Chapter 2

Materials and Methods

All reagents were purchased from Sigma-Aldrich unless stated otherwise.

2.1 Parasitology

2.1.1 Rodents

The following rodents were used in this study:

Strain	Description
Theiler's Original (TO) mice	For routine work. Outbred, 6-8 weeks of age, male and female.
BALB/c mice	Used as recipients of STM experiments. Inbred, 6-8 weeks of age, male and female.
Wistar rats	Used to propagate parasites to be transfected in STM experiments. Outbred, 200 g – 250 g (~ 8weeks), female.

Animals were provided by Harlan Laboratories, UK. All animal research was conducted under licences issued by the UK Home Office using protocols reviewed by the ethics committee of the Wellcome Trust Sanger Institute.

2.1.2 Parasite lines

The following *P. berghei* parasite lines were used in this dissertation.

Line	Description
ANKA 2.34	WT line. For routine work and used as background for generation of transgenic parasites and as control for phenotypic analysis.
Clone RMgm-7	Selectable marker–free reporter strain expressing the mu3 variant of green fluorescent protein (GFP). This was generated in the *P. berghei* ANKA cl15cy1 background. Also used as background for generation of transgenic parasites and as control for phenotypic analysis.
gsk3 KO	Validation of STM results.
cdpk1 KO	Validation of STM results and used as background in the genetics interaction screen.
cdpk3 KO	Used as background in the genetics interaction screen.
cdpk4 KO	Used as background in the genetics interaction screen.
cdpk6 KO	Used as background in the genetics interaction screen.
pkg^{T619Q}	Used as background in the genetics interaction screen. (Provided by M.

	Brochet [154])
rio1 KO	Validation of STM results.
rio2 KO	Validation of STM results.
PBANKA_082960 KO	Validation of STM results.
tkl3 KO	Validation of STM results.

2.1.3 Parasite maintenance

All parasite lines were propagated in the above mentioned rodents. These were infected either by intraperitoneal (i.p.) or intravenous (i.v.) injection of *P. berghei* infected blood, using 30-gauge (G) needles. The latter route was administered in the tail vein and only used to infect the transfection recipients. Infections were monitored on thin blood smears made from one drop (~3 µL) of tail blood and stained with 10 % Giemsa stain solution modified diluted in water. Parasitaemia was counted under 1000x magnification and was expressed as the percentage of infected RBC per total number of RBC.

Parasite harvesting was always a terminal procedure. For that the rodents were anesthetised by i.p. injection of 10 mL/Kg of a solution containing 12 % ketamine and 0.16 % xylazine in phosphate-buffered saline (PBS). Blood was collected by cardiac puncture using syringes pre-loaded with 100 µL of heparin (30 U/mL).

2.1.4 Parasite cloning by limiting dilution

Limiting dilution was used to clone transgenic parasite populations. To this end, a TO mouse was infected with the mutant line. When parasitaemia reached 0.3-1 %, the mouse was sacrificed and the blood was diluted in RPMI1640 (Gibco) by successive dilutions to a final concentration of 1.5 parasites/100 µL. Next, 200 µL of this suspension, i.e. three parasites, were injected i.p. into each of a set of ten mice. Under *in vivo* conditions this procedure usually results in two to three positive infections. Parasitaemia was checked on day 9 post-infection and parasites were genotyped by PCR.

2.1.5 Selection marker recycling

All targeting vectors contained a recyclable positive/negative selection cassette (*hdhfr-yfcu*) [54]. After dilution cloning the selection marker was removed by negatively selecting

parasites with 5-FC (1 mg/mL) in drinking water, for seven days, from day 1 post-infection onwards. In the presence of negative selection, only parasites that lose the selection cassette as a result of homologous recombination between the direct repeats flanking the fusion gene are able to survive. On day 7-10 post-drug treatment the parasites obtained were genotyped and re-cloned.

2.1.6 Parasite phenotyping

2.1.6.1 Asexual growth curves of *P. berghei* parasites.

This method was used to perform pairwise comparisons of growth curves without using a barcode strategy. For each mutant, three mice were infected with 10^6 parasites i.p. Blood smears were taken daily to monitor parasitaemia. A total of 1500 RBCs were counted per replicate, per time-point to calculate growth curves for each mutant. These experiments were performed under a "severe" protocol according to our animal project licence.

2.1.6.2 Exflagellation assay

Approximately 5 µL of blood was taken by tail bleed and mixed with ookinete media (section 2.1.6.3.1). After 5 min, 10 µL of this mix was loaded on a haemocytometer, and the number of exflagellating microgametocytes per 1000 RBCs was counted. A minimum of five fields per replicate were counted.

2.1.6.3 Ookinete phenotyping

2.1.6.3.1 Ookinete culture

In order to induce reticulocytosis and hence gametocyte production [155], mice were injected i.p. with 0.2 mL phenylhydrazine (6 mg/mL) three days prior to infection with *P. berghei*. On day 4 post-infection, infected blood was collected and re-suspended in ookinete medium: RPMI-1640 Medium HEPES modification, with L-glutamine and 25 mM HEPES, without sodium bicarbonate (Gibco); 50 mg/L hypoxanthine; 50 units/mL of Pen/Strep; 100 µM xanthurenic acid in 6 mM $NaHCO_3$; filter-sterilised; final pH 7.4. Just before the blood was added, the medium was supplemented with 20 % FBS (gibco). The culture was incubated for 18 h at 19 °C. On the following day, cultures were checked for the presence of ookinetes on Giemsa stained smears.

2.1.6.3.2 Ookinete conversion rate calculation

To calculate the ookinete conversion rate, 18-20 hour ookinete cultures were sampled (50-100 µL) and spun for 1 min at 500 g. The pellets were stained with 13.1 antibody, an anti-P28 Cy3-labeled monoclonal antibody [156,157], diluted 1:500 in PBS, at room temperature for 10 min. The presence of 0.4 % Hoechst (bisBenzimide H33342 trihydrochloride) enabled the detection of the nuclei but this was not essential. Parasites were checked immediately on a Leica DM2500B and the ratio ookinetes : activated macrogametocytes, i.e. banana shaped : round forms, was calculated. Images were processed using ImageJ software v.1.44o.

2.1.6.3.3 Ookinete purification with magnetic beads

The ookinete cultures were centrifuged for 5 min at 500 g. Then, pellets were re-suspended in 8 mL of ookinete medium, in 15 mL Falcon tubes, and incubated with 5 µL of 1:50 dilution of an anti-P28 antibody coated with magnetic beads, for 5 min, on a rotating mixer. Next, tubes were placed on a magnetic Dynarack (Dynal Biotech) for 2 min or until the solution was clear. The culture medium was then removed and, without removing the tube from the stand, PBS was added to wash the bound ookinetes. After this, the tubes were removed from the magnetic stand and ookinetes were re-suspended in an appropriate volume of PBS.

2.1.6.4 Oocyst dissection and counts

Twelve days after infection, mosquitoes were anesthetised (10 min, at -20°C) and midguts were dissected out into PBS, using fine point dissection forceps and a 30 G needle. Dissections were carried out in sets of 10 midguts per strain, which were then covered with a coverslip. The number of oocysts present in each midgut was counted under phase contrast.

2.1.6.5 Sporozoite dissection and counts

Twenty-one days after infection, mosquitoes were anesthetised (10 min, at -20 °C) and salivary glands were dissected out into PBS, using fine point dissection forceps and a 30 G needle. Dissections were carried out in sets of 10 mosquitoes per strain. In the event that individual glands were damaged or lost during dissection, an equivalent number of extra mosquitoes would be dissected. Glands were transferred into a 1.5 mL tube and homogenised

with a micro-pestle in 100 µL. Next, the number of sporozoites was counted on a haemocytometer. For this a sample of 5 µL of the homogenate was diluted in 5 µL of PBS.

2.2 Generation of targeting vectors

2.2.1 Single tube protocol

All vectors not obtained from the *Plasmo*GEM resource (19 out of 54) were assembled using the small scale tube protocol as previously described [73]. Briefly, the *Pb*G library clone was chosen by the *Plasmo*GEM database as the optimal clone containing the gene of interest (GOI). This was amplified and electroporated (Bio-Rad Gene Pulser Xcell; parameters: 1800 V, 10 µF, 600 Ω) with the recombineering plasmid (pSC101-BAD-gbaA-tet). The transformed bacteria were incubated overnight at 30˚C in 4 mL of TB medium, with 30 µg/mL of kanamycin and 5 µg/mL of tetracycline. On the next day, this culture was diluted in fresh medium to an optical density (OD600) of 0.05. When the OD 600 reached 0.3-0.4, expression of the recombineering proteins was induced by addition of L-arabinose (0.2% w/v) and by a temperature switch to 37 ˚C. This allowed efficient expression of the proteins and concomitant loss of the plasmid which contained a thermo-sensitive origin of replication. After 40 min of induction, the cells were washed three times with ice-cold ultrapure water (3 min, 5000 g) and electroporated with 1 µg of a PCR product containing a *zeo-pheS* cassette flanked by Gateway attR1-attR2 sites [79]. This amplicon was generated with primers that included the 50 bp located immediately upstream and downstream of the start and stop codons, respectively. The sequence of these primers was provided by the *Plasmo*GEM database and the cycling programme was as follows: 95 °C 5’ // 95 °C 30’’ / 50 °C 30’’ / 68 °C 1’ (x30) // 68 °C 10’ //. The resulting culture was allowed to recover in 4 mL of TB medium for 70 min before zeocin (50 µg/mL) and kanamycin (30 µg/ mL) were added. This was incubated overnight at 37 ˚C. On the following morning, the recombineered library clone (i.e intermediate vector) was extracted using a mini-prep kit (Qiagen) according to the manufacturer’s instructions. This was followed by dialysis as high purity is crucial for the success of the gateway step. In the second step of this protocol, an *in vitro* Gateway reaction was used to replace the *zeo-pheS* cassette by the *Plasmodium* drug resistance cassette. The Gateway reaction was set up in 20 µL using 2 µL of LR clonase (Invitrogen), 100 ng Gateway Entry plasmid (pR6K-attL1-3xHA-hdhfr-yfcu-attL2), LR clonase buffer and 300 ng purified intermediate vector. The final vector was transformed into electrocompetent *E. coli* TSA and

plated on YEG-Cl agar with kanamycin (30 µg/ mL). Colonies were verified by PCR to ensure the presence of the cassette. Four of them were sequenced together with the vectors produced by the resource, to check the integrity of the homology arms and barcode sequences. Prior to transfection, constructs were digested with *Not*I to release the bacterial regulatory regions.

2.2.2 Vectors provided by the *Plasmo*GEM resource

The *Plasmo*GEM vectors were generated by recombinase mediated engineering in continuous liquid culture on 96-well plates as previously described [73], with the following modifications:

At the first step, the PCR product consisting of a *zeo-pheS* marker and 50 bp primer extensions homologous to the GOI was purified using the High Pure 96 UF Cleanup Kit (Roche) to improve Lambda Red recombination efficiency in *E. coli*. The resulting intermediate vectors were selected in liquid culture containing 30 µg/mL kanamycin and 50 µg/mL zeocin. After two rounds of antibiotic selection cultures were re-inoculated into fresh selective medium and incubated for no longer than 16 hours at 37 °C. Intermediate vector DNA was obtained using a Qiagen Plasmid Plus 96 Miniprep kit and eluted into 20 µL Tris-EDTA buffer.

At the second step, the Gateway reaction was set up in 20 µL using 2 µL of LR clonase (Invitrogen), 100 ng Gateway Entry plasmid (e.g. pR6K-attL1-3xHA-hdhfr-yfcu-attL2), LR clonase buffer and 300 ng purified intermediate vector. Gateway reactions were purified using the High Pure 96 UF Cleanup Kit. Electrocompetent *E. coli* TSA were transformed, plated on YEG-Cl agar and four colonies picked to verify the sequence of their homology arms.

2.3 Generation of mutant *P. berghei* parasites

2.3.1 Generation of single mutants

Single mutants were generated using a standard transfection protocol [44]. Briefly, one donor mouse was infected and monitored until blood parasitaemia reached 1-3 %. At this point the infected blood was harvested and used to prepare an overnight culture of schizonts. Each millilitre of blood was diluted in 50 mL of schizont medium (RPMI 1640 medium containing 25 mM glutamine and 25 mM HEPES (Gibco), 10 mM $NaHCO_3$, 100 U/mL

penicillin/streptomycin (Gibco) and supplemented with 25 % FBS), gassed for 90 seconds with malaria gas (1 % O_2, 3 % CO_2, 96 % N_2) and incubated at 36.5 °C with gentle shaking.

On the following morning mature schizonts were purified on a 55 % Nycodenz/PBS (v/v) cushion with low acceleration and brakes, for 20 min. The brown layer of schizonts was collected with a Pasteur pipette and washed in schizont medium (500 g, 2 min). The pellet of schizonts was then mixed with the transfection reagent (16 µL of P3 primary cell) and 2 µg of vector DNA (~5 µL), and electroporated using the 4D Nucleofector System (Lonza) and the pulse programme FI-115. Electroporated parasites were immediately injected i.v. into 6-8 week-old TO mice. Drug selection was initiated on the following day with pyrimethamine (70 mg/L in drinking water). Parasitaemia was monitored daily for five days from day 5 post-transfection.

In positive infections blood was harvested when it reached > 2 % parasitaemia and parasite gDNA was genotyped (sections 2.4.2, 2.4.3.2, 2.4.4).

*Plasmo*GEM identification numbers for vectors used in these experiments are listed in appendix I.

2.3.2 STM protocols

2.3.2.1 Parallel transfection

STM transfections were performed by electroporation of purified schizonts largely as described in section 2.3.1, with the following modifications: parasites for the schizont culture were propagated in female Wistar rats to achieve maximal transfection efficiency. Purified schizonts were washed in complete medium, mixed with 16 µL of the P3 transfection reagent (Lonza) and then with the pool of targeting vectors. This transfection mix was electroporated using the 4D Nucleofector System (Lonza) in 16-well strips according to the pulse program FI-115. DNA pools contained 100 ng of each of the *Plasmo*GEM vectors. Prior to transfection, the pool was digested with *Not*I to release the bacterial vector arms, precipitated with isopropanol and re-suspended in a volume not exceeding 6 µL.

Electroporated parasites were immediately injected i.v. into 6-8 week-old Balb/c inbred mice. Three batches of schizonts were transfected with the same vector pool. Resistant parasites were selected by pyrimethamine (70 mg/L in the drinking water). Infections were monitored daily by counting Giemsa stained thin blood films under 1000 x magnification.

*Plasmo*GEM identification numbers for vectors used in these experiments are listed in appendix I.

2.3.2.2 Collection and processing of STM time-points.

A small sample of blood (≤ 30 μL) was collected at exactly the same time each day from the tail vein on days 4, 5, 6, 7 and 8 post-transfection, in 200 μL of PBS. Blood was lysed by adding 1 mL of 1:10 dilution in dH_2O of pre-chilled lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 1 mM Na_2EDTA; pH 7.4), followed by centrifugation for 3 min at 1000 g. Next, gDNA was extracted using the phenol-chloroform method described in section 2.4.3.1 and re-suspended in 50 μL of water (days 4, 5 and 6) or in 100 μL (days 7 and 8). Giemsa stained thin blood smears were performed daily to monitor infection.

2.4 DNA preparation and genotyping methods

2.4.1 White blood cell (WBC) removal

Collected blood was diluted 1:5 in PBS and then applied to a cellulose powder column previously equilibrated with PBS. More PBS was added to assist the blood moving through the column. The flow-through was collected into a 50 mL Falcon tube and then spun for 10 min at 1000 g. Supernatant was discarded and the parasite pellet was re-suspended in an appropriate volume that depended on the downstream application.

2.4.2 Blood lysis

After blood collection by tail bleed or cardiac puncture, RBCs were lysed using lysis buffer (0.15 M NH_4Cl, 0.01 M $KHCO_3$, 1 mM Na_2EDTA; pH 7.4). Blood was diluted in lysis solution at a 1:10 ratio in dH_2O and incubated on ice for 5 min. Then, the suspension was centrifuged at 1000 g and supernatant was discarded. The black pellet of parasites was re-suspended either in PBS or in TNE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA) depending on the extraction method chosen.

2.4.3 DNA extraction methods

The extraction method was chosen according to the table below.

Table 2.1| Choice of DNA extraction method depending on starting material.

Stage	Method	White blood cell removal
Blood for PCR genotyping	DNeasy Blood & Tissue Kit (Qiagen)	No
Blood for Southern blot/ WGS/ or from STM time-points	Phenol-chloroform	Yes

2.4.3.1 Phenol-chloroform extraction of blood samples

Lysed parasite pellets were re-suspended in 600 µL of TNE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA) containing 1 % SDS and 3 µL of RNAse A (20 mg/mL) and incubated at 37 °C, for 10 min. Next, 10 µL of proteinase K (20 mg/mL) were added and the mix was incubated at 37 °C for 45 min. At this point, 800 µL of buffered phenol:chloroform:isoamylalcohol (25:24:1) were added and samples were inverted several times and centrifuged at 10000 g for 5 min, at room temperature. The resulting aqueous upper phase was transferred to a new tube and 800 µL of chloroform:isoamylalcohol (24:1) were added, mixed and centrifuged in the same way. The new aqueous upper phase was transferred to a new tube and DNA was precipitated overnight at -20 ˚C with isopropanol at a v/v ratio of 1:1. On the following day, DNA was pelleted by centrifugation, air-dried and re-suspended in 50-100 µL of DNAse-free water.

2.4.3.2 DNeasy Blood & Tissue Kit

Blood was lysed according to section 2.4.2 and re-suspended in 200 µL of PBS. Then samples were processed according to the manufacturer's instruction and re-suspended in 100 µL of EB buffer.

2.4.4 PCR genotyping

Parasites were genotyped to check for correct integration of the *Plasmo*GEM vectors. Unless stated otherwise in the Figure, seven PCRs were performed on gDNA, for each line: (1) a positive control that targeted *rna polymerase II* (primers arg84/arg85); (2) a positive control for presence of the selection cassette (primers arg80/arg81); (3) 5' end of the WT locus; (4) 3' of the WT locus; (5) 5' end of the modified locus; (6) the 3' end of the modified locus, and (7) a long-range integration PCR. For reaction seven, a gene-specific primer annealed to the chromosome just outside of the vector's homology arm and paired with a primer annealing to the selection cassette within the targeting vector (either arg216 or arg218, depending on the orientation of the selection cassette relative to the first oligonucleotide).

All primers are listed in appendices II and III. PCR reaction mixes were prepared using Gotaq mix (Promega) according to manufacturer's guidelines. The cycling program was: 95 °C 5' // 95 °C 30'' / 55 °C 30'' / 68 °C 1'/kb (x30) // 68 °C 10' //. The elongation time varied according to the size of the longest amplicon, i.e. 1 min per kb.

2.4.5 Pulsed-Field Gel Electrophoresis (PFGE) of *P. berghei* chromosomes

Highly infected blood was collected by cardiac puncture and WBCs depleted (section 2.4.1) prior to lysis (section 2.4.2). The parasite pellets were subsequently re-suspended in 1 % low melting point agarose in TNE buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA) at 42 °C. While liquid, the mixture of parasites and agarose was loaded into plug moulds and left to settle for 20 min. These were then digested in 5 mL of SE buffer (0.5 M EDTA, pH 8; 1 % sarcosyl) with 100 µg/mL of proteinase K for 48 h at 37 °C.

A 1 % agarose gel was prepared in 0.5 x TBE and run in 0.5 x TBE, in a Chef Chiller DR III system from BioRad, according to the manufacturer's instructions and Pfander *et al* [73]. After the run, the gel was stained with ethidium bromide and imaged to assess chromosome migration. Then, the gel was depurinated in 0.25 M HCl for 20 min and denatured for another 20 min in denaturing buffer (1.5 M NaCl, 0.5 M NaOH). Finally, the DNA was transferred and hybridised according to section 2.4.7 to a Hybond XL membrane by capillarity and hybridised with a probe that targeted the *Pbdhfr* 3' UTR. This sequence was chosen as it naturally occurs in chromosome 7 and can therefore be used as positive control. Additionally, it is present in all *Plasmo*GEM vectors as a direct repeat, flanking the drug cassette. The probe was amplified from gDNA using primers arg496 and arg497 and the

following cycling programme: 95 °C 5' // 95 °C 30'' / 55 °C 30'' / 68 °C 30'' (x30) // 68 °C 10' //. Probe labelling and hybridisation were performed using a standard Southern blot protocol detailed in section 2.4.7.

2.4.6 DNA preparation for Southern blot analysis

DNA extracted from blood stage parasites was digested with restriction enzymes to provide suitable sized DNA fragments for analysis (Table 2.2). A minimum of 3 µg was digested for each hybridisation. All enzymes were purchased from NEB and reactions were prepared according to the manufacturer's guidelines.

Table 2.2| Summary of restriction enzymes used to digest parasite DNA for Southern blot analysis.

Parasite line	Enzymes	Expected sizes (WT/Mutant, bp)
***cdpk1* KO**	*Sna*BI + *Xba*I	3870/2771
***cdpk3* KO**	*Spe*I + *Eco*RI	5538/4098
***cdpk4* KO**	*Hind*III	5153/4083
***cdpk6* KO**	*Pst*I, + *Bgl*II	4706/2794
***gsk3* KO**	*Cla*I + *Xba*I	2707/ 821

After digestion, the DNA was separated on a 0.7 % agarose gel (Biorad laboratories), at 35V for 16 h. Once the run was finished, the gel was stained with ethidium bromide and imaged to assess DNA migration. This was followed by incubation in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 20 min. Finally, the DNA was transferred onto a Hybond N^+ membrane (Amersham Biosciences) overnight by capillary action transfer. On the next day, the membrane was cross-linked by a UV cross-linker.

2.4.7 Southern blotting hybridisation

The membrane was pre-incubated in hybridisation buffer (0.25 M Na_2HPO_4, pH 7; 20% SDS; 0.25mM EDTA; 1 % BSA (w/v)) for 30 min, at 55 °C, while the probe was end-labelled with High Prime DNA labelling kit (Roche) and [^{32}P] γATP according to the manufacturer's guidelines. Next, the probe was purified using ProbeQuantTM G-50 Micro Columns according to the manufacturer's instructions (Amersham Biosciences) and denatured at 95 °C for 5 min. After denaturation, it was cooled down on ice for 5 min and added to the

hybridisation buffer. These were incubated at 55 °C, overnight, in a rotating oven. On the next morning, the buffer was removed and the membrane was washed three times in 2x SSC, for 20 min, at 55°C. The Southern blot was visualised by exposure to BioMaxTM MR film (Kodak) at -80°C.

2.5 Illumina sequencing

2.5.1 Library preparation

2.5.1.1 Adaptor ligation

2.5.1.1.1 Whole genome sequencing

Blood from three highly infected mice (parasitaemia >10 %) was collected and gDNA was prepared according to sections 2.4.1, 2.4.2 and 2.4.3.1. A minimum of 2 µg was sheared using a Covaris ultrasonicator (model E220) and settings suggested by the manufacturer to obtain ~ 500 bp fragments. Libraries were prepared using the TruSeq DNA PCR-Free LT Sample Prep Kit (Illumina) according to the manufacturer's guidelines. Prior to sequencing each library was quantified by qPCR using a Library Quantification kit (Kapa Biosystems – kk4834). Equimolar amounts of each library were pooled and the final library was diluted to 4 nM.

As the library preparation (i.e. steps from DNA fragmentation onwards, see 1.3.1.2) and quantification steps are available as a service provided by the Illumina C team at the Sanger institute, they were delegated, once I was fully trained on these protocols.

2.5.1.1.2 STM time-points

The initial library preparation protocol for STM samples was based on an adaptor ligation strategy. The kit NEBNext DNA Library Prep Master Mix Set for Illumina (NEB) was used for this purpose, according to the manufacturer's instructions, but starting from the "dA-Tailing of End Repaired DNA" step (see 1.3.1.2). A minimum of 500 ng of PCR amplicon sample was required to initiate this protocol.

Prior to sequencing each library was quantified by qPCR using a Library Quantification kit (Kapa Biosystems – kk4834). Equimolar amounts of each library were pooled (multiplexing) and the final library was diluted to 1 nM.

As the library preparation (i.e. from dA-Tailing onwards) and quantification steps are available as a service provided by the Illumina C team at the Sanger institute, they were delegated, once I was fully trained on these protocols.

2.5.1.2 Direct amplification

For reasons detailed in chapter 3, the direct amplification method became the standard method to prepare libraries to sequence the vector-specific barcodes. It relied on a nested PCR approach. Briefly, 1 μL of each DNA sample served as template for a PCR reaction using Advantage 2 Taq polymerase (Clontech) with primers arg444 and arg445 (95 °C 5' // 95 °C 30'' / 55 °C 20'' / 68 °C 8'' (x35) // 68 °C 10' //), which bind to constant annealing sites flanking each barcode. This generated a 167 bp amplicon that was further extended in a second PCR reaction using oligonucleotides that in their 5' extensions contain Illumina adaptors and sample-specific barcodes for multiplexing up to 32 samples in one run of a MiSeq instrument. For sample specific indexing, 5 μL of the first amplicon served as template for a further 10 amplification cycles (95 °C 5' // 95 °C 30'' / 68 °C 15''(x10) // 68 °C 5' //) using one generic oligonucleotide (PE1.0) and one of a set of 32 index oligonucleotides (listed in appendix IV). The size and quality of the resulting amplicons were verified on a 1.5 % agarose gel. Each library was purified using the MinElute PCR Purification Kit (Qiagen) and eluted in 50 μL of EB buffer. Then, these were quantified in a Qubit system and 100 ng of each library were pooled. The final pool was quantified and quality controlled by qPCR for the presence of sequencing adaptors using a Library Quantification kit (Kapa Biosystems- kk4834). Prior to loading, the final multiplexed library was diluted to 1 nM.

Quantification by qPCR and MiSeq loading steps were performed by the Illumina C team at the Sanger institute.

2.5.2 MiSeq run conditions

All samples were loaded and run on a MiSeq instrument by the low-throughput Illumina Bespoke team at the Sanger Institute.

2.5.2.1 Whole genome sequencing

These libraries were diluted to 4 nM and mixed with 1 % PhiX for internal control purposes and run at normal cluster density (8-10 x 10^5 clusters/mm^2). Reads were paired-end and 150 bp-long. Data were mapped with BWA, handled with SAMtools, and visualised in Artemis.

2.5.2.2 Barcode sequencing

Due to their low complexity, PCR amplicon libraries were diluted to 1 nM and then spiked with 40-50 % of PhiX before being loaded at low cluster density (4 x 10^5 clusters/mm^2). Reads were paired-end and 150 bp-long.

2.6 Data analysis

2.6.1 Fitness calculation by barcode counting

Using a Perl script written by Frank Schwach, barcode sequences were extracted from the sequencer output files (*fastq*) and counted for each gene, for every time point and input samples.

Then, the relative abundance of each barcode within the pool was determined using my own R script. The quantitation was considered reliable for barcodes accounting for at least 0.1 % of all counts.

The input sample, i.e. an aliquot of the pool of vectors used in the transfection, was used as positive control for the presence of vectors in the pool and also as an internal control for sample cross-contamination. Parasitaemia curves for these mutants were inferred by combining the relative abundance of each barcode with the observed total parasitaemia, determined by a Giemsa stained thin blood film. This was done by multiplying the daily proportion of each mutant by the corresponding overall parasitaemia.

The relative fitness (w) of a mutant represented by a barcode on a given day (d) was calculated according to Mani *et al* [101] by comparing the daily change in its relative abundance (A) to that of the reference genes (A_{Ri} ... A_{Rn}) with normal growth:

$$w_{gene\ d} = \frac{A_d}{A_{d-1}} : \frac{\sum_{i=1}^{n} \frac{A_{Ri\ d}}{A_{Ri\ d-1}}}{n}$$

where n=4 as the reference genes in these experiments were *soap*, *p25*, *p28* and *p230p*. This was done by diving the daily fold change of the relative abundance of each mutant (A_d / A_{d-1}, where *d-1* is the day of infection preceding day *d*) by the average of the equivalent daily fold change of the normal growth references $\frac{\sum_{i=1}^{n} \frac{A_{Ri\ d}}{A_{Ri\ d-1}}}{n}$. Statistical analyses compared the growth rate (G_r), i.e. the daily fold change, of each mutant against the normal-growth reference vectors as given by:

$$Gr_{gened} = \frac{A_d}{A_{d-1}} \text{ and } Gr_{Rd} = \frac{\sum_{i=1}^{n} \frac{A_{Ri\ d}}{A_{Ri\ d-1}}}{n}$$

using a two tailed T-test (unequal variance, p-values adjusted according to the false discovery rate method). A given mutant was considered viable when consistent growth of its barcode was observed for all time points in at least 2 of 3 replicates.

2.6.2 Genetic interaction coefficients

Genetic interaction coefficients were calculated for each day post-transfection as the difference between the observed and the expected fitness of a double mutant ($M_{1,2}$). The expected value was calculated from the observed fitness of the single mutants (M_1 and M_2) using a multiplicative model [101] as follows:

$$w_x(\text{obs}) * w_y(\text{obs}) = w_{xy}(\text{exp}),$$

where w_x(obs) is the observed fitness for mutant *x*; w_y(obs) is the observed fitness for mutant *y*, both calculated as described in section 2.6.1. and w_{xy}(exp) is the expected fitness of the double mutant *xy* assuming that genes *x* and *y* do not interact.

Uncertainty of the predicted fitness of the double mutant for a given day was determined from the observed standard deviations (SD) of the fitness values for the single mutants, according to the rules of error propagation for SD:

$$\sqrt{\left(\frac{SD_x}{w_x}\right)^2 + \left(\frac{SD_y}{w_y}\right)^2} \times w_{xy}(\text{exp}) .$$

2.7 Western blotting

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a NuPAGE Novex 4-12 % Bis-Tris pre-cast gel (Life Technologies), at a constant voltage of 200 V for 50 min, according to manufacturer's instructions. After electrophoresis was complete, the proteins were transferred to Immobilon®-P PVDF membranes (Sigma-Aldrich) using the XCell II™ blot module (Life Technologies) and 1X NuPAGE® transfer buffer (Life Technologies) (with 10 % methanol), at a constant voltage of 30 V for 1 hour, according to manufacturer's instructions. After the transfer was complete, the PVDF membrane was blocked overnight in 5 % milk (Marvel)/PBS, at 4 °C.

On the next day, the membrane was probed with primary antibodies for 1 hour at room temperature with rotation, followed by 3 washes with PBST. In this project I only used the anti-HA tag rabbit monoclonal antibody (Cell Signalling Technology), diluted 1:400, in

2 % milk/0.1% Tween-20/PBS (Sigma-Aldrich) (PBST). The membranes were then probed with horse radish peroxidase (HRP)-conjugated secondary antibodies (Cell Signalling Technology), diluted in PBST, for 1 hour at room temperature with rotation, followed by another 3 washes with PBST. The presence of the HRP-conjugated secondary antibodies on the membranes was detected using enhanced chemi-luminescence (Amersham ECL, GE Healthcare) and Biomax MR films (Kodak).