

A practical guide to genome-engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti*

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Abstract

The following protocol is designed to help researchers generate precise genomic alterations in the mosquito *Aedes aegypti* using the CRISPR-Cas9 system of RNA-guided nucleases. A companion pre-print can be found at <http://biorxiv.org/content/early/2014/12/30/013276>.

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Goals

- Manipulation of the *Ae. aegypti* genome through the precise induction of a genomic double-strand break via CRISPR-Cas9
- Efficient generation of CRISPR-Cas9 reagents: sgRNAs, Cas9, donor DNA
- Isolation of mutant alleles generated by non homologous end joining (NHEJ) or homology-directed repair (HDR)

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General considerations

- Work under RNase free conditions!
 - Use clean nuclease-free filter tips, tubes, and other consumables.
 - Wipe down benches and pipets with RNase Away or RNaseZap before beginning work.
- Avoid excess freeze-thaw cycles for your mRNA and sgRNAs.
 - Make small aliquots of each component and of the final injection mix and store at -80°C.
- We recommend designing and testing multiple sgRNAs to maximize the probability of achieving high levels of modification at your target.

Selected Kits & Reagents

- Cas9 recombinant protein, PNA Bio (catalog #CP01)

PCR and DNA purification

- Beckman Coulter Agencourt Ampure XP beads (catalog #A63880)
- KOD Hot Start DNA Polymerase (catalog #71086-3)
- Life Technologies AccuPrime SuperMix II (catalog #12341012)

in vitro transcription of Cas9 mRNA and sgRNAs

- Ambion mMessage mMachine T7 Ultra (catalog #AM1345) – *Cas9 mRNA in vitro transcription*
- Ambion MegaScript T7 (catalog #AM1334) – *sgRNA in vitro transcription*
- Ambion MegaClear Kit (catalog #AM1908) – *Purification of Cas9 mRNA and transcribed sgRNA*
- Agilent Bioanalyzer 2100 and RNA 6000 Nano Kit (catalog #5067-1511)

Deep-sequencing of PCR amplicons

- Illumina MiSeq Desktop Sequencer and Illumina MiSeq Reagent Kit v3 (catalog #MS-102-3001)
- Illumina MiSeq Nextera XT indexed primers (catalog #FC-131-1002)
- Life Technologies Qubit Fluorometer and Qubit dsDNA HS Assay Kit (catalog #Q32854)
- Bio-Rad iQ5 iCycler or other qPCR platform
- KAPA Biosciences Illumina Library Quantification kit (catalog #KK4844)

Target design and selection

The CRISPR-Cas9 system relies on RNA-DNA base-pairing interactions between the sgRNA and a complementary sequence of genomic DNA to guide the Cas9 nuclease to a specific genomic target. The recognition site for *Streptococcus pyogenes* Cas9 takes the following form: N₁₇₋₂₀ (NGG).

NGG is the protospacer associated motif, or PAM, and **is not** contained within the sgRNA DNA template or transcribed RNA, but is required to be immediately adjacent to your target sequence in the genome for Cas9 nuclease activity.

Putative target sites can be identified by simply scanning the region of the particular genomic location that you want to target (for example, the first exon of a given target gene) for a GG on either strand, or by using an online tool such as ZiFit (<http://zifit.partners.org/ZiFiT/ChoiceMenu.aspx>) or CRISPR Design (<http://crispr.mit.edu>). Each of these tools has pre-loaded the *Ae. aegypti* and *Anopheles gambiae* genome to assist in designing sgRNAs and predicting potential off-target binding and mutagenesis.

If you choose to scan for sites manually, you should still check your sgRNA design for potential off-target effects by using ZiFit, CRISPR Design Tool, or another algorithm designed to predict off-target binding and mutagenesis. We try to avoid sgRNAs that have potential off-target sites with fewer than 3 mismatched bases (or fewer than 2 mismatched bases for a shorter, 17bp guide).

In the example given in Figure 1, the primary target sequences are listed below with the PAM indicated by **bold**:

- Target #1: GATCGGCCCATCCCCACCAG **CGG**
- Target #2: CATTTCCGCTGGTGGGGAT **GGG**
- Target #3: GCATTTCCGCTGGTGGGGA **TGG**

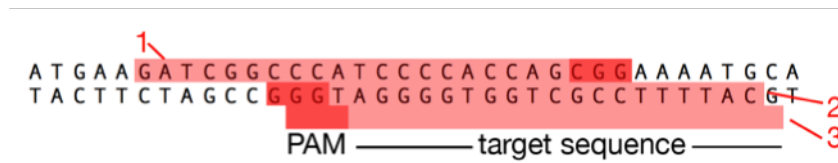


Figure 1: An example target region with CRISPR target sites highlighted in red, and the PAM highlighted in darker red:

For this protocol, the sgRNA target sequence ideally starts with GG - these are the first two obligate nucleotides transcribed by the T7 RNA polymerase. If your target sequence does not start with GG, you may extend it to 21 or 22 base pairs (ie, add the 'GG' to the 5' end of your 20bp recognition sequence). This advice does not apply if you're using a different amplification strategy (ie, expressing the sgRNA from a plasmid off of a U6 promoter, or using a different RNA polymerase).

- In the example given in Figure 1, the target sequences become the following, with added bases indicated by *italics*:
 - Target #1: GGATCGGCCCATCCCCACCAG **CGG**
 - Target #2: GGCATTTCCGCTGGTGGGGAT **GGG**
 - Target #3: GGCATTTCCGCTGGTGGGGA **TGG**

sgRNA template generation and RNA production

- To generate the template for each sgRNA, you need to order the following oligonucleotides (we order them as PAGE-purified oligos from Integrated DNA Technologies [IDT]). The bold portion of the sequences represent complementary sequences that allow the two primers to anneal.
 - CRISPR_R: (this primer can be used for all targets)
5'–AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAACTT **GCTATTTCTAGCTCTAAAAC**–3'
 - CRISPR_F: (contains the T7 promoter and the specific target sequence).
5'–GAAATTAATACGACTCACTATA GGN_{XX} **GTTT TAGAGCTAGAAATAGC**–3'
- GGN_{XX} represents your target sequence including the GG necessary for T7 transcription but **not including the PAM**. See above section for details.
- Perform a brief template-free PCR to anneal and fill in these two overlapping oligonucleotides and produce a full-length dsDNA template. We use KOD polymerase, with the following reaction conditions, but this should work with other polymerases as well.

PCR reaction setup		PCR program	
10x PCR buffer	10µL	95°C	2 min
dNTPs (2mM each)	10µL	35 cycles of	
25mM MgSO ₄	6µL	95°C	20s
CRISPR_F primer (10µM)	5µL	58°C	10s
CRISPR_R primer (10µM)	5µL	70°C	10s
KOD Hot Start Polymerase	2µL		
nuclease free water	62µL		
TOTAL	100µL		

- Run 2µL of this reaction on a gel to verify the size and specificity of amplification. You should see a single band of approximately 100bp in size.
- Purify the remaining 98µL with Ampure XP beads according to the following protocol. Alternatively, you can purify your template DNA by any method laid out in the MegaScript Kit handbook (e.g. PCR purification column, phenol/chloroform precipitation).
 - Add 1.8x volume of Ampure XP beads to the PCR reaction in a 1.5ml Eppendorf tube
 - Vortex to mix well
 - Incubate for 5min at room temperature
 - Place on magnet stand until solution is clear; approximately 5 minutes
 - Remove supernatant
 - Rinse beads with 300µL freshly made 80% EtOH (make with RNase free water)
 - Remove supernatant and repeat rinse step
 - Remove all remaining ethanol and air dry for 5 min
 - Remove from magnet stand and resuspend beads in 30µL of nuclease free water
 - Incubate 10 minutes at room temperature and place on magnet stand
 - Transfer supernatant to separate tube and check the concentration with a spectrophotometer
- Check the purity and concentration of your purified sgRNA template with a spectrophotometer - we shoot for > 100ng/µL so that it will be concentrated enough for *in vitro* transcription.

***in vitro* transcription of sgRNAs**

1. To generate sgRNA, set up the following *in vitro* transcription reaction with the T7 Megascript Kit following the manufacturer's protocol.
2. Briefly, thaw all ribonucleotides and 10x reaction buffer and vortex to ensure they are well mixed.
3. After thawing, keep nucleotides on ice, but the reaction buffer at room temperature.
4. Add to a 200 μ L PCR tube in the following order and mix gently but thoroughly by pipetting up and down:

IVT reaction components	Volume	Incubation
nuclease free water	to 20 μ L	2 hours (minimum) to overnight
free ribonucleotides (ATP,CTP,GTP,UTP)	2 μ L each (8 μ L total)	(12-16h max.) @ 37°C
10x reaction buffer	2 μ L	
sgRNA PCR template	300ng	then add 1 μ L of Turbo DNase and
T7 enzyme mix	2 μ L	incubate for an additional 15min @ 37°C
TOTAL	20μL	

5. Purify RNA using the MegaClear column purification kit following the manufacturer's protocol. You should get anywhere from 5-100 μ g of total RNA from this reaction depending on template quality and incubation time.
6. Check the concentration of RNA with a spectrophotometer, or if possible, check size and concentration with an Agilent Bioanalyzer or Tapestation. sgRNA should appear as a single band without obvious degradation products - the sizing is not always accurate due to the secondary structure of the sgRNAs, but you're still shooting for a sharp peak at approximately the correct size with little evidence of degradation.
7. Divide each sgRNA into 10 μ g or other conveniently sized aliquots and freeze at -80°C.

Choosing a source of Cas9

- We have obtained the best results when injecting recombinant Cas9 protein from PNA Bio (<http://www.pnabio.com>); catalog #CP01). In our hands and our system, it is more mutagenic and more stable than Cas9 mRNA, and much more mutagenic than injecting plasmids encoding Cas9 (but it is more expensive than either of these methods).
- Other commercial sources of Cas9 include NEB (catalog # M0386).
- There is a recently published protocol for producing your own Cas9 protein in *E. coli*, but we have not yet tried this. The protocol can be found in the supplemental materials of Gagnon et al., 2014 (full citation available in the References section).

Cas9 mRNA restriction digest and *in vitro* transcription

- We use pMLM3613 (<http://www.addgene.org/42251/>) as a source of Cas9, but any plasmid containing Cas9 with an upstream T7 promoter will work. Alternatively, you can purchase Cas9 mRNA from companies such as TriLink Biotech (<http://www.trilinkbiotech.com>) or PNA Bio (<http://www.pnabio.com>).
- To make Cas9 mRNA from pMLM3613:
 1. Linearize 10 μ g of plasmid with the restriction enzyme PmeI in a 50 μ L reaction according to the following protocol. Mix reaction components well before incubation.

Restriction Digest		Incubation
CutSmart buffer	5 μ L	2 hours @ 37°C
plasmid DNA	10 μ g	
nuclease free water	to 45 μ L	
PmeI enzyme	5 μ L	
TOTAL	50μL	

1. Run 2 μ L on a gel next to 400ng of uncut plasmid to verify that digestion is complete.
2. Purify remaining 48 μ L of reaction with 1.8x Ampure XP beads (as described in the *sgRNA template generation and RNA production* section above). Alternatively, perform sodium acetate precipitation as outlined in the mMessage mMachine T7 Ultra Protocol Handbook). Elute in 10 μ L of nuclease free water and check concentration by spectrophotometer.
3. Perform *in vitro* transcription using the Ambion mMessage mMachine T7 Ultra kit. Read the protocol from the kit for full details.
4. Thaw NTP/CAP mix and 10x Reaction buffer; once thawed, keep the NTP/CAP mix on ice but the reaction buffer at room temperature.
5. Add the following, in order and mix gently but thoroughly by pipetting up and down:

IVT reaction setup	single reaction	double reaction	Incubation
nuclease free water	to 20 μ L	to 40 μ L	2h @ 37°C
2x NTP/CAP mix	10 μ L	20 μ L	
10x reaction buffer	2 μ L	4 μ L	
purified, digested pMLM3613	X μ L (1 μ g)	X μ L (2 μ g)	
T7 enzyme mix	2 μ L	4 μ L	
TOTAL	20μL	40μL	

1. After incubation, immediately proceed to polyadenylation by thawing the reagents and adding the following, in order, and mixing gently but thoroughly by pipetting up and down:

polyadenylation setup	single reaction	double reaction	Incubation
Cas9 <i>in vitro</i> transcription reaction	20µL	40µL	45min @ 37°C
nuclease free water	36µL	72µL	
5x EPAP buffer	20µL	40µL	
25mM MnCl ₂	10µL	20µL	
10mM ATP	10µL	20µL	
E-PAP enzyme mix	4µL	8µL	
TOTAL	100µL	200µL	

2. Purify RNA with a MegaClear column following the manufacturer's protocol.
3. Check RNA concentration with a spectrophotometer, or better, check size and concentration with an Agilent Bioanalyzer or Tape station.
4. Divide Cas9 mRNA into 10µg or other conveniently sized aliquots and store at -80°C.

Donor plasmid or ssODN production for homology-directed repair

- Homology-directed repair occurs at much lower frequency than NHEJ-mediated mutagenesis. Typical rates are 1-2% of G_0 (meaning 1 to 2 of 100 injected and outcrossed G_0 animals will give rise to mutant progeny containing the donor cassette).
- HDR templates can be double-stranded plasmid DNA or single-stranded oligonucleotides (ssODNs).
- If using double stranded DNA as an HDR template, prepare the DNA via an endotoxin-free maxiprep prior to microinjection. We use the Qiagen EndoFree Plasmid Maxi Kit (Qiagen, catalog #12362).
 - A good backbone for creating a dsDNA donor plasmid with a fluorescent marker is pSL1180-HR-PUBeCFP, available at Addgene (<http://www.addgene.org/47917/>) and described in Liesch et al., 2013 and McMeniman et al., 2014 or PSL1180polyUBdsRED (<http://www.addgene.org/49327/>).
 - Into this or a similar construct, insert homology arms of at least 500bp on either side of the cut site. The longer these arms, the better, and in general, we aim for ~2kb of unique sequence on either side of the target recognition sequence.
- If using an oligonucleotide donor, the following guidelines apply:
 - a ssODN donor is comprised of 200bp total sequence due to the limits of commercially available oligo synthesis - we use IDT as an oligo synthesis provider.
 - Homologous arms of at least 60bp on either side are recommended, but the longer, the better.
 - This limits the payload of a single-strand donor to 80bp maximum; this can be used to deliver novel restriction sites (to add in genotyping), STOP codons to ensure disruption of the mRNA, an attP site, or LoxP sites as a framework for further site-directed engineering.
 - We typically order oligos as Ultramers from IDT with standard desalting - no special purification is necessary.
- For both types of donors, ensure that your cassette does not contain the target recognition sequence to prevent CRISPR-Cas9-mediated editing of the donor cassette pre- or post-integration. Cas9 cleaves 3bp 5' of the PAM; when designing ssODN donors, your arms should extend to this site.
- For a more detailed discussion of donor design for homology-directed repair with other genome editing tools in *D. melanogaster* (but should translate to CRISPR), see Beumer et al., 2013 (full citation in References section).

Final clean-up and injection

This step is critical to remove any leftover salt/ethanol/other junk from the various components.

- Calculate the final desired weight of each component. We use the following concentrations:

Cas9 mRNA or protein	300 ng/ μ L
sgRNA (each)	40 ng/ μ L
ssDNA donor	125 ng/ μ L
dsDNA plasmid donor	500-700 ng/ μ L

- Thaw sgRNA and Cas9 mRNA aliquots, and mix together with DNA donor (if using protein, **do not co-purify** with the sgRNA and the donor; instead, mix together with *purified* sgRNA and donor).
- If total volume is less than 50 μ L, bring up to 50 μ L with nuclease free water before purifying according to the following protocol:
 1. Add 1/10th volume of 3M sodium acetate (pH 5.2)
 2. Add 3 volumes of 100% ethanol and mix thoroughly
 3. Place at -20°C or -80°C for at least thirty minutes
 4. Spin in a microcentrifuge at top speed for 45 minutes at 4°C
 5. Repeat the following wash steps twice:
 - a. Carefully remove supernatant, taking care not to disrupt or dislodge the pellet
 - b. Wash pellet by adding 100 μ L of freshly made 70% EtOH
 - c. Spin in a microcentrifuge at top speed for 3 minutes at 4°C
 - d. Carefully remove supernatant, taking care not to disrupt or dislodge the pellet
 6. Switch to a small pipet tip and carefully remove any remaining liquid
 7. Air dry with lid open for a few minutes to allow residual ethanol to evaporate
 8. Resuspend pellet in desired volume of nuclease free water (accounting for the volume of protein that you'll need to add, if applicable!)
 9. Add Cas9 recombinant protein if using, and mix thoroughly
 10. Proceed to microinjection immediately, or aliquot injection mix and freeze at -80°C

Screening for modification

To determine the efficacy of a particular sgRNA, we use Illumina's MiSeq platform to quantify the rate of insertions and deletions generated by non-homologous end-joining in G_0 animals. This approach will give you an idea of the levels of somatic modification in the injected individuals, which we believe represents a reasonable proxy for the likelihood of germline transmission of mutant alleles. For a discussion of alternative methods of screening sgRNA efficacy and our reasons for the decision to use next-generation sequencing, see the companion pre-print (see References).

Screening G_0 animals: "test" injections

We often perform small "test" injections designed to test the efficacy of a number of sgRNAs. These injections contain a cocktail of sgRNAs designed against different genomic targets. G_0 animals are sacrificed as pupae and used strictly for molecular analysis of sgRNA efficacy. This helps to determine the most effective sgRNA for a particular genomic target and can maximize the likelihood of obtaining favorable results for more difficult injections involving homology-driven repair.

1. Hatch injected G_0 embryos.
2. Collect small pools of pupae (we typically collect 3 pools of 3 pupae each).
3. Extract genomic DNA from each pool.
4. Prepare a 150-500bp amplicon from this genomic DNA using primers on either side of the presumptive cut site. See "Preparing Sequencing Libraries" subsection below for detailed methods.
5. Assess the level of NHEJ-produced indels by sequencing your amplicon with the Illumina MiSeq.

Screening G_1 individuals for stable germ-line transmission of mutant alleles

Our strategy for isolating stable mutant alleles depends on the type of modification desired. The general strategy for recovering each type of mutation is found below, while more detailed discussion and example(s) of each type of mutation can be found in the companion pre-print (see References). We typically work with female G_0 s only for ease of rearing, and because we have observed an overwhelming majority of HDR-induced mutations are derived from female founders.

- Insertions and deletions generated by non-homologous end-joining occur at high frequencies but can be laborious to screen for. To isolate these types of mutations:
 1. Outcross G_1 females to an excess of wild-type males.
 2. Blood-feed G_1 females and split into small pools of 3 animals for egg-laying. Once eggs have been collected, freeze G_1 females.
 3. Extract genomic DNA from the retained G_1 females and generate PCR amplicons for next-generation sequencing (see "Preparing Sequencing Libraries" section below) or identification of mutant alleles by other means.
 4. Once a desired allele has been identified, hatch the G_2 eggs from the corresponding pool. Collect female pupae, mate to wild-type males *en masse*. Collect eggs from single females 3 days after blood-feeding.
 5. Collect eggs and genotype the G_2 female from each cross to identify containing mutant individuals. Continue by hatching eggs from only with those crosses with a mutant parent.
 6. Continue out-crossing for >5 generations, mating and blood-feeding mutant females *en masse* and collecting eggs from and genotyping single females at each generation.
- Larger deletions generated by injection of two sgRNAs can be screened for by designing PCR primers that span the locus and running the PCR products on a gel. Pools of G_1 individuals containing at least one mutant chromosome should result in the presence of a smaller band.
 1. Outcross G_1 females to an excess of wild-type males.
 2. Blood-feed G_1 females and split into small pools of 3 animals for egg-laying. Once eggs have been collected, freeze G_1 females.

3. Extract genomic DNA from the retained G_1 females and generate PCR amplicons from PCR primers spanning both sgRNA cut sites.
 4. Run these PCR amplicons on a gel and identify pools with an additional band (typically smaller, corresponding to a deletion between the two sgRNAs).
 - In addition to precise deletions between two sgRNA recognition sites, we have also observed inversions, insertions, deletions plus insertions, as well as other more complex genomic rearrangements. Thus, we recommend gel-extracting and Sanger sequencing putative mutant bands at this step to characterize the precise molecular nature of each lesion.
 5. Once a desired allele has been identified, hatch the G_2 eggs from the corresponding pool and set up single-pair outcrosses with wildtype individuals of the opposite sex.
 6. Collect eggs and genotype the G_2 female from each cross to identify containing mutant individuals. Continue by hatching eggs from only with those crosses with a mutant parent.
 7. Continue out-crossing for >5 generations, mating and blood-feeding mutant females *en masse* and collecting eggs from and genotyping single females at each generation.
- Using homology-driven repair to insert a novel restriction site results in a precise repair and insertion of exogenous sequence. We typically screen for these events by designing PCR primers across the cut site and digesting them with the restriction enzyme corresponding to the novel restriction site.
 1. Outcross G_1 females to an excess of wild-type males.
 2. Blood-feed G_1 females and split into small pools of 3 animals for egg-laying. Once eggs have been collected, freeze G_1 females.
 3. Extract genomic DNA from the retained G_1 females and generate PCR amplicons of at least 500bp surrounding the sgRNA target site.
 4. Split each PCR reactions in half; reserve half and digest the other half with the appropriate restriction enzyme.
 5. Run both halves side-by-side on a gel. Pools containing a mutant chromosome should result in the release of two smaller fragments in the digested half of the reaction.
 6. Once a desired allele has been identified, hatch the G_2 eggs from the corresponding pool and set up single-pair outcrosses with wildtype individuals of the opposite sex.
 7. Collect eggs and genotype the G_2 female from each cross to identify containing mutant individuals. Continue by hatching eggs from only with those crosses with a mutant parent.
 8. Continue out-crossing for >5 generations, mating and blood-feeding mutant females *en masse* and collecting eggs from and genotyping single females at each generation.
 - Using homology-driven repair to insert a visible marker is the easiest to screen but occurs at low frequency (typical founder rates are 1-2%). When screening for a visible marker, you need to outcross every surviving female G_0 and screen every single G_1 offspring from these crosses. Even when a G_0 is a successful founder, only a subset of their offspring will contain the integrated cassette. In our experience, mutants generated by homology-driven repair are almost always derived from female G_0 founders.
 1. Outcross G_0 females to an excess of wild-type males and G_0 males to an excess of wild-type females.
 2. Blood-feed G_0 females and collect eggs individually. Blood-feed wild-type females and split into large pools of 10-20 animals for egg-laying. Once eggs have been collected, freeze G_0 animals.
 3. Hatch all G_1 eggs (separately, keeping track of parents), and screen for fluorescence as larvae or pupae under a dissecting microscope with a fluorescent light source. We immobilize larvae and pupae for screening by placing them on a moist piece of filter paper.
 4. After isolating fluorescent G_1 individuals, rear them to adults and outcross them to three wildtype individuals of the opposite sex to establish a stable line.
 5. At this point, you must verify that the insertion of the fluorescent marker is directional via PCR across the homology arms (see companion pre-print for a discussion).
 6. Continue out-crossing for >5 generation by collecting fluorescent female offspring from each successive out-cross.

Preparing Sequencing Libraries

- We use the Illumina's MiSeq platform to deep sequence pools of G₀ (or G₁) DNA.
- Sequencing libraries are generated via a two-step PCR. The first PCR will amplify a 100-500 bp amplicon surrounding the CRISPR site and add small overhangs to each end of this amplicon. These overhangs complement with Illumina's Nextera XT indexed primers, which you will use in the second PCR.

Design and order primers:

1. Design primers that will generate a small amplicon of 100-500bp surrounding your CRISPR target site. Design the amplicon so that the CRISPR site is about 75bp (50-100bp is OK) from one primer.
2. Test primer sets with wild-type DNA and KOD Hot Start Polymerase (or any other polymerase).
3. Select a primer set that amplifies a single band of the expected size and verify the sequence identity via Sanger sequencing. This is a good opportunity to ensure that the sgRNA binding site doesn't contain polymorphisms in your injection strain.
4. Once you have a primer set that amplifies the correct wild-type amplicon surrounding your CRISPR site, add overhangs to your custom primers. The primer around 75bp from your CRISPR site will be used to create the "MiSeq_F" primer. Order the following oligonucleotides to use as primers for MiSeq PCR#1:

MiSeq_F: (use the sequence of the primer ~75bp from your CRISPR site)

5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-**YOURsequenceHERE**-3'

MiSeq_R:

5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-**YOURsequenceHERE**-3'

MiSeq PCR #1:

This PCR includes a Hold step at 4°C after 20 cycles. At this step you will remove 35µL from the reaction to set aside for use as the template for MiSeq PCR #2. The low PCR cycle number is chosen to allow representation of rare events found in G₀ DNA by preventing exponential amplification of the predominant wild-type amplicons. The remaining 15µL of the reaction will undergo an additional 20 cycles. Run the 15µL of 40-cycle PCR product on a gel to verify amplification.

PCR reaction setup		PCR program	
10x KOD PCR buffer	5µL	95°C	2 min
dNTPs (2mM each)	5µL	20 cycles of	
25mM MgSO ₄	3µL	95°C	20s
MiSeq_F primer (10µM)	1.5µL	(T _m -2)°C	10s
MiSeq_R primer (10µM)	1.5µL	70°C	15s
G ₀ genomic DNA	1µL	4°C	Hold and Remove 35 µL
Nuclease free water	32µL	20 cycles of	
KOD polymerase	1µL	95°C	20s
TOTAL	50µL	(T _m -2)°C	10s
		70°C	15s

MiSeq PCR #2:

After verifying successful amplification in PCR #1 by running the 40-cycle product on a gel, use the 20-cycle product from PCR #1 as template for PCR #2. In this PCR, you will use Illumina's S5 and N7 Nextera primers to barcode each sample. All samples will end up pooled before being loaded on the

MiSeq for sequencing, so barcodes allow you to identify separate samples in the sequencing results. In each kit, there are 8 different S5 primers and 12 different N7s, which allow you to create a library with up to 96 uniquely-barcoded samples. Recently, Illumina has been selling additional indexed primers that allow for multiplexing of up to 384 samples in a single run.

PCR reaction setup		PCR program	
2X Accuprime Supermix II	12.5µL	94°C	2 min
Illumina S5 adapter	2µL	20 cycles of	
Illumina N7 adapter	2µL	95°C	30s
20-cycle PCR #1 product	2µL	52°C	30s
Nuclease free water	6.5µL	68°C	45s
TOTAL	25µL		

Purify PCR #2 with Ampure beads:

1. Bring the 25µL reaction product up to 50µL by adding 25µL of nuclease free water.
2. Purify the PCR #2 product using 0.6X (30µL) Ampure beads. Ampure beads bind to DNA in a size selective manner, and a concentration of 0.6X is chosen to remove primers from the reaction.
3. Complete Ampure clean-up as described under the *sgRNA template generation and RNA production* section of these methods.
4. Elute in 15µL EBT (Buffer EB + 0.05% Tween-20).

Quantify samples

Quantify samples with Life Technologies Qubit Fluorometer dsDNA High-Sensitivity kit or other system to accurately quantify low concentrations of double-stranded DNA.

Pool samples to ~12nM

1. Pool all samples of the same amplicon based off relative concentrations. The goal is to equally represent each sample in the final library. For example, if sample 1 is 10 ng/µL and sample 2 is 20 ng/µL, the pool should contain twice as much volume of sample 1 as sample 2. If you are sequencing multiple amplicons, create separate pools for each amplicon at this point.
2. Dilute pools to around 12nM. The following formula offers a rough conversion from DNA concentration in ng/µL to molarity:

$$\frac{\text{Concentration of DNA } \left(\frac{\text{ng}}{\mu\text{L}}\right)}{660 \frac{\text{g}}{\text{mol}} \times \text{length of amplicon (bp)}} \times 10^6 = \text{concentration of DNA (nM)}$$

Verify and quantify amplicon libraries

Verify product identity on Agilent 2100 Bioanalyzer

1. Follow manufacturer's protocol to run 1µL of the purified and diluted pool on a High-Sensitivity DNA Analysis Bioanalyzer chip to confirm the removal of primers and the presence of a single product.

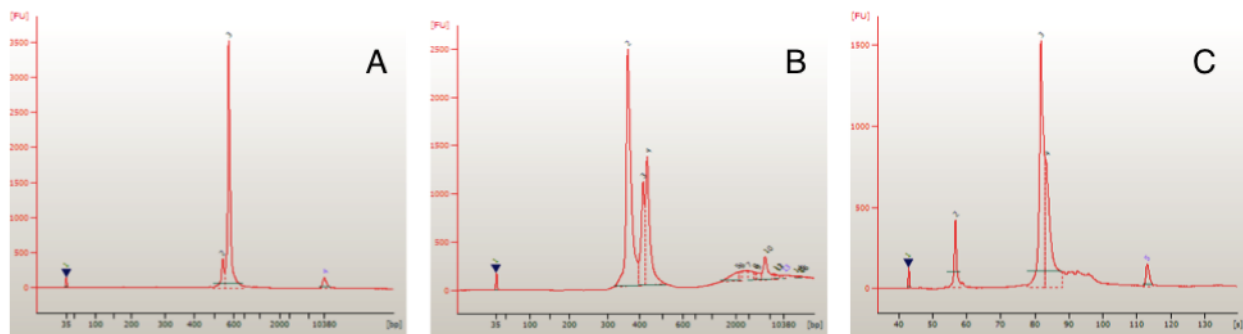


Figure 2: Example traces of **A)** desired Bioanalyzer results showing a single product and complete removal of primers, **B)** Bioanalyzer results showing multiple products either due to off-target amplification during PCR #1 or incomplete addition of Nextera adapters in PCR #2, **C)** Bioanalyzer results showing the presence of excess primers.

Library quantitation by qPCR

At this stage, we use the Bio-Rad iQ5 iCycler qPCR platform and KAPA Biosciences Illumina Library Quantification kit to determine the molarity of sequencing libraries. KAPA also sells kits for Illumina Library Quantification using other qPCR platforms.

1. Program the following cycling program into the Bio-Rad iQ5 software and create a plate on the software that mimics the actual 96-well plate (see manufacturer's protocol for instructions).

qPCR reaction setup (per reaction)		qPCR program	
qPCR Master Mix with Illumina Primer Mix	12 μ L	95°C	5 min
Nuclease free water	4 μ L	35 cycles of	
Standard or diluted library	4 μ L	95°C	30s
TOTAL	20μL	60°C	45s

2. Add Illumina Library Quantification Primer Premix (10x) to KAPA SYBR FAST qPCR Master Mix (2x) and mix well.
3. Use 1 μ L of each sample library to make serial dilutions of 1:1000, 1:10000, and 1:20000. The qPCR measures the molarity of your libraries by interpolating from the standards curve, which ranges from 20pM to 2x10⁻⁴pM. Correct quantification of libraries relies on accurate pipetting in this and the following steps.
4. Make a master mix of water and qPCR Master Mix with Illumina primers. The master mix will be used for all 6 standards and all library dilutions. Remember that all samples and standards are run in triplicate. We recommend preparing the master mix for 5-10 extra reactions to account for volume loss during pipetting.
5. Add 16 μ L master mix to each well of an iCycler iQ qPCR 96-well plate that will be used for standard or sample reactions.
6. Add 4 μ L of each standard and each library dilution to the appropriate wells. Set up all reactions in triplicate.
7. Cover plate with film and spin-down.
8. Load and run your qPCR plate. When the qPCR run is finished, the software will automatically create a standard curve and interpolate the molarity of your samples.

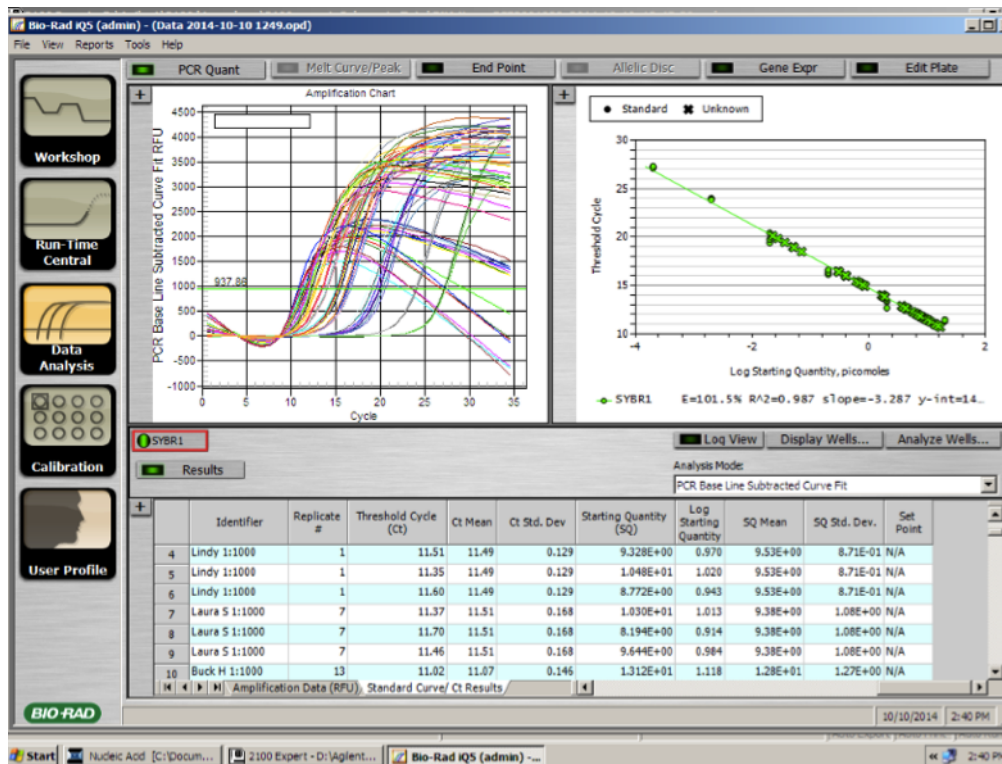


Figure 3: Example of the BioRad iQ5 software following a successful qPCR run showing raw traces and replicated diluted samples fit to a standard curve.

Make final pool

1. Dilute all pools to 10nM based off of qPCR readings
2. Combine equal volumes of each 10nM pool to create a final pool, which will be used for sequencing on the MiSeq. All amplicons can be pooled together once they are diluted to equal molarity.

Sequence Library on MiSeq

To create the final pooled and diluted, follow Illumina's protocol (we use Illumina MiSeq v3 reagents).

1. Thaw Illumina hybridization buffer HT1, and keep on ice.
2. In a 1.5mL eppendorf tube, combine 4µL of purified and pooled 10nM PCR products produced in the previous section with 1µL PhiX DNA.
3. Add 5µL 0.2 N NaOH and mix well by pipetting up and down.
4. Let DNA/NaOH mixture sit at room temperature for 5 minutes.
5. Immediately add 990µL HT1 and mix well to create a 50pM pool.
6. In a new 1.5mL eppendorf tube, mix 120µL of the 50pM pool with 480µL HT1 to get 600µL of 12.5pM pool. Keep this final mixture on ice until loaded into the Reagent Cartridge. Note that 12.5pM is a conservative concentration for the final pool and can be adjusted.
7. Follow manufacturer's protocol to load and run the Illumina MiSeq Desktop Sequencer.

Sequencing reads are aligned to the reference (wild-type) sequence and analyzed for the presence of variants, especially insertions and deletions. Example code for such analysis of deep-sequencing data can be found at: https://github.com/bnmtthws/crispr_indel under the 'tutorial' section.

References and other resources

This protocol is a companion to a publication containing data on our work on genome modification in *Ae. aegypti*. The work can be found in pre-print form at bioRxiv:

- Genome-engineering with CRISPR-Cas9 in the mosquito *Aedes aegypti* Kathryn E Kistler, Leslie B Vosshall, Benjamin J Matthews (2014) bioRxiv. <http://dx.doi.org/10.1101/013276>

This protocol was based on two papers in particular:

- Bassett, A. R., Tibbit, C., Ponting, C. P., & Liu, J.-L. (2013). Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 system. *Cell reports*, 4(1), 220–228. doi:10.1016/j.celrep.2013.06.020
- Hwang, W. Y., Fu, Y., Reyon, D., Maeder, M. L., Tsai, S. Q., Sander, J. D., et al. (2013). Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature biotechnology*, 31(3), 227–229. doi:10.1038/nbt.2501

Two excellent general overview of practical considerations of the CRISPR-Cas9 system as it applies to genome engineering:

- Mali, P., Esvelt, K. M., & Church, G. M. (2013). Cas9 as a versatile tool for engineering biology. *Nature methods*, 10(10), 957–963. doi:10.1038/nmeth.2649
- Ran, F. A., Hsu, P. D., Wright, J., Agarwala, V., Scott, D. A., & Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. *Nature protocols*, 8(11), 2281–2308. doi:10.1038/nprot.2013.143

Other references referred to in the text:

- Beumer, K. J., Trautman, J. K., Mukherjee, K., & Carroll, D. (2013). Donor DNA Utilization during Gene Targeting with Zinc-finger Nucleases. *G3* (Bethesda, Md.). doi:10.1534/g3.112.005439
- Liesch, J., Bellani, L. L., & Vosshall, L. B. (2013). Functional and genetic characterization of neuropeptide Y-like receptors in *Aedes aegypti*. *PLoS neglected tropical diseases*, 7(10), e2486. doi:10.1371/journal.pntd.0002486
- Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M. & Joung, J. K. Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat. Biotechnol.* (2014). doi:10.1038/nbt.2808
- Gagnon, J. A., Valen, E., Thyme, S. B., Huang, P., Ahkmetova, L., Pauli, A., et al. (2014). Efficient Mutagenesis by Cas9 Protein-Mediated Oligonucleotide Insertion and Large-Scale Assessment of Single-Guide RNAs. *PLoS One*, 9(5), e98186. doi:10.1371/journal.pone.0098186

There are many active and helpful webpages and online communities detailing protocols, results, and new approaches and modifications to the CRISPR-Cas9 system in multiple organisms. In no particular order:

- <http://groups.mrcfgu.ox.ac.uk/liu-group/useful-links/oxfcrispr/oxfcrispr>
- <http://flycrispr.molbio.wisc.edu>
- <http://www.crisprflydesign.org>
- <http://www.genome-engineering.org/crispr/>
- <http://www.crispr-cas.org>
- <https://groups.google.com/forum/#!forum/crispr>
- <https://groups.google.com/forum/#!forum/talengineering>
- <https://groups.google.com/forum/#!forum/flycrispr-discussion-group>
- <https://www.addgene.org/CRISPR/>