# Chapter 5

# RNAi in the Malaria Vector, *Anopheles gambiae*

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## Abstract

Malaria is a disease that kills more than a million people each year in tropical and subtropical countries. The disease is caused by *Plasmodium* parasites and is transmitted to humans exclusively by mosquitoes of the genus *Anopheles*. The lack of functional approaches has hampered study of the biological networks that determine parasite transmission by the insect vector. The recent discovery of RNA interference and its adaptation to mosquitoes is now providing crucial tools for the dissection of vector–parasite interactions and for the analysis of aspects of mosquito biology influencing the vectorial capacity. Two RNAi approaches have been established in mosquitoes: transient gene silencing by direct injection of double-stranded RNA, and stable expression of hairpin RNAs from transgenes integrated in the genome. Here we describe these methods in detail, providing information about their use and limitations.

**Key words:** Malaria, mosquito, RNAi, transgenesis, immunoblotting, q-PCR.

## 1. Introduction

Human malaria persists today as one of the most widespread and devastating infectious diseases in the world. *Plasmodium* parasites are transmitted to humans when an infected *Anopheles* mosquito takes a blood meal. The relationship between the mosquito vector and the malaria parasite is shaped by a complex network of biological interactions determining whether a given mosquito species will be capable of sustaining parasite development. Moreover, the vector competence to transmit disease is also governed by a series of factors intrinsic to mosquito biology, such as host preferences, longevity, immunity, and reproductive rates. However, while efficient genetic methods to study gene function in *Plasmodium* have long been established *(1)*, functional studies in the

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mosquito vector are lagging behind, and until recently little was known about the factors influencing *Plasmodium* development in the *Anopheles* vector.

Since the recent discovery of RNA interference, the "RNAi revolution" has reshaped the field of functional genomics, allowing characterization of genomes previously recalcitrant to targeted gene manipulation. This revolution has also hit mosquito research. Combined with the availability of the genome sequence of the major malaria vector, *Anopheles gambiae*, RNAi provides a new tool for malaria research, permitting study of molecules and mechanisms of mosquito biology that are relevant to disease transmission. Here, we describe two approaches for RNAi-based silencing in *Anopheles* for performing functional analysis in the mosquito vector. Further development of these methods might ultimately lead to new ways of controlling and perhaps even eradicating this devastating disease.

## 2. Materials

### 2.1. dsRNA Synthesis

1. pLL10 (**Fig. 5.1A**): A pBluescript-based plasmid with two T7 promoter sequences flanking the polylinker region in opposite directions *(2)*.
2. Proteinase K stock solution: 20 mg/mL in sterile 20 mM Tris (pH 8); 1.5 mM $CaCl_2$, 50% glycerol. Aliquots can be stored at –20°C. Proteinase K buffer: 10 mM Tris-HCl (pH 8); 10 mM EDTA (pH 8); 5 mM NaCl; 2 mM $CaCl_2$. Proteinase K final solution: add 1 μL of proteinase K (20 mg/mL) to 150 μL of buffer. Store in 50 μl aliquots (one tube for two reactions) at –20°C.
3. Linearized plasmid and RNA purification: RNase-free (DEPC-treated) water; phenol/chloroform/isoamyl alcohol (25:24:1); chloroform; isopropanol; 70% ethanol. All reagents should be RNase-free.
4. Synthesis and purification of single-stranded RNAs (ssRNAs): T7 MEGAscript kit (Ambion, Applied Biosystems, Foster City, CA, USA).

### 2.2. Plasmids for Stable RNAi and Injection Mixture

1. Transformation vector pBac[3xP3-EGFPafm] or similar, which contains a fluorescent protein selectable marker under control of the artificial *3xP3* promoter cloned within the inverted repeats of the *piggyBac* transposable element (**Fig. 5.1B**).
2. Helper plasmid phsp-pBac, which contains the piggyBac transposase gene driven by the *hsp70* promoter from *Drosophila melanogaster (3)* (**Fig. 5.1B**).

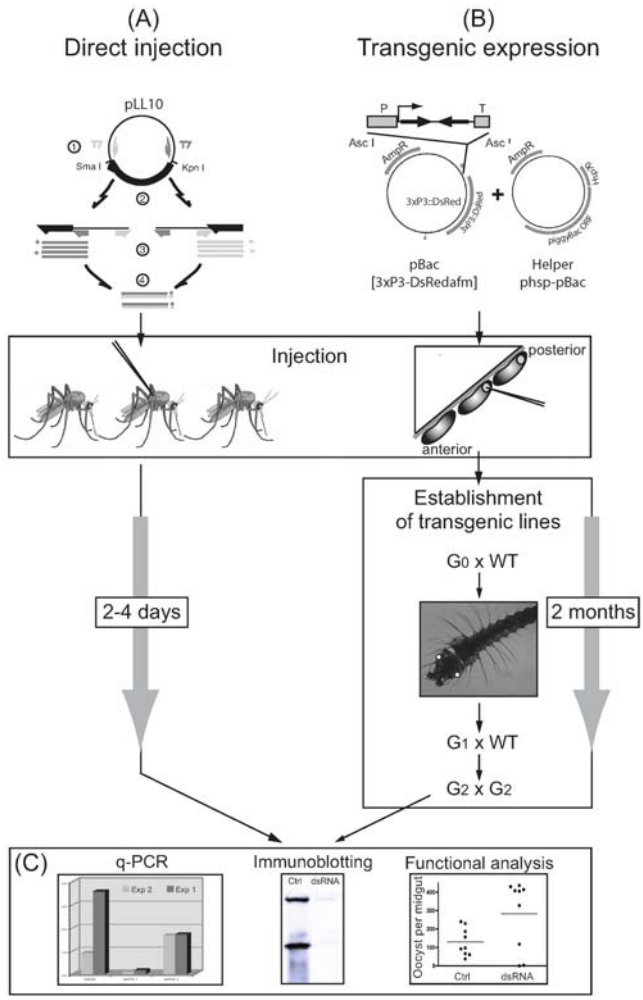


Fig. 5.1. **Methods for RNAi gene silencing in the mosquitoes**. (**A**) Direct injection of dsRNA. Map of pLL10 and position of the T7 promoters. The main steps in dsRNA synthesis are described: (1) cloning of the gene of interest in pLL10 between the two T7 promoters, using polylinker enzymes; (2) linearization of the plasmid on both sides of the insert; (3) synthesis of sense (+) and antisense (–) ssRNAs independently using the MEGAscript kit; (4) annealing of ssRNAs to form dsRNA (±). In vitro synthesized dsRNA is injected into the mosquito thorax (here shown for adults). Efficiency of dsRNA silencing can be estimated 12–24 h later by q-PCR or 2–4 days later by immunoblotting. The persistence of silencing often depends on the targeted gene, but in general it lasts for at least 5 days. (**B**) Stable RNAi gene silencing in transgenic mosquitoes. Cloning of the inverted fragments of the target gene into a *piggyBac* transformation vector. Injection of the transformation mixture, comprising the transformation vector and a transposase-expressing "helper" plasmid, into mosquito embryos. Expression of the selectable marker (in this case, expression of a red fluorescent protein is driven by the *Pax3* gene promoter in the eyes and nervous system (white)). Fluorescent $G_1$ individuals are outcrossed with wild-type mosquitoes (WT), and $G_2$ fluorescent progeny is intercrossed to amplify numbers of transgenic mosquitoes. Homozygous lines are then established by selection and intercross of homozygous individuals. (**C**) The efficiency of RNAi silencing in the injected and transgenic mosquitoes can be analyzed by q-PCR (transcriptional level) and immunoblotting (translational level). To date, a number of functional tests have been established to reveal the involvement of the targeted gene in the process of interest (in this example, development of the malaria parasites in the mosquito midgut was dramatically increased in the dsRNA-treated mosquitoes).

3. Injection mixture: 400 μg/mL of transformation vector and 150 μg/mL of helper plasmid phsp-pBac in injection buffer (*see* **Section** 2.4, Step 2). Prepare three or more aliquots of 20 μL each. Keep at –20°C until use.

### 2.3. Injection of dsRNA into Adult Mosquitoes

1. Strains of *A. gambiae* can be obtained from the MR4 (*see* **Note 1**). Mosquitoes are bred at 28°C and 70% humidity, with a day/night period of 12 h/12 h.
2. Waxed paper cartons (e.g., ice-cream or drink containers); filter paper circles of a matching diameter to fit in the bottom of the cartons (#1, Whatman, Kent, UK); fine nylon or cotton netting; tape; small elastic bands.
3. Mosquito aspirator.
4. Absorbent cotton wool; 10% sugar solution in water; small-sized Petri dish bottoms.
5. $CO_2$ bottle, $CO_2$ distributor, and pad (InjectMatic, Geneva, Switzerland).
6. Injector Nanoject II (Drummond, Broomall, USA); capillaries (FT330B); a syringe (1 mL) and needle (G24) filled with mineral oil; a paintbrush, forceps.
7. Micropipette puller (P-97, Sutter Instrument Company, Novato, USA).

### 2.4. Establishment of Transgenic Mosquitoes Expressing Hairpin RNAs

1. Mosquito embryos.
2. Injection buffer (5 mM KCl, 0.5 mM sodium phosphate, pH 6.8), filter paper, a fine paintbrush, glass slides.
3. Isotonic buffer (150 mM NaCl, 4.9 mM KCl, 10.7 mM, Hepes, 2.4 mM $CaCl_2$, pH 7.2) (*see* **Note 2**).
4. Stereoscope MZ6 (Leica, Wetzlar, Germany) with cold light source CLS150, or similar.
5. Reverted microscope Diaphot or similar, with 5x, 10x, and 20x objectives; NT-88NE three-dimensional micromanipulator (Narishige, Tokyo, Japan) or similar; microinjector Eppendorf femtojet (Eppendorf AG, Hamburg, Germany) or similar; sterile femtotip capillaries (Eppendorf AG, Hamburg, Germany); microloaders (Eppendorf AG, Hamburg, Germany).
6. Small Petri dishes (60 mm), 3MM Whatman filter paper (Whatman, Kent, UK), blotting paper.
7. Fluorescence microscope Nikon Eclipse TE200 (Nikon, Melville, USA) or similar; fluorescence filter sets (Texas Red/Cy3.5 and Blue GFP Bandpass).
8. Incubator at 28°C, 70% humidity with a day/night period of 12 h/12 h.

### 2.5. Analysis of Efficiency of RNAi Silencing

1. Hand-held homogenizer, TRIzol reagent (Invitrogen, Cergy Pontoise, France), DEPC-treated water, sterile

RNase-free labware (tips, microtubes), chloroform, isopropanol, 75% ethanol, spectrophotometer.
2. SuperScript III reverse transcriptase and reaction buffer (Invitrogen, Cergy Pontoise, France) or similar, dNTPs, random hexamer primers, RNase OUT (Ambion, Applied Biosystems, Foster City, CA, USA), PCR machine.
3. SybrGreen reaction mix, primers specific for the target gene (*see* **Note 3**).
4. Quantitative PCR machine (*see* **Note 4**).
5. Polyacrylamide gel electrophoresis, running buffer, 6x protein loading buffer (350 mM Tris-HCl (pH 6.8); 10.28% SDS; 36% Glycerol; 5% ß-Mercaptoethanol; 0.012% Bromphenol blue), PageRuler$^{TM}$ Prestained Protein Ladder (Fermentas International Inc, Burlington, Canada).
6. Membrane for protein transfer (Amersham Hybond-P, GE Healthcare UK Ltd, Buckinghamshire, England), wet protein transfer unit (Bio-Rad Laboratories, Hercules, USA), transfer buffer.
7. Washing solution: Phosphate-buffered saline (PBS): prepare a 10x stock (130 mM NaCl; 7 mM, $Na_2HPO_4$; 3 mM $NaH_2PO_4$) and autoclave. For working solution, dilute one part with nine parts of water. Blocking solution: PBS, 5% nonfat milk powder (Nestlé S.A., Vevey, Switzerland)).
8. Secondary antibody solution: Use the recommended dilution of anti-rabbit, anti-mouse, or anti-rat IgG antibodies conjugated with horseradish peroxidase (HRP).
9. Amersham ECL$^{TM}$ Western Blotting Detection Reagents (GE Healthcare UK Ltd, Buckinghamshire, England).
10. X-ray film (Fuji Photo Film CO., Ltd, Tokyo, Japan), cassette.

## 3. Methods

In *Anopheles* mosquitoes, RNAi-mediated silencing can be achieved by two different methods: (a) injecting double-stranded RNA (dsRNA) directly into the body cavity of adult mosquitoes *(2)* (**Fig. 5.1A**); and (b) expressing dsRNA in situ from a stably integrated transgene *(4, 5)* (**Fig. 5.1B**). The method of choice often depends on the application. Direct injection of dsRNA permits a fast assessment of the function of the target gene at a selected developmental stage, allowing rapid medium-scale screens. The relatively high number of mosquitoes needed for injections and the transient nature of silencing of some genes are among the most common limitations of this method.

The generation of transgenic lines expressing stable RNAi transgenes is more labor intensive; however, it provides an inexhaustible supply of mutant mosquitoes for in-depth phenotypical and biochemical analyses, and allows the time and tissue-specific knockdown of the target genes through the use of appropriate promoters.

Direct injection of dsRNA includes several steps: (a) a candidate gene is selected; (b) the optimal target sequence is cloned into an appropriate vector for dsRNA synthesis; (c) dsRNA is injected into adult mosquitoes and the efficiency of gene silencing is examined 1–4 days after injection. In general, the whole process, from the selection of a gene of interest to elucidation of its function in the particular biological process, may be achieved in 1 month. Understandably, establishment of transgenic mosquitoes requires longer periods before any functional tests can be performed. This includes the sometimes delicate cloning into the *piggyBac* expression vectors; injections of a large number of embryos; efficient crossing methods to obtain a sufficiently large $G_1$ progeny; screening of $G_1$ individuals, normally at the larval stage; identification of positive, transgenic individuals; and the establishment of transgenic lines. Initial functional analysis can be performed with heterozygous lines, provided a screening is carried out to ensure the transgenic origin of the individuals selected for the experiments. In all, it might take 2–3 months from the identification of the gene of interest to the establishment of the transgenic line(s).

### 3.1. dsRNA Synthesis

1. Clone a fragment of a gene of interest in pLL10 (**Fig. 5.1A**). The optimal size of the fragment is determined by the efficiency of the RNA synthesis reaction, and is between 100 and 1000 bp for the T7 MEGAscript kit. To avoid off-target effects, it is important to design dsRNA constructs for at least two highly specific regions of the gene of interest. Potential gene cross-silencing can be gauged using the DEQOR software (http://cluster-1.mpi-cbg.de/Deqor/deqor.html) *(6)*.
2. Prepare DNA of the constructed plasmid (*see* **Note 5**).
3. For the synthesis of sense and antisense ssRNAs, 2 × 10 μg of plasmid are linearized separately with two different restriction enzymes, one on each side of the insert, in 50 μL (**Fig. 5.1A**). Confirm that digestion is complete by loading 1 μL of the reaction on 1% agarose gel (*see* **Note 6**).
4. Add 25 μL of proteinase K final solution and 4 μL of 10% SDS to each digest. Incubate at 50°C for 30 min.
5. Add 80 μL of phenol/chloroform/isoamyl alcohol. Vortex and incubate 2 min at room temperature (RT). Centrifuge

for 5 min at maximal speed and collect the aqueous phase in a fresh tube.

6. Add 80 μL of chloroform. Vortex and incubate for 2 min at RT. Centrifuge at maximal speed and collect aqueous phase in a fresh tube.
7. Add 56 μL of isopropanol, mix by inverting the tube, and incubate at 4°C for 15 min. Centrifuge for 15 min at 4°C at maximal speed and discard supernatant.
8. Wash with 100 μL of 70% ethanol, centrifuge 5 min at maximal speed, discard supernatant and allow the DNA pellet to air dry on the bench.
9. Dissolve pellet in 20 μL of water. Check DNA quality and concentration (should be around 0.5 μg/μL) on a 1% agarose gel. Linearized plasmids can be stored at –20°C.
10. Thaw reagents of the T7 MEGAscript kit (Ambion, Applied Biosystems, Foster City, CA, USA). Keep ribonucleotides on ice, and transcription buffer at RT.
11. For each plasmid, add in order: (8–*x*) μL of water; 2 μL of each NTP; 2 μL buffer; *x* μL linearized plasmid (1 μg), and 2 μL enzyme mix. If several reactions are assembled in parallel, prepare a master mix with all reagents except linearized plasmids, aliquot calculated volume in each tube, then add plasmids. Incubate overnight at 37°C (8–14 h). *See* **Notes 7** and **8**.
12. DNA template is then digested by adding 1 μL of DNase I to each reaction. Incubate 15 min at 37°C.
13. Purification of ssRNAs: To the incubation mix add 115 μL of water and 15 μL of ammonium acetate stop solution and mix thoroughly. Extract RNA with 150 μL of phenol/chloroform/isoamyl alcohol, followed by 150 μL of chloroform (*see* Steps 5 and 6). Recover aqueous phase and transfer it to a fresh tube.
14. Precipitate RNA by adding 150 μL of isopropanol, mix well by inverting the tube and incubate 15 min at –20°C. Centrifuge 15 min at 4°C at maximal speed and discard supernatant. Air dry pellet for a few minutes on the bench and dissolve it in 20 μL of water.
15. While samples are drying, boil water in a 1–3 L beaker covered with aluminum foil.
16. Measure concentration of ssRNAs using a UV spectrophotometer. Dilute 1 μL of each ssRNA in 9 μL of 10 mM Tris buffer (pH 8). Take 1 μl of this dilution into 100 μl of the same 10 mM Tris buffer for measurement, and keep the remaining 9 μl at –20°C. To calculate the concentration, use the following formula: Concentration of ssRNA in μg/μL = $OD_{260}$ × dilution factor × 40/1000. The dilution factor is 1000 in the example given.

17. Adjust concentration of sense and antisense ssRNAs to 3 μg/μL each and mix equal volumes (the rest of the ssRNAs can be stored at –20°C or –80°C). Close tubes tightly and boil samples for 5 min in the beaker. Allow samples to slowly cool down to RT.
18. Check dsRNA quality on a 1.8% agarose gel. For this, denature the ssRNA dilutions from Step 16 at 95°C for 3 min and immediately cool on ice. Spin briefly. Run 1 μl of these and 1 μl of dsRNA from Step 17 (in 5 μL of DNA loading buffer) on gel. A clear shift should be observed in the migration patterns of ssRNAs and dsRNA: dsRNA migrates slower than the corresponding ssRNAs.
19. dsRNA is quite resistant to multiple freezing/thawing cycles. Store dsRNA –20°C or –80°C.

### 3.2. dsRNA Injection into Adult Mosquitoes

1. Mosquito breeding: *See* "Anopheles Culture" by M.Q. Benedict, CDC Atlanta, USA at the MR4 website (http://www2.ncid.cdc.gov/vector/vector.html).
2. Needles for injection: Pull glass capillaries using the needle puller. Each capillary gives two needles in which elongated tip is sealed.
3. Prepare mosquito pots. Cut a cross-shaped opening in the side of a pot and seal it with tape. Place a filter paper circle at the bottom of the pot; it will blot out excess of sugar or mosquito droppings. Stretch netting over the top of the pot and secure with an elastic band.
4. Place around sixty 1–2-day-old female mosquitoes into the pot using an aspirator. Fill a small-sized Petri dish bottom with a cotton pad soaked in 10% sugar solution and place it on the netting.
5. Break the tip of the glass capillary with forceps, so that the tip is rigid enough, but the opening not too large. Fill the capillary with mineral oil using a syringe and a needle. Assemble the Nanoject injector and fill capillary with the dsRNA solution. Verify settings on the control block of the Nanoject and set the desired injection volume and speed. To analyze one gene, we usually inject each mosquito with 69 nL of dsRNA at the highest speed.
6. Immobilize mosquitoes in the pot by $CO_2$ treatment and align them with dorsal side up on the $CO_2$ pad. Using the injector and a brush, carefully inject dsRNA solution into the dorsal plate of the mosquito thorax (*see* **Notes 9** and **10**). During injections, limit exposure of mosquitoes to $CO_2$ using the pedal-controlled distributor (*see* **Note 11**).
7. After all mosquitoes are injected, gently place them back in paper pots using a brush, stretch the netting, fix it with an elastic band, and place a sugar cotton pad on the top. Keep

mosquitoes until further analyses in the humidified 28°C incubator (usually for 4 days). *See* **Note 12**

### 3.3. Design and Cloning of the RNAi Expression Plasmid

1. Clone 300–800 bp sense and antisense sequences of the target gene in a tail-to-head fashion, under the control of the appropriate promoter, into an intermediate plasmid of choice. Insert the whole RNAi DNA cassette into the single *Asc* I site of transformation vector pBac[3xP3-EGFPafm] or similar (**Fig. 5.1B**). *See* **Note 13**

### 3.4. Establishment of Transgenic Lines Expressing Hairpin RNAs

1. For standard techniques of mosquito breeding, see above.
2. Blood feed 3–5-days-old female mosquitoes (approximately 100–150 per cage). Starve females for a few hours prior to blood feeding by depriving them of sugar.
3. At 48–72 h post blood feeding, place an oviposition cup (60 mm Petri dish containing Whatman 3MM filter paper soaked in isotonic buffer) into the cage (*see* **Note 14**).
4. Collect embryos every 30 min, each time placing a new oviposition cup into the cage to allow females to lay more embryos. After removing the cup containing the embryos from the cage, place it at RT to slow down development and hence hardening of the chorion.
5. Cut a piece of membrane and a piece of blotting paper with a razor blade at a 135° angle, as shown in **Fig. 5.1B**. Place the membrane (top) and the blotting paper (bottom) onto a glass slide, with the blotting paper slightly exposed, and moisten them with isotonic buffer.
6. At 80–100 min after oviposition, when embryos have started to darken slightly, use a fine paint brush to align 20–40 embryos on the glass slide against the moistened blotting paper, with their posterior poles oriented towards the top of the glass slide, as shown in **Fig. 5.1B**. All embryos must be oriented in the same direction. Remove excess of liquid; however, always keep the membrane moist adding buffer from time to time to avoid embryo desiccation.
7. Transfer the embryos to the microscope for injection. Fill an Eppendorf femtotip glass needle with 2 μL of injection mixture, using microloaders. Make sure the needle is open before injection, by checking for the presence of a little drop at the tip of the needle after injection (*see* **Note 15**).
8. Inject the posterior part of the embryo, using an injection pressure (pi) of 700–1000, and a counter pressure (pc) of 300–500, depending on the needle. When injected successfully, a slight clearing of the embryo yolk should be visible. Take care to remove the embryos that have not been injected, as these are the most likely to survive and therefore their presence will increase the screening efforts (*see* **Note 16**).

9. After injection, place the slide directly onto a tray containing water and larval food, placing strips of 3MM paper around the side of the tray to avoid embryos getting dry. After 2–4 days, count the embryos that have hatched.
10. Separate females from males immediately after emergence or by sexing them at the pupal stage in order to ensure they are virgins ($G_0$ generation). Cross $G_0$ adults with wild-type (WT) individuals, placing 10–15 $G_0$ females with 30–50 WT males and 10–15 $G_0$ males with 30–50 WT virgin females in separate cages (**Fig. 5.1B**). Blood feed 4–6 days after the crosses have been set up to provide sufficient time for mating, and collect $G_1$ progeny. In order to provide an estimate of fertility of the $G_0$ individuals, females can be forced to lay eggs individually in Petri dishes or in 50 mL Falcon tubes in which the conical bottom part has been cut off and replaced by a fine netting (to allow sugar feeding through the use of cotton pads or paper soaked in 5% sucrose solution), while the lid can function as a small oviposition cup when filled with water. Repeat the feedings/egg collections a minimum of three times per cross, especially with the crosses involving $G_0$ males, in order to increase the number of progeny screened.
11. Screen $G_1$ progeny at the larval stage with the appropriate fluorescence filter, separate transgenic individuals from negative ones and backcross them to WT individuals of the opposite sex to propagate the line (**Fig. 5.1B**).
12. Sequence some individuals from each transgenic line to ensure the integrity of the RNAi cassette.

### *3.5. Analysis of Efficiency of RNAi Silencing at the Transcriptional Level*

1. Collect 10–15 mosquitoes from the control and experimental groups at different time points after dsRNA injections or at the desired developmental stage in the case of transgenic individuals (*see* **Note 17**), and extract total RNA using TRIzol reagent or equivalent according to the manufacturer's instructions. Measure the concentration of the RNA solution.
2. Convert 2 μg of total RNA into cDNA using SuperScript reverse transcriptase or similar as recommended by the manufacturer (*see* **Note 18**).
3. Perform quantitative polymerase chain reaction (q-PCR) using primers for the gene of interest and SybrGreen PCR master mix. Compare levels of the target gene expression in the control and experimental mosquitoes (**Fig. 5.1C**) (*see* **Note 19**).

### *3.6. Analysis of Efficiency of RNAi Silencing at the Protein Level*

1. Immunoblotting can be performed on hemolymph extracts or on other relevant tissues (**Fig. 5.1C**). For hemolymph collection, clip the proboscis of 10 mosquitoes and collect

clear drops of blood directly into a 6x protein-loading buffer. As an example, for total tissue extracts, grind five mosquitoes in 250 μL of protein extraction buffer, centrifuge the sample at 4°C at 3,000 rpm to clear the extract from remaining tissues, and mix 5 μL of the cleared sample with 5 μL of the 6x protein loading buffer (*see* **Note 20**).

2. Run protein extracts on SDS-PAGE using an appropriate polyacrylamide concentration until the loading buffer dye reaches the bottom of the gel.
3. Transfer proteins from the gel to a membrane according the instructions provided with the protein transfer unit, and incubate the membrane with the primary antibody against the protein of interest.
4. Detect the primary antibody using a secondary HRP-conjugated antibody according to general protocols, and develop the membrane using ECL™ Western Blotting Detection Reagents according to the manufacturer's instructions.
5. Wrap the wet membrane in plastic and expose it on an X-ray film for 30 s. Adjust the exposure time according to the strength of the signal.

## 4. Notes

1. MR4 (Malaria Research and Reference Reagent Resource) stores and provides reagents to the malaria research community; *see* http://www.malaria.mr4.org/.
2. Solutions are stored at RT, unless otherwise stated.
3. The choice of the reaction mix depends on the q-PCR machine; in our experience, testing a number of primer concentrations greatly improves efficiency.
4. Similar results are obtained with different brands of q-PCR machines.
5. Sequencing of an insert cloned in pLL10 can be done using universal M13 forward and reverse primers.
6. All plasmid DNA should be digested as RNA polymerases are very processive and will generate long heterogeneous transcripts from circular plasmids.
7. When transcription is optimal, the reaction at the end is rather viscous.
8. Sense and antisense ssRNAs can be produced in a single reaction, e.g., from a PCR fragment that was amplified with primers bearing T7 promoters. However this does not ensure that both strands are synthesized with the same efficiency. We therefore prefer to prepare sense and antisense

ssRNAs separately and measure their respective concentrations before annealing, to make sure equal quantities of both strands are mixed and to obtain reproducible quantities of dsRNA.

9. The maximum volume that can be injected at once is 69 nL. To inject larger volumes, repeated injections (up to four) can be performed.
10. With one filled needle, about 60 mosquitoes can be injected with 69 nL each.
11. Excess of $CO_2$ treatment is toxic to mosquitoes.
12. Humidity improves survival of injected mosquitoes. Make sure to place mosquitoes into a humidified incubator or chamber immediately after injection.
13. To facilitate cloning of the sense and antisense inverted repeats in *E. coli*, it may be necessary to insert a small linker region or an intron between them *(5)*.
14. In the case of *Anopheles stephensi* mosquitoes, it is advisable to soften their chorion before injection to facilitate the injection procedure. To this end, embryos are laid in a 0.1 mM *p*-nitrophenyl *p′*-guanidinobenzoate (pNpGB) (Sigma-Aldrich, St. Louis, USA) solution, dissolved in isotonic buffer, and kept there until injection *(7)*.
15. Many laboratories prefer to pull their own capillaries and use quartz needles for the injection procedure *(8)*.
16. During a set of injections, it is likely that the needle will get clogged due to cytoplasm uptake. It may then be necessary to use the "Clean" function on the Femtojet (reaching high pressure values) to try to unclog the needle, and in some cases the same function may be used for the actual injection procedure.
17. Collected samples can be kept at –80°C for several weeks until further use.
18. To obtain sufficient amounts of cDNA the reaction volume can be scaled up to 100 μL.
19. Not all genes show significant differences at the transcriptional level. In some instances (e.g., *Cactus*) reduction of 20% in the transcriptional level is sufficient to trigger a prominent phenotype *(9)*.
20. Do not store the remaining samples, as protein extracts are not stable and rapidly degrade.

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## References

1. van Dijk, M. R., Waters, A. P., and Janse, C. J. (1995) Stable transfection of malaria parasite blood stages *Science* **268**, 1358–1362.
2. Blandin, S., Moita, L. F., Köcher, T., Wilm, M., Kafatos, F. C., and Levashina, E. A. (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene *EMBO Rep* **3**, 852–856.
3. Handler, A. M., and Harrell, R. A., 2nd (1999) Germline transformation of *Drosophila melanogaster* with the piggyBac transposon vector *Insect Mol Biol* **8**, 449–457.
4. Brown, A. E., Bugeon, L., Crisanti, A., and Catteruccia, F.(2003) Stable and heritable gene silencing in the malaria vector *Anopheles stephensi Nucleic Acids Res* **31**, e85.
5. Brown, A. E., Crisanti, A., and Catteruccia, F.(2003) Comparative analysis of DNA vectors at mediating RNAi in Anopheles mosquito cells and larvae *J Exp Biol* **206**, 1817–1823.
6. Henschel, A., Buchholz, F., and Habermann, B.(2004) DEQOR: a web-based tool for the design and quality control of siRNAs *Nucleic Acids Res* **32**, W113–W120.
7. Catteruccia, F., Nolan, T., Loukeris, T. G., Blass, C., Savakis, C., Kafatos, F. C., and Crisanti, A.(2000) Stable germline transformation of the malaria mosquito *Anopheles stephensi Nature* **405**, 959–962.
8. Lobo, N. F., Clayton, J. R., Fraser, M. J., Kafatos, F. C., and Collins, F. H.(2006) High efficiency germ-line transformation of mosquitoes *Nat Protoc* **1**, 1312–1317.
9. Frolet, C., Thoma, M., Blandin, S., Hoffmann, J. A., and Levashina, E. A. (2006) Boosting NF-kappaB-dependent basal immunity of *Anopheles gambiae* aborts development of *Plasmodium berghei*. *Immunity* **25**, 677–685.