Optimization of Genetic Material Recovery for Downstream Analyses

By

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Thesis

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List of Abbreviations

dried blood spots	DBS
newborn screening	NBS
primary immunodeficiencies	PID
severe combined immunodeficiency	SCID
x-linked agammaglobulinemia	XLA
T-cell receptor excision circles	TRECs
T-cell receptor	TCR
polymerase chain reaction	PCR
kinetic outlier detection	KOD
polyethylene	PE
polystyrene	PS
polyethylene terephthalate	PET
Synechococcus sp. PCC 7002	PCC 7002
American Type Culture Collection	ATCC
polyethylene microparticles	PEμPs
polyethylene nanoparticles	PENPs

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Introduction

The effective detection and analysis of nucleic acids is critical for many fields, including clinical diagnostics, environmental testing and forensic science. Intracellular nucleic acids hold essential genetic information that can be used for a myriad of reasons, such as identifying the presence of biological traces or to diagnose, treat, prevent and cure illnesses [1]. However, the analysis of nucleic acids can be complicated and involve several multi-step processes. The process for detection of unique genetic sequences, in particular, relies on the efficient extraction and purification of RNA or DNA from downstream inhibitors. Additionally, many sample-dependent factors need to be considered, such as cell lysis, DNA versus RNA, sample background, sample volume, appropriate preparation of chemicals, and downstream analysis requirements [2].

Over the past 20 years, efforts in nucleic acid analysis have focused towards translating sophisticated biomolecular analytical procedures from a laboratory setting toward processes that can be conducted in large-scale and/or multiplex automated systems. These efforts are motivated by a desire to develop cost-effective and simplified versions of existing processes and because current methodologies are limited when detecting smaller nucleic acids, such as plasmid DNA and gene regulating molecules (tRNA, mRNA, etc.) [3]. This is as a result of conventional nucleic acid extraction methods being developed with the aim of extracting large nucleic acids from whole blood [4].

The following chapters in this thesis include two distinct projects in which robust genomic extraction and recovery procedures were conducted from microsamples of whole

blood or bacterial cells. These nucleic acid extraction procedures were optimized to allow for maximum extraction of genomic material with simple and time-effective procedures.

Chapter 1: Electrophoretic Extraction of Episomal DNA from Dried Blood Spots for Neonatal Screening

1. Introduction

Dried blood spot (DBS) sampling is a minimally invasive and cost-effective technique involving the preservation of minute amounts of whole blood onto filter paper cards. Recorded as early as the 1960s by Guthrie and Susi [5], DBS are prepared by collecting a sample of peripheral blood from a finger, hell, toe, or earlobe puncture onto dried filter paper. In newborn screening (NBS), DBS are collected and used to detect metabolic, hematologic, and other inherited disorders. Currently, tests for over 60 inherited disorders are available in the United States [6].

Major efforts in NBS are focused on developing methods to detect primary immunodeficiencies (PIDs). In inborn errors of immune function, such as those involved in severe combined immunodeficiency (SCID) and X-linked agammaglobulinemia (XLA), mutations in a single gene can lead to life-threatening infections [7]. A great deal of attention has been devoted to developing rapid, effective detection methods for PIDs and the measurement of signal joint T-cell receptor excision circles (TRECs) by quantitative PCR holds significant promise.

T-cell receptor excision circles are episomal DNA molecules that are produced as a result of the T cell maturation process [6][7][10]. The circularized DNA fragments form through the rearrangement of the T-cell receptor (TCR) α-locus and do not further replicate in dividing cells [9, 10]. Because of this lack of replication, the molecules can serves as a measure of T-cell proliferation and replication as well as of thymic function [9]. Although SCID can be caused by

multiple disease mechanisms, all patients with SCID have been shown to have a characteristically reduced number of TRECs [9], [11]–[15].

Many of the methodologies currently used to analyze episomal DNA in DBS are not suitable for large scale genetic analysis or for automation. Commercially available DNA extraction kits were developed to extract genomic DNA from whole blood[4], which has much larger sample size that of DBS [16]. Small sample size, impurities, and spotting variations in the DBS can lead to significant variations in the absolute TREC copy number, even in samples taken from the same DBS [17][18]. Many protocols currently used for the extraction of TREC from DBS rely on expensive materials such as Proteinase K, solid phase purification solutions, and silica column supports [12][19][20]. Other commonly used protocols require numerous, lengthy preparation steps, often over an hour for cell lysis and DNA extraction alone and become too time-consuming for large scale testing [4][21]. Even in more rapid methods of analysis, such as FTA cards, microwave-based extraction and direct PCR, extraction can become complicated in samples where DNA template concentration is low, such as TREC. Direct PCR from FTA cards or Whatman filter paper is perhaps the most simple and rapid of these methods, skipping the extraction step entirely. However, it still requires wash steps and can be significantly limited due to the presence of natural inhibitors in these samples [22][23]. Removal of these inhibitors often requires complicated wash and elution steps that can add at least 15-30 minutes to procedure time [22][24][25]. Another complication occurs during sample handling, where FTA card can also be easily contaminated due to incorporation of lysis chemicals in the paper [26]. Because of the limited amount of sample available on these spots, the amount of genetic material is also limited [22]. This is especially important in low copy DNA such as TREC and can

lead to effects such as non-specific amplification, stochastic effects, and large variability in yields [26][27][28].

The objective of this thesis project was to investigate the effect of temperature, electric field strength and time on blood spots for extracting DNA from DBS. We worked to optimize a protocol for extracting and quantifying DNA from neonatal dried blood spots.

2. Methods & Materials

2.1. Creation of standardized DBS samples. To develop this robust method of DNA extraction, this investigation used well characterized methods shown to reduce potential variability in blood samples such as hematocrit adjustment, washing, and pipetting onto filter card [29][30][31][28]. Blood cells were washed and re-suspended in PBS instead of serum [29] or charcoal treated plasma to limit interferences in downstream processes such as PCR [28]. Briefly, cord blood (PerkinElmer Waltham, MA) was prepared by centrifuging at 2500 x g for 10 minutes at 4 ˚C. The plasma was then discarded. Saline was added to the remaining blood mixtures and hematocrit was measured and adjusted to 48-53. 85 µl blood spots were then pipetted onto dried blood spot cards (PerkinElmer 226 Sample Collection Device, PerkinElmer, Waltham, MA). DBS cards were aged overnight at room temperature and then punched into a 96 well plate using a Wallac DBS puncher (PerkinElmer, Waltham, MA) [32][30].

2.2. Extraction of DNA from DBS

2.2.1. Wash Steps - 3.2 mm dried blood spot samples were then placed into the wells of a 96-well PCR plate using clean forceps. DBS then underwent a series of wash steps with a detergent buffer. In short, 80 µl of wash buffer (1X PBS/ 0.1% Tween-20) was added to each well of the 96 well plate. The plate was vortexed at 25˚C for 10 minutes at 700 rpm on a temperature-controlled shaker (Thermoshake, Inheco, Martinsried, Germany). After the first incubation, the eluate was discarded, and the wash step was repeated. A third wash step was performed by adding 80 µl of a lysis buffer (10mM KOH/30mM Tris buffer) to each well and incubating for another 10 minutes at 25˚C and 700 rpm. The lysis buffer had a pH of 12.25.

2.2.2. Non-vortex procedure (0 rpm wash steps) - An alteration was made to the vortexing wash protocol in order to create a procedure that did not require vortexing steps. 3.2 mm dried blood spot samples were added to a 96 well plate and washed 2 times with a detergent wash buffer (1X PBS/ 0.1% Tween-20) and then once with a lysis buffer (10mM KOH/30mM Tris buffer). After each wash step the plate was incubated at 25˚C for 5 min without agitation (0 rpm).

2.2.3. Manual lysis extraction procedure - The same wash steps were used in both the manual lysis procedure (PerkinElmer) and novel electric field procedure, however after this point the protocols differed significantly (Figure 1B). To perform the manual lysis procedure an additional 80 µl of lysis buffer (10mM KOH/30mM Tris

buffer) was added to each well containing a DBS. The plate was then incubated at 70˚C and 700 rpm on a temperature-controlled shaker for 30 minutes. After this incubation the eluate from PCR plate was removed and analyzed immediately using qPCR.

2.2.4. Electric field extraction procedure - DBS undergoing the electric field extraction procedure were removed from the PCR plate using clean forceps and placed in a Sigma-Aldrich© electroporation cuvette with a gap width of 0.1 cm. Cuvettes containing DBS and 80 µl of lysis buffer (10mM KOH/30mM Tris buffer) were then either subjected to a five- minute heating step or processed in the electric field immediately. Electric fields were then applied to the DBS containing cuvette. At the end of each extraction, the supernatant was removed from the cuvette and analyzed immediately using qPCR.

2.3. Analysis of Extracted DNA

2.3.1. Detection of T-cell Receptor Excision Circles - To detect TREC extracted from DBS, real time quantitative PCR was performed. All experimental conditions were tested with at least three experimental samples and two technical replicates per sample were performed to assess experimental accuracy. Each PCR run of 20 µL contained 4 µL of sample, TaqMan 2× PCR Master Mix, and OneTaq® Hot Start DNA Polymerase (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA). The probes used for this assay were labeled with FAM for TREC and HEX for the Ribonuclease P/MRP

Subunit P30 gene (RPP30). RPP30 was used as a reference gene to assess PCR amplification and DNA extraction. Reactions were carried out on a PikoReal Real Time PCR System (Thermo Fisher Scientific, Waltham, MA) using the following protocol, a 5 minute denaturation at 94°C followed by 44 cycles of 15 seconds at 94°C, 30 seconds at 60°C and 15 seconds at 68°C. Fluorescence data from PCR amplification was analyzed using the Picoreal software and the R project statistical computing qpcR library. C_t values were determined by two separate methodologies. First C_t was calculated using a fixed threshold value of 170 for RPP30 and a fixed threshold value of 50 for TREC. Further analysis of the raw fluorescence data included a sigmoidal fitting of the amplification curves with a five-parameter log-logistic curve. Kinetic Outlier Detection (KOD) was used to detect and remove cases of unusual amplification in two steps. A test on sigmodal structure identified runs that failed to amplify, by requiring that the difference between the first and second derivative maxima be less than 10 cycles [33]. Samples that were not eliminated as outliers were tested against the efficiencies of a standard reference set using a z-test [34]. The second derivative maximum, cpD2, and Cy0 numbers were also obtained. Copy number was obtained from the raw C_t values. TREC and RPP30 copy numbers were calculated by a tenfold dilution series of 105 copies of linearized plasmid (PerkinElmer, Waltham, MA, USA). A standard curve was created for both plasmids by plotting the C_t numbers against the number of copies of the plasmid. Extraction efficiency was assessed using percent yield, which was calculated from literature

values. For an extraction of 100% efficiency, a 3-mm dried blood spot was considered to contain 3.47 ng/ µL of genomic DNA [35][36].

2.3.2. Picogreen quantitation of DNA extracted from DBS - Double stranded DNA was quantified with a Quant-iT™ PicoGreen® dsDNA kit (Invitrogen, Eugene, OR, USA). Each experimental condition was tested using three experimental samples (or DBS) and two technical replicates per sample. The procedure consisted of the following. PicoGreen reagent was prepared as a 1:200 dilution with TE buffer (pH 7.5). Experimental samples were diluted in a 1:10 dilution in TE buffer to reduce inhibitors in the sample. 20 µL of each sample was added to 20 µL of the PicoGreen reagent working solution in a black 384-OptiPlate (PerkinElmer, Waltham, MA, USA) and was read using a fluorescence plate reader (EnVision 2105 multimode plate reader (PerkinElmer, Waltham, MA, USA)) at an excitation wavelength of 485 nm and an emission wavelength of 535. Lambda DNA (supplied by the manufacturer) at a concentration of 100 µg/ mL was used to construct a standard curve. Blank values and background were subtracted from experimental data.

2.3.3. Statistical evaluation of experimental optimization - Statistical analysis of electric field extraction of DNA from dried blood spots was performed using multi-factor ANOVA. The Tukey HSD test was used to evaluate the statistical significance of interactions between each factor's levels. The three factor ANOVA to evaluate the experimental optimization had the following factors and levels: KOH concentration

(5 mM, 10 mM and 15 mM), Applied voltage (10V, 25V, 50V) and time of pulse (30s, 60s, 300s). A p- value of 0.05 was considered to be statistically significant. Statistical analysis was performed using a the XLSTAT statistical software package (Addinsoft, New York, NY).

3. Results

To evaluate the feasibility of extraction of genomic DNA from DBS using the electric field cuvette device, a proof of concept experiment was performed (Figure 1). In this experiment DBS were oriented in one of two directions, either with side of the filter paper spotted with cord blood facing the negative electrode or with the side of the filter paper spotted with cord blood oriented toward the positive electrode. The results of this experiment showed that no RPP30 or TREC was detected when the blood spotted on the paper was facing the negative electrode. A control where a DBS and buffer were added to the cuvette also showed no amplification of RPP30 (results not shown). This suggests that the electric field alone was responsible for the DNA extracted from the surface.

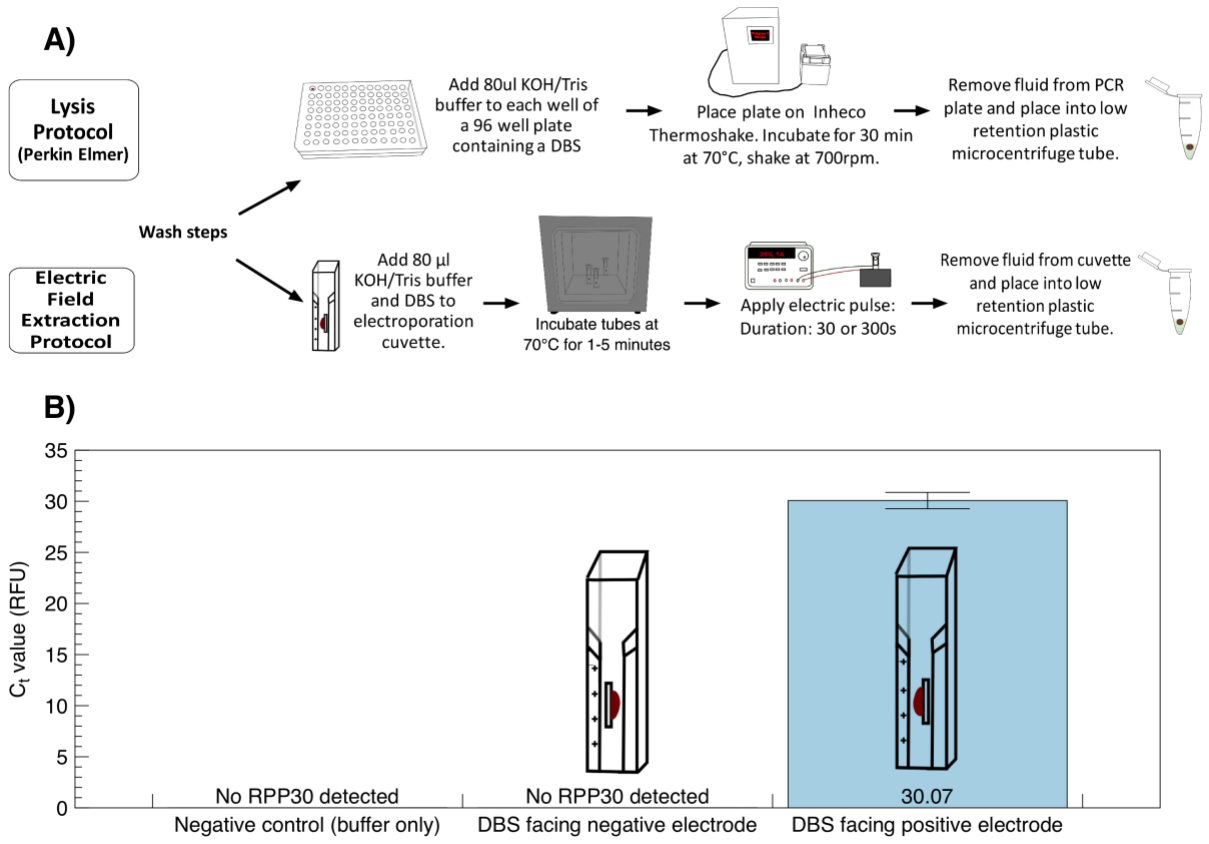


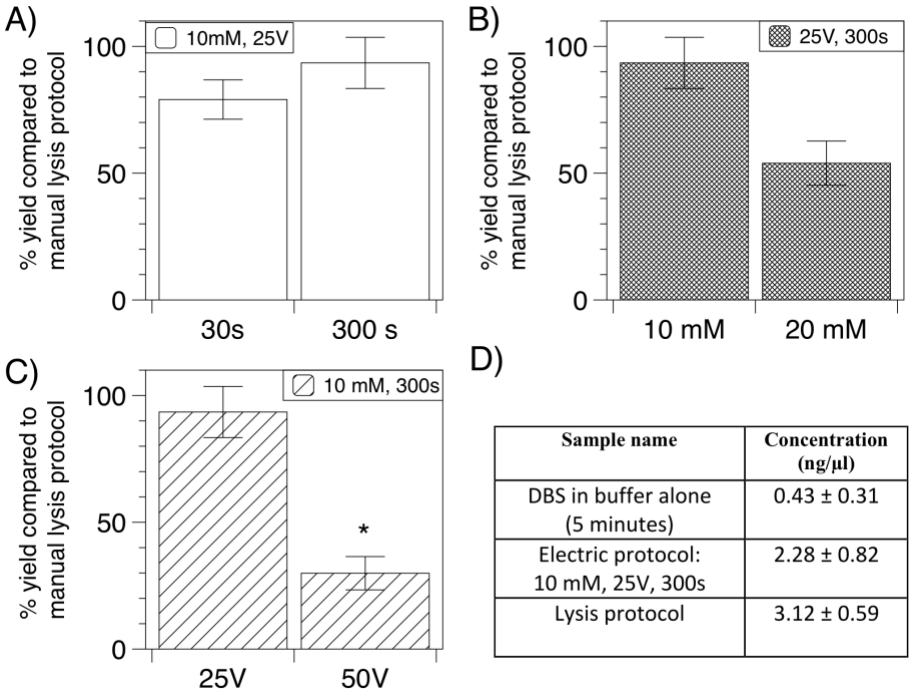
Figure 1.

Proof of Concept Experiment & DNA Extraction Procedures. A) Proof of concept experiment to evaluate feasibility of dried blood spot system. C_t values of DBS placed in electroporation cuvette and oriented in one of two directions: i) blood spot oriented toward negative electrode ii) blood spot oriented toward positive electrode. Values were compared to blank control with buffer only. B) Diagram highlighting differences between proprietary lysis procedure and novel electric field extraction procedure

Optimization of electric protocol for DNA recovery

Evaluation of DNA retrieval from electric field procedure - The optimization of the system was continued with an in depth look at three parameters of the electric field procedure, time of the pulse applied across the cuvette plates, strength of the voltage applied across the cuvette plates, and finally the concentration of KOH in the lysis buffer solution. The housekeeping gene, RPP30 was used to measure the variation in genomic DNA extracted from the DBS and as a

preliminary method of assessing its quality. Three biological replicates for each experimental condition were used for real-time PCR analysis and two technical replicates were analyzed for each biological replicate to assess experimental accuracy. As seen in Figure 2a, evaluations of pulse time at 10 mM and 25V showed decreases in C_t with increasing pulse time. Evaluation of the molar concentration of KOH in the buffer showed that KOH in excess of 10mM lead to slight decreases in in the yield of DNA from the electric extraction. (Figure 2b) Optimal recovery was seen at 10mM, 25V and 300s, recovering 93% of DNA captured by the lysis procedure.



Sample name	Concentration (ng/µl)
DBS in buffer alone (5 minutes)	0.43 ± 0.31
Electric protocol: 10 mM, 25V, 300s	2.28 ± 0.82
Lysis protocol	3.12 ± 0.59

Figure 2.
Comparison of Percent Yield with Manual Extraction. Comparison of the percent yield of RPP30 from electric procedure with selected parameters with the manual lysis procedure A) Pulse time (30s, 300s) B) KOH concentration in Tris buffer (10 mM, 20 mM) C) Voltage applied to cuvette (25V, 50V) D) Measurement of DNA concentration by PicoGreen® reagents in samples from both proprietary lysis and electric field procedures. Data = mean +/SEM; N=3; *p < 0.05

Additionally, this procedure reduced the time of the manual lysis procedure by a total of 25 minutes. Higher voltages and molar concentration of KOH in the buffer lead to significant decreases in the recovery of DNA from DBS ($p=0.050$, two-way ANOVA) (Figure 2c). This suggests that recovery of DNA at 10 mM and 25V may be unaffected by harmful changes in the environment of the cuvette such as bubble formation, heating and dramatic pH changes caused by hydrolysis. These effects have been linked to pulse time in electroporation [37]. Further quantification of dsDNA was performed using a Picogreen assay and can be seen in Figure 2d. Three biological replicates per experimental condition were analyzed using the Picogreen assay. Two technical replicates were performed for each biological replicate. The Picogreen assay confirmed that the presence of dsDNA within the 10mM, 25V, 300s electric sample was significantly different than samples exposed to buffer alone ($p=0.050$).

Development of a non-vortex protocol to aid in DNA recovery - Scale up to automation is another important part of the creation of a viable methodology for DNA extraction from dried blood spots. The high throughput analysis of an automated DNA extraction would be especially beneficial for large scale genetic testing such as newborn screening for primary immunodeficiencies. Automation increases efficiency and accuracy, standardizes sample handling, and limits human contact with potentially infected materials making it highly effective strategy to process clinical samples like DBS. To create a fully automated system, certain requirements of the manual lysis procedure needed to be changed. Most significantly, vortexing needed to be removed from this protocol. Thus, an optimized electric procedure without vortexing in the wash steps was developed. Samples were analyzed using real-time PCR

with three biological replicates for each experimental condition and two technical replicates to assess experimental accuracy. As seen in Figure 3, the yield from this improved procedure for automation was 81% of that of the manual lysis procedure. The removal of vortexing from the wash steps, reduced the time of the wash steps by half, not only simplifying the procedure but saving an additional 15 minutes over the original electric procedure. The total time for genomic DNA extraction using the optimized electric procedure without vortexing was 20 minutes.

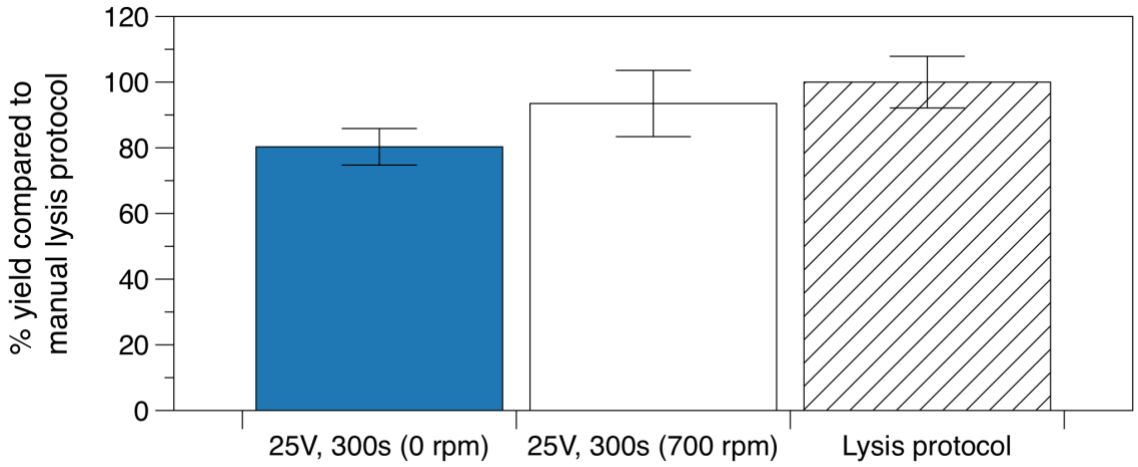


Figure 3.
Analysis of Vortexing on Wash Steps. Comparison of percent yield of RPP30 from electric procedure (wash steps at 700 rpm, wash steps at 0 rpm) and proprietary lysis procedure. Data = mean+/SEM; N=3

Optimization of episomal DNA recovery - To design a procedure without vortexing that would preferentially yield episomal DNA from the DBS, optimizations to the previous protocol were necessary. High temperature extraction methodologies are commonly used for plasmid isolation and preparation within the literature and are particularly suited to episomal DNA recovery. Initial testing on vortexed samples showed the addition of a 5-minute heating

extraction step at 70°C caused a significant decrease in the C_t of RPP30 (p=0.032, two-way ANOVA). Thus, to increase episomal DNA recovery, a 5-minute heat-based extraction was added to the novel electric protocol. The temperature, location and duration of the heat-based extraction was assessed using qPCR.

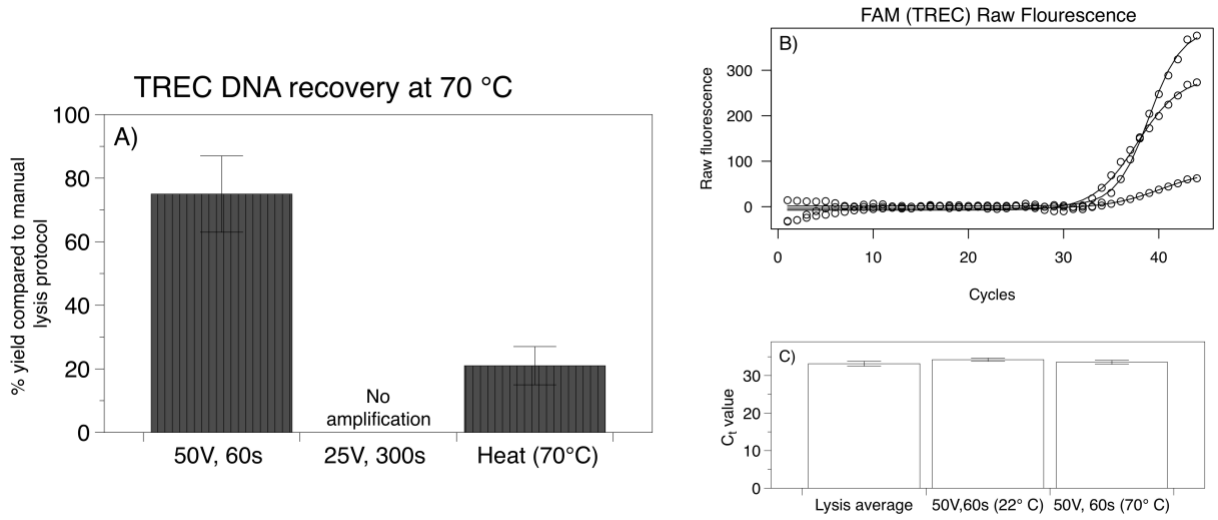


Figure 4.
Episomal DNA Extraction Optimization. Addition of 70˚C heating step to non-vortexed electric procedure (0 rpm wash steps) to increase episomal DNA yield. A) Comparison of the percent yield of TREC from optimized electric procedure (wash steps at 0 rpm, 70˚C heating step) with proprietary lysis procedure B) Raw fluorescence curves for FAM (TREC) of analyzed samples C) Ctvalues of non-vortexed electric procedure (wash steps at 0 rpm) with and without 70˚C heating step. Data = mean +/SEM; N=3

Figure 4A shows the average yields of the optimized 70°C heating procedure for non-vortexed samples at two conditions 25V, 300s and 50V, 60s. No amplification was seen from the 25V samples for TREC episomal DNA. A substantial decrease in the C_t value of the non-vortex samples occurred at 50V, 60s. These results were statistically different than that of the control, heating alone (p=0.006). As seen in Figure 4A, samples processed at 50V, 60s yielded 75% of the total yield of the manual lysis procedure. Figure 4C compares the C_t values the 50V, 60s samples

at 70°C and samples without the added heating step (22 °C (room temperature)). By combining the heating procedure and the electric extraction, yields were increased above the yield of either method alone with no greater than a 5-minute increase in the total time.

Analysis of electric field - Simulation of the electric field within the extraction device was carried out using the AC/DC module of COMSOL Multiphysics (v5.3, COMSOL Group, Burlington MA). Two distinct 2D model geometries representing the experimental conditions tested within the model system were designed (Figure 5). Each model consisted of three parts, two parallel stainless-steel electrodes, an electrolyte buffer region, and a model of the dried blood spot.

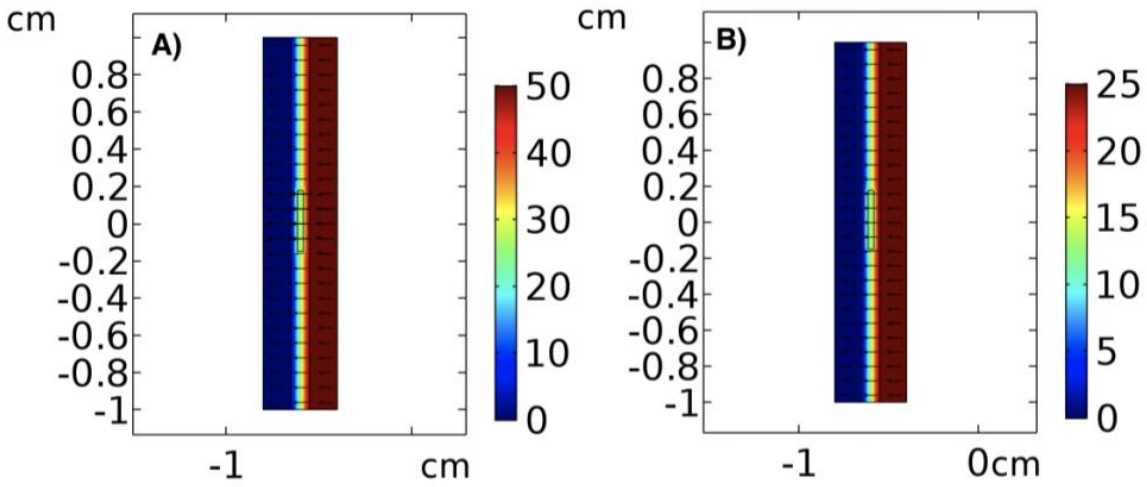


Figure 5.
Finite Element Analysis of Electroporation Cuvette. Highlights of finite element analysis of electroporation cuvette A) Electric potential distribution across cuvette at 50V B) Electric potential distribution across cuvette at 25V.

To perform the finite analysis, each area was meshed with a free triangular mesh. The minimum element size of this mesh was 0.001 cm and a grid independence study confirmed that the calculated solution was independent of the mesh size. Boundary conditions for the model were

prescribed as follows, potential at the edge of the first stainless steel electrode was set as the voltage applied during the experiment (25V or 50V). The remaining electrode was then defined as a ground. The DBS can be seen as a 3.2 mm x 0.33 mm rectangular region located in the center of the diagram. The conductivity of this region was approximated as 0.7 S/m the conductivity of blood [36]. This approximation neglects the contribution of the filter paper to the total conductivity of the DBS. Recent studies suggest that the filter paper makes little difference in the value of the conductivity of a dried blood spot [38]. The relative permittivity of the DBS was set as 5260 [39]. The conductivity of stainless steel was set at 1.45x 106 S/m and the conductivity of the KOH/Tris buffer was determined to be 0.133 S/m using a conductivity meter (Horiba Laquatwin B-771 Horiba Ltd.) [40]. An LCR meter was used to calculate the dielectric constant of the KOH/Tris buffer from the measured capacitance from between the plates of the electroporation cuvette. Values obtained (6.92) were in good agreement with literature values for KOH buffers. Diffusion coefficients for OH- and K+ ions were taken from the literature and were as follows 5.23 x10-9 and 1.9 x10-9 [41]. Results of this simulation can be seen in Figure 5 for voltages of 50V (Figure 5 A) and 25V (Figure 5 B). The simulation results showed a roughly uniform electric gradient generated across the plates of the cuvette with an electric field strength of 500 V/cm for the 50V experiment and 250 V/cm for the 25V experiment. This proves that the cuvette is suitable for DNA removal from the DBS and for DNA free fluid electrophoresis. Importantly, other than the strength of the electric gradient generated across the longitudinal gap, there were no significant differences between the model systems. This suggests that forces from the electric field are the main factor in removal of DNA from the DBS.

4. Discussion

This study performed an in-depth analysis and optimization of a novel electric field extraction to remove DNA from DBS. Currently, methodologies available for DNA extraction from DBS are laborious and time consuming. This newly optimized procedure not only reduces the time of the manual procedures by more than 30 minutes, it negates the need for costly treatment and wash steps, while retaining the recovery of episomal DNA from the DBS.

In the present work, we have created a novel methodology for the extraction of DNA from DBS using electric fields and optimized this process. We performed a proof of concept experiment which showed the feasibility of the DNA extraction by electric field. Additionally, computational analysis of the electroporation cuvette showed uniform electric gradients generated across the plates of the model suggesting that the electric field is the main factor in DNA removal from the DBS.

Optimization of the extraction process for TREC episomal DNA achieved genomic and episomal DNA recoveries in the 25V, 300s and 50V, 60s procedures that were not significantly different than the recoveries of the manual lysis procedure. To our knowledge, no study within the literature has used an electric field methodology to remove DNA from DBS. The 25-minute extraction time from our optimized electric field DBS lysis procedure is one of the lowest procedural times for episomal DNA extraction currently found within the literature.

In addition to assessing the feasibility of the novel electric field system this study developed and optimized an electric field extraction procedure suitable for scale-up to automation. The novel electric field extraction methodology allowed for the removal of time-consuming wash steps which rely heavily on vortexing to improve DNA yield. Not only were these new procedures able to remove these time and energy- consuming steps, but to retain more than 80% of the yield of RPP30 and 75% of the yield of TREC of the manual lysis procedure respectively. Perhaps more encouragingly, early results suggest that TREC DNA can be recovered from DBS exposed to a dual electric field extraction (25V, 300s and then 50V, 60s (Ct 34.87)). As seen in our investigations of the orientation of the DBS, the cellulose fiber matrix of the dried blood spot, has a direct effect on the migration of DNA out of the filter paper. Long DNA molecules, like TREC episomal DNA, have been shown in the literature to remain trapped within the network of the filter paper even after cell lysis and wash steps. This effect is thought to be caused by DNA entanglement with fiber matrix of the paper and is dependent on the pore size of the paper [42].

The purity of the DNA retained on the fiber matrix of the paper is influenced by the porosity. Filter papers with larger pores release DNA, while filter papers with small pores may retain inhibitors from blood samples [42]. Using the matrix of the filter paper to hold the episomal DNA might provide a unique method to remove inhibitors and pre-purify DBS samples and should be explored in further detail.

Several factors may have affected the overall yield from the electric field extraction system. Quantification of DNA has been shown to be strongly affected by adhesion to standard plastics [43][44]. Results of an in-depth analysis of adhesion showed substantial increases

(p=0.012) in average Ct value after exposure to the plastic cuvette. Continued testing within the experimental system should address the problems of adhesion. Future tests should consider pre-treatment of the cuvette with carrier RNA [44] or the addition of BSA to the buffer media.

Amplification inhibition was another concern for accurate quantification. Amplification inhibition has been shown to frustrate interpretation and analysis of low copy DNA [42] [43]. Inhibition can lead to target underestimation, and ultimately to false negatives [47]. An analysis of inhibition within our samples by serial dilution showed results suggestive of inhibition in individual samples including variations in ΔCt below what would be expected at 100% efficiency. These results suggest that inhibition may be a cause of target underestimation in DBS samples [40]. However, confounding factors such as natural variation in DNA amount and quality on DBS remain significant in the variation of amplification efficiency among DBS [47]. Thus, a robust method of assessing similarity and excluding outliers in the data (as was used within this paper) is necessary. Future work should focus on population-based investigations and on the development of statistical tools and methodologies to better account for this natural variation.

Future designs should adapt the novel electric field methodology for the extraction and purification of other charged analytes (such as RNA and proteins) from DBS. Most importantly, an on-plate extraction system should be designed for the scale up to automation of the rapid electric field extraction methodology. Automation of this procedure will not only increase efficiency and accuracy but will standardize the processing of DBS. Increased throughput and reduced processing time from an automated system would significantly improve clinical diagnostics where large numbers of DBS are required, like newborn genetic screening.

5. Conclusions

This study showed the feasibility of DNA retrieval from dried blood spots using an innovative electric field methodology. An in-depth analysis of the electric field methodology showed significant recovery of both genomic and episomal DNA from dried blood spots. Optimization of this procedure showed voltage across the plates had a significant effect on DNA retrieval further confirming the utility of this process. Electric field extraction not only retrieves episomal and genomic DNA but it produces a notable reduction in process time from standard lysis procedures, a time savings of over 30 minutes. These results prove that, electric field extraction provides a rapid and effective method to remove episomal DNA on patient samples such as dried blood spots, and as such provides a valuable tool for clinical genetic screening.

Chapter 2: Analysis of total RNA extracted from cyanobacterial cells for biotechnology applications

1. Introduction

Plastic waste pollution in the ocean is a large global environmental concern. The accumulation of plastic litter in the ocean is increasing with an estimated 4.8 to 12.7 million metric tons (MT) of generated plastic waste from 192 coastal countries entering the ocean in 2010 [48]. Petrochemical plastics, including polyethylene (PE), polystyrene (PS), and polyethylene terephthalate (PET), make up approximately 85% of the total global plastic usage [49]. Degradation of petroleum-based plastics is severely limited under conventional decomposition by long-lasting fragmentation under physical factors and a lack of assimilation by biodegradative factors [50]. Recently, research efforts have focused on identifying alternative methods for detecting and mitigating the impact of plastics on the ecosystem.

An area of emerging interest is the use of microorganisms as genetic and biotechnological tools for bioremediation. *Synechococcus* sp. PCC 7002 (PCC 7002) is a robust marine strain and model organism for biotechnological applications. The cyanobacterial strain has a fully published genome sequence available and the highest growth rate among all cyanobacteria investigated so far [51]. Furthermore, PCC 7002 has been shown to interact with polystyrene foam and other hydrocarbons [52], although the interactions and exact mechanisms involved in this phenomenon remain largely unknown [50].

By identifying changes in the transcriptome of PCC 7002 when exposed to plastic particulates, we hope to gain insight into the mechanism of hydrocarbon metabolism by PCC

7002. However, extraction and isolation of genetic material from cyanobacteria can be difficult due to their tough-to-lyse cell walls. Cyanobacterial cells are gram-positive and have a strong peptidoglycan barrier due to the higher quantity and thickness, length distribution, and degree of crosslinking in its peptidoglycan layer [53]. Conventional methods for cellular wall disruption in cyanobacteria include enzymatic/chemical methods (lysis with EDTA-lysozyme, Triton X, or phenol-chloroform lysis) and mechanical techniques (use of sonication or bead beating). Typically, temperature-dependent steps are also included in the extraction processes (freeze/thaw cycles, autoclaving, incubating/heating, lyophilization).

The aim of this study was to optimize the extraction of genomic material from PCC 7002 cells in order to facilitate downstream genetic analysis of cultures when exposed to polyethylene micro- and nano-sized particulates. An analysis of total RNA integrity and purity was conducted using microfluidic chips and a nanodrop as a result of integrating heating and beat beating to a TRIreagent-based RNA extraction. The final goal of this project is to identify the enzymatic process through which PCC 7002 degrades polymer particulates, as preliminary findings from our collaborators suggest [54].

2. Methods & Materials

2.1. Growth of bacterial cultures

2.1.1. Stock Cell Culture Conditions - PCC 7002 was acquired from the American Type Culture Collection (ATCC) and grown at 25 °C in a photobioreactor (Grofizz, Austin, TX) containing A+ medium. The culture was kept under constant air bubbling and 12-hour diurnal fluorescent lighting. Cell density was measured using a direct counting method.

The bacterial cells were stained with 0.33% neutral red and measured with a Neubauer-improved disposable hemocytometer (Bulldog Bio, Portsmouth, NH) via phase-contrast/brightfield microscopy (VWR, Radnor, PA).

2.1.2. Growth Monitoring - A growth analysis of PCC 7002 in response to polyethylene micro and nanosized particles, and to no particle exposure (control) was conducted by performing direct cell counts at 24-hour intervals and wet weight measurements at day 0, 5 and 10. Biomass calculations were conducted by extracting 1 mL aliquots and centrifuging at 4500 rpm for 10 min. The resulting supernatant was discarded, and the mass of the bacterial cell pellet was measured using a Mettler Toledo balance (Columbus, OH). Bacterial cells were then resuspended in *RNAlater* (ThermoFisher) solution and frozen in -20 °C until required.

2.2. Preparation of experimental samples

2.2.1. Polyethylene particulate suspension - Prior to sample preparation, polyethylene particulates were suspended in Tween20 (Sigma-Aldrich, St. Louis, MO) prior to exposure to bacterial cell culture. The suspensions were created by adding polyethylene nanospheres (200 – 9900 nm; density 0.953 g/cc) or clear polyethylene microspheres (1 – 4 µm; density 0.96 g/cc) acquired from Cospheric (Santa Barbara, CA) to a freshly made 0.1% Tween20 solution and centrifuging twice at 12000 RPM for 10 min to get particles into the solution. The mixtures were then sonicated at room temperature for

30 min in a water-bath sonicator (VWR, Radnor, PA) to make a homogenous particle mixture.

2.2.2. Experimental sample collection procedure - For sample collection, four independent cultures were grown under three experimentally-induced conditions: exposure to 0.1% Tween20, 20% (w/v) polyethylene nanoparticle, or 20% (w/v) polyethylene microsphere solution. The independent batch-cultures were created by adding 3 mL of stock algal culture to glass scintillation vials. For the particulate-exposed samples, A+ medium and particulate suspension amounts were added to a final concentration of 0.00050 g/mL microparticle or nanoparticle solution [55]. The sample exposed only to 0.1% Tween20 was prepared by adding an equal volume of only 0.1% Tween20 solution. The fourth scintillation vial was a control. The four batch-cultures were incubated under fluorescent light at 30 °C and under continuous shaking of 118 rpm in an Innova 4080 shaking incubator (Eppendorf, New York, NY) for 10 days.

2.3. RNA Extraction

2.3.1. Total RNA extraction optimization procedure – Total RNA was extracted using the Direct-zol RNA kit (ZYMO) as directed, or with addition of a 95°C heating step or bead beating step. To begin, aliquots of 2 mL of bacterial cells were centrifuged at 4500 rpm for 10 min, and the supernatant was removed and discarded. The cells were resuspended in 300 µL of TRIzol reagent (ThermoFisher) and mixed to homogenization. Samples were placed in a BeadBeating tube and pulsed twice at the highest speed in a homogenizer for 20s, incubated at 95 °C for 5 min, or neither (control). Samples were

than centrifuged at 12000 rpm for 30s. The supernatant was removed and placed into a RNAse-free tube before addition of 100% ethanol in a 1:1 ratio. After mixing using a pipette-technique, the samples were each placed into a Direct-zol columns for extraction. A DNAse I digestion treatment step was conducted in-column by addition of 85 µL of DNAse I and DNA Digestion Buffer mix and room-temperature incubation for 15 min. 400 µL of RNA pre-wash was added to the column and centrifuged at 12000 rpm for 30s, this step was done twice. 700 µL of RNA Was Buffer was added to the column and centrifuged at 12000 rpm for 2 min, also twice. Finally, RNA was eluted from column by adding 50 µL of nuclease free water to column and letting it stand for 1 min before final centrifugation of 12000 rpm for 30s.

2.3.2. Total RNA extraction from experimental samples – Bacterial cell aliquots in same growth phase were exposed to nano- or micro-sized polyethylene particulates. Total RNA extraction was conducted from 1.5 mL aliquots of bacterial cells in *RNAlater* (ThermoFisher) solution. Cells were centrifuged at 4500 rpm for ten minutes and resuspended in TRIzol reagent in control procedure described above.

2.4. Analysis of extracted total RNA

2.4.1. RNA quantitation and evaluation of RIN - RNA concentration and purity were measured using the NanoDrop ND-1000 UV/Vis spectrophotometer according to manufacturer's instructions (NanoDrop Technologies, USA). The mean and standard deviation from three biological replicates were calculated for each experimental condition to quantitate the total RNA concentration (ng/µL) and absorbance ratios 260/280 and 230/280. RNA

integrity was verified from RIN values provided by the Agilent Bioanalyzer RNA Total Prokaryote 2100 Pico system. Peak and fragment analysis information was also taken from the system.

3. Results

RNA Extraction

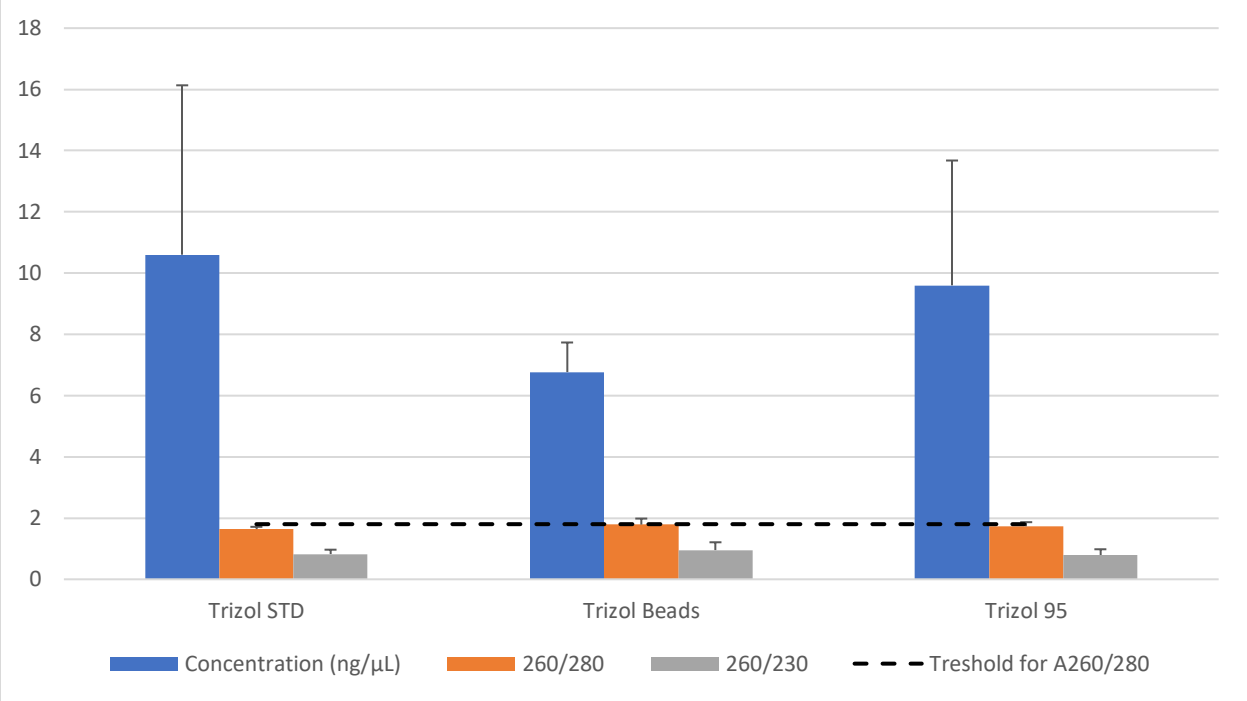


Figure 6.
Extracted RNA Yield and Purity. Yield and absorption ratios for the different extraction methods were determined. For each method, 3 cyanobacteria aliquots were used for RNA extraction and 2 µl analyzed using the NanoDrop ND-1000 UV/Vis spectrophotometer. The resulting bars shown are the average, and the standard deviation, from values obtained for each extraction method.

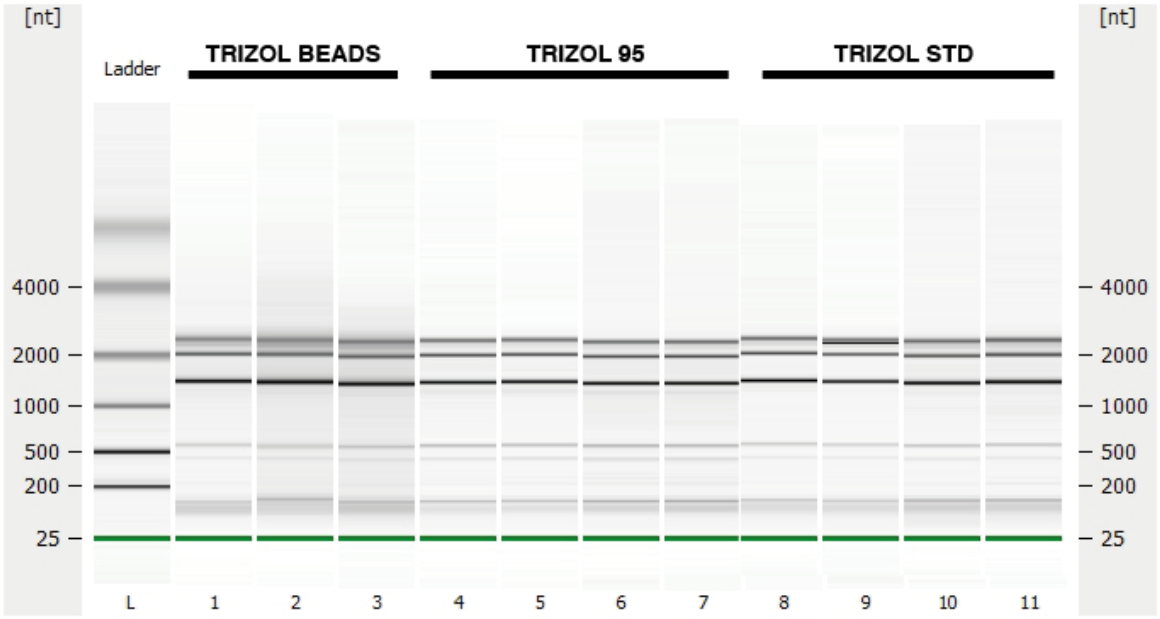


Figure 7.

RNA Integrity. Gel image generated by automated Bioanalyzer system (Agilent). First lane shows RNA ladder supplied with the Pico RNA High Sensitivity Assay. For lanes, 0.5 µl of extracted RNA following distinct RNA extraction procedure following a guanidinium thiocyanate-phenol-chloroform based extraction procedure combined with BeadBeating Lysis Beads (ZYMO) [TRIZOL BEADS], an additional 95 °C incubation step [TRIZOL 95], or with following only the manufacturer's instructions [TRIZOL STD].

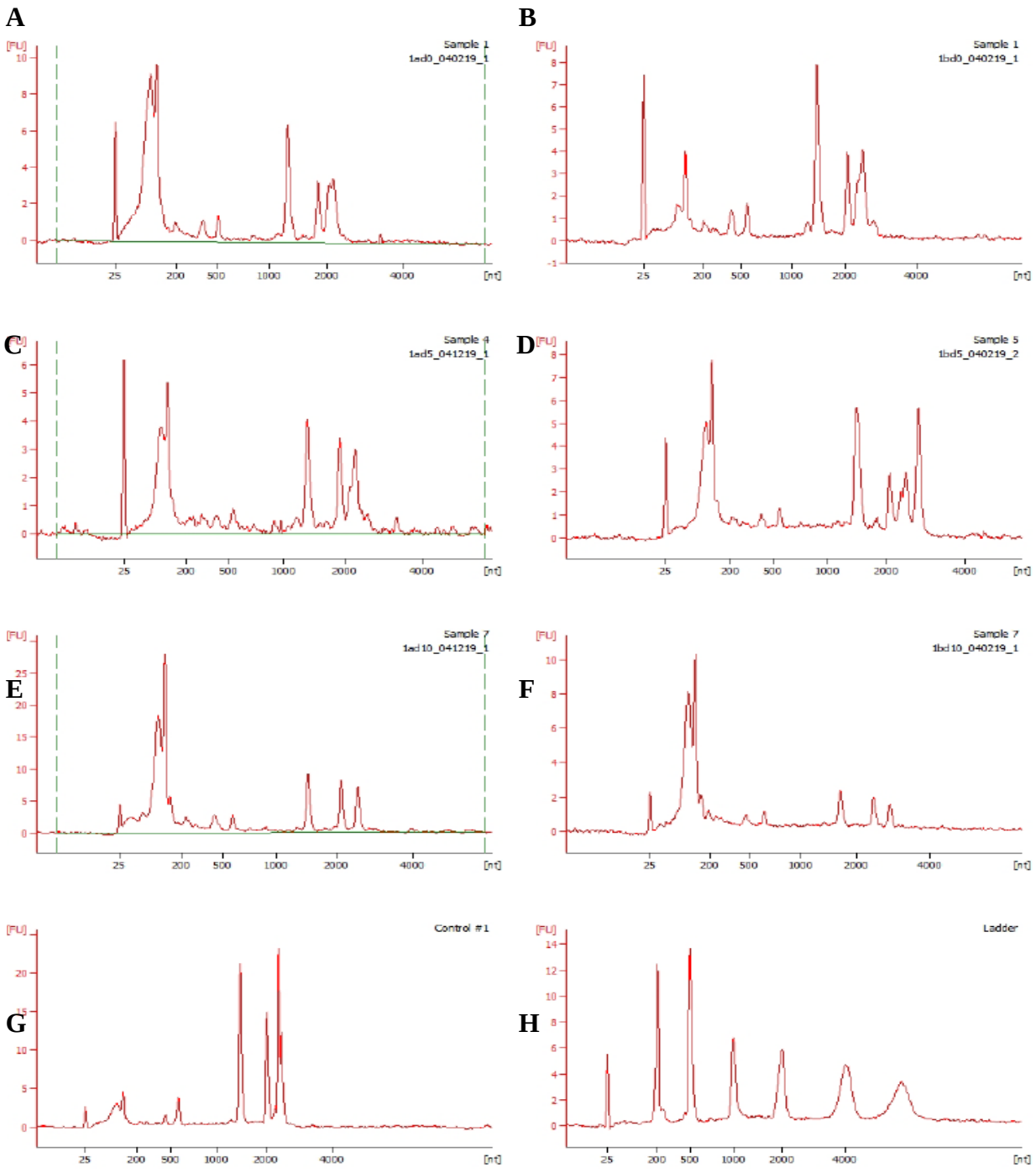


Figure 8 A-H.

RNA Quality after Microparticle Exposure. Electropherograms generated by bioanalyzer (Agilent) of total RNA extractions from *Synechococcus sp.* 7002 bacterial cells exposed to micro- and nanosized particles after 0, 5 and 10 days. All electropherograms were capture from 0.5 µL aliquots of total RNA from distinct experimental conditions. A) Total RNA sample extracted immediately after initial exposure to PENPs. B) Total RNA sample extracted immediately after initial exposure to PEµPs. C) Total RNA sample extracted 5 days after exposure to PENPs. D) Total RNA sample extracted 5 days after exposure to PEµPs. E) Total RNA sample extracted 10

days after exposure to PENPs. F) Total RNA sample extracted 10 days after exposure to PEµPs. G) Total RNA sample from sp. 7002 culture without exposure to polymer particulates. H) RNA ladder

4. Discussion

This study performed a preliminary analysis of the RNA molecules present after extraction from PCC 7002 bacterial cells placed under stress by introduction to plastic particulates. Our results indicate an increased presence of small RNA molecules in the bacterial cells that have been exposed to the polymer particulates, as suggested by the much larger peak in the 5s region on the electropherograms. Additionally, we conducted an optimization of the total RNA extraction process by analyzing the effects on RNA purity and concentration when adding a heating or mechanical lysis step to the extraction. Currently, methodologies available for RNA extraction from gram-positive bacteria are laborious and include highly toxic and volatile chemicals.

Additionally, commercially available kits are not suitable for cyanobacteria with tough to lyse cellular membranes, as these organisms can decrease RNA concentration and purity and make downstream analysis difficult. This investigation optimized the concentration and purity of a commercially available kit by adding an additional 5 min bead beading step.

Several factors may have affected the overall yield from the RNA extraction process. RNA extraction and isolation from cyanobacteria is particularly challenging because they possess an extended array of secondary metabolites [56] that impair cell lysis and nucleic acid purification [57]. Additionally, research suggests that TRIzol could selectively cause small RNAs with a low GC content to be inefficiently recovered [58] from low density bacterial cell cultures.

Downstream analysis of regulatory miRNA molecules could be severely limited from RNA extracted from low cell density amounts, such as those taken for this study.

The purity of the RNA relies on the effective removal of proteins and genomic DNA. Results from the nanodrop absorbance measure for the 260/230 ratio suggest high salt concentrations that could be removed by ethanol precipitation; however, this could result in further loss of total RNA molecules. Additionally, the low yield of total RNA concentration could affect the spectrophotometer reading, causing a magnified representation of any salt contamination in the samples. The guanidine thiocyanyte based column extraction from the Direct-zol kit could be leaving those contaminates in the sample. To analyze samples for this source of contamination, an additional step involving washing the extracted RNA with water-saturated 1-butanol could remove the contaminants [58]. However, it is reported that up to 100mM of guanidine thiocyanate in RNA sample do not compromise the reliability of downstream analysis [59]. Future studies should analyze the effect of 260/230 ratios on RNA-seq and RT-qPCR processes. Absorbance ratios of 260/280 at approximately 1.8 are suggestive of putative DNA contamination. Of the extraction processes analyzed, only the bead beating procedure displayed a purity ratio at that level. For definitive findings if gDNA is contaminating the samples, a picogreen assay could be conducted for the selective binding of gDNA in the sample.

Future studies will include RT-PCR and RNA-seq analysis to characterize changes in gene expression regulation of PCC 7002 when exposed to polymer particulates. By identifying changes in the transcriptome of PCC 7002 when exposed to plastic particulates, we hope to gain insight into the mechanism of hydrocarbon metabolism by PCC 7002.

5. Conclusions

This study showed the optimization of RNA isolation from PCC 7002 bacterial cells by adding a mechanical lysis step prior to extraction. An analysis of the resulting total RNA fragments showed significant presence of small RNA molecules from bacterial cells exposed to plastic particulates. This study's findings show that RNA extraction from tough to lyse cells can be optimized to produce RNA purities sufficient for sensitive downstream analysis, such as RT-PCR and RNAseq.

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