Chapter 3 : Specific *Anopheles* Techniques

3.1 Determining the sex of Anopheles *pupae and larvae*

MR4 Staff

Introduction

There is often an experimental need to separate the sexes before they emerge e.g. in order to preserve unmated status of females, to obtain material for molecular analysis, or to determine male/female larval ratios. *Anopheles spp.* differ from many other mosquitoes in that there is often no easily discernable difference in the female/male larval or pupal size or pigmentation though some, but not all, anophelines L4 females can be identified based on the generally darker color and larger size. Here we present three methods for determining the sexes based on larval and pupal characteristics.

Larval sex determination: Option 1

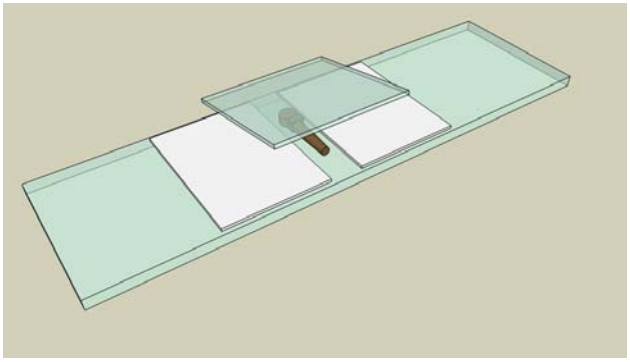

Figure 3.1.1. Cartoon of a 'sandwich' slide which works well to position and immobilize a larva for viewing without causing injury.

An early method for sexing *Anopheles* larvae based on the form of the imaginal antennal lobes has been reported (Jones 1956), but the graphics in the manuscript can be difficult to interpret – particularly in copies. Here we offer a refinement of the method and new images developed for *An. gambiae*. (Note: This method is not very useful with *An. stephensi* because the imaginal disks are difficult to see.) The best results are usually obtained with 2nd day L4s as the pre-antennal lobe is almost fully formed. All observations and photographs were made on a compound microscope, and it is important to use the dark-field setting.

Materials

- Standard glass microscope slide
- 0.3 - 0.5 mm thick plastic spacer e.g. a thin plastic laboratory ruler cut into 1 X 1.5 cm pieces. The thickness must be selected to support a coverslip over the gap so that a larva is held firmly but not crushed. A stack of plastic coverglasses may be stacked and glued together to obtain the appropriate thickness.
- Epoxy glue

Constructing the viewing slide:

1. Clean the slide with ethanol and dry.
2. Apply a small drop of epoxy glue to the plastic spacers.
3. Glue the spacers onto the slide 0.8 - 1cm apart from each other and allow to cure.

Protocol

Place a larva, dorsum upward, between the spacers. Add sufficient water to fill the gap, and place a coverglass on top such that it bridges the spacers. The pre-antennal lobes can be seen between the imaginal eyes while viewing the dorsal side of the head. In males, the lobe is large, circular and easier to see. The female's lobe is smaller and it is only easily seen in the second and third days of the fourth instar. Males are typically a bit easier to identify than females (see **Figures 3.1.2** and **3.1.3**).

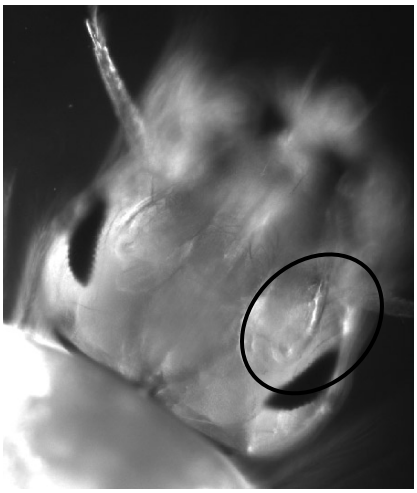

Figure 3.1.2. L4 *Anopheles gambiae* male. Region of interest for sex determination is circled.

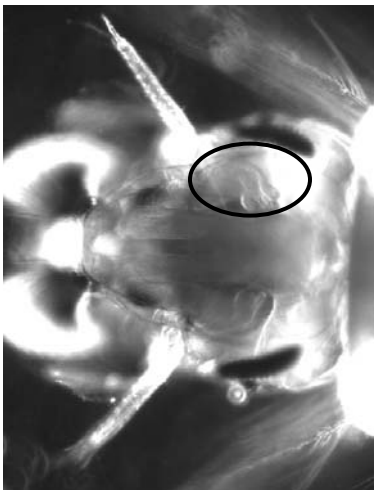

Figure 3.1.3. L4 *Anopheles gambiae* female. Region of interest for sex determination is circled.

Larval sex determination: Option 2

In *An. gambiae* and *arabiensis,* the "Red stripe" character can be visualized in L3s through mid pupae (Benedict et al. 2003) and provides a character that can be used to positively identify females with high certainty. When the *collarless* alleles (see Morphological Characteristics, Chapter 4) are heterozygous (c+ / c), a red stripe is evident on the female dorsum. The *collarless* trait is polymorphic in most colonies and wild populations and appears to have little if any effect on vigor. In **Figure 3.1.3**, the dorsum of this L4 larva has both white and red pigment characteristic of a c+ / c heterozygous female. While the *presence* of the red stripe can be used to select females with high certainty, the *absence* of the red stripe does not necessarily indicate a male in a polymorphic population. Though this method does not allow one to distinguish males, a cross between a homozygous c+ / c+ and c / c individuals would create F1 heterozygotes in which both sexes could be distinguished with good certainty. X-chromosome markers could also be used in a genetic scheme to produce progeny whose sex could be determined as early as the L1 stage. (Stocks suitable for such crosses are available from the MR4.)

Figure 3.1.3. Red stripe character indicates a female larva.

Pupal sex identification

Anopheles pupae are much simpler to sex than larvae. The pupa should be lying on its side and semi-dry in order to see the genitalia easily, and it may be necessary to use a small brush or forceps to gently lift the paddles.

1. Using a pipette, gently transfer 1 pupae to either a depression well plate or a piece of damp filter paper. If using a depression well plate, remove as much water as possible so that the pupa is lying on its side.
2. Under a stereoscope, observe the prominent genitalia for comparison with **Figure 3.1.4**.

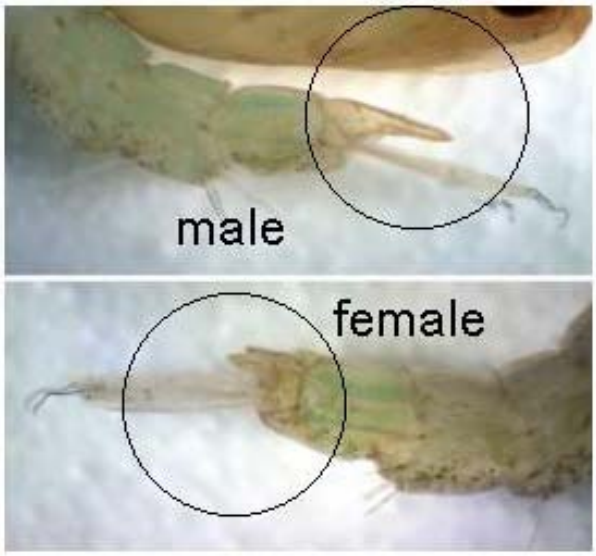

Figure 3.1.4. The terminalia of the pupae are very distinctive. However, the paddles can easily get in the way making it difficult to distinguish. Gently poking the pupa will usually make them change position to reveal the terminalia.

References

Benedict MQ, McNitt LM, Collins FH (2003) Genetic traits of the mosquito *Anopheles gambiae*: Red stripe, frizzled, and homochromy1 J Hered 94 227-235

Jones JC (1956) A Simple Method for sexing living *Anopheles* larvae (Diptera, Culicidae). Annals Entomol Soc Am 50:104-106

3.2 Microinjection Methods for Anopheles Embryos

Mark Benedict

Introduction

We present two methods that have been successful (and a variation of Method 1 using oil). The first was developed by John R. (Randy) Clayton for injection of *An. gambiae* embryos, and it has been used to obtain high frequency egg hatching and EGFP transient expression rates as described by Grossman et al., 2001 (Grossman et al. 2001). This method is similar to that used by Dave O'brochta's group at the Univ. Maryland. They successfully transformed *An. gambiae* using a similar method by covering the embryos with halocarbon oil prior to injection (Kim et al. 2004). Using oil provides better visibility of the DNA solution flow rate.

The second method is fast and requires less judgment than those above. It was developed by Hervé Bossin and Mark Benedict for *An. arabiensis* and *An. gambiae*. However, it should be useful for many mosquito species. Anecdotally, mosquito species vary in the ease with which they can be microinjected. Both of the methods above have been used successfully with *gambiae* s.l. which is supposed to be one of the more difficult to inject.

We recommend mounting the injection needle in a fixed position and moving the slide holding the aligned embryos using the stage controls to the appropriate position for injection. This allows one to use a rather simple needle positioner mounted on or by the microscope.

Anopheles embryos cannot be dechorionated, so use of rigid needles and firm positioning of the embryos is necessary. Quartz glass injection needles are by far preferable to aluminosilicate or borosilicate needles. These require higher pulling temperatures than the other glasses and therefore a laser needle-puller must be used. If you are unable to afford one, at least use aluminosilicate needles.

Materials:

- Mated adult females bloodfed 3-5 days post-eclosion.
- Clean water in a wash bottle
- Pipettor e.g. P20
- Fine paint brushes[1] and forceps
- Filter paper
- 2X Na phosphate Injection buffer (see below for preparation; requires KCl and di- and mono-basic sodium phosphate)
- Minimum fiber filter paper e.g. (Whatman 1450-090, 'Hardened circles')
- Eppendorf Microloader tips (no. 5242-956-003)
- Ultrafree-MC filters (no. UFC30HV00)
- Quartz glass capillaries, 1 mm OD, 0.7 mm ID X 10 cm length (e.g. Sutter no. QF100-70-10)
- Double-sided adhesive tape. 3M type 415 has been tested for vertebrate toxicity and is a good choice (Method 1 only)
- 22 x 22 glass or plastic coverglasses with a strip of pre-cut double-sided tape attached (Method 1 only)

[1] Select the brushes carefully from among the finest at an art or craft store. Sable brushes are excellent and more expensive, but regardless, a very fine pointed tip is essential.

- 25 mM NaCl (or 10-50 mM range for testing, Method 1 only)
- Millipore (or other) mixed cellulose ester membranes (e.g. HAWP02500- US or HAWP03700, Method 2 only)[2]

Equipment

- Either a compound or high-quality dissecting microscope can be used for injections. It is preferable if it can be dedicated to this purpose.
- Sutter P-2000 Micropipette puller or similar device[3]
- Needle positioner and holder
- pH meter
- Eppendorf Femtojet or similar device equipped with a foot pedal
- Dissecting scope and illuminator for embryo alignment

Solutions

This recommendation is for *Drosophila melanogaster* from Bill Engels lab, but it seems suitable for mosquitoes. Prepare two 0.1 M solutions of monobasic and dibasic sodium phosphate. Mix the two and adjust pH with one or the other to pH 6.8-7.8. Prepare a solution of 0.5 M KCl in purified water.

2X injection buffer is:

0.2 mM Na phosphate

10 mM KCl

Filter sterilize and store at room temperature or lower.

Starting procedures common to both methods

1. Prepare the capillaries by flushing them several times with purified water followed by ethanol to remove lint and glass chips. Blot the remaining ethanol and flame the capillaries briefly to remove all liquid. It is convenient to store them in a covered glass culture tube.
2. Immediately before use, thaw the DNA and filter through a 0.2 micron Millipore Ultrafree-MC filter filter to remove particulates. This latter measure (suggested by D. O'brochta) is simple and effective. Store on ice until use.
3. Harvest eggs 60-96 hours after females are bloodfed by placing 10 to 15 females in a transparent cylindrical container (~40 ml) open at one end and covered with rubber dental dam at the other (**Figure 3.2.1**). Alternatively, eggs may be collected on damp filter paper in a small Petri dish[4].

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[2] Any non-fibrous membrane that is very thin, hydrophilic and does not contain detergent would probably work for this e.g. Southern blotting membrane. If in doubt regarding the presence of wetting agents etc. rinse the membrane well before use. These types of membranes are perfect because when wet, they adhere closely to a microscope slide so that the embryos don't slide beneath the membrane, and they are about the same thickness as an embryo so visualization is easy.

[3] The program we use for the P-2000 is: HEAT: 650, FIL: 4, VEL: 40, DEL: 150, PUL: 157. However, conditions necessary to produce suitable needles may differ on your device and may require slight adjustment, even from day to day.

[4] Cooling the eggs will extend their useful time, but MQB's experience has been that this also reduces survival.

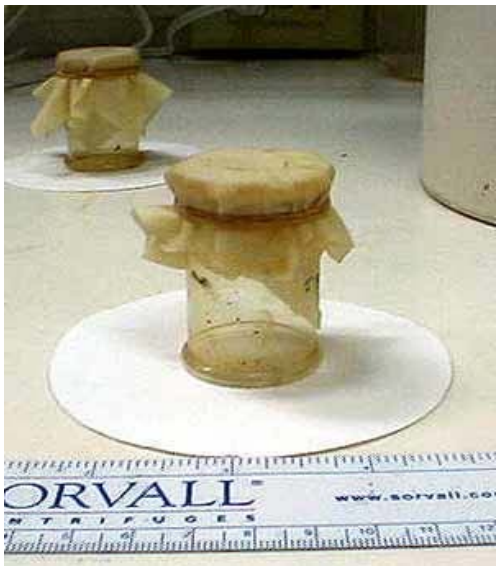

Figure 3.2.1. Cylindrical container (~40 ml) containing 10-15 previously bloodfed females open at one end and covered with rubber dental dam at the other

Figure 3.2.2. Container with females resting over a single water-filled well on a cobalt blue ceramic depression plate. Embryos deposited in single depression shown (inset).

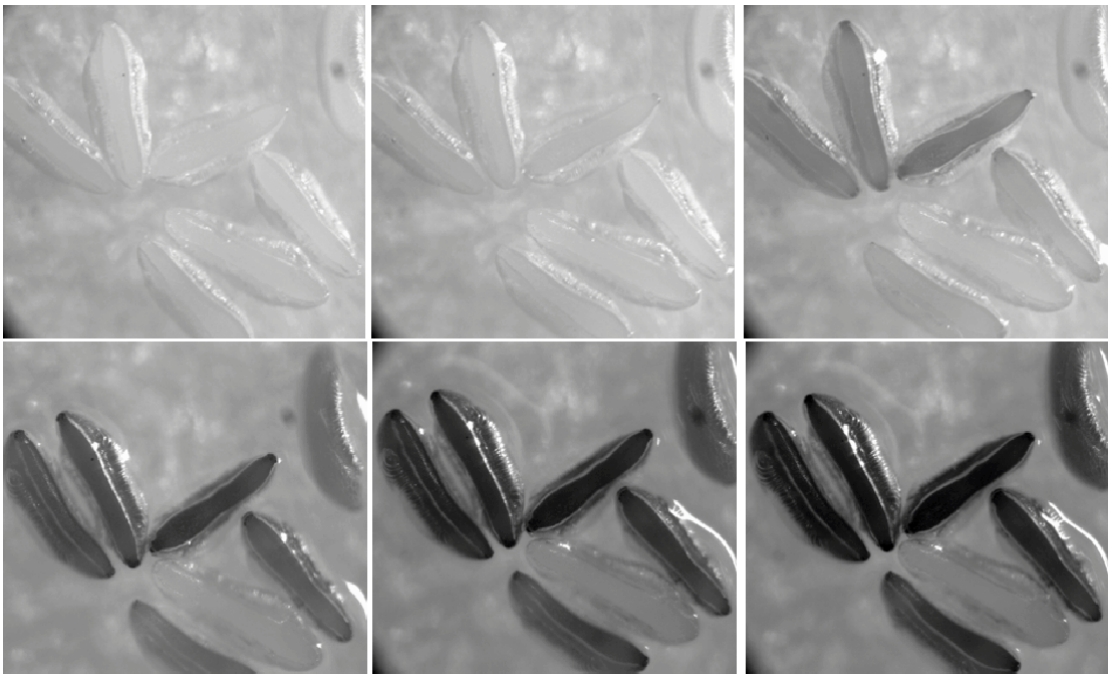

Figure 3.2.3. Egg darkening at 20 min intervals (top left to bottom right) beginning approximately 15 m after oviposition and incubated at room temperature (~22°C). Most of the darker eggs in the lower two left panels are suitable for injection. Note that one egg did not darken. It will not hatch. When eggs have fully darkened (as in the lowest right panel) but are often still at the blastoderm stage, they are more difficult to inject (photographs courtesy of G. Labbe, Oxitec, used with permission). Non-melanized eggs as in panels 1-3 cannot be handled without suffering mortality.

4. Slide the container over a single water-filled well on a depression plate such that the females can access the water (see **Figure 3.2.2**). Cover to darken for 30 minutes and then remove the females.
5. Age the new embryos for at least 30 minutes at insectary conditions (28°C; 80% humidity) or at room temperature. After this time, the eggs should be medium gray (**Figure 3.2.3)**.

Method 1:

The distinctive feature is that embryos are injected under saline, the concentration of which is determined empirically to balance internal pressure. The balance pressure must be such that the turgidity is sufficient for needle penetration yet low enough that oozing and needle backflow are minimized. The salt concentration can be adjusted as needed to achieve this for each species.

1. At room temperature (~ 24^{o}C), transfer eggs from the depressions to a glass slide with a fine paintbrush and align with the dorsal (flattened, concave) surface facing up.
2. Align the anterior ends of 25-35 eggs in 25 mM NaCl against a strip of reduced-fiber filter paper.
3. Remove the filter paper by tugging it away sharply, so as not to disturb the alignment of the embryos.
4. Allow eggs to desiccate slightly.
5. Press a taped coverslip gently against the eggs' dorsal surface and immediately invert. Getting the embryos to stick to the tape is the most difficult part of this procedure. They cannot be too wet - they will not stick - or too dry - in which case they die.
6. Cover with a solution of 25 mM NaCl (or Halocarbon oil) to prevent drying and place eggs in a humid box at room temperature until injection. Eggs are appropriate for injecting around 2 hours after deposition when they have are medium dark. Choosing eggs for injection is discussed in **Figure 3.2.3**.
7. Immediately prior to injection, add more 25 mM NaCl to the coverslip. A large volume surrounding the embryos is desirable as it reduces distortion of the image. Attach the coverslip to a glass slide with a bit of double-stick tape along the edge and place on the stage.
8. Inject embryos on the ventral surface, near the posterior end, with the embryo turned at an angle of about 15-25 degrees. The horizontal angle of the needle can vary, but should be roughly within 30 degrees vertical from the plane of the stage. Take care to avoid injection into the periplasmic space. Instead, inject immediately anterior to the periplasmic space and posterior to the egg floats. Injections should be carried out at 100X magnification.
9. Immediately after all of the embryos on a slide are injected, remove the slide from the scope and and place the coverslip carrying the eggs into a cup of reverse-osmosis/deionized sterilized (RO/DI) H_20 at room temperature to recover.
10. When all injected coverslips from a cohort have been aligned and injected, placed all coverslips in a cup of 50 ml RO/DI H_20 under insectary conditions to hatch. It is not necessary that the eggs float for hatching.
11. Hatching will begin in approximately 48 hours after which the larvae can be handled as described in the chapter on family culture.

Method 2:

This method differs in that embryos are aligned against a thin membrane and injected semi-dry. No adhesive tape is necessary.

1. Cut pieces of membrane with a scalpel or razor blade at an approximately 45° angle so that the posterior edge for injection will be perpendicular to the needle. A cleanly cut edge is desirable.
2. Cut a piece of filter or blotter paper smaller than the height of the slide. You may wish to stack a couple of pieces to provide a larger water reservoir.

3. Assemble the membrane and filter paper as shown (**Figure 3.2.4**) and wet with water so that all paper is wet and the membrane is moist but not dripping.
4. Using a brush, transfer 30-50 embryos to the edge of the membrane.
5. Distribute them as shown (**Figure 3.2.5**) with the narrower posterior end toward the bottom. When aligning the embryos, roll them over so that the ventral side (convex) is upward, and they will nest nicely in the 90º niche between the membrane and slide.
6. Orient all in the same direction. As you work, keep the papers moist by adding small volumes (10 µl) of water to the blotter paper. You should maintain a meniscus of water around the eggs, but do not wet excessively causing the eggs to become dislocated.
7. When you have filled the edge of the membrane with eggs (~50), transfer to the scope for injection. Keep in mind that when using this technique, the needle will not be submerged in liquid, so keep sufficient back-pressure on the needle to keep it cleared. Frequently check the needle flow by withdrawing the needle and 'inject' into air. You should see a small droplet appear or run back up the needles into a larger droplet that often hangs on the needle shaft. Add small volumes of water to the blotter paper as the eggs dry during injection.
8. After injection rinse the eggs off into a Petri dish using water and incubate as in Method 1.

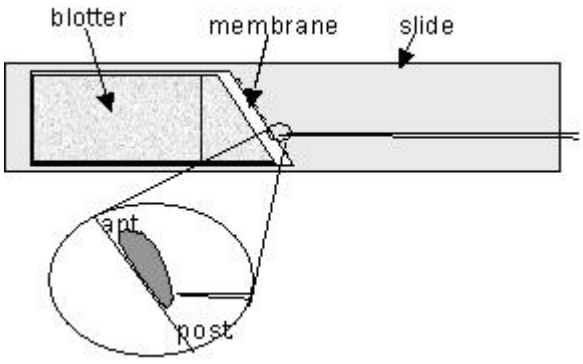

Figure 3.2.4. Diagram of microinjection method using membranes as opposed to the adhesive double-stick tape.

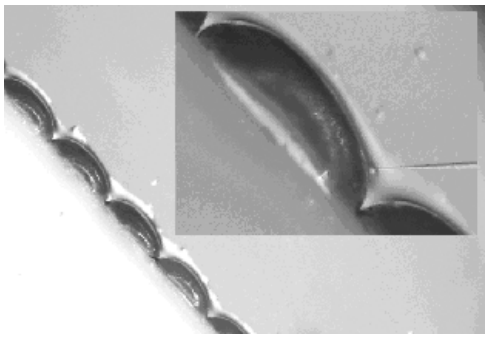

Figure 3.2.5. Eggs aligned for injection with a closeup of the needle approaching perpendicular to the injection area (photograph courtesy of G. Labbe, Oxitec).

Other things you might want to know

Q: Do you remove the chorion before injection as has been described in *Drosophila*?

A: No. Endochorion removal in anophelines has not been accomplished. This is why the quality of the needles and turgor of the eggs is crucial.

Q. What does a good *An. gambiae* injection look like?

A: Larval hatch rates vary between 10% and 50% using either method. The most probable cause of this variation is physical wounding of the embryo during injection. If the needle does not slide easily through the chorion of the egg during injection then something is wrong. It is the ease of penetration that allows continuous injection without needle clogging or breakage.

Under good conditions, the needle will slide in and out of the egg with little effort. Slight resistance to penetration is apparent when entering the egg and a small volume of yolk can sometimes be seen flowing into the tip of the capillary, only to be expelled immediately during injection. Although visibility is worse injecting under aqueous solution rather than halocarbon or mineral oil, a slight clearing of the yolk is often

seen, even through the dark chorion. Injected eggs sometimes recoil and bulge briefly and slightly when a sufficient volume has been released into them and this is also a good sign as long as a minimal amount of yolk escapes from the wound site.

Q: I don't have a laser needle puller. Will this method work with boro- or aluminosilicate needles?

A: In principle, there is no reason why this method would not work with a softer glass but with frequent needle replacement; however, an attempt at this has not been published. Quartz needles may simply allow a larger degree of error on the part of the person injecting. Aluminosilicate glass needles are preferable to borosilicate because of their greater hardness.

Q: Have you used a chorion hardening inhibitor?

A: No. Many inhibitors have been tested of the prophenoloxidase activation cascade (pNpGB, benserazide, PTU), but we have found nothing that clearly resulted in an increase in embryo injectability.

Q: Do you bevel your needles?

A: No. Non-beveled quartz is hard and sharp enough so that needles can be pulled and used immediately.

Q: How do you prepare your DNA for injection?

A: DNA was prepared with a Qiagen Endo-Free kit and resuspended in injection buffer. It was then stored at -80^{o}C until use.

Q: What do you feed your hatching larvae?

A: We feed L1 larvae two drops of 2% w/v baker's yeast on day two post-injection and another two drops on day four. Beyond day four, we feed as appropriate with our standard food mixture of finely ground Koi Floating Blend.

Q: I thought anopheline eggs floated when they hatched. Aren't your injected embryos submerged when they hatch in Method 1?

A: Yes. While *Anopheles* eggs typically do float, submerging eggs post-injection does not seem to have a strong effect on mortality relative to floating controls. In addition, this method avoids the large degree of mortality which was inflicted when attempting to remove the eggs from the adhesive surface of the tape.

Q: How hard do you press the coverslip down on the embryos when you're picking them up in Method 1?

A: Delicately but firmly (!?). Just hard enough to see that the eggs have come into contact with the tape and bulge slightly.

Q: What happens if I inject the embryos earlier than you describe?

A: Younger embryos are difficult to inject due to sensitivity to handling. Simply moving them early in development kills them.

References

Grossman GL, Rafferty CS, Clayton JR, Stevens TK, Mukabayire O, Benedict MQ (2001) Germline transformation of the malaria vector, *Anopheles gambiae*, with the piggyBac transposable element. Insect Mol Biol 10:597-604

Kim W et al. (2004) Ectopic expression of a cecropin transgene in the human malaria vector mosquito *Anopheles gambiae* (Diptera: Culicidae): effects on susceptibility to *Plasmodium*. J Med Entomol 41:447-455

3.3 Plasmodium *Sporozoite ELISA*

Robert Wirtz, Melissa Avery, Mark Benedict

Introduction

Enzyme-linked immunosorbent assays (ELISAs) were developed to detect *Plasmodium falciparum*, *P. vivax*-210, and *P. vivax*-247 circumsporozoite (CS) proteins in malaria-infected mosquitoes. The sensitivity and specificity of the ELISAs are based on the monoclonal antibodies (Mabs) used. The ELISAs detect CS proteins, which can be present in the developing oocysts, dissolved in haemolymph, and on sporozoites present in the haemocoel or in the salivary glands.

Therefore, a positive ELISA may detect CS in organs other than the salivary glands and does not establish that species as a vector. ELISA results also may not be concordant with detection of sporozoites from salivary gland dissections.

ELISAs can be carried out on fresh, frozen, or dried mosquitoes. If specimens are to be dried, they must be processed quickly and kept dry (stored with desiccant) to prevent microbial growth that can result in high background values. Before collection of the mosquitoes is initiated, consideration should be given to the possibility of conducting other tests (e.g., molecular, host blood meal, etc.) that may require different storage conditions or extraction buffers. Voucher specimens should also be collected and saved.

The "sandwich" ELISA is begun by adsorption of the capture Mab to the wells of a microtiter plate (**Figure 3.3.1**). After the capture Mab has bound to the plate, the well contents are aspirated and the remaining binding sites are blocked with blocking buffer. Mosquitoes to be tested are ground in blocking buffer containing IGEPAL CA-630, and an aliquot is tested. Positive and negative controls are also added to specific plate wells at this time. If CS antigen is present (depicted as diamond in Fig. 1.B) it will form an antigen-antibody complex with the capture Mab. After a 2-hour incubation at room temperature, the mosquito homogenate is aspirated and the wells are washed. Peroxidase-linked Mab is then added to the wells, completing the formation of the "sandwich" (Fig. 1.C). After 1 hour, the well contents are aspirated, the plate is washed again and the clear peroxidase substrate solution is added (Fig 1.D). As the peroxidase enzyme reacts with the substrate, a dark green product is formed (Fig 1.D), the intensity of the color is proportional to the amount of CS antigen present in the test sample.

Results are read visually or at 405-414 nm using an ELISA plate reader 30 and/or 60 minutes after the substrate has been added. ELISA positive mosquitoes should be retested to: a) confirm positives and b) estimate the amount of CS protein per mosquito if desired.

Because the volume of mosquito homogenate is not sufficient for numerous tests, users may choose to ‘recycle’ the sample by transfering the homogenate to a second plate containing a different antibody.

For technical advice or recommendations regarding the use of this protocol, please contact:

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Acknowledgments

Development of these assays was a cooperative effort among the National Academy of Sciences, National Institutes of Health, Centers for Disease Control and Prevention, Naval Medical Research Institute, New York University, Walter Reed Army Institute of Research and the World Health Organization. These instructions were developed by Robert Wirtz, Melissa Avery and Mark Benedict.

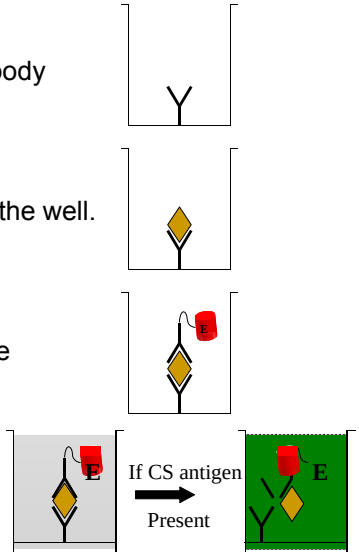
Figure 3.3.1. The "sandwich" ELISA for detection of *Plasmodium falciparum* and *P. vivax* circumsporozoite proteins.

A. Anti-sporozoite monoclonal antibody adsorbed to the plate.

B. Mosquito homogenate added to the well.

C. Peroxidase-linked anti-sporozoite monoclonal antibody added.

D. ABTS substrate added.



Preparation of Sporozoite ELISA Solutions

The following solutions should be prepared prior to performing the procedure. Do not add sodium azide to solutions as it is a peroxidase inhibitor. We also no longer add thimerosal to the solutions, as this is mercury-based and presents problems with proper disposal. Keep all solutions in the refrigerator when not in use and adhere to shelf lives to prevent problems related to microbial growth in working solutions.

Order supplies from the suggested vendors or insure that they are identical to those recommended. Be aware too that different 1.5 ml tubes have internal dimensions in which the pestles may not work, microtiter plates have different binding qualities, and different caseins are less efficient in blocking and may result in decreased sensitivity or higher background OD values.

1. **PBS** – phosphate buffered saline, pH ~7.4: Use stock laboratory PBS OR Dulbecco's PBS (Sigma #D5773). Adjust pH if necessary. Add 10 mg phenol red or 100 µl of phenol red stock solution (1 g/10 ml water) per 1 liter PBS. Store at 4°C. Shelf life is 2 weeks.
2. **BB** – blocking buffer: BB can be prepared in two ways (A or B). Shelf life is 1 week at 4°C; BB may be frozen. Use only ELISA grade bovine serum albumin (BSA) and casein prepared from bovine milk (Sigma C7078).

A.

BB - BSA / casein:	½ liter	1 liter
PBS, pH 7.4	500 ml	1 liter
BSA (1.0%)	5.0 g	10.0 g
casein (0.5%)	2.5 g	5.0 g
phenol red[1]	100 µl	200 µl

[1] Preparing a stock solution of phenol red (1 g / 10 ml water) eliminates the need to weigh small amounts.

1. Suspend bovine serum albumin (BSA) and casein in PBS and mix for 2 hours or until dissolved. Some casein may not dissolve.
2. Add the phenol red.

B.

Boiled casein (BB):	½ liter	1 liter
PBS, pH 7.4	450 ml	900 ml
casein	2.5 g	5.0 g
0.1 N NaOH	50 ml	100 ml
phenol red[1]	100 µl	200 µl

1. Suspend casein in 0.1 N NaOH and bring to a boil.
2. After casein is dissolved, slowly add the PBS, allow to cool, adjust the pH to ~7.4 with HCl, and add the phenol red.

BB:IG-630 – blocking buffer with IGEPAL CA-630: This is the mosquito grinding solution.

to 1 ml BB add 5 µl IGEPAL[2] CA-630

5 ml BB add 25 µl IGEPAL CA-630

Mix well to dissolve the IG-630 in the BB. Shelf life at 4°C is 1 week.

PBS:Tw – wash solution: PBS plus 0.05% Tween 20. Add 0.5 ml Tween 20 to 1 liter of PBS. MIX WELL. Store at 4°C. Shelf life is 2 weeks.

MAb stock – Monoclonal antibody stock solution: Dissolve the lyophilized MAb in diluent (1:1 distilled water and glycerol) to give stock solutions of 0.5 mg / ml (0.5 µg / µl). The water:glycerol solution prevents freezing during routine storage at -20°C.

P. falciparum = 0.20 µg / 50 µl PBS

P. vivax-210 = 0.025 µg / 50 µl PBS[3]

P. vivax-247 = 0.025 µg / 50 µl PBS[3]

[2] IGEPAL CA-630 (Sigma I3021) replaces NONIDET P-40 which is no longer available from Sigma-Aldrich. If available, NONIDET P-40 can be used.)

[3] Mabs for *P. vivax* will be made available in this kit at a future date.

Capture MAbs:

MAb	µg / 5 ml	µl MAb stock / 5 ml PBS
Pf	20 µg	40 µl stock
Pv-210	2.5 µg	5 µl stock
Pv-247	2.5 µg	5 µl stock

Peroxidase conjugated MAbs:

MAb	µg / 5 ml	µl MAb stock / 5 ml BB
Pf	5.0 µg	10 µl stock
Pv-210	5.0 µg	10 µl stock
Pv-247	5.0 µg	10 µl stock

Positive controls

The following table describes the volumes and amounts necessary for the dilution series from the stock (Vial I) starting with the lyophilized positive control. Add the volume of BB listed for Tube I and mix until the lyophilized positive control is dissolved. Using the volumes of BB recommended, perform 2 serial dilutions with thorough mixing between steps. Tube III is used as your single positive control in your initial test. If needed, further dilutions will be performed on the plate during confirmational testing.

Dissolved control solutions should be frozen for later use.

	Tube	vol. BB	Pf	conc. Pf
P. falciparum	I (stock)	200 µl +	5 µg =	25 ng / µl
	II	992 µl +	8 µl tube I =	0.2 ng / µl
	III	990 µl +	10 µl tube II =	2.0 pg / µl
	Tube	vol. BB	Pv0210	conc. Pv-210
P. vivax-210	I (stock)	200 µl +	5 µg =	25 ng / µl
	II	990 µl +	10 µl tube I =	250 pg / µl
	III	996.8µl +	3.2 µl tube II =	0.8 pg / µl
	Tube	vol. BB	Pv247	conc. Pv-247
P. vivax-247	I (stock)	200 µl +	880 µg =	4.4 µg / µl
	II	975µl +	25 µl tube I =	110 ng / µl
	III	990 µl +	10 µl tube II =	1.1 ng / µl

Mosquito Sample Preparation

1. Place the mosquito[4] in a labeled 1.5 ml micro centrifuge tube and grind in 50 µl BB:IG-630.
2. Rinse pestle with two 100 µl volumes of BB, catching the rinses in the tube (Total vol. = 250 µl).[5]
3. Before grinding the next mosquito, rinse pestle in PBS-Tw twice; dry with tissue to prevent contamination.
4. Samples may be used immediately or frozen for later analysis.

Preparation of Negative Controls

At least 7 or 8 laboratory reared female mosquitoes that are known to be uninfected[6] (same as test species if possible) should be prepared in the same way as the test samples. Use these in the same way as the test sample instructions in negative control wells. Be sure that you use a clean pestle so that contamination with a positive sample does not occur.

Developing Standard Curves

A negative cut-off value below which sporozoites will not be considered present must be determined by analysis of negative controls. Calculate the mean OD of at least 7 negative individuals after the ELISA procedure. If any has a value two times higher than the mean absorbance, retest the sample. That is, in order to determine if a sample is positive, negative, or questionable, multiple negative controls should be run in the plate with the samples. After the ELISA is completed and the OD's have been determined by the plate reader, take the average of the OD's for those negative controls. Multiply the average by 2. This is the "cutoff" value. If your sample's OD is greater than this cutoff value, they are considered positive. If sample's OD is below this cutoff value, they are considered negative. If your sample's OD is close in value to the cutoff value, run the sample in the confirmation test.

For each *Plasmodium* species, a standard positive control curve should be developed. In a microtiter plate, add 50 µl BB to wells in a column. Perform a 2 X dilution series by adding 50 µl from the appropriate Tube III to row A. Mix and transfer 50 µl to the next row well. Continue to the bottom of the plate. Perform the dilution series in triplicate. If desired, plots of the absorbance of these standards can be plotted as follows.

[4] You may grind one mosquito, one head + thorax only, or up to five pooled mosquitoes in each tube in 50 µl of BB:IG-630.

[5] The total resulting grinding volume is ideally 200 µl because CSP will be more concentrated and therefore the test more sensitive. However, each test requires 50 µl. If both an initial and confirmational test are needed, then 100 µl will be needed for each CSP. If testing for Pf, Pv210, & Pv247, then 300 µl could be needed. The likelihood of this for happening for all three is low, so 250 µl would be enough. If only testing for 1 CSP (like Pf) 200 µl will be enough and more ideal than 250 µl.

[6] This is ideal, but if not available, one can use male mosquitoes, or, if not those, then blocking buffer.

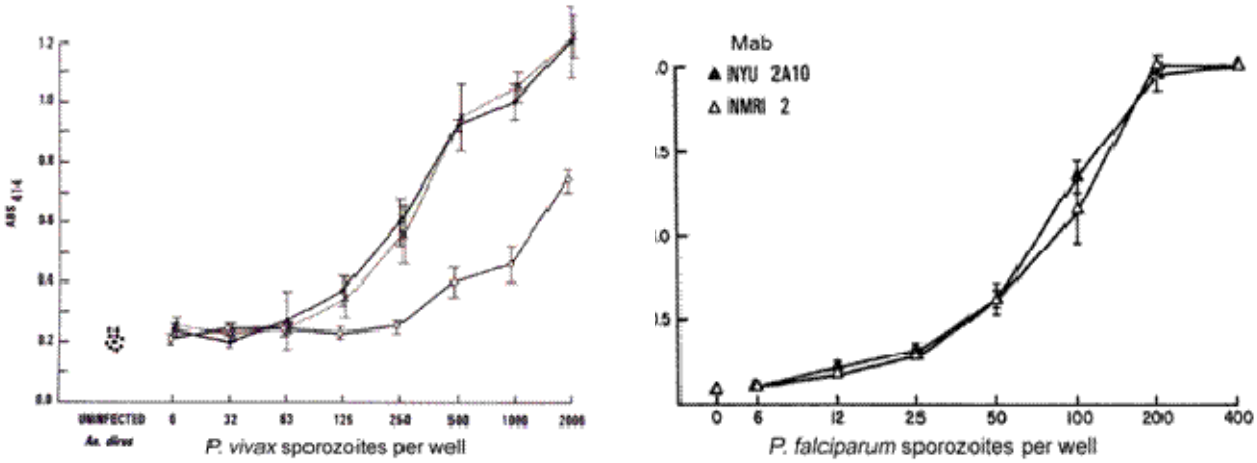


Figure 3.3.2. Examples of standardization curves developed for *P. vivax* (left) and *P. falciparum* (right). Note that near an OD of 2, the *P. falciparum* curves are not linear and would prevent correct estimates of the number of sporozoites present.

Sporozoite ELISA Directions

1. Place 50 µl of capture Mab solution in each well of the ELISA plate[7] and incubate for at least 30 min or as long as overnight at room temperature. (During this incubation, mosquito test samples and negative controls may be prepared. Alternatively, prepare the samples prior to analysis and freeze.)
2. Use a separate plate for each sporozoite species.
3. Cover plate with another plate or lid during all incubations to prevent evaporation.
4. Remove well contents by aspiration[8] and fill wells with BB.
5. Incubate 1 hour. (Alternative mosquito test sample preparation interval.)
6. Drain well contents by aspiration or banging plates sharply and add 50 µl mosquito homogenate per well.
7. Add 50 µl positive and negative control solutions to wells.
8. Incubate 2 hours at room temperature.
    a. Near the end of the incubation period prepare the ABTS - Substrate solution - This solution should be prepared fresh. Mix Solution A (ABTS from Kirkegaard Perry, www.kpl.com) and Solution B (hydrogen peroxide) 1:1. Prepare enough to add 100 µl / well. If you are doing a full plate, prepare 10 ml (5 ml of Solution A and 5 ml of Solution B).
    b. Near the end of the incubation period, mix Mab-peroxidase Conjugate in blocking buffer: 0.05 µg / 50 µl BB. Prepare enough for 50 µl / well. Hereafter we will refer to this simply as 'conjugate.'
    c. In a 1.5 ml microfuge tube, **confirm enzyme activity** by mixing 5 µl of the conjugate above with 100 µl ABTS. There should be a rapid color change indicating that the peroxidase enzyme and the substrate are functional.

[7] To fill each of the 96 wells on a plate with 50 µl requires 4.8 ml. It is convenient to make up 5.0 ml of each Mab solution and 10.0 ml of substrate (100 µl/well) per plate

[8] If a vacuum aspiration system is not available, trays should be held firmly by their sides, inverted over a sink or tray, and banged gently to remove the solutions. Follow this by blotting on clean absorbent paper.

9. Remove homogenate.
10. Wash wells 2 X with PBS-Tw by filling and emptying the wells.[9]
11. Add 50 µl conjugate to each well
12. Incubate for 1 hour
13. Remove conjugate
14. Wash wells 3 X with PBS-Tw.
15. Add 100 µl ABTS per well.
16. Incubate for 30 and / or 60 min.
17. Read visually, or determine OD at 405-414 nm[10] at both 30 and 60 minutes.

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9 If background has been observed in previous analyses, a third wash may be performed.

10 Absorbance values of OD > ~ 2 are in the non-linear portion of the standard curve. If quantitative information is required, dilute the strong positives 1:1 by adding 150 µl BB before retesting (Original grinding volume was 200 µl, minus 50 µl removed for testing). Repeat if necessary to insure that the absorbance value for the test sample is in the linear portion of the positive control curve (0.5 to 1.5 OD units) before calculating the number of sporozoites.

SPOROZOITE ELISA NO:_____

(Sample analysis)

DATES: Coat:_____ Test:_____

	1	2	3	4	5	6	7	8	9	10	11	12
A	pos	neg										
B												
C												
D												
E												
F												
G												
H												

SPOROZOITE ELISA NO:_____

(Standard Curves or 'Confirmation test')

DATES: Coat:_____ Test:_____

			+ control dilutions rep 1	rep 2	rep 3							
	1	2	3	4	5	6	7	8	9	10	11	12
A	neg 1		2 X	2 X	2 X							
B	neg 2		4 X	4 X	4 X							
C	neg 3		8 X	8 X	8 X							
D	neg 4		16 X	16 X	16 X							
E	neg 5		32 X	32 X	32 X							
F	neg 6		64 X	64 X	64 X							
G	neg 7		128 X	128 X	128 X							
H	neg 8		256 X	256 X	256 X							

3.4 Family Culture

MR4 Staff

Introduction

Family – or single family - culture is useful for many experimental plans or for the development of new strains. It is particularly important when the mother's genetics must be known, in genetic crossing, and when genotypic frequencies are of interest. If specific knowledge of the father's genetics is important to studies, pair matings must be used (Chapter 3) or inferred from progeny analysis. Otherwise, *en masse* - mated females can be isolated for individual egging. It some cases, the genetics of the mother will be determined based on the genotypes/phenotypes of her progeny.

Another application of family culture is to establish wild colonies. In species complexes such as *An. gambiae, An. funestus* and *An. dirus* where sympatric forms co-exist, it is essential to isolate the species of interest (Mpofu et al. 1993). By utilizing family rearing techniques, individual females are separated so that eggs from a single pair are segregated, thus allowing the establishment of pure-breeding lines that become laboratory colonies.

It is best to culture individual families in the dish in which the eggs were laid for the first couple of days to avoid moving fragile eggs/larvae. The Qorpak vial shown in **Figure 3.4.1** are suitable for 100 or fewer progeny, but larger numbers should be collected in larger cups to prevent early larval mortality. The larvae should be progressively transferred to larger containers and water volumes as they develop. It is not uncommon to culture a family in three different containers before pupation to ensure good survival.

A common error is to conclude that one's family culture method is suitable even in the absence of hatching data based on total egg hatch. Because L1s may die and decay rapidly, their presence can only be known based on a count of the *total number of eggs that hatched*. Particularly for frequency and survival data this is essential. Do not rely on counts of larvae even one day after hatching for quantitative data.

Obtaining eggs from reluctant females

Low rates of oviposition often hinder successful family culture. Several methods have been developed that have been shown to increase oviposition within the laboratory. The use of a dark oviposition dish is more attractive than a clear or white dish in *An. quadrimaculatus* (Lund 1942), *An. gambiae* (Huang et al. 2005), and *An. arabiensis* (MQB pers. comm.). In *An. albimanus,* wild caught blood fed females that were allowed to oviposit in a 5 dram vial laid more eggs than those allowed to oviposit in a large cage (Bailey and Seawright 1984). The complete removal of one wing of a gravid female that is lightly anesthetized will promote oviposition soon thereafter, though this is time consuming for the technician. This method is also a last resort because mortality results.

Rearing schedule for individual families

If you are starting with bloodfed material, begin with schedule at day 4.

Day 1- Blood feed females.

Day 2- No attention is required.

Day 3- No attention is required.

Day 4- Transfer gravid females to vials lined with filter paper (for example: Qorpack Bridgeville, PA. No. 3891 containing strips of filter paper cut to size, **Figure 3.4.2**) and containing 1-2 cm of water.

Day 5-Remove the females from their vials.

Day 6-add 2 drops of a 2% w/v yeast slurry to each vial.

Day 7-count hatch rate and transfer larvae to a larger container (**Figure 3.4.2**) containing 0.02% w/v final concentration of yeast (see Determining Egg Hatch Rates, Chapter 3).

Day 8-observe, but feeding is usually not needed

Day 9 through pupation-feed ground fish food or other larval diet. Monitor water quality carefully in these smaller pans as it is easier to accidentally overfeed such a small number of larvae. Transfer to larger containers as needed.

Figure 3.4.1. This particular individual family oviposition tube is modified from a chamber purchased through Qorpack Bridgeville, PA. No. 3891.

Figure 3.4.2. Examples of family rearing trays.

References

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3.5 Determining Egg Hatch Rates

MR4 Staff

Introduction

Egg hatching rates vary between stocks depending on intrinsic fertilization rates, semisterility due to crossing type or presence of chromosomal aberrations and the methods used to handle eggs after oviposition. Egg hatch rates >80% are typical. Counting hatched larvae is not a proxy for determining the hatch rate as mortality may occur in the L1 stage and these larvae are very difficult to detect. Typically, anopheline eggs that sink to the bottom of the pan do not hatch, however, they should be inspected and included in hatch rate data.

Unhatched eggs fall into several classes which may be of interest to record: (1) Unmelanised eggs are often observed but will inevitably fail to hatch; (2) Unhatched melanised eggs in which no indication of an embryo can be seen; (3) Unhatched melanised eggs in which a developing embryo is seen but never hatches; (4) Unhatched melanised eggs in which an embryo is alive but has not hatched.

The last category is problematic. In some species, hatch is very synchronous, but in others – *An. gambiae* – it occurs over several days even when eggs are moist (Lehmann et al. 2006). We have often observed egg batches in which most eggs have hatched a day earlier, but the activity of counting the eggs stimulates further hatch. These various types and timings of hatching should be taken into account when collecting and interpreting hatching data.

Materials

- Filter paper
- Wash bottle containing water
- Fine probes

Equipment

- Stereoscope
- 2-place denominator (counter)

Determining hatch rates for eggs collected from individual females

1. Assuming eggs have been collected in tubes, these should have been lined previously with filter paper (See Family Culture). If the eggs are not all at the edges, carefully touch the center of the water. The oil on your fingers will usually cause the eggs to move to the side. If not, tease them to the edge with a probe.
2. Very slowly and smoothly slide the papers up the side of the tube. The eggs should adhere to the paper. Transfer to a rigid, movable, flat surface. A small piece of Plexiglass (10 X 20 cM) is ideal for this.

Figure 3.5.1. An unhatched (left) and hatched egg (right) from the same female, laid on the same day. It is apparent the lower one has hatched because of the dislocated operculum. Un-melanised eggs do not hatch.

3. Count the eggs *in situ* under a stereoscope recording hatched vs. unhatched on the denominator. You may need to prod or burst the egg with a probe to determine whether it is hatched. Typically the operculum of hatched eggs will be dislodged somewhat (**Figure 3.5.1**). If only a sample of eggs is needed for rate information, counting 50 eggs is sufficient. Otherwise, count all the eggs.

Determining hatch rates for *en masse* egg collections

In this case, the container from which eggs are taken may or may not be lined with filter paper. If it is, skip to step 2.

1. Slide a small piece of filter paper (approx 2 X 6 cm) down the side of the tray above the eggs to be collected. When the paper begins to wet, slowly slide it behind the eggs until the paper is well-submerged.
2. Slowly slide the paper on which the eggs are resting up the side of the tray until it is above the water. The eggs should adhere to the paper as it is raised.
3. Transfer the paper to the counting board as described above and count.

References

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3.6 Anopheles *Mating*

3.6.1 Mating : General considerations

Paul Howell

Introduction

Mating in most anopheline mosquitoes occurs during the early evening and is believed to occur primarily in swarms. *Anopheles* male mosquitoes aggregate just before dusk and commence swarming at the onset of sunset. Swarming males use their erect antennal fibrillae to detect a nearby female mosquito's wing beat frequencies (Nijhout and Sheffield 1979; Ikeshoji et al. 1985; Leemingsawat 1989; Clements 1992). In *Toxorhynchites* it was found that males will actually harmonize their wing beat with females as they near, possibly as a form of species recognition, before mating commences (Gibson and Russell 2006). In many species, copulation is initiated in flight as males and females meeting within the swarms (Clements 1992). Once a male anopheline has grasped a receptive female, it reorients itself so it is in the venter-to-venter position allowing the reproductive organs to meet. After coitus commences, the male moves into an end-to-end position with the female as the pair falls (Charlwood and Jones 1979). Copulation may continue for a short period of time after alighting, but in most genera it is a very quick process which ceases before the pair reaches the ground.

Newly emerged anophelines are not sexually mature. Male mosquitoes require about 24 hours before their terminalia have rotated and their fibrillae are mature enough to become erect and detect females (Clements 1992). Female mosquitoes, however, typically need 48-72 hours before they become receptive to males - usually prior to blood feeding in the wild. *Anopheles* males can mate several times, but females become refractory to re-insemination and re-mating is rare (Villarreal et al. 1994; Yuval and Fritz 1994). In *An. culicifacies*, it was found that a proportion of colonized females had multiple inseminations, but this was attributed to the laboratory setting and not a natural behavior (Mahmood and Reisen 1980).

In the laboratory, it is often not feasible to maintain a colony in a large enough cage to promote natural swarming behavior. Instead, selection of a stenogamous colony - one that breeds in a small cage - is performed. During colonization, only a proportion of individuals will respond to the novel environment, and a genetic 'bottleneck' occurs - loss of heterozygosity. Norris et al. showed that even a newly colonized strain has an extreme loss of heterozygosity compared to field samples from the same area (Norris et al. 2001). Often, fixation of particular alleles is a quick inevitable process and cannot be remedied without the introduction of new field material.

Although little is known about cues that are needed to stimulate mating within the laboratory, some experiments have been done to develop methods to improve colony mating. The addition of a simulated sunrise and sunset has been shown to have a positive effect on colonization efforts – presumably due to improved mating (Charlwood and Jones 1980; Panicker and Bai 1980). There are also reports of researchers utilizing a low watt colored light prior to the dark period to stimulate mating (Pan et al. 1982; Villarreal et al. 1998). Cage size also has an impact on the success of a colony. Some species will not mate within a small cage due to some unknown parameter, such as eurygamy, and require larger cages to complete mating (Pan et al. 1982; Marchand 1985). Marchand (1985) and Peloquin (1988) utilized an artificial sky and horizon to promote mating.

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3.6.2 Forced Copulation

MR4 Staff

Introduction

Adults from several *Anopheles* - *An. dirus*, *An. funestus*, and *An. darlingi* - have proved difficult species from which to establish a *stenogamous* laboratory colony. The main obstacle has been to stimulate mating. In the late 1950's, McDaniel and Horsfall (1957) developed an induced copulation technique to produce *Aedes* mosquitoes that are intractable in the insectary. Their method was based on reported observations on the mating behavior of European mantids which decapitated their mates prior to copulation. When the male mantis was decapitated, the suboesophageal ganglion was severed thereby overcoming the innate inhibition of copulatory muscles in the male (Baker 1964).

This method is sometimes necessary - induced, or 'forced,' copulation - in order to initiate and even to maintain a colony (**Table 3.6.2.1**) or to obtain matings with rare or specific individuals. Often, colonies initiated by forced copulation will eventually become stenogamous. Specific adaptations increase the success rate for mating of anophelines (Baker et al. 1962), (Ow Yang et al. 1963). Caravaglios (1961) found that males did not need to be decapitated and could be simply held with a small suction pipette, mated, and then returned to the colony without harm. Conditioning the males by placing them in a 15 C room for 12-24 hours was found to increase copulation rates (Baker et al. 1962).

***Anopheles* species**	**Reference**	**Stenogamy established**
annularis	(Choochote et al. 1983)	F10
dirus	(Lianzhu et al. 1986)	F8
earlei	(Kreutzer and Kitzmiller 1969)	F5
lesteri	(Oguma and Kanda 1976)	F7
Pseudopuncti-pennis	(Darsie and Lopez 1980)	F8
sinensis	(Oguma and Kanda 1976)	F27

Table 3.6.2.1. Anopheline mosquito colonies initiated utilizing the induced copulation technique that eventually became stenogamous.

Materials

- Minutien pins mounted on small wooden sticks (10-20)
- Ethyl ether
- 50 ml Falcon tubes modified to hold mosquitoes (or similar)
- Vacuum source with modified tip to prevent mosquitoes from being damaged
- Glass container with lid (e.g. staining jar)
- Cotton balls

Method - modified from the *An. maculatus* technique (Ow Yang et al. 1963)

Success depends on the preparation of high-quality males and females. Both sexes should be of an appropriate age that reflects when the species mates. Males should be at least 72 hours old and females at least 48-72 hours old to ensure the reproductive organs have fully matured.

1. Two to four hours prior to mating, bloodfeed 3-4 day old females to repletion. Using bloodfed females is not absolutely necessary, but it has been reported that the engorged abdomen of the female makes forced copulation more successful (McCuiston and White 1976). Moreover, this ensures that at least one of the requisites for obtaining progeny has been accomplished!

2. Separate males from females and place them in separate containers.[1]
3. Gently aspirate approximately 10 blood fed females into an anesthetizing container (**Figure 3.6.1**). Before proceeding, see Mosquito Anesthesia section. The anesthetizing container can be made from a 50 ml Falcon tube with the tip removed and both ends covered in mesh held in place with rubber bands or any container that can withstand ether. In a glass container with a lid, place 10-20 cotton balls in the bottom and pour in 5-10 ml of ether. Nitrogen or carbon dioxide gas can be used as well to anesthetize the females with little to no affect on the rate of successful copulation (Fowler 1972).

Figure 3.6.2.1. An example of an anesthetizing tube that can be placed in a larger container containing ether vapor.

Figure 3.6.2.2. Proper vacuum pressure aspiration of a male prior to pinning.

4. Prepare un-anesthetized males by attaching them to a fine pipette attached to a mild vacuum, the tip of which is just large enough to hold the male by the thorax without damaging it (**Figure 3.6.2.2**). The optimal place to capture a male is on the mesonotum. However, if the vacuum is weak you can capture the male by slipping the pipette over the male's abdomen. Caution should be used when capturing males in this manner as the vacuum may damage the male's claspers.
5. Once the male is captured, gently pierce the side of the thorax with a minutien pin mounted on a small wooden stick (**Figures 3.6.2.3 and 3.6.2.4**) e.g. the stick from an oral cotton swab could be modified with a pin for this purpose. It will be necessary to support the male against a firm surface to enable the pin to penetrate. Prepare 5-10 males at a time in this manner. Use only males that are still moving for matings.

[1] In some *Anopheles* species, it is has been found that "seasoning" the males by placing them at approximately 15-20° C at least 12 hours prior to copulation increases the mating success rate (Ow Yang CK, Sta Maria FL, Wharton RH (1963) Maintenance of a laboratory colony of *Anopheles maculatus* Theobald by artificial mating. Mosq News 23:34-35). This is not true for all species.

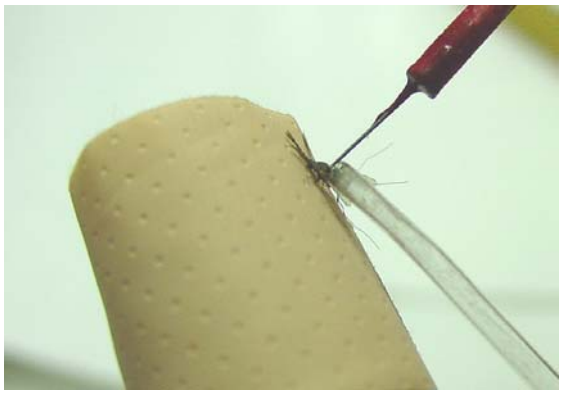

Figure 3.6.2.3. Holding male with vacuum pressure, gently stab the male through the thorax taking care to not crush the head.

Figure 3.6.2.4. Male prepared for mating.

6. Place one container of 10 females into the anesthetizing chamber and leave it for 6-10 seconds (depending on the species and concentration of ether).
7. Watch the females closely, and once they have all fallen from the sides, remove them from the chamber. Do not leave females in the ether too long or they will not recover from the treatment.
8. Gently disperse the females onto a piece of filter paper and position ventral side up. Take a mounted male, pinch off the head and hind-tarsi, and then gently stroke the abdomen of the male over the female's abdomen to stimulate the claspers to open.
9. Place the male at a 45-90° angle venter-to-venter with the female until the male clasps the female. Leave clasped for 1-2 seconds then pick up both using the male on the pin. If mated successfully, they will remain attached for several seconds.[2] Successful mating has usually occurred if they remain attached for 3-5 seconds.
10. Place male and female together into a new cage to allow female to recover from the anesthesia.
11. Ensure that the females are recovering from the anesthesia by gently blowing into the recovery cage. If females are not waking up within 10 minutes, anesthetize more lightly.

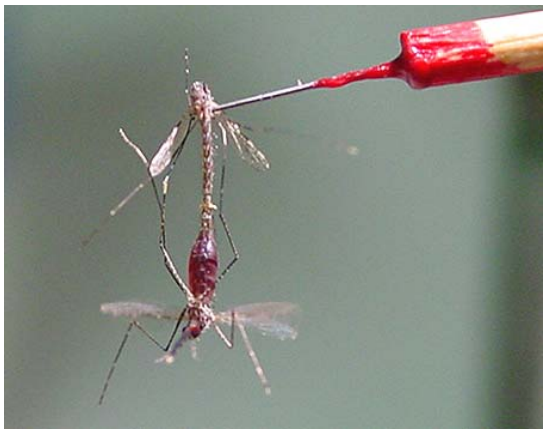

Figure 3.6.2.5. Successful mating. The decapitated male is attached to the bloodfed anesthetized female.

[2] It is possible to reuse males to mate more than one female.

References

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3.6.3 Pair Matings

MR4 Staff

Background

Pair mating (or single-pair mating) is most commonly used when knowledge of the genetic makeup of both parents is important. The isolation of specific phenotypes within *Anopheles* mosquitoes is useful in determining vector population species/genetic composition (Rabbani et al. 1976). Phenotypes such as insecticide resistance as well as other novel genetic mutations can be quickly isolated and purified using pair mating (Collins et al. 1986).

Beside forced copulation (Chapter 3), mating between a particular male and female can be obtained by free-mating in small numbers in small containers. Allowing a male and a female to mate freely is less invasive and time consuming than the method of forced copulation. Neither method is efficient, however.

Benedict and Rafferty (2002) reported a method for obtaining reasonable frequencies of free matings. They observed that mating did not occur until later than is typical, so we recommend keeping the pair in the mating tube for 7 or 8 days.

Materials

- Qorpack tubes with modified lids, or similar
- Cotton balls
- 10% sucrose solution
- Rack for holding the single pair mating tubes
- Aspirator

Figure 3.6.3.1. This particular choice of tube that we use for family oviposition is modified from a chamber purchased through Qorpack (Bridgeville, PA. No. 3891). Many similar tubes are suitable. The bottom may be covered with a filter paper disk to absorb fluids.

Procedure

1. Ensure the lighting in the space in which the mosquitoes will be held is adjusted to obtain sunset and sunrise periods to entrain the larvae before adulthood.
2. After emergence, place a male and female in an individual rearing container and leave for 5-8 days. Additional females may be added if desired – 5 would be a reasonable maximum.
3. Maintain the adults by placing a sugar pad on top of the tube and keeping it wet.
4. Obtain eggs as described in Chapter 2 under Family Culture.

References

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3.6.4 An. gambiae *and* arabiensis Mating Status Determination

K.R. Ng'habi and Greg Lanzaro

Introduction

The *An. gambiae* mosquito has a karyotype consisting of two pairs of autosomes (Chromosome 2 and 3) and one pair of sex chromosomes (Chromosome X and Y). The Y chromosome constitutes ~10% of the whole genome and contains a male determining factor which, when present in a XX/XY system, induces male development (Clements 1992). Y-chromosome linked DNA fragments have been characterized and Y-chromosome specific PCR markers have been developed (Krzywinski et al. 2004; Krzywinski et al. 2005). Previously, detection of mating success among females relied on microscopic dissection of female ovaries or examination of sperm in the female spermatheca. This method is reliable and robust but with the limitations that it is time consuming, labor intensive, and requires fresh specimens. A simple and rapid method to determine the mating status of dried female *An. gambiae* is therefore required in order to analyze large sample sizes within a short period of time.

Prepare PCR mixture for 96, 48 or 1 50µl PCR reactions[1]. Add reagents in the order presented.

96	48	1	Reagent
3724.8 µl	1862.4 µl	38.8 µl	Sterile water
18624 µl	9312 µl	5 µl	*Taq* 10X PCR Buffer with $MgCl_2$ (1.5 mmol/L)
3724.8 µl	1862.4 µl	0.2 µl	dNDP (2.5 mmol/L)
931.2 µl	465.6 µl	0.25 µl	S23 (F, 25pmol) [CAAAACGACAGCAGTTCC]
232.8 µl	116.4 µl	0.25 µl	S23 (R, 25pmol) [TAAACCAAGTCCGTCGCT]
116.4 µl	58.2 µl	0.5 µl	*Taq* polymerase
27.354 ml	13.677 ml	45 µl	Total (To each 45µl add 5µl of DNA template)

Table 3.6.4.1. F and R indicate forward and reverse primers, respectively. DNA extractions of males may also be used as positive controls.

96	48	1	Reagent
3724.8 µl	1862.4 µl	38.8 µl	Sterile water
18624 µl	9312 µl	5 µl	*Taq* 10X PCR Buffer with $MgCl_2$ (1.5 mmol/L)
3724.8 µl	1862.4 µl	0.2 µl	dNDP (2.5 mmol/L)
931.2 µl	465.6 µl	0.25 µl	128125I (F, 25pmol) [GGCCTTAACTAGTCGGGTAT]
232.8 µl	116.4 µl	0.25 µl	128125I (R, 25pmol) [TGCTTTCCATGGTAGTTTTT]
116.4 µl	58.2 µl	0.5 µl	*Taq* polymerase
27.354 ml	13.677 ml	45 µl	Total (To each 45µl add 5µl of DNA template)

Table 3.6.4.2. F and R indicate forward and reverse primers, respectively. DNA extractions of males may also be used as positive controls.

PCR cycle conditions

94°C/3min x I cycle
(94°C/20sec, 55°C-64°C/30sec and 72°C/1 min)* x 35 cycles
72°C/10min x 1 cycle
4°C hold

Run samples on a 3.0 % agarose EtBr gel for visualization. *The primers have different amplification temperatures but within this range should work.

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns compensate for imprecise measurements.

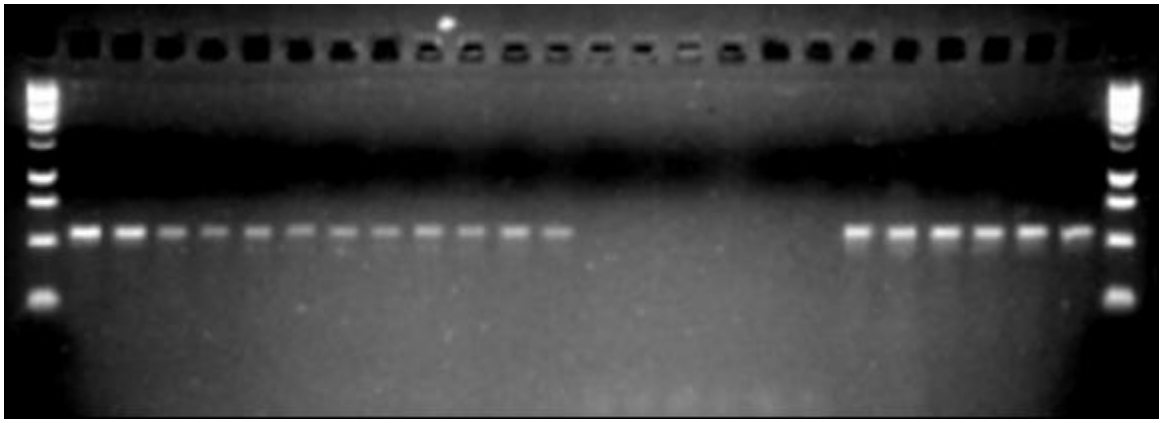

Figure 3.6.4.1: Agarose gel electrophoresis showing amplification of Y chromosome sequences in *An. arabiensis* (primer 128125I) males and mated females. There was no amplification in virgin females. Lanes 1 and 26, ladder, lanes 2-13 mated, lanes 14-19 unmated, lanes 20-25 males. (Ng'habi et al. 2007), used with permission.

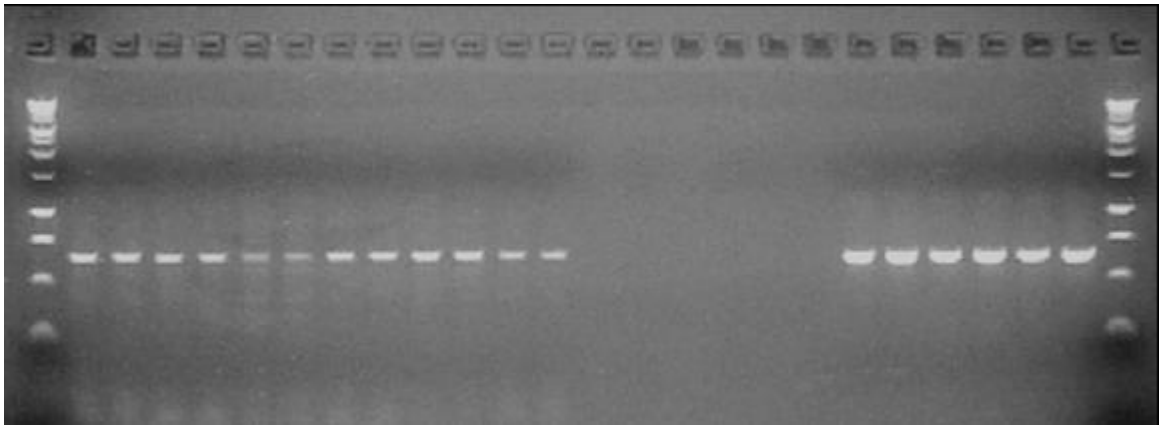

Figure 3.6.4.2: Agarose gel electrophoresis showing amplification of Y chromosome sequences in *An. gambiae s.s.* (Primer S23) males and mated females. There was no amplification in virgin females. Lanes 1 and 26, ladder, lanes 2-13 mated, lanes 14-19 unmated, lanes 20-25 males. (Ng'habi et al. 2007), used with permission.

References:

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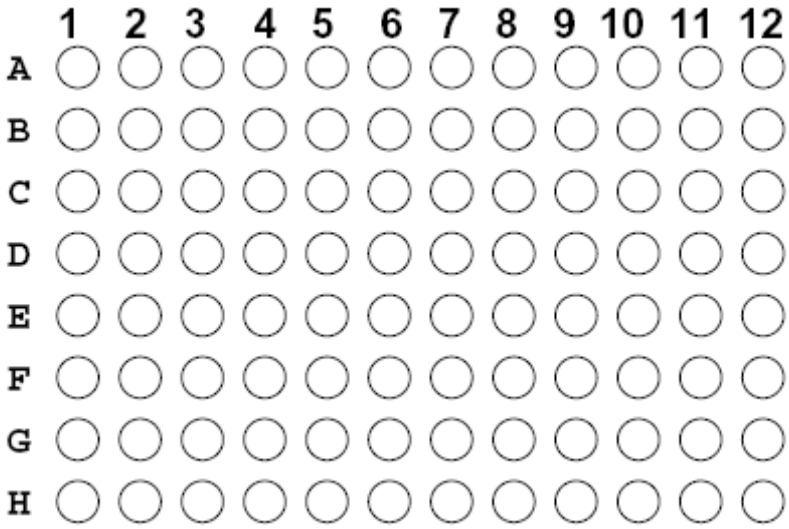
Ng'habi KR, Horton A, Knols BG, Lanzaro GC (2007) A new robust diagnostic polymerase chain reaction for determining the mating status of female Anopheles gambiae mosquitoes. Am J Trop Med Hyg 77:485-487

96 well sample preparation template



3.7 Anopheles Embryo Fixation

MR4 Staff

Introduction

The following embryo fixation method is suitable for preparing *An. gambiae* embryos for immunostaining and may be suitable for other anophelines or even genera of mosquitoes. It was developed and used by Yury Goltsev (2004) and further tested by John Yoder (2006). After fixation, embryos of the proper developmental stage must be selected from the pool for analysis. Removal of the relatively impermeable chorion of *An. gambiae* requires a method different from that used for *Drosophila melanogaster*.

Solutions

- purified water e.g. distilled or reverse-osmosis/deionized
- 25% household bleach diluted in purified water
- heptane
- 9% formaldehyde in purified water, adjust to pH 7 with NaOH.
- methanol

Materials

- glass vials e.g. scintillation vials
- 100 micron nylon mesh or similar (for device in **Figure 3.7.1**)
- Pasteur pipettes

Procedure:

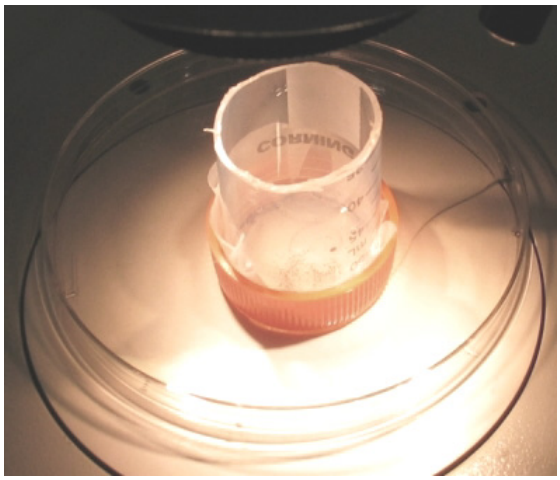


Figure 3.7.1. Eggs must be contained in a manageable mesh container in which solutions can be added and removed by draining and rinsing. Shown is an example of a possible container improvised from a 50 ml disposable tube that was cut. The lid clamps the mesh and has a hole to allow solutions to pass.

1. Remove egging cup containing newly laid embryos from mosquito cage and hold at 20^{o}C. Embryos can be collected for about 3 hours and then held until the desired developmental stage.
2. Rinse eggs into a fine mesh basket (e.g. 100 micron nylon mesh) with deionized water. Place mesh with eggs into an empty Petri dish under a stereo microscope. An example of a possible container for eggs is shown in **Figure 3.7.1**.
3. While watching the embryos through the microscope, gently add bleach solution to the egg container until the eggs are floating. This step washes away the exochorion. Swirl gently 1-2 times; remove the mesh container when approx 50% of the eggs sink.
4. Rinse eggs and mesh container thoroughly with deionized water.

5. Place eggs in a new Petri dish containing purified water while you “test crack” a sample of the embryos. This step is necessary to ensure that the exochorion was removed during the bleaching step. If this layer is not removed, the fixatives will not permeate the embryo and fixation will fail.

    a. Aliquot approx. 25 test embryos into a scintillation vial. Remove the water with a pipette and add 5 ml heptane.

    b. Incubate at room temperature for 5 minutes while occasionally swirling gently.

    c. Add 5 ml of methanol and vigorously swirl once to mix. Place scintillation vial on its side under the stereoscope and watch for cracking (**Figure 3.7.2).** It is normal for the embryos to seep out of their chorion. If the embryos crack, discard these test eggs and proceed with the protocol. If they do not, longer bleaching is needed.

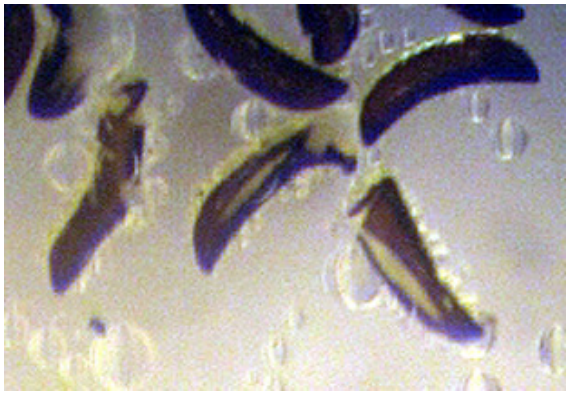


Figure 3.7.2. Examples of eggs cracking properly. Cracking is an indication that the process is proceeding correctly, and you can continue. Discard these test eggs.

6. Rinse embryos into a new scintillation vial with deionized water. Remove as much water as possible using a Pasteur pipette.

7. Add 5 ml heptane and shake 3-4 times gently by hand to mix. Remove as much remaining water as possible. Add 5 ml formaldehyde. Shake on rotary platform for 25 minutes on a medium speed setting. Eggs will accumulate between layers as shown in **Figure 3.7.3**.

Figure 3.7.3. Non-damaged eggs will accumulate at the interface between two layers.

8. Remove formaldehyde phase only (leaving heptane phase) using a fresh pipette. Replace with a large volume of deionized water. Briefly shake 3-4 times gently by hand and remove only the water phase (leaving heptane phase). Add 10ml of fresh deionized water.

9. Shake on platform an additional 30 minutes on a medium speed setting.

10. Remove only water phase (leaving heptane phase). Fill vial to the top with boiling deionized water. Incubate for 30 seconds.

11. Remove hot water phase (leaving heptane phase) and replace with ice-cold deionized water.

12. Place vial on ice for 10 minutes.

13. Remove water phase using a glass pipette.

14. Remove as much of the heptane phase as possible.

15. Add 5ml of fresh heptane. Remove as much water as possible.

16. Add 5ml methanol and swirl vigorously once, place scintillation vial on its side under stereo scope to watch for cracking, a sign the fixation has been successful to this

point.

17. Let stand 15-20 minutes.
18. Remove as much of both phases as possible. Rinse with 5 ml methanol twice removing any excess liquid, then add 5 ml fresh methanol.
19. At this point, the embryos can be stored at -20^{o}C in methanol for several months.
20. The endochorion must be manually peeled away using fine needles and double stick tape before staining.

References

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3.8 Mosquito Anesthesia

Mark Benedict and Paul Howell

Introduction

Adult mosquitoes can be immobilized by chemical anesthesia or by chilling. In the following, we describe simple methods and apparatuses to accomplish this. Be aware that in excess, all of these methods result in mortality and must be tested before routine use. Generally, highest survival is obtained with the minimum exposure sufficient to immobilize the mosquitoes. A common indicator of stress due to anesthesia - short of failure to recover - is tarsi falling off and abdominal swelling.

CO_2 and N_2

Carbon dioxide and nitrogen gas are both useful for anesthesia and are very safe for human exposure. They can be supplied as compressed gas, as vapor from sublimation of dry ice or evaporation of liquid nitrogen. CO_2 has the distinct advantage over nitrogen that, its density being greater than air, it pools in trays where mosquitoes are placed. Furthermore, its smell is distinct and unpleasant making detection simple. Nitrogen has no odor and regulation of the amount and its presence is more difficult to determine. However, given the apparent disadvantages of N_2, I have heard reports that nitrogen anesthesia is preferable to maintain some behaviors e.g. for forced copulation (for which see McCuiston and White 1976).

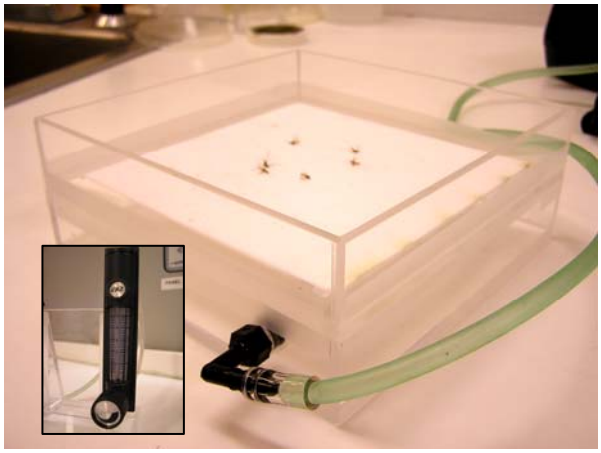

Figure 3.8.1. This improvised chamber provides sufficient area for as many mosquitoes as is safe to keep anesthetized at one time. It requires a flow rate of approximately 3 l / min which can be regulated with a simple flow valve (inset).

Shown is a small chamber made for anesthesia of mosquitoes (**Figure 3.8.1**). It is constructed of Plexiglas and has a white plastic platform made of porous polyethylene (e.g. Small Parts no. SPE-040-20) through which gases can pass. The gas is fed into the lower portion from which it suffuses up onto the mosquitoes lying on the tray. The raised walls of the upper portion help retain a reservoir of CO_2. This same container can be used for nitrogen anesthesia. Similar simple improvised anesthesia containers can be constructed using fine nylon mesh and plastic containers at hand.

A simple modification is to bubble the gases through water to humidify them. A device that will perform this can be made from a flask and rubber stoppers. We have no information indicating whether this measure increases longevity and/or recovery, but it is a prudent measure.

Ethyl Ether and Chloroform

Vapors of both of these are useful for anesthesia when used safely (see safety measures at the end of this section). Their volatility and combustibility combined with their effects on humans make them a second choice to CO_2 and N_2. However, they are very portable and require little equipment in use. Moreover, their effects are generally longer-lasting than those of CO_2 and N_2 meaning that more working time with an immobilized mosquito can be achieved. Chloroform kills mosquitoes more readily.

Both can be administered by pouring the minimum effective amount (e.g. 1 ml) onto an absorbent material such a sponge or cotton wool. The container holding this should be air tight and sufficiently large to introduce a mosquito holding tube. Mosquitoes are blown into the tube which is then placed in the chamber until the adults are knocked down. The tube is then removed and the adults shaken out.

Triethylamine

This chemical is used for *Drosophila* quite successfully because it is extremely safe. It is available for this purpose from Carolina Biological Supply as “FlyNap.” Our limited experience with this is that it ‘anesthetizes‘ *Anopheles* mosquitoes irreversibly. In this regard, it is similar to chloroform but would be a good choice when extended immobilization but not recovery is acceptable. Normal biological activities of several types during anesthesia have been confirmed in *Culex* (Kramer et al. 1990), but we are not aware of similar observations of *Anopheles*.

Chilling

Figure 3.8.2. A small Peltier cold table on which damp filter paper is placed to conduct cold and prevent mosquitoes from sticking to the condensation.

All mosquitoes with which I have had experience will withstand some degree of chilling on ice followed by resting on a near-freezing surface. Shown is a small chilling table that can be used for this purpose. It is usually necessary to cover the cold surface with a thin piece of *damp* paper to prevent the mosquitoes from sticking to the condensate on the platform. The slightly damp paper adheres uniformly to the plate and increases heat transfer. Even this must be changed frequently as it becomes sticky. Entire cages or cups of mosquitoes can be placed briefly in a freezer in order to knock them down and then transferred to a chilled surface, but make the time as short as possible since most anophelines will not survive total freezing.

Ether and Chloroform Safety

Exposure to ether and chloroform should be minimized by keeping containers sealed, dispensing minimum amounts, and using them for the shortest possible durations of time.

Diethyl ether should be stored in a flammable storage cabinet or an explosion proof refrigerator not longer than 3 - 6 months. This cabinet should not be used to store oxidizing agents. Explosive peroxides can form with long term storage, so purchase and store only enough for immediate needs. The occupational exposure limit is 400 ppm as a time-weighted average (TWA, 8 hour exposure) and it has a short term limit exposure (15 minutes) of 500 ppm. Keep the anesthesia chamber closed as much as possible. Review the specific MSDS of the manufacturer you purchase ether from for any additional handling and storage recommendations, as well other relevant health and safety information. Some manufacturers recommend that you do not open unless contents are at room temperature or below, and that after opening the container, any unused ether be discarded or disposed of after 2-3 days. Only dispense ether in a chemical fume hood. Avoid agitation and sparks during all phases of use.

Chloroform is considered a known animal carcinogen with unknown relevance to humans. Its occupational exposure limit for an 8-hour TWA exposure is 10 parts per million (ppm). However, exposures to chloroform should never exceed 50 ppm at any time. This is referred to as OSHA’s ceiling occupational exposure limit. Chloroform has a low odor threshold of 85 ppm, so by the time you smell chloroform the ceiling concentration would have already been exceeded. Anesthetizing procedures

should be performed in an area with good ventilation, preferably a chemical fume hood or other form of local exhaust enclosure.

Employees using this chemical should be trained to recognize the acute and chronic health effects associated with an over-exposure that can occur by inhalation, absorption through the skin and by ingestion. Selection of gloves is of particular importance since permeation of some nitrile gloves can occur within as little as 3 minutes. Contact the glove manufacturer for specific selection recommendations. Employees should always be informed of glove limitations and trained accordingly even for incidental use. Chloroform should not be stored with caustics. Review the MSDS for additional safe handling, storage and disposal information.

Acknowledgments

Thanks to Paul Vinson and Cheryl Connell of the CDC Office of Health and Safety for safety advice.

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McCuiston LJ, White DJ (1976) Laboratory colonization of *Aedes sollicitans* (Walker) with a review of the technique of induced copulation. Proc N.J. Mosq Control Assoc.:164-175

3.9 Eye Color Mutant Screening

Mark Benedict

Introduction

Anopheles mosquitoes undergo induced color change (called homochromy) based on perception of the background against which they are cultured.[1] When larvae are reared on either a dark black or white background, they become pigmented dark or pale respectively as shown by the pair of *Anopheles albimanus* larvae in **Figure 3.9.1**. The degree of darkening depends in part on the length of time the larvae have been cultured in a black container and the degree of fat body development. Therefore, larvae cultured during their entire development in a dark container at a low density show this change most dramatically. This color change depends on the normal eye pigmentation and, presumably, on the proper function of any pigmentation and signaling pathways involved in the response.

The method requires simply culturing the larvae from at least the second stage in black or dark-colored containers that are illuminated. (Larvae cultured in darkness will develop typical pale pigmentation.) The source of illumination does not appear to be critical. Occasional transfers of a few minutes to white containers for feeding or correcting the density does not interfere with the effect.

At the L3 or L4 stage, larvae are scanned *en masse* in the dark tray in a well-illuminated location for those that appear lighter in color. They are usually quite apparent as demonstrated by the two larvae in **Figure 3.9.2**, but purposely seeding a sample of dark larvae with a few that are pale will demonstrate the degree of effect that can be expected. These individuals are transferred to a dish for microscopic examination. Leaving the larvae undisturbed during examination provides better visualization since the dorsal side coloration is a more consistent indicator of general color. After this initial selection, it is also helpful to transfer the larvae to a white tray and scanning for pale individuals. Usually, no more than approximately 25 larvae per thousand cultured in this way require individual examination.

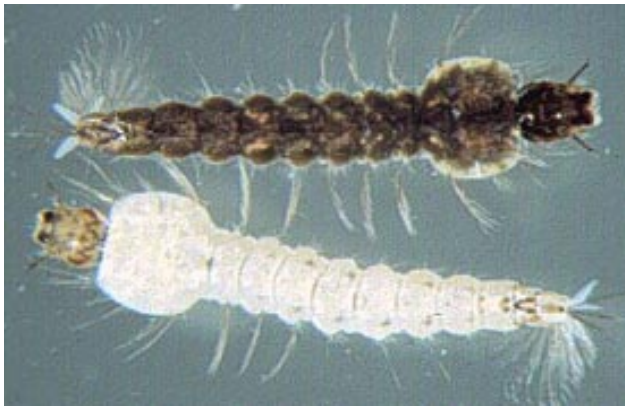

Figure 3.9.1. *Anopheles albimanus* larvae reared on a black background (top) and a white background (bottom).

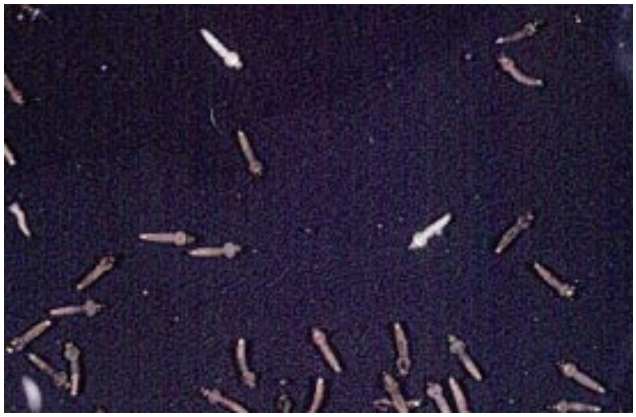

Figure 3.9.2. Eye color mutants are easier to detect on a dark background as they will not change pigmentation and will appear much lighter in color.

[1] Before beginning a large screen, it is advisable to culture a thousand or so larvae of the species of choice in dark containers. While all individuals of most species change color, some laboratory stocks have a low frequency of individuals that do not.

References

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Benedict MQ, Chang H (1996) Rapid isolation of anopheline mosquito eye-colour mutants based on larval colour change. Medical and Veterinary Entomology 10:93-96

3.10 Establishing Cell Lines from Anopheles spp. Embryonic Tissues

Ulrike Munderloh

Materials

Anopheles eggs

Mosquitoes are commonly reared at ~28°C; other temperatures are suitable, but will influence the timing of egg production and embryonic development. Female mosquitoes are provided a blood meal from a suitable host in the afternoon of day 0. The afternoon/evening of day 2, a dish with clean water is placed in the cage, to allow females to deposit eggs over night.

Eggs aged 24-36 hrs are collected using a transfer pipette, strip of screen, or filter paper, and added to a 35 mm diameter Petri dish containing 70% ethanol with a drop of Tween 80 (e.g., Sigma-Aldrich catalog Nr. P4780). The eggs will sink, and should be agitated by swirling the dish. The ethanol is replaced with 0.5% benzalkonium chloride (e.g., Sigma-Aldrich catalog Nr. 09621) with a drop of Tween 80, and the dish again agitated for 5 min. The benzalkonium chloride is removed, and the eggs are rinsed 2-3 times in sterile, distilled water. 50~100 eggs are transferred to a new dish containing 0.2 ml of culture medium supplemented with 10-20% fetal bovine serum (FBS, heat-inactivated), 5-10% tryptose phosphate broth (TPB), and a mixture of penicillin (50-100 units/ml) and streptomycin (50-100 µg/ml; e.g., Invitrogen catalog Nr. 15140-122) and fungizone (0.25 – 0.5 µg/ml; e.g., Invitrogen catalog Nr. 15290-018).

Media

We have used Leibovitz's L-15 medium successfully, as well as a modification thereof, L-15B, diluted to ~300 mOsm/L using sterile cell culture grade water (Munderloh and Kurtti 1989; Munderloh et al. 1999). Other media may be substituted, such as RPMI1640, Medium 199, Eagles' MEM, or Ham's F12 (e.g., from Invitrogen, http://www.invitrogen.com/site/us/en/home/Applications/Cell-Culture/Mammalian-Cell-Culture.reg.us.html) with 10% - 20% FBS (Invitrogen or Sigma) and 5-10% TPB (Becton Dickinson, catalog Nr. 260300), but should be tested for their ability to sustain primary and established cell lines. The pH of the medium should be adjusted to 7.0 to 7.2 using either sterile 1-N NaOH or 1-N HCl, as needed. If the medium pH drifts up beyond 7.8, it may be useful to add a buffer such as HEPES (e.g., Invitrogen catalog Nr. 15630) or MOPS (e.g., Sigma-Aldrich catalog Nr. M1442) at ~25 mM concentration

Methods

Eggs are crushed by applying gentle downward pressure using a sterile glass or plastic plunger from a 3 or 5-ml syringe, the flattened end of a sterile glass rod, or similar device. Crushed eggs and tissues are collected with a 2-ml pipette, and transferred to a 5.5 cm^2 flat-bottom tube (Nunc, catalog Nr. 156758) in 1-2 ml of complete medium containing antibiotics and antifungal solution as above, and the tubes are tightly capped. Cultures are incubated flat side down at 28-31°C. Use of a CO_2 incubator is not necessary and not recommended.

Cultures are fed approximately once a week by removing as much of the medium as possible without aspirating any tissue fragments or cells and replacing it with 2 ml of fresh medium. Antibiotics/antifungals should be included in the medium for the first few weeks, and can be omitted subsequently. If it is desired to continue using antibiotics, the antifungal component should be omitted. A mixture of penicillin and streptomycin is preferable over gentamycin as the latter may adversely affect mosquito cell lines in the long term.

The progress of the cultures is best monitored using an inverted phase contrast microscope. During the first days after adding the embryonic fragments, most tissue clumps will remain non-adherent, and organs such as guts and Malpighian tubules should show active peristaltic movements. Within days to weeks, cells should be migrating out from the torn tissue ends, and will often anchor to the bottom of the tube. Commonly, hollow balls consisting of a "monolayer" of cells surrounding a fluid-filled interior, will be seen

sprouting from embryonic fragments. Once cells in a primary culture have replicated sufficiently, a portion (~1/2 to 1/3) of the tissues can be removed by pipetting, and transferred to a new tube to set up a subculture. It is advisable to keep the other portion of cells in the parent flask or tube, as it is common that they will remain vigorous even if the subculture should fail.

This process is repeated many times until cultures can be subcultured or split on a regular basis, and the culture is considered established. During this process increasingly larger culture vessels will be used, e.g., 12.5-cm^2 flasks, then 25-cm^2 flasks, etc. Although the first several subcultures are usually made by transferring 30 – 50% of the cells to a new culture vessel, with time it is advisable to "push" a cell line by using higher dilutions of 1:10 or more. Some mosquito cell lines can be diluted up to 100-fold. Seeding subcultures at relatively high densities (dilutions of 1:2 or 1:3) will depress cell replication and slow growth, often resulting in cultures of poor condition.

It is not uncommon that a single primary culture will give rise to sublines displaying differing morphologies. Some sublines may continue growth as adherent cells, and others may become established as suspension cultures. In particular, the "hollow ball" or "vesicle" phenotype frequently develops, and may become fixed. Sublines with particular, desired characteristics can also be selected by culture manipulation (e.g., adherent lines can be developed by continuously discarding non-adherent cells during medium changes). Once cells are growing reliably, it is a good idea to try to reduce the amount of FBS, and TPB. Established mosquito cell lines commonly grow quite well with only 5% of FBS. Adding a lipoprotein supplement (such as the one from Rocky Mountain Biologicals, Missoula, MT, or the CellPro-LPS from Fisher Scientific), if available, can further reduce the requirement for FBS, and reduce cost.

These methods can equally be applied to other mosquito species, keeping in mind the length of time required for embryonic development. Eggs should be at least at their half point before hatching, all the way up to just before hatching. Although open culture vessels can be used, such as small Petri dishes or multi-well plates, they require a humidified atmosphere as well as a CO_2 incubator when media containing bicarbonate are employed. Also, open culture vessels are far more susceptible to contamination than closed ones.

Additional references: (Munderloh et al. 1982) (Mazzacano et al. 1991)

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3.11 Molecular Identification of Plasmodium spp. in Anophelines

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Introduction

The identification of *Plasmodium* species in whole mosquitoes by PCR is difficult because of the presence of reaction inhibitors from the insects (Schriefer et al. 1991). The present protocol is based on a chelex extraction which overcomes the PCR inhibition phenomenon. The semi-nested multiplex PCR technique detects and distinguishes among the four human *Plasmodium* species in single mosquitoes and in pools of up to 100 mosquitoes. The extraction and PCR technique presented here can be useful to: (1) estimate by mosquito pooling *Plasmodium* prevalence in *Anopheles* populations in low prevalence areas where large numbers of individual mosquitoes would need to be processed to obtain a reliable estimate; (2) incriminate *Anopheles* species as malaria vectors; (3) identify at one time all the circulating *Plasmodium* species in vectors from an area; (4) detect mixed infections in mosquitoes; and (5) detect mosquitoes with low-level parasite infections.

DNA extraction (Lardeux et al. 2008)

Homogenize mosquito heads + thoraces in saline solution (NaCl 0.9%), add Chelex 100X (**Table 3.11.1**) and vortex. Incubate at 100°C for 10 min and centrifuge at 13 000 rpm for 5 min. Mix the supernatant with 1:1 vol. phenol-chloroform and centrifuge at 10 000 rpm for 5 min 3 times. Mix the supernatant with 1:1 vol. 70% ethanol and centrifuge 14 000 rpm for 20 min. Dry the pellet at 37°C, suspend it in 100 µl sterile H_2O (nuclease-free H_2O). Keep at 4°C until PCR processed.

Mosquito pool size	Volume NaCl (µl)	Concentration Chelex 100X (w/v)°	Volume Chelex 100X (µl)
1-10	50	5 %	240
20	100	5 %	480
30	150	5 %	750
40	200	10 %	800
50	250	10 %	800
60	300	10 %	900
70	350	10 %	900
80	400	10 %	1000
90	450	10 %	1000
100	500	10 %	1000

Table 3.11.1. Concentration of Chelex 100X (%), volumes (µl) of chelex 100X and of saline solution used in the preparation of the DNA template in accordance with the size of the pool of mosquitoes (n° of mosquitoes processed)

Semi-nested multiplex PCR for Human *Plasmodium* species identification (Lardeux et al. 2008)

Prepare PCR Master Mix for 96, 48 or 1 20 µl PCR reactions[1]. Add reagents in the order presented.

96	48	1	Reagent
259.2 µl	129.6 µl	2.7 µl	Nuclease free H_2O
384.0 µl	192.0 µl	4.0 µl	Taq 1X PCR Buffer with $MgCl_2$
96.0 µl	48.0 µl	1.0 µl	dNTP's (0.5 mM mix)
96.0 µl	48.0 µl	1.0 µl	*Universal reverse*. UNR (R, 0.05 µM) GAC GGT ATC TGA TCG TCT TC
96.0 µl	48.0 µl	1.0 µl	*Plasmodium.* PLF (F, 0.05 µM) AGT GTG TAT CAA TCG AGT TTC
28.8 µl	14.4 µl	0.3 µl	Go Taq DNA Polymerase (5U/µl)
960 µl	**480 µl**	**10 µl**	**Total (to each 10µl reaction add 10 µl template DNA)**

Table 3.11.2. F and R indicate forward and reverse orientation.

Prepare PCR 2 Master Mix for 96, 48 or 1, 20 µl PCR reactions; Add reagents in the order presented

96	48	1	Reagent
912.0 µl	456.0 µl	9.5 µl	Nuclease free H_2O
384.0 µl	192.0 µl	4.0 µl	Taq 1X PCR Buffer with $MgCl_2$
96.0 µl	48.0 µl	1.0 µl	dNTP's (0.5 mM mix)
153.6 µl	76.8 µl	1.6 µl	PLF (F, 0.08 µM) AGT GTG TAT CAA TCG AGT TTC
38.4 µl	19.2 µl	0.4 µl	*P. falciparum.* FAR (R, 0.04 µM) AGT TCC CCT AGA ATA GTT ACA
38.4 µl	19.2 µl	0.4 µl	*P. vivax.* VIR (R, 0.04 µM) AGG ACT TCC AAG CCG AAG C
38.4 µl	19.2 µl	0.4 µl	*P. malariae.* MAR (R, 0.04 µM) GCC CTC CAA TTG CCT TCT G
38.4 µl	19.2 µl	0.4 µl	*P. ovale*. OVR (R, 0.04 µM) GCA TAA GGA ATG CAA AGA ACA G
28.8 µl	14.4 µl	0.3 µl	Go Taq DNA Polymerase (5U/µl)
1728 µl	**864 µl**	**18 µl**	**Total (to each 18 µl reaction add 2 µl of PCR1 amplicon)**

Table 3.11.3. F and R indicate forward and reverse orientation.

PCR cycle conditions

PCR 1 : UNR-PLF

94°C/ 5 min x 1 cycle
(94°C/ 1 min, 60°C/ 1 min, 72°C/ 90 sec) x 40 cycles
4°C hold

PCR 2: PLF-MAR, FAR, VIR, OVR

94°C/ 5 min x 1 cycle
(94°C/ 30 sec, 62°C/ 30 sec, 72°C/ 60 sec) x 35 cycles
72°C/ 10 min x 1 cycle
4°C hold

Run samples on a 1.5 % agarose EtBr gel for visualization.

If needed, positive samples from agarose can also be run on an 8% polyacrylamide gel, staining with 0.2% silver nitrate and revealed with a 2:1 volume of 30 g / l sodium carbonate: 0.02% formaldehyde.

Primers create fragments of 269 bp (*P. malariae*) , 395 bp (*P. falciparum*), 436 bp (*P. ovale*), 499 bp (*P. vivax*)

[1] Amounts for larger master mixes have been adjusted upwards to be sufficient for 50 and 100 rxns compensate for imprecise measurements.

References

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96 well sample preparation template