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Preparing and injecting embryos of Culex mosquitoes to generate null mutations using CRISPR/Cas9
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1   **In TITLE:**
2   Preparing and Injecting Embryos of *Culex* Mosquitoes to Generate Null Mutations Using
3   CRISPR/Cas9
4
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17 **KEYWORDS:**
18 Designing guide RNAs, micromanipulation, microinjection, mosquito embryo, mutation
19 screening, Northern house mosquito, genome editing
20

21 **SUMMARY:**
22 CRISPR/Cas9 is increasingly used to characterize gene function in non-model organisms. This
23 protocol describes how to generate knock-out lines of *Culex pipiens*, from preparing injection
24 mixes, to obtaining and injecting mosquito embryos, as well as how to rear, cross, and screen
25 injected mosquitoes and their progeny for desired mutations.
26

27 **ABSTRACT:**
28 *Culex* mosquitoes are the major vectors of several diseases that negatively impact human and
29 animal health including West Nile virus and diseases caused by filarial nematodes such as canine
30 heartworm and elephantasis. Recently, CRISPR/Cas9 genome editing has been used to induce
31 site-directed mutations by injecting a Cas9 protein that has been complexed with a guide RNA
32 (gRNA) into freshly laid embryos of several insect species, including mosquitoes that belong to
33 the genera *Anopheles* and *Aedes*. Manipulating and injecting *Culex* mosquitoes is slightly more
34 difficult as these mosquitoes lay their eggs upright in rafts rather than individually like other
35 species of mosquitoes. Here we describe how to design gRNAs, complex them with Cas9 protein,
36 induce female mosquitoes of *Culex pipiens* to lay eggs, and how to prepare and inject newly laid
37 embryos for microinjection with Cas9/gRNA. We also describe how to rear and screen injected
38 mosquitoes for the desired mutation. The representative results demonstrate that this technique
39 can be used to induce site-directed mutations in the genome of *Culex* mosquitoes and, with slight
40 modifications, can be used to generate null-mutants in other mosquito species as well.
41

42 **INTRODUCTION:**
43 *Culex* mosquitoes are distributed throughout the temperate and tropical regions of the world
44 and transmit several deadly viruses including West Nile virus[1], St. Louis encephalitis[2] as well as

45   filarial nematodes that cause canine heartworm[3] and elephantiasis[4]. Members of the *Culex*
46   *pipiens* complex, which includes *Cx. quinquefasciatus*, *Cx. pipiens pipiens* and *Cx. pipiens*
47   *molestus*, show striking variations in many aspects of their biology. For example, while *Cx.*
48   *quinquefasciatus* and *Cx. pipiens molestus* are incapable of entering an overwintering
49   dormancy[5,6], *Cx. pipiens pipiens* display robust seasonal responses and enter diapause in
50   response to short days[7,8]. Additionally, *Cx. pipiens molestus* tend to be more anthropophilic while
51   *Cx. pipiens* and *Cx. quinquefasciatus* are more zoophilic[6]. However, in the United States and
52   throughout many other places in the world, these species interbreed, which has strong
53   implications for disease transmission as hybrids of the *Cx. pipiens pipiens* and *Cx. pipiens molestus*
54   are opportunistic feeders and will bite both birds and humans[9], thereby serving as bridge vectors
55   for West Nile virus. Studying these and other fascinating aspects of the biology of *Culex*
56   mosquitoes has been hampered, in part, because *Culex* mosquitoes are slightly more difficult to
57   rear in the lab than *Aedes* mosquitoes, which produce quiescent and desiccation-resistant eggs[10]
58   and because functional molecular tools are not as well developed for *Culex* species.
59
60   CRISPR/Cas9 genome editing is a powerful technology that has been used to evaluate the biology
61   of several important mosquito species[11-13], including the Southern house mosquito, *Culex*
62   *quinquefasciatus*[14-16]. This technology, developed by Jennifer Doudna and Emmanuelle
63   Charpentier, exploits a natural bacterial defense against viruses by bacterially-derived, CRISPR-
64   associated endonucleases (Cas proteins; see review by Van der Oost et al.[17]). When injected into
65   animal embryos, the Cas9 proteins in combination with an appropriate guide RNA can produce
66   double-stranded breaks within the genome. This is most frequently done by using the Cas9
67   protein that is complexed with guide RNAs, which directs endonuclease activity to a specific
68   region of the genome. After the Cas9 protein has created a site-specific double-stranded break,
69   the cellular machinery attempts to repair the break using one of two mechanisms. The first
70   entails ligating the two ends together through non-homologous end joining (NHEJ), which is
71   error-prone and often produces out-frame insertions and deletions in the genome that can result
72   in non-functional proteins, thereby generating a knock-out mutation. Alternatively, the cellular
73   machinery might use homology-directed repair (HDR) by finding similar sequences to correctly
74   repair the break. The similar sequence may be provided by the second chromosome within the
75   organism (see review[18]). However, if the repaired sequence exactly matches the original
76   sequence, the Cas9 protein will be able to again cut the DNA. Alternatively, researchers can also
77   include a donor plasmid that contains homologous sequences on either side of the cut site of the
78   target sequence with an alternative repair sequence—often a fluorescent marker protein,
79   modified version of the original gene, or other modification—that can be copied and inserted
80   into the genome, or "knocked-in."
81
82   Timing is critical when injecting embryos, and this is especially the case when using CRISPR/Cas9
83   genome editing to create mutations in insects. This is because the Cas9 protein and gRNAs have
84   the greatest capacity to generate mutations only when the embryo is in its syncytial state, before
85   cellular membranes have formed and when multiple nuclei are accessible within the embryo. For
86   mosquitoes, nuclei reach the periphery ~2-4 hours after oviposition, depending on
87   temperature[19], and therefore successful microinjection must occur before this time. Additionally,
88   the Cas9 protein will cut any nuclear DNA that it can access, such that the individual resulting

89   from the injection will contain a mosaic of cells, some having the desired mutation, and others
90   not. In order for these mutations to be successfully inherited, the Cas9 protein must cut DNA that
91   resides in the germline that will give rise to the future eggs and sperm. To ensure that mutations
92   are generated in the germline it is best to inject all materials close to the location of the pole cells
93   within the embryo, which are the progenitors of the insect germline. The pole cells are located
94   near the posterior end of *Culex* embryos[20]. In addition to injecting embryos, it is imperative to
95   develop a careful plan for crossing and screening offspring in order to detect the desired
96   mutation.
97
98   This protocol describes how to generate gRNAs and complex them with Cas9 protein to prepare
99   injection mixes, as well as how to induce female mosquitoes of *Culex pipiens* to lay eggs and how
100 to prepare and inject those eggs for CRISPR/Cas9-mediated genome editing. Additionally, we
101 describe how to rear, cross and screen injected embryos and their progeny to confirm that the
102 desired mutation has been obtained. Using this protocol, we generated null mutations for a gene
103 of interest, *cycle*, in the Buckeye strain of *Culex pipiens*. This strain was originally established in
104 2013 from field-collected mosquitoes in Columbus, Ohio and is maintained by the Meuti lab. This
105 protocol can be used for additional studies that require CRISPR/Cas9 genome editing in *Culex*
106 mosquitoes, as well as other mosquito species, and, more generally, is relevant to employing
107 CRISPR/Cas9 genome editing to any insect species.
108
109 **PROTOCOL:**
110
111 In most research institutions, an approved Biosafety Protocol must be in place before transgenic
112 insects are generated or maintained to ensure that genetically modified organisms will not
113 escape or be removed from the laboratory facility. Additional government regulations might also
114 apply. Before beginning a project of this nature, check all institutional policies and procedures to
115 determine what documents and approvals are required.
116
117 **1.   Designing gRNAs and preparing injection mixes**
118
119 1.1.   Design 2-5 gRNAs for each target gene as some gRNAs work better than others. gRNAs
120 that target a gene of interest can be either manually designed or free programs, such as Chop-
121 Chop, can be used.
122
123 1.2.   Design and order primers that will amplify 100-200 bp fragments around each gRNA.
124 Ideally, the cut site should be located approximately in the middle third to half of the PCR
125 product. Note how many bp on each site of the cut will be produced.
126
127 1.3.   Obtain gRNAs.
128
129 1.3.1   Purchase gRNAs from commercial companies such as Integrated DNA Technologies,
130 Fisher Scientific, Genescript and others. This is the easiest option.
131
132 1.3.2   Alternatively, create gRNAs in the lab using PCR amplification to generate the templates

133   for subsequent isothermal synthesis. See the protocol described by Port et al.[21] and Kistler et
134   al.[22].

135

136   1.3.3   Provide gRNAs via plasmid that is injected into embryos. In this case, the gRNA should be
137   driven by the U6 promotor (see [23,24] for details).

138

139   1.4   Obtain the Cas9 protein.

140

141   1.4.1   Order Cas9 commercially from several companies including Fisher Scientific. This is the
142   easiest option.

143

144   1.4.2   Make Cas9 protein and purify in the lab according to Basu et al.[25] and Kistler et al.[22].

145

146   1.4.3   Inject *Cas9* mRNA into embryos, where it will later be translated into active Cas9 protein.
147   *Cas9* mRNA can either be synthesized in the lab using in vitro transcription[22] or purchased from
148   vendors such as Sigma.

149

150   NOTE: The translated Cas9 protein must contain a nuclear localization signal (NLS) on the C-
151   terminus, and therefore the NLS must also be present in the injected *Cas9* mRNA.

152

153   1.4.4   Express Cas9 protein in vivo through plasmid-based expression. In this case, it is best to
154   have the expression of Cas9 on the plasmid driven by an embryonic promoter that has been well-
155   characterized in the target species.

156

157   NOTE: To date, embryonic promoters have not been well-characterized in *Culex* mosquitoes, and
158   therefore injecting purified protein or Cas9 mRNA is recommended. Complexing the gRNAs with
159   the Cas9 protein, adapted from Kistler et al.[22].

160

161   1.4.5   If injecting Cas9 protein with the gRNA together (recommended), ensure that the final
162   concentrations of Cas9 protein is 300 ng/µL and the final concentration of a single gRNA is 80
163   µg/µL. Mix the protein and an individual gRNA in a 0.2 mL PCR tube.

164

165   1.4.6   Incubate for 20 min on ice.

166

167   1.6.   Testing gRNAs with an in vitro assay (e.g., Guide-it sgRNA Screening Kit).

168

169   1.6.1.   Amplify the fragments around the cut site for each gRNA using PCR.

170

171   1.6.2.   Run the PCR products on a gel to ensure that they are the correct size. Then, purify PCR
172   products and sequence them to ensure that they target the correct gene region.

173

174   1.6.3.   Mix 200 ng of the leftover purified PCR product with 1 µL of Cas9 reaction buffer, 1 µL of
175   BSA, 1.5 µL of complexed Cas9:gRNA and bring the total reaction volume to 15 of. Incubate for 5
176   min at 37°C.

177

178 1.6.4. Run the product on a gel again. If the Cas9 protein successfully cut the PCR product, the
179 primary band should appear to be more faint and additional smaller bands should be present.
180 Note the reduction in size of the original PCR product, and, if desired, calculate the reduction in
181 band intensity to approximate the cutting efficiency.

182

183 1.7.   Preparing the final injection mix

184

185 1.7.1. For each gRNA that successfully cut its targeted PCR product, mix volumes of each Cas9
186 and gRNA into a 0.5 mL tube such that the final volume remains 300 ng/µL of Cas9 protein and
187 80 ng/µL of each gRNA.

188

189 1.7.2.   Store the complexed Cas9 and gRNAs at -80 °C until they are needed for injection.

190

191 **2.   Pulling and beveling needles**

192

193 NOTE: Successful injections and survival of embryos requires sharp needles (**Figure 1**).

194

195 2.1.   Use either 1 mm outer diameter borosilicate or quartz glass microcapillaries. Siliconize
196 the microcapillaries in a chemical fume hood before being pulled.

197

198 2.1.1. In brief, place 3 mL of Sigmacote into a glass beaker and vacuum draw into a second
199 beaker containing the glass microcapillaries for at least 4 h, allowing the siliconizing solution to
200 vaporize and settle on all surfaces of the microcapillaries.

201

202 2.1.2. Afterwards, slowly allow the vacuum chamber to equalize to ambient pressure by
203 gradually opening the stopcock valve and allowing air to return into the chamber. The needles
204 are then ready to be pulled.

205

206 2.1.3. Always read the instruction manual of needle pullers before use.

207

208 NOTE: The instructions below detail how to use a P-2000 Laser Needle Puller to pull glass needles.
209 It is important to note that all needle pullers even of the same brand are not calibrated to pull
210 exactly the same needle across different units. The key to getting good needles is to start at a
211 given set of parameters then pull needles using parameters that are above and below these
212 values. Test the needles at each of these parameters by evaluating how well the needle pierces
213 embryos and how well the injection mix flows from the needle. Refine the parameters until a
214 needle is obtained that is easy to open or bevel, that pierces embryos smoothly and that delivers
215 injection mix easily.

216

217 2.2.   Pulling needles using the P-2000 laser needle puller

218

219 2.2.1. Turn on the laser needle puller and allow the laser to warm up for 15 min before the first
220 pull.

221

2.2.2. Program the machine for the type of glass microcapillary tube (Remember any needle puller parameters are a starting point since needle pullers are not calibrated to produce the same needle across all pullers):
Quartz: Heat = 730; Fil = 4; Vel = 40; Del = 122; Pul = 156

2.2.3. After programming the needle puller, load a microcapillary and clamp it in place, taking care to only touch the end of the microcapillary tube and not the area that will be heated by the laser.

2.2.4. After clamping the microcapillary in place, place thumb and forefinger on the finger bars and then release the pull bars by pressing the spring releases one at a time.

2.2.5. Squeeze thumb and forefinger to pull bars fully together.

2.2.6. Position the other side of the microcapillary tube in its clamp such that a small portion of the tube extends from both sides. Tighten the clamps making sure that both clamps are finger-tight and that they hold the "pull bars" firmly in place.

2.2.7. Close the protective shroud on the puller.

2.2.8. Press the **Pull** button and the laser will start to heat the glass, indicated by the red "laser on" light located under the shroud. The pull is completed when the pull bars are completely separated, accompanied by a metallic thud.

2.2.9. Ensure that the "laser on" light is not illuminated before lifting the shroud.

2.2.10. Hold the small end of the microcapillary tube that extends beyond the clamp with thumb and forefinger and loosen the clamp. Then lift the microcapillary tube out of the clamp.

2.2.11. Use forceps to carefully place the microcapillary tube into a square Petri dish lined with double-sided tape. Ensure when placing the needle into the holding box that the back end of the needle touches first and the needle is gently lowered into place so that the sharp tip is not damaged.

2.2   Pulling needles using a PC-10/PC-100 needle-puller

2.2.1   Set up PC-10 puller using 4 weights and set the temp for 50.4 °C for a single-step pull.

2.2.2   Place a borosilicate glass microcapillary into the top clamp. Then raise the weighted bottom clamp into position and clamp the bottom portion of the microcapillary, making sure that the capillary is in position to pull two good needles. This is a rather long pull cycle, but it produces good needles for beveling.

264

265   2.3   Beveling needles.

266

267   NOTE: This method of wet beveling gives real time feedback on relative opening size of the
268   needle.

269

270   2.3.1   Assemble the abrasive plate and retaining ring of the needle beveler. Ensure that the gray
271   side of the abrasive plate is oriented upward between the upper and lower retaining ring. Tighten
272   the screws on the retaining ring, alternating from screw to screw to ensure that each screw is
273   tightened evenly. The abrasive plate and retaining rings will hereafter be referred to as the
274   grinding assembly.

275

276   2.3.2   Place 13 drops (~ 520 μL) of pedestal oil onto the optical flat surface, and then place the
277   grinding assembly on top of the plate. Place the assembly beneath a dissecting microscope, and
278   then turn on the beveler. A little extra oil compared to Sutter's recommendation is used, as this
279   keeps the abrasive plate spinning for a longer period when used in conjunction with this wet
280   beveling method.

281

282   2.3.3   Add 1% Photo-Flo solution to the grinding surface and move the wick into position so that
283   it is immersed in the Photo-Flo solution.

284

285   2.3.4   Turn on the air at the main source. Then place a pulled borosilicate needle into the holder
286   and tighten the retaining ring. Turn on the air at the regulator and increase until the pressure has
287   reached 26 PSI.

288

289   2.3.5   Watching from the side, lower the needle using the coarse adjustment knob until it almost
290   touches the Photo-Flo liquid surface.

291

292   2.3.6   Adjust the microscope to locate the needle in the field of view. Start at the lowest
293   magnification and increase the magnification. Carefully adjust the position of the needle using
294   the coarse adjustment knob until it just touches the surface of the Photo-Flo liquid.

295

296   2.3.7   Using the coarse adjustment knob and continuing to look under the microscope, lower
297   the needle until it is close to the abrasive surface but not touching it.

298

299   2.3.8   Using the fine adjustment knob, carefully and slowly lower the needle, paying close
300   attention to the needle and the shadow it leaves on the abrasive surface of the beveler. When
301   the needle and its shadow look like they are about to touch the abrasive surface, continue to
302   lower the needle even more slowly and carefully.

303

304   2.3.9   Alternate between letting the needle "bevel" by lowering it on to the abrasive surface,
305   and then raising it. Before raising the needle, quickly note the setting on the micrometer on the
306   fine adjustment knob so that the needle can be lowered to the same position or, if necessary, to
307   a slightly lower position. Remember to keep the needle below the Photo-Flo liquid layer. Once
308   the needle has been raised above the abrasive surface, quickly stop and restart the rotation of

309 the beveler to check for bubbles. If the needle has been adequately beveled, air bubbles should
310 flow out from the needle and into the Photo Flo. If the plate is not stopped, bubbles will likely
311 not become visible until the needle opening is too large.
312
313 2.3.10 If no bubbles appear when the needle is raised above the abrasive surface and the beveler
314 has stopped, repeat the beveling procedure, lowering the needle to a slightly lower position on
315 the micrometer located on the fine adjustment knob, raising the needle, stopping the beveler
316 plate and checking for air bubbles. Repeat until a small but steady stream of bubbles appear.
317
318 2.3.11 Once air bubbles are present, check the relative opening size by stopping the abrasive
319 plate and lowering the air pressure, noting the PSI at which bubble formation stops, as this is a
320 relative indication of the size of the opening of the beveled needles. The lower the pressure at
321 which air stops escaping from the needle tip, the larger the needle opening. Needles beveled to
322 the point at which bubbles stop escaping from the needle at 18-20 PSI indicates that the needle
323 has a relatively small opening and should cause minimal damage to an embryo when used for
324 microinjections.
325
326 2.3.12 After checking the needle opening, increase the air pressure to 26 PSI to prevent liquid
327 from entering the needle. Then raise the needle up and out of the Photo-Flo layer using the
328 coarse adjustment knob. It is important to note that as long as air is flowing out of the needle the
329 Photo-Flo does not enter the needle, and therefore the inside of the needle is protected from
330 contamination.
331
332 2.3.13 Once the needle is out of the Photo-Flo, turn off the air and remove the needle from the
333 needle holder.
334
335 2.3.14 Using forceps, place the newly beveled needle into a Petri dish that has either double
336 sided tape or modeling clay to keep it in place. Repeat the process until 10-20 beveled needles
337 have been produced.
338
339 **3.   Blood-feeding the parental generation of mosquitoes**
340
341 3.1.   Rear larvae of *Cx. pipiens* mosquitoes under unambiguous long day conditions (>13 h of
342 light/day) at 25-27 °C to ensure that mosquitoes do not enter their overwintering dormancy or
343 diapause.
344
345 3.2.   Seven to ten days after peak adult emergence, prepare the Hemotek Membrane feeding
346 system by plugging the heating units into the power supply. Adjust the temperature of each unit
347 to 37 °C (human internal body temperature) or 41 °C (chicken internal body temperature) using
348 the adjustment screw on the top of the unit. Ensure that the correct temperature has been
349 reached using an electric thermometer.
350
351 3.3.   Prepare the blood-meal reservoir for feeding.
352

353 3.3.1. Cut one square of parafilm for each blood-feeding cylinder, and rub parafilm against bare
354 feet. This provides scents that are attractive to the mosquitoes.

355

356 3.3.2. Stretch the parafilm as thinly as possible, and wrap the edges tightly around the meal
357 reservoir. If desired, secure in place with a rubber O-ring.

358

359 3.3.3. Add approximately 200 µL of 0.1 M ATP solution to 15 mL of fresh or thawed whole,
360 sodium citrate-treated chicken blood. The ATP helps stimulate blood feeding, and sodium citrate
361 prevents the blood from coagulating.

362

363 3.3.4. Hold the reservoir so that the parafilm side is facing down, and carefully use a disposable
364 pipette to fill the reservoir. Each reservoir holds ~3 mL of blood. Seal the filling ports with plastic
365 plugs.

366

367 3.3.5. Screw the meal reservoirs into the heating units and place on top of the mosquito cage
368 such that the parafilm side is facing down and the mosquitoes can feed through the mesh.

369

370 3.4. Cover the cages with black trash bags to simulate dusk and blow vigorously into each cage
371 as the increased CO_2 concentration stimulates blood-feeding.

372

373 3.5. Keep the feeder on the cage for 2-8 h to maximize blood feeding. Check periodically and
374 note the proportion of females that have taken a blood meal (evident by their red and distended
375 abdomens).

376

377 **4. Inducing egg-laying in adult mosquitoes**

378

379 4.1. Four to five days after blood feeding, prepare a 50 mL conical tube for mosquito
380 oviposition.

381

382 4.1.1. Create a ~20 mm whole near the bottom of the conical tube.

383

384 4.1.2. Cover the hole with two squares (35 mm^2) of dental dam rubber, one with a horizontal
385 slit and another with a vertical slit. Use tape to attach the squares of dental dam to the conical
386 tube.

387

388 4.1.3. Place a 4.25 cm piece of filter paper onto a 35 mm x 10 mm Petri dish and wet the filter
389 paper with 750 µL of distilled water.

390

391 4.1.4. Invert the conical tube onto the filter paper and twist back and forth a few times to ensure
392 that it is secure.

393

394 4.2. Use a mouth aspirator to carefully remove ~10 females of *Cx. pipiens* from their cage and
395 transfer them to the prepared conical tube.

396

397 4.3. Place an opaque cylinder over the conical tube to provide darkness to the mosquitoes as
398 this stimulates egg-laying, and wait 20-25 minutes.
399
400 **5.** **Micro-manipulating freshly-laid *Culex* eggs**
401
402 5.1. Prepare the slide for attaching the mosquito embryos for microinjection.
403
404 5.1.1. Attach a piece of double-sided tape to the width of a cover glass.
405
406 5.1.2. Attach a thin strip of transparent medical dressing (e.g., Tegaderm, 2-3 mm wide) to the
407 double-sided tape such that it runs parallel to the short end of the cover glass and is positioned
408 approximately 5 mm from the end of the cover glass.
409
410 5.1.3. Remove excess double-sided tape with a sharp razor blade, and remove 2-3 mm of
411 medical dressing and double-sided tape from each end of the cover glass. This allows a layer of
412 oil to remain on the slide after the eggs have been mounted to the cover glass.
413
414 5.1.4. Using a wax pencil, draw a "D" shape around the double-sided tape and strip of medical
415 dressing. This will act as a barrier to hold the water around the eggs after injection.
416
417 5.2. After the slide has been prepared and 20-25 minutes have elapsed, remove the opaque
418 tube and determine whether or not the mosquitoes have laid any eggs.
419
420 5.2.1. If not, cover them for another 5-10 minutes.
421
422 5.2.2. If the mosquitoes have laid eggs, carefully but quickly lift the conical tube and cover with
423 a cap. Place the mosquitoes in a separate cage, and record the time that eggs were collected on
424 a spreadsheet. Then add 50-100 μL of DI water to the filter paper.
425
426 5.3. Under a dissecting microscope line up the eggs.
427
428 5.3.1. Position a piece of filter paper (10 mm x 30 mm rectangles cut from coarse filter paper
429 with fast flow rate) under a 24 mm x 40 mm cover glass such that the cover glass covers 50-60%
430 of the left hand side of the filter paper.
431
432 5.3.2. Wet the filter paper with 50 μL of DI water. The water will spread under the cover glass
433 slowly, creating a reservoir to keep the filter paper and the eggs wet during micromanipulation.
434 A meniscus will form between the cover glass and the filter paper when the correct amount of
435 water is applied.
436
437 5.3.3. Separate the eggs from their egg rafts using a fine paint brush, and position so that the
438 narrow, posterior end of the embryo is touching the cover glass (left) and the wider, anterior end
439 is to the right.
440

441   5.3.4. Align eggs for 20 min, carefully observing the change of their color from a milky white to
442   a very light gray. Routinely inspect the water film under the cover glass to ensure that there is an
443   adequate amount of water. If the eggs appear to be drying out, add a small amount of DI water
444   (20-30 µL) to the filter paper.

445

446   5.3.5. After 20 min, discard all remaining eggs.

447

448   5.4.   Transfer the eggs to the microinjection slide.

449

450   5.4.1. Use a small (10 mm x 30 mm) piece of coarse filter paper to wick water from the side of
451   the saturated filter paper, and remove the wicking filter paper with forceps. Repeat 2-3 times to
452   ensure that the filter paper with the eggs is as dry as possible.

453

454   5.4.2. Lay a fresh, dry rectangle of filter paper and hold it in place with a pair of forceps. Carefully
455   pull away the cover glass to the left, away from the aligned eggs.

456

457   5.4.3. Dry the excess water on the stage without disturbing the small filter paper that contains
458   the aligned eggs. Continue to dry the wet filter paper with the eggs several more times, pressing
459   on the small rectangles of filter paper with thumbs and/or forceps, to ensure that the eggs are
460   as dry as possible before transferring them to the injection slide.

461

462   5.4.4. Using forceps, remove the strip of medical dressing from the mounting slide.

463

464   5.4.5. Hold the mounting slide with a thumb and middle finger and the exposed medical
465   dressing facing down, and lower at a 45° angle onto the line of aligned eggs. Position the slide
466   such that the wider, anterior end of the eggs (left) is hanging slightly off the end of the medical
467   dressing, as this enhances the ability of the *Culex* larvae to successfully hatch from the mounted
468   eggs.

469

470   5.4.6. Press index finger to the underside of the cover glass, while continuing to hold the cover
471   glass between the thumb and middle finger, to ensure that all of the aligned eggs are firmly
472   attached to the medical dressing.

473

474   5.4.7. Immediately invert the cover glass so that the eggs are facing up, and apply a thin layer
475   of oil Halocarbon oil (2-3 drops; ~20 µL) to the eggs. This prevents the eggs from desiccating
476   during microinjection. Remove any eggs that are not attached to the medical dressing or are not
477   covered by the oil.

478

479   5.4.8. Place the glass cover with the mounted eggs facing up inside of a square Petri dish with a
480   large square of wet filter paper for transport for microinjection.

481

482   **6.   Injecting *Culex* embryos**

483

484   6.1.   Back-filling the injection needles with the injection mix

485

6.1.1.   Choose a needle filler or gel loading tip that will fit easily into the back end of the selected injection needle, and will have a bit of space between the end of the needle filler and the injection needle.

6.1.2.   Carefully place a single and small drop of injection mix (~0.5-1 µL) into the injection needle, using the needle filler to back-fill the needle. Place the drop of injection mix near where the injection needle begins to taper.

6.2.   Attach the injection needle to the micro-injector.

6.3.   Place the slide containing the embryos onto the stage of the microscope.

6.4.   Opening the needle

NOTE: If using beveled needles, this is not necessary.

6.4.1.   Use a starting injection pressure of ~30 PSI and adjust as necessary to deliver an appropriate volume of injection mix.

6.4.2.   Under a dissection microscope, position the needle above the embryo, and carefully use the micromanipulator to lower the needle onto the embryo. Once the needle is barely touching the embryo, quickly move the embryo perpendicular to the long axis of the needle.

6.4.3.   To determine if the needle was successfully opened, press the injection trigger and see whether any bubbles/injection mix escape from the needle. If not, repeat moving the embryo against the needle until the needle is open and injection mix escapes when the trigger is pressed.

6.5.   Injecting the embryos

6.5.1.   Position the first embryo in line in the center of view in the microscope and ensure that it is in focus.

6.5.2.   Place the needle over the first egg, also within the center of the field of view within the microscope.

6.5.3.   Progressively increase the magnification on the microscope, carefully lowering the needle to keep it just above the first embryo, centered and in focus. Proceed until the microscope has reached its highest magnification and both the embryo and the needle are in focus.

6.5.4.   Carefully move the embryo on the stage slightly to the left and lower the needle slightly so that the needle and the embryo are now in the same plane of view.

6.5.5.   Using the microscope stage to move the embryo, carefully and gently touch the embryo

to the tip of the needle. The narrow, posterior end of the embryo should deflect slightly. Adjust the height of the needle if it is too high or too low.

6.5.6.   Using the microscope stage, move the posterior end of the embryo onto the needle and observe the needle penetrating the chorion of the mosquito egg. The needle should just penetrate the membrane in the approximate location of the pole cells within *Culex* embryos. Once this occurs, pull the injection trigger 1-3 times to shoot injection mix into the embryo. Deliver a small amount of injection mix, just enough that a small clearing in the embryoplasm can be detected.

6.5.7.   Using the stage, move the embryo to the right, away and off the tip of the needle. If the injection went well, little to no fluid should leak from the embryo.

6.5.8.   Move the stage down so that the next embryo in the line is positioned in front of the needle. Then again, move the stage to the left, positioning the embryo onto the injection needle and pull the injection trigger.

6.5.9.   If injection mix does not appear to be coming out and/or if a large amount of fluid escapes from the embryo after the needle is removed, replace the injection needle and start again.

6.5.10. Destroy any un-injected or damaged eggs.

6.6.   Tending to the embryos post-injection

6.6.1.   After all embryos on the slide have been injected, wash off the Halocarbon oil by applying reverse osmosis water with a squirt bottle, such that the stream of water is directed onto the top of the glass coverslip above the injected embryos and allowing the water to flow down the coverslip and over the embryos to rinse the oil away. Make sure to not direct the water stream directly onto the embryos as this may damage or detach them from the Tegederm. This process removes most, but not all, of the Halocarbon oil.

6.6.2.   Cover the slide with 150 μL of DI water.

6.6.3.   Place the slide with the injected embryos on wet filter paper inside of a square Petri dish.

6.6.4.   Place the Petri dish containing the injected embryos in an environmental chamber set to 25-27 °C, 70-80% RH with a long day photoperiod (>13 h light/day).

7.   Rearing injected embryos (F_0) to adulthood and setting up crosses

7.1.   Examine the slides of injected embryos daily, expecting that 1st instar larvae should emerge 1.5 days after injection.

7.2.   Count the number of 1st instar larvae, and place 100-200 larvae into a shoebox-sized

573 Tupperware container with 450 mL of DI water, and 50 mg of ground fish food. At this time,
574 create 1-5 times more containers containing wild-type or un-injected larvae that will be used to
575 cross with the injected mosquitoes.
576
577 7.3.   Feed the larvae daily, slightly increasing the amount of food they receive each day until
578 reaching 300-500 mg on the 6th day of larval life.
579
580 7.4.   Once the pupae appear, use a transfer pipette to place one pupae into a 2 mL
581 microcentrifuge tubes filled 50-75% full of DI water. Repeat for all pupae, both the pupae that
582 were injected as embryos as well as uninjected, wild-type pupae. Carefully press the lids so that
583 they are slightly ajar, preventing the mosquitoes from escaping but still allowing for a small
584 amount of gas exchange.
585
586 7.5.   Once the adult mosquitoes emerge inside their microcentrifuge tubes, release injected
587 female mosquitoes in a cage with virgin wild-type male mosquitoes, achieving a ratio of at least
588 1 wild-type male for every injected female. Similarly, release injected male mosquitoes in a cage
589 with virgin wild-type females, achieving a ratio of approximately 5-10 virgin wild-type females for
590 every injected male. A higher ratio of wild-type females to injected males is recommended as
591 each male mosquito can mate multiple times. Therefore, having higher numbers of wild type
592 females that mate with injected males increases the number of F1 progeny that can be produced.
593
594 **8.   Obtaining and rearing F$_1$ mosquitoes and screening them for mutations**
595
596 8.1.   Seven to ten days after adult emergence, offer a blood meal to the females in each cage
597 as described above (step 3).
598
599 8.2.   Four days after blood-feeding, place oviposition water into each cage. Each egg raft
600 represents the progeny of a single female mosquito, and therefore should be placed into its own
601 plastic, shoebox-sized rearing container shortly after oviposition. Label each container with a
602 unique identifier, and also indicate that the larvae belong to the F$_1$ generation for the gene of
603 interest, whether the male or female parent was injected, the date the pan was established,
604 rearing conditions, and the experimenter's initials.
605
606 8.3.   Monitor the pans of larvae daily. As soon as larvae hatch in the pans, provide 50 mg of
607 ground fish food. Increase the food daily for 6 days as described above (step 7.3).
608
609 8.4.   Transfer individual pupae to 2 mL microcentrifuge tubes containing between 1-1.5 mL of
610 DI water, label each tube, and crack the lids.
611
612 8.5.   Once the adult mosquitoes emerge, carefully open the 2 mL microcentrifuge tube,
613 covering with an index finger, and using the other hand, place a 3 dram glass vial over the top of
614 the microcentrifuge tube. The mosquito's natural instinct should be to fly up and into the glass
615 vial. Once this happens, slide a finger over the opening of the glass vial, and quickly invert it,
616 placing a small ball of cotton that has been slightly wetted with 10% sucrose solution into the top

617 of the vial. This prevents the mosquito from escaping and provides a necessary food and water
618 source.

620 8.6.   Label both the tube containing the pupal exuvia and the glass vial containing the adult
621 mosquito to indicate the larval pan sex of the mosquito and the unique identifier for that
622 individual. Ex: II.C.M32, where "II" represents that the male parent was injected, "C" represents
623 the pan/egg raft from which the individual originated, and "M32" designates that was the 32nd
624 male to emerge from that larval rearing container.

626 8.7.   Place adult mosquitoes within their individual glass vials back into the environmental
627 chamber, and add a small amount (~20 µL) of 10% sucrose solution to their cotton daily. Be sure
628 not to overfeed or saturate the cotton as the mosquitoes will get stuck in the sucrose solution
629 and die.

631 8.8.   Screening mosquitoes for the desired mutation

633 8.8.1. Extract genomic DNA from 5-10 pupal exuvia from each egg raft/pan of larvae using the
634 Phire Direct PCR Kit according to the manufacturer's instructions, as well as 1-2 wild-type
635 individuals which will serve as a control.

637 NOTE: As the offspring from each raft are likely full siblings, screening 5-10 larvae from each pan
638 will determine whether the mutation has been passed on to the offspring. If none of the screened
639 larvae from one pan have the desired mutation, do not screen additional adults. If some of the
640 larvae have the mutation, then every individual from that pan should be screened.

642 8.8.2. Using the previously designed PCR primers (step 1.2), amplify the fragment around the
643 predicted location of your mutation using the Phire PCR kit in both the wild type and F1 progeny
644 and run PCR fragments on a 3-4% agarose gel to determine if there are any visible insertions or
645 deletions (indels).

647 NOTE: Any individuals that have the desired mutation will be heterozygous and should display 2
648 bands, one wild type and one either slightly shorter or longer at the desired position. To
649 distinguish these, compare the position and thickness of the bands of the wild type individuals
650 with those in the bands in the F1 progeny. Ideally 2 discrete bands will be visible, but if the indel
651 is very small, the band might appear to be wider and/or blurrier.

653 8.8.3. Purify the PCR product either by excising the band containing the suspected indels
654 (recommended) or by purifying the remaining PCR product that was not loaded into the gel.
655 Submit the purified PCR for sequencing to determine whether the desired mutation is present.

657 **9.   Obtaining and rearing F_2 and F_3 mosquitoes and screening them for mutations**

659 9.1.   Release all F_1 adult mosquitoes for which the desired mutation was found by screening
660 its pupal exuvia from their individual glass vials and into a cage containing wild-type, un-injected

661 mosquitoes of the opposite sex. Backcrossing for this second generation should remove all
662 deleterious effects of injection and inbreeding.

663

664 9.2.   Blood feed the cages of the mutant F_1 mosquitoes and their wild-type counterparts as
665 previously described. Four to five days later, collect the resulting F_2 egg rafts.

666

667 9.3.   Label and rear the F_2 larvae as described above, placing each egg raft into a separate,
668 labeled container. Upon pupation, again separate individual pupae into individual 2 mL
669 microcentrifuge tubes, and transfer each adult into separate glass vials capped with sucrose-
670 soaked cotton upon emergence. Then screen the pupal exuvia of each F_2 individual the desired
671 mutation as previously described (steps 8.5-8.8).

672

673 NOTE: Here all individuals that have the desired mutation will be heterozygous, and therefore
674 the PCR product should ideally produce 2 distinct bands when run on a 3-4% agarose gel.

675

676 9.4.   Once mutant F_2 larvae have been identified, place all males and females that have the
677 mutation into the same cage to mate. Blood feed 7-10 days after adult emergence, and 4-5 days
678 later, collect the F_3 egg rafts.

679

680 9.5.   Place each F_3 egg raft into a separate larval rearing container, and label and feed as
681 described above.

682

683 9.6.   Separate the F_3 pupae into individual 2 mL microcentrifuge tubes. Once adults emerge,
684 transfer them to individual glass vials capped with 10% sucrose-soaked cotton. Screen the pupal
685 exuvia as previously described. This time, approximately 25% of the offspring in each pan should
686 be homozygous for the null mutation. Place these individuals into a single cage, and use them to
687 create and maintain the newly-generated mutant line of mosquitoes.

688

689 NOTE: If a low number of homozygous mosquitoes are present, heterozygous F_3 males and
690 females can be crossed with each other to generate additional homozygous-null mosquitoes in
691 the F_4 generation.

692

REPRESENTATIVE RESULTS:
694 Using the described protocol, we were able to successfully inject embryos of *Cx. pipiens*, and
695 observed a high rate of survival among the injected embryos (~55%, **Figure 1**). Earlier trials had a
696 lower percentage of survival, likely because the anterior of the egg follicle was attached to the
697 medical dressing strip, preventing mosquito larvae from escaping from the chorion and
698 successfully swimming into the water. Ensuring that the anterior end extends beyond the strip of
699 medical dressing greatly increased larval survival, and results in high quality offspring that are
700 capable of developing to adulthood and reproducing (**Figure 2B**).

701

702 Subsequent experiments and screening indicate that the null mutation for the circadian clock
703 gene *cycle* (*cyc*; KM355981) made it into the germline of the injected embryos (**Figure 3**). Given
704 that our primers amplified a large region of the *cyc* gene near the cut-sites, it was difficult to

705　observe two separate bands in heterozygous, F_1 mosquitoes. This demonstrates the utility of
706　designing primers that will amplify fragments that are ~100-200 bp around the cut site, and also
707　the importance of sequencing all suspected mutant mosquitoes. Sequencing revealed that
708　approximately 10% of the screened mosquitoes showed a small insertion or deletion near the
709　Cas9 cut site of the first guide RNA that we had designed (**Figure 3**), suggesting that this was a
710　highly effective gRNA and that we successfully targeted the pole cells during microinjections. The
711　F_1 individuals successfully mated and produced viable offspring (F_2 generation), some of which
712　also contained the mutation.
713
714　**FIGURE AND TABLE LEGENDS:**
715　**Figure 1: Example of a beveled borosilicate needle.** This needle was fabricated from a siliconized
716　borosilicate microcapillary tube, pulled using a PC-10 vertical needle puller, and then beveled on
717　a BV-10 Beveller. The needle is viewed under ~63x magnification.
718
719　**Figure 2: Survival of the F_0 generation across their development.** (**A**) Image of 1st instar larvae
720　that hatched from 3 slides containing injected embryos. (**B**) Graphical representation of the
721　number of individuals at each life stage. Of the 801 embryos that were injected, 441 1st instar
722　larvae hatched, and of these 149 individuals reached pupation, resulting in a total of 121 adults
723　(73 males and 43 females).
724
725　**Figure 3: Representative results showing the transmission of the desired mutation.** The top two
726　sequences represent the sequence of the *cycle* gene in *Culex quinquefascaitus* (Cx.quinq;
727　XP_001865023.1) and in wild type females of *Cx. pipiens* (WT.F; KM355981). The sequences
728　below represent sequences in three male F_1 offspring (I.DM1; II.JM4; II.K10M). The green box
729　represents the PAM sequence recognized by the Cas9 protein (CGG) while the green arrow
730　represents where the Cas9 protein cleaved genomic DNA. These results show that short deletions
731　of 2 or 4 nucleotides were inherited in the F_1 males.
732
733　**DISCUSSION:**
734　This protocol presents methods to introduce specific mutations into the genome of *Culex*
735　mosquitoes and can be used to edit the genome of other mosquitoes as well. The protocol is
736　significant in that it provides specific details of not only how to prepare the injection materials,
737　but also a detailed video overview of how to induce mosquitoes to lay eggs, as well as how to
738　prepare and inject those eggs. We also summarize of how to take advantage of the biology of
739　female *Cx. pipiens* to lay eggs in individual rafts and thereby screen a smaller proportion of
740　offspring from each female for desired mutations. The methods presented here have been
741　optimized for *Cx. pipiens* but can be adapted, with small adjustments, to edit the genomes of
742　other mosquitoes or insects. Additionally, the micromanipulation and microinjection protocols
743　described here are amenable to injecting several different materials into insect embryos,
744　including transposons, dsRNA or other endonucleases such as TALENs or Zinc-Finger nucleases.
745　Moreover, the beveling protocol generates microinjection needles with variable opening sizes
746　therefore creating needles that can be used for injecting a wide variety of injection materials.
747　The open size of the needle can be inferred by slowly decreasing the pressure of air after the
748　needle has been beveled, and noting the pressure at which bubbles of air stop flowing from the

749   tip of the newly opened needle. The less air pressure that is needed to produce bubbles indicates
750   that the needle has a larger the opening size, and conversely, higher air pressures indicate that
751   the needle has a smaller opening size. Needles with a larger opening sizes are better for injecting
752   larger particles and more viscous materials, but will likely cause greater damage to the embryo
753   and therefore reduce survival.
754
755   Microinjection experiments are in many ways a race against the clock. First, one must inject
756   individual embryos within the first 2 hours of oviposition, before the chorion hardens and
757   blastoderm formation occurs. Therefore, it is imperative to note when the mosquitoes were first
758   placed in the oviposition chamber, how long it takes to manipulate the eggs for injection (we
759   recommend no more than 20-30 minutes), and how long it takes to inject all embryos. Time is
760   also limited when screening adult mosquitoes for mutations as *Cx. pipiens* are quite sensitive to
761   their surroundings and mosquitoes generally only survive for 3-5 days in individual glass vials.
762   Therefore, it is imperative to screen mosquito pupal exuvia as quickly as possible. Alternatively,
763   one could also extract genomic DNA from a single mosquito leg[14]. In order to do so, however, the
764   mosquitoes should first be anesthetized on ice. As we were weary of how the cold treatment and
765   loss of limbs might impact mosquito survival and their ability to mate and lay viable eggs, we
766   decided to instead screen the discarded pupal exuvia for mutations. The level of gDNA within the
767   exuvia is likely lower than inside a mosquito leg, and this does add extra effort and time to
768   separate mosquitoes at the pupal stage, but also ensures that all of the adults are unmated when
769   they are released into their appropriate cages, which is absolutely vital. We feel that the results
770   are worth it, but encourage others to consider if removing a leg might be a better course of action
771   for their experimental needs.
772
773   The best way to expedite screening for mutations is to inject a plasmid containing a genetic
774   marker, such as a fluorescent protein, flanked by homologous sequences on either side of the
775   cut site. Rates of homology directed-repair (HDR), knock-in mutations are generally lower than
776   non-homologous end-joining (NHEJ), knock-out mutations (HDR=0.71%; NHEJ=24.87%;[22]).
777   Therefore, the insertion of the marker will likely occur with a much lower frequency, but the
778   time-savings offered by being able to quickly screen mosquito larvae under a fluorescent
779   microscope may be worth the risk, depending on the project. Additionally, rates of HDR and
780   knock-in mutations are enhanced when one also injects a plasmid containing the genetic
781   sequence of the Cas9 protein, driven by the a well-characterized embryonic promotor, and the
782   gRNA driven by the U6 promotor[24,26]. This is likely because early in embryonic development,
783   when nuclei are rapidly dividing (S phase of the cell cycle), the error-prone but expedient NHEJ
784   mechanism seems to be the preferred method of repairing double-stranded breaks (reviewed[18]).
785   However, as embryogenesis progresses the cellular machinery is primed to use the more accurate
786   HDR mechanism to repair double-stranded breaks (late S phase and G2 phase). Injecting a
787   plasmid that contains the Cas9 sequence early in embryonic development allows the Cas9 protein
788   to reach most target cells while the embryo is still in its syncytial state and before cellular
789   membranes have formed, but the slight delay required to transcribe and translate the Cas9
790   protein seems to increase the likelihood that the endonucleasewill be active at a time when the
791   developing embryo is more likely to use HDR to accurately repair double-stranded breaks in
792   genomic DNA. For example, Lin et al.[27] discovered that rates of HDR were enhanced to ~33%

793 when the Cas9 protein complexed with gRNA were injected in human cell lines arrested in the M
794 phase of the cell cycle. An additional benefit of delivering Cas9 and gRNAs on plasmids is that
795 they are less viscous and therefore less likely to clog the needle during injections (R. Harrell,
796 personal observation). However, until embryonic promoters are characterized and validated in
797 *Culex* mosquitoes, we recommend injecting Cas9 protein or mRNA as described here.
798
799 Additionally, given the amount of time that one must dedicate to rearing and screening
800 mosquitoes, we recommend having at least one full-time technician, student or researcher
801 devoted to this task. We also recommend strategically considering how many null mutants one
802 needs for a given experiment and the importance of genetic diversity within the null line. While
803 we were able to identify mosquitoes in the F_1 and F_2 generations that had the mutation, only a
804 handful of the F_2 mosquitoes survived to adulthood the heterozygous mutant mosquitoes failed
805 to produce viable offspring. We think that this is much less to do with the mutation, and more
806 likely is a result of the lengthy period of time that both male and female mosquitoes were
807 confined to glass tubes while we were screening them. This prolonged period of isolation most
808 likely interfered with their ability to successfully mate and, in the case of females, obtain a blood
809 meal and lay viable eggs. Outcrossing for a single generation and/or having optimized screening
810 techniques in place should prevent this issue from occurring in the future. Therefore, by following
811 this protocol we are confident that researchers will be able to successfully generate null lines of
812 *Culex* mosquitoes and, with minor adjustments, additional insects.
813

823

824 **DISCLOSURES:**
825 RH works for the Insect Transformation Facility, which provides services in insect genetic
826 modification.
827

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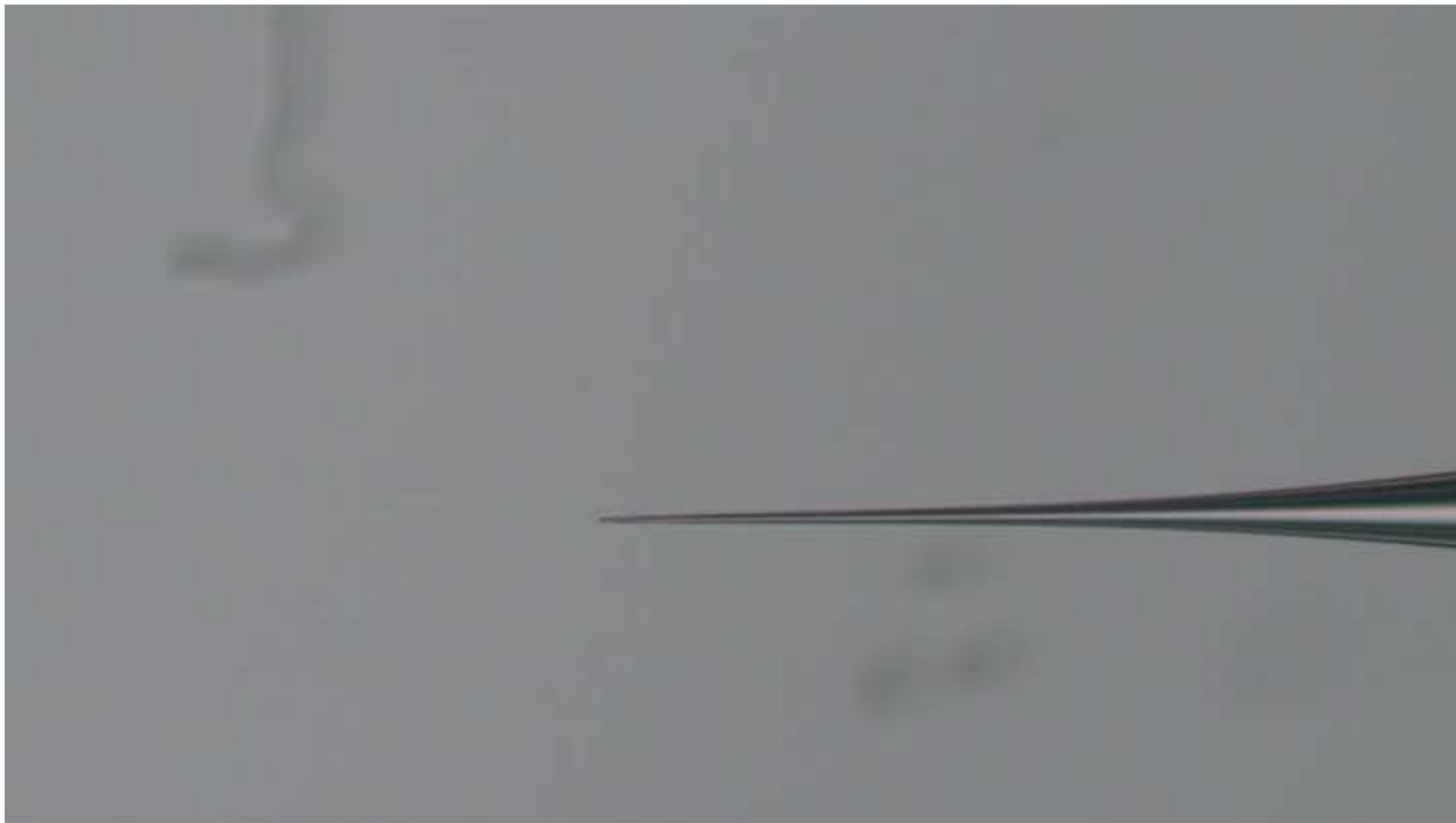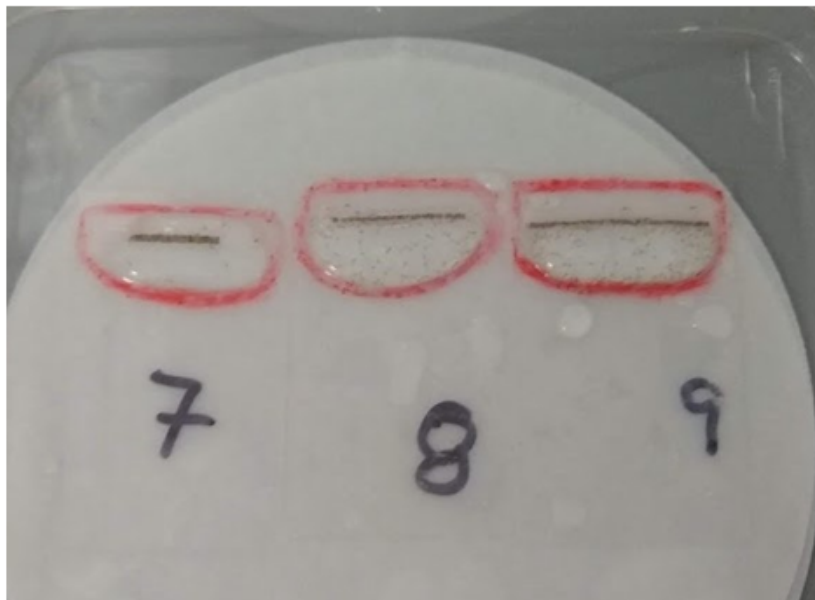892 engineering by controlled timing of CRISPR/Cas9 delivery. *eLife*. **3,** p.e04766 (2014).

893

Figure 1

Figure 2

A

B
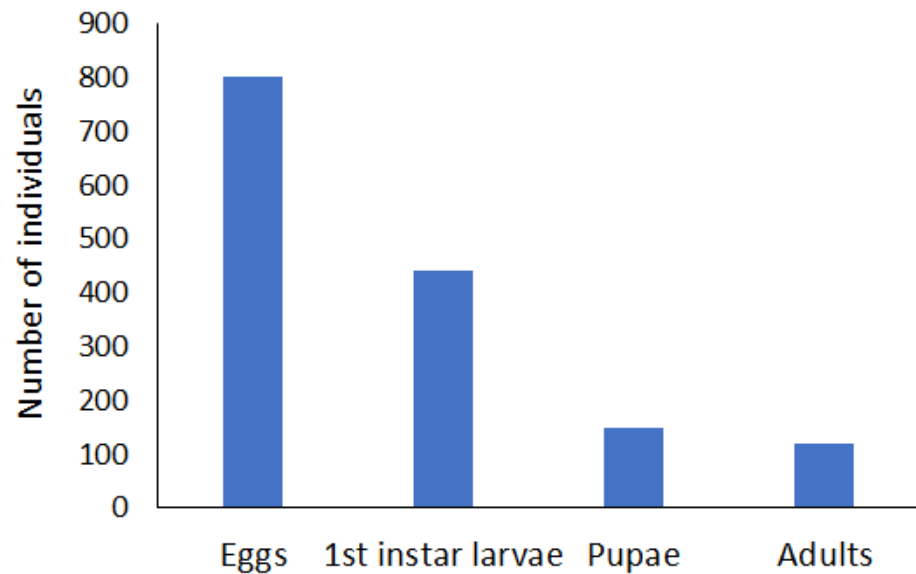
Figure 3

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Artificial Membrane Feeder	Hemotek	SP5W1-3	Company location: Blackburn, UK
ATP	Invitrogen	18330019	Company location: Carlsbad, CA, USA
Borosilicate glass mirocapillary tubes, 1 mm outer diameter	World Precision Instruments	1B100-6	Company Location: Sarasota, FL, USA
BV10 Needle Beveler	Sutter Instruments	BV-10-B	Company Location: Nobato, CA, USA
Whatman Circular filter paper (12.5 cm)	Sigma Aldrich	WHA1001125	Company Location: St. Louis, MO, USA
Conical tube (50 mL)	Thermo Fisher Scientific	339652	Company Location: Waltham, MA, USA
Fisherbrand course filter paper with fast flow rate	Thermo Fisher Scientific	09-800	Company Location: Waltham, MA, USA
Cover glass (24 x 40 mm)	Thermo Fisher Scientific	50-311-20	Company Location: Waltham, MA, USA
Dental dam	Henry Schein Inc	1010171	Company Location: Melville, NY USA
Scotch double-sided tape	Thermo Fisher Scientific	NC0879005	Company Location: Waltham, MA, USA
FemtoJet 4i microinjector	Eppendorf	5252000021	Company Location: Hamburg, Germany
Glass vial (2 dram)	Thermo Fisher Scientific	033401C	Company Location: Waltham, MA, USA
Halocarbon oil	Sigma Aldrich	H8898-50ML	Company Location: St. Louis, MO, USA
P-2000 Laser Needle Puller	Sutter Instruments	P-2000/G	Company Location: Nobato, CA, USA
Parafilm	Thermo Fisher Scientific	50-998-944	Company Location: Waltham, MA, USA
PC-100 Weighted Needle Puller	Narishige	PC-100	This is compatabile with the earlier PC-10 model,
Phire Direct PCR Kit	Thermo Fisher Scientific	F140WH	Company Location: Waltham, MA, USA

Kodak Photo-Flo (1%)	Thermo Fisher Scientific	50-268-05	Company Location: Waltham, MA, USA
Quartz glass mirocapillary tubes, 1 mm outer diameter	Capillary Tube Supplies Limited	QGCT 1.0	Company Location: Cornwall, UK
Guide-it™ sgRNA Screening Kit	Takara, Bio USA	632639	This kit allows you to determine if gRNAs cut
Sigmacote	Sigma Aldrich	SL2-100ML	Company Location: St. Louis, MO, USA
Small petri dishes (35X10 mm)	Thermo Fisher Scientific	50-190-0273	Company Location: Waltham, MA, USA
Sodium citrate chicken blood	Lampire biologicals	7201406	Company Location: Everett, PA, USA
Fisherbrand Square petri dish (10 cm x 10 cm)	Thermo Fisher Scientific	FB0875711A	Company Location: Waltham, MA, USA
Tegaderm	Henry Schein Inc.	7771180	Company Location: Melville, NY USA
Tropical fish food	Tetramin	N/A	
Whatman filter paper	Thermo Fisher Scientific	09-927-826	Company Location: Waltham, MA, USA
Whatman filter paper, 4.25 cm	Sigma Aldrich	1001-042	Company Location: St. Louis, MO, USA

Reviewer #1:
Manuscript Summary:
In the protocol "Preparing and injecting embryos of Culex mosquitoes to generate null mutations using CRISPR/Cas9", the authors present a clear protocol for the use of CRISPR/Cas9 in Culex pipiens (and Culex quinquefasciatus). Overall, the protocol is comprehensive and detailed. City and State/Country information for the sourcing of materials would be helpful for the final table. In addition, the strain of Culex pipiens used must be included. Also, information on the strain sharing needs to be included as to where the mosquitoes can be sourced. Below are a few minor suggestions/comments that will hopefully strengthen an already well-written protocol.

We thank this reviewer for his/her insightful comments and critiques. We have added the City, State and Country information for sourcing materials into the Excel table. We also now indicate the strain of mosquitoes that we used and where they are housed in the last paragraph of the introduction (lines 101-104).

Major Concerns:
No major concerns.

Minor Concerns:
1.1 - CHOP CHOP is great, but maybe cite it as an example of good design programs. In our lab we've sometimes had better success manually designing sgRNAs.

We appreciate the suggestion and have modified statement 1.1 to reflect that manually designing gRNAs is an option and that there are other free programs available.

1.3.1 - I would recommend against stating that ordering sgNRA is the most expensive. Genscript now offers 2ng of sgRNAs for <$30 (academic), this is actually cheaper than the cost of a forward oligo and synthesis using the NEB ENgen kit with a cleanup column.

Thank you for the tip! We have added Genescript as an option and removed the phrase that directly ordering gRNAs is the most expensive option.

1.3.2 - gRNAs are not made by PCR amplification, the templates for isothermal synthesis are. In addition, it might read confusingly for some people since in vitro synthesis is usually driven by the T7 viral promoter, or SP6.

We agree that this is a very helpful and important distinction. We have now modified this section to state gRNAs can be created in the lab using PCR amplification to generate the templates for subsequent isothermal synthesis, and then refer readers to the Port et al. and Kistler et al. manuscripts for details.

1.3.3 - In cases where a gene reference is given, please also indicate the CPIJ accession from Vectorbase.

We agree with this statement, but do not mention any specific gene in this portion of the protocol.

1.4.1 - Same comment as previously regarding cost. For a protocol, simply list the possible sources of reagents, let the user determine expense.

Thank you. We have removed the statement regarding the expense.

1.4.2 - This comment links with (1.3.3), if the use of on-plasmid sgRNA expression is considered for the protocol, then alternatives to the use of Cas9 protein should also be given, eg/ mRNA, plasmid

We agree and do state provide information on how researchers might use a plasmid to express the Cas9 protein. We now also describe how researches might inject Cas9 mRNA into embryos without getting into the details of which specific promoters work best in each species (now section 1.4.3).

1.4.3 - Cas9 is not delivered by a plasmid unless the Cas9 protein contains a sequence binding motif present on the plasmid. Consider "Cas9 may also be expressed in vivo through plasmid-based expression" or similar. Also, the vas2 promoter is characterized for An. gambiae, while in Ae. aegypti, the vasa promoter region contains a large 5'-UTR intron (>10KB), Incidentally, in our lab, we have built and used polyubiquitin Cas9 constructs (with large T antigen SV40 terminator), it is functional in embryo for knock-ins, so it must work, perhaps not as efficiently as protein for indels, but for knock-in, it may be advantageous to have plasmid-based expression of Cas9 since the donor also needs to be present at the time of cleavage (protein has NLS, plasmids rely on nuclear membrane dissolution). The timing of protein vs plasmid helper for knock-in may be due to many factors. Unless you have a characterized promoter for Cas9 expression on plasmid in Cx. quinquefasciatus, it may be best to simply mention plasmid-based expression without the discussion of promoters in other species, or to avoid mention of plasmid entirely.

Thank you very much for this important distinction. We have modified what is now 1.4.4 to state "Cas9 protein may be expressed in vivo Cas9 protein may also be expressed in vivo through plasmid-based expression. In this case, it is best to have the expression of Cas9 on the plasmid driven by an embryonic promoter that has been well-characterized in the target species.

Note: To date, embryonic promoters have not been well-characterized in Culex mosquitoes, and therefore injecting purified protein or Cas9 mRNA is recommended."

1.5.1 - Indicate that these are final concentrations.

Thank you. We have done this.

Line 163 - City and State/Country for Sutter Instruments.
Line 198 - Company, City and State/Country for PC-10

Thank you. We have included the location for both Sutter Intstruments and Narishige Intl., which is the company that makes the PC-10.

Section 2 - Are the capillary tubes treated prior to pulling?

The capillary tubes are siliconized before they are pulled, and we have indicated this in the text. Thank you for asking!

2.3.2 - Requires an actual volume (or estimate). Note that the manual for the Sutter BV-10 recommends 1-2 drops, 13 drops is roughly 450 µL, which is considerably more than 'a little extra'.

We thank the reviewer for this comment and have indicated that the approximate value of pedestal oil is 520 uL. We have also included a photo of the BV-10 manual below, indicating that the manual does recommend applying 10 drops of oil. Therefore, adding 13 drops is slightly more than the protocol recommends.

for pipette tips in the .05 um to ~ tips in the .5um to 1um range. Diamond coated plates are ... tips and can be ordered from Sutter Instrument Co.

Installation

The following installation instructions are repeated from the Setup section (page 2):

1) Unscrew the three screws holding the upper retaining ring to the lower retaining ring.

2) Identify the abrasive surface on the grinding plate. The top abrasive surface has a speckled or grayish finish and is marked with a part number. Place grinding plate between the two retaining rings with abrasive surface facing the top retaining ring as per Figure 2. Secure in place by screwing upper retaining ring to lower retaining ring. Tighten screws down until you feel a slight resistance and then turn them another 1/8 turn.

3) Place ~10 drops of pedestal oil onto the optical flat surface of the pedestal.

4) Place grinding plate assembly onto pedestal. The magnets (embedded in the rotating base and the lower retaining ring) will attract each other and squeeze out the excess oil between the two glass surfaces to form a parallel oil bearing. After several hours, this oil will be forced out and the optical flat must be re-oiled. Indication of insufficient oil is a jerky movement of the abrasive plate or failure of the plate to turn. *Do not allow the beveler to be operated under these conditions, or damage to the optical flat surface may occur.*

5.4.7 - Halocarbon 27? Also, estimate drops in µL.

Thank you for this good catch. We now indicate that the approximate volume is 20 ul.

6.6 - Please describe briefly how oil is washed off.

Thank you for this suggestion. We have broken 6.6 into 4 sub-steps and have provided more information on how to rinse off the Halocarbon oil without damaging the embryos (now section 6.6.1).

7.5 - Please provide approximate ratios of G0 to wild type for the backcrosses. Also, for G0 male backcrosses, one male could inseminate 5-10 females (assuming this is similar to Ae. aegypti in Helsinki and Harrington), so as males eclose, they need to go into virgin cages of females or they don't have an opportunity to mate. Please provide additional details on the backcrossing.

Thank you for this recommendation. We have added additional information to step 7.5 to clarify how we set-up the back-crosses. We did indeed use a higher ratio of virgin female mosquitoes in cages with injected male mosquitoes to increase the number of F1 progeny that were produced.

Line 489 - Outcrossing refers to a cross to an individual of a different genetic background. Backcrossing refers to a cross to an individual of the same/similar

background. Why is outcrossing used here instead of backcrossing to the parental lab strain?

This is another helpful catch. Thank you! The mosquitoes were backcrossed to the non-injected parental strain, and we have corrected this in the text.

Line 540 - Please provide Vectorbase CPIJ accession number, or include the NCBI genome build version and where it can be obtained.

Thank you for this catch. We included this in the figure legend, but missed it in the results section. The cycle sequence in Cx. pipiens (KM355981) was obtained from targeted RACE and submitted to NCBI for the publication of Meuti et al. 2015 "Functional circadian clock genes are essential for the overwintering diapause of the Northern house mosquito, Culex pipiens."

Line 557 - TALENs.

This has been changed as well. Thank you.

Reviewer #2:
Manuscript Summary:
The manuscript submitted by Meuti (Nicol) et al. describe detailed protocol for gene knockout experiment using CRISPR/Cas9 Culex pipiens mosquito. It is known that some basic operations, especially microinjection, at this mosquito genera involves technical obstacles (such as the hard chorion), which might be a reason for the far fewer number of publicated studies in which transgenic or genome editing was attempted to this mosquito compare to other mosquito genera. The manuscript by Meuti (Nicol) et al. provides detailed protocol from very setting up stage of experiments to genetic crossing to obtain desired mutants in step-by-step manner. I see many merits of this protocol accompanied by visual instructions to be available for many researchers for deep understanding of Culex mosquitoes biology.

Major Concerns:
Along with the detailed description for the method to obtain good needle. there will be huge benefit to show an example picture of that. First comers usually do not know how the ideal shape of needle-end looks like during the optimization step.

Thank you for this suggestion. We now include an image of a properly pulled and beveled borosilicate needle (new Figure 1).

A good planing of the genetic cross after injection is important to ensure retrieving mutated allele and fix it in population. Although the description about this process is well written, there seems to be more improvements needed. For instance, in Step 7.5, I do not see the reason why only females of F0 generation are used as a founder of mutant line. Male mosquito can also transmit mutated alleles, and sometime it is more effective than using female (crossing with multiple WT females is possible). Also authors should note the importance of separating each sex before or immediately after the emergence to prevent inter-crossing, and elaborate how to achieve it.

We fully appreciate this critique and suggestion. We have modified section 7.5 to make it more clear that we recommend (and did!) setting up crosses between both virgin injected male offspring and virgin un-injected (wild type) female offspring as well as virgin injected female offspring and virgin un-injected (wild type) male offspring, and include information on the ratios that we used and recommend. We have also clarified how we ensured that all adults were unmated before introducing them into their cages.

Also, in Step 8.8.3, please describe how to infer the sequence of mutated allele in heterozygous genotype in more detail.

Thank you for this helpful suggestion. We have added additional details in 8.8.1-8.8.3 to describe how to identify suspected indels. Specifically, we recommend also extracting gDNA from wild-type individuals, performing PCR and using these as a control to compare the band position and size with the PCR products that were generated in the F1 progeny.

L556 although the author say the protocol are "amenable to injecting any material into insect embryos", this argument is exaggerated. There still be difficult material to inject such as large particle and highly viscous fluids.

We greatly appreciate this point and have modified the text to state that this protocol is amenable to injecting several different materials into insect embryos. We also now point out that by increasing the bevel of the needle, one can generate needles with various opening sizes that can allow researchers to inject larger particles and more viscous materials, but that this might cause more damage to insect embryos.

Minor Concerns:
I was not able to find "sgRNA Screen Kit It kit" in lineup of TAKARA Bio products. Probably, "Guide-it"?

Thank you very much for catching this. We have fixed this in the text and table.

L566 The study cited as Ref 14 actually used a leg for genotyping.

Thank you for pointing this out. We have added this reference here.