GeneWeld: a method for efficient targeted integration directed by short homology

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Gene Targeting Protocol for Integrations with pGTag Vectors using CRISPR/Cas9

Targeting strategy (Figure 1):

- **A. Selection of a CRISPR/spCas9 target site downstream of the first AUG in the gene of interest**
- **B. Synthesize sgRNA and spCas9 mRNA**
- **C. Injection of sgRNA and spCas9 mRNA**
- **D. Testing for indel production/mutagenesis**
- **E. Design short homology arms**
- **F. One Pot Cloning of Homology Arms into pGTag Vectors**
- **G. Injection of GeneWeld reagents (spCas9 mRNA, Universal sgRNA (UgRNA), genomic sgRNA and pGTag homology vector) into 1-cell zebrafish embryos**
- **H. Examine embryos for fluorescence and junction fragments**

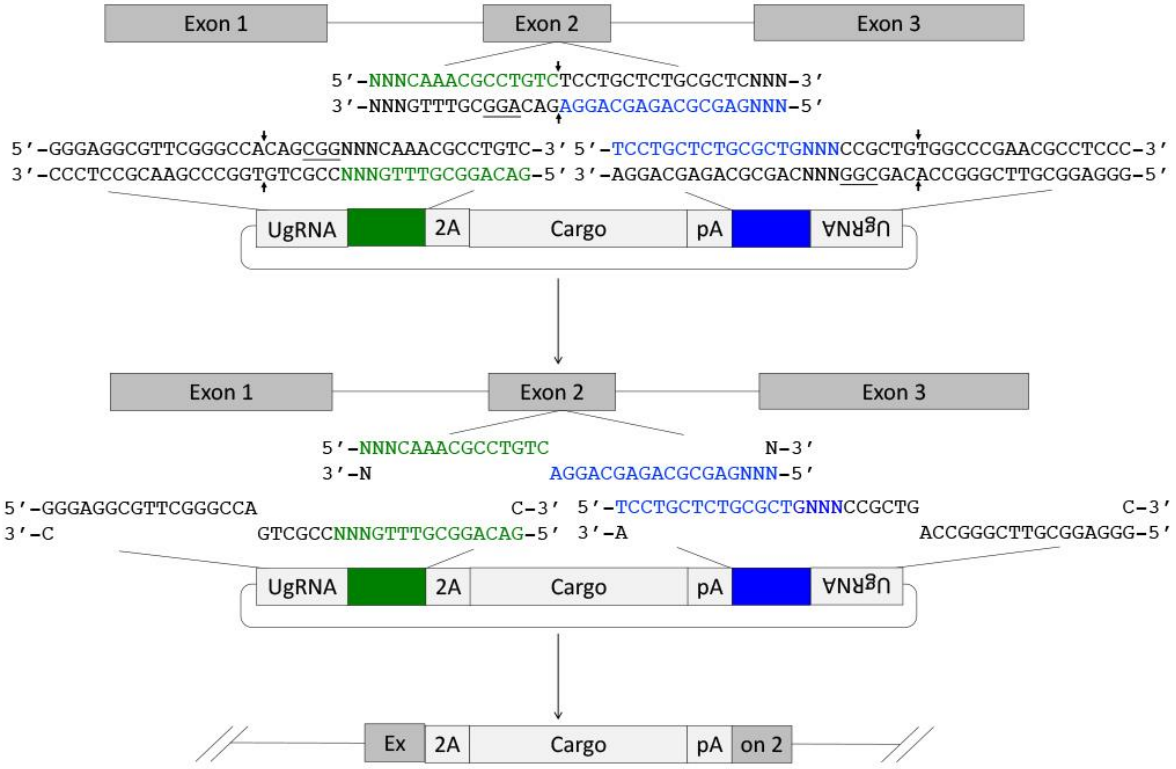


Figure 1. Targeting integration of the pGTag vectors into the 5' region of a gene. Upon CRISPR/Cas9 targeting and cutting of both the genome (with a sgRNA) and plasmid donor (with UgRNA), the genomic and plasmid DNA likely undergo end resection mediated by the MRN complex and *ExoI*, resulting in annealing of complementary homology arms. This promotes precise homology-directed integration of cargo DNA at the CRISPR/Cas9 double-strand break.

A. Selection of a CRISPR/spCas9 target site downstream of the first AUG in the gene of interest

1. To select a CRISPR/Cas9 target site in a 5' exon, find and download the targeted gene's genomic and coding sequences.

    a. At <ensemble.org> Search for the gene name of interest for the species of interest and open the Transcript page.
    b. In the left-hand side bar click on "Exons" to find the first coding exon and initiation ATG. If there are alternative transcripts for the gene, make sure there are not alternative initiation ATGs. If there are alternative start codons, target the first 5' exon that is conserved in all transcripts to generate a strong allele.
    c. Download the transcript and 5' exon to be targeted as separate sequence files.
    d. Using ApE: <http://biologylabs.utah.edu/jorgensen/wayned/ape/> annotate the coding sequence with the exons.

2. Use CRISPRScan (http://www.crisprscan.org/) (Moreno-Mateos et al., 2015) to efficiently identify target sites and generate oligos for sgRNA synthesis for the target gene.

    a. Select the “Predict gRNAs” on the lower right-hand side of the home page of the CRISPRScan website.
    b. Paste the 5’ exon sequence into the indicated box. If the exon is very large, start with a small amount of sequence. Ideally exon sequence of ~200 bp near the desired target site. Do not design CRISPRs to intron/exon borders. If there are problems with the copy and pasting of exon sequence, first paste the sequence into a new ape file, save, then copy and paste from the new file.
    c. Select “Zebrafish (Danio rerio)” as the species
    d. Select “Cas9 – nGG” as the enzyme.
    e. Select “In vitro T7 promoter”.
    f. Click on “Get sgRNAs.” Examine the output. The generated targets are ranked by CRISPRScan from high to low. Select a target site (the 20 bp that are capitalized in the oligo column) from those given by CRISPRScan using the following criteria (The best gRNAs will have all of these):

        i. An exact match to the genomic locus., When an oligo is clicked on the page will display additional information to the right. In the section called “Site Type” any mismatches in the oligo are displayed. Exact matches including 5’GG- are ideal for in vitro transcription and 100% genomic target match.
        ii. The target is in the desired location of the gene.
        iii. The Target is on the reverse (template) strand. Reverse strand guides are more favorable, but either will work
        iv. A high CRISPRScan score, and a lower CFD score. However, lower score sgRNA targets may work fine.

    g. Annotate the selected target sequence in the transcript sequence files.
    h. For sgRNA synthesis the entire oligo sequence from CRISPRscan containing the selected target will need to be synthesized. This oligo is represented as “Oligo A” in Figure 2.

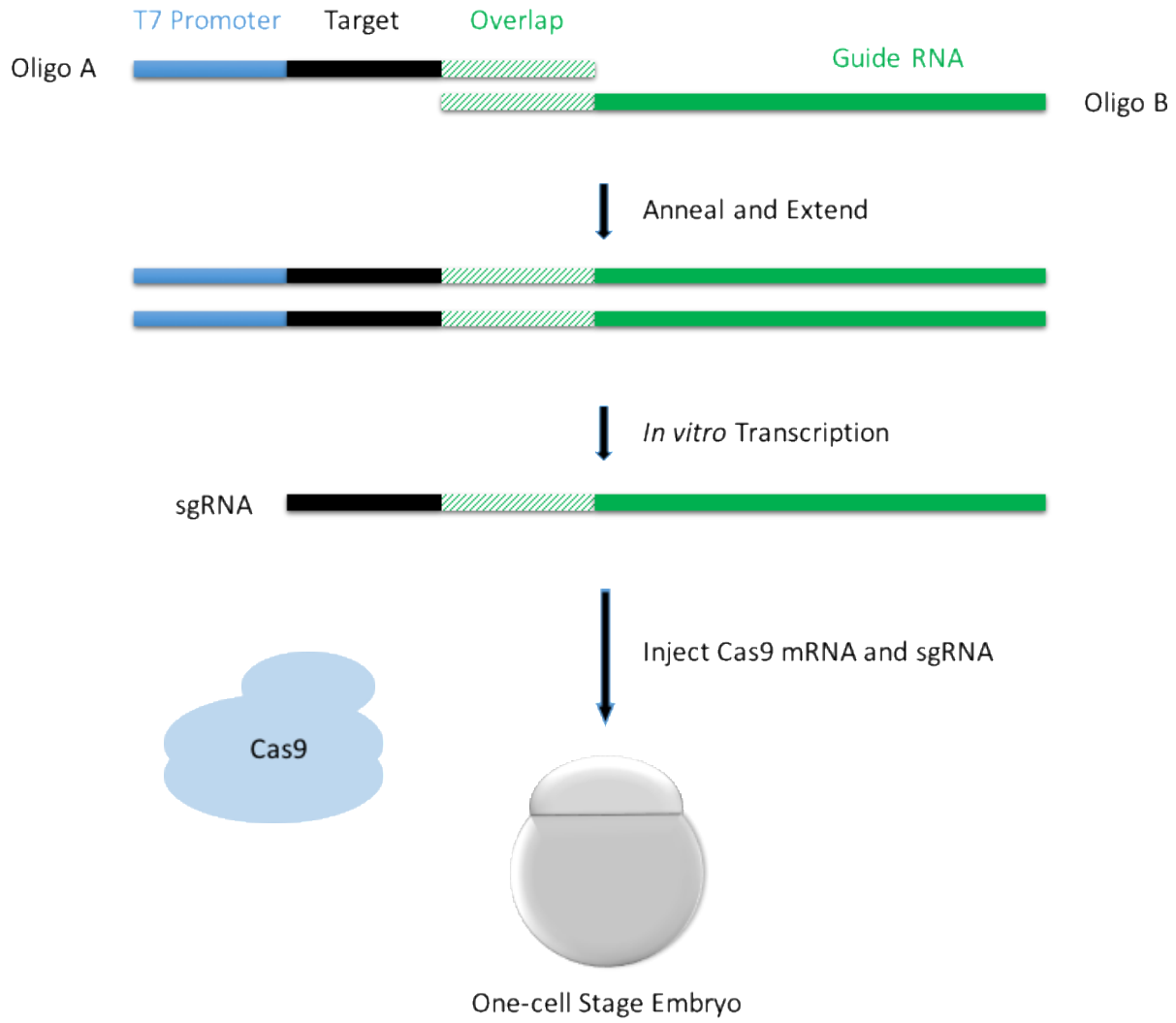


Figure 2. Cloning-free gRNA synthesis. Oligo A is composed of the T7 promoter at the 5' end, target sequence for gRNA, and gRNA overlap sequence for gRNA synthesis. CRISPRScan provides direct output for Oligo A. The strategy for gRNA production using Oligo A is based on (Varshney et al., 2015).

3. **Alternative to CRISPRScan:** Designing "CRISPR Oligo A" from a genomic target sequence. Skip this section if Oligo A was designed with CRISPRScan.

If the target sequence was identified using tools other than CRISPRScan, Oligo A can be designed manually. (Note: CRISPRScan will use a shorter overlap region but this does not affect template production). Add T7 and Overlap sequences (see Figure 2) to the 20 bp of target sequence without the PAM. Oligo A for the targeted gene will look like the example below:

5'-TAATACGACTCACTATAGGNNNNNNNNNNNNNNNNGTTTTAGAGC TAGAAATAGC-3'

The sequences in blue (first 17 characters) are the T7 promoter, the grey GG are part of the T7 promoter and ideally are part of the target sequence (see below), the Ns are the target

sequence, and the sequence in green (last 20 characters) are the overlap region to synthesize the non-variable part of the sgRNA. The T7 promoter works optimally with the two grey GGs, however, these GGs will be transcribed by T7 and thus become a part of the sgRNA. Target sequences that contain the GGs may work better, but there are differing reports in the literature on the importance of this (Moreno-Mateos et al., 2015). If possible, select a target that starts with GG. Refer to Moreno-Mateos et al., 2015 for other gRNA architectures with variations on the 5'GG motif.

a. If the target sequence did not have two Gs at the beginning, additional G's will need to be added to the start of the target sequence for efficient transcription as outlined below:

*The lower case 'g' is an extra 'G' not in the genomic sequence; the upper-case G *is* in the genomic. Lower case gs will not base pair with the genomic target.

i. without GG: ggN NNN NNN NNN NNN NNN N (22 bp) – 2 bases are added,
ii. with one G: gGN NNN NNN NNN NNN NNN NNN (21 bp) – one base is added, G is part of the target sequence.
iii. with two G: GGN NNN NNN NNN NNN NNN NN (20 bp) – no bases are added, GG is part of the target sequence.

Oligo A is made by taking this target sequence with 5'GG and pasting it into a clean file.

b. Copy and paste the T7 promoter sequence to the 5' end of the target sequence: TAATACGACTCACTATA
c. Copy and paste the Overlap sequence to the 3' end of the target sequence: GTTTTAGAGCTAGAAATAGC
d. Check the sequences to ensure they are correct and that the PAM is NOT present in this oligo.

4. Oligo B design (Figure 2) contains the conserved guide RNA sequence: All Oligo Bs will be the same and can be ordered in large quantities.

5'-GATCCGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTT AACTTGCTATTTCTAGCTCTAAAAC-3'

5. To increase yield of the sgRNA synthesis the primers "T7 primer" (5'-TAATACGACTCACTATA-3') and "3'gRNA primer" (5'- GATCCGCACCGACTCGGTG-3') are also required.

6. For checking for mutagenesis at the target site, design ~20 bp DNA primers for PCR amplification to amplify at least 130 bp of DNA surrounding the target site. Mutagenesis is estimated through comparison of PCR products from injected and uninjected embryos, by visualizing small insertions and/or deletions (Indels) using electrophoresis, or by sequencing.

a. Primer 3 is used for primer design: http://biotools.umassmed.edu/bioapps/primer3_www.cgi
b. Paste DNA sequence surrounding the target site into the web interface. It is recommended to use 160 – 300 bp of exon sequence centered on the cut site for

primer design. Intron sequence can be used, but this often contains polymorphisms that can lead to amplification failure.

c. Locate the target sequence, including the PAM sequence (*italicied* below), and predict the cut site (3 bp into the target sequence from the PAM represented here by the 'x'). Mark the targeted exon sequence approximately 65-150 bp on both sides of the cut site by putting [square brackets] around it. Primer3 will design primers outside this sequence. This design allows the primers to be used for both checking of mutagenesis and for junction fragment analysis when checking for integration.

Example:
CGGCCTCGGGATCCACCGGCC**[**AGAATCGATATACTACGATGAACAGAGCAAATTT
GTGTGTAATACGGTCGCCACCATGGCCTxCCT*CGG*TTTGCTACGATGCATTTGCAC
CACTCTCTCATGTCCGGTTCTGGG**]**AGGACGTCATCAAGGAGTTCATGCGCTTCAA
GGTGCGCATGGAGGGCTCCGTGAAC

d. Set the "Primer Size" variables to Min = 130, Opt = 170, and Max = 300. Everything else can be left at the defaults.
e. Click on "Pick Primers"
f. Select primers from the output. Note the "product size" expected and the "tm" or melting temperature of each primer/pair. Smaller product sizes are easier to visualize mutagenesis.

B. Synthesize the sgRNA

General guidelines and good laboratory practices for working with DNA and RNA. DNA, RNA and the enzymes are sensitive to contamination from dust and skin. Following these guidelines will prevent the degradation of the DNA and RNA you are trying to make:

- Be clean. Clean the workbench, pipetmen, racks, and centrifuges with RNase Away or something equivalent.
- Wear gloves and change when contaminated. Contamination will occur when gloves contact hair, face, skin, or the floor.
- Keep everything on ice unless the protocol indicates otherwise.
- Centrifuge components to the bottom of the tube before use, after mixing, after use, and after incubation steps.
- Do not vortex enzymes. Gently flick the tube or pipet up and down to mix samples.
- Avoid touching the walls of the tube when pipetting.
- Use a new pipette tip for each new dip.
- Dispense solutions from a pipet to the bottom of the tube, or into the liquid at the bottom of the tube when setting up reactions.
- Only remove 1.5 ml centrifuge tube and PCR tubes from their package while wearing gloves. Reseal the tube package after tubes are removed.

Assembly of CRISPR Oligos A + B into a Transcription Template

1. For synthesis of the gRNA from Oligo A and B, make a 100 µM freezer stock and 1 µM working stock for each oligo. All oligos are described in Section A starting on page 7.

2. Centrifuge ordered oligos briefly before opening, to move all dried DNA flakes to the bottom of the tube.

3. Add a volume (x µL) of RNase-free water to make a 100 µM stock. The tubes should be labeled with the gene name as well as the number of nmol in the tube. The amount of water to be added will need to calculated based on the nanomoles of material contained within.

4. Vortex for 30 seconds.

5. Centrifuge briefly.

6. Make a 100-fold dilution of each 100 µM stock Oligo A and B in separate 1.5 ml tubes.
   a. Label one 1.5 mL centrifuge tube per Oligo A with name of oligo, date, and "1 µM" to indicate working stocks.

   1 µL of 100 µM Oligo A stock or Oligo B
   99 µL of RNF-water
   100 µL total

   b. Vortex.
   c. Briefly centrifuge.
   d. Store all stocks in freezer at -20 ^{o}C for long-term storage.

7. Set up the following reaction in PCR tubes. The next two steps will generate a short segment of DNA (gDNA or guideDNA Template) which will be used as a template for synthesis of RNA:

   12.5 µL 2X KOD Master Mix
   1 µL Oligo A (1 µM)
   1 µL Oligo B (1 µM)
   1 uL T7 primer (10 µM)
   1 uL gRNA 3' primer (10 µM)
   8.5 µL RNF-water
   25 µL total

8. Run PCR under the following conditions:

   Denature at 98°C for 2 minutes
   Denature at 98 °C for 30 sec.
   Anneal at 50 °C 30 sec.
   Extend at 70 °C 30 sec.
   Go to (step 2) nine times.
   Extend at 70 °C 2 min
   Hold 4 ^{o}C forever.

9. Run 1.2% agarose gel in 1X TAE to check that the template was synthesized:

   a. Remove 3 µL of the reaction and place in a 1.5 ml tube.
   b. Mix in 1 µL of 6x loading buffer.
   c. Load all 4 µL of the sample on the gel. Run the gel at 125 V for 30 minutes. Be sure

to load a molecular weight marker.
d. Check on the transilluminator and image the gel.
e. A single 120 bp band should be detected when 3 µL is loaded on gel.

In vitro transcription (IVT) using the gRNA template

1. Use the Ambion T7 Megascript Kit for transcription reagents, but follow the instructions below.

2. Thaw the T7 10X Reaction Buffer and RNF-water at room temperature, and thaw the ribonucleotides solutions on ice.

3. Vortex the T7 10X Reaction Buffer to make sure all DTT is solubilized. No white flecks should be visible.

4. Microcentrifuge all reagents briefly before opening to prevent loss of reagents and/or contamination by materials that may be present around the rim of the tube(s).

5. Keep the T7 Enzyme Mix on ice or in a -20 ^{o}C block during assembly of the reaction.

6. Make a master mix for each reaction. Assemble the reaction at room temperature on the bench. Add reagents from largest to smallest volume, adding the 10X Reaction Buffer second to last and the T7 Enzyme Mix last.

Note: Components in the transcription buffer can lead to precipitation of the template DNA if the reaction is assembled on ice. If the reaction precipitates, the synthesis reaction will not fully occur.

7. Reagent list:

   10 µL of RNF-water
   5 µL of gDNA template (100 to 500 ng total)
   4 µL of NTP (1 µl of each; A, U, C, G)
   1 µL of 10x transcription buffer – must be fully resuspended at room temp
   1 µL of T7 polymerase enzyme mix

8. Incubate at 37 ^{o}C for 4 to 16 hours. Longer incubations result in considerably better yields.

9. Add 1 µL of Turbo DNAse and incubate for 15 min at 37 ^{o}C. This will digest the template DNA in the sample.

10. Optional quality control step: Run 2 µL of sample on a 1.2% gel in 1X TAE.

    a. Clean the gel box, comb and tray with RNase Away, rinse with DI water.
    b. Remove 2 µl of sample into a clean 1.5 ml (Keep RNA on ice!)
    c. Add 3 µL of RNF-water and 5 uL of Ambion RNA loading buffer with formamide.
    d. Vortex briefly.
    e. Spin down samples briefly.
    f. Run all of this mixture on a 1.2% agarose gel/1X TAE, at 100 V for 1 hour.
    g. Image gel. 2 bands should be visible at ~100 and 120 bp.

Purification of guide RNA

1. Use the miRNeasy Qiagen kit for purification of gRNAs according to the manufacturer's instructions.
2. After Purification verify presence of RNA by running a 1.2% gel in 1X TAE.
3. Clean the gel box, comb and tray with RNase Away, rinse with DI water. Run on a 1.5% agarose gel/1X TAE, at 100 V for 1 hour as above.
4. Image gel. 2 bands should be visible at ~100 and 120 bp.
5. Nanodrop the RNA sample to determine the concentration.
6. Store RNA at -20 °C.

Preparation of SpCas9 mRNA

1. Digest ~5-10 µg pT3TS-nCas9n plasmid with Xba1 (plasmid Addgene #46757 (Jao et al., 2013)).

2. Purify digested DNA with Qiagen PCR cleanup kit or Promega PureYield Plasmid Miniprep System. Elute in RNF-water.

3. Run 100-500 ng on 1.2% agarose gel in 1X TAE to confirm the plasmid is linearized.

4. Use 100 ng to 1 µg DNA as template for in vitro transcription reaction.

5. Use mMESSAGE mMACHINE T3 kit Life Technologies (AM1348) and follow the instructions in the manual.

6. Use the miRNeasy Qiagen kit for purification of nCas9n mRNA according to the manufacturer's instructions.

7. Verify mRNA integrity by mixing 1 uL of purified Cas9, 4 µL of RNF water, 5 µL glyoxl dye (Ambion).

8. Heat mixture at 50 ^{o}C for 30 minutes, then place on ice.

9. Clean the gel box, comb and tray with RNase Away, rinse with DI water.

10. Run all 10 µL of RNA mixture on 1.2% agarose gel in 1X TAE at 100 V for 1 hour as above. One band should be visible at 4.5 kb.

11. Nanodrop the RNA sample to determine the concentration. Concentrations between 0.45 and 1 µg/µL are expected.

12. Aliquot and store RNA at -80 °C.

C. Injection of sgRNA and spCas9 mRNA

The injections here are designed to deliver 25 pg of gRNA and 300 pg of Cas9 mRNA in 2 nL of fluid to embryos at one-cell stage.

Injection trays are cast with 1.2% agarose with 1X embryo media (Zebrafish Book; zfin.org) in polystyrene petri dishes (Fisher No. FB0875713). Injection trays can be used multiple times and stored at 4*C for up to three weeks between use.

1. Trays are pre-warmed to 28.5 ^{o}C prior to injection by placing them in the 28.5 ^{o}C incubator. Try to mitigate tray cooling while not in use.

2. Glass needles are pulled from Kwik-Fil borosilicate glass capillaries (No. 1B100-4) on a Flaming/Brown Micropipette puller (Model P-97).

Injection samples are made to contain the following diluted in RNF water or injection buffer (final concentration: 12.5 mM HEPES pH 7.5, 25 mM Potassium Acetate, 37.5 mM Potassium Chloride, 0.0125 % glycerol, 0.025 mM DTT ph 7.5)

   a. 12.5 ng/µL of genomic gRNA
   b. 150 ng/µL of mRNA for Cas9

3. Needles are loaded with 1.5 to 2.5 µL of sample, and then loaded onto a micro-manipulator attached to a micro injector (Harvard Apparatus PLI - 90) set to 30-40 PSI with an injection time of 200 msec.

4. Needles are calibrated by breaking the end of the tip off with sterile tweezers, ejecting 10 times to produce a droplet of fluid, collecting the droplet into a 1 µL capillary tube (Drummond No. 1-000-0010), and measuring the distance from the end of the capillary to the meniscus of the droplet. This distance is converted to volume (where 1 mm = 30 nL) and adjusted to achieve an effective injection volume of 2 nL. Volumes are adjusted by changing the injection time. There is a linear relationship between volume and time at a set pressure. Avoid injection times less than 100msec and over 400 msec.

5. One cell embryos that have been collected from mating cages are pipetted from collection petri dishes to the wells on the injection tray.

6. Use the micro-manipulator and microscope to pierce the one-cell of embryos on the injection tray at an angle of 30^{o} with the needle. Inject 2 nL of sample in the one-cell near the center of the cell-yolk boundary.

7. After embryos have been injected, wash them from the injection tray into a clean petri dish with embryo media.

8. Keep 20 - 40 embryos separate as uninjected controls. Treat and score the control embryos in the same way as the injected embryos.

9. At 3 - 5 hrs post injection remove any unfertilized or dead embryos from the dishes. This will prevent death of the still developing embryos.

D. Testing for indel production/mutagenesis

Phenotypic scoring of embryos

1. The gRNA itself may be toxic to the developing embryos. Injection toxicity can be estimated by the number dead embryos from a round of injection compared to the un-

injected control dish. Count and remove any brown/dead embryos from injected and un-injected dishes. If there are significantly more dead embryos in the injected dish then the guide may be toxic, impure, or very effective at disrupting a required gene. Reducing the amount of guide or Cas9 mRNA injected may help reduce toxicity.

2. Score and document embryonic phenotypes on days 1 - 4 post fertilization (dpf). Under a dissection microscope examine the un-injected controls and injected embryos, and sort the embryos into categories.

3. Scoring categories

    - -Severe- These embryos have some parts that look like a control embryos, but are missing key features. Examples: embryos that lack their head, eyes, or tail, or embryos that have an unnaturally contorted shape or are asymmetric.

    - -Mild- These embryos appear mostly normal, but have slight defects such as small eyes, pericardial edema, shortened trunk/tail, or curled/twisted tails.

    - -Normal- appears normal and similar to controls.

Digestion of embryos for isolation of genomic DNA for mutagenesis analysis

Genomic DNA (GDNA) can be isolated from zebrafish embryos aged between 1 and 5 dpf using this protocol. Embryos can be analyzed as individuals or as pools (maximum 5) from the same injection.

1. Dechorionate embryos, if they have not emerged from the chorion.

2. It is recommended to screen a minimum of 3 embryos from each scoring category for mutagenesis. Place each embryo, including controls, into separate PCR tubes. Remove as much of the fish water as possible. If needed, spin briefly and remove additional water.

3. Add 20 µL of 50 mM NaOH <u>per embryo</u>.

4. Heat the embryos at 95^{o}C in a thermocycler for 15 minutes.

5. Vortex samples for 10 seconds. Be sure that the tubes are sealed to prevent sample loss while vortexing.

6. Spin samples down and heat for an additional 15 min at 95 ^{o}C in a thermocycler.

7. Vortex samples and then spin the tubes down again. The embryos should be completely dissolved.

7. Neutralize the samples by adding 1 µL of 1 M Tris pH 8.0 per 10 µL NaOH. Mix by vortexing then spin down.

8. Genomic DNA should be kept at 4 ^{o}C while in use and stored at -20^{o}C.

Analysis of CRISPR/Cas9 mutagenesis efficiency at targeted gene locus.

1. Set up the following PCR reactions for each tube of digested embryos using the primers designed at the end of section A, page 10.

    12.5 µL of 2x GoTaq Mastermix
    1 µL of Forward Primer (10 uM)
    1 µL of Reverse Primer (10 uM)
    1 µL of gDNA template (digested embryos)
    9.5 µL of nuclease-free water
    25 µL total

2. Vortex and briefly spin down the PCR reactions.

3. Run the following PCR program to amplify the targeted locus.

95°C	2 minutes	
95°C	30 seconds	]
55°C*	30 seconds	] x 35 cycles
72°C	30 seconds	]
72°C	5 minutes	
4°C	hold	

    *if the primers were designed with higher or lower tm's than the annealing temperature in line three, then that temperature will need to be adjusted to 2°C below the designed primer tm.

4. Run up to 7 µL of PCR product on a 3.0% agarose gel, 1X TAE, for 1 hr at 80-100V.

5. Analyze the gel for DNA bands that appear diffuse or different in size from the control lane. This indicates that the presence of indels in the gene of interest

6. Alternatively clone and sequence PCR products or sequence them directly to verify the presence of indels.

E. Design short homology arms

Homology directed gene targeting allows the integration of exogenous DNA into the genome with precision to the base pair level. However, designing and cloning individual targeting vectors and homology arms for each gene of interest can be time consuming. The pGTag vector series provides versatility for ease of generation of knockout alleles (Figure 3). The vectors contain BfuAI and BspQI type II restriction enzymes for cloning of short homology arms (24 or 48 bp) using Golden Gate cloning. The pGTag vectors require in-frame integration for proper reporter gene function. The reporter gene consists of several parts. First, a 2A peptide sequence causes translational skipping, allowing the following protein to dissociate from the locus peptide. Second and third, eGFP, TagRFP, or Gal4VP16 coding sequences for the reporter protein have a choice of sequence for localization domains, including cytosolic (no) localization, a nuclear localization signal (NLS), or a membrane localization CAAX sequence. Finally translation is terminated by one of two different polyadenylation sequences (pA); a β-actin pA from zebrafish or the SV40pA.

For many genes, the signal from integration of the report protein is too weak to observe. In these cases the Gal4VP16 vector allows for amplification of the report to observable expression levels in F0s and subsequent generations. A 14XUAS/RFP Tol2 plasmid is provided to make a transgenic line for use with the Gal4VP16 vector.

Sequence maps for these plasmids can be downloaded at www.genesculpt.org/gtaghd/

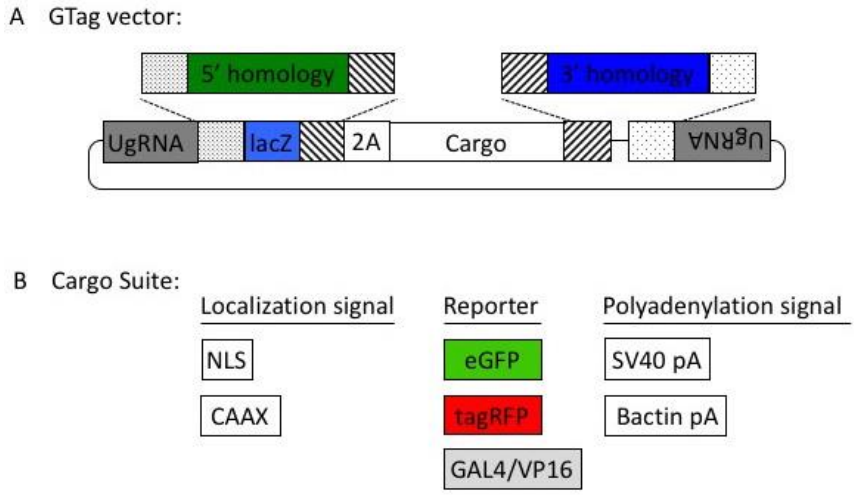


Figure 3. The pGTag vectors allow one step cloning of homology arms.

All vectors can be obtained through Addgene (www.addgene.org). Because the pGTag plasmids contain repeated sequences, they may be subject to recombination in certain strains of bacteria. **It is strongly recommended that they are propagated at 30ºC to reduce the possibility recombination.**

The web tool, GTagHD www.genesculpt.org/gtaghd/, allows for quick design of cloning ready homology arm oligos for a gene of interest.
To use the tool, choose the "Submit Single Job" tab. Follow the instructions in the tab.

There should be 4 oligos (two pairs that will be annealed) generated that should be ordered for cloning. If there are any problems with the sequences and values that were entered, the web page will display the errors and give advice on how to fix them.

The following protocol describes how to design homology arm oligos manually:

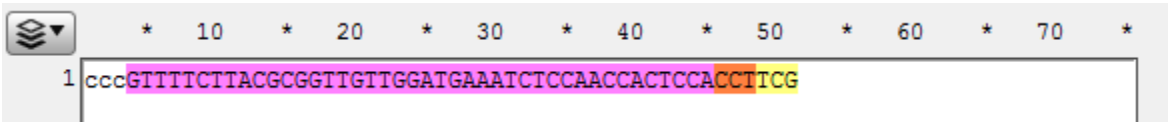
Note In the following section when orientation words are used, they are used in the context of the reading frame of the genetic locus of interest. Example: A 5' template strand CRISPR means that the target site for the CRISPR is on the template strand at the locus and is toward the 5' end of the gene. Upstream homology domains are 5' of the CRISPR cut and downstream homology domains are 3' of the cut with respect to the gene being targeted. Also note: Upper case and lower case bases are not specially modified; they are typed the way they are as a visual marker of the different parts of the homology arms.

For the Upstream Homology Domain

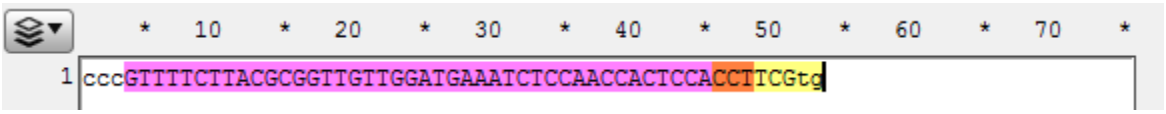
1) Open the sequence file for the gene of interest and identify the CRISPR site. (In this example it is a Reverse CRISPR target in Yellow, the PAM is in Orange, coding sequence is in purple)

Copy the 48 bp 5’ of the CRISPR cut (the highlighted section below) into a new sequence file; this is the upstream homology.

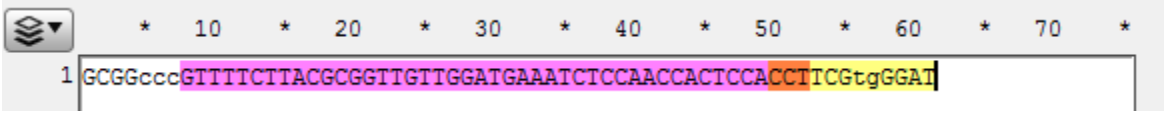
2) Observe the next three bases immediately upstream of the 48 bp of homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the three bases are “GGA” so “ccc” was chosen for the spacer)
Add the spacer to the new file 5’ (in front) of the homology, see below. The spacer acts a non-homologous buffer between the homology and the eventual 6 bp flap from the universal guide sequence that will occur when the cassette is liberated and may improve intended integration rates over MMEJ events.



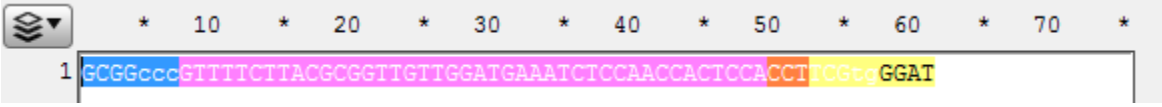
3) Determine where the last codon is in the homology. Here the 3’ G in the homology domain is the first base in the codon cut by this CRISPR target. Complete the codon by adding the remaining bases (called padding on GTagHD) for that codon from your sequence to ensure your integration event will be in frame.



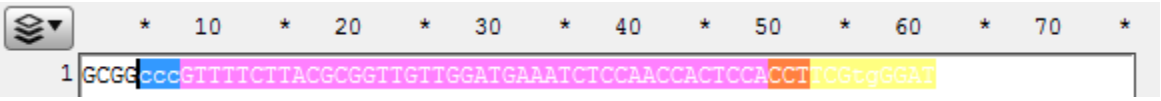
4) Add the BfuAI enzyme overhang sequences for cloning, to the ends of the homology domain. 5’-GCGG and 3’-GGAT. (Here both overhangs are added to prevent errors in copying sequence for the oligos in the next two steps.)



5) The Upstream Homology Oligo A will be this sequence from the beginning to the end of the last codon (see highlighted below). Copy and paste this sequence into a new file and save it. In this example this oligo sequence is 5’-GCGGcccGTTTTCTTACGCGGTTGTTGGATGAAATCTCCAACCACTCCACCTTCGtg-3’.



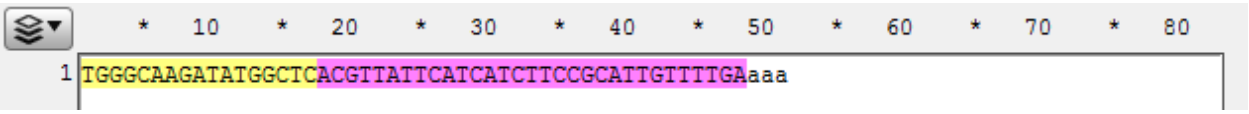
6) The Upstream Homology Oligo B will be the reverse compliment of this sequence from beginning of the spacer to the end of the sequence (see highlighted below). Copy the reverse compliment, paste it into a new file, and save it. In this example this oligo sequence is 5'-ATCCcaCGAAGGTGGAGTGGTTGGAGATTTCATCCAACAACCGCGTAAGAAAACggg-3'.



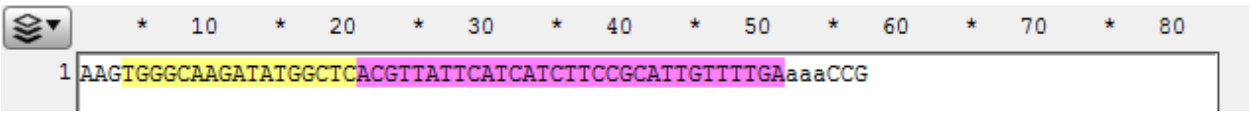
For the Downstream Homology Domain

7) Open sequence file for the gene of interest and identify the CRISPR site. (Reverse CRISPR target in Yellow, PAM in Orange, coding sequence is in purple)
Copy the 48 bp 3' of the CRISPR cut into a new sequence file; this is the downstream homology.

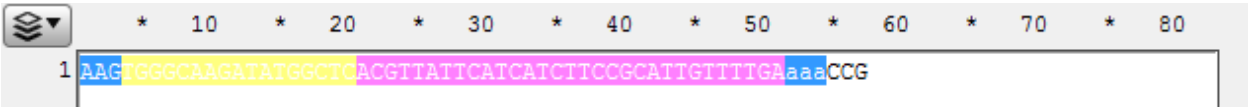
8) Observe the next three bases downstream of the 48 bp of homology, and pick a base not present to be the 3 bp spacer between the homology and the Universal PAM in the vector. (Here the bases are "CTG" so "aaa" was chosen for the spacer.)
Add the spacer to the new file 3' of (after) the homology.



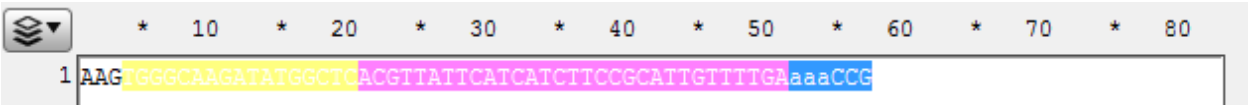
9) Add the BspQI enzyme overhang sequences for cloning, to the ends of the homology domain. 5'-AAG and 3'-CCG. (Here both overhangs are added to prevent errors in copying sequence for the oligos in the next two steps.)



10) The Downstream Homology Oligo A will be this sequence from the beginning of the sequence to the end of the spacer (see highlighted below). In this example this oligo sequence is 5'-AAGTGGGCAAGATATGGCTCACGTTATTCATCATCTTCCGCATTGTTTTGAaaa-3'.



11) The Downstream Homology Oligo B (will be the reverse compliment of this sequence from the beginning of the homology to the end of the sequence (see highlighted below). In this example this oligo sequence is 5'-CGGtttTCAAAACAATGCGGAAGATGATGAATAACGTGAGCCATATCTTGCCCA-3'



F. One Pot Cloning of Homology Arms into pGTag Vectors

**Note if the homology arm oligos contain either the sequence "5'-ACCTGC-3'" or "5'-GAAGAGC-3'" (or their compliments) the cloning reaction will be less efficient.
*Note some sequences just don't work very well. **Ligation is more efficient with annealed homology arms and the purified ~1.2 kb and ~2.4kb fragments from vectors that have been digested with BfuAI and BspQI. If problems are encountered, one homology arm can also be cloned sequentially.**

1. Homology Arm Annealing

Anneal upstream and downstream homology oligo pairs separately:

4.5 µL oligo A at 10 uM
4.5 µL oligo B at 10 uM
4 µL 10x Buffer 3.1 from NEB
27 µL dH20
total = 40 µL

Incubate at 98°C for 4 min, 98°C 45 sec x 90 steps decrementing temp 1°C/cycle, 4°C hold

(Alternatively heat in 95-98°C water for 5 minutes, and then remove the boiling beaker from the heat source and allow it to cool to room temp for 2 hours, before placing samples on ice.)

2. 1-Pot Digest

Assemble the following:

4.0 µL dH2O
2 µL Plasmid at 50 ng/uL
1 µL 10x 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 uL total

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

3. Ligation

Add the following:

3 uL 5x T4 quick ligase buffer
1.5 uL dH2O
0.5 uL T4 quick ligase
15 uL total

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

4. Transformation

a. On ice, thaw 1 (one) vial competent cells (50 µL) for every 2 ligation reactions. (approx. 5 min). It is recommended to use NEB Stable Competant E. coli (C3040H) cells to limit recombination.
b. While cells are thawing, label the microcentrifuge tubes for each ligation and put on ice.
c. Once the cells are thawed, use a pipette to transfer 25 µL of the competent cells into each labeled tube.
d. Add 1.5 µL of a ligation reaction into competent cells to transform.
    a. Amount of ligation reaction added should be less than 5% of volume of competent cells.
e. Mix by tapping the tube several times or gently mixing with the pipet tip.
    a. Do NOT mix by pipetting, this will lyse the cells.
f. Incubate on ice for 5 to 20 minutes.
g. Heat shock the cells by submerging the portion of the tube containing the cells in a 42°C water bath for 40 - 50 seconds.
h. Incubate cells on ice for 2 minutes.
i. Add 125 µL of room temperature LB to each transformation.
j. Incubate cells at 30°C for 1- 1.5 hour(s) in a shaking incubator.
k. While the transformed cells are recovering, spread 40 µL of X-Gal solution, and 40 µL IPTG 0.8 M on LB Kanamycin selection plates.
    a. X-Gal is lethal to cells while wet, it is recommended to first label the plates and then place them in a 30°C incubator to dry.
l. After recovery and the X-Gal is dry, Plate 150 µL of each transformation on the corresponding correctly labeled plate.
m. Incubate plates overnight at 30°C.

5. Growing colonies

Pick 3 white colonies from each plate and grow in separate glass culture tubes with 3 mL LB/Kanamycin.

Or to pre-screen colonies by colony PCR:

a. Pick up to 8 colonies with a pipet tip and resuspend them in separate aliquots of 5 µL dH2O. Place the tip in 3 ml of LB/Kan, label, and store at 4ºC.
b. Make a master mix for your PCR reactions containing the following amounts times the number of colonies you picked.

    7.5 µL 2x Gotaq mastermix
    5.5 µL dH2O
    0.5 µL primer at 10 uM "F3'-check" 5'- GGCGTTGTCTAGCAAGGAAG -3'
    0.5 µL primer at 10 uM "3'_pgtag_seq"5'-ATGGCTCATAACACCCCTTG-3'
    14 µL total

c. Aliquot 14 µL of mixed master mix into separate labeled PCR tubes.
d. Add 1 µL of colony to each reaction as template.
e. or 20 ng purified plasmid as control.
f. Cycle in a thermocycler

95ºC	2 minutes	
95ºC	30 seconds	]
57ºC	30 seconds	] x 35 cycles
72ºC	30 seconds	]
72ºC	5 minutes	
4ºC	hold	

g. Run 5 µL of PCR product on a 1% agarose gel. You should get bands that are a different size than the control.

6. Mini Prep Cultures

   Follow Qiagen Protocol

7. 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 from (Jao et al., 2013)) as described above (page 14).

2. Synthesize UgRNA and purify as described above (page 11) using the following oligo A:

5'-TAATACGACTCACTATAGGGAGGCGTTCGGGCCACAGGTTTTAGAGCTAGAAATAGC-3'

Corresponding to the universal target sequence: GGGAGGCGTTCGGGCCACAG

Alternatively, the UgRNA can be directly ordered form IDT and resuspended in RNF water.

5’-GGGAGGCGUUCGGGCCACAGGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGG
CUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCGGAUC-3’

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 and eluted in RNF water.

Embryo Injections for Integration of pGTag vectors

Injections are performed as previously described in 2 nl per embryo with the addition of the UgRNA and targeting pGTag DNA.

Final per embryo:	Injection mixture:
150 pg of nCas9n mRNA	75 pg/nl of nCas9n mRNA
25 pg of genomic gRNA	12.5 pg/nl of genomic gRNA
25 pg of UgRNA	12.5 pg/nl of UgRNA
10 pg of pGTag DNA	5 pg/nl of pGTag DNA

H. Examine embryos for fluorescence and junction fragments

Embryos are examined for fluorescence under a Zeiss Discovery dissecting microscope with a 1X objective at 70-100X magnification. If weak signals are observed, embryos are manually dechorionated, and viewed on glass depression well slides. If no or weak signals were observed, Gal4VP16 integrations are attempted in a 14XUAS-RFP background. Embryos displaying widespread fluorescence in expression domains consistent with the targeted gene are examined for junction fragments or raised to adulthood for outcrossing.

F0 Junction fragment analysis between the genomic locus and the targeting vector is carried out by isolating DNA from embryos followed by PCR. The following primers are used for junction fragment analysis and must be paired with gene specific primers (5’ to 3’):

5’ pGTag junctions:

R-Gal4-5'juncM	GCCTTGATTCCACTTCTGTCA	with a gene specific forward primer
R-RFP-5'junc	CCTTAATCAGTTCCTCGCCCTTAGA	
R-eGFP-5'-junc	GCTGAACTTGTGGCCGTTTA	

3’ pGTag junctions:

F-Gal4-3'juncM	GCAAACGGCCTTAACTTTCC	with a gene specific reverse primer
F-Gal4-3'juncJ	CTACGGCGCTCTGGATATGT	
F-RFP-3'junc	CGACCTCCCTAGCAAACTGGGG	
F-eGFP-3'junc	ACATGGTCCTGCTGGAGTTC	

PCR amplification of junction fragments can be a result of artifacts (Won and Dawid, 2017), so it is important to carryout control amplifications with injected embryos that lack the genomic gRNA. F0 analysis by PCR of junction fragments is carried out to examine correct targeting. F-Gal4-3'juncM and F-Gal4-3'juncJ are two alternate primers for amplification of junction fragments from the Gal4 cassette due to gene specific mis-priming depending on the target loci.

7.5 µL 2x Gotaq mastermix
5.5 µL dH2O
0.5 µL primer at 10 uM genomic primer
0.5 µL primer at 10 uM pGTag primer
14 µL total

1. Aliquot 14 µL of mixed master mix into separate labeled PCR tubes.
2. Add 1 µL of genomic DNA to each reaction as template.
3. Cycle in a thermocycler with the following steps:

95°C	2 minutes	
95°C	30 seconds	]
55°C	30 seconds	] x 35 cycles
72°C	30 seconds	]
72°C	5 minutes	
4°C	hold	

4. Run 5 µL of PCR product on a 1.2 % agarose gel in 1XTAE. Putative junction fragments should give bands that are of predicted size.

F0 animals that are positive for the reporter gene are raised to adults then outcrossed and examined for fluorescence as above. The Gal4VP16 system can lead to silencing resulting in mosaic patterns in F1 embryos. F1 embryos displaying fluorescence are examined for junction fragments as above, raised to outcross to make F2 families or sacrificed at 3 weeks post fertilization for Southern-Blot analysis of integrations. F0 and F1 identified fish can be incrossed or backcrossed to get an initial impression of the homozyogous phenotypes. It is recommended that lines are continuously outcrossed once established.

References:

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Moreno-Mateos, M.A., Vejnar, C.E., Beaudoin, J.D., Fernandez, J.P., Mis, E.K., Khokha, M.K., and Giraldez, A.J. (2015). CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nat Methods *12*, 982-988.
Varshney, G.K., Pei, W., LaFave, M.C., Idol, J., Xu, L., Gallardo, V., Carrington, B., Bishop, K., Jones, M., Li, M.*, et al.* (2015). High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. Genome Res *25*, 1030-1042.
Won, M., and Dawid, I.B. (2017). PCR artifact in testing for homologous recombination in genomic editing in zebrafish. PLoS One *12*, e0172802.