

# Chapter 2

## Current Overview of TALEN Construction Systems

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### Abstract

Transcription activator-like effector (TALE) nuclease (TALEN) is the second-generation genome editing tool consisting of TALE protein containing customizable DNA-binding repeats and nuclease domain of FokI enzyme. Each DNA-binding repeat recognizes one base of double-strand DNA, and functional TALEN can be created by a simple modular assembly of these repeats. To easily and efficiently assemble the highly repetitive DNA-binding repeat arrays, various construction systems such as Golden Gate assembly, serial ligation, and ligation-independent cloning have been reported. In this chapter, we summarize the current situation of these systems and publically available reagents and protocols, enabling optimal selection of best suited systems for every researcher who wants to utilize TALENs in various research fields.

**Key words** TALEN, Golden Gate assembly, Pre-assembled library, Serial ligation, Ligation-independent cloning

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## 1 Introduction

The rapid emergence of clustered regularly short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9) caused a paradigm shift in genome editing study [1]; however, TALEN is still an important genetic tool for functional genomics study, because various characteristics of these two tools differ from each other [2]. For example, CRISPR-Cas9 normally works as a monomer nuclease, while TALEN works as a dimer. CRISPR-Cas9 produces blunt ends, while TALEN produces cohesive ends. CRISPR-Cas9 consists of a complex of protein and RNA, while TALEN consists of protein only. These differences enable complementary usage of these two technologies.

Functional TALEN protein can be created by the assembly of DNA-binding repeats, in which the specificity of base recognition is defined by repeat-variable di-residue (RVD) [3, 4], into the backbone of TALEN harboring N- and C-terminal domains. Each single repeat consists of 34 amino acids, where the 12th and 13th residues are called RVD. Amino acid composition other than RVD among repeats is basically the same, but some non-RVD variations

on particular positions such as 4th and 32nd residues have been determined to be important for TALEN activity [5]. The lengths and amino acid sequence of N- and C-terminal domains of TALE are also identified as important factors for the activity and specificity of TALENs [6–8]. In addition, other contexts such as promoter, UTRs, poly-A signal sequence, and codon usage of TALEN-coding sequence are also important for the appropriate use in various cells and organisms. Therefore, proper choice of TALEN structure and vector backbone is needed in terms of the best practical application as well as the selection of construction systems described below.

Typically, 15–20 repeats have to be assembled in a defined order to construct functional TALENs. To achieve this, various one- or two-step modular assembly methods using single or multiple repeat libraries have been developed, improved, and applied by many research groups. In this chapter, we describe an overview of the current construction systems of TALEN, providing brief introduction of each system and detailed information of publically available plasmid kits and protocols.

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## 2 Summary of TALEN Construction Kits

Generally, the custom-made TALEN is created using a set of plasmids packaged for its construction. Such plasmid kits can be obtained from Addgene, the nonprofit plasmid repository [9]. Table 1 summarizes a current list of TALEN kits available from Addgene. There are various differences among these kits, including the method of modular assembly, the number of plasmids, and framework of TALEN scaffold. Regarding the construction method, a one-pot cloning strategy called Golden Gate assembly is most widely used. This method enables the simultaneous digestion and ligation of multiple plasmids in a single tube with high efficiency and accuracy, without requiring any special equipment other than a standard thermal cycler. Thus, the current TALEN kits are mostly based on this method. Other methods, however, are adopted in some kits, and they have unique characteristics that might be advantageous for a particular use. The Joung Lab TAL Effector Engineering Reagents are for the simple restriction digestion and ligation of modules in a serial manner, which can commonly be utilized for a low-/middle-/high-throughput generation of custom TALENs. The LIC TAL Effector Assembly Kit is based on a ligation-independent cloning (LIC) method, which does not require a recombinant ligase, but depends on the annealing of relatively long overhangs. The following sections are the detailed information of these methods and systems to provide the best understanding.

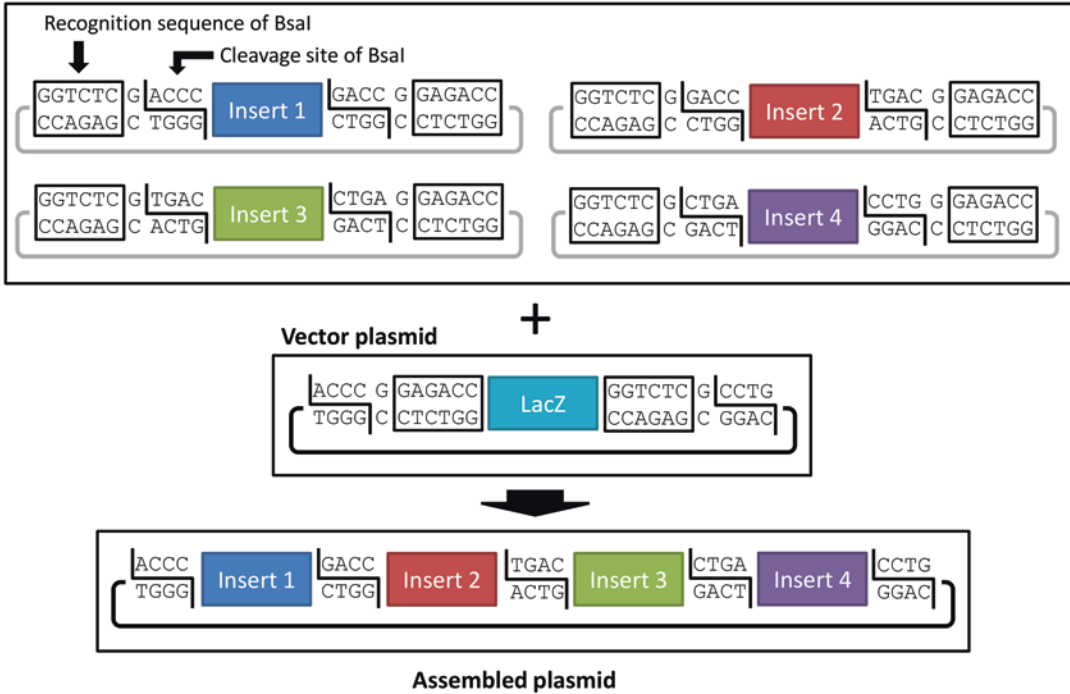
**Table 1**  
**Summary of TALEN kits available from Addgene**

Kit name	Kit ID	Depositor	Assembly method	Number of plasmids	Repeat number
Golden Gate TALEN and TAL Effector Kit 2.0	1,000,000,024	Daniel Voytas and Adam Bogdanove	Golden Gate	86	12–31
Platinum Gate TALEN Kit	1,000,000,043	Takashi Yamamoto	Golden Gate	35	6–21
TALE Toolbox	1,000,000,019	Feng Zhang	PCR/Golden Gate	12	13, 19, 25
Musunuru/Cowan Lab TALEN Kit	1,000,000,034	Kiran Musunuru and Chad Cowan	Golden Gate	834	15
Ekker Lab TALEN Kit	1,000,000,038	Stephen Ekker	Golden Gate	256	15
FusX TALEN assembly system	1,000,000,063	Stephen Ekker	Golden Gate	336	15, 16
REAL Assembly TALEN kit	1,000,000,017	Keith Joung	Serial ligation	32	Any number
LIC TAL Effector Assembly Kit	1,000,000,023	Veit Hornung	Ligation-independent cloning	76	10–19

### 3 Golden Gate Assembly-Based Systems

#### 3.1 Introduction of Golden Gate Assembly

Golden Gate assembly was first reported in 2008 [10], and soon after the initial publication, the high capacity of simultaneous assembly of many fragments (nine inserts in the acceptor vector) was shown [11]. In this assembly method, type IIS restriction enzymes such as BsaI and BsmBI are used to generate various patterns of cohesive ends at the same time by using a single enzyme, enabling simultaneous ligation of multiple modules in a defined order (Fig. 1). The recognition sequence of the enzyme on the insert plasmid is placed outside the module sequence, while that on the acceptor vector is placed inside the vector backbone sequence. It results in not only a seamless cloning, but also a prevention of re-cutting the assembled products. In addition, a lacZ cassette for the blue/white selection in the cut-out region of the acceptor vector and different antibiotic selection marker on the insert vectors can reduce the false-positive clones. Thus, the Golden Gate assembly method is a very sophisticated protocol for tandem

**Insert plasmids**

**Fig. 1** Example of Golden Gate assembly. The assembly of four inserts mediated by BsaI enzyme is shown. The four inserts are tandemly ligated into the vector plasmid without carrying BsaI recognition sequences

ligation of multiple modules, which is quite suited for the TALE repeat assembly.

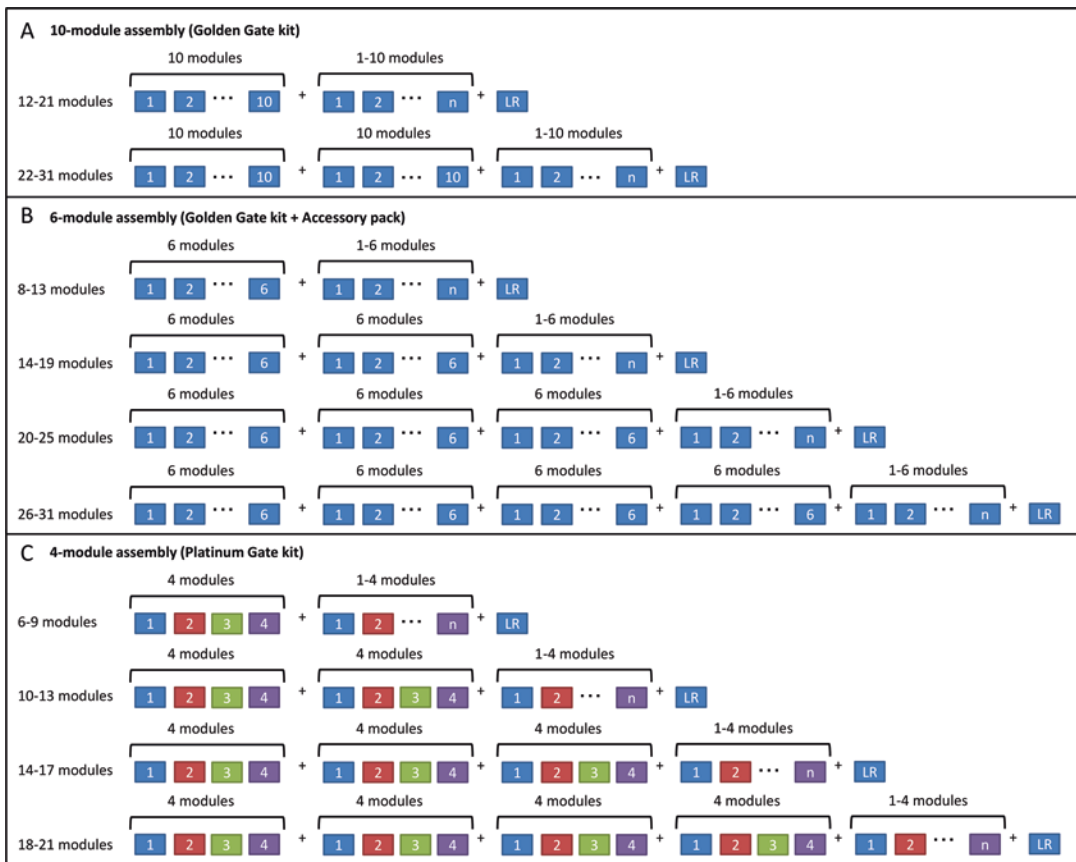
### 3.2 Typical Protocol for the Golden Gate Assembly-Based TALEN Construction

1. Prepare all the plasmids needed and design TALEN target sequences.
2. Mix the insert and vector plasmids with a restriction enzyme and a DNA ligase in a single PCR tube, and perform 1st-step Golden Gate assembly in a thermal cycler. Typically, up to ten modules are tandemly assembled in one intermediate array vector.
3. Perform bacterial transformation with blue/white selection. Screen the intended clones by colony PCR. Culture the clones and extract the plasmid DNA.
4. Perform second-step Golden Gate assembly, transformation, colony screening, and plasmid preparation in a similar way. All the procedures can be conducted in 5 days [12, 13].

### 3.3 Original Golden Gate Kit from Voytas/Bogdanove Lab

The first TALEN kit appeared in Addgene is the Golden Gate TALEN and TAL Effector Kit, deposited by Daniel Voytas and Adam Bogdanove [12]. Currently, the version 2.0 of the kit is available (Kit #1000000024). The kit contains 86 plasmids,

including 60 module vectors, 5 last repeat vectors, 13 intermediate array vectors, and 6 destination vectors to create TALEN vectors containing 12–31 DNA-binding repeats with no non-RVD variations (Fig. 2a). The functionality of the TALEN vector created using this kit has been widely validated in many cells and organisms; however, the vector often cannot be used directly and needs additional plasmids, because the original kit only contains yeast expression vectors as destination vectors. In addition, the reaction of 10-module assembly is relatively difficult and not always successful. In this regard, the add-on plasmids compatible with the Golden Gate kit have been created and deposited in Addgene by many groups.



**Fig. 2** Summary of 10-, 6-, and 4-module assembly needed for the Golden Gate kit (a), the Golden Gate kit with the Accessory pack (b), and the Platinum Gate kit (c), respectively. Up to 31 (a, b) or 21 (c) modules can be assembled with the 2-step Golden Gate assembly systems. In the systems based on the Golden Gate kit, all the DNA-binding modules consist of the same amino acid composition excluding RVDs (a, b), whereas those in the TALENs created with the Platinum Gate kit consist of variable repeats (c). See the main text for details. LR last repeat

### 3.4 Accessory Plasmids for the Golden Gate Kit

To make the most of the Golden Gate kit, various add-on plasmids have been developed (Table 2). All of them are destination vectors and distributed as single plasmids, excluding the TALEN Construction Accessory Pack (Kit #1000000030), developed by ours [13]. This accessory pack contains nine plasmids including seven intermediate array vectors and two destination vectors. The unique intermediate array vectors are, of course, compatible with the Golden Gate kit, and they enable six-module assembly, which results in efficient and accurate concatemerization of the TALE repeats without any additional cloning steps (Fig. 2b). The destination vectors are for in vitro transcription using T7 RNA polymerase and expression in mammalian cells via CMV or CAG promoters, harboring truncated TALEN scaffolds with enhanced activity and specificity (47 amino acids for C-terminal domain) [7, 13].

Regarding other plasmids, RCIscrip-GoldyTALEN and pC-GoldyTALEN (Plasmid #38142 and #38143), pCS2TAL3-DD and pCS2TAL3-RR (Plasmid #37275, #37276, #48636, and #48637), and pCAG-T7-TALEN(Sangamo)-Destination Constructs (Plasmid #37184, #40131, and #40132) are in vitro transcription and expression vectors with CMV or CAG promoters for TALENs. TAL5-BB and pTAL6-BB (Plasmid #36033 and #36034) are yeast expression vectors for TALE-based transcriptional repression. TALE-transcription activation destination vectors (Plasmid #47388 and #47389) are mammalian expression/in vitro transcription vector for TALE-based transcriptional activation. Destination vectors for TALE-mediated Genome Visualization (TGV) (Plasmid #47874 and #47875) are mammalian expression vector to visualize the dynamics of the chromosome. pTAL7a and pTAL7b (Plasmid #48705 and #48706) are destination vectors for the TALEN application in human pluripotent stem cells. pBlue-TAL (Plasmid #49401) is for the generation of germline mutations in *Bombyx mori* and *Drosophila melanogaster*.

### 3.5 Platinum Gate Kit from Yamamoto Lab

Although the convenience of the Golden Gate kit has become a higher level by utilizing various additional plasmids, the activity of resultant TALENs is not always sufficient, requiring improvement of amino acid composition of the TALE repeats. We previously found that periodically patterned variable repeats can enhance the DSB-inducing activity of TALENs [5, 14]. The Platinum Gate TALEN kit (Kit #100000004) is the only one that highly active TALENs with the variable repeats can be created using Golden Gate assembly method. The versatility of TALENs constructed with this kit, referred to as Platinum TALENs, has been proven in a number of publications, reporting various applications in a wide variety of cells and organism, such as human iPS cells [14, 15], nematodes [16], sea urchins [17], ascidians [18], water flea [19], zebrafish [20], newts [21], frogs [5], mice [22], rats [5], and marmosets [23].

**Table 2**  
**Summary of Golden Gate add-ons**

<b>Kit/plasmid name</b>	<b>Kit/plasmid ID</b>	<b>Depositor</b>	<b>Contents</b>	<b>Number of plasmids</b>
TALEN Construction Accessory Pack	1,000,000,030	Takashi Yamamoto	Intermediate array vectors and destination vectors for in vitro transcription and mammalian expression	9
RCIScript-GoldyTALEN and pC-GoldyTALEN	38,142, 38,143	Daniel Carlson	Destination vectors for in vitro transcription and expression of TALENs	2
TAL5-BB and pTAL6-BB	36,033, 36,034	Tom Ellis	Destination vectors for yeast expression of TALE-based transcriptional repressors	2
pCS2TAL3-DD and pCS2TALEN3-RR	37,275, 37,276, 48,636, 48,637	David Grunwald	Destination vectors for in vitro transcription and expression of TALENs	4
pCAG-T7-TALEN(Sangamo)-Destination Constructs	37,184, 40,131, 40,132	Pawel Pelczar	Destination vectors for in vitro transcription and expression of TALENs	3
TALEN-transcription activation destination vectors	47,388, 47,389	Charles Gersbach	Destination vectors for mammalian expression of TALE-based transcriptional activators	2
Destination vectors for TALE-mediated Genome Visualization (TGV)	47,874, 47,875	Maria-Elena Torres-Padilla	Destination vectors for mammalian expression of fluorescent TALE to visualize chromosome dynamics	2
pTAL7a and pTAL7b	48,705, 48,706	Boris Greber	Destination vectors for the application of TALEN technology in human pluripotent stem cells	2
pBlue-TAL	49,401	Michal Zurovec	Destination vector for the generation of germline mutations in <i>Bombyx mori</i> and <i>Drosophila melanogaster</i>	1



The kit contains 35 plasmids including 16 module vectors, 11 intermediate array vectors, and 8 destination vectors. TALENs with 6–21 repeats can be constructed using 4-module assembly method (Fig. 2c). Fewer numbers of assembled modules compared with previous systems (4 vs. 10 or 6) result in an easy, robust, and efficient assembly reaction. Destination vectors are designed to be applicable in mammalian expression and in vitro transcription, similar to the previous ones. Moreover, two truncation patterns of N- and C-terminal domains of TALE based on different TALE architecture (+153/+47 of PthXo1 and +136/+63 of AvrBs) are included. These two scaffolds have different preferences on the TALEN activity; +153/+47 TALEN is active with limited spacer lengths, while +136/+63 TALEN is active with broad spacer lengths [5]. Users can choose the appropriate scaffold from these two options depending on the intended use. The detailed protocol to construct Platinum TALEN was described in a previous publication [24].

### **3.6 TALE Toolbox from Zhang Lab (Combined Method of Golden Gate Assembly and PCR Amplification)**

Another popular TALEN kit based on Golden Gate assembly is the TALE Toolbox (Kit #1000000019) developed by Feng Zhang [25]. The TALE Toolbox consists of a relatively small number of plasmids: four module vectors and eight destination vectors. Among them, four destination vectors are for TALE-based transcriptional activation; thus, only eight plasmids are needed to create TALENs. However, this kit requires several additional procedures including PCR amplification, purification of PCR products, exonuclease treatment, another round of PCR amplification, and gel extraction and purification. Therefore, although the initial cost and labor is low compared with the other methods, users have to consider the running cost and labor. Two rounds of PCR amplifications might introduce unintended errors. Variable repeat TALENs cannot be created, because there are only four module vectors.

### **3.7 Multiple Module Library-Mediated Systems from Ekker Lab and Musunuru/Cowan Lab**

As described so far, Golden Gate assembly-based construction of TALENs basically depends on the two-step assembly process. However, there are several systems enabling one-step construction of TALEN vectors by using pre-assembled module libraries. Musunuru/Cowan Lab TALEN Kit (Kit #1000000034) contains 834 plasmids including 58 three-module library, 768 four-module library, and 2 destination vectors (pTAL\_GFP and pTAL\_RFP) [26]. Using these large-scale libraries, 15-repeat TALENs can be constructed with a single cloning step. Stephen Ekker lab first deposited the partial library containing 256 four-module plasmid sets (Egger Lab TALEN Kit; Kit #1000000038), which is compatible with the Golden Gate kit [27]. This plasmid set can help in creating 15-repeat TALENs, but requires additional construction of intermediate array plasmids. Subsequently, Ekker lab has



developed another system called FusX TALEN Assembly System (Kit #1000000063) [28]. The FusX system contains 336 plasmids, enabling one-step construction of TALENs without additional intermediate constructs. This system is also compatible with the Golden Gate kits; thus, all the accessory plasmids for the Golden Gate kit can also be used. These multiple module library-mediated methods are suitable for high-throughput production of TALEN vectors, while there also are some limitations. First, hundreds of pre-assembled plasmids are needed and have to be maintained. Second, assemblable repeat length is very limited (15 repeats in the Musunuru/Cowan system and 15 or 16 repeats in FusX system). Since the N-terminal domain of TALE recognizes a thymine, this limitation narrows the target range of TALENs. Third, incorporation of repeat variations such as variable repeats and noncanonical RVD repeats [29] is very difficult.

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## 4 Other Systems

### **4.1 TAL Effector Engineering Reagents from Joung Lab (Serial Ligation Method)**

Keith Joung lab has provided the REAL Assembly TALEN kit (Kit #1000000017) that consists of 32 plasmids including 28 module vectors and 4 destination vectors [30]. Since the REAL assembly method depends on the standard serial digestion and ligation procedure, it requires several cloning steps to create functional TALENs. To improve the throughput, the pre-assembled module library can be used, similar to the Golden Gate assembly-based systems (REAL-Fast method). The detailed procedures for creating TALENs using REAL or REAL-Fast method were described in a previous publication [31]. In addition, the fast ligation-based automatable solid-phase high-throughput (FLASH) method can also be applicable with the same reagents [32, 33]. The FLASH system enables automated or manual TALEN assembly in a 96-well format. Moreover, TALENs constructed with these systems contain variable repeats similar to the Platinum TALENs. However, the pre-assembled module library for the REAL-Fast and FLASH systems has not yet distributed via Addgene.

### **4.2 LIC Assembly Kit from Hornung Lab (Ligation-Independent Cloning Method)**

Veit Hornung lab has reported another approach for TALEN assembly. They developed a ligation-independent cloning (LIC) assembly-mediated system (LIC TAL Effector Assembly Kit; Kit #1000000023) [34]. In this system, a unique procedure named chew back reaction is required. The chew back reaction is a programmed DNA end resection mediated by T4 DNA polymerase, resulting in unique 5' overhangs for the annealing of multiple DNA fragments in a defined order. The kit contains 76 plasmids, creating 10- to 19-repeat TALENs with two assembly steps. The detailed procedures for LIC-based TALEN construction were described in a previous publication [35].

### 4.3 Other Systems Currently Unavailable from Addgene

Besides the Addgene kits, there are a number of alternative TALEN construction systems reported, such as iterative capped assembly (ICA) system [36], uracil-specific excision reagent (USER) cloning-based methods [37, 38], STAR method based on isothermal assembly [39], synthetic oligonucleotide-mediated system [40]. It is highly desired for these interesting systems to be available via Addgene in the future.

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