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Product Information

CompoZr® Targeted Integration Kit – rRosa26

Catalog Number **CTIR-1KT**

Technical Bulletin

Product Description

The CompoZr Targeted Integration Kit – rRosa26 is designed to integrate a user specified gene of interest into the Rosa26 locus on rat chromosome 4. The use of a well-validated pair of zinc finger nucleases (ZFNs) engineered to target the rRosa26 locus enables highly efficient targeted integration (TI) of exogenous transgenes. When the provided rRosa26 ZFN pair is co-delivered into a rat cell along with a donor plasmid coding for a user specified gene, the cell is stimulated to activate homology directed repair (HDR). HDR directs integration of the user specified gene of interest into the Rosa26 genomic locus (see Figure 1). The provided donor plasmid (pZDrRosa26) contains a multiple cloning site (MCS) for inserting a user specified gene expression construct or any other genetic element. Once modified to contain a user desired transgene, the donor plasmid can be used in conjunction with the ZFNs to integrate the transgene at the rRosa26 locus. ZFN-aided TI of transgenes ranging from 1–8 kb in size can routinely achieve efficiencies of 1–30% in variety of mammalian cell types. [1-3]

A primary advantage of using ZFN-mediated TI is to eliminate the effects that genomic context has on the expression of delivered transgenes. For instance, most stable cell lines are constructed via random integration of a plasmid. This results in a collection of clones with greatly varied expression levels and expression stability relative to methods that employ TI.[4] Furthermore, unlike loxP or FRT recombinase sites, it is likely the rRosa26 ZFN recognition site is present in every rat cell type, enabling immediate use in a broad range of research applications for rat genetics. ZFNs are composed of an engineered zinc finger DNA binding domain fused to the non-specific nuclease domain of the restriction enzyme FokI, the development of which is reviewed elsewhere.[5] Each zinc finger interacts with approximately three nucleotides and multiple fingers can be assembled together to specifically bind larger composite DNA sequences.

Importantly, the FokI nuclease domain must dimerize to achieve double strand cleavage of the target DNA. This means that a heterodimeric pair of ZFNs is required to bind and cleave the targeted site. ZFNs are used to create a targeted double strand break (DSB) that stimulates the process of HDR by several orders of magnitude.

The rRosa26 ZFNs provided in this kit cleave the following sequence on rat chromosome 4:

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5′– CTCGTGATCTGCAACTGGAGTCTTTCTGGAAGATAGGCGGGA –3′
3′– GAGCACTAGACGTTGACCTCAGAAAGACCTTCTATCCGCCCT –5′
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The ZFN target site above is written left-to-right in the direction of transcription by the rat Rosa26 promoter. Underlined sequence denotes zinc-finger protein binding regions. Recent qRT-PCR experiments on rat cell lines suggest that the rat Rosa26 promoter drives expression at levels similar to mouse Rosa26 when compared with housekeeping genes.[6] We can thus imply that transgene expression from the rat Rosa26 promoter could be just as efficient as at the mouse Rosa26 site, but no in house experiments have been conducted to that effect.

To date, the rat Rosa26 locus has not been utilized due to the lack of genome editing technology for rats. While mouse genetics has relied on the facile genome editing methods developed for mouse ES cells over the last three decades[7], the isolation and efficient manipulation of rat ES cells has remained extremely difficult. Recently, ZFNs were successfully applied to rat embryos to produce knockouts[8] and targeted integration events[9], bypassing the need for rat ES cells. These ZFN-based developments have opened the door to begin transferring many standard mouse genetic tools to the rat, with the Rosa26 locus being a primary tool represented in >2,000 publications on mouse genetics.

The mouse Rosa26 locus has a long history of development and application. Mice modified at the Rosa26 locus were initially derived from pools of ES cells infected with the retroviral gene trap vector at a low multiplicity of infection.[10] The Rosa26 locus was cloned and the provirus was shown to interrupt transcripts which encode a nuclear RNA expressed in a broad variety of tissues.[11] The generalized lacZ expression at this site suggested that the targeting of genes to the Rosa26 locus would be a desirable method to achieve ubiquitous transgene expression.[12] From the time of its discovery, hundreds of transgenic mice and cell lines expressing a variety of transgenes including reporters, site specific recombinases, and noncoding RNAs have been successfully created using the Rosa26 locus.[13] Importantly, a majority of studies employing TI into the Rosa26 locus relied on the high rate of homologous recombination that is characteristic of mouse ES cells.[7] However, mouse somatic cells have remained recalcitrant to efficient TI methods, largely prohibiting the use of the Rosa26 locus in established mouse cell lines. Application of ZFN-stimulated TI has been shown to elevate the rate of TI to a level suitable for routine experimentation in mouse embryos and somatic cells.[6,9] Furthermore, despite a relatively high rate of homologous recombination, TI rates in mouse ES cells are also greatly stimulated by ZFN-induced DSBs, significantly reducing the extent of single cell cloning efforts.[1] Very recently, rat ES cells have been cultured and manipulated in a fashion similar to mouse ES cells to produce a p53 knockout rat.[14] However, it is still unclear if the rates of homologous recombination in rat ES cells enable the same, reliable genome editing methods that mouse ES cells are capable of. As with mouse ES cells[1], it is very likely genome editing efficiencies in rat ES cells will also be greatly increased by ZFNs, and may also benefit from ZFN-stimulated targeted integration.

References

1. Meyer, M., de Angelis, M.H., Wurst, W. and Kuhn, R. (2010) Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc Natl Acad Sci U S A*, **107**, 15022-15026.
2. DeKelver, R.C., Choi, V.M., Moehle, E.A., Paschon, D.E., Hockemeyer, D., Meijsing, S.H., Sancak, Y., Cui, X., Steine, E.J., Miller, J.C. *et al.* (2010) Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res*, **20**, 1133-1142.
3. Moehle, E.A., Rock, J.M., Lee, Y.L., Jouvenot, Y., DeKelver, R.C., Gregory, P.D., Urnov, F.D. and Holmes, M.C. (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.
4. Smith, J.R., Maguire, S., Davis, L.A., Alexander, M., Yang, F., Chandran, S., ffrench-Constant, C. and Pedersen, R.A. (2008) Robust, persistent transgene expression in human embryonic stem cells is achieved with AAVS1-targeted integration. *Stem Cells*, **26**, 496-504.
5. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S. and Gregory, P.D. (2010) Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*, **11**, 636-646.
6. Sigma-Aldrich. (2011) Unpublished data.
7. Capecchi, M.R. (1989) Altering the genome by homologous recombination. *Science*, **244**, 1288-1292.
8. Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X. *et al.* (2009) Knockout rats via embryo microinjection of zinc-finger nucleases. *Science*, **325**, 433.
9. Cui, X., Ji, D., Fisher, D.A., Wu, Y., Briner, D.M. and Weinstein, E.J. (2011) Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat Biotechnol*, **29**, 64-67.
10. Friedrich, G. and Soriano, P. (1991) Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev*, **5**, 1513-1523.
11. Zambrowicz, B.P., Imamoto, A., Fiering, S., Herzenberg, L.A., Kerr, W.G. and Soriano, P. (1997) Disruption of overlapping transcripts in the ROSA beta geo 26 gene trap strain leads to widespread expression of beta-galactosidase in mouse embryos and hematopoietic cells. *Proc Natl Acad Sci U S A*, **94**, 3789-3794.
12. Soriano, P. (1999) Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet*, **21**, 70-71.
13. Casola, S. (2010) Mouse models for miRNA expression: the ROSA26 locus. *Methods Mol Biol*, **667**, 145-163.
14. Tong, C., Li, P., Wu, N.L., Yan, Y. and Ying, Q.L. (2010) Production of p53 gene knockout rats by homologous recombination in embryonic stem cells. *Nature*, **467**, 211-213.

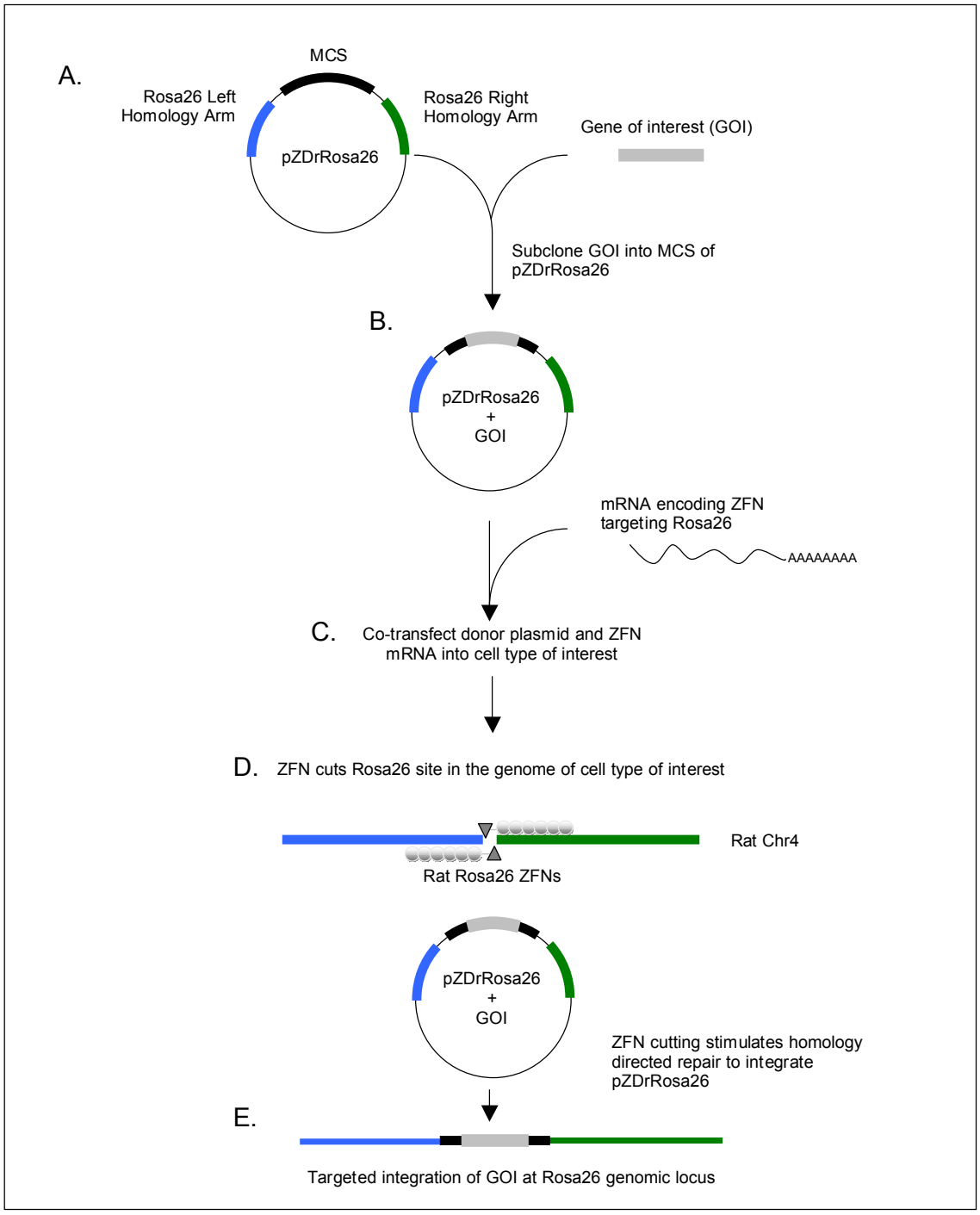

Figure 1. Schematic of CompoZr Targeted Integration Kit – rRosa26 workflow. (**A**) The supplied donor plasmid (pZDrRosa26) contains a multiple cloning site (MCS) to subclone in your gene of interest (GOI). (**B**) GOI-modified donor plasmid, which contains the left and right homology arms to the rat Rosa26 genomic integration site. (**C**) Co-transfection of the donor plasmid + GOI and supplied mRNA encoding a pair of ZFNs targeting the genomic integration site of rat Rosa26. (**D**) ZFNs bind and cut the genomic rat Rosa26 site in the cell type of interest. (**E**) ZFN stimulated homology directed repair leads to targeted integration of GOI into the genome.

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.

Storage and Stability
All components can be stored at -20 °C for up to 12 months. For better stability of the mRNA component, store at -80°C.
RNases are ubiquitous and very stable proteins, which are a primary concern for any researcher attempting to manipulate RNA. Employ precautionary measures to avoid introduction of RNases. We recommend using RNase-free pipette tips, preferably those having an aerosol barrier, to wear latex gloves and change them frequently, and keep bottles and tubes closed when not in use.

Kit Reagents Provided	Catalog Number	Supplied
ZFN mRNA – rRosa26	M7324	10 × 1 each
Reverse Genotyping Primer-rRosa26	R4283	25 µM
Forward MCS Primer-rRosa26	F7934	25 µM
Integration Control – rRosa26*	I3286	1 vial
pZDonor-rRosa26	D2072	100 µg

Note: The exact concentrations of reagents will vary from lot to lot and are contained on the Certificate of Analysis.

* The Integration Control is a genomic DNA preparation from a targeted NRK-52E clone, in which a multiple cloning site (MCS) from pZDonor Rat Rosa26 is integrated into one copy of the Rosa26 genomic locus. It is used as a positive control for Junction PCR (see Procedures, Section C). NRK-52E is derived from rat kidney epithelial cells.

Reagents and Equipment Recommended But Not Provided
- Nucleofection® reagents
- Cell Line Nucleofector® Kit V (Lonza Catalog Number VCA-1003)
- GenElute™ Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70)
- Expand™ High Fidelity^{PLUS} PCR System (Roche Catalog Number 03 300 242 001)
- dNTPs (Catalog Number D7295)
- SeaKem LE Agarose (Lonza Catalog Number 50002)
- Hank's Balanced Salt Solution (HBBS, Catalog Number H6648)
- DirectLoad™ WideRange DNA Ladder (Catalog Number D7058)
- CEL-I Enzyme + Enhancer (Transgenomics Surveyor® Kit, Catalog Number 706025)
- Forward rRosa26 CEL-I Primer: 5′-GGCGGATCACAAGCAATAAT
- Reverse rRosa26 CEL-I Primer: 5′-CAGTGGAGTAGGCGGAGAAA
- Forward junction PCR primer specific for customer gene of interest (GOI)

Procedures

A. Preparation of Plasmid Donor DNA
A donor plasmid, pZDrRosa26, is included in the Rat Rosa26 TI kit to be used as a cloning vector to harbor the gene of interest (GOI). This vector contains a 221 bp homologous DNA sequence upstream of the rRosa26 ZFN cleavage site (Appendix B) and a 622 bp homologous DNA sequence downstream of the ZFN cleavage site, separated by a 50 bp multiple cloning site (MCS). pZDrRosa26 is ampicillin resistant, and the MCS is included to allow for cloning of your GOI. See the Appendix B for additional donor plasmid sequence information. Please note that the homology arms in pZDrRosa26 were designed using sequence data from the Brown Norway rat.

1. Unmodified pZDrRosa26
To determine if the rRosa26 TI Kit is effective for a cell type of interest, the MCS from pZDrRosa26 can be integrated into the Rosa26 genomic locus with ready-to-use reagents provided in the kit. A simple junction PCR can be used to detect positive integration events (see Figure 2). This analysis can be performed rapidly (2-3 days) on a pool of cells, and serves as a check for ZFN and TI activity in your particular cell type prior to modification of pZDrRosa26 to contain your gene of interest. Protocol details on the junction PCR assay are given in Section C below.

2. pZDrRosa26 Containing Gene of Interest (GOI)

The GOI can be cloned into the MCS of pZDrRosa26, which consists of the following unique sites: XmaI, SalI, PmeI, XhoI, AgeI, BamHI, EcoRI, SacII. For gene expression, a promoter, a DNA encoding a protein or non-coding mRNA, and a transcriptional termination signal (e.g. polyA or other) need to be included. Preferably, your gene expression construct should be tested for functionality on an episomal plasmid prior to attempting genome integration. The best direction for transcription may depend on the specific aspects of your GOI. Even though preliminary qRT-PCR data suggest the rat Rosa26 promoter is ubiquitously expressed, no experiments have yet been conducted to show transgene expression driven by the endogenous Rosa26 promoter using a splice acceptor format. We highly recommend re-sequencing your GOI in the modified pZDrRosa26 plasmid if PCR-based subcloning was used. As a general rule, we recommend the use of 2 μg/kb of donor sequence in transfection. For instance, pZDrRosa26 is ~3.7 kb and the use of 7.4 μg of plasmid DNA in transfections is recommended. If the GOI cassette is 5 kb long, which makes the GOI-containing donor ~9 kb in size, use of 18 μg of plasmid DNA is needed.

B. Co-Delivery of ZFN mRNA and Plasmid Donor

Cellular delivery is best performed by nucleofection (Lonza). Please note that lipid-based transfection has not been validated for use with this kit, and has generally been less effective for delivery of ZFN mRNA. Electroporation has not been validated for use with this kit. However, successful mRNA delivery for other ZFNs has been demonstrated using the cell type specific electroporation instructions.

1. General Requirements

Cells should be in log phase at the time of transfection and should not be used immediately after thawing or at very high passage numbers. Extra caution should be taken when dealing with mRNA. Cells should be washed twice in a serum-free medium or saline before mixing with mRNA. Avoid exposing mRNA to cells or plasmid DNA unnecessarily. During electroporation or nucleofection, mix mRNA with cells immediately before nucleofection.

Note: when integrating pZDrRosa26 containing your GOI, make sure to adjust the amount of DNA transfected according to the size of the donor plasmid to 2 μg/kb. Unmodified pZDrRosa26 is 3.7 kb. Endotoxin-free DNA preparations are preferred.

2. Transfection via nucleofection

Note: The following procedure has been successfully applied to NRK-52E cells. For cells other than NRK-52E, please follow Lonza's cell type-specific instructions.

Day before transfection:

i. Seed the cells at a density of 40,000 cells/cm^2.

Day of transfection:

i. 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 or until needed in step ix.
ii. Count the cells. Cell density should be between 50,000 to 100,000 cells/cm^2.
iii. Centrifuge 1-1.5 million cells per transfection at 200 × *g* for 5 minutes.
iv. Aspirate supernatant. Resuspend cell pellet in 13 ml of HBSS gently. Centrifuge at 200 x *g* for 5 minutes.
v. Repeat step iv.
vi. Prepare experimental tubes as described below.

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
ZFN mRNA	–	5 μl	–	5 μl**
Donor Plasmid	–	–	5 μl	5 μl
GFP Control Plasmid*	2.5 μg*	–	–	–

- Total volume of insert for each nucleofection should be no more than 10 μl.

*** GFP Control Plasmid is user supplied.**

**** ZFN mRNA should be added in step viii after the cells have been added to the donor-containing tube and immediately before nucleofection to avoid exposure to traces of RNase potentially carried over from plasmid preparations**.

vii, Resuspend cells in 100 μl of Nucleofection Solution V (Lonza) for each reaction. (for 4 reactions, resuspend in 400μl)

viii. One reaction at a time, add 100 μl of cells to each DNA-containing tube. For reaction 4, after you add the 100 μl of cells to the donor-containing tube, add ZFN mRNA at this point, just prior to nucleofection, transfer the mixture to a 2 mm nucleofection cuvette, and nucleofect on a Nucleofector with program T-027 for NRK-52E cells. Different cell lines may require different nucleofection solutions and programs.

ix. Immediately after nucleofection of each sample, use a transfer pipette to add ~500 μl of the prewarmed medium from the 6-well plate in step (i) to the cuvette. Then, carefully transfer cells from the cuvette to the remaining prewarmed medium in the 6-well plate.

x. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

C. Junction PCR assay for detection of chromosomal integrations.

Note: to provide consistency for various TI kits (human, mouse, and rat), junction PCR is the preferred assay for estimating TI efficiencies using the provided pZDrRosa26 plasmid (unmodified, MCS only). Alternative integration detection strategies can be attempted using RFLP assays, but past experience has shown that junction PCR assays are generally more reliable due to the smaller sizes of PCR amplicons. For users who prefer RFLP assays, rat Rosa26 primer sequences that have been validated for RFLP use are provided in Appendix B.

Targeted integration (TI) does not occur at the same efficiency in all cell types. Several parameters can affect TI efficiency including: (1) transfection efficiency, (2) ZFN expression levels, and (3) the homologous recombination rate for particular cell types. In order to determine the rate of ZFN-mediated TI in a particular cell type, run the junction PCR assay (Figure 2) using reagents provided in the kit. Performing dilutions of the Integration Control (IC) and running them next to your experimental samples and undiluted IC during the junction PCR is recommended. Dilution of the IC genomic DNA into unmodified genomic DNA (e.g. the GFP control gDNA as indicated below) simulates decreasing levels of TI and serves as a rough quantitation of TI efficiency. For example, the undiluted IC genomic DNA represents a sample with a 50% TI rate since this genomic DNA was prepared from an NRK-52E clone in which one of the two Rosa26 alleles contains an integration of the MCS from pZDrRosa26. The results of this experiment will serve as a guide to determine the efficiency of integration in your cell type and provide a rough estimate for how many clones need to be screened to find a correctly modified cell in which the GOI has been integrated.

Note: The procedure outlined below is performed using the unmodified pZDrRosa26 donor plasmid provided in the kit. Once you have modified the donor plasmid with your GOI cloned into the MCS, a similar junction PCR assay can be developed by substituting a new primer for the Forward MCS Primer provided in the kit.

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

2. PCR amplify the genomic DNA with the Reverse Genotyping Primer and the Forward MCS Primer. A DNA polymerase that efficiently amplifies mammalian genomic DNA is preferred to amplify the ~1kb amplicon. The following procedure is for using the 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 from section B, part 2 above, it is recommended to include control reactions using the supplied IC as a template. The IC is the positive control for the junction PCR reaction and IC dilutions serve as a rough estimate of decreasing TI frequencies.

PCR templates for junction PCR:

A. gDNA from cells transfected with GFP plasmid.
B. gDNA from cells transfected with ZFN mRNA alone.
C. gDNA from cells transfected with pZDrRosa26 alone
D. gDNA from cells transfected with ZFN mRNA + pZDrRosa26.
E. IC control DNA (undiluted, simulates a 50% integration rate)
F. 1:10 dilution of IC gDNA. Add 1 μl IC gDNA to 9 μl GFP gDNA and mix well by pipetting (only use 5 μl for PCR).
G. 1:40 dilution of IC gDNA. Add 1 μl IC gDNA to 39 μl GFP gDNA and mix well by pipetting (only use 5 μl for PCR).
H. 1:100 dilution of IC gDNA. Add 1 μl of the 1:10 dilution ("F" above) to 9 μl GFP gDNA and mix well by pipetting (only use 5 μl for PCR).

Note: Make sure that the genomic DNA concentration of all samples above is as similar as possible. Preferably similar to the concentration of the IC gDNA provided in the kit (20-50 ng/μl). This will ensure the most accurate quantitation of TI efficiencies via junction PCR.

PCR reaction set-up

Reagent	Volume / Reaction
Water, PCR Reagent Grade	Adjustable
5× PCR buffer	10 μL
dNTPs (10 mM)	1 μL
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 μL
Reverse Genotyping Primer (25 μM)	1 μL
Forward MCS Primer (25 μM)	1 μL
Genomic DNA (or 5 μL of IC and IC dilutions)	200 ng
Total volume	50 μL

Cycling conditions

Step	**Temp.**	*Time*	**Cycles**
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	15 cycles
Annealing/ Extension	72 °C	1 min, decrease 0.5 °C every cycle	
Denaturation	95 °C	30 sec	20 cycles
Annealing	62 °C	30 sec	
Extension	72 °C	1 min	
Final Extension	72 °C	5 min	1
Hold	4 °C	Indefinitely	

3. Run the PCR products on a 0.8% agarose gel (freshly made is best) with proper markers, such as DirectLoad WideRange DNA Ladder (Sigma Cat. No. D7058). The expected size for product is ~1kb.
4. Only samples in which integration has occurred will give a product at the expected size. Below is an example (Figure 2).

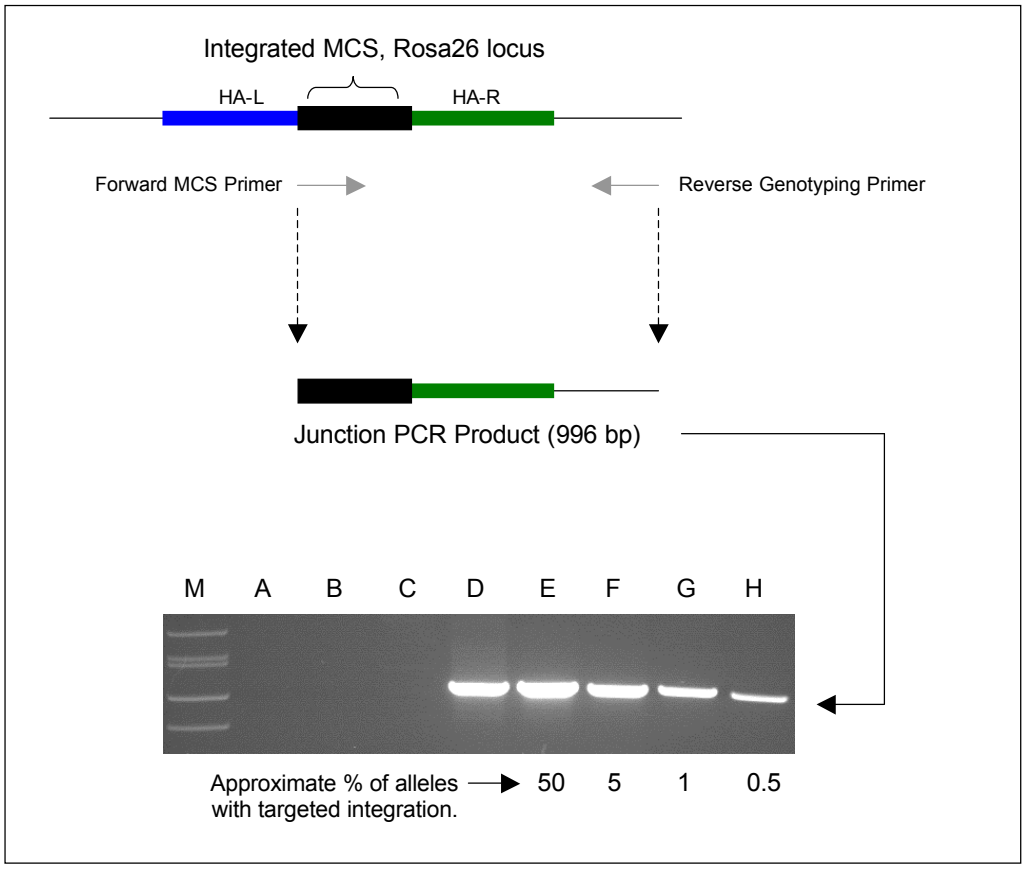

Figure 2. Junction PCR to detect insertion of the pZDrRosa26 MCS into rat NRK-52E cells. The Forward MCS Primer and Reverse Genotyping Primer were used to detect insertion of the 50 bp MCS from pZDrRosa26. Lanes: (A) genomic DNA from a mock nucleofection with GFP plasmid, (B) ZFN mRNA only, (C) pZDrRosa26 only, (D) ZFN mRNA + pZDrRosa26, (E) undiluted IC control genomic DNA from an NRK-52E clone harboring an MCS insertion in one of two alleles, (F) IC gDNA diluted 1:10 into wild-type NRK-52E gDNA, (G) IC gDNA diluted 1:40, (H) IC gDNA diluted 1:100.

D. Cell Cloning

Approximately seven days after transfection, cells are ready for cloning. Cloning can be done earlier, however, waiting for seven days is likely to increase cloning efficiency (i.e. single cell survival rates). It is important to maintain optimal cell growth between transfection and cloning by avoiding overgrowth of cells.

Note: prior to single cell cloning, verify that the TI event has occurred within the population of transfected cells using the junction PCR assay (Section C).

1. Limiting dilution
   a. Manually

      For suspension cells: Count cells and dilute cells to 4 cells/ml. Seed 200 µl of cells/well in 96-well plates.

      For adherent cells: Trypsinize the cells and neutralize with serum-containing medium. Pellet cells, resuspend in complete medium, dilute, and seed as with suspension cells.

   b. Sorting single cells by using FACS - Make cell suspension and sort single cells into each well of 96-well plate.

2. Screening of clones - Regardless which method is used for limiting dilution, not all of the wells will have a clone. After clones grow up, consolidate them into new 96-well plates. Make triplicates of the consolidated 96-well plates, one for frozen stocks, one for genomic DNA preparation, and one for maintenance. Harvest genomic DNA from each well of one set of plates.
3. PCR amplify with the Reverse rRosa26 Primer and a gene-specific forward primer (not included) to detect the insertion junction. A DNA polymerase that efficiently amplifies mammalian genomic DNA is preferred. The following procedure is for using the Roche Expand High Fidelity^{PLUS} PCR System. Optimization of the conditions may be necessary if another polymerase is used. Extension times may need to be increased depending on the length of the amplicon.

Reagent	Amount
Water, PCR Reagent Grade	Adjustable
5× PCR buffer	10 µL
dNTPs (10 mM)	1 µL
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µL
Reverse Genotyping Primer (25 µM)	1 µL
Forward MCS Primer (not provided) (25 µM)	1 µL
Genomic DNA	200 ng
Total volume	50 µL

Step	**Temp.**	*Time*	**Cycles**
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	15 cycles
Annealing/ Extension	72 °C	1 min (may need to be extended) decrease 0.5 °C every cycle	
Denaturation	95 °C	30 sec	20 cycles
Annealing	62 °C	30 sec	
Extension	72 °C	1 min (may need to be extended)	
Final Extension	72 °C	5 mins	1
Hold	4 °C	Indefinitely	

4. Run samples on a 1% agarose gel. Only integration clones will give a product at the expected size.

5. Positive clones identified by junction PCR should be subjected to further analyses to confirm correct integration, including Southern blot detection and assays to detect protein expression.

Troubleshooting Guide

Problem	Cause	Solution
Integration Control amplifies, but no amplification from transfected samples.	Quality of genomic DNA preparation	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 Integration Control did not amplify.	Most likely, the DNA polymerase used is not suitable for the amplification.	Try a different DNA polymerase enzyme and/or PCR buffer reagents.
No integration detected using Junction PCR on a pool of cells.	The homology arms in pZDrRosa26 were designed using sequence data from the Brown Norway rat.	Sequence the Rosa26 region in your rat cell line to make sure it is the same.
	Transfection efficiency is too low.	Always make sure GFP plasmid control is included in every transfection experiment. Optimize the transfection procedure to increase the efficiency. A transfection efficiency of higher than 50% is preferred.
	RNA degradation	Run a CEL-I assay (see Appendix C). 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 (see Storage and Stability). To make sure the mRNA is not degraded due to improper storage, check RNA integrity on a gel (see RNA gel running procedure in Appendix D).
	The cell line of interest has 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)
	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 ºC. Then run the mixture out 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.
	Junction PCR conditions are not optimal.	Try the targeting experiment in NRK-52E cells, which has been shown to support highly efficient targeted integration. Use the targeted NRK-52E DNA or the Integration Control (included in the kit) as template to work out junction PCR conditions.

Problem	Cause	Solution
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 the 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 out several pairs of primers both at the 5′ and the 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 integration 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 a suspected growth disadvantage caused by the GOI overexpression, 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. Rat Rosa26 ZFN Recognition Sequence

5′– CTCGTGATCTGCAACTGGAGTCTTTCTGGAAGATAGGCGGGA –3′
3′– GAGCACTAGACGTTGACCTCAGAAAGACCTTCTATCCGCCCT –5′

The ZFN target site above is written left-to-right in the direction of transcription by the rat Rosa26 promoter. Underline denotes zinc-finger protein binding regions.

B. Plasmid and primer sequence information

Homology arm and MCS sequences:
Note: the homolgy arms in pZDrRosa26 were designed based on the November 2004 genome assembly, version 3.4 produced by the Atlas group at Baylor Human Genome Sequencing Center (HGSC) as part of the Rat Genome Sequencing Consortium. The sequence represents the Brown Norway rat, and it is possible that sequences may vary in other rat strains.

The sequences below are written 5'-3' in the direction of transcription from the rat Rosa26 promoter, and in order of occurrence in the pZDrRoas26 plasmid:

>HA-L
TTGGAGACAAGAAGCACTTGCTCTCCAAAAGTCGGTTTGAGTTATCATTAAGGGAGCTGCAGTGGAGTAGG
CGGAGAAAAGGCCGCACCCTTCTCAGGACGGGGGAGGGGAGTGTTGCAATACCTTTCTGGGAGTTCTCTGC
TGCCTCCTGTCTTCTGAGGACCGCCCTGGGCCTGGAAGATTCCCTTCCCCCTTCTTCCCTCGTGATCTGCA
ACTGGAGT

>MCS
CCCGGGAGAGTCGACGTTTAAACCTCGAGACCGGTGGATCCGAATTCAGACCGCGG

>HA-R
CTTTCTGGAAGATAGGCGGGAGTCTTCTGGGCAGGCTTAAAGGCTAACCTGGTGCGTGGGGCGTTGTCCTG
CAGAGGAATTGAACAGGTGTAAAATTGGAGGGGCAAGACTTCCCACAGATTTTCGATTGTGTTGTTAAGTA
TTGTAATAGGGGCAAATAAGGGAAATAGACTAGGCACTCACCTGGGGTTTTATGCAGCAAAACTACAGGTT
ATTATTGCTTGTGATCCGCCCTGGAGAATTTTTCACCGAGGTAGATTGAAGACATGCCCACCCAAATTTTA
ATATTCTTCCACTTGCGATCCTTGCTACAGTATGAAATTACAGTATCGTGAATTAGAATATATAAGCAGAA
TTTTAAGCATTTTAAAAGAGCCCAGTACTTCATGTCTGTCTCTCCCACTTCTGCAGCCCTATCAAAGGGTA
TTTTAGCACACTCATTTTAGTCCCATTTTCATTTGTTGTACTGGCTTATCCAATCCCTAGACAGAGCACTG
GCATTCCCTCTCTCCTGATCTTAGAAGTCCGATGACTCATGAAACCAGACAGATTAGTTTCATACACCACA
AATCGAGGCTGTAGCTGGGGCCTTTAACATTGCAGTTTTTTTATTCTTCAGTAC

Restriction Sites within the MCS:

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          SalI          XhoI       BamHI          SacII
CCCGGGAGAGTCGACGTTTAAACCTCGAGACCGGTGGATCCGAATTCAGACCGCGG
 XmaI           PmeI         AgeI        EcoRI
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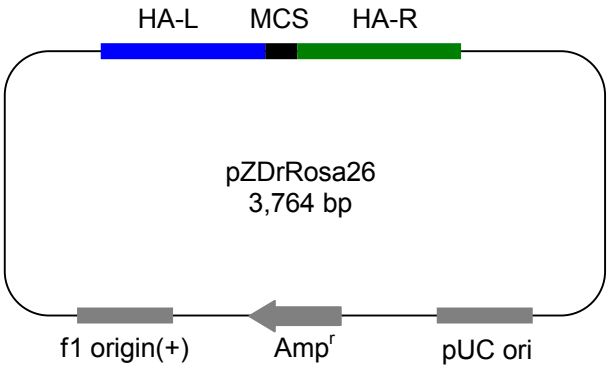
Overall Donor Plasmid Schematic:



Primer Sequences:

Reverse Genotyping Primer:
5′- TGCACTCATGAAATCCACTGAAGCA
(anneals to the rat Rosa26 chromosomal locus, downstream of the right homology arm)

Forward MCS Primer:
5′- CCTCGAGACCGGTGGATCCGA
(anneals within the MCS of the pZDrRosa26)

RFLP Forward Primer:
5’- AGGCCCAAGTGTGGAACACCA

RFLP Reverse Primer:
5’- GAGTTGGGGCTCAGTCGGGT

Cel-I Forward Primer:
5’- GGCGGATCACAAGCAATAAT

Cel-I Reverse Primer:
5’- CAGTGGAGTAGGCGGAGAAA

C. General Considerations for Embryo Microinjection of ZFNs and Donor Plasmids

This kit was developed for targeted integration applications using cell lines and has not been optimized for use with embryo microinjection methods used to make genetically modified animals. However, a few published articles suggest targeted integration can be achieved by microinjection of ZFNs and donor plasmids in embryos. In animal applications, ZFN mRNA is typically diluted to 1-10 ng/µl and donor plasmids are typically diluted to 1-15 ng/µl following purification prior to embryo microinjection.[1,8,9] Please note that optimal concentrations may vary significantly depending on species (rat, mouse, etc.) or strain specific needs. Prior to attempting microinjection of ZFN mRNA and donor plasmids into embryos, we highly recommend performing test injections to be sure the nucleic acid preparations do not clog the microinjection needle. One step typically performed to minimize needle clogging is to spin the nucleic acid preparation at high rpm, and load the injection needle with liquid from the top of the sample.

D. CEL-I Assay

Background

After the ZFN makes a double strand break at the target site, eukaryotic cells use two main mechanisms to repair the chromosome: homology directed repair (HDR) and non-homologous end joining (NHEJ). NHEJ is more efficient across a variety of cell types, occurs throughout the cell cycle, and is often error-prone, creating small deletions and insertions (~1-20 bp) at the cleavage site. To measure the cleavage efficiency of ZFN in the cell, use the CEL-I assay, which detects small mutations created during error-prone NHEJ. 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 modified 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 modified strands hybridize to form dsDNA with mismatches around the cleavage site. These mismatches can be cleaved by an enzyme called CEL-I (see Figure 3).

CEL-I Assay Procedure

1. PCR amplify the genomic DNA from the GFP and ZFN alone mRNA transfected samples from Procedures, section C with the Forward rRosa26 CEL-I Primer (not supplied; 5′ GGCGGATCACAAGCAATAAT 3′) and the Reverse rRosa26 CEL-I Primer (not supplied; 5′ CAGTGGAGTAGGCGGAGAAA 3′). A DNA polymerase that efficiently amplifies mammalian genomic DNA is preferred to amplify the ~400 bp amplicon. The following procedure is for using the 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 Integration Control as template. The Integration Control is the positive control for both the PCR reaction and the CEL-I assay.

Reagent	Volume
Water, PCR Reagent Grade	Adjustable
5× PCR buffer	10 µL
dNTPs (10 mM)	1 µL
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µL
Forward rRosa26 CEL-I Primer (not provided) (25 µM)	1 µL
Reverse rRosa26 CEL-I Primer (not provided) (25 µM)	1 µL
Genomic DNA	200 ng
Total volume	50 µL

Step	**Temp.**	*Time*	**Cycles**
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	30 cycles
Annealing	62 °C	30 sec	
Extension	72 °C	45 sec	
Final Extension	72 °C	5 min	1
Hold	4 °C	Indefinitely	

2. Take 10 µL of PCR reaction from GFP and ZFN mRNA transfected samples 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 Transgenomics User Guide for the Transgenomic SURVEYOR Mutation Detection Kit for Standard Gel Electrophoresis.

3. Add 1 µL of enhancer and 1 µL of Nuclease S (from Transgenomics Catalog Number 706025) to each reaction and incubate at 42 °C for 20 minutes.
4. Run the digestions on a 10% PAGE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Cat.No.D7058).

E. Checking ZFN mRNA Quality

1. Mix 1 µl of the ZFN mRNA and 1 µl of formamide loading buffer (0.05% xylene cyanol and 0.05% bromophenol blue in formamide, Cat. No. F9037).
2. Heat to 70 °C for 3 minutes and place on ice.
3. Load to 1% precast gel (Cat. No. P5472) and run in 1× TBE buffer at 240 V for 20 minutes, using DirectLoad 1 kb Ladder (Cat. No. D3937) as a size marker. Intact ZFN mRNA runs at ~1 kb as one defined band with minor smearing below the major band.

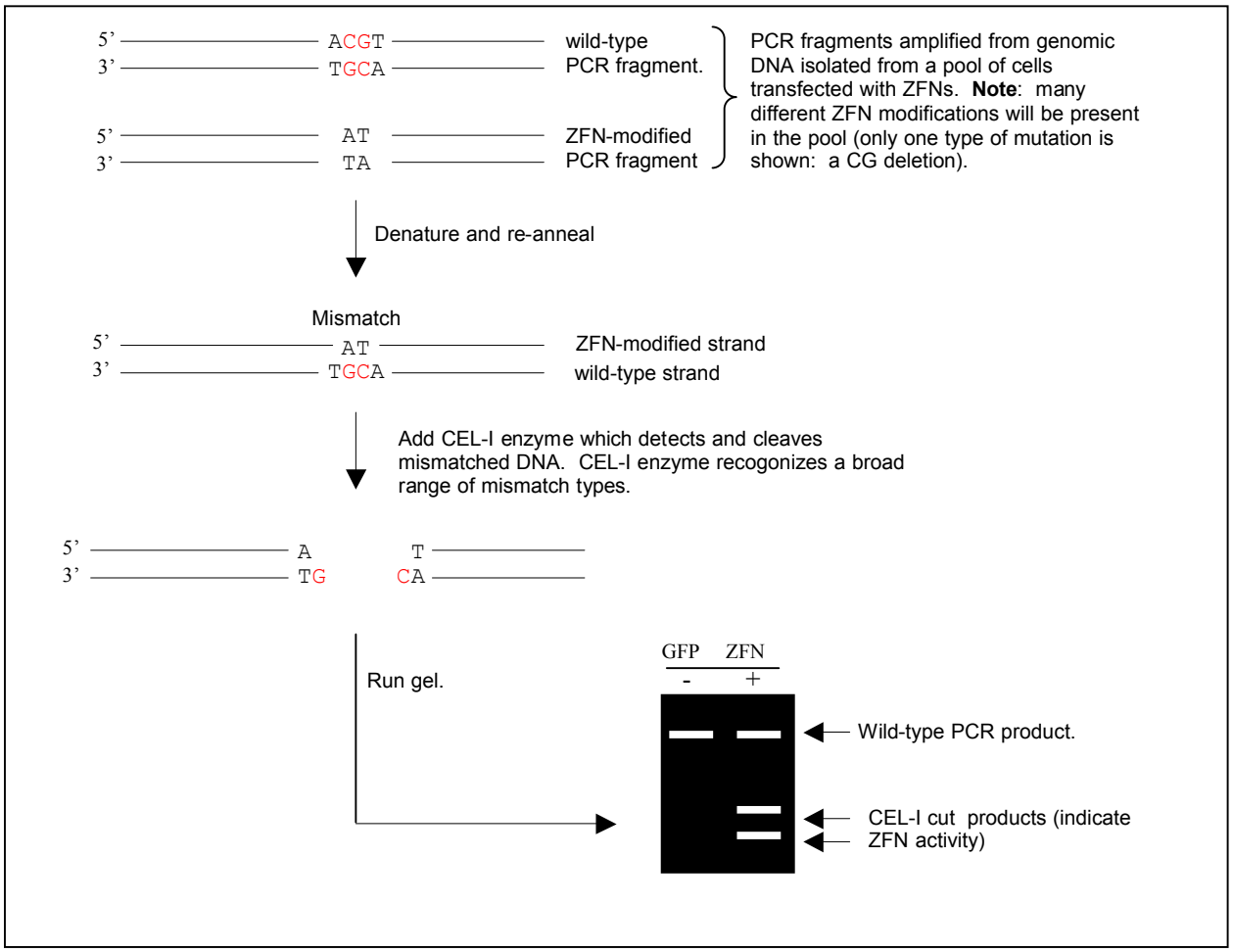


Figure 3. Detection of small insertions and deletions created by NHEJ errors following treatment of cells with ZFNs.

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