimentary copy of cDNA. Depending on the purpose of the experiment, there are three options for primers for cDNA first-strand synthesis: (1) Oligo(dT): a specific primer for eukaryotic cells. (2) Random hexamer primers: non-specific primers used to copy full-length mRNA. (3) Specific primers: only the required cDNA can be produced. In this process, it is necessary to prevent RNA degradation due to excessive temperature, and the subsequent product can be used for PCR amplification, and the high temperature during denaturation during cycling will degrade the remaining RNA and inactivate the reverse transcriptase, and the final product is the cDNA corresponding to the mRNA.

2.4. dPCR

dPCR, which stands for Digital PCR, is a quantitative PCR technique. The basic principle of dPCR is to evenly divide the target molecule in the sample into many tiny reaction cells and perform PCR amplification in each reaction unit. By numerically counting the positive and negative results of the reaction unit, the presence or absence of the target molecule can be determined, allowing for precise quantification.

3. Advantages and limitations

Table 1: An overview of the advantages and disadvantages of different PCR techniques

method	advantages	Limitations
PCR	The cost is low, the standard is perfect The product can be recycled for other experiments	The operation is cumbersome The specificity and sensitivity are low It is easy to be contaminated It can only be qualitative but not quantitative
method	advantages	Limitations
qPCR	Higher specificity and sensitivity than PCR Can be use for quantitative analysis	The cost is high The product is not recoverable Non-specific amplification and false-positive results may be present
method	advantages	Limitations
RT-PCR&RT-qPCR	Can be used with RNA	RNA is unstable The RT step increases the likelihood of contamination and increases time-consuming
method	advantages	Limitations
dPCR	Absolute quantitation, higher specificity and sensitivity	It is expensive Complex and time-consuming to operate

3.1. PCR

We can see this from Table 1 that PCR is a mature technology, its equipment, pharmaceuticals, raw materials have a mature industrial chain, the operation details have good specifications, the design of primers also has many samples that can be used for reference, but as the first generation of PCR technology, it can only be qualitative detection and cannot be quantitatively detected, can only operate DNA.

DOI: 10.54254/2753-8818/2025.23394

3.2. qPCR

qPCR combines PCR technology with fluorescence quantification technology, and collects fluorescent signals by adding fluorescent dyes or fluorophores to the PCR amplification reaction system, so as to realize real-time monitoring of the changes in the amount of amplified products in each cycle throughout the PCR process, but it still does not completely solve the problem of unstable results caused by contamination and low specificity in PCR technology, such as the occurrence of false positive and false negative results.(Tab.1)

3.3. RT-PCR&RT-qPCR

The addition of reverse transcription technology allows PCR to be used in RNA detection in the form of cDNA production, but the instability of RNA reduces the accuracy of experimental results, and the reverse transcription process increases the time used in the experiment and further increases the possibility of direct and indirect contamination. (Tab.1)

3.4. dPCR

dPCR uses nanotechnology to ensure that there is, on average, only one copy or no target DNA molecule in each reaction chamber, amplifying it with fluorescent signals to achieve the absolute count of target nucleic acid molecules, thereby improving detection sensitivity and accuracy. However, the precision and even customization of the instruments significantly increase costs, and the complexity of the operations also leads to substantial time costs, which means it cannot completely replace traditional PCR technology. (Tab.1)

4. Problems & optimizations

In the experiment, PCR often does not get satisfactory results, and the causes of failure and existing problems are analyzed from both the operation and the materials. Summarize improvement plans.

4.1. Operation

Excluding human operational errors, improper selection of consumables, and unreasonable temperature and time settings are the main reasons for PCR failure, most of the volumes of PCR tubes can meet the requirements of the PCR reaction. However, apart from meeting the experimental requirements, low-capacitance tubes are preferentially recommended. Because the low-volume reaction tube has a small upper space, it can increase the thermal conductivity and reduce evaporation. And when dispensing, it is necessary to avoid adding too much or too little. Excess can lead to decreased thermal conductivity, spillage, and cross-contamination, while too little sample can result in sample evaporation loss.

Denaturation temperature and time: this part is directly related to the unwinding of DNA, usually 50% CG proportion of DNA 90~95 °C is enough, higher CG content requires higher temperature, but at the beginning of the whole cycle, pre-denaturation is required, large fragment (>1 kb) template 30s is not enough to completely denature, 5-10min pre-denaturation can greatly enhance the amplification efficiency of the first cycle, pre-denaturation and denaturation time temperature is insufficient will lead to the failure of the whole PCR process.

Annealing temperature and time: Annealing temperature is an important factor affecting the specificity of PCR, which greatly affects the binding and mismatch probability of primers, and is easy to measure and modify, which is an important research parameter for PCR optimization. Here is an example. Using mixtures of templates, biased and nonbiased PCRs were carried out at annealing temperatures ranging from 47 to 61°C, which was conducted on a 1:1 mismatch pair mixture of A.

DOI: 10.54254/2753-8818/2025.23394

hydrophilia (AH) and B. subtilis (BS) DNA templates, employing 27F and 63F primer sets. The PCR products were digested by the Hin6I restriction enzyme, yielding two T - RFs with a size difference of 25 bp. The reproducibility was within a margin of error of 5%.

During amplification with the mismatch primer, the deviation from the nearly 1:1 template ratio increased almost exponentially as the annealing temperature rose. Due to this correlation, at annealing temperatures of 59.9°C and 61°C, the detection of the mismatch template led to relatively high SD values for the peak area ratios. When lower annealing temperatures were used, the original ratio was approximated.

Conversely, for the perfectly matching 27F primer, such an almost exponential relationship between the annealing temperature and the distortion from the template ratio, which exceeded the margin of error, was not observed. Notably, even an extremely low annealing temperature of 47°C did not adversely affect the PCR product ratios in nonbiased amplifications. To find the annealing temperature more accurately, can set a transverse gradient and perform 12 rows, and to succeed as soon as possible, can try touch-down PCR [2].

Elongation temperature and time: The elongation temperature of PCR reaction is generally controlled between 70~75 °C, the commonly used temperature is 72 °C, too high elongation temperature will hinder the binding of primers and templates, the time of PCR elongation reaction mainly depends on the length of the fragment to be amplified, for DNA fragments within 1kb, about one minute is enough for polymerase to complete the work, 3~4 kb target sequence needs 3~4 min, and for fragments of 10 kb and above length, the amplification time needs to be extended to 15 minutes. It is important to note that an excessively long extension time will lead to the appearance of non-specific bands.

Cycle time: It determines the number of PCR amplification results, however, due to its exponential growth, too many cycles will lead to an increase in non-specific amplification

4.2. Material

4.2.1. Template

Concentration and quality are two important indicators of the template, too high and too low concentration will affect PCR, usually recommended 50-500 ng/ul, for genomic DNA needs to have a large molecular weight, usually need to meet the level of gel greater than 15000 marker of a single band during the detection, no drag, for cDNA according to different species there are multiple bands, need to meet the diffusion is not serious, for the plasmid it is mostly supercoiled, need to meet the wirelessness (open loop), no drag [3].

4.2.2. Primers

The length of primers is generally 15-30 bp, the commonly used is 18-27 bp, but should not be greater than 38, too short will lead to multiple binding sites on the template, resulting in non-specific amplification, too long will lead to an elongation temperature greater than 74 °C, not suitable for Taq DNA polymerase reaction and increase the probability of the occurrence of secondary structure, the upstream and downstream primers should not be too different, the end of the primer is preferably G or C, and the GC content of the primer is generally 40%-60%, The Tm value of the template sequence corresponding to the primer should be about 50-65 °C, the 3' end of the primer can not be modified, the 5' end of the primer can be modified, the bases should be randomly distributed, and there can be no 4 consecutive bases between the primer itself and the primer to complement each other to form a dimer or hairpin structure. To further increase specificity, nested PCR can be used [4].

DOI: 10.54254/2753-8818/2025.23394

4.3. Enzyme & buffer

As an important factor affecting the success of PCR, there are two main types of enzymes, Taq enzymes and high-fidelity enzymes. Buffers provide dNTPs with the desired ions, which, together with sterile water, constitute the reaction conditions, and due to the maturity of PCR technology, buffers have been greatly commercialized, but it is still necessary to pay attention to the optimal reaction conditions for templates, primers, and enzymes when selecting.

4.3.1. Taq

The Taq DNA polymerase gene is 2496 bases in length, encodes 832 amino acids, and has a molecular weight of 94 KD. The enzyme has the highest biological activity at 70~75 °C. Under the condition of 75~80°C, each enzyme protein molecule can be extended by about 150 bases per second. At 70°C, the elongation rate of enzyme molecules is more than 60 bases per second. When the temperature decreases, the elongation rate decreases significantly. 22 bases per second at 55°C, about 1.5 bases per second at 37°C, and about 0.25 bases per second at 22°C. When the temperature exceeds 80°C, the synthesis rate also decreases significantly, which may be related to the disruption of the stability of the primer or primer-template complex.

Taq DNA polymerase has good thermostability. The test showed that the biological half-lives of Taq DNA polymerase were 130 minutes, 40 minutes and 5~6 minutes at 92.5°C, 95°C and 97.5°C, respectively. Compared with E. coli DNA polymerase I, Taq DNA polymerase has 5'→3' polymerase activity and 5'→3' exonuclease activity, but lacks 3'→5' exonuclease activity. Therefore, if a mismatch of certain amino acids occurs in a PCR reaction, the enzyme has no corrective function. The chance of base mismatch in PCR reactions using Taq DNA polymerase is around 2.1×10-4. Like many other polymerases, Taq DNA polymerase is a Mg2+-dependent enzyme whose catalytic activity is very sensitive to Mg2+ concentrations. Using salmon sperm DNA as a template, when the concentration of dNTP was 0.7~0.8 mmol/L, the PCR reaction was carried out with different concentrations of Mg2+ for 10min, and the results showed that the catalytic activity of the enzyme was the highest when the concentration of MgCl2 was 2.0 mmol/L, which could maximize the activation of the activity of Taq DNA polymerase, and when the concentration of Mg2+ was too high, the enzyme activity could be inhibited by 40~50% when the concentration of MgCl2 was 10 mmol/L. Since Mg2+ can bind to dNTPs and affect the free Mg2+ concentration in the PCR reaction, the concentration of MgCl2 should be appropriately adjusted in different reaction systems. In general, the concentration of Mg2+ in the reaction should be at least 0.5~1.0mmol/L higher than the total concentration of dNTP.

4.3.2. High-fidelity enzymes

The main difference between high-fidelity enzymes and Taq enzymes is that high-fidelity enzymes possess a very strong 3'→5' exonuclease activity, which provides excellent proofreading capabilities. They can also perform high-efficiency and high-fidelity amplification of GC-rich templates. However, because of this, high-fidelity enzymes require simultaneous proofreading during extension, resulting in a slower elongation rate compared to Taq enzymes. For example, the synthesis rate of Pfu DNA polymerase is less than half that of Taq DNA polymerase. When using high-fidelity enzymes, one can increase the elongation time and add antibodies that inhibit DNA polymerase activity and 3'→5' exonuclease activity at room temperature to prevent primer mismatches and primer degradation before the PCR reaction. Additionally, some high-fidelity enzymes have higher annealing efficiencies than Taq enzymes, which can partially compensate for their slower synthesis rate. Combined with an appropriate buffer, the synthesis speed can be further improved.

DOI: 10.54254/2753-8818/2025.23394

For example, high-fidelity PCR increased the accuracy of detection of Candida solanacearum in potato tubers, which is difficult to detect with conventional methods, by 30-40% [5] Naturally occurring proofreading DNA polymerases, such as Pfu and KOD, have approximately 10-fold higher fidelity than Taq polymerases. However, the "next generation" of high-fidelity DNA polymerases has been specifically engineered to have ultra-high fidelity, approximately 50-100 times that of Taq polymerases [6].

5. Application

5.1. PCR

Traditional PCR techniques can be used for food safety testing due to their simplicity and speed, such as the identification of live and dead bacteria based on the integrity of cell membranes, where vPCR technology consists of two reaction steps, nucleic acid intercalation dye pretreatment and PCR amplification, to quickly identify live bacteria from dead bacteria in a sample. Due to the incomplete cell membrane of damaged or dead bacteria, light-activated nucleic acids embedded in dyes, including ethidium monoazide (EMA) or propidium monoazide (PMA), can penetrate the cell to form irreversible binding to DNA molecules, thereby inhibiting subsequent PCR amplification and enabling the identification of live and dead bacteria [7].

5.2. qPCR

qPCR has a wide range of applications in medicine because of its quantitative properties, which can provide detection methods for diseases with atypical clinical manifestations, including pathogen detection, cancer gene mutation analysis, drug metabolism testing, and infectious disease screening, such as leishmaniasis, whose diagnosis relies on clinical manifestations, epidemiology, and laboratory data. With regard to laboratory methods, the lack of gold-standard for human patients or animals compromises accurate epidemiological data collection [8], thereby limiting disease control. qPCR technology provides an efficient molecular diagnostic tool.

5.3. RT-PCR&RT-qPCR

The combination of reverse transcription technology and PCR technology has created RT-PCR and RT-qPCR, and its RNA-usable characteristics have brought the convenience of PCR technology to more fields, such as its irreplaceable role in the detection of coronaviruses such as SARS-CoV-2, RT-PCR nucleic acid detection is the main means of diagnosing COVID-19 using respiratory samples, and RT-qPCR detection is the gold-standard for COVID-19 diagnosis [9].

5.4. dPCR

The absolute quantification of dPCR provides greater precision in the assay, making it a precise molecular diagnostic tool in precision medicine, for example, in cancer treatment, where accurate detection of each patient's targeted DNA sequence is critical due to the heterogeneity of the genetic phenotype. In the early days, most applications of dPCR in oncology focused on detecting molecules from tumor tissue samples, which is known as the gold-standard for tumor genotyping, which can track the development of a patient's disease (before, during, and after treatment) through dPCR through a series of blood or other readily available liquid biopsy samples, enabling precision medicine optimized for specific patients [10,11].

DOI: 10.54254/2753-8818/2025.23394

6. Conclusion

PCR as a biological in vitro amplification of DNA technology, for the detection and identification of nucleic acids provides a strong help, so far has developed three generations of PCR technology, although the most widely used first and second generation PCR technology is still not high specificity, false positives and other characteristics, but its technology is mature, simple operation, low cost, as an efficient detection means in agriculture, food, medicine and other areas play an irreplaceable role, through the improvement of the details of each step of PCR, The accuracy of traditional PCR technology is constantly improving, and the emerging PCR technology is combined with digital technology and nanotechnology to further improve the specificity and accuracy of PCR from the mechanism level, although these new technologies are generally more complex and expensive, but in the foreseeable future, with the continuous maturity of digital and nanotechnology, the advantages of emerging PCR technology will be amplified, in the early diagnosis of diseases, detection of rare sequences and rare mutations, single-cell analysis and gene expression analysis, Fields that require high sensitivity and specificity, such as measuring viral load, will inevitably have a wider range of applications.

References

[1] *Hong Liu, Huazhong Agricultural University, Research on polymerase chain reaction and gene chip technology and its application in the quarantine and monitoring of viruses in major aquatic animals(2019) cnki.net*

[2] *FEMS Microbiology Ecology, Volume 60, Issue 2, May 2007, Pages 341–350, Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targetting bacterial community analysis, Rita Sipos, Anna J. Székely, Márton Palatinszky, Sára Révész, Károly Márialigeti, Marcell Nikolausz, https://doi.org/10.1111/j.1574-6941.2007.00283.x.*

[3] *Front.Microbiol 10 Novenmber2022 Sec.Microbiotech https://doi.org/10.3389/fmicb.2022.1024827 ,A simple and rapid technique of template preparation for PCR*

[4] *Chinese Journal of Veterinary Medicine,54,12(2018) Comparative analysis of different PCR methods for the detection of Babesia ovale. bovine Tian Wannian1, Xue Shujiang2, Jia Lijun2, Zhang Shoufa2 College of Animal Science and Technology, Jilin University of Agricultural Science and Technology, Jilin, Jilin Province, 1321012. College of Agriculture, Yanbian University, Yanji, Jilin Province, 133002*

[5] *Short Communication Published: 11 February 2015 Volume 92, pages 451–454, (2015) High-Fidelity PCR Improves the Detection of 'Candidatus Liberibacter solanacearum' in Potato Tubers, Cating, R.A., Rondon, S.I. & Hamm, P.B. High-Fidelity PCR Improves the Detection of 'Candidatus Liberibacter solanacearum' in Potato Tubers. Am. J. Potato Res. 92, 451–454 (2015). https://doi.org/10.1007/s12230-015-9434-z*

[6] *Potapov, V., & Ong, J. L. (2017). Examining Sources of Error in PCR by Single_Molecule Sequencing. PloS one, 12(1), e0169774. doi:10.1371/journal.pone.0169774 PMID: 28060945*

[7] *Nogva, H.K.; Drømtorp, S.M.; Nissen, H.; Rudi, K. Ethidium monoazide for DNA-based differentiation of viable and dead bacteria by 5'-nuclease PCR. BioTechniques 2003, 34, 804–813. [Google Scholar]*

[8] *Galluzzi, L., Ceccarelli, M., Diotallevi, A. et al. Real-time PCR applications for diagnosis of leishmaniasis . Parasites Vectors 11, 273 (2018). https://doi.org/10.1186/s13071-018-2859-8*

[9] *Zhen Huang, Di Tian, Yang Liu, Zhen Lin, Christopher J. Lyon, Weihua Lai, Dahlene Fusco, Arnaud Drouin, Xiaoming Yin, Tony Hu, Bo Ning,*

[10] *Ultra-sensitive and high-throughput CRISPR-p owered COVID-19 diagnosis,Biosensors and Bioelectronics,https://doi.org/10.1016/j.bios.2020.112316.(https://www.sciencedirect.com/science/article/pii/S0956566320303110)*

[11] *Lexiang Zhang, Rokshana Parvin, Qihui Fan, Fangfu Ye,Emerging digital PCR technology in precision medicine,Biosensors and Bioelectronics,Volume 211,2022,114344,ISSN 0956-5663,https://doi.org/10.1016/j.bios.2022.114344.(https://www.sciencedirect.com/science/article/pii/S0956566322003840)*