Chapter 3: Assessing *Plasmodium falciparum* fitness and antimalarial resistance using Barcode sequencing

3.1 Summary

The aim of this chapter is to assess how barcoded parasite lines could be used to measure the fitness and antimalarial resistance of *P. falciparum*. To investigate how the genetic background of parasites affect these phenotypes, I used barcoded *P. falciparum* strains from different geographic locations, described below, in pooled growth competition assays. Parasite growth in the presence and absence of drugs was measured over time and each barcode proportion in the pools were measured. These parameters were measured at different time points using Barcode sequencing (BarSeq).

3.2 Background

3.2.1 *Plasmodium falciparum* genetic background, fitness and resistance

Increasing artemisinin resistance remains a major challenge in malaria treatment but despite this being a widely studied area there is not yet an established understanding of the mechanism. However, point mutations in *Pfkelch13* are associated with resistance, with one of the major *PfKelch13* mutations, C580Y, linked with decreased haemoglobin uptake by the endocytic machinery (Birnbaum *et al,* 2020; Sharma *et al,* 2020). Mutations in *Pfcrt,* the determinant of chloroquine resistance, are now also associated with resistance to other antimalarials currently in use and in development such as piperaquine (Martin, 2020). The association of parasite fitness, that is the parasite's ability to survive and replicate to the next generation, with drug resistance has been reported in various studies. The presence of acquired mutations leading to decreased susceptibility of *P. falciparum* to

antimalarials has been observed to negatively affect parasite fitness in the absence of drug pressure (Froberg *et al,* 2013).

The fitness cost of *P. falciparum* incurred by resistance-associated mutations has been observed with polymorphisms of different genes including *pfmdr1, pfcrt and* other genes, in various endemic locations. This has led to a drastic decrease in prevalence of resistant parasites over time in the case of chloroquine where the drug had been withdrawn from malaria treatment for a long period (Hayward *et al,* 2005; Nursing *et al,* 2009; Fohl and Roos, 2005; Kublin *et al,* 2003). However, observations of enhanced fitness in the presence of acquired drug resistance associated mutations as a result of strong selective pressure have been reported in other studies (Gabryszewski *et al,* 2016). The co-existence and interaction of these multiple genes in epistasis can confer varying phenotypic outcomes. This can also be distinctly impacted by parasite genetic backgrounds that can demonstrate varying tolerance to resistance-associated polymorphisms. Thus, understanding the aspects of parasite genetic backgrounds and co-segregating compensatory mutations is vital. This can have varying impacts on the observed fitness costs of artemisinin resistant mutations which is important in understanding mechanisms of resistance to artemisinin and partner drugs in ACTs (Tirrell *et al,* 2019; Amato *et al,* 2018).

3.2.2 Measuring *Plasmodium falciparum* fitness

Various approaches that are based on parallel comparison of different strains are applied in assays to evaluate resistance and fitness of *P. falciparum*. The quantification of different protein markers, metabolic products or DNA that are overall indicators of parasite biomass are mostly relied on in current in vitro phenotypic assays. This is aided with the use of intercalating fluorescence dyes or radioactive

markers, microscopic assessment of parasite development and detection of parasite specific proteins (Molnar *et al,* 2020). In most studies a maximum of two lines are mixed in head-to-head competition assays in multiple experiments performed in parallel with two competing parasite lines compared in each experiment.

Several studies have assessed parasite growth and resistance by fluorescence microscopy and quantitative analysis using genetically edited *P. falciparum* lines labelled with fluorescent and bioluminescent proteins such as Green Fluorescent Protein (GFP) and mCherry (Marin-Mogollon *et al,* 2019; Mbengue *et al,* 2015; Istvan *et al,* 2019). A study by Gabryszewski *et al* (2016) utilised wild type and transgenic Dd2 and Cam734 lines edited with *pfcrt*-specific zinc-finger nuclease in co-culture assays with a GFP-tagged wild type competitor line. This study revealed the impact of *pfcrt* mutations on parasite physiology and haemoglobin catabolism, and the capacity of compensatory mutations in neutralizing parasite fitness costs and associated drug resistance impact (Gabryszewski *et al,* 2016).

Recent studies have made use of growth competition assays with sequencing to assess parasite fitness and resistance *in vitro*. In a study by Straimer *et al* (2017) assessing the impact of *Pfkelch13* mutations on ozonide susceptibility and parasite fitness, pyrosequencing was used to determine growth rates of *Pfkelch13* mutant lines i*n vitro*. The fitness costs conferred by the resistance-associated polymorphisms in genetically edited resistant mutants was validated in this study (Straimer *et al,* 2017). A similar approach was performed in another study by Nair *et al,* (2018) investigating fitness outcomes in *Pfkelch13* mutations, that utilised genome editing strains in competition assays and deep sequencing. This study validated the higher fitness cost of C580Y mutation compared to the R561H *Pfkelch13* allele (Nair *et al,*

2018). However, one limitation of these co-culture methods is that only two lines, the test line and control competitor, can be examined at once.

Drug sensitivity assays remain a crucial aspect in surveillance and monitoring of drug efficacy and resistance, and for the identification of important resistance-associated molecular markers (Dhingra *et al,* 2019). These antimalarial drug susceptibility techniques are mainly based on development and maturation of parasites in the presence of a titration of drug concentrations for which several methods have been developed and measured *in vitro* or *ex vivo* (Maji, 2018). An essential basis for drug discovery by mimicking the *in vivo* situation is facilitated by application of *in vitro* drug sensitivity assays of parasites that allows the effective penetration of compounds inside cellular membranes of parasites to a measurable level (Sinsha *et al,* 2017). Assays that have been developed to specifically target the ring stage of the parasites include the ring-stage survival assay ($RSA^{0–3\,h}$) that measures parasite survival rates after a short-term exposure to artemisinin. This method, which aims to simulate the short half-life of artemisinin in vivo, has been vital in determining delayed parasite clearance linked with *Pfkelch13* and the current goal standard for identifying artemisinin resistance (Niaré, *et al,* 2018; Witkowski *et al* 2013). For the majority of antimalarials, however, standard 48-hour or 72-hour drug sensitivity assays are widely used to monitor overall drug susceptibility phenotype of *P. falciparum* parasites for current antimalarial drugs (Chaorattanakawee *et al,* 2015). Understanding drug resistance dynamics and evolution patterns in a competitive environment that is crucial for drug discovery and surveillance, is determined by measuring parasite fitness and drug resistance experiments in which *in vitro* assays are mostly performed (Rosenthal *et al,* 2013).

In this chapter, I assessed a method to capture both the fitness and drug sensitivity profiles in genetically edited strains of different genetic backgrounds. These lines had been CRISPR-edited with unique molecular barcodes, allowing their co-culture and quantification using barcode sequencing (BarSeq) by next generation sequencing.

3.3 Methods and Results

3.3.1 Making a Pool of Barcoded Parasites

Six *P. falciparum* lines with unique barcodes were previously generated by Manuela Carrasquilla (PhD thesis, 2019), as described in section 2.2. The thawed lines were maintained in culture until growing well and then mixed to generate a pool with equal representations for barcode sequencing. The lines were each edited with a unique barcode inserted at the *Pfrh3* locus, which is a non-essential gene that does not have any impact on parasite growth (Duraisingh *et al,* 2002). Three independent mixes (pools) were made to perform experiments using the barcoded *P. falciparum* strains (Table 3.1) of different genetic backgrounds with varying drug resistance profiles. The parasitaemia of each line was measured as described in section 2.2. The individual barcoded lines were synchronised as described in 2.1.4 and equal proportions of each were added in each pool following the descriptions in section 2.2. The parasitaemia of each pool was adjusted to 1% parasitaemia and synchronisation of the pools done in 50mL culture flasks. The co-cultures of the independent pools were maintained in the absence and presence of chloroquine, mefloquine and piperaquine at different concentrations as described below and samples were harvested on different days over an 18-day period. Drug pressure was applied when parasitaemia was at least 1% and maintained for the duration of the experiment.

Cultures maintained in the absence and presence of drugs were adjusted to a 1% parasitaemia after collection of 10mL that was then lysed for DNA isolation and library preparation. Parasitaemia was monitored and cells were harvested at intervals on different days. Genomic DNA was extracted from harvested parasites as described in section 2.5.1.

3.3.2 Generating libraries for Illumina Sequencing

To amplify barcodes for library generation for Illumina sequencing (MiSeq), a nested PCR reaction (Figure 3.1) was performed on gDNA isolated from the harvested cells using reagents and cycling conditions described in section 2.5.2. 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 performed using primers p191/p194, producing a 3kb product (figure 3.2 A) that extends outside the homology arms of the original *Pfrh3* editing donor, to ensure amplification of only integrated barcodes and not episomal donors. A second PCR described in section 2.5.2 was performed on the purified 3kb products to generate a short 150bp amplicon, using the primers p1356/p1357 (figure 3.2 B). Illumina indexes were introduced in a third PCR using forward primers i501 or i502 (p2085/p2086) and Illumina index primers (1-96, p1359-p1454) with conditions previously described in section 2.5.2. The libraries generated from this PCR were purified and diluted to 4nM for Illumina sequencing. BarSeq samples were multiplexed in one MiSeq run.

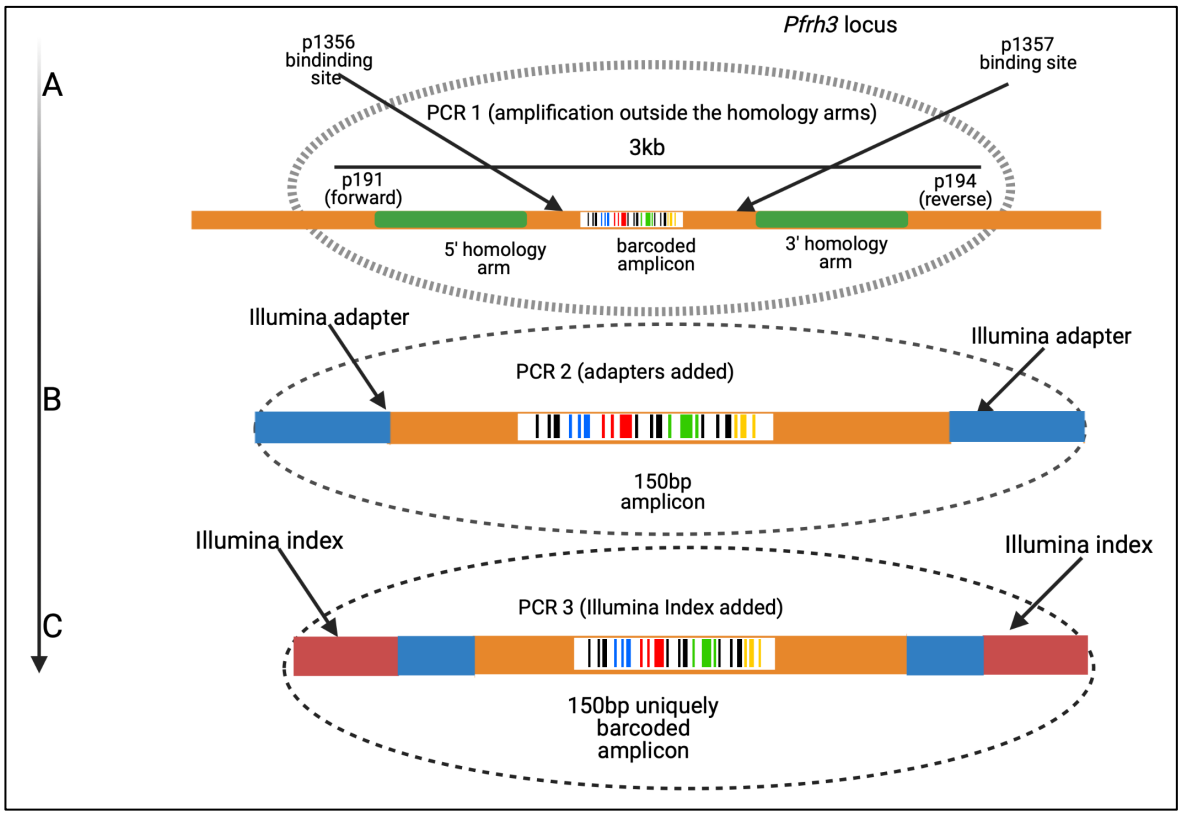


Figure 3.1 Library generation workflow for Barseq A) The 3kb product from the first PCR using primers 191/194 that extends outside the homology region to avoid episomal amplification. **B)** A short 150bp amplicon is produced from the second PCR using primers p1356/p1357 that flank the barcode. **C**) Illumina adapters added in the third PCR using i501 or i502 (p2085/p2086) and Illumina index primers (1-96, p1359-p1454).

Table 3.1 *P. falciparum* strains used in BarSeq assay. Each clone is barcoded with a unique sequence (barcode ID) from chloroquine-sensitive and multidrug-resistant strains of different backgrounds (Pinheiro *et al,* 2018).

Strain	Clone	Barcode Sequence	Resistance Profile
3D7	A	TAGCAGAAGTT	Sensitive
3D7	B	TAATAGGGAGC	Sensitive
3D7	C	GTCTCCGTATT	Sensitive
V1/S	A	TCTCTGAATCA	Chloroquine, pyrimethamine
V1/S	B	GACATGGGATT	Chloroquine, pyrimethamine
PH0212-c(CAM)	A	GTGAGAGTATC	Artemisinin, pyrimethamine, chloroquine

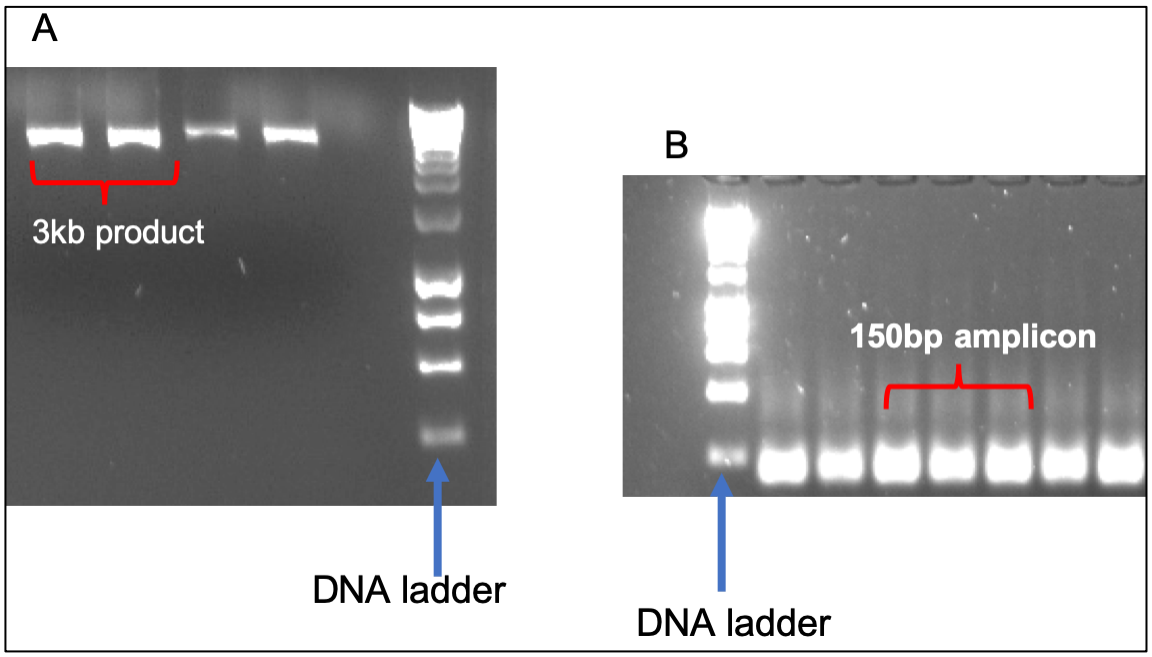


Figure 3.2 PCR products from the nested PCR in Illumina library generation. A) The 3kb product from the first PCR using primers 191/194. B) The 150bp amplicon produced from the second and third PCR described above. 1kb DNA hyperLadder (Bioline) was loaded alongside products.

3.3.3 Drug sensitivity assays to determine drug concentrations for BarSeq

One of each strain (3D7, V1/S and CAM), were used to perform standard 72-hour drug sensitivity assays to determine IC_{50} values of the different drugs listed in Table 2.2. The drug sensitivity assays were performed in biological replicates following methods described in section 2.3.1 and 2.3.2 to determine the concentration range of the drugs used in *in vitro* assays for the BarSeq assays. The standard inhibitory curves are shown below in figure 3.3. As expected, there was a >10-fold shift in IC_{50} for chloroquine between the sensitive 3D7 strain and the resistant V/1S and CAM strains. In contrast, there were smaller differences in IC_{50} fold-change across strains for mefloquine and piperaquine.

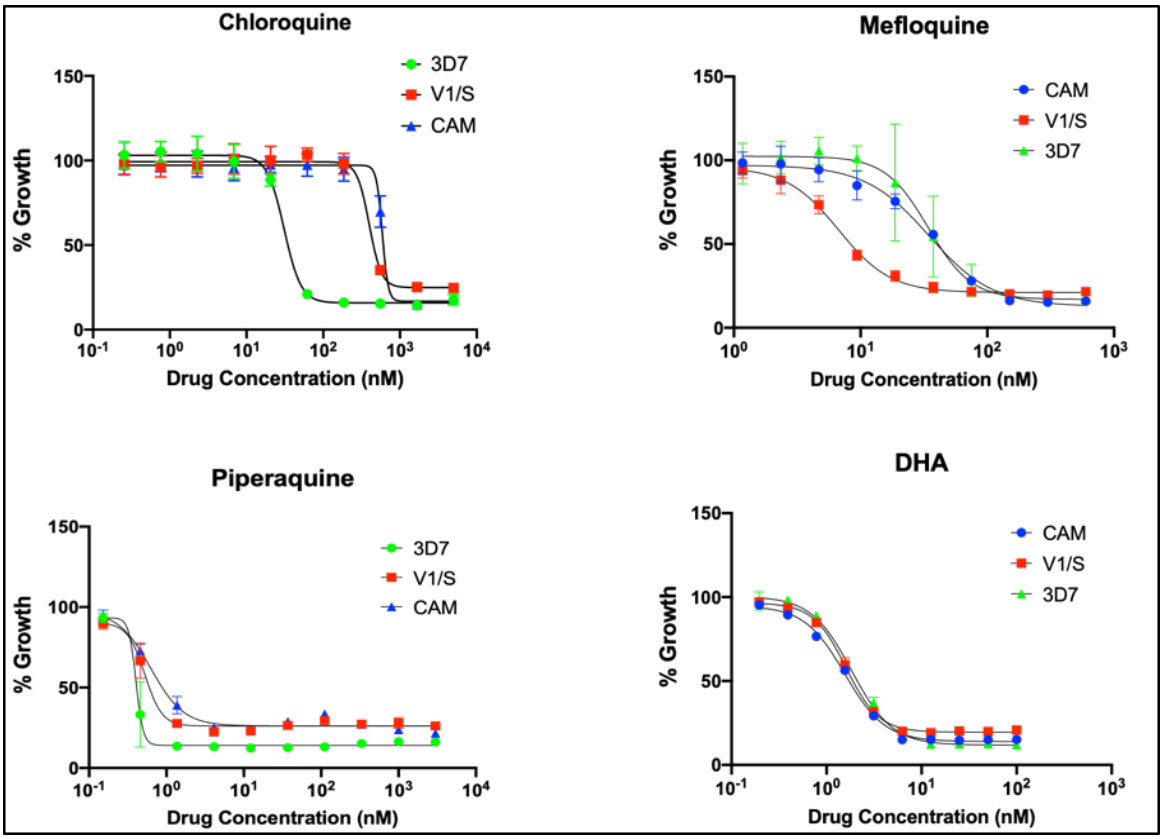


Figure 3.3 Standard dose response curve for chloroquine, mefloquine, piperaquine and DHA. Dose response curve for strains used in competition assays. IC50 curves obtained from assays performed in triplicate. The IC_{50} values are shown in table 3.2 below

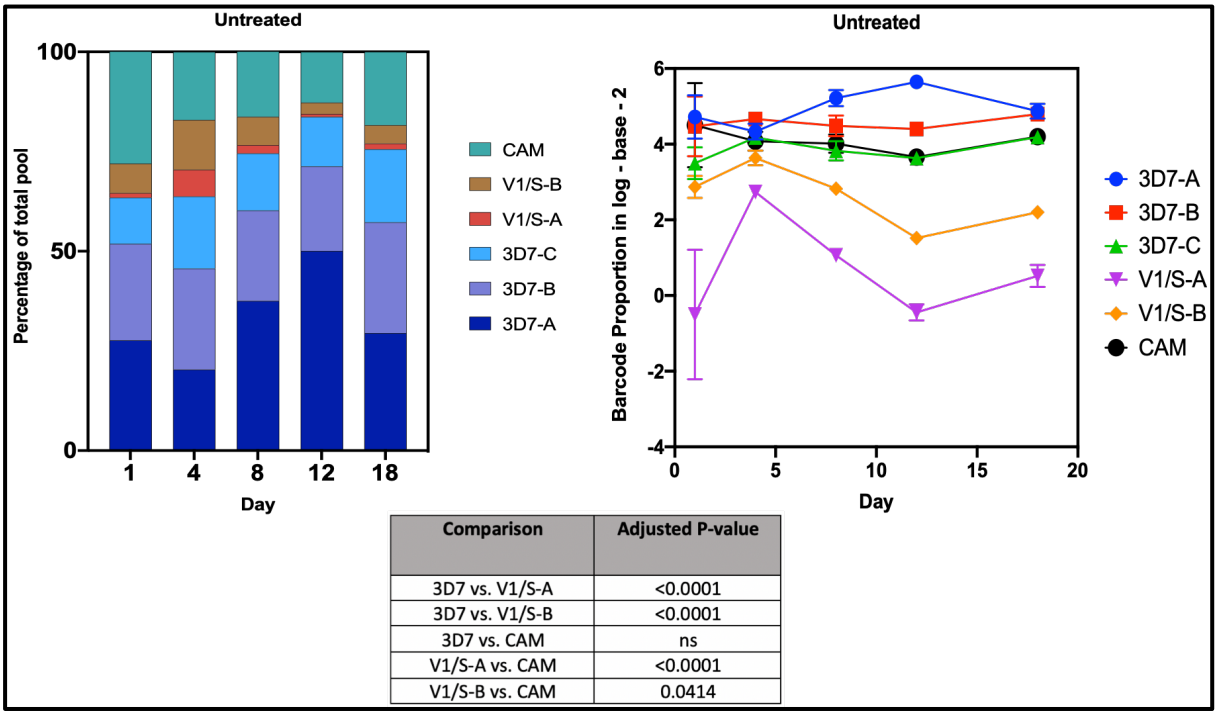
Table 3.2 The IC_{50} values obtained for chloroquine, mefloquine, piperaquine, DHA. Standard 72-hour drug assays were performed and the mean IC_{50} values and standard deviation from three biological replicates of each drug.

Drug	3D7 IC50 (nM)	V1/S IC50 (nM)	CAM IC50 (nM)
Chloroquine	23.2 ± 6.8	332.4 ± 57.2	576.4 ± 64.3
Mefloquine	32.7 ± 4.8	7.2 ± 3.8	29.2 ± 5.2
Piperaquine	0.4 ± 0.03	0.9 ± 0.7	2.7 ± 3.3
DHA	1.9 ± 0.2	1.6 ± 0.6	2.1 ± 1.7

3.3.4 Drug resistance phenotypes determined by BarSeq

To determine if the BarSeq co-culture method could detect differences in fitness and drug resistance phenotypes across strains *in vitro*, competition assays were carried out in triplicates as described in section 3.3.1.

In the absence of drugs, in normal culture conditions, the 3D7 and CAM strains moderately outcompeted the V1/S strains in an 18-day co-culture, although both V1/S lines were present at a lower initial proportion in the pool (figure 3.4). The growth rates of the two independently barcoded V1/S clones (V1/S-A and V1/S-B) was similar across the 18 days co-culture. Similar growth rates were also observed across the independent internal replicates of 3D7 clones (3D7A-C). One-way analysis of variance (ANOVA) with Tukey test was performed using the barcode proportion change over time to determine the mean difference between strains. There was a significant difference between V1/S and the other strains. No significant difference was observed between 3D7 and CAM when the barcode proportion change was compared across all three strains over time.



Comparison	Adjusted P-value
3D7 vs. V1/S-A	<0.0001
3D7 vs. V1/S-B	<0.0001
3D7 vs. CAM	ns
V1/S-A vs. CAM	<0.0001
V1/S-B vs. CAM	0.0414

Figure 3.4 Growth rates of all strains over 18 days in absence of drug. Competition assay of three strains with two internal replicates for V1/S and three internal replicates of 3D7 each uniquely barcoded. The percentage of each barcode in the total pool at different days shown in the left bar graph and log barcode proportion over time shown in the right plot. The statistical difference of barcode proportion changes over time across the different strains using one-way analysis of variance (ANOVA) with Tukey test is shown in the bottom panel.

To examine how the presence of different drug selections impacted growth, the pool was exposed to different concentrations of chloroquine, mefloquine and piperaquine. Drug resistance phenotypes were determined in triplicate cultures using BarSeq as described above. The relative growth of each barcoded line in the presence of different concentrations of chloroquine is shown in figure 3.5. The growth curves are shown as the log proportion of each barcode over time. The range of chloroquine concentrations was selected to start at the equivalent of approximately 1X the IC_{50} of 3D7 (20nM) up to the approximate 1X IC_{50} of the CAM strain (500nM). The differences in drug IC_{50} correlated with the phenotypes observed by BarSeq showing a significant difference in the means of barcode proportion change between the sensitive and

chloroquine resistant strains, p-values obtained by performing ANOVA with Tukey to measure differences across strains is shown in table 3.3. There was no significant difference between the internal 3D7 clones at exposure to all different concentrations and between the internal V1/S lines across the concentration range of 1X IC_{50} to 10X. A significant difference was observed between the 3D7 and both V1/S and CAM strains at the lowest concentration of chloroquine of 1X IC_{50}. Between the two resistant strains, V1/S demonstrated intermediate tolerance with increasing chloroquine doses compared to the CAM strain that showed a higher tolerance even at 5X and >10X IC_{50}. The 3D7 strain showed relative sensitivity to chloroquine at the lowest concentration of 1X IC_{50}, as expected. At higher doses of chloroquine of up to >10X IC_{50} of 3D7, the CAM strain maintains a strong relative growth, consistent with the high IC_{50} for this strain demonstrated in the standard drug assays (Table 3.2).

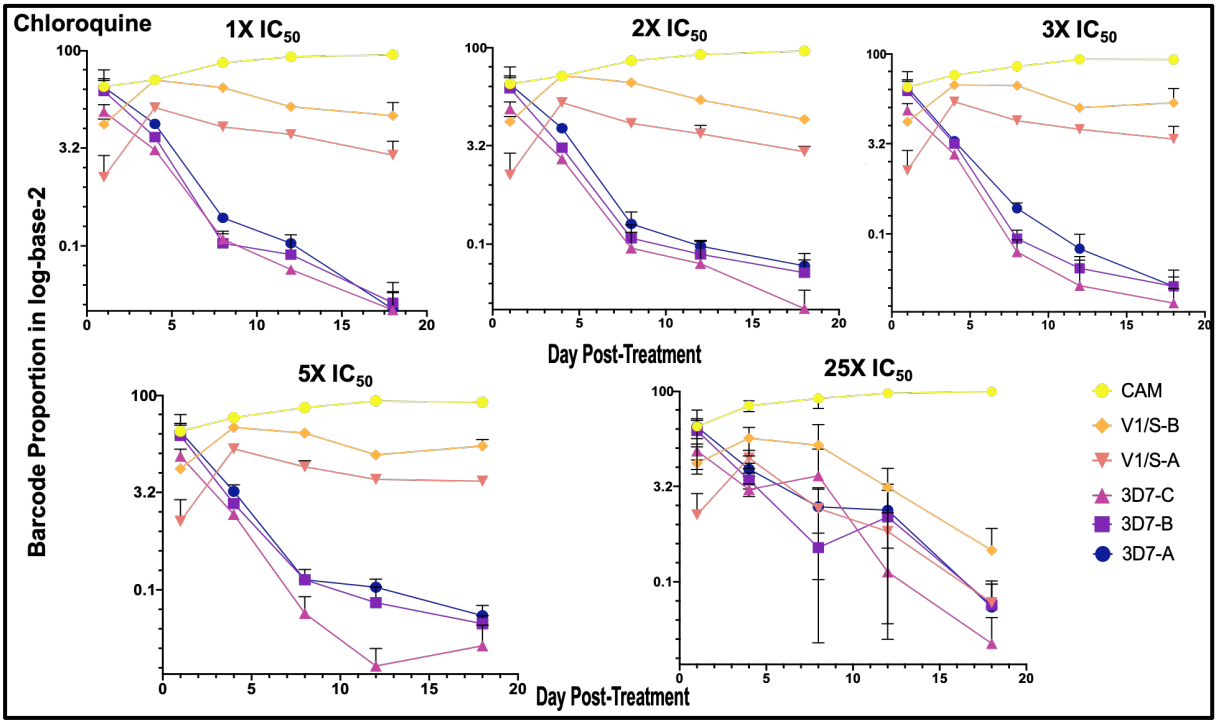


Figure 3.5 BarSeq of barcoded clones of *P. falciparum* strains with increasing concentrations of chloroquine. Competition assays of barcode pools in the presence and absence of chloroquine at different concentrations. The barcode proportion in the total count at every time point is represented on the y-axis

Table 3.3 Mean comparison of barcode proportion change across strains exposed to different chloroquine concentrations. The statistical difference between the change in proportion relative to day 1 of the different strains in presence and absence of chloroquine. ANOVA with Tukey test showed significant difference between chloroquine sensitive and resistant strains in the presence of CQ. Non-significant (ns) difference between groups is p>0.05. There was no significant difference between CAM and V1/S

Mean Comparison	Chloroquine concentration (Adjusted P-value)					
	Untreated	20nM	50nM	60nM	100nM	500nM
3D7-V1/S-A	ns	0.0005	0.0004	0.0002	0.0002	ns
3D7-V1/S-B	ns	0.0025	0.0019	0.0036	0.0010	0.0414
3D7-CAM	ns	0.0021	0.0018	0.0013	0.0009	ns

Based on the IC_{50} value obtained for mefloquine in the standard drug assays, the BarSeq assays for mefloquine were set up with concentrations ranging from a sublethal concentration for 3D7 to >10xIC50. The relative growth curves from the proportions of each barcode are shown in figure 3.6. Contrary to results obtained for chloroquine, V1/S showed a sensitive phenotype even at low concentrations of mefloquine and was outcompeted by the other strains. The 3D7 and CAM strains showed higher tolerance to mefloquine than V1/S at sub-lethal doses which correlates with the differences in drug IC_{50} values for mefloquine. A relatively unchanging barcode proportion over time was observed across the different strains at >10X $1C_{50}$, and the assay was stopped at day 8 due to the cultures not growing. This reflects the amplification of DNA present from dead parasites and does not represent genuine growth change. The statistical summary of ANOVA with Tukey test for mean comparison across groups is shown in table 3.4.

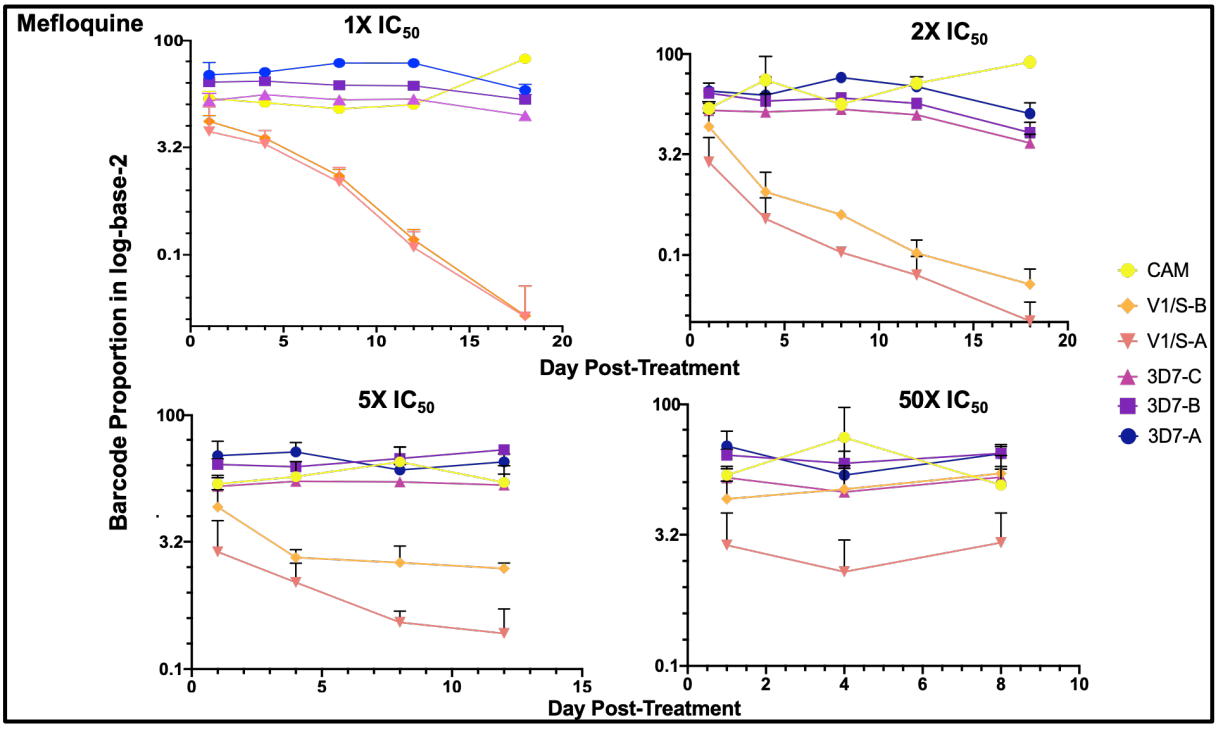


Figure 3.6 BarSeq of barcoded clones of *P. falciparum* strains with increasing mefloquine concentrations. Competition assays of barcode pools without drug treatment and with mefloquine treatment at different concentrations. The log2 of each barcode proportion in the total count across different time points is represented. Note that at 500nM, all strains are killed.

Table 3.4 Mean comparison of barcode proportion change between strains exposed to different mefloquine doses. The statistical difference between change in barcode proportion relative to day 1 across strains measured by one-way analysis of variance with Tukey test. Non-significant (ns) difference between groups is p>0.05

Mean Comparison	Mefloquine concentration (Adjusted P-value)			
	10nM	20nM	50nM	500nM
3D7-V1/S-A	ns	ns	ns	ns
3D7-V1/S-B	ns	0.0418	ns	ns
3D7-CAM	ns	ns	ns	ns
V1/S-A-CAM	ns	0.0178	ns	ns
V1/S-B-CAM	ns	0.0057	ns	ns

The BarSeq approach was performed to determine phenotypes of the different strains in the absence and presence of piperaquine concentrations ranging from 10nM to 500nM which is approximately 25X IC_{50} to 1250X IC_{50} of 3D7, respectively, over a 13-day period shown in figure 3.7. This concentration range was based on previous IC_{50} data derived in the lab with a piperaquine stock that was subsequently discovered to be significantly less potent. Consistent with the results shown in figure 3.4, there was a significant difference between V1/S and 3D7 in the competition assays in the absence of the drug with V1/S outcompeted by 3D7 and CAM strains. In the presence of the lowest piperaquine concentration tested (25X IC_{50}), the change in proportion over time observed does not correlate with differences in drug IC_{50} across the strains in the standard 72-hour assay. The barcode proportion for the CAM strain, which had the highest IC_{50} for piperaquine, showed a greater drop by day 13 indicating greater sensitivity of CAM at this concentration and higher doses. Similar barcode proportion to the total input over time was observed across the different strains from 75X IC_{50} and dead parasites were confirmed by microscopy. This suggests these assays are not meaningful and represent the presence of DNA from dead parasites. As noted above, the initial concentration range was selected based on historical lab data for piperaquine IC_{50}, however the stock used for these BarSeq assays was subsequently discovered to be considerably more potent, reflected in the sub-nanomolar IC_{50} values shown in Table 3.2. Thus, all the concentrations tested were already several fold above the IC_{50} of the strains in the pool.

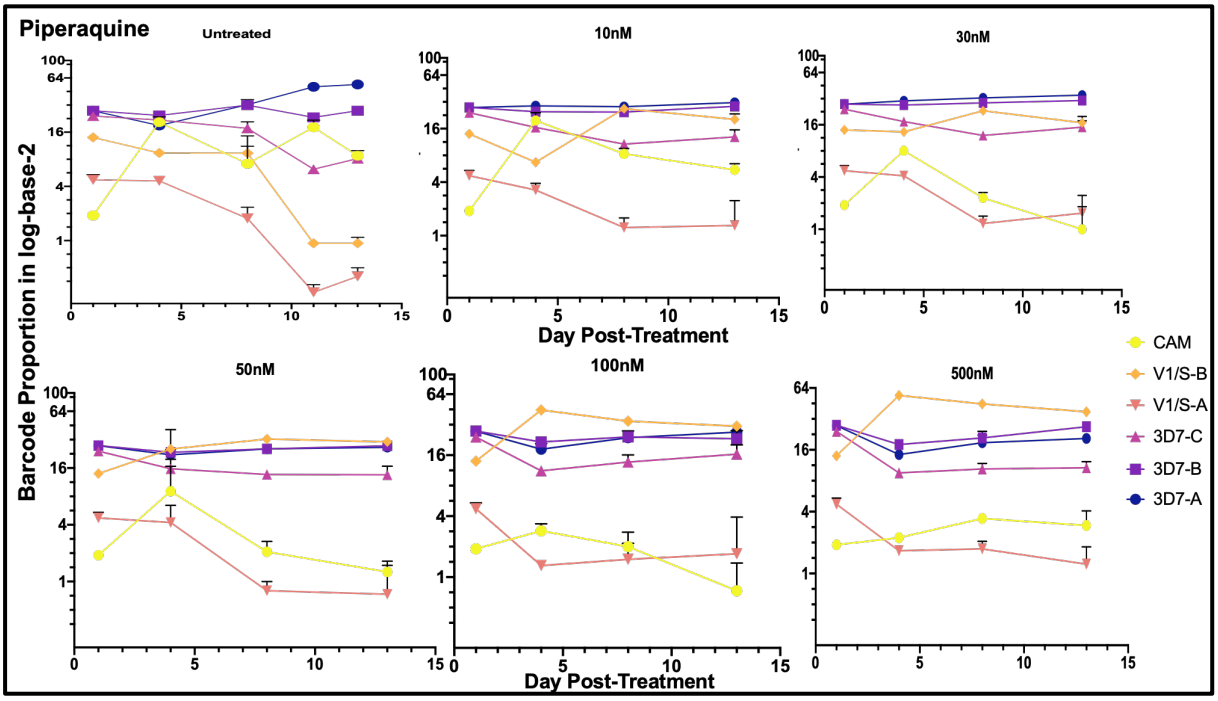


Figure 3.7 BarSeq of barcoded clones of *P. falciparum* strains with increasing piperaquine concentration. Competition assays of barcode pools without drug treatment and with piperaquine at different concentrations with each barcode proportion in the total count at every time point represented.

Table 3.5 Mean comparison of barcode proportions across strains in the different assays. The statistical difference between the means of the different groups and clones in presence and absence of CQ, measured by one-way analysis of variance with Tukey test. There was statistical difference in mean comparison across groups with a p-value of <0.0001 of the ANOVA summary. Non-significant (ns) difference between groups is p>0.05. Note that at 500nM, all strains are dead

Mean Comparison	Piperaquine BarSeq (Adjusted P-value)					
	Untreated	10nM	30nM	50nM	100nM	500nM
3D7-V1/S-A	ns	ns	ns	ns	ns	ns
3D7-V1/S-B	ns	ns	ns	ns	ns	0.0110
3D7-CAM	ns	ns	ns	ns	ns	ns
V1/S-A-CAM	0.0045	0.0108	ns	ns	ns	0.0224
V1/S-B-CAM	0.0050	ns	ns	ns	ns	ns

3.4 Discussion

Studies involving *in vitro* selection of drug resistant *P. falciparum* remains a crucial technique for identification of the mechanisms and genetic markers of antimalarial drug resistance and parasite fitness (Mwai *et al,* 2012). Informative analyses of genetic markers of drug resistance and fitness cost associated with resistance have been facilitated by the combination of *in vitro* assays, gene editing and genome sequencing (Ng and Fidock, 2019, Tirell *et al,* 2019; Hott *et al,* 2015). The results obtained from the experiments in this chapter demonstrate the ability of assessing the resistance and fitness profiles of multiple *P. falciparum* strains using the BarSeq approach. The V1/S strain demonstrated a fitness disadvantage relative to the CAM strain, which in turn appears less fit than 3D7, which has been cultured in the lab for decades. It is evident from the results that there is a fitness advantage in some strains over others in the absence of drug pressure *in vitro*. Clearer differences in overall fitness may be revealed by extending the assay period. This BarSeq approach allows studying the fitness profile of multiple strains at the same time at a large-scale contrary to previous methods that independently measure fitness in head to head assays mostly with two strains co-cultured at a time, limiting scalability.

The expected phenotypes for the strains that have been well profiled with regards to chloroquine sensitivity was observed, as were the drug sensitivity profiles of these strains for mefloquine and piperaquine that are partner drugs in the ACTs. An increase in copy number of the *Pfmdr1*, resulting in an amplification of cellular transcripts had been suggested to directly modulate mefloquine resistance (Mwai *et al,* 2012; Llinàs *et al,* 2006). This highlights the importance of the sensitivity of V1/S to low concentration of mefloquine that is observed in the results. V1/S has *Pfdhfr* and *Pfcrt* mutations causing resistance to pyrimethamine and chloroquine, respectively.

However, the absence of *Pfmdr1* copy number amplification that affect sensitivity to several drugs including mefloquine is indicated by the phenotype observed in the results (Yongkiettrakul *et al,* 2020; Ding *et al,* 2012, Fidock *et al,* 2000). In a study by Chugh *et al* (2015) identifying cross-resistance signals from antimalarial compounds using multidrug-resistant strains, the *Pfmdr1* copy number for V1/S was 1 (Chugh *et al,* 2015). However, *Pfmdr1* copy number is unlikely to be the sole reason for differences in sensitivity, as 3D7 was less sensitive than V1/S despite also having a single copy of *Pfmdr1*. The intermediate phenotype of the artemisinin resistant CAM strain to mefloquine and piperaquine obtained by the Barseq approach highlights the importance of its ability to assess multiple strains of different resistance profiles. The results obtained shows sensitivity of CAM to mefloquine by BarSeq demonstrating efficacy of mefloquine which is one of the main partner drugs in ACTs to counteract the challenge of multi drug resistance in Southeast Asia where the CAM strain originated (Hamilton *et al,* 2019; Ménard and Fidock 2019). However, there is a lack of correlation between the standard drug assay and BarSeq approach in the drug profiles across the strains for piperaquine, likely due to the high concentrations of piperaquine used relative to the IC_{50} values of the strains. This illustrates that the BarSeq approach may not give meaningful data at concentrations appreciably above the IC_{50}, with counts coming from dead parasites. Alternate approaches would be to use a version of the *in vitro* piperaquine survival assay (PSA) in the BarSeq approach for piperaquine sensitivity, as PSA has been reported to detect piperaquine resistance and treatment failure more reliably than classic dose–response assays (Duru *et al,* 2015).

In subsequent work, I aim to assess the phenotype of sensitivity to artemisinin which remains the frontline antimalarial. This will be done by performing DHA drug

response by BarSeq with RSA (0-3h) in which parasites are left to grow into the subsequent cycle before microscopy assessment after tightly synchronised early rings (0–3h) are exposed to a ‘pulse’ of DHA and washed (Witkowski *et al,* 2013). I also aim to perform this approach with an additional selection of strains of other backgrounds to have a greater diversity representation of strains.

For longer term growth experiments or maintenance of drug-screening pools, rapid out-competition of slow growing mutants by strains that grow faster has been pointed out by Bushell *et al,* (2017) in a *P. berghei* gene function study using BarSeq (Bushell *et al,* 2017). This point will be considered in future work by ensuring that the different strains to be mixed in a pool for competition assays will have relatively comparable growth rates.