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TECHNICAL NOTE

Criminalistics

A quantitative method to assess DNA extraction efficiency

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Abstract

Obtaining an accepted minimum amount of extracted and purified DNA is critical for generating complete forensic short tandem repeat (STR) profiles. Certain sample types encountered in forensic laboratories may pose challenges in obtaining the desired target amount of extracted DNA, which can be compounded when an extraction yields low recovery, resulting in a loss of the genetic material necessary for downstream forensic DNA typing. While multiple studies have evaluated the impact of extraction chemistries on yield, they often focus on "end-to-end" profiling success rather than quantifying the discrete loss of DNA during the extraction step. This study presents a quantitative framework using digital PCR (dPCR) to benchmark extraction efficiency by comparing known amounts of DNA pre- and post-extraction. Extraction efficiency was evaluated using silica spin column and magnetic resin-based protocols across five input amounts, with three sample types: whole blood, human cells, and pre-extracted DNA. Results demonstrate significant differences in efficiency between protocols for cellular samples, with both methods exhibiting increased variability at the 1 ng threshold. This data also revealed that previously extracted DNA (SRM 2372a) fails to accurately represent cellular extraction dynamics, serving instead as a control for purification-related loss (e.g., column retention) rather than lysis efficiency.

KEYWORDS
digital PCR, DNA extraction, extraction efficiency, extraction efficiency measurement, magnetic bead-based extraction, silica-based extraction

Highlights

- Extraction efficiency is studied to determine how much DNA is recovered during the extraction process.
- Extraction efficiency can differ depending on the protocol used and the source of the DNA.
- Offers a standardized benchmarking method for assessing forensic DNA extraction efficiency.

1 | INTRODUCTION

Since the onset of DNA evidence being collected, processed, and analyzed in forensic laboratories, an extensive amount of research has been published evaluating common extraction protocols used in forensic DNA analysis. However, most of these evaluations are based on the extent to which a complete STR profile can be obtained following subsequent PCR amplification and capillary electrophoresis of extracted samples (i.e., counting recovered alleles). These studies do not examine solely the effects of extraction on the amount of DNA

successfully purified and retained for downstream processes, but rather the workflow as a whole [1, 2]. These types of studies provide valuable information and are more reflective of casework, as the amount of DNA present before extraction is typically unknown; however, they limit the ability to gauge the extraction kit's isolated efficiency when chemistry alone is used in these scenarios. Using known amounts of DNA and isolating the extraction step while obtaining recovery measurements can offer substantial insight into how extraction efficiency is affected by a chosen set of variables and help pinpoint the root cause of large amounts of sample loss and variability [3, 4]. Once a DNA sample is collected and has entered the laboratory, research has shown that extraction is generally where the sample is most susceptible to significant loss in quantity [5]. However, the extent of this loss is dependent on many variables, including sample source, quantity, and extraction method, making further knowledge of the extent to which these variables impact extraction efficiency crucial [6, 7].

This study aims to provide a method to assess the extraction efficiencies of techniques commonly employed in forensic DNA laboratories with three extraction variables to ascertain their impact(s) on recovery: (1) two different protocols utilizing different chemistries (manual and automated), (2) three DNA sources (fibroblast cells, whole blood, and previously extracted DNA), and (3) a range of starting amounts of the estimated DNA quantity (1, 5, 10, 20, and 50 ng). To systematically approach this, known amounts of input DNA were examined in a broad range and were not meant to replicate casework samples directly. By isolating the extraction step, we provide laboratories with a reproducible approach to assess how specific protocols perform across various sample types. Furthermore, the use of pre-extracted DNA provides a baseline for isolating the inherent DNA loss occurring during the chemical and mechanical phases of the extraction process. By distinguishing between the loss of "naked" DNA and the loss associated with cellular samples, laboratories can more accurately troubleshoot whether recovery issues stem from protocol chemistry or inefficient sample preparation. Other factors, including substrate, sample collection method, sample storage conditions, and degradation levels, were not examined in this study. While a broad body of work examines some of these factors, this study aimed to control additional variables to examine extraction efficiencies [8–10]. By extracting samples containing known amounts of DNA, an average extraction efficiency can be obtained. Quantitative data on specific extraction step losses allow forensic laboratories to pinpoint process inefficiencies separate from sampling or amplification limitations. The framework proposed here serves as a diagnostic tool that decouples the extraction process from downstream amplification.

2 | MATERIALS AND METHODS

2.1 | DNA sources

Whole blood was purchased from Interstate Blood Bank (Memphis, TN) with a reported white blood cell count. The blood bank determined the concentration of white blood cells using flow cytometry and reported it as 2.2×10^6 cells/mL. The blood was diluted in 1 mol/L Tris–HCl and 0.1 mmol/L EDTA (TE^{-4}).

The human cell line CRL-1486 (HEPM, Human Fibroblast) was purchased from ATCC (Manassas, VA) and cultured at the National Institute of Standards and Technology (NIST). This human cell line was selected for its stability and prior characterization (data not shown) in the development of Standard Reference Material 2391d [11]. Cells were grown in Eagle's Modified Essential Medium (ATCC) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and Dulbecco's Modified Essential Medium (ATCC) supplemented with 10% fetal bovine serum, respectively. Cells were harvested at passage six and counted using a Multisizer M3 Coulter Counter (Beckman Coulter, Brea, CA) to determine the average number of cells per flask. Briefly, CRL-1486 growth media was removed from the flask, and cells were washed with 15 mL of Dulbecco's Phosphate Buffered Solution (DPBS, Invitrogen). The DPBS was removed, 2 mL of 0.25% Trypsin/0.53 mmol/L EDTA (ATCC) was added to the cells, and the flask was incubated at 37°C, 5% CO_2 for 3–4 min or until cells detached. CRL-1486 growth media (8 mL) was added to the detached cells and pipetted up and down to break up cell clumps. Cells were washed twice with DPBS. Between washes, cells were spun down using a tabletop centrifuge at room temperature, 800 *g*, for 10 min. The supernatant was removed, and the cell pellet was resuspended in DPBS for measurement. A 100 µL aliquot of the CRL-1486 cells was added to 9.9 mL Isoton II solution (Beckman) in Coulter counter cups. The cells were swirled to mix, and the cup was placed into the Coulter counter. The cell count program was initiated (with a dilution factor of 100), and the counts were recorded. The cells were counted five times, and the average was used to determine the final number of cells to prepare dilutions for the study. The number of cells required to prepare working stocks at each of the targeted DNA inputs was calculated under the assumption that each cell contained 6 pg of nuclear DNA. The undiluted cells, at a concentration of 40,231 ± 140 cells/µL, were added to TE^{-4} buffer to create stock tubes of 600 µL for the 50 and 20 ng input amounts. For the lower DNA input amounts (10, 5, and 1 ng), a 1:50 solution of cells to DPBS was prepared, and this was added to the calculated volume of TE^{-4} to make 600 µL for each targeted DNA input amount. These 600 µL stocks were aliquoted into individual sample tubes in 50 µL volumes for extraction.

Component A of the Human DNA Quantitation Standard (Standard Reference Material 2372a) was used as a control group for this study, as the DNA recovered will not be impacted by cell lysis or cell debris [12]. This sample source will be referred to herein as SRM 2372a. For each DNA input amount, the DNA was diluted with TE^{-4} buffer 1 day prior to extraction.

Each DNA source was diluted to 50, 20, 10, 5, and 1 ng total DNA input in a volume of 50 µL. Five replicates per amount were extracted for 25 samples per each extraction protocol and DNA source. For the estimated amount of DNA present in blood and cells, an assumption of 6 pg of DNA per cell was made (e.g., 4000 cells = 24 ng of genomic DNA).

2.2 | Extraction protocols

Two extraction protocols were assessed using two different kits and techniques. Protocol 1 was used to extract samples with resin-based magnetic beads utilizing an automated extraction instrument. This was done with the QIAGEN EZ1 Advanced XL purification robot and the DNA EZ 1&2 Investigator kit (QIAGEN, Germantown, MD). Protocol 2 was used for manual extractions with silica spin columns using the QIAGEN QIAamp DNA Investigator kit (QIAGEN, Germantown, MD). These techniques and kits will be referred to as protocol 1 and protocol 2 for the remainder of this report.

For both protocols, a sample volume of 50 µL was extracted at each input amount for each extraction protocol in replicates of five.

Protocol 1 materials were extracted according to the manufacturer's recommended protocols for the EZ 1&2 DNA Investigator kit [13]. To maintain consistency in incubation times between the two protocols, the samples were incubated for 1 h at 56°C in a thermomixer, rather than the standard minimum required time of 15 min. Additionally, 1 µL of carrier RNA was added at a concentration of 1 µg/µL. After purification, the samples were eluted in 50 µL TE^{-4} and stored at 4°C in perfluoroalkoxy polymer (PFA) vials (Savillex Corporation, Eden Prairie, MN) until analyzed by digital PCR (dPCR).

Protocol 2 samples were extracted according to the ATF-LS-FB09 General QIAamp Investigator DNA Extraction protocol with the QIAamp DNA Investigator extraction kit, following the Purification from Blood or Body Fluids Spin Protocol [14]. The samples were incubated for 1 h at 56°C per the protocol's recommended incubation time for reference samples. After incubation, 1 µL of carrier RNA at 1 µg/µL was added. Samples were eluted in 50 µL TE^{-4} Buffer and stored at 4°C in PFA vials until analyzed by digital PCR (dPCR).

2.3 | Digital PCR

Digital PCR (dPCR) was performed using a Bio-Rad QX200 Droplet Digital PCR System (Bio-Rad, Hercules, CA) within 24 h post-extraction. Each extract was quantified using dPCR with a NIST-developed dPCR assay targeting the EIF5B gene on chromosome 2, which was determined to be a single-copy target, 67 base pairs in length [15]. The assay consists of a forward primer (gccaaacttcagccttctcttc, Eurofins, Huntington, AL), reverse primer (ctctggcaacatttcacactaca, Eurofins), and FAM-labeled BHQ+ probe (tcatgcagttgtcagaagctg, LGC Biosearch Technologies, Novato, CA).

The dPCR setup consisted of 12.5 µL Bio-Rad Supermix for Probes (no dUTPs) (Bio-Rad), 1.9 µL forward primer (5 µmol/L), 1.9 µL reverse primer (5 µmol/L), 1.2 µL BHQ+ FAM-labeled probe (5 µmol/L), 2.5 µL PCR grade water, and 5.0 µL sample. dPCR measurements were performed in triplicate for each sample.

For all dPCR samples, droplets were generated on the Auto Droplet Generator (Bio-Rad) using the Droplet Generation Oil for Probes (Bio-Rad). Droplets were thermal cycled on a ProFlex thermal cycler (Applied Biosystems, Waltham, MA) for 95°C for 10 min, followed by 60 cycles of 94°C for 0.5 min and 60°C for 1 min, then 98°C for 10 min before a 4°C hold until the plate was removed. Droplets were read on the QX200 Droplet Reader (Bio-Rad), and analysis was performed using the QX Manager Software version 2.1.0 (Bio-Rad). The numbers of positive and negative droplets at the end of 60 cycles were determined by visually setting an assay-specific intensity threshold for each run; then, the data was exported into Excel (Microsoft, Redmond, VA) for further analysis. Original extracted stock concentrations were determined by converting the calculated lambda value to nanograms per microliter [16].

2.4 | Statistical analysis

Two-sample *t*-tests were used to compare the efficiencies of the two protocols for each of the three source types. The resulting *p*-values were used to assess whether the two protocols produced statistically significant differences in efficiency. Additional *p*-values were then calculated to determine if there was a statistically significant relationship between input amount and efficiency for each source.

A multifactor analysis of variance (ANOVA) was initially evaluated to assess the interaction between protocol and sample source. However, data inspection indicated potential heteroscedasticity (nonconstant variance) among the sample types, with the biological samples displaying variances up to eight times higher than the SRM 2372a reference samples. While a multifactor model can offer advantages, relying on a pooled variance estimate in this context carries the risk of artificially underestimating the error associated with the high-variability biological samples. To ensure that our conclusions were robust to such modeling assumptions, stratified Welch's two-sample *t*-tests were selected to compare extraction efficiencies within each sample type. This conservative approach prevents the possibility of inflating statistical significance through variance pooling and ensures that the reported differences reflect genuine performance disparities between the extraction protocols.

3 | RESULTS AND DISCUSSION

3.1 | Calculation of average extraction efficiencies obtained across protocols and sample sources

Extraction efficiency (%) was calculated as the ratio of the amount of DNA recovered to the known amount of DNA before extraction for each sample. Five replicates were prepared of each starting amount from each source for extraction (e.g., five aliquots of 50 ng of blood, five aliquots of 20 ng of blood, etc.). This was done for both protocols, and then the samples were quantified in triplicate. The resulting 15 measurements were then converted into extraction efficiency values and used to calculate an average efficiency. This calculation was done for each set of five samples per source, amount, and protocol. A summary of the extraction efficiency results from the 5, 10,

TABLE 1 Summary of average extraction efficiencies all across sources & extraction protocols. The average extraction efficiencies plus or minus their standard deviations for samples extracted using protocol 1 (automated extraction, pink) and the samples extracted using protocol 2 (manual extraction, blue) are shown for each source at starting amounts of 5, 10, 20, and 50 ng. The average extraction efficiency values for each protocol and sample source across all starting amounts can be seen in gray at the bottom of the table.

	Blood		Cells		SRM 2372a	
Amount (ng)	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2
50	69.1% ± 7.9%	32.2% ± 5.7%	84.8% ± 7.7%	60.4% ± 5.1%	57.6% ± 10.3%	50.3% ± 7.4%
20	60.1% ± 9.6%	34.9% ± 5.0%	71.0% ± 22.6%	56.6% ± 11.9%	53.7% ± 11.2%	56.9% ± 4.3%
10	57.9% ± 10.3%	32.4% ± 3.8%	77.2% ± 12.5%	62.2% ± 11.5%	56.1% ± 13.4%	53.4% ± 8.6%
5	65.1% ± 9.4%	32.1% ± 8.5%	82.8% ± 12.9%	50.0% ± 7.8%	45.9% ± 7.7%	50.5% ± 6.1%
Overall average	63.0% ± 10.1%	32.9% ± 6.0%	78.9% ± 15.5%	57.3% ± 10.4%	53.4% 10.7%	52.8% ± 7.2%

TABLE 2 Summary of Average Extraction Efficiencies All Across Sources and Extraction Protocols for 1 ng Dataset. The average extraction efficiencies, plus or minus their standard deviations, for samples extracted using protocol 1 (automated extraction, pink) and those extracted using protocol 2 (manual extraction, blue), are shown for the 1 ng starting amount only.

	Blood		Cells		SRM 2372a	
Amount (ng)	Protocol 1	Protocol 2	Protocol 1	Protocol 2	Protocol 1	Protocol 2
1	94.6% ± 32.6%	42.6% ± 26.2%	70.6% ± 32.5%	51.3% ± 21.8%	53.9% ± 15.2%	52.6% ± 18.3%

20, and 50 ng samples across all sources and extraction protocols is presented in Table 1.

3.1.1 | Average extraction efficiencies for 1 ng samples

The average extraction efficiencies for the 1 ng dataset are shown in Table 2. These results are presented independently from the larger starting amounts due to their high variability. The statistical analysis was performed with and without the highly variable 1 ng data (as described below in Figure 1; Figure S1). However here, describe solely the 1 ng data as it may be most relevant to lower DNA template casework-type samples. The standard deviations are noticeably higher within the 1 ng dataset for both protocols when compared to the data in Table 1. This increased variability is most noticeable in the blood extraction measurements, where the standard deviation of the 1 ng data for protocols 1 and 2 is 32.6% and 26.2%, respectively, which is over 17% higher than the standard deviations within the datasets for the larger starting amounts. These higher rates of variability are consistently observed throughout the data, regardless of the source or protocol.

3.2 | Analysis of the statistical significance of extraction efficiency across various sample sources and protocols

A two-sample *t*-test was performed to determine if a statistically significant difference was observed between the extraction efficiencies obtained using two different protocols. This statistical significance was evaluated for each source to provide insight into whether the DNA source impacts extraction efficiencies, in addition to the protocol used. This was done by using the average efficiency values at each starting amount to assess whether significance may change with DNA input.

Figure 1 shows three plots, each displaying the average efficiencies obtained using either protocol 1 or protocol 2. These tests for significance were performed to determine if there was a significant difference between the efficiencies of the protocols from each of the three source types at alpha (α) = 0.05. The resulting *p*-values are indicated at the bottom of each graph. The solid lines on each graph represent the average efficiency for each protocol and source across the range of starting amounts, from 1 to 50 ng, and the dotted lines indicate a 95% prediction interval. The greatest difference in average efficiency between the two protocols was observed with blood, resulting in an average difference of 35.1%. The SRM 2372a control samples showed the least variability across protocols, with an average efficiency difference of 0.72%.

Significant differences in average efficiencies were observed between protocols for both blood and cells, indicating rejection of the null hypothesis for these sources. The null hypothesis cannot be rejected for SRM 2372a as no significant difference was observed.

Two-sample *t*-test hypotheses:

Null Hypothesis (H_0): There is no significant difference between average extraction efficiencies obtained using protocol 1 versus samples extracted using protocol 2 ($\mu_1 = \mu_k$).

Alternative hypothesis (H_1): There is a significant difference between average extraction efficiencies obtained using protocol 1 versus samples extracted using protocol 2 ($\mu_1 \neq \mu_k$).

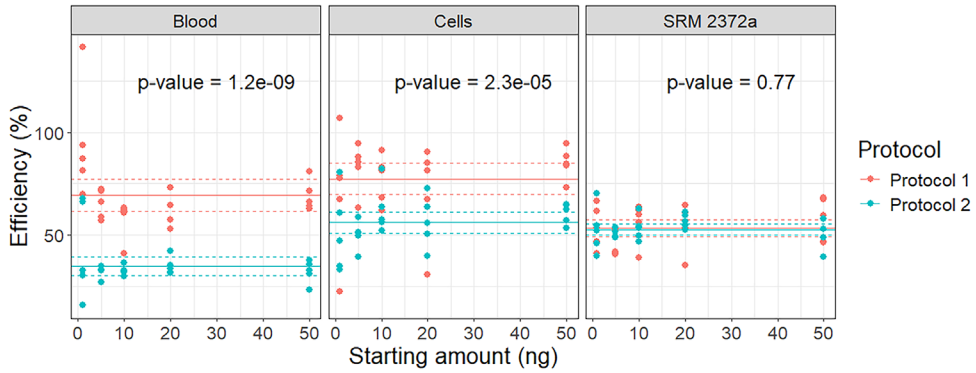

FIGURE 1 Linear regression plots showing the average extraction efficiency obtained from extracting blood, cells, and SRM 2372a across starting amounts of 1, 5, 10, 20, and 50 ng. The solid horizontal line depicts the sample average for the corresponding protocol, and the dashed lines provide a 95% confidence interval for the mean based on a one-sample *t*-test. The *p*-value shown in each panel is derived from a two-sample *t*-test comparing the averages of the two protocols within each source type. Average efficiency values (%) of each replicate quantitation from protocol 1 are shown in pink, and values from extractions done with protocol 2 are shown in blue. The solid lines indicate the average efficiency for a protocol across the range of starting amounts, and the surrounding dotted lines indicate the 95% prediction interval.

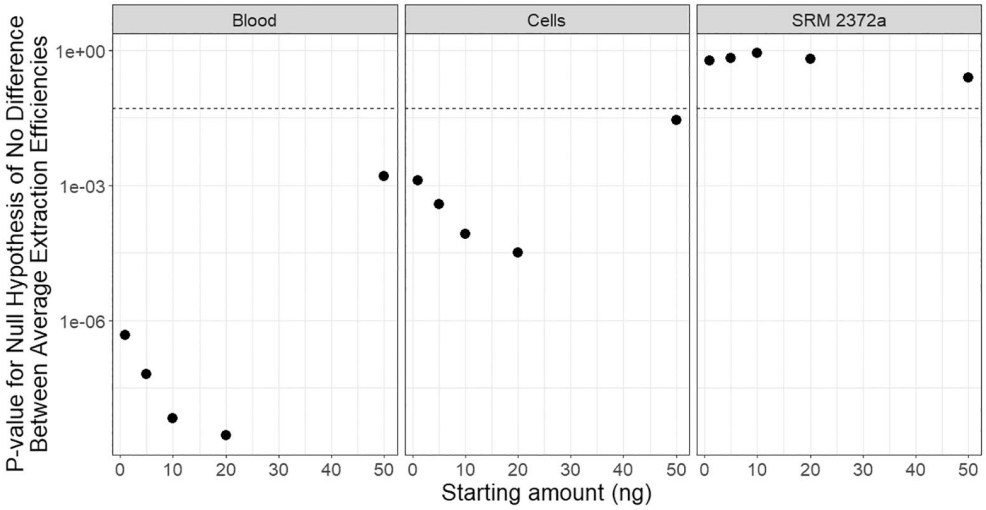

FIGURE 2 *p*-Values for starting amounts of 1, 5, 10, 20, and 50 ng were calculated in order to measure if there was support for the null hypothesis, which stated that there is no significant difference between efficiencies obtained from protocol 1 and protocol 2. The *y*-axis is the calculated *p*-value on a logarithmic scale. The dotted line represents alpha (α), which is equal to 0.05.

3.3 | Statistical significance of average extraction efficiencies across estimated DNA input amount

In order to further assess the impact that starting amount has on extraction efficiencies between two protocols, *p*-values were calculated to determine if there was a significant difference in efficiencies between the two methods for all three extraction sources used in this study for starting amounts of 1, 5, 10, 20, and 50 ng at $\alpha = 0.05$ (Figure 2). This was specifically done to determine if the rejection or acceptance of the null hypothesis remained the same regardless of the starting amount (i.e., if the starting amount of DNA affected statistical significance). The statistical testing showed a significant difference in efficiencies between blood and cells across all starting amounts, indicating that the null hypothesis should be rejected. However, the null hypothesis cannot be rejected for the SRM 2372a samples, as there was no significant difference across all amounts.

Hypotheses for the determination of statistically significant differences between input amount and average extraction efficiency across the DNA sources:

Null Hypothesis (H_0): There is no statistically significant difference between the input amount of DNA and the average extraction efficiency for each source.

Alternative Hypothesis (H_1): There is a statistically significant difference between the input amount of DNA and the average extraction efficiency for each source.

4 | CONCLUSIONS

The methodology presented here, of using a dPCR-based comparison of absolute DNA recovery from defined cellular inputs, provides a standardized framework for quantifying the efficiency of the extraction step in isolation. The results can serve as a baseline for the expected variability in DNA extraction efficiency when examining different protocols, DNA sources, and starting amounts. Additional factors that impact extraction efficiency, such as substrate, sample collection, sample storage, and environmental conditions, were not evaluated in this work. However, similar studies indicate that introducing additional variables, such as sample age and substrate, can also yield highly variable results and should be further studied and considered when validating an extraction method in a forensic DNA laboratory [17]. The strength of this study lies in our examination of highly characterized initial sources of DNA with minimal variables; however, future studies may utilize this quantitative framework to assess the specific impacts of environmental degradation and chemical inhibition on DNA recovery.

The extraction of blood and cells indicated a significant difference in recoveries between protocols. This suggests that certain extraction kit chemistries may be more efficient in recovering DNA from a particular source than others. In similar studies, researchers also observed significant differences in recovery rates when using different extraction kits [18, 19]. Many published studies evaluating the differences in recovery rates between various extraction methods also considered additional variables, such as substrate and environmental factors. By conducting a study with fewer variables that affect the extraction method (e.g., surface effects, swab collection, swab release), we can draw more confident conclusions about the impact of the extraction protocol and the DNA source on recovery rate [17]. These findings highlight the importance of quantifying protocol-specific DNA recovery, providing a metric for laboratories to evaluate the technical performance of extraction methods under controlled conditions.

The NIST SRM 2372a resulted in statistically insignificant variation across methods and starting amounts. Additionally, the recovery data revealed distinct performance trends based on the biological source. SRM 2372a consistently exhibited the lowest intrasample variability across all tested protocols. This serves as a critical benchmark for the mechanical efficiency of the extraction process, indicating that physical loss during bead-binding and elution is minimal in this study. However, it is worth noting that previously extracted DNA is the least representative of samples encountered in casework and is intended for use as a manipulation control. It allows us to differentiate between loss due to physical handling and loss due to biological extraction mechanics. Thus, validating against a standard such as NIST Standard Reference Material 2372a without the addition of well-characterized DNA sources that undergo the extraction process and are representative of casework samples may not provide accurate information on the extraction efficiency of a particular method.

Data for the 1 ng starting amounts of DNA displayed a wide range of variability across both protocols and all three DNA sources, ranging from 0% to over 100%. Although the 1 ng of DNA input amounts may be the most similar to what may be expected in casework samples, the results did not allow for reliable conclusions to be drawn based exclusively on those values. (See Data S1 for quantitation values).

Some of this variation may have been due to initial cell counting uncertainties or the nature of extracting an amount of DNA at the lower bound of this study's dataset (approximately 1 ng, equivalent to 150 cells' worth of DNA) with both protocols. Studies have shown that uncertainty rates of cell counting depend on method, instrumentation, concentration, and cell type; however, it has been found that WBC count uncertainties can range from approximately 1.4%–8.1% [20]. As the observed variability was highest with the blood extractions, it is essential to note that these uncertainties were not taken into account when initial calculations were performed to produce dilutions at the targeted starting amounts. Furthermore, the extraction efficiency variability for 1 ng of SRM 2372a was lower than that of blood and cells, further suggesting that the variability may be due to cell-related sources (e.g., initial cell dilution, incomplete cell lysis, or varying loss of cell material) during the extraction process.

When examining the efficiencies greater than that of the 1 ng data, more robust conclusions can be drawn about the impact of protocol and source extraction efficiencies. Comparative statistical testing was performed as described in Section 3.2, in which the 1 ng dataset was excluded (See Figure S1). Though the overall conclusions of significance did not change for each source, the results indicated stronger support when excluding the 1 ng data. The support for rejecting the null hypothesis for blood and cells increased by a magnitude of six and one, respectively. This observation suggests that it may be challenging to accurately assess extraction efficiency when using this method for samples of low quantity. This, however, is valuable information that should be kept in mind when assessing extraction efficiencies at lower input amounts, as even in a highly controlled study with minimal variables, this variability was unavoidable.

Further studies should be conducted to determine if other methods yield more consistent results when the starting amount of DNA is less than 5 ng, as these may be more suitable for laboratories working with challenging samples that provide only minimal amounts of genetic material. These results also underscore the need for further research on optimizing standard extraction methods to achieve higher and more consistent efficiencies across various sample types and DNA amounts.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to report.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

DISCLAIMER

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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