Studies using transgenic rodent malaria parasites to improve live attenuated malaria vaccines

Othman, A.S.B.

CHAPTER 6

EXPRESSION OF FULL-LENGTH *PLASMODIUM FALCIPARUM* P48/45 IN *P. BERGHEI* BLOOD STAGES: A METHOD TO EXPRESS AND EVALUATE VACCINE ANTIGENS

Ahmad Syibli Othman[1,2], Jing-wen Lin[3], Blandine M Franke-Fayard[1], Hans Kroeze[1], Fiona J.A. van Pul[1], Séverine Chevalley-Maurel[1], Jai Ramesar[1], Catherin Marin-Mogollon[1], Matthijs M. Jore[4], Merribeth J. Morin[5], Carole A. Long[6], Robert Sauerwein[4], Ashley Birkett[5], Kazutoyo Miura[6], Chris J. Janse[1] and Shahid M. Khan[1]*

[1] Leiden Malaria Research Group, Parasitology, Leiden University Medical Center (LUMC), Leiden, The Netherlands
[2] Faculty of Health Sciences, Universiti Sultan Zainal Abidin, Terengganu, Malaysia
[3] Division of Pediatric Infectious Diseases, State Key Laboratory of Biotherapy, West China Second Hospital, Sichuan University and Collaboration Innovation Center, Chengdu, China
[4] Department of Medical Microbiology, Radboud University Medical Center, Nijmegen, The Netherlands
[5] PATH's Malaria Vaccine Initiative, Washington DC, USA
[6] Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, NIH, Rockville, Maryland, USA.
* The corresponding author

Molecular and Biochemical Parasitology, 2018; 224 : 44-49

ABSTRACT

The transmission-blocking vaccine candidate Pfs48/45 from the human malaria parasite *Plasmodium falciparum* is known to be difficult to express in heterologous systems, either as full-length protein or as correctly folded protein fragments that retain conformational epitopes. In this study we express full-length Pfs48/45 in the rodent parasite *P. berghei*. Pfs48/45 is expressed as a transgene under control of the strong *P. berghei* schizont-specific *msp1* gene promoter (Pfs48/45@PbMSP1). Pfs48/45@PbMSP1 schizont-infected red blood cells produced full-length Pfs48/45 and the structural integrity of Pfs48/45 was confirmed using a panel of conformation-specific monoclonal antibodies that bind to different Pfs48/45 epitopes. Sera from mice immunized with transgenic Pfs48/45@PbMSP1 schizonts showed strong transmission-reducing activity in mosquitoes infected with *P. falciparum* using standard membrane feeding. These results demonstrate that transgenic rodent malaria parasites expressing human malaria antigens maybe used as means to evaluate immunogenicity and functionality of difficult to express malaria vaccine candidate antigens.

INTRODUCTION

Efficient and conformationally-accurate expression of *Plasmodium* proteins in heterologous systems, such as yeast or bacteria, is frequently problematic resulting in misfolded or incorrectly modified proteins, which are often poorly expressed [1, 2]. This hampers the screening of *Plasmodium* antigens in immunization studies for their suitability as vaccine candidate antigens. Preclinical evaluation of *Plasmodium* antigens often involves immunizing rodents with recombinant *Plasmodium* proteins followed by an examination of induced immune responses, either *in vivo* using rodent models of malaria or *in vitro* by performing functional assays with human malaria parasites incubated with immune sera [3]. Multiple factors contribute to inefficient expression of *Plasmodium* proteins, such as the high AT content of *Plasmodium* genes, large size and often unique protein structure (i.e. encoding repeated stretches of amino acids) and unique post-translational modifications [1, 4]. This is particularly evident for cysteine-rich proteins where correct folding depends on accurate formation of disulfide bridges to form domains specific for *Plasmodium* proteins [5-7]. Transgenic rodent malaria parasites (RMP) expressing human malaria parasite (HMP) proteins are increasingly used to evaluate and rank order candidate malaria vaccines before investing in scalable manufacture to support advancement to clinical testing [3]. Such transgenic RMP have been used in preclinical assays to evaluate vaccine potential of HMP proteins, both *in vivo* where mice are immunized with HMP antigens and subsequently challenged with transgenic RMP expressing the cognate HMP or in *in vitro* assays where immune sera or antibodies are evaluated for inhibition of parasite growth or invasion. Both the functional complementation of RMP genes by the HMP orthologs [3] and analysis of HMP expression using antisera, provide evidence for correct expression of functional HMP proteins in transgenic RMP [8]. Based on these studies, we reasoned that transgenic RMP can be used as expression systems to more efficiently express, screen, validate and down-select HMP antigens as potential novel malaria vaccine candidates [2, 9]. Further, the expression of conformationally-accurate *Plasmodium* proteins could be used to generate epitope-specific monoclonal antibodies, which in turn can be used to better characterize the vaccine antigen. The use of RMP would circumvent many of the above-mentioned problems associated with expression in heterologous expression systems including, but not limited to, peculiarities of post-translational modifications and *Plasmodium*-specific domains involved in protein trafficking and cellular location. As a proof of concept, we generated transgenic *P. berghei* (*Pb*) parasites that express full length Pfs48/45 from *P. falciparum* (*Pf*). The Pfs48/45 protein is expressed in *Plasmodium* gametocytes and gametes [10, 11] and contains multiple cysteine-rich domains with multiple disulfide bonds [12-14]. These constitute distinct conformational B cell epitopes that can be recognized by several monoclonal antibodies some of which have transmission-blocking (TB) activity [15]. Pfs48/45 becomes exposed on the surface of gametes once the parasite is taken up in blood meal by a mosquito and here the antigen can be targeted by antibodies and other components of the blood meal [16]. Expression of Pfs48/45 for TB

immunization studies has been problematic in most commonly used expression systems, mainly due to incorrect or insufficient protein folding, which is dependent on the correct formation of disulfide bridges in this cysteine-rich protein [17, 18]. The limited reactivity of recombinant Pfs48/45 with monoclonal antibodies against conformational epitopes of Pfs48/45 has indicated this misfolding [19, 20].

MATERIAL AND METHODS

Experimental animals and parasites

Female OF1 and C57Bl/6 mice (6 to 8 weeks old; Charles River/Janvier) and Wistar rats (HsdCpb:WU; 175-199 gr, Harlan Netherlands BV) were used. All animal experiments of this study were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042, 12043). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All experiments were performed in accordance with relevant guidelines and regulations. The following reference lines of the ANKA strain of *P. berghei* (*Pb*) were used in this study: line cl15cy1 (Janse *et al.*, 2006) and line $GIMO_{pbANKA}$ (1596cl1; RMgm-687 in www.pberghei.eu; referred to as *Pb*WT). The $GIMO_{pbANKA}$ (1596cl1) was generated in the cl15cy1 parent line and this line expresses a fusion of a drug resistance gene *hdhfr (human dihydrofolate reductase*) and a drug sensitivity gene *yfcu* (*yeast cytosine deaminase and uridyl phosphoribosyl transferase*), the so called positive-negative selectable marker (SM), constitutively expressed by the *P. berghei eef1*α promoter stably integrated into the *230p* locus [21].

Generation and genotyping of the transgenic parasite line, Pfs48/45@ PbMSP1

To introduce the *Pfs48/45* gene (PF3D7_1346700) into the redundant *p230p* gene locus (PBANKA_0306000) of the *Pb* genome, we generated DNA construct pL1706. The basic gene insertion construct pL0046 was used, which contains the 5' and 3' *230p* targeting regions, the *tgdhfr/ts* selectable marker (SM) cassette and an mCherry expression cassette under the control of the *eef1*α promoter with 3' terminal sequence of *pbdhfr/ts*. The *eef1*α promoter was replaced by the *msp1* promoter (PBANKA_0831000) using *Afl*II and *Bam*HI digestion. The *msp1* promoter was amplified from genomic *Pb* ANKA DNA using primers 6145 and 6146. In addition the mCherry coding sequence (CDS) was replaced by the *Pfs48/45* CDS using *Bam*HI and *Sgr*AI digestion. The *Pfs48/45* CDS was amplified from genomic DNA of the *Pf* NF54 strain using primers 5583 and 5584. This resulted in construct pL1706. In order to introduce the expression construct in the genome of the parent GIMO *Pb*ANKA line (1596cl1) , we next removed the *tgdhfr/ts* SM by digestion of the plasmid with *Sbf*I and *Afl*II. The ends of the linearized constructs were then rendered blunt using Klenow enzyme treatment, and re-ligated. This final construct (pL1707) were analyzed via restriction digestions to confirm correct assembly. Before transfection, the construct pL1707 was linearized by digesting the plasmid with *Ksp*I.

Parasites of line 1596cl1 were transfected with this construct (exp. 1807) using standard transfection technologies and transformed parasites selected by negative selection with 5-fluorocytosine (5-FC) [21, 22]. Selected parasites were cloned by limiting dilution. Three independent clones have been obtained after the cloning and correct integration of the construct was confirmed by Southern Analysis of PFG-separated chromosomes (data not shown). Mutant 1807cl2 was used for further genotype and phenotype analysis. Correct integration of the construct into the *p230p* gene locus was performed by diagnostic PCR-analysis and Southern analysis of pulsed field gel (PFG) separated chromosomes as described previously [23]. For Southern analysis, PFG-separated chromosomes were hybridized to a mixture of two probes, one recognizing *hdhfr* and one control probe, recognizing the *p25* gene on chromosome 5 [22].

Western and IFA analyses of Pfs48/45 expression

Transgenic schizonts were obtained from short-term overnight cultures of infected blood obtained by cardiac puncture from rats or mice as previously described [24]. Leucocytes were removed from the infected blood using Plasmodipur filters before the parasites were put into short-term overnight culture. Schizonts from the short-term cultures were purified using Nycodenz gradient centrifugation, resulting in parasite populations consisting of >90% schizonts [24].

For Western analysis, purified schizont preparations were extracted in 25 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1.0% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride. Insoluble debris was removed by centrifugation at 13,000 g for 5 min at room temperature (RT) and the supernatant was used for Western analysis [15]. Parasite proteins were separated by electrophoresis on a 12% SDS-PAGE gel and transferred to Hybond ECL nitrocellulose membrane (Amersham Biosciences) for 2 h at 200 mAh. Membranes were blocked for non-specific binding in phosphate-buffered saline (PBS) with 0.1% Tween 20 (PBST) containing 3% skim milk (Elk, Campina, The Netherlands) overnight at 4°C. Blots were hybridized with 4 anti-*Pf*s48/45 monoclonal antibodies that recognize *Pf*s48/45 epitopes I, IIb, III and V (antibodies 85RF45.1, 85RF45.2b, 85RF45.3, 85RF45.5) [14]. One microgram of protein was loaded in each lane and for reduced reaction, the DTT was added at final concentration of 10 mM [25]. After incubation with the monoclonal antibodies the membranes were washed with PBST and incubated for 1 hour at RT with horseradish peroxidase (HRP)-conjugated goat anti-rat IgG secondary antibody (Sigma-Aldrich) and developed in Amersham ECL Western Blotting Detection Kit according to the manufacturer's instructions (GE Healthcare). As a loading control, the membranes were also incubated with rabbit anti-*P. yoelii* MSP1 antibody [26], followed by incubation with HRP-conjugated goat anti-rabbit IgG secondary antibody (GE Healthcare).

The amount of *Pfs48/45* protein in total schizont extract was estimated by quantitative Western blot analysis. Protein extracts of the schizont and gametocyte lysates (see above) and R0.10C recombinant protein were quantified using Pierce™ BCA protein assay kit (Thermo Fisher Scientific). Protein extracts (500 ng) were loaded on the SDS-PAGE gel and

a serial dilution series (50, 25, 12.5, 6 and 3 ng) of the recombinant *P. falciparum* P48/45 fused to GLURP R0 domain (R0.10C) was loaded on the gel. Proteins were separated by electrophoresis and transferred to nitrocellulose membrane as described above and the blot was probed with antibody 85RF45.1 (1:2000 dilution) as the primary antibody. The X-ray film was exposed to the membrane for 30 sec and developed using HQ 350XT X-ray Film Processor. The optical intensity (or Optical Densitometry (OD) values) of the signals were quantified with a BioRad GS-800™ Calibrated Densitometer using Quantity One software (Bio-Rad).

For immunofluorescence analyses (IFA), schizonts-infected red blood cells (RBC) were collected from short-term overnight cultures of infected mice blood described above [24]. The schizont-infected RBC were washed 3 times in PBS and 5 µl of packed cells resuspended in 1 ml PBS. 15-20 µl of this suspension was placed in a well of a 10-well black cell-line diagnostic microscope slide (Thermo Scientific) and allowed to air dry. The slides were fixed with 4% paraformaldehyde in PBS for 30 min and cells were permeabilized with 1% Triton X-100 in PBS for 30 min at RT. The slides were incubated overnight at 4°C with the four different rat anti-*Pf*s48/45 antibodies and rabbit anti-MSP1 antibody (described above) in 10% fetal calf serum in PBS, washed 3 times with PBS at RT, followed by incubation for 1 hour with secondary conjugated antibodies anti-rabbit IgG Alexa Fluor®488 (Invitrogen) or anti-rat IgG Alexa Fluor®594 (Invitrogen). Nuclei were stained with the DNA-specific dye Hoechst-33342 at a final concentration of 10 µM (Sigma, The Netherlands) for 30 min at RT. Fixed cells were covered with 1-2 drops of an anti-fading agent (Vectashield), and a coverslip placed onto of the cells and sealed with nail polish. Stained cells were analysed for fluorescence using a Leica fluorescence MDR microscope (100x magnification). Pictures were recorded with a DC500 digital camera microscope using Leica LAS X software with the following exposure times: Alexa: 0.7 s; Hoechst 0.136 s; bright field 0.62 s (1x gain).

Immunization with schizont-extracts and purification of IgG from immunized mice

For generation of the schizont-extracts for immunization, 10 Wistar rats were infected with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 parasites and at a parasitemia of 1-3% infected blood was collected by cardiac puncture. Leucocyte removal, short-term overnight culture of infected RBC and Nycodenz gradient purification of schizonts was performed as described above. Purified schizont-infected RBC were divided into samples containing 1.1×10^9 schizont-infected RBC cells. The cells were pelleted by centrifugation (450 *g*, 8 minutes) and stored at −80°C after removal of the supernatant.

Groups of 10 C57BL/6 mice were immunized with either *Pb*WT (c15cy1) or Pfs48/45@PbMSP1 schizonts by intravenous injection of 1×10^8 schizont-infected red blood cells (in 200 µl RPMI). Mice were immunized a total of four times, at 2 week intervals. Before injection, schizonts were subjected to 3 freeze (dry ice) - thaw (RT) cycles, to ensure parasites were killed. Fourteen days after the last immunization blood was collected from all mice by cardiac puncture and serum collected after centrifugation (1500×g for 10

min). Serum was stored at −20°C until further analysis. IgG was purified from the pooled serum from 10 mice, by protein G affinity chromatography (Pierce, Rockford, IL) according to the manufacturer's instructions and adjusted to a final concentration of 4 mg/ml in phosphate-buffered saline (PBS).

Standard Membrane Feeding Assay (SMFA)

IgG purified from sera obtained from immunized mice was assessed for transmission reducing (TR) activity in SMFA as previously described [27, 28] using *P. falciparum* (*Pf*) gametocytes. Briefly, *Pf* gametocyte cultures (16 to 18 days old) of *P. falciparum* NF54 (originally provided by Steve Hoffman, Sanaria, Rockville, MD) were adjusted to 0.15 to 0.2% stage V gametocytemia at 50% hematocrit. Sixty microliters of a test sample (with a defined concentration of purified mouse IgG) in 1x PBS was mixed with 100 µl of the gametocyte mixture, and the final mixture was immediately fed to 50 female *Anopheles stephensi* (Nijmegen strain, 3 to 6 days old) mosquitoes through a membrane-feeding apparatus. Mosquitoes were kept for 8 days and dissected (20 per sample) to count the number of oocysts. As assay controls both malaria-naïve human sera and an anti-Pf25 monoclonal antibody (4B7; [29]) were used to establish background and complete inhibition of oocyst formation, respectively. Significance of inhibition (% inhibition in oocyst intensity) was determined by the zero-inflated negative binomial model described previously [28].

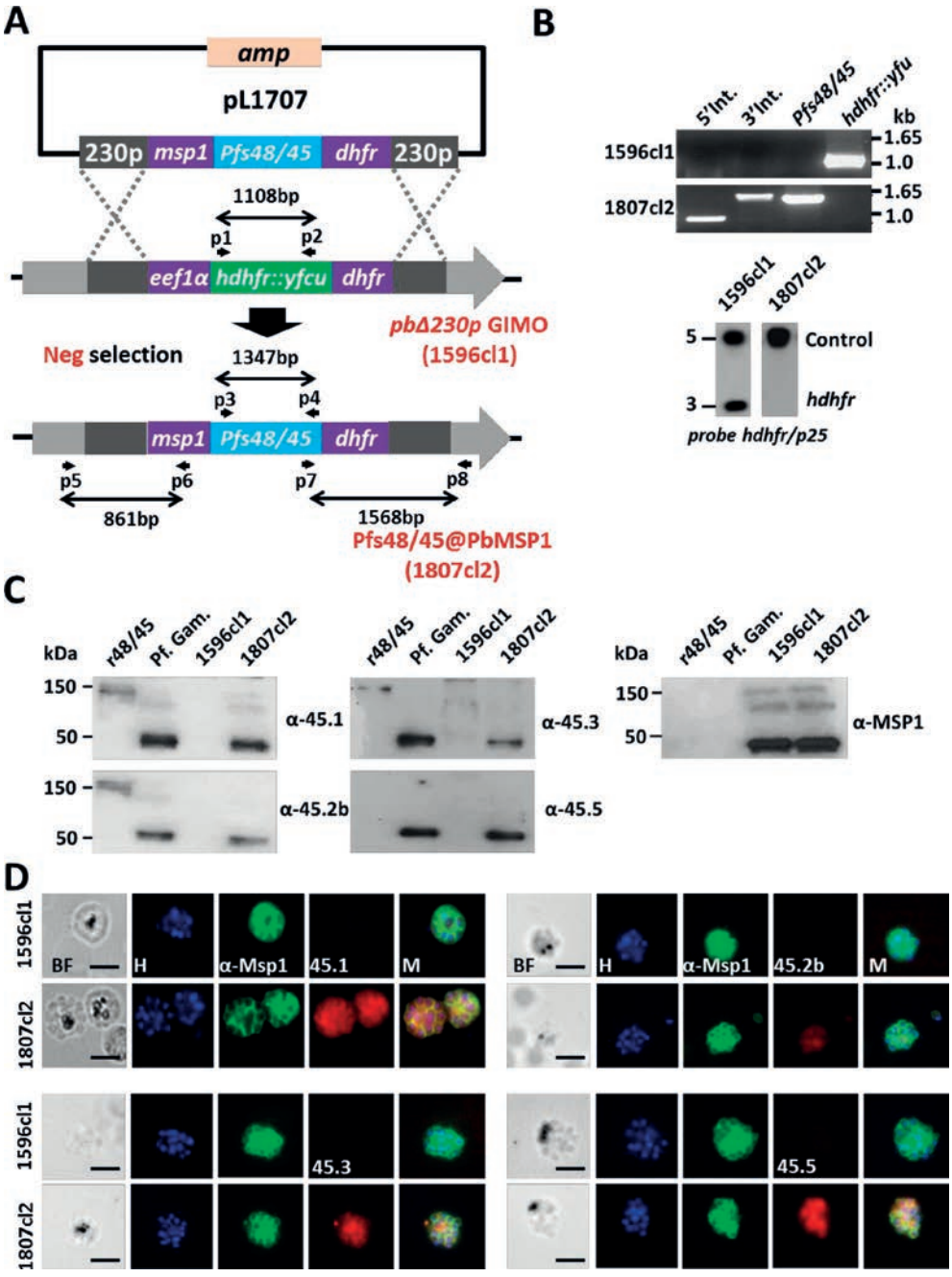
RESULTS AND DISCUSSION

In this study the coding sequence of the gene encoding *Pfs48/45* (PF3D7_1346700) was introduced into the redundant *P. berghei p230p* gene locus (PBANKA_0306000) [3, 21, 30]. The *Pfs48/45* gene was placed under control of 1.3 kb of the promoter region of the schizont-specific *Pb msp1* gene (PBANKA_0831000). This promoter was chosen since *msp1* is one of the highest transcribed genes in developing *Pb* schizonts [31] and the *Pb* schizont stage can be easily produced and purified in large quantities [24]. The transgenic parasite (Pfs48/45@PbMSP1) was generated by the method of GIMO transfection and selection [21]. Using this method transgenes can be rapidly introduced into the *p230p* gene locus in a $GIMO_{pbANKA}$ parent line by replacing the positive-negative selectable marker expression cassette by the transgene expression cassette (**Supplementary M&M** and **Figure 1A**). Correct replacement of the selectable marker cassette and insertion of the Pfs48/45 expression cassette in a cloned line of Pfs48/45@PbMSP1 (1807cl2) was confirmed by diagnostic PCR and Southern analysis of chromosomes separated by pulsed-field gel electrophoresis (**Figure 1B**). Analysis of the growth rate of transgenic Pfs48/45@PbMSP1 parasites during the cloning period demonstrated normal growth of blood stages, comparable to wild type (WT) *Pb*ANKA parasites (i.e. all mice (n=3) achieved a 0.5-2% parasitemia on day 8, after inoculation with a single infected red blood cell). To obtain transgenic schizonts, parasites were cultured overnight using standard methods to produce and purify *Pb* schizonts (**Supplementary M&M**).

Figure 1. Generation, genotype and phenotype analyses of Pfs48/45@PbMSP1, a transgenic *P. berghei* parasite expressing *P. falciparum* P48/45 in schizonts. (A) Schematic representation of the introduction of the *Pfs48/45*-expression cassette into the $GIMO_{pbANKA}$ parasite (line 1596cl1). Construct pL1707 contains the *Pfs48/45* gene flanked by the *msp1* promoter region and the *3' pbdhfr UTR*. This construct is integrated into the modified *P. berghei 230p* locus of $GIMO_{pbANKA}$ that contains the *hdhfr::yfcu* selectable marker (SM) cassette by double cross-over homologous recombination at the homology regions (*230p*; grey boxes). Negative selection with 5-FC selects for parasites that have the *SM* cassette replaced by the *Pfs48/45* expression cassette. Location of primers used for PCR analysis and sizes of PCR products are shown. (B) Diagnostic PCR (upper panel) and Southern analysis of PFG-separated chromosomes (lower panel) confirm correct integration of construct pL1707 in line 1807cl2 parasites. PCR shows the absence of the *hdhfr::yfcu* marker and the presence of the *Pfs48/45*. 5' integration PCR (5' int; primers p5/p6), 3' integration PCR (3' int; primers p7/p8), *hdhfr::yfcu* (primers p1/p2), *Pfs48/45* (primers p3/p4). Primer locations and product sizes are shown in A and primer sequences in Table S1). Hybridization of PFG-separated chromosomes with a mixture of two probes (the *hdhfr* probe and a control probe recognizing *p25* gene on chromosome 5) shows the removal of the SM cassette marker in the *230p* locus on chromosome 3 in 1807cl2 parasites. (C) Western analysis of Pfs48/45 expression in protein extracts of purified gametocytes of *P. falciparum* (Pf Gam), purified schizonts of wild type *P. berghei* (1596cl1) and purified schizonts of Pfs48/45@PbMSP1 (1807cl2). As a positive control, recombinant *P. falciparum* P48/45 fragment fused to GLURP R0 domain (R0.10C) was included (expected molecular size is 150 kDa). Blots were stained with 4 different anti-*Pf*s48/45 antibodies (45.1-3, 45.5) that recognize different epitopes. Anti-*Py*MSP1 antibody staining was used as a loading control. (D) Immuno-fluorescence analyses of Pfs48/45 expression in purified schizonts of Pfs48/45@PbMSP1 (1807cl2), and the reference parent *P. berghei* GIMO line (i.e. WT; 1596cl1). Fixed parasites were stained with four different rat anti-*Pf*s48/45 mAbs (45.1-3, 45.5) and rabbit anti-*Py*MSP1 antibody followed by secondary conjugated antibodies anti-rabbit IgG Alexa Fluor ® 488 (green) or anti-rat IgG Alexa Fluor ® 594 (red). Nuclei stained with the DNA-specific dye Hoechst 33342 (H). All pictures were recorded with the same exposure/gain times; anti-rabbit IgG Alexa Fluor ® 488 (green) 0.7 s; anti-rat IgG Alexa Fluor ® 594 (red) 0.6s; Hoechst (blue) 0.136 s; bright field 0.62 s (1x gain). BF: bright field; M: merged. Scale bar: 2 µm.

We confirmed expression of Pfs48/45 in the transgenic schizonts by Western and immuno-fluorescence analysis using four anti-Pfs48/45 monoclonal antibodies 85RF45.1 (45.1), 85RF45.2b (45.2b), 85RF45.3 (45.3), and 85RF45.5 (45.5). Three of these (45.1, 45.2b and 45.3) recognize conformational epitopes (epitopes I, IIb and III respectively) in the C terminal region of Pfs48/45 [14].

In Western analysis all the antibodies recognize a protein of the expected size (48 kDa) in protein extracts from Pfs48/45@PbMSP1 schizonts and WT *Pf* gametocytes but not in extracts from a *Pb* line that does not express Pfs48/45 (i.e. $GIMO_{pbANKA}$ line 1596cl1). As a positive control, recombinant protein that contains a fragment of *P. falciparum* Pfs48/45 fused to the GLURP R0 domain (R0.10C) was included and as expected a 150 kDa band was present after probing with monoclonal antibodies 45.1, 45.2b and 45.3 but was not present after probing with 45.5 (**Figure 1C**) [32]. We next examined the presence of Pfs48/45 epitopes using the anti-*Pf*s48/45 antibodies by immuno-fluorescence assay (IFA) (**Figure 1D**). All antibodies recognized Pfs48/45 produced in the Pfs48/45@PbMSP1 schizonts and did not react with proteins of WT *Pb* schizonts (**Figure 1C and 1D**). These results demonstrate that transgenic *Pb* schizonts can effectively express full length



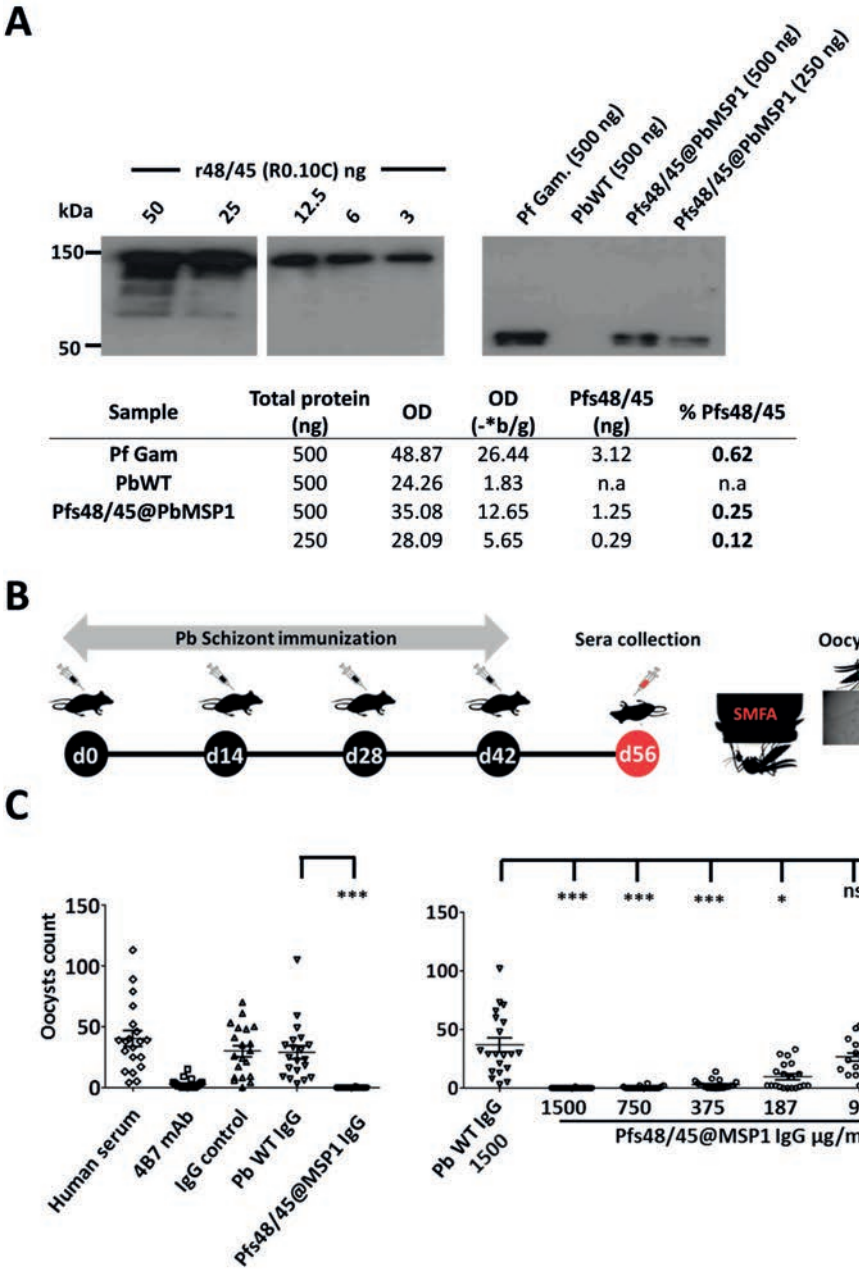
Pfs48/45, which retains a number of conformational epitopes. Pfs48/45, like MSP1, contains a GPI anchor and is present at the plasma membrane of *Pf* gametocytes/gametes [33]. The immuno-fluorescence analyses indicate that Pfs48/45 was located in the cytoplasm of the transgenic merozoites as the fluorescence signals did not completely overlap with

fluorescence signals obtained with anti-MSP1 antibodies, which stain MSP1 at the merozoite plasma membrane in mature schizonts (**Figure 1D**). Possible reasons for this observation is that GPI attachment may be different between rodent and human *Plasmodium* parasites, or that the attachment of Pfs48/45 onto *P. berghei* merozoites would require the presence of other *Plasmodium* proteins normally present in gametocytes/gametes.

In order to estimate the proportion of Pfs48/45 present in the protein lysates of Pfs48/45@PbMSP1 schizonts we performed a quantitative Western Blot analysis. Densitometry analysis of signals obtained after probing known amounts of Pfs48/45@PbMSP1 protein schizont lysates and a dilution series of recombinant Pfs48/45 with anti-*Pf*s48/45 monoclonal 45.1, revealed that the intensity of the schizont lysate signals corresponds to less than 1 ng of recombinant Pfs48/45 (**Supplementary Figure S1**), indicating that is between 0.25 - 0.12% of the total schizont lysate is Pfs48/45 (**Figure 2A**).

Next, we examined if Pfs48/45@PbMSP1 transgenic schizont lysate could be used to raise sera that could block *Pf* transmission in mosquitoes, presumably by Pfs48/45 specific antibodies. Two groups of 10 C57BL/6 mice were immunized 4 times (2 week interval) with lysates of $1x10^8$ schizonts of either Pfs48/45@PbMSP1 or WT (c15cy1) parasites (**Figure 2B**). Purified schizonts were inactivated by three rounds of freezing on dry ice followed by thawing at room temperature before immunization and schizont lysates were injected intravenously. Two weeks after the final immunization, serum was collected from all animals and a serum pool made for each group. Total IgG was isolated from the pooled sera and tested for transmission-reducing activity (TR activity) in standard membrane feeding assays (SMFA) using *Pf* gametocytes (**Supplementary M&M**). *Pf* gametocytes were fed to *A. stephensi* mosquitoes in the presence of IgG obtained from mice immunized with

Figure 2. Quantification of Pfs48/45 protein in Pfs48/45@PbMSP1 schizont lysate and transmission reducing (TR) activity of IgG isolated from mice immunized with Pfs48/45@PbMSP1 schizont lysates. **A.** Pfs48/45@PbMSP1 schizont lysates (500 & 250 ng), *P. falciparum* gametocytes (Pf Gam.; 500 ng) and *P. berghei* WT schizont lysate (*Pb*WT; 500 ng) were analyzed in Western blot analysis using anti-*Pf*s48/45 monoclonal 85RF45.1 (1:2000). Densitometry analysis was performed on signals after probing Pfs48/45@PbMSP1 schizont lysate (500 ng) and a dilution series (50, 25, 12.5, 6 and 3 ng) of recombinant Pfs48/45 (r48/45; R0.10C) with antibody 45.1. The Table shows the calculated Pfs48/45 protein content (ng) and the percentage of Pfs48/45 protein in parasite samples; see **Supplementary Figure S1** for determination of Pfs48/45 in samples. *quantification performed after subtraction of background (b/g) Optical Densitometry (OD) values and **quantitation based on regression curve calculations (see **Supplementary Figure S1**). **B.** Timeline showing the immunization of mice with extracts of Pfs48/45@PbMSP1 and *Pb*WT schizont lysates and collection of sera for isolation of IgG that is tested for TR activity in standard membrane feeding assays (SMFA) of *P. falciparum* gametocytes to *Anopheles stephensi* mosquitoes (see **C**). **C.** Left panel: First SMFA with IgGs from mice immunized with purified schizonts of Pfs48/45@PbMSP1 and *Pb*WT. TR activity was determined by the mean number of oocysts 8 days after feeding, and significance of inhibition was determined by the zero-inflated negative binomial model described previously [28]. Right panel: Second SMFA with serially diluted IgGs. IgG from mice immunized with purified schizonts of Pfs48/45@PbMSP1 was titrated resulting in the concentrations shown in the Figure. Significant TR activity was detected until a concentration of 187 µg/ml (*p=0.014). Significant; *$p < 0.05$, ***$p < 0.001$.



Sample	Total protein (ng)	OD	OD (-*b/g)	Pfs48/45 (ng)	% Pfs48/45
Pf Gam	500	48.87	26.44	3.12	**0.62**
PbWT	500	24.26	1.83	n.a	n.a
Pfs48/45@PbMSP1	500	35.08	12.65	1.25	**0.25**
	250	28.09	5.65	0.29	**0.12**

schizonts of either Pfs48/45@PbMSP1 or WT. In the first experiment, IgG (1500 µg/ml) from Pfs48/45@PbMSP1-immunized mice showed 99.8% inhibition in oocyst density (p=0.001) compared to the IgG obtained from WT immunized (**Figure 2C**). Next, TR activity was determined in SMFA using a dilution series of the IgG obtained from Pfs48/45@PbMSP1-immunized mice. Significant TR activity with IgG from Pfs48/45@PbMSP1-immunized mice

was still observed at a concentration of 187 µg/ml (p=0.014) compared to the control IgG (Figure 2C). The quantitative Western blot analysis (Figure 2A) indicated that is between 0.25-0.12% of the total Pfs48/45@PbMSP1 schizont lysate was Pfs48/45 and therefore it is likely that the majority of the IgG from the immunized mice is not directed against Pfs48/45. The failure to induce TR activity of IgG of mice immunized with WT schizont lysate indicates that the small proportion of anti-*Pf*s48/45 antibodies are mediating the TR activity after Pfs48/45@PbMSP1 schizont lysate immunization. The strong TR activity mediated by the total IgG isolated from Pfs48/45@PbMSP1 immunized mice (Figure 2C), indicates that Pfs48/45 expressed in *P. berghei* can induce antibodies with potent TR activity.

Combined, our proof-of concept studies demonstrate that transgenic *Pb* schizonts can be used as a system to produce a difficult to express HMP protein that is correctly folded and retains conformational epitopes of the native protein. This opens possibilities to use this expression system to evaluate the immunogenicity of other difficult to express antigens or specific domains of these parasites. Studies using sera obtained from mice immunized with *Pf* proteins expressed by transgenic *Pb* parasites could be used to rank-order novel vaccine candidate antigens, not only in TB studies but also for blood-stage antigens using blood stage growth inhibition assays (GIA) or sporozoite-antigens using inhibition of sporozoite invasion (ISI) assays [3]. Moreover, the expression in transgenic schizonts of HMP proteins with affinity tags will allow for the purification of these HMP proteins from whole parasite lysate preparations and immunization with purified protein will mean that all of the raised immune response is due to the target antigen and will permit a more detailed analyses of antigen immunogenicity, for example to examine and clone potent inhibitory and cross-reactive B-cells/antibodies after rodent immunization [34]. The creation of transgenic parasites that express antigens from multiple life-cycles that can induce potent immune responses is also of interest to the development of whole organism vaccines [35]. For example, genetically attenuated sporozoite vaccines could be further modified to induce immune responses against multiple life cycle stages by expression in sporozoites and liver stages antigens of blood- or transmission–stages to produce a multi stage-vaccine.

ACKNOWLEDGEMENTS

We would like to thank Dr. Michael Theisen and Dr. Susheel K. Singh, Statens Seum Institute, Copenhagen Denmark, for kindly providing us with the recombinant *P. falciparum* P48/45 protein fragment (R0.10C). A.S. Othman is supported by a Skim Latihan Akademik IPTA - SLAI (Ministry of Higher Education, Malaysia). C. M. Mogollon was supported by Colciencias Ph.D. fellowship (Call 568 from 2012 Resolution 01218 Bogotá, Colombia). The SMFA works were supported in part by the intramural program of the National Institute of Allergy and Infectious Diseases/NIH. This work was funded by PATH's Malaria Vaccine Initiative under Grant No. OPP48782 from the Bill & Melinda Gates Foundation.

REFERENCES

1. Flick, K., et al., *Optimized expression of Plasmodium falciparum erythrocyte membrane protein I domains in Escherichia coli.* Malaria Journal, 2004. **3**.
2. Tuju, J., et al., *Vaccine candidate discovery for the next generation of malaria vaccines.* Immunology, 2017. **152**(2): p. 195-206.
3. Othman, A.S., et al., *The use of transgenic parasites in malaria vaccine research.* Expert Review of Vaccines, 2017. **16**(7): p. 685-697.
4. Ntege, E.H., et al., *Blood-stage malaria vaccines: post-genome strategies for the identification of novel vaccine candidates.* Expert Review of Vaccines, 2017. **16**(8): p. 769-779.
5. Klein, M.M., et al., *The cysteine-rich interdomain region from the highly variable Plasmodium falciparum erythrocyte membrane protein-1 exhibits a conserved structure.* Plos Pathogens, 2008. **4**(9).
6. Thompson, J., et al., *Plasmodium cysteine repeat modular proteins 1-4: complex proteins with roles throughout the malaria parasite life cycle.* Cellular Microbiology, 2007. **9**(6): p. 1466-1480.
7. MacDonald, N.J., et al., *Structural and Immunological Characterization of Recombinant 6-Cysteine Domains of the Plasmodium falciparum Sexual Stage Protein Pfs230.* Journal of Biological Chemistry, 2016. **291**(38): p. 19913-19922.
8. Longley, R.J., et al., *Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates.* Scientific Reports, 2015. **5**.
9. Miguel-Blanco, C., et al., *Hundreds of dual-stage antimalarial molecules discovered by a functional gametocyte screen.* Nature Communications, 2017. **8**.
10. van Dijk, M.R., et al., *A central role for P48/45 in malaria parasite male gamete fertility.* Cell, 2001. **104**(1): p. 153-164.
11. van Dijk, M.R., et al., *Three Members of the 6-cys Protein Family of Plasmodium Play a Role in Gamete Fertility.* Plos Pathogens, 2010. **6**(4).
12. Annoura, T., et al., *Two Plasmodium 6-Cys family-related proteins have distinct and critical roles in liver-stage development.* Faseb Journal, 2014. **28**(5): p. 2158-2170.
13. Mistarz, U.H., et al., *Expression, Purification and Characterization of GMZ2'.10C, a Complex Disulphide-Bonded Fusion Protein Vaccine Candidate against the Asexual and Sexual Life-Stages of the Malaria-Causing Plasmodium falciparum Parasite.* Pharmaceutical Research, 2017. **34**(9): p. 1970-1983.
14. Theisen, M., M.M. Jore, and R. Sauerwein, *Towards clinical development of a Pfs48/45-based transmission blocking malaria vaccine.* Expert Review of Vaccines, 2017. **16**(4): p. 329-336.
15. Outchkourov, N., et al., *Epitope analysis of the malaria surface antigen Pfs48/45 identifies a subdomain that elicits transmission blocking antibodies.* Journal of Biological Chemistry, 2007. **282**(23): p. 17148-17156.
16. Vermeulen, A.N., et al., *Sequential Expression of Antigens on Sexual Stages of Plasmodium-Falciparum Accessible to Transmission-Blocking Antibodies in the Mosquito.* Journal of Experimental Medicine, 1985. **162**(5): p. 1460-1476.
17. Milek, R.L.B., et al., *Plasmodium falciparum: Heterologous synthesis of the transmission-blocking vaccine candidate Pfs48/45 in recombinant vaccinia virus-infected cells.* Experimental Parasitology, 1998. **90**(2): p. 165-174.
18. Milek, R.L.B., H.G. Stunnenberg, and R.N.H. Konings, *Assembly and expression of a synthetic gene encoding the antigen Pfs48/45 of the human malaria parasite Plasmodium falciparum in yeast.* Vaccine, 2000. **18**(14): p. 1402-1411.
19. Milek, R.L.B., et al., *Immunological properties of recombinant proteins of the transmission blocking vaccine candidate, Pfs48/45, of the human malaria parasite Plasmodium*

falciparum produced in Escherichia coli. Parasite Immunology, 1998. **20**(8): p. 377-385.

20. Outchkourov, N.S., et al., *Correctly folded Pfs48/45 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in mice.* Proceedings of the National Academy of Sciences of the United States of America, 2008. **105**(11): p. 4301-4305.

21. Lin, J.W., et al., *A Novel 'Gene Insertion/Marker Out' (GIMO) Method for Transgene Expression and Gene Complementation in Rodent Malaria Parasites.* Plos One, 2011. **6**(12).

22. Salman, A.M., et al., *Generation of Transgenic Rodent Malaria Parasites Expressing Human Malaria Parasite Proteins*, in *Malaria Vaccines: Methods and Protocols*, A. Vaughan, Editor. 2015, Springer New York: New York, NY. p. 257-286.

23. Janse, C.J., J. Ramesar, and A.P. Waters, *High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei.* Nature Protocols, 2006. **1**(1): p. 346-356.

24. Janse, C.J. and A.P. Waters, *Plasmodium-Berghei - the Application of Cultivation and Purification Techniques to Molecular Studies of Malaria Parasites.* Parasitology Today, 1995. **11**(4): p. 138-143.

25. Singh, S.K., et al., *A Plasmodium falciparum 48/45 single epitope R0.6C subunit protein elicits high levels of transmission blocking antibodies.* Vaccine, 2015. **33**(16): p. 1981-1986.

26. Holder, A.A. and R.R. Freeman, *Biosynthesis and Processing of a Plasmodium-Falciparum Schizont Antigen Recognized by Immune Serum and a Monoclonal-Antibody.* Journal of Experimental Medicine, 1982. **156**(5): p. 1528-1538.

27. Miura, K., et al., *Functional Comparison of Plasmodium falciparum Transmission-Blocking Vaccine Candidates by the Standard Membrane-Feeding Assay.* Infection and Immunity, 2013. **81**(12): p. 4377-4382.

28. Miura, K., et al., *Transmission-blocking activity is determined by transmission-reducing activity and number of control oocysts in Plasmodium falciparum standard membrane-feeding assay.* Vaccine, 2016. **34**(35): p. 4145-4151.

29. Barr, P.J., et al., *Recombinant Pfs25 protein of Plasmodium falciparum elicits malaria transmission-blocking immunity in experimental animals.* J Exp Med, 1991. **174**(5): p. 1203-8.

30. van Dijk, M.R., et al., *Three members of the 6-cys protein family of Plasmodium play a role in gamete fertility.* PLoS Pathog, 2010. **6**(4): p. e1000853.

31. Otto, T.D., et al., *A comprehensive evaluation of rodent malaria parasite genomes and gene expression.* Bmc Biology, 2014. **12**.

32. Theisen, M., et al., *A multi-stage malaria vaccine candidate targeting both transmission and asexual parasite life-cycle stages.* Vaccine, 2014. **32**(22): p. 2623-2630.

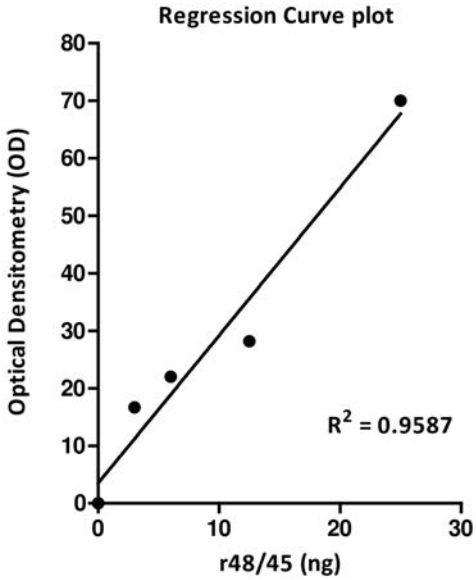
33. Eksi, S., et al., *Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production.* Molecular Microbiology, 2006. **61**(4): p. 991-998.

34. Triller, G., et al., *Natural Parasite Exposure Induces Protective Human Anti-Malarial Antibodies.* Immunity, 2017. **47**(6): p. 1197-+.

35. Bijker, E.M., et al., *Novel approaches to whole sporozoite vaccination against malaria.* Vaccine, 2015. **33**(52): p. 7462-7468.

SUPPLEMENTARY DATA

A



B

Sample	Total protein (ng)	OD	OD (-*b/g)	**Pfs48/45 (ng)	% **Pfs48/45
r48/45 (R0.10C)	50	102.99	80.56	n.a	35
	25	92.45	70.02	n.a	35
	12.5	50.63	28.20	n.a	35
	6	44.43	22.00	n.a	35
	3	39.12	16.68	n.a	35
	0	22.43	0	n.a.	n.a
Pf Gam	500	48.87	26.44	3.12	**0.62**
PbWT	500	24.26	1.83	n.a	n.a
Pfs48/45@PbMSP1	500	35.08	12.65	1.25	**0.25**
	250	28.09	5.65	0.29	**0.12**
***Background**	0	22.43	0.00	n.a	n.a

Supplementary Figure S1. Quantification of Pfs48/45 protein in Pfs48/45@PbMSP1 schizont lysate by Western blot analyses as shown in Figure 2A. Densitometry and regression curve analysis was performed on signals after probing Pfs48/45@PbMSP1 schizont lysate (500 ng) and a dilution series (50, 25, 12.5, 6 and 3 ng) of recombinant Pfs48/45 fusion protein (r48/45; R0.10C) with antibody 45.1. Regression analysis based on the dilution series of recombinant Pfs48/45 fusion (R0.10C). Optical densitometry (OD) values of signals before and after *background (b/g) subtraction and the calculated protein content and the percentage of Pfs48/45 protein of the total amount of protein in the *P. falciparum* gametocyte (*Pf* Gam), P. *berghei* WT schizont (*Pb*WT) and Pfs48/45@*Pb*MSP1 schizont lysates. ** To calculate the amount and percentage of Pfs48/45 in the protein samples we adjusted for the proportion of Pfs48/45 (approximately 35%) in the recombinant protein R0.10C (a GLURP::Pfs48/45 fusion protein).

Supplementary Table S1. List of primers used in this study

Primer ID	Leiden code	Gene ID	Sequence	Product (bp)	Description
Primers for confirmation PCR analysis					
P1	4698		GTTCGCTAAACTGCATCGTC	1108	Forward h*dhfr*
P2	4699		GTTTGAGGTAGCAAGTAGACG		Reverse h*dhfr*
P3	6324	PF3D7_1346700	CATGCCATGGATGATGTTATATATTTCTGCGAAAAAGGC	1347	Forward *Pfs48/45*
P4	6325	PF3D7_1346700	ATAGTTTAGCGGCCGCCTAAATATATAATAATTGCTACAATTAGG		Reverse *Pfs48/45*
P5	5510		GCAAAGTGAAGTTCAAATATGTG	861	Forward 5′ Integration
P6	6192		TTAATTTGCACTTCAACATCAC		Reverse 5′ Integration
P7	6196		TTTTGGCTAAAACATTTATATTCC	1568	Forward 3′ Integration
P8	5511		AGTGACTTTCAGTGAAATCGC		Reverse 3′ Integration

Pf = *P. falciparum*, h = human