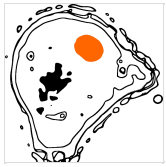

Nov 19, 2020

Plasmodium berghei ookinete culture

In 1 collection

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1 *Works for me* dx.doi.org/10.17504/protocols.io.bmm8k49w

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ABSTRACT

Plasmodium berghei is one of the main non-human malaria models used in research. It readily infects laboratory strains of mice and rats as well as mosquitoes. Because of this, it is safe to manipulate and it does not require human fluids/tissues. This document provides the Operational Procedure for producing and culturing ookinetes of *P. berghei in vitro*. The method has been optimized for the *P. berghei* ANKA train clone 2.34 and the derived GFP-expressing clone, but should work for other strains.

This method is adapted and modified from:

Rodríguez MC, Margos G, Compton H, Ku M, Lanz H, Rodríguez MH, Sinden RE (2002). Plasmodium berghei: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. Experimental parasitology.

Carter, V., Cable, H. C., Underhill, B. A., Williams, J., & Hurd, H. (2003). Isolation of Plasmodium berghei ookinetes in culture using Nycodenz density gradient columns and magnetic isolation.. Malaria Journal. https://dx.doi.org/10.1186%2F1475-2875-2-35

Nacer A, Underhill A, Hurd H (2008). The microneme proteins CTRP and SOAP are not essential for Plasmodium berghei ookinete to oocyst transformation in vitro in a cell free system.. Malaria journal. https://doi.org/10.1186/1475-2875-7-82

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GUIDELINES

Although *Plasmodium berghei* ookinetes have been successfully cultured for more than 30 years, several improvements have been made to the basic protocol. One of them is the use of CF11 cellulose powder to remove white blood cells from the culture. This is advisable since it has been shown that the white blood cells can induce programmed cell death in the ookinetes. Furthermore, it is also a recommended step if DNA or RNA has to be extracted from the culture because eliminates nucleic acid contamination from the white blood cells. However, we have found the use of CF11 columns troublesome since, in our hands, it causes the culture yield to decrease (Fig. 1) probably because of a reduction in gametocyte viability.

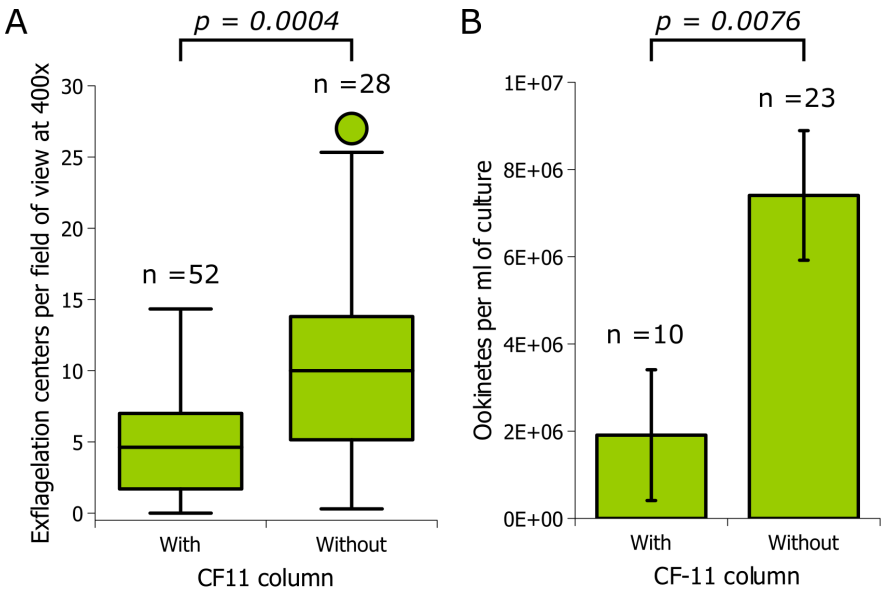

Figure 1. Decrease in yield after passing the infected blood through CF11 cellulose powder columns to eliminate white blood cells. Error bars represent the standard deviation of the mean, n = number of cultures, p = two-tailed t-test.

Working schedule to obtain ookinetes every week.

	Sun.	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.	
9:00-10:00			Ookinetes ready to use					
10:00-12:00			Phenylhydrazine			Parasite pass for ook. culture and for next week's pass.		
12:00-14:00								
14:00-16:00		Ookinete culture						

DISCLAIMER:

This protocol involves working with mice which, by the nature of the procedure, will end being sacrificed.

ABSTRACT

Plasmodium berghei is one of the main non-human malaria models used in research. It readily infects laboratory strains of mice and rats as well as mosquitoes. Because of this, it is safe to manipulate and it does not require human fluids/tissues. This document provides the Operational Procedure for producing and culturing ookinetes of *P. berghei in vitro*. The method has been optimized for the *P. berghei* ANKA train clone 2.34 and the derived GFP-expressing clone, but should work for other strains.

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BEFORE STARTING

Requirements:
1.- *Plasmodium berghei* cryopreserved stock.
2.- Laboratory mice (we use 6 to 8 weeks old male BALB/c mice).
3.- CO_2 euthanasia chamber.
4.- Brightfield microscope.
5.- Microscope slides and coverslips.
6.- Newbauer chamber (haemocytometer).
7.- 0.6 and 1.5 ml microcentrifuge tubes.
8.- Incubator at 20°C (Temp. range 18 to 21°C).
9.- 1 ml syringes.
10.- Cotton balls.
11.- Bunsen burner.
12.- T25 culture flasks.
13.- Common cell culture labware.

Reagents:
1.- Saline solution (0.9%).
2.- Methanol.
3.- Ethanol (70%).
4.- Giemsa stain (30% in water).
5.- Immersion oil.
6.- Phosphate-buffered-saline (PBS).
7.- Phenylhidrazine (6 mg/ml of saline).
8.- Heparin (250 IU/ml of saline).
9.- Ookinete culture medium: RPMI 1640 medium at pH 8.3 supplemented with 23.81 mM sodium bicarbonate, 0.37 mM hypoxanthine, 25 mM HEPES, 5000 U/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin, and 20% heat-inactivated fetal bovine serum.

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Mice infections for passages.

1 Mice are inoculated via intraperitoneally (IP) with no more than **200 µl** either from a cryopreserved stock or by passage from other mice.
From cryopreserved stock: allow the blood-cryopreservation solution to thaw slowly and inoculate **200 µl** IP.
Passage form another mouse: The number of parasites per ml of blood has to be determined to always inoculate a constant amount.

2 To determine the number of parasites per ml of blood, collect a drop of tail blood from the infected mouse and make a thin smear. Let it dry and fix with methanol.

3 Prepare **1 mL** of 30% Giemsa per slide and stain for **00:10:00** .

4 Rinse slide with distilled water and dry by gently applying pressure with an absorbent paper towel. Do not rub the paper towel on the slide.

5 Determine the parasitemia by counting at least 500 red blood cells (RBC) at 1000x magnifications and determine the percentage of infected RBC.

$$parasitemia = (iRBC/RBC)(100)$$

where *iRBC* = infected RBC counted, *RBC* = total number of RBC counted.

Infected RBC with more than one parasite count as one.

6 Collect an additional **1 µl** of tail blood and dilute it to **1 mL** with PBS with **1 µl** of heparin.

7 Mix well and determine the number of RBC per ml of blood with the Neubauer chamber.

7.1 Fill the two slots of the Neubauer chamber with ~ **10 µl** of blood solution (which is diluted 1/1000). Do not overflow the chambers.

7.2 View under the microscope first with the 10x objective to locate the grid, and then with a 40x objective to zoom into the centre grid (the one with the smallest squares). The centre square is a 5 by 5 grid, each of these smaller squares are a 4 by 4 grid. Count the number of RBC in 5 squares of the 5 by 5 grid (the 4 squares in the corners and the centre one). Cells touching the top and right margins are counted, while cells touching the bottom and left margins are not counted.

7.3 $$RBC/ml = (xRBC/20 \times 10^6)(1 \times 10^{12})(1000)$$

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8 Determine the number of infected RBC per ml of blood using the parasitemia obtained previously.

$$iRBC/ml = (parasitemia/100)(RBC/ml)$$

9 Euthanize the mouse in the CO_2 chamber and collect the blood via cardiac puncture with a 1 ml syringe preloaded with **10 µl** of heparin. Before puncture, clean the puncture site with a cotton ball soaked in 70% ethanol.

10 For maintenance passages, dilute the blood with PBS to obtain 4 x 10^5 infected RBC per **200 µl** and inoculate IP the mice.

Make no more than 8 passages as gametocytes lose the ability to mature into gametes. Also, keep infected mice for no more than a week as they will suffer unnecessarily. With this inoculum, the parasitemia should reach between 15 to 25% in one week.

Mice infections for ookinete culture

11 For ookinete culture, inoculate mice with **200 µl** of phenylhydrazine IP three days before the inoculation of the parasites to induce reticulocytosis.

12 Determine the parasitemia and infected RBC concentration of the donor mouse as explained in steps 2 to 8.

13 Collect the infected blood as explained in step 9.

14 Dilute the infected blood with PBS to obtain 4 x 10^7 infected RBC per **200 µl** and inoculate the donor mouse IP.

With this inoculum, the parasitemia must reach between 15 to 25% in three days.

Ookinete culture

15 Three days after the inoculum of 4 x 10^7 infected RBC verify the parasitemia as explained in steps 2 to 5, and verify the viability of the gametocytes by performing an exflagellation test.

15.1 For the exflagellation test, collect an additional **2 µl** of tail blood and place in a 0.6 ml microcentrifuge tube with 7 µl of ookinete medium and **1 µl** of heparin. Mix very gently. Take the resulting **10 µl** and place them on a microscope slide, cover with a coverslip and incubate for **00:15:00** at **20 °C** .

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15.2 Determine the number of exflagellation centres in 10 random fields of view.

We only use mice with 15 to 25% parasitemia and >7 exflagellation centres per field of view.
We find it not useful to determine the gametocytaemia because the correlation of parasitemia vs gametocytaemia, and the correlation between gametocytaemia vs exflagellation is low (Fig 2).

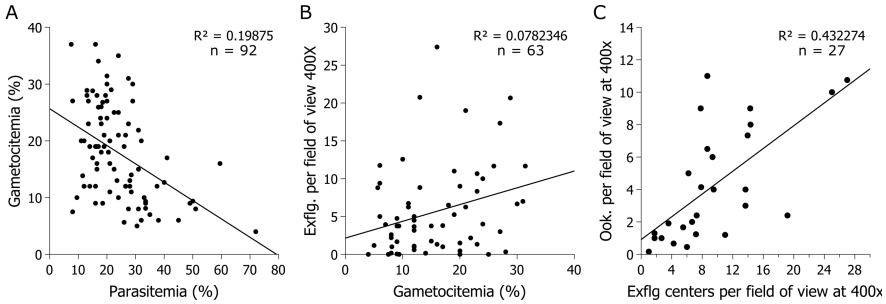

Figure 2. Correlation between parasitemia and gametocytaemia (**A**), gametocytaemia and exflagellation (**B**), and exflagellation and number of ookinetes obtained (**C**).

16 ⚠

Prepare a T25 culture flask with 4 ml of ookinete medium. Sacrifice the mice and collect the blood as explained in step 9.

About 1 ml of blood is obtained.
It is critical to collect the blood in a gentle manner but fast. Do not pull the syringe plunger too much as to generate vacuum. Mix the blood with the heparin very well. Try to succeed in the cardiac puncture in the first attempt. Rough manipulation of the blood at this step decreases considerably the ookinete culture yield.

17 Add the blood to the culture flask. Again treat the blood gently, do not push the syringe plunger too hard.
Mix slightly and incubate at **20 °C** for **18:00:00** .

18 ✱

After incubation, determine the number of ookinetes in the culture by taking a sample of **50 µl** . Dilute in **950 µl** of PBS and count in the Neubauer chamber.

18.1 Add ~ **10 µl** of the ookinete culture dilution on each chamber and count the number of ookinetes in the four big squares in the corners. For reference, these big squares have a 4 by 4 grid.

$$Ookinetes/ml = (xOokinetes/4squares)(20)(10000)$$

This is the number of ookinetes over the number of squares counted, times the dilution factor, times a

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conversion factor.

Usually, between 25 and 35 million ookinetes are obtained in total per culture (from one mouse).

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