Improving Genetic Editing Efficiency in *Aedes aegypti* Mosquitoes via Germline Cas9

Ivan Lo

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Abstract

Aedes aegypti are one of the most prevalent vectors in the world. Previous population control methods have utilized genetic editing technology to alter the genome of mosquitoes. With the expansion of the CRISPR/Cas9 toolbox, Cas9 technology has been adapted to be used in *Ae. aegypti*. Once a Double Strand Break (DSB) is generated by Cas9, the conserved DNA repair pathways will go towards Non-Homologous End Joining (NHEJ) or Homology Directed Repair (HDR). The high efficiency and error-prone NHEJ is biased compared to the robust, error-free HDR. However, genome editing relies on HDR to repair DSB based on the desired donor template. Therefore, we seek to improve the HDR efficiency by utilizing germline gene promoter regions and altering the expression of Cas9 *in vivo*. We found *zpg* and *nanos* as two candidate germline genes to express Cas9. This thesis focuses on the optimization of HiFi assembly of germline Cas9 designs and the possible future directions of this project. We hypothesized that plasmid design and higher concentration of DNA would affect HiFi assembly efficiency. While both factors resulted in higher mean number of colonies, we did not find a statistically significant difference. We recommend avoiding small DNA fragments during the design of assembly reactions and using the highest concentration of DNA possible to generate the most colonies. *Nanos-Cas9* was successfully cloned and verified by sequencing reads, while *zpg-Cas9* sequences returned plasmids without any inserts. This study is the first step in the effort to generate an improved germline Cas9 for genetic editing in *Ae. aegypti*.

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Acknowledgements

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Introduction

Introduction to *Aedes aegypti* mosquitoes

The yellow fever mosquitoes *Aedes aegypti* are a globally prevalent vector for arboviruses like yellow fever, dengue, chikungunya and zika (Li et al, 2020). *Ae. aegypti* are responsible for 390 million dengue infections annually (Bhatt et al., 2013), causing significant mortality among humans (Dong et al., 2015). These viruses are transmitted through blood-feeding female mosquitoes. Once a mosquito bites an infected human, the virus goes through an incubation period before getting passed on to the next individual during blood feeding (Fuller, 1961). *Ae. aegypti* mosquitoes are anthropophilic, meaning that they prefer humans as a blood source. Combined with their ability to lay eggs in small bodies of fresh water (Fuller, 1961; Matthews, Younger & Vosshall, 2019), this makes them dangerous vectors that must be controlled to prevent population outbreaks.

Genetic Control in *Ae. aegypti*

Traditional population controls through chemical insecticides have been used to reduce the number of *Ae. aegypti* worldwide. Despite the success of these methods, insecticides are unsustainable because of resistance development and high costs of application. Therefore, various genetic population controls have been implemented in *Ae. aegypti*. Wolbachia *pipientis* are intracellular bacteria that have been utilized to manipulate mosquito biology and prevent viral infection (Alphey, L., 2014). This method is relatively less invasive compared to *in vivo* genetic disruption and editing. Sterile Insect Technique (SIT) relies on the mass-rearing and subsequent release of sterile male mosquitoes (Phuc et al., 2007). Through utilizing transposons

like *piggyBac* elements, mosquitoes can be transformed into transgenic lines that carry dominant lethal constructs (Lobo et al., 2006; Phuc et al., 2007; Kokoza et al., 2001); Homing endonucleases genes (HEGs) with *Fok*I and synthetic *Medea* system are DNA elements that were first used to target and cleave genomic DNA (Alphey, L., 2014; Aryan et al., 2013; Kim & Chandrasegaran,1994). Further methods were tailored for *in vivo* gene disruption, including zinc finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN). These genetic elements were able to produce gene knockout or editing in a target specific manner (Aryan et al., 2013**;** Degennaro et al., 2013; McMeniman et al., 2014). However, they are often time consuming, with huge effort required to engineer and optimize these genetic tools (Dong et al., 2015). With the current expansion in the utilization of Clustered Regularly Interspaced Palindromic Repeats (CRISPR) systems, CRISPR/Cas9 has been proposed as a novel method for population control in *Ae. aegypti*. CRISPR/Cas9 does not require the lengthy multistep assembly of the protein domains in ZFNs and TALENs (Sentmanat et al., 2017), and provides relative ease for use in model and non-model organisms.

Introduction to the CRISPR-Cas9 System

CRISPR/Cas9 is a powerful genetic editing tool which was first identified in *Streptococcus pyogenes* bacteria. A part of the bacterial defense against virus and plasmids, the RNA-guided system cleaves invading DNA (Ren et al., 2013; Jinek et al., 2018). This system requires a few components. First, two non-coding CRISPR RNAs (crRNA) must be present, namely the trans-activating crRNA (tracrRNA) and precursor crRNA (pre-crRNA) (Bassett et al., 2013; Jinek et al., 2018). The pre-crRNA contains a spacer sequence, which is interspaced with direct repeats and guides the Cas9 protein to a complementary target. These two components have been combined into a chimeric, single-guide RNA (sgRNA) able to direct Cas9

to gene targets (Cong et al., 2013; Wang et al., 2013). Second, a protospacer adjacent motif (PAM) sequence (5'-NGG-3') is needed on the target sequence as well as a 10 – 12 nucleotide seed sequence that the sgRNA binds via Watson-Crick base pairing (Cong et al., 2013; Li et al., 2017a; Ma et al., 2016; Sternberg et al., 2014). Once the Cas9 protein binds to the sgRNA, it is directed to the target sequence to make a double-strand break (DSB) in the DNA. The DSB is fixed by the cell machinery through DNA repair pathways that are conserved in eukaryotes (Tang et al., 2019).

DSBs are repaired by the cell in two distinct pathways, Non-homologous End Joining (NHEJ) and Homology Directed Repair (HDR) (Ren et al., 2013). NHEJ is an error-prone pathway that is predominant in the cell. The DNA Ligase IV complexes with various components to repair the DSB and cause insertion or deletion mutations. On the other hand, the HDR pathway is able to repair the DNA in an error-free manner as long as homologous DNA sequence is present (Paix et al., 2017). While the NHEJ is not restricted in the cell cycle, HDR mainly happens in the S- or G2- phases (Liu et al., 2019). The mechanism behind which pathway is chosen to repair the DSBs is currently unknown.

Genetic editing methods have utilized the HDR pathway by providing the cell with a donor template with homology arms complementary to the target sequence. Donor templates that are single or double stranded linear DNAs (Paix et al., 2017), circular double stranded DNA (Li et al., 2017a) and single-stranded oligodeoxyribonucleotides (ssODNs), can carry the sequence that researchers wish to edit into the genome. The desired sequence can range from single-base pair changes to large exogenous sequences of up to 10kb (Ballabio, 2016). HDR-editing has been widely utilized in model organisms including mice (Koreman et al., 2020; Xue et al., 2014), *Drosophila melanogaster* (Yu et al., 2013), zebrafish (Chang et al., 2013) and even extends to

non-model organisms like *Anopheles gambiae* mosquitoes (Hammond et al., 2020), jewel wasps (Li et al., 2017b), sea squirts (Stoff et al., 2014) and mites (Dermauw et al., 2013).

CRISPR/Cas9 in *Ae. aegypti*

Previous studies have successfully optimized the use of CRISPR/Cas9 in *Ae. aeypti*, but have noted the low survival rates and low HDR editing rates. An injection mix including recombinant Cas9 protein, sgRNA and donor templates are injected into the pre-blastoderm embryos of *Ae.aegypti*. The embryo syncytium of nuclei enables the CRISPR/Cas9 components to permeate to both somatic and germline cells (Kistler, Vosshall & Matthews, 2015), which were used to generate knock-in mutants in sensory receptor genes (Matthews, Younger & Vosshall, 2019). Thus, the improvement of HDR editing has great implication for further genetic manipulation in *Ae. aegypti*. Various work has been generated to increase HDR editing in cell culture including optimization of donor templates (Paix et al., 2017), pharmacological inhibition of DNA Ligase IV, *RAD52* protein restriction of Cas9 expression (Gutierrez-Triana et al., 2018), small interfering RNA (siRNA) targeting NHEJ complex proteins (Liu et al., 2019), *p53* tumor suppressor (Tang et al., 2017) and optimization of tRNA to increase gRNA production (Koreman et al., 2020). However, these methods require exogenous elements that might negatively impact organismal biology. Therefore, the aim of this project will be to restrict Cas9 expression during development to bias HDR pathway during DSB repair.

Use of CRISPR/Cas9 in Gene Drives

The use of CRISPR/Cas9 system in mosquitoes can genetically modify populations through gene drives. Gene drives are genetic constructs that bias the inheritance of specific genetic elements autonomously so that desired traits can spread in a population. Combined with

HDR editing, traits that inhibit reproduction or limit the spread of vectors can be integrated into the genomes of lab-reared mosquitoes (Li et al., 2020). Through inheritance of CRISPR components, desired gene drive elements can spread via HDR editing through Super-Mendelian inheritance that turns heterozygous carriers to homozygous individuals (Hammond et al., 2020). Despite the extreme risk that *Ae. aegypti* pose on human population, confinable gene drives have only recently been tested in these mosquitoes (Li et al., 2020). Gene drives have been established in the malaria mosquito *An. gambiae*, with notable development of a dominant lethal genetic element used by commercial company Oxitec Ltd. (Evans et al., 2019; Hamond et al., 2020). The spread of gene drive elements relies on HDR using CRISPR/Cas9 technology. Resistance to gene drive elements often occur from NHEJ disruption, as sites repaired with indels are resistant to further DSB and HDR repair. This effectively prevent it from further gene drive editing (Hammond et al., 2020). The improvement of HDR editing can therefore help reduce population resistance.

Project Approach and Significance

Previous methods of maternally inherited Cas9 or insertion of recombinant Cas9 protein could be active throughout embryo development; It is possible that DSBs from these active Cas9 proteins are mostly repaired by NHEJ. The restriction of Cas9 expression to the germline could restrict Cas9 activity and DSBs at certain developmental stages. Such DSBs could be more prone to HDR pathway instead of NHEJ, thereby providing higher genetic editing efficiency. Drawing from the work done to improve HDR editing in *An. gambiae* (Hammond et al., 2020), we hypothesize that Cas9 expression through germline elements can bias the HDR pathway towards DNA repair, increasing HDR editing rates. It should be noted that tightly linked genes have been conserved only in some chromosomal arms between *An. gambiae* and *Ae. aegypti*. Previous

efforts in optimizing HDR editing in *Drosophila melanogaster* and *An. gambiae* needs to be carefully translated to *Ae. aegypti* as there is significant chromosomal element reshuffling between these genomes (Severson et al., 2014).

Hammond et al. utilized regulatory elements in the 5' and 3' untranslated regions (UTR) of *An. gambiae* germline genes (*zpg* and *nanos*) to drive Cas9 expression *in vivo*, with notable increase in HDR editing efficiency (Hammond et al.,2020) (for functions of *zpg* and *nanos*, see **Discussion**). We decided to test these analogous genetic elements in *Ae. aegypti* by constructing a Cas9 expression cassette with 5'UTR, 3'UTR and any introns that could have regulatory activity. The main focus of this thesis is on the HiFi Assembly reaction for generating the appropriate germline Cas9 designs. The HiFi Assembly is a commercially available cloning reaction kit that allows the joining of DNA fragments together into a single plasmid. We hypothesized that plasmid design and concentration both alters the number of colonies generated by assembly reactions. Germline Cas9 plasmid designs will need to be validated and amplified in order to test their efficiency in *Ae. aegypti* genome editing.

In the future, separate sgRNAs constructs driven by *Ae. aegypti* specific U6 promoter (Li et al., 2020) will be added to plasmids of donor templates with flanking homology arms to the target sequence. These Cas9 expression constructs and sgRNA – donor template constructs will be injected into *Ae. aegypti* embryos, which could decrease toxicity compared to previous methods that utilized a high concentration of recombinant Cas9 protein (Kistler, Vosshall & Matthews, 2015). We hope to use various genetic screening methods including fluorescence microscopy and gel electrophoresis to isolate outcrossed mosquitoes with stable germline HDR edits that can be passed on to the next generations.

The results generated in this study can help guide HiFi assembly reactions for construction of desired plasmids. Future outcomes can provide insight into the genetic similarity between *An. gambiae* and *Ae. aegypti*, which are two of the most prevalent mosquito vectors for pathogens that cause human disease. As *Ae. aegypti* has increasing significance in the field of neurobiology as a novel model organism, the result of this work could aid in the generation of reporter strains across many genes through precise genetic manipulation (Matthews & Vosshall, 2020). Moreover, the use of germline regulatory elements could give insight to the mechanisms behind DSB repair pathways during CRISPR/Cas9 systems; Improvements towards the HDR editing rates can be implemented in gene drive methods to reduce population resistance to gene drive elements.

STAR★Methods

Key Resources Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BACTERIAL AND VIRUS STRAINS		
E. coli DH5α	NEB	Cat#2987H
CHEMICALS, PEPTIDES, AND RECOMBINANT PROTEINS		
Ampicillin	Sigma-Aldrich	Cat#171257
Kanamycin	Sigma-Aldrich	Cat#60615
Q5 Hot Start High-Fidelity 2X Master Mix	NEB	Cat#M0493S
OneTaq Hot Start 2X Master Mix	NEB	Cat#M0484S
NEBuilder HiFi DNA Assembly Master Mix	NEB	Cat#E2621L
1kb DNA ladder	NEB	Cat#N3232S
Gel Loading Dye Purple (6X)	NEB	Cat#B7024S
Agarose	BioBasic	Cat#D0012
2xYT medium broth	Sigma-Aldrich	Cat#Y2377
Tryptone	Sigma-Aldrich	Cat#T7293
Yeast Extract	Sigma-Aldrich	Cat#Y1625
Sodium Chloride	Sigma-Aldrich	Cat#S3014
Trizma Base	Sigma-Aldrich	Cat#T1503
Acetic Acid	VWR	Cat#CABDH3092
EDTA	NEB	Cat#7011V
Glycerol	UBC Chemstores	Cat#OR-43190-4
SOC outgrowth medium	NEB	Cat#B9020S
Ethanol	UBC LSI Stores	Cat#P016EAAN
BamHI-HF	NEB	Cat#R3136S
EcoRV-HF	NEB	Cat#R3195S
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific	Cat#S33102
CRITICAL COMMERCIAL ASSAYS		
NucleoSpin Plasmid Midi kit	Macherey-Nagel	Cat#740410
NucleoSpin Plasmid Mini kit	Macherey-Nagel	Cat#740588
NucleoSpin Gel and PCR Clean-up	Macherey-Nagel	Cat#740609
EXPERIMENTAL MODELS: ORGANISMS/STRAINS		
Aedes aegypti	Nene et al., 2007	Cat#LVP-IB12
OLIGONUCLEOTIDES		
All primer sequences listed Table S1	This manuscript	N/A
RECOMBINANT DNA		
pUC19	NEB	N/A
pRGEB32-BAR	Addgene	Plasmid #126072

Zpg 5'	TWIST Bioscience	N/A
Zpg 3'	IDT	N/A
Nanos 5'	TWIST Bioscience	N/A
Nanos 3'	TWIST Bioscience	N/A
SOFTWARE AND ALGORITHMS		
BLAST	Altschil et al., 1990	N/A
Vectorbase v. 49 beta	https://vectorbase.org/vectorbase/app/	N/A
FlyBase v. FB2020_05	https://flybase.org/	N/A
Benchling	https://benchling.com	N/A
R v. 4.0.3	http://www.r-project.org/	N/A
NEBuilder assembly tool	https://nebuilder.neb.com/	N/A
NEB Tm Calculator	https://tmcalculator.neb.com/#!/main	N/A
OTHER		
Sheep blood	Cedarlane Labs	Cat#CL2581-100D

Contact for Reagents and Resource Sharing

Any requests for the resources, reagents and further information should be directed to and will be fulfilled by the Contact in Matthews Lab, Nicholas Tochor. (ntochor@gmail.com)

Experimental Model and Subject Details

Mosquito maintenance and rearing

Aedes aegypti Liverpool IB12 (LVP-IB12) strain (Nene et al., 2007) was used in both the embryo injection and as wild-type for subsequent out-crossing. Mosquitoes were maintained with 25-28˚C, 80% relative humidity and a photoperiod with 14 hours light to 10 hours dark following pervious work (DeGennaro et al., 2013). Adult female mosquitoes were fed with sterile sheep blood, while all adult mosquitoes were provided with 10% sucrose ad libitum. Mosquito rearing was approved by University of British Columbia (Biosafety permit #B19-0156).

Methods Details

Germline gene sequence identification

Germline Cas9 constructs were designed by combining non-genic regions of germline genes with the *sp*Cas9 sequence. Both *zpg-Cas9* and *nos-Cas9* were constructed using the Benchling online platform (https://benchling.com). *Zpg* and *nos* from *Drosophila* were obtained on FlyBase (https://flybase.org/). I then obtained the *zpg* and *nos* DNA sequence from Vectorbase (https://vectorbase.org/vectorbase/app/) through a BLASTp search for *An. gambiae* and *Ae. aeypti* analogs. Untranslated Region (UTR), exon and introns were manually annotated using the alignment tool on Benchling.

Germline Cas9 design and synthesis

Each construct contains (1) upstream region of chosen germline gene, (2) genic region of germline gene depending on expression, (3) *NLS-Cas9-3xFLAG-NLS* sequence and (4) downstream region of chosen germline gene. These components were inserted in a commercially available vector backbone containing an antibiotic resistance selection cassette, pUC19.

To generate *zpg-Cas9*, the construct contains (1) 5' UTR of *zpg*, (2) a truncated version of the coding gene with 78 amino acid deletion at the C-terminus (see **Discussion: Germline Cas9 Designs and Reasoning**), (3) *T2A NLS-Cas9-3xFLAG-NLS*, and (4) 3' UTR of *zpg*. Insert was combined using Benchling assembly tool and sent for synthesis. The 5' region was 2.1 kb in length while the 3' region was 253 bp. The 5' region was synthesized using the TWIST Bioscience gene synthesis service (https://www.twistbioscience.com/) and included the T2A sequence, while the 3' region was synthesized by IDT DNA oligo synthesis. NLS-Cas9-3xFLAG-NLS was amplified from pRGEB32-BAR (Addgene #126072).

To generate *nanos-Cas9*, the construct contained (1) the non-coding region 2Kb upstream of *nanos* including 5'UTR, (3) *NLS-Cas9-3xFLAG-NLS*, and (4) non-coding region 1.974Kb downstream of *nos* including 3'UTR. Note that (2) coding regions of *nanos* was excluded from this construct. Insert was combined using Benchling assembly tool and sent for synthesis. Both 3' and 5' fragments were synthesized using the TWIST Bioscience gene synthesis service, while NLS-Cas9-3xFLAG-NLS was amplified from pRGEB32-BAR (Addgene #126072).

Amplification of DNA fragments

HiFi Assembly primers were generated using the NEBuilder Assembly Tool (https://nebuilder.neb.com/) for downstream assembly. All PCR reactions were done using assembly primers (Table S1). All PCR reactions were done using the Q5 polymerase. All reaction annealing temperatures were verified with the NEB Tm Calculator (https://tmcalculator.neb.com/#!/main)

Synthesized germline gene fragments (5' and 3') were PCR amplified independently. pUC19 backbone was first digested with BamHI-HF to generate linearized DNA and subsequently PCR amplified. Cas9 fragment was PCR amplified from *pRGEB32-BAR*.

Gel electrophoresis and DNA purification from Agarose Gel

Gel electrophoresis was done on all samples after PCR amplification. DNA was purified from the agarose gel using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Cat#740609). I followed the procedure listed on the company website (https://www.takarabio.com/documents/User%20Manual/NucleoSpin%20Gel%20and%20PCR%20Clean/NucleoSpin%20Gel%20and%20PCR%20Clean-up%20User%20Manual_Rev_04.pdf).

Bacterial transformation

All purified fragments were subsequently transformed in competent E. coli following the protocol listed on the company website (https://international.neb.com/products/c2987-neb-5-alpha-competent-e-coli-high-efficiency#Product%20Information). Bacterial cells were grown on agar plates with associated antibiotic resistance.

DNA extraction

Bacteria were grown in liquid culture in LB medium with associated antibiotics after transformation. Plasmids were extracted from liquid bacterial culture using the NucleoSpin Plasmid, mini kit (Macherey-Nagel, Cat#740588) for assembly and sequencing. I followed the procedure listed on the company website for the Miniprep protocol (https://www.mn-net.com/nucleospin-plasmid-mini-kit-for-plasmid-dna-740588.50).

For purposes of full plasmid sequencing validation and future mosquito embryo injection, Midiprep was done to extract higher amount of DNA. Bacteria were grown in liquid culture 2xYT medium with associated antibiotics after transformation. Plasmids were extracted from culture using the NucleoSpin Plasmid Xtra Midi kit (Macherey-Nagel, Cat#740410). I followed the procedure listed on the company website for the Midiprep protocol (https://www.mn-net.com/nucleobond-xtra-midi-kit-for-transfection-grade-plasmid-dna-740410.50).

HiFi Assembly of DNA fragments

HiFi Assembly is a commercially available reaction kit that includes exonuclease, polymerase and ligase for the joining of DNA fragments. HiFi Assembly of all 4 components were done following the guidelines from NEBuilder Assembly Tool and procedure listed on the company website (https://international.neb.com/products/e2621-nebuilder-hifi-dna-assembly-

master-mix#Product%20Information). Primers were generated with at least 30bp overlap (Supp. Table 3). Each assembly reaction was done twice with two different concentrations of DNA each. A negative control was included with only the PCR amplified pUC19 fragment and assembly master mix. Products of the assembly reactions were transformed in bacteria (see **Bacterial Transformation**).

Colony PCR

Colonies from agar plates were picked randomly to check for successful assembly DNA fragments. A set of forward and reverse primers spanning the 5' UTR and Cas9 fragment were used to PCR amplify the desired junction. All reaction annealing temperatures were verified with the NEB Tm Calculator (https://tmcalculator.neb.com/#!/main). I followed the procedure listed on the website for the OneTaq Hot Start 2X Master Mix for colony PCR with bacterial cells (https://international.neb.com/protocols/2012/09/05/one-taq-hot-start-dna-polymerase-m0481). Gel electrophoresis was done to ensure correct banding size and positive colonies were grown in liquid culture.

Sequencing

Primers for sequencing were designed manually or generated by the Benching primer design wizard. All primers were between 18 to 24 base pairs in length. Primers were designed in the forward direction across the entire 5' UTR – Cas9 – 3' UTR insert to validate the sequence. Subsequent reverse fragments were generated for areas that require further sequencing. Sequences were done using the GENEWIZ Sanger Sequencing service (https://www.genewiz.com/Public/Services/Sanger-Sequencing/). The primer sequences are listed in Supp. Table 3.

Statistical analysis

Data was analyzed with R studio (Version 4.0.3). All boxplots were generated with the ggplot2( ) function. For the mean number of colonies for the comparison of all HiFi assembly reactions, an ANOVA test and Tukey HSD test were done to test if there is a significant difference between samples. For pairwise comparisons between concentrations and germline cas9 design, an F test was done to test for homogeneity of variance. The comparison between design had unequal variance and a Welch's two sample t test was performed; The comparison between DNA concentrations had equal variance and an independent t test was performed.

Data and Software Availability

Gene DNA sequences for *Aedes aegypti* were obtained from Vector base v. 49 beta (https://vectorbase.org/vectorbase/app/) and genic components were manually annotated.

Results

Germline Cas9 plasmid design

Analogs were identified from *An. gambiae* to *Ae. aegypti*. Names of gene analogs are provided in Supplementary Table 1. The final design of germline Cas9 constructs are shown in **Figure 1** (for plasmid design, see **Supp. Fig. 4**). *Nanos-Cas9* included the 2Kb fragment upstream and 1.974Kb downstream sequences of the *Ae. aegypti nanos* gene. *Zpg-Cas9* included the 256bp 5' UTR and the 253bp 3'UTR segments of the *Ae. aegypti zpg* gene. The *zpg* gene region spans from exon 1 to a truncated version of exon 3 that is a 78 amino acid terminal deletion, followed by a T2A ribosomal skip sequence and the Cas9 sequence. The *Cas9* sequence included a NLS sequences at the 5' end and another NLS sequence at the 3'end. The T2A sequence will cause ribosomes to translate the truncated *zpg* protein and Cas9 independently.

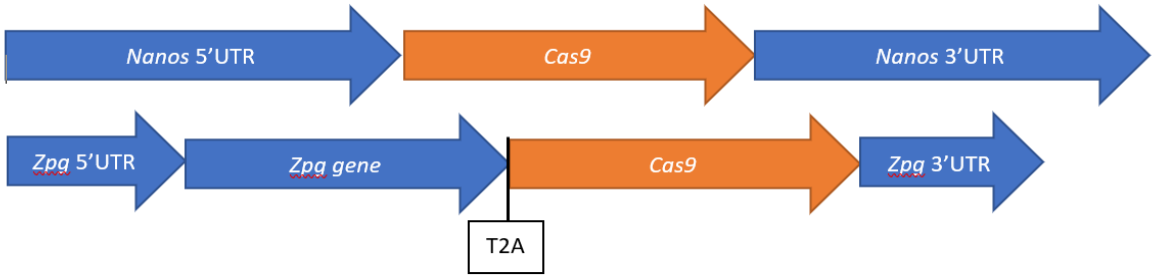
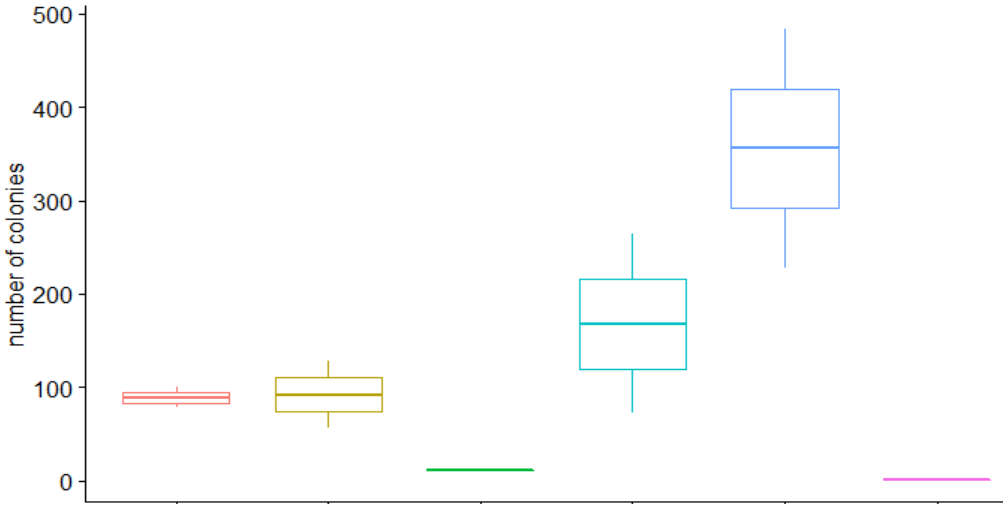


Figure 1. Schematic of Germline Cas9 DNA insert designs of *nanos-Cas9* (top) and *zpg-Cas9* (bottom). T2A is the ribosomal skip sequence inserted between 3' end of the *zpg* gene and the 5' end of *Cas9.*

HiFi Assembly Result

For the purpose of this thesis project, the assembly efficiency across the two germline Cas9 designs were assessed. Number of colonies were counted for all assembly reactions, including the two different concentrations of total DNA used and negative control, as detailed in **Figure 2**. Mean number of colonies were generated between two reactions. Overall, the ANOVA test did not generate a significant difference for the mean number of colonies across all reactions ($p = 0.0671$). The number of colonies between concentration and design were not significantly different. A Tukey HSD test also generated no significant difference in number of colonies across all pairwise comparisons (Supp. Table 2).

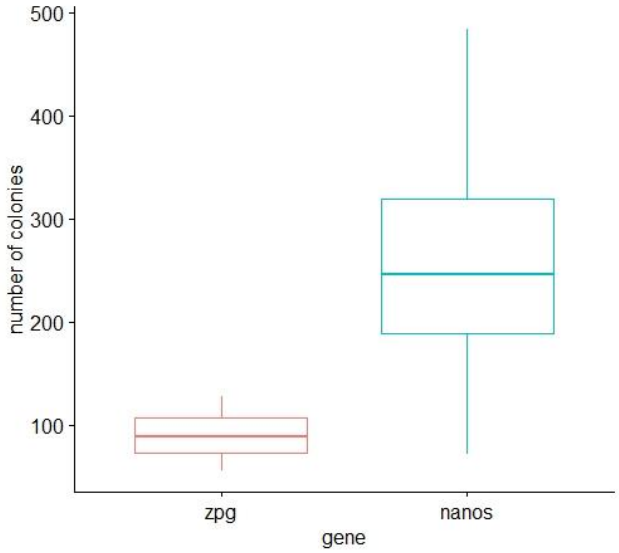


Germline Cas9 inserts	+	+		+	+	
pUC19 backbone	+	+	+	+	+	+
HiFi assembly	+	+	+	+	+	+
Concentration (pmol)	0.2	0.35		0.2	0.35	
Mean	89	92	11	168	356	1
Design	*Zpg Cas9*			*Nanos Cas9*		

Figure 2. Mean number of colonies in HiFi Assembly reaction for total DNA concentrations of 0.2pmol, 0.35pmol and negative control without germline Cas9 inserts. Both *zpg* and *nanos* designs were incubated in the same conditions for the assembly reactions.

Comparison between *zpg Cas9* and *nanos Cas9* designs

In order to test for specific differences in design regardless of concentrations, the number of colonies were averaged between both concentrations of DNA (0.2pmol and 0.35pmol). An F-test was done to check for homogeneity for variance ($p < 0.05$), which shows that variance across designs are not equal. The results of the Welch's Two Sample t-test is summarized in **Figure 3**. Nanos design did have a higher mean number of colonies, but the difference between mean number of colonies for *zpg* and *nanos* plasmid designs were not statistically significant ($p = 0.1354$).

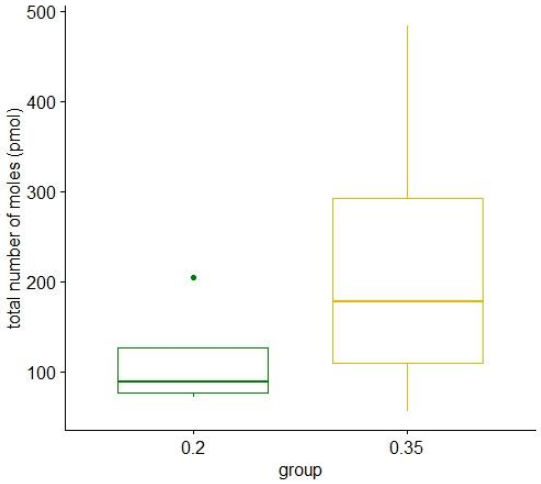


Germline Cas9 design	*zpg*	*nanos*
Mean number of colonies	90.5	262.0
t-value	1.9871	
p-value	0.1354	

Figure 3. Mean number of colonies in HiFi Assembly reaction for each germline Cas9 design.

Comparison between DNA concentrations

The NEB HiFi Assembly guideline recommends a 0.2 to 0.5 pmols range for total amount of fragment in the assembly reaction. Based on the concentration of DNA fragments after PCR reactions, 0.2pmol and 0.35pmol reactions were done to encompass the least and most amount of DNA fragments. The mean number of colonies were averaged based only on reaction concentration. An F test was done to check for homogeneity in variance (p = 0.100), which showed that the two samples had equal variance. The independent t-test results are summarized in **Figure 4**. The higher concentration results generated higher number of colonies, but the difference of means based on concentration was also not statistically significant (p = 0.3048).



DNA concentration (pmol)	0.2	0.35
Mean number of colonies	114.0	224.0
t-value	-1.1219	
p-value	0.3048	

Figure 4. Mean number of colonies in HiFi Assembly reaction for different concentrations of DNA fragments.

Colony PCR verification of DNA insertion

Colonies were randomly picked in each Assembly reactio to verify the correct insertion of DNA fragments (5'UTR - Cas9 - 3' UTR). The DNA fragment amplified spans the junction from 5'UTR of the germline gene to the start of the Cas9 sequence with a varying lengths (*nanos*: 700bp, *zpg*: 1000bp). 7 out of the 8 colonies from *Nanos Cas9* assembly showed a positive band, while only 4 out of 11 colonies from *Zpg Cas9* showed a faint positive band. Other colonies show an offtarget bands at 500bp or below (**Figure 5**).

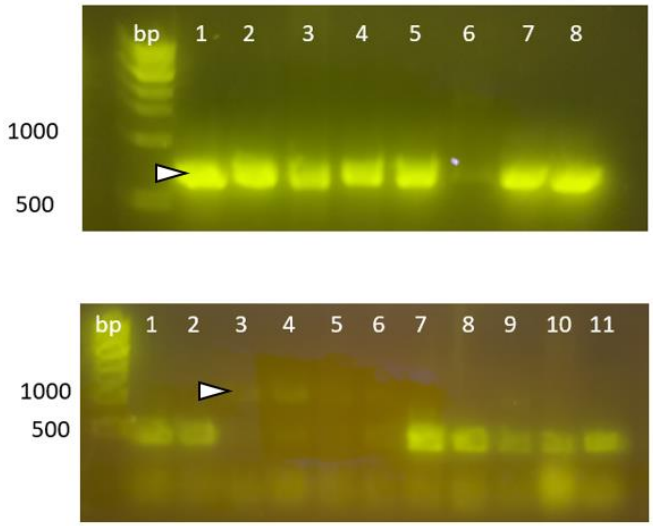


Figure 5. Agarose gel images showing colony PCR reaction for random colonies picked from HiFi Assembly reactions of *nanos Cas9* (top) and *zpg Cas9* (bottom). *Nanos Cas9* gel electrophoresis was done in a 1% gel while *zpg Cas9* gel electrophoresis was done in a 2% gel. Arrowhead shows the expected gel banding size if DNA junction was successfully amplified.

Discussion

Germline Cas9 Designs and Reasoning

The choice of *nanos* and *zpg* as germline promoters was based on previous germline gene driver work done by Hammond et al. (2020), which showed transmission rates of gene drive to the next generation to be above 90%. The *zpg* gene in *D. melanogaster* functions in the formation of gap junctions for germline and cyst cells (Tazuke et al., 2002) while *nanos* are maternal genes that are transcribed in the oocyte (Magnusson et al., 2011). The deposited mRNA of both genes concentrates at the germ plasm, furthering restricting the expression of genes in the germline cells of embryos (Tazuke et al., 2002; Rangan et al., 2009). Only *nanos* has been shown to generate a robust split-gene drive in *Ae. aegypti* (Li et al., 2020) while *zpg* has not been studied. The *Ae. aegypti* genome has been extensively reshuffled compared to that of *D. melanogaster* and *An. gambiae*, while the two mosquito species do share extensive homology in certain regions of the chromosome arms (Severson et al., 2004). Thus, functional conservation of the germline genes between *D. melanogaster*, *An. gambiae* and *Ae. aegypti* is still an active area of research.

The *nanos-Cas9* and *zpg-Cas9* designs were done to encompass possible promoter activities that could be found in untranslated regions (UTRs) and genic regions. Based on the expression data on *Ae. aegypti* (data unpublished from Matthews Lab), promotional activity was restricted in the UTR of *nanos*. 2Kb segments were used indiscriminately both upstream and downstream without inclusion of the next gene (Fig. 1). On the other hand, unique sequences in intron 1 of *zpg* possibly encompasses promoter activity for the gene. Therefore, the germline-Cas9 design includes part of the *zpg* gene (Fig. 1). As the overexpression of wildtype *zpg* protein could have

unintentional deleterious effects to the mosquitoes, a non-functional version of *zpg* with a 78 amino acid sequence terminal deletion was used. In *D. melanogaster*, the C-terminus has been shown to be essential for the localization and function of *zpg*, and the proposed deletion is enough to generate non-functional *zpg* (Smendziuk, C. M., 2016). Whether the same truncation can render *Ae. aegypti zpg* gene non-functional has yet to be confirmed by the generation of a healthy transgenic line using *zpg-Cas9* (generating transgenic line: see **Limitations and Future Directions**).

HiFi Assembly Optimization

Overall, comparisons of the HiFi Assembly reactions did not generate a significant difference between the mean number of colonies (Fig. 2, 3 & 4). This could point to the small sample size of the data analyzed, as only two reactions were done for each condition of the design assemblies. A higher number of repeating reactions could decrease the variance of each sample and produce a better comparison between assembly conditions.

In terms of the overall comparison of all assembly conditions, neither negative control generated a significant difference compared to the samples. The negative controls tested for left over pUC19 backbone that would be transformed into bacteria or self-annealing action as HiFi Assembly joins both ends of the pUC19 backbone back together. Although a lower number of colonies is observed for both designs, none of the comparisons were statistically significant (Supp. Table.2). This is surprising especially for the comparison of *nanos-Cas9* 0.35pmols, which had 356 colonies compared to only one colony for the negative control (Fig. 2). The comparison between these samples generated the lowest adjusted p-value of all comparisons ($p = 0.0646$, Supp. Table. 2). The colony numbers are highly variant even within the same conditions,

so it is likely that repeated sampling will show a significant difference for this particular comparison.

In general, *nanos-Cas9* at 0.35pmols had the higher mean number of colonies followed by *nanos-Cas9* at 0.2pmols (Fig. 2). However, the pairwise comparison between *nanos-Cas9* and *zpg-Cas9* did not generate a statistically significant difference (Fig. 3). It is unlikely that the specific sequence of the designs had any effect on the assembly reactions, which points to the varying lengths of the DNA fragments. While *nanos-Cas9* combined four fragments of similar size (~2Kb), *zpg-Cas9* included one particularly small segment for the 3'UTR that is only 253bp in length (Fig. 1). During the assembly, the *zpg* 3'UTR segment was added in three times excess of the other DNA fragments. Assembly reactions done with similarly sized fragments could result in a higher mean number of colonies.

Similarly, the comparison between DNA concentration of fragments did not yield a statistically significant result. The 0.35pmol concentration assembly had a higher mean number of colonies than the lower concentration, which is expected as more DNA fragments are available for the polymerase to extend on. Based on the results of this thesis, the recommendation for future HiFi Assembly reactions are as follows:

- DNA fragments can be designed to be approximately similar in sequence length for highest efficiency. Avoid having particularly short sequences if possible.
- Reactions should be done with the highest possible total pmols of DNA as recommended by the protocol without exceeding the reaction volume.

Correct Insertion of DNA fragments and Sequencing

Besides the efficiency of HiFi Assembly reaction to generate desired plasmids, the correct insertion of DNA fragments to backbone is equally important. For the downstream purposes of mosquito embryo injection, sequences must be verified to ensure that germline Cas9 is correctly expressed without introduction of mutations. At the time of submission for this thesis, all seven colonies from *nanos-Cas9* that showed the desired band across the 5'UTR and Cas9 junction had promising sequencing results (Fig. 5). Specifically, *nanos-Cas9* 7 had been fully sequenced and only two junctions of less than 100bp requires further validation. For *zpg-Cas9*, much of the colonies generated off-target bands which could be due to binding of primers to other areas of the plasmid. Of the four colonies with faint bands of the correct size, sequencing result only showed the pUC19 backbone sequence (Fig. 5). Thus, the assembly reactions were not successful in the colonies sampled. Repeated sampling from the same reaction could still generate a correct plasmid, as only one sample is needed for downstream DNA amplification and extraction. All germline plasmid designs must be fully validated before any work can be done with *Ae. aegypti*, as CRISPR-Cas9 has the ability to make lasting genome edits through NHEJ and HDR.

Limitations and Future Directions

Although germline Cas9 strategies have been hypothesized and shown to improve HDR editing (e.g. Hammond et al., 2020; Li et al., 2020), the reason for this bias is unclear. Maternal deposition of nuclease in *An. gambiae* gene drive have been shown to be the major source of NHEJ (Gantz et al., 2015), while the purposed strategy in this thesis will allow expression of Cas9 restricted to certain developmental stages. Future research could also be directed to expression of *zpg* and *nanos* during different embryonic life stages. Whether Cas9 will be

restricted to HDR activity during certain times of development could be impactful for future genetic editing approaches.

Due to the unprecedented time during which this thesis is completed, the validation of germline Cas9 as a strategy to improve genome editing in *Ae. aegypti* will require continued effort in the future. The validated plasmid sequences can be co-injected with a separate plasmid containing sgRNA and donor template into *Ae. aegypti* embryo at early stage of development. The donor template will be a fluorescent protein sequence (e.g. ECFP) which will be inserted into the DSB through HDR. Fluorescence insertion will allow subsequent screening of embryos with HDR activity. Compared to previous methods of injection with exogenous Cas9 protein (Kistler, Vosshall & Matthews, 2015), in vivo expression of Cas9 will hopefully increase survival of embryos. Germline Cas9 relies heavily on cell machinery to express Cas9 and restrict expression to certain developmental stages. Thus, results of future experiments could also show possible functional conservation between analogs with *An. gambiae* and *D. melanogaster*.

If HDR rates is significantly increased in the F0 generation, the mosquitoes will be outcrossed to wildtypes to ensure that DNA edits in the germline can be passed on. Sequencing and PCR can again be done on heterozygous individuals of the F1 generation. Ultimately, continued crossing through multiple generations will allow the formation of transgenic lines of mosquitoes. With a more efficient CRISPR-Cas9 strategy relying on germline gene expression, transgenic lines can be generated with lower effort of embryo injection and validation. By altering the sgRNA and donor template, the implications of a more efficient Cas9 can be used across any genomic target and any desired DNA insert. Finally, varying designs of the *zpg* and *nanos* are currently being tested by our collaborators in hopes of finding regions of promoter activity that best promotes HDR activity.

Conclusion

HiFi Assembly reactions was not significantly different between varying germline Cas9 plasmid designs and concentrations of DNA fragments. It is recommended that DNA fragments of similar length and highest possible concentration of DNA be used for higher efficiency of assembly reactions. While *nanos-Cas9* and *zpg-Cas9* currently requires sequencing validation, these designs serve as candidates to be injected into *Ae. aegypti* embryos to generate transgenic organisms. This study will rely on future efforts to verify the hypothesis of increasing HDR efficiency via germline Cas9.

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Supplementary Materials

Species	*Anopheles gambiae*	*Drosophila melanogaster*	*Aedes aegypti*
Zpg	*AGAP006241 / Innexin 2*	*Inx5-PB*	*AAEL006726 / Inx 4*
Nanos	*AGAP006098*	*Nos-PA*	*AAEL012107*

Supplementary Table 1. Names of analogous genes identified from BLASTp searches via genes identified in Vector Base and Fly Base.

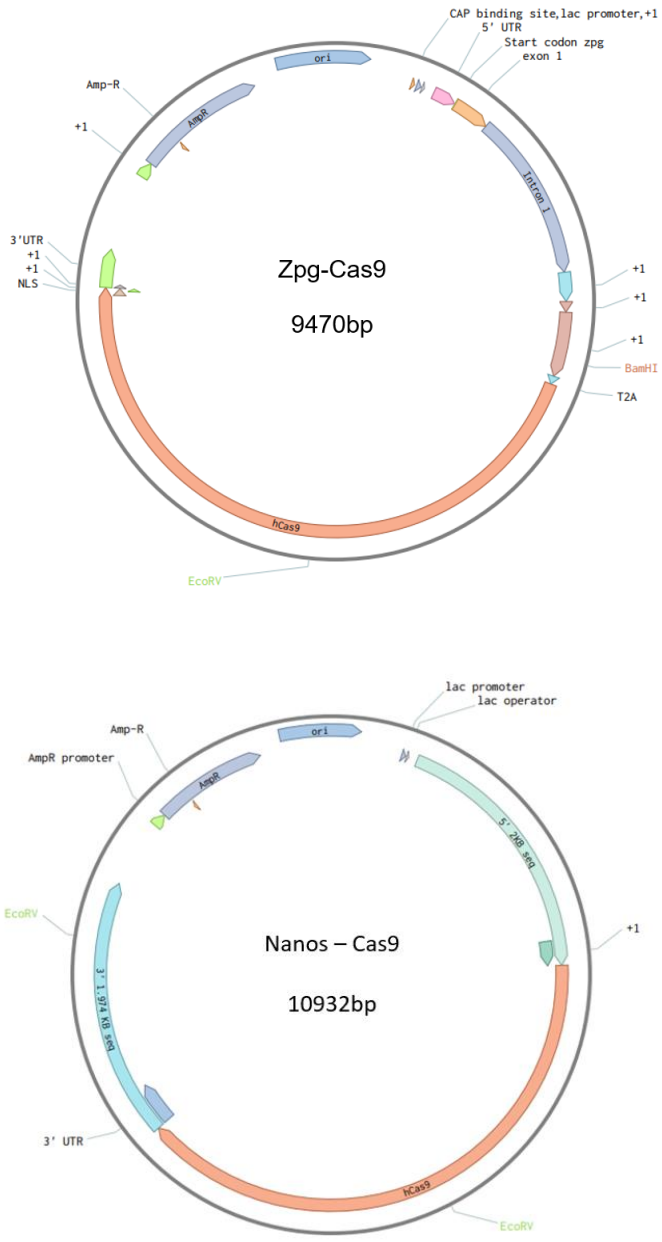
Interaction	Difference	Adj-p value
Nanos 0.35 – Nanos 0.2	188	-0.4425
Nanos negative - Nanos 0.2	-167	-0.5450
Zpg0.2 – Nanos 0.2	-79	0.9501
Zpg0.35 – Nanos 0.2	-76	0.9572
Zpg negative – Nanos0.2	-157	0.5983
Nanos negative – Nanos0.35	-355	0.0646
Zpg0.2 – Nanos0.35	-267	0.1806
Zpg0.35 – Nanos0.35	-264	0.1871
Zpg negative – Nanos0.35	-345	0.0724
Zpg0.2 – Nanos negative	88	0.9253
Zpg0.35 – Nanos negative	91	0.9158
Zpg negative – Nanos negative	10	1.0000
Zpg0.35 – Zpg0.2	3	1.0000
Zpg negative – Zpg0.2	-78	0.9526
Zpg negative – Zpg 0.35	-81	0.9452

Supplementary Table 2. Results of the Tukey HSD tests between all samples of *zpg-Cas9* and *nanos-Cas9* with 0.2pmol and 0.35pmol total concentration of DNA fragments, as well as negative controls

Supplementary Table 3. Primer sequences used in this study

Primer Name	**Sequence (5'-3')**
Nanos HiFi Assembly primers	
nanos 5'_fwd	5' – GACTCTAGAGCCATTGGATTTTTCAAACTTTC – 3'
nanos 5'_rev	5' – TATAGTCCATTTTGTTCGTTGATCTCGATC – 3'
hcas9_fwd	5' – AACGAACAAAATGGACTATAAGGACCAC – 3'
hcas9_rev	5' – AAAAAAACAATTACTTTTTCTTTTTTGCCTG – 3'
nanos 3'_fwd	5' – AGAAAAAGTAATTGTTTTTTTTTATGAAATGTTTCTG – 3'
nanos 3'_rev	5' – CCCGGGGATCTGAGTAGTGTTCTGTCCG – 3'
Nanos backbone_fwd	5' – ACACTACTCAGATCCCCGGGTACCGAGC – 3'
Nanos backbone_rev	5' – AATCCAATGGCTCTAGAGTCGACCTGCAGG – 3'
Zpg HiFi Assembly primers	
zpg 5'_fwd	5' – GACTCTAGAGCCCACCTCAATCCAAACTCG – 3'
zpg 5'_rev	5' – TATAGTCCATTGGGCCGGGATTTTCCTC – 3'
hcas9_fwd	5' – TCCCGGCCCAATGGACTATAAGGACCAC – 3'
hcas9_rev	5' – CTTTTATCGATTACTTTTTCTTTTTTGCCTG – 3'
zpg 3'_fwd	5' – GAAAAAGTAATCGATAAAAGTATCGTCCTAAG – 3'
zpg 3'_rev	5' – CCCGGGGATCTTATGTTTCACATACTTTATTCTATATTTG – 3'
Zpg backbone_fwd	5' – TGAAACATAAGATCCCCGGGTACCGAGC – 3'
Zpg backbone_rev	5' – TTGAGGTGGGCTCTAGAGTCGACCTGCAGG – 3'
Nanos sequencing primers	
nanos seq 2 fwd	5' - CCCAGGATGAATAACTGTAAC - 3'
nanos seq 3 fwd	5' - CACCTGAAGATATTGCTG - 3'
nanos seq 4 fwd	5' - CGATTCTGATTAGCACGC - 3'
nanos seq 4 rev	5' - ACAGAGTTGGTGCCGATGTC - 3'
nanos 3 seq 2 fwd	5' - TTTTGGGGTTGCTTGGGC - 3'
nanos 3 seq 3 fwd	5' - CACATCACTCAAGTCGAG -3'
nanos 3 seq 4 fwd	5' - CATTTGGTTGGAGATCTG -3'
nanos 3 seq 5 rev	5' - AAAGACTTCCCTCCCCATG - 3'
nanos hcas9 seq 1 rev new	5' - TGGCGTTGATGGGGTTTTCCTC - 3'
Cas9 sequencing primers	
hCas9 seq 1 fwd	5' - CCAAAGAAGAAGCGGAAG - 3'
hCas9 seq 2 fwd	5' - AAGCTGTTCATCCAGCTGG- 3'

hCas9 seq 3 fwd	5’ - GCCAGGAAGAGTTCTACAAGTTC - 3’
hCas9 seq 4 fwd	5’ - CTGCTGTTCAAGACCAACC - 3’
hCas9 seq 5 fwd	5’ - CACAAGCCCGAGAACATC - 3’
hCas9 seq 6 fwd	5’ - GACGAGAATGACAAGCTGATC - 3’
hCas9 seq 7 fwd	5’ - TGGGACCCTAAGAAGTAC -3’
hCas9 seq 8 fwd	5’ - TTACCCTGACCAATCTGGGAG - 3’
Zpg sequencing primers	
zpg seq 1 fwd	5’ - TCATTCGACTCGACCAACACGG - 3’
zpg seq 2 fwd	5’ - AAACCGAAGAGGGCCTATCTGG - 3’
zpg seq 3 fwd	5’ - TGAATCGAAGCCAAACCTCCCC - 3’
zpg seq 4 fwd	5’ - TTTTGCGAACGCGGTCGTCTTG - 3’
zpg seq 4 rev	5’ - ACCTTGGCCATCTCGTTGCTGAAG - 3’
Colony PCR primers	
zpg 3’ cas9 c fwd	5’ - AAAGTGCTGAGCATGCCCCAAG - 3’
zpg 3’ cas9 c rev	5’ - AAACGACGGCCAGTGAATTCGAGC- 3’
nanos seq 4 fwd	5’ - CGATTCTGATTAGCACGC - 3’
nanos seq 4 rev	5’ - ACAGAGTTGGTGCCGATGTC - 3’
zpg seq 4 fwd	5’ - TTTTGCGAACGCGGTCGTCTTG - 3’
zpg seq 4 rev	5’ - ACCTTGGCCATCTCGTTGCTGAAG - 3’



Supplementary Figure 4. Maps of *Zpg-Cas9* and *Nanos-Cas9* plasmid used in this study.