Chapter 23

A Rapid TALEN Assembly Protocol

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Abstract

Owing to their modular and highly specific DNA recognition mode, transcription activator-like effector nucleases (TALENs) have been rapidly adopted by the scientific community for the purpose of generating site-specific double-strand breaks (DSBs) on a DNA molecule. A pair of TALENs can be used to produce random insertions or deletions of various lengths via nonhomologous end-joining or together with a homologous donor DNA to induce precise sequence alterations by homologous recombination (HR). Here, we describe a method for TALEN assembly (easyT) and a strategy for genome engineering via HR.

Key words TALEN, Genome engineering, Site-specific gene integration

1 Introduction

TALENs are fusion proteins containing a transcription activator-like effector (TALE) domain and a FokI nuclease domain [1]. FokI is a nonspecific nuclease that can generate DSBs in a DNA molecule upon homodimerization. The need for two FokI domains to dimerize requires that TALENs be used as a pair (left and right), binding two sequences separated by 14–32 bp and in a nuclease-to-nuclease orientation. Our TALE domain is a truncated derivative of the AvrXa7-FN TALEN [2] that retains 207 N-terminal amino acids and 63 C-terminal amino acids. TALE repeats within the TALE domain are responsible for the recognition of a specific DNA sequence—one repeat recognizing a single nucleotide. Repeats within the repeat domain contain 34 amino acids (the last "half-repeat" contains 17 amino acids and can also recognize a single nucleotide) that differ only in amino acids at positions 12 and 13. These two amino acids, also known as repeat variable diresidue (RVD), confer the sequence preference to each repeat by following the simple RVD code: diresidue HD recognizes cytosine, NI recognizes adenine, NG recognizes thymine, and NK recognizes guanine [3, 4]. By following the RVD code, a pair of TALENs can be designed to recognize a target DNA sequence and introduce a DSB at that site.

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In our lab, we are interested in studying downregulation of silencing established by Polycomb group (PcG) proteins observed in regenerating *Drosophila* imaginal discs following disc fragmentation [5]. For regeneration of a damaged imaginal disc, cells at the site of injury need to activate specific genes that are normally under PcG-mediated silencing. It is thought that Jun N-terminal kinase (JNK) signaling pathway is responsible for specific PcG target gene reactivation in these cells [6]. At the molecular lever, however, it is still not clear how this reactivation is accomplished. One attractive hypothesis is that transcription factor (TF) AP-1, which acts downstream of JNK, binds to the regulatory elements of these specific PcG target genes and either modulates PcG function or reactivates gene expression regardless of PcG silencing.

Thus, in order to investigate whether AP-1-binding sites found within the regulatory DNA regions of *unpaired* (*upd*) and *wingless* (*wg*) genes, two PcG target genes, are necessary for their reactivation during imaginal disc regeneration, we synthesized two TALEN pairs with the easyT assembly method and used them together with a homologous donor DNA to delete two AP-1-binding sites and insert a selectable marker gene to identify these mutants among F1 adults [7]. We used green fluorescent protein under the control of 3xP3 promoter to identify mutants by fluorescence in the eye.

2 Materials

2.1 easyT Unit Library Preparation

1. easyT kit (22 plasmids): Temp_type-a-NI, Temp_type-a-NG, Temp_type-a-NK,Temp_type-a-HD,Temp_type-b-NI,Temp_type-b-NG, Temp_type-b-NK, Temp_type-b-HD, Temp_type-c-NI, Temp_type-c-NG, Temp_type-c-NK, Temp_type-c-HD, Temp_type-d-NI, Temp_type-d-NG, Temp_type-d-NK, Temp_type-d-HD, Temp_unit1(a′)-NI, Temp_unit1(a′)-NG, Temp_unit1(a′)-NK, Temp_unit1(a′)-HD, Temp_tail unit, TALEN backbone plasmid. The easyT kit is available from our lab upon request.
2. Phusion® High-Fidelity DNA Polymerase.
3. QIAquick PCR Purification Kit.
4. Agarose.
5. Tris-borate-EDTA buffer: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3.

2.2 TALEN Synthesis with easyT

1. Restriction enzymes: BaeI.
2. T4 DNA ligase.
3. QIAquick Gel Extraction Kit.
4. DNA Clean & Concentrator™-5.
5. *Taq* DNA Polymerase with ThermoPol® Buffer.

6. GenElute™ Plasmid Miniprep Kit.
7. DH5α competent *E. coli*.

2.3 *Drosophila* Embryo Microinjection

1. QIAGEN Plasmid Midi Kit.
2. Fly lines: w^{1118} and w^{1118} $lig4^{169}$.
3. UV microscope.
4. QIAamp DNA Micro Kit.

3 Methods

The method described in this chapter can be divided into three parts: easyT unit library preparation, TALEN synthesis with easyT, and homologous donor DNA cloning and *Drosophila* embryo microinjection.

3.1 easyT Unit Library Preparation and TALEN Backbone Plasmid Pre-digestion

1. Use Phusion® High-Fidelity DNA Polymerase and set up 48 PCR reactions following the manufacturer's recommendations. Use plasmids from easyT kit as a template for PCR reactions. Consult Tables 1 and 2 for plasmid template and primer pair information. Use 5 ng of plasmid template per each 50 μL PCR reaction.
2. Run 5 μL of each PCR reaction on 0.8% agarose gel to confirm a single band of the correct size in each reaction (*see* **Note 1**). Amplified unit sizes: unit 1—137 bp, unit 2, 6, 10, 14, 18—158 bp, unit 3, 7, 11, 15, 19—157 bp, unit 4—142 bp, unit 5—148 bp, unit 8—145 bp, unit 9—144 bp, unit 12—146 bp, unit 13—143 bp, unit 16—148 bp, unit 17—141 bp, tail unit 17—136 bp, tail unit 18—143 bp, tail unit 19—139 bp, tail unit 16 and 20—136 bp.
3. Purify DNA from PCR reactions using QIAquick PCR Purification Kit and dilute DNA to 5 ng/μL. Label tubes with nucleotide recognized by the RVD and unit number. For example, unit 1 generated from template Temp_uit1(a′)-NI is labeled as A1 (tail unit is labeled as tail 16, tail 17, etc.). Units are ready to be used for TALEN assembly.
4. Digest TALEN backbone plasmid (easyT kit) with BaeI. Use 4 μL of 10× T4 DNA ligase buffer, 0.4 μL of 100× BSA, 2.5 μL of 0.32 mM *S*-adenosylmethionine (SAM), 4 μL of BaeI, and 1 μg of TALEN backbone plasmid, and dilute with sterile water to 40 μL. Incubate at 25 °C for 2 h.
5. Run digestion reaction on 0.8% agarose gel. Excise the 5.2 kb band and extract DNA using QIAquick Gel Extraction Kit. Pre-digested TALEN backbone plasmid is ready to be used for TALEN assembly (*see* **Note 2**).

Table 1
Primers for easyT TALEN assembly method and genomic DNA PCR

Primers for easyT unit library	
TALunit1-F(MluI)	AAAAGTACCAATATGATTCACGCGTGGCGCAATGCACTGA
TALagcct-R	GATAGGTACTAAGGTAGCCTGTACTAGGCTTGCTTGCCGCC
TALagcct-F	CTTGACTTTAGTACCTAAGCACAGCCTTGGAGACGGTGCAGC
TALcttga-R	GATAGGTACTAAGGTAGCCTGTACTTCAAGAGCTTGCTTGCCGCC
TALcttga-F	CTTGACTTTAGTACCTAAGCACCTTGAGACGGTACAGCGG
TALgaaac-R	GATAGGTACTAAGGTAGCCTGTACTGTTTCCAGCGCTTGCTTGCCGCC
TALgaaac-F	CTTGACTTTAGTACCTAAGCACGAAACGGTACAGCGGCTG
TALunit4-R(HindIII)	TACGGGGGTATAGTGTACTAAGCTTGCTTGCCGCC
TALunit5-F(HindIII)	AAAAGTACCAATATGAAGCTTTGGAGACAGTACAGCGG
TALunit8-R(XbaI)	TACGGGGGTATAGTGTACTTCTAGAGCTTGCTTGCCGCC
TALunit9-F(XbaI)	AAAAGTACCAATATGTCTAGAGACGGTACAGCGG
TALunit12-R(XhoI)	TACGGGGGTATAGTGTACTCTCGAGCGCTTGCTTGCCGCC
TALunit13-F(XhoI)	AAAAGTACCAATATGCTCGAGACGGTACAGCGG
TALunit16-R(BsrGI)	TACGGGGGTATAGTGTACAGTTTCCAGCGCTTGCTTGCCGCC
TALunit17-F(BsrGI)	AAAAGTACCAATATGAGAAACTGTACAGCGGCTGTTG
TALtail17-F(BsrGI)	AAAAACAAAAGTACCAATATGAGAAACTGTACATGCCCAGTTATCTCGCCCTGA

TALtail18agcct-F	CTTGACTTTAGTACCTAAGCACAGCCTTGGAGACGATTGTTGCCCAGTTATC
TALtail19cttga-F	CTTGACTTTAGTACCTAAGCACCTTGAGACGATTGTTGCCCAGTTATC
TALtail20gaaac-F	CTTGACTTTAGTACCTAAGCACGAAACGATTGTTGCCCAGTTATC
TALtail-R(AatII)	TACGGGGGTATAGTGTACTGGACGTCCGCCGAGG
Primers for 4-mer amplification	
TAL4mer-F	AAAAACAAAAGTACCAATATG
TAL4mer-R	GGGGGGTACGGGGGTATAGTGTAC
Primers for colony PCR and sequencing	
TALrepeat-F	GTTACAGTTGGACACAGG
TALrepeat-R	ACGTGCGTTCGGGAATG
TALrepeat-F2	ACTGACGGGTGCCCCCCT
TALrepeat-R2	GGGCGAGATAACTGGGC
Primers for genomic PCR of modified wg locus	
wg-F(outside)	CAACCAAATAGTACTCCTCCCTTTCTCCTG
wg-R(on EGFP)	GTTCTTCTGCTTGTCGGCCATGATATAG

Table 2
Templates and primer pairs for easyT unit library

Unit	NI (A)		NG (T)		NK (G)		HD (C)	
	Template	Primer pair	Template	Primer pair	Template	Primer pair	Template	Primer pair
Unit 1	Temp_unit1(a′)-NI	TALunit1-F(MluI) TALagcct-R	Temp_unit1(a′)-NG	TALunit1-F(MluI) TALagcct-R	Temp_unit1(a′)-NK	TALunit1-F(MluI) TALagcct-R	Temp_unit1(a′)-HD	TALunit1-F(MluI) TALagcct-R
Unit 2, 6, 10, 14, 18	Temp_type-b-NI	TALagcct-F TALcttga-R	Temp_type-b-NG	TALagcct-F TALcttga-R	Temp_type-b-NK	TALagcct-F TALcttga-R	Temp_type-b-HD	TALagcct-F TALcttga-R
Unit 3, 7, 11, 15, 19	Temp_type-c-NI	TALcttga-F TALgaaac-R	Temp_type-c-NG	TALcttga-F TALgaaac-R	Temp_type-c-NK	TALcttga-F TALgaaac-R	Temp_type-c-HD	TALcttga-F TALgaaac-R
Unit 4	Temp_type-d-NI	TALgaaac-F TALunit4-R(HindIII)	Temp_type-d-NG	TALgaaac-F TALunit4-R(HindIII)	Temp_type-d-NK	TALgaaac-F TALunit4-R(HindIII)	Temp_type-d-HD	TALgaaac-F TALunit4-R(HindIII)
Unit 5	Temp_type-a-NI	TALunit5-F(HindIII) TALagcct-R	Temp_type-a-NG	TALunit5-F(HindIII) TALagcct-R	Temp_type-a-NK	TALunit5-F(HindIII) TALagcct-R	Temp_type-a-HD	TALunit5-F(HindIII) TALagcct-R
Unit 8	Temp_type-d-NI	TALgaaac-F TALunit8-R(XbaI)	Temp_type-d-NG	TALgaaac-F TALunit8-R(XbaI)	Temp_type-d-NK	TALgaaac-F TALunit8-R(XbaI)	Temp_type-d-HD	TALgaaac-F TALunit8-R(XbaI)
Unit 9	Temp_type-a-NI	TALunit9-F(XbaI) TALagcct-R	Temp_type-a-NG	TALunit9-F(XbaI) TALagcct-R	Temp_type-a-NK	TALunit9-F(XbaI) TALagcct-R	Temp_type-a-HD	TALunit9-F(XbaI) TALagcct-R
Unit 12	Temp_type-d-NI	TALgaaac-F TALunit12-R(XhoI)	Temp_type-d-NG	TALgaaac-F TALunit12-R(XhoI)	Temp_type-d-NK	TALgaaac-F TALunit12-R(XhoI)	Temp_type-d-HD	TALgaaac-F TALunit12-R(XhoI)

Unit 13	Temp_type-a-NI	TALunit13-F(XhoI)	Temp_type-a-NG	TALunit13-F(XhoI)	Temp_type-a-NK	TALunit13-F(XhoI)	Temp_type-a-HD	TALunit13-F(XhoI)
		TALagcct-R		TALagcct-R		TALagcct-R		TALagcct-R
Unit 16	Temp_type-d-NI	TALgaaac-F	Temp_type-d-NG	TALgaaac-F	Temp_type-d-NK	TALgaaac-F	Temp_type-d-HD	TALgaaac-F
		TALunit16-R(BsrGI)		TALunit16-R(BsrGI)		TALunit16-R(BsrGI)		TALunit16-R(BsrGI)
Unit 17	Temp_type-a-NI	TALunit17-F(BsrGI)	Temp_type-a-NG	TALunit17-F(BsrGI)	Temp_type-a-NK	TALunit17-F(BsrGI)	Temp_type-a-HD	TALunit17-F(BsrGI)
		TALagcct-R		TALagcct-R		TALagcct-R		TALagcct-R
Tail unit	Template	Primer pair						
Tail_unit17	Temp_tail unit	TALtail17-F(BsrGI)						
		TALtail-R(AatII)						
Tail_unit18	Temp_tail unit	TALtail18agcct-F						
		TALtail-R(AatII)						
Tail_unit19	Temp_tail unit	TALtail19cttga-F						
		TALtail-R(AatII)						
Tail_unit20 (Tail_unit16)	Temp_tail unit	TALtail20gaaac-F						
		TALtail-R(AatII)						

3.2 TALEN Synthesis with easyT

Here we use synthesis of a TALEN pair targeting the *wg* locus as an example to describe TALEN assembly (*see* **Note 3**). These TALENs are 20-mers, i.e., containing 20 units, and recognize 19 bp sequences (tail unit is not involved in DNA recognition). *See* Fig. 1 for a schematic overview of easyT TALEN assembly.

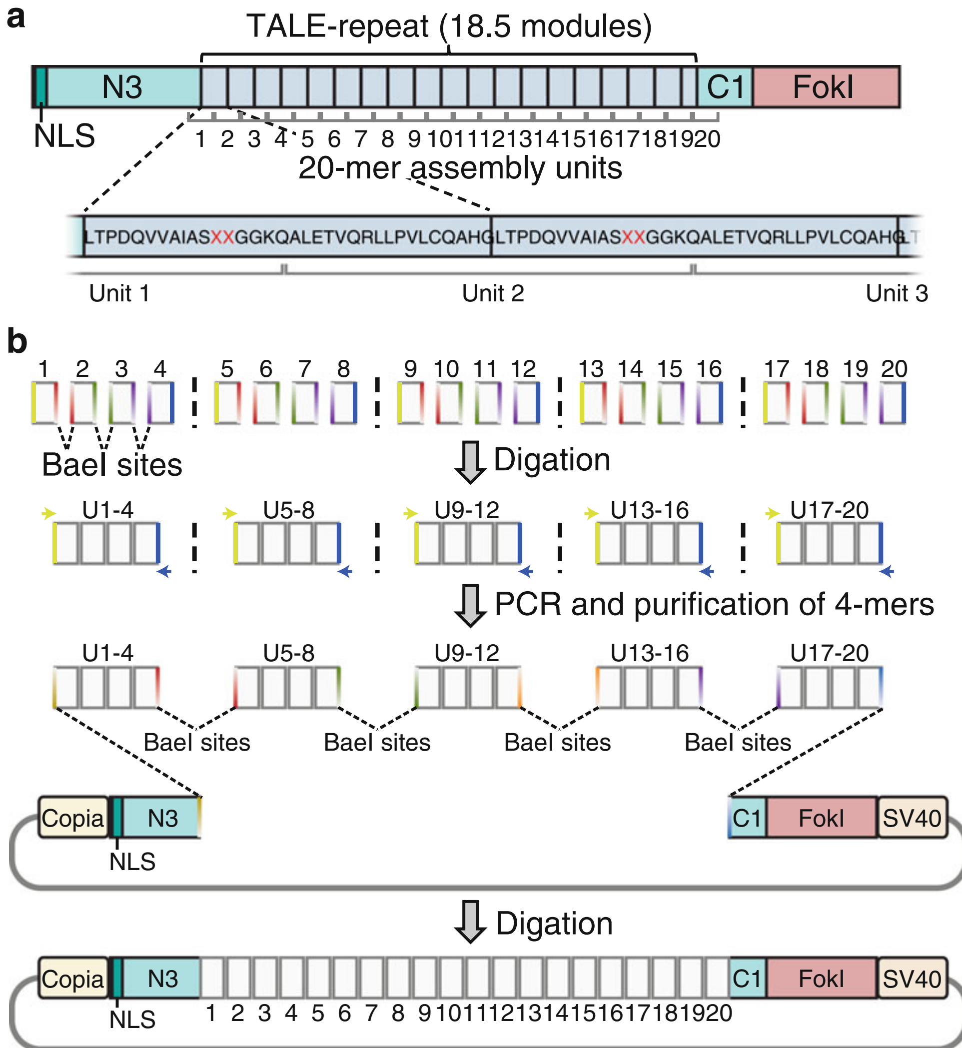

Fig. 1 Construction of TALENs with the easyT protocol. (**a**) A schematic representation of a TALEN with a TALE-repeat length of 18.5 modules. The TALE-repeat is assembled from 20 monomer units. The boundaries of monomer units were shifted from those of the TALE-repeat modules. (**b**) Overview of TALEN cloning. In the first step, four units are assembled into 4-mers in a "digation" reaction. In the second step, 4-mers are PCR-amplified, run on an agarose gel, gel-extracted, and concentrated. Finally, 4-mers were assembled into the TALEN backbone plasmid in the second digation reaction. *Yellow* and *blue arrows* indicate primers used for 4-mer amplification. Reproduced from Katsuyama, T. et al., An efficient strategy for TALEN-mediated genome engineering in Drosophila, Nucleic Acids Research, 2013, 41, 17, e163-e163, by permission of Oxford University Press

1. Use the following guidelines to select TALEN target site: TF-binding site to be deleted should be positioned in the spacer region between left and right TALEN-binding sequences, TALEN-binding sequences should be preceded by the nucleotide thymine, and the spacer region between left and right TALEN should be about 20 bp. According to these guidelines, the sequence 5′-tcatctgatgcttcacagaatcagtagc**tgactca**ctccgattcagtttcaggaattca-3′ was selected for the deletion of a putative AP-1-binding site within the *wg* locus (underlined—TALEN -binding sequences, in bold—the putative AP-1-binding site).
2. Setting up ten digation reactions. A digation reaction contains both BaeI restriction enzyme and T4 DNA ligase, and hence both DNA digestion and ligation are taking place. Thus, if digation reaction contains units 1–4, at the end of digation the reaction will contain monomers of units 1–4; 2-mers of units 1–2, 2–3, and 3–4; 3-mers of units 1–2–3 and 2–3–4; and 4-mers of units 1–2–3–4. Take ten PCR tubes (five per each TALEN) and add the following in each tube: 2 μL of 10× T4 DNA ligase buffer, 0.2 μL of 100× BSA, 1.25 μL of 0.32 mM SAM, 2 μL of 2.5 mM dNTPs, 2 μL of BaeI, 1 μL of T4 DNA ligase, and 7.55 μL of sterile water. Label PCR tubes L1 through L5 for left TALEN and R1 through R5 for right TALEN. In tube L1 add 1 μL of units C1, A2, T3, and C4; in tube L2 add 1 μL of units T5, G2, A3, and T8; in tube L3 add 1 μL of units G9, C2, T3, and T12; in tube L4 add 1 μL of units C13, A2, C3, and A16; and in tube L5 add 1 μL of units G17, A2, A3, and tail 20. Correspondingly, in tube R1 add 1 μL of units G1, A2, A3, and T4; in tube R2 add 1 μL of units T5, C2, C3, and T8; in tube R3 add 1 μL of units G9, A2, A3, and A12; in tube R4 add 1 μL of units C13, T2, G3, and A16; and in tube R5 add 1 μL of units A17, T2, C3, and tail 20. Mix by pipetting up and down several times and incubate at 25 °C for 1 h.
3. Purify DNA from digation reaction using DNA Clean & Concentrator™-5 kit and elute in 6 μL of sterile water.
4. Set up ten 50 μL PCR reactions using Phusion® High-Fidelity DNA Polymerase and primers TAL4mer-F and TAL4mer-R (Table 1). Use 1 μL of eluate from previous step to amplify 4-mers L1, L2, L3, L4, L5, R1, R2, R3, R4, and R5.

PCR program:					
98 °C	<98 °C	55 °C	72 °C>	72 °C	4 °C
3 min	10 s	10 s	15 s	7 min	∞
		<32 cycles>			

5. Run PCR reactions on 2.0% agarose gel until 4-mers are separated from 3-mers. Cut the bands corresponding to 4-mers and purify DNA using QIAquick Gel Extraction Kit (*see* **Note 4**).
6. Check concentration of 4-mers. Use DNA Clean & Concentrator™-5 kit to concentrate DNA if concentration of 4-mers is below 15 ng/μL.
7. Setting up second digation reaction: In the second digation reaction, purified 4-mers from the previous step are digated into pre-digested TALEN backbone plasmid prepared in Subheading 3.1, **step 5**. Take two PCR tubes and label them R and L for left and right TALEN. In each tube add 2 μL of 10× T4 DNA ligase buffer, 0.2 μL of 100× BSA, 1.25 μL of 0.32 mM SAM, 2 μL of 2.5 mM dNTPs, 2 μL of BaeI, 1 μL of T4 DNA ligase, and 30 ng of pre-digested TALEN backbone plasmid. In tube L add 30 ng of each of the 4-mers L1, L2, L3, L4, and L5 from Subheading 3.2, **step 5**. In tube R add 30 ng of each of the 4-mers R1, R2, R3, R4, and R5 from Subheading 3.2, **step 5**. Mix by pipetting up and down several times and incubate at 25 °C for 1 h.
8. Use 10 μL of second digation reactions to transform competent *E. coli* cells. We use DH5α-competent *E. coli*. Spread transformed *E. coli* cells on agar plates selecting for ampicillin resistance.
9. Identify colonies containing full-length TALENs by colony PCR. We recommend picking at least ten individual colonies per TALEN. Set up 20 PCR reactions. In each PCR tube add 2.5 μL of 10× ThermoPol buffer, 2 μL of 2.5 mM dNTPs, 1 μL of 10 mM primer TALrepeat-F, 1 μL of 10 mM primer TALrepeat-R, 0.1 μL of Taq DNA polymerase, 8.4 μL of sterile water, and a single *E. coli* colony dissolved in 10 μL sterile water.

PCR program:					
94 °C	<94 °C	55 °C	72 °C>	72 °C	4 °C
5 min	30 s	30 s	3 min	7 min	∞
		<25 cycles>			

10. Run completed colony PCR reactions on 0.8% agarose gel. For ease of identification of positive colonies, we recommend running on a gel a PCR reaction with a previously confirmed TALEN (control) as a template. Figure 2 is an example of a gel picture with 1 kb DNA ladder, control TALEN PCR reaction, and eight colony PCR reactions (lanes 1–10, respectively). The band pattern of a control 20-mer TALEN shows a characteristic five-band pattern, with bands corresponding to 4-mer, 8-mer, 12-mer, 16-mer, and 20-mer. Colonies with this five-band pattern are positive colonies containing full-length TALENs. Culture positive colonies overnight in LB medium supplemented with ampicillin.

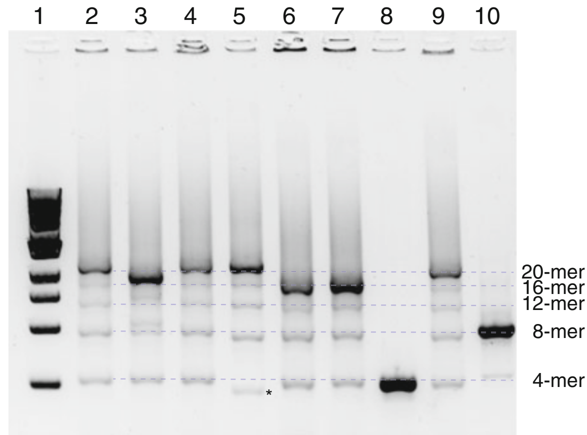

Fig. 2 Identifying positive clones by the pattern of PCR bands. The amplicons of colony PCR were run on a 0.8 % agarose gel. 1 kb DNA ladder (NEB) was loaded on lane 1. Positive clone having full 20-mer units (lane 2) shows a strong 20-mer band around 2 kb and additional four weak bands at the size of 4-, 8-, 12-, and 16-mer. Based on the pattern of these bands, we can estimate the number of 4-mers and identify possible positive clones: lanes 4 and 9. While lane 5 shows a strong band at 20-mer size, it is a negative because the size of the lowermost band (*) is obviously different. Reproduced from Katsuyama, T. et al., An efficient strategy for TALEN-mediated genome engineering in Drosophila, Nucleic Acids Research, 2013, 41, 17, e163-e163, by permission of Oxford University Press

11. Purify plasmid DNA from overnight cultures of positive colonies using GenElute™ Plasmid Miniprep Kit. Sequence plasmids using primers TALrepeat-F and TALrepeat-R and discard clones containing PCR-induced mutations (*see* **Note 5**).

3.3 Homologous Donor DNA and *Drosophila* Embryo Microinjection

1. 2 kb of genomic DNA sequences upstream and downstream of the putative AP-1-binding site targeted for deletion were amplified and cloned upstream and downstream of 3xP3-EGFP expression cassette. Thus, a selectable marker gene replaces AP-1-binding site on the donor DNA.
2. Next, donor DNA plasmid and two TALEN plasmids were midi-preped with QIAGEN Plasmid Midi Kit and injected into embryos of w^{1118} $lig4^{169}$ strain following standard embryo microinjection protocol (*see* **Note 6**). As a starting point, we recommend injecting 50 ng/μL per each TALEN plasmid and 100 ng/μL for donor DNA plasmid (*see* **Note 7**).
3. The adults of microinjected embryos were crossed to w^{1118}. Progeny of this cross was scored via green fluorescence in the eye for mutants that have integrated donor DNA.
4. Isolate genomic DNA of mutant flies using QIAamp DNA Micro Kit.

5. PCR reaction on genomic DNA of mutant flies using primers aligning on EGFP and outside of homology arms can be performed to check for a band of expected size and to confirm targeted integration (*see* **Note 8**).

4 Notes

1. If more than one band appear on the gel, use QIAquick Gel Extraction Kit to excise the band of the correct size and proceed to Subheading 3.1, **step 3**.
2. In our experience, complete digestion and clean extraction of the TALEN backbone plasmid are crucial to increase frequency of full-length TALENs in Subheading 3.2, **step 10**. To test whether TALEN backbone plasmid is completely digested, a ligation reaction containing only pre-digested TALEN backbone plasmid can be carried out. Completely digested and properly extracted TALEN backbone plasmid would not form self-ligation products and would not produce ampicillin-resistant *E. coli* colonies after transformation.
3. Our computation analysis indicated that sequences as short as 13 bp could be sufficient to specify uniquely a position within non-repetitive euchromatic regions of the *Drosophila* genome [7]. We also tested TALEN pairs recognizing 15 and 19 bp sequences and found no evidence that longer sequences result in higher frequency of targeted genome modification. Additionally, synthesis of TALENs recognizing 15 bp sequences is more efficient than synthesis of TALENs recognizing 19 bp sequences. Thus, if there are no additional restrictions/considerations on TALEN size or recognition sequence length, we recommend synthesizing TALENs recognizing 15 bp target sequences (i.e., 16-mers).
4. It is important to separate 4-mers from 3-mers on the gel. Presence of 3-mers in the second digation reaction (Subheading 3.2, **step 7**) would reduce the frequency of full-length TALENs in Subheading 3.2, **step 10**.
5. The sequencing company that performed TALEN sequencing for us can generate sequence read lengths of up to 1100 bp. Thus, 20-mer TALEN is the largest TALEN size we can generate and sequence through all units using forward and reverse primers aligning on TALEN backbone plasmid (outside of unit sequences). If the average sequence read length produced by your sequencing service provider is shorter than 1100 bp, consider synthesizing TALENs of smaller size (i.e., 16-mer, 17-mer).
6. We used homozygous $lig4^{169}$ mutant flies as recipients of microinjections. w^{1118} $lig4^{169}$ strain had been previously shown to increase donor DNA integration via HR [8].

7. We believe that optimal concentration of TALEN plasmids and donor DNA plasmid is a function of affinity of TALEN pair to their target site, TALEN toxicity due to off-target binding, toxicity associated with genomic regions on the donor DNA, and toxicity due to high concentration of plasmid DNA. As a result, optimal concentration of TALEN plasmids and donor DNA plasmid has to be determined empirically for each new experimental setup.
8. We perform genomic DNA PCR with primers aligning within EGFP and outside of homology arm sequences present on donor DNA to confirm targeted integration of 3xP3-EGFP and deletion of AP-1-binding site. In our experience, 2 kb regions can be easily amplified from genomic DNA. Consequently, the length of the homology arm sequences is constrained by the ability to amplify this region from genomic DNA.

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