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Original Article

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Determination of the shelf life of primers used in conventional polymerase chain reaction for pathogen detection

Abstract

Aim: Polymerase chain reaction (PCR) is an essential tool for the detection of many pathogens, relying on the use of primers. However, there is limited literature on the shelf life of these nucleotides, which is crucial for meeting the stability requirements of European regulations for in vitro diagnostic medical devices (IVDR) (Regulation (EU) 2017/746). This study aims to determine the shelf life of primers used in conventional PCR.
Materials and Methods: Over the past 15 years, we have conducted numerous PCR experiments for various pathogens to estimate the shelf life of dissolved oligonucleotides stored at -20°C. This study focuses on the shelf life of primers used in conventional PCR for two pathogens: *Chlamydia trachomatis* and *Streptococcus mutans*.
Results: The findings suggest that primers stored at -20°C typically have a shelf life of five years, even after enduring at least 20 freeze-thaw cycles.
Conclusion: This study contributes valuable insights to the limited literature on primer stability, demonstrating that primers stored at -20°C remain viable for five years. These findings can help laboratories and research institutions reduce costs by minimizing the need for frequent primer replacement and ensure compliance with legal stability requirements.

Keywords: Primer, oligonucleotide, polymerase chain reaction, shelf life, chlamydia trachomatis, streptococcus mutans

INTRODUCTION

Polymerase chain reaction (PCR) has been widely used for decades in various molecular analyses. Primers, essential components of PCR, are either designed manually or generated using a variety of software tools available on the market and online platforms. PCR plays a crucial role in detecting genetic mutations and identifying pathogens such as influenza virus, coronavirus, *Mycobacterium tuberculosis*, HCV, and tick-borne pathogens (1-10).

Primers, which are short sequences of nucleotides, are integral to PCR reactions and are stored by all molecular laboratories. However, there is a notable lack of literature on the shelf life of these oligonucleotides. For kit manufacturers and laboratories, determining the shelf life of primers is essential for ensuring stability and compliance with regulatory standards.

There are numerous oligonucleotide synthesizing companies, including Thermo Fisher Scientific, IDT, Eurofins, Microsynth, Metabion, and Bioneer, based in the USA and Europe. According to their websites, these companies recommend storing oligonucleotides at -20°C, with suggested shelf lives ranging from six months to two years (11-17). However, despite this guidance, there is a significant lack of publicly available data from manufacturers of ready-to-use PCR kits, diagnostic kit producers, universities, or diagnostic laboratories regarding the actual shelf life of primers.

The shelf life of oligonucleotides is a critical issue, as it directly affects the quality of research and diagnostic outcomes. Many diagnostic companies, such as those holding FDA and CE approvals, are involved in producing molecular diagnostic products. The World Health Organization (WHO) also regularly publishes PCR primers for pandemic-related pathogens on its

website, alongside its own approval system for diagnostic kits. Furthermore, hundreds of laboratories worldwide, certified to conduct genetic and diagnostic testing, rely on the accuracy and stability of their reagents. Yet, it appears that few, if any, of these institutions have attempted to systematically determine the shelf life of oligonucleotides, raising an important question: how do manufacturers of approved kits or diagnostic laboratories ensure the quality and longevity of their in-house assays?

The recent European regulations for in vitro diagnostic medical devices (IVDR), specifically Regulation (EU) 2017/746, mandate the validation and verification of molecular tests. These regulations require performance tests for sensitivity, specificity, intra-assay and inter-assay precision, linearity, and, where applicable, method comparisons. Such factors significantly influence the performance of diagnostic kits, further underscoring the importance of determining the shelf life of primers used in PCR-based assays.

These institutes, laboratories, and diagnostics manufacturers are certified according to DIN EN ISO 13485:2016, which sets the standards for quality management systems in the design and manufacture of medical devices. If the shelf life of critical components, such as oligonucleotides, is not determined, how can they meet all the requirements of these certifications? The stability of these components is vital, as many of the kits developed under these standards are used in the development and monitoring of new therapies and vaccines.

Without clear data on the shelf life of primers, there is a risk that the quality and reliability of diagnostic assays could be compromised, potentially impacting the outcomes of research and clinical diagnostics. As such, determining the shelf life of oligonucleotides is not only a matter of scientific inquiry but also of regulatory and clinical importance.

Furthermore, incorrectly determining the shelf life of essential components places a financial strain on the healthcare system. Over the past 15 years, we have put significant effort into collecting vital data from our experiments conducted during the development and production of ready-to-use kits supplied worldwide. This research mirrors other cases of resource mismanagement, such as the widespread use of 10-20% fetal calf serum (FCS) in cell cultures, despite our findings demonstrating that 2-3% is sufficient for various applications. These practices represent significant wastage of public funds, as unnecessary expenditures could be reduced with proper data and validation (18).

In this research, we aimed to determine the shelf life of oligonucleotides. While we conducted hundreds of experiments targeting various pathogens and genetic markers, this study focuses on a select few examples to illustrate the findings. These examples are representative of the broader conclusions drawn from our extensive experimental work.

MATERIALS AND METHODS

In this study, the results for two pathogens, *Chlamydia trachomatis* and *Streptococcus mutans*, are presented.

Conventional PCR Protocol

DNA was isolated using a mini-column DNA isolation kit (SB0001 - Genekam Universal DNA Isolation Kit (INT); Genekam, Germany) following the manufacturer's protocol. Buccal swabs were used for the dental pathogen *Streptococcus mutans*, as previously described (19). Each individual sample was anonymized and represented by a number, thus no ethics committee approval was required. The DNA positive control for *Chlamydia trachomatis* (Genekam) was used.

PCR was performed using a thermocycler (Biometra, Germany). Negative controls were included in each run to ensure accurate pipetting and to monitor for potential false-positive results. For each reaction, 2 µl of isolated DNA was added to 18 µl of a mastermix solution (Genekam) containing primers, resulting in a total reaction volume of 20 µl per microtube.

These experiments were repeated at least 20 times with the same primers over a period of five years or more. Primers were dissolved in DPEC-treated water to achieve a concentration of 100 pmol/µl (100 µM) and were stored at -20°C after each thawing and use.

Cycling Conditions

The PCR cycling conditions for each pathogen were as follows:

- ***Streptococcus mutans:***

Initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 1 minute. A final extension step at 72°C was performed for 5 minutes.

- ***Chlamydia trachomatis:***

Initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. A final extension step at 72°C was performed for 5 minutes.

Electrophoresis

The PCR products were subjected to electrophoresis on a 1.5% agarose gel (Carl Roth, Germany) for 40 minutes at 120 volts. The gel was stained with ethidium bromide (Carl Roth) to visualize the DNA bands, which were observed at 479 bp for *Streptococcus mutans* and 188 bp for *Chlamydia trachomatis*.

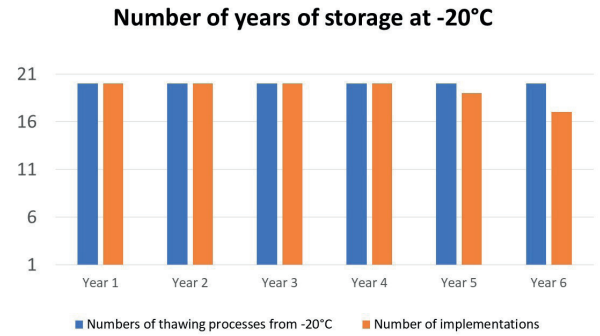
Primer Information

The exact sequences of the primers cannot be disclosed in this work, as they are part of the commercial kits developed by our

laboratory. The oligonucleotides used in this study were not purified by HPLC or similar methods.

RESULTS

The results are summarized in Graph 1. The primers stored at -20°C for both *Chlamydia trachomatis* and *Streptococcus mutans* remained functional in all tests for at least five years, even after undergoing a minimum of 20 freeze-thaw cycles. Throughout this period, the PCR tests consistently produced clearly visible bands on the agarose gel (Figures 1 and 2).



Graph 1. Performance of *Chlamydia trachomatis* and *Streptococcus mutans* Oligonucleotides Over the Storage Period at -20°C

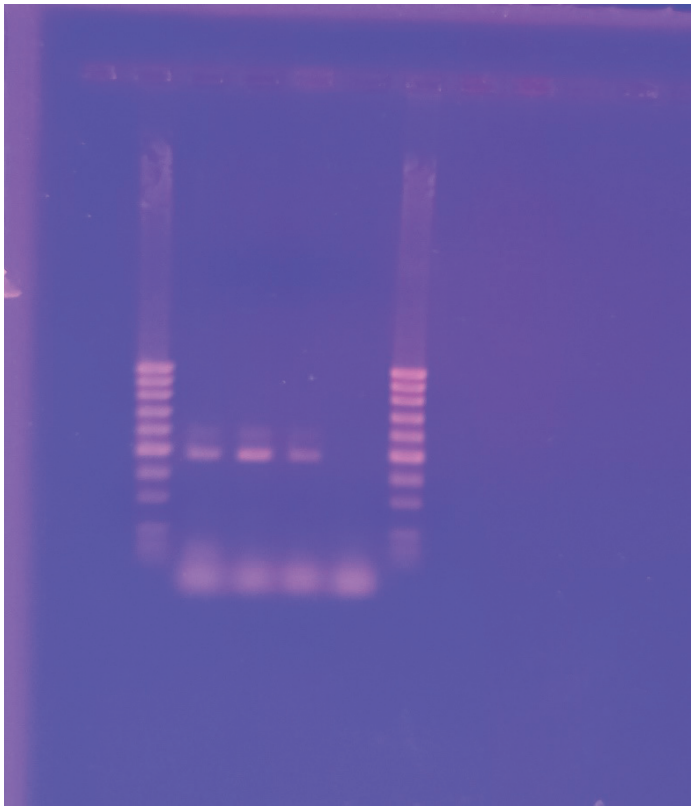


Figure 1. Agarose gel electrophoresis results for *Streptococcus mutans*; ***Lane 1-3:*** Positive samples showing clear bands at 479 bp; ***Lane 4:*** Negative control, showing no band, confirming the absence of cross-contamination or false-positive results

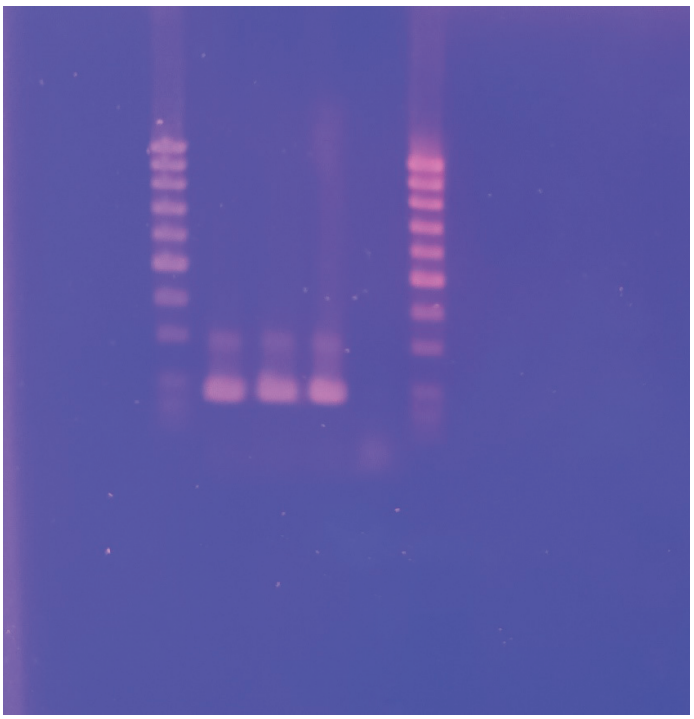


Figure 2. Agarose gel electrophoresis results for *Chlamydia trachomatis*; ***Lane 1-3:*** Positive samples showing clear bands at 188 bp; ***Lane 4:*** Negative control, showing no band, confirming the absence of cross-contamination or false-positive results

After five years, the primers began to show signs of reduced activity, as evidenced by weak or diffused bands in positive samples on the agarose gel. However, upon synthesizing new primers, the repeated PCR tests once again produced strong bands of the correct size, indicating full restoration of PCR performance.

The results of the negative control showed no bands, confirming the absence of cross-contamination or false-positive results.

Over the course of five years, with at least 20 freeze-thaw cycles, a decrease in the sensitivity of the PCR tests was observed, indicating a gradual loss of primer activity. After multiple cycles of thawing and freezing, the primers began to produce diffuse or no bands on the agarose gel, highlighting the diminished effectiveness. This loss of activity was particularly noticeable after the primers had been thawed and frozen beyond their shelf life, where even one or two thaw cycles resulted in suboptimal PCR performance.

Despite this, the primers for *Streptococcus mutans* were successfully used to conduct diagnostics on an oral swab taken during the SARS-CoV-2 illness phase of the testing individual. The primers produced strong bands on the agarose gel, which were subsequently isolated and sequenced (data not shown, as it is part of another publication). This indicates that older primers may still be functional for gene sequencing, even after extended storage.

DISCUSSION

In this study, we demonstrated that the shelf life of primers used in conventional PCR exceeds five years. These findings contrast with the claims of various primer synthesis companies, which typically state that dissolved primers have a shelf life of six months to three years when stored at -20°C (10-17). Despite the importance of this issue, it has largely been overlooked by laboratories that are certified to perform diagnostics and genetic tests, such as parentage testing.

This study specifically focused on primers used in conventional PCR, and as such, the findings may not be applicable to other oligonucleotides, such as probes for real-time PCR, antisense oligos, or other molecular applications. Therefore, these results should be primarily considered for research and diagnostics involving conventional PCR.

Our findings indicate that some primers can be used for up to seven years without any issues, provided they are properly thawed and consistently stored at -20°C. In contrast, many primers lost their activity after being stored at 4°C for several days and repeatedly thawed and refrozen during that period (data not shown). Therefore, the results of this study specifically apply to dissolved primers at a concentration of 100 pmol/µl stored at -20°C.

Additionally, we have observed that some primers, over 10 years old, are still functional in ongoing research, suggesting that primer longevity may extend beyond five years under optimal conditions. A critical factor contributing to this stability is the rarity of electricity outages in Germany, which ensures consistent storage temperatures. In regions where electricity cuts are more frequent, fluctuations in storage temperature may negatively impact primer shelf life, potentially shortening their effective use.

Based on our findings, we recommend thawing primers and using them within approximately 45 minutes. If additional studies are required, primers can be temporarily stored in a dedicated refrigerator for 4-5 hours, after which they should be returned to -20°C for long-term storage.

This research has the potential to help many institutions and companies reduce costs. Diagnostic companies, in particular, may benefit by extending the shelf life of their ready-to-use PCR kits, though further testing of other components is necessary to ensure overall kit stability.

We strongly recommend that test kit manufacturers and certified laboratories conduct their own studies to determine the shelf life of their oligonucleotides, as individual conditions and formulations may vary.

In future research, we plan to investigate the shelf life of probes used in real-time PCR testing.

CONCLUSION

In this study, we tested the shelf life of primers used for conventional PCR. Our results demonstrate that frozen primers can be effectively used for up to five years, contradicting the general belief that primers remain stable for only one year. These findings can help molecular laboratories optimize their resources and reduce unnecessary expenses.

Conflict of Interests
The authors declare that there is no conflict of interest in the study.

Financial Disclosure
The authors declare that they have received no financial support for the study.

Ethical Approval
Ethics committee Approval is not needed as each individual sample was anonymized and represented by a number.

Additional Contributions
Ms. Kornelia Niklis assisted with laboratory work, and Mr. Helge Schreiner contributed to graphic design.

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