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ABSTRACT

The advent of the CRISPR / Cas9 technology permits the targeted editing of mosquito genomes, ranging from site-directed mutagenesis of genes of interest yielding knockout mutations (which arise by insertion / deletion of a few nucleotides) to site-specific insertion of exogenous DNA sequences such as fluorescence markers or even large gene drive cassettes, themselves encoding the components of the CRISPR / Cas9 system.

To obtain these heritable targeted changes, genome editing requires the delivery of Cas9 protein and its guide RNA(s) to the developing germ tissue of an embryo. Different species require adaptation of this basic principle to accommodate for their specific biology. Here, we describe a technical pipeline based on delivering the CRISPR/Cas9 components in the form of injected plasmid or as transgenes, resulting in highly efficient gene editing in *Anopheles* malaria vector mosquitoes. We have reliably employed these methods to mutagenize > 20 different loci of interest in *Anopheles coluzzii* to date.

Introduction

Elucidating the function of genes of interest in model organisms has greatly benefited from the study of knockout mutants. In the fields of mosquito biology and mosquito vector / pathogen interactions, candidate mosquito genes arising from genetic or transcriptomic screens, or from mining genomic data, can now be further characterized by mutagenesis. For example, a mutant phenotype resulting in higher pathogen loads will reveal a pathogen-antagonistic function of the disrupted mosquito gene (antiviral, antiparasitic), while reduced pathogen loads will denote an agonistic (pro-pathogen) function (Smidler et al., 2013; Dong et al., 2018). Following its publication as a programmable genome editing tool in 2012 (Jinek et al., 2012) the CRISPR / Cas9 system has enabled precise targeted modifications of mosquito genomes in a manner unthinkable until then, supplanting other alternatives then in development such as TALENs (Aryan et al., 2013; Smidler et al., 2013) and Zinc Finger Nucleases (DeGennaro et al., 2013; Liesch et al., 2013; McMeniman et al., 2014).

Beyond creating simple knockout mutants, gene editing can also consist in inserting desired sequences into a locus of choice, such as docking sites enabling subsequent ΦC31 integrase-mediated transgene integration; a fluorescence marker to track a mutation; or even the larger DNA sequences necessary to encode the components of a gene drive (Gantz et al., 2015; Hammond et al., 2016; Kyrou et al., 2018). Further applications, such as the substitution of alleles; or fluorescent protein / epitope tagging of endogenous genes in their native genomic contexts to enable imaging / biochemistry analyses, are also within reach using variations of currently established protocols, and are under development (e.g., Chen et al., 2020).

We and others have developed CRISPR/Cas9 tools for editing *Anopheles* mosquito genomes (Hammond et al., 2016; Gantz et al., 2015; Dong et al., 2018). While embryo microinjection of recombinant Cas9 protein and synthetic gRNAs allows successful gene editing in several insect species (including *Anopheles stephensi* and *Aedes aegypti*: Gantz et al., 2015; Kistler et al., 2015), we prefer delivering the CRISPR / Cas9 components by transgenic expression, or in the form of injected expression plasmids, as commonly performed in *Drosophila* (e.g., Gratz et al., 2015). The advantages of this DNA-only approach are (i) the stability of plasmid-based injection mixes, compared to the uncertainty on the stability of RNA-protein complexes or stored batches of Cas9 protein; (ii) to alleviate the labor and cost of gRNA synthesis and the precautions that are necessary when handling RNA; (iii) to confine Cas9 expression to developing germ cells thanks to germline-specific promoters such as *vasa* (Papathanos et al., 2009), which are expected to reduce somatic tissue mutagenesis in injected individual and the associated fitness loss if targeting essential genes (Quinn et al., 2021). Finally, we empirically observed that the success rate of gene editing in *Anopheles* is superior when injecting plasmids rather than recombinant Cas9 and synthetic gRNA, perhaps due to a longer temporal window of Cas9 and gRNA expression upon plasmid injection, increasing genome exposure to the mutagenic activity.

Here we describe a kit of plasmids, deposited at Addgene (Addgene #173668—173675 ; #174530—174538), and a plasmid assembly protocol based on Golden Gate Cloning (Engler and Marillonnet, 2014) for easy construction of gRNA expression vectors and of knock-in

plasmids for efficient integration of desired sequences into a chosen locus in the *Anopheles* genome. These tools can be used for CRISPR-Cas9 genome editing in the following ways:

- introduce small insertions / deletions (indels) in the target genomic locus of choice (e.g., olfaction genes in Hinze et al., 2023; Pelletier et al., 2023). This approach exploits the non-homologous end joing (NHEJ) DNA repair pathway. Resulting mutants must be genotyped by PCR to establish mutant lines;

- insert a transgenic cassette carrying a fluorescence marker gene, such as GFP, within the target genomic locus, causing gene loss-of-function if inserted within coding sequences (e.g., Kyrou et al., 2018; Kalita et al., 2023; Krzywinska et al., 2023). This approach exploits the DNA repair pathway mediated by homologous recombination. Compared to the above approach, this one has the great advantage of coupling the mutation with a visual selection marker, alleviating the screening process thanks to fluorescence selection (Marois, 2023) rather than labor-intensive genotyping PCRs. Besides a fluorescence reporter gene, the inserted cassette may contain additional genetic cargo, such as the Cas9 and gRNA expressing genes themselves in the case of gene drive constructs (e.g., Carballar-Lejarazú et al., 2020; Green et al., 2023).

- insert a fluorescence marker gene, such as GFP, downstream of an endogenous promoter in order to generate a transcriptional fusion and characterize the expression pattern of that promoter (e.g., the *Saglin* gene in Klug et al., 2022). The GFP reporter may also be inserted in-frame within a coding sequence, resulting in the expression of a fluorescently-tagged fusion protein, as was reported for an *Aedes* Cadherin gene (Chen et al., 2020) enabling imaging studies of the subcellular localization of the protein of interest.

- knock out the expression of a chosen gene in a tissue-specific manner, by crossing mosquitoes expressing gRNAs to mosquitoes expressing Cas9 in desired tissues under the control of a tissue-specific promoter. This is particularly useful when the goal is to knock out a gene that is essential during development, which cannot be achieved by heritable mutagenesis as loss-of-function mutations will be lethal (Keller Valsecchi et al., 2021; Mela-Lopez, Marois and Blandin, manuscript in preparation).

The procedure outlined here focuses on the making of necessary plasmids for CRISPR/Cas9 genome editing. Injecting these plasmids in the embryos of a Cas9-expressing transgenic mosquito line, or together with a Cas9 expressing helper plasmid, will result in genome editing without a requirement for purified Cas9 protein or synthetic gRNAs, hence it is a "DNA-only" based approach. All necessary plasmids have been made available from Addgene (Table 1).

For the process of micro-injection in *Anopheles* eggs and transgenesis, refer to Fuchs et al., 2013; Pondeville et al., 2014; Volohonsky et al., 2015; Carballar-Lejarazú et al., 2021.

Materials

Reagents

Appropriate plasmid vectors (Addgene numbers listed in Table 1)

*Bbs*I (or *Bpi*I), *Bsa*I (or *Eco*31I) restriction enzymes and their buffers

PCR reagents (polymerase, dNTPs, buffers)

T4 DNA ligase

ATP (10 mM stock solution)

TE buffer (10 mM TRIS pH 7.5-8, 1 mM EDTA)

Scr7 (APExBio, make 50 mM stock solution in DMSO then 50 μM in H_2O)

Equipment

Thermocycler

Electrophoresis chamber and UV imager for DNA analysis

Standard equipment for *Escherichia coli* transformation

DNA sequence analysis software

Insectary equipment for mosquito breeding

Micro-injection platform for transgenesis

Fluorescence binocular microscope

Table 1: Plasmid vectors available from Addgene for mosquito CRISPR applications

Purpose	*Plasmid name*	**Bsa*I* *overhangs**	*Addgene #*	*Properties*
Vector to clone Golden Gate Cloning modules (such as 5' and 3' RH), lacks any *Bsa*I site in the backbone	pKSB-	-	62540	Ampicillin resistant, blue/white selection, pBluescriptKSII+ with endogenous *Bsa*I site removed. Clone *Bsa*I-flanked PCR products in the MCS (using standard or Gibson cloning methods)
Destination vectors to assemble multiple inserts by Golden Gate Cloning	pENTRR4 ATCC LacZ GCTT	ATCC GCTT	173668	Kanamycin resistant, blue/white selection, Assemble multiple inserts by Golden Gate Cloning using the ATCC, GCTT BsaI cassette. No fluorescence marker in the backbone.
	pDSAG pDSAT pDSAY pDSAR pDSARN pDSAP	ATCC GCTT	62289 62290 62291 62292 62295 62293	Kanamycin resistant, blue/white selection, Assemble multiple inserts by Golden Gate Cloning using the ATCC, GCTT *Bsa*I cassette. Backbone has *att*B site and selection marker cassette (G = 3xP3-GFP, T= 3XP3-mTurquoise, Y = 3xP3-YFP, R = 3xP3-DsRed, RN= 3xP3-DsRedNLS, P = OpIE2-pac (puromycin resistance) (Volohonsky et al., 2015)
	pPiggyBac ATCC LacZ GCTT lox AttP	ATCC GCTT	173496	Kanamycin resistant piggyBac vector for random insertion transgenesis. Assemble multiple inserts by Golden Gate Cloning, using the ATCC GCTT *Bsa*I cassette.
gRNA cloning vectors	pKSB- gRNA1 pKSB- gRNA2 pKSB- gRNA3 pKSB- gRNA4 pKSB- gRNA 2/3	ATCC GGAA GGAA AGAG AGAG AACA AACA GCTT GGAA AACA	173671 173672 173673 173675 173674	Ampicillin resistant, contain a U6-gRNA expression template, clone protospacer linkers in the *Bbs*I sites (overhangs CCTT GTTT)
Fluorescent marker modules	pKSB- 3xP3-YFPsv40 pKSB- 3xP3-GFPsv40 pKSB- 3xP3-DsRedsv40		174530 174531 174532	Ampicillin resistant, promoter—fluorescent marker modules can be

	pKSB- 3xP3-mTurqSV40 pKSB- attP3xP3-GFPsv40 pKSB- PUb-NLSmTurqsv40 pKSB- PUb-GFPsv40 pKSB- PUb-GFPTub56D pKSB- PUb-DsRedsv40	GGGG AAGA	174533 174538 174535 174536 174537 174538	used as selection markers in knock-in experiments with 3xP3 (universal) or *Aedes aegypti* PUb promoter (not for *Anopheles*)
Cas9 transgenesis and helper plasmids	pDSAY-vasa-Cas9 pDSARN-vasa-eSpCas9	-	173669 173670	Kanamycin resistant helper plasmids for Cas9 expression or for docking site transgenesis (contain *att*B site)

* For simplicity and to avoid confusion, *Bsa*I overhangs are always indicated as the 5'-overhang nucleotides of the top DNA strand

PROCEDURE

1. Choosing the gRNA targets

Manually scan the coding sequence of your gene of interest on either DNA strand for gRNA target sites matching the sequence: **G**$(N)_{17\text{-}19}$NGG.

G*$(N)_{17\text{-}19}$ is the protospacer motif that defines the specificity of Cas9 cutting, a double-stranded break occurring between the fourth and third nucleotides preceding NGG. The protospacer motif may be 18 to 20 nucleotides in length, with shorter gRNAs potentially increasing specificity (Fu et al., 2014). The underlined NGG constitutes the CRISPR Protospacer Adjacent Motif (PAM), without which no Cas9 cleavage can occur. In the protospacer, the first **G** (bold) will also serve as the transcription start for RNA polymerase III (PolIII) in the gRNA expression vectors, which are based on a PolIII-specific U6 promoter that uses **G** as transcriptional start. If no suitable gRNA target beginning with a G can be found, it is theoretically possible to add an ectopic G at the beginning of the protospacer without compromising efficiency, though we have never tested this in Anopheles.*

Discard potential target sequences harboring poly-T stretches (4 Ts or more), which mimick a PolIII transcription terminator and could decrease gRNA transcription.

It may be preferable to also discard potential targets with strongly unbalanced GC contents; we favor targets with a balanced nucleotide composition and ending with G or Cs before the PAM. All four possible Cas9 PAMs (AGG, GGG, CGG, TGG) work efficiently in Anopheles*.*

Select gRNA target sequences best positioned relative to the location in the gene where Cas9 cleavage is desired.

For Approach 2 below, PCR genotyping of mutants may be greatly facilitated by positioning the gRNA on the recognition site of a restriction enzyme, which will be mutated as a result of gene editing.

Run the best-positioned target sites in a bioinformatics analysis program such as CRISPOR (Haeussler et al., 2016) or Cas-OFFinder (Bae et al., 2014), to discard potential guides matching off-target sites elsewhere in the genome.

In our empirical experience based on sequencing predicted off-target sites in subsets of mutant mosquitoes, putative off-target sites harboring two mismatches or more remain intact despite exposure to Cas9/gRNA and are not a concern. Systematic investigations of off-target activity in Anopheles suggest that it is generally very low (Garrood et al., 2021). To further decrease the risk of off-target mutations, some of the tools utilized here (Cas9-expressing mosquito line R9-BC8, helper plasmid pDSARN-vasa-eSpCas9sv40) express the more stringent eSpCas9 mutant (Slaymaker et al., 2016).

Retain 3 (up to 4 for simple mutagenesis) different gRNAs targeting the locus of interest.

Early experiments have shown that a single gRNA is usually sufficient. The number of gRNAs can be restricted to 1 or 2 especially if off-target mutagenesis is to be minimized. However, multiplexing the gRNAs protects against the risk of failure due to the unlucky selection of an inefficient gRNA, or to the existence of unexpected target site polymorphism in the chosen mosquito strain. For simple knock-out mutagenesis, several gRNA targets can be distributed along the coding sequence; though concentrating them at the beginning of the gene may better ensure full disruption by generating early frameshifts (unless alternative ATG translation start sites exist downstream). gRNA multiplexing is particularly interesting for tissue-specific knockout of target genes, as combinations of multiple mutations ensure the production of null mutants in the majority of cells.

For insertion of an exogenous sequence (Approach 1 below), the gRNA targets should be clustered at the desired insertion region.

If generating indel mutations is the goal (Approach 2 below), closely spaced gRNAs may stimulate deletion of the intervening sequence, whereas longer spacing between gRNAs may favor NHEJ repair of each cut independently.

We do not pay particular attention to the gRNA efficiency scores predicted by bioinformatics programs, since in our experience about 9 gRNAs out of 10 will be efficient in Anopheles regardless of predictions. Some guides predicted to be efficient may not work, and vice-versa. We hypothesize that inefficiency is primarily due to poor accessibility of target DNA to the Cas9 protein, dependent on nucleosome and chromatin structure. This hypothesis is strongly supported by our observation that a gRNA targeting a fluorescence marker showed an efficacy ranging from 0 to 100 % depending on the genomic position of that marker across a library of transgenic lines. For tissue-specific knockout applications, it is also possible that the target site of a given gRNA may be accessible to Cas9 in some cell types, but not in others, due to cell type-specific chromatin packaging.

2. Designing oligonucleotide linkers for gRNA expression

Once all protospacer sequences have been selected, purchase two complementary oligonucleotides to generate a linker corresponding to each protospacer.

The NGG PAM must be absent from this linker.

The two primers must be complementary to each other over the 18 to 20bp protospacer nucleotides. Additionally, to allow linker cloning in the BbsI restriction sites of our gRNA expression vectors, they should harbor the following overhangs:
5'-CCTT (followed by the 18-20 nucleotides preceding the PAM)
5'-AAAC (followed by 18-20 nucleotides complementary to the above)
Here is one example:

*5'-cctt**G**ATTCCACGCACAGTCGACC*
*5'- aaacGGTCGACTGTGCGTGGAAT**C***

which will anneal as:

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5'-cctt G ATTCCACGCACAGTCGACC
        CTAAGGTGCGTGTCAGCTGGcaaa-5'
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Each gRNA expression linker is cloned into an individual gRNA-expression plasmid module (pKSB-gRNA$_{1-4}$, Addgene #173671—173675) to express single guide RNAs in mosquitoes. It is helpful to prepare a scheme of the final desired Golden Gate assembly, based on Figures 1 and 2, to decide in which plasmid each gRNA linker will be cloned.

These steps are in preparation for Golden Gate Cloning plasmid assembly described in Step 6, below. The pKSB-gRNA$_{1-4}$ modules contain a standard gRNA-coding template composed of the widely used CRISPR-tracr fusion (Jinek et al., 2012), under control of an Anopheles coluzzii U6 promoter (AGAP013557) within a pBluescriptKSII+ vector backbone, in which we mutated the endogenous BsaI site (Addgene #62540). We thus created a series of 5 plasmids in which the gRNA expression template is flanked by two BsaI restriction sites.

To insert the target-specific sequence encoding each gRNA, two *Bbs*I restriction sites allow directional ligation of the linker as follows:

3. Ligating protospacer linkers into the gRNA expression vectors

- Prepare a 100 µM stock solution of each primer. Combine 10 µl of each of two linker primers together into a new 1.5 ml tube and add 80 µl of TE buffer.

- Denature the mixture for 3-4 minutes at 95°C. Return it slowly to room temperature to allow proper linker annealing.

This constitutes a 10 µM stock of annealed linker.

- Pepare a 333x dilution of this linker by mixing 2 µl of the 10 µM stock with 664 µl of H2O.

- In a PCR tube, mix:

    10 ng pKSB-gRNA of a given plasmid (Addgene #173671—173675)

    1 µl BbsI buffer

    1 mM ATP

    1 µl diluted linker

    0.6 µl BbsI enzyme (*Bbs*I can be substituted for its isoschizomer *Bpi*I)

    0.4 µl T4 DNA ligase

    Complete to 10 µl with H2O

Place the tube in a thermocycler and run the linker ligation program:

    37°C 10 min (favors *Bbs*I digest)

    20°C 10 min (favors ligation)

    Repeat 3-10 times from step 1

    37° 5 min

    72°C 15 min (to denature ligase).

- Add 5 µl of a mix containing: 0.5 µl *Bbs*I buffer, 0.5 µl fresh *Bbs*I enzyme, 4 µl H2O

Incubate 30 min at 37°C to re-open unreacted clones still harboring *Bbs*I sites (sabotage reaction).

- Transform 5 µl of the resulting reaction in *Escherichia coli* competent cells. Plate on LB plates supplemented with ampicillin (100 µg/ml).

Usually this cloning reaction is very efficient and yields at least one good clone out of two miniprepped and sequenced E. coli *clones.*

The next steps are dependent on which of Approach 1 or Approach 2, below, is chosen.

4. Approach 1: Assembling a plasmid for combined gRNA expression and repair template for knock-in

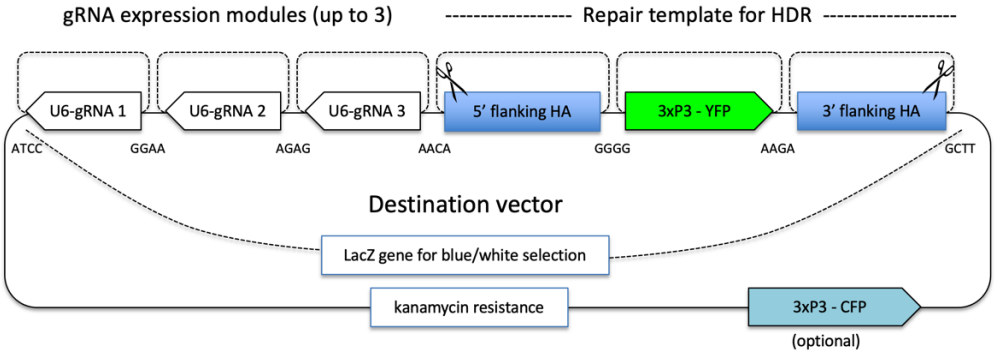

Figure 1: Plasmid map of the gRNA expression and repair template vector for injection into Cas9-expressing mosquito embryos. Inserts (gRNA expression modules, regions of homology, fluorescence marker) are assembled from ampicillin-resistant plasmid modules or PCR products in the destination vector by Golden Gate Cloning (GGC) using *Bsa*I sites whose variable 4-base overhangs are indicated (top strand nucleotides). The single-tube restriction-ligation GGC reaction removes a LacZ cassette allowing blue-white screening, resulting in white positive colonies. Ampicillin-resistant backbones that are lost upon the GGC reaction are represented as dashed lines. The number of gRNAs may be reduced to 2 by short-circuiting the assembly with module pKSB- gRNA 2/3 (Addgene #173674), or to 1 by short-circuiting the assembly with a short linker. HA = homology arms to promote homologous recombination-mediated repair of the genomic Cas9 break. Scissors show the position of gRNA target sites that can be incorporated at the extremities of the HAs to promote plasmid linearization by Cas9 upon injection. U6-gRNA1, 2, 3 plasmids are available from Addgene (#173671—3) as well as destination vectors (#173668; 62289—95). A fluorescence marker in the backbone of the vector, different from the marker in the repair template, can help identify non-canonical integration events that integrate the entire plasmid in the mosquito genome.

The plasmid assembly presented here (Figure 1) yields a single vector for gRNA expression and for DNA repair template delivery, to be injected in Cas9-expressing mosquito embryos. Mosquitoes having integrated a fluorescence marker in the target sequence will be identified in the progeny of the injected mosquitoes by screening for fluorescent individuals. An advantage of this single vector strategy is to ensure that all mosquito cells receiving injected material simultaneously possess all ingredients mediating homology directed repair, i.e., gRNA expression units and repair template, preventing the possible segregation of co-injected plasmids into different cells, observed previously (Volohonsky et al., 2015). Alternative approaches are possible, such as cloning of the gRNA expression cassette(s) on a Cas9-expressing plasmid for injection into wild-type embryos (e.g. Quinn et al., 2021) along with a DNA repair template provided on a different plasmid or as synthetic DNA. The strategy adopted here is optimized for injection in mosquito embryos transgenically expressing Cas9, and also works with some efficiency when Cas9 is expressed from a helper plasmid (Addgene #173669).

PCR amplify 5' flanking and 3' flanking homology arms (HA):

Design PCR primers to amplify about 1000 bp of genomic sequence on either side of the Cas9 cut site(s), excluding the gRNA recognition sequences or at least their PAMs.

Like in other model systems (e.g. C. elegans, Paix et al., 2015) shorter HA may suffice, but we have so far not attempted to reduce HA size below 800-1000 bp. HAs should preferably be devoid of internal BsaI restriction sites for best performance during Golden Gate Cloning.

For subsequent Golden Gate Cloning assembly into the vector, the PCR primers are designed to contain appropriate BsaI restriction sites at their extremities. The variable sticky BsaI overhang sequences are dictated by the order in which all inserts should sequentially assemble as shown in Figure 1, i.e. compatible with the gRNA$_3$ module on the left side, and with the fluorescence marker in the middle.

In addition, we incorporate a target site for one of the gRNAs, along with its PAM, on the 5' extremity of the 5'RH PCR product, and / or on the 3' extremity of the 3'RH product (red asterisks in Figure 1). Although we have not systematically tested the necessity of this, it is intended to promote linearization of the injected plasmid by Cas9 after injection, yielding a relaxed repair template that may more easily take part in the break repair process. In addition, these cuts in the injected plasmid may reduce the frequency of whole-plasmid integration, including plasmid backbone and gRNA-coding units, which is a commonly encountered event.

In summary, the forward primer for the 5'HA should have the following structure:

GGTCTCN*AACA*G(N)$_{17\text{-}19}$CGG + annealing region

underlined bases being the recognition site of one of the guides, including a CGG PAM, Bsa*I restriction site in bold generates a 5'-AACA overhang compatible with the preceding insert in the Golden Gate Cloning assembly.*

Alternatively (or in addition), the reverse primer for the 3'HA can contain a gRNA target site:

GGTCTCN*AAGC*G(N)$_{17\text{-}19}$CGG + annealing region.

Besides the presence of the underlined bases corresponding to the recognition site of one of the guides including PAM, the Bsa*I restriction site (bold) will generate an overhang compatible with the 5'-GCTT overhang on the right side of the destination vector in the Golden Gate assembly.*

The 5'HA reverse primer, as well as the 3'HA forward primer, should be designed with correct *Bsa*I sites at their extremities, the overhangs of which are dictated by compatibility with the fluorescence marker cassette, for example (Figure 1):

5'HA reverse: 5'-**GGTCTC**N*CCCC* + annealing region

3'HA forward: 5'-**GGTCTC**N*AAGA* + annealing region.

We have deposited a small library of fluorescence markers at Addgene, each including promoter, transcription terminator, and the *Bsa*I overhangs shown in the above example

(Addgene #174530—174538). Besides the *3xP3* promoter that is particularly useful in *Anopheles* mosquitoes and a number of other insects, this library of plasmids also includes fluorescence markers under control of the *Aedes aegypti Polyubiquitin* (*PUb*) promoter, to facilitate the development of similar approaches in other species (of note, the *Aedes* PUb promoter unfortunately does not work well in *Anopheles*). One GFP marker module (Addgene #174534) additionally contains an *attP* docking site, so that knock-in mosquitoes can be used as docking strains for subsequent PhiC31 integrase-mediated transgenesis.

The PCR products of the 5' flanking and 3' flanking RH may be purified and used as such in the Golden Gate cloning reaction (section 6 below), or cloned in a plasmid and sequence-verified prior to the Golden Gate Cloning reaction, if introducing PCR mutations at the knock-in site must be avoided. For PCR cloning of these PCR products, we employ the Takara In-Fusion® cloning kit using the *Bsa*I site-free pBluescript derivative pKSB- (Addgene #62540) as destination vector, which we amplify by PCR. This implies to further extend the 5' sequence of each primer to display 15 bp of homology to the extremities of the PCR amplified pKSB-. Alternative PCR cloning options are also valid, such as cloning blunt PCR products in the *Sma*I, *Eco*RV, or *Ecl*136II sites of pKSB-.

Once these flanking HA modules have been obtained, they should be assembled together with the gRNA expression modules and fluorescence marker module in a destination vector such as pENTR ATCC-LacZ-GCTT (Addgene #173668) which is marker-free, or in a vector of the pDSAG/T/Y/R series (Volohonsky et al., 2015, Addgene #62289—62292). Proceed to section 6.

5. Approach 2: Assemble a transgenesis plasmid for expression of 1, 2, 3 or 4 gRNAs

The purpose of this simpler transgenesis plasmid, depicted in Figure 2, is to produce indel mutant mosquitoes by crossing a mosquito line expressing Cas9 in its germ line to a second transgenic mosquito line ubiquitously expressing one or several gRNAs. Mutagenesis will occur in F1 mosquitoes. Those will be back-crossed to wild-type, which separates Cas9 from the gRNAs if both transgenes are in the same docking site, and heritable mutations are identified by PCR in the progeny of the backcross (Figure 3).

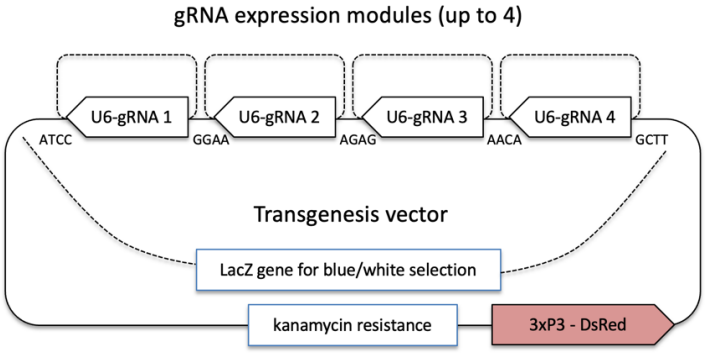

Figure 2: Plasmid map of a gRNA expression vector for transgenesis and subsequent mosquito line crossing to a Cas9-expressing line. The fluorescence marker of the transgenesis vector (here 3xP3-DsRed)

should preferably be different from that in the Cas9 expressing line. The number of gRNAs can be reduced to 3 by short-circuiting the assembly with module pKSB- gRNA 2/3, or further reduced by short-circuiting the assembly with a short linker.

A variant of Approach 2 is to generate the same gRNA-expressing plasmid and transgenic mosquito line, and cross it to a second line expressing Cas9 in a restricted tissue of choice. This will result in tissue-specific gene knockout in the F1 progeny. For example, the germ line can be mutagenized using *vasa-Cas9* expressing mosquitoes as fathers in the cross (Keller Valsecchi et al., 2021). Conversely, when crossing males of the gRNA-expressing line to females expressing Cas9 from the vasa promoter, F1 embryos undergo somatic mutagenesis in all or most embryonic cells due to maternal deposition of Cas9 protein and/or mRNA. If the target gene is essential for viability, this results in embryo lethality. The reverse cross (Cas9 males x gRNA females) results in later lethality (mainly during metamorphosis and adult emergence from the pupa), this time due to some degree of leaky expression of the vasa promoter in a subset of somatic cells. For tissue-specific knockout of the target gene in tissues other than the germ line, transgenic strains expressing Cas9 in the desired tissue(s) will be necessary.

The gRNA expression modules are assembled by Golden Gate Cloning as in Approach 1, but leaving out DNA repair template modules. The destination vector must be a transgenesis plasmid. For docking site mediated transgenesis, plasmids of the pDSAG/T/Y/R series (Volohonsky et al., 2015, Addgene #62289—62292) are ideal, with their built-in fluorescence selection markers. A plasmid for piggyBac transgenesis is also available (Addgene #173496), but a selection marker must be added during Golden gate assembly. The fluorescence marker gene of the destination vector should be chosen to be different from that in the Cas9-expressing transgenic line, in order to better identify trans-heterozygous F1 mosquitoes and to discriminate each transgene in subsequent generations when establishing mutant lines.

In case additional DNA sequences should be included in the final plasmid assembly, each of these can be prepared as a PCR product or plasmid module harboring appropriate *Bsa*I sites at its extremities. For tips on the design of *Bsa*I overhangs for Golden Gate Cloning, see Box.

Box 1: tips for BsaI overhang design for additional Golden Gate cloning:

- When designing *Bsa*I modules, creating a methylation site overlapping the *Bsa*I recognition or cleavage sequence should be avoided. The *dcm* methylation sequence is CC(A/T)GG; the *dam* methylation consensus is GATC.

- In a single Golden Gate cloning reaction, do not combine several overhangs that are reverse complement of each other: this would produce unwanted irreversible ligations.

- Do not use any palindromic overhang (they would allow irreversible head-to-head self ligation of a given insert).

- Overhangs used for a given assembly reaction should be as different as possible to each other, to avoid cases of illegitimate ligations.

- the *Bsa*I site in ampicillin resistant plasmid backbones has a CGGT overhang. Avoid this overhang when constructing modules and prefer using the *Bsa*I-free ampicillin resistant pKSB- to clone modules (Addgene #62540).

- A Golden Gate cloning assembly can be closed using a short linker with appropriate four-base overhangs, made by annealing two complementary primers.

- Prepare dilutions of all plasmid and PCR product modules at 40 fmol/µl using the formula:

plasmid size x 0.0264 = ng/µl for 40 fmol/µl

- Combine them in a single PCR tube :

40 fmol of destination plasmid

40 fmol of each module

1 mM ATP

2 µl BsaI buffer

1 µl ligase

1 µl BsaI enzyme

Complete to 20 µl with H_20

- Place the PCR tube in a thermocycler and perform the following cycle :

Step1 : 37°C for 5 min *(this step favors* Bsa*I cutting)*

Step 2 : 20°C for 5 min *(this step favors ligation)*

Repeat 10-30 times from Step 1

Step 9 : 20°C for 50 min

Step 10 : 50°C for 20 min

In this sabotage step, Bsa*I recuts unreacted plasmids —omit the sabotage step in case one insert contains an internal* Bsa*I site!*

Step 11 : 75°C 20 min *(this step inactivates ligase —unnecessary if one insert contains an internal BsaI site)*

12°C forever

- Transform 5 µl of this Golden Gate reaction mixture into competent *E. coli* cells. Use blue-white screening to exploit the destination plasmid's *LacZ* cassette, screen white clones by colony PCR using primers spanning two modules, miniprep 5-10 positive colonies, and verify plasmid structure by restriction digest and sequencing. From a validated colony, grow a 100 mL culture in LB medium supplemented with kanamycin, and purify the plasmid DNA using an endotoxin-free midiprep kit according to the manufacter's instructions. Proceed to embryo micro-injection.

7. Next steps: micro-injection, mosquito crosses and screening

The gRNA expression plasmids described above must be injected (at a DNA concentration of 400 to 500 ng/µl) in Cas9-expressing *Anopheles* embryos for Approach 1, or in an *Anopheles att*P docking line along with a PhiC31 integrase-coding helper plasmid (e.g., Addgene #62299) for Approach 2 (Fuchs et al., 2013; Pondeville et al., 2014; Volohonsky et al., 2015). In approach 1, we include 2 µM of the drug Scr7 in the injection mix, which potentially boosts homologous recombination repair by inhibiting ligase IV involved in non-homologous end joining (we haven't compared efficiency in the presence and absence of Scr7).

We have started to distribute two transgenic lines of *Anopheles coluzzii* expressing Cas9 under the control of the *vasa* promoter. One (called YC9), in the G3 background of *An. gambiae x coluzzii*, expresses the native form of Cas9 codon-optimized for human expression (Cong et al., 2013). We subcloned the *Cas9* gene under control of the *A. gambiae vasa2* promoter (Papathanos et al., 2009) in transgenesis vector pDSAY (Volohonsky et al., 2015) to obtain a YFP-expressing transgenic line with Cas9 inserted on chromosome 2 (Dong et al., 2018; Keller Valsecchi et al., 2021) in the X1 docking locus (Volohonsky et al., 2015). The second line (called R9-BC8), in the Ngousso *An. coluzzii* background, is almost identical except that its Cas9 version is eSpCas9 (Slaymaker et al., 2016), engineered to reduce off-target activity, and that the selection marker is DsRedNLS from transgenesis vector pDSARN (Volohonsky et al., 2015). We haven't noticed any strong difference in efficiency of mutagenesis or knock-in recovery between the two lines. The two Cas9 expression vectors used to generate these lines, along with their DNA sequences, have been deposited at Addgene (#173669, #173670) to enable the generation of additional transgenic lines in other desired genetic backgrounds for which attP docking lines have been established. In addition, these vectors can also be used as helper plasmids for co-injection with the gRNA plasmid in wild-type mosquitoes, alleviating the need to generate novel Cas9-expressing lines. Although we prefer injecting Cas9-expressing lines, the helper plasmid approach is also working (e.g., Quinn et al., 2021 in *Anopheles funestus* and we have used it successfully in *Anopheles arabiensis* for fluorescent marker knockin).

In both approaches 1 and 2, mosquitoes undergoing mutagenesis are back-crossed to the wild-type. In Approach 1, progeny expressing the knocked-in fluorescence marker will be readily identified by screening groups of neonate larvae under the fluorescence microscope (Marois, 2023). Positive larvae can be further backcrossed to amplify the desired genetic change and establish mutant lines, each backcross being an opportunity to dilute out potential off-target mutations while positively selecting for fluorescence. Proper integration of the transgene at the predicted site should be verified by genotyping using PCR primers binding in and outside the cloned sequences. Inadvertent co-integration of the plasmid backbone frequently occurs due to unexpected recombination events during DNA repair, and should also be verified by genotyping PCRs. A fluorescence marker in the plasmid backbone, expressing a different color, will greatly help identify such events. When inadvertent integration of the gRNA-expressing backbone occurs (which can generate a split gene drive in combination with the Cas9 transgene), the desired event can still easily be obtained in the next generation by crossing once more to the Cas9 line, which triggers gene conversion of the newly inherited wild-type locus, this time most frequently using the correct homology arm

for recombination. The desired event is often characterized by a decrease in marker gene fluorescence compared to mosquitoes carrying the entire plasmid backbone.

The crossing scheme used to recover small, unmarked mutations from Approach 2 is outlined in Figure 3. We prefer to cross gRNA-expressing female mosquitoes to males expressing Cas9, in order to minimize somatic mutagenesis due to maternal Cas9 deposition. Among F1 mosquitoes, it is again preferable to use males (undergoing mutagenesis in their germ line) in a backcross to wild-type females. If performing the reverse backcross, a new round of mutagenesis will frequently occur in those zygotes inheriting the gRNA transgene, due to maternal deposition of Cas9, which might lead to confusion in identifying mutations because of the appearance of several novel mutations (somatic and/or heritable) in the backcross progeny.

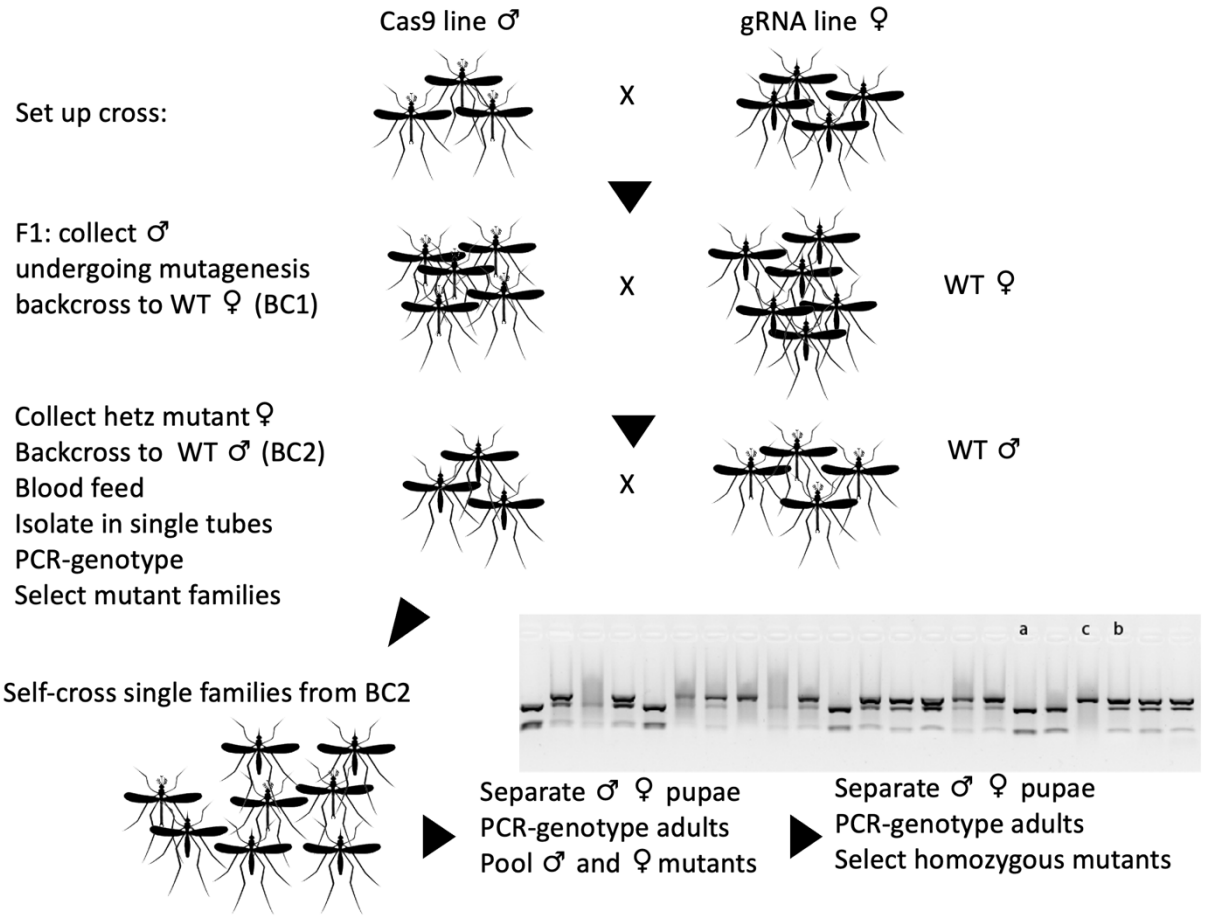

Figure 3: crossing scheme to create and isolate unmarked *Anopheles* mutants. Cas9-expressing males are crossed to gRNA-expressing females (F0 cross). F1 males undergoing mutagenesis in their germ line are crossed to wild-type females (Backcross 1). Progeny candidate mutant females are backcrossed to wild-type males (Backcross 2), individually isolated to lay eggs and genotyped. The gel inset shows genotyping PCR products digested with a restriction enzyme whose restriction site is mutated in the target gene, **a**-type products represent the fully-digested wild-type, **b**-type heterozygous individuals, and **c**-type homozygous mutants. Chosen "monoclonal" families from individual mutant females are self-crossed to amplify the mutation. PCR

genotyping of the progeny is used once to enrich for mutants, once more in a subsequent generation to homozygotise the mutation.

Next, the most convenient way to isolate pure mutant families is as follows (Figure 3): backcross putative heterozygous mutant females engendered by F1 males to new WT males *en masse*. Blood feed these females until they are fully engorged. A second blood feeding 3 days later may help increase the proportion of females laying eggs. 3 days after blood feeding, isolate each female to lay her eggs in individual tubes (Drosophila vials of length 6.4 cm, diameter 2.5 cm) provided with wet filter paper around the sides and 1.5 mL of water. Extract DNA and perform genotyping PCR on those females that laid eggs, using PCR primers that span the target region. Send the PCR product for Sanger sequencing. Successfully mutagenized females will yield two overlapping sequence chromatograms from the point of the mutation on, expected in the vicinity of the gRNAs' PAM. The mutant sequence can be deduced by subtracting the WT sequence from the chromatogram, a task that is easily performed manually. Mutations resulting in small insertions/ deletions that change the reading frame may be preferentially selected, as mutations resulting in frame shifts and premature stop codons are more likely to disrupt gene function. Mutations that result in a restriction site polymorphism may be preferred, to facilitate PCR genotyping of the mutants by combining PCR with restriction digest analysis. For this, positioning the gRNA targets on existing restriction sites can be planned at the step of gRNA design. Alternatively, deletion mutants producing a significant size difference in the PCR product may be chosen.

Selected progenies from single females carrying a desired mutation are amplified for one generation. To homozygotise the mutation, we separate male and females offspring in distinct cages at the pupal stage and leg-genotype about 96 individual males and females using the Phire Direct Animal Tissue kit (ThermoFisher) on one leg (a hind leg for females, a middle leg for males) while keeping each mosquito alive in a Drosophila tube kept in a humid atmosphere to avoid desiccation (genotyping should be completed within 24h to avoid mortality, or a piece of filter paper wet with 10% sugar can be put in each vial). Alternatively, to minimize mosquito damage, genotyping PCR can be performed on pupal cases post emergence of isolated adults.

After the identified heterozygous mutant males and females are pooled and allowed to mate, a similar genotyping experiment must be repeated in the following generation, now to select for homozygous mutants[1]. A sufficiently large number of mosquitoes must be screened, as reproductive success in *Anopheles* is favored by a critical mass of male mosquitoes (swarming behavior). In addition, fitness costs conferred by some mutations can complicate mutant line amplification.

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[1] At this step, it can be relevant to also isolate a wild-type control mosquito line using the non mutant mosquitoes identified during genotyping. This control line and the mutant share a similar genetic background, will carry the same Cas9 off-target mutations if any exist, and therefore represents the best negative control for phenotypic characterization of the mutant.
Mutant and control line will still carry one of the two transgenes used for mutagenesis, i.e. Cas9 or gRNA, if both were initially inserted in the same docking site (which allows to separate them more easily and avoid further rounds of mutagenesis). This residual transgene can be counter-selected by fluorescence screening in subsequent generations, or retained to visually identify mutant and wild-type mosquitoes in experiments where mutants should be mixed with wild-type controls.

As these mutant selection steps by PCR genotyping are tedious, we now strongly favor Approach 1, whereby a selection marker is used instead of PCR to track and homozygotise the mutation. Fluorescence-based selection can be greatly facilitated by automated fluorescence sorting of neonate mosquito larvae, permitting to rapidly sort large numbers of homozygous, heterozygous mutant and control mosquito populations (Marois, 2023; Marois et al., 2012). In the case of a fluorescently tagged mutation, it may even become unnecessary to homozygotise the mutant line, as homozygous mutant and negative controls can be extracted punctually for the needs of an experiment. This approach has the added benefit of avoiding any genetic drift between mutant and control mosquitoes, here maintained within a single population. Genetic drift may otherwise confound phenotype analyses, especially when establishing mutant and control lines from a small number of founder mosquitoes.

Nevertheless, a main advantage of Approach 2 is its very high efficiency. Depending on the target locus and gRNAs, we have obtained mutagenesis efficiencies ranging from 20 to 100%, the latter value being frequently reached when using more than one gRNA. Approach 2 plasmids can also be extremely useful for tissue-specific gene inactivation, by crossing gRNA-expressing transgenic mosquitoes to mosquitoes expressing Cas9 in a tissue of choice.

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