

# Infecting *Anopheles stephensi* With Rodent Malaria Parasites

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### A. Reagents:

1. DMEM or RPMI

DMEM (4.5g/L glucose)  
RPMI 1640

Cellgro #MT-10-017-CM  
Cellgro #MT-10-040-CM

2. Giemsa stain, modified

Sigma GS-500

Dilute 1:5 with ddH<sub>2</sub>O before use. Make fresh just before use.

3. Heparin solution

For a 100 U/ml stock solution:

1 ml heparin @ 1000 U/ml  
9 ml 1x PBS pH 7.4  
10 ml

Store at 4°C.

IMPORTANT: Heparin cannot contain preservatives.

4. Ketamine/Xylazine solution

1.4 ml ketamine @ 100 mg/ml  
0.6 ml xylazine @ 20 mg/ml  
8.0 ml 1x PBS pH 7.4  
10 ml

Store at 4°C.

5. Sugar pads

Cotton balls saturated with 10% dextrose. Make fresh daily.

### Start infection:

Infections can be started either with a vial of frozen infected blood, or by bite of infected mosquitoes. This will depend on the particulars of your situation. What is not advisable is to use parasites that have been cycled through mice

for long periods of time without having gone through the mosquito as they lose the ability to make gametocytes. If you are doing cycles routinely then it's best to use infected mosquitoes to begin your new cycle. If you are doing a cycle with a mutant that cannot complete the mosquito cycle, then you must start each new cycle with a vial of frozen blood. Below are instructions for each.

#### By Mosquito Bite:

1. Turn up sugar pads from infected mosquito cages at least 1 hr before feeding on mice. Use mosquitoes that are at least 14 to 15 days post-bloodmeal for *P. yoelii* and at least 18 days post-bloodmeal for *P. berghei*.
2. Place two anesthetized naïve Swiss Webster female mice (6-8 weeks) on the cage for 10 - 15 min, rotating every 5 min. Return the mice to Animal Facility when they have recovered from anesthesia.
3. Start checking blood smears of these mice 5 to 7 days after the feed. When they are positive for parasites will depend on how infective the mosquitoes were and how many mosquitoes you used for this feeding. We usually feed with 10 to 20 well-infected mosquitoes and check mice on day 6 or 7 post-feed.

#### By Frozen Blood

1. Quickly thaw a vial of infected blood.
  - ⇒ An equal volume of DMEM or RPMI medium can be added to the frozen blood.
2. Inject 2 mice i.p. with 150 – 200 µl blood.
  - ⇒ Usually 4 – 5 week old female Swiss Webster mice (25 – 30 g), but any mouse strain that is susceptible to infection can be used.
3. Check parasitemia of mice by Giemsa-stained blood smear 4 days after injection.
4. If parasitemia is > 1%, transfer blood to recipient mice (see below). If parasitemia is 1%, mouse should be ready the next day. If parasitemia is <1, keep checking mouse every day until parasitemia reaches >1%.
  - ⇒ Ideally, the parasitemia should be between 2 – 5%.

#### B. Blood Transfer:

This will usually be about 1 week after mice are infected by mosquito bite or 4 days after mice are infected by frozen blood. Again, this will depend on many factors including how many mosquitoes were used, how many parasites were in the frozen blood, etc. We do blood smears on these mice and do the blood transfer when parasitemia is between 2 - 5%.

1. Prepare a 3 ml syringe by pre-loading with heparin solution.

⇒ Heparin solution at 100 units/ml in PBS at 4°C.

⇒ Heparin amount needed is 10% of expected blood volume.

→ A 25 -30 g mouse will yield approximately 1 ml blood.  
Therefore, load syringe with 100 µl of heparin.

2. Anesthetize the mouse starting with 200 µl Ketamine/Xylazine administered i.p. Give more if necessary.

3. Exsanguinate by cardiac puncture.

4. Mix the blood with the heparin solution and place on ice.

5. Quickly dilute the blood to 1% parasitemia with DMEM or RMPI on ice.

⇒ You will need 200 µl of diluted blood per mouse. (Make extra.)

6. Inject mice i.p. with 200 µl of diluted blood.

⇒ The number of mice needed for blood transfer depends on the number of mosquitoes to be infected.

small cage (50 – 100 mosquitoes) → 2 – 3 mice

large cage (100 – 250 mosquitoes) → 4 – 5 mice

### C. Infecting Mosquitoes:

1. Check mice (from step C6) for presence of mature gametocytes by Giemsa-stained blood smear 3 days after blood transfer.

2. If mice have 1 – 2 mature gametocytes per field, they are ready for mosquito feeding.
  - ⇒ If there are very few or mostly immature gametocytes, check the mouse again later in the day. If there still few or immature gametocytes check the next day both in the morning and evening.
  - ⇒ Do not allow parasitemia to get too high! Usually parasitemia should be ~3% (but not >5%). If parasitemia is too high, inflammatory mediators in blood will affect gamete fusion in the mosquito and result in low or no infections.
  - ⇒ Not all mice may be infected or be ready for feeding. Use only those mice that are optimal.
  - ⇒ Below is a picture of a female gametocyte:



**Figure 1:** Mature female gametocyte

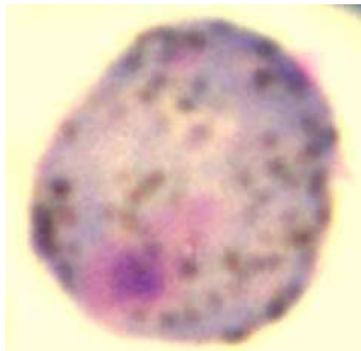
- ⇒ Should see both male and female gametocytes; female gametocytes outnumber male gametocytes
3. Obtain a cage of 3 – 5 day old *An. stephensi* female mosquitoes for feeding.
    - ⇒ Make sure that there aren't too many mosquitoes in the cage. More is not necessarily better! If there are too many mosquitoes and too few mice, mosquito infectivity will be low and the mice will die during the feeding.
  4. Starve the mosquitoes by removing the sugar pad from the top of the cage and placing the cage in the *P. berghei* or *P. yoelii* incubator for 2 hr.
  5. Anesthetize the mouse with 150 – 200  $\mu$ l Ketamine/Xylazine administered i.p.

6. Place the mice on the top of the cage and put the cage (with the mice on top) back in the incubator for 7 min. For *P. berghei*, the incubator should be at 19°C because fertilization will not occur at higher temperatures. For *P. yoelii* the incubator is at 24°C. All incubators should have between 70 and 80% humidity.
  - ⇒ Remain by the incubator and make sure the mice have not awoken during the feeding.
  - ⇒ Place the cages away from other cages in the incubators. (This is so that if a mouse awakens, it will not fall or climb onto another cage.)
7. Rotate the mice and incubate for another 7 min.
8. Replace the sugar pad on top of the cage and leave in the incubator.
  - ⇒ Put mice back in their cages on a paper towel and wait for them to wake up. You will need them the next day for the second feeding.
9. The next day, do a second feeding by repeating steps D5 – D8.
  - ⇒ The second feeding ensures that any mosquitoes that could not or did not feed the day before, will feed.
  - ⇒ This step can be omitted. If it is, extend the time of the first feeding.
  - ⇒ This is day 1 post-feeding.
10. Keep the cage in the *P. berghei* or *P. yoelii* incubator.
11. For *P. berghei*, on day 10 – 11 post-feeding, dissect 10 – 20 mosquitoes to obtain midguts. Look for oocysts on midgut wall to determine infectivity. For *P. yoelii* this should be done on day 8 - 10 post-feeding.
12. For *P. berghei*, on day 18 post-feeding, dissect 10 – 20 mosquitoes to obtain salivary glands. Determine number of sporozoites per mosquito. Salivary gland sporozoites are good for use between days 18 - 22. After this you have lower numbers and somewhat lower infectivity. For *P. yoelii* this should be done on day 14 post-feeding. Salivary gland sporozoites are good for use between days 14 - 16.

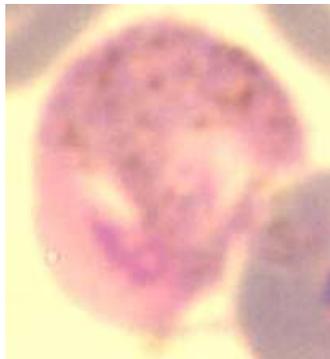
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## Microscopic appearance of gametocytes – Giemsa Staining:

**Mature FEMALE Gametocyte:** eccentric (compact) nucleus, scattered pigment granules and blue staining cytoplasm. The gametocyte is completely filling its host cell and the membrane of the erythrocyte is hardly visible.



**Mature MALE Gametocyte:** large nucleus (red DNA spot in a large pink areola) at the edge of the cell, scattered pigment granules and pink staining cytoplasm. The pink color (compared to the blue cytoplasm of female gametocytes) is the result of the less basic pH of the male cytoplasm, most probably due to the much lower number of ribosomes.



hpi	characteristics	morphology		
0-4	invasion (merozoites)			
4-8	intracellular growth			
8-12	intracellular growth			
12-18	intracellular growth			
18-20	schizogony sexuality manifest			
20-22	sexual dimorphism not visible			
22-23	schizogony completed			
23-26	sexual dimorphism visible			
26-27	gametocyto- genesis completed			
30-48	stable gametocytemia			
48-57	degeneration of gametocytes			

**Figure 2:** Morphology of blood stages of *P. berghei* (from: Mons, B (1986) Acta Leiden. 54, 1-124). Stages shown are from synchronized blood stage infections of the ANKA strain of *P. berghei* at several time points after invasion (hpi) of merozoites into erythrocytes.