Chapter 2: General Methodology

2.1 Cultivation of *Plasmodium falciparum*

Parasites were cultivated *in vitro* following the methods developed by Trager and Jensen with minor modifications (Trager and Jensen, 1976). All cell culture work was conducted in a sterile biological safety cabinet and the mandatory safety precautions of each institution were followed (United States, Biosafety Level-2/BSL-2; United Kingdom, Containment Level-3/CL3).

2.1.1 *In vitro* culture maintenance

Asexual stages of *P. falciparum* isolates adapted for cell culture were grown in complete media (CM) consisting of Roswell Park Memorial Institute medium (RPMI)-1640 (Thermo Fisher Scientific) supplemented with 0.5-1% Albumax II (a serum substitute), GlutaMAX (Gibco, L-glutamine substitute), HEPES (20 mM, pH 7.0), 2.0 g/L sodium bicarbonate, and gentamicin (25 μg/ml) with 2-3% O+ human erythrocytes, more commonly referred to as red blood cells (RBCs). All parasite lines used in this study were adapted for continuous culture in Albumax II, a human serum substitute, but when specified, field isolates were also supplemented with 1.25-5% pooled human AB serum (Innovative Research). Unless noted otherwise, parasites were maintained in routine continuous culture at 0.5-2% parasitemia in CM at 2-3% hematocrit and stored at 37°C in non-vented tissue culture flasks (Corning) after addition of mixed gas containing 1% O_2, 3% CO_2, and 96% N_2.

2.1.2 Determination of parasite culture hematocrit and parasitemia

Hematocrit is defined as the proportion (% volume) of red blood cells per volume of blood or culture. For the purposes of this thesis and as widely standardized for *in vitro* cultivation of *P. falciparum* (Trager and Jensen, 1976), hematocrits ranged from 1-5% depending on the parasite line and experimental procedure. *P. falciparum* can invade and grow in all human blood types, but O+ RBCs were used preferentially due to their compatibility with any serum type (Jensen, 1988, Schuster, 2002). Leukocyte-depleted RBCs were obtained from healthy O+ donors weekly (Interstate Blood Bank, USA or National Health Service (NHS), UK) and added fresh to cultures at least once per week. Before use in cultures, the blood was aliquoted and washed twice with incomplete media (ICM) (RPMI-1640, with no additional supplements) by centrifugation at 3000 RPM for 10 minutes, without brake to ensure separation of the medium from the packed RBCs (pRBCs). After the final wash, the top layer

of ICM was aspirated and CM was added to dilute the packed cells to 50% hematocrit. Aliquots were stored at 4°C and used to maintain cultures for up to two weeks. When specified, 10% citrate-phosphate-dextrose-adenine (CPDA), an anticoagulant/whole blood preservative was added to the 50% hematocrit aliquot to prolong RBC integrity in long-term culture experiments (see **sections 2.3-2.4**).

Parasitemia denotes the quantification of parasites in the blood. Parasites can be measured in the blood using light microscopy or flow cytometry. The methods used for this study were based on the degree of quantitative accuracy required. Flow cytometry was utilized when more accurate parasite counts were necessary or to detect very low parasitemias (see section 2.7 on flow cytometry). Light microscopy was preferred for day-to-day culturing and accurate staging of the 48-hour *P. falciparum* life cycle.

To view and quantify *P. falciparum* parasitemia in cell cultures, medium was aspirated from the culture and ~1.5-2 µl of the pRBCs were spread on a glass microscope slide to create a thin film or "smear" of blood. Thin smears of every culture were made at least once weekly to quantify the percent parasitemia. The blood smears were fixed in 100% methanol, stained with 10% Giemsa (Sigma-Aldrich) for 10 minutes, then viewed with the 100X objective under oil immersion. Components in the Giemsa staining solution attach to DNA, making the stain particularly useful for detecting malaria parasites in cell culture, since their RBC hosts are devoid of a nucleus. Thus, any Giemsa stained cells on the blood smear can be viewed and counted as a parasite. (Note this restricted to *in vitro* parasite cultures or other samples depleted of leukocytes; in patient blood, leukocytes and any circulating reticulocytes (younger RBCs) contain DNA and would stain positive as well.) Parasitemia was calculated by dividing the number of parasite-infected red blood cells (iRBCs) by the total number of RBCs (iRBCs) + uninfected RBCs) times one hundred (iRBCs/total RBCs*100= % parasitemia). For each smear, at least 500 cells were counted to obtain the % parasitemia.

Parasitemias of continuous cultures ranged between 0.5-5%, depending on the parasite line and experiment. For specific experimental protocols, parasitemias were grown above 5%, but culture medium was changed at least once daily to avoid nutrient depletion and stress. High parasitemias and other environmental conditions of *in vitro* cultures have been shown to stress asexual stage parasites and promote the irreversible conversion of asexual stages to gametocytes, the non-replicating, sexual stage of parasites (Carter and Miller, 1979). Parasitemias of cultures were therefore monitored carefully and the higher the parasitemia,

the more frequent medium changes and blood replenishment occurred to prevent increased gametocyte production.

2.1.3 Thawing and cryopreservation of parasites

To minimize effects from long-term *in vitro* cultivation, parasites were grown in culture for up to 2-3 months then discarded after a fresh aliquot of the isolate was thawed. Parasite lines adapted for continuous culture were cryopreserved as described below and stored in a -80°C freezer or in liquid nitrogen. Frozen cryovials were thawed for 2-3 minutes in a 37°C incubator. All thawing solutions were warmed to 37°C before use. After thawing, the volume of the isolate was measured and transferred to a 50 mL centrifuge tube. Slowly, 0.1x the volume of 12% NaCl was added dropwise to the culture with gentle, intermittent shaking/swirling between drops. The tube was left standing at room temperature for 5 minutes. Next, 10x the volume of 1.6% NaCl was added to the isolate dropwise, while shaking/swirling. The mixture was left at room temperature undisturbed for an additional 5 minutes. Then the tube was centrifuged at 1500 RPM, room temperature for 5 minutes. The supernatant was aspirated and 10x the volume of 0.9% NaCl/0.2% dextrose was added dropwise to the pellet while slowly swirling the tube. The culture was centrifuged as before at 1500 RPM for 5 minutes. After aspirating the remaining thawing solution, the pellet was resuspended in pre-warmed CM and transferred to a culture flask and fresh RBCs were added, depending on the volume of thawed blood, for a final hematocrit of 1-3%.

Cryopreservation of the parasites was performed as soon as the cultures contained at least 1-2% of ring-stage parasites. Such ring-stage parasitemia is necessary because the method for freezing-down the cultures only preserves ring-stages. The culture was centrifuged at 2500 RPM for 5 minutes. The medium was aspirated and the volume of the packed culture was estimated and 1.66x the pellet volume of pre-warmed Glycerolyte 57 (Fenwal) solution was added to the pellet dropwise, with gentle shaking. The solution was transferred to a labelled cryovial (up to 1 mL per vial) and stored in a -80°C freezer or liquid nitrogen.

2.1.4 Synchronization of parasites: sorbitol and Percoll gradients

In *P. falciparum*-infected patients, the populations of parasite stages may be asynchronous or synchronous (Hawking et al., 1968, Hawking, 1970, Garcia et al., 2001). Ring stages predominate in circulation because mature trophozoite and schizont stages are sequester in the vasculature or are removed by splenic filtration (Miller et al., 2002). Ring stages survive

better than mature stages during cryopreservation, so that freshly established cultures tend to be synchronous, but these will become asynchronous after several life cycles (Trager and Jensen, 1976, Haynes et al., 1976). For many experimental procedures, stage-specificity is crucial, so two main methods of synchronization were employed by this study: sorbitol lysis and Percoll gradient separation.

2.1.5 Sorbitol synchronization

Sorbitol synchronization was used to obtain ring-stage populations of parasites (Lambros and Vanderberg, 1979). A stock solution of 5% D-sorbitol was prepared in deionized water and filtered (0.2 μm). Cultures containing mostly rings (less than 10-12 hours post-invasion) were centrifuged at 2500 RPM for 5 minutes, medium aspirated and the volume of the pellets/pRBCs was measured. The pellets were resuspended in10x the pellet volume of pre-warmed 5% sorbitol, vigorously mixed and left at 37°C for 10-15 minutes. After incubation, the tubes were centrifuged at 2500 RPM for 5 minutes and resuspended in 10x volume of ICM. Cells were again centrifuged at 2500 RPM for 5 minutes, media was aspirated and cells were resuspended in CM to maintain the culture volume at 2-3% hematocrit.
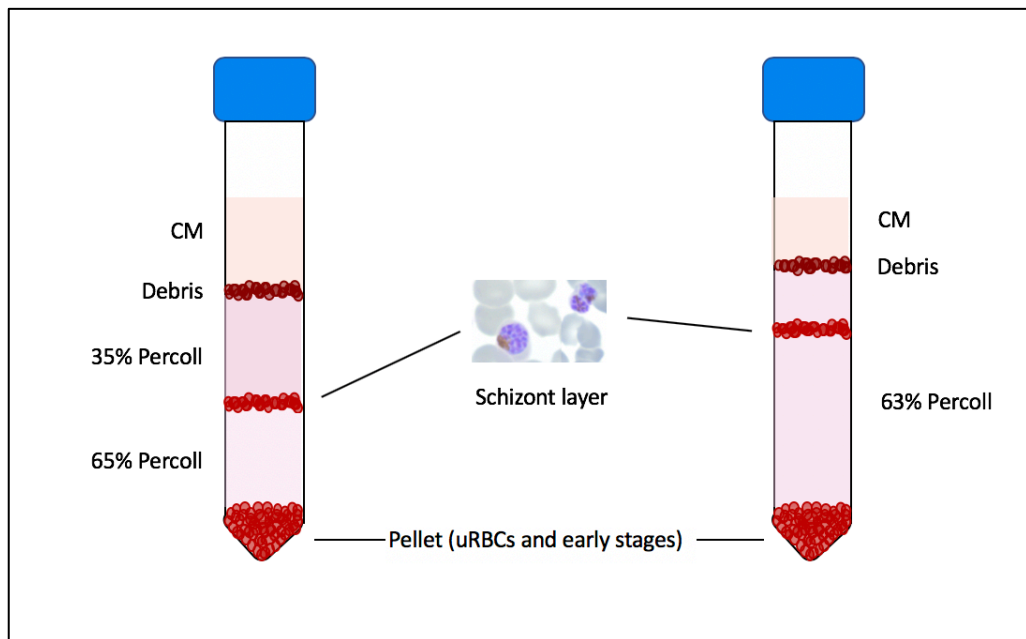
2.1.6 Percoll gradients for parasite synchronization

Percoll gradients were used to separate late-stage parasites from early stages (Kramer et al., 1982, Stanley et al., 1982). Percoll enrichment was performed using either a 35%/65% gradient when at the National Institutes of Health (USA) or using a 63% single gradient when at the Wellcome Sanger Institute (UK). Variations in the protocols at each institute were based only on the availability of resources at each location and no differences were observed in experimental results from either Percoll procedure.

For the 35%/65% gradient procedure (Miao and Cui, 2011), a 90% stock of Percoll (GE Lifesciences) was made by diluting 9 volumes of Percoll in 1 volume of 10X PBS. The 90% Percoll stock was kept at 4°C for up to 1 month before making a fresh stock. On the day of synchronization, a solution of 7.5 U/mL heparin (Sigma) in ICM, referred to as heparinized RPMI, was prepared and warmed at 37°C. Aliquots of 35% and 65% Percoll were made by diluting the pre-warmed 90% Percoll stock with heparinized RPMI. Cultures to be synchronized were centrifuged at 2500 RPM for 5 minutes and resuspended in heparinized RPMI and left standing at 37°C for 15 minutes. While parasites were incubating, Percoll gradients were prepared in 15 mL centrifuge tubes by pipetting 65% Percoll into each tube,

then layering 35% Percoll on top, being careful not to create any bubbles or disturb the separation between the two solutions. The culture was then added on top of the 35% layer and a distinct separation between the culture and the Percoll layers was observed. The gradients were layered in 3's with 3 mL of 65% Percoll, followed by 3 mL of 35% Percoll, and 3 mL of culture consisting of ~300 µL packed RBCs. After adding all three layers the tubes were centrifuged for 15 minutes at 1000g with no brake. After centrifugation the top layer containing debris was discarded (see **Figure 2.1**), the next layer containing the enriched, mature parasitized RBCs was saved and added to a tube containing 10 mL of pre-warmed ICM. The bottom pellet containing early stages and uRBCs was washed in 10 mL of ICM or discarded as necessary for the particular experiment. The tubes were centrifuged at 1000g for 5 minutes then resuspended in the desired volume of CM and/or RBCs.

The 63% Percoll procedure was performed by diluting Percoll to 63% with ICM and 10X PBS. This protocol does not use heparin. The culture was centrifuged at 800g for 5 minutes while 5-6 mL of pre-warmed 63% Percoll was added to 15 mL tubes. After centrifugation, the pellet (at least 1-1.5 mL of pRBCs) was resuspended in 5 mL of CM and layered on top of the 63% Percoll. The gradient was centrifuged at 1200g for 12 minutes, 0 break. After centrifugation, the layers were treated the same as for the aforementioned 35%/65% gradient, with the top debris layer aspirated and discarded, the middle late-stage layer saved and bottom pellet saved, if desired. The separated stages were washed in 10 mL of prewarmed CM by centrifugation at 800g for 5 minutes and resuspended in the volume of CM and RBCs needed for subsequent experiments.

Figure 2. 1. Schematic of Percoll gradients. A.) Schematic of a 35%/65% Percoll gradient. The topmost cell suspension layer is aspirated, the distinct band in the middle between the 35% and 65% layers are enriched late-stage parasites, which can be seen in the Giemsa smear. The bottom pellet contains uninfected RBCs (uRBCs) and early-stage parasites. **B.**) Schematic of a 63% Percoll gradient.

2.2 Parasite lines

2.2.1 Cambodian field isolates

The patient isolates used for this study were obtained from cohort studies performed from September 2012 through December 2013 in three Cambodian provinces (ClinicalTrials.gov number, NCT01736319) (Amaratunga et al., 2016). All samples were collected from patients aged 2-65 years with acute, uncomplicated *P. falciparum* malaria. The isolates were adapted to *in vitro* parasite culture (Amaratunga et al., 2016) using previously published methods (Trager and Jensen, 1976, Haynes et al., 1976) and cultivated with the same culture conditions used for established parasite lab lines (**see Section 2.1**). The field isolates used during the course of this thesis research are summarized in Table 1.
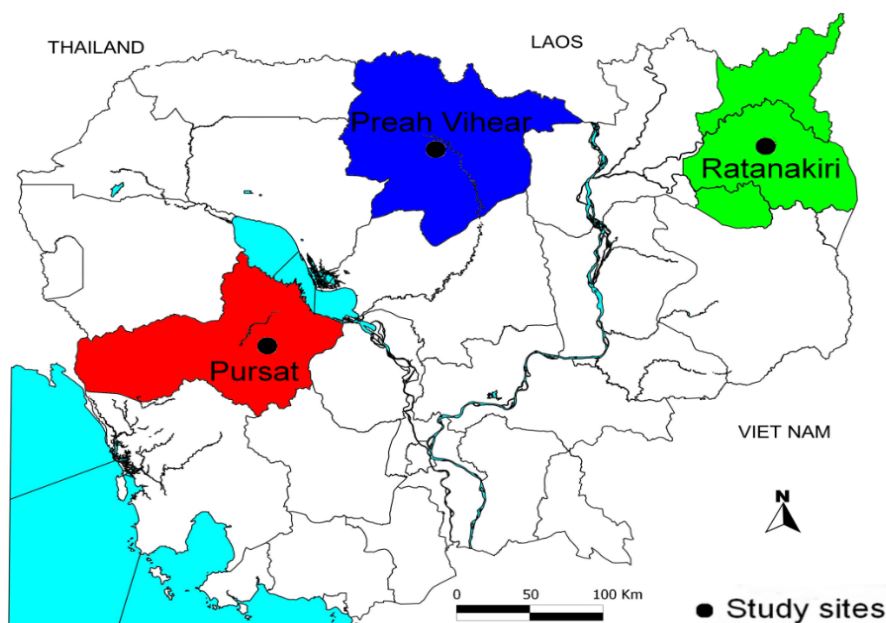
The isolates referred to as piperaquine-resistant (PPQ-R) in this study are from the Western Cambodian province of Pursat or the Northwestern province of Preah Vihear. Isolates referred to as piperaquine-sensitive (PPQ-S) are from the Eastern province of Ratanakiri or Preah Vihear (**Figure 2.2**). Since PPQ-S parasites may recrudesce for various reasons and not all PPQ-R infections will recrudesce in immune patients, final categorization of lines as PPQ-R or PPQ-S was established by parasite susceptibility to piperaquine as determined in *ex vivo, in vitro,* and piperaquine survival assays (PSAs) (Duru et al., 2015, Amaratunga et al., 2016, Amato et al., 2017). Recrudescence vs. recurrent infections were determined by PCR

genotyping *msp1*, *msp2*, and *glurp*, the genetic markers used to distinguish recurrent infections from new infections (Snounou and Beck, 1998). Using both genotypic and phenotypic data, we were then further able to distinguish both the PPQ-R and PPQ-S populations of parasites (**Table 2.1**). The parasites referred to as PPQ-R have non-synonymous single nucleotide polymorphisms (SNPs) in the genes encoding the following proteins: K13 C580Y, MCP-N252D, exo-E415G, and several contain mutations in PfCRT including H97Y, M343L, and A366T. They also have two or more copies of *plasmepsin 2* and *plasmepsin 3* and one copy of *pfmdr1* (**Table 2.1**). In contrast the PPQ-S group of parasites are wildtype for: K13 C580, MCP-N252, exo-E415, and they have a single copy of *plasmepsin 2* and *plasmepsin 3*; similarly, they have one copy of *pfmdr1* (**Table 2.1)** but none of the PfCRT mutations noted in the PPQ-R parasites (Amaratunga et al., 2016). Details of these lines were published after whole genome sequencing in Amato *et al.* 2017 (Amato et al., 2017).

The Cambodian field isolate, PH-0212-C, was also used in this study and detailed in Table 2.1. It was obtained from Pursat, Cambodia in 2010 (Amaratunga et al., 2012) ClinicalTrials.gov, number NCT00341003) and has an intermediate resistance to PPQ and some of the mutations present in the isolates from 2012-2013 (**Table 2.1**).

Table 2. 1 Summary of Cambodian field isolates and laboratory strains used in this study

Oxford code (Sanger ID)	Code/Name	Region	PPQ status	Year	Recrudescence after DHA-PPQ treatment	Kelch13	mdr1 CN	PM2-3 CN	exo-E415G SNP	mcp-N252D SNP	PfCRT SNPS
PH1150-C	163-KH2-40	Preah Vihear	PPQ-S	2013	No	WT	1	1	E	N	WT
PH1034-C	163-KH2-007	Preah Vihear	PPQ-S	2012	No	WT	1	1	E	N	WT
PH1310-C	163-KH3-056	Ratanakiri	PPQ-S	2013	No	WT	1	1	E	N	WT
PH1058-C	163-KH3-012	Ratanakiri	PPQ-S	2012	No	WT	1	1	E	N	WT
PH0971-C	163-KH3-005	Ratanakiri	PPQ-S	2012	No	WT	1	1	E	N	WT
PH1097-C	163-KH2-033	Preah Vihear	PPQ-S	2012	No	WT	1	1	E	N	WT
PH1008-C	163-KH1-01RME	Pursat	PPQ-R	2012	Yes	C580Y	1	2	G	D	M343L
PH1345-C	163-KH1-004	Pursat	PPQ-R	2012	Yes	C580Y	1	3	G	D	WT
PH1224-C	163-KH1-081	Pursat	PPQ-R	2013	Yes	C580Y	1	2	G	D	A366T
PH1387-C	163-KH2-021	Preah Vihear	PPQ-R	2012	Yes	C580Y	1	2	G	D	H97Y
PH1265-C	163-KH1-060RME	Pursat	PPQ-R	2013	Yes	C580Y	1	3	G	D	H97Y
PH1263-C	163-KH1-059RME	Pursat	PPQ-R	2013	Yes	C580Y	1	3	G	D	H97Y
Dd2	Dd2	Southeast Asia	PPQ-S	N/A	N/A	WT	2	1	E	N	WT
3D7	3D7	Africa	PPQ-S	N/A	N/A	WT	1	1	E	N	WT

Figure 2. 2. **Map of Cambodia highlighting the three provinces where samples were collected during clinical trials.** From West to East, Pursat (red) is identified as "KH1" in the NIH sample identification code, Preah Vihear (blue) is "KH2," and Ratanakiri (green) is "KH3."

2.2.2 Long-term laboratory-adapted parasite lines

The laboratory strains (lab strains) used in this study are differentiated from the contemporary Cambodian isolates in that the lab lines are widely used parasite lines that have been maintained *in vitro* for many years. The lab strains, 3D7 and Dd2, were used for the purposes of this research and are included in Table 2.1.

The 3D7 parasite is a clone of NF54, which originated from a patient living near the Schiphol Airport, Amsterdam in the Netherlands, who had never been abroad (Ponnudurai et al., 1981, Walliker et al., 1987). The exact origin of the NF54 isolate is still unknown, but genetic studies suggest that it is from Africa (Preston et al., 2014, Su, 2014).

The Dd2 parasite is a Southeast Asian line derived as a clone from the W2-MEF line (a mefloquine pressured clone of W2) (Oduola et al., 1988a) which was obtained from an Indochina/CDC *P. falciparum*-infected patient isolate (Oduola et al., 1988b, Guinet et al., 1996).

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2.3 Transfection of *P. falciparum* field isolates and laboratory lines

2.3.1 Transfection of P. falciparum using DNA-loaded red blood cells

Transfections of pre-loaded RBCs were performed using previously published methods (Wu et al., 1995, Deitsch et al., 2001) and based on the findings that *P. falciparum* takes up pre-loaded DNA from within its host RBC. Approximately 350 μL of 50% uninfected RBCs (uRBCs) or 175 μL of packed RBCs were aliquoted for each transfection and spun down in 15 mL centrifuge tubes for 5 minutes at 2500 RPM and washed twice with 5 mL of cytomix. During the washing steps, late-stage parasites (schizonts) to be transfected were enriched from cultures by Percoll gradient (**Section 2.1.4**) then gassed and incubated at 37°C. After the final wash of uRBCs, all cytomix was aspirated from the pellet and 50-100 μg of plasmid DNA in cytomix was added to each pellet for a final volume of 400 μL per tube. The contents of each 15 mL tube were transferred to 0.2 cm cuvettes and electroporated using a Bio-Rad GenePulser Xcell (Bio-Rad) set for 0.310 kV, 975 μFD, infinity resistance. Time constants were monitored with the desired range between 10-14 ms. Only 1-2 cuvettes were electroporated at a time and immediately following the pulse, contents were transferred to 15 mL culture tubes with 10 mL of CM and were incubated in the cell culture hood or at 37°C for 15-30 minutes. After recovering, the tubes of electroporated RBCs were centrifuged at 2500 RPM for 5 minutes and supernatant was aspirated. RBC lysis is expected in the supernatant, so it appears redder in color than normal. The cells were washed with ICM and then resuspended in ~5 mL CM. The Percoll enriched parasites (~30-50 μL pellet) were resuspended in CM for a final concentration of ~10 μL pRBCs/mL and 1 mL was added to each transfection for a final 2-3% hematocrit culture. Cultures were gassed and incubated at 37°C. The next day (day 1 post-transfection), all transfections were smeared to ensure parasitemia was not too high (>4-5%) or too low (unobservable or <0.5%). If the parasitemia was too high, the cultures were cut and if parasitemia was too low, the cultures were watched closely or discarded. On day 1 or day 2 post-transfection, parasites were selected with drug-supplemented CM, containing either 1-10 nM WR99210 (Jacobus Pharmaceuticals) or 2 μg/mL blasticidin (BSD) (Thermo-Fisher Scientific) depending on the plasmid used and parasite transfected. The drug concentrations used for selection were based on the IC_{50} values for the particular drug for each distinct parasite line. Optimally, parasites were selected with a drug concentration just above the IC_{50} value.

Transfections were smeared daily until they were observed to die off due to drug pressure (about 4-6 days post-transfection, depending on the drug). After that point, transfections were smeared only 1-2 times per week until around Day 18. If field isolates were not up by days 50-60, they were discarded.

Due to the challenges associated with the transfection of field isolates, variations of this general transfection protocol were performed to enhance transfection efficiency and are detailed within each data chapter when relevant.

2.3.2 Transfection of ring stage *P. falciparum*

Transfection of ring stage parasites was performed similar to DNA-loaded RBCs (**section 2.3.1**). In ring stage transfections, ring stage parasites (iRBCs) are directly electroporated. This procedure was predominantly performed using lab lines (Dd2), rather than field isolates, because this method causes direct stress to the parasites upon electroporation and lab lines are well-adapted to culture conditions and procedures.

Ring-stage parasites were collected at 5-6% ring-stage parasitemia, with earlier rings preferred. For every transfection, 3-5 mL of a culture at 2-3% hematocrit and 5-6% ring-stage parasitemia was centrifuged at 2500 RPM for 5 minutes and resuspended and washed in 5 mL cytomix. After the wash, 50-100 μg of plasmid DNA was added to each 15 mL tube in a total volume of 400 μL of cytomix and transferred to a 0.2 cm cuvette and electroporated under the same conditions described above (**section 2.3.1**). Immediately following electroporation, transfections were transferred to 15 mL tubes containing 10 mL of prewarmed CM then left in the 37°C incubator to recover for 1 hour. After recovery, transfections were centrifuged and washed in ICM at 2000 RPM for 5 minutes. Cultures were resuspended in medium for a final 2-3% hematocrit, gassed, and incubated overnight. As detailed above (**2.3.1**), cultures were smeared the following day and cut if parasitemias were too high. Drug was added on Day 1 post-transfection, using 1-5 nM WR99210 (Jacobus Pharmaceuticals) or 2 μg/mL blasticidin (BSD) (Thermo-Fisher Scientific) depending on the plasmid and parasite transfected.

Ring-stage transfections were supplemented with fresh blood containing 10% citrate-phosphate-dextrose-adenine (CPDA), an anticoagulant/whole blood preservative used to prolong RBC integrity since lab lines could take approximately 15-25 days to come up. If transfection experiments did not show parasites by days 40-45, they were discarded.

2.4 Parasite cloning by limiting dilution

To obtain isogenic parasite lines after positive transfections were confirmed, the bulk cultures were cloned by limiting dilution to obtain individual parasites (Rosario, 1981). Samples were plated in 96-well plates at 0.6 parasites per well and 1% hematocrit. For all cloning procedures, the cloning plates were set up in freshly washed blood prepared with 10% CPDA. Field isolates were plated at 0.8-1.2 parasites/well at 1% hematocrit in conditioned medium due to their slower growth rate. Conditioned medium was prepared by filtering spent medium from the specific isolate and diluting 1:2 or 1:4 with fresh CM. Conditioned medium was stored at 4°C for up to 3 days then discarded. Weekly, 0.5% fresh RBCs and CM or conditioned CM were added to each plate. Around days 17-18, plates were screened for parasite growth using a 1X SYBR Green I solution in lysis buffer. This technique takes advantage of the properties of SYBR to fluoresce upon intercalating between DNA bases. Since only parasitized RBCs contain DNA, only wells that contain living parasites will produce a measurable level of fluorescence when exposed to SYBR Green I. Aliquots from the cloning plates (~20 µl) were resuspended thoroughly in the lysis buffer solution (consisting of 10 mM Tris-HCl, 5 mM EDTA, 0.1% w/v saponin, and 1% v/v Triton X-100) and 1X SYBR Green I (Invitrogen, 10,000x stock) and incubated in the dark at 37°C for 20 minutes then read on a microplate reader (FLUOstar Omega) using a 485/535 nm excitation and emission filter. Positive wells with values higher than the background wells containing CM and RBCs-only were screened by smear. If parasites were observed, the clones were expanded and saved for genomic DNA (gDNA) extraction, cryopreservation, and continuous cultures were maintained for functional assays.

2.5 Saponin lysis and genomic DNA extraction from cultivated parasites

Parasites were collected for genomic DNA using a saponin solution to selectively permeabilize infected RBC membranes to remove host cell hemoglobin content. When cultures reached at least 2% parasitemia of late stages (trophozoites and schizonts) at 2-3% hematocrit in 10 mL-12 mL volume, cultures were spun down at 2500 RPM for 5 minutes and resuspended in 0.15% saponin (Sigma-Aldrich) in 1X phosphate buffered saline solution (PBS). Saponin solutions were made fresh on the day of the experiment from 1% or 10% stocks stored at 4°C for several months. Tubes were left to rest for 3-5 minutes at room temperature then were centrifuged at 4000 RPM, washed at least twice with 1X PBS, until the sample turned from red to a brownish/black color and the pellets were stored at -20°C or used immediately for DNA extraction. A DNeasy Blood & Tissue Kit (Qiagen) was used to

extract genomic DNA from thawed saponin pellets. DNA was eluted using the kit buffer (buffer AE) or water, depending on the experimental procedure.
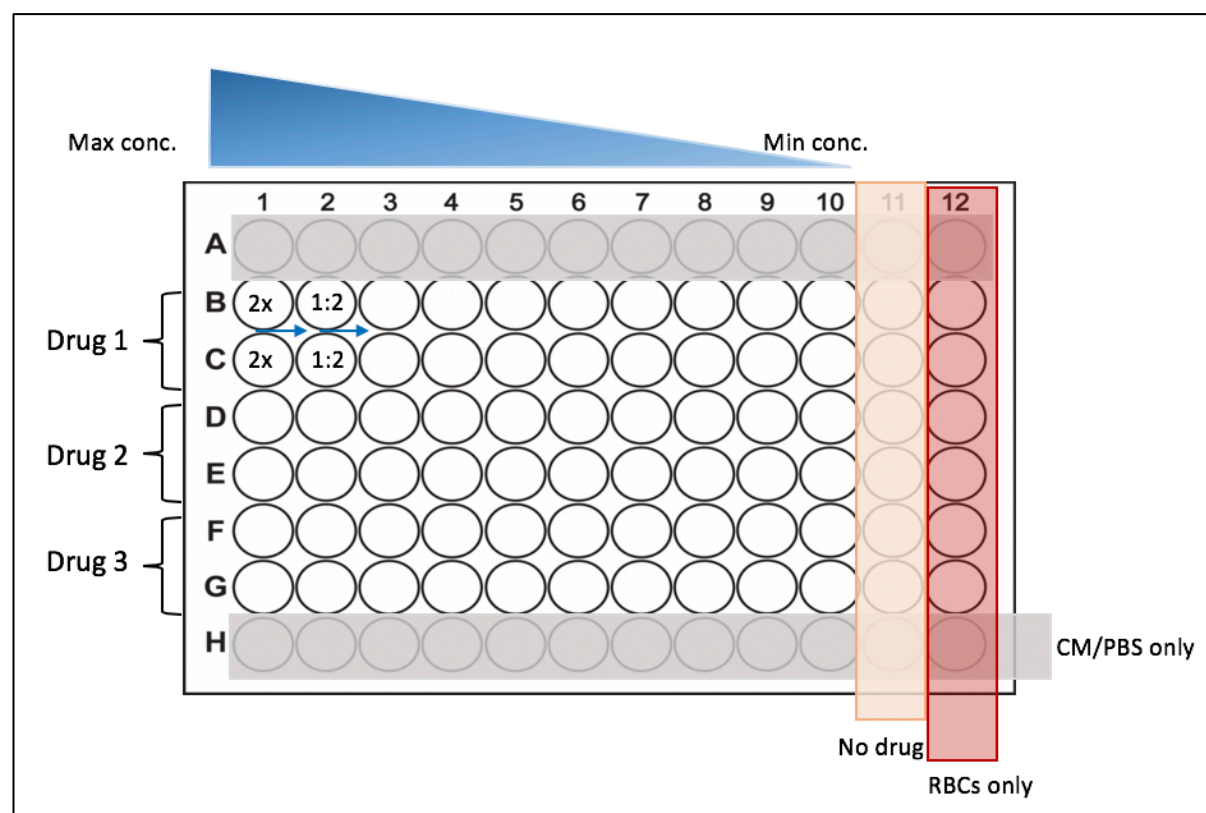
2.6 *In vitro* parasite survival assays

2.6.1 Antimalarial drug preparation

Antimalarial drugs were obtained from the Worldwide Antimalarial Resistance Network (WWARN) and Sigma. Stocks were prepared according to solubility as follows: piperaquine in 0.5% lactic acid in water; dihydroartemisinin (DHA) and lumefantrine (LUM) in dimethyl sulfoxide (DMSO); and chloroquine (CQ) and mefloquine (MQ) in water.

2.6.2 Drug sensitivity assays

The *in vitro* susceptibility of parasites to several commonly used antimalarial drugs was assessed using standard 72-hour dose-response SYBR Green I fluorescence-based assays (Bacon et al., 2007, Smilkstein et al., 2004). Drug stocks were prepared in CM at 2x the desired concentration and 1:2 serial dilutions were made by adding 50 μl per well in duplicate in a 96-well plate (**Figure 2.3**). The second to last wells in column 11 served as a no drug control (CM only) to measure maximum parasite growth and the last wells in column 12 contained RBCs only (1.5% hematocrit) to measure background fluorescence of the RBCs (**Figure 2.3**). Synchronous ring-stage parasites (0-10 hours) were added (50 μl per well at 3% hematocrit) to obtain a 1% parasitemia and 1.5% hematocrit in a final volume of 100 μl per well. The concentrations for each drug were: PPQ and MQ: 400 nM; CQ: 10 μM; DHA: 100-400 nM; and LUM: 100 nM. The drug assay plates were incubated in a chamber with mixed gas at 37°C for 72 hours. After the incubation period, parasites were lysed using a SYBR Green I solution. A 2x SYBR-lysis buffer solution was prepared by adding 2x SYBR (added fresh from a 10,000x stock) to a 2x solution of lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 0.016% w/v saponin, 0.08% v/v Triton X-100). After 72 hours, an equal volume of the SYBR-lysis buffer solution (100 μL) was added directly to the volume in the drug assay plates and mixed thoroughly. Plates were incubated in the dark at 37°C for 20 minutes then measured on a microplate reader (FLUOstar Omega) using a 485/535 nm excitation and emission filter. For all experiments in this study, the half maximal inhibitory concentration (IC_{50}) was taken as the drug concentration at which the SYBR green fluorescence was 50% of the value measured in the no drug (control) wells. Parasite survival (% growth) was determined by comparing fluorescence of the parasites exposed to drug (exposed) to fluorescence of parasites in the no drug control (non-exposed). All values were normalized

by subtracting the background fluorescence of RBCs-only. GraphPad Prism 8 Version 8.0.2 was used to generate dose-response curves using a non-linear regression model for log(inhibitor) vs. normalized response(variable slope) to calculate IC_{50} values with standard deviation (SD). All assays were performed in duplicate in three independent experiments. Statistical comparisons between parasite lines were performed using Mann-Whitney U tests.
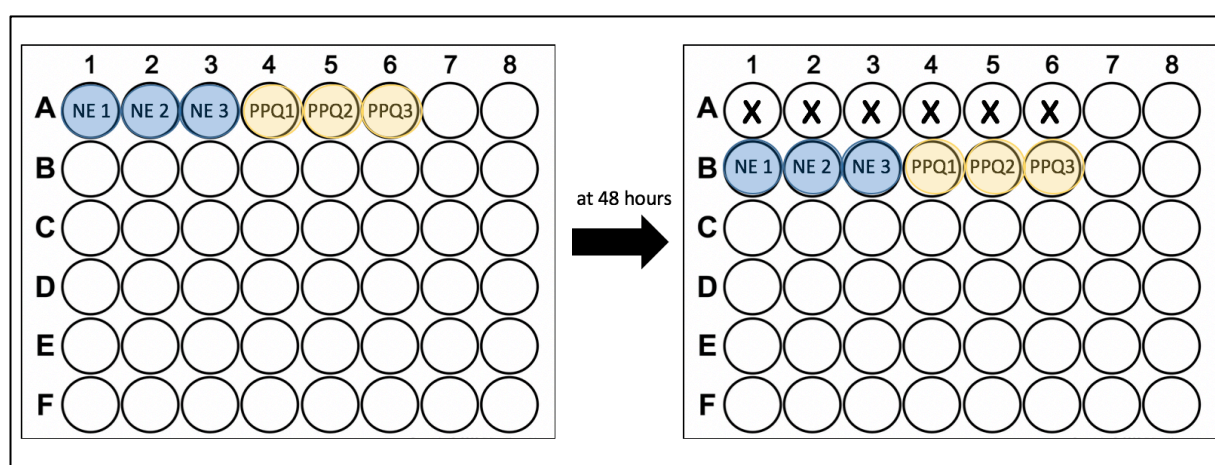

Figure 2. 3. **Schematic for IC_{50} drug assay 96-well plate layout.** Maximum drug concentration is plated in column 12 (2x the desired concentration) and serial 1:2 dilutions are plated in the following wells up to column 10. Column 11 (orange) contains no drug (parasites only) and column 12 contains RBCs only. Rows A and B (grey) contain CM or PBS only to account for any edge effects due to evaporation. Up to three drugs can be assayed on one plate.

2.6.3 Piperaquine survival assays (PSAs)

Piperaquine survival assays (PSAs) were performed following the procedure detailed by Duru *et al*. 2015 (Duru et al., 2015). Parasite lines were tightly synchronized 1-2 weeks prior to the assay by 5% sorbitol lysis and when parasitemia reached 3-5%, a Percoll gradient was performed on ~45 hour schizonts (segmented schizonts) and allowed to invade fresh RBCs for up to 3 hours. After incubation, the parasites were immediately sorbitoled and adjusted to 0.8–1.5% parasitemia with a 2% hematocrit. The cultures were then plated in a 48-well plate (in triplicate) and exposed to 200 nM piperaquine (from the Worldwide Antimalarial Resistance Network (WWARN) or Sigma) or the control vehicle (0.5% lactic acid in water) (**Figure 2.4**). The plates were incubated for 48 hours in a chamber with mixed gas containing

1% O_2, 3% CO_2, and 96% N_2 at 37°C. After 48 hours, cultures were washed once with ICM (10 mL) resuspended in complete medium and placed in a new well and cultured for an additional 24 hrs (**Figure 2.4**). After 72 hours, smears of all samples were prepared and flow cytometry was performed to measure viability. The proportion of viable parasites in exposed and non-exposed cultures was evaluated by counting 250,000 events per sample. Based on the accuracy of flow cytometry for measuring parasitemia (Amaratunga et al., 2014), the flow cytometry data was taken to represent viable parasites per well rather than counting 10,000 RBCs per treatment (Duru et al., 2015). However, the slides for microscopy were still counted after performing flow cytometry (at least 1000 cells per slide) to ensure the values obtained via flow cytometry were similar to the number of viable cells viewed and counted by microscopy. The parasite growth rate was defined as the non-exposed (NE) parasitemia at 72 hours/initial parasitemia (INI) at time zero (growth rate= NE/INI). The percent survival was defined as the number of viable parasites in PPQ-exposed (PPQ)/# of viable parasites in non-exposed (NE) x 100 (% survival=PPQ/NE*100). The % survival data was graphed and analysed using GraphPad Prism Version 8.0.2. Statistical comparisons between parasite lines were performed using Mann-Whitney U tests.

Figure 2. 4. **PSA 48-well plate setup.** Each treatment condition non-exposed (NE) and treated (PPQ) is performed in triplicate. After 48 hour incubation with PPQ, the drug is washed off and the cultures are plated in a new well in the 48-well plates.

2.7 Quantification of viable parasites using flow cytometry

Flow cytometry was utilized to measure parasitemia following the methods described by Amaratunga *et al*. (Amaratunga et al., 2014). This procedure utilizes two fluorescent dyes: SYBR green I, which binds DNA (as described above) and MitoTracker Deep Red (Thermo
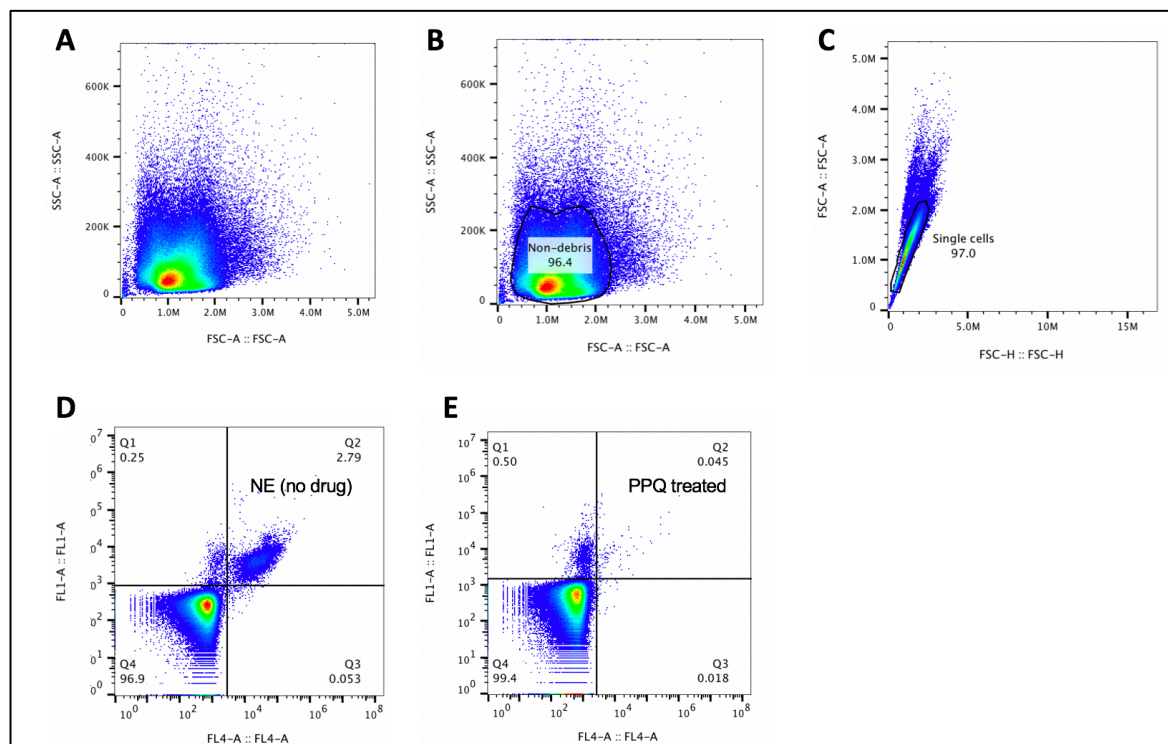
Fisher Scientific), which stains mitochondria and is a marker for actively metabolizing (live) cells.

For quantification of viable parasites in PSAs, 100 μL of culture from each treatment condition was collected in a 96-well plate and washed twice with 1X PBS. For measuring the parasitemia of other cell cultures, at least 20 μL of packed iRBCs were collected. Two controls were included for each flow cytometry assay: a stained-RBC only sample (2% hematocrit) and an unstained-RBC only sample to control for background fluorescence. After aspirating the final wash for all samples, the cells (except for the unstained-RBC only control) were resuspended in 100 μL of a SYBR Green I (0.4x)/MitoTracker Deep Red (0.6 μM) solution and incubated for 20 minutes at 37°C in the dark. After incubation, the cells were washed twice in 1X PBS and resuspended in 200 μL of 1X PBS. A 1:10 dilution of the stained samples was made in a final volume of 200 μL PBS and read on a flow cytometer (BD Accuri, NIH and CytoFLEX and Beckman Coulter CytoFLEX, Sanger).

The excitation and emission spectra for SYBR Green I is 497/520 nm and MitoTracker Deep red is 644/665 nm enabling the fluorescent dyes to be measured using the FITC (FL1) and APC (FL4) filters, respectively. A range of 200,000-250,000 events were recorded for each sample and subsequent data was analyzed and gated using FlowJo (Version 10.5.3).

Samples that stained positive with both SYBR and MitoTracker were recorded as live cells. Samples that stained with only SYBR green were counted as dead and only the percentage from the SYBR + MitoTracker gate was used to calculate the parasitemia for each culture (**Figure 2.5**). Samples were gated using the side scatter (SSC) vs. forward scatter (FSC) view (**Figure 2.5A**) of all events to obtain a population of "non-debris" by removing all debris outside of the gate (**Figure 2.5B**). Using the non-debris population, a single cell (singlet) population was obtained by gating the population in the forward scatter-area (FSC-A) by forward scatter-height (FSC-H) view (**Figure 2.5C**). Parasitemias were then quantified by gating the quadrants in FL1 (FITC/SYBR) vs. FL4 (APC/MitoTracker) (**Figure 2.5 D-E**). Quadrant 1 (Q1, upper left) shows cells that are SYBR positive only and counted as dead since SYBR fluorescence only indicates the presence of DNA. Quadrant 2 (Q2, upper right) shows SYBR + MitoTracker positive cells, which is taken to be the parasitemia or percentage of all live cells (**Figure 2.5 D-E**).

Figure 2. 5. Flow cytometry quantification of parasitemia using SYBR Green I and MitoTracker Deep Red. A.) Side-scatter (SSC) and forward scatter (FSC) view of all 250,000 events. **B.)** The population of cells was gated to remove debris. **C.)** The non-debris population was further gated to obtain single cells only (singlets). **D.)** Example of a non-exposed (NE) sample from a PSA. The parasitemia is represented in the top right corner of quadrant 2 (Q2). Q2 contains cells that stained positively for SYBR and MitoTracker. **E.)** Example of a PPQ-treated sample from a PSA with a PPQ-sensitive isolate. The population in Q1 (upper left) represents SYBR-positive parasites and the population in Q2 shows few live cells, as expected in a PPQ-sensitive isolate. The flow cytometry data was analyzed using FlowJo (Version 10.5.3).

2.8 Molecular cloning techniques

2.8.1 Transformation of competent bacteria cells

Bacterial transformations were carried out using competent *E. coli* cells (XL10-Gold Ultracompetent cells, Agilent Technologies or 10-beta Electrocompetent *E.* coli, NEB). Plasmid DNA (~1-50 ng of DNA in 2-10 μL) was added directly to competent cells on ice and incubated for 30 minutes. After incubation, the transformations were heat-shocked at 42°C for 30 seconds, placed back on ice for 2 minutes then recovered in 1 mL of SOC broth for 1-2 hours at 37°C. The transformations were plated on LB agar plates containing 100 μg/mL ampicillin (LB + ampicillin plates) and incubated at 37°C overnight. The following day, colonies were inoculated in LB broth supplemented with 100 μg/mL ampicillin and incubated overnight, at 37°C, shaking (~180 RPM). Cultures were harvested the next day at 4000 RPM for 20 minutes at 4°C and plasmids were isolated.

2.8.2 Plasmid preparation: mini, midi, and maxi preparations

Plasmids for all experiments were prepared using mini, midi, or maxi preparation (prep) kits (Qiagen, NIH; Macherey-Nagel, Sanger). For molecular cloning work, less plasmid DNA was needed, so mini preps were performed and DNA was eluted using water or the elution buffer supplied with the kit. For transfections, large amounts of plasmid DNA were required and were obtained via maxi preps. Plasmid DNA for use in transfections was resuspended in cytomix. Glycerol stocks of bacterial cultures were made by combining a 1:1 mixture of glycerol and the bacteria culture in a cryovial. Stocks were stored at -80°C.

2.8.3 Polymerase chain reaction (PCR) protocols

PCR reactions were performed using SapphireAmp Fast PCR Master Mix (Takara Bio Inc) or CloneAmp HiFi PCR Premix (Takara Bio Inc) and reaction conditions were set based on the manufacturer's recommendations. Each reaction contained 10 μM of the forward and reverse primers, 2x SapphireAmp Fast PCR Master Mix or CloneAmp HiFi PCR, the DNA template (1-2 μl for approximately ~20 ng DNA), and up to 10 or 20 μL of water. For the Sapphire mix, the thermocycler (GeneAmp 9700 PCR machine, Thermo Fisher) conditions were as follows: 92°C for 2 minutes, followed by 30 cycles of 92°C for 30 seconds, 55-59°C for 30 seconds (temperature was dependent on primers), 66°C for 1.5 minutes, followed by a 1 minute extension at 66°C. For CloneAmp: denaturation at 98°C for 2 minutes, followed by 30-35 cycles of 98°C for 10 seconds; 55°C for 30 seconds, and elongation at 68°C for one minute.

2.8.4 DNA sequencing

PCR products were purified using a PCR purification kit (Qiagen or Macherey-Nagel) and sent for sequencing by Genewiz (USA) when at the NIH or to Eurofins (Europe) when at the Sanger Institute.