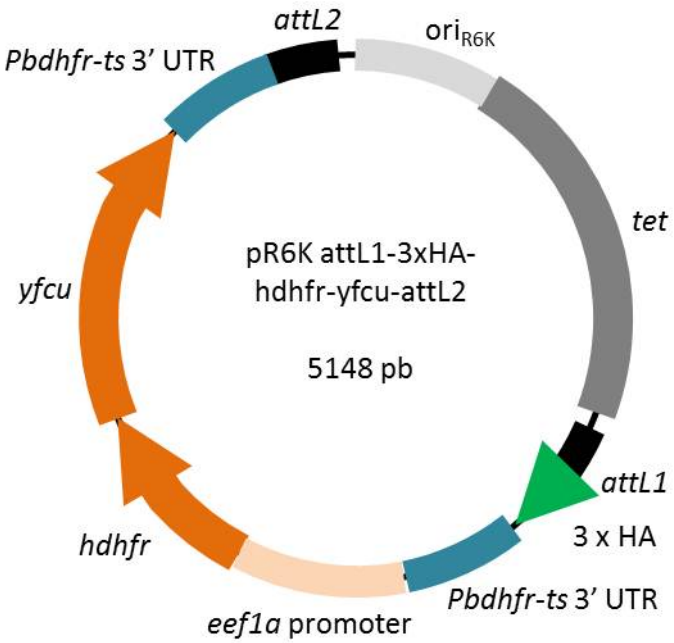

Supplementary Figure 1. Map of plasmid pR6K attL1-3xHA-hdhfr-yfcu-attL2. ori$_{R6K}$ – origin of replication; *tet* – tetracycline resistance gene; *Pbdhfr-ts* – *P. berghei* dihydrofolate reductase – thymydilate synthase; *eef1a* – *P. berghei* elongation factor 1a; *hdhfr* – human dihydrofolate reductase; *yfcu* – *Saccharomyces cerevisiae* cytosine deaminase-uracil phosphoribosyltransferase fusion gene.

Supplementary Table 1. Genes selected for optimisation. Genes shown were used to optimise the production of knockout (KO) and HA tagging vectors by recombineering (Fig. 2) and for subsequent validation of recombineered vectors in *P. berghei* (Fig. 4)

Gene ID	Annotation	Vector type(s)
PBANKA_133780	DHHC-type zinc finger protein, putative	KO + HA tag
PBANKA_144390	conserved *Plasmodium* protein, unknown function	KO + HA tag
PBANKA_143660	inner membrane complex protein 1h	KO + HA tag
PBANKA_132430	conserved *Plasmodium* protein, unknown function	KO + HA tag
PBANKA_120200	membrane skeletal protein, putative	KO + HA tag
PBANKA_133370	phosphodiesterase delta	KO
PBANKA_051200	DHHC-type zinc finger protein, putative	KO + HA tag
PBANKA_093900	ion channel protein, putative	KO
PBANKA_135150	calcium dependent protein kinase 5	HA tag
PBANKA_082340	phosphoglycerate kinase, putative	HA tag

Supplementary Table 2. Genes selected for scaling up recombineering to 96-well plate format.

Plate position	Pb identifier	description	Pf ortholog	Vector type	recombineering successful	Gateway successful
A01	PBANKA_124300	DHHC	PFF0485c	KO	Y	N
A02	PBANKA_135150	PK - CDPK5	PF13_0211	KO	Y	N
A03	PBANKA_051280	MTRAP	PF10_0281	KO	Y	Y
A04	PBANKA_140960	Conserved eukaryotic spermatogenesis	PF13_0060	KO	Y	Y
A05	PBANKA_121090	parafusin homologue	PF10_0122	KO	wrong clone	not done
A06	PBANKA_083520	AP2 domain protein	PFI1665w	KO	Y	Y
A07	PBANKA_141570	AP2 domain protein	PF13_0097	KO	Y	Y
A08	PBANKA_135400	RNA lariat debranching enzyme, putative - PPP group	PF13_0222	KO	Y	Y
A09	PBANKA_051180	EF - centrin, putative	PF10_0271	KO	Y	Y
A10	PBANKA_124300	DHHC	PFF0485c	3xHA Tag	N	not done
A11	PBANKA_051280	MTRAP	PF10_0281	3xHA Tag	wrong clone	not done
A12	PBANKA_135150	PK - CDPK5	PF13_0211	3xHA Tag	Y	Y
B01	PBANKA_133780	DHHC - eight TM domains, DHHC domain intracellular; exp. in shzts	MAL13P1.126	KO	Y	Y
B02	PBANKA_061520	PK - CDPK4	PF07_0072	KO	Y	Y
B03	PBANKA_041290	CTRP	PFC0640w	KO	Y	Y
B04	PBANKA_131040	EF - centrin, putative - highest in gametocytes according to Pf transcriptome and	PF14_0443	KO	Y	Y
B05	PBANKA_111510	pH sensitive transporter	PFE0775c	KO	N	not done
B06	PBANKA_093230	AP2 domain protein	PF11_0163	KO	Y	Y
B07	PBANKA_050250	EF - erythrocyte membrane-associated antigen 7.7E-04	PF10_0177	KO	Y	Y
B08	PBANKA_103550	vacuolar protein sorting 29, putative - PPP group	PF14_0064	KO	Y	Y
B09	PBANKA_093840	EF - endoplasmic reticulum-resident calcium binding protein	PF11_0098	KO	Y	Y
B10	PBANKA_133780	DHHC - eight TM domains, DHHC domain intracellular; exp. in shzts	MAL13P1.126	3xHA Tag	Y	Y
B11	PBANKA_041290	CTRP	PFC0640w	3xHA Tag	Y	Y
B12	PBANKA_132640	glyceraldehyde-3-phosphate dehydrogenase-1	PF14_0598	3xHA Tag	Y	Y
C01	PBANKA_144390	palmitoylated - conserved hypothetical protein	PFL1415w	KO	Y	Y
C02	PBANKA_101980	PK - CDPK-related w/o EF hands	PF14_0227	KO	wrong clone	not done
C03	PBANKA_143760	GAP45	PFL1090w	KO	Y	Y
C04	PBANKA_143660	membrane skeletal protein	Pfl1030w	KO	Y	Y
C05	PBANKA_111270	conserved transporter	PFE0645w	KO	Y	Y
C06	PBANKA_100180	AP2 domain protein	PFD0200c	KO	Y	Y
C07	PBANKA_131540	EF - protein phosphatase 2b regulatory subunit, putative 8.3E-03	PF14_0492	KO	wrong clone	not done
C08	PBANKA_133240	hypothetical protein - PPP group	PF14_0660	KO	wrong clone	not done
C09	PBANKA_101060	EF - calmodulin	PF14_0323	KO	Y	Y
C10	PBANKA_144390	palmitoylated - conserved hypothetical protein	PFL1415w	3xHA Tag	Y	Y
C11	PBANKA_143760	GAP45	PFL1090w	3xHA Tag	Y	Y
C12	PBANKA_112290	hexokinase	PFF1155w	3xHA Tag	Y	Y
D01	PBANKA_132430	palmitoylated - conserved hypothetical protein—Myristoylated and Palmitoylated	PF14_0578	KO	Y	Y
D02	PBANKA_121710	PK - At CDPK-like-like no good homologues	PFC0945w	KO	Y	Y
D03	PBANKA_130860	Aldolase	PF14_0425	KO	Y	Y
D04	PBANKA_123130	putative transporter	PFE0825w	KO	Y	Y
D05	PBANKA_061460	gametocyte specific permease	PF07_0070	KO	Y	Y
D06	PBANKA_101550	AP2 domain protein	PF14_0271	KO	Y	Y
D07	PBANKA_132950	protein serine/threonine phosphatase - PPP group	PF14_0630	KO	Y	Y
D08	PBANKA_132800	hypothetical protein - PPP group - UIS2	PF14_0614	KO	Y	Y
D09	PBANKA_130810	EF - hypothetical protein	PF14_0420	KO	Y	Y
D10	PBANKA_132430	palmitoylated - conserved hypothetical protein—Myristoylated and Palmitoylated	PF14_0578	3xHA Tag	Y	Y
D11	PBANKA_130860	Aldolase	PF14_0425	3xHA Tag	Y	Y
D12	PBANKA_145950	MTIP	PFL2225w	3xHA Tag	Y	Y
E01	PBANKA_136520	palmitoylated - thioredoxin-related protein	PF13_0272	KO	Y	Y
E02	PBANKA_145340	PK - CaMK2 Kinase domain most similar to cdpk2	PFL1885c	KO	Y	Y
E03	PBANKA_134010	lactate dehydrogenase-1	PF13_0141	KO	Y	Y
E04	PBANKA_121070	gametocyte specific	PF10_0120	KO	Y	Y
E05	PBANKA_124020	putative GPCR	PFE1265w	KO	Y	Y
E06	PBANKA_103430	AP2 domain protein	PF14_0079	KO	Y	Y
E07	PBANKA_102010	PP1-like protein serine/threonine phosphatase - PPP group	PF14_0224	KO	N	not done
E08	PBANKA_131870	Protein phosphatase 2C, putative - PPM group	PF14_0523	KO	wrong clone	not done
E09	PBANKA_094140	EF - caltractin (centrin)	PF11_0066	KO	Y	Y
E10	PBANKA_136520	palmitoylated - thioredoxin-related protein	PF13_0272	3xHA Tag	Y	Y
E11	PBANKA_134010	lactate dehydrogenase-1	PF13_0141	3xHA Tag	Y	Y
E12	PBANKA_120200	palmitoylated - IMC1 related protein	PF10_0039	3xHA Tag	Y	Y
F01	PBANKA_103540	palmitoylated - conserved membrane protein	PF14_0065	KO	Y	Y
F02	PBANKA_141980	PDEb	MAL13P1.118	KO	Y	Y
F03	PBANKA_112560	pyruvate kinase-1	PFF1300w	KO	Y	Y
F04	PBANKA_061040	Ca transport non-SERCA	PFL0590c	KO	Y	Y
F05	PBANKA_010950	AP2 domain protein	PFF0550w	KO	Y	Y
F06	PBANKA_131320	AP2 domain protein	PF14_0471	KO	Y	Y
F07	PBANKA_050250	erythrocyte membrane-associated antigen - PPP group	PF10_0177	KO	Y	Y
F08	PBANKA_100770	protein phosphatase 2C - PPM group	PFD0505c	KO	Y	Y
F09	PBANKA_051480	EF - calmodulin, putative	PF10_0301	KO	Y	Y
F10	PBANKA_103540	palmitoylated - conserved membrane protein	PF14_0065	3xHA Tag	Y	Y
F11	PBANKA_112560	pyruvate kinase-1	PFF1300w	3xHA Tag	N	not done
F12	PBANKA_140960	Conserved eukaryotic spermatogenesis	PF13_0060	3xHA Tag	Y	Y
G01	PBANKA_071120	palmitoylated - conserved hypothetical protein—ubiquitin-like domain	MAL8P1.62	KO	N	not done
G02	PBANKA_141990	PDEg	MAL13P1.119	KO	Y	Y
G03	PBANKA_120200	palmitoylated - IMC1 related protein	PF10_0039	KO	Y	Y
G04	PBANKA_070470	gametocyte high	PF08_0023	KO	wrong clone	not done
G05	PBANKA_011210	AP2 domain protein	PFF0670w	KO	wrong clone	not done
G06	PBANKA_131970	AP2 domain protein	PF14_0533	KO	wrong clone	not done
G07	PBANKA_122740	protein phosphatase, putative - PPP group	PF08_0129	KO	Y	Y
G08	PBANKA_040720	dual-specificity protein phosphatase, putative - PTP group	PFC0380w	KO	Y	Y
G09	PBANKA_020630	EF - centrin, putative	PFA0345w	KO	Y	Y
G10	PBANKA_071120	palmitoylated - conserved hypothetical protein—ubiquitin-like domain	MAL8P1.62	3xHA Tag	N	not done
G11	PBANKA_082340	Phosphoglycerate kinase	PFI1105w	3xHA Tag	Y	Y
G12	PBANKA_131040	EF - centrin, putative - highest in gametocytes according to Pf transcriptome and	PF14_0443	3xHA Tag	Y	Y
H01	PBANKA_145230	palmitoylated - conserved membrane protein	PFL1825w	KO	N	not done
H02	PBANKA_133370	PDEd	PF14_0672	KO	Y	Y
H03	PBANKA_051200	DHHC - exp. predominantly in gametocytes	PF10_0273	KO	Y	Y
H04	PBANKA_093900	mechanosensitive ion channel	PF11_0092	KO	Y	Y
H05	PBANKA_021440	AP2 domain protein	PF07_0126	KO	Y	Y
H06	PBANKA_135600	AP2 domain protein	PF13_0235	KO	Y	Y
H07	PBANKA_082850	serine/threonine protein phosphatase, putative - PPP group	PFI1360c	KO	Y	Y
H08	PBANKA_050620	hypothetical protein - NIF group - UIS12	PF10_0124	KO	Y	Y
H09	PBANKA_102440	EF - calmodulin, putative 5.8E-04	PF14_0181	KO	Y	Y
H10	PBANKA_145230	palmitoylated - conserved membrane protein	PFL1825w	3xHA Tag	N	not done
H11	PBANKA_051200	DHHC - exp. predominantly in gametocytes	PF10_0273	3xHA Tag	Y	Y
H12	PBANKA_143660	membrane skeletal protein	Pfl1030w	3xHA Tag	Y	Y
				total	79/87	77/79

Supplementary Protocol 1. 96-well Recombineering and Gateway Reaction

All bacterial liquid cultures are grown in 1 x TB (11.8 g Bacto-Tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (anhydrous), 2.2 g potassium dihydrogen phosphate (anhydrous) per litre) with 0.4% glycerol (added after autoclaving) and the indicated antibiotics. All water used is HPLC grade. All reagents were purchased from Sigma-Aldrich unless otherwise indicated. Primers were ordered from Invitrogen.

Day 0

1. Inoculate PbG01 clones from glycerol stock in 1ml TB + 30 µg ml^{-1} kanamycin in a 96-deepwell plate and shake overnight at **37 °C**.

Day 1

Confirmation of library clones

2. The identity of library clones can be confirmed directly from the stationary overnight culture by PCR with target specific primers ① and ②. (See also Fig. 2a).
3. Add 5 µl of stationary culture to 30 µl of H_2O in a PCR plate and lyse cells by incubation at 90 °C for 10 min. Use 1 µl as template in subsequent PCR reaction.

H_2O	9.5 µl
Template	1.0 µl
Primer ① (2 µM)	1.0 µl
Primer ② (2 µM)	1.0 µl
2 x GoTaq Green	12.5 µl
Total	25.0 µl

95 °C 5’ // 95 °C 30’’ / 50°C 30’’ / 68 °C 1’ (x 30) // 68 °C 10’ // 4 °C hold

Transformation of recombinase plasmid pSC101gbdA

4. Dilute cultures ~1:100 to an OD_{600} of 0.05 in fresh TB buffer + kanamycin in the morning and resume shaking at **37 °C** until OD_{600} reaches 0.6 - 0.8.
5. Place a sterile 96-well electroporation cuvette (Harvard Apparatus) on a piece of saran wrap on ice for at least 30 min.
6. Place the deep well plate on ice. Let cool down for ~15 min. Centrifuge 4000 rpm for 5 min at 4 °C in a table top Eppendorf centrifuge. Remove supernatant by quickly inverting the plate into container with Virkon. Wash cell pellet by resuspending in 1 ml ice cold water.
7. Repeat wash step three times. The pellet will become more lose during subsequent washes, so care must be taken not to lose the pellet. After the last wash drain inverted plate by padding onto some tissue paper.
8. Resupend bacterial pellets in 50 µl of ice-cold water containing 50 ng of pSC101gbdA and transfer to cold electroporation cuvette.

9. Electroporate at 2400 V, 70 Ω, 25 µF (BTX electroporator).
10. Immediately add 50 µl of TB to each well and transfer well content into a 96-deepwell plate with 900 µl TB.
11. Let cells recover during shaking for 70 min at **30 °C**.
12. Add 500 µl of TB medium with 15 µg ml^{-1} tetracycline and 90 µg ml^{-1} kanamycin (final concentration 5 and 30 µg ml^{-1} respectively) and continue shaking overnight at **30 °C**.

PCR amplification of zeo-pheS cassette

13. The *zeo-pheS* cassette and flanking Gateway *attR1* and *attR2* sites are amplified with recombineering primers containing 50 base pair (bp) extensions with homology to the target region (~1.8 kb).

H_2O	15.5 µl
pR1R2 plasmid (12 ng/µl)	1.0 µl
10x buffer	2.5 µl
Primers R1 and R2 (2 µM) each	2.5 µl
dNTPs (10 mM each)	0.5 µl
AdvantageTaq2 (Clontech)	0.5 µl

95 °C 5’ // 95 °C 30’’ / 58 °C 30’’ / 72 °C 1’30’’ (x30) // 72 °C 10’ // 4 °C hold

R1 primer	50 bp homology forward + 5’-aaggcgcataacgataccac-3’
R2 primer	50 bp homology reverse complement + 5’-ccgcctactgcgactataga-3’

14. Digest template for 1 h at 37 °C with *Dpn*I (5 µl of 4 U $µl^{-1}$ diluted in 1 x reaction buffer)
15. Dialyse PCR product against HPLC water in 96-well disposable dialyser (Harvard Apparatus) for 1 h.
16. Load 2 µl of dialysed PCR product on agarose gel to check concentration.
17. Transfer 250 ng - 1 µg of dialysed PCR product to a fresh tube, add H_2O to 50 µl and store at -20 °C until use.

Day 2

Recombineering of bacterial selection cassette

18. Dilute cultures ~1 in 100 to an OD_{600} of 0.05 in fresh TB + kanamycin + tetracycline (1 ml) and resume shaking at **30 °C** until OD_{600} reaches 0.3 - 0.4.
19. Glycerol stocks of bacteria containing pSC101gbdA and the library clone can be made at this stage.
20. Induce with 20 µl L-arabinose (10%, final 0.2%) per well for 40 min at **37°C**.
21. Place a sterile 96-well electroporation cuvette on a piece of saran wrap on ice for at least 30 min.
22. Place the deep well plate on ice. Let cool down for ~15 min. Centrifuge 4000 rpm for 5 min at 4 °C in a table top Eppendorf centrifuge. Remove supernatant by quickly inverting the plate into container with Virkon. Wash cell pellet by resuspending in 1 ml ice cold water.

23. Repeat wash step three times. After the last wash drain inverted plate by padding onto some tissue paper.
24. Resupend bacterial pellets in 50 µl of ice-cold *zeo-pheS* PCR product and transfer to cold electroporation cuvette.
25. Electroporate at 2400 V, 70 Ω, 25 µF (BTX electroporator).
26. Immediately add 50 µl of TB to each well and transfer well content into a 96-deepwell plate with 900 µl TB.
27. Let cells recover during shaking for 70 min at **37 °C**.
28. Add 500 µl of TB medium with 150 µg ml^{-1} zeocin (Invitrogen) (final concentration 50 µg ml^{-1}) and continue shaking overnight at **37 °C**.

Day 3

Verification of recombineering

29. Dilute cultures to OD_{600} = 0.1 in 1 ml of fresh TB + zeocin in two 96-well plates (Qiagen) at midday and resume shaking overnight at 37 °C. This eliminates some background observed after initial incubation.
30. The success of the recombineering reaction can be monitored at this stage by PCR directly from the overnight culture with primer ① and a primer specific for the *zeo-pheS* cassette (primer ③: 5'-tcattcttcgaaaacgatct-3'). Add 5 µl of stationary culture to 30 µl of H_2O in a PCR plate and lyse cells by incubation at 90 °C for 10 min. Use 1 µl as template in subsequent PCR reaction.

H_2O	9.5 µl
Template	1.0 µl
Primer ① (2µM)	1.0 µl
Primer ③ (2µM)	1.0 µl
2 x GoTaq Green	12.5 µl
Total volume	25.0 µl

95 °C 5' // 95 °C 30'' / 50 °C 30'' / 68 °C 1' (x30) // 68 °C 10' // 4 °C hold

Day 4+5

Gateway reaction

31. Prepare glycerol stocks from overnight cultures and store at -80 °C.
32. Prepare minipreps with 96-well miniprep kit (Qiagen) according to manufacturer's instructions with slight modifications: twice the recommended volume for buffers P1, P2 and P3 are used and DNA is eluted with 75 µl TE, giving ~30 µl eluate.
33. The LR Clonase reaction is performed overnight at 25 °C in a PCR machine:

Gateway Donor plasmid (25 ng/µl)	1.0 µl
Clone miniprep DNA (30 ng $µl^{-1}$)	3.0 µl
LR clonase buffer	1.0 µl
LR clonase (Invitrogen)	1.25 µl

In the morning, add 0.5 µl Proteinase K and incubate the reaction at 37 °C for 10min.

34. Add 13 µl of H_2O to each sample, transfer to a 96-well dispolyzer and dialyze against H_2O for 60 min. Cover the dispolyzer with a lid from a tissue culture plate to avoid evaporation.
35. Transfer dialyzed DNA directly to 96-well plate with electrocompetent TSA cells (Lucigen; 50 µl aliquots) and continue with transformation or store at -20 °C until use.
36. Mix well and transfer into sterile 96-well electroporation cuvette pre-cooled in ice as before.
37. Electroporate at 2400 V, 70 Ω, 25 µF (BTX electroporator).
38. Immediately add 50 µl of TB to each well and transfer well content into a 96-deepwell plate with 900 µl TB.
39. Let cells recover during shaking for 70 min at **37 °C**.
40. Centrifuge 4000 rpm for 5 min at 4 °C in a table top Eppendorf centrifuge.
41. Resuspend cells in 100 µl TB medium and plate onto YEG-Cl plates (5 g Yeast Extract, 5 g NaCl, 2 g 4-Chloro-DL-phenylalanine, 15 g Agar and 0.4% glucose) with 30 µg ml^{-1} kanamycin. Incubate plates at 37 °C overnight or RT during weekend.

Day 6 and 7

Verification of Gateway reaction

42. Inoculate 4 clones per YEG-Cl plate in 1ml of TB + kanamycin in 96-deepwell plates (4 plates if doing 96 clones) and shake overnight at 37 °C.
43. Positive Gateway clones can be identified by PCR directly from the overnight culture with primers ① and a primer specific for the Gateway cassette (primer ④: 5'-ctttggtgacagatactac-3'). Add 5 µl of stationary culture to 30 µl of H_2O in a PCR plate and lyse cells by incubation at 90 °C for 10 min. Use 1µl as template in subsequent PCR reaction.

H_2O	9.5 µl
Template	1.0 µl
Primer ① (2 µM)	1.0 µl
Primer ④ (2 µM)	1.0 µl
2 x GoTaq Green	12.5 µl

95 °C 5' // 95 °C 30'' / 50 °C 30'' / 68 °C 1' (x30) // 68 °C 10' // 4 °C hold

44. Inoculate positive clones in 3.5 ml TB + kanamycin + 0.2% arabinose in 48-deepwell plates and shake overnight at 37 °C. The addition of arabinose increases pJAZZ copy number to 15-20 per cell. 3.5 ml of culture are required for miniprep to obtain enough DNA for transfection of parasites.

Day 8

Preparation of constructs for transfection

Note: This protocol is for transfection of *P. berghei* parasites with the 96-well Nucleofector Shuttle system (Lonza). For transfection by other methods, larger amounts of DNA are required and midipreps for each construct from a minimum of 50 ml culture are suggested.

45. Prepare glycerol stocks from overnight cultures and store at -80 °C.
46. Prepare minipreps with 96-well miniprep kit (Qiagen) according to manufacturer's instructions with slight modifications: twice the recommended volume for buffers P1, P2 and P3 are used and DNA is eluted with 100 µl TE, yielding ~50 µl eluate at 5-10 ng μl^{-1}.
47. Digest DNA with *Not*I at 37 °C for min. 4 h in 100 µl total volume.
48. Transfer to 1.5 ml Eppendorf tubes and precipitate DNA by addition of 4 µl glycogen (5 mg ml^{-1}), 10 µl of 3 M NaOAc, pH 4.6, and 300 µl ethanol (stored at -20 °C). The addition of glycogen is optional, but improves precipitation of small DNA amounts and does not interfere in our experience with transfection into *P. berghei*. Spin 20,000 x g for 15 min at 4 °C.
49. Pour off supernatant and wash by adding 250 µl of 70% EtOH. Spin 20,000 x g for 5 min at 4 °C
50. Let samples dry and resuspend pellet in 10 µl of H_2O. Store at -20 °C until transfection.

Supplementary Protocol 2. Transfection of *P. berghei* schizonts

The transfection protocol is based on the published protocol (ref. 6). Parts written in black are for transfection using the 96-well Shuttle system, parts written in red mark the changes in the protocol for transfection using the Nucleofector II and individual cuvettes.

All *P. berghei* infections are carried out in female TO mice, 6-8 weeks of age in accordance with a valid Home Office project licence. Higher transfection efficiencies can be achieved by using rats. DNA for transfection is prepared as outlined in Supplementary Protocol 1.

Day -3

1. A mouse with a *P. berghei* infection around 5% is used for mechanical passage into new mice for schizont culture. Parasitaemia is monitored by Giemsa staining.
2. The mouse is bled by cardiac puncture into a syringe filled with 50 µl heparin (300 U ml^{-1}) and 100 µl of infected blood injected intra-peritoneal (ip) into each new mouse. One mouse yields ~ 2 – 4 x 10^7 schizonts. ~2 – 4 x 10^6 (4 – 8 x 10^6) schizonts are used per transfection.
3. Inject mice i. p. with 200 µl sterile phenylhydrazine at 6 mg ml^{-1} in PBS. One mouse per transfection and one additional mouse for every 5 transfections are required.

Day -1

4. Prepare schizont medium: RPMI 1640 with 25 mM glutamine and HEPES + 10 mM $NaHCO_3$, 20% foetal bovine serum and 100 U ml^{-1} *penicillin*, 100 µg ml^{-1} streptomycin.
5. For cultivation of schizonts, blood is collected at a parasitaemia between 0.5 and 2%, which is usually 1-2 days after infection, to ensure optimal development of schizonts *in vitro*.

6. Infected mice are bled by cardiac puncture into a syringe filled with 50 µl heparin (300 U ml^{-1}) and the blood is transferred to 50 ml pre-warmed schizont medium in a 250 ml polyethylene Erlenmeyer flask with screw cap. The culture is gassed for 90 s with a stream of malaria gas (1% O_2, 3% CO_2, 96% N_2) and then closed tightly. The culture is placed into a 36.5 °C shaking incubator at a slow speed that just prevents the blood from settling.

Day 0

7. 22 h after setting up the culture, monitor schizont development by Giemsa staining. Continue with step 8 if $\geq$ 70% of schizonts are mature.
8. Harvest red blood cells by centrifugation at 300 x g for 10 min at room temperature with slow acceleration and deceleration.
9. Prepare a 55% Nycodenz gradient by mixing 2.75 ml of Nycodenz solution with 2.25 ml of schizont medium.
10. Remove supernatant until the last 2 ml and resuspend the cells. Add resuspended cells slowly on top of the Nycodenz. Spin at 300 x g for 30 min at room temperature in a table top centrifuge with slow acceleration and deceleration.
11. During this time, collect blood from phenylhydrazine-treated mice by cardiac puncture into a syringe filled with 50 µl heparin (300 U ml^{-1}) and aliquot into 200 µl aliquots (one per transfection) in 2 ml round-bottom Eppendorf tubes. Place blood into a thermomixer at 37 °C.
12. Collect schizonts from the interphase and transfer to a 15 ml Falcon tube. Carefully add schizont medium to 10 ml and spin 300 x g for 10 min at room temperature with slow acceleration and deceleration. For 96-well shuttle, continue with step 13. For Nucleofector II, add medium to 5 ml and aliquot 1 ml into 1.5 ml Eppendorf tubes. Spin at 500 x g for 2 min at room temperature. For Nucleofector II, continue with step 17.
13. Prepare 10 µl DNA aliquots for transfection. If DNA was prepared by miniprep as outlined in **Supplementary Protocol 1**, the complete DNA from one digested miniprep is used. Otherwise, 250 - 500 ng of DNA is used per transfection.
14. Prepare Lonza 96-well shuttle. Program F1-115 was used in this study, but several other programs are also recommended by the manufacturer. Resuspend schizonts carefully in up to 200 µl of Lonza T cell solution (20 µl per transfection).
15. Add 20 µl of cell suspension to the DNA, mix gently and immediately transfer 20 µl to the electroporation plate.
16. Prepare all wells and pulse. No more than 8 wells are pulsed at the same time, since parasites are very fragile at this stage and should be transferred quickly after electroporation. Continue with step 22.
17. Prepare 10 µl of DNA containing 2 µg of digested DNA per transfection.
18. Prepare Nucleofector II. Program U-033 is recommended.
19. Add 100 µl of Basic Parasite Solution 2 to 10 µl of DNA and mix well.
20. Remove supernatant from parasite pellet and resuspend in DNA solution.
21. Transfer to electroporation cuvette and pulse. Continue with step 22.
22. Transfer transfected parasites into 200 µl of warm blood and pipet vigorously to break up schizonts and enable invasion of reticulocytes.

23. Incubate at 37 °C with slight shaking (300 rpm) for up to 30 min, until 200 µl of infected reticulocytes can be injected intraperitoneally into phenylhydrazine-treated mice. (The highest transfection efficiency can probably be achieved by injecting electroporated schizonts directly intravenously, however we find *in vitro* culture of reticulocytes followed by intraperitoneal injections more convenient when performing large numbers of transfections).

Day 1 post infection

24. Add pyrimethamine for selection of a single resistance marker to drinking water (70 mg L^{-1}) and maintain drug selection for 3 - 9 days.
25. Alternatively, for selection of *hdhfr* in the presence of *Tgdhfr-ts* resistance marker, inject WR99210 (16 mg kg^{-1}) intraperitoneally at 24 h, 48 h and 72 h post infection.

Day 6 post infection

26. Start monitoring parasitaemia on Giemsa-stained blood films.

Supplementary Protocol 3. Genotyping by Pulsed-Field Gel Electrophoresis of *P. berghei* Chromosomes

For pulse-field gel electrophoresis, we use the Chef Chiller DR III system from BioRad. Run conditions have to be optimized for other systems.

1. Pellets of free parasites harvested from whole blood by NH_4Cl lysis after removal of white blood cells are resuspended in 1% low melt agarose in TNE (50 mM Tris, pH 8.0, 100 mM NaCl, 5 mM EDTA) and plugs for pulse-field gel electrophoresis are cast according to manufacturer's instructions.
2. Proteins are digested by incubating plugs in 5 ml SE buffer (0.5 M EDTA, pH 8.0, 1% sarcosyl) with 100 µg ml^{-1} of Proteinase K for 2 x 12 h at 37 °C.
3. Gels are 0.8% agarose in 0.5 x TBE and run in 0.5 x TBE.
4. Run conditions:

Blocks of runs	I	II	III
Buffer temperature	5.5 °C	5.5 °C	5.5 °C
Run angle	106°	106°	106°
Voltage	3.5 V	3.5 V	3.5 V
Start switch time	500 s	300 s	200 s
Final switch time	700 s	500 s	300 s
Run time	60 h	25 h	8 h

5. Pulse-field gels are then stained with ethidium bromide and a picture is taken under UV-light.
6. Gels are blotted and hybridised using standard Southern blot protocols.