Development of a Transfection System for the Free-Living Amoeba Naegleria fowleri Using the piggyBac Vector

Kati Räsänen
University of South Florida, KRASANEN@MAIL.USF.EDU

Follow this and additional works at: http://scholarcommons.usf.edu/etd
Part of the Public Health Commons

Scholar Commons Citation

This Thesis is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
Development of a Transfection System for the Free-Living Amoeba **Naegleria fowleri** Using the piggyBac Vector

by

Kati E. Räsänen

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Public Health Department of Global Health with a concentration in Global Communicable Diseases College of Public Health University of South Florida

Major Professor: Dennis Kyle, Ph.D.
Thomas Unnasch, Ph.D.
Patricia Maher, Ph.D.

Date of Approval:
March 17, 2017

Keywords: Molecular Cloning, Transposon, Electroporation, Drug Screening

Copyright © 2017, Kati E. Räsänen
Dedication

I would like to dedicate this thesis to my father, Dr. Jukka Räsänen, who reminds me that things will get worse, and supports me when they do.
Acknowledgements

I would like to thank my major professor, Dr. Dennis Kyle, for providing me the opportunity to work with him on a project that represents all I stand for in public health, medicine, and science. I would also like to thank Dr. John Adams and Dr. Shulin Xu, for their contributions and guidance in the development of the piggyBac vector. Thank you also to Dr. Thomas Unnasch, who allowed me to continue work in his lab that contributed to key developments of my project. Lastly, special thanks to Dr. Christopher Rice, Johan Chabanon, and Hassan K. Hassan, for their guidance and assistance.
# Table of Contents

List of Tables ................................................................................................................................. ii

List of Figures ................................................................................................................................. iii

Abstract ........................................................................................................................................ iv

Chapter One: Introduction .............................................................................................................. 1
- Pathogenic Free-living Amoeba .................................................................................................... 1
  - *Naegleria fowleri* .................................................................................................................. 1
- Primary Amebic Meningoencephalitis ........................................................................................ 3
- Transfection Studies .................................................................................................................... 6
- The piggyBac Transfection System ............................................................................................ 7
- Molecular Cloning ........................................................................................................................ 8
- Utilizing piggyBac to Establish Transfection in *N. fowleri* ....................................................... 9

Chapter Two: Methods ................................................................................................................... 11
- Cell Culturing .............................................................................................................................. 11
- Drug Screening for Selectable Markers ....................................................................................... 12
- Generation of Genetic Clones ..................................................................................................... 13
- Primer Design and PCR conditions ............................................................................................ 14
- PCR Cloning ................................................................................................................................ 16
- Restriction Enzyme Digests of Untranslated Regions ................................................................ 18
- Extraction and Purification of Vectors and Inserts ..................................................................... 19
- Cloning Inserts Into piggyBac and Helper Plasmids .................................................................. 19
- Electroporation ............................................................................................................................. 21

Chapter Three: Results ................................................................................................................... 23
- Drug Screening for Selectable Markers ....................................................................................... 23
- Generation of Genetic Clones ..................................................................................................... 24
- Primer Design and PCR conditions ............................................................................................. 25
- PCR Cloning ................................................................................................................................. 26
- Cloning Inserts into piggyBac and Helper Plasmids ................................................................... 27
- Electroporation ............................................................................................................................. 29

Chapter Four: Discussion ............................................................................................................... 30

References ....................................................................................................................................... 35
List of Tables

Table 1: Recipe for Nelson’s Complete Media ................................................................. 11

Table 2. Primers Designed to Amplify N. fowleri Gene Regulatory Regions ......................... 15

Table 3. Reaction Components for Amplification of N. fowleri Inserts ............................... 16 from Genomic Template

Table 4. Thermocycling conditions for Amplification of N. fowleri Inserts .......................... 16 from Genomic Template

Table 5: Reaction Components for PCR Screening of Transformed Recombinant DNA ....... 17

Table 6: Thermocycling conditions for PCR Screens of Recombinant DNA .......................... 17

Table 7: Restriction Enzyme Digest of 5’ UTR Regions in piggyBac and Helper Plasmids ..... 18

Table 8: Restriction Enzyme Digest of 5’ UTR Region Inserts ............................................. 18

Table 9: Ligation Scheme of piggyBac and helper plasmids with T4 Ligase ......................... 20

Table 10: Electroporation Buffer Recipe ............................................................................. 21

Table 11: IC50 Values for Drugs Screened for Use as Selectable Markers ............................ 23

Table 12: Voltages and Time Constants for Electroporated Samples ................................. 29
List of Figures

Figure 1. Lifecycle Stages of *Naegleria fowleri* ......................................................... 2

Figure 2. Lifecycle of *Naegleria fowleri* ................................................................. 2

Figure 3. Distribution of PAM infections in the United States ....................................... 5

Figure 4. New England Biolabs Traditional Cloning Workflow ....................................... 9

Figure 5. Maps of piggyBac and Helper Plasmids provided Dr. John Adams .................... 10

Figure 6: Dose-response Curves Generated for Drugs Screened for Use as Selectable Markers

Figure 7: Observed Growth in Single Cell Cloning for *N. fowleri* ..................................... 24

Figure 8: Amplification of *N. fowleri* Untranslated Regions ........................................ 25

Figure 9: Amplification of *N. fowleri* Untranslated Regions from StrataClone Vector .......... 26

Figure 10: PiggyBac and Helper Plasmid Constructs ..................................................... 28

Figure 11: PiggyBac Construct Designed for Further Testing .......................................... 33
Abstract

*Naegleria fowleri* is a free-living amoeba that causes primary amoebic meningoencephalitis (PAM). In the United States, there are between 0-8 cases of PAM per year, with approximately 98% of cases resulting in death. High case fatality and limited treatment options highlight the need for better understanding of this organism in terms of its biology and pathogenicity. Transfection is a useful tool that allows for the study of gene function, but at present no transfection systems have been established for *N. fowleri*. This study attempts to establish a transfection system for *N. fowleri* using the piggyBac vector, with the hope of eventually using the piggyBac transposon system to identify novel genes related to pathogenicity in *N. fowleri*. To accomplish this, 5’ and 3’ regulatory regions for genes in the *N. fowleri* genome were amplified and inserted into a piggyBac vector with a GFP reporter gene via molecular cloning, and vectors introduced to the amoeba via electroporation. Although no GFP was visualized after transfection, there are several routes for optimization of the transfection system that could be explored. Development of a transfection system could allow for the study of pathogenicity *in vivo*, by either utilizing the transposon system of piggyBac or the expression of reporter genes for visualization of amoeba during the course of infection. Further elucidating *N. fowleri* pathogenicity factors could reveal new drug targets, give new information about the organism’s biology, and help better define an effective treatment regimen to combat PAM.
Chapter One:
Introduction

Pathogenic Free-living Amoeba

Free-living amoebae (FLA) are opportunistic protozoa found ubiquitously and with wide distribution in nature. Habitats include soil, sediment, dust, fresh water lakes, seawater, sewage, drinking water, contact solutions, and air conditioning units among many others. Of the FLA, there are four genera currently known to contain species capable of human infection; *Acanthamoeba, Naegleria, Balamuthia*, and *Sappinia* (Trabelsi et al., 2012). Unlike other parasitic protozoa, pathogens of these genera are able to complete their life cycles without an intermediate human or animal host, feeding on bacteria or other microorganisms in their habitat. Despite their ability to live freely, some species are able to cause fatal central nervous system diseases in humans and animals, with some species of *Acanthamoeba* and *Balamuthia* also infecting sites other than the CNS (Marciano-cabral, 2009). The majority of FLA infections are fatal and recognized postmortem, highlighting the importance of developing both rapid diagnostic methods and treatment. Pathogenesis and mode of infection differs between genera of FLA, requiring individual study (Rocha-azevedo, 2009).

*Naegleria fowleri*

*Naegleria fowleri* is currently the only species of the *Naegleria* genus known to be a human pathogen. Like the other free-living amoeba, this organism is distributed worldwide in
both soil and water, although it is most commonly found in fresh, warm water. The *Naegleria* species are classified as amoeboflagellates, due to their ability to transform into a flagellate in response to changes in ionic strength. Alternatively, the amoeba can also be found in trophozoite form as well as cyst form (to survive adverse environmental conditions) (Jonckheere, 2011) (Figure 1). Feeding, cell division, and infection occurs in the trophozoite stage. Cell division occurs via binary fission with a specific growth rate between .03 - .23 h⁻¹ (Goudot et al., 2012). Trophozoites infect humans and animals by penetrating the nasal mucosa and migrating to the brain, causing primary amebic meningoencephalitis (Figure 2).

**Figure 1:** Lifecycle stages of *Naegleria fowleri*. A) Trophozoite stage B) Cyst Stage C) Flagellate stage. Magnification 1000x (CDC, 2016)

**Figure 2:** Lifecycle of *Naegleria fowleri* (CDC, 2015)
Primary Amebic Meningoencephalitis

The first documented case of an infection caused by *N. fowleri* was in 1965, published in the British Medical Journal by Dr. M. Fowler and Dr. R. F. Carter. This report detailed the case of a nine-year-old boy in South Australia who died from meningitis in 1961, as well as three other cases of similar pathology in 1965. The only pathologically remarkable finding in these cases was the presence of amoebae in the brain and meninges, which were morphologically distinct from *Entamoeba histolytica* (the only amoeba recognized to invade human tissues at the time). Fowler and Carter proposed the causative agent of these meningitis cases to be an *Acanthamoeba* species, based on animal studies performed a few years earlier demonstrating pathogenicity of *Acanthamoeba* (Fowler & Carter, 1965). *Naegleria* would not be identified as the causative agent of PAM until 1968 by Dr. Cecil Butt (Butt et al., 1968), who had also coined the phrase “Primary Amebic Meningoencephalitis” two years prior to describe the meningitis caused by the unidentified amoebae (Butt, 1966).

PAM occurs when water contaminated by *N. fowleri* flagellates enters the nasal cavity. There the amoeba convert to the trophozoite form and attach to the olfactory mucosa, crossing through the cribriform plate via the olfactory nerves into the olfactory bulbs of the brain (Jamerson et al., 2012). Presence of amoeba in the brain prompts an innate immune response, where macrophages will recruit an influx of neutrophils. Release of cytolytic molecules such as hydrolases, phospholipases, neuraminidases, proteases, and phospholipolytic enzymes causes significant nerve and CNS tissue damage, which often results in death of the host (Grace et al., 2015). In addition to immune response, structures on the surface of the amoeba termed “amoebastomes” (John et al., 1984) or “food cups” enable the
amoeba to ingest human tissue. This has earned *N. fowleri* it’s colloquial name, “The Brain-eating Amoeba”.

The incubation period of PAM ranges from 2-15 days. The disease lacks distinctive symptoms, and often presents in a similar way to bacterial or viral meningitis. As such, misdiagnosis is of great concern, and the majority of patients with PAM are diagnosed post-mortem (Rocha-azevedo, 2009). Patients in early stages of infection have shown symptoms like headache, nausea, fever, and fatigue. Later stage infection includes symptoms such as nuchal rigidity, confusion, photophobia, lethargy, seizures, and coma. Diagnosis is made via visualization of amoeba in the cerebral spinal fluid after lumbar puncture, and confirmed by a real-time multiplex PCR assay developed by the CDC (Visvesvara, 2013).

Information regarding appropriate treatment regimens for PAM has been based on case reports or in *vitro* studies. The current drug of choice for treatment is the antifungal agent Amphotericin B in conjunction with other drugs such as fluconazole, miconazole, miltefosine, azithromycin, and rifampin. Due to the rarity of PAM, no clinical trials to evaluate the efficacy of treatment regimens has been performed, and treatment has been based on previous case reports and in *vitro* studies of drug efficacy.

As of 2011, there were 235 cases of PAM reported worldwide (Jonckheere, 2011). Although cases of PAM occur worldwide due to the distribution of *N. fowleri*, the thermophilic nature of the amoeba makes infection most conducive in tropical, subtropical, and temperate zones particularly during the summer months. The majority of case reports come from developed countries in subtropical and temperate zones. Lack of case reports from tropical and developing countries may be due to underreporting, possibly from misdiagnosis or lack of
awareness of the disease (John, 1982). Cases have also been associated with religious and cultural practices such as ritual ablution, purifications, and nasal irrigation with neti pots (Siddiqui & Khan, 2014).

Between 1962-2015 there have been 138 reported cases of PAM in the United States, with three known survivors in this time period. The majority of cases have occurred in young, previously healthy males after recreational water exposure in warm-weather states. Risk factors associated with contracting PAM include swimming and diving in fresh water sources (such as ponds or lakes), submersion in untreated water, and exposure to hot springs (Yoder et al., 2010) (Figure 3). The Centers for Disease Control and Prevention reports between 0-8 cases of PAM in the United States per year (CDC, 2015).

Although there are relatively few cases of PAM annually, the high fatality rate in the United States (approximately 98%) and limited treatment options highlight the need for better understanding of this organism in terms of its biology and pathogenicity.
**Transfection Studies**

Transfection is a useful analytical tool that allows for the study of gene function in both prokaryotic and eukaryotic cells. Transfection utilizes biological, chemical, or physical methods to introduce foreign genetic material (transgenes) into cells. Examples of these methods include lipid-mediated introduction of transgenes, microinjection via microneedles, or formation of temporary pores in cells using electric shocks (electroporation). This introduction can either result in long-term expression of transgenes (stable transfection) or temporary expression where genetic material is not integrated into the genome and is lost due to environmental conditions or cell replication (Kim & Eberwine, 2010). To make transfection stable in a target cell, transgenes are introduced along with a marker gene, encouraging sustained expression even after cell replication. Marker genes can either act as a selectable marker, which grants a cell a beneficial trait (commonly drug resistance) or a marker for screening that causes cells containing transgenes to look different than wild-type (natural or otherwise unaltered) cells (commonly fluorescent proteins) (Liu, 2013). Transgenes can be introduced to cells via a multitude of vectors, among the most common of these being plasmid vectors.

Among the multitude of applications for transgenic technology is gene knockout, a technique that renders a host’s gene inoperative by mutation, allowing for study of gene function when compared to a wild-type host (Liu, 2013). One method of inducing such a mutation is by transposon mediated mutagenesis. Transposons (also called transposable elements) are pieces of genetic material that have the ability to move within or between genomes with the help of an enzyme called a transposase. Transposases will recognize specific sequences in a host’s genome and will cut DNA at this site, inserting the transposon in this
break (Opjnen & Camilli, 2013). With this technique a library of cell strains, each with a single randomly inserted transposon, can be constructed to link the observed phenotypes with the mutated genotype, thereby identifying a gene function. If the DNA sequence of disrupted genes is unknown, transposons can also serve as the starting point for gene sequencing, allowing for identification of unknown sequences (Opjnen & Camilli, 2013).

The piggyBac Transfection System

The piggyBac transposable element has been used for enhancer trapping, gene discovery, and identifying gene function in organisms such as Drosophila and yeast (Balu et al., 2005). First derived from the cabbage looper moth in 1980, piggyBac is a member of the TTAA site-specific class of transposons. It has unique advantages of exclusively inserting into TTAA sequences, inserting and excising precisely, and non-preferential integration into transcriptional units within the genome (leading to truly random mutations, rather than biases for certain regions) (Balu et al., 2005). One of the features that makes piggyBac particularly useful however is the control of the transposon movement by the helper plasmid, which contains the transposase required to move piggyBac. By having the transposon and transposase on different plasmids, movement of the transposable element can be induced or restricted with introduction or removal of the helper plasmid (Fraser et al., 1995). As such, successful utilization of the piggyBac system requires expression of both the piggyBac and helper plasmids. To maximize this expression, piggyBac may be modified via molecular cloning to contain gene regulatory regions specific for the organism of interest.
Molecular Cloning

Molecular cloning is the process by which a DNA fragment of interest (often termed insert) is isolated, amplified, added to a vector (forming recombinant DNA), then transformed into living cells where the recombinant DNA is replicated with each cell division. In this way multiple copies of recombinant DNA can be generated, or cloned (NEB, 2016). This process utilizes two major classes of enzymes: restriction endonucleases (also called restriction enzymes) and ligases. Restriction endonucleases recognize short, palindromic DNA sequences in double-stranded DNA and cut DNA specifically at this site (restriction site). This generates two ends of DNA that are either “blunt” (no overhang of nucleotides) or “sticky” (containing an overhang of nucleotides). Ligases are a class of enzyme that catalyze formation of phosphodiester bonds between the 5’ phosphate of one nucleotide and the 3’ hydroxyl of another, effectively joining together two pieces of DNA (Mullis, 1990).

In traditional cloning, an insert is generated by amplification from source DNA using polymerase chain reaction (PCR); adding restriction sites on the ends of the insert. Both the insert and the desired vector are digested by restriction enzymes, purified (by physical or chemical means), and joined together with a DNA ligase. After recombinant DNA has been generated it can be transformed into bacterial cells and cloned (Tirabassi, 2016) (Figure 4). This is generally done using competent Escherichia coli (E. coli) cells, utilizing the organism’s rapid doubling time (approximately 20-30 minutes) and property of >80% of cells to uptake only one plasmid molecule during transformation (Weston et al., 1979). Copies of recombinant DNA can then be collected from E. coli cells for use in further applications, such as transfection.
Utilizing piggyBac to Establish Transfection in *N. fowleri*

Although there are transfection systems in place for a variety of protozoa, including other species of amoeba, there have been two published works exploring the establishment of a transfection system in *Naegleria*. In 2005, Jeong et al. cloned a gene found in the pseudopodia of *N. fowleri* and chemically transfected it into a non-pathogenic *Naegleria* species, *N. gruberi*, to assess whether the gene was related to pathogenicity (a gain-of function mutation if the new gene could cause the non-pathogen to become pathogenic). This group used a eukaryotic vector pEGFP-C2 containing gene regulatory sequences (promoters- a sequence of DNA that initiates transcription of a gene) from a cytomegalovirus (CMV), and a green fluorescent protein (GFP) screening marker (Jeong et al., 2005). A year later, the same group attempted to optimize expression of the vector by altering the promoters used in the

*Figure 4:* Workflow of traditional molecular cloning, with insert amplification by PCR and restriction enzyme digest of insert fragments from an existing vector as sources of insert DNA (Tirabassi, 2016)
vector, as viral or bacterial promoters are generally not active in protozoan parasites (Song et al., 2006). Promoters used in this study were a CMV promoter, a promoter cloned from the gene nfa1 in N. fowleri, and a promoter for a polyubiquitin gene originating from an Acanthamoeba species.

At present, there have been no studies attempting to establish a transfection system in N. fowleri. The aim of this study is to first identify a selectable marker that can be used for stable transfection, based on drugs currently used as selectable markers in other protozoa. Native host regulatory regions from N. fowleri genes actin, alpha-tubulin, and polyubiquitin (both 5’ untranslated and 3’ untranslated regions) amplified via PCR will be cloned into a piggyBac plasmid and helper plasmid provided by Dr. John Adams of the University of South Florida after removal of existing 5’ and 3’ untranslated regions which are designed for use in Plasmodium falciparum (Figure 5). The modified piggyBac plasmid will then be transiently transfected via electroporation into N. fowleri trophozoites to evaluate expression of the recombinant plasmid using a GFP screening marker.

Figure 5: Plasmid maps of piggyBac and helper plasmids provided by Dr. John Adams of the University of South Florida. PiggyBac plasmid contains an EF1a5’ untranslated region, human dihydrofolate reductase selectable marker, green fluorescent protein marker, and HRP2 3’ untranslated region. Helper plasmid contains CAM and P.c. DHFR 5’ untranslated regions, piggyBac transposes, and HSP86 3’ untranslated region.
Chapter Two:

Methods

Cell Culturing

Prior to culturing, Nelson’s Complete Media (NCM) was prepared according to the recipe outlined in Table 1. Media was filtered using a 1000mL PES .22μm vacuum-driven filter system (Genesee Scientific, Cat. No. 25-229).

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>2mg/L</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>4mg/L</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>142mg/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>136mg/L</td>
</tr>
<tr>
<td>NaCl</td>
<td>120mg/L</td>
</tr>
<tr>
<td>Liver Infusion Broth</td>
<td>1.7g</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.7g</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1L</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>100mL</td>
</tr>
</tbody>
</table>

*Table 1: Nelson’s Complete Media*

*N. fowleri* (Nf69) cells were obtained from frozen stocks of cells stored in 10% DMSO with 10% Penicillin-Streptomycin (10,000 U/mL). Immediately after thawing 1mL of stock culture was added to a 50mL conical tube. 10mL of NCM was slowly added allowing gradual dilution of DMSO to 1%. 5mL of culture was added to each of two T25 non-vented flasks and incubated at 37 °C until cells reached approximately 80-90% confluence.
Flasks were placed on ice for 25-30 minutes to induce cell detachment from flasks. Cells were transferred to a 50mL conical tube and centrifuged at 1620xg in an Eppendorf 5810R for five minutes. Supernatant was removed and cell pellet was resuspended in 1mL of NCM. From this, 10μL was added to 990μL of NCM in a 1.5mL eppendorf tube and mixed. 10μL from the eppendorf tube was placed on a hemocytometer and cells were counted under the 10x microscope objective. From this the culture was diluted to a concentration of 4 x 10^6 cells/mL, and 1mL of culture was added to a T75 non-vented flask containing 10mL of NCM seeding 4 x 10^6 cells per flask. Cultures were passaged twice weekly using this protocol.

**Drug Screening for Selectable Markers**

Drugs chosen for screening were pyrimethamine, WR99210 (malaria dihydrofolate reductase inhibitor), blasticidin, hygromycin, and G418 (geneticin). Blasticidin, hygromycin, and G418 were weighed to starting concentrations of 5mg/mL in cell culture grade water to generate stock solutions. Stock dilutions of drugs were made on a mother plate (clear non-culture treated). 100μL of NCM-I was added to rows 2-6 and 8-12 of a 96-well mother plate. 200μL of the 5mg/mL drug stock was added to column 1 and 7 in the mother plate in duplicate rows, allowing for a total of four drugs to be tested from columns 1-6 and three drugs from columns 7-12. Due to lack of solubility in water, pyrimethamine and WR9920 were first dissolved in DMSO at concentrations of 10mg/mL via sonication. Drugs were further diluted in microfuge tubes with 50μL of drug into 450μL of Nelson’s Incomplete Media (NCM-I) to generate stocks of 1mg/mL drug with 10% DMSO. Drugs were added to mother plates in the same manner as drugs treated with water. Serial dilutions were performed for all drugs by
transferring 100\(\mu\)L from column 1 down to column 6, and from column 7 down to column 12. Plate-to-plate transfers were performed by moving 10\(\mu\)L from the mother plate to the corresponding wells on a daughter plate (clear- culture treated), giving a starting concentrations of 500\(\mu\)g/mL for blasticidin, hygromycin, and G418, and 100\(\mu\)g/mL for pyrimethamine and WR99210. 90\(\mu\)L of \textit{N. fowleri} cell culture at a concentration of 5.5x10^4 was added to each well containing drug on the daughter plate. Daughter plate was incubated for 72 hours at 37 °C. Controls included a blank of only NCM, a positive growth control (90\(\mu\)L cells, 10\(\mu\)L NCM), and a negative growth control (50\(\mu\)L cells, 50\(\mu\)L amphotericin B).

After incubation for 72 hours, plates and reagent were allowed to come to room temperature in a laminar flow hood. At the 72 hour mark, 25\(\mu\)L of CellTiter-Glo® reagent (Promega, Cat. No. G7570) was added to all wells. Plates were shaken on plate shakers for two minutes. After shaking, plates were allowed to sit for ten minutes. Plates were read with Softmax® Pro software using endpoint luminescence settings and analyzed using TriFox analysis software. Biological replicates were performed twice, giving a total of three independent drug screens.

**Generation of Genetic Clones**

Cells were harvested and counted according to procedure outlined above. Cells were diluted to concentrations allowing for each of three conditions in a 96-well plate: .5 cells/well, .25 cells/well, and .125 cells/well. 100\(\mu\)L of culture was added to each well in each of the three conditions. 70% media changes were performed every week for the duration of observation. Confluence estimations were made every three days by visualization.
Based on results, cells from the .25 cells/well plate were chosen for passaging. When cell growth in wells was observed to reach 90-100% confluence, cells from six wells were passaged into a 12-well plate. To determine whether putting cells on ice affected the efficacy of passaging from a well plate, 100μL from six wells of the 96 well plate were first added to 1mL in each of six of the wells of the 12-well plate. 96-well plate wells were washed x2 with 100μL of media. The plate was then wrapped in parafilm and placed on ice for 30 minutes before the process was repeated with the same wells.

After wells in the 12-well plate had reached 90-100% confluence, cells from two wells were added to 2mL NCM in each of two T25 flasks. Wells were washed x2 with 1mL NCM, giving a final volume of 5mL in the T25 flasks. Cultures were passaged as outlined above until they reached 90-100% confluence in a T75 flask. Frozen stocks of cells were generated by harvesting cells and diluting to the seeding density concentration of 4 x 10^6 cells/mL, then adding penicillin/streptomycin to a concentration of 10%, and slowly adding DMSO to a concentration of 10%. Aliquots of 1mL were added to CryoTube™ vials and frozen in an ethanol bath before storage at -80 °C.

**Primer Design and PCR conditions**

PCR targets were identified from sequences acquired from Eukaryotic Pathogen Database Resources (EuPathDB). Amplicon lengths were chosen based on recommendations by Dr. Shulin Xu; approximately 1500-2000 for 5’ UTRs and 1000-1500 for 3’ UTRs. Primers were designed to amplify the 5’UTR and 3’ UTR regions of *Naegleria fowleri* actin 2 (geneID NF0013260), α-tubulin n-acetyltransferase (geneID NF0045270_2), and polyubiquitin 5 (geneID
NF0029300_1). Primers were designed using Primer3Plus bioinformatics software (Weston et al., 2014). Prior to order, nucleotides for restriction enzyme cut sites were added to the 5’ end of primers. For 5’ UTRs, EcoRI (GAATTC) was added. For 3’ UTRs HindIII (AAGCTT) was added. Primers were also designed according maps of the pL-BACII-EDGH (piggyBac) and pCDTH (helper) plasmids provided by Dr. John Adams flanking the 5’ UTR and 3’ UTR insert regions to verify presence of insert as well as directionality. Primers were ordered from Integrated DNA Technologies® (Table 2).

Table 2: Primers Designed to Amplify N. fowleri Gene Regulatory Regions

<table>
<thead>
<tr>
<th>Primer Target</th>
<th>5’ – 3’ Sequence (Forward)</th>
<th>5’ – 3’ Sequence (Forward)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin 5’ UTR</td>
<td>GAATTCGAAAGGATATTTGGGCTTCACT</td>
<td>GAATTCGTTGTGGTGGTTCTTCTACGACG</td>
<td>1751</td>
</tr>
<tr>
<td>Actin 3’ UTR</td>
<td>AAGCTTTTGGATGACACATGTAAGCT</td>
<td>AAGCTTTTGGATGACACATGTAAGCT</td>
<td>1075</td>
</tr>
<tr>
<td>α-tubulin 5’ UTR</td>
<td>GAATTCGAAATGAGATAGGCTCAGTG</td>
<td>GAATTCGTTGAAAGGAGAAGCAGGTGAGAAG</td>
<td>1421</td>
</tr>
<tr>
<td>α-tubulin 3’ UTR</td>
<td>AAGCTTTGCAATGAACTCGAATACGACG</td>
<td>AAGCTTTGCAATGAACTCGAATACGACG</td>
<td>1161</td>
</tr>
<tr>
<td>Polyubiquitin 5’ UTR</td>
<td>GAATTCTGGAAATGAGATAGGCTCAGTG</td>
<td>GAATTCGTTGAAAGGAGAAGCAGGTGAGAAG</td>
<td>1563</td>
</tr>
<tr>
<td>Polyubiquitin 3’ UTR</td>
<td>AAGCTTTGCAATGAACTCGAATACGACG</td>
<td>AAGCTTTGCAATGAACTCGAATACGACG</td>
<td>1400</td>
</tr>
<tr>
<td>piggyBac 5’ UTR Flanking</td>
<td>TGAGCTGCACTGTTTGGGATGACACG</td>
<td>GCTCTGGGAGACAGCGGACG</td>
<td>-</td>
</tr>
<tr>
<td>piggyBac 3’ UTR Flanking</td>
<td>TGAGCTGCACTGTTTGGGATGACACG</td>
<td>GCTCTGGGAGACAGCGGACG</td>
<td>-</td>
</tr>
<tr>
<td>Helper 5’ UTR Flanking</td>
<td>ACTGTCAATTCCTCCCAAACAGCG</td>
<td>GCTCTGGGAGACAGCGGACG</td>
<td>-</td>
</tr>
<tr>
<td>Helper 3’ UTR Flanking</td>
<td>ACTGTCAATTCCTCCCAAACAGCG</td>
<td>GCTCTGGGAGACAGCGGACG</td>
<td>-</td>
</tr>
</tbody>
</table>

Template genomic DNA was extracted from N. fowleri trophozoites using the Promega Wizard® SV Genomic DNA Purification System following manufacturer’s protocol with a starting cell concentration of 4 x 10^6 cells/mL (Promega, 2012). Due to addition of adenine residues to the 3’ end of DNA molecules, taq-based polymerase OneTaq® Hotstart DNA polymerase from
New England Biolabs (Cat. No. M0481S) was used for amplification of inserts from genomic DNA. PCR components and reaction conditions are outlined in Tables 3 and 4 respectively.

**Table 3:** Reaction Components for Amplification of *N. fowleri* Inserts from Genomic Template

<table>
<thead>
<tr>
<th>Component</th>
<th>50μL Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Q5 OneTaq Standard Reaction Buffer</td>
<td>10μL</td>
<td>1X</td>
</tr>
<tr>
<td>10 mM dNTP’s</td>
<td>1μL</td>
<td>200μM</td>
</tr>
<tr>
<td>10 μM Forward Primer</td>
<td>2.5μL</td>
<td>0.2μM</td>
</tr>
<tr>
<td>10 μM Reverse Primer</td>
<td>2.5μL</td>
<td>0.2μM</td>
</tr>
<tr>
<td>OneTaq Hot Start DNA Polymerase</td>
<td>.25μL</td>
<td>1.25U/Rxn</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1μL</td>
<td>118.8ng</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>32.75</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4:** Thermocycling conditions for Amplification of *N. fowleri* Inserts from Genomic Template

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98</td>
<td>30s</td>
</tr>
<tr>
<td>x30 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>98</td>
<td>8s</td>
</tr>
<tr>
<td>Annealing</td>
<td>57</td>
<td>10s</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1m 42s</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5m</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

Amplicons were generated in a MyCycler™ thermocycler. Amplicons were visualized by running 10μL of PCR product on a 1.2% agarose gel with 3.5μL SYBR® Gold stain (ThermoFisher, Cat. No. S11494) at 110V for 45 minutes alongside a 1kb ladder (NEB, Cat. No. N3232S).

**PCR Cloning**

To clone inserts, amplified inserts were ligated into the Agilent Strataclone PCR Cloning Vector following the procedure outlined in the StrataClone PCR Cloning Kit (Agilent, 2015).
Transformed *E. coli* cells were spread in volumes of 100μL on Luria-Bertani (LB) plates (Difco™ LB Agar, deionized water) supplemented with ampicillin (100mg/L), and 100μL 2% X-gal spread on each plate. Colonies were selected based on blue-white screening. Selected colonies, as well as *E. coli* containing piggyBac and helper plasmids, were growth in 3mL LB liquid media (Difco™ LB Broth, deionized water) supplemented with ampicillin (100mg/L) overnight in a Forma Orbital shaker at 37 °C. Inserts were verified by PCR screen using forward and reverse primers used in initial insert amplification following the PCR reaction set up outlined in Table 5.

**Table 5:** Reaction Components for PCR Screening of Transformed Recombinant DNA

<table>
<thead>
<tr>
<th>Component</th>
<th>20μL Reaction</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phusion High-fidelity PCR Master Mix</td>
<td>10μL</td>
<td>1X</td>
</tr>
<tr>
<td>10 μM Forward Primer</td>
<td>1μL</td>
<td>0.5μM</td>
</tr>
<tr>
<td>10 μM Reverse Primer</td>
<td>1μL</td>
<td>0.5μM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>2μL</td>
<td>-</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>To 20μL</td>
<td></td>
</tr>
</tbody>
</table>

Template DNA was extracted by first aliquoting 100μL of overnight growth liquid culture into a 1.5mL Eppendorf tube. Tubes were then placed in a heating block and heated at 100°C for ten minutes. Cells were then pelleted by centrifugation at 13k rpm for five minutes in a 5424 centrifuge. Supernatant containing DNA was added to PCR reaction mixtures. PCR was run according to conditions outlined in Table 6.

**Table 6:** Thermocycling conditions for PCR Screens of Recombinant DNA

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>30s</td>
</tr>
<tr>
<td>x20 cycles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>8s</td>
</tr>
<tr>
<td>Annealing</td>
<td>57</td>
<td>10s</td>
</tr>
<tr>
<td>Extension</td>
<td>72</td>
<td>1m 42s</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72</td>
<td>5m</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>
Plasmids were extracted from cells using QIAprep® Spin Miniprep Kit following manufacturer’s instructions (Qaigen, 2015).

**Restriction Enzyme Digests of Untranslated Regions**

Treatment for cloning of 5’ and 3’ UTRs was the same for both piggyBac and helper plasmids. To prepare vectors for ligation, extracted plasmids were digested according to reaction components listed in Table 7, added sequentially as listed in .6mL eppendorf tubes.

**Table 7:** Restriction Enzyme Digest of 5’ UTR Regions in piggyBac and Helper Plasmids

<table>
<thead>
<tr>
<th>Component</th>
<th>piggyBac</th>
<th>Helper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>To 100μL</td>
<td>To 100μL</td>
</tr>
<tr>
<td>10X Buffer EcoRI</td>
<td>10μL</td>
<td>10μL</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>5μg</td>
<td>5μg</td>
</tr>
<tr>
<td>EcoRI Enzyme (10U/μL)</td>
<td>10μL</td>
<td>10μL</td>
</tr>
</tbody>
</table>

Reactions were incubated at 37 °C for 90 minutes. After 60 minutes, 1μL of calf intestinal alkaline phosphatase (CIAP, Invitrogen, Cat. No. 18009-027) was added to each reaction before incubation was resumed for the remaining 30 minutes. After incubation, reactions were heat inactivated at 65 °C for 20 minutes.

Inserts were prepared by first digesting StrataClone plasmids according to reaction setup listed in Table 8, added sequentially as listed.

**Table 8:** Restriction Enzyme Digest of 5’ Untranslated Region Inserts

<table>
<thead>
<tr>
<th>Component</th>
<th>Actin</th>
<th>α-tubulin</th>
<th>Polyubiquitin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-free water</td>
<td>To 50μL</td>
<td>To 50μL</td>
<td>To 50μL</td>
</tr>
<tr>
<td>10X Buffer EcoRI</td>
<td>5μL</td>
<td>5μL</td>
<td>5μL</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>10μg</td>
<td>10μg</td>
<td>10μg</td>
</tr>
<tr>
<td>EcoRI Enzyme (10U/μL)</td>
<td>10μL</td>
<td>10μL</td>
<td>10μL</td>
</tr>
</tbody>
</table>
Reactions were incubated at 37 °C for 90 minutes. After incubation, reactions were heat inactivated at 65 °C for 20 minutes.

**Extraction and Purification of Vectors and Inserts**

For 5’ UTR regions, vectors were extracted and purified via phenol/chloroform extraction followed by ethanol precipitation. Equal volumes of phenol-chloroform (Sigma, CAS No. 136112-00-0) were added to each of the reaction tubes and vortexed for 45 seconds. Tubes were spun in an Eppendorf 5424 centrifuge for five minutes at 13604xg. The aqueous DNA layer was removed from the tube by pipette and placed in a new .6mL eppendorf tube. This process was repeated once more with phenol, then again with pure chloroform. After extraction, 1/10 volume of Na-acetate was added to each tube, followed by x3 volumes of 100% ethanol. Tubes were placed at -80 °C for 30 minutes to precipitate DNA. Tubes were centrifuged for 30 minutes at 13604xg at 4°C. Supernatant was removed and DNA pellet was washed with cold 70% ethanol. Tubes were centrifuged at room temperature for five minutes at 13k rpm. Pellets were dried by inverting over a kimwipe for ten minutes before being redissolved in 1X TE. Samples were stored at -20 °C. For cloning of 3’ UTRs, Vectors were purified by gel extraction using Promega’s Wizard SV Gel and PCR Clean-Up System following manufacturer’s protocol (Promega, 2010) on a 1.2% agarose gel run at 120V for 60 minutes.

Inserts for 5’ and 3’ UTRs were extracted and purified by gel purification (1.2% agarose, 120V, 60 minutes).

**Cloning Inserts into piggyBac and Helper Plasmids**

Vectors and inserts were ligated using NEB T4 DNA Ligase as outlined in Table 9.
Volumes used for vectors and controls were calculated using NEBiocalculator using a starting vector mass of 50ng. After ligation, 3μL of product was transformed into Subcloning Efficiency™ DH5α™ Competent Cells following manufacturer’s guidelines (Invitrogen, 2006). Cells were allowed to recover in 250μL S.O.C medium (Invitrogen, Cat. No. 15544-034). 100μL transformed cells were spread onto LB+Amp selective media plates and incubated overnight at 37 °C.

25 colonies from each vector + insert transformation plate were screened at a time. LB+Amp screening plates were designed with 5x5cm grids drawn onto the bottom of each plate with each square labeled 1-25. Corresponding culture tubes containing 3mL LB+Amp were labeled accordingly. Colonies were picked from transformation plates with a sterile pipette tip, streaked onto one square of the screening plate, then used to inoculate the corresponding culture tube. Plates and tubes were incubated overnight at 37 °C. PCR screening for inserts in the piggyBac and helper plasmids was performed as described above (PCR cloning, Table 5, Table 6). Forward and reverse primers used for inserts in the piggyBac and helper plasmids were insert specific forward and piggyBac/helper UTR flanking region reverse respectively to screen for insert directionality. Positive directionality hits were regrown using screening plate cells in 3mL LB+Amp culture tubes incubated overnight at 37 °C. From this 500μL of cells were
added to 500μL 50% glycerol and frozen to generate stock cultures, and the remainder used for plasmid extraction as described above. Plasmid DNA was sequenced via overnight sequencing by Eurofins Genomics with UTR flanking region forward primers for insert verification.

After successful ligation and cloning of 5’ UTRs into piggyBac and helper plasmids, 3’ UTRs were inserted. The same methodology was used to insert these regions, with the exception of restriction enzyme used. Plasmids and inserts were digested with HindIII in 10X Buffer R. Reactions were heat-inactivated at 80 °C rather than 65 °C.

**Electroporation**

Extracted piggyBac plasmids containing *N. fowleri* specific 5’ UTR regions (Actin, α-tubulin, and polyubiquitin) as well as a piggyBac plasmid with the 5’UTR region removed were prepared and sterilized for electroporation via phenol extraction and ethanol precipitation to a concentration of approximately 5μg/μL. Electroporation buffer was prepared with components listed in Table 10.

**Table 10: Electroporation Buffer Recipe**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>8.94g/L</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt; x 2H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>0.02g/L</td>
</tr>
<tr>
<td>Na&lt;sub&gt;2&lt;/sub&gt;HPO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>1.30g/L</td>
</tr>
<tr>
<td>KH&lt;sub&gt;2&lt;/sub&gt;PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>.12g/L</td>
</tr>
<tr>
<td>HEPES</td>
<td>5.96g/L</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.76g/L</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt; x 6H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>1.02g/L</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>1L</td>
</tr>
</tbody>
</table>

Buffer was brought to a pH of 7.8 prior to vacuum filter sterilization. Prior to electroporation, Gene Pulser® .2mm electroporation cuvettes, electroporation buffer, and
sterile DNA were placed on ice. 1mL NCM media was aliquoted into each of 12 wells on a 12-well plate, and pre-warmed in a 37 °C incubator. Cells were harvested in late-log phase from flasks using methods described above (cell passaging). After centrifugation, cells were resuspended in 10mL cold electroporation buffer and counted. Cells were pelleted again and resuspended to a working stock of 2.5 x 10^6 cells/mL in electroporation buffer and placed on ice. 1mL of cell stock solution was aliquoted to 50μL conical tubes for each of four reaction condition, with an additional tube for a control sample without DNA. 20ug of DNA for each of the four experimental conditions was added conical tubes and swirled to mix. 400μL of mixtures, as well as a control sample of cells without addition of DNA, were transferred to .2cm cuvettes. Program used to deliver shocks was based on a protocol for transfection of Leishmania, as no published protocol exists for *N. fowleri* (Beverzey & Clayton, 1993). BioRad Gene Pulser Xcell™ was programed to deliver exponential decay pulses at .1kV with a capacitance of 500μF. After pulse delivery, cuvettes were placed on ice for five minutes, then pulsed a second time. Cuvettes were again placed on ice for ten minutes before 200μL aliquots were transferred in duplicate to a 12 well plate. 200μL of stock cells was plated in the remaining two wells to serve as a non-electroporated control. The plate was incubated overnight at 37 °C prior to visualization of cells with a Zeiss microscope.
Chapter Three:

Results

Drug Screening for Selectable Markers

At a starting concentration of 500μg/mL, TriFox analysis software was unable to generate an IC₅₀ value for G418. A reliable IC₅₀ value was not pursued, as it was shown to have little efficacy in the application of the study. Average IC₅₀ values of biological replicates, given in μg and μM, are summarized in Table 11 for the remainder of drugs. IC₅₀ of pyrimethamine was found to be 63.6μM (15.8μg), WR99210 82.9μM (32.7μg), Blasticidin 62.6μM (28.7μg), and hygromycin 333.0μM (175.6μg). Dose-response curves generated for each drug can be seen in Figure 6.

<table>
<thead>
<tr>
<th>Drug Name</th>
<th>IC₅₀(μg)</th>
<th>IC₉₀(μg)</th>
<th>IC₅₀(μM)</th>
<th>IC₉₀(μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyrimethamine</td>
<td>15.8</td>
<td>43.0</td>
<td>63.6</td>
<td>173.0</td>
</tr>
<tr>
<td>WR99210</td>
<td>32.7</td>
<td>57.2</td>
<td>82.9</td>
<td>144.9</td>
</tr>
<tr>
<td>Blasticidin</td>
<td>28.7</td>
<td>168.9</td>
<td>62.6</td>
<td>368.3</td>
</tr>
<tr>
<td>Hygromycin</td>
<td>175.6</td>
<td>&gt;500</td>
<td>333.0</td>
<td>-</td>
</tr>
<tr>
<td>G418</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 11: IC₅₀ Values for Drugs Screened for Use as Selectable Markers
Generation of Genetic Clones

After one week of growth in a 96-well plate growing cells were visualized in wells as per Figure 7. The .5 cell/well plate showed a total of 35 wells with growth (36.5% of wells). The .25 cells/well plate showed growth in 20 wells (20.8% of wells). The .125 cells/well plate showed growth in a total of 9 wells (9.4% of wells).

Figure 6: Dose-response curves for single biological replicate screens of pyrimethamine (A), WR99210 (B), Blasticidin (C) and Hygromycin (D). X-axis displays drug concentration on a logarithmic scale in units of µM/mL. Y-axis shows growth taking into account control wells as measured by luminescence output (relative light units).

Figure 7: Observed growth in wells for single cell cloning of N. fowleri using three dilution conditions: .5 cells/well, .25 cells/well, and .125 cells/well. Wells showing growth highlighted in green.
Variation in subjective growth rate existed between wells in the .5 cells/well plate, but not in the .25 cells/well and .125 cells/well plates. It took 10 days for the majority of wells with cells to reach 100% confluence, indicating necessity for passage to a 12-well plate. Cells passaged after the plate had been placed on ice reached 100% confluence one day faster than those which had not been placed on ice (three days and four days respectively). After passage to a T25, it took four days for cells placed on ice to reach 100% confluence, and three more after passage to a T75. Cloning process was determined to take approximately three weeks from seeding of a 96-well plate to a fully confluent T75 flask.

**Primer Design and PCR Conditions**

After trials with several polymerases, amplification of *N. fowleri* UTRs was best achieved using Phusion® High-fidelity PCR Master Mix (ThermoFisher Cat. No. F531S) and OneTaq® Hotstart DNA polymerase (Figure 8).

![Image](image.png)

**Figure 8:** 1.2% agarose gel run at 110V for 45 minutes showing amplicons of *N. fowleri* untranslated regions. Lanes 1, 8, and 15 contain a 1kb DNA ladder. Lanes 2-7 contain amplicons from PCR using Phusion® High-fidelity PCR Master Mix. Lanes 9-14 contain amplicons from PCR using OneTaq® Hotstart DNA polymerase. Lanes 2 and 9 contain amplicons of Actin 5’ UTR reaction, lanes 3 and 10 contain Actin 3’ UTR, lanes 4 and 11 contain α-tubulin 5’ UTR, lanes 5 and 12 contain α-tubulin 3’ UTR, lanes 6 and 13 contain polyubiquitin 5’ UTR, and lanes 7 and 14 contain amplicons from polyubiquitin 3’ UTR.
α-tubulin 3’ UTR was not successfully amplified, however amplification of this region was not pursued further due to presence of a HindIII cut site within the sequence.

**PCR Cloning**

All transformation plates showed both blue and white *E. coli* colonies, with the majority of colonies white or light blue. Transformation of the StrataClone SoloPack Competent Cells was evaluated by transformation of a pUC18 control plasmid. >50 colonies were observed on the transformation plate, indicating a transformation efficiency of greater than $5 \times 10^7$ cfu/ug pUC18 DNA. Kit reagent integrity was evaluated by ligation of StrataClone Control Insert with the StrataClone Vector Mix. As expected, >100 cfu were observed with greater than 97% white colonies. Seven colonies from each transformation plate were screened for presence of insert before miniprep (Figure 9), although these were not sent for sequencing. All colonies screened showed bands at appropriate lengths for their respective inserts (Table 2), with the exception of polyubiquitin 3’ UTR colony #1 which showed no amplification of insert.

![Figure 9](image)

*Figure 9:* 1.2% agarose gel run at 110V for 45 minutes showing amplicons of *N. fowleri* untranslated regions. Lanes 1 and 20 in both rows contain 1kb ladder. Lanes 2-8 row 1 contain samples of amplified actin 5’ UTR, lanes 9-15 contain actin 3’ UTR, and lanes 16-19 contain α-tubulin 5’ UTR. Row 2 lanes 2-4 contain the remainder of α-tubulin 5’ UTR samples, lanes 5-11 contain polyubiquitin 5’ UTR, and lanes 12-19 contain polyubiquitin 3’ UTR.
Cloning Inserts into piggyBac and Helper Plasmids

Digested plasmids and inserts were visualized on a FisherBiotech™ Ultraviolet Transilluminator (Cat. No. FB-TIV-88A) before excision for gel extraction. After digestion with EcoRI, inserts showed two bands of expected lengths indicating removal of the insert from the StrataClone vector, with the exception of polyubiquitin 5’ UTR which showed a band at approximately 800bp. Phenol extraction of piggyBac and helper plasmids resulted in DNA in appropriate concentrations to continue with ligation.

After transformation of ligation reactions of piggyBac and helper 5’ UTR inserts, all transformation plates showed growth between 25-100 colonies. All colonies grew when restreaked on screening plates, as well as in LB+Amp liquid media. Positive directionality was confirmed for at least one transformed colony per insert and plasmid combination by screening with the flanking forward primer and insert specific reverse primer. These colonies were chosen for plasmid extraction and sequencing.

For piggyBac 5’ UTR regions sequenced, all showed high numbers of identities matched when sequenced regions were aligned via NCBI Basic Local Alignment Search Tool (BLAST) with sequences obtained from EuPathDB. For the first 250bp, actin 5’ UTR showed a 99% match, α-tubulin 5’ UTR showed a 100% match, and polyubiquitin showed a 99% match (with actin and polyubiquitin each showing a single point mutation from adenine to guanine). Helper plasmid 5’ UTR regions also showed high identity matches for the first 250bp (Actin 5’ 99%, α-tubulin 5’ 100%, polyubiquitin 100%).

PiggyBac 3’ UTRs showed 99% identity match when comparing the first 250bp of each insert in each construct with EuPathDB sequences. Helper plasmid 3’ UTRs for the first 250bp
also showed high identity match percentages: 99% for both actin and polyubiquitin 3’ UTRs.

PCR screening results as well as sequencing data indicate completion of 12 proposed constructs (Figure 10).

Figure 10: PiggyBac and Helper plasmids constructs. PiggyBac plasmids (A–f) and helper plasmids (G–L) contain combinations of actin, α–tubulin, and polyubiquitin 5’ untranslated regions. 3’ untranslated regions are combinations of actin and polyubiquitin untranslated regions.
Electroporation

Prior to harvesting, amoeba were observed to be approximately 80% confluent in the T75 flask. No extracellular debris was observed. Applied voltages and time constants for each sample cuvette are summarized in Table 12.

Table 12: Voltages and Time Constants for Electroporated Samples

<table>
<thead>
<tr>
<th>Sample (5’ Insert)</th>
<th>Pulse 1</th>
<th></th>
<th>Pulse 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Applied Volts (V)</td>
<td>Time Constant (ms)</td>
<td>Applied Volts (V)</td>
<td>Time Constant (ms)</td>
</tr>
<tr>
<td>Actin</td>
<td>98</td>
<td>4.0</td>
<td>98</td>
<td>4.5</td>
</tr>
<tr>
<td>α-tubulin</td>
<td>99</td>
<td>3.0</td>
<td>99</td>
<td>5.5</td>
</tr>
<tr>
<td>Polyubiquitin</td>
<td>99</td>
<td>3.0</td>
<td>99</td>
<td>5.5</td>
</tr>
<tr>
<td>piggyBac, no 5’</td>
<td>98</td>
<td>4.5</td>
<td>100</td>
<td>5.5</td>
</tr>
<tr>
<td>Control (No DNA)</td>
<td>98</td>
<td>4.0</td>
<td>98</td>
<td>4.0</td>
</tr>
</tbody>
</table>

24 hours after electroporation, non-electroporated controls showed healthy cells with no extracellular debris as approximately 30% confluence. All other wells, containing cells that had undergone electroporation, showed a mix of cell health. Many cells appeared unhealthy, being of small size with few visual vacuoles. Some trophozoites appeared to have encysted. Additionally, significant amounts of what appeared to be cell debris was present in the media surrounding cells. There did not seem to be a significant difference in cell appearance between duplicate samples, nor between sample types. Presence of DNA in the electroporation reaction did not appear to influence cell health at this stage. GFP emission was not detected when visualized with a Zeiss microscope. Electroporated cells reached 95-100% confluence in five days, whereas non-electroporated cells reached confluence in three days.
Chapter Four:

Discussion

Although pyrimethamine had the lowest IC$_{50}$ value of drugs screened, WR99210 was chosen as the selectable marker. Both pyrimethamine and WR99210 target dihydrofolate reductase (DHFR), however the antiparasitic effect of WR99210 has been shown to be reversed in *Plasmodium falciparum* when a human dihydrofolate reductase (hDHFR) is introduced to the parasite (Fidock & Wellems, 1997). This implies that the drug is specific for the parasite enzyme, and introduction of hDHFR to amoeba via piggyBac may confer resistance to WR99210. Considering the presence of hDHFR within the piggyBac plasmid obtained from Dr. John Adams, WR99210 will be used to select for transfectants in future work. Nevertheless, elucidation of other drugs shown to have efficacy against *N. fowleri* could be useful in other applications that require a selectable marker, such as cotransfections.

In generating genetic clones, estimations in growth rate were limited to subjective observation as living cells could not be quantified over a long period of time. Additionally, distribution of cells within a well limited the accuracy of confluence estimations, as cells tend to congregate around the edges of a flat-bottom well. Nevertheless, there appeared to be variations in growth rate between individual wells on a plate, indicating that more than one initial cell had been seeded in a single well. Because this variation seemed to decrease as the initial seeding stock was diluted, the .125 cell/well dilution condition was deemed the most
ideal for future generation of clones. Once piggyBac is transfected into cells, cell lines of amoeba expressing GFP would need to be obtained via the dilution scheme outlined above so that cultures of clones all expressing GFP could be obtained. Establishing methodology for generating genetic clones is not only useful for obtaining a pure culture of GFP transfectants, but also has application for transposon generated clones. Once a mutant is made via transposon mediated mutagenesis, a pure culture of clones all containing the same mutation could be used for phenotype screening or other gene function analyses.

Promoter choice in transfection is dependent on host cells, with cell specific promoters being a common route for expression of foreign genes. In this study untranslated regions approximately 1.5kb upstream of genes necessary for cell function were chosen in the hopes of capturing promoter regions of those genes, so that GFP would be expressed by *N. fowleri*. After digestion of polyubiquitin 5’ untranslated regions, the presence of an EcoRI cutsite was discovered within the insert sequence, leading to a shorter product length than expected. Because the site lay approximately halfway through the target sequence, the cut was not visualized via gel electrophoresis, due to the size similarity of fragments. Construction of plasmids with this 5’ untranslated region was continued, however, as the sequence inserted may contain the necessary promoter sequences despite truncation. Because inserts are not comprised of coding sequences, and because the sequence required for proper gene regulation is unknown, point mutations present in insert sequences when compared to sequences from EuPathDb are not likely to affect functionality of regulatory regions. The extent to which the genome of *N. fowleri* has been annotated is also limited.
Presence of living amoeba after electroporation indicates that the voltage used was not too high as to cause complete cell death, although there were signs of cell stress and cell death. Both presence of cysts and extracellular material (possibly bacteria, which the amoeba may harbor and release) indicate poor cell health immediately following electroporation. No GFP expression was visualized on initial electroporation. As this study was exploratory in methodology, the next steps would be an attempt to optimize electroporation. In this trial no drug pressure was applied to parasites following electroporation. In a second round of electroporation, drug pressure using WR9920 should be applied to parasites both immediately and after 24 hours of recovery. A recovery period without drug pressure is recommended in many transfection protocols (Beverzey & Clayton, 1993). However, due to the relative speed of \textit{N. fowleri} cell division, a second condition of immediate drug pressure should be explored as well. Beyond drug pressure, optimization of voltage applied to cells, time constant of pulse, and number of pulses may also be influential in successful electroporation. To evaluate whether genetic material was transfected into cells at all, a DNA extraction and sequencing may be performed in an attempt to detect any of the introduced plasmid DNA. Other methods of transfection that may be attempted if electroporation continues to be unsuccessful. Some reagent-based methods, including lipid-mediated gene delivery, calcium phosphate gene introduction, and polymer transfection, may be explored. Method selection depends on a variety of factors including sensitivity of cells, cell type, and media conditions.

After failure to visualize GFP expression, the GFP sequence in the piggyBac was sequenced and analyzed via BLAST analysis. The sequence had 100% match to other GFP sequences present in a multitude of cloning vectors, with the exception of six base-pairs not
present in the piggyBac vector: ATGAGT. It is possible that this sequence was removed from piggyBac in previous manipulations of the plasmid. Removal of this sequence means the removal of the start codon ATG, which may account for lack of GFP expression, as there would be no start codon to initiate translation of the protein.

If stable transfection of *N. fowleri* is achieved, future studies utilizing piggyBac in *N. fowleri* could take advantage of both the transposon mediated mutagenesis system, as well as expression of screening markers. For example, substitution of GFP for a luciferase gene may be used for quantification of parasite load in *in vivo* mouse models, allow for tracking of amoeba throughout the course of infection, explore infectious dose of amoeba, or visualize a treatment response in drug trials. In order to remove GFP, further work must be performed to both introduce a restriction enzyme cut site in piggyBac that can be used to ligate a new marker gene, and the new marker must be cloned into the vector. For this, a piggyBac vector with both 5’ and 3’ untranslated regions removed via restriction enzyme digests (generated in this study) could be used, as this vector could be used to test different promoter regions and construct vectors with different markers with ease (Figure 11).

![Proposed piggyBac plasmid](image)

*Figure 11*: Proposed piggyBac plasmid that could be used for further testing of successful promoters allow for insertion of various screening markers. NheI cut site must be introduced to existing constructs.
Primary amebic meningoencephalitis is a disease with rapid onset and rapid mortality for afflicted persons, usually otherwise healthy children and young adults. Development of a transfection system could allow for the study of pathogenicity in vivo, by introducing knockout mutations via transposon mediated mutagenesis or by allowing for visualization and quantification of amoeba in mouse models. Further elucidating N. fowleri pathogenicity factors could reveal new drug targets, give new information about the organism’s biology, and help better define an effective treatment regimen.
References


dynamic of *Naegleria fowleri* in a microbial freshwater biofilm. Water research, 46(13), 3958-3966.


