INFRAVEC2 PROTOCOL

Standardization protocol for *Anopheles coluzzii* infections with *Plasmodium falciparum*

Protocol Information

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Comments:

Project Information

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PROTOCOL

Protocol version 1, based on infection of *A. coluzzii* mosquitoes as drafted by partner MPIIB. Protocols will be updated as reagents and data become available.

1. PARASITE CLONES

The *P. falciparum* NF54 clone originated from Prof. Sauerwein's laboratory (RUMC) and was authenticated for *Pfs47* genotype by PCR on genomic DNA. *P. falciparum* asexual cultures were monthly tested for *Mycoplasma* contamination.

2. GAMETOCYTE CULTURES (25 cm^2 culture flasks)

- Spin-down asynchronized asexual cultures of *P. falciparum* (2% parasitemia) at 1,500 rpm for 5 min.

- Spin-down separately washed O^+ red blood cells (RBCs) less than one week old at 2,500 rpm for 5 min.

- Dilute the collected RBCs to 1% of total parasitemia in 250 µl volume and resuspend in 6 ml of complete culture medium containing 10% pooled A^+ human serum without gentamicin in a T25 cm^2 flask. Incubate at 3% O_2, 4% CO_2 at 37 °C.

- From the following day, replace the culture medium (6 ml) every day at around the same time for 14-16 days. Make the biggest effort to reduce the time and avoid the temperature drop as it may induce precocious gametogenesis. To this end, warm the medium carefully before change, prepare all needed materials before taking the culture out of the incubator and change the medium on a heated plate (37°C) to minimize the temperature drop.

3. COUNTING GAMETOCYTES CULTURES

- Smear the culture on a microscope slide on day 7 (1 week after initiation). To this end, spin-down the culture for 16 - 20 s at 11,000 rpm. Smear 1 - 2 µl the culture pellet and keep the slide at 37 °C to avoid gametocyte egress until the smear is dry. Fix the smear with 100% methanol and stain with Giemsa.

- Check for the presence of stage II gametocytes (typically "half-moon" or "lemon" shaped). There are also asexual stages and other stages of gametocytes.

- Trash the cultures inefficient levels of gametocyte induction (fewer that 2 stage II gametocytes per a macroscopic field).

4. MOSQUITO REARING

A. coluzzii Ngousso strain was established by IRD in Youndé, Cameroon, and was selected for *TEP1*S1/S1* genotype by MPIIB, where it is maintained since 2013. Eggs are immersed into dechlorinated water for hatching. Larvae is distributed in pans of 250 individuals and supplied with 2% liver food preparation (2 parts Liver powder: 2 parts tuna meal: 1 part Vanderzant Vitamin Mix). Store the mixture at 4°C. Keep in the fridge at all times. Once the water is added the food can be used for 3 days. Mix well before and during use (liver food sediments very quickly). For L1 and L2 instar larvae use 2-2.5 ml per pan, for L3 and L4 instar larvae use 3-4 ml per pan.

5. PREPARATION of MOSQUITOES

Pay a particular attention to the safety as mosquito cages will be used in the secured BSL3 space on the day of infection.

One day before the planned infection:

Select a cage the desired format. While choosing your cage for infection, consider the following issues that could decrease infection efficiency and compromise the outcome of your experiment: (i)

seams in the region where the feeder will be applied, (ii) loose cage wall netting, (iii) thick net tissue (for more details see the section below).

- Put into the cages the exact number of female mosquitoes you intend to infect.

- Remove all males from the cages to facilitate sorting of blood fed mosquitoes after infection in BSL3 conditions.

- Remove sugar pads from the cages in the morning of the infection day.

6. STANDARD INFECTION PROCEDURES (BSL3)

- On the day of infection, mosquito cages are transferred in the secured BSL3 area by the authorized personnel.

- Mosquitoes are exposed to infected blood through a feeder filled with *P. falciparum* gametocyte culture for 15 min at 26°C The feeder is warmed up to 37°C by circulating water.

- The authorized personnel remove unfed females into 70% ethanol on the same day and estimate the approximate number of blood fed mosquitoes.

- Infected mosquitoes are placed into an incubator at 26°C 80% humidity until the end of experiment.

- Sugar pads are changed by the authorized personnel every 2-3 days.

- On the planned date, the desired number of mosquitoes are collected from the cages into 70% ethanol, washed 3 times in PBS and only then released from the BSL3 containment.

7. MIDGUT DISSECTION

- Use gloves for dissections of *P. falciparum*-infected mosquitoes.

- Dissect the midgut on a slide in a drop of 1% mercurochrome solution in water.

- Remove the surrounding tissues as much as possible (including the Malpighian tubules) that could interfere with the oocysts counting.

- Incubate the midguts in mercurochrome solution for 3 - 5 min.

- Put a cover slip.

- Observe the dissected midguts under a light microscope (10X/20X objective) and take pictures if possible for further analyses.

- Count the oocysts either directly under the microscope or digitally on collected images. Note: *P. falciparum* oocysts on day 12 post infection have 3 typical features: (1) sharp contour, (2) darker color than the midgut background and (3) black pigment spots of hemozoin.

- After dissection, clean the bench surface with ethanol.

- Wipe out mercurochrome from the dissecting tools and incubate them for 10 - 20 min in 70% ethanol before washing them with deionized water.

- All mosquito leftover materials should be discarded into the autoclaving bin.

8. INFECTION READOUTS:

Infection rate: number of oocysts per a single mosquito midgut;

Sporogonic index: mean or median diameter of each oocyst in a single mosquito midgut multiplied by mean/median number of oocysts per midgut;

Infection prevalence: percentage of mosquitoes with at least one oocyst out of total mosquitoes taken into experiment.