Responses to questions from IZFS Webinar, 9/28/2021.

Do you inactivate the ISce-I prior to injection?
We do not inactivate ISce-I prior to injection.

What was survival rate for different conditions used for injection (predigested, in vivo-digested, circular)?
Survival rate depends on the optimal doses for the template and CRISPR RNPs. We first identify the optimal dose for the two Cas12a RNPs (each done separately) – these are usually not toxic, but can cause some level of lethality. We then co-inject both RNPs with template and ISce-I followed by PCR across 5' and 3' homology junctions in individual embryos. We then adjust DNA template dose to optimize knock-in and/or increase survival based on this initial result (optimal DNA doses usually range between 20pg to 50pg per embryo). We typically see good knock-in results in cases where survival of normal embryos is about 50%. In our hands, it seems that pre-digested and in vivo-digested give similar survival rates; note in this case that "pre-digested" templates are not cleaned up at all and are digested immediately prior to injection. Therefore, it is likely that the ISce-I stays in association with the free DNA ends, possibly reducing toxicity versus free DNA ends.

How do you troubleshoot junction PCRs? If they don't work how do you know if it's a PCR problem or an HDR problem?
The junction PCRs are among the more challenging steps of the protocol. We usually test a number of different primer pairs. While there is usually not an obvious positive control in these cases, you can test primer pairs in different combinations on the endogenous locus and plasmid template to identify which individual primers are likely to work together for your knock-in allele. You can also run a general PCR positive control on the endogenous locus with primers expected to give a similar size to make sure the reaction worked and the DNA is good. We also rely on KAPA2G FAST hotstart polymerase, which seems to do a good job for fragments ranging from 1 to 3kb.

How do you perform the junction PCR sequencing? Do you clone?
Yes, we always perform sequence verification on cloned fragments. We use the KAPA2G FAST hotstart polymerase to amplify the fragment from individual embryos. This enzyme leaves 3'-A tails, so we then shotgun clone these fragments into a TA-compatible plasmid and sequence resulting clones.

For Cas12a or Cas9, do you inject mRNA or protein?
We routinely inject recombinant protein pre-complexed with guide RNAs. Cas12a mRNA injection does not work so it can only be used as recombinant protein. In our hands Cas12a works for every target we have tried. Also, since it's PAM is TTTN, and does not seem to be as sensitive to GC content in the spacer, it is more likely that you will find active targets in intronic sequence. For crRNAs, we use in vitro synthesis and rely on an optimized hairpin sequence (see our website for a protocol, https://www.umassmed.edu/lawson-lab/reagents/genome-editing---cas12a/).

Have you tried Cas nickase?
We have not tried Cas nickase for stimulating homology-directed repair.

If you use individual injected embryos for PCR screening, how do you take it to the next generation?
We inject a sufficient number of embryos to allow us to remove about 8 to 12 embryos to verify recombination at 5' and 3' ends. If positive, their remaining siblings are then grown to adulthood.

Responses to questions from IZFS Webinar, 9/28/2021.

Do you find that you need different homology arms for each fish that you use? If so, does this seem to affect efficiency?
In our wild type strains, there are usually only a couple of haplotypes across the target region. Therefore, we usually can identify a small group of fish isogenic for a particular target sequence that can be used for generating embryos for injection. A caveat here is that they may not be isogenic for another locus, so if you are targeting another gene, that needs to be separately sequenced. We have not carefully measured efficiency if using unmatched fish. However, we have certainly seen cases where the Cas12a used for knock-in will cut in one wild type strain, but not in another.

Distance between the loxP sites impacts recombination efficiency? What distances are acceptable?
Yes, the efficiency of any type of recombination is inversely proportional to distance. What distances are “acceptable” is hard to answer as there will also be locus-specific effects. I would recommend keeping the distance between the loxP sites less than 2 kb if possible. I would also point out an issue in our design was that we inadvertently put the loxP sites outside of the cryaa:Venus marker cassette, making the deleted fragment unnecessarily long. We are currently removing the marker cassette, which will allow us to directly test how size differences might impact recombination at the same locus.

How long (in generations) does it take from the first CRISPR injections to do the real experiment?
Not sure what is meant by “the real experiment” here, but I assume it means phenotypic analysis of the conditional knockout. With the single step approach, you can identify founders from injected embryos after about 3 months (depends on average time to maturity in your system). If you get founders, at this point you can outcross to an appropriate CreERT carrier. Ideally, that CreERT carrier already bears a Switch line that will allow you to visualize knockout cells (and isolate them to validate knockout). If so, then 3 months later you will have fish to incross for embryos bearing the conditional allele and CreERT (and possibly the switch line). I think a conservative estimate – and assuming you are seeing efficient 5’ and 3’ knock-in at the target in injected embryos – would be about 8 to 12 months until you have sufficient numbers of fish with the appropriate CreERT and switch lines in the background.

What is the optimized cutting efficiency for doing HDR?
We usually determine this empirically by performing a dose response for each individual Cas12a RNP. We are currently using the dose just under that which gives 100% cutting. Note that this is based on loss of restriction digest since all of our targets have restriction sites that will be lost from indels introduced by the Cas12a.

Have you tried to introduce a conditional splice acceptor/reporter cassette into early introns to cause truncating mutations?
We have not tried this, but this approach has been used successfully in zebrafish (https://elifesciences.org/articles/24635). A caveat here is that splice traps may not be 100% efficient, allowing some wild type transcript to be made and resulting in a hypomorphic phenotype.

Are you using radioactivity for your Southerns?
We are using DIG-labeled DNA probes. The chemiluminescent substrates that are now available are very stable and therefore sensitive enough to easily detect single copy loci in a large genome.

Responses to questions from IZFS Webinar, 9/28/2021.

Have you considered using shorter homology arms to reduce non-specific integration? What are your thoughts on the Geneweld protocol that utilizes shorter homology arms in fish?
There is limited definitive data on longer homology arms leading to increased non-specific integration. When using transgenes with long homology arms, those sequences can certainly contribute to ectopic transgene expression, which has been interpreted as off-target integration, although additional flanking genomic sequence can also simply be providing some level of enhancer or promoter activity. Experiments looking into germline transmission and unbiased assessment of off-target integrations (e.g. through Southern analysis) has not been comprehensively done, as far as I know. The high efficiency of the Geneweld approach, which uses shorter homology arms, is somewhat biased by the fact that it is introducing screenable markers (e.g. EGFP or GAL4). Therefore, you can see which embryos have a correct fusion with precise editing. We are finding in separate unpublished studies that the Geneweld approach with a non-screenable knock-in sequence leads to mostly imprecise edits on both sides of the homology cassette, including those that affect the knock-in sequence itself. We have not observed this issue when using longer homology arms; an obvious caveat here may be that our PCR screen is relatively stringent and may not be picking up imprecise edits that affect the knock-in cassette. We have also noted reduced knock-in rates when using a 300 bp 3' arm for at least one target. For these reasons, we have tried to us between 500 and 1000 bp homology arms.

Have you seen any issue with using a negative selection marker in the vector backbone that impacts KI efficiency?
We have not definitively tested this, but it should not have any impact given that the cassette (along with several kb of plasmid vector) are outside of the homology arms.

Have you tried to inject linearized, purified HDR template alone?
We have not tried this.

For the beta-actin switch embryos that did not switch, did the switch come from the mother and could this be simply due to maternal contribution seen from a het carrier?
The embryos used for analysis in this experiment were from group crosses, so this is certainly a possibility since the ubb element can drive maternal expression.

What is your opinion on conditional knock-out by regulating intracellular CRISPR/Cas9 expression?
I am not a huge fan of this approach. Inducing a double strand break in somatic cells is not a good thing and will result in a typical DNA damage response, including induction of p53. Any resulting phenotypes will be modified by this response so rescue transgene controls are essential to confirm a specific effect.

Have you been using a codon-optimized Cre? Do you notice a significant difference?
The Cre sequence in the CreERT we are using (PMID: 21138979) appears to be derived from the original sequence used in mouse. It is therefore possible that codon optimization for zebrafish may improve its activity.

Responses to questions from IZFS Webinar, 9/28/2021.

Have you looked at Gata2a protein levels in your conditional mutants? How do you distinguish between an early window for gata2a requirement vs. masked later onset phenotypes because of perdurance of Gata2a protein that was made prior to recombination? To this point – will rapid zerbafish development (compared to mouse) complicate Cre/lox recombination strategies?

This is a very important consideration when interpreting outcomes from conditional knockout experiments (e.g. different phenotypes with different windows of tamoxifen treatment). Investigation of Gata2a protein levels is currently underway, as is assessment of transcript levels in FACS-isolated cells positive for the switch marker gene. These will be essential steps in characterization of conditional alleles. Inevitably, some proteins with long half-lives may be challenging to eliminate in the short timeframe of embryonic development; in these cases it may be possible to generate conditional alleles that also incorporate a degron to facilitate cell-specific analysis.

Do you think your approach would be suitable for floxing multiple exons?

It is certainly feasible to flox multiple exons, while trying to limit the distance between the loxP sites.

Could you please name the kits you mentioned as an alternative to Hot Shot DNA extraction for long amplicons?

We have found that the Zebrafish Genotyping Kit from In Vivo Biosystems works well to give long amplicons and allows rapid genomic DNA isolation from individual embryos.
https://invivobiosystems.com/product/zebrafish-genotyping-kit/

Could you comment on the rationale for removing the marker cassette post-integration?

In some cases, the cryaa:Venus marker cassette may interfere with endogenous gene function (similar to what occasionally happens with neo cassettes in mouse knock-ins). This can be tested as soon as founders are identified by crossing to a known null allele (if available) and checking the phenotype (and then genotype) of resulting cryaa:Venus-positive embryos. If they are normal and viable, then you can probably leave the cassette. If transheterozygous embryos at this stage display a phenotype, you can re-cross the founder and remove the cassette using FLP or CRISPR. Of course, you will then have to use finclip PCR to identify conditional mutant carriers.

Do you have any comparisons with mRNA vs. protein for knock-in efficiency? With either donor vector or oligo knock-in?

We have been relying on Cas9 and Cas12a protein so we have not done a side-by-side comparison with mRNA. In the case of Cas12a, only protein can be used. In this case, we use an improved Cas12a and optimized crRNA backbone that improves efficiency (see our website for protocols, https://www.umassmed.edu/lawson-lab/reagents/genome-editing---cas12a/). For protein, we recommend optimizing the dose so that you are not over-cutting the site to rapidly. If you use mRNA, you may want to perform a similar optimization, just in case your CRISPR is too active. The approaches we use for oligo knock-in and conditional allele knock-in are hard to compare since the templates look quite different, and are targeting different sites. For knock-in of loxP sites, we prefer using a plasmid HDR donor since it is possible to knock in both loxP sequences, along with a marker gene, in a single step. Using the existing oligo-based approach takes multiple generations.