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Preface

This is the fourth edition of *Methods in Malaria Research*. The first two were our “in-house” collections of protocols that we have found very useful in the lab. The fourth edition is a revised and expanded version of the third, which was produced with the help of the MR4 of ATCC, who also distributed the book online globally (www.malaria.mr4.org/publication.html). We hope you find our collection of malaria protocols useful.

You will find some innovations in this edition such as new chapters (animal models, mosquitoes), ten new sections, including more than 30 new protocols. New headings are found in each protocol such as Equipment and Materials and reagents. A list of contributors and the names and addresses of the authors of the various protocols will be of help if you have questions or comments about the methods.

In assembling this book, we have tried to maintain a broad angle on what protocols to include that are relevant to malaria research, be it culturing of parasites, immunological assays, or molecular biological techniques. One criterion we have set is that the methods be well-tried by those sending us protocols. We have intended to give enough detail in the methodology that someone who is not an expert in the field of malaria will still be able to make use of these protocols. Naturally we hope those of you who are expert malariologists will also find this book beneficial and interesting.

We acknowledge the work of Jane Edwards, Ramona McClatchey, and Fran Bass at ATCC for copyediting the text, writing the index, and compiling the lists of abbreviations and suppliers.

We consider this book a “working document” of malaria protocols that, with the help of our readers/users, will continuously grow and evolve as new and improved methods are communicated to us. If you find mistakes or have a method you think is suitable for the next edition, please write to:

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>2-ME</td>
<td>2-mercaptoethanol (β-mercaptoethanol)</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection, Manassas, VA, USA</td>
</tr>
<tr>
<td>BrB</td>
<td>breaking buffer</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>Chloramphenicol acetyltransferase</td>
</tr>
<tr>
<td>CGMRC</td>
<td>Centre for Geographic Medicine Research Coast</td>
</tr>
<tr>
<td>CHEF</td>
<td>clamped homogeneous electric field</td>
</tr>
<tr>
<td>CMCM</td>
<td>complete malaria culture medium</td>
</tr>
<tr>
<td>CPD</td>
<td>citrate phosphate dextrose</td>
</tr>
<tr>
<td>cpm</td>
<td>count per minute</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>edetic acid; ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(β-aminooethyl ether)-N,N,N′,N′-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EMEAS</td>
<td>erythrocyte membrane staining by enzyme linked antibodies</td>
</tr>
<tr>
<td>EMIF</td>
<td>erythrocyte membrane immunofluorescence</td>
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<td>FBS</td>
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<td>LB</td>
<td>Luria broth</td>
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<tr>
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<td>red blood cell(s)</td>
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<td>ring-stage erythrocyte surface antigen</td>
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<td>reverse transcription polymerase chain reaction</td>
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<td>sodium dodecyl sulfate</td>
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<td>Tris/acetate (buffer)</td>
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<td>transmission blocking assay</td>
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<td>Tris</td>
<td>tris(hydroxymethyl)amino methane</td>
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<td>TSE</td>
<td>Tris/sodium chloride/EDTA solution</td>
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<td>USP</td>
<td>United States Pharmacopeia</td>
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<td>WB</td>
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<td>WBC</td>
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<td>X-gal</td>
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PARASITES

I. Culturing of erythrocytic asexual stages of *Plasmodium falciparum* and *P. vivax*

I:A. The candle-jar technique of Trager–Jensen

by Malin Haeggström¹, Martha Schlichtherle¹, and Ahmed Bolad²

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Equipment

- incubator (37 °C)
- glass desiccator (e.g., candle jar)
- cell culture flask
- candles
- centrifuge
- sterile pipettes
- sterile tubes
- glass slides and coverslips
- microscope, fluorescence or light

Materials and reagents

- purified erythrocytes (or human blood type O⁺ in CPD-adenine (Terumo) or S.A.G.M. (“Sagman” solution or EDTA)
- MCM (see below)
- Tris (Sigma)
- Albumax II (Gibco)
- RPMI 1640 (Gibco)
- gentamicin
- 1 M HEPES (Gibco)
- Hanks’ balanced salt solution (Gibco)
- acridine orange (10 µg/mL) or Giemsa 5%

optional:
- human serum
- glucose
- hypoxanthine (Sigma)
- Tris-buffered Hanks’ (TH)

Preparations

Prepare malaria culture medium (MCM) and Tris-buffered Hanks’ (TH) for washing cells.

*Albumax complete medium:*

10.43 g RPMI 1640 powder (Gibco)
25 mL 1 M HEPES solution or 6 g HEPES (Gibco)
2 g NaHCO₃
0.5 mL gentamicin (from 50 mg/mL stock)
5 g Albumax II
Add distilled water to 1 liter. Filter-sterilize.
Use within 10 days, store at −20 °C.

Comment: For growing parasites from patient blood, use 10 g of Albumax for 1 liter of complete MCM. The vast majority of cultures will survive at least 2 weeks. It is also important to avoid serum in the culture for preparation of crude parasite antigen (see SEROLOGY, section III:B). Not all strains can be adopted to Albumax II medium.

**Alternative MCM:**
- 10.43 g RPMI 1640 powder (Gibco)
- 25 mL 1M HEPES or 6 g HEPES
- 2 g NaHCO₃
- 0.5 mL gentamicin (from 50 mg/mL stock)
Add distilled water to 1 liter. Filter-sterilize and store at −20 °C in 45-mL aliquots.

For complete MCM (cMCM), add 5 mL of human blood type AB⁺ serum (inactivated at 56 °C for 60 min; then stored at −20 °C) to 45 mL of medium. Complete MCM can be used for up to one week if stored at 4 °C.

MCM can also be made from commercial liquid RPMI with sodium bicarbonate and HEPES buffer (Gibco). Just add 5 mL of 100× L-glutamine (Gibco) and 0.25 mL gentamicin (Gibco) to a 500 mL bottle of the RPMI.

**TH (0.15 M Tris-buffered Hanks’)** *(pH 7.2):*
- 2.11 g Tris–HCl
- 0.2 g Tris-base
- 7.88 g NaCl
Dissolve in distilled water and bring volume to 1 liter.
Mix 1 volume of Tris buffer with 1 volume of Hanks’.

**In vitro cultures in tissue-culture flasks**
- Wash the erythrocytes 3 times in TH or RPMI 1640 to remove CPD, serum, and leukocytes if present. Dilute to 5% hematocrit with cMCM in small flasks of 25 cm² (0.2 mL of packed cells to 4 mL of cMCM) or in 75-cm² flasks (1.0 mL to 20 mL).
- Add parasites to an appropriate parasitemia (see below).
- Put the flask in a candle jar and loosen the screw cap. Produce low oxygen by burnt out candle and place the jar at 37 °C.
- Replace the MCM every day (not necessary the day after subcultivation).
- Subculture the cultures 2 times/week.

**Subcultivation**
- Stain a drop of the culture with acridine orange (10 µg /mL) on a glass slide and put on a coverslip or by Giemsa staining of a thin smear (see PARASITES, section III:A or B).
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- Count the parasitemia (i.e., the percentage of infected cells, see PARASITES, section III:C).
- Prepare freshly washed O⁺ blood in cMCM (5% hematocrit) and add it to the culture to obtain a parasitemia of not more than 1%, preferably 0.1 to 0.5% if two cycles until next subculturing, 0.5 to 1% if one cycle. Parasitemia should never exceed 15%.

References

I:B. Establishment of long-term in vitro cultures of \textit{Plasmodium falciparum} from patient blood  
by Morten A. Nielsen and Trine Staalsoe  
Centre for Medical Parasitology, Department of Infectious Diseases M7641, Rigshospitalet, Copenhagen, Denmark  
e-mail: mncmp@rh.dk

Careful adherence to the procedures described below should allow a very high success rate with immediate culture (provided parasites are not already damaged by antimalarial drugs), and even when using cryopreserved parasite stabilates, a success rate of >70% should be within reach.

**Equipment**
- centrifuge
- incubator

**Materials and reagents**
- culture medium:  
  - 500 mL RPMI 1640 (Gibco)  
  - 25 mg gentamycin (Gibco)  
  - 91.6 mg L-glutamine (Sigma)  
  - 2.5 g Albumax II (Invitrogen) (See comments below.)  
  - 10 mL normal human serum  
  - 10 mg hypoxanthine (Sigma) (See comments below.)
- washing medium:  
  - 500 mL RPMI 1640  
  - 25 mg gentamycin (Gibco)
- freezing solution:  
  - sterile, distilled water  
  - 3.0% sorbitol (Sigma)  
  - 28.0% glycerol (Sigma)  
  - 0.65% NaCl
- thawing solution:  
  - sterile, distilled water  
  - 3.5% NaCl
- Giemsa stain:  
  - 10% Giemsa in phosphate buffer
- gas mixture:  
  - 2% O\textsubscript{2}, 5.5% CO\textsubscript{2}, 92.5% N\textsubscript{2} (Special gas mixtures such as this one can be bought from suppliers of compressed gases.)
- red blood cells (RBC) for subcultivation:  
  - Blood type O Rh\textsuperscript{-} or O Rh\textsuperscript{+} RBC in CPD buffer, washed 3 times in washing medium to remove the Buffy coat (See comments below.)

**Step-by-step manipulations**
- Collect heparin or CPD anticoagulated venous blood from patient. Do not use EDTA blood, which does not support parasite survival. The collected blood can be used for cultivation immediately (“straight out of the arm”) or after cryopreservation.
Preparation for immediate cultivation ("straight out of the arm") (optional)

- Wash the anticoagulated blood sample 3 times in prewarmed (37 °C) washing medium (centrifuged for 8 min at 600 × g). Remove buffy coat after each centrifugation.
- Continue as described under Cultivation of fresh patient isolates below.

Cryopreservation (optional—Do only if immediate culture is not possible or desirable.)

- Spin the anticoagulated blood sample (centrifuged for 8 min at 600 × g) and remove plasma.
- Mix equal amounts of ring-stage parasitized packed RBC and freezing solution (see comments below).
- Distribute the mixture immediately into 1.8-mL screw-cap tubes and snap-freeze in liquid nitrogen.
- Store the tubes in liquid nitrogen until use.
- On day of use, thaw the RBC suspension in a 37 °C-water bath.
- Spin the tube (8 min at 600 × g) immediately after thawing (see comments below).
- Discard the supernatant, add an equal amount of thawing solution, mix well, and incubate the cells for 1 min (see comments below).
- Fill the tube with prewarmed (37 °C) washing medium and repeat centrifugation as above.
- Wash the RBC 2 times in prewarmed (37 °C) washing medium (centrifuge for 8 min at 600 × g).
- Continue as described under Cultivation of fresh patient isolates below.

Cultivation of fresh patient isolates

- Suspend RBC at ~4% hematocrit in prewarmed (37 °C) culture medium.
- Add 5 mL of the RBC suspension to a 50-mL cell culture flask (T flask) or 25 mL to a 250-mL cell culture flask.
- Flush 50-mL flasks for ~30 s with gas mixture (250-mL flasks for ~90 s) at 1.5- to 2-bar pressure (see figure and comments below).
- Incubate the flasks at 37 °C for 24 h.
- Remove the culture flask gently from the incubator and remove spent supernatant.
- Remove a tiny amount of RBC with a sterile Pasteur pipette and use these RBC to make a Giemsa-stained thin smear.
- Add new (prewarmed) culture medium to the flask, gas it as above, and return it to incubation at 37 °C.
- Subcultivate the parasites when necessary (see comments below).

Comments

- The Albumax II and hypoxanthine we use cannot be considered sterile. We thus make a stock solution of 100 g of Albumax II and 400 mg of hypoxanthine in 2 liters of RPMI 1640 and filter the solution through 0.8-µm and 0.2-µm filters. We then use 50 mL of this stock solution per 500-mL bottle of culture medium.
- It is important to keep all media preheated to 37 °C and to minimize handling time outside the 37 °C incubator. (No coffee breaks while handling cultures!)
- When gassing the culture flasks, fit the gas hose with a 0.2-µm filter unit, and use a sterile needle (preferably blunt to avoid accidents) (see the figure).
• If initial patient parasitemia is high (>0.4%), change the medium the next day, otherwise leave it for 48 to 72 h before the first change of culture medium.
• Subcultivate patient isolates to keep parasitemia below 1 to 2% by adding fresh uninfected RBC. Although many laboratory-adapted parasite lines tolerate 5% parasitemia or more, this is not the case for most patient isolates.
• Do not leave parasites in freezing or thawing solution longer than absolutely necessary, as these solutions are harmful to the parasites.
• Washed uninfected RBC for subculture should be kept in the refrigerator for 24 h before first usage (to discourage any remaining leukocytes), and can be kept in the refrigerator for up to 14 days.

References


Figure
I:C. Short-term cultivation of *Plasmodium falciparum* isolates from patient blood
by Malin Haeggström and Martha Schlichtherle
Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: malin.haeggstrom@smi.ki.se

We give here a protocol for growing *P. falciparum* from patient blood. The vast majority of cultures will survive only the first cycle (i.e., from the ring stage to trophozoites/schizonts) and will not be able to reinvoke new RBCs. In our hands, about 90% of patient isolates grew for at least one cycle. For establishment of long-term cultivation, see PARASITES section I:B.

**Equipment**
- 37 °C incubator
- centrifuge for 500-3000 x g
- candle-jar or gas (92% N₂, 5% CO₂, 3% O₂)

**Materials and reagents**
- venous blood from malaria patient
- heparinized or EDTA tubes
- Polymorphprep (Axis-Shield)
- RPMI 1640 (optimal HEPES-buffered)
- O⁺ RBCs (or autologous blood group)
- MCM with AB⁺ serum
- culture flasks or plates

**Procedure**
- Collect 2 to 5 mL of venous blood into heparinized/EDTA tubes and keep at 4 °C. Process the sample within 1 hour.
- Carefully layer the blood over 5 mL of Polymorphprep and centrifuge at 500 x g for 15 min.
- Collect the cell layer of interest. Erythrocytes are in the bottom of the tube.
- Add 10 mL of sterile RPMI 1640 (37 °C) to the cells, resuspend, and centrifuge at 500 x g for 5 min.
- Aspirate the supernatant and repeat the wash twice.
- Estimate the pellet volume and mix with equal amount RPMI 1640.

If you wish to keep the monocytes, transfer the cell layer containing monocytes into a sterile 15-mL tube (see the manufacturer’s protocol for Polymorphprep). Wash once with RPMI 1640 by centrifugation at 3,000 to 5,000 x g for 2 min. Remove the supernatant and add 1 mL of 10% DMSO in fetal calf serum. Freeze the cells immediately at −70 °C and then transfer to liquid nitrogen.

**Reference:**
I:D. Growing *Plasmodium falciparum* cultures at high parasitemia

*by Malin Haeggström and Martha Schlichtherle*

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: malin.haeggstrom@mtc.ki.se

By using increasing volumes of culture medium, one can grow *P. falciparum* to higher parasitemia (above 10%). This protocol may be especially suited for maintaining high parasitemias of wild isolates, allowing maturation of a maximum number of ring stage parasites to trophozoites.

**Procedure**

- Set up cultures as described in PARASITES, section I:A, but use the table below as a guide for medium volumes.
- Increase the parasitemia gradually using the indicated volumes until a desired parasitemia is reached, then maintain the culture in the appropriate volume.
  
  For example, for a 200-µL packed cell culture, use 4 times the volume of medium in the table for any given parasitemia.

<table>
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<th>% Parasitemia</th>
<th>Volume of medium (mL)/50 µL of packed culture</th>
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**Reference**

KEMRI, CGMRC / Wellcome Trust Research Unit, Kilifi, Kenya
I:E. Arresting *Plasmodium falciparum* growth at the trophozoite stage with aphidicolin

by Malin Haeggström

Microbiology and Tumor Biology Center (MTC), Karolinska Institutet and Swedish Institute for Infectious Disease Control, Box 280, SE-171 77 Stockholm, Sweden
e-mail: malin.haeggstrom@mtc.ki.se

This is a method to get fresh/frozen isolates or in vitro cultures to arrest in late trophozoite stages by inhibiting DNA synthesis.

Materials and reagents
- malaria culture (only young stages! see PARASITES, section I:A) or a vial of frozen malaria parasites
- aphidicolin (Sigma-Aldrich)
- DMSO

Preparations
- Prepare a 1.5 mg/mL stock-solution of aphidicolin in DMSO.
- Dilute stock-solution 1:1000 into your malaria culture (4 µL in a 4-mL flask).
- Let the culture continue to grow until the parasites reach trophozoite stage (around 24 h after adding the aphidicolin). See PARASITES, section I:A.

Analyse or use your culture within 12 h after trophozoite stages are reached. Even though the parasites may look fine for some more hours, it is NOT recommended to use them after a longer time in aphidicolin.

Note: If you add the aphidicolin to a culture of later stages, the cells will not arrest.

References
- Bull P, KEMRI, CGMRC, Kilifi, Kenya (personal communication)
I: F. Culturing erythrocytic stages of *Plasmodium vivax*
by Rachanee Udomsangpetch
Department of Pathobiology, Faculty of Science, Mahidol University, Thailand
e-mail: scrud@mahidol.ac.th

**Equipment**
- CF11-column (Cellulose powder CF11, Whatman)
- syringe
- centrifuge
- orbital shaker (Leigh Labs)
- fluorescence or light microscope

**Materials and reagents**
- PBS
- McCoy’s 5A medium (Sigma–Aldrich)
- reticulocyte-enriched red blood cells (may be obtained from cord blood or from hematochromatosis patients)
- human AB serum
- ethidium bromide (Sigma)
  - CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
- brilliant cresyl blue (ALD, Sigma–Aldrich)
- slides and coverslips

**Procedure**
- Collect 5 mL of heparinised blood from *P. vivax*-infected patients. Prepare thin and thick blood films for examination of parasite stages and parasitemia.
- Dilute the blood with 5 mL of PBS.
- Pack a sterile CF11-column having the same volume as the diluted blood (10 mL) in a syringe and equilibrate it with PBS before use. Add the diluted blood to the column and elute the red blood cells (RBC, reticulocyte enriched) with PBS. The leukocytes will stick to the CF11 cellulose.
- Collect the RBC passing through the column (reticulocyte enriched), centrifuge at 2,000 rpm, and wash once in McCoy’s 5A medium at room temperature.
- Resuspend the RBC to 5% hematocrit in McCoy’s 5A medium containing 25% human AB serum and maintain the culture in a candle jar or use a gas mixture containing 5% CO₂, 5% O₂, 90% N₂.
- In order to enhance growth of parasites, shake the culture gently and continuously at 75 rpm in the orbital shaker.
- Check the blood smears daily to confirm maturation of the parasite and add reticulocyte-enriched RBC when required.
- Enumerate the infected RBC using one of the following techniques:
  - Mix 1 drop of ethidium bromide (10 μg/mL) with one drop of the culture, mount the mixture on a glass slide with a coverslip, examine it under a UV-light microscope, and count the number of the parasitised RBC from 10,000 RBC.
  - Mix 1 volume of 1% brilliant cresyl blue in 5 volumes of the culture. Mount and examine as described above.
References

II. Freezing and thawing of asexual *Plasmodium* spp.

We give several methods below for cryopreserving and thawing *Plasmodium* strains and wild isolates. In general, parasites frozen according to one method can be thawed using another method’s thawing protocol. For convenience, we generally freeze parasite strains using the Stockholm Sorbitol Method, while thawing using the simpler NaCl Method or the 5% & 27% Sorbitol Method. It appears that the lengthier thawing protocol of the Stockholm Sorbitol Method gives lower initial parasitemia (killing of the later stages) and a more synchronous culture during the first two cycles.

When parasites are cultured in Albumax II MCM to avoid adhering IgG, no addition of serum is required.

II:A. **Stockholm Sorbitol Method**

*by Malin Haeggström and Martha Schlichtherle*

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e-mail: malin.haeggstrom@mtc.ki.se

**Equipment**
- tabletop centrifuge (Beckman)
- liquid nitrogen tank
- immufuge

**Materials and reagents**
- fetal bovine serum (Gibco)
- freezing medium:
  - 28% glycerol
  - 3% sorbitol
  - 0.65% NaCl
  - distilled water

To make 250 mL: Mix 180 mL of 4.2% sorbitol in 0.9% NaCl with 70 mL of glycerol. Filter-sterilize; store frozen.

**Freezing**
- Transfer a 4-mL culture into a test tube.
- Pellet erythrocytes by centrifugation (immufuge on low for 1 min) and discard the supernatant; the pellet is approximately 0.2 mL.
- Add 0.3 mL of serum (of complementary blood group) (40% hematocrit). For Albumax II cultures (see PARASITES, section I:A), add 0.3 mL of fetal bovine serum (40% hematocrit).
- Add 0.5 mL of the freezing medium, drop-by-drop, while shaking the vial gently; the addition should take approximately 1 min.
- Transfer the medium into a sterile cryovial.
- Drop the vial gently into the liquid nitrogen tank.

**Thawing**
- Prepare the sorbitol solutions listed below, have MCM and heat-inactivated serum ready (see PARASITES, section I:A). For Albumax II MCM, see also the comment above.
• Take a malaria culture vial from the liquid nitrogen tank.
• Thaw it quickly, approximately 1 min in a 37 °C-water bath.
• Put the vial on ice and transfer the contents into chilled 50-mL centrifuge tubes and centrifuge gently to pellet the cells (250 × g for 5 min at 4 °C in a Beckman tabletop centrifuge).

• Remove the supernatant and add the following, drop by drop, while shaking the vial gently:
  1 mL 17.5% sorbitol in ice-cold PBS, then
  2 mL 10% sorbitol, and finally
  2 mL 7.5% sorbitol

• Centrifuge as before, remove the supernatant, then add:
  1 mL 10% sorbitol, then
  2 mL 7.5% sorbitol, and finally
  2 mL 5% sorbitol

• Centrifuge, then add:
  1 mL 7.5% sorbitol, then
  2 mL 5% sorbitol, and finally
  2 mL 2.5% sorbitol

• Centrifuge, then add:
  1 mL 5% sorbitol, then
  2 mL 2.5% sorbitol, then
  2 mL MCM with 15% serum (at room temperature)

• Centrifuge, then add:
  1 mL 2.5% sorbitol, then
  2 mL MCM with 15% serum
  Transfer to a 5-mL tube.

• Centrifuge in the immufuge.
  Add 4 mL of MCM containing 15% serum (see comment for Albumax II MCM above) and packed erythrocytes of a complimentary blood group (see also PARASITES, section I:A).
II:B. Freezing of patient isolates and strains with glycerolyte

by Malin Haeggström and Martha Schlichtherle

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e-mail: malin.haeggstrom@mtc.ki.se

This protocol is useful for all primate malaria parasites tested (Plasmodium falciparum, P. vivax, P. knowlesi, P. coatneyi, P. fragile, and P. cynomolgi).

Cryoprotective solution
glycerolyte:
- 57% glycerol USP
- 16 g/liter of sodium lactate
- 300 mg/L of KCl USP
- 25 mM sodium phosphate (pH 6.8)
(This solution may be purchased from Baxter/Fenwal.)

Procedure
All steps are at room temperature unless otherwise stated.

- Take heparinized blood containing very young ring stages of malaria and centrifuge it for 10 min at 700 × g (approximately 3,000 rpm).
- Remove the supernatant and estimate volume of packed cells, V.
- SLOWLY add 0.33 × V of glycerolyte through gentle mixing. Let the tube stand for 5 min.
- Add dropwise 1.33 × V of glycerolyte, mixing gently.
- Label cryotubes with the parasite’s code/name and date.
- Distribute 1 mL of the preparation per cryovial.
- Freeze at −70 °C for at least 18 h.
- Transfer vials to liquid nitrogen for long-term storage.
II.C. Thawing of glycerolyte-frozen parasites with NaCl

by Malin Haeggström and Martha Schlichtherle

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Materials and reagents
- sterile 1.6% NaCl (Baxter/Fenwal, 4B7870)
- sterile 12% NaCl (Baxter/Fenwal, 4B7874)
- malaria culture medium (MCM, see PARASITES, section I:A)
- 15% AB+ serum

Procedure
- Remove a vial from cold storage and thaw it at 37 °C for 1 to 2 min.
- Transfer blood to 50-mL centrifuge tubes with a sterile pipette. Measure blood volume, V.
- Add 0.1 \times V of 12% NaCl slowly, dropwise, while shaking the tube gently.
- Let the tube stand for 5 min.
- Add 10 \times V of 1.6% NaCl slowly, dropwise, swirling the tube.
- Centrifuge the tube at 500 \times g at 20 °C for 5 min.
- Aspirate the supernatant and add 10 \times V of MCM slowly, dropwise, while shaking the tube.
- Centrifuge the tube at 1,500 rpm at 20 °C for 5 min and aspirate the supernatant.
- Resuspend pelleted blood cells in MCM with 15% AB+ serum and transfer to a culture flask and desiccator. For continued culturing, see PARASITES, section I.
II:D. Thawing of cryopreserved *Plasmodium falciparum* using sorbitol

by Malin Haeggström and Martha Schlichtherle

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Equipment
- water bath (37 °C)
- table-top centrifuge (Beckman)

Materials and reagents
- 27% sorbitol in PBS, filter-sterilized, stored at 4 °C
- 5% sorbitol in PBS, filter-sterilized, stored at 4 °C
- 15% AB+ serum

Procedure
- Remove a vial from liquid nitrogen and thaw it quickly in a 37 °C water bath; shake it lightly.
- Transfer thawed parasites into a round-bottomed 13-mL tube (max. 1 mL/vial).
- Slowly add 2 volumes of 27% sorbitol, the first volume over 8 min, the second over 5 min. Slowly stir the tube constantly on a vortex.
- Let it stand for 5 min.
- Add 2 volumes of 5% sorbitol over 10 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ (1,250 rpm on a table-top Beckman centrifuge) for 3 min; discard the supernatant.
- Add 2 volumes of 5% sorbitol over 8 min.
- Let it stand for 5 min.
- Centrifuge the tube at $400 \times g$ for 3 min; discard the supernatant.
- Add 1 to 2 mL of MCM over 1 min. Centrifuge the tube at $400 \times g$ and discard the supernatant. The wash may be repeated once.
- Resuspend the pellet in 15% AB+ serum in MCM. Add washed, packed RBC's to 5% hematocrit.
- Change the medium the next day. Continue culturing as usual (see PARASITES, section I).

Reference
III. Staining of parasite culture or patient blood and estimation of parasitemia

III:A. Acridine orange (AO) vital stain of cultures

by Malin Haeggström¹, Martha Schlichtherle¹, and Hedvig Perlmann²

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Equipment
UV-equipped microscope

Materials and reagents
Acridine orange (AO) (Fluka)

Stock solution:
Dissolve 10 mg of AO in 10 mL of distilled water. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Working solution:
Add 0.1 mL of stock solution to 9.9 mL of PBS (pH 7.2). Store in the dark at 4 °C.

Alternate acridine orange hydrochloride solution (Fluka)
Dilute 1:5000 in PBS. Add sodium azide to a final concentration of 0.02%. Store in the dark at 4 °C.

Procedure
- Mix one drop of culture with a small drop of AO on a slide.
- Put on a coverslip.
- Analyse the slide immediately or keep it in the dark.
- Count 5 to 10 fields of vision to estimate the parasitemia (40× objective, 10× ocular).
III:B. *Giemsa staining of thick or thin blood films*  
*by Marianne Lebbad*  
*Svenska institution för infektionsjägning och mikrobiologi och tumörbiologi (MTC), Karolinska Institutet SE-171 82 Solna, Sweden*  
e-mail: marianne.lebbad@smi.ki.se

**Equipment**
- light microscope

**Materials and reagents**

**Giemsa stain:**  
For best and most reproducible results, buy ready-made Giemsa stain from, for example, Sigma–Aldrich. It is quite difficult to prepare a batch that works as well as the commercial one.  
Check every new batch of Giemsa stock solution before using it. **Prepare a new working solution for each staining session.**

phosphate buffer, 6.7 mM (pH 7.1):

- 0.41 g KH$_2$PO$_4$
- 0.65 g Na$_2$HPO$_4$·H$_2$O

Bring to 1 liter with distilled water.

methanol (reagent grade for analysis, Merck)

**Giemsa staining of thick blood films from cultures**
- Leave the thick film to dry properly, preferably overnight. In case of hurry, leave it to dry in an incubator at 37 °C for 1 h. Do not fix.
- Prepare a fresh 2% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Leave it to stain for 30 min. (A stronger Giemsa solution will over-stain parasites from culture.)
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

**Giemsa staining of thick blood films from patients**
- Leave the thick film to dry properly. In case of hurry, leave it to dry in an incubator at 37 °C for 30 min.
- Prepare a fresh 4 to 5% Giemsa solution in phosphate buffer (pH 7.1).
- Put the slide on a staining rack and pour the Giemsa solution on the slide. Do not fix.
- Leave the slide to stain for 20 min.
- Rinse very carefully and gently. Tap water may be used, but do not let the stream run directly on the blood film.
- Leave the slide in an upright position to dry.
- Observe with immersion oil and objective at 100×.

**Giemsa staining of thin blood films from patients or cultures**
- Air-dry the thin film.
• Fix the film in methanol for about 30 s.
• Prepare a fresh 5 to 10% Giemsa solution in phosphate buffer (pH 7.1).
• Put the slide in a staining jar or on a staining rack and pour the Giemsa solution on the slide.
• Leave it to stain for 20 min.
• Rinse it carefully and thoroughly under running tap water.
• Leave the slide in an upright position to dry.
• Observe the film with immersion oil and objective at 100×.

Reference
III:C. Estimation of the percentage of erythrocytes infected with *Plasmodium falciparum* in a thin blood film

*by Marianne Lebbad*

*Swedish Institute for Infectious Disease Control and Microbiology and Tumor Biology Center (MTC), Karolinska Institutet SE-171 82 Solna, Sweden*

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**Equipment**

Use a light microscope with a special ocular (i.e., a reticulocyte ocular) which has a large and a small square where the small square is 10% of the large one (see the figure below, although proportions are not maintained).

**Procedure**

- Use the 100× objective to view cells under oil immersion.
- Choose an area of a Giemsa-stained thin blood film where the erythrocytes are evenly distributed.
- Count all erythrocytes in the small square plus two of the borders.
- Without moving the slide, also count the number of infected erythrocytes in the whole area of the big square plus two borders (i.e., including those in the area of the small square).
- Move the slide to “randomly adjacent” fields and continue counting as above (i.e., all erythrocytes of small fields and parasites of accompanying large fields). Continue counting fields until you reach the sum of 100 erythrocytes in the small fields. By extrapolating, the equivalent of 1000 erythrocytes have been examined (regarding parasites).
- Repeat the counting at least twice for a total examination of three different parts of the slide. Take the mean number of infected erythrocytes per 1000 erythrocytes and divide by 10 to get the percent infected erythrocytes.

Please note: If one erythrocyte contains ≥ 2 parasites it is still counted as one infected erythrocyte.

**Reference**

III:D. Evaluation of parasitemia by lactate dehydrogenase assay
by Makoto Hirai¹² and Ingrid Faye⁷
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²Department of Medical Zoology, Jichi Medical School, 3311-1, Minamikawachi, Tochigi, 329-0498, Japan
e-mail: m.hirai@jichi.ac.jp

Evaluation of parasitemia by lactate dehydrogenase assay is a high throughput method for screening of antimalarial factors.

**Equipment**
- microplate reader
- flat-bottomed 96-well plate

**Materials and reagents**
- Malstat reagent (Flow Incorporated)
- red blood cells
- nitro blue tetrazolium (NBT) stock solution:
  - 100 mg NBT
  - 50 mL distilled water
  - Dissolve NBT in distilled water. Store in the dark at 4 °C.
- phenazine ethosulphate (PES) stock solution:
  - 5 mg of PES
  - 50 mL distilled water
  - Dissolve PES in distilled water. Store in the dark at −20 °C.

**Procedure**
- Add 100 µL of Malstat reagent to 96-well microplate.
- Add 20 µL of infected or noninfected red blood cells (hematocrit is 1 to 2%).
- Incubate the plate at room temperature and shake gently several times during incubation to solubilize red blood cells.
- During incubation, mix equal volumes of NBT and PES in the dark and add 20 µL of the mixture to the wells.
- Keep the plate in the dark.
- After 30 to 60 min, check the color development (dark purple).
- Read the plate at 650 nm. Use uninfected red blood cells as reference.
- This assay can be used in 0 to 7% of parasitemia (non-synchronized parasite in 2% of hematocrit).

If you deal with a large number of samples, 20 µL of red blood cells in 96 wells can be stored in 96-well plates at −80 °C before the enzyme assay.

**References**

IV. Purification and synchronization of erythrocytic stages

IV:A. Enrichment of knob-infected erythrocytes using gelatine sedimentation

by Victor Fernandez
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Equipment
immufuge blood centrifuge

Materials and reagents
mature parasite stages
gelatine (Sigma G2625)
RPMI 1640 with HEPES

Procedure
• Make 0.7% gelatine in RPMI 1640 with HEPES.
• Warm and stir the solution at 37 °C until the gelatine is dissolved.
• Filter-sterilize the solution and store it in the refrigerator. Warm it to 37 °C before use.
• Use mature parasite stages. (Ring forms, knobless late stages, and rosettes will sink to the bottom.)
• Wash the culture once using RPMI 1640.
• Resuspend the pellet, approximately 0.2 mL, in 2 mL of 0.7% gelatine solution.
• Incubate the pellet for 1 h at 37 °C.
• Carefully remove the supernatant, which contains the “knobby” parasites, without touching the pellet.
• Centrifuge the supernatant to pellet the parasitized RBC (PRBC) (1 min on high setting in the immufuge blood centrifuge).
• Wash the cells 2 or 3 times using RPMI 1640.
• Subculture the cells using fresh blood and media (see PARASITES, section I).

Reference
IV:B. Sorbitol-synchronization of *Plasmodium falciparum*-infected erythrocytes

*by Victor Fernandez*

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**Equipment**
- centrifuge

**Materials and reagents**
- parasite culture of >5% parasitemia
- sorbitol
- malaria culture medium (MCM)

**Procedure**
- Take parasites when they are mostly at the ring stage. They must not be later than 10 to 12 h postinvasion when the sorbitol treatment is done.
- Spin down the parasite culture at $600 \times g$ to a pellet (take 4 mL of a culture of >5% parasitemia).
- Add 4 mL of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Shake 2 or 3 times.
- Centrifuge the culture at $600 \times g$, and wash it 3 times in malaria culture medium, and dilute it to 5% hematocrit.
- Count parasitemia and subculture as usual.
- Repeat the procedure after one cycle (approximately 48 h).
- To keep the parasites synchronized, the sorbitol treatment must be performed once a week.

**Reference**

IV:C. **Enrichment of late-stage infected erythrocytes in 60% Percoll**

*by Victor Fernandez*

_Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden_

e-mail: victor.fernandez@smi.ki.se

**Equipment**

- centrifuge, swing-out rotor, refrigerated

**Materials and reagents**

- mature parasite stages
- malaria culture medium (MCM) with the human serum replaced by 10% FBS

**60% Percoll solution:**

- 60 mL Percoll
- 7 mL $10\times$ PBS
- 33 mL PBS

The solution is enough for 40 tubes of 2.5 mL each, which is enough to enrich 80-mL of culture. Make fresh solutions before each use. Keep all solutions on ice throughout the preparation.

**PBS**

**Procedure**

- Wash a mature-stage *Plasmodium falciparum* culture 3 times in MCM with the human serum replaced by 10% FBS and resuspend it to 10% hematocrit in cold MCM with 10% FBS.
- Distribute the culture in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge in a swing-out rotor at $1,500 \times g$ and $4^\circ C$ for 15 min ($2,000$ rpm in Sorvall or Beckman centrifuge).
- Withdraw carefully the cells at the interphases, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.

**Reference**

IV:D. Separation of *Plasmodium falciparum* mature stages in Percoll/sorbitol gradients

*by Victor Fernandez*

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Equipment
- centrifuge, fixed angle rotor (Sorvall SS-34 or Beckman JA-20)
- benchtop centrifuge

Materials and reagents
- heparin
- PBS (pH 7.2)
- Percoll
- sorbitol
- RPMI 1640
- distilled water
- malaria culture medium (MCM)
- Corex tubes
- 23 G 0.6-mm ∅ needle
- tuberculin syringe

Preparation of gradients (for 4 gradients):

<table>
<thead>
<tr>
<th>Volume</th>
<th>Percoll</th>
<th>Solution L</th>
<th>Solution H</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mL</td>
<td>45 mL</td>
<td>5 mL</td>
<td></td>
</tr>
<tr>
<td>(90% Percoll, 5% sorbitol)</td>
<td>(0% Percoll, 5% sorbitol)</td>
<td>45 mL 10× RPMI 1640</td>
<td>27 mL distilled water</td>
</tr>
<tr>
<td>5 mL</td>
<td>10× RPMI 1640</td>
<td>2.5 g sorbitol</td>
<td>1.5 g sorbitol</td>
</tr>
</tbody>
</table>

Prepare gradients:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Percoll</th>
<th>Solution L</th>
<th>Solution H</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mL</td>
<td>40%</td>
<td>8.3 mL</td>
<td>6.7 mL</td>
</tr>
<tr>
<td>15 mL</td>
<td>60%</td>
<td>5.0 mL</td>
<td>10.0 mL</td>
</tr>
<tr>
<td>15 mL</td>
<td>70%</td>
<td>3.3 mL</td>
<td>11.7 mL</td>
</tr>
<tr>
<td>20 mL</td>
<td>80%</td>
<td>2.2 mL</td>
<td>17.8 mL</td>
</tr>
</tbody>
</table>

Procedure
- For each gradient, fill a 15-mL Corex tube as indicated below. Using a syringe, and starting with the lightest solution (40%), carefully layer the next denser layer under the previous one:
Separation of parasite stages

- Wash the PRBC and resuspend them in 1 mL of Solution L.
- Add 100 U/mL of heparin and pass the suspension 5 times through a 23 G 0.6-mm Ø needle using a tuberculin syringe (to disrupt rosettes).
- Very carefully overlay the cell suspension on top of the gradient.
- Centrifuge the gradients in a fixed angle rotor at 10,000 rpm for 30 min at 20 °C. Do not use a brake to decelerate.
- Discard the top of the gradient.
- Collect the parasites (floating as described in the diagram above) in a fresh centrifuge tube, no more than 5 mL/tube in a 10-mL tube. Fill the tube with PBS, mix very gently, and spin at 3,000 rpm for 1 min in a benchtop centrifuge.
- Resuspend the PRBC in MCM (see also PARASITES, section I:A).

Reference

IV:E. Obtaining free parasites

by Denise Mattei

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724

Paris, France

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Equipment

centrifuge (15-mL tube and microfuge)

Materials and reagents

- Plasmion (Fresenius France)
- 5% solution (w/v) of Gly-Ser (Sigma G3252)
- 40 mM HEPES
- 10 mM D-glucose in distilled water

Procedure

- Enrich parasitized red blood cells with Plasmion. Recover them by centrifugation at 2,000 rpm for 2 min. Resuspend the pellet at 37 °C in 1.5 volumes of a 5% solution (w/v) of Gly-Ser made with 40 mM HEPES (pH 7.2) and 10 mM D-glucose in distilled water.
- Incubate at 37 °C for 10 to 30 min.
- Add 5 volumes of culture medium.
- Centrifuge at 1,000 rpm for 3 min. Free parasites are in the supernatant; intact cells are in the pellet. The pellet of intact PRBC can be re-incubated with Gly-Ser.

Reference

IV:F. Obtaining semi-intact cells
by Denise Mattei
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e-mail: dmm@pasteur.fr

In eukaryotic cells this method extracts soluble cytoplasmic components with the membranes retained.

Equipment
refrigerated microfuge

Materials and reagents
Plasmion (Fresenius France)
washing buffer (WB):
  10 mM HEPES (pH 7.2)
  15 mM KCl
breaking buffer (BrB):
  50 mM HEPES (pH 7.2)
  90 mM KCl

Procedure
- Enrich parasitized red blood cells with Plasmion. Wash 1 volume of Plasmion (without centrifugation) 3 times in PBS and resuspend it in the same volume of ice cold washing buffer, WB. For example, if you start with 250 µL of Plasmion, resuspend the pellet in 250 µL of WB.
- Centrifuge the cells in a refrigerated microfuge at 15,000 rpm for 10 min at 4 °C.
- Wash the pellet once in WB and resuspend it in the original volume of WB.
- Incubate it on ice for 10 min. Recover PRBC by centrifugation at 15,000 rpm for 10 min at 4 °C.
- Resuspend the pellet in the original volume of breaking buffer, BrB.
- Centrifuge as above and resuspend the pellet in one tenth of the original volume of BrB. Semipermeable parasites can be used in immunofluorescence studies or as membrane-enriched extracts on Western blots or immunoprecipitations.

Reference
IV:G. Alanine synchronization of *Plasmodium falciparum*-infected erythrocytes

by Catherine Braun-Breton

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e-mail: cbb@pasteur.fr

Only early trophozoites and noninfected erythrocytes will survive this treatment.

**Equipment**

centrifuge

**Materials and reagents**

- early trophozoites
- noninfected erythrocytes
- 0.3 M alanine
- 10 mM HEPES (pH 7.5)
- culture medium (RPMI 1640 with 10% heat-inactivated human serum)

**Procedure**

- Prewarm a solution of 0.3 M alanine, 10 mM HEPES (pH 7.5) at 37 °C.
- Spin down the parasite culture to a pellet (5 min at 2,000 × g at 20 °C).
- Resuspend the pellet in 5 volumes of 0.3 M alanine, 10 mM HEPES (pH 7.5).
- Incubate the solution for 3 min at 37 °C.
- Add 10 volumes of culture medium.
- Centrifuge the tube for 5 min at 2,000 × g at 20 °C and resuspend the pellet in culture medium at a 5% hematocrit.

**Reference**

Methods in Malaria Research

PARASITES

IV:H. Selection of trophozoites by using magnetic cell sorting (MACS)

by Anna Vogt and Fredrik Pettersson

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Equipment

- Vario-MACS magnet (Miltenyi Biotec)
- MACS CS-column (Miltenyi Biotec)
- 3-way stopcock (Miltenyi Biotec)
- 10-mL syringe
- 5-mL syringe
- 0.8-mm needle
- 0.6-mm needle
- 50-mL Falcon tubes
- microscope
- centrifuge

Materials and reagents

- 4 to 6 mL malaria culture, 5 to 10% parasitemia
- PBS
- 150 mL 2% BSA/PBS
- heparin (25000 IU/mL, Lövens)

Preparation

- Connect the 3-way stopcock and the column and place it in the magnet (fig. 1).
- Fill the 10-mL syringe with 2% BSA/PBS and connect it to the left opening of the 3-way stopcock. Open the way between the syringe and the column by closing the way between the syringe and the tap way out (fig. 2). Load the column with 2% BSA/PBS by carefully pushing the connected syringe (fig. 3). Fill up to 10 mm above the column matrix. Close the 3-way stopcock totally (fig. 4). Refill the syringe with 2% BSA/PBS and reconnect it to the column. Leave the column for incubation for at least 5 min.
- Preparing the malaria culture: Centrifuge the malaria culture. Resuspend the pellet in 2 mL of 2% BSA/PBS. If using rosetting parasites, add heparin (100 U/mL) and push the culture through a 0.6-mm needle five times. Check in the microscope that all the rosettes are disrupted.
- Cut the top of the cover of a 0.8-mm needle and connect it to the 3-way stopcock (fig. 1). Open the way between the column and the needle and let the excess of loaded 2% BSA/PBS elute. Do not let any air into the column.
- Load the parasites on the top of the column. Open the way between the column and needle. Let the parasites migrate into the column. Close when 1 mm of solution is left. Add some 2% BSA/PBS and let the cells sediment following a quick opening of the tap to let the last cells immigrate. Do not let blood go through the needle. Leave for incubation for 10 min.
- Wash the column by adding 50 mL of 2% BSA/PBS to the top of the column and open between column and the needle. Leave some millimeters of the solution before closing the tap.
- Disconnect the needle from the 3-way stopcock. Disconnect the column from the magnet. Elute the bound material by holding the column upside down over a
Falcon tube and flush through 50 mL of 2% BSA/PBS using the syringe connected to the 3-way stopcock.

- Centrifuge the eluted material at 1,500 rpm for 5 min at room temperature. If using heparin, wash the pellet 5 times in 50 mL of PBS.
- Count the parasitemia.
- The column can be reused immediately. Place the column in the magnet and follow the protocol from the beginning. If reused later, please use the instructions for this procedure delivered together with the columns.

Comments
Approximately 50% of the trophozoites originally found in culture will be an enriched suspension. The following factors have been shown to affect the results:

- The culture should have a parasitemia of 6 to 10%, well synchronized trophozoites.
- The volume of culture used should not be larger than 6 mL or $10^9$ cells.
- There should be no (or very few) extracellular parasites in the culture, as they seem to attach better to the matrix.
- A good looking, well-synchronized, culture without extracellular parasites seems to be the most important thing for a good result on the MACS.
- If heparin-free cultures are necessary, rosettes can be disrupted mechanically by passing the culture through the needle without heparin (see above), but make sure all rosettes are disrupted before loading the column.

Reference

Figures
Fig 1 – Assembly of the MACS column
IV.I. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

*by James G. Beeson and Stephen J. Rogerson*

Department of Medicine, University of Melbourne, Post Office Royal Melbourne Hospital, Parkville VIC 3050, Australia

e-mail: beeson@unimelb.edu.au

See PARASITES, section VII:A

Data for MACS-column:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
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<td>6.3 mL</td>
</tr>
<tr>
<td>Reservoir volume</td>
<td>6.5 mL</td>
</tr>
<tr>
<td>Maximum capacity total cells</td>
<td>$10^9$</td>
</tr>
<tr>
<td>Maximum capacity retained cells</td>
<td>$2 \times 10^8$</td>
</tr>
<tr>
<td>Rinse volume</td>
<td>60 mL</td>
</tr>
<tr>
<td>Wash volume</td>
<td>30 mL</td>
</tr>
<tr>
<td>Elution volume</td>
<td>30 mL</td>
</tr>
</tbody>
</table>
V. Micromanipulation cloning of *Plasmodium falciparum*  
by Victor Fernandez  
Swedish Institute for Infectious Disease Control, SE-171 82 Solna, Sweden  
e-mail: victor.fernandez@smi.ki.se

**Equipment**
- inverted microscope (Diaphot 300, Nikon)  
- micromanipulator system (NT-88, Nikon/NARISHIGE)  
- micropipette puller (Sutter Instrument, Model P-87)  
- pipette filling accessories: tubing, needle, syringe, MicroFil (World Precision Instruments)

**Materials and reagents**
- capillaries, Boroglass, outer diameter = 1.0 mm, inner diameter = 0.5 mm (Sutter Instrument)  
- glass slides  
- 12- or 24-well microplates  
- 0.2-µm syringe filter  
- culture flasks, 25 cm²  
- parasite culture of 5 to 10% parasitemia  
- malaria culture medium (MCM; see PARASITES, section I:A)  
- human serum, blood type AB⁺ (HS, see PARASITES, section I:A)  
- human erythrocytes, blood type O⁺ (see PARASITES, section I:A)  
- PBS (pH 7.2)  
- RPMI 1640  
- acridine orange (see PARASITES, section III:A)  
- Acrylease (Stratagene)

**Preparation**
- Pull pipettes in the micropipette puller. We use program 0 (heat: 392, vel: 100, pressure: 599). Each capillary gives two micropipettes. Break the tip with tweezers to get an inside diameter of 3 to 5 µm. Check in the microscope that the pipette tip edge is blunt.  
- Mix MCM with human serum to get 90% MCM/10% HS and filter through a 0.2-µm syringe filter.  
- Fill a pipette with filtered 90% MCM/10% HS. Attach the pipette to a tube connected to a needle/syringe. Put the tip of the pipette in filtered 90% MCM/10% HS and fill the tip by pulling the syringe. Hold for 1 to 2 min until the liquid is above the narrow part of the tip. Detach the syringe. Fill the rest of the pipette by using a MicroFil. Coat the outside of the filled micropipettes by dipping the tip in filtered 90% MCM/10% HS for at least 10 min.  
- Attach the micropipette to the micromanipulator system.

**V:A. Micromanipulation cloning of parasites**
- Add 1 mL of filtered 90% MCM/10% HS to well A1 of a microplate. Make sure that the entire surface of the bottom is covered.  
- Incubate the plate for 1 h at room temperature.  
- Add 1 or 2 mL of 85% MCM/15% HS to the rest of the wells. Use the smaller volume for 24-well plates and the larger for 12-well plates.
• Add 2 to 8 µL of parasite culture to well A1. Dispense and leave it for 15 min to sediment.
• Pick individual erythrocytes with one parasite each and transfer them to the other wells of the microplate, placing only one parasite-infected erythrocyte in each well. If rosetting parasites are picked, strip off the uninfected erythrocytes and make sure that only one parasite is picked.
• Wash erythrocytes three times with RPMI 1640 and add them to all wells (except A1) to get a hematocrit of 1%; 20 µL to 12-well plates and 10 µL to 24-well plates.
• Culture the cells in candle jars as described below in section C below (see also PARASITES, section I:A).

V:B. Micromanipulation cloning of *Plasmodium falciparum* for single-cell RT-PCR

• Siliconize several slides with Acrylease.
• Put a drop of PBS on one of these slides.
• Exchange the filtered 90% MCM/10% HS in the lower part of the micropipette tip with PBS.
• Put 0.2 to 0.3 mL of PBS on another siliconized slide.
• Add 1 to 2 µL of the parasite culture and mix.
• Let the cells sediment for 5 to 10 min.
• On a siliconized slide, dispense 10 µL of PCR mix (see also MOLECULAR BIOLOGY, section V).
• Transfer 1 infected erythrocyte (with 1 parasite) from the parasite-containing drop to the PCR mix drop.
• Pipette the 10-µL drop containing the infected erythrocyte back into the PCR tube and immediately freeze the tube on dry ice.
• Pick uninfected erythrocytes as controls.

V:C. Expansion of *Plasmodium falciparum* clones

• *Day 0:* Cloning (see sections A and B above).
• *Day 3:* Change the medium. Carefully remove the supernatant and add fresh 85% MCM/15% HS. Add 1 mL to 24-well microplates and 2 mL to the 12-well plates.
• *Day 6:* Change the medium and add 1% hematocrit of washed erythrocytes (10 µL to 24-well plates, 20 µL to 12-well microplates).
• *Day 9:* Change the medium.
• *Day 12:* Change the medium and add 1% hematocrit.
• *Day 15:* Change the medium.
• *Day 17:* Change the medium and add 1% hematocrit.
• *Day 19:* Check the clones. Take a drop from the bottom of the well and put it on a slide. Add a drop of acridine orange (see PARASITES, section III:A) and use the microscope to check for parasites. Examine at least 30 fields. If the parasitemia is >1%, move the clone to a 25-cm² culture flask with 90% MCM/10% HS and continue to incubate the culture (see PARASITES, section I:A). Change the medium in wells with very low or no detectable parasitemia.
• *Day 21:* Check wells and/or flasks and record the phenotype of the parasites. Discard empty wells and transfer clones with parasites to culture flasks as above.
(see Day 19). When the parasitemia in the flasks is higher than 5% and the parasites are in the ring stage, they can be frozen (see PARASITES, section II).

References


VI. Cytoadhesion and rosetting assays

VI:A. Basic cell media

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CHO and L cell medium, solution for 500 mL:
- 5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)
- 3.0 g HEPES (Gibco)
- 0.4 g geneticin
- 450 mL distilled water
- 50 mL fetal calf serum
- 0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and filter-sterilize; store at 4 °C for up to one month.

Alternative CHO and L cell medium, solution for 500 mL:
- 450 mL commercial RPMI 1640 (Gibco with 20 mM HEPES, without L-glutamine)
- 50 mL fetal calf serum
- 8 mL geneticin (Gibco stock concentration, 50 mg/mL)
- 50 mL L-glutamine (Gibco stock concentration, 20 mM, 100×)
- 0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)

Mix components and store at 4 °C for up to one month.

HUVEC medium, solution for 500 mL:
- 5.84 g MCDB-131 (Sigma)
- 3.0 g HEPES (Gibco)
- 1.0 g NaHCO₃
- 10 ng/mL (final concentration) epidermal growth factor
- 1 µg/mL (final concentration) hydrocortisone
- 0.5 mL penicillin/streptomycin (Gibco stock concentrations: penicillin, 10,000 IU/mL; streptomycin, 10,000 µg/mL)
- 25 mL fetal calf serum

Mix components and filter-sterilize; store at 4 °C for up to a month.

COS-7 cell medium:

Dulbecco’s Modified Eagle Medium (DMEM, Gibco, 41966-029)
- 10% fetal calf serum

Mix components. Store the medium at 4 °C. Once the serum has been added, use this complete medium within one week.

Binding medium (pH 6.8), solution for 500 mL:
- 5.2 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)
- 3.0 g HEPES (Gibco)
- 450 mL distilled water
- 50 mL human serum, inactivated, nonimmune AB⁺ (10%)

Mix components and set pH to 6.8 immediately before use.
Alternatively, RPMI 1640 and HEPES can be mixed, filter-sterilized, and kept at 4 °C for up to a month. Immediately before use, add 10% human serum and set the pH to 6.8.

10× RPMI, solution for 100 mL:
- 10.43 g RPMI 1640 (with glutamine, without NaHCO₃; Gibco)
- 6 g HEPES (Gibco)
- 2 g glucose
- 100 mL distilled water
Mix components and filter-sterilize. Set pH to 6.8.
VI:B. Thawing melanoma and other cell lines

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Equipment

- water bath (37 to 40 °C)
- centrifuge (Beckman Allegra 6R)
- 15-mL Falcon tube (BD Labware)
- 25-cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
- incubator (37 °C, 5% CO₂)

Materials and reagents

- appropriate cell medium (see PARASITES, section VI:A)
- gelatine (2% gelatine in PBS, warmed for 30 min at 37 °C)

Thawing of cells

- Remove the vial with the frozen cells from the liquid nitrogen container and put it into a water bath at 37 to 40 °C. Thawing should be rapid (40 to 60 s).
- As soon as the ice is melted, remove the vial from the water bath.
- Transfer the contents of the vial to a sterile test tube and centrifuge it at 300 × g for 5 min.
- Discard the supernatant.
- Dilute the cell suspension with an appropriate volume of the recommended culture medium.
- Transfer the suspension to a culture flask and incubate it at 37 °C in 5% CO₂. The flask top should be loose. When cultivating HUVEC cells, the bottom of the flask should be coated with warm gelatine before the cells are added.
- In order to expedite the removal of the protective freezing additive (glycerol or dimethyl sulfoxide) from the culture medium, change the culture medium 24 h after thawing unless stated otherwise in the specific instructions for a particular cell line.
VI:C. Freezing of cell lines

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Equipment
- freezing vials (Nunc Cryo Tube Vials; Nalge Nunc International)
- 15-mL Falcon tube (BD Labware)
- Neubauer chamber
- microscope (40× lens)
- 4 °C-refrigerator
- −70 °C-freezer
- liquid nitrogen tank for long-term storage

Materials and reagents
- freezing medium:
  - 7 mL RPMI 1640 medium
  - 1 mL dimethyl sulfoxide (DMSO)
  - 2 mL fetal calf serum (FCS)
- EDTA–trypsin solution:
  - EDTA, 2 mg/mL
  - trypsin, 2.5 mg/mL
- appropriate cell medium (see PARASITES, section VI:A)
- trypan blue

Freezing of cells
- Prepare the freezing medium.
- Detach the cells from the cell culture flask with 2 mL of EDTA–trypsin solution and resuspend them in 10 mL of fresh medium (see PARASITES, section VI:D below).
- Count a sample of cells in trypan blue (1:2 dilution) using a Neubauer chamber.
- Centrifuge the sample at 4 °C (300 × g for 5 min) and resuspend the cells with freezing medium at 1 × 10^6 cells/mL.
- Aliquot the cells into freezing vials and keep them at 4 °C for 2 h.
- Place the vials in −70 °C overnight.
- Place them in liquid nitrogen for long-term storage.
VI:D. Cultivation of CHO, COS, HUVEC, melanoma, and L cells

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Equipment
- centrifuge (Beckman Allegra 6R)
- 15-mL Falcon tube (BD Labware)
- 25-cm² culture flask (treated, nonpyrogenic, polystyrene; Corning)
- incubator (37 °C, 5% CO₂)

Materials and reagents
- RPMI 1640 medium
- EDTA–trypsin solution
  - EDTA, 2 mg/mL
  - trypsin, 2.5 mg/mL
- appropriate medium (see PARASITES, section VI:A)
- 0.5% gelatine in PBS for HUVEC cells

Passage of cell culture
- Aspirate the old medium from the culture.
- Wash the cells once with RPMI 1640.
- Add 2 mL of EDTA–trypsin.
- Leave the cells for approximately 5 min.
- Shake the flask sharply and check the flask in the inverted microscope to see that the cells have detached.
- Add 10 mL of fresh medium.
- Mix and transfer the suspension to a 15-mL Falcon tube and centrifuge the suspension for 5 min at 300 × g.
- Remove the supernatant by aspiration and resuspend the pellet in 1 mL of culture medium.
- Determine the number of cells in a Neubauer chamber.
- Add 5 mL of fresh culture medium to a new 25-cm² flask (for HUVEC cells use a gelatine-coated flask, see note below) and add approximately 10⁵ cells.
- Incubate the cells at 37 °C in 5% CO₂. The flask top should be loose.

Note: Before seeding the HUVECs, coat the culture flasks with 1% gelatine in sterile PBS sterile for 1 h at 37 °C:
- Add 2 to 3 mL of gelatine in PBS to the cell culture flask.
- Incubate the flasks for 30 min at 37 °C.
- Remove the excess gelatine by aspiration and add cells as above.
VI:E. Formaldehyde-fixation of melanoma cells
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Equipment
24-well microplates
cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc International)
incubator (37 °C, 5% CO₂)
inverted microscope (125×)
microscope (1000×)

Materials and reagents
melanoma C32 cells in culture
appropriate medium (see PARASITES, section VI:A)
EDTA–trypsin solution:
EDTA, 2 mg/mL
trypsin, 2.5 mg/mL
RPMI 1640 medium
PBS
1% formaldehyde

Fixation of cells
• Place cell culture coverslip with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
• Add 1 mL of appropriate cell cultivation medium.
• Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding melanoma cells in cell culture bottles, add approximately 50 × 10³ cells to each of the wells.
• Leave the melanoma cells to attach to the coverslip in the incubator (37 °C, 5% CO₂) overnight.
• Remove the medium from the melanoma cells and wash twice with PBS.
• Add 0.5 mL of 1% formaldehyde to each well and incubate the cells for 1 h at room temperature.
• Wash them twice with PBS.
• Add PBS and cover the wells; store at 4 °C until use. The maximum storage time is approximately 1 month.

Reference
VI:F. Binding assays to endothelial and melanoma cells

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Equipment

24-well microplates
- cell culture-treated coverslips (Thermanox plastic coverslip; Nalge Nunc Int.)
- centrifuge (Beckman Allegra 6R)
- 15-mL Falcon tube (BD Labware)
- incubator (37 °C)
- glass slides
- inverted microscope (125×)
- microscope (1000×)

Materials and reagents

- melanoma or endothelial cells in culture
- malaria culture of 8% parasitemia or higher with a majority of the parasites at trophozoite stage
- EDTA–trypsin solution:
  - EDTA, 2 mg/mL
  - trypsin, 2.5 mg/mL
- RPMI 1640
- appropriate medium (see PARASITES, section VI:A)
- 5% Giemsa solution in phosphate buffer (see PARASITES, section III:B)
- 1% glutaraldehyde in PBS
- glycerol
- binding medium (see PARASITES, section VI:A).

Procedure

- Place cell culture coverslips with the cell culture-treated side upwards at the bottom of the wells of a 24-well microplate.
- Add 1 mL of appropriate cell culture medium to each well.
- Follow the protocol for subcultivation of cells (see PARASITES, section VI:D), but instead of seeding cells in culture bottles, add approximately 50 × 10^3 cells to each of the wells with coverslips.
- Leave the cells to attach to the coverslip in the incubator (37 °C, 5% CO2) overnight. For melanoma cells, please see note at the end of the protocol.
- Inspect cells that have been plated on coverslips; each cell should be separated from other cells for easy reading of binding.
- Centrifuge the malaria culture at 500 × g for 5 min at room temperature.
- Wash the culture 3 times (500 × g) with binding medium and resuspend in binding medium to a hematocrit of about 2%.
- Aspirate medium from the coverslips in the 24-well plate and rinse once with binding medium. Be careful not to let the coverslip with the attached cells dry.
- Add 0.5 mL of malaria culture suspension to each coverslip.
- Incubate the cells at 37 °C for 60 min, gently rocking by hand every 15 min. Do not use a CO2 incubator.
• Check the binding of malaria-infected erythrocytes to the cells using an inverted microscope.
• Wash away unbound red cells by dipping the coverslip carefully in each of 3 beakers of binding medium or RPMI 1640.
• Fix the coverslips in 1% glutaraldehyde for 60 min or overnight. Alternatively, fix in 1% methanol for 30 min.
• Rinse coverslips with distilled H₂O and stain with 1% Giemsa for 20 to 30 min.
• After staining, rinse the coverslips with water and let them dry.
• Mount the coverslips on glass slides with glycerol.
• Examine the slides under a microscope (oil, 1,000×) and count 300 cells.
• Calculate the average number of infected erythrocytes bound per target cell.

Note: Melanoma cells can be used fresh or fixed with 1% formaldehyde if fixation insensitive receptors are to be studied, such as CD36 (see PARASITES, section VI:E).

Reference
VI:G. Selection of cytoadherent parasites by passage over C32 melanoma cells, CHO, HUVEC, or other endothelial cells

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Equipment

centrifuge (Beckman Allegra 6R)
incubator (37 °C)
15-mL Falcon tube (BD Labware)
inverted microscope (125×)

Materials and reagents

malaria culture of 8% parasitemia or higher (e.g., enriched) with a majority of the parasites in trophozoite stage
binding medium (see PARASITES, section VI:A above)
25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence
complete malaria culture medium (see PARASITES, section I:A)
human erythrocytes blood type O⁺ (see PARASITES, section I:A)

Selection of cytoadherent parasites

• Use malaria cultures with a parasitemia exceeding 8%; lower parasitemias are not worthwhile, because the binding will be too low. The majority of the parasites should be in the trophozoite stage.
• Transfer the malaria culture to a 15-mL Falcon tube and centrifuge at 500 × g for 5 min at room temperature.
• Wash the culture 3 times (500 × g) in binding medium and resuspend in binding medium at a hematocrit of 2 to 3%.
• Use a 25-cm² culture flask covered with melanoma/endothelial cells at approximately 80% confluence.
• Remove growth medium and wash the melanoma/endothelial cells once with binding medium.
• Overlay the cells with the malaria culture suspension.
• Incubate the cells at 37 °C for 1 to 1.5 h. Do not use a CO₂ incubator.
• Resuspend the cells by gently rocking the flask every 15 min.
• Wash the flask gently 3 times with binding medium to remove unbound erythrocytes.
• Check binding of erythrocytes using an inverted microscope.
• Remove binding medium and add complete malaria culture medium and washed fresh human erythrocytes (see PARASITES, section I:A) at 5% hematocrit to the culture flask.
• Transfer the culture to a new flask the next day (after merozoite reinvasion of RBC).
• Continue culturing (see PARASITES, section I).

Reference

VI:H. Binding of fluorescent receptors heparin, blood group A, and PECAM-1/CD31 to
*Plasmodium falciparum*-infected erythrocytes

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Equipment

- PCR tubes
- centrifuge (500 × g)
- microcentrifuge (500 × g)
- glass slides
- coverslips
- UV light microscope (100× magnification)

Materials and reagents

- malaria culture (with parasites in trophozoite stage)
- RPMI 1640
- ethidium bromide
  
  **CAUTION:** This chemical is toxic and mutagenic. Wear protective clothing and
  use only in a chemical fume hood.

for heparin:

- fluorescein-conjugated heparin (2 mg/mL, Molecular Probes)

for blood group A:

- fluorescein-conjugated blood group A (1 mg/mL A<sub>α</sub>-PAA-flu, Syntosome)

for CD31:

- recombinant PECAM-1/CD31 (800 µg/mL, R&D Systems)
- Alexa Fluor 488 Protein Labeling Kit (Molecular Probes)

Binding of fluorescent receptors

PECAM-1/CD31 is bought unlabelled and must therefore be labelled with a fluorophore
before the experiment. Use the Alexa Fluor 488 Protein Labeling Kit to label 400 µg of
CD31 in 0.5 mL of PBS. Follow the protocol provided by Molecular Probes.

- Wash the culture (5% hematocrit) twice in RPMI 1640 (500 × g) and resuspend
to original volume.
- Aliquot 30 µL of washed culture in PCR tubes (0.6 mL). Make one tube for each
receptor to be investigated.
- Centrifuge the PCR tubes in a microcentrifuge (500 × g), remove the
supernatant, and resuspend the cells to a volume of 26 µL with RPMI 1640.
- Add 4 µL of the fluorochrome-conjugated receptor to each PCR tube and mix
(regarding CD31 see note below).
- Incubate the cells for 30 min in the dark with resuspension after 15 min.
- Wash the cells 3 times with RPMI 1640 (500 × g) and resuspend them to 30 µL in
RPMI 1640.

When using heparin-FITC or blood group A-FITC, the fluorescence might be very weak.
A possibility is to amplify the signal by using Alexa 488 labelled αFITC (antifluorescein,
goat IgG fraction, Alexa Fluor 488 conjugate from Molecular Probes) in a second
incubation (dilution 1:8, 30 min at room temperature in the dark). After this second
incubation, wash the cells 3 times as before and continue as described below.
• Resuspend the cells in 30 µL of RPMI 1640 and mix 10 µL of cells with 0.5 to 1 µL of ethidium bromide (1 µL/ml) on a glass slide. Alternatively, to reduce the amount of background, incubate the cells in the PCR tube with ethidium bromide (1 to 2 µL; 1 µg/mL) for 5 to 10 s before the last wash.

If fluorescence is weak, ethidium bromide may be skipped altogether as the infected cells can be detected in the microscope by the presence of pigment.

• Add a coverslip and count the fluorescence rate at 100× magnification with oil in a UV-light microscope.

• Calculate the fluorescence rate as \[ \frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}} \]

References

VI:I. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized receptors

*by James G. Beeson*

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**Equipment**

- microscope
- Class 2 safety cabinet (laminar flow)
- centrifuge

**Materials and reagents**

- marking pens, indelible and suitable for plastic
- 150-mm plastic (polystyrene) tissue culture (petri) dishes
- Dako Pen for Immunohistochemistry (Dako Corp) or similar, OR plastic sealing film (e.g., Parafilm, Nescofilm)
- liquid paraffin oil
- glass coverslips
- phosphate-buffered saline (pH 7.2) OR Tris-buffered saline (pH 7.4)
- RPMI–HEPES (pH of 6.8 to 7.4) without bicarbonate added
- bovine serum albumin
- pooled nonimmune human serum OR nonimmune AB serum
- 2% glutaraldehyde in PBS
- Giemsa stain
- purified receptors
- adhesion medium: RPMI–HEPES (pH of 6.8 to 7.4), plus EITHER 10% human serum (pooled or AB serum from nonexposed donors) OR 1% BSA

**Procedure** (Beeson et al. 1999)

- On the underside of a plastic petri dish, mark approximately 10-mm diameter circles for the required number of test spots (usually 3 spots for each receptor being tested.) Number or label the circles accordingly.
- Inside the petri dish, on the base, use a Dako pen for immunohistochemistry to copy circles over those drawn above. This creates a circle of plastic that will prevent the parasite suspension being tested (see step 8) from spreading. Alternatively, cut holes in a layer of plastic sealing film using a hole punch and carefully press the plastic film onto the base of the petri dish (see Beeson et al. 1998). Use a blunt instrument (e.g., top of a plastic pen) to press the film on completely, taking care around holes to form a good seal with the plastic dish.
- Place 5 to 10 µL of each receptor, diluted in PBS or TBS, in the center of each circle.
- Incubate the dishes overnight at 4 °C in a humid box to ensure the receptor spots do not dry out.
- Before performing the adhesion assay, block nonspecific binding by placing a drop or two (50 to 100 µL) of 1% BSA in PBS over each receptor spot. Incubate for at least 30 min at room temperature.
- Prepare the parasites in a Class 2 cabinet:
  - Spin down parasites from culture or patient sample at low speed.
  - Wash parasite-RBC pellet once with RPMI–HEPES.
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- Remove the supernatant and resuspend the pellet in cytoadherence medium (RPMI–HEPES plus either 10% human serum OR 1% BSA) to the required hematocrit, usually 1 to 5%. We typically use mature pigmented trophozoite-infected RBCs at a parasitemia of 1% or higher.
  - Wash the plate with either PBS or RPMI–HEPES by adding 25 mL of buffer, agitating, and discarding the liquid. Repeat once.
  - Aspirate, using suction, the remaining buffer from each spot and then add 40 µL of parasite suspension to each well (performed in Class 2 safety cabinet).
  - Incubate the cells at 37 °C for 30 min (no agitation).
  - Gently wash off unbound cells with RPMI–HEPES (pH of 6.8 to 7.4) or PBS (pH of 7.2 to 7.4).
    - Add 25 mL of buffer carefully to one side of the dish using a pipette. Do not add the buffer directly over receptor spots.
    - Rock the dish gently to remove unbound cells and aspirate the supernatant.
    - Wash the dish 4 to 6 times in total.
  - Fix bound cells with 2% glutaraldehyde in PBS for at least 2 h.
  - Stain the cells with 10% Giemsa for 10 min, wash off stain with water, and allow to dry. Do not overstain as it becomes difficult to distinguish infected from uninfected RBCs.
  - Once they are dry, cover the spots with liquid paraffin oil (or clear vegetable oil) and a glass coverslip.
  - Count bound infected-RBCs microscopically, expressing counts as bound parasitized RBCs/mm². Compare adhesion to receptors to that of BSA-coated control spots to account for any nonspecific adhesion.

Comments
Steps 6 – 10 should be performed in a Class 2 safety cabinet.

Purified chondroitin sulfate A (CSA) – We used CSA from bovine trachea at 10 µg/mL, or from porcine rib at 50 µg/mL. This works well for most isolates. CSA can be coupled to phosphatidylethanolamine to improve immobilization on plastic (Rogerson et al. 1995).

Different plastic culture dishes can be used in these assays. We generally use Falcon 1058, but others also work.

Various compounds, serum, or antibodies can be tested for inhibitory activity by incubating parasites with the inhibitor for 5 to 45 min prior to step 7 (see Beeson et al. 1998, Reeder et al. 1999).

References


VI:J. Adhesion of *Plasmodium falciparum*-infected erythrocytes to immobilized hyaluronic acid
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Hyaluronic acid (HA) preparations
HA from bovine vitreous humor (Sigma) is isolated by strong anion exchange chromatography and generally has little or no detectable chondroitin sulfates (Chai et al. 2001). Other sources of HA, but not all, also support adhesion. Some preparations of HA may contain significant amounts of copurified chondroitin sulfates.

Immobilization of HA onto plastic
Immobilization is generally effective with PBS or TBS (pH of 7.2 to 7.4) as the coating buffer, and parasite adhesion is enhanced by including calcium in the coating buffer (and in all other steps). HA effectively adsorbs to the surface of Falcon 1058 plastic dishes (tissue culture treated, Becton–Dickinson).

Optimum conditions for adhesion
- Adhesion of parasites to HA is highest at pH 7.4.
- Include calcium in all steps.
- Use mature pigmented trophozoites in assays rather than schizonts.
- Fixation of bound parasites with glutaraldehyde (see PARASITES, section VI:A) may decrease observed adhesion to HA. It may be necessary to count numbers of cells bound to HA prior to fixation.

Materials and reagents
- hyaluronate lyase (hyaluronidase) from *Streptomyces hyalurolyticus*
- hyaluronic acid isolated from bovine vitreous humor
- phosphate-buffered saline

Step-by-step procedure for preparation and use of lyase-treated hyaluronic acid as a control in adhesion assays
- Incubate HA (1 mg/mL) with and without hyaluronate lyase (10 to 20 TRU/mL) in PBS, at 60 °C for 4 h or overnight.
- Stop incubation by boiling HA samples for 10 min at 100 °C. Cool samples and centrifuge them at high speed to remove any insoluble aggregates.
- Make aliquots of treated and untreated HA samples and store them at −20 °C.
- To measure parasite adhesion to HA, dilute lyase-treated and untreated HA samples 1:10 (i.e., a final HA concentration of 100 µg/mL) in PBS and coat plastic surfaces with it as described above (see PARASITES, section VI:A).
- Perform adhesion assay as described above (see PARASITES, section VI:A).
- Interpretation: Adhesion of parasites to nonlyase-treated HA but not to lyase-treated HA indicates specific adhesion to HA.
References


VI:K. Binding of Plasmodium falciparum-infected erythrocytes to placental tissue sections
by Kirsten Flick and Carin Scholander
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e-mail: kirsten.flick@mtc.ki.se

See PARASITES, section VII:B
VI:L. Enrichment of rosetting parasites using Ficoll–Isopaque (Pharmacia)

by Victor Fernandez and Antonio Barragan

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Equipment
immufuge blood centrifuge

Materials and reagents
Ficoll–Isopaque (FIP) (Pharmacia)
RPMI 1640
MCM with 10% heat-inactivated serum

Protocol
• Use 2 mL of culture per tube, mature and rosetting parasites, with a 5 to 10% parasitemia and layer it very carefully on 2 mL of sterile, ice-cold Ficoll–Isopaque (FIP).
• Centrifuge the tube in the immufuge blood centrifuge for 12 s on the high setting at room temperature.
• Quickly suck up the pellet using a Pasteur pipette.
• Wash the pellet 3 times using RPMI 1640.
• Subculture (see PARASITES, section I) using fresh, washed erythrocytes and MCM with 10% heat-inactivated serum.

Reference
VI:M. Reversion of rosettes

by Victor Fernandez and Antonio Barragan

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Equipment
UV-light microscope (40×)

Materials and reagents
96-well microtiter plate
Parafilm
acridine orange

Procedure
• Keep the Plasmodium falciparum culture under standard conditions with 10% normal serum added to the buffered medium (see PARASITES, section I:A).
• Perform the studies on cultures with 5% hematocrit, 4 to 8% parasitemia at late stage, and >50% rosetting rate.
• Mix an aliquot of 25 to 50 µL of the rosetting P. falciparum culture (1:1) with various dilutions (e.g., 1:5) of sera to be tested. Also prepare a control using nonimmune human sera or MCM (see PARASITES, section I:A).
• Incubate the cultures at 37 °C for 60 min in a 96-well microtiter plate. Cover the plate with Parafilm.
• Use two separate aliquots from each well, mix each with a small amount of acridine orange, and mount them on glass slides. Count 25 consecutive fields of vision per slide using a 40× lens and incident UV light (2 slides/well, approximately 50 fields). Count fields of vision diagonally over the slide, from one corner to the other, in order to compensate for a possible uneven distribution of rosettes on the slide. If the cell concentration is low, it may be necessary to count in both diagonal directions.

Comments
Infected erythrocytes (except ring forms) within a rosette and infected erythrocytes not connected to a rosette are scored separately.

Infected erythrocytes that have bound two or more noninfected cells are scored as rosettes. If the rosette contains more than one infected erythrocyte, the cells are scored separately.

Ring forms within a rosette are not counted.

Late-stage (trophozoite and schizont, but not ring forms) infected erythrocytes are scored and added to the total number of late infected erythrocytes.

Since it can be hard to tell the difference between a ring form and an early trophozoite, only parasites with a diameter that covers at least one-third of the diameter of the erythrocyte are counted.

The number of infected erythrocytes in large rosettes (where it can be difficult to determine the exact number of infected cells) are counted as 3.
If it is difficult to determine the number of cells (usually 2 or 3) bound to an infected erythrocyte, the coverslip can be pressed lightly (using a pencil) to induce a slight movement. This is important, because quite often it is found that only 1 erythrocyte is actually bound to the infected cell, when others that may appear to be are not.

Use two controls, one before counting the samples and one after.

Reference
VII. Placental malaria

VII:A. Isolation of *Plasmodium falciparum*-infected erythrocytes from the placenta

_by James G. Beeson and Stephen J. Rogerson_

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Equipment

- microscope
- safety cabinet (laminar flow)
- centrifuge
- rotating wheel or tube roller
- protective eye wear and face mask

Materials and reagents

- 50-mL plastic tubes
- scalpel or scissors, forceps
- phosphate buffered saline with 50 mM EDTA

Procedure (Beeson et al. 1999)

- Collect a placenta as soon as possible after delivery, and determine whether it is infected by performing a blood smear. Orient the placenta so the maternal side is facing up. Clean the surface with PBS if necessary.
- From apparently healthy areas of the maternal surface, cut several pieces of placental tissue (approximately 1.5 to 2 cm in each dimension, up to 6 cm³) from different areas using either a scalpel or scissors and forceps. Immediately put cut pieces of placental tissue into 50-mL tubes containing PBS with 50 mM EDTA. Several pieces can be placed into a single tube, but the placental tissue should occupy no more than one-third of the volume. Wear protective eyewear and a face mask during this procedure.
- Mix tubes containing placental tissue on a rotating wheel or tube roller for 1 h at room temperature. To increase yield of placental blood and parasites, placental tissue can be cut into smaller pieces or finely chopped after 30 min, and the incubation then continued for a further 30 min. Doing this does not appear to significantly influence the parasitemia or parasite phenotypes of the preparation.
- Stop incubation, then compress the placental tissue with a blunt instrument to further remove any blood and parasites. Let tubes stand for one minute to allow tissue pieces and debri to settle.
- Collect the supernatant, then centrifuge and wash the pellet 3 times with PBS.
- Prepare a thin smear and determine the parasitemia of the preparation.
- Leukocytes can be depleted by passing a suspension of the placental blood over a cellulose column or over Percoll density gradients.
- If necessary, the parasitemia can be enriched by passage over Percoll density gradients or by other methods, as described elsewhere.

References

VII:B. Binding of Plasmodium falciparum-infected erythrocytes to placental tissue sections

by Kirsten Flick and Carin Scholander

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e-mail: kirsten.flick@mtc.ki.se

Equipment
- cryostat at −20 °C

Materials and reagents
- binding medium:
  - 5.2 g RPMI powder
  - 3.0 g HEPES
  - 500 mL distilled H₂O
- liquid nitrogen
- methanol
- distilled H₂O
- hyaluronidase: 10 µg/mL enzyme in PBS (pH 7.2)
- chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6)
- heparinase: 0.5 units/mL in PBS
- PBS
- slides with 8 wells of 8 mm or 10 wells of 6 mm each (Novakemi AB)
- coverslips
- 50-mL Falcon tubes

Placenta sections:
- Cut fresh placental tissue into small cubes (≈ 1 × 1 cm) and freeze immediately in liquid nitrogen. Transfer them later to −70 °C for storage.
- Adapt the placental tissue cubes to −20 °C for 1 h before use.
- Cut 5-µm thick sections with a cryostat at −20 °C.
- Transfer the sections to 8- or 10- well slides (see below).
- Keep the slides with sections at −70 °C. The sections should not be older than 1 week when used in experiments.
- For use, remove a slide from −70 °C, transfer it immediately into cold PBS, and keep it in PBS until use.

Preparation of PRBC:
- Enrich the infected cells for late trophozoites in a Percoll-gradient or by MACS purification.
- Keep the cells as packed cells on ice after purification.
Binding of PRBC to placenta sections:
- Resuspend 1 to 2 µL of packed cells in 23 to 24 µL of binding medium.
- Add those 25 µL to one well containing a placental section, forming a drop on top of the section.
- Incubate the section for 1 h at 37 °C.
- Tip off the drop carefully.
- Wash the section 3 times for 5 min in binding medium: Use three 50-mL Falcon tubes with 40 mL of binding medium each; let the slide stand in each for 5 min.
- Fix the sections on a slide for 15 s in methanol.
- Let them air dry.
- Stain the sections with 5% Giemsa for 30 min (overlay sections with a drop of Giemsa stain).
- Wash away the Giemsa stain with distilled H2O.
- Put a coverslip over the sections.

Counting of bound PRBC in the microscope:
- Count from upper left corner to lower right corner of the section, field next to field at 400× magnification.
- Repeat from upper right corner to lower left corner.
- Determine bound PRBC/field, calculate to PRBC/mm².

Binding of PRBC to placenta sections under influence of inhibitor molecules:
- Incubate the placenta sections with 25 µL of binding medium or PBS containing the inhibitor for 1 h at 37 °C.
- Wash the sections for 3 min with PBS in a petri dish placed on a slow-moving shaker (see below).
- AND/OR: Resuspend PRBC in binding medium containing the inhibitor and incubate them for 1 h at 37 °C.
Perform the binding assay as described above. Include at least 2 wells with a positive control (i.e., with no inhibitor present) on each slide to control the quality of the experiment.

**Enzyme treatment of placenta sections:**
- Use a volume of 25 μL on each section:
  - hyaluronidase: 10 μg/mL of enzyme in PBS (pH 7.2); incubate at 37 °C for 45 min.
  - chondroitinase: 0.5 units/mL in Tris–HCl (pH 8.6); incubate at 37 °C for 10 to 30 min.
  - heparinase: 0.5 units/mL in PBS, incubate at 37 °C for 30 min.
- Wash the sections for 3 min with PBS in petri dishes.
- Include at least 2 wells with a positive control (i.e., no enzyme treatment) on each slide to control the quality of the experiment.

Perform the binding assay as described above.

**Reference**
VIII. Detection of antibodies to the infected erythrocyte surface

VIII:A. Reversion of rosettes

By Victor Fernandez and Antonio Barragan
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See PARASITES, section VI:M

VIII:B. Agglutination assay using purified trophozoite-infected erythrocytes. Measurement of antibodies to *Plasmodium falciparum* variant antigens on the surface of infected erythrocytes

By James G Beeson and Emily J. Mann
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Materials and reagents
- phosphate-buffered saline (PBS)
- microscope slide
- glass coverslips
- mounting medium (e.g., Depex)
- 96-well microtitre plates (Eppendorf tubes [1.5 mL], or similar)
- Giemsa staining (see PARASITES, section III:B)
- human serum
- methanol

Procedure
- Isolate trophozoite-infected RBCs (not schizonts) from culture using Percoll density gradients, gelatin, or other methods, described elsewhere.
- After harvesting parasites, carefully and gently wash 3 times with warm PBS.
- Make a smear, stain it with Giemsa, and examine the parasite preparation. Check for intact parasite-infected RBCs. Aim for at least 50% parasitemia (higher is better).
- Incubate the parasites with human serum diluted 1:10 for 45 min on a rotating wheel at room temperature. This is best done by putting 2.5-µL aliquots of each test serum into individual wells of a 96-well microtitre plate (or using Eppendorf tubes). Add 22.5 µL of parasite suspension (at a 2% hematocrit) to each well containing test serum. Always include a negative control serum. Parasites harvested from 25 mL of culture (5 to 10% parasitemia, 3% hematocrit) can usually be resuspended in 0.5 to 1.0 mL of PBS.
- Following incubation, pipette out 10 µL of the suspension and spread it gently with a plastic pipette tip onto a glass slide, making a circle of about 1.5-cm diameter. Make 2 smears for each sample on the same or separate slides. [Alternatively, to examine cells as ‘wet preps’, place a coverslip over a 10-µL drop (do not smear out) and examine by microscopy (see below)].
- Allow the smear to dry for 5 min.
- Fix the smears in fresh methanol.
• Stain the smears for 5 to 10 min with 10% Giemsa, but do not over-stain. Once
the smears are dry, mount coverslips using a permanent mounting medium (e.g.,
Depex). Slides can then be stored (protected from light) for long periods and
examined or re-examined at any time.
• Examine both smears for each sample by light microscopy.
  – Initially scan for agglutinates using a 10× objective.
  – Examine any agglutinates at 40×.
  – Consider as positive only those agglutinates of 5 infected cells or more.
  – Record the percentage of parasitised RBCs in agglutinates and the size of
    the largest agglutinate observed.

Comments
Count only agglutinates of parasitised RBCs. Don’t count agglutinates of parasites that
have broken out of the RBC membrane (which frequently and easily agglutinate). Make
sure you can see an intact RBC membrane around cells in agglutinates.

Watch out for agglutinates of cells with debri. Agglutinated cells must be in contact with
each other.

Do not count linear agglutinates. Cells lined up in a row are rarely true agglutinates. Find
an area where cells are evenly distributed.
VIII:C. Serum micro-agglutination of infected erythrocytes
by Antonio Barragan
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Equipment
UV light microscope
rotator

Materials and reagents
RPMI 1640/HEPES/sodium bicarbonate/ gentamycin solution (MCM; see PARASITES, section I:A)
acridine orange (see PARASITES, section III:A)
polystyrene round-bottomed tubes

Procedure
• Culture parasitized red blood cells (PRBC) to the late trophozoite and/or early schizont stage and wash the cells 3 times in MCM; resuspend the PRBC at 20% hematocrit.
• Dispense aliquots of 25 µL in prelabelled polystyrene round-bottomed tubes.
• Add 25 µL of serum to the parasite suspension (1:2 dilution). For a 1:5 dilution serum test, add prediluted serum at 1:2.5 in MCM.
• Incubate the PRBC and serum mixture at 37 °C for 1 h with constant rotation.
• Mount an aliquot from each polystyrene tube, mix with a small amount of acridine orange on a glass slide, and count 50 consecutive fields of vision diagonally using a 40× lens and incident UV-light microscopy.
• Always use negative and positive control sera along with the test sera.

Interpretation
• Score the assay as negative when no agglutinates of 4 or more PRBC are detected in the examined slide.
• Use the following semiquantitative scoring scale for agglutination analysis:
(-) for no agglutinate of 4 or more PRBC
(1+) for 1 to 5 agglutinates of 4 to 10 PRBC
(2+) for >5 agglutinates of 4 to 10 PRBC or 1 to 5 agglutinates of 11 to 20 PRBC
(3+) for >5 agglutinates of 11 to 20 PRBC or 1 to 5 agglutinates of >20 PRBC
(4+) for >5 agglutinates of >20 PRBC
(UA) for unspecific agglutination
• When using rosetting strains, note the presence or absence of rosettes.

References

VIII:D. Flow cytometry detection of surface antigens on fresh, unfixed red blood cells infected with *Plasmodium falciparum*

by Helene Jouin

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The indirect immunofluorescence labeling is performed as usual on living cells in suspension, and parasitized erythrocytes are selected by gating the fluorescence intensity of either Hoechst 33342 or thiazole orange (TO) dyes, which reflect the erythrocyte content of parasitic DNA or RNA. TO allows identification of only mature stages of the parasite. Nevertheless, since we have not observed surface immunostaining of the young stages of the parasite when using Hoechst, TO is a good reagent because it allows performing the flow cytometric analysis with a single laser analyser.

**Materials and reagents**

- Hoechst 33342 (Sigma)
- thiazole orange (TO) (Retic-COUNT, BD Immunocytometry Systems)
- FACSFlow solution (BD Immunocytometry Systems) containing 2% fetal calf serum

**General procedures for DNA or RNA staining, surface immunofluorescence staining, and flow cytometry analysis**

When Hoechst is used, the DNA staining is performed first, followed by surface immunofluorescence staining (using FITC or phycoerythrin conjugated second antibody). It is necessary to use two lasers because of the differing excitatory wavelengths of the two fluorochromes.

When TO is used, surface immunofluorescence staining is performed before the RNA staining. The samples can be analysed on a single laser (488 nm). Since TO fluorescence is read using the FITC channel, FITC conjugates cannot be employed for surface immunofluorescence.

**Intraerythrocytic parasite DNA staining with Hoechst 33342 (before surface immunofluorescence staining)**

- Dissolve Hoechst 33342 at 1 mg/mL of distilled water.
- Add the solution directly to the culture medium at a final concentration of 20 µg/mL.
- Incubate the culture for 30 min at 37 °C in the dark.
- Wash the culture in PBS and then perform the immunofluorescence staining.

**Intraerythrocytic parasite DNA staining with TO (after immunofluorescence staining)**

- Resuspend the washed immunofluorescent stained parasitized red blood cells directly in 1 mL of a solution of TO.
- Keep in the dark for 30 min prior to flow cytometric analysis.

**Surface immunofluorescence staining**

- Resuspend a pellet of $5 \times 10^6$ parasitized red blood cells in 100 µL of immune serum diluted 1:50 in FACSFlow solution containing 2% fetal calf serum.
• Incubate the pellet at room temperature for 30 min.
• Wash it 3 times with FACSFlow-FCS.
• Resuspend the pellet in the appropriate fluorescent-conjugated antibody.
• Incubate it at room temperature for 30 min.
• Wash it twice with FACSFlow-FCS.
• Resuspend it in 1 mL of FACSFlow.

**Flow cytometric analysis**
Parasitized red blood cells that have been stained with Hoechst 33 342 must be analysed on a cell sorter equipped with two lasers: a UV laser tuned to 320 nm for the excitation of the Hoechst dye and a second laser tuned to 488 nm for the excitation of FITC and phycoerythrin.

Parasitized red blood cells which have been stained with TO and phycoerythrin conjugates can be analysed on a standard flow cytometer equipped with only one laser tuned to 488 nm. The TO emission is detected in the FITC channel. The color compensation between TO and phycoerythrin fluorescences is set up using a control sample (without surface immunofluorescence staining).

Erythrocytes are gated on the basis of their forward scatter and side scatter signals using logarithmic scales. Parasitized erythrocytes are gated on the basis of their positive staining with Hoechst 33 342 or TO. All fluorescence parameters are recorded with logarithmic amplification. Process list mode data from 10,000 gated cells.

**Reference**
VIII:E. Analysis of plasma antibodies to variant surface antigens by flow cytometry

by Trine Staalsoe
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Equipment
2-color flow cytometer

Materials and reagents
PBS + 2% FCS (PBS2)
Plasmodium falciparum late-stage infected erythrocytes purified by magnetic separation (see PARASITES, section IV:H) at 2 × 10^6 erythrocytes/mL in PBS2
ethidium bromide (0.1 mg/mL in PBS)
   CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
human plasma or serum
_goat anti-human IgG antibody (DAKO A473)
FITC-conjugated rabbit anti-goat Ig antibody (DAKO F250)

Procedure
Antibody Labeling
• Add ethidium bromide to the erythrocyte suspension (20 µL/mL).
• Mix 100 µL of ethidium bromide-labelled erythrocyte suspension with 5 µL of test plasma/serum and incubate the tube for 30 min at 5 °C.
• Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 µL of PBS2.
• Mix the erythrocyte suspension with anti-human antibody (diluted 1:250) and incubate it as above.
• Wash the suspension twice in 3 mL of PBS2 and resuspend the sample in 100 µL of PBS2.
• Mix the erythrocyte suspension with anti-goat antibody (diluted 1:25), and incubate it as above.
• Wash the suspension once in 3 mL of PBS2 and resuspend the sample in 200 µL of PBS2.
• After antibody Labeling, samples may be kept at 5 °C overnight before analysis.

Data acquisition by flow cytometry
• Adjust flow cytometer settings to achieve a clear separation of uninfected (ethidium bromide-negative) and infected (ethidium bromide-positive) erythrocytes in an FSC/ethidium bromide dot plot (see Fig. 1 in Staalsoe et al. 1999).
• Acquire and store data on FCS, SSC, FL1 (FITC), and FL2 (ethidium bromide).

Data analysis
• Flow cytometry data files (FCS format) can be analysed in any of the many software packages available. We usually use either the freeware WinMDI program (which is good for generating overlays and other graphics for publication) or WinList (which is a very powerful analysis program with wonderful
possibilities for creation of macros that make a breeze of the analysis of the hundreds of files you will often need).

- Set a gate around late-stage infected erythrocytes in an FSC/ethidium bromide diagram plot (similar to R3 in Fig. 1D in Staalsoe et al. 1999).
- Use the FITC fluorescence of the cells within this gate to quantify antibody recognition of variant surface antigens. This can be expressed either as the mean/median fluorescence or as the percentage of cells with FITC fluorescence above that of a control sample incubated without plasma/serum or with control plasma/serum (viz., Fig. 2 in Staalsoe et al. 1999).

Reference
IX. Immunoglobulin- or serum protein-binding to infected erythrocytes

IX:A. Stripping erythrocytes of bound serum proteins and reformation of rosettes

by Fredrik Pettersson and Carl Johan Treutiger

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Equipment
sterile test tubes
centrifuge (500 × g)
96-well plates
microscope (400×)

Materials and reagents
500 µL of a rosetting malaria culture (5% hematocrit, rosetting phenotype with a majority of the parasites in trophozoite stage)
PBS (pH 7.2)
heparin, 25000 IU/mL (Leo Pharma)
sodium citrate

RPMI 1640–HEPES medium (50 mL):
0.52 g RPMI 1640 medium (with glutamine, without NaHCO₃; Gibco)
0.3 g HEPES (Gibco)
45 mL distilled water
Mix and set pH to 6.8.

Disruption of rosettes with heparin
Since the rosettes of every parasite strain differ in their heparin-sensitivity, you will have to titrate the necessary heparin-concentration (e.g., FCR3S1.2: 100 U/mL).

• Transfer 500 µL of culture to an appropriate test tube and add the necessary amount of heparin.
• Incubate the culture for 15 min before the next step.

Treatment with sodium citrate to disrupt heparin resistant rosettes

• Centrifuge the culture at 500 × g and remove the supernatant.
• Add 500 µL of PBS with 100 mM sodium citrate (pH 7.4) to the pellet and incubate it for 5 min.
• Wash the pellet twice with PBS; centrifuge it each time (500 × g) and remove the PBS.
• Add 0.25 mL of RPMI 1640–HEPES to the pelleted malaria culture (the hematocrit will be 10%).

You may include 10% H-Albumin (Sigma), but this can interfere or block cell interactions and the binding to solid surfaces.

Rosette reformation

• Prepare the test substance (e.g., sera or proteins) in 2× concentration of above RPMI 1640 medium.
• Mix 25 µL of the washed culture (10% hematocrit; as above) with 25 µL of the test substance in a 96-well plate. The final hematocrit will be 5% (H-Albumin concentration, if included, will be 5% as well).
• Incubation probably works as well at room temperature as it does at 37 °C for 15 min.
• Read the rosetting rate (=reformed rosettes) using acridine orange (see PARASITES, section III:A)

Reference
IX:B. Detection of serum proteins on the surface of *Plasmodium*-infected erythrocytes

by Fredrik Pettersson and Carl Johan Treutiger

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Equipment

- Centrifuge (500 × g)
- Appropriate test tubes for centrifuge
- Microfuge tubes
- Glass slides
- Coverslips
- UV-light microscope (100×)

Materials and reagents

- *Malaria* cultures (5 to 10% trophozoites)
- Antibody against serum protein to be detected (e.g., αIgM)
- Fluorochrome conjugated secondary antibody
- RPMI 1640
- Ethidium bromide (1 µg/mL)

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

**Immunofluorescence**

- The cultures to be tested should have a parasitemia of 5 to 10% with a majority of parasites in trophozoite stages.
- Transfer the culture to appropriate test tubes and wash (500 × g) it twice in RPMI 1640. Add RPMI 1640 to the initial volume.
- Dilute antibody to double final concentration of desired dilution in RPMI 1640. (A dilution factor of two will be introduced during the assay because of cell suspension.)
- Aliquot 50 µL of each dilution of antibody in small tubes (e.g., microfuge tubes) and add 50 µL of resuspended culture.
- Mix by shaking the tubes gently and incubate them for 30 min in the dark at room temperature with careful resuspension after 15 min.
- Shake the tube gently after 15 min.
- Wash the cells 3 times (500 × g) in 0.5 mL of RPMI 1640.
- Remove the supernatant carefully and add 100 µL of secondary antibody diluted to the final concentration. Mix gently.
- Incubate the cells for 30 min in the dark with careful resuspension after 15 min and wash them as before.
- Resuspend the cells in 40 µL of RPMI 1640 and mix 10 µL of cells with 0.5 to 1 µL of ethidium bromide (1 µg/mL) on a glass slide.
- Add a coverslip and count the fluorescence rate at 100× magnification with oil in a UV-light microscope.

- Calculate the fluorescence rate as: \[ \text{fluorescence rate} = \frac{\text{number of fluorescent cells}}{\text{total number of late trophozoites}} \]
Note: To reduce the amount of background the cells can be incubated with ethidium bromide (1 to 2 µL; 1 µg/mL) for 5 to 10 s before the last wash instead of being mixed with ethidium bromide on the slide.

Note: Count all nonfluorescent cells with the corresponding size of the parasite as the parasites in the fluorescent cells. This is important to achieve the correct fluorescence rate, which could otherwise be too high.

Reference
IX:C. Binding and eluting antibodies from the *Plasmodium*-infected RBC surface

by Fredrik Pettersson and Martha Schlichtherle

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Equipment
- immufuge blood centrifuge
- round-bottomed test tubes (e.g., 5-mL snap-cap tubes)
- microfuge tubes
- high speed microcentrifuge with cooling system
- high quality pH paper

Materials and reagents
- *P. falciparum* culture with a parasitemia of >8%. (If rosetting is of interest, the rosetting rate should be >50%.)
- serum or antibody solution to be investigated
- RPMI 1640
- PBS (optional)
- acridine orange

Solution A:
- 0.2 M glycine / 0.2 M NaCl:
  - 3.75 g glycine
  - 2.9 g NaCl
  - Bring to 250 mL in distilled water.

Solution B: (0.2 M HCl)
- Mix 1.64 mL of concentrated HCl into 98.4 mL of distilled water.
  **Note:** Mix acid into water, never vice versa with concentrated acids.

Glycine–HCl elution solution (pH 3):
- Mix 5 mL of solution A and 1.3 mL of solution B.
- Adjust to pH 3 using a high quality pH paper or pH meter.
- Make fresh for each day of elution.

Neutralization solution: (2 M Tris-base)
- 9.69 g Tris-base
- Bring to 40 mL; pH will be ~11.

Regarding disruption of rosettes and stripping of serum bound proteins, see Materials and reagents in PARASITES, section IX:A..

If using Percoll/sorbitol gradients to separate trophozoites for binding antibodies to the parasite, see Materials and reagents in PARASITES, section IV:D.

Procedure
- **Use at least 1 mL of culture where the parasitemia is >8 to 10%. (If rosetting is of interest, the rate should be >50%; see PARASITES, sections VI:L, M.)**
• If purified trophozoites are desired for the antibody binding, use Percoll/sorbitol gradients as in PARASITES, section IV:D. Have a large prep ready with either 12 small bottles (25-cm²) or 3 large ones (75-cm²) of culture for 8 Percoll gradients. Follow the protocol for gradients in section IV:D, then resuspend purified cells in 0.5 to 1 mL of RPMI 1640 before the addition of serum/antibodies, followed by the protocol below from the incubation/elution step onward.

• If using the culture straight as it is without trophozoite purification, place the culture in a round-bottomed tube holding a few milliliters and fitting an appropriate blood centrifuge (e.g., a 5-mL snap-cap tube in an immufuge blood centrifuge). Disrupt rosettes using heparin and a syringe, then strip cells of serum-bound proteins (see PARASITES, section IX:A above).

• After the sodium citrate treatment of cells, wash them once with PBS and then with RPMI 1640 (see section IX:A above).

• Add an equal volume of serum or antibody solution to the cell pellet as the starting volume of culture (i.e., mg/mL concentrations of antibody).

• Gently mix and slant the tube so that the blood solution almost reaches the surface of the tube. Carefully roll to mix the settling RBCs every 15 min or so. Incubate the tube for 2 to 3 h at room temperature.

• While the tube is incubating, prepare the glycine–HCl elution solution. At the end of the incubation, check that RBCs look normal through acridine orange vital staining (see PARASITES, section III:A).

• Spin down the cells (500 × g), remove the supernatant, and wash the cells twice with PBS.

• Elute bound antibodies by adding glycine–HCl elution solution to cells in the amount of the starting culture volume. Incubate them for 1 to 1.5 min only! (It is possible that the bound antibodies can be eluted with less elution buffer for increased antibody concentration; this would have to be tested empirically.)

• Quickly transfer the cells to 2 microfuge tubes and centrifuge them at 8,000 to 10,000 rpm in a chilled centrifuge for 1 min.

• Taking care not to touch the cell pellets, remove the supernatant with the eluted antibodies and pool them into one tube.

• To each milliliter of elution, add 26 to 30 µL of 2 M Tris-base. Mix carefully and use 4 µL to spot on a high quality filter paper. Check the pH. Add more Tris-base, 1 µL at a time, until the pH reaches 7.0 to 7.2. Check the pH after each addition. If the eluate becomes too basic, lower the pH by adding 10 to 20 µL of 0.2 M HCl (= solution B).

• Remove the precipitate that may form by spinning the eluate for a minute in the blood centrifuge.

• If you wish, you may dialyze the eluate overnight against a few changes of PBS in the cold, stirring it slowly.

For storage, you may add sodium azide to 0.02% and a “tiny tad” of pure human serum albumin (e.g., Sigma A3782) if this does not interfere with your subsequent assays.

You can use these eluted antibodies in surface immunofluorescence (see PARASITES, section IX:B above) or in standard Western blotting. Typically, 1:2 or 1:4 dilution of the eluted antibodies (from serum) would be visible in surface immunofluorescence, and 1:10 to 1:20 dilutions in Westerns.
IX.D. Enrichment of immunoglobulin binding parasites using Dynabeads
by Fredrik Pettersson and Andreas Hedini
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Equipment
Magnetic Particle Concentrator (MPC-1, Dynal)
haemocytometer
cryotubes
rotator for incubating cryotubes
25-cm² cell culture flask
centrifuge for washing RBC (immunofuge II, Baxter)
microscope (400×)

Materials and reagents
uncoated Dynabeads M-450 Tosylactivated (90 mg/mL, Dynal)
appropriate antibodies for coating the Dynabeads
0.1 M borate
PBS 0.1% HSA (50 mL PBS + 50 mg albumine, filter-sterilized)
PBS 10% HSA (50 mL PBS + 5 g albumine, filter-sterilized)
acridine orange
RPMI 1640
MCM without serum (see PARASITES, section I:A)
MCM with 10% serum (see PARASITES, section I:A)

Enrichment of the parasites
• Coat uncoated Dynabeads M-450 Tosylactivated with antibodies using the protocol provided by Dynal.
• Count the parasitemia of the \textit{Plasmodium falciparum} culture and determine the stages of the parasites using acridine orange (see PARASITES, section III:A). It should have a parasitemia of approximately 10% and the parasites should be in trophozoite stages.
• Determine the number of erythrocytes per milliliter using a haemocytometer. Calculate the number of target cells (=infected RBCs) by multiplying the number of cells with the parasitemia. One or two milliliters of parasite culture (approximately $5 \times 10^7$ target cells) is usually enough to achieve a good parasitemia after enrichment.
• Calculate the volume of Dynabeads to have 6 Dynabeads per target cell in the solution.
• Wash the Dynabeads 3 times with 3 mL of RPMI 1640 using a 3.6-mL cryotube and a Magnetic Particle Concentrator.
• Wash the culture 3 times ($500 \times g$) with MCM without serum (see PARASITES, section I:A).
• Resuspend the erythrocytes in approximately 2 mL of RPMI 1640 and add the RBCs to the Dynabeads in the cryotube.
• Immediately incubate the tube on a rotator at room temperature for 1.5 to 2.0 h, during which time the trophozoites will attach to the coated Dynabeads.
• After the incubation, before the beads/erythrocytes have time to settle, use the Magnetic Particle Concentrator to remove the medium while the PRBC attached to magnetic beads are bound to the MPC-1.
• Wash the beads/erythrocytes very gently, 1 or 2 times with RPMI 1640 using the Magnetic Particle Concentrator.
• Resuspend the beads/erythrocytes in MCM with 10% serum (see PARASITES, section I:A) in a 25-cm² cell culture flask at a hematocrit of 5% (i.e., 3.8 mL of MCM with serum, 0.2 mL of packed erythrocytes, and the Dynabead-bound *Plasmodium falciparum* infected erythrocytes in a 25-cm² flask).

During the next day, remove the beads from the culture using the Magnetic Particle Concentrator. The parasitized erythrocytes should by then have infected the free erythrocytes in the culture. Continue culturing the remaining erythrocyte mixture as described in PARASITES, section I.

Reference
X. Fixation protocols for transmission electron microscopy

X:A. Fixation of tissue samples

by Anne von Euler

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Please note: Use only glutaraldehyde of high purity, “EM-grade”. Purchase only small amounts at any one time, store them at −20 °C, and use them as soon as possible. Also, use only paraformaldehyde powder. It is important to dissolve the paraformaldehyde in alkaline buffer before adjusting the cacodylate buffer to neutral pH.

Please note: ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC.

Solutions

For the solutions below:
- Dissolve paraformaldehyde in double-distilled water in a FUME HOOD.
- Stir and heat the solution until it reaches 60 °C.
- Add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.
- Cool this solution before adding cacodylate and glutaraldehyde.
- Adjust the pH with 1.0 M NaOH or 2 M HCl.
- Store solutions at 4 °C in dark bottles and use within 1 month or before a white precipitate appears.

Fixation buffer, 100 mL:
0.1 M sodium cacodylate (dimethylarsinic acid sodium salt trihydrate for synthesis), 1% glutaraldehyde, 1% paraformaldehyde (pH 7.4):
- 1 g paraformaldehyde (> 95% pure)
- 2.2 g sodium cacodylate
- 1.4 mL 70% glutaraldehyde (1.96 mL if 50% GA stock), EM-grade
For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water

Fixation storage buffer, 100 mL:
0.1 M sodium cacodylate, 0.1% glutaraldehyde, 0.1% paraformaldehyde (pH 7.4):
- 0.1 g paraformaldehyde (> 95% pure)
- 2.2 g sodium cacodylate
- 0.14 mL 70% glutaraldehyde (0.2 mL if 50% GA stock), EM-grade
For adjusting the pH to 7.4, use 2 M HCl. Adjust volume to 100 mL using double-distilled water.

Procedure

- Cut pieces of tissue to a maximum size of 0.5 cm³.
- Immediately immerse the tissue cube in fixation buffer and leave it for 30 min.
- Aspirate the fixation buffer and fill the tube with fixation storage buffer. Fill the tube to the top to avoid contact with oxygen which will cause formalin crystals in the sample.
- Close the tube carefully and seal it with Parafilm.
- Store the samples at 4 °C. When being transported, the samples do not need to be at 4 °C if that is not possible.
Reference
X:B. Embedding samples for electron microscopy

by Anne von Euler

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Equipment

Oven placed in a fume hood or connected to special ventilation

Materials and reagents

- Durcupan (Fluka)
- acetone
- First Durcupan solution:
  1 part Durcupan
  2 parts acetone
- Second Durcupan solution:
  2 parts Durcupan
  1 part acetone
- 0.1 M sodium cacodylate (EM-buffer)
- 1% OsO4 in 0.1 M EM-buffer
- All ethanol used should be diluted from 99.5% ethanol.
- 99.5% ethanol
- 70% ethanol
- 50% ethanol
- 95% ethanol plus 2% uranyl acetate

Cell samples should be in small tubes, which allow good mixing. Use short glass Pasteur pipettes and break off the thin tip when handling the Durcupan. Use a special waste container for the Durcupan, one that allows the acetone to evaporate in the fume hood and the Durcupan to polymerize and detoxify.

Use 0.1 M sodium cacodylate as a buffer since phosphate buffer can produce precipitates in some samples. It is referred to as EM-buffer in the protocol.

Please note: ALL CHEMICALS used in this protocol ARE EXTREMELY TOXIC. Work in a fume hood at all times and use proper waste containers for the chemicals.

Day 1:

- Aspirate the supernatant and rinse the cells 3 times for 5 min each in 0.1 M EM-buffer.
- To give contrast, add enough 1% OsO4 in 0.1 M EM-buffer to cover the sample.
- Incubate the sample for 45 min at 37 °C or for 1.5 h at room temperature. The OsO4 evaporates easily, so seal the tubes properly.
- Aspirate the OsO4 and discard it in a proper waste container for OsO4. Rinse the sample 3 times for 5 min each in EM-buffer.
- For dehydration, add each of the reagents listed below, one at a time for 5 min each at room temperature. Aspirate and discard the supernatant after each 5-min interval. All ethanol used should be diluted from 99.5% ethanol.
  - 50% ethanol
  - 70% ethanol
  - 95% ethanol plus 2% uranyl acetate
95% ethanol plus 2% uranyl acetate
99.5% ethanol
acetone

- Add the first Durcupan solution and leave the sample for 2 to 5 h at room temperature.
- Aspirate the Durcupan/acetone solution and add the second Durcupan solution and leave the sample overnight at room temperature.

- **Day 2:**
  - Aspirate the Durcupan/acetone solution, add pure Durcupan, and leave the sample overnight at room temperature.

- **Day 3-4 or 3-5:**
  - Change Durcupan daily until polymerization.
  - Put a little label in the Durcupan, for identification, quite close to the sample itself.
  - Polymerize the samples in a 50 °C oven for at least 20 to 30 h. The polymerization can be done over a weekend. The oven should be placed in a fume hood or connected to special ventilation.

Reference
X:C. **Fixation of erythrocytes for immuno-electron microscopy**

by Martha Schlichtherle and Anne von Euler

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**Equipment**
centrifuge

**Materials and reagents**
- parasite culture with desired parasitemia
- RPMI 1640
- 0.1 M phosphate buffer (pH 7.4)

**Fixation solution**, 100 mL (1% paraformaldehyde, 0.2% glutaraldehyde, 0.1 M phosphate buffer (pH 7.4)):
- Prepare phosphate buffer stocks (Na₂HPO₄ and NaH₂PO₄), then mix for 0.2 M (pH 7.4) according to Maniatis or “Current Protocols in Molecular Biology”.
- Mix 1.0 g paraformaldehyde in 50 mL of double-distilled water. In a FUME HOOD, stir and heat the solution until it reaches 60 °C, add 1.0 N NaOH dropwise (1 to 2 drops) until the milky white solution turns clear.
- 50 mL 0.2 M phosphate buffer
- 0.4 mL glutaraldehyde (50% stock)

This fixing solution lasts for 2 months when stored at 4 °C.

**Procedure**
- Grow a parasite culture until desired parasitemia is reached.
- Cool RPMI 1640, 0.1 M phosphate buffer, and fixing solution to 4 °C.
- Wash cells in RPMI 1640 twice and once in cold 0.1 M phosphate buffer (pH 7.4).
- Fix samples in cold fixation solution for 10 min at 4 °C.
- Wash the sample three times in cold 0.1 M phosphate buffer. If using microfuge tubes, spins at 2,500 rpm for 1 min at 4 °C suffice.
- If shipping samples, fill the tubes completely with 0.1 M phosphate buffer, cap them well, and ship them on ice by express.
- If shipping antibodies, add sodium azide for a final concentration of 0.01% and send them on ice by express.

**References**
Method developed by Dr. Masamichi Aikawa.


XI. In vitro reinvasion and growth inhibition assays

XI:A. In vitro reinvasion and growth inhibition assay by microscopy of erythrocyte monolayers by Birgitta Wahlin-Flyg
Octapharma AB, SE-112 75 Stockholm, Sweden
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Equipment
centrifuge
UV-microscope

Materials and reagents
human type O+ RBCs
parasitized red blood cells (PRBC)
TCM:
  HEPES-buffered (20 mM) RPMI 1640 medium
  10% normal human serum
  2 mM glutamine
gentamycin (25 µg/mL)
  0.2% NaHCO₃
Tris-buffered Hanks’ solution (TH)
  0.06 M bicarbonate buffer (pH 9.6)
  1% glutaraldehyde in PBS
acridine orange (10 µg/mL)
96-well flat-bottomed tissue culture plates
conical tubes
8-well multitest slides
coverslips

Procedure
- Using normal human type O+ RBCs, dilute parasitized red blood cells (PRBC) from *Plasmodium falciparum* cultures infected with late trophozoites and schizonts to a parasitemia of approximately 1% and adjust the hematocrit to 2% with TCM.
- Set up quadruplicate tests in 96-well flat-bottomed tissue culture plates. Use 100 µL of parasite culture mixed with 100 µL of TCM or various dilutions of antibodies.
- Incubate the plate at 37 °C for 18 to 20 h in a candle jar (see PARASITES, section I:A).
- Transfer the quadruplicate tests separately to 4 conical tubes, wash them by centrifugation 2 times with 1 mL of Tris-buffered Hanks’ solution (TH), followed by a dilution to 1% hematocrit, by adding 150 µL of TH.
- Make monolayers in duplicates from each tube on 8-well multitest slides that have been pretreated for 30 min with 0.06 M bicarbonate buffer (pH 9.6).
- After 30 min, wash the slides in TH, fix them by two treatments for 10 s each with 1% glutaraldehyde in PBS, wash them with distilled water, and air-dry them.
- Store the slides at room temperature until they are analysed in the UV-microscope.
Analysis of percent parasitemia by UV-microscopy

- To analyse the parasites, stain by adding one drop of acridine orange (10 µg/mL) per well for a few seconds, then wash the slides with distilled water and mount a coverslip.
- Screen 25 microscope fields per well (200 visual fields/slide). Since the number of RBCs/microscope field has been estimated to be 200/field, the percent parasitemia can be calculated from a total of 40,000 RBCs/culture:
- Calculate the percent parasitemia as:

\[
\text{percent parasitemia in control} - \text{percent parasitemia in sample} \times 100
\]

per cent parasitemia in control

Reference

XI.B. In vitro reinvasion and growth inhibition assay by flow cytometric measurement of parasitemia using propidium iodide (PI) staining

by Alice Nyakeriga

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Moi University FHS, Department of Immunology, Eldoret, Kenya
e-mail: anyakeriga@hotmail.com

This method allows for quantification of parasitemia and for quantification of parasites in different stages of differentiation according to DNA content. Many similar protocols have been published using different intercalating dyes such as acridine orange, thiazole orange, hydroethidine, or YOYO-1. Each of them has advantages and disadvantages. Below we describe our variant that we find easy and reliable. Care should be taken to distinguish nucleated cells from parasitized erythrocytes when analyzing material containing nucleated cells. The protocol can be extended by additional labeling of cells using FITC-, PE-, and APC-labeled antibodies.

Equipment
- centrifuge
- flow cytometer
- FACScan (Becton-Dickinson)
- FACScalibur (Becton-Dickinson)

Materials and reagents
- PBS
- PBS containing 0.025% (v/v) glutaraldehyde
- PBS containing 0.01% saponin
- PBS containing 2% FCS
- propidium iodide (PI)

Procedure
- Take 1 × 10^6 to 2 × 10^6 red blood cells (RBCs) from a culture or a drop of blood from an infected individual collected into heparinized isotonic buffer (e.g., PBS or 0.9% sodium chloride).
- Wash the cells in PBS twice.
- To fix the cells, resuspend them in 1 mL of PBS containing 0.025% (v/v) glutaraldehyde, and incubate them at room temperature for 20 min. Alternatively, incubate the cells at 4 °C for 30 to 45 min. Cells may be stored in the fixative for several weeks before proceeding.
- Centrifuge the cells for 5 min at 450 × g. Aspirate the supernatant followed by two washes in PBS.
- Permeabilization:
  - Resuspend the cells in 0.5 mL of PBS containing 0.01% saponin.
  - Incubate them at room temperature for 5 min.
- Wash the cells in PBS twice at 450 × g for 5 min.
- Resuspend the cells in 0.5 to 1.0 mL of FACS buffer. (We use PBS containing 2% FCS.).
- Staining with propidium iodide: To the above cell suspension, add PI to a final concentration of 10 µg/mL. Incubate the cells at 37 °C for 1 to 2 h.
- Analyze the cells in a flow cytometer with a 488-nm laser for excitation, and detect emission from intercalating PI in the 670-nm long pass filter (red FL3
channel). We use the FACScan and the FACScalibur from BD and detect emission in FL3.

References


XI.C. \( ^3 \text{H}-\text{hypoxanthine incorporation assay for the study of growth inhibition by drugs} \)

by Berit Aydin Schmidt

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Apparatus
- speed vac (Savant Plus SC 110 A)
- Harvester (Tomtec Mach III)
- scintillation counter (1450 Microbeta Liquid)
- Candle jar
- Bag sealer

Equipment
- 96-well cell culture plates, flat-bottomed with lid (Costar)
- Eppendorf tubes
- Falcon tubes, 14-mL
- pipettes, tips 5- to 200-µL
- dispenser (Eppendorf Multipette plus)
- Eppendorf Combitip Plus for 0.5 mL
- filter papers (Printed Filtermat A Wallac)
- sample bags (Wallac)
- gloves
- racks

Materials and reagents
- parasite strain (FCR 3 S1) in culture (unsynchronized)
- complete culture medium: RPMI 1640 with 10% human serum from blood group AB+ = MCM (see PARASITES, section I:A)
- human erythrocytes, blood type O
- test compounds dissolved in DMSO (in conc. 5 mM)
- DMSO (dimethyl sulfoxide)
- acridine orange (see PARASITES, section III:A)
- Beta-plate scintillation fluid
- chloroquine diphosphate, in two concentrations, 40 nM and 640 nM, as a control drug
  - stock solution for control drug 1 (64 × 10\(^{-5}\) M chloroquine diphosphate (MW: 515.9 g/mol):
    - 33 mg chloroquine diphosphate
    - 100 mL distilled water.
    - Prepare the stock solution. Store at 4 °C in the dark in a plastic flask. (The drug adheres to glass.)
  - working solution for control drug 1: Prepare the working solutions of control drug 1 by diluting the stock solution by 1:100 in RPMI 1640 (containing 1% DMSO); 64 × 10\(^{-7}\) M = control drug 1
  - working solution for control drug 2: Make two-fold dilutions of the working solution for control drug 1 in 4 steps; 4 × 10\(^{-7}\) M = control drug 2
8-3H-hypoxanthine (Amersham), 1 mCi/mL in 50% ethanol
Aliquot 100 µL (in 100-µL Eppendorf tubes) and store at −20 °C.
Prepare the working solution by evaporating the alcohol in a speed vac.
Resuspend the isotope in 5 mL of RPMI 1640 (i.e., 20 µL isotope/mL of RPMI 1640).
Prepare a new working solution for each assay.

Preparation

Day 1
Work in a sterile hood and use sterile tips and tubes.
• Dilute the test compounds (25 per microplate)
  A: 1/1005 µL in 0.5 mL RPMI
  B: 1/100050 µL of A in 0.5 mL RPMI
Final concentrations will be: A: 5 µM, B: 0.5 µM
• Prepare a dilution of washed, uninfected human type O+ erythrocytes at 2% hematocrit in MCM as background.
  Example: 20 µL O+ blood in 1 mL MCM. Add 1 µL DMSO (to achieve the same concentration as in the test samples).
• Estimate the parasitemia (X%) in the malaria culture and prepare a parasite suspension with 0.2% parasitemia and 2% hematocrit. A 20-mL suspension is enough for 3 plates. (Calculate the amount of parasite culture to add for a 0.2% parasitemia in 20 mL of MCM, add 0.4 mL of O+ blood for 2% hematocrit.)

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• Fill all wells around the border of the 96-well plate with a few drops of RPMI.
• Add 10 µL of control drug I to each of the wells E2 and E3.
• Add 10 µL of control drug II to each of the wells F2 and F3.
• Add 10 µL of the test compounds in dilutions A and B above to each of the wells 1A, 1B (G2, G3) to 25 A, 25B (G10, G11), resulting in 25 compounds in two dilutions per plate.
• Add 90 µL of the uninfected erythrocytes to each of the wells B2 and B3 for background.
• Add 90 µL of the parasite suspension to each of the wells C2, C3, D2, and D3 for controls.
• Add 90 µL of the parasite suspension to each of the remaining wells with control drugs and test compounds.
Methods in Malaria Research

• Continue in the same way with the next plate.
• Note the date, starting time, and number of the plate on the lids.
• Put the plates in a candle jar and incubate them at 37 °C for 24 h. Use a thin layer of sterile water on the bottom of the jar.

Day 2
• Add 25 µL of the ³H-hypoxanthine working solution to each well (0.5 µL/well) and continue the incubation for another 18 h.

Day 3
• Harvest the cells to labeled filter papers in a cell harvester and dry them in an incubator for 1 h.
• Put the filters in sample bags and add 3.2 mL of scintillation fluid.
• Seal the bags and see that the fluid is evenly distributed over the filters.
• Read the cpm in a MicroBeta counter.
• Estimate the inhibition:

% inhibition = 100 – \left(\frac{cpm \text{ in test well} - cpm \text{ in B2,3} \times 100}{cpm \text{ in C2,3 D2,3} - cpm \text{ in B2,3}}\right)

Control drug 1 inhibits the growth of FCR3 S1 by >90%
Control drug 2 inhibits the growth of FCR3 S1 by 10 to 30%

References

XI:D. *Plasmodium falciparum* hemoglobin formation assay

by Mats Wahlgren

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Equipment

- high speed Immufuge blood centrifuge
- centrifuge with Beckman JA-20 rotor
- vortex
- spectrophotometer (560-nm)

Materials and reagents

- malaria culture
- 4-mL Falcon tubes (BD Labware, 2058)
- 1.5-mL microcentrifuge tube
- 5% sorbitol, prepared in distilled water and filtered through a 0.22-µm filter
- MCM with 10% serum
- Triton X-100
- distilled water
- NaOH
- pyridine
- 2.5 mM potassium ferricyanide, K₃Fe(CN)₆
- sodium hydrosulfite, Na₂O₄S₂
- ferriprotoporphyrin IX chloride (Fluka or Sigma) (optional)

Procedure

Grow *Plasmodium falciparum* in MCM medium containing 10% human type AB⁺ serum and 5% hematocrit (type O⁺ blood) according to the candle-jar method of Trager and Jensen (see PARASITES, section I:A).

Synchronization of parasites (see also PARASITES, sections IV:B, G)

- Perform 1 cycle (or more if necessary) of synchronization by sorbitol lysis. Choose a culture with a majority of rings.
- Spin the 4-mL culture for 1 min at “high” speed in an immufuge blood centrifuge using 4-mL Falcon tubes.
- Add 4 mL of 5% sorbitol to the pellet.
- Mix the culture thoroughly by inversion and let it stand for 10 min.
- Spin again for 1 min.
- Wash the pellet 3 times in MCM with 10% serum.
- Return the cells to a culture flask.
- Wait until next ring stage period. Repeat the synchronization 2 more times if necessary.

Growth of parasites with drugs

- Use cultures at the ring stage (12 to 18 h) and a parasitemia of ~5%.
- Change the cultures to fresh MCM (with 10% serum) before adding the drugs.
- Add test drugs to a final concentration of 1 or 10 µg/mL (or concentrations in between).
• Controls:
  – untreated PRBC culture (as background)
  – PRBC culture treated with chloroquine, or other anti-malarial drugs (e.g., Fansidar), at 1 and 10 µg/mL
• Mix by inversion and split the culture into flasks or wells in a cell culture plate.
• Grow a 4-mL culture for each time point in a 25-cm² cell culture flask or a 2.5-mL culture for each time point in a 6-well plate.
• Allow the culture to mature for ~30 h with or without an inhibitory drug.

Pyridine-hemochrome method for the measurement of haem incorporation in hemozoin
• Harvest the culture at various time points spread over ~30 h and measure hemozoin content; e.g., use cultures at: 0 h, 18 h, 20 h, 25 h, and 30 h.
• Transfer contents of the flasks/wells to 10-mL centrifuge tubes which hold for the centrifugation below.
• Add Triton X-100 to a final concentration of 1% (4 mL of a 2% Triton X-100 solution to a 4-mL culture). Leave a small volume of culture for 5% Giemsa staining (see also PARASITES, section III:B) and for counting the parasitemia.
• Spin at 4 °C and 13,000 rpm for 45 min (Beckman JA-20 rotor).
• Discard the supernatant and save the pellet.
• Resuspend the pellet with 1 mL of distilled water and transfer it to a 1.5-mL microcentrifuge tube.
• Spin it at 4 °C and 13,000 rpm for 15 min in a microcentrifuge. This step can be repeated to further wash away any free haem.
• Discard the supernatant and save the pellet.

Do the following steps in a VENTILATED HOOD since pyridine and potassium ferricyanide are VERY TOXIC. Collect waste in a flask, label it appropriately, and send it as organic waste.

• If one wishes to set up a standard curve for the assay, one can use hemin which is ferriprotoporphyrin IX chloride (Fluka or Sigma). Make standard working solutions in distilled water from a stock solution of hemin prepared in DMSO, as hemin is not directly soluble in water. Then take 520 µL of these prepared hemin standard solutions and treat them exactly as the dissolved hemozoin pellet below.
• Add 520 µL of distilled water to the tube with the culture hemozoin pellet, followed by 62 µL of N NaOH and 123 µL of pyridine (for a 4-mL sample). Use half of these volumes for a 2.5-mL sample. Vortex to dissolve the pellet. Add the same volumes of NaOH and pyridine to the hemin standard tubes.
• Split the mixture into equal parts in 2 microcentrifuge tubes. Add 20 µL (or 10 µL for a 2.5-mL sample) of 2.5 mM potassium ferricyanide to one tube to oxidize haem. Add “a pinch” of sodium hydrosulfite to the other tube to reduce haem. Mix by inversion.
• Measure the absorbance in a spectrophotometer at the wavelength of 560 nm.
• Calculate the relative amount of hemozoin at each time point as well as for the hemin standards:
  $$\Delta \text{OD}_{560} = \text{OD}_{560} (\text{reduced sample}) - \text{OD}_{560} (\text{oxidized sample})$$
• Plot hemozoin content in the culture versus time and compare relative to culture without drugs and to culture with chloroquine and/or Fansidar, as well as to the standards.

References


I. Rearing of Anopheles stepheni mosquitoes

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See MOSQUITOES AND PARASITES, section II:C

II. Culturing of sexual, oocyst, and sporozoite stages

II:A. Plasmodium falciparum gametocyte culture, purification, and gametogenesis

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Equipment
- Incubator (37 °C)
- Centrifuge
- Culture flasks (25-cm², 75-cm², and 150-cm², Corning, Canted neck flasks)

Materials and reagents
- Human RBC washed in incomplete medium
- Incomplete medium
- RPMI 1640
- 25 mM HEPES
- 0.37 mM hypoxanthine (pH 7.4)
- Complete medium (incomplete medium) containing 10% heat-inactivated pooled normal human serum
- 29 mM NaHCO₃
- 60% Percoll: 9 volumes of Percoll stock, 1 volume of 10× RPMI, 5 volumes of 1× RPMI
- Exflagellation-inducing medium:
  - 10 mM Tris (pH 7.6)
  - 170 mM NaCl
  - 10 mM glucose
  - 10% heat-inactivated normal human serum
  - 25 mM NaHCO₃
  - 50 to 100 µM xanthurenic acid (optional)
  - Nycodenz (as step gradient of 6%, 11%, and 16%)
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**Procedure**

- Spin down freshly drawn O+ human blood (not more than 2 to 4 days old) in 15-mL centrifuge tubes at 1,000 rpm for 10 min. Discard the supernatant including the WBC containing buffy coat. Wash the RBC pellet 3 to 4 times using incomplete medium and resuspend it with an equal volume of incomplete medium to give rise to a final suspension of 50% RBC.

- Start cultures for gametocytes at 6% hematocrit and 0.3% parasitemia. Example: In a 75-cm² flask, add 1.8 mL of 50% washed RBC, estimated volume of parasites from a donor culture, and complete medium to make up the volume to 15 mL.

- Change medium (12 mL) every day. Make sure that the temperature of the medium is 37 °C and culture temperature does not fall below 37 °C during the medium change. A slide warmer set at 37 °C can be used inside the hood to maintain temperature of cultures during medium change.

- Monitor the parasite growth every other day and when parasitemia reaches 3 to 5%, start changing the medium by double the medium volume (25 mL). On the subsequent days keep changing 25 mL of medium on a daily basis.

- It takes around 14 to 18 days to obtain matured gametocyte cultures.

**Comments**


**Enrichment using Percoll centrifugation**

- Prepare the 60% Percoll.

- Spin down the parasite culture and resuspend it to ~25% hematocrit in incomplete medium.

- Carefully layer 1 volume of parasite suspension over 2 to 2.5 volumes of 60% Percoll in a 15-mL (13 × 100 mm) polypropylene or Corex glass tube.

- Centrifuge the suspension at 10,000 rpm for 20 min at room temperature.

- Collect parasites at the interphase, wash them 3 or 4 times in incomplete medium, and check them by Giemsa stain.

**Gametogenesis and purification of *P. falciparum* gametes/zygotes**

Mature gametocytes are stimulated to undergo gametogenesis for 30 min at room temperature (25 to 26 °C) and gametes/zygotes are purified by centrifugation.

- Resuspend mature *P. falciparum* cultured parasites at 20% hematocrit in the exflagellation-inducing medium at a pH of 8.1 to 8.3 at room temperature for 30 min. Addition of 50 to 100 µM xanthurenic acid can also enhance the parasite yield.

- Layer the parasite suspension over a discontinuous step gradient of Nycodenz (6%, 11%, 16%) and spin it at 16,000 × g for 15 min at room temperature.

- Gametes/zygotes sediment at the interface of 6% and 11%.

**References**

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II:B. Cultivation of *Plasmodium falciparum* gametocytes for mosquito infectivity studies

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Gametocytes of *P. falciparum* are established from continuously maintained cultures of asexual blood stage parasites. Gametocytes begin to form in significant numbers in blood culture only following daily dilution with fresh RBC and medium for several days. In order for gametocytes to comprise a substantial proportion of PRBC, it is necessary for asexual PBRC to reach a high density and become stressed in vitro before conversion to gametocyte production. This occurs typically 6 to 8 d after the dilution of a culture. Gametocytes then require at least a further 8 d to reach maturity. Cultures for gametocyte production are therefore maintained for a minimum of 14 d without further dilution with fresh RBC in order to obtain a large proportion of mature and infectious gametocytes. We routinely perform gametocyte feeds to mosquitoes using cultures propagated continuously for the preceding 14 to 17 d.

**Materials and reagents**

Refer to Appendix of this protocol for details of materials and generic methods.

**Propagation of asexual blood cultures**

- Retrieve an isolate of *P. falciparum* cloned line 3D7A from liquid nitrogen and place it in a 25-cm² tissue culture flask along with 0.25 mL of freshly washed RBC and 6 mL of complete medium. Gas the culture for 30 s and tighten the culture flask cap. Keep it in a dedicated 37 °C dry incubator.

- At the same time every day the culture requires a medium change. Tip the flask(s) at 30 degrees to the horizontal, which results in good separation between RBC and the medium that forms the over-layer. As flasks are settled, warm the appropriate volume of complete medium to 37 °C. Settling of flasks and warming of medium takes 40 to 60 min. Remove the spent medium from each culture, discard and replace it with an equal volume (6 mL) of fresh medium, and gas the flask for 30 s. Perform all operations on a warming plate set to 37 °C in order to minimize heat loss to cultures during the time they are out of the 37 °C incubator.

**Growth of blood cultures for gametocyte production**

- In order to produce gametocytes on a long term basis, it is necessary to maintain a stock of 'asexual' cultures from which the gametocyte cultures are drawn. Every 5 d, flasks which have reached or exceeded a parasitemia of 1% may be subcultured ("split") by adding fresh RBC in order to reduce the parasitemia to about 0.5%. In this way, from one flask at a high parasitemia a number of ‘daughter’ flasks may be started at a lower parasitemia. Some of these flasks may be used for further stock asexual cultures and some may be used for gametocyte culture. The new asexual stock cultures are grown for another 5 d before being split again when a minimum parasitemia of 1% is reached. This guarantees a continuous supply of cultures for later gametocyte preparation. Existing gametocyte cultures are not subcultured but continue to have their medium changed daily.

- After 6 to 8 d post-subculture, developmental stage I and II gametocytes will start to appear (following asexual PRBC ‘stress’ once a threshold parasitemia is reached) and from 14 d post-subculture flasks should contain mature, stage V,
gametocytes. By 17 d, 1 to 4% of PRBC should be stage V gametocytes and the gametocytemia of all stages of development (I-V) may be up to 12%. Progress can be checked by examining thin blood films taken periodically post-subculture (usually on Days 8, 12, and 15).

- Although it is possible to attain much higher gametocytemias by enriching for gametocytes by a variety of treatments (Percoll or Nycodenz density gradient centrifugation or selective drug treatment are the most commonly used), this is not recommended for mosquito infectivity studies. In the experience of ourselves and others, artificially increasing the gametocytemia is often counterproductive to satisfactory transmission of the parasite to the mosquito vector.

- Development of male sexual stage parasites is more rapid than that of females, so mature male gametocytes may be seen from 14 d but mature females usually only from 16 d post-subculture. In order to ensure a good balance of male and female gametocytes for the purposes of transmission, it is common practice to mix cultures of different ages at the time of the blood feed (see MOSQUITOES AND PARASITES, section II:C).

References


Appendix
Safety precautions

- Plasmodium falciparum is classified as a Category 3 pathogen and all appropriate health and safety measures must be carried out when maintaining this parasite in human blood in vitro, in keeping with local regulations. Perform all work in a Class II safety cabinet. Exclude all nonessential staff from the laboratory while work is in progress. Always wear a Howie-style laboratory coat and latex gloves.

- Any isolate of P. falciparum that is used routinely should not be resistant to standard antimalarial drugs.

- Discard spent medium and all disposable plasticware in a hypochlorite disinfectant (e.g., Chloros or Presept) to a concentration of 10% free chlorine before disposal. Swab all working areas with 70% ethanol at the conclusion of any work. Provide a wash bottle of 70% ethanol to flood any spillage of infective material.
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- Obtain blood and serum from a reliable sources only, such as your local Blood Transfusion Service, where it is prescreened for the presence of HIV and hepatitis viruses.

Complete medium
RPMI 1640 medium is purchased as sterile 1-liter bottles (Gibco, 041-91187A). This medium contains 5.96 g/L HEPES buffer, 3.60 g/L glucose, and 50.0 g/L hypoxanthine. To each 1-liter bottle, add 42 mL of freshly prepared filter-sterilized 5% sodium bicarbonate (Sigma S-5761) (this is referred to as ‘incomplete’ medium). Following the further addition of 100 mL of heat-inactivated pooled human serum, the liquid is now ‘complete’ medium. Store the medium at 4 °C and use it within 7 d, during which time the pH should be monitored (optimum pH 7.3).

Retrieval of parasites from liquid nitrogen
- Thaw cryotube(s) in a 37 °C water bath for 2 min.
- Transfer the thawed contents to a sterile microcentrifuge tube and centrifuge it at 10,000 \( \times \) g for 1 min, then remove the supernatant.
- Resuspend the pellet in 1 mL of PBS + 10% sorbitol; add this slowly dropwise with continuous mixing.
- Repeat steps 2 and 3 twice more (i.e., total of 3 washes).
- Wash and resuspend the pellet in complete medium when the cells are ready for culture.

Washing human RBC
- Use group O, Rhesus group positive whole blood taken into adenine-CPD (citrate phosphate dextrose). Blood used should be less than 7 d old.
- Centrifuge the blood at 800 \( \times \) g for 5 min.
- Remove the white blood cells by aspiration of theuffy coat.
- Add an equal volume of incomplete medium and centrifuge it at 800 \( \times \) g for 5 min.
- Discard the incomplete medium.
- Repeat steps 4 and 5 twice more (i.e., total of 3 washes).
- After washing, the packed RBC may be used directly (i.e., at a 100% hematocrit) and any remainder discarded. Alternatively, the packed RBC may be resuspended in an equal volume of complete medium (i.e., at a 50% hematocrit), which should be stored at 4 °C and used within 7 d.

Gas mixture
1% oxygen, 3% carbon dioxide, and 96% nitrogen (BOC Specialty Gases, or your local specialist gas supplier). Pass the gas mixture from the cylinder via a gas-reducing valve into the safety cabinet by a silicon tube. Filter the gas through an in-line Whatman gamma 12 sterilizing unit with a 0.3-µm filter tube. Connect the filter to a sterile Millex GS 0.22-µm disc filter, the end of which is attached to a 19G \( \times \) 1.4-inch blunt-ended needle fitted to a 1-mL syringe. When gassing a culture flask, the flow rate should be moderate but sufficiently strong to ruffle the surface of the liquid.

Pooled serum
Pool human group O, Rhesus group positive serum from a minimum of 10 donors to minimize variations of quality between individuals. Packs of serum are routinely heat-inactivated at the source, but if not they should be held at 56 °C for 1 h. Test the pools
before full-scale use to ensure that they are sterile and support parasite growth, in particular that of gametocytes. Aliquots may be stored at –20 °C for up to 6 months.

Parasites
In order to maximize the likelihood of satisfactory gametocyte production, it is best to use a line or clone of *P. falciparum* that is known for its production of gametocytes. Good producers include NF54, 3D7A, and HB3A, obtainable from the MR4 repository. Restart cultures from frozen stocks every 2 months or less since gametocytogenesis in a continuously cultured line will start to wane after this period.

Parasite staining
Use improved R66 Giemsa (BDH 350864X) diluted 1:10 with Sorensen's buffer (HD Supplies; HDS 20, pH 7.2). Fix thin blood films in 100% methanol and stain for 20 min in the 10% Giemsa solution. Wash them with tap water for 20 s and allow to air dry before viewing by light microscopy using oil immersion at 1000× magnification.

Thin blood film
Small volume of RBC removed from culture flask and smeared across a glass slide to give a monolayer of RBC.

Sterile plasticware
- 25-cm² cell culture flasks (Iwaki 3100-025 or similar)
- 30-mL Universal containers (Sterilin 128A or similar)
- Plastic pipettes, singly wrapped, disposable, graduated (2-, 5- and 10-mL; Sterilin or similar)
II:C. Gametocyte Culture Protocol: Membrane Feeding Gametocytes to Mosquitoes  
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1. Gametocyte culture protocol

**Erythrocytes:** Human O+ erythrocytes from banked blood (collected in CPD-adenine), as fresh as possible (not more than 7 days after the bleed).

Wash the red cells three times (or more if necessary) with incomplete MCM, removing any buffy coat, and resuspend with an equal volume of complete MCM. This 50% hematocrit blood should be stored at 4 °C.

**Incomplete malaria culture medium (IMCM)** as for the asexual cultures, but with the addition of 50 mg/liter hypoxanthine (Sigma) and the omission of NaHCO₃. Adjust the pH to 7.2.

**Complete malaria culture medium (CMCM):** Add 10 ml of human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56°C for 1 hour, and stored in aliquots at −70°C) and 4.2 ml of a sterile, freshly made 5% solution of NaHCO₃ to 100 ml IMCM, made as incomplete medium not more than 2-3 weeks ago. Incubate at 37 °C overnight to check for contamination before use. Use within one week and discard if pH increases to >7.6 (pink in color).

**A gametocyte-producing stock culture between 4 and 10%:** Thaw fresh stock parasites every 2-3 months because the gametocyte-producing ability (and infectiousness of gametocytes) drops after a time.

The culture must be in good health and growing quickly. Ideally, it will need diluting down 2-3 times a week. Do not dilute cultures below 1% as this can stop them from growing well for a few days, and do not let the culture get too high (>6-8%) before you dilute it down, as it may ‘crash’. The presence of gametocytes in a stock culture is a good thing for gametocyte culture!

**Method**

Set up cultures in tissue culture flasks
- Small culture flasks of 25 cm² have an initial volume of 5 ml which will increase to 7.5 ml; 75-cm² flasks have an initial volume of 15 ml which will increase to 25 ml.
- Set up cultures at 0.5-0.7% parasitemia, 6% hematocrit in complete MCM. If the culture is mainly rings, set it up at 0.7%; if mainly schizonts, at 0.5%; if mixed, at 0.6%.
- Prewarm the flasks and complete MCM to 37 °C before use.
- Gas the flasks with a mixture of 1% O₂, 3% CO₂, balance N₂ (candle jars or CO₂ incubators are not recommended) for a minimum of 10 seconds (25-cm² flasks) or 20 seconds (75-cm² flasks) at a pressure of around 5 lb/in².
- Place in a 37 °C incubator with the flasks lying down. Replace complete MCM daily and agitate cultures gently after gassing.
It is very important to keep the cultures as warm as possible at all times. When removing the spent medium, do not take off any of the black pigment that comes off just before you start to take off blood.

**Bulking up the gametocyte culture:**
Once the culture has reached a high parasitemia it is 'bulked up'. This means that the volume of medium added is increased to 7.5 ml (or 25 ml) without the addition of more blood cells.

- Make thin blood smears on the fourth day after setting up the culture. Fix and stain with Giemsa’s stain as previously described.
- Examine under 100x oil objective. You should look for a high parasitemia and ‘stressed looking’ parasites such as triangular ring stages and hazy or faint-looking trophozoites and schizonts.
- If these are not present, do not bulk up. Make blood smears on the following days and bulk up when appropriate (usually 4 or 5 days after set-up).
- As soon as the culture is judged ready, bulk up by adding extra medium to each flask to give a final volume of 25 ml (75-cm² flasks) or 7.5 ml (25-cm² flasks).
- Thereafter maintain the cultures on the increased volume of medium, changing daily and gassing.
- Prepare smears once or twice a week to check the state of your cultures and to look out for contamination.
- Gametocytes are mature from 14-17 days after the start of culture.

**References**


2. Membrane feeding gametocytes to mosquitoes

This protocol is for infection of Anopheles mosquitoes with P. falciparum gametocytes, grown according to the gametocyte culture protocol. Gametocytes should NOT be grown with gentamycin, as this can have adverse effects on the infection rates.

**Note:** *P. falciparum* is classified as a category 3 pathogen in some countries, which means that there are regulations regarding its safe use. Familiarize yourself with any relevant local or national safety regulations in your country before beginning work, and take steps to minimize the risk of accidental infection. This is particularly important when mosquitoes are being infected. It is necessary to have an approved secure insectary and to take every step to ensure that potentially infected mosquitoes do not escape.

**Materials**
- 14 - 17 day old gametocyte cultures
- Human blood type O+ serum (from a blood bank, pooled from several donors, heat inactivated at 56 °C for 1 hour)
- Human O+ erythrocytes from banked blood (collected in CPD-adenine), washed to remove the anticoagulant before use, as fresh as possible (no more than a few days since collection)
- Glass membrane feeders and plastic tubing to connect them
- Clamps and stands to which to attach the feeders
- Circulating waterbath at 37 °C
- Baudruche membrane or Parafilm sealer and small elastic bands
- 1-pint waxed-paper cartons (e.g., ice-cream containers), filter paper circles to fit in the bottom of the cartons, latex sheeting, nylon netting, tape.
- Mosquito aspirator (‘pooter’)
- *Anopheles* mosquitoes, 3-5 days postemergence
- 5% glucose in 0.05% PABA (4-amino benzoic acid) solution
- Absorbent cotton wool

**Preparation for a membrane feed**

- Prepare sufficient mosquito pots for the experiments you are planning. Cut a small hole (approximately 2 cm square) in the side of a waxed paper carton. Cover this on the outside of the carton with two squares of latex sheeting (e.g. dental dam) with a slit cut into each one, and tape securely over the hole to form the leak-proof
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inlet/outlet port. Fix a filter paper circle to the base of the pot. Stretch netting over the top of the pot and secure with an elastic band and tape.

• Collect female mosquitoes two days before you are going to carry out the feed itself. These mosquitoes should be between 3 and 5 days postemergence on this day (so they will be between 5 and 7 days post emergence on the day of feed).

• Place a small piece of cotton wool soaked in distilled water on top of the netting. This should be replaced daily until the day of feed.

• Attach baudruche membrane or finely stretched Parafilm to the glass feeders with an elastic band. If using baudruche it is easier to apply it wet.

• Connect the feeders to a circulating waterbath set at 37 °C, and clamp at the correct level for the mosquito pots.

Preparing the infectious feed
Work swiftly and take steps to prevent the temperature of the parasite material from dropping below 37 °C.

• Prewarm washed uninfected fresh blood and serum to 37 °C.

• Resuspend the washed uninfected fresh blood in the serum to give a final packed cell volume (pcv) of 40%. Keep warm at 37 °C.

• Remove medium from culture flasks and resuspend the cells by gentle shaking. Transfer to tubes and centrifuge to pellet (5 minutes at 1800 x g at 37 °C).

• Remove supernatant, measure the volume of the pellet (approximately), and add an equal volume of serum to the pellet. Mix the pellet and serum very well (remember the gametocytes stick to the side of the tube).

• Dilute the parasite/serum mix with 3 to 9 times its volume of the fresh washed uninfected fresh blood/serum.

• Mix with a Pasteur pipette and place approximately 1 ml (depends on membrane feeder size) into each feeder.

• Place the mosquito pot underneath and allow to feed for approximately 20 minutes.

• Remove the unfed mosquitoes 2-3 hours after the feed and kill by freezing at –20 °C.

To check for exflagellation
• Place a small drop of the prepared parasite/blood/serum mixture on a microscope slide, breathe on a coverslip, and place over the drop of blood. Examine microscopically (phase or Nomarski 40x objective are best) as soon as possible (always within 10 minutes).

Maintenance and dissection
• Keep infected mosquitoes in a secure insectary at 26 °C/80% relative humidity.

• Place clean cotton wool pads soaked in 5% glucose/0.05% PABA solution on top of the netting; replace these daily.

• Kill mosquitoes with chloroform, and then dip briefly into ethanol. Dissect in 1x PBS.

• Oocysts will be visible from 7 days postfeed, but are easiest to see 9-10 days postfeed (depending on the temperature of the insectary).

• Sporozoites may be seen in the salivary glands from day 14 postfeed onwards.
References

This protocol is summarized from the reference below. Please refer to this chapter for original references.

II:D. Production of *Plasmodium falciparum* oocysts and sporozoites

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The production of different sexual stages of *P. falciparum* and their isolation from infected mosquitoes facilitates a variety of cellular, molecular, immunochemical, and transmission-blocking studies that examine the parasite-vector relationship. The procedures of mosquito rearing, parasite cultivation, and infection and dissection of mosquitoes are highly specialized skills requiring considerable experience to perform with competence. Additionally, the timing of the two life cycles, that of the parasite and that of the mosquito, requires precise planning so that the mosquitoes are the correct age to infect during the brief period in which the parasites are viable for infection.

Mosquitoes capable of transmitting *P. falciparum* should be maintained within a limited access, dedicated, high security humidified insectary. It is recommended that all rooms be painted in a light color and all cages be constructed of white materials in order to make highly visible any mosquito that may temporarily escape. A nonresidual insecticide spray should also be available in the event of an emergency. Infected mosquitoes to be dissected are first stunned or anesthetized by chloroform and then rendered incapable of movement (dewinged and delegged). Any potentially infective mosquitoes that are not to be dissected are frozen by placing the complete container at −20 °C prior to autoclaving and disposal.

**Equipment required for blood feed of mosquitoes**

(A) *Pooter*—A pooter is a device used to transfer mosquitoes by aspiration. We use a polycarbonate plastic-tubed pooter, but any toughened material is suitable provided that it is transparent. Ours are custom-made (contact details upon request) but an efficient pooter can equally well be modified from a 10-mL plastic pipette. This is prepared for use as follows:

- Insert a stainless steel (or nylon) fine-meshed disc of the internal dimensions of the pooter tube 2 cm from one end of a 20- to 25-cm tube. A ring may be etched in the internal surface of the tube in which the disc can sit securely.
- Attach a length of clear, transparent silicon rubber tubing (approximately 30 cm) over the meshed end of the polycarbonate tube.
- Use the pooter by placing the free end of the silicon rubber tubing in your mouth and aspirating by a ‘suck-blow’ technique. Transfer the mosquitoes by sucking them into the open end of the polycarbonate tube and then blowing them out into the container.

(B) *Mosquito container*—The ideal container should minimize the risk of mosquito escape or injury or of the handler being bitten. The container used successfully by ourselves and several other research groups is customized from a 500-mL cylindrical ice-cream container made of waxed cardboard (of the type used by Ben & Jerry’s and Häagen-Dazs). This is prepared for use as follows:

- Cut out a circular piece of filter paper (Whatman no. 1, or similar) to the internal dimensions of the container and tape it to the floor of the container to soak up any spillage that may occur (from nutrient feed, diuresis drops, etc.) when in use.
- Cut a 3-cm square in the side and cover the hole with 2 layers of dental latex (HCM rubber dental dam or similar, available from your local dental supplies distributor) into which two 1-cm cuts are made, one of which is then placed...
perpendicular to the other. Hold both squares of latex firmly in place with electrician’s insulating tape. This results in a cross-shaped entry port through which, with the aid of a pooter, mosquitoes can be safely introduced or removed.

- To complete the container, secure a double layer of ‘bridal veil’ netting over the mouth of the container with elastic bands and insulating tape.
- Other than during the actual blood feed, keep the containers inside a gauze-covered cage that acts as a secondary security barrier.

(C) Blood feeder—A glass membrane feeder is used to deliver the infectious blood meal to the mosquitoes. Blood feeders consist of a water-jacketed chamber into which the infectious blood feed can be introduced. Ours are custom-made by a professional glass blower (contact details upon request). This is prepared for feeding use as follows:

- Cover the blood feed chamber with a Baudrouch membrane derived from bovine intestine (Joseph Long Inc.). Wet the membrane with tap water to ensure that, when dry, it is taut. This will encourage the mosquitoes to feed. If washed and dried thoroughly after each use, a membrane can be used 5 or 6 times before it loses its integrity. In fact, in our experience, previously used membranes are preferred to new membranes because their use results in a greater proportion of mosquitoes taking a blood meal.
- Hold the membrane in place with an elastic band.
- Pass tap water at 38 °C through the water jacket via a recirculating pump (water inlet via the lower tube, outlet via the higher tube).

Rearing of *Anopheles stephensi* mosquitoes

- Keep each stock colony of *An. stephensi* in a gauze-covered, plastic-covered wire-framed cage. We use cages of frame dimensions 30 cm³, which are covered with mosquito netting or ‘bridal veil’ netting to incorporate a 15-cm front sleeve.
- Keep cages at 26.0 ± 0.5 °C and 80.0 ± 2.0% relative humidity. Maintain all colonies on a reverse light cycle with 12 h artificial lighting from 2000 to 0800 h. This enables all manipulations to be performed in keeping with the nocturnal activity of the mosquito during normal working hours.
- Feed adult female mosquitoes twice a week on uninfected rodent blood. We routinely feed directly by bite of naive outbred mice, but rats or guinea pigs are also suitable. Anesthetize the mouse by intraperitoneal injection of 0.1 mL per 10 g body weight of 10% Hypnorm (Janssen Pharmaceutica) and 20% Valium (Roche) in sterile distilled water. Place the mouse abdomen-down, with the four limbs stretched out, on top of the cage in which the mosquitoes to be fed are kept, and allow feeding for 20 min. The abdomen may be shaved of fur to facilitate access for feeding. We use mice under terminal anesthesia in accordance with project and personal licences issued by the UK Home Office and following institutional guidelines. Your local regulatory authority may permit rodents to be reused for the purposes of mosquito feeding, in which case they can be allowed to recover from the anesthetic.
- Eggs are laid by fed female mosquitoes 2 to 3 d after a blood feed. Place a small bowl lined with filter paper (Whatman no. 1, or similar) and filled with tap water on the floor of the stock cage overnight. The following morning, remove the bowl to another cage, and cover it with a square of transparent Perspex until the eggs begin to hatch (within 2 d).
• Rear the larvae in clean plastic washing-up bowls (preferably colored white) containing 3 inches of water. Carefully transfer the contents of the hatching bowl into a larval rearing bowl (approximately 300 larvae per bowl) and discard the filter paper. The water must not contain large amounts of chlorine so it may be necessary to allow your local tap water to stand for a minimum of 24 h before use to allow any chlorine to dissipate (‘chlorine-free’ water). We recommend the continuous maintenance of a reservoir of chlorine-free water ready for use. Alternatively, water can be boiled before use or bottled water can be used. Whatever the source and treatment of the water, it must be at room temperature before eggs are added.
• Anopheline mosquito larvae are primarily surface feeders and must be provided with a form of food that floats on water. We use koi carp pellets (Wardley Corporation premium koi staple, or a suitable alternative supplied by your local aquarist). Baby weaning formulation milk powder (‘Farex’ or similar) may also be used but should to be finely sieved before sprinkling on the water.
• Feed the larvae morning and evening with fresh pellets (a ‘little but often’ regime). Remove and discard any surplus ‘old’ food. In this way, the larvae receive sufficient food, but not so much that the bowl becomes contaminated with uneaten food.
• After 2 to 3 d, reduce the mosquito numbers. By keeping the larvae numbers between 200 and 250 per bowl, large well-nourished mosquitoes are produced.
• Larvae should pupate 6 to 8 d post emergence from eggs if kept at 26 °C and 80% relative humidity. Pupation can be delayed by 2 or 3 d by reducing the ambient temperature by 1 or 2 °C. Kill surplus larvae by immersion in hot water. Do not discard them live down the sink.
• Collect pupae and place them in a bowl of chlorine-free water. Cut an inch off of the point of a filter-paper cone and place the cone over the bowl. This system allows emergence of adult mosquitoes (2 to 3 d postpupation), but also deters emergent mosquitoes from flying back into the bowl and drowning and keeps fed females from laying eggs in the pupae bowl. Place the pupae bowl in the stock cage.
• Provide a solution of 5% glucose (Sigma) and 0.05% p-aminobenzoic acid (PABA; Sigma) in chlorine-free water for all adult mosquitoes. Filter-sterilize this nutrient solution through a 0.22-µm filter (Sterilin or similar) before soaking a cotton wool pad and placing it in the cage. The pad should be moist but not dripping as this leads rapidly to fungal contamination, and should be changed daily.

On the day before the feed
• Prepare the mosquito container(s) and place into each, with the aid of a pooter, 4 to 6-d-old virgin female mosquitoes (i.e., they should be 5 to 7 d postemergence from pupae on the day of the feed). Add a suitable number of mosquitoes for the volume of the container to be used; for a 500-mL cylindrical container, we add between 100 and 250 mosquitoes. Up to 20 mosquitoes can be accommodated comfortably within a pooter at any one time to enable counting during the transfer procedure. It is important to handle the mosquitoes carefully so as not to damage them.
• Test mosquitoes for their readiness for a blood feed by evaluating their attempt to feed on a warmed, water-filled, tissue culture flask placed on top of the gauze-
covered stock cage in which they are housed. If the pooter is used for transfer to the feed container while the culture flask is in place, those mosquitoes that are attracted by the warm object can be positively selected. This procedure acts not only to select older individuals more eager for blood among a population of slightly mixed ages (due to variation in the timing of emergence from pupation) but also to segregate female from male mosquitoes. Only females are attracted by the warmed flask, as its temperature mimics that of a human (from which they require blood in order to lay eggs); male mosquitoes do not blood feed and are ambivalent to the presence of the flask.

- Feed mosquitoes on a cotton wool pad soaked in 5% glucose/0.05% PABA which is placed on top of the mosquito container. Remove the cotton wool pad 12 h before the blood feed. Starving the mosquitoes for 12 h prompts the vast majority to feed to engorgement on blood.

On the day of the feed
- Wash fresh group O, Rhesus group positive whole blood and warm it to 37 °C.
- Warm group O, Rhesus group positive serum to 37 °C.

For details of preparation of human RBC and pooled serum, refer to the Appendix of MOSQUITOES AND PARASITES, section II:B.

- Place a Baudrouch membrane on the blood feeder, wet it, and allow it to dry. Once the membrane is dry (approximately 20 min), switch on the water pump and allow the water jacket to warm up to 38 °C (approximately 60 min).
- Take *P. falciparum* to be used for the feed from 14- to 17-d-old cultures which contain mature, stage V gametocytes. As male gametocytes mature more rapidly than do females, in practice it is best to use either: a) exclusively cultures that are 17-d post-subculture, which should contain a sufficient number of gametocytes of both sexes; or b) a preparation of blood of different ages (such as a 1:1 mix of 14- and 16-d-old cultures), which will show a sex ratio bias in favor of male or female parasites, respectively. For method of cultivation, refer to “Growth of blood cultures for gametocyte production” of MOSQUITOES AND PARASITES, section II:B.

If at all possible, perform the following procedures entirely at 37 °C and as rapidly as safe handling and good microbiological practice permits in order to minimize the possibility of gametocytes committing to activation/exflagellation before the blood meal is taken up by the mosquito.

- Transfer infective cultures to centrifuge tube(s) prewarmed to 37 °C.
- Centrifuge the cultures at 800 × g for 2 min in a centrifuge prewarmed to 37 °C.
- Remove and discard the supernatant.
- Measure the volume of the remaining pellet, containing packed PRBC, and add an equal volume of serum.
- Make the volume of infective blood/serum from step 8 up to that of the blood feeder to be used (1 to 2 mL) by diluting the suspension up to 3× volume with a mixture of 1:1 washed human RBC/serum. Mix gently but thoroughly. We routinely dilute blood in this manner because: a) better infection rates are achieved using blood that contains a high but submaximal gametocytemia; b) addition of fresh blood/serum to the 17-d-old cultured PRBC promotes mosquito
feeding, in terms of both extending the feeding time of individual mosquitoes (qualitative) and increasing the number that feed (quantitative).

- Rapidly transfer the diluted infective blood from step 9 into the blood feeder using a prewarmed syringe with a blunt-ended needle, ensuring that there are no air bubbles present.
- Remove a mosquito container from its outer cage. Place the container underneath the blood feeder, ensuring that the blood feeder and the netting are in intimate contact.
- Turn off any artificial lighting and allow the mosquitoes to feed undisturbed for 12 to 15 min. We recommend that the high security insectary in which the mosquitoes are held be without windows; this enables feeding to take place in the dark, mimicking feeding behavior in the wild.
- Remove the mosquito container from underneath the blood feeder, thus terminating feeding, and return it to a gauze-covered cage. Most mosquitoes that have been starved of nutrients for the previous 12 h will feed to engorgement within a few minutes. These will have a very distended, red abdomen and can be easily distinguished from mosquitoes that have not fed. If the mosquitoes have fed well, the floor of the container will be covered with red splashes of fluid passed by diuresis. Depending on the ease with which the pooter can be manipulated within the container, it may be possible at this stage to remove individual unfed mosquitoes.
- During the time the blood feed is taking place, examine any infective blood/serum mix that was not added to the membrane feeder for gametocyte activation and/or exflagellation. Exflagellation is the explosive production of male gametes of the malaria parasite, which happens in the mosquito midgut within a few minutes of a blood meal. The cytoplasm of the male gametocyte becomes agitated, followed rapidly by the sudden and vigorous protrusion and detachment of up to 8 flagellated gametes (spermatozoa) from the surface of the parasite cell body. This phenomenon also occurs spontaneously in vitro and thus may be observed in cultures of fresh infected blood under the light microscope (1000× magnification under oil immersion). It is controlled in vitro solely by the change from 37 °C to the ambient laboratory temperature, the pH rise this brings being mediated by a fall in CO₂ tension as the blood equilibrates with the atmosphere. Viewing of gametogenesis (typically between 10 and 25 min after removal of a blood culture from the 37 °C incubator) is a strong indicator that oocysts and sporozoites will be produced following a blood feed. However, in our experience, observable exflagellation is not a prerequisite for successful mosquito infectivity.

On days after the feed

- Subsequent to a blood feed, feed mosquitoes daily on a 5% glucose/0.05% PABA diet, as previously described. Delaying the start of feeding by 24 h post blood feed enriches for blood-fed mosquitoes as the vast majority of “die off” mosquitoes under these conditions are those that did not blood feed and therefore were starved of nutrients for at least 36 h.
- Mosquitoes may be dissected for the presence of oocysts at 10 to 12 d postblood feed. Dissection for sporozoites may take place from 14 d postblood feed. The number of live mosquitoes in each container at the time of dissection must be verified and recorded by 2 people independently (usually by observing carefully the pootering process). The total number of mosquitoes either dissected or
undissected at the end of the experiment should tally with the records made. The protocol for dissections is described below.

Dissection of blood-fed mosquitoes

**Dissection of mosquito midgut to determine the presence of oocysts**

- For the detection of *P. falciparum* oocysts, dissect mosquitoes 10 to 12 d postinfective blood feed.
- Remove mosquitoes from the container via a pooter, the end of which is immediately plugged with cotton wool and sealed with masking tape to prevent escape. Mosquitoes may be collected one at a time, but up to 5 mosquitoes can be held in the cavity of the pooter without compromising handler safety.
- Strike the pooter against the palm of the hand firmly several times to knock the mosquitoes out for a sufficient period of time to allow their safe removal onto a dissection board. In order to stop each mosquito from flying, and also as an aid to dissection of the body, remove its legs and one wing with a scalpel blade.
- Place dewinged and deheaded mosquitoes in a watch glass containing dissection medium (PBS + 0.0001% FCS). Using a pair of fine forceps, pick up each mosquito by its one remaining wing and dip it into 70% ethanol to ensure both sterility and killing of the mosquito.
- Remove individual mosquitoes, mount them on glass microscope slides onto which a drop of dissection medium had been previously placed, and remove the remaining wing.
- Dissect the mosquito under a binocular light microscope using a 40× objective (total magnification 400×).
- Separate the abdomen from the rest of the mosquito. Remove the gut by holding the anterior of the abdomen with one dissecting needle while at the same time making a small cut with a second needle in the tegument on each side of the seventh abdominal segment. Using the second needle, gently pull on the apex of the abdomen until the gut and Malpighian tubules are exposed.
- Sever the alimentary canal sufficiently far forward to bring away (and discard) all of the foregut except for the section immediately proximal to the midgut.
- Anchor the remainder of the gut by placing the point of one needle on the posterior section of foregut. Using the other needle, cut through the alimentary canal at the junction of the midgut and the hindgut, simultaneously severing the Malpighian tubules. Discard the hindgut and Malpighian tubules, leaving only the midgut on the slide.
- When an especially rapid dissection is required (with least chance of disruption to the oocysts), once the gut has emerged completely from the abdomen, cut off and remove the hindgut only, leaving the midgut exposed but with the foregut and esophagus still attached.
- Using a needle, pick up the dissected midgut and transfer it to a fresh slide onto which has been placed a drop of dissecting medium. Lower a coverslip gently onto the moistened midgut, which then can be viewed for the presence of oocysts. The volume of medium bathing the preparation is important as too little will cause the midgut to rupture (so releasing oocysts and thereby preventing their examination *in situ*) and too much will prevent adequate flattening of the
midgut for optimal viewing. Draw off excess medium by holding a filter paper to the edge of the slide.

- View guts by under 400× magnification with reduced light when oocysts, if present, should be easily identified as circular refractive bodies on the gut wall of the dissected mosquito.
- In our experience, the number of oocysts recovered per infected mosquito varies between 1 and >200, average 30. Mosquitoes that are not infected, as determined by a lack of oocysts, are usually those that did not blood feed and will not contain eggs. Occasionally, an infected mosquito (with oocysts) that has no eggs may also be seen.

**Dissection of mosquito salivary glands to determine the presence of sporozoites**

- Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.
- Sporozoites may first be observed in the salivary glands 14 to 17 d postinfective blood feed. The junction of the mosquito head and thorax contains a pair of glands, each of which is tri-lobed (or very occasionally four-lobed).
- Repeat steps 1 through 6 as described above for oocyst dissection.
- Place a dissecting needle gently on the thorax of the mosquito, just below the region where the salivary glands lie.
- Place a second needle on the ‘neck’ of the mosquito (apex of head and thorax) without cutting it, then with a gentle pulling action detach the head.
- The salivary glands should now be exposed and can be detached from the head. If the salivary glands are not apparent, then press with a needle again on the thorax, when the salivary glands should emerge from the thorax and can be detached.
- Transfer the dissected glands and a small volume of dissection medium into a 0.5-mL microcentrifuge tube using a 100- to 200-µL Gilson pipette. Keep the microcentrifuge tube on ice.

**Liberation of sporozoites from salivary glands**

- Cover the microcentrifuge tube containing the salivary glands with Parafilm to reduce the chance of leakage. Release the sporozoites by gently rupturing the glands using a laboratory benchtop whirlimixer set to maximum for 3 min or by vigorous pipetting using a 200-µL Gilson pipette.
- Once liberated from the salivary glands, the sporozoite yield can be estimated by counting using an hemocytometer. Place 10 µL of the sporozoite suspension into the hemocytometer chamber and then using 200 to 400× magnification phase-contrast microscopy to visualize the sporozoites, count the number present in a 4 × 4 grid: this number relates to the number of sporozoites per mm² × 10⁴.
- If a qualitative examination for sporozoites is all that is required, transfer salivary glands directly to a glass microscope slide. Lower a coverslip carefully onto the moistened glands and tap it gently to disrupt the glands and release any sporozoites present. These should be easily identified as comma-shaped, motile bodies.
- In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.
References

III. Transmission blocking and sporozoite invasion assay

III:A. Transmission blocking assay (TBA) – membrane feeding

by Mrinal Kanti Bhattacharyya and Nirbhay Kumar

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Equipment

membrane feeder (glass), Lillie Glassblower, USA (Tel: +1-404-436-8959)
silicon tubing to connect the feeders
aspirator tube
pint containers (Nptune Brand Round containers, Kahn Paper Co.)
dental dam, hygienic
nylon net, bridal net (any fabric store).
Parafilm
water pump, constant temperature circulator (Polystat, Cole-Parmer, or any other equivalent)
light microscope (e.g., Olympus)
dissecting microscope (e.g., Olympus)
dissecting forceps
dissecting glass slide
glass slides and coverslips
Eppendorf tubes
sterile pipettes
insectaries approved for handling Plasmodium falciparum-infected mosquitoes

Materials and reagents

parasite culture:
Maintain Plasmodium falciparum gametocyte culture for 14 to 18 days making blood smears on alternate days. From Day 12 onward, make blood smears on a daily basis and look for mature C-shaped gametocytes as well as in vitro exflagellation of male gametocytes.

mosquitoes:
Maintain and breed Anopheles stephensi according to the standard procedure (see MOSQUITOES AND PARASITES, section II:C). Use 3- to 5-d-old mosquitoes for the experiment.

human RBC
heat-inactivated normal human serum (NHS)
heat-inactivated antiserum
incomplete medium used for P. falciparum cultures
mercurochrome solution

Methods

Important notes before starting

- Plasmodium falciparum cultures of at least 0.5% mature gametocytes and at least 1 to 2 exflagellation centers per field should be used for mosquito feeding.
- Before preparing blood meal, have mosquitoes and feeders ready.
- Take care to maintain the parasite and the blood always at 37 °C.
Exflagellation assay
- Using a sterile pipette, take out 1 mL of parasite culture and transfer it to an Eppendorf tube.
- Spin down the tube for 15 s at high speed and discard the supernatant.
- Add an equal volume of normal human serum to resuspend the pellet and allow it to stand for 10 min at room temperature.
- Place 10 µL of this suspension on a glass slide and cover it with a coverslip.
- Determine the average number of exflagellation centers per field using a light microscope at 400× magnification. A polarizer filter may help in locating the parasitized RBC as in this configuration the malaria pigments look shiny. Scan at least 10 to 20 fields to get a proper estimate of exflagellation centers.

Making dilution of antibodies in 50% RBC
Premix the antibodies to be tested for transmission blocking activities with 50% red blood cells in heat-inactivated normal human serum at various dilutions (1:5 to 1:20). Keep these diluted antibodies at 37 °C until the final feeding.
- Place 100 µL of 50% RBC (in incomplete medium) in a microfuge tube, harvest the cells at 3,000 rpm for 1 minute, and wash them with heat-inactivated NHS.
- Resuspend the cells at 50% hematocrit in NHS and antiserum to obtain desired serum dilution.
- Incubate the tubes at 37 °C.

Preparation of mosquito pint
- Make a hole (2-cm diameter) in the paper can. Cover the hole with two squares (2 in × 2 in) of dental rubber with perpendicular cuts on top of each other using adhesive tapes.
- Cover the top of the paper can with nylon net of appropriate size and securely tape the net to the paper can.

Collecting female mosquitoes in pint
Distribute 30 to 40 female mosquitoes per pint. The easiest way to collect female mosquitoes from a mixed pool of male and female is to place a warm water bottle (39 to 40 °C) against the wall of the mosquito container. The female mosquitoes are attracted to the warmer side of the cage, whereas the male mosquitoes will be all over. Starve the mosquitoes for 5 to 6 h.

Preparation of feeders
- Connect the glass feeders to each other and to the circulation water pump with silicon tubing to generate a continuous flow of 39 °C hot water through the water jackets of the membrane feeders.
- Stretch Parafilm membrane (approximately 2 in × 2 in) in both directions and place on the glass feeder. Maintain the temperature of the membrane feeder by a glass water jacket (39 °C) and a circulating water bath.
- Place the pint with female mosquitoes under the warmed membrane feeder.

Membrane feeding
This step is the most crucial and must be performed as quickly as possible. During the whole procedure, take care not to allow the temperature of the *Plasmodium falciparum*
gametocyte culture to drop below 37 °C. A drop in temperature promotes exflagellation. The success of the experiment depends on the maximization of exflagellation as well as gametogenesis within the mosquito, not outside. Allow the mosquitoes to engorge for 15 min.

Experimental steps
- Place 10 mL of matured *Plasmodium falciparum* gametocyte culture in a 50-mL conical tube.
- Harvest at 1,000 rpm for 2 min and then allow the rotor to stop naturally (without the break).
- Remove the supernatant and resuspend the pellet at 50% hematocrit with prewarmed NHS.
- Keep the tube warm at 37 °C.
- Lower the membrane feeder over the nylon net covering the pint. Make sure that the mosquitoes are attracted to the membrane.
- Add 50 µL of gametocyte suspension kept warm at 37 °C to the diluted antibody and immediately transfer the mixture to the feeders.
- After 15 min, separate the unfed mosquitoes from the fed ones. It is very easy to separate the fed mosquitoes from unfed mosquitoes as the fed mosquitoes have swollen red abdomens.
- Feed sugar and water to the mosquitoes and maintain them for 7 to 9 more days in a room maintained at 26 °C and 60 to 80% relative humidity.

Scoring and statistical analysis
Perform any TBA experiment with at least 2 or 3 different concentrations of the test serum. The controls may include preimmune sera, irrelevant antiserum (raised against asexual stage specific antigens), no antiserum added, and a serum known to possess transmission blocking activity.
- After 7 to 9 days, dissect the midgut of each and every individual mosquito from different groups under the dissection microscope and look for the presence of oocysts. Mosquitoes can be knocked down by dropping them in 70% ethanol and then transferred to a container with 1× PBS. Transfer the mosquitoes to glass slides and carefully pull the posterior part of mosquito with dissecting forceps, holding the anterior part of mosquito at the same time with another forceps. Treat the mosquito midguts thus separated with drops of 0.1% Mercurochrome solution and allow them to stain for a few minutes under coverslips.
- The oocysts will stain red on a light pink background of the midgut. Count the number of oocysts per midgut.
- Calculate the infection rates (number of mosquitoes infected per total number dissected) and number of oocysts (geometric means) in mosquitoes.
- Compare the infection rates and the number of oocysts in mosquitoes fed test serum to those obtained from feeds using appropriate control sera.
- Perform the Mann–Whitney test to assess the statistical significance.

Comments:
Apart from assessing the potency of different transmission blocking antibodies, the same experimental procedure can be used to address other biologically important questions. For example, effects of some chemotherapeutic drugs as transmission blocking agents.
can easily be scored with the help of this assay. Also the involvement of different agents on parasite infectivity can be quantitated. A similar experimental design is also used to perform genetic crosses using different parasite strains.

Reference
III:B. **Sporozoite invasion assay**

*by Christopher J. McCormick¹, Michael R. Hollingdale², and Andrew W. Taylor-Robinson¹*

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The techniques used for assessing *Plasmodium berghei* and *P. falciparum* sporozoite invasion are identical, although the latter parasite obviously poses a substantial risk to any individual exposed to it, so due care should be taken in handling infectious mosquitoes and viable sporozoites. Sporozoites are layered over hepatoma (HepG2-A16) cells or primary human hepatocytes and recognized by monoclonal antibody or serum raised to circumsporozoite (CS) protein, which bind the cell surface of sporozoites and block sporozoite invasion into hepatoma cells in vitro.

The reagent that is recommended for sporozoite detection is an IgM monoclonal antibody (MAb 36) which reacts with the CS proteins of both *P. falciparum* and *P. berghei*, and which was isolated from *P. falciparum* sporozoite-immunized mice (Sina et al. 1992; available on request from MR Hollingdale). In assays of biological activity, Mab 36 induces the CS precipitation reaction with live sporozoites and blocks the invasion of hepatoma cells by sporozoites in vitro at concentrations much lower than those observed for other reported CS protein-specific MAb. However, suitable alternatives are available in the MR4 repository: rabbit antiserum against *P. falciparum* CS protein (MRA-24) and rat antiserum against recombinant *P. falciparum* CS protein region I to C-terminus (MRA-21).

**Equipment**

- CO₂-incubator
- 200-µL Gilson pipette
- hemocytometer
- phase-contrast microscope

**Materials and reagents**

- immortalized human hepatocyte cell line HepG2 (available from MR4 as either a frozen ampoule or a growing culture)
- entacin–collagen–laminin (ECL)
- serum-free RPMI 1640 medium
- mosquitoes (see MOSQUITOES AND PARASITES, section II:C)
- PBS
- PBS + 0.0001% FCS
- PBS + 1% BSA
- MEM, minimal essential medium (Sigma)
- 10% heat-inactivated FCS (referred to as ‘binding medium’)
- 100% methanol
- MAb 36
- goat anti-mouse peroxidase conjugate (DAKO)
- distilled water
- DAB tablet (Sigma ‘Fast’ DAB kit)
- H₂O₂ (Sigma ‘Fast’ DAB kit)
- DPX mountant (HD Supplies)
Methods in Malaria Research

MOSQUITOES AND PARASITES

8-well Nunc slides

Producing a confluent HepG2 monolayer to study sporozoite invasion

The growth of HepG2 cells on 8-well Nunc coverslips is achieved using standard culture techniques, splitting confluent cultures between 1:3 and 1:6. However, the surface of slide wells should be precoated with an extracellular matrix (entacin–collagen–laminin; ECL) to promote cell attachment.

- Dilute a 40-µL frozen aliquot of ECL (stock concentration at 1 mg/mL) in 4 mL of serum-free RPMI 1640 medium. Add 200 µL of this solution to each well of the 8-well slides.
- Leave at 37 °C for a minimum of 2 h.
- Aspirate off the ECL solution and add a 250-µL suspension of HepG2 cells to each well.
- Place slides in an incubator (37 °C, CO2) for 2 to 3 d to allow the cells to attach and grow before using them in an invasion assay.

Invasion assay

Consider freshly dissected salivary glands containing *P. falciparum* sporozoites as potentially infectious and handle them with appropriate caution. Always wear a Howie-style laboratory coat and latex gloves.

- Dissect the mosquitoes for sporozoites between 14 and 17 d after an infectious feed, as described in detail previously (see MOSQUITOES AND PARASITES, section II:C).
- Dissect out both salivary glands from the apex of head and thorax of each mosquito and remove any unwanted body tissue. Collect salivary glands into ice-cold PBS + 0.0001% FCS. In our experience, the number of sporozoites recovered per infected mosquito varies between 4,000 and 30,000, average 15,000.
- Using a 200-µL Gilson pipette, vigorously pipette the suspension containing mosquito salivary glands up and down to disrupt the glands in order to release free sporozoites.
- Determine the density of sporozoites in this suspension using a hemocytometer. Place 10 µL of the sporozoite suspension into the hemocytometer chamber, use phase-contrast microscopy to visualize the sporozoites, and count the number present in a 4 × 4 grid. This number relates to the number of sporozoites per mm² × 10⁴.
- Dilute and divide the sporozoite suspension so that there are between 10,000 and 20,000 sporozoites per 300 µL of minimal essential medium (MEM) + 10% heat-inactivated FCS (referred to as ‘binding medium’). Ensure that this density is the same for all groups that are to be compared.
- Remove 8-well slides containing HepG2 cells from the 37 °C CO2 incubator, wash each well once with 300 µL of binding medium, and add 300 µL of sporozoite suspension. Place slides in a loose-lid container and return them to the 37 °C CO2 incubator for 3 to 4 h.
- Gently aspirate off the sporozoite suspension, add 300 µL of warmed MEM (without FCS), and leave the slides for 5 min.
- Repeat the wash.
- Aspirate off the MEM and flood the wells with 100% methanol. After 5 min, remove the methanol, add PBS, and leave them at 4 °C overnight.
Visualization of sporozoites
Visualize the sporozoites using immunohistochemical techniques.

- Discard the PBS solution and remove the plastic well surrounds and the rubber gaskets from the 8-well slides.
- Block each monolayer with a 100-µL solution of PBS + 1% BSA for 10 min.
- Aspirate off the solution and add 100 µL of PBS + 1% BSA + 5 µg/mL of MAb 36 (shows immunological cross-reactivity between \textit{P. falciparum} and \textit{P. berghei} CS proteins).
- Incubate the slides in a humidified chamber at room temperature for 30 min.
- Aspirate off the primary antibody solution and wash the cell monolayers by placing the slides in a Coplin jar containing PBS for 2 min. Repeat this step twice more.
- Add 100 µL of PBS + 1% BSA containing a 1:200 dilution of goat anti-mouse peroxidase conjugate. Incubate the slides in a humidified chamber at room temperature for 30 min.
- Repeat the PBS washes.
- In 1 mL of distilled water, dissolve one DAB tablet and one H\textsubscript{2}O\textsubscript{2} tablet from the Sigma ‘Fast’ DAB kit. Add 100 µL of this solution to each cell monolayer. Allow the reaction to proceed for 2 min and then terminate it by washing off the DAB solution with tap water. Note: \textbf{DAB is very toxic.} Always wear gloves when handling it and use copious amounts of water to wash solutions containing DAB down a suitable laboratory sink.
- Allow slides to dry and then mount them using DPX mountant and glass coverslips. Visualize sporozoites under either 200× or 400× magnification using phase contrast microscopy.

References

ANIMAL MODELS

I. Infection of monkeys with *Plasmodium* spp.

by William E. Collins

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Equipment

- compound microscope
- centrifuge
- water bath (37 °C)
- liquid N2 freezer
- Neubauer chamber
- Monolet

Materials and reagents

- syringes
- 16g, 19g, 21g, and 27g needles
- 25 × 75-mm microscope slides
- cryovials (Nunc)
- Glycerolyte 57 (Baxter Health Care - Fenwall Division)
- Giemsa stain
- heparinized vacutainer tubes
- phosphate buffer (pH 7.0)
- RPMI 1640
- 50-mL centrifuge tubes
- 12% NaCl
- 1.6% NaCl
- 0.9% NaCl + 2% dextrose solution

Preparations

Infection with fresh parasitized erythrocytes

- Collect blood from donor animal (using femoral vein) into a heparinized tube.
- Based on the parasite count, aseptically dilute blood in RPMI 1640 to give the required number of parasites in 1 mL or less.
- Restrain (New World) or anesthetize (larger Old World) monkeys.
- Inject blood slowly into femoral vein using a 25 g needle; maintain pressure on the needle exit hole until bleeding stops.

Infection with frozen parasitized erythrocytes

- Thaw vial(s) of frozen blood rapidly in 37 °C water bath.
- Rapidly transfer cells to a 50-mL tube by sterile pipette (note volume).
- Poke a hole in the cap of the 50-mL tube with a 16g needle.
- Draw 0.2× blood volume of 12% NaCl into a syringe and 21g needle.
- Add 12% NaCl dropwise through the hole while shaking the tube.
- Let the tube stand at room temp for 5 min without shaking.
- Take up 10× volume (of cells) of 1.6% NaCl.
- Add the NaCl solution dropwise through the hole while shaking the tube.
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- Spin the tube at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 10× cell volume of 0.9% NaCl and 2% dextrose solution as above.
- Spin at 1,400 rpm for 10 min.
- Remove the supernatant by aspiration and suspend the pellet by gentle shaking into 1 mL of RPMI 1640.
- Inoculate recipient monkey via the femoral vein using a syringe with 25g needle.

**Comment**
Viable parasites have been stored frozen for >25 years; thawing and refreezing has markedly reduced their viability.

**Infection via mosquito bite**
- Cage infected mosquitoes in a small container covered with nylon netting.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps (the center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage).
- Allow mosquitoes to feed to engorgement (usually 10 to 15 min).
- After feeding, dissect mosquito salivary glands and examine them for presence of sporozoites to determine number of infected mosquitoes.
- Determine the number of sporozoites remaining in the glands:
  - 1+ = 1 to 10 sporozoites
  - 2+ = 11 to 100 sporozoites
  - 3+ = 101 to 1,000 sporozoites
  - 4+ = >1,000 sporozoites
- Record the total number of +’s for all mosquitoes fed on the animal to determine the exposure index.

**Infection with sporozoites harvested by dissection**
- Kill mosquitoes by exposure to chloroform.
- Remove legs and wings.
- Transfer bodies to a clean slide (wiped with alcohol just prior to use).
- Remove salivary glands from the thorax by gently pulling the base of the head from the body using either a fine needle or a 27g needle on a 1-mL syringe into a drop of 20% heat-inactivated monkey serum/saline (homologous species).
- Cut the salivary duct and remove the body and head. Ten or more sets of glands can be harvested into one drop of serum/saline.
- Add a coverslip and apply gentle pressure to rupture glands.
- Gently remove the coverslip and wash sporozoites from coverslip onto the slide with several drops of serum/saline.
- Aspirate the sporozoite suspension into a syringe with 25g needle.
- Transfer the suspension to a Nunc vial.
- Adjust the volume to approximately 1 mL.
- Repeatedly aspirate and express the suspension from the syringe to further dissociate sporozoites from salivary gland tissue.
- Transfer a small volume to a Neubauer cell counting chamber and allow it to set for 15 min.
- Calculate the number of sporozoites available.
- Dilute the suspension to the desired number of sporozoites in 1 mL serum/saline.
• Inject into the femoral vein using a 25g needle.

Comments
Infection of New World monkeys with *P. falciparum* and *P. vivax* via sporozoites has required the injection of large numbers of sporozoites, either via the bites of many heavily infected mosquitoes or via needle-injection of dissected sporozoites. Increasing the number of sporozoites injected has resulted in greater transmission rates and shorter prepatent periods.

Monitoring parasitemia in the infected monkey
• Remove the animal from the cage and immobilize it.
• Thoroughly clean either an area of the tail or the back of the leg.
• Prick with a "Monolet", carefully avoiding the vein.
• From the drop of blood expressed, make thick and thin blood films on a precleaned, prelabeled microscope slide. Label the slide with the animal number and date.
• After the thin film dries, fix it with methanol.
• Stain the slide with Giemsa stain for 35 to 45 min.
• Examine the slide for presence of parasites.
• Express the parasite counts as
  1) parasites per 10,000 RBC,
  2) parasites per 100 WBC, or
  3) parasites per µL.
• If the latter, the method of Earle and Perez is preferred. Using a 5-µL pipette, collect the blood and express it onto a 15-mm × 5-mm area of a precleaned, prelabeled microscope slide (area is usually prescribed on the slide tray under the slide). Add a second drop of blood to the same slide to make the thin film.

Freezing parasitized erythrocytes
• Collect blood from the femoral vein into a heparinized tube.
• Spin the tube for 10 min at 1,200 rpm.
• Estimate the packed cell volume.
• Remove the plasma.
• Wash the cells 2 times with RPMI.
• Calculate 2× RBC volume (volume of Glycerolyte needed).
• Dropwise add one-fifth of the Glycerolyte volume to the tube at 1 or 2 drops per second with shaking, then let the tube stand for 5 min.
• Add the remaining Glycerolyte dropwise at same rate with shaking.
• Aliquot 0.5 mL to 1.0 mL per labeled Nunc vial.
• Transfer the vials to a −70 °C freezer overnight; transfer them to a vapor phase liquid nitrogen freezer for storage.

References

II. Infection of mosquitoes with *Plasmodium* spp. in monkeys

*by William E. Collins*

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**Equipment**
- enamelware or plastic pans
- incubator (25 °C)

**Materials and reagents**
- deionized water
- baker’s yeast
- mosquito larval food
- monkey/rat chow
- lactalbumen (Sigma L-7252)
- brewer's yeast (Sigma YBD)
- 10% sugar solution
- cotton balls
- blood source (rabbit/Guinea pig)
- Parafilm

**Preparation**

**Rearing anopheline mosquitoes**
- Grind monkey or rat chow in a mill and sieve through a 60-mesh screen.
- Combine lactalbumen, inactive brewer's yeast, and chow (1:1:1).
- Store the mixture in a refrigerator.
- Allow mosquito eggs to hatch in a pan lined with strips of paper toweling.
- Feed a “pinch” of active baker’s yeast on Day 1.
- On succeeding days, feed increasing amounts of mosquito larval food.
- Maintain the water depth at 2 to 3 cm.
- Every 3 or 4 days, split pans until Day 8 to give approximately 1 larva/cm² of water surface.
- When pupae appear, strain and suspend larvae and pupae in ice water.
- Pour floating pupae into an emergence cup.
- Return the larvae to the rearing pan.
- Place the pupae in a cage for emergence.
- After they emerge, feed them a 10% sugar solution daily on a cotton ball.
- Beginning 3 days after emergence, offer them a blood meal from an anesthetized rabbit or guinea pig every 3 or 4 days. (Shave hair from the feeding area.).
- Three days after each blood meal, put a small bowl with water (1 to 2 cm depth) into the cage overnight to collect eggs.

**Comments**

Most commonly reared anopheline mosquitoes such as *Anopheles stephensi*, *A. atroparvus*, *A. quadrimaculatus*, *A. gambiae*, and *A. albimanus* will mate in cages; others such as *A. dirus* require force mating. The most suitable vector depends on the species and strain of *Plasmodium* being investigated.
Infection of mosquitoes by feeding on monkey
- Transfer female mosquitoes (3 to 7 days after emergence) to infection cages.
- Concentrate female mosquitoes by placing a warm hand to the side of the holding cage.
- Aspirate and transfer the mosquitoes to the infection cage.
- Partially starve mosquitoes overnight with 5% sugar solution.
- Anesthetize and immobilize the monkey on a restraining board using Velcro straps. (The center of the restraining board is cut out to allow the belly of the monkey to rest directly on top of the feeding cage.)
- Allow mosquitoes to feed to repletion (usually 10 to 15 min); remove and discard unfed mosquitoes.
- Store cartons of mosquitoes in a 25 °C incubator.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.
- Seven to 10 days after feeding (depending on the species of *Plasmodium*), dissect aliquots of mosquitoes to determine presence and numbers of oocysts.
- If oocysts are present, return undissected mosquitoes to the incubator.
- Dissect and examine salivary glands 10 to 18 days after feeding (depending on the temperature and species of *Plasmodium*).

Comments
Development of oocysts depends on the temperature and the species of *Plasmodium*. When incubated at 25 °C, the quotidian parasite *P. knowlesi* completes sporogonic development in 10 days; the tertian parasites *P. vivax*, *P. ovale*, *P. cynomolgi*, *P. gonderi*, *P. coatneyi*, *P. fragile*, *P. fieldi*, *P. simium*, and *P. simiovale* in 10 to 15 days; the quartan parasites *P. malariae*, *P. brasilianum*, and *P. inui* in 15 to 20 days.

Infection of mosquitoes by membrane feeding
- Transfer mosquitoes to infection cage (as above).
- Collect blood in heparin from the donor animal.
- Centrifuge blood at 1,500 rpm for 10 min.
- Remove plasma and replace it with serum or heparinized blood from an unexposed animal or human.
- Cover the feeding surface of a water-jacketed feeding bell with stretched Parafilm.
- Place the blood in the chamber.
- Commence circulation of water (37 °C).
- Lower the bell onto the netting of the feeding cage and allow mosquitoes to feed to repletion for 5 to 10 min.
- Remove and discard unfed mosquitoes.
- Feed mosquitoes a 10% sugar solution during extrinsic incubation.

Comments
Blood is collected in heparin, ACD, or is defibrinated with glass beads; EDTA inhibits infection. Transmission-blocking immunity often develops rapidly in monkeys. Removal of plasma and its replacement with serum or blood from an uninfected host allows continued mosquito infection if infectious gametocytes are present. Membrane feeding provides a means of assessing the transmission-blocking activity of immune sera by combining dilutions of sera from immunized
monkeys with infectious gametocytes. Reduction in oocyst densities measures transmission-blocking activity.

References


III. Culturing erythrocytic stages of *Plasmodium chabaudi*: a mouse malaria model

*by Masashi Hayano*¹,² and *Helena Helmby*³

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**Equipment**
- hemocytometer

**Materials and reagents**
- 0.05% *p*-aminobenzoic acid (PABA, Sigma)
- Giemsa stain (Gurr's improved Giemsa, BDH Laboratory Supplies)
- acridine orange (AO) (optional)
- heparinized tubes
- methanol

**Choice of mouse strain**
- C57Bl/6-resistant mice (clear the parasitemia and become immune)
- BALB/c-semisusceptible mice (some die, survivors become immune)
- A/J 100%-susceptible mice (all die)

For optimal parasite growth, add *p*-aminobenzoic acid to the drinking water of the mice.

**Frozen stabilates**
- Bleed mice with high parasitemia (>20%) into heparinized tubes.
- Aliquot blood (100 µL/tube) in cryotubes and freeze immediately in liquid nitrogen.
  (For short periods, the tubes can be frozen and stored at −70 °C.)

Since parasite virulence can increase after a number of in vivo passages, it is important to start from a fresh stock of stabilates when mortality starts to increase. (Mortality is best observed in a semisusceptible strain.) Freeze new stabilates regularly.

**To start infection**
- Thaw a cryotube of parasites.
- Inject undiluted contents immediately i.p. into a naive recipient mouse.
- After 7 to 14 days, parasites appear in the blood.

**Counting parasitemia**
- Make a thin blood film from tail blood, fix with methanol for 2 min, and stain with Giemsa.
- Count percent infected cells (100× oil immersion).
  Instead of Giemsa staining, acridine orange (AO) staining of thin blood films is possible. After methanol fixing, add a small drop of AO on a slide, add a
coverslip, and count parasitemia under a light and UV-light microscope (see PARASITES, sections V:A, C).

Passage of infection into new mice

- Bleed the donor mouse.
- Count parasitemia as above and the number of erythrocytes per microliter in a hemocytometer.
- Dilute the blood in sterile PBS to $10^7$ infected erythrocytes per milliliter.
- Inject 100 $\mu$L i.p. per mouse ($10^6$ infected erythrocytes per dose).
- Parasites appear after 2 to 3 days, and after 8 to 10 days the parasitemia is around 30-50%.
- Mice succumbing to the infection usually die on Days 9-12.

Higher infectious doses give an earlier peak of parasitemia, while lower doses give a delayed peak; i.e., a 10× concentrated dose gives a peak approximately 1 day earlier, while a dose diluted 1:10 causes a peak 1 day later.

If you inject i.v., only one-tenth of the i.p. dose is needed to obtain similar parasitemias.

Reference
IMMUNOCHEMISTRY

I. Studies of the Plasmodium falciparum-infected erythrocyte surface

I:A. Surface iodination of PRBC (Lactoperoxidase method)

by Victor Fernandez
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Materials and reagents
parasite culture at 5% hematocrit and >5% parasitemia
\(^{125}\text{I}\)-Na 1 mCi/10 \(\mu\)L (Amersham IMS.30)
PBS (pH 7.2) (stored at 4 °C)
lactoperoxidase, 2 mg/mL (Sigma L8257) (stored at −20 °C)
30% \(\text{H}_2\text{O}_2\) (stored at 4 °C)

Labeling buffer:
100 mL PBS (pH 7.2)
0.087 g \(\text{K}_2\text{HPO}_4\)
0.1 mL 1 mM KI

washing buffer:
100 mL PBS (pH 7.2)
0.83 g KI

Procedure (On ice at all times)
- Use cells from a culture of 10 to 20% parasitemia (not lower than 5% at any case) with a majority of trophozoite stages.
- Wash the cells 3 times with PBS, then transfer 200 \(\mu\)L of the washed packed PRBC into a fresh centrifuge tube and add 800 \(\mu\)L of Labeling buffer.
- Add 0.5 to 1 mCi of \(^{125}\text{I}\)-Na to the cell suspension.
- Add 100 \(\mu\)L of lactoperoxidase stock solution (2 mg/mL).
- Dilute the stock solution of \(\text{H}_2\text{O}_2\) 1,000-fold in PBS just before use.
- Add 25 \(\mu\)L of the diluted \(\text{H}_2\text{O}_2\) to the cells; mix gently.
- Wait 1 min and repeat the addition 3 times (4 times altogether).
- At 1 min after the last addition of diluted \(\text{H}_2\text{O}_2\), wash the cells 4 times in ice-cold washing buffer.
- The cells are now ready for separation/enrichment of mature stages in Percoll/sorbitol gradients as described in PARASITES, section IV:D.

Reference
I:B. **Solubilization/extraction of surface-radiolabelled proteins**

*by Victor Fernandez*

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**Equipment**

- microfuge, refrigerated

**Materials and reagents**

Solution containing:
- 1% Triton X-100
- 1 mM PMSF
- 1 mM EDTA
- 1 µM leupeptin
- 2 µM pepstatin
- 1% ε-amino-n-caproic acid in PBS

SDS–PAGE sample buffer:
- 2% SDS
- 5% 2-mercaptoethanol

**Procedure**

- Use the pellet of labelled PRBC enriched for mature stages on Percoll/sorbitol gradient as described above (see PARASITES, section IV:D).
- Resuspend the pellet in 0.25 to 0.5 mL of a solution containing 1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 1 µM leupeptin, 2 µM pepstatin, and 1% ε-amino-n-caproic acid in PBS. Incubate the pellet on ice for 1 h with periodic vortexing.
- Centrifuge the samples in a microfuge at 4 °C, at maximum speed for 15 min. Separate and save the supernatant (the fraction soluble in Triton X-100).
- Resuspend/extract the pellet in SDS–PAGE sample buffer, mix well by pipetting, boil for 4 min, mix again, and centrifuge it for 3 min at maximum speed in a microfuge.
- Separate the supernatant (the fraction soluble in SDS).
- Run aliquots of Triton X-100 and SDS-soluble fractions on SDS–PAGE gels (6% homogeneous or 5 to 8.5% gradients for proteins above Band 3; i.e., M.W. ~100,000, 7.5 to 17.5% gradients for proteins below Band 3).

**Reference**

Methods in Malaria Research

I: C. Alternative solubilization protocol
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Equipment
- SDS–PAGE gel apparatus
- microfuge

Materials and reagents
- 2% SDS
- PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors
- PBS containing 1% Triton X-100, 1 mM PMSF and 1% ε-amino-n-caproic acid
- protein A–Sepharose (Amersham Biosciences 17-0780-01 or Sigma)

Preparation
- Solubilize ~200 µL of erythrocytes (the pellet from one flask) in 4 to 8 mL of 2% SDS (dissolved in PBS containing 0.1 mg/mL of leupeptin and 0.1 mg/mL of chymostatin protease inhibitors).
- Incubate the sample at room temperature for 30 to 90 min. Any possible background radiation from the protein A–Sepharose can be adsorbed by mixing washed protein A–Sepharose with the solubilized sample and letting it incubate for 1 h prior to the immunoprecipitation.

Solubilization
- Extract surface-radioiodinated proteins by using 0.2 mL of pellet plus 1.8 mL of PBS containing 1% Triton X-100, 1 mM PMSF, and 1% ε-amino-n-caproic acid and incubating the tube at 4 °C on ice for 1 h.
- Centrifuge the extract in a microfuge at maximum speed for 15 min. Remove the Triton X-100 soluble supernatant, then freeze the supernatant at −70 °C.
- Extract the insoluble pellet in SDS–PAGE sample preparation buffer.
- Separate the proteins on a 7.5 to 17.5% linear gradient SDS–PAGE gel.

Reference
I:D. Immunoprecipitation of $^{125}$I-labelled PfEMP1

by Victor Fernandez

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Equipment
- microcentrifuge
- PhosphorImager

Materials and reagents
- $^{125}$I-labelled SDS extract, preferably use 100,000 or more cpm per sample
- $^{125}$I-labelled Triton X-100, if desired
- 2% Triton X-100 in PBS
- Hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors
- Protein A-Sepharose (Amersham Biosciences 17-0780-01)

NETT buffer:
- 150 mM NaCl, MW 58.44 (8.77 g/liter)
- 5 mM EDTA, MW 380.2 (1.9 g/liter)
- 50 mM Tris-base, MW 121.1 (6.06 g/liter)
- 0.02% sodium azide
- 0.5% Triton X-100
- Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT with final 0.3 M NaCl
NETT with 10 mg/mL Ig-free BSA containing protease inhibitors (1 mM PMSF, 1 mM EDTA, 1 mM leupeptin, 2 mM pepstatin, and 1% ε-amino-n-caproic acid in PBS)
NETT with 10 mg/mL BSA Fraction V secondary antibodies as appropriate
protein A-Sepharose equilibrated with NETT with Ig-free BSA without protease inhibitors, make up as a 1:1 suspension (i.e., 50 μL of Sepharose gel : 50 μL of suspension)
5% SDS Sample Buffer (= 2× concentration)

Procedure

Day 1
- Reconstitute SDS extract by adding an equal volume of 2% Triton X-100 in PBS and 20 volumes of NETT plus Ig-free BSA containing protease inhibitors.
- Aliquot the reconstituted SDS extract in microfuge tubes allowing about 100,000 cpm per tube (or more if it is available).
- Add primary antibody:
  - For sera, use 10 μL/sample for monoclonal antibodies (you can go up to 20 μL with human or Aotus sera if desired), but rabbit sera generate a high MW aggregate which messes up this region on the gel. Use 2 to 5 μL of hybridoma supernatant to which has been added 25 mM of HEPES (pH 7.4), 0.5% Triton X-100, 5 mM EDTA, and protease inhibitors.
- Incubate the sample overnight at 4 °C with slow rotation/mixing.
Day 2

- Add secondary antibodies if using primary antibodies not binding protein A. Leave the tubes for 1 h at room temperature.
- Add 100 µL of the above equilibrated protein A–Sepharose.
- Leave it at room temperature for 1 h with intermittent mixing.
- Take off the supernatant and put in $^{125}$I liquid waste. Wash the gel–slurry with the following solutions and quick, ~30-s spins in a microcentrifuge on the high setting:
  - 1 mL NETT–BSA
  - 1 mL NETT
  - 1 mL NETT with 0.3 M NaCl
- Leave the slurry for 20 min to allow NaCl to equilibrate into the beads. Then wash again:
  - 1 mL NETT with 0.3 M NaCl
  - 1 mL NETT
- Take off all supernatant.
- Add 50 µL of 2× Sample Buffer to the pellet. Mix and freeze at −70 °C until use in SDS–PAGE gel electrophoresis.

Notes on the capacity of protein A–Sepharose
According to the manufacturer's specification, the capacity is 20 mg of human-IgG per milliliter of gel or 3 to 10 mg of mouse-IgG per milliliter of gel. Taking 10 mg/mL as above, then 50 µL of gel used for immunoprecipitation has a maximum capacity of 500 µg of IgG. Based on the principle that it is best to use less than 20% of capacity to get complete binding, one should aim to use maximally 100 µg of IgG.

  e.g., 10 µL of rat serum = 100 µg of IgG. Add 50 to 100 µg of secondary antibody IgG.

Autoradiography for the measurement of γ rays
Stain, destain, and dry the SDS–PAGE gel. Expose a PhosphorImager screen (or an X-ray film using an intensifying screen) at room temperature.

Reference
II. Metabolic Labeling of *Plasmodium falciparum*

II:A. Metabolic Labeling of *Plasmodium falciparum*-infected erythrocytes

by Victor Fernandez  
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Materials and reagents

- RPMI custom medium, containing everything including serum, but without methionine  
- healthy parasites at ring-stage  
- 0.5 mCi of $^{35}$S-methionine (Amersham SJ1015)  
- RPMI 1640

Procedure

- Use ring-form parasites to label, then let them develop into late stages (20 to 30 h) for analysis.  
- Use RPMI custom medium, containing everything including serum, but without methionine.  
- Use as high a parasitemia as possible (i.e., healthy parasites at ring-stage).  
- Use 0.5 mCi of $^{35}$S-methionine per small culture flask (25 cm$^2$).  
- Remove the old medium, as usual, leaving a few drops of the old medium in the flask.  
- Mix 0.5 mCi of $^{35}$S-methionine with 5 mL of medium and add to the flask.  
- Incubate the flask as usual at 37 °C for approximately 30 h, until late parasite stages develop.  
- Wash the parasites 3 times with RPMI 1640.  
- Freeze the parasite pellet at −20 °C.

Comments on autoradiography for measuring β rays

- Stain and destain the gel. If it will be exposed on an X-ray film, use Amplify (Amersham) after the last destaining bath. Use enough Amplify for the gel to float freely and let it soak for 15 to 20 min. This step is not needed if a PhosphorImager screen will be used.  
- Dry the gel. Expose the X-ray film to the gel at −70 °C using an intensifying screen, or simply use a PhosphorImager screen at room temperature.

Reference

II:B. Immunoprecipitation using metabolically labelled parasite extracts
by Victor Fernandez
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Equipment
immufuge

Materials and reagents
NETT-buffer 1 (NETT-1):
- 150 mM NaCl, MW 58.44 (8.77 g/L)
- 5 mM EDTA, MW 380.2 (1.9 g/L)
- 50 mM Tris-base, MW 121.1 (6.06 g/L)
- 0.02% sodium azide
- 0.5% Triton X-100
Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

NETT-buffer 2 (NETT-2):
same as above except use 500 mM NaCl (29.22 g/liter).
Adjust to pH 8.1 if mouse antibody is used, otherwise use pH 7.4.

- 10% Triton X-100
- 1% BSA
- 50% protein A–Sepharose (washed with NETT-buffer 1)
SDS–PAGE sample buffer:
- 100 mM Tris-HCl, pH 6.8
- 10% Glycerol
- 5% SDS
- 2% 2-mercaptoethanol
- bromophenol blue
- 0.25% Coomassie blue R-250
- Amplify (Amersham)

Procedure
- Mix in a conical centrifuge tube:
  300 µL NETT-1
  100 µL 10% Triton X-100
  25 to 100 µL antibodies
  100 µL solubilized sample
  1% BSA
- Incubate the mix for 90 min at room temperature or 4 °C, slowly rotating/mixing.
- Add 75 µL of 50% protein A–Sepharose. Incubate the mix for 30 to 60 min at room temperature. Shake the tube a little now and then.
- Wash the Sepharose mixture and centrifuge it in a blood centrifuge (Immufuge) for 15 s on low setting:
  twice with NETT-1
  once with NETT-2
  twice with NETT-1
- Add 50 µL of sample prep. buffer and boil the solution for 2 min. Run the sample on SDS–PAGE.
- Stain and destain the gel, use Amplify for no more than 20 min (see IMMUNOCHEMISTRY, section II:A above), then dry the gel at 60 °C.
- Put the dried gel on film, without intensifying screen, in a cassette at −70 °C for at least two weeks or simply use a PhosphorImager screen at room temperature.

Reference
III. Resolution of giant proteins (200 kDa to 1 MDa) of *Plasmodium falciparum* on polyacrylamide–agarose composite gels

*by Denise Mattei and Artur Scherf*

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**Equipment**

SDS–PAGE gel apparatus

**Materials and reagents**

polyacrylamide–agarose composite gel:
- 375 mM Tris–HCl (pH 8.8)
- 0.5% agarose
- 3% acrylamide (29:1, acrylamide:bisacrylamide)
- 0.1% SDS

ammonium persulfate

*N,N,N′,N′*-tetramethyl-ethylenediamine (TEMED)

sample buffer:
- 10% SDS
- 250 mM Tris–HCl (pH 8.8)
- 20% glycerol
- 0.1% bromphenol blue

running buffer:
- 25 mM Tris–HCl (pH 8.3)
- 250 mM glycine
- 0.1% SDS

transfer buffer:
- 48 mM Tris
- 39 mM glycine
- 0.0375% SDS
- 3% 2-mercaptoethanol (optional)

**Preparation**

- Prepare the polyacrylamide–agarose composite gel. Dissolve the agarose by boiling it in the Tris buffer, cool it to 50 °C, and then add the acrylamide and SDS.
- Initiate the polymerization by adding ammonium persulfate and TEMED to a final concentration of 0.1% and 1 μL/mL of gel, respectively. Cast the gel in a standard horizontal chamber without a stacking gel layer.
- Boil the protein extracts for 5 min in sample buffer and load them on the gel. The addition of 3% 2-mercaptoethanol to the sample buffer is optional. In the presence of 2-mercaptoethanol, samples are denatured by heating to 50 °C for 15 min.
- Size-fractionate the proteins at 15 V/cm in running buffer for 1 to 2 h.
- Transfer the size-fractionated proteins to a nylon membrane using a semidry transfer device at 3.5 mA/cm² in transfer buffer for 2 h.

**Reference**

IV. Recovery of *Plasmodium falciparum* native proteins and active enzymes

IV:A. Solubilization in the presence of non-detergent sulphobetaines, mild solubilization agents for protein purification

*by Catherine Braun-Breton*

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**Equipment**

- centrifuge

**Materials and reagents**

- RPMI 1640
- 10% Triton X-100 (stock solution)
- mixed ion-exchange beads AG 501-X8 (Bio-Rad)
- 3 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka) dissolved in warm water and repurified on mixed ion-exchange beads; kept at −20 °C
- 2% Triton X-100, 2 M NDSB 201 (extraction solution; kept at −20 °C)
- leupeptin and pepstatin (10 µg/mL) (optional) (stock solutions)
- double-distilled water
- sulphobetaine SB3-14 (optional)

**Preparation**

- Wash pelleted PRBC twice in RPMI 1640.
- Resuspend the pellet in 1 volume of double-distilled water.
- Freeze at −20 °C and thaw at room temperature.
- Add 2 volumes of:
  - 2% Triton X-100 (or detergent sulphobetaine SB3-14)
  - 2 M NDSB 201
  - leupeptin and pepstatin (10 µg/mL), if needed
- no Tris buffer (usually 10 mM [pH 7.5]) if followed by isoelectric focusing
- Incubate the pellet for 30 min at room temperature.
- Centrifuge the pellet for 30 min at 15,000 × g at 4 °C; keep the supernatant (extract) frozen at −20 °C.

**Reference**

The Rotofor cell is a preparative scale, free solution, isoelectric focusing apparatus useful in a protein purification scheme. The cell can achieve 25-fold purification of a protein in a single 4-h run. This technique is especially advantageous when protein activity must be maintained. Bioactivity is maintained because the proteins are in solution in their native conformation.

**Equipment**
- Rotofor cell (Bio-Rad)
- pH meter

**Materials and reagents**
- 0.1 M NaOH
- 0.1 M H₃PO₄
- 1% Triton X-100
- 1 M NDSB 201 (3-{1-pyridinio}-1-propanesulfonate) (Fluka)
- 2% ampholytes (Pharmalytes of the appropriate pH range, usually between pH 3 and pH 10, 40% stock solution, Pharmacia)

**Preparation**
- Equilibrate the ion exchange membranes by overnight incubation in the appropriate electrolyte solution: 0.1 M NaOH for the anion exchange membrane and 0.1 M H₃PO₄ for the cation exchange membrane.
- Cool the Rotofor cell by connecting the cooling finger to a source of circulating coolant.
- Assemble the focusing chamber according to the instruction manual.
- Fill the electrolyte chambers with electrolytes immediately after assembly to prevent the membranes from drying: 25 mL of 0.1 M NaOH for the cathode chamber (–) containing the anion exchange membrane and 25 mL of 0.1 M H₃PO₄ for the anode chamber (+) containing the cation exchange membrane.
- Fill the focusing chamber with 45 mL of focusing solution, using a 50-mL syringe with a needle:
  - 1% Triton X-100 (or SB3-14; not necessary for soluble proteins)
  - 1 M NDSB 201
  - 2% ampholytes
- Prerun the chamber for 1 h at 4 °C, 11 W, 34 mA, and 995 V to preform the pH gradient.
- Load 1 to 5 mL of the sample (protein solution or extract) in 1 M NDSB 201, 2% ampholytes.
- Add the sample in the central channels of the Rotofor focusing chamber to prevent exposure of the proteins to very low or high pH.
- Protein concentration should be adjusted for desired yield. Assuming each component will focus in 1 to 3 fractions; preparative fractionation of 1 to 2 g of proteins has been successfully performed!
- Run the sample for 3 h at 4 °C, 11 W, 34 mA, and 995 V.
• Collect the 20 fractions on ice.
• Measure the pH of each fraction. Take a 100-µL aliquot, add 1 mL of double-distilled water, and keep it at room temperature for 10 min before measuring the pH.
• Store the fractions at –20 °C.

Reference
SEROLOGY

I. Antibody staining of *Plasmodium falciparum*-infected erythrocytes

I:A. Erythrocyte membrane immunofluorescence (EMIF)

by Hedvig Perlmann

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden
e-mail: hedvig.perlmann@imun.su.se

Equipment

- centrifuge
- humid chamber (wet filter paper in a plastic box)
- microscope (UV and light)

Materials and reagents

*Plasmodium falciparum* cultures in human type O+ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)

- glutaraldehyde (GDA)
- biotinylated anti-Ig of the appropriate specificity (Vector Labs)
- FITC–Avidine D, F/P 4.9 (Vector Labs).
- ethidium bromide (Sigma)
  - CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
- Tris (Sigma)
- Hanks’ balanced salt solution (Gibco)
- multitest slides (8 wells; ICN Biomedicals)
- Pasteur pipettes
- micropipettes

**TH-buffer (0.15 M Tris-buffered Hanks’, pH 7.2):**

- 2.11 g Tris–HCl
- 0.2 g Tris-base
- 7.88 g of 0.9% NaCl
  - Dissolve the ingredients in distilled water and bring volume to 1 liter.
  - Mix 1 volume of Tris buffer with 1 volume of Hanks’ BSS.
  - Add 0.02% NaN₃ (200 mg NaN₃/1 liter).

**coating buffer (pH 9.6):**

- 1.59 g Na₂CO₃
- 2.93 g NaHCO₃
- 200 mg NaN₃
  - Dissolve the ingredients in distilled water and bring the volume to 1 liter.

**Monolayers**

- Wash cultures (5 to 10% parasitemia) in TH and resuspend them to 1% hematocrit.
- Pretreat multitest slides, 8 wells with one drop (~20 μL) per well of coating buffer for 30 min.
• Add one drop per well of the 1% *P. falciparum* culture directly after aspiration of the coating buffer and leave the slides to settle for 30 min.
• Rinse off unbound erythrocytes by gently shaking the slides immersed upside down in TH.

**Glutaraldehyde (GDA)-fixation of monolayers**
• Quickly but gently cover the wet monolayers with 1 to 2 mL of 1% GDA (in PBS) on top of the slide.
• Decant the GDA after about 10 s and repeat the GDA fixation once.
• Wash the slides with distilled water and air-dry them. Slides can then be stored long-term in a freezer (>10 years).

**Staining**
All incubations below should be done at room temperature in a humid chamber for 30 min.
• Treat the slides sequentially with one drop (~20 µL) of serial dilutions of sera containing malaria antibodies.
• Use 15 µg/mL of biotinylated anti-Ig of the appropriate specificity followed by 50 µg/mL of FITC–avidin D.
• Wash the slides between the incubations with TH or PBS.
• For counter-staining, use one drop of ethidium bromide solution (10 µg/mL) in each well and rinse the slides with distilled water after a few seconds.

**Reference**
I:B. Erythrocyte membrane staining by enzyme linked antibodies (EMEAS)

by Rachanee Udomsangpetch
Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th

Prepare glutaraldehyde-fixed monolayers as in the protocol for EMIF (see SEROLOGY, section I:A).

Equipment
- light microscope with a 100× oil-immersion lens
- centrifuge
- humid chamber (wet filter paper in a plastic box or dish)

Materials and reagents
- Plasmodium falciparum cultures in human type O+ erythrocytes, 5 to 10% parasitemia (see PARASITES, section I:A)
- multitest slides (8 wells; ICN Biomedicals)
- Pasteur pipettes
- micropipettes
- biotinylated anti-Ig (Vector Labs)
- streptavidin/ALP (Mabtech)
- PBS
- ALP-conjugated Substrate Kit (Bio-Rad 170-6432)

coating buffer (pH 9.6):
- 1.59 g Na₂CO₃
- 2.93 g NaHCO₃,
- 200 mg NaN₃
Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Staining
All incubations below should be done at room temperature in a humid chamber for 30 min.

- Treat the slides sequentially with serial dilutions of immune sera.
- Use biotinylated anti-Ig of the appropriate specificity and streptavidin/ALP.
- Wash the slides between the incubations with PBS.
- Add freshly prepared substrate.
- Wash slides under distilled water after 20 min, then air-dry them.
- Examine the slides under a light microscope with a 100× oil-immersion lens.

Reference
I:C. Parasite immunofluorescence (PARIF)
by Rachanee Udomsangpetch
Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand e-mail: scrud@mahidol.ac.th

This protocol allows for antibody staining of intracellular *Plasmodium falciparum* using air-dried monolayers of infected erythrocytes. For staining of the live, intact PRBC surface, please refer to PARASITES, section IX:B.

Equipment
- fan

Materials and reagents
- *Plasmodium falciparum* cultures in human type O+ erythrocytes, 5 to 10% parasitemia of primarily late stages
- PBS coating buffer (pH 9.6):
  - 1.59 g Na₂CO₃
  - 2.93 g NaHCO₃
  - 200 mg NaN₃
  - Add distilled water up to 1 liter.

Monolayers
- Use *Plasmodium falciparum* cultures in human type O+ erythrocytes, 5 to 10% parasitemia of primarily late stages.
- Wash cells in PBS and resuspend them to 1% parasitemia.
- Pretreat multitest slides with one drop (10 to 20 µL) per well of a coating buffer for 30 min.
- Aspirate the wells and immediately add one drop of the washed culture to each well.
- Leave it to settle for 30 min.
- Gently aspirate the excess of red cells from each well and air-dry the slides under a fan.
- The slides can be stored in a freezer; dry them well before staining.
- For staining, see the protocol for EMIF in SEROLOGY, section I:A above.

Reference
II. Antibody selection on immobilized antigen or peptide

by Denise Mattei

Unité de Biologie des Interactions Hôte-Parasite, CNRS URA 1960, Institut Pasteur, 75724 Paris, France

e-mail: dmm@pasteur.fr

Equipment

PD10 column equilibrated in PBS

Materials and reagents

- 0.45-µm nitrocellulose membrane
- PBS
- PBS plus 5% powdered skim milk
- 3 M MgCl₂, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol

Preparation

- Prepare 5 mL of a 5 to 10 µg/mL solution of peptide or protein in PBS.
- Soak a 0.45-µm nitrocellulose-membrane filter in this solution for 30 min at room temperature with gentle agitation, and then soak a second filter in this solution.
- Incubate the filters for saturation in PBS plus 5% powdered skim milk for 90 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Incubate each filter in 5 mL of a 1:20 dilution of appropriate serum in PBS plus 5% powdered skim milk for 60 min at room temperature (or overnight at 4 °C) with gentle agitation.
- Wash the filters in PBS plus 5% powdered skim milk for 30 min at room temperature with gentle agitation.
- Wash the filters twice in PBS.
- Soak the filters in 5 mL of a solution of 3 M MgCl₂, 75 mM HEPES/NaOH (pH 7.2), 25% ethylene glycol.
- Incubate the filters for 20 min at room temperature with gentle agitation.
- Recover the solution and load it on a PD10 column equilibrated in PBS for collection of 500-µL fractions.
- Check for the presence of IgG in the fractions by dot blot analysis (5 µL of each fraction).

Reference

III. ELISA

III:A. Equipment, materials, and reagents

by Hedvig Perlmann
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e-mail: hedvig.perlmann@imun.su.se

Equipment
- micropipette, multichannel pipette (e.g., Thermo Electron)
- microplate washer (e.g., Thermo Electron, Skatron Instruments)
- microplate reader (e.g., V max kinetic microplate reader with computer program SOFTmax Cat. No. 79-200 105, 79-200 100)
- centrifuge, swing out rotor

Materials and reagents
With many proteins or peptides, PBS can be used as a coating solution at least with the above plates; test your system first.

- flat-bottomed microtiter plates (Maxisorp from Nunc A/S or High Binding from Costar, Cat. No. 3590)
- disposable pipette tips
- tubes for preparaions of dilutions (Bio-Rad, Cat. No. 223-9391)
- cell culture flasks
- RPMI–HEPES (see PARASITES, section I:A)
- Hanks' balanced salt solution (Gibco)
- PBS 10× stock solution:
  - 40 g Na₂HPO₄ · 12 H₂O
  - 5 g KH₂PO₄
  - 81 g NaCl
  - 1.0 mL 20% NaN₃
  - Dissolve in distilled water and bring volume to 1 liter.
- Coating buffer (pH 9.6):
  - 1.59 g Na₂CO₃
  - 2.93 g NaHCO₃
  - 1.0 mL 20% NaN₃
  - Dissolve the ingredients in distilled water and bring the volume to 1 liter.
- Blocking buffer:
  - 0.5% BSA in coating buffer
- Tween buffer for dilutions and incubations:
  - 100 mL PBS stock (10×, see above)
  - 5 g BSA
  - 0.5 mL Tween
  - 1.0 mL 20% NaN₃
  - Dissolve the ingredients in distilled water and bring the volume to 1 liter.
Methods in Malaria Research

**SEROLOGY**

**Tween wash:**
- 45 g NaCl
- 2.5 mL Tween

Dissolve the ingredients in distilled water and bring the volume to 5 liters.

**Enzyme substrate buffer:**
- 97 mL diethanolamine
- ~700 mL distilled water
- 1 mL 20% NaN₃

Adjust the pH to 9.8 with 1 M HCl (~100 mL), then add 101 mg of MgCl₂·H₂O. Bring buffer volume to 1 liter.

**Substrate:**
- NPP (kept in freezer)

Dissolve 1 tablet of NPP in 5 mL of enzyme substrate buffer; keep the solution in the dark. Incubate plates with the substrate at room temperature and keep them in the dark until you are ready for a reading at OD₄₀₅ (usually in 20 to 40 min).

**TH (0.15 M Tris-buffered Hanks’, pH 7.2):**
- 2.11 g Tris–HCl
- 0.2 g Tris-base
- 7.88 g NaCl

Dissolve the ingredients in distilled water and bring the volume to 1 liter.

Add 1.0 mL of 20% NaN₃. Mix 1 volume of Tris buffer with 1 volume of Hanks’ balanced salt solution.

**Sera:**
For antibody determinations, sera may be kept in the refrigerator diluted 1:10 in Tris-buffered Hanks’ with 0.02% NaN₃ for many months.

**Conjugate:**
- ALP-conjugated or biotinylated anti-Ig of appropriate specificity at recommended concentration
- Streptavidine–ALP (Mabtech) when biotinylated antibody has been used.

**60% Percoll solution:**
- 60 mL Percoll
- 7 mL 10× PBS
- 33 mL PBS
- 0.3 M NH₄OH
III:B. Antigens for coating

by Hedvig Perlmann

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Crude parasite antigen
Late stage-infected erythrocytes enriched by gradient centrifugation. For 40 tubes, 80-mL of culture:

- Prepare fresh 60% Percoll solutions before each use. Keep all solutions on ice throughout the preparation.
- Wash a late stage *Plasmodium falciparum* culture once in RPMI–HEPES and resuspend it to 10% hematocrit in cold RPMI–HEPES.
- Distribute in centrifuge tubes, 2 mL/tube.
- Using a Pasteur pipette, gently add 2.5 mL of cold 60% Percoll to the bottom of each tube.
- Centrifuge the tubes in a swing-out rotor at 1,500 \( \times \) 4 °C for 15 min (2,000 rpm in Sorvall or Beckman centrifuge).
- Withdraw the cells at the interphases with care, pool them, and wash them 3 times with cold PBS (centrifuging each time for 7 to 8 min at 2,000 rpm). The interphases contain trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Freeze-thaw or sonicate the isolated interphases, determine protein after centrifugation, and coat the plate at 10 \( \mu \)g/mL in coating buffer.
- Contaminating HGG in this antigen is avoided by using Albumax to replace serum in the medium for the parasite cultures (see PARASITES, section I:A).

Comment: 12 medium size cultivation flasks (75 cm\(^2\), 20 mL of culture) with late stages of 10% parasitemia give about 1.5 mg of protein in the extract.

Air-dried monolayers of human erythrocytes (of the same type as used for parasite cultures)

- Pretreat the ELISA plate with 50 \( \mu \)L of coating buffer for 30 min.
- Add 50 \( \mu \)L of freshly washed human erythrocytes (in TH or PBS) at 1% hematocrit.
- After 30 min gently and carefully rinse off any unbound erythrocytes and leave the plates to air-dry.
- Proceed as usual (see SEROLOGY, section III:C, ELISA procedure).

Peptide/BSA conjugation

- Dissolve 1 mg of peptide in 0.1 mL of distilled water.
- Add 0.3 M NH\(_4\)OH, if necessary, until the peptide is dissolved, then add PBS to a total volume of 0.5 mL.
- Dissolve 0.5 mg of BSA in the peptide solution.
- Add 0.5 mL of 0.25% glutaraldehyde in PBS dropwise while stirring.
- Mix the solution gently on a roller drum in the cold, overnight.
- Dialyse against PBS, then store at −20 °C. We use 10 \( \mu \)g/mL for coating.

Comment: Several malaria peptides are commercially available from Bachem. Bigger peptides as MAPs can be used for coating without BSA conjugation.
III:C. **ELISA procedure**

_by Hedvig Perlmann_

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**Procedure**

- Coat the plate with 50 µL of peptide solution or crude parasite antigen at 10 µg/mL in coating buffer.
- Keep the plate at 4 °C overnight. With many proteins or peptides, PBS can be used as a coating solution. Test your system first.
- Block with 100 µL of 0.5% BSA in coating buffer for 3 to 4 h at 37 °C.
- Wash 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 µL of serum samples diluted 1:1000; leave them for 1 h at 37 °C.
- Wash them 4 times with 0.9% NaCl plus 0.05% Tween.
- Add 50 µL of ALP-conjugated or biotinylated anti-Ig of appropriate specificity at the recommended concentration in Tween-buffer; leave for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- If biotinylated antibody has been used, add 50 µL of streptavidin–ALP diluted 1:2000 in Tween-buffer; leave the sample for 1 h at 37 °C.
- Wash the sample 4 times with 0.9% NaCl plus 0.05% Tween.
- Develop the sample with 50 µL of NPP (1 tablet/5 mL of substrate buffer) (see SEROLOGY, section III:A) under Materials and reagents and read at OD$_{405}$.

**References**


CELLULAR IMMUNOLOGY

I. Preparation of human peripheral blood mononuclear cells (PBMC)

by Marita Troye-Blomberg and Jacob Minang

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Equipment
- centrifuge (swing out rotor)
- Burker chamber
- light microscope

Materials and reagents
- heparinized tubes (CPT sodium heparin blood collection tubes, BD Vacutainer Systems)
- Pasteur pipettes
- conical centrifuge tubes
- Ficoll-Paque (Pharmacia)
- RPMI 1640 with 20 mM HEPES (Gibco)
- L-glutamine (Gibco)
- penicillin–streptomycin combination (PEST) (Gibco)
- fetal bovine serum (FBS) (Gibco)

Tissue culture medium (TCM):
- RPMI 1640–HEPES
- 2 mM L-glutamine
- penicillin–streptomycin combination (final concentration of 100 units/mL of penicillin G sodium and 100 µg/mL of streptomycin sulfate)
- 10% heat-inactivated FBS

Turk gentian violet staining solution:
- 40 mg gentian violet
- 12.5 mL acetic acid
- Make up to 200 mL with distilled water.

Procedure
- Collect venous blood into heparinized 10-mL tubes.
- Save and freeze a small fraction of the plasma in Eppendorf tubes (1 to 2 mL for each tube) for antibody determinations.
- Mix blood in a 1:1 ratio with RPMI 1640 containing 20 mM HEPES solution and carefully layer 7 mL of the resultant suspension on top of 3 mL of Ficoll-Paque in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 2,800 rpm for 20 min. Collect the interphase with a Pasteur pipette.
- Pool and dilute (~10 times) the cells in RPMI 1640 containing 20 mM HEPES.
- Wash the cells 2 times, first at 1,800 rpm for 10 min and then at 1,200 rpm for 7 min and resuspend the cells in each 10-mL tube in 3 to 5 mL of TCM depending on the density/size of the pellet.
• Pipette 10 µL of the resultant cell suspension in TCM into 40 µL of TURK for counting of the lymphocytes in a Burker-chamber.

Alternative preparation of whole blood cells for cultures
• Collect 10 mL of venous blood as above.
• Pipette 10 µL of cell suspension into 30 µL of Turk for counting of the lymphocytes.
• For the cultures, dilute the blood 2, 5, or 10 times in TCM.

Reference
II. Antigen preparations and peptides for cellular work

II:A. Crude parasite antigen (Percoll-band)

*by Marita Troye-Blomberg*

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*e-mail: marita@imun.su.se*

For working with parasites and Percoll, please refer also to PARASITES and for crude antigen, please refer to SEROLOGY, section III, ELISA.

**Equipment**

- centrifuge (swing out rotor)
- sonicator
- freezer at −70 °C

**Materials and reagents**

- RPMI 1640–HEPES
- PBS
- conical centrifuge tubes
- Pasteur pipettes
- 0.45-µm filters (Millipore)

60% Percoll:

<table>
<thead>
<tr>
<th>Volume</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mL</td>
<td>Percoll</td>
</tr>
<tr>
<td>7 mL</td>
<td>10× PBS</td>
</tr>
<tr>
<td>33 mL</td>
<td>1× PBS</td>
</tr>
</tbody>
</table>

**Procedure**

- Prepare parasite extracts from late stage cultures. Wash them once in RPMI 1640–HEPES and suspend them to 10% hematocrit in cold RPMI 1640–HEPES.
- Layer 2 mL of extract gently on top of 2.5 mL of cold 60% Percoll in 10-mL conical centrifuge tubes.
- Centrifuge the tubes at 1,500 × g for 15 min at 4 °C. Collect the interphase with a Pasteur pipette, pool and wash the cells 3 times with cold PBS (1,500 × g for 7 to 8 min), and resuspend them in a small volume of PBS. The interphase contains trophozoites and schizonts, while rings and uninfected erythrocytes are found in the pellet.
- Sonicate (25w) the cells with short intervals for 2 min, determine protein content, and dilute to about 2 mg/mL.
- Centrifuge the sample at 1,500 × g for 10 min, filter-sterilize it, make a final protein determination, then store it in aliquots of 1 mg/mL in a −70 °C freezer.

**Reference**

II.B. Desalting of synthetic peptides
by Marita Troye-Blomberg
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e-mail: marita@imun.su.se

Equipment
speed-vac

Materials and reagents
Sep-Pak C\textsubscript{18} cartridge (Waters)
5-mL syringe
distilled water
methanol

Procedure
• Use a Sep-Pak C\textsubscript{18} cartridge connected with a 5-mL syringe to desalt peptides. Prewet the cartridge 3 times with 1.5 mL of methanol and wash it 2 times with 5 mL of water before use.
• Dissolve 10 mg of peptide in 10 mL of distilled water (or 10% methanol in distilled water) and pass the solution 3 times through the cartridge.
• Wash out the salts 2 times with 5 mL of distilled water, elute the peptide 2 times with 1.5 mL of 60% methanol and 2 times with 1.5 mL of 100% methanol.
• Pool the eluates, evaporate them in a speed-vac, weigh them, dissolve them in distilled water at 1 mg/mL, freeze them, and store them frozen.

Reference
III. T-cell proliferation

by Marita Troye-Blomberg

Immunology, Stockholm University, SE-106 91 Stockholm, Sweden

E-mail: marita@imun.su.se

Equipment

- incubator (37 °C, 5% CO₂ in a humid atmosphere)
- scintillation counter
- cell harvester (TomTec Cell Harvester)

Materials and reagents

- round-bottomed 96-well microplates (Costar 3799, Corning)
- (6-³H)-thymidine (specific activity 2 Ci/mmol; Amersham)
- TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
- peripheral blood mononuclear cells (PBMC)

Procedure

- Dilute PBMC in TCM to $1 \times 10^6$ cells/mL and seed $1 \times 10^5$ cells/well in 96-well round-bottomed microplates in triplicates per test.
- Add antigens (parasite extracts or recombinant proteins) or peptides at 2 to 10 µg/mL representing epitopes of interest at the initiation of the culture.
- Incubate the plates for 5 days at 37 °C in an atmosphere of 5% CO₂.
- Harvest 100 µL for cytokine determination at the time of pulsing, and then pulse with 1 µCi [6-³H]-thymidine in 100 µL of TCM for 18 h.
- Harvest the cells and measure the thymidine incorporation by liquid scintillation.
- Results are expressed as stimulation index (SI), defined as mean cpm of test triplicates divided by mean of cpm of control triplicates. An SI $> 2.5$ is considered positive.

Reference

The following description should be considered as a guideline on how to stimulate and analyse cells for IFN-γ production by the ELISPOT technique. Conditions for cell preparation, stimulation, and the ELISPOT may be changed to fit with the test situation.

**Equipment**
- incubator (37 °C, 5% CO₂ in a humid atmosphere)
- multi-channel pipette
- dissection microscope (40×)

**Materials and reagents**
- nitrocellulose plates (Millipore Corp.)
- mAb anti-human IFN-γ, 7-B6-1-Biotin (Mabtech)
- biotinylated mAb anti-human IFN-γ, 7-B6-1-Biotin (Mabtech)
- streptavidin–alkaline phosphatase (Mabtech)
- BCIP/NBT substrate (Bio-Rad Laboratories)
- TCM (tissue culture medium), see CELLULAR IMMUNOLOGY, section I
- 5-mL round-bottomed tissue culture tubes (Falcon 2058, BD Labware)
- PHA (phytohemagglutinin)
- PBS (filter-sterilized phosphate buffer saline) (pH 7.2)
- FCS (fetal calf serum)

**Procedure**
Stimulation of cells can be carried out directly in the ELISPOT plate, but better results may be obtained if cells are first stimulated for a shorter period in culture tubes. In the following description cells are incubated for 2 to 4 h in tubes and thereafter transferred to ELISPOT plates for further incubation.

- Prepare sterile PBMCs.
- Count cells and suspend them in TCM to a concentration of 2 × 10⁶ cells/mL.
- Transfer 0.5 to 1 mL of cell suspension to 5-mL round-bottomed tissue culture tubes.
- Add antigens (parasite extracts or recombinant proteins) or peptides representing epitopes of interest at a concentration of 2 µg/mL at the initiation of the culture. As a positive control, add phytohemagglutinin (PHA) at a concentration of 5 µg/mL.
- Incubate cells at 37 °C for 2 to 4 h.

**Coating of ELISPOT plates**
- Dilute the coating antibody (mAb anti-human IFN-γ, 7-B6-1-Biotin) to 15 µg/mL in filter-sterilized phosphate buffer saline (PBS).
- Add 100 µL/well to the nitrocellulose plates and incubate them overnight at 4 °C.
Transfer of cells to the nitrocellulose plate (sterile)
Before transferring stimulated cells to the coated ELISPOT plates, wash them 4 times with sterile PBS (200 µL) to remove unbound coating antibody.
- Add stimulated cells to the plates. For IFN-γ, a cell concentration of 20,000 to 50,000 cells/well is usually suitable. If the number of cells exceeds 250,000 cells/well, this may result in blurry spots due to multiple cell layers (especially in the wells with cells stimulated with PHA).
- Incubate cells for 10 to 40 h at 37 °C in a humid atmosphere.

Addition of the secondary antibody (nonsterile)
- Remove cells by washing them 6 times (200 µL/well) using PBS (multi-channel pipette).
- Dilute biotinylated mAb (anti-human IFN-γ 7-B6-1-Biotin) to 1 µg/mL in PBS with 0.5% fetal calf serum (PBS-0.5% FCS) and add 100 µL/well.
- Incubate the plates for 2 to 4 h at room temperature.
- Wash them in PBS 6 times (200 µL/well).
- Add 100 µL of streptavidin–alkaline phosphatase (diluted 1:1000 in filtered PBS-0.5% FCS) and incubate the plates for 1 to 2 h at room temperature.
- After washing them in PBS 6 times (200 µL/well), add 100 µL of substrate and incubate them until spots emerge (may be up to 1 h).
- Stop color development by washing the plates under running tap water (3 times at 200 µL/well).
- Leave the plate to dry; inspect and count the spots with a dissection microscope (40×).

One-Step ELISpot kits:
Very sensitive and easy to use kits are available at Mabtech AB.

Reference
V. Reverse transcription PCR for detection of cytokines

by Ankie Söderlund

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Equipment
−70 °C freezer
incubator (37 °C, 5% CO2 in a humid atmosphere)
RT-PCR kit with recombinant AmpliTaq DNA polymerase (Applied Biosystems)
PCR using cytokine specific primers

Materials and reagents
Ficoll-Paque (Amersham Biosciences)
round-bottomed tubes
cryotubes
liquid nitrogen
PBS
acid guanidinium thiocyanate–phenol–chloroform
DEPC-treated distilled water

Procedure
• Isolate PBMC from venous blood by Ficoll-Paque centrifugation as described in CELLULAR IMMUNOLOGY, section I.
• To detect any spontaneous cytokine production in the controls, wash unstimulated PBMC 4 times in PBS. Before the last wash, transfer cells to a cryotube, centrifuge them at 300 × g, and carefully remove all remaining PBS. Immediately freeze the cells in liquid nitrogen.
• To detect cytokines after antigen stimulation, place cells in round-bottomed tubes, one (or two) for each time point, and add the desired antigen. Place the tubes at 37 °C, 5% CO2, for 4, 7, and 22 h.
• Wash the cells 4 times in PBS as described above, remove the supernatant, and freeze the cells in liquid nitrogen.
• For total RNA extraction, either use commercial kits available or the acid guanidinium thiocyanate–phenol–chloroform method by Chomczynski and Sacchi, 1987 (see also MOLECULAR BIOLOGY, section II).
• Dilute the RNA in 50 µL of DEPC-treated distilled water and thereafter keep it stored at −70 °C.
• Use an RT-PCR kit with recombinant AmpliTaq DNA polymerase for reverse transcription and subsequent PCR using cytokine specific primers.

References

VI. Detection of cytokine-gene polymorphism

VI:A. Extraction of human DNA from peripheral blood or buffy coat

by Ben Gyan
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Equipment
- incubator
- centrifuge
- gel electrophoresis apparatus
- speed-vac
- gel drier or oven
- spectrophotometer
- camera
- automated thermal cycler
- microscope with UV light

Materials and reagents
- lysis buffer:  
  - 1.6 M sucrose
  - 50 mM Tris–HCl (pH 7.5)
  - 25 mM MgCl₂
  - 5% Triton X-100

- DNA buffer:  
  - 10 mL 1 M Tris–HCl (pH 7.5)
  - 2.5 mL 0.5 M NaCl
  - 2.5 mL 0.5 M EDTA
  - 1.25 mL 20% SDS
  - Adjust volume to 50 mL using double distilled H₂O.

- sterile vacutainers with EDTA (ethylamine tetra-acetic acid)
- Falcon tubes, 50-mL
- proteinase K solution (Invitrogen 25530 049)
- 20% SDS
- sterile double-distilled H₂O
- Pasteur pipettes
- Eppendorf tubes
- phenol
- chloroform
- 3 M sodium acetate
- absolute ethanol
- 70% ethanol

Procedure
- Draw 1 to 8 mL of venous blood into sterile vacutainers containing EDTA and keep it at room temperature (or frozen).
- Transfer blood into a 50-mL Falcon tube and add 10 to 40 mL of lysis buffer.
- Turn the tube gently upside down for 2 to 3 min.
- Centrifuge the tube at 2,000 rpm for 10 min (with brake off) and discard the supernatant.
- Add 40 µL of proteinase K solution, 40 µL of 20% SDS, 180 µL of DNA buffer, and 340 µL of sterile double-distilled H₂O.
- Mix the sample well by gently pipetting it up and down until it becomes gelatine-like.
- Digest the blood by incubating it overnight at 37 to 42 °C.
- Transfer the sample into Eppendorf tube and add 600 µL of phenol.
- Extract the DNA by revolving/mixing the tubes until the solution becomes milky.
- Centrifuge the tube at 13,000 rpm for 10 min at room temperature.
- Transfer the upper aqueous phase (DNA) into a new Eppendorf tube.
- Add 800 µL of chloroform.
- Extract the DNA and centrifuge the tube at 6,000 rpm for 10 min.
- Transfer the upper phase into a new Eppendorf tube.
- Precipitate the DNA by adding one-tenth volume of 3 M sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at −20 °C. The DNA can be stored this way as well.
- Centrifuge the precipitate for 30 min at 4 °C, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 µL of distilled water, depending on its size.
- Determine the DNA concentration at OD₂₆₀; 2 mL of culture may yield ~2 µg of genomic DNA. Visualize ~0.5 µg on a 0.8% agarose gel to see that the DNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.
VI:B. PCR for amplification of nucleotide repeat (VNTR) polymorphisms of cytokine genes

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Equipment
automated thermal cycler
UV-transilluminator
camera

Materials and reagents
dNTPs
primers
Taq polymerase
PCR buffer with MgCl₂
sterile H₂O
Taq gold (Applied Biosystems)
agarose gel
0.1% ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

TE buffer (pH 8.0):
10 mM Tris Cl (pH 8.0)
1 mM EDTA (pH 8.0)

Procedure
• Prepare the mix at the following final concentration (total volume 25 µL):
  100-250 ng DNA
  200 nM dNTPs
  0.1 µM of each primer
  1.5 U Taq polymerase
  PCR buffer (with MgCl₂), 8% (v/v)
  sterile H₂O, 46.4% (v/v)
• Program the automated thermal cycler according to manufacturers’ instructions. Use the following guidelines to set annealing temperature and extension time according to primer and product considerations.
  • Hot start at 95 °C for 10 min when using Taq Gold.
  • Denature the DNA for 1 min at 94 °C. If the GC content is ≤50%, anneal it at 55 °C; if it’s >50%, anneal at 65 °C. Extend for 5 min at 72 °C. Run the program for 30 cycles.
  • Prepare 2% agarose gel in TE buffer. Run the gel at 80 to 100 V and stain it with 0.1% ethidium bromide. Visualize the gel under UV light. Take a photograph.
VI:C. **PCR for amplification of single nucleotide polymorphisms or RFLP of cytokine genes**

by Ben Gyan

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**Procedure**

- Use the same procedure as for VNTR polymorphisms, CELLULAR IMMUNOLOGY, section VI:B. The PCR product will give the same band for all samples. To detect polymorphisms, further digest the PCR product with digestive enzymes according to the manufacturers' instructions.
- Run the digested PCR product on 3% agarose gel (use NuSieve GTG) electrophoresis at 80 V and stain the gel with 0.1% ethidium bromide. Visualize the gel under UV light and take a photograph.
VI:D. Amplification Refractory Mutation System (ARMS) PCR for detection of previously characterised mutations in cytokine genes

by Ben Gyan

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Equipment
- automated thermal cycler
- agarose gel electrophoresis
- UV-transilluminator

Materials and reagents
- Tris–HCl (pH 8.8)
- MgCl₂
- Taq polymerase
- dNTP
- Tween 20 (ABgene)
- primers
- ethidium bromide

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

PCR master mix:
- 25 mM Tris–HCl (pH 8.8)
- 1.5 to 4.0 mM MgCl₂
- 0.25 Taq polymerase
- 200 µM dNTP
- 0.01% (v/v) Tween 20 (ABgene)

Primer mix:
- specific primer mix (10 µM of generic primer and 10 µM of specific primer)
- 5 µM of positive internal control (10 µM of internal control forward and 10 µM of reverse primers).

Procedure
- Put 100 to 250 ng of each DNA into two tubes or wells.
- Add other PCR reagents as indicated in the master mix above.
- Program the automated thermal cycler according to manufacturers’ instructions. Use guidelines in CELLULAR IMMUNOLOGY, section VI:B for VNTR polymorphisms to set annealing temperature and extension time according to primer and product considerations. Run the program for 30 cycles.
- Analyze the PCR products by agarose gel electrophoresis followed by ethidium bromide staining and visualisation under UV light. Amplification should occur in only one tube if the DNA template contains wild-type sequence or mutation sequence. Heterozygous individuals show amplification in both tubes. A positive control sequence is required to measure that absence of product is not due to PCR failure.
Reference
VII. Haptoglobin phenotyping using PAGE

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Equipment
- gel electrophoresis apparatus
- gel drier or oven

Materials and reagents
- polyacrylamide gel
- Tris–HCl
- Tris-base
- glycine
- distilled water
- 10% ammonium persulphate
- TEMED (tetramethyl-ethylenediamine)
- haptoglobin
- erythrocyte haemolysate
- benzidine stain
- filter paper

Buffers:
- 1.5 M Tris–HCl (pH 8.8)
  - 54.5 g Tris-base
  - 150 mL distilled H₂O
- 0.5 M Tris–HCl (pH 6.8)
  - 6 g Tris-base
  - 60 mL distilled H₂O
- 10× running buffer
  - 15.5 g Tris-base
  - 72.1 g glycine
  - 500 mL distilled H₂O

Procedures and preparations
- Cast a polyacrylamide gel with separation gel of concentration 4.7% and stacking gel of 2.5%.

<table>
<thead>
<tr>
<th></th>
<th>separating gel (4.7%)</th>
<th>stacking gel (2.5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% bis acrylamide</td>
<td>7 mL</td>
<td>1.3 mL</td>
</tr>
<tr>
<td>Tris–HCl</td>
<td>11.25 mL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>distilled water</td>
<td>26.7 mL</td>
<td>6.0 mL</td>
</tr>
<tr>
<td>10% ammonium persulphate</td>
<td>150 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>TEMED</td>
<td>37.5 µL</td>
<td>18 µL</td>
</tr>
</tbody>
</table>

- Add 3 µL of erythrocyte haemolysate to 10 µL of serum or plasma and control haptoglobin.
• Mix the solution well and incubate it for 5 min to enable haptoglobin in serum to bind to hemoglobin.
• Add 10 µL of loading buffer. Load the sample onto the gel using a template.
• Run electrophoresis at a constant voltage of 80 to 120V using 1× running buffer.
• Stain the gel for 5 to 15 min with benzidine stain.
• Wash the gel in distilled water.
• Transfer the gel onto filter paper and dry it using a gel dryer or oven.

References

MOLECULAR BIOLOGY

I. Small-scale genomic DNA isolation from Plasmodium falciparum

by Martha Schlichterle and Mats Wahlgren

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Equipment

- centrifuge (4 °C)
- speed-vac
- agarose electrophoresis unit
- spectrophotometer

Materials and reagents

- infected erythrocytes (a few mL with about 10% parasitemia)
- phosphate-buffered saline (PBS) (pH 7.2)
- 5% saponin solution
- lysis buffer:
  - 40 mM Tris–HCl (pH 8.0)
  - 80 mM EDTA (pH 8.0)
  - 2% SDS, 0.1 mg/mL
  - proteinase K (Add the proteinase K just before using the buffer.)
- phenol equilibrated with 0.1 M Tris–HCl (pH 7.0)
- chloroform
- RNase (Stratagene, RNace-It Ribonuclease Cocktail)
- 3 M sodium acetate (pH 5.0)
- absolute ethanol
- 70% ethanol
- TE-buffer:
  - 10 mM Tris–HCl (pH 8.0)
  - 1 mM EDTA (pH 8.0)

Procedure

- Centrifuge the infected erythrocytes at 3,000 × g for 2 min. Wash the cells once in cold PBS.
- Resuspend the cells from one microfuge tube (1.7 mL) in 1 mL of PBS.
- Add and gently mix 10 μL of 5% saponin (for a final concentration of 0.05%).
- Immediately centrifuge the tube at 6,000 × g for 5 min after lysis is observed.
- Remove the supernatant.
- Add 25 μL of lysis buffer and 75 μL of distilled water to the pellet.
- Incubate the tube at 37 °C for ~3 h with intermittent stirring by hand.
- Add 100 μL of distilled water, then 200 μL of phenol. Mix well and centrifuge at 2,000 × g for 8 min.
- Extract likewise with 200 μL of chloroform.
- Add 2 μL of RNace-It cocktail for 30 min at 37 °C.
- Extract with phenol and chloroform as above.
• Precipitate the gDNA by adding one-tenth volume of sodium acetate and 2.5 volumes of absolute ethanol; allow the tube to sit for 2 hours or overnight at −20 °C. The DNA can be stored this way as well.
• Centrifuge the precipitate at 2,000 \( \times \) \( g \) for 30 min at 4 °C, wash it gently once with 70% ethanol, dry it in a speed-vac, and gently resuspend the pellet in 25 to 100 \( \mu \)L of distilled water, depending on its size.
• Determine the DNA concentration at OD\textsubscript{260}; 2 mL of culture may yield \(~2 \mu\text{g}\) of gDNA. Visualize \(~0.5 \mu\text{g}\) on a 0.8% agarose gel to see that the gDNA runs as a high-molecular weight, somewhat broad band and is thus unsheared and free of RNA.
II. RNA extraction from *Plasmodium falciparum*

II:A. RNA extraction from *Plasmodium falciparum* using guanidine thiocyanate and acidic phenol

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Equipment
- centrifuge (4 °C)
- JA-20 Beckman rotor
- small-blade Polytron homogenizer (Brinkmann)

Materials and reagents (see also Solutions below)
- PRBC (Check PRBC to determine the parasitemia and rosetting rate. Usually eight 75-cm² bottles with a parasitemia of 7 to 10% will yield ~200 to 300 µg of RNA.)
- cold sterile PBS (pH 7.2)
- complete solution D (with 72 µL 2-mercaptoethanol (2-ME)/10 mL of Solution D or 288 µL of 2-ME/40 mL of Solution D). Remove 1 mL into a microcentrifuge tube; store the rest at 4 °C for Day 2. Solution D with 2-ME added is stable for 2 weeks at 4 °C.)
- 2 M sterile sodium acetate (pH 4)
- acidic phenol:chloroform:isoamylalcohol (125:24:1) (Ambion 9722)
- ice cold 2-propanol
- distilled water
- 50-mL Falcon tubes (BD Labware)

Solutions
- Solution D for RNA isolation:
  - 100 g guanidine thiocyanate
  - 117.2 mL sterile distilled water
  - 7 mL 0.75 M sodium citrate (pH 7.0) (autoclaved)
  - 10.6 mL 10% N-lauroyl-sarcosine (Sigma L9150; filtered; store stock at room temperature)
  - Check the pH, which should be ~7.0.
  - The solution can be stored for 3 months at room temperature.

  Working solution:
  - 720 µL 2-mercaptoethanol/100 mL of Solution D; keeps for 2 weeks if stored at 4 °C.

  2 M sodium acetate, 50 mL:
  - Add 8.2 g of anhydrous sodium acetate to about half the final volume of distilled water; as the salt is dissolving, adjust the pH to 4.0 with ~16 mL of concentrated acetic acid; bring to volume and autoclave.
Tris–EDTA buffer (TE) (see also Sambrook et al. 1989):
- 10 mL 1 M Tris–HCl
- 2 mL 0.5 M EDTA (pH 8.0)
- 988 mL water
Combine Tris and EDTA in water; adjust pH to 7.4; autoclave. Store the solution at 4 °C; it is stable for 6 months.

10× electrophoresis buffer (0.2 M MOPS and 10 mM EDTA):
- 40 mL 1 M MOPS (pH 7.4) (autoclaved; 209.3 g/mol)
- 4 mL 0.5 M EDTA (pH 8.0) (autoclaved)
- 156 mL distilled water (autoclaved)

RNA loading buffer, deionized formamide:
- 25 g mixed-bead ion exchange resin (Bio-Rad, 142-6424)
- 250 mL of formamide (Sigma F7503)
Wrap an Erlenmeyer flask in foil. Add resin beads and formamide. Stir the mixture for 60 min in the hood. Filter through Whatman No. 1 filter paper. Store aliquots in dark bottles at −20 °C (stable for 1 year).

RNA loading buffer:
- 3.37 mL deionized formamide
- 1.08 mL formaldehyde (37%)
- 0.5 mL 10× electrophoresis buffer
- 20 μL 0.5 M EDTA (pH 8.0)
- 285 μL glycerol (sterile)
- 50 μL 10% SDS
- 50 μL bromphenol blue, 0.5% (or 5 mg)
- 50 μL xylene cyanole, 0.5% (or 5 mg)
Aliquot, then store at −20 °C.

Agarose gel (small 40 mL):
- 0.4 g agarose (Sigma A9539)
- 29 mL distilled water
Add agarose to sterile water, heat to boiling to dissolve the agarose, then cool to 65 °C.
Add:
- 4 mL 10× electrophoresis buffer
- 50 μL 0.5 M EDTA
In the hood, quickly add 7 mL of formaldehyde (or less if using Sigma’s buffers). Pour the gel solution into a clean, taped gel tray containing a comb. Remove bubbles and allow the gel to cool.

20× SSC stock (5 L):
- 876.5 g sodium chloride
- 441.0 g sodium citrate
Add salts to distilled water and mix to dissolve. Add distilled water to total of 5 liters, adjust the pH to 7.0 with HCl, and autoclave.
Prehybridization/hybridization fluid:
0.5 M Na$_2$HPO$_4$ (pH 7)
1 mM EDTA
7% SDS

Probe removal solution
0.1% SDS
Place a nylon filter in the boiling solution and remove from heat. After 15 min
remove and check the filter. Either repeat the treatment or simply expose the
filter to check that the probe is really removed. Keep the filter moist at all times by
placing it in a sealed hybridization bag. It may be stored at −20 °C.

Isolation of RNA, Day 1

- Cool the centrifuge for 50-mL Falcon tubes.
- Pool cells (PRBC) into two 50-mL tubes on ice.
- Centrifuge the cells at 1,800 rpm for 5 min at 4 °C.
- Remove the supernatant. Wash the cells with ~30 mL of cold PBS. Gently
resuspend the cells. Centrifuge them again.
- Remove the supernatant. Resuspend the cells in cold PBS to ~20 mL/tube and
transfer into centrifuge tubes that have lids (i.e., not Corex tubes) and that
withstand 15,000 × g and organic solvents. Centrifuge them as above.
- Remove the supernatant and set the cells on ice.
- Rinse and clean a small-blade Polytron homogenizer by running distilled water in
it, followed by some solution D.
- Add 10 mL of solution D per centrifuge tube and suck up and down to disrupt
cells.
- Homogenize the cells on “medium” setting for 15 s. Avoid frothing. Keep the cells
on ice.
- Clean the homogenizer by running solution D in it, then running distilled water,
with changes of water until no trace of homogenized material appears on the
blades.
- To each tube add:
  1.0 mL 2 M sodium acetate (pH 4) (× 0.1 volume)
  10 mL acidic phenol (× 1 volume)
  2.0 mL chloroform:isoamyl alcohol (× 0.2 volume)
- Cap the tubes with lids and vortex well for ~1 min. Set them on ice for 15 to 30
min. Meanwhile cool the centrifuge and rotor (JA-20 Beckman rotor) to 4 °C.
- Invert and vortex to mix the samples, then immediately centrifuge the tubes at
11,000 rpm for 30 min at 4 °C.
- Remove and save the supernatant in two new centrifuge tubes (Nalgene 3110).
Use lids. Avoid material from the interphase.
- Add an equal volume of 2-propanol (at −20 °C), mix, and set the sample at
−20 °C overnight or at least for a few hours.
Further purification of RNA, Day 2

- Cool the JA-20 Beckman rotor. Mark the side of each tube where the “invisible” RNA pellet is expected to be. Centrifuge the tubes at 11,000 rpm for 30 min at 4 °C.
- In a ventilated hood, carefully remove the supernatant with a 10-mL pipette, being careful to go down the opposite side of the tube from where the pellet is expected. There should be an almost transparent, gel-like pellet. Mark the tube where the pellet is.
- Dry the tubes some in the hood, but do not over-dry them.
- Resuspend both tubes’ RNA contents in a total of 0.5 mL of Solution D.
- Transfer the contents into a microcentrifuge tube. Precipitate it with an equal amount of 2-propanol overnight at −20 °C (or at least for a few hours).

References

II:B. Measurement of RNA yield and purity, and visualization on agarose–formaldehyde gel

by Bobo Mok and Martha Schlichtherle
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Equipment
microfuge

Materials and reagents
premixed RNA electrophoresis buffer (Sigma M5755)
RNA gel loading buffer (Sigma R4268)

10× electrophoresis buffer:
23.4 mL distilled water
600 µL 0.5 M EDTA (pH 8)
6 mL 1 M MOPS (pH 7.4)
This makes 30 mL total, approximately the amount needed for a regular small gel system.

formaldehyde
agarose
1 M EDTA (pH 8.0)
distilled water (or DEPC-treated water)
3 M sodium acetate (pH 5.2)
ethidium bromide
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
bromphenol dye

Procedure
• Extract and purify the RNA as instructed in MOLECULAR BIOLOGY, section II:A, Days 1 and 2.

Day 3:
• Microfuge the RNA for 30 min at 12,000 to 14,000 rpm and 4 °C.
• Meanwhile prepare buffers and gel. Use EITHER:
  premixed RNA electrophoresis buffer (Sigma M5755) and RNA gel loading buffer (Sigma R4268). The gel should then contain a lower amount of formaldehyde of 2.4%.
  OR:
  10× electrophoresis buffer
• Have formaldehyde ready in the hood.
• Prepare a 1% agarose gel:
  0.4 g agarose
  29 mL of distilled water
  Mix, heat, and cook briefly. Cool gel liquid some. Then add:
  4 mL 10× electrophoresis buffer
  25 µL 1 M EDTA (pH 8.0)
• Bring the gel liquid to the hood, add 7 mL of formaldehyde, mix, then quickly pour the gel. Use a comb which is set aside for RNA-work so that no RNases from fingers are present on it.

• Allow the gel to solidify in the hood, but do not over-dry.

• Continue with the RNA sample. Wash precipitated RNA 2 times with 900 µL of 70% ethanol at –20 °C. Allow it to dry some in the hood.

• Set a small water bath to 60 to 65 °C.

• Dissolve the RNA in ~100 µL of autoclaved distilled water (or DEPC-treated water) depending on pellet size. If the RNA does not dissolve well with pipetting, heat it briefly at 65 °C. Pipette to dissolve, then keep the RNA on ice.

• Use ~1:100 dilution of RNA for spectrophotometric measurement, preferably in a microcuvette to minimize the amount of RNA which is lost to absorbance measurements.

• Use the program for fixed wavelength at 260 and 280 nm.

• Take absorbance readings after blanking with distilled water.

• Calculate:

\[
\text{RNA concentration of stock: } (\text{Abs}) (\text{dil.}) (40 \mu g/mL)/1000 = \text{RNA } \mu g/\mu L \\
\text{total yield: } (\text{above } #) (\mu L \text{ stock RNA}) = \mu g \text{ RNA} \\
\text{purity: } \frac{\text{Abs}_{260}}{\text{Abs}_{280}} = \text{should be ~1.8 to 2.0 if not too much protein contamination is present.}
\]

• Take out 5 µg or less of RNA to run in a lane on the gel.

• The RNA left to precipitate is: total yield – spec. reading – gel loading.

• Precipitate the RNA left by adding one-tenth volume of 3 M sodium acetate (pH 5.2), mix, then add 3 times volume of –20 °C absolute ethanol. Store RNA at –70 °C.

• If RNA volume taken out for gel is >10 µL, speed-vac it down to dryness. Otherwise add RNA loading buffer to 19 µL (or less if using Sigma’s premade solution, see manufacturer’s suggestions). Mix and heat for 5 to 10 min at 60 to 65 °C. Set on ice

• Add 1 µL of ethidium bromide (at ~250 ng/ml); mix. Pour 1× electrophoresis running buffer over the gel and load.

• Run the gel at ~50 V for more than 1 h with the bromphenol dye running about half way. Use commercial RNA marker(s) if needed (i.e., Gibco). For best results the gel should be run at 4 °C. View and photograph the gel under UV-light. Two RNA bands should be quite visible corresponding to rRNAs. A third low rRNA band may at times also be visible; it is usually fuzzy. After a distilled-water rinse, the gel may be transferred to a nylon filter and be Northern probed using manufacturer’s protocols.

References

II:C. Reliable RNA preparation for *Plasmodium falciparum*

by Sue Kyes

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Equipment

- benchtop centrifuge, refrigerated
- microfuge
- water baths/heating blocks (37 °C and 60 °C)
- spectrophotometer/cuvettes for reading absorbance at 260 nm and 280 nm (optional)

Materials and reagents

- parasite-infected red blood cells (iRBC): 0.5 mL packed cell volume at 10% parasitemia, expected yields:
  - rings 50–150 µg total RNA
  - trophozoites, schizonts, gametocytes 250–750 µg total RNA
- gloves
- TRIzol Reagent: (Invitrogen) 15596-026 (Follow manufacturer’s suggestions for safety and storage.)
- chloroform (AnalAR from BDH, or Sigma C5312, or similar)
- 2-propanol (AnalAR from BDH, or similar)
- formamide (molecular biology grade, Sigma F9037, or similar. It is not necessary to deionize before use. Open bottle in chemical fume hood.)
- RNAse-free, chloroform/phenol-resistant plastics
- screw-top test tubes*
- 1.5-mL microfuge tubes*
- pipette tips*
  *Straight from manufacturer is usually ok; autoclave if not sterile, taking care to wear gloves in handling.

optional:

- RNase-free water (Sigma W4502 or Milli-Q purified or similar)
- RNase-free 3M sodium acetate solution (pH 5.2) (Sigma S7899 or similar)
  Either buy RNase-free water and sodium acetate solution or treat distilled water and sodium acetate solution with 0.1% diethyl pyrocarbonate (DEPC; Sigma, D5758) as described in Sambrook et al. 1989; p. 7.3; this requires autoclaving.
- RNase-free 75% ethanol/25% RNase-free water
- RNase-free absolute ethanol (AnalAR from BDH, or Sigma E7023, or similar)

Protocol

Key RNA advice: Wear gloves for everything, and use RNAse-free plastics.

Preserve RNA in TRIzol

- Spin cells gently at 1,800 rpm (600 × g) for 4 min at room temperature. *(example: 0.5 mL of packed infected RBC, about 10% parasitemia)*¹,²
- Remove supernatant (s/n)³. Tap the tube to loosen the cell pellet⁴.
• Add TRIzol, prewarmed to 37 °C⁵.
  For RINGS: add 10 pellet volumes of TRIzol.
  (example: 5 mL of TRIzol to 0.5 mL cells)
  For all other stages: add 20 pellet volumes of TRIzol.
  (example: 10 mL of TRIzol to 0.5 mL cells)
• Shake the sample to dissolve any clumps.
• Incubate the sample at 37 °C for 5 min.
• Either store the sample at −70 to −80 °C⁶ or continue.

Continue extraction and precipitate RNA
• (Thaw samples at 37 °C, if necessary.)
• Add 0.2 TRlzol volumes of chloroform.
  (example: sample in 5 mL TRIzol, add 1 mL of chloroform)
  (example: sample in 10 mL TRIzol, add 2 mL of chloroform)
• Vigorously shake the sample; let it stand at room temperature for 2 to 3 min.
• Spin the tube at 4 °C for 30 min (microfuge = maximum 9,000 × g; or for 15-mL tubes in benchtop centrifuge, 1,000 to 1,400 × g is fine)⁷.
• Remove the aqueous (top, clear, RNA-containing) layer to a new tube⁸. Expect to recover up to 0.6 TRIzol volumes, but avoid the interface!
  (example: from sample in 5 mL of TRIzol, expect 3 mL of aqueous layer)
• Add 0.5 TRIzol volumes of 2-propanol to the RNA; invert the tube several times to mix.
  (example: to 3 mL of aqueous layer, add 2.5 mL 2-propanol)
• Transfer/split large samples to 1.5-mL snap-cap microfuge tubes.
• Precipitate the samples for AT LEAST 2 h (up to several days) at 4 °C.

Finish preparation, check quantity/quality of RNA
• Spin the sample at 12,000 × g (13,000 to 14,000 rpm, microfuge) for 30 min at 4 °C or room temperature⁹.
• Remove as much as possible of the supernatant¹⁰ (“Optional 75% ethanol wash¹¹”).
• Air dry the sample, inverted, at room temperature, for no more than 5 min.
• Add formamide (usually a total of 100 µL for a starting pellet of 0.5 mL of infected RBC)¹².
• Heat the sample at 60 °C for 10 min; place it on ice.
• Resuspend the pellet by pipetting¹³.
• Check the absorbance at 260 nm¹⁴.
• Store the pellet at −70 to −80 °C.

Notes to the above procedure:
1: Especially for rings, cells pellet much more efficiently in 15-mL tubes than in 50-mL tubes. Subsequent steps are also easiest in 15-mL tubes, so harvest large volumes of cells directly in 15-mL tubes.
2: Use phenol/chloroform-resistant tubes.
3. After removing tubes, adjust centrifuge temperature down to 4 °C for the next spin.

4. Do not wash cells or lyse them with saponin. It is important to lyse the infected red blood cells directly in TRIzol as soon as possible after removing them from culture.

5. TRIzol seems to work best if it is prewarmed to 37 °C. Make sure lids are tightly sealed before shaking the tubes. Do the 37 °C incubation before freezing the samples to ensure complete complexing of nucleoproteins. The TRIzol can cope with extracting the RNA from hemoglobin, of which there is obviously plenty. TRIzol does not cope with separating RNA from DNA very well. Trophozoites/late stage parasites synthesize lots of DNA, and therefore require excess TRIzol.

6. Because the RNA is very stable at this point (if stored at −70 to −80 °C), collecting lots of samples is easy. Store them until you can process them all at once.

7. Do NOT spin at room temperature. If using a microfuge, use screw-cap tubes. Snap-cap tubes tend to leak and make a horrible mess of the inside of the centrifuge.

8. Avoid the interface to avoid DNA contamination. It is easier to recover more of the aqueous layer from 15-mL tubes than from 50-mL tubes. The DNA seems to collect right above the interface, so do not try to collect every last microliter of aqueous layer. The manufacturer’s protocol for retrieving DNA from this does not seem to work due to massive amounts of hemoglobin. If anyone manages, please let me know!

9. Give tubes a quick vortex before spinning. Place tubes in the microfuge with hinges pointing outwards (for future identification of RNA pellet). Room temperature spin is fine, but 4 °C is ideal.

10. Remove supernatant in two stages:
   a. Keeping pipette tip away from the putative pellet (directly beneath hinge, at bottom of tube), remove nearly all of the supernatant. Save the supernatant to a new tube if you are worried about losing your sample.
   b. Give the tube a quick ‘flick spin’, again with hinges pointing outwards. This gets all those dribbles on the side of the tube down to the bottom again. Remove last bits of supernatant, as much as possible, taking care to avoid touching the very tiny, glassy pellet. If there is a large pellet, there is probably DNA or protein in it.

11. At this point, it is optional to add a 75% ethanol wash step: Add 0.5 mL of 75% ethanol: 25% DEPC–water, ice-cold; spin for ~5 min in a microfuge, and remove all supernatant. This gets rid of all traces of phenol and is particularly useful for cDNA preparations (see next note).

12. Add the formamide to the tube, avoiding touching the ‘pellet’. Split the total formamide volume among the microfuge tubes if it was a large sample split to several tubes. RNA is very stable for a long time in formamide. However, the formamide affects downstream enzymatic applications. If you plan to make cDNA, it is worth considering resuspending the RNA pellet in DEPC–water, if you have it. If you have RNA samples in DEPC–water, store them in aliquots at −80 °C, but beware that the RNA is not very stable in water, and does not survive many freeze-thaw cycles. One way around this is to precipitate the RNA (add one-tenth volume of RNase-free 3M sodium acetate [pH 5.2], and 2.5 volumes of ethanol), then store at −80 °C. This is very stable, and will last ‘forever’.
you need some RNA, just thaw the tube, vortex it, and remove the volume necessary to give the desired amount; spin it down in the microfuge for ~30 min.

13: By pipetting up and down, mix each sample thoroughly, making sure that nothing is stuck to the bottom of the tube. The pellet should have completely dissolved. Pool tubes of the same sample-type back together at this point. Set aside small volumes for measuring absorbance or running on gel.

14: Spectrophotometric measurement of quantity:
Check absorbance at 260 nm: Try 1 µL in 500 µL of water, remembering to blank against 1 µL of formamide in 0.5 mL of water.
Conversion: OD 1 = 40 µg RNA
Compare to OD 280. The ratio OD260/OD280 should be close to 2.0 for pure RNA. If there is any contaminating phenol, this number will be lower and the quantification will not be accurate.
If this of great concern, at the point marked *above, add the 75% ethanol wash step. It does not seem to make much difference, but gives some people peace of mind that the RNA is really clean.

Agarose gel measure of quality:
Using the procedure outlined for Northern blots below (see MOLECULAR BIOLOGY, section II:D), run a small gel, with small lanes. Run 1 to 2 µL of each sample.
There should be two main ribosomal bands and a few minor bands of unknown provenance. Good RNA preparations have roughly equal amounts of both main ribosomal bands. If the RNA is degraded, there will be a ‘comet tail’ and no discrete bands. If there is plenty of DNA, there will also be a faint smudge close to the wells. Sometimes this does not matter, but sometimes it is really annoying. There are methods for getting rid of the DNA, but they are expensive and not fun.
Finally, if your sample had human white blood cells in it, you’ll see more than two ribosomal bands!

References


TRIzol is based on the following:
II:D. Simple Northern

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NO DEPC-treatment anywhere! Just wear gloves for everything.

Equipment
agarose gel tank, combs, and power pack
deep plastic trays for soaking gels (rinsed with 1× distilled water)
UV transilluminator and gel photography equipment
heating block/water bath (60 to 65 °C)
ic to chill samples

Materials and reagents
gloves
hydrogen peroxide, 30% (Sigma H1009)
deionized water (or 1× or 2× distilled water), but not autoclaved or DEPC-treated, for all gel buffers and solutions, except as noted*.
No solutions are autoclaved

1× TBE (as for DNA gels):
0.089 M Tris
0.089 M boric acid
2 mM EDTA

5× TBE:
54 g Tris-base
27.5 g boric acid
20 mL 0.5 M EDTA
Combine ingredients; makes 1 liter.

agarose (normal grade as for DNA gels)
1 M guanidine thiocyanate (Sigma G6639; MW=118.2); i.e., 118 mg/mL of sterile* water (non-DEPC, as used for DNA work is fine)
Make only as much as you need on day of use (e.g., ~0.5 to 0.6 mL).
TOXIC; be sure to dispose of waste properly.

DNA loading dye containing bromophenol blue
formamide (Sigma F9037, molecular biology grade).
Not necessary to deionize. Open in chemical fume hood.

fresh, dilute ethidium bromide solution (~0.1 to 0.5 µg/mL, ~200 mL)
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

fresh 7.5 mM NaOH, ~1 to 1.5 liter (fresh, made on same day)
for blotting (see Sambrook et al.)
Hybond N+ (Amersham)
Whatman 3M paper
towel
2 glass plates, one big enough to fit over a shallow plastic tray
shallow plastic tray
2× SSC:
0.3 M NaCl
0.03 M sodium citrate
(1 liter of 20× SSC = 175.3 g NaCl, 88.2 g sodium citrate, to pH 7 with
HCl; dilute 1:10 for 2× SSC)

**Protocol**

*Electrophoresis of RNA and capillary transfer to nylon membrane*

- Clean gel apparatus by soaking everything including combs in 3% H$_2$O$_2$ for 10
  min (1:10 dilution of 30% stock in 1× distilled H$_2$O) or in 0.5 M NaOH for 2 h, or in 1% SDS for 2 h then rinsing in 1× distilled H$_2$O (which is not necessarily sterile or RNAse-free!).
- For a 100-mL, 1% agarose gel, using a relatively clean bottle, combine 100 mL of 1× TBE + 1 g agarose, and heat, with caution, in a microwave oven. The best resolution depends on the expected transcript size. Use the following guide for agarose concentration:
  - 0.8% agarose for large transcripts (greater than 7 kb)
  - 2% agarose for short transcripts (1.5 to 3 kb)
- Cool the gel to 55 to 60 °C. Check this with a relatively clean thermometer.
- Add 0.5 mL of freshly-made 1 M guanidine thiocyanate (final concentration = 5 mM G-SCN).
- Pour the gel and let it set for at least 30 min at room temperature.
- Prepare the samples while the gel sets.

**amount of RNA:**
- For 0.8% agarose gel, use 6 to 10 µg of total RNA per lane.
- For 2% agarose gel, use 3 to 5 µg per lane.

**volume of RNA to load:**
- total, approximately 5 to 20 µL per lane

**loading dye:**
- Not necessary if samples are in formamide.
- If your samples are in water, add an equal volume of formamide (final 50% formamide or greater).
- NOTE: Don’t put formaldehyde in your samples. It does not work on this type of gel!
  - If you absolutely need loading dye, try formamide with final 1× TBE, 0.5% bromophenol blue. Add this AFTER denaturing your RNA in formamide.
- Denature ~2 µg of size-standard RNA, in ~10 µL of formamide for ~10 min; place it on ice.
- Denature samples at 60 to 65 °C for 2 min; place it on ice.
- Having rinsed the gel tank, place the set gel in it, pour in 1× TBE to cover it, then remove the comb and any end blocks.
- Load the gel. Load a blank lane with DNA loading dye between the size marker and the samples to track the running of the samples. This also keeps your
marker from spilling into a sample lane, which sometimes makes a mess of hybridizations.

- Electrophorese samples into the gel for approximately 10 to 15 min at 110 volts, then run at 70 to 80 volts (3.5 v/cm) until the bromophenol blue is approximately 8 to 10 cm from the well. The process takes about 3 to 4 h.
- Stain the samples in fresh, dilute, ethidium bromide solution for ~10 min.
- Destain the for a few minutes in 1× TBE using buffer left over from the gel box.
- Photograph them with a ruler next to the gel. Leave the gel on UV transilluminator for ~2 min. This nicks the RNA, helping with transfer of larger transcripts.
- Soak the gel in ~200 mL of 7.5 mM NaOH, twice, for 10 min each time.
- Set up the capillary transfer to Hybond N+ in 7.5 mM NaOH to run overnight (not less than 8 h, especially important for large transcripts). (See Sambrook et al. 1989.)

**Disassemble blot and start hybridization**

- Neutralize the filter in 2× SSC for 5 min.
- Air dry the filter. Put the filter on UV light box at this point and draw size marker positions directly on the filter with a pencil.
- UV-crosslink, if you have a cross-linker, but this is not necessary.
- Prehybridize and hybridize as usual (see Kyes et al. 2000 for solutions and conditions). To start, try 55 °C hybridization, 60 °C wash in 0.5× SSC/0.1% SDS.

**References**


III. *Plasmodium falciparum* cDNA library construction

by Martha Schlichterle and Mats Wahlgren

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Equipment
- centrifuge (4 °C)
- agarose electrophoresis unit
- 50-mL Falcon tubes (BD Labware)
- Polytron homogenizer (Brinkmann)
- centrifuge (Beckman)
- speed-vac

Materials and reagents
Consider using kits from Stratagene for extraction of RNA and construction of cDNA libraries. They are generally more commonly employed than the Pharmacia kits used below. However, we did successfully make several cDNA libraries from two different strains using the Pharmacia kits.

Throughout the following protocols (MOLECULAR BIOLOGY III:A–E), there are references to the appropriate protocols from the companies from which library materials were bought. Further changes in these general “company-protocols” have been outlined below to facilitate *Plasmodium falciparum* library construction.

III:A. Cell preparations at desired cell stage(s)

Grow parasites in 75-cm² large flasks (12 mL per flask). You will need several sets of large flasks to be able to purify a workable amount of mRNA. We usually used 8 bottles/preparation where 8 bottles with ~10% parasitemia would yield 100 to 200 µg (sometimes even 300 µg) of total RNA. If rosetting parasites are desired, use parasites with ≥50% rosetting rate. See also protocols on cell culturing in PARASITES, section I, and synchronization of erythrocytic stages if that is desired (PARASITES, section IV).

III:B. RNA preparation (total RNA)

See separate protocol for RNA preparation in MOLECULAR BIOLOGY, section II above. Store RNA as a precipitate in 3× volume of ethanol with 0.3 M sodium acetate at −70 °C in microfuge tubes.

III:C. mRNA selection of pooled total RNA

See general protocol from 5Prime → 3Prime for mRNA isolation using oligo(dT) cellulose spin columns. (See also other companies for smaller amounts of mRNA isolation.)

Procedure
- Centrifuge microcentrifuge tubes with RNA in microfuge at 4 °C for 30 min.
- Wash samples with 70% cold ethanol and centrifuge them again for ~20 min.
- Dry tubes upside down in a hood with good airflow or in a speed-vac.
• Add 50 to 100 µL of pure “kit-H2O” to each tube and resuspend the RNA. Pool the RNA into one tube.
• Heat the sample at 65 °C for a few minutes, then place it on ice.
• Load 1 to 5 mg of total RNA per column. See company protocol (5Prime → 3Prime) for the rest.

Examples of mRNA yields:
One sample of ~2 mg total RNA gave:
~ 87 µg mRNA after the 1st selection (~3.5%)
~ 61 µg mRNA after the 2nd selection (~2.5%)

Another sample of ~1.05 mg total RNA gave:
~71 µg mRNA after the 1st selection (~6.8%)
~42 µg mRNA after the 2nd selection (~4%)

From gel analysis it was clear that a second mRNA selection is not necessary since no rRNA bands were shown after one round of selection.
mRNA was stored as a precipitate according to the protocol with muscle glycogen, sodium acetate, and ethanol.

Store pure mRNA in aliquots of 1 µg, 5 µg, and maybe more.
Run a few microgram-per-lane samples to visualize mRNA as a faint smear in the range of the two larger rRNA bands. The front of these two bands should however be sharp if unselected total RNA is also run in a separate lane along with mRNA to indicate lack of degradation. See MOLECULAR BIOLOGY, section II:C for RNA-gel protocol. If using pure mRNA for a gel, you may want to blot and fix it to nylon for possible later use in a Northern.
III:D. **cDNA synthesis**  
*by Martha Schlichtherle and Mats Wahlgren*  
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**Pharmacia’s kits:** Timesaver cDNA Synthesis Kit was used with some components replaced by the Directional Cloning Toolbox.

Additional reagents needed are indicated in the protocol booklet. If one has good quality distilled water, there is no need to use DEPC which is very hazardous (and can be destructive to RNA if it remains after autoclaving DEPC-treated water).

One needs to have access to the following water bath temperatures:
- 12 °C overnight (next day at 16 °C)
- room temperature
- 37 °C
- 65 °C

Optional: dry ice for dry ice/ethanol bath.

**Procedure**
- Cool the lyophilizer trap and have spin columns ready in autoclaved, sterile Corex tubes.
- Use 4 µg of very pure mRNA per cDNA synthesis (both for FCR3S1 and TM284 strains). One could use 5 µg to possibly increase the recombinant phage.
- Centrifuge the mRNA in an Eppendorf microfuge for 30 min at 4 °C, then wash it gently once with 70% ethanol to remove salts from the small white mRNA pellet. Centrifuge it again for 20 min, then dry it briefly.
- Follow Pharmacia's general protocol; use 1 mL of *Not*I dT₁₈ primer from the Directional Cloning Toolbox as the cDNA primer. (See the Pharmacia protocol.)
- Where phenol/chloroform extraction is indicated, use 50 µL of each, followed by an extra step of pure chloroform extraction.
- Ligate the adaptor for 2 to 2.5 h at 16 °C.
- Digest the adaptor with *Not*I for 1.5 h (not 1 h), then extract it again with chloroform/phenol and pure chloroform.
- Precipitate the cDNA in an ethanol/dry ice bath for 30 min.
- If one uses two purification steps with organics (i.e., phenol/chloroform, then chloroform), as opposed to one combined step of phenol/chloroform, one loses more cDNA material.
- It may be appropriate to use 20 µL of column effluent with 2 µg of vector to do the ligations, rather than the 15-10-5 µL scheme suggested in the protocol, since 15 µL of effluent gave more recombinant phage than 10 µL, which gave more than 5 µL. One strain did not yield any recombinant phage with 5 µL of column eluate.
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III:E. cDNA insertion, propagation, and amplification in phage using Pharmacia's λ ExCell NotI/EcoRI /CIP kit

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See Pharmacia's Timesaver cDNA Synthesis Kit protocol along with comments for cDNA and phage ligation. Use the phage λ vector to set up and do ligation reactions with Plasmodium falciparum cDNA.

Use extra microfuge spin times (20 min, not 10 min), 70% ethanol washing, centrifugation, and drying of DNA. cDNA/phage may be heated for a few minutes at 50 °C to sufficiently resuspend the cDNA before ligation reactions are done. Incubate ligation reactions for 30 to 40 min at 16 °C, then at 4 °C overnight.

Use Stratagene's Gigapack II packaging extracts to package ligated products into phage. (Now there is the Gigapack III kit, which is easier to use).

Use 3 to 4 µL of the cDNA-λ ligation per packaging. See the Gigapack II protocols.

Control packagings are not done. It is imperative to work very quickly once the extracts are removed from −70 °C to thaw. Package for 1 h 45 min.

Procedure

• Use Escherichia coli strain NM522 as the host for phage grown on special minimal plates (see Pharmacia protocols). Inoculate a few colonies in 10 to 20 mL of Luria broth (LB) with 0.2% maltose and 10 mM MgSO₄.
• Shake the inoculum at 30 °C overnight.
• The next day, centrifuge the cell suspension at ~2,000 rpm for 8 min and remove the LB medium.
• Resuspend the cells in 10 mM MgSO₄ for an OD₆₀₀ of 0.5.
• Cool the cells at 4 °C until use; use within 5 days with phage.
• Restreak stock plates of NM522 about every 3 weeks. Freeze extra bacterial stock at −70 °C as described in the manual using ~20% glycerol in the medium.
• Titer these primary libraries, 3 primary libraries/strain since there were 3 ligation mixtures set up for each strain. Phage dilutions of 1:10 and 1:100 of the primary libraries are recommended to titer.
• For the 1:10 dilution titering, mix 1 µL of phage with 9 µL of SM-buffer (see Stratagene's picoBlue Immunoscreening kit protocol).
• Make another such serial 1:10 dilution to achieve 1:100 phage dilution.
• Of each dilution, mix 1 µL with 200 µL of NM522 bacteria in 10 mM of MgSO₄ (see above) and place at 37 °C for ~15 min.
• Add 3 mL of LB low-melt agarose (at 49 °C) to the mixture and pour it on a prewarmed LB plate.
• Incubate the plate at 37 °C overnight.
• About 100 to 250 phage may be visible on the lower dilution plates. Approximately 0.5 × 10⁶ to 1 × 10⁶ independent clones may be generated per “ligation-library”.

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**Amplification of primary libraries**
- One needs about 40 to 50 large petri dishes with LB agar (poured fresh 1 to 2 days previously and evenly on a FLAT surface) for a library amplification (~13-cm plates). Each plate can take 20,000 to 40,000 phage; maybe 25,000 is a good number. Follow the rest of the Gigapack protocol for phage amplification.
- Allow phage plaques to grow for 7 to 8 h until they are pinhead sized (not too large).
- Overlay the plates with 9 to 10 mL of SM buffer overnight. It is very important that the plates lie very flat so that there is liquid on the whole plate into which the phage can elute. If the plates don't lie flat, parts will dry up as the buffer soaks into the agar, thus causing some uneven amplifications of phage populations.
- Recover and pool about 7 mL of SM-buffer/plate into 1-liter sterile bottles.
- Remove the bacteria with chloroform as described in the protocol, and aliquot the phage suspension into sterile 50-mL Corex tubes for centrifugation. Freeze most of the amplified libraries as aliquots at −70 °C with some chloroform/DMSO as described.
- Test both primary and secondary libraries for blue/clear phage indicating ratios of phage with no cDNA insert to those with an insert. Those without an insert should have the LacZ gene intact and thus produce β-galactosidase that in the presence of inducer (IPTG) and substrate X-gal would form blue plaques. Phage with insert are clear.
- Mix 1 µL of unamplified phage or 5 to 8 µL of a 10⁵-dilution of amplified library with 200 µL of bacteria to get 200 to 300 phage plaques per plate.
- Incubate the phage and bacteria for ~20 min at 37 °C, quickly mix with 3.0 mL of 49 °C top agarose, 7.5 µL of 1 M IPTG, and 50 µL of a solution of 250 mg of X-gal per mL of dimethylformamide (DMF), and pour on a prewarmed LB plate.
- Allow the top agarose to harden without a lid for 5 min, then incubate the plate upside down overnight at 37 °C.
- Remove the plates the following day and place them at 4 °C for the day (or alternatively longer) for the blue color to fully develop. The primary libraries have 2 to 3% of phage that are blue (dark or light blue) indicating no insert. The rest should contain inserts. Amplified libraries are only 3 to 5% nonrecombinant.
- For initial titering of primary libraries, 1 µL of 10-fold dilutions is enough to use (see above).
- Dilute amplified libraries in steps of 1:100, where 10 µL of 10⁻⁶, 10⁻⁷, 10⁻⁸ (and possibly more) are plated and a range of about 800 down to 50 phage are seen on the increasingly diluted plates.

Examples of titres achieved:
FCR3S1 library: ~3⁹ to 10⁹ pfu/mL; TM 284 was at ~9⁹ to 10⁹ pfu/mL.
Libraries were aliquoted as 1-mL samples in microfuge tubes and stored with 50 µL of chloroform, along with some larger aliquots stored in screw-cap glass tubes with some chloroform at 4 °C for library screening. The rest were kept for long-term storage as aliquots in 15-mL tubes with DMSO/chloroform at −70 °C (see also the Gigapack protocol).

**Testing excision of inserts from libraries**
To get an idea of insert sizes in the libraries, we used recombinant phage directly from the libraries to excise, or release, clones as phagemids. Best results were obtained with
pExCell release directly from the library as opposed to plaque-purified phage clones. For the in vivo excision, we used NP66 *E. coli* accompanying the kit as well as the specified medium and temperature shift from 32 to 39 °C (See Pharmacia λ.ExCell protocol).

- Pick colonies the following day.
- Incubate them in LB-amp overnight.
- Perform plasmid mini-preps using the alkaline lysis method from “Current Protocols” with suggested modifications, such as phenol extraction, etc. (Do not use boiling mini-preps.)
- Use *Pvu*II enzyme to cut around insert sites. Use about half of the digest on a gel. *Pvu*II-cut sites are ~380 bp apart on the vector (i.e., without insert).
- The vector arms should appear as one band of ~2.5 kb. Insert sizes from a few random clones tested were seen from ~0.5 to 2.2 kb. Or simply test insert size by PCR using vector arm primers.

References

Protocols from the following companies’ kits:
- 5Prime → 3Prime’s Maxi oligo (dT) Cellulose spin column for mRNA purification
- Pharmacia’s Timesaver cDNA Synthesis
- Pharmacia’s Directional Cloning Toolbox
- Pharmacia’s λ. ExCell NotI/EcoRI/CIP
- Stratagene’s Gigapack Packaging Extracts
- Stratagene’s picoBlue Immunoscreening

IV. PCR of AT-rich genomes/regions

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Noncoding regions of *Plasmodium falciparum* tend to be extremely rich in AT content (>90%). PCR amplification of these regions is significantly improved by lowering the *Taq* polymerase extension temperature from 72 to 60 °C. The other parameters do not need to be changed.

Note: The AT content of the entire genome is estimated to be ~82% while coding regions are thought to be 60 to 70% AT.

References


V. Single cell RT-PCR of var (DBL-1) mRNA from *Plasmodium falciparum*

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Equipment

PCR machine with hot lid

Materials and reagents

- GeneAmp RNA PCR Kit (Applied Biosystems)
- RNase-free DNase, 10 U/µL (Stratagene)
- DNase
- RNase inhibitor
- Opti-Prime PCR buffer 3 and buffer 4, mixed 1:1 (Stratagene)
- *Taq*Start antibody (Clontech)
- *Taq* DNA polymerase, 5000 U/µL (Pharmacia)

- Prepare PCR mix for *n* samples:
  - deoxyadenosine triphosphate: 2 µL × *n*
  - deoxycytidine triphosphate: 2 µL × *n*
  - deoxyguanosine triphosphate: 2 µL × *n*
  - deoxythymidine triphosphate: 2 µL × *n*
  - GeneAmp 10× PCR Buffer II: 2 µL × *n*
  - MgCl₂: 4 µL × *n*
  - distilled water: 1 µL × *n*

- Aliquot the PCR mix into *n* thin-walled PCR tubes (15 µL/tube).
- Place a single infected erythrocyte into each tube and immediately freeze it on dry-ice (see PARASITES, section V:B).
- Release the DNA and RNA from the parasite by heating the tubes to 93 °C for 3 min in the PCR machine. Cool them on ice.
- Add 1 µL of DNase and 1 µL of RNase inhibitor to each tube. Degrade the DNA at 37 °C for 30 min in the PCR machine. Inactivate the DNase at 93 °C for 3 min.
- Add 1 µL of RNase inhibitor, 1 µL of random hexamers, and 1 µL of reverse transcriptase to each tube. Perform the reverse transcription at 42 °C for 30 min and inactivate the reverse transcriptase at 93 °C for 3 min.
- Prepare the *Taq*Start mix for *n* reactions:
  - *Taq* polymerase: 4.4 µL × *m* (where *m* = 4.8 × *n* / 25.4)
  - *Taq*Start antibody: 4.4 µL × *m*
  - *Taq*Start buffer: 17.6 µL × *m*
  - Mix and incubate for at least 5 min at room temperature.

- Prepare the PCR master mix for *n* reactions:
  - Opti-Prime PCR buffer 3+4: 8 µL × *n*
  - Specific primer DBL1-1 (DBL1-3): 2 µL × *n*
  - Specific primer DBL1-2: 2 µL × *n*
  - *Taq*Start mix: 4.8 µL × *n*
  - distilled water: 63.2 µL × *n*
• Add 80 µL of PCR master mix to each tube. The tubes already contain 20 µL from the previous steps; the final volume in each tube should thus be 100 µL.
• Amplify the var sequences using the following PCR conditions:
  93 °C for 20 s, 55 °C for 30 s, and 72 °C for 1 min.
  Run 50 cycles followed by a last extension step at 72 °C for 7 min.
• Run 20 µL of the PCR products on a 1.2% agarose gel with TBE buffer.

Primer sequences
DBL1-1: 5′-GGW GCW TGY GCW CCW TWY MG-3′
DBL1-2: 5′-ARR TAY TGW GGW ACR TAR TC-3′
DBL1-3: 5′-GCA CGA AGT TTY GCA GA-3′

Reference
VI. Fluorescent in situ hybridization (FISH) for Plasmodium falciparum

(Adapted from Ersfeld, K. & Gull, K, 1997)

by Lucio Freitas-Junior and Artur Scherf

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Equipment
thermal cycler with in situ adapter (Eppendorf)
water baths (37 °C, 50 °C, and 60 °C)
humid chamber
microscope that allows analysis of different fluorochromes, CCD camera attached (e.g., Nikon 800)

Materials and reagents:
saponin
incomplete RPMI:
RPMI 1640
25 mM HEPES
0.37 mM hypoxanthine (pH 7.4)
standard microscope slides
In situ Frame for 25 µL (Eppendorf)
hybridization solution (HS):
50% formamide (Roche Applied Science)
10% dextran sulfate
2× SSPE
250 µg/mL herring sperm DNA
Probes: To prepare the probes, use the “Fluorescein High-prime kit” (Roche Applied Science) for Fluorescein signals and “Biotin high-prime kit” (Roche Applied Science). For detection of the biotinylated probes use avidin-conjugate with rhodamine (Roche Applied Science).
M solution (pH 7.5):
100 mM maleic acid
150 mM NaCl
1% blocking reagent (Roche Applied Science)
4% bovine serum albumin (BSA) (SIGMA)

TNT solution (pH 7.5):
100 mM Tris–HCl
150 mM NaCl
0.5% Tween 20 (v/v)

20× SSC
0.1% NP 40 in PBS
2% paraformaldehyde in PBS (pH 7.2)
formamide (Roche Applied Science)
50%, 70%, 90%, and 100% ethanol
VECTASHIELD mounting medium with DAPI (Vector Laboratories)
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**Procedure**

**Parasite fixation:**
Before starting, mark the region where the parasites will be deposited on the slide with a diamond marker: This makes it easier to visualize them later. This area should be about 5 mm × 5 mm.

- Treat the parasites with saponin to lyse the erythrocyte membrane.
- Wash them twice in incomplete RPMI.
- Deposit a monolayer of parasites in the area previously marked on the microscope slides.
- Air dry the slides for 30 min (or overnight) at room temperature.
- Fix the slides in 2% paraformaldehyde in PBS (pH 7.2) for 5 min.
- Wash the slides three times in PBS and dehydrate them through a series of ethanol washes (50%, 70%, 90%, and 100%) for 5 min each.
- Air dry the slides for 30 min. At this point you can use the slides or store them at −80 °C (in aluminium foil).

**In situ hybridization:**

- Remove the slides from −80 °C just before use and leave them at room temperature for 10 min before removing the aluminium foil.
- Permeabilize the parasites by incubating them in 0.1% NP 40 in PBS for 5 min at room temperature.
- Wash the slides 3 times in PBS for 5 min each at room temperature.
- Fix the plastic frame around the area containing the parasites.
- Apply the HS solution (denatured at 95 °C for 5 min) on this area.
- Incubate the slides for 30 min at 42 °C in a humid chamber.
- For hybridization, remove as much of the HS solution as possible and add fresh HS containing the probes. (For biotin-labeled probes, use a final concentration of 1 ng/µL and for fluorescein probes use a final concentration of 0.5 ng/µL.)
- Cover the area with the In situ Frame coverslip.
- Denature the slides in a thermal cycler with in situ adapter for 2 min at 94 °C followed by hybridization at 37 °C overnight.

**Washing:**

- Remove the In situ Frame coverslip and the HS solution.
- Wash the slides in 2× SSC/50% formamide for 30 min at 37 °C, followed by 2× SSC for 10 min at 50 °C, 2× SSC for 10 min at 60 °C, and 4× SSC for 10 min at room temperature.
- Equilibrate the cells in M solution for 5 min at room temperature in a humid chamber protected from light.
- Remove the M solution and replace it with M solution plus avidin–rhodamine (final concentration: 50 ng/mL) for detection using biotin probes. Incubate the slides for 30 min at room temperature.
- Wash the slides in M solution for 10 min at room temperature, and twice in TNT solution for an additional 10 min each at room temperature.
- Dry the slide and mount using Vectashield with DAPI.
- Analyse the slide by fluorescence microscopy.
Comments
Always wear gloves!
In optimal conditions the FISH technique will give a clean result without any background.
Never let the slides dry out after the hybridization process has begun.
This technique works nicely for other *Plasmodium* species and also works for *Leishmania* however for *Leismania* the fixation is performed in 100% ethanol for 10 min at room temperature.

References

VII. Separation and mapping of chromosomes of malaria parasites using pulsed-field gel electrophoresis (PFGE)

VII:A. PFGE Protocol 1
by Rosaura Hernandez-Rivas and Artur Scherf

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Equipment
- Pulsaphor/Gene Navigator PFGE apparatus (Pharmacia) or CHEF-DR system (Bio-Rad)
- centrifuge
- water bath
- 15-mL Falcon tubes (BD Labware)

Materials and reagents
- SeaKem GTG agarose (Cambrex) for agarose gels at a concentration of >0.6%
- chromosomal grade agarose (Bio-Rad) for agarose gels ≤0.6%
- low-melting point agarose (Cambrex) for embedding parasites in agarose blocks and preparative isolation of chromosomes
- plastic gel molds (2 × 5 × 10 mm, 100-µL volume; Pharmacia)
- nylon membrane: Hybond-N+ (Amersham) or Biotrans(+) (ICN Biomedicals)
These membranes allow the rapid alkaline transfer of DNA and their physical characteristics make them especially useful for multiple hybridization cycles (usually 5 to 10 cycles).
- erythrocyte lysis buffer: 0.15% saponin (Sigma) in phosphate-buffered saline (PBS).
- ethidium bromide in water: 10 mg/mL
CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.

- alkaline transfer buffer: 0.4 M NaOH
20× SSC:
- 3 M sodium chloride
- 0.3 M sodium citrate
- TE buffer:
    10 mM Tris-HCl
    1 mM EDTA (pH 8.0)
    1 mM phenylmethylsulphonyl fluoride (PMSF) in TE

- molecular weight size standards:
  5 kb ladder (4.9 to 120 kb)
  ladder (0.05 to 1 Mb)
  Saccharomyces cerevisiae (0.2 to 2.2 Mb)
  Hansenula wingei (1 to 3.1 Mb) (Bio-Rad)
cell lysis buffer (CLB):
0.5 M EDTA (pH 8.0)
10 mM Tris–HCl
1% sodium lauryl sarcosinate (Sigma)
Store at room temperature. Before cell lysis, add 2 mg/mL of proteinase K from a stock solution.

prehybridization and hybridization buffer:
0.5 M NaPO4 (pH 7.2)
7% SDS
1% BSA

hybridization buffer:
7% SDS
0.5 M EDTA (pH 8.0)
1% BSA

saturated 2-butanol:
Mix 2-butanol with 1 M NaCl in TE (4:1 v/v). Add 9 volumes of saturated 2-butanol and 1 volume of the aqueous phase to the gel blocks. This is important to avoid gel shrinking!

10× TBE buffer:
108 g Tris-base
54 g boric acid
8.35 g disodium-EDTA per liter (pH ~8.5)

Preparation of DNA for PFGE
The ability to preserve the intact size of large DNA molecules is critical for the success of PFGE. To overcome the problem of DNA shearing, intact cells are embedded in low-melting agarose blocks. Cells are lysed and proteins are removed by proteinase K treatment. This procedure yields DNA that is both intact and susceptible to restriction enzyme digestion. The agarose block can be loaded directly into the well of a pulsed-field gel.

The host cells of the intracellular blood stage form of *Plasmodium falciparum* need to be lysed by saponin before the parasite cells are embedded into agarose blocks.

- Estimate the volume of the erythrocyte pellet and add 1.5 volumes of 0.15% saponin in PBS at room temperature.
- Resuspend the pellet and incubate it for 3 to 5 min. The liquid will clarify as the red blood cells lyse.
- Add 5 volumes of cold PBS and centrifuge the pellet at 5,000 rpm for 10 min.
- Resuspend the dark pellet which contains the parasite material in 2 mL of PBS.
- Pellet the cells by centrifugation at 3,000 rpm for 10 min.
- Resuspend the pellet at a concentration of approximately $5 \times 10^8$ parasites/mL of PBS.
- Equilibrate the cell suspension at 37 °C and add an equal volume of 1.6% melted low-melting point agarose (37 °C).
- Mix the cell suspension gently and dispense it immediately into plastic gel molds.
- Allow the gel to solidify at 4 °C for about 20 min.
• Place up to 20 solidified blocks in 10 mL of cell lysis buffer (CLB) and incubate
  them for 24 h at 42 °C.
• Replace the CLB with an equal volume of fresh CLB.
• Incubate the blocks for an additional 24 h.
• The blocks can be stored indefinitely in TE buffer at 4 °C.

Electrophoresis conditions
Optimal separation of the different chromosome size classes usually requires the use of
a range of different PFGE conditions. Table 1 shows the running conditions of large DNA
fragments of various sizes using the CHEF (clamped homogeneous electric field)
system. These PFGE methods have been used in studies of *Plasmodium falciparum* and
*Trypanosoma cruzi*, but also apply to other parasitic protozoa. The described
electrophoresis conditions have been developed for the Pulsaphor/Gene Navigator
PFGE apparatus (Pharmacia) based on the CHEF design. Similar results have been
obtained with the CHEF-DR system (Bio-Rad).

Table 1. Electrophoresis conditions for separation of DNA fragments by PFGE (CHEF
system)

<table>
<thead>
<tr>
<th>Efficient separation</th>
<th>Pulse time (ramping)</th>
<th>Voltage</th>
<th>Run time</th>
<th>Agarose concentration</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5–100 kb</td>
<td>0.5 s – 1.0 s</td>
<td>400 V</td>
<td>7 h</td>
<td>1.3%</td>
<td>0.2× TBE</td>
</tr>
<tr>
<td>50–2000 kb</td>
<td>50 s – 90 s</td>
<td>200 V</td>
<td>22 h</td>
<td>1.0%</td>
<td>0.5× TBE</td>
</tr>
<tr>
<td>500–4000 kb</td>
<td>90 s – 300 s for 24 h</td>
<td>95 V</td>
<td>48 h</td>
<td>0.7–0.8%</td>
<td>0.5× TBE</td>
</tr>
<tr>
<td></td>
<td>followed by 300 s – 720 s for 24 h</td>
<td>85 V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2000–6000 kb</td>
<td>120 s – 720 s</td>
<td>85 V</td>
<td>36 h</td>
<td>0.4–0.6% high gel strength agarose</td>
<td>0.5× TBE</td>
</tr>
</tbody>
</table>

• The agarose concentration and type of agarose used depends on the DNA size
  range to be separated. For example, pulsed field separations larger than 2
  megabases are improved by using high gel-strength agarose (chromosomal
  grade agarose, Bio-Rad) allowing preparations of very low-percentage agarose
gels (0.4 to 0.6%), which give significantly better separation of large
chromosomes in the range of 2 to 5 megabases..

• For a 15 cm × 15 cm × 0.5 cm 1% agarose gel, dissolve 1.1 g of SeaKem GTG
  agarose in 110 mL of 0.5× TBE buffer by heating it in a microwave oven. Make
  sure that the volume has not changed. If it has, adjust again to 110 mL.

• Cool the agarose in a 50 °C water bath and pour it into the casting stand.

• After the gel is solidified, fill the wells with 0.5× TBE buffer and insert the blocks
  containing parasite DNA. About 2 to 5 mm of a block (1 × 10^7 to 2.5 × 10^7
  parasites) generally contains enough material to visualize the chromosomes after
  ethidium bromide staining. (This number may also work for other parasite
species.) However, for best PFGE results it may be necessary to determine the optimal number of cells/block.

- Seal the blocks into the well with 1% low-melting point agarose.
- Fill the electrophoresis chamber with 2.2 liters of TBE buffer. Do not overfill; the buffer should just cover the top of the agarose gel. Keep the temperature of the running buffer (18 °C) constant during the run.

**Staining of chromosomes**

- After completing the PFGE run, the separated chromosomes can be visualized by staining the agarose gel for 15 min in ethidium bromide (1 µg/mL in water).
- Destain the gel by two washes in 0.5× TBE for 1 h with gentle agitation, and photograph it (do not forget the ruler) using a shortwave UV light (254 nm).

**ATTENTION!** If chromosomes are intended for use in subsequent restriction mapping studies, longwave UV light (360 nm) must be used to avoid nicking the DNA.

**Southern hybridization**

- Transfer the DNA from the agarose gel to a nylon membrane such as Hybond-N or Biotrans. In order to ensure efficient transfer of large DNA fragments, nick the chromosomal DNA by exposing the stained agarose gel for 5 min on a UV-light table (254 nm).
- The alkaline transfer procedure using 0.4 N NaOH as transfer buffer works fine for chromosomal DNA blots. We routinely set up the capillary transfer (standard molecular biology procedure) for at least 24 h. Make sure that the weight on top of the absorbent paper stack does not exceed 1 kg. There is no need to fix DNA after alkali blotting.
- For 32P-labelled DNA probes, the random hexamer priming method in combination with the following hybridization buffer usually works well to give a good signal-to-background ratio with nylon membranes: 7% SDS, 0.5 M EDTA (pH 8.0), 1% BSA. Single-copy genes of *P. falciparum* are generally detected after 6 h to overnight exposure on X-ray film.
- Remove probes by standard protocols or as recommended by the manufacturer. Stripped blots can be stored for prolonged periods at room temperature.

**Restriction enzyme digestion of embedded chromosomes**

Generally, restriction enzymes diffuse into agarose blocks and are thus suited for chromosome mapping studies. The most useful restriction endonucleases are those with 8-base recognition sites which cut only a few times in chromosome-sized DNA fragments. However, in genomes which are biased in their AT content, such as *P. falciparum*, certain enzymes which recognize GC-rich 6-base pair sequences (*Sma*1, *Bgl*1) can also be used as “rare” cutters in these AT-rich genomes.

- For restriction enzyme digestion of agarose-embedded total parasite DNA, remove the lysis buffer by extensive washes in TE buffer (2 to 3 washes in 20 mL of TE).
- Inactivate any remaining proteinase K by treating the blocks with 1 mM phenylmethylsulphonyl fluoride (PMSF) in TE (10 mL for 20 blocks) for 2 h at room temperature, followed by three washes in 10 mL of TE for 30 min each.
Individual chromosomes or DNA fragments that have been cut out of a stained agarose gel after a PFGE run must be treated with 2-butanol to extract any remaining ethidium bromide prior to restriction enzyme digestion.

- Add 9 volumes of 2-butanol saturated in 1 M NaCl/TE and 1 volume of the aqueous phase to the gel block (5:1, v/v).
- Agitate the mixture at room temperature for 30 min.
- Repeat the extraction twice and wash the block five times in 10 mL of TE buffer for 30 min each.
- Store the blocks at 4 °C.
- Equilibrate the blocks with 5 volumes of restriction buffer containing 100 µg/mL of nuclease-free BSA for 30 to 60 min at room temperature. Remove the buffer and add 2 volumes of fresh buffer and restriction endonuclease (approximately 100 to 200 U/mL). Incubate the blocks at the recommended temperature for 4 h or overnight.
- Partial digestion of chromosomal DNA is useful for mapping studies. In this case, set up separate digestions of serial dilutions of the restriction enzyme for 2 h each (0.01 U/mL, 0.1 U/mL, 1 U/mL, 10 U/mL, and 100 U/mL). Stop the reactions by adding EDTA to a final concentration of 50 mM.
- Depending on the expected fragment size, restricted DNA can be separated by PFGE according to the run conditions described in Table 1.

Two dimensional PFGE

Most chromosomes in *P. falciparum*, as in other protozoan parasites, are heterogeneous in size and can be separated from each other by PFGE. Two-dimensional (2D) PFGE studies of a karyotype can give useful information concerning genetic markers located on several chromosomes. Multigene families are often observed in pathogenic protozoa.

- Separate chromosomes in the first dimension and stain them with ethidium bromide as described above.
- Photograph the gel using longwave UV light (360 nm).
- Use a razor blade to excise strips of gel (~2 mm) that contain all chromosomes.
- Extract ethidium bromide using 2-butanol in a 15-mL Falcon tube (as described above).
- Perform restriction enzyme digestions as described above.
- Embed the slice of gel containing the digested chromosomes and size markers (*λ* ladder or 5 kb ladder) into a precut slot in the top of a 1.3% agarose gel and seal it with 1% low-melting point agarose.
- Carry out standard PFGE separation using the run conditions described in Table 1. For Southern hybridization, continue as described above.

References


VII:B. PFGE Protocol 2

by Mats Wahlgren

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Equipment
- centrifuge
- incubator
- Plug mold (Bio-Rad)
- PosiBlot 30-30 pressure blotter (Stratagene)
- Stratalinker UV crosslinker (Stratagene, model 2400)
- Falcon tubes (BD Labware)

Materials and reagents
- 10% saponin in PBS
- 10 mM EDTA (pH 7.6)
- PBS
- low-melt agarose
- 0.5 M EDTA, 1% N-lauryl sodium sarcosinate (sarkosyl), proteinase K
- chromosomal grade agarose (Bio-Rad)
- ethidium bromide
  CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood.
- 0.25 M HCl
denaturation buffer:
  - 0.5 M NaOH
  - 1.5 M NaCl
neutralization buffer:
  - 1 M Tris
  - 1.5 M NaCl
10× SSC
nylon membrane Hybond-N (Amersham)
TSE:
  - 10 mM Tris–HCl (pH 8.0)
  - 100 mM NaCl
  - 1 mM EDTA

Sample preparation
- Calculate the parasitemia of the culture to be used.
- Add 1/100 volume of 10% saponin to the culture. Incubate the culture for 5 min at room temperature.
- Pellet the free parasites by centrifuging them at ~1,000 rpm for 5 min (i.e., a “gentle spin”).
- Dissolve the pellet in PBS and spin it again. When dissolving the pellet, use a broad tip.
- Wash the pellet two more times with PBS.
Methods in Malaria Research

After the last centrifugation, dissolve the pellet in TSE to get a concentration of $8 \times 10^8$ parasites/mL.

Mix the parasites with an equal volume of 1.6% low-melt agarose to get a final concentration of $4 \times 10^8$ parasites/mL. The agarose should be between 37 and 42 °C.

Dispense the mix into plug molds and let the agarose solidify, first at room temperature and then at 4 °C for 20 to 30 min.

Put the DNA/agarose plugs in 0.5 M EDTA, 1% N-lauryl sodium sarcosinate, 2 mg/mL of proteinase K; 10 mL of solution will be enough for 20 DNA plugs in a Falcon tube.

Incubate the plugs at 37 °C for 48 h.

Store the DNA plugs at 4 °C in the same solution as above (or possibly in TE).

Electrophoresis conditions

See also the section on electrophoresis conditions in MOLECULAR BIOLOGY, section VII:A, for details.

<table>
<thead>
<tr>
<th>Separation</th>
<th>Pulse time (ramping)</th>
<th>Voltage</th>
<th>Run time</th>
<th>Included angle</th>
<th>Buffer/agarose *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quick separation 500–2500 kb</td>
<td>Blk 1 5–12 min</td>
<td>2.0 V/cm</td>
<td>30 h</td>
<td>53°</td>
<td>1× TAE/</td>
</tr>
<tr>
<td></td>
<td>Blk 2 3–5 min</td>
<td>4.0 V/cm</td>
<td>17.5 h</td>
<td>53°</td>
<td>0.7%</td>
</tr>
<tr>
<td>Good separation 500–1800 kb</td>
<td>Blk 1 6–8 min</td>
<td>2.5 V/cm</td>
<td>80 h</td>
<td>60°</td>
<td>0.5× TBE/</td>
</tr>
<tr>
<td></td>
<td>Blk 2 4–6 min</td>
<td>2.5 V/cm</td>
<td>64 h</td>
<td>60°</td>
<td>1%</td>
</tr>
<tr>
<td></td>
<td>Blk 3 2–4 min</td>
<td>2.5 V/cm</td>
<td>20 h</td>
<td>60°</td>
<td></td>
</tr>
</tbody>
</table>

* The agarose used is chromosomal grade agarose (Bio-Rad). Buffer temperature is 14 °C for quick separation and 10 °C for good separation.

Southern blot

Stain the gel for 30 min in 1 µg/mL of ethidium bromide. Destain the gel for 10 min in distilled water. Look at the gel under UV light and take a photograph. Destain the gel for another hour in distilled water.

Prepare the gel for Southern transfer by depurinating it in 0.25 M HCl for 30 min.

Rinse the gel in distilled water and denature it in denaturation buffer for 30 min.

Rinse the gel in distilled water and neutralize it in neutralization buffer for 30 min.

Rinse the gel in 10× SSC. Wet the nylon membrane in distilled water and let it soak for 5 min in 10× SSC.

Transfer the chromosomes from the gel to the nylon membrane in a PosiBlot 30-30 pressure blotter. Assemble the pressure blotter according to the manual and use 10× SSC as the transfer buffer. One hour at 75 to 80 mm Hg is enough to transfer the chromosomes to the membrane.

Quickly rinse the filter in 2× SSC to get rid of any gel pieces sticking to the membrane. Put the membrane on a Whatman paper to dry off a little.
• While the membrane is still moist, cross-link the DNA to the membrane by exposing it to UV light in a crosslinker. Use setting “auto cross-link”.

References

VIII. Recombinant expression of *Plasmodium falciparum* var- gene domains

by Qijun Chen

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**Equipment**
- bacterial culture flask
- bacterial culture incubator
- sonicator/French press
- centrifuge (4 °C)
- spectrophotometer

**Materials and reagents**
- pGEX vector (Pharmacia)
- BL21-CodonPlus-RIL competent cells (Stratagene) or BL21 competent cells (Pharmacia Biotech)
- LB plates (ampicillin 100 µg/mL)
- PBS (pH 7.2)
- PBS plus 0.2 M NaCl and 0.05% Tween-20
- 2× YT/amp medium:
  - 16 g/L tryptone
  - 10 g/L yeast extract
  - 5 g/L NaCl
  - ampicillin 100 µL/mL
- EDTA
- PTG (1 M)
- Pefabloc (Roche Applied Science)
- lysozyme (Pharmacia Biotech)
- Triton X-100
- glutathione-Sepharose (Pharmacia Biotech)
- glutathione solution
- thrombin (Pharmacia Biotech)

**Procedure**
- Clone the sequence of var-domains into the pGEX vector in the correct reading frame.
- Transform BL21-CodonPlus-RIL competent cells or BL21 competent cells and select the positive transformed bacteria with ampicillin in LB (100 µg/mL) using manufacturer’s conditions. The former strain appears to result in less degradation during expression.
- Pick several colonies and culture them separately overnight in 20 mL of LB medium with 100 µg/mL of ampicillin at 37 °C.
- Inoculate the overnight culture in prewarmed (room-temperature) 2× YT/amp medium in a ratio of 1:100. Use 20 mL of medium for the initial expression test, and 1 to 10 liters for large scale expression. Continue shaking the culture at room temperature until the OD₆₀₀ of the culture is 0.4 to 0.6. Precool the centrifuge to 4 °C.
• Check the induction system for your vector. If you are using the pGEX system and thus the tac-promoter, add IPTG to 0.1 mM (using a 1 M stock) and induce the expression at a temperature lower than 30 °C:
  overnight for the ATS-region
  less than 4 h for DBL-domains
• Sediment the bacteria by centrifugation at 1,500 × g for 20 min at 4 °C if using large-volume centrifuge flasks. For small volumes, sediment for 10 min.
• Resuspend the bacteria with cold PBS (20 times the pellet volume), then add:
  EDTA to 0.5 mM (do NOT use if using a his-tag vector)
  Pefabloc to 1 mM (or 1 mg/mL)
  lysozyme to 1 mg/mL
  (DTT to 5 mM, optional)
• Mix completely and sonicate the bacteria five times for 18 s each, or use a French press to release the fusion protein from the bacteria. You may have to pass the bacterial solution through the press twice to release protein.
• Add 20% Triton X-100 to a final concentration of 1% and shake it at 4 °C for 30 min. If using a GST-vector, prepare glutathione–Sepharose according to manufacturer’s instructions and follow the protocol below. Otherwise, refer to the manufacturer’s recommendations for the expression system you have chosen.
• Centrifuge bacterial lysis at 2,000 × g for 15 min at 4 °C.
• Remove and save the supernatant, add to it at least 0.5 mL of bead volume of glutathione–Sepharose per liter of original culture.
• Allow the binding of the fusion protein and matrix to proceed for 1 h with gentle shaking at 4 °C.
• Wash the adsorbed beads twice with cold PBS plus 0.2 M NaCl and 0.05% Tween-20 and once with cold PBS only.
• Sediment the suspension by centrifuging (2,000 × g). Remove the supernatant.
• Add an equal volume of glutathione solution to the beads and release the protein for 60 min at room temperature, or overnight at 4 °C with slow shaking.
• Sediment the beads and measure the yield of fusion protein in the supernatant with the spectrophotometer at 280 nm.
• The fusion protein can be digested either directly on the glutathione–Sepharose beads or in the eluent solution. Use 30 U of thrombin per 500 µL of bead volume of beads or 1 mL of eluent solution. The digestion is usually carried out overnight at 4 °C.
• Aliquot, then store the fusion protein at −70 °C.

Reference
IX. Transfection of *Plasmodium falciparum* within human red blood cells

by **Yimin Wu**

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There are 3 basic steps in asexual stage *P. falciparum* transfection:

A. Preparing the materials, parasite culture, and DNA constructs

B. Transfecting DNA into parasites by electroporation

C. Assaying the expression of transfected genes

This protocol includes the chloramphenicol acetyl-transferase (CAT) assay, luciferase assay, and the selection of stable transformants by the anti-folate pyrimethamine.

IX:A. **Materials**

**Parasite culture**

Prepare $1 \times 10^9$ to $2 \times 10^9$ parasitized RBCs (PRBCs) for each transfection. A synchronized ring-stage parasite (<20 h) culture with high parasitemia (>15%) is essential for an improved transfection efficiency. For cultivation, synchronization, and purification see PARASITES, sections I through IV. Because washed human RBCs deteriorate after 4 weeks at 4 °C (even sooner at 37 °C), the parasite culture should be replenished with fresh RBCs before transfection, especially when a stable selection is desired.

**DNA constructs**

The design of transfection constructs is specific to the goal of each individual experiment. Plasmodial flanking regions are required for the expression in parasites; see also “References” at the end of this protocol. Use 50 to 100 µg of DNA for each transfection. The plasmid may be dissolved in TE buffer at 1 to 3 mg/mL.

**Buffers**

Incomplete Cytomix:

- 120 mM KCl
- 0.15 mM CaCl$_2$
- 2 mM EGTA
- 5 mM MgCl$_2$
- 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$
- 25 mM HEPES

PBS:

- 1.7 mM NaH$_2$PO$_4$
- 5 mM Na$_2$HPO$_4$
- 150 mM NaCl
- 10% saponin/PBS stock solution

**Equipment**

- GenePulser (Bio-Rad)
- Capacitance Extender (Bio-Rad)
- electroporation cuvettes (0.2-cm and 0.4-cm gap)
- table-top centrifuge (Sorvall)
Reagents, buffers, and equipment for CAT assay

TEN:
- 1 mM EDTA
- 150 mM NaCl
- 40 mM Tris–HCl (pH 7.6)
- 250 mM Tris–HCl (pH 7.6)
- 110 mCi/mmol, Moravek Biochemicals, diluted to 2.5 nCi/µL
- [14C]chloramphenicol
- acetyl coenzyme A (20 mg, Calbiochem, dissolved in 250 mM Tris–HCl [pH 7.6] to 40 mM)
- ethyl acetate
- chloroform
- methanol
- TLC plate (aluminium-backed, thin-layer silica gel 60A plates, Whatman)
- glass chromatography chamber
- X-ray film and film cassettes, alternatively Phosphorimager cassettes and scanner (Molecular Dynamics)

Reagents, buffers, and equipment for luciferase assay

Luciferase Assay System (Promega):
- cell lysis buffer
- luciferase substrate
- substrate solvent
- purified luciferase as positive control
- luminometer (Turner)

Reagents for stable selection

Pyrimethamine stock solution is made in dimethyl sulfoxide (DMSO, 50 mg/mL). Make the selection culture medium without gentamycin. The final content of DMSO in the culture medium should be less than 0.5%.

IX.B. Transfection

Transfecting parasites, Day 1

- Change the parasite culture supernatant with fresh medium and make a smear to ensure the quality of the culture. Return the culture to the incubator for at least one h while preparing for transfection.
- Chill the electroporation cuvette and the Cytomix solution on ice. Set the GenePulser electroporator at 2.5 kV, 25 µF, 200 Ω.
- After everything is in place, spin down the cells at 1,500 rpm for 5 min in a tabletop centrifuge. Resuspend the cells (about 100 to 200 µL) with 500 µL of Cytomix and not more than 100 µL of the DNA construct*. Mix well by pipetting up and down several times.
  *Note: The DNA construct solution should be further concentrated if the volume to be added exceeds 100 µL in order to reach the desired final concentration in the transfection mix.
- Transfer the mixture into a 0.4-cm cuvette and execute the electroporation. Take a note of the set voltage, the actual voltage, capacitance, and time constant. A
slight deviation of actual voltage is acceptable. The time constant should be 0.7 to 0.9 ms.

- Immediately return the cuvette to ice for 5 min, then transfer the cells from the cuvette to a culture flask together with 10 mL of culture medium (rinse the cuvette with culture medium to ensure a complete transfer). Return the flask to the incubator.
- An alternative electroporation setting is 0.31 kV/960 uF with the Bio-Rad GenePulser, using a 0.2-cm cuvette. The total volume allowed in the 0.2-cm cuvette is 400 µL. The time constant is about 12 to 15 ms.

Changing medium, Day 2

- Change the culture medium and add 50 µL of fresh blood. Check the parasite smear. Don’t panic if the smear contains lots of extracellular, dying parasites or cell debris, as many parasites normally die during this procedure. Recovering parasites should have developed to trophozoites or schizonts by now.

Starting selection for stable transformants or changing medium for transient expression assay, Day 3

- For stable transformation, this is the day to start drug pressure. Feed the culture with medium containing pyrimethamine (100 ng/mL). On Day 4, continue to feed the culture with medium containing pyrimethamine (100 ng/mL). Most parasites die after 2 days of drug treatment, as can be seen in a Giemsa-stained thin smear. The pyrimethamine pressure may thus be dropped to 40 ng/mL. Continue this pressure until transformants are detected in Giemsa-stained thin smear (usually 3 to 5 weeks). Add 25 µL of fresh RBCs into the culture each week during the selection procedure.
- For transient CAT and luciferase expression assays, continue to change the culture supernatant with 10 mL of fresh medium. Check the smear. New rings should emerge by Day 3.

C. Transfectant harvest and expression assays, Day 4

- The luciferase assay substrate is supplied as a powder and accompanied by a vial of solvent. Once the powder is dissolved, aliquot the substrate solution and store it at −20 °C. Before harvesting the cells, thaw and equilibrate the substrate to room temperature. Transfer 100 µL of the substrate into an assay tube and cover it with a piece of aluminium foil to avoid light.
- Spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it into a microfuge tube. Add 10 µL of 10% saponin/PBS stock to bring the final concentration of saponin to 0.1%; incubate the culture at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min and aspirate the supernatant and RBC ghosts.
- Wash the pellet 2 or 3 times with PBS to remove hemoglobin which interferes with the luciferase assay. Resuspend the pellet completely in 30 µL of 1× lysis buffer by pipetting up and down. Freeze/thaw the sample for one cycle, then vigorously pipette up and down again. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Take 20 µL of the supernatant for the luciferase assay.
• Because there is a slight delay of the photon peak after luciferase is added to the substrate, the timing of sample measurement is crucial if a quantitative comparison is desired. A practice optimized for Turner Luminometer measurements is to wait until the Luminometer is available (you are the only user at the time) and ready (the baseline has returned to zero). Set the timer for 10 s upon mixing the substrate and the lysate. Insert the tube into the chamber and start the measurement when the timer goes off. The set-up of the Turner Luminometer is usually done by a specialist from the vendor company. This measuring protocol should be revised if a different luminometer is used.

• An alternative positive control for the luciferase assay is *Escherichia coli* cells carrying the tester plasmid.

• For the CAT assay, spin down the parasite culture, resuspend the pellet in 1 mL of PBS and transfer it to a microfuge tube. Add 10 µL of 10% saponin/PBS stock to make the final concentration to 0.1% and incubate the pellet at room temperature for 5 min. Spin it at top speed in a microfuge for 3 min, then aspirate the supernatant and RBC ghosts. Wash the pellet once with 1 mL of PBS and once with 1 mL of TEN. Resuspend the cell pellet in 120 µL of 250 mM Tris–HCl (pH 7.6) with PMSF (1 mM); pipette up and down vigorously. Freeze/thaw the sample for 3 cycles. The lysis of parasites is hardly discernible by eye because a large portion of the pellet is hemozoin. Centrifuge the lysate in a microfuge for 3 min at top speed. Incubate the lysate for 10 min at 60 °C to inactivate cellular acetylases. Clear the lysate by centrifugation in a microfuge at 15,000 × g for 2 min.

• In a microfuge tube, mix 10 µL of lysate, 20 µL of acetyl-CoA (40 mM), and 1 µL of \([^{14}C]\)chloramphenicol (2.5 nCi). Incubate the mixture at 37 °C for 3 h, then add a second 20-µL aliquot of acetyl-CoA. Continue the incubation overnight.

• Handle all the samples, reagents, and waste according to radiation safety regulations from this point onward.

Continue the CAT assay, Day 5

• Momentarily spin down condensations of the reaction mix, then add 350 µL of ethyl acetate. Vortex to thoroughly mix and spin briefly to separate the organic and aqueous phases. Transfer 300 µL of the upper organic layer to a clean microfuge tube and dry the extract in a speed-vac centrifuge (30 to 60 min at 45 to 60 °C).

• Redissolve the sample with 25 µL of ethyl acetate. Mark the origin of a thin-layer chromatography (TLC) plate (Whatman) with a soft lead pencil 1.5 to 2 cm from the edge. Apply 10 µL of sample to the origin. Prepare a TLC chamber containing 200 mL of chloroform:methanol (20:1). Place the TLC plate in the chamber and allow the solvent front to move ~15 cm. Remove the plate from the chamber and air-dry the plate.

• Place the TLC plate in a film cassette and align the plate and the X-ray film with radioactive ink. A 3- to 5-day exposure is required due to the low level expression (or low transfection efficiency). Develop the film and align it with the plate. Alternatively, expose the TLC plate to a Phosphorimager screen for overnight and scan it with the Phosphorimager. Three radioactive spots should be observed. The two faster migrating spots represent the 1- and 3-acetylated chloramphenicol. The spot that has migrated the least distance from the origin represents the nonacetylated form.
• Liquid scintillation may be used to quantify the CAT activity. This involves cutting the radioactive spots from the plate for measurement of the amount of radioactivity they contain.

References


X. Alternative protocol using DNA-loaded red blood cells for transfection  

by Kirk Deitsch  
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**Equipment**  
Electroporator (Bio-Rad)

**Materials and reagents**  
Incomplete Cytomix (see Wu et al. 1995):  
- 8.95 g KCl  
- 0.017 g CaCl₂  
- 0.76 g EGTA  
- 1.02 g MgCl₂  
- 0.871 g K₂HPO₄  
- 0.68 g KH₂PO₄  
- 7.08 g HEPES  
Dissolve ingredients in water, adjust pH to 7.6 by adding NaOH, and adjust final volume to 1 liter. Filter-sterilize and use.

**Preparation**  
The procedure outlined below is a modification of the above protocol and takes advantage of the observation that malaria parasites actively take up DNA from the cytoplasm of DNA-loaded red blood cells.

- Using a stock of red blood cells stored at 50% hematocrit, transfer 350 µL of cells to a 10- or 15-mL centrifuge tube and spin them down. Remove the supernatant and wash the pellet with 5 mL of Incomplete Cytomix. Spin down the cells again and remove the supernatant.
- Resuspend the cells in Cytomix containing 50 to 100 µg of plasmid DNA. The final volume should be 400 µL. Transfer the cells to a 0.2-cm cuvette and place it on ice.
- Electroporate the cells using the conditions outlined above. We are using the following conditions: voltage: 0.31 kV; capacitance: 960 µF; time constants should be in the 10 to 14 ms range.
- Rinse the cells from the cuvette with 5 mL of culture medium. Two consecutive washes of 2.5 mL each works well. Transfer the cells to a centrifuge tube.
- Spin down DNA-loaded red blood cells and remove the supernatant. The supernatant should be quite red from cell lysis, however if nearly all of the red cells have lysed, check the pH of the Cytomix. It should be ~7.6.
- Transfer the cells to a culture flask and inoculate with parasites. Typically the red cells from two electroporations are used for a 5-mL culture. Parasites will take up and express DNA as they invade the DNA-loaded red blood cells.

**Advantages of using DNA-loaded red blood cells**  
- Flasks containing DNA-loaded red blood cells can be inoculated with 100 to 200 µL of an established culture. Such parasites tend to be healthier because they have not been electroporated and give higher levels of transient expression compared to the same starting numbers of parasites transfected directly by electroporation.
• High levels of expression can be obtained by first loading red cells as described above, then inoculating the cells with mature stage parasites purified by Percoll/sorbitol gradients as outlined in PARASITES, section IV:D. As the cells infected by the mature stages rupture, the resulting merozoites invade the DNA-loaded red blood cells resulting in very high numbers of synchronous, transfected parasites.

• If extremely high levels of transient expression are desired, a culture of parasites can be continuously maintained with DNA-loaded red blood cells. After four generations (8 days) of parasite multiplication in DNA-loaded cells, maximal levels of expression are observed. These levels can be up to 20-fold higher than that observed after direct electroporation of parasite-infected cells.

References


XI. Selectable episomal transfection of asexual stage \textit{Plasmodium falciparum} parasites

\textit{by Amar Bir Singh Sidhu and David A. Fidock}

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Equipment

Gene Pulser electroporator with Capacitance Extender (Bio-Rad)
electroporation cuvettes (0.2-cm gap, Bio-Rad)
centrifuge
incubator (37 °C)
microscope with 100× lens
modular incubator chambers (Billups–Rothenberg)

Materials and reagents for plasmid preparation

electrocompetent or chemically competent \textit{Escherichia coli} (We prefer XL10-Gold or DH5α for propagation of plasmids containing \textit{P. falciparum} DNA.)

TE buffer:
- 1 mM EDTA
- 10 mM Tris–HCl (pH 7.6)

incomplete Cytomix buffer:
- 120 mM KCl
- 0.15 mM CaCl$_2$
- 2 mM EGTA
- 5 mM MgCl$_2$
- 10 mM K$_2$HPO$_4$/KH$_2$PO$_4$
- 25 mM HEPES

Plasmid Maxiprep columns (Qiagen)
Amicon Centricon 100 columns (Millipore)

Materials and reagents for \textit{P. falciparum} propagation

\textit{P. falciparum} strains
gas mixture of 5% O$_2$, 5% CO$_2$, and 90% N$_2$
human red blood cells (RBC) washed free of leukocytes
RPMI 1640 with L-glutamine (Invitrogen, Catalog No. 31800)
hypoxanthine (Sigma)
HEPES (Sigma)
sodium bicarbonate (Invitrogen)
gentamycin (Invitrogen)
Albumax (I or II, Invitrogen)

complete medium:
- RPMI 1640 with L-glutamine
- 50 mg/liter hypoxanthine
- 10 mg/liter gentamycin
- 25 mM HEPES
- 0.225% NaHCO$_3$
- 0.5% Albumax

Parafilm
Methods in Malaria Research

sterile 6-well tissue culture plates
sterile glass pipettes and other tissue culture plastic ware
WR99210 (available upon request from David Jacobus, Jacobus Pharmaceuticals, Princeton, NJ, USA, e-mail: dpjacobus@aol.com)
blasticidin S HCl (Sigma)
pyrimethamine (Sigma)

This protocol describes fundamental steps for selecting episomally transfected asexual-stage *P. falciparum* parasite lines:

A. Preparation of parasite cultures and DNA constructs
B. Electroporation of circular plasmid DNA into parasites
C. Maintenance of transfected parasite lines

XI:A. **Preparation of parasite cultures and DNA constructs**

**Parasite cultures**
For each transfection, use $1 \times 10^7$ to $4 \times 10^7$ ring stage parasitized RBCs (PRBCs).
When preparing parasite cultures for transfection, decrease the hematocrit of the culture from the normal 4% to between 2.5 and 3.0%. Reducing the hematocrit and providing regular medium changes will help obtain higher percentages of ring stage parasites in culture. Because washed human RBCs deteriorate after 4 weeks at 4 °C, infuse the parasite cultures with fresh RBCs shortly before transfection. Feed the parasite cultures regularly to provide nutrients and to prevent buildup of toxic wastes.

The day before transfection, adjust the parasite cultures to between 1.8 and 2.5% parasitemia, rich in trophozoite stages, with addition of fresh RBCs. This parasitemia usually gives 4 to 7% ring stage cultures the next day. Feed the parasite cultures in the late evening again to get healthy ring stage parasites the next day. On the day of transfection, smear and feed the parasites early in the morning and allow them to grow for about 3 to 4 h prior to electroporation. Check that the parasite cultures provide healthy rings and RBCs for transfection and determine the parasitemia (best is about 4 to 6%).

**DNA constructs**
Prepare pure supercoiled plasmid DNA using Maxiprep columns. Dissolve the DNA in TE buffer at a concentration of 1 to 2 mg/mL. Before transfection, exchange the TE buffer with incomplete Cytomix buffer using Amicon Centricon 100 columns (see below). Store the plasmid DNA equilibrated with incomplete Cytomix buffer at 4 °C, so that whenever parasites are ready, transfection can be performed. Typically, use 50 to 100 µg of plasmid DNA per transfection.

- To exchange TE buffer with incomplete Cytomix buffer for transfection, load approximately 300 µg of plasmid DNA onto an Amicon 100 column.
- Make the volume up to 2.0 mL with filter-sterilized incomplete Cytomix buffer. Cover the top of the column with Parafilm and puncture the Parafilm several times with a sterile 21-23 gauge needle.
- Spin the loaded column in an Amicon collection tube at 3,000 rpm at 4 °C. It usually takes about 40 to 60 min to concentrate.
- After the spin, add 100 to 200 µL of incomplete Cytomix buffer to the column membrane and gently pipette up and down with the residual incomplete Cytomix
buffer in the column, avoiding rupture of the column membrane. Remove the
column from the collection tube, flip it upside down into a sterile Eppendorf tube
and spin at 3,000 rpm for 5 min to recover the plasmid DNA.

- Measure the volume of eluted plasmid DNA and determine the volume required
to transfect the parasites with 100 µg of plasmid DNA.

The design of the construct, the choice of selectable marker, and the selection of the
initial transformation plasmid all depend upon the transfection strategy. Plasmids are
most often designed to achieve gene disruption (“knockout”) or allelic replacement (e.g.,
to exchange polymorphic codons). Selectable markers established for stable episomal
transfection of *P. falciparum* include *Toxoplasma gondii* dihydrofolate reductase–
thymidylate synthase (dhfr-ts), human dihydrofolate reductase (dhfr, with or without the
gene encoding green fluorescent protein as a fusion product), blasticidin S deaminase
(from *Aspergillus terrus*), neomycin phosphotransferase II (transposon Tn5), and
puromycin-N-acetyltransferase (from *Streptomyces alboniger*). These markers confer
resistance to pyrimethamine, WR99210, blasticidin S HCl, geneticin/G418, and
puromycin respectively. When using *T. gondii* dhfr-ts, be careful to transfec
t pyrimethamine-sensitive parasites. The other selectable markers listed above can be
used against antimalarial drug-resistant and drug-sensitive parasite strains.

Transfection plasmids are available for both allelic exchange and transgene
expression strategies. One recent addition is the development of constructs expressing
thymidine kinase as a negative selectable marker. The use of this marker, in conjunction
with the positive marker human dhfr, reportedly enables the selection of parasites in
which the plasmids have integrated via double crossover events. This recent approach
will help in selecting for recombination into the desired locus (as compared to
recombination into genomic sequences that have homology to noncoding, regulatory
elements present elsewhere on the transfection plasmids). Further, this approach should
help in selecting for integration events that confer a deleterious growth phenotype
relative to episomally transfected or wild-type parasites.

While not covered by this protocol, techniques and plasmids also exist for transient
transfection assays in *P. falciparum*, for example to study regulatory elements. These
plasmids typically use luciferase or chloroamphenicol acetyl-transferase (CAT) for signal
detection.

X: B. Electroporation of circular plasmid DNA into parasites

Before starting the transfection experiment, label all electroporation cuvettes and tissue
culture plates for parasite culture. Set the Bio-Rad Gene pulser to 0.31 kV, 950 µF, and
infinity resistance. Prepare a stock of RBC at 4% hematocrit in complete medium. The
whole transfection procedure should be finished in about 30 to 60 min from the time the
parasites are harvested to the time the transfected parasites are put back into the
incubator.

We note that the procedure listed below relies on transfection of parasitized RBC. An
alternative approach recently published by Kirk Deitsch and colleagues involves
transfecting *P. falciparum* by first electroporating uninfected RBC with plasmid DNA,
then adding mature parasite stages and allowing them to invade the plasmid DNA-
preloaded RBC. This is an effective means of obtaining relatively high levels of
luciferase activity and may well represent an improvement for selectable episomal transfection. Nonetheless, the protocol listed below is reliable and works well.

- For each transfection, harvest 2.5 to 3.0 mL of predominantly ring stage parasite culture into a sterile centrifuge tube and spin down the culture at 2,200 rpm for 4 min at room temperature.
- Aspirate the supernatant using a sterile glass pipette.
- Gently resuspend the parasite culture pellet in 3 mL of sterile incomplete Cytomix buffer and spin the culture again at 2,200 rpm for 2 min at room temperature. Aspirate the supernatant as described above.
- After washing the parasite culture with incomplete Cytomix buffer, resuspend the parasite pellet in the required volume of incomplete Cytomix buffer containing plasmid DNA. Measure the volume of this parasite culture–plasmid DNA suspension and make the final volume up to 400 µL with incomplete Cytomix buffer.
- Transfer the suspension into a Bio-Rad Gene Pulser cuvette (0.2-cm), avoiding any air bubbles.
- Perform the electroporation (using the 0.31 kV, 950 µF conditions described above). Time constants between 7 and 11 milliseconds give good parasite transfection efficiency.
- Immediately after the electroporation, aseptically add 1 mL of fresh complete medium into the Gene Pulser cuvette. Add the medium gently down the side of the cuvette as the RBC are fragile at this point.
- When all the electroporations are completed, transfer the Gene Pulser cuvettes back to the tissue culture hood. Using a 1-mL pipette, gently remove the cuvette contents into 6-well tissue culture plates (one well per transfection). Pipette 1 mL of complete medium into the cuvette and gently remove most of culture, leaving behind the lysed and dried culture that accumulates around the top of the cuvette.
- Add 3.0 mL of the pre-prepared RBC/complete medium mixture (4% hematocrit) into the well containing the 2 mL of culture from the electroporated material. Mix gently with a 5-mL pipette.
- When all the electroporations are finished, place the 6-well tissue culture plate into a modular chamber, gas the chamber with 5% O2, 5% CO2, and 90% N2, and place it back into a 37 °C tissue culture incubator.
- Three to four hours after electroporation, remove the cultures from the wells and spin them at 2,000 rpm for 4 min at room temperature. Discard the supernatants.
- Resuspend each parasite pellet in 5 mL of fresh complete medium, put it back into the appropriate well of the tissue culture plate, gas, and return it to the incubator.
- The following day, feed each well with 5 mL of complete medium.

**XI:C. Maintenance of transfected parasite lines**

- On the second day post-transfection, smear the electroporated parasite cultures to determine their parasitemia. A parasitemia of 0.8 to 2.4% at this time point is usually indicative of relatively good transfection efficiency. For most plasmids, this typically means that epismally transfected parasite lines will be first observed in 2 to 3 weeks. From this day onward, feed parasites with medium
containing the selectable agent. To select for human dhfr, blasticidin S-deaminase, or T. gondii dhfr-ts, we recommend using complete medium supplemented with 2.5 nM WR99210, 2.5 µg/mL of blasticidin S HCl, or 20 ng/mL of pyrimethamine respectively.

- Feed the parasites daily on Days 3-5.
- On Day 6, smear the lines. There should be no live asexual stage parasites. Large numbers of gametocytes are a sign of excessive stress during the transfection and/or subsequent handling procedures. Reasons for this can include poor parasite health prior to transfection, too much time spent outside of the incubator, or poor RBC condition. Similarly, it is not good to see too many “blebby” RBC. (If needed, these RBC can be diluted out by replacing 20 to 30% of the culture with fresh uninfected RBC at this time.)
- Resuspend the Day 6 cultures in 5 mL of complete medium containing the drug and transfer them into a sterile centrifuge tube. Spin the tube at 1,800 rpm for 3 min at room temperature; this centrifugation helps to remove toxic lysis products and dead parasite material. Aspirate and discard the supernatant. Resuspend the pellet in 5 mL of medium containing both drug and an extra 0.1 mL of RBC (stock of 50% hematocrit).
- Skip Day 7.
- Feed again on Day 8.
- Skip Day 9.
- Feed again on Day 10. Repeat the smearing, centrifugation, and addition of fresh RBC procedures performed on Day 6. There should be minimal parasite debris left with few gametocytes and the RBC should be in good condition. Note: From Day 10 onwards, it is possible to reduce culture volumes from 5 mL down to 1 mL per line until parasites are observed, then the lines can be expanded back out to 5 mL again for freezing.
- Feed every second day (Days 12, 14 etc.) and smear every 4 days until parasites become microscopically detectable on Giemsa-stained slides. Typically, parasites are first visualized on Days 14 to 24. To prevent the parasite cultures from lysing, discard 30 to 40% of the cultures and replace them with fresh RBC every 6 or 7 days.
- Once parasites have been seen, begin feeding them daily. Freeze the transfected parasite lines as soon as the parasitemia reaches ≥ 2% with >50% rings. Note the growth rate of the episomal transfected cultures, which grow slower than the parental nontransfected lines.
- Expand and harvest the remaining cultures for genomic DNA extraction from trophozoite mature stage parasites to confirm transfection by plasmid rescue. Subsequently, parasite lines can be maintained in 1-mL volumes for long-term propagation.
- Make frozen parasite stocks every month. It is also convenient to prepare genomic DNA once a month to screen for integration by PCR.
- Stable integrants resulting from homologous recombination and single-site crossover of the plasmid into the genome can often be observed between 45 and 90 days post-transfection. These stable integrant parasites typically grow faster than episomally transfected lines. PCR with primers specific to the vector and the plasmid can be used in combination to efficiently screen for the presence in the bulk culture of parasites that underwent the desired recombination event. PCR positive data can be confirmed by Southern hybridization. Once the desired
integration has been detected, the corresponding parasite lines can be cloned by limiting dilution and detected using the Malstat lactate dehydrogenase reagent.

References


XII. Telomere repeat amplification protocol in *Plasmodium falciparum* (PfTRAP)  
by Nasséra Bakhsis¹, Emanuell Bottius¹, Luísa M. Figueiredo², and Artur Scherf¹  
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**Equipment**  
- homogenizer (Kontes; size 19)  
- slides and coverslips  
- optical microscope  
- centrifuges (15-mL tube and microfuge)  
- PCR machine  
- vertical electrophoresis system (10 × 8 cm)

**Materials and reagents**  
- parasite culture of 10% parasitemia containing ~10⁹ parasites  
  (mostly 16- to 30-h cultures)  
- Buffer B:  
  - 10 mM Tris–HCl (pH 7.5)  
  - 1 mM MgCl₂  
  - 1mM EGTA  
  - 5 mM 2-mercaptoethanol  
  - 10% glycerol  
  - 10 µg/mL leupeptin  
  - 10 µg/mL pepstatin  
- TRAP buffer:  
  - 200 mM Tris–HCl (pH 8.3)  
  - 15 mM MgCl₂  
  - 630 mM KCl  
  - 0.05% Tween 20  
  - 10 mM EGTA  
  - 500 µM dNTP  
  - 1 mg/mL BSA  
- Oligonucleotides:  
  - PfTS (5′ AATCCGTCAGCAGAGTTCA 3′)  
  - PfCX (5′ GGCGCGT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT G/A AACCCT 3′)  
  - a GC-clamp followed by 3 telomere repeats

**Preparation**  
- Wash the homogenizer carefully and rinse it with 0.4 M NaOH, 0.25 M HCl, 0.4 M NaOH, and distilled water successively. Put it on ice before use.  
- Use only RNase-free solutions and materials for the TRAP assay.  
- Wear gloves all the time and keep parasite extracts on ice.
Preparation of a semipurified protein extract (~3 h)

- Do a saponin lysis of a freshly recovered culture (described elsewhere in this book) containing ~10^9 parasites.
- Wash the pellet in 5 volumes of PBS to eliminate the majority of erythrocyte contaminants.
- Wash the pellet in Buffer B.
- Resuspend the parasite pellet in 200 µL of Buffer B.
- Transfer parasites to the homogenizer and lyse the parasitophorous vacuole and cytoplasmic membrane by applying 80 strokes on ice.
- Carefully, take ~2 µL of the lysate and put it between the slide and a coverslip.
- Using a phase contrast optical microscope, check that the parasitophorous vacuole and cytoplasmic membrane have been disrupted (resulting in debris in the suspension). If not, apply 80 more strokes. Repeat this procedure until more than 90% of the parasites have been lysed.
- Transfer the lysate to a prechilled 1.5-mL tube.
- Centrifuge the lysate for 1 hr at 17,600 × g at 4 °C.
- The supernatant consists of a semipurified cytoplasmic fraction at a concentration of 5 × 10^6 parasite equivalents per microliter. Aliquot it in 2-, 4-, and 12-µL amounts and store it at −80 °C.
- The pellet containing the nuclei can be resuspended in 200 µL of Buffer B, lysed by sonication, and centrifuged as described above.
- Both cytoplasmic and nuclear fractions contain telomerase activity. For practical reasons we usually use the cytoplasmic fraction.

TRAP assay (~6 h)

The TRAP assay consists of an elongation and an amplification step. In the elongation step, an oligonucleotide (PfTS) that mimics a chromosome extremity is used as a substrate for telomerase. The amplification step consists of a PCR in which the products from the elongation step are amplified and labelled with ^32P.

Elongation step:
- Use enough DNA in the reaction (equivalent to about 10^7 parasites) to get a positive reaction.
- In an RNase-free PCR tube, prepare the following mix:
  - 2 µL of protein extract
  - 5 µL of TRAP buffer
  - 100 ng of PfTS oligonucleotide
  - H₂O to 48.5 µL
- Incubate the mix at 37 °C for 1 h.

Amplification step:
- To each tube containing the elongation products add:
  - 100 ng of PfCX oligonucleotide
  - 2.5 µCi ^32P-dNTP
  - 2.5 U Ampli Taq Gold DNA polymerase (Applied Biosystems)
  - H₂O up to final volume of 50 µL
- Amplify as follows:
  - 1 cycle: 95 °C for 10 min
  - 35 cycles: 95 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min
• Run 2 controls for nontelomerase-mediated incorporation. Before starting the elongation step, (i) pretreat semipurified protein extract for 30 min at 37 °C with 10 µg of RNase A and (ii) denature it at 95 °C for 10 min.
• Resolve PCR products on a 15% nondenaturing 8 × 10-cm polyacrylamide gel, at 150V (~2 h), in 1x TBE.
• At the end of electrophoresis, disassemble apparatus, leaving the gel stuck to one of the glass plates. Cover it with Saran wrap and expose it (without drying) to Kodak film for a few hours.

Reference
XIII. Genotyping of *Plasmodium falciparum* parasites by PCR: *msp1*, *msp2*, and *glurp*

*by Georges Snounou*¹ and *Anna Färnert*²

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**Equipment**
- micropipettes and tips
- thermal cycler
- apparatus for agarose gel electrophoresis
- photographic equipment and UV transilluminator
- refrigerator (4 °C) and freezer (−20 °C) for reagent storage
- microcentrifuge

**Materials and reagents**
- *Taq* polymerase (store at −20 °C) with appropriate buffer (store at 4 °C)
- MgCl₂ stock solution (often provided by enzyme supplier)
- dNTP: a working solution with a concentration of 5 mM for each of dATP, dCTP, dGTP and dTTP (store at −20 °C)
- oligonucleotide primers (sequences are given below): a working solution with a concentration of 2.5 µM for each oligonucleotide primer (store at −20 °C)
- mineral oil
- loading buffer (5×):
  - 50 mM Tris (pH 8.0)
  - 75 mM EDTA (pH 8.0)
  - 0.5% SDS
  - 30% w/v sucrose
  - 10% Ficoll (w/v, average molecular weight 400 000)
  - 0.25% orange G dye (w/v approximately)
- 10× TBE buffer:
  - 1 M Tris
  - 1 M boric acid
  - 50 mM EDTA
  - A pH of approximately 8.3 should be obtained without any adjustment.

**Agaroses:**
- normal agarose
- high resolution agaroses (such as NuSieve or MetaPhor, Cambrex)
- ethidium bromide solution containing 10 mg per mL of water

CAUTION: This chemical is toxic and mutagenic. Wear protective clothing and use only in a chemical fume hood. Store in the dark at 4 °C.

pure water

Genotyping of *P. falciparum* infections permits characterization of distinct subpopulations present in an isolate. The genetic markers merozoite surface protein (*msp1*) (block2),
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msp2, and glutamate rich protein (glurp) have been chosen due to their extensive polymorphism in size and sequences. The different allelic types, i.e., families, identified for msp1 (K1, MAD 20, and RO33 types) and msp2 (FC27 and IC/3D7) are detected with specific primers in a second nested PCR.

The amplification strategy used for genotyping P. falciparum parasites is the nested PCR. In the first amplification reaction oligonucleotide primer pairs, which will hybridize to conserved sequences flanking the repeat polymorphic regions of the genes, are used. The product of this first reaction is then used as a DNA template for separate second amplification reactions in which the oligonucleotide primers used recognise sequences contained within the DNA fragment amplified in the first reaction.

Setting up the first amplification reaction

- Calculate the total volume of reaction mixture required: 20 µL per reaction x (total number of reactions + 1).
- Calculate the amount of:
  - buffer (final concentration 1×)
  - MgCl₂ (final concentration 1.5 mM)
  - dNTP (final concentration 125 µM)
  - oligonucleotides (final concentration 250 nM)
  - Taq polymerase (final concentration 2 units per 100 µL)
  - and water to make up the total volume

NB: If all three genetic markers (msp1, msp2, and glurp) are to be analysed, then a mixture of the corresponding 3 oligonucleotide primer pairs can be added to the reaction. Thus one first amplification reaction only is required for each sample.

- Add, in order, the correct volumes of: water, buffer, MgCl₂, oligonucleotide primers, dNTP, and the Taq polymerase. Mix by a short vortex pulse.
- Aliquot 20 µL of the reaction mixture per labelled tube.
- Add 40 µL of mineral oil overlay to each tube.
- Add 1 µL of DNA template to each tube (water for the negative controls).
- Place in the cycler and run the PCR reaction.

Setting up the second amplification reactions

Steps 1 – 5 as above.

- Remove 1 µL from the first amplification reaction tube from under the oil overlay and add it to the oil overlay of the second amplification reaction tube.
- Place the tube in the cycler and run the PCR reaction.

NB: Each second amplification reaction must be performed with a single oligonucleotide pair. Thus, per sample, 3 separate reactions will be required for the analysis of msp1, two for msp2, and one for glurp.

Cycling parameters

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<thead>
<tr>
<th>Step</th>
<th>Temperature/Time</th>
<th>Function</th>
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<tbody>
<tr>
<td>Step 1</td>
<td>95 °C for 5 min</td>
<td>Initial denaturation</td>
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<tr>
<td>Step 2</td>
<td>X °C for 2 min</td>
<td>Annealing</td>
</tr>
<tr>
<td>Step 3</td>
<td>72 °C for 2 min</td>
<td>Extension</td>
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<tr>
<td>Step 4</td>
<td>94 °C for 1 min</td>
<td>Denaturation</td>
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<tr>
<td>Step 5</td>
<td>Repeat Steps 2–4 a total of 25 cycles (Nest 1) or 30 cycles (Nest 2)</td>
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<tr>
<td>Step 6</td>
<td>X °C for 2 min</td>
<td>Final annealing</td>
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<tr>
<td>Step 7</td>
<td>72 °C for 5 min</td>
<td>Final extension</td>
</tr>
<tr>
<td>Step 8</td>
<td></td>
<td>The reaction is completed by reducing the temperature to 25 °C.</td>
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</tbody>
</table>
Methods in Malaria Research

\[ X = 58 \, ^\circ \text{C} \] for Nest 1 (M1-OF/M1-OR; M2-OF/M2-OR; G-OF/G-OR) and the Nest 2 reactions of *glurp* (G-NF/G-OR).

\[ X = 61 \, ^\circ \text{C} \] for Nest 2 reactions of *msp1* (M1-KF/M1-KR; M1-MF/M1-MR; M1-RF/M1-RR) and of *msp2* (M2-FCF/M2-FCR; M2-ICF/M2-ICR).

### Sensitivity and specificity
- The parameters provided above (cycling parameters and final concentrations) may have to be altered in order to obtain optimal sensitivity and specificity. The use of different temperature cyclers and enzymes has an influence on the efficiency of the PCR reaction. The nested PCR for the 3 genetic markers should be capable of detecting about 10 parasite genomes per microliter of blood.
- Optimization of the PCR conditions requires the preparation of a standard set of genomic DNA templates. These are prepared from a defined quantity of in vitro cultured cloned parasite lines. Cloned lines must be used since the haploid genome contains a single copy of each of the 3 genes. If needed the conditions are varied in such a way that a single band is obtained for each of the specific second amplification reactions for a range of parasite genomic DNA concentrations.
- Sensitivity is mainly dependent on the number of cycles, thus these can be increased if needed.
- Specificity is most affected by the annealing temperature and eventually the MgCl₂ concentration. A common problem is the generation of two PCR products for the specific second amplification reactions. This is due to carryover of oligonucleotide primers and PCR product from the first reaction, which can be minimized by reducing the oligonucleotide concentrations and/or the number of cycles in the first amplification reaction.

### Minimizing contamination
- The risks of contamination are enormously increased when nested PCR is performed. Thus the transfer of the product of the first amplification reaction to the second amplification reaction mixture should be performed with extreme care. In this context the oil that is used to overlay the reaction mixtures acts as a very efficient contamination barrier, and it is strongly suggested to retain the practice even when a heated lid is available.
- Ideally, setting up the first and second amplification reactions should be performed in a separate room from the one where the gels are migrated. Moreover, the transfer of the template from the first to the second amplification reactions must be performed with a dedicated pipette in yet another room, preferably with filter tips.

### Analysis of the PCR product
- Add 5 µL of the loading buffer to the PCR product.
- Load 12 µL of sample on a suitable agarose gel and migrate (1× TBE buffer).
- Stain the gel in TBE buffer containing ethidium bromide (final concentration 1 µg per mL) for 30 min.
- Destain in TBE or water for 5 min.
- Visualise on a UV transilluminator.
Normal agarose is suitable for the analysis of all 3 genetic markers. However, given the small size of the bands which will result from the amplification of \textit{msp1}, and the small variations in the sizes of the different allelic variants, the use of agarose type which give higher resolution is advised. The best results are obtained if the gel is kept cold before and during electrophoresis. The high cost of such agarose types (NuSieve or MetaPhor) can be compensated by the fact that the gels can be reused at least 10 times without significant loss of resolution. Used gels can be stored in TBE buffer, and when needed for further use, they are just reboiled. To compensate for condensation, add up water to the original gel volume when reboiling.

References


Oligonucleotide sequences
Merozoite Surface Protein 1: \textit{msp1}
First reaction: M1-OF 5'-CTAGAAGCTTCTAGAGATGCAGTATTG-3'
M1-OR 5'-CTTAATAGTATTCTAAATTCAAGTGGATCA-3'
Second reaction: K1-type M1-KF 5'-AAATGAAGAAAGAATTACTAAAAAGGTGC-3'
M1-KR 5'-GCTTGCATCAGCTGGAGGGCTTGCACCAGA-3'
MAD20-type M1-MF 5'-AAATGAAGAAAGAAGTGAACAGCTGTAC-3'
M1-MR 5'-ATCTGAAGGATTGACTGCTTGAATTACC-3'
RO33-type M1-RF 5'-TAAAGGATGAGAATATTCAAGTGGATCA-3'
M1-RR 5'-CATCTGAAGGATTGCGACCTGGAGATC-3'
Merozoite Surface Protein 2: msp2
First reaction:M2-OF 5’- ATGAAGGTAATTAAACATTGTCTATTATA -3’
   M2-OR 5’- CTTTGTACCATCGTACATTCTT -3’

Second reaction:
FC27-type M2-FCF 5’- AATACTAAGAGTGTAGGTGCARATGCTCCA -3’
   M2-FCR 5’- TTTATTTGATCGCATTGCAGAACTTGCA -3’
IC/3D7-type M2-ICF 5’- AGAAGTATGGCGAGAAAGTAAKCCCTYCTACT -3’
   M2-ICR 5’- GATTGTAATTCGGGGGATTCAGTTTGGTG -3’

Glutamate Rich Protein: glurp
First reaction:G-OF 5’- TGAATTTGAAAGATGTTCCACTGAAC -3’
   G-OR 5’- GTGGAATTTGCTTTTCTTCAACACTAA -3’

Second reaction:
   G-NF 5’- TGTCACACTGAACAATTAGATTTAGATCA -3’
   G-OR 5’- GTGGAATTTGCTTTTCTTCAACACTAA -3’
FIELD BLOOD SAMPLING

I. Finger prick blood sampling

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I:A. Blood collection on filter paper

Equipment
heating block (Techne)

Materials and reagents
heparinizised capillary tubes, 75-µL (Drummond Scientific)
chromatography filter paper, ET31CHR (Whatman).
EDTA tubes (Microtainer, Becton Dickinson)

Procedure
• Draw finger prick blood samples into 1 to 3 heparinized, 75-µL capillary tubes and transfer them to chromatography filter paper.
• Alternatively, allow 1 drop of blood to fall onto a filter paper directly from the finger. The finger must not touch the filter paper (see the photo).
• Soak the drop into the filter paper until the paper is evenly red without spreading to the underlying surface.
• Dry the filter-paper samples completely in air (see the photo) and place them in small plastic bags.
• We can keep the dried filter-paper samples at room temperature for at least several weeks in field conditions before extracting antibodies, human and parasite DNA, and postdose drugs.
• Store the filter-paper samples at –20 °C for long term.
I:B. Blood collection in EDTA tubes

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A better quality and quantity of DNA can be obtained by EDTA tubes than by filter paper.

Materials and reagents
- EDTA tubes (Microtainer, Becton Dickinson)

Procedure
- Draw blood samples taken from the finger tip into 2 to 5 heparinized capillary tubes as mentioned above and transfer into an EDTA tube.
- We can keep the EDTA-tube samples at room temperature for at least one week in field conditions before extracting DNA.
- Store the EDTA-tube samples at –80 °C for long term.
II. Serum extraction from filter-paper samples

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Equipment
- paper punch
- shaker (Vortex)

Materials and reagents
- 10-mL tubes
- PBS containing 0.05% Tween and 0.5% BSA
- Pasteur pipettes

Procedure
- Use a paper punch with a diameter of about 6 mm for punching out discs. Be careful to use areas in the middle of the spot where the blood is evenly spread.
- Put the discs in 10-mL tubes.
- Add 500 µL of PBS containing 0.05% Tween and 0.5% BSA.
- Incubate the discs for 2 h at room temperature on a shaker.
- Vortex them for a few seconds.
- Withdraw the liquid with a Pasteur pipette and aliquot it.
- Store at −20 °C until analysis.
- The extract corresponds to a serum dilution of ~1:100. The dilution factor is however dependent on the quality of the filter paper.
III. DNA extraction

III: A. DNA extraction from filter-paper samples

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Microscale isolation of Plasmodium falciparum or human DNA from filter-paper samples has been conventionally done by brief boiling (Wooden et al. 1992; Uchida et al. 1995), which is sufficient for obtaining short DNA.

Equipment

- centrifuge

Materials and reagents

- sterile water containing 15 µL of Chelex-100 (Bio-Rad)
- HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin (Merck)
- 1.5-mL tube
- QIAamp DNA Mini Kit (Qiagen)
- ethanol
- DNA elution buffer AE:
  - 0.5 mM EDTA
  - 10 mM Tris–HCL (pH 9.0)

Procedure

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 x 3-mm pieces.
- Heat the pieces in 205 µL of sterile water containing 15 µL of Chelex-100 at 100 °C for 8 min.
- After cooling them on ice for 2 min, collect the supernatant containing DNA (200 µL) by centrifugation and store them at −20 °C.

However, the DNA samples thus obtained show a limited PCR template activity with little amplification of DNA longer than 1 kb. Considering the extremely high A + T content of P. falciparum DNA, boiling may cause fragmentation of DNA and would no longer be a method of choice for DNA isolation when a long PCR product covering an entire gene is required. To solve this problem the following alternative method has been developed (Sakihama et al. 2001):

- Cut one quarter or half of the dried filter blot, equivalent to 19 or 38 µL of blood, into 3 x 3-mm pieces.
- Incubate the pieces in 1 mL of HEPES-buffered saline (HBS) containing 0.5% (w/w) saponin at room temperature for 1.5 h in a 1.5-mL tube.
- Wash them twice with 1 mL of HBS.
- Use a QIAamp DNA Mini Kit to isolate DNA remaining on the filter paper, according to the manufacturer’s instructions with the following modifications.
- To avoid shearing DNA, minimize vortexing and substitute by repeated inversions.
• Before the addition of ethanol to the extract, thoroughly remove the filter-paper debris by centrifugation at 9,000 × g for 5 min.
• Reduce the volume of DNA elution buffer AE to 50 µL.
• Prolong the preincubation before DNA elution to 5 min to maximize the elution recovery.
• Store the eluted DNA at 4 °C until PCR amplification.

References


III:B. DNA extraction from filter-paper discs previously used for serum extraction
by Manijeh Vafa, Masashi Hayano, and Marita Troye-Blomberg
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Equipment
- vortex
- PCR machine
- centrifuge

Materials and reagents
- Eppendorf tubes
- saponin
- PBS
- Chelex-100
- PCR tubes
- distilled water

Procedure
- After serum extraction as described in FIELD BLOOD SAMPLING, section II,
  transfer the discs to new Eppendorf tubes containing 1 mL of 0.5% saponin in
  PBS
- Incubate the tubes at 4 °C overnight.
- Discard the solution.
- Wash the discs with 1 mL of PBS at 4 °C for 15 to 30 min.
- Discard supernatant.
- Transfer the discs into 0.2-mL PCR tubes containing 50 µL of 20% Chelex-100 in
  water + 150 µL of distilled water.
- Vortex the tubes for 30 s.
- Incubate the mix at 99 °C for 15 min in the PCR machine.
- Vortex for 30 s.
- Centrifuge the tubes at 10,000 rpm for 2 to 3 min.
- Collect the supernatant (using pipette) in new microtubes.
- Use 5 µL for PCR, or store at –20 °C.
III:C. DNA extraction from EDTA-tube samples
by Akira Kaneko
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Japan and Malaria Research Laboratory, Department of Medicine, Karolinska Hospital, SE-
171 76 Stockholm, Sweden
e-mail: akirak@research.twmu.ac.jp

Equipment
- water bath
- Generation Capture Plate kit (Gentra Systems)

Materials and reagents
- whole blood frozen in EDTA tube
- EDTA tubes

Procedure
- Use 150 to 375 µL of frozen (−80 °C) whole blood collected in an EDTA tube.
- Thaw it quickly in a 37 °C water bath and keep it on ice until use.
- Use Generation Capture Plate kit according to the manufacturer’s instructions.
III:D. Fast methanol-based DNA extraction from blood spots in filter paper.

by Pedro Gill and Sandor Bereczky

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Equipment
- heating block (Techne)
- micropipette, 1000-µL

Materials and reagents
- blood spots on filter paper (Whatman 3MM)
- new surgical blades
- tips
- 1.5-mL Eppendorf tube
- methanol
- distilled, sterile water

Procedure
- Cut a blood spot (about 3 × 5 mm) from the filter paper. (Note: Not all filter papers work when making the blood spot collection. Make sure you are using the right one, usually Whatman 3MM.) Do not touch the blood with the fingers when manipulating the filter paper. Use new surgical blades, one for each sample, to avoid contamination. The cuts should be done on a disposable surface, the best choice being small yellow stickers (note pads).
- Use the blade also for transferring the cut-out to a 1.5-mL Eppendorf tube. The filter papers with the remaining blood spots should be protected (e.g., in separate plastic bags) until a new DNA extraction. After each blood sample, remove 2 or 3 note pad sheets (due to the fact that the blade usually cuts through at least two sheets of the note pad).
- Add 125 µL of methanol (room temperature) and wait 15 min. Make sure that the whole paper-cut is soaked in the methanol. Keep at room temperature until further processed (at least 15 min).
- Remove the methanol with a 1000-µL micropipette (using a new tip for every sample).
- Let the paper dry at room temperature for 15 min with the lid open. Close the lid and rotate the tube gently. If the paper moves freely it is dry, if it sticks even slightly, dry it for 5 to 10 more minutes.
- Add 65 to 75 µL of distilled, sterile water and smash the paper in the water with the micropipette. The water will become slightly red.
- Heat the tube at 95 to 100 °C in a heating block or a water bath for 15 min.
- Ready for PCR! Use 3 to 10 µL in a 50-µL total reaction volume. (Attention: This extraction is rather dirty. Too much template can give rise to inhibition of polymerase.)

Note: FIELD BLOOD SAMPLING, section III:A for DNA extraction is suitable for long, intact fragments over 700 bp, and is particularly efficient for fragments more than 1000 bp, while FIELD BLOOD SAMPLING, section III:D is particularly efficient for short fragments up to 500 bp and when the filter paper cut is small (2 × 2 mm).
References


IV. Postdose drug level monitoring

by Akira Kaneko
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Using the filter-paper samples, chromatographic analytical methods were developed to monitor capillary blood levels of the drug and its metabolites after administration by Bergqvist and colleagues. Currently methods for the following drugs are available:

References


Suppliers

5 Prime → 3 Prime
(see Eppendorf – 5 Prime)

ABgene
Web: www.abgene.com

Amersham Biosciences
(also referred to as Amersham, Pharmacia, and Pharmacia Biotech in this manual)
Web: www.amershambiosciences.com

Applied Biosystems
Web: www.appliedbiosystems.com

ATCC
Web: www.atcc.org

Axis-Shield PoC AS
Web: www.axis-shield-poc.com

Bachem
Web: www.bachem.com

Baxter Healthcare
(referred to as Baxter/Fenwal in this manual)
Web: www.baxter.com

BD Biosciences
Web: www.bdbiosciences.com

BD Immunocytometry Systems
Web: www.bdbiosciences.com

BD Labware
Web: www.bdbiosciences.com

BD Vacutainer Systems
Web: www.bd.com/vacutainer

BDH Laboratory Supplies
Web: www.bdh.com

Beckman Coulter
(referred to as Beckman in this manual)
Web: www.beckmancoulter.com

Becton Dickinson
Web: www.bdbiosciences.com

Billups-Rothenberg
Web: www.brincubator.com

Bio-Rad Laboratories
Web: www.bio-rad.com

BOC Specialty Gases
Web: www.boc.com

Brinkmann Instruments
Web: www.brinkmann.com

Calbiochem
Web: www.emdbiosciences.com

Cambrex
Web: www.cambrex.com

Clontech
Web: www.bdbiosciences.com/clontech/

Cole-Parmer
Web: www.coleparmer.com

Corning Life Sciences
Web: www.corning.com/lifesciences/

Dako Corp
Web: www.informagen.com

Drummond Scientific
Web: www.drummondsci.com

Eppendorf
Web: www.eppendorf.com

Eppendorf – 5 Prime
(referred to in this manual as 5 Prime → 3 Prime)
Web: www.5prime.com

Flow Incorporated
Web: www.malariatest.com

Fluka
Web: www.sigma-aldrich.com/Brands/Fluka_Riedel_Home.html

Fresenius France
Web: www.biam2.org
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