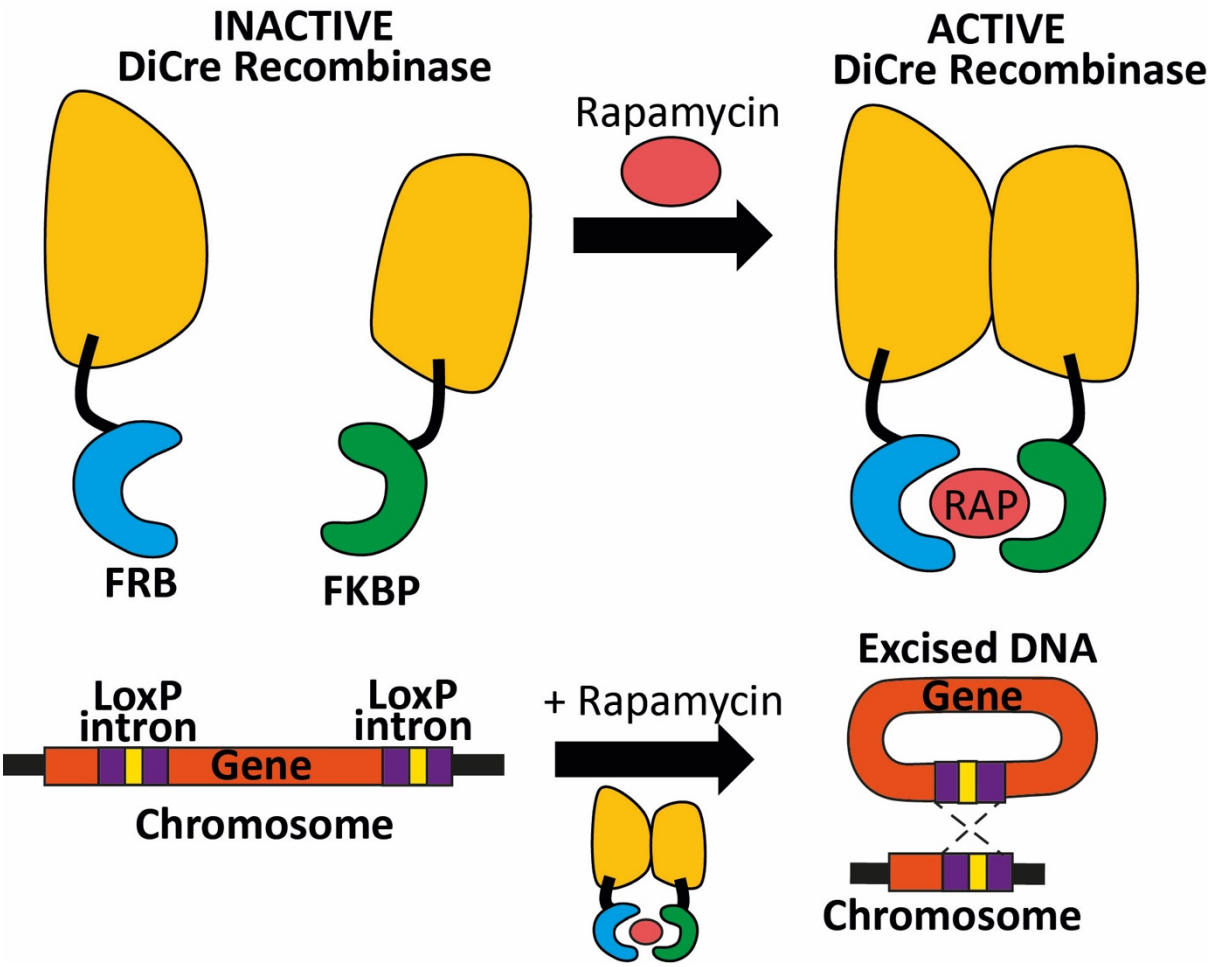
The LoxP system allows for the conditional deletion of a gene or chromosomal region (1). The region is flanked by loxP sequences which can be placed within the *P. falciparum* SERA2 intron (LoxPint), allowing them to be inserted anywhere within a gene of interest. The parasite line expresses two halves of the cre-recombinase, which come together upon addition of rapamycin to create an active recombinase, which excises the region between the two loxP sequences. Our vectors and the NF54 DiCre line are available on addgene.(2,3)

<u>Below you will find:</u>

- **Tips on where to put the LoxPint and designing the plasmid.**
- **Protocols for integrating the sequences into the parasite genome with selection linked integration (SLI) or CRISPR Cas9.**
- **Schematics of different ways to use the LoxPint, including conditional mutation.**
- **Advantages and disadvantages of different methods.**
- **A list of papers using the different methods.**



Why use the LoxPint system for gene/domain deletion

- Conditional – the region between the two LoxP sequences is deleted only upon addition of rapamycin. Conditional knockout is crucial for essential genes and for analysing phenotypes which are affected by parasite life stage (e.g. cell rigidity, phosphorylation, egress, cytoadhesion, etc.)
- Highly efficient – 99% of parasites excise the gene of interest (in some cases the excised plasmid may re-insert into the loxP site but this is entropically unfavourable and therefore very rare).
- Total gene knockout – unlike degron-based methods for conditional deletion, the gene will be 100% deleted.
- Flexible – as the LoxP sequence is placed inside an artificial intron it can be placed anywhere within a gene of interest with no effect on the protein sequence.
- Adaptable – can include various tags, selectable markers and methods of chromosomal editing (selection linked integration or Cas9).

What you need

- Parasite line expressing DiCre (contact us for our NF54-DiCre line which forms gametocytes and is transmissible through mosquitoes).
- Plasmids for Cas9 gene editing or selection linked integration (available on request).
- Compounds of choice for selection (WR and G418 for SLI, WR or Puromycin for Cas9).
- Transfection systems, e.g. AMAXA.

Cas9 Method (4)

- We use a Cas9 plasmid made by Ellen Kneupfer. The plasmid contains the Cas9 nuclease gene, the CRISPR RNA sequence under an U6 promoter, and two back-to-back BbsI restriction sites for inserting the guide sequence. There is a hDHFR-FCU cassette which can be positively selected for with WR, and negatively selected for with Ancotil.
- The repair plasmid contains the homology arms and insertion, which has no drug selection. We use a pMK-RQ plasmid from IDT but any empty vector will do.
- Linearize the repair plasmid with a restriction enzyme which can be heat inactivated, and ethanol precipitate 20ug of the Cas9 plasmid and 3x molar ratio of the repair plasmid for transfection.
- Add 5nM WR99210 to the parasites from one day after transfection, for 4 days.
- WR resistance may then be regained through cloning the parasites or negative selection with ancotil.

Selection Linked Integration (SLI) Method (5)

- We use a modified pARL vector from Tobias Spielmann's lab. It contains a HA tag, the T2A slip peptide, neomycin/G418 resistance cassette, LoxPint and GFP sequence. There is an additional hDHFR cassette for WR resistance. The 5' homology arm, LoxPint, and recodonised sequence are cloned upstream of these.
- Transfect 100ug of this plasmid.
- Add 2.5 nM WR99210 to the parasites from 48h post-transfection, until parasites appear. Once a sample of the WR-selected parasites have been frozen, select with 225µg/ml G418 until a doubly-resistant population appears.

Designing the Plasmid

- Because the loxPint is so AT rich you may struggle to get it synthesised as short DNA sequences (e.g. Gblocks). Some companies will make the sequence, however (e.g. IDT's custom gene synthesis.
- We just PCR amplify the LoxPint sequence using 60bp primers to make it long enough to gel purify, then Gibson/overlap PCR it together with the below fragments.

Homology region

- For SLI use a 5' homology region of at least 450bp, and ideally about 700-800bp.
- For Cas9, use 5' and 3' homology regions of at least 100bp, ideally 300-500bp.
- For SLI, avoid including the start ATG and 5'UTR in the homology region. This may lead to expression of the protein and Neo resistance cassette episomally from the plasmid, reducing the need for integration into the endogenous locus.

Where to insert the 1st LoxPint

- Before region of interest (e.g. kinase domain, essential motif, transmembrane seq.)
- Insert the intron into a common splice junction such as between an AG and a GT. See here for a list of common ones: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3209046/
- Try to avoid another GTA too close upstream of the intron or an AG too close downstream in case of splicing at these points instead.
- Just upstream of Cas9 cut site (within 100bp ideally).
- We don't recommend inserting the loxP into any uncharacterised intron due to the difficulty in predicting branch sites.

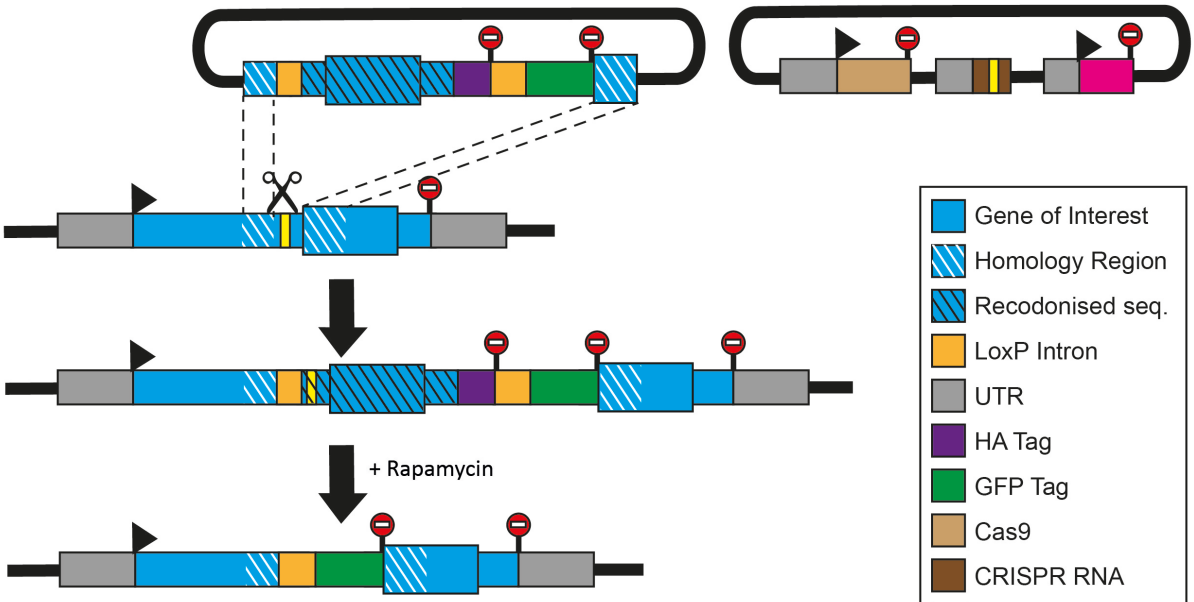
Recodonised region

- Make sure the recodonisation is quite thorough with the manual recodonisation feature here: https://eu.idtdna.com/CodonOpt (Map codons>manual optimization). Change all codons (apart from methionine and tryptophan) to another common *Plasmodium* codon.
- If using Cas9, make sure the guide sequence is either recodonised thoroughly or split by the insertion of the loxP.
- Avoid changing the codons so that the intron splice junction is very rare (see above) or there is an 'AG' too close to the intron, to avoid incorrect splicing.

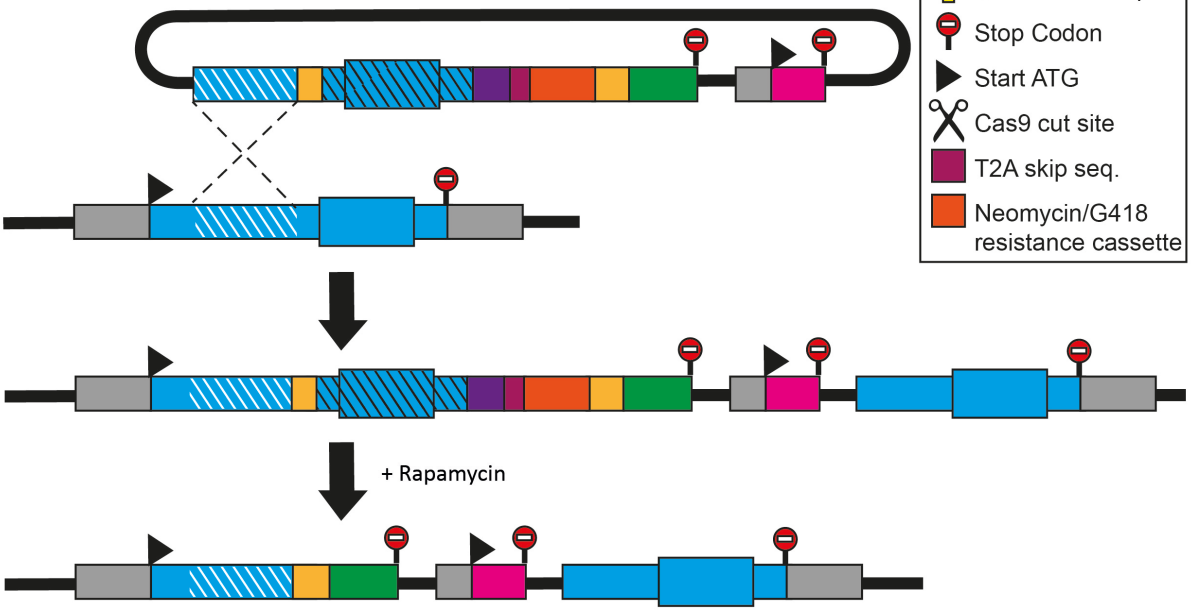
After gene of interest.

- HA tag for western blot/IFA (optional).
- T2A Neomycin - essential for SLI but may also be included when using Cas9 to select for expression of the gene of interest or as an additional selection for positive integration events (optional for Cas9).
- 2nd LoxP intron.
- GFP to come into frame with the N-terminus post-excision. This can confirm correct excision on a single cell level and indicate the localisation of the N-terminus. However, removal of the C-terminus often results in misfolding of the N-terminus and the GFP is rarely visible (optional).
- Recodonised region of interest (WT or mutant) which will come into frame upon rap-treatment. This can give you a conditional mutant or WT complemented line. The region must be recodonised differently to the original recodonisation above.

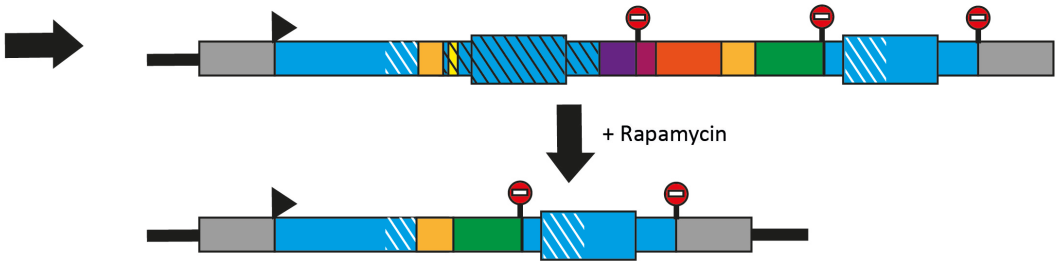
1. Domain Knockout 1 - Cas9 Method *



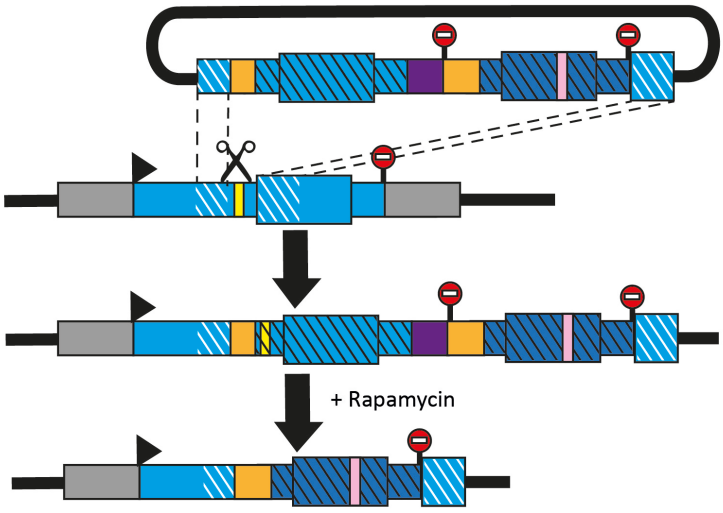
2. Domain Knockout 2 - Selection linked integration (SLI) Method

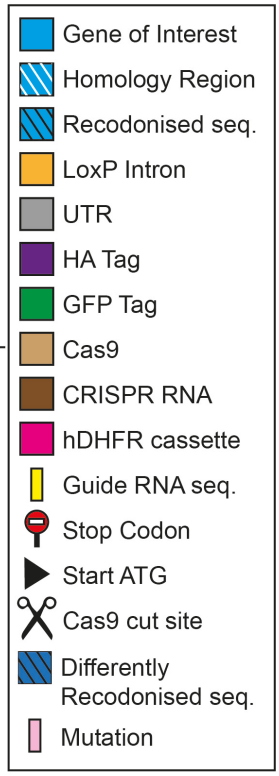


*Optional - Combine selection linked integration (T2A Neo) with the Cas9 method.

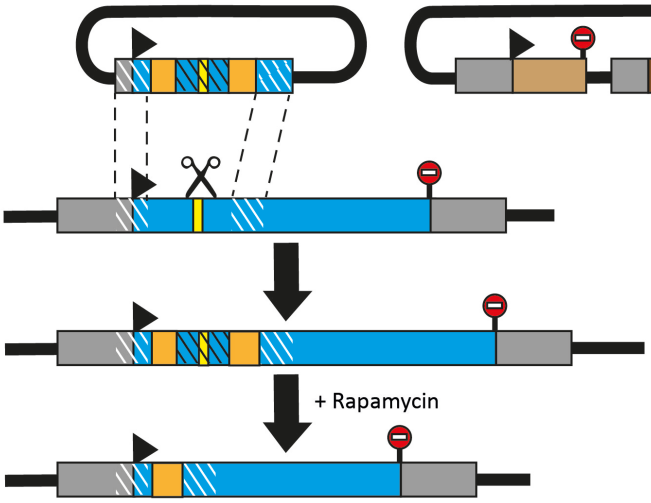


3. Conditional introduction of point mutations



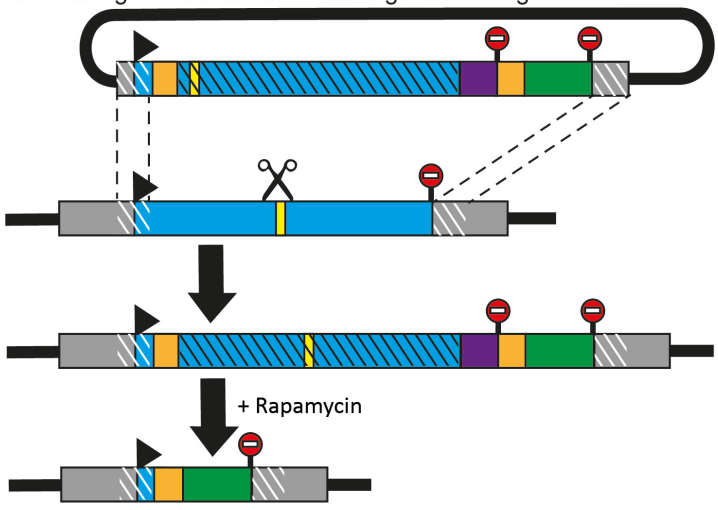


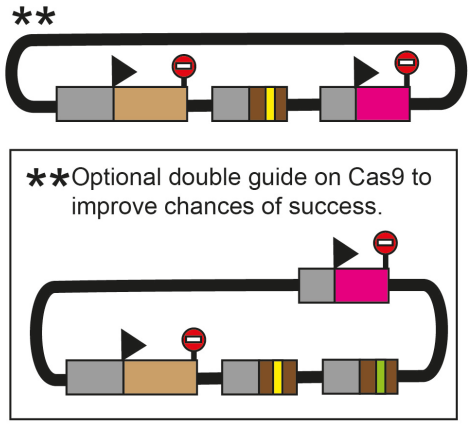
4. Whole gene knockout 1 - frame shift method

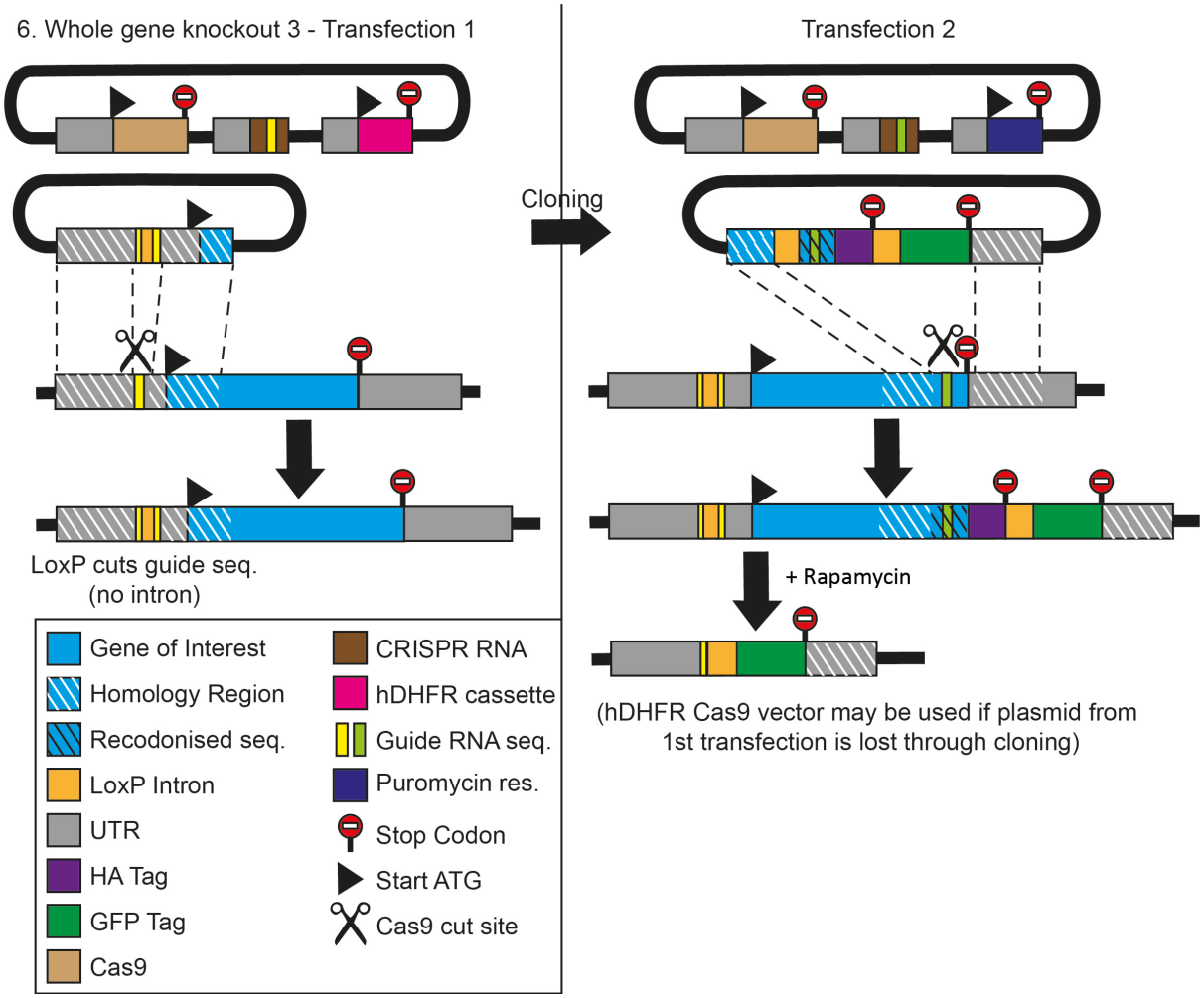


(Out of frame upon excision)

5. Whole gene knockout 2 - whole gene flanking method






6. Whole gene knockout 3 - Transfection 1
Transfection 2
Cloning
LoxP cuts guide seq.
(no intron)
+ Rapamycin
(hDHFR Cas9 vector may be used if plasmid from
1st transfection is lost through cloning)
Gene of Interest
Homology Region
Recodonised seq.
LoxP Intron
UTR
HA Tag
GFP Tag
Cas9
CRISPR RNA
hDHFR cassette
Guide RNA seq.
Puromycin res.
Stop Codon
Start ATG
Cas9 cut site

Method + refs	Advantage	Disadvantage
1 (1) Domain cKO 1 (Cas9)	• Parasites appear in 2-4 weeks • Marker free after cloning or negative selection of Cas9 plasmid. • Short homology arms (100-700bp) • Endogenous protein levels. • Irreversible Integration	• Does not knock out whole gene. • No selection for integration or protein expression (but T2A-Neo can be included if desired) • Protein level may be very low if variably expressed.
2(5–7) Domain cKO 2 (SLI)	• Selection for integration into the genome. • Can select for expression of gene of interest. • Low chances of wild type parasites surviving due to double selection.	• Long homology arms needed (450-1000bp) • Requires two selection steps, first with WR then with G418. • Not marker-free. • Parasites appear in 3-8 weeks. • May be reversible as the duplicated homology region can recombine. • May unnaturally overexpress gene of interest leading to mislocalisation/aggregation. • T2A may not always skip, resulting in about 30% GOI-NEO population. • Cannot place LoxPint within ~450bp of N-terminus.
3(8) cMutate	• Complement deleted domain with mutated sequence.	• Two different recodonisations may be needed.
4 (13) Frame shift cKO	• Knocks out all but the very N-terminus of gene • One transfection • Minimal recodonisation required.	• Cannot include a C-terminal tag.
5 (9–12) Gene flanking cKO	• Knocks out all but the very N-terminus of gene • One transfection • Can use a second Cas9 guide to improve the chances of success. • Can include a tag.	• Requires recodonisation of whole gene - expensive for long genes and may not be possible for repetitive sequences.
6 (14,15) Whole gene Double transfect cKO	• Knocks out entire gene. • Minimal recodonisation required. • Can include a tag.	• Two transfections required, ideally with cloning in-between. • LoxP in 5' UTR may influence protein expression. • May require two Cas9 plasmids with different resistance cassettes if 1st is not lost through cloning/negative selection.

Papers

- Paper detailing the use of the DiCre system in *Plasmodium*:

1. Collins CR, Das S, Wong EH, Andenmatten N, Stallmach R, Hackett F, et al. Robust inducible Cre recombinase activity in the human malaria parasite *Plasmodium falciparum* enables efficient gene deletion within a single asexual erythrocytic growth cycle. Mol Microbiol. 2013 May;88(4):687–701.

- Paper from our Lab detailing the insertion of LoxP sites intro the SERA2 intron:

2. Jones ML, Das S, Beldal H, Collins CR, Blackman MJ, Treeck M. A versatile strategy for rapid conditional genome engineering using loxP sites in a small synthetic intron in *Plasmodium falciparum*. Scientific Reports. 2016 Feb;6:9.

- Our NF54 DiCre line:

3. Tibúrcio M, Yang ASP, Yahata K, Suárez-Cortés P, Belda H, Baumgarten S, et al. A Novel Tool for the Generation of Conditional Knockouts To Study Gene Function across the *Plasmodium falciparum* Life Cycle. MBio. 2019 Sep 17;10(5).

- Details of Ellen Knuepfer's Cas9 plasmid (both WR and Puromycin resistant):

4. Knuepfer E, Napiorkowska M, van Ooij C, Holder AA. Generating conditional gene knockouts in *Plasmodium* – a toolkit to produce stable DiCre recombinase-expressing parasite lines using CRISPR/Cas9. Sci Rep [Internet]. 2017 Jun 20 [cited 2020 May 22];7. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5478596/

- Papers using a method similar to method 2:

5. Birnbaum J, Flemming S, Reichard N, Soares AB, Mesén-Ramírez P, Jonscher E, et al. A genetic system to study *Plasmodium falciparum* protein function. Nat Methods. 2017 Apr;14(4):450–6.

- Our FIKK KO paper, using method 2 and a combination of 1 and 2 (Cas9 with T2A Neo):

6. Davies H, Belda H, Broncel M, Ye X, Bisson C, Introini V, et al. An exported kinase family mediates species-specific erythrocyte remodelling and virulence in human malaria. Nat Microbiol. 2020 Apr 13;

- Paper using a method similar to method 2:

7. Robert-Paganin J, Robblee JP, Auguin D, Blake TCA, Bookwalter CS, Krementsova EB, et al. *Plasmodium* myosin A drives parasite invasion by an atypical force generating mechanism. Nat Commun. 2019 23;10(1):3286.

- A paper detailing mutant complementation using multiple Lox sequences.

8. Koussis K, Withers-Martinez C, Baker DA, Blackman MJ. Simultaneous multiple allelic replacement in the malaria parasite enables dissection of PKG function. Life Sci Alliance. 2020 Apr;3(4).

- Papers using a similar method to method 5:

9. Suarez C, Lentini G, Ramaswamy R, Maynadier M, Aquilini E, Berry-Sterkers L, et al. A lipid-binding protein mediates rhoptry discharge and invasion in *Plasmodium falciparum* and Toxoplasma gondii parasites. Nat Commun. 2019 06;10(1):4041.

10. Henrici RC, Edwards RL, Zoltner M, van Schalkwyk DA, Hart MN, Mohring F, et al. The *Plasmodium falciparum* Artemisinin Susceptibility-Associated AP-2 Adaptin μ Subunit is Clathrin Independent and Essential for Schizont Maturation. mBio. 2020 25;11(1).

11. Bui HTN, Niederwieser I, Bird MJ, Dai W, Brancucci NMB, Moes S, et al. Mapping and functional analysis of heterochromatin protein 1 phosphorylation in the malaria parasite *Plasmodium falciparum*. Sci Rep. 2019 13;9(1):16720.

12. Hill RJ, Ringel A, Knuepfer E, Moon RW, Blackman MJ, van Ooij C. Regulation and Essentiality of the StAR-related Lipid Transfer (START) Domain-containing Phospholipid Transfer Protein PFA0210c in Malaria Parasites. J Biol Chem. 2016 Nov 11;291(46):24280–92.

- A paper using a similar method to method 4:

13. Sherling ES, Perrin AJ, Knuepfer E, Russell MRG, Collinson LM, Miller LH, et al. The *Plasmodium falciparum* rhoptry bulb protein RAMA plays an essential role in rhoptry neck morphogenesis and host red blood cell invasion. PLoS Pathog. 2019;15(9):e1008049.

- Papers using a similar method to method 6 (either multiple transfections or inserting the lox sequence into the promoter):

14. Patel A, Perrin AJ, Flynn HR, Bisson C, Withers-Martinez C, Treeck M, et al. Cyclic AMP signalling controls key components of malaria parasite host cell invasion machinery. PLoS Biol. 2019;17(5):e3000264.

15. Boonyalai N, Collins CR, Hackett F, Withers-Martinez C, Blackman MJ. Essentiality of *Plasmodium falciparum* plasmepsin V. PLoS ONE. 2018;13(12):e0207621.