Published in final edited form as:

Methods Mol Biol. 2010 ; 634: 87–100. doi:10.1007/978-1-60761-652-8_6.

A Method for Rapid Genetic Integration into *Plasmodium falciparum* Utilizing Mycobacteriophage Bxb1 Integrase

Sophie H. Adjalley, Marcus C.S. Lee, and David A. Fidock

Abstract

Genetic manipulation of the human malaria parasite *Plasmodium falciparum* has presented substantial challenges for research efforts aimed at better understanding the complex biology of this highly virulent organism. The development of methods to perform gene disruption, allelic replacement or transgene expression has provided important insights into the function of parasite genes. However, genomic integration studies have been hindered by low transfection and recombination efficiencies, and are complicated by the propensity of this parasite to maintain episomal replicating plasmids. We have developed a fast and efficient site-specific system of integrative recombination into the *P. falciparum* genome, which is catalyzed by the mycobacteriophage Bxb1 serine integrase. This system has the advantage of providing greater genetic and phenotypic homogeneity within transgenic lines as compared to earlier methods based on episomal replication of plasmids. Herein, we present this methodology.

Keywords

Plasmodium falciparum; Malaria; Bxb1 integrase; *attB* and *attP* sites; Integrative recombination; Transfection; Transgene expression

1. Introduction

The clinical and public health impacts of malaria are enormous, causing disease in over 500 million individuals and resulting in the death of approximately one million young children each year (1). In recent years, substantial efforts have been devoted to developing tools to genetically manipulate *Plasmodium falciparum*, the most lethal etiologic agent of malaria. Knockouts, allelic replacements, and transgene expression are now established techniques for investigating *Plasmodium* gene function (2). Procedures to manipulate the *P. falciparum* genome, however, have been hampered by the low efficiencies of transfection and recombination, as well as the persistence of self-replicating episomal concatemers, which limit the frequency of integration events into the parasite genome (2).

Integrative recombination constitutes an effective method to target exogenous DNA into specific genomic locations. For bacteriophages, integration into the bacterial host chromosome is typically mediated by tyrosine or serine integrases that catalyze site-specific recombination between phage *attP* and bacterial *attB* sites (3). These phage integrases, which mediate integration as well as excision reactions, can differ in their requirements for accessory proteins (4). Integration events are highly directional, thus excluding the possibility of spontaneous reversion. Serine integrases of the mycobacteriophage Bxb1 as well as of *Streptomyces* sp. phage ϕ C31 have been extensively used for in vitro integration.

In particular, the stability of the *attB* × *attP* locus generated by the ϕ C31 system has been well documented in yeast and bacteria (5, 6).

Mycobacteriophage Bxb1 is a temperate phage that integrates its genome at an *attB* site located within the *Mycobacterium smegmatis* *groEL1* gene. Recombination occurs between a 48-bp *attP* and a 38-bp *attB* site, each of which contains interrupted inverted repeats that flank a conserved 5'-GT dinucleotide (7). The non-palindromic nature of this central 5'-GT dinucleotide determines the directionality of integration. Unlike the tyrosine integrase systems, serine integrase-based recombination only requires the recombinase to efficiently catalyze the reaction, with no requirement for bacterial host cofactors, divalent cations, or DNA super-coiling (7). These properties make the serine integrase-mediated recombination system an ideal tool for targeted gene delivery in heterologous systems.

We have applied this serine integrase-driven recombination to *P. falciparum*, and find that transiently expressed mycobacteriophage Bxb1 integrase can efficiently catalyze recombination between an *attP*-containing plasmid and an *attB* site integrated into the parasite genome. This system was first established to achieve rapid integration of a *pfenr*-GFP gene fusion. *pfenr* (also known as *pffabI*) encodes *P. falciparum* enoyl acyl carrier protein ACP reductase, which is a component of the type II fatty acid synthesis pathway that functions in the parasite apicoplast. Microscopic studies localized the corresponding PfENR-GFP protein product to this intracellular organelle (8).

The integrase-based recombination offers several advantages over episomal-based transgene expression: namely, a higher proportion of fluorescent parasites when the fluorescently tagged transgene is integrated and a more uniform intensity of fluorescence (Fig. 1a–c). This provides for a more genetically and phenotypically homogeneous expression system as compared to episomal expression, where plasmid copy numbers and transgene phenotypes display higher variability (8, 9). This system has now become a standard tool in our lab to permit studies of transgene expression in phenotypically homogeneous parasite cultures.

Below, we describe the materials and methods of our integrase-based dual-plasmid co-transfection approach, which we now routinely utilize when conducting studies of transgenes expressed as a single copy from the *P. falciparum* genome. The simplicity of this integrase-based reaction, which can function without a requirement for exogenous host factors, makes it likely that such a system could be adapted to many heterologous cell systems.

2. Materials

2.1. Parasite Cultures

1. Dd2^{*attB*} and 3D7^{*attB*} parasite lines harboring an *attB* site integrated at the non-essential *cg6* locus. These lines are available from the Malaria Research and Reference Reagent Resource Center (MR4, Manassas, VA – www.malaria.mr4.org). We note that Dd2^{*attB*} propagates more rapidly and is our preferred host line.
2. RPMI 1640 with L-glutamine (Invitrogen, Carlsbad, CA), 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic (HEPES; CalBiochem, San Diego, CA), 50 mg/L hypoxanthine (Sigma-Aldrich, St. Louis, MO) medium is supplemented with 0.5% Albumax II (Invitrogen), 0.25% Na₂CO₃ (Sigma-Aldrich), and 0.01 mg/mL gentamicin (Invitrogen) to constitute complete media (CM). Store at 4°C.

3. Human red blood cells (RBCs) and human sera are obtained from Interstate Blood Bank (Memphis, TN).

2.2. Parasite Transfection and Selection of Recombinant Lines

1. Plasmids: Integrase-expressing plasmid pINT, which contains a Neomycin selectable marker that confers G418 resistance; *attP*-containing plasmid pLN-ENR-GFP, which harbors a *pfenr-gfp* expression cassette as well as a *bsd* (blasticidin S-deaminase) selectable marker that confers resistance to blasticidin hydrochloride. pLN-ENR-GFP can be digested with *Afl*III and *Avr*II enzymes (New England Biolabs, Ipswich, MA) to replace the *pfenr-gfp* fusion with the gene of interest. Digestion with *Avr*II and *Bsi*WI (New England Biolabs) can also be performed to place the gene of interest in frame with *gfp*, whose product is positioned at the C-terminus of the corresponding fusion protein. Both the pINT and pLN-ENR-GFP plasmids are available from the Malaria Research and Reference Reagent Resource Center (MR4). Sequence files are available from GenBank (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide>) under the identifiers DQ813653 and DQ813654 for pLN-ENR-GFP and pINT, respectively. Plasmid maps are illustrated in Fig. 2a, b.
2. Cytomix (1×): 120 mM KCl, 0.2 mM CaCl₂, 2 mM EGTA, 10 mM MgCl₂, 25 mM HEPES, 5 mM K₂HPO₄, 5 mM KH₂PO₄; pH 7.6. Store at room temperature.
3. Gene-Pulser II (Bio-Rad, Hercules, CA) and 0.2-cm cuvettes (Bio-Rad).
4. Blasticidin HCl (Invitrogen) is dissolved in tissue culture water at 10 mg/mL, sterile-filtered and stored in aliquots at −80°C. Add to parasite cultures at a final concentration of 2.5 µg/mL.
5. G418 (Cellgro, Manassas, CA) is dissolved in tissue culture water at 200 mg/mL, sterile-filtered and added to parasite cultures at a final concentration of either 125 µg/mL (Dd2 line) or 250 µg/mL (3D7 line).
6. WR99210 (Jacobus Pharmaceuticals, Princeton, NJ; molecular weight 394.35) is dissolved in CM or tissue culture water at 25.4 mM (10 mg/mL), sterile-filtered and stored in aliquots at −80°C. From this, we prepare working stocks of 25 µM in CM that we keep for up to 4 weeks at 4°C. Add to parasite cultures at a final concentration of 2.5 nM. This antifolate inhibits *Plasmodium* dihydrofolate reductase and selects for recombinant parasites expressing human dihydrofolate reductase (9, 10).

2.3. Plasmid Rescue

1. 0.1-cm cuvettes (Bio-Rad).
2. XL1-Blue (or other suitable) Electroporation-Competent Cells (Stratagene, La Jolla, CA).
3. SOC media (Sigma-Aldrich).
4. Luria Broth Base (Becton, Dickinson and Company, Franklin Lakes, NJ) and adequate antibiotic. Both the pINT and the *attP*-containing plasmids possess a carbenicillin resistance marker, which we select with 50 µg/mL carbenicillin disodium (Sigma-Aldrich).

2.4. Cloning Parasites by Limiting Dilution

1. CritSpin Microhematocrit Centrifuge System (StatSpin, Westwood, MA).

2. Malstat reagent: 100 mM Tris–HCl, 0.2 M L-lactic acid (Sigma-Aldrich), 0.2% (v/v) Triton X-100, and pH 9.1. Store at 4°C.
3. 3-Acetylpyridine adenine dinucleotide (APAD, Sigma-Aldrich; molecular weight 662.44) is dissolved in sterile water at 50 mg/mL, stored at –20°C, and added to Malstat reagent at 10 µM final concentration.
4. Nitroblue tetrazolium (NBT, Promega, Madison, WI) 50 mg/mL is stored at –20°C and used at a final concentration of 200 µg/mL.
5. Diaphorase (Sigma-Aldrich) is dissolved in sterile water at 8 mg/mL, stored at –20°C and added to Malstat reagent at a final concentration of 64 µg/mL.

2.5. Extraction of *P. falciparum* Genomic DNA from Cloned Parasites for Subsequent PCR Screening

1. Phosphate buffered saline (PBS): A 10× stock solution is prepared with 1.37 M NaCl, 26.8 mM KCl, 43 mM NaH₂PO₄, 14.7 mM KH₂PO₄, and pH 7.4. Autoclave, then store at room temperature. Use at 1× concentration by diluting with double-distilled water (ddH₂O).
2. Saponin (Sigma-Aldrich) is dissolved in ddH₂O at 10% (w/v) and used at a final concentration of 0.1% (w/v).
3. Lysis Buffer: 0.5 M Sucrose, 1 M Tris–Cl pH 7.6, 1 M MgCl₂, and 1% Triton X-100. Autoclave and store at 4°C.
4. Down Scale Prep (DSP) Buffer (4×): 1 M Tris–Cl pH 8.0, 1 M KCl, and 1 M MgCl₂. To prepare working solution, dilute to 1× and include Tween 20 and Proteinase K (Sigma-Aldrich) at final concentrations of 0.5% and 2.5 µg/mL, respectively. Our stock of Proteinase K is dissolved in sterile water at 10 mg/mL and stored at –20°C. Note that the working solution has to be made fresh each time.
5. DNeasy Blood and Tissue kits (Qiagen, Valencia, CA).

3. Methods

3.1. Overall Procedure

Parasites to be transfected are thawed from cryopreserved stocks and placed into culture for 5–7 days to obtain sufficient parasites (see below). During this period, DNA is prepared from a small aliquot (1 mL at 3–6% parasitemia, with a hematocrit of ~3%; see below) and subjected to microsatellite typing (11, 12) to confirm the identity of the strains to be used (see Subheading 3.2, step 2 and Note 1).

Transfection involves the use of two plasmids, one expressing the gene of interest (which can be prepared by modifying the *attP*-containing pLN-ENR-GFP plasmid to replace the *pfenr* coding sequence with the desired transgene; Fig. 2a) and the other expressing the integrase (such as pINT, Fig. 2b). Co-transfection of these plasmids into an *attB* line, such as Dd2^{attB} or 3D7^{attB}, results in transgene integration into the *cg6-attB* site (Fig. 2c).

Recombination mediated by mycobacteriophage Bxb1 integrase usually yields a predominant population of integrants, which can be assessed by PCR screening and plasmid

¹We recommend, if applicable, verifying the phenotype of the pre-transfection culture (by performing drug assays, adhesion assays, etc.), when the purpose of the genetic manipulation is to modify this phenotype. It is important that a new stock of parasites be cryopreserved at the time the transfection is performed, to provide the proper control for future analyses of the recombinant lines.

rescue experiments. However, we have also experienced instances where integrative recombination did not occur in a uniform manner, but instead led to a mixed population of parasites that either underwent integration (integrants) or continued to replicate the plasmids episomally (episomal replicants). This has been observed with 3D7^{attB}, which propagates more slowly than Dd2^{attB}, leading us to use the latter whenever there is a choice. In such a case of mixed parasite population, it becomes essential to obtain clones by limiting dilution so as to isolate the expected integrants and obtain a genetically homogeneous population. Clones are detected by assaying for parasite-specific lactate dehydrogenase (LDH) activity (see Subheading 3.5). The analysis of the recombinant locus is typically performed by Southern Blot to confirm the predicted genome integration and evaluate the number of plasmid copies that can integrate in tandem.

3.2. Preparation of Cultures for Transfection

1. *P. falciparum* Dd2^{attB} and 3D7^{attB} cultures are propagated in human RBCs at a 3% hematocrit in CM under reduced oxygen conditions (90% N₂, 5% O₂, and 5% CO₂) as described (10, 13). These lines have been engineered by the introduction of an acceptor *attB* site (from *M. smegmatis*) into the non-essential *cg6* locus. This recombinant locus is maintained by continuous selection with 2.5 nM WR99210 (9).
2. In order to verify, by microsatellite typing, the identity of the lines to be transfected, genomic DNA is extracted after saponin lysis of parasite-infected RBCs. As we generally culture parasites in 6-well plates at a volume of 5 mL per well, we usually perform the extraction from a similar volume of culture. Cells are transferred into a 15-mL sterile conical tube and spun for 3 min at 650 × g in a tabletop centrifuge. The supernatant is aspirated and the cell pellet resuspended in 5 mL of 0.1% saponin in PBS (1×) for 5 min at room temperature, then spun at 3,150 × g for 5 min. The parasite pellet is washed twice in PBS before proceeding to genomic DNA extraction using a DNeasy Blood & Tissue Kit (Qiagen). We note that this kit contains two types of lysis buffer. We typically lyse parasites with the AL buffer. Also, we elute DNA from the Qiagen column in a smaller volume than suggested in the user manual, i.e. usually twice 50 µL.

3.3. Transfection and Selection

Transfection procedures are carried out in Cytomix and the final volume of infected cells and plasmids in the buffer should be 450 µL.

1. 50–100 µg of each plasmid are required per transfection. These have to be equilibrated in Cytomix beforehand to reach a final volume of 150 µL per transfection. When preparing plasmids, the DNA pellet can be either directly dissolved in 1× Cytomix, or resuspended in sterile water or 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA pH8.0) and diluted 1:1 in 2× Cytomix.
2. The day before transfecting, *P. falciparum* cultures are diluted in freshly drawn, uninfected RBCs to obtain predominantly ring-stage parasites at 5–8% parasitemia on the following day.
3. On the day of transfection, parasites are first fed with fresh medium and smeared to confirm that the culture contains predominantly ring-stage parasites at greater than 5% parasitemia. Parasite-infected cells are then harvested (2.5 mL per transfection) and centrifuged in sterile conical tubes for 3–4 min at 640 × g at room temperature.
4. Cell pellets are washed once in 1 × Cytomix (2.5 mL per transfection) and then resuspended in 300 µL of 1 × Cytomix.

5. Plasmid DNA and infected cells are then mixed together and transferred to electroporation cuvettes.
6. Settings for electroporation of infected RBCs are the following: voltage of 0.31 kV and capacitance of 950 μ F (when set on maximum capacitance).
7. Immediately after electroporation, 1 mL of complete media (CM) is gently and aseptically added to each cuvette. Once all electroporations are completed, the contents of the cuvettes are transferred to 6-well plates that have been prepared beforehand (each well contains 3.5 mL of CM with 4% hematocrit and receives the contents of one transfection). Cuvettes are then washed with another 1 mL of CM that is added to the well, and the plates are placed at 37°C.
8. Approximately 1–4 h after transfection, cultures are transferred to sterile conical tubes and spun for 4 min at $450 \times g$ at room temperature. Pellets are then gently resuspended in 5 mL of complete media (with WR99210) and returned to the 6-well plate.
9. Selection with the agents BSD and G418 (14, 15) starts the day after transfection (i.e. day 1). Cultures are smeared that day to check the post-electroporation parasitemia, which is typically between 1 and 3% (see Note 2).
10. Cultures are fed every day for the first 6 days, then every other day, with fresh medium containing the selection agents. Drug-mediated clearance of live parasites is microscopically confirmed on day 6. Integrase-mediated recombination is thought to be a rapid event, and it is assumed that maintenance (using G418 drug pressure) of the pINT plasmid expressing the integrase is only required for the first 5–6 days. After that time, G418 can be removed.
11. On day 6, a 100- μ L aliquot of fresh RBCs (50% hematocrit) is added to each well. On day 12, 30–40% of each culture is replaced with fresh RBCs and media by diluting the culture 3:5. This dilution is essential as lysis of aging RBCs can occur after a prolonged period of culture. Transfections are then diluted 3:5 every 6 days and monitored by Giemsa stain every 4–5 days for reemergence of the transfected population. In our experience, Dd2^{attB}/ENR-GFP parasites (generated by transfecting the Dd2^{attB} line with the pLN-ENR-GFP plasmid) appeared within 18 days of electroporation. In general, transfected parasites that have undergone *attB* $\times$ *attP* recombination are observed 15–25 days post-electroporation. In most experiments, the recovered parasites are predominantly integrants; however, it is not unusual to detect a subpopulation of parasites containing episomal replicating plasmids in the bulk culture.

3.4. Plasmid Rescue

This procedure can be performed to help discriminate between parasites expressing the transgene of interest either from an integrated plasmid or from episomes.

1. Proceed to genomic DNA extraction from transfected parasite cultures as described in Subheading 3.2, step 2.
2. 100 ng of genomic DNA is used to transform 50 μ L of *Escherichia coli* competent cells (e.g. XL1) by electroporation. Settings for bacteria electroporation are as follows: voltage of 1.8 kV, resistance of 200 Ω , and capacitance of 25 μ F.

²If the parasitemia is below 1%, it might be necessary to repeat the transfection, as the probability of having transfected parasites is reduced. If the parasitemia is above 3%, cultures have to be closely monitored for the first few days following drug pressure, and culture dilution might be required to prevent parasites dying from overgrowth rather than drug selection.

3. Electroporated bacteria are recovered in 500 μ L of SOC media (2% bacto-tryptone, 0.5% bacto-yeast extract, 9 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose, and pH 7.0) at 37°C for 1 h. 250 μ L of recovered bacteria culture are plated on LB/agar with the appropriate antibiotic. We use 50 μ g/mL carbenicillin, which selects for expression of betalactamase by the pLN-ENR-GFP and pINT plasmids and their derivatives. As an alternative, 100 μ g/mL ampicillin can also be used, although this tends to produce more resistant satellite colonies upon extended bacterial culture. Quantification of the rescue efficiency provides a reasonably accurate measure of the extent to which the *attP*-containing plasmids have integrated into the *attB* site, versus their episomal replication (see Note 3).

3.5. Limiting Dilution of Parasite Clones and the Lactate Dehydrogenase Assay

As mentioned earlier, although the Bxb1 integrase-mediated recombination system is highly efficient and generally yields a predominant population of recombinants, detection of a subpopulation of parasites that express the transgene of interest as an episome is not uncommon. In the event that a highly homogeneous population of transgenic parasites is required, isolating integrant parasites from those that harbor episome-replicating plasmids can be achieved by limiting dilution cloning.

1. Before proceeding, the parasitemia of the culture has to be assessed accurately, both by Giemsa-stained smear (providing the percentage of infected RBCs), and by determining the hematocrit of the culture (indicating the percentage of RBCs per mL of culture). For the latter, we use a CritSpin Microhematocrit Centrifuge system or a standard hemocytometer. We typically consider the number of RBCs to be around 10^{10} per mL of packed cells. Consequently, 3% hematocrit, for instance, corresponds to 3×10^8 RBCs/mL.
2. Once the exact parasitemia is determined, cultures are diluted so as to inoculate the equivalent of 0.5–0.8 parasites per well. This would be predicted to yield approximately 50–80 parasitized wells per plate, but in general we tend to recover about 70% of the expected numbers of clones (i.e. approximately 30–50 clones per plate). We generally incubate 200 μ L of parasite culture per well in 96-well microtiter plates with a hematocrit of 1.8%, and always use fresh blood to prevent any RBC lysis during the course of the procedure.
3. Cultures are fed once a week with a mix of media and fresh RBCs at 0.4% hematocrit. This provides additional fresh RBCs for parasite reinvasion. If parasites are cultured with medium that is supplemented with 10% human serum instead of 0.5% Albumax, cultures are fed twice a week.
4. Growth of parasite clones is assessed after 2–3 weeks using an LDH assay based on the Malstat reagent (16, 17) (see Note 4).
  - a. One 96-well plate requires 12 mL of Malstat reagent, to which 158.4 μ L of APAD (50 mg/mL) is added. 6 mL of this mixture is then transferred to a 15-mL conical tube (tube 1) that is kept on ice and wrapped in aluminum foil. To this, we add 24 μ L of NBT (50 mg/mL). The other 6 mL of Malstat reagent/APAD is transferred to another 15-mL conical tube (tube

³The rescue efficiency, considered as the number of cfu (colony-forming units) per μ g of genomic DNA, correlates with the number of episomal replicating plasmids. We generally estimate that integrated plasmids will produce 10^2 cfu/ μ g DNA (because of a subpopulation of integrated plasmids “looping out” of the recombinant locus (9)), whereas episomal replicating plasmids will likely generate 5×10^3 to 10^5 cfu/ μ g DNA.

⁴This assay relies on the specific detection of parasite LDH and is based on the principle that the *P. falciparum* LDH enzyme can substitute APAD (3-acetylpyridine adenine dinucleotide) for NAD (nicotinamide adenine dinucleotide) as a cofactor, whereas the human erythrocyte LDH does not have this ability.

2), also kept on ice and wrapped in aluminum foil. To that mix, we add 48 μL of diaphorase (8 mg/mL).

- b. 20 μL of parasite-infected cell suspension from each well is then transferred into a new round-bottom 96-well microtiter plate.
- c. Tubes 1 and 2 are mixed together and 120 μL of this mixed Malstat solution is added to each well using a 12-well pipetting device.
- d. The plate is then placed in the dark (or wrapped in aluminum foil) for 30–45 min.
- e. The purple color associated with parasitized RBCs can be detected by measuring the absorbance at 650 nm in a plate reader. If the parasitemia is high enough, the color change in positive wells can be clearly distinguished visually from the negative wells, appearing more purple than uninfected wells. We also note that this assay can distinguish the level of parasitemia in positive wells. As a rule of thumb, we tend to avoid the wells that have the strongest LDH activity as we predict that these result from multiply infected RBCs, which could therefore represent a mixture of genetically distinct parasites (see Note 5).

3.6. Extraction of *P. falciparum* Genomic DNA from Cloned Parasites

Once positive clones have been detected by the LDH assay and have reached an adequate parasitemia (1–2%), PCR screening can be performed to confirm the isolation of recombinant parasites. Below we list a protocol that we use to extract genomic DNA directly from the parasites that have been cloned and cultured in 96-well microtiter plates.

1. A 96-well PCR plate is prepared by aliquoting 15 μL of 1% saponin (diluted from a 10% stock in $1\times$ PBS) in each well.
2. 150 μL of each parasite culture is transferred to the corresponding well in the saponin plate. 150 μL of fresh media with RBCs is added to the remaining volume of culture, such that parasites have been diluted 1:4, and the culture plate is returned to the incubator. The saponin plate is incubated at room temperature for 5 min and spun at $3,150 \times g$ in a tabletop centrifuge using microplate adaptors.
3. Supernatants are discarded by inverting the plate and the remaining pellets are washed by resuspending in 100 μL of PBS. The plate is centrifuged once again and the supernatants are removed, as above.
4. Pellets are then resuspended in 40 μL of lysis buffer. The plate is centrifuged and the supernatants discarded, as before. The step is repeated once.
5. Finally, pellets are resuspended in 40 μL of DSP working solution and heated at 50°C for 2 h, followed by 10 min at 95°C .
6. After cooling the plate at 4°C for 10 min, 4 μL can be used as a template for PCR, and the remainder is stored at -20°C .

The PCR strategy is generally designed to confirm the generation of *attL* and *attR* flanking sites upon integrase-mediated recombination between the genomic *attB* site and the plasmid *attP* site, as well as the absence of the wild-type locus and of plasmid episomes. Primers

⁵To reduce the prevalence of multiply infected RBCs, the cultures can be incubated for 48 h prior to cloning with gentle rocking to maintain the RBCs in suspension. If after 3 weeks of culture, parasites are still below the detection threshold or at a density not sufficient to perform parasite extraction, cultures can be diluted 1:2 with fresh RBCs to avoid any cell lysis and cultured for an extra week.

used for these PCR screenings are sketched in Fig. 2c (see figure legend for primer sequences).

Acknowledgments

We thank Heather Schneider for help with the figures. This work was supported by P01 AI060342 (PI. James Sacchettini)

References

1. Snow RW, Guerra CA, Noor AM, Myint HY, Hay SI. The global distribution of clinical episodes of *Plasmodium falciparum* malaria. *Nature*. 2005; 434:214–217. [PubMed: 15759000]
2. Crabb BS, Rug M, Gilberger TW, Thompson JK, Triglia T, Maier AG, et al. Transfection of the human malaria parasite *Plasmodium falciparum*. *Methods Mol Biol*. 2004; 270:263–276. [PubMed: 15153633]
3. Groth AC, Calos MP. Phage integrases: biology and applications. *J Mol Biol*. 2004; 335:667–678. [PubMed: 14687564]
4. Ghosh P, Wasil LR, Hatfull GF. Control of phage Bxb1 excision by a novel recombination directionality factor. *PLoS Biol*. 2006; 4:e186. [PubMed: 16719562]
5. Thorpe HM, Wilson SE, Smith MC. Control of directionality in the site-specific recombination system of the *Streptomyces* phage phiC31. *Mol Microbiol*. 2000; 38:232–241. [PubMed: 11069650]
6. Thomason LC, Calendar R, Ow DW. Gene insertion and replacement in *Schizosaccharomyces pombe* mediated by the *Streptomyces* bacteriophage phiC31 site-specific recombination system. *Mol Genet Genomics*. 2001; 265:1031–1038. [PubMed: 11523775]
7. Ghosh P, Kim AI, Hatfull GF. The orientation of mycobacteriophage Bxb1 integration is solely dependent on the central dinucleotide of *attP* and *attB*. *Mol Cell*. 2003; 12:1101–1111. [PubMed: 14636570]
8. Nkrumah LJ, Muhle RA, Moura PA, Ghosh P, Hatfull GF, Jacobs WR Jr, et al. Efficient site-specific integration in *Plasmodium falciparum* chromosomes mediated by mycobacteriophage Bxb1 integrase. *Nat Methods*. 2006; 3:615–621. [PubMed: 16862136]
9. Fidock DA, Wellems TE. Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci USA*. 1997; 94:10931–10936. [PubMed: 9380737]
10. Fidock DA, Nomura T, Wellems TE. Cycloguanil and its parent compound proguanil demonstrate distinct activities against *Plasmodium falciparum* malaria parasites transformed with human dihydrofolate reductase. *Mol Pharmacol*. 1998; 54:1140–1147. [PubMed: 9855645]
11. Su XZ, Wellems TE. *Plasmodium falciparum*: a rapid DNA fingerprinting method using microsatellite sequences within *var* clusters. *Exp Parasitol*. 1997; 86:235–236. [PubMed: 9225776]
12. Anderson TJ, Haubold B, Williams JT, Estrada-Franco JG, Richardson L, Mollinedo R, et al. Microsatellite markers reveal a spectrum of population structures in the malaria parasite *Plasmodium falciparum*. *Mol Biol Evol*. 2000; 17:1467–1482. [PubMed: 11018154]
13. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science*. 1976; 193:673–675. [PubMed: 781840]
14. Mamoun CB, Gluzman IY, Goyard S, Beverley SM, Goldberg DE. A set of independent selectable markers for transfection of the human malaria parasite *Plasmodium falciparum*. *Proc Natl Acad Sci USA*. 1999; 96:8716–8720. [PubMed: 10411941]
15. Wang P, Wang Q, Sims PF, Hyde JE. Rapid positive selection of stable integrants following transfection of *Plasmodium falciparum*. *Mol Biochem Parasitol*. 2002; 123:1–10. [PubMed: 12165384]
16. Makler MT, Hinrichs DJ. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *Am J Trop Med Hyg*. 1993; 48:205–210. [PubMed: 8447524]

17. Goodyer ID, Taraschi TF. *Plasmodium falciparum*: a simple, rapid method for detecting parasite clones in microtiter plates. *Exp Parasitol*. 1997; 86:158–160. [PubMed: 9207746]
18. Staalsoe T, Giha HA, Dodoo D, Theander TG, Hviid L. Detection of antibodies to variant antigens on *Plasmodium falciparum*-infected erythrocytes by flow cytometry. *Cytometry*. 1999; 35:329–336. [PubMed: 10213198]

Figure 1.

Generation of homogeneous parasites by integrase-mediated *attB* × *attP* recombination. (a) Live cell imaging of Dd2^{attB} parasites expressing an mRFP-tagged cytosolic protein (PfVps4) encoded on a plasmid integrated at the *cg6-attB* locus. Bright field (*left panel*) and merged fluorescence images (*right panel*) showing mRFP-PfVps4 (*red*) and Hoechst 33342-stained nuclei (*blue*). Prior to imaging, infected RBCs were enriched by magnet-activated cell sorting (MACS, Miltenyi Biotec Inc., Auburn, CA) (18). Bar = 10 μm. (b) and (c) Flow cytometry analysis of parasites expressing GFP either from a plasmid integrated at the *cg6-attB* locus (Dd2^{attB}/GFP) or from episomes (Dd2/GFP). Parasites were incubated with Hoechst 33342 dye and analyzed at 16- to 20-h post-invasion. Cells were gated for nuclear staining to distinguish infected from uninfected RBCs. (b) GFP expression profiles show a higher percentage of GFP-expressing parasites in the integrant Dd2^{attB}/GFP population (94%) than in the episomal Dd2/GFP (74%). (c) Dd2^{attB}/GFP integrants show a unique peak of GFP expression with negligible variance, whereas Dd2 parasites expressing GFP from episomes display multiple peaks with a significantly larger variance. Panels (b) and (c) were derived from Fig. 4 in ref. 8 with the permission of the Nature Publishing Group.

Figure 2.

Dual-plasmid approach for integrase-mediated *attB* × *attP* recombination. **(a)** Map of the *attP*-carrying plasmid pLN-ENR-GFP that can be used as a template for engineering the stable site-specific integration of a gene of interest. Digestion with *AvrII* and *AflIII* can be performed to replace the *enr-gfp* fusion by the gene of interest. Alternatively, digestion with *AvrII* and *BsiWI* can place the gene of interest in frame with *gfp*. The plasmid carries the blasticidin S-deaminase (*bsd*) selectable marker. **(b)** Map of the integrase-expressing plasmid pINT, which harbors a neomycin selectable marker. The plasmid is usually maintained under G418 selection pressure only for the first 6 days following transfection. **(c)** Schematic representation of the *attB* × *attP* recombination allowing integration of a gene of interest (*yfg*) at the *cg6-attB* locus. Dd2^{attB} or 3D7^{attB} parasites are co-transfected with pINT and an *attP*-plasmid carrying the gene of interest, and selected with 2.5 μg/mL BSD, 2.5 nM WR99210 (to select for the human dihydrofolate reductase (*dhfr*) marker in the *cg6-attB* locus) and 125 or 250 μg/mL G418 (for the Dd2^{attB} and 3D7^{attB} parasite lines respectively). Integrase expressed from the pINT plasmid catalyzes insertion of the *attP*-plasmid harboring the gene of interest into the *attB* site. PCR reactions using p1 + p2 and p3 + p4 primers as well as Southern blots analyzing digested-DNA hybridization to *cg6* or *yfg* probes (designated as open boxes), can be performed to confirm integration of the gene of interest at the *cg6-attB* locus. Sequences of the primers for PCR screening are as follows: p1: 5'-GAAAATATTATTACAAAGGGTGAGG, p2: 5'-TTAGCTAATTCGCTTGTAAG, p3: 5'-CTCTTCTACTCTTTCGAATTC, p4 is designed based on the sequence of the gene of interest. pBS refers to the pBluescript backbone. *UTR*: untranslated region. Panel (c) was adapted from Fig. 3 in ref. 8 with the permission of the Nature Publishing Group.