RESEARCH ARTICLE

TALENs Construction: Slowly but Surely

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Abstract

Cancer is thought to be a direct result of transcriptional misregulation. Broad analysis of transcriptional regulatory elements in healthy and cancer cells is needed to understand cancer development. Nucleases regulatory domains are recruited to bind and manipulate a specific genomic locus with high efficacy and specificity. TALENs (transcription activator-like effector nuclease) fused to endonuclease FokI have been used widely to target specific sequences to edit several genes in healthy and cancer cells. This approach is promising to target specific cancer genes and for this purpose it is needed to pack such TALENs into viral vectors. There are some considerations which control the success of this approach, targeting appropriate sequences with efficient construction of TALENs being crucial factors. We face some obstacles in construction of TALENs; in this study we made a modification to the method of Cermk et al 2011 and added one step to make it easier and increase the availability of constructs.

Keywords: TALENs - RVDs - Golden gate construction - cancer therapy

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Introduction

Genome engineering enables researchers to modify genomic loci of interest in a precise manner, which has various applications in industry and human therapeutics. Site-specific DNA double-strand break can guarantee an efficient genome editing which will be repaired by homologous recombination (HR) or non-homologous end joining (NHEJ) generating desired genetic modifications (Perez et al., 2012). The outcome of NHEJ is variable, includes nucleotide insertions, deletions or substitutions in the broken region. HR restores the double-strand breaks using homologous template DNA, and as a consequence the outcome of repair is controllable (Li et al., 2011; Wei et al., 2013). DNA domains bind to specific to DNA sequence and carry a protein with nuclease activity were developed to ease the genome manipulation.

Many diseases in particular cancer is thought to be a direct result of transcriptional misregulation (Lee and Young, 2013; Youns et al., 2013; Kumar et al., 2016). Genome wide analysis of transcriptional regulatory elements in healthy and cancer cells is mandatory not only to understand but also to overcome the cancer development. Nucleases or transcriptional regulatory domains are recruited to bind and manipulate a specific genomic locus with efficacy and specificity (Grimmer and Farnham, 2014). Zinc finger proteins (ZFP), which recognize different sets of nucleotide triplets, were used as a magnificent genomic editing tool. Specific zinc finger DNA binding domains and the endonuclease Fok I (ZFN) were generated to target specific DNA sequences (Urnov et al., 2010). ZFNs were efficiently applied but with considerable drawbacks, not all nucleotide triplets have corresponding zinc fingers, the coast and time consuming (Bibikova et al., 2003; Cradick et al., 2011).

TALEN (transcription activator-like effector nuclease)-was introduced as a more advantageous genome editing tool much more than ZFNs (Hockemeyer et al., 2011, Huang et al., 2011; Tesson etal., 2011). TAL effectors (TALEs) discovered in Xanthomonas species as an invasive factor to infect plant (Bonas et al., 1989). TALEs are injected into plant cells via the bacterial type III secretion system (TTSS), transfecting the plant cell nuclei to activate transcription of desired genes by targeting their effector gene promoters (Kay et al., 2007; Romer et al., 2007). TALEs consist of special effector proteins and a central domain which binds specifically to DNA sequence and comprises of a variable number of corresponding monomer repeats. These monomer repeats are varying from 5 to over 30 and each one contains 34 amino acids that specifically recognize one target nucleotide. Interestingly, that the sequence of amino acids of these repeats is highly conserved with unique variation in the residues at position 12 and 13, which called "repeat variable di-residues" (RVDs) which simply determines the nucleotide binding specificity of each TALE repeat (Boch and Bonas, 2010; Miller et al., 2011). The specificity of DNA binding is determined by the RVDs with A, C, G,

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T recognized by NI, HD, NN /NK and NG respectively (Moscou and Bogdanove, 2009). Recently, NH showed a higher specificity than NN to target nucleotide G (Streubel et al., 2012). Fok I nuclease was fused to an artificial TALE to create a specific TALEN, which is used widely as a powerful genome editing tool in a wide range of model systems and cultured human cells (Figure 1), reviewed at (Streubel etal., 2012; Gaj et al., 2013; Sun and Zhao et al., 2013; Wei et al., 2013).

Numerous factors should be considered when selecting the most appropriate vector system to deliver TALENs targeting cancer. Several factors include promoter, selection marker, host cell, length of ORF and fusion tags are considered (Shearer and Saunders, 2015). Cloning of TALENs is a technical challenge because of extensive identical repeat sequences; several methods were developed to enable rapid and proper assembly of TALENs. These approaches include high-throughput solid-phase assembly (Briggs et al., 2012; Reyon et al., 2012) and ligation-independent cloning techniques (Schmid et al., 2013). Golden Gate molecular cloning was the earliest method used widely to construct TALENs (Figure 2) (Cermak et al., 2011), however the construction of ten RVDs together was a challenging step and it was very tedious and difficult to be achieved in several labs. In this work we aimed to modify Golden Gate molecular cloning to enable easier assembly of TALENs. Inspite the addition of one more step, the yield of positive constructed TALENs was increased significantly.

Materials and Methods

Plasmids, Primers, Enzymes and Plasmid extraction kits

The plasmids for RVDs are provided from Golden Gate TALEN assembly kit as glycerol stock at -80ºC. The primers are ordered from Sigma Aldrich (USA) as summarized in Table 1. PCR polymerase, dNTPs, High Fidelity polymerase and DNA ladder were provided from NEB (New England Biolabs).

Plasmid extraction and digestion

Loop-full from E coli glycerol stock harboring plasmids for RVDs were cultivated overnight in Luria Broth (LB) both containing antibiotic (spectinomycin or tetracycline or ampicillin, Sigma-Aldrich) and plasmids were extracted by Mini-prep Qiagen kit. Plasmids were digested by restriction endonuclease enzymes in suitable provided buffers (New England Biolabs).

Competent cells and Plasmid Transformation

The constructed plasmids were transformed to E.coli Sur2 competent cells (Lab stock). Competent cells were prepared by heat shock; briefly overnight cultured cells were grown for 1-2 hours in LB broth (Sigma, USA) to optical density 0.2-0.8. The cells were collected by centrifugation and the re-suspended in glycerol water (equal amounts) and kept in ice for 5 minutes. The plasmids were added to the suspended cells then heat shocked by sudden transfer to hot water path at 42ºC, diluted with 1 ml of Super Optimal Broth (SOC) broth media and shacked for 1 hour at 37ºC. White blue selection was done to select the proper constructs (while colonies) on LB-agar plates containing X-gal and antibiotic (spectinomycin or tetracycline or ampicillin) and covered by Isopropyl β-D-1-thiogalactopyranoside (IPTG).

Modified Method of construction

Cermak et al, 2011 showed the TALEN assembly method (Figure 2) (Cermak et al., 2011). Briefly the plasmids for the chosen RVDs 1-10 and pFUSA or pFUSB were extracted and digested with BsaI, then the RVDs will be ligated to digested pFUS in thermocycler 10x (37°C/5min + 16°C/10min) + 50°C/5min + 80°C/5min. To destroy all un-ligated linear dsDNA fragments including incomplete ligation products, plasmid-Safe nuclease

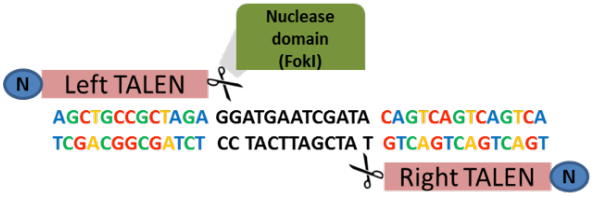

Figure 1. TALEN Construction. TALENs consist of a central domain typically composed of tandem TALE for recognition of a specific DNA sequence and C-terminal domain of the functional endonuclease Fok I

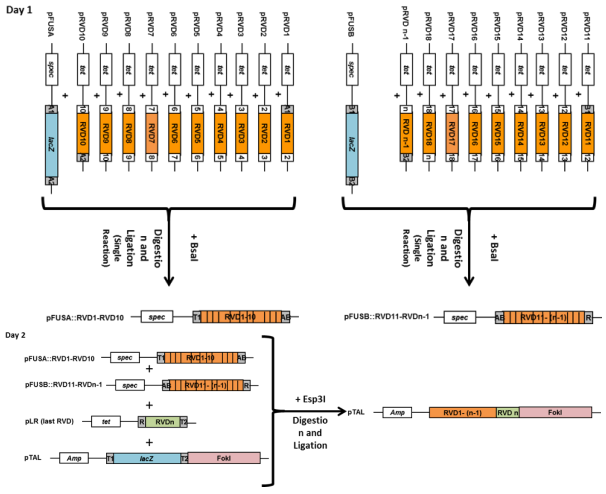

Figure 2. Golden Gate Assembly Kit (Cermak et al., 2011)

Table 1. Sequence of Primers and Plasmids Used in this Study

Primer	Sequence	Source/ Reference
pCR8_F1	ttgatgcctggcagttccct	Ceramk et al., 2011
pCR8_R1	cgaaccgaacaggcttatgt	Ceramk et al., 2011
RVD-1-BsaI-For	agaggtctcgctatcgccagcca	This study
RVD-5-BsaI-Rev	gagggtctcctgcaccgtttcg	This study
Plasmids for RVDs	Golden Gate kit	Ceramk et al., 2011
pFUSA and PFUSB	Golden Gate kit	Ceramk et al., 2011

treatment was done for 1 hour at 37°C. The ligated plasmids will be transferred to competent cells by heat shock and proper white clones will be selected on X-gal/ Spectinomycin plates (Figure 3). The selected clones will be further confirmed by colony PCR using primers pCR8_F1 and pCR8_R1 (Table 1), anneal at 55°C, extend 1.75min, cycle 30-35X. The expected should be around 1.2KB for vectors with 10 repeats with smearing and a 'ladder' of bands starting at 200bp and every 100bp up to ~500bp, which is the sign of a correct clone and is the result of the repeats in the clones (Figure 4).

In our modified method (Figure 5), we mixed the plasmids for RVDs 1-5 and pFUSB5, then digestion with BsaI and ligation were done in thermocycler 10x (37°C/5min + 16°C/10min) + 50°C/5min + 80°C/5min. RVD 1-5 was amplified using primers RVD-1-BsaI-For and RVD-5-BsaI-Rev (Table 1) using High fidelity polymerase, anneal at 55°C, extend 0.6 min, cycle 30-35X (Figure 6). The PCR amplified RVD 1-5 was mixed with pFUS plasmid and digested with BsaI and ligated in thermocycler 10x (37°C/5min+16°C/10min) + 50°C/5min+80°C/5min. Plasmid-Safe nuclease treatment was done and the ligated plasmids were transferred to competent cells. White blue selection was done on X-gal/ spectinomycin plates where white colonies were selected. The selected clones were colony PCR confirmed using primers pCR8_F1 and pCR8_R1 (Figure 7A). And for further confirmation, double diagnostic digestion using XbaI and AfLII was done (Figure 7B). The typical procedure and our modification were used in parallel to construct six different sequences of RVDs and the percentage of positive clones was calculated for each

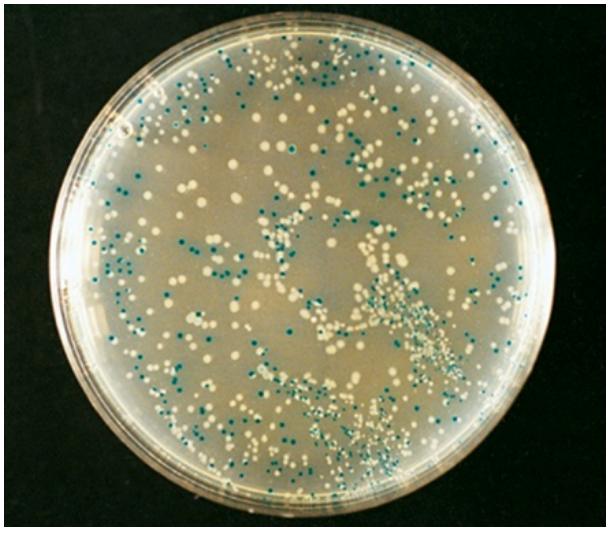

Figure 3. Blue White Selection. The transferred cells were grown in the presence of X-gal and IPTG. Cells transformed with vectors containing RVDs (replace lac Z operon) will produce white colonies; cells transformed with non-recombinant plasmids (i.e. only the vector) grow into blue colonies

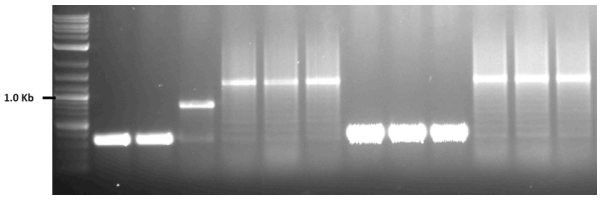

Figure 4. Colony PCR results for pFUS Vectors. Lanes 2 and 3 are negative pFUS clones (empty). Lanes 5,6,7,11,12 and 13 show the correct clones for pFUS ligated to 10 RVDs and show the laddering effect

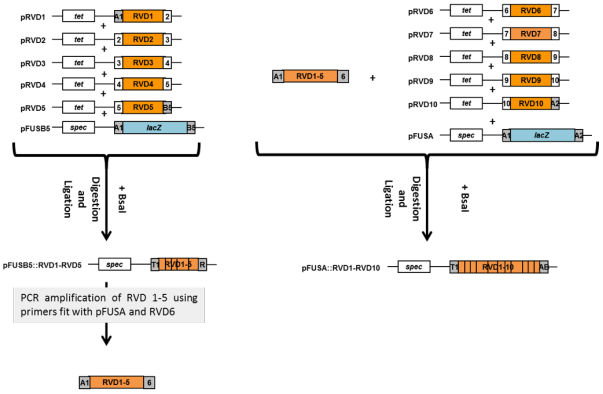

Figure 5. Modified method. RVDs (1:5) were ligated to pFUSB5. RVD 1-5 DNA segment was amplified by high fidelity PCR polymerase using primers with BsaI cut ends fitting with RVD 6 and pFUS. Finally, RVD1-5 and plasmids for RVD 6:10 were digested by BsaI and the cuts were ligated by T4 ligase enzyme

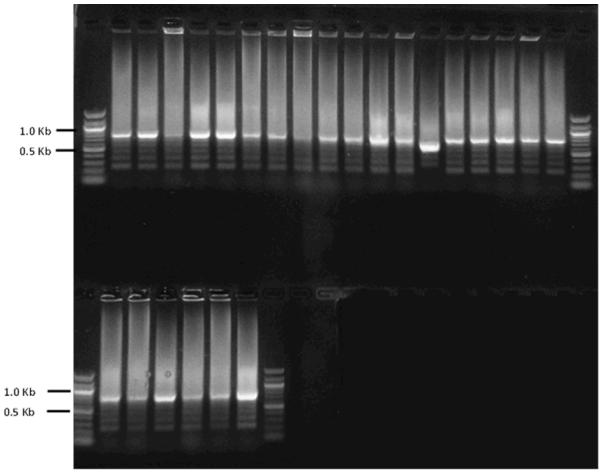

Figure 6. RVD 1-5 Amplification. RVD 1-5 was amplified using primers RVD-1-BsaI-For and RVD-5-BsaI-Rev using High fidelity polymerase, anneal at 55oC, extend 0.6 min, cycle 30-35X

Table 2. Sequence of RVDs Used in this Study

TALEN NR.	Targeted sequence	RVD sequence
1	CCCCAGCTTT	HD1 HD2 HD3 HD4 NI5 NH6 HD7 NG8 NG9 NG10
2	TTGGGAGGTG	NG1 NG2 NH3 NH4 NH5 NI6 NH7 NH8 NG9 NH10
3	CCCCAGACTG	HD1 HD2 HD3 HD4 NI5 NH6 NI7 HD8 NG9 NH10
4	GGACTCATGA	NH1 NH2 NI3 HD4 NG5 HD6 NI7 NG8 NH9 NI10
5	AGATCCCACA	NI1 NH2 NI3 NG4 HD5 HD6 HD7 NI8 HD9 NI10
6	TCAGAAGGAA	NG1 HD2 NI3 NH4 NI5 NI6 NH7 NH8 NI9 NI10

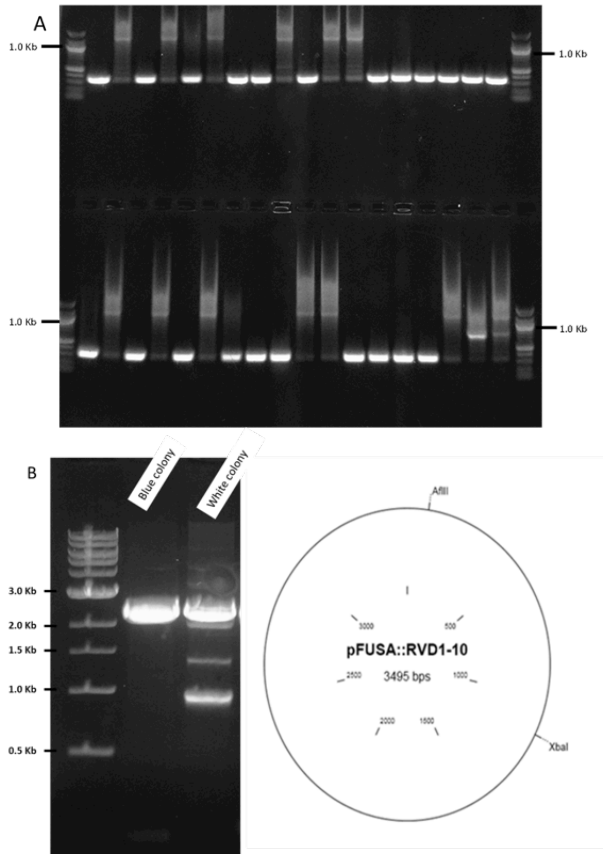

Figure 7. Results of Modified Method. A) Colony PCR of selected white colonies. B) Diagnostic digestion of pFUSA::RVD1-10 by XbaI and AflII

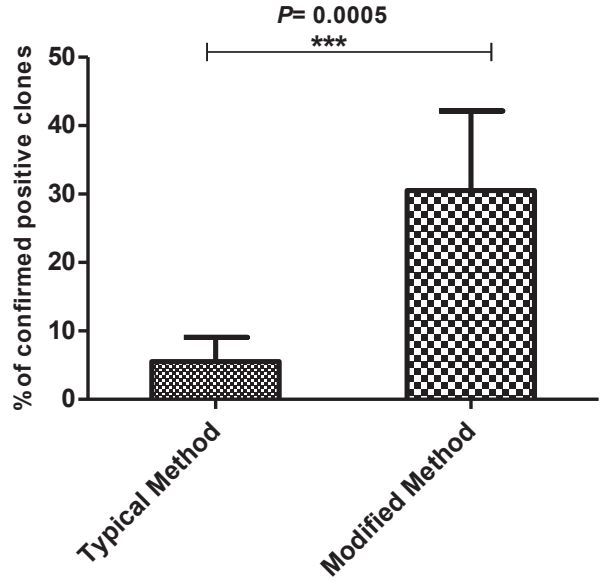

Figure 8. Significance of Modification. Positive confirmed clones increased by adding one more step significantly (P=0.0005)

construct.

Results

For construction of TALEN more than 10 RVDs, it needs 5 days (Figure 1) (Cermak et al., 2011), the most important and difficult step is to digest and ligate 10 RVDs with pFUS and the positive clones were very few. In our modified procedure, we added one more step; we fused RVD 1-5 to pFUSB5 and then amplified RVD 1-5 using ends which can by digested to fit pFUS and RVD 6 (Figure 5). RVD 1-5 was digested with BsaI and ligated to pFUS and transformed to competent cells. The positive white clones were selected from X-gal/spectinomycin plates and confirmed by colony PCR and diagnostic digestion. Six different 10 RVDs sequences were tested (Table 2), the modification used increased the confirmed positive clones significantly more than traditional method (Table 3 and Figure 8). The average of positive clones was increased from 4.4% to about 30%.

Table 3. Number of Positive Clones to Tested Clones and their Percentage.

TALEN NR.	Typical method		Modified method	
1	1/36	2.7%	16/36	44.4%
2	2/36	5.5%	8/36	22.2%
3	1/36	2.7%	12/36	33.3%
4	3/36	8.3%	10/36	27.7%
5	1/36	2.7%	5/36	13.8%
6	4/36	11.11%	15/36	41.6%

Discussion

DNA specific domains which carry a protein with nuclease activity are efficient as genome editing tool. New approaches were developed to target cancer genome using nuclease domains (Grimmer and Farnham, 2014; Shearer and Saunders, 2015). ZNF was the first developed approach using nuclease activity to target specific sequence of DNA but suffered from several drawbacks (Cradick et a., 2011). That is why the scientists worked on developing more advantageous approaches; TALENs met these needs to target any sequence of DNA on contrast to ZNF which do not bind to all nucleotide triplets (Bibikova et al., 2003; Cradick et a., 2011). TALENs were extensively used in genome manipulation (Streubel etal., 2012; Gaj et al., 2013; Sun and Zhao et al., 2013; Wei et al., 2013).

Recently, clusters of regularly interspaced short palindromic repeats (CRISPER) system and the Cas9 endonuclease is the newest site-specific genomic editing tool (Gaj et al., 2013; Wei et al., 2013). It gives a technical advantage over TALENs that it is easier constructed and introduction to animal models; however, the cas9 size was large to be packed in suitable viral vector which usually has limited room. In contrast to TALENs, we can control the size of construct by controlling the number of RVDs, which gives the chance to be packed in suitable viral vector. So for targeting specific DNA sequence using viral vector, we worked in parallel on constructing efficient TALENs with suitable size and engineering Cas9 to smaller size.

TALENs were widely used to target specific genomic sites of normal and cancer cells (Gaj et al., 2013). There are several approaches were developed to construct TALENs include high-throughput solid-phase assembly (Briggs et al., 2012) and ligation-independent cloning techniques (Schmid et al., 2016). Inspite Golden Gate molecular cloning approach was the earliest one; it is still widely used (Figure 2) (Cermak et al., 2011). The most critical step

in TALEN construction is to ligate ten RVDs to suitable pFUS plasmid vector. To overcome this difficulty we went through several approaches, first one was amplifying RVDs by PCR and try to ligate them separately without ligation to vector plasmid. It was very difficult to gel purify these RVDs (74bp-98bp) and to ligate them, as a result the possibility of success was low. Second approach, we amplified RVDs by PCR and ligate them directly with vector without using plasmids carrying RVDs, to skip the step of these plasmids digestion which may ease the construction. We found no great difference between this approach and the Golden Gate approach, moreover, it was tedious and coast to amplify all RVDs using high fidelity polymerase enzyme and then purify these PCR products. The third approach (Figure 5); we ligated first five RVDs with pFUSB5 which have complementary ends with the first RVD and fifth RVD. Then, amplify RVD (1-5) by unique primers which universally fit with any RVD 1 and any RVD 5 which means that we can use these primers for any RVDs we want. Moreover we designed primers with ends fit with pFUSA and RVD6 after BsaI digestion of PCR amplified RVD (1-5). Mixture of PCR amplified RVD (1-5), Plasmids for RVDs 6 to 10 and pFUSA was digested by BsaI endonuclease and then ligated with T4 Ligase enzymes. Surprisingly, the results were promising and the positive clones were significantly increased. We repeated the same procedure on sex TALENs and by both Golden Gate approach and our modified approach, we found a significant increase of positive clones. We can explain this success by decreasing the number of DNA segments which should be ligated in one step which reflects as a consequence on success. The most interesting that we needed only to use a couple of primers which can be used to any RVDs. This modified approach needs one more day but the yield of positive clones is increased significantly, it is slowly but surely.

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