Chapter 2: Methods

2.1 In vitro cultures of *P falciparum*

2.1.1 Thawing and Freezing of Parasites

Parasite stocks in glycerolyte kept in -80^{o}C were thawed at room temperature and transferred to a 50mL falcon tube. Approximately 100uL of 12% NaCl was slowly added to the thawed parasites dropwise whilst gently swirling the tube to mix. Then 5ml of 1.6% NaCl was slowly added to the parasites whilst swirling and left to sit for 5 minutes before centrifugation at 1500rpm for 3 minutes. The liquid was aspirated and 5ml of 0.9% NaCl, 0.2% Dextrose was then slowly added dropwise to the pellet whilst swirling to mix and centrifuged at 1500rpm for 3 minutes. The pellet was resuspended in 5mL of complete media and supplemented with addition of fresh RBCs.

Parasites stocks were generated by freezing at parasitaemia of ≥ 3% with parasites mostly at ring stage. Parasite cultures were centrifuged at 2000rpm for 5 minutes and the media aspirated. The pellet was resuspended in 1ml of glycerolyte freezing solution (made up of 456 ml Glycerol, 16 g L-Sodium lactate, 300mg KCl, 516.6mg Monobasic sodium phosphate(monohydrate), 1242mg Dibasic sodium phosphate (monohydrate), 544mL MilliQ water, at pH6.8 with Sodium phosphate solution). The glycerolyte was added to the pellet dropwise whilst gently agitating and transferred to a cryovial and stored in -80^{o}C immediately.

2.1.2 Parasite Culture Maintenance

Different *P. falciparum* parasite strains of various genetic background (Table 2.1) were maintained *in vitro* in routine culture adapted from Trager and Jensen (1976) for the experiments performed in this study. Parasites were routinely cultured with

complete growth medium at a final haematocrit of 3% in O+ red blood cells (RBCs) obtained from healthy donors with informed consent from the National Health Services Blood and Transfusion service (NHSBT). Human RBCs were used in accordance with approval from the NHS Cambridgeshire Research Ethics Committee and the Wellcome Sanger Institute Human Materials and Data Management Committee. Parasites were kept in incubators at 37^{o}C and routinely supplied with a gas mixture with concentrations of 1% oxygen, 3% carbon dioxide and 96% nitrogen. The parasitaemia of all parasites in routine culture was kept at under 5% to keep parasites fit and growing. Parasitaemia of cultures was measured by making smears from 2uL of culture on glass microscope slides which were fixed with 100% methanol and then stained with filtered 10% Giemsa solution for 10 minutes. The dried smear was observed by light microscopy at 100X magnification and parasitaemia was obtained in percentage by counting the number of parasitized RBCs (iRBC) against the total RBCs in 10 fields.

Table 2.1 *Plasmodium falciparum* strains used for experiments performed in the project. The origin and year that the different strains were isolated and adapted into culture (Lee and Fidock 2016; Walliker *et al,* 1987; Green *et al,* 1985; BEI Resources, 2020; Heinberg *et al,* 2013).

Strain	Origin	Year Isolated
3D7	West Africa, Isolated in Netherlands (NF54 clone)	1970s
Dd2	Laos, Southeast Asia (W2 clone)	1982
FCR3	Gambia, West Africa	1976
Tanzania (200708)	Tanzania, Africa	2006
V1/S	Vietnam, Southeast Asia	1980
PH0212-c (CAM)	Pursat, Western Cambodia	2010

2.1.3 Red Blood Cells and Culture Medium

Complete growth medium used in the routine culture of parasites was prepared using Roswell Park Memorial Institute (RMPI)-1640 as supplement. RPMI-1640 containing Albumax was made to completion by supplementing 0.5M HEPES, 1X Glutamax and 25ug/mL gentamicin. Alternative supplements were made in the media preparation for drug assays and selecting transfections where the relevant drug was added to the growth medium.

Red blood cells of blood group O and rhesus positive (O+) were washed twice with growth medium by centrifugation at 3000rpm for 5 mins during each wash to pellet RBCs. RBCs were then re-suspended in an equal volume with growth medium at 50% haematocrit supplemented with 10% (v/v) citrate-phosphate-dextrose plus adenine (CPDA) to maintain fresh RBCs kept at 4^{o}C. RBC stocks for culture maintenance were replaced with a new batch of O+ blood on a weekly basis with every batch undergoing the same routine wash steps previously mentioned.

2.1.4 *Plasmodium* Stage Synchronisation

Loss of synchrony in *P. falciparum* erythrocytic stages occurs after a few life cycles *in vitro*, leading to asynchronous growth with the different asexual stages present. The elimination of distinct parasite stages by various parasite synchronisation methods are described to address these challenges and allow synchronisation of parasites (Ranford-Catwright *et al,* 2010).

Synchronisation aimed at the isolation of mature segmenting schizonts using a Percoll gradient was performed to enrich late trophozoites or schizonts (Ressurreição *et al.* 2020). A 63% (v/v) Percoll solution was made from neat Percoll in filter-sterilised 10X PBS and serum-free RPMI-1640 and pre-warmed. Parasite pellet from 3% haematocrit culture was collected by centrifugation at 900g for 5 minutes and resuspended in complete media to haematocrit of 30-40% and maximum volume of 5mL. The resuspended pellet was then gently layered onto 10mL of the Percoll cushion in a 15mL falcon tube avoiding mixture of the two phases. The Percoll gradient was then centrifuged at 1300g for 11 minutes with zero brake to obtain efficient gradient and separation of the stages. The thin brown band between the Percoll and media, containing the late stages, was carefully collected. The collected late stages were washed and resuspended in pre-warmed complete media and returned to culture. Cultures were assessed microscopically over the next few hours, and reapplied to a fresh Percoll cushion once substantial invasion of RBCs was seen. After centrifugation, the late stages and media were discarded, and the pellet consisting of uninfected RBCs and early rings was collected and sorbitol treated to further tighten synchronisation.

Synchronisation by enrichment of ring stages was done using the sorbitol lysis method originally described by Lambros and Vanderberg (1979) in which culture was

treated with filter-sterilised 5% (w/v) sorbitol in water, killing the mature forms of the asexual stages. The infected RBC pellet was obtained by centrifugation at 2000rpm for 5 minutes in Falcon tubes. Parasite pellets were then resuspended in 10 volumes of pre-warmed 5% sorbitol and incubated for 10 minutes at 37ºC. Sorbitol was removed by decantation upon centrifugation at 1500g for 3 minutes followed by 2 wash steps with pre-warmed complete media centrifuged at 1500rpm for 3 minutes. The washed pellet was resuspended in pre-warmed complete media and adjusted to the appropriate haematocrit by addition of fresh RBCs to make up for RBCs ruptured with matured parasites.

2.2 Making a Pool of Barcoded Parasites

Six *P. falciparum* lines (Table 3.1), previously barcoded at the *Pfrh3* locus by Manuela Carrasquilla in her thesis work (Carrasquilla, 2019) and described in section 3.3.1, were maintained in culture and mixed to generate a pool with equal representations for barcode sequencing. Three independent mixes (pools) were made to conduct experiments in triplicate. The parasitaemia of each line was measured by flow cytometry using the CytoFLEX Flow Cytometer (Beckman Coulter). Staining of cells was done with both SYBR green and mitotracker deep red (ThermoFisher) so that live parasites can be distinguished from dead parasites for accurate counting. The haematocrit of each culture was measured using the cell counter (Cellometer auto 1000; Nexcelom Bioscience). To ensure that equivalent proportions for the different lines were added, the number of parasites per mL of each culture was calculated by multiplying the parasitaemia obtained on the flow cytometer by the number of cells per mL obtained with the cell counter. Equal absolute number of parasites for each strain was mixed and the parasitaemia of the pool was adjusted to 1% parasitaemia

Synchronisation of these pools were performed and continuous culture in the absence and presence of drugs at different concentrations, listed in Table 2.2, was carried out. Drug pressure was applied when parasitaemia was at least 1% over a duration of time. Parasitaemia was monitored and cells were harvested at intervals.

Table 2.2 List of compounds used for Barcode sequencing phenotypic assays and the range of concentrations. The pools of the different strains were grown in different concentrations of each these drugs in the range listed, in triplicates.

Drug	Concentration range (nM)
Chloroquine	20-500
Piperaquine	10-500
Mefloquine	10-500

2.3 Parasite Phenotypic Assays

2.3.1 Antimalarial Drug Sensitivity Assay

To assess parasite growth inhibition in the presence of drugs, standard malaria drug sensitivity assays were performed on parasites using the malaria SYBR Green I-based fluorescence (MSF) assay developed by Smilkstein *et al* (2004). Different concentrations of the relevant drugs (Table 2.3) were prepared in a two-fold serial dilution or 3-fold serial dilution in a 96 well tissue culture plate format accommodating 10 dilution points of each drug as shown in figure 2.1. Parasites in routine culture were prepared by diluting with uninfected RBCs to a haematocrit of 2% and parasitaemia of 1% and assessed in duplicates for each dilution point. A column of no drug and uninfected RBCs of 2% haematocrit were included in the plate as control wells which were used for the calculation of parasite growth inhibition as well as growth medium only wells as control for background fluorescence. 50uL of each of the drug

concentration dilutions done with growth medium was added to each well except the uninfected RBCs wells and the no drug wells. 50uL of prepared parasite culture was added to all the wells except the media only wells containing 100uL of media. The assay plates containing the drug suspension and parasites with a total volume of 100uL in each well were then put into routine culture gas and incubation conditions previously described for 72 hours.

Table 2.3 List of compounds used for 72-hour drug assays to obtain inhibitory concentrations and the range of concentrations used in the serial dilution for drug plate set up. (A) Drug concentration used for Barcode Sequencing experiments (B) Compounds and concentration used in testing *P. falciparum* parasites with Keap1-Kelch inhibitors

A

Drug	Concentration range (nM)
Chloroquine	0-5000
Piperaquine	0-3000
Mefloquine	0-600
Dihydroartemisinin	0-100

B

Drug	Concentration range (µM)
KI696	0-250
RA839	0-500
ML334	0-500

2.3.2 SYBR Green Assay

The drug assay plates were removed from routine culture conditions after 72 hours. A 2X SYBR lysis buffer with SYBR green stain was prepared for the assessment of parasite growth. The lysis buffer containing 10mM Tris pH 7.5, 3mM EDTA, 0.1% saponin solution and 2% Triton X-100 solution was used to make a 1:5,000 dilution of the 10,000X stock SYBR Green to 2X. 100uL of this solution was added to the 96 well drug-parasite plate, and then incubated for 30 minutes at 37^{o}C in the absence of light. Fluorescence from stained DNA in parasitised RBCs correlating to growth was measured on the FLUOstar Omega microplate reader (BMG Labtech) using a 485-nm excitation filter and a 535-nm emission filter. Parasite growth and inhibitory concentration (IC50) values were determined by results derived from the analysis of drug assays reads as well as statistical tests done on GraphPad Prism.

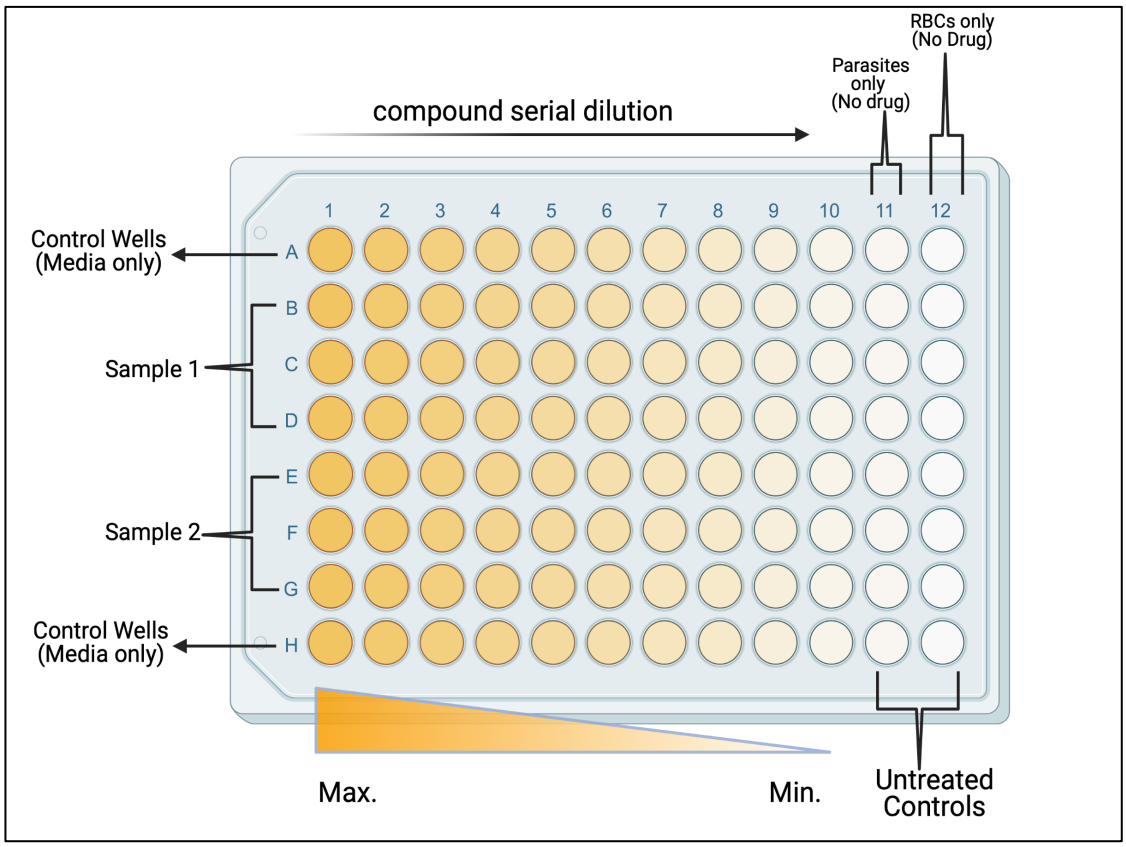

Figure 2.1 Plate layout up for drug sensitivity assays. Sample are loaded in triplicate. Maximum concentration of the selected drug is loaded on column 1 and a 2-fold serial dilution per column is done up to column 10. Column 11 loaded with untreated parasites and column 12 loaded with RBCs only are the negative controls used for the analysis of the dose response curve.

2. 4 Transfection of *P. falciparum* by Electroporation of Infected Red Blood Cells

Standard episomal transfections were carried out for CRISPR/Cas9 genetic editing of parasites listed in table 2.1 using methods adapted from (Fidock and Wellems, 1997). Parasite cultures at >5% parasitaemia were harvested and pelleted by centrifugation of 1500pm for 3 minutes to discard growth medium. <150uL pellet was resuspended in a volume of cytomix that was equal to volume of harvested culture. The suspension was centrifuged and the supernatant discarded. The parasite pellet was resuspended in cytomix with 50ug of pooled plasmid DNA, described in 2.5.3, 2.5.4 and table 4.1, in a total volume of <450uL. The suspension was loaded into

0.2cm Biorad electroporation cuvettes and cells were electroporated at 0.31 kilovolts (Kv), 950 microfarad (μF) and maximum capacitance using the BioRad Gene Pulser Xcell electroporator and immediately resuspended in complete growth media followed by a 1-hour incubation to allow cells to recover. The suspension was then washed and resuspended in complete media with fresh RBCs added and put in routine culture conditions previously described. Transfected cells were continuously cultured in complete media with WR99210 drug at 5nM for Dd2 and 2.5nM for FCR3 and Tanzanian strains, for selection at day 1 of post-transfection. Transfected parasites were continuously cultured in the presence of the WR99210 until recovery of parasites occurred and routinely monitored by microscopy at least twice a week.

2.5 Molecular Assays

2.5.1 Genomic DNA Extraction from Parasite Cultures

Parasites cultures were harvested for extraction of genomic DNA (gDNA) using 5 to 10mL of culture volumes, at 3 to 5% parasitaemia and 3% haematocrit. Parasite cultures were centrifuged and the pellet was lysed by resuspension in 0.15% saponin in PBS and incubated for 5 minutes at room temperature. Washing of the lysed cells was done twice with 10mL of sterile PBS and gDNA extraction was carried out directly from the pellets using Qiagen's DNeasy Blood and Tissue Kit. The pellet was first resuspended in 200μL PBS and incubated at 56^{o}C for 10 minutes following addition of 20μL of proteinase K and 200μL of lysis buffer AL. The sample was applied to the column, which was washed twice with 500μL buffers AW1 and AW2 respectively provided in the kit and eluted in 50μL of Elution Buffer. The gDNA was stored in -20˚C for subsequent library preparation for Barcode Sequencing

2.5.2 Barcode Sequencing

To amplify barcodes for library generation for Illumina sequencing (MiSeq), a three-step PCR was performed on gDNA isolated from the cultures using CloneAmp 2X Mix (CloneAmp™ HiFi PCR Premix, 639298), nuclease-free water and relevant primers at 10μM, as described below. The PCR was done in a total reaction volume of 25μL for 30 cycles of 98°C for10 s, 55°C for 15 s, 72°C for 45 s, finishing at 4°C. A water-only control was included in all PCR runs to check for potential contamination of reagents. An aliquot of all PCR products was separated on a 2% agarose gel to confirm amplification of the barcode amplicon, and the remainder purified using AMPure SPRI paramagnetic bead-based chemistry (Beckman Coulter). An initial PCR was done using primers p191/p194 for amplification of outside the homology arms of pfrh3 to ensure amplification of only integrated barcodes. A second PCR was performed on the purified products using adapter primers p1356/p1357 (150bp) for BarSeq at 30 cycles of 98°C for10 s, 55°C for 15 s, 72°C for 45 s, finishing at 4°C in 25uL reaction volume. A third PCR was performed to introduce Illumina indexes using forward primers i501 or i502 (Paired-end 1.0 from Illumina, p2085/p2086) and Illumina index primers (1-96, p1359-p1454) listed in chapter 3. The reaction was performed for 10 cycles of 98 °C/10 s, 55 °C/15 s, 72°C/15 s, finishing at 25°C in a 10uL reaction volume. These products from this PCR were quantified using the PicoGreen dsDNA quantitation fluorescence assay kit by Invitrogen in a 96 well plate format. Fluorescence was measured using the FLUOstar Omega microplate reader and diluted to 4nM for Illumina sequencing.

2.5.3 Deconvolution of Plasmid Pool for Plasmid Selection

Plasmids used in this project were selected from a complex pool of pDC2-CAM-Cas9-U6-hDHFR plasmids containing a codon-optimised Cas9 under the control of the *P. falciparum* calmodulin promoter and a single guide RNA (gRNA), expressed from the U6 promoter, designed to target *Pfkelch13* at position 580 as shown in figure (2.3.1). The plasmids were generated by a former PhD student, Manuela Carrasquilla (PhD, 2019) as a complex pool with up to 64 different Pfkelch13 donors, with each plasmid differing by a single codon at position C580 of the locus. As part of my project, I intended to generate defined sub-pools of these plasmids, and thus needed to isolate individual plasmids. Deconvolution of this complex pool of plasmids with a common pDC2 backbone encoded with 64 possible codons, was carried out in order to isolate each possible individual donor with the aim of constructing sub-pools of donors with only viable amino acids that has been previously seen to be more efficient in replacing cysteine at position 580. The bacterial glycerol stock of the complex plasmid pool was grown overnight and then several dilutions were plated to isolate single colonies. Approximately 96 single colonies were picked and cultured overnight in 37^{o}C in 96 deep well plate in NZY + Ampicillin at 240rpm illustrated in figure 2.5 Minipreps of these individual colonies were carried out using the standard protocol as per the QIAprep 96 Plus Miniprep Kit. Sanger sequencing with primer p282 was performed on the isolated plasmid DNA.

2.5.4 DNA Isolation of Plasmid with single codons

Chromatograms from the Sanger sequencing results was used to identify plasmids with single codons at position 580 encoding for the amino acids of interest described in chapter 4. Plasmids harbouring these codons were further expanded into bigger

bacterial cultures in 200mL of Luria-Bertani (LB) broth + Ampicillin (100µg/ml) and cultured overnight. Midipreps were performed using the manufacturer's protocol (NucleoBond Xtra Midi/Maxi) kit by MACHEREY-NAGEL. The final plasmid pellet was eluted in sterile cytomix and quantified using the nanodrop and then stored in -20˚C for use in transfections of parasite cultures.

2.6 Data Analysis

The data generated from the standard drug assays was analysed on GraphPad Prism and curves were fit using the non-linear regression model (variable slope).

For barcode sequencing of parasite lines, the barcodes, day, assay condition and the barcode read counts were included in the sequencing output file. The barcode proportion was used to measure the abundance of each line in a pool, and the change in barcode proportion over time used to measure the growth rate of each parasite over time, either in the presence or absence of drug. The BarSeq data presented in section 3.3.4 was analysed on GraphPad Prism to plot the relative growth curves of the parasites and analysis of variance (ANOVA) with Tukey test was performed to compare the mean difference of the change in barcode proportion over time across the different parasites.