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CRISPR–Cas9 Methods and Key Considerations in the Production of *Aedes aegypti* Mutant Strains

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Abstract

Since the characterization of the CRISPR–Cas9 system in prokaryotes, it has become the prime choice in gene editing because of its exceptional flexibility, ease of use, high efficiency, and superior specificity. As a result, CRISPR–Cas9-mediated gene-editing technologies have enabled researchers not only to engineer transgenic animal strains with site-directed insertions more efficiently but also to generate desired mutants for previously intractable species. One such species is the invasive yellow fever mosquito, *Aedes aegypti*, which is notorious for its ability to transmit many blood-borne human pathogens. Methods for developing new transgenic strains of the yellow fever mosquito may aid in the effort to control its populations and provide significant benefits for the public. Here, we provide an overview of injection and noninjection methods for generating transgenic mosquitoes and also high-light important experimental design features.

BACKGROUND

Gene Editing with CRISPR–Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR) is a class of bacteriophage-derived DNA sequences found in the genomes of prokaryotic organisms (Jinek et al. 2012). Prokaryotes use CRISPR sequences coupled with an RNA-guided DNA endonuclease enzyme called CRISPR-associated protein 9 (Cas9) to recognize and cleave foreign DNA fragments complementary to the CRISPR sequences (Jinek et al. 2012). The CRISPR–Cas9 system has enabled a wide range of gene editing applications since its functional characterization. During editing, the endonuclease Cas9 first forms a ribonucleoprotein complex with a single-guide RNA (sgRNA)—an RNA segment complementary to a 20-bp-long genomic DNA sequence. Next, this ribonucleoprotein complex induces a double-stranded DNA break at the targeted genomic locus. The DNA break then activates the endogenous cellular repair machinery, which repairs the break via one of the following two main mechanisms: end-joining (EJ) repair or homology-directed repair (HDR) (Capecchi 1989; Takata et al. 1998; Hefferin and Tomkinson 2005). The EJ repair mechanism is error prone and tends to incur small base pair insertions or deletions

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(indels), whereas HDR promotes high-fidelity repairs, as it uses DNA templates with homology to the breakpoint to repair breaks, thereby facilitating site-specific insertion of desired exogenous DNA fragments.

CRISPR–Cas9-Based Gene Editing in *Aedes aegypti*

The yellow fever mosquito, *Ae. aegypti*, is one of the world's most widespread mosquito species. It is notorious for its ability to transmit many blood-borne human pathogens, such as yellow fever, dengue, Zika, and chikungunya viruses (Powell 2018). Due to the significant public health burden caused by *Ae. aegypti*, effective control of its populations has long been desired. The CRISPR–Cas9 system offers potential genome engineering solutions for reducing *Ae. aegypti* populations, which may prevent the spread of various pathogens (Li et al. 2020a, 2021b).

Several CRISPR–Cas9-based gene-editing methods have been developed for *Ae. aegypti* and several other mosquito species (Kistler et al. 2015; Li et al. 2017, 2018, 2020b, 2021a; Gantz and Akbari 2018; Bui et al. 2020). Based on differences in the Cas9 endonuclease and sgRNA delivery to mosquitoes, these methods can be grouped into the following two categories: injection-based methods and non-injection-based methods.

The injection-based methods involve the direct delivery of the CRISPR–Cas9 system components (e.g., sgRNAs, Cas9, and donor DNA) into mosquito embryos via microinjection (Kistler et al. 2015). During this process, the injection mixture containing all or some of the CRISPR–Cas9 editing components is injected into early mosquito embryos during the presyncytial blastoderm stage while the embryo is one large multinucleated cell. It is worth noting that when wild-type mosquito embryos are used for injection, the injection mixture needs to include all components, whereas if Cas9-expressing mosquito embryos are used for injection, only non-Cas9 components are required (e.g., sgRNAs and donor DNA if HDR-mediated insertion is desired). Importantly, the injected components can be of different forms. Specifically, either recombinant Cas9 protein or purified Cas9 mRNA can be used as the Cas9 component. The sgRNA sequence encoding an in vitro–transcribed single-stranded RNA or double-stranded DNA plasmid (generated from a U6 backbone plasmid) can be used as the sgRNA component (Li et al. 2021a). If HDR repair is desired, donor DNA must be included as the third component for embryonic microinjection. Donor DNA containing sequence homology adjacent to the cut site can be supplied as short single-stranded DNA (ssDNA) oligonucleotides or plasmid DNA.

In addition to embryonic injection, an alternative injection method called receptor-mediated ovary transduction of cargo (ReMOT control) can also induce heritable germline gene editing in mosquitoes without the need to inject embryos (Chaverra-Rodriguez et al. 2018). In the ReMOT control method, an injection mixture containing CRISPR–Cas9 system components is instead injected into the thorax of blood-fed adult mosquito mothers, which results in gene editing in the mothers' ovaries, ultimately producing transgenic progeny. ReMOT control has been applied successfully for generating heritable EJ mutations in several mosquito species; however, it has not yet been adapted for inserting DNA via HDR (Chaverra-Rodriguez et al. 2018).

In the non-injection-based methods, both Cas9 and/or sgRNA are genetically encoded in the mosquito genome. Cas9 is expressed under the control of promoters specific to the germline or soma, and sgRNAs are expressed by small nuclear RNA promoters that are expressed ubiquitously. Editing can be achieved by crossing genetically encoded sgRNA-expressing mosquitoes with Cas9-expressing mates, and their offspring thus inherit both components of the CRISPR–Cas9 system and are capable of very high rates of self-editing (Li et al. 2017, 2020a). The transgenic Cas9-expressing and/or sgRNA-expressing mosquito strains are usually generated beforehand via the direct delivery method. Depending on project needs, some of the transgenic mosquito lines (such as Cas9-expressing mosquito strains) may be readily available to the research community and work quite efficiently (we have several such strains available in house for interested parties, Li et al. 2017), whereas others (such as mosquitos expressing an sgRNA of interest) may need to be generated using the embryonic microinjection method.

Here, we focus on the embryonic injection-based method that uses genetically encoded Cas9 mosquito strains to generate site-directed mutants/HDR-mediated insertions, as it is the foundational method that can accommodate most project requirements about the generation of mutant *Ae. aegypti* lines.

KEY PROJECT PLANNING DECISIONS

Repair Mechanism

Two predominating DNA mechanisms are used to repair DNA breaks generated by the CRISPR–Cas9 system, namely, the EJ and HDR mechanisms. EJ repair is generally error prone and induces small base-pair repairs that can be a deletion, an imprecise insertion, or point mutations (Hefferin and Tomkinson 2005). This mechanism is suitable when the only desired feature in generated mutant lines is a simple disruption of a target gene in an unspecified way.

The HDR mechanism is required if precise editing, such as a precisely mutated sequence or precise insertion of certain DNA fragments, is desired. In addition, HDR allows for the inclusion of a positive marker (e.g., 3xP3-dsRed), which enables fast postediting genotyping and screening. During HDR-based repair, the cellular repair machinery uses a donor DNA fragment as the template for repairing double-stranded breaks on the genome (Capecchi 1989). The desired sequence provided by the donor DNA template is inserted into the genome at the breakpoint, leading to precise editing of the targeted gene.

Whether DNA is repaired by EJ or HDR is heavily dependent on the cell cycle. For example, the HDR mechanism required for donor DNA insertion is favored in certain stages of meiosis and nearly absent in other stages (Branzei and Foiani 2008). Therefore, the timing of Cas9 expression or delivery is important for increasing HDR efficiency, which is typically very low in *Ae. aegypti*. High HDR editing efficiency has been reported in certain species, such as *Anopheles stephensi*, offering hope for the future improvement of the HDR technique (Gantz et al. 2015). Many research groups are working on methods to improve HDR efficiency to promote more accurate HDR-mediated genome editing by manipulating the cell cycle, adding or stimulating factors that support HDR, or inhibiting factors that

promote EJ (Liu et al. 2018; Li and Margolis 2021; Manoj et al. 2021); however, most of these new methods are still proof-of-concept and would need further development and validation in *Ae. aegypti*.

CRISPR–Cas9 Component Forms

Cas9 may be supplied in the injection mixture either in the form of a recombinant protein (enzyme), which offers the freedom to work in arbitrary genetic backgrounds, or in the form of mRNA. Alternatively, genetically encoded Cas9-expressing mosquito embryos can be used for injection, avoiding the need to include Cas9 in the injection mixture altogether. Both the Cas9 protein and Cas9 mRNA can be generated in-house or purchased commercially. Although both forms provide reliable editing efficiency, they are challenging to produce in-house. Also, the Cas9 mRNA is more unstable than the Cas9 protein. Using the genetically encoded Cas9-expressing mosquito embryos directly for injection, in contrast, offers reproducible high editing efficiency compared to using Cas9 mRNA and Cas9 protein directly (Li et al. 2017). The challenge is that generating true-breeding Cas9-expressing mosquito lines is time-consuming and nontrivial. Many of these lines, however, are readily available to the research community, allowing researchers to streamline their efforts. As there is a limited need to customize Cas9 sequences or expression for most applications, using Cas9 mosquito embryos is the recommended method to achieve consistent high editing efficiency.

The sgRNA may be provided in the injection mixture as RNA or as a DNA plasmid. Of the two options, the RNA form tends to deliver higher editing efficiency. The RNA form can also be rapidly synthesized either in-house or via commercial vendors, but it is generally unstable and requires −80°C storage and minimal freeze–thaw handling, which means additional effort, care, and cost for projects. A DNA plasmid of sgRNAs offers good stability but suffers from lower editing efficiency. In addition to these two options, it is possible to use genetically encoded sgRNA-expressing mosquito embryos for injection with Cas9 solutions or by crosses with Cas9-expressing mosquitoes. Yet, generating such transgenic lines is time-consuming and different projects often require different sgRNA designs, such as target sequence and copy number of the selected sgRNAs. It is also common for a project to undergo several rounds of sgRNA design before an optimal version is chosen. Therefore, it is more cost effective to use sgRNA in the form of either an RNA or DNA plasmid.

HDR-mediated insertions require a third component, namely, a donor DNA template. This template can be designed in the form of either ssDNA or DNA plasmids. The ssDNA can be rapidly synthesized, and its size is usually limited to ~200 nt (Bier et al. 2018; Kanca et al. 2019), which can impede the insertion of many genes and markers. In comparison, DNA plasmids do not have size limitations and are more stable, but they take longer to generate. Similar to the choice of sgRNA, donor DNA templates can also be provided via genetically encoded transgenic mosquitoes, although the efforts involved are significant.

Target Site Selection and sgRNA Design

Target site selection to generate mutations in the *Ae. aegypti* genome first requires choosing a target gene region and then identifying putative target sites within the target gene region. The choice of a target gene is usually project specific. The target genes can be selected using the *Ae. aegypti* reference genome and/or reference genomes of other closely related species (other mosquito species or *Drosophila melanogaster*). Confirmation of the selected genes' transcription profile in *Ae. aegypti* is recommended and can be achieved by performing NCBI-BLAST searches against available *Ae. aegypti* transcriptional databases (Vector Caltech, vectorbase.org/vectorbase/app; Akbari et al. 2013). Once target genes are defined, sgRNA target sites within the target gene sequence can be selected by scanning both the sense and antisense strands for 20-nt-long sequences that include an NGG sequence (either inspect visually or use available software) (Xie et al. 2014; Labun et al. 2016). The NGG sequence is called a protospacer-adjacent motif (PAM) sequence, N represents any of the four nucleotides (A, T, C, and G), and G refers to guanine. Online sgRNA design programs are also available, such as CHOPCHOP V3.0.0 and CRISPR software, to aid in the design of effective sgRNAs (Labun et al. 2016).

We recommend using NCBI-BLAST or BLAT to check for any potential off-target effects of the sgRNAs (Bhagwat et al. 2012; Stover and Cavalcanti 2014). Select the most specific sgRNAs within the specified target regions with the least potential off-target binding sites. In addition, to increase the chance of successful editing, it is recommended to design several different sgRNAs for each target gene and coinject these different sgRNAs during microinjection.

Once the target sequences are chosen, one can design the sgRNAs. An sgRNA consists of the following two segments: a scaffold sequence and target sequence. The scaffold sequence is kept constant, whereas the target sequence needs to have the following features: (1) a 20-nt sequence complementary to the selected target site sequence, (2) NGG present at its 3′ end, and (3) guanine padding at its 5′ end if it does not already have two guanines as a T7 RNA polymerase-preferred initiation motif.

Donor DNA Design (for HDR Only)

The donor DNA plasmid and genetically encoded template components (for HDR-mediated insertion) minimally contain the following three elements: user-defined DNA insertion fragment, a marker sequence, and two homology arms of 500–1000 bases flanking the target cleavage site. For gene knockout experiments, it is common to insert a DNA fragment (typically a fluorescent protein) into the open reading frame of the target gene (Basu et al. 2015; Kistler et al. 2015; Li et al. 2017). The marker sequence is used to track transgenesis, which is optional if the DNA insertion fragment already includes a fluorescent marker. For the homology arms, one arm end should be near the cleavage site, and the other arm should exclude the six nucleotides 3′ to this cleavage site (inclusive of the PAM). The sgRNA sequence is often left out of the donor arms. In addition to the basic framework discussed here, more sophisticated tricks in donor plasmid design, such as in-frame fusion, have also been reported using HDR (Fig. 1; Shankar et al. 2021).

The ssDNA templates, sometimes referred to as single-stranded oligo donors, facilitate HDR-mediated insertions of small DNA fragments, such as short peptide tags or base pair edits, but require only short homology arms, typically of the same length (Renaud et al. 2016). These templates are routinely designed so the insertion site and double-stranded break site are in close proximity (<10 nt), which was shown to improve HDR efficiency (Elliott et al. 1998; Yang et al. 2013). In *Ae. aegypti*, this approach generated low frequencies of somatic and germline modifications (Kistler et al. 2015) and has also been used to modify the expression of endogenous genes in this same organism (Ling et al. 2017).

CONCLUSION

The CRISPR–Cas9 system offers exceptional flexibility, ease of use, high efficiency, and superior specificity for editing DNA sequences on almost any genomic location. As a result, it has been used in numerous species, of which many were previously intractable for genome engineering, including *Ae. aegypti*. One of the important applications for the CRISPR–Cas9-mediated gene-editing technologies is generating mutant strains. Here, we provided a method overview of CRISPR–Cas9-based *Ae. aegypti* mutant strain generation and discussed some of the key design considerations based on our experience. In the accompanying protocol (see Protocol: **Generating *Aedes aegypti* Mutant Strains with Transgenic Cas9** [Sun et al. 2023]), we present a detailed step-by-step CRISPR–Cas9-based genome-editing procedure for inducing desired heritable edits in *Ae. aegypti*. Notably, this protocol describes a ready-to-use method that accommodates most of the common needs for mutant generation while serving as a starting point for further customization for more sophisticated project requirements. Using this tool in mosquitoes will help researchers unravel the genes important for basic physiological functions and also may prove useful for generating innovative population control strategies that can leverage genetically encoded CRISPR reagents such as gene drives (Champer et al. 2016; Li et al. 2020a; Raban et al. 2020; Verkuijl et al. 2020) and precision-guided sterile insect techniques (Kandul et al. 2019; Li et al. 2021b) to develop transformative technologies for preventing the spread of deadly mosquito-borne pathogens.

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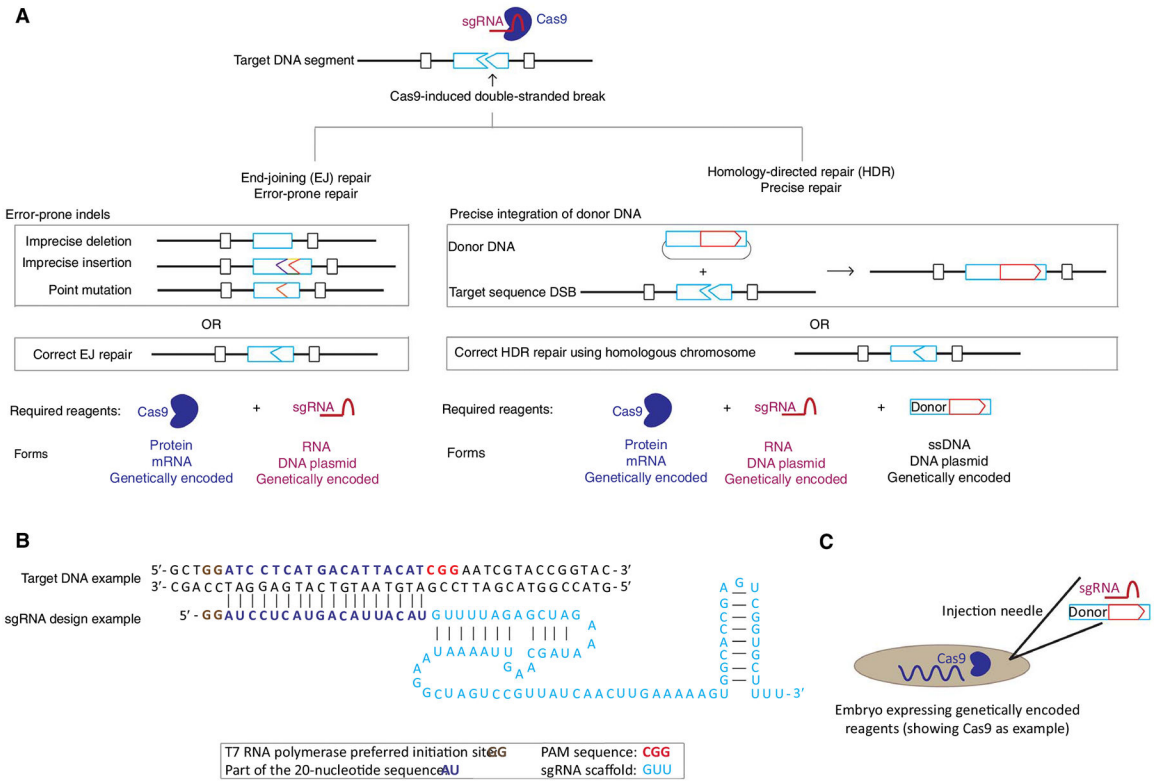

FIGURE 1.

Basic principles in using CRISPR–Cas9 to generate mutant mosquitoes. (*A*) The repair mechanisms and their required reagents enabled by the CRISPR–Cas9 system. After Cas9-induced genome cleavage, the double-stranded break is repaired by either end-joining (EJ) or homology-directed repair (HDR). Both repair mechanisms required the Cas9 and single-guide RNA (sgRNA) components to induce and direct cleavage of a specific site in the genome. EJ repair is the more common repair pathway but is error-prone, often resulting in gene knockout because of the generation of deletions, insertions, and point mutations that disrupt gene expression. HDR, on the other hand, can use a donor DNA template to precisely repair the break (knock-in). The HDR mechanism, therefore, requires donor DNA supplied as ssDNA or a DNA plasmid, or it can be genetically encoded to serve as a template for this process. The DNA donors are designed with the desired modification and homology arms flanking the cleavage site. (*B*) An sgRNA design example. (*C*) Schematic of non-genetically encoded component delivery into embryos expressing genetically encoded Cas9 via microinjection. (DSB) Double-stranded break, (PAM) protospacer-adjacent motif.