

Chapter 21

Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins

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Abstract

We describe methods for the rapid generation of transgenic rodent *Plasmodium berghei* (*Pb*) parasites that express human malaria parasite (HMP) proteins, using the recently developed GIMO-based transfection methodology. Three different genetic modifications are described resulting in three types of transgenic parasites. (1) Additional Gene (AG) mutants. In these mutants the HMP gene is introduced as an “additional gene” into a silent/neutral locus of the *Pb* genome under the control of either a constitutive or stage-specific *Pb* promoter. This method uses the GIMO-transfection protocol and AG mutants are generated by replacing the positive–negative selection marker (SM) *hdhfr::yfcu* cassette in a neutral locus of a standard GIMO mother line with the HMP gene expression cassette, resulting in SM free transgenic parasites. (2) Double-step Replacement (DsR) mutants. In these mutants the coding sequence (CDS) of the *Pb* gene is replaced with the CDS of the HMP ortholog in a two-step GIMO-transfection procedure. This process involves first the replacement of the *Pb* CDS with the *hdhfr::yfcu* SM, followed by insertion of the HMP ortholog at the same locus thereby replacing *hdhfr::yfcu* with the HMP CDS. These steps use the GIMO-transfection protocol, which exploits both positive selection for *Pb* orthologous gene-deletion and negative selection for HMP gene-insertion, resulting in SM free transgenic parasites. (3) Double-step Insertion (DsI) mutants. When a *Pb* gene is essential for blood stage development the DsR strategy is not possible. In these mutants the HMP expression cassette is first introduced into the neutral locus in a standard GIMO mother line as described for AG mutants but under the control elements of the *Pb* orthologous gene; subsequently, the *Pb* ortholog CDS is targeted for deletion through replacement of the *Pb* CDS with the *hdhfr::yfcu* SM, resulting in transgenic parasites with a new GIMO locus permissive for additional gene-insertion modifications.

The different types of transgenic parasites can be exploited to examine interactions of drugs/inhibitors or immune factors with HMP molecules in vivo. Mice either immunized with HMP-vaccines or treated with specific drugs can be infected/challenged with these transgenic mutants to evaluate drug or vaccine efficacy in vivo.

Key words Malaria, *Plasmodium berghei*, Human parasites, Transgenic rodent parasites, GIMO-transfection, Challenge model, Vaccine-candidates, Drug-targets

1 Introduction

Rodent malaria parasites are used as models for human malaria and in particular to aid drug discovery and vaccine development. These models are used to study drug action and to identify targets for protective immune responses, *in vivo*. Although a high level of orthology exists between the genes of *Plasmodium* species that infect rodents and humans [1, 2], critical differences often exist in the sequence and structure between the encoded proteins. In addition, human malaria parasites (HMP; e.g., *P. falciparum* and *P. vivax*) express genes that are absent from rodent parasite genomes. These genetic differences complicate the analysis of drugs/inhibitors or immune factors in rodent models and the effective translation of findings in model systems to human malaria. “Humanizing” rodent parasites by introducing HMP genes into rodent parasite genomes can help to circumvent problems of interpreting data from different interventions, arising from structural differences that exist between functional HMP and rodent malaria parasite orthologs. In addition, it increases the possibilities of analyzing HMP-specific proteins *in vivo* [3].

Here we describe methods to generate “humanized” rodent malaria parasites in the *P. berghei* (*Pb*) model. Efficient methods exist for *Pb* genetic modification including a recently described method for GIMO-transfection (Gene Insertion-Marker Out; [4]) that greatly simplifies and speeds up the generation of transgenic parasites expressing heterologous proteins, free of drug-selection marker genes. Three different genetic modifications are described here resulting in three types of transgenic parasites that express HMP proteins. (1) *Additional Gene* (AG) mutants. In these mutants the HMP gene is introduced as an additional gene into a silent/neutral locus of the *Pb* genome under the control of a constitutive or stage-specific *Pb* promoter. This method is mainly used when a functional ortholog of the HMP gene is absent in the rodent malaria parasite genome. This method makes use of the GIMO-transfection method and AG mutants are created by replacing the positive–negative selection marker (SM) *hdhfr::yfcu* cassette in a neutral locus of a standard GIMO mother line with the HMP gene expression cassette, resulting in SM free transgenic parasites. (2) *Double-step Replacement* (*DsR*) mutants. In these mutants the coding sequence (CDS) of the *Pb* gene is replaced with the CDS of the orthologous HMP gene, in a two-step GIMO transfection. First, the *Pb* gene is deleted by double crossover homologous recombination with a construct having the *hdhfr::yfcu* SM flanked by the 5′ untranslated region (UTR; containing the gene promoter) and 3′ UTR (containing the transcriptional terminator sequences) of the *Pb* gene as gene targeting regions (TRs), thereby creating an “GIMO locus”. Next, this GIMO locus is replaced by the HMP gene expression cassette with the HMP

homolog flanked by the same 5' UTR and 3' UTR regions of the orthologous *Pb* gene. This method creates transgenic parasites expressing an HMP gene free of SM. (3) *Double-step Insertion (DsI)* mutants. When a *Pb* gene is essential for blood stage development the DsR approach is not suitable, as it is not possible to first create a GIMO-based deletion mutant in the first step. Therefore, in DsI mutants the HMP gene is first introduced into the neutral locus of an existing GIMO mother line under the control of 5' UTR and 3' UTR of the *Pb* ortholog. This step, in common with the AG method, results in a transgenic mutant expressing an additional copy of HMP but under the control elements of the *Pb* ortholog in a neutral locus, free of SM. Subsequently, the *Pb* ortholog is deleted. This method generates transgenic parasites not only expressing the HMP gene but also containing a GIMO locus (in the *Pb* gene). The absence of SM in the DsR and AG mutants and the GIMO locus created in DsI mutants facilitates rapid additional genetic modifications in these lines, for example to introduce additional HMP genes or reporter genes encoding fluorescent or luminescent proteins.

These different transgenic parasites can be exploited to examine interactions of drugs/inhibitors or induced immune factors against HMP molecules, in vitro and in vivo. Mice either immunized with candidate HMP-vaccines or treated with specific antibodies or drugs/inhibitors can be infected or challenged with these transgenic parasites to evaluate the efficacy of drugs/inhibitors or vaccines in vivo [3]. Humanized rodent parasites have been used to assess the efficacy of candidate vaccines against the *P. vivax* TRAP protein [5], the *P. falciparum* and *P. vivax* CS proteins [6, 7], the *P. falciparum* and *P. vivax* P25 proteins [8, 9] and the *P. falciparum* MSP1 gene [10, 11]. In addition, humanized *Pb* parasites expressing *P. falciparum* cyclic GMP-dependent protein kinase (PKG) and *P. falciparum* hexose transporter 1 have been generated, permitting in vivo screening of inhibitors interfering with PKG and antimalarial sugar analogs respectively [12, 13].

In this chapter we principally describe the generation of GIMO-based mutants in the rodent malaria parasite, *P. berghei*; however, similar reagents (e.g., a GIMO mother line and constructs) also exist for the rodent parasite *P. yoelii* [4].

2 Materials

2.1 Standard Parasites Lines Used for Generation Transgenic Parasites Expressing HMP Genes

1. Wild type (WT) lines of *P. berghei* ANKA or NK65; the most frequently used is the reference ANKA line, cl15cy1 [14].
2. The reporter *Pb* ANKA parasite line *PbGFP-Luc_{con}* (676m1cl1) expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the constitutive *eef1a* promoter and is SM free [15]. The reporter cassette is integrated into the neutral 230p locus (PBANKA_030600). For details of

PbGFP-Luccon, see RMgmDB entry #29 (<http://www.pberghei.eu/index.php?rmgm=29>) and **Note 1**.

3. The reporter *Pb* ANKA parasite line *PbGFP-Luc_{ama1}* (1037mlflmlcl1) expresses a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV) under the schizont-specific *ama-1* promoter and is SM free [16]. The reporter cassette is integrated into the neutral *230p* locus (PBANKA_030600). For details of *Pb* GFP-Luc_{ama1}, see RMgmDB entry 32 (<http://www.pberghei.eu/index.php?rmgm=32>) and **Note 1**.
4. The standard PbANKA-*230p* GIMO mother line (GIMO_{PbANKA} 1596cl1). The line contains a positive–negative selection marker (SM) cassette, a fusion gene of *hdhfr* (human dihydrofolate reductase; positive SM) and *yfcu* (yeast cytosine deaminase and uridyl phosphoribosyl transferase; negative SM) under control of the constitutive *eefla* promoter, stably integrated into the neutral *230p* locus (PBANKA_030600) through double crossover recombination [4]. The GIMO mother line is used for introduction of transgenes into the modified *230p* locus through transfection with constructs that target the *230p* locus. These constructs insert into the *230p* locus (“gene insertion”), thereby removing the *hdhfr::yfcu* SM (“marker out”) from the genome of the mother line. Transgenic parasites that are SM free are subsequently selected by applying negative drug selection using 5-FC. This selection procedure is performed in vivo in mice. For details of the PbANKA-*230p* GIMO mother line, see RMgmDB entry #687 (<http://www.pberghei.eu/index.php?rmgm=687>). We have generated a comparable standard GIMO mother line for the rodent parasite *P. yoelii* XNL Py17XNL-*230p* GIMO mother line (GIMO_{Py17X}, 1923cl1; see **Note 2**).
5. The standard PbANKA-*sI* GIMO mother line (2149cl1). The line contains a positive–negative *hdhfr::yfcu* selection marker (SM) cassette, under control of the constitutive *eefla* promoter stably integrated into the neutral *sI* gene-locus (PBANKA_120680) through double crossover recombination. The neutral *sI* locus has also been used in *P. yoelii* to introduce reporter proteins [17]. In addition, it contains GFP under the constitutive *eefla* promoter integrated into the neutral *230p* locus (PBANKA_030600). This line is currently unpublished but is available from the Leiden Malaria Research Group. The GIMO mother line is used for introduction of transgenes into the modified *sI* locus through transfection with constructs that target the *sI* locus. These constructs insert into the *sI* locus (“gene insertion”), thereby removing the *hdhfr::yfcu* selectable marker (“marker out”) from the genome of the mother line. Transgenic parasites that are marker-free

are subsequently selected by applying negative drug selection using 5-FC. This selection procedure is performed in vivo in mice. The PbANKA-*sI* GIMO mother line contains a GFP-luciferase fusion expression cassette in the *230p* locus and therefore transgenic parasites made in this line express both GFP and luciferase under the constitutive *efl1α* promoter. In contrast, transgenic parasites made in the PbANKA-*230p* GIMO do not express a reporter protein.

2.2 Laboratory Animals and Mosquitoes

2.2.1 Laboratory Animals

In our laboratory, we mainly use Swiss Webster mice (6 weeks old; 25–26 g) for generation of the transgenic parasite lines. However, other mouse strains such as C57BL/6 and BALB/c can also be used. All experiments using mice must be performed according to the appropriate National guidelines and regulations.

2.2.2 Laboratory Mosquitoes

Anopheles stephensi. For procedures of maintenance/rearing of mosquitoes and infection of mosquitoes, *see* ref. [18].

2.3 Basic DNA Constructs/Plasmids

1. Plasmid pL0043 [4]. This is a basic construct that is used to rapidly introduce transgenes into the *230p* locus (PBANKA_030600) of the PbANKA-*230p* GIMO mother line. This construct integrates by double crossover recombination and replaces the positive–negative SM (*hdhfr::yfcu*) cassette with the transgene-expression cassette (*see* **Note 3** for details of the generation of pL0043). We have generated a similar construct (pL1849) to rapidly introduce transgenes into the *230p* locus (PY17X_0306600) of the PyXNL-*230p* GIMO mother line. This construct integrates by double crossover homologous recombination and replaces the positive–negative SM with the transgene-expression cassette. The construct pL1849 was generated using a modified 2-step PCR method (*see* **Note 4**).
2. Plasmid pL2023. This is a basic construct that can be used to rapidly introduce transgenes into the *Pb sI* locus (PBANKA_120680) of the PbANKA-*sI* GIMO mother line. This construct integrates by double crossover recombination and replaces the positive–negative (*hdhfr::yfcu*) SM. *See* **Note 5** for details of the generation of pL2023 (pL2023 is currently unpublished but is available from the Leiden Malaria Research Group).
3. Plasmid pL0034. This construct contains the positive–negative (*hdhfr::yfcu*) SM cassette. It can be used to clone any specific 5' and 3' gene targeting region (TR) to generate a targeted gene-deletion mutant in the *Pb* genome by double crossover homologous recombination; replacing the *Pb* target gene with the *hdhfr::yfcu* SM, thereby generating a gene-specific GIMO locus (selected by positive selection using pyrimethamine).

The generated gene-specific GIMO line can then be used, in a subsequent transfection to introduce the orthologous HMP gene into this GIMO locus. Specifically, by using a modified DNA construct that contains the *Pb* gene 5' and 3' TRs (these TRs must include the complete *Pb* gene promoter and transcription terminator sequences), replacing the SM with a HMP CDS, thereby creating a HMP expression cassette. The GIMO protocol can then be employed to insert the HMP expression cassette (gene insertion) and replacing the introduced *hdhfr::yfcu* SM cassette (marker out), using negative selection. This results in generation of DsR mutants (Subheading 3.2) without SM.

2.4 Reagents

For reagents for in vitro cultivation of schizonts and transfection of *P. berghei* see ref. [14].

1. Glycerol. Stock solution: 30 % (vol/vol) glycerol in PBS. Store at 4 °C.
2. Phosphate-buffered saline (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.
3. Heparin, Grade I-A, cell culture tested, 140 mUSP units/mg. 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 RPMI 1640 culture medium without FBS to create a final solution of 200 units/ml. Store at 4 °C.
4. Pyrimethamine solution (Sigma). 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 during the complete selection period.
5. 5-FC solution. Negative selection of mutants is performed by providing 5-fluorocytosine (5-FC) in the drinking water of mice, 1 day after the mice have been infected with transfected parasites. The 2 mg/ml final concentration of 5-FC diluted with drinking water is provided to mice during the complete selection period (see ref. 19 for details of the 5-FC drinking water protocol).
6. DNA constructs (5–10 μg) in 5–10 μL TE buffer or water used for transfection (stored at –20 °C).

2.5 Equipment

For equipment for in vitro cultivation of schizonts and transfection of *P. berghei* see ref. [14].

1. Nucleofector device (Lonza) (<http://www.lonza.com/>).
2. Anesthesia induction chamber: Fluovac isoflurane–halothane scavenger (Stoelting Co.). Mice are anesthetized in the “induction chamber,” which is prefilled with the anesthetic vapor (a mixture of isoflurane and air) via the vaporizer unit. The injection of parasites or collecting blood by heart puncture is performed in mice that are kept under anesthesia by holding their muzzles to the small mask that is connected to the vaporizer unit.
3. Insulin syringes.

3 Methods

Three different genetic modifications are described resulting in three types of transgenic parasites that express HMP proteins: (1) Additional Gene mutants (AG; Subheading 3.1); (2) Double-step Replacement mutants (DsR; Subheading 3.2); and (3) Double-step Insertion mutants (DsI; Subheading 3.3). All mutants are generated using GIMO-transfection method [4]. For GIMO-transfection constructs are used, which contain the positive–negative drug-selection marker (SM) *hdhfr::yfcu*. This marker is a fusion of a positive SM (human *hdhfr* conferring resistance to pyrimethamine) and the negative SM (*yfcu* conferring sensitivity to 5-fluorocytosine; 5-FC). Parasites expressing *hdhfr::yfcu* are resistant to the pyrimethamine but are sensitive to 5-FC. GIMO-transfection uses positive selection for targeted gene-deletion and negative selection to rapidly generate gene-insertion transgenic parasites free of SM.

3.1 Transgenic Parasites with an HMP Gene in a Neutral Locus: Additional Gene (AG) Mutants

When a functional ortholog of an HMP gene is absent in the *Pb* genome, the HMP gene can be introduced into a neutral/silent locus of the *Pb* genome. Moreover, this approach can be used when the stage specificity and/or level of HMP expression needs to be altered. See Table 1 for different promoter regions used to drive transgene expression. The HMP gene is introduced into a standard GIMO mother line (Subheading 2.1) using a modified GIMO construct (Fig. 1; Subheading 2.3). The standard constructs target the *230p* or *sI* gene locus, as they contain targeting regions (TRs) to these genes as well as containing a multiple cloning site, where the HMP expression cassette can be introduced. The HMP expression cassette consists of the HMP CDS flanked upstream by the desired 5' UTR (containing the *Pb* promoter) and downstream by the 3' UTR (containing the transcriptional

Table 1
Different *P. berghei* regulatory regions (promoter and 3' UTR transcription terminator sequences) used to control transgene expression

Gene name	Gene product	Reference ^a or primer ^b	Timing of expression
Promoter regions			
<i>eflα</i>	PBANKA_113330	RMgm-7; [15]	Constitutive
<i>hsp70</i>	PBANKA_071190	RMgm-928 and [31]	Constitutive (Strong)
<i>ama1</i>	PBANKA_091500	RMgm-32; [16]	Schizonts (blood, liver), sporozoites
<i>Soap</i>	PBANKA_103780	ATAAGAATGCGGCGCGGTATCTAAAAAGCCTAATATTTC/CCATGCCATGGTTTGATTAAATAAATACAAAAAGG	Ookinetes
<i>Dynein heavy chain, putative</i>	PBANKA_041610	AAGGATOCCTTTTATCAATTTGGGATAATTAATTC/AAGATATCATATTTAAACAGATTAAAGTACCG	Male gametocytes
<i>lap4/ccp2</i>	PBANKA_131950	AAGATATCTTTATAAATGAAGCAATACCCAC/TTGGATCCTTCTATTATAAAAAATATAATATATG	Female gametocytes
<i>wis4</i>	PBANKA_050120	TATCCTGCAGGGTGATAGTGTAGATTTTGTGTTGAC/ATAAGAATGCGGCGCGAGACGTAATAATTATGTGCTGAAAGG	Sporozoites (early) liver stages
<i>cs/csp</i>	PBANKA_040320	GCTCCTTAAGACATAAAAGGGAATATGGAATATACTAGC/CGGGATCCAAATATATGCGGTGTATATATAGATTTTG	Sporozoites, liver stage

<i>Trap</i>	PBANKA_134980	CATGGGCCCCGAAAGGGAATGGCAAAATTATGTGTC/ ACTGTTAAACGGGAAATTGTCTTACCCATATTCTCTAC	Sporozoites
<i>sequestrin/lisp2</i>	PBANKA_100300	CATGGGCCCCGTCCTTATGGGCATATAACATCG/ AAGGCCTATGTGTAAAAAGTAAATGATTATAATAGAAAGTG	Middle/late liver stages
<i>3' UTR regions</i>			
<i>pbdhfr/ts</i>	PBANKA_071930	CGGGATTCCGTTTTTCTTACTTATATATTATATACC/ GCTCGGTACCCCGAAATTGAAGG	NA
<i>Cam</i>	PBANKA_101060	TGCTCTAGAAATTATTAAATATATATGAATATATACATCGTTGTATGCG/ CGGGGTACCCGACCATATAAGAAATTAACCC	NA
<i>dhfr-fpgs</i>	PBANKA_134000	RMgm-757; [31]	NA
<i>dhfr-pplk</i>	PBANKA_142670	RMgm-757; [31]	NA
<i>uis4</i>	PBANKA_050120	CGGATATCTATAATTCAATATGAGTAGTGAATTTCAG/ GGCCGGGTACCTTTCGCTTAAATGCTTGTGTCATC	NA
<i>trap</i>	PBANKA_134980	CGGGATCCAACTTAAGAGATATTATTTTGTTCG/ ATAAGAATGGGCGCCCATATATCTAGATGATTATTCTTATGTTAC	NA
<i>sequestrin/lisp2</i>	PBANKA_100300	CCGGGGTACCATGCATATAAGAAAAAGCCCCAACCC/ ATAAGAATGGGCGCGCTCGAAGAAATTCGAGTTAAGAAATTAAATGATGG	NA

^aReference to a line where gene regulatory sequence has been used to regulate transgene expression either as an entry in the RMgm Database (www.pberghei.eu; i.e., RMgm-#ID) or in a publication

^bThe primer sequences (i.e., Forward/Reverse) surrounding the regulatory region are shown with the restriction site used (*bold and underlined*) to introduce the region into the transfection construct

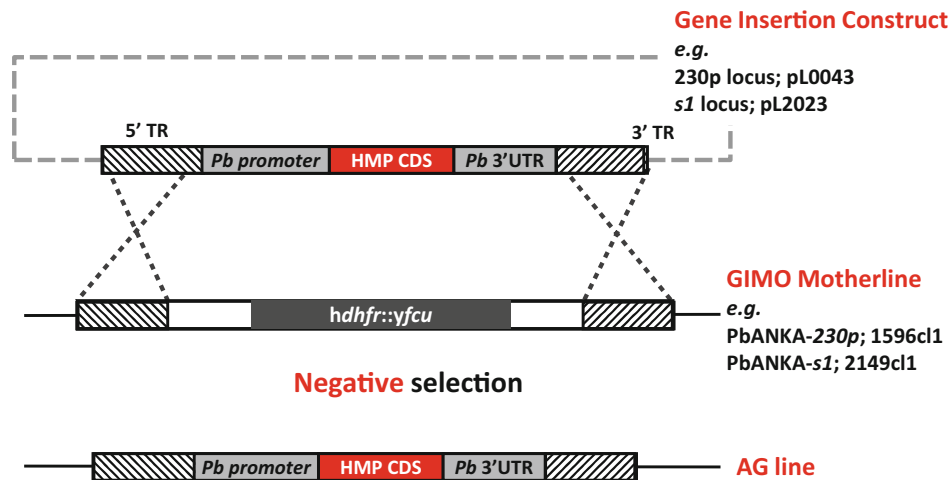


Fig. 1 Generation of AG mutants. Additional Gene (AG) mutants have a human malaria parasite (HMP) gene introduced into a neutral locus (*230p* or *s1* locus) of the *P. berghei* GIMO mother line genome by GIMO-transfection. Schematic representation of the construct to introduce the human malaria parasite (HMP) gene into the *230p* or *s1* locus of GIMO mother lines, resulting in the generation of the AG mutant after negative selection with 5-FC is shown. Two basic constructs can be used to rapidly introduce transgenes into neutral *Pb* loci by GIMO transfection; specifically pL0043 to introduce transgenes into the *230p* locus (for transfection into the *PbANKA-230p* GIMO mother line; 1596cl1) and pL2023 for the *s1* locus (for transfection into the *PbANKA-s1* GIMO mother line; 2149cl1). These basic constructs contain 5' and 3' TRs for these loci as well as a multiple-cloning site. The final Gene Insertion construct contains the HMP CDS is flanked upstream by the 5' UTR (containing the gene promoter region) and downstream by 3' UTR (containing the transcription terminator sequence) regions of the *Pb* ortholog, both of which can be PCR-amplified from *Pb* gDNA. The selection of the *Pb* promoter region is dependent on the timing and strength of HMP gene expression required (see Table 1). The HMP CDS can be PCR-amplified from HMP genomic DNA or cDNA. The construct is targeting the *230p* or *s1* target regions (hatched boxes) by double crossover homologous recombination (DXO), resulting in replacement of the *hdhfr::yfcu* selectable marker cassette that is present in the GIMO parasites with the HMP gene expression cassette (see Note 6 for details)

terminator sequence) regions. See Fig. 1 and Note 6 for details on the generation of these constructs.

Two different standard *Pb* GIMO lines exist containing the *hdhfr::yfcu* SM cassette either in the neutral *230p* locus (the *PbANKA-230p* GIMO mother line [4]), or the silent *s1* locus (the *PbANKA-s1* GIMO mother line) see Subheading 2.1. The latter line is currently unpublished but is available from the Leiden Malaria Research Group. This line exhibits normal development throughout the complete life cycle (CJJ and SMK, unpublished observations). The neutral *s1* locus has also been used in *P. yoelii* to introduce reporter proteins [17]. For introducing an HMP gene as an additional copy in *P. yoelii* XNL, a GIMO line exists (the *PyXNL-230p* GIMO mother line; [4]), which contains the *hdhfr::yfcu* SM cassette into the neutral *230p* locus (see Note 2).

3.1.1 Transfection of Parasites and Injection into Mice

1. Prepare 5–10 µg of linearized DNA construct in 5–10 µL water or TE buffer. This construct is based on a standard GIMO construct and contains the 5' and 3' TRs of the *230p* or *sI* targeting regions of the GIMO mother line and an HMP gene expression cassette (i.e., the HMP gene CDS flanked by 5' UTR (promoter) and 3' UTR of a chosen *Pb* gene; Fig. 1, **Note 6**).
2. Collect cultured (in vitro matured) *P. berghei* schizonts of the standard PbANKA-*230p* or PbANKA-*sI* GIMO mother lines (Subheading 2.1) for transfection as described in ref. [14].
3. Transfect the schizonts with the DNA construct using a Nucleofector device using the transfection protocol and conditions as described in ref. [14].
4. Inject the transfected parasites into a mouse tail vein using insulin syringes under anesthesia.

3.1.2 Negative Selection of Parasites (5-FC) and Cloning

1. Select transfected parasites by negative selection with 5-FC: provide the mouse with drinking water containing 5-FC one day after infection with transfected parasites. Provide the water for a period of 8–11 days up to the collection of infected blood.
2. Collect transfected and selected parasites between day 8 and 11 after infecting mice with transfected parasites. In successful transfection experiments, the parasitemia usually increases to 2–5 % between days 8 and 11. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 2–5 % by heart puncture under anesthesia.
3. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for cloning transfected parasites): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
4. Use the remainder of the blood (0.4–0.8 ml) to genotype the transfected parasite population by Southern analysis of separated chromosomes (see **Note 7a**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
5. If the genotyping shows correct deletion of the *hdhfr::yfcu* SM cassette, clone the parasites by the method of limiting dilution. Infect a mouse with transfected parasites from the liquid nitrogen storage and collect blood at a parasitemia of 0.2–1 % for limiting dilution of parasites and injecting 10 mice with 0.3 parasites per mouse using insulin syringes under anesthesia as described in ref. [20].
6. Collect cloned parasite lines at day 8/9 after cloning. The parasitemia usually increases to 0.5–2 % at day 8. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 1–3 %

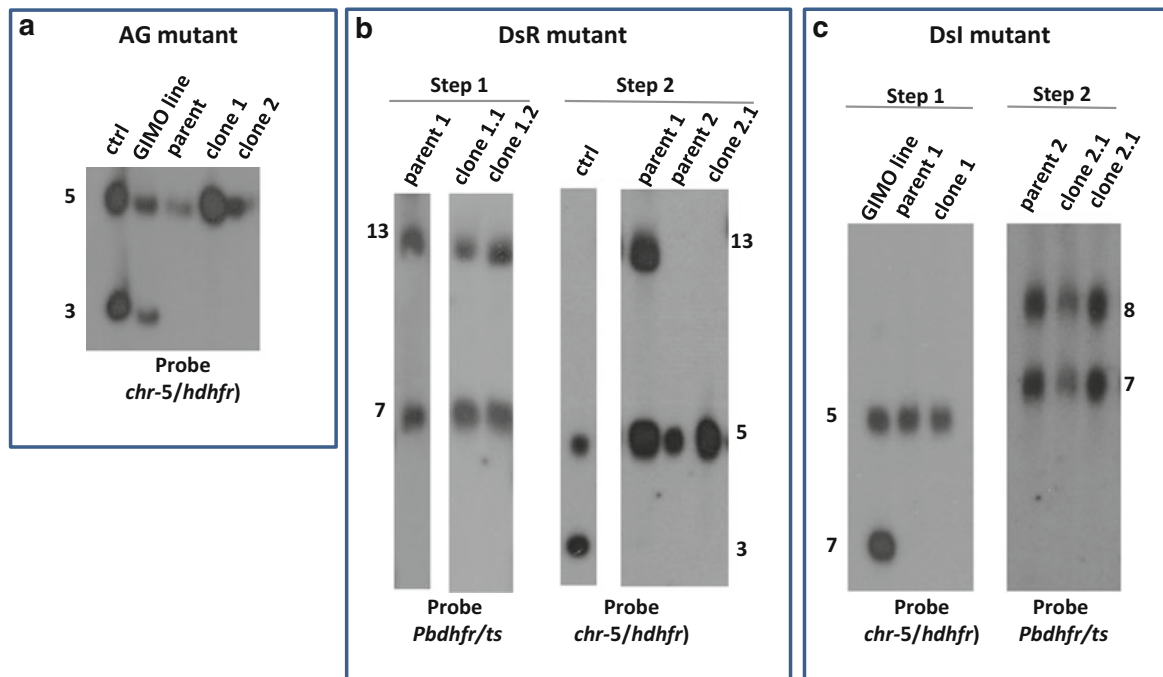


Fig. 2 Genotyping of AG, DsR, and DsI mutants. **(a) AG mutants:** Southern analysis of chromosomes (chrs) separated by pulsed-field gel electrophoresis (PFGE) to confirm integration of the DNA construct in the GIMO locus by showing the removal of the *hdhfr::yfcu* SM cassette both in uncloned (parent) and cloned parasites. The Southern blot is hybridized with a mixture of two probes: one recognizing *hdhfr* and a control probe recognizing chr 5. As an additional control (ctrl), parasite line 2117cl1 is used with the *hdhfr::yfcu* SM integrated into chr 3. **(b) DsR mutants:** Southern analysis of PFGE-separated chrs to confirm integration of the DNA construct into the target *Pb* gene both in uncloned (parent 1) and cloned parasites selected in step 1 of the DsR transfection protocol. The chrs are hybridized using a probe recognizing the 3'UTR *Pbdhfr/ts* of the SM of the integrated construct which also hybridizes to the endogenous *Pbdhfr/ts* on chr 7. For genotyping of selected parasites in step 2 of the DsR protocol (i.e., the uncloned parent 2 and cloned parasites): Southern analysis of PFGE-separated chrs to confirm integration of the orthologous HMP gene in the GIMO locus by showing the absence of the *hdhfr::yfcu* SM cassette. Separated chrs are hybridized with a mixture of two probes: one recognizing *hdhfr* and a control pb25 probe recognizing chromosome 5 as described above for the AG mutants. **(c) DsI mutants:** Southern analysis of PFGE-separated chrs to confirm integration of the DNA construct in the GIMO locus by showing the absence of the *hdhfr::yfcu* SM cassette both in uncloned (parent 1) and cloned parasites in step 1. Chrs are hybridized with a mixture of two probes: one recognizing *hdhfr* and a control probe recognizing pb25 on chr 5 as described above for the AG mutants. For genotyping of selected parasites in step 2 (the uncloned parent 2 and cloned parasites) we usually perform Southern analysis of PFGE-separated chromosomes to confirm integration of the DNA construct in the target *Pb* gene both in uncloned (parent 1) and cloned parasites selected with positive selection in step 1. The chrs are hybridized using a probe recognizing the 3'UTR *Pbdhfr/ts* of the SM of the integrated construct which also hybridizes to the endogenous *Pbdhfr/ts* on chr 7 as described above for step 1 of the DsR protocol

by heart puncture under anesthesia. Use 0.2 ml of infected blood for storage in liquid nitrogen: transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.

7. Use the remainder of the blood (0.4–0.8 ml) to genotype the cloned parasite population by Southern analysis of separated chromosomes (*see* **Note 7a**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
8. The cloned AG parasites with a correct genotype are used for phenotype analysis to (1) determine (normal) development throughout the life cycle and (2) to confirm correct HMP expression (*see* Subheading 3.4).

3.2 Transgenic Parasites Expressing HMP Proteins: Double Step Replacement (DsR) Mutants

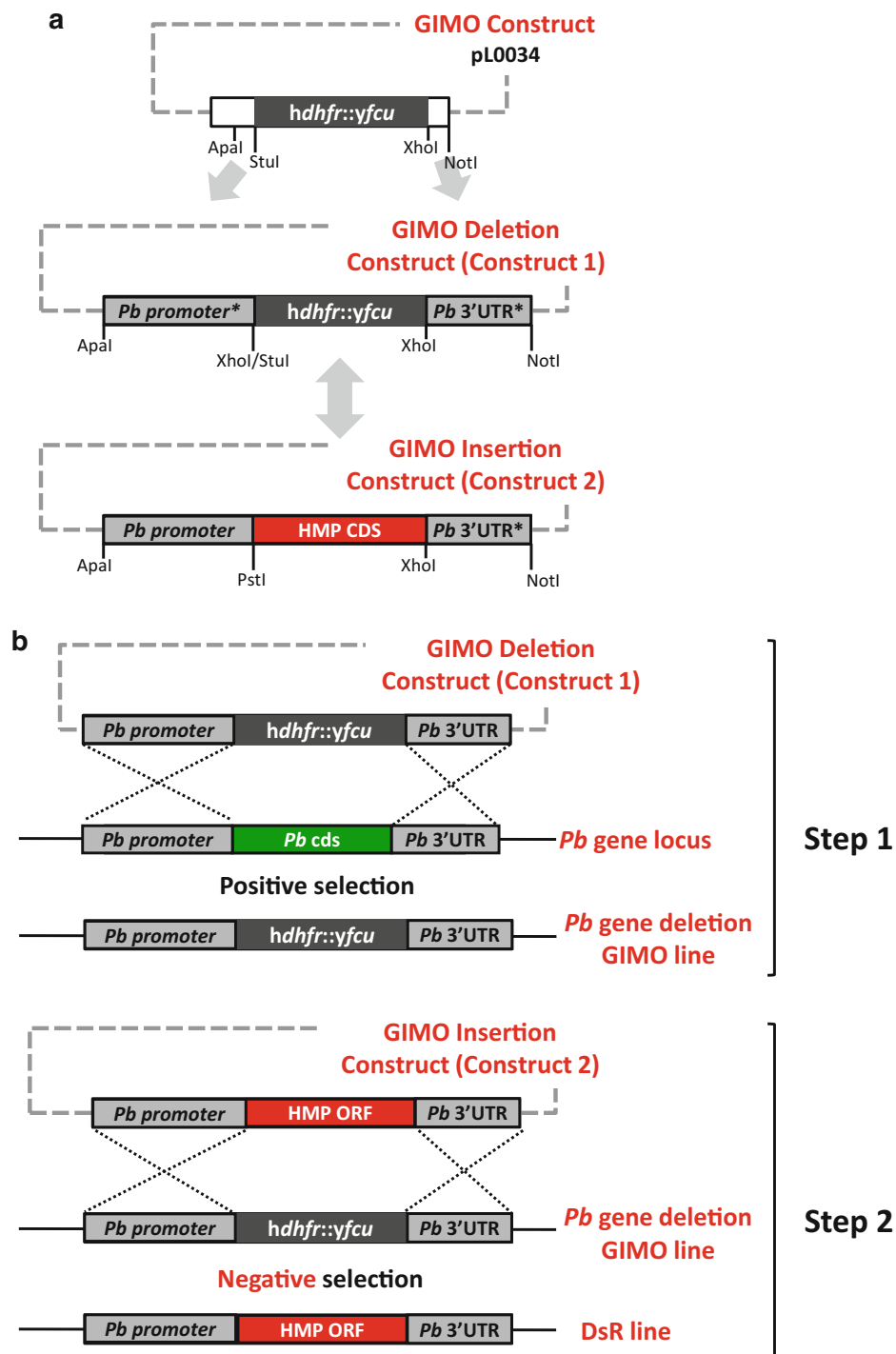
In DsR mutants the CDS of the *Pb* gene is replaced with the CDS of the HMP ortholog in a two-step transfection procedure (Fig. 3, **Note 8**). First, parasites are transfected with a gene-deletion construct designed to replace the *Pb* CDS with the *hdhfr::yfcu* SM cassette. This results in deletion of the *Pb* gene; however, selection of gene-deletion mutants (by positive selection with pyrimethamine) is only possible if the gene is not essential for blood stages. In the transfected parasites the locus of interest will contain unaltered 5' and 3' sequences upstream and downstream of the CDS. Clonal lines of these gene-deletion mutants will then be transfected with a second gene-insertion constructs that leads to replacement of the *hdhfr::yfcu* SM cassette with the orthologous HMP gene. Transfected parasites will be selected with 5-FC, which will kill the non-transfected parasites that still harbor *hdhfr::yfcu*. This method creates parasites that express an HMP gene under the control of regulatory sequences of the *Pb* ortholog, which are free of SM, permitting additional genetic modifications.

3.2.1 Transfection of Parasites and Injection into Mice: Transfection Step 1

1. Prepare 5–10 µg of linear DNA construct in 5–10 µL water or TE buffer. This construct (*Construct 1*) is a gene-deletion construct containing the *hdhfr::yfcu* SM cassette flanked by the 5' and 3' TRs of the *Pb* gene and is based on standard construct pL0034 (Subheading 2.3; Fig. 3, *see* **Note 8**).
2. Collect cultured (in vitro matured) *P. berghei* schizonts of wild type (WT; Subheading 2.1) or reference reporter lines (e.g., *PbGFP-Luc_{con}* or *PbGFP-Luc_{ama1}*; Subheading 2.1, *see* **Note 1**) for transfection as described in ref. [14].
3. Transfect the schizonts with *Construct 1* using a Nucleofector device using the transfection protocol and conditions as described in ref. [14].
4. Inject the transfected parasites into a tail vein of a mouse using insulin syringes under anesthesia.

3.2.2 Positive Selection of Parasites (Pyrimethamine) and Cloning: Transfection Step 1

1. Select transfected parasites by positive selection with pyrimethamine: provide the mouse with drinking water containing pyrimethamine 1 day after infection with transfected parasites. Provide the water for a period of 8–11 days up to the collection of infected blood.



2. Collect transfected and selected parasites between day 8 and 11 after infecting mice with transfected parasites. In successful transfection experiments, the parasitemia usually increases to 2–5 % between days 8 and 11. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 2–5 % by heart puncture under anesthesia.
3. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for cloning transfected parasites): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
4. Use the remainder of the blood (0.4–0.8 ml) to genotype the transfected parasite population by Southern analysis of separated chromosomes (*see Note 7b*, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
5. If genotyping shows correct deletion of the *Pb* CDS, the parasites are cloned by the method of limiting dilution. Infect a mouse with transfected parasites from the liquid nitrogen storage and collect blood at a parasitemia of 0.2–1 % for limiting dilution of parasites and injecting 10 mice with 0.3 parasites per mouse using insulin syringes under anesthesia as described in ref. [20]. Genotyping and cloning of the transfected parasites is an essential step before proceeding with transfection step 2 (*see Note 9*).

Fig. 3 (continued) 5'-TR created with an *XhoI* restriction site at the 5' end and a *PstI/XhoI* double restriction sites at the 3' end. Similarly the 3'-TR has been generated with an *XhoI* restriction site at the 5' end and *NotI* restriction sites at the 3' end. Therefore *Construct 1* has been constructed with a specific restriction site (*XhoI*) both just before and after the *hdhfr::yfcu* SM cassette, and digestion with this enzyme releases the *hdhfr::yfcu* SM cassette from the plasmid resulting in a linear DNA construct containing both 5' and 3' TRs with complementary ends. Self-ligation can then be performed to generate a "marker-free" construct with only the 5'- and 3'-TRs and a multiple-cloning site in between these TR which can in turn be used to insert the orthologous HMP gene and thereby create *Construct 2* (GIMO Insertion construct). The HMP CDS being flanked upstream by the orthologous *Pb* 5' UTR (containing the gene promoter region) and downstream by *Pb* 3' UTR (containing the transcription terminator sequence), *see Note 8* for details. **(b)** *Generation of DsR mutants* where the *Pb* target gene (TG) is replaced with the orthologous gene of a human malaria parasite (HMP). *1st step*: The *GIMO Deletion construct* (*Construct 1*) is used to replace the *P. berghei* gene with the positive/negative selectable maker (*hdhfr::yfcu*) cassette, resulting in the generation of the TG GIMO line after positive selection with pyrimethamine. *Construct 1* targets the TG at the target regions (*hatched boxes*) by double crossover homologous recombination. After genotyping and confirming the correct construct integration, this line is cloned. *2nd step*: The *GIMO insertion construct* (*Construct 2*) is used to replace the SM in the GIMO line with the HMP CDS, under negative (5-FC) selection, resulting in DsR mutants. *Construct 2* integrates by double crossover homologous recombination using the same TRs employed in *Construct 1*, resulting in the introduction of the HMP gene under the control of the *Pb* target gene promoter and transcriptional terminator sequences.

6. Collect cloned parasite lines at day 8/9 after cloning. The parasitemia usually increases to 0.5–2 % at day 8. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 1–3 % by heart puncture under anesthesia. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for “transfection step 2” (see below)): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
7. Use the remainder of the blood (0.4–0.8 ml) to genotype the cloned parasite population by Southern analysis of separated chromosomes (*see* **Note 7b**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].

3.2.3 Transfection of Parasites and Injection into Mice: Transfection Step 2

1. Prepare 5–10 µg of linear DNA construct in 5–10 µL water or TE buffer. This construct (*Construct 2*) contains the HMP-CDS flanked by the same *Pb* 5' UTR and 3' UTR sequences present in *Construct 1* (*see* Fig. 3, **Note 8**).
2. Collect cultured (in vitro matured) schizonts of cloned gene-deletion parasites (as generated in transfection step 1) for transfection as described in ref. [14].
3. Transfect the schizonts with *Construct 2* using an Nucleofector device using the transfection protocol and conditions as described in ref. [14].
4. Inject the transfected parasites into a tail vein of a mouse using insulin syringes under anesthesia.

3.2.4 Negative Selection of Parasites (5-FC) and Cloning: Transfection Step 2

1. Select transfected parasites by negative selection with 5-FC: provide the mouse with drinking water containing 5-FC one day after infection with transfected parasites. Provide the water for a period of 8–11 days up to the collection of infected blood.
2. Collect transfected and selected parasites between day 8 and 11 after infecting mice with transfected parasites. In successful transfection experiments, the parasitemia usually increases to 2–5 % between days 8 and 11. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 2–5 % by heart puncture under anesthesia.
3. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for cloning transfected parasites): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.

4. Use the remainder of the blood (0.4–0.8 ml) to genotype the transfected parasite population by Southern analysis of separated chromosomes (*see* **Note 7**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
5. If the genotyping shows correct deletion of the *hdlhfr::yfcu* SM cassette clone the parasites by the method of limiting dilution. Infect a mouse with transfected parasites from the liquid nitrogen storage and collect blood at a parasitemia of 0.2–1 % for limiting dilution of parasites and injecting 10 mice with 0.3 parasites per mouse using insulin syringes under anesthesia as described in ref. [20].
6. Collect cloned parasite lines at day 8/9 after cloning. The parasitemia usually increases to 0.5–2 % at day 8. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 1–3 %. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for “transfected step 2” (see below)): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
7. Use the remainder of the blood (0.4–0.8 ml) to genotype the cloned parasite population by Southern analysis of separated chromosomes (*see* **Note 7**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described [14].
8. The cloned DsR parasites with a correct genotype are used for phenotype analysis to (1) determine (normal) development throughout the life cycle and (2) to confirm correct HMP expression (*see* Subheading 3.4).

3.3 Transgenic Parasites Expressing HMP Proteins: Double-Step Insertion (Dsl) Mutants

When a gene is essential for blood stages it is not possible to use the DsR approach as described in Subheading 3.2, since the initial gene deletion step will yield no deletion mutants. In DsI mutants the HMP CDS is therefore first introduced into the neutral locus of an existing GIMO mother line (Subheading 2.1) as was described for AG mutants using a construct (*Construct 1*) copy of HMP but under the control elements of the *Pb* ortholog in a neutral locus, free of SM (*see* Fig. 4, **Note 10**). Subsequently, the *Pb* ortholog is targeted for deletion through replacement of the *Pb* gene with the *hdlhfr::yfcu* SM, resulting in transgenic parasites with a new GIMO locus permissive for rapid additional gene-insertion modifications (*see* Fig. 4, **Note 10**).

3.3.1 Transfection of Parasites and Injection into Mice: Transfection Step 1

1. Prepare 5–10 µg of linear DNA construct in 5–10 µL water or TE buffer. This construct (*Construct 1*) is based on a standard GIMO construct and contains an HMP flanked by the 5' UTR and 3' UTR regulatory sequences of the orthologous *Pb* gene (*see* Fig. 4, **Note 10**).

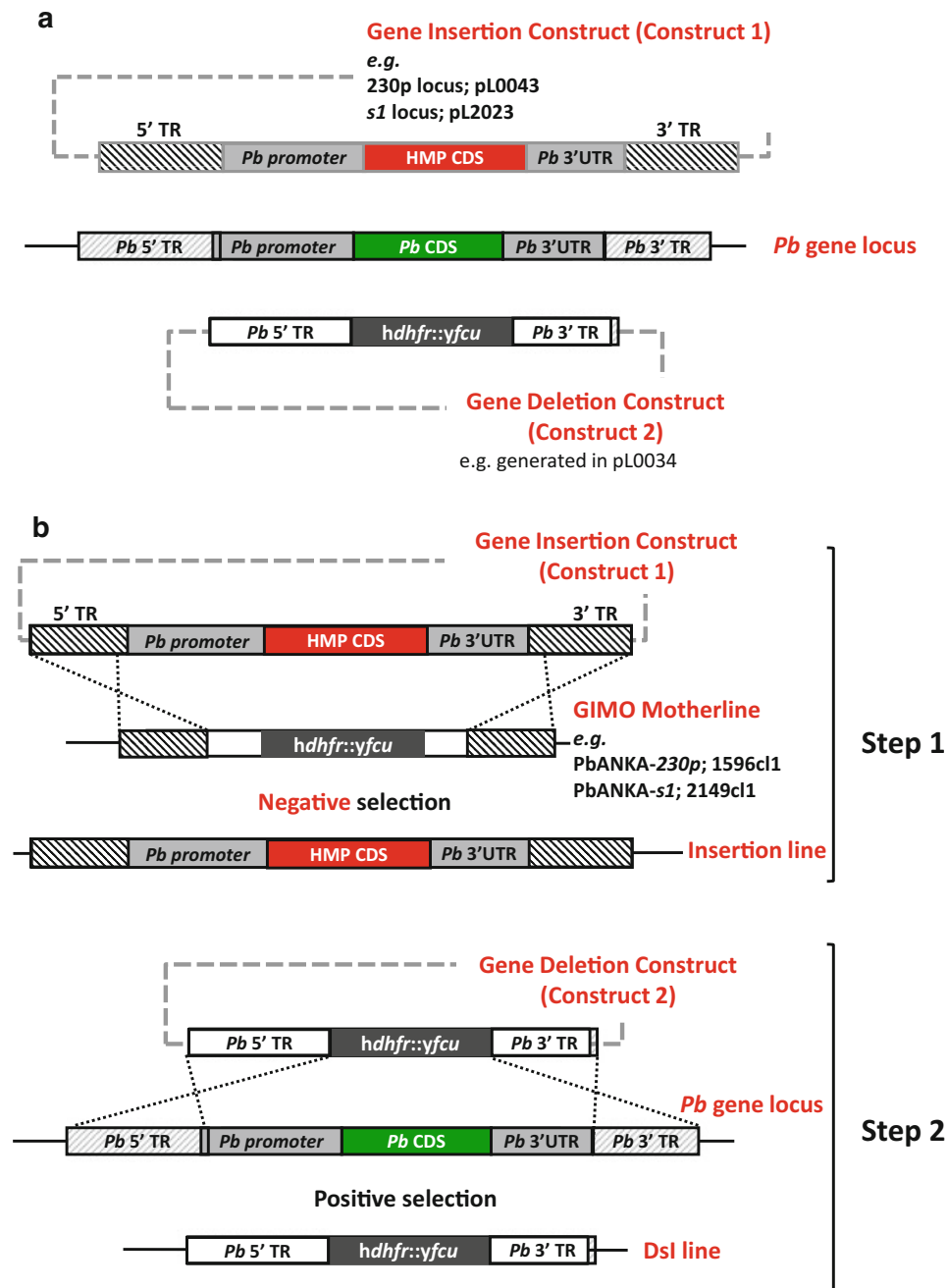


Fig. 4 Generation of Dsl mutants. **(a)** Generation of the two constructs for generation of Dsl mutants. Schematic representation describing the generation of *Construct 1* (*Gene Insertion construct*). Two basic constructs can be used to rapidly introduce transgenes by GIMO transfection into neutral *Pb loci*; specifically pL0043 to introduce transgenes into the *230p* locus (for transfection into the PbANKA-230p GIMO mother line; 1596cl1) and pL2023 for the *s1* locus (for transfection into the PbANKA-s1 GIMO mother line; 2149cl1). See Fig. 1 for details on the generation of constructs using these plasmids and the 5' and 3' Targeting regions (5' and 3' TR). The HMP CDS is flanked upstream by the gene promoter region and transcription terminator sequence of the *Pb* ortholog, both of which can be PCR-amplified from *Pb* gDNA. *Construct 1* is linearized using appropriate restriction enzymes outside of these 5' and 3' TRs before transfection. *Construct 2* (*Deletion construct*). This construct can be generated by cloning 5' and 3' TRs of the *Pb* gene into the cloning sites of basic construct pL0034, upstream and downstream of the SM, respectively. Both TRs can be PCR-amplified from *Pb* gDNA.

3.3.2 Negative Selection
of Parasites (5-FC)
and Cloning:
Transfection Step 1

2. Collect cultured (in vitro matured) *P. berghei* schizonts of a standard GIMO line (Subheading 2.1) for transfection as described in ref. [14].
 3. Transfect the schizonts with the DNA construct using an Nucleofector device using the transfection protocol and conditions as described in ref. [14].
 4. Inject the transfected parasites into a tail vein of a mouse using insulin syringes under anesthesia.
1. Select transfected parasites by negative selection with 5-FC: provide the mouse with drinking water containing 5-FC one day after infection with transfected parasites. Provide the water for a period of 8–11 days up to the collection of infected blood.
 2. Collect transfected and selected parasites between day 8 and 11 after infecting mice with transfected parasites. In successful transfection experiments, the parasitemia usually increases to 2–5 % between days 8 and 11. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 2–5 % by heart puncture under anesthesia.
 3. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for cloning transfected parasites): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
 4. Use the remainder of the blood (0.4–0.8 ml) to genotype the transfected parasite population by Southern analysis of separated chromosomes (see Note 7c, Fig. 2) or digested genomic DNA or by diagnostic PCR as described [14].

Fig. 4 (continued) *Note: It is important to choose at least one (ideally both) Pb TR (here shown as hatched boxes) that are different from the promoter/terminator regions used in Construct 1 to avoid targeting the HMP gene when it has been introduced into the neutral locus.* The generated construct is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs. See Note 10 for details. **(b) Generation of Dsl mutants,** the HMP is introduced into a neutral locus and the *Pb* ortholog is subsequently deleted; *1st step:* the HMP expression cassette is introduced into a neutral locus (the *230p* or *s1* locus) in a standard GIMO mother line as described for AG mutants but under the control elements of *Pb* orthologous gene using the *Construct 1 (Gene Insertion construct)*. The construct targets the *230p* or *s1* target regions by double crossover homologous recombination, under negative (5-FC) selection, resulting in replacement of the *hdhfr/yfcu* selectable marker cassette present in the GIMO Mother line. After genotyping and confirming the correct construct integration this line is cloned. *2nd step:* The essential *Pb* ortholog gene CDS is targeted for deletion through replacement of the *Pb* CDS with the *hdhfr:yfcu* SM using *Construct 2 (Deletion construct)*, by homologous double crossover recombination performed under positive selection with pyrimethamine resulting in the generation of the *Dsl mutant*

5. If the genotyping shows correct deletion of the *hdhfr::yfcu* SM cassette clone the parasites by the method of limiting dilution. Infect a mouse with transfected parasites from the liquid nitrogen storage and collect blood at a parasitemia of 0.2–1 % for limiting dilution of parasites and injecting 10 mice with 0.3 parasites per mouse using insulin syringes under anesthesia as described in ref. [20]. Genotyping and cloning of the transfected parasites is an essential step before proceeding with transfection step 2 (see **Note 9**).
6. Collect cloned parasite lines at day 8/9 after cloning. The parasitemia usually increases to 0.5–2 % at day 8. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 1–3 % by heart puncture under anesthesia. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for “transfected step 2” (see below)): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
7. Use the remainder of the blood (0.4–0.8 ml) to genotype the cloned parasite population by Southern analysis of separated chromosomes or digested genomic DNA (see **Note 7c**, Fig. 2) or by diagnostic PCR as described in ref. [14].

**3.3.3 Transfection
of Parasites and Injection
into Mice: Transfection
Step 2**

1. Prepare 5–10 µg of linear DNA construct in 5–10 µL water or TE buffer. This construct (*Construct 2*) contains *hdhfr::yfcu* SM cassette flanked by 5' and 3' UTR regions outside of the *Pb* gene regulatory sequences (see Fig. 4, **Note 10**).
2. Collect cultured (in vitro matured) schizonts of cloned transgenic HMP-expressing parasites (as generated in transfection step 1) for transfection as described in ref. [14].
3. Transfect the schizonts with *Construct 2* using an Nucleofector device using the transfection protocol and conditions as described in ref. [14].
4. Inject the transfected parasites into a tail vein of a mouse using insulin syringes under anesthesia.

**3.3.4 Positive Selection
of Parasites
(Pyrimethamine)
and Cloning:
Transfection Step 2**

1. Select transfected parasites by positive selection with pyrimethamine: provide the mouse with drinking water containing pyrimethamine 1 day after infection with transfected parasites. Provide the water for a period of 8–11 days up to the collection of infected blood.
2. Collect transfected and selected parasites between day 8 and 11 after infecting mice with transfected parasites. In successful transfection experiments, the parasitemia usually increases to 2–5 % between days 8 and 11. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 2–5 % by heart puncture under anesthesia.

3. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for cloning transfected parasites): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
4. Use the remainder of the blood (0.4–0.8 ml) to genotype the transfected parasite population by Southern analysis of separated chromosomes (*see* **Note 7**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
5. If genotyping shows correct deletion of the *Pb* CDS clone the parasites by the method of limiting dilution. Infect a mouse with transfected parasites from the liquid nitrogen storage and collect blood at a parasitemia of 0.2–1 % for limiting dilution of parasites and injecting 10 mice with 0.3 parasites per mouse using insulin syringes under anesthesia as described in ref. [20].
6. Collect cloned parasite lines at day 8/9 after cloning. The parasitemia usually increases to 0.5–2 % at day 8. Collect 0.6–1 ml of blood from the mouse with a parasitemia of 1–3 % by heart puncture under anesthesia. Use 0.2 ml of infected blood for storage in liquid nitrogen (these parasites are used for “transfection step 2” (see below)): transfer 0.2 ml of the suspension to an Eppendorf tube containing 0.3 ml glycerol-PBS stock solution; distribute this suspension equally among two cryogenic vials, 0.25 ml per tube, and store these tubes within 20 min in liquid nitrogen.
7. Use the remainder of the blood (0.4–0.8 ml) to genotype the cloned parasite population by Southern analysis of separated chromosomes (*see* **Note 7**, Fig. 2) or digested genomic DNA or by diagnostic PCR as described in ref. [14].
8. The cloned DsI parasites with a correct genotype are used for phenotype analysis to (1) determine (normal) development throughout the life cycle and (2) to confirm correct HMP expression (*see* Subheading 3.4).

3.4 Phenotype Analysis of Transgenic Parasites Expressing HMP Genes

Before using transgenic *Pb* parasites expressing HMP genes in assays for drug- or vaccine evaluation, the phenotype is analyzed to (1) determine whether HMP gene expression affects normal development and (2) to confirm correct timing and level of HMP gene expression. Below we briefly mention several assays that are used for such analyses and refer to published methods.

3.4.1 Transgenic Parasites Expressing HMP Genes in Asexual Blood Stages

1. Determination of blood stage growth. A standard method to analyze growth is determined from the multiplication rate of asexual blood stages in vivo during the cloning procedure of mutants [16] and is calculated as follows: the percentage of infected erythrocytes in Swiss OF1 mice injected with a single parasite is quantified at days 8–11 by examination of

Giemsa-stained blood films. The mean asexual multiplication rate per 24 h is then calculated assuming a total of 1.2×10^{10} erythrocytes per mouse (2 ml of blood). The percentage of infected erythrocytes in mice infected with a single parasite of a *Pb* ANKA reference lines ranges between 0.5 and 2 % at day 8 after infection, resulting in a mean multiplication rate of 10 per 24 h [16].

A second frequently used method to determine asexual growth rate is by examining the course of parasitemia after infecting mice with a standard dose of infected red blood cells (irbc, i.e., 10^3 – 10^4 irbc). The course of parasitemia can be determined by counting irbc in Giemsa stained blood films of tail blood samples or, when parasites express GFP or Luciferase, by standard FACS [21] or simple bioluminescence assays [22] respectively.

2. Determination of HMP expression. This is performed using standard Northern analysis of transcription, Western and or immunofluorescence analysis of protein expression methods.

3.4.2 Transgenic Parasites Expressing HMP Genes in Transmission Stages (Gametocytes, Zygotes, Oocysts, and Sporozoites)

1. Gametocyte production is usually determined by counting mature gametocytes in tail blood of mice that have either synchronized or non-synchronized blood stage infections [23]. Ookinete production is usually determined in standard in vitro cultures of ookinetes [24] and is usually defined as the percentage of female gametocytes/gametes that develop into mature ookinetes. The ratio of unfertilized female gametocytes/gametes to ookinetes is determined in Giemsa stained slides of samples taken from the ookinete cultures at 16–24 h [25]. Oocysts and sporozoites production is determined in *A. stephensi* mosquitoes that are infected on gametocytemic mice. This is usually performed by microscopic analysis of dissected midguts and salivary glands [26].
2. Determination of HMP expression. This is performed using standard Northern analysis of transcription, Western and or immunofluorescence analysis of protein expression methods.

3.4.3 Transgenic Parasites Expressing HMP Genes in Liver Stages

1. Sporozoite infectivity and liver stage development can be determined in cultures of hepatocytes (e.g., HepG2, Huh7) or in mice infected with sporozoites, either by mosquito bite or by intravenous injection of sporozoites [27]. Sporozoites are obtained by standard methods of dissection of salivary glands of infected mosquitoes [27]. Sporozoite infectivity and liver parasite loads can be quantified by qPCR [28] or, when parasites express Luciferase, by bioluminescence assays (both in vitro and in vivo imaging; [27]) or by measuring the prepatent period (time taken, in days, to achieve a parasitemia in the blood of 0.5–2 % after infection of mice with defined numbers of sporozoites; [29]).

2. Determination of HMP expression. This is performed using standard Northern analysis of transcription, Western and or immunofluorescence analysis of protein expression methods.

4 Notes

1. *PbGFP-Luc_{amal}* (1037mlf1mlcl1) and the *PbGFP-Luc_{con}* (676mlcl) are reporter parasites expressing a fusion protein of GFP (mutant3) and firefly luciferase (LUC-IAV). These lines are suitable reporter lines for use in simple and sensitive in vitro and in vivo screening assays to test inhibitors and chemicals for antimalarial activity against blood stages [22]. These assays are based on the determination of luciferase activity (luminescence) in small blood samples containing transgenic blood-stage parasites that express luciferase under the control of a promoter that is either schizont-specific (*ama-1*) or constitutive (*eef1a*). The reading of luminescence assays is rapid, requires a minimal number of handling steps and no experience with parasite morphology or handling fluorescence-activated cell sorters, produces no radioactive waste and test plates can be stored for prolonged times before processing. In addition, the *PbGFP-Luc_{con}* (676mlcl) line can be used to determine the number of parasites in the liver (i.e., liver loads) in live animals over time, using in vivo imaging methodologies [27, 30].
2. The standard PyXNL-230p GIMO mother line (GIMO_{Py17X}, 1923cl1). The line contains a positive–negative selectable marker (SM) cassette, a fusion gene of *hdhfr* (*human dihydrofolate reductase*; positive SM) and *yfcu* (*yeast cytosine deaminase and uridyl phosphoribosyl transferase*; negative SM) under control of the constitutive *eef1a* promoter stably integrated into the neutral 230p locus (PY17X_0306600) through double crossover recombination [4]. The GIMO mother line is used for introduction of transgenes into the modified 230p locus through transfection with constructs that target the 230p locus. These constructs insert into the 230p locus (“gene insertion”), thereby removing the *hdhfr::yfcu* selectable marker (“marker out”) from the genome of the mother lines. Transgenic parasites that are SM free are subsequently selected by applying negative drug selection using 5-FC. This selection procedure is performed in vivo in mice. For details of the PyXNL-230p GIMO mother line, see RMgmDB entry #688 (<http://www.pberghei.eu/index.php?rmgm=688>).
3. To generate pL0043, the 230p TRs as well as the ampicillin resistance gene were amplified from plasmid pL1063 (MRA-852, www.mr4.org) using primers 5116/5117 (GGGGTACCGAGCTCGAATTCTCTTGAGC;

GGGGTACCGAGCTCGAATTCTCTTGAGC). A multiple cloning site (MCS) was amplified from pCII-Blunt-TOPO vector (Zero Blunt TOPO PCR Cloning Kit, Invitrogen, Groningen, The Netherlands) using M13 forward and reverse primers. The two PCR products were digested with *Asp*718I and *Not*I restriction enzymes and ligated together creating the targeting construct pL0043.

4. pL1849 was generated using a modified 2-step PCR method (*see Note 5*). In the first PCR reaction, 5'- and 3'-TRs (both 1 kb) of 230p were amplified from *P. yoelii* 17XNL genomic DNA with the primer set 6523/6524 (GAAGTCGTACT CCTTGGTGACGGGTACCGTGATGGAATGGCAACA TCTG; CATCTACAAGCATCGTCGACCTCGGTTGGAC AATGTAATGCTAC) and 6525/6526 (CCTTCAATTTT GGATCCACTAGAAAGTAAAAGGGGTAAGACAGC; CCTTCAATTTTCGGATCCACTAGAAAGTAAA GGGGTAAGACAGC). These primers contain 59-extensions homologues to the *hdhfr::yfcu* selection marker cassette and 59-terminal extensions with an anchor-tag suitable for the second PCR reaction. A 55 nt oligo (oligo 6598; GAGGTCGACGATGCTTGTAGATGCCCCGGGCCTT CAATTTTCGGATCCACTAG) containing a *Xma*I restriction site flanked by 2 sequences homologues to the *hdhfr::yfcu* selection cassette was used to join the two 230p TR regions. In the second PCR reaction an fragment containing both 230p TRs interrupted by the *Xma*I site was amplified, using the external anchor-tag primers 4661/4662 (GAAGTCGTACT CCTTGGTGACG; GAAGTCGTACTCCTTGGTGACG), resulting in the PCR product of 2 kb. The PCR product was cloned into TOPO TA vector (TOPO TA Cloning Kit, Invitrogen, Groningen, The Netherlands) resulting in construct pL1849.
5. To generate plasmid pL2023, the 5'- and 3'-TRs (each about 0.8 kb) of the *sI* locus were PCR-amplified *Pb* genomic DNA using primer pairs 1003/1004 (5'TR; CATGGGCCC ACCATGCTTTGTCTGAGAGTG and AAGGCCTGGTA CCATACTGTTCTTCCCAATGGATC) flanked by *Apa*I restriction site at the 5' end and both *Stu*I/*Kpn*I restriction sites at its 3' end of the PCR fragment and the primers 1005/1006 (3'UTR; ATAAGAATGCGGCCGCCTGCAG CATTCAAATGCTTGAAGGCGATG and ACATGGCGCC AAGCTTATGGCACATGGATCGAACAG), flanked by *Not*I/*Pst*I restriction sites at the 5' end and *Kas*I/*Hind*III restriction sites at its 3' end of the PCR fragment. The 5' and 3' TRs were subsequently cloned into the sites of *Apa*I/*Stu*I and *Not*I/*Kas*I of pL0034, upstream and downstream of the *hdhfr::yfcu* SM cassette, respectively, resulting construct

pL1928, which has been used to generate PbANKA-*sI* GIMO mother line, 2149cl1, using positive selection (Subheading 2.1).

Currently pL1928 is unpublished but is available from the Leiden Malaria Research Group. Plasmid pL1928 has been constructed with a specific restriction site (*KpnI*) both just before and after the *hdhfr::yfcu* SM cassette, therefore digestion with this enzyme releases the *hdhfr::yfcu* SM cassette from the plasmid resulting in a linear DNA construct containing both 5' and 3' TRs of the *sI* locus with complementary ends. Self-ligation was performed to generate a "marker-free" *sI* targeting construct (pL2023) with only the 5'- and 3'-*sI* TRs flanking a multiple-cloning site. Therefore, pL2023 is a basic construct that can be used to rapidly clone HMP genes into the neutral *sI* *Pb* locus and introduced into a PbANKA-*sI* mother line that also expresses a fluorescent-bioluminescent reporter (i.e., GFP-luciferase; PbANKA-*sI* GIMO mother line, 2149cl1).

6. Constructs to generate AG mutants (Subheading 3.1):

Two basic constructs (Subheading 2.3) can be used to rapidly introduce transgenes into neutral *Pb* loci by GIMO transfection; specifically pL0043 to introduce transgenes into the *230p* locus (for transfection into the PbANKA-230p GIMO mother line; 1596cl1) and pL2023 for the *sI* locus (for transfection into the PbANKA-*sI* GIMO mother line; 2149cl1). These basic constructs contain 5' and 3' TRs for these loci as well as a multiple-cloning site. This multi-cloning site is used to introduce the HMP CDS, which can be PCR-amplified from HMP genomic DNA (gDNA) or cDNA. The HMP CDS is flanked upstream by the 5' UTR (containing the gene promoter region) and downstream by 3' UTR (containing the transcription terminator sequence) regions of a chosen *Pb* gene, both of which can be PCR-amplified from *Pb* gDNA (Fig. 3a). The selection of the *Pb* promoter region is dependent on the timing and strength of HMP gene expression required, and a variety of standard *Pb* promoter regions have been characterized and are available (see Table 1 for details). The regions critical for HMP gene expression (i.e., the promoter and HMP CDS) of the final construct must be sequenced to ensure that the HMP gene will be correctly expressed in the AG mutant. In addition, an expression cassette containing a reporter gene can also be introduced into this construct if required. For example the GFP::luciferase expression cassette can be PCR-amplified from construct pL1063 [15]. In case of using PbANKA-*sI* (2149cl1) GIMO mother line there is no need to include the GFP-Luciferase cassette into the *sI* targeting construct, since this line already contains the GFP-Luciferase cassette integrated into the neutral *230p* locus. The final *Construct I* is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs.

7. Genotyping of AG, DsR, and DsI mutants (*see* Fig. 2)

- (a) AG mutants: We usually perform Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (PFGE) to confirm integration of the DNA construct in the GIMO locus by showing the absence of the *hdhfr::yfcu* SM cassette both in uncloned (parent) and cloned parasites. Separated chromosomes are hybridized with a mixture of two probes: one recognizing *hdhfr* (recognizing the SM in chr 3 and chr 12 for the 230p and *sl* mother lines respectively) and a control probe (we frequently use *pb25* gene probe that recognizes *Pb* chromosome 5). As an additional control (ctrl), parasite line is used with the *hdhfr::yfcu* SM integrated into a known *Pb* chromosome (we frequently use line 2117cl1 that has the SM in chr 3).
- (b) DsR mutants: We usually perform Southern analysis of PFGE-separated chromosomes to confirm integration of the DNA construct in the target *Pb* gene both in uncloned (parent 1) and cloned parasites selected with positive selection in step 1. The chromosomes are hybridized using a probe recognizing the *Pbdhfr/ts* 3'UTR of the SM of the integrated construct which also hybridizes to the endogenous *Pbdhfr/ts* gene on chromosome 7. The ratio between the hybridization signal of the target chromosome and of chromosome 7 is informative for both the success of vector integration, the contamination with wild-type parasites and the possible presence of plasmids that usually migrate at the height of chromosome 9–11. For genotyping of selected parasites in step 2 (uncloned/parent 2 and cloned) we usually perform Southern analysis of PFGE-separated chromosomes to confirm integration of the orthologous HMP gene in the GIMO locus by showing the absence of the *hdhfr::yfcu* SM cassette. Separated chromosomes are hybridized with a mixture of two probes: one recognizing *hdhfr* and a control probe (for example recognizing chr 5; *pb25*) as described under (a) for the AG mutants.
- (c) DsI mutants: We usually perform Southern analysis of PFGE-separated chromosomes to confirm integration of the DNA construct in the GIMO locus by showing the absence of the *hdhfr::yfcu* SM cassette both in uncloned (parent 1) and cloned parasites in step 1. Separated chromosomes are hybridized with a mixture of two probes: one recognizing *hdhfr* and a control (*pb25*) probe recognizing chromosome 5 as described under (a) for the AG mutants. For genotyping of selected parasites in step 2 (uncloned/parent 2 and cloned) we usually perform

Southern analysis of PFGE-separated chromosomes to confirm integration of the DNA construct in the target *Pb* gene both in uncloned (parent 1) and cloned parasites selected with positive selection in step 1. The chromosomes are hybridized using a probe recognizing the 3'UTR *Pb**dhfr/ts* of the SM of the integrated construct which also hybridizes to the endogenous *Pb**dhfr/ts* on chromosome 7 as described under (b) for step 1.

8. Generation of the two constructs for generation of DsR mutants (Subheading 3.2).

Construct 1 (GIMO Deletion construct): This construct can be generated by cloning 5' and 3' TRs of the *Pb* gene (these regions need to include the complete *Pb* gene promoter and transcriptional terminator sequences) into the cloning sites of the basic construct pL0034 (Subheading 3.2), upstream and downstream of the *hdhfr::yfcu* SM, respectively. Both TRs can be PCR-amplified from *Pb* gDNA. The targeting regions need to be chosen outside of the *Pb* CDS sequence and preferably >0.6 kb in size (Fig. 2). The generated construct is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs. The first transfection with *Construct 1* is aiming to replace the *Pb* CDS with the *hdhfr::yfcu* SM cassette. *Construct 1* can be transfected into a standard reporter parasite line *Pb*GFP-Luc_{con} (676m1cl1; Subheading 2.1, item 1) to generate a *Pb* gene deletion GIMO mother line that also encodes the GFP-Luciferase expression cassette.

Construct 2 (GIMO Insertion construct): This construct is generated based on *Construct 1*, replacing the *hdhfr::yfcu* SM cassette in *Construct 1* with HMP gene CDS sequence, which can be PCR-amplified from HMP gDNA or cDNA, resulting a HMP gene expression construct under the control of the 5' and 3' regulatory elements of the *Pb* ortholog. *Construct 1* can be constructed with specific restriction sites just before and after the *hdhfr::yfcu* SM cassette; therefore digestion with these enzymes will release the *hdhfr::yfcu* SM cassette from the plasmid resulting in a linear DNA construct containing only both 5' and 3' TRs of the target *Pb* gene. This linear construct can be used as a vector to clone/insert the orthologous HMP into it to generate *Construct 2* which contains the HMP CDS flanked upstream by the orthologous *Pb* 5' UTR (containing the gene promoter region) and downstream by *Pb* 3' UTR (containing the transcription terminator sequence) (Fig. 2).

The generated *Construct 2* is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs. A second transfection is performed in the gene-deletion GIMO mother line with *Construct 2* resulting in a replacement of the *hdhfr::yfcu* SM cassette with the orthologous HMP gene.

This method creates parasites that express an HMP gene under the control of the *Pb* ortholog regulatory sequences, these mutants are also SM free, facilitating easier additional genetic modifications.

9. Both for the generation of the DsR and DsI mutants, genotyping and cloning of the transfected parasites in step 1 is an essential step before proceeding with transfection step 2. In our experience, the use of parasites from uncloned populations (selected in step 1) for transfection in step 2 increases the chance to select for incorrect mutants in step 2.
10. Generation of the two constructs for generation of DsI mutants (Subheading 3.3).

Construct 1 (Gene Insertion construct): Two basic constructs can be used to rapidly introduce transgenes by GIMO transfection into neutral *Pb* loci (Subheading 2.3); specifically pL0043 to introduce transgenes into the *230p* locus (for transfection into the PbANKA-230p GIMO mother line; 1596cl1) and pL2023 for the *sI* locus (for transfection into the PbANKA-sI GIMO mother line; 2149cl1). These basic constructs contain 5' and 3' TRs for these loci as well as a multiple-cloning site. This multi-cloning site is used to introduce the HMP CDS, which can be PCR-amplified from HMP genomic DNA or cDNA. The HMP CDS is flanked upstream by the 5' UTR (containing the full gene promoter region) and downstream by 3' UTR (containing the transcription terminator sequence) regions of the *Pb* ortholog, both of which can be PCR-amplified from *Pb* gDNA (Fig. 3a). The regions critical for HMP gene expression (i.e., the promoter and HMP CDS) of the final construct must be sequenced to ensure that the HMP gene will be correctly expressed in the DsI mutant. For stage-specific expression or overexpression, other control elements of *Pb* genes could also be used (*see* Table 1). In addition, an expression cassette containing a reporter gene can also be introduced into this construct if required. For example GFP::luciferase expression cassette, which can be PCR-amplified from construct pL1063 [15]. However, when using construct pL2023 there is no need to include the GFP-Luciferase cassette into this construct, since PbANKA-sI (2149cl1) GIMO mother line already contains the GFP-Luciferase cassette integrated into the neutral *230p* locus. The final *Construct 1* is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs.

Construct 2 (Deletion construct): This construct can be generated by cloning 5' and 3' TRs of the *Pb* gene into the cloning sites of basic construct pL0034 (Subheading 3.2),

upstream and downstream of the *hdhfr:yfcu* SM, respectively. Both TRs can be PCR-amplified from *Pb* gDNA. It is important to choose at least one (ideally both) *Pb* TR that is different from the promoter/terminator regions used for HMP gene expression, to avoid removing the HMP gene introduced into the neutral locus. For small or medium sized genes (<3 kb), the targeting regions could be chosen outside of the *Pb* gene (*see* Fig. 3). For genes >3 kb in size, or where the regions between the neighboring genes are small (i.e., <1 kb), the 5' and/or 3' TR could also be designed within the *Pb* CDS. The generated construct is linearized using appropriate restriction enzymes outside of the 5' and 3' TRs.

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