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Targeted Genome Modification via Triple Helix Formation

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Abstract

Triplex-forming oligonucleotides (TFOs) are capable of coordinating genome modification in a targeted, site-specific manner, causing mutagenesis or even coordinating homologous recombination events. Here, we describe the use of TFOs such as peptide nucleic acids for targeted genome modification. We discuss this method and its applications and describe protocols for TFO design, delivery, and evaluation of activity in vitro and in vivo.

Keywords

Homologous recombination; Mutagenesis; Peptide nucleic acid (PNA); Triplex; Triplex-forming oligonucleotide (TFO); Site-specific gene editing

1 Introduction

1.1 Triplex-Forming Oligonucleotides

While double helices are key to the understanding and study of the biological sciences, nucleic acids are also capable of forming triple helices. In fact, before the establishment of the double-helical nature of DNA, Linus Pauling proposed a triple-helix structure [1]. Felsenfeld et al. demonstrated the possibility of triple helix formation when they noted that polyU and polyA RNA strands could bind in a 2:1 ratio [2]. Triplex-forming oligonucleotides, or TFOs, can form similar triple helices. TFOs can bind in the major groove of duplex DNA in a polypurine/polypyrimidine run, with reverse Hoogsteen hydrogen bonds antiparallel to a polypurine strand of a DNA duplex or with Hoogsteen bonds in a parallel orientation to the purine strand (Fig. 1a).

While both DNA and RNA can form triple-helix structures, novel synthetic nucleic acid analogues can also be used for TFO formation (Fig. 1b). Peptide nucleic acids (PNAs) are synthetic compounds with a neutral polyamide rather than charged phosphodiester backbone. They are more resistant to protease and nuclease degradation and can bind more tightly to DNA and RNA [3, 4]. Gamma PNA molecules, which feature a pre-organized conformation, have increased binding to target DNA [5, 6]. Mini-PEG or other modifications may also increase PNA binding to target DNA [7]. Locked nucleic acids (LNAs) are another synthetic oligonucleotide, with a bridge between the 2' oxygen and 4' carbon, "locking" the ribose in a fixed 3'-endo configuration [8–10]. This rigid conformation reduces barriers to binding by lowering the binding entropy [11].

PNAs and other molecules are capable of forming unique structures with DNA (Fig. 1c). These structures include triplexes consisting of PNA binding with DNA through Hoogsteen base pairs at homopurine/pyrimidine stretches. In addition, two PNA molecules connected by a flexible linker can form a bis-PNA “clamp” that can form a PNA/DNA/PNA triplex at the target site [12]. Tail clamp PNAs (tcPNAs) have an extended 5–10 bp “tail” that forms a PNA/DNA duplex in addition to a PNA/DNA/PNA “clamp,” allowing for more specific binding without the need for a long (15–17 mer) homopurine/pyrimidine stretch [13, 14]. Novel duplex structures are also possible, such as PNA strand-invasion duplexes and pseudocomplementary PNAs (pcPNAs) capable of double-duplex invasion [15]. Strand invasion and cellular uptake can be enhanced by addition of positively charged lysine residues [16], and cell-penetrating peptides (CPPs) can be added to molecules to enhance the uptake [17, 18]. The base substitute pseudoisocytosine (J) can be used in place of cytosine to encourage Hoogsteen bond formation in a pH-independent fashion [12]. These unique synthetic molecules have numerous applications, some of which we describe below.

1.2 Applications of TFOs

TFO binding has been shown to inhibit transcription, replication, and protein binding to DNA [19–21]. In addition, TFOs tethered to mutagens can promote DNA damage in a sequence-specific fashion and induce mutagenesis [22–25]. More recently, researchers have demonstrated that TFOs can mediate site-specific gene modification, both in vitro and in vivo [26–29]. TFOs can also be used for splice site correction, for example in Duchenne muscular dystrophy [30] or beta-thalassemia [31]. Triplex-forming molecules have also recently been used for the suppression of oncogenes and proto-oncogenes to reduce cancer cell growth. For example, TFOs have been used to decrease MET expression and induce cell death in hepatoma cells [32], reduce cell proliferation by binding to Ki-ras [33] and bcl-2 [34–36], and HER-2/neu [37].

In this chapter we focus on the use of triplex-forming molecules to mediate gene modification and novel methods for TFO delivery that can be used for transfer of diverse nucleic acids. While introduction of an oligonucleotide homologous to a target gene may lead to recombination at low levels, use of TFOs can enhance recombination frequencies, leading to targeted, specific editing of endogenous human genes. PNA TFOs have recently been used to mediate site-specific gene editing in a beta-thalassemia-associated site, leading to heritable modification in primary human hematopoietic stem cells [26, 38, 39]. tcPNA molecules have been used to modify the human *CCR5* gene, leading to the production of cell lines resistant to HIV-1 infection [27]. Conjugation of PNA molecules to CPPs has allowed for direct in vivo targeting of genes for site-specific mutagenesis in hematopoietic stem cells [28], and encapsulation of PNA molecules in biodegradable polymer nanoparticles has allowed for editing of primary human cells with lower toxicity and direct in vivo gene editing [29, 40]. This type of directed gene modification could also be used for cancer gene therapy or creation of selectively edited cell lines for the study of cancer biology.

1.3 Mechanism of Triplex-Induced Genome Modification

PNA TFOs form triplexes by binding with high affinity and specificity in the major groove of a complementary strand of duplex DNA. The stable, abnormal PNA/DNA/PNA triple

helix is recognized by a cell's own DNA repair machinery, sensitizing the surrounding DNA for homologous recombination [38]. PNAs exhibit stable, high-affinity binding to DNA and genetic specificity [41], making them advantageous for creating heritable changes in targeted genes. Intracellular delivery of a site-specific PNA has also been shown to induce recombination of a short, single-stranded donor DNA molecule, encoding a desired modification, with a nearby genomic site, shown schematically in Fig. 2 [38].

Mechanisms of TFO-induced DNA repair and recombination have been previously reviewed [42, 43]. Multiple investigations indicate that the nucleotide excision repair (NER) pathway has a role in recognizing and repairing triplex structures [25, 26, 38, 44]. Evidence suggests that the NER factors xeroderma pigmentosum group A (XPA) and replication protein A (RPA) bind specifically as a complex to cross-linked triplex structures [44]. XPA deficiencies have also been shown to decrease TFO-induced plasmid recombination [45], decrease PNA-induced plasmid repair synthesis, and decrease PNA-induced plasmid recombination with an ssDNA donor [38]. While there appears to be a clear association between the NER pathway and TFO-induced repair and recombination, some evidence suggests that other repair pathways may be involved in metabolizing triplex structures [45]. Additionally, it was found that overexpression of Rad51, a homologous recombination factor, increased TFO-induced recombination of tethered donor DNA into a shuttle vector [46]. Furthermore, Rad51 deficiency resulted in no TFO-induced recombination of tethered donor DNA [46].

In addition to being an important tool for instigating DNA repair, triplex-forming molecules may also prove to be an important tool to study DNA repair machinery at different target sites. Current evidence suggests that the repair of TFO-associated lesions is dependent on the NER pathway, while other repair pathways such as homologous recombination and transcription-coupled repair may also be involved in TFO-induced recombination. Although the mechanisms by which PNA TFOs are able to augment recombination frequencies of donor DNA fragments into genomic targets are not clearly defined, this is an ongoing area of research. Further studies aimed at understanding how triplex structures are recognized and repaired will be instrumental in improving the efficacy of targeted genome modification of disease-causing genes.

1.4 Overview of Techniques

In this chapter, we discuss the use of triplex-forming oligonucleotides and novel delivery vehicles for site-specific genome editing. We describe the selection of target sites for gene editing, evaluation of binding affinity of TFOs, creation of delivery tools for oligonucleotide transfer into target cells, and evaluation of mutagenesis and site-specific genome editing.

2 Materials

1. Oligonucleotides: Oligonucleotides discussed here can be ordered from Midland Certified Reagent Company Inc. (Midland, TX) or other vendors. Single-stranded donors should be protected by three phosphorothioate internucleoside linkages at both the 5'- and 3'-end to prevent degradation

and should be purified by reversed-phase high-performance liquid chromatography.

2. PNAs: PNAs can be ordered from Bio-Synthesis (Lewisville, TX) or Panagene (Daejeon, South Korea).
3. Gel shift assays: Taq polymerase—Invitrogen (Carlsbad, CA). Qiagen Gel extraction kit and QIAquick PCR purification kit (Venlo, Limburg), T4 DNA ligase, Tris–EDTA buffer—pH 8. 10 mM Tris, bring to pH 8.0 with HCl and 1 mM EDTA, and polyacrylamide gel—40 % 19:1 bis:acrylamide, TBE buffer (1 L of 5× stock—54 g Tris base, 27.5 g boric acid, 20 mL of 0.5 M EDTA pH 8.0), EDTA, 10 % ammonium persulfate, TEMED.
4. Silver staining: Ag solution—0.1 % silver nitrate in dH₂O, developer solution—3 % potassium carbonate plus 250 µL formalin and 125 µL 10 % sodium thiosulfate per liter.
5. Nucleofection: Amaxa Nucleofection Kits—Lonza Group (Basel, Switzerland), Geneporter 2—Gene Therapy Systems (San Diego, CA), or BTX Electro Square Porator—BTX (Holliston, MA). StemSpan culture media—STEMCELL Technologies Inc. (Vancouver, Canada), RPMI media, FBS, and L-glutamine.
6. Peptide–PNA conjugates: Penetratin 1—Qbiogene, 0.1 M DTT, NAP5 filtration column—GE Healthcare Life Sciences (Piscataway, NJ).
7. PLGA: 50:50 ester-terminated PLGA, 0.95–1.2 g/dl, can be ordered from LACTEL absorbable polymers—DURECT corporation (Birmingham, AL).
8. Reporter systems: DMEM—Life Technologies (Carlsbad, CA), 10 % FCS, phenol, chloroform, isoamyl, Qiagen Gel Extraction Kit (Venlo, Limburg), RPMI media—Sigma Aldrich (St. Louis, MO), Ficoll-Paque—GE Healthcare Life Sciences (Piscataway, NJ).
9. Mouse model: EGFP-654 transgenic mice from the laboratory of Ryszard Kole (Chapel Hill, NC) [31].

3 Methods

3.1 TFO Design and Synthesis

3.1.1 Selection of Target Site and TFOs

1. As noted above, Hoogsteen bonding for triplex formation requires homopurine/pyrimidine stretches. These should be identified near the site targeted for genome modification within a few hundred base pairs of the target [47].
2. TFO-binding sites should be 13–30 bp and PNA-binding sites should be 8–10 bp, with 5–10 bp clamp for added specificity and increased binding

affinity if desired [27]. TFOs will bind in the parallel orientation to the purine-rich strand of the target or in the antiparallel orientation with the polypurine strand.

3. Several modifications are also available. Psoralen can be used to induce cross-links [48] and can be conjugated to the TFO with phosphoramidates. bis-PNA clamps consist of two PNAs connected by a linker such as 8-amino-3,6-dioxaoctanoic acid (O). Lysine residues can be added to either or both ends to enhance the uptake and binding, and pseudoisocytosine (J) can be used to reduce pH dependence of cytosine N3 protonation. An example of a bis-PNA tail clamp with terminal lysines is given in Fig. 3.

3.1.2 Choice of Donor DNA

1. Single-stranded DNA donors should be homologous to the site desired for modification, except for the desired base pair change(s). Antisense or sense donors can be designed and can be between 30 and 100 nucleotides in length. Six base pair changes are preferred to easily detect the change by allele-specific PCR (AS-PCR).
2. First and last three bases should use phosphorothioate linkages to inhibit exonuclease degradation.

3.1.3 Oligonucleotides

1. PNA may be purchased or synthesized using Boc-protected monomers on solid support as described by Christensen et al. [49]. Molecules can be purified by reverse-phase HPLC and characterized by MALDI-TOF.

3.2 Evaluation of Binding with Gel Shift Assays

A gel shift assay can be used to confirm the binding of PNA molecules to their desired target sequences of DNA. To perform this assay, plasmids that contain a 150–200 base pair section of the target gene including one or two of the potential PNA target sequences should be created. Select the 150–200 base pair region of interest, and select primers to amplify this region. Amplify using Taq DNA polymerase as per the manufacturer's protocol. Run PCR products on 1.1 % agarose gel. Extract PCR products using Qiagen Gel Extraction Kit as per the manufacturer's protocol. Identify restriction enzyme sites around the region of interest that are also present in a plasmid of your choice (3–4 kb). Digest both the PCR products and the plasmids separately overnight. Purify the PCR products and digested plasmid using the QIAquick PCR Purification Kit as per the manufacturer's protocol. Ligate the PCR products and plasmid using T4 DNA ligase (or other ligases of choice). Submit plasmids for sequencing to verify insert.

3.2.1 Binding Reaction—To perform the binding reaction and allow the PNA to bind to its target sequence, combine various amounts of PNA diluted to 10 μ M for final PNA concentrations of 0, 0.2, 0.4, 0.8, and 1.2 μ M, 5 μ L of 400 ng/ μ L of plasmid DNA, 6.7 μ L of 30 mM of KCl for a final concentration of 10 mM, and enough TE (pH 8) to bring the final reaction volume to 20 μ L. These reactions should be left to incubate overnight at 37 °C.

3.2.2 Digest—Once the PNA has bound its target on the plasmid, a 150–200 base pair fragment of the target gene is cut out from the plasmid so that it can be run on a gel (see Note 1). To perform this digestion reaction, the 20 μL of the binding reaction should be combined with 1 μL of each enzyme used to insert the target gene into the plasmid, 3 μL of the appropriate buffer for these enzymes, 0.3 μL of 100 $\times$ BSA, and 4.7 μL of dH_2O to bring the final reaction volume to 30 μL . The digestion reaction should be incubated at 37 $^\circ\text{C}$ for 2 h.

3.2.3 Running the Gel—To detect binding, digested DNA must be run on a polyacrylamide gel, which allows DNA fragments to be separated based on size and charge. To make the 8 % native gel, mix 10 mL of 40 % 19:1 bis:acrylamide, 10 mL 5 $\times$ TBE buffer, 200 μL 0.5 M EDTA, 29.25 mL dH_2O , 500 μL 10 % ammonium persulfate (APS), and 50 μL TEMED for a final volume of 50 mL. Using a large pipette, transfer this mixture to an assembled gel apparatus. Allow approximately 30 min for the gel to solidify, and add TBE to flush the wells. To 15 μL of each digest reaction, add 3 μL 6 $\times$ DNA loading dye, and load the 18 μL onto the gel, attempting to keep the samples as close to the middle of the gel as possible, as the sides of the gel may become distorted. Load 100 and 10 bp DNA ladders onto the gel on either end of the samples. Run the gel at 33 mA for 30 min to allow the loaded DNA to settle into the wells and then run at 20 mA for approximately 4 h to ensure good separation.

3.2.4 Visualizing the Gel—Once the gel has run, it must be stained to visualize the DNA fragments present. When disassembling the gel apparatus, leave the gel on one glass plate for support and immerse this in fresh Ag solution (0.1 % silver nitrate in dH_2O) for 10 min, making sure not to agitate it. Pour off the Ag solution into a bottle for future use (can be used up to six times). Rinse the gel with dH_2O and develop for 1 min with freshly made developer solution (3 % potassium carbonate plus 250 μL formalin and 125 μL 10 % sodium thiosulfate per liter). If bands cannot be seen, the gel can be left to develop for more time, though this does not usually take more than 15 min. Once developed, developer solution should be poured out and the gel can be “picked up” with Whatman filter paper by laying the paper on the gel and allowing the gel to adhere to it. The gel can then be covered in Saran wrap and dried so that it can be preserved.

When examining the stained gel, one, two, or several bands may be observed. A lower band indicates unbound DNA, whereas successful binding is demonstrated by a shift of the DNA band upward on the gel, since the charged PNA molecule slows down the transit of the complex. Therefore, the absence of a lower band indicates that all the DNA has been bound by PNA and reflects optimal binding. Several bands may be present due the fact that PNA can infiltrate the DNA molecule to bind in different configurations, each of which migrates differently through the gel.

¹Gel shift assay: A 90–100 base pair segment for binding may also be created by PCR for binding assay, rather than use of plasmid. This may change optimal binding conditions, however.

3.3 Delivery Methods

Several options exist for delivery of TFOs to cells, including nucleofection, use of CPPs, and delivery in biodegradable nanoparticles. Nucleofection can be accomplished using commercially available kits, but can only be used in vitro and may lead to high levels of cell death compared to nanoparticle delivery or other methods [40]. Conjugation of TFOs to CPPs or delivery in biodegradable nanoparticles allows for transfection with lower cell death and direct in vivo nucleic acid delivery [18, 28, 29, 40].

3.3.1 Nucleofection

1. Nucleofection can be used for delivery of TFOs to certain cell types, and optimal procedures will depend on cell type and molecules to be delivered. Amaxa Nucleofection Kits can be used for nucleofection of primary human hematopoietic cells with PNA and DNA [26, 27]. Amaxa nucleofection may be superior to some other reagents in several different cell systems [50]. Geneporator 2 or BTX Electro Square Porator can also be used according to the manufacturer's instructions for delivery of TFOs.
2. For primary human CD34+ cells, 1×10^6 cells can be nucleofected in 100 μ L complete media with 0.2 nmol DNA + 0.8 nmol PNA using the Amaxa Human CD34+ Nucleofection Kit as per the manufacturer's protocol. Cells can then be resuspended in StemSpan culture media with cytokines for expansion.
3. THP1 and K562 cells can be electroporated with TFO in 100 μ L PBS using a BTX Electro Square Porator ECM 830 at 350 V, 12 ms, and 1 pulse. Cells can be resuspended in RPMI media with 10 % FBS and L-glutamine for expansion.

3.3.2 Peptide–PNA conjugates

1. Conjugation of TFOs to CPPs may greatly enhance the uptake—we present one strategy here. PNAs can be conjugated to a CPP such as Antp (Penetratin 1) via a reducible disulfide linkage, with activation of the peptide with a pyridyl disulfide at the N-terminus. A 3'-thiol modified TFO [18, 28].
2. Dissolve a 3'-thiol-modified TFO in 0.1 M DTT and leave overnight at 37 °C. Apply the solution to a NAP5 filtration column equilibrated with dH₂O and elute into a siliconized Eppendorf with activated Antp. Add methanol dropwise if precipitation is observed, and vortex. Heat the reaction at 65 °C for 15 min, incubate for an additional hour at 37 °C, and evaporate methanol if needed.

3.3.3 Nanoparticles—The use of an engineered biodegradable polymer delivery system for site-specific genome editing was initially described in the literature in 2011 when McNeer et al. developed methods to formulate poly(lactic-co-glycolic acid) (PLGA) nanoparticles containing PNA and DNA to be used for enhanced delivery to hematopoietic

stem and progenitor cells [40]. PLGA is an FDA-approved biocompatible and biodegradable polymer used clinically for delivery of drugs, including drugs for treatment of prostate cancer (Lupron Depot and Trelstar). Previous work has shown that PLGA nanoparticles can provide intracellular delivery of nucleic acids and oligomers, including plasmid DNA for transfection of cell lines [51, 52] and siRNAs for sustained gene silencing [53]. McNeer et al. first demonstrated that PLGA nanoparticles could be used as an intracellular delivery system for triplex-forming PNAs and short, donor DNAs in primary human CD34+ cells. They showed that the PNA/DNA PLGA nanoparticle delivery system was efficient and nontoxic but also could be used for specific PNA-mediated recombination in the human β -globin locus. In addition, they demonstrated modification in the human *CCR5* gene using a *CCR5*-targeted PNA with a *CCR5* donor DNA [40]. Site-specific genome editing of the *CCR5* and β -globin genes in human hematopoietic cells via triplex-forming PNA and donor DNA nanoparticles has also been described in vivo in chimeric mice [29]. PLGA nanoparticles containing an anti-microRNA-155 (anti-miR-155) PNA have additionally been shown to slow the growth of miR-155 “addicted” tumors in vivo in a mouse model of lymphoma [54].

Nanoparticles for these studies were formulated using a previously described [55] double-emulsion solvent evaporation technique that was modified to allow for the encapsulation of PNA alone or PNA and DNA [40]. General methods, as well as an example of specific instructions for making a 40 mg batch of PLGA particles, are described below (*see* Note 2).

1. Dissolve 50:50 ester-terminated PLGA, 0.95–1.2 g/dl, in an organic solvent such as dichloromethane or another organic solvent partially miscible in water (e.g., ethyl acetate, benzyl alcohol, propylene carbonate) (1 mg polymer/10 μ L organic phase). For example, dissolve 40 mg of PLGA in 400 μ L of dichloromethane overnight. Note: A different polymer blend containing PLGA and poly(beta-amino)ester (PBAE) can also be used as a PNA/DNA delivery vehicle [56].
2. Add the encapsulant, PNA/DNA in dH₂O, dropwise to the polymer solution while stirring. For example, make a mixture of 10 μ L of donor DNA in a 2 mM solution with 10 μ L of PNA in a 1 mM solution with 10.8 μ L water. This 30.8 μ L solution would be added to the 400 μ L polymer solution above (*see* Note 2).
3. Probe sonicate the first emulsion for 30 s at 38 % amplitude to further emulsify. Amplitude and frequency settings should be optimized for the sonicator probe used.
4. Add the first emulsion dropwise to 5 % polyvinyl alcohol (use twice the initial volume of organic solvent) while stirring. Any desired surface modifiers (e.g., DSPE-PEG, avidin) should be dissolved in 5 % polyvinyl alcohol (second emulsion).

²Use of nanoparticles: Different cell types may require different dosages of nanoparticles, so an initial experiment should include a dose–response curve, including cell survival measures. Fluorescent dyes such as Coumarin 6 in nanoparticles (dissolved in the DCM phase of creating particles) can be used to track the uptake of particles in cells by FACS or confocal imaging (Fig. 4).

5. Probe sonicate the second emulsion for an additional 30 s.
6. Transfer the final mixture to a stirring beaker of 0.3 % polyvinyl alcohol. For a 40 mg batch, use 20 mL of 0.3 % PVA. Stir at room temperature for 3 h to evaporate the dichloromethane.
7. Collect the nanoparticles by spinning for 10 min at $16,000 \times g$ at 4 °C.
8. Wash the nanoparticles in cold dH₂O. The number of washes can vary depending on the intended use of the nanoparticles. Three washes are normally sufficient for cell culture and animal studies. Mix the nanoparticles with 10 mL of cold dH₂O and spin at $16,000 \times g$ for 10 min for each wash.
9. After washing the nanoparticles, resuspend in dH₂O, and freeze at –80 °C for at least 1 h.
10. Lyophilize the nanoparticles for 2–3 days.
11. Morphology of the nanoparticles can be analyzed using an XL-30 scanning electron microscope (FEI, Hillsboro, Oregon) or comparable instrument. To image the particles, smear them on an imaging stub, and sputter-coat with palladium or gold. ImageJ software analysis can be used to determine particle diameter. The hydrodynamic diameter of particles in aqueous solution can also be determined by light scattering using instruments such as the Malvern Zetasizer (*see Note 3*).

3.4 Evaluation of Mutagenesis

3.4.1 Reporter Systems—A mouse cell line (AV16) with a multiple chromosomally integrated λ supFG1 shuttle vector carrying the *supFGI* mutation reporter gene can be used to assess mutagenesis mediated by TFO AG30 (AGG AAG GGG GGG GTG GTG GGG GAG GGG GAG) or variations [18, 57, 58]. We have also described the use of a luciferase-based assay in a previous *Methods* chapter [59] Subheading 3.6. Evaluation of Induced Recombination: Luciferase Assay.

1. Cells are grown in DMEM with 10 % FCS with G418 at 0.8 mg/mL.
2. Add TFO to the cells, and collect genomic DNA after allotting time for mutation induction (normally 2–4 days posttreatment).
3. Prepare high-molecular-weight DNA by lysis with 10 mM Tris (pH 8), 100 mM EDTA, 0.1 % SDS, and 50 μ g/mL proteinase for 3 h at 37 °C, followed by phenol extraction (25 phenol: 24 chloroform:1 isoamyl) and ethanol precipitation.
4. Rescue phage vector DNA into phage particles using in vitro packaging extracts made from restriction deficient lysogen (NM759). Grow the phage on *E. coli* containing an amber mutation in the *lacZ* gene with 5-bromo-4-

³Nanoparticle formulation: SEM imaging and controlled-release profiles will help determine whether nanoparticles have appropriate morphology and loading.

chloro-3-indolyl- β -D-galactopyranoside and isopropyl- β -D-thiogalactopyranoside.

5. Phage with functional *supF* genes will produce blue plaques, but phage with mutations will produce colorless plaques. The ratio of colorless to total plaques gives an estimation of mutation frequency.

3.4.2 Sequencing Strategies

1. Deep sequencing may be used for analysis of mutagenesis in a gene of choice.
2. Bar-coded primers, with a 6 bp unique barcode per sample, should be used to amplify a 100 bp region of interest using high-fidelity platinum taq polymerase with provided buffers (*see* Note 4). Run the PCR products on a 1 % agarose gel, and extract using the Qiagen gel extraction kit. Samples with different barcodes may be pooled for sequencing.
3. Samples can be ligated to adapters and sequenced on Illumina HiSeq platform using 75 base pair paired-end reads. Preparation for sequencing will vary depending on the sequencing platform used.

3.5 Evaluation of Recombination

3.5.1 Reporter Systems—A mouse reporter system developed by Roberts et al. [31] that ubiquitously expresses a modified eGFP pre-mRNA containing an aberrantly spliced β -globin intron (IVS2-654) can be used to evaluate PNA/DNA-mediated recombination in vivo. Nanoparticles containing PNA and DNA directed at the incorrect 5' splice site can be delivered to the EGFP-654 transgenic reporter mice in an attempt to correct the aberrant splice site via site-specific gene modification. Correction of the aberrant splice site would result in the expression of eGFP in a cell that was correctly modified. This mouse model has previously been used to demonstrate splice switching using LNA molecules to bind at the aberrant splice site [31].

1. Design desired PNA and DNA molecules (described in Subheadings 3.1.1 and 3.1.2, respectively) to target the aberrantly spliced β -globin intron (IVS2-654). The DNA should contain the sequence for the correct splice site. We have recently used the following molecules [29]: donor DNA, 5'-AAAGAATAACAGTGATAATTTCTGGGTTAAGG
CAATAGCAATATCTCTGCATATAAATAT-3'; 654PNA1, N terminus-KKK-JTTTJTTTJTTJ-OOO-TCTCTT TCTTTCAGGGCA-KKK-C terminus; and 654PNA2, N terminus-KKK-JJTJTTTJTTJ-OOO-TCTTCCTCCCACAGCTCC-KKK-C terminus.
2. Formulate and image nanoparticles as described above in Subheading 3.3.3 and deliver to mice via preferred delivery route (e.g., tail vein injection, retro-orbital injection, intraperitoneal, intranasal).

⁴Deep sequencing: Double bar-coding will allow the researcher to discard chimeric reads from analysis. In addition, it is important to examine the background level of mutation in non-treated samples.

3. After delivering desired dose of nanoparticles, sacrifice the mice and harvest organs or cells of interest.
4. Mechanically disrupt the organs in RPMI-1640 media, and strain the cells through a cell strainer into a 50 mL conical tube.
5. Next, separate any red blood cells from the harvested organs by Ficoll-Paque separation (2:1 ratio of cells to Ficoll-Paque). Slowly layer the cells onto the Ficoll-Paque in a 50 mL conical tube. Spin at $1,000 \times g$ for 20 min. Collect the mononuclear cell layer and wash with dPBS. Collect the cell pellet, and fix desired amount of cells by resuspending in 4 % paraformaldehyde.
6. Look for the expression of eGFP (which indicates correction of aberrant splice site by recombination) by using fluorescent-associated cell sorting (FACS).
7. Harvested cells can also be co-stained using fluorescently labeled antibodies directed against a cell population of interest. Any antibodies used for co-staining should be detected in a channel other than the channel used to detect eGFP fluorescence.
8. Recombination can also be detected by using an allele-specific mRNA PCR technique, described below in Subheading 3.5.2.
9. Recombination can additionally be evaluated by imaging cells or tissue sections for expression of the eGFP modification using a fluorescence microscope.

3.5.2 Allele-Specific PCR—To detect different DNA sequences corresponding to the original and corrected gene, an allele-specific PCR method can be used.

1. **Primer design**—When selecting a set of two primers for allele-specific PCR, the same gene-specific reverse primer should be used, though the forward primers should be designed such that their 3' ends differ and correspond to either the original or the corrected gene sequence. Thus, under the correct conditions, each primer will only be able to amplify its corresponding sequence of DNA. Primers are usually about 20–30 base pairs in length, with roughly equal salt-adjusted melting temperatures between 60 and 70 °C. Increasing the G/C content of the primers raises the melting temperature, since there are stronger hydrogen-bonding interactions between these bases. Alternately, the same gene-specific forward primer may be used with an allele-specific reverse primer. Forward and reverse primers should be selected with a span of hundreds of base pairs between them.
2. **PCR testing**—To determine the optimal AS-PCR conditions, a gradient technique can be used to select the correct annealing temperature that will allow for each primer set to only amplify its corresponding DNA sequence (see Note 5). To test PCR conditions, plasmids should be designed to

contain a segment of DNA corresponding to the entire section of DNA to be amplified, with either the original or the corrected DNA sequence present. It may be necessary to use site-directed mutagenesis to create one sequence from the other. Using the gradient technique four reactions can be set up; two primers sets can be paired with either the matching or the mismatched plasmid. Then these four reactions can be run simultaneously using a variety of annealing temperatures, usually from about 10 °C below to 10 °C above the primer melt temperature. Running the PCR products on an agarose gel and staining DNA with ethidium bromide for visualization under UV light can allow for the best conditions to be selected. This technique can lead to the detection of DNA sequences at plasmid concentrations as low as 0.002 ng/μL.

3. mRNA PCR—To modify this technique so that it can be used to detect specific mRNA sequences, the same allele-specific forward primers can be used, but the gene-specific reverse primer should be selected such that it corresponds to an exon that is separated from the location of the forward primer by an intron. This will ensure that only the mRNA sequence is short enough to be amplified by the primers for a given elongation time, with the result that the amplified product will only correspond to mRNA and not DNA.
4. qRT-PCR—To convert this technique into a real-time method to analyze levels of gene correction in a more quantitative fashion, fluorescent dyes that bind to DNA can be added to the PCR reaction mixture. Using a thermal cycle with the ability to read levels of fluorescence emitted by dyes such as SYBR Green, which is used to monitor DNA accumulation, and ROX, used as a reference dye, one can plot the relative amounts of DNA amplified over time.
5. When attempting to optimize allele-specific PCR conditions, many problems may be encountered. A lack of PCR products may point to annealing temperatures that are too high, whereas nonspecific products indicate an annealing temperature that is too low. Another important factor that can be manipulated is Mg^{2+} concentrations, since this is an important cofactor for the DNA polymerase enzyme. To circumvent problems, a touchdown technique can be utilized, in which the annealing temperature is set to a high temperature during the PCR cycle but then decreased every cycle until landing on a lower temperature that is used for the remainder of the cycles. This helps ensure that the first PCR products amplified, and thus those that are preferentially amplified during future cycles, have the highest specificity.

⁵AS-PCR: It may be necessary to attempt multiple primer concentrations and PCR conditions to optimize the protocol.

3.5.3 Sequencing Strategies

1. Cells may be cloned using a limiting dilution strategy to identify clones with the desired modification using AS-PCR. Positive clones can then be harvested for genomic DNA isolation and PCR of a 100–200 bp region surrounding the modification site and amplicons submitted for regular sequencing.
2. Deep sequencing can be used for analysis of modification in the target gene and off-target sites. Primers should flank a 100 bp region with the targeted modification in the center. As described above, bar-coded amplicons can be sequenced on the Illumina HiSeq platform (*see Note 5*).

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Fig. 1. Chemical structures

(a) Example of Hoogsteen bond formation in triple helix, purine (AAT and GGC) and pyrimidine (TAT and CGC) motif. From Gorman et al. (2001) *Current Molecular Medicine* reprinted in Schleifman et al. *Methods in Molecular Biol* Vol. 435. (Schleifman, Chin et al. [59]). (b) Examples of novel synthetic nucleic acid analogues. From Briones et al. *Anal Bioanal Chem* 2012. (c) Examples of triplex structures

Fig. 2. Schematic of genome modification using triplex-forming oligonucleotides

Triplex formation by site-specific intracellular TFO binding recruits factors that can induce recombination of a single-stranded donor DNA, encoding a desired modification, at a nearby genomic location

Fig. 3. Example of target site and gel shift assay

Sample sequence and tail clamp PNA binding to a site in the cystic fibrosis transmembrane receptor gene. Reprinted from Schleifman et al. (Schleifman, Bindra et al. [27])

Fig. 4. Schematic of nanoparticle formulation

PNA and DNA were loaded into nanoparticles using a previously described double-emulsion solvent evaporation technique (Fahmy, Stamstein et al. [55]; McNeer, Chin et al. [40]). A scanning electron micrograph shows a sample batch of PNA/DNA-loaded PLGA nanoparticles