Engineering essential genes with a “jump board” strategy using CRISPR/Cas9

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Abstract

Figure 1: A-B. Schematic diagrams of the jump board strategy for engineering the let-7 locus. To generate the jump board strain (A), hermaphrodites of the strain EG9615(oxSi1091[mex-5p::Cas9(smu-2 introns) unc-119(+)] II; unc-119(ed3) III) carrying a transgene expressing the Cas9 protein were injected with a crRNA targeting let-7 (purple), tracrRNA and dsDNA HDR donor to replace the 64 bp sequence corresponding to the precursor-let-7 transcript with the 23 bp jump board sequence (blue). To permit recovery of F1 progeny carrying a lethal jump board let-7 null allele, injected P0 hermaphrodites were crossed to males carrying the mnDp1 [umnIs25] genetic balancer, which contains a wild type let-7 locus, as well as an integrated myo-2p::GFP transgene. The resulting jump board strain, VT3742(oxSi1091 II;
Here, we describe a platformed “jump board” strategy and its application in systematically engineering the essential microRNA let-7 (Fig. 1A-E) and protein coding gene lin-28 (Fig. 1F) in *C. elegans*. We chose the jump board protospacer sequence (INPP4A) which is (1) comprised of a PAM site and a protospacer antisense to a crRNA with experimentally confirmed high editing efficiency (INPP4A-crRNA), and (2) non-homologous to *C. elegans* genome, including the genetic balancer we used (*mnDp1*). Notably, the jump board protospacer contains an EcoRV restriction site, which can be utilized for rapid large-scale genotyping by which HDR events can be identified in the F1 generation (Fig. 1C). Using the jump board strategy, we have so far created 28 let-7 alleles for various experimental purposes, among which 15 alleles showed lethality and require rescue by *mnDp1*. Note that the let-7 jump board allele (*ma393*) itself is a new let-7 null allele in which the precursor-let-7 is completely removed.

We have also used the jump board strategy to mutate the two CCHC motifs at the zinc finger domain of lin-28 (Fig. 1F) (Mayr and Heinemann, 2013; Van Wynsbergh et al., 2011). *lin-28*(lf) phenotypes include near-sterility, which could have been mitigated by using a genetic balancer for chromosome I (analogous to the approach for let-7), but in this case we employed a *lin-46*(lf) genetic background which suppresses *lin-28*(lf) phenotypes (Pepper et al., 2004). We suggest that *lin-28* represents an example of how the jump board strategy offers significant advantages for systematic mutagenesis of protein coding genes, as well as microRNAs or other non-coding sequences.

The jump board strategy described here can facilitate the targeted mutagenesis at essential genes for a number of reasons: 1) for genetic loci that lack convenient and efficient protospacer sites, the jump board provides a highly efficient foreign crRNA/protospacer platform; 2) the jump board protospacer sequence contains a convenient restriction site that facilitates rapid large-scale genotyping; and 3) the targeting of a foreign sequence protospacer enables the use of genetic balancers unconfounded by undesired editing of the balancers. Conventionally, essential genes can also be edited by targeting the wild type locus, and employing a balancer to rescue the mutations, either by crossing the P0s with balancer-carrying
males, or by including the balancer from the outset. In the first instance, the jump board strategy eliminates the need to cross the P0s, and in the second instance, the fact that only the jump board sequence is targeted eliminates the complications associated with unwanted editing of the balancer, which include loss of homozygous edited F1s, and extra crossing step and/or genotyping to distinguish balancer edits from chromosomal edits. We thus suggest that the jump board strategy, although requiring an extra step to establish the platform, offers particular advantages for repeated mutagenesis of individual loci of interest, where throughput is promoted by the highly efficient INPP4A protospacer and the ease of screening for desired edits. We also note that the jump board strategy also prevents cleavage of dsDNA HDR donor molecules or re-cleavage of converted loci, both of which can impact HDR efficiency. Finally, *C. elegans* gene editing technology using CRISPR continues to improve through innovation, for example, to the design of donors and the compositions and preparation of reagent mixtures (Dokshin et al., 2018; Ghanta and Mello, 2020; Richardson et al., 2016; Song et al., 2016). These improvements can be applied to the jump board HDR approach for even greater throughput of targeted mutagenesis.

**Methods**

**Request a detailed protocol**

**Genome editing at the let-7 genomic locus**

Sequences corresponding to the WT precursor-let-7 and its 500 bp flanking regions were cloned into pCR2.1-TOPO vector. The Q5 mutagenesis kit (NEB, Cat:E0554) was used to generate the mutant plasmids. dsDNA donors were generated from the mutagenized plasmids by PCR with 73/106 bp flanking the precursor-let-7 and purified by ethanol precipitation. Injection mixtures containing final concentrations of 30 ng/µl AltR_Cas-9_crRNA_let-7 (step 1) or AltR_Cas-9_crRNA_INPP4A (step 2) (PAM sites shown in Fig. 1D), 10 ng/µl AltR_Cas-9_crRNA_dpy-10_cn64 as co-CRISPR marker (Arribere et al., 2014), 75 ng/µl Alt-R tracrRNA (IDT, Cat:1072532), 10 ng/µl each dsDNA donor and 1X duplex buffer (IDT, Cat: 11010301) were incubated at room temperature for 10 min for pre-annealing and injected into the gonad of young adults of strain EG9615(oxSi1091[mex-5p::Cas-9(smu-2 introns) unc-119(+)] II; unc-119(ed3) III) (step 1) or VT3742(oxSi1091 II; mnDp1[umnIs25] (X;V)/+ V; let-7(ma393) X) (step 2). We suggest injecting 4-8 P0 for single HDR donor and 8-16 P0 for multiplexed HDR donors.

**Genotyping the products of HDR at the let-7 locus**

For both steps (Fig. 1A and B), F1 dumpy/roller animals with *mnDp1[umnIs25]* (detected by pharyngeal GFP) were picked and genotyped by PCR after egg laying, and the PCR products were analyzed by restriction digestion using EcoRV (NEB, Cat:R3195S) at 1 unit/µl PCR product for 30 min. In the first step (Fig. 1A), samples from the broods with jump board HDR should have EcoRV cleavage. In the second step (Fig. 1B), samples containing either homozygous HDR or homozygous InDel should have no EcoRV cleavage. Samples with candidate HDR (with EcoRV cleavage in Fig. 1A and with no EcoRV cleavage in Fig. 1B) were subjected to Sanger sequencing, and F1 broods with homozygous HDR were selected and outcrossed. For the genotype screen at the second step, we suggest screening 48 F1 for single HDR donor and 96 F1 for multiplexed HDR donors.

**Genome editing and genotyping at the lin-28 genomic locus**

In the first step of editing, the Ultramer single-strand DNA donor with 35/35 nt flanking homology was obtained from IDT. The injection mixture containing final concentrations of 15.3 ng/µl AltR_Cas-9_crRNA_lin-28_1, 15.1 ng/µl AltR_Cas-9_crRNA_lin-28_2 (PAM sites shown in Fig. 1F), 7.2 ng/µl AltR_Cas-9_crRNA_dpy-10_cn64, 82.6 ng/µl Alt-R tracrRNA, 159.1 ng/µl ssDNA donor, 0.57 ng/µl AltR-S.p.Cas9 nuclease (IDT, Cat: 1081058) and 1X duplex buffer were incubated at 4 °C for 15 min for pre-annealing and injected into VT3711(oxSi1091[oxSi1091 II; unc-119(ed3) III]) (step 1) or VT3742(oxSi1091 II; mnDp1[umnIs25] (X;V)/+ V; let-7(ma393) X) (step 2). We suggest injecting 4-8 P0 for single HDR donor and 8-16 P0 for multiplexed HDR donors.

**Genotyping the products of HDR at the lin-28 locus**

For both steps (Fig. 1A and B), F1 dumpy/roller animals with *mnDp1[umnIs25]* (detected by pharyngeal GFP) were picked and genotyped by PCR after egg laying, and the PCR products were analyzed by restriction digestion using EcoRV (NEB, Cat:R3195S) at 1 unit/µl PCR product for 30 min. In the first step (Fig. 1A), samples from the broods with jump board HDR should have EcoRV cleavage. In the second step (Fig. 1B), samples containing either homozygous HDR or homozygous InDel should have no EcoRV cleavage. Samples with candidate HDR (with EcoRV cleavage in Fig. 1A and with no EcoRV cleavage in Fig. 1B) were subjected to Sanger sequencing, and F1 broods with homozygous HDR were selected and outcrossed. For the genotype screen at the second step, we suggest screening 48 F1 for single HDR donor and 96 F1 for multiplexed HDR donors.

**Reagents**

VT3742(oxSi1091 II; *mnDp1[umnIs25]* (X;V)/+ V; *let-7(ma393) X*), VT3914(*lin-28(ma416ma487) I; *lin-46(ma164) V*) strains and oligo sequences are available upon request.

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References

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