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Optimized enzymatic cell lysis protocols for single-cell genomic analysis

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Abstract

Optimizing cell lysis is essential for reliable single-cell genomic analysis. This study sought to refine enzymatic lysis conditions to minimize incubation time while maintaining DNA integrity. Microdissected human leukocytes were digested with proteinase K under various incubation durations (2–16 h) and temperatures (37–60 °C), and genomic DNA recovery was assessed through Y-chromosome–specific PCR amplification. Shorter incubation periods combined with elevated temperatures, particularly at 60 °C, achieved amplification efficiencies comparable to or greater than those obtained with the conventional overnight digestion at 37 °C. Statistical analysis revealed no significant differences between the optimized and standard conditions. These results indicate that enzymatic lysis duration can be substantially reduced without compromising DNA yield or quality. The proposed approach enables same-day genomic preamplification and enhances the throughput of single-cell workflows, offering practical advantages for clinical diagnostics and forensic genetics.

Keywords: Single-cell analysis, Enzymatic lysis, Proteinase K, PCR amplification, DNA extraction.

1. Introduction

Cell lysis is a critical step in extracting genetic material from cells for downstream applications, such as PCR-based genomic analysis.

Recent advancements in single-cell lysis techniques have focused on improving efficiency, reducing lysis time and increasing the sensitivity of downstream genomic analysis. Notable innovations include microfluidic devices and electroporation-based lysis approaches, which have been used to enhance single-cell genomic analysis, including minimal DNA degradation and compatibility with automated systems for high-throughput analysis [1, 2].

A research study introduced a lysis-on-screen approach for microorganisms, facilitating the discovery of beneficial enzymes from environmental samples. This system allows controlled cell disruption, enabling researchers to selectively lyse target cells while preserving intact cells for subsequent analyses [3].

However, current methods of Droplet microfluidics on single cells require multiple microfabricated devices for enzymatic cell lysis and PCR reagent addition [4].

In addition to microfluidic and electroporation, expensive techniques, new research has also focused on optimizing direct cell lysis protocols for single-cell gene expression analysis. A 2013 study compared several direct cell lysis protocols and identified bovine serum albumin (BSA) as an effective agent for preserving RNA integrity in single cells [5].

The study found that BSA-lysis allowed for efficient RNA extraction and subsequent quantitative PCR, making it an excellent choice for gene expression studies from single cells. This development has implications for applications such as transcriptomics and single-cell RNA sequencing, where maintaining RNA integrity is crucial for accurate results.

A study published in 2024 evaluated the impact of enzymatic lysis on DNA extraction and microbial community analysis. Using MetaPolyzyme, a chemical cell lysis treatment, researchers observed significant shifts in the detectability of microbial composition in saliva samples. The treatment favored the detection of Gram-positive bacteria such as Streptococcus and the distribution of antimicrobial resistance genes. These findings highlight the importance of optimizing cell lysis methods to accurately characterize microbial communities [6].

A combined enzymatic and alkaline lysis method was evaluated for separating sperm and epithelial cells in forensic samples. The study demonstrated that this approach

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is expedited, cost-effective and improves DNA isolation efficiency in forensic investigations [7].

A comparative evaluation of lysis methods revealed that enzymatic lysis yields higher DNA quantities but may compromise quality compared to mechanical methods. This underscores the need for optimization based on specific experimental requirements [8].

In recent years, various novel technologies have been employed to develop new and innovative approaches to cell lysis, such as freeze-thawing, bead beating, incursion in liquid nitrogen, sonication, enzymatic approaches and chemical-based approaches. Despite the significant progress in this field, numerous challenges remain requiring continued enhancements of available tools [9].

Studies in this field aim to introduce more precise and efficient tools or to reduce the costs of cell lysis procedures.

Lysis protocols vary depending on the type of cells, the purpose of the study and the subsequent use of the extracted DNA. This focuses on enzymatic methods of cell lysis used in single-cell genomic research, on their applications to microdissected cells genome amplification efficiency.

This study aimed to optimize enzymatic lysis for DNA extraction by minimizing incubation time, thereby reducing risks to DNA integrity and downstream analyses. Shorter lysis periods provided sufficient cell disruption and DNA recovery, enabling reliable genomic amplification. This approach is particularly relevant for single-cell studies, where low DNA yield and degradation often cause allele dropout in PCR.

2. Materials and methods

The two primary methods of cell lysis for genomic extraction are alkaline lysis and enzymatic lysis. Both techniques aim to break down the cellular membrane and release the genomic DNA while minimizing degradation and contamination.

2.1. Lysis of the single cell

Lysis could be achieved by different approaches. Alkaline lysis was performed by applying 5 µl of a solution containing 200 mM KOH and 50 mM dithiothreitol to the cell, and the assembly was subjected to a 10-minute incubation at 65 °C. The lysate was then neutralized with a buffer (900 mM Tris-HCl, 300 mM KCl). Enzymatic lysis was carried out in a final volume of 15 µl (100 mM Tris-HCl, pH 8 and 1 mg/ml of proteinase K (Thermo Fisher Scientific, Waltham, MA, USA)). Both approaches released DNA from their protein environment; however, extraction was deliberately omitted in order to minimize genomic loss.

To access the genetic information of the cell, protein lysis of each microdissected cell was performed. The product of microdissection was directly contacted with the lysis solution. Indeed, the two main methods of cell lysis performed on a single cell were alkaline lysis (KOH) and enzymatic lysis (proteinase K). The first method was the method of choice for performing lysis on fresh cells, which were obtained for a genetic test, because the time required was very short (10 minutes at 65 °C) [1].

This lysis was normally performed at the bottom of the PCR tube, since the recovered single cell was visualized under a microscope and transferred to the bottom of the tube, which ensured direct contact with the lysis buffer even if the final volume of lysis buffer was small (5 µl or 3 µl in some laboratories). However, in the case of fixed cells on a membrane or filter, we were forced to perform lysis of the cell attached to the cap (adhesive membrane) of the tube in an incubator, and the tubes were turned upside down, hence the need for using a higher volume of lysis buffer (15 µl) to ensure contact between the lysis medium and the cell. Furthermore, the isolated cells were not fresh, which led to a prolonged time to achieve a "complete" cell lysis.

2.2. Alkaline lysis

Alkaline lysis was a commonly used technique, particularly for fresh cells, due to its simplicity and speed. This method involved the application of a lysis buffer containing 200 mM KOH and 50 mM dithiothreitol (DTT) to the microdissected cell. The solution was then incubated at 65 °C for 10 minutes. This process effectively disrupted the cell membrane, releasing DNA into the solution. Following lysis, the reaction was neutralized with a buffer containing 900 mM Tris-HCl and 300 mM KCl.

The alkaline environment allowed for the rapid extraction of DNA, minimizing the time for genome degradation, a critical element when working with single cells.

Alkaline lysis was ideal for fresh cells, especially when rapid genomic analysis was required. This method had become the standard for single-cell studies, as the lysate could be directly used for PCR amplification. The protocol was particularly effective for cells that could be easily accessed, such as those recovered from microdissection. The process involved transferring the cell to the PCR tube where it directly contacted the lysis solution, ensuring complete lysis in a minimal volume (typically 5 µl). However, when cells were fixed on membranes or filters, larger volumes of lysis buffer (15 µl) were used to ensure adequate contact between the buffer and the cells.

2.3. Enzymatic lysis

Enzymatic lysis was another widely used method, especially when dealing with more complex cellular structures or fixed cells. In this approach, proteinase K was used to digest proteins and other cellular components, thereby releasing DNA. The lysis buffer contained 100 mM Tris-HCl (pH 8) and 1 mg/ml of proteinase K, with a final reaction volume of 15 µl. Enzymatic lysis was typically performed at 37 °C for 16 hours.

While this method was highly effective, the prolonged incubation period required for proteinase K to act on the cellular proteins was a drawback. Extended incubation could lead to DNA degradation, reducing the quality and quantity of the genetic material obtained, which could result in allele dropout during PCR amplification. To compare the effectiveness and applicability of different single-cell lysis techniques, we summarized the key characteristics of alkaline and enzymatic lysis in Table 1. This comparison highlighted differences in buffer composition, sample type compatibility, incubation conditions, advantages, limitations, and typical applications. Such information could guide the choice of lysis method depending on the nature of the sample and the downstream genomic analysis.

The study was performed on leukocyte cells fixed on a filter (porous polycarbonate membrane), and peripheral blood samples were collected from male individuals in EDTA tubes. Red blood cells were lysed using a stan-

Table 1. Comparison of two common single-cell lysis methods.

Feature	Alkaline Lysis	Enzymatic Lysis
Buffer composition	200 mM KOH + 50 mM DTT	100 mM Tris-HCl (pH 8) + 1 mg/mL proteinase K
Sample type	Fresh cells	Fixed or complex cells
Incubation conditions	65°C for 10 min	37 °C for 16 h
Volume	5 µl (fresh cells), 15 µl (fixed cells)	15 µl
Advantages	Rapid, simple, minimal genome degradation, lysate ready for PCR	Effective for fixed or structurally complex cells, high DNA recovery
Limitations	Not suitable for fixed cells, may require larger volumes for filters	Long incubation time, risk of DNA degradation and allele dropout
Applications	Standard single-cell PCR, rapid genomic analysis	Single-cell genomic studies require robust lysis

Alkaline lysis and enzymatic lysis, highlighting their buffer composition, compatible sample types, incubation conditions, reaction volumes, advantages, limitations, and typical applications in genomic studies.

dard buffer, and leukocytes were concentrated by filtration through an 8 µm porous polycarbonate membrane.

After filtration, membranes were rinsed with 1 mL PBS, air-dried, and stained with hematoxylin-eosin (HE) (Sigma-Aldrich, USA) to allow visualization of leukocytes under the microscope.

Prepared membranes were stored at –20 °C until analysis. Target leukocytes were isolated by laser microdissection using a NIKON microscope (Nikon Instruments Inc., Tokyo, Japan). Interesting cells may be subject to molecular analysis by PCR to clarify conclusively the origin and nature of the cell for the diagnostic or scientific study.

The study of enzymatic lysis by proteinase K applied to single cells comprises the composition of the lysis buffer, the lysis time and temperature of incubation. Enzymatic lysis was performed in 15 µl final volume at 37 °C for 16 h [10, 11].

Despite the demonstrated effectiveness of the lysis protocol, we have realized that the incubation period was overnight. This can reduce and alter the cellular genome, and the risk of allele dropout during PCR was the possibility to reduce the duration of lysis in order to perform genomic preamplification directly after cell lysis on the same day and improve the quality of amplification. To do this, several protocols were used without changing the composition of the lysis buffer (15 µl). The tested cells derived from the same male individuals for whom blood was treated as described above, leukocytes "selected" on the filter were stained with hematoxylin-eosin (HE) and microdissected by the NIKON system.

To minimize DNA loss, no separate extraction step was performed. Instead, lysates were used directly for PCR amplification. Y-chromosome–specific PCR (Y-PCR) was applied to evaluate genome recovery. The percentage of successful Y-PCR amplifications was recorded as the main measure of lysis efficiency.

For each protocol, 100 cells and evaluated to determine the efficiency of genomic amplification by quantifying the number of successfully amplified genomes using Y PCR. On the other hand, while enzymatic lysis provided high-quality genomic material, the longer incubation period required for proteinase K digestion (16 hours) posed challenges in terms of timely results and DNA integrity. To address this, we explored modified enzymatic protocols with reduced incubation times.

3. Results

Enzymatic lysis of cells (n = 100 per condition) was performed for 2, 4, or 16 hours at 37 °C, 50 °C, or 60 °C. The proportion of cells yielding a positive Y-chromosome-specific PCR (Y-PCR) signal was consistently high across all incubation times and temperatures (Table 2). Across all conditions, enzymatic lysis resulted in comparable Y-PCR detection rates, with no significant differences observed between treated and control groups. These results indicate that variations in incubation time and temperature within the tested range did not substantially affect the success of enzymatic lysis for Y-PCR detection.

3.1. Descriptive statistics

Statistical Analysis of genomic amplification by quantifying the number of successfully amplified leucocyte

Table 2. Enzymatic cell lysis protocol with different conditions.

No of cells	Duration (hours)	Temperature(C°)	Nb of positive cells by Y PCR (%)	Difference of control tube
100	16 h	37	80	NS
100	4 h	37	70	NS
100	2 h	37	63	NS
100	4 h	50	74	NS
100	2 h	50	71	NS
100	4 h	60	82	NS
100	2 h	60	85	NS
				NS: non significance

Cells (n = 100 per condition) were subjected to enzymatic lysis for different durations (2, 4, or 16 h) at temperatures of 37 °C, 50 °C, or 60 °C. The efficacy of lysis was assessed by quantifying the percentage of cells yielding a positive Y-chromosome–specific PCR (Y-PCR) signal. Control tubes consisted of untreated cells processed under the same PCR conditions. Differences between test and control groups were statistically evaluated; no significant (NS) differences were observed.

genomes using Y PCR. This analysis represents the effect of temperature and duration on the percentage of positive Y PCR results in DNA leucocyte cells.

The mean percentage of positive PCR tends to increase with temperature. Variability is higher at 37 °C compared to 50 °C and 60 °C (Table 2). Statistical analysis revealed no significant differences between any of the test conditions and the untreated controls, indicating that lysis duration and temperature within the tested ranges did not significantly influence PCR detection efficiency. Hypothesis Testing (ANOVA); Effect of Temperature: p-value = 0.185 (Not statistically significant); Effect of Time: p-value = 0.803 (Not statistically significant)

3.2. Graphical analysis

The following diagrams (Figures 1 and 2) illustrate the impact of temperature and time on PCR positivity: The effect of incubation temperature on enzymatic lysis efficiency was first assessed by combining data from 2 h and 4 h treatments. Boxplot analysis (Figure 1) indicated that the percentage of Y-PCR–positive cells was comparable across 37 °C, 50 °C, and 60 °C, with no statistically significant differences observed (NS, $p > 0.05$).

The combined influence of incubation time and temperature was further examined using scatter plot analysis (Figure 2). Cells lysed for 2, 4, or 16 hours at 37 °C, 50 °C, or 60 °C all yielded similarly high percentages of Y-PCR–positive cells. Color-coded data points representing different temperatures showed no significant trends or differences between conditions (NS, $p > 0.05$), indicating that variations in both incubation duration and temperature within the tested ranges did not significantly affect enzymatic lysis efficiency.

3.3. Statistical analysis

A statistical evaluation was conducted to determine the influence of incubation temperature and duration on genomic amplification efficiency in single leukocytes subjected to enzymatic lysis. The proportion of Y-chromosome–positive PCR reactions was used as the dependent variable, while temperature (37 °C, 50 °C, 60 °C) and incubation duration (2–16 h) were considered independent variables.

Descriptive analysis indicated that the mean percentage of positive PCR reactions increased with higher lysis temperatures. At 37 °C, amplification success ranged from 63% after 2 h to 80% after 16 h. Increasing the temperature to 50 °C resulted in positivity rates between 71% and 74%, while 60 °C yielded the highest recovery (82–85%) even at shorter incubation times. Variability in PCR positivity was greater at 37 °C and decreased progressively with increasing temperature, suggesting improved consistency under elevated thermal conditions (Table 2).

Graphical analysis confirmed these observations. The boxplot (Figure 1) demonstrated a positive association between temperature and amplification success, whereas the scatter plot (Figure 2) revealed no clear correlation between incubation time and PCR outcome, indicating that shorter lysis periods can be as effective as prolonged ones when higher temperatures are applied.

Inferential testing was performed using one-way analysis of variance (ANOVA) to compare the effects of temperature and incubation time on PCR positivity. The results showed no statistically significant effect of temperature ($p = 0.185$) or duration ($p = 0.803$) on amplification efficiency. These findings suggest that increasing lysis temperature to 50–60 °C permits a marked reduction in incubation time—from 16 h to as little as 2 h—without compromising DNA recovery or amplification quality.

All statistical analyses were carried out using SPSS version 27.0 (IBM Corp., Armonk, NY, USA). A significance threshold of $p < 0.05$ was adopted for all tests.

4. Discussion

Researchers have explored combining enzymatic lysis with mechanical methods to enhance lipid extraction or highlighting the potential of combining enzymatic lysis with physical aids for rapid cell disruption [12, 13].

However, the adequacy of cell lysis is important factor affecting the number and types of proteins identified in single-cell proteomic research [14].

The choice of protocol depends on the cell type, the condition of the cells (fresh or fixed), and the need for

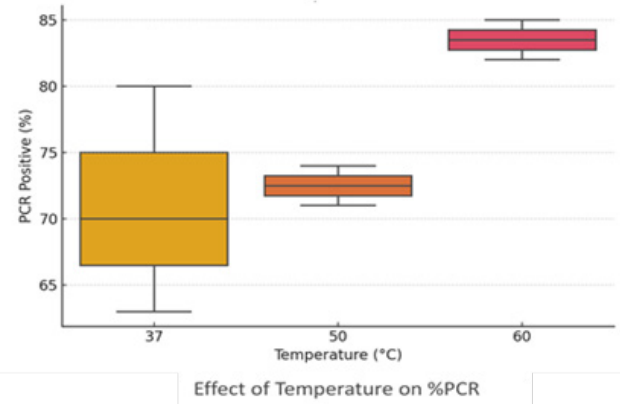


Fig. 1. Effect of temperature on the percentage of Y-PCR positive cells following enzymatic lysis. Boxplot analysis showing the distribution of Y-PCR–positive cells (%) after enzymatic lysis at 37 °C, 50 °C, and 60 °C. Each temperature group includes combined data from 2 h and 4 h incubation times. Median values, interquartile ranges, and data ranges are shown. No statistically significant differences were observed between conditions (NS, $p > 0.05$).

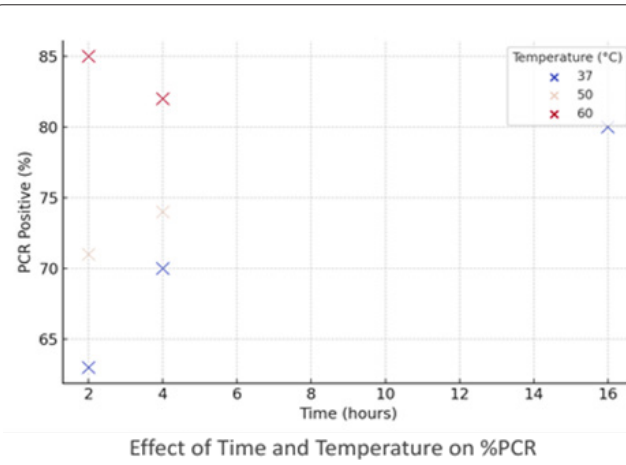


Fig. 2. Combined effect of incubation time and temperature on Y-PCR positivity following enzymatic lysis. Scatter plot showing the percentage of Y-PCR–positive cells after enzymatic lysis of 100 cells under various time (2, 4, and 16 hours) and temperature (37 °C, 50 °C, and 60 °C) conditions. Data points are color-coded by temperature. Each point represents a single experimental condition. No statistically significant differences were observed (NS, $p > 0.05$).

rapid results. The efficiency of genome amplification from single cells is influenced by the lysis protocol, as incomplete or delayed lysis can lead to inconsistent results. Enzymatic lysis with proteinase K is beneficial for fixed or more complex samples. However, optimizing the lysis time, especially for enzymatic protocols, is critical to minimize DNA degradation and improve the accuracy of genetic analyses.

The results showed that by decreasing the enzymatic lysis time and increasing temperature to 60 °C while maintaining the buffer composition, we could still achieve satisfactory amplification efficiency. These optimized protocols are crucial for improving the quality of genomic data obtained from single-cell samples. Despite the demonstrated effectiveness of the lysis protocol, we have realized that if the incubation period is long (overnight), this can reduce and alter the cellular genome and the risk of allele dropout during PCR.

The observed robustness of enzymatic lysis across different temperatures and incubation times can be attributed to the intrinsic stability of proteinase K, which remains active over a broad range of conditions. Additionally, the structural composition of the cell membrane and fixation-induced cross-linking may require only partial enzymatic digestion for effective lysis, making the process less sensitive to minor variations in temperature or incubation duration. This highlights the reliability of enzymatic lysis for fixed or structurally complex cells in single-cell genomic studies.

While only moderate temperature and incubation time ranges were tested in this study (37–60 °C; 2–16 h), future work could explore broader conditions and alternative enzymes or cell types. Such studies would help to define the limits of enzymatic lysis efficiency and further optimize the method for diverse single-cell applications.

This approach has improved the efficiency of PCR in order to maximize the number of genomes amplified and eliminate the risk of contamination. The genetic test result (from a single cell) based on PCR techniques has more accuracy and reliability of genomic information. We believe that this molecular technique of fixed single cells can be applied to try the clinical and molecular diagnosis of cancer or fetal cells.

The efficiency of single-cell genomic analysis depends strongly on the choice of lysis protocol. Our results demonstrate that shorter incubation times combined with higher temperatures (up to 60 °C) can maintain or even improve amplification efficiency compared with the standard overnight protocol at 37 °C. The data suggest that increasing temperature and duration may improve PCR positivity, but the statistical significance is weak.

Conflict of interest
All authors have to declare their conflicts of interest.

Consent for publications
All authors have to write this sentence that they read and approved the final manuscript for publication.

Ethical approval
The authors declare that the study did not involve human participants or animal experimentation. Human-derived cells were used exclusively for in vitro analyses. All experimental procedures were conducted in accordance with the ethical principles of the Declaration of Helsinki and institutional guidelines for the use of human biological materials in research.

Availability of data and material
The authors declare that they have incorporated all data into the manuscript.

Author contributions
Conceptualization; H.M., H.R.; methodology, all authors; validation, H.M., H.R., H.B.S., A. A., H.A; formal analysis, all authors; investigation, All authors; resources, all authors; data curation, all authors; writing—original draft preparation, H.M., H.R., H.B.S., A. A., H.A; writing—review and editing, H.M., H.R., H.B.S., A. A., H.A; supervision, H.M., H.R.; project administration, H.R. All authors have read and agreed to the published version of the manuscript.

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