GeneWeld: Efficient Targeted Integration Directed by Short Homology in Zebrafish

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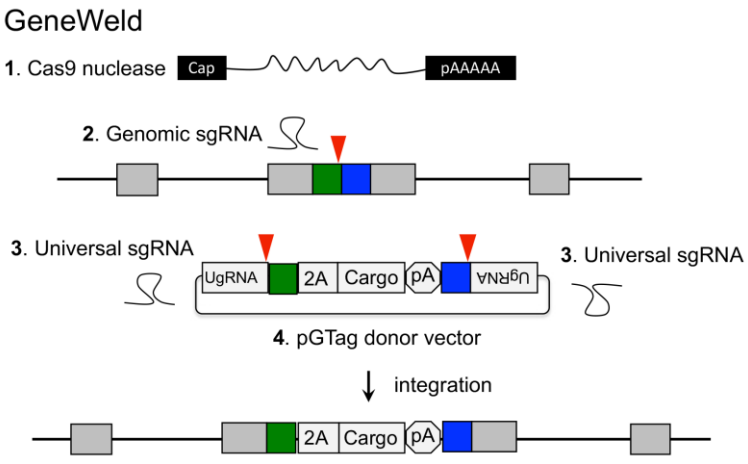
This 07-01-2024 updated document is derived from (Wierson et al., 2020) and (Welker, 2021).

Overall aim:

This protocol describes the GeneWeld CRISPR/Cas9 method for generating precision targeted integration alleles in zebrafish. GeneWeld uses short homology of 24-48 bp to drive targeted integration of DNA reporter cassettes by homology-mediated end joining (HMEJ) at a CRISPR/Cas9 induced DNA double-strand break (Welker, 2021; Wierson et al., 2020). We provide a suite of donor vectors, called pGTag, pPRISM, and pUFlip that can be easily engineered to isolate alleles expressing fluorescent reporters, fluorescent fusion proteins, Gal4 and CreERT2 drivers, or floxed conditional alleles in a gene of interest. The following detailed protocol outlines steps for gRNA selection, homology arm design, vector construction, CRISPR/Cas targeting, and recovery of targeted integration alleles. pGTag, pPRISM, and pUFlip vectors are available through Addgene (https://www.addgene.org/kits/essner-geneweld/) or contacting jessner@iastate.edu or mmcgrail@iastate.edu.

Graphical abstract:



GeneWeld method for CRISPR/Cas9 targeted integration.

Materials and Reagents

1. Polystyrene Petri dishes (Thermo Fisher, catalog number: FB0875713)
2. Borosilicate Glass Capillaries (World Precision Instruments, catalog number: 1B100-04)
3. Microloader tips (Eppendorf, catalog number: 920001007)
4. Commercially available molds for injection plates available from https://www.agnthos.se/index.php?id_product=204&controller=product
5. Kwik-Fill borosilicate glass capillaries (World Precision Instruments, catalog number: 1B100-4)
6. EasyStrip Plus Tube Strip with Attached Ultra Clear Caps (Thermo Fisher Scientific catalog number: AB2005)
7. pGTag vectors are available through Addgene (https://www.addgene.org/kits/essner-geneweld/)
8. NEB Stable Competent *E. coli* (New England Biolabs, catalog number: C3040I)
9. One Shot TOP10 Chemically Competent *E. coli* (Thermo Fisher, Invitrogen, catalog number: C404010)
10. pT3TS-nCas9n expression vector (Addgene, catalog number: 46757)
11. PureYield Plasmid Miniprep System (Promega, catalog number: A1223)
12. Ambion mMessage Machine T3 Transcription Kit (Thermo Fisher, catalog number: AM1348)
13. miRNeasy Mini Kit (Qiagen, catalog number: 217004)
14. NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, catalog number: E7645L)
15. Zebrafish Tg (*miniTol2<14XUAS:mRFP, γcry:GFP>*)^{tl2} (Balciuniene et al., 2013)
16. Zebrafish wildtype WIK strain (Zebrafish International Resource Center, catalog number: ZL84, https://zebrafish.org/home/guide.php)
17. Agarose (Thermo Fisher, catalog number: BP160-500)
18. Ethidium Bromide (Fisher Scientific, catalog number: BP1302-10)
19. Ethyl 3-aminobenzoate methanesulfonate, Tricaine MS-22 $C_9H_{11}NO_2 \cdot CH_4SO_3$ (Millipore Sigma catalog number 886-86-2)
20. 1-phenyl-2-thiourea $C_7H_8N_2S$ (Thermo Fisher, catalog number: AC207250050)
21. NorthernMax-Gly Sample Loading Dye (Thermo Fisher, catalog number: AM8551)
22. Decon ElIMINase (Fisher Scientific, catalog number: 04-355-31)
23. Molecular Grade RNase/DNase-Free water (*e.g.*, Invitrogen, catalog number: 10977023)
24. XbaI restriction endonuclease (New England Biolabs, catalog number: R0145S)
25. BfuAI restriction endonuclease (New England Biolabs, catalog number: R0701S)
26. BspQI restriction endonuclease (New England Biolabs, catalog number: R0712S)
27. GoTag Green 2× MasterMix (Promega, catalog number: M7123)
28. X-Gal solution, ready-to-use, 20mg/ml (Thermo Fisher Scientific, catalog number: R0941)
29. LB broth (Fisher Scientific, catalog number: BP9723-500)
30. LB agar (Fisher Scientific, catalog number: BP9724-2)
31. SOC medium (Thermo Fisher Scientific, catalog number: 15544034)
32. Kanamycin (Fisher Scientific, catalog number: BP9065)
33. T4 Quick Ligase, Rapid DNA Ligation Kit (Thermo Fisher Scientific, catalog number: K1422)
34. Sodium Hydroxide NaOH (Millipore, Sigma-Aldrich, catalog number: 30620)
35. Tris Base (Fisher Scientific, catalog number: BP152-500)
36. UgRNA

5'-GGGAGGCGUUCGGGCCACAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGGAUC-3' and gene specific sgRNAs ordered from Synthego or IDT (see Section B.)

37. Primers, can be ordered from IDT:
    F3'-check: 5'-GGCGTTGTCTAGCAAGGAAG-3'
    R3' pgtag seq: 5'-ATGGCTCATAACACCCCTTG-3'
    R-Gal4-5'juncM: 5'-GCCTTGATTCCACTTCTGTCA-3'
    R-RFP-5'junc: 5'-CCTTAATCAGTTCCTCGCCCTTAGA-3'
    R-eGFP-5'-junc: 5'-GCTGAACTTGTGGCCGTTT-3'
    F-Gal4-3'juncM: 5'-GCAAACGGCCTTAACTTTCC-3'
    F-Gal4-3'junc: 5'-CTACGGCGCTCTGGATATGT-3'
    F-RFP-3'junc: 5'-CGACCTCCCTAGCAAACTGGGG-3'
    F-eGFP-3'junc: 5'-ACATGGTCCTGCTGGAGTTC-3'
38. Zebrafish embryo E2 Medium (see Recipes)

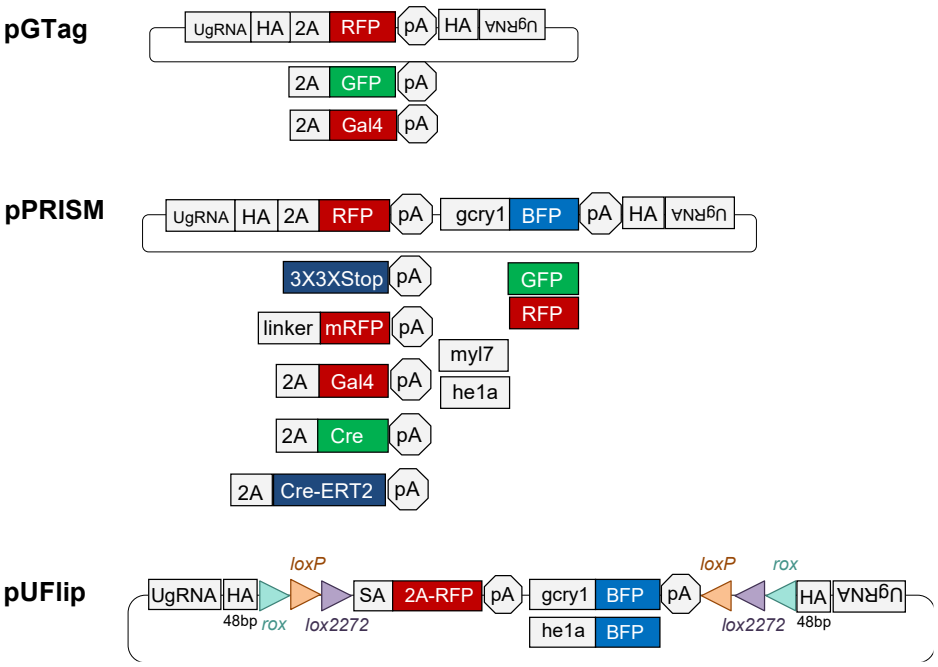
Equipment

1. Microcap Microliter Pipets (Drummond Scientific, catalog number: 1-000-0010)
2. Flaming/Brown Micropipette Puller (Sutter Instrument, model: P-97)
3. X-Cite 120W Metal Halide lamp (Excilitas Technologies, model: X-Cite 120Q)
4. Pico-Injector (Harvard Apparatus, model: PLI-100)
5. MM-3 Micromanipulator (Narishige, model: MM-3)
6. Nitrogen gas tank, or, JUN-AIR Oil-lubricated Piston Air Compressor (Cole-Parmer, catalog number: 1152000)
7. Nanodrop (Thermo Fisher Scientific, model: NanoDrop 2000)
8. iBright FL1500 Imaging System (Thermo Fisher Scientific, model: A44241)
9. Shaking Incubator (Thermo Scientific, model: MaxQ8000)
10. Isotemp Standard Laboratory Incubator (*e.g.*, Thermo Scientific, model: 51-028-065HPM)
11. Precision General Purpose Baths (*e.g.*, Thermo Scientific, model: TSGP02)
12. Thermal Cycler (Eppendorf, 6335000020)
13. Zeiss SteREO Discovery.V8 Stereomicroscope or similar and epi-illumination X-Cite 120W metal halide light source with fiber optic cable (Excilitas Technologies)
14. Owl EasyCast B2 Mini Gel Electrophoresis Systems (Thermo Fisher Scientific, model: B2-12)

Software:

1. Ensembl https://www.ensembl.org/Danio_rerio/
2. UCSC https://genome.ucsc.edu/cgi-bin/hgTracks?db=danRer11
3. SnapGene 30 day free trial https://www.snapgene.com/free-trial
4. Primer 3 http://biotools.umassmed.edu/bioapps/primer3_www.cgi
5. Synthego ICE Analysis https://ice.synthego.com/#/

GeneWeld Vector suite at Addgene:



GeneWeld vectors for CRISPR/Cas9 targeted integration to isolate alleles with fluorescent reporters (pGTag), Gal4 and Cre/ERT2 drivers, fluorescent fusion proteins, loss of function alleles (pPRISM), and floxed conditional alleles (pUFlip).

Protocol:

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A. Introduction

The GeneWeld strategy (Figure 1) and pGTag/pPRISM/pUFlip vector series are designed for straightforward assembly of vectors containing short homology arms for efficient recovery of CRISPR/Cas9 precision targeted

integration alleles.

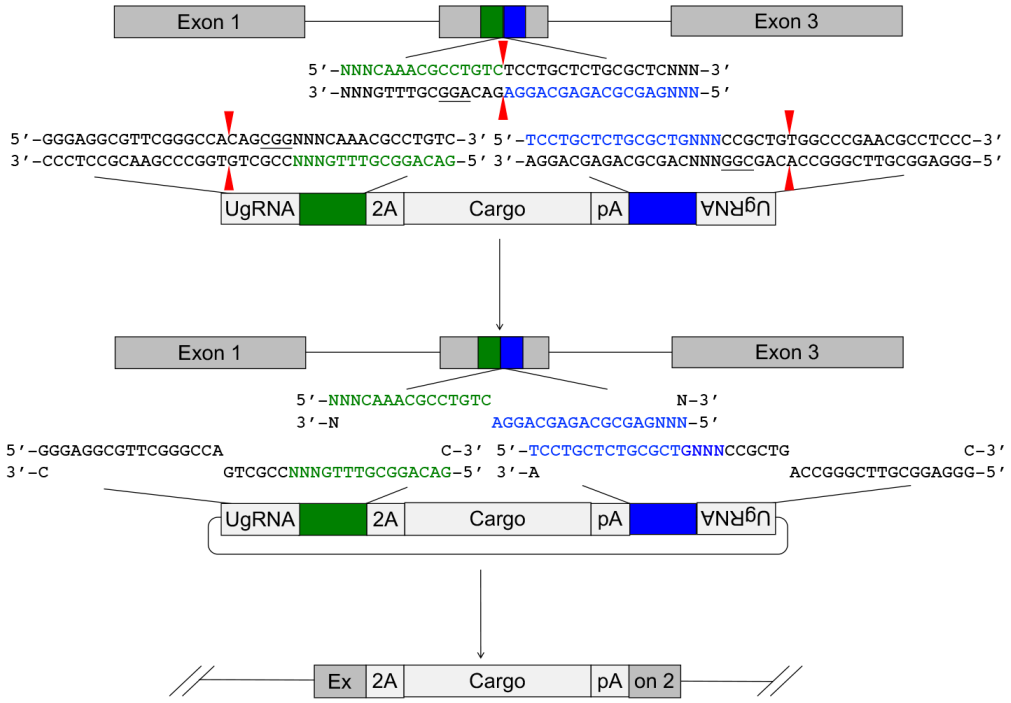

Figure 1. Targeted integration of pGTag vector cargo DNA into a coding exon. Short homology arms complementary to the 5' (green) and 3' (blue) sequences of the genomic target site are cloned on the 5' and 3' sides of the vector cargo DNA. The short homology arm cargo cassette is flanked by two universal guide RNA UgRNA sites. CRISPR/Cas9 simultaneously targets double strand breaks at the sgRNA genomic target site and at the UgRNA sites flanking the cargo on the plasmid donor. Exonuclease end resection liberates single stranded DNA in the vector homology arms that is complementary to the resected strands on the 5' and 3' sides of the genomic double strand break. The complementary sequences direct homology mediated end joining integration of the cargo DNA at the exon target site. PAM sequences are underlined and small black arrows indicate Cas9 cut sites in the genome and vector.

B. Selection of a CRISPR/spCas9 target site in the gene of interest

1. Zebrafish wild type strains in common use are polymorphic.
   Note: It is highly recommended to first sequence the target sequence in the genomic DNA from your fish strain and use this sequence to design gRNAs.
2. To identify an sgRNA site for targeting, first, view the gene model on a genome browser, and download the gene sequences.
   a. At <ensembl.org> Search for the gene name of interest for the species of interest and open the Transcript page (Figure 2).

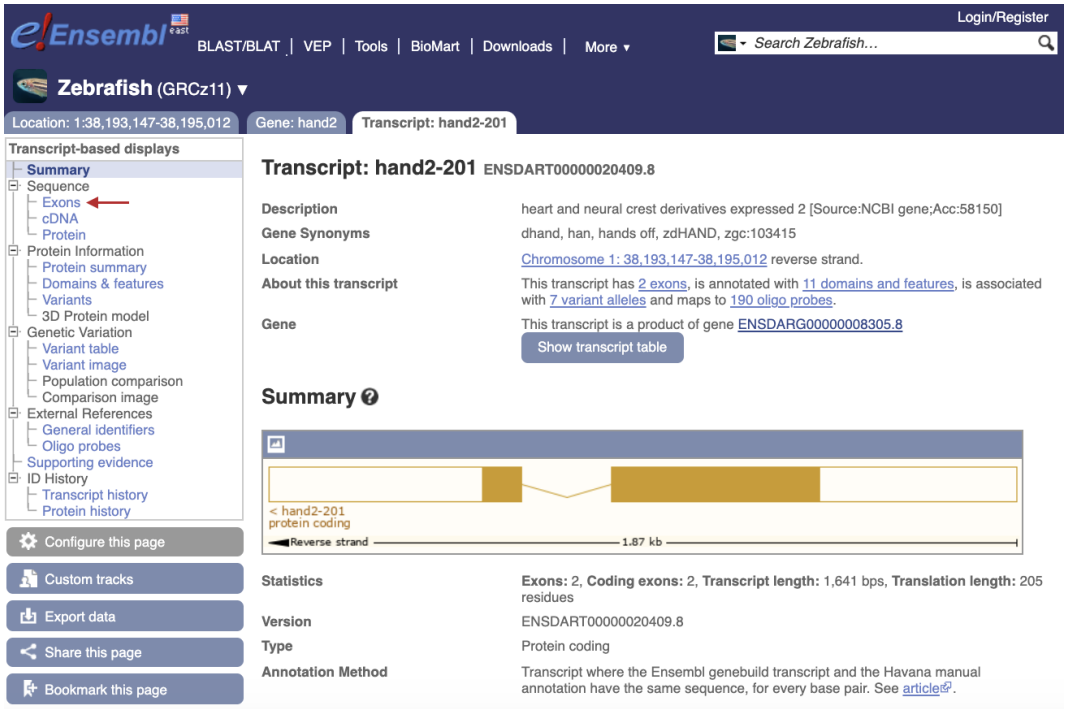

Figure 2. Screenshot of zebrafish *hand2* Transcript page on the ensembl.org genome browser (https://useast.ensembl.org/Danio_rerio/Transcript/Summary?db=core;g=ENSDARG00000008305;r=1:38193147-38195012;t=ENSDART00000020409)

Click on Download to download the genomic sequence for the gene.

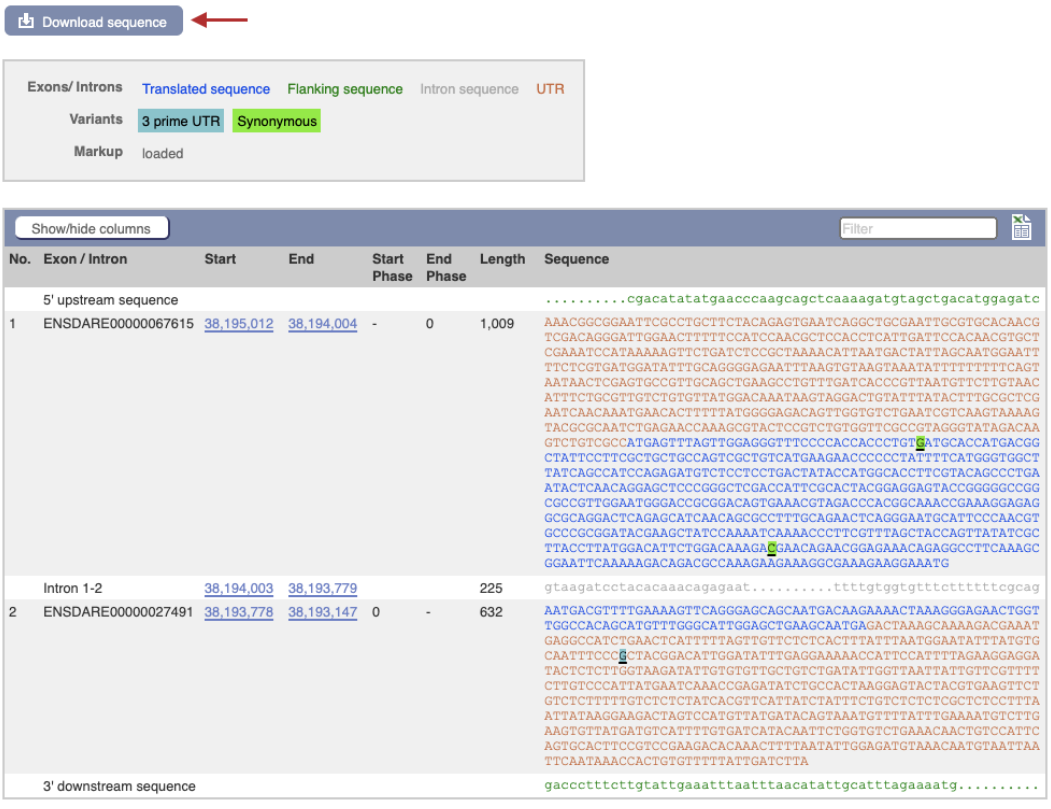

Figure 3. Exon sequences and Download page for zebrafish *hand2* gene on the ensembl.org genome browser

b. Open the genomic sequence in SnapGene and annotate the 5'UTR, exons, introns and 3'UTR.
c. Design primers to amplify the target exon(s) or intron(s) from fin clip genomic DNA in your strain of fish. Sequence the amplicon. Use this sequence to identify gRNA sites by searching for PAM sequences near the site of your intended knockin allele.

   Annotate the selected gRNA target sequence and NGG PAM in the SnapGene gene file.

3. Design ~20 bp DNA primers for a PCR amplicon of ~150-200 bp surrounding the sgRNA target site. Annotate the primer sequences in the gene file. The primers will be used to amplify genomic DNA from embryos after injection of CRISPR reagents to test for mutagenesis at the target site. The presence of indels at the target site can be detected in the PCR products in multiple ways, including gel electrophoresis to visualize heteroduplex formation, resistance to restriction enzyme digestion at a site overlapping the sgRNA target, or direct sequencing followed by ICE Analysis.

4. Synthetic sgRNAs can be ordered from Synthego (https://orders.synthego.com/products/crisprevolution-sgrna-ez-kit-13/#/tubes?mod_code=1) or IDT (https://www.idtdna.com/site/order/oligoentry/index/crispr). On the Synthego ordering page select the tab for Modifications 2'-O-Methyl at first and last bases, 3' phosphorothioate bonds between first 3 and last 2 bases.

   Primers can also be ordered from IDT.

5. Preparation of SpCas9 mRNA
   Digest ~5-10 µg pT3TS-nCas9n plasmid (plasmid Addgene #46757) (Jao et al., 2013) with Xba1 to linearize the vector.Use 100 ng to 1 µg DNA as template for in vitro transcription reaction with the mMESSAGE mMACHINE T3 kit Life Technologies (AM1348). Follow the manufacturer's instructions provided with the kit. Save a 1 ul aliquot of the *in vitro* synthesis reaction.

C. Injection of sgRNA and spCas9 mRNA
Deliver 25 pg sgRNA and 300 pg Cas9 mRNA in a 2 nl volume to embryos at the one-cell stage. Below is a step-by-step protocol for zebrafish embryo injection. A detailed video of zebrafish embryo injection can be found in Rosen *et al.* (2009) in the Journal of Visualized Experiments publication (Rosen et al., 2009) (doi: 10.3791/1115).

1. Cast zebrafish embryo injection trays with custom molds that create 45 degree troughs for lining up and holding embryos (Figure 4A). Molds are also available commercially (for example https://www.agnthos.se/index.php?id_product=204&controller=product).
   Melt 1.2% agarose in 1× E2 Medium (https://wahoo.cns.umass.edu/book/export/html/867) and pour into polystyrene Petri dish (Fisher No. FB0875713). The mold is set on top (Figure 4B), and once the plates have set gently remove the mold (Figure 4C). Injection trays can be used multiple times and are stored inverted at 4°C for up to three weeks between use.

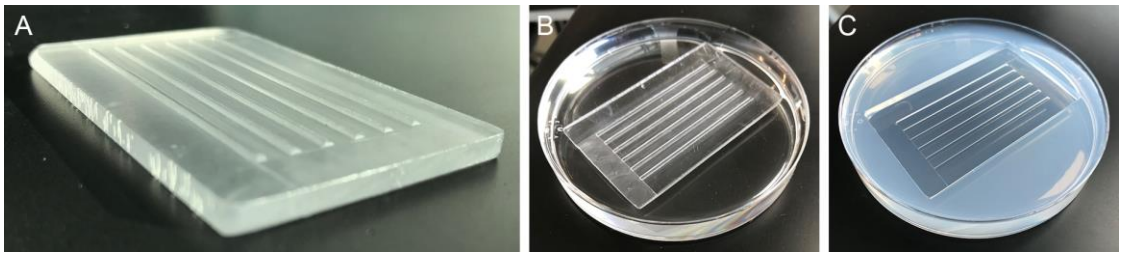

Figure 4. Injection tray mold. (A) is set on top of melted 1.2% agarose (B). Solidified injection plate with troughs to hold embryos (C).

2. Before injection, pre-warm trays to 28.5°C prior to injection by placing them in a 28.5°C incubator for 20 min.
3. Pull microcapillary glass needles using Kwik-Fill borosilicate glass capillaries (No. 1B100-4) on a Sutter Instrument Flaming/Brown Micropipette Puller (Model P-97).
4. Prepare injection samples containing the following diluted in Molecular Grade RNase/DNase-free water:
   12.5 ng/µl of genomic sgRNA
   150 ng/µl of mRNA for Cas9
   Keep injection solution on ice
5. Calibrate injection needle by first breaking the end of the tip off with sterile tweezers (Figure 5A, B). Use the pedal to expel 10 droplets and capture each droplet with a 30 mm long capillary tube that represents a volume of 1 µl (Drummond No. 1-000-0010) (Figure 5C). Measure the distance from the end of the capillary to the meniscus of the liquid and convert to volume: 1 mm = 30 nl, therefore 2/3 of a mm = 20 nl. The volume of each droplet is adjusted by changing the injection time in order to achieve 2 nl/droplet. There is a linear relationship between volume and time at a set pressure. Avoid injection times less than 100 ms and over 400 ms.
6. Transfer one-cell embryos from collection Petri dishes to the troughs on the pre-warmed agarose injection tray (Figure 5D). Each embryo is encased in a chorion. As the embryos near the first cell division at 45 minutes after fertilization, the single cell is clearly visible atop the yolk (Figure 5D).
7. Use the micro-manipulator to pierce the needle through the chorion and into the embryo. Inject 2 nl of sample at the center of the yolk interface/boundary between the single cell and the yolk (Figure 5E, white arrow points to interface where needle tip is placed). Inject embryos before the first cell division begins.

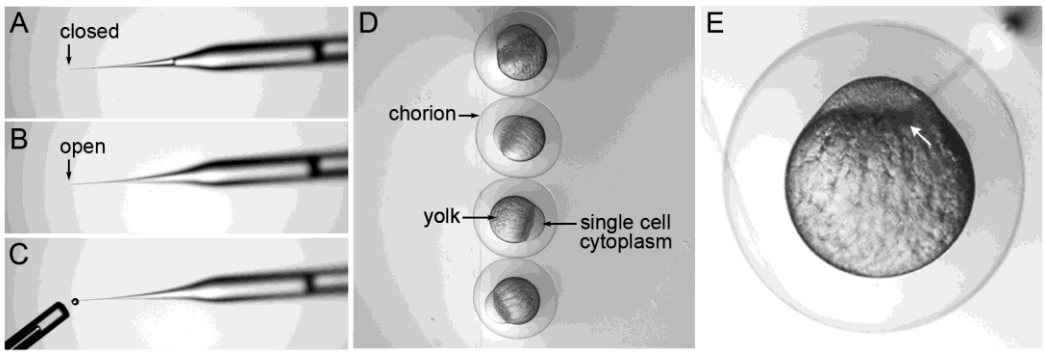

Figure 5. Microinjection needle calibration and zebrafish single cell embryo microinjection. (A) Image of backloaded injection needle with closed tip. (B) A small amount at the tip of the needle is removed using forceps to create an open end. (C) A single droplet of injection solution is expelled by

pressing on the injection apparatus pedal. The tip of a 1 µl Drummond capillary tube is shown that was used to capture 10 drops. (D) embryos lined up in an injection tray trough with labels indicating the chorion, yolk and single cell embryo. (E) Image of needle inserted through the chorion and into the embryo. The tip of the injection needle is positioned at the yolk interface (white arrow) between the single cell on top and the yolk below.

8. After embryos have been injected, wash the embryos from the injection tray into a clean Petri dish with embryo media. Keep 20-40 embryos separate to use as uninjected controls. At 3-5 h post injection remove any unfertilized or dead embryos from the dishes.

D. Test for sgRNA mutagenesis efficiency and indel production

1. Biallelic inactivation can lead to loss of function phenotypes that may be lethal for essential genes. After injection count and remove dead embryos from the dish of injected embryos. If all of the embryos are defective and unlikely to survive, reduce the amount of guide sgRNA that is injected to 12.5 pg. If embryos still fail to survive, reduce the amount of sgRNA further to 6.25 pg. As we reported previously, for a ubiquitously expressed, essential gene such as the tumor suppressor *rb1*, the amount of injected sgRNA needs to be reduced to 6.25 pg, in order to recover viable juvenile fish that survive to adulthood and transmit germline gene edited alleles (Solin et al., 2015).
2. Digestion of embryos for isolation of genomic DNA and analysis of CRISPR/Cas9 mutagenesis efficiency at targeted gene locus.

   Extract genomic DNA from zebrafish embryos aged between 1 and 5 dpf, either individual embryos or pools of 5 embryos from the same injection.

   a. Dechorionate embryos, if they have not emerged from the chorion.
   b. Place embryo into a PCR tube and remove as much of the fish water as possible. Collect at least 3 injected embryos and 1 uninjected control embryo.
   c. Add 20 µl of 50 mM NaOH per embryo.
   d. Heat the embryos at 95°C in a thermocycler for 30 min.
   e. Vortex samples and spin the tubes down. The embryos should be completely dissolved.
   f. Neutralize the samples by adding 1 µl of 1 M Tris pH 8.0 per 10 µl NaOH. Mix by vortexing then spin down.
   g. Set up the PCR reactions for each tube of embryo digested genomic DNA using the gene specific Forward and Reverse primers that were designed to create an amplicon around the CRISPR/Cas9 target site.
   h. Run up to 7 µl of PCR product on a 2.5 to 3% agarose gel. An example of gRNA injection and validation targeting exon 2 of the *hand2* gene is shown below (Figure 6, 7). The control uninjected embryo PCR amplicon runs as a single, tight band on the gel (U). The amplicons from 5 injected embryos are diffuse in appearance, in comparison to the U control. This indicates heteroduplex formation in the PCR product caused by the presence of indel mutations at the CRISPR target site in the gene of interest.

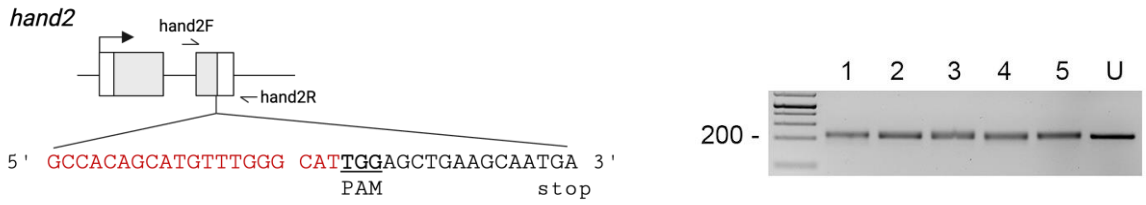

Figure 6. *hand2* exon 2 gRNA validation. *hand2* exon 2 forward strand gRNA in red. PAM underlilned. Space in sequence indicates cut site. Gel shows PCR amplicons generated with primers flanking the gRNA target site. Diffuse bands in injected embryos represent heteroduplex DNA caused by indel mutations at the target site.

i. For quantitative analysis of mutagenesis efficiency, Sanger sequence PCR products to verify the presence of indels. % indel formation can be analyzed using Synthego's ICE Analysis https://ice.synthego.com/#/. Alternatively, Illumina MiSeq multiplex next generation sequencing can be used to test the efficiency of multiple gRNAs in parallel.
   An example of ICE analysis of amplicons from the *hand2* exon 1 targeted embryos #3 and #6 from Figure 6 is shown below in Figure 7. Embryo #3 and #6 show 84% and 80% indel alleles after targeting, respectively, indicating high mutagenesis efficiency of the sgRNA.

Figure 7. Validation of sgRNA mutagenesis efficiency by ICE analysis. PCR amplicon from *hand2* exon 2 targeted embryo #3 was Sanger sequenced and the results analyzed with Synthego ICE Analysis. The results show a mutagenesis efficiency of 96% and and a range of indel mutations around the target site.

E. Design short homology arms for pGTag targeting vector assembly
   Homology directed targeted integration allows the seamless integration of exogenous DNA into the genome with precise repair events at the target site. The pGTag/pPRISM/pUFlip vector series provides tools to

generate fluorescent reporters, fusion proteins, Gal4 and CreERT2 drivers, and conditional alleles (Figure 8). pGTag and pPRISM are designed for targeted integration into the coding sequence of a gene (Almeida et al., 2021; Wierson et al., 2020).

pUFlip is designed for targeted integration of a floxed cassette into an intron to create a conditional allele that results in transcriptional termination and expression of mRFP when Cre recombinase is present (Liu et al., 2022).

The vectors contain BfuAI and BspQI type II restriction enzymes for cloning of short homology arms (24 or 48 bp). The reporter gene consists of several parts. A 2A peptide sequence causes translational skipping, allowing the following protein to dissociate from the locus peptide. The fluorescent reporter coding sequences have a number of options for localization signals, including cytosolic (no signal), a nuclear localization signal (NLS), or a membrane localization CAAX sequence. Translation is terminated by one of two different transcription termination polyadenylation (pA) sequences; the 3'UTR region of the zebrafish β-actin gene or the SV40 viral transcription termination sequence. For many genes, the level of endogenous gene expression is not high enough to produce a detectable fluorescence signal from the integrated reporter gene. The Gal4 pGTag vector in combination with the transgenic Tol2<14XUAS/RFP> reporter line (Balciuniene et al., 2013) allows for amplification of the signal.

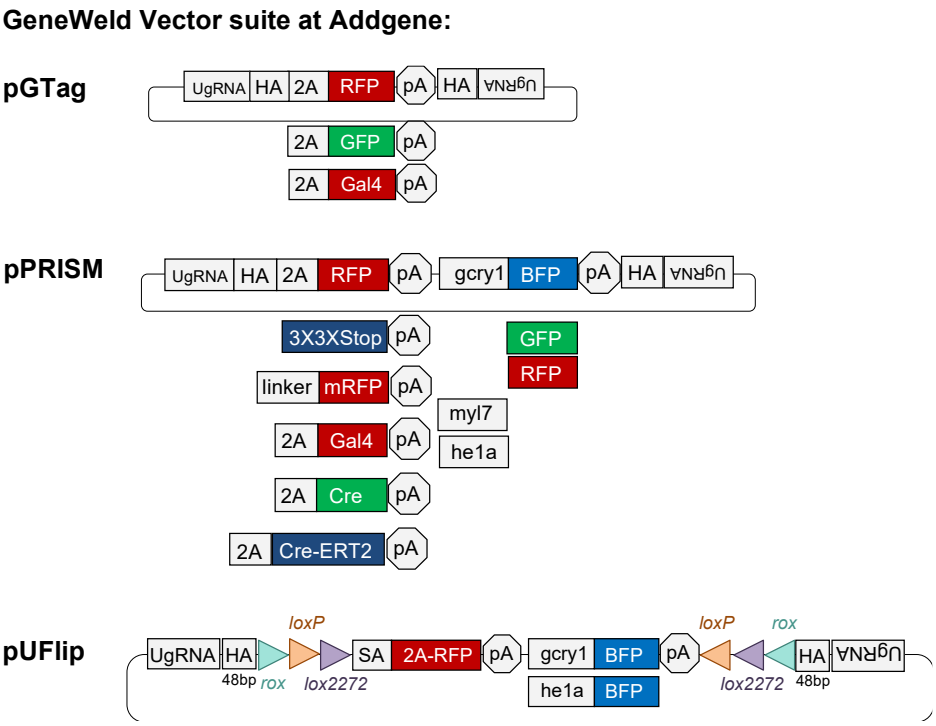

Figure 8. The pGTag, pPRISM, and pUFlip vectors allow one step cloning of homology arms. Vectors can be obtained from Addgene (www.addgene.org) or by contacting jessner@iastate.edu or mmcgrail@iastate.edu.

Manual Homology Arm Design

Two complementary oligos, Oligo A and Oligo B, are annealed to generate the double stranded homology arms. The Oligos are designed with 5' overhangs that are complementary to the Type IIS restriction enzyme sites for cloning into the GeneWeld pGTag, pPRISM and pUFlip vectors.

The following protocol describes how to design homology arm oligos manually:
Note: In the following section, orientation of target sites and homology is in the context of the reading frame of the gene of interest. Example: A forward strand CRISPR gRNA means that the gRNA and PAM are encoded on the sense strand of the gene. 5' homology arms are 5' to the CRISPR/Cas9 cut site and 3' homology arms are 3' to the cut site with respect to the reading frame of the gene being targeted.

Example: Designing 5' and 3' homology arms to a CRISPR/Cas9 gRNA target site in exon 2 of *hand2* gene

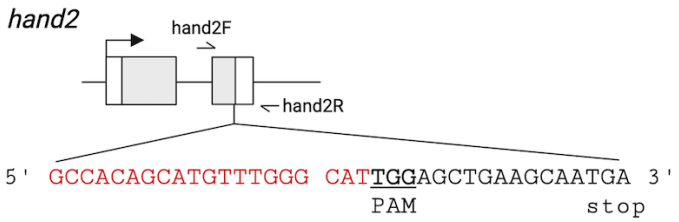

Figure 9. Diagram of CRISPR/Cas9 gRNA (red), PAM (underlined), and cut site (space)

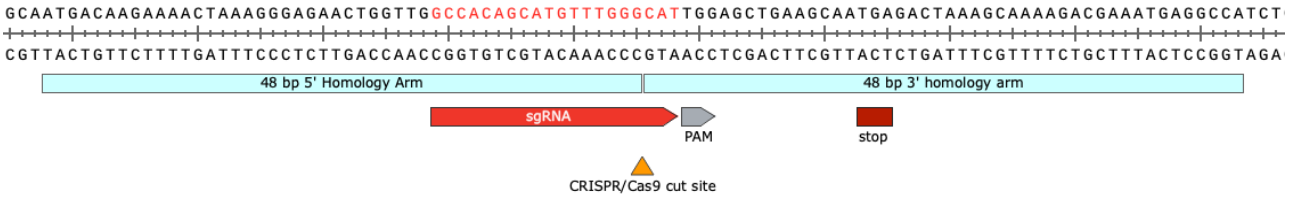

Figure 10. Snapshot of SnapGene file showing location of CRISPR sgRNA, cut site, PAM, and 48 bp 5' and 3' homology arms

5' Homology Arm Design

1. The 48 bp 5' to the CRISPR cut site is used to build the 5' Homology arm – text highlighted in blue (Figure 9).

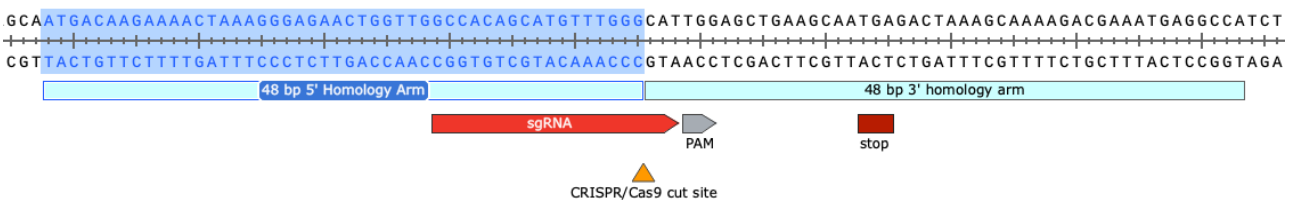

Figure 11. SnapGene screenshot showing 48 bp 5' homology arm highlighted in blue.

2. To design Oligo A for the 5' Homology Arm, copy the sequence of the 48bp homology arm top strand. Add back nucleotides to the 3' end of the oligo to complete the coding sequence. Determine where the last codon is in the homology arm. Here the 3' G in the homology domain is the first base in the codon cut by the gRNA – GCA coding for Alanine. The cut site removes the last 17 bp from the 3' end of the coding sequence. Complete the GCA codon by adding the bases "ca". Add back the last 15 nucleotides, however, to prevent the gRNA from cutting the homology arm, scramble the last 15 codons using redundant codons so the sequence is no longer complementary to the gRNA, but the encoded amino acids remain the same – "ctcgaactgaacaa". (Figure 12). Addition of these nucleotides will ensure the reading frame is intact and the coding sequence is complete.

5′ ATGACAAGAAAACTAAAGGGAGAACTGGTTGGCCACAGCATGTTTGGGcactcgaactgaacaa 3′

Figure 12. 48 bp 5' homology arm shown in blue with 17 nucleotide scrambled sequence "cactcgaactgaacaa" at 3' end to complete the coding sequence and prevent recognition by gRNA.

3. Add the BfuAI enzyme overhang sequences for cloning, to the end of the homology domain: 5'-GCGG. This is the sequence of Oligo A (Figure 13).

5′ GCGGATGACAAGAAAACTAAAGGGAGAACTGGTTGGCCACAGCATGTTTGGGcactcgaactgaacaa 3′

Figure 13. Sequence of single stranded Oligo A for making the 5' homology arm. 5'-GCGG nucleotides provide overhang sequences for BfuAI Type IIS restriction enzyme cloning of the 5' homology arm.

4. The 5' Homology Arm Oligo B will be the reverse complement of Oligo A starting at "A". 5'-ATCC is added to the 5' end to provide the BfuAI overhang sequence for cloning (Figure 14).

3′ TACTGTTCTTTTGATTTCCCTCTTGACCAACCGGTGTCGTACAAACCCgtgagcttgacttgttCCTA 5′

Figure 14. Sequence of single stranded Oligo B for making the 5' homology arm. 5'-ATCC nucleotides provide overhang sequences for BfuAI Type IIS restriction enzyme cloning.

5. When Oligo A and Oligo B are annealed, the 5' homology arm will have 5' overhangs complementary to the BfuAI enzyme site overhangs in the GeneWeld pGTag vector (Figure 15).

```
5′ GCGGATGACAAGAAAACTAAAGGGAGAACTGGTTGGCCACAGCATGTTTGGGcactcgaactgaacaa     3′
3′     TACTGTTCTTTTGATTTCCCTCTTGACCAACCGGTGTCGTACAAACCCgtgagcttgacttgttCCTA 5′
```

Figure 15. Complete sequence of the 48 bp 5' homology arm with scrambled remaining codons to complete the coding sequence and BfuAI overhangs.

3’ Homology Arm Design

6. The 48 bp 3’ to the CRISPR cut site is used to build the 3’ Homology arm – text highlighted in blue (Figure 16).

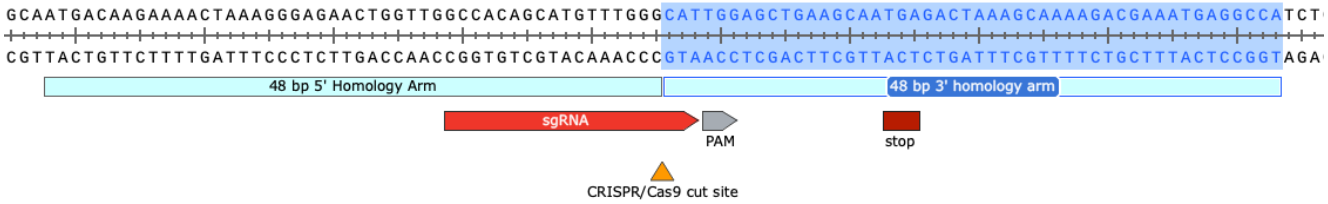

Figure 16. SnapGene screenshot showing 48 bp 3’ homology arm highlighted in blue.

7. To design Oligo A for the 3’ Homology Arm, copy the sequence of the 48bp homology arm top strand. Add the BspQI enzyme overhang sequence 5’ – AAG to the 5’ end. (Figure 17).

5’ AAGCATTGGAGCTGAAGCAATGAGACTAAAGCAAAAGACGAAATGAGGCCA 3’

Figure 17. Sequence of single stranded Oligo A for making the 3’ homology arm. 5’-AGG nucleotides provide overhang sequences for BspQI Type IIS restriction enzyme cloning of the 3’ homology arm.

8. The 3’ Homology Arm Oligo B will be the reverse complement of Oligo A starting at “C”. 5’-CGG is added to the 5’ end to provide the BspQI overhang sequence for cloning (Figure 18).

3’ GTAACCTCGACTTCGTTACTCTGATTTCGTTTTCTGCTTTACTCCGGTGGC 5’

Figure 18. Sequence of single stranded Oligo B for making the 3’ homology arm. 5’-CGG nucleotides provide overhang sequences for BspQI Type IIS restriction enzyme cloning.

9. When Oligo A and Oligo B are annealed, the 3’ homology arm will have 5’ overhangs complementary to the BspQI enzyme site overhangs in the GeneWeld pGTag vector (Figure 15).

5’ AAGCATTGGAGCTGAAGCAATGAGACTAAAGCAAAAGACGAAATGAGGCCA 3’
3’ GTAACCTCGACTTCGTTACTCTGATTTCGTTTTCTGCTTTACTCCGGTGGC 5’

Figure 19. Complete sequence of the 48 bp 3’ homology arm with BspQI overhangs.

10. An example of homology arm design with complementary overhangs for cloning into the pGTag and pPRISM BfuAI and BspQ1 sites is shown below *hand2* gRNA site (Figure 20).

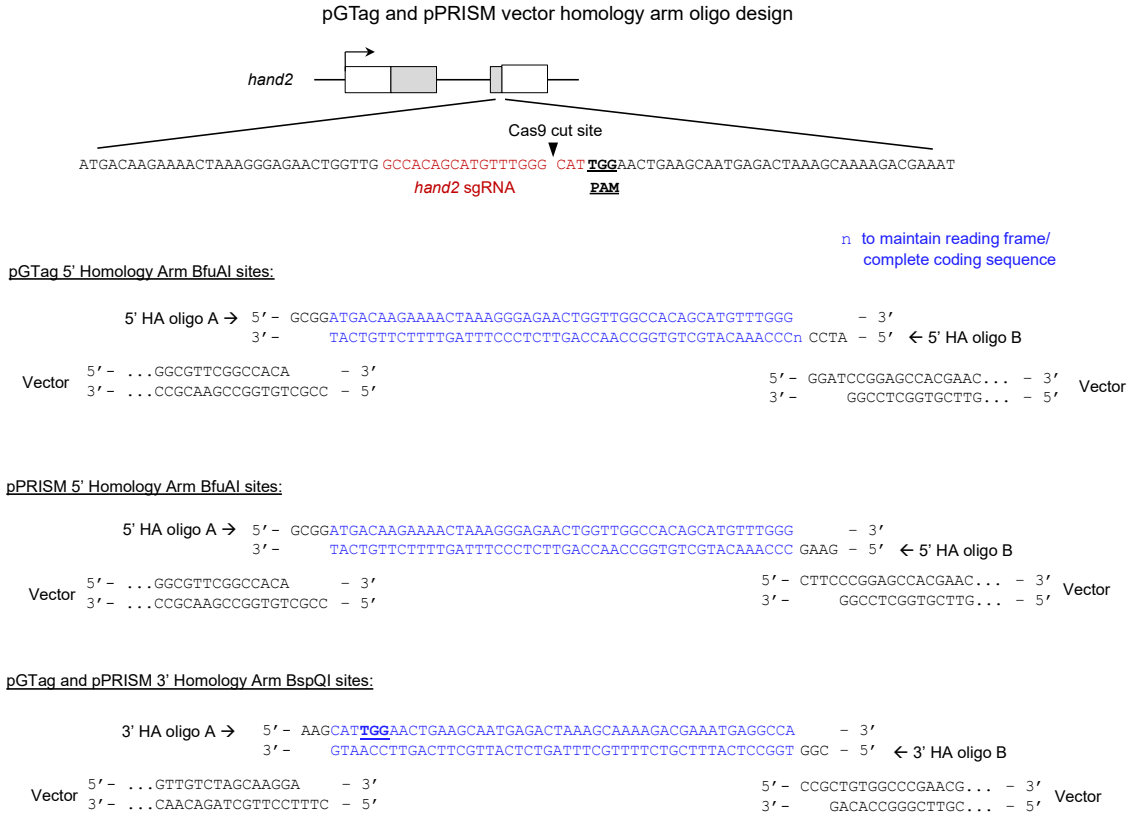

Figure 20. Example of pGTag and pPRISM vector homology arm design showing complementary 5' overhangs for cloning into the BfuAI and BspQI type II restriction enzyme sites. Diagram of CRISPR/Cas9 target site in the *hand2* gene. gRNA sequence in red, PAM sequence underlined and in bold. Annealed homology arm oligos A and B are shown with overhangs (green) complementary to the vector overhangs after enzyme digestion. n = nucleotides included to maintain the reading frame/complete coding sequence of integration alleles.

F. One Pot Cloning of Homology Arms into pGTag Vectors

Notes:

a. *If the homology arm oligos contain the sequence "5'-ACCTGC-3'" or "5'-GAAGAGC-3'" (or their complements) the cloning reaction will be less efficient.*

b. *If One Plot cloning is unsuccessful, the 5' and 3' homology arms can be cloned sequentially into the vector using gel purified linear plasmids digested with the appropriate enzyme.*

1. Homology Arm Annealing

   Anneal upstream and downstream homology oligo pairs separately:

   4.5 µl oligo A at 10 µM

   4.5 µl olio B at 10 µM

   4 µl 10× Buffer 3.1 from NEB

   27 µl dH_2O

   40 µl total

To anneal the oligos, run the following program in a thermal cycler: Step 1, incubate at 98°C for 5 minutes; Step 2 incubate at 97°C for 45 seconds; repeat Step 2 for 90 cycles during which the temperature is decreased by 1°C/cycle; hold at 4°C for the final step. Alternatively, boil water in a glass beaker on a hot plate, and incubate the tube in the boiling water for 5 minutes. Remove the beaker from the heat source and allow it to cool to room temperature. Store the annealed homology arms on ice or in a -20°C freezer.

2. 1-Pot Digest

   Assemble the following:

   4.0 µl dH_2O

   2 µl Plasmid at 50 ng/µl

   1 µl 10× Buffer 3.1 from NEB

   1 µl 5' annealed homology arm

   1 µl 3' annealed homology arm

   0.5 µl BfuAI enzyme from NEB

   0.5 µl BspQI enzyme from NEB

   10 µl total

   Incubate at 50°C for 1 h, place on ice.

3. Ligation

   Add the following:

   3 µl 5× T4 quick ligase buffer

   1.5 µl dH_2O

   0.5 µl T4 quick ligase

   15 µl total

   Incubate 8-10 min at room temperature (to overnight). Store at -20°C.

4. Transformation – To prevent recombination at repetitive elements in the plasmid, grow transformations and overnight cultures at 30°C. Our standard protocol uses NEB Stable Competent *E. coli* (C3040H) cells for cloning and propagation of the GeneWeld pGTag and pPRISM plasmid series to limit recombination.

5. Growing colonies

   Pick 3 white colonies from each plate and grow in separate glass culture tubes with 3 ml LB/Kanamycin, overnight at 30°C, or to pre-screen colonies by colony PCR:

6. Mini Prep Cultures and sequencing of plasmids

   The 5’ homology arm can be sequenced by the 5'_pgtag_seq primer:

   5'-GCATGGATGTTTTCCCAGTC-3’.

   The 3’ homology arm can be sequenced with the “3’_pgtag_seq”primer:

   5'-ATGGCTCATAACACCCCTTG-3'.

G. Injection of GeneWeld Reagents (spCas9 mRNA, Universal sgRNA (UgRNA), genomic sgRNA and pGTag homology vector) into 1-cell zebrafish embryos

Prepare and collect the following reagents for injection:

1. Prepare nCas9n mRNA from pT3TS-nCas9n (Addgene #46757) (Jao et al., 2013)
2. The UgRNA and genomic sgRNA can be directly ordered form IDT or Synthego and resuspended in Molecular Grade RNase/DNase-free water.

3. The pGTag homology vectors should be purified a second time prior to microinjection under RNase free conditions with the Promega PureYield Plasmid Miniprep System beginning at the endotoxin removal wash. Plasmid DNA is eluted in Molecular Grade RNase/DNase-Free water.
4. Embryo Injections for Integration of pGTag vectors.

   Injections are performed into single cell embryos at a volume of 2 nl per embryo containing the following concentration of RNAs and vector:

In injection solution	In embryo
75 pg/nl of nCas9n mRNA	150 pg of nCas9n mRNA
12.5 pg/nl of genomic sgRNA	25 pg of genomic sgRNA
12.5 pg/nl of UgRNA	25 pg of UgRNA
5 pg/nl of pGTag DNA	10 pg of pGTag DNA

H. Test injected embryos for evidence of precision on-target integration

1. Examine injected embryos for fluorescence under a Zeiss Discovery dissecting microscope with a 1× objective at 70-100× magnification. If no or weak signal is observed, integration of pGTag-Gal4VP16 can be used to amplify reporter expression in the 14XUAS-RFP transgenic line (Balciuniene *et al.*, 2013). High resolution confocal live imaging can also be carried out, as shown in Wierson *et al.* (2020) (Figure # below).

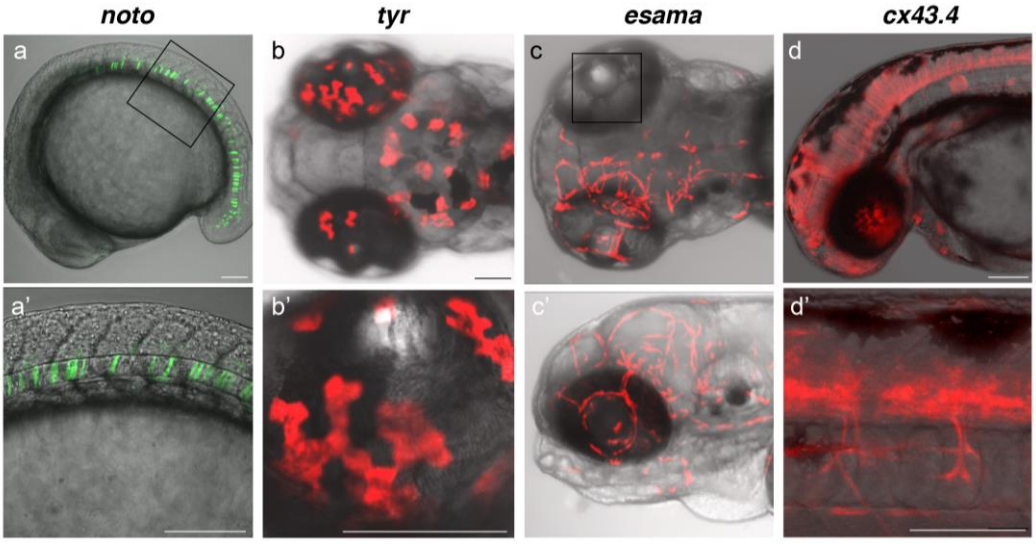

Figure 21. Examples of 2A-GFP and 2A-mRFP targeting into F0 embryos injected with pGTag vectors targeting *noto*, *tyr*, *esama*, and *cx43.4* genes.

2. Perform junction fragment PCR analysis on positive embryos that display widespread fluorescence in expression domains consistent with the targeted gene. Isolate genomic DNA from individual embryos and a control embryo (See Section D 2. Digestion of embryos for isolation of genomic DNA for mutagenesis analysis). PCR amplify the genomic DNA-integrated cassette 5' and 3' junctions fragments. An example of F0 embryo junction analysis is shown in Figure #.

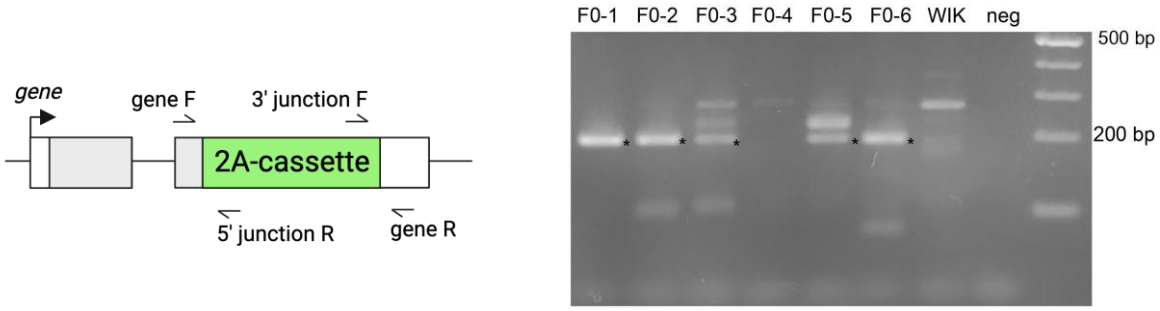

Figure 22. Examples of 5' and 3' PCR junction analysis of individual F0 embryos injected with GeneWeld pGTag targeting vector, genomic gRNA, universal UgRNA, and Cas9 mRNA.

I. Establish a new transgenic line of a precision targeted integration allele.

1. Raise to adulthood fluorescence reporter expressing F0 siblings of injected embryos that showed positive bands for 5' and 3' junction analysis indicating precision targeted integration. Outcross F0 adults to wild type and examine the progeny for reporter gene fluorescence as above in order to identify F1 embryos that have inherited a stable germline integration allele. For Gal4Vp16 integration alleles, cross the F0 adults to the 14XUAS:RFP reporter line. Silencing of the 14XUAS:RFP reporter may result in mosaic expression patterns in Gal4Vp16 targeted F1 embryos.
2. Test F1 fluorescence positive embryos for precise transgene integration by junction fragment PCR analysis as described above. Raise F1 siblings to adulthood and fin-clip to identify individuals with precise targeted transgene integration.
3. Outcross a single positive F1 adult to establish F2 families. F1s can also be sacrificed at 3 weeks post fertilization to the confirm location and precision of targeted integrations by genomic Southern Blot RFLP analysis. Continue to maintain lines by outcrossing to wild type in subsequent generations.

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