UDC 577.21:578.1 https://doi.org/10.15407/biotech18.02.086

EVALUATION OF NUCLEIC ACID EXTRACTION METHODS FOR PATHOGEN DETECTION USING PCR

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Received 2025/03/06
Revised 2025/03/16
Accepted 2025/04/30

This work focuses on evaluating nucleic acid extraction methods and optimizing lysis buffer components to enhance nucleic acid yield and minimize the impact of potential inhibitors on target gene amplification to improve the detection of causative pathogens and facilitate their integration into diagnostic practice.

Aim. To compare nucleic acid extraction methods from patient biological samples and to optimize lysis buffer composition to enhance nucleic acid yield.

Methods. Spectrophotometric and fluorometric methods were used to assess the efficiency and quality of nucleic acid extraction. The concentration of the obtained nucleic acids was measured using a Qubit 4 fluorometer. The quality of the extracted nucleic acids was analyzed by electrophoretic separation in agarose gel, which allowed for the assessment of their integrity and the presence of degradation. To evaluate the inhibition of target genes by extraction agents, real-time PCR was applied.

Results. Solid-phase extraction using silica-coated magnetic particles demonstrated superior performance compared to liquid-liquid extraction. Optimal lysis conditions were achieved using 2.7 M guanidinium thiocyanate with 30% isopropanol, which enhanced DNA recovery and inhibitor removal. The addition of glycogen-linear polyacrylamide improved DNA precipitation in the liquid-liquid method. Overall, the solid-phase approach showed better amplification efficiency and nucleic acid yield.

Conclusions. It was established that solid-phase extraction methods based on silica-coated magnetic carriers are optimal for isolating nucleic acids from patients' biological samples for pathogen differentiation.

Keywords: nucleic acid extraction, pathogen identification, polymerase chain reaction, diagnostics.

Given the increasing importance of pathogen diagnostics, particularly under conditions of active warfare, there is a need to refine and implement rapid and selective methods for pathogen identification applicable at the early stages of infection. Compared to traditional bacteriological methods, which require 24 to 72 hours, real-time polymerase chain reaction (PCR) enables significantly faster results — within a few hours, which is critically important for patients in severe condition [1, 2]. PCR is a highly sensitive method capable of rapidly detecting pathogens, however, its effectiveness depends on the quality of the prepared nucleic acid sample. Despite the widespread use of solid-phase and liquid-liquid extraction of nucleic acids in clinical practice, both methods require adaptation and optimization according to the biological properties of the specific pathogen and the source of biological material.

Citation: Prokhorenko, D. K., Grynyuk, I. I. (2025). Evaluation of nucleic acid extraction methods for pathogen detection using PCR. *Biotechnologia Acta*, 18(2), 86–88. https://doi.org/10.15407/biotech18.02.086

Aim. To compare nucleic acid extraction methods from patient biological samples and to optimize lysis buffer composition to enhance nucleic acid yield.

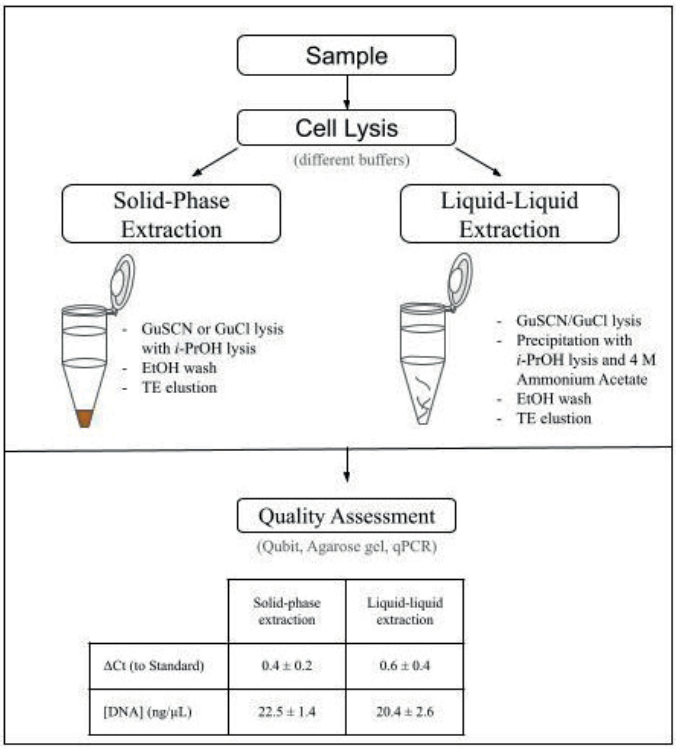
Methods. The concentration of the obtained nucleic acids was measured using a Qubit 4 fluorometer (Thermo Fisher Scientific, USA) [3]. The quality of the extracted nucleic acids was analyzed by electrophoretic separation in agarose gel [4]. To evaluate the inhibition of target genes by extraction agents, real-time PCR was applied. Nucleic acid amplification was performed using the Bio-Rad CFX96 Touch^{TM} Real-Time PCR Detection System in a two-phase thermal cycling mode. The amplification protocol included an initial denaturation at 94 °C for 3 minutes, followed by 9 cycles consisting of denaturation at 94 °C for 20 seconds, annealing at 58 °C for 30 seconds, and elongation at 72 °C for 30 seconds. This was followed by 38 additional cycles with shortened durations: 94 °C for 15 seconds, 58 °C for 15 seconds, and 72 °C for 15 seconds, with fluorescence measurement conducted during the elongation step. The total number of cycles was 47. Fluorescence analysis was performed during the elongation phase of the second block-phase.

Results and Discussion. The study compared two nucleic acid extraction methods of solid-phase extraction using silica-based sorbents on magnetic particles and liquid-liquid extraction — to assess their efficiency and potential for automation.

A human plasma sample spiked with a known quantity of target DNA (10^4 copies) was used for extraction. Real-time PCR was performed with a reference standard equivalent to 10^4 copies of the target fragment, providing a benchmark Ct value of 22.4 under the defined threshold conditions. An analysis of how the extraction method affects target gene amplification (see Table) showed that solid-phase extraction using silica-coated magnetic particles resulted in higher nucleic acid copies count and less inhibition of probe (Ct = 22.8 ± 0.2), improved purity (A260/280 = 1.91), less time consumption and was more suitable for PCR automation.

Comparison of solid-phase and liquid-liquid nucleic acid extraction methods (n = 5)

Extraction method	A260/280	Ct (qPCR)	Time (min)	DNA Degradation Level
Solid-phase extraction	1.91	22.8±0.2	40	Low
Liquid-liquid extraction	1.73	23.0±0.4	60	Moderate



Schematic representation of the research results

To ensure efficient disruption of the cell membrane and release of nucleic acids, different types of lysis buffers containing chaotropic agents – solutions of guanidinium thiocyanate (1.5 M–6 M) and guanidinium chloride (1.5 M–5 M) in isopropanol (0–40%) were tested. Real-time PCR analysis showed that the absence of an organic phase significantly reduced the amount of recoverable target DNA template.

The use of urea and alkali metal thiocyanates had no notable effect on DNA yield, while alkaline conditions negatively affected RNA stability. The most effective lysis buffers contained 2.7 M guanidinium thiocyanate in 30% isopropanol, which facilitated both the removal of PCR inhibitors and the precipitation of nucleic acids. In the case of liquid-liquid extraction, the addition of glycogen-linear polyacrylamide (4 mg/mL) proved to be an efficient co-precipitant for improving DNA recovery.

The figure presents the main steps of solid-phase and liquid-liquid DNA extraction methods, including cell lysis using guanidinium-based buffers, washing steps, elution, and subsequent quality assessment using qPCR and spectrophotometry.

Comparative evaluation of solid-phase and liquid-liquid nucleic acid extraction methods based on ΔCt values (relative to standard) and DNA concentration (ng/μL) confirmed the advantage of the solid-phase method in both the quantity of recovered nucleic acids and their amplification efficiency.

Conclusions. It was established that solid-phase extraction using silica-coated magnetic particles is optimal for isolating nucleic acids from patient biological samples for downstream pathogen identification. The optimal extraction conditions include the use of lysis buffers containing 2.7 M guanidinium thiocyanate and 30% isopropanol, which increase DNA yield and reduce PCR inhibition.

Authors' contribution

D.K. Prokhorenko conducted biochemical studies, processed and analyzed the obtained results, wrote the abstract, I. I. Grynyuk analyzed the data and edited the text.

Funding source

The work was supported by the LLC "XEMA", Ukraine.

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