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Product Information

CompoZr® Targeted Integration Kit – mRosa26

Catalog Number **CTIM-1KT**

Technical Bulletin

Product Description

The CompoZr® Targeted Integration Kit – mRosa26 is designed to integrate a user specified gene of interest into the Rosa26 locus on mouse chromosome 6. The use of a well-validated pair of zinc finger nucleases (ZFNs) engineered to target the Rosa26 locus enables highly efficient targeted integration (TI). When the provided Rosa26 ZFN pair is co-delivered into a mouse cell along with a donor plasmid coding for a user specified gene, the cell is stimulated to go through the natural process of 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 (pZDonor -mRosa26) contains a multiple cloning site (MCS) for inserting any user specified gene expression construct or exogenous DNA. 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 Rosa26 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 for 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 Rosa26 ZFN recognition site is present in every mouse cell type, enabling immediate use in a broad range of research applications for mouse genetics.

ZFNs are composed of an engineered zinc finger DNA binding domain fused to the non-specific nuclease domain of the restriction enzyme *Fok*I, 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 *Fok*I 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 Rosa26 ZFNs provided in this kit cleave the following sequence on mouse chromosome 6 (zinc finger binding regions underlined):

Direction of mRosa26 promoter →

5′-TGCAACTCCAGTCTTTCTAGAAGATGGGCGGGAGTC-3′
3′-ACGTTGAGGTCAGAAAGATCTTCTACCCGCCCTCAG-5′

The mouse Rosa26 locus has a long history of development and application in mouse genetics. 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.[6] 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.[7] 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.[8]

From the time of its discovery, hundreds of transgenic animals and cell lines expressing a variety of transgenes including reporters, site-specific recombinases, and noncoding RNAs have been successfully created using the Rosa26 locus.[9] 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.[10] However, mouse embryos, and both primary and transformed cells have remained recalcitrant to efficient TI methods.[11] 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.[12,13] 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]

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, preferable 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 – mRosa26	M4574	10 × 1 each
Forward Genotyping Primer - mRosa26	F0933	25 μM
Reverse MCS Primer - mRosa26	R2158	25 μM
Integration Control – mRosa26*	I2036	1 vial
pZDonor -mRosa26	D9196	100 μg

Note: The exact concentrations of reagents will be lot specific. Please see the Certificate of Analysis.

* The Integration Control is a genomic DNA preparation from a targeted Neuro-2a clone, in which a multiple cloning site (MCS) from pZDonor - mRosa26 is integrated into one of three copies of the Rosa26 genomic locus. It is used as a positive control for Junction PCR (see Procedures, Section C).

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 mRosa26 CEL-I Primer: 5′-TAAAACTCGGGTGAGCATGT
- Reverse mRosa26 CEL-I Primer: 5′-GGGGAGTGTTGCAATACCTTT
- Reverse junction PCR primer specific for customer gene of interest (GOI).

References

1. Meyer, M., et al., Gene targeting by homologous recombination in mouse zygotes mediated by zinc-finger nucleases. *Proc Natl Acad Sci U S A*, **107**, 15022-15026 (2010).
2. DeKelver, R.C., et al., 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 (2010).
3. Moehle, E.A., et al., 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).
4. Smith, J.R., et al., Robust, persistent transgene expression in human embryonic stem cells is achieved with AAVS1-targeted integration. *Stem Cells*, **26**, 496-504 (2008).
5. Urnov, F.D., et al., Genome editing with engineered zinc finger nucleases. *Nat Rev Genet*, **11**, 636-646 (2010).
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7. Zambrowicz, B.P., et al., 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 (1997).
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9. Casola, S., Mouse models for miRNA expression: the ROSA26 locus. *Methods Mol Biol*, **667**, 145-163 (2010).
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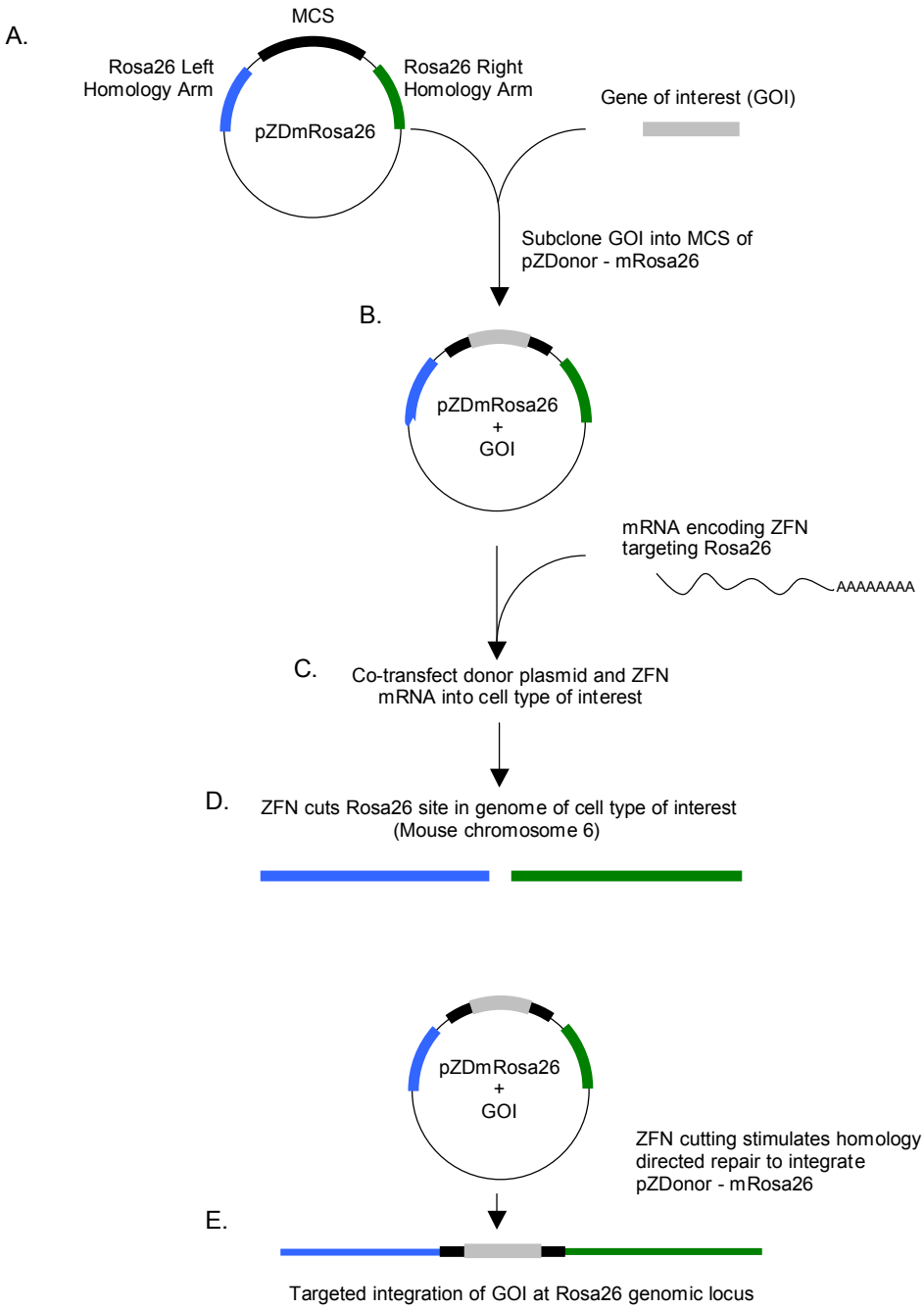
11. Brinster, R.L., et al., Targeted correction of a major histocompatibility class II E alpha gene by DNA microinjected into mouse eggs. *Proc Natl Acad Sci U S A*, **86**, 7087-7091 (1989).
12. Cui, X., et al., Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat Biotechnol*, **29**, 64-67 (2011).
13. Sigma-Aldrich. (2011) Unpublished data.

Figure 1.
Schematic of CompoZr Targeted Integration Kit – mRosa26 workflow



A. The supplied donor plasmid (pZDonor - mRosa26) 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 mouse 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 mouse Rosa26.
D. ZFNs bind and cut the genomic mouse Rosa26 site in the cell type of interest.
E. ZFN stimulated homology directed repair leads to targeted integration of GOI into the genome.

Procedures

A. Preparation of Plasmid Donor DNA

A donor plasmid, pZDonor - mRosa26 (Catalog Number D9196, pZDmRosa26 for shorthand), is included in the Mouse Rosa26 TI kit to be used as a cloning vector to harbor the gene of interest (GOI). This vector contains two 800 bp sequences flanking the mRosa26 ZFN cleavage site, separated by a 56 bp multiple cloning site (MCS). (See appendix for plasmid map)
The MCS is included to allow easy cloning of your GOI into the donor vector.

1. Unmodified pZDmRosa26

To determine if the mRosa26 TI Kit is effective for a cell type of interest, the MCS from pZDmRosa26 can be integrated into the Rosa26 genomic locus using reagents provided in the kit. A simple junction PCR to detect the MCS insertion, using a Rosa26-specific primer (Forward Genotyping Primer-mRosa26 Cat. No. F0933) and a MCS-specific primer (Reverse MCS Primer-mRosa26Cat. No. R2158) provides a result, which is easy to interpret, as only positive integration events will yield an amplicon (see Figures 2 and 3). This analysis can be performed rapidly (2–3 days) on a pool of cells prior to modification of pZDmRosa26 to contain your gene of interest.

2. pZDmRosa26 Containing Gene of Interest (GOI)

The GOI can be cloned into the MCS of pZDmRosa26, which consists of the following unique sites: *Sac*I*, Eco*R*I, BamH*I*, Age*I*, Xho*I*, Pme*I*, Sal*I*, Xma*I. For gene expression, a promoter, a DNA containing an ORF (protein coding or non-coding mRNA), and a transcriptional termination signal (e.g. polyA or other) need to be included. The best direction for transcription may depend on the specific aspects of your GOI. The endogenous mouse Rosa26 promoter may be used if a splice acceptor is included with your GOI (a splice acceptor example for *lacZ* is shown in Meyer et al., 2010, PNAS, 107: 15022). We highly recommend re-sequencing your GOI in the modified pZDmRosa26 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, pZDmRosa26 is ~4.6 kb and the use of 9 μg of plasmid DNA in transfections is recommended. If the GOI cassette is 5 kb long, which makes the GOI-containing donor 10 kb in size, use of 20 μg of plasmid DNA is needed.

B. Co-Delivery of ZFN mRNA and Plasmid Donor

Cellular delivery can be performed by nucleofection (Lonza). Please note that lipid-based transfection has not been validated for use with this kit. However, successful mRNA delivery for other ZFNs has been demonstrated using TransIT-mRNA (Mirus Bio Cat. No. MIR2225, LLC).

1. General Requirements

Cells should be in log phase at the time of transfection. 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 DNA plasmid unnecessarily. During electroporation or nucleofection, mix mRNA with cells immediately before nucleofection.

Note: when integrating pZDmRosa26 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 pZDmRosa26 is 4.6 kb. Endotoxin-free DNA preparations are preferred.

2. Transfection via nucleofection

Note: The following procedure has been successfully applied to Neuro-2a cells. For cells other than Neuro-2a, please follow Lonza's cell type-specific instructions.
Day before transfection:

i. Seed the cells at a density of 2×10^5 cells/ml the day before transfection.

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 $2.5–5 \times 10^5$ cells/ml.
iii. Centrifuge 1–1.5 million cells per transfection at $200 \times g$ for 5 minutes.
iv. Aspirate supernatant. Resuspend cell pellet in 13 ml of HBSS gently. Centrifuge at $200 \times g$ for 5 minutes.
v. Repeat step iv.
vi. Prepare experimental tubes as described on the next page.

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
ZFN mRNA – mRosa26 (M4574)	–	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 400 µl)

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 electroporation cuvette, and nucleofect on a Nucleofector with program T-024 for Neuro-2a cells. Different cell lines may require different nucleofection solutions and programs.

ix. Immediately after nucleofection of each sample, use a transfer pipette to add a pipettefull (~500 µl) of the prewarmed medium from the 6-well plate in step vi 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: For mouse Rosa26 ZFN TI reagents, junction PCR is the preferred assay for estimating TI efficiencies. Alternative quantitations can be attempted using RFLP assays (e.g. PCR using primers outside the homology arms on pZDmRosa26, followed by restriction digestion), but PCR amplification of the required 2 kb region around the Rosa26 locus has not been sufficiently reproducible to establish a reliable assay.

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 3) 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 33% TI rate since this genomic DNA was prepared from a Neuro-2a clone in which one of three Rosa26 alleles contains an integration of the MCS from pZDmRosa26. 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 pZDmRosa26 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 Reverse MCS Primer provided in the kit.

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

2. PCR amplify the genomic DNA with the Forward Genotyping Primer and the Reverse MCS Primer. A DNA polymerase that efficiently amplifies mammalian genomic DNA is preferred to amplify the ~890 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 from section B, part 2 above, it is recommended to include control reactions using the supplied IC as 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 pZDmRosa26 alone
D. gDNA from cells transfected with ZFN mRNA + pZDmRosa26.
E. IC control DNA (undiluted, simulates a 33% 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 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
Forward Genotyping Primer	1 µL
Reverse MCS Primer	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; decrease 0.5 °C every cycle
Annealing/ Extension	72 °C	1 min	
Denaturation	95 °C	30 sec	20 cycles
Annealing	58 °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 Catalog Number D7058). The expected size for product is ~890 bp.
4. Only samples in which integration has occurred will give a product at the expected size. Below is an example (Figure 2).

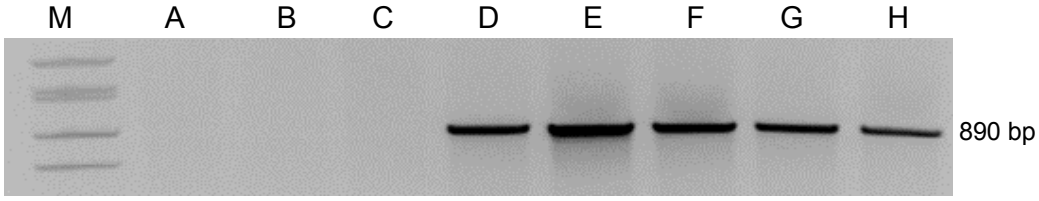
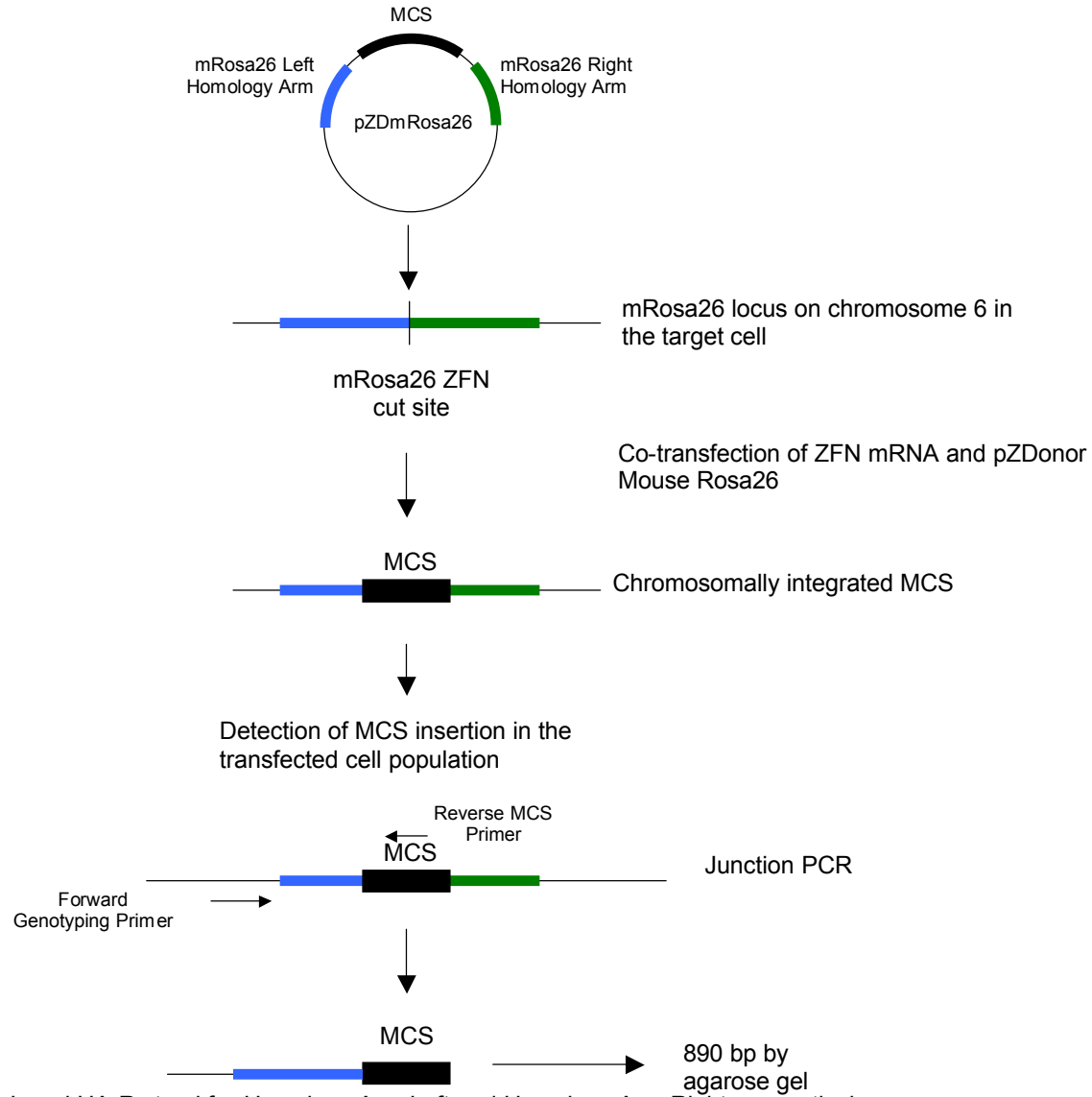


Figure 2. Junction PCR using templates A-H described above. This experiment was performed using Neuro-2a cells (similar results have been obtained in NIH3T3 cells). M= marker.

5. The TI efficiency for your cell type can be estimated by comparing the intensity of the junction PCR bands with that of the IC and IC dilutions (Figure 2). This can be done visually, but for more accurate quantitation, use a densitometry program, such as the freeware ImageJ (http://rsbweb.nih.gov/ij/) or more advanced qPCR methods if available. If the PCR band intensity for your experimental donor + ZFN is equal to or greater than the 1:40 dilution of the IC gDNA, then it is recommended to proceed to single cell cloning (section D) by screening approximately 100–200 clones for targeted insertions. If your experimental sample is below the 1:40 dilution, single cell cloning may still be feasible, but a significantly larger number of clones (>500) may have to be screened or selection methods maybe needed (e.g. GFP or antibiotic resistance). If no amplification is seen, do not proceed to single cell cloning and consult the Troubleshooting section.

Figure 3.
Schematic of the Junction PCR Assay or Integration at the mRosa26 site.



HA-L and HA-R stand for Homology Arm-Left and Homology Arm-Right, respectively. MCS stands for Multiple Cloning Site. Only samples in which integration has occurred will yield a product at the expected size (~890 bp).

D. Cell Cloning

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) because some cells may not survive transfection, yet will not die in the first couple of days after transfection. 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 Forward mRosa26 Primer and a gene-specific reverse primer (not included) to detect the insertion junction. A DNA polymerase that efficiently amplifies human genomic DNA is preferred to amplify the 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. Extension times may need to be increased depending on the length of the amplicon.

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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
Forward Genotyping Primer (25 µM)	1 µL
Reverse 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; decrease 0.5 °C every cycle
Annealing/ Extension	72 °C	1 min (may need to be extended)	
Denaturation	95 °C	30 sec	20 cycles
Annealing	58 °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.	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). 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).
	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 Neuro-2a cells, which has been shown to support highly efficient targeted integration. Use the targeted Neuro-2a DNA or the Integration Control (included in the kit) as template to work out junction PCR conditions.

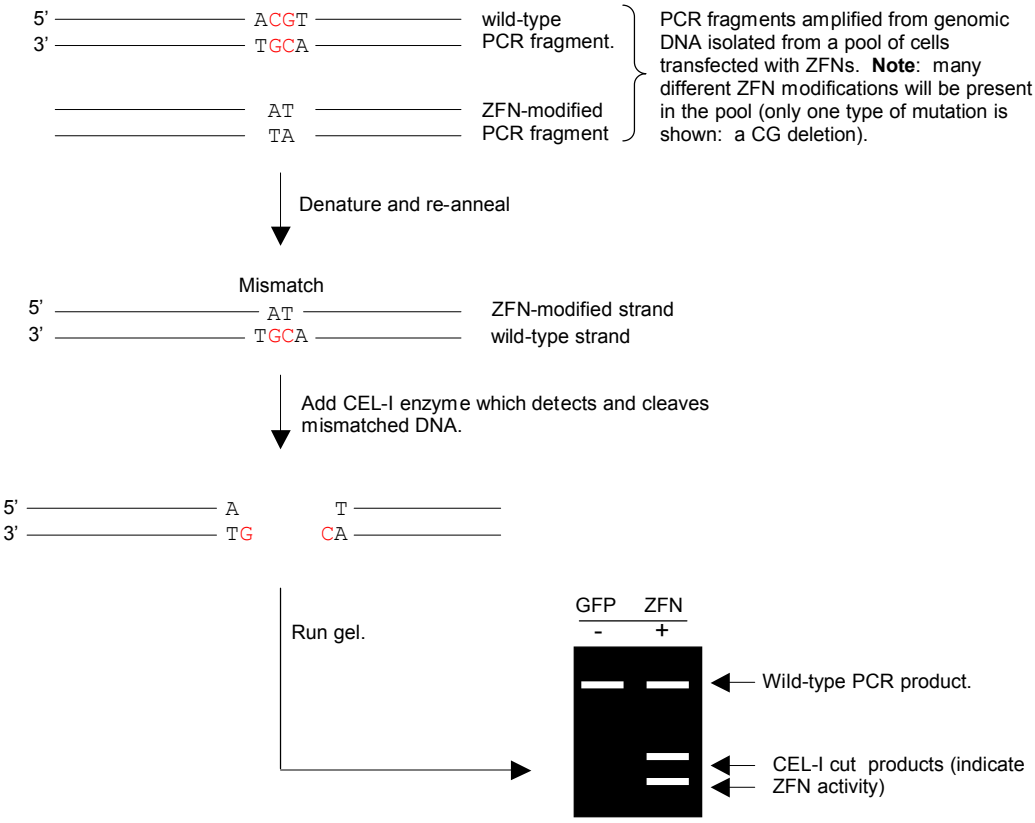
Problem	Cause	Solution
No integration detected by junction PCR at the single cell clone level.	Genomic DNA is not pure.	Use a 96-well genomic DNA purification kit to yield higher quality DNA. The genomic DNA method stated previously is a quick method for extracting DNA, but it does not include any DNA purification steps. It is possible that unpurified DNA may make PCR amplification difficult. 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 the suggested 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

CEL-I Background

After a ZFN makes a double strand break at the target site, eukaryotic cells uses 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 4).

Figure 4.
Schematic of CEL-I assay used to analyze ZFN activity.



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 mRosa26 CEL-I Primer (not supplied; 5′ TAAAACTCGGGTGAGCATGT 3′) and the Reverse mRosa26 CEL-I Primer (not supplied; 5′ GGGGAGTGTTGCAATACCTTT 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
10× PCR buffer	10 µL
dNTPs (10 mM)	1 µL
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µL
Forward mRosa26 CEL-I Primer (not provided) (25 µM)	1 µL
Reverse mRosa26 CEL-I Primer (not provided) (25 µM)	1 µL
Genomic DNA	200 ng
Total volume	50 µL

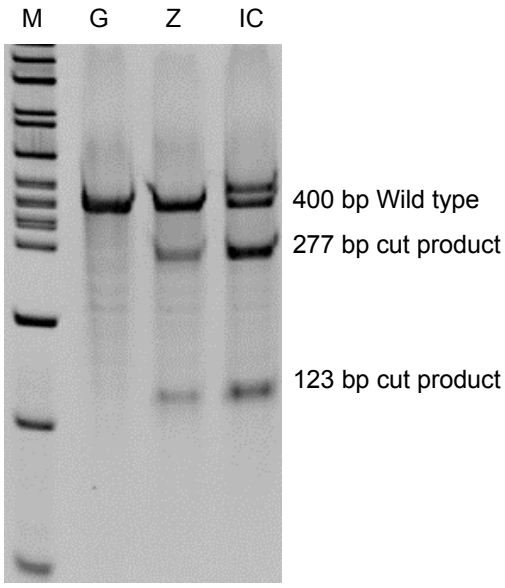
Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 min	1
Denaturation	95 °C	30 sec	30 cycles
Annealing	58 °C	30 sec	
Extension	72 °C	45 sec	
Final Extension	72 °C	5 min	1
Hold	4 °C	Indefinitely	

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 (Catalog Number D7058) (see Figure 5).

Figure 5.
An example of the CEL-I Assay in Neuro-2a cells



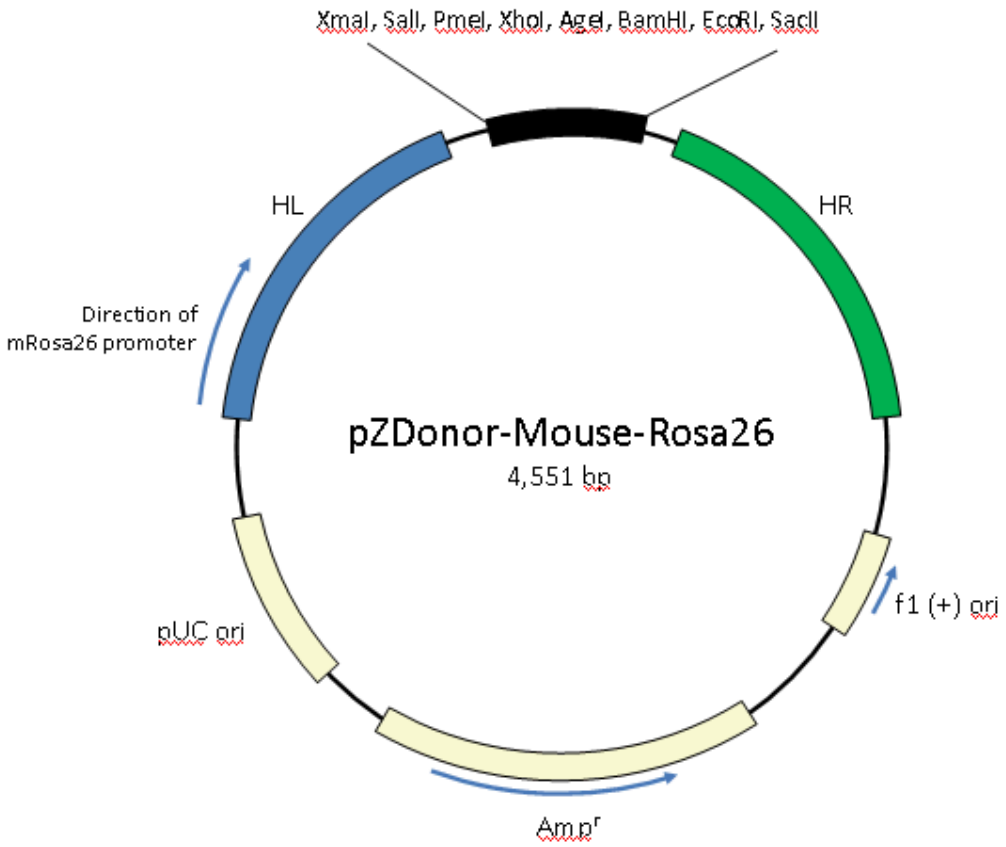
Cells were transfected via nucleofection and harvested 48 hours after transfection. gDNA was prepared, and PCR and digestions were done as previously described.

M: DirectLoad WideRange DNA Marker (Catalog Number D7058)
G: GFP transfected pool of cells
Z: Pool of cells transfected with ZFN alone
IC: Cells that have been isolated from a single cell clone that has the MCS integrated in only one of the three alleles present.

RNA Gel Running Procedure

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, Catalog Number F9037).
2. Heat to 70 °C for 3 minutes and place on ice.
3. Load to 1% precast gel (Catalog Number P5472) and run in 1× TBE buffer at 240 V for 20 minutes, using DirectLoad 1 kb Ladder (Catalog Number D3937) as a size marker. Undegraded ZFN mRNA runs at ~1 kb as one defined band with minor smearing below the major band.

Map of the donor plasmid



Multiple cloning site:

Direction of mRosa26 promoter →

XmaI	SalI	PmeI	XhoI	AgeI	BamHI	EcoRI	SacII	ZFN-R

5′-TGCAACTCCAGTCTTTCTCCCGGGAGAGTCGACGTTTAAACCTCGAGACCGGTGGATCCGAATTCAGACCGCGGTGGGCGGGAGTC

3′-ACGTTGAGGTCAGAAAGAGGGCCCTCTCAGCTGCAAATTTGGAGCTCTGGCCACCTAGGCTTAAGTCTGGCGCCACCCGCCCTCAG

ZFN-L

Reverse MCS Primer Binding Site

Primer Sequences:

Forward mRosa26 Primer (anneals to the Rosa26 chromosomal locus, beyond the region of homology in the donor plasmid arms):

5′–TTGGGGGAGGAGACATCCACCTGG

Reverse MCS Primer (anneals within the MCS of the donor plasmid):

5′-ACCTCGAGACCGGTGGATCCGA

mRosa26 ZFN Recognition Sequence:

5′-TGCAACTCCAGTCTTTCTAGAAGATGGGCGGGAGTC-3′
3′-ACGTTGAGGTCAGAAAGATCTTCTACCCGCCCTCAG-5′

Underlined sequence corresponds to ZF binding regions. The DNA sequence is cleaved between the ZF binding regions.

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