Video Article

Evaluation of Exon Inclusion Induced by Splice Switching Antisense Oligonucleotides in SMA Patient Fibroblasts

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Abstract

Spinal muscular atrophy (SMA), a lethal neurological disease caused by the loss of *SMN1*, presents a unique case in the field of antisense oligonucleotide (AON)-mediated therapy. While *SMN1* mutations are responsible for the disease, AONs targeting intronic splice silencer (ISS) sites in *SMN2*, including FDA-approved nusinersen, have been shown to restore SMN expression and ameliorate the symptoms. Currently, many studies involving AON therapy for SMA focus on investigating novel AON chemistries targeting *SMN2* that may be more effective and less toxic than nusinersen. Here, we describe a protocol for *in vitro* evaluation of exon inclusion using lipotransfection of AONs followed by reverse transcription polymerase chain reaction (RT-PCR), quantitative polymerase chain reaction (qPCR), and Western blotting. This method can be employed for various types of AON chemistries. Using this method, we demonstrate that AONs composed of alternating locked nucleic acids (LNAs) and DNA nucleotides (LNA/DNA mixmers) lead to efficient *SMN2* exon inclusion and restoration of SMN protein at a very low concentration, and therefore, LNA/DNA mixmer-based antisense oligonucleotides may be an attractive therapeutic strategy to treat splicing defects caused by genetic diseases. The *in vitro* evaluation method described here is fast, easy, and sensitive enough for the testing of various novel AONs.

Video Link

The video component of this article can be found at <https://www.jove.com/video/57530/>

Introduction

Spinal muscular atrophy (SMA) is a fatal neuromuscular disorder inherited in an autosomal recessive pattern. It is characterized by the degradation of motor neurons and progressive trunk and limb muscle paralysis^{1,2}. The majority of SMA occurrences are due to a homozygous mutation in the *survival of motor neuron 1 (SMN1)* gene³. The *survival of motor neuron 2 (SMN2)* gene is an inverse duplicate of *SMN1*, and has a nearly identical sequence differing by only five bases^{4,5}. A C-to-T transition in *SMN2* located in exon 7 makes the gene nearly nonfunctional because the mutation leads to the essential exon 7 being excluded in nearly 90% of *SMN2* transcripts (**Figure 1a**). *SMN2* mRNAs missing exon 7 cannot compensate for the *SMN1* function because its protein product is unstable and is rapidly degraded.

Antisense therapy recently emerged as a very promising strategy for treating SMA⁶. The recent approval of nusinersen by the U.S. Food and Drug Administration (FDA) made it the first drug available for treating SMA⁷. Nusinersen is an 18-mer antisense oligonucleotide (AON) with a 2'-O-methoxyethyl modification (MOE) and a phosphorothioate backbone. The drug targets the intronic splicing silencer N1 (ISS-N1) located in intron 7 of the *SMN2* gene. Binding of nusinersen to ISS-N1 promotes the recovery of functional full-length SMN protein expression from the endogenous *SMN2* gene by inducing exon 7 inclusion (**Figure 1a**)^{8,9,10}. Currently, many studies involving AON therapy for SMA focus on investigating novel AON chemistries that may be more effective and less toxic than nusinersen. It has been demonstrated that AONs with other chemistries also efficiently induce exon inclusion in *SMN2* exon 7 both *in vitro* and *in vivo*^{11,12,13}.

Locked nucleic acids (LNAs) are chemically modified RNA analogs containing a methylene bridge connecting the 4'-Carbon with the 2'-Oxygen within the furanose structure (**Figure 1b**)^{14,15}. Compared to DNAs or RNAs, LNAs have an increased affinity for binding to complementary RNA sequences and have the added benefit of being highly resistant to endogenous nucleases. The LNA chemistry has been applied for use as probes for fluorescence *in situ* hybridization (FISH) and in qPCR^{16,17}. Also, it is utilized to regulate gene expression both *in vitro* and *in vivo*. GapmeR AONs are a combination of single-stranded DNA molecules flanked by several LNAs at the 5' and 3' ends. They knock down gene expression by binding complementary to targeted mRNAs, causing them to be degraded by the activated RNase H¹⁸. LNA/DNA mixmers are AONs which are composed of DNA nucleotides integrated in between LNAs. Presented in this orientation they can bind miRNA to inhibit its function (LNA-antimiR)¹⁹. Some LNA-antimiRs have reached clinical development. For examples, miravirsin (AntimiR-122) is an LNA-antimiR that inhibits miR-122 to treat hepatitis C infection and several Phase II clinical trials are currently ongoing^{19,20}. Recently, it has been

demonstrated that LNA/DNA mixmers can also modulate RNA splicing²¹. It can bind a specific sequence of mRNA and induce exon skipping in dystrophin mRNA and exon inclusion in *SMN2* mRNA *in vitro*^{13,22}.

In this article, we outline a methodology for the induction of exon inclusion using AONs and the evaluation of efficacy at the RNA and protein levels. To exemplify this method, we used the LNA/DNA mixmers targeting ISS-N1 in intron 7 of *SMN2*. AONs transfected into SMA patient cells by lipotransfection induced exon 7 inclusion in the final *SMN2* transcript and upregulation of SMN protein production. One of the advantages of using LNA/DNA mixmers to induce the exon inclusion is that the effective concentration for the transfection is significantly lower than other chemistries¹³. This method can be used for many other AONs except for phosphorodiamidate morpholino oligomers (PMOs), which, due to their neutral charge, need to enter cells through endocytosis induced by the Endo-Porter co-transfection reagent.

Protocol

1. Cell culture

1. Culture SMA patient fibroblasts in the growth medium (Dulbecco's modified Eagle medium 1:1 F-12 (Ham) with 10% fetal bovine serum (FBS) and 0.5% penicillin/streptomycin) in a CO₂ incubator at 37 °C.
2. Determine cell concentration using an automated cell counter and dilute cells to 1 x 10⁵ cells/mL in the growth medium, then seed on the appropriate plate. Use 12-well plates (1 mL per well) for RT-PCR or qPCR.
3. Incubate the plates for 24 h in a CO₂ incubator at 37 °C.

2. LNA/DNA mixmer transfection

1. Ensure cell confluency of around 65 - 80% for optimal transfection efficiency.
2. Thaw 10 µM LNA/DNA mixmers slowly on ice.
3. In a 1.5-mL tube, prepare a 20x mixmer solution by adding the mixmers to serum-deprived media so that the final volume reaches 50 µL.
4. Mix the mixmer solution with 50 µL of serum-deprived media containing 3% transfection reagent (1:1 ratio).
5. Incubate for 10 minutes at room temperature.
6. Add 900 µL serum-deprived medium with 5% FBS to each tube.
7. Aspirate the old media from the plate and replace with 1 mL LNA/DNA mixmer solution containing the transfection reagent.
8. Incubate the cells for 24 - 48 h at 37 °C in a CO₂ incubator.

3. RNA extraction

1. Remove the medium and add 1 mL of guanidinium thiocyanate-phenol-chloroform reagent to the cells. Wash the bottom of the well several times with guanidinium thiocyanate-phenol-chloroform and collect it into 1.5-mL tubes.
2. Vortex at high speed for 30 s and store at -80 °C for 1 h or overnight.
3. Thaw the samples at room temperature and vortex well.
4. Add 200 µL chloroform, shake vigorously for 15 s, and incubate at room temperature for 3 min.
5. Centrifuge at 12,000 x g for 15 min at 4 °C.
6. Collect the top clear aqueous phase into a new tube. Make sure to avoid collecting any of the white interphase that forms between the upper and lower phases.
7. Add 500 µL isopropanol and 1 µL 8 µg/µL glycogen (RNA grade). Vortex for 10 s and incubate at room temperature for 10 min or at -20 °C overnight.
8. Centrifuge at 12,000 x g for 10 min at 4 °C and remove all of the supernatant. A white pellet will form at the bottom of the tube.
9. Add 1 mL cold 75% ethanol and centrifuge at 7,500 x g for 5 min at 4 °C.
10. Remove all the ethanol and dry the pellet at room temperature until no ethanol is left in the tube.
11. Dissolve the pellet in 30 µL DNase/RNase free water, and incubate for 10 min at 60 °C.
12. Measure RNA concentration using a spectrophotometer (at 260 nm), and adjust RNA concentration to 50 ng/µL.

4. One step RT-PCR to measure the exon inclusion rate of *SMN2*

1. Mix 12.5 µL of 2x RT-PCR reaction buffer, 1.0 µL of one-step RT-PCR enzyme mix, 0.5 µL of each primer (*SMN2* forward: 5'-CTGCCTCCATTTCCTTCTG-3', *SMN2* reverse: 5'-TGGTGTTCATTTAGTGCTGCTC-3', *GAPDH* forward: 5'-TCCCTGAGCTGAACGGAAG-3', *GAPDH* reverse: 5'-GGAGGAGTTTGGTCGCTGT-3'), 2 µL of total RNA (50 ng/µL), and 8.5 µL of RNase/DNase-free distilled water.
2. After cDNA is synthesized at 50 °C for 15 min, start the PCR reaction. Amplify the *SMN2* exon 6-8 with 30 PCR cycles (94 °C for 15 s, 60 °C for 30 s, 68 °C for 25 s). Amplify the *GAPDH* with 20 PCR cycles (94 °C for 15 s, 60 °C for 30 s, 68 °C for 20 s).
3. Add 5 µL 6x loading dye to the PCR product, and load 5 µL of the mixture into a 2% agarose gel and run for 40 min at 100 V.
4. Image the gel and analyze it using ImageJ software.
5. Measure the intensity of full-length bands and $\Delta 7$ bands. Calculate the rate of exon 7 inclusion by the intensity value of (full-length)/(full-length + $\Delta 7$ *SMN2*).

5. Quantitative PCR (qPCR) to measure the exon inclusion rate of *SMN2*

1. For cDNA synthesis, mix 1 µL Oligo dT, 1 µL 10 mM dNTPs, and 11 µL total RNA (50 ng/µL). Incubate at 65 °C for 5 min.
2. Place the samples on ice and add 4 µL 5x buffer, 1 µL 0.1 M DTT, 1 µL RNase inhibitor, and 1 µL reverse transcriptase to each sample.

3. Incubate at 50 °C for 60 min, and heat up to 80 °C for 10 min to stop the reaction. The cDNA can be stored at -20 °C.
4. Dilute the cDNAs with RNase-free water (1:5 dilution). Mix 10 µL 2x SYBR Green qPCR enzyme mix, 0.8 µL 10 µM each primer (full-length *SMN2* forward: 5'-GCTATCATACTGGCTATTATATGGGTTT-3', full-length *SMN2* reverse: 5'-CTCTATGCCAGCATTTCCTTAAT-3', $\Delta 7$ *SMN2* forward: 5'-TCTGGACCACCAATAATTCCC-3', $\Delta 7$ *SMN2* reverse: 5'-ATGCCAGCATTTCATATAATAGCC-3', *GAPDH* forward: 5'-GCAAATTCATGGCACCCT-3', *GAPDH* reverse: 5'-AGGGATCTCGCTCCTGGAA-3'), 3 µL diluted cDNA, and 5.4 µL RNase-free water.
5. Start the qPCR reaction. Use the conditions: 95 °C for 30 s, then a 40 cycle repeat at 95 °C for 5 s and 60 °C for 20 s.
6. Calculate the relative expression of full-length *SMN2* to *GAPDH* or $\Delta 7$ *SMN2*, and normalize to the non-treated cells with the $\Delta\Delta C_t$ algorithm.

6. Western blotting

1. Remove transfection medium from cells and wash with phosphate-buffered saline (PBS) once.
2. Add 100 µL lysis buffer with a protease inhibitor.
3. Detach cells from the bottom of each well using a sterile cell scraper, and collect into 1.5-mL tubes. Incubate samples on ice for 30 min.
4. Pass cell lysate through 21-G needle 10 times to crush the cells.
5. Centrifuge at 20,800 x g for 15 min at 4 °C and collect the supernatant into new 1.5-mL tubes. The sample can be stored in a -80 °C deep freezer.
6. Determine protein concentration using a BCA Protein Assay Kit, and adjust the concentration to 1.0 µg/µL.
7. Mix 5 µL samples and 5 µL 2x sodium dodecyl sulfate (SDS) sample buffer (10% SDS; 14% 0.5M Tris-HCl solution, pH 6.7; 1% 0.5M EDTA-2Na stock solution, pH 8.0; 20% Glycerol; 0.004% bromophenol blue dye, 5% β -mercaptoethanol) and heat at 70 °C for 10 min.
8. Load the samples into a 4-12% Bis-Tris Protein gels and run for 1 h at 150 V.
9. Soak a thick filter paper in each buffer: (Concentrated anode buffer: 0.3 M Tris-HCl, 20% methanol; anode buffer: 0.03 M Tris-HCl, 20% methanol; cathode buffer: 25 mM Tris, 20% methanol, 40 mM 6- amino-n-hexanoic acid, 0.01% SDS).
10. Soak a polyvinylidene difluoride (PVDF) membrane in methanol for 20 s, then transfer it into the anode buffer.
11. Equilibrate the gel after SDS-PAGE with the cathode buffer for 5 - 10 min under agitation.
12. Place the filter papers, PVDF membranes and the gel on a semi-dry blotting machine, as follows: concentrated anode buffer paper/anode buffer paper/PVDF membrane/protein gel/cathode buffer paper (**Figure 2**).
13. Cover the stack and start the transfer at 20 V for 30 min.
14. After the transfer, rinse the PVDF membrane with PBST (PBS with 0.05% Tween 20) once.
15. Block the membrane at room temperature for 30 min in 5% skim milk powder dissolved in in PBST or at 4 °C for overnight.
16. Dilute a purified mouse anti-SMN antibody and a rabbit anti-Cofilin antibody to 1:10,000 in 5% skim milk powder in PBST. Incubate the membrane in it for 1 h at room temperature or at 4 °C overnight.
17. Wash 3 times with PBST for 10 min.
18. Dilute secondary antibodies (HRP-conjugated goat anti-mouse and anti-rabbit IgG (H + L)) to 1:10,000 in 5% skim milk powder in PBST. Incubate for 1 h at room temperature in the dark.
19. Wash the membrane 3 times with PBST for 10 min.
20. Detect the band signal using the chemiluminescence Western blotting detection kit.

Representative Results

Using SMA patient fibroblasts, we targeted the ISS-N1 silencer region in intron 7 of the *SMN2* gene with eight different antisense LNA/DNA mixmers that were transfected at a 5 nM concentration (**Figure 3**). Each of the mixmers contained a modified phosphorothioated backbone which enabled them to resist nuclease degradation. To evaluate the efficacy of the mixmers, the rate of *SMN2* exon 7 inclusion was quantified by RT-PCR and qPCR using primers specific to *SMN2*. The inclusion efficiency of exon 7 ranged from 78 - 98% when transfected with mixmers #1 - 5 in the patient cells (**Figure 4a, b**), while transfection utilizing mixmers #6-8 at the same dose did not result in any significant difference when compared to the control. The results obtained from qPCR analysis also displayed that the quantity of full-length *SMN2* mRNA transcript was significantly increased by the mixmers #1 - 3 and #5 treatments when compared to the control (**Figure 4c**).

In addition, the result of the Western blotting showed that transfection using mixmers #1 - 5 enabled higher levels of SMN protein expression in the patient fibroblasts (a 1.5 - 1.9-fold increase from the control, **Figure 5**). The treatments using mixmers #6 - 8 did not result in a change in the SMN protein expression level in the cells. These data demonstrate that transfection of LNA/DNA mixmers #1-5 induces the *SMN2* exon 7 inclusion efficiently in SMA patient fibroblasts.

Figure 1: Antisense-mediated exon inclusion strategy to treat SMA. (a) A C-to-T transition in exon 7 of *SMN2* leads to the skipping of exon 7 in approximately 90% of *SMN2* transcripts. These transcripts are out-of-frame and are rapidly degraded in the cells. Antisense oligonucleotides (AONs) bind the intronic splicing silencer N1 (ISS-N1), which induces exon inclusion in *SMN2*. The exon 7-included *SMN2* mRNAs can produce functional SMN protein. **(b)** Chemical structures of RNA and phosphorothioated LNA. The entire backbones of the mixmers we used in this article are phosphorothioated to prevent degradation. This figure has been modified from Touznik *et.al.*¹³. [Please click here to view a larger version of this figure.](#)

Figure 2: Semi-dry transfer method for Western blotting. A thick filter paper soaked in concentrated (Con.) Anode buffer, a thick filter paper soaked in Anode buffer, a PVDF membrane, a gel and a thick filter paper soaked in Cathode buffer are stacked between the two plates of a semi-dry blotting machine. [Please click here to view a larger version of this figure.](#)

Mixmer	Positions (from Ex7 3' end)	Mixmer sequence	Length (mer)
1	10, 39	C*+A*A*+A*A*+G*T*+A*A*+G*A*+T*T*+C*A*+C*T*+T* T*+C*A*+T*A*+A*T*+G*C*+T*G*+G	30
2	10, 27	T*+C*A*+C*T*+T*T*+C*A*+T*A*+A*T*+G*C*+T*G*+G	18
3	10, 27	T*+C*A*+C*+T*+T*+C*+A*T*+A*A*+T*+G*+C*T*+G*+G	18
4	10, 22	+T*T*+C*A*+T*A*+A*T*+G*C*+T*G*+G	13
5	10, 22	+T*T*+C*A*+T*A*+A*T*+G*+C*T*+G*+G	13
6	10, 17	A*+A*T*+G*C*+T*G*+G	8
7	10, 17	+A*+A*T*+G*+C*T*+G*+G	8
8	10, 17	+A*+A*T*+G*+C*+T*+G*+G	8

Figure 3: LNA/DNA mixmer sequence for SMN2 exon 7 inclusion. DNA base: G, A, T, C. LNA base (red): +G, +A, +T, +C. Phosphorothioated DNA base: G*, A*, T*, C*. Phosphorothioated LNA base (red): +G*, +A*, +T*, +C*. This figure is reproduced from Touznik *et.al.*¹³. [Please click here to view a larger version of this figure.](#)

Figure 4: Representative results of RT-PCR and qPCR to evaluate the efficacy of the LNA/DNA mixmers. Transfection of mixmers #1 - 5 leads to *SMN2* exon 7 inclusion at a significantly higher rate than the mock transfection. (a) RT-PCR of *SMN2* and *GAPDH* in SMA patient fibroblasts following transfection at 5 nM concentration. Top band: exon 7-included full-length *SMN2* mRNA. Bottom band: exon 7-excluded *SMN2* mRNA. (b) Quantitative analysis of *SMN2* exon 7 inclusion in the mixmer-treated SMA fibroblasts using RT-PCR. (c) Relative expression analysis of full-length *SMN2* to *GAPDH* measured using qPCR. The data were normalized to non-treated control cells. Error bars represent mean $\pm$ standard deviation (three independent experiments). One-way ANOVA with Dunnett's multiple comparison test was performed. M: mock control, NT: non-treated, H: healthy control fibroblast cells, B: blank. This figure is reproduced from Touznik *et al.*¹³. [Please click here to view a larger version of this figure.](#)

Figure 5: Representative results of Western blotting to measure SMN protein expression. Transfection of the LNA/DNA mixmers increases SMN protein production. (a) Western blotting of SMN and Cofilin (loading control) of healthy and mixmer-treated SMA patient fibroblasts. (b) Fold increase in SMN protein levels of mixmer-treated SMA fibroblasts. Transfection of mixmers #1 - 5 at 5 nM concentration significantly increases the production of SMN proteins. The data were normalized to the ratio of SMN/Cofilin in non-treated SMA fibroblasts. Bars represent mean $\pm$ standard deviation (four independent experiments). One-way ANOVA with Dunnett's multiple comparison test was performed. M: mock control, NT: non-treated. This figure is reproduced from Touznik *et al.*¹³. [Please click here to view a larger version of this figure.](#)

Discussion

The *in vitro* evaluation method described here is fast, easy, and sensitive enough for the testing of various AONs. This protocol is widely applicable to exon skipping and inclusion of other genes and various cell types. In addition, most AON chemistries (except PMOs) can be

transfected using this method. AONs can regulate splicing of pre-mRNA from both endogenous and artificially introduced genes, and can be co-transfected with plasmid vectors carrying targeted genes.

A critical step of this method is that the cell confluency should be around 65 - 80% when AONs are transfected into fibroblasts. This condition might be different for other cell types. The best concentration of AONs for transfection can be also different (0.5 - 100 nM), depending on targeted sequences and cell types.

This method is not without potential pitfalls. For example, the efficacy of mixmers is determined by the sequences of mixmers and LNA/DNA composition. For ISS-N1, a mixmer, which is composed of LNAs with DNA being substituted for LNA at every third nucleotide position, was more effective than a mixmer with an equivalent sequence with DNA substitution at every other nucleotide or all-LNA oligonucleotide¹³. However, this may be different for other target sequences. Therefore, it is necessary to optimize sequences of LNA/DNA mixmers and evaluate the efficacy of mixmers at RNA level before going further. The entire backbones of mixmers should be phosphorothioated to prevent degradation. In addition, although this lipotransfection-mediated transfection method can be used for most AON chemistries, it cannot be used for charge-neutral phosphorodiamidate morpholino oligomers (PMOs), since lipotransfection requires negatively charged oligonucleotides. For PMO transfection methods, see elsewhere^{23,24,25}.

Although this method is useful for testing novel AONs, the SMA fibroblast cell model does not recapitulate *in vivo* conditions in its entirety. Animal models can serve as an important source to test the *in vivo* efficacy and safety²⁶.

For exon skipping/inclusion, PMO and 2'-O-methoxyethyl modification (2'MOE) can be used instead of LNA/DNA mixmers^{27,28}. However, the optimal concentration of these chemistries for transfection are typically much higher^{29,30}. Because of the better efficacy compared to other antisense chemistries, the use of LNA/DNA mixmer-based antisense oligonucleotides may be an attractive therapeutic strategy to treat splicing defects caused by genetic diseases in the future.

Disclosures

The authors have nothing to disclose.

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