

Triplex-Mediated Gene Modification

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Summary

Gene targeting with DNA-binding molecules such as triplex-forming oligonucleotides or peptide nucleic acids can be utilized to direct mutagenesis or induce recombination site-specifically. In this chapter, several detailed protocols are described for the design and use of triplex-forming molecules to bind and mediate gene modification at specific chromosomal targets. Target site identification, binding molecule design, as well as various methods to test binding and assess gene modification are described.

Key Words: Antigene; homologous recombination; mutagenesis; peptide nucleic acid (PNA); triplex; triplex-forming oligonucleotide (TFO).

1. Introduction

Triplex formation was first cited in the literature in 1957, when Felsenfeld et al. (1) described the ability of polyU and polyA RNA strands to bind in a 2:1 ratio. Triplex-forming oligonucleotides (TFOs) are capable of forming similar structures by binding in the major groove of duplex DNA to polypurine/polypyrimidine runs. These molecules bind to DNA in a sequence-specific manner in either a parallel or antiparallel orientation. In the antiparallel (purine) motif, a polypurine TFO binds through reverse Hoogsteen hydrogen bonds antiparallel to the polypurine strand of the DNA duplex. In the parallel (pyrimidine) motif, a polypyrimidine TFO binds through Hoogsteen base pairing in a parallel orientation to the purine strand of the duplex DNA. Through the manipulation of the cell's own repair machinery, TFOs have been used as tools to effectively inhibit transcription (2), inhibit the binding of proteins to DNA (3–6), inhibit DNA replication (7,8), promote site-specific DNA damage by delivery of a mutagen (9–12), induce

mutagenesis (13,14), and enhance recombination in both chromosomal and episomal targets (15–17).

Another class of molecules capable of triplex formation is peptide nucleic acids (PNAs). PNAs are oligonucleotide analogs in which the phosphodiester backbone of DNA is replaced with an uncharged polyamide backbone (18). Because of its hybrid nature, PNA is resistant to both nucleases and proteases, increasing its stability when transfected into cells (19). PNAs can bind to DNA or RNA through Watson-Crick hydrogen bonds with high thermodynamic stability. Similar to TFOs, PNAs can form triplexes through Hoogsteen base pairing at polypurine:polypyrimidine sites. Triplexes can form when two PNA strands bind to the homopurine strand in a homopurine/homopyrimidine stretch of DNA. Whereas one PNA strand binds through Watson-Crick base pairing in an antiparallel orientation, the second strand binds to the PNA/DNA duplex through Hoogsteen bonding to form the triplex (20). By linking two PNA molecules through a flexible linker, a *bis*-PNA (clamp) is formed, which can be used to bind sequence specifically to a DNA target with an extremely high melting temperature. PNAs have been shown *in vitro* to block DNA polymerization, inhibit transcription initiation and elongation, and prevent sequence-specific protein binding. In addition, the formation of the D-loop has been shown to create an artificial transcriptional promoter (21–25).

There still exist many challenges in the implementation of triplex technology *in vivo*. TFO and PNA binding can be inhibited by such cellular conditions as potassium and magnesium concentrations. A reduction in binding may also be observed depending on the availability of a target site as a result of its location in chromatin. Efficient delivery of both TFOs and PNAs into cells still remains a challenge. Delivery of PNAs can be improved through the addition of positive lysine residues and conjugation to cell-penetrating peptides such as Antennapedia and trans-activator of transcription (TAT) (26). Furthermore, conjugation of a nuclear localization signal has been shown to increase targeting of both TFO and PNA molecules to the nucleus (27).

Modification of TFO backbones and/or bases has been used to increase cellular uptake, improve binding affinity, and prevent degradation on entry into cells. For example, backbone modifications, including replacing the phosphate backbone with cationic phosphoramidate linkages such as N, N-diethylethylene-diamine or N, N-dimethyl-aminopropylamine can increase the binding affinity of TFOs *in vitro* (28). The use of N, N-diethylethylene-diamine-modified bases may also eliminate G-quartet formation in G-rich oligonucleotides. To overcome the pH dependence of pyrimidine triplexes, pseudoisocytosine, a base modification which mimics the N3 protonation of cytosine, is readily utilized in both TFOs and PNAs. Other base modifications, 5-methyl-2'-deoxyuridine and 5-methyl-2'-deoxycytidine, as well as the sugar modification 2'-O-(2-aminoethyl)-ribose have all been shown to increase the binding affinity of TFOs in the pyrimidine motif (29,30).

Triplex formation stimulates mutagenesis by provoking the cell's own DNA repair pathways, primarily the nucleotide excision repair machinery. TFO-induced mutagenesis can lead to heritable and permanent changes in specific genes. TFOs are used to direct site-specific mutagenesis by either delivering a mutagen or directly inactivating a gene of interest. By conjugating a nonspecific mutagen such as psoralen (pso) to a TFO, the mutagen can be directed to a specific target site. Pso-TFOs have been shown to induce mutations in a target site on plasmids *in vitro* and when transfected in mammalian cells, as well as in chromosomal targets (9,13,31). Majumdar et al. (32) showed that by synchronizing cells and treating them in late S-phase targeted mutagenesis could be increased 5.5-fold over those in G₁, and 2.5-fold over cells in early S-phase. By manipulating cell synchronization or transcriptional state, it may be possible to increase the efficiency of triplex-induced mutagenesis.

A reporter system was constructed to detect and quantify TFO-induced mutagenesis. The *supFG* reporter gene, which encodes an amber suppressor tyrosine tRNA and contains a TFO-binding site was cloned into an SV40 vector. In bacteria with an amber mutation in the *lacZ* gene, a functional copy of the *supF* gene produces blue colonies; whereas a nonfunctional (mutated) copy produces white colonies when plated in the presence of 4-chloro-5-bromo-3-indolyl- β -D-galactopyranoside and isopropyl- β -D-1-thiogalactopyranoside. The frequency of mutagenesis can then be calculated by comparing the number of white colonies with blue (9,33). Using this reporter system, pso-TFOs have been found to induce mutagenesis in mouse cells containing a chromosomally integrated copy of the *supFG* reporter gene (34). Whereas pso has been shown to induce mutations, triplex formation alone using either TFOs or PNAs has also been shown to be sufficient to stimulate mutagenesis in target genes (20,34). In transgenic mice containing the same reporter gene integrated in the chromosome, intraperitoneal injection of the TFO, AG30, also led to site-specific mutations (13).

Another strategy that can be implemented to modify, correct, or replace genes is homologous recombination. Normally in cells, homologous recombination occurs at a low frequency; however, studies have shown that DNA damage at the target site can increase the frequency of homologous recombination (35). To exploit this idea, several labs have used pso-TFOs to create site-specific damage in order to sensitize a target site for homologous recombination (36,37). In these experiments it was found that not only could DNA damage by pso increase intrachromosomal recombination (38), but also a TFO alone was sufficient to induce homologous recombination (16,17,39). Triplex formation elevated the recombination level at a target site and led to the correction of a specific mutation (16). Using a plasmid with two tandem *supF* genes, each containing different point mutations, intramolecular recombination was shown to be increased on binding of a TFO, resulting in gene correction of one copy of the gene (36). When TFOs with or without pso were microinjected into the nuclei of mouse cells containing two mutant copies of the *herpes simplex virus thymidine kinase* gene in

tandem, homologous recombination was stimulated at a frequency of 1%, 2000-fold over background (17). It was hypothesized that by linking a TFO to a short donor fragment, the TFO would act to position the donor over the target area whereas triplex formation would sensitize the site for homologous recombination (15). The designed donor fragment would be completely homologous to the site adjacent to the triplex-binding site (TBS) except for the mutation or base correction desired in the target gene. Some studies have determined that antisense (homologous to the transcribed strand) donors are preferred over sense (matching the nontranscribed strand) donors at certain target sites (39). Further studies of this method have shown that the donor does not need to be tethered to the TFO or be adjacent to the TFO-binding site. Homologous recombination has been detected at sites up to 750 bp away from the triplex site (40). It has also been shown that TFOs can stimulate intermolecular recombination at a single-copy genomic locus in mammalian cells. In this study, both pyrimidine and purine motif TFOs were able to induce site-specific recombination in a dose-dependent manner up to a frequency of 0.11% (39).

Triplex formation has many applications in chromosomal gene targeting. This chapter will discuss the use of TFOs or PNAs to induce a site-specific mutation in a gene of interest. To use this method of gene targeting, one must first identify potential binding sites (polypurine stretch) within the gene of interest (*see Subheading 3.1.*). A TFO or *bis*-PNA, which can bind to the target, must be designed (*see Subheading 3.2.*) and evaluated for binding affinity to the target site using **Subheadings 3.3.** and **3.4.** Once a TFO or PNA has been validated by showing a strong binding affinity *in vitro* and *in vivo*, experiments can be designed to optimize delivery into the target cells. Gene correction or insertion of a specific mutation can be detected by various means. If the target is a reporter gene, then the relevant phenotypic assay can be used. If not, allele-specific polymerase chain reaction (PCR) can be used to detect a sequence change in the genomic DNA of the target cells.

2. Materials

All solutions and buffers can be stored at room temperature unless otherwise noted.

1. Oligonucleotides: most oligonucleotides discussed here, including *ps*-conjugated TFOs and single-stranded donors, can be ordered from Midland Certified Reagent Company Inc. (Midland, TX) or other vendors. Single-stranded donors should be modified with the first three (5' end) and last three (3' end) base pairs containing phosphorothioate linkages to prevent nuclease degradation. The same protection can be achieved with TFOs with the inclusion of 3' amine groups. All oligonucleotides should be purified using either high-pressure liquid chromatography or gel purification.
2. PNAs: PNAs (*bis*-PNAs) can be ordered from Bio-Synthesis (Lewisville, TX), or Applied Biosystems (Foster City, CA).

3. Cells: Chinese hamster ovary cells.
4. Vectors: *FLuc*⁺ from pGL3-Basic Vector (Promega, Madison, WI).
5. Cell media—for Chinese hamster ovary cells: Ham's F12 medium with 10% of fetal bovine serum, 2 mM of L-glutamine.
6. For TFO-binding assay, **Subheading 3.3.**: triplex-binding buffer: 10 mM of Tris-HCl (pH 7.6), 0.1 mM of MgCl₂ (*see Note 1*), 1 mM of spermine, and 10% of glycerol (with or without 140 mM potassium).
7. For restriction protection assay, **Subheading 3.4.**: lysis buffer: 50 mM of Tris-HCl (pH 7.5), 20 mM of EDTA, 100 nM of NaCl, 0.1% of sodium dodecyl sulfate; TE pH 8.0: 10 mM of Tris-HCl (pH 8.0), 1 mM of EDTA (pH 8.0).

3. Methods

3.1. Target Site Identification

TFOs and PNAs bind with high affinity to polypurine/polypyrimidine sequences. The target sequence should therefore be a homopurine run (~80%) with few mixed sequences (these decrease binding affinity). By scanning the gene of interest, these homopurine stretches can be identified and TFOs that bind in either a parallel or antiparallel orientation can be designed as discussed in **Subheading 3.2.1. (Fig. 2A)**. TFO-binding sites should be typically 14–30 bp in length, and PNA-binding sites should be at least 8–10 bp in length. It may be necessary to identify several possible target sites as some may be more accessible to triplex formation than others owing to chromatin structure. It is also important to note that if pso is to be conjugated to the TFO (to site-specifically induce crosslinks) then the target site should contain a 5' TpA site at either the 5'- or 3'-end of the polypurine run. Target sites may include regulatory regions of the gene of interest, which may be effective for modulating gene expression.

3.2. Design and Synthesis

After selecting a target site, several molecules can be designed and synthesized to bind or alter the target. Below are the guidelines that will help to determine which molecule will work best (**Fig. 2B**).

3.2.1. Triplex-Forming Oligonucleotide (See **Note 2**)

1. For A-rich target sites, the pyrimidine motif is preferred and requires TFOs containing C and T or their analogs (C⁺ will form Hoogsteen bonds with a G in a G:C base pair, and T with the A in an A:T base pair [**Fig. 1**]). These TFOs will bind in an orientation parallel to the purine-rich strand of the target duplex.
2. For G-rich target sites, the purine motif is preferred and requires TFOs containing A (or T) and G or their analogs (G forms Hoogsteen bonds with the G in a G:C base pair and A or T with the A in an A:T base pair). These oligos will bind in an antiparallel orientation, forming reverse Hoogsteen bonds, with the polypurine strand of the duplex.
3. To induce targeted crosslinks at the triplex site, pso can be conjugated on the 5'- or 3' end of the TFO using pso phosphoramidates.

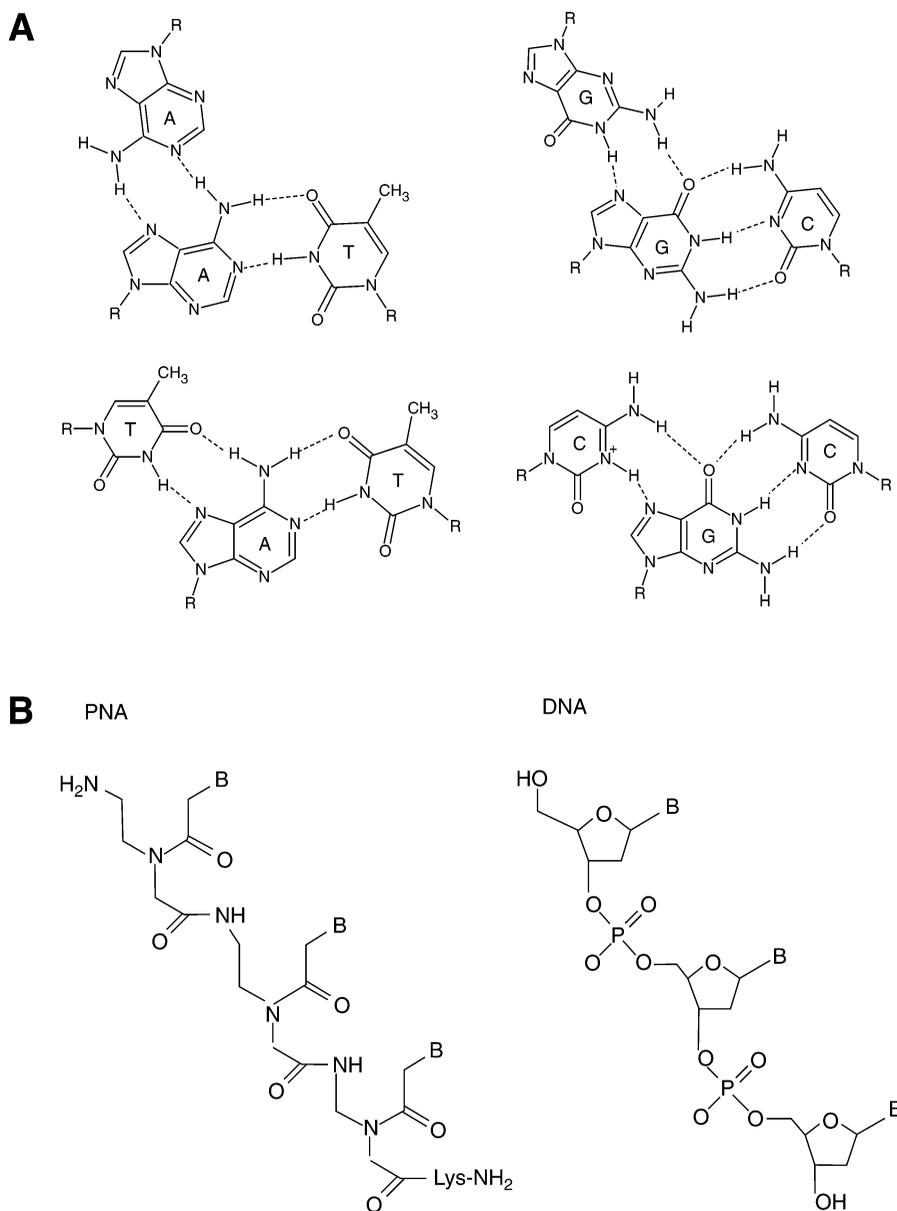


Fig. 1. (A) Schematic of triplet base pairing during triplex formation in both the purine (A:A:T and G:G:C) and pyrimidine motif (T:A:T and C⁺:G:C). (B) Chemical structure of PNA vs DNA (reprinted with permission from [ref. 49](#)).

4. TFOs containing backbone or base modifications as described in **Subheading 1**, can also be synthesized to create a molecule with a high binding affinity to the target site.

3.2.2. Peptide Nucleic Acid

1. Most often a *bis*-PNA clamp containing two pyrimidine PNAs connected by a flexible linker (8-amino-3,6-dioxaoctanoic acid [O]) is used for targeting. This molecule is oriented N-terminus to C-terminus (**Fig. 2B**).
2. To aid in strand invasion, several lysine residues can be put on the duplex-forming strand of the *bis*-PNA. These positive charges have also been shown to facilitate uptake into cells (**41**). Other conjugates such as the cell-penetrating peptides transactivator of transcription, the HIV transactivator of transcription, and antennapedia, the third helix of the *Drosophila* homeodomain transcription factor, have also been shown to increase uptake of PNA (**42**).
3. To overcome the pH dependence of N3-protonation of cytosine, pseudoisocytosine (J) can be used in place of cytosine in the Hoogsteen strand. This J base forms two Hoogsteen bonds with a G:C base pair (**43**).

3.2.3. Donors

1. Single-stranded donors can be 30–2000 bases in length and can be homologous to any region within 750 bp of the triplex site (*see Note 3*) (**39**).
2. Antisense (binding to the sense strand of the DNA) or sense (binding to the antisense strand of the DNA) donors can be designed (*see Note 4*).
3. Donors can be synthesized by Midland Certified Reagent Company (*see Subheading 2., step 1*) or long double-stranded donors can be synthesized by PCR amplification of a plasmid. If donors are ordered from Midland, the first and last three bases should be attached through phosphorothioate linkages to inhibit nuclease degradation.
4. When designing a donor, desired sequence changes should be kept toward the center of the oligo, and a sufficient number of bases must be on either side of the mismatch to allow for homologous recombination within the target (**Fig. 2A,B**).

3.3. Evaluation of Binding Under Physiological Conditions

3.3.1. TFO-Binding Assay

1. To evaluate the binding of a TFO, a gel mobility shift assay is used. For this, a synthetic duplex containing the potential TFO-binding site should be designed. To do this, complementary oligomers containing the target sequence can be annealed to form duplexes.
2. The duplex DNA can then be 5' end-labeled using T4 Polynucleotide kinase and (γ -³²P) dATP in the reaction. Typical reactions contain 10⁻⁶ M duplex (final concentration) in a total volume of 20 μ L.
3. Next electrophorese the duplex on a 15% polyacrylamide gel to purify. Following electroelution of the duplex, add this purified duplex to a Centricon-3 column (Millipore, Bedford, MA) to concentrate (as per the manufacturer's instructions).

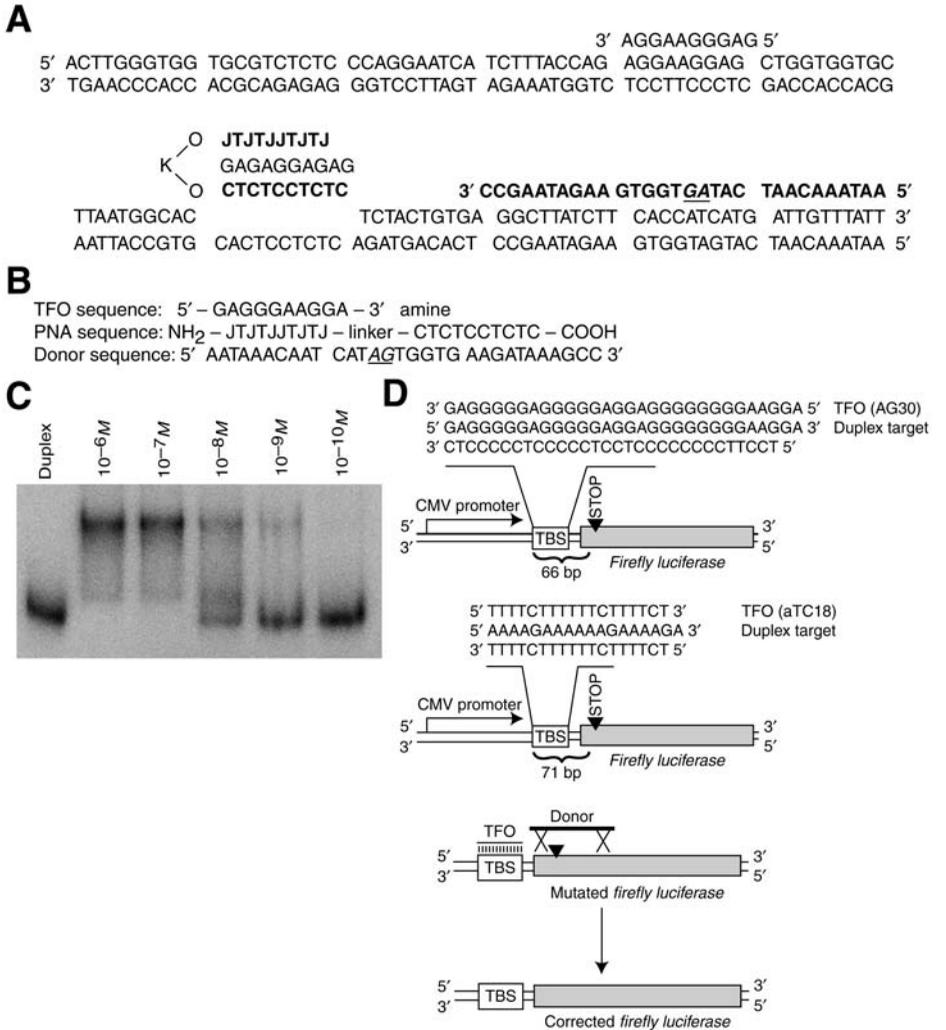


Fig. 2. (A) Sample sequence depicting binding of a purine-rich TFO, a *bis*-PNA, and an antisense donor. (B) Sequence and orientation of the TFO and *bis*-PNA that would be synthesized to bind to the target sites in A. The single-stranded DNA donor depicted would be used to mutate the underlined site from TC to GA. (C) Example of a TFO-binding gel-shift assay. Duplex DNA containing the target site was radiolabeled with ³²P and incubated with the indicated amounts of the corresponding TFO. (D) Schematic diagram of the luciferase assay. The diagram depicts AG30, a polypurine TFO, and aTC18, a polypyrimidine TFO, binding to their respective target sites. When a donor containing the wild-type sequence of the *luciferase* gene is introduced, it can correct the stop codon mutation (as indicated by the inverted triangle [▼]) by triplex-induced homologous recombination (reprinted with permission from ref. 50).

4. Incubate the duplex DNA at 37°C for approx 12–24 h in a series of reactions containing increasing concentrations of TFO in triplex-binding buffer. A typical reaction is 20 μL , with 2 μL (10^{-6} M) of labeled duplex and 2 μL of 10-fold dilutions of TFO (10^{-12} – 10^{-7} M).
5. Next, run the binding reactions on a 12% native (19:1 acrylamide:*bis*-acrylamide) gel at 60–70 V in 89 mM Tris, 89 mM of boric acid with 0.1 mM of MgCl to achieve separation of the triplex structures from the duplex.
6. The gel can then be imaged with a PhosphorImager (Amersham Biosciences, Piscataway, NJ) to quantify the band shift. This data can be used to calculate the dissociation constant of the TFO-binding to the target site (**Fig. 2C**), which represents the concentration at which binding is half-maximal.

3.3.2. PNA-Binding Assay

1. In Eppendorf tubes add the PNA at concentrations ranging from 0 to 1 μM , 2 μg of plasmid DNA containing the target site flanked by known restriction enzymes, KCl to a final concentration of 10 μM , and TE to a final volume of 10 μL .
2. Place these tubes at 37°C overnight.
3. The next day, digest the entire reaction (tube) in a 20 μL digest with the restriction enzymes flanking the binding site (*see Note 5*). Allow the reaction to digest for 1–2 h at the enzyme-specific temperature.
4. Add DNA loading dye to each sample and run them on a 10% of polyacrylamide gel until bands are well separated. To visualize the resulting gel shift, stain with silver stain (sodium borohydrate 0.1% silver nitrate [Sigma Aldrich, St. Louis, MO]) (*see Note 6*).
5. Expected result: at 0 μM , only a single band representing the duplex fragment containing the target site should be seen. As concentrations of PNA increase, there should be a shift in the band corresponding to binding.

3.4. Evaluation of Intracellular Binding: Restriction Protection Assay/Southern

Several strategies exist to physically evaluate intracellular binding of a TFO or PNA to the target site. If pso is conjugated to the TFO or PNA, a restriction protection assay, a denaturation resistance assay or a PCR-based assay (**44**) can be used. In this chapter the first two methods will be described in detail.

1. For this assay, a restriction enzyme (e.g., *Bam*HI) recognition site overlapping the target site is necessary.
2. Cells containing your target site should be used.
3. Design and synthesize a pso-TFO that will bind to the target site and direct a crosslink to a position within the restriction enzyme recognition site. Transfect 2–5 μg of this pso-TFO into cells containing the chromosomal target site (*see Note 7*).
4. Incubate cells at 37°C for 2–6 h posttransfection and then irradiate for a total dose of 1.8 J/cm² of ultraviolet A (UVA) irradiation (UVA light source centered at 365 nm; Southern New England Ultraviolet, Branford, CT). This will allow pso photoactivation to crosslink the pso-TFO to the duplex DNA.

5. Immediately following UVA irradiation, wash the cells with phosphate-buffered saline and resuspend in heated (60°C) lysis buffer at a concentration of 5×10^6 cells/mL for 15 min.
6. Treat with proteinase K at a concentration of 100 µg/mL overnight at 37°C.
7. Extract the genomic DNA by addition of phenol to the lysates once, followed by two extractions with chloroform/isoamyl alcohol (24:1). Ethanol precipitate the DNA and resuspend in TE pH 8.0 and 100 mM of KCl (~100 µL).
8. Incubate samples at 60°C for 2 h to disrupt noncovalent triplexes and remove by filtration through a Centricon column (Millipore) as per manufacturer's instructions (*see Note 8*).
9. Digest the genomic DNA with the appropriate restriction enzyme (e.g., *Bam*HI) and two restriction enzymes that will cut out a defined fragment containing the target site.
10. Analyze the DNA by Southern blot using a designed probe to identify the fragment.
11. Expected result: if crosslinking occurred at the target site, the pso adduct in the *Bam*HI site will block cleavage at this site. The product resulting from the restriction protection of the *Bam*HI site can be visualized and quantitated in comparison with the extent of the *Bam*HI digestion products. This can then be used to quantify TFO-directed pso adduct formation in the target.

3.5. Evaluation of Intracellular Binding of Pso-TFOs: Denaturation-Resistance Assay (45)

1. Cells containing the endogenous gene or a chromosomally integrated exogenous gene can be used in the following assay.
2. Design and synthesize a pso-TFO, which binds to the target, and transfect 2–5 µg into cells (*see Note 7*).
3. Repeat **steps 4–7** as written in **Subheading 3.4**.
4. Digest 10–20 µg of genomic DNA with flanking restriction enzyme(s) that will cut out the target region.
5. Denature the digested DNA by heating in a solution of 90% formamide (to a final concentration of ~80%) to 80°C for 15 min.
6. Electrophorese the samples in a neutral 1.5% TAE agarose gel.
7. Transfer the DNA from the gel to a nylon filter/membrane by Southern blot and hybridize the membrane with a ³²P-labeled probe that contains the target region.
8. Expected results: once denatured, uncrosslinked DNA fails to reanneal after it enters a neutral environment, whereas crosslinked DNA will “snap back” to reform a duplex. Denatured crosslinked DNA when run in a neutral gel will migrate at the same rate as nondenatured DNA, whereas uncrosslinked DNA will migrate faster. The relative amount of the denatured (fast migrating) and the snap back (slow migrating) bands can then be quantified as a measure of target site crosslinking.

3.6. Evaluation of Induced Recombination: Luciferase Assay (40)

A luciferase assay was created to evaluate triplex-induced gene correction by homologous recombination. The *Fluc* reporter gene can be engineered to contain the target site from any gene (TBS) upstream of the coding region of luciferase.

This wild-type construct can then be mutated through site-directed mutagenesis to insert a stop codon mutation within the *Fluc* coding region, which will prevent full translation of the gene. When a TFO or PNA binds to the TBS, it can stimulate recombination between an introduced DNA donor and the *luciferase* gene to correct the mutation. Site-specific recombination can then be evaluated based on the luciferase enzyme activity in the cell lysates when compared with a standard curve, which can be created by mixing cell populations containing the chromosomally integrated wild-type or mutant construct. This reporter system can be used as a model for recombination at any chromosomal gene of interest. By placing the TBS of the target gene upstream of the *luciferase* gene, a construct is created that can be chromosomally integrated to create stable cell lines. These cell lines allow for systematic testing of various DNA-binding molecules for their effects on recombination at chromosomal targets. This assay can be used as an intermediate to evaluate induction of homologous recombination at a chromosomal target by a DNA-binding molecule at a specific target site and allows for quantification of this induction. **Figure 2D** depicts how this reporter is used to measure triplex-stimulated recombination by TFOs.

1. A construct containing the TFO target site upstream of the start site of the wild-type firefly *luciferase* gene, *FLuc*⁺. A stop codon can then be created at varying distances from the target site (24–750 bp) (**40**) using site-directed mutagenesis (QuikChange Site-Directed Mutagenesis Kit, Stratagene, LaJolla, CA). Vector containing the wild-type *Fluc*⁺ should be used as a control.
 - a. Donors are designed containing correct wild-type sequence (~50-mer (**39**))—see **Subheading 3.** for synthesis and design).
 - b. TFO designed to bind to target site.
2. Wild-type and mutant cell lines can be stably created using the above constructs.
 - a. Determine that the construct is integrated in a single copy by Southern blot.
3. Mix wild-type and mutant cells in fixed ratios to establish standard curves.
 - a. Dilute mutant cells to a concentration of 1×10^5 cells/mL and seed 50,000 mutant cells per well into a 12-well plate. Dilute wild-type cells to 5×10^4 cells/mL, serially dilute cells and add to the wells containing the mutant cells.
 - b. Grow cells 48 h and then harvest and lyse. Analyze lysates as described in **step 8.**
 - c. This standard curve can be used to normalize the experimental data and allow for quantification of recombination frequencies.
4. 24 h before transfection seed 12-well plates with wild-type or mutant cells so they will be approx 50% confluent the day of transfection.
5. The following day cells are transfected using Geneporter 2 (Gene Therapy Systems, San Diego, CA) according to manufacturer's instructions delivering various concentrations of donor and TFO to each well (see **Note 9**).
6. Incubate the cells for 24–48 h at 37°C to allow for cellular repair and recombination processes to occur.
7. 48-h posttransfection, rinse the cells two times with phosphate-buffered saline (Gibco BRL, Carlsbad, CA) and then lyse cells with 250 μ L of 1X passive lysis buffer (Promega).

8. Assay luciferase activity (gene correction) using the Promega Dual Luciferase Kit (Promega) and a luminometer (Berthold Technologies [Oak Ridge, TN]), as per manufacturer's instructions.
9. Expected result: if TFO-induced recombination between the donor and the gene occurred, correction of the stop codon would have taken place. This would restore translation of a fully functional *Luciferase* gene, resulting in luciferase activity above levels seen in mock transfected cells or cells treated with donor alone.

3.7. Evaluation of Target Gene Correction/Mutagenesis: Genotype Analysis by Allele-Specific PCR (46,47)

After treating cells containing the target gene with TFO, PNA, and/or donor DNA, gene correction or mutation can be detected by allele-specific PCR. Harvest genomic DNA from the treated cells using Promega's Wizard SV Genomic DNA Purification System and dilute it to approx 50 $\mu\text{g}/\mu\text{L}$. A forward primer can be designed with its 3' end containing the desired mutation, and the reverse primer should be similar in length and T_m . Add 50 ng of the genomic DNA to a 25 μL PCR reaction and a gradient run to determine the optimal annealing temperature of the primers. Plasmids containing the wild-type and mutant gene can be used as control templates (mutations can be introduced into the wild-type gene with site-directed mutagenesis [QuikChange Site-Directed Mutagenesis Kit, Stratagene]). Expected results: a band should be present on the gel in lanes with mutant template and no bands for wild-type DNA (*see Note 10*).

4. Notes

1. These conditions are used to test binding under physiological conditions.
2. All TFOs should be synthesized with a 3' end cap of amine groups to prevent degradation by 3' exonucleases.
3. Optimal donor length will vary from site to site often making it necessary to try various lengths (**39**).
4. Certain targets can be more sensitive to recombination with a sense or an antisense donor. In some cases it may be necessary to test both types of donors for recombination efficiencies.
5. For best results the digest should result in a 150–200-bp band.
6. Multiple bands may be seen in the gel shift owing to the formation of various PNA–DNA triplex structures (**48**).
7. Any transfection method can be used for this, for example, digitonin permeabilization, electroporation, or cationic lipids. It may be necessary to test several methods to determine which will give the highest efficiency and lowest toxicity in your specific cell type.
8. The Centricon column size will vary depending on the size of the duplex and/or TFO. Please check the manufacturer's specifications to determine which column size is right for you.

9. Control reactions containing only donor DNA should also be run to evaluate the effect of the TFO.
10. When testing for a mutation by allele-specific PCR using genomic DNA, 40 cycles should be used. Alternatively, real-time PCR can be used for a more sensitive measure of PCR products.

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