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TITLE:

Effective Oral RNA Interference (RNAi) Administration to Adult *Anopheles gambiae* Mosquitoes.

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SUMMARY:

The oral administration of dsRNA produced by bacteria, a delivery method for RNA interference (RNAi) that is routinely used in *Caenorhabditis elegans,* was successfully applied here to adult mosquitoes. Our method allows for robust reverse genetics studies and transmission-blocking vector studies without the use of injection.

ABSTRACT:

RNA interference has been a heavily utilized tool for reverse genetic analysis for two decades. In adult mosquitoes, double-stranded RNA (dsRNA) administration has been accomplished primarily *via* injection, which requires significant time and is not suitable for field applications.

To overcome these limitations, here we present a more efficient method for robust activation of RNAi by oral delivery of dsRNA to adult *Anopheles gambiae*. Long dsRNAs were produced in *Escherichia coli* strain HT115 (DE3), and a concentrated suspension of heat-killed dsRNA-containing bacteria in 10% sucrose was offered on cotton balls *ad-libitum* to adult mosquitoes. Cotton balls were replaced every 2 days for the duration of the treatment. Use of this method to target *doublesex* (a gene involved in sex differentiation) or *fork head* (which encodes a salivary gland transcription factor) resulted in reduced target gene expression and/or protein immunofluorescence signal, as measured by quantitative Real-Time PCR (qRT-PCR) or fluorescence confocal microscopy, respectively. Defects in salivary gland morphology were also observed. This highly flexible, user-friendly, low-cost, time-efficient method of dsRNA delivery could be broadly applicable to target genes important for insect vector physiology and beyond.

INTRODUCTION:

Many diseases are transmitted by mosquitoes, making the study of mosquito physiology and genetics an important undertaking. The use of RNAi in these organisms has been prominent in the last 20 years and has allowed for the functional characterization of many mosquito genes[1–5]. The most commonly used technique for dsRNA delivery has been microinjection, which has the drawbacks that it can injure the mosquitoes and requires significant time and effort. Oral delivery methods for RNAi have been tested, but mainly in the larval stage of the mosquitoes[6–9]. Oral delivery of dsRNA in adult mosquitoes has not been fully explored and could be a useful tool for the study of vector biology and vector control.

Malaria is transmitted by *Anopheles* mosquitoes when an infected female mosquito takes a blood meal from an uninfected host and injects saliva containing malarial parasites[10]. To ultimately be transmitted in the saliva of a mosquito, the parasite must overcome many hurdles, including evading the mosquito immune system, traversal of the midgut barrier, and invasion of the salivary glands[11]. Mosquito salivary gland (SG) architecture is key to parasite invasion and that architecture is controlled both by key salivary gland-expressed transcription factors as well as determinants of sexual dimorphism. Several highly conserved transcription factors are required for cellular specification and homeostatic maintenance of the salivary glands and for the production and secretion of salivary proteins that function in blood-feeding[12–14]. Fork head (Fkh) is a winged helix transcription factor that functions as a major regulator of insect SG structure and function (based on studies in fruit flies and the silkworm moth)[15–20]. In the *Drosophila* SGs, Fkh functions with Sage, an SG-specific basic helix-loop-helix (bHLH) transcription factor, to promote SG survival and saliva production[19]. An important, positive co-regulator of saliva production in *Drosophila* is CrebA, a well-studied leucine zipper transcription factor that upregulates the expression of secretory pathway genes[21–23]. There is also a strong degree of morphological differentiation in female salivary glands that likely plays a key role, not only in blood-feeding but also in the ability of parasites to invade this tissue[24].

Many of the genes involved in determining salivary gland survival, structure, physiology, and sexual dimorphism have complex spatiotemporal expression profiles[25–27], and the traditional delivery methods of dsRNA to induce RNAi are not always efficient at targeting these kinds of

genes in this or other tissues. However, oral delivery of dsRNA in the larval stage *Aedes aegypti* and *An. gambiae* mosquitoes has been used successfully to silence the female-specific form of the *dsx* gene[9,28]. Previous studies using dsRNA in mosquito salivary glands found that, although large amounts of dsRNA were required, the silencing effect was relatively long-lasting (at least 13 days)[29]. Here, the ability of heat-killed *E. coli* strain HT115 (DE3) expressing sequence-specific dsRNA for *dsx*, *fkh,* or *CrebA* to induce RNAi silencing of these genes in adult female mosquitoes was tested. Oral administration of dsRNA induced gene knockdown in *An. gambiae*, with clear reductions in mRNA levels and with phenotypes consistent with the loss-of-function of these genes. Thus, this approach will likely work to knock down the function of a variety of salivary gland genes.

PROTOCOL:

1. Cloning dsRNA into *E. coli* expression vector

1.1. Select the target gene sequence to insert into an appropriate vector for the expression of dsRNA. Retrieve the expression values from Vectorbase.org using the following method.

1.1.1. Search for a gene of interest (e.g., **Table 1**) on the homepage search box.

1.1.2. In the resulting gene page, navigate to the **8. Transcriptomics** section.

1.1.3. Look for the listed relevant RNA-seq and microarray gene expression experiments.

1.1.4. Transcribe values of interest into the spreadsheet software and create a data table.

1.2. Select a commercially available plasmid with at least one T7 promoter to be used. If the selected plasmid has only one T7 promoter (as most commercial plasmids do), include a second T7 promoter in the reverse primer to be used for the amplification of the dsDNA for the gene of interest.

NOTE: The dsRNA sequence for the target genes can be selected using the web application E-RNAi for the design of RNAi reagents[30]. Either long dsRNA (approximately 400 bp) or short-hairpin dsRNA (shRNA) can be designed based on specific gene sequences. These sequences should be amplified and sequenced for identity confirmation before cloning. The selected gene regions, plasmids, and promoters used in this study are listed in **Supplementary File 1**.

1.3. Perform cloning according to a simple one-step procedure described previously[9,31]. For this purpose, purify the PCR product and ligate to the linearized plasmid DNA. Use the product of the ligation for the heat-shock transformation of competent *E. coli* cells[32]. Select the transformed cells through blue/white screening. Confirm the orientation of the insert using a T7-primer PCR and confirm the sequence using M13 primers.

NOTE: White/blue screenings can be used when the plasmid selected for transformation carries the lacZ gene that codes for β-galactosidase. White colonies should contain the desired insert within the lacZ and can be selected to further confirm the presence and orientation of the target sequence[33].

1.4. Purify the plasmid from the first transformation and use it to transform competent *E. coli* HT115 (DE3) as previously described[34]. After confirmation that the plasmid with the insert is present in the competent *E. coli* HT115 (DE3), make glycerol stocks of bacteria for single use.

NOTE: An appropriate non-related control dsRNA should be acquired or prepared to use in every experiment. In this case, the sequence for the unrelated gene *aintegumenta (ant)* from *Arabidopsis thaliana* is used.

2. Preparation of heat-killed bacteria expressing dsRNA

2.1. Grow a culture from a single bacterial colony of *E. coli* strain HT115 (DE3) containing the dsRNA expressing plasmid in 50 mL of Luria Broth (LB) containing 100 µg/mL of ampicillin and 12.5 µg/mL of tetracycline, on a platform shaker (180 rpm) at 37 °C for 12 h.

2.2. Dilute the bacterial culture (1:1000) into 2x Yeast Tryptone (2x YT) media containing 100 µg/mL of ampicillin and 12.5 µg/mL of tetracycline.

2.3. Induce dsRNA production by adding 40 µM (final concentration) isopropyl β-D-1-thiogalactopyranoside (IPTG).

2.4. When the cells reach an $O.D._{600}$ = 0.4, approximately after 2 h of induction at 37 °C with agitation at 180 rpm, prepare a concentrated suspension of heat-killed bacteria as described by Taracena et al [9]. Pellet the cells by centrifugation (4000 x *g,* 4 °C, 10 min) and wash cells in one volume of sodium phosphate buffer (PBS).

2.5. Spin again under the same conditions, re-suspend in PBS to 1/100 of the initial volume, and place at 70 °C for 1 h.

2.6. Make 400 µL aliquots of the heat-killed bacteria and store these aliquots at -20 °C until further use (do not store for more than a week). This suspension of heat-killed bacteria contains the specific dsRNA for the RNAi experiments. Carry out this procedure both for the target-gene dsRNA-bacteria and for the un-related dsRNA-control to be used in each experiment.

3. Feeding mosquitoes with heat-killed bacteria expressing dsRNA

3.1. Defrost one aliquot of dsRNA (HT115 (DE3) bacteria suspension) and mix with 1.6 mL of 12% sugar solution containing 0.2% methylparaben.

3.2. Soak a small cotton ball in this solution and place the soaked cotton ball inside a cage containing 5-day-old mosquitoes. Ensure that the mosquitoes feed on this solution, picking up both the sugar and the dsRNA-containing bacteria simultaneously.

3.3. Change the cotton ball soaked in dsRNA-sugar solution every other day for 8 consecutive days.

3.4. Keep mosquito cages under constant conditions, i.e., 27 °C and 80% relative humidity with a photoperiod of 12 h:12 h light: dark photocycle, separated by a 30 min dawn and 30 min dusk period.

4. Assay target gene expression levels

4.1. Cold-anesthetize the mosquitoes by placing the container on ice for a min or until the mosquitoes stop moving. Once the mosquitoes are anesthetized, place them on a cold surface to isolate females for dissection.

4.2. Spray 70% ethanol to the mosquitoes and place them on a glass surface with PBS. With a pair of forceps, secure the mosquito head steady and pull the thorax very slowly, allowing the salivary glands to be released into the PBS.

4.3 Keep the salivary glands in ice-cold PBS until 10 individuals have been dissected. Pool Ten SGs for RNA extraction using the guanidinium thiocyanate-phenol-chloroform method. Suspend the RNA pellet in 30 µL of RNase-free water.

4.4. Use 1 µL aliquot of the RNA extracted from the SG in the previous step, to read absorbance at 260 and 280 nm and calculate the RNA concentration of each sample by multiplying with the dilution factor. A 260/280 ratio of ~2.0 indicates good quality RNA.

4.5. Use 1 µg of the purified RNA to synthesize complementary DNA (cDNA) using a commercial reverse transcription kit.

4.6. Make a 1:10 dilution of the cDNA to prepare an RT-PCR reaction according to the manufacturer's recommendations. For each sample, prepare a reaction for the target gene and in parallel, set up a reaction with the housekeeping (HK) gene. Set each gene reaction in a technical triplicate to eliminate the impact of random variation from the method.

NOTE: Here, the *An. gambiae* ribosomal *S7* gene (GeneBank: L20837.1) and *actin* (VectorBase: AGAP000651) have been used as HK genes.

4.7. Use all primers at a final concentration of 300 nM, following the SYBR-green manufacturer's indications. Amplify with standard PCR conditions: 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C.

NOTE: To quantify gene expression, the delta-delta-Ct method (ΔΔCt) is used. Delta Ct (ΔCt) is the difference between the Ct of the target gene and the Ct of the housekeeping gene. ΔΔCt is the difference between the ΔCt of the experimental group and the ΔCt of the control group[35].

5. Phenotypic evaluation: successful blood-feeding

5.1. To evaluate the ability to blood-feed, set groups of 15 female mosquitoes treated with target and control dsRNA on small cages (12 cm diameter) and starve them for 4 h.

5.2. Using a circulating water bath set to 37 °C, glass mosquito feeders (24 mm diameter) and parafilm membrane, offer defibrinated sheep blood to the mosquitoes.

NOTE: Blood can be acquired from a commercial vendor who aseptically draws it from healthy, donor animals of U.S. origin and manually defibrinates without anticoagulants or additives.

5.3. By direct observation, count and record the number of probing attempts to successfully acquire a blood meal from the first five females to become fully engorged in each group.

NOTE: To avoid significant metabolic changes in the mosquitoes, that could interfere with energy resources impacting blood-seeking behavior, starvation was kept to the minimum (4 h). As a result, not every mosquito would avidly seek the blood-meal and we limited the count of the engorged females to five (a third of each group's total), to reduce the effect of time variables such as exposure to human odor, temperature change between the chambers and the feeding surfaces, etc.

6. Phenotypic evaluation: Salivary gland morphology and down-regulation of relevant proteins

6.1. Isolate fresh tissue in 1x Phosphate-buffered saline (PBS) as described in step 4.2 and fix in ice-cold acetone for 90 s. Rinse several times in 1x PBS after removing the acetone. Incubate with primary antibodies overnight at 4 °C with antiserum (see **Table of Materials**) diluted into 1x PBS.

NOTE: See **Table of Materials** for identification of the primary antibodies used for saliva proteins (*Anopheles* anti-platelet protein, AAPP; Mucin 2, MUC2), SG transcription factors (Fork Head, fkh; Sage, sage; Cyclic-AMP response element-binding protein A, CrebA), and a marker of secretory vesicles (Rab11). These antibodies are used as readouts for SG form and function. However, any antibody suitable for immunofluorescence should be suitable for this protocol.

6.2. Wash in 1x PBS several times. Add secondary antibodies (fluorescent) diluted in 1x PBS, and incubate in the dark at room temperature for 2 h. Add any counterstain [such as 4',6-diamidino-2-phenylindole (DAPI; DNA), wheat germ agglutinin (WGA; for chitin), phalloidin (for F-actin), and/or Nile Red (for lipids)] 30 min before the end of the 2 h incubation.

6.3. Wash three times in 1x PBS. Then, mount the tissues in 100% glycerol on a standard microscope slide with a 1 mm thick coverslip and store at -20 °C until imaging using a fluorescence confocal microscope.

NOTE: To obtain quantitative data, imaging settings must be held constant. Here, only maximum intensity projection images through the entire 3D volume of the tissue were included, and all image quantification was normalized between treatments (within an experiment) based on DAPI signal in non-SG tissue remnants (fat body, cuticle, or head) also present on the slide.

REPRESENTATIVE RESULTS:

To begin, microarray expression data from VectorBase was used to scan potential targets across developmental stages[36,37] to determine the expression status of all genes relevant to the current study (**Table 1**). As expected, all our chosen target genes showed expression in adult SGs. Levels of *aapp* and *sage* were particularly high (**Table 1**). Also of note were the high levels of expression of *f-Agdsx* in adult female SGs[9].

Specific segments from each gene were evaluated for use as dsRNA using the web application *E-RNAi for the design of RNAi reagents*[30]. The ~400 bp regions containing sequences unique to each target gene were then cloned (**Figure 1A**), transformed into the appropriate bacterial strains, and used to prepare suspensions of heat-killed bacteria, which were induced to produce dsRNA. Adult mosquitoes were fed for 8 days on the sucrose-soaked cotton balls containing the bacterial suspensions of dsRNA for *f-Agdsx, fkh*, or *ant* (the unrelated negative control).

For the analysis of RNAi feeding of female mosquitos, it was first determined whether *f-Agdsx* or *fkh* dsRNA-feedings induced gene silencing. A 98.8% reduction (±2.1) in *fkh* transcript levels was observed in the group fed with *fkh*-dsRNA (**Figure 1B**), indicating that the dsRNA very effectively reduced the abundance of *fkh* transcripts in SGs. Surprisingly, *fkh* mRNA levels were reduced by 82.0% (±18.9) in the mosquitoes treated with dsRNA for *f-Agdsx,* which had an 89.86% (±4.48) of *f-Agdsx* reduction, suggesting that *fkh* could be a target of F-Dsx in the salivary gland. Concomitant with the significant reduction in *fkh* expression levels, the *fkh*-knockdown mosquitoes exhibited a significant increase in the number of probing attempts needed to blood-feed. These mosquitoes exhibited, on average, five times more feeding attempts than the control group or *f-Agdsx* dsRNA fed mosquitoes to be completely engorged with blood (**Figure 1C**). This led to asking whether the *fkh* knockdown RNAi treatments caused changes in localization and/or distribution of key transcriptional regulators (SG TFs Sage and CrebA) (**Figure 2**), secreted proteins (AAPP and mucin) (**Figure 3**), and secretory machinery [Nile Red (lipids) and Rab11 (secretory vesicles)] (**Figure 4**). Importantly, substantial differences in staining intensity were observed across different lobe regions, lobes, and individual SGs.

As predicted, levels of *sage* and *CrebA* staining were markedly reduced in all SG lobes following *fkh* RNAi (**Figure 2B**) compared to *ant* control RNAi (**Figure 2A**). Reductions in both the highest maximum intensity values (red dashed lines and numeric labels) and lowest maximum intensity values (blue dashed lines and numeric labels) in line scan profiles suggested reductions in areas

of both high and low signal within the tissue (**Figures 2A,B**). These data suggest that *An. gambiae* *fkh* RNAi is effective and that *fkh* regulates the production and/or stability of the SG TFs *Sage* and *CrebA* in *An. gambiae*, analogous to their genetic relationship in *Drosophila* SGs[19,38,39].

When considering highly abundant saliva-component proteins, levels of *Anopheles* anti-platelet protein (AAPP)[40,41] were reduced in all three SG lobes following *fkh* RNAi, compared to control RNAi treatment (**Figure 3A,B**; green). On the other hand, no changes in levels of Mucin were observed (**Figure 3A,B**; purple). These data suggest that Fkh contributes differently to the expression of different saliva protein genes.

Finally, two markers of secretion were observed (**Figures 4A,B**): Rab11 (vesicles associated with apical recycling endosomes)[42] and Nile Red (lipids). Reduced Rab11 fluorescence was observed in distal lateral (DL) lobes following *fkh* RNAi treatment (**Figure 4A v** vs. **4B v**; green). However, increased Rab11 signal in the medial (M) and proximal lateral (PL) lobes (**Figure 4A vii, ix** vs. **4B vii, ix**; green) also occurred. No discernible difference was observed in Nile Red signal (**Figures 4A,B**; purple) after *fkh* RNAi compared to the control RNAi treatment. These data suggest that *fkh* reduction may alter some secretory machinery action in a complex manner that differs between SG lobes.

FIGURE AND TABLE LEGENDS:

Table 1: Mean log2 microarray expression profiles for *An. gambiae* genes of interest. Shown are gene names, functional category, Vectorbase (AGAP) identifiers, and mean log2 microarray expression data gathered from Vectorbase. These data indicate that our genes of interest (involved in salivary gland (SG) cell biology and secretion) are expressed and enriched in larval stage 3 (L3) and adult SGs, as compared to whole individuals.

Figure 1: *f-Agdsx* and *fkh* knockdown in adult *An. gambiae* reduces *fkh* mRNA levels in the SGs and affects the female ability to blood-feed. (**A**) Representative image of the plasmid design utilized for dsRNA production in this methodology. The second T7 promoter sequence is added to the plasmid by including it in the 3' primer used to amplify the insert to be cloned into the pGEMT plasmid. The plasmid is then transformed into *E. coli* HT115 (DE3) bacteria and a feeding solution is made of a suspension of induced heat-killed bacteria in 10% sugar water. (**B**) Animals fed with a dsRNA feeding solution for either *f-Agdsx* or *fkh,* showed significantly lower levels of *fkh* transcripts (one-way ANOVA with multiple comparisons; n=15). However, only the group fed with *fkh* dsRNA (**C**) showed a significant difference in the number of biting attempts needed to acquire a blood meal. Mosquitoes in this group needed, on average, five times the number of probing attempts to obtain a successful blood meal than needed by the control or the *dsx*-dsRNA fed groups (one-way ANOVA with multiple comparisons; n=15). Error bars indicate the Standard Error of the Mean (SEM). Each experiment was conducted in three separate biological replicates.

Figure 2: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG transcription factor levels. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5–13) of oral exposure to either (**A**) non-related dsRNA control (*ant*) or (**B**) dsRNA targeting the SG TF *fork*

head (*fkh*, AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), antisera against the SG TFs Sage (green) and CrebA (purple). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes, and labeled "inset") indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each zoomed lobe are plotted (always from left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dotted lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct.

Figure 3: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG secreted protein levels. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5–13) of oral exposure to either (**A**) non-related dsRNA control (*ant*), or (**B**) dsRNA targeting the SG TF *fork head* (*fkh*,AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), and the saliva proteins AAPP (green) and Mucin (MUC2, purple). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes) indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each lobe are plotted (always from left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dashed lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct. Italic "*DL*" labels (Bi) indicate two visible regions of the same DL lobe.

Figure 4: *fkh* knockdown in adult *An. gambiae* salivary glands reduces SG secretion markers. Shown are representative images from day 13 adult female *An. gambiae* SGs after 8 days (days 5–13) of oral exposure to either (**A**) non-related dsRNA control (*ant*), or (**B**) dsRNA targeting the SG TF *fork head* (*fkh*, AGAP001671) in 10% sucrose stained with the dyes DAPI (DNA; red), labeled wheat germ agglutinin (WGA, chitin/ O-GlcNAcylation; blue), Nile Red (lipids; purple), and antisera against the recycling endosome vesicle marker Rab11 (green). Scale bar lengths shown are microns. SGs (i) are outlined with white dashes. Yellow lines in zoomed lobe images (of the regions enclosed by yellow boxes) indicate where the line scans of signal intensity were conducted. Green and purple channel intensities corresponding to line scans for each lobe are plotted (always left to right in the SG) in the graphs below the images; X-axis = distance (in pixels) and Y-axis = gray unit (pixel intensity). The pixel intensity's dynamic range is delimited by red (maximum) and blue (minimum) dashed lines and the corresponding values are shown on each graph. MIP = maximum intensity 3D projection through the entire SG depth. DL: distal lateral lobe; M: medial lobe; PL: proximal lateral lobe; SD: salivary duct.

DISCUSSION:

The ability to effectively deliver dsRNA to *An. gambiae* mosquitoes by oral feeding has broad implications for studies of vector biology both in the laboratory and in the field. Microinjection has long been accepted as the preferred mode of delivery of chemicals, antibodies, RNAi, and genetic modification strategies in mosquitoes[43,44]. The consequence of substantial physical manipulation, cellular damage, and stress can be avoided by the use of oral delivery, which could also be potentially suitable for large-scale or field applications. Previous work has suggested that RNAi acts ubiquitously within an individual adult mosquito[29], allowing for effects in all tissues, including salivary glands. By feeding mosquitoes with large numbers of dsRNA-expressing *E. coli* that are digested asynchronously over a long timeframe, one can potentially achieve consistent and uniform exposure to the RNAi across all individuals in a cage. This method allows to feed large numbers of mosquitoes and analyze potential variability of the resulting phenotypes depending on the target gene. However, one important consideration is the possibility of heterogeneous distribution of the bacteria, and hence dsRNA, in the cotton fiber. The 400 µL of bacteria used daily for mosquito sugar-feeding would contain approximately ≤4.6 µg of dsRNA, as described and calculated previously[9] but the amount of dsRNA ingested by each mosquito was not individually determined. If building dsRNA constructs becomes routine, this simple treatment protocol allows for rapid assimilation of this technique by any mosquito researcher. *A priori*, the time expenditure during treatment (30 min per day) is trivial compared to the time taken to learn and apply microinjection to similar sample sizes.

Feeding dsRNA is routinely used for reverse genetics studies in the model organism *Caenorhabditis elegans*[45]. This heavy level of use underscores the value of the oral delivery approach. Construction of an *An. gambiae* genome-wide library in transformed *E. coli*, similar to that which exists in *C. elegans*[46,47], would allow for rapid reverse genetic screening in mosquitoes at an increased scale. However, it is important to note that the efficiency of the method depends in great measure on the endogenous levels of transcript and if the expression is not limited to the target tissue but expressed more broadly[4,8,44]. Additionally, there is evidence that some insecticides could induce behavioral avoidance from mosquitoes[48], and feeding with bacteria that potentially induce adverse effects in them could trigger similar patterns of avoidance. In the controlled setting of the laboratory, where the mosquitoes did not have an alternative food source, they did not have a choice to avoid the sugar water with *E. coli* and the need for a nutritious source would probably override the instinct to avoid the bacteria. However, this should be considered if the strategy were meant to be used in less controlled settings.

It may be possible to target multiple genes simultaneously (using one construct, multiple constructs, or a mixture of transformed bacterial isolates), but further studies are needed to assess effectiveness. Another important consideration to this point is the evaluation of possible off-target or synergistic effects when using single or multiple targets. The establishment of appropriate control genes and groups is an important part of the experimental design. Further, it is tempting to speculate that this approach could be used to target other pathogens or viruses[49]. Previous work toward RNAi induction in mosquitoes was performed under conditions where the reagent was directly injected, so *E. coli* were not present. The *E. coli* may provide a

protective compartment allowing for the slower release of dsRNA over time, ensuring that exposure is more or less continuous over a much longer period[29].

Finally, these results show that the effects of this technique are tunable by adjusting the time frame (length and starting day) of exposure and the quantity of *E. coli* used. This feature allowed us to study the functions of essential genes (*dsx* and *fkh*) by identifying optimal knockdown conditions by trial and error. This greatly enhances the likelihood that target genes of interest can be investigated using this technique.

In summary, it was found that oral delivery of RNAi to adult mosquitoes can be simple, versatile, and a powerful approach to studying mosquito gene function and for the creation of novel and malleable tools for vector control of mosquito-borne diseases.

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DISCLOSURES:

The authors report that they have no conflicts of interest to disclose.

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Figure 1

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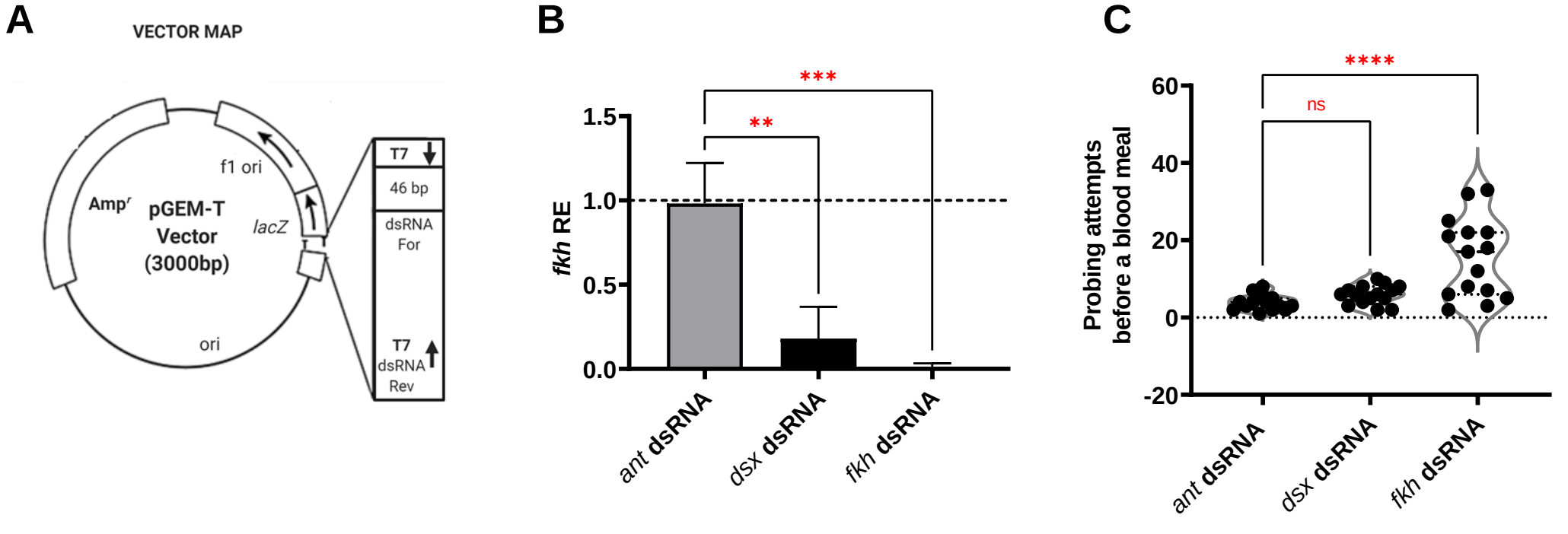

Figure 2

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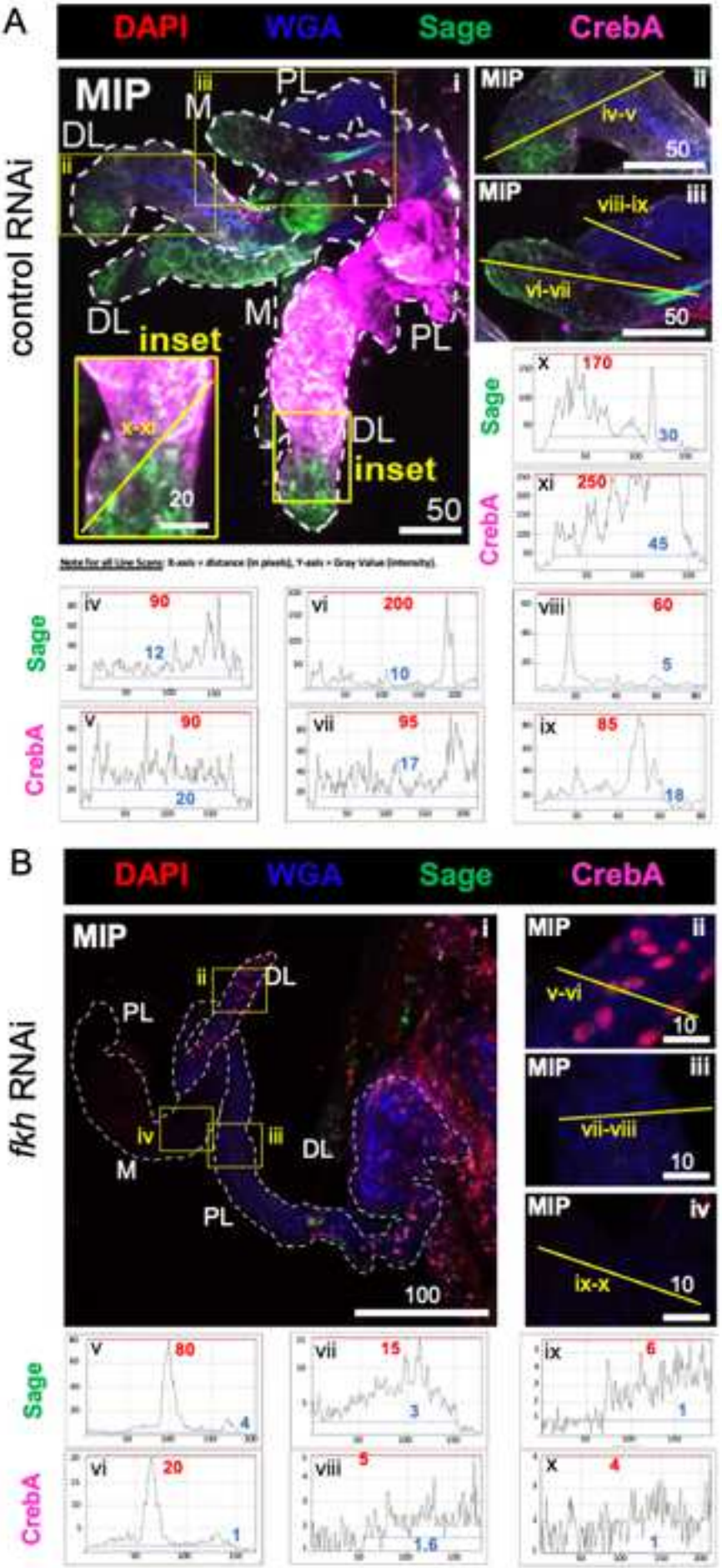

Figure 3

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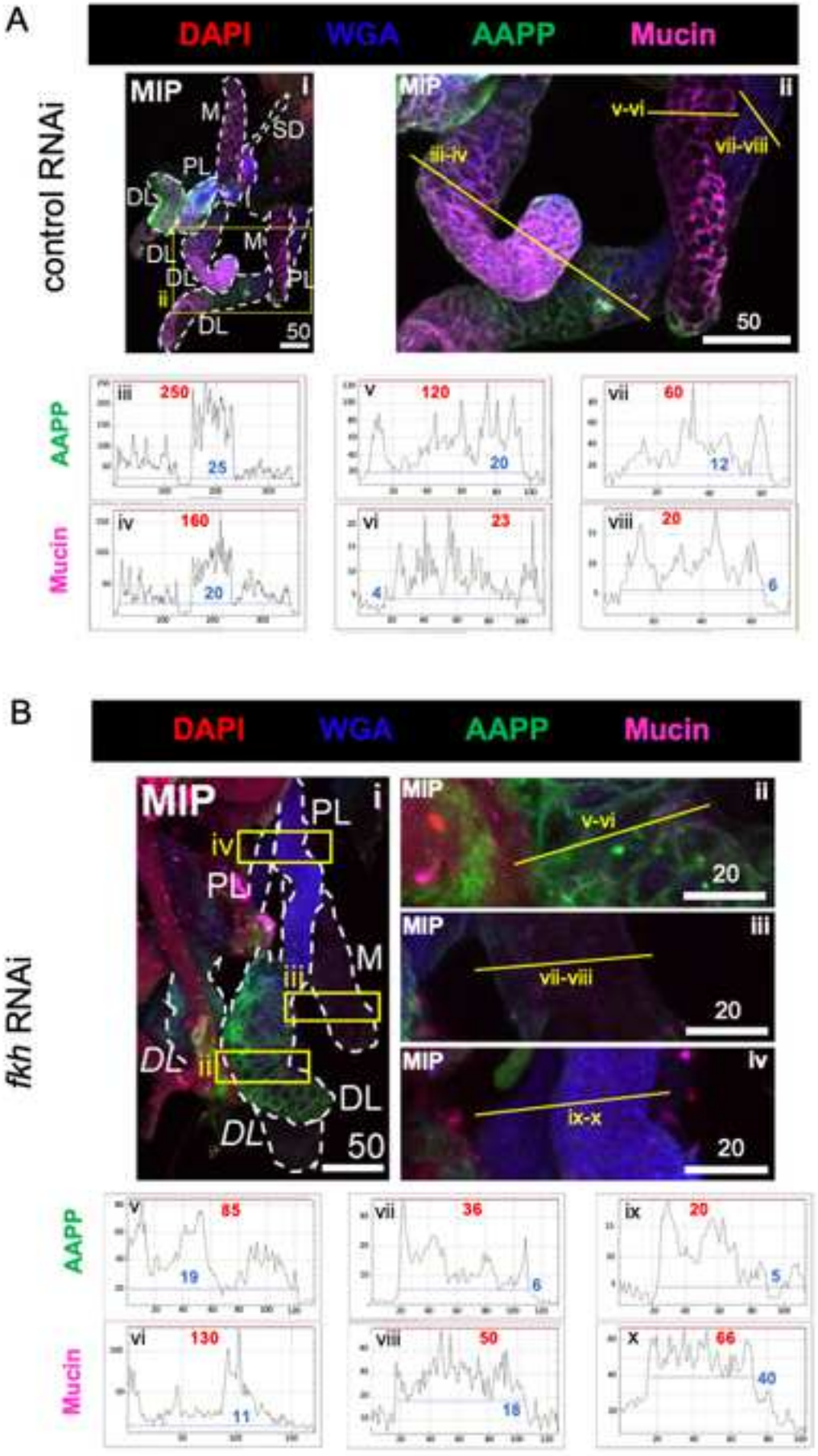

Figure 4

Click here to access/download;Figure;MT MW RNAi Methods - Vec Fkh Images - v3_Fig4.tiff

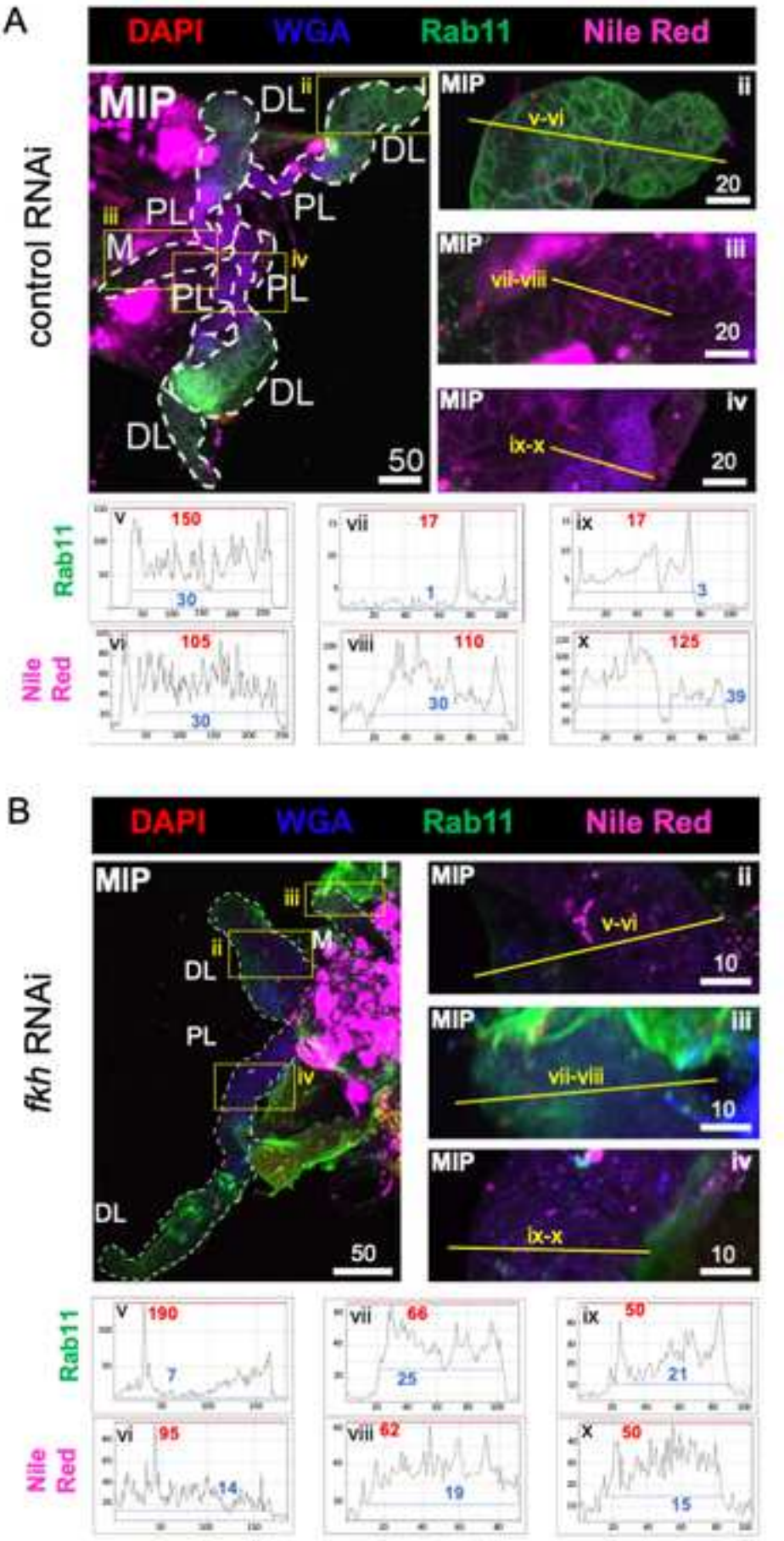

Table 1. Mean log2 microarray expression profiles for Anopheles gambiae genes of interest.

		Dataset:	*Goltsev*	*Neira Oviedo*	*Neira Oviedo*
gene symbol	**function**	**AGAP ID**	**embryo (25 hr.)**	**L3 larvae**	**L3 SG**
AAPP	saliva protein	AGAP009974	3.92	4.38	4.33
CrebA	txn factor	AGAP001464	6.28	5.22	5.92
"	txn factor	AGAP011038	4.50	4.46	5.23
dsx	txn factor	AGAP004050	4.91	5.39	5.55
fkh	txn factor	AGAP001671	5.18	4.67	5.25
MUC2	saliva protein	AGAP012020	4.59	5.53	5.63
Rab11	vesicular trafficking	AGAP004559	10.21	7.47	8.60
sage	txn factor	AGAP013335	5.32	5.96	8.89

Baker	*Baker*	*Baker*	*Baker*
adult female body (3 day)	**adult male body (3 day)**	**adult female SG (3 day)**	**adult male SG (3 day)**
3.81	2.46	11.92	2.69
2.99	2.96	3.27	3.13
2.96	2.86	3.05	2.88
3.72	4.00	4.57	4.01
2.99	3.09	3.21	3.05
2.96	3.07	3.08	3.26
4.90	3.79	3.38	2.96
3.40	3.33	7.37	7.23

October 6th, 2021

Response to the revision made for the manuscript JoVE63266 "Effective oral RNA interference (RNAi) administration to adult Anopheles gambiae mosquitoes."

We would like to thank the editor and the reviewers for all the work done to make the manuscript acceptable for publication. Our response, actions, or comments to each individual point are as follows:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   Spelling and grammar has been reviewed
2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
   Personal pronouns have been removed throughout the text.
3. Please define all abbreviations at first use.
   All abbreviations have been defined at first use now.
4. Introduction: Line 66-68 – Please include citations to support the statements.
   Citations have been included.
5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
   For example: Trizol, Zeiss
   Trademarks have been removed.
6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.
   Numbering in the protocol section has been adjusted and bullets have been eliminated.
7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
   The protocol section has been modified to be compose entirely of discrete steps with each step containing less than 4 sentences.
8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
   The structure of the protocol has been reviewed for it to contain only action items with the specifications to perform each action.
9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that

cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

Protocol section is now written in imperative tense with a few notes on it.

10. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

    Details have been added where they were missing to contain enough detail to explain how the method was performed.

11. Please add more details to your protocol steps:

    Line 102: Please provide a NOTE on blue/white screening. Please include citations in the NOTE if needed.

    A note on blue/white screening has been added with the reference for the method.

    Line 127: Please mention the centrifugation temperature.

    Temperature has been added

    Line 149: Please mention how the mosquitoes were anesthetized. Please include citations if any previously published method was adopted.

    The mosquitoes are anesthetized by placing them in ice, this has been further clarified.

    Line 153: Please include details on the gene quantification method.

    Details regarding the gene quantification method have been added.

    Line 164-166: Please provide details on the methods of blood-feeding and recording. Since we film the protocol, all these details are essential.

    Details on the blood-feeding method have been extended.

12. Please provide a list of all the gene sequences used, plasmids, and promoters used in the study in a separate Supplementary File. Please reference the file in the manuscript text and include a legend in the Figure/Table legend section.

    A supplementary file for this purpose has been created and referenced in line 196.

13. Please provide details of the antibodies used in the study in the table of materials.

    Antibody details have been added in the table of materials.

14. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and it should also be in line with the Title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

    Protocol has been highlighted.

15. Please provide Table 1 as xlsx file. Also, please include a legend in the Figure/table legend section.

    Table 1 has been changed to xlsx file and the legend has been added in the appropriate section.

16. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

    a) Critical steps within the protocol

    b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The discussion has been modified according this and the comments from the reviewers to include this information.

17. Please do not abbreviate the journal names in the References.
Journal names abbreviations have been removed.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
Good manuscript, missing data regarding mosquito survival which is in discussion but not presented.
We thank the reviewer for the revisions. Unfortunately, we did not have all the data recorded with enough replicates to include the survival as a full result for this manuscript and due to the pandemic in the last year, repetition of the experiments for this purpose was impossible. We do realize that quoting the results without showing the numbers with full statistics is not ideal, and therefore we have removed the mention of this result in the discussion.

Major Concerns:
Needs to include missing data. Insets in Figure 2 wholly unreadable, no idea what I'm supposed to get from reading these if I can't see the Y axis number or X axis clearly at all and there's no clear distinction between these and controls.

Figure 2 and its legend have been significantly modified to make the results easier to obtain. The inserts in this figure show the pixel intensity's dynamic range, delimited by red (maximum) and blue (minimum) dashed lines and numbers on each graph (Y axis, gray value). The X axis denotes the position along the line scan in pixels. Numbers and axes have been adjusted for better representation of our data.

Minor Concerns:
Text needs editing in places.

Errors noted on:

Multiple lines - citation needs to go after period. ***This has been corrected.***
Line 35 - dsRNA defined before definition below. ***Definition of the abbreviation has been corrected.***
Line 36 - talk about damage to organism is unnecessary and would be better to talk about how oral RNAi is field applicable while injection is not. ***The applicability to larger and field applications has been noted.***
Line 40 italicize ad-libitum. Italics needed for Anopheles on line 60 and other places to.

Formatting has been corrected.
Line 64 - Question mark in SG ***Question mark was removed.***
Line 110 - No need to say Timmons 2000 with citation there. ***The form of the citation was corrected.***
Line 164 - give a bit more detail about blood feeder setup, was parafilm used? How long was feeding allowed? ***Details about the blood feeder were added.***
Figure 2 - please give more detail about insets i-ix. ***Insert details have been included in the legend of the figure.***
Line 283 - Text is red coloured in places. ***Formatting has been corrected.***
Line 289-290 - talks about mortality but no data shown, either remove comment or include data. ***The mention to this data has been removed.***

Reviewer #2:
Manuscript Summary:
The manuscript by Taracena et al., entitled "Effective oral RNA interference (RNAi) administration to adult Anopheles gambiae mosquitoes" describes an effective, efficient technique of delivering double-stranded RNAs to adult *Anopheles gambiae*. Bacteria expressing dsRNA targeting genes essential to salivary gland function were heat-killed and mixed with sucrose solutions and fed to adult mosquitoes for 8 days. Treated mosquitoes demonstrated significant knockdown of the target gene fork head (FKH; determined through qPCR) and morphological defects to salivary glands. The authors provide clear methodologies and this technique is much easier to perform than microinjections, which are typically used in many mosquito RNAi experiments. The manuscript will be of interest to many mosquito researchers, and with only a few minor recommended edits, listed below, this manuscript will be suitable for publication.

We thank the reviewer for the positive summary and the acknowledgment of the use of the method for mosquito researchers.

Major Concerns:
1. The authors fed mosquitoes bacteria expressing bacteria expressing dsx dsRNA, and noted that fkh transcripts were reduced. They suggested that dsx transcript reduction impacted fkh, indicating possible regulation of fkh by dsx, but they neglected to report the extent of dsx transcript knockdown. Can they provide the data for dsx transcript reduction?
We thank the reviewer for noticing that this information was missing. We have added the value of the f-Agdsx expression within the text (89.86% (±4.48)), line 392.

2. Can the authors estimate the dosage of dsRNA that applied to the cotton wicks? How much dsRNA was produced by the mass of E. coli applied to those wicks? I anticipate that dsRNA doses may vary with only slight differences in IPTG/bacterial density/temperature, etc, so it would be helpful to know if there was a way to ensure precise dsRNA dosings using this method.
This is an excellent point made by the reviewer, and we have now added a comment in the discussion touching the subject. We have clarified that even if we could estimate the

amount of dsRNA which was placed in the cotton wicks, with the current method we could not estimate the specific amount of dsRNA ingested by each mosquito.

3. The line scan analyses in Figure 2 are difficult to interpret, as the axes' labels are too small to read, and they are not too meaningful as they are only sample reads. The sample images provide a good visual difference between control and fkh feedings, but the line scan data would be better summarized in graphical form, providing a quantitative presentation based on an appropriate number of replicate samples.

Figure 2 has been modified to make the interpretation easier for the readers.

Minor Concerns:
4. Do mosquitoes feed as much on sucrose when it is contaminated with E. coli? i.e. Can the authors comment on whether the bacteria could be a feeding deterrent, and if the mosquitoes might have acquired bigger doses of dsRNA if purified dsRNA was simply mixed with the sucrose?

This is another excellent question raised by the reviewer, given that there is evidence that some insecticides can induce behavioral avoidance from mosquitoes. If mosquitoes were to sense the adverse effects of the E. coli contaminated sugar meal, it is not difficult to imagine they could develop a similar pattern. Maybe, if given the choice, the mosquitoes could prefer to feed from a clean sugar. In the case of our experiments, the laboratory setting used lacked an alternative food source for the mosquitoes, so they were forced to eat the contaminated sugar. If they ate less was not measured at this time, since the concentration offered seemed to induce sufficient effect. This considerations have now been added in the discussion and we believe they greatly contribute to the interpretation of the data.

5. In the introduction, the authors listed several published studies of mosquitoes fed dsRNA, but reference 7 doesn't describe mosquitoes. I think they meant to refer to another Whyard et al. publication that describes feeding of dsRNA to mosquito larvae: (parasitesandvectors.biomedcentral.com/articles/10.1186/s13071-015-0716-6).

This has been corrected.

Reviewer #3:
Manuscript Summary:
This manuscript describes a protocol for oral delivery of dsRNA produced by *E. coli* for gene silencing in mosquitoes. The method was exemplified by gene silencing in the salivary gland. The efficacy of gene silencing was evaluated by expression abundance at both mRNA and protein level. The loss-of-function phenotypes by the gene silencing were presented as well.

We thank the reviewer for the feedback and the comments on the minor concerns section have been addressed.

Major Concerns:
None

Minor Concerns:
Protocol Step 4 describes the qPCR methods for quantification of gene expression level, and Table 1 was specified to represent the data. However, in Representative Results, Table 1 represents microarray data that were extracted from published data (gathered in Vectorbase). It would be appropriate to describe this data extraction from datasets in the public domain in Protocol Step 4. The qPCR data were actually presented in Figure 1B. Please correct.
We thank the reviewer for noticing thing, the protocol step 4 has been modified and the mention of table 1 and figure 1 have been cited properly inside the text.

Reviewer #4:
Manuscript Summary:
The title of the manuscript refers to RNAi in general. However, despite the fact that at some point (line 106) the possibility of using short-hairpin dRNA (shRNA) instead of long dsRNA is mentioned, the information is mostly focused on the use of dsRNA.
Given that it is possible for beginners and researchers unfamiliar with RNAi technology to have access to this work; I think it might not hurt to specify some aspects that I allow myself to respectfully mention below.
There is no mention of the fact that the amount of dsRNA administered to each mosquito is not controlled, but on the contrary, it is only called attention (ie line 288) that "one can potentially achieve consistent and uniform exposure to the RNAi across all individuals". Not to mention drawbacks of oral feeding.

We thank the reviewer for this observation, and an appropriate mention of this limitation of the method has been added in the discussion in line 596

It does not mention the fact that regardless of whether "An appropriate non-related control dsRNA" (line 113) is used, this does not rule out the possibility of nonspecific effects of the dsRNA used. The foregoing is not commented, not even as a remote possibility (line 211) when a decrease in the levels of the fkh transcript was observed when administering dsRNA for f-Agdsx.

To this point, we have added this consideration in the discussion, line 561

The recommendation to group 10 SGs (line 150) is practical to determine the expression level of the specific gene, however, the determination in individual organs allows validating the homogeneous level of silencing that is being achieved with the form of administration that is being recommending.In this case, I would not so easily recommend blind trial and error without knowing, since the levels of dsRNA that are being administered individually are not known at any time and can vary significantly from one experiment to another. Although the opposite can be assumed, perhaps the scenario of observing off-target or toxic effects due to the administration of excessive RNAi can be reached.

We thank the reviewer for this helpful comments, we believe that with the modifications made in the discussion (lines 596, 652, and 661)

Line 254, the fkh silencing values with the f-Agdsx specific dsRNA are not shown or mentioned.

This value has been added in the results section, line 392.

The above in order to know how much is the difference in the level of silencing observed, if there was one, between both dsRNAs (fkh and f-Agdsx) and to know if this difference in the level of silencing could explain the difference in the number of biting attempts needed to acquire a blood meal.

Congratulations on the job.
Thank you very much.

Major Concerns:

Minor Concerns:

Reviewer #5:
Manuscript Summary:
The authors describe a method to deliver dsRNAs to mosquitoes via oral feeding. This method is able to achieve efficient knock down of the expression of genes ion mosquito body, particularly of salivary glands (which are the target of the presented study).

Major Concerns:
I have no major concerns. The approach is nice, well designed and clearly explained.

We would like to thank the reviewer for the positive feedback and the interesting questions posed. We have tried to address them both in our response here and in the discussion in the manuscript.

Minor Concerns:
I have few questions that the authors might consider during the revision.

Line 64: (?SG)...I think it is a typo error.

This was in fact a typo, it has been corrected.

Line 79: actually, to my best knowledge, salivary glands are one of the tissues in which it is "easy" to achieve knockdown via dsRNA injections; there are other tissues that are more resilient (such as reproductive tissues). I think the point here is the amount of dsRNA needed, the cost and the time for the experiment.

We thank the reviewer for this observation, our intention in this sentence was to point out that RNAi by injection is not always efficient at targeting genes with complex spatiotemporal expression profiles in the salivary gland or in other tissues. The sentence has been modified to better reflect this point.

Line 140; line 311: the authors say that they used 5 days old mosquitoes and that the system is tunable. Why don't use mosquito that are 1 day old (with SG this is particularly relevant, as at 1 day old they do not have mature SG)? Do the authors have more data about the time when the bacteria are fed to the mosquitoes and the effects on the knockdown?

This is an excellent point made by the reviewer, unfortunately, we do not have more data about early timepoints. We did some preliminary tests, that didn't result on striking

phenotypes, our hypothesis is that after emergence the mosquitoes emerge with enough reserves that do not feed enough in the sugar water with dsRNA in time for us to observe an effect. However, as discussed before, these genes we targeted are complex spatiotemporal expression profiles, it is possible that genes that are exclusively expressed in the gut, or in other tissues, with different levels of expression, can be silenced in earlier timepoints.

Supplementary file

Gene sequences used

Cloned sequence of Forkhead for dsRNA

>FKH

ATGTCGGGCAACTGTCTCACCTCGACGCCGATCGGGTACAGCTCGATGGGGTCGCCGATCAGCAACATGG
GCTCGTGCATGGGCGGCAACGGCATGAGCACGATGGCGGCCATGTCCGGGTACTCGAGCGTGGCCGGCAG
CCGGGAGGTGCTGGGCGATCCGAGCTCGCCAAACTCGGTCGCGCTGCAGCGGGCCCGCACGGAGAAGCCG
GCCGCGACGTACCGGCGGAACTACACGCACGCCAAGCCGCCCTACTCGTACATCAGCCTGATCACGATGG
CGATCCAGAACAAAA

```
AGAP001671      ATGCAAAAGCTCTACCCGGAAAGCTCGATCAATACGAGCAGTAGCATGGCGGTGGGCGGT   60
FKH2            ------------------------------------------------------------   0

AGAP001671      GGAGGCGGAGGCGGCGGCGGCGGCGGTGGCGGCAACATGAGCCCGATGGCCACCACGTAC   120
FKH2            ------------------------------------------------------------   0

AGAP001671      AGCAGCATGAACAGTATGGGGATGGCGGTCGGCGGCATGACGTCCGTGTCGCCCCAGGGC   180
FKH2            ------------------------------------------------------------   0

AGAP001671      GGCGGGTTCGGCGCGACCGTGCTCGGCAGCCCGGGCATGGGCGGGATGGGCGCCGCCATG   240
FKH2            ------------------------------------------------------------   0

AGAP001671      AACAGCATGTCGGGCAACTGTCTCACCTCGACGCCGATCGGGTACAGCTCGATGGGGTCG   300
FKH2            ------ATGTCGGGCAACTGTCTCACCTCGACGCCGATCGGGTACAGCTCGATGGGGTCG   54
                      **************************************************

AGAP001671      CCGATCAGCAACATGGGCTCGTGCATGGGCGGCAACGGCATGAGCACGATGGCGGCCATG   360
FKH2            CCGATCAGCAACATGGGCTCGTGCATGGGCGGCAACGGCATGAGCACGATGGCGGCCATG   114
                ************************************************************

AGAP001671      TCCGGGTACTCGAGCGTGGCCGGCAGCCGGGAGGTGCTGGGCGATCCGAGCTCGCCCAAC   420
FKH2            TCCGGGTACTCGAGCGTGGCCGGCAGCCGGGAGGTGCTGGGCGATCCGAGCTCGCCAAAC   174
                ************************************************************ ***

AGAP001671      TCGGTCGCGCTGCAGCGGGCCCGCACGGAGAAGCCGGCCGCGACGTACCGGCGGAACTAC   480
FKH2            TCGGTCGCGCTGCAGCGGGCCCGCACGGAGAAGCCGGCCGCGACGTACCGGCGGAACTAC   234
                ************************************************************

AGAP001671      ACGCACGCCAAGCCGCCCTACTCGTACATCAGCCTGATCACGATGGCGATCCAGAACAAC   540
FKH2            ACGCACGCCAAGCCGCCCTACTCGTACATCAGCCTGATCACGATGGCGATCCAGAACAAA   294
                ************************************************************

AGAP001671      CCGCACAAGATGCTGACGCTGGCCGAGATCTACCAGTTCATCATGGATCTGTTCCCGTTC   600
FKH2            A-----------------------------------------------------------   295

AGAP001671      TACCGGCAGAACCAGCAGCGGTGGCAGAACTCGATCCGGCACTCGCTCAGCTTCAACGAC   660
FKH2            ------------------------------------------------------------   295

AGAP001671      TGCTTCGTGAAGGTGCCCCGCACGCCGGACAAGCCGGGCAAGGGGTCGTTCTGGACGCTC   720
FKH2            ------------------------------------------------------------   295
```

```
AGAP001671     CATCCCGACTCGGGCAACATGTTCGAGAACGGGTGCTACCTGCGGCGCCAGAAGCGGTTC   780
FKH2           ------------------------------------------------------------   295

AGAP001671     AAGGACGAGAAGAAGGAGGTGCTCCGGTCGCTGCACAAGAGCCCGGCGCACGGCGGCAGC   840
FKH2           ------------------------------------------------------------   295

AGAP001671     CTCGATGCGGTCGGCAGCCCGGACAAGAAGGACCCGAACGAGGAGCACCACCATCACCAT   900
FKH2           ------------------------------------------------------------   295

AGAP001671     CACCATCACAGCCACAGCCACCACAGCCACCGGACGGAGCACGTGTCCAAGCTGAGCGCG   960
FKH2           ------------------------------------------------------------   295

AGAP001671     GCAGTCGACACGCACGGCATGCTGAACAGTGCGCACGGTAAAGACGCGGACGCGCTCGCG  1020
FKH2           ------------------------------------------------------------   295

AGAP001671     ATGCTGCACGCGACGGCCGACTTATGCTTAGCCCAACAATCTCATTCACAGCATGGTGGT  1080
FKH2           ------------------------------------------------------------   295

AGAP001671     TCGCACCACCCTACGCACCACCACCACCACCATCCGGCCCACCAGCAGCTGCAGCAGGAG  1140
FKH2           ------------------------------------------------------------   295

AGAP001671     GAATTGACGGCTATGGTAAATCGCTGCCACCCTTCGCTGCTTGGTGAATATCACTCGATG  1200
FKH2           ------------------------------------------------------------   295

AGAP001671     CACCTGAAGCAGGAACCGGCGGGCTACACGCCCTCTAGTCACCCGTTCTCGATCACCCGG  1260
FKH2           ------------------------------------------------------------   295

AGAP001671     CTGCTGCCGACCGAGTCGAAGGCGGACATCAAGATGTACGAGATGAGCCAGTACGCCGGC  1320
FKH2           ------------------------------------------------------------   295

AGAP001671     TACAACGGGCTCAGCCCGCTGCCGAACTCGCACGCGGCCGCGGCCGCCCTCGGGCAGGAC  1380
FKH2           ------------------------------------------------------------   295

AGAP001671     TCGTACTATCACCAGAGCCTCGGCTACCACCATGCGTCCACGGGCACGACCAGCTTGTGA  1440
FKH2           ------------------------------------------------------------   295
```

Cloned sequence of Doublesex for dsRNA

>DSX
CAAGCGGTGGTCAACGAATACTCACGATTGCATAATCTGAACATGTTTGATGGCGTGGAGTTGCGCAATA
CCACCCGTCAGAGTGGATGATAAACTTTCCGCACCACTGTAACTGTCCGTATCTTTGTATGTGGGTGTGT
GTATGTGTGTTTGGTGAAACGAATTCAATAGTTCTGTGCTATTTTAAATCAAGCCGCGTGCGCAACTGAT
GCCGATAAGTTCAAACTAGTGTTTAAGGAGTGGAGCGAGAGAGCCGCACC

```
DSX              --CAAGCGGTGGTCAACGAATACTCACGATTGCATAATCTGAACATGTTTGATGGCGTGG  58
AGAP004050-RB    GTCAAGCGGTGGTCAACGAATACTCACGATTGCATAATCTGAACATGTTTGATGGCGTGG  60
                   ************************************************************

DSX              AGTTGCGCAATACCACCCGTCAGAGTGGATGATAAACTTTCCGCACCACTGTAACTGTCC  118
AGAP004050-RB    AGTTGCGCAATACCACCCGTCAGAGTGGATGATAAACTTTCCGCACCACTGTAACTGTCC  120
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                ************************************************************

DSX             GTATCTTTGTATGTGGGTGTGTGTATGTGTGTTTGGTGAAACGAATTCAATAGTTCTGTG  178
AGAP004050-RB   GTATCTTTGTATGTGGGTGTGTGTATGTGTGTTTGGTGAAACGAATTCAATAGTTCTGTG  180
                ************************************************************

DSX             CTATTTTAAATCAAGCCGCGTGCGCAACTGATGCCGATAAGTTCAAACTAGTGTTTAAGG  238
AGAP004050-RB   CTATTTTAAATCAAGCCGCGTGCGCAACTGATGCCGATAAGTTCAAACTAGTGTTTAAGG  240
                ************************************************************

DSX             AGTGGAGCGAGAGAGCCGCACC----------------------------------------  260
AGAP004050-RB   AGTGGAGCGAGAGAGCCGCACCACGGTACAGAAGGGCAGCAGAATGGGTCGGCAGCCTAG  300
                **********************

DSX             ----------------------------------------------------------  260
AGAP004050-RB   CTGCACTGGTGCGGTGCGTCCGGCGTCTCGGGGGGGAGGGCGAGGAAATTCTAGTGTTAAA  360


DSX             ----------------------------------------------------------  260
AGAP004050-RB   TCGGAGCAGCAAAAACAAAACAGTGGTCGTCCCGTTCAAGAAACGGCCTGTACACACACA  420


DSX             ----------------------------------------------------------  260
AGAP004050-RB   CAGAAAACACTGCAGCATGTTTGTACATAGTAGATCCTAGAGCAGGTGGTCGTTGCTCCT  480


DSX             ----------------------------------------------------------  260
AGAP004050-RB   CGAACGCTCTGGACGCACGGCTTCGCGCGTATTTGCGTAGCGTTCCGCCGATCGTGGGTA  540


DSX             ----------------------------------------------------------  260
AGAP004050-RB   TTCGTACTGCCACAAGCCCGCTTTCTCCCATGCAATCTCTGCAACCAAACCAACAAACAA  600


DSX             ----------------------------------------------------------  260
AGAP004050-RB   CAACAAAAAACCAATCGACAAAATGAATCACACCCCTTTTGTATCATCTGTATATTCTTG  660


DSX             ----------------------------------------------------------  260
AGAP004050-RB   TTCTTTGCGTTCTTTTCTATGTGGCCCACGCCCCGGCGGGTACGTAATTGCGTCGAAAAC  720


DSX             ----------------------------------------------------------  260
AGAP004050-RB   CCCGAAAACCCCGGCACATACAGTGTACATACGGTTTGAGGACAACTTTGACCTGCAGCC  780


DSX             ----------------------------------------------------------  260
AGAP004050-RB   CTTCTGGGGTTGCCACGTGTAGCTATACTTGTGAGATCGGGCGCCGACGGTGTAAAGCGC  840


DSX             ----------------------------------------------------------  260
AGAP004050-RB   GAATGGCCGCCACACAGTGTGTCCACTCCAACACTACCCCTCTGGAACTACCCCGTCCAG  900


DSX             ----------------------------------------------------------  260
AGAP004050-RB   GGATGCACCGGCTCGGCTCATGCCCCTGCAAAACAGTCCGGGCTCCACTGTAGTAGCTCC  960


DSX             ----------------------------------------------------------  260
AGAP004050-RB   GGCGTTGCTCTGAGAGAAGGATGCCCTTCGAAGTGTCGAAAGCGTGCATTGGGCGTTCAA  1020


DSX             ----------------------------------------------------------  260
AGAP004050-RB   GTGTGTGTGTGTGTGTTAGGTTTAGCGAGAAACAGCAGCAGTTGCGTGTGCTGAAAAGCG  1080


DSX             ----------------------------------------------------------  260
```

```
AGAP004050-RB      AAGGAGTAATAGAGTGCATAATGAAAATGAAAATGAAAATGAAGCAAAAGTAGAAGGCGG  1140

DSX                ------------------------------------------------------------  260
AGAP004050-RB      AGGAGAGCAACCTGTGTTCCACTAGTAGCGAATAGTTTAGTCTAGTTTCGTCACCAATCA  1200

DSX                ------------------------------------------------------------  260
AGAP004050-RB      ACCTTCCAACCATCGTTCAACCAATACCTGAGTCAACATCGTCATCGTTATCGTGCCACA  1260

DSX                ------------------------------------------------------------  260
AGAP004050-RB      ACTTTATTAAAAATGAACCTTGTCCGCGCCACCGTAGGGTGATCTAAGGCGACCTTTCTT  1320

DSX                ------------------------------------------------------------  260
AGAP004050-RB      ACGGGCGCGACCCACATGCCATCGTCACCTTCTCCAATCAAAACCAACAGCCTGTACCGA  1380

DSX                ------------------------------------------------------------  260
AGAP004050-RB      TGGTGTGCAATTGTGCGTGCGTGTGTGTTATTAGCAAAAAAAGAGAAAGAGTCGACGAGA  1440

DSX                ------------------------------------------------------------  260
AGAP004050-RB      GAGAGATAGATCGAGATCGAGAGTACAAAAGAGCAGTAGAAATGTTCGTTGTTTGTTTTT  1500

DSX                ------------------------------------------------------------  260
AGAP004050-RB      CGTAACACAGTTGTTTAGCCAAAATGGGAATTTCCAATAATCCCGGGGGCGGGGAAATGC  1560

DSX                ------------------------------------------------------------  260
AGAP004050-RB      GGGAATACTGCGTACACACATACATCAATCAAAAAGAAAAATCCTTGCGCTACATCACTA  1620

DSX                ------------------------------------------------------------  260
AGAP004050-RB      CCGTTTGCGCGGTGCTGATCTAGAGCAGACCACTTTCCACTCCACTCTACAATCAATCAA  1680

DSX                ------------     260
AGAP004050-RB      TCTGTGCAGAAG     1692
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Plasmids

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>pGEM-T vector
GGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCCGCGGGATATCACTAGTGCGGCCGCCTGCAGGTCGAC
CATATGGGAGAGCTCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATG
GTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG
CCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCG
TGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAAT
CAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCG
TTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACT
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GCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA
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CCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCC
```

GAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTC
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TCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCT
CCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCAT
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Promoters

T7 Promoter TAATACGACTCACTATAGGG