# TALEN expression vectors
# Yasunori Sasakura 7th May, 2018

(Caution: These days our group uses TALENs much more frequently than CRISPR/Cas9, we only provide the protocols for TALENs now.)
(Figure 1 below shows the overview of this protocol)

**1) TALEN kit**

Currently the recommended TALEN construction kit is the Platinum Gate TALEN kit (Sakuma et al., 2013, Scientific Reports 3, 3379). You can purchase the basic set of this kit from Addgene.

https://www.addgene.org/kits/yamamoto-platinumgate/?gclid=Cj0KCQjw4eXPBRCtARIsADvOjY2SQXgDuHaRgHLpuLY9KyyKmIrT2J5oaUniSiFJ33H75cE7qE2re6EaAmfPEALw_wcB

**2) Design of TALENs**

After you select the gene to knockout, please determine the region of the gene to introduce mutations. We usually choose the region corresponding to the core domain critical for the protein function. For example, DNA-binding domain for transcription factors and kinase domain for kinases. Transmembrane domain can also be a good target for transmembrane proteins. If such a domain is not present or obscure in your protein encoded by the target gene, then we recommend to target around the start codon of the gene. Please notify that there might be several translational start sites or cryptic start sites in a gene. In such cases the efficiency of yielding phenotypes would be reduced when you target the site around the start codon.

Once you choose the DNA region for targeting, copy and paste the DNA region (200 nt long or a single exon is usually sufficient. Long DNA region will yield too many TALEN candidates) onto TALEN targeter 2.0 (https://tale-nt.cac.cornell.edu/node/add/talen). The parameter we use is as follows:

Select the “Provide Custom Spacer/RVD Lengths” tab.
Minimum spacer length: 15
Maximum spacer length: 18

Minimum repeat array length: 16
Maximum repeat array length: 17

G substitute: NN

Filter options: Show all TALEN pairs (including redundant TALENs)

Streubel et al. guidelines: On

Pre-loaded sequence: default (-none-)
Scoring Matrix: Default (Doyle et al.)
Upstream Base: Default (T only)

After entering the parameters, push the Submit button.

The program will result in several pairs of TALENs that may be good for knocking out the gene. Please paste the results on the Excel. Then select one or two pairs for construction. By the historical reason, we prefer to choose the hetero-lengthened pairs with one side has 16 repeats and the other side 17 repeats, and the last repeat is NG, but you may choose pairs that does not obey the rule. The historical reason just mentions that we have more experience to knockout genes with TALENs having the condition than other pairs.

A TALEN pair selected above have ~70% of probability to be a good TALEN pairs. Therefore, we usually create two pairs for a single target to assure the success of knockouts.

**3) 1st assembly**
The selected TALENs will be shown as follows:

```
NI NG NN NN NN NG NN HD NN NI NN NI NI HD NI HD NG (Left repeat)
NN NN NN NG NG HD NI NG NN NN NI HD NG NG NG NG (Right repeat)

T ATGGGTGCGAGAACACT acaggattgctcgtatgt AAAAGTCCATGAACCC A
```

(The DNA sequence of the target site of the above TALENs. Lower case exhibits spacer. The first and last nucleotides are zero repeat/upstream base that are not

included in the TALEN array.)

Please separate the array data as follows:

```
NN NN NN NG =a3a cassette
NG HD NI NG =a3b cassette
NN NN NI HD =a3c cassette
NG NG NG    =b3 or b4 cassette
NG          =last repeat
```

In the first assembly, you need to create the a3a, a3b, a3c and b3/b4 plasmids that have three or four aligned repeats as described above. The method for the 1st assembly is completely the same as the one described in the kit. Therefore, we will not mention it here. The 1st assembled clones can be shared between TALENs.

**4) 2nd assembly**

Once you obtain the a3a, a3b, a3c and b3/b4 plasmids that are compatible with your TALENs, then ligate the 1st assembled repeats to make full TALENs.

After the assembly, you have to check the activity of your TALENs. For this reason, the 2nd assembly should use pBSEF1a>SanTAL::2A::mCherry construct as the backbone vector, because the assembled TALENs can be expressed under the control of EF1a promoter, which is a good ubiquitous promoter for testing the activity of TALEN in Ciona.

The protocol of the 2nd assembly is as follows:

Adjust the concentration of 1st assembled clones to 50-100ng/ul.

Make the 1:1:1:1 mixture of 1st clones.

Make the following mixture

| | |
|---|---|
| 1st clone mixture | 1.0 ul |
| 50ng/ul backbone vector | 0.3 ul |
| 2x QuickLigase buffer | 3.6 ul |

| | |
|---|---|
| Esp3I (Fermentas) | 0.2 ul |
| QuickLigase (NEB) | 0.2 ul |
| Water | 1.9 ul |
| | 7.2 ul |

10-13 cycles of 5 min at 37ºC, 10 min at 16 ºC (please use a thermal cycler).
Increasing cycle number will yield more colonies.

After the cycle is over, then add the following materials in the reaction mixture:

| | |
|---|---|
| 10x Tango buffer (Supplied with Esp3I) | 1.0 ul |
| 20mM DTT | 1.0 ul |
| Water | 0.6 ul |
| Esp3I | 0.2 ul |

30 min-1 hr at 37 ºC
5 min at 80 ºC to inactivate Esp3I

This reaction will reduce the background colonies.

Transform E. coli and plating on LB-amp plate.
We use hand-made E. coli (titer is $10^7$-$10^8$ colony forming unit/ug DNA). Usually 1 ul of the reaction mixture yields sufficient number of colonies.

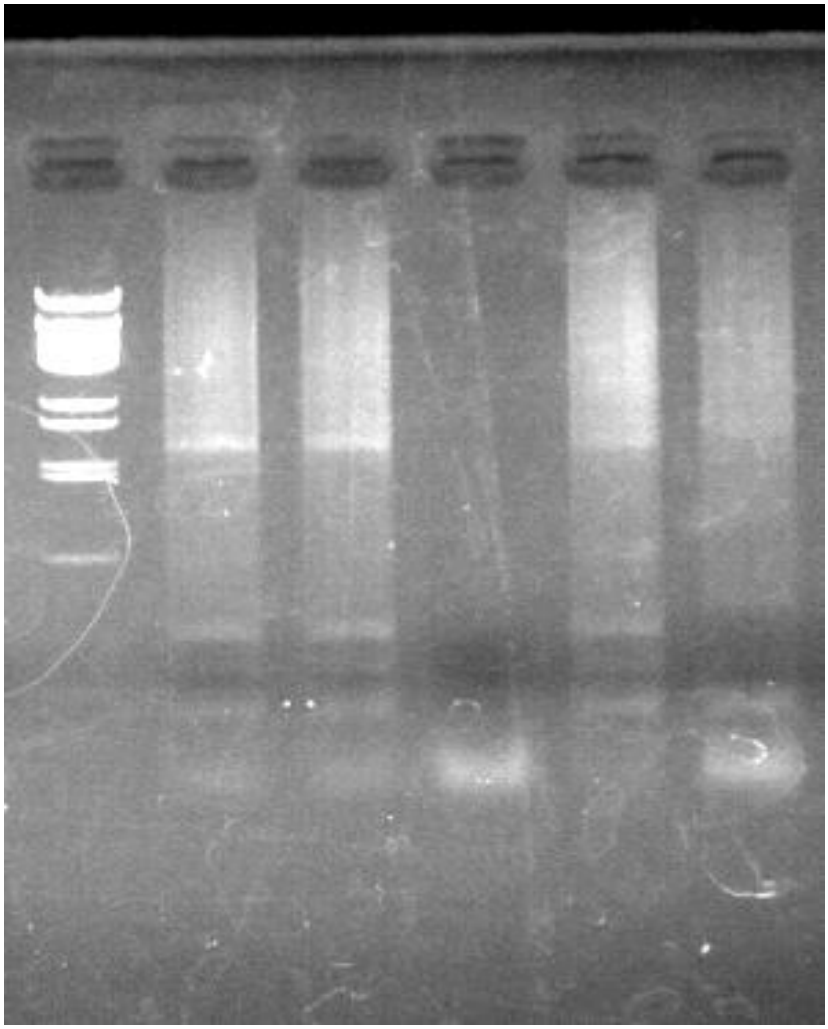
**5) Insert check**
Insert check of the 2nd assembled clones should be done with the following primers and PCR conditions.

TALE-F: GCACCCCTCAACCTGACCCCAG
TALE-R: CTCGAAAGCTGGGCCACGATTG

PCR conditions:
5 min at 95 ºC
30 cycles of 94-30s, 65-30s, 72-50s
5-10 min at 72 ºC

4-10 ºC forever

Successfully assembled colonies will show a smear band (see the photo below). Pick up the clones and culture in 2 ml LB-amp liquid.

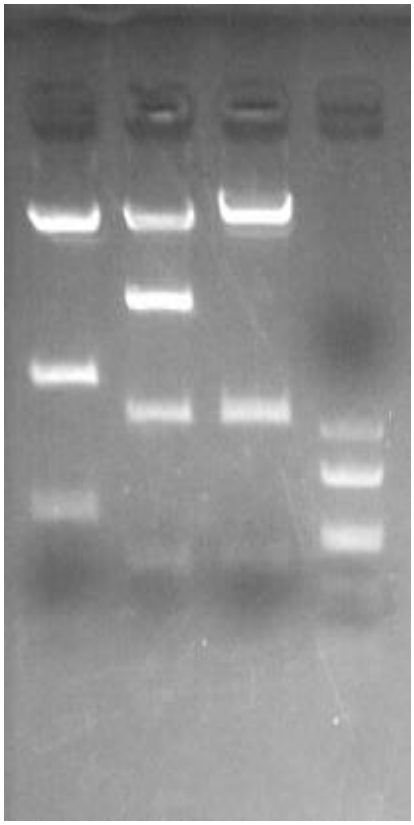


Example of the insert check: 1st lane, marker; 2-6 lanes, TALEN clones. Lane 2, 3 and 5 and 6 suggest the success of assembly. The 4th lane will be a wrong clone and do not pick the colony.

Miniprep with your laboratory method.

Check the plasmids with restriction enzyme NruI. The NN (G) repeats have one NruI site and you may confirm the success of the assembly based on the position of

NN modules. The photo below is an example of three different TALENs digested by NruI. The 2nd assembly does not usually fail.



1st lane: 700+300
2nd lane: Promoter band+600+200
3rd lane: 600+200 (in basepair)
4th lane: Marker (pBluescript digested with HaeIII)
For each lane, the top band is the backbone vector and please ignore it.

**6) Checking activity**

Minipreped EF1a>TALEN::2A::mCherry plasmids are sufficient for checking its mutation activity. In our case, the method is as follows:

Elute minipreped DNA in 50ul elution solution.
4 ul of the plasmid solution will be used for NruI check.

Remain 10 ul of plasmid, and use the rest plasmid solution for electroporation.

Mix L and R plasmids and the mixture will be used for the single round of electroporation. For the details please see Yoshida and Treen (2018).

Collect embryos with bright mCherry fluorescence. The shape of embryos may be bad but it is not necessary to take care the development.

Spin down embryos, and discard seawater.

Isolate genomic DNA with Wizard genomic DNA purification kit (promega).

Add 600 ul Nuclei lysis solution. Incubate for 1-2 hours at 50 ºC. Rotation is better.

Cool down at room temperature.

Add 200 ul Protein precipitation solution. Incubate on ice for 5 min.

Centrifuge 13,500 rpm for 4 min at 4 ºC.

Collect supernatant to a new tube.

Centrifuge 13,500 rpm for 4 min at 4 ºC.

Collect supernatant to a new tube.

Add 1 ul glycogen (Roche).

Add 600 ul Isopropanol.

Centrifuge 1 min at **room temperature**.

Discard supernatant by decantation. Pellets may be invisible.

Add 100 ul 70% ethanol.

Centrifuge 1 min at **room temperature**.

Discard 70% Ethanol and dry up.

Add 10-20 ul water.

PCR amplification of the TALEN target site.

| | |
|---|---|
| genome DNA | 1.0 ul |
| 10x PCR buffer | 2.0 ul |
| dNTPs | 1.6 ul |
| 10pmol/ul primer | 2.0 ul |
| 10pmol/ul primer | 2.0 ul |
| ExTAQ hot start version | 0.2 ul |
| D3W | 11.2 ul |
| | 20.0 ul |

5 min at 95 ºC, 50 cycles of (94-30s, 55-30s, 72-1~3min) 5-10 min at 72 ºC, 10ºC forever.

Electrophoresis 5 ul of PCR mixture on 15% polyacrylamide gel (AA:Bis = 29:1). The voltage should be 100v at constant voltage. Higher voltage will reduce the resolution of the bands.

If mutations are introduced, shifted bands will appear. The typical examples can be seen in our knock out database (http://marinebio.nbrp.jp/ciona/sequenceFile/knockOut/gsTalens/Raldh2/Raldh2-HMA.pdf).

When the mutation bands appear, then subclone the bands into an arbitral vector for sequencing analysis. 70% or higher mutation frequency will be OK for further analyses.

**7) Switching vectors**

When you obtain a TALEN pair that has a good mutation frequency, you may have to transfer the repeat arrays to appropriate vectors for further analyses. There are mainly two ways of the transfer: expression vectors for tissue-specific expression, and mRNA synthesis vector.

The easiest way is repeating the second assemblies on the appropriate vectors. Because Golden gate method is reproducible and high efficient, the assembly is usually successful. However, there is a possibility that new TALENs are wrongly assembled, and the wrongness could not be checked completely due to the repeat structure. By this reason, we recommend the following ways.

**7-1) Repeat switching method**

Isolate assemblies from EF1a>TALEN vectors with SalI and BamHI.

| | |
|---|---|
| Minipreped solution | 5ul |
| 10x T buffer (TAKARA) | 3ul |
| SalI | 1ul |
| BamHI | 1ul |
| Water | to 20ul |

Inverse PCR the acceptor vectors with the following primers:

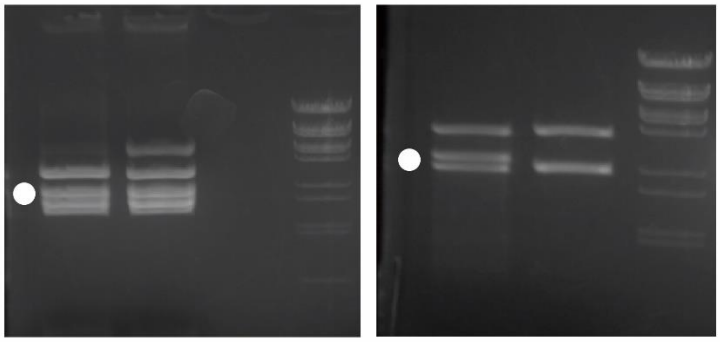
ACCAACAGAAGGATCCCCGAGAGGACATCACAT
CTGCGCGACTGTCGACCTCACTTTGGGCTTGATC

(Please use high fidelity DNA polymerase. We use PrimeStar HS DNA polymerase from TAKARA bio. 30 cycles are sufficient).

Add 1 ul of DpnI restriction enzyme (TAKARA) to the PCR solution after PCR is finished. This enzyme digests the template vector that have methylated sites done in E. coli.

Gel extraction of the restriction digested plasmids and PCR products. The restriction fragments of TALEN repeats will not be separated well from other DNA bands (see the photos below). Please do not mind it, because In-fusion reaction is high efficient and highly specific.

Several examples of the isolation of TALEN repeats from vectors



Isolate the bands around the dots (Marker, BstEII digested Lambda DNA).

After elution, the DNAs are mixed for In-fusion reaction.

The In-fusion reaction almost always successfully yields appropriate vectors. However, the success rate on the pHTB-TALEN vector is lower than other vectors and a few nucleotides are sometimes lost at the joining site. We check the success of the In-fusion by sequencing with the following primers:

AATGGACTATAAGGACCAC (5' junction site)
TTTTCGTGATCCACCGAGATGC (3' junction site)

**7-2) Promoter switching method**

The promoter region of TALEN expression vector can be removed with NotI. A new promoter can be inserted at the NotI site to switch promoter for tissue-specific expression.

Our TALEN expression vectors were constructed on pBluescript, which is different from that used in our pSP vector series. Therefore, promoter fragments for pSP vectors are not compatible with pBluescript. If you like to isolate promoter for TALEN expression from pSP vector, amplify the promoter with the following primer set:

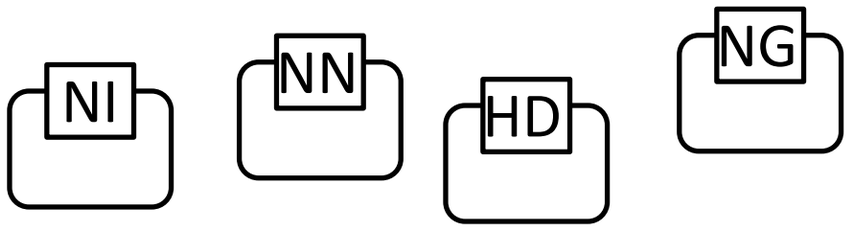
| | |
|---|---|
| CCGAGGGTGGCGGCCTAGGTGACACTATAGAACTC | pSP-TALEN-promoter-switch-XhoF |
| CCGAGGGTGGCGGCCCTGCAGGTCGACTCTAGAggatc | pSP-TALEN-promoter-switch-BamF |
| TAGTCCATTGCGGCCGCAAGGGGATC | pSP-TALEN-promoter-switch-R |

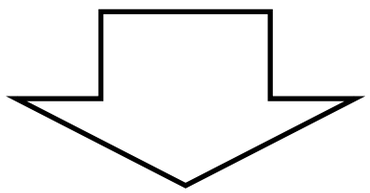
When the promoter is inserted in the BamHI/BamHI site of pSP vector, please use pSP-TALEN-promoter-switch-BamHI. When the promoter is inserted in the XXXX/BamHI site (where XXXX is an arbitral restriction site that is located at the 5’ upstream of BamHI site), please use pSP-TALEN-promoter-switch-XhoF.

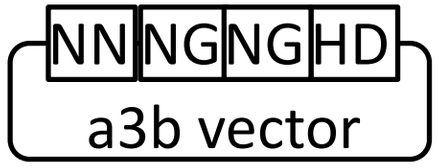
The PCR fragments have the homology arm for In-fusion reaction for the NotI site of pBS-TALENs.

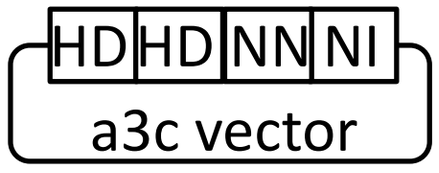
(Caution: The forward primer could not be used for a few TALEN vectors. In this case, the homology arm should be 5’-ACCGCGGTGGCGGCC-3’)

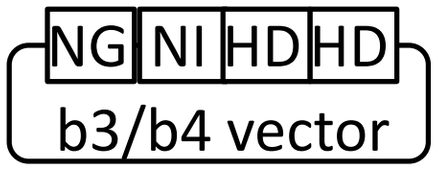
# 1st Assembly of TALEN repeats




etc...

Day 1
- Mix repeat units.
- Golden gate reaction.
- Post digestion of unassembled plasmids.
- Transformation overnight.

Day 2
- Insert check.
- Incubate appropriate clones.

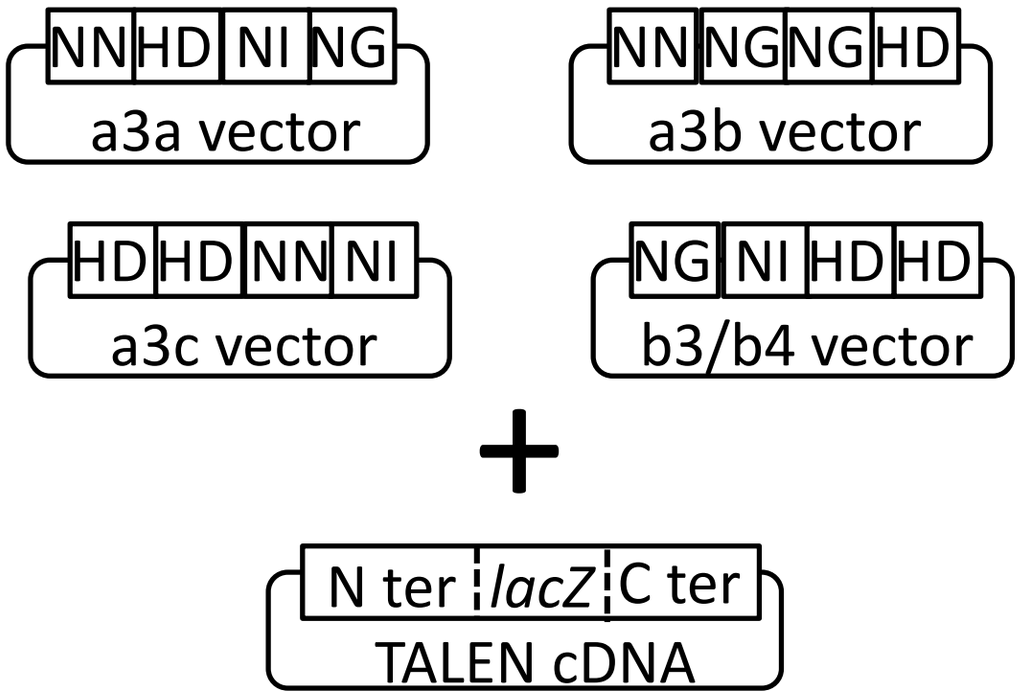
Day 3
- Mini-prep.
- (optional) sequencing to confirm the assembly.

The combination of the 1st assembled clones is about 1,000 for creating TALENs with 16~17 repeats.

→We could skip this step when we finish construction of the 1,000 clones.

＊Our group recommends using Platinum TALEN kit (Sakuma et al., 2013).

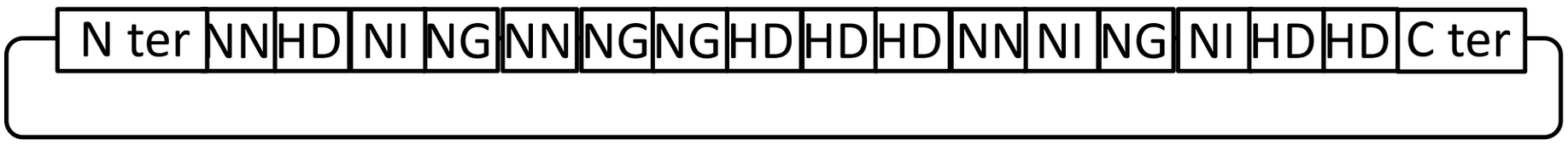
# 2nd Assembly of TALEN repeats




Day 1
- Mix 1st assembled clones.
- Golden gate reaction.
- Post digestion of unassembled plasmids.
- Transformation.

Day 2
- Insert check.
- Incubate appropriate clones.

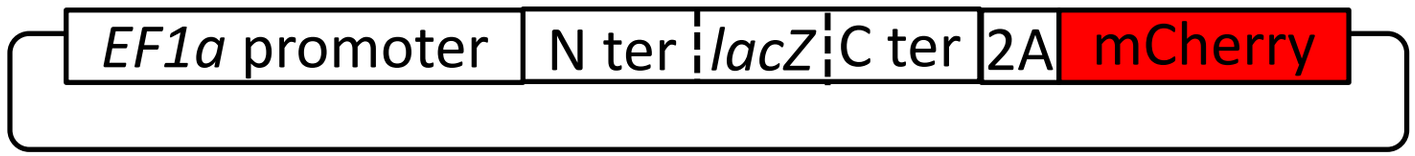
Day 3
- Mini-prep.
- Restriction digestion to confirm the assembly.
- (optional) sequencing of 5’ and 3’ ends of assembly.




Full TALEN cDNA (approx. 3,000bp)

*Once repeat is assembled, you can replace the repeat in any vector with restriction enzymes.

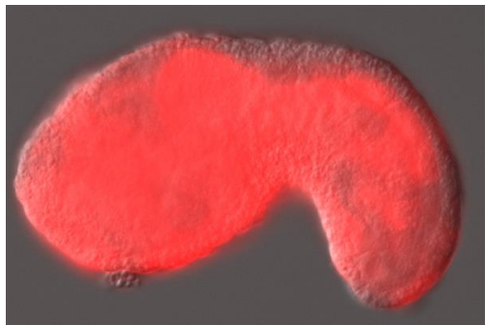
# Checking mutation efficiency of designed TALEN



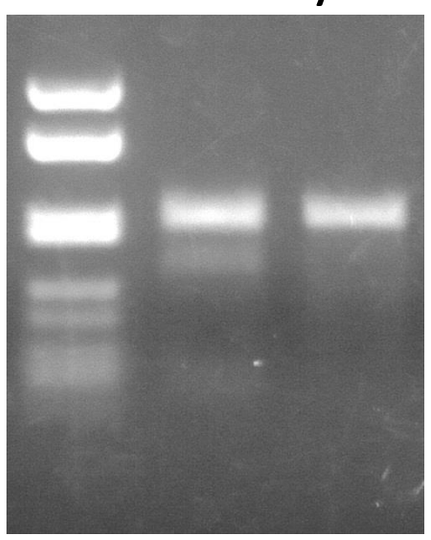

Ubiquitous promoter>TALEN::2A::mCherry

After assembly, electroporation into embryos.

We can see the expression of TALENs by red fluorescence.



Cell assay



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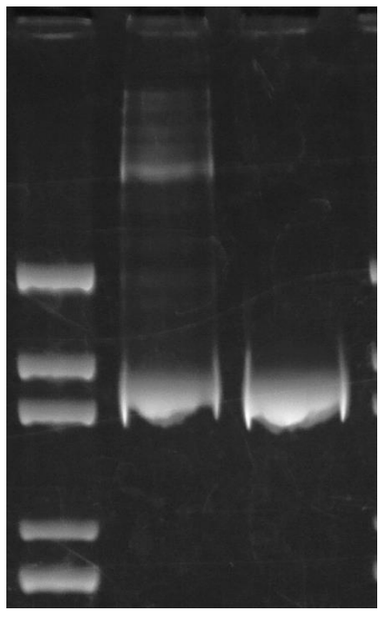


Checking occurrence of mutations by electrophoresis

・2nd assembly of your designed TALEN into the backbone vector of TALEN with *EF1α* promoter.

・Midiprep and electroporation of the vectors.

・Expression of TALENs could be monitored with the fluorescence of mCherry.

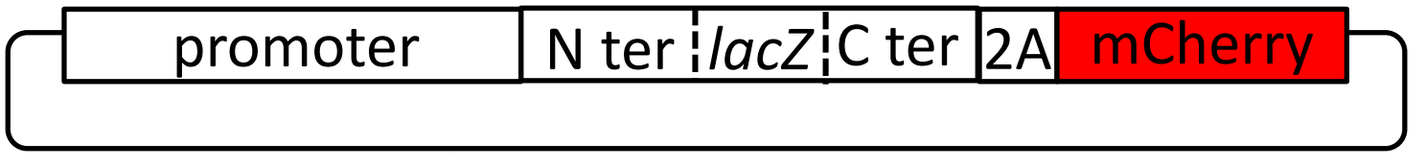
・Collect tailbud embryos with ubiquitous mCherry fluorescence.

・Genome isolation and PCR amplification of the target site.

・Digestion with Cell or polyacrylamide gel electrophoresis.

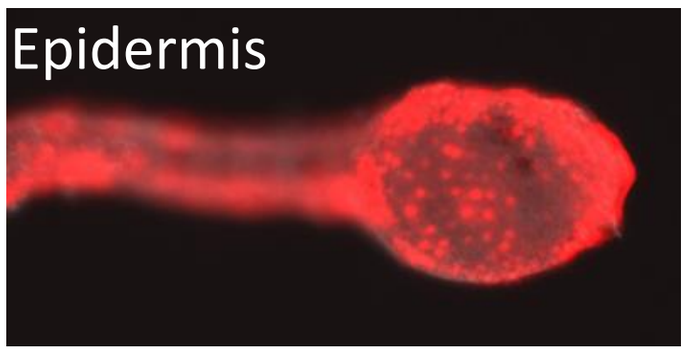
・(Optional) Cloning of PCR bands and sequencing to see the efficiency of mutations.

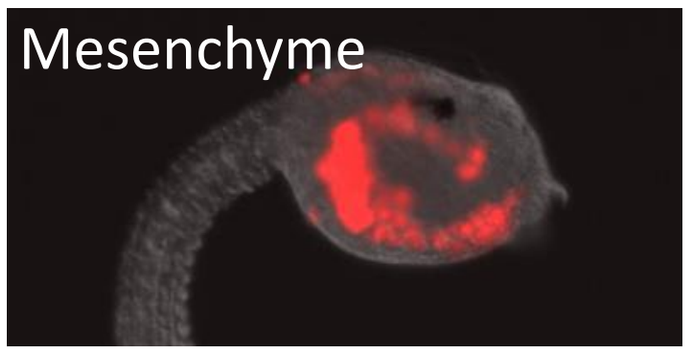
# Tissue-specific TALEN vector series

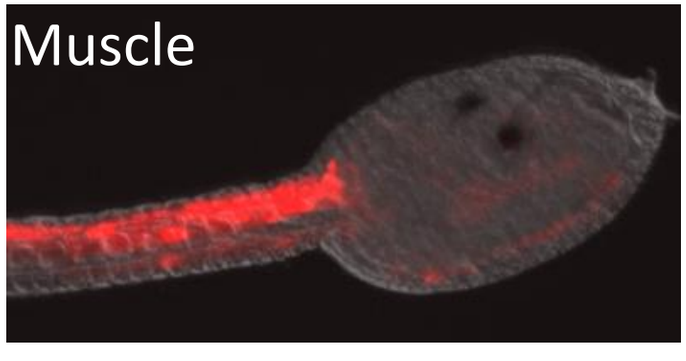


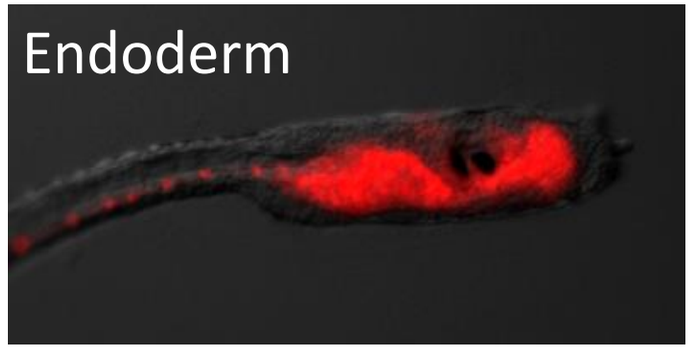

Tissue-specific promoter>TALEN::2A::mCherry

After assembly, electroporation into embryos.



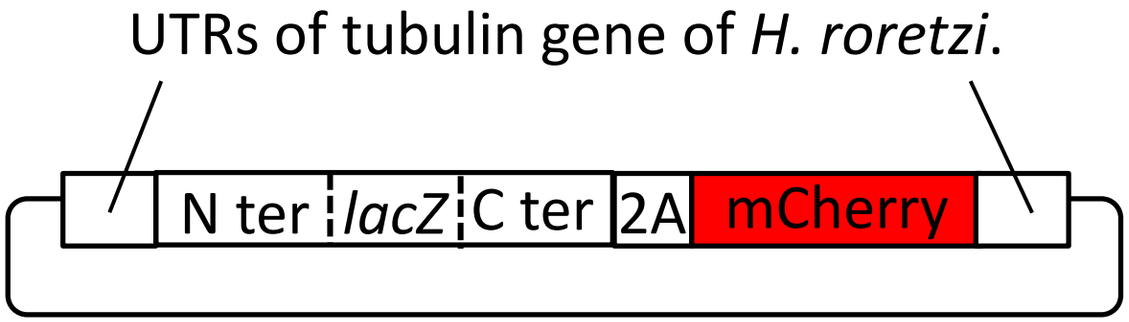

・Tissue-specific disruption of genes gives us important information for elucidating gene functions. TALENs can be used for this purpose easily, because they are gene-encoded proteins.

・Insert your repeats into a TALEN backbone vector with a tissue-specific promoter.

・Expression of TALENs could be monitored with the fluorescence of mCherry.

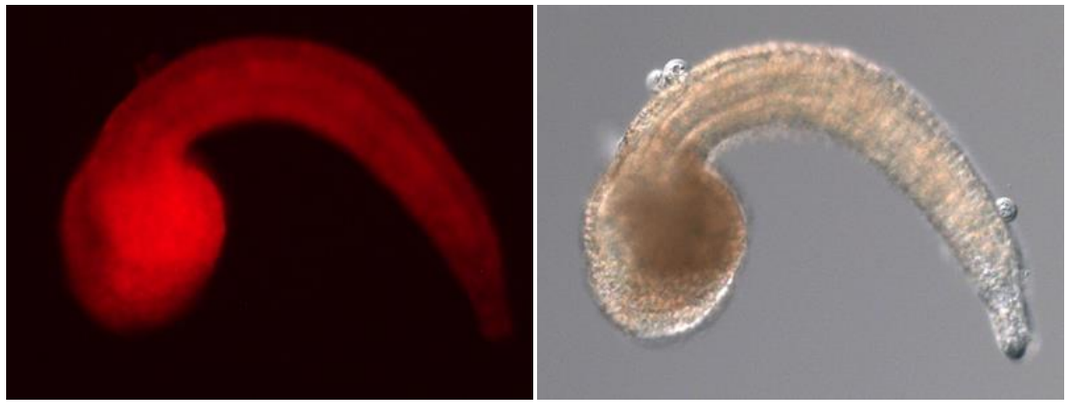
・We already created vectors for epidermis, muscle, neural tissue, endoderm, mesenchyme. These vectors could be shared among researchers.

# TALEN mRNA for less mosaic knockouts and germline mutations

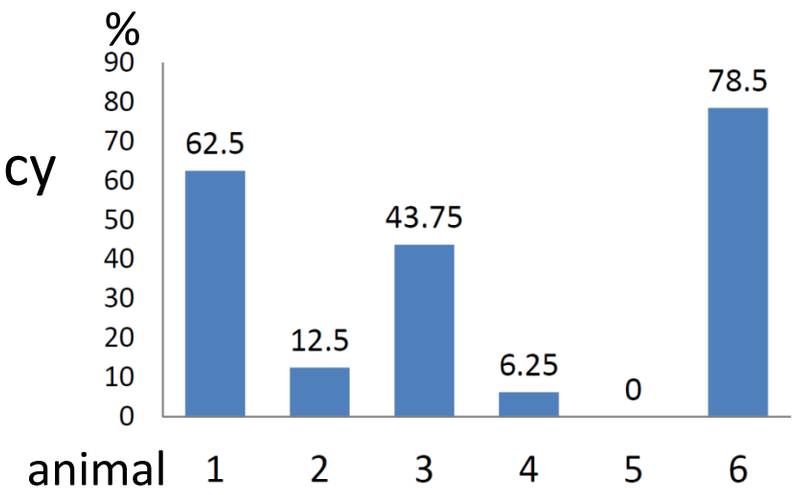



pHTB TALEN::2A::mCherry

mRNA synthesis and microinjection



Mutation frequency in sperm genome




・Microinjected mRNA disperses rapidly in eggs. Therefore, TALEN mRNA likely to be uptaken by most cells of an embryo.

・Insert your repeats into the backbone vector of TALEN with UTRs for increasing translation efficiency.

・*In vitro* transcription of mRNA is necessary after construction.

・This method enables to introduce mutations in germ cell genome as well as somatic cell genome.