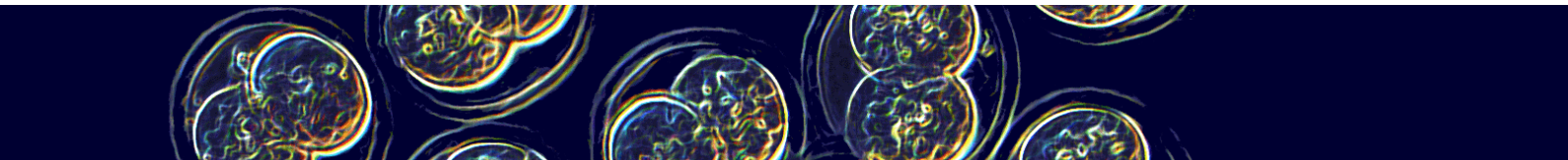
University of Basel

Centre for Transgenic Models
University of Basel
Mattenstrasse 22
4002-Basel, Switzerland

Centre for Transgenic Models
CTM

CTM guidelines for the generation, validation and maintenance of GMO mice with CRISPR-generated alleles.

Content



Guidelines

Overview of genome engineering approaches

CTM uses CRISPR-Cas/9-based gene targeting techniques to generate genetically modified animals. Depending on the desired genetic modification (see Table 1), Cas9 RNPs alone or in combination with DNA repair template, will be introduced into a mouse preimplantation embryo. On a molecular level, the Cas9 RNP will introduce targeted DNA double-stranded breaks (DSBs) at the selected target sequence (Figure 1). These DSBs can subsequently be repaired by the embryo DNA repair machinery either through error-prone non-homologous end joining (NHEJ) or use the DNA repair template and the more-precise homology-directed repair (HDR). Consequently, genome engineering utilizes the NHEJ to introduce targeted deletions (knockouts) and HDR to obtain site specific insertions (knock-ins). It must be noted that both NHEJ and HDR are intrinsic DNA repair mechanisms that co-exist in the developing embryo therefore a desired outcome must be selected for by genotyping at the F0 and F1 mouse breeding stage.

*Table 1: Generalized overview of genome engineering approaches used **in combination with Cas9 RNPs** for generating targeted modifications of various sizes.*

Modification Type	*Repair Template*	*Delivery method*	*Embryo stage*
Small deletion	none	Electroporation	Zygote
Lage deletion	none	Electroporation	Zygote
Insertion ≤ 200 bp	ssDNA	Electroporation	Zygote
Insertion 200-2000 bp	long-ssDNA	Microinjection	Zygote/2-cell
Insertion1-4 kb	rAAV	Transduction + Electroporation	Zygote/2-cell
Insertion ≥ 4kb	linear- dsDNA	Microinjection	2-cell

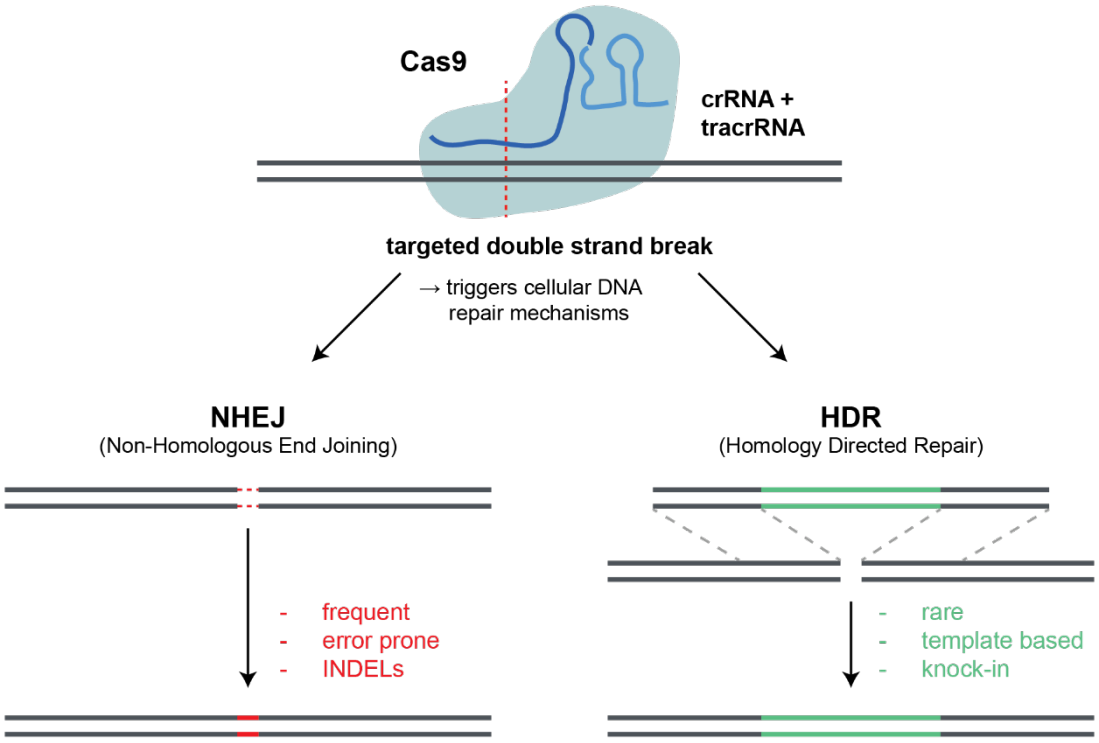


Figure 1: Overview of CRISPR/Cas9-based genome engineering

Founder (F0) Mosaicism

For most projects, the CTM will inject or electroporate the modifying reagents (such as the Cas9-RNP and a repair template) at the one- or two-cell embryonic stage. While this allows efficient modification, these reagents can persist in the cells beyond these early embryonic stages. This means not all cells of the embryo will be subjected to the same or any modification frequently giving rise to complex mosaic founder (F0) animals (Figure 2). When these founder animals are biopsied, the isolated gDNA often contains a combination of multiple alleles of the target locus in addition to the desired modification and/or wildtype sequence. It is important to keep in mind that attempts to PCR-amplify multiple alleles in a mosaic gDNA sample can lead to unforeseen PCR artefacts. Furthermore, the obtained toe biopsy might not be representative of the genetic modifications in all cells of all organs – especially the germline. It is therefore possible that the germ cells do not (all) have the same modification(s) as those detected in a toe biopsy. It is therefore essential to also thoroughly analyze and validate the modified region in the offspring (F1) generated by crossing the F0 animals with wildtype (wt) partners.

Overall, genetic characterization of novel GMO mouse lines is a complex process. For these reasons, CTM strongly favors the delivery of validated F1 animals instead of potentially mosaic F0 founders.

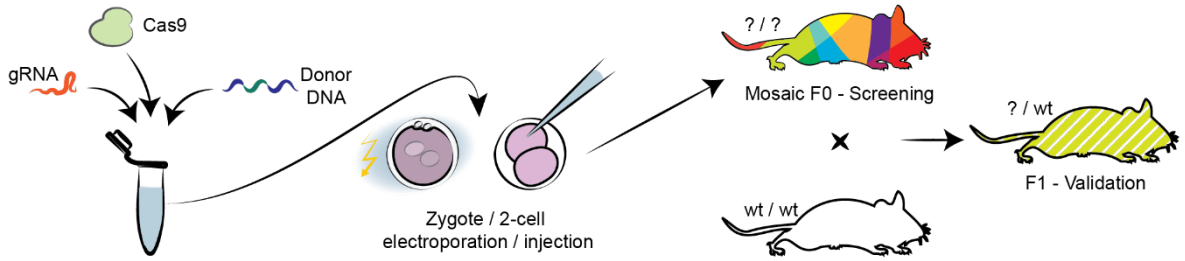


Figure 2: Simplified scheme of mouse transgenesis by CRISPR/Cas9 genome engineering.

Genotyping Strategies

DNA isolation and storage

- Biopsies should be stored at -20°C prior to DNA isolation.
- We generally isolate gDNA from toe clips using a proteinase K based tissue lysis followed by isopropanol precipitation (see Protocols section). This provides sufficiently pure and long DNA fragments for most analysis.
- gDNA extracts should be stored at either 4°C for short term storage (i.e. < 30 days) or at -80°C for prolonged storage (e.g. founder DNA).
- Avoid freeze-thaw cycles as they lead to DNA fragmentation.

Detection of NHEJ-based and small modifications

For modifications without DNA template or where the template is <200 bp, where the resulting PCR fragments are not distinguishable by gel electrophoresis, we suggest the following:

1) Ideally, design a restriction digest and PCR based assay to pre-select potentially positive animals before sending amplicons for sanger sequencing.
2) Sequence the PCR products directly.

Detection of Larger targeted alleles (knock-ins)

Screening:

- If you have an established PCR of any part of the transgene (e.g. GFP, mCherry, Cre, …), you may use this to screen all samples for its presence. This allows you to reduce the number of samples in the following steps.

- Keep in mind that presence of part of the transgene does not mean the transgene is…
    - … integrated in the correct location
    - … integrated in the correct orientation, or
    - … integrated as a single copy!

Validation of insertion location and orientation:

- To validate the correct localization and orientation of the transgene, you need to design your PCRs such that the complete sequence is covered.
    - This sequence not only entails the sequence of the homology arms and insertion but should also include genomic sequences on either end of the homology arms.
    - As a rule of thumb, we generate outside PCR primers such that they are located at least 100 bp outside either homology arm.
- Depending on the design, insertion size, and strategy used you may wish to proceed either with multiple overlapping PCR amplicons (see Figure 3A, PCR a1/a2) or directly with a single (long-range) "out-out" PCR (see Figure 3A, PCR b).
- If you wish to start with overlapping PCR amplicons design the primers as follows:
    - b1) Forward primer outside of 5' homology arm +
      Reverse primer inside the transgene/insertion
    - b2) Forward primer inside the transgene/insertion+
      Reverse primer outside of 3' homology arm
    - *b3) Optional: Should amplicons a and b not overlap, create additional internal amplicons to cover the complete insertion.*
- Ideally the outermost primers designed for the overlapping PCR can also be used together for the "out-out" PCR (see Figure 3A, PCR b).
- For primer design we recommend NCBI primer BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast.
    - Primers may also be designed manually but should be checked for specificity by performing BLAST against the mouse genome.
- Multiple overlapping PCR amplicons are a useful first validation step. Nevertheless, overlapping amplicons by themselves can never be seen as complete validation and can lead to misleading results. For example, if multiple, concatemeric insertions occurred (see Figure 3B) both amplicons will appear in PCR reactions. Concatemeric insertions are relatively common. The template DNA can be integrated in any possible direction and any combination can occur.
- Once you validated correct 5' and 3' insertion you will need to follow up with a "out-out" PCR spanning the complete modified region (see Figure 3A, PCR b).
- If it is not possible to amplify the complete modified region (e.g. the PCR is > 15kb or you have a difficult sequence in your insertion such as a CAG promoter or IRES), we suggest the following:
    - Perform qPCR to assess the copy number of your transgene (results of more than 1n may be indication of multimeric insertions)
    - Perform targeted nanopore sequencing (ask us for more information).
- Note: if the out-out PCR only amplifies a single band of the size expected for the correct insertion and the wild-type band is not visible, the mouse most likely carries a bi-allelic insertion. In the case of an F0 animal this does not mean that the insertion is homozygous and identical on both alleles as the insertions represent two distinct transgenic events. Therefore, all F1 offspring must also be validated.
- If the out-out PCR only amplifies the WT band, there could be multiple reasons:
    - The WT sequence gets amplified preferentially due to shorter amplicon length. This is frequently observed with insertions of larger transgenes.
    - The mouse is mosaic and only a low percentage of cells have the desired modification.
    - Multiple copies of your templated DNA have been inserted. Therefore, the elongation time is too short to amplify the complete concatemeric sequence.

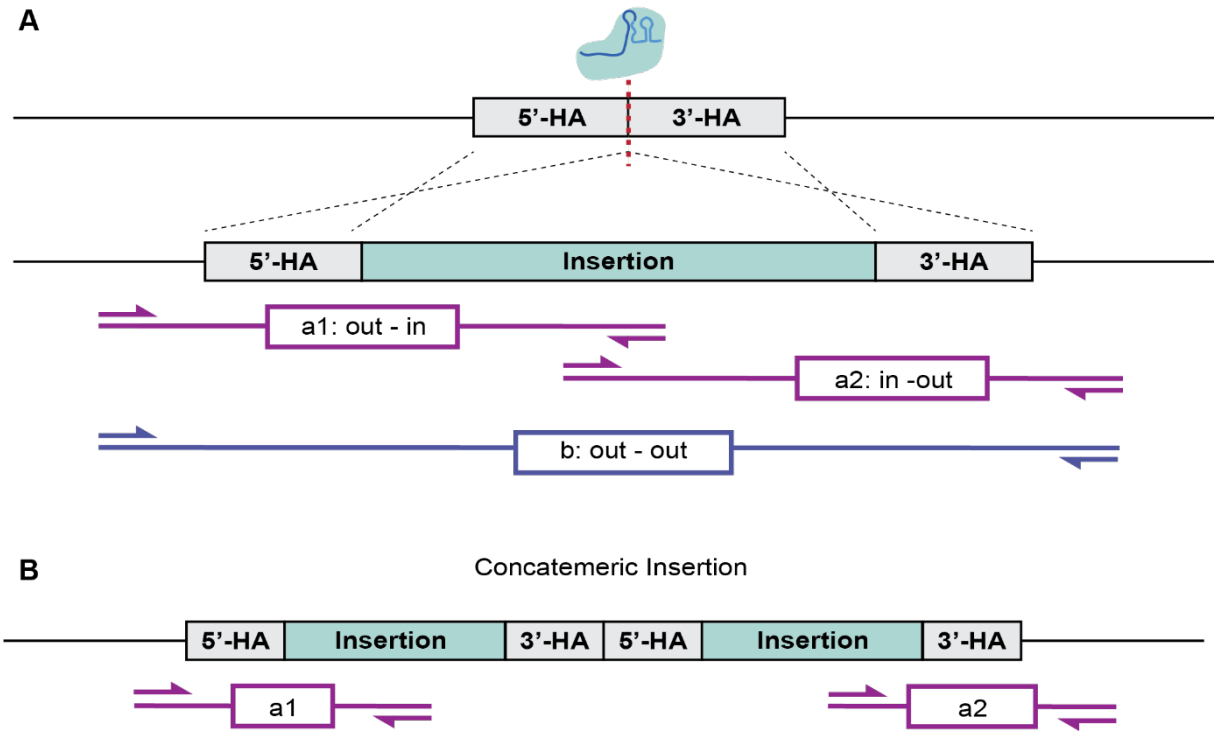


Figure 3 Schematic representation of genotyping strategies for large, targeted insertions. A) The Cas9 RNP cleavage will produce a double strand break between the 5' and the 3' homology arms (HA). By providing a corresponding DNA repair template, a desired non-endogenous (transgene) sequence can be inserted between the HAs. Overlapping (a1/a2, out-in +in-out) PCR amplicons (purple) can provide a first step for sequence validation. The integrity of the complete sequence can only be fully validated by PCR, if the complete region can be amplified in one PCR reaction (b: out-out, blue). B) Representation of a concatemeric head-to-tail insertion highlights the pitfall of using overlapping PCR amplicons for validation. Note that in this case, both PCRs will lead to amplicons of the expected size and sequence while the modification did not result in the desired outcome.

Sequencing

- Short PCR amplicons (<1kb) should be column purified and sent to a commercial service (Microsynth) for Sanger sequencing.
    - If your PCR leads to multiple products, the target band may be excised form the gel and purified.
- Longer amplicons can be sent to a commercial service for ONT long read sequencing (CTM also uses the Microsynth service).
    - Be aware that commercial services often use an approach that randomly integrates sequencing adapters into the DNA fragments.
- Given the potential admixture of wild-type and transgenic sequences, it is essential to analyze not just a potentially provided consensus sequence but to align the individual reads. Autoalign (https://www.bioinformatics.babraham.ac.uk/autoalign/) is an accessible online tool for long read sequence alignment.
- We recently established a native targeted sequencing method for cases where complete, outside-outside PCR amplification of the target region is not possible. **Contact us for more information.**

Data Analysis

- While you might get a consensus sequence from your sequencing service, it is advisable to ask your commercial supplier for raw reads (.fastq format).
- A step-by-step guide can be found in the protocols section ("Analysis of ONT Amplicon Sequencing Data")

Breeding

The CTM recommends breeding the most promising F0 candidate(s). Once germline transmission from a particular F0 is achieved, use only that single F0 founder animal for establishing a breeding line. If you wish to proceed with breeding more than one founder, the offspring of each founder must be treated as individual sub-lines.

Founder animals should be outbred, i.e. back-crossed with wild type animals of the appropriate background. Founder animals should not be inbred with other founder animals, as this increases the chances of unintended passenger mutations (such as off-target modifications) to manifest in the line.

Bear in mind that due to mosaicism, a single founder can still pass on more than just two alleles and therefore the integrity of the modification needs to be validated in all F1 animals used for breeding. We advise continuing using out-in/in-out genotyping PCRs at least until F2 to make sure one is not accidentally breeding animals with “passenger” insertion(s) at other genomic locations. Take deviations from mendelian segregation seriously as it can indicate the presence of passenger insertion(s).

Ideally any validated F1 animals are bred once more with wild type animals. Alternatively, F1 can also be crossed with other F1 animals originating from the same founder animal or they can be mated with F1 animals of any other established line of your choice.

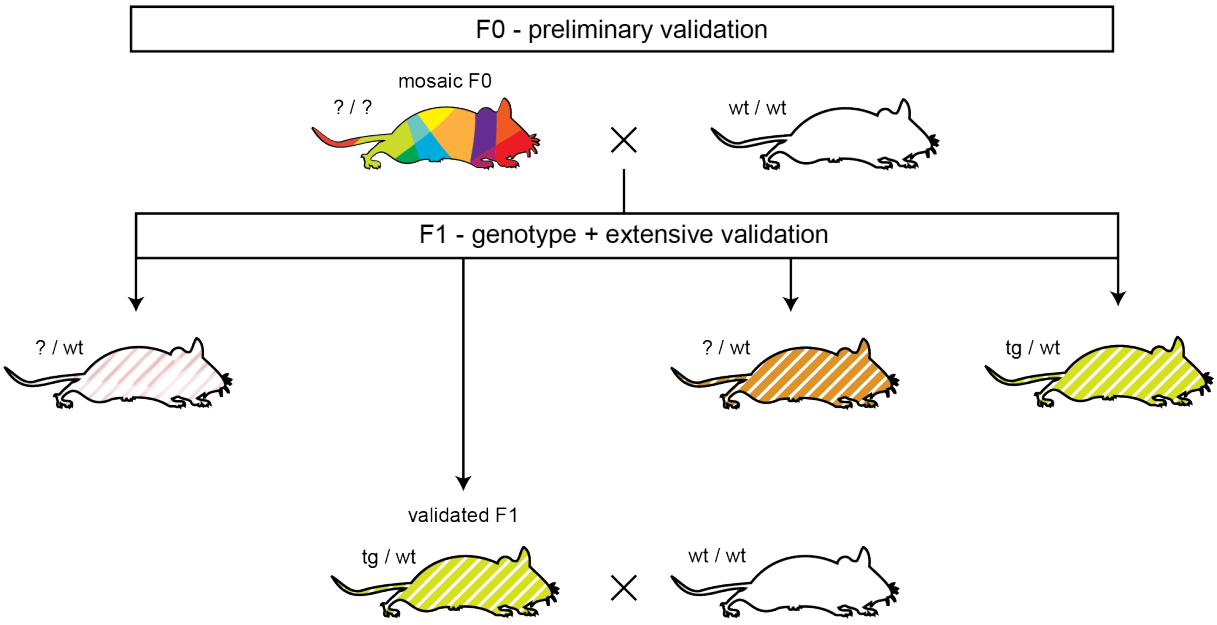
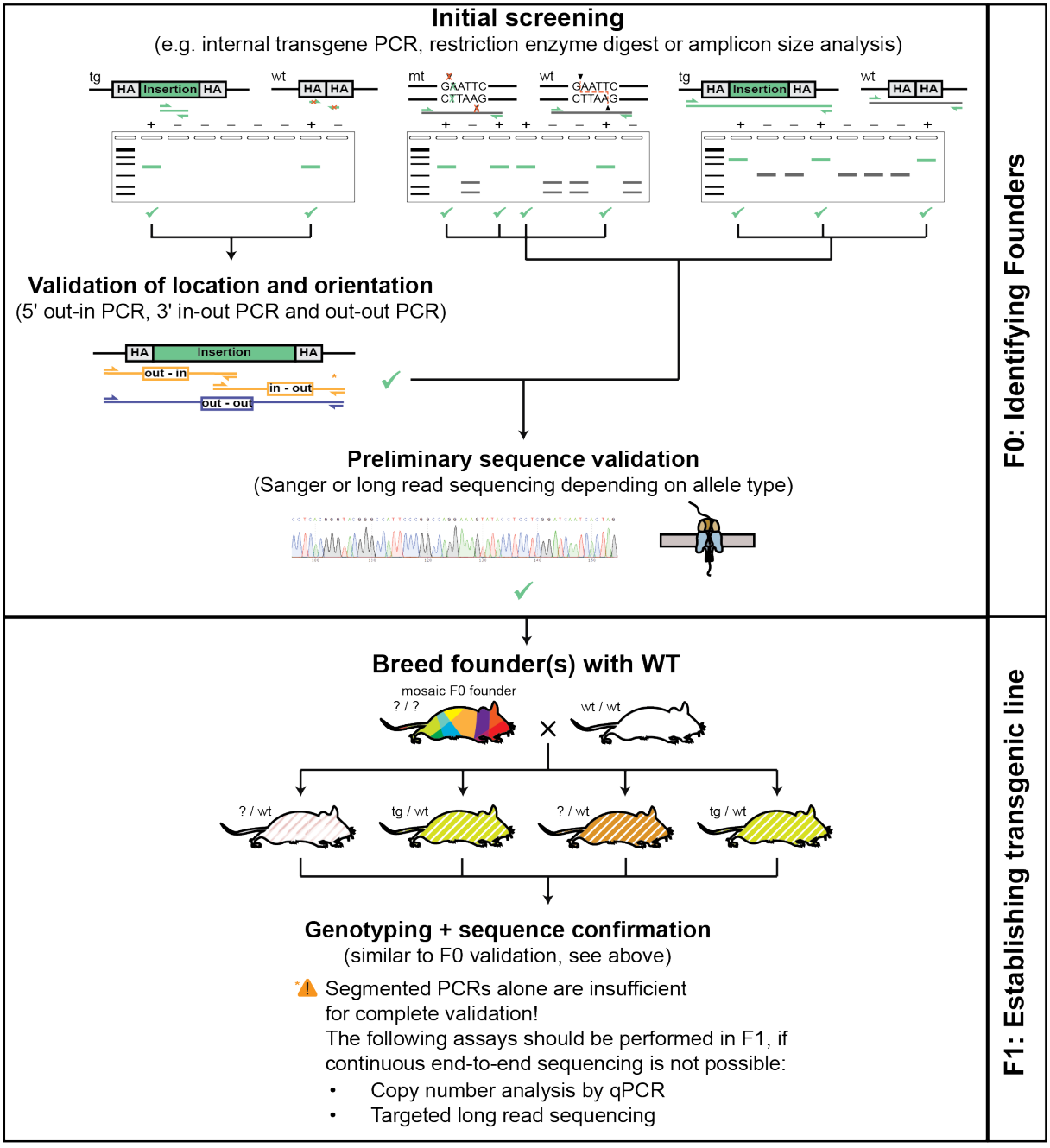


Figure 4 F0 to F1 Breeding Scheme for CRISPR-generated mice

Overview of validation strategies

The strategy chosen depends on the type of modification. Please consult the guidelines for more detail.



Protocols

DNA isolation

Materials:

- ☐ 1.5mL Eppendorf Tubes
- ☐ Pipets + Tips
- ☐ Heating Block
- ☐ Rotary mixer
- ☐ Centrifuge

Stock solutions and Buffers:

Reagent	Supplier	Cat-No.	Concentration	Diluent/Solvent
Proteinase K (PK)	Carl ROTH	7528	20 mg/ml	H2O
Tris-HCl pH 8.0	ThermoFisher	AM9855G	1 M	-
EDTA, pH 8.0	ThermoFisher	AM9261	0.5 M	-
NaCl	Merck	1.06404.1000	5 M	H2O
NaCl	Merck	1.06404.1000	Saturated (6M)	H2O
SDS	Merck	L3771	10 %	H2O
Isopropanol	Merck	1.09634.1011		
Ethanol	Merck	1.00983.1000	70 %	H2O
Nuclease-free water	Bioconcept	3-07F04-H		

	Final concentration	mL stock for 500 mL buffer
Lysis Buffer		
Tris-Cl	10 mM	5
EDTA	50 mM	50
NaCl	100 mM	10
SDS	0.5 %	25
TE Buffer		
Tris-Cl	10 mM	5
EDTA	1 mM	1

Procedure:

- ☐ Add 390 µL Lysis Buffer + 10 µL PK to your biopsy (toe, or ear)
- ☐ Incubate o/n at 55°C, 400 rpm
    - Faster lysis can be achieved by using 360 µL lysis buffer + 40 µL PK and incubating for 2-3 h at 55°C, 1500-2000 rpm
- ☐ Add 350 µL Lysis Buffer, mix by inverting the tube
- ☐ Add 250 µL saturated(6M) NaCl, mix by inverting for 2min on a rotary mixer
- ☐ Centrifuge at max speed (>15’000 x g) for 10 min at room temperature (20-25°C)
- ☐ Transfer 750 µL of Supernatant into a new and labeled 1.5 mL Eppendorf tube,
    - avoid transferring any proteins etc. floating on the surface
    - This will leave behind some liquid (~200 µL). We suggest setting the tubes aside until the remaining extraction has been successfully completed. You can use it as a backup in case something goes wrong.
- ☐ Add 500 µL isopropanol, mix by inverting for 2 min on a rotary mixer
- ☐ Centrifuge at max speed (>15’000 x g) for 10 min at room temperature (20-25°C)
- ☐ Discard the supernatant
- ☐ Wash the pellet with 700 µL 70% ethanol
- ☐ Centrifuge at max speed (>15’000 x g) for 10 min at room temperature (20-25°C)
- ☐ Remove ethanol and dry pellet
- ☐ (Don’t over dry the pellet otherwise, it will be very hard to get it back into solution)

- ☐ Add 100 µL TE-Buffer or H2O
    - DNA can be stored at 4°C and used for PCR the next day. You can store the DNA at -80°C for long-term storage. Avoid freeze-thaw cycles.
- ☐ To use the DNA the same day, add 100 µL of TE-Buffer after removing the ethanol, put the tube with an open lid for 5-10 min at 55°C close the lid and incubate it for 1h

Remarks: It is important that the centrifugation steps occur at room temperature (20-25°C). If your centrifuge heats up noticeably or the ambient temperature is elevated, we suggest using a cooling centrifuge set to 20°C.

Standard PCR

PCR-Mix:

For most PCRs up to 5 kb we use the “KAPA2G Fast HotStart Genotyping mix” (Sigma Aldrich, KK5621):

COMPONENT	***Stock conc.***	***unit***	***µl / reaction***	***Final conc.***	***unit***
2X KAPA2G mix	2	X	12.5	1	X
Forward Primer	10	µM	0.5	0.2	µM
Reverse Primer	10	µM	0.5	0.2	µM
Template DNA			1	0.4-4	ng/ul
Nuclease-Free Water			10.5		
Total			***25***		

Program:

The KAPA mix is optimized to anneal at 60°C. As a rule of thumb, we start off by using 60°C and continue with a gradient PCR if this does not work.

95ºC	3 min	
95ºC	15 sec	35 X
60°C (55-65ºC)	30 sec	
72ºC	15 sec/kb	
72ºC	1 min/kb	

Long-Range PCR

For PCR amplicons above 4-5 kb we suggest using the NEB LongAmp Hot Start Taq 2X Master Mix (NEB M0533).

PCR-Mix:

COMPONENT	***Stock conc.***	***unit***	***µl / reaction***	***Final conc.***	***unit***
2X NEB LongAmp MM	2	X	12.5	1	X
Forward Primer	10	µM	1	0.4	µM
Reverse Primer	10	µM	1	0.4	µM
Template DNA			2	0.4-8	ng/ul
Nuclease-Free Water			8.5		
Total			***25***		

Program:

94ºC	3 min	
94ºC	30 sec	35 X
55-65ºC	40 sec	
65°C	50 sec/kb	
65°C	1 min/kb	