# Selection by flow-sorting of genetically transformed, GFP-expressing blood stages of the rodent malaria parasite, *Plasmodium berghei*

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**This protocol describes a methodology for the genetic transformation of the rodent malaria parasite *Plasmodium berghei* and the subsequent selection of transformed parasites expressing green fluorescent protein (GFP) by flow-sorting. It provides methods for: transfection of the schizont stage with DNA constructs that contain *gfp* as the selectable marker; selection of fluorescent mutants by flow-sorting; and injection of flow-sorted, GFP-expressing parasites into mice and the subsequent collection of transformed parasites. The use of two different promoters for the expression of GFP is described; these two promoters require slightly different procedures for the selection of mutants. The protocol enables the collection of transformed parasites within 10–12 days after transfection. The genetic modification of *P. berghei* is widely used to investigate gene function in *Plasmodium* sp. The application of flow-sorting to the selection of transformed parasites increases the possibilities of parasite mutagenesis, by effectively expanding the range of selectable markers.**

## INTRODUCTION

Research into malaria parasites has been greatly improved through the application of reverse genetics in the past 10 years. However, the use of transfection in the study of the biology of malaria parasites has been limited due to poor transfection efficiencies and a paucity of selection markers[1–3]. Here, we describe a methodology for high-efficiency transfection of the rodent malaria parasite *Plasmodium berghei* and subsequent selection of genetically modified parasites by flow-sorting of parasites expressing the green fluorescent protein (GFP). This protocol is based on the methodology described by Janse *et al.*[4] with some (minor) modifications.

Mature schizonts containing fully developed merozoites are the most suitable target cells for the transfection of *P. berghei*. The introduction of DNA into schizonts has proven to be far more efficient than transfection of the other blood stages, such as ring forms and trophozoites. Transfection is performed using the non-viral Nucleofector® technology, which requires 1–2 $\times$ $10^7$ schizonts per transfection experiment. The collection of schizonts is not described in this protocol, but is performed as described in the Transfection Protocol, Steps 1–22 (ref. 5).

Flow-sorting of transformed parasites is not performed directly after the transfection procedure. The transfected parasites are first injected into a mouse and allowed to re-invade new erythrocytes and to grow for at least 48 h (two developmental cycles). This time period allows for integration of the linear DNA vectors into the genome. In most transfection experiments, 0.1–1% of the parasites receive (linear) DNA constructs. However, of these parasites, less then 10% will incorporate the construct into their genome[4] and parasites can express the fluorescent marker from non-integrated linear DNA vectors. If flow-sorting took place shortly after transfection, many fluorescent-positive parasites containing non-integrated linear DNA constructs would also be collected. Subsequently, most of these parasites would lose these constructs and revert back to wild-type parasites. In addition, the timing of the collection of parasites for flow-sorting during the day is important. The mice will have been injected with the transfected schizonts at day 1 between 11:00 and 12:00 a.m. Injection is followed by the immediate rupture of schizonts and invasion of erythrocytes by the merozoites, resulting in the start of a synchronous infection. The synchronicity of development is maintained for at least two subsequent cycles. Because *P. berghei* has an asexual (blood-stage) cycle of 22–24 h, a new cycle of invasion occurs every day between 9:00 and 12:00 a.m. To obtain the ring/trophozoite stage for the overnight cultures and subsequent flow-sorting, infected tail blood is collected between 3:00 and 5:00 p.m. at day 3.

A total number of 20–200 fluorescent, GFP-expressing parasites are selected by flow-sorting from the overnight cultures of infected blood, which are immediately injected into a mouse after the sorting procedure. The actual flow-sorting procedure is generally performed by an experienced operator who prepares the settings of the fluorescence-activated cell sorting (FACS) machine and runs the machine. The settings that are important for sorting GFP-expressing parasites are described in EQUIPMENT SETUP. If flow-sorting and injection of the GFP-expressing parasites into a mouse have been successful, the parasitemia in the mouse will usually rise to levels of 0.05–3% by days 6–8 after injection. Analysis of GFP expression in these parasites is an important first step to assess the success of the flow-sorting procedure. As indicated before, even after two cycles of blood-stage development, flow-sorting can result in selection of GFP-positive parasites that contain non-integrated DNA constructs that are subsequently lost during the growth phase in the mouse. Therefore, the parasite population in the mouse may consist of mixtures of stably transformed, GFP-positive parasites and wild-type, GFP-negative parasites. If GFP-expressing parasites are present, the infected blood is collected for storage of the parasites in liquid nitrogen (cryopreservation) and for genotype analysis of the parasites. This protocol does not describe the methods for genotype analysis of genetically transformed parasites, as these have been well described in previous reviews[6,7].

A variety of fluorescent markers are available for flow-sorting but, up until now, the procedure for flow selection of genetically modified malaria parasites has been described only for GFP. Two different promoters have been used for the expression of GFP in flow-sorted parasites; the constitutive *eef1aa* promoter[8] and the schizont-specific *ama1* promoter[9]. The use of these promoters requires slightly different procedures for the selection of mutants. The type of fluorescent marker and the promoter that is used to drive its expression are important considerations for both the flow-sorting procedure and for any subsequent experiment with the selected mutant parasites. Further experiments may require additional fluorescent markers to be introduced into the mutant parasite — for example, for use in protein-tagging experiments. Therefore, before starting transfection experiments with fluorescent proteins as selectable markers, it is important to critically assess the usefulness of the fluorescent marker and its promoter.

This protocol should result in the generation and selection of genetically transformed *P. berghei* parasites, which can be used for multiple research purposes (for reviews, see refs. 1–3). As there are only a few drug-selectable markers available for the transformation of *P. berghei*, the use of fluorescent markers, such as GFP, to select genetically transformed parasites expands the possibilities of performing sequential genetic modifications on the same parasite clone. Furthermore, mutant parasite lines that express fluorescent markers can be useful tools for investigating host–parasite interactions using *in vivo* imaging technologies (for reviews, see refs. 10, 11). **Table 1** shows the characteristics of a number of transgenic *P. berghei* lines, which express GFP or the fusion protein GFP–Luciferase, that have been selected by flow-sorting and that are available for the research community and can be obtained on request from the Leiden Malaria Research Group (http://www.lumc.nl/1040/research/malaria/accesspage.html#paraline).

## MATERIALS

### REAGENTS

- **Parasite lines** *P. berghei*, clone 15cy1 of the ANKA strain is used as a reference 'wild-type' line. *P. berghei*, mutant clone 354cl4 (*PbGFP-LUC$_{SCH}$*)[3] is a transgenic parasite line that expresses the fusion protein GFP-luciferase. The *gfp-luciferase* gene is under control of the schizont-specific promoter of the *ama1* gene of *P. berghei*. This line is available on request from the Leiden Malaria Research Group (http://www.lumc.nl/1040/research/malaria/malaria.html).
- **Parasite stages** Schizonts of parasite 354cl4. The protocol requires the generation of 0.5 to $1\times10^8$ cultured and purified schizonts to establish a synchronous infection in four mice. These schizonts are obtained as described in the protocol detailing transfection of the schizont stage of *P. berghei* (Steps 1-22)[9].
- **Mice** In our laboratory, mice of the following two strains are used: Swiss (OF1 ico, Construct 242, aged 6 wk, 25-26 g) and C57BL/6 (C57Bl/6Jico OF1, Construct 1, aged 6 wk, 20-25 g). The mice are obtained from Charles River.
- Foetal bovine serum (FBS), heat-inactivated (Invitrogen; Cat. no.: 10108-165); store at –20 °C
- 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)
- 24-well culture plate (Costar; Cat. no.: 3526)
- 5 ml Falcon tube; polystyrene round-bottom tubes; 12 × 75 mm style (Becton Dickinson; Cat. no.: 352054)
- Sterile buffered 0.9% NaCl solution (154 mMol/l NaCl; 1.4 mMol/l P; pH 7.5)
- Neomycin-Sulfate, USP grade (Invitrogen; Cat. no.: 21810-031) (Prepare a stock solution of 10 mg $ml^{-1}$ Neomycin-Sulfate in distilled water; store at –20 °C)
- Glycerol stock solution (for freezing of parasites): 30% (v/v) glycerol in phosphate-buffered saline (PBS; Roche; Cat. no.: 1.09204.0500); store at 4 °C
- Gas mixture of 5% $CO_2$, 5% $O_2$, 90% $N_2$ (in a gas bottle/cylinder)
- TE buffer (10 mM Tris, pH 8.0; 1 mM $Na_2EDTA$); store at room temperature
- Giemsa solution (Merck; Cat. no: 1666-789); working solution: 10% Giemsa solution in Sörensen staining buffer
- Cryogenic vials (2 ml; Corning Life Sciences; Cat. No.: 430488)
- Culture medium RPMI1640 (Invitrogen; Cat. no.: 13018-015)
- Nucleofector® solution 88A6 (Amaxa GmbH)
- Hoechst (Bisbenzimide H) 33258 (Sigma; Cat. no.: B1155)
- Heparin, Grade I-A, cell culture tested, 140 m USP units $ml^{-1}$. Store at 4 °C

### REAGENT SETUP

**Parasite line.** In our laboratory, the reference line 'cl15cy1' of the ANKA strain of *P. berghei* is used. However, parasite lines of other commonly used strains of *P. berghei,* such as NK65, can also be used.

**Parasite stages.** Schizonts of parasite cl15cy1. The protocol requires the generation of cultured, purified schizonts for transfection. In the Transfection Protocol, Steps 1–22 (ref. 5), the collection of 0.5–1 × $10^8$ schizonts is described; these are sufficient for 10 independent transfections.

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

▲ CRITICAL Diets of laboratory rodents with low contents of total protein, energy and *p*-aminobenzoic acid can negatively influence *P. berghei* infections[12]. In our laboratory, we therefore provide diets with a high protein content (20–25% of total diet) and gross energy content (18,000–20,000 kJ/$kg^{-1}$).

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

**Phosphate-buffered saline** 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 9 volumes of distilled water. Adjust the pH to 7.2 with 1 M HCl and sterilize by autoclaving for 20 min at 120 °C.

**Sörensen staining buffer** Working solution: dissolve 2.541 g $KH_2PO_4$ and 8.55 g $Na_2HPO_4.2H_2O$ in 5 l of distilled water at pH 7.2. Store at room temperature.

**Culture medium RPMI1640** Add the RPMI1640 powder (with L-glutamine and 25 mM HEPES, without $NaHCO_3$) to 1 l of distilled water. In addition, add 0.85 g $NaHCO_3$ and 5 ml of Neomycin-Sulfate stock solution. Filter-sterilize (0.2 μm) and store at –20 °C in 100 ml aliquots.

**Complete culture medium RPMI1640** Culture medium RPMI1640 supplemented with FBS to a final concentration of about 20% (v/v). ▲ CRITICAL For optimal *in vitro* growth of the parasites, a relatively high percentage (~20%) of FBS is required.

**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. For a working solution, dilute the stock solution with 9 volumes of distilled water. Store at 4 °C.

**Nucleofector® solution 88A6** Used for the transfection of the schizonts by the Nucleofector® device. Store at 4 °C. The kit includes 2.5 ml of the 88A6 solution with supplementary solution (for 25 transfections), 25 cuvettes and 25 plastic pipettes.

**Hoechst (Bisbenzimide H) 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.

### EQUIPMENT

- Table-top centrifuge; Beckman Coulter Allegra (Beckman)
- Eppendorf microcentrifuge (16,000*g*)
- Vortex shaker (Ika Labortechnik)
- Biohazard Class II safety cabinet ! CAUTION Most manipulations with blood that is infected with genetically modified *P. berghei* parasites are performed in a Class II safety cabinet.
- Incubator or shaking water bath (37 °C) (as cultivation occurs in closed culture flasks that are flushed once with a gas mixture, no specific requirements are needed for the regulation of the gas mixture in the incubators)

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- Glass desiccator (e.g., candle jar)
- Nucleofector® device (Amaxa GmbH)
- Infra-red heat lamp (home-made or from Science Products)
- Fluovac isofluorane/halothane scavenger (Stoelting Co)
- Light microscope, Carl Zeiss Standard 25 (Zeiss)
- Fluorescence microscope, Leica DMRA HC 'upright' microscope (Leica)
- Fluorescence-activated cell sorting (FACS) machine (Becton Dickinson)

**EQUIPMENT SETUP**

**Fluovac isofluorane/halothane scavenger** Used for the anesthesia of mice. Mice are anesthetized in the 'induction chamber', which is pre-filled with the anesthetic vapor (isofluorane/oxygen) via the vaporizer unit. Injection of parasites 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** All light microscopes with a 100× oil-immersed objective are suitable.

**Fluorescence microscope, Leica DMRA HC 'upright' microscope** Used for the analysis of parasites expressing GFP and for counting infected erythrocytes/ parasites that are stained with the DNA-specific dye Hoechst 33258. Most fluorescence microscopes that are 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 dyes are suitable for this purpose.

**Fluorescence-activated cell sorting (FACS) machine** In our laboratory, the FACS Vantage, Diva or Aria from Becton Dickinson are used for the analysis, counting and sorting of parasites expressing fluorescent markers.

The suggested set up for the FACS machine is as follows:

1. Temperature: the complete process of flow-sorting should be performed at room temperature
2. Solutions for sorting and collection of cells: the flow-sorted parasites should be collected in complete culture medium, but the sorting process within the FACS should be done with a buffered 0.9% NaCl solution
3. 'Deflection Plate Voltage and drop charge' should be set in such a way that deflected, sorted drops are collected at the bottom of the tube (and not at the sides of the tube)
4. Sorting should be performed for 'purity and not for yield' (FACS DIVA, ARIA from Becton Dickinson: use 'purity mask' as 'sort-mask')
5. Sorting speed: 10,000 events/s
6. Photomultiplier tube (PMT) voltage should be set at maximum sensitivity
7. Sideward/forward gating
   - i. For flow-sorting of parasites obtained from tail blood (that express GFP under the control of the *eef1aa* promoter), erythrocytes should be selected by gating on forward scatter (FSC) and sideward scatter (SSC), as shown in **Fig. 1**. This results in selection of erythrocytes and excludes smaller particles, such as platelets, free parasites and cell debris from sorting
   - ii. For flow-sorting parasites from schizont cultures, no gate should be set in forward/sideward scatter, resulting in the selection of all cells, including platelets, free parasites and cell debris (**Fig. 1**). This is performed to be able to collect the small, free merozoites that are present as a result of the rupture of schizonts. Flow-sorted merozoites are highly infective to mice when intravenously injected (C.J.J. and B.F.F., unpublished observations)
8. Excitation of GFP should be performed at a wavelength of 488 nm and emission of the fluorescence should be detected with a band pass filter of 530/30 nm (FL1)
9. GFP-positive cells should be selected by gating on GFP fluorescence intensity (=FL1), as shown in **Fig. 1** (= Gate R2; FL2 is empty). The R2 gate should be set to flow-sort GFP-positive cells on FL1/FL2 (to exclude aspecific fluorescence; FL2 channel 575/26 nm)

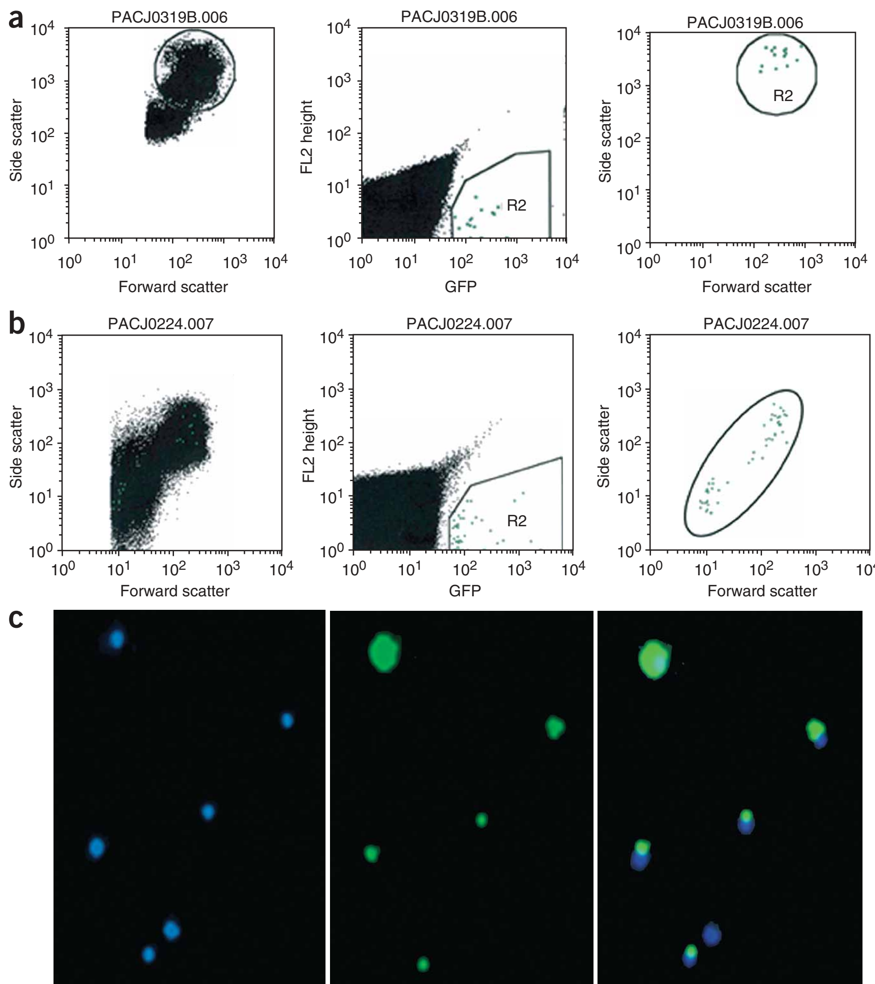


**Figure 1 | The procedure for the selection of fluorescent, GFP-expressing blood stages of *Plasmodium berghei* by flow-sorting.** Erythrocytes containing green fluorescent protein (GFP)-expressing parasites are selected by flow-sorting from uninfected erythrocytes and from erythrocytes containing wild-type parasites by setting a gate R2, as shown in the second dot plot in **a** and **b**. (**a**) Flow-sorting of GFP-expressing parasites (*eef1aa* promoter) obtained from tail blood. Erythrocytes are selected for analysis by setting a gate (circle) on forward/sideward scatter, as shown in the first dot plot. In this way, small particles, such as platelets and debris, are excluded from sorting and only GFP-positive erythrocytes are selected (see the selected GFP-positive cells with a high forward and sideward scatter in the third dot plot). (**b**) Flow-sorting of GFP-expressing parasites (*ama1* promoter) obtained from overnight cultures. No gate is set in the forward/ sideward scatter (first dot plot). Without a gate, small particles, such as platelets, debris and free merozoites, are included in the sorting procedure. In this way, the small, GFP-expressing merozoites will be selected, which are present in the overnight culture as a result of rupture of mature schizonts (see the selected GFP-positive cells in the right-hand side dot plot with the low forward and sideward scatter). (**c**) Visualization of GFP-expressing blood stages that are selected by flow-sorting. The parasites are (counter) stained with the DNA-specific fluorescent dye Hoechst-33258. The right-hand side picture shows a merged picture of GFP, Hoechst and the light field of (infected) erythrocytes.

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## PROCEDURE

**Transfection of purified schizonts and injection into mice** (Day 1, 11:00–12:00 a.m.)

**1|** Distribute the 10 ml schizont suspension containing 0.5–1 × $10^8$ schizonts among 10 Eppendorf tubes (1 ml per tube) and proceed immediately with step 2. The parasites from one Eppendorf tube are used for one transfection.

▲ CRITICAL STEP About 5–10 min before electroporation of the parasites, place a mouse under an infra-red heat lamp. The tail veins swell at the higher temperature, simplifying the intravenous injection procedure.

**2|** 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.

**3|** Discard as much as possible of the supernatant.

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

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

**6|** Place the cuvette in the Amaxa Nucleofector® device and transfect using program U33.

**7|** Remove the cuvette from the Nucleofector® device and immediately add 50 μl of complete culture medium.

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

**9|** 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 0.1–1% can be observed soon after this time). Repeat steps 2–9 for the remaining 9 Eppendorf tubes.

**Collection of parasites (infected blood) for flow sorting (Day 3)**

**10|** Make a thin blood smear from one droplet of tail blood (between 2:00–4:00 p.m. on day 3) from the mouse injected on day 1 with transfected schizonts (see **Box 1**). If the parasitemia is higher than 0.5%, proceed with Step 11 (see 'CRITICAL STEP' below if the parasitemia is lower than 0.5%).

▲ CRITICAL STEP If the parasitemia is lower than 0.5%, flow-sorting is less optimal as a result of the low number of fluorescent parasites. It is best to wait 24 h for the collection of infected blood and then continue the following day when the parasitemia has increased to 0.5–3%. A parasitemia that is higher than 3–5% is also suboptimal. At higher parasitemia, many erythrocytes will become multiply infected or parasites will reside in the 'older' erythrocytes (normocytes) and not in the reticulocytes. In both cases, the development of parasites is impaired.

**? TROUBLESHOOTING**

**11|** Prepare 10 ml of complete culture medium RPMI1640 by adding 2 ml of freshly thawed FBS to 8 ml RPMI1640 culture medium.

**12|** Collect one droplet of infected tail blood in 1 ml complete culture medium RPMI1640. The blood is collected between 3:00 and 5:00 p.m.

**13|** If the parasites are transfected with constructs that contain *gfp* under control of the *ama1* promoter, incubate the infected blood in culture overnight as described in (A). If the parasites are transfected with constructs that contain *gfp* under control of the *eef1aa* promoter, incubate the infected blood in culture overnight as described in (B).

▲ CRITICAL STEP In the overnight cultures, the ring forms and (young) trophozoites develop into mature trophozoites or schizonts. Mature schizonts do not rupture spontaneously and remain viable for several hours, allowing for flow-sorting of

### BOX 1 | MAKING THIN BLOOD SMEARS

i) Make a thin blood smear on a microscope slide.
ii) Fix the smear for 2 s with methanol and stain with a fresh Giemsa working solution for 10 min.
iii) Wash the slide with tap water.
iv) 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.

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mature schizonts that contain fully developed merozoites. For optimal development of the parasites, the correct gas conditions (lowered oxygen concentration compared to air), the pH of the culture medium (7.2–7.4) and the temperature are critical. The temperature is critical 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 22–24 h. A temperature of 37 °C is optimal to collect viable, mature schizonts between 10:00 and 12:00 a.m. at day 4 (A) and a temperature of 34 °C is optimal to collect viable, mature trophozoites and young schizonts between 10:00 and 12:00 a.m. at day 4 (B).

**(A) Parasites expressing GFP under control of the *ama1* promoter**

▲ CRITICAL STEP As the *ama1* promoter drives the expression of GFP only in the mature schizont stage, early-stage parasites need to first be collected from the mice and cultured *in vitro* overnight for the generation and collection of schizonts. The schizont stage is not present in the peripheral blood circulation of the animals because this stage sequesters in the capillaries of deep organs. *In vitro* cultures are, therefore, the only source for these stages.

(i) Transfer the 1 ml cell suspension containing the infected blood in culture medium, to a well of a 24-well culture plate.
(ii) Place the culture plate in a small glass desiccator (e.g., candle jar; **Fig. 2**).
(iii) Flush the desiccator for 2 min at 1.5–2 bar pressure with the following gas mixture: 5% $CO_2$, 5% $O_2$, 90% $N_2$ (see **Fig. 2**).
(iv) Place the desiccator on a shaker in an incubator at 37 °C. Switch on the shaker at a speed that is just fast enough to keep the cells in suspension.
(v) Incubate the cultures (shaken) at 37 °C until the next day (day 4) and proceed with Step 14.

**(B) Parasites expressing GFP under the *eef1aa* promoter (Fig. 3)**

▲ CRITICAL STEP The *eef1aa* promoter drives the expression of GFP in all blood forms, including the stages found in the peripheral blood circulation. Therefore, parasites that are obtained directly from the tail blood of a mouse can be used for flow-sorting. However, the young blood stages, such as the ring forms, have a relatively low GFP fluorescence and are difficult to select from non-GFP-expressing erythrocytes (**Fig. 3**). Therefore, for more optimal flow-sorting conditions, it is recommended to, first, culture the blood stages overnight before starting flow-sorting. Overnight cultivation at 34 °C allows for the collection of old trophozoites and young schizonts that have the higher fluorescence intensities (**Fig. 3** and ref. 4).

(i) Transfer the 1 ml cell suspension containing the infected blood in culture medium, to a well of a 24-well culture plate.
(ii) Place the culture plate in a small glass desiccator (e.g., candle jar; **Fig. 2**).
(iii) Flush the desiccator for 2 min. at 1.5–2 bar pressure with the following gas mixture: 5% $CO_2$, 5% $O_2$, 90% $N_2$ (see **Fig. 2**).
(iv) Place the desiccator on a shaker in an incubator at 34 °C. Switch on the shaker at a speed that is just fast enough to keep the cells in suspension.
(v) Incubate the cultures (shaken) until the next day at 34 °C (day 4) and proceed with Step 14.

**Flow-sorting of GPF-expressing parasites and injection into mice (Day 4)**

**14|** Between 10 and 12 am, transfer the 1 ml culture suspension from the overnight culture to a 5 ml Falcon tube.

**Figure 2 |** A 24-well culture plate containing infected tail blood in culture medium, placed in a glass desiccator (candle jar). The infected blood is cultured overnight in the desiccator, which is placed on a shaker and gassed with a gas mixture of 5% $CO_2$, 5% $O_2$ and 90% $N_2$.

**15|** Take the 5 ml Falcon tube containing the cultured parasites to the FACS machine for flow-sorting and prepare the settings of the FACS machine (see CRITICAL STEP below). Place the tube in the FACS machine and start the sorting process.

**16|** Flow-sort with 0.9% NaCl solution at room temperature.

▲ CRITICAL STEP The actual flow-sorting procedure should be performed by an experienced operator who prepares the settings of the FACS machine for flow-sorting and runs the FACS machine. The settings that are important for sorting GFP-expressing infected erythrocytes from non-infected erythrocytes and also from erythrocytes containing non-GFP-expressing parasites are described in EQUIPMENT SETUP. It is recommended that the correct settings and gates of the FACS machine are defined using the blood stages of the existing GFP-expressing mutant lines (**Fig. 1**). Several *P. berghei* mutant lines are available that express GFP under the control of the *eef1aa* or *ama1* promoters (**Table 1**).

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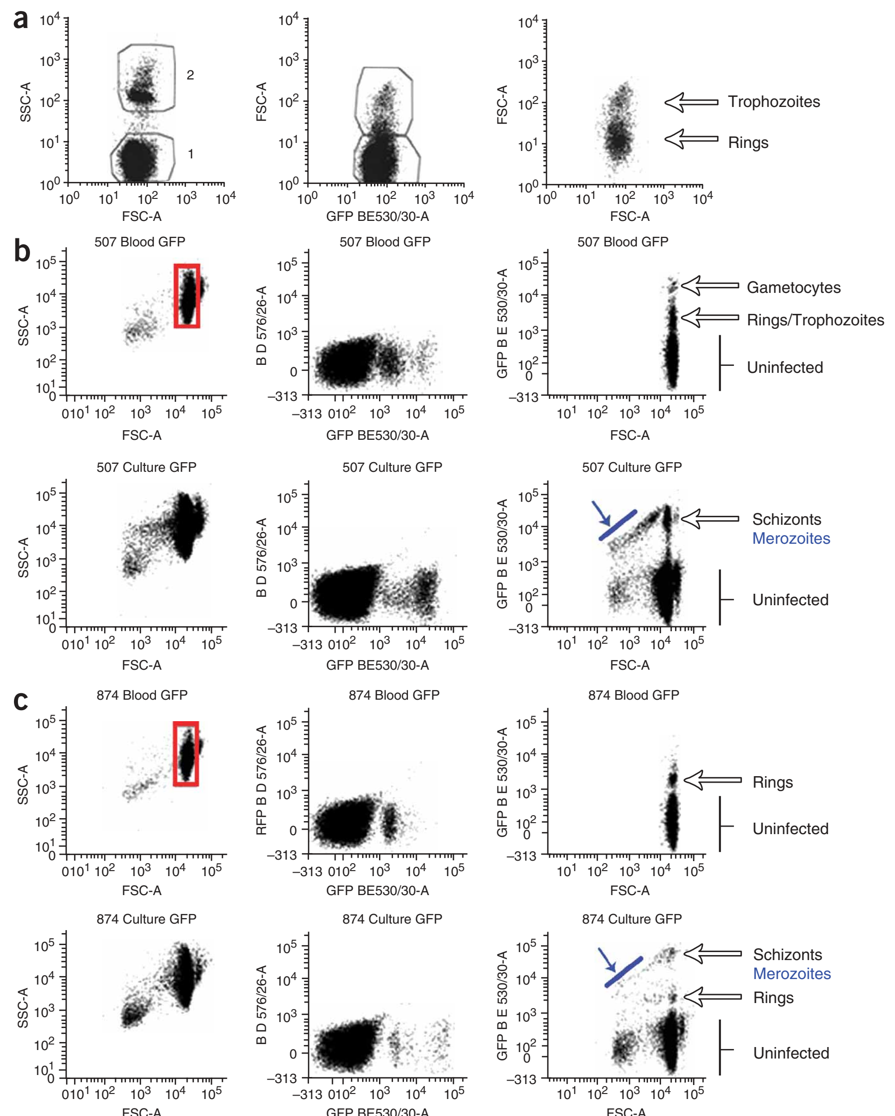


**Figure 3 |** Analysis of GFP fluorescence intensity by flow cytometry of the blood stages of transgenic *Plasmodium berghei* lines. These parasites express green fluorescent protein (GFP) under the control of either the constitutive promoter *eef1aa* (**a**,**b**) or the schizont-specific *ama1* promoter (**c**). (**a**) Infected tail blood is stained with the fluorescent, DNA-specific dye Hoechst 33258 (first dot plot) to visualize all uninfected (population 1) and infected (population 2) erythrocytes. The second dot plot shows the GFP fluorescence of all erythrocytes, demonstrating an overlap in GFP fluorescence between uninfected and infected erythrocytes. The third dot plot shows the GFP fluorescence from only the infected erythrocytes (as gated on Hoechst fluorescence, as shown in the first dot plot). The overlap in GFP fluorescence between infected and non-infected erythrocytes is the result of the low fluorescence intensity of ring forms when they express GFP from a single copy *gfp* gene under the control of the *eef1aa* promoter. (**b**) Comparison of GFP fluorescence intensity of parasites (*eef1aa* promoter) from tail blood (upper panel) or from overnight cultures of the blood stages (lower panel). In the upper panel, erythrocytes are gated on forward/sideward scatter (see red gate in the first dot plot). In this way, small particles, such as platelets and debris, are excluded from sorting. In the lower panel, no gate is set in forward/sideward scatter to include free merozoites in the analysis, which are present in the cultures as a result of the rupture of schizonts. Note the increased fluorescence intensity of the cultured schizonts (lower panel, second and third dot plots) compared with the ring forms and trophozoites in tail blood (upper panel, second and third dot plots) and the presence of (clusters) of GFP-positive merozoites with low forward/sideward scatter (blue arrow). (**c**) Comparison of GFP fluorescence intensity of parasites (*ama1* promoter) from tail blood (upper panel) or from overnight cultures of the blood stages (lower panel). In the upper panel, erythrocytes are gated on forward/sideward scatter (see red gate in the first dot plot). In the lower panel, no gate is set in forward/sideward scatter to include free merozoites in the analysis, which are present in the cultures as a result of the rupture of schizonts. Note the increased fluorescence intensity of the cultured schizonts (lower panel, second and third dot plots) compared with the ring forms in tail blood (upper panel, second and third dot plots) and the presence of (clusters) of GFP-positive merozoites with low forward/sideward scatter (blue arrow).

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**17|** Collect 20–200 GFP-positive cells in 300 μl complete culture medium in a 5 ml Falcon tube (wet the sides of the tube) or in a well of a 24-well culture plate at room temperature.
▲ CRITICAL STEP The total time required to flow-sort 20–200 GFP-positive cells depends on the parasitemia of the infected blood and the efficiency of the transfection procedure. Usually, it takes 10–30 min to collect 20–200 GFP-positive cells from a total of 10–20 × $10^6$ cells.

**18|** Inject the complete suspension of 300 μl, containing the flow-sorted cells in complete culture medium, into a tail vein of a mouse under anesthesia using an insulin syringe.

**19|** Repeat Steps 17 and 18 2–3 times for the injection of a total of 2–3 mice with 20–200 flow-sorted parasites from one transfection experiment.
▲ CRITICAL STEP About 5–10 min before injection of the parasites, place the mice under an infra-red heat lamp. The mice tail veins swell at the higher temperature, simplifying the intravenous injection procedure. In addition, prepare the Fluovac Anesthetic Scavenging Unit in advance and fill the 'induction chamber' with the anesthetic vapor (isofluorane/oxygen) to rapidly inject the parasites into the mice after flow-sorting. We prefer to inject 2–3 mice with flow-sorted parasites because the

**TABLE 1** Transgenic *Plasmodium berghei* parasite lines expressing GFP, or the fusion protein GFP–Luciferase, which have been obtained by flow-sorting and do not contain a drug-selectable marker.

| Parasite line | Transgene | Promoter[1] | Insertion locus[2] | Double, single cross-over[2] | Expression of GFP or GFP–Luc[1] | Reference |
|---|---|---|---|---|---|---|
| 507cl1 | GFP | *eef1aa* | *230p* | Double | All blood stages | 4 |
| 440cl1 | GFP | *eef1aa* | *c/d-ssu-rrna* | Single | All blood stages | 4 |
| 676cl1 | GFP–Luc | *eef1aa* | *230p* | Double | All blood stages | 4 |
| 662cl1 | GFP–Luc | *eef1aa* | *c/d-ssu-rrna* | Single | All blood stages | 4 |
| 874 | GFP | *ama1* | *c/d-ssu-rrna* | Single | Schizonts | Unpublished |
| 875 | GFP–Luc | *ama1* | *c/d-ssu-rrna* | Single | Schizonts | Unpublished |

[1] GFP or GFP–Luciferase (GFP–Luc) under control of the *eef1aa* promoter are expressed in all blood stages. Expression is low in rings/young trophozoites and increases in older trophozoites/young schizonts and mature female gametocytes show a high expression level. GFP or GFP–Luciferase under the control of the *ama1* promoter are only expressed in the maturing schizont. [2] GFP or GFP–Luciferase is integrated into the genome by using constructs for single or double cross-over integration by homologous recombination. The targets in the genome used for homologous recombination are: the non-essential *230p* gene; and the *small subunit (ssu) ribosomal rna* gene of the non-essential c- or d-unit. Single cross-over recombination can result in the integration of multiple copies of the constructs, resulting in higher expression levels of GFP or GFP-Luciferase.

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flow-sorting and injection procedure often results in infection that consists of a mixture of GFP-expressing mutant parasites and wild-type parasites (for an explanation, see 'ANTICIPATED RESULTS'). The availability of more than one mouse offers the possibility of choosing the mouse with the highest percentage of mutant parasites for further analysis.

**Collection of genetically transformed parasites on days 10–12 (= days 6–8 after flow-sorting)**

**20|** Make a thin blood smear from one droplet of tail blood from the mice injected with the flow-sorted parasites 6–8 days after flow-sorting (see **Box 1**). If the parasitemia is lower than 2%, repeat the blood smear on consecutive days until it reaches 2–5%. If the parasitemia is between 2 and 5%, proceed with the next step.

**? TROUBLESHOOTING**

**21|** If the parasites are transfected with constructs that contain *gfp* under control of the *eef1aa* promoter, proceed with (A). If the parasites are transfected with constructs that contain *gfp* under control of the *ama1* promoter, proceed with (B).

**(A) For parasites transfected with constructs that contain *gfp* under control of the *eef1aa* promoter**

(i) Collect one droplet of tail blood in 100 μl PBS of each of the mice.
(ii) Add 2 μl Hoechst to this suspension and leave for 5 min at 37 °C.
(iii) Put 5 μl of the suspension on a microscope slide under a cover slip.
(iv) Check for fluorescent parasites (both Hoechst and GFP fluorescence using a fluorescent microscope (objective at 40×). If GFP-positive parasites are present, proceed with the collection of parasites for cryopreservation and genotype analysis (Step 21; see TROUBLESHOOTING if low numbers or no GFP-positive parasites are present).

▲ CRITICAL STEP The DNA-specific dye Hoechst 33258 is used to stain all parasites. Analysis of both the Hoechst and GFP fluorescence of the parasites provides information about the level of contamination with wild-type parasites, which are Hoechst-positive but GFP-negative.

**(B) For parasites transfected with constructs that contain *gfp* under control of the *ama1* promoter**

▲ CRITICAL STEP As the *ama1* promoter drives the expression of GFP only in the mature schizont stage and not in the other blood stages, parasites must first be collected from the mice and cultured overnight for the generation of schizonts. The schizont stage is not present in the peripheral blood circulation of the animals because it sequesters in the capillaries of deep organs and therefore *in vitro* cultures are the only source for these stages. In the overnight cultures, the ring forms and (young) trophozoites develop into viable schizonts that reach maturity (early) in the morning of the next day.

(i) Prepare 10 ml complete culture medium RPMI1640 by adding 2 ml of freshly thawed FBS to 8 ml RPMI1640 culture medium.
(ii) From each of the mice, collect one droplet of tail blood in 1 ml complete culture medium RPMI1640.
(iii) Transfer these cell suspensions to wells in a 24-well culture plate.
(iv) Place the culture plate in a small glass desiccator (e.g., candle jar; **Fig. 2**).
(v) Flush the desiccator for 2 min at 1.5–2 bar pressure with the following gas mixture: 5% $CO_2$, 5% $O_2$, 90% $N_2$ (**Fig. 2**).
(vi) Place the desiccator on a shaker in an incubator at 36.5 °C. Switch on the shaker at a speed that is just fast enough to keep the cells in suspension.
(vii) Incubate the parasite cultures until the next day at 36.5 °C.
(viii) Between 9:00 and 10:00 a.m., add 20 μl Hoechst to each well and leave for 5 min at 37 °C.
(ix) Put 5 μl of the suspension on a microscope slide under a cover slip.
(x) Check for fluorescent parasites (both Hoechst and GFP fluorescence) using a fluorescent microscope (objective at 40×). If GFP-positive parasites are present, proceed with the collection of parasites for cryopreservation and genotype analysis (Step 21; see TROUBLESHOOTING if low numbers or no GFP-positive parasites are present).

▲ CRITICAL STEP The DNA-specific dye Hoechst 33258 is used to stain all parasites. Analysis of both the Hoechst and GFP fluorescence of the parasites provides information about the level of contamination with wild-type parasites, which are Hoechst-positive but GFP-negative.
? TROUBLESHOOTING

**22|** 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.

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

**24|** Transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol/PBS stock solution.

**25|** Distribute this suspension among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min of the initial cardiac heart puncture 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 2–4 naive mice. To re-infect mice, one cryogenic vial is removed from the liquid nitrogen tank and thawed at room temperature or at 37 °C. Immediately after thawing, the suspension is injected intraperitoneally into 2–4 mice using a 1 ml syringe. In these mice, the parasitemia usually rises to 1–10% within 3–7 days.

**26|** 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.

**27|** 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).

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

**29|** Discard the supernatant and store the parasite pellet at –20°C for further genotype analysis. The parasite pellet is used for the isolation of genomic DNA and for preparing agarose blocks for separation of the chromosomes by pulsed-field gel electrophoresis[5].

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● TIMING

**Day 1: Transfection of purified schizonts (1 h)**

**Day 3: Collection of parasites (infected blood) for flow-sorting**
1 h for determination of the parasitemia of the infected mice
1 h to prepare the overnight cultures of the infected blood

**Day 4: Flow-sorting of GPF-expressing parasites and injection into mice**
1–2 h to flow-sort 2–3 times 20–200 parasites from one transfection experiment and subsequent injection into mice

**Days 10–12: Collection of genetically transformed parasites**
Parasites with GFP under the control of the *eef1aa* promoter:
1 h per day for checking the parasitemia of the mice and GFP expression
2.5 h for collection of the parasites
Parasites with GFP under the control of the *ama1* promoter:
1 h per day for checking the parasitemia of the mice and preparation of the overnight cultures
2.5 h for checking GFP expression and collection of the parasites

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

**TABLE 2** Troubleshooting table.

| STEP | PROBLEM | POSSIBLE REASON | SOLUTION |
|---|---|---|---|
| 10 | The parasitemia of the mice is <0.5% at day 3 | The mice have been injected at day 1 with too few transfected parasites | Check the next days if the parasitemia increases to levels of 1–3% and then proceed with the protocol Step 11 |
| | The parasitemia of the mice is >3% at day 7 | The mice have been infected at day 1 with too many transfected parasites | Proceed with the protocol (Step 11). However, in the next experiments, adjust the number of schizonts that are injected into the mouse |

**TABLE 2** Troubleshooting table (continued).

| | | | |
|---|---|---|---|
| **19** | The parasitemia of the mice is $< 0.5\%$ | a. Flow-sorting and injection of the parasites was not successful and the mouse did not become infected | a. Repeat and optimize the transfection and flow-sorting procedure |
| | | b. The introduction of the DNA construct results in growth retardation of the mutant parasites | b. Monitor the blood of the mice for an extended period, as mutant parasites may appear after day 8. Collect the parasites at the correct parasitemia of 2–5% |
| **21** | Parasites are not expressing GFP | a. The gating during the flow-sorting was not correct and infected, non-GFP-positive erythrocytes have been selected | Adjust the gates during flow-sorting such that only GFP-positive cells are selected |
| | | b. GFP-positive erythrocytes have been flow-sorted and injected but the parasites lost the (non-integrated) constructs during further growth | b.1. Make sure that parasites are transfected with only linear DNA and not with (contaminated) circular constructs |
| | | | b.2. Start the flow-sorting 1–2 days later after transfection. Most parasites with non-integrated constructs will have lost this DNA (and GFP expression) |
| | The parasite population consists of a mixture of GFP-positive and GFP-negative parasites | a. GFP expression is not detected in parasites that do express GFP: young ring forms, with *gfp* under the control of the *eef1aa* promoter, have a low, not easily detectable fluorescence intensity; parasites with *gfp* under the *ama1* promoter express GFP only in the mature schizonts | Check carefully that GFP expression is in the correct stages of the parasites |
| | | The population is a mixture of wild-type and mutant parasites as a result of flow-sorting of parasites with and without integrated DNA constructs. The latter parasites can lose the DNA constructs and revert to wild type | In many flow-sorting experiments, this will happen. The desired mutants can be selected by an additional selection using flow-sorting or by cloning of the parasites by the method of limiting dilution |

GFP, green fluorescent protein.

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## ANTICIPATED RESULTS

Flow-sorting of GFP-expressing parasites will result in the selection of genetically transformed *P. berghei* parasites that can be used for multiple research purposes (for reviews, see refs 1–3). An essential first step when using transgenic parasites is the analysis of the genotype of the selected parasites to confirm the correct genetic modification. These analyses are performed as described for genetically modified parasites that are selected by drug selection[5–7]. The collection of desired parasite mutants using flow selection is less straightforward than standard drug-selection methods and, therefore, careful subsequent analysis of the flow-sorted population is essential. The reduced efficiency of selection by flow-sorting is due to two main factors. First, the low GFP fluorescence intensity of young blood stages expressing GFP under the *eef1aa* promoter and the background fluorescence of red blood cells affects the clear distinction between erythrocytes not containing parasites or containing young forms (see **Fig. 3**). Second, the inability, initially, to distinguish between parasites that express GFP from the linear (episomal) DNA vector or from the *gfp* gene that is integrated into the genome of the parasite. It has been calculated that 0.1–1% of parasites transfected with the Nucleofector® technology take up DNA[4]. These parasites can express the fluorescent marker from non-integrated linear DNA vectors, but only in a small proportion of them is the linear DNA integrated into the genome. We have found that integration of vectors by single cross-over integration occurs in 1–10% of the parasites that have taken up DNA, whereas the figure is closer to 0.1–1% by double cross-over integration. In our laboratory, flow selection of fluorescent parasites and injection into a mouse always results in the establishment of an infection, but in more than 50% of these infections the parasite population consists of a mixture of stably transformed, GFP-expressing mutants and GFP-negative, wild-type parasites (the proportion of mutant parasites ranges between 10 and 90% in different experiments). To obtain pure lines of the desired mutants, transformed parasites are cloned from these mixed populations using the standard method of limiting dilution.

Notwithstanding the fact that flow selection is currently less efficient than drug selection, the methodology described in this protocol has been used to generate a number of stably transformed, transgenic parasites that express GFP or the fusion protein

GFP–Luciferase without a drug-selectable marker (**Table 1** and ref. 4). This not only demonstrates that fluorescent markers can be used as selectable markers, but also provides useful fluorescent, reference parasite lines that are still amendable to further genetic modifications using the existing drug-selectable markers.

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