

Original Article

Evaluation of the efficacy of two methods for direct extraction of DNA from *Mycobacterium tuberculosis* sputum

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Abstract

Introduction: Whole genome sequencing (WGS) has shown superiority over other bacterial typing methods and can be used to monitor disease transmission. The long culture period hinders use of WGS as a diagnostic tool for TB. The ideal situation would be to efficiently sequence directly from clinical specimens such as sputum. Attempts to sequence directly from *Mtb* clinical samples have achieved very low coverage (less than 0.7X). We compared DNA extraction methods for direct extraction from *Mycobacterium tuberculosis* positive sputum and assessed their suitability for Single Molecule Real Time sequencing.

Methodology: We evaluated the extraction efficiency of the PrimeXtract kit and an in-house CTAB method by extracting DNA from *Mtb* sputum. We evaluated the methods on these parameters: ease of use, efficiency (quantity and purity) and the cost per extraction.

Results: The PrimeXtract kit was able to isolate $5.93 \mu\text{g/mL} \pm 0.94$, (Mean \pm SEM) concentration of DNA and a yield of $0.2975 \mu\text{g} \pm 0.04723$, (Mean \pm SEM). Comparatively, the CTAB method isolated $1.88 \mu\text{g/mL} \pm 0.38$ DNA and a yield of $0.09 \mu\text{g} \pm 0.02$. Both concentration and yield from the kit were significantly ($p = 0.0002$) higher than those from CTAB. The PrimeXtract kit had a DNA purity ratio of 1.69 ± 0.09 compared to the CTAB's 1.73 ± 0.14 and this difference was not statistically different.

Conclusion: PrimeXtract kit has a superior extraction efficiency than the CTAB method on *Mtb* sputum in terms of DNA yield although no significant difference by DNA purity was seen.

Key words: *Mycobacterium tuberculosis*; SMRT sequencing; PrimeXtract; CTAB.

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Introduction

Tuberculosis (TB) is the ninth leading cause of mortality worldwide whose impact has been exacerbated by coinfection with HIV/AIDS. Most TB deaths could be prevented with early diagnosis and appropriately timed treatment [1]. The advent of high throughput DNA sequencing platforms has led to unprecedented use of whole genome sequencing (WGS) to better understand the genome and epigenome of *Mycobacterium tuberculosis* (*Mtb*) the causative agent for TB [2]. WGS has shown superiority over other bacterial typing methods and can be used to monitor disease transmission [3]. The long culture period hinders use of WGS as a diagnostic tool for TB [4]. The ideal situation would be to efficiently sequence directly from clinical specimens such as sputum.

Attempts to sequence from *Mtb* clinical samples have achieved very low coverage (less than 0.7X) as a consequence of high human DNA contamination [4]. In the study, a NucleoSpin Tissue-Kit for hard to lyse bacteria was used [5]. Some DNA extraction methods including Infection Diagnostic Inc (IDI) lysis tubes, Prepman Ultra, Qiagen QIAamp, Tris-EDTA, Sodium dodecyl sulfate (SDS)-Triton X and Tris-EDTA (TE) buffer have been evaluated for direct extraction of *Mtb* sputum for polymerase chain reaction (PCR). The Cetyltrimethylammonium bromide (CTAB) method was found to be superior for direct extraction from sputum in a study of seven DNA extraction protocols [6]. The Primextract, a commercially available DNA extraction kit has shown great promise in the extraction of DNA directly from clinical samples [7].

Single Molecule Real Time (SMRT) Sequencing yields highly accurate consensus sequences as it offers long read lengths and random error profiles [8]. Additionally, SMRT sequencing lacks GC bias and can be used to detect DNA base modifications [8]. However, unlike other sequencing technologies, SMRT sequencing has stringent DNA requirements. Pure high molecular weight double stranded native DNA is required as there is no amplification step during sequencing [9]. Some studies have sequenced *Mtb* DNA from commercial kits using SMRT sequencing but this has largely been from culture [2]. To date and to the best of our knowledge, no study has attempted SMRT sequencing directly on clinical samples either through a commercial kit or an in-house DNA extraction method.

We hereby report an evaluation of a commercially available DNA extraction kit; Primextract kit (Longhorn Vaccines and Diagnostics, USA) and an in-house Cetyltrimethylammonium bromide (CTAB) based method directly from confirmed *Mtb* positive sputum. The two methods were evaluated on the ease of use, efficiency (quantity and purity) and the cost per extraction. We further assessed suitability for SMRT sequencing.

Methodology

Samples and Bacteriological Methods

Sputum samples were collected from a total of 120 TB patients and frozen on the same day of collection under a different TB study. From these, a total of forty TB confirmed 3+ positive samples were selected for extraction. *Mtb* was confirmed by both BD MGIT TBC ID test device (Becton Dickinson, Maryland, U.S.A) following manufacturer's instructions and Ziehl-Neelsen staining.

Prior to processing all samples were kept frozen at -20°C. Samples were decontaminated with sodium hydroxide and N-acetyl-L-cysteine (NaOH/NALC) with 1% NaOH final concentration. Following centrifugation each pellet was re-suspended in 1 mL of buffer and used for direct DNA extraction. Each sample was homogenized by vortexing at maximum speed for 5 minutes. A 50 µL aliquot and three tenfold dilutions were drawn from each sample before the extraction and used to determine cell density. Colony forming units (CFU) were calculated using the formula:

CFU = Number of colonies × 20 (50 µL was inoculated into each segment) × dilution factor

Genomic DNA Isolation

The Primextract DNA extraction kit (Longhorn Vaccines and Diagnostics, San Antonio, USA) was evaluated following manufacturer's instructions. A 200 µL aliquot of each decontaminated sample was processed according manufacturer's protocol. As a positive control, a H37Rv loopful that had been re-suspended in 200 µL molecular biology grade water was processed in a similar way for the kit. The Primextract kit is a spin-column based kit with ready to use lysis and wash solutions. It uses no heated incubation and has short centrifugation times. As a comparator the in-house CTAB method, samples were processed as previously described [6] with modifications. In brief to a 200 µL aliquot of the same sample where the kit sample was drawn, an equal amount of TE buffer was added and boiled at 85°C for 10 minutes to kill the *Mtb*. This was followed by immediate freezing at -20°C for 15 minutes and addition of 40 µL (20mg/mL) lysozyme. Following incubation at 37°C for 1 hour, bacterial membrane was disrupted by increasing the temperature to 65°C. Proteinase K (250µg/mL final concentration) and 1% SDS were added with continuous agitation. This was followed by addition of 10% CTAB and 0.7 M to a combined final concentration of 1%. Following incubation at 65 °C for 20 minutes, DNA was extracted with chloroform:isoamyl alcohol (24:1) and precipitated using 0.6 volumes isopropanol. All DNA was eluted in 50 µL of elution solution/ Tris-EDTA (TE) buffer and concentrations were measured using Qubit 3.0 fluorometer (Life Technologies, Austin, USA) whereas DNA purity was determined using a NanoDrop Spectrophotometer (ND-1000, NanoDrop Technology, Wilmington, Delaware, USA). A 260/280 ratio of 1.8-2.0 was considered to be high purity DNA. To ensure reproducibility all samples were processed in triplicate.

Statistical Analysis

The efficiency of two DNA extraction methods for forty samples was compared. Prism 7.0 software (Graph Pad La Jolla, CA, USA) was used for statistical analysis of the data. Means of concentrations, yield and purity of DNA were calculated. The Welch corrected t-test was used to test statistical significance of the data with a p value of < 0.05 indicating a significantly statistical difference between parameters being compared.

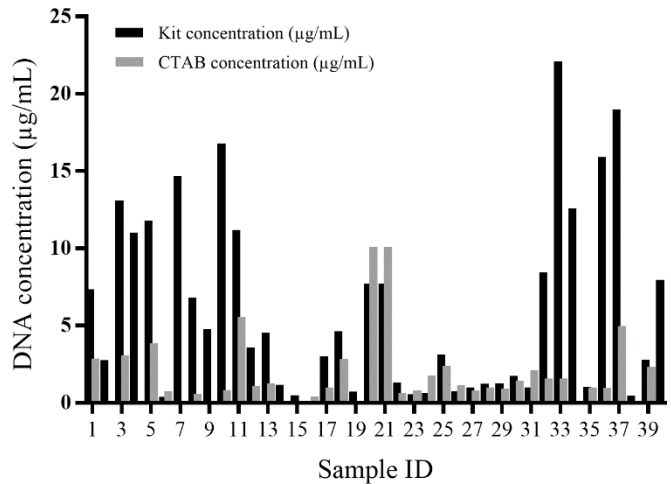
Results

The study compared the efficiency of the two extraction methods. Ease of use, total time, DNA yields (μg), concentration ($\mu\text{g}/\text{mL}$) and purity obtained from extraction using two different methods were compared. Cell densities of samples used in the study were determined by plating a $50 \mu\text{L}$ aliquot and three tenfold dilutions for CFU calculation. Despite being positive for DNA, majority of samples had zero CFU counts as they could not be revived. These were recorded as $< 80 \text{ cfu}/\text{mL}$ as this was the lowest recorded CFU count although we were mindful this DNA could as well be via host contamination. The kit uses a single sample preparation step with 5 centrifugation times of 60 seconds each with a total working time of < 1 hour. Comparatively, CTAB uses several reaction steps and longer centrifugation steps, the longest lasting up to 15 minutes. Furthermore, the CTAB method has a total working time of ~ 7 hours.

DNA concentrations were determined using a qubit fluorometer and results were presented as Mean \pm SEM for each method. The yield was calculated from the concentration in $50 \mu\text{L}$ of DNA sample. The yield was also presented as Mean \pm SEM. Significant differences were observed in both the concentration and yield.

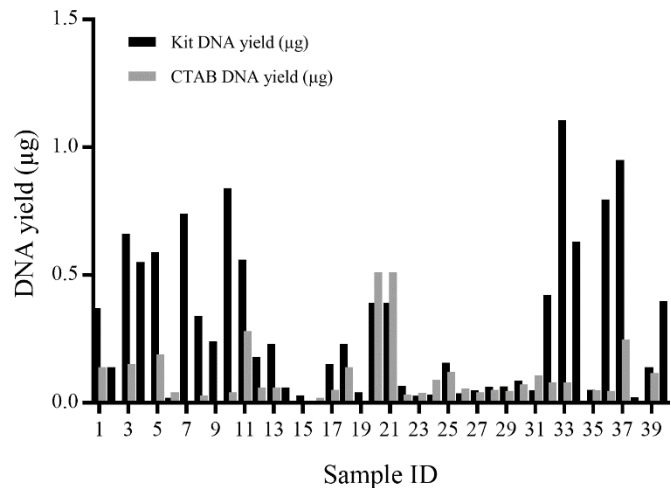
DNA purity was determined on a Nanodrop and results were also presented as Mean \pm SEM. Results from this study indicate that the PrimeExtract kit produced both higher yield and concentration (Table 1 and Figures 1 and 2). The kit had a concentration of $5.93 \mu\text{g}/\text{mL} \pm 0.94$, (Mean \pm SEM) and DNA yield of $0.2975 \mu\text{g} \pm 0.04723$, $n = 40$ (Mean \pm SEM). Comparatively, the CTAB method produced a concentration of $1.88 \mu\text{g}/\text{mL} \pm 0.38$, (Mean \pm SEM) and DNA yield of $0.09 \mu\text{g} \pm 0.02$, (Mean \pm SEM). Both concentration and yield from kit were significantly ($p = 0.0002$) higher than those from CTAB. The PrimeExtract kit had a DNA purity (260/280) ratio of 1.69 ± 0.09 (Mean \pm SEM) compared to the CTAB's 1.73 ± 0.14 , $n = 40$ (Mean \pm SEM). The kit purity was lower than that from CTAB (Figure 3) although this result was not statistically significant ($p = 0.76$). Purity values for the CTAB method, however, were much closer to the target purity range of 1.8-20.

Figure 1. Comparison of Concentrations of DNA extracted using two different methods.



DNA concentrations from two different extraction methods; the PrimeExtract kit (Longhorn Vaccines, USA) and an in-house CTAB method ($n = 40$). Concentration was measured using a qubit fluorometer ($\mu\text{g}/\text{mL}$).

Figure 2. Comparison of Yields of DNA extracted using two different methods.



Yields of DNA obtained using two different extraction methods: The PrimeExtract kit (Longhorn vaccines, USA) and an in-house method CTAB ($n = 40$). Yields were calculated from concentrations ($\mu\text{g}/\text{mL}$).

Table 1. Summary of results of DNA extraction using PrimeExtract and CTAB method ($n = 40$).

Method	Concentration ($\mu\text{g}/\text{mL}$)	Yield (μg)	Purity 260/280	Cost/sample	Total time
	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM	Mean \pm SEM
PrimeExtract	5.93 ± 0.94	0.29 ± 0.047	1.69 ± 0.09	5.76	0.5
CTAB	1.88 ± 0.38	0.09 ± 0.02	1.73 ± 0.14	0.39	7

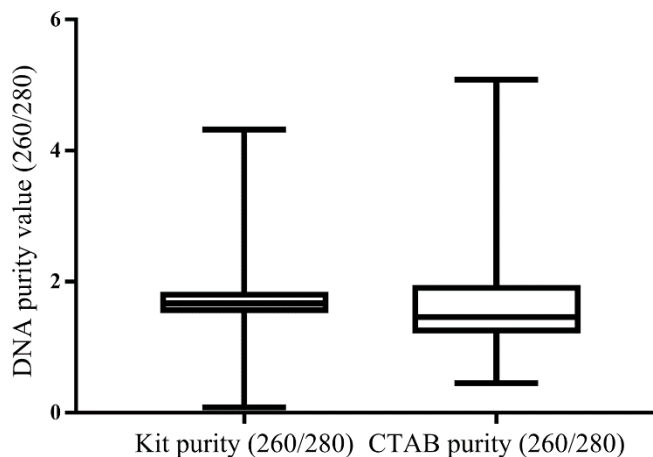
Discussion

The success of molecular downstream application very much depends on the quality and quantity of the DNA. Choice of a DNA extraction method is critical to this success. Unlike other WGS technologies, the PrimeXtract kit has been highly recommended for recovery of high molecular weight quality DNA directly from *Mycobacterium tuberculosis* clinical specimens [7]. Since we had previously successfully sequenced *Mtb* from a solid culture using SMRT, we then wanted to investigate if we could sequence directly from clinical specimens. To do this we had to find a DNA extract method that could efficiently extract DNA for such a purpose.

In the present work, we have demonstrated that the PrimeXtract DNA extraction kit is superior to the CTAB method for the isolation of *Mtb* genomic DNA in a 200 μ L sputum sample in terms of yield. The kit was capable of recovering higher quantities DNA than the comparator in the 200 μ L sample under consideration. The superiority of the kit is demonstrated in almost all samples (Figures. 1 and 2 and Table 1) where despite an equal input material, it has higher DNA output. The kit uses a specialized lysis solution and wash buffers and aid to release the DNA and wash off any cell impurities. These are critical in release and clean-up of DNA. Further the kit uses a spin column that in some ways limits the input material. The sensitivity of the kit is enhanced by a silica-based spin column that traps the DNA during centrifugation. The other advantage of the kit is that it is easy to use with short centrifugation times (60 seconds each) and total time \pm 1 hour. In the present study the kit, however, was deemed unsuitable for SMRT sequencing. It could only recover less than 2 μ g of DNA required for a 5Kb insert SMRTbell library preparation. Despite this observation, the kit was able to isolate high DNA yields compared to other kits within its range namely Qiagen (28.2 pg) and Prepman (30.4 pg)

The CTAB method has been shown to recover higher yields of DNA with good purity from clinical samples compared to other in house methods [6]. This is the first study to compare this method against the PrimeXtract kit. In our hands, the CTAB was inferior in terms of yield. Some investigators have suggested that CTAB method is efficient for DNA extraction from solid *Mtb* cultures [10]. Successful isolation of *Mtb* DNA especially from clinical samples requires harsh treatment to weaken the cell wall. In this study we used heating and immediate freezing to disrupt connections holding lipid contents together. It is obvious that this did not work very well. One study proposed heating at

Figure 3. Comparison of Purity of DNA extracted using two different methods.



Purity of DNA obtained using two different extraction method. The PrimeXtract kit (Longhorn Vaccines, USA) and an in-house CTAB method (n = 40). Purity was obtained using an ND-1000 Spectrophotometer Nanodrop. A 260/280 value of 1.8-2.0 was considered a pure preparation. Error bars represent standard error of the mean (SEM).

100°C in an appropriate buffer to achieve the results [11]. It has been suggested that use of phenol and chloroform helps in improving the yield [12]. We avoided using phenol as residual phenol is incompatible with Pac bio library preparation. Chloroform aids in denaturation of proteins and formation of a separating layer between aqueous and organic phases [6]. From our experience, the protein removal and DNA precipitation steps were appropriate as we have successfully applied them before. We used isopropanol to selectively remove DNA leaving RNA and other impurities. We then used 70% ethanol to wash off any remaining impurities from the DNA.

There were a lot of variations on the purity obtained from the samples using the two methods (Figure 3). This could be due the different samples containing varying amounts of impurities. The CTAB, however, had better mean purity value 1.73 which is closer to the required range of 1.8-2.0 compared to the kit's 1.69 which is much lower. As we did not use phenol, the lower 260/280 ratio would most likely indicate protein contamination. This is suggestive of the fact that the chloroform step did not work properly.

In addition to being labour-intensive, the CTAB method has a higher sample turn over time (total time 7 \pm hours) as compared to the kit's < 1 hour. The CTAB method, however, has the advantage of being flexible. It is possible to increase the cell density and successfully recover higher amounts of DNA. To

improve purity however a eukaryotic DNA, clean up step may be necessary. Another point to consider in the choice of an extraction method would be the cost. The PrimeXtract kit costs \$5.76 per extraction [7]. Compared to other DNA extraction kits, \$2.30 for Qiagen and \$1.05 for Prepman [5], the PrimeXtract costs well over double as much as the other methods. Comparatively the CTAB method costs ~\$0.39 per extraction [13]. Evidently the PrimeXtract despite being more efficient in terms of time, labour and quantity of DNA, is much more expensive than the CTAB method. We were unable to accurately quantify cell densities as most of our frozen strains could not be resuscitated for CFU counts. This is due to the fact that freezing kills some of the cells.

Conclusion

Our results suggest that the PrimeXtract kit is superior to the CTAB method for direct DNA extraction from *Mtb* sputum. With proper optimization such as additional sample decontamination and DNA purification steps, the PrimeXtract kit holds the potential to be used in direct extraction from sputum for the purpose of SMRT sequencing. We have previously sequenced cultured isolates using the CTAB method. If CTAB is to be used for direct extraction, a much high starting sample than one used in this study will be required. For resource-poor settings where kits are not readily available, CTAB remains the method of choice. Choice of an extraction method should ultimately be based on cost and ease of use. The study had some limitations. There is need for direct correlation between DNA yield and CFUs. In our case, it was not possible to accurately determine the CFUs. Additionally, the level of impurities in each sample could not be accurately quantified.

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Role of Funding Source

The funding sources played no role in the study design, data analysis or writing of the report. The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication.

Authors' Contributions

VN, GD and MC conceived the study. VN, GD and MK performed the investigations. VN analyzed the data. VN, WLM, DS, GD, MC wrote the report. VN and GD oversaw the research. All authors contributed to the study design and reviewed the report.

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