Development of Tools for Stable Transfection in the Human Filarial Parasite Brugia malayi via the piggyBac transposon system

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Development of Tools for Stable Transfection in the Human Filarial Parasite *Brugia malayi* via the *piggyBac* transposon system

by

Johan Chabanon

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

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Dedication

To my grandpa Freddy, for nurturing my love of science and medicine. To my grandma Evelyn, whose support I can always count on. To my brother/cousin Solal, who always keeps me motivated. To my siblings Ben, Nina, and Maya, who make me look forward to going home everytime. And most importantly, to my mother, for being the only one I can always depend on.
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Thank you to Dr. Canhui Liu for helping me with the biolistic transfection and answering so many of my questions. I would like to thank all my lab mates that I’ve had—Kathryn, Juan, Kati, and Kristi—who have made lab so much better in the tough times. I would especially like to thank Hassan Hassan for his limitless patience in fielding all my questions and helping me find my way around the lab.
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Abstract

*Brugia malayi* is one of three species of nematode known to cause lymphatic filariasis (LF) in humans. LF infects over 120 million people, causing debilitating disease. Various global programs have been launched in the past 20 years to eliminate LF. These programs have greatly scaled up the resources and efforts allocated to halting the transmission and reducing disease burden. Only a few drugs are used to treat LF, and resistance is thus a devastating possibility. Research aimed at identifying new drug targets could therefore prove essential in elimination of LF.

Genetic manipulation of *B. malayi* has been limited to transient transfections. A transfection system allowing for stable integration of transgenic sequences into the nuclear genome of this parasite would enable more robust studies that could lead to identification of novel drug targets and vaccine candidates. The *piggyBac (pB)* transposon system has been successfully applied to develop a stable transfection system in a variety of species. This system involves two plasmids, a helper and a donor. The donor plasmid contains the target DNA and a selectable marker flanked by specific inverted terminal repeat (ITR) regions. The helper plasmid expresses the *pB* transposase that will catalyze the precise integration of any DNA report tools necessary to adapt the *pB* system in *B. malayi*. Three versions of the donor plasmid were constructed, each containing a *Gaussia* Luciferase (GLuc) selectable marker but differing only by the fluorescent protein expressed. The construct containing a YFP gene was used to transf ect embryos via biolistics to test whether YFP and GLuc are expressed.
Chapter One: Introduction

Lymphatic filariasis – the disease and its control

Filarial parasitic nematodes threaten hundreds of millions of people worldwide, mostly in developing areas. Lymphatic filariasis (LF), caused by *Wuchereria bancrofti* and two *Brugia* species, is a neglected tropical disease that commonly leads to elephantiasis, hydrocele, and lymphedema (WHO, 2017).

Filarial parasites have a complex life cycle, involving stages in both a mosquito vector and mammalian host. The infectious third-stage filarial larvae (L3) are injected into a person by an infected mosquito during a blood meal. These larvae develop into adults that reside in the lymphatics, most commonly in the extremities and male genitalia. The adults will produce an average of 10 thousand microfilariae a day that migrate from the lymph to the blood channels where they can be ingested by a mosquito, facilitated by a periodicity that causes peak concentrations to coincide with the local feeding habits of the mosquito vector. Once inside a mosquito, the microfilariae molt twice to become L3 larvae and can infect another person (CDC DPDx, 2016; Taylor et al., 2010).

In the year 2000, over 120 million people worldwide were infected with lymphatic filariasis (LF) and approximately 1 billion people in 54 countries were at risk (WHO, 2017). This led to the launch of the Global Programme to Eliminate Lymphatic Filariasis (GPELF) by the WHO in 2000. The GPELF, visualized in Figure 1, utilizes a multimodal approach consisting of mass drug administration (MDA), vector control and integrated vector management (VC/IVM),
and Morbidity management and disability prevention in populations with LF (MMDP) (Ichimori et al., 2014).

Since the launch of GPELF, the London declaration was signed in 2012 and committed to eliminating LF by 2020, the Bill & Melinda Gates Foundation called for eradication by 2030, and the UN General assembly specifically targeted all neglected tropical diseases (NTDs) for elimination by 2030 (Rebollo & Bockarie, 2016). All of these initiatives have dramatically scaled up the resources and efforts to reduce the incidence and burden of LF.

Between 2000 and 2012, the MDA program resulted in 4.45 billion treatments being consumed by people living in endemic areas, leading to an estimated 96.7 million (Ramaiah & Ottesen, 2014). Seven billion treatments have been consumed as of today. The economic benefit of GPELF, in terms of amount saved over the lifetimes of the benefit cohorts, has been estimated to be between US$69.30–150.7 billion (Turner et al., 2016).

There are four main classes of drugs used to treat filarial diseases: diethylcarbamazine (DEC), benzimidazoles (notably, albendazole), avermectins, and oxytetracyclines (which affect
the Wolbachia endosymbiont); the first three affect the primarily the microfilariae in the blood (Scott & Ghedin, 2009). The current recommendation by the WHO is annual DEC and albendazole, though research has shown that including ivermectin (a derivative of avermectin) may improve microfilariae clearance (Ismail et al., 2001). The WHO also recommends preventive chemotherapy and transmission control (PCT) as the first-line strategy to achieve transmission interruption.

MDA will lead to stronger selective pressure on these parasites, potentially leading to the emergence of drug-resistant strains. New treatments could provide essential to eliminating LF, should substantial resistance arise.

**Genetic manipulation of Brugia malayi**

The filarial genome project was launched in 1994, with the goal of developing a framework to study the genome of human filarial parasites. *Brugia malayi* was selected as the organism of choice for this project, as it was the only filarial parasite readily maintained in small laboratory animals (Unnasch, 1994).

In following few years, over 22,000 expressed sequence tags (ESTs), short fragments of cDNA used to identify genes, had been produced from *B. malayi* and deposited in the public databases by laboratories from all over the world (Blaxter et al., 2002). While these studies were useful for gene discovery, new methods were needed to study gene function and regulation for *B. malayi*.

Reverse genetic approaches have been successfully used to study the free-living nematode *Caenorhabditis elegans*. Transient transfection by microinjection and particle bombardment (biolistics) were successfully used to introduce exogenous DNA into *C. elegans*, and later in *B. malayi* (Higazi et al., 2002). In a biolistic transfection, concentrated DNA is
precipitated onto small gold particles and propelled into embryos under high pressure; this method has been employed to study gene regulation in *B. malayi* (Shu et al., 2003; Higazi and Unnasch, 2004; Higazi et al., 2005; de Oliveira et al., 2008; Liu et al., 2010). Transient transfections are particularly limiting for studying parasites with complex life cycles, as the exogenous DNA introduced is not inherited. In a biolistic transfection, the transfected embryos are rendered developmentally incompetent. The genome of *B. malayi* was sequenced and compared to that of *C. elegans*, providing a foundation for future genetic studies (Ghedin et al., 2007).

Xu et al. (2011) demonstrated a novel method of transfecting developmentally competent *B. malayi* larvae, using a method of chemical transfection in cell culture while inducing the molting of L3 larvae to increase parasite permeability. Reporter activity was detected from adults and microfilariae retrieved from jirds (mammalian host used to maintain *B. malayi*). While this study demonstrated a method of transfecting the parasites without rendering them developmentally incompetent, the transgenic sequences introduced were probably not inherited in a Mendelian fashion. A stable method of transfection allowing the integration of transgenic DNA into the nuclear genome of *B. malayi* would enable more robust genetic studies.

The *piggyBac* (*pB*) transposon system has been applied to a wide variety of invertebrates and vertebrates (Lok, 2013). Of particular relevance, the *pB* system was utilized to integrate transgenes into the genome of the parasitic nematode *Strongyloides ratti* (Shao et al., 2012). Large inserts (up to 14 kb) have been delivered without a significant loss in efficiency (Vargas et al., 2016).

The *pB* transposon system utilizes two plasmids to enable stable insertional mutagenesis: a donor and a helper. The donor plasmid contains the DNA targeted for integration, flanked by
the inverted terminal repeats (ITR) region. The helper plasmid expresses the \textit{pB} transposase, which catalyzes that integration of DNA flanked by ITRs into the TTAA sites of the chromosomes (Balu & Adams, 2006). Adapting the methods from Xu et al. (2011) to introduce the proper donor and helper plasmids could lead to the development of a stable transfection system using the \textit{pB} system.

**Objective**

The goal of this project was to design a donor plasmid that could be used in a \textit{piggyBac} transfection system. A plasmid designed for \textit{piggyBac} transfection with a secreted \textit{Gaussia} luciferase (GLuc) selectable marker flanked by ITRs, with its expression driven by the HSP70 promoter and its 3’UTR was the starting point for this process. While GLuc is a sensitive reporter assay that can confirm a successful transfection, it does not allow for positive selection of transfected parasites or a direct inspection to observe localization. For this reason, we planned to use a fluorescent protein as a reporter assay. Due to reported autofluorescence, three different fluorescent proteins were chosen: GFP, YFP, and CherryRed (Table 1); all were purchased from addgene (Cambridge, MA).

**Table 1.** Fluorescent proteins chosen.

<table>
<thead>
<tr>
<th>Name</th>
<th>Ex</th>
<th>Em</th>
<th>Backbone plasmid</th>
<th>Expression spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP</td>
<td>488</td>
<td>507</td>
<td>pCS2+8NeGFP</td>
<td>\textit{C. elegans}</td>
</tr>
<tr>
<td>YFP</td>
<td>516</td>
<td>529</td>
<td>pCS2+8NmCitrine</td>
<td>\textit{C. elegans}</td>
</tr>
<tr>
<td>CherryRed</td>
<td>587</td>
<td>610</td>
<td>pCS2+8NmCherry</td>
<td>\textit{C. elegans}</td>
</tr>
</tbody>
</table>

The \textit{BmRPS12} promoter, which drives the expression of the 12 kDa small subunit ribosomal protein gene (de Oliveira et al., 2008), was picked to drive the expression of the fluorescent proteins. This promoter, like the HSP70 promoter, has been mapped in detail. The donor plasmid was then transfected into embryos by biolistic transfection to test the expression efficacy.
Chapter Two: Materials & Methods

General Methods

Polymerase chain reaction (PCR)

PCR was used to produce large quantities of the desired inserts and to confirm that an insert was present.

Restriction enzyme (RE) digest

Restriction enzymes (REs) are endonucleases that recognize specific nucleotide sequences and cleave the DNA in a predictable manner. In order to engineer an insert into a vector, restriction enzymes are used to linearize the insert and vector, and produce complementary nucleotide overhangs. These complementary overhangs will allow the insert and vector to be annealed together via hydrogen bonding. As will be discussed later, DNA ligase can then join the adjacent strands in a ligation reaction.

DNA extraction

Following RE digest, DNA was extracted from solutions using either a phenol-chloroform extraction or an agarose gel extraction. For a phenol-chloroform extraction, an equal volume of phenol:chloroform is added to a DNA sample. The phenol:chloroform denatures the proteins (i.e. RE enzymes), which are then separated from DNA by centrifugation. The DNA is then purified by an ethanol precipitation, which involves precipitating DNA out of a solution by
concentrated salt and cold temperature. Once precipitated, the DNA can be pelleted by centrifugation and further washed by 70% ethanol. The DNA is then dissolved in 1X TE.

An agarose gel extraction is used to separate and purify two strands of DNA. This method is used when an insert is amplified from plasmid DNA and to remove an insert from an undesired vector. The sample is run on an agarose gel, which separates linear strands of DNA by size, and extracted using the Wizard® SV Gel and PCR Clean-Up System kit (Promega).

**Heat-shock transformation**

A heat-shock reaction allows the introduction a plasmid into engineered *E. coli* cells to produce large, stable quantities of the plasmid or confirm that a ligation reaction was successful. The protocol varies according to cells used.

**PCR screen**

Following a transformation, up to 40 colonies can be screened for presence of a plasmid containing the desired insert. Colonies are picked and grown overnight in LB containing 100 ng/µL of ampicillin. Following incubation, 100µL of solution is transferred to 1.7 mL eppendorf tube and boiled at 100°C for 10 minutes on a heating block, and subsequently centrifuged for 5 minutes; this method allows for quick production of a DNA-containing solution that can be used as a template in a PCR.

Two different types of PCR screens can be run: directional or non-directional, differing only by primers used in reaction. A non-directional PCR screen uses a forward and reverse primer flanking the multiple cloning site, while a directional PCR will use a flanking primer and an insert-specific primer, each of opposite directions.

PCR samples are run on an agarose gel to confirm the presence of an insert.
High quality plasmid miniprep

The plasmid from a liquid bacterial suspension of less than 5 ml is isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen).

Sequencing

The plasmid from a liquid bacterial suspension of less than 5 ml is isolated and purified using the QIAprep Spin Miniprep Kit (Qiagen). The purified plasmids are then sent to Eurofins Genomics for sequencing, according to their instructions. Analysis of the sequencing results is conducted via BLAST search (NIH NCBI BLASTn).

Preparation of bacterial glycerol stock

Bacterial cultures containing a desired plasmid will be stored with 25% glycerol at -80°C for long-term storage. This stock is prepared by mixing 500µL of 50% glycerol with 500µL of bacterial solution in a cryopreservation vial.

Inserting Multiple Cloning Site (MCS)

Designing and preparing MCS insert for cloning

The sequence of the parental donor plasmid was analyzed to find all non cutters, or restriction enzymes whose recognition site was absent in the plasmid. Eight non-cutters were chosen, one of which (BclI) was chosen for the MCS to be cloned into. The rest were arranged to allow maximal overlap, shortening the total length (Table 2).

<table>
<thead>
<tr>
<th>BclI</th>
<th>NdeI</th>
<th>AatII</th>
<th>SmaI</th>
<th>Clal</th>
<th>MluI</th>
<th>BglII</th>
<th>BclI</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGATCA</td>
<td>CATATG</td>
<td>GACGTC</td>
<td>CCCGGG</td>
<td>ATCGAT</td>
<td>ACGCGT</td>
<td>AGATCT</td>
<td>TGATCA</td>
</tr>
</tbody>
</table>
Two oligonucleotides, containing the full MCS, were designed to form 4 base-pair 5’ overhangs when annealed (Fig. 2). Approximately 5 µM of these individual oligonucleotides were phosphorylated with 10 units of T4 polynucleotide kinase (NEB) in a solution containing 1 mM of ATP and incubated at 37ºC for 30 minutes. After inactivating the kinase, the phosphorylated oligonucleotides were annealed in a thermocycler by heating to 100ºC for 3 minutes and subsequently cooling to 25ºC at a rate of -1ºC every 30s.

1. Primers designed and ordered
   Fwd: 5’ GATCATATGACGTCCCGGGATCGATACGCGTAGATCT 3’
   Rev: 5’ GATCAGATCTACGCGTATCGATCCCGGGACGTCATAT 3’

2. Oligonucleotides individually phosphorylated

3. Annealed to leave 4bp overhangs:
   5’ GATCATATGACGTCCCGGGATCGATACGCGTAGATCT 3’
   3’ TATACTGCAGGGCCCTAGCTATGCGCATCTAG 5’

**Figure 2.** Generating MCS insert

Preparation of the vector for insertion of MCS

Site-directed mutagenesis was used to introduce a BclI site downstream of the HSP70 3’ UTR and upstream of the ITR2 site. Two overlapping 40bp primers with a central mutation site were designed to introduce a 2bp change in the sequence (Fig. 3). The reagents and protocol were provided by the GENEART® Site-Directed Mutagenesis System kit (ThermoFisher, USA).

Original: 5’ ACGCGGTCTGGTTATAGTTCAAAATCAGTGACACTTACCGCA 3’
Mutg_Fwd: 5’ ACGCGGTCTGGTTATAGTTCAAGATCGTAGTACGACACTTACCGCA 3’
Mutg_Rev: 5’ TGCGGTAAGTGTCACACTGGAATACGACACTTACGACACTTACCGCA 3’

**Figure 3.** Mutagenesis primers.
Red represents the mutated base-pairs

The linearized vector was then transformed into DH5α-T1 cells provided by the manufacturer. Six colonies were picked, grown overnight in LB media containing 100 ng/µL of ampicillin, purified, and sent for sequencing to confirm presence of desired mutation and
absence of undesired mutations. Once confirmed, 5µg of the vector was digested with BclI and dephosphorylated with 5 units of calf intestinal alkaline phosphatase. The vector was then extracted via phenol/chloroform extraction and purified by ethanol precipitation.

**MCS ligation**

The MCS insert was ligated into the vector at a 3:1 molar ratio by incubating at 16°C for 16 hours. The mixture, listed in (Table 3), was assembled in an RNase-free, thin-walled tube along with 1X ligation buffer. Two control reactions were run: one with no insert, to determine how much self-ligation is present, and one with no insert and no T4 ligase, to determine how much uncut vector is in the plasmid.

**Table 3. MCS ligation components**

<table>
<thead>
<tr>
<th></th>
<th>Ligation (µL)</th>
<th>Control 1 (µL)</th>
<th>Control 2 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>26.3</td>
<td>29.3</td>
<td>27.3</td>
</tr>
<tr>
<td>10X Ligase buffer</td>
<td>3.5</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Vector</td>
<td>2.2</td>
<td>2.2</td>
<td>2.2</td>
</tr>
<tr>
<td>Insert</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>T4 Ligase</td>
<td>2.0</td>
<td>0.0</td>
<td>2.0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>35.0</td>
<td>35.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>

**Confirmation of Ligation**

A PCR screen was run on selected colonies against a control to analyze relative size difference. Positive hits were sent for sequencing, one colony was stored in 25% glycerol at -80°C.
Figure 4. General overview of construct designs. Six different ligations were conducted to generate 5 different constructs.

Primer design and PCR amplification

The primers used in this study are provided in Table 4. The primers for the RPS12 promoter were designed with synthetic NdeI sites, while its 3’ UTR was amplified with a
synthetic BglII site to be cloned into the corresponding site. All the primers for the fluorescent proteins were designed for the fluorescent protein genes to be cloned into the MluI site of the MCS. The sequences of CherryRed and YFP had identical 5’ and 3’ 30 bp sequences, allowing them to be amplified using the same primers. All fluorescent proteins were amplified from the fluorescent protein backbones purchased from Addgene (Cambridge, MA).

Insert DNA was amplified via PCR according to the components and program in Table 5 and Table 6.

**Table 4. Primers used for sequencing and amplification of inserts**

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pB-Screen_Fwd</td>
<td>5’T CGTATGAGTTAAATCTTTAAGTCACG 3’</td>
</tr>
<tr>
<td>pB-Screen_Rev</td>
<td>5’T GTGCATTTAGGACATCTCAGTC 3’</td>
</tr>
<tr>
<td>RPS12-Pro_Fwd</td>
<td>5’T GGGCATATGGCTTAAGGAGAATTTTTAAAAAACTATAGAG 3’</td>
</tr>
<tr>
<td>RPS12-Pro_Rev</td>
<td>5’T GGGCATATGCATGTTCAGCAGTTGTCCTCGAT 3’</td>
</tr>
<tr>
<td>RPS12-3UTR_Fwd</td>
<td>5’T GGGAGATCTTGAATTTTTATTATTAAGAAG 3’</td>
</tr>
<tr>
<td>RPS12-3UTR_Rev</td>
<td>5’T GGGAGATCTGCCCAAGCAATTTCCAATGA 3’</td>
</tr>
<tr>
<td>Cherry/YFP_Fwd</td>
<td>5’T GGGACGCATGTGACTCTTTGGACATCTCAGTC 3’</td>
</tr>
<tr>
<td>Cherry/YFP_Rev</td>
<td>5’T GGGACGCAGCGCTTTGGAGCAATTTGATTTAGG 3’</td>
</tr>
<tr>
<td>GFP_Fwd</td>
<td>5’T GGGACGCCTGGTGGGTCTTTGCTGCCAATTCATCA 3’</td>
</tr>
<tr>
<td>GFP_Rev</td>
<td>5’T GGGACGCAGCGCTTTGGATTTTGCTCCAAACTCATCA 3’</td>
</tr>
</tbody>
</table>
Table 5. PCR components for insert amplification

<table>
<thead>
<tr>
<th>Components (Starting Concentration)</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-free water</td>
<td>27.5</td>
</tr>
<tr>
<td>10X PCR buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>MgCl2 (50mM)</td>
<td>4.0</td>
</tr>
<tr>
<td>dNTPs (2 mM)</td>
<td>10.0</td>
</tr>
<tr>
<td>Forward Primer (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td>Reverse Primer (50 µM)</td>
<td>0.5</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (5 U/µL)</td>
<td>0.5</td>
</tr>
<tr>
<td>DNA template</td>
<td>2.0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>50</td>
</tr>
</tbody>
</table>

Table 6. PCR thermocycler program for insert amplification

<table>
<thead>
<tr>
<th></th>
<th>Temperature (°C)</th>
<th>Duration</th>
<th># of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>3 min</td>
<td>1</td>
</tr>
<tr>
<td>Amplification</td>
<td>94°C (Tm-5)°C 68°C</td>
<td>3 sec 40 sec 1.5 min</td>
<td>30</td>
</tr>
<tr>
<td>Final extension</td>
<td>68°C</td>
<td>20 min</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4°C</td>
<td>Until storage</td>
<td>1</td>
</tr>
</tbody>
</table>

Strata ligation

All of the inserts were amplified to be cloned into the linear StrataClone PCR cloning vector. This vector contains topoisomerase-charged ends with modified uridine overhangs (that binds to 3’ adenosine overhangs of PCR products) and a loxP recognition sequence that enables recombination to create a circular DNA molecule containing the PCR product.
The PCR product and vector were incubated for 5 minutes at room temperature and subsequently transformed into Cre recombinase-expressing cells provided by manufacturer. Cre recombinase catalyzes the recombination between the two loxP sites, resulting in a circular plasmid containing the PCR product. The vector contains a lacZ’ α-complementation cassette, which enables blue-white screening. Up to 40 white colonies are grown in liquid media and screened by PCR for presence of insert.

Preparation of insert and vector

Two separate colonies containing the proper vector and the proper insert in a strata vector were purified using the QIAGEN Plasmid Midiprep kit. Once purified, 10µg of insert and 5µg of vector were separately digested by the same restriction enzyme (to produce complementary overhangs). The insert was extracted via agarose gel extraction to separate it from the Strata vector and the vector was purified via phenol/chloroform extraction, followed by an ethanol precipitation.

Ligation of insert into vector

Two different protocols were used for ligations. The first protocol was used to ligate the RPS12 promoter & its 3’ UTR, as well as YFP into the final vector. The second protocol was used to ligate CherryRed and GFP into the vector. In the first protocol, the insert to vector molar ratio was 3 to 1. The components added were according to Table 7. The solutions were incubated at 16°C for 16 hours and the T4 ligase was inactivated at 65°C for 10 minutes (Table 7).
This protocol did not yield any success for the ligations of CherryRed and GFP; it was therefore modified in the hopes of increasing ligation efficiency. The insert:vector ratio was increased to 6:1 and the total volume was reduced to 20 μL. The insert and vector were heated at 65°C for 5 minutes prior to adding ligase buffer and ligase to disrupt an insert-to-insert or vector-to-vector sticky end interactions. The 16°C incubation was shortened to 15 hrs and preceded by 30 minutes at 4°C, while followed by 30 minutes at 22°C (Table 8).

### Table 7. Ligation 1 components

<table>
<thead>
<tr>
<th></th>
<th>Ligation (µL)</th>
<th>Control 1 (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H2O</td>
<td>To 35µL</td>
<td>To 35µL</td>
</tr>
<tr>
<td>10X Ligase buffer</td>
<td>3.5</td>
<td>3.5</td>
</tr>
<tr>
<td>Insert:Vector</td>
<td>3:1</td>
<td>No insert</td>
</tr>
<tr>
<td>T4 Ligase</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>TOTAL</td>
<td>35.0</td>
<td>35.0</td>
</tr>
</tbody>
</table>

Following ligation, 3µL of the ligation solution was transformed into Subcloning Efficiency™ DH5α™ Competent Cells (ThermoFisher) according to the manufacturer’s protocol. Up to 40 colonies were picked and screened by PCR for the presence of insert in the

### Table 8. Ligation 2 thermocycler program

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>30 min</td>
</tr>
<tr>
<td>16°C</td>
<td>15 hrs</td>
</tr>
<tr>
<td>22°C</td>
<td>30 min</td>
</tr>
<tr>
<td>65°C</td>
<td>10</td>
</tr>
<tr>
<td>4°C</td>
<td>Infinite</td>
</tr>
</tbody>
</table>
proper orientation. Up to 3 positive hits were sent for sequencing and one confirmed colony was stored in glycerol at -80°C.

**Biolistic transfection**

Approximately 30 mg of gold beads (0.6µm diameter) were washed with 1ml of 70% ethanol once followed by three washes with 1ml nuclease free water. The beads were then resuspended in 500µL of 50% glycerol, giving a concentration of 60 mg/ml, and stored at 4°C. The beads were resuspended in the solution by placing on a platform vortex for 15 minutes; 50 µL of this solution are transferred to another centrifuge tube for use in a single transfection.

The new tube was placed on the platform vortex while the remaining components were added in order: 10µL of 2µg/µL purified DNA in water, 55µL of 2.5 M CaCl₂, and 22µL of 0.1 M of spermidine. After the sample was vortexed for 3 more minutes and allowed to settle, the beads were washed once with 280µL of 75% ethanol and once with 280µL of absolute ethanol. The beads were then resuspended in 18µL of absolute ethanol, with 6µL loaded onto macrocarriers.

The embryos are isolated by dissection of adult female worms. Five female worms were used per transfection. The adult worms are visible with the naked eye and can be easily isolated on a 3.5 cm petri dish in a flow hood. The worms were vigorously diced using a curved bladed scalpel to release the embryos. Embryos were collected into a 1.7 mL tube in around 500µL of CF-RPMI. The embryos were then centrifuged at 6000 x g and resuspended in 30µL of CF-RPMI. These embryos are then spread in the center of a 3.5 cm petri dish.

The DNA/bead-coated macrocarrier, the embryo-containing dish, and an 1100 PSI break disk were all properly loaded onto a biolistic unit (BioRad, Hercules, CA). The embryos were
then bombarded with the beads at 1100 PSI under a vacuum of 12.5 in of mercury. The embryo-containing dish was then placed in a humid box and covered for 5 minutes. Following incubation, 1.6 ml of CF-RPMI was added to the dish and placed in a 37ºC (5% CO2) incubator for 48 hours.

**Analysis of Transfected Embryos**

**Secreted luciferase assay**

Following the 48-hour incubation, 50µL of BioLux GLuc substrate was mixed with 5ml of BioLux GLuc Assay buffer in a glass culture tube. One blank was run to assess background along with 20µL of each sample mixed with 50µL of the GLuc assay solution. The luciferase activity was measured using an FB12 Tube Luminometer (Berthold). The activity is measured in relative light units (RLU).

**Fluorescence microscopy assay**

Following incubation, 20µL of embryos in CF-RPMI were pipetted onto a microscope slide and fixed with coverslip. Various slides were observed under varying emission wavelengths using an automated fluorescence microscope (Zeiss).
Chapter Three: Results

The site-specific mutagenesis, with the goal of generating a 2-bp mutation to create a BglII site was confirmed by sequencing (Fig. 5). This BglII site allowed for the insertion of a multiple cloning site, which in turn enabled fluorescent proteins to be cloned in with the BmRPS12 promoter and its 3’ UTR to drive the expression. The plasmid with only the MCS was named pBACII-BmGluc (Fig. 6); this plasmid was transfected via biolistics and analyzed via luciferase assay (Fig. 12).

**Figure 5.** Sequencing result of mutagenesis. Highlighted region is BglII site.

**Figure 6.** Sequencing result of MCS ligation. Highlighted region are BglII sites flanking the MCS.
Prior to being cloned into the MCS, all individual inserts were cloned into the Strata vector as described in the methods section and confirmed by sequencing.

The first insert to be ligated into the MCS was the RPS12 3’UTR. This insert was amplified from the genomic DNA of *B. malayi*. A directional PCR screen was run on 38 colonies, with two positive clones detected; both of these were confirmed by sequencing.

![Image](image.png)

**Figure 7.** PCR screen for RPS12 3’ UTR ligation

Next, the RPS12 promoter was ligated into the MCS. Only one colony grew from the ligation reaction, and it was confirmed by PCR screen to have the insert in the correct orientation (Fig. 8). The plasmid containing the RPS12 promoter and its 3’UTR was named pBACII-BmGluc-RPS12 and stored at -80ºC; this plasmid was used as a control for a fluorescence microscopy assay and a luciferase assay (Fig. 13)
All of the fluorescent protein inserts were individually ligated into the MluI site, flanked by the RPS12 promoter and its 3’ UTR. A directional PCR screen was run for all the individual ligations with varying levels of success: the YFP ligation had 12 positive hits out 18 (Fig. 9); the GFP ligation had 6 out of 16 (Fig. 10); and the cherryRed had 1 out of 13 (Fig. 11). Only the construct containing YFP was transfected via biolistics into *B. malayi* embryos and analyzed by luciferase assay (Fig. 13) and fluorescence microscopy assay.

The pBACII-BmGluc-YFP construct was analyzed by fluorescence microscopy using the pBACII-BmGluc-RPS12 as a control; both of these constructs were identical with the exception of the YFP gene. Autofluorescence was observed equally in both samples, with no significantly greater fluorescence in the YFP channel.
Figure 9. Directional PCR screen for YFP ligation
Expected size: 1919 bp

Figure 10. Directional PCR screen for GFP ligation
Expected size: 2128 bp

Figure 11. Directional PCR screen for CherryRed ligation
Expected size: 1910 bp
Figure 12. pBACII-BmGluc Luciferase assay.
The two samples of embryos were transfected via biolistics with pBACII-BmGluc

Figure 13. pBACII-BmGluc-YFP Luciferase assay.
pBACII-BmGluc-RPS12 served as a control. Both samples transfected via biolistics
Chapter Four: Discussion

The luciferase assays for the three different constructs demonstrate that they were all successfully transfected into the embryos. The inconclusive results from the fluorescent microscopy assay could be due to a number of reasons. One reason may be that the RPS12 promoter and its 3’UTR did not successfully drive the expression of the YFP. Expression of the YFP might have also been at a very low level, rendering it difficult to discern between autofluorescence and YFP-derived fluorescence. No autofluorescence was detected in undisturbed microfilariae and L3s. It is possible that the stress induced to prepare the embryos for transfection resulted in high levels of autofluorescence. Studies in the lab are currently ongoing to test a modified version of the in vitro transfection described in Xu et al. (2011) that uses a lipofection instead of a calcium phosphate precipitate-mediated transfection. This transfection, conducted on the more robust L3s, does not require much stress to be induced and could provide a method to detect fluorescence without the impeding autofluorescence. If no significant fluorescence is detected, a different promoter could be used or introns specific for BmRPS12 could be cloned in to increase the efficiency of expression.

These constructs could be used for spatial and temporal analysis of gene expression, which could elucidate gene function. In an organism with a complex life cycle like B. malayi, different genes will be expressed at various times throughout the life cycle. Fluorescent protein reporters allow researchers to monitor the gene expression in a specific cell or tissue and at certain times in a life cycle. Hunt-Newbury et al. (2007) used a GFP construct to generate spatial
and temporal tissue expression profiles for 10% of all genes in *C. elegans*, analyzing each stage of development.

MicroRNAs (miRNAs) play an important role in regulating posttranscriptional gene expression. These small RNA molecules generally form a protein complex that binds to sequences in the 3’UTR of mRNAs, and could be targeted for the development of novel treatments (Poole et al., 2014). Different miRNAs are restricted to different life cycle stages in nematodes, suggesting a potential role in their development.

miRNA activity has been analyzed using GLuc as a reporter, demonstrating that miR-71 decreased reporter activity when inserted into the 3’UTR (Liu et al., 2015). Using GLuc as a reporter, however, does not all for monitoring miRNA according to different tissue types. Using a GFP as a reporter, Brown et al. (2006) were able to demonstrate that miR-142 was suppressed only in hematopoietic lineages. This result showed that GFP was an effective reporter to analyze how miRNAs could efficiently differentiate gene expression among different tissue types.

Kato et al. (2009) developed a vector system encoding two different fluorescent proteins (GFP & RFP). The GFP was connected to a specific miRNA (miR-133), while the RFP was not. The researchers demonstrated in real-time the reduction of the GFP with no effect on RFP. This study demonstrates how different fluorescent protein constructs could be utilized in tandem to more efficiently study gene regulation in varying tissue and cell types.

Future tools for genetic studies of *Brugia malayi* could be adapted from studies of other nematodes, especially *C. elegans*. CRISPR/Cas9 has been used to effectively modify the genome of various species to inactivate genes or knock-in desired sequences (Wei et al., 2013). Lo et al. (2013) demonstrated that CRISPR/Cas9 was highly effective at inducing precise indels or mutations that are heritable in a diverse number of nematodes, including *Pristionchus pacificus*.
and *C. elegans* which are diverged 300 million years ago. This system also requires exogenous plasmids to be introduced into an organism without harm to the host. If a stable system is developed to introduce plasmids designed for *piggyBac* transfection into *B. malayi*, it could be adapted for targeted genome engineering with CRISPR/Cas9.

These studies could be applied to research into various drug targets. As previously mentioned, the current drugs mostly target the larval stages or the *Wolbachia* and do not have much of an effect on the adult stages. Several targets have been proposed, such as the nematode molting pathways and the nervous system (Scott & Ghedin, 2009). Filarial parasite biology is also of interest to human transplant research, due to the adult’s ability modulate the host immune reaction and persist in individuals for years without being detected by any immune reaction (Maizels & Yazdanbakhsh, 2003).
References


