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Product Information

CompoZr® Targeted Integration Kit – AAVS1

Catalog Number **CTI1**

Technical Bulletin

Important

This protocol is optimized for use with the Expand™ High Fidelity^{PLUS} PCR System (Roche).

Other suggested Taq polymerases include:
JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich)
JumpStart AccuTaq™ LA DNA Polymerase (Sigma-Aldrich)
Accuprime™ *Taq* DNA Polymerase System (Invitrogen)
Accuprime *Taq* DNA Polymerase High Fidelity (Invitrogen)

Any of these four polymerases may require additional optimization of the amplification protocol. The use of Taq polymerases other than those suggested is not supported.

Product Description
The CompoZr Targeted Integration Kit – AAVS1 is designed to integrate a user specified Gene of Interest (GOI) into the adeno-associated virus integration site 1 (AAVS1) on human chromosome 19. The use of a well-validated pair of zinc finger nucleases (ZFNs) engineered to target the AAVS1 locus enables the efficient targeted integration. When the provided AAVS1 ZFN pair is co-delivered into a human cell line 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 the targeted integration of the user specified gene of interest into the AAVS1 locus (see Figure 1). ZFN-aided targeted integration can achieve 20% efficiency in some cell types,[1] representing an improvement of three to four orders of magnitude in efficiency over normal homologous recombination events.[2,3]

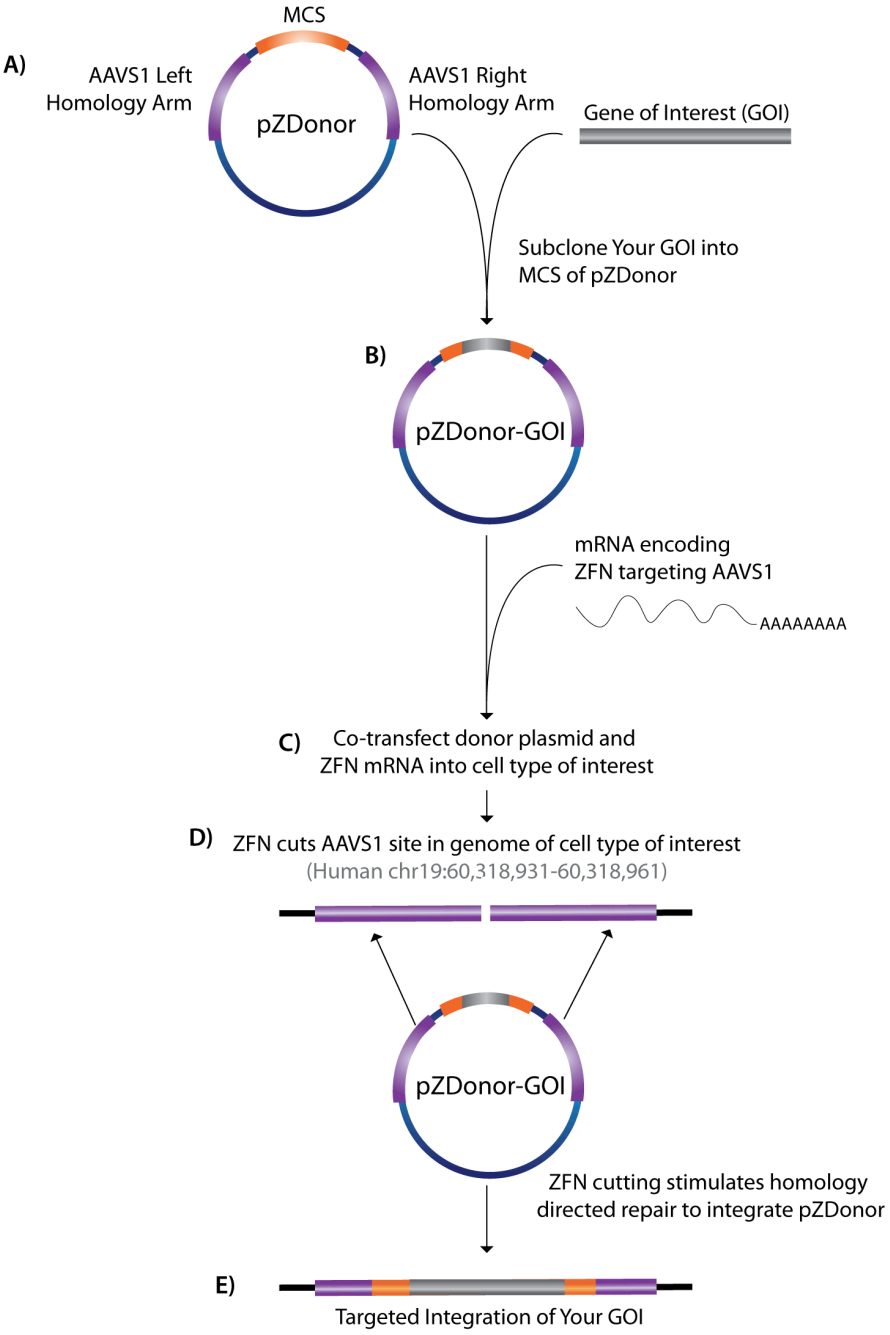
Zinc finger nucleases are composed of an engineered zinc finger DNA binding domain fused to the non-specific nuclease domain of the restriction enzyme *Fok*I, the development of which is reviewed elsewhere.[4] Each zinc finger interacts with three nucleotides and multiple (N) fingers can be assembled together to specifically bind a targeted sequence of 3N bases. Importantly, the *Fok*I nuclease domain must dimerize to achieve a double strand cleavage in the DNA. This means a ZFN pair is required to bind and cut the targeted site (see Figure 2). ZFNs are used to create a targeted double strand break that stimulates the process of HDR.

A primary advantage for using ZFN-mediated targeted integration is to eliminate the effects genomic context has on the expression of delivered transgenes. For instance, most transgenic 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. Furthermore, it is likely the AAVS1 site is present in every human cell line, unlike phage recombination sites such as Cre and FLP. This provides a consistent site for transgene delivery and reduces the likelihood of expression variability across experiments.

The AAVS1 locus is the only adeno-associated virus type 1 integration site in the human genome.[5] This site constitutively expresses a protein named p84 with no known function and the locus provides a safe, stable site for transgene integration and expression. Multiple genes of various lengths have been successfully integrated into this site, including GFP under human CMV or PGK promoters. Stable expression of GFP was maintained for up to three months. Having a defined safe harbor site for integration eliminates the variability of expression affiliated with cells lines created using random integration. This allows for an accurate comparison between clones when the monitoring of changes in gene expression is important. For example, assessing the level of knockdown afforded by different shRNAs or changes in phenotypes following the integration of various mutant forms of a protein of interest.

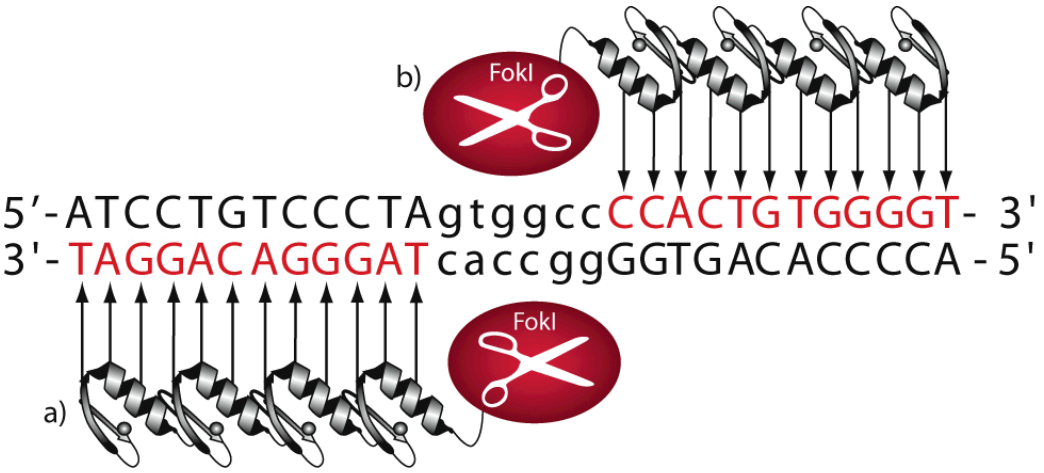
The AAVS1 ZFNs are highly efficient and have comparable cutting efficiencies across all cell types. However, integration efficiency can range between 3 and 30%. This was determined by inserting a 50 bp multiple cloning site into the AAVS1 locus in various human cell lines. As the insert size increases, the integration efficiency decreases. For large inserts, selection may be necessary.

Figure 1.
Schematic of CompoZr Targeted Integration Kit – AAVS1 workflow.



A) Supplied pZDonor with a multiple cloning site (MCS) is easily used to subclone in your Gene of Interest (GOI).
B) GOI-modified pZDonor, which contains a left and right homology arm to the AAVS1 genomic integration site.
C) Co-transfection of the GOI-modified pZDonor and supplied mRNA encoding a pair of ZFNs that target the genomic integration site of AAVS1.
D) ZFNs bind and cut the genomic AAVS1 site in the cell type of interest.
E) ZFN stimulated homology directed repair leads to targeted integration of the GOI into the genome of the cell type of interest.

Figure 2.
Schematic of a pair of four-finger ZFN binding to a target DNA



Each of the two ZFNs bind to a 12 bp sequence on the opposite strand flanking the cleavage site. The two binding sequences are 6 bp apart.

References

1. Lombardo, A., et al., Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat. Biotechnol*., **25**, 1298-1306 (2007).
2. Porteus, M.H., and Baltimore, D., Chimeric nucleases stimulate gene targeting in human cells. *Science*, **300**, 763 (2003).
3. Bibikova, M., et al., Enhancing Gene Targeting with Designed Zinc Finger Nucleases. *Science*, **300**, 764 (2003).
4. Chandrasegaran, S., and Smith, J., Chimeric restriction enzymes: what is next? *Biol. Chem.*, **380**, 841-848 (1999).
5. Linden, R.M., et al., Site-specific integration by adeno-associated virus. *PNAS, USA*, **93**, 11288-11294 (1996).

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. Care must be taken not to introduce RNases. It is recommended to use 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.

Reagents

Kit Reagents	Catalog Number	Supplied
ZFN mRNA – AAVS1	M7947	10 × ~0.6 µg/µl
Forward MCS Primer	F7555	25 µM
Reverse AAVS1 Primer	R5905	25 µM
Integration Control – AAVS1*	I2410	~35 ng/µl
pZDonor – AAVS1	Z3027	~1.75 µg/µl

Note: The exact concentrations of reagents will vary from lot to lot. The exact concentrations for the purchased kit are printed on the Certificate of Analysis.

* The Integration Control is a genomic DNA preparation from a targeted HCT116 clone, in which a multiple cloning site is integrated into one of two copies of the AAVS1 site. It is used as a positive control for the Junction PCR reaction (see Procedures, Section C), the RFLP assay for integration efficiency, and CEL-I assay (see Appendix).

Reagents and Equipment Recommended But Not Provided

Nucleofection® reagents
Electroporation reagents
*Trans*IT®-mRNA Transfection Reagent (Mirus Bio Catalog Number MIR 2225, LLC)
GenElute™ Mammalian Genomic DNA Miniprep Kit (Catalog Number G1N70)
GenElute PCR Clean-Up Kit (Catalog Number NA1020)
Expand High Fidelity^{PLUS} PCR System (Roche Catalog Number 03 300 242 001)
dNTPs (Catalog Number D7295)
Restriction enzyme, *Hin*d III (Catalog Number R1137)
Agarose
EX-CELL® GTM-3 (Catalog Number G9916) - If using electroporation
Hank's Balanced Salt Solution (HBSS, Catalog Number H6648)
Cell Line Nucleofector® Kit V (Amaxa Catalog Number VCA-1003)
DirectLoad™ WideRange DNA Ladder (Catalog Number D7058)
DirectLoad 1 kb DNA Ladder (Catalog Number D3937)
Lysis Solution for Blood (Catalog Number L3289)
Neutralization Solution for Blood (Catalog Number N9784)
AlumaSeal™ II (Catalog Number A2350)
Forward AAVS1 Primer: 5′ GGCCCTGGCCATTGTCACTT 3′
Nuclease S (CEL-I Enzyme) + Enhancer (Transgenomic SURVEYOR® Kit, Catalog Number 706025)
Forward AAVS1 CEL-I Primer: 5′ TTCGGGTCACCTCTCACTCC 3′
Reverse AAVS1 CEL-I Primer: 5′ GGCTCCATCGTAAGCAAACC 3′

Procedures

A. Preparation of Plasmid Donor

A donor plasmid, pZDonor (Catalog Number Z3027), is included in the CompoZr Targeted Integration Kit to be used as both a MCS donor for the Junction PCR Assay for Integration and RFLP Assay for Integration Efficiency, and as a cloning vector to insert your Gene of Interest (GOI). This vector contains two 800 bp sequences flanking the AAVS1 ZFN cleavage site, separated by a 50 bp multiple cloning site (MCS).

1. Unmodified pZDonor

To determine if the CompoZr Targeted Integration Kit works in the cell line of interest, the MCS from the pZDonor should be integrated into the AAVS1 site. A simple PCR to detect the MCS insertion junction using a MCS-specific primer (Forward MCS Primer, Catalog Number F7555) and an AAVS1-specific primer (Reverse AAVS1 Primer, Catalog Number R5905) provides an easy to interpret result, as only positive integration events will yield an amplicon (see Figure 3).

The integration efficiency of the MCS can be obtained by performing the RFLP Assay for Integration Efficiency (see Appendix). This assay provides an estimate for the number of clones to screen in order to obtain a positive integration of the GOI (see Figures 4 and 5, Appendix).

2. Donor Containing your Gene of Interest (GOI)

The GOI can be cloned into the MCS of pZDonor, consisting of the following unique sites: *Hin*d III, *Eco*R I, *Xba* I, *Xho* I, *Pme* I, *Sal* I, and *Not* I. For gene expression, a promoter, the ORF for the gene of interest, and a poly(A) signal need to be included. Direction of transcription may not matter. However, in most of the tested constructs, the expression cassettes are in the same transcriptional orientation as the endogenous gene, which is from left to right. The expression of the same gene in opposite orientations at this locus has not been compared. Additionally, the AAVS1 ZFN target intron 1 of the p84 gene. The endogenous promoter may be used if a splice acceptor is provided. However, if a stand-alone protein (without fusions) expressed from the GOI is desired, an additional genetic element, such as an internal ribosome entry site (IRES) or a 2A peptide, is needed downstream of the splice acceptor. Sequencing the construct is recommended if cloning was performed using PCR amplification of the gene of interest. As a general rule, the use of 2 μg/kb of donor sequence in transfection is recommended. For instance, pZDonor is ~5 kb and the use of 10 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 in transfections is needed.

B. Co-Delivery of ZFN mRNA and Plasmid Donor

Cellular delivery can be performed by nucleofection, standard electroporation, or using lipid based transfection reagents. Please use the specific procedure described for the preferred method of delivery.

1. General Requirements

Cells should be in log phase at the time of transfection. Extra caution should be taken when dealing with RNA. Cells should be washed twice in a serum-free medium or saline before mixing with RNA. Avoid exposing RNA to cells or DNA plasmid unnecessarily. During electroporation or nucleofection, mix RNA with cells immediately before zapping.

Note: For integrating the GOI, simply replace the pZDonor with the cloned GOI-containing donor and make sure to adjust the amount of DNA according to the size of the donor plasmid to 2 μg/kb. Endotoxin-free DNA preparations are preferred.

2. Transfection

a. Via nucleofection

Note: The following procedure has been successfully applied to K562 cells. For cells other than K562, please follow Amaxa's cell line-specific instructions.

i. Seed the cells at a density of 2×10^5 cells/ml the day before transfection.
ii. On the day of transfection, count the cells. Cell density should be between $2.5–5 \times 10^5$ cells/ml.
iii. Centrifuge 2 million cells per transfection at $200 \times g$ for 5 minutes.
iv. Wash cells twice with 20 ml of HBSS.
v. Prepare experimental tubes:

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
ZFN mRNA – AAVS1 (M7947)	–	5 µl	–	5 µl**
Plasmid Donor	–	–	5 µl	5 µl
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied. Total volume in the nucleofection should be less then 10 µl.
** 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.

vi. 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.
vii. Resuspend cells in 100 µl/reaction of Nucleofection Solution V (Amaxa).
viii. One reaction at a time, add 100 µl of cells to each DNA-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-016 for K562 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 pipetteful (~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 to the CO_2 incubator at 37 °C.

b. Via electroporation

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

i. Seed the cells 1–3 days before transfection so the cells are ~80% confluent on the day of transfection.
ii. On the day of transfection, trypsinize the cells and neutralize the trypsin with complete medium.
iii. Count the cells and centrifuge 8 million cells (2 million cells per transfection) at 100 × *g* for 5 minutes.
iv. Wash cells twice with 20 ml of EX-CELL GTM-3 (Catalog Number G9916)
v. Prepare experimental tubes:

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
ZFN mRNA – AAVS1 (M7947)	–	5 µl	–	5 µl**
Plasmid Donor	–	–	5 µl	5 µl
GFP Control Plasmid*	2.5 µg*	–	–	–

* 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 electroporation to avoid exposure to traces of RNase potentially carried over from plasmid preparations.

vi. Fill 4 wells in a 6-well plate with 2 ml of medium each and prewarm in a CO_2 incubator at 37 °C.
vii. Resuspend cells in 800 µl of EX-CELL GTM-3 medium.
viii. One sample at a time, add 200 µl of cells to each DNA-containing tube, add ZFN mRNA at this point, just prior to electroporation, transfer the mix to a 2 mm cuvette and electroporate with the following conditions:
voltage: 115 V, capacitance: 950 µF
ix. Immediately after electroporation of each sample, use a transfer pipette to add a pipetteful of prewarmed medium to the cuvette to help transfer the cells to the medium in the 6-well.
x. Finish all reactions and return the 6-well plate to the CO_2 incubator at 37 °C.

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

i. Cells should be at 60–90% confluency at the time of transfection. The day before transfection, seed 8×10^5 cells in 2 ml of complete medium in a 6-well plate. Remove the medium and add 1 ml of fresh complete medium to cells prior to transfection.

ii. Prepare experimental tubes. Mix reagents by pipetting.

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
***Trans*IT mRNA Reagent**	2.5 µl	2.5 µl	2.5 µl	2.5 µl
Serum-Free Medium	100 µl	100 µl	100 µl	100 µl
MRNA Boost Reagent	1.25 µl	1.25 µl	1.25 µl	1.25 µl

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

Tube - Label / Reagent	1 - GFP	2 - ZFN Only	3 - Donor Only	4 - ZFN + Donor
ZFN mRNA – AAVS1 (M7947)	–	5 µl	–	5 µl
Plasmid Donor	–	–	5 µl	5 µl
GFP Control Plasmid*	2.5 µg*	–	–	–

* GFP Control Plasmid is user supplied.

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

C. Junction PCR Assay for Integration

Note: This assay is used to determine if integration of the MCS from the unmodified pZDonor has occurred as described in Procedures, Section A, Part 1 (also see Figure 3).

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

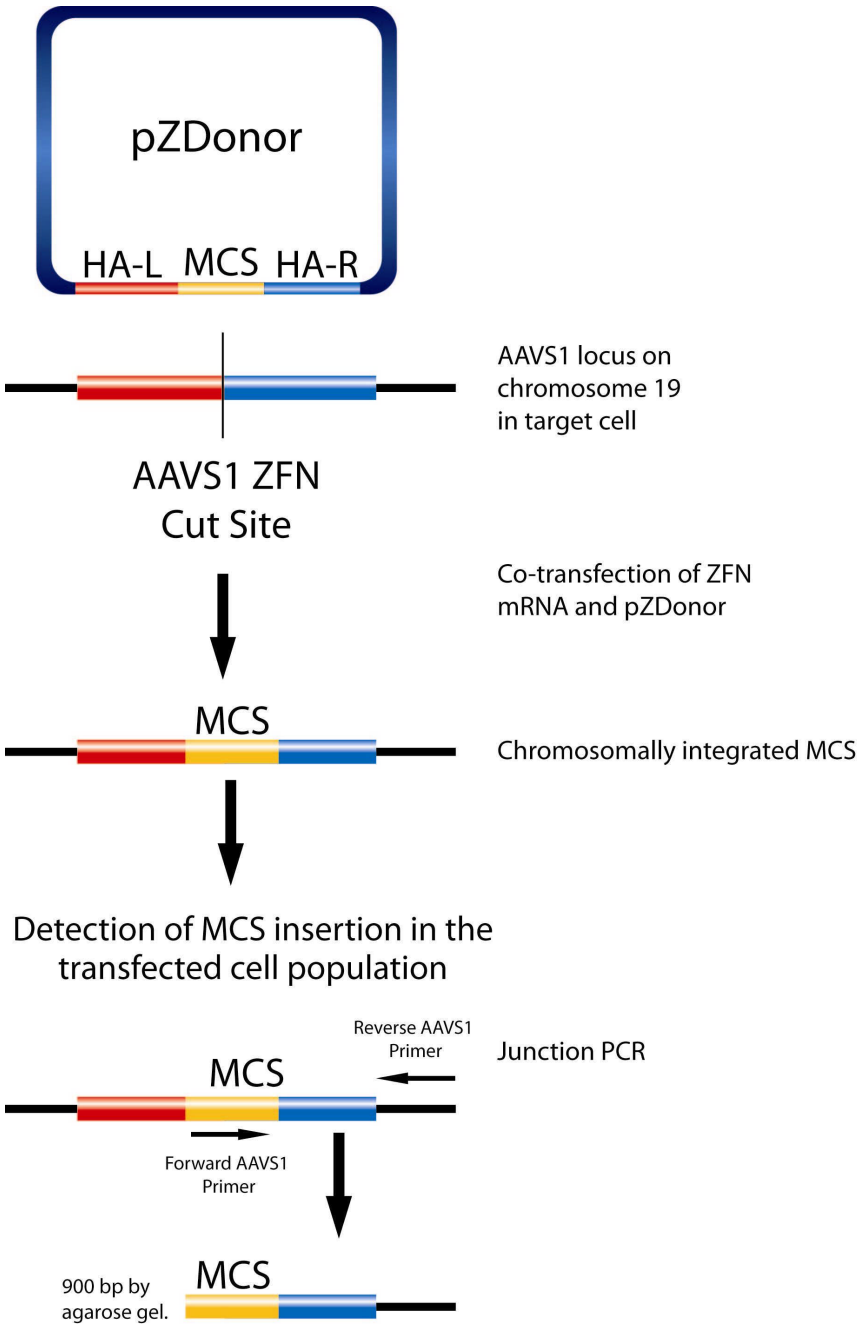
2. PCR amplify the genomic DNA with the Forward MCS Primer and the Reverse AAVS1 Primer. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred to amplify the ~900 bp amplicon. The following procedure uses the Roche Expand High Fidelity^{PLUS} PCR System. 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 supplied Integration Control as template. The Integration Control is the positive control for the PCR reaction.

Reagent	Volume / Reaction
Water, PCR Reagent	Adjustable
5× PCR buffer	10 µl
dNTPs (10 mM)	1 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µl
Forward MCS Primer	1 µl
Reverse AAVS1 Primer	1 µl
Genomic DNA (or 5 µl of integration control)	200 ng
Total volume	50 µl

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	15 cycles; decrease 0.5 °C every cycle
Annealing/Extension	68 °C	1 minute 30 seconds	
Denaturation	95 °C	30 seconds	20 cycles
Annealing	58 °C	30 seconds	
Extension	72 °C	1 minute 30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

3. Run the PCR products on a 1% agarose gel or 5% PAGE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058). The expected size for product is ~900 bp.
4. Only samples in which integration has occurred will give a product at the expected size.
   Note: There is the potential to observe a faint band in the donor alone lane because of amplification of the donor plasmid from the MCS specific forward primer and amplification of the genomic DNA from the AAVS specific reverse primer. In subsequent rounds of amplification complementary pairing and amplification could occur between the two aforementioned amplicons resulting in a faint band in the donor alone lane. If the donor + insert band is brighter than the donor alone band, integration has most likely occurred. To reduce the chances of a faint donor alone band, wait longer before harvesting genomic DNA to allow for the donor plasmid to get washed out and/or degraded.

Figure 3.
Schematic of the Junction PCR Assay for Integration at the AAVS1 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 (~900 bp).

D. Cell Cloning

Do not proceed to single cell dilution cloning until performing a junction PCR on a pool of cells as previously described with an AAVS1 genomic specific primer (included or designed by enduser) and a gene specific primer (instead of the MCS specific primer). A junction PCR product from a pool of cells specific to the GOI provides confidence that it is appropriate to move onto single cell dilution cloning. **It is also recommended to design several pairs of junction primers both to the 3′ junction of the GOI and at the 5′ junction of the GOI** to ensure a pair works well prior to doing single cell dilution cloning. This is also recommended because it provides confidence that the entire GOI has been integrated.

Seven days after transfection, cells are ready for cloning. Cloning can be done earlier. However, waiting for 7 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 days after transfection. It is important to maintain optimal cell growth between transfection and cloning by avoiding overgrowing the cells.
Note: It is highly recommended to determine approximately how many clones will be needed to screen prior to screening by following the RFLP Assay for Integration Efficiency (see Appendix).

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 a 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.
3. Harvest genomic DNA for each well:
   Note: The following example is optimized for adherent HCT116 cells and extraction efficiencies may vary for different cell types.
   a. Aspirate the medium from cells (cells should be confluent).
   b. Wash cells with 100 µl of HBSS (Catalog Number H6648).
   c. Add 20 µl of Lysis Solution for Blood (Catalog Number L3289) to each well, then rock back and forth to ensure the entire well is covered.
   d. Seal 96-well plate with AlumaSeal II (Catalog Number A2350).
   e. Incubate at 75 °C for 15 minutes.
   f. Add 180 µl of Neutralization Solution for Blood (Catalog Number N9784) and mix by pipetting.
   g. DNA can now used directly for PCR or stored at 4 °C for up to 6 months.

4. PCR amplify with either the Reverse AAVS1 Primer and a gene-specific forward primer (not included) or the primer set that gave the most robust amplification on a pool of cells to detect the insertion junction. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred to amplify the amplicon. The following procedure uses 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	Volume
Water, PCR Reagent	Adjustable
5× PCR buffer	4 µl
dNTPs (10 mM)	0.4 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.2 µl
Forward Gene-specific Primer (not provided) (25 µM)	0.4 µl
Reverse AAVS1 Primer (25 µM)	0.4 µl
Genomic DNA (~2 µl if harvested as previously described)	200 ng
Total volume	20 µl

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

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	15 cycles; decrease 0.5 °C every cycle
Annealing/Extension	68 °C	1 minute 30 seconds (may need to be extended)	
Denaturation	95 °C	30 seconds	20 cycles
Annealing	58 °C	30 seconds	
Extension	72 °C	1 minute 30 seconds (may need to be extended)	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

5. Run samples on a 0.8% agarose gel. Only integration clones will give a product at the expected size.

6. 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.
No integration detected using junction PCR on a pool of cells.	Transfection efficiency is too low.	Optimize the transfection procedure to increase the efficiency, >50% is preferred.
	RNA integrity	Run a CEL-I assay (see Appendix). If the ZFN mRNA transfected samples with reasonable transfection efficiency are negative for CEL-I assay, the mRNAs were likely degraded before or during transfections. Follow all proper procedures on handling RNA (see Storage and Stability). To make sure the mRNAs are 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 good cleavage signal, yet no integration was detected, it is probably best to try a different cell line if possible. See Table 1 (Appendix) for cell lines that have been tested so far.
	The cells used are at a high passage number.	Low passage cells should be used. Low passage is generally considered less than 20 passages.
	Residual RNAse from donor is degrading the ZFN mRNA.	Add additional washes to the endotoxin free DNA isolation kit being used. Two to four times the number of washes is recommended. To test if there is RNAse contamination in the donor preparation, mix equal amounts of mRNA with donor (1–2 μl) for 1.5 hours at 37 °C. Then run the mixture 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 RNA is being degraded.
No integration detected by junction PCR at the single cell clone level.	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.
	Primers are not optimized.	Test out several pairs of primers both at the 5′ and 3′ junction of the 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.	Amplification of donor and 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 a band in the donor + ZFN band 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 clone because the donor plasmid will have been washed out during the time it takes to grow a colony of cells from a single cell.
RFLP Assay for Integration Effeciency gave good integration efficiency, but unable to obtain GOI integration clones.	Transfection efficiency is too low.	Always make sure GFP plasmid control is included in every transfection experiment.
	RNA degradation	Perform CEL-I assay on the pooled DNA. If CEL-I is negative, most likely the ZFN mRNA was degraded during or before the transfections. Make sure all the proper procedures are followed.
	Junction PCR conditions are not optimal.	Try the targeting experiment in K562 cells, which has been shown to support highly efficient targeted integration of insertion up to 8 Kb. Use the targeted K562 DNA as template to work out junction PCR conditions.
	Overexpression of the 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. Decreased efficiencies with increased insertion size have been observed.	Screen more clones if junction PCR showed positive.

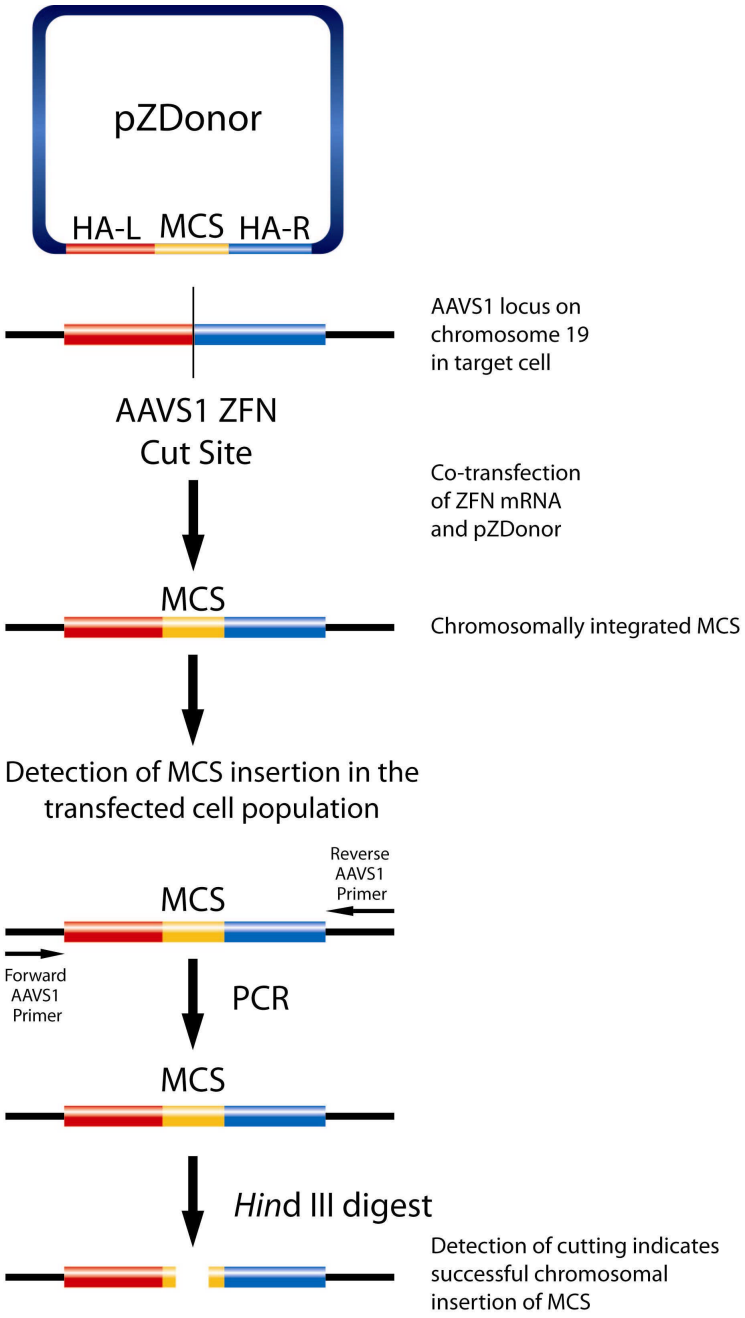
Appendix

A. Determining Integration Efficiency in the Cell Line

When transfected into different human cell lines, the AAVS1 ZFNs have comparable cutting efficiencies across all lines tested. However, integration efficiency ranges between 3 and 30% when inserting a 50 bp multiple cloning site into the AAVS1 locus in various human cell lines. This indicates HDR does not occur at the same efficiency in all cell lines. In order to determine the rate of integration in your cell type of interest, performing the RFLP Assay for Integration Efficiency is recommended. The results of this experiment will serve as a guide to determine the efficiency of integration in the cell line and how many clones need to be screened to find a cell clone in which the GOI has been integrated.

The RFLP Assay for Integration Efficiency consists of co-transfecting ZFN mRNA – AAVS1 (Catalog Number M7947) and the pZDonor (Catalog Number Z3027) to integrate the 50 bp multiple cloning site between the two homologous arms in the donor upon cleavage of the AAVS1 site by the ZFNs. A pair of primers outside of the homology arms is used to amplify the genomic DNA from the transfected pool of cells. The pool of cells as well as the PCR product made from that pool will include wild type DNA as well as fragments that have had the multiple cloning site integrated at the AAVS1 locus. The rate of integration can be determined by digesting the PCR products with one of the restriction enzymes in the multiple cloning site. The fragments with the multiple cloning site integrated will then be cleaved and the wild-type DNA will remain intact (see Figure 4). The resulting digestion product can then be run on a gel and the efficiency of integration determined by analyzing the percentage of cut DNA as compared to uncut DNA.

Figure 4.
Schematic of the RFLP Assay for Integration Efficiency.



HA-L and HA-R stand for Homology Arm-Left and Homology Arm-Right, respectively. MCS stands for Multiple Cloning Site. The location of the restriction enzyme *Hind* III site is indicated. Integration efficiency can be determined by analyzing the percentage of cut DNA as compared to uncut DNA.

B. RFLP Assay for Integration Efficiency

Note: This assay is used to determine integration efficiency of the MCS from the unmodified pZDonor as previously described.

1. One to three days after transfection, collect the cells to prepare chromosomal DNA using a GenElute Mammalian Genomic DNA Miniprep Kit.

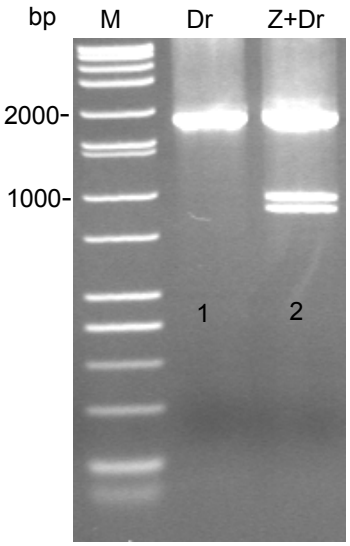
2. PCR amplify the genomic DNA with the Forward AAVS1 Primer (not supplied; 5′ GGCCCTGGCCATTGTCACTT 3′) and the Reverse AAVS1 Primer. A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred to amplify the ~1.9 kb amplicon. The following procedure uses the Roche Expand High Fidelity^{PLUS} PCR System. Optimization of 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 restriction digest assay.

Reagent	Volume / Reaction
Water, PCR Reagent	Adjustable
5× PCR buffer	10 µl
dNTPs (10 mM)	1 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µl
Forward AAVS1 Primer (not supplied)	1 µl
Reverse AAVS1 Primer	1 µl
Genomic DNA	200 ng
Total volume	50 µl

Step	Temp.	Time	Cycles
Initial Denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	15 cycles; decrease 0.5 °C every cycle
Annealing/Extension	68 °C	3 minutes 30 seconds	
Denaturation	95 °C	30 seconds	20 cycles
Annealing	58 °C	30 seconds	
Extension	72 °C	3 minutes	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

a. Use a PCR cleanup kit to purify the PCR reactions: Donor Plasmid Alone, Donor Plasmid plus ZFN mRNA, and the Integration Control.
b. Digest 17 µl of cleaned up products with 2 µl of *Hin*d III buffer and 1 µl of *Hin*d III at 37 °C for 1 hour.
c. Run the digestions on a 0.8% agarose gel or 5% PAGE gel with proper markers, such as DirectLoad WideRange Ladder (Catalog Number D7058). The expected size for uncut product is ~1,900 bp, and those for *Hin*d III-cut products are ~1,000 bp and ~900 bp.
d. Determine integration efficiency by using a densitometry program, such as the freeware ImageJ (http://rsbweb.nih.gov/ij/). The integration control should show roughly 50% cleavage (see Figure 5).

Figure 5.
An example of the RFLP Assay for Integration Efficiency in K562

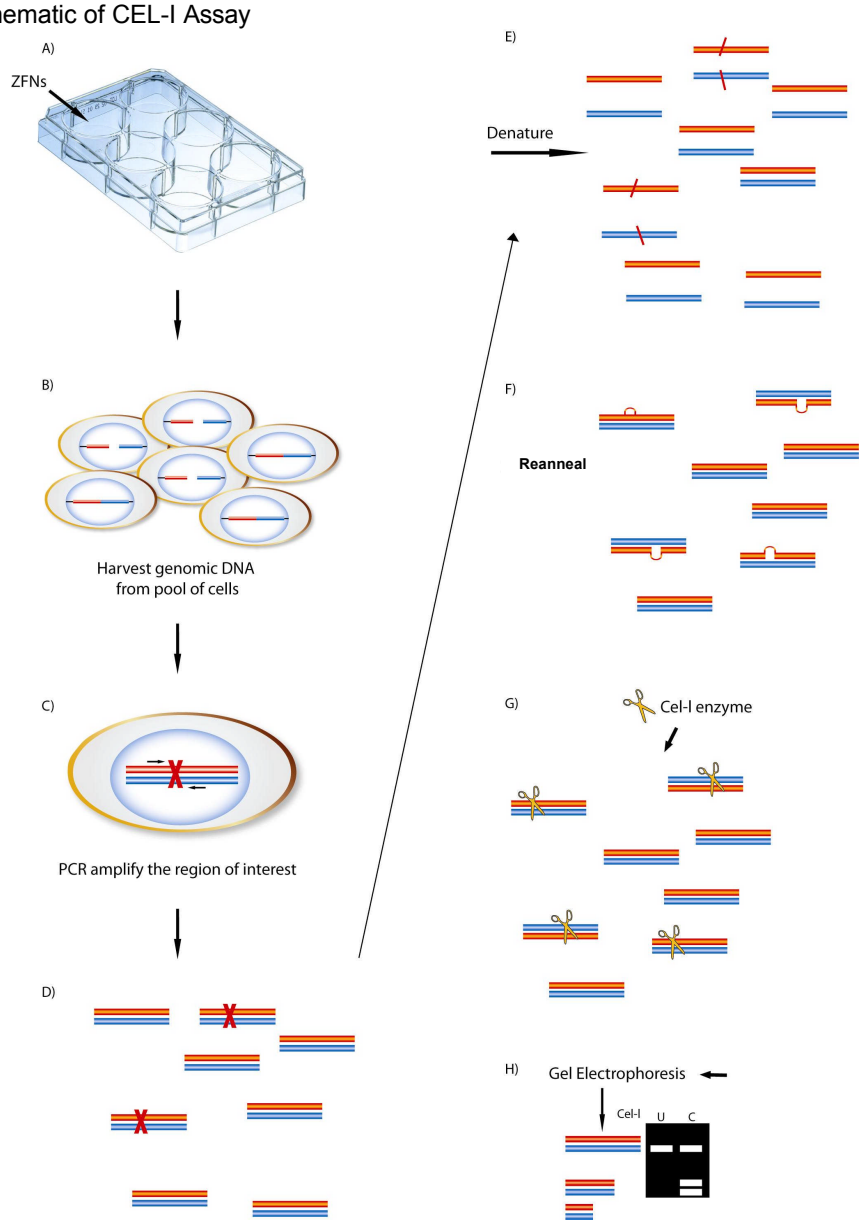


Cells were transfected via nucleofection and harvested 3 days after transfection. DNA was prepared, amplified, and digested as described in Appendix, Procedure B, step c. Lanes 1 and 2 are results from the PCR Assay, comparing donor alone versus donor plus ZFN. Samples were resolved on a 0.8% agarose gel.
M: Marker
Dr: Donor alone
Z+DR: ZFN+ Donor

C. 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, i.e., homologous directed recombination and non-homologous end joining (NHEJ). NHEJ is more efficient in most cell types and an error-prone process that often introduces deletions and insertions at the cleavage site. To measure the cleavage efficiency of ZFNs in the cell, use the CEL-I assay, which takes advantage of the NHEJ process. In the assay, the target region is amplified in a PCR reaction using genomic DNA from the transfected cell pool as template. If ZFNs are active, the genomic DNA will be a mixture of wild-type and NHEJ 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 (see Figure 6).

Figure 6.
Schematic of CEL-I Assay



CEL-I assay is used to analyze ZFN activity. After transfection of the ZFN mRNA into cells (A), the ZFNs bind and cut their target sequence creating a double-strand break in a portion of the cells (B). If the double-strand break is repaired by aberrant nonhomologous end joining (NHEJ), insertions or deletions (depicted by red X) will be made (C). Genomic DNA is then harvested from the transfected pool of cells. PCR is performed to amplify the target site from the genomic DNA of the transfected pool (C-D). The PCR product is denatured (E) and reannealed to allow for heteroduplex formation between the wild-type and modified amplicons (F). Then, the CEL-I enzyme, which specifically cleaves mispaired regions in double strand DNA, is added (G). The CEL-I enzyme digests are resolved using gel electrophoresis (H). The relative intensity of the smaller bands compared to the parental band indicates the frequency of NHEJ events, hence, efficiency of ZFN. On top of the black box (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.

D. CEL-I Assay Procedure

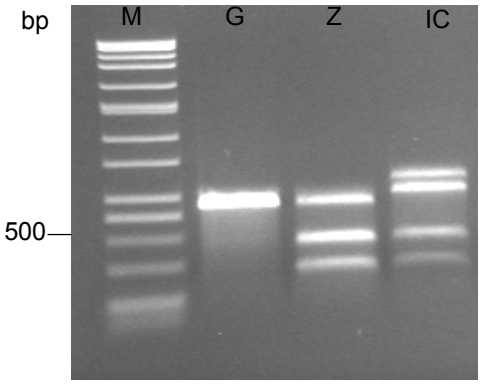
1. PCR amplify the genomic DNA from the GFP and ZFN alone mRNA transfected samples from Procedures, Section C with the Forward AAVS1 CEL-I Primer (not supplied; 5′ TTCGGGTCACCTCTCACTCC 3′) and the Reverse AAVS1 CEL-I Primer (not supplied; 5′ GGCTCCATCGTAAGCAAACC 3′). A DNA polymerase that efficiently amplifies human genomic DNA with proofreading capability is preferred to amplify the ~500 bp amplicon. The following procedure uses 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	Adjustable
5× PCR buffer	10 µl
dNTPs (10 mM)	1 µl
Roche Expand High Fidelity^{PLUS} Polymerase	0.5 µl
Forward AAVS1 CEL-I Primer (not provided) (25 µM)	1 µl
Reverse AAVS1 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 minutes	1
Denaturation	95 °C	30 seconds	30 cycles
Annealing	55 °C	30 seconds	
Extension	72 °C	30 seconds	
Final Extension	72 °C	5 minutes	1
Hold	4 °C	Indefinitely	

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 (CEL-I) (from Transgenomic Catalog Number 706025) to each reaction and incubate at 42 °C for 20 minutes.
4. Run the digestions on a 1% agarose gel or 5% PAGE gel with proper markers, such as DirectLoad WideRange DNA Ladder (Catalog Number D7058) (see Figure 7).

Figure 7.
An example of the CEL-I Assay in K562



Cells were transfected via nucleofection and harvested 1 day after transfection. DNA was prepared, and PCR and digestions were done as previously described.
M: DirectLoad WideRange DNA Marker (Catalog Number D7058)
G: GFP transfected cells
Z: Cells transfected with ZFNs alone
IC: Integration control from HCT116 cells - Integration control is a genomic DNA preparation from a targeted HCT116 clone, in which a multiple cloning site is integrated into one of the two copies of AAVS1 site. For this reason, two top bands are observed in the IC control CEL-I assay.

E. 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.

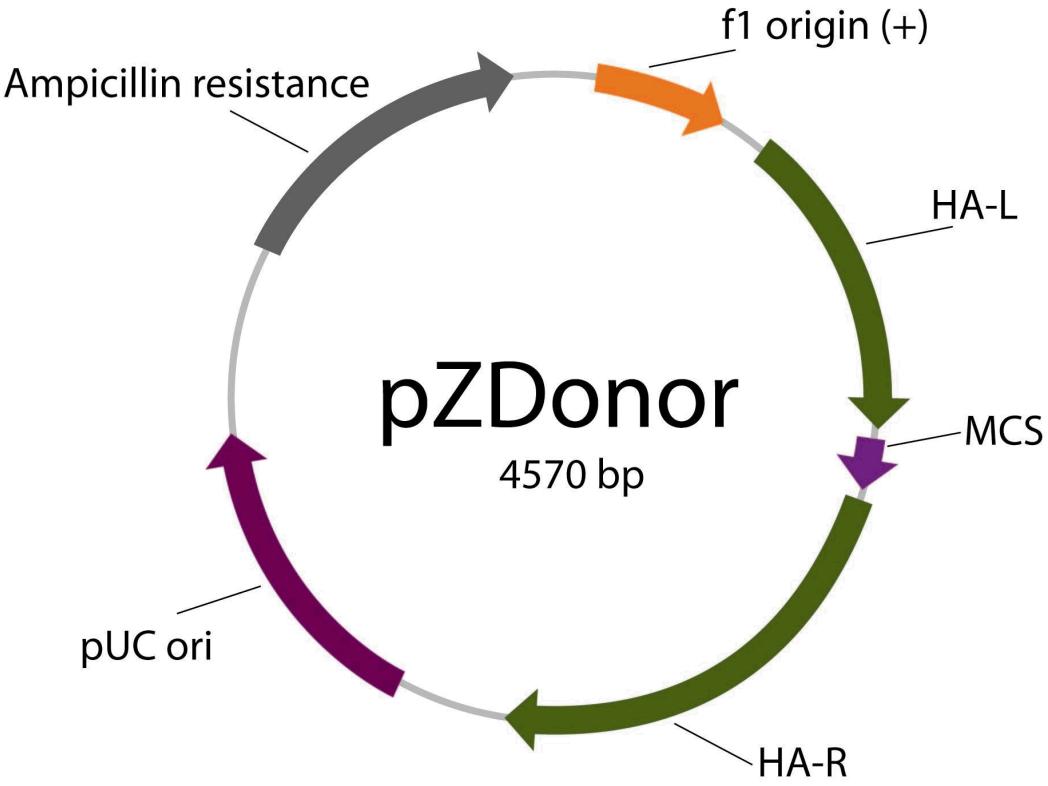
Table 1.
Targeted Integration in Various Cell Lines Using Different Delivery Methods

Cell Line	Nucleofection	Electroporation	*Trans*IT (lipid)
K562	+	NA	NA
HEK293	+	+	+
HeLa	+	+	+
A549	+	+	+
MCF7	+	+	NA
HCT116	+	+	NA
U-2 OS	+	NA	NA
IMR 90	+	NA	NA
LNCap	+	NA	NA
DU145	+	NA	NA
HepG2	+	NA	NA

In each experiment, 10–20 μg of plasmid donor and 4 μg of ZFN mRNA were used.

(+) indicates >1% integration for the given cell type and transfection method.
NA stands for not assayed.

Map of the donor plasmid



Multiple cloning site:
AAGCTTGAATTCTCTAGAAATATTCTCGAGGTTTAAACGTCGACGCGGCCGC
Hind III *Eco*R I *Xba* I *Xho* I *Pme* I *Sal* I *Not* I

Primer Sequences:

Forward MCS Primer:
5′ AGCTTGAATTCTCTAGAAATATTCTCGAGGTTTAAACGTCGACGC 3′

Reverse AAVS1 Primer:
5′ GGAACGGGGCTCAGTCTG 3′

AAVS1 ZFN Recognition Sequence:

5′- ACCCCACAGTGGggccacTAGGGACAGGAT 3′

The 12 bp in red uppercase is the complimentary sequence of the recognition site of the left ZFN, the 12 bp in blue uppercase is the binding site of the right ZFN, and the 6 bp in black lowercase is the space between the two ZFN recognition sites, over which the *Fok*I domains dimerize and cleave the DNA to make a double strand break.

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