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Modifications to Bacterial Growth Conditions and Mini-Prep Procedure for Maximizing DNA Yield

Elisa Hemmati[1]*and Michelle Huang[1]

[1]Simon Fraser University, *Department of Biomedical Physiology & Kinesiology*

Abstract

Plasmid DNA purification from *Escherichia coli* (*E. coli*) bacterial cells is essential for standard molecular biology experiments such as transfection, polymerase chain reactions, and restriction enzyme digests. To improve yield, we have modified the growth broth, miniprep protocol precipitation step and elution buffer to optimize DNA yield and purity. We hypothesized that cells under the condition of Terrific Broth (TB), ethanol, and double distilled water (ddH_2O) will generate the highest DNA yield and purity. Six experimental conditions were used to test for growth differences between TB and Lysogeny Broth (LB). The quality of DNA samples that were isolated using ethanol and High Salt Wash Buffer (HBC) were compared, as was the elution quality using Tris-EDTA (TE buffer) and ddH_2O. The DNA concentration and purity were measured, and *E. coli* cells grown in TB with no mini prep modifications had a significantly higher DNA yield. This suggests that TB should be used in place of LB for transformed bacterial amplification when large quantities of plasmid are required.

Keywords — Terrific Broth, bacterial growth, plasmid isolation, plasmid purification

1. Introduction

Plasmid DNA purification from bacteria cells removes cellular components such as proteins, lipids, and the bacterial chromosome. A successful purification that gives a high yield DNA is beneficial for further experimental uses, such as transfection, restriction enzyme digests and polymerase chain reaction experiments. The media used for bacterial culture, the buffer used in miniprep procedures, the size of spin columns and the pH of the eluent can all influence DNA yield and purity. The quality of isolated DNA can also be compromised by nuclease activity, free radical oxidation and UV light exposure [1]. In the process of purifying plasmid DNA from bacterial cells, it is also important to keep in mind that the lipopolysaccharides in the membrane of bacterial cells, called 'endotoxins', can impair purity and disrupt later experiments [1].

Liquid media are used to grow bacterial culture from single colonies of bacteria that have been transformed with plasmid DNA. Growth media contains tryptone, a source of nucleic and amino acids, which provides bacteria with nutrients that support cell division and maturation [2]. Lysogeny broth (LB) is used most commonly for

*Corresponding Author: ehemmati@sfu.ca

Escherichia coli (*E. coli*) because it promotes proliferation and gives consistent plasmid DNA yields [2]. Another growth medium that contains higher yeast extract compared to LB is Terrific broth (TB). TB contains glycerol and potassium phosphates to provide the bacterial cells with an additional carbon source and prevent premature cell death due to inhospitable pH values [2, 3]. In a recent study by Wood et al. [3], *E. coli* growth in TB gave the highest yield of plasmid. A greater yield resulting from TB was found to be solely caused by the higher concentration of yeast in the media [3].

A miniprep begins with inoculation of bacteria into liquid media followed by overnight culture. Once the bacteria are harvested, a resuspension buffer that contains RNAse A is added. Afterwards, an alkaline lysis solution is added to the bacterial cells that contain sodium hydroxide which breaks down the phospholipid bilayer and disrupts hydrogen bonds between DNA bases. As a result, plasmid DNA will exit the bacterial cell [4]. A neutralization solution is added to preserve the plasmid DNA and promote the precipitation of the bacterial DNA [4]. HBC buffer is then added, to reduce the solubility of the DNA in water, as it contains guanidine hydrochloride and isopropanol [5]. Guanidine hydrochloride is a strong chaotropic agent that affects the hydrogen bonds in water molecules, so the DNA can easily bind to the silica filter in the column [1, 6]. However, isopropanol may not be the best precipitant because sucrose and salts are less soluble in isopropanol and may precipitate and contaminate the DNA [7, 8]. Ethanol also contains chaotropic properties which allow DNA to bind to the silica filters without disrupting the solubility of salt [9].To further purify the DNA from RNA and protein residue, a wash buffer is required prior to elution. For the final step of the miniprep, the DNA must unbind from the column so that it can be collected. For plasmid DNA purification methods, such as those found in the the Mira-prep [6] or Omega BioTek protocol [10], the elution step calls for the use of water at a neutral pH or a common elution buffer like Tris-EDTA, which serves to prevent oxidation of DNA by free radicals as well as prevents DNA degradation by DNAses [1].

The plasmid pCDH1.2_EF1_vGpH_ELKS1_shRNA ("pCDH1.2) is classified as a high copy number plasmids because it is derived from a pUC vector [11]. High copy number plasmids have hundreds of plasmid DNA molecules per cell and thus give high DNA yields when extracted from bacterial cells by utilizing plasmid miniprep kits [3]. In an upper-division cellular physiology lab course with an expected enrollment of 40-70 students per semester, it is essential to develop a protocol that would result in high DNA yields while also being time-efficient and straightforward for novice bench scientists. The purpose of this experiment is to modify the procedure from the OmegaBioTek E.Z.N.A Plasmid Miniprep Kit I lab manual to enhance the yield of the plasmid pCDH1.2.

In this experiment, we modify three factors to potentially maximize DNA yield of the miniprep procedure and compared the results to the standard miniprep protocol. TB was used for bacterial amplification instead of LB, HBC buffer was modified with ethanol and Tris-EDTA (TE) buffer was substituted with ddH_2O with a pH of 7.0 to investigate whether they are equally sufficient for DNA elution. With these modifications, we hypothesize that DH5α *E. coli* bacteria cells grown in TB with miniprep substitutions using ethanol as a buffer and water as an eluent will produce the highest DNA yield compared to our control condition of using LB, HBC buffer, and

Tris-EDTA buffer.

2. Methods

The miniprep protocol was adapted from weeks 3 and 4 of the BPK 408W Lab Manual [12]. The growth media were Terrific Broth (TB; 2.4% (w/v) yeast extract, 2% (w/v) tryptone, 0.4% (v/v) glycerol, 10% (v/v) Phosphate buffer) and Lysogeny Broth (LB; 0.5% (w/v) yeast extract, 1% (w/v) tryptone, 1% (w/v) NaCl). The bacterial cells were inoculated into 18 mL of TB or LB, with either broth containing 100 µg/mL ampicillin. After being grown in a shaking incubator overnight, the samples were divided into six different experimental conditions as seen in Table 1. Purification of the plasmid DNA was completed by following the Omega BioTek E.Z.N.A Plasmid Miniprep Kit I protocol [10]. Then, either HBC buffer or ethanol was added to the columns containing plasmid DNA followed by a wash buffer. Finally, the DNA was eluted with either ddH_2O or TE buffer.

2.1. Experimental Miniprep Procedure

Omega BioTek E.Z.N.A Plasmid Miniprep Kit I was used for plasmid DNA purification from the bacterial cells (refer to Figure 1 for general experimental procedure). The Omega BioTek protocol [10] recommends using an endA negative strain of *E. coli* such as DH5α for plasmid isolation. DH5α cells were transformed with pCDH1.2 and grown overnight in either 18 mL LB or TB with 18 µL of ampicillin (100 µg/mL) on a shaking incubator at 37°C. The following day, 1 mL of the culture was transferred to the appropriate 1.5 mL Eppendorf tubes for each condition and spun at 10,000 *x g* for 1 minute. The media was decanted and another 1 mL of culture was added for a total of 2 mL per tube, which was subsequently spun again for another minute. 250 µL of resuspension buffer was added and the cells were vortexed followed by 250 µL of lysis buffer. The tubes were inverted fifteen times and left to incubate for 3 minutes. 350 µL of a neutralization solution was added and the tubes were inverted until a white precipitate formed. The tubes were then spun for 10 minutes at 10,000 *x g*. The lysate was then transferred to mini-columns, spun for 1 minute at 13,000 *x g* and the filtrate discarded. 450 µL of HBC buffer or ethanol was added to the columns and they were spun again at 13,000 *x g*. Wash buffer was added in two intervals at volumes of 700 and 400 µL and the columns were spun for 1 minute at 13,000 *x g* after each time the wash buffer was added. Empty columns were spun at 13,000 *x g* for 2 minutes to ensure the column was dry. TE buffer was diluted in water (1:10) and heated to 55°C. 55 µL of TE buffer or ddH_2O was added and incubated for 1 minute. The DNA was eluted from the column and they were spun at 13,000 *x g* for 1 minute, and the eluate was reapplied to the column and subsequently spun again for another minute. A Thermo Scientific^{TM}NanoDrop Lite Spectrophotometer was blanked with 1 µL of TE buffer or water. 1 µL of the plasmid DNA was placed on the spectrophotometer to obtain the DNA concentration and the A_{260}/A_{280} ratio.

2.2. Experimental Conditions

The experiment consists of six total conditions: control, terrific broth, water eluate, ethanol, terrific broth/ water eluate/ ethanol, and terrific broth/ TE buffer/ ethanol condition (refer to Table 1).

Table 1: *Experimental Conditions and Miniprep Modifications.*

Condition	Control	Water Eluate	Ethanol	(TB)	TB Water Ethanol	TB, TE Buffer, Ethanol
Bacterial Growth Media	LB			TB		
Wash Reagent (450 µL)	HBC	HBC	Ethanol	HBC	Ethanol	Ethanol
Elution Reagent (55 µL)	TE	Water	TE	TE	Water	TE
Number of Trials	3	3	3	3	3	2

TE:10 mM Tris-EDTA pH 8.5
HBC: Guanidine Hydrochloride; pH 3.0-5.0
Water: Double-distilled

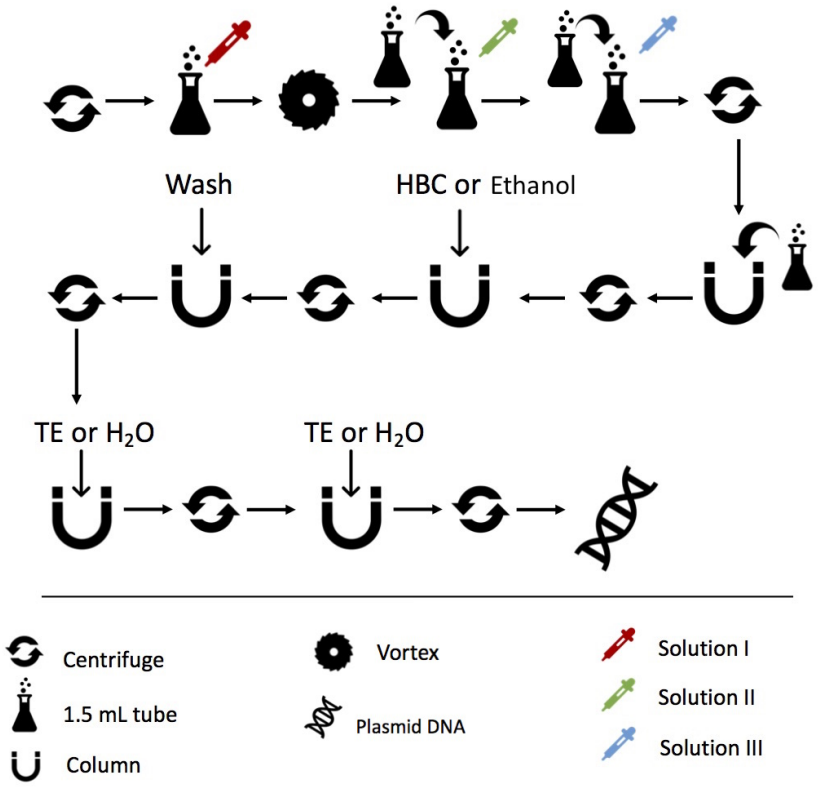

Figure 1: *General experimental mini-prep procedure. The procedure outlines modifiations that give rise to the 6 experimental conditions in Table 1. A legend of the symbols is shown at the bottom of the figure.*

3. Results

The bacterial cells transformed with plasmid pCHD1.2 and amplified in TB with no miniprep modifications had the highest DNA yield (8.52 ± 0.59 µg) when compared to the other 5 experimental conditions (Fig. 2). The average A_{260}/A_{280} ratio for condition 2 was also equivalent to the control condition, 1.77 ± 0.05 and 1.76 ± 0.15 respectively. In contrast, pCHD1.2 DNA precipitated with ethanol (condition 4) had the lowest DNA yield (2.85 ± 0.44 µg). The TB, water and ethanol (condition 5) had a higher DNA yield (6.14 ± 0.41 µg) compared to the control condition (4.76 ± 1.60 µg). The sixth condition, TB, TE buffer and ethanol, produced the highest purity with a A_{260}/A_{280} ratio of 1.85 ± 0.01. To test for a quantitative significant difference between our conditions and the control, an Analysis of Variance (ANOVA) test with the Tukey-Kramer method was used ($\alpha < 0.05$). There was a significant increase in the DNA yield when LB was replaced with TB. As shown in Fig. 2, these increases in DNA yield are only significant for the TB condition. The ethanol condition (condition 4) had the lowest DNA yields (2.85 ± 0.44 µg). The plasmid DNA was pure in all six conditions (Fig. 2). In general, we would expect for high copy number plasmids such as pCDH1.2 a yield of 15-25 µg for 5 mL of starting culture grown in LB [10].

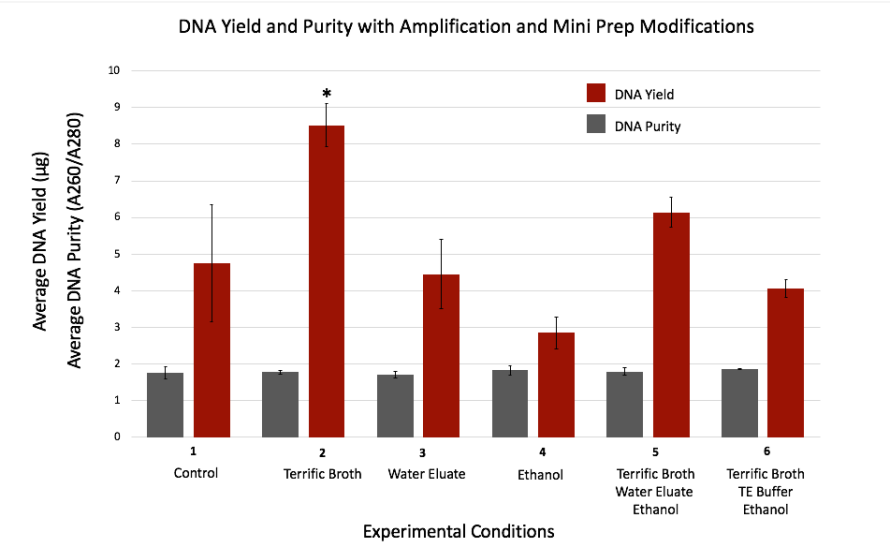

Figure 2: *DNA Yield with Amplification and Mini Prep Modifications. Bar graph of average DNA yield (ng) from all six experimental conditions. Error bars represent standard deviation and significance is based on these error bars. n=6 for conditions 1-5 and $n = 4$ for condition 6. * = significant increase compared to control condition.*

4. Discussion

4.1. DNA Purity

The pCHD1.2-transformed *E. coli* cells grown in TB had the same DNA purity when compared to the control. The purity of our DNA yield was determined by the A_{260}/A_{280}

ratio, in which a value of 1.75 -1.95 is accepted as pure. Values greater than 1.95 suggests RNA contamination while less than 1.7 suggests protein contamination [6]. All six conditions had ratios ranging between 1.71-1.85, indicating acceptable purity of the plasmid isolation. Our hypothesis was refuted as the condition containing three modifications (TB broth, ethanol, and water) did not generate the highest DNA yield. Based on the data obtained from the DNA yield and purity of the fourth condition (HBC buffer substituted with ethanol), we speculate that the volume of ethanol used was not sufficient at precipitating our plasmid DNA. According to BiteSizeBio [9], higher volumes of ethanol are required to produce similar results to isopropanol. In addition, another DNA precipitation protocol recommended using ice-cold ethanol over isopropanol; however, in our protocol the 95% ethanol was at room temperature [8]. Colder temperatures of ethanol may result in an increased isolation yield as it promotes the flocculation of nucleic acids [9]. Our data also showed that TB conditions had a higher purity than the control, which makes the TB amplifications protocol suitable for experiments such as transfection, restriction enzyme digests, and polymerase chain reaction.

4.2. DNA Yield

In comparing the amount of DNA purified from cells grown in different media, we demonstrated that *E. coli* cells grown in TB had the highest DNA yield compared to the control condition. This is due to the larger amounts of nutrient found in TB when compared to LB broth. TB contains five times more yeast, two times more tryptone than LB, and the presence of glycerol. This extends bacteria growth and plasmid amplification by supplying *E. coli* bacteria cells with larger amounts of amino acids. One study found that when the same amount of yeast extract was added to LB as TB, similar DNA yields resulted [3]. Therefore, the high yeast content of TB is likely the key factor in achieving higher yields.

When considering the plasmid isolation reagents, the DNA yields were lower than the control when HBC buffer was substituted with ethanol and when TB and ethanol were substituted with LB and HBC buffer respectively. Based on these results, it is likely that ethanol contributed to a lower DNA yield in both conditions 4 and 6. Therefore, ethanol may not be an acceptable alternative for HBC buffer in the plasmid DNA miniprep procedure. We propose that TE buffer may have contributed to a higher DNA yield than water because DNA is more stable at an alkaline pH, and TE buffer has a higher efficiency at dissolving DNA than water [13]. In general, our DNA yields across all six conditions were not as high as expected. According to the product manual for the E.Z.N.A Plasmid Mini Kit I [14], for 5 mL of inoculated culture high copy number plasmids have an expected yield of 15-25 µg. This could be due to poor cell lysis, overgrown culture or low elution efficiency. The protocol warns against leaving the lysing solution for more than 5 minutes however, due to our many samples it could be possible that the solution what left on too long. The cells also may not have been dispersed evenly before adding Solution II or Solution II had to be left longer for a clear lysate. Finally, another potential explanation is that the cultures were incubating for almost 18 hours which is more than the 16-hour maximum time recommendation [14]. In future experiments, tight regulation of time restrictions and appropriate temperature

and pH of solutions will hopefully result in the expected DNA yields.

4.3. Other Considerations

With further experimentation in mind, plasmid DNA storage is also crucial as these experiments may not be performed simultaneously. According to Oxford Gene Technology [15], DNA stored in Tris-EDTA buffer were stable at 2-8°C for 8 years, but DNA stored in water experienced degradation. Furthermore, Tris-EDTA chelates magnesium and other divalent metals which suppresses DNA degradation by DNAses. Therefore, the usage of Tris-EDTA for elution may be more suitable if long term storage is required. Further research should test whether larger volumes of ethanol and TB modifications will result in a higher DNA yield and purity. The cost of TB and LB are both 62 dollars per litre on ThermoFisher [16, 17], therefore there are no cost restraints for using TB.

4.4. Endotoxin Contamination

With a higher plasmid DNA yield, the presence of endotoxin may affect processes such as transfection. Endotoxin is found on the cell wall of *E. coli* bacteria and is produced during plasmid purification [18]. When bacterial cells are lysed, they release a large amount of endotoxin to their surroundings. Endotoxin forms large micelles that are negatively charged and can be purified along with plasmid DNA [18]. This could lead to a higher yield because the endotoxin is being co-purified with the DNA. On the other hand, this toxin is toxic to mammalian cells and can result in a reduction in cell viability and transfection efficiency. There are currently photometric tests available that can identify the presence of endotoxins in purified plasmid DNA samples [18]. There is also an 'Endo-Free' version of the plasmid purification kit that was used in this experiment that includes an endotoxin removal step in the purification process [19].

5. Conclusion

TB broth is more efficient at promoting bacterial growth and plasmid amplification than LB. The DNA miniprep procedure with TB generated equivalent purity as the control. Therefore, TB should be used in substitution of LB in bacterial growth for plasmid amplification. Further research is still required to understand the effects of endotoxin on mammalian transfection, and the effects of TE buffer and water on DNA degradation.

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