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Product Information

CompoZr® Custom Zinc Finger Nuclease (ZFN)

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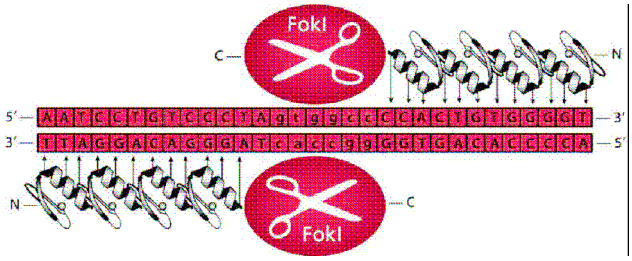
Product Description

CompoZr Zinc Finger Nucleases (ZFNs), a class of engineered endonucleases, facilitate targeted genome editing by binding to a user-specified genomic locus and causing a double-strand break (DSB). The cell then employs endogenous DNA repair processes, either non-homologous end joining (NHEJ) or homology-directed repair (HDR), to repair this targeted DSB. These repair processes can be channeled to generate precisely targeted genomic edits resulting in an organism or cell lines with specific gene disruptions (knockouts), integrations, or modifications such as disease-associated SNPs.

CompoZr ZFNs consist of two functional domains: a DNA-binding domain comprised of a chain of zinc finger proteins and a DNA-cleaving domain comprised of the nuclease domain of F*okI*.



Each zinc finger DNA binding protein recognizes approximately 3 base pairs of DNA sequence. By combining 4-6 zinc finger proteins together, each ZFN can target and specifically bind a 12-18 base pair sequence. Importantly, the endonuclease domain of F*ok*I has been reengineered to function as an obligate heterodimer in order to cleave DNA (Doyon et al., 2011). This means a *pair* of ZFNs is required to bind and cut the genomic DNA at the targeted site and this property is used to ensure specificity. The target sequences for each ZFN must be separated by 5-7 base pairs to allow formation of the catalytically active F*ok*I dimer. The 24-36 base pair DNA binding specificity and additional positional constraints drive a very high degree of precision in genome editing. Each set of CompoZr ZFNs has been validated by Sigma to cleave at the genomic site of interest.

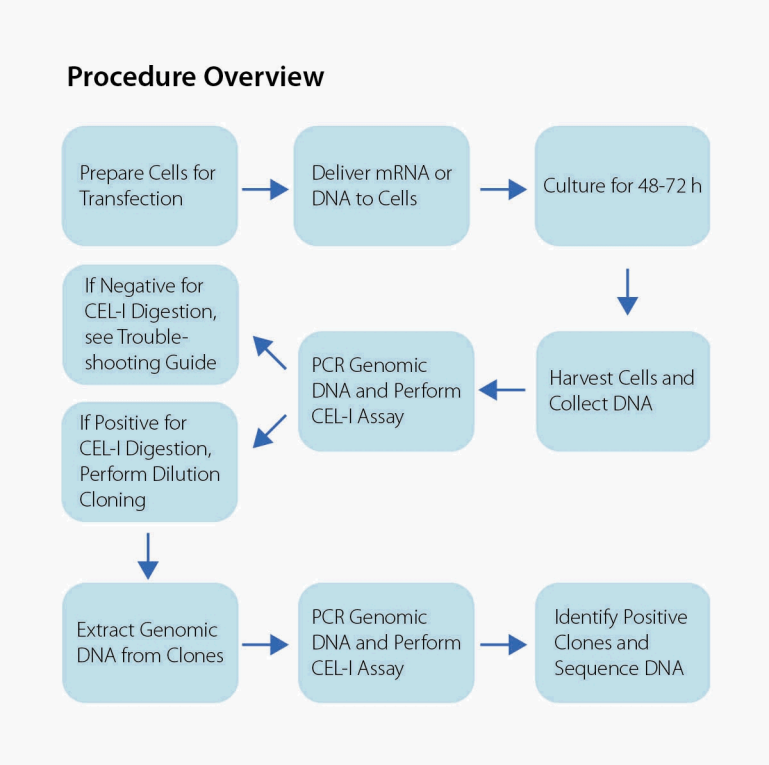
Provided in the kit are the ZFNs designed against the user-specified gene in the target organism. We provide two plasmids encoding the ZFN pair for the best performing ZFN and optional ready-to-deliver mRNA for the same ZFN pair. Included in the kit for ZFNs targeted to *human, rat, mouse and hamster* are primers for screening as well as genomic DNA from samples where the supplied ZFN has been tested. For ZFNs against all other organisms, plasmid DNA for the next two highest efficiency pairs of ZFNs is also provided.

The ZFNs can be delivered to cells as either mRNA, plasmid DNA, or lentiviral particles; preferably integration deficient lentivirus (IDLV). The major advantage of mRNA is that it eliminates the need to use different promoters for ZFN expression in different cell types, as mRNA is universal to all cell types. For instance, mRNA tends to work better in HeLa cells than plasmid since the CMV promoter on ZFN plasmids is not as active in HeLa as in other common cell types. *Please make sure the utmost caution is taken when handling mRNA to avoid degradation and loss of activity.* There are advantages to using plasmid DNA as well. ZFN expression is prolonged when originating from plasmid DNA, and may help in cell types where lower frequencies of ZFN cutting are observed. Additionally, plasmid DNA provides a source for generating more mRNA when existing mRNA stocks are used up.

For cell types that are not compatible with nucleofection or lipid based transfection methods, we recommend trying IDLV. To overcome lower transgene expression levels typically associated with IDLV, all IDLV-ZFN expression cassettes include built-in GFP and RFP reporters to help in rapid optimization of transduction conditions and/or enrichment of successfully transduced cells via FACS.

Cell line modification using ZFNs is simple and relies on standard processes such as transfection, dilution cloning, and genotyping. We have successfully used CompoZr ZFNs for targeted gene editing in a variety of cell types including K562, HEK293, HeLa, A549, MCF-7, C6, Neuro-2A, LNCaP and CHO cells (Santiago et al., 2008; DeKelver et al., 2010; Chen et al., 2011), as well as iPS cells (Hockemeyer et al.,2010) and animal models (Geurts et al., 2009). The CompoZr technology is compatible with standard methods of DNA delivery into cells, including microinjection, lipid-based transfection, electroporation, and nucleofection. Following delivery into the cell, ZFN-mediated editing will occur in as little as three days. Cells harboring the desired genome edits can then be isolated by dilution cloning and screening of individual clones by genotyping or phenotypic assays (Santiago et al., 2008). The targeted deletion and integration events that happen in ZFN-treated cells give rise to a population of cells containing multi-allelic, monoallelic modification, or unmodified gene sequences. From this pool it is possible to rapidly isolate several cell lines containing either heterozygous or homozygous mutations. Aneuploid cell lines are also conveniently modified since triploid to hexaploid loci have all been successfully modified using ZFNs in the absence of antibiotic selection.

Previous methods for generating modified cell lines have relied heavily on random integration of a plasmid construct and required extensive screening approaches to generate a single, usable cell line. Much of the work with targeted genome modifications has been limited to mouse models and still requires several rounds of reproduction to generate progeny for experiments. The limited applicability of prevalent techniques prohibits numerous laboratories from attacking important biological questions. CompoZr ZFNs provide a new and exciting way to target many organisms and cell types for modification at a single defined locus, allowing mutations, correction, and deletions within the natural chromosomal context. In addition, the high efficiency of gene modification using CompoZr ZFNs greatly reduces the time it takes to generate a clonal cell line for research use.



Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices. Sigma's CompoZr zinc finger nucleases have been designed for maximum target specificity, however off targeting may occur in certain instances and should be assessed by whole genome sequencing.

Storage and Stability

Store the kit at –70 °C immediately upon arrival. When removing kit components, ensure the tubes containing mRNA, which will not be immediately used, remain frozen. Please avoid repeated freeze thawing of the pure mRNA component (if applicable to your order) and remove only as many vials from the kit for thawing as are needed for immediate experiments. All components can be stored at –80 °C for up to 12 months.

Practice aseptic techniques to avoid RNase contamination of the components. Caution must be taken to avoid RNase contamination during ZFN mRNA preparation, especially during the RNA elution and post-elution handling steps. The work area and the pipette set must be free of RNases. Use RNase*ZAP*™ to decontaminate the work area and the pipette set if necessary. Use RNase-free pipette tips, preferably those with an aerosol barrier. Always wear gloves and change them often. Keep reagent vials and sample tubes closed when not in use.

Kit Components

Component	Catalog Number	Quantity
ZFN mRNA	MRZFN	1 each
pZFN1	D1ZFN	20 µg
pZFN2	D2ZFN	20 µg
ZFN Primer F	PFZFN	25 µmoles
ZFN Primer R	PRZFN	25 µmoles
ZFN Control DNA	CDZFN	1 vial

Note: For detailed information about the products please refer to the CofA

Note: Included in the kit for ZFNs targeted to human, rat, mouse and hamster are primers for genotyping by the CEL-I assay as well as genomic DNA from samples where the supplied ZFN has been tested. For ZFNs against all other organisms, plasmid DNA for the next two highest efficiency pairs of ZFNs is also provided.

Protocols

Delivery of ZFNs for Gene Knockout

Reagents and Equipment Recommended But Not Provided

Nucleofection® reagents
Electroporation reagents
*Trans*IT®-mRNA Transfection Reagent (Mirus Bio Catalog Number MIR 2225, LLC)
EX-CELL® GTM-3 (Catalog Number G9916) - If using electroporation
Hank's Balanced Salt Solution (HBSS, Catalog Number H6648)
Cell Line Nucleofector® Kit V (Lonza Catalog Number VCA-1003)
Hexadimethrine Bromide (Polybrene, Catalog Number H9268).
Integration Deficient Lentivirus (IDLV) Packaging Mix (Sigma Catalog Number I3411)

a. Nucleofection
Note: The following procedure has been successfully applied to K562 cells. For cells other than K562, please follow cell line-specific instructions which can be found at http://www.lonzabio.com

i. Seed the cells at a density of 2×10^5 cells/ml the day before transfection.
ii. On the day of transfection, take out Cell Line Nucleofector Kit V and let warm to room temperature.
iii. Add the supplement to the Nucleofection Solution V according to the manufacturer's protocol.
iv. Count the cells. Cell density should be between $2.5–5 \times 10^5$ cells/ml.
v. Fill a 6-well plate with 2 ml of medium in each well and prewarm in a CO_2 incubator at 37 °C for at least 20 minutes prior to nucleofection.
vi. Centrifuge 2×10^6 cells per transfection (8×10^6 total) at $200 \times g$ for 5 minutes.
vii. Wash cells twice with 20 ml of HBSS.
viii. Prepare experimental tubes:

Tube - Label / Reagent	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µl (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**Δ	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied. Total volume in the nucleofection should be less then 10 µl.
**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.
Δ Optimal concentration of the ZFN plasmid may need to be determined based on your cell line of interest.

ix. Remove the 6-well plate containing media from step (v) from incubator.
x. Resuspend cells in 400 µl (100 µl/reaction) of Nucleofection Solution V.
Note: Do not leave cells in nucleofection solution longer than 15 minutes as this will greatly diminish transfection efficiency. To minimize exposure time of the mRNA/DNA to the cells and the cells to the cuvette, perform the reactions separately and quickly.
xi. One reaction at a time, add 100 µl of cells to each DNA or mRNA-containing tube. Transfer the mixture to a 2 mm electroporation cuvette and nucleofect on a Nucleofector with program T-016 for K562 cells.
Note: Nucleofection solutions and programs are cell line specific.

xii. Immediately after nucleofection of each sample, use a transfer pipette to add a pipetteful (~500 µl of the prewarmed medium from the 6-well plate in step (ix) to the cuvette. Then, carefully transfer cells from the cuvette to the remaining prewarmed medium in the 6-well plate.
xiii. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

b. Electroporation
Note: The following procedure has been successfully applied to HEK293 cells. Each cell line should be optimized for electroporation conditions using a reporter construct, such as a GFP expressing plasmid.

i. Seed the cells 1–3 days before transfection so the cells are ~80% confluent on the day of transfection.
ii. On the day of transfection, harvest and count cells.
iii. Fill a 6-well plate with 2 ml of medium in each well and prewarm in a CO_2 incubator at 37 °C for at least 20 minutes prior to electroporation.
iv. Centrifuge 2×10^6 cells per transfection (8×10^6 total) at $200 \times g$ for 5 minutes
v. Wash cells twice with 20 ml of EX-CELL GTM-3 (Catalog Number G9916)
vi. Prepare experimental tubes:

Tube - Label / Reagent	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µL (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied. Total volume in the electroporation should be less then 10 µl.
**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.

vii. Remove the 6-well plate containing media from step (iii) from incubator.
viii. Resuspend cells in 800 µl of EX-CELL GTM-3 medium.
ix. One sample at a time, add 200 µl of cells to each DNA or mRNA-containing tube. Transfer the mix to a 2 mm cuvette and electroporate with the following conditions: voltage: 115 V, capacitance: 950 µF
x. Immediately after electroporation of each sample, use a transfer pipette to add a pipetteful of prewarmed medium to the cuvette to help transfer the cells to the medium in the 6-well.
xi. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

c. Lipid-based transfection reagents
Note: The following procedure was designed for use with the TransIT-mRNA Transfection Kit. A549, HCT116, HeLa, and HEK293 cells have been successfully transfected using this procedure. Conditions may have to be optimized if other transfection reagents and cell lines are used.

i. Cells should be at 60–90% confluency at the time of transfection. The day before transfection, seed 8×10^5 cells in 2 ml of complete medium in a 6-well plate.
ii. The day of transfection, remove the medium and add 1 ml of fresh complete medium to cells prior to transfection.
iii. Prepare experimental tubes. Mix reagents by pipetting.

Tube - Label / Reagent	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
***Trans*IT-mRNA Reagent**	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Serum-Free Medium	100 µl	100 µl	100 µl	100 µl
mRNA Boost Reagent	1.25 µl	1.25 µl	1.25 µl	1.25 µl

iv. To the appropriate tubes, add the following additional reagents:

Tube - Label / Reagent	1 - GFP	2 - ZFN mRNA	3 – ZFN DNA	4 – cells only
ZFNs (mRNA)	–	5 µl (1 vial)	–	–
ZFNs (DNA)	–	–	2.5 µg each**	–
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied.
**ZFN plasmids are provided as separate reagents (2 plasmids per ZFN pair). Each pair of plasmids must be pooled together and added to the reaction to express the full pair of ZFNs.

v. Incubate the mixture at room temperature for 2 minutes. **DO NOT** let samples incubate for more than 5 minutes.
vi. Add the entire mix (~103 µl) of *Trans*IT-Boost-Medium-mRNA to the cells dropwise. Gently rock the plate to mix the complexes, do not swirl.
vii. Incubate in a CO_2 incubator at 37°C.

d. Transduction using integration-deficient lentivirus (IDLV).
Note: To avoid random and permanent integration of ZFN transgenes into the host-cell genome, we recommend the use of integration-deficient lentivirus (IDLV). IDLV particles can be supplied by Sigma or made in your lab using Sigma's lenti-formatted ZFN plasmids along with Sigma's IDLV packaging mix (Cat. No. I3411). All Sigma lentiviral plasmids implement fluorescent protein (FP) reporters to aid in overcoming the reduced infection efficieny of IDLV vs. standard integrating lentivirus. The following procedure was developed for use with U2OS cells in a 24-well plate format. U2OS, MSC, SH-SY5Y, IMR-90, HUVEC cells have been transduced successfully using this procedure. Conditions may have to be optimized if other cell lines or formats are used. Additionally, standard IDLV-ZFN plasmids supplied by Sigma Aldrich drive expression from the CMV promoter which may not be optimal for all cell types. To circumvent the issue, the CMV promoter is flanked by 2 restriction enzymes, SpeI and EcoRI to enable convenient promoter swaps, if needed. If transducing suspension cells please refer to spinoculation protocols, keeping in mind the guidelines given below.

It is highly recommended that for each new cell type to be transduced, a range of MOI 25-100 is tested using the GFP control provided in the kit.
To calculate MOI
Multiplicity of infection (MOI) is the number of transducing IDLV particles per cell.
(total number of cells per well) x (desired MOI) = total transducing units needed (TU)
(total TU needed) / (TU/ml reported on C of A) = total ml of IDLV particles to add to each well

Note: To avoid significant cell death, the volume of virus in the transduction mix should not exceed 10% of the total volume.

Day 1

i. Plate 4 x 10^4 U2OS cells/well in complete medium 24 hours prior to transduction. Typically cells are transduced at 50-80% confluency.
ii. Incubate overnight at 37˚C.

Day 2

iii. It is best to leave the virus on the cells as long as possible, so plan accordingly (start of the work day is recommended).
iv. Thaw the IDLV stock quickly on the bench, and then transfer it to ice until ready for use. Gently (around 50 x g) spin down material in tubes before opening. Thaw only what is needed since each freeze thaw cycle reduces functional titer!
v. Label sterile tubes for each transduction. Add the reagents in the following order:
    a. Complete Media (enough for a total of 300µl reaction).
    b. Polybrene to a final concentration of 8 µg/ml.
        Note: Polybrene enhances transduction of most cell types. However, some cells, such as primary neurons, are sensitive to polybrene and may require alternate transduction methods like ExpressMag (Sigma-Aldrich Cat. No. SHM01).
    c. Desired volume of IDLV. Transduction rates of IDLVs are reduced 7 to 17-fold relative to integrating lentivirus (Wanisch et al., 2009. Mol. Ther., 17:1316) and thus often require higher MOI. It is highly recommended that for each new cell type to be transduced, a range of MOI 25-100 is tested using the GFP control provided in the kit.

vi. After adding all the reagents in the respective tube(s), take the 24-well plate seeded on Day 1 out of the incubator.
vii. Carefully aspirate all the media from the well(s) that have been seeded on Day 1.
viii. Add the entire volume (300 µl) of the IDLV-containing media onto the cells.
ix. Gently rock the plate to mix the components.
x. Place the plate in the incubator for at least 6 hours.
xi. Add 1 ml of pre-warmed complete media to each well(s) and incubate overnight (approx. 15-17 hours)
    Note: When long incubation presents a toxicity concern, cells may be incubated for as little as 4 hours before changing the medium.

Day 3

xii. Remove the viral particle-containing medium and replace it with fresh, pre-warmed complete culture medium.

Day 4

xiii. Harvest cells and plate for pooled cell analysis or single cell cloning.
    Around 30% of the cells are used for extraction of genomic DNA, genotyping using CEL-I, some other PCR assay or reporter-based assays.
    Around 70% of the cells are used for sorting GFP and RFP positive cells. Allow sorted cells to grow for approximately 2-4 weeks depending on the growth rate of your cell line of interest, then genotype by CEL-I, or some other PCR assay or reporter-based assays.

Harvesting Genomic DNA after Delivery of ZFNs

Reagents and Equipment Recommended But Not Provided
GenElute™ Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70)
Hank's Balanced Salt Solution (HBSS, Catalog Number H6648)
Lysis Solution for Blood (L3289)
Neutralization Solution for Blood (N9784)
AlumaSeal™ II (A3250)

a. 6-well plates
Note: Do NOT harvest ALL of your pooled cells. It is important to maintain the culture in order to have cells to single cell dilution clone after you confirm that you have CEL-I digestion products. We recommend subculturing about 1/5 of the pooled cells, and harvesting genomic DNA from the rest of the population.

i. One to three days after transfection, collect the cells to prepare chromosomal DNA using the GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70).

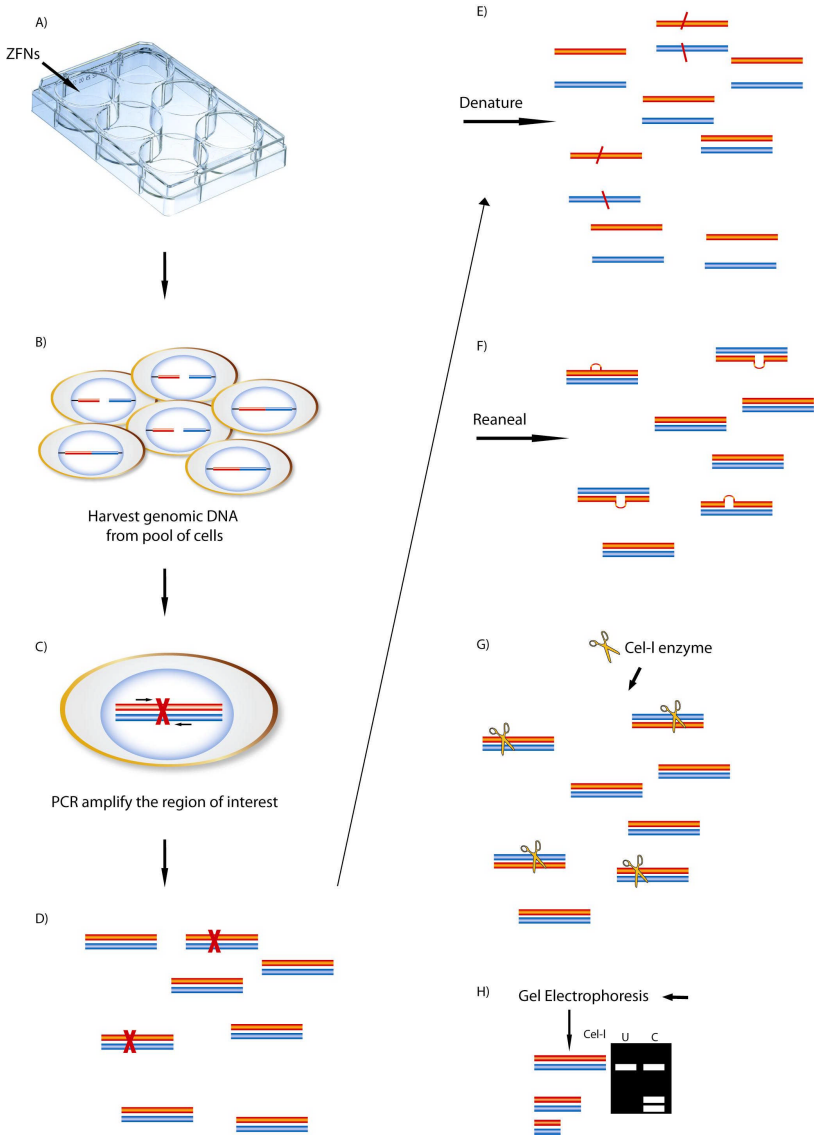
b. 96-well plates
Note: Prior to harvesting genomic DNA from single cell derived clones in a 96-well plate, you should consolidate clones to reduce the number of plates that will be manipulated. Some wells will have no cells prior to consolidation. After you make your consolidation plate, you should make 3 replica plates. One plate will be used as a glycerol stock, one plate will be used to harvest genomic DNA and perform the CEL-I assay, and one plate will be used as a working plate.

ii. Harvest genomic DNA for each well:
Note: The following example is optimized for adherent HCT116 cells and extraction efficiencies may vary for different cell types.

a. Aspirate the medium from cells (cells should be confluent).
b. Wash cells with 100 µl of HBSS (Catalog Number H6648).
c. Add 20 µl of Lysis Solution for Blood (Catalog Number L3289) to each well, then rock back and forth to ensure the entire well is covered.
d. Seal 96-well plate with AlumaSeal II™ (Catalog Number A3250).
e. Incubate at 75 °C for 15 minutes.
f. Add 180 µl of Neutralization Solution for Blood (Catalog Number N9784) and mix by pipetting.
g. DNA can now be used directly for PCR or stored at 4 °C for up to 6 months.

SURVEYOR Mutation Detection Assay (CEL-I Assay)

CEL-I Background
After ZFNs make a double strand break at the target site, the cell uses two main mechanisms to repair the broken chromosome – homology directed repair (HDR) and nonhomologous end joining (NHEJ). NHEJ is more efficient across a variety of cell types than HDR and can often be an error-prone process that introduces deletions and insertions at the cleavage site. To measure the cleavage efficiency of ZFNs in the cell, we recommend using the CEL-I or SURVEYOR assay, which can detect and quantify NHEJ-errors. In the assay, the target region is amplified in a PCR reaction using genomic DNA from the transfected cell pool as template. If ZFNs are active, the genomic DNA will be a mixture of wild-type and NHEJ-error products (insertions or deletions at the target site). The PCR product is then denatured under high temperatures. When the temperature is gradually lowered, some wild-type and NHEJ-error products hybridize to form double strand DNA with mismatches around the cleavage site, which can be cleaved by an enzyme called CEL-I or SURVEYOR (see figure below).



Schematic of the CEL-I Assay used to detect ZFN activity. (A) ZFN plasmid or mRNA is delivered to cells. (B) Expressed ZFNs bind and cut their target sequence creating a double-strand break (DSB) in a portion of the cells. (C) Aberrant repair of some DSBs by NHEJ results in insertion, deletion or substitution (depicted by red X). (C, D) Genomic DNA is harvested from the transfected pool of cells and amplified at the locus of interest. (E-F) PCR product is denatured and re-annealed creating heteroduplex formation between wild type and modified amplicons. (G) The CEL-I mismatch endonuclease assay results in cleavage of heteroduplex molecules. (H) CEL-I enzyme digests are resolved by PAGE. The observed ratio of cleavage product to parental band indicates the fraction cut, and hence, efficiency of ZFNs. On top of the black box in (H) representing an electrophoresis gel on the right, lane U stands for a CEL-I-uncleavable sample indicative of no heteroduplex formation, and lane C stands for a CEL-I-cleavable sample indicative of heteroduplex formation and, therefore, ZFN cleavage.

CEL-I Protocol

Reagents and Equipment
Expand High Fidelity^{PLUS} PCR System (Roche Catalog Number 03 300 242 001)
dNTPs (Sigma Catalog Number D7295)
DirectLoad™ WideRange DNA Ladder (Sigma Catalog Number D7058)
Nuclease S (CEL-I enzyme) + Enhancer (Transgenomic SURVEYOR Kit, Catalog Number 706025)
10% PAGE-TBE gel
Thermocycler
Genomic DNA (ZFN-treated, untreated, and control*)
Validated PCR primers*

*Supplied with CSTZFN kits for human, mouse, rat, and Chinese hamster gene targets; gene targets for other species require user-supplied primers and control genomic DNA

a. PCR optimization
Success of the CEL-I assay hinges on the quality of the initial PCR amplification at the locus of interest. We highly recommend optimizing PCR conditions according to the specific polymerase, genomic DNA isolation method, and cycling conditions used by the investigator. ZFN kits for human, mouse, rat and Chinese hamster gene targets include both validated primers and control genomic DNA (ZFN-treated). ZFN kits for other species require primers and control DNA to be supplied by the user. In these cases, the following guidelines may be used as a starting point for primer design and PCR optimization:

- Limit amplicon size to between ~200bp and ~400bp for optimal resolution in PAGE and to reduce background
- Design primers such that the ZFN cut site is located approximately one-third of the total distance from either end of the amplicon (cleavage occurring too close to one end results in a digested fragment not easily seen in the gel)
- Check primer specificity by using on-line tools such as BLAST, ePCR (NCBI), or "PCR" at UCSC Genome Bioinformatics.
- Use primers with high annealing temperatures in order to achieve specificity of amplification (56-60 $^{\circ}$C is suggested)
- Order two or three primers for both forward and reverse annealing and choose the best combination
- Amplification of normal genomic DNA, followed by CEL-I digest, will help you identify primers resulting in the cleanest CEL-I assay (the less smear or banding after digest, the better)

b. Genomic DNA isolated using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70).

i. PCR amplify the genomic DNA from the 4 transfected samples. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Roche Expand High Fidelity^{PLUS} PCR System. Optimization of the conditions may be necessary if another polymerase is used.
Note: In addition to the transfected samples, it is recommended to include a control reaction using the included control genomic DNA (CDZFN).

Reagent	Volume
Water, PCR Reagent	Adjustable
5× PCR buffer	10 µl
dNTPs (10 mM)	1 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µl
ZFN primer F (provided) (25 µM)	1 µl
ZFN primer R (provided) (25 µM)	1 µl
Genomic DNA	200 ng
Total volume	50 µl

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30 cycles
Annealing	50-60 °C*	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

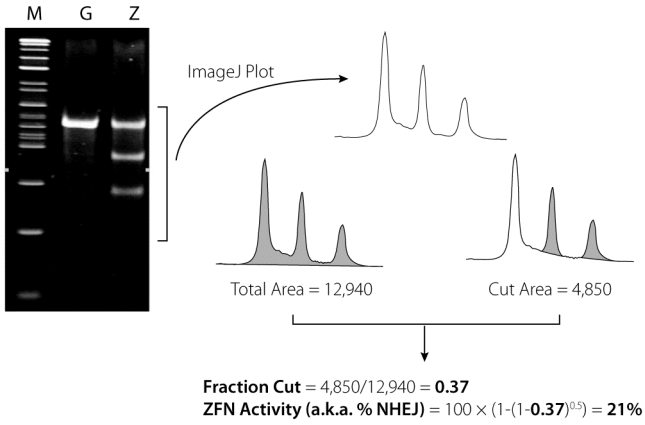
* Check CofA for the specific annealing temperature for your primers

ii. Take 10 µl of PCR reaction from each sample (4 experimental + control) and use the following program on a thermocycler:
95 °C, 10 minutes
95 °C to 85 °C, –2 °C/second
85 °C to 25 °C, –0.1 °C/ second
4 °C, indefinitely
Note: This step can also be performed without a thermocycler. For specific instructions, see the Transgenomic User Guide for the Transgenomic SURVEYOR Mutation Detection Kit for Standard Gel Electrophoresis.

iii. Add 1 µl of enhancer and 1 µl of Nuclease S (from Transgenomic Catalog Number 706025) to each reaction and incubate at 42 °C for 20-40 minutes.
Note: A master mix of enhancer and nuclease S can be made just prior to digest. Do not allow enhancer and nuclease S to incubate together for extended periods prior to digest.

iv. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058) (see figure below).

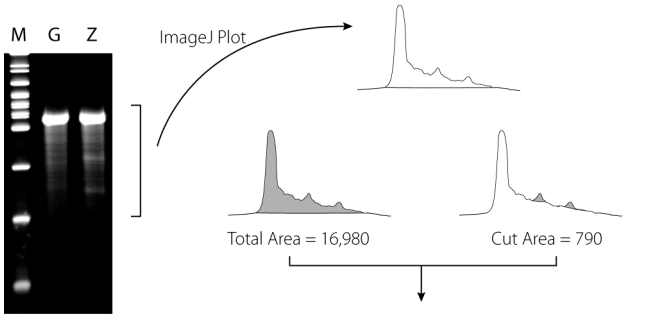
An example of a highly active ZFN pair in the CEL-I Assay in K562 cells.



Definitions:
Fraction Cut = estimate of frequency of mismatched PCR fragments.
ZFN Activity (a.k.a. % NHEJ) = estimate of mutated alleles in pooled cell population (reported in product Certificate of Analysis).

Cells were transfected via nucleofection and harvested 1 day after transfection. Genomic DNA was Harvested using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70), and PCR and CEL-I digestions were done as described above. Samples were run on a 10% PAGE-TBE gel. M: DirectLoad WideRange DNA Marker (Catalog Number D7058). G: GFP transfected cells Z: Cells transfected with ZFN alone.

An example of a ZFN pair with lower activity in the CEL-I Assay in K562 cells.



Definitions:
Fraction Cut = estimate of frequency of mismatched PCR fragments.
ZFN Activity (a.k.a. % NHEJ) = estimate of mutated alleles in pooled cell population (reported in product Certificate of Analysis).

Cells were transfected via nucleofection and harvested 1 day after transfection. Genomic DNA was Harvested using GenElute Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70), and PCR and CEL-I digestions were done as described above. Samples were run on a 10% PAGE-TBE gel. M: DirectLoad WideRange DNA Marker (Catalog Number D7058). G: GFP transfected cells Z: Cells transfected with ZFN alone.

Note: If the CEL-I assay on a pool of cells yields a CEL-I digestion product for your "ZFN alone" sample, you can move on to single cell dilution cloning in order to isolate a clone that has been correctly modified by the ZFNs. After about 3-4 weeks, single cells should have grown into a colony that covers most of a well in a 96-well plate. At this time, you should consolidate clones to reduce the number of plates that will be manipulated. Some wells will have no cells prior to consolidation. After you make your consolidation plate, you should make 3 replica plates. One plate will be used as a glycerol stock, one plate will be used to harvest genomic DNA and perform the CEL-I assay, and one plate will be used as a working plate.

c. CEL-I Assay Procedure using DNA harvested from a 96-well plate

i. PCR amplify the genomic DNA. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Roche Expand High Fidelity^{PLUS} PCR System. Optimization of the conditions may be necessary if another polymerase is used.

Note: In addition to the transfected samples, it is recommended to include a control reaction using the included control genomic DNA (CDZFN).

Reagent	Volume
Water, PCR Reagent	Adjustable
5× PCR buffer	4 µl
dNTPs (10 mM)	0.4 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.2 µl
Forward Gene-specific Primer (not provided) (25 µM)	0.4 µl
Reverse AAVS1 Primer (25 µM)	0.4 µl
Genomic DNA (~2 µl if harvested as previously described for 96-well)	200 ng
Total volume	20 µl

Note: Volumes are small and should be used to make a master mix for a larger number of samples.

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30 cycles
Annealing	55 °C	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

ii. Take 10 µl of PCR reaction from each well and use the following program on a thermocycler:
95 °C, 10 minutes
95 °C to 85 °C, –2 °C/second
85 °C to 25 °C, –0.1 °C/ second
4 °C, indefinitely
Note: This step can also be performed without a thermocycler. For specific instructions, see the Transgenomic User Guide for the Transgenomic SURVEYOR Mutation Detection Kit for Standard Gel Electrophoresis.

iii. Add 1 µl of enhancer and 1 µl of Nuclease S (from Transgenomic Catalog Number 706025) to each reaction and incubate at 42 °C for 40 minutes.
Note: A master mix of enhancer and nuclease S can be made just prior to digest. Do not allow enhancer and nuclease S to incubate together for extended periods prior to digest.

iv. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058).

Troubleshooting Guide

Problem	Cause	Solution
Genomic Control DNA (CDZFN) amplifies, but no amplification from transfected samples.	Quality of genomic DNA preparation is poor	Use a high quality genomic DNA isolation kit.
	Quantity of template	Make sure DNA concentration is measured accurately and use 200 ng of input template DNA.
The Genomic Control DNA did not amplify.	Most likely, the DNA polymerase used is not suitable for the amplification.	Try a different DNA polymerase. We highly suggest using the recommended polymerase and PCR conditions.
No CEL-I signal detected in your cell type.	Transfection efficiency is too low.	Optimize the transfection procedure to increase the efficiency, >50% is preferred.
	RNA integrity	Follow all proper procedures on handling RNA. To make sure the mRNAs are not degraded due to improper storage, check RNA integrity on a gel.
	The cells used are at a high passage number.	Low passage cells should be used. Low passage is generally considered less than 20 passages.
	Cell-to-cell variation in ZFN expression	Perform anti-FLAG Western blot analysis to assess ZFN expression.
No CEL-I PCR product at the single cell clone level from 96-well plate.	DNA is not pure.	Use a 96-well genomic DNA purification kit to yield higher quality DNA. The genomic DNA method stated in the Genomic DNA harvesting protocol for a 96-well plate is a quick method for extracting DNA, but it does not include any DNA purification steps. It is possible that unpurified DNA may make PCR amplification difficult.
Targeted Integration of Exogenous DNA		
No integration detected using Junction PCR on a pool of cells	The donor homology arms aren't homologous at the genomic insertion site.	Sequence the genomic locus of insertion to make sure it's the same as the donor.
	Transfection efficiency is too low.	Optimize the transfection procedure to increase the efficiency. A transfection efficiency of at least 50% is preferred.
	RNA degradation	Run a Cel-I assay. If the ZFN mRNA transfected samples with reasonable transfection efficiency are negative for Cel-I assay the mRNA was likely degraded before or during transfections. Follow all proper procedures on handling RNA. To make sure the mRNA is not degraded due to improper storage check RNA integrity on a gel.
	The cell line of interest has a very low rate of homologous recombination.	If CEL-I gave a good cleavage signal, yet no integration was detected, consider using an antibiotic selection cassette within the donor plasmid MCS.
	The cells used are at a high passage number.	Low passage cells should be used. (low passage is generally considered <20 passages)

No integration detected using Junction PCR on a pool of cells	Residual RNase from donor is degrading the ZFN mRNA.	Make sure you use an endotoxin-free DNA isolation kit. If that is not sufficient to get rid of RNases add additional washes to the endotoxin-free DNA isolation kit being used. Two to four times the number of washes is recommended. To test if there is RNase contamination in the donor preparation mix equal amounts of mRNA with donor (1-2 µl) for 1.5 hours at 37°C. Then run the mixture on a 1% agarose gel. If RNase contamination is not a problem both an mRNA band and a larger DNA band will be seen. If only one band is observed the mRNA is being degraded.
No integration detected by junction PCR at the single cell clone level.	Genomic DNA is not pure.	Use a 96-well genomic DNA purification kit to yield higher quality DNA. It is possible that unpurified DNA may make PCR amplification difficult. It is recommended to include a PCR control with primers that are known to amplify another region of genomic DNA for each genomic preparation.
	Genomic DNA is too concentrated.	When using a quick extraction method one may get too much DNA. It has been found that diluting the genomic DNA by 5-100 fold in neutralization buffer or water can increase the PCR efficiency. This is particularly true if genomic extract is very viscous prior to neutralization.
	Problem with extracting genomic DNA from 96-well plate.	Allow desired clones to grow up in a 6-well plate and then use the GenElute™ Mammalian Genomic DNA Miniprep Kit (Cat. No. G1N70) to extract and purify the genomic DNA. This will ensure quality genomic DNA.
	Primers are not optimized.	Test several pairs of primers both at the 5' and 3' junction of your GOI. Do this first on a pool of transfected cells to ensure integration.
Junction PCR yielded a band in both the donor alone and the donor + ZFN lanes at the pool level.	Amplification of donor genomic DNA.	Allow a longer period of time to pass prior to harvesting genomic DNA in order to allow the donor plasmid to be degraded or washed out. If the band in the donor + ZFN sample is brighter than the donor alone band, one most likely has a real integration event. This should not be a problem when analyzing single cell clones because the donor plasmid will have been washed out during the time it takes to grow a colony of cells from a single cell.
Junction PCR assay for integration efficiency gave good efficiency but unable to obtain GOI integration clones.	Overexpression of GOI is toxic or causes a growth disadvantage.	If the GOI expression is toxic a weaker promoter should be used. If there is suspected growth disadvantage caused by the GOI over expression limiting dilution should be done one or two days after transfection.
	Low efficiency due to long insert.	Screen more clones if junction PCR showed positive.
	Junction PCR conditions are not optimal.	Design new primer pairs and test out different conditions.
	Cells do not survive single cell sorting.	Make serial dilutions and seed various numbers of cells in 100 mm or 150 mm dishes. Let colonies form and then pick them into 96-well plates.

Appendix

A. General Considerations for targeted integration using donor DNA

Targeted integration (TI) of exogenous DNA is greatly enhanced with ZFN-mediated homology directed repair (Urnov et al., 2005). A donor plasmid with left and right homology arms of approximately 400-800bp is recommended. Donors with smaller homology arms (50-100bp) have been shown to work but at a lower efficiency relative to longer arms when applied to various cell types (Orlando et al., 2010). The use of drug selection may enhance efficiency when smaller homology arms are utilized. As for a donor plasmid backbone, it is recommended to use small pUC-based plasmids (<2,600 bp) to limit the mass of dsDNA that is transfected. Ideally, the plasmid backbone will be as small as possible, lack common restriction sites, and not have significant sequence homology to the target region of interest.

The goal of most TI projects is to make a nucleotide change at a location distal to the ZFN cuts-site, avoiding unwanted mutations via NHEJ-errors in an open reading frame, or other functional genetic element directly at the ZFN cut site. To reduce the frequency of unwanted secondary NHEJ-derived mutations, it is recommended, though not absolutely necessary, to incorporate silent mutations in the ZFN binding site within the donor plasmid. This will minimize ZFN cleavage of the donor plasmid or re-cleavage of the target site post-integration of the exogenous DNA. At least two ZFN-blocking mutations (ZBMs) should be incorporated into the ZFN binding site separated by at least 3 bp if they are within the same ZFN arm. Alternatively, one ZBM can be placed in both arms.

To assess the rate of mutation in the pooled cell population prior to single cell cloning several different methods may be used:

1. Out-out PCR, where primers sit outside the region of DNA sequence homology harbored on the exogenous DNA and amplify across the point of modification. Large transgene insertions can be detected this way using gel electrophoresis. This method can often be challenging due to the need to amplify large fragments (> 2kb) from complex genomic DNA, but has been successfully implemented up to 10 kb (Moehle et al., 2007).
2. Junction PCR, where one primer is located in the transgene (or is specific to a small SNP site on the exogenous donor), and the other primer is located on a region outside sequence homology harbored on the exogenous donor DNA. This method is very sensitive when analyzing a pooled cell population. The lower limit of detection is often approximately 1 in 30,000 alleles. Considering this, it should not be used as a decision tool to proceed to single cell cloning unless it is applied using a carefully developed quantitative PCR approach. In general it is useful as a "yes/no" assay for detecting HR events in a pool of cells, and very useful in clonal cell genotyping.
3. Out-out PCR, followed by restriction digestion or Cre-loxP modification (a.k.a. RFLP assay). When a restriction site is added (or lost) as the result of the integration of exogenous DNA, it is very useful in characterizing out-out PCR products. Relative to junction PCR, this method can be very quantitative when using restriction enzymes. Additionally, when other genetic elements are added which enable enzymatic detection (such as detecting loxP sites by in vitro Cre recombinase treatment), those enzymes can be used to help genotype clonal cells prior to full genotyping via DNA sequencing.

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Moehle, E.A. *et al*. (2007) Targeted gene addition into a specified location in the human genome using designed zinc finger nucleases. *Proc Natl Acad Sci* U S A **104**, 3055-3060 (2007).

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Suga, H., Kadoshima, T., Minaguchi, M., Ohgushi, M., Soen, M., Nakano, T., Takata, N., et al. Self-formation of functional adenohypophysis in three-dimensional culture. *Nature*, **480**(7375), 57-62 (2011).

B. ZFN mRNA Production

The following protocols are intended for CompoZr ZFN users to produce additional ZFN mRNA in large quantity (60-80 μg) from a CompoZr ZFN plasmid construct provided by Sigma-Aldrich. Before the ZFN mRNA production, users first need to transform each of the two paired-ZFN plasmid constructs into an *E. coli*. strain and perform a midi or maxi scale plasmid purification for each construct. Purified plasmid is then digested into a linear form with XbaI and purified by phenol/chloroform extraction to generate a high quality DNA template for *in vitro* transcription.

Capped ZFN mRNA is produced from linearized plasmid DNA template by *in vitro* transcription with a MessageMAX™ T7 ARCA-Capped Message Transcription Kit. A poly(A) tail is then added to each ZFN mRNA by polyadenylation with a Poly(A) Polymerase Tailing Kit. Poly(A) tailed ZFN mRNA is then purified by spin column with a MEGAclear™ Kit. Finally, the two ZFN mRNAs are combined in equal amounts for use in gene knockout or target integration experiments.

Caution must be taken to avoid RNase contamination during ZFN mRNA preparation, especially during the RNA elution and post-elution handling steps. The work area and the pipette set must be free of RNases. Use RNase*ZAP* to decontaminate the work area and the pipette set if necessary. Use RNase-free pipette tips, preferably those with an aerosol barrier. Always wear gloves and change them often. Keep reagent vials and sample tubes closed when not in use.

Reagents Required but Not Provided

- XbaI (New England Biolabs, Catalog Number R0145S)
- Phenol/Chloroform/Isoamyl Alcohol (Sigma-Aldrich, Catalog Number P2069)
- 3 M Sodium Acetate Buffer Solution (Sigma-Aldrich, Catalog Number S7899)
- 100% Ethanol (Sigma-Aldrich, Catalog Number 459884)
- MessageMAX T7 ARCA-Capped Message Transcription Kit (Epicentre, Catalog Number MMA60710)
- Poly(A) Polymerase Tailing Kit (Epicentre, Catalog Number PAP5104H)
- ScriptGuard™ RNase Inhibitor (Epicentre, Catalog Number SRI6320K)
- MEGAclear Kit (Ambion, Catalog Number AM1098)

Reagents Recommended but Not Provided

- GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, Catalog Number NA0400)
- RNase-free Water (Sigma-Aldrich, Catalog Number 95284)
- RNase*ZAP* (Sigma-Aldrich, Catalog Number R2020)
- Agilent RNA 6000 Nano Reagent Part 1and 2 (Agilent, Catalog Number 5067-1511)
- Agilent 2100 Bioanalyzer (Agilent, Catalog Number G2938C)

a. DNA Template Preparation

i. Plasmid purification

a. Transform each of the two paired-ZFN plasmid constructs provided by Sigma-Aldrich into an *E. coli* strain using kanamycin at 25 μg/ml for selection.
b. Prepare a liquid culture for each plasmid construct from an isolated colony. LB is the preferred medium. Supplement the medium with kanamycin at 25 μg/ml. Perform a midi or maxi scale plasmid purification. It is highly recommended to use an endotoxin-free plasmid purification kit, such as GenElute HP Endotoxin-Free Plasmid Maxiprep Kit, to ensure low levels of endotoxins and residual RNase in purified plasmid preparations.

ii. Restriction enzyme digestion and post-digestion purification

a. Set up a restriction enzyme digestion for each of the two plasmid constructs in a 1.5-ml microcentrifuge tube according the table below.

Plasmid DNA	20 μg
10X Buffer 4 (NEB)	10 μl
100X BSA	1 μl
XbaI (20 U/ μl)	8 μl
Sterile distilled water	Bring to total 100 μl
Total reaction volume	100 μl

Incubate at 37 °C for 1-2 hours.

b. Add 100 μl of phenol/chloroform/isoamyl alcohol (the bottom layer) to each digestion and vortex vigorously for at least 30 seconds. Note: When using Sigma's phenol/chloroform/isoamyl alcohol, add the equilibration buffer into the phenol/chloroform/isoamyl alcohol and mix thoroughly and place the bottle in a refrigerator for at least 4 hours to separate the phases before use.
c. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature.
d. Use a P-100 pipette and carefully transfer 50 μl of the supernatant into a clean 1.5-ml microcentrifuge tube. Note: Place the pipette tip about half way into the supernatant layer and slowly aspirate the aqueous phase into the tip. Do not touch the inter-phase. If desired, up to 80 μl of the supernatant may be recovered. If more than 50 μl of the supernatant is recovered, increase the volume of 3 M sodium acetate and 100% ethanol proportionally in the next step; also increase the volume of RNase-free water proportionally to resuspend the DNA pellet.
e. Add 5 μl of 3 M sodium acetate solution and mix briefly. Add 150 μl of 100% ethanol and mix thoroughly to precipitate DNA.
f. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature. Carefully pipette off the liquid. Note: Place the tubes in a fixed orientation in the centrifuge, such as with the cap hinge outwards and check for the white pellet after centrifugation. Always remove the liquid from the side opposite the pellet. Keep the same orientation in the subsequent centrifugation steps.
g. Add 150 μl of 70% ethanol to wash the pellet. Centrifuge at maximum speed (~20,000 xg) for 5 minutes at room temperature. Carefully remove all the liquid. Note: If the pellet is too loose for all the liquid to be removed, centrifuge the tube again for 2 minutes before removing the remaining liquid.
h. Air-dry the pellet for 5 minutes and then resuspend in 8 μl of RNase/DNase free water. Vortex the tube to resuspend and then centrifuge briefly to collect the liquid.

Note: you may proceed immediately to in vitro transcription or store the sample at -20 °C for later use.

iii. In Vitro Transcription with MessageMAX T7 ARCA-Capped Message Transcription Kit

a. Set up a restriction enzyme digestion for each of the two plasmid constructs in a 1.5-ml microcentrifuge tube according the table below.
b. Thaw RNase-free Water, 10X Transcription Buffer, ARCA Cap/NTP PreMix, 100 mM DTT, and plasmid DNA template, and centrifuge the tubes briefly. Warm these reagents to room temperature before assembling the reactions.
c. Set up an in vitro transcription reaction for each of the two plasmid constructs. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature in the order given below:

RNase-free Water	10 µl
Plasmid DNA template (~1 µg/µl)	2 µl
10X Transcription Buffer	4 µl
ARCA Cap/NTP PreMix	16 µl
100 mM DTT	4 µl
MessageMAX T7 Enzyme Solution	4 µl
Total reaction volume	40 µl

d. Incubate at 37 °C for 30 minutes. Note: Extending the incubation time to 1 hour may increase the RNA yield in some instances.
e. At the end of the in vitro transcription incubation, centrifuge the tube briefly and add 2 µl of DNase I. Mix gently and centrifuge briefly. Note: Do not vortex the DNase I or the DNase I digestion.
f. Incubate the DNase I digestion at 37 °C for 15 minutes. Note: At the end of the DNase digestion, you may immediately proceed to the poly(A) tailing reaction or store the reaction at -20 °C overnight before proceeding to poly(A) tailing.

b. Poly(A) Tailing with Poly(A) Polymerase Tailing Kit
i. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature according to the order given below:

RNase-free Water		109 µl
10X Reaction Buffer		20 µl
10 mM ATP		20 µl
ScriptGuard RNase Inhibitor	(40 U/µl)	5 µl
In vitro transcription reaction		42 µl
Poly(A) Polymerase		4 µl
Total volume		200 µl

ii. Incubate the reaction at 37 °C for 1 hour. Note: At the end of the poly(A) tailing reaction, you may proceed immediately to RNA purification or store the reaction at -20 °C overnight before purification.

c. RNA Purification with MEGAClear Kit

i. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature according to the order given below:
ii. Add 700 µl of Binding Solution Concentrate to each poly(A) tailing reaction (200 µl) and mix thoroughly by vortex.
iii. Add 500 µl of 100% ethanol and mix thoroughly by vortex.
iv. Insert a Filter Cartridge into a Collection Tube and add 700 µl of the RNA sample into the Filter Cartridge.

v. Centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube. Note: Centrifugation in this and all subsequent steps is performed at room temperature.
vi. Add the remaining RNA sample into the same Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
vii. Add 500 µl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
viii. Add another 500 µl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 xg for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
ix. Dry the Filter Cartridge by centrifugation at ~16,000 xg for 1 minute.
x. Transfer the Filter Cartridge into a new Collection Tube. Add 50 µl of Elution Solution directly onto the center of the filter inside the Filter Cartridge. Close the cap of the tube immediately.
xi. Heat the tube at 65 °C for 10 minutes on a clean heat block or in a clean water bath.
xii. Centrifuge the tube at ~16,000 xg for 1 minute to elute RNA.

d. Determination of RNA quantity and quality, and RNA pooling
i. Combine 1 µl of eluted RNA sample with 99 µl of TE buffer to make a 100X dilution.
ii. Measure the RNA concentration and the A_{260}/A_{280} ratio of the diluted sample with a spectrophotometer or a NanoDrop instrument. When a spectrophotometer is used, RNA concentration and yield can be calculated as follows:

RNA concentration: A_{260} x dilution factor x 40 = µg/ml
RNA yield: 0.05 ml x RNA concentration (µg/ml) = µg

RNA yield should be ≥ 40 µg per transcription reaction. A_{260}/A_{280} should be ≥ 2.2.

iii. To assess RNA quality, prepare a 10-µl dilution aliquot at 80 ng/µl in a clean 1.5 ml microcentrifuge tube. Heat the aliquot at 65 °C for 5 minutes to denature, and run 1 µl of the sample on an Agilent bioanalyzer using the mRNA Nano Assay. The full length ZFN mRNA band should fall between the 1,000 and 2,000 nt markers. Poly(A) tailed CompoZr ZFN mRNAs are between 1,200 and 1,500 nt in length, dependent on how many zinc fingers the construct contains. Alternatively, RNA quality can be evaluated on agarose gel along with appropriate RNA markers as reference.
iv. Combine the two paired-ZFN mRNAs in an RNase-free tube in a concentration of 400 µg/ml each (standard formulation) or in a higher concentration if desired, following the examples below:

Example 1: Standard formulation (400 µg/ml each)

ZFN mRNA #1 (1400 µg/ml)	29 µl (40 µg)
ZFN mRNA #2 (1500 µg/ml)	27 µl (40 µg)
RNase-free Water	**44 µl**
Total volume	100 µl

Example 2: Higher concentration formulation (700 µg/ml each)

ZFN mRNA #1 (1400 µg/ml)	50 µl (70 µg)
ZFN mRNA #2 (1500 µg/ml)	47 µl (70 µg)
RNase-free Water	**3 µl**
Total volume	100 µl

The combined ZFN mRNA is now ready for use. For storage, keep the combined ZFN mRNA at –20 °C for short-term storage (less than 6 months) or at –80 °C for long-term storage (longer than 6 months).

C. Monitoring cellular delivery of ZFN mRNA and plasmids.

Following delivery of ZFN plasmids or mRNA to cells, several options exist for monitoring delivery efficiency. All CompoZr ZFNs contain an N-terminal FLAG tag which can be used in Western blots to monitor ZFN expression. Alternate ZFN vector formats provide a fluorescent protein (FP) between the promoter and start of the ZFN coding region. Cells which have been transfected with FP-linked ZFN vectors can be subsequently inspected by microscopy or FACS to monitor transfection efficiency and ZFN expression levels.

D. Enriching cell populations for ZFN-modified cells using fluorescent reporters

If you are using ZFN expression vectors which encode a fluorescent protein (FP), fluorescence-activated cell sorting (FACS) can be used to isolate cell populations with significantly increased frequencies of ZFN-induced modifications. This FACS-enrichment approach is particularly useful in scenarios where delivery efficiencies and/or ZFN expression levels are low. Within CompoZr FP-ZFN expression vectors, the FP and ZFN protein coding regions are linked by a small sequence encoding a 2A-peptide. The 2A peptide is a ‘‘self-cleaving’’ peptide which allows production of two individual proteins from one transcript and utilizes “ribosomal skipping” rather than proteolytic cleavage mechanism to generate two individual proteins. The average length of 2A peptides is approximately 18–22 amino acids (Ryan et al., 1991; Donnelly et al., 2001). In CompoZr FP-ZFN expression constructs, TagGFP2 or TagRFP (hereafter referred to as GFP or RFP) is linked to the N-terminus of the left ZFN (ZFN1) or right ZFN (ZFN2) by the 2A peptide.

Tips for FACS enrichment of cells expressing FP-ZFNs

Note 1: ZFNs in plasmid or mRNA form can be used for delivery to cells, although it is recommended that ZFN mRNA be used since some cell types are sensitive to dsDNA delivery.

Note 2: If mRNA is used, cells should be incubated at 30˚C for 2-3 days after nucleofection or transfection. Cold-shock will maximize FP-ZFN expression from the transcript and has been found to be a critical if mRNA is to be used for delivery. If plasmid DNA is used, cells can be incubated at 37˚C or 30˚C. Following delivery and prior to FACS sorting or single cell cloning, cells should be checked by microscopy to observe fluorescence.

Note 3: Cells can be harvested for FACS according to commonly used protocols. We suggest that cells be sorted into low, medium, and high FP expression levels in regions of equivalent GFP and RFP expression. Cell populations with the highest GFP/RFP expression have been shown to enrich genome edits with the extent of the “high” fraction ranging from 1 to 30% of the total cell population. The optimal size of the “high” and “medium” populations may vary, depending on particular ZFNs, genomic loci, cell types, delivery methods, and other experimental variables. A good start is to divide the cell population into three low, medium, and high fractions each of which comprises 1 to 20% of the total cell population.

Properties of fluorescent proteins in CompoZr ZFN vectors

Sigma FP-linked ZFN expression vectors use Evrogen’s TagGFP2 and TagRFP fluorescent proteins. TagGFP2 is the improved variant of TagGFP, a mutant of the *Aequorea macrodactyla* GFP-like protein (Xia et al., 2002, Subach et al., 2008). TagGFP2 possesses bright green fluorescence with excitation/emission maxima at 483 and 506 nm, respectively. TagRFP is a monomeric red (orange) fluorescent protein generated from the wild-type RFP from sea anemone *Entacmaea quadricolor* (Merzlyak et al., 2007). It possesses bright fluorescence with excitation/emission maxima at 555 and 584 nm, respectively (Shaner et al., 2004).

E. Single-vector formats for ZFN delivery and expression

In situations of low transfection efficiency, the chances of delivery of two individual ZFN plasmids become exponentially less likely. To address these scenarios, CompoZr ZFN expression vectors have the capability to be changed from the standard two-vector format, to a single vector via a simple subcloning scheme. This capability is an option for both standard and FP-linked ZFN expression vectors. The following protocol can be used to subclone to single vector format (refer to vector maps in section D above):

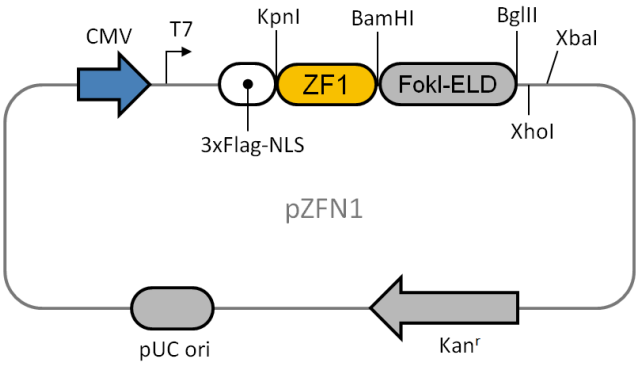
1. Digest pZFN1-GFP with restriction enzymes BglII/XhoI and recover the 4.8kb vector fragment.
2. Digest pZFN2-RFP with restriction enzymes BglII/XhoI and recover the 1.2kb small fragment that contains the 2A-ZFN2 coding region.
3. Ligate the 4.8kb vector fragment and 1.2kb fragment to yield a single GFP-tagged construct, where GFP, ZFN1 and ZFN2 will be expressed as individual proteins from one transcript.

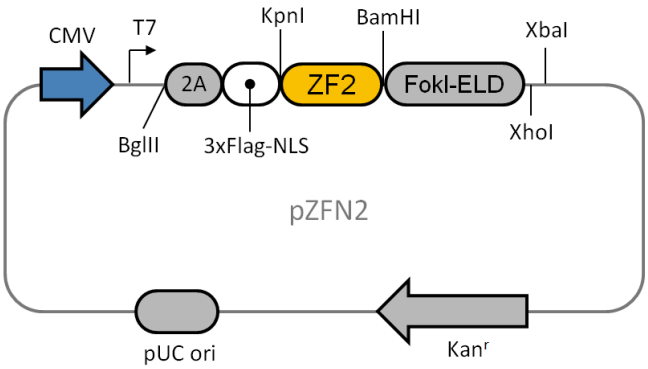
Note: the above single vector protocol can also be used for standard untagged CompoZr ZFN constructs. The same BglII/XhoI restriction sites are used, but the fragment size for ZFN1 will be smaller (4.2 kb).

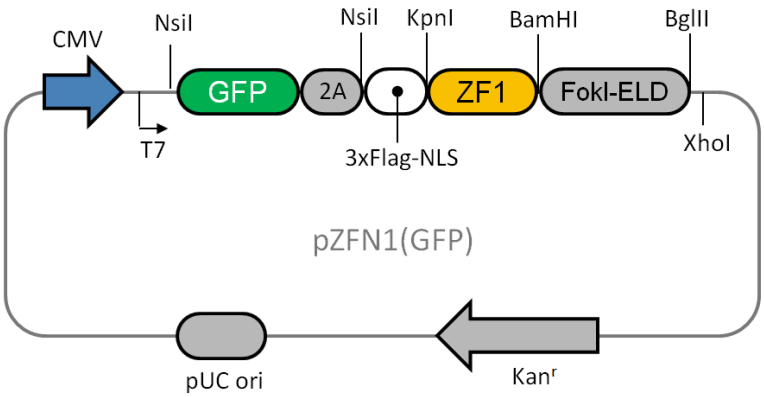
F. Useful restriction sites

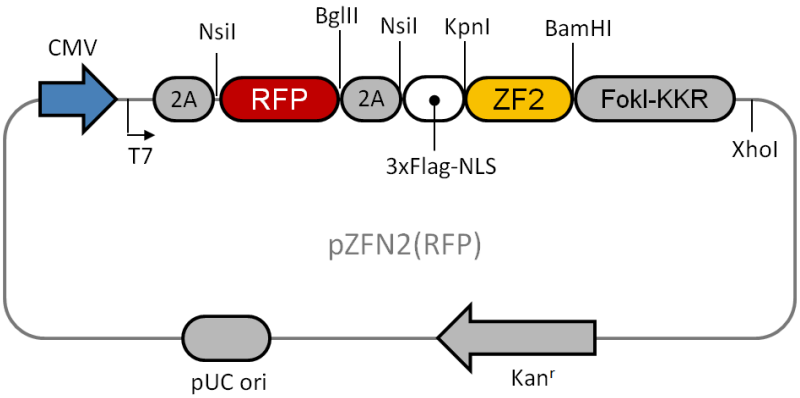
For all CompoZr ZFN expression vectors, the ZFN ORF is subcloned into KpnI/BamHI restriction sites. Both GFP-2A and RFP-2A encoding sequences are flanked by two NsiI restriction sites which allows removal or replacement of GFP-2A or RFP-2A by a single NsiI digestion. Once the FP is removed by religation at NsiI, the reading frame is preserved to maintain expression of the downstream ZFN open reading frame.

G. Vector Maps





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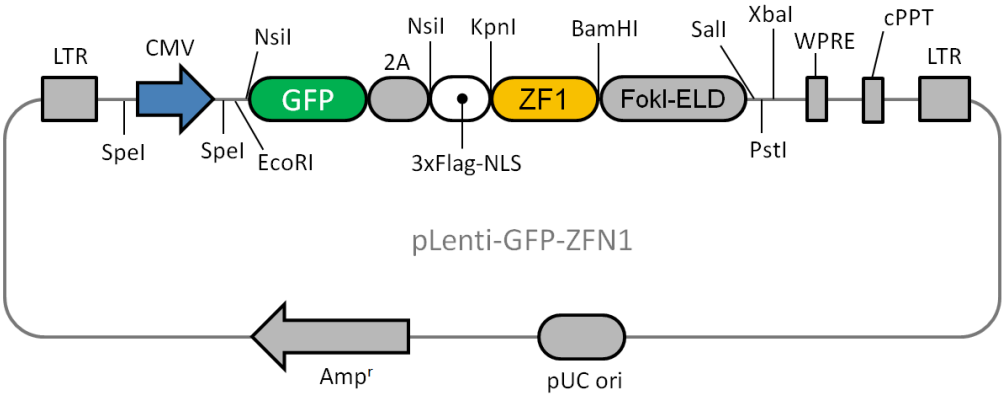
pUC ori
Kanr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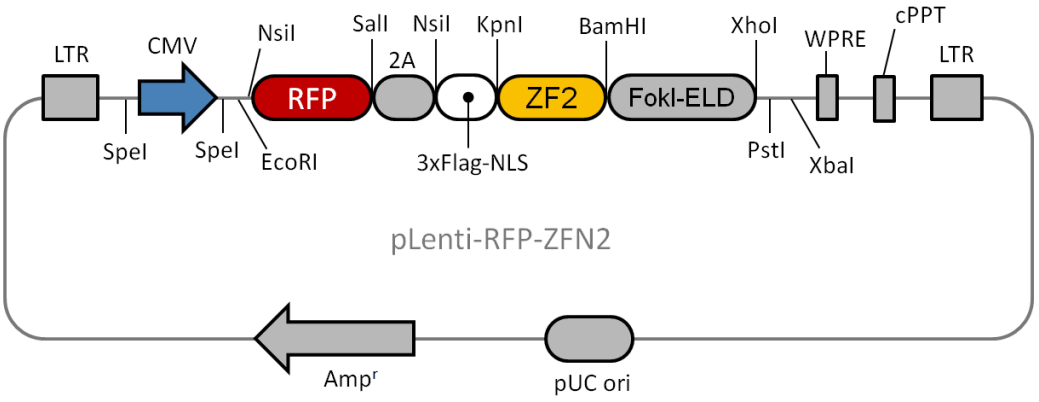
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SpeI
EcoRI
3xFlag-NLS
PstI
XbaI
pLenti-RFP-ZFN2
Ampr
pUC ori

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For the most up to date reference list, please visit our website:
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