A protocol for membrane feeding assays to determine the infectiousness of *P. falciparum* naturally infected individuals to *Anopheles gambiae*

1 Direct membrane feeding assay protocol outline

This protocol describes procedures to conduct membrane feeding assays to assess the infectiousness of individuals living in malaria endemic areas. Humans may be infectious to mosquitoes if they have mature male and female gametocytes in their peripheral blood. Because of the limited sensitivity of microscopy to detect gametocytes, epidemiological studies that aim to enroll all potentially infectious individuals may choose to include all individuals with evidence of malaria infection (gametocytaemic or not) or enroll individuals regardless of blood film results. Studies that do not aim to include all potentially infectious individuals may be more restrictive and impose a minimum gametocyte density prior to membrane feeding.

The protocol below describes the whole blood membrane feeding assay wherein a venous blood sample is offered to mosquitoes directly. Procedures where the autologous plasma of gametocyte carriers is replaced by malaria-naïve control serum may require optimisation to prevent a loss of gametocyte infectiousness compared to the whole blood membrane feeding assay described below. A description of an approach to serum replacement membrane feeding assays is described in section 5.6 of this protocol. An example of a data-recording sheet is shown in section 6.

2 Staff

- Nurse or other clinically qualified personnel who can safely perform venipunctures
- At least one entomologist as assisting staff

3 Materials

3.1 Durables

- Temperature and humidity controlled insectary that is equipped to securely hold malaria-infected mosquitoes
- Safety hood for mercurochrome preparation
- –20°C freezer
- Thermostatic circulator bath with pump [e.g., Julabo ED Heating Immersion Circulator]
- Incubator or water bath (to maintain disposables and blood samples at 37°C)
- Weighing scale with 0.5g precision
- Dissection microscope [e.g., Leica S6 E stereozoom with optic light source]
- Conventional binocular light compound microscope
- Membrane feeder set up, typically 5-20 glass feeders or more [e.g., www.coelenglastechniek.nl] connected to each other by plastic tubing, which is also connected to an immersion circulating water bath [e.g. various models of the Thermo Fisher Scientific Isotemp Immersion Circulator or similar]
- Mosquito cages or long-term mosquito storage cups for holding mosquitoes [e.g., size 25x25x25cm] and selecting fed mosquitoes [e.g., size 35x35x35cm]
- 1000 ml pipette [for serum replacement procedure only]
- Heated centrifuge [for serum replacement procedures only, e.g., Eppendorf Centrifuge 5702RH]
- Cup holders [optional]
- Personal protection: protective clothing should be worn at all times (e.g. a laboratory coat or smock) as well as clothing to cover exposed skin

3.2 Consumables for venous sampling

- Gloves
- Ethanol swabs
- Cotton wool
- Heparinised or Citrate Phosphate Dextrose tubes (≥4 ml) [e.g., Vacutainer® Lithium Heparin Green 10.0 ml with Hemogard Closure]
- Tourniquet
- Vacutainer needle/butterfly needle [e.g., Blood Collection Set Safety-Lok™ 21G 0.75in 7in Luer Green]
- Sticking plaster (band-aid)

3.3 Consumables mosquito husbandry and membrane feeding

- Plastic bag or heated water bottle for selection
- Cotton wool or cotton wool pads
- 6-10% glucose solution; or sugar cubes used along with wet (water) cotton balls
- Gloves
- Parafilm [e.g., 10.2 cm x 3658cm (4 inch x 125 ft)] or Baudruche membrane or hemotek membrane (rubber bands will be needed to hold the hemotek membranes on a traditional glass membrane feeder)
- Scissors
- Safety goggles to prevent blood exposure incidents
- Paper cups (500 ml) for short-term mosquito storage and feeding (ice cream pint cups, hard walled, cardboard)
- Sticker labels
- Blunt needles [e.g., Kendall Monoject aluminum hub blunt needles 0.7mm x 38.1mm]
- Syringes [e.g., BD Plastipak™ 10 ml syringes]
- 1000 µl pipette tips
- Dark cloth for covering feeders
- 10% Bleach
- Cleaning basins
- Towel (wet towel to cover cups containing mosquitoes to maintain a good relative humidity in case holding

*MalariaWorld Journal*, www.malariaworld.org. ISSN 2214-4374 1 November 2013, Vol. 4, No. 16
rooms do not allow relative humidity to be controlled
- Nylon netting (with small holes)
- Rubber bands
- Tape (medical or labelling tape)
- Mouth aspirator or other suction device for handling mosquitoes
- AB serum from a blood bank from a non-endemic setting, e.g., European control serum (for serum replacement procedures only)
- Eppendorf tubes (for serum replacement procedures only)

3.4 Consumables mercurochrome solution and midgut dissection/examination
- Merbromine [Mercurybromide fluorescein Sigma # 63869]
- 100 ml bottles
- Droppers to dispense mercurochrome on dissection slides
- Distilled water
- Microscope slides [e.g. Menzel-GLäser 76x26mm microscopic slides]
- Cover slips
- Plastic disposable pipets
- No. 5 Forceps (X2)
- Surgical needle or surgical knife [e.g. Micro feather 30° disposable ophthalmic scalpel with plastic handle]

4 Preparations
4.1 Preparing a 1% mercurochrome solution (some groups use 0.4% mercurochrome)
- Place scale in the safety hood
- Put 100 ml bottles in the hood without the lids
- Weigh 0.5 g merbromine
- Add 0.5 g merbromine in 100 ml bottle and close lid
- Clean the hood with distilled water
- Add 50 ml distilled water (preferably with needle through the lid)
- The 1% mercurochrome solution is now ready for use
- Protect from light with foil or use dark bottles
- The mercurochrome solution can be stored at room temperature in the dark and used for 8 months
- If you prefer to use 0.4% mercurochrome, add 100 ml of distilled water to 0.4 g merbromide following the same procedures

4.2 Preparation of mosquitoes for membrane feeding assays (start on the day of feeding)
- Select mosquitoes of an appropriate age. For Anopheles gambiae, 2-7 day old mosquitoes are commonly used. For An. dirus 5-7 day old mosquitoes are used. The optimal age range will depend on setting. The age range that is chosen should be sufficiently narrow to avoid large variations in mosquito survival rates between experiments. Mosquitoes should preferably be selected for membrane feeding, which can be achieved by using membrane feeders for blood feeding during colony maintenance
- Collect the mosquitoes
  o Apply a water bottle or thick plastic bag containing water at ~37-40°C to one side of the cage, preferably opposite the opening. Females that are seeking a blood meal will be attracted to the heat and probe the warmed water bottle. Avoid selecting non-aggressive females; variation in aggressiveness may result in variation in feeding rates between cups
  o Open the ‘trunk’ of the cage
  o Collect the female mosquitoes that are probing the bottle: gently aspirate using a mouth aspirator or other suction device and transfer them into paper cups (ice cream pint cups) of about 500 cm³ covered on the top with netting that is held with rubber bands and strong labelling tape
  o Twist the ‘trunk’ of the cage to prevent mosquitoes escaping every time you transfer mosquitoes into a cup
- Starve mosquitoes by removing glucose sources. The time that mosquitoes need to be starved to make them aggressive for the experiment depends on the mosquito species. Some laboratories starve from early morning (e.g., 5-6 am) for experiments conducted after 10 am. The best approach will depend on the local setting and some laboratories use starvation overnight or even for 24 hours during which mosquitoes are provided with water only using soaked cotton wool. Water-soaked cotton wools should be removed at least an hour before feeding starts
- A maximum number of mosquitoes should be determined per feeder. This depends on the feeder size and the size of the cup. For An. gambiae, 50-100 mosquitoes are commonly used per cup for mini-feeders (see below); for An. dirus as many as 100 mosquitoes are used per mini-feeder. A balance has to be found to avoid crowding in cups but allow a sufficiently high number of mosquitoes per feeder. As long as feeding is complete (few partially fed mosquitoes) and feeding rates are acceptable (see considerations on number of observations below), flexibility is acceptable between labs and between experiments. Feeding rates should ideally be reported to facilitate inter- and intra-lab comparisons
- The optional use of cup and feeder holder can stabilise contact between cups and mini-feeders
- If cups are being re-used, it is advisable to add absorbing filter paper at the bottom of the cup to absorb blood droplets (mosquito diuresis) that may appear during the feeding process (depending on mosquito species). In this way cups stay cleaner and can be used repeatedly. A simple 70% ethanol wash of the sides of used cups followed by air-drying prolongs its use. Used nets should be cleaned in 10% bleach, rinsed thoroughly
with water and air dried for > 24 hrs. Consider cups and nets as potentially contaminated by blood even if apparently clean, always wear gloves, even for manipulation of cleaned material.
- Properly label mosquito cups, e.g., date/species/origin/experiment number/operator/lab
- Close any opening of cups (used to transfer mosquitoes) with a cotton pad, which you stick with tape. Dental dams can also be used.
- Close the ‘trunk’ of the cage by making a knot.
- If not enough female mosquitoes are present in one stock cage the remaining mosquitoes can be collected from another stock cage with the same rearing and eclosion date. Either mix mosquitoes in the different cages or record the mosquito source to take any unforeseen variation between mosquito populations into account in data analysis.

4.3 Preparation of membrane feeding equipment (start one hour before feeding)

- Attach the feeders to the water pump.
- Switch on the water bath (preset to reach 37-38°C in the feeders).
- Check that the temperature in the water bath is constant and that the water is running through to the tubing and membrane feeders.
- Check to make sure there are no leaks and then turn off the unit.
- Wearing gloves, cut at least enough pieces of Parafilm or Hemotek membrane of approximately 2x2cm for each mini feeder. Although the Parafilm when stretched carefully will readily attach to the glass feeder, the hemotek membrane piece needs to be held in place with a rubber band. Ensure rubber bands are available for each feeder.
- Goldbeater membrane / Baudruche membrane: This type of membrane is thin and can be kept dry in the refrigerator for years. Cut approximately 2x2cm pieces for each of the mini feeders. Ensure rubber bands are available for each feeder, the membrane can be attached to the feeder by rubber bands to tighten the membrane to the edge. Some laboratories dip the edge of the feeder in PBS then attach the membrane on the feeder to improve adherence.
- Now indicate to the person doing the venipuncture that they can start the sampling process. Once this has started, continue with the next step. When using Parafilm, the longer the stretched parafilm covering the feeder is heated, the higher the risk of breakage. Thus it is best to keep the immersion circulator off until 5-10 minutes before delivery of the blood.
- Once the blood is being drawn, stretch the Parafilm (or Baudruche membrane) squares against the light of a fluorescent lamp till the point of breaking in two directions (approximately twice the size). At this point the Parafilm turns from opaque to semi-transparent. For Hemotek membranes or Goldbeater membranes, the square needs to be held in place with a rubber band. These membranes do not change colour and do not need to be stretched.
- Press the Parafilm onto the outer rim of the blood chamber, the overlapping Parafilm is stuck onto the water jacket of the feeder. Careful wrapping makes it unnecessary to use rubber bands.

5 Procedures

5.1 Venous sampling

- While the membrane feeding apparatus is running, the nurse or other clinically qualified medical personnel will perform the venous sampling.
- Ensure that you have all materials (needle, cotton wool, tube, tourniquet) ready, the sharps container is accessible and the person in the insectary is ready to receive the tube.
- Ensure that the heparin tubes are correctly labelled. Do not use EDTA tubes, anticoagulants can affect the feeding quality and heparin is most widely used.
- Remove the tube when filled with the required amount of blood and mix by gently inverting 5-6 times.
- Give this tube to the person performing the membrane feeding assay. If the blood is not used immediately, put it in the water bath to keep it at 37-38°C. Even if the tube is transported between rooms, a thermos flask with 37-38°C water, or a dry bath insert pre-warmed to 37-38°C can be used to minimise the drop in temperature. If the blood is used immediately, these precautions are not needed. Time it so that the duration from blood collection to feeding is as short as possible, preferably within 10 minutes. In the period between bleeding and feeding, tubes should be kept at 37-38°C.

5.2 Whole blood membrane feeding assay

- Wear gloves and goggles to avoid blood exposure incidents.
- Once you receive the Heparin tube from the person who performed the bleeding, quickly take 1 ml of blood from the tube using a blunt needle or a transfer pipet. Do not use sharp needles at any point during the membrane feeding procedures after the venous sample has been taken.
- In case there is time lag between blood draw and the feed, place the Heparin tube containing the whole blood in a 37°C water bath.
- Return the Heparin tube to the lab technician who may take plasma and whole blood aliquots for additional analyses.
- Dispense the whole blood to two feeders. Mini feeders can take 350-500 µl of blood. A minimum of 150-200 µl is required for feeding 40 mosquitoes. Make sure the tip of the blunt needle or pipet is passed the neck of the glass feeder and that you are dispensing directly unto the membrane, not to the neck or the side of the feeder. Using more than 1 feeder per sample (e.g., per partici-
- Discard these unfed females by killing them by cold (-20 °C overnight)
- Store the remaining (fully fed) mosquitoes in the cage at 26-28°C at 80% humidity
- Record the number of remaining mosquitoes
- Especially the first 24 hrs after feeding, it is advisable to keep the fed mosquitoes undisturbed. After 24 hrs you may transfer mosquitoes from the selection cage to a storage cage or beaker. Some laboratories keep fed mosquitoes undisturbed for a minimum of three days. If mortality is low, you may also choose to keep mosquitoes in the original feeding cup until dissection; the recommendation to move mosquitoes to clean cups is based on the unpublished observation that mortality is higher in cups that are contaminated with blood products after the feeding procedure
- From 24 hrs after the feed, the selected mosquitoes are maintained on a 6-10% glucose solution, soaked on pads
- Soak the pads daily
- Change the pads three times per week (e.g., Mondays, Wednesdays and Fridays) to minimise the risk of fungal and bacterial contamination affecting your mosquito population. To further minimise the risk of fungal and bacterial contamination, you can also use a water-soaked cotton ball that is placed on the top of the mosquito cage/cup (under a small medicine cup to delay evaporation) and a sugar cube. The mosquitoes can take their sugar meal directly from a sugar cube and can take water from the soaked cotton ball to avoid desiccation
- Record mosquito mortality on a daily basis

5.4 Cleaning the feeders
- Dismantle the membrane feeders
- Using gloves transfer them immediately after the feeding into a basin with 4% bleach and tap water
- Carefully remove all of the Parafilm or membrane
- Leave the feeders in this basin for a minimum of 30 minutes
- Transfer the feeders carefully into a flow-through basin with tap water
- Make sure the feeders are completely below the water level
- Leave the feeders in this basin for a minimum of 30 min
- Repeat the previous three steps at least one more time. It is critical to completely remove residual bleach during the wash
- Transfer them into a second basin with distilled water; make sure the feeders are completely below the water level and leave for 10 min
- Rinse the feeders
- Blow dry the feeder using a pressure air tap or dry by air overnight

- Once the 15-20 min feeding is over, move the cups with fed mosquitoes to the mosquito handling room
- Place the cup inside a small clean cage
- Empty the cup carefully by removing the netting and gently tapping the cup
- Allow mosquitoes to settle for a minimum of 5 min
- Visually check the abdomen of each mosquito to see if the mosquitoes are fully blood fed
- Remove the unfed and partially fed mosquitoes with the suction device or mouth aspirator; place them in another cup or large cage for subsequent killing
- Make sure to have adequate lighting and be patient in selecting and aspirating out the unfeds
5.5 Dissection of mosquito midguts for microscopic examination and enumeration of oocyst numbers

- Mosquito midguts are examined 6-9 days post infection. Day 8 is best since earlier days have smaller oocysts making identification and enumeration difficult, and on later days oocysts may already have ruptured. Dissection at these days ensures that sporozoites have not yet migrated into the salivary glands and mosquitoes are therefore not infectious, i.e., do not pose a direct health hazard for laboratory personnel. However, all mosquitoes that have taken a potentially infectious blood meal should be handled with utmost care and should be analysed or destroyed by day 10 after feeding to avoid a direct health hazard of infectious mosquitoes for laboratory staff or other laboratory users.
- Immobilise mosquitoes by placing them in a -20°C freezer for 10 min.
- Prepare a slide that may hold multiple mosquito midguts:
  - Place droplets of 0.4-1% mercurochrome on a non-frosted slide.
  - Under a dissection microscope, remove the midgut from the mosquitoes using a surgical knife and forceps in one of the droplets.
  - Hold mosquitoes at the thorax and the posterior abdomen (see Fig. 1 in main article).
  - Pull the abdomen apart until the midgut is exposed.
  - The midgut is a translucent tissue shaped like a mandolin or guitar.
  - Use forceps to remove the Malpighian tubules that are attached to the posterior end of the midgut.
- Cover the midgut and mercurochrome with a coverslip.
- Continue until you have three midguts in mercurochrome, covered with coverslips.
- Place the slide in a wet chamber for a minimum of 5 min before examination. Lower concentrations of mercurochrome will require a longer staining time. 20 Min staining is recommended at a 0.4% mercurochrome solution.
- Examine mosquito midguts for the presence of oocysts at 20x magnification; if uncertain confirm with 40x magnification.
- Similar to slide reading, double reading of slides should be done for quality control. If a single microscopist is examining all slides, it is recommended to have oocysts confirmed by an independent reader.
- The exact number of mosquitoes that need to be examined depends on the study questions. However, since mosquito infection rates can be as low as 5% when feeding on natural gametocyte densities, a minimum of 25 and preferably ≥50 mosquitoes should be examined per individual experiment.
- Record the number of oocysts for each individual mosquito (only recording summary figures significantly reduces the sensitivity of your statistical analyses) and allow data to be analysed per cup/feeder. A template for recording is provided on the last page.
- The following endpoints can be determined using membrane feeding assays:
  - i) the proportion of infectious individuals: the proportion of individuals infecting ≥1 mosquito
  - ii) the proportion of infected mosquitoes: the number of infected mosquitoes divided by the total number of examined mosquitoes. This can be calculated per experiment or for groups of participants. If calculated for groups of participants, analyses have to adjust for clustering of observations derived from the same feeder and from the same individual.
  - iii) the oocyst density in infected mosquitoes. This should be recorded for individual mosquitoes (and not summarized per experiment) to allow the most powerful statistical analysis.

5.6 Serum replacement membrane feeding

Please note: there is considerably less experience with serum replacement experiments compared to whole blood membrane feeding assays and procedures require optimisation at the laboratory performing the assay. The availability of a heated centrifuge is preferred but good results have been achieved with non-heated centrifuges or non-heated centrifuges with warmed centrifuge rotors. Below is the procedure as it is performed in Ouagadougou, Burkina Faso:
- Prepare a record sheet for each patient, and each serum replacement condition to note down the volume of autologous plasma that is removed per sample. Often samples are offered in three conditions: whole blood (see section 5.2), AB replacement serum (below) and autologous plasma control (below).
- Label 1.5 ml Eppendorf tubes and corresponding mosquito cups with the patient identifying number and the serum replacement condition.
- Set the water bath, dry incubator, and heated centrifuge to 37°C and allow them to come to temperature.
- Insert the labelled 1.5 ml Eppendorf tubes, blunt needles, syringes, heparinized vacutainers and 1000 ml pipette tips into the dry incubator. Give them at least 15 min of incubating time before use.
- Approximately 15 min before needed, insert an aliquot of AB naïve serum into the dry incubator to allow it to come to 37°C.
- Collect the patient’s blood in a heparinised vacutainer as outlined in section 5.1 using a heated vacutainer for collection.
- For the following steps, work as quickly and accurately as possible to minimise the time that the aliquots are handled at room temperature, and to aim to have no more than 15 min between taking the blood and feeding it to the mosquitoes.
- Aliquot two times 400 µl of whole blood from the vacutainer into a heated Eppendorf tube using a heated pipette tip. Centrifuge the aliquot at 3000 rpm for 3 min in the heated centrifuge. Make sure the plasma is yellow, not red. (If it is red centrifuge at a lower speed). One tube will be used as control for the experi-
ment (autologous plasma tube). The cells and plasma are later mixed again and the sample is offered to mosquitoes to determine whether there was loss in gamocyte infectivity due to the sample handling.

- If working with multiple serum replacement conditions, keep the Eppendorf tubes in the 37°C dry incubator in a rack while processing the other aliquots.
- From one of the tubes (AB replacement serum tube), remove as much of the patient’s plasma as possible using a 200 ml tip. Transfer the plasma to an Eppendorf for storage and note down the volume removed.
- Take out the AB naive serum aliquot from the heated incubator. Take the same volume of AB control serum as that of the patient’s plasma that was removed in the previous step and add it to the cell pellet.
- Invert (not shake) the ‘AB replacement serum tube’ and the ‘autologous plasma tube’ 10 times to mix.
- Using a heated blunt needle and heated syringe take the entire volume from the Eppendorf tubes and transfer it to the membrane feeder. If doing multiple serum replacement conditions, use a new blunt needle and syringe to load each sample.
- Move the corresponding labelled cups of mosquitoes underneath the feeders and allow the mosquitoes to feed, as previously outlined.
- A comparison of mosquito infection rates between the AB replacement serum tube and the autologous plasma tube will allow an estimate of the transmission-reducing properties of components in the patient’s plasma.

Citing this protocol:

6. Recording sheet (example)

Part 1. Information related to the study participant and the experiment. An example is given for two experiments where two cups were used.

<table>
<thead>
<tr>
<th>ID</th>
<th>Gam_µl</th>
<th>Date_exp</th>
<th>Start_time_bleed</th>
<th>End_time_bleed</th>
<th>Start_time_feed</th>
<th>End_time_feed</th>
<th>No_fed_cup1</th>
<th>No_unfed_cup1</th>
<th>No_survived_cup1</th>
<th>No_fed_cup2</th>
<th>No_unfed_cup2</th>
<th>No_survived_cup2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT_003</td>
<td>32</td>
<td>01/04/2013</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>11</td>
<td>30</td>
<td>34</td>
<td>8</td>
<td>29</td>
</tr>
<tr>
<td>ACT_007</td>
<td>16</td>
<td>03/04/2013</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>35</td>
<td>9</td>
<td>31</td>
<td>29</td>
<td>16</td>
<td>28</td>
</tr>
</tbody>
</table>

ID=personal ID number; Gam_µl=gametocytes/microlitre; Start_time_bleed=timepoint when bleeding started; End_time_bleed=timepoint when bleeding was completed; Start_time_feed=timepoint that mosquitoes were placed in contact with the feeder(s); End_time_feed=timepoint when feeding was stopped; No_fed=number of mosquitoes fully engorged; No_unfed=number of mosquitoes not or partially fed; No_survived=number of mosquitoes that survived until the day of dissection.

Part 2. Information for each mosquito. A weight can be used to avoid repetition in negative samples. An example is given for an experiment what used two cups with 31 and 28 mosquitoes examined, respectively. In the first cup 4 mosquitoes were infected with 2, 3, 3 and 7 oocysts (27 negatives); in the second cup 2 mosquitoes were infected with 1 and 4 oocysts (26 negatives). The weight variable indicates how many mosquitoes had a certain oocyst density and therefore what weight should be attached to a certain observation in the statistical analysis. In Stata, the weight variable can be used to obtain individual mosquito observations by using the expand command (i.e. expand weight).

<table>
<thead>
<tr>
<th>Participant_id</th>
<th>Date_examined</th>
<th>Cup</th>
<th>No_oocyst</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>1</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>1</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>1</td>
<td>0</td>
<td>27</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>2</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>ACT_003</td>
<td>08/04/2013</td>
<td>2</td>
<td>0</td>
<td>26</td>
</tr>
</tbody>
</table>