Epitope Tag Knock-In

This protocol is for inserting small epitope sequences into the zebrafish genome.

Part I – Target selection and sgRNA test

☐ Do a bit of background research on your favourite protein and see if anyone has successfully tagged it on the N or C terminus whilst retaining function!

- If nothing is known, be prepared to do some validation experiments!
  (e.g. rescue of a LOF mutant with tagged mRNA)

☐ Find your genes ENSEMBL ID and copy that into CHOPCHOP/CRISPRscan to identify gRNA targets close to the start or stop codon (depending on where you're inserting the tag)

- If using synthetic sgRNAs (e.g. Synthego or IDT), the only gRNA requirement is the PAM motif (NGG). Otherwise, inclusion of a 5'GG or G is necessary for T7 transcription of gRNA and can reduce efficiency of sgRNA activity *in vivo*.

☐ Identify primer sites ~200-350 bp away upstream and downstream from your target cutsite that will be used for genotyping and testing efficiency of sgRNA cleavage.

☐ Order oligos for sgRNA synthesis (if you're producing via *in vitro* transcription) / synthetic gRNAs, as well as genotyping oligos.

☐ If synthesizing sgRNA from an oligo, see Appendix B. If using synthetic gRNA, resuspend in RNase-free H2O and nanodrop (I resuspend Synthego sgRNAs in 25 µL H2O). Store stocks at -80°C.

☐ Make a working stock of gRNA at 250 ng/µL in RNAse-free H2O and store at -80°C.

☐ On day of test injection, assemble the following components:

Reagent	Final Conc.	Volume (5 µL)
gRNA (250 ng/µL)	50 ng/µL	1 µL
2M KCl	300 mM	0.75 µL
EnGen Spy Cas9 NLS (~3.22 ng/uL)	~ 500 ng/µL	0.75 µL
H2O	to 5 µL	2.5 µL

☐ Incubate at 37°C for 5 minutes and store at room temperature.

- The glycerol in the Cas9 protein is viscous so storing on ice can make it more challenging to pipette and get in needle. The ribonucleoproteins are stable for the duration of your injection at room temperature.

☐ Load your needle and inject 1 nL of mix into wildtype embryos at the 1-cell stage.

- Chorionated injections are preferred because the dying/exploding embryos won't make their siblings ill within the plate.
- **IMPORTANT: keep uninjected wildtype embryos for your control!**

☐ At the end of the day, clean up embryos (fresh water, removal of exploded/sick embryos) and incubate overnight.

☐ The following day, manually dechorionated ~15 embryos from your injected and ~5 embryos from your uninjected.

☐ Prepare the following lysis buffer mix.

Reagent	**Volume (for 500 µL)**
10x PCR Buffer	50 µL
Proteinase K (20 mg/mL)	12.5 µL
H2O	437.5 µL

☐ Cut an 8 strip tube in half and pipette 50 µL of lysis buffer into each tube.

☐ In the first tubes, pipette 3 wildtype embryos into lysis buffer without transferring excessive egg water (suck them up and let them fall to the end of the pipette tip, then touch the tip to the lysis buffer.

☐ Repeat in tubes 2-4 but pull 3 embryos from your injected embryos.

☐ Put tubes in thermocycler and run the genomic DNA extraction program.

DNA extraction program

55ºC for 1 hour

95ºC for 10 minutes

12ºC hold

☐ After program is completed, vortex tubes briefly and spin down.

- Embryo should be completely dissolved after vortexed and debris will pellet with a pulse in the spinner.

Quick Note: You can run your genotyping PCR any way you like. I prefer the GoTaq Master Mix because it's easy and cheap!

☐ Run a PCR using the genotyping primers on the 4 samples using the following mix.

Reagent	**Volume (1 reaction)**	**Volume (6 reactions)**
2X GoTaq Green Master Mix	6.25 µL	37.5 µL
Forward Primer (10 µM)	0.625 µL	3.75 µL
Reverse Primer (10 µM)	0.625 µL	3.75 µL
H2O	4 µL	24 µL
DNA	1 µL	X

- Samples being run are 1 wildtype, 3 mutants and 1 negative control (water)

☐ Verify amplification by running 2.5 µL of your PCR on a 2% gel.

- High levels of mutagenesis may be observed by multiple bands / smeary bands in your injected samples compared to the wildtype.

☐ Once verified that you amplified something, perform a PCR cleanup using whatever kit your lab has and send fragments for Sanger sequencing using the forward and reverse primer.

☐ Once Sanger sequencing has returned, go to ice.synthego.com and upload your wildtype and mutant samples.

- Do this one at time for each mutant sample compared to the wildtype.

☐ Take the average mutagenesis efficiency (pool 1% + pool 2% + pool 3% / 3)

- I find an efficiency over 50% will yield successful knock-ins! If less than 50%, you may need to screen more F0 fish.

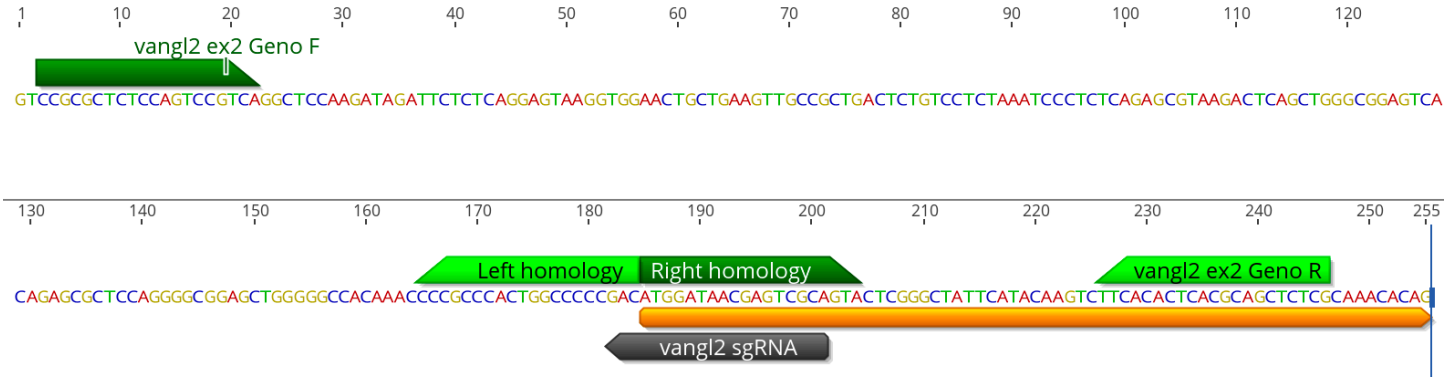
☐ Got a guide that works? Time to move onto the next step!

Part II – Tag design

☐ Using your favourite sequence analysis software, identify the cutsite where your gRNA cleaves.

- As a reminder, Cas9 cleaves bluntly between -3 and -4 from the PAM site.

☐ Mark 20 bp of sequence flanking this region on either side of the cutsite, like shown below:



☐ Copy this homology sequence to a new file, and insert your epitope tag of choice into your sequence in-frame.

- Note: ATG start codon was included in right homology so the right arm was extended 3 bp.
- Here, I'm inserting an ALFA tag, but this approach is amenable to whichever tag you'd like.
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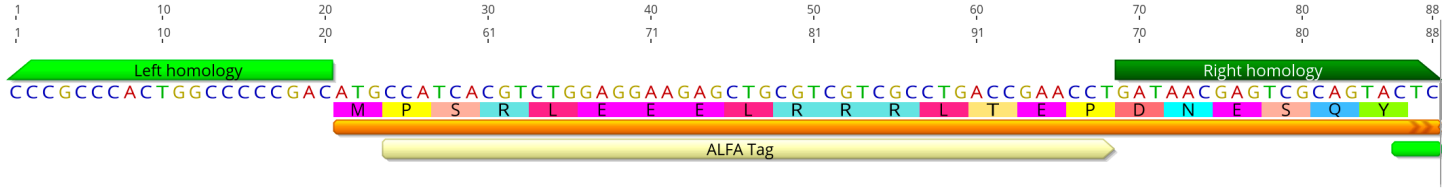


☐ Order this sequence as it is as a **PAGE purified** (CRITICAL!!!) desalted oligo. This is your template!

Part III – Knock-in injection and F0 test

This portion of the protocol involves injecting embryos twice: (1) with a donor oligo into the yolk and then (2) with assembled Cas9 nuclease into the cell. In my hands, this method has a two key benefits. First, the embryos experience reduced toxicity of having ssDNA directly injected into the cell (which can cause developmental defects) and second, the ssDNA excluded from the nuclease mix will ensure that the Cas9 is performing optimally and not 'stuck' to any ssDNA.

☐ On the day of knock-in injections, prepare ribonucleoprotein complexes as described above:

Reagent	Final Conc.	Volume (5 µL)
gRNA (250 ng/µL)	50 ng/µL	1 µL
2M KCl	300 mM	0.75 µL
EnGen Spy Cas9 NLS (~3.22 ng/uL)	~ 500 ng/µL	0.75 µL
H2O	to 5 µL	2.5 µL

☐ Incubate at 37°C for 5 minutes and store at room temperature.

☐ Prepare donor oligo by first resuspending the PAGE purified oligo to 50 µM in RNase-free H2O, and then dilute a working stock of oligo to 2 µM (you only need about 5-10 µL of working stock).

☐ Load your first needle with the donor oligo mix, put that into the rig and calibrate to 1 nL volume.

☐ Load your second needle with the Cas9 mix and keep that aside.

☐ Pull your gates on wildtype fish and collect embryos ideally within 5-10 minutes.

☐ Load an entire plate (6 full lanes) with chorionated embryos and, as quickly as you can, inject 1 nL of your oligo mix into the **yolk** of the embryos.

- At this stage, speed is more important than precision. Just rapid fire as quickly as possible!
- Remember to keep wildtype uninjected controls!

☐ Squirt your embryos into a new plate and label it as 'DONOR OLIGO ONLY'.

☐ Suck up some of these embryos and return them to the injection plate (ensure you leave ~15-25 embryos as oligo only control embryos for PCR verification).

☐ Load your second needle containing Cas9 mix into the rig and calibrate to 1 nL volume.

☐ Re-inject your embryos with the Cas9 mix, ensuring you hit the cell as closely as possible.

☐ Put embryos in the incubator and let them grow until the end of the day.

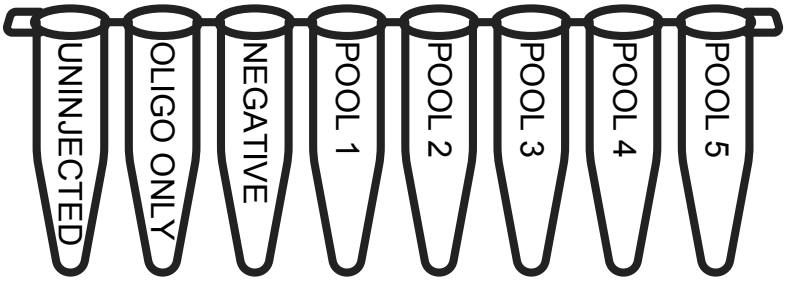
☐ Prior to going home, clean up your plate and purge any sick/exploded embryos.

☐ The following day, manually dechorionated ~20 embryos from your dual injected, ~5 embryos of your donor oligo only injected and ~5 embryos from your uninjected plates.

☐ Prepare the following lysis buffer mix.

Reagent	Volume (for 500 µL)
10x PCR Buffer	50 µL
Proteinase K (20 mg/mL)	12.5 µL
H2O	437.5 µL

☐ Label an 8 strip tube in the following way:



☐ Pipette 50 µL lysis buffer into each tube.

☐ In the first tube, pipette 3 uninjected wildtype embryos into lysis buffer without transferring excessive egg water.

☐ In the second tube, pipette 3 donor oligo only injected embryos into lysis buffer without transferring excessive egg water.

☐ Leave the third tube.

☐ In the 4th-8th tube, pipette pools of 3 dual injected embryos into each tube.

☐ Put tubes in thermocycler and run the genomic DNA extraction program.

DNA extraction program

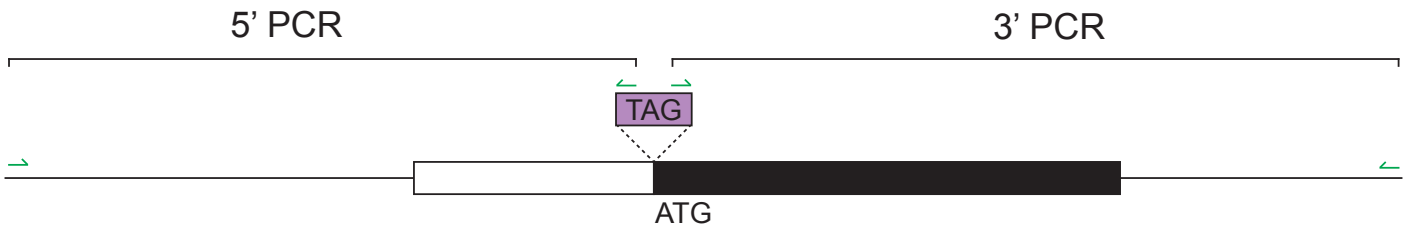
55ºC for 1 hour

95ºC for 10 minutes

12ºC hold

☐ After program is completed, vortex tubes briefly and spin down.

☐ Set up **two** PCR master mixes using the PCR strategy that was originally used for gRNA testing, albeit with the addition of tag-specific primers. The strategy for F0 tag detection is outlined below:



Mix 1 (5’ PCR)

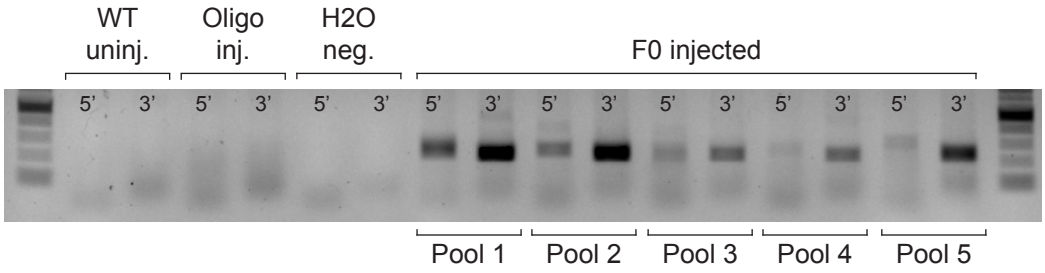
Reagent	Volume (1 reaction)	Volume (8 reactions)
2X GoTaq Green Master Mix	6.25 µL	50 µL
Genotyping Forward Primer (10 µM)	0.625 µL	5 µL
Tag Reverse Primer (10 µM)	0.625 µL	5 µL
H2O	4 µL	32 µL
DNA	1 µL	X

Mix 2 (3’ PCR)

Reagent	Volume (1 reaction)	Volume (8 reactions)
2X GoTaq Green Master Mix	6.25 µL	50 µL
Tag Forward Primer (10 µM)	0.625 µL	5 µL
Genotyping Reverse Primer (10 µM)	0.625 µL	5 µL
H2O	4 µL	32 µL
DNA	1 µL	X

☐ Check amplification by running half (6.25 µL) of your PCR on a 2% gel.

- Bands for 5’ and 3’ PCRs should only be present in the dual injected embryos, such as in this example:
- Band intensity doesn’t necessarily correlate here as the PCR may be inefficient for a given junction. If there’s any evidence of integration, grow those fish regardless.



☐ Got bands? NICE. Grow up your fish to adulthood!

Part IV – Screening for germline transmission

The goal for rapid germline screening is to set up incrosses of F0 fish to quickly check for integration events, and then to isolate those pairs and do further PCR screening and growth. Using this screening, I observe ~20% integration of tags with about half of those being precise integration events.

☐ **Optional**: To speed up growth, at approximately 1 month old split to very low density (~10 per 2L tank). This will let them reach sexual maturity closer to 2 months.

☐ At 1.5 months of age, separate the male and female siblings.

- This step makes more likely to incross successfully when placed together.

☐ When fish are ready to mate, set up incrosses of F0 fish and collect eggs from pairs.

- To expedite this process, set up as many pairs as possible and only collect from pairs that produce >60 embryos.
- Pairs that produce <60 embryos can be returned to tanks to repeat mating the following week

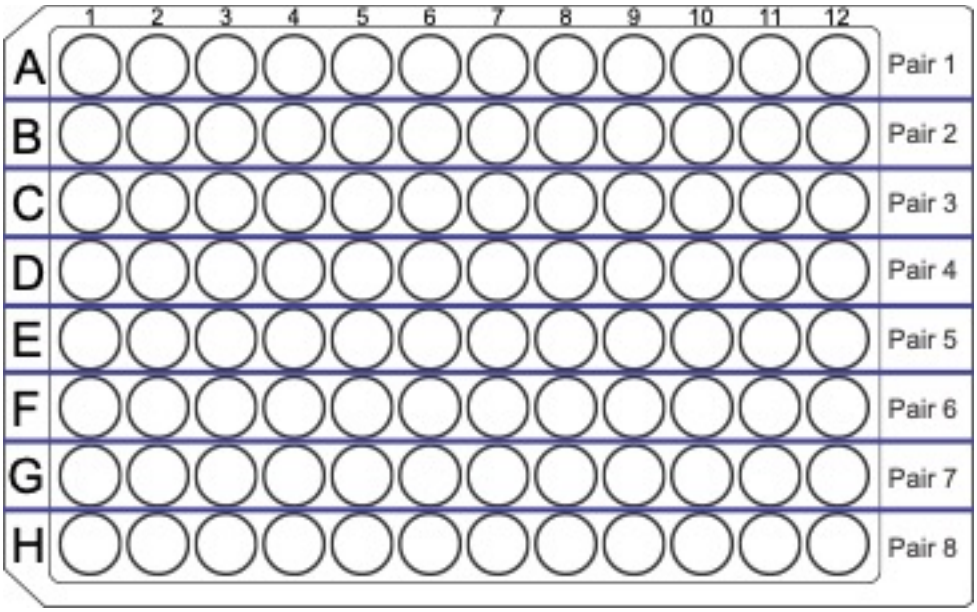
☐ Label the pair of fish and their babies with an appropriate number (e.g. Pair 1) and store parents while babies are growing in the incubator.

- Ideally, 8 or more pairs will provide sufficient embryos such that you can fill a 96-well plate.

☐ **Optional**: At 24 hpf, dechorionate babies by adding pronase to plates and returning to incubator (~30 minutes) until the chorions are shed off by gentle pipetting.

- This step helps get the embryos out of their chorions for easier collection at 48 hpf.

☐ At 48 hpf, anesthetize the embryos with MESAB and prepare a 96 well plate as follows:



☐ From each corresponding pair, pipette up pools of 5 embryos and place into each well.

- 12 pools of 5 embryos each will yield 60 embryos screened
- This number of embryos has been sufficient for detecting integration events in my hands

☐ Briefly spin embryos to bottom of plate wells in centrifuge

☐ Prepare the following lysis buffer mix.

Reagent	Volume (for 5 mL)
10x PCR Buffer	500 µL
Proteinase K (20 mg/mL)	125 µL
H2O	4375 µL

☐ Remove as much water from the wells of the plate as possible, being careful not to suck up any embryos.

- I find that the easiest way to do this is to attach a P200 tip to a glass pipette end by wrapping a seal with Parafilm. You can use this tool to carefully remove water from the wells.

☐ Add 40 µL lysis buffer to each tube.

☐ Put plate in thermocycler and run the genomic DNA extraction program.

DNA extraction program

55ºC for 1 hour

95ºC for 10 minutes

12ºC hold

☐ After program is completed, vortex plate briefly and spin down.

☐ Set up two PCR master mixes corresponding to checking 5' and 3' junctions while the embryos are lysing.

- These are the same PCRs that were performed in the pre-screen F0 stage.
- I scale down these PCRs to quarter reactions to save on mix.

Mix 1 (5' PCR)

Reagent	Volume (1 reaction)	Volume (96 reactions)
2X GoTaq Green Master Mix	3.125 µL	300 µL
Genotyping Forward Primer (10 µM)	0.3125 µL	30 µL
Tag Reverse Primer (10 µM)	0.3125 µL	30 µL
H2O	1.5 µL	144 µL
DNA	1 µL	X

Mix 2 (3’ PCR)

Reagent	Volume (1 reaction)	Volume (8 reactions)
2X GoTaq Green Master Mix	6.25 µL	50 µL
Tag Forward Primer (10 µM)	0.625 µL	5 µL
Genotyping Reverse Primer (10 µM)	0.625 µL	5 µL
H2O	4 µL	32 µL
DNA	1 µL	X

☐ Pipette master mixes into two plates (keeping track of which plate contains the 5’ PCR mix and which plate contains the 3’ PCR mix) and keep cool until embryo lysis is complete.

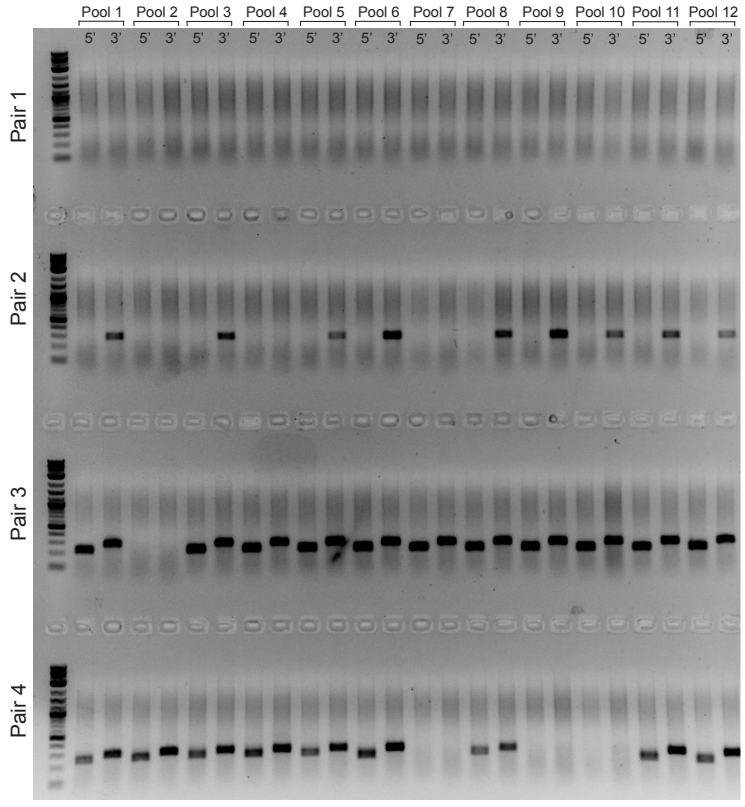
☐ Using a multichannel pipette, add 1 µL of embryo lysis to each of the corresponding wells on the 5’ and 3’ plates.

☐ Spin down DNA into the master mix and run genotyping PCR on those plates.

☐ While the PCR is running, pour one 100 mL 1.5% agarose gels and use four 25-well combs.

- See reagent list for product numbers on these items.

☐ When PCR is complete, load your gels using the multichannel pipette such that each **row** contains samples from a single pair, and that the 5’ and 3’ PCRs are side-by-side. Your gel should look something like this:



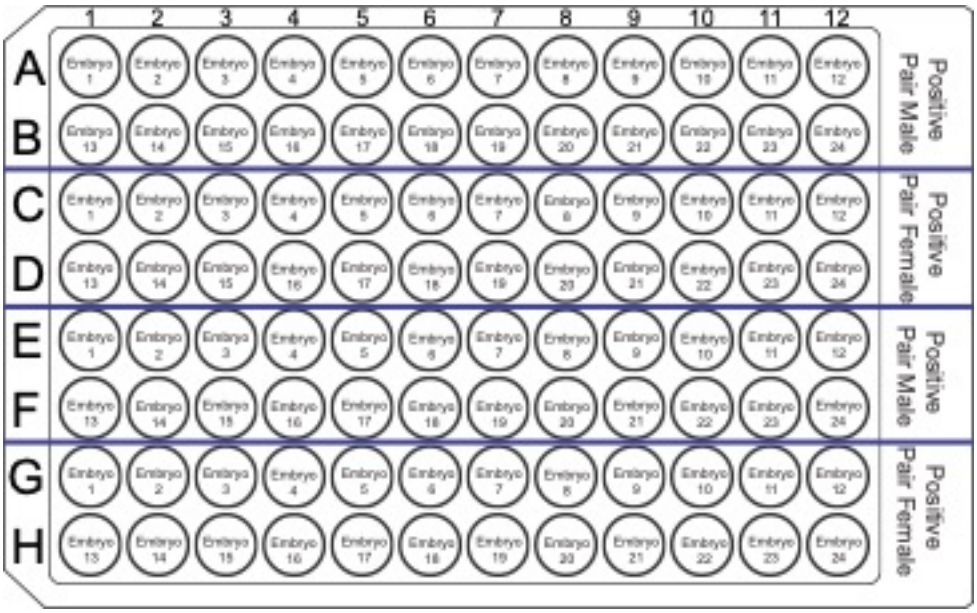
☐ Pairs that produce no positive PCRs can be euthanized (pair 1 in this example).

☐ Pairs that produce a single positive junction can also be euthanized, as they often have incomplete tag integration events or damage to the locus that prevents screening (pair 2 in this example).

☐ Pairs that produce double positive junction PCRs should be isolated from each other (pairs 3 and 4 in this example) and returned to the system for subsequent rescreening the following week.

☐ Using a similar strategy as above, collect embryos from the male and females crossed to wildtype fish and grow to 48 hpf.

☐ Rather than doing pooled PCRs on embryos, the rescreening PCR strategy will instead use individual embryos. Set up your PCR plate in the following way:



☐ Prepare a lysis buffer mix as follows:

Reagent	**Volume (for 5 mL)**
10x PCR Buffer	500 µL
Proteinase K (20 mg/mL)	125 µL
H2O	4375 µL

☐ Pipette 25 µL of lysis buffer into each well.

☐ Anaesthetize embryos from the individual crosses and and pluck into their corresponding well into the plate using forceps.

☐ Set up two PCR master mixes corresponding to checking 5’ and 3’ junctions while the embryos are lysing.

- I scale these PCRs to **half** reactions to such that I can run out a small amount on a gel, and then PCR cleanup for Sanger sequence verification.

Mix 1 (5’ PCR)

Reagent	Volume (1 reaction)	Volume (96 reactions)
2X GoTaq Green Master Mix	6.25 µL	600 µL
Genotyping Forward Primer (10 µM)	0.625 µL	60 µL
Tag Reverse Primer (10 µM)	0.625 µL	60 µL
H2O	4 µL	288 µL
DNA	1 µL	X

Mix 2 (3’ PCR)

Reagent	Volume (1 reaction)	Volume (8 reactions)
2X GoTaq Green Master Mix	6.25 µL	600 µL
Tag Forward Primer (10 µM)	0.625 µL	60 µL
Genotyping Reverse Primer (10 µM)	0.625 µL	60 µL
H2O	4 µL	288 µL
DNA	1 µL	X

☐ Pipette master mixes into plates and keep cool until embryo lysis is complete.

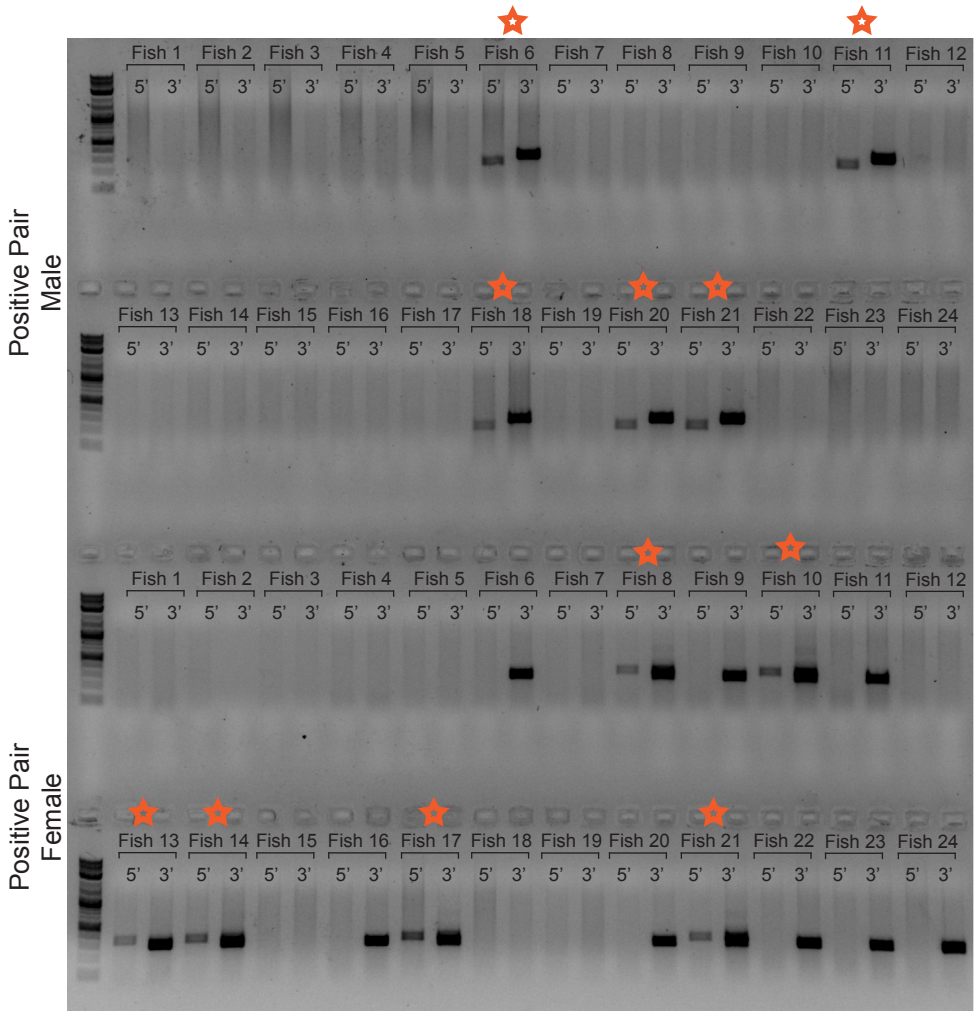
- At this stage, I typically do the 5’ and 3’ PCRs on the same plate for a given pair that I’m screening.

☐ Using a multichannel pipette, add 1 µL of embryo lysis to each of the corresponding wells on the 5’ and 3’ plates.

☐ Spin down DNA into the master mix and run genotyping PCR on those plates.

☐ While the PCR is running, pour one 100 mL 1.5% agarose gels and use four 25-well combs.

☐ When PCR is complete, load your gels using the multichannel pipette such that each **row** contains samples from a single fish, and that the 5’ and 3’ PCRs are side-by-side. Load 2.5 µL of your PCR reaction into each lane and keep the rest for subsequent sequencing. Your gel should look something like this:



- Orange stars indicate embryos that are dual positive for both junctions. We will be using these samples while ignoring the ones that are only single positive.

☐ Identify the dual positive samples and perform a PCR cleanup on the remainder of the reaction.

☐ Send the positive 5' and 3' PCRs for sequencing using the genotyping forward and reverse primer, respectively.

☐ Analyze sequence alignment in your favourite sequencing software, taking note of the homology junctions to which the oligo integrated. Identify fish that produce positive junctions and grow up remainder of its offspring.

- Only grow up fish that have successful integrations with no mutations around the homology sites!

Part V – Establishing lines

☐ Once F1 fish have reached appropriate size, fin clip and run the same 5' and 3' PCRs as described above to identify positive heterozygotes.

☐ Sequence verify all positive heterozygotes junction PCRs and group ones that have the same alleles.

- F0 fish can transmit multiple different alleles so ensure you've got the same alleles moving forward!

☐ Once those fish reach sexual maturity, incross and perform a PCR using the genotyping primers.

- In this cross, you will get 25% homozygous knock-in, 50% heterozygous knock-in and 25% wildtype fish.
- The epitope tags are often large enough to resolve alleles by running on a 2% gel.

☐ Sequence verify precise integrations in the homozygous fish, and grow the remainder of the incross to adulthood.

Congratulations! You just made your tagged allele! Wasn't that easy?

Appendix A – GEAR Tags and Primers

ALFA tag (my favourite and best tag)

Protein sequence:	PSRLEEELRRRLTEP
DNA sequence:	CCATCACGTCTGGAGGAAGAGCTGCGTCGTCGCCTGACCGAACCT
Genotyping forward primer:	CACGTCTGGAGGAAGAGCTG
Genotyping reverse primer:	GTTCGGTCAGGCGACGAC

VHH05 tag

Protein sequence:	PQADQEAKELARQISP
DNA sequence:	CCGCAGGCTGATCAGGAGGCTAAAGAGCTGGCAAGACAGATTAGCCCC
Genotyping forward primer:	TGGCAAGACAGATTAGCCCC
Genotyping reverse primer:	TTAGCCTCCTGATCAGCCTG

127d01 tag

Protein sequence:	PSFEDFWKGEDP
DNA sequence:	CCATCCTTCGAAGATTTCTGGAAGGGTGAGGATCCT
Genotyping forward primer:	TCTGGAAGGGTGAGGATCCT
Genotyping reverse primer:	TCACCCTTCCAGAAATCTTCGA

Appendix B – sgRNA Synthesis (Using EnGen sgRNA Synthesis Kit)

☐ Resuspend target oligos to 100 µM (as standard for any oligo).

☐ Dilute target oligo to reaction working concentration of 1 µM (1 µL oligo + 99 µL H_2O).

☐ Thaw EnGen 2X sgRNA Reaction Mix and 0.1 M DTT at room temperature and vortex before use.

☐ Combine the following in a tube at room temperature:

****NOTE: If making multiple guides, do NOT set up a master mix as the reactions tend to fail. Instead, make a mastermix of H_2O and DTT, and aliquot those into tubes and add the remainder of the components.*

Reagent	**Full Reaction**	**½ Reaction**	**¼ Reaction**
Nuclease-free H_2O	2 µL	1 µL	0.5 µL
EnGen 2X sgRNA Reaction Mix	10 µL	5 µL	2.5 µL
Target-specific DNA Oligo (1 µM)	5 µL	2.5 µL	1.25 µL
DTT (0.1 M)	1 µL	0.5 µL	0.25 µL
EnGen sgRNA Enzyme Mix	2 µL	1 µL	1 µL
TOTAL	20 µL	10 µL	5 µL

☐ Mix by vortexing, spin down and incubate at 37°C for 1-2 hours.

☐ Bring reaction volume to 25 µL with nuclease-free H_2O.

☐ Add 1 µL DNase I, mix by vortexing, spin down and incubate at 37°C for 15 minutes.

☐ Add 24 µL of nuclease-free H_2O to reaction (total volume is 50 µL) and proceed with column cleanup using Monarch RNA Cleanup columns.

Appendix C – Product Info

EnGen Spy Cas9 NLS	NEB, item ref. M0646T
EnGen sgRNA synthesis kit	NEB, item ref. E3322S
GoTaq Green 2X Master Mix	Promega, item ref. M7123
25-well comb, 1.0 mm thickness	VWR, item ref. 490001-320
Gel cast, 4 slots	VWR, item ref. 490001-336