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Melting dsDNA donor molecules potentiates precision genome editing in *C. elegans*

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14   **Running Title:** High HDR efficiencies using melted dsDNA donors.

15   **Key Words:** CRISPR, HDR, Genome Editing, Donor DNA

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24 **ABSTRACT**

25 **CRISPR genome editing has revolutionized genetics in many organisms. In the nematode**

26 ***Caenorhabditis elegans* one injection into each of the two gonad arms of an adult**

27 **hermaphrodite exposes hundreds of meiotic germ cells to editing mixtures, permitting the**

28 **recovery of multiple indels or small precision edits from each successfully injected animal.**

29 **Unfortunately, particularly for long insertions, editing efficiencies can vary widely,**

30 **necessitating multiple injections, and often requiring co-selection strategies. Here we show**

31 **that melting double stranded DNA (dsDNA) donor molecules prior to injection increases the**

32 **frequency of precise homology-directed repair (HDR) by several fold for longer edits. We**

33 **describe troubleshooting strategies that enable consistently high editing efficiencies**

34 **resulting, for example, in up to 100 independent GFP knock-ins from a single injected**

35 **animal. These efficiencies make *C. elegans* by far the easiest metazoan to genome edit,**

36 **removing barriers to the use and adoption of this facile system as a model for understanding**

37 **animal biology.**

38

39   In the nematode worm *C. elegans*, genome editing can be achieved by direct injection of

40 Cas9 guide-RNA ribonucleoprotein (RNP) complexes into the syncytial ovary (CHO *et al.* 2013;

41 PAIX *et al.* 2015; DOKSHIN *et al.* 2018). In the worm germline, such injections afford the editing

42 machinery simultaneous access to hundreds of meiotic germ nuclei that share a common

43 cytoplasm. Under optimal conditions the frequency of F1 progeny with indels caused by non-

44 homologous end joining (NHEJ) can be greater than 90% of those progeny expressing a co-

45 injection plasmid marker gene (DOKSHIN *et al.* 2018). Leveraging these high cutting efficiencies,

46 precise genome editing is readily achieved using short (under ~200 nucleotide [nt]), single-

47 stranded oligodeoxynucleotide (ssODN) donors, permitting insertions of up to ~150 nt in length

48 (ARRIBERE *et al.* 2014; ZHAO *et al.* 2014; PAIX *et al.* 2015; PRIOR *et al.* 2017; DOKSHIN *et al.*

49 2018). However, with longer dsDNA donors (~1kb), HDR events are recovered at lower

50 frequencies, require more complex protocols, high concentrations of the donor DNA, and typically

51 require screening the broods of multiple injected animals (TZUR *et al.* 2013; ARRIBERE *et al.* 2014;

52 KIM *et al.* 2014; WARD 2015; PAIX *et al.* 2016; SCHWARTZ AND JORGENSEN 2016; PAIX *et al.* 2017;

53 DOKSHIN *et al.* 2018; FARBOUD *et al.* 2019; SILVA-GARCIA *et al.* 2019; VICENCIO *et al.* 2019).

54   There are multiple reasons why longer repair templates may be less efficient as donors for

55 HDR compared to ssODNs. First, empirical studies suggest that long dsDNA is more toxic than

56 short single-stranded DNA (MELLO *et al.* 1991), limiting safe donor concentrations to less than

57 200 ng/µl for ~1kb donors. Second, upon injection into germline cytoplasm, dsDNA molecules

58 quickly form large extra-chromosomal arrays via both end-joining and homologous recombination

59 pathways, and appear to do so while sequestered away from genomic DNA (STINCHCOMB *et al.*

60 1985; MELLO *et al.* 1991). Concatenation of donor molecules into large arrays would have the

61 effect of lowering the number of individual molecules available to access and to template repair at

62 the target site double strand break (DSB). Moreover, if injected DNA assembles concatenates

63 while sequestered from the nuclear DNA—perhaps within de novo nucleus-like organelles

64 (FORBES *et al.* 1983)—this process could preclude templated repair of a genomic target site until

65 after the sequestered concatenates gain nuclear access after nuclear envelope breakdown occurs

66 post-fertilization.

67   In a recent study, we showed that CRISPR-mediated HDR could be increased ~4-fold by

68 mixing, melting, and re-annealing overlapping donor molecules to create donor templates with

69 single-stranded overhangs (DOKSHIN *et al.* 2018). In those previous studies, we limited our

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70   analysis to a cohort of F1 'Roller' progeny that express the co-injection marker gene *rol-6*

71   *(su1006)*. Here, to explore editing efficiency outside the Roller cohort, we scored the entire brood

72   of each injected animal for precisely edited progeny that incorporate and express fluorescent

73   protein markers (GFP or mCherry). We show that the vast majority of insertions occurred later in

74   the brood, after the cohort of progeny that express the Roller phenotype. Whereas overhangs

75   improved the frequency of editing among the F1 Rollers (DOKSHIN *et al.* 2018), they had no benefit

76   within this latter segment of the brood. Instead, melting the donor molecules, alone, sufficed to

77   increase the HDR frequency to as high as 50% of the post-injection progeny. We provide a protocol

78   and troubleshooting strategies that enable even a novice injector to achieve their editing goals and

79   to optimize editing efficiencies.

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89   **MATERIALS AND METHODS**

90   Detailed editing protocol is provided in Supplemental Material, **File S1**.

91   Strains and genetics

92   All the strains were generated in the Bristol N2 background unless specified otherwise and

93   cultured on normal growth media (NGM) plates seeded with OP50 bacteria (BRENNER 1974).

94   Strains used in this study are listed in Supplemental Material, **Table S1**.

95   At CSR-1 locus, GFP was introduced between FLAG::linker(9bp) and TEV in

96   FLAG::linker::TEV::CSR-1 strain.

97   Scoring methodology

98   Injected P0 animals were individually cultured on NGM plates at room temperature (22°C- 23°C)

99   unless specified otherwise. P0 animals with more than 100 post-injection progeny and at least 20

100   Rollers were selected— except at 100 ng/µl and 200 ng/µl of dsDNA donor where number of

101   Rollers can be lower than 20 due to toxicity— and their F1 progeny were scored between 72 and

102   90 hours post-injection. All the F1 progeny from each brood were mounted onto 2% agarose pads

103   and screened under fluorescence microscope for GFP or mCherry expression. GraphPad Prism

104   (Version 8.4) was used to perform statistical tests and calculate P-values.

105   Oligos and donors

106   End-modified donors were generated by PCR using oligos with 5′ SP9 modifications (IDT). Oligos

107   used for to generate *hrde-1* and *F53H1.1 gfp* donors also contain 15bp linkers on either end of *gfp*

108   which also serve as PCR primers. Sequences of all the crRNAs and oligos are provided in

109   Supplemental Material, **Table S2** and **Table S3** respectively.

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114 <u>Data availability</u>

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116 The authors state that all data necessary for confirming the conclusions presented in the manuscript

117 are represented fully within the manuscript. All the reagents are available upon request.

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119   **RESULTS**

120   **Melting the donor dramatically stimulates HDR for longer edits**

121   We recently showed that melting and reannealing donor molecules to create asymmetric donors

122   with single-stranded homology arms can improve the frequency of CRISPR-mediated homology-

123   directed insertions among transformants that were positive for a transformation marker (DOKSHIN

124   *et al.* 2018). Because transformation markers can cause confounding effects or toxicity, we decided

125   to conduct an initial study in which markers were omitted altogether. For this purpose, we chose

126   to target the insertion of *gfp* into the easily scored *glh-1* locus, which encodes a VASA-related

127   DEAD-box protein that localizes robustly to germline perinuclear foci known as P granules or

128   nuage.

129   We prepared the *gfp* donor by PCR using primers tailed with 35 nt of homology to the *glh-*

130   *1* locus (**Figure 1A**). In order to separately analyze the consequences of melting and of generating

131   single-stranded overhangs we prepared three types of donor, (i) PCR products that were never

132   melted, "unmelted donors," (ii) "melted donors" that were heated and allowed to reanneal, and

133   (iii) "asymmetric melted donors" that were prepared by heating a mixture of two overlapping *gfp*

134   PCR products (one with 35-bp homology to *glh-1* at each terminus and one without (DOKSHIN *et*

135   *al.* 2018). For simplicity, we refer to denaturing and quickly cooling the donor as "melting," (see

136   Methods). We injected each type of donor along with Cas9-guide-RNPs targeting *glh-1* into the

137   core cytoplasm of the pachytene syncytium just distal to the gonad turn. Ideal injections result

138   when the flow of the injection solution extends bilaterally from the injection site into the queue of

139   oocytes at the proximal end and into mitotic region at the distal end (MELLO *et al.* 1991). Only

140   animals with two such injections—one per arm—were analyzed.

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141   As previously shown (DOKSHIN *et al.* 2018) the asymmetric melted donor outperformed

142   the unmelted donor. The asymmetric *gfp::glh-1* donor yielded 381 GFP-positive transformants

143   among 900 F1 progeny, or 42% of total post injection progeny. The unmelted symmetric donor

144   in contrast yielded half as many edits, 161 GFP-positive transformants among 740 post-injection

145   progeny, (22%). Surprisingly, the symmetric melted donor was just as effective as the asymmetric

146   melted donor, yielding 331 GFP positives among 906 F1 progeny, (37%). Thus, when the entire

147   brood is scored a melted symmetric donor was as effective as its asymmetric counterpart. For

148   melted donors, the number of GFP positive edits equaled approximately two-fifths of all post

149   injection progeny exceeding the total number of Roller transgenics typically recovered per injected

150   animal (**Figure S1 and see below**).

151

152   **Efficient HDR occurs over a broad range of donor concentrations**

153   To explore how the frequency of *gfp* edits varied over a range of donor concentration, we injected

154   unmelted or melted *gfp::glh-1* donor at concentrations of 6.25 ng/µl, 12.5 ng/µl, 25 ng/µl, 50 ng/µl,

155   100 ng/µl and 200 ng/µl (25 ng = 0.04 pmol). In order to control for injection quality, each injection

156   mix included 40 ng/µl of the *rol-6(su1006)* co-transformation marker. For each donor mix, we

157   injected 5 to 7 worms, singled those receiving optimal bilateral injections, and further analyzed

158   two worms that made at least 100 post-injection progeny, including at least 20 Rollers. We then

159   screened all the post-injection progeny—Roller and non-Roller—for germline GFP expression.

160   We noted that the overall percentage of *gfp* insertions per injected animal (40%–50% for melted

161   donors) (**Figure 1B**) was similar to levels achieved when the *rol-6* marker was omitted (**Figure**

162   **S1**), suggesting that the *rol-6* marker does not interfere with the overall efficiency of editing.

163   Surprisingly, the frequency of GFP-positive progeny per injected animal remained similar over a

164   32-fold range of donor concentrations. Melted donors consistently outperformed unmelted donors

165   at every concentration (**Figure 1B**). These results suggest that, even at the donor concentration of

166   6.25 ng/µl, the HDR efficiency may be near saturation. At donor concentrations above 25 ng/µl,

167   the frequency of Rollers per injected animal declined, suggesting that these higher concentrations

168   cause toxicity (**Figure S2**). Taken together, these findings suggest that melted donors provide high

169   rates of HDR with low toxicity over donor concentrations in the range of 6.25 ng/µl (0.01 pmol/µl)

170   to 25 ng/µl (0.04 pmol/µl). Based on these findings we chose to use 25 ng/µl of donor in further

171   investigations.

172   We next wished to examine how editing efficiencies vary among the Roller and non-Roller

173   cohorts of post-injection progeny. We found that melted donors out-performed unmelted donors

174   in both Roller and non-Roller cohorts (**Figure 1, C-E**), yielding several dozen *gfp* edited progeny

175   per injected animal (as shown in two representative broods, **Figure 1C**). Strikingly, the fraction of

176   GFP expressing progeny was much higher among non-Rollers (49%) (**Figure 1E**) compared to

177   Rollers (15%) (**Figure 1D**).

178   To confirm the generality of these findings, we targeted two additional germline-expressed

179   genes, *csr-1* and *znfx-1* (**Figure 1, F-K**). In both cases, melted donors consistently outperformed

180   unmelted donors for *gfp* and *mCherry* insertions respectively (**Figure 1, F and I**). When melted

181   donors were used, the fraction of animals with precision insertions was approximately ~10-fold

182   higher than levels obtained with unmelted donors. This enhancement was observed in both the

183   Roller (**Figure 1, G and J**) and non-Roller cohorts (**Figure 1, H and K**). We also explored whether

184   melted donors were beneficial for editing with Cas12a (CPF1) (EBBING *et al.* 2017) RNPs —

185   which recognize an AT rich TTTV protospacer adjacent motif (PAM) sequence. Indeed, Cas12a

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186   editing yielded high HDR efficiencies comparable to those achieved with Cas9 RNPs for *gfp*

187   insertion at both the *glh-1* and *F53H1.1* loci (**Figure S3**) (See **File S1** for protocol).

188

189   **Editing efficiency peaks later in the brood after the Roller cohort of progeny are produced**

190   The finding that HDR events are more prevalent among non-Roller progeny might reflect different

191   developmental competencies of germ nuclei to form these distinct types of transgenics. For

192   example, distal pachytene germ nuclei may be more receptive to recombination between the target

193   chromosomal locus and the *gfp* donor, whereas more proximal germ nuclei may be more receptive

194   to forming extra-chromosomal transgenes driven by recombination between co-injected DNA

195   molecules (see Discussion) (MELLO *et al.* 1991). To examine these possibilities, we followed the

196   production of Roller and GFP-positive progeny over the entire post-injection brood. Worms

197   receiving 'ideal' bilateral injections of an editing mix prepared with melted *gfp*::*glh-1* donor (25

198   ng/μl) and *rol-6* co-injection marker (40 ng/μl) were cultured in two groups of 4 injected animals.

199   Each group of animals was transferred every 4 hours to fresh plates to divide their broods into 12

200   segments over the next two days. Animals were transferred one more time on the third day (64

201   hours post-injection) thus dividing the progeny into 14 groups (**Figure 2A**). We then scored the

202   frequency of Roller progeny and GFP-positive progeny in each segment.

203   Consistent with the idea that Roller extra-chromosomal transgenes assemble in more

204   proximal germ cells, nearly 100% of the Roller progeny were produced within the first 28 hours

205   post injection. The frequency of Rollers peaked between 8 and 12 hours post-injection where

206   Rollers comprised 81% of the 47 progeny produced in the interval. The frequency of Roller

207   progeny remained above 60% until 20 hours post injection, declining to ~30% then 13% over the

208   next two 4-hour intervals. Rollers were virtually absent among progeny produced after 28 hours

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209   (**Figure 2B**). In striking contrast, the frequency of precision editing events was low within the first

210   24 hours and then appeared to plateau and remain high during the entire remainder of the brood

211   (**Figure 2B**). For example, only 20% of the 306 progeny produced in the first 24 hours were GFP

212   positive while an average of 54% were positive among the progeny produced thereafter (n=1327).

213   Importantly, while GFP precision editing was less frequent within the first 24 hours (where Roller

214   transgenics were found), precision editing was not under-represented within the Roller cohort. For

215   example, we found that 24% of Rollers vs 20% of all animals produced in the first 24 hours were

216   GFP positive (**Figure 2B**). Moreover, among GFP positive animals produced in this interval 60%

217   were Rollers. Thus, the Roller marker positively correlates with *gfp* editing but does so within a

218   cohort of progeny that precedes the optimal editing window for *gfp* insertion (See Discussion).

219

220   **Donor purity is crucial for best HDR efficiencies**

221   Although *rol-6* transformation precedes the optimal window of *gfp* insertion (as shown above), we

222   nevertheless found that the *rol-6* marker provides a valuable troubleshooting metric (**Figure S2**).

223   For example, while attempting to knock-in *gfp* at two different loci (*hrde-1* and *F53H1.1*), *gfp*

224   insertions were unexpectedly rare. These experiments were conducted using melted TEG-modified

225   donors (GHANTA *et al.* 2018), which typically yield as many as 100 GFP+ progeny per injected

226   worm. However, despite ideal injections that produced high numbers of Roller progeny, only 2

227   (average) Rollers were GFP positive per brood (spin-column, **Figure 3, A** and **D**). Scoring entire

228   broods for GFP, we only obtained a maximum of 18 (*hrde-1*) (**Figure 3A**, P0# 2) and 13 (*F53H1.1*)

229   (**Figure 3D**, P0#s 1 and 2) GFP-positive progeny per injected worm. The fraction of Rollers (spin-

230   column, **Figure 3, B and E)** or non-Rollers (spin-column, **Figure 3, C and F**) expressing GFP

231   stayed below 8% at both the loci. Because the number of Rollers per injected animal was near the

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232   optimal range, we reasoned that the injection quality was good, injected animals were healthy, and

233   the injection mixture was non-toxic.

234   To understand why editing was so infrequent, we randomly sequenced the target site in 25

235   F1 Rollers. In 21 of 24 Rollers, we identified non-wildtype sequences at the target site (**Figure**

236   **3G**), indicating that double-strand breaks were not the limitation. Importantly, none of these 21

237   Rollers contained *gfp* insertions (**Figure 3G**). Upon reading the sequencing trace, we found that

238   13 F1 animals contained a 15-bp insertion precisely where GFP sequences should have inserted

239   (**Figure 3G and 3H**). To our surprise, this short sequence perfectly matched a segment of the PCR

240   oligo sequences (**Figure 3I**), and thus could be explained by insertion of a primer fragment or

241   primer-dimer that was produced inadvertently during donor preparation. To test this possibility,

242   we purified the *gfp* donors by size-exclusion using SPRI paramagnetic beads or by gel-extraction.

243   Purifying the *hrde-1* donor with SPRI beads (optimized to exclude fragments smaller than 300 bp)

244   modestly increased the percentage of GFP-positive progeny to 10% of F1 Rollers (n=212; **Figure**

245   **3B**) and 32% of non-Roller progeny (n=625; **Figure 3C**). By contrast, gel-purified *hrde-1* donor

246   dramatically increased the percentage of GFP-positive progeny to 29% of F1 Rollers (n=163;

247   **Figure 3B**) and 49% of non-Rollers (n=538; **Figure 3C**), with as many as 95 GFP-positive

248   progeny from one injected worm (**Figure 3A**, P0#5). Similar results were obtained after gel

249   purification of the *F53H1.1* donor (**Figure 3, D-F**). These findings demonstrate the utility of the

250   Roller marker as a metric for troubleshooting the editing protocol and reveal the importance of

251   removing PCR-based contaminants from donor preparations to achieve best knock-in efficiencies.

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254 **DISCUSSION**

255   We initiated these investigations to explore why long (~1-kb) DNA donors were less

256 efficacious than short single-stranded oligonucleotide (ssODN) donors in *C. elegans*. We have

257 shown that melting the donor DNA dramatically enhances precision editing, enabling efficient

258 editing with shorter homology arms and at significantly lower donor DNA concentrations than

259 previously recommended (PAIX *et al.* 2015; PAIX *et al.* 2017; DOKSHIN *et al.* 2018). We show that

260 as many as 100 precisely edited progeny can be obtained from a single injected animal, an editing

261 efficiency of nearly 50% of post injection progeny, and far exceeding the typical frequency of

262 progeny transformed with simple extrachromosomal arrays (**Figure S2**) (MELLO *et al.* 1991).

263   Importantly, whereas the production of Roller transgenic progeny peaks during the first 24

264 hours post-injection, *gfp* edits peak after 24 hours and remain high through the remainder of the

265 injected animals brood. Previous studies also reported that most *gfp*-edited animals are produced

266 on the second day after injection (PAIX *et al.* 2014). These findings suggest that developmental

267 differences between distal (less mature) and proximal (more mature) germ nuclei may favor

268 formation or acquisition of distinct transgene types. For example, perhaps the large *rol-6* plasmid

269 molecules are excluded from germ nuclei, and instead rapidly assemble into cytoplasmic

270 extrachromosomal arrays that are swept by the germ plasm into developing oocytes, and only enter

271 nuclei after fertilization (as previously suggested (MELLO *et al.* 1991)). A size limitation on nuclear

272 uptake may explain why we and others have found that donors over 2 kilo-basepairs yield few

273 editing events (unpublished results) (PAIX *et al.* 2016; FARBOUD *et al.* 2019).

274   The observation that *gfp* editing peaks later, approximately 28 hours post injection, and

275 then remains high, suggests either that proximal germ nuclei tend to exclude the donor, or that

276 more distal nuclei are more receptive to recombination. Based on an ovulation rate of 23 minutes

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277 (MCCARTER *et al.* 1999), the appearance and persistence of GFP-positive progeny is consistent

278 with editing in nuclei that were in pachytene (i.e., undergoing meiotic recombination) at the time

279 of injection. Whatever the reason for the HDR enhancement caused by melting the donor it is

280 striking that the extrapolated rates of precision *gfp* insertion within these pachytene nuclei range

281 as high as 70%.

282 Donor purity is crucial to achieve high knock-in efficiencies of long inserts. Contaminating

283 primer dimers that contain homology arms can compromise HDR efficiency by integrating at the

284 target site. Removing these contaminants by gel-extracting the donors dramatically increased *gfp*

285 knock-in efficiencies. Similarly, as a time saving alternative to gel-extraction we found that

286 purification using SPRI paramagnetic beads also improves HDR efficiencies, however using the

287 optimal ratio of beads to PCR reaction was critical to removing the shorter contaminants (See

288 protocol **File S1**).

289 We do not know why melting the donor stimulates HDR. We obtained similar HDR rates

290 across the entire range of donor concentrations, indicating that donor concentrations were saturated

291 (or nearly so) at the lowest dose tested. Yet, melting the donor increased the HDR rate several fold

292 at each concentration. Thus, melting stimulates recombination by acting on events or mechanisms

293 that are independent of donor concentration. Conceivably, melting induces structural changes—

294 e.g., denaturation bubbles caused by incomplete reannealing—that promote active nuclear uptake

295 or directly stimulate repair. For example, single-stranded regions from incomplete re-annealing

296 could promote strand invasion or act as damage signals that recruit trans-acting factors that

297 facilitate HDR. Indeed, preliminary studies suggest that when we slow the cooling step to promote

298 re-annealing, melted donors perform about half as well (**Figure S4**). Interestingly, melting did not

299 stimulate the already high HDR efficiency of a shorter 400 nt donor (Data not shown). Clearly

300   more work is needed to fully explore and understand how donor-melting promotes HDR

301   efficiency.

302   Undoubtedly, the high efficiencies of precision editing achieved here owe both to the easy

303   access of worm pachytene germ cells to microinjection and to the remarkable receptiveness of

304   these cells to HDR. A parallel study suggests that editing is enhanced even further when donor 5′

305   ends are modified with tri-ethylene glycol (TEG) (GHANTA *et al.* 2018). Importantly, the

306   combination of melting and TEG modifications increases the proportion of *gfp*-sized edits among

307   the easily identified Roller progeny cohort by approximately twenty-fold from 1-2% to 20-40%.

308   For experienced injectors, a single optimally injected animal can yield more than 100 GFP knock-

309   ins (nearly two thirds of post-injection progeny), dramatically enhancing the ease and efficiency

310   of genome editing. Given these high HDR efficiencies even researchers with little worm

311   experience can now readily adopt this facile genetic animal model.

312

318   **Competing interests:** C.C.M is a co-founder and Scientific Advisory Board member of CRISPR

319   Therapeutics. Some of the findings described here are part of the patent applications filed by the

320   University of Massachusetts Medical School on which the authors are inventors.

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379

380   **Figure Legends**

381   **Figure 1**. **Melting dsDNA donors potentiates homology directed repair in *C. elegans*.** (**A**)

382   Schematic representation to insert *gfp* at the N-terminus of a protein coding gene immediately

383   down stream of start codon (atg) using symmetric melted dsDNA donors and Cas9-guideRNA

384   ribonucleoproteins (RNPs) is shown; grey segment represents sequence upstream of the start

385   codon. Precise repair (HDR) enables fluorescent protein expression. (**B**) HDR efficiencies at the

386   *glh-1* locus using symmetric unmelted (grey bars) or melted donors (black bars) with *rol-6*

387   injection marker at indicated concentrations (n=2 broods) is plotted as percentage of F1s

388   expressing GFP per injected animal (P0). Using unmelted and melted donors, HDR efficiencies at

389   the *glh-1* locus is plotted as (**C**) number of fluorescence+ animals among Rollers and non-Rollers

390   from two representative broods. Percentage of animals expressing fluorescence among, (**D**) Rollers

391   and (**E**) non-Rollers, is plotted as percentage (n = 3 or 4 broods) for *glh-1* locus. Similarly,

392   improvement in fluorescent protein insertion efficiencies with melted donors are shown for (**F-H**)

393   *csr-1* and (**I-K**) *znfx-1* loci. Each data point represents the percentage of animals expressing

394   fluorescent protein among F1s scored in each cohort per brood. Bars represent median. Number of

395   fluorescence+ animals over number of animals scored is shown above the bars. Green dots

396   represent GFP and red dots represent mCherry insertions.

397   **Figure 2. Editing occurs later in the brood after roller cohort.** (**A**) Schematic representation of

398   the experiment is shown. 4 Injected animals placed on a single plate were moved at indicated post

399   injection time points and F1 embryos laid during the time-intervals were scored for GFP as adults.

400   (**B**) Fraction of the progeny produced in each time window that are Rollers (open black bars),

401   GFP+ Rollers (open green bars) and GFP+ progeny (Rollers and non-Rollers, solid green bars) are

402   plotted as percentage. Bars represent mean value of two replicates and each replicate consists of 4

20

403   P0 animals injected with 25 ng/µl of symmetric melted donors and 40 ng/µl of *rol-6* co-injection

404   marker. Animals were cultured at 18°C-20°C.

405   **Figure 3. Purity of donor DNA is crucial for best HDR efficacy.** HDR efficiencies of donors

406   prepared by different methods of purification are plotted for *hrde-1* and *F53H1.1*. (**A**) Number of

407   GFP+ F1 Rollers and non-Rollers from two representative broods are plotted. GFP+ animals

408   among, (**B**) Rollers and (**C**) non-Rollers is plotted as percentage of animals scored in each cohort

409   per brood. Similarly, (**D-F**) HDR efficiencies are plotted for *F53H1.1* locus. (**G**) Insertions and

410   deletions identified at *hrde-1* target site in F1 Rollers from two P0s (spin-column), (**H**) sanger

411   sequencing trace of the 15bp non-random insert for a homozygous F2 animal. Partial homology

412   arms of the donor are shown in blue and the sequence that got inserted into the genome is shown

413   in red. (**I**) Schematic representation of predicted primer dimer formation is shown with 6bp perfect

414   match and mismatched 3′ tails. Part of each oligo that is homologous to the PCR template plasmid

415   is shown in black (linkers on either end of *gfp*) and the homology arms are shown in blue and the

416   sequence (Insert) that would get inserted through HDR is shown in red. All the donors were 5′

417   TEG-modified and melted. Gel-purified donors were further cleaned-up with SPRI beads (See **File**
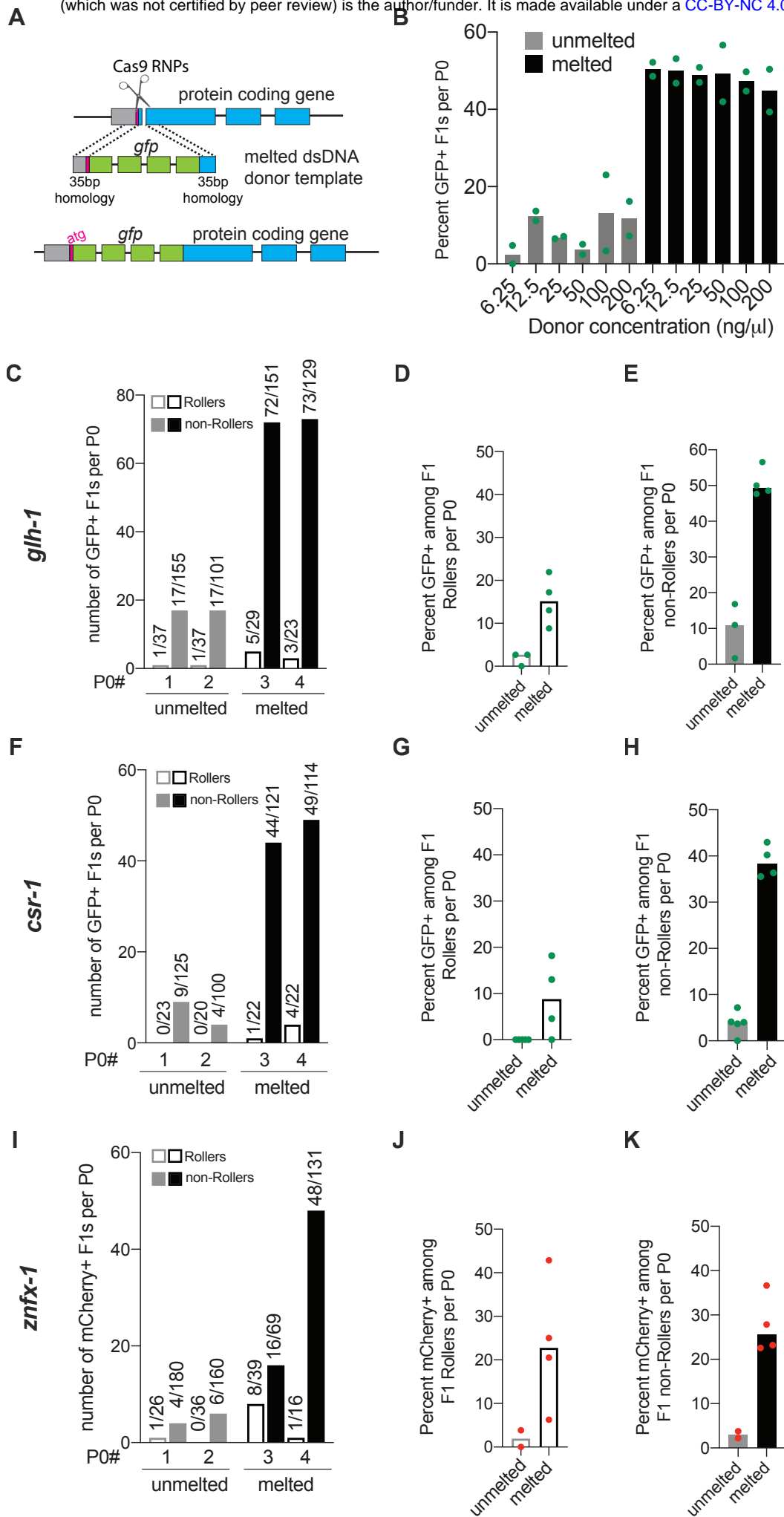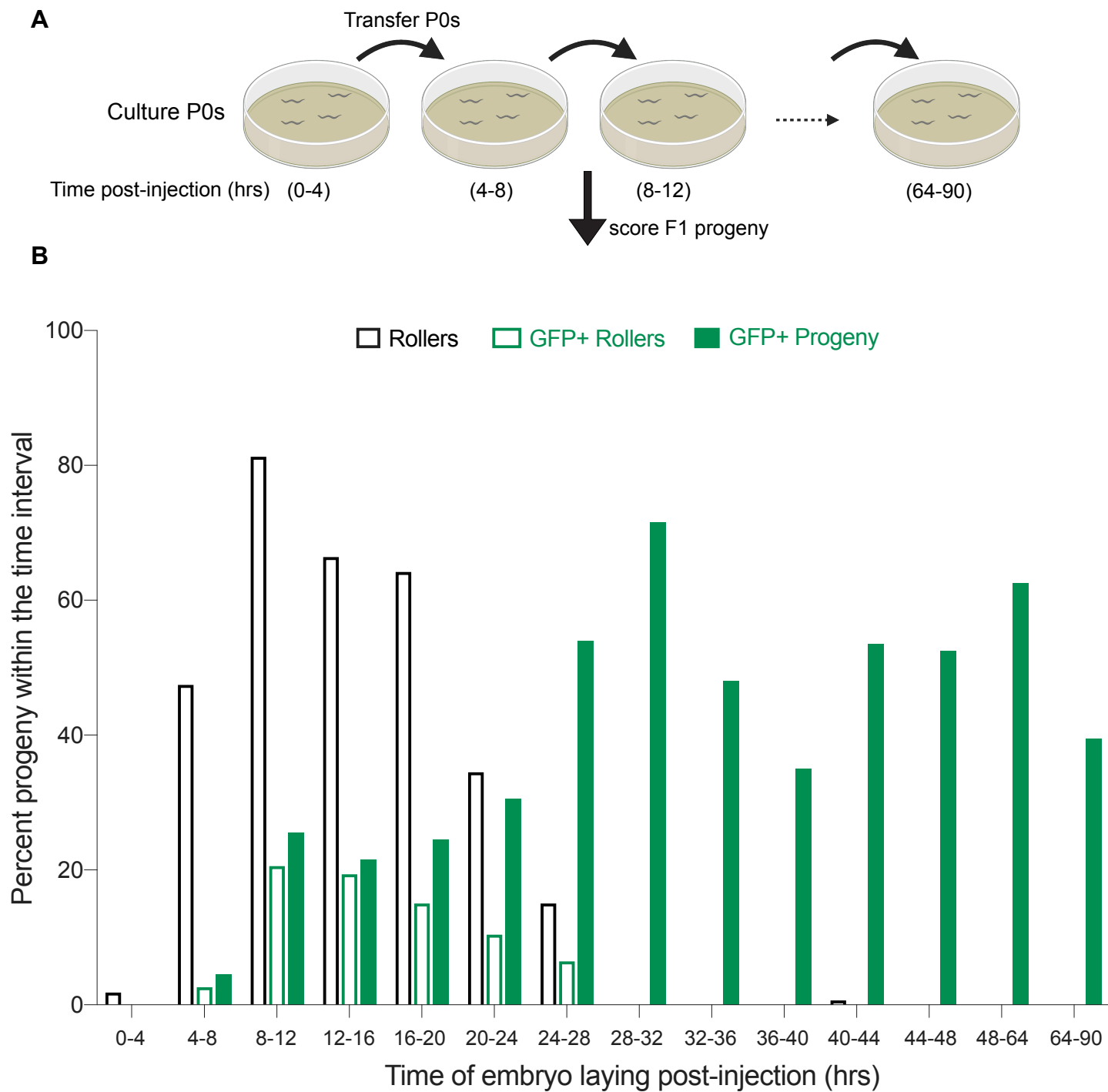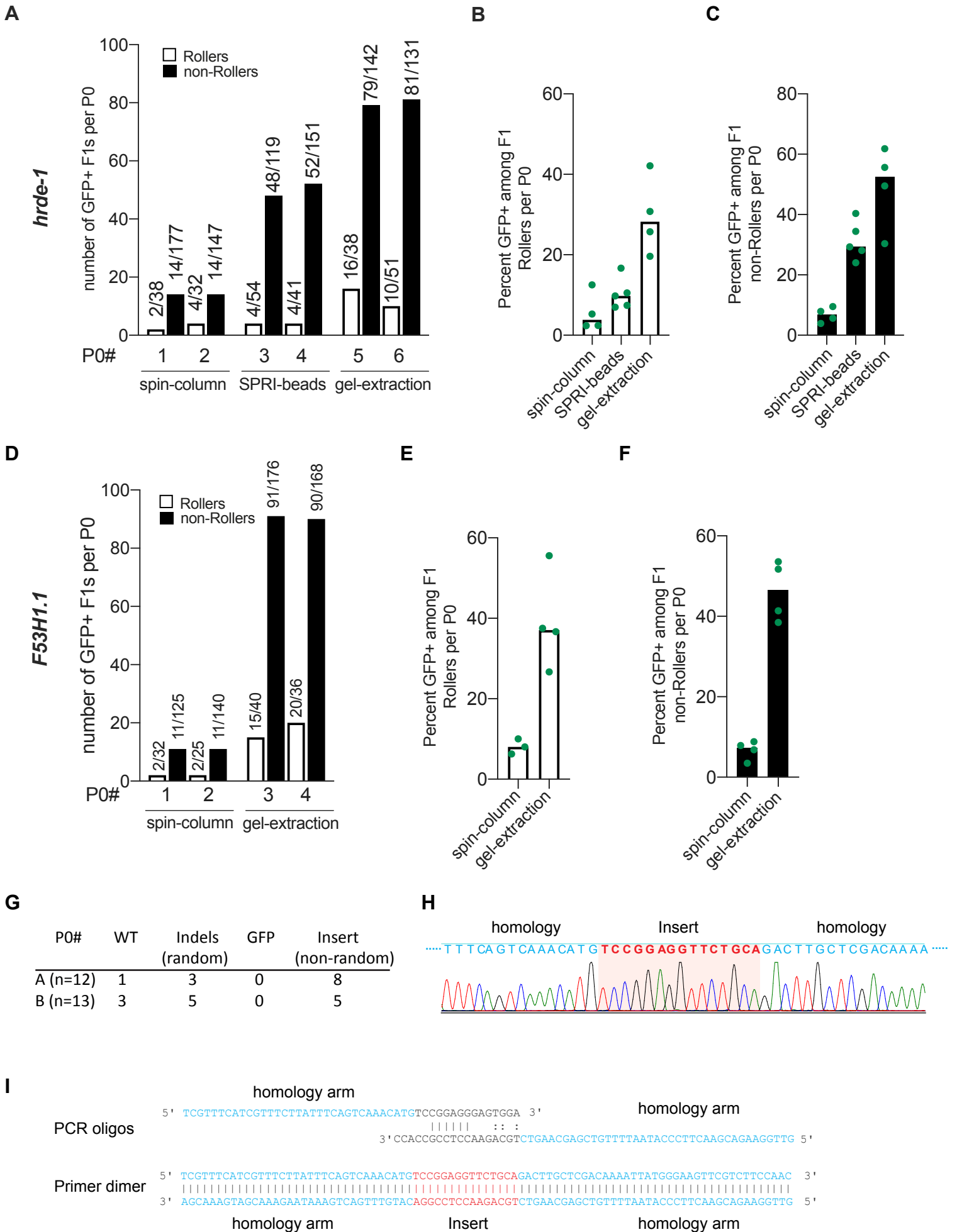
418   **S1**).

419

Figure 1

Figure 2

A

Transfer P0s

Culture P0s

Time post-injection (hrs)   (0-4)   (4-8)   (8-12)   (64-90)

score F1 progeny
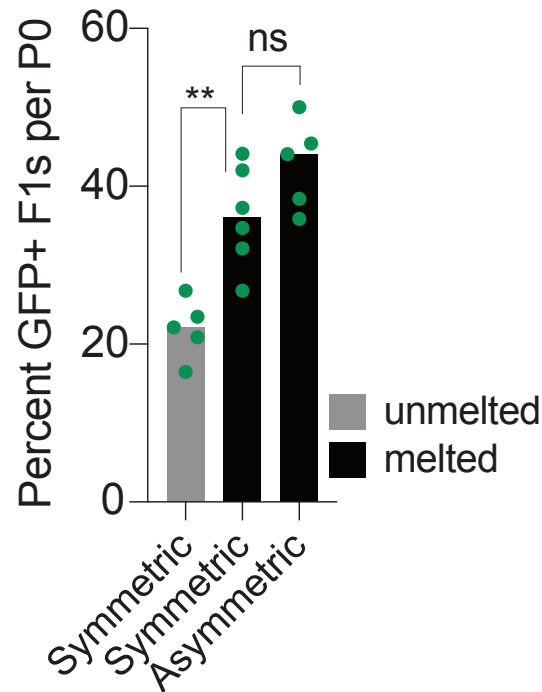
B

Figure 3

Supplementary Figures

Figure S1: Melted dsDNA donors promote homology directed repair. HDR efficiencies at the *glh-1* locus using symmetric (unmelted or melted) and asymmetric donors (n=5 or 6 broods) without *rol-6* injection marker. Each data point (green) represents the percentage of animals expressing GFP among F1s scored per brood. Bars represent median.P-values (**, 0.0087 and ns, 0.1255) were determined by Mann Whitney test (unpaired, non-parametric)
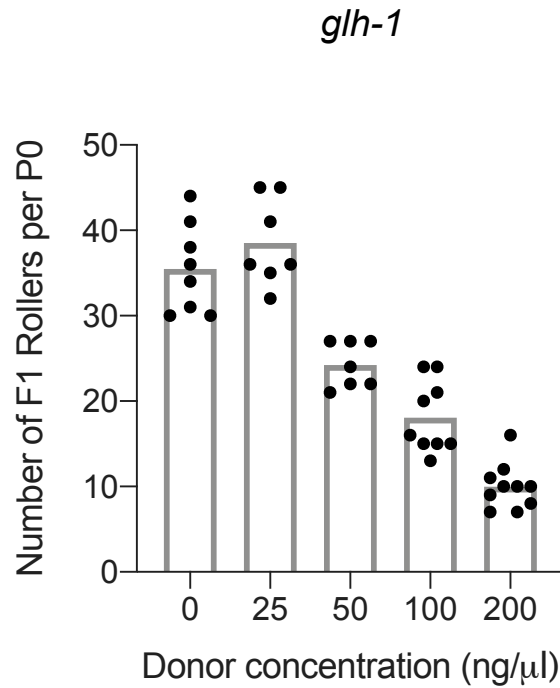
Figure S2. High donor concentrations are toxic and reduce the number of Rollers. Injection mixes contain Prf4::*rol-6(su1006)*, Cas9 protein, crRNA targetting *glh-1* locus and *gfp::glh-1* dsDNA donor with ~35bp homology arms at indicated doses. Each dot represents the number of F1 rollers obtained per P0 animal and the bar represents mean; (n=7 to 10 broods per condition). dsDNA donors were not melted.
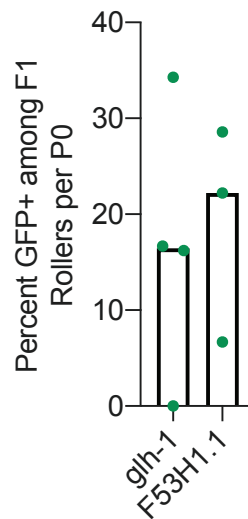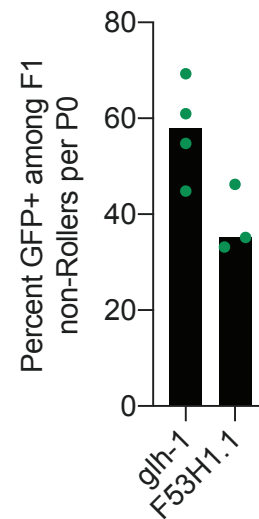
Cas12a based genome editing

A
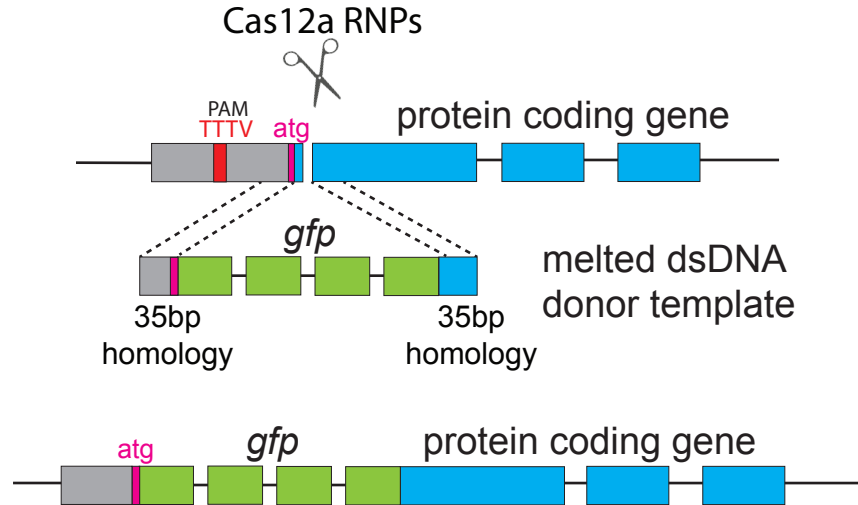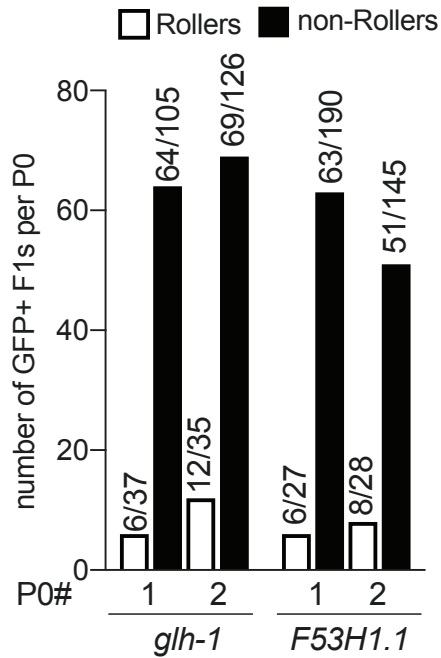
Figure S3. Precise genome editing using Cas12a nuclease and melted dsDNA donors. (A) Schematic representation of template dependent editing with Cas12a and melted dsDNA donor to insert *gfp* at the start codon (atg). Protospacer adjacent motif (PAM) for Cas12a system is TTTV, where V is A, C or G. HDR efficiencies at *glh-1* and *F53H1.1* loci are plotted as (**B**) number of GFP+ F1 animals among two representative broods, (**C**) percentage of GFP+ animals among F1 Rollers and (**D**) percentage of GFP+ animals among F1 non-Rollers; n= 3 or 4 broods. Number of GFP+ animals over number of animals scored are shown above the bars. Each data point represents the percentage of animals that are GFP+ among F1s scored in each cohort per brood and bars represent the median.
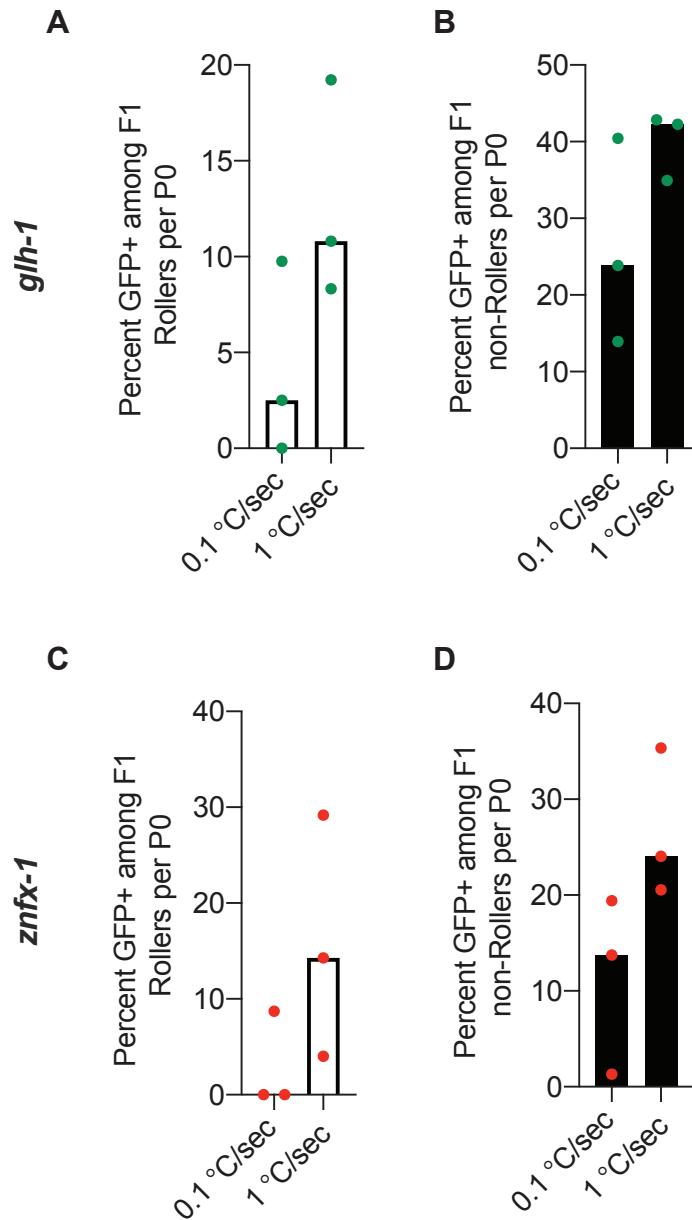
Figure S4. Quickly cooled donors act as better repair templates than slowly cooled donors. *gfp* (green dots) insertion efficiencies at *glh-1* loci are plotted for (**A**) Rollers and (**B**) non-Rollers using slow (0.1 ºC/sec) and quick (1 ºC/sec) cooled donors as percentage. (**C and D**) *mCherry* (red dots) insertion efficiencies at *znfx-1* locus. Each data point represents an F1 brood and bars represent median. Thermal cycler program for slow cooling: 95 ºC - 2:00 min; 85 ºC - 1:00 min; 75 ºC - 1:00 min, 65 ºC - 1:00 min, 55 ºC - 1:00 min, 45 ºC - 1:00 min, 35 ºC - 1:00 min, 25 ºC - 1:00 min, 4ºC- hold. Ramp down rate: 0.1 ºC/sec. See **File S1** for quick cooling conditions.

Supplemental Information

1. 1. Figure S1- Melted dsDNA donors promote homology directed repair

2. 2. Figure S2- High donor concentrations are toxic and reduce the number of Rollers

3. 3. Figure S3- Precise genome editing using Cas12a nuclease and melted dsDNA donors

4. 4. Figure S4- Quickly cooled donors are better repair templates than slowly cooled donors

5. 5. Table S1- List of *C. elegans* strains

6. 6. Table S2- Sequences of crRNAs

7. 7. Table S3- Sequences of Oligos

8. 8. File S1- Detailed editing protocol

10 **Supplemental Table S1: List of *C. elegans* strains**

Genotype	Strain Name
F53H1.1(ne4815[gfp::F53H1.1]) IV	WM703
glh-1(ne4816[gfp::glh-1]) I	WM704
csr-1(ne4483[flagx3::linker::tev] IV	WM705
znfx-1(ne4817[mcherry::znfx-1]) II	WM706
hrde-1(ne4818[gfp::hrde-1]) III	WM707
csr-1(ne4819[flagx3::linker::gfp::tev::csr-1]) IV	WM708

11

12 **Supplemental Table S2: Sequences of crRNAs**

ID#	Target locus	Sequence (5′→3′)	Notes
CMG-18	*hrde-1*	CATAATTTTGTCGAGCAAGT	To insert N-terminal tag
CMG-25	*csr-1*	AAGATGTTCAGGGCAAGTCT	To insert N-terminal tag
CMG-33	*Flagx3::linker::tev*	TATAAAGACGATGACGATAA	To insert gfp at csr-1 locus
CMG-34	*glh-1*	TTTTCTGCGAAAATGTCTGA	To insert N-terminal tag
CMG-35	*glh-1*	TGCGAAAATGTCTGATGGTTG	To insert N-terminal tag; (A.s. Cpf1)
CMG-77	*F53H1.1*	TTCCAGTTTTCGATGGGTCG	To insert N-terminal tag
CMG-79	*F53H1.1(A.s. Cpf1)*	CAGTTTTCGATGGGTCGCGGC	To insert N-terminal tag
CMG-88	*znfx-1*	AGGTTTCTGACCATTGAATA	To insert N-terminal tag

13

14 **Supplemental Table S3: Sequences of oligos**

ID#	Sequence (5′→3′)	Notes
cmo-KG475F	ATTTTCTGGAAAAATCTTAA	*gfp::glh-1* donor
cmo-KG476R	TTAGCAGCACTTTCGCTATC	
cmo-KG830F	/SP9/TCGTTTCATCGTTTCTTATTTCAGTCAAACATGTCCGGAGGGAGTGGA	*gfp::hrde-1* donor
cmo-KG831R	/SP9/GTTGGAAGACGAACTTCCCATAATTTTGTCGAGCAAGTCTGCAGAACCTCCGCCACC	
cmo-KG832F	/SP9/ATCCAAAAATCCCCAATTTTTTCCAGTTTTCGATGTCCGGAGGGAGTGGA	*gfp::F53H1.1* donor
cmo-KG833R	/SP9/ACGCTTTCGTTTGTGCTCTTTGTGCTCGCCGCGACCAGAACCTCCGCCACC	
cmo-T1193F	CTTGTTTCAGACCAATTCGCCAACCGTATTCAATGGTCTCAAAGGGTGAAGAAGA	*mCherry::znfx-1* donor
cmo-T1194R	GGCGGCGGGAGCCCTGGGGGGGCGAGGTTTCTGACCTTATACAATTCATCCATGC	
cmo17648	AATCTCAATCAGGACGGTAAAG	*hrde-1* indel detection
cmo17649	GAACTCCTAGGCATAATGTTGA	
cmo-JG55F	ACATAAAACGATAAATCGGC	*F53H1.1* indel detection
cmo-JG56R	TTCCGTGACTCTTCCATTTC	
cmo-KG825R	CGCCGTTTTACTCTCTTT	
cmo17659	CGATTGGAAGTAGAGGTTCT	*gfp::csr-1 donor*
cmo17660	ATCATGATATTGACTATAAA	
cmd-25	gaactatacttttttcaggacttaactctgacatgGATTACAAAGACCATGATGGTGACTATAAGGATCATGATATTGACTATAAAGACGATGACGATAAGGGTGGCGGAGAGAACCTCTACTTCCAATCGaaCcaAaaAcaGaaCccTagGctAgcActAaacatcttcgggcttgagctctctgagcgaacgat	ssODN donor to knock-in flagx3::linker::tev at *csr-1* locus

15

Melting dsDNA donor molecules potentiates precision genome editing in *C. elegans*

Cas9 Based Genome Editing

I. **Materials:**

1. *S. pyogenes* Cas9 3NLS (10 μg/μl, IDT)
2. tracrRNA (IDT)
3. crRNA (2 nmol or 10 nmol, IDT)
4. ssODN 4 nmol Ultramer (standard desalting, IDT)
5. PRF4::*rol-6(su1006)* plasmid (high quality Midi or Maxiprep)
6. SPRI paramagnetic beads (AMPure XP, Beckman Coulter)

Re-suspension (Stock Solutions):

1. Aliquot 0.5 μl (5 μg or 30 pmol) of Cas9 protein and store at -80°C (avoid freeze/thaw cycles)
2. tracrRNA – 0.4 μg/μl (18μM) in IDT nuclease free duplex buffer, store at -20°C (aliquots at -80°C)
3. crRNA – 0.4 μg/μl (34 μM) in TE PH 7.5 (IDT), store at -20°C (aliquots at -80°C)
4. ssDNA oligo donor – 1 μg/μl in ddH2O, store at -20°C
5. PRF4::*rol-6 (su1006):* 500 ng/μl, store at -20°C

II. **Injection mixture preparation:**

Add components of the injection mixture to the tube containing Cas9 in the following sequence:

1. Cas9 – 0.5 μl of 10 μg/μl stock (30 pmol)
2. Add tracrRNA – 5 μl of 0.4 μg/μl stock (90 pmol)
3. Add crRNA – 2.8 μl of 0.4 μg/μl stock (95 pmol) (if two guides are needed add 1.4 μl of each)
4. Pipette the mixture gently several times and incubate @37°C for 15 minutes. In our experience adding any double stranded DNA before RNP complex formation reduces HDR efficiency.
5. Add ssODN donor – 2.2 μl of 1 μg/μl stock (or)
   Add melted dsDNA – 500 ng (final concentration: 25 ng/μl for ~1kb donors or 45 fmol/μl)
6. Add PRF4::*rol-6 (su1006)* plasmid – 1.6 μl of 500 ng/μl stock
7. Add nuclease free water to bring the final volume to 20 μl and pipette gently several times.
8. To avoid needle clogging, centrifuge the mixture @14000rpm for 2 min, transfer about 17 μl of the mixture to a fresh tube and keep the tube on ice; proceed to loading the needles.

Notes:
- *All the above steps in section II can be performed at room temperature*
- *Aggregation is not an issue under these Cas9 concentrations.*
- *Final injection mixture can be stored at 4°C and re-used for several months (up to 6 months) without compromising efficiency; we have not yet tested mixes that are older than 6 months.*

Cas12a (Cpf1) Based Genome Editing

I. Materials:

1. A.s. Cas12a Ultra (10 μg/μl, IDT)
2. Cpf1-crRNA 21 bases long (2 nmol or 10 nmol, IDT)
3. ssODN 4 nmol Ultramer
4. PRF4::*rol-6(su1006)* plasmid (high quality Midi or Maxiprep)
7. SPRI paramagnetic beads (AMPure XP, Beckman Coulter)

Re-suspension (Stock Solutions):

1. Aliquot 0.5 μl (5 μg or 32 pmol) of Cas12a protein and store at -80°C (avoid freeze/thaw cycles)
2. Cas12a-crRNA – 40 μM in TE PH 7.5 (IDT), store at -20°C (aliquots at -80°C)
3. ssDNA oligo donor – 1 μg/μl in ddH2O, store at -20°C
4. PRF4::*rol-6 (su1006):* 500 ng/μl, store at -20°C

II. Injection mixture preparation:

Add components of the injection mixture to the tube containing Cas9 in the following sequence:

1. Cas12a – 0.5 μl of 10 μg/μl stock (32 pmol)
2. Add cas12a-crRNA – 2.5 μl of 40 μM stock (100pmol)
3. Add TE PH 7.5 – 3.0 μl
4. Pipette the mixture gently several times and incubate @37°C for 15 minutes
5. Add ssODN donor – 2.2 μl of 1 μg/μl stock (or)
    Add melted dsDNA – 500 ng (final concentration: 25 ng/μl for ~1kb donors or 45 fmol/μl)
6. Add PRF4::*rol-6 (su1006)* plasmid – 1.6 μl of 500 ng/μl stock
7. Add nuclease free water to bring the final volume to 20 μl and pipette gently several times.
8. To avoid needle clogging, centrifuge the mixture @14000rpm for 2 min, transfer about 17 μl of the mixture to a fresh tube and keep the tube on ice; proceed to loading the needles.

Notes:
- *All the above steps in section II can be performed at room temperature*
- *TE is added in step 3 for easier pipetting; by further diluting the crRNA stock this step can be omitted.*

III.   Donor Design and Generation

ssODN donors:

To generate ssODN donor, add 35 bases of 5′ homology sequence in front of the tag (or mutations) and 35 bases of the 3′ homology sequence at the end. Mutate the PAM site or the guide binding sequence if it is not already disrupted by the insert. If the guide binding sequence is mutated or if silent mutations are introduced between the guide cleavage site and the desired insertion site, length of homology sequence should be 35bp from the last mutation.

dsDNA donors:

Generate dsDNA donors by PCR either by using unmodified oligos or 5′ SP9-modified oligos.

1. Order unmodified (or 5′ SP9 modified) oligos with standard desalting (IDT); 35nt as homology arms and 20nt complementary to insert (eg: GFP). SP9 modifications are available at 100nmol scale from IDT.

2. Perform PCR with an insert-containing plasmid as the template for amplification; use High-Fidelity polymerase.

3. Run a few microliters of PCR on agarose gel to check if a single bright band is obtained. If non-specific amplification is observed, set up a temperature gradient and find the optimal temperature.

4. PCR clean-up: use one of the following three options depending on your experimental conditions.

    a. Purify the PCRs using spin-columns and elute DNA in 20 μl of nuclease free water. Generally, column purification is sufficient, and you may proceed to step 5. However, some primer pairs produce long (~80bp) primer dimers that may contain the entire homology arms. Spin-columns may not be able to remove dimers of this length completely. We found that these short "dimer donors" are preferentially used as templates over full-length donors with the desired insert (such as GFP). *Note*: Dimers may or may not be visible on the agarose gel.

    b. If dimer formation is a concern, use 0.6x SPRI beads (AMPure XP) to perform the clean-up instead of spin-columns. For example: add 60 μl of beads to 100μl of PCR, wash with 70% ethanol twice, elute in nuclease free water (refer to the bead manufacturer's protocol for further details).

    c. If primer dimers are clearly visible on the gel, then it is best to gel-extract the DNA. However, gel extracted DNA can be toxic, presumably due to the presence of guanidine hydrochloride (component of binding buffer) in the final elute. To reduce salt contamination, incubate the column with wash buffer for 10 min before centrifugation; repeat washes 2-3 times. Strong absorbance at 230nm on Nanodrop suggests GuHCl contamination. For best results, gel-extracted DNA should be further purified with 1x to 1.5x AMPure XP beads (strongly recommended).

5. After purification, dilute a portion of dsDNA PCR donor to 100 ng/μl and transfer about 5.5 μl to a PCR strip tube and proceed to the heating step.

6. Heat to 95 ℃ and cool to 4 ℃ using thermal cycler (95 ℃-2:00 min; 85 ℃-10 sec, 75 ℃-10 sec, 65 ℃-10 sec, 55 ℃-1:00 min, 45 ℃-30 sec, 35 ℃-10 sec, 25 ℃- 10 sec, 4 ℃-hold. Ramp down at 1 ℃/sec at every step).

7. Add melted donor DNA to rest of the injection mixture only after pre-incubating RNP complexes.

    Note: we store purified donors at -20 ℃ and melt them right before adding to the injection mix. We have not explored storage and re-use of melted donors.

IV.   <u>Micro-injection and Screening:</u>

1. Inject 5 to 10 young adults and transfer them onto individual plates. If both arms of the hermaphrodite gonad are injected, a good injection should yield 20 to 40 F1 Rollers.

2. After about 72 hours post injection, score for number of F1 Rollers and choose 2 plates with the highest number of Rollers.
   Note: We generally culture the injected animals at room temperature (~22°C-23°C).

3. a. For ssODN-based editing: choose 2 P0 plates that segregate the highest number of F1 Rollers; pick about 24 F1 Rollers from these 2 plates and place them onto separate plates.

   b. For dsDNA-based editing: Choose 2 plates that segregate the highest number of F1 Rollers and from these 2 plates, pick ~24 non-Rollers that are younger than Rollers and place them onto separate plates. Younger animals among the Roller cohorts can also be picked. For inexperienced injectors, we recommend using 5′ end-modified dsDNA donors and picking F1 Rollers.

4. To avoid false positives due to mosaicism in F1 animals, pick several F2s from each plate, perform pooled lysis and genotype. Genotyping primers should lie outside of the homology arms to avoid amplification from transiently retained donor molecules.

5. Alternatively, correct insertions of fluorescent tags can be screened under a fluorescence dissecting scope or by using high magnification fluorescence microscope. For high magnification screening, mount several F2 animals onto 2% agarose pads and immobilize with levamisole.