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RESEARCH ARTICLE

Breaking Boundaries in Malaria Research: Design of a Genetic Tool for High-Throughput Gametocidal Drug Screening

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ABSTRACT

Malaria, a devastating illness remains a global health concern with an estimated 249 million malaria cases in 85 malaria-endemic countries around the world. Malaria elimination, challenged by drug resistance, requires strategic interventions that could be the implementation of antimalarials with selective actions on the different phases of the parasite life cycle. Of particular relevance is gametocytocidal drugs that could be used to prevent transmission of malaria infection to the mosquito.

Finding drugs with gametocidal effect thus are limited by the technical challenges of large-scale production and quantification of parasite transmission stage, gametocytes. To surmount these

obstacles, our study endeavors to design a genetic engineering strategy (a vector construct) to further deliver nucleic acid information through transfections-based systems in the form of a plasmid into Plasmodium falciparum. This approach will enable us to engineer a transgenic parasite line for multi-stage drug screening, targeting the symptomatic intra-erythrocyte parasite stage and gametocytes.

Genetic engineering tools such as selected linked integration system and attB-attP site-specific recombination will be used in our vector construct aiming the genetic integration process into the P. falciparum genome. These systems will accommodate strategies for easy and accurate stage-specific quantification such as RFP-luciferase fusion cloned downstream stage-specific promoters leading to reporter products with optical outputs and for efficient production of gametocytes at large scale using a riboswitch-based inducible gene expression system. Such technology is of major need and will pave the way for scaling up the capacity for high-throughput drug screening, leading to improved strategies to find drugs capable of blocking malaria transmission.

KEYWORDS: Malaria; drug screening; gametocytes; genome-editing strategies; *Plasmodium falciparum*.

1· INTRODUCTION

1.1· The current Malaria placement as a tropical disease

Malaria illness is one of the major concerns in the world, being an epidemic in 85 countries [1]. The disease comprises two hosts: the human and the mosquito. The Life cycle in humans starts when the female mosquito Anopheles is infected with Plasmodium parasites and takes a blood meal in humans[2]. Common antimalarials are losing efficacy due to rising resistance and do not tackle the form of transmission (gametocytes) [1]. Currently, only two drugs from the 8 - 8-aminoquinoline family (primaquine and tafenoquine) target gametocytes across all stages [3], [4], however, the treatment is very toxic for patients with glucose – 6 – phosphate dehydrogenase deficiency (G6PD)[5], therefore there is a need to screen and discover novel drugs that not only affect asexual parasites but also gametocytes. Nevertheless, studying gametocytes pose challenges. Current in vitro production methods are labor-intensive [6]. The standard approach involves subjecting parasites to stress conditions such as high parasitemia and nutrient starvation [7]. Achieving a balance in parasitemia is crucial for generating healthy gametocytes and decreasing cell death [7]. The assessment of parasitemia in gametocytes is subjective once it is user-dependent on a light microscope, and there is almost no human-friendly method to detect and quantify gametocytes [3], [8]. Given these limitations, there is a significant need to screen and discover new drugs capable of targeting both asexual parasites (responsible for symptoms) and gametocytes (responsible for the disease transmission).

1.2· Unraveling the role of gametocytes: key players in Malaria's puzzle

Gametocytes are the form of transmission [9]. The major key player in sexual commitment is the AP2 – G family [9], which promotes a cascade of genes to develop and commit the neutral forms into gametocytes [10]. Gametocytes have five stages to fully develop, mature, and be functional[11]. To follow gametocytes, the *Plasmodium falciparum* sexual–specific protein 16 (*pfs16*) seems to play a major role with expression starting 35 hours post–merozoite invasion, with the signal expression being like the five stages of gametocytes [12]. More recently, it was discovered that pfs16 promotes vector transmission of the disease by silencing the mosquito's immune system [13].

1.3· Fluorescence and luminescence

The fluorescence [19], [20], and luminescence [21] have been implemented as optical readouts of several biological markers in *Plasmodium falciparum.* Comparing fluorescence to luminescence, luminescence involves the emission of light by a substance without the need for an external light source whereas fluorescence specifically refers to the absorption and re-emission of light at a longer wavelength. Luminescence assays tend to be highly sensitive and stable, while fluorescence offers high brightness and spatial resolution, making it ideal for imaging applications. Marin-Mogollon investigators genetic–engineer a *P. falciparum* NF54 reporter line to be expressed in gametocytes, sporozoites, and liver stage through CRISPR – Cas9 for fluorescence and luminescence readings [22]. Our goal is to develop a genetic-based strategy in a form of a plasmid further to be transfected into Plasmodium falciparum for identifying and quantifying gametocytes using fluorescence and/or luminescence. This genetic tool comprises the pfs16 gametocytes-promoter (for expression of a protein that exhibits fluorescence (EmiRFP670) and luminescence (Luciferase) solely at the gametocyte stage of the parasite).

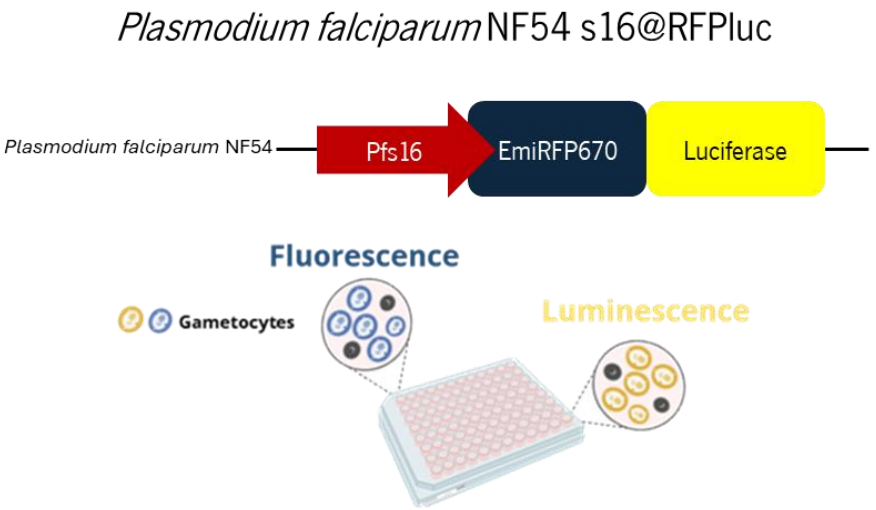

Figure 1 — Engineering a *Plasmodium falciparum* NF54 transgenic line to detect and quantify Gametocytes for high–throughput gametocidal drug screening by fluorescence and luminescence through episomal expression.

2. MATERIALS AND METHODS

2.1. *In silico* design

The genetically encoded EmiRFP670Luc@s16 tool is designed to be episomally expressed and/or integrated into the genome of *P. falciparum*, under the *pfs16* promoter (specific to gametocytes) (Figure 2). In this tool, the N-terminal *pfs16* promoter (specific of gametocytes) is in frame with an EmiRFP670 - enhanced red fluorescent protein, linked to luciferase through a linker (GGGATCCTGGCTAGCCAGTCGACCTGCAGGC). The sequence of the promoter *pfs16* was obtained from the PlasmoDB database with the PF3D7_0406200 code, luciferase sequence was obtained from *P. falciparum* NF54-GFP-luc strain (MRA 1217, BEI Resources), and the fluorescent reporter protein EmiRFP670 was obtained from the addgene plasmid pLAMP1-emiRFP670 (#136570). The plasmid pDC2 – $pfcam_p$ – Snf7 – GFP – BSD – attP was used as the backbone plasmid and was kindly provided by David Fidock and Marcus Lee. The sequence of the cloning vectors pJET 1.2 and pNZY28 were supplied by ThermoFisher Scientific and NZYTech, respectively. The ApE – A Plasmid Editor v3.0.9 and SnapGene version 6.2.1 were used for the *in silico* design of EmiRFP670Luc@s16.

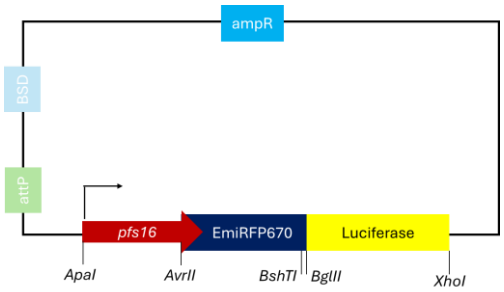

Figure 2 — Gametocytes under the *pfs16* promotor, and blasticidin-S deaminase (*bsd*) selection cassette to select the parasites for episomal expression.

2.2. Engineering the EmiRFP670Luc16 cassete

Genomic DNA (gDNA) of *P. falciparum* 3D7 and NF54-GFP-luc strains was extracted using the NZY Tissue gDNA isolation kit (NZYTech). The *pfs16* gene was amplified from *P. falciparum* 3D7 with primer P1 and P2, comprising the recognition sites for *ApaI* and *AvrII*, respectively. The *P. falciparum* NF54-GFPluc was used to amplify luciferase with primers P3 and P4, with restriction recognition sites for *BglII* and *XhoI*, respectively. The plasmid DNA (pDNA) from pLAMP1-emiRFP670 was extracted with the NZYMiniprep kit (NZYTech). The EmiRFP670 was amplified from the pLAMP1 - emiRFP670 pDNA with primers P5 and P6, with specific restriction sites for *BshTI* and *AvrII*, respectively. The *pfs16* gene promoter was amplified with a Supreme NZYTaq II 2× Green Master Mix (NZYTech), while *luciferase* and EmiRFP670 were amplified with 2X

Phusion Green Hot Start II High-Fidelity PCR Master Mix (ThermoFisher Scientific). The PCR programs and reaction conditions are depicted in Tables 1 and 2.

Table 1 — PCR reaction conditions for the three amplifications: *pfs16*, *emiRFP670*, and *luciferase*.

Fragment	PCR Reaction Mix
pfs16	2x Supreme Green Master Mix, 10 µM Forward primer, 10 µM Reverse primer, 100ng of 3D7 DNA, and water in a total volume of 20 uL
luciferase	2X Phusion Green Hot Start II High-Fidelity PCR Master Mix, 10 µM Forward primer, 10 µM Reverse primer, 100 ng *Plasmodium falciparum* MRA 1217, 0.6 µL DSMO and water in a total volume of 50 µL.
emiRFP670	2X Phusion Green Hot Start II High-Fidelity PCR Master Mix, 10 µM Forward primer, 10 µM Reverse primer, 100 ng pLAMP1 – EmiRFP670, 0.6 µL DSMO and water in a total volume of 20 µL.

After amplification, the three fragments were inserted into cloning vectors; pfs16 was introduced into the pNZY28 commercial plasmid from the NZY-A PCR cloning kit (NZYTech), while luciferase and EmiRFP670 were inserted into pJET1.2 commercial plasmid from the CloneJET PCR Cloning kit (ThermoFisher Scientific) as shown in Figure 3. These constructs were then transformed into NZYStar competent cells (NZYTech) through the heat shock method. Positive recombinants were screened through restriction enzymes, followed by Sanger sequencing (STAB VIDA, Lda).

Table 2 — Primers used for pfs16, EmiRFP670, and luciferase amplifications with the respective PCR programs, restriction recognition sites, and amplicon size.

Fragment	Restriction enzyme – recognition site	Primer sequences (5′- 3′)	Settings	Amplicon size (bp)
pfs16	*AvrII* *ApaI*	P 1- **CCTAGG**gttgaagaaagtataaatagaaaaatggc P 2 - **GGGCCC**gtagctatccaaaaataaatatcc	95 º C – 5`; 40 Cycles: 94 ºC – 30``, ºC – 30``, 72 ºC -; 72 ºC – 10′.	1296
luciferase	*BglII* *XhoI*	P 3 - gca**GATCT**gggatcctggctagcca P 4 - gc**CTCGAG**ttacacggcgatctttccgc	98 º C – 2`; 40 Cycles: 98º C –	1686

			10‟, 59ºC – 20‟, 72º C - 45‟; 72ºC – 10`	
emiRFP670	*AvrII* *BshTI*	P 5 - taagca**CCTAGG**atggcggaaggctccg P 6 - taagca**ACCGGT**gctctcaagcgcggtg	98ºC – 30‟; 35 Cycles: 98ºC – 7‟, 66.6ºC – 25‟, 72ºC – 27‟; 72ºC – 10`;	933

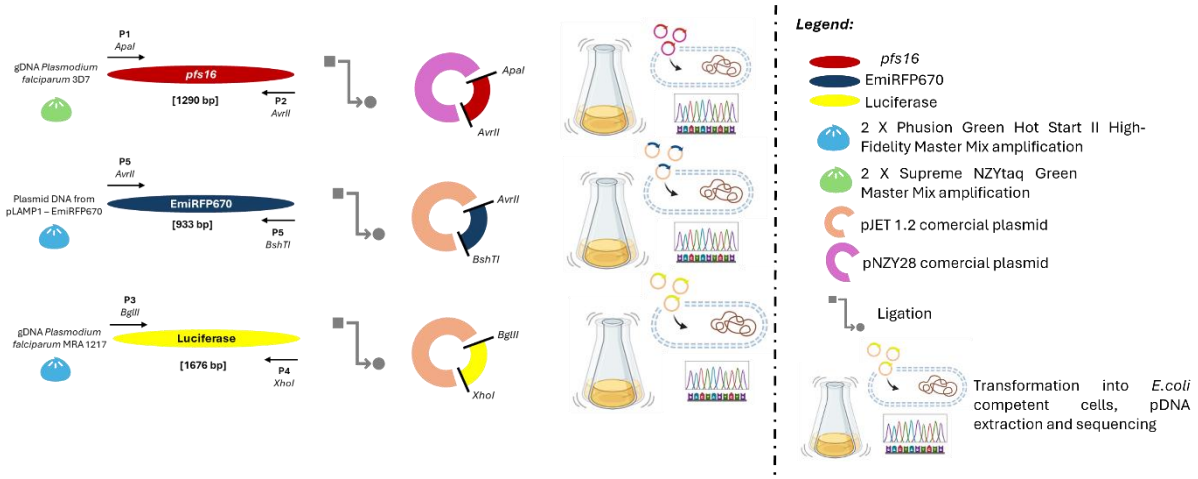

Figure 3 — An overview of the strategy for amplifying and ligating the fragments (*pfs16*, EmiRFP670, and Luciferase), which were then transformed into the pJET and pNZY28 commercial plasmids.

Restriction and ligation cloning of each fragment into the backbone plasmid pDC2 – $pfcam_p$ – Snf7 – GFP – BSD – attP was possible using *ApaI* and *AvrII*, *BshTI* and *AvrII*, and *BglII* and *XhoI* restriction sites as illustrated in Figure 4. The pDC2 – pfcamp – EmiRFP670 – GFP – BSD – attP plasmid, the pDC2 – $pfcam_p$ – EmiRFP670 – Luciferase – BSD – attP plasmid, and the final plasmid pDC2 – *pfs16* – EmiRFP670 – Luciferase – BSD – attP were screened by colony PCR with the primers and PCR program displayed in Table 3, and restriction enzymes to find the positives recombinants.

Table 3 — Colony PCR primers and settings for confirmation of pDC2 – $pfcam_p$ – EmiRFP670 – GFP – BSD – attP, pDC2 – $pfcam_p$ – EmiRFP670 – Luciferase – BSD – attP plasmid, and the final plasmid pDC2 – *pfs16* – EmiRFP670 – Luciferase – BSD – attP plasmids.

Plasmid Name	**Primers (5` - 3′)**	**PCR Settings**
pDC2 – $pfcam_p$ – EmiRFP670 - GFP	P 7– ATGATCTGGGTATCTCGCAAAGCA P8 - GAATACCAGGCCGTGGCCT	95 °C – 5′; 35 cycles: 94 ºC – 30‟,57ºC – 30‟, 72 ºC – 20‟;72 ºC – 10`

pDC2 – *pfcam*$_p$– EmiRFP670 - Luciferase	P9 – ATGGTGTATCGTTTCGATGAGCAA P10 - CCTAGGGTTGAAGAAAGTATAAATAGAAAAATGGC	95 °C – 3′; 35 cycles: 94 ºC – 30`, 50.6ºC – 30``, 72 ºC – 55``;72 ºC – 10`
pDC2 – *pfs16* – EmiRFP670 - Luciferase	P11 - ATGATAAACCGGGCGCGGT P12 - GGTCAAGAGGTCAGGCTGCC	95 °C – 3′; 35 cycles: 94 ºC – 30`, 50.6ºC – 30``, 72 ºC – 55``;72 ºC – 10`

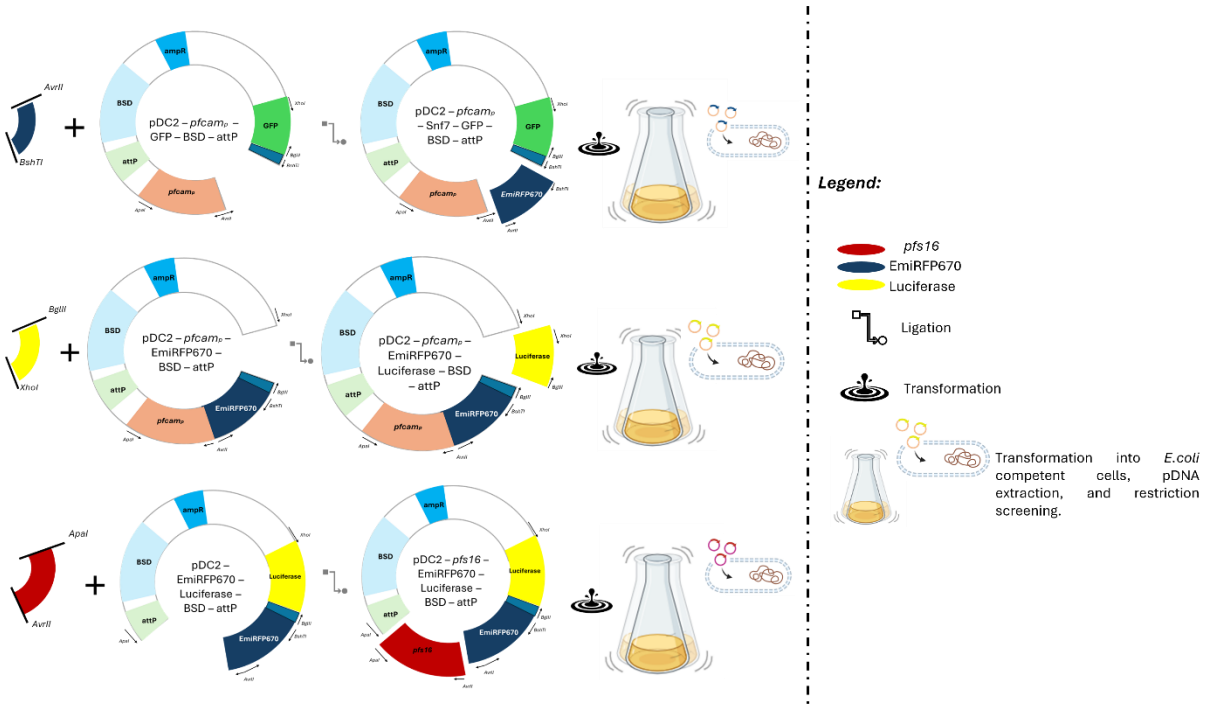

Figure 4 — Hands-on strategy to engineer the final plasmid pDC2 – pfs16 – EmiRFP670 – Luciferase – BSD – attP.

3. RESULTS AND DISCUSSION

3.1. Selection of genes, their amplification, and subsequent vector cloning

The EmiRFP670Luc@s16 genetic tool aims at the identification and quantification of gametocytes in P. falciparum. To achieve this, we selected the *psf16* promoter for the tool to be specific to gametocytes. The *psf16* gene is only expressed upon sexual commitment and is similarly expressed following 35 hours post-invasion until the rest of the gametocytes' cycle (around 15 days) [13]. This allows the identification of all gametocyte stages, including the early stage I, whose morphology resembles the one of asexual mature parasites [8], [23]. The *pfs16* was designed to be in frame with the EmiRFP670 protein linked to luciferase. These reporter proteins were selected to allow the detection and quantification of the gametocytes. EmiRFP670, derived from *Rhodopseudomonas*

palustris, presents an enhanced signal, when compared to other red fluorescent proteins, due to a increase on photostability, and brightness [24]. Specifically, this one displays an excitation peak of around 642 nm and an emission of around 670 nm [24]. The detection of this protein can be performed by any fluorescence reading equipment such as through flow cytometry, fluorimeter detection, and fluorescent microscopy, the latest also allowing localization studies [22], [25], [26]. To improve the readout capacities, we also include a protein that emits luminescence, in this case, we chose *luciferase*. Luciferase is more sensitive (present low signal-to-noise ratio) and allows the test of frozen samples [9].

The PCR amplification of each gene was confirmed through visualization of agarose gel electrophoresis under ultraviolet light with the expected amplicon sizes enunciated in Table 2. Afterward, each gene was cloned into an intermediate commercial plasmid (pJET and pNZY28) to facilitate the final cloning through restriction–ligation reactions (Figure 3A). Positive recombinants were verified with restriction enzymes (Figure 3B) before being outsourced for Sanger Sequencing.

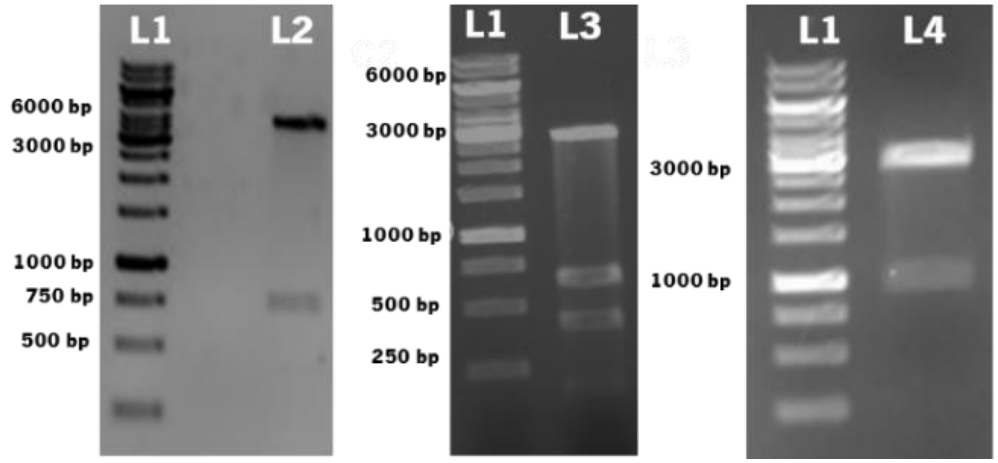

Figure 5 — Agarose Gel of the restrictions of the plasmid of interest. Lane 1: Gene Ruler™ 1 kb DNA Ladder; Lane 2 (L2): pJET – Luciferase plasmid restriction with *BstBI*: 3799 bp; 788 bp; Lane 3 (L3): pNZY28 - *pfs16* plasmid restriction with *BamHI* and *NsiI*: 2862 bp, 666 bp, 457 bp, and 184 bp; Lane 4 (L4): pJET – EmiRFP670 plasmid restriction with *BshTI* and *AvrII*: 2974 bp, and 933 bp.

3.2· Engineering the Pdc2 – *pfs16* – EmiRFP670 – Luciferase – BSD – attP

The pDC2 – *pfcam*$_p$ – Snf7 – GFP – BSD – attP plasmid was used as the backbone plasmid for our vector construct, EmiRFP670Luc@s16 cassette. The pDC2 plasmid was selected for its capacity to enable the expression of fluorescent and luminescent proteins under the control of a gametocidal promoter (pfs16). Additionally, this plasmid contains the attP sequence, facilitating genome integration through recombination of the attPsite in the plasmid with agenomic attB site,
facilitated by the bxb1 integrase system [17]. Furthermore, this plasmid presents a promoter (*pfcam*$_p$), followed by two genes (*snf7* and *gfp*) with sites for restriction enzymes (*ApaI* and *AvrII*, *BshTI* and *AvrII,* and *BglII* and *XhoI*). Thus, these restriction enzymes were used to allow the replacement of the original genes (*pfcam*$_p$, *snf7,* and *gfp)* by our genes of interest (*pfs16, emiRFP670,*

and linker + *luciferase*), avoiding open reading frame errors. The pDC2 – $pfcam_p$ – EmiRFP670 – GFP – BSD – attP plasmid, the pDC2 – $pfcam_p$ – EmiRFP670 – Luciferase – BSD – attP plasmid, and the pDC2 – *pfs16* – EmiRFP670 – Luciferase – BSD – attP plasmid screening and positive results are depicted in Figure 6.

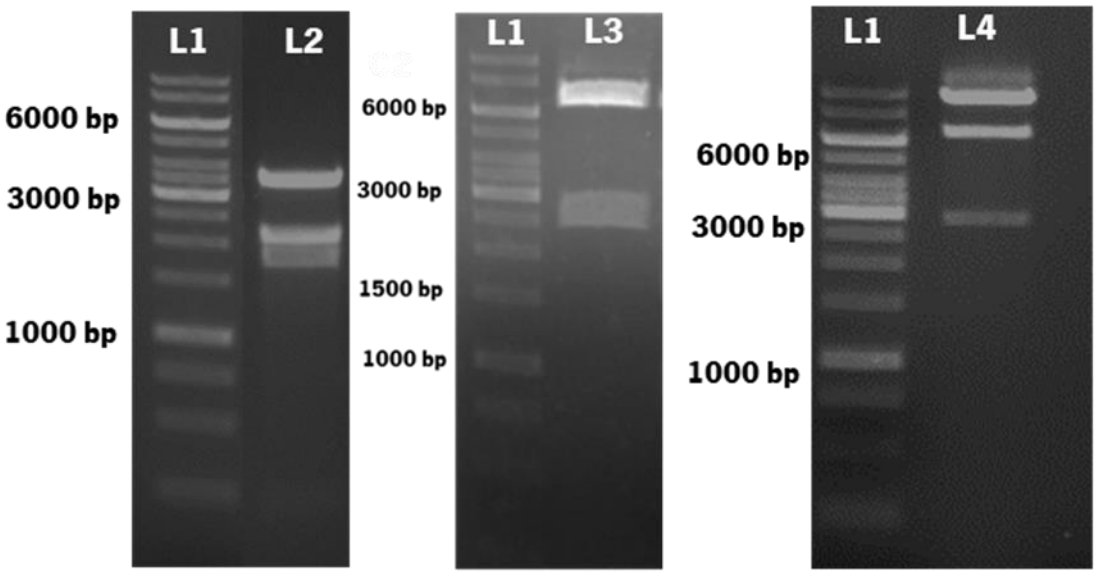

Figure 6 — 0.8% Gel agarose of the restriction: Lane 1(L1): Gene Ruler™ 1 kb DNA Ladder; Lane 2 (L2): pDC2 – $pfcam_p$ – EmiRFP670 – GFP – BSD – attP plasmid restriction with *AatlII* and *BamHI*: 3605 bp, 2210 bp, 2000 bp and 239 bp ; Lane 3 (L3): pDC2 – $pfcam_p$ – EmiRFP670 – Luciferase – BSD – attP plasmid restriction *SdaI* and *NheI*: 6512 bp, 2525 bp, 10 bp; Lane 4 (L4): pDC2 – *pfs16* – EmiRFP670 – Luciferase – BSD – attP plasmid restriction with *Xbal*: 6419 bp; 2839 bp.

4 · CONCLUSION

In this article, we report EmiRFP670Luc@s16 as a genetic tool to detect and quantify gametocytes through the expression of a red fluorescent protein and luciferase. By using pfs16, a specific sexual promoter, the EmiRFP670Luc@s16 cassette will target the 5 *P. falciparum* stages of gametocytes with almost constant expression levels throughout their lifecycle. The EmiRFP670Luc@s16 was designed to express the EmiRFP670, which allows live cell-imaging and quantification by optical readouts, including fluorescence microscopy, fluorimeter and flow cytometry, and luciferase for the sensitive detection of frozen samples through luminescence. The herein described a genetic tool is designed to allow both episomal and genomic integration expression, through parasite selection with BSD. Genomic integration of this tool, as previously mentioned, can occur through the bxb1 integrase system, however this requires an attB-engineered parasite strain. The episomal plasmid is more versatile as it does not require the time-consuming genome integration of recombinants, nor specific strains. We are confident that the vector construction we have developed represents a significant advancement in the field. This innovative tool enables rapid and precise optical readout of gametocytes, making it ideal for high-throughput screening of gametocidal drugs. Additionally, it offers the opportunity to assess the function of proteins involved in gametocyte development. By

revolutionizing the screening process for anti-gametocyte drugs, this genetically engineered tool also has the potential to identify key players in gametocyte development, thus informing the discovery of new transmission blocking antimalarial drugs.

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Author Contributions. Beatriz Fonseca has done the investigation, lab work, and writing. Vitória Baptista has contributed to the writing review, Leyre and Carla have contributed to the *in silico* design, and Isabel Veiga has made conceptualization, writing –review, and editing. All the authors revised and approved the manuscript.

Conflict of Interest. The authors declare no conflict of interest.

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