Chapter 5

Assembly of Customized TAL Effectors Through Advanced ULtiMATE System

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Abstract

Transcription activator-like effectors (TALEs) have been widely applied in gene targeting. Here we describe an advanced ULtiMATE (*U*SER-based *Liga**t**i*on-*M*ediated *A*ssembly of *T*AL *E*ffector) system that utilizes USER fusion technique and archive of 512 tetramer templates to achieve highly efficient construction of TALEs, which takes only half a day to accomplish the assembly of any given TALE construct. This system is also suitable for large-scale assembly of TALENs and any other TALE-based constructions.

Key words TAL effectors, TALENs, ULtiMATE system, USER, Assembly

1 Introduction

TAL effectors (TALEs), originally identified from bacteria *Xanthomonas*, contain a central DNA-binding region of tandem repeats of 33–35 amino acids, with each repeat specifically targeting a nucleotide through repeat variable diresidues (RVDs) [1, 2]. This modular DNA binding feature has inspired custom designed TALE proteins for use in gene editing [1, 3–5]. It is important to have a robust cloning strategy in order to apply this powerful genetic tool. Here we describe an advanced ULtiMATE (*USER-based Liga**t**i*on-*M*ediated *A*ssembly of *T*AL *E*ffector) system for TALE construction, a method upgraded from our prior report [6] and different from other published protocols [4, 7–10]. ULtiMATE utilizes USER fusion technique [11] to assemble DNA fragments, which are obtained from PCR reactions using special uracil-containing primers and unique polymerases that could incorporate a deoxyadenine opposite a dU, such as PfuTurbo Cx Hotstart DNA polymerase. The USER™ enzyme mixture of glycosidase (UDG) and DNA glycosylase-lyase endo VIII was used to remove the dU residues to generate 3′-protruding sticky ends in PCR products [11].

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We designed four types of basic TALE repeat unit that differ in their DNA sequences flanking the RVD-coding region but encode the same amino acid sequence except for RVD, designated as W-, X-, Y-, and Z-type, respectively. Based on this design, total of 16 TALE repeat units were commercially synthesized in such a way that every type harbors the coding sequence of one of the four RVDs that recognize a particular DNA base (NI→A, HD→C, NN→G and NG→T). With these 16 units, we preassembled all combinations of tetramers in either **WXYZ** or **XYZW** format to make a pool of 512 templates. Time to generate 512 tetramer templates using ULtiMATE system is about 4 weeks.

With tetramer templates, construction of customized TALEs becomes much faster and easier to operate. The protocol consists of PCR reactions, DNA identification through gel electrophoresis (an optional procedure), purification of PCR products, USER fusion, and transformation. It takes only about 6 h for the construction procedure. As the PCR primers are fixed, about two dozens of TALE arrays could be assembled in one 96-well plate simultaneously, and the number of TALE arrays for assembly is only limited by the capacity of PCR reactions.

Golden Gate method could also be used for TALE assembly based on the same 512 tetramer archive. Primers for both the ULtiMATE system and the Golden Gate method are listed.

2 Materials

2.1 PCR Templates

1. Synthesizing 16 TALE monomers (*see* **Note 1**).
2. Constructing 512 TALE tetramers as PCR templates, 256 in **WXYZ** format and 256 in **XYZW** format (*see* **Note 2**).

2.2 PCR Primers

1. Normal and uracil primers for PCR reaction.
2. Primers for colony PCR and sequencing analysis.

2.3 TALE Expressing Vectors

pIRES-EGFP-TALEN (*see* **Notes 2** and **3**) (Fig. 1).

2.4 Enzymes, Chemicals, and Kits

1. PfuTurbo Cx Hotstart DNA Polymerase and buffer (Agilent Technologies).
2. dNTP mix (2.5 mM each).
3. USER™ enzyme (New England Biolabs).
4. PCR product purification kit.
5. BsmBI restriction enzyme (Thermo Fisher Scientific) (*see* **Note 4**).
6. Tango buffer (Thermo Fisher Scientific).
7. T4 DNA ligase.

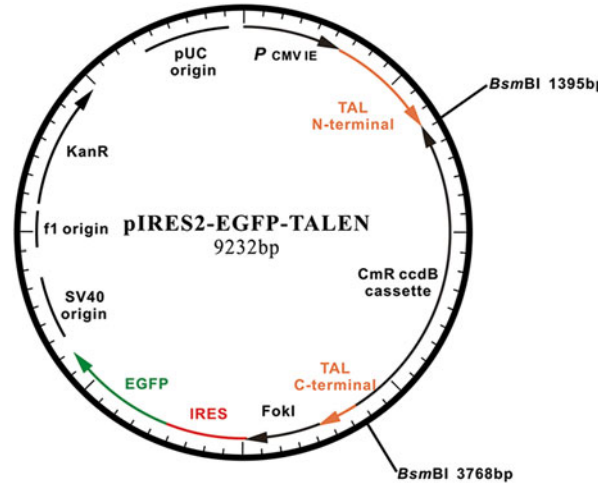

Fig. 1 pIRES2-EGFP-TALEN: Vector for TAL nuclease construction

8. 10 mM ATP.
9. 50 mM DTT.
10. Trans1-T1 competent cells.
11. 2× Easymix Taq enzyme.

3 Methods

3.1 Construction of 512 Tetramer Templates

1. Performing PCR reactions using the 16 TALE monomers (Table 1) as templates and select ULtiMATE primers (Table 2) based on Table 3. For each PCR reaction, mix the following into 0.2 mL PCR tube: TALE monomer (5 ng/μL, 3 μL), forward primer (1 μM, 3 μL), reverse primer (1 μM, 3 μL), PfuTurbo Cx DNA Polymerase (2.5 units/μL, 0.15 μL), PfuTurbo Cx Buffer (10×, 1.5 μL), dNTP mix (1.5 μL), and ddH_2O to a total volume of 15 μL. PCR steps are as follows: 95 °C, 3 min for hot start; thermal cycling (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; 30×); 72 °C, 10 min; and hold at 4 °C.
2. Combine four PCR reactions for one tetramer into one tube. Add 1.5 μL USERTM enzyme (1000 units/mL) into mixed PCR product, incubate at 37 °C for 15 min, and cool on ice.
3. Add 1.5 μL T4 DNA ligase (100 units) and 7 μL T4 DNA ligase buffer into USER-digested PCR products, and incubate at 16 °C for 1 h.
4. Gel-purify (2 % agarose) ligated products, extract the ~400-bp DNA band, and ligate them with a blunt vector with no kanamycin-resistant gene (*see* **Note 2**). Verify clones through Sanger sequencing.

Table 1
Archive of 16 TALE monomers

W *type*: 5′-CTGACACCAGAGCAAGTAGTGGCTATTGCAAGTNNNNNNGGTGGCAAACAAGC GCTGGAGACCGTGCAGAGGCTCCTTCCGGTGCTCTGCCAAGCACACGGT
X *type*: 5′-CTCACTCCGGAACAGGTGGTCGCAATCGCGAGCNNNNNNGGCGGCAAGCAAGC CCTTGAGACAGTCCAAAGACTTTTGCCTGTCCTTTGTCAGGCGCATGGC
Y *type*: 5′-CTTACGCCTGAGCAAGTCGTTGCGATCGCCTCCNNNNNNGGCGGAAAACAGGCT TTGGAAACCGTGCAGCGGTTGCTGCCCGTTTTGTGCCAAGCCCACGGA
Z *type*: 5′-TTGACCCCCGAACAGGTTGTAGCCATAGCTTCTNNNNNNGGAGGTAAGCAGGCA CTGGAAACCGTGCAGCGCCTGCTCCCAGTACTGTGTCAGGCTCATGGG

RVD-coding sequence NNNNNN = AACATC (NI, for A-recognition), AACGGC (NG, for T-recognition), CACGAC (HD, for C-recognition), or AACAAC (NN, for G-recognition)

Table 2
ULtiMATE primers for tetramer construction

Primer	Sequence
F-Yx	AGCAAGTCGUTGCGATCGCCTCC
F-Zy	ACGGATUGACCCCCGAACAGGTTGTAGCC
F-X-new	CTCACTCCGGAACAGGTGGTCG
F-W-new	CTGACACCAGAGCAAGTAGTGGCTATTG
R-Xy	ACGACTTGCUCAGGCGTAAGGCCATGCGCCTGACAAAGGACA
R-Yz	AATCCGUGGGCTTGGCACAAAACGGGC
R-Z-new	CCCATGAGCCTGACACAGTACTGG
R-W-new	ACCGTGTGCTTGGCAGAGC

Table 3
Selection of templates and primers for PCR reaction for TALE tetramer construction

		1st PCR	2nd PCR	3rd PCR	4th PCR
WXYZ	TALE monomer template	W	X	Y	Z
	Forward primer	F-W-new	F-Xw	F-Yx	F-Zy
	Reverse primer	R-Wx	R-Xy	R-Yz	R-Z-new
XYZW	TALE monomer template	X	Y	Z	W
	Forward primer	F-X-new	F-Yx	F-Zy	F-Wz
	Reverse primer	R-Xy	R-Yz	R-Zw	R-W-new

Table 4
ULtiMATE primers

Primer	Sequence
F-W5	tataCGTCTCaGAACCTGACACCAGAGCAAGTAGTGGCTATTG
F-Wz	ACCGUGCAGCGCCUGCUCCCAGTACTGTGTCAGGCTCATGGGCTGACA CCAGAGCAAGTAGTGG
F-Xz	AGGCTCAUGGGCTCACUCCGGAACAG
F-Xw	AACAGGTGGUCGCAAUCGC
F-Ww	AGAGCAAGUAGTGGCTAUTGCAA
F-Wy	AGCAAGUAGTGGCTATTGCAAG
F-Xy	ACTCACUCCGGAACAGGTGGTCGCAATCG
R-Zw	AGCAGGCGCUGCACGGUTTCCAGT
R-Zx	AGUGAGCCCAUGAGCCUGACACAGTA
R-Wx	ATUGCGACCACCUGTUCCGGAGTGAGACCGTGTGCTTGGCAGAG
R-Ww	ATAGCCACUACTTGCTCUGGTGTCAGACCGTGTGCTTGGCAGAGC
R-Yw	ACTTGCUCTGGTGTCAGTCCGTGGGCTTGGCACAAAACGG
R-Yx	AGTGAGUCCGTGGGCTTGGCACAAAACGG
R-W3	tataCGTCTCaTGCTCTCCAGCGCTTGTTTGCCACC
R-X3	tataCGTCTCaTGCTCTCAAGGGCTTGCTTGCCGC
R-Y3	tataCGTCTCaTGCTTTCCAAAGCCTGTTTTCCGCC
R-Z3	tataCGTCTCaTGCTTTCCAGTGCCTGCTTACCTCC

3.2 PCR Amplification for TALE Array Construction

1. Based on target sequence, select templates and primers for the PCR reaction (*see* **Notes 5–8**, Tables 4 and 5).
2. For each PCR reaction, mix the following into 0.2 mL PCR tube: tetramer template (5 ng/μL, 3 μL), forward primer (1 μM, 3 μL), reverse primer (1 μM, 3 μL), PfuTurbo Cx DNA Polymerase (2.5 units/μL, 0.15 μL), PfuTurbo Cx Buffer (10×, 1.5 μL), dNTP mix (1.5 μL), and ddH_2O to a total volume of 15 μL.
3. Perform PCR reaction as follows: 95 °C, 3 min for hot start; thermal cycling (95 °C, 30 s; 60 °C, 30 s; 72 °C, 30 s; 30×); 72 °C, 10 min; and hold at 4 °C.
4. Optional step: DNA electrophoresis with 1 μL PCR product for quality check. The size of PCR product containing N repeats is about $(102 \times N - 42)$ bp.

Table 5
Selection of templates and primers for PCR reaction to assemble TALE arrays containing 7.5–22.5 repeats

Tetramer templates		1st PCR	2nd PCR	3rd PCR	4th PCR	5th PCR	6th PCR
		WXYZ	WXYZ	XYZW	XYZW	WXYZ	WXYZ
Primers	22.5-mer					F-Ww/R-Yw	F-Wy/R-Z3
	21.5-mer						F-Wy/R-Y3
	20.5-mer						F-Wy/R-X3
	19.5-mer				F-Xw/R-Ww	F-Ww/R-Z3	–
	18.5-mer					F-Ww/R-Y3	–
	17.5-mer			F-Xz/R-Wx		F-Ww/R-X3	–
	16.5-mer					F-Ww/R-W3	–
	15.5-mer	F-W5/R-Zw	F-Wz/R-Zx		F-Xw/R-W3	–	–
	14.5-mer				F-Xw/R-Z3	–	–
	13.5-mer				F-Xw/R-Y3	–	–
	12.5-mer				F-Xw/R-X3	–	–
	11.5-mer			F-Xz/R-W3	–	–	–
	10.5-mer			F-Xz/R-Z3	–	–	–
	9.5-mer			F-Xz/R-Y3	–	–	–
	8.5-mer			F-Xz/R-X3	–	–	–
	7.5-mer		F-Wz/R-Z3	–	–	–	–

3.3 USER Digestion and Purification of PCR Products

1. Mix all the PCR products of the same TALE array. Mix all the PCR products of the same TALE array into one tube. Add 1.5 μL USER™ enzyme (1000 units/mL) directly into 60–75 μL mixed PCR product, and incubate at 37 °C for 15 min and cool on ice.
2. Add 1.5 μL T4 DNA ligase (100 units) and 7 μL T4 DNA ligase buffer into USER digested PCR products, and incubate at 16 °C for 1 h.
3. Purify USER-digested PCR products.

3.4 Cycles of BsmBI Digestion, DNA Ligation, and Transformation

1. Mix the purified PCR fragments with the following into one tube: BsmBI (Esp3I) (7.5 units), T4 DNA ligase (100 units), ATP (10 pmol), DTT (10 pmol), Tango buffer (1×), TALE expression vector (~20 ng), and ddH_2O to a total volume of 10 μL.
2. Perform thermo cycles (37 °C, 5 min; 16 °C, 5 min; 6×), and hold at 4 °C.

Table 6
Primers for colony PCR and sequencing analysis

Primer	Sequence
TAL-F	AAGAGGGGAGGCGTGACGGC
TAL-R	CAAGCCAGGGCCACCAGGT

3. Transform 5 μL products into 100 μL Trans1-T1 competent cells, and plate onto solid LB medium with 25 μg/mL of kanamycin. Other types of competent cells (except DB3.1 or any other ccdB-resistant strains) can also be used here.

3.5 Validation of TALE Clones

1. Perform colony PCR using the thermo cycles (95 °C, 30 s; 60 °C, 30 s; 72 °C, 2 min; 30×) with TAL-F and TAL-R primer pair (Table 6). If all steps are correctly performed, analyzing 5–10 colonies will be enough (*see* **Note 9**).
2. Examine the size of PCR products by electrophoresis. A DNA band from a right clone should be around the size of $(102 \times N + 107)$ bp (N indicates number of repeats in the TALE, including the last half repeat).
3. Sequence the TALE constructs using TAL-F and TAL-R primers (Table 6).

4 Notes

1. Archive of 16 TALE monomers.
2. Besides pIRES-EGFP-TALEN, other TALE vectors with non-ampicillin selection marker could be modified to perform ULtiMATE synthesis, as long as the cloning vector contains the ccdB cassette flanked by the BsmBI sites as indicated (Fig. 2). Because the tetramer templates are maintained in a vector containing ampicillin-resistant gene, ampicillin cannot be used as the selection marker for TALE vectors. All plasmids carrying tetramer templates are available to academic researchers upon request.
3. TALE expression vector pGL3-TALEN and pLentiLox3.7-TALE [11] can also be used if the purification step in Subheading 3.2 is substituted by DNA electrophoresis and gel purification.
4. Make sure that your BsmBI enzyme works at 37 °C. BsmBI from different producers may work at different temperatures.

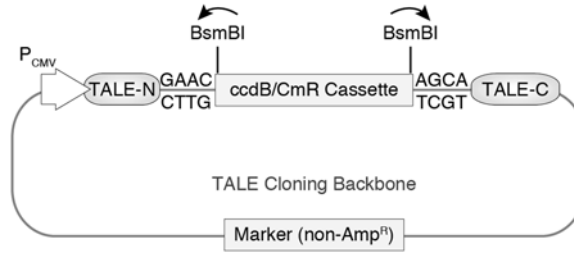

Fig. 2 Universal structure of TALE backbone vectors suitable for ULtiMATE cloning. N-terminal non-repeat region of TALE should be ended with GAAC in sense strand and a following recognition site of BsmBI, because after BsmBI digestion, a 5′-GAAC sticky end needs to be exposed to ligate the TALE repeats into backbone vector. Similarly, C-terminal non-repeat region should be beginning with AGCA. A backbone vector should contain a selection marker except ampicillin

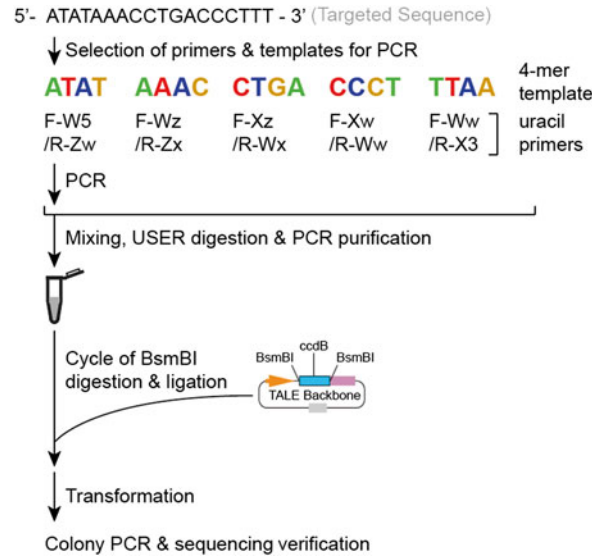

Fig. 3 Flow chart of TALE construction by ULtiMATE system with tetramer templates. Flow chart of the cloning process of a 17.5-mer TALE targeting ATATAAACCTGACCCTTT. The selection of primers are based on Table 5. The PCR amplification of tetramers can be finished within 1.5 h. Without the need of purification, the PCR products of all reactions are mixed for subsequent USER enzyme digestion. After purification, digested products are mixed with TALE cloning backbone for the cycles of BsmBI digestion and ligation, followed by the bacterial transformation (~1.5 h). The candidate clones are isolated based on colony PCR results, and verified by sequencing analysis

Table 7
Example of the assembly of a TALE array with 25.5 repeats (target sequence: TACCGTACCCGAGTTTGAACTTGTGT)

	1st PCR	2nd PCR	3rd PCR	4th PCR	5th PCR	6th PCR	7th PCR
Tetramer templates	**TACC**	**GTAC**	**CCGA**	**GTTT**	**GAAC** (or **GAAG/A/T**)	**CTTG** (or **CTTC/A/T**)	**GTGT**
Primers	F-W5/ R-Zw	F-Wz/ R-Zx	F-Xz/ R-Wx	F-Xw/ R-Ww	F-Ww/R-Yw	F-Wy/R-Yx	F-Xy/ R-W3
Expected PCR products	**TACC**	**GTAC**	**CCGA**	**GTTT**	**GAA**	**CTT**	**GTGT**

5. Select the PCR tetramer templates and primers (Table 4) for PCR reaction to construct each TALE array. TALE constructs with TALE repeat numbers from 7.5 to 22.5 are summarized in Table 5. Notably, the tetramers for the 1st, 2nd, 5th, and 6th PCR are in the WXYZ format, and the tetramers for the 3rd and 4th PCR are in the XYZW format. Figure 3 shows a schematic of a cloning process of a 17.5-mer TALE using ULtiMATE protocol.
6. For TALE constructs containing more than 22.5 repeats, the selection of tetramer templates and primers (Table 4) should be adjusted, as long as there are no identical primers used in the same ligation reaction. Table 7 shows an example of the assembly of a TALE array with 25.5 repeats.
7. With the same tetramer templates, end primers (both 5′ and 3′), and the ccdB cloning vectors, the TALE construction could also be conducted with modified Golden Gate or LIC (ligase-independent cloning) methods.
8. For the Golden Gate method, the principle of templates and primers selection, as well as the TALE assembly are the same as in Subheading 3, except that the primers are different and listed in Table 8, and the USER™ digestion step is not required. For each PCR reaction, mix the following into 0.2 mL PCR tube: tetramer template (5 ng/μL, 1 μL), forward primer (2 μM 1 μL), reverse primer (2 μM, 1 μL), TransTaq™ HiFi DNA Polymerase (5 unit/μL, 0.2 μL), TransTaq HiFi Buffer (10×, 1 μL), dNTPs (0.8 μL), and ddH_2O to a total volume of 10 μL. Perform PCR reaction as follows: 94 °C, 5 min; thermal cycling (94 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s; 30×); 72 °C, 10 min; and hold at

Table 8
Primers for Golden Gate method

Primer	Sequence
F-W5	tataCGTCTCaGAACCTGACACCAGAGCAAGTAGTGGCTATTG
F-Wz′	TCTCGTCTCTCTGACACCAGAGCAAGTAGTGG
F-Xz′	GTCCGTCTCATGGGCTCACTCCGGAACA
F-Xw′	ATACGTCTCACAAGCACACGGTCTCACTCCGGAACAGGTGGT
F-Ww′	GTCCGTCTCCAGAGCAAGTAGTGGCTAT
F-Wy′	GTCCGTCTCCCCACGGACTGACACCAGAGCAAGTAGT
F-Xy′	GTGCGTCTCCCACGGACTCACTCCGGAACAGGTGG
R-Zw′	TATCGTCTCATCAGCCCATGAGCCTGACACAGTACT
R-Zx′	CCGCGTCTCGCCCATGAGCCTGACACAG
R-Wx′	AGACGTCTCGCTTGGCAGAGCACCGGAA
R-Ww′	TATCGTCTCACTCTGGTGTCAGACCGTGTGCTTGGCAGAGCA
R-Yw′	TATCGTCTCCGTGGGCTTGGCACAAAACGGGCAGC
R-Yx′	GGGCGTCTCCCGTGGGCTTGGCACAAAACGGGCAGCAACCG
R-W3	tataCGTCTCaTGCTCTCCAGCGCTTGTTTGCCACC
R-X3	tataCGTCTCaTGCTCTCAAGGGCTTGCTTGCCGC
R-Y3	tataCGTCTCaTGCTTTCCAAAGCCTGTTTTCCGCC
R-Z3	tataCGTCTCaTGCTTTCCAGTGCCTGCTTACCTCC

4 °C. Figure 4 shows a schematic of a cloning process of a 17.5-mer TALE using a Golden Gate protocol (in BsmBI digestion and ligation step, at least 16 cycles are needed).

9. When TALE construction is correctly performed, positive rate of colony PCR usually achieves 6/6 or 11/12. If positive rate is too low, please check whether there are any mistakes in the steps.

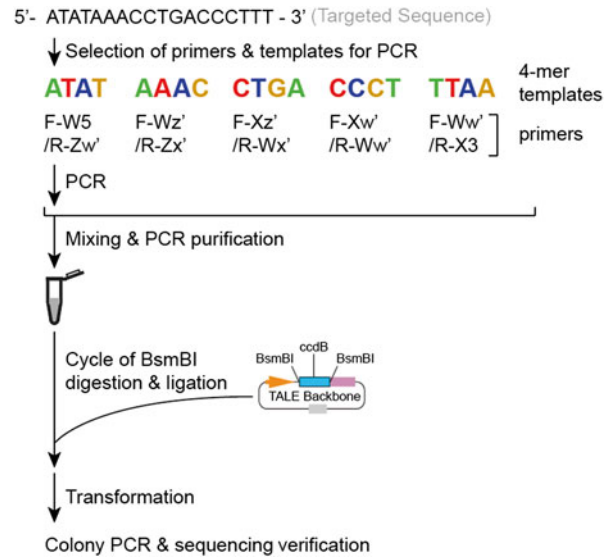

Fig. 4 Flow chart of TALE construction by Golden Gate method with tetramer templates. Flow chart of the cloning process of a 17.5-mer TALE targeting ATATAAACCTGACCCTTT. Primers are also selected based on Table 5 with a small change that Golden Gate primers are named with an inverted comma and their sequences can be found in Table 8. After PCR amplification, the PCR products of all reactions are purified and mixed with TALE cloning backbone for the cycles of BsmBI digestion and ligation

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