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Comparison and Optimization of Simple DNA Extraction Methods for LAMP-Based Point-of-Care Applications Employing Submillimeter Skin Biopsies

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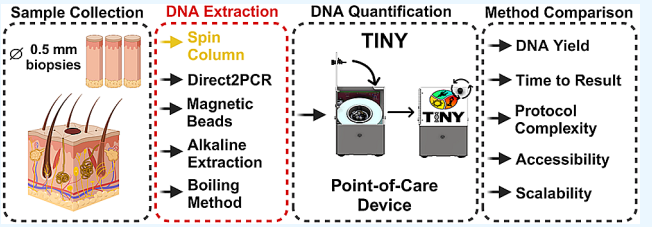
ABSTRACT: Loop-mediated isothermal amplification (LAMP) has gained particular attention for point-of-care (POC) applications due to its advantages over traditional nucleic acid testing approaches. However, a prevailing limitation of LAMP in POC applications is nucleic acid extraction from the sample prior to analysis. This is particularly true for complex samples such as submillimeter skin biopsies where lysis and digestion involve intricate and lengthy procedures. The objective of this study was to compare alternative methodologies against the spin-column laboratory standard and evaluate them based on the World Health Organization ASSURED criteria for POC testing. Four methods—magnetic bead extraction, alkaline extraction, proteinase K-heat inactivation extraction, and boiling method extraction—were optimized utilizing porcine skin submillimeter punch biopsies and subsequently validated on human skin. Results show that both alkaline extraction and proteinase K-heat inactivation produce DNA yields equivalent to or higher than the spin-column method in porcine and human skin. When evaluated against the ASSURED criteria, both methods demonstrated low complexity while being highly scalable and readily accessible. Overall, this comparative study established a robust framework for selecting DNA extraction methods for submillimeter skin biopsies in POC applications. It also underscored the performance of the alkaline extraction method based on the ASSURED criteria, providing equivalent DNA yields to laboratory standards with reduced complexity and potential for cost-effective scalability.



1. INTRODUCTION

Nucleic acid amplification tests (NAATs) are widely used in medical diagnostics due to their high levels of sensitivity and specificity.[1,2] For point-of-care (POC) and remote setting applications, various isothermal NAATs have been developed, thus removing the need for thermal cycling and thereby the need for sophisticated equipment.[3−5] Compared to traditional approaches, these techniques provide more rapid results with simpler equipment and could thus improve decentralized testing capabilities.[6,7] Recently, loop-mediated isothermal amplification (LAMP) has become a particularly attractive method and accounts for more than 60% of all applied isothermal NAATs.[6,8] In LAMP-based methods, the target DNA/RNA sequence is amplified at ∼65 °C and detected at the end point through colorimetry or monitored throughout via fluorescence (real-time LAMP).[3,9−11] Additionally, LAMP demonstrates increased resistance to sample impurities compared to other methods, making it well-suited for applications where centralized healthcare is atypical.[11−13] However, there is a significant bottleneck in the DNA extraction of solid tissue samples, including skin biopsies, which typically require hours for tissue digestion, thus adding time to an otherwise expedient approach.

In 2006, the World Health Organization (WHO) issued the ASSURED criteria for the development of practical POC tests.[14] To meet these criteria, molecular diagnostics must be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users. To this end, several devices have been created to simplify LAMP diagnostics at the POC, including hand-held microfluidic devices,[15] miniature devices with several wells,[16−19] and a 96-well device for high-throughput applications.[20] Another example is "TINY", which was originally designed for POC diagnosis of Kaposi's sarcoma using skin punch biopsies, capable of maintaining 68 °C by either electricity, solar power, or flame.[21,22]

Though TINY and other simple devices are capable of performing LAMP at the POC with comparable detection performance to commercial analyzers, they still rely on laboratory standard techniques for DNA extraction, preventing

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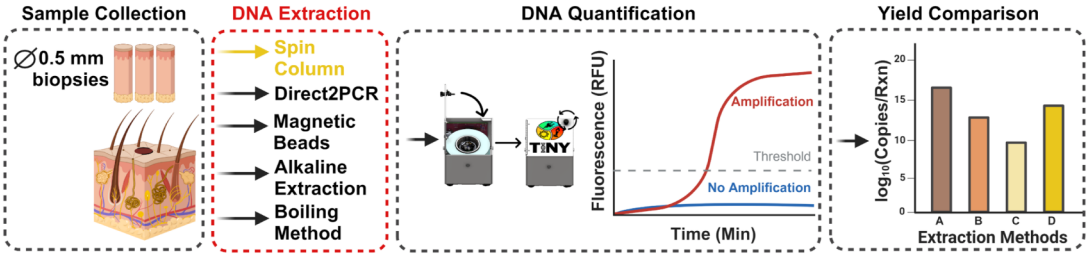

Figure 1. Overview of the study design. In the quadruplet, three 0.5 mm punch biopsies were collected from either porcine or human skin and then subjected to DNA extraction. Four methods were optimized and compared to the DNeasy kit (shown as the gold standard)—Direct2PCR, magnetic bead extraction, alkaline extraction, and the boiling method. After extraction, the number of copies was quantified by loop-mediated isothermal amplification (LAMP) using the TINY device. Comparisons between the methods include DNA yield, cost, number of steps, time to result, and protocol complexity.

their full realization at POC.[23,24] A standard extraction method via a silica membrane spin column requires significant time, skill, and equipment.[25−27] In this method, samples are first chemically digested by proteinase K and a strong anionic detergent at 55 °C. Digestion times can range from minutes for nonsolid biopsies—saliva, blood, urine, fine needle aspirates, etc.,—to hours for punch biopsies. Next, DNA is reversibly bound to a silica membrane within the spin column followed by subsequent washing and centrifugation steps to remove cellular contaminants. Finally, the stabilizing elution buffer releases the purified DNA from the membrane. While spin-column extraction methods are abundant and well-documented, the need for sophisticated equipment (for heating and centrifugation), time requirements, and expertise poses significant challenges for decentralized healthcare applications.[23,24]

The challenges imposed by skin biopsy processing and DNA extraction in POC and decentralized applications could be alleviated by employing variations of existing rapid DNA extraction methods typically reserved for simpler samples. These include magnetic bead-based extractions,[24,28] alkaline extractions,[29,30] proteinase K digestion-based extractions,[31,32] and extraction via thermal lysis.[33,34] In magnetic bead extraction, specialized beads have been developed to selectively bind to DNA under specific pH conditions, enabling the separation of DNA from a lysis solution. Following lysis, the DNA-laden beads are separated by applying a magnetic field and removing the supernatant. Subsequent washing and elution steps are common for downstream applications. In alkaline extraction, NaOH hydrolyzes cellular and nuclear membranes to release DNA into solution;[29,30] samples are incubated in a lysing solution of sodium hydroxide at 95 °C, and extraction is terminated by cooling the solution and adding a neutralizing buffer with Tris.[29,30] The proteinase K digestion method utilizes proteinase K in a buffered solution to break down cells and releaseDNA. To terminate the digestion, the solution is briefly heated above 90 °C to inactivate proteinase K.[31,32] The boiling method or extraction through thermal lysis is not new among the DNA extraction techniques. Exposure to high temperatures is known to damage the cell membranes and disrupt cellular integrity, ultimately releasing DNA into solution without the need for harsh chemicals.[33,34] Although these methods have been shown to work for DNA extraction of tissue, none have been optimized for submillimeter skin biopsies or assessed for their compliance with the ASSURED criteria.

In this study, a series of rapid DNA extraction protocols were evaluated for accuracy and practicality in limited resource settings. To accomplish the evaluation, the objectives of this study were to (i) optimize and compare the DNA yields of the alternative methods against the spin-column method (laboratory standard) and (ii) evaluate each method based on the ASSURED criteria for POC compatibility. Multiple submillimeter biopsies were collected from porcine and human skin tissue and subjected to DNA extraction utilizing four different methods—magnetic bead-based extraction, alkaline extraction, proteinase K digestion, and the boiling method. Optimization was first performed utilizing porcine skin and then tested on human skin for comparison. Each method was assessed on scalability, accessibility, performance, and complexity. This manuscript represents the first attempt to optimize POC-compatible methods for DNA extraction from submillimeter skin biopsies as well as establishes a framework upon which these methods can be selected in accordance with specific applications.

2. MATERIALS AND METHODS

2.1. Experimental Design. Four replicates of porcine and human skin tissue samples, each using three 0.5 mm punch biopsies, were used to compare the DNA yields of four rapid, POC-compatible DNA extraction methods: magnetic bead extraction, alkaline extraction, Direct2PCR proteinase K extraction, and the boiling method (Figure 1). The DNA yield of each extraction was calculated using real-time LAMP in TINY. To normalize the results, the final volumes of extracted DNA samples were diluted to 100 μL. For each of these methods, the durations of the time-critical steps were varied to optimize DNA yields. The optimized methods were compared to each other as well as to the spin-column method. Finally, an assessment based on the ASSURED criteria was done to evaluate the applicability of these methods for POC applications.

2.2. Target Detection LAMP Assays. Two LAMP assays targeting regions of the GAPDH gene were used to amplify extracted target DNA. As the GAPDH gene is found at a rate of two copies per cell, the LAMP assays herein provide a robust means to quantify DNA yield and can approximate the number of cells' worth of DNA obtained. The first assay was designed in-house to target a 500 bp region at the beginning of the porcine GAPDH gene (*Sus scrofa*, NCBI GenBank accession number NC_010447.5). The target sequence was aligned against *Sus scrofa* (NCBI taxid: 9821), *Homo sapiens* (NCBI taxid: 9606), viruses (NCBI taxid: 10239), and bacteria (NCBI taxid: 2) to ensure the specificity of the region. The primers were designed using the New England Biolabs Primer Design tool and were blasted against the rest of the *Sus scrofa*

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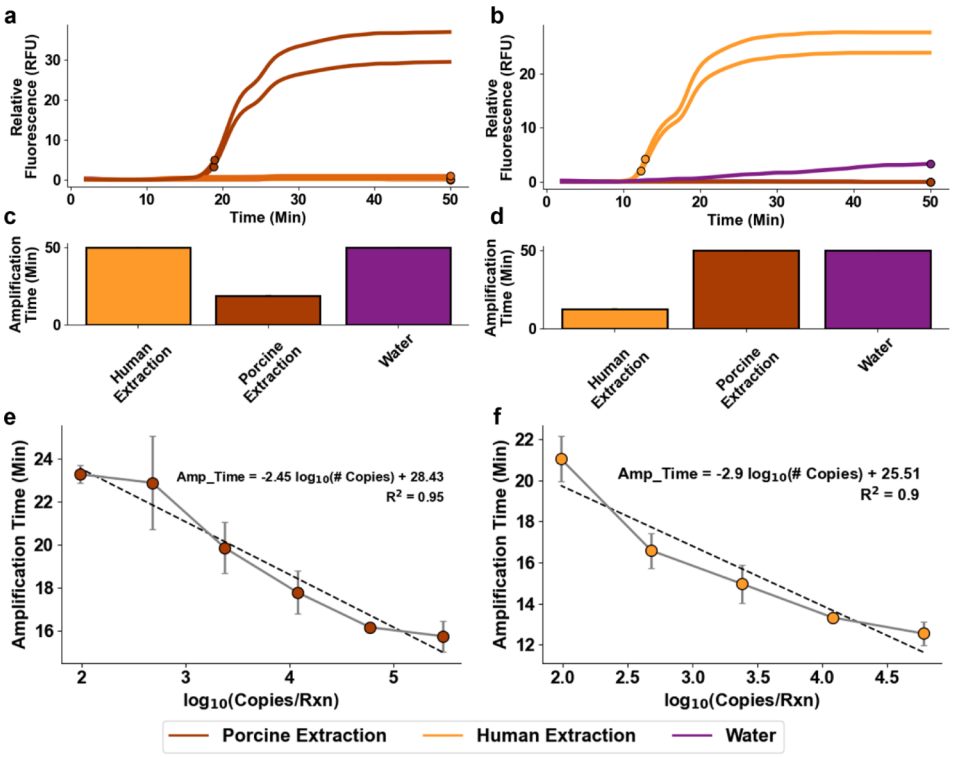

Figure 2. LAMP assays for quantification of the number of copies for porcine and human biopsies. (a,b) Amplification curves for porcine GAPDH and human GAPDH assays, respectively. Each assay was compared against each other and water for specificity. (c,d) Calculated amplification times from (a,b) amplification curves. 50 min is assigned to negative samples (total run time). (e,f) Standard amplification curves for porcine and human GAPDH assays. The porcine standard curve ranged from 96 to 300000 target copies per reaction, while the human standard curve ranged from 96 to 60000 target copies per reaction.

genome to ensure the region's specificity. The second LAMP assay included primers targeting a sequence within the human GAPDH gene.[20] All primer sequences (Tables S1 and S2) were ordered from Integrated DNA Technologies (IDT) and tested against pig skin DNA extractions, human skin DNA extractions, KSHV (ORF26) plasmids, and water using Real-Time LAMP (Figure 2a−d).

The LAMP assay composition (Table S3) includes 10× isothermal buffer (NEB B0537S), 100 mM $MgSO_4$ (NEB B1003S), 10 mM dNTPs (NEB N0447L), and BST 2.0 WarmStart polymerase (NEB M0538S), which were purchased from New England Biolabs. 20× EvaGreen (89138−984) was obtained from VWR International. Nuclease-free water (AM9915G) and 50× ROX (12223012) were purchased from Invitrogen. All assay preparation was done inside biosafety cabinet Class II. Genomic DNA samples were prepared to create standard curves for both assays (Figure 2e,f). Porcine DNA samples were prepared through serial dilutions starting at 300000 Cp/Rxn down to 19. The same procedure was done for human DNA samples starting at 60000 Cp/Rxn.

All LAMP reactions were performed using TINY.[22] The TINY device comprises optical filters compatible with EvaGreen and ROX dye emission channels. During the reaction, the device was maintained at 68 °C and monitored for fluorescence changes. All real-time data were subjected to a series of Hampel filters to remove unwanted intensity drops, followed by smoothing. Amplification times were calculated using the threshold-based algorithm described in[20] using the Python programming language. Since all LAMP reactions were run for 50 min, the amplification time for nonamplified samples was set to 50. All results are reported in copy number per reaction (Cp/Rxn), which was calculated from the equations in Figure 2e,f.

2.3. Tissue Collection and Sampling. Tissue samples used were stored at −20 °C until use. Porcine skin was obtained from a local butcher. Prior to use, the skin was shaved and dissected for storage. Human skin was sourced from Weill Cornell Medicine. All DNA extractions were performed using 3 × 0.5 mm tissue punch biopsies (Electron Microscopy Sciences 69039-05).

2.4. Extraction Methods. *2.4.1. Spin-Column Extraction Method.* To establish a baseline, the spin-column method (DNeasy Blood & Tissue Kit, #69504, QIAGEN) was used as the laboratory standard method. Following the kit's instructions, the submillimeter biopsies underwent thorough digestion at 56 °C submerged in lysis buffer and proteinase K. Upon completion, the resultant liquid was transferred to spin columns and subjected to centrifugation for 1 min. Two subsequent washing steps were performed in a similar fashion. To elute the extracted DNA, 100 μL of AE buffer was added (as opposed to 200 μL per kit's instructions) to the column and incubated for 1 min at room temperature prior to final centrifugation and elution.

2.4.2. Magnetic Bead Extraction Method. Magnetic bead extraction was carried out employing the DynaBeads DNA DIRECT Universal kit (Cat. 63006) from Thermo Fisher Scientific. Following the manufacturer's protocol, the washing buffer was diluted to 1× using DEPC-treated water (Thermo Fisher Scientific, Cat. AM9915G). An additional lysis solution was prepared by mixing the lysis solution with a 20:1 volume of 10 M NaOH following the protocol for an enhanced lysis

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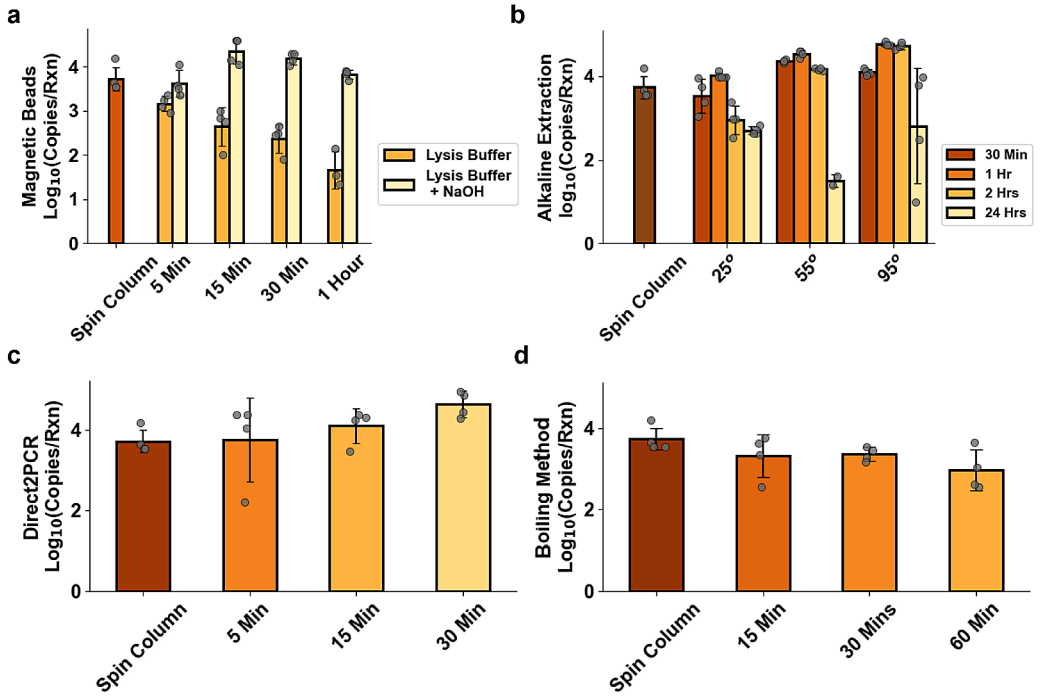

Figure 3. Optimization of the DNA extraction methods using porcine skin tissue (four replicates for all trials). (a) Assay time optimization of the magnetic bead DNA extraction with and without NaOH. Assay times range from 5 min to 1 h. The highest yield was at 15 min with NaOH present. (b) Time and temperature optimization for alkaline extraction. Temperature optimization ranged from 25 to 95 °C, and time optimization ranged from 30 min to 24 h. The highest yield was recovered at 1 h and 95 °C. (c) Direct2PCR time optimization from 5 to 30 min. Highest yield at 30 min. (d) Boiling method optimization. All trials were performed at 95 °C from 15 to 60 min. Highest yield at 30 min.

solution. For all trials, three 0.5 mm pig skin microbiopsies were added into 200 μL of either the base lysis solution or lysis solution with NaOH and incubated at room temperature for periods ranging from 10 min to 2 h. Once finished, the tubes were placed in a magnetic separation rack (New England Biolabs, Cat. S1506S) for 2 min. The supernatant was removed, and two consecutive washing steps were performed using 200 μL of washing buffer. The beads were then suspended into 100 μL of elution buffer and incubated at 65 °C for 10 min to elute the DNA from the beads. To remove the beads, the solution was placed in the rack for 2 min and the supernatant containing the extracted DNA was transferred to a 200 μL PCR tube.

2.4.3. Alkaline Extraction Method. For the alkaline extraction, tissue samples were incubated in an alkaline lysis solution for a set duration before the addition of a neutralizing buffer to end extraction.[29,30] The alkaline lysis solution was a 25 mM NaOH solution prepared from a 1 M NaOH stock solution (Sigma-Aldrich 1310-73-2). The neutralizing buffer consisted of 100 mM Tris-HCL and 0.5 mM EDTA and was prepared from stock solutions of 0.5 M Tris-HCl at pH 8 (Thermo Fisher J67510.AE) and TE buffer at pH 8 (10 mM Tris-HCl, 1 mM EDTA, Promega V6231). DPEC-treated water was used for all required dilutions. Tissue biopsies were placed in 50 μL of the lysis solution for periods of 15, 30, 60, and 120 min at either ambient temperature, 55 °C, or 95 °C. After incubation, 50 μL of the neutralizing buffer was added to the solution. DNA quantification via LAMP was then performed using 5 μL of the neutralized solution.

2.4.4. Proteinase K-Heat Inactivation Method (Direct2PCR). The commercially available Phire Tissue Direct PCR kit (Thermo Fisher, F170S) was used for Direct2PCR proteinase K digestion following the published dilution protocol.[31] To ensure that the tissue biopsies were sufficiently immersed, solution volumes were adjusted. In a 200 μL PCR tube, the biopsies were immersed in 40 μL of the dilution buffer and 1 μL of DNARelease Additive. The samples were left at ambient temperature for 15, 30, and 60 min prior to a 98 °C incubation for 2 min to denature proteinase K. After the solutions cooled to ambient temperature, 59 μL of TE buffer was added to bring the final volume of the solution to 100 μL. DNA quantification via LAMP was then performed using 5 μL of the final solution.

2.4.5. Boiling Method (Thermal Lysis). In 200 μL PCR tubes, the tissue biopsies were submerged in 100 μL of DEPC-treated water and incubated at 95 °C for 15, 30, and 60 min. After incubation, the samples were allowed to cool to ambient temperature to prevent premature activation of the BST polymerase and initiation of the LAMP reaction. DNA quantification via LAMP was then performed using 5 μL of the resulting solution.

2.5. Protocol Complexity Analysis. Each of the protocols was broken down into its respective number of steps and equipment requirements. Protocol complexity (PC) was calculated to estimate the intricacy of each protocol utilizing eq 1 (derivation and assumptions in Supporting Information).

$$PC = MS + IDS \times TI + TI \quad (1)$$

The equation utilizes the number of manual steps (MS), instrument-dependent steps (IDS), and the number of technical instruments (TI) to estimate the intricacy or complexity of the protocol. PC is proportional to MS as more manual steps increase complexity. Since the technical instrument is necessary to perform steps on it, IDS is dependent on TI. This term accounts for steps performed with technical instruments. PC increases with more technical

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instruments and steps performed with them. Finally, the more technical instruments required in the protocol, the greater its PC. This is due to the complexity of acquiring, introducing, and maintaining such instruments.

3. RESULTS AND DISCUSSION

3.1. Validation of LAMP Assays. To validate the porcine GAPDH assay, DNA extractions from the spin-column method were run in duplicate using the TINY device. Negative controls, including either nontemplate sequences or water, were used in each reaction. The real-time curve revealed successful amplification of porcine skin extractions (containing ∼12000 Cp/Rxn), whereas other samples did not show any amplification (Figure 2a). These findings affirm the specificity of the in-house designed primers targeting the porcine GAPDH gene. The mean amplification time for both replicates was 18.9 min with a standard deviation of 0.1 min or 6 s (Figure 2c). These results indicate consistent assay performance and minimal variability between measurements. The human GAPDH assay was similarly tested for specificity (Figure 2b). Using ∼60000 Cp/Rxn, the assay demonstrated a mean amplification time of 12.6 min with an SD of 0.3 min or 18 s (Figure 2c).

Collectively, these results endorse the specificity of these assays for their respective target sequences in real-time LAMP. For each assay, standard curves were generated to quantify the number of copies in experimental samples as well as to evaluate the sensitivity of each assay (Figure 2e,f). The porcine GAPDH assay's standard curve shows an R^2 value of 0.95, reflecting high linearity down to ∼100 Cp/Rxn. Below this limit, a nonlinear relationship between the amplification time and the number of copies was observed, so the limit of quantification and detection was set to 100 Cp/Rxn. The human GAPDH curve exhibited an R^2 value of 0.9, with a similar loss of linearity below ∼100 Cp/Rxn, consistent with previous findings.[20] While the true detection limit is below 100 Cp/Rxn, the nonlinear relationship between the amplification time and copy number must be considered.

3.2. Optimization of Extraction Methods. The incubation periods and temperatures for each extraction method were optimized using porcine skin tissue samples. For the same quantity of tissue, the DNA yield from each method was compared to that of the spin-column method which yielded an average of ∼5000 copies of DNA (3.7 $\log_{10}$(Cp/Rxn)) with a standard deviation of 435 copies.

3.2.1. Magnetic Bead Optimization. The magnetic bead extraction protocol was optimized by extending the lysis time and adding NaOH, an optional step in the protocols (Figure 3a). The recommended time from the protocol is 5 min; however, it was extended up to an hour to explore the impact on tissue penetration. The lysis buffer without NaOH produced its maximum yield, ∼ 3 $\log_{10}$(Cp/Rxn) or ∼1400 copies, at 5 min after which DNA degraded as indicated by the decreasing yields. The low yield, as compared to that of the spin-column method, indicates that significant DNA remained in the remaining tissue fragments. However, adding NaOH to the lysis buffer demonstrated an optimal yield at 15 min with ∼4 $\log_{10}$(Cp/Rxn) or ∼24000 copies, a yield approximately 10-fold higher than without NaOH and higher than the spin column's ∼5,000 copies. Although both a change in lysis time and the addition of NaOH influenced the results, the DNA yield was most influenced by the addition of NaOH. The presence of NaOH increases the permeability of the tissue by disrupting cellular structures and breaking down proteins, leading to more DNA being released into the solution.[35] The optimal lysis time for the magnetic bead method was 15 min of lysis with NaOH.

3.2.2. Alkaline Extraction Optimization. The alkaline extraction method was optimized for lysis time and temperature (Figure 3b). Regardless of the extraction temperature, a similar trend for different lysis times was observed; DNA yields peaked after 1 h of lysis time before decreasing. The later decrease indicates that DNA degradation occurred after the peak at 1 h. Except for the 24 h lysis time, increased temperatures produced higher DNA yields, with the best yields at 95 °C with >40000 copies. These results were expected since temperature increases molecular mobility and increases the permeability of tissues, increasing the release of DNA into the solution. Heating samples for 24 h caused many of the tubes to pop open under the increased pressure; a noticeable loss of the sample due to evaporation was observed, leading to highly variable results. Most variations of this method produced comparable DNA yields to spin-column extraction, and 1 h lysis produced higher yields for all temperatures. When implementing alkaline extraction, the incubation time and temperature must be sufficient to release sufficient DNA into the solution without allowing excessive DNA degradation to occur. For instance, increasing the temperature from room temperature to either 55 or 95 °C generally produced higher yields for time points less than 24 h. After 24 h, DNA degradation, indicated by reduced yields, was observed at all temperatures. The optimal DNA yield was observed at 1 h of lysis time at 95 °C.

3.2.3. Direct2PCR Optimization. Direct2PCR is a straightforward method that provides a simple and fast one-pot extraction mixture. Since the extraction method utilizes proteinase K, heating the samples to 98 °C is critical to inactivate proteinase K thereby enabling PCR and LAMP reactions. As a result, the DNA yield was optimized by altering the incubation time only (Figure 3c). Although the protocol calls for a 5 min digestion time, 15 and 30 min periods were performed to ensure the maximum DNA yield. After 30 min, the tissue had been fully digested, so no additional time points were considered. For all three time points, this method produced comparable DNA yield to spin-column extraction. Higher DNA yields were observed with longer digestion periods, with the optimal DNA yield after 30 min with ∼44000 copies.

3.2.4. Boiling Method Optimization. The boiling method works by disrupting cell membranes and denaturing proteins through heat, removing the need for any additional chemicals. The extraction process was performed at 95 °C for periods ranging from 15 to 60 min using only water (Figure 3d). Due to practical challenges, longer times were excluded as prolonged heating caused sample tubes to pop open and the sample was lost to evaporation. Though all heating periods produced similar DNA yields, 30 min of heating resulted in the least amount of variability with a mean copy number of ∼2200. As no decline in DNA yield was observed with longer heating times, no DNA degradation was suspected. However, none of the heating times yielded more DNA than the spin-column method, suggesting subpar performance and DNA release from the tissue.

3.3. Evaluation of Methods for POC DNA Extraction from Human Tissue. The optimized protocols for each method were tested using human skin tissue, and successful

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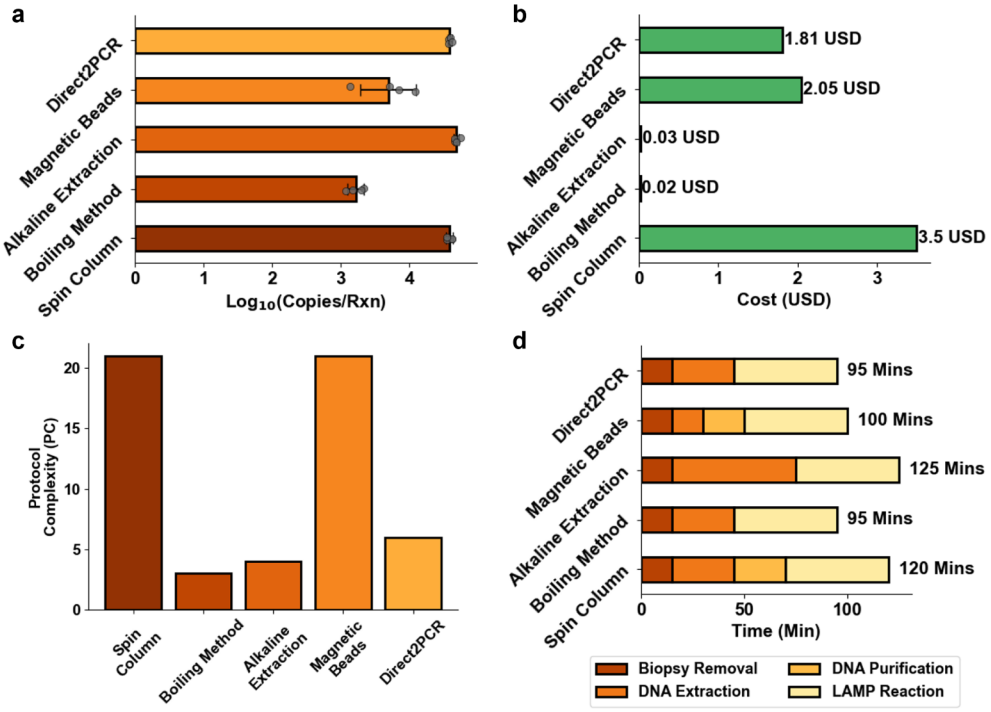

Figure 4. Summary plots for optimized methods evaluated with human tissue. (a) Average yield of the four replicates from optimized methods (Table 1)—Direct2PCR (30 min), magnetic beads (15 min with NaOH), alkaline extraction (1 h at 95 °C), and boiling method (30 min). (b) Estimated unit cost (USD) per reaction for each method. Estimations were performed considering bulk quantities and reagent costs alone; equipment is not accounted for in the estimation. (c) Protocol complexity for each method. Complexity was calculated using eq 1 based on the number of steps and equipment requirements. (d) Time to result for all methods, including biopsy removal, DNA extraction, DNA purification (if applicable), and the LAMP reaction.

DNA extraction was observed for each method (Figure 4a, Table 1 and Table 2). Direct2PCR and alkaline extraction exhibited higher DNA yields ~4.5 $\log_{10}$(Cp/Rxn) which were comparable to the yields observed in porcine tissue. Additionally, the narrow standard deviations observed in these methods attest to their high precision and reliability. In contrast, the boiling method yielded the lowest DNA quantity, aligning with previous results. The magnetic bead method produced ~3.5 $\log_{10}$(Cp/Rxn), falling short of the expected yield and exhibiting increased variability. The difference in yield between the porcine and human tissue suggests that further optimization is required for human tissue applications. While the magnetic beads and the boiling methods exhibited lower DNA yields than the spin-column method, Direct2PCR and alkaline extraction yielded similar DNA quantities, demonstrating their viability as alternatives for tissue DNA extraction.

Table 1. Optimized Incubation Parameters

method	temperature	lysis/digestion time	addition
Direct2PCR	25 °C	30 min	-
magnetic beads	25 °C	15 min	NaOH
alkaline extraction	95 °C	60 min	-
boiling method	95 °C	30 min	-

An important consideration when selecting an extraction technique is the end use of the extracted DNA. In this study focusing on DNA extractions for POC diagnostics, no downstream applications of the amplified DNA were considered, as LAMP, the chosen NAAT, does not permit such applications. Additionally, LAMP is known to be more resistant to sample impurities, and many of the POC-compatible extraction methods tested lacked DNA purification measures.[6,13] Since no evidence of LAMP inhibition was observed, and the purification steps in the spin-column and magnetic bead methods did not provide increased DNA yields, these results further support the enhanced performance of the LAMP reaction when amplifying DNA from skin tissues. Even

Table 2. Evaluation of POC-Compatible DNA Extraction Methods for Solid Tissue

method	no of steps	required equipment	purification	optimal time to result (min)	optimal lysis time (min)	cost per reaction (USD)	% of yield compared to DNeasy	tissue digestion
Direct2PCR	5	heat block	no	95	30	1.81	100.13	yes
magnetic beads	12	heat block magnet	yes	100	15	2.05	80.56	no
alkaline extraction	3	none (optional: heat block)	no	125	60	0.03	102.16	no
boiling method	1	heat block	no	95	30	0.02	70.30	no
spin-column	14	centrifuge heat block	yes	120	30	3.5	100	yes

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though the impure skin DNA samples have not inhibited the reaction here, further considerations are necessary when dealing with other sample types.

3.4. ASSURED Criteria Assessment. Each extraction method was assessed for adherence to the World Health Organization's ASSURED criteria. Affordability and deliverability were assessed by per-sample costs and availably of the reagents. Rapidness was assessed by the time to result required. The number of steps and required supplies were used to gauge user-friendliness and whether the protocol was equipment-free. While sensitivity and specificity are application-dependent and could not be directly measured, the sensitivity of a NAAT diagnostic largely depends on the assay's limit of detection and the quantity of target DNA in the sample. Thus, the DNA yields quantified in Sections 3.2 and 3.3 were used as proxy for relative sensitivity.

3.4.1. Affordable and Deliverable. Whether a solution is affordable and economically viable plays a pivotal role in its adoption, especially when the financial burden is borne by the user. To gauge affordability, the cost per sample was systematically computed in bulk for each extraction method (Figure 4b). Across the methods used, a significant difference in cost per sample was calculated. For alkaline and boiling extraction, the per-sample cost was less than 0.05 USD as opposed to a few US dollars for the other methods. This difference is largely due to the simplicity of the reagents required and that the formulations are in the public domain and easily purchased worldwide. In contrast, spin-column, magnetic bead, and Direct2PCR methods employ proprietary formulations or equipment available exclusively through their respective vendors, resulting in elevated costs per sample and limitations in deliverability. Though these methods offer commendable quality control and technical support, their availability highly depends on the vendor's stock and shipping capabilities. Collectively, the broad accessibility and affordability of the alkaline extraction and boiling methods underscore their suitability for field and remote setting applications.

3.4.2. Rapid and Robust. The total time to result is crucial in POC applications, where timely results are needed for clinical decision-making. Additionally, providing a clinical diagnosis during the same visit could improve patient retention, especially for patients who must travel to receive care. For evaluation, the duration from biopsy removal to completion of the LAMP reaction was considered (Figure 4c, Table 2). The Direct2PCR and boiling methods were the fastest at 95 min, followed closely by the magnetic bead method at 100 min and then spin-column and alkaline extraction at 120 min. For solid tissue samples, the DNA extraction times are contingent on the size and geometry of the tissue. The 0.5 mm microcores in this study exhibited a high surface-to-volume ratio, thereby needing shorter digestion and lysis times. In contrast, larger samples will require longer extraction times and application-specific optimization.

3.4.3. User-Friendly and Equipment-Free. Since user-friendliness is dependent on user outcomes, protocol complexity (PC) was used as a proxy to quantify simplicity and equipment dependency (Figure 4c). The value estimated by the equation provides an unbiased estimate of intricacy that does not depend on user inputs. Instead, the equation encompasses only the interconnection between the different components within each protocol, providing an estimate as to the number of points where failure has the potential to occur. In field and remote settings, where access to technical equipment and trained personnel is limited, simple and equipment-free protocols are favored for optimal results; thus, a protocol with low complexity is favored. From the results, the spin-column and magnetic bead methods possess the highest complexity with values of 21. The increased complexity of these methods is due to the need for multiple specialized equipment as well as the number of steps that are performed on them. Conversely, the boiling method possesses the lowest complexity with a value of 3, followed by alkaline extraction and Direct2PCR with values of 4 and 6, respectively. Because these three methods only rely on 1 technical instrument and only for a single step (Table S4), only the number of steps accounts for their different complexities. Even though these methods possess low complexity values compared to the spin-column method, there is no threshold complexity for adequacy for POC applications. Further investigation would be needed to establish a threshold value. Moreover, alkaline extraction stands out as it can be run at room temperature, eliminating the need for significant equipment. In this regard, its updated protocol complexity is 3, achieving the lowest complexity along with the boiling method.

Eq 1 does have limitations due to its assumptions as it weighs every step and technical instrument the same and does not account for processing time or multiple samples. While these assumptions may not hold in every case, they enable the estimation of complexity for any given protocol. For more specific instances, eq 1 could be modified to account for these factors.

4. CONCLUSIONS

This study optimized several tissue DNA rapid extraction methods for skin submillimeter biopsies and compared them against the spin-column laboratory standard. Following the comparison, each method was evaluated based on the WHO's ASSURED criteria for POC testing. To simplify the challenges currently posed by skin tissue sample processing, this study establishes a framework for selecting the appropriate skin tissue DNA extraction method for POC applications, ergo, a method that produces equivalent DNA yields to laboratory standards while also streamlining the intricate processes and equipment burden.

The methods were compared using both porcine and human skin submillimeter punch biopsies. During initial optimization on porcine samples, all four methods successfully extracted DNA, thereby demonstrating their feasibility as alternative extraction methods despite their different mechanisms. The magnetic bead, alkaline extraction, and Direct2PCR methods produced comparable or even higher DNA yields than spin-column extraction, while the boiling method yielded significantly less DNA. Once optimized, the methods were then applied to human tissue to assess their applicability to POC applications. Here, only the Direct2PCR and alkaline extraction methods yielded comparable DNA to spin-column extraction.

For each method, the time to result, DNA yield, calculated cost per sample, and protocol complexity were evaluated against the ASSURED criteria to assess POC practicality. Overall, the alkaline extraction method is shown to be the most adequate for POC applications. While producing equivalent DNA yields to laboratory standards, this method also possesses significantly lower protocol complexity, making it more suited for decentralized applications. Since the required reagents are

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ACS Omega 2024, 9, 38855−38863

readily accessible and the formulation is part of the public domain, alkaline extraction can be scaled quickly and cost-effectively. Furthermore, our results indicate that it can be run at room temperature, eliminating the need for the heat block and thus fulfilling the equipment-free criteria of ASSURED. While this study provides evidence, further optimization of this method is necessary for maximum DNA yield at room temperature. Direct2PCR also demonstrated similar performance to the spin-column method. While Direct2PCR produced faster results, it cannot be performed at room temperature due to the presence of proteinase K, which inhibits amplification if not inactivated or removed. Nonetheless, the digestion mechanism employed by this method may be advantageous for larger and more compact tissue samples where other methods fail because of penetration limitations. Conversely, the magnetic beads and boiling methods did not produce comparable DNA yields to the spin-column method, thus failing to demonstrate applicability to submillimeter skin biopsies.

Collectively, this comparative study established a robust framework for selecting rapid DNA extraction methods for submillimeter skin biopsies in POC applications. When considering the WHO's ASSURED criteria, the results of this study underscore the superior performance of the alkaline extraction; in a simple three-step protocol, it produced equivalent DNA yields to the laboratory standard with reduced complexity and potential for rapid scalability.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05025.

Derivation of protocol complexity equation; primer sequences for porcine GAPDH LAMP assay; primer sequences for human GAPDH LAMP assay; LAMP reaction composition for human and porcine assays; breakdown of components for protocol complexity calculations (PDF)

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Notes

The authors declare no competing financial interest.

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