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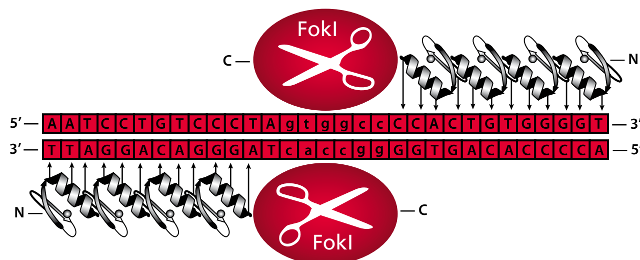
Technical Bulletin

CompoZr® Knockout ZFN Kit - CHO GS

Catalog No. ZFNGS

Product Description

CompoZr Zinc Finger Nucleases (ZFNs), a class of engineered DNA-binding proteins, facilitate targeted genome editing by binding to a user-specified 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 heal 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.



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 FokI. Each zinc finger DNA binding protein recognizes a 3 base pair target. 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 FokI has been reengineered to function as an obligate heterodimer in order to cleave DNA (Miller et al., 2007). 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 FokI 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 SAFC to cleave at the genomic site of interest.

Cell line modification using ZFNs is simple and relies on standard processes such as transfection, dilution cloning, and genotyping. 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, followed by dilution cloning and screening of individual clones. The targeted deletion and integration events that happen in ZFN-treated cells give rise to a population of cells containing biallelic or monoallelic modification, or unmodified gene sequence. 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.

Welcome to the future of genetics!

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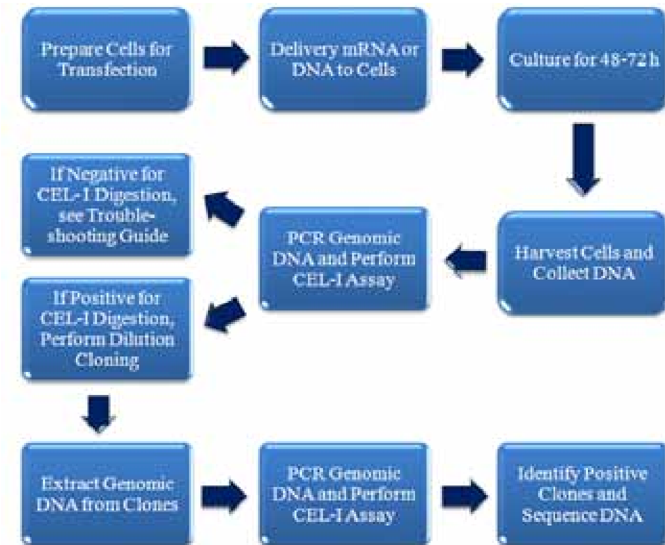
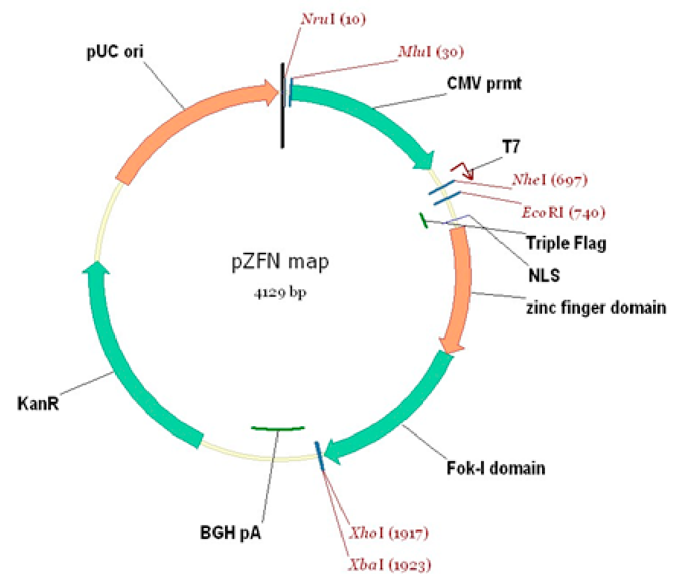
Procedure Overview



Kit Components

Cat. No.	Component	Quantity
ZFNGSA9075	ZFN Plasmid A	50 μg
ZFNGSB9372	ZFN Plasmid B	50 μg



Map of ZFN Plasmid

Precautions and Disclaimer

The CompoZr Zinc Finger Nuclease Kit 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.

Storage and Stability

Store the kit at –20 °C immediately upon arrival.

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Protocols

Part 1: CompoZr® ZFN mRNA Production

Purpose

The following protocol is intended for CompoZr ZFN users to produce ZFN mRNA in large quantity (60-80 μg) from a CompoZr ZFN plasmid construct provided by SAFC. Before the ZFN mRNA production, it is recommended that users first transform the ZFN plasmid construct into an *E. coli.* strain and perform a midi or maxi scale plasmid purification for the 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 the 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 RNaseZAP 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 and Equipment

GenElute HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich, **Cat. No. NA0400**)

XbaI (New England Biolabs, Catalog Number R0145S)

Phenol/Chloroform/Isoamy Alcohol (Sigma-Aldrich, **Cat. No. P2069**)

3M Sodium Acetate Buffer Solution (Sigma-Aldrich, **Cat. No. S7899**)

100% Ethanol (Sigma-Aldrich, **Cat. No. 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)

RNase-free Water (Sigma-Aldrich, **Cat. No. 95284**)

RNaseZap (Sigma-Aldrich, **Cat. No. R2020**)

Agilent RNA 6000 Nano Reagent Part 1and 2 (Agilent, Catalog Number 5067-1511)

Agilent 2100 Bioanalyzer (Agilent, Catalog Number G2938C)

**Note: These reagents are also available in animal-component free forms. Please contact Epicentre for more information.*

Procedures

I. DNA Template Preparation

1. Plasmid purification
   a. Transform each of the two paired-ZFN plasmid constructs provided by SAFC 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.
2. 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/isoamy alcohol (the bottom layer) to each digestion and vortex vigorously for at least 30 seconds.
Note: When using Sigma's phenol/chloroform/isoamy alcohol, add the equilibration buffer into the phenol/chloroform/isoamy 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 x g) 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 x g) 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 x g) 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.

II. *In Vitro* Transcription with MessageMax T7 ARCA-Capped Message Transcription Kit

1. Thaw RNase-free Water, 1X 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.

2. 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 (~1µg/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**

Incubate at 37 °C for 30 minutes.

Note: Extending the incubation time to 1 hour may increase the RNA yield in some instances.

3. 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.

4. 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.

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III. Poly(A) Tailing with Poly(A) Polymerase Tailing Kit

1. Add the reaction components into a clean 1.5-ml microcentrifuge tube at room temperature according to the order given below:

Component	Volume
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**

2. 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.

IV. RNA Purification with MegaClear Kit

1. Add 700 μl of Binding Solution Concentrate to each poly(A) tailing reaction (200 μl) and mix thoroughly by vortex.
2. Add 500 μl of 100% ethanol and mix thoroughly by vortex.
3. Insert a Filter Cartridge into a Collection Tube and add 700 μl of the RNA sample into the Filter Cartridge.
4. Centrifuge at ~16,000 x g 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.

5. Add the remaining RNA sample into the same Filter Cartridge and centrifuge at ~16,000 x g for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
6. Add 500 μl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 x g for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
7. Add another 500 μl of alcohol diluted Wash Solution to each Filter Cartridge and centrifuge at ~16,000 x g for 30 seconds. Decant the flow-through liquid and return the Filter Cartridge to the Collection Tube.
8. Dry the Filter Cartridge by centrifugation at ~16,000 x g for 1 minute.
9. 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.
10. Heat the tube at 65 °C for 10 minutes on a clean heat block or in a clean water bath.
11. Centrifuge the tube at ~16,000 x g for 1 minute to elute RNA.

V. Determination of RNA quantity and quality, and RNA pooling

1. Combine 1 μl of eluted RNA sample with 99 μl of TE buffer to make a 100X dilution.
2. 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.

3. 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 GS ZFN mRNA bands should be ~1,200 nucleotides in length. Poly(A) tailed CompoZr ZFN mRNAs are between 1,400 and 1,700 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.
4. Combine the two paired-ZFN mRNAs in an RNase-free tube in a concentration of 500 μg/ml each (standard formulation) or in a higher concentration if desired, following the example on the next page:

Example 1: Standard formulation (500 μg/ml each)

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ZFN mRNA #1 (1400 µg/ml)	36 µl (50 µg)
ZFN mRNA #2 (1500 µg/ml)	33 µl (50 µg)
RNase-free Water	31 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).

Part II: Transfection of ZFN into Cell Line

Purpose

The following protocol is intended for CompoZr ZFN users to produce a population that has been successfully transfected with a ZFN pair against the gene of interest.

Note: Either ZFN DNA or ZFN RNA may be transfected into the host cell line. However, we prefer the use ZFN RNA for cell line engineering. With RNA, there is no chance of the ZFN coding sequence integrating into the host genome. Additionally, following the transfection protocol below, higher ZFN activity is typically achieved with ZFN RNA.

Reagents and Equipment

Electroporator (Biorad Genepulser)

0.2 cm Electroporation Cuvettes (Sigma-Aldrich, **Cat. No. Z706086**)

ZFN DNA or RNA

Growth Media for cells being transfected

6-well tissue culture plate

Procedures

I. Prepare Cells One Day Prior to Transfection

1. Pass cells at 0.5 × 10^6 cells/mL

II. Prepare Reagents for Transfection

1. Pre-chill electroporation cuvettes on ice at least 10 minutes prior to electroporation.
2. Aliquot 5-15 µg of pooled sterile ZFN DNA or RNA into a 1.5-mL microfuge tube and set on ice until ready.
   a. The total volume of nucleic acid should be no more than 50 µl for electroporation.
3. Set the electroporation conditions on the Biorad Genepulser.
   a. Exponential Protocol.
   b. Voltage = 115V.
   c. Capacitance = 950 µF.
   d. Resistance = ∞.
4. Place 2 mL of growth media in one well of a 6-well plate (1 well for each transfection), and place the plate in the incubator to warm prior to electroporation.

III. Preparing Cells for Transfection

1. Count cells to be transfected to determine the viable cell density.
2. Determine the amount of cells needed for electroporation.
   a. A total of 1e6 cells in 150µl will be needed for each transfection.
3. Spin down cells for 5 minutes at 1,000 rpm and bring up in 150 µl of growth media per transfection.

IV. Electroporation

1. In a biosafety cabinet, mix 150 μl of cells with prealiquoted ZFN DNA or RNA, and transfer the entire volume to the 2 mM electroporation cuvette.
2. Cap the cuvette and place in the Genepulser electroporation pod. Check to be sure the conditions are set, and press the pulse button to electroporate the cells.
   a. Record the time constant and voltage. Time constants should range from 30-40ms. Voltage reads range from 110-115V.
3. Bring the cuvette back into the hood and carefully transfer the pulsed cells to one well in the 6-well plate.
4. Repeat steps 1-3 for any remaining conditions and then place the 6-well plate into a 37 °C, 5% CO_2 incubator.

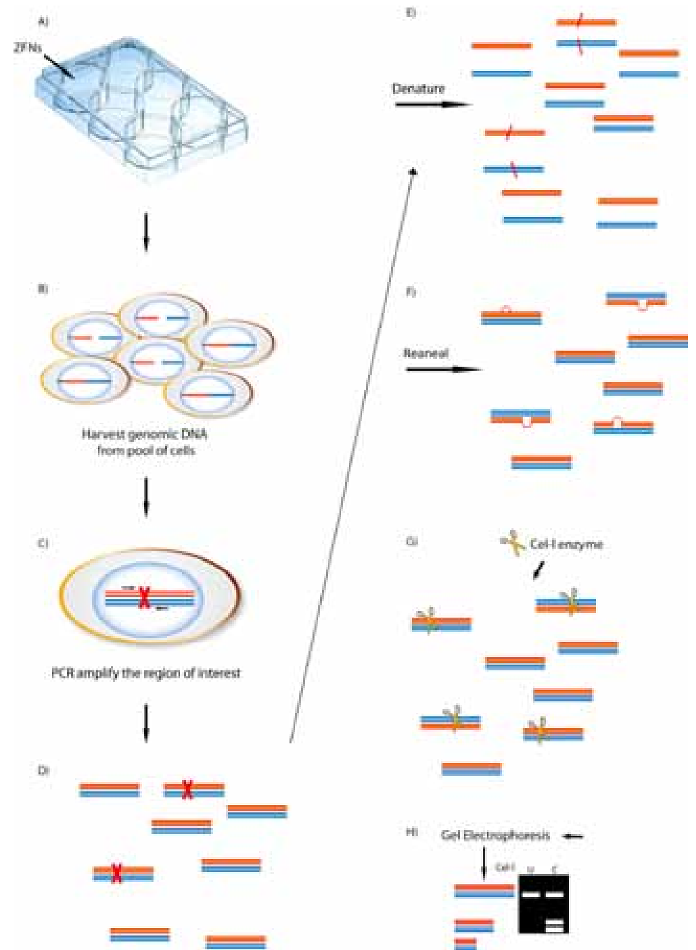
Part III: Surveyor Mutation Detection Assay (CEL-I Assay)

Purpose

This assay will determine the cutting efficiency of the ZFN pair on the gene of interest. This assay is typically done on the transfected pools 3 and 7-10 days post ZFN electroporation.

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 - HDR and NHEJ. NHEJ is more efficient in most cell types and can 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 takes advantage of the NHEJ process. In the assay, the target region is PCR amplified using genomic DNA purified from the transfected pool as the template. If ZFNs are active, the genomic DNA will be a mixture of wild-type and NHEJ 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 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 on right).



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 non-homologous end joining (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.

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Reagents and Equipment

GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma, **Cat. No. G1N70**)

Direct Load Wide Range DNA Ladder (Sigma, **Cat. No. D7058**)

10% TBE Gel (Bio-Rad, Catalog Number 161-1128)

1X TBE (Sigma, **Cat. No. T4073**)

Ethidium Bromide (Sigma, **Cat. No. E1510**)

CEL-I Primers (Sigma Genosys)

GS Cel I F Primer Sequence: 5´-gggtggcccgtttcatct-3´

GS Cel I R Primer Sequence: 5´-cgtgacaactttcccatatcaca-3´

AccuTaq LA DNA Polymerase (Sigma, **Cat. No. D8045**)

AccuTaq LA 10X Buffer (Sigma, **Cat. No. B0174**)

Deoxynucleotide Mix (Sigma, **Cat. No. D7295**)

Transgenomics Surveyor Nuclease Kit – Nuclease S + Enhancer (Transgenomics, Catalog Number 706020)

Gel Loading Buffer (Sigma, **Cat. No. G2526**)

Molecular Grade Water (Sigma, **Cat. No. W4502**)

Quick Extract Buffer (Epicentre, Catalog Number QE-09050)

Procedures

CEL-I Assay from 6-well transfected pools

I. Prepare Chromosomal DNA.

1. If sampling day 3 population, remove 1 mL of cells from each of the transfected cultures for DNA isolation. The remaining 1mL can be scaled up to a T25 flask for expansion. If sampling day 10, remove 1-2e6 cells for isolation.
2. Prepare DNA according to provided protocol in GenElute DNA miniprep kit.

II. CEL-I Assay

1. PCR amplify the genomic DNA purified from the transfected samples. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Sigma's AccuTaq high fidelity polymerase. Optimization of the conditions may be necessary if another polymerase is used.

dNTPs (10 mM)	1.25 μl
AccuTaq 10 × Buffer	2.5 μl
AccuTaq Polymerase	0.5 μl
ZFN primer F (10 μM)	1 μl
ZFN primer R (10 μM)	1 μl
Genomic DNA	200 ng
Total volume	**25 μl**

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	40 Cycles
Annealing	58 °C*	30 seconds	
Extension	68 °C	30 seconds	
Final Extension	68 °C	5 minutes	1
Hold	4 °C	Indefinitely	

*Annealing temperature is specific for GS CEL-I primers.

2. Check PCR on 4% agarose gel.
3. Take 10 μl of PCR reaction from each sample and use the following program on a thermocycler:

   95 °C, 10 minutes

   95 °C to 85 °C, –2 °C/second

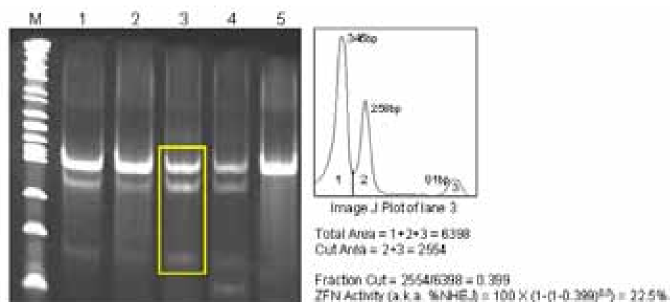
   85 °C to 25 °C, –0.1 °C/ second

   4 °C, indefinitely
4. Add 1 μl of enhancer and 1 μl of Nuclease S (Transgenomics) 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.
5. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Sigma) (see figure on next page).
6. Stain in 100 ml 1xTBE with 3 μl of 10 mg/ml EtBr for 3 min and destain with 1xTBE.
7. Visualize with UV illuminator.

   a. A band the size of the PCR fragment obtained with the CEL-I primers will be present. If ZFN activity occurred, two smaller bands will also be present representing ZFN activity with in that region of the genome.

An example of GS CEL-I assay results in suspension CHO K1 cells



Lane	Transfection Condition	Fraction Cut	Percent NHEJ
1	5 μg DNA	0.313	17.1
2	10 μg DNA	0.244	13.1
3	5 μg RNA	0.399	22.5
4	10 μg RNA	0.367	20.5
5	Mock	0.0	0.0

Cells were transfected via electroporation and harvested 3 days after transfection. Genomic DNA was harvested using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma), 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 (Sigma). 1: Cells transfected with 5 μg of ZFN DNA. 2: Cells transfected with 10 μg of ZFN DNA. 3: Cells transfected with 5 μg of ZFN RNA. 4: Cells transfected with 10 μg of ZFN RNA. 5. Mock transfected cells.

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 cloning in order to find a pure clone with the desired genotype/ phenotype. 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 2 replica plates. 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.

CEL-I Assay from 96-well single cell clones

I. Prepare cell extracts for chromosomal DNA.

1. Transfer the entire contents of 96-well tissue culture plate containing the single-cell clones to a 96-well PCR plate.
2. Centrifuge for 5 minutes at 270 x g and aspirate off supernatant, being careful not to disturb the cell pellets.
3. Add 50 μl of Quick Extract solution to each well and mix vigorously.
4. Using a PCR machine, heat to 65 °C for 15 minutes and then 95 °C for 5 minutes. Extracts may be used immediately for the CEL-I assay or frozen for future use.

II. CEL-I Assay

1. PCR amplify the genomic DNA purified from the transfected samples. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred. The following procedure is for using Sigma's AccuTaq high fidelity polymerase. Optimization of the conditions may be necessary if another polymerase is used.

dNTPs (10 mM)	2.5 μl
AccuTaq 10X Buffer	5 μl
AccuTaq Polymerase	1 μl
ZFN primer F (10 μM)	2.5 μl
ZFN primer R (10 μM)	2.5 μl
Cell extract	2.5 μl
Total volume	**50 μl**

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	40 Cycles
Annealing	58 °C*	30 seconds	
Extension	68 °C	30 seconds	
Final Extension	68 °C	5 minutes	1
Hold	4 °C	Indefinitely	

*Annealing temperature is specific for GS CEL-I primers.

2. Check PCR on 4% agarose gel.
3. Take 10 μl of PCR reaction from each sample 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
4. Add 1 μl of enhancer and 1 μl of Nuclease S (Transgenomics) 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.
5. Run the digestions on a 10% PAGE-TBE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Sigma).
6. Stain in 100 ml 1xTBE with 3 μl of 10 mg/ml EtBr for 3 min and destain with 1xTBE.
7. Visualize with UV illuminator.
8. A band the size of the PCR fragment obtained with the CEL-I primers will be present. If ZFN activity occurred, two smaller bands will also be present representing ZFN activity with in that region of the genome.

Troubleshooting Guide

Problem	Cause	Solution
No PCR amplification of target sequence	Quality of genomic DNA 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
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.

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References

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