# Engineering genomic deletions and inversions in mouse ES cells using custom designed nucleases (Prot XX)

Elphège P. Nora and Edith Heard

Mammalian Developmental Epigenetics Group
INSERM U934 / CNRS UMR3215
Institut Curie - Centre de Recherche
Pole de Biologie du Developpement et Cancer
26, rue d'Ulm 75248 PARIS Cedex 05 FRANCE

e-mail:
elphege.nora@gladstone.ucsf.edu
edith.heard@curie.fr

**Publication Date**:

**Reviewed on**:

**Keywords**: Genome engineering, mouse embryonic stem cells, deletions, inversions, Transcription Activator-Like Nucleases (TALENs), Zinc-Finger Nucleases (ZFNs)

## Introduction

The recent development of custom designed nucleases, such as Zinc-Finger Nucleases (ZFN), Transcription Activator-Like Effector Nucleases (TALENs) and the Clustered Regularly Interspaced Short Palindromic Repeat Associated system (CRISPR/Cas9) has opened up exciting opportunities to edit genomes in a wide range of organisms (Joung and Sander, 2013 for review). Knocking out protein-coding genes can be easily achieved by using just one pair of such dimeric nucleases, to target the first coding exon, thereby introducing short indels that result in a translational frameshift. Several reports have also demonstrated the possibility to target larger genomic rearrangements by using two pairs of nucleases (Carlson et al., 2012; Gupta et al., 2013; Lee et al., 2011). Although homologous recombination mediated genetic engineering is feasible in some systems, such as mouse embryonic stem cells, this approach requires multiple steps, including the selection of drug-resistant clones, and can be laborious depending on the target and nature of the targeting. This approach is being rapidly superceded by the advent of custom ZFN, TALEN and CRISPR/Cas9 technologies, which enable the extremely rapid and efficient disruption of not only coding, but also non-coding elements, by creating deletions, or by changing local genomic organization by creating inversions.

### *Protocol Applications*

We present here a protocol allowing efficient generation of targeted deletions and inversions in mouse Embryonic Stem Cells (mESCs). Using TALENs, we have successfully isolated deletions and inversions ranging from 1kb to 50kb, but even larger rearrangement should be feasible (Lee et al., 2011). Possible applications of this technology include: complete silencing of coding or non-coding RNAs by

promoter deletion; deletion of coding or non-coding exons; knock-out of enhancers; deletion or inversion of large clusters of regulatory elements; disruption of intronic microRNAs, *etc*…

Unlike classical homologous recombination-based methods, this protocol does not make use of drug selection, thus enabling the one-step isolation of mutant mESCs clones within a couple of weeks only. There is no need to build any donor construct, as homologous recombination is not used. Thus for each new region to target, the only specific reagents to prepare are custom designed nucleases and PCR primers for genotyping.

We present here results obtained using TALENsin, as these custom designed nucleases can be easily produced using available kits by any laboratory with basic molecular biology skills (see http://www.addgene.org/TALEN/). In principle this protocol could also be used with ZFNs (see http://www.addgene.org/zfc/) or any other custom nucleases such as those based on the CRISPR/Cas9 system (see http://www.addgene.org/CRISPR/).

### *Limitation of the Approach*

The efficiency of the approach varies depending on the size of the intended deletion/inversion. Deletions of 1-3kb are typically obtained at frequencies of 1-5%, while we observed 0.5-1% frequencies for 50kb deletions/inversions. We note that inversions tend to occur at a lower frequency (3 to 5 fold) than the corresponding deletions (Gupta et al., 2013; Lee et al., 2011). Note that the frequencies obtained using classical homologous recombination with selection markers are typically in the range of .1% to 1%, and these alleles need further engineering such as the removal of the selection cassette.

We suggest genotyping strategies based on clone-pooling, in order to speed-up the screening procedure particularly for infrequent mutations.

The cleavage efficiency by the custom designed nucleases is also critical. It is recommended to test the ability of the nucleases to induce mutations before embarking on large-scale experiments (e.g. the T7 Endonuclease I assay (Kim et al., 2009)or the Surveryor nuclease assay (Sanjana et al., 2012)).

It is important to keep in mind that targeted cells will be constitutively mutated. If the intended modification is lethal, one may need to resort to homologous recombination techniques in order to engineer conditional alleles. The use of one pair of custom designed nucleases may also help for this, as they can greatly enhance homologous recombination efficiencies (Hockemeyer et al., 2011).

If the deletion/inversion is required in the homozygous state it may be necessary to proceed with two serial rounds of targeting.

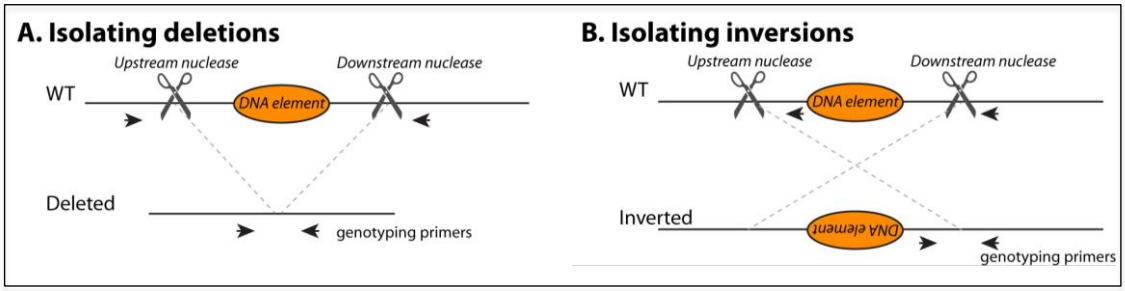


**Figure 1: Overview of the strategy.** Two pairs of custom designed nucleases with target sites separated by 1-50kb are transiently expressed in mESCs. Single clones are isolated and genotyped by PCR using primers that can discriminate Wild-Type (WT) versus deleted or inverted alleles.

## Procedure

### *I-Designing of two pairs of TALENs*

1. Locate the DNA element to delete/invert using a Genome Browser (GB), *e.g.* UCSC GB at http://genome.ucsc.edu or ENSEMBL GB at www.ensembl.org

2. Retrieve the DNA sequence from a 300-500 base-pair stretch directly upstream of the region ton delete/invert.
   *e.g.* Use the function "VIEW/DNA" in the UCSC GB or "EXPORT DATA" in the ENSEMBL GB.

3. Use this as an input sequence to design a pair of TALENs *e.g.* using the online tool TAL Effector Nucleotide Targeter 2.0 (Doyle et al., 2012) at https://tale-nt.cac.cornell.edu

4. Choose one pair of TALENs (see notes).

5. Repeat steps 2-4 with a 300-500 base-pair stretch directly downstream of the region ton delete/invert.

*Notes*:

Online softwares can typically identify tens of TALEN target sites within a 300-500bp sequence. We recommend following these guideline when choosing a pair of TALENs:

A) Small deletion/inversion are most efficiently engineered, so try reducing intended inversion/deletion size as much as possible.

B) Avoid CpG motifs within TALEN target sites, as cytosine methylation may decrease TALEN binding efficiency – unless you are constructing TALENs with methylation-insensitive monomers (Valton et al., 2012). In our experience

CpGs in the spacer between the left and right TALENs do not prevent nuclease activity.

C) If working with cells with a different genetic background than the reference genome (C57BL/6J), check that your TALEN target sites do not contain any SNP or short indel. List of SNPs/indels for popular strains can be found on the website of the Sanger Institute: http://www.sanger.ac.uk/cgi-bin/modelorgs/mousegenomes/snps.pl

D) Avoid designing TALENs within repeats (either derived from transposons, satellites or segmental duplications). These elements can be located using genome browsers (see Figure 2).

E) Minimize off-target scores, if allowed by the design software (this is possible for the mouse genome with TAL Effector Nucleotide Targeter 2.0).

F) After choosing a pair of TALENs, it is safe to perform a genome-wide *in silico* search using each TALEN target site as entries to verify that they are not present at multiple positions in the genome. *e.g.* use the BLAT tools in the UCSC GB in TOOLS/BLAT.

G) Make chosen TALENs and design genotyping primers as described in figure 3.

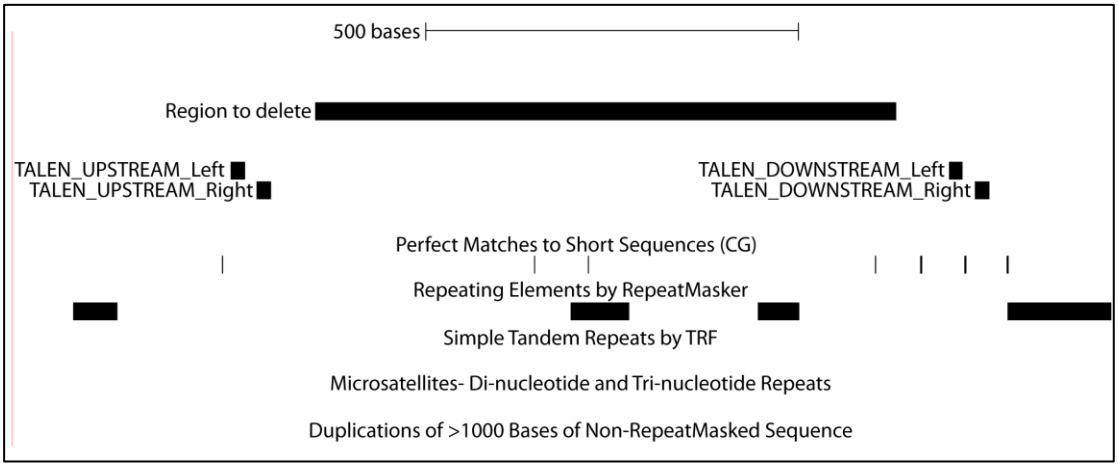


**Figure 2: TALEN design results displayed on the UCSC genome browser.** The position of the region to delete/invert is depicted alongside with the upstream and downstream pairs of TALENs. CpG dinucleotides are visualized by activating the "Short Match" track with "CG" as the entry search. Transposon-derived repeat are highlighted upon activation of the "RepeatMasker" track. Segmental duplications, simple repeats (minisatellites) and microsatellite can be displayed using the activating eponymous tracks, and are absent from the region presented here.

## *II-Production of TALENs*

Several freely available TALEN assembly kits exist (*e.g.* trhough http://www.addgene.org/TALEN/). For example, those based on Golden-Gate cloning typically allow assembling several pairs of TALENs in less than a couple of weeks. Our lab has successfully used the method described by Sanjana et al., 2012

in mESCs with a Fok-I backbone containing a pCAGGS promoter instead of the default pCMV.

*Notes*: It is also possible to obtain custom TALENs or ZFNs from commercial sources or form the transgenesis core of various universities and institutes.

### *III-Delivery of TALENs into mESCs*

As no selection for transfected cells is used, it is critical to achieve maximum transfection efficiency. Using the Amaxa 4D-Nucleofector system (Lonza) our lab typically achieves >75% transfection efficiency with reporter plasmids, which is higher than when using lipofection or classical electroporation.

1. Prepare TALEN plasmid DNA for the four constructs (upstream Left & Right, Downstream Left & Right) by Midi or Maxiprep. Concentrate DNA at 1µg/µL or higher.
   *e.g.* using the NucleoBond Xtra Midi Plus kit from Macherey-Nagel.

2. Have >5 million mESCs happily growing. Change culture medium few hours prior to nucleofection to ensure optimal growth.

3. Nucleofect 5 million mESCs mESCs with 2.5µg of each TALEN, so 10µg of DNA in total, according to the manufacturer's protocol (see notes).

4. Resuspend nucleofected cells in 10mL of 37°C mESC culture medium.

5. Seed 2mL in a 25cm² gelatinized flask (do not use feeder cells here). These cells will be used to check that the engineered allele is present in the population of transfected cells, before isolating single mutant clones.

6. Seed 10mL of a 1/10 dilution of transfected cells in two feeder-containing round ~60cm² petri dishes (10mL each). Use gelatinized dishes if the mESC line is feeder-independent.

7. Seed 10mL of a 1/1000 dilution of transfected cells in two round ~60cm² petri dishes (10mL each).

8. Seed 10mL of a 1/1000 dilution of transfected cells in two round ~60cm² petri dish (10mL each). Plating serial dilutions ensures optimal clone density for picking.

9. Change medium every 1-2 days until clones are ready to be picked.

*Notes*:

A) Only use plasmid DNA extracted by Midi or Maxiprep – Miniprep columns yield DNA that is not suitable for transfection.

B) Ensure that the nucleofection solution is a single-cell suspension, as clumps of cells will result in non-clonal colonies.

C) It is advised to first determine the optimal nucleofection program using a reporter plasmid, as conditions can differ from cell line to cell line.

D) Additional information on Nucleofection can be found at http://bio.lonza.com/fileadmin/groups/marketing/Downloads/Protocols/Generated/Optimized_Protocol_309.pdf

## *IV- Assessing the presence of mutant alleles before screening single clones*

It is possible to check that the intended mutation is present in the pool of transfected cells prior to picking single clones. Deletions and inversions larger than a few hundred nucleotides can easily be detected with the following PCR design:

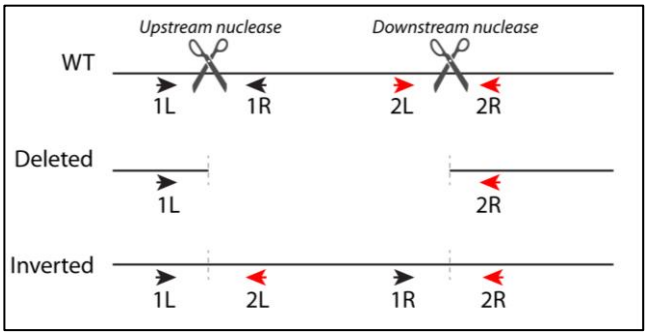


**Figure 3. Design of genotyping primers.** Two pairs of PCR primers are designed to encompass each TALEN target site. Deletions or inversions rearranging the target locus can be identified simply by using distinct combinations of PCR sprimers.

1. Design two pairs of primers, each encompassing a TALEN target site, and each amplifying around 500bp of genomic DNA (see figure 3 and notes)
   *e.g.* using the online Primer3 website (Rozen and Skaletsky, 2000) http://frodo.wi.mit.edu/

2. Grow transfected cells in the 25cm² of the previous step for 48 to 72 hours.

3. Harvest cells by trypsinization, spin them down and discard supernatant to leave a dry pellet that can be stored at -80°C if needed.

4. Extract genomic DNA from this cell pellet
   e.g. using the GenElute mammalian miniprep kit

5. Use this genomic DNA to run a PCR aiming at detecting deleted or inverted alleles. Running two separate PCRs aiming at detecting the unmodified alleles can be used as a positive control to check primer efficiency. Genomic DNA from untransfected cells can be used as a negative control.

   **Unmodified alleles**: Primers 1L+1R and Primers 2L+2R
   **Deleted allele**: Primers 1L+2R
   **Inverted allele**: Primers 1L+2L and Primers 1R+2R

When using default Primer3 settings to design primers, we routinely use the following PCR conditions:

| Reagent | µL per reaction |
|---|---|
| gDNA (100ng/µL) | 1 |
| 10X custom PCR buffer | 2 |
| 50mM MgSO4 | 1.8 |
| 10mM dNTPs | 0.8 |
| Primer A (10µM) | 0.8 |
| Primer B (10µM) | 0.8 |
| Taq Polymerase | 0.15 |
| H2O | 12.65 |
| ***TOTAL*** | ***20 µL*** |

| | | |
|---|---|---|
| 95°C | 3 min | |
| 95°C | 30 sec | **X 35** |
| 60ºC | 30 sec | |
| 68ºC | 20-40 sec | |
| 68ºC | 5 min | |
| 15ºC | hold | |

**6.** Run a 2% agarose gel electrophoresis and ensure that modified alleles are detected in the pool of transfected cells.

*Notes*:

A) When performing smaller (<2kb) deletions, elongation time during PCR should be reduced to 20-30 sec to avoid amplifying unmodified alleles with primers 1L+2R.

B) Annealing temperature during PCR may be changed to optimize yield and specificity.

C) Primers can be designed so that amplicons corresponding to unmodified, deleted and inverted alleles respectively are all of different size. This allows running a single PCR reaction with three primers to detect at once the presence of any type of allele, provided primers to not cross-react. Be aware that DNA extracted from the population of transfected cells mostly contain unmodified alleles, so it may be difficult to detect both unmodified and modified alleles at once at this step. We recommend using such triplex PCR design only when screening DNA from single clones.

D) It is important to include positive and negative controls in for the PCR to make sure genotyping primers work as intended – especially when using three primers at once.

E) If no deleted/inverted allele is detected it may be because the transfection efficiency is low, in which case it is useful to run transfection tests with a reporter plasmid. Another source of failure can come from poor TALEN-mediated cleavage. One can assess TALEN cleavage efficiency using for example using the T7 Endonuclease I assay (Kim et al., 2009) or the Surveryor assay (Sanjana et al., 2012). It is useful to include a TALEN pair that is known to work as a positive control when running such tests.

## *V- Isolating single mutant clones*

### CLONE PICKING

Once single (clonal) ESC colonies can be picked, which is typically 5 to 7 days after Nucleofection, transfer them to a gelatinized 96well plate (or with feeder cells if mEScs are feeder-dependent), according to standard mESC culture procedures. Avoid picking only the largest colonies as these may carry karyotypical abnormalities conferring fast growth. Use of a binocular or dedicated picking instrument can help picking clones at early stages using a 10µL or 20µL pipet tip. Deletions <5kb are typically obtained at frequencies ranging from 1% to 5%. This means that picking one or two 96-well plates is usually sufficient to isolate at least one mutant clone.

For deletions or inversions larger than 5kb it is wise to pick an additional 96-well plate consisting of pools of clones (so each well contains a mixture of 5 colonies or so). For this take a 200µL tip, aspirate the first colony, do not eject cells but instead keep on picking a second colony etc… If the mutation event is very rare pools of up to 20 colonies can easily be picked that way. Once a pool containing the desired allele is identified it can be subcloned by limiting dilution to isolate a homogenous mutant clones.

## GENOMIC DNA EXTRACTION FROM CLONES

1. Cells are usually ready 2-5 days after picking colonies. For each 96-well plate to be split, two 96-well plates should be ready with gelatin (or one gelatinized and one with feeders if mESCs are feeder-dependent).

2. Aspirate culture medium from the clone-containing 96-well plate

3. Add 150µL 1X PBS

4. Aspirate PBS

5. Add 50µL of Trypsine and incubate 15min at 37°C

6. Replace medium of the gelatin-coated / feeder-containing 96-well plate with 100µL of mESC culture medium

7. Resuspend thoroughly the trypsinized cells with 100µL of mESC medium. Transfer around 25µL in a gelatinized/feeder-containing plate: these cells will be kept in culture. Transfer around 125µL in a gelatinized plate (without feeder cells): these cells will be used for genotyping.

8. After overnight growth the plate for genotyping should be almost confluent and can be used to prepare genomic DNA. The other plate can be left in culture for 1-2 days, changing medium daily.

9. Aspirate culture medium from the genotyping 96-well plate and add 150µL 1X PBS

10. Aspirate PBS and add Proteinase K to Lysis buffer at 1mg/mL final. Dispense 50µL of supplemented buffer in each well. Seal firmly with a plastic film and incubate in a humidified chamber at 60°C 4hrs to overnight.

11. Leave plate on ice for 5min to cool it down.

12. Add 150µL of ice-cold precipitation solution and Incubate 30min at room temperature.

13. Spin plate 30min at 4°C at 4000rpm.

14. Invert the plate to decant liquid and blot excess liquid on paper towels.

15. Add 150µL of 70% Ethanol and spin 15min at 4000rpm.

16. Invert the plate to decant liquid and blot excess liquid on paper towels.

17. Leave the plate open to dry completely at room temperature (>15min)

18. Add 200µL of H2O and incubate at least 1h at 37°C to 45°C to resuspend DNA. This is the "undiluted DNA" plate, which can be stored at -20°C.

19. Prepare a 1/10 dilution of the DNA by distributing 45µL of H2O in a fresh 96-well plate and adding 5µL of resuspended genomic DNA. This is the "diluted DNA" plate, which can be stored at -20°C.

GENOTYPING PCR

1. Assemble on ice following reaction in a 2mL tube:

| **Reagent** | Vol per reaction | **For 102 reactions** |
|---|---|---|
| 10X custom PCR buffer* | 2 | 204 |
| 50mM MgSO4 | 1.8 | 183.6 |
| 10mM dNTPs | 0.8 | 81.6 |
| PrimerA | 0.8 | 81.6 |
| PrimerB | 0.8 | 81.6 |
| Taq NEB | 0.16 | 16.3 |
| H2O | 11.64 | 1187.3 |
| ***TOTAL*** | *18µL* | ***1836 µL*** |

2. Distribute 18µL in a 96-well PCR plate

3. Add 2µL of genomic DNA of each clone in the corresponding well of the 96-well PCR plate

4. Run the following PCR cycle:

| | | |
|---|---|---|
| 95°C | 3 min | |
| 95°C | 30 sec | X 35 |
| 60ºC | 30 sec | |
| 68ºC | 20-40 sec | |
| 68ºC | 5 min | |
| 15ºC | hold | |

5. Run the whole reaction on a 2% agarose gel electrophoresis to identify clones harboring mutant alleles

6. Identify mutant clones and select them for further amplification from the 96-well plate kept in tissue culture.

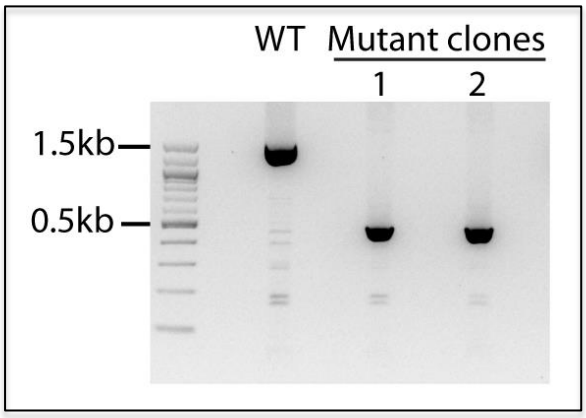


**Figure 4. Example of genotyping results for a 1kb deletion induced with TALENs.** Primers flanking the deletion were used (1L and 2R, see figure 3), with a 2' elongation time to amplify both wild-type (1.5kb) and deleted (0.5kb) alleles. Targeted deletion was here on the X chromosome in male mESCs so mutants are hemizygous and do not harbor any wild-type allele.

*Notes*:

A) When screening short deletions it may be convenient to extend the PCR elongation time in order to detect the presence of the undeleted (WT) and deleted alleles simultaneously.

B) If PCR design allows three primers to be used at once it is possible to simultaneously screen for unmodified, deleted and inverted alleles (See Note IV-B)

C) If no mutant clone is isolated make sure that the PCR set-up is working, for example by re-running a genotyping with primers amplifying unmodified alleles (1L+1R or 2L+2R).

D) DNA concentration is critical for optimal genotyping PCR. In case no specific PCR product is observed even when trying to amplify unmodified alleles, try adjusting

the amount of DNA used as template. If a smear is visible in the wells, reduce DNA concentration. If lanes show no signal at all, increase DNA concentration.

E) It is not unusual to isolate alleles with deletion/inversion slightly larger or shorter than expected. These alleles might still create interesting mutations so it is advisable to select the corresponding clones for further analysis as well.

## *Characterizing mutated alleles*

The most straightforward way to characterize mutant alleles is to use the PCR product from the genotyping reaction for Sanger sequencing (Figure 4). If direct sequencing from the DNA extracted from the 96-well plates is gives noisy signal, prepare clean genomic DNA (for example using the GenElute mammalian miniprep kit). Re-run the genotyping PCR, and gel extract the DNA (using e.g. the Qiagen MinElute gel extraction kit) to generate high-quality.

It is rare but not unusual to isolate mutants where the deletion/inversion occurred a few tens of bp away from the TALEN target site. It is also useful to check the integrity of the non-deleted/ non-inverted allele in heterozygous clones (Figure 5). In some cases the non-rearranged allele harbors short indels at the TALEN target sites. Be aware that serial targeting, in order to produce homozygous mutants for example, must be performed in heterozygous clones harboring intact TALEN target sites on the remaining allele.

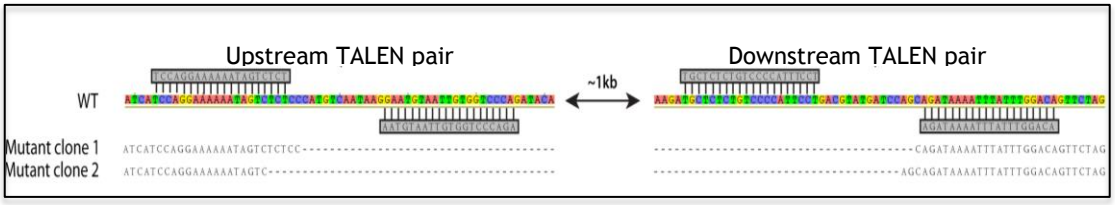


**Figure 5. Sequencing of the engineered alleles.** PCR product from figure 3 was gel extracted and used for Sanger sequencing with one of the primers used for amplification. Alignment with reference sequence reveals the exact position of deletion the at the nucleotide precision, which typically vary from clone to clone.

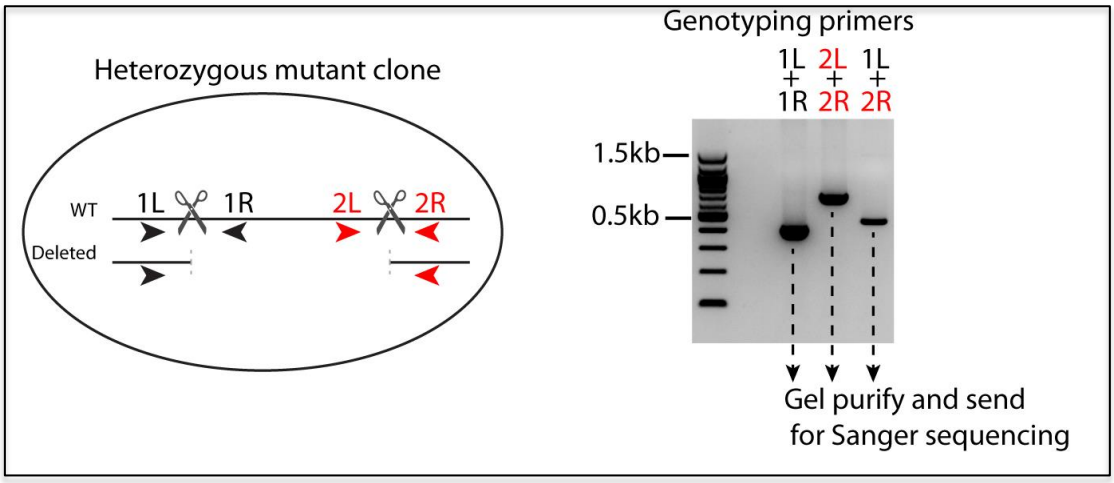


**Figure 6. A strategy to check the integrity of non-deleted/non-inverted alleles.** When isolating heterozygous deletions/inversions it is important to check whether non rearranged alleles harbor small indels that would not have been detected by PCR. For this non rearranged alleles are specifically amplified using primers 1L+1R and 2L+2R in separate reactions, while 1L+2R would specifically amplify deleted alleles and 1L+2L or 1R+2R inverted alleles. PCR products are gel extracted after electrophoresis and used for Sanger sequencing with one of the primers used for amplification.

*Notes*: It may be useful to ultimately perform additional characterization, such as Southern analysis, to ensure proper engineered alleles have been created.

## List of Reagents

### *Production of TALENs*

- TALEN tool box kit (Addgene cat#1000000019) or any other source

### *Delivery of TALENs into mESCs*

- NucleoBond Xtra Midi Plus kit (Macherey-Nagel cat# 740412.10)
- Amaxa 4D-Nucleofector system (Lonza cat# AAF-1001B and AAF-1001X)
- P3 primary cell 4D Nucleofection reagents (Lonza cat# V4XP-3024)
- Standard mESCs culture reagents

### *Assessing the presence of mutant alleles before screening single clones*

- GenElute mammalian genomic DNA miniprep kit (Sigma G1N70)
- Taq polymerase (NEB M0267S)
- dNTPs (Fisherscientific cat# 10610851)
- Two pairs of Primers designed to encompass each TALEN target site
- *10X Custom PCR buffer: 600mM Tris H2S04 pH8.9, 180mM ammonium sulfate – stored at -20°C
- Conventional agarose gel electrophoresis apparatus and imaging system

### *Isolating single mutant clones*

- Proteinase K (Roche 03115879001)
- Lysis buffer without proteinase K, stored at room temperature:

| Reagent | Final concentration | For 50mL |
|---|---|---|
| 1M Tris-HCl (pH 7.5) | 10 mM | 500 µL |
| 0.5M EDTA | 10 mM | 1 mL |
| 10% SDS | 0.5% | 2.5mL |
| 5M NaCl | 10 mM | 100 µL |
| H2O | | 45.9 mL |

- Precipitation solution: 75mM NaCl in Ethanol.

### *Characterizing mutated alleles*

- MinElute gel extraction kit (Qiagen 28604)
- Access to a Sanger sequencing facility

## Additional sources of information about TALENs and genome engineering:

- TAL Effectors Discussion Forum
  https://groups.google.com/forum/#!forum/taleffectors

- Genome Engineering Newsgroup
  https://groups.google.com/forum/#!forum/talengineering

## Abbreviations

ZFNs: Zinc-Finger Nucleases
TALENs: Transcription Activator-Like Nucleases
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
Indel: Insertion or deletion
mESCs: mouse Embryonic Stem Cells
dNTPs: deoxyribonuclotide triphosphates

## Reviewer's comments:

Name, title (PhD)
Address

(1) text

(2) text

## References

Carlson, D.F., Tan, W., Lillico, S.G., Stverakova, D., Proudfoot, C., Christian, M., Voytas, D.F., Long, C.R., Whitelaw, C.B.A., and Fahrenkrug, S.C. (2012). Efficient TALEN-mediated gene knockout in livestock. PNAS *109*, 17382–17387.

Doyle, E.L., Booher, N.J., Standage, D.S., Voytas, D.F., Brendel, V.P., Vandyk, J.K., and Bogdanove, A.J. (2012). TAL Effector-Nucleotide Targeter (TALE-NT) 2.0: tools for TAL effector design and target prediction. Nucleic Acids Res. *40*, W117–122.

Gupta, A., Hall, V.L., Kok, F.O., Shin, M., McNulty, J.C., Lawson, N.D., and Wolfe, S.A. (2013). Targeted Chromosomal Deletions and Inversions in Zebrafish. Genome Res.

Hockemeyer, D., Wang, H., Kiani, S., Lai, C.S., Gao, Q., Cassady, J.P., Cost, G.J., Zhang, L., Santiago, Y., Miller, J.C., et al. (2011). Genetic engineering

of human pluripotent cells using TALE nucleases. Nature Biotechnology *29*, 731–734.

Joung, J.K., and Sander, J.D. (2013). TALENs: a widely applicable technology for targeted genome editing. Nature Reviews Molecular Cell Biology *14*, 49–55.

Kim, H.J., Lee, H.J., Kim, H., Cho, S.W., and Kim, J.-S. (2009). Targeted genome editing in human cells with zinc finger nucleases constructed via modular assembly. Genome Res. *19*, 1279–1288.

Lee, H.J., Kweon, J., Kim, E., Kim, S., and Kim, J.-S. (2011). Targeted chromosomal duplications and inversions in the human genome using zinc finger nucleases. Genome Research.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Methods Mol. Biol. *132*, 365–386.

Sanjana, N.E., Cong, L., Zhou, Y., Cunniff, M.M., Feng, G., and Zhang, F. (2012). A transcription activator-like effector toolbox for genome engineering. Nature Protocols *7*, 171–192.

Valton, J., Dupuy, A., Daboussi, F., Thomas, S., Maréchal, A., Macmaster, R., Melliand, K., Juillerat, A., and Duchateau, P. (2012). Overcoming Transcription Activator-like Effector (TALE) DNA Binding Domain Sensitivity to Cytosine Methylation. J. Biol. Chem. *287*, 38427–38432.