Plasmodium berghei: general parasitological methods

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1a Solutions and buffers (2004; see some updates below in 1b)

- **PBS (phosphate buffered saline) normal**
  The 10x stock is the commercial available PBS from Roche (Cat. nr: 1666 789).
  Components of the 10x PBS: 0.01M KH_2PO_4, 1.37M NaCl, and 0.027M KCl, pH 7.0
  Working solution:
  - Dilute the stock 10 x with demineralised water
  - Adjust the pH to 7.2 with 1M HCl solution
  - Autoclave for 20 minutes.
- **PBS (phosphate buffered saline) 'rich'**
  20mM Hepes, 20mM Glucose, 4mM $NaHCO_3$, 0.1% BSA
  Working solution:
  - Dilute the stock PBS solution 10 times (see above) and add the above mentioned components
  - Adjust the pH to 7.25 with 5M NaOH
  - Sterilise the solution by passing it through a 0.2µm filter
  - Store the solution at 4ºC
- **Heparine**
  Stock solution: 200 I.U./ml
  Dissolve the content of one ampulla (0.2ml = 5000 I.U.) in 25ml RPMI1640 culture medium (pH 7.2) without fetal calf serum.
- **Phenylhydrazine- HCl (Merck)**
  Stock solution (Merck): 25mg/ml
  Dissolve 250mg phenylhydrazine (phz) in 10 ml 0.9% NaCl and store at -20°C in eppendorf tubes. Caution: phz-powder is a toxic substance.
- **Giemsa-solution and staining buffer**
  For dilution of the stock Giemsa solution (Merck Cat. nr. 1.09204.0500) we use the Sörensen staining buffer.
  Sörensen staining buffer:
  - KH_2PO_4 2,541g per 5 liter
  - $Na_2HPO_4 .2 H_2O$ 8,5507g per 5 liter
  - Adjust the pH to 7.2 with NaOH

  We use a 10 % Giemsa-buffer solution for staining slides for a period of 10 minutes.
- **Complete culture medium: RPMI1640 pH 7.3 containing 25% foetal calf serum**
  Culture medium: RPMI1640, with L-glutamine and 25mM HEPES, without $NaHCO_3$ (Invitrogen).
  Preparation of medium:
  - Dissolve 15,89g powder of RPMI1640 medium in 1l demineralised water. Add powder slowly under continuous stirring.
  - Add 0.85g $NaHCO_3$
  - Add 50.000 ug Neomycin (stock-solution of 10.000 ug./ml; Gibco)
  - Sterilise by filtration through a 0,2µm sieve (see below)
  - Store at -20C in 100-200ml bottles
  - Immediately prior to use, add foetal calf serum (FCS) to a final concentration of 25% (v/v) to give complete culture medium.

  Sterilisation of culture medium
  - Fill reservoir with medium and close lid
  - Connect reservoir to N2-gas bottle and to the filter container which is placed in the flow hood

- Open gas bottle (0.5 bar)
- At the same time open the small tap on the filter container until the air has escaped.
- Open the other tap and collect the medium

Sterilisation of the filter container

- Remove the upper part of the filter container by removing the three screws
- Place the Metricel membrane (0.2 um) with the shiny side downwards on the metal sieve. The blue side of the metal sieve should be facing upwards
- Place the polypropylene sieve on the Metricel membrane
- Place the glassfiber filter on top and close the filter container
- Autoclave for 20 min. Taps and in-and outlets covered with aluminium foil.

Filters

1. Metricel Membrane filter G/A-8; 0.2um, 142mm
2. Polypropylene filter, 127mm
3. Glassfiber filter A/E, 127mm

- **Neomycin**

  Stock-solution of 10.000 ug./ml (Gibco)

- **Ookinete culture medium: RPMI1640 pH 8.0 containing 10% fetal calf serum**
  - RPMI1640 medium prepared and stored as complete culture medium (see above).
  - The pH is adjusted to 8.2 with 1N NaOH prior to sterilisation.
  - Immediately prior to use, foetal calf serum (FCS) is added at a final concentration of 10% (v/v) to give ookinete culture medium with a pH of about 8.0.

- **Nycodenz stock solution**

  Nycodenz powder (Lucron Bioproduct BV) is obtained from Life Technologies. Store at room temperature.
  - Dissolve 138g Nycodenz-powder in 500ml Buffered Medium (see below) (density 1.15g/ml at 20ºC)
  - Autoclave for 20min at 120ºC and store at 4ºC

  Buffered medium:
  - 5mmol/l Tris/HCl 605.7mg/l, pH 7.5
  - 3mmol/l KCl 223.7mg/l
  - 0.3 mmol/l Ca Na $_2$EDTA 112.3mg/l

- **Erythrocyte-lysis buffer:**

  10x stock-solution:
  - 1.5M NH_4Cl 80.23g/l
  - 0.1M $KHCO_3$ 10.012g/l
  - 0.01M EDTA 3,72g/l

- **Sulfadiazine**

  Sulfadiazine for treatment of mice is dissolved in the drinking water (30mg/l)

- **Aphidicolin**

  Mol mass: 348,6 (Sigma Cat. nr: A 0781: 5mg)

  Working concentration: 5.10^{-4} M for 1ml parasite cultures in 24 wells plates.

  Stock solution (100x): 2 mg in 114,7µl DMSO.

  Add 10µl stock solution to1 ml culture medium to get a final concentration of 5.10^{-4} M.

1b Solutions and buffers (2020)

Aphidicolin

Sigma, Cat.nr. A0781 (5 mg), Mw 348.6
Final concentration in cell culture: 5.10^{-4} M
100X stock solution: 2 mg Aphidicolin in 114 µl DMSO
Add 10 µl stock solution to 1 ml culture medium to reach the final concentration

WR99210 Drug

Sigma, Cat.nr. W1770-5mg
Synonym:1,6-Dihydro-6,6-dimethyl-1-[3-(2,4,5-trichlorophenoxy)propoxy]-1,3,5-triazine-2,4-diamine
Depending on the selection pressure, take a 10 ml tube and combine 6, 12, 16, or 20 mg of WR99210 with 3.5 ml of DMSO. Dissolve by vortex until clear. Add 1.5 ml of demineralized water to obtain the final WR99210 solution in 70% DMSO. The stability of these solutions is guaranteed for 4 days.
These solutions are suitable for mice with a bodyweight of approximately 20 grams (e.g. 0.1 ml subcutaneous injection (in the neck) of a 12 mg WR99210 solution results in a treatment of 12 mg/kg bodyweight)
Treatment of mice with 20 mg/kg bodyweight WR99210 for selection of parasites with the hDHFR selectable marker and the Pyrimethamine selection marker from a previous transfection
Prepare a 20 mg WR99210 solution as described above. On 4 consecutive days, under anaesthesia, subcutaneously inject the mice with 1 dose of 0.1 ml WR99210 in the neck.

Pyrimethamine

MP Biomedicals, Cat.nr. 194180, Mw 248.7
For a 100X stock solution (7 mg/ml), Pyrimethamine is dissolved in DMSO or in 0.5% lactic acid (we prefer the DMSO solution). Take a 50 ml tube and combine 70 mg of Pyrimethamine with 10 ml of DMSO or 10 ml of 0.5% lactic acid . Dissolve by vortex for obtaining a clear solution.
Pyrimethamine drug selection of malaria parasites in mice with pyrimethamine in drinking water
Prepare a 70 µg/ml Pyrimethamine work solution by combining 10 ml of stock(7mg/ml) solution with 990 ml of tap water (pre-adjust the pH of the water to 4.0-5.0, using a 1M HCl solution (DMSO method) or lactic acid). Provide as drinking water to the mice and continue the treatment for at least 5 days until the selected parasites are collected.
Note: pH adjustment of the water is essential. At a pH >5, Pyrimethamine will start to precipitate.

Nycodenz

Lucron Bioproduct B.V. (ELITechGroup Benelux), Product code AXI-1002424, Mw 820

A Nycodenz solution is prepared by dissolving 138 grams of Nycodenz powder in 500 ml of buffered medium (see below). The solution is autoclaved at 121°C for 20 minutes and stored at 4°C.
Buffered medium, pH 7.5
5 mM Tris-HCl, pH 7.5 (605.7 mg/L)
3 mM KCl (233.7 mg/L)
0.3 mM $CaNa_2EDTA$ (112.2 mg/L)

Erythrocyte Lysis Buffer
10X stock solution, pH 7.4:
1.5 M $NHCl_4$, pH7.4 (80.23 g/L)
0.1 M $KHCO_3$ (10.12 g/L)
0.01 M EDTA (3.72 g/L)
For lysis of erythrocytes, dilute the stock 10 times in demineralized water and store at 4°C.

10X Phosphate Buffered Saline (PBS)
Roche, Cat.nr. 1666 789
Components:
0.01 M KH_2PO_4
1.37 M NaCl
0.027 M KCl
pH7.0
Prepare a work solution by diluting 10 times in demineralized water. Adjust the pH to 7.2 with a 1 M HCl solution and autoclave at 121°C for 20 minutes. Store at room temperature.

Enriched PBS
(PBS with Hepes/Glucose/BSA for collecting parasites)
Prepare a 1X PBS solution and supplement with:
20 mM Hepes
20 mM Glucose
4 mM $NaHCO_3$
0.1% BSA
Adjust the pH to 7.25 with 5 M NaOH. Sterilize the solution by passing it through a 0.2 µm filter. Store at 4°C.

Low Melting Agarose
(Preparing Pulsed Field Gel blocks)
Sigma, Cat.nr. A9414 10g
In a 50 ml tube, combine 200 mg of Low Melting Agarose with 10 ml of TNE buffer (see below). Place the tube in a microwave and bring to the boil. Boil for 30-60 seconds. Keep at 37°C if used directly. Store at room temperature if needed at a later stage. The agarose is stable for several months and can be re-melted in boiling water or by heating it in the microwave for ~10 seconds.

TNE buffer
50 mM Tris, pH 8.0
100 mM NaCl
5 mM EDTA

Giemsa
Merck, Cat.nr. 1.09204.0500
Giemsa Dilution Buffer (Sorenson's Staining Buffer)
3.73 mM KH_2PO_4 (2.541 g/5L)
9.61 mM Na_2HPO_4-$2H_2O$ (8.55 g/5L)
pH 7.2
For staining of blood smears, prepare a 10% Giemsa solution in Sorenson's buffer

Sugar solution
(Feeding of mosquitos)
Prepare a 5% (w/v) solution of glucose in tap water or mineral water. Sterilize the solution by passing it through a 0.2 µm filter.

Rompun/Ketamine Anaesthetic
(Rompun/Ketamine mix in PBS for mice and rats)
Rompun (LUMC Pharmacy), Cat.nr. 939692, 25 ml
Ketamine (LUMC Pharmacy), Cat nr. 912042, 5x10 ml
1X PBS
Mix Rompun, Ketamine and PBS 1:1:1
Intraperitoneally inject 40-50 µl per mouse and 120-160 µl per rat

From DEC: '*Infected mice are anesthetized with Rompun/Aescoket (a ratio of 15:11, Rompun:Aescoket; 30 µl/mouse) and placed in a 'mouseholder' which is placed for a period of 20 minutes on a mosquito cage containing 20-30 mosquitoes*'.

Update May 2015
Mosquito infection experiments are performed with infected mice under Sedamun/Anesketin anaesthesia (Sedamun contains 2% Xylazine and Anesketin 100mgKetamin/ml).
Mice are anesthetized with Sedamun/Anesketin (a ratio of 15:11, Sedamun/Anesketin; 30 µl/mouse)

Neomycin Sulphate
(Supplement for RPMI medium)
In a 50 ml tube, prepare a 200X (10 mg/ml) stock solution by combining 0.5 gram of Neomycin with 50 ml of demineralized water. Add 5 ml of this stock solution to 1 litre of RPMI parasite culture medium. Alternatively, dissolve 50 mg of Neomycin Sulphate in 1 litre of RPMI directly. The stock solution is stored at -20°C.

Sulfadiazine solution
Sigma, Cat.nr. S-3549

The concentration of the sulfadiazine can vary between 15 and 35 mg/L. For collecting pure populations of gametocytes, we standardly use 30 mg/L in the drinking water of the mice.

Phenyl hydrazine (Phenylhydrazinium chloride)
Merck Chemicals, Cat.nr. 1072530100 (100 g), Mw 144.6. Caution; extremely harmful!
Prepare a 25 mg/ml work solution by combining 1 gram of phenyl hydrazine with 40 ml of physiological salt solution (0.9% (w/v) NaCl in demineralized water). Store aliquots of 1 ml at –20 °C.
Intraperitoneally inject 0.1 ml into mice with a bodyweight of approximately 20 grams.

Hoechst
(Nucleic acid staining)
BisBenzimide H 33258. Sigma, Cat.nr. B1155, Mw 533.9
Prepare a 500 µM (13.35 mg) stock solution in 50 ml water for cell staining and store aliquots of 1 ml at –20 °C.

FACS analysis of Glutaraldehyde (0.25%) fixed parasites
Add 4 µl of Hoechst stock solution to 1 ml of cells suspended in PBS, mix and stain in the dark for 1 hour. The final staining concentration is 2 µM Hoechst.

FACS sorting of live parasites
The staining is performed in culture medium (or enriched PBS) at 37C 9for one hour). Add 20 µl of the Hoechst stock solution to 1 ml of parasite suspension in culture medium (5-10ul of blood in 1 ml of culture medium). The final concentration is 10 µM Hoechst.
In case of cell sorting, the parasites are collected in culture medium with FCS (or enriched PBS)

Hoechst 33342
(Nucleic acid staining)
BisBenzimide H 33342. Sigma, Cat.nr. B2261, Mw 561,93, **water soluble**
Prepare a 500 µM (14.0 mg) stock solution in 50 ml water for cell staining and store aliquots of 1 ml at –20°C.
Used for staining of fixed or live parasites as described above.

Mercurochrome (Mercury dibromofluorescenin disodium salt)
(Oocysts staining in dissected mosquito)
Stain with a 2-10% Mercurochrome solution in PBS.

Negative selection of mutants in mice by providing 5-fluorocytosine (5-FC) in the drinking water
5-fluorocytosine (5-FC), LUMC Pharmacy, 250 ml, 10 mg/ml
Recently, a simple procedure has been described for *in vivo* selection of marker-free parasites that have spontaneously (or actively, by the GIMO method) lost the

h*dhfr*::y*fcu* marker from their genome by providing 5-fluorocytosine (5-FC) in drinking water (see Orr et. al., Malaria J, 2011).
'Dilute the 10 mg/ml stock solution 5 times to a concentration of 2 mg/ml in the appropriate amount of drinking water and provide to the mice in their water bottles. Treatment with 5-FC starts 1 day post infection and continues throughout the selection period. Drinking bottles used were opaque white plastic which hold between 250-300 mls with rubber bungs using ball bearing drip nozzles (North Kent plastics). 5-FC water should be changed every 4 days, mainly for the sake of animal welfare. A mouse will drink 5-10 ml per day. Bioavailability of 5-FC when given orally is 76-89%. (journal of antimicrobial chemotherapy (2000) 46, 171-9 A. Vermes et al Flucytosine: a review……)'

In Leiden we use the following procedure to efficiently select the required mutants with 5-FC:
Negative selection of mutants is performed by providing 5-fluorocytosine (5-FC) in the drinking water of mice, 1 day after the mice have been infected with transfected parasites. The 2,5 mg/ml final concentration of 5-FC diluted with drinking water (we use a 4X stock 5-FC solution that has been formulated by the LUMC pharmacy; stock is 10mg/ml; usually 37 ml of the stock solution + 113 ml drinking water). 5-FC-containing drinking water is provided to mice during the complete selection period.

Alternative sources of 5-FC
InvivoGen, Cat. Code sud-5fc, 250 mg at 10 mg/ml. Store at room temperature in a cool and dark place.
Sigma, Cat.nr. F7129-1G. Store at 2-8°C. A stock solution of 10 mg/ml can be prepared by dissolving 5-FC in demineralized water shaking vigorously or by using a vortex. Apply heat if necessary. Store in a darkened bottle at room temperature in a cool and dark place.

Chloroquine (in drinking water for mice, MBC paper 2011, 4262)
Sigma, Cat.nr. C6628, Mw 515.86
Chloroquine powder is dissolved in normal tap water to a concentration of 0.288 mg/ml (288 mg/l) and provided to the mice in their water bottles. In order to make the solution more palatable for the animals, glucose is added at a concentration of 15 g/l). The daily uptake of water is between 4 and 10 ml.

Artesunate
Intraperitoneal injection
Prepare a solution of 6.25 mg/ml Artesunate in 5% $NaHCO_3$ (sodium bicarbonate). Treat the mice for 3 consecutive days with one dose of 200 µl Artesunate, injected intraperitoneally (1.25 mg/25gram bodyweight = 50 mg/kg). From experience we know that this treatment results in a radical cure of mice infected with 1-3% parasites.

In drinking water
Add 5 ml NaHCO3 (5% solution) per 60 mg of artesunate (1 bottle: old batch of artesunate or SIGMA: 3731-500MG)

Add 200 ml tap water
(= 1,2 mg/mouse/day)

Old methods

Pyrimethamine solution for treatment of mice by i.p. injection.
Dissolve 20 mg pyrimethamine in 10 ml DSMO in a tube of 50 ml by stirring on a vortex stirrer
Inject 0.2 ml intraperitoneal (i.p) to a mouse of about 20g.
For storage, wrap the tube with aluminium foil against light exposure and keep it at room temperature. Prepare this pyrimethamine solution every week fresh.

WR99210
WR99210 from JACOBS PHARMACEUTICAL COMPANY INC (Cleaveland Lane 37, P.O. Box 5290, Princeton)
Depending on the 'selection pressure', dissolve 6, 12, 16, or 20 mg WR99210 in 3,5ml DMSO in a 10 ml tube by vortexing and subsequently, add 1,5 ml of distilled water to have a final solution in 5ml (70% DMSO and 30% water).
Treatment of mice of approximately 20g bodyweight.
For instance : Subcuteaneously injecton (in the neck) of 0.1ml WR99210 solution of 12mg in 5ml is applied for a treatment of 12mg/kg bodyweight.

WR99210 solution in a mix of Ethanol/ Benzyl alcohol/Myglyol (ratio; 57:40:3)
Weight 16 mg of WR99210
Take the WR99210 in 10 ml tube
Add to this tube 2ml ethanol of 98% and 0.15 ml Benzyl alcohol
Mix by stirring on a Vortex until you have a clear solution
Finally add 2.85 ml of Myglyol (final volume is 5 ml)
For treatment of P.berghei malaria parasite inject intra peritoneal 0.1ml into a mice of 20 gram (16 mg/kg body weight

WR99210 solution in a mix of Ethanol/ Benzyl alcohol/water (ratio; 57:40:3)
12 mg or 16 mg/ kg body weight
Weight 16 mg of WR99210
Take the WR99210 in 10 ml tube
Add to this tube 2ml ethanol of 98% and 0.15 ml Benzyl alcohol
Mix by stirring on the Vortex until you have a clear solution
Finally add 2.85 ml demineralised water (final volume is 5 ml)
Inject 0.1ml of this solution to a mouse for treatment of P.berghei malaria parasites (16mg/kg Body weight) for double selection cassette(pyrimethamine and hDHFR)

2. Cryopreservation of blood stage parasites

Blood stages of parasite lines/clones are stored in liquid nitrogen. These blood stages are obtained from infected mice.

1. Collect 0.8-1ml of infected blood by cardiac-puncture from a mouse with a parasitemia of 1-10%
2. Mix the blood with 1ml of a glycerol/PBS solution (30% glycerol; v/v), containing 0.05ml of Heparin stock-solution.
3. Transfer the suspension to 4 cryotubes (Nunc), 0.5ml per tube.
4. Leave the tubes for 5-15min at 4°C and transfer the vials gently into the liquid nitrogen tank

The blood suspension of one tube (0.5ml) can be used to infect 2-10 mice. By intraperitoneal injection of 0.1ml of this suspension into a mouse the parasitemia usually rises to 1-10% within 3-5 days: see 3

3. Infection of laboratory animals with blood stage parasites

*We infect mice/rats by **intraperitonal injection (i.p.)** of infected erythrocytes (blood stages). Only in the cloning procedure or to establish synchronized infections we infect animals by injecting blood stages **intravenously (i.v.)** into the tail veins.*

*Blood stages are either obtained from cryopreserved stocks (see **2**) or directly from heart- or tail-blood from infected animals. For most purposes we do not determine exactly the number of parasites injected. For our standard infections, maintenance and propagation of the parasites we use Swiss mice (20-25g) and Wistar Rats (180-220g). Swiss mice are bigger, easier to handle and cheaper than most other mice strains.*

For maintenance and propagation of parasites in vivo the following characteristics are important: 1µl of blood contains approx. $6x10^6$ erythrocytes; P. berghei has a mean multiplication rate in mice (and rats) of 10 per 24 hour during the period of increasing parasitemia of 0 to 5-10%; at a parasitemia of more than 5-10% mice can die from cerebral complications; the course of parasitemia and disease characteristics are less well-described in rats compared to mice; in our hands P. berghei has a stronger preference for reticulocytes in rats than in mice. In cloned lines of P. berghei, parasites arise (by mutation) during asexual multiplication, which have lost the capacity to produce gametocytes. These non-producer parasites can overgrow the gametocyte producer parasites. To prevent the loss of gametocyte production we maintain parasites in mice (by weekly mechanical passage) only for a maximum period for eight weeks. After this period we start new infections from cryopreserved stocks from the parent clone.

Mouse infection with cryopreserved blood stages, usually on Friday

1. Thaw the content of one cryotube containing 0.5ml blood suspension (see **2**) at room temperature.
2. Inject 0.02-0.5 ml of the suspension i.p. into a mouse

Mouse infection with blood stages obtained from an infected mouse (mechanical passage), usually on Thursday

1. Collect one droplet of tail blood (5µl) from an infected animal with a parasitemia of 5-15% in 10ml PBS
2. Inject 0.1ml of the suspension i.p. into a mouse

3. On day 4-7 after injection the parasitemia will increase from 0.1 to 5-20%

This method is used to weekly passage parasites from mouse to mouse. Passage is performed 1-2 days before disease characteristics, such as cerebral complications, becomes obvious.

Rat infection with blood stages obtained from an infected animal

1. Collect 5-8 droplets of tail blood (30-40µl) from an infected animal with a parasitemia of 5-15% in 1ml PBS
2. Inject the 1ml suspension i.p in a rat. Usually we give two i.p. injections of 0.5ml of the suspension using a 2ml syringe.
3. On day 4 or 5 after injection the parasitemia ranges between 0.5-3%

This method is used to collect parasites for transfection or for establishment of synchronous infections

4. Treatment of laboratory animals with phenylhydrazine-HCl

P. berghei has a strong preference for reticulocytes. For certain purposes we treat animals with phenylhydrazine-HCl (phz) to induce reticulocytosis. One single dose of phz, injected i.p. (mice:125mg/kg bodyweight; rats 35mg/kg bodyweight) results in the presence of 30-50% reticulocytes at day 4-6 after injection. We treat animals with phz for: 1) collection of large numbers of blood stages for molecular/biochemical studies, 2) establishment of synchronous infections in rats, 3) for obtaining large(r) numbers of gametocytes.

Mice

1. Inject 0.1ml of the phz stock solution per mouse (20-25g) using an 1ml syringe
2. Infect the treated mice with parasites 1-2 days after the phz-treatment

Rats

1. Inject 0.3 ml phz stock solution per rat (200g) using an 1 ml syringe
2. These rats are used for i.v. injection of schizonts/merozoites at day 4-5 after phz-treatment (see chapter 6)

5 Giemsa stained blood films: investigation of (the course of) the parasitemia

The course of parasitemia (=percentage of infected cells) is determined in Giemsa stained blood films made from tail blood. The infection of P. berghei is (relatively) asynchronous compared to for example the synchronous development of blood stages of P. chabaudi. However, there is a certain level of synchronous development of the parasites during the period of increasing parasitemia from 0 to 5-10%. When the laboratory animals are kept under a regular day-night light regime, more than eighty percent of the parasites will become mature schizonts in the early morning between 2.00 and 6.00 a.m. Therefore, in slides made between 9.00 a.m. and 12.00 a.m. most parasites appear as rings/young trophozoites. The asexual cycle of P. berghei takes 22-24h. Trophozoites are 'mature' 16 hours after invasion of the merozoite, entering schizogony. At this time, the parasites disappear from the peripheral blood circulation, sequestering in the capillaries of inner organs till maturity of schizonts.

1. Make 1-3 separate thin blood films from tail blood on a standard microscope slide.
2. Air-dry the thin films

3. Fix the slide for 1s in methanol
4. Prepare a fresh Giemsa solution in 'Giemsa-staining buffer' (10%, v/v)
5. Pour the Giemsa solution on the slide (approx. 3-4ml per slide)
6. Leave to stain for 10 min (we also use a 5% (v/v) Giemsa solution for a staining period of 20-30 min.)
7. Rinse carefully with tap water
8. Leave the slide in an upright position to air-dry
9. Examine the stained blood films under a standard light microscope with immersion oil and objective at 100x.

6. Establishment of synchronized blood infections in rats

*The development of blood stages of P. berghei in animals is relatively asynchronous. We can establish infections in rats and mice that are highly synchronous during two developmental cycles. These synchronous infections are used to collect the different developmental bloodstages (rings, young and old trophozoites, young and mature schizonts, young and mature gametocytes) for molecular/biochemical studies and for in vitro testing of drugs. Synchronous infections are established by injecting purified mature schizonts/merozoites directly in the tail veins of the animals. Within 3-4 hours after injection all merozoites have invaded a new erythrocyte resulting in a (ringform) parasitemia of 0.5-10% (depending on the number of parasites injected). We here describe the establishment of a synchronous infection in a rat. In **7** we describe synchronous infections in mice.*

Infection of a rat (day 0)

A rat is infected with parasites to serve as a source of blood stage parasites for the culture and purification of schizonts. A Wistar rat (180-220g) is intra-peritoneally infected on day 0, typically a Thursday, with 2-3x10^7 infected erythrocytes

1. Collect 5-8 droplets of blood (25-40μl) from either the tail or the heart of a mouse with a parasitemia of 5-15% in 1ml PBS at room temperature
2. Inject the 1ml suspension immediately i.p. into a rat. Usually we give two injections of 0.5ml of the suspension using a 2ml syringe.
3. In general the rat will have a parasitemia between 0.5 and 3% on day 4 or 5.

Phenylhydrazine treatment of a rat (day 1)

A rat is treated with phenylhydrazine-HCl (phz) to induce reticulocytosis. This rat will be used later (day 5-6) for the intravenous injection of the cultured, purified schizonts. Since P. berghei has a strong preference for reticulocytes, invasion of the parasites is more efficient in phz-treated rats than in untreated rats. Moreover in phz-treated rats the number of multiple infected erythrocytes is reduced. This is important since multiple infected erythrocytes containing more than two parasites will not support the growth of these parasites into healthy mature schizonts.

1. Inject i.p. a Wistar rat (180-220) on day 1 with 0.30-0.35ml of a stock-solution of phenylhydrazine using an 1ml syringe.

In vitro culture of schizonts (day 4 or 5)

Blood stages of P. berghei are cultured in RPMI1640 medium (pH 7.3) containing foetal calf serum (FCS). In general the parasites are maintained in vitro for only one developmental

cycle: ring forms/young trophozoites are allowed to develop into mature schizonts during a period of 16-23 hours. Blood is collected from the positive rat (infected at day 0) at a parasitemia of 1-3% at day 4 (Monday) or 5 (Tuesday) between 10.00h and 16.00h. Higher parasitemia's are suboptimal since many erythrocytes will become multiple infected. In animals that are kept under the normal day/night light regime the development of the parasites is relatively synchronous. In these animals bursting of the schizonts and invasion by merozoites occur in the early morning between 4.00h and 6.00h. Therefore, most parasites are in the ring form/young trophozoite stage when the infected blood is collected. The infected blood is then cultured overnight at 36.5-37°C. By 9.00h the next day all parasites have developed into mature schizonts, which do not burst under our in vitro conditions. Schizonts of P. berghei containing mature merozoites can survive for several hours and can be manipulated without bursting and the loss of viability.

2. Collect 5-8ml infected blood by cardiac puncture using a 10ml syringe from a rat with a parasitemia of 1-3%. Usually the blood is collected between 10.00 and 16.00h at day 4 or 5.
3. Transfer the blood to 5-7ml complete culture medium to which 0.3ml stock-solution of Heparin is added
4. Pellet the cells by centrifugation (8 min at 1500 rpm)
5. Discard the supernatant.
6. Resuspend the cells in 150ml complete culture medium in a 500ml Erlenmeyer flask
   We use these flasks in our 'automatic' continuous gassing system in which the cultures are continuously gassed throughout the complete culture period. Continuously gassing is however not necessary for these cultures. Cultures can be maintained therefore in closed, plastic 250ml culture flasks that have been gassed once at the beginning of the culture period.
7. Put the flask on a shaker in a 37°C water bath, incubator or climate room and connect it to the gas-system with the following gas mixture: 5% CO_2, 5% O_2, 90% N_2.
8. Switch on the shaker after 5min at a minimal speed to keep the cells in suspension.
9. Leave the parasites in culture at 37ºC until the next morning 9.00h.
   The culture temperature is critical since the developmental rate of the parasites is dependent on the temperature. Above 38,5C parasites will degenerate. Lower than 37ºC the parasites will develop into healthy parasites but the developmental time will be extended. Even at a temperature of 30ºC the parasites will reach the mature schizont stage, but the development of ring forms into schizonts will take more than 48 hours.
10. Take a small sample (0.5ml) from the cultures in an eppendorf tube at 9.00h to determine the 'quality' of the parasites.
11. Pellet the cells (maximum speed, 5s) and discard the supernatant
12. Make a thin blood smear of the cells, fix with methanol and stain with Giemsa (see chapter **5**)
13. Examine schizont development using a light-microscope at a 1000x magnification (100x objective, immersion oil).
    Healthy, viable schizonts are distinguished by the presence of 12-24 'free' merozoites within one red blood cell and one cluster of pigment (haemozoin). Smearing the cells on the microscope slides often damages the red cell membrane and the merozoites

are visible as more or less clustered free parasites. A purple (red) defined compact nucleus and a dot of blue cytoplasm is characteristic of the viable merozoites. About 15-25% of the parasites in these smears are single-nucleated (young) gametocytes. Degenerate schizonts often show a compact morphology in which the separate merozoites are difficult to recognise. Be careful not to mistake developing schizonts (which are still in the process of nuclear division prior to budding off of the merozoites) for degenerated schizonts.

14. Start with the purification of the schizonts (see below)

Purification of mature schizonts (day 5 or 6)

Schizont-infected cells (1-3% of the total cell population) are separated from uninfected cells by Nycodenz-density gradient centrifugation. We always start with the purification procedure between 9.00 and 10.00h. Starting later in the morning results in a higher percentage of degenerated schizonts. For the density gradients we use Nycodenz instead of Percoll. Percoll is used by many workers to separate parasite stages, but our experience is that in contrast to Percoll, Nycodenz does not affect the viability of parasites.

1. Prepare a 55% Nycodenz/PBS solution (v/v). See chapter **1** for the preparation of the Nycodenz stock solution. In general a total volume of 50 ml (27.5ml Nycodenz stock-solution, 22.5 ml PBS) is required for a culture suspension of 150ml.
2. Transfer the culture suspension containing the schizonts, to four 50ml tubes (35ml per tube).
3. Using a 10ml pipette, gently add 10ml of the Nycodenz-solution in each tube **under** the culture suspension, so that a sharp contrasting division is visible between the two suspensions.
4. Centrifuge 20-30 min at 1500 rpm in a swing out rotor at room temperature (no brake)
5. Collect carefully the 'brown' (grey) layer at the interface between the two suspensions. In general a total volume of about 30-40 ml is collected from the four tubes. *The schizonts (and leucocytes, platelets, gametocytes and old trophozoites if present) will collect at the interface of the two suspensions, while the uninfected cells will pellet on the bottom of the tubes.*
6. Pellet the schizonts by centrifugation (1500rpm, 8min). For this 'washing' step we add to the schizont-suspension approx. 20ml culture medium, obtained from the top of the gradients.
7. Discard the supernatant.
8. Resuspend the schizont pellet with complete culture medium to a total volume of 400-500µl.
9. Proceed with injection of the purified schizonts into the rat that has been treated with phenylhydrazine at day 1 (see next section)

Injection of purified schizonts/merozoites into a rat (day 5 or 6): start of the synchronous infection

1. Put the rat (that has been treated with phenylhydrazine at day 1) at 37°C, 10min before injection of schizonts. The rat veins swell at this temperature, simplifying the i.v. injection procedure (swollen veins).
2. Inject the complete 400-500µl schizont-suspension into a tail vein under isofluorane anaesthesia using Insulin-syringes (Becton Dickinson, Micro-Fine +, 0.5ml;

0.30mm(30G) x 8mm, cat.nr. 324870). Usually we inject the animal between 10.30 and 11.00h.

3. Determine the parasitemia of the rat in Giemsa stained blood films (see chapter **5**) 3-4 hours after infection.
   In general the parasitemia ranges between 0.5-3% consisting of mainly ring forms 3-4 hours after infection. T*he major part of the ring forms develops within 22-24 hours into mature schizonts. A small part of the ringforms develops within 26-30 hours into mature gametocytes. In the rats the young and mature schizonts sequester in the capillaries of inner organs*

7. Establishment of synchronized blood stage infections in mice

*The first part of the procedure, i.e. culture and purification of schizonts, is the same as for the establishment of synchronous infections in rats (see chapter **6**). The purified schizonts, collected from the culture of infected blood of one rat, are i.v. injected into the tail veins of 3-5 mice. We inject 100-150µl of the schizont suspension per mouse using* Insulin-syringes *(Becton Dickinson, Micro-Fine +, 0.5ml; 0.30mm(30G) x 8mm, cat.nr. 324870). The mice are placed at 37°C 10min before injection of the schizonts. The veins swell at this temperature, simplifying the i.v. injection procedure (swollen veins). When we use the synchronized parasites for testing the efficacy of drugs the mice are not pre-treated with phenylhydrazine. Four hours after injection of schizonts the parasitemia ranges between 0.5 and 10% dependent on the amount of purified schizonts injected and reticulocytes present.*

8. Removal of leucocytes from infected blood

We remove leukocytes from the infected blood by using commercially available leukocyte filters (Plasmodipur filters, Euro-Diagnostica). These are small filters that effectively remove leukocytes from a maximum of 10-15ml of blood diluted with culture medium or PBS (minimal dilution 1:1). We use RPMI1640 culture medium in the filtering process if we use the parasites for further in vitro cultivation. We use PBS if we use the parasites for collection of DNA/RNA/protein

1. 'Prewash' the Plasmodipur filter (Euro-Diagnostica, www.eurodiagnostica.com) with 10ml culture medium or PBS using a 20ml syringe which is placed on top of the filter
2. Pass the infected blood suspension through the filter using a 20ml syringe
3. Elute with 15-20ml of culture medium or PBS
4. Pellet the infected erythrocytes by centrifugation (8min; 1500rpm)
5. Discard the supernatant.
   The parasites are then ready for in vitro cultivation or for further manipulation for DNA/RNA/protein

9. Short-term *in vitro* culture of asexual blood stages:

- Purification of asexual blood stages from synchronized parasites

- Testing the susceptibility of asexual blood stages to drugs/inhibitors

*For different research purposes collection of large numbers of the different developmental stages without contamination with other stages or with leucocytes is necessary. We use short-term in vitro cultures of synchronized infections as described in chapter **6** to collect rings, young/old trophozoites and immature/mature schizonts. Usually we use synchronized*

infections that are established in rats because a larger volume of infected blood can be obtained from rats than from mice. The infected blood is usually cultured in Erlenmeyer-flasks or 250ml culture flasks.
For testing the susceptibility of asexual blood stages to drugs/inhibitors we use short-term in vitro cultures of synchronized infections as described in chapter ***7****. We incubate infected blood from mice containing ring forms for a period of 20-24h in 24-well plates in the presence of the drugs/inhibitors*

Establishment of synchronized infections in rats/mice
- See chapter **6** and **7**

- Collection of infected blood (ring forms) and removal of leucocytes
Because old trophozoites and schizonts disappear from the peripheral blood circulation in vivo, we collect blood from the infected animals when the parasites are ring forms. These ring forms are placed in culture for further development into trophozoites and schizonts. We collect infected blood either 3-4 hours after injection of the schizonts (ring forms from the first cycle) or 24-26 h after injection of the schizonts (ring forms from the second cycle). Dependent on the numbers of parasites required we collect infected blood from one or more rats.

A: infected blood from one rat
1. Collect 5-8ml infected blood from one rat by cardiac puncture under isofluorane-anaesthesia 3-4h after injection of the schizonts or 24-26h after injection of the schizonts.
2. Dilute the blood in 5-7ml complete culture medium to which 0.3ml stock-solution of Heparin is added.
3. Remove the leucocytes suspension using a Plasmodipur-leucocyte filter as described in chapter **8**.
4. Pellet the cells by centrifugation (1500rpm, 8min) and discard the supernatant.

B: infected blood from one mouse
1. Collect 0.8-1ml infected blood one mouse by cardiac puncture under isofluorane-anaesthesia 3-4hours after injection of the schizonts or 24-26 hours after injection of the schizonts
2. Dilute the blood in 5ml complete culture medium to which 0.2ml stock-solution of Heparin is added
3. Remove the leucocytes suspension using a Plasmodipur-leucocyte filter as described in chapter **8**.
4. Pellet the cells by centrifugation (1500RPM, 8min) and discard the supernatant

Short term in vitro culture of the blood stages
Blood stages of P. berghei are cultured in RPMI1640 medium (pH 7.3) containing foetal calf serum (FCS). The parasites are maintained in vitro for only one developmental cycle: ring forms are allowed to develop into mature schizonts during a period of 20-24 hours. In these cultures reinvasion of merozoites is limited to a minimum due to the inability of mature schizonts of P. berghei to burst spontaneously and the low number of reticulocytes present in

the cultures. Mature schizonts of P. berghei containing merozoites can survive for several hours and can be manipulated without bursting and loss of viability.

a: infected blood from one rat

1. Resuspend the pelleted erythrocytes (containing mainly ring forms) in 150ml complete culture medium in a 500ml Erlenmeyer flask
   We use these flasks in our 'automatic' continuous gassing system in which the cultures are continuously gassed throughout the complete culture period. Continuously gassing is however not necessary for these cultures. Cultures can also be maintained in closed, plastic 250ml culture flasks that have been gassed once at the beginning of the culture period.
2. Put the flask on a shaker in a 37°C water bath, incubator or climate room and connect it to the gas-system, using the following gas mixture: 5% CO_2, 5% O_2, 90% N_2.
3. Switch on the shaker after 5min at a minimal speed to keep the cells in suspension.
4. Leave the parasites in culture at 37°C until the parasites have developed into mature schizonts.
   The culture temperature is critical since the parasite developmental rate is dependent on the temperature. Above 38.5ºC parasites will degenerate and at a lower temperature than 37ºC the parasites will develop into viable, mature schizonts but the developmental time will be extended. Even at a temperature of 30ºC the parasites will reach the mature schizont stage, but the development of ring forms into schizonts will take more than 48 hours.
5. Collect parasite samples at the appropriate time points from the culture for Giemsa stained films (0.5ml in eppendorf tube) and for collection of the different blood stages

Young trophozoites	6-7h after start of the culture (= 11 hpi)
Old trophozoites	12-13h after start of the culture (= 16 hpi)
Immature schizonts	16-17h after start of the culture (= 20 hpi)
Mature schizonts	21-22h after start of the culture (= 26 hpi)

   *The rate of development is dependent on the temperature of the cultures. Even differences of half a degree Celsius significantly influence the developmental rate. The time schedule shown above is therefore not fixed but depends on the experimental temperature conditions of the different culture systems/rooms/incubators used. In 'gametocyte-producer' clones of the ANKA strain of P. berghei about 10-20% of the ring forms develop into gametocytes that cannot be separated from the other stages. To obtain pure asexual blood stages we therefore often use 'non-producer' clones (chapter **10**). Gametocytes are usually purified from synchronous in vivo infections of the 'gametocyte-producer' clones (see chapter **12**)*

b: infected blood from one mouse (for drug-testing)

For drug/inhibitor sensitivity tests we usually incubate infected blood in 24 well culture plates. The infected blood is obtained from synchronized in vivo infections and contains (mainly) ring forms (3-4h after invasion) at a parasitemia between 1 and 3%. For the drug/inhibitor tests we do not remove the leucocytes from the infected blood. We use a final cell suspension in culture of 1% (v/v; blood/culture medium). Serial dilutions of the drugs/inhibitors are made in complete culture medium. The 24 well plates are placed in a Candle Jar on a shaker at 37°C for a period of 20-24h to allow the development of the ring forms into mature schizonts. The

Candle Jar is either continuously gassed or the oxygen concentration is lowered by burnt out candles.

1. Resuspend the pelleted cells (containing mainly ring forms) in complete culture medium to a 2% (v/v) cell-suspension
   The total volume is dependent on the number of wells in the drug/inhibitor dilution series and the number of drug tests in the experiment. Usually we have 2-3 wells per drug/inhibitor concentration.
2. Distribute 0.5ml of the cell-suspension per well in a 24-well plate
3. Add to each well 0.5ml complete culture medium containing the drug/inhibitor, resulting in a total volume of 1ml per well.
   The serial dilutions of the drug/inhibitor are made in complete culture medium containing 2x the final concentration of the drug.
4. Put the plate in a Candle Jar type of device on a shaker at 37°C and connect it to the gas-system, using the following gas mixture: 5% CO_2, 5% O_2, 90% N_2.
5. Switch on the shaker after 5min at a speed to keep the medium moving.
6. Leave the parasites in culture at 37°C for a period of 20-24h, until the ring forms have developed into mature schizonts.
7. Collect parasite samples at the appropriate time points from the culture-wells for quantification of schizont maturation and/or DNA replication either in Giemsa stained blood films or using flow cytometry.
   To determine the effect of drugs/inhibitors on blood stage development we usually collect samples 20-24hour after start of the cultures. Samples are used to prepare Giemsa stained blood films or blood cells are fixed for flow cytometry. In Giemsa stained blood films the development of the ring forms into mature schizonts is monitored (counting of the ratio/percentage/number of (arrested) rings, trophozoites, immature and mature schizonts). By flow cytometry, the rate of DNA replication in the parasites (schizonts) is determined after staining the parasites with DNA specific fluorescent dyes. We prefer this method above determination of growth by the incorporation of radioactive nucleic acid precursors into the DNA of parasites because in principal the same data is generated by flow cytometry and there is no need to work with radioactive precursors. In addition, flow cytometry generates data from individual cells allowing more detailed analysis of the growth characteristics of the parasites in culture (Janse and Vianen (1994) in Methods in Cell Biology: Flow cytometry (Vol. 42)(Darzynkiewicz, Z., Crissman, H.A. and Robinson, J.P., eds.), pp295-318, Academic Press). From the data of examination of Giemsa stained slides and/or flow cytometric analysis drug dose-response curves are generated and C_{50} inhibition values calculated

10. Purification of ring forms, trophozoites and schizonts

a) Ring forms

1. Ring forms are collected from synchronized infections in rats at 3-4h (rings from the first cycle) or at 24-28h (rings from the second cycle) after injection of the schizonts (see chapter **6**).
2. The rings are collected after removal of the leucocytes from the infected blood (see chapter **8**).

Ring forms of the second cycle are contaminated with (nearly) mature gametocytes that originate from rings of the first cycle. Ring forms of the first cycle are contaminated with low numbers of mature gametocytes which are injected into the animals together with the schizonts. To circumvent the contamination with gametocytes, one can separate the ring forms from the gametocytes by Nycodenz-density gradient centrifugation (see below). Another possibility is to use non-gametocyte producer clones for collection of ring forms.
Ring forms are always collected from in vivo infections and not from in vitro cultures since invasion of erythrocytes in vitro is less efficient than in vivo in mice and rats. Moreover in vivo the schizonts are sequestered in micro capillary blood vessels of inner organs, resulting in relatively pure populations of young ring forms in the blood circulation without contamination with schizonts.

Purification of ring forms from synchronized infections using Nycodenz-gradient centrifugation

To remove the low percentage of contaminated gametocytes and schizonts from populations of ring forms we perform the following Nycodenz gradient-centrifugation step.

1. Dilute 5-8ml of infected blood (containing mainly ring forms) from one rat in 100-140ml of PBS
2. Prepare a 50% Nycodenz/PBS solution (v/v)(see chapter **1** for the preparation of the Nycodenz stock solution). In general a total volume of 50ml (27.5ml Nycodenz stock-solution, 22.5ml PBS is required) for a 100-140ml cell-suspension.
3. Transfer the culture suspension containing the schizonts, to four 50ml tubes (35ml per tube).
4. Using a 10ml pipette, gently add 10ml of the Nycodenz-solution in each tube **under** the culture suspension, so that a sharp contrasting division is visible between the two suspensions.
5. Centrifuge 20-30 min at 1500 rpm in a swing out rotor at room temperature (no brake)
6. Discard first carefully the 'brown' (grey) layer at the interface between the two suspensions, containing the gametocytes/schizonts. Then discard the remaining supernatant (PBS and the Nycodenz solution).

   The schizonts and gametocytes (and old trophozoites if present) will collect at the interface of the two suspensions, while the uninfected and ring-infected cells will pellet on the bottom of the tubes.
7. Wash the pellet once with PBS (1500 rpm, 8 min)
8. Discard the supernatant

   The ring forms are then ready for further manipulation, for example purification of DNA, RNA or protein. Ring-infected erythrocytes cannot be separated from the non-infected cells by density-gradient centrifugation. Usually we lyse the red blood cells to get rid of the red blood cells and subsequently pellet the ring forms by centrifugation (8min, 2000rpm)

b) 'Young' and 'old' trophozoites

1. Trophozoites are collected from *in vitro* culture of synchronized ring forms (see chapter **9**). *The ring forms develop into mature trophozoites in 16h at 37.5ºC. At around 16h the trophozoites start nuclear division*.
2. 'Young' trophozoites are collected at 6-7hours after the start of the cultures (=11 hours post invasion);
3. 'Old' trophozoites are collected at 12-13h after start of the cultures (= 16hpi)
   The trophozoites are then ready for further manipulation, for example purification of DNA, RNA or protein. Trophozoite-infected cells can be separated from the non-infected cells by Nycodenz density-gradient centrifugation. Usually we lyse the red blood cells to get rid of the red blood cells and subsequently pellet the trophozoites by centrifugation (8min, 2000rpm)

c) Immature and mature schizonts

1. Schizonts are collected from the *in vitro* culture of synchronized ring forms (see chapter **9**).
2. Immature schizonts are collected at 16-17hours after the start of the cultures (=20 hpi);
3. Mature schizonts are collected at 21-22 after start of the cultures (=26hpi).
   Fully mature schizonts of P. berghei do not burst spontaneously in vitro in gently shaken culture flasks. Schizonts of P. berghei containing mature merozoites can survive for several hours and can be manipulated without bursting and the loss of viability
   The schizonts are then ready for further manipulation, for example purification of DNA, RNA or protein. Schizont-infected erythrocytes can be separated from the non-infected cells by Nycodenz density-gradient centrifugation. Usually we lyse the red blood cells to get rid of the red blood cells and pellet the schizonts by centrifugation (8min, 2000rpm).

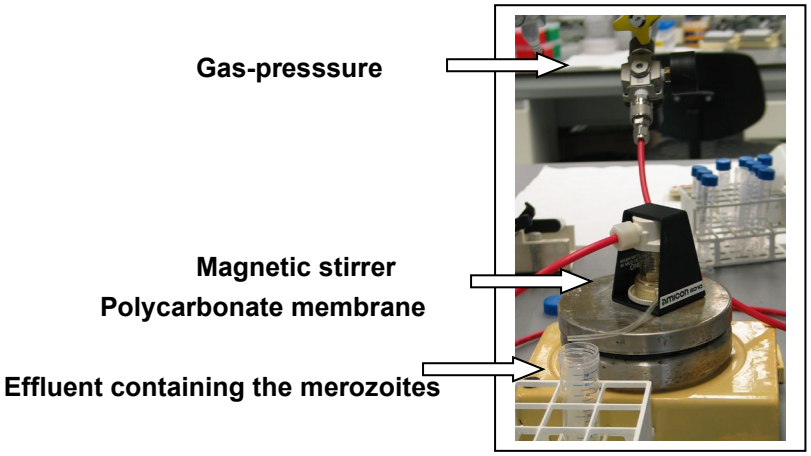
11. Purification of merozoites

Large numbers of free merozoites can easily be obtained since mature schizonts of P. berghei do not burst spontaneously in in vitro cultures. Schizonts can survive for several hours and can be manipulated without bursting and the loss of viability. We perform the purification at room temperature. Collected free merozoites remain viable (=are able to invade red blood cells) for at least 15-30min.

1. Purify cultured mature schizonts using Nycodenz density gradient centrifugation as described in chapter **6**
   It is possible to run a second Nycodenz gradient of 44% (v/v, Nycodenz/PBS) for 15min, 2000rpm using the purified schizonts in order to remove platelets; the purified schizonts will pellet at the bottom of the tube; many platelets will be collected at the interface)
2. Resuspend the purified schizonts in culture medium to a 5-10% cell suspension to a total volume of 10-20 ml
3. Transfer the schizont-suspension to a culture chamber (ultra-filtration cell) loaded at the base with a 1.5 or 2µm pore-size polycarbonate membrane (Nuclepore Corp)

We use an 'ultrafiltration cell' (stirred cell) of Amicon (Amicon 8010) as the culture chamber. This culture chamber contains a spinning bar, which 'vigorously' stirs the schizont-suspension in order to rupture schizonts

4. Pass the suspension through the filter by applying sufficient gas-pressure (N_2)
5. Collect the effluent containing the merozoites
6. Collect the merozoites by centrifugation (2000rpm, 8min or in eppendorf centrifuge: 5s, max. speed) or add the merozoites immediately to uninfected cells for reinvasion studies



12 Purification of gametocytes

a) Gametocytes from synchronized infections

*There are no simple methods to collect large numbers of gametocytes, free from other developmental stages. Gametocytes have the same density as mature trophozoites and schizonts which hampers the purification of gametocytes from asynchronous infections by density gradient centrifugation. Our standard procedure is to collect gametocytes from synchronized infections in rats. Synchronous in vivo infections, established as described in chapter **6**, contain at 26-30hpi (mainly) ring forms and (immature) gametocytes. These two stages can be separated on density gradients. The course of parasite development in synchronous infections is as follows: purified schizonts are intraveneously injected into a rat (=0 hpi). Between 0hpi and 3hpi all schizonts burst and merozoites invade red blood cells resulting in a 1-3% parasitemia of ring forms. Ring forms develop within 22-24h into mature schizonts, which give rise to the second developmental cycle of the parasites. A 'small' percentage of the ring forms do not develop into schizonts but differentiate in 26-30h into mature schizonts. In our reference 'high producer' clone this percentage is 15-25% in synchronized infections in rats. Thus at 24-26hpi ring forms of the second cycle and (immature) gametocytes of the first cycle are present. We usually collect blood at 26hpi for separation of the gametocytes from the uninfected erythrocytes and ring-infected erythrocytes. These are 'immature' gametocytes. To obtain mature gametocytes we incubate the purified, immature gametocytes for a period of six hours at 37ºC under standard in vitro culture conditions. It is at this moment not yet possible to purify younger gametocytes. Up to 16-20hpi the young gametocytes cannot be distinguished from asexual 'old' trophozoites. It is possible to collect and purify gametocytes from the second cycle in synchronized infections. At 48hpi ring forms of the third cycle and 'immature' gametocytes of the second cycle are*

present in the blood of the rat. In addition mature gametocytes of the first cycle are present. Collection and purification of gametocytes at 48hpi result in higher yields of gametocytes (5-10x higher) but less pure preparations.

The procedure described here is for the purification of gametocytes from three rats. We usually collect blood from 3-6 rats. All procedures are performed at 37°C to prevent gametocyte activation. The procedure consists of 1) collection of infected blood, 2) removal of leucocytes, 3) separation of gametocytes from uninfected cells and ring-infected erythrocytes by density gradient centrifugation.

1. Collect heart blood from 3 rats under isofluorane-anaesthesia.
2. Dilute the blood from each rat (5-8ml) in 5-7ml complete culture medium containing 0.3ml heparin-stock solution in a 50ml tubes at 37ºC (in a water bath in the animal room).
3. Pass the blood cell-suspension from each rat through a Plasmodipur-filter to remove leukocytes as described in chapter **8**. Elute with complete culture medium. This step and the following steps are carried out in a 37ºC climate room.
4. Mix the cell suspensions from the three rats and add culture medium to a total volume of 280ml
5. Distribute the cell suspension of schizonts in eight 50ml tubes (35ml per tube)
6. Using a 10ml pipette, gently add 10ml of the Nycodenz-solution in each tube **under** the culture suspension, so that a sharp contrasting division is visible between the two suspensions.
7. Centrifuge 20-25 min at 1500 rpm in a swing out rotor at room temperature (no brake)
8. Collect carefully the 'brown' (grey) layer at the interface between the two suspensions (usually a total volume of about 30-40ml per 4 tubes is collected).
   The gametocytes will collect at the interface of the two suspensions, while the uninfected cells and ring infected cells will pellet on the bottom of the tubes.
9. Pellet the gametocytes by centrifugation (1500 rpm, 8min)
10. Discard the supernatant
    These young gametocytes are then ready for further manipulation, either for further maturation in vitro (see below) or for purification of DNA, RNA or protein.

In vitro maturation of the purified, young gametocytes

1. Resuspend the gametocytes in 10ml of complete culture medium
2. Distribute the cell-suspension in two wells of a six-well plate
3. Put the plate in a Candle Jar type of device at 37°C and connect it to the gas-system using the following gas mixture: 5% CO_2, 5% O_2, 90% N_2
4. Leave the parasites for a period of six hours at 37°C.
   During this period all immature gametocytes will develop into mature gametocytes.

b) Gametocytes from asynchronous infections in mice: 'sulfadiazine method'

A technique for purification of gametocytes from asynchronous infections has been described by Beetsma et al. (Exp. Parasitol. 1998, 88, 69-72). It is based on the selective killing of asexual blood stages by the antimalarial drug sulfadiazine while (mature) gametocytes are unaffected. This method yields relatively pure preparations of fully mature (and degenerated)

gametocytes. We have made some small adaptations to this method to decrease the percentage of degenerated gametocytes. We use these preparations for in vitro ookinete development and we routinely obtain fertilization rates of female gametes of 40-80%.

1. Day 0 (normally Tuesday): inject a mouse i.p. with 0.1ml phenylhydrazine (phz) stock-solution.
   This treatment is performed to induce reticulocytosis in the mice.
2. Day 2 (normally Thursday): infect the phz-treated mouse with approx. 10^7 infected erythrocytes. Infection occurs as follows:
   - Collect 15µl tail blood of an infected mouse with a parasitemia of approx. 5-15% in a heparinized capillary
   - Dilute the infected blood in 0.2 ml of PBS
   - Inject this suspension i.p into a mouse
3. Day 5 (normally Sunday): Start treatment of the mouse with sulfadiazine (in the morning) for a period of three days. Sulfadiazine is dissolved in the drinking water (30mg/l).
   This treatment will kill and remove asexual parasites from the blood while the (mature) gametocytes will be unaffected.
4. Day 7 (normally Tuesday): Check early in the morning (8.30 am) the presence of gametocytes in a Giemsa stained blood film, made from tail blood. If the gametocytemia is >1% (= more then 3 gametocytes per field in a thin blood film at a 1000x magnification) proceed as follows:
5. Collect 0.8-1ml of heart blood from the mouse for the collection of the gametocytes.
   Since gametocytes have a survival time of only 26-30h it is important to collect the gametocytes as early as possible at this day (between 9.00 and 10.00); collection of gametocytes one day earlier is not useful since many (degenerated) blood stages are still present in the blood.
6. Proceed with the infected blood as described below for: 1) gametocyte purification or 2) ookinete cultures

1) Gametocyte purification by Nycodenz-density gradient centrifugation

All procedures are performed at 37°C to prevent activation of gametocytes. The purification can be performed using complete culture medium or PBS.

1. Collect 0.8-1ml of heart blood from a mouse under isofluorane-anaesthesia using an 1ml syringe containing 0.05ml heparin-stock solution
2. Dilute the blood in 5ml of complete culture medium or PBS at 37°C (a water bath in the animal room).
   When more mice are needed the blood of up to 5 mice can be collected in 5ml of culture medium or PBS and heparin can be added to the culture medium or PBS; 0.2-0.3ml stock-solution Heparin for 2-5 mice.
3. Remove leucocytes by passing the blood suspension through a Plasmodipur filter as described in chapter **8**. This procedure is performed at 37°C in a climate room.
4. Add complete culture medium or PBS to a total volume of 70ml
5. Distribute this suspension in two 50ml tubes (35ml per tube).
   If blood is collected from 3-5 mice we add complete culture medium or PBS to a total volume of 140ml that is distributed over 4 tubes

6. Using a 10ml pipette, gently add 10ml of the 49% Nycodenz/PBS-solution (v/v) in each tube **under** the culture suspension, so that a sharp contrasting division is visible between the two suspensions.
7. Centrifuge 15-20 min at 1500 rpm in a swing out rotor at room temperature (no brake)
8. Collect carefully the 'brown' (grey) layer at the interface between the two suspensions (in general a total volume of about 30-40ml is collected from the four tubes).
   The gametocytes (and old trophozoites if present) will collect at the interface of the two suspensions, while the uninfected and ring-infected cells will pellet on the bottom of the tubes.
9. Pellet the gametocytes by centrifugation (1500 rpm, 8 min). For this 'washing' step we add approx. 20ml culture medium, obtained from the top of the gradients.
10. Discard the supernatant

2) Ookinete culture

See chapter 13 for a more detailed description of the in vitro cultivation of ookinetes

1. Collect 0.8-1ml of heart blood from a mouse under isofluorane-anaesthesia using an 1ml syringe containing 0.05ml heparin-stock solution
2. Dilute the infected blood in 5ml of ookinete culture medium at 21°C, pH 8.0 (a water bath in the animal room).
3. Remove the leucocytes by passing the suspension through a Plasmodipur leucocyte filter as described in chapter **8** (all culture medium at a temperature of 21˚C) :
   a. 'Prewash' the filter with 10 ml ookinete culture medium using a 20ml syringe which is placed on top of the filter
   b. Pass the 5ml cell suspension through the filter using a 20 ml syringe
   c. Elute with 15ml ookinete culture medium
   d. Collect and dilute the cells in a total volume of 70-80 ml of ookinete culture medium in a 100ml bottle

   It is important to perform the removal of leucocytes rapidly within 1-1.5 min because the gametocytes are induced to undergo gamete formation after adding the blood to the ookinete culture medium)
4. Distribute the 70-80ml cell suspension in two 200ml flasks and incubate for 16-24h at 21˚C (leave the flasks in a horizontal position)
5. Repeat this procedure for every mouse
6. Take a small sample (20-40µl) after 10-12min of the cell suspension and count the number of exflagellating male gametocytes in a Bürker heamacytometer. *Counting is performed under a light-microscope using a 400x magnification. Exflagellation centres are easily recognized by the actively moving male gametes that are still attached the 'gametocyte-body'*
7. Take a small sample (20-40µl) after 16-24h of culture and count the number of ookinetes in a Bürker heamacytometer.
   Counting is performed under a light-microscope using a 400x magnification. Unstained, free ookinetes and clusters of ookinetes are easily recognized by their banana-shape, moving activity and presence of clusters of pigment.

c) Enriched populations of gametocytes from asynchronous infections in mice treated with phenylhydrazine

We use enriched populations of gametocytes that are obtained from asynchronous infections in mice, which have been pre-treated with phenylhydrazine for the following purposes:
a) To analyse transcription of genes and expression of proteins in gametocytes
b) To analyse transcription of genes and expression of proteins during ookinete development
c) To analyse the rate of gamete formation, fertilization and ookinete formation in vitro
In these infections relatively large numbers of gametocytes are present, mixed with asexual blood stages

Method 1 of infection of mice

1. Inject a mouse on day 0, usually Wednesday with 0.1ml of phenylhydrazine stock solution
2. Collect on day 1, usually Thursday 15µl of tail blood (3 droplets) in 0.2ml PBS of a mouse with a parasitemia of 5-15%
   *We collect this tail blood from a mouse that has been infected the previous week by mechanical passage, either from mouse to mouse on a Thursday or directly from liquid nitrogen storage on Friday; see chapter **3**.*
3. Inject the 0.2ml blood/PBS-suspension i.p. immediately into the phenylhydrazine-treated mouse
4. Proceed with step 8

Method 2 of infection of mice

5. Inject a mouse on day 0, usually Thursday with 0.1ml of phenylhydrazine stock solution
6. Infect this mouse i.p. on day 1, usually Friday, with 0.1ml of a blood-suspension from cryo-preserved parasites (take one cryotube from the liquid nitrogen, thaw at room temperature)
7. Proceed with step 8

Checking gametocytemia and exflagellation

8. Determine the parasitemia and gametocytemia in Giemsa stained blood films from tail blood at day 4 and 5
9. If gametocytemia is >1% (= more then 3 gametocytes per field in a thin blood film at a 1000x magnification) proceed as follows:
10. Count the number of exflagellating male gametocytes as follows
    This check is often performed to select the mouse with the highest number of gametocytes that are able to form gametes
11. Collect 10µl of tail blood in a heparinized capillary-tube
12. Dilute the blood in 1ml of ookinete culture medium at 21˚C
13. Take a small sample (20-40µl) after 10-12 min. of culture and count the number of exflagellating male gametocytes in a Bürker heamacytometer.
    Counting is performed under a light-microscope using a 400x magnification. Exflagellation centres are easily recognized by the actively moving male gametes that are still attached the 'gametocyte-body'
14. Proceed as follows:

a) Collecting blood for collecting enriched populations of gametocytes

All procedures are performed at 37°C to prevent activation of gametocytes.

1. Collect 0.8-1ml of heart blood from a mouse under isofluorane-anaesthesia using an 1ml syringe containing 0.05ml heparin-stocksolution
2. Dilute the infected blood in 5ml PBS at 37°C (a water bath in the animal room).

When more mice are needed the blood of up to 5 mice can be collected in 5ml of PBS, heparin can be added directly to the PBS; 0.2-0.3ml stock-solution for 2-5 mice.

3. Remove leucocytes by passing the blood suspension through a Plasmodipur filter as described in chapter **8**. This procedure is performed at 37°C in a climate room
4. Add PBS to a total volume of 70ml
5. Distribute the suspension in two 50ml tubes (35ml per tube).
   If blood is collected from 3-5 mice we add PBS to a total volume of 140ml that is distributed over 4 tubes
6. Using a 10ml pipette, gently add 10ml of the Nycodenz-solution in each tube **under** the culture suspension, so that a sharp contrasting division is visible between the two suspensions.
7. Centrifuge 20-30 min at 1500 rpm in a swing out rotor at room temperature (no brake)
8. Collect carefully the 'brown' (grey) layer at the interface between the two suspensions (in general a total volume of about 30-40 ml is collected from the four tubes). *The gametocytes (and old trophozoites if present) will collect at the interface of the two suspensions, while the uninfected and ring-infected cells will pellet on the bottom of the tubes.*
9. Pellet the gametocytes/trophozoites by centrifugation (1500 rpm, 8 min). For this 'washing' step we add approx. 20ml PBS
10. Discard the supernatant

b) Collection of blood for a 'large-scale' ookinete culture

(For analysing gene transcription, protein expression)

1. Collect 0.8-1ml of heart blood from a mouse under isofluorane-anaesthesia using an 1ml syringe containing 0.05ml heparin-stock solution
2. Dilute the infected blood in 5ml of ookinete culture medium at 21°C, pH 8.0 (a water bath in the animal room).
3. Remove the leucocytes by passing the suspension through a Plasmodipur leucocyte filter as described in chapter **8** (all culture medium at a temperature of 21˚C):
   a. 'Prewash' the filter with 10 ml ookinete culture medium using a 20ml syringe which is placed on top of the filter
   b. Pass the 5ml cell suspension through the filter using a 20 ml syringe
   c. Elute with 15ml ookinete culture medium
   d. Collect and dilute the cells in a total volume of 70-80 ml of ookinete culture medium in a 100ml bottle

   It is important to perform the removal of leucocytes rapidly within 1-1.5 min because the gametocytes are induced to undergo gamete formation after suspension in ookinete culture medium.
4. Distribute the 70-80ml suspension in two 200 ml flasks and incubate for 16-24h at 21˚C (leave the flasks in a horizontal position)
5. Repeat this procedure for every mouse
6. Take a small sample (20-40µl) after 10-12 min. of the cell suspension and count the number of exflagellating male gametocytes in a Bürker heamacytometer. *Counting is performed under a light-microscope using a 400x magnification. Exflagellation*

centres are easily recognized by the actively moving male gametes that are still attached the 'gametocyte-body'

7. Take a small sample (20-40µl) after 16-24h of culture and count the numbers of ookinetes in a Bürker heamacytometer.
   Counting is performed under a light-microscope using a 400x magnification. Unstained, free ookinetes and clusters of ookinetes centres are easily recognized by their banana-shape, moving activity and presence of clusters of pigment.
8. Pellet the cells by centrifugation (1500rpm, 8 min)
9. Discard the supernatant

c) Collection of blood for 'small scale' cultures

(For analysing the rate of gamete formation, fertilization and ookinete formation)

1. Collect 10µl of tail blood in a heparinized capillary-tube
2. Dilute the blood in 1ml of ookinete culture medium at 21˚C in an eppendorf tube.
3. Take a small sample (20-40µl) after 10-12 min. of culture and count the number of exflagellating male gametocytes in a Bürker heamacytometer.
   Counting is performed under a light-microscope using a 400x magnification. Exflagellation centres are easily recognized by the actively moving male gametes that are still attached the the 'gametocyte-body'
4. Take a small sample (20-40µl) after 16-24h of culture and count the numbers of ookinetes in a Bürker heamacytometer.
   Counting is performed under a light-microscope using a 400x magnification. Unstained, free ookinetes and clusters of ookinetes centers are easily recognized by their banana-shape, moving activity and presence of clusters of pigment

13 *In vitro* cultivation of ookinetes

Large numbers of P. berghei ookinetes can be produced in relatively simple culture systems. Cultured ookinetes can be used to infect mosquitoes via membrane feeding or used for molecular/biochemical analysis. In vitro production of ookinetes can be used to determine the functional maturity of gametocytes, to analyse phenotypic effects on fertilization and zygote development resulting from disruption of genes and to test inhibitory compounds.

Different sources of gametocytes have been used in our laboratory for the culture of ookinetes:

a) *Purified gametocytes from synchronized infections (see 12a). These gametocyte preparations are not used on a regular basis because the method of gametocyte purification is laborious.*
b) *Purified gametocytes obtained by the 'sulfadiazine-method' described by Beetsma et al. (1998) (see chapter **12**). Using these gametocytes relatively pure preparations of ookinetes/unfertilised gametes can be obtained that are free from asexual blood stages (a contamination of about 10% asexual blood stages). We regularly obtain a 50-90% conversion rate of female gametocytes into ookinetes in culture.*
c) *Infected blood from mice with asynchronous infections. These mice have been treated with phenylhydrazine and contain a high number of gametocytes (see chapter **12**). In our laboratory this is the most frequently used source for ookinete cultures. In these cultures high numbers of ookinetes can be produced, which are mixed with high numbers of asexual blood stages (in erythrocytes).*

No simple procedures are available for separation of zygotes/ookinetes from the asexual blood stages. In our hands density gradient centrifugation was not successful and flow cytometric separation was hampered by the formation of clusters of ookinetes/unfertilised gametes in vitro.
Other groups have used mAbs against an ookinete surface protein (Pbs21, mAb 13.1) coupled to magnetic beads to separate ookinetes from the asexual blood stages (personal communication J. Thompson, Edinburgh). With this method ookinetes are purified together with unfertilised female gametes, because these latter stages also express Pbs21 on their surface. Other groups have used magnetic isolation procedures (Carter et al. (2003) Isolation of Plasmodium berghei ookinetes in culture using Nycodenz density gradient columns and magnetic isolation. Malaria J. 2(1), 35)
Conversion rates of female gametocytes into ookinetes range between 20-90%, partly depending on the maturity/quality of the gametocytes. A high proportion of immature or older, degenerating gametocytes results in lower conversion rates. In our hands, by selecting preparations with a high proportion of mature gametocytes, conversion rates range between 40-90%.

See chapter 12 for more detailed protocols for cultivation ookinetes using gametocytes from different sources

1. Incubate infected blood or purified gametocyte preparations that are kept at 37°C immediately in ookinete culture medium (RPMI1640, pH 8.0) at 21-22°C at a final cell suspension of 1-2% (v/v; for example 1ml of blood in 80-100ml medium).
2. For some purposes removal of leucocytes is desired. To remove leucocytes from 1-5ml of infected blood, we resuspend the blood in 5ml of ookinete culture medium and pass this suspension as quickly as possible through a Plasmodipur leucocyte filter and collect the cells in ookinete culture medium.
3. The filter is pre-washed and eluted with ookinete culture medium at a temperature of 21°C (see chapter **12** for more details on leucocyte removal from infected blood using Plasmodipur filters).
4. The cell suspension is transferred to either 24 well or 6 well plates or to culture flasks (50-100ml flasks) in such a way that the medium depth is no greater than 5mm *(whether this low medium depth is essential for optimal ookinete production is not clear; we also obtain high gametocyte-ookinete conversion rates when we incubate 10µl of infected blood in 1ml of ookinete culture medium in eppendorf tubes).*
5. The plates/flasks are stored in air for a period of 16-24 hours at 21-22°C.
6. We usually count the number of exflagellations in culture (a sample of 20-40µl) after 10-12min using a simple cell-counter under a light microscope (400x magnification).
7. After 16-24h we count the number of mature ookinetes in the cell-counter under a light microscope (400x magnification) for calculation of the gametocyte-ookinete conversion rate (see below).
   Unstained, live ookinetes (mature and retort forms) are easily recognized under the light microscope at a 400x magnification.
8. We try to perform the procedures of collecting and incubation of gametocytes as quickly as possible and prefer to avoid changes in the temperature other than the rapid drop from 37°C to 21°C. *In our hands temperature changes, for example short periods of storage of gametocytes at 4°C, results in 'pre-activation' of gametocytes to*

undergo gametogenesis that will result in lower gametocyte-ookinete conversion rates.

Calculation of gametocyte conversion rate, gamete conversion rate, ookinete conversion rate

To determine the capacity of gametocyte production of cloned lines we measure the gametocyte conversion rate. For determination of maturity and fertility of gametocytes and gametes, respectively, we measure the gamete and ookinete conversion rates. The following methods are used for counting gametocytes and ookinetes:

1. *Counting gametocytes in Giemsa stained blood films using a light microscope at a 1000x magnification*
2. *Counting exflagellating male gametocytes in live preparations in a Bürker cell counter using a light microscope at a 400x magnification. Exflagellation is induced by adding infected blood to ookinete culture medium.*
3. *Counting ookinetes in live preparations in a Bürker cell counter using a light microscope at a 400x magnification. Ookinetes are counted 16-24 hour after adding infected blood to ookinete culture medium. Unstained, live ookinetes (mature and retort forms) are easily recognized under the light microscope at a 40x magnification (see also chapter 5 for pictures of live ookinetes).*
4. *Counting ookinetes and unfertilised female gametes in live preparations after staining with monoclonal mAb13.1, that is specific for the surface protein Pbs21. This protein is present on female gametes, zygotes and ookinetes. Live ookinetes (mature and retort forms) and gametes are counted using a fluorescence microscope at a 400x magnification.*

Gametocyte conversion rate

*The gametocyte conversion rate is defined as the percentage of blood stage ring forms that develop in synchronized infections under standardized conditions into gametocytes (see chapter **6** and **12a** for the characteristics of synchronous infections).*

1. Thin blood films are made from cells from synchronized infections at 4 hours and 30h after injection of schizonts and stained with Giemsa.
2. The parasitemia (=percentage of ring-infected cells) is determined by counting a total of 100-200 ring forms at 4h.
3. At 30h the gametocytemia (=percentage of gametocyte infected cells) is determined by counting of a total of 30-100 gametocytes.

Gamete conversion rate

The conversion of male gametocytes into male gametes (the process of exflagellation) is defined as the gamete conversion rate and is a measure for the functional maturity of the gametocytes.

1. The number of male gametocytes is determined by counting 30-70 males in Giemsa stained thin blood films.
2. The number of exflagellating males is determined by counting the number of exflagellations in a cell-counter, 10-12min after *in vitro* incubation of infected blood in ookinete culture medium.

3. The male gamete conversion rate is the number of exflagellations per 10^5 erythrocytes divided by the number of male gametocytes per 10^5 erythrocytes and multiplied by 100.

It is also possible to determine the conversion rate of female gametocytes into gametes. Because both unfertilised female gametes and zygotes express the surface protein Pbs21 on their surface whereas female gametocytes do not, it is possible to determine the percentage of female gametocytes that developed into gametes by staining gametes/zygotes with monoclonal antibody (mAb13.1) that is specific for Pbs21.

1. The number of female gametocytes is determined by counting 30-70 females in Giemsa stained thin blood films.
2. The number of gametes/zygotes is determined by counting the number of Pbs21-positive gametes/zygotes/ookinetes in a cell counter, 10-22h after *in vitro* incubation of infected blood in ookinete culture medium.
3. The female gamete conversion rate is the number of Pbs21 positive gametes/zygotes per 10^5 erythrocytes divided by the number of female gametocytes per 10^5 erythrocytes and multiplied by 100.

Ookinete conversion rate

The conversion of female gametocytes/gametes into ookinetes is defined as the ookinete conversion rate.

1. The number of female gametocytes is determined by counting 30-70 females in Giemsa stained thin blood films.
2. The number of ookinetes is determined by counting the number of live ookinetes in a cell counter, 10-22h after *in vitro* incubation of infected blood in ookinete culture medium.

Another, simple, way to determine the conversion rate of female gametes into ookinetes is to count all Pbs21-positive cells after staining with mAb13.1 (ookinetes and unfertilised female gametes stain with the Pbs21 specific mAb13.1) in a cell counter, 16-22h after in vitro incubation of infected blood in ookinete culture medium. The ookinete conversion rate is the percentage of ookinetes (mature and retort forms) of the total number of Pbs21-positive cells (ookinetes and the unfertilised gametes).

14. *In vitro* cross-fertilization assays: testing fertility of male and female gametes

We have used in vitro cross-fertilization between different clones of P. berghei for testing the fertility of either male or female gametes of parasite clones that were able to produce gametes but were unable to form zygotes (van Dijk et al. 2001, Cell, 104, 153-64). To determine female and/or male fertility we use the following approaches.

- We use the inhibitor aphidicolin to specifically block male gamete formation in the clone from which we want to determine the fertility of female gametes (clone A). Aphidicolin is added to the ookinete culture medium during the first twelve minutes after incubation of the gametocytes in ookinete culture medium (during the process of exflagellation. Aphidicolin is an inhibitor of polymerase-α and specifically blocks the formation of male gametes while female gamete formation is unaffected by aphidicolin (Janse et al., 1986, Mol. Biochem. Parasitol. 20, 173-182). Aphidicolin is removed from the culture medium after 12min by one wash step.

- We use a mutant clone defective in expression of the gamete/ookinete surface protein Pbs21 (clone B) (Tomas, et al. (2001) EMBO J. 20, 3975-3983; van Dijk et al. 2001, Cell, 104, 153-64). The female gametes of clone A and the male/female gametes of clone B are mixed and 16-24h later the number of Pbs21-positive female gametes and ookinetes (resulting from cross-fertilization of female gametes of clone A by male gametes of clone B) and the number of Pbs21-negative ookinetes (resulting from self-fertilisation of clone B) are counted. The ookinete conversion rate of clone A is a measurement for the fertility of female gametes of clone A.
- We use in crossing experiments two defined clones that are defective in either male or female fertility. Parasite clones in which the Pb48/45 gene is disrupted are defective in male fertility wheras the females are fertile. Parasite clones in which the Pb47 is disrupted are defective in female fertility, whereas the males are fertile
- We use the ookinete conversion rate (see chapter **13**) as a measure for the fertility of either male or female gametes

An example of the protocol for cross-fertilization between gametes of two clones (A and B) in which the inhibitor aphidicolin is used is described below. In this example clone B is the parasite mutant line that lacks the expression of the surface protein Pbs21 that is normally present on female gametes and ookinetes.

Method 1 of infection of mice

1. Inject 4 mice on day 0, usually Wednesday, with 0.1ml of phenylhydrazine (phz) stock solution
2. Collect on day 1, usually Thursday 30µl of tail blood (6 droplets) in 0.4ml PBS of a mouse with a parasitemia of 5-15% infected with clone A and
3. Collect on the same day 30µl of tail blood (6 droplets) in 0.4ml PBS of a mouse with a parasitemia of 5-15% infected with clone A
   *We collect this tail blood from mice that have been infected the previous week by mechanical passage, either from mouse to mouse on a Thursday or directly from liquid nitrogen storage on Friday; see chapter **3**)*
4. Inject i.p. 0.4ml blood/PBS-suspension of clone A immediately into 2 phz-treated mice (0.2ml per mouse) and
5. Inject i.p. the 0.4ml blood/PBS-suspension of clone B immediately into 2 phz-treated mice (0.2ml per mouse).
6. Proceed with step 8

Method 2 of infection of mice

7. Inject 4 mice on day 0, usually Thursday with 0.1ml of phenylhydrazine (phz) stock solution
8. Infect on day 1, usually Friday, two of the phz treated mice i.p. with 0.1ml of a blood-suspension from cryo-preserved parasites of clone A, and
9. Infect on day 1, usually Friday, two of these mice i.p. with 0.1ml of a blood-suspension from cryo-preserved parasites of clone B
   Take one cryotube from the liquid nitrogen and thaw at room temperature
10. Proceed with step 8

Checking gametocytemia and exflagellation

11. Check the parasitemia and gametocytemia in Giemsa stained blood films at day 4 and 5
12. If gametocytemia is >1% (= more then 3 gametocytes per field in a thin blood film at a 1000x magnification) proceed as follows:
13. Count the number of exflagellating male gametocytes as follows:
    (this check is often performed to select the mouse with the highest number of gametocytes that are able to form gametes)
    - Collect 10µl of tail blood in a heparinized capillary-tube
    - Dilute the blood in 1 ml of ookinete culture medium at 21˚C
    - Take a small sample (20-40µl) after 10-12 min. of the cell suspension and count the number of exflagellating male gametocytes in a Bürker heamacytometer. *Counting is performed under a light-microscope using a 400x magnification. Exflagellation centres are easily recognized by the actively moving male gametes that are still attached the 'gametocyte-body'*
14. Proceed as follows with those mice from clone A and B that have comparable numbers of exflagellation

Cross-fertilization between clone A and B and ookinete formation

15. Collect 5µl of tail blood from a mouse infected with clone A in a heparinized capillary-tube
16. Dilute the infected blood immediately into 1ml of ookinete culture medium at 21°C, in an eppendorf tube to induce gamete formation. This medium contains aphidicolin at a concentration of $5x10^{-4}$M.
    Male gamete formation is blocked by aphidicolin whereas female gametes are produced normally.
17. Collect at the same time 5µl of tail blood from a mouse infected with clone B in a heparinized capillary-tube
18. Dilute this blood immediately into 1ml of ookinete culture medium (pH 8.0) at 21°C without aphidicolin in an eppendorf tube to induce gamete formation.
19. Pellet cells of clone A after 12min for 5s at 10,000 rpm in an eppendorf centrifuge.
20. Discard the supernatant carefully
21. Add the 1ml of cell suspension containing clone B to the pellet of clone A and resuspend the cells of clone A.
    This result in the mixing of the female gametes of clone A with the male and female gametes of clone B at 12-14min after induction of gamete formation.
22. Count 16-18 hours after mixing the parasites, the number of Pbs21 positive female gametes and Pbs21 positive and negative ookinetes in a Bürker heamacytometer after staining the parasites with FITC-labelled Pbs21 specific mAb13.1.
23. The ookinete conversion rate (see chapter **13**) is determined from:
    a) The female gametes of clone A, fertilized by males of clone B (Pbs21 positive ookinetes)
    b) The female gametes of clone B, fertilized by males of clone B (Pbs21 negative ookinetes)

Cloning of blood stage parasites

Parasites are cloned using the method of limiting dilution of blood stage parasites and injection of single parasites into laboratory animals. The method described here is adapted to

our specific laboratory conditions and it is possible that other laboratories have to make small changes in order to get reliable results with the cloning procedure. For example we standard inject a calculated 2 parasites per mouse intra-venously into 10-20 mice, resulting in an infection rate of 20-50% of the mice. This means that either not all parasites survive the cloning procedure or that we (in a reproducible way) calculate a too high number of parasites and thus instead of injecting two parasites we inject in fact only 0.2-0.5 parasites per mouse. We consider a cloning experiment successful if less then 50% of the mice become positive.

Method 1 of infection of mice

1. Collect one droplet of tail blood (5µl) from an infected animal with a parasitemia of 5-15% in 10ml PBS (= day 0, usually on Thursday)
2. Inject 0.1ml of the suspension i.p. into two mice
3. Check the parasitemia on day 4 (Monday) and 5 (Tuesday) after infection in Giemsa stained blood films
4. Proceed with step 8

Method 2 of infection of mice

5. Infect two mice i.p. with 0.01ml of a blood-suspension from cryo-preserved parasites (=day 0, usually on Friday)
   Take one cryotube from the liquid nitrogen, thaw at room temperature
6. Check the parasitemia on day 3 (Monday) and 4 (Tuesday) after infection in Giemsa stained blood films
7. Proceed with step 8

Limiting dilution of blood stages and infection of mice with a single parasite

8. Start the cloning procedure within 1-2 hours after preparation of the Giemsa stained slides.
   If one waits longer there is a change that the parasitemia increases as a result of invasion of new parasites. A higher parasitemia in the starting material will of course affect the cloning procedure.
9. Use a mouse with a parasitemia between 0.3 and 1%, with a minimum of multiple infected erythrocytes
10. Collect 5 µl of infected tail blood in a heparinized capillary tube
11. Dilute the blood in 1ml of complete culture medium in an eppendorf tube
12. Take a small sample (20µl) and count the red blood cell density in a Bürker cell counter (usually 12-18 erythrocytes per small square: (16x144x no of rbc)/0.576 = number of erythroctes/ul of blood)
13. Calculate the number of erythrocytes and the number of parasites (infected erythrocytes) per µl of the cell suspension
14. Dilute the suspension with culture medium to a final concentration of 2 parasites/ 0.2ml culture medium
15. Inject from this suspension 0.2ml/mouse intravenously (tail veins) into 10-20 mice. *The mice are placed at 37°C 10-20min before injection of the parasites. The mice veins swell at this temperature, simplifying the i.v. injection procedure (swollen veins).*
16. Check the parasitemia of these mice in Giemsa stained blood films at day 8 after infection. In a successful experiment 20-50% of the mice become positive and will show a parasitemia of 0.3-1% at day 8.

16 Preparation of (purified) parasites for isolation of DNA, RNA and protein: lysis of red blood cells

For isolation of DNA, RNA or protein from the parasites, we usually first lyse the erythrocytes in order to remove the (uninfected) red blood cells. In the lysis buffer erythrocytes lyse whereas parasites, leucocytes and platelets remain 'intact'. We usually do not use this lysis procedure for Nycodenz-purified populations of parasites/infected erythrocytes.

1. Resuspend pelleted erythrocytes in cold (4°C) erythrocyte lysis buffer:
   Cells from up to 1ml of blood: use 10ml of buffer
   Cells from 1-5ml of blood: use 50ml of buffer
2. Leave this suspension on ice for 3-5min (the clarity of the suspension is an indicator of the state of the lysis procedure i.e. when fully clear lysis is complete)
3. Pellet the parasites by centrifugation (8min, 2000rpm)

17 Additional Protocols

Purification of *Plasmodium* blood stages with the VARIOMACS (magnetic field)
Purification is based upon retention of cells, containing iron-based pigments, in a strong magnetic field. It can be used for separation of gametocytes from ring-infected and uninfected erythrocytes or for separation of trophozoites and schizonts from ring-infected and uninfected erythrocytes and from leucocytes and platelets. In our laboratory we have only very limited experience with the VARIMACS and we did only some preliminary experiments for the purification of schizonts from uninfected cells, leucocytes and platelets

1. The system can be operated either in the 37°C climate room or in the ML-II lab at room temperature.

2. Defrost the appropriate amount of culture medium (and place in the 37°C climate room).
   Per purification, 75 ml of culture medium is needed. Serum components have no effect on the purification and will contribute to the condition and viability of the cells. Including serum is especially recommended when the purified cell are to be used to set up new cultures.

3. Spin down your culture or blood sample for 8 minutes at 1500 rpm (in the 37°C room).
   Per mouse, 1 ml of blood can be extracted by hart puncture. Up to 3 ml of blood (~10^{10} erythrocytes) can be loaded on the column.

4. Culture pellets are resuspended in 3 ml of there own supernatant. After spinning down blood samples, the serum is discarded and the erythrocytes resuspended in 3 ml of clean culture medium (keep the samples at 37°C). Prepare a smear.

5. Fill the column (MACS column CS, Miltenyi Biotec) **from below** with clean culture medium. Pump speed 89%
   Make sure the distance between the top needle in the column and the white column filter is as big as possible. When the sample is applied to the column, some of the cells will pass the white filter. When there is a large distance between the filter and the needle, a volume is created big enough to allow sedimentation of those cells. If the needle is close to the filter, passing cells will be sucked up and discarded.
   At this stage, air bubbles in the tubes can be ignored. They will disappear when they reach the column.

6. Allow 10 ml of culture medium to pass the column from below.

7. Stop the pump and reset the speed at 49%. Place the intake needle in the sample and switch on the pump (**the sample is loaded from below**)
   Allow some of the sample to be retained in order to prevent formation of air bubbles.

8. Stop the pump and place the intake needle in clean culture medium. Reset the pump speed at 39%.

9. Continue pumping until the sample is in the column.
   Some of the sample may pass the white filter on top of the column but that's nothing to worry about (see step 5)

10. Stop the pump and place the column inside the magnet (Vario MACS, Miltenyi Biotec)

11. **Change the pump direction** and place the former outlet needle in clean culture medium. Start the pump and collect 10 ml of flow through. Stop the pump.
    This first fraction contains uninfected and parasite infected erythrocytes. Of the infected cells, 90% contain ring stage parasites. Spin down 100 µl and use the pellet to prepare a smear.

12. Start the pump and collect 20 ml of flow trough. Stop the pump.

This second fraction is not so important. Its purpose is to clear the sample from erythrocytes. Spin down 200 µl and use the pellet to prepare a smear.

13. Remove the column from the magnet and place it in a clamp.

14. Start the pump and collect 10 ml of flow through. Stop the pump.
    This third fraction contains mainly trophozoites, schizonts, and gametocytes (if present in your sample) Spin down 500 µl and use the pellet to prepare a smear.

15. Spin down the first and third fraction for 8 minutes at 2000 rpm (in the 37°C climate room).

16. Suspend the pellets in 1 ml of the supernatant.

17. Transfer the cells into a 1.5 ml Eppendorf tube.

18. Spin down the cells and discard the supernatant.

19. Store the pellets at -80°C.

20. Reset the pump speed at 99% and drain the system by air.

21. **Change the direction of the pump** and fill the system with deionised water. Allow 20 ml to run through.

22. **Change the direction of the pump** and drain the system by air.

23. **Change the direction of the pump** and fill the system with ethanol absolute. Allow 20 ml to run through.

24. **Change the direction of the pump** and drain the system by air.

25. Take the column and blow-dry it from each side using an air outlet.