Yamamoto lab TALEN construction protocol

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This is an efficient TALEN construction protocol described in Sakuma *et al.*, Genes to Cells, 2013 (http://dx.doi.org/10.1111/gtc.12037). Since this method is based on the "Golden Gate TALEN and TAL Effector Kit" (Addgene), see Cermak paper (http://dx.doi.org/10.1093/nar/gkr218) and Voytas lab protocol (http://www.addgene.org/TALeffector/goldengateV2/) before reading this.

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■Materials

○Plasmids

Golden Gate TALEN and TAL Effector Kit
(http://www.addgene.org/TALeffector/goldengateV2/)

Module plasmids: **pHD1-6, pNG1-6, pNI1-6, pNN1-6***

*You can also use pNK1-6 or pNH1-6 instead of pNN1-6.

Array plasmids: **pFUS_B1-6**

Last repeat plasmids: **pLR-HD, NG, NI, NN**

Takashi Yamamoto lab Golden Gate Supplemental Kit
(http://www.addgene.org/TALeffector/goldengate/add-ons/)

Array plasmids: **pFUS_A1A, A2A, A2B, A3A, A3B, A4A, A4B**

Destination vector plasmids: **pcDNA-TAL-NC2, pCAGGS-TAL-NC2***

*pcDNA-TAL-NC2 is almost the same as pcDNA-TAL-NC (Sakuma *et al.*, 2013) except codon-optimized FokI and existence of Flag tag.
pcDNA-TAL-NC2 has CMV/T7 promoters, whereas pCAGGS- has a CAG promoter.

○Reagents (excluding standard PCR enzymes and cell culture reagents)

Maker	Product name	Cat. No.	unit
NEB	Quick Ligation Kit	M2200S	30 reactions
		M2200L	150 reactions
NEB	BsaI-HF	R3535S	1000 units
		R3535L	5000 units
Fermentas	Esp3I	ER0452	1000 units
NEB	BspEI	R0540S	1000 units
Life Tech	ChargeSwitch-Pro Plasmid Mini Kit	CS30250	250 reactions
Promega	Wizard SV Gel and PCR Clean-up System	A9282	250 reactions

○Primers

pCR8_F1, pCR8_R1, TAL_F1, TAL_R2 (described in the original protocol)

■Methods

1. TALEN design

Design TALEN target sequence using “TALEN Targeter”.

TALEN Targeter

https://tale-nt.cac.cornell.edu/

Choose “TALEN Targeter (old version with design guidelines)”.

According to our past results, it is preferable that spacer length is around 15 bp (12-16 bp) and number of repeat array is 16-20 when using pcDNA-TAL-NC2 or pCAGGS-TAL-NC2 as a destination vector. “Require a T at position N” checkbox is not a must.

2. 6-module assembly -STEP 1-

Assemble RVD repeats for the chosen target sequences. Using our methods, you can make TALENs bearing 8-31 repeats. It is necessary that we choose appropriate combinations of vectors for the desired module numbers. The correspondence table of module number and vector composition is indicated below.

	pFUS_A1A	pFUS_B1-B6	pLR-NG**
8-13 modules :	6 +	(1-6) +	1

	pFUS_A2A	_A2B		
14-19 modules :	6 +	6 +	(1-6) +	1

	pFUS_A3A	_A3B	_A3C*		
20-25 modules :	6 +	6 +	6 +	(1-6) +	1

	pFUS_A4A	_A4B	_A4C*	_A4D*		
26-31 modules :	6 +	6 +	6 +	6 +	(1-6) +	1

* A3C, A4C and A4D are just expediential names. **In fact: A3C=A2B, A4C=A3B, A4D=A2B**

** Select any one of pLR-HD, NG, NI and NN if you uncheck the checkbox “Require a T at position N”

As an example, here I describe the procedures for constructing a TALEN pair targeting a human gene, *HPRT1* (*HPRT1*_B TALEN). The target site is indicated below. Numbers of modules are 20 (left) and 17 (right). Spacer length is 15 bp.

Left TALEN

5’ -CCATTCCTATGACTGTAGAT TTTATCAGACTGAAG AGCTATTGTGTGAGTAT-3’

3’ -GGTAAGGATACTGACATCTA AAATAGTCTGACTTC TCGATAACACACTCATA-5’

Right TALEN

In this case, combinations of vectors and inserts are as the following table. Note that you don’t have to take the last half repeat into account here, because it will be added in the STEP 2.

List of vector/insert combinations when constructing *HPRT1*_B TALEN（STEP 1）

Left	vector	pFUS_A3A	pFUS_A3B	pFUS_A2B (A3C)	pFUS_B1
	insert	HD HD NI NG NG HD	HD NG NI NG NN NI	HD NG NN NG NI NN	NI
Right	vector	pFUS_A2A	pFUS_A2B		pFUS_B4
	insert	NI NG NI HD NG HD	NI HD NI HD NI NI		NG NI NN HD

You can adopt two ways of module assembly. One way is the Golden Gate method described in the original protocol. Although this method requires no preliminary preparation, there are several problems; long reaction time is needed, and success rate of Golden Gate reaction is easily influenced by reduction of activities of ligase and restriction endonucleases. Due to these difficulties, simple ligation method is adopted in our lab. It requires BsaI-digested inserts and vectors prepared in advance, but the protocol is much simpler and the reaction is more robust than the Golden Gate method. In this document, I will introduce the ligation method for STEP1.

2-1. Preparation of BsaI-digested pFUS vectors and module fragments

Prepare BsaI-digested vectors and modules for STEP1 with the following reaction condition. This treatment does not have to be done each time. You only have to prepare once for all 13 vectors and all 24 modules, then you can use them repeatedly if they are stored at -20℃.

Plasmid	5-10 μg	
BsaI-HF	0.5-1 μl	
NEBuffer 4	2 μl	
SDW (Sterile Distilled Water)	up to 20 μl	37℃, O/N

pFUS vectors should be dephosphorylated; in our lab, alkaline phosphatase is directly added in the digested solution after the overnight digestion, and additional 1 hr incubation is performed.

Separate digested DNA fragments by agarose gel electrophoresis, excise appropriate bands, and collect them in microtubes.

Extract DNAs using Wizard SV Gel and PCR Clean-up System (Promega). Elution should be performed with 30-40 μl of the nuclease-free water containing in the kit.

2-2. 6-module assembly using ligation method

Mix the prepared DNA solutions and ligate them as follows.

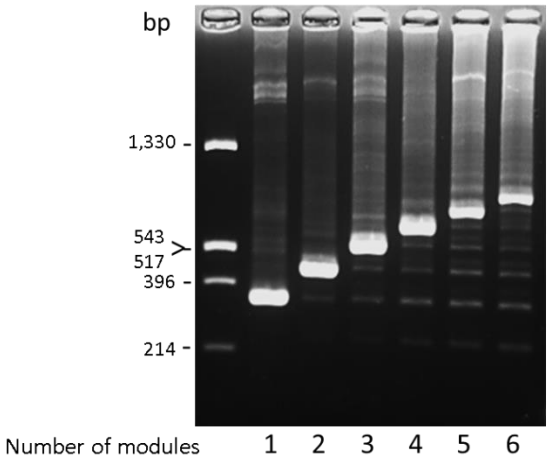
Vector	0.6 μl	
Module	0.6 μl × 1-6	
Quick ligase	0.3 μl	
10X T4 DNA ligase buffer *	0.5 μl	
SDW	up to 5 μl	16℃, 30 min.

* supplied with T4 DNA ligese (NEB)

You can scale down the above reaction if you want to (see Appendix). Transform 0.5-1 µl of the reaction product directly to chemical competent *E. Coli* such as XL1-Blue and streak the transformant on Spectinomycin/X-gal/IPTG LB plate. Culture O/N at 37°C. Plasmid-Safe nuclease treatment is not needed.

2-3. Colony PCR of STEP1 clones

Select the desired clones using colony PCR (see Appendix for detailed reaction conditions). The Following is an example of colony PCR results.



As indicated in the panel, you can see the strong band at the target length and the faint ladder bands at intervals of approximately 100 bp.
Culture the desired clones with 100 ng/µl Spectinomycin/LB media.

3. 6-module assembly -STEP 2-

Perform 2nd step assembly using the 6-module ligated plasmids constructed in the section 2-3. Although you can adopt two assembly ways similar to STEP1 (pp4), we recommend the Golden Gate method in this step because constructed plasmids can directly be used for the reaction.

3-1. 6-module assembled plasmid preparation

Purify the plasmids from bacteria-saturated liquid culture using Miniprep kit. Of course you can extract the plasmids from each clone separately, but you also can collect 6-module clones in one tube to minimize costs and reduce labor (culture separately then collect them up together). For example, in the case of *HPRT1*_B TALENs, extract the plasmids from the multiple clones indicated by the same color (see table below) using one mini-prep column.

Left	vector	pFUS_A3A	pFUS_A3B	pFUS_A3C	pFUS_B1
	insert	HD HD NI NG NG HD	HD NG NI NG NN NI	HD NG NN NG NI NN	NI
Right	vector	pFUS_A2A	pFUS_A2B		pFUS_B4
	insert	NI NG NI HD NG HD	NI HD NI HD NI NI		NG NI NN HD

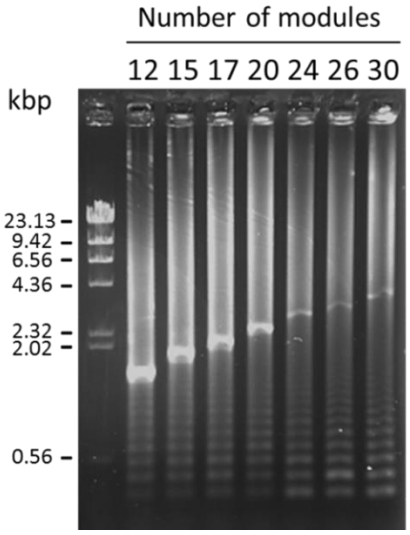
pFUS_B1-6 clones can be collected together with pFUS_A clones, but individual extraction is suggested for them because they are useful for many TALEN constructs. Also, it is not suggested that you culture various clones in the same vial, because bacterial yields of each clone can be varied dramatically if you do so.
Column-purified plasmids should be adjusted to 100 ng/μl.

3-2. Golden Gate cloning into pcDNA/pCAGGS-TAL-NC2 vector

Golden Gate reaction followed by adding more Esp3I should be performed as described in Appendix. This additional digestion dramatically reduces blue colonies.
Transform the reaction product to XL1-Blue and streak the transformant on Ampicillin/X-gal/IPTG LB plate. Culture O/N at 37°C.

3-3. Colony PCR of STEP2 clones

Screen for correctly assembled clones by colony PCR. You can use TAL_F1/TAL_R2 primers both for pcDNA-TAL-NC2 and pCAGGS-TAL-NC2 vectors. The detailed reaction condition is described in Appendix.



Just like STEP1, you can see the strong band at the target length and the faint ladder bands at intervals of approximately 100 bp.
Culture the desired clones and purify the plasmids using Miniprep kit (e.g. ChargeSwitch-Pro Plasmid Miniprep Kit, Life Tech).

3-4. Assembly validation by BspEI digestion (optional)

Usually you can proceed to SSA assay when colony PCR shows correctly-sized amplification. However, if you run the TALEN construction for the first time or you get some unreliable results in any steps, you can validate the final TALEN constructs easily by BspEI digestion described in the original protocol. Because BspEI cuts HD modules only, you can check the HD-assembled pattern for each TALEN plasmids.

Important notes:

Since last repeat and HD1 do not have BspEI site, TALEN plasmids constructed using 6-module assembly method will NOT be cut at the 1st, 7th, 13th, 19th and 25th HD.

In the example below, HD in red letters will be cut and HD in blue letters won't be cut.

Left	vector	pFUS_A3A	pFUS_A3B	pFUS_A3C	pFUS_B1
	insert	HD HD NI NG NG HD	HD NG NI NG NN NI	HD NG NN NG NI NN	NI
Right	vector	pFUS_A2A	pFUS_A2B		pFUS_B4
	insert	NI NG NI HD NG HD	NI HD NI HD NI NI		NG NI NN HD

-Appendix-
Bench protocol for STEP1 & 2 (Yamamoto Lab)

STEP1 (μl)

■Ligation

Vector	0.18		
Module	0.18 × 1-6		
Quick ligase	0.09		
10X T4 DNA ligase buf.	0.15	premix	16℃, 30min.

■Colony PCR

10X buf. for Hybripol	0.8						
dNTPs	0.64						
10μM primer	0.16+0.16						
Hybripol DNA polymerase (BIOLINE)*	0.04						
$MgCl_2$	0.24	95℃	95℃	55℃	72℃	72℃	4℃
SDW	5.96	30s.	15s.	15s.	15s.	15s.	∞
Total	8			×27			

*Any other standard Taq polymerase can be used.

STEP2

■Golden Gate reaction	A1A	A2A-A2B	A3A-A3C	A4A-A4D			
100ng/μl pFUS_Amix	0.3	0.6	0.9	1.2			
100ng/μl pFUS_B	0.3						
50ng/μl pLR	0.6						
100ng/μl vector	0.15						
10×T4 DNA ligase buf.	0.4						
Esp3I	0.2	premix			37℃	16℃	4℃
Quick ligase	0.2				5min.	10min.	∞
SDW	1.85	1.55	1.25	0.95	×6		
Total	4						

→ Additional digestion: add 10×Tango buf. 0.5 μl, 10mM DTT 0.5 μl, Esp3I 0.2 μl.
37℃, 1hr / 80℃, 5min. / 4℃, ∞

■Colony PCR

10X buf. for Hybripol	0.8						
dNTPs	0.64						
10μM primer	0.16+0.16						
Hybripol DNA polymerase	0.04						
$MgCl_2$	0.24	95℃	95℃	65℃	72℃	72℃	4℃
SDW	5.96	30s.	15s.	15s.	50s.	50s.	∞
Total	8			×27			