Chapter 6

Engineering Customized TALENs Using the Platinum Gate TALEN Kit

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Abstract

Among various strategies for constructing customized transcription activator-like effector nucleases (TALENs), the Golden Gate assembly is the most widely used and most characterized method. The principle of Golden Gate assembly involves cycling reactions of digestion and ligation of multiple plasmids in a single tube, resulting in PCR-, fragmentation-, and purification-free concatemerization of DNA-binding repeats. Here, we describe the protocols for Golden Gate assembly-based TALEN construction using the Platinum Gate TALEN Kit, which allows generation of highly active Platinum TALENs.

Key words Platinum TALEN, Platinum Gate TALEN Kit, Golden Gate assembly, Modular assembly, 4-Module assembly

1 Introduction

Transcription activator-like effector nucleases (TALENs) recognize target DNA sequence via highly repetitive DNA-binding repeats; therefore, the construction of customized TALENs requires a sophisticated method for serial modular assembly [1]. Currently, various systems for constructing TALENs have been reported by many independent groups [2]. Among them, Golden Gate cloning is one of the most accomplished systems for laboratory-based production of TALENs. Golden Gate cloning was first reported in 2008 as a one-tube and one-step seamless cloning method [3]. In this cloning method, one only has to mix the vector plasmid and one or more insert plasmid(s) with DNA ligase and type IIs restriction enzyme in a single microtube, and perform a thermal cycling reaction, followed by additional digestion. The products can then be directly used for bacterial transformation.

Cermak et al. originally reported Golden Gate assembly-based TALEN construction in 2011 [4]. The system comprises two-step Golden Gate cloning for assembling 12–31 modules. All the plasmids for this system have been deposited in Addgene (Cambridge, MA,

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USA) as "Golden Gate TALEN and TAL Effector Kit" (cat#1000000024). We previously modified some parts of the kit and supplied the "Yamamoto Lab TALEN Accessory Pack" (cat#1000000030) through Addgene as a supplemental package of the Golden Gate kit [5]. The accessory pack contains alternative intermediate vectors for more efficient repeat assembly.

Recently, we have found that repeating patterns of non-repeat-variable di-residue (non-RVD) variations potently enhanced TALEN activity [7]. These highly active TALENs were named Platinum TALENs, and the Golden Gate-based system for construction of Platinum TALENs was established (Platinum Gate system). Materials for the Platinum Gate system have also been distributed from Addgene as the "Platinum Gate TALEN Kit" (cat#1000000043). Thus far, Platinum TALENs have been proven to work very well compared to conventional Golden Gate TALENs in various cells and organisms, such as HEK293T cells [6], HCT116 cells [7, 8], nematodes [9], sea urchins [10], ascidians [11], newts [12], frogs [6, 13], mice [14, 15], and rats [6]. In this chapter, we describe the step-by-step protocols for constructing Platinum TALENs.

2 Materials

1. Platinum Gate TALEN Kit (Addgene; *see* **Note 1**).
2. Lysogeny broth (LB) liquid media and LB-agar plate containing 100 μg/ml ampicillin or spectinomycin.
3. Plasmid Miniprep Kit (*see* **Note 2**).
4. Thermal cycler.
5. Quick Ligation Kit (New England Biolabs, Beverly, MA, USA; *see* **Note 3**).
6. T4 DNA Ligase Reaction Buffer (New England Biolabs).
7. BsaI-HF (New England Biolabs; *see* **Note 4**).
8. Esp3I (Thermo Scientific Japan, Tokyo, Japan; *see* **Note 5**).
9. MscI.
10. Dithiothreitol (DTT).
11. 5-Bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal)/isopropyl β-d-1-thiogalactopyranoside (IPTG) solution for blue/white selection.
12. Chemically competent bacterial cells, such as XL1-Blue or XL10-Gold.
13. Standard Taq DNA polymerase.

14. Primers for colony PCR (*see* **Note 6**).
    pCR8_F1: 5′-TTGATGCCTGGCAGTTCCCT-3′
    pCR8_R1: 5′-CGAACCGAACAGGCTTATGT-3′
    TALE-Fv2: 5′-GAGCACCCCTCAACCTGACCCC-3′
    TALE-R: 5′-CTCGAAAGCTGGGCCACGATTG-3′

3 Methods

3.1 Setting Up the TALEN Construction System

1. Culture all 35 clones contained in the Platinum Gate TALEN Kit in liquid LB media with 100 μg/ml ampicillin or spectinomycin overnight at 37 °C with shaking.
2. Extract the plasmids using Plasmid Miniprep Kit.
3. Measure the DNA concentration and adjust it to 50 ng/μl for module plasmids and final destination vectors, and 25 ng/μl for intermediate array vectors.

3.2 Design of TALENs

1. Access "TALEN Targeter" [16] at Cornell University's website (https://tale-nt.cac.cornell.edu/; *see* **Note 7**).
2. Paste genome sequence in the FASTA format into the sequence box (*see* **Note 8**).
3. Select the "Provide Custom Spacer/RVD Lengths" tab and set an appropriate spacer length and repeat array (*see* **Note 9**).
4. Set "G Substitute," "Filter Options," and "Streubel et al. guidelines" as "NN," "Show all TALEN pairs (include redundant TALENs)," and "On," respectively (*see* **Note 10**).
5. Press "submit" to obtain the result (*see* **Note 11**).

3.3 First-Step Golden Gate Assembly

Assemble corresponding DNA-binding modules in the intermediate array vectors. Using the Platinum Gate TALEN Kit, TALENs with 6–21 modules can be constructed. Note that the correct combination of array vectors used in the first assembly step must be selected in accordance with the number of modules, as shown in Fig. 1. As examples, the target sequence and correspondence table for constructing *eGFP* TALEN, described previously [6, 14], are shown in Fig. 2a, b, respectively.

1. Prepare the following reaction mixture in a PCR tube. Solutions in the black box should be prepared as a premix, and 0.4 μl added to each sample (*see* **Note 12**).

First-step Golden Gate assembly

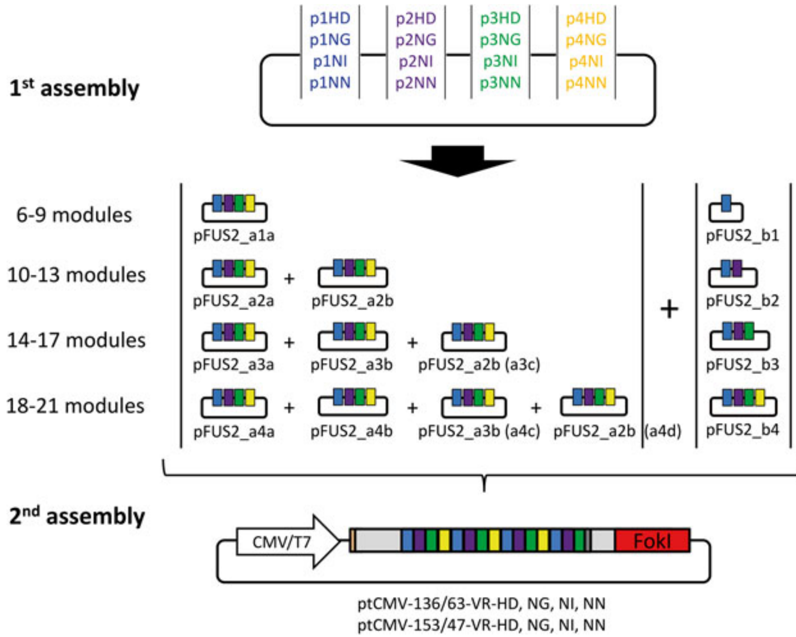

Fig. 1 Schematic overview of the Platinum Gate TALEN Kit. In the first assembly step, four or fewer modules are ligated into intermediate array vectors. In the second step, constructed arrays are joined into a mammalian expression/in vitro transcription vector. *CMV* cytomegalovirus promoter

A

Left TALEN

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T CTTCAAGGACGACGGCAACT acaagacccgcgccg AGGTGAAGTTCGAGGGCG A
A GAAGTTCCTGCTGCCGTTGA tgttctgggcgcggc TCCACTTCAAGCTCCCGC T
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Right TALEN

B

Left TALEN	Vector	pFUS2_a4a	pFUS2_a4b	pFUS2_a3b	pFUS2_a2b	pFUS2_b3
	Insert	p1HD p2NG p3NG p4HD (C-T-T-C)	p1NI p2NI p3NN p4NN (A-A-G-G)	p1NI p2HD p3NN p4NI (A-C-G-A)	p1HD p2NN p3NN p4HD (C-G-G-C)	p1NI p2NI p3HD (A-A-C)
Right TALEN	Vector	pFUS2_a4a	pFUS2_a4b	pFUS2_a3b	pFUS2_a2b	pFUS2_b1
	Insert	p1HD p2NN p3HD p4HD (C-G-C-C)	p1HD p2NG p3HD p4NN (C-T-C-G)	p1NI p2NI p3HD p4NG (A-A-C-T)	p1NG p2HD p3NI p4HD (T-C-A-C)	p1HD (C)

Fig. 2 Examples of TALEN target sequence (**a**) and corresponding table of vectors and inserts for the first assembly (**b**). Target sequences of left and right TALENs are *underlined*. Note that the innermost bases should be ignored in this step, because the last DNA-binding repeat is included in the destination vector for the second assembly step. *Letters in brackets* indicate recognition sequences

Number of modules	1	2	3	4
Intermediate array vector (25 ng/μl)	0.3			
Module plasmid(s) (50 ng/μl)	0.3 × 1	0.3 × 2	0.3 × 3	0.3 × 4
T4 DNA Ligase Reaction Buffer	0.2			
BsaI-HF	0.1	Premix		
Quick Ligase	0.1			
Water	1	0.7	0.4	(0.1)[a]
Total	2 (μl)			

[a]Consider this as zero

2. Place the tube in a thermal cycler and perform the following program: 37 °C for 5 min and 16 °C for 10 min for three cycles (*see* **Note 13**).
3. Remove the tube from the thermal cycler and add the following premixed solutions to each tube: 0.25 μl of NEBuffer 4, 0.25 μl of 10× BSA, and 0.1 μl of BsaI-HF (*see* **Note 14**).
4. Return the tube to the thermal cycler and perform the following program: 50 °C for 30 min, followed by 80 °C for 5 min.
5. Remove the tube and transform the product directly into competent bacterial cells.
6. Plate the transformants onto an LB/spectinomycin plate containing X-gal/IPTG.
7. Culture the plate overnight at 37 °C.

3.4 Colony PCR Screening for the First-Step Clones

1. Pick white colonies and perform colony PCR using pCR8_F1 and pCR8_R1 primers (*see* **Note 15**). Examples of band patterns amplified from correctly assembled clones are shown in Fig. 3a.

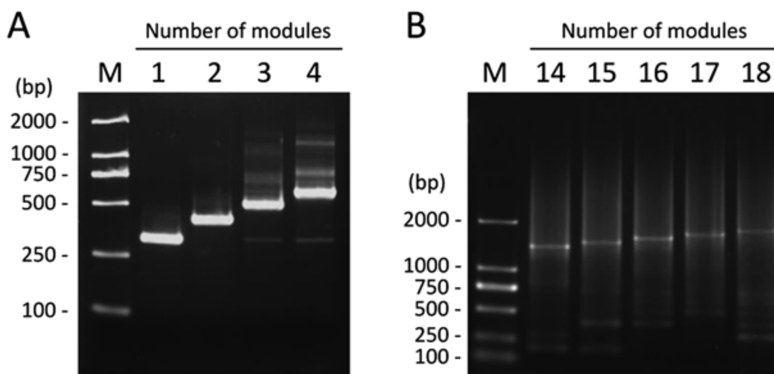

Fig. 3 Gel images of PCR screening in the first step (**a**) and the second step (**b**). In these experiments, plasmid DNAs were used as templates. Minor bands were caused by repetitive sequences in the DNA-binding repeats. *M* wide-range DNA ladder (100–2000 bp) (Takara Bio, Shiga, Japan)

2. Culture the intended clones in liquid LB media with 100 μg/ml spectinomycin overnight at 37 °C with shaking.
3. Extract the plasmids using Plasmid Miniprep Kit.
4. Measure the DNA concentration and adjust it to 50 ng/μl.

3.5 Second-Step Golden Gate Assembly

Assemble 1- to 4-module-assembled plasmids in the destination vectors. Destination vectors contain the last half repeat; therefore, you should choose the appropriate vector in accord with each target sequence. Although the basic procedures are similar to the first assembly step, enzymes and reaction conditions differ slightly.

1. Prepare the following reaction mixture in a PCR tube. Solutions in the black box should be prepared as a premix, and 0.8 μl added to each sample (*see* **Note 16**).
   Second-step Golden Gate assembly

Number of modules	6–9	10–13	14–17	18–21
pFUS2_a plasmid(s) (50 ng/μl)	0.6×1	0.6×2	0.6×3	0.6×4
pFUS2_b plasmid (50 ng/μl)	0.6			
Destination vector (50 ng/μl)	0.3			
T4 DNA Ligase Reaction Buffer	0.4			
Esp3I	0.2	Premix		
Quick Ligase	0.2			
Water	1.7	1.1	0.5	(−0.1)[a]
Total	4 (μl)			

[a]Consider this as zero

2. Place the tube in the thermal cycler and perform the following program: 37 °C for 5 min and 16 °C for 10 min for six cycles (*see* **Note 17**).
3. Remove the tube from the thermal cycler and add the following premixed solutions to each tube: 0.5 μl of 10× Tango buffer, 0.5 μl of 10 mM DTT, and 0.2 μl of Esp3I (*see* **Note 18**).
4. Return the tube to the thermal cycler and perform the following program: 37 °C for 1 h, followed by 80 °C for 5 min.
5. Remove the tube and transform the product directly to competent bacterial cells.
6. Plate the transformants onto an LB/ampicillin plate containing X-gal/IPTG.
7. Culture the plate overnight at 37 °C.

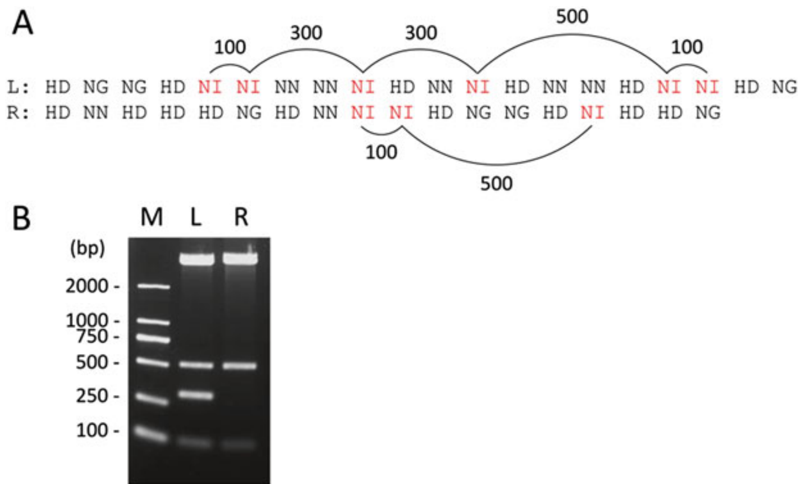

Fig. 4 Schematic illustration (**a**) and gel image (**b**) of MscI digestion. *Red letters* indicate positions of DNA-binding modules harboring NI as the repeat-variable di-residue (RVD), which are cut by MscI. Note that NI modules at the last repeat cannot be cut. Approximate sizes of fragments generated by MscI digestion are illustrated in panel **a**. *L* left TALEN, *R* right TALEN, *M* wide-range DNA ladder (100–2000 bp)

3.6 Colony PCR Screening for the Second-Step Clones

1. Pick white colonies and perform colony PCR using TALE-Fv2 and TALE-R primers (*see* **Note 19**). Examples of band patterns amplified from correctly assembled clones are shown in Fig. 3b.
2. Culture the intended clones in liquid LB media with 100 μg/ml ampicillin overnight at 37 °C with shaking.
3. Extract the plasmids using Plasmid Miniprep Kit.
4. Measure the DNA concentration and adjust it appropriately.

3.7 Checking the TALEN Plasmid by MscI Digestion (Optional)

The Golden Gate cloning enables highly accurate modular assembly; therefore, colony PCR screening is generally sufficient for checking the clones. Nonetheless, restriction digestion may be used as another simple verification method. As shown in Fig. 4a, b, MscI enzyme cuts modules harboring NI as RVD only, and we can easily verify the accuracy of assembly.

4 Notes

1. The Platinum Gate TALEN Kit contains 16 module plasmids (p[1–4]HD, p[1–4]NG, p[1–4]NI, p[1–4]NN), 11 intermediate array vectors (pFUS2_[a1a, a2a, a2b, a3a, a3b, a4a, a4b, b1, b2, b3, b4]), and 8 destination vectors (ptCMV-136/63-VR-[HD, NG, NI, NN], ptCMV-153/47-VR-[HD, NG, NI, NN]) (Fig. 1). Module plasmids and destination vectors

contain an ampicillin resistance gene, whereas intermediate array vectors contain a spectinomycin resistance gene.

2. The success rate of Golden Gate assembly is greatly affected by the quality of the plasmid DNA. We have confirmed that the GenElute™ HP Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) and the ChargeSwitch® Pro Plasmid Miniprep Kit (Life Technologies, Carlsbad, CA, USA) could be used.
3. The Quick Ligation Kit contains Quick Ligase and 2× reaction buffer. In our protocol, only the Quick Ligase is used for the first and second assembly, and the separately sold 10× T4 DNA Ligase Reaction Buffer is used in these assembly steps instead of 2× buffer.
4. New England Biolabs sells conventional BsaI and high-fidelity BsaI-HF. Both enzymes can be used for the first assembly, but we recommend the use of BsaI-HF (#R3535S or #R3535L), because conventional BsaI is likely to become less active during long storage in our experience.
5. Thermo Scientific sells conventional Esp3I and FastDigest Esp3I. FastDigest enzymes are not suitable for Golden Gate cloning; therefore, you should choose the conventional enzyme (#ER0451 or #ER0452).
6. The optimal annealing temperature is 55 °C for pCR8_F1 and pCR8_R1, and 66 °C for TALE-Fv2 and TALE-R. Note that TALE-Fv2 and TALE-R primers are designed to amplify constructed TALEN plasmids and cannot be used for empty destination vectors before assembling DNA-binding repeats.
7. TALEN Targeter is one of the tools on the TAL Effector Nucleotide Targeter (TALE-NT) web resource. Two versions of the TALEN Targeter program, with or without design guidelines, are currently available on TALE-NT. We have previously used the old version with design guidelines; however, because of the high efficiency of Platinum TALENs, we use the newer version of TALEN Targeter without design guidelines currently (https://tale-nt.cac.cornell.edu/node/add/talen).
8. The required length of the genomic region depends on the base sequence and conditions set, such as spacer region and repeat number. Typically, several hundred bases are enough to design multiple TALEN sets.
9. Regarding spacer length, we recommend 15–19 (around 17) for ptCMV-136/63 vectors and 12–16 (around 15) for ptCMV-153/47 vectors. Optimal numbers of repeat arrays are 15–20 (around 17) for both scaffolds.
10. The Platinum Gate TALEN Kit contains only NN modules for guanine recognition. Turn the Streubel et al. guidelines off when no results matching this criterion are found.

11. Other parameters such as “Upstream Base” and “Expires” need not be changed if there is no reason to do so. After submission, you will find the table of search results on the web browser. However, the full information is not displayed on the table; therefore, we strongly recommend the text file labeled as “Result File (Tab-Delimited)” is saved and opened with spreadsheet software such as Microsoft Excel.
12. If the assembly does not work well with these conditions, increase the total reaction volume up to 10 μl.
13. Increase the number of cycles if the efficacy of the assembly is low.
14. This additional digestion with fresh enzyme and buffer dramatically reduces the number of blue colonies.
15. Any standard DNA polymerase can be used. Two colonies for each sample are sufficient to screen the correct clones.
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