High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*

Chris J Janse, Jai Ramesar & Andrew P Waters

Leiden University Medical Center, P.O.Box 9600, 2300 RC Leiden, The Netherlands. Correspondence should be addressed to C.J.J. (c.j.janse@lumc.nl).

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This protocol describes a method of genetic transformation for the rodent malaria parasite *Plasmodium berghei* with a high transfection efficiency of 10^{-3}–10^{-4}. It provides methods for: (i) *in vitro* cultivation and purification of the schizont stage; (ii) transfection of DNA constructs containing drug-selectable markers into schizonts using the nonviral Nucleofector technology; and (iii) injection of transfected parasites into mice and subsequent selection of mutants by drug treatment *in vivo*. Drug selection is described for two (antimalarial) drugs, pyrimethamine and WR92210. The drug-selectable markers currently in use are the pyrimethamine-resistant dihydrofolate reductase (*dhfr*) gene of *Plasmodium* or *Toxoplasma gondii* and the *DHFR* gene of humans that confer resistance to pyrimethamine and WR92210, respectively. This protocol enables the generation of transformed parasites within 10–15 d. Genetic modification of *P. berghei* is widely used to investigate gene function in *Plasmodium*, and this protocol for high-efficiency transformation will enable the application of large-scale functional genomics approaches.

INTRODUCTION

The generation and characterization of genetically modified *Plasmodium* parasites in the last 10 years has greatly improved our understanding of the biology of malarial parasites. The use of transfection in the study of the biology of malarial parasites has been limited in the past, however, because of poor transfection efficiencies (frequency of 10^{-6}–10^{-9}) and the paucity of selection markers[1–3]. Here we present a standard protocol for genetic transformation of the rodent malarial parasite *Plasmodium berghei* using the nonviral Nucleofector technology for transfection[4]. The improvement using this method over standard methodologies results in transfection efficiencies in the range of 10^{-3}–10^{-4} (targeted integration of DNA vectors into the genome)[4]. High transfection efficiency confers the benefits of reduced time, a reduced number of laboratory animals and a reduced amount of materials required to generate transformed parasites compared to previously published technologies for genetic transformation of *P. berghei*[2,3,5–8]. Moreover, it facilitates the application of new experimental strategies for reverse genetics, such as direct selection by flow sorting of stable transformed parasites expressing fluorescent proteins[4]. This technology has already been used for the development of methods for larger-scale manipulation of the genome of *P. berghei*[9,10].

The Nucleofector technology is based on electroporation and is believed to achieve a more efficient transfer of DNA into the nucleus through a combination of a range of undisclosed electrical parameters and proprietary transfection solutions. It has proved to be an efficient DNA delivery system for many cell types that are difficult to transfect[11–13]. It has also been successfully used to transfect the rodent malaria parasites *P. yoelii*[14] and *P. chabaudi* (personal communication, J. Thompson). Transfection of the human malarial parasite *P. falciparum* with the Nucleofector technology has not yet been reported.

This protocol describes the methods for the collection of blood-stage schizonts of *P. berghei*, transfection of these schizonts and subsequent selection of genetically transformed parasites. Mature schizonts containing fully developed merozoites are the most suitable target cells for transfection of *P. berghei*. Introduction of DNA into schizonts has proved to be far more efficient than transfection of the other blood stages, such as ring forms and trophozoites. Compared to other species of *Plasmodium*, *P. berghei* schizonts can be collected far more easily. This is because erythrocytes containing mature *P. berghei* schizonts are the end product of *in vitro* maturation of blood-stage parasites, do not rupture spontaneously and can survive in culture for prolonged periods. For each transfection experiment, $0.5–1 \times 10^7$ schizonts are needed. Laboratory mice are infected with *P. berghei* to serve as a source of blood-stage parasites for the *in vitro* growth and purification of the schizonts. The protocol describes the collection of $0.5–1 \times 10^8$ schizonts obtained from infected blood of two mice, which is sufficient for up to ten independent transfections. The success of transfection is less dependent on the absolute number of schizonts transfected than it is on the 'viability' of these schizonts, and therefore the protocol emphasizes the viability more than the absolute numbers of parasites to be transfected. Transfections can be performed within a range of 10^6–10^8 schizonts per transfection.

The procedures for the collection of the schizonts are usually performed in a standard laboratory, separated from the animal facilities housing the mice to be infected with the transfected schizonts. Cultured and purified schizonts are relatively stable for a few hours at room temperature (19–23 °C); however, after transfection, the schizonts and merozoites rapidly degenerate. It is therefore recommended to perform the actual transfection procedure as close as possible to the animal facility where infection of the mice will take place. Moreover, it is recommended that transfected schizonts are immediately injected into a mouse before proceeding with the next transfection.

The *in vivo* selection procedure is based on the treatment of rodents with drugs, which selects for parasites expressing

drug-resistant selectable markers. So far, only three selectable markers exist for the transformation of *P. berghei*, all of which confer resistance to pyrimethamine via the *dhfr* gene from either *Plasmodium* (P-*dhfr*) or *T. gondii* (Tg-*dhfr*) or the human *DHFR* gene[2,3]. The human *DHFR* gene confers resistance not only to the drug pyrimethamine but also to the antimalarial drug WR99210. Introduction of all three genes into pyrimethamine-sensitive *P. berghei* parasites gives rise to a large increase (approximately 1,000 times larger) in pyrimethamine resistance. Pyrimethamine selection *in vivo* is preferred over WR99210 selection, as the former can be provided in the drinking water. The WR99210-*DHFR* selection system can be used in conjunction with the pyrimethamine–Tg-*dhfr* selection system, allowing for two sequential manipulations of the genome[15]. This can be used, for example, to knock out two genes in the same parasite line or for the complementation of knockout parasites. As *DHFR* is also resistant to pyrimethamine, the WR99210-*DHFR* selection system can only be used as the second selectable marker system when both selectable markers are required.

Drug treatment of the animals always starts 1 d after transfected parasites have been injected to allow the parasites to complete one full developmental cycle in the absence of drug pressure. One day after injection of the transfected parasites, parasitemia will usually rise to levels between 0.05 and 3%. After the first 2 d of drug treatment, a rapid drop in parasitemia occurs (to undetectable levels), indicating that most of the parasites are not transformed. In successful experiments, the parasitemia increases again to levels of 0.1–5% between days 5 and 7 after transfection. At a parasitemia between 2 and 5%, blood is collected from the mouse for storage of blood-stage parasites in liquid nitrogen (cryopreservation) and also for the first genotype analysis of the genetically transformed parasites. The genotype analysis consists of PCR and Southern analysis of digested genomic DNA or separated chromosomes to establish the correct integration of linear DNA constructs and the presence of circular plasmids or the contamination with wild-type parasites. If the parasites are transformed with constructs expressing fluorescent markers such as green fluorescent protein (GFP), expression of these markers in the blood stages is monitored in live parasites using a standard fluorescence microscope.

This protocol describes neither the basic technologies for construction of DNA vectors necessary for genetic modification nor protocols for genotype analysis of genetically transformed parasites, as these have been well described in previous reviews[7,8]. At the end of the protocol, a number of examples are shown of genotype analyses which are routinely conducted on genetically transformed parasites.

MATERIALS

Laboratory animals In our laboratory, we use Swiss mice (OF1 ico, Construct 242; age, 6 weeks (25–26 g); Charles River). However, also other mouse strains such as C57BL/6 and BALB/c can be used.

▲ **CRITICAL** Diets of laboratory rodents with low contents of total protein, energy and *p*-aminobenzoic acid (PABA) can negatively influence *P. berghei* infections[16]. In our laboratory, we therefore provide diets with a high protein content (20–25% of total and gross energy content (18,000–20,000 kJ/kg).

! **CAUTION** All experiments using mice must be performed according to the applicable national guidelines and regulations.

Parasites In our laboratory, we use the reference line 'cl15cy1' of the ANKA strain of *P. berghei*[4]. However, parasite lines of other commonly used strains of *P. berghei*, such as NK65, can also be used.

REAGENTS

- Fetal bovine serum, heat inactivated (FBS; Invitrogen; cat. no. 10108-165); store at –20 °C.
- Giemsa solution (Merck, cat. no. 1666 789); working solution, 10% Giemsa solution in Sörensen staining buffer
- Neomycin sulfate, USP grade (Invitrogen, cat. no. 21810-031); store at –20 °C (see REAGENT SETUP)
- Phosphate-buffered saline (PBS; Roche, cat. no. 1.09204.0500)
- Heparin, Grade I-A, cell culture tested, 140 mUSP units/mg (Sigma, cat. no. H3149)
- Culture medium RPMI1640 (Invitrogen; cat. no. 13018-015; see REAGENT SETUP)
- Nycodenz density-gradient solution (Lucron Bioproducts, cat. no. 1002424; see REAGENT SETUP)
- Nucleofector solution 88A6 (Amaxa, GmbH; see REAGENT SETUP)
- Pyrimethamine solution (Sigma, cat. no. P-7771; see REAGENT SETUP)
- WR99210 solution (Jacobus Pharmaceutical Company; see REAGENT SETUP)
- Hoechst (Bisbenzimide H) 33258 (Sigma, cat. no. B1155; see REAGENT SETUP)

EQUIPMENT

- Vortex shaker (Ika Labortechnik)
- Biohazard Class II safety cabinet

! **CAUTION** Most manipulations with blood infected with genetically modified *P. berghei* parasites are performed in a Class II safety cabinet.

- Nucleofector device (Amaxa GmbH) (http://www.amaxa.com)
- Infrared heat lamp (home-made or from Science Products)
- Insulin syringes, MicroFine +, 0.5 ml; 0.30 mm (30G) × 8 mm (Becton Dickinson, cat. no. 324870)
- Cell-culture flask (75 cm^2 growth area and canted neck (Corning Life Sciences, cat. no. 430720))
- Table-top centrifuge (Beckman Coulter Allegra); most table-top centrifuges with a swing-out rotor are suitable
- Eppendorf microcentrifuge (12,000 r.p.m. or 16,000*g*); most microcentrifuges are suitable
- Incubator or water bath (37 °C)

▲ **CRITICAL** As cultivation occurs in closed culture flasks that are flushed once with a gas mixture, no specific requirements are needed for regulation of the gas mixture in the incubators. Also, shaking water baths (37 °C) are suitable for the cultivation of schizonts.

- Fluovac isofluorane-halothane scavenger (Stoelting Co) http://www.stoeltingco.com/physio/store/ViewLevel3.asp?keyword3=509

▲ **CRITICAL** Mice are anesthetized in the 'induction chamber,' which is prefilled with the anesthetic vapor (a mixture of isofluorane and oxygen) via the vaporizer unit. Injection of parasites or drugs is performed in mice that are kept under anesthesia by holding their muzzles to the small mask that is connected to the vaporizer unit.

- Light microscope, Carl Zeiss Standard 25 (Zeiss); all light microscopes with an oil-immersed ×100 objective are suitable
- Fluorescence microscope, Leica DMRA HC 'upright' microscope (Leica); most fluorescence microscopes, equipped with the right filter combinations for excitation of GFP (BP450-490) and Hoechst 33258 (UV, BP340-380) and suppression filters (GFP, LP515; Hoechst 33258, LP425) to detect the fluorescence of the dyes are suitable

REAGENT SETUP

Glycerol stock solution 30% (vol/vol) glycerol in PBS. Store at 4 °C.

Neomycin sulfate stock solution Prepare a stock solution of 10 mg/ml neomycin sulfate in distilled water.

PBS stock solution 0.01 M KH_2PO_4, 0.1 M Na_2HPO_4, 1.37 M NaCl, 0.027 M KCl; pH 7.0. For a working solution, dilute the stock solution with nine volumes of distilled water. Adjust the pH to 7.2 with 1 M HCl and sterilize by autoclaving for 20 min at 120 °C.

Heparin stock solution Dissolve the heparin powder in distilled water to a concentration of 25,000 units/ml. Filter sterilize (0.2 μm) and store at 4 °C.

For a working solution, add 0.2 ml of the stock solution to 25 ml RPMI1640 culture medium without FBS to create a final solution of 200 units/ml. Store at 4 °C.
Sörensen staining buffer For a working solution, dissolve 2.541 g KH_2PO_4 and 8.55 g $Na_2HPO_4.2H_2O$ in 5 L distilled water, pH 7.2. Store at room temperature (19–23 °C).
RPMI1640 culture medium Add the RPMI1640 powder (with L-glutamine and 25 mM HEPES, without $NaHCO_3$) to 1 L distilled water. In addition, add 0.85 g $NaHCO_3$ and 5 ml neomycin-sulfate stock solution. Filter sterilize (0.2 µm) and store at –20 °C in 100-ml aliquots.
Complete RPMI1640 culture medium Culture medium RPMI1640 supplemented with FBS to a final concentration of about 20% (vol/vol).
▲ **CRITICAL** For optimal *in vitro* growth of the parasites a relatively high percentage (~20%) of FBS is needed.
Nycodenz density-gradient stock solution Dissolve 138 g Nycodenz powder in 500 ml Buffered Medium (density of 1.15 g/ml at 20 °C) and sterilize by autoclaving for 20 min at 120 °C. Store at 4 °C.
Buffered medium for preparation of the Nycodenz density-gradient solution 5 mM Tris/HCl, 3 mM KCl, 0.3 mM $CaNa_2EDTA$; pH 7.5. Store at –4 °C.
Erythrocyte lysis buffer stock solution 1.5 M NH_4Cl, 0.1 M $KHCO_3$, 0.01 M Na_2EDTA; pH 7.4. Store at room temperature (19–23 °C). For a working solution, dilute the stock solution with nine volumes of distilled water. Store at 4 °C.
Nucleofector solution Store at 4 °C. The kit includes 2.5 ml of the 88A6 solution with supplement solution (for 25 transfections), 25 cuvettes and 25 plastic pipettes. http://www.amaxa.com
Pyrimethamine solution Dissolve pyrimethamine powder (5-4-Chlorophenyl-6-ethyl 2,4-pyrimidinediamine) in DMSO to a final concentration of 7 mg/ml (stirring on a vortex) and dilute 100 times with normal tap water with an adjusted pH of 3.5–5.0 (with 1 M HCl). Store at 4 °C and use the solution for the drinking water of mice for a maximum of 7 d.
WR99210 solution Add 16 mg WR99210 powder to 2 ml ethanol (98%) and 0.15 ml benzyl alcohol. Mix by stirring on a vortex to dissolve WR99210. Add up to 5 ml with 2.85 ml distilled water. Store at room temperature (19–23 °C) and use the solution for a maximum of 7 d.
Hoechst 33258 stock solution Dissolve Hoechst 33258 in distilled water to a final concentration of 500 µM. Store at –20 °C.
! **CAUTION** Weigh the Hoechst 33258 powder in a biological safety cabinet using gloves and a mask, as it is moderately cytotoxic.
Gas mixture 5% CO_2, 5% O_2, 90% N_2 (in a gas bottle or cylinder).
TE buffer 10 mM Tris, pH 8.0; 1 mM Na_2EDTA. Store at room temperature (19–23 °C).
DNA vectors DNA constructs (5–10 µg) in 5 µl TE buffer or distilled water used for transfection (stored at –20 °C). These constructs contain one of the following selectable markers: P-*dhfr*, Tg-*dhfr*, *DHFR*. The details of the construction of these vectors are not described in this protocol. Many of the standard plasmids for transfection of *P. berghei* are available from Malaria Research and Reference Reagent Resource Center (TRIS-EDTA). (http://ww.malaria.mr4.org/

PROCEDURE

Collection of *P. berghei* schizonts for transfection (Days 1–5)

1| On Day 0, collect one to four droplets (4–16 µl) of tail blood from a *P. berghei*–infected mouse in 0.4 ml PBS. The parasitemia (i.e., the percentage of infected erythrocytes) in this mouse must be in the range of 5–15%.
▲ **CRITICAL STEP** The start of the procedure at day 0 requires the availability of a mouse infected with wild-type *P. berghei* parasites with a parasitemia of 5–15%, obtained by mechanical passage or started from cryopreserved parasite stocks. The parasitemia is determined as described in **Box 1.**

2| Immediately inject the suspension intraperitoneally into two mice, 0.2 ml per mouse.
■ **PAUSE POINT** After infection of the two mice, it will take several days before the parasitemia reaches the required level for the transfer to *in vitro* culture for the cultivation of schizonts. Usually blood is collected from the infected mice at day 4 after infection when the parasitemia is between 1 and 3%.

3| Between 10:00 and 14:00 on day 4, make a thin blood smear from one droplet of tail blood from the two mice infected on day 0, as described in **Box 1**. If the parasitemia ranges between 1% and 3% proceed with Step 4.
▲ **CRITICAL STEP** If the parasitemia is lower than 1%, it is best to wait 1 d for the collection of infected blood and continue with Step 4 the next day if the parasitemia has increased to 1–3%. A parasitemia higher than 3% is suboptimal because many erythrocytes will become multiply infected or parasites will reside in the 'older' erythrocytes (normocytes) and not in reticulocytes. In both cases, the development of schizonts in culture is (greatly) impaired.
? TROUBLESHOOTING

4| Prepare 125 ml complete RPMI1640 culture medium by adding 25 ml freshly thawed FBS to 100 ml RPMI1640 culture medium.

5| Collect a total of 1.5–2 ml infected blood from the two infected mice by cardiac puncture under anesthesia between 13:00 and 15:00. Immediately add the blood to a 50-ml tube containing 5 ml complete culture medium supplemented with 0.3 ml heparin stock solution.

BOX 1 MAKING OF THIN BLOOD SMEARS

1. Make a thin blood smear on a microscope slide.
2. Fix the smears for 2 s with methanol and stain with a fresh Giemsa working solution for 10 min.
3. Wash the slide with tap water.
4. Air-dry the slide and determine the parasitemia using a light microscope (with immersion oil and objective at ×100) by counting 20–30 fields of 300–400 erythrocytes per field.

▲ **CRITICAL STEP** It is important that the infected mice are kept on a normal day-night light cycle. *P. berghei* has a 22–24-h asexual blood-stage cycle which is partly 'synchronized' in mice with the normal day-night light regime. In these mice, the rupture of schizonts and invasion of erythrocytes mainly occur between 02:00 and 04:00 every 24 h. As a result, most parasites are at the ring or young trophozoite stage when the infected blood is collected between 13:00 and 15:00.

6| Harvest the (infected) erythrocytes by centrifugation for 8 min at a speed that will generate 450*g* and discard the supernatant.

7| Resuspend the (infected) erythrocytes in 50 ml complete culture medium.
▲ **CRITICAL STEP** Leucocytes or platelets are not removed from the infected blood. There is no indication that these cells affect *in vitro* growth of schizonts or transfection rates.

8| Distribute the 50 ml culture suspension equally among two 250-ml culture flasks and add 25 ml complete culture medium to each flask.

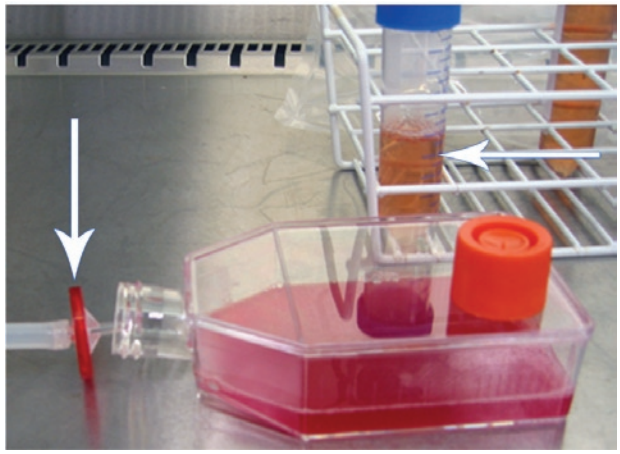

Figure 1 | Flushing of 250-ml cell-culture flasks containing *P. berghei*–infected blood in complete RPMI1640 medium with a gas mixture of 5% CO_2, 5% O_2, 90% N_2 using a 0.2-µm filter unit connected to the gas hose (arrow). The yellow-orange color of the culture medium in the 50-ml tube (arrow) is an indication of the correct pH. A more pinkish color indicates a pH that is too high and detrimental for the growth of the schizonts.

9| Flush the flasks for 90 s at 1.5–2 bar pressure with the gas mixture (**Fig. 1**). Close the flasks tightly immediately after gassing.

10| Put the flasks on a shaker and incubate at 36.5 °C (water bath or incubator). Shake at a speed just enough to keep the cells in suspension.

11| Leave the parasites in the cultures (shaken) at 36.5 °C until the next day.
▲ **CRITICAL STEP** In the overnight cultures, the ring forms and (young) trophozoites develop into schizonts that reach maturity in the morning of the next day. The mature schizonts do not rupture spontaneously and remain viable for several hours, allowing for the isolation of synchronized mature schizonts that contain fully developed merozoites. For optimal development of the schizonts, the correct gas conditions (lowered oxygen concentration compared to air), the pH of the culture medium (7.2–7.4) and the temperature are crucial. The temperature is crucial because it influences the rate of development of the schizonts. Above 38.5 °C, parasites will degenerate. Lower than 37 °C, the parasites will develop into healthy parasites but the developmental time of one complete cycle will be longer than the standard 22–24 h. A temperature of between 36 °C and 37 °C is optimal to collect viable, mature schizonts between 09:00 and 11:00 on day 5.

Day 5
12| On day 5 at 09:00, collect a small sample (0.5 ml) from the overnight culture in an Eppendorf tube to determine the 'quality' of the schizonts (see CRITICAL STEP below).

13| Harvest the cells of the sample for 5 s at 16,000*g* and discard the supernatant.

14| Make a thin blood smear, as described in **Box 1**, of the cells harvested and examine the morphology of the parasites (schizonts) using a light microscope (with immersion oil and objective at x100). If 70–80% of the parasites are morphologically 'viable', mature schizonts (see CRITICAL STEP below and **Fig. 2**), proceed with the purification of the schizonts (Step 15). If there are still many young, developing schizonts (i.e., parasites in the process of nuclear division), wait for 1–2 h before proceeding with Step 15.
▲ **CRITICAL STEP** Viable schizonts are distinguished by the presence of 12–16 merozoites within one erythrocyte and one cluster of pigment (hemozoin; **Fig. 2a**). Smearing the cells on a microscope slide often damages the red-cell membrane, and the merozoites are visible as more or less clustered but free parasites. A purple (red) defined compact nucleus and a dot of blue cytoplasm are characteristic for viable merozoites. About 15–25% of the parasites in these smears are singly nucleated (young) gametocytes (**Fig. 2b**). Degenerate schizonts often show a compact morphology in which the separate

merozoites are difficult to recognize. Take care not to mistake developing schizonts (which are still in the process of nuclear division prior to budding off of the merozoites; **Fig. 2c**) for degenerated schizonts.
? TROUBLESHOOTING

15| Prepare a 50% Nycodenz-PBS solution (vol/vol) in a 50-ml tube by adding 25 ml Nycodenz stock solution to 25 ml PBS.
▲ CRITICAL STEP Before electroporation, separate the schizonts (1–3% of the total cell population) from the uninfected erythrocytes by density-gradient centrifugation. Usually, this purification procedure starts between 09:00 and 10:00. Starting at a later time point results in a higher percentage of degenerated schizonts. For the density gradients, Nycodenz is used instead of Percoll. Percoll is often used to separate parasite stages, but the experience from our laboratory is that in contrast to Percoll, Nycodenz does not affect the viability of schizonts (see also **Supplementary Video 1** online for Steps 16–21).

16| Distribute the 100-ml culture suspension containing the schizonts equally among three 50-ml tubes (30–35 ml per tube).

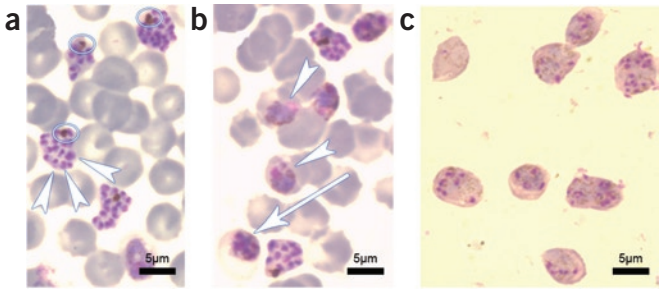

Figure 2 | Images of cultured *P. berghei* schizonts in Giemsa-stained thin blood smears (light microscope, ×100 objective). (**a**) Fully mature schizonts. Viable schizonts are recognized by the presence of 12–16 'free' merozoites (arrowheads) within the red blood cell and one dot of clustered malaria pigment (hemozoin; circles). Often these schizonts burst during preparation of the slide, resulting in merozoites found as more or less clustered free parasites. (**b**) Most cultures contain not only mature schizonts but also immature schizonts (white arrow) that are still in the process of merozoite formation and single-nucleated gametocytes (arrowheads). (**c**) Immature schizonts during the process of nuclear division that have been purified on a Nycodenz density gradient. If the majority of the schizonts are still at this stage, it is best to wait 2–3 h before starting the procedure of collecting schizonts. These young forms are sometimes difficult to distinguish from degenerated schizonts that are present in culture if the culture conditions, such as pH or gas conditions, were not correct.

17| Using a 10-ml pipette, gently underlay 10 ml of the Nycodenz-PBS solution to each tube under the culture suspension in such a way that a well-defined division is visible between the two suspensions (**Fig. 3a**).

18| Centrifuge for 20 min at 450*g* in a swing-out rotor at room temperature (19–23 °C) without brake. The centrifugation takes 20 min. During this period, prepare the DNA solution, which will be introduced into the schizonts by electroporation (Steps 28–30; i.e., add 100 μl Nucleofector 88A6 solution to 5–10 μg DNA dissolved in 5–10 μl water or TE buffer). In addition, make all necessary preparations for the anesthesia and injection of rodents (Steps 29–33).

19| Using a Pasteur pipette, carefully collect in a 50-ml tube the brown-grayish layer of schizonts at the interface between the two suspensions (**Fig. 3b**, **Fig. 4** and **Supplementary Video 1**). Usually a total volume of about 20–25 ml is collected from the three tubes.
? TROUBLESHOOTING
▲ CRITICAL STEP The schizonts (and leukocytes, gametocytes and old trophozoites, if present) will collect at the interface of the two suspensions (**Fig. 4**), whereas the uninfected cells will pellet at the bottom of the tube. Erythrocytes containing mature schizonts are fragile, and all manipulations to collect these cells must be performed with care.

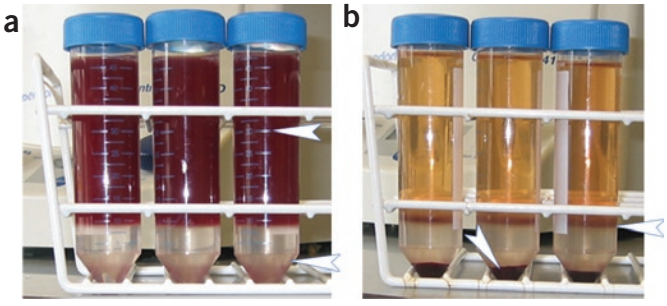

Figure 3 | One-step Nycodenz density-gradient purification of schizont-infected erythrocytes from uninfected erythrocytes. (**a**) The Nycodenz gradient solution (10 ml) is layered under the culture suspension (35 ml) containing the schizont-infected erythrocytes in a 50-ml tube. (**b**) The density gradients after centrifugation. The schizont-infected erythrocytes collect at the interface between the Nycodenz solution and the culture suspension, whereas the uninfected erythrocytes collect at the bottom of the tubes.

20| Add complete culture medium to the collected cell suspension up to a volume of 40 ml. The addition of culture medium 'dilutes' the Nycodenz solution, which will have been collected together with the schizonts (one may use the 'used' culture medium, which is present on top of the Nycodenz solution from the three tubes from which the schizonts have been collected).

21| Harvest the schizonts by centrifugation for 8 min at a speed that will generate 450*g*.

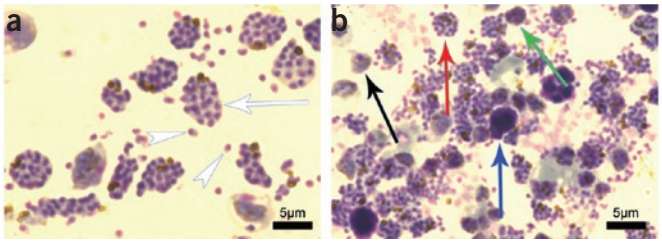

Figure 4 | Images of Nycodenz-purified *P. berghei* schizonts in Giemsa-stained thin blood smears (light microscope, x100 oil-immersed objective). (**a**) Fully mature schizonts containing 12–16 merozoites (white arrow) and free merozoites (arrowheads) from schizonts that ruptured during purification and/or during preparation of the blood smear on the slide. (**b**) An overview of Nycodenz-purified cells from a *P. berghei* schizont culture, showing the presence of mature schizonts (red arrow) and white blood cells (blue arrow), gametocytes (black arrow) and immature schizonts (green arrow).

22| Discard the supernatant.

23| Carefully resuspend the schizonts in 1 ml of complete culture medium (with care because the schizonts are fragile).

24| Add 9 ml complete culture medium to the schizont suspension (this medium may be obtained from the three tubes from which the schizonts have been collected).

25| Distribute the 10-ml schizont suspension among ten Eppendorf tubes (1 ml per tube). The parasites from one Eppendorf tube are used for one transfection.

Transfection of purified schizonts and injection into mice (day 5)

▲ **CRITICAL STEP** Five to ten minutes before electroporation of the parasites, place the mice under an infrared heat lamp. The tail veins swell at the higher temperature, simplifying the intravenous injection procedure

26| Fill the 'induction chamber' of the anesthesia apparatus with the anesthetic vapor (isofluorane-oxygen) and harvest the schizonts in the Eppendorf tube by centrifugation for 5 s at 16,000*g* in a microcentrifuge. See also video 2 for steps 26-33.

27| Discard as much as possible of the supernatant.

28| Add 100 μl of the Nucleofector solution 88A6 which already contains 5–10 μl of the DNA solution (5–10 μg DNA construct in water or TE buffer) and resuspend the schizonts.

29| Transfer the mixture to an electroporation cuvette and put a mouse in the 'induction chamber' for anesthesia.

30| Place the cuvette in the Amaxa Nucleofector device and transfect using program U33.

31| Remove the cuvette from the Nucleofector device and immediately add 50 μl complete culture medium.

32| Transfer the solution (150 μl) from the cuvette to an Eppendorf tube using a plastic pipette.

33| With an insulin syringe, inject the complete transfection solution (150 μl) into a tail vein of a mouse under anesthesia (injection of the schizonts results in rapid invasion of new erythrocytes within 2–4 h after injection and a parasitemia of between 0.1 and 1% can be observed soon after this time). Repeat steps 23–33 for the remaining Eppendorf tubes.

Drug selection of genetically transformed parasites (days 6–15)

34| If wild-type parasites are transfected with DNA constructs that contain only one of the selectable markers Tg-*dhfr*, P-*dhfr* or *DHFR,* select with the drug pyrimethamine (Option A). If mutant parasites, already containing the T*g-dhfr* or P-*dhfr* marker, are transfected with DNA constructs with the *DHFR* selectable marker, select with the drug WR99210 (Option B).

(A) Pyrimethamine treatment of mice

(i) Provide the mice with drinking water containing pyrimethamine, 1 d after infection with transfected parasites (between 12:00 and 16:00).

(ii) Provide the water for a period of 4–9 d up to the collection of infected blood. Proceed with Step 35 on day 10.

(B) WR99210 treatment of mice

(i) Subcutaneously inject a single dose of 0.1 ml WR99210 (equal to 16 mg/kg body weight in mice of 20 g) 1 d after infection with transfected parasites (between 12:00 and 16:00).

(ii) Repeat the same treatment on the following 3 d (a total of four injections) and proceed with Step 35 on day 10.
▲ **CRITICAL STEP** WR99210 is injected subcutaneously and is not provided in the drinking water because WR99210 is poorly water soluble.

The difference in resistance between parasites containing only the Tg-*dhfr* and parasites containing both the Tg-*dhfr* and the *DHFR* is only in the order of 10. As a result, selection with WR99210 of parasites with both selectable markers from a population of parasites that already contains the Tg-*dhfr* selectable marker is not straightforward. To increase the chance to select the desired mutants with both selectable markers from a population with only T*g-dhfr*, it is recommended to perform the WR99210 drug-selection procedure in not one but in two to three mice.

Collection of drug-resistant, genetically transformed parasites (days 10–15)

35| On day 10, make a thin blood smear (see **Box 1**) from one droplet of tail blood of the mouse injected with transfected schizonts at day 5. If the parasitemia is lower than 2%, make another blood smear on subsequent days until parasitemia is between 2 and 5%. If the parasitemia is between 2 and 5%, proceed with Step 36.

▲ **CRITICAL STEP** In successful transfection experiments, the parasitemia usually increases to 2–5% between days 10 and 15. Occasionally, however, this level of parasitemia is reached only after day 15, and several different causes may explain the slower growth rate of the parasites: (i) the transfection was unsuccessful, but during the selection period parasites arose with a spontaneous mutation that confers resistance against pyrimethamine or WR99210; (ii) the transfection was unsuccessful but the drug treatment did not kill all wild-type parasites; (iii) the transfection was successful but the introduced genetic modification affected parasite growth rate; (iv) the transfection was successful, but most of the parasites contain the introduced constructs as episomes (circular plasmids). Parasites containing episomal constructs usually grow more slowly under drug selection than transgenic parasites containing DNA integrated into their genome. The slower growth rate is principally due to unstable segregation of episomes during schizogony and the uncoordinated partitioning of episomes into the resulting merozoites; hence the merozoites that contain no episome are sensitive to the drugs during their subsequent development and are killed by the treatment.

As it is not possible to distinguish between successful and unsuccessful transfections when the parasitemia increases at a lower rate than expected, it is recommended to proceed with the collection of parasites and to analyze the genotype as described below.

? TROUBLESHOOTING

36| If parasites are transfected with constructs that contain fluorescent markers (such as GFP, RFP or proteins tagged with fluorescent markers) check first for expression of the fluorescent marker, as described below. If not, proceed with Step 37.

(i) Collect one droplet of tail blood (4–8 µl) in 200 µl PBS.
(ii) Add 4 µl stock solution of the DNA-specific dye Hoechst 33258 and leave for 5 min at 37 °C.
(iii) Place 5 µl of the suspension on a microscope slide under a cover slip.
(iv) Check for fluorescent parasites using a fluorescent microscope (objective at x40).
(v) If fluorescent parasites are present, proceed with Step 37.

▲ **CRITICAL STEP** The analysis for fluorescent parasites is an additional step to check whether a transfection has been successful. If no fluorescent cells are detected, it is recommended to proceed with the collection of parasites and to analyze the genotype as described below. A lack of fluorescent cells does not necessarily mean that the transfection has been unsuccessful. It can be due to a low level of expression of the fluorescent markers, undetectable by microscopy.

Because nonfluorescent, live parasites within red blood cells cannot easily be distinguished under the microscope, it is recommended to (counter) stain the parasites with a fluorescent dye to be able to detect all blood stages. In our laboratory, we usually use the DNA-specific dye Hoechst 33258 (**Fig. 5**).

37| Collect 0.6–1 ml of blood from the mice with a parasitemia of 2–5%. Collect the blood (as much as possible) by cardiac puncture under anesthesia using a 1-ml syringe containing 0.1 ml of heparin stock solution.

38| Transfer the blood of each mouse to separate 10-ml tubes in order to mix the blood with the heparin.

39| Transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution.

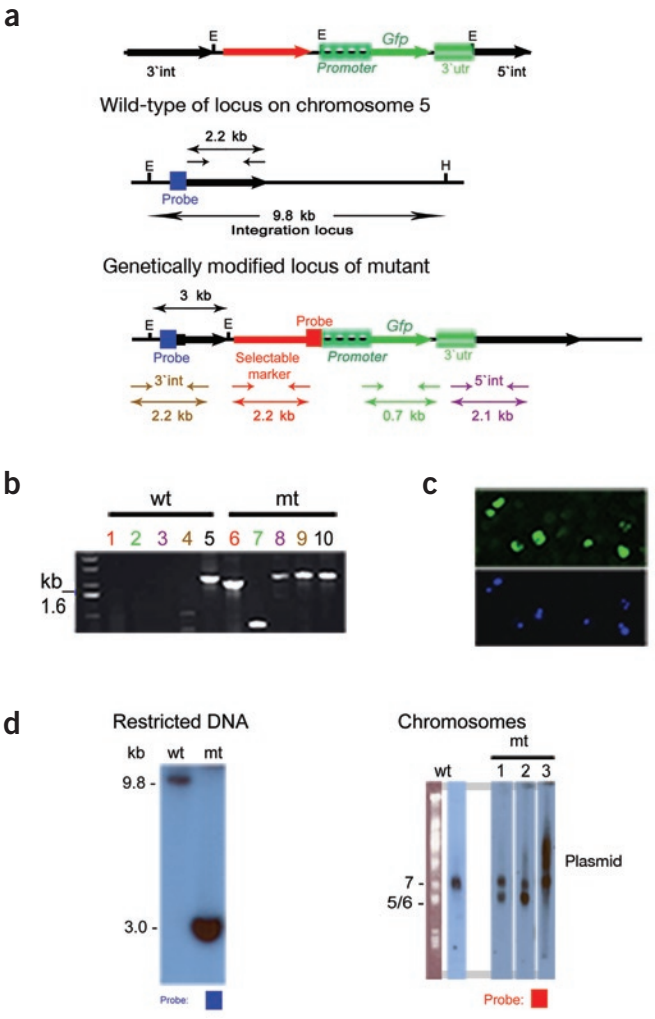

Figure 5 | Examples of standard analyses of the genotype of parasites selected by drug treatment after transfection. An example is shown for a genetically transformed mutant parasite (mt) that has been transfected with a construct containing the transgene *gfp* and is designed to achieve targeted integration by homologous recombination into the small subunit ribosomal RNA *(ssu-rrna*) gene on chromosome 5. (**a**) The DNA vector, integration locus and the resultant locus after vector integration. The vector construct contains the drug-selectable marker, the fluorescent marker (*gfp*) and sequences for targeted integration into the *ssu-rrna* locus. Restriction sites and probes used for Southern analysis and primers for PCR are indicated. (**b**) PCR analysis of genomic DNA to establish correct integration of the construct into the mutant (mt) parasite genome. Different primer combinations are used, as indicated by the colors (see **a**). Wild-type (wt) parasites are used as a control. (**c**) Fluorescence microscopy to analyze GFP expression in the blood stages of the mt parasites that express *gfp* under control of a blood-stage promoter (*eef1aa*). The parasites are counterstained with the DNA-specific dye Hoechst 33258. (**d**) Southern analysis of restricted genomic DNA to show correct integration of the DNA-vector and for the possible presence of untransformed, wild-type parasites. (**e**) Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (PFGE) to confirm integration of the DNA construct into the correct chromosome and also the possible presence of circular plasmids and/or wild-type parasites. The chromosomes are hybridized using a probe recognizing the 3′-UTR of the selectable marker which is also present in the genome (as the 3′-UTR of the *dhfr* gene of *P. berghei* on chromosome 7). Therefore, the ratio between the hybridization signal of the target chromosome and of chromosome 7 is informative for both the number of copies of the vector integrated (lower intensity of chromosome 7; see lane 2), the contamination with wild-type parasites (higher intensity of chromosome 7; not shown) and the possible presence of plasmids that usually migrate at the height of chromosome 9-11 (lane 3).

40| Distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.

▲ **CRITICAL STEP** The glycerol solution containing infected blood can be directly transferred into the liquid nitrogen storage without additional steps. For future experiments, these samples can be used to infect two to four naive mice. To re-infect mice, remove one cryogenic vial from the liquid nitrogen tank and thaw it at room temperature (19–23 °C) or at 37 °C. Immediately after thawing, inject the suspension intraperitoneally into two to four mice using a 1-ml syringe. In these mice, the parasitemia usually rises to 1–10% within 3–7 d.

41| To the remainder of blood in the 10-ml tube, add 10 ml of cold (4 °C) erythrocyte lysis buffer for lysis of the erythrocytes.

42| Leave this suspension on ice for 3–5 min (the clarity of the suspension is an indicator of the state of the lysis procedure, i.e., when fully clear, lysis is complete).

43| Harvest the parasites by centrifuging for 8 min at a speed that will generate 500*g*.

44| Discard the supernatant and store the parasite pellet at −20 °C for further genotype analysis. The parasite pellet is used for isolation of genomic DNA and for preparing agarose blocks for separation of the chromosomes by pulsed-field gel electrophoresis (see **Fig. 5** for examples of the analyses of the genotype).

● TIMING

Days 0-5: Collection of *P. berghei* schizonts for transfection
Day 0: Infection of 2 mice (1.5 h)
Day 4: Preparing overnight cultures of infected blood (2 h)
Day 5: Collection of schizonts (2 h)
Day 5: Transfection of purified schizonts (1 h)
Days 6–15: Drug selection of genetically transformed parasites
1 h to set up on day 6 for pyrimethamine and WR99210 treatment
0.5 h per day on days 7–9 for WR99210 treatment of mice
Days 10–15: Collection of drug-resistant, genetically transformed parasites
0.5 h per day for checking the parasitemia of the mice
2.5 h for collection of the parasites

? TROUBLESHOOTING

See **Table 1** for troubleshooting guidance.

TABLE 1 | Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
3	The parasitemia of the mice is >3% at day 4	The mice have been infected at day 0 with too many parasites	Infect new mice and adjust the infection dose; do not proceed with the transfection protocol
	The parasitemia of the mice is <1% at day 4	The mice have been infected at day 0 with too few parasites	Check the next day whether the parasitemia increases to levels between 1 and 3% and then proceed with the transfection protocol
14	Many degenerated schizonts are present in the overnight culture	(i) The pH of the culture medium exceeded 7.4	(i) Check the preparation procedure of the culture medium and check the 'gassing procedure' of the cultures
		(ii) The parasitemia of the infected blood is too high	(ii) Do not culture infected blood with a parasitemia higher than 3%
	Most schizonts are not yet mature	The culture temperature is too low or at the start of the cultures the parasites were mainly very young ring forms	This is not a serious problem, as one can wait several hours until the schizonts are mature and then start the collection procedure.
	Not many mature schizonts are present in the smears but there are many free merozoites	The mature schizonts ruptured during preparation of the slide	The presence of merozoites on the slides confirms the presence of mature schizonts in culture, and one can proceed with the collection of the schizonts. Prepare the slides more carefully.
19	No distinct 'brown-grayish' band of schizonts is present at the interface between the Nycodenz and culture medium	(i) Too few healthy, mature schizonts are present in the culture to show distinct bands	(i) Optimize the culture conditions for the cultivation of schizonts
		(ii) The centrifugation conditions disturbed the gradients, for example, centrifugation with the brake on	(ii) Remove the supernatant and resuspend the erythrocytes in complete culture medium to run a second Nycodenz gradient with the correct centrifugation conditions
		(iii) Incorrect Nycodenz concentration	(iii) Remove the supernatant and resuspend the erythrocytes in complete culture medium to run a second Nycodenz gradient with the correct Nycodenz concentration

TABLE 1 | Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
35	The parasitemia is >5% at day 10 after drug treatment with WR99210	Most probably the drug treatment failed and many wild-type parasites are present (see CRITICAL STEP about WR99210 treatment)	Improve and repeat the subcutaneous injection
	Still no parasites at day 15 and also not at the following 5 d (see also below)	The introduction of the DNA construct in the parasite is unsuccessful or lethal (for example, as a result of disruption of an essential gene for blood stages)	Repeat transfection; check the DNA constructs for defects
	Still no or very low numbers of parasites at day 15	(i) The introduction of the DNA construct results in growth retardation of the mutant parasites	(i) Monitor the blood of the mice for an extended period, as mutant parasites may appear after 15 d. Collect the parasites at the correct parasitemia of 2–5%
		(ii) Drug selection killed all parasites and only a wild-type parasite with a spontaneous mutation that confers pyrimethamine resistance survived	(ii) This may occasionally occur and cannot be prevented. This can only be confirmed by genotype analysis of the parasites
		(iii) Parasites that survived drug selection grow slowly because they contain the drug-selectable marker on an episome (see CRITICAL STEP for an explanation of the slow growth of parasites with episomes)	(iii) Wait and collect the parasites at the correct parasitemia

ANTICIPATED RESULTS

The procedure described here should result in the generation and selection of genetically transformed *P. berghei* parasites that can be used for multiple research purposes (reviewed in refs. 1–3). These purposes include, for example: (i) analysis of gene function by gene disruption, modification or replacement and also by protein overexpression or protein-tagging studies; (ii) analysis of processes or structural elements in gene transcription and post-transcriptional regulation of gene expression; (iii) transgene expression of fluorescent markers to visualize parasite-host interactions; (iv) transgene expression of human parasite (e.g., *P. falciparum*) proteins in *P. berghei* to analyze their function and which can be used as immunological tools.

An essential first step when using transgenic parasites is the analysis of the genotype of the selected parasites to confirm the correct genetic modification. The methodologies of such analyses have been published in previous reviews[7,8] and consist of the following standard technologies: (i) diagnostic PCRs to show the presence of the introduced DNA (for example, by amplification of the drug-selectable marker) or to demonstrate correct integration of the construct into the target DNA locus within the genome; (ii) Southern analysis of genomic DNA to show the presence of the introduced DNA constructs as either episomes or as integrants within the genome and eventually the number copies of the integrated constructs; (iii) plasmid-rescue experiments to confirm the integrity of introduced episomes and to recover genomic DNA flanking the insertion site (single cross-over experiments only); (iv) Southern analysis of separated chromosomes using pulsed-field gel electrophoresis. This last analysis is particularly useful, as it provides quantitative information on the episomal or integrated nature of the DNA constructs, the chromosomal location of the DNA constructs after integration and also on the possible contamination of the genetically transformed parasite population with wild-type parasites. Examples of the above genotype analyses are shown in **Figure 5**. After confirmation of the correct genotype of the selected mutant parasites, it is strongly recommended to obtain 'pure lines' of these mutants by cloning. Cloning of the blood stages is performed using the method of limiting dilution.

The most commonly used standard method of transfection of *P. berghei* involved electroporation of 10^{-8}–10^{-9} schizonts with 20–50 µg of DNA construct using single high-voltage pulses (0.8–1.5 kV) and low capacitance settings (25 µF). Typical transfection efficiency ranged between 10^{-7} and 10^{-9} using different (linear) DNA constructs aimed at targeted integration into the genome. Previously, variables tested, such as voltage and capacitance settings, and parasite stage and numbers, did not result in an increased efficiency, including low voltage and high capacitance settings as used in *P. falciparum* transfections[3,7]. In order to combat the low transfection efficiencies in *Plasmodium,* alternative transfection methods for *P. falciparum* have been developed that include the electroporation of noninfected red blood cells to allow spontaneous uptake of DNA[17] and the use of polyamidoamine dendrimers to transfer DNA across membranes[18]. These attempts resulted in higher transient transfection efficiency but not obviously in significantly improved rates of selecting stably transformed parasites[19].

Because of low transfection efficiency and transfection success rate of the previous methods of transfection of *P. berghei*, at least two mice per transfection experiment (standard) were injected for subsequent drug selection of transfected parasites and treated for 3–4 consecutive days with pyrimethamine[7]. Usually parasites became apparent in the blood (with a parasitemia of 0.01–1%) 7–10 d after infection of the mice[7,8]. To prevent contamination with wild-type parasites that survived the drug treatment, the standard procedure is to transfer these parasites by intraperitoneal injection of 10^6–10^7 into two to three naive mice for a second round of drug selection for 3–4 d before parasites are collected for genotype analysis and cloning. Using this method, the total time to select transfected parasites is therefore 13–15 d, making use of four to five mice per transfection experiment[7,8]. In contrast, with the high transfection efficiency and success rate with the methodology described here, only one mouse per transfection experiment is required for selection of transfected parasites. Under optimal conditions, transformed parasites reach a parasitemia of 0.1–1% on day 5 or 6 after injection. Combined with the 10–100 times smaller number of parasites used per transfection and the smaller amount of DNA constructs, the methodology described in this protocol strongly reduces the time, number of laboratory animals and materials required to generate transfected parasites.

This Nucleofector technology of transfection used in this protocol is based on electroporation and has been claimed to achieve a more efficient transfer of DNA into the nucleus. Other conditions of the Nucleofector procedure are the same as those used in the conventional methodology (such as the transfected parasite stage and selection of transfected parasites), therefore, the increase in transfection rate probably results from differences in electroporation parameters. It is unclear whether the DNA was more efficiently or rapidly delivered to the nucleus of the parasite, but it may be anticipated that enhanced access to the nucleus might indeed improve the effective rate of integration of linear DNA into the genome. The improved integration rate may result from several factors: (i) increased amounts of DNA reaching the cytoplasm followed by nuclear uptake; (ii) improved direct delivery of DNA to the nucleus; or (iii) a shorter time span between electroporation and integration and thereby avoiding the observed degradation of the DNA fragments by exonuclease activity. 'Natural transfer' of DNA fragments from the cytoplasm into the nucleus might be an inefficient process in *Plasmodium* because genome replication and nuclear division during mitosis occur without breakdown of the nuclear envelope.

Whatever the underlying mechanism, the increased transfection rate has a number of clear advantages for the current genetic modification protocols of *P. berghei*. As long-term *in vitro* cultures of the blood stages of *P. berghei* are not easily established, one is dependent on laboratory animals for both the multiplication and selection of transfected parasites. Therefore, the high transfection efficiency with the 100% success rate and the small numbers of parasites required for transfection significantly reduces the number of laboratory animals used. Combined with the short period of only 5–6 d to select the transfected parasites, this greatly simplifies the transfection technology, and high-efficiency transformation will enable the application of large-scale functional genomics approaches, such as promoter traps and functional complementation.

Note: Supplementary information is available via the HTML version of this article.

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