VII. Constructing *Plasmodium berghei* gene targeting vectors using recombineering

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1. Background

This protocol describes a restriction-ligation free method for engineering AT-rich *Plasmodium* DNA using the Red/ET recombination system of bacteriophage lambda (Zhang *et al*., 1998; Wang *et al*. 2006). We here use this method to generate vectors for gene knock-out and 3' tagging in *Plasmodium berghei* (Pfander *et al*. 2011). However, individual modules of the protocol can be combined and extended to generate more complex genetic modification and complementation vectors, even for large *P. berghei* genes. Recombineered vectors have two significant advantages over standard genetic modification constructs: 1) They are produced without PCR-based amplification of *Plasmodium* DNA (AT-rich and highly error-prone), and 2) they display an increased recombination frequency in *P. berghei* due to the augmented length of their homology arms of typically 6-10 kb.

This protocol uses clones from an arrayed large-insert library of (PbG) *P. berghei* genomic DNA, available from the Wellcome Trust Sanger Institute. The library has been end-sequenced and mapped, so individual library clones carrying a given gene of interest (GOI) can be identified and retrieved from frozen stocks. These clones are then converted into genetic modification vectors by using a combination of Red/ET recombineering and Gateway technology. Figure 1 outlines the steps involved. The PbG library currently contains >9000 clones, allowing for the design of knockout (ko) and c-terminal tagging vectors for >90% of all *P. berghei* predicted open reading frames. For PbG library clones and reagents contact plasmogem@sanger.ac.uk.

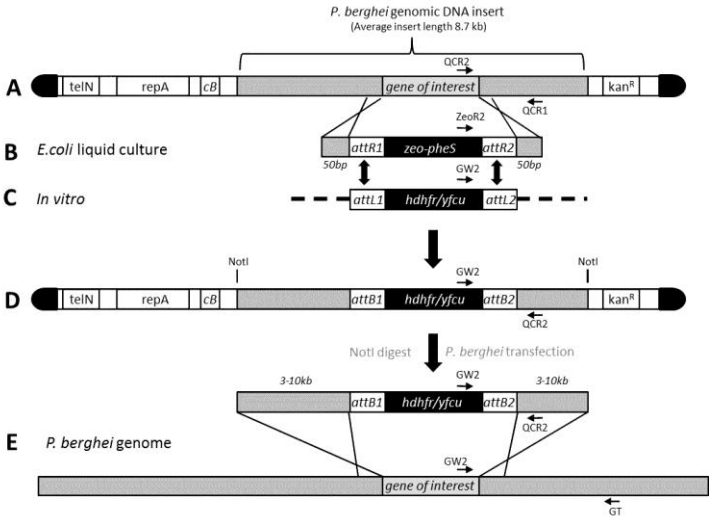

Figure 1. Schematic overview of the generation of *P. berghei* gene targeting vectors using recombineering. The strategy shown is that employed for targeted disruption. For c-terminal tagging the selection marker is inserted directly downstream of the gene of interest, replacing the stop codon. **(A)** Schematic of the pJAZZ vector showing hairpin telomers (black), telomerase gene (*TelN*), replication factor and origin (*repA*), and kanamycin resistance gene (kan^R). The *P.*

berghei genomic DNA insert carries the gene of interest (GOI). (**B**) The GOI is replaced by a bacterial selection (*zeo-pheS*) cassette by recombineering facilitated by 50 bp homology regions flanking the GOI, and present on a PCR product carrying the *zeo-pheS* cassette. (**C**) An *in vitro* Gateway (GW) reaction step exchanges the *zeo-pheS* marker for the *P. berghei hdhfr/yfcu* selection cassette, as mediated by the attR sites associated with the *zeo-pheS* marker, and attL sites flanking the *hdhfr/yfcu* cassette. (**D**) The completed gene targeting vector is prepared prior to *P. berghei* transfection by NotI digestion, which releases the modified *P. berghei* insert from the flanking vector sequences. **(E)** Following transfection, the gene targeting vector facilitates the modification the *P. berghei* genome by integration at the GOI through double homologous recombination.

2. Methodology summary:

A. Electroporation of pSC101gbaA plasmid and subsequent induction of recombineering competency proteins from this plasmid.
B. Electroporation of PCR product carrying the *zeo-pheS* bacterial selection cassette flanked by attR Gateway sites and 50 bp *P. berghei* homology regions. Recombination between homologous sequences is facilitated by the recombineering competency proteins, resulting in the replacement of the GOI with the *zeo-pheS* cassette, and generation of an intermediate vector (here denoted PbG zeo-pheS).
C. *In vitro* Gateway reaction using the pR6K attL1-3xHA-hdhfr-yfcu-attL2 (Pfander *et al*. 2011) as a donor plasmid. Exchange between attR sites on the PbG *zeo-pheS* intermediate vector and attL sites on the pR6K attL1-3xHA-hdhfr-yfcu-attL2 donor mediates the replacement of *zeo-pheS* with the *Plasmodium* positive negative selective marker *hdhfr/yfcu* (Braks *et al*. 2006).

3. Reagents and equipment

Electroporation events are performed using the Gene Pulser Xcell (Bio-Rad) or BTX ECM 630 (Harvard Scientific), and 1 mm electroporation cuvettes (Bio-Rad).

A. Selective media and agar

	Final conc.	Stock conc.	Solvent
Kanamycin	30 µg/ml	10 mg/ml	dH_2O
Tetracycline	5 µg/ml	5 mg/ml	ethanol
Zeocin (Source BioScience)	50 µg/ml	10 mg/ml	dH2O

Terrific broth (TB): For 1 L TB use 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (anhydrous) and 2.2 g potassium dihydrogen phosphate (anhydrous), supplemented with 0.4% glycerol after autoclaving.

YEG-Cl kanamycin plates: Per 1 L dH_2O, add 5 g Yeast Extract, 5 g NaCl, 2 g 4-chloro-DL-phenylalanine and 15 g agar and autoclave. Allow to cool to < 55°C, add 0.4 % (v/v) sterile glucose and kanamycin.

Protocol Update: zeocin and kanamycin plates: Per 1 L dH_2O, add 5 g yeast extract, 5 g NaCl and 15 g agar and autoclave. Allow to cool to < 55°C and add zeocin and kanamycin.

B. Miscellaneous reagents

10% L-arabinose, sterile filtered.
Gateway LR Clonase enzyme (Invitrogen), (kit contains buffer and Proteinase K).

BigEasy-TSA Electrocompetent Cells (SOLOS), (Lucigen).
100µM pore filters for DNA dialysis (Millipore).
Sterile HPLC-grade H_2O is used throughout the protocol.
MilliQ ultrapure H_2O used for media.
Qiaprep Spin Mini Prep Kit (Qiagen).
PCR reagents: Proof-reading polymerase (e.g. Advantage 2 polymerase, Clontech).
2xGoTaq Green master mix (Promega), screening polymerase.
DpnI restriction enzyme.

C. Plasmids

PbG clones: PbG is a *P. berghei* ANKA clone 15cy1 genomic DNA library. The library was constructed using the pJAZZ-OK blunt low copy (five per cell) vector from Lucigen (Ravin *et al.* 2003, Godinska *et al*. 2010). These clones and their derivatives can only be propagated in TSA *E.coli* (ampicillin^R, Lucigen). This vector confers kanamycin resistance.

pSC101gbdA: This plasmid encodes recombination and proofreading activities from lambda phage and recA. This vector converts any *E. coli* strain into a competent strain for Red/ET recombination (Wang *et al.* 2006) and confers tetracycline resistance. The pSC101 origin of replication restricts replication of this plasmid to 30°C. In contrast, 37°C is non-permissive. L-arabinose induction and temperature switching together tightly regulate Red/ET protein expression.

pR6K attR1-zeo-pheS-attR2: The template for the PCR reaction amplifying the *zeo-pheS* selection marker with flanking attR1 and attR2 sites for Gateway recombination. This vector confers tetracycline resistance and can only be replicated in *pir*$^+$ *E.coli*.

pR6K attL1-3xHA-hdhfr-yfcu-attL2: The Gateway donor plasmid that facilitates the replacement of the *zeo-pheS* marker with the *hdhfr-yfcu* casette. This plasmid is available with different flavour tags for c-terminal addition of GFP or 3xHA tags; other tagging vectors are in development. This vector confers tetracycline resistance and can only be replicated in *pir*$^+$*E. coli*.

D. Primers

Primer sequences are available from http://plasmogem.sanger.ac.uk.

Primers for amplification of the *zeo-pheS* selection cassette: Primers contain 50 bp of sequence specific for GOI, followed by 20 bp annealing to the selection cassette.

Primer R1: 50 bp homology region specific for GOI + 5'-aaggcgcataacgataccac-3'
Primer R2: 50 bp homology region specific for GOI (Reverse complement) + 5'-ccgcctactgcgactataga-3'

Primers for amplification of the *wt* GOI allele: Primers are designed to span the insertion site for the *zeo-pheS* alternatively, *hdhfr-yfcu* cassette. Prior to starting, the primer combination below can be used to confirm the presence of the GOI in the PbG clone used as starting material.

QCR1: 20 bp primer annealing within the modified or deleted GOI
QCR2: 20 bp primer annealing outside modified or deleted GOI

Primers for assessment of successful integration of selection cassettes into PbG clone, when used in combination with the gene specific QCR2 primer:

zeo-pheS cassette: ZeoR2 5'-tcattcttcgaaaacgatct-3'
hdhfr-yfcu cassette: GW2 5'-ctttggtgacagatactac-3'

Protocol

***Day 0**. Start PbG clone culture*

0.1 Inoculate PbG clone from glycerol stock into 4.0 ml TB-kanamycin.
0.2 Grow overnight at 37°C, shaking at ~250 rpm.

***Day 1**. Transformation of recombinase plasmid pSC101gbdA*

Before start, chill H_2O and electroporation cuvettes on ice and cool centrifuge to 4°C.

1.1 Dilute overnight cultures to an OD_{600} of 0.05, in 4 ml TB-kanamycin.
1.2 Resume shaking at **37°C** until OD_{600} reaches 0.6-0.8 (check OD_{600} after 2 hours).
1.3 During incubation:
  a. Set up *zeo-pheS* cassette PCR with gene specific homology arms (1.12).
  b. Dilute pSC101gbdA plasmid to 10 ng of plasmid in 50 µl H_2O. Keep on ice.
1.4 When at an OD_{600} of 0.6-0.8, place the tube with the culture on ice for 15 min. Keep cells cold from this point onwards.
1.5 Transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Discard the rest of the culture.
1.6 Spin for 3 min at 5,000 g at 4°C.
1.7 Make cells electrocompetent by sequential washes in ice cold HPLC-grade H_2O:
  1.7.1 Carefully aspirate supernatant
  1.7.2 Wash the cell pellet in 1 ml ice cold H_2O.
  1.7.3 Spin 3 min at 5,000 g in a cold microfuge.
  1.7.4 Repeat 2 more washes (step 1.7.1-1.7.3) with 1 ml ice cold H_2O.

The cell pellet becomes looser with each wash. Make sure cells are drained well after final wash.

1.8 Keeping the tube on ice, re-suspend cell pellet in 50µl of diluted pSC101-gbdA plasmid, and transfer to a chilled 1 mm gap-width electroporation cuvette.
1.9 Electroporate bacteria:
  1.9.1 Settings: BTXECM 630 electroporator (1800 V, 25 µF, 200 Ω), or Bio-Rad Gene Pulser Xcell (1800 V, 10 µF, 600 Ω).
  1.9.2 Immediately add 950 µl of TB, and transfer cells to a 14 ml culture tube.
1.10 Allow cells to recover at **30°C**, shaking at 225 rpm for 70 min. Total volume at this stage is 1 ml.
  DO NOT CULTURE AT 37°C AT THIS STAGE.
1.11 Add 3 ml TB supplemented with kanamycin and tetracycline (final concentration; 30 µg/ ml kan and 5 µg/ml tet), incubate o/n at **30°C** shaking.
  DO NOT CULTURE AT 37°C AT THIS STAGE.

1.12 PCR amplification of *zeo-pheS* cassette with homology arm extensions for recombineering:

1.12.1 PCR reaction

H_2O	15.5µl
pR6K attR1-zeo-pheS-attR2 plasmid template(12 ng/µl)	1.0µl
10x PCR buffer	2.5µl
Primers R1 and R2 (2 µM), each	2.5µl
dNTPs (10 mM each)	0.5µl
AdvantageTaq2 (Clontech), or other proof-reading Taq polymerase	0.5µl

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

1.12.2 Visualise 2.5 µl of PCR product by gel electrophoresis. Expected size is 2.0 kb.

1.12.3 Digest the rest of the PCR reaction with 1 µl of DpnI (stock 20 U/µl) at 37°C for 1 h. This is done to eliminate template plasmid before transformation.

1.12.4 Dialyse PCR product against HPLC-grade H_2O (0.1 µM pore filters, Millipore) for 1h.

1.12.5 Transfer dialysed product from the filter to an Eppendorf tube, quantify DNA by spectrophotometry and store at -20°C.

Day 2. *Transformation of zeo-pheS bacterial selection cassette (recombineering)*

Before start, chill H_2O and electroporation cuvettes on ice and cool bench top centrifuge to 4°C.

2.1 Dilute overnight cultures to an OD_{600} of 0.05, in 4 ml TB with kanamycin and tetracycline.

2.2 Resume shaking at **30°C** until OD_{600} reaches 0.3-0.4 (check OD_{600} after 2 h). DO NOT CULTURE AT 37°C AT THIS STAGE.

2.3 During incubation:

2.3.1 Prepare attR1-zeo-pheS-attR2 PCR product for transformation by diluting 250ng – 1µg of purified PCR product in 50 µl of cold ddH_2O.

2.4 At OD_{600}=0.3-0.4, add 80 µl 10% L-arabinose (0.2% final concentration) and incubate:

2.4.1 In water bath for 5 min at **37°C.**

2.4.2 In shaking incubator for 35 min at **37°C.**

2.5 Chill tube on ice for 15 min. Keep cells cold from this point onwards.

2.6 Transfer 1.4 ml of culture to 1.5 ml centrifuge tube. Discard the rest of the culture.

2.7 Spin for 3 min at 5,000 g at 4°C.

2.8 Make cells electrocompetent by sequential washes in ice cold HPLC-grade H_2O as outlined in step 1.7.

2.9 Keeping the cells on ice, re-suspend cells in 50 µl of diluted attR1-zeo-pheS-attR2 PCR product, and transfer to a chilled 1 mm gap-width electroporation cuvette.

2.10 Electroporate cells as outlined in step 1.9.

2.11 Allow cells to recover at **37°C**, shaking for 70 min. Total volume at this stage is 1 ml.

2.12 Add 3 ml of TB supplemented with zeocin (final concentration 50 µg/ml) and incubate o/n at **37°C** shaking. Protocol Update: Select using zeocin (50 µg/ ml) and kanamycin 30 µg/ ml.

Important note. One of the strengths of recombineering is its high efficiency, which allows selection to take place through serial liquid culture. However, transformations can be plated out at this stage to allow for screening of single colonies. This ensures that only successfully recombineered clones are taken forward to the next step. This approach may be favoured when first establishing the protocol in your lab, or when a particular construct proves difficult to generate.

Single colony screening is thus not routinely necessary, but if it is the preferred method, after step 2.11; first remove 100 µl culture and keep to one side. Spin remaining culture volume for 2 minutes at 6000 rpm, remove ~800 µl of the supernatant and then re-suspend cells in the remaining 100µl. Spread the two dilutions of cells onto pre-warmed zeocin and kanamycin agar plates, incubate o/n at 37°C, then screen individual

colonies by PCR (as outlined in step 3.3) for successful insertion of the *zeo-pheS* cassette. Single *zeo-pheS* positive colonies can then be grown in 2 ml TB-zeocin-kanamycin and the protocol resumed at step 3.1.

Day 3. *Gateway mediated replacement of zeo-pheS selection cassette with hdhfr-yfcu marker*

3.1 Use the Qiaprep Spin Mini Prep Kit (Qiagen) according to manufacturer's instructions to isolate the intermediate vector containing *zeo-pheS.* Use 2 ml culture volume and double P1, P2, N3 buffer volumes to account for growth in rich TB medium. Elute in 50 µl of TE pH 8.0.

3.2 Quantify DNA and visualise PbG *zeo-pheS* intermediate vector by gel electrophoresis. A single, or two very closely migrating, band(s) of >12kb should be present.

3.3 Verify the recombineering reaction by PCR:

3.3.1 PCR reaction

H_2O	6.5 µl
Template (plasmid DNA)	1.0 µl
2xGoTaq Green master mix	12.5 µl
Primer QC2 (Gene specific), (2 µM)	2.5 µl
Primer ZeoR2 (Generic), (2 µM)	2.5 µl

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

3.3.2 Visualise 10 µl of PCR product by gel electrophoresis. The resulting PCR product should migrate as a single band of ~400-800 bp.

3.4 Set up the Gateway LR Clonase reaction in a thin-walled PCR tube:

PbG zeo-pheS intermediate vector (~30 ng/µl)	10.00 µl
pR6K attL1-3xHA-hdhfr/yfcu-attL2 (100 ng/µl)	1.00 µl
LR clonase buffer 5X	4.00 µl
LR clonase enzyme mix	2.00 µl
TE	3.00 µl

3.5 Incubate the Gateway mixture o/n at 25°C in a PCR machine using heated lid function.

Day 4. *Transformation of Gateway product*

4.1 Add 0.5 µl of proteinase K to inactivate Gateway reaction and incubate at 37°C for 10 min.

4.2 Dialyse the Gateway product as described in step 1.12.4.

4.3 Transform 5-10 µl of dialysed Gateway product into 50 µl BigEasy-TSA electrocompetent cells, as described in step 1.9.

4.4 Allow cells to recover at **37°C**, shaking for 70 min. The volume at this stage is 1 ml.

4.5 Remove 100 µl culture, and keep to one side.

4.6 Transfer remaining culture volume to 1.5 ml Eppendorf tubes.

4.7 Spin remaining culture volume for 2 minutes at 6000 rpm, remove ~800 µl of the supernatant, and re-suspend cells in the remaining 100 µl culture medium.

4.8 Spread the two dilutions of cells onto pre-warmed YEG-Cl kanamycin agar plates.

4.9 Incubate plates overnight at **37°C**.

Day 5. *PCR based screening for Gateway hdhfr-yfcu positive PbG clones*

5.1 Pick >4 colonies and perform colony PCR to verify the Gateway reaction:

5.1.1 Pick single colonies and inoculate 4 ml TB-kanamycin and 10 µl H_2O:

5.1.1.1 Grow inoculated selective medium o/n at 37°C for glycerol stocks.

5.1.1.2 Boil 10 µl colony lysate for 10 min at 95°C.

5.1.2 PCR Reaction

H_2O	5.0 µl
Template(colony lysate)	2.5 µl
2xGoTaq Green master mix	12.5µl
Primer QC2 (gene specific), (2µM)	2.5 µl
Primer GW2 (generic), (2µM)	2.5 µl

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

Important note. Verified clones can be prepared for *P. berghei* transfection by growing medium or large scale cultures in TB supplemented with kanamycin. Qiagen Midi or Maxi prep kit can then be used to purify the DNA (using double buffer volumes as described in step 3.1). Prior to transfection, the entire *P. berghei* genomic DNA fragment, which contains the modified GOI and acts as the targeting vector, is released by NotI digestion. *P. berghei* transfections are performed using 1-5 µg NotI digested DNA, purified by standard ethanol precipitation. There is no need to purify the insert away from the pJazz flanking arms that are also released by NotI digestion.

References

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