XTN™ TALENs
Site-Specific Nucleases

User Manual

Version 2.0

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1.1 Introduction and Background

Transcription Activator-Like Effector Nucleases (TALENs) are proteins that are secreted from the bacteria *Xanthamonas*. TALENS derived from *Xanthomonas* (XTN™ TALENs) have two important domains. The first is a DNA-binding domain. The second is a nuclease domain. The DNA binding domain consists of highly conserved 33-35 amino acid repeat sequences that recognize specific DNA sequences. The amino acids in positions 12 and 13 of the amino acid repeats vary and convey the specific DNA-binding properties. For example, the amino acid sequence NI binds to A, HD binds to C, NG binds to T, NN binds to G (see figure 1). XTN™ TALENS can be designed to bind to specific locations in the genome just by knowing the sequence of the DNA, and using this code to assemble a plasmid clone that expresses the appropriate amino acids at residues 12 and 13 within the repeated domains. Once bound to the DNA, the dimerized nuclease domain cuts the DNA.
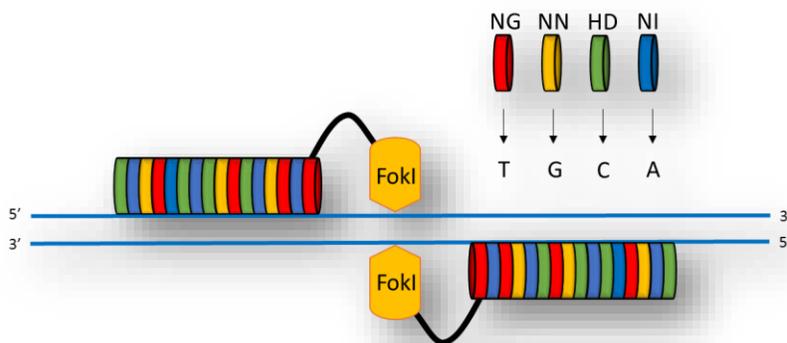

Figure 1. XTN™ TALENs consist of a DNA-binding domain and FokI nuclease domain. Variable amino acid repeats create predictable, specific DNA-binding patterns.

XTN™ TALENs been engineered to have the FokI and other nuclease domains expressed at the C-terminus. The XTN™ TALENs work as a pair of heterodimers at the respective recognition sites to induce double strand DNA breaks. One XTN™ TALEN is engineered to the top strand of DNA; the other XTN™ TALEN is engineered to the bottom strand of DNA. Subsequent non-homologous end joining (NHEJ) repair can introduce deletions or insertions, which cause the reading frame to shift, thus resulting in knockout mutations. Co-transfection of an XTN™ TALEN pair with a targeting vector can result in incorporation of the donor sequences through homologous recombination (HR) to repair the cut site, thus resulting in knock-in mutations (see Figure 2).
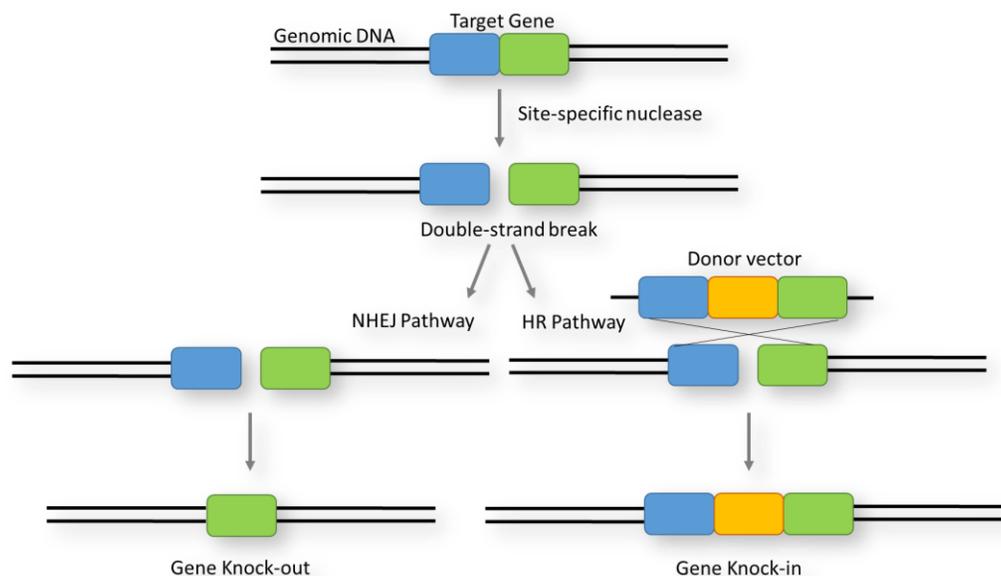
***Figure 2.** Mechanism of XTN™ TALEN mediated mutagenesis. The DNA-binding domain binds at a specific nucleotide target sequence. The FokI domain then induces a double-stranded DNA break. (Middle) Knockout mutations are created via non-homologous end joining, which occurs naturally in the cells, causing small deletions or insertions that disrupt the reading frame of the DNA. Knock-in mutations can be created through homologous recombination along with a donor vector that has the desired DNA sequence.*

Transposagen's proprietary XTN™ TALEN assembly system provides the fastest turn-around time in the industry. We are also able to create targeting vectors for knock-in and gene editing studies. The XTN™ TALEN technology can be used in conjunction with Transposagen's other core technology, the *piggyBac*™ transposon vectors, which provides the only method for Footprint-Free™ Genetic Engineering down to a single base pair. For additional information, visit the Transposagen website at http://www.transposagenbio.com/gek-footprint-free.

1.2   Design and Construction of XTN™ TALENs

After submission of your target gene sequence, our scientists will design and construct your custom XTN™ TALENs. Transposagen delivers 10µg – 50µg of sequence verified, transfection-ready plasmids. More information about pricing and ordering can be found on the Transposagen website at http://www.transposagenbio.com/xtn.

Note: Transposagen does not perform cleavage assays/activity assays for standard orders. For an additional fee-for-service, this assay can be performed. Contact your local Gene Editing Specialist or email info@transposagenbio.com for more information.

1.3 **Choosing a Donor Plasmid**
If you are planning a knock-in or gene editing experiment, you will also need to choose a donor plasmid. In addition to the sequence needed to make the desired mutations, a donor plasmid may contain a selectable marker such as an antibiotic resistance or a fluorescence gene. The use of selection can reduce the number of clones that must be screened for correct insertion of the mutation. Transposagen has many pre-validated selection cassettes available in the Multivector™ series**.** These include, but are not limited to:

Puromycin/TK   Multivector™ Catalog# PB-MV1Puro-TK
Neomycin/TK   Multivector™ Catalog# PB-MV1Neo-TK
GFP/Puromycin   Multivector™ Catalog# PB-MV1CMV-GFP-Puro
Neomycin   Multivector™ Catalog# PB-MV1Neo
Hygromycin/TK   Multivector™ Catalog# PB-MV1Hygro-TK

1.4 **Protocol – Introduction of XTN™ into Target Cells in Culture.**
XTN™ TALEN can be introduced into target cells using the method that is most appropriate for the target cells. Below are example conditions others have used for transfecting cells with site-specific nuclease technologies. Nucleofection or electroporation usually results in the highest cleavage efficiencies, so it is recommended to use an electroporation or nucleofection delivery method if possible. For gene editing and targeted integration purposes, you will also want to include a donor plasmid in the transfection/ electroporation of the XTN™ TALEN pair into the target cells.

A. Transfection into Cell Lines in Culture
Lipid-based transfection works in many cases, and many researchers choose to use Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions, especially for commonly available cell lines such as CHO, 293, HT1080, HepG2, and NIH 3T3. Transposagen recommends using 3 µg of each XTN™ TALEN for transfections using lipid-based transfections.

B. Transfection of CHO-K1 cells using Amaxa Nucleofector II
1. Mix $1x10^6$ CHO-K1 cells with 1 µg of each XTN™ TALEN and 100 µL Amaxa Solution T.
2. Transfect cells in an Amaxa Nucleofector II using program U-23
3. Recover into 1.4 ml warm F-12 medium + 10% FCS.
4. Grow as described in Chaderjian et al., 2005.

C. Transfection of Mouse Embryonic Stem Cells using Lipofectamine 2000
1. Transfect ES cells using Lipofectamine 2000 (Invitrogen) with 3 µg of each XTN™ TALEN.
2. 15 hours after transfection, change to ESLX media containing ES-DMEM, 20% ES qualified FBS, 1× nonessential amino acids, 1× nucleosides, 1,000 U/ml ESGRO LIF (Chemicon), 2 mmol/l l-glutamine, 1× Pen/Strep (Invitrogen), and 0.12 mg/ml sodium pyruvate and 0.1 mmol/l BME (Sigma-Aldrich). (Connelley et al., 2010)

D. Electroporation of Cells in Culture
1. We recommend using $2x10^6$ cells in 400 µl OptiMEM (Invitrogen), 10 µg of each XTN™ TALEN, and 40 µg of donor vector.
2. Electroporation conditions should be optimized for each cell line and may depend on the electroporation instrumentation being used. (Perez-Pinera et al., 2012)

E. Direct delivery of XTN™ TALEN proteins into Cells
Gaj et al., 2012 have published that it is possible to directly introduce ZFNs in the protein form into fibroblasts. This method may prove useful for XTN™ TALENs as well.

1.5   Creation of Transgenic Animals with XTN™ TALENs

A. Creation of Transgenic Rat Spermatogonia with XTN™ TALENs using Nucleofector LONZA
We recommend using the protocol described in Izsvák et al. (2010) for creation of transgenic rat spermatogonia. This protocol uses the Amaxa Cell Line Nucleofector Kit L to introduce the DNA.
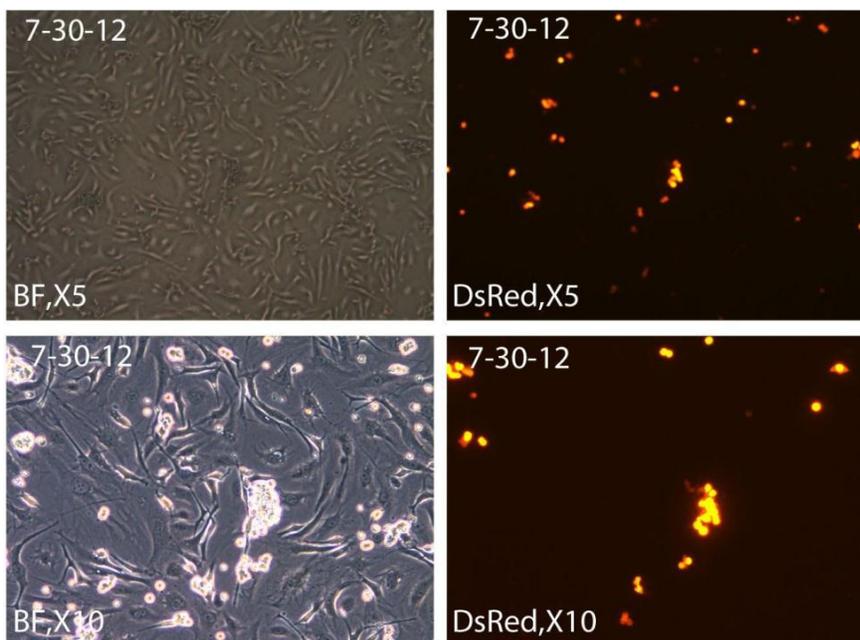

Figure 3. *This image shows the results of nucleofection in rat SSCs. Spermatogonial stem cells from WT Sprague Dawley (SD) rats were used for transfection with highly purified plasmid DNA by Nucleofection. Nucleofection was perfomed with Nucleofector device (Lonza) and Cell line Nucleofector Kit from Amaxa. DNA amount: 3μg L-XTN™ TALEN +3μg R-XTN™ TALEN +3μgDsRed. Transfection efficiency is 50-60%.*

B. Injection into Rat Embryos
For injection into embryos, we recommend the method published in Guerts et al. (2010). For more details, we recommend reading the reference. We have summarized the protocol here. The XTN™ TALENS can be delivered to embryos as supercoiled plasmid DNA or as mRNA.
1. Prepare plasmid DNA using the GenElute HP midiprep kit (Sigma-Aldrich) following the manufacturer's instructions.
2. Resuspend in DNA Injection Buffer at 10 ng/μL and store at −20°C until use.
3. Harvest the rat embryos and keep in a 37°C incubator with 5% CO_2 until needed.
4. Thaw the prepared DNA and dilute it to the desired concentration using DNA Injection Buffer.
5. Using a microinjector, deliver the XTN™ TALENs to one-cell fertilized embryos. The XTN™ TALENs should be delivered to the pronucleus.

C. **Introduction into Xenopus oocytes**
1. The XTN™ TALEN expression vectors can be linearized with *PmeI.*
2. The linearized vectors can then be transcribed and polyadenylated*in vitro* using the mMESSAGE mMACHINE t7 Ultra kit (Ambion).
3. The resulting mRNA can be injected into Xenopus oocytes.

1.6 Cleavage Analysis

A. **Enrichment for cells containing Nuclease-Induced Mutations**
An effective protocol for determining efficient cleavage has been described by Kim et al., 2011. This method includes an enrichment step for cells containing nuclease-induced mutations. The protocol is summarized here.
1. In this method, a reporter plasmid is also transfected into cells that contains monomeric RFP-enhanced GFP (mRFP-eGFP) as a fusion protein. The eGFP, however is out-of-frame with the mRFP due to a nuclease target site that has been inserted between the two.
2. When the XTN™ TALEN has not cleaved the fusion protein, the cells fluoresce only red. If an XTN™ TALEN cleaves the fusion protein, the cells will fluoresce both red and green.
3. This method enables one to determine whether a given pair of XTN™ TALENs has a high rate of cleavage. Cells can be sorted by FACS analysis or clonal selection, and then further sequenced to verify the presence of the desired genetic changes.

B. **CelI Mutation Detection Assay Using Fluorescent Primers**
The CelI mutation detection assay is another reliable way to look for genetic changes mediated by XTN™ TALENs (Kulinski et al., 2000). CelI is a nuclease isolated from celery that is stable over a wide range of conditions. It can recognize areas of DNA mismatch and cleave the DNA at those locations.
1. PCR is used to amplify both the normal and mutant alleles, using primers that have a 6-carboxyfluorescein (FAM) tag on one and a 4,7,2',7'-tetrachloro-6-carboxyfluorescein (TET) tag on the other.
2. When the DNA is denatured and then renatured, mismatches occur and the CelI enzyme recognizes the mismatches and cuts them on only one strand.
3. The resulting digested DNA is run on a DNA sequencer such that a digestion product will fluoresce either blue (FAM) or green (TET). The sizes of the two fragments indicate the location of the mismatch, and the sum of the sizes of the fragments equals the entire length of the PCR product.

C. **CelI Mutation Detection Assay using PAGE**
The CelI Mutation Detection Assay can also be performed without using fluorescent primers (Hibbs et al. Sigma-Aldrich). The reaction works the same way, except that visualization of the fragments is performed using polyacrylamide gel electrophoresis as shown in Figure 4.
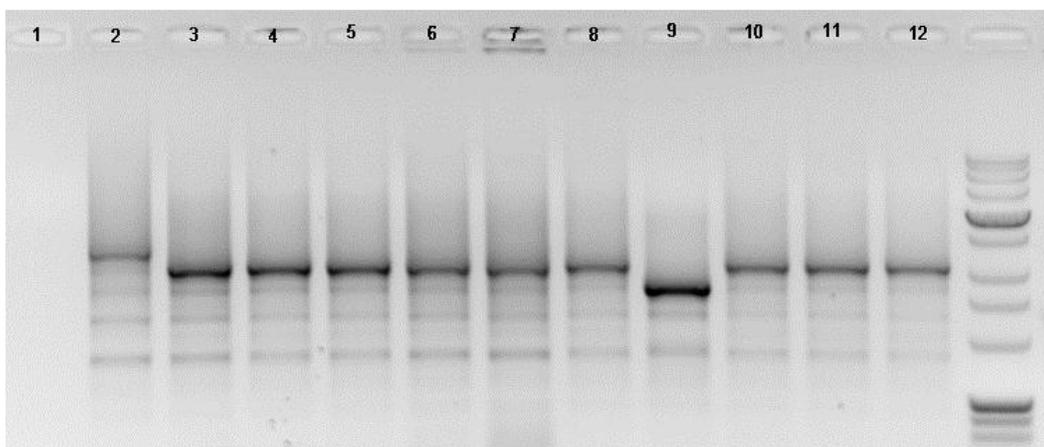
Figure 4. *Individual bacterial colonies were picked, and PCR was used to verify the presence of the correct sized bands. Clones that had the correct band were further sequence verified.*

D. PCR and Sequencing for Cleavage Analysis

Another method for cleavage analysis is to do successive rounds of PCR and sequencing. This method is more time-consuming because often many clones need to be analyzed in order to find one that has the desired cleavage efficiency. (Kim et al., 2010)

1. After 2-3 days of culture, isolate genomic DNA from the cells. Perform a PCR using primers that flank the deleted chromosomal sequence, and that result in a PCR product that is less than 1 kb. The Tm should be ~60°C and the GC content should be 40-60%. Run the PCR for about 30-35 cycles. Then run the PCR out on an agarose gel.
2. The next step is for the PCR product to be sequenced. Subclone the PCR fragment into a blunt vector and then transform into bacterial cells. Pick colonies the next day, run a miniprep, and sequence the inserts using a primer that is included in the vector. Align the sequences with the known wild-type sequence and identify any deletions or insertions within the newly created sequence.
3. Estimate the frequency of the genetic change within the genomic DNA from the original samples by doing nested PCR.
4. Once you have a pair of XTN™ TALENs that have a high cleavage efficiency, use those sequences to further transfect or electroporate the plasmids into your target cells.

E. Cell Line Generation

In general, there are two ways to generate cell lines including knockout cell lines or transgenic cell lines.

1. If you use a homologous recombination vector (donor plasmid) with an antibiotic resistance marker (Neo, puro, etc.) that inserts near the XTN™ TALEN cleavage site, you can select for resistant cells to greatly enrich for knockouts or transgenics. However, some copies of the donor vector will undergo random integration, so that not all cells that express the antibiotic resistance will have the desired genetic change. We recommend isolating clones by dilution cloning or single cell sorting. Then use PCR to screen for targeting at the cut site. One primer should anneal within the resistance marker or promoter and one in the genome *outside* the arms of homology. Note that the vector should not contain the entire recognition sequence of the XTN™ TALENs to avoid having the vector cleaved inadvertently.

2. The other way is to not use a homologous recombination vector, but rely on nonhomologous end joining to disrupt the coding sequence (see Figure 5). Again you'll want to isolate clones, then perform PCR with oligonucleotides that flank the cut site by a couple hundred bases. The PCR product should show a deletion or may sometimes show small insertions. It is also possible to see a much shorter PCR product in some cases. However, both alleles may not have cut, so the sequence results will be noisy near the cut site, and clean before it. If all alleles did cut, the sequence results will be clean but will be different from the wild-type. This method may require screening hundreds of clones to find a clone with the desired genetic change. With either method, if not all alleles are disrupted, another round of transfections may be required.

Figure 5. *Sequencing results of several knockout clones using XTN™ TALENs. Green letters indicate the engineered XTN™ TALEN left and right binding sites. Grey letters indicate native sequence. Red letters indicate mutations. In this case, the mutation frequency was 16%.*

1.7 Frequently Asked Questions

Q. How do I know where to cut my DNA?
A. The answer depends on your particular application. However, a general design parameter is to BLAST your sequence to be sure it is not repetitive. Also, if you plan to knockout a gene by cutting with XTN™ TALENs, a common strategy is to cut in the first coding exon, usually 50-100 bases from the start codon. Please note that this strategy does not necessarily result in completely non-functional gene product(s) and you should do the proper bioinformatics analysis before ordering.

Q. Can the XTN™ TALEN be subcloned into a different vector backbone?
A. Yes, there is a unique restriction enzyme site just before the ATG and after the stop codon. Please note that the destination vector must have a promoter and polyA tail (or whatever other signals are necessary for the species to express the XTN™ TALEN appropriately).

Q. Can I make RNA of the XTN™ TALEN from these plasmid backbones?
A. Yes. There is a T7 promoter for *in vitro* RNA synthesis using kits such as mMESSAGE mMACHINE T7 Ultra kit from Ambion. The vector can be linearized using PmeI.

Q. Do the XTN™ TALENs employ Fok1 nucleases that can homodimerize?
A. Our current XTN™ TALENs use the wild-type Fok1 nuclease and it can form homodimers. The long recognition sequences employed (generally 16-18 bp per XTN™ TALENs) help minimize any concerns about off-site cutting.

Q. Is the DNA prep I receive suitable for immediate transfection of my cells?
A. Yes, it is endotoxin-free and ready to use.

Q. If I need to make more DNA, are there any special instructions?
A. We recommend using recombination-deficient cells, STBL3 or the equivalent, to minimize the risk of recombination of the repetitive elements inherent in XTN™ TALEN-encoding DNA.

Q. Do you BLAST or otherwise analyze the requested cut sequence for specificity within the genome?
A. No. It is the responsibility of the end user to determine the suitability of the sequence for his/ her own research purposes. We make no attempt to discern the intended use of the XTN™ TALEN you are ordering.

Q. What is percentage of my transfected cells will actually have the gene-of-interest knocked out or modified?
A. It is difficult to predict the percentage of cells that will be effectively knocked out. There are many factors that affect the outcome:
      1. The transfection efficiency
      2. The percentage of cells receiving both constructs
      3. The cutting efficiency
      4. Only a certain percentage of cells are cut at both alleles
      5. There may be more than 2 alleles, especially in transformed cell lines
      6. Some loci are unavailable, presumably due to chromatin structure.

Q. What if my XTN™ TALEN doesn't cut?
A. There are many factors that affect the cleavage efficiency of any nuclease (see the answer to the above question). However, we do offer a limited guarantee. If, after repeated good faith attempts with proper transfection controls, you do not see any evidence of cleavage activity, we will make another XTN™ TALEN to a nearby site from the original sequence you provided to us in your order form free of charge. You will only be responsible to pay for shipping.

Q. If I want to create an animal model or cell line, can you help us with that?
A. Yes. Transposagen offers custom cell line and animal model production. When a custom service order is received we always provide the XTN™ TALENs to the customer for in-house use as the first milestone for the project.

1.8 References

1. Chaderjian et al. (2005) *Biotechnol. Prog.*,21:550-553.
2. Connelley et al. (2010) *Molecular Therapy*, 18(6):1103–1110.
3. Gaj et al. (2012) *Nature Methods,* 9:805-807.
4. Geurts et al. (2010) Chapter 15: Generation of Gene-Specific Mutated Rats Using Zinc Finger Nucleases in *Methods in Molecular Biology*, pg. 211-225.
5. Hibbs et al. http://www.sigmaaldrich.com/etc/medialib/docs/Sigma-Aldrich/Posters/1/knockout_cancer_lines_poster.Par.0001.File.tmp/knockout_cancer_lines_poster.pdf
6. Izsvák et al. (2010) *Nature Methods*, 7:443-445.
7. Kim et al. (2010) *Cold Spring Harb Protoc.* 8:August, 1-7.
8. Kim et al. (2011) *Nature Methods*, 8(11):941-943.
9. Kulinski et al. (2000) *BioTechniques*, 29:44-48.
10. Perez-Pinera et al. (2012) *Nucleic Acids Res.* 40(8):3741-3752.
11. Reyon et al. (2012) *Nature Biotechnology,* 30(5):460-465.
12. Yusa et al. (2011) *Nature*, 478:391-394.