

## **TALEN expression vectors**

**Yasunori Sasakura 7th May 2018**

**Modified on 26th January 2023**

(Caution: These days our group uses TALENs much more frequently than CRISPR/Cas9. We provide the protocols only 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=Cj0KCQjw4eXPBRCTARIsADvOjY2SQXgDuHaRgHLpuLY9KyyKmlrT2J5oaUniSiFJ33H75cE7qE2re6EaAmfPEALw\\_wcB](https://www.addgene.org/kits/yamamoto-platinumgate/?gclid=Cj0KCQjw4eXPBRCTARIsADvOjY2SQXgDuHaRgHLpuLY9KyyKmlrT2J5oaUniSiFJ33H75cE7qE2re6EaAmfPEALw_wcB)

### **2) Design of TALENs**

After you select a gene to knock out, determine the region of the gene in which to introduce mutations. We usually choose the region corresponding to the core domain critical to the protein function, such as a DNA-binding domain for transcription factors or a kinase domain for kinases. A transmembrane domain can also be a good target for transmembrane proteins. If such a domain is not present or is obscure in your protein encoded by the target gene, then we recommend targeting around the start codon of the gene. Note 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; a long DNA region will yield too many TALEN candidates) onto TALEN targeter 2.0 (<https://tale-nt.cac.cornell.edu/node/add/talen>). We use these parameters:

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 identify several pairs of TALENs that may be good for knocking out the gene. Paste the results in an Excel spreadsheet, then select one or two pairs for construction. For a historical reason, we prefer to choose the hetero-lengthened pairs in which one side has 16 repeats and the other side 16 or 17.

A TALEN pair selected in this manner has ~70% probability of being a good TALEN pair. Therefore, we usually create two pairs for a single target to assure the success of knockouts.

### 3) First assembly

The selected TALENs will appear 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 ATGGGTGCGAGAACT acaggattgctcgatatgt AAAAGTCCATGAACCC A
```

(The DNA sequence of the target site of the above TALENs. Lowercase letters are spacers. The first and last nucleotides are zero repeat/upstream bases that are not included in the TALEN array.)

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 (this example is a b3)
NG           =last repeat
```

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

#### **4) Second assembly**

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

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

The protocol of the second assembly is as follows:

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

Make a 1:1:1:1 mixture of the first clones.

Make the following mixture:

First clone mixture	1.0 ul
50 ng/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 37°C, 10 min at 16 °C (use a thermal cycler).  
Increasing the cycle number will yield more colonies.

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

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

30-60 min at 37 °C  
5 min at 80 °C to inactivate Esp3I

This reaction will reduce the number of background colonies.

Transform *E. coli* and plate on an LB-amp plate.

We use handmade *E. coli* (titer is 10<sup>7</sup>-10<sup>8</sup> colony forming units/ug DNA). Usually, 1 ul of reaction mixture yields a sufficient number of colonies.

### 5) Insert check

The insert check of the second assembled clones should be done with the following primers and PCR conditions.

TALE-F: GCACCCCTCAACCTGACCCCAG

TALE-R: CTCGAAAGCTGGGCCACGATTG

PCR conditions:

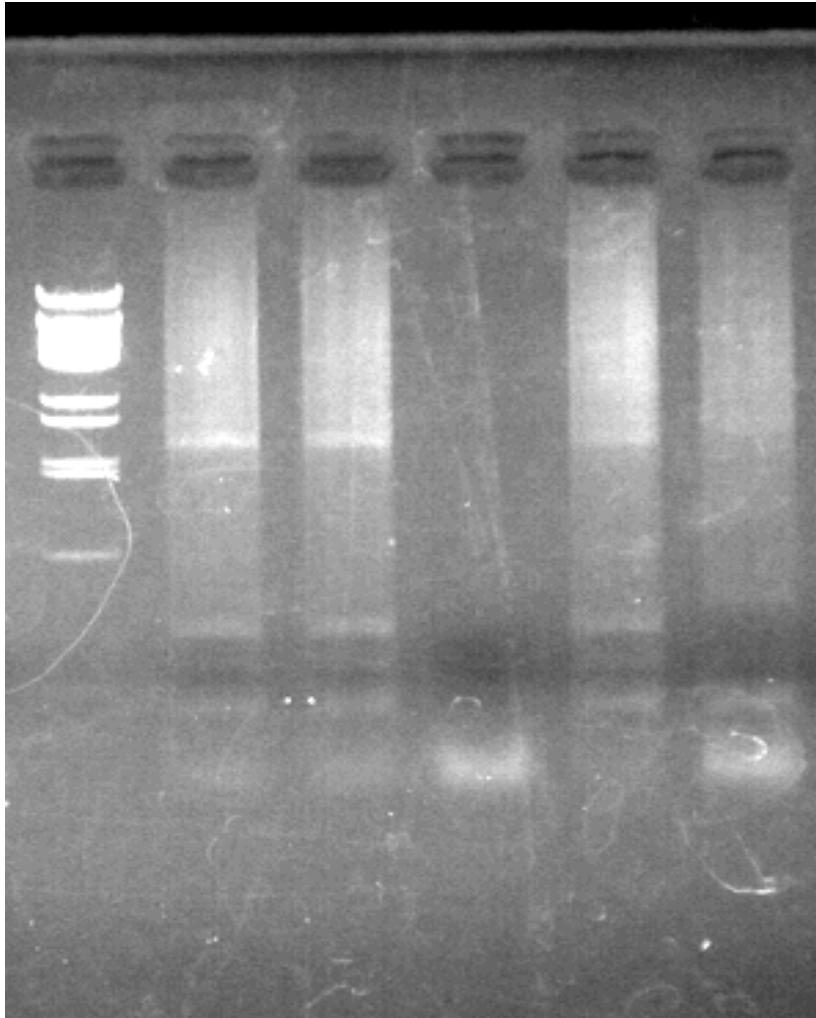
5 min at 95 °C

30 cycles of 94-30s, 65-30s, 72-50s

5-10 min at 72 °C

4-10 °C

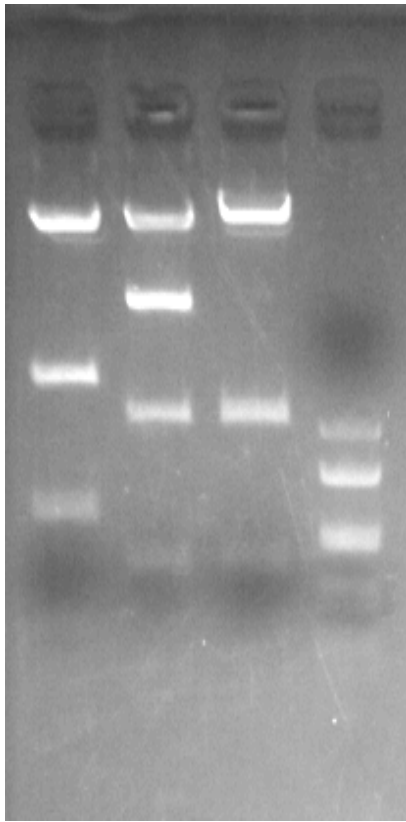
Successfully assembled colonies will show a smear band (see the photo below). Pick up the clones and culture them in 2 ml LB-amp liquid.



Example of an insert check (from the left): first lane, marker; 2-6 lanes, TALEN clones. Lanes 2, 3, 5, and 6 suggest the success of the assembly. The fourth lane will be the wrong clone; do not pick the colony.

Miniprep using your laboratory method.

Check the plasmids with restriction enzyme NruI. The NN (G) repeats have one NruI site, and you can confirm the success of the assembly based on the positions of the NN modules. The photo below is an example of three different TALENs digested by NruI. The second assembly does not usually fail.



First lane: 700+300

Second lane: Promoter band+600+200

Third lane: 600+200 (in base pairs)

Fourth lane: Marker (pBluescript digested with HaeIII)

For each lane, the top band is the backbone vector. Please ignore it.

## 6) Checking activity

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

Elute miniprepmed DNA in 50 ul elution solution.

4 ul of plasmid solution will be used for the NruI check.

Use the remaining 10 ul of plasmid solution for electroporation.

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

Collect embryos that exhibit bright mCherry fluorescence. The shape of the embryos may be bad, but it does not influence on the estimation of mutation rate.

Spin down the embryos and discard the seawater.

Isolate genomic DNA with the Wizard genomic DNA purification kit (Promega).

Add 600 ul nuclei lysis solution. Incubate for 1-2 hours at 50 °C, preferably with rotation.

Cool to room temperature.

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

Centrifuge at 13,500 rpm for 4 min at 4 °C.

Collect the supernatant into a new tube.

Centrifuge for 13,500 rpm for 4 min at 4 °C.

Collect supernatant into a new tube.

Add 1 ul glycogen (Roche).

Add 600 ul isopropanol.

Centrifuge for 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.

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
10 pmol/ul primer	2.0 ul
10 pmol/ul primer	2.0 ul
ExTAQ hot-start version	0.2 ul
<u>D3W</u>	<u>11.2 ul</u>
	20.0 ul

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

Electrophoresis 5 ul of PCR mixture on 15% polyacrylamide gel (AA:Bis = 29:1). The voltage should be a constant 100 V. 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 knockout database

(<http://marinebio.nbrp.jp/ciona/sequenceFile/knockOut/gsTalens/Raldh2/Raldh2-HMA.pdf>).

When the mutation bands appear, subclone the bands into a conventional vector for sequencing analysis. A 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 to transfer them: expression vectors for tissue-specific expression and mRNA synthesis vectors.



The easiest way is to repeat the second assemblies on the appropriate vectors. Because the Golden Gate method is reproducible and highly efficient, the assembly is usually successful. However, there is a possibility that new TALENs are incorrectly assembled in which case that the incorrectness cannot be checked completely due to the repeat structure. For this reason, we recommend the following methods.

### 7-1) Repeat switching method

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

Miniprep solution	5 ul
10x T buffer (Takara)	3 ul
SalI	1 ul
BamHI	1 ul
Water	to 20 ul

Inverse PCR acceptor vectors with the following primers:

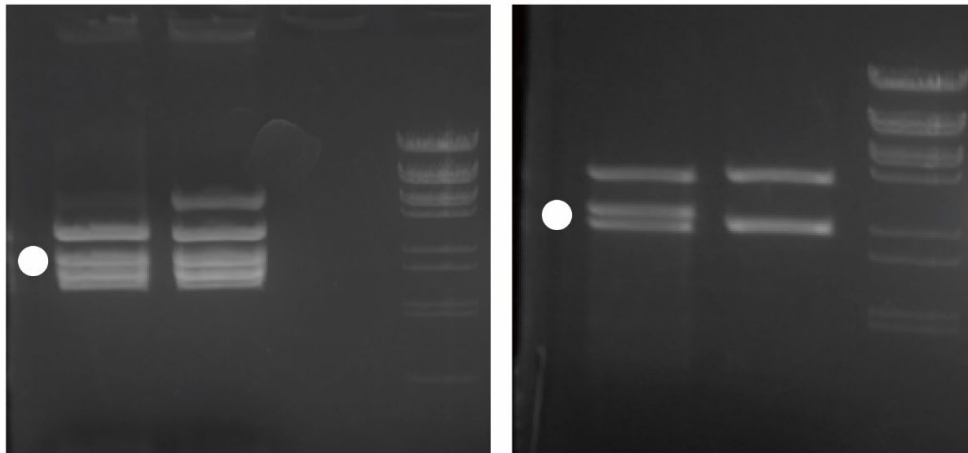
```
ACCAACAGAAGGATCCCCGAGAGGACATCACAT
CTGCGCGACTGTCGACCTCACTTTGGGCTTGATC
```

(Use a high-fidelity DNA polymerase. We use PrimeStar HS DNA polymerase from TAKARA Bio. 30 cycles is sufficient).

Add 1 ul of DpnI restriction enzyme (Takara) to the PCR solution after PCR is finished. This enzyme digests the template vector that are methylated in *E. coli*.

Extract the gel from the restriction-digested plasmids and PCR products. The restriction fragments of TALEN repeats (approx. 2,000 bp) do not separate well from other DNA bands (see the photos below). Don't worry about this, because the In-fusion reaction is highly 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, mix the DNAs for the 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 that of other vectors, and a few nucleotides are sometimes lost at the joining site. We check the success of the In-fusion reaction by sequencing with the following primers:

AATGGACTATAAGGACCAC (5' junction site)

TTTTCGTGATCCACCGAGATGC (3' junction site)

## 7-2) Promoter switching method

The promoter region of the TALEN expression vector can be removed with NotI. A new promoter can be inserted at the NotI site to switch the 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 wish to isolate a promoter for TALEN expression from the 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

The above shows two forward primers. When the promoter is inserted into the BamHI/BamHI site of the pSP vector, use pSP-TALEN-promoter-switch-BamHF. When the promoter is inserted in the XXXX/BamHI site (where XXXX is an arbitrary restriction site that is located 5' upstream from the BamHI site), use pSP-TALEN-promoter-switch-XhoF.

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

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

# First Assembly of TALEN repeats

Day 1

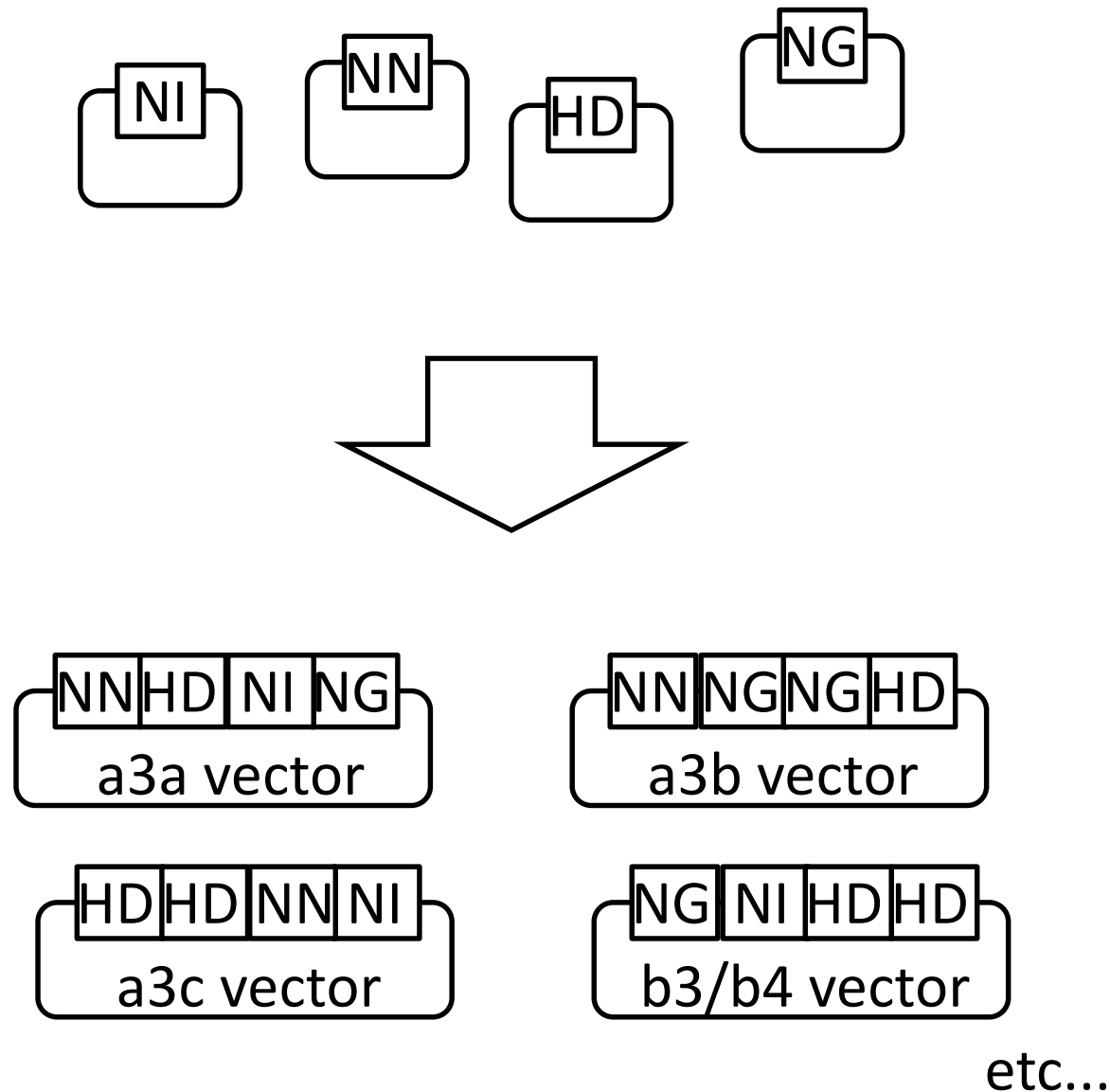
- Mix repeat units.
- Golden Gate reaction.
- Post digestion of unassembled plasmids.
- Transformation overnight.

Day 2

- Insert check.
- Incubate appropriate clones.

Day 3

- Mini-prep.
- (Optional) Sequencing to confirm the assembly.

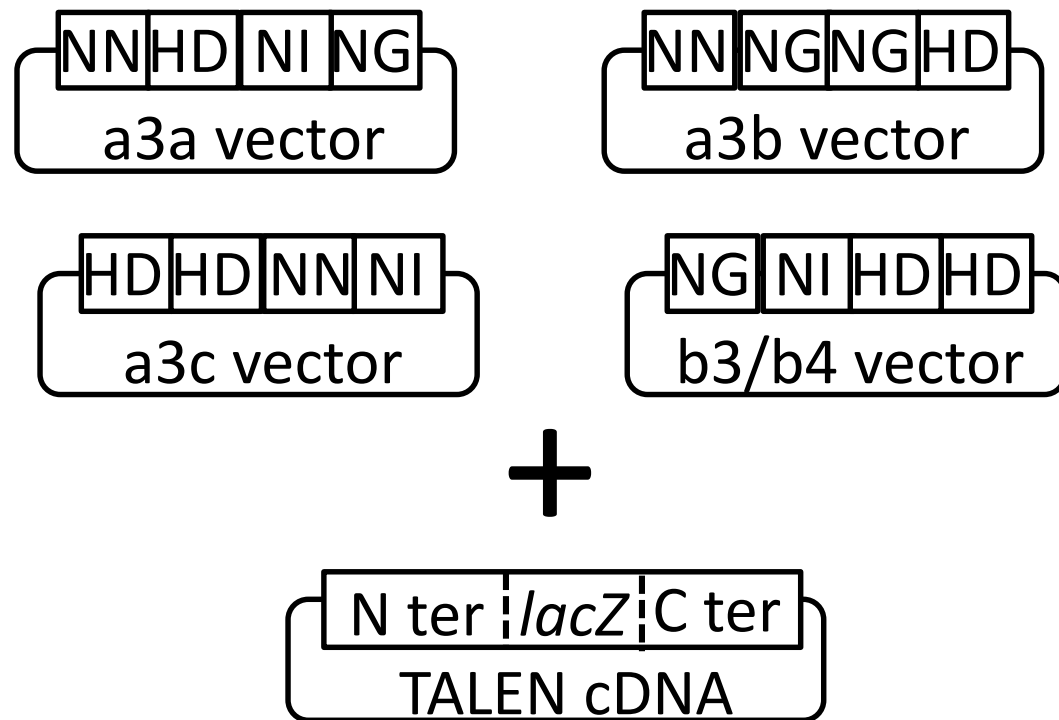


There are approx. 1,000 combinations of the first assembled clones for creating TALENs with 16~17 repeats.

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

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

# Second Assembly of TALEN repeats



## Day 1

- Mix the first assembled clones.
- Golden Gate reaction.
- Post digestion of unassembled plasmids.
- Transformation.

## Day 2

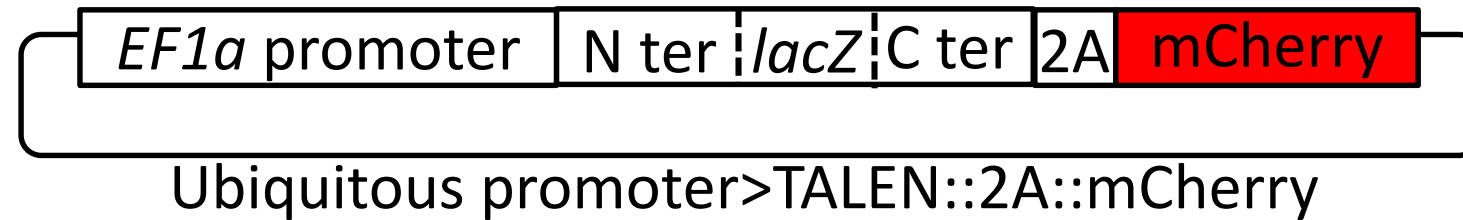
- Insert check.
- Incubate appropriate clones.

## Day 3

- Mini-prep.
- Perform restriction digestion to confirm the assembly.
- (optional) sequencing of 5' and 3' ends of assembly.

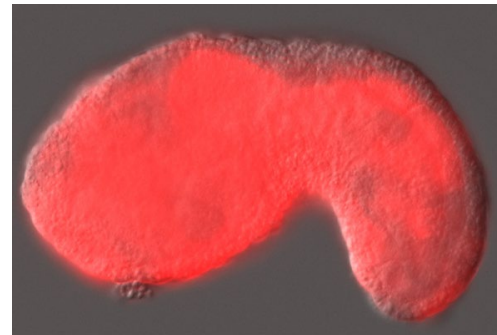
\*Once the repeat is assembled, you can replace it in any TALEN backbone vector with restriction enzymes.

# Checking mutation efficiency of designed TALEN

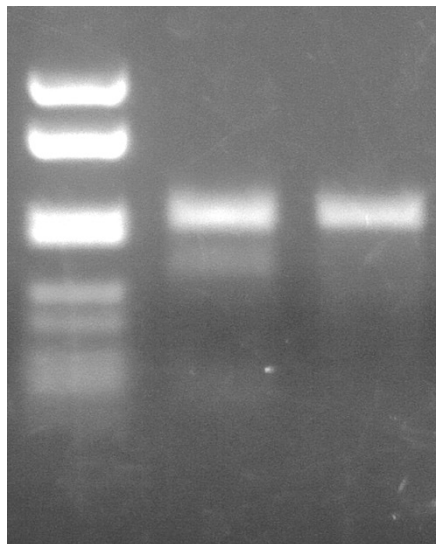


After assembly, electroporate into embryos.

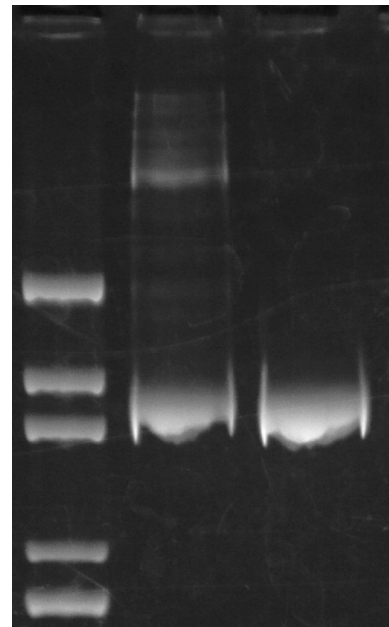
We can see the expression of TALENs by red fluorescence.



Cell assay



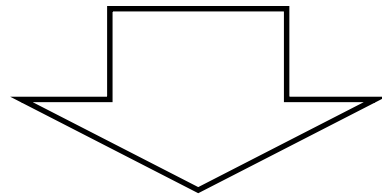
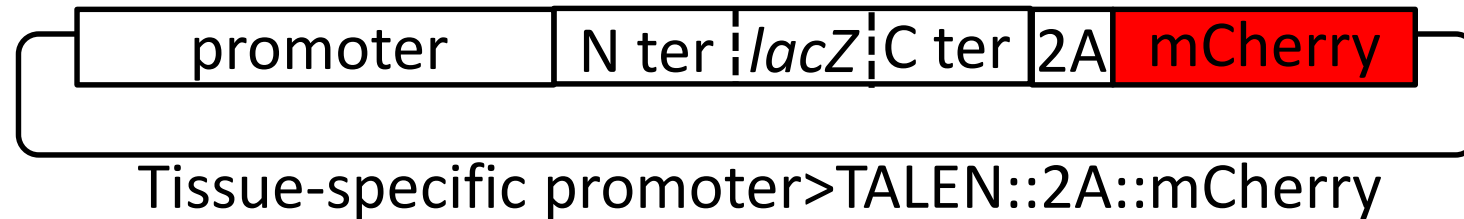
PAGE



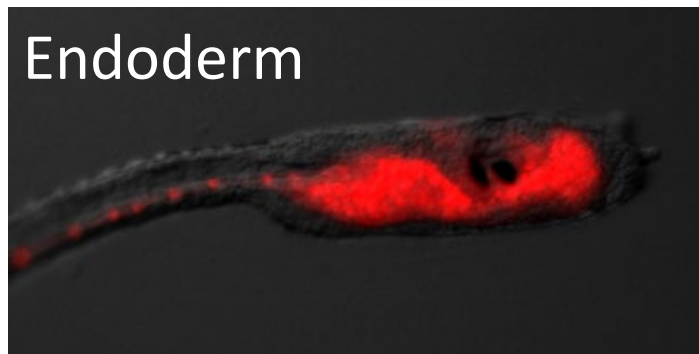
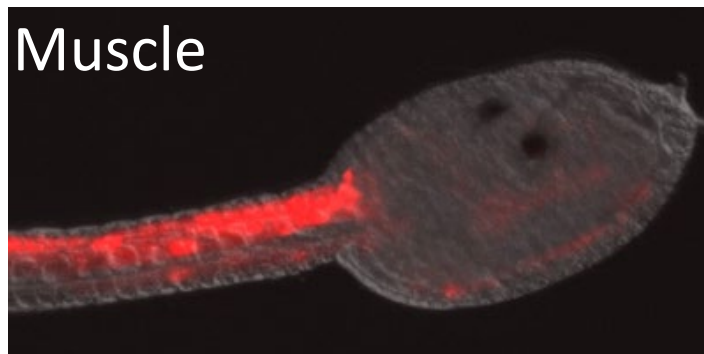
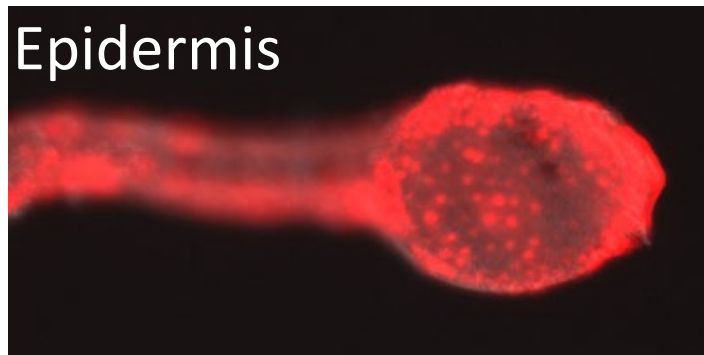
Checking the occurrence of mutations by electrophoresis

- 2nd assembly of your designed TALEN into the backbone vector of TALEN with the *EF1α* promoter.
- Miniprep and electroporation of the vectors.
- Expression of TALENs can 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



After assembly, electroporation into embryos.



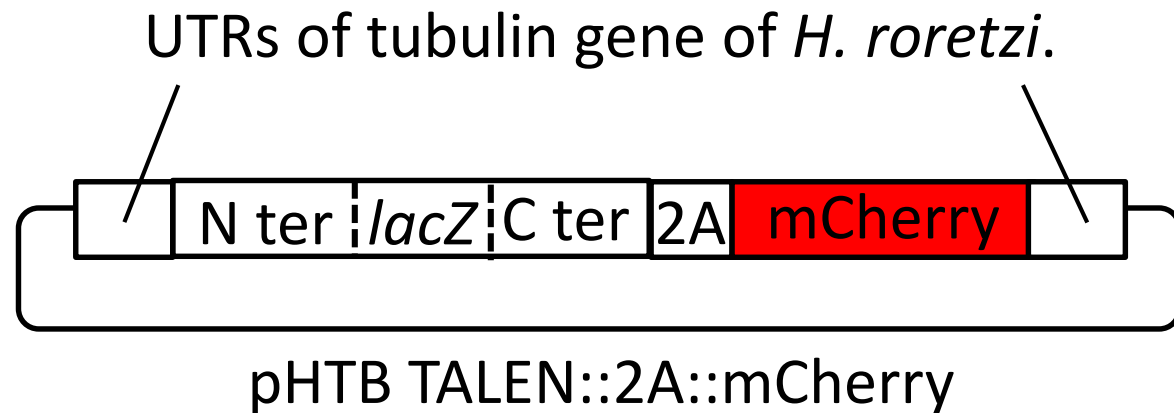
- Tissue-specific disruption of genes gives us important information for elucidating gene functions. TALENs can be used for this purpose easily, because they are gene-encoded proteins.

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

- Expression of TALENs can be monitored with the fluorescence of mCherry.

- We have already created vectors for epidermis, muscle, neural tissue, endoderm, and mesenchyme. These vectors are available from the NBRP project

# TALEN mRNA for knockout with reduced mosaicism and germline mutations



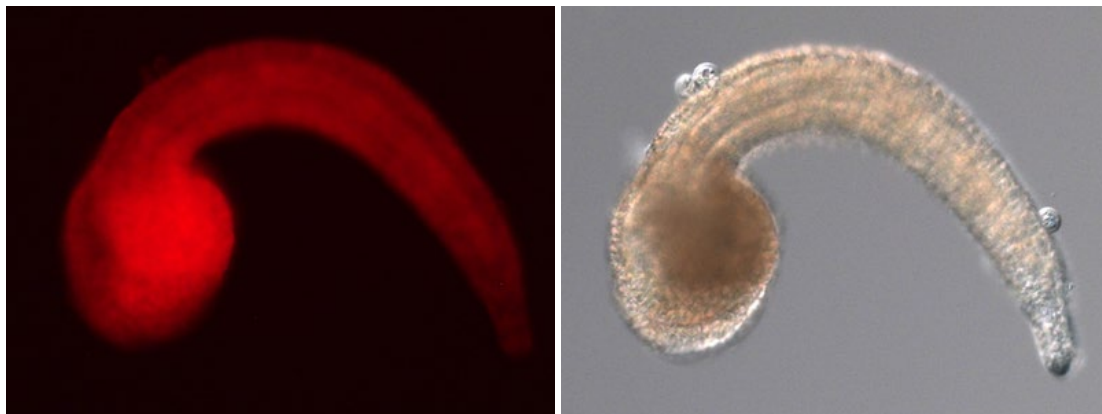
- Microinjected mRNA disperses rapidly in eggs. Therefore, TALEN mRNA is likely to be uptaken by most cells of an embryo.

- Insert your repeats into the backbone vector of TALEN with UTRs to increase translation efficiency.

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

- This method enables you to introduce mutations in a germ cell genome as well as a somatic cell genome.

mRNA synthesis and microinjection



Mutation frequency  
in sperm genome

