THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

OPTIMIZATION OF *ESCHERICHIA COLI* GROWTH CONDITIONS TO MAXIMIZE PLASMID YIELD

JOSHUA MCANULTY

SPRING 2012

A thesis
submitted in partial fulfillment
Of the requirements
for a baccalaureate degree
in Science
with honors in Biochemistry and Molecular Biology

Reviewed and approved* by the following:

Dr. Song Tan
Professor of Biochemistry and
Molecular Biology
Thesis Supervisor

Dr. C.-P. David Tu
Professor of Biochemistry and
Molecular Biology
Honors Advisor

Wendy Hanna-Rose
Associate Department Head for Undergraduate Studies
Department of Biochemistry and Molecular Biology

*Signatures are on file in the Schreyer Honors College

Abstract

Plasmids are circular double-stranded DNA that replicate independently of chromosomal DNA. Additional genes encoded by the plasmid provide phenotypical traits that increase versatility and adaptability of the host organism. In molecular biology, plasmids are often used as vectors for producing recombinant DNA and proteins. The Tan laboratory uses bacterial plasmids to produce nucleosome DNA fragments for reconstituting nucleosomes used in biochemical and structural studies of chromatin enzymes. However, the protocol used to grow *E. coli* containing such plasmids has not been examined systematically to optimize plasmid yield. The aim of this thesis is to determine the optimal *E. coli* growth conditions to maximize plasmid yield. Quantitative Polymerase Chain Reaction (qPCR) of culture samples were used to determine the concentration of plasmids grown under different growth conditions. The effect of three different growth media (2xTY, PDMR, and TB) on plasmid yield was examined using both ampicillin and kanamycin resistant plasmids. The effect of growth temperature was also examined. My results suggest the use of kanamycin resistant plasmid grown at 37º C in TB media produced the largest plasmid yield 3.7x the yield using currently used growth conditions in our laboratory.

Table of Contents

List of Figures

List of Tables

Acknowledgments

I would like to thank all current and past members of the Tan Lab for the advice and help over past two and a half years including: postdoctoral fellows, graduate students, research technicians, and fellow undergraduates. In particular, I would like to thank Sam Teicher for his preliminary work on this project and Jiehuan Huang for his mentorship on this and other projects. Above all I would like to thank Dr. Song Tan for giving me the opportunity to develop my skills as a researcher and guiding me throughout the process.

Introduction

1.1. Plasmids in Nature

Plasmids are double-stranded circular DNA that replicate independently of chromosomal DNA. They are found in all kingdoms of life, but the majority of plasmid containing organisms are bacteria (Phillips and Funnell 2004). Since plasmids are not part of the chromosomal DNA, they are not essential for life. However, the additional genes in plasmids can provide unique phenotypical traits that increase adaptability and versatility of the host organism. For example, some plasmids encode for antibiotics that kill other organisms surrounding the host while others provide antibiotic resistance. Only an organism with an appropriate antibiotic resistant gene will survive in the presence of the antibiotic (Phillips and Funnell 2004). There is a repertoire of genes a plasmid may carry, and the functions they confer range from nitrogen fixing to the virulence of anthrax producing *Bacillus anthracis* adding to the diversity of microbial life (Helgason et. al. 2000).

1.2. Plasmid Use in Molecular Biology

Plasmids are used extensively in molecular biology research. Since the advent of gene cloning, plasmids have played a critical role in DNA recombinant technology as vectors (Lederberg 1998). Vectors serve as a vehicle for recombinant DNA cloning and expression. To create a recombinant plasmid, restriction enzymes can be used to digest the vector DNA at specific locations resulting in a gap in the vector DNA sequence. The gene to be inserted is also digested with restriction enzymes producing ends of the DNA fragment compatible with the gap in the vector. The vector and gene insert fragments are mixed together in the presence of DNA ligase which covalently links the gene into the vector (See Figure 1.1.). The resulting recombinant

DNA is transformed into bacterial cells and cultured to produce large quantities of the gene or protein it encodes (Cohen 1993).

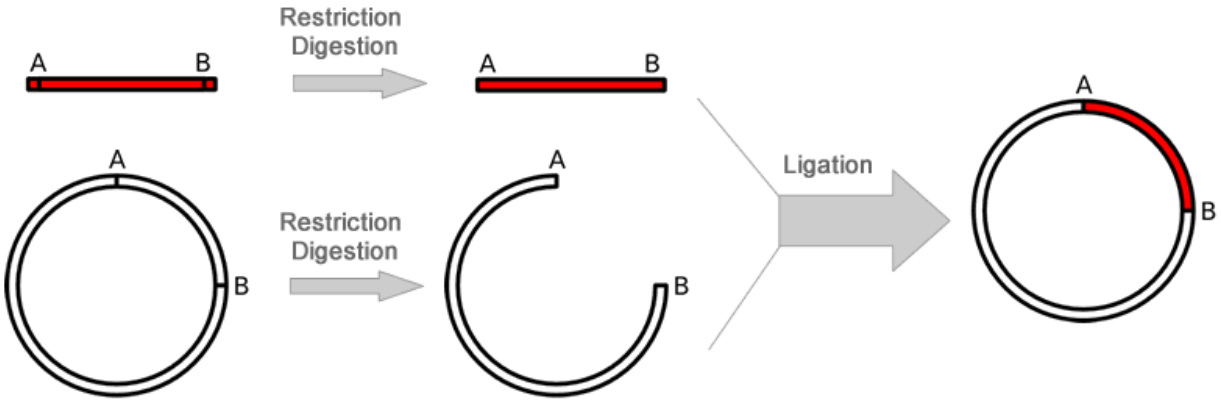


Figure 1.1. Basic Subcloning Schematic. The vector and gene are cut with restriction enzymes and the gene is ligated into the vector. (Image obtained from http://bioinforx.com/lims/bxseqtools/ultimate-molecular-cloning-guides/Subcloning)

1.3. Criteria for Effective Plasmid Vectors

The convenience of DNA manipulation is an important consideration for plasmid vectors. Small vectors are advantageous for manipulations, purification of restriction fragments, and effective insertion of larger genes (Mandecki et al. 1990). The restriction sites present in vectors play a pivotal role directing the insertion of a gene. A multiple cloning site (MCS), a segment of the DNA that contains several unique restriction sites, is incorporated into vectors to control where the gene is inserted. Conversely, many restriction sites outside of the MCS are unnecessary and may be excluded to simplify subcloning (Mandecki et al. 1990).

The presence of a selection marker is needed to discriminate between cells that contain the plasmid and those that do not. Antibiotic resistance is often used as a selectable marker. The

antibiotic that the plasmid confers resistance to is added to an agar plate and incubated. Cells that contain the plasmid are resistant to the antibiotic and are able to form colonies (Nair 2007). These colonies can be cultured, also in the presence of antibiotic, to produce DNA or protein without growth of cells that do not contain the correct plasmid.

Finally, a high copy number plasmid is desirable for producing large quantities of recombinant DNA or protein. Most naturally occurring replicons have low-copy numbers below 20 copies per cell. High-copy number ColE1-type plasmid derivatives such as pUC plasmids (See Section 1.4.) can reach 500-700 copies per cell (Prather et. al. 2003).

A derivative of the pWM-type plasmids was used in this thesis (Mandecki et al. 1990). The pWM plasmids, created by Mandecki and colleagues, were the first totally synthetic *E. coli* plasmids. Previously, plasmid vectors were spliced versions of plasmids in nature without optimized DNA sequences for an effective plasmid vector. The pWM series of plasmids were based on the pUC-type plasmids and created by the *FokI* method of gene synthesis (Mandecki and Bolling 1988). The pWM528 plasmid contains half of the restriction sites of the equivalent pUC18 plasmid and is 636 bp shorter in length (2050 bp vs 2686 bp). Only seven restriction sites for restriction enzymes recognizing 6-bp sequences are present compared to 24 in the pUC-type plasmids. The low number of restriction sites in the plasmid backbone allows for flexibility in selecting enzymes sites for cloning because most 6-bp recognizing restriction enzyme could be used. In addition, the pWM-type plasmids maintained a high copy number of 500-700 copies per cell (Mandecki et al. 1990).

1.4. Plasmid DNA Production

One use of plasmids is to produce large quantities of DNA. A plasmid with a DNA segment of interest is transformed into bacteria and cultured to replicate the plasmid within the cells. The amount of plasmid produced is not only dependent on the number of cells grown, but also the number of plasmid copies in each cell. Selecting the right plasmid construct and optimizing growth conditions can be critical for high plasmid yields.

The high copy number pUC plasmids, named after the University of California where they were created, are a derivative of ColE1-type plasmids (Vieira and Messing 1982). Since plasmids replicate independently of chromosomal DNA, they have their own origin of replication that controls the rate of DNA replication. ColE1-type plasmid replication is initiated by the RNA primer RNAII. RNAII anneals with the template DNA and is cleaved by RNase H providing a 3' OH for DNA Polymerase I to begin DNA synthesis. This process is regulated by RNAI which binds to RNAII causing a conformational change that reduces its effectiveness as a primer for DNA synthesis. The RNA one Modulator (ROM) protein accelerates the binding of RNAI to RNAII further repressing DNA synthesis (See Figure 1.2.). The pUC plasmids' high copy number is attributed to a point mutation in RNAII and deletion of ROM. The mutation increases the efficiency of RNAII as a primer for DNA synthesis and the deletion of ROM decreases the binding of the RNA I to RNAII. However, the mutation is temperature sensitive. At temperatures of 30º C or below the mutation effects are suppressed, but at 42º C the plasmid copy number is increased (Lin-Chao, 1992).

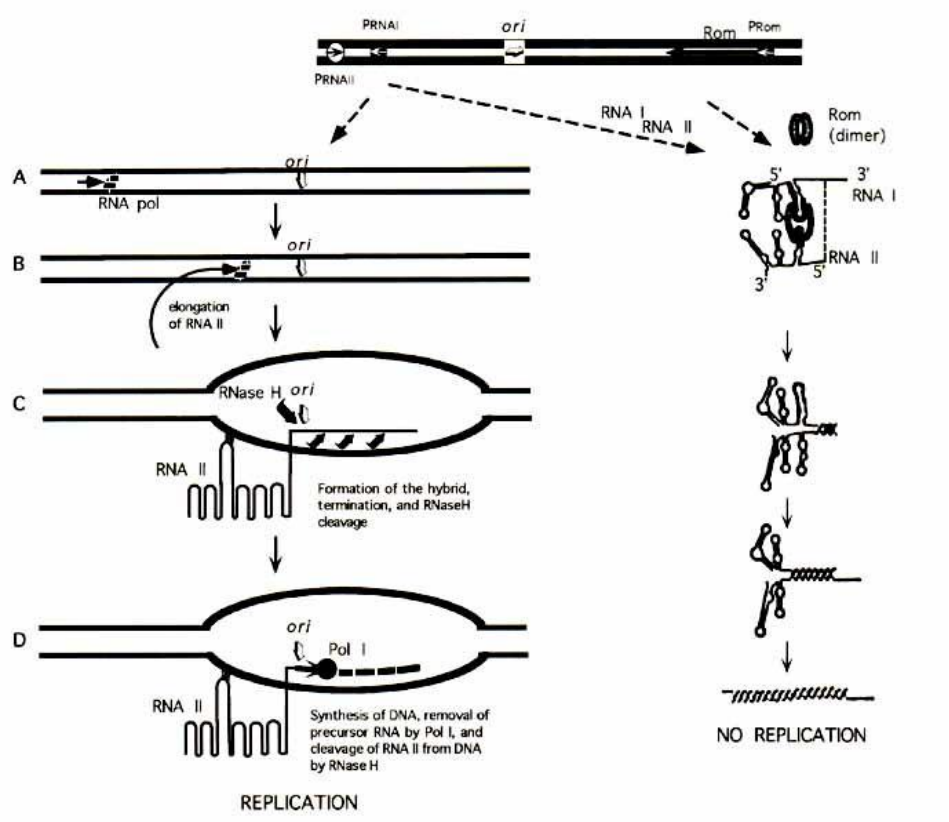


Figure 1.2. ColE1-type Plasmid Replication Regulation. RNA II acts as a primer for DNA replication. The binding of RNA I to RNA II is mediated by ROM which causes decreased DNA replication. (Modified from http://www.bioscience.org/1999/v4/d/actis/fulltext.htm)

The composition of the growth medium can affect bacterial growth and plasmid production. Sufficient amounts of nutrients are required for the cells to grow, divide, and replicate plasmids. A medium rich in nutrients can support higher cell densities and longer growth periods. Media can be formulated with known concentrations of nutrients, defined media, or contain substances with unknown nutrient concentrations, complex media. Defined media is advantageous for standardizing nutrient sources to increase plasmid yield (Wang et. al. 2001). However, complex media usually requires fewer components, is easier to prepare, and produces high cell densities similar to defined media (Durland and Eastman, 1998). Three complex growth media (2xTY, PDMR, and TB) were used in this thesis. 2xTY media is the standard media used for growing *E. coli* in the Tan laboratory. 2xTY media contains the complex nutrient sources bacto tryptone and

yeast extract which provides amino acids, minerals, proteins, amino acids, trace elements, and vitamins essential for biosynthesis of cellular matter. Sodium chloride is another component of 2xTY media added for maintenance of culture osmolarity (Danquah and Forde, 2007). PDMR and TB media are supplemented with additional nutrients that have been shown to produce high plasmid yields (Danquah and Forde, 2007). TB (terrific broth) media contains larger concentrations of bacto tryptone and yeast extract providing a rich nutrient environment. Glycerol is supplemented in TB media for an additional carbon and energy source for improved cell growth (O'Kennedy et al. 2000). Phosphates are also supplemented to buffer pH change and increase availability of phosphates for DNA synthesis. PDMR (Plasmid DNA medium optimized for carbon/nitrogen ratio) media contains less bacto tryptone and yeast extract than 2xTY and TB media. However, glucose and ammonium chloride are added to optimize the C:N ratio for plasmid yield. (Danquah and Forde, 2007). In addition, the medium is supplemented with phosphate salts and magnesium sulfate.

Selective growth of cells containing antibiotic resistant plasmid is important for plasmid yield. Ampicillin is a β-lactam antibiotic that inhibits cell wall production causing cell lysis. β-lactamase, which hydrolyzes β-lactam, is produced by ampicillin resistant plasmids and secreted into extracellular space (Mayers, 2009). Increased extracellular concentration of β-lactamase, especially in high cell density cultures, may degrade enough β-lactam for cells without the antibiotic plasmid to survive. More cells that do not contain the antibiotic resistant plasmid can grow decreasing plasmid yields. In contrast, kanamycin is an amino-glycoside antibiotic which causes cell death by inhibiting ribosomal translation. Kanamycin resistant plasmids encode an amino-glycoside-modifying enzyme that inactivates the amino-glycoside (Davies and Wright,

1997). In this process, antibiotic resistance is not provided to cells without the antibiotic resistant plasmid. Thus, kanamycin is more effective than ampicillin at selecting cells containing an antibiotic resistant plasmid.

Finally, the stage of cell growth in the bacterial culture can affect plasmid yield. Bacterial cultures show distinct growth phases. The initial lag phase of bacterial growth is characterized by zero growth rate. Bacterial cells begin to rapidly divide during the exponential phase increasing cell density in the culture. Lack of nutrients, accumulation of metabolic products, and changes in pH or ion equilibrium inhibit bacterial growth and growth rate reaches zero during the stationary phase. Finally, the number of bacterial cells decreases during the phase of decline (Monod, 1983). The amount of cells and plasmid copy number per cell determines plasmid yield. Plasmid copy number increases during the late exponential phase and early stationary phase (Reinikainen, 1988). However, a decrease in copy number is found in later stages of the stationary phase (Pradyumna, 1992). Therefore, the optimal time to harvest plasmids is during the early stationary phase when the cell density and copy number is highest.

1.5. Purpose of Research Project

The Tan Lab investigates how chromatin enzymes and factors interact with the nucleosome through biochemical and structural experiments to better understand how our genes are regulated. Recombinant nucleosome DNA fragments and histone proteins are used to reconstitute nucleosomes for these experiments. To prepare sufficient amounts of nucleosome DNA fragments (typically 145-160 bp in length), plasmids containing sixteen tandem repeats of the nucleosome DNA fragment are transformed into *E. coli* cells and cultured to produce large

amounts of plasmid. The plasmids are isolated and digested with restriction enzymes to obtain individual nucleosome DNA fragments for nucleosome reconstitution. The current protocol for growing *E. coli* to produce plasmids that contain the nucleosome DNA fragments has not been optimized for plasmid yield. A considerable amount of time is devoted to plasmid production to provide adequate amounts of recombinant nucleosomes used for experimentation (each preparation requires about 40 hours of work). The aim of my project was to optimize *E. coli* growth conditions to improve the yield of plasmid production in a reproducible manner. To achieve this, I grew *E. coli* cultures under varying conditions and quantified the amount of plasmid in the bacterial culture by Quantitative Polymerase Chain Reaction (qPCR). The effects of media, temperature, antibiotic resistance, and length of culture growth were examined. Ampicillin and kanamycin resistant plasmids were grown in three different growth media (2xTY, PDMR, and TB media) at 37º C and plasmid content was compared. The effect of temperature induction, growing initially at 23º C or 30º C then increasing to 42º C, was examined with ampicillin resistant cells in each media type. Samples were collected over a time course to identify the appropriate time for plasmid harvest.

Materials and Methods

2.1. Bacteriological Methods

2.1.1. Bacteriological Strains:

HB101 (Source Applied Biosystems) cells were used for each transformation. The plasmids pST55-16xNCP601a and pST89-16xNCP601a (obtained from Dr. Song Tan) were transformed into the HB101 cells to confer ampicillin and kanamycin resistance, respectively. HB101 cells are suggested for plasmid preparation of nucleosome DNA fragments (Pamela 2003).

HB101 Genotype:

F-, *hsd*S20(rB-, mB-), *xyl*5, □-, *rec*A13, *gal*K2, *ara*14, *sup*E44, *lac*Y1, *rps*L20(strr), *leu*B6, *mtl*-1, *thi*-1

2.1.2. Bacteriological Media

Three types of liquid media were used to optimize the growth of *E. coli* cells. 2xTY, PDMR, and TB liquid media was used for the preculture, culture, and dilution of culture samples when measuring the cell density (OD_{600}).

2xTY liquid media consisted of 1.6% bacto tryptone (w/v), 1.0% yeast extract (w/v), and 0.5% sodium chloride (w/v) in deionized water. The media was autoclaved and stored at room temperature before use. Ampicillin (50 μg/ml) or Kanamycin (15 μg/ml) were added when appropriate before the start of the preculture or culture growth (See Appendix A for 1 L example).

PDMR liquid media (Danquah and Forde, 2007) was created from three separate solutions. The first solution consisted of 0.988% Bacto tryptone (w/v) and 0.55% Yeast Extract (w/v) in deionized water. The second solution consisted of 6.78% anhydrous disodium phosphate (w/v), 3% potassium diphosphate (w/v), and 1.06% ammonium chloride (w/v) in deionized water. The third solution consisted of 10% glucose (w/v) and 0.24% magnesium sulfate (w/v) in deionized water. The solutions were autoclaved separately, allowed to cool to room temperature, and combined to create the final PDMR liquid media. The first, second, and third solutions volume contributed 80%, 10%, and 10% of the total combined volume, respectively. PDMR liquid media was stored at room temperature before use. Ampicillin (50 μg/ml) or Kanamycin (15 μg/ml) were added when appropriate before the start of the preculture or culture growth (See Appendix A for 1 L example).

TB liquid media (Danquah and Forde, 2007) was created from two separate solutions. The first solution consisted of 1.33% bacto tryptone (w/v), 2.66% yeast extract (w/v), and 0.556% glycerol (w/v) in deionized water. The second solution consisted of 9.4% dipotassium phosphate (w/v) and 2.0% potassium diphosphate (w/v) in deionized water. The solutions were autoclaved separately, allowed to cool to room temperature, and combined to create the final TB liquid media. The first and second solution volume contributed 90% and 10% of the final combined volume, respectively. TB liquid media was stored at room temperature before use. Ampicillin (50 μg/ml) or Kanamycin (15 μg/ml) were added when appropriate before the start of the preculture or culture growth (See Appendix A for 1 L example).

The solid media used for transformation was TYE. TYE consisted of 1.5% agar (w/v), 1.0% bacto tryptone (w/v), 0.5% yeast extract (w/v), and 0.8% sodium chloride (w/v) in deionized water. The media was autoclaved and cooled before adding ampicillin (100 µg/ml) or kanamycin (50 µg/ml) when appropriate. The media was then poured into Petri dishes, solidified overnight, and stored at 4º C.

2.2. Culture Growth

2.2.1. Plasmid Transformation

Frozen competent HB101 (Source Applied Biosystems) cells were thawed on ice. 1 µl of 100 ng/µl plasmid was added to 100 µl thawed HB101 cells and incubated on ice for 15-40 minutes. The cells were heat shocked in a 42º C water bath for 30 seconds and placed on ice for 10-20 seconds. 500 µl 2xTY liquid media was added to the cell mixture and the eppendorf tubes were transferred to a 37º C shaking incubator (200 rpm) for 15-40 minutes. 75 µl of the cell solution was spread on TYE plates containing the appropriate antibiotic. The plates were incubated at 37º C for 20-24 hours.

2.2.2. Colony Restreak

Single colonies from the plasmid transformation plate were restreaked with a sterile inoculation loop onto another TYE plate containing the same antibiotic. The restreak was performed immediately after removing the plasmid transformation plate for the 37º C incubator. The restreak plates were incubated at 37º C for 20-24 hours.

2.2.3. Preculture Inoculation

5 ml of liquid media in 50 ml round-bottom Pyrex tubes was inoculated with 3-5 medium sized colonies from the restreak plate and placed in a 37º C shaking incubator (200 rpm) after adding the appropriate antibiotic resistance. The preculture was grown until the OD_{600} reached between 0.05 and 0.5. The OD_{600} of the preculture was recorded before it was used to inoculate the 500 ml cultures.

2.2.4. 500 ml Culture Inoculation

500 ml of liquid media in a 2 liter glass flask without baffles was inoculated with 200 µl preculture of the same media type. The appropriate antibiotic resistance was added and the 500 ml culture was placed in a 37º C shaking incubator (200 rpm).

2.2.5. Culture Samples

1 ml of culture was removed at 2 hour intervals from 18 to 28 or 30 hours after 500 ml culture inoculation. The OD_{600} was recorded after 1:10 culture/media dilution to keep within the effective range of the spectrophotometer. 200 µl of the culture sample was flash-frozen with liquid nitrogen and stored at -80 C for future qPCR analysis.

2.3. Growth Conditions

The growth conditions of E. coli were varied to determine the optimal method for producing high plasmid yield. The growth length of 18 to 28 or 30 hours (if feasible) was used for every growth condition. Both ampicillin and kanamycin resistance cells were grown at 37º C, but only

ampicillin resistant cells were used for temperature induction. Temperature induction started the culture growth at 23º C or 30º C and increased the temperature to 42º C at 18 hours. 2xTY, PDMR, and TB media was used for every growth condition. Three biological replicates were attempted for each condition, but not always successful. Table 2.1. summarizes the different conditions examined and the number of biological replicates completed.

	2xTY Media	PDMR Media	TB Media
Amp^r 37º C	3 Cultures	2 Cultures*	3 Cultures
Kan^r 37º C	3 Cultures	3 Cultures	3 Cultures
Amp^r 23º C to 42º C	3 Cultures	3 Cultures	1 Culture**
Amp^r 30º C to 42º C	3 Cultures	3 Cultures	3 Cultures

Table 2.1. Summary of Experiments Performed.

*Contaminated media for one culture

**No growth after 28 hours for two cultures

2.4. Quantitative Polymerase Chain Reaction

2.4.1. Reagents and Setup

Quantitative Polymerase Chain Reaction (qPCR) was used to assay the amount of plasmid DNA in *E. coli* cell cultures. Florescence probes are incorporated in the DNA amplified by qPCR. The number of PCR cycles required, threshold cycle (Ct), to reach a florescence threshold is measured (See Figure 2.1.). Higher concentration of DNA in a sample will produce higher concentrations of PCR products with florescent probes. The Ct will be lower because the threshold florescence will be met at a lower number of PCR cycles. A standard curve of Ct

values is created with known concentrations of initial plasmid DNA. The sample concentration is calculated by comparing the sample Ct values to the Ct values of the standard curve.

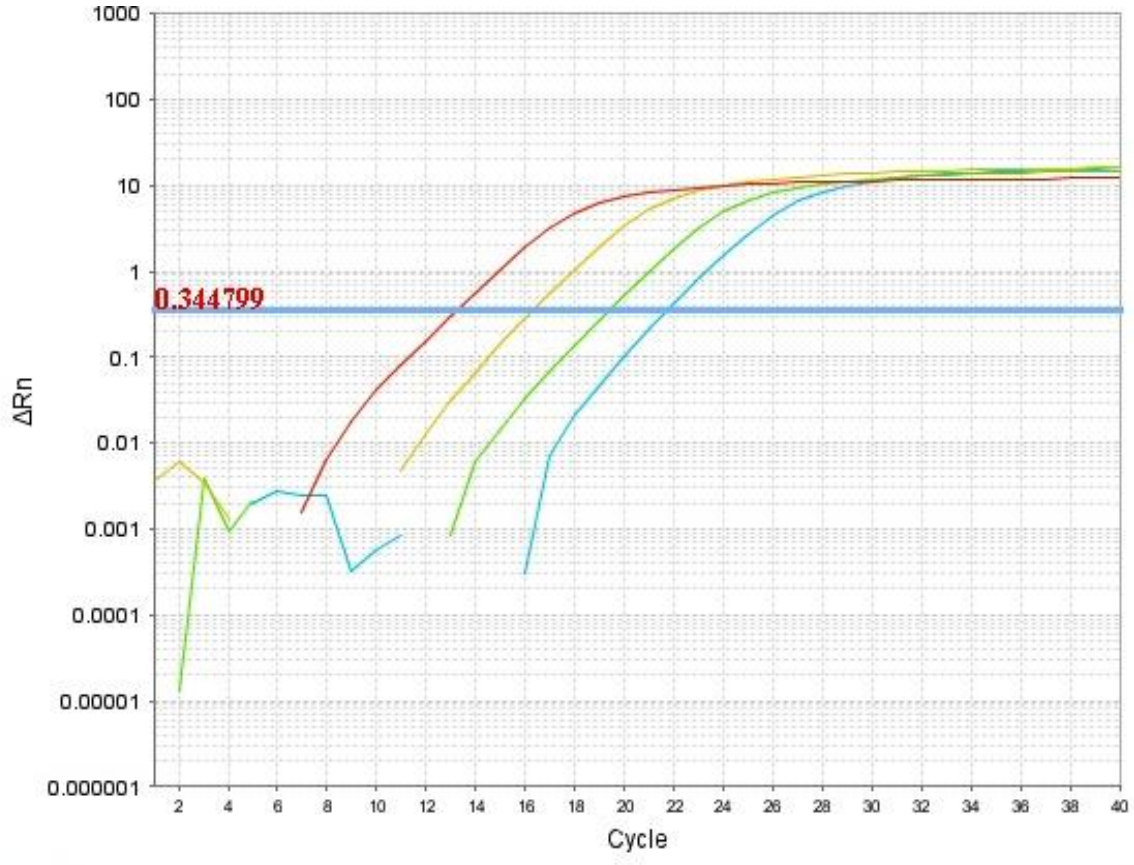


Figure 2.1. Example of Raw qPCR Data. The number of PCR cycles to reach the threshold florescence, blue horizontal line, is measured. The y-axis (ΔRn) is florescence of sample minus the background florescence obtained before the qPCR reaction. The colored lines represent a standard curve dilution of known plasmid concentration. The red line represents the highest plasmid concentration reaching the threshold cycle (Ct) at approximately 13.5 cycles. The light blue line represents the lowest concentration of plasmid with a Ct value of approximately 21.8.

The qPCR reaction consisted of 6 µl SYBR® Advantage® GC qPCR Premix, 0.3 µl forward primer, 0.3 µl reverse primer, and 3.4 µl, and 2 µl of sample dilution or standard curve DNA. Each 12 µl qPCR reaction was added to one well in the Micro AMP^{tm} fast optical 96-well reaction plate and Micro AMP^{tm} Optical Adhesive Film was applied over the reaction plate before the qPCR reaction. Culture samples were diluted 1:24000 culture/MilliQ water before addition to the qPCR reaction. The ampicillin resistant plasmid pST5516xNCP601a (Created by

Dr. Song Tan) used the forward primer STO3283 and reverse primer STO3284. The kanamycin resistant plasmid pST5516xNCP601 (Created by Dr. Song Tan) used the forward primer STO3285 and reverse primer STO3286 (See Appendix A for additional primer information). Each qPCR reaction was duplicated on the same qPCR plate and the Ct values were averaged for accuracy.

The reaction plate was placed in the Stepone Plus Real-Time PCR System (Applied Biosystems) with the following program: holding phase (2 minutes at 95 C), 40 cycles (15 seconds at 95 C, 1 minute at 60C, 1 minute at 72 C), meltcurve (15 seconds 95 C, 1 minute 60 C, 15 seconds 95 C). The data was compiled and analyzed by Step One Software v2.1. The threshold florescence was calculated by the Stepone Plus Real-Time PCR System from the background and sample florescence.

2.4.2. Standard Curve

A standard curve was created for every qPCR reaction by diluting the pST5516xNCP601a plasmid to concentrations of 0.1 ng/µl, 0.01 ng/µl, 0.001 ng/µl, 0.0001 ng/µl, and 0.00001 ng/µl. The Ct values vs. negative inverse log of plasmid concentration was plotted to produce the standard curve.

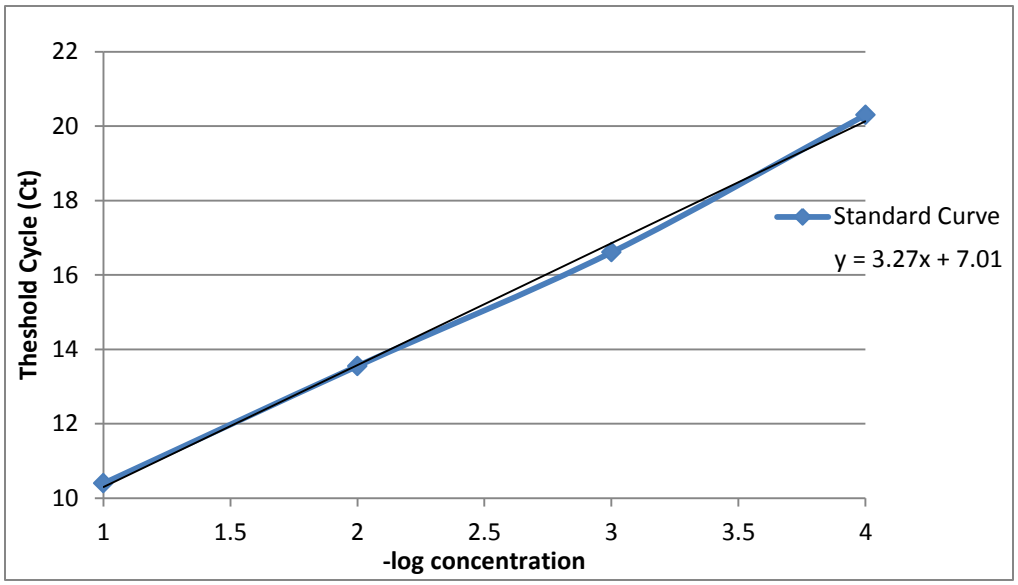
Since the standard curve was created from plasmid only, the effect of the culture on the qPCR reaction was assessed. A culture of HB101 cells without transformed plasmid was grown in TB media. The cells were diluted 1:24000 and plasmid concentration of 0.1 ng/µl, 0.01 ng/µl, 0.001

ng/µl, 0.0001 ng/µl, and 0.00001 ng/µl was added. The qPCR calculated concentrations of the culture/plasmid and only plasmid were compared.

The efficiency of the standard curve was calculated to determine if the slope reflected the 10 fold dilution by the equation: Efficiency = [10^ (1/slope)] – 1. Every standard curve used for quantification of plasmid concentration was between 90% and 110% efficient.

2.4.3. Plasmid Concentration Calculation

The standard curve plotting Ct values vs. the negative inverse log of plasmid concentration was used to calculate plasmid content in cell cultures. The linear standard curve equation was calculated. The Ct values of the sample were inserted into the linear standard curve equation. The inverse log of the negative value obtained from the equation produced the concentration. The concentration found was multiplied by 24000 to account for the dilution prior to the qPCR reaction (See Figure 2.2.).



Sample Ct = A

B = (A– 7.02)/ 3.27

C = 10 ^ (-B)

C * 24000 = Plasmid Concentration (ng/µl)

Figure 2.2. Plasmid Concentration Calculation from qPCR Standard Curve. The sample Ct values is inserted in the standard curve equation. The inverse log of the negative number obtained is found. This number is multiplied by 24 000 to account for the dilution before the qPCR reaction.

Results

3.1. Quantitative Polymerase Chain Reaction Assay

The Quantitative Polymerase Chain Reaction (qPCR) was used to quantify to amount of plasmid DNA produced in each culture. Previous attempts in the Tan laboratory to optimize the E coli growth protocol for increased plasmid yields used a different method for plasmid quantification. The plasmids were first isolated from the cell culture and spectrophotometry was used to quantify the amount of plasmid. The plasmid yields obtained were variable and not reproducible. For more accurate and reproducible results, qPCR was used in this thesis to quantify plasmid concentration.

The qPCR assay conditions needed to be optimized for accuracy and reproducibility before it was used for quantification of plasmid yields. The culture samples used for development of the qPCR assay were grown by Samuel Teicher, a former Tan laboratory member. Culture samples must be diluted before the qPCR reaction because the high plasmid concentrations in the culture are outside the qPCR limits of quantification. Initially the culture samples were diluted 1:2 400 culture/milliQ water before the qPCR reaction. The threshold cycle (Ct) values for the TB media culture diluted 1:2 400 (See Table 3.1.) were close or below the lowest threshold value of the standard curve. Plasmid concentrations are overestimated and inaccurate when the Ct values are close or below the lowest value of the standard curve (See Figure 3.1.).

Standard Curve		2xTY Media		TB Media	
Plasmid Concentration (ng/µl)	Threshold Cycle (Ct)	Hours After Inoculation	Threshold Value	Hours After Inoculation	Threshold Cycle (Ct)
0.1	10.12	18	12.83	18	10.55
0.01	13.78	20	12.91	20	10.54
0.001	17.42	22	12.54	22	**9.83**
0.0001	20.11	24	12.69	24	10.13
		26	12.73	26	**9.79**

Table 3.1. Ct values for 1:2 400 Diluted Culture Samples. Ampicillin resistant plasmid grown at 37 C in 2xTY and TB media by Sam Teicher diluted to 1:2 400 with deionized water before addition to qPCR reaction.

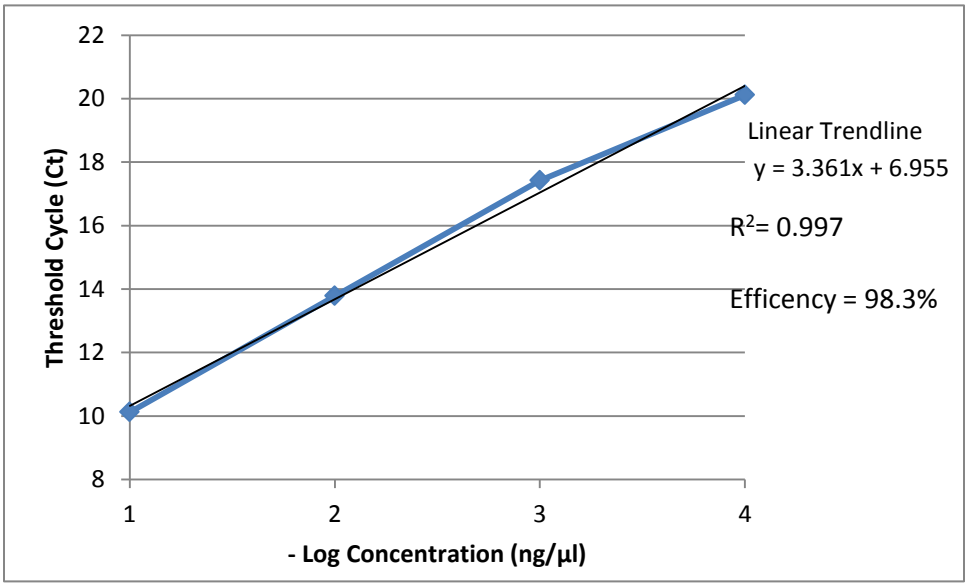


Figure 3.1. Standard Curves for 1:2 400 Diluted Culture Samples. The plasmid pST5516xNCP601 was diluted to concentration of 0.1, 0.001, 0.0001, and 0.00001 ng/µl before the qPCR reaction. The inverse long of concentration was calculated and plotted versus the Ct values.

To correct for the low threshold values of 1:2 400 culture/MilliQ water dilutions, a larger dilution of 1:24 000 culture/MilliQ water was performed to decrease the plasmid concentration for higher Ct values. The Ct values (See Table 3.2.) obtained from this dilution were higher and within the middle of the standard curve (See Figure 3.2.). The plasmid concentration of TB

media culture for the 1:24 000 dilution were lower and the 2xTY media culture plasmid concentration was similar. This dilution was used for all qPCR experiments.

Standard Curve		2xTY Media		TB Media	
Plasmid Concentration (ng/µl)	Threshold Cycle (Ct)	Hours After Inoculation	Threshold Cycle (Ct)	Hours After Inoculation	Threshold Cycle (Ct)
0.1	8.16	18	14.64	18	12.57
0.01	11.49	20	13.45	20	12.32
0.001	14.99	22	13.87	22	12.77
0.0001	18.33	24	13.51	24	12.32
		26	14.01	26	12.93

Table 3.2. Threshold Values of 1:24 000 Diluted Culture Samples. Ampicillin resistant plasmid grown at 37 C in 2xTY and TB media by Sam T diluted to 1:24 000 with deionized water before addition to qPCR reaction.

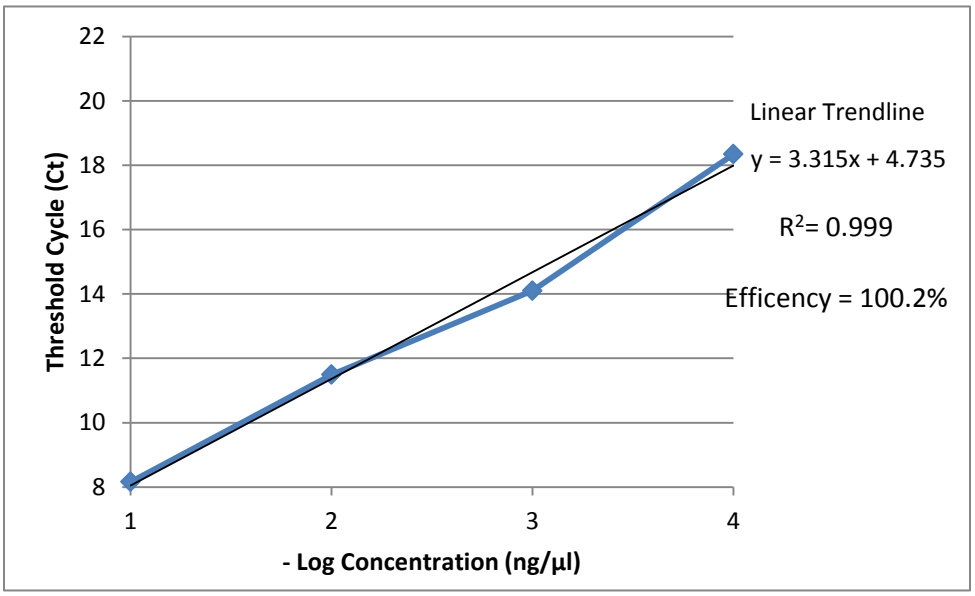


Figure 3.2. Standard Curve for 1:24 000 Diluted Culture. The plasmid pST5516xNCP601 was diluted to concentration of 0.1, 0.001, 0.0001, and 0.00001 ng/µl before the qPCR reaction. The inverse long of concentration was calculated and plotted versus the Ct values.

The standard curve used purified plasmid at known concentrations. The cultures contained media, antibiotics, and the *E. coli* cell contents in addition to plasmid. To determine if the these additional components had an effect on the qPCR reaction, a culture of HB101 cells without plasmid was grown and known concentrations of purified plasmid was added. The culture grew rapidly with no antibiotic selection and a sample was collected when the OD_{600} reached 10. The culture was diluted 1:24 000 culture/milliQ water and the standard curve plasmid concentrations were added to compare to the normal standard curve by qPCR (See Figure 3.3.). The diluted culture standard curve produced R^2 and efficiency values similar to the standard curve from purified plasmid. Efficiency measures accuracy of the standard curve by comparing the slope of the 10 fold diluted plasmid standard curve to the slope of a perfectly efficient 10 fold plasmid dilution qPCR reaction. The culture decreases the efficiency by about 1% which will not have an effect on plasmid concentration calculations, especially because the OD_{600} of 10 was higher than any cultures grown.

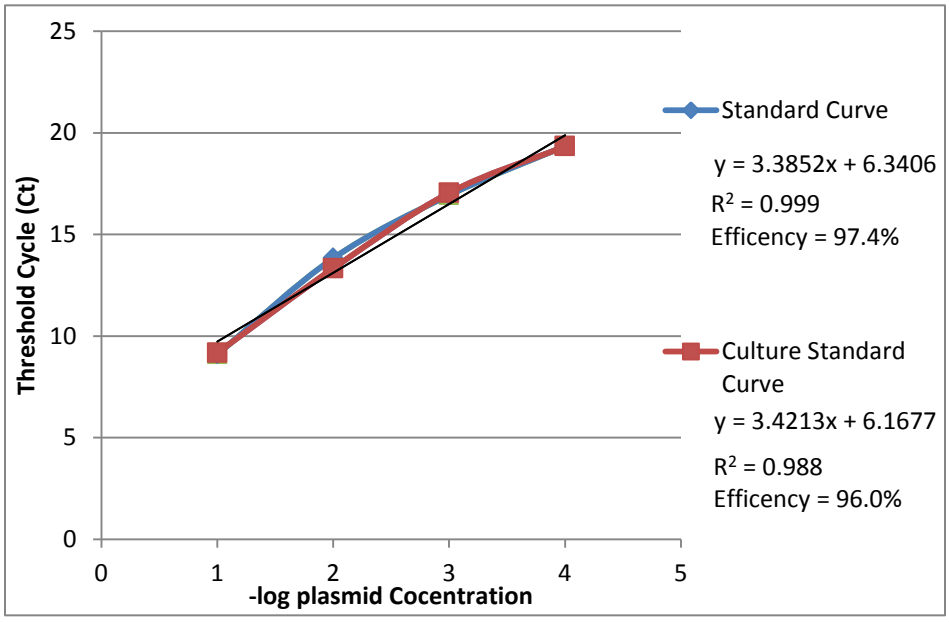


Figure 3.3. Culture Effect on qPCR Reaction. The culture standard curve has a very similar R^2 and efficiency as the purified plasmid standard curve.

3.2. Current Growth Protocol

The following current standard growth protocol was followed and the plasmid concentration was calculated for a reference to the other experimented growth conditions. The ampicillin resistance plasmid pST55-16xNCP601a (Created by Dr. Song Tan) containing 16 nucleosome DNA fragment inserts was transformed into HB101 cells (Source Applied Biosystems) and grown in 2xTY media at 37º C. The OD_{600} of the preculture was measured before inoculation of the 500 ml culture. Samples of the cultures were taken every 2 hours from 18 to 28 or 30 hours after inoculation and the OD_{600} was measured (See Figure 3.4.). The plasmid concentration of each culture sample was calculated by qPCR (See Figure 3.5.).

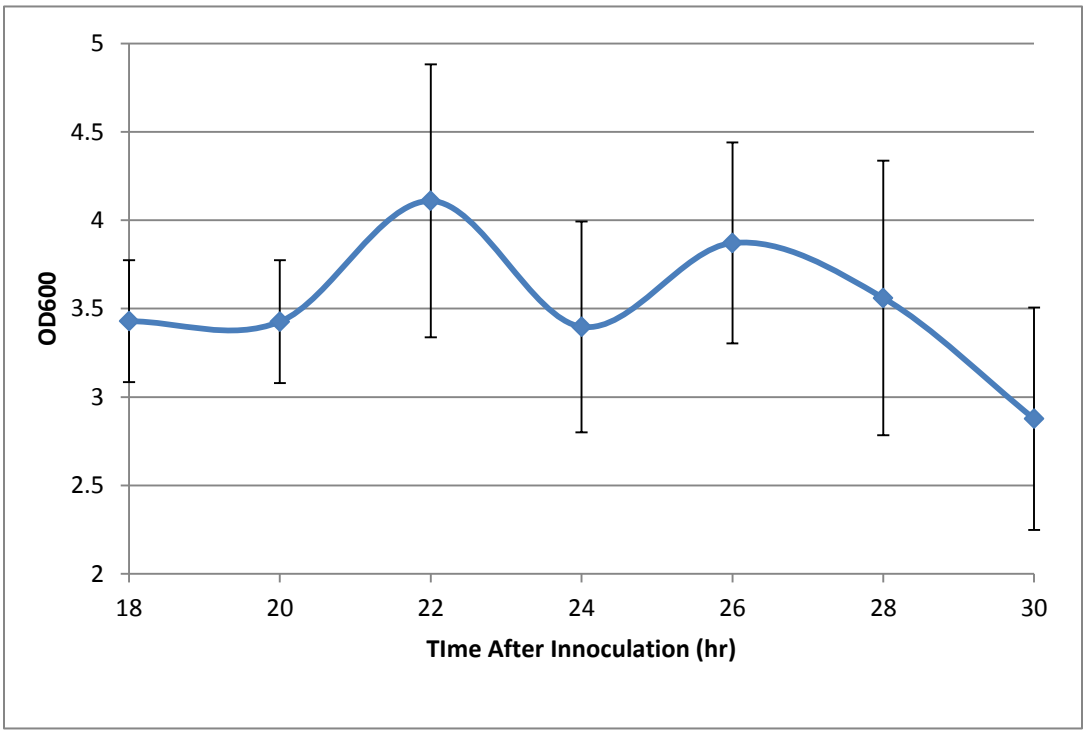


Figure 3.4. Average OD_{600} of Ampicillin Resistant Plasmid Grown at 37º C in 2xTY media. The cultures were diluted 1:10 with 2xTY media before OD_{600} measurement. Mean and error bars calculated from three separate experiments.

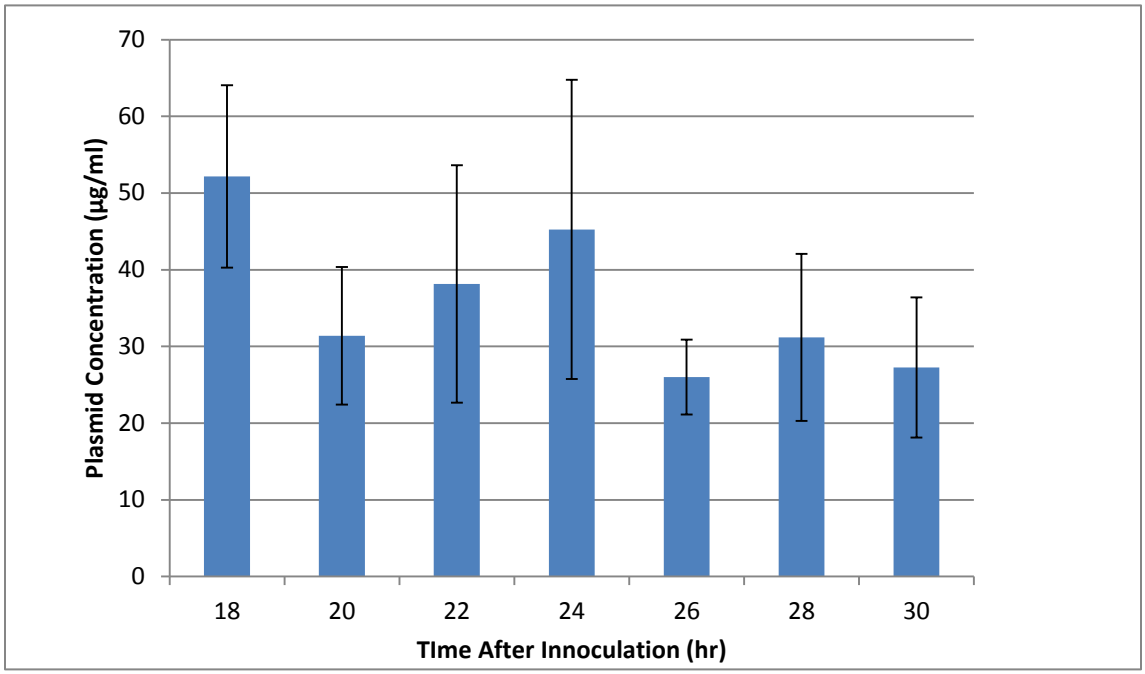


Figure 3.5. Average Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 37º C in 2xTY Media. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments.

The cell density and plasmid concentration varied similarly with time. The highest plasmid concentration obtained was 52 µg/ml at 18 hours. The large error of both cell density and plasmid concentrations showed the variably between each of 3 replicates (See Table 3.3.). Plasmid concentration was consistently lower after 18 hours and averaged approximately 35µg/ml. The actually plasmid yields obtained by the current protocol are between 10 to 20 µg/ml quantified by spectrophotometry after plasmid isolation. Loss of plasmid during isolation may partly explain the higher plasmid concentration calculated by qPCR.

2xTY Media OD_{600} and Plasmid Concentration						
Preculture OD_{600}	0.31		0.34		0.34	
	OD_{600}	Plasmid Concentration	OD_{600}	Plasmid Concentration	OD_{600}	Plasmid Concentration
18 hour	3.9	40 µg/ml	2.8	41 µg/ml	3.5	78 µg/ml
20 hour	4.1	14 µg/ml	2.9	42 µg/ml	3.3	40 µg/ml
22 hour	5.2	8.0 µg/ml	2.6	46 µg/ml	4.5	60 µg/ml
24 hour	4.4	18 µg/ml	2.3	34 µg/ml	3.4	83 µg/ml
26 hour	4.9	16 µg/ml	2.9	32 µg/ml	3.8	29 µg/ml
28 hour	4.9	10 µg/ml	2.2	46 µg/ml	3.6	38 µg/ml
30 hour			2.1	16 µg/ml	3.6	38 µg/ml

Table 3.3. Plasmid Concentration and OD_{600} of Current Growth Protocol. The concentration was calculated from the standard curve produced by each individual qPCR plate.

3.3. Ampicillin Resistant Plasmid Grown at 37º C in PDMR and TB Media

PDMR and TB media have been formulated to produce high cell densities and plasmid yields (Danquah and Forde, 2007). These media were used and compared to 2xTY media to determine if similar results could be obtained. The ampicillin resistance plasmid pST55-16xNCP601a containing 16 nucleosome DNA fragment copies was transformed into HB101 cells and grown in PDMR and TB media at 37º C. The OD_{600} of the preculture was measured before inoculation of the 500 ml culture. The OD_{600} was measured for PDMR and TB media cultures every 2 hours from 18 to 28 or 30 hours after inoculation and compared to 2xTY media (See Figure 3.6.). One PDMR media culture was contaminated before inoculation and therefore excluded.

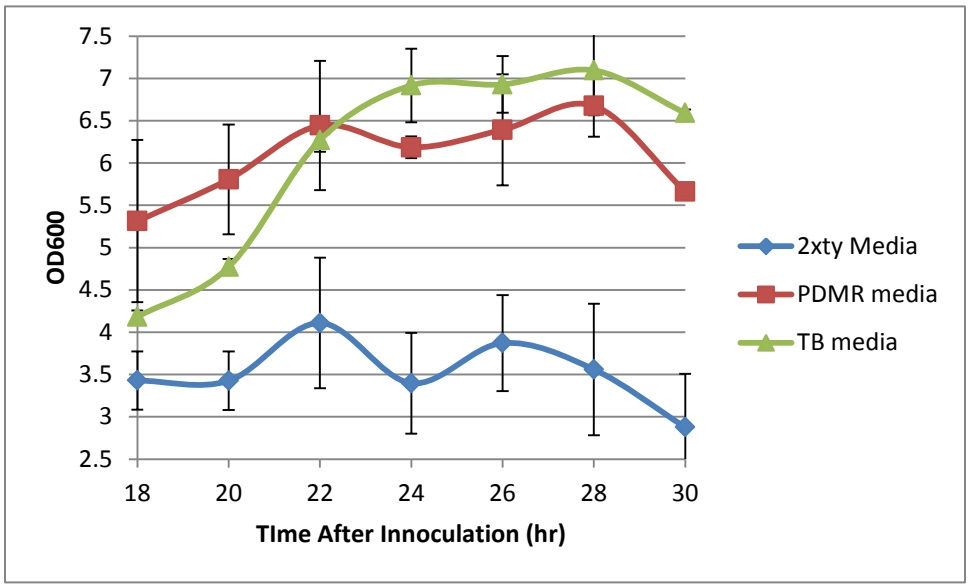


Figure 3.6. Average OD_{600} of Ampicillin Resistant Plasmid Grown at 37 C in 2xTY, PDMR, and TB media. Three separate 2xTY and TB cultures and two separate PDMR cultures (1 culture excluded due to contamination of media before inoculation) were grown and diluted 1:10 with corresponding media before OD_{600} measurement. Mean and error bars calculated from three separate experiments in 2xTY and TB media, only two PDMR media experiments are represented.

The TB media culture reached the highest cell density followed closely by the PDMR media culture. The TB media culture showed a large increase in cell density until 24 hours not seen in 2xTY or PDMR media. The TB media precultures OD_{600} were consistently lower than 2xTY and PDMR media precultures and might have caused the delay in exponential cell growth.

The plasmid content of PDMR and TB cultures was calculated by qPCR and the average concentration for each media was found (See Figure 3.7.). TB media produced higher plasmid concentrations than the other media types with a maximum of 134 µg/ml at 28 hours. 2xTY and PDMR media culture's plasmid concentrations fluctuated around 35 µg/ml over the entire time period sampled. The increase in OD_{600} was related to the increase in plasmid concentration for

the TB media cultures. I do not have a simple explanation for the large variation in plasmid concentration from 26 to 28 to 30 hours.

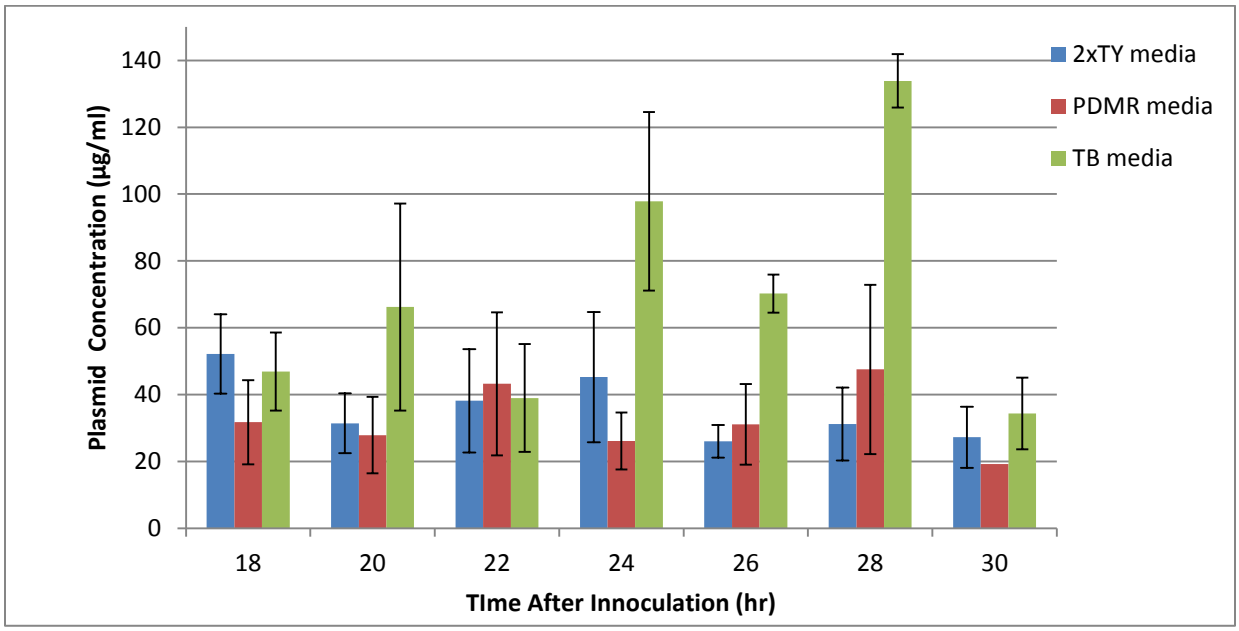


Figure 3.7. Average Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 37º C in 2xTY, PDMR, and TB media. The concentrations were calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments in 2xTY and TB media, and two separate experiments in PDMR media.

PDMR and TB Media Plasmid Concentrations (µg/ml) and OD_{600}										
	PDMR Media				TB Media					
Preculture OD_{600}	0.4656		0.1933		0.2738		0.1755		0.1755	
	OD_{600}	(µg/ml)	OD_{600}	(µg/ml)	OD_{600}	(µg/ml)	OD_{600}	(µg/ml)	OD_{600}	(µg/ml)
18 hour	4.3	19	6.2	44	4.2	42	4.0	30	4.2	69
20 hour	5.2	16	6.4	40	4.6	22	4.7	126	4.9	50
22 hour	5.6	22	7.2	64	6.52	12	6.0	36	6.2	68
24 hour	6.3	18	6.0	35	7.6	47	6.1	112	6.8	135
26 hour	7.1	19	5.7	43	7.3	64	6.2	82	7.1	65
28 hour	7.0	22	6.3	73	8.1	120	6.6	148	6.4	134
30 hour			5.6	42			6.5	47	6.6	21

Table 3.4. Plasmid Concentration and OD_{600} of Ampicillin Resistant Plasmid grown at 37º C in PDMR and TB Media. The concentration was calculated from the standard curve produced by each individual qPCR plate.

3.4. Kanamycin Resistant Plasmid Grown at 37º C in 2xTY, PDMR, and TB Media

Plasmids that confer resistance to the antibiotic ampicillin encode the enzyme β-lactamase. Ampicillin, which causes cell lysis, is degraded by β-lactamase produced and secreted extracellularly by cells containing the ampicillin resistant plasmid (Mayers, 2009). If β-lactamase concentrations reach high levels as in a saturated culture, much of the ampicillin will be degraded allowing for cells without the antibiotic plasmid to grow decreasing plasmid yields. Kanamycin resistance is conferred by a different mechanism maintaining effective concentrations of kanamycin in the culture (Davies and Wright, 1997). Kanamycin resistant plasmids were grown in each media type at 37º C and compared to ampicillin resistant plasmid grown in the same conditions to determine if the antibiotic resistance mechanism had an effect on plasmid yield.

The kanamycin resistant plasmid pST89-16xNCP601a (Created by Dr. Song Tan) containing 16 nucleosome DNA fragment copies was transformed into HB101 cells and grown in 2xTY, PDMR, and TB media at 37º C. The OD_{600} of the preculture was measured before inoculation of the 500 ml culture. Samples of the 500 ml cultures were taken every 2 hours from 18 to 28 hours after inoculate and the OD_{600} was measured.

The average cell density of kanamycin resistant plasmid grown in 2xTY media was lower than ampicillin resistant plasmids over the entire time course (See Figure 3.8.). However, kanamycin resistant plasmids produced higher average plasmid concentrations at every time point (See Figure 3.9.).

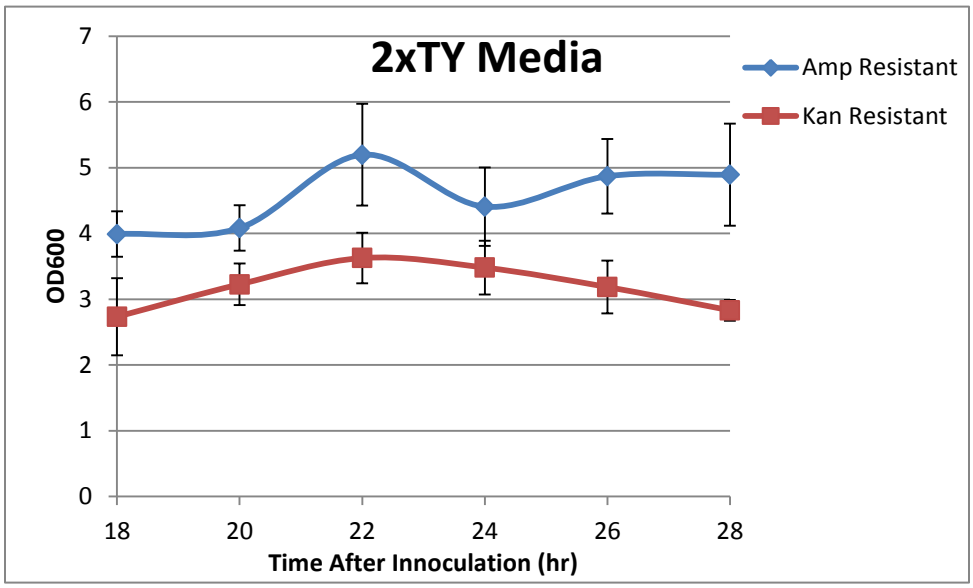


Figure 3.8. Average OD_{600} of Kanamycin and Ampicillin Resistant Plasmids Grown at 37º C in 2xTY Media. Three separate 2xTY cultures were grown and diluted 1:10 with 2xTY media before OD_{600} measurement. Mean and error bars calculated from three separate experiments.

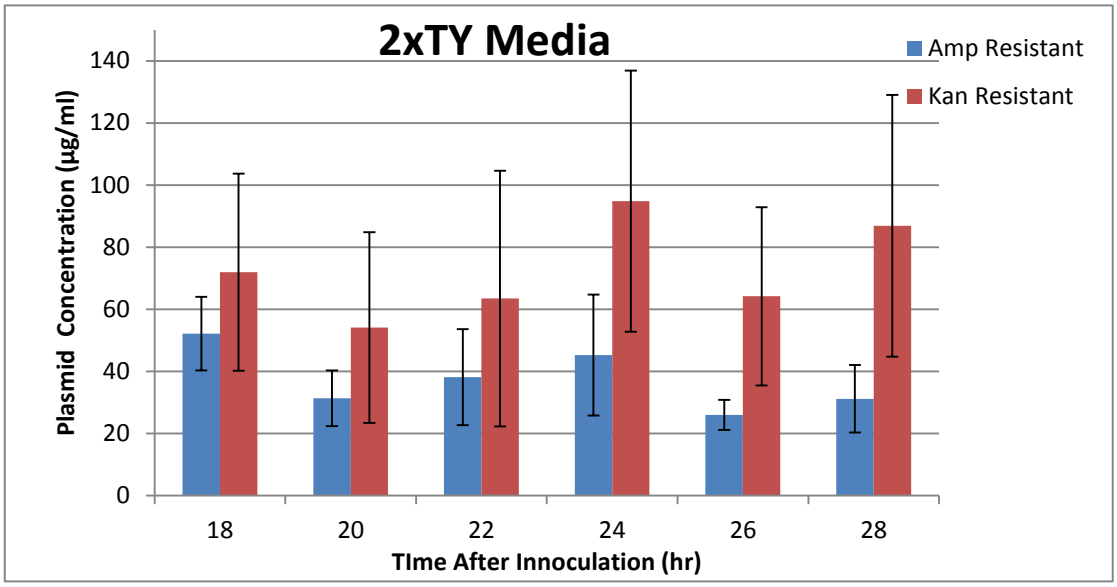


Figure 3.9. Average Plasmid Concentrations of Kanamycin and Ampicillin Resistant Plasmids Grown at 37º C in 2xTY Media. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments.

This may be attributed to the antibiotic resistance mechanism. Cells without the ampicillin resistant plasmid may have grown creating a denser culture, but did not contain plasmid decreasing overall plasmid concentration. The highest average plasmid concentrations for kanamycin and ampicillin resistant plasmids grown in 2xTY media were 95 µg/ml and 52 µg/ml, respectively. One replicate with low kanamycin resistant plasmid concentrations compared to the other two replicates caused a large amount of error (See Table 3.5., compare left from middle and right columns). The replicate with the lower plasmid concentration was inoculated with a lower cell density preculture than the other replicates. Also, the cell densities were higher in the replicates with the higher OD_{600} precultures.

2xTY Media OD_{600} and Plasmid Concentrations (µg/ml)						
Preculture OD_{600}	0.1764		0.3642		0.3642	
	OD_{600}	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**
18 hour	1.7	9.6 µg/ml	3.7	113 µg/ml	2.6	92 µg/ml
20 hour	2.6	1.7 µg/ml	3.6	52 µg/ml	3.5	108 µg/ml
22 hour	2.9	2.0 µg/ml	3.7	141 µg/ml	4.2	47 µg/ml
24 hour	2.6	11 µg/ml	3.9	143 µg/ml	3.8	130 µg/ml
26 hour	2.5	15 µg/ml	3.1	64 µg/ml	3.9	114 µg/ml
28 hour	2.67	7.4 µg/ml	2.6	102 µg/ml	3.1	151 µg/ml

Table 3.5. Plasmid Concentrations and OD_{600} of Kanamycin Resistant Plasmid grown at 37° C in 2xTY Media. The concentration was calculated from the standard curve produced by each individual qPCR plate.

The average cell density of kanamycin resistant plasmids grown in PDMR media was higher than the ampicillin resistant plasmids from 18 to 24 hours (See Figure 3.10.). After 24 hours, the cell density decreased and was lower than the ampicillin resistant plasmid culture. The average

plasmid concentration of kanamycin and ampicillin resistant plasmids grown in PDMR was calculated by qPCR (See Figure 3.11.). Kanamycin resistant plasmids produced higher average plasmid concentrations throughout the entire time course. The highest average plasmid concentrations for kanamycin and ampicillin resistant plasmids were 102 µg/ml and 47 µg/ml, respectively. Again, the antibiotic resistance mechanism may explain the similar cell density between both ampicillin and kanamycin resistant plasmids, but much higher plasmid yield in the kanamycin resistant plasmids.

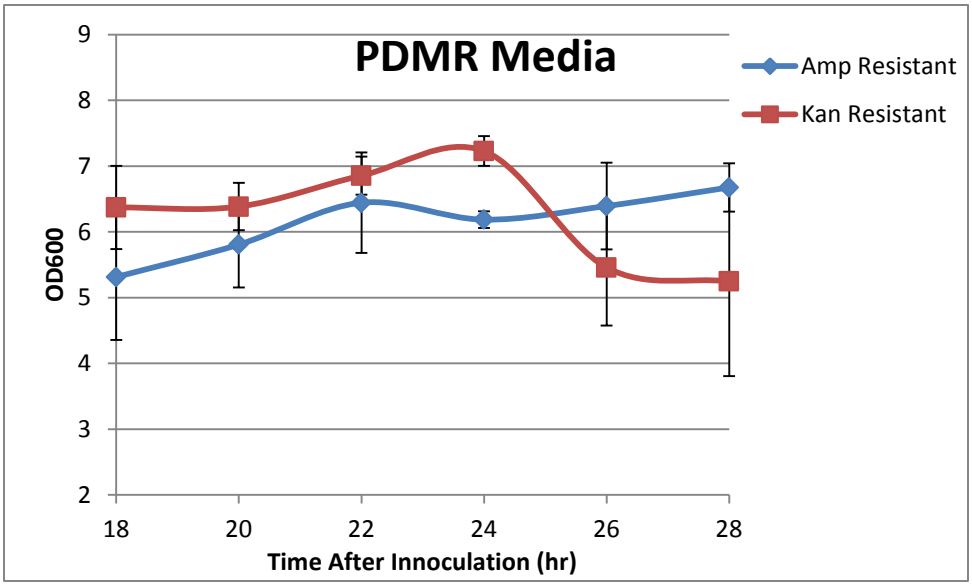


Figure 3.10. Average OD_{600} of Kanamycin and Ampicillin Resistant Plasmid Grown at 37º C in PDMR Media. Three separate PDMR cultures were grown and diluted 1:10 with PDMR media before OD_{600} measurement. Mean and error bars calculated from three separate experiments.

The large error found with kanamycin resistant plasmids is partially attributed too much lower plasmid concentrations of one replicate (See Table 3.6.).

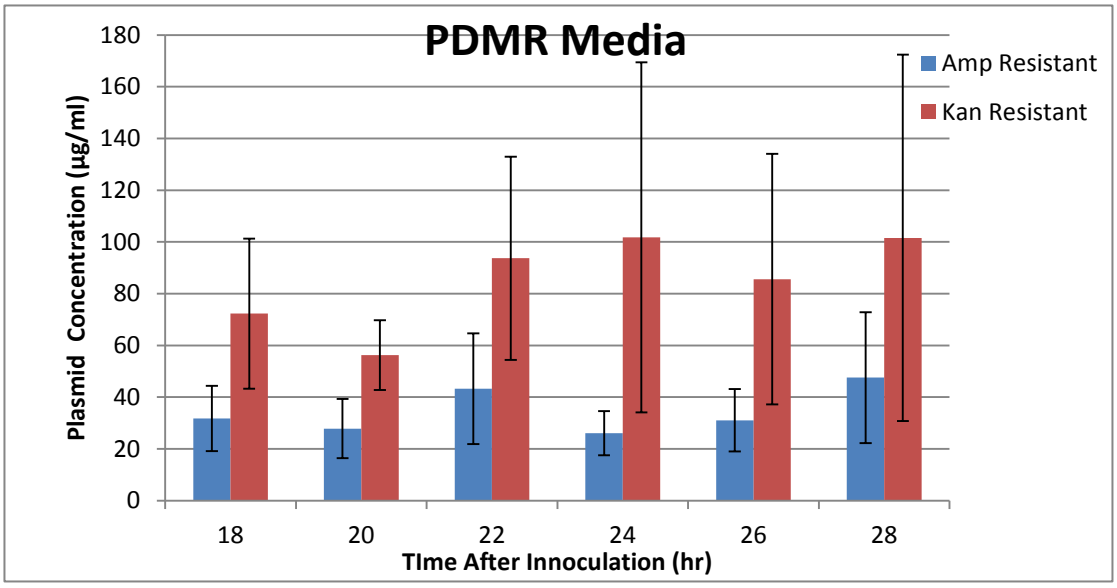


Figure 3.11. Average Plasmid Concentrations of Kanamycin and Ampicillin Resistant Plasmids Grown at 37º C in PDMR Media. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments.

PDMR Media OD_{600} and Plasmid Concentrations (µg/ml)						
Preculture OD_{600}	0.40		0.47		0.47	
	OD_{600}	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**
18 hour	5.2	17 µg/ml	7.3	114 µg/ml	6.6	86 µg/ml
20 hour	5.7	30 µg/ml	6.4	72 µg/ml	7.0	68 µg/ml
22 hour	6.9	19 µg/ml	6.3	111 µg/ml	7.3	151 µg/ml
24 hour	6.9	18 µg/ml	7.7	51 µg/ml	7.2	236 µg/ml
26 hour	7.2	14 µg/ml	4.5	65 µg/ml	4.7	178 µg/ml
28 hour	7.9	15 µg/ml	3.0	48 µg/ml	4.8	242 µg/ml

Table 3.6. Plasmid Concentrations and OD_{600} of Kanamycin Resistant Plasmid Grown at 37º C in PDMR Media. The concentration was calculated from the standard curve produced by each individual qPCR plate.

The cell density and plasmid concentration showed similar change with time for kanamycin resistant plasmid grown in TB media (See Figure 3.12.). The average cell density was lower than the ampicillin resistant plasmid after 22 hours. The average plasmid concentration was

significantly greater than the ampicillin resistant plasmid from 18 to 22 hours and similar or less after 22 hours (See Figure 3.13.). The decrease in cell density of kanamycin resistant plasmids may have indicated phase of decline growth causing decreased plasmid copy number. The highest average plasmid concentrations for kanamycin and ampicillin resistant plasmids were 186 µg/ml and 133 µg/ml, respectively. The large error found with kanamycin resistant plasmids is partially attributed to much lower plasmid concentration of one replicate (See Table 3.7.). This replicated was inoculated with lower cell density preculture. However, it is not clear whether the preculture cell density is sufficient to account for the large difference in plasmid yields.

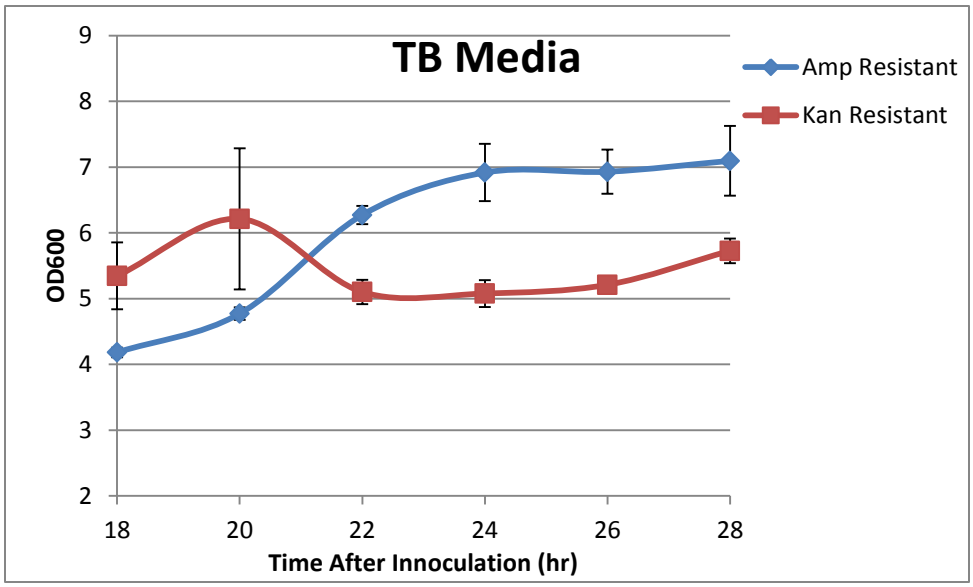


Figure 3.12. Average OD_{600} of Kanamycin and Ampicillin Resistant Plasmids Grown at 37º C in TB Media. Three separate TB cultures were grown and diluted 1:10 with TB media before OD_{600} measurement. Mean and error bars calculated from three separate experiments.

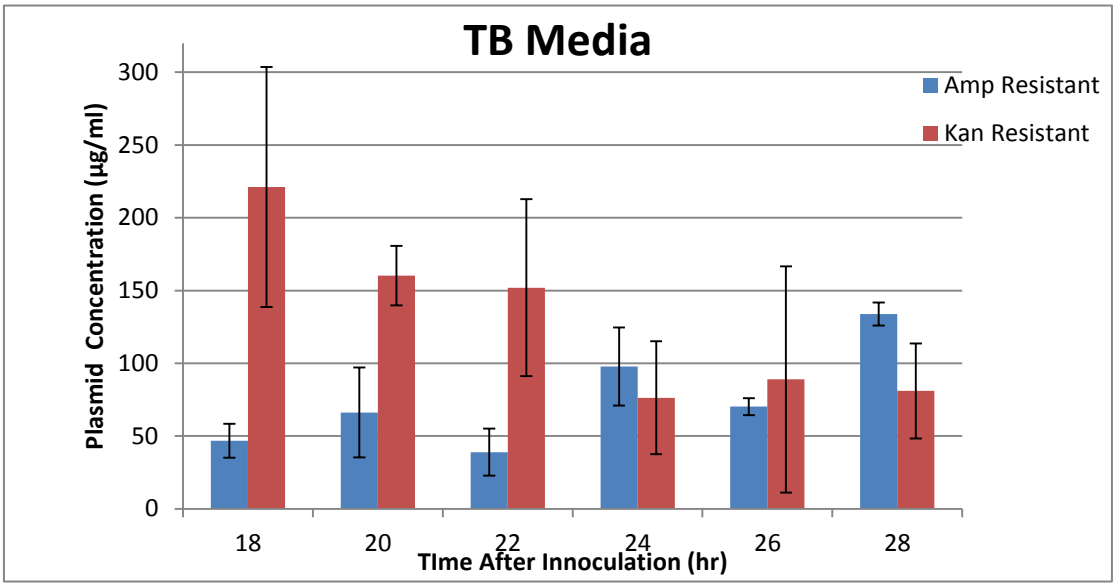


Figure 3.13. Average Plasmid Concentrations of Kanamycin and Ampicillin Resistant Plasmid Grown at 37º C in TB Media. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments.

TB OD_{600} and Plasmid Concentrations						
Preculture OD_{600}	0.25		0.42		0.42	
	OD_{600}	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**	**OD_{600}**	**Plasmid Concentration**
18 hour	4.3	33 µg/ml	5.9	139 µg/ml	5.7	304 µg/ml
20 hour	4.1	12 µg/ml	7.6	140 µg/ml	6.9	180 µg/ml
22 hour	4.8	16 µg/ml	4.9	280 µg/ml	5.4	159 µg/ml
24 hour	4.7	11 µg/ml	5.0	70 µg/ml	5.4	148 µg/ml
26 hour	5.3	5 µg/ml	5.2	53 µg/ml	4.9	208 µg/ml
28 hour	5.4	40 µg/ml	5.6	69 µg/ml	6.0	134 µg/ml

Table 3.7. Plasmid Concentration of Kanamycin Resistant Plasmid Grown at 37º C in TB Media. The plasmid concentration was calculated from the standard curve produced by each individual qPCR plate.

3.5. Temperature Induction of Ampicillin Resistant Plasmid in 2xTY, PDMR, and TB Media

The plasmid used in this thesis was a ColE1-type plasmid with a point mutation in RNAII and deletion of ROM which increases plasmid copy number within a cell. However, the mutation is temperature sensitive. At temperatures of 30º C or below the mutation effects are suppressed, but at 42º C the plasmid copy number is increased (Lin-Chao, 1992). Temperature induction was used to take advantage of the plasmids temperature sensitivity. Cultures were grown at or below 30º C to increase cell density then increased to 42º C for increased plasmid replication.

The ampicillin resistant plasmid pST55-16xNCP601a was transformed into HB101 cells and grown in 2xTY, PDMR, and TB media cultures. The 500 ml cultures were grown at 23º C for 18 hours when the temperature was increased to 42º C. Three replicates for 2xTY and PDMR media were grown, but two of the TB media replicates did not grow over the time course. The OD_{600} of the preculture was measured before inoculation of the 500 ml culture. Samples of the 500 ml cultures were taken every 2 hours from18 to 28 hours after inoculate and the OD_{600} was measured.

The OD_{600} of the cultures was measured and the average of the replicates was determined (See Figure 3.14.). The average cell densities of each media started out low and increased with the increase in temperature to 42º C. PDMR media culture had the highest average OD_{600} over the entire time course. 2xTY media showed a gradual increase in OD_{600} while the single TB media culture examined did not begin increasing until 22 hours.

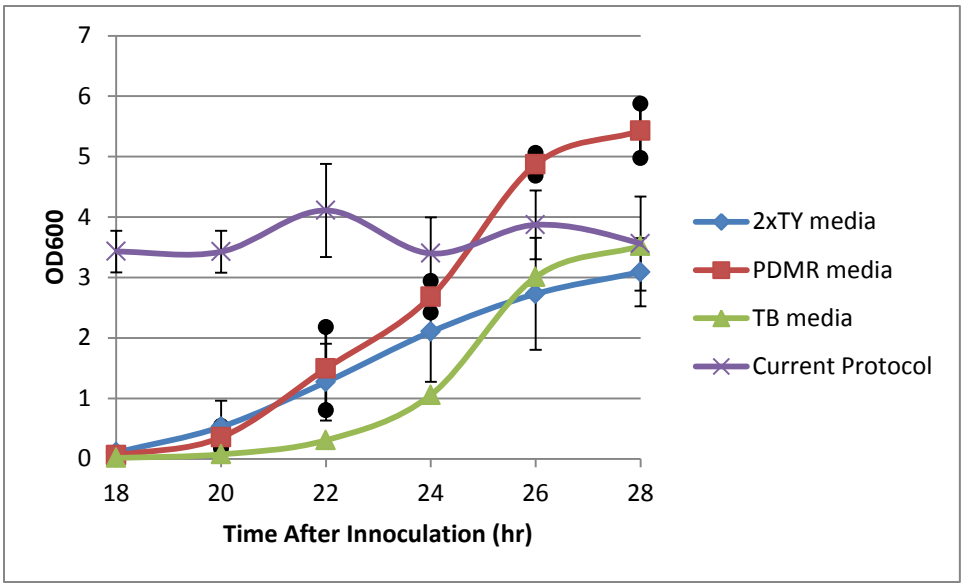


Figure 3.14. Average OD_{600} of Ampicillin Resistant Plasmid Grown at 23ºC then 42ºC in 2xTY, PDMR, and TB Media. Three separate cultures were grown in 2xTY and PDMR, but only one TB cultures grew. Cultures were diluted 1:10 with corresponding media before OD_{600} measurement. Mean and error bars calculated from three separate experiments in 2xTY and PDMR media, only one TB media experiment is represented.

The concentration of each culture was calculated by qPCR and the average of the replicates was found (See Figure 3.15.). The plasmid concentration in each culture increased with time similarly to the cell density. A large amount of error was found because one replicate in 2xTY and PDMR media culture reached higher plasmid concentrations compared to the other replicates (See Table 3.8.). Plasmid concentrations reached 130 µg/ml and 137 µg/ml in one 2xTY and PDMR culture, respectively. The other 2xTY and PDMR cultures only reached plasmid concentrations of 10 µg/ml.

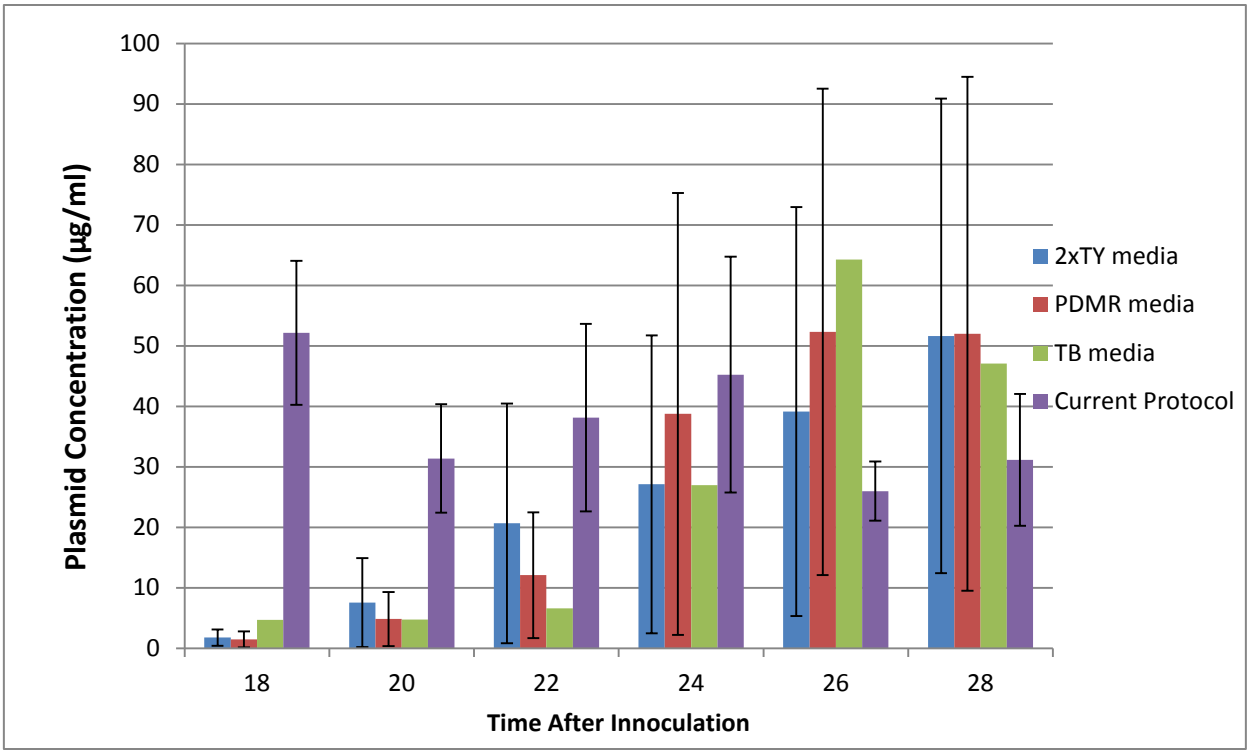


Figure 3.15. Average Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 23º C then 42ºC in 2xTY, PDMR, and TB Media. Only one TB media culture was successfully grown. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments, only one TB media experiment is represented.

Plasmid Concentration (µg/ml)							
	2xTY Media			PDMR Media			TB Media
Preculture OD_{600}	0.37	0.27		0.42	0.33		0.21
18 hour	4.4	0.57	0.27	4.1	0.18	0.10	4.7
20 hour	22	0.25	0.16	13.7	0.28	0.44	4.7
22 hour	60	1.2	0.49	32.8	1.7	1.65	6.5
24 hour	76	2.5	2.5	110	2.6	1.82	26
26 hour	100	6.4	4.2	130	18	6.1	64
28 hour	130	10	14	137	10	9.1	47

Table 3.8. Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 23º C then 42ºC in 2xTY, PDMR, and TB Media. The concentration was calculated from the standard curve produced by each individual qPCR plate.

Temperature induction of 23º C to 42º C increased plasmid concentration after temperature switch in one replicate of each media type. The plasmid concentrations reached by this replicate at 28 hours were high relative to the current growth protocol. However, the 23º C initial growth temperature produced low cell densities after 18 hours (i.e. just before temperature induction) which could have limited maximum plasmid yields. The initial growth temperature of 30º C produced higher cell densities at 18 hours to possibly induce greater plasmid yields upon temperature induction.

The ampicillin resistant plasmid pST55-16xNCP601a was transformed into HB101 cells and grown in 2xTY, PDMR, and TB media cultures. The 500 ml cultures were grown at 30º C for 18 hours when the temperature was increased to 42º C. The OD_{600} of the preculture was measured before inoculation of the 500 ml culture. Samples of the 500 ml cultures were taken every 2 hours from18 to 28 hours after inoculate and the OD_{600} was measured.

The OD_{600} of the cultures was taken from 18 to 30 hours and the average of each media type was calculated (See Figure 3.16.). The temperature of 30º C induced much larger 18 hours cell densities than 23º C and reached OD_{600} values higher than any other growth condition. TB media cultures were lower than 2xTY and PDMR at 18 hours, and then increased with time. 2xTY and PDMR media cultures showed high cell densities at 18 hours and remained high over the time course.

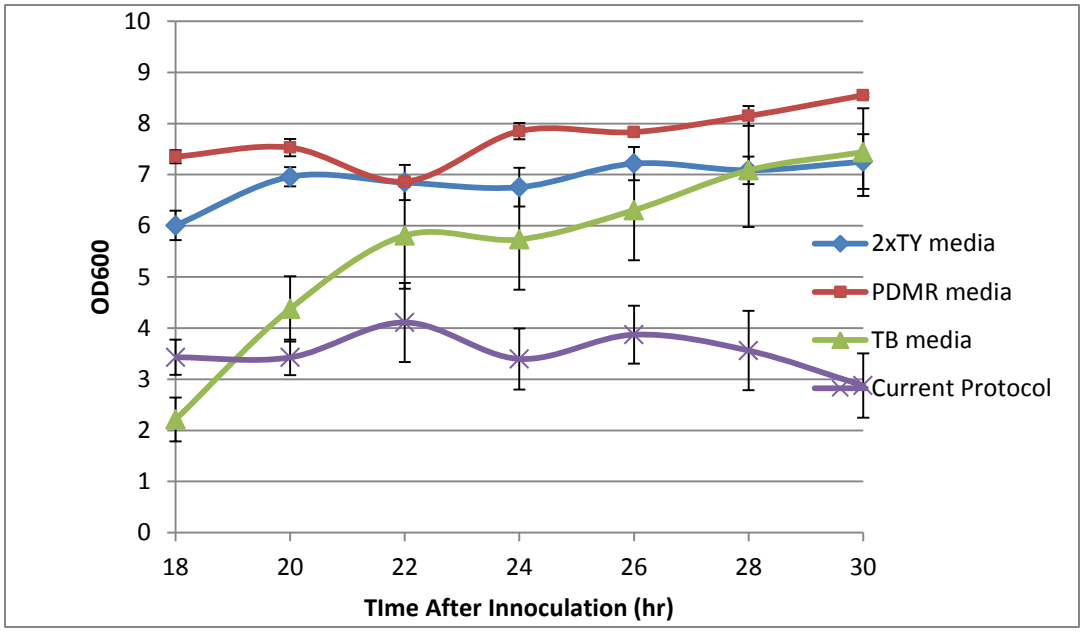


Figure 3.16. Average OD_{600} of Ampicillin Resistant Plasmid Grown at 30º C then 42ºC in 2xTY, PDMR, and TB Media. Cultures were diluted 1:10 with corresponding media before OD_{600} measurement. Mean and error bars calculated from three separate experiments.

The plasmid concentration for each culture was determined by qPCR (See Figure 3.17.). 2xTY media average plasmid concentration increased from 18 to 20 hours, and remained approximately 70 µg/ml after 20 hours. TB media produce the largest plasmid concentration of 90 µg/ml at 22 and 28 hours. PDMR media highest plasmid concentration was 66 µg/ml at 18 hours.

Temperature induction of 30º C to 42º C did not significantly increase the plasmid concentration shown in 28º C to 42º C temperature induction. It is possible that the culture was already in late stationary phase growth and the temperature increase was unable to induce an increase in plasmid replication.

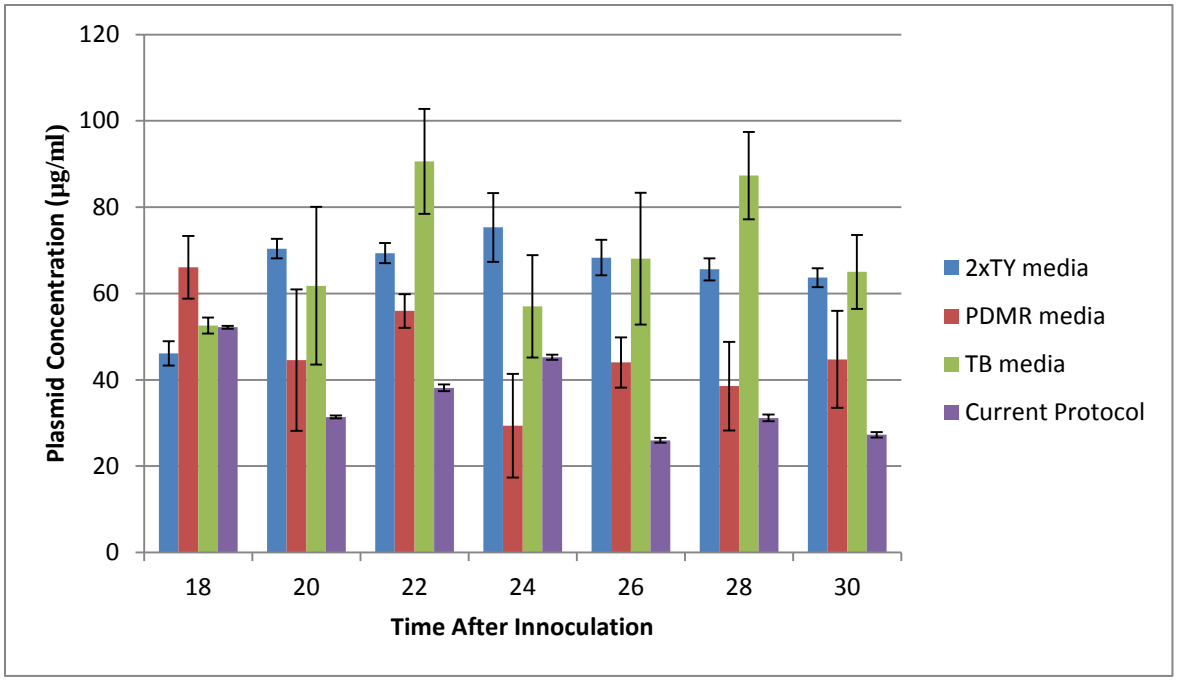


Figure 3.17. Average Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 30º C then 42ºC in 2xTY, PDMR, and TB Media. The concentration was calculated from the standard curve produced by each individual qPCR plate. Mean and error bars calculated from three separate experiments.

Plasmid Concentration (µg/ml)									
	2xTY Media			PDMR Media			TB Media		
Preculture OD_{600}	0.2509			0.1468			0.1718		
18 hour	33	58	45	69	92	62	55	53	48
20 hour	39	94	77	34	59	39	98	47	39
22 hour	64	77	66	58	59	53	110	92	68
24 hour	97	71	56	19	25	39	66	70	33
26 hour	71	56	76	49	27	38	85	81	37
28 hour	75	45	76	35	11	41	133	108	19
30 hour	78	41	70	42	7.0	47	63	51	80

Table 3.9. Plasmid Concentration of Ampicillin Resistant Plasmid Grown at 30º C then 42ºC in 2xTY, PDMR, and TB Media. The concentration was calculated from the standard curve produced by each individual qPCR plate.

Discussion

4.1. Optimal Media Type

TB was the optimal media type experimented for growing *E. coli* cells to produce large plasmid yields. TB media had significantly larger plasmid concentrations than 2xTY and PDMR media in every experiment except for the 23º C to 42º C temperature induction experiment. PDMR supported culture densities that were as high or higher than TB, but the plasmid concentrations were only slightly larger than 2xTY media exemplifying the importance of plasmid copy number per cell. The average plasmid concentration of every sample taken for ampicillin resistant plasmids grown in 2xTY, PDMR, and TB at 37º C was 36 µg/ml, 32 µg/ml, and 70 µg/ml, respectively.

4.2. Optimal Growth Temperature

High plasmid concentrations using temperature induction were found, but not reproducible. Only one culture grown at 23 ºC to 42ºC in each media showed a large plasmid yield. Ampicillin resistant plasmid grown in PDMR media at 23 ºC to 42ºC produced the highest single culture sample concentration of 137 µg/ml at 28 hours. The average plasmid concentration of three cultures using ampicillin resistant plasmid grown in TB media at 37ºC is 134 µg/ml at 28 hours. Temperature induction of 30ºC to 42ºC produced consistent plasmid concentration for each replicate reaching 91 µg/ml in TB media. The expected large increase in plasmid concentration with temperature increase to 42ºC was not found. The temperature induction protocol needs to be optimized for both reproducibility and plasmid yield. The growth

temperature of 37ºC provides consistent plasmid concentrations similar to the single highest temperature induction plasmid concentration and is therefore suggested for plasmid preparation.

4.3. Optimal Antibiotic Resistance:

Kanamycin resistant plasmids reached higher plasmid concentrations than ampicillin resistant plasmids in every sample. Average concentration for every sample taken using kanamycin plasmid in 2xTY, PDMR, and Tb media were 73 µg/ml, 85 µg/ml, and 130 µg/ml, respectively. These concentrations are approximately 2x that of ampicillin concentrations in the same media type.

4.4. Optimal Growth Length

The optimal length of growth to produce the highest plasmid yield varied between the different growth conditions. Growth rate was variable between cultures and designating a specific time to harvest plasmids would result in variable plasmid yield. Therefore, the OD_{600} value of the cultures should be used to determine the time of plasmid harvest. In general, the highest plasmid concentrations were obtained at the beginning of the stationary phase when the cell densities stopped increasing. The OD_{600} should be monitored closely and form multiple cultures when performing large plasmid preps because of the variable culture growth.

4.5. Preculture Cell Density Effects

Higher preculture cell density usually increased 500 ml culture OD_{600}, plasmid concentration, and rate of growth. The exact effects of preculture density can only be estimated because it was not systematically varied. My data suggests precultures should be grown to a high OD_{600},

approximately 0.4 to 0.5, for the largest plasmid yield. The optical density of the preculture is a parameter that needs to be examined more thoroughly for its effects on plasmid yields.

4.6. Growth Variability

There was a large amount of plasmid concentration variability between cultures. Some but not all variability can be attributed to preculture cell density. Culture grown under the same conditions inoculated with the same preculture still produced variable plasmid concentrations. The intra-qPCR precision may also account for the plasmid concentration variability, but this seems unlikely given that the standard curves of each qPCR reaction had efficiencies between 90% and 100%.

4.7. Increase in Plasmid Yield from Optimal Condition

The optimal condition for plasmid yield was kanamycin resistant cells grown at 37º C in TB media. The current growth protocol of ampicillin resistant plasmids grown at 37º C in 2xTY media produced an average plasmid concentration of 35 µg/ml for every sample taken. The highest average plasmid concentration of the optimal growth condition was 221 µg/ml at 18 hours. The average plasmid concentration of every sample taken was 130 µg/ml reflecting a 3.7x increase in plasmid yield compared to the current growth protocol. Since 18 hours was the earliest time point for my experiments, future experiments should examine earlier time points including 14 and 16 hours for growing the kanamycin resistance plasmid in TB media at 37°C.

4.8. Trouble Shooting

4.8.1. qPCR Assay

An extensive amount of time was taken developing a functional qPCR assay. The efficiency of the standard curve was improved by using larger dilution volumes and careful pipetting. The 10 fold dilutions were created by 5:45 volume:volume plasmid solution/milliQ water dilutions using a P10 pipetman. The dilution of the cultures was also improved by larger serial dilution volumes of 2:118, 2:78, and 5:45 culture solution/milliQ water to obtain 1:24 000 diluted cultures. Plating of the qPCR reaction was done carefully, but as fast as possible to prevent bleaching of the florescent probe. Duplicate or triplicate qPCR reactions are needed for accuracy because a small difference in CT values can translate to large variations in plasmid concentrations.

4.8.2. Culture Growth

Although culture growth was variable, steps were taken to produce consistent results. Restreaked plates can be refrigerated up to 48 hours before inoculation of the preculture. However, fresh restreaked plates were always used to eliminate difference from refrigeration. Preculture growth was unpredictable, taking anywhere from 4 to 9 hours to reach the appropriate OD_{600}. This was further complicated by growing multiple precultures and attempting to obtain similar OD_{600} between them. The size and amount of colonies used was carefully selected for more consistent preculture growth. The variable preculture growth time is troubling and deserves study in the future.

4.9. Future Experiments

Other conditions could be examined to determine the effect on plasmid yield. Many other types of media are used for bacterial growth. While TB media produced large plasmid yields, it could be supplemented with additional nutrients to see if they affect plasmid yield. Temperature induction produced large increases in plasmid concentration, but the results were not reproducible. More experimentation to determine the right initial temperature before increasing to 42º C could be found. Finally, the effects of preculture OD_{600} needs to be analyzed more thoroughly for better understanding of why the time of preculture growth was so variable.

References

1. Phillips, G, and B. Funnell. *Plasmid Biology*. Washington, DC: American Society for Microbiology, 2004. 1-16.
2. Helgason, E., O. Okstad, D. Caugant, et al. "*Bacillus anthracis, Bacillus cereus, and Bacillus thuringiensis* – one species on the basic of genetic evidence." *Applied Enviromental Microbiology* 66 (2000): 2627-2630.
3. Cohen, S. N. "Bacterial plasmids: their extraordinary contribution to molecular genetics." *Gene* 135 (1993): 67-76.
4. Mandecki, W, M. Hayden, M. Shallcross, and Elizabeth Stotland. "A totally synthetic plasmid for general cloning, gene expression and mutagenesis in *Escherichia coli*." *Gene* 94 (1990): 103-07.
5. Nair, A. J. *Principles of Biotechnology*. Hingham, MA: Laximi Publications, 2007.646-647.
6. Prather, K., S. Sagar, J. Murphy, and M. Chartrain. "Industrial scale production of plasmid DNA for vaccine and gene therapy: plasmid design, production, and purification." *Enzyme and Microbial Technology* 33.7 (2003): 865-83.
7. Mandecki, W., T.J. Bolling. "*fok*I method of gene synthesis." Gene 68 (1988): 101-107
8. Lederberg, J. "Plasmid (1952-1997)." *Plasmid* 39 (1998): 1-9.
9. Vieira, J., and J. Messing. "The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers." *Gene* 19.3 (1982): 259-68.
10. Lin-Chao, S., W.T. Chen, and T.T. Wong. "High copy number of the pUC plasmid results from a Rom/Rop-suppressible point mutation in RNA II." *Molecular Biology* 6.22 Nov. (1992): 3385-93.
11. Wang, Z., L. G. Le, Y. Shi , G. Wegrzyn "Medium design for plasmid DNA production based on stoichiometric model." *Process Biochem* 36 (2001):1085–93.
12. Danquah, M., and G. Forde. "Growth Medium Selection and Its Economic Impact of Plasmid DNA Production." *Journal of Bioscience and Bioengineering* 104.6 (2007): 490-97.
13. O'Kennedy, R., C. Baldwin, and E Keshavarz-Moore. "Effects of growth medium selection on plasmid DNA." *Journal of Biotechnology* 76 (2000): 175-83
14. Mayers, D. *Antimicrobial Drug Resistance: Mechanisms of Drug Resistance*. Vol. 1. New York: Humana Press, 2009. 70-71.
15. Davies, J., and G. Wright. "Bacterial resistance to aminoglycoside antibiotics." *Trends in Microbiology* 5.6 (1997): 234-40
16. Monod, J. "The Growth of Bacterial Cultures." *Annual Review of Microbiology* 3 (1983): 513-536
17. Reinikainen, P. "Escherichia *coli* production in fermenter". *Biotechnol Bioeng* 33(1989): 386–93.
19. Pradyumna, N., N.Irwin, B.G. Thompson, M. Gray. "Effects of oxygen fluctuations on recombinant *Escherichia coli* fermentation." *Biotechnology and Bioengineering* 41.6 (1993)
20. Pamela, N et. al. "Reconstitution of Nucleosome Core Particles from Recombinant Histones and DNA" *Methods of Enzymology* 375 (2003): 23-44

Appendix A

2xTY Media 1 Liter Example:

16 g Bacto tryptone
10 g Yeast extract
5 g NaCl

Add 1 liter deionized water and Autoclave

TB Media 1 Liter Example:

Solution 1:

12 g Bacto tryptone
24 g Yeast extract
5 g 100% Glycerol

Add 900 ml deionized water

Solution 2:

9.4 g Dipotasssium Phosphate (K_2HPO_4)
2 g Potassium Phosphate (KH_2PO_4)

Add 100 ml deionized water

Autoclave solutions separately and combine after cooling to room temperature.

PDMR Media 1 Liter Example:

Solution 1:

7.9 g Bacto tryptone
4.4 g Yeast extract

Add 800 ml deionized water

Solution 2:

6.78 g Anhydrous Disodium Phosphate (Na_2HPO_4)
3 g Potassium Phosphate (KH_2PO_4)
1.06 g Ammonium Chloride (NH_4Cl)

Add 100 ml deionized water

Solution 3:
10 g Glucose
0.24g Magnesium Sulfate ($MgSO_4$)

Add 100 ml deionized water

Autoclave solutions separately and combine after cooling to room temperature.

Quantitative Polymerase Chain Reaction Primers:

STO 3283: Forward qPCR Primer for pST55-16xNCP601a

Sequence: CCTGACGAGCATCACAAA

STO 3284: Reverse qPCR Primer pST55-16xNCP601a

Sequence: AGCGAACGACCTACACC

STO 3285 Forward qPCR Primer for pST89-16xNCP601a

Sequence: CTCCTGTTCCGACCCTG

STO 3286 Reverse qPCR Primer for pST89-16xNCP601a

Sequence: GCACCGCCTACATACCTC

Academic Vita

Joshua Thomas McAnulty

Education:

The Pennsylvania State University 2008-2012
Major: Bachelor of Science in Life Science *University Park, PA*
Minor: Kinesiology
Honors in Biochemistry and Molecular Biology

Activities:

High School Football Coaching Assistant Summers 2008-2011
- Organized workouts and drills *Harborcreek, PA*
- Supervised weight room

Volunteer at Corry Memorial Hospital Summer 2009
- Assisted transporting patients *Corry, PA*
- Shadowed in ER, OR, PT, and Radiology

Research Assistant in Dr. Song Tan's Lab September 2009-present
- Express and purify proteins *University Park, PA*
- Perform plasmid DNA mutation and purification

Peer Tutor Biology 240W, Functional Anatomy Spring Semester 2010
- Created weekly quizzes *University Park, PA*
- Guided student studying

Work Experience:

Country Fair Summers 2009-2011
- Promoted to shift manager *Erie, PA*
- Trained new employees
- Assisted with inventory

Organizations:

Premed Mentor Program, Vice President August 2009-present
- Mentor freshmen premed students *University Park, PA*
- Direct meetings and logistics
- Recruit mentors and mentees

Alpha Epsilon Delta Premedical Honors Society April 2010-present
- Volunteer at blood drives *University Park, PA*
- Raise money for dance marathon (THON) benefiting pediatric cancer
- Attend medical related presentations