A Southern blot protocol to detect chimeric nuclease-mediated gene repair

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Running head: PCR-based Southern for gene repair

Summary

Gene targeting by homologous recombination at chromosomal endogenous loci has traditionally been considered a low-efficiency process. However, the effectiveness of such so-called genome surgery has recently been drastically improved through technical developments, chiefly the use of designer nucleases like zinc-finger nucleases (ZFNs), meganucleases and transcription activator-like effector nucleases (TALENs). These enzymes are custom-designed to recognize long target sites and introduce double-strand breaks (DSBs) at specific target loci in the genome, which in turn mediate significant improvements in the frequency of homologous recombination. Here, we describe a Southern blot-based assay that allows detection of gene repair and estimation of repair frequencies in a cell population, useful in cases where the targeted modification

itself cannot be detected by restriction digest. This is achieved through detection of a silent restriction site introduced alongside the desired mutation, in our particular example using integration-deficient lentiviral vectors (IDLVs) coding for ZFNs and a suitable targeting template matrix.

Key words: Zinc-finger nucleases, integration-deficient lentiviral vectors, homologous recombination, Southern blot.

1. Introduction

Gene targeting is a process in which information from a DNA molecule introduced into a cell replaces that in the corresponding chromosomal segment. The process relies on homologous recombination, one of the major cellular pathways for DSB repair. Gene targeting is a very low efficiency process that can be considerably enhanced by the introduction of a DSB at the target locus. This major development was initially achieved using the *S. cerevisiae* homing endonuclease I-*Sce*I and pre-engineered target sites (reviewed in [1]). Practical application had to wait for the development of target site-specific chimeric nucleases, initially ZFNs and I-*Cre*I-based meganucleases, and more recently TALENs. These enzymes can introduce DSBs at specific target sites, which in turn stimulate the cell's endogenous homologous recombination machinery [2, 3]. The first example of targeted genome manipulation *in vitro* with engineered nucleases at a human endogenous locus was based on the use of ZFNs [4]. These enzymes have also mediated the first example of *in vivo* genome surgery [5]. The following references provide recent reviews of engineered nuclease technology [6-8].

An important requirement to assess the efficiency of gene targeting is the availability of an *in vitro* assay to detect the phenomenon and estimate its frequency. While deep-sequencing technologies have facilitated this considerably, normally through custom services, it is still useful to have a gel-based assay for in-house estimation of gene targeting frequencies. Here, we describe in detail a Southern blot-based protocol that allows detection and quantification of a surrogate restriction site, to estimate correction of a linked targeted point mutation in cell lines and primary cells.

The protocol we will describe here does not require a particular type of designer nuclease, but was developed using a ZFN, and for this reason a brief description of this particular type of enzymes follows. ZFNs are heterodimeric enzymes that combine the non-specific cleavage domain from *Fok*I restriction endonuclease with DNA-binding zinc-finger domains. The monomers are composed of several tandemly arranged zinc-finger domains (each recognizing a tri-nucleotide in the target site) and a nuclease domain. The enzyme dimerizes through the *Fok*I domains, providing a tool to recognize a specific site in which a spacer of about six nucleotides where the cutting will occur is flanked by the sites recognized by the two monomers (**Figure 1**). Because the recognition specificities of the zinc-finger domains can be easily manipulated experimentally, ZFNs offer a general way to induce targeted site-specific DSBs in the genome [9].

A major issue with designer nucleases is off-target cutting-mediated mutagenesis or toxicity, whereby unintended DSBs introduced in the target genome lead to unwanted effects [10]. The development of obligate heterodimeric ZFNs and further refinements of the nuclease domain have helped to address this problem [11-15], but generic concerns with nuclease off-target cutting remain. In any case, targeted nuclease technology has become a powerful tool for genetic manipulations, not only for site-specific genome surgery in plant and mammalian genomes, but potentially also for human therapy.

Firstly, once a strategy for targeted modification has been decided upon, a target site for the nuclease must be chosen. As gene conversion tracts are generally short [16, 17], the nuclease recognition site should be chosen as close as possible to the target site to be modified. The ZiFiT software package identifies potential target sites in DNA sequences for which ZFNs maybe engineered [18]. Once the ZFN is obtained, its cutting efficiency can be tested using the surveyor *Cel*I assay as described by Guschin *et al*. [19] or deep sequencing, in both cases detecting modifications introduced at the target site by non-homologous end-joining repair of the induced DSBs.

Secondly, a targeting construct (repair matrix) must be obtained. Our goal was the correction of a point mutation in a mouse gene. To avoid the presence of heterologies that can reduce the frequency of gene targeting [20], we prepared a 1.7 kb corrective template using high-fidelity PCR amplification of genomic DNA from wild-type fibroblasts of the same mouse strain, followed by DNA sequencing to check for absence of unwanted sequenced changes. Afterwards, using standard site-directed mutagenesis, we introduced a silent diagnostic restriction site on the template, as close as possible to the site that would correct the genomic mutation (**Figure 2**). As our ZFN target site was located 3' to the mutation on the target gene, we placed our silent site 5' to the wild-type site on the correcting template. In this configuration, incorporation of the silent restriction site on the targeted gene would ensure the presence of the linked wild-type nucleotide and hence the reversion of the mutation (**Figure 2**).

Delivery of the corrective cassette and the genes encoding the ZFN monomers may be achieved by several methods, in our case integration-deficient lentiviral vectors (IDLVs) [21, 22]. Once the target cells have been transfected or transduced with the relevant vectors, the cellular DNA is extracted to analyze for the presence of the desired change. For this the DNA sequence around the target site is PCR-amplified with primers external to the corrective template, to prevent amplification of template molecules not yet diluted out or randomly integrated in the cells (**Figure 3A**). The PCR product is then digested overnight with the diagnostic restriction enzyme. The digestion mixture is loaded onto an agarose gel, electrophoresed, transferred onto a membrane and subsequently hybridized, using the initial PCR product as a labeled probe (**Figure 3B**). In this manner, we have been able to visualize the three bands expected if homologous recombination-mediated gene repair occurs between the cellular DNA and the donor DNA template. Using Image J, it was also possible to quantify by densitometry the 1.7 kb band from untargeted alleles and the 1 kb band diagnostic for gene repair, and to estimate the percentage of gene targeting from

their relative values. We chose not to include the diagnostic 0.7 kb band on the densitometry measurements because of the overlap with non-specific smear at low molecular weight (**Figure 3C**). This method has allowed us to estimate frequencies of correction of about 2% of alleles in the population.

2. Materials

2.1: *In vitro* transduction of fibroblasts with IDLVs coding for the ZFN monomers and the repair template

1. Mouse fibroblasts.
2. Culture medium:Dulbecco's Modified Eagle Medium (DMEM with high glucose, Gibco/Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco/Invitrogen) and 1X penicillin/streptomycin (100X solution, Gibco/Invitrogen).
3. Trypsin solution (0.25% trypsin/EDTA, Gibco/Invitrogen).
4. IDLVs encoding ZFN1, ZFN2 and template. A complete protocol for producing and titrating lentiviral particles has been described by Giry-Laterrière *et al.*[23]. Suggestions pertaining to IDLVs have been published before [22].

2.2: Genomic DNA extraction and PCR amplification of the targeted region

1. DNeasy Blood & Tissue Kit (Qiagen).
2. 100% EtOH.
3. PCR reaction: 10X LongAmpTaq reaction buffer (NEB), 10 mM dNTPs, external primers to the template diluted to10 μM, LongAmpTaq polymerase (NEB), dH2O.
4. 0.7% agarose gel in 1X TAE. 50X TAE: In 900ml dH2O 242g Tris base, 57.1ml Glacial Acetic Acid, 18.6 g EDTA. Adjust pH to 8 and the volume to 1L.
5. Appropriate waste disposal for ethidium bromide-contaminated materials following local H&S guidelines.

2.3: Restriction enzyme digestion of PCR product, agarose gel electrophoresis and salt transfer

1. Appropriate restriction enzyme (NEB), 10X NEB buffer, 10X BSA if required according to manufacturer's instructions.
2. DNA size markers without loading dye.
3. 10X Nick Translation buffer (NTB): 0.5M TrisHCl pH 7.5, 0.1 M MgSO4, 1 mM DTT, 500 μg/ml BSA
4. CTG: 0.5 mMof each dCTP, dTTP and dGTP in 10 mM TrisHCl pH 7.5.
5. [α-^{32}P]dATP.
6. DNA Polymerase I, Large (Klenow) Fragment (NEB, M0210S).
7. TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).
8. Pharmacia Microspin S-300HR columns.

9. 0.7% agarose gel in 1X TAE, cast in large electrophoresis tray (~200 ml) in order to obtain a good separation of the digested fragments.
10. 0.25 N HCl: 975 ml ddH2O with 25ml of 37% HCl added drop-wise (make up in a fume hood).
11. Denaturing solution: 0.4 N NaOH (16 g), 0.6 M NaCl (35.08 g), ddH2O to 1 l.
12. Neutralizing solution: 1.5 M NaCl (87.66 g), 0.5 M TrisHCl (60.55 g), 32 ml HCl, ddH2O to 1 l.
13. GeneScreen Plus membrane.
14. 20XSSC: 3M NaCl (175.35 g), 0.3 M tri-sodium citrate dehydrate (88.23 g), ddH2O to 1 l.
15. Whatmann 3MM paper.
16. Blotting paper.
17. Glass plate (same size as agarose gel).
18. Bottle weighting about 500 g.
19. 0.4 N NaOH: 16 g NaOH, ddH2O to 1 l.
20. 0.2 M TrisHCl pH 7.5 or 8, 1XSSC: 40 ml 1M Tris, 10 ml 20XSSC, 150 ml ddH2O.
21. Appropriate waste disposal for radioactively contaminated materials, according to local H&S guidelines.

2.4: Probe labeling and hybridization

1. TE buffer.
2. 60 ng/μl dN6 (random hexanucleotides) (Sigma, H-0268).
3. 10X random priming buffer (RP): 0.5 M TrisHCl pH 6.9, 0.1 M MgSO4, 1 mM DTT (store at -20°C).
4. CTG: 0.5mM of each dCTP, dTTP and dGTP in 10 mM TrisHCl pH 7.5.
5. [α-^{32}P]dATP
6. DNA Polymerase I, Large (Klenow) Fragment (NEB, M0210S).
7. Pharmacia Microspin S-300HR columns.
8. Church mix (1% BSA, 7% SDS, 0.5 M Phosphate buffer): prepare solutions 1,2 and 3, mix them and make up to 500 ml with ddH2O.
9. Solution 1 for Church mix: 35 g SDS dissolved in ddH2O and made up to 300 ml.
10. Solution 2 for Church mix: 5 g BSA dissolved in ddH2O and made up to 50 ml.
11. Solution 3 for Church mix: 28.4 g Na2HPO4, 6.9 g NaH2PO4.H2O dissolved in water (warming up in microwave oven) and made up to 125 ml.
12. 2XSSC 0.5% SDS: 100 ml 20XSSC, 5 g SDS, ddH2O to 1 l.
13. Plastic bag suitable for heat sealing.

3. Methods

3.1: *In vitro* transduction of fibroblasts with IDLVs coding for ZFN monomers and the repair template

Carry out all procedures under sterile cell culture conditions.

1. Wash a 75-cm^2 flask of fibroblasts (70-80% confluent) with 15 ml PBS, and trypsinize the cells using 1.5 ml trypsin-0.25% EDTA.
2. Resuspend the cells in 10 ml of DMEM 10% FBS and transfer them to a 15 ml conical tube.
3. Centrifuge the cells at 478 *g* for 5 min, remove the supernatant and resuspend the pellet in 10 ml of DMEM 10% FBS.
4. Determine the number of cells/ml using a haemocytometer.
5. Plate out 10^5 cells per well in a 6-well plate. Place the plate in an incubator at 37°C, 5% CO2 overnight (**Note 1**).
6. The next day, replace the medium with a minimum volume of fresh medium, ensuring that there is enough to cover the cell monolayer (e.g.: 0.5 ml/well for a 6-well plate). This will maximize the chances of interaction between vectors and cells (**Note 2**).
7. Transduce with appropriate MOI (Multiplicity of infection) of ZFN1, ZFN2 and template vectors, using a relative ratio of 1:1:2, respectively (**Note 3**).
8. Return the plate to the incubator for 3-4 h, then top-up each well with 1.5 ml of fresh medium. Incubate the plate for 5 more days.

3.2: Genomic DNA extraction and PCR amplification of the targeted region

1. Remove media from each well, wash with 2 ml PBS and trypsinize the cells using 100 µl trypsin-0.25% EDTA.
2. Resuspend the cells in 1 ml DMEM 10% FBS and then transfer to 1.5 ml eppendorf tubes.
3. Centrifuge the cells at 478 *g* for 5 min, remove supernatant and resuspend pellet in 1 ml PBS.
4. Centrifuge the cells again at 478 *g* for 5 min, remove supernatant and resuspend cells in 200 µl PBS.
5. Proceed to genomic DNA extraction according to DNeasy Blood & Tissue Kit (Qiagen):
6. Add 20 µl proteinase K and 200 µl Buffer AL and mix thoroughly by vortexing.
7. Incubate the samples at 56°C for 10 min.
8. Add 200 µl ethanol (96–100%) and mix thoroughly by vortexing.
9. Transfer the mixture onto a DNeasy Mini spin column placed in a 2 ml collection tube.
10. Centrifuge at 4,720 *g* for 1 min. Discard the flow-through and collection tube.
11. Place the spin column in a new 2 ml collection tube.
12. Add 500 µl Buffer AW1.
13. Centrifuge for 1 min at 4,720 *g.*
14. Discard the flow-through and collection tube.
15. Place the spin column in a new 2 ml collection tube.
16. Add 500 µl Buffer AW2.
17. Centrifuge for 3 min at 14,460 *g*. Discard the flow-through and collection tube.
18. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

19. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at 4,720 *g* and keep DNA-containing eluate.
20. Measure DNA concentration by optical density at 260 nm (Nanodrop or similar).
21. Perform a PCR amplification using primers external to the template DNA region:
22. Prepare the PCR mix as follow:
    - 100 ng of genomic DNA
    - 1X LongAmpTaq reaction buffer
    - 300 µM dNTPs
    - 0.4 µM forward primer
    - 0.4 µM reverse primer
    - 2.5 U LongAmpTaq DNA polymerase (NEB, M0323S)
    - Complete to 25 µl with nuclease-free water
23. Gently mix the reaction on ice and transfer tubes to PCR machine with the block preheated to 94°C.
24. Begin thermocycling (94°C 30 sec, 30X (94°C 30 sec, 59°C 60 sec, 65°C 1 min 20sec), 65°C 10 min, hold 4°C).
25. Load 5 µl of the reaction in a 0.7% TAE-agarose gel containing 0.5 µg/ml ethidium bromide and run for 50 min at 50 V.
26. Expose the gel to UV light to verify specificity of amplification and size of the amplified product.

3.3: Restriction enzyme digestion of PCR product, agarose gel electrophoresis and salt transfer

<u>Reminder</u>: a diagnostic restriction site will have been introduced next to the corrected site if homologous recombination-mediated gene repair has taken place. Take standard precautions when handling radioactive materials and dispose of waste according to local regulations.

1. Measure the DNA concentration of each PCR product by optical density at 260 nm.
2. Transfer 500 ng into clean 1.5 ml eppendorf tubes.
3. Add:
    - 5 µl 10X enzyme buffer
    - 5 µl 10X BSA (if required)
    - 1 µl of the appropriate enzyme
    - Top-up to 50 µl with sterile distilled water.
4. Incubate at 37°C for 16 hours.
5. Prepare radioactive size markers ("hot ladder") as follows (**Note 4**):
6. Make up mix of:
    - 1 µg suitable DNA size marker (without loading dye)
    - 2 µl 10X nick-translation buffer (NTB)
    - 4 µl CTG
    - 1 µl (10 µCi) [α-^{32}P]dATP
    - 1 µl Klenow (5U/µl)
    - Top up with water to 20 µl.

7. Mix gently and incubate at room temperature for 90 min.
8. Add 40 µl of TE buffer and mix.
9. Count 1 µl in Biomax Cerenkov Geiger counter.
10. Pack Pharmacia Microspin S-300HR column by spinning at 660 *g* for 1 min.
11. Load sample onto column, spin at 660 *g* for 2 min, keep eluate.
12. Count 1 µl of eluate in Biomax Cerenkov Geiger counter (incorporation should be around 50%).
13. Keep the hot ladder (eluate) frozen for up to several weeks if necessary.
14. Cast a 200 ml 0.7% agarose gel in 1XTAE containing 0.5 µg/ml ethidium bromide.
15. Load lanes with 15-45 Cerenkov Geiger counts of hot ladder or the totality of the digested products.
16. Run gel overnight at 35 V in 1XTAE-buffer with 0.5 µg/ml ethidium bromide, in cold room.
17. Take and save a picture.
18. Shake the gel in 0.25 N HCl for 10min.
19. Wash gel twice with dH_2O.
20. Shake the gel in denaturing solution for 30 min.
21. Wash gel twice with ddH_2O.
22. Shake the gel in neutralizing solution for at least 30 min.
23. Cut GeneScreen Plus membrane about 1cm longer and wider than the gel (gel stretches when smoothed on transfer platform). Cut a corner as an orientation reference.
24. Pre-wet the membrane in water for a few seconds and equilibrate it in 10XSSC for 15 min.
25. Set-up a capillary transfer blot using 10XSSC (remove air bubbles by rolling pipette). Order from bottom:
    - Inverted gel (isolate the gel edges with plastic film)
    - GeneScreen Plus membrane
    - Two 10XSSC-soaked Whatman 3MM paper pieces (same size as gel)
    - Stack of blotting paper about 10 cm high (same size as gel)
    - Glass plate
    - Bottle weighting about 500 g
26. Transfer overnight.
27. Dismount the transfer blot.
28. Shake the membrane in 0.4 N NaOH for 1 min.
29. Shake the membrane in 0.2 M Tris-HCl pH 7.5 or 8 for 1 min.
30. Wash membrane quickly in 2XSCC.
31. Place the membrane DNA side up on 2XSCC-soaked Wathman 3MM paper.
32. Fix in Stratagene UV cross-linker using auto cross-link function (the membrane can be dried on filter paper for storage or kept in 2XSSC if proceeding to hybridization).

3.4: Probe labeling and hybridization

Reminder: to identify the bands of interest we probe the membrane with the starting PCR product. This fragment will hybridize with the 1.7 kb PCR product as well as with the 1 and 0.7 kb digested fragments (**Note 5**). Take standard precautions when handling radioactive materials and dispose of waste according to local regulations.

1. Transfer 50-100 ng of the PCR product into a 1.5 ml eppendorf tube (ideally screw-cap) and top-up to 4 µl with TE buffer.
2. Add 1 µl of 60 ng/µl dN6 and boil for 5 min in a heat-block.
3. Transfer onto ice for 2 min and afterwards spin down for a few seconds at full speed.
4. Add 12 µl of the following mixture:
    - 12 µl water
    - 3 µl 10X random priming buffer (RP)
    - 3 µl CTG.
5. Add 2 µl (20 µCi) [α-32P]dATP (**Note 6**).
6. Add 1 µl of Klenow (5U/µl).
7. Incubate at room temperature for 1 h.
8. Add 40 µl TE buffer.
9. Count 1µl in Biomax Cerenkov Geiger counter.
10. Pack Pharmacia Microspin S-300HR column by spinning at 660 *g* for 1 min.
11. Load sample onto column and spin at 660 *g* for 2 min, keep eluate.
12. Count 1 µl of eluate in Biomax Cerenkov Geiger counter (incorporation should be around 50%).
13. Wet the DNA membrane from step 3.3-32 in 2XSSC if it was stored dry and spread it in a hybridization tube. Drain 2XSSC from tube.
14. Add 15 ml of Church mix.
15. Pre-hybridize rotating for at least one hour at 68°C.
16. Boil the labeled probe for 5 min in a heat-block and transfer it to ice for 2 min.
17. Add the boiled probe to the hybridization tube.
18. Hybridize at 68°C rotating overnight.
19. Discard the hybridization solution.
20. Perform 3 quick washes and 3x10min washes with 2XSSC, 0.5% SDS at 65°C (**Note 7**).
21. Seal the membrane in a plastic bag.
22. Expose to a Phosphoimager screen.
23. Acquire image by using a laser scanner (**Figure 3**).

3.5: Estimation of the percentage of corrected alleles using Image J

Image J software can be freely downloaded from:
http://rsbweb.nih.gov/ij/download.html

1. Using Image J, quantify the intensity (I) of the relevant bands (1.7 kb, 1 kb and 0.7 kb) on the Phosphoimager image (**Note 8**).

2. The percentage of corrected alleles = $(100 \times (I_{1kb} + I_{0.7kb})) / (I_{1.7kb} + I_{1kb} + I_{0.7kb})$. Or if 0.7 kb band is not scanned for densitometry (**Note 5**), percentage of corrected alleles = $(100 \times (1.7 \times I_{1kb})) / (I_{1.7kb} + 1.7 \times I_{1kb})$.

4. Notes

1. It is also possible to plate the cells out on the day of transduction, waiting for them to attach before vector is added.
2. Another way to maximise transduction is to add vectors to the cells in suspension, before plating out. Briefly, put the same number of cells in 1.5 ml eppendorf tubes in a minimum volume, add the vectors, mix gently and incubate at 37°C for 1 to 2 h (mix every 15 min as the cells will drop to the bottom of the tube). Then, either wash the cells once, centrifuge and resuspend them in an appropriate volume of medium for plating in a 6-well plate well or add medium to the eppendorf tube and plate out the mixture directly if a longer transduction time is preferred.
3. MOI refers to the number of vector particles added per cell. As a rule of thumb, we would normally start with MOI 100 for the ZFN monomer vectors and 200 for the repair matrix. Try different ratios, keeping 1:1 for the ZFN monomers but increasing the amount of DNA donor template, it may improve the rate of correction.
4. The use of non-radioactive size markers may be possible, as the bands corresponding to the different sizes some times show-up with varying intensity due to non-specific hybridization with the radiolabeled probe.
5. In the example shown we chose not to include the diagnostic 0.7 kb band on the densitometry measurements because of the overlap with non-specific smear at low molecular weight (**Figure 3C**).
6. We use radiolabeled dATP because it is the most frequent nucleotide in our DNA template. You should identify the most suitable nucleotide in your own template.
7. If the signal is too strong and a higher stringency wash is needed, proceed step-wise with 1-3x10 min washes at 65°C in:
   - 0.5XSSC, 0.5% SDS
   - 0.2XSSC, 0.5% SDS
   - 0.1XSSC, 0.5% SDS
8. There are other program packages that allow densitometric quantifications, including Adobe Photoshop and Odyssey.

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Figure Legends

Figure 1. Schematic of ZFN bound to target DNA. A standard ZFN is a heterodimeric enzyme. Each monomer is made up of several zinc-fingers linked to a non-specific nuclease domain from *Fok*I endonuclease. Dimerization through the nuclease domains allows recognition of two hemi-sites of about 12 nucleotides each, separated by a spacer of about 6 base-pairs where cutting will occur.

Figure 2. Gene targeting strategy. A ZFN target site has been selected 3' to the point mutation to be corrected in the chromosomal locus. As this particular correction event cannot be detected by restriction analysis, a diagnostic restriction site made up of silent mutations has been introduced 5' to the wild-type site on the repair template. Upon successful homologous recombination (blue crossovers) the gene is repaired and simultaneously the diagnostic restriction site is transferred to the chromosome. Note that the target site for the ZFN could be removed from the template (for instance by site-directed mutagenesis), but that would decrease the homology between target gene and template and likely reduce the frequency of homologous recombination.

Figure 3. Detection and estimation of gene targeting. (A, B) Schematic representations of targeted locus and expected restriction products. Successful gene correction will incorporate a diagnostic restriction site next to the corrected point mutation. Amplification of the chromosomal target with primers external to the targeting construct (arrows), followed by restriction digest should produce three bands if gene targeting has been successful. (C) Example image showing detection and quantification of gene repair. Genomic DNA samples were PCR-amplified, digested, transferred and hybridized as described. Mock corresponds to non-transduced fibroblasts, and plasmid control is the original plasmid containing the donor template modified by site-directed mutagenesis. Other controls included fibroblasts transduced with IDLV-template alone (MOI 1,000) or IDLV-ZFN1 alone (MOI 5,000). For gene targeting experiments fibroblasts were transduced with IDLVs carrying the ZFN monomers (at the indicated MOIs) and template (at twice the indicated MOI). Five days after transduction, genomic DNA was isolated and tested for the presence of the diagnostic restriction site for gene correction. The values corresponding to the percentage of corrected alleles are indicated in white.

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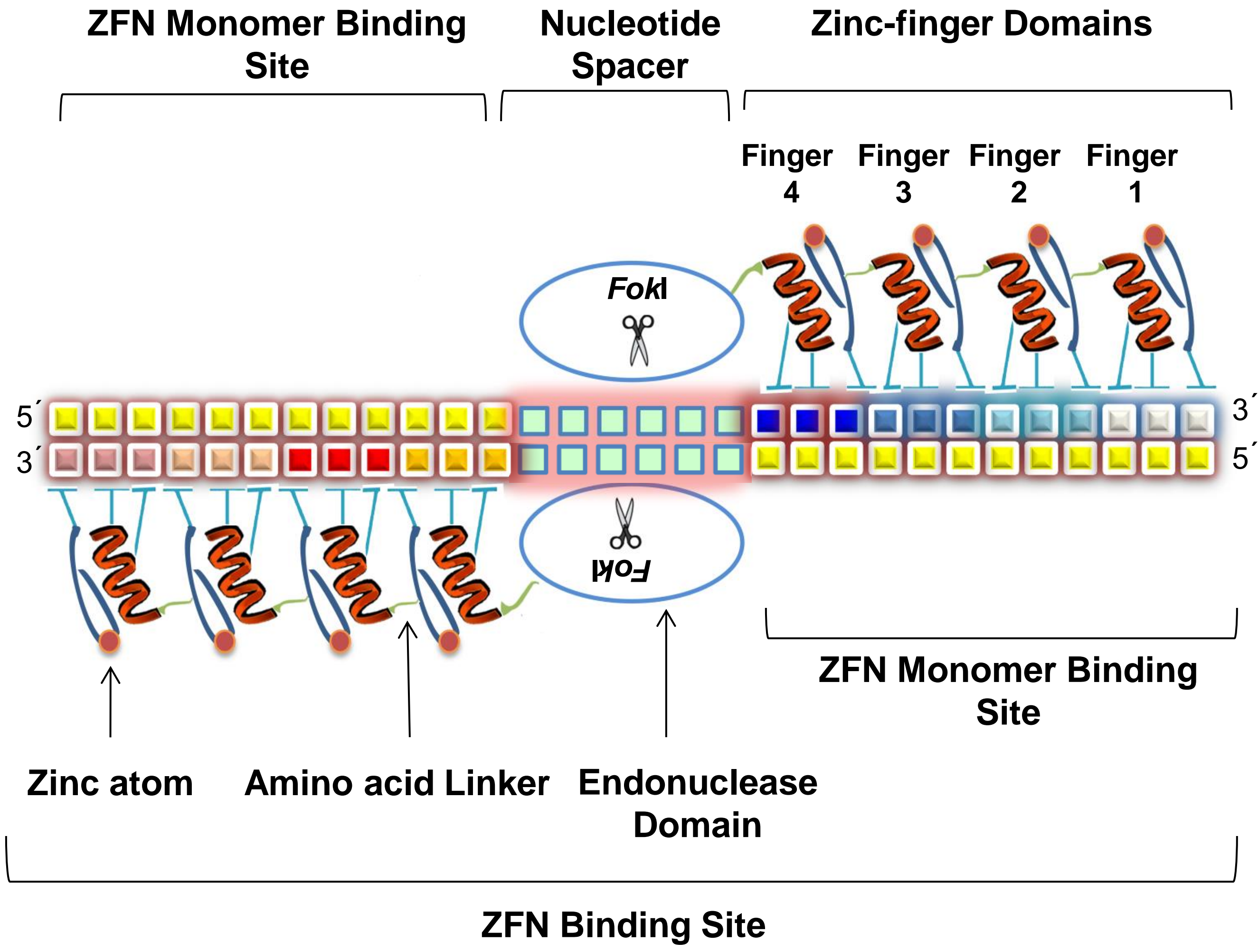
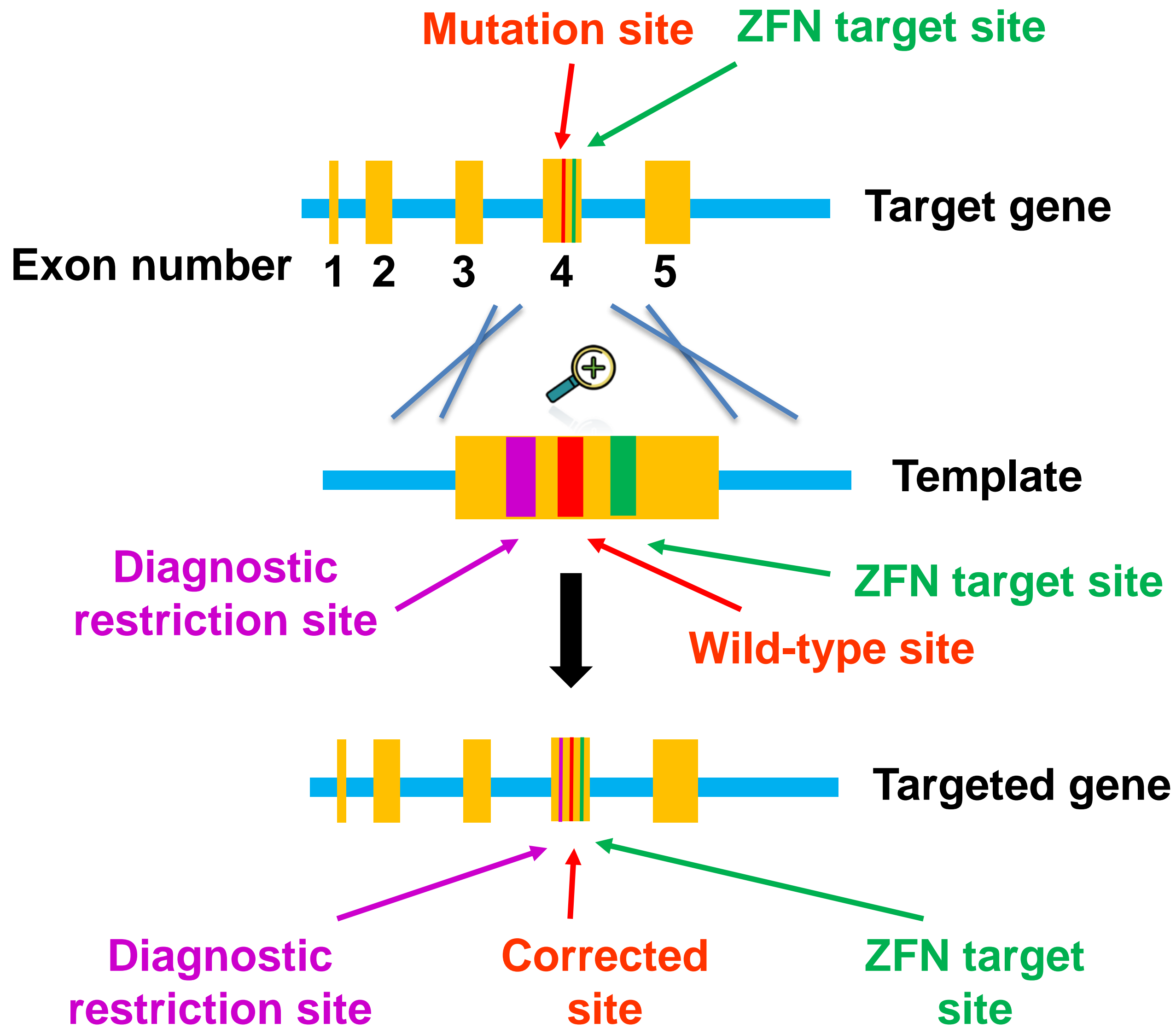
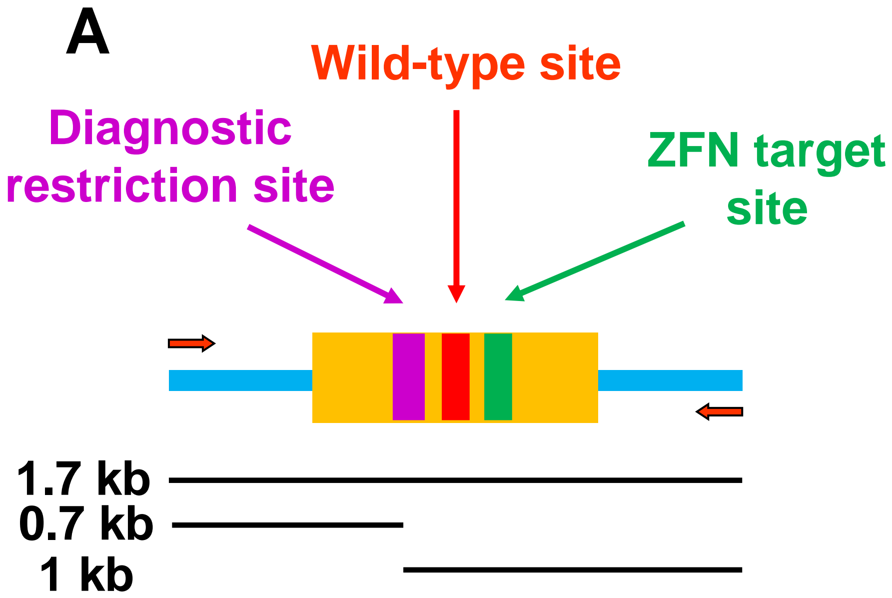
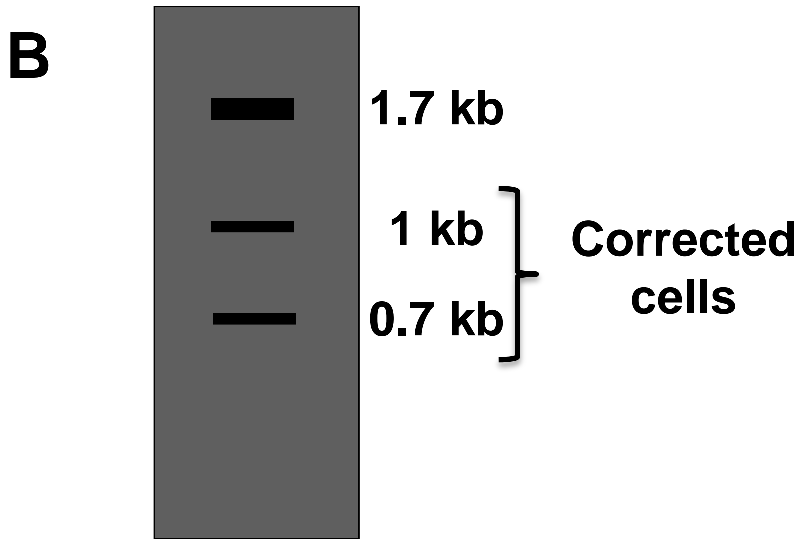


ZFN Monomer Binding Site
Nucleotide Spacer
Zinc-finger Domains
Finger 4
Finger 3
Finger 2
Finger 1
FokI
FokI
5´
3´
3´
5´
ZFN Monomer Binding Site
Zinc atom
Amino acid Linker
Endonuclease Domain
ZFN Binding Site


Mutation site
ZFN target site
Target gene
Exon number
1
2
3
4
5
Template
Diagnostic
restriction site
ZFN target site
Wild-type site
Targeted gene
Diagnostic
restriction site
Corrected
site
ZFN target
site





C

84.9

1.1 1.9 2.7 4.7

Mock
Control plasmid
Template/1000
ZFN1/5000
500
1000
2500
5000