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Pre-mRNA Splicing Modulation by Antisense Oligonucleotides

Natalia N. Singh, Diou Luo, and Ravindra N. Singh

Department of Biomedical Sciences, College of Veterinary Medicine (2034 Vet Med Bld.), Iowa State University, Ames, IA 50011, USA.

Abstract

Pre-mRNA splicing, a dynamic process of intron removal and exon joining, is governed by a combinatorial control exerted by overlapping *cis*-elements that are unique to each exon and its flanking intronic sequences. Splicing *cis*-elements are usually 4-to-8-nucleotide-long linear motifs that provide binding sites for specific proteins. Pre-mRNA splicing is also influenced by secondary and higher order RNA structures that affect accessibility of splicing *cis*-elements. Antisense oligonucleotides (ASOs) that block splicing *cis*-elements and/or affect RNA structure have been shown to modulate splicing in vivo. Therefore, ASO-based strategies have emerged as a powerful tool for therapeutic manipulation of splicing in pathological conditions. Here we describe an ASO-based approach to increase the production of the full-length *SMN2* mRNA in spinal muscular atrophy patient cells.

Keywords

Antisense oligonucleotide (ASO); Survival motor neuron (SMN); Pre-mRNA splicing; Intronic splicing silencer N1 (ISS-N1); Transfection; Nucleofection; Spinal muscular atrophy (SMA); 2'-*O*-methyl modification; Phosphorothioate backbone; Phosphoroamidate morpholino oligonucleotide (PMO)

1. Introduction

Most human genes are split between coding (exonic) and noncoding (intronic) sequences. Pre-mRNA splicing is a process during which introns are removed and exons are joined together to make messenger RNA (mRNA). The process is catalyzed by a very complex and dynamic macromolecular complex known as the spliceosome (1). To distinguish between exons and introns, the spliceosome relies on sequences at exon-intron junctions, namely the 3' and the 5' splice sites. Due to the degenerate nature of the splice sites, assistance of other splicing regulatory elements (*cis*-elements) located within exons and introns is required for correct splicing. These *cis*-elements can either facilitate exon recognition, in which case they are called enhancers, or make exons “invisible” to the spliceosomal machinery, in which case they are called silencers (1–4). Splicing enhancers and silencers are binding sites for protein factors, mainly SR and hnRNP proteins, that assist or interfere with the recognition of the splice sites by the basic components of the spliceosome. Splicing enhancer and

*Corresponding author. Phone: (515) 294-2451. Fax: (515) 294-2315. natalias@iastate.edu.

4.Notes

silencer operate under the influence of unique contexts furnished by varying sizes of introns and exons. RNA structure modulates splicing by influencing the accessibility of the splicing regulatory *cis*-elements and their contexts (5–7). Coupling of pre-mRNA splicing to transcription adds yet another level of control to the splicing regulation. For example, RNA polymerase II elongation rate influences the decision whether a given exon will be included or skipped (8,9). Also, transcription of protein-coding genes is controlled by local chromatin structure which in turn is affected by multiple factors, including nucleosome positioning, histone modifications, and DNA methylation. Therefore, factors involved in the abovementioned processes have potential to effect splicing outcomes (8–11). Use of minigenes has been very helpful in deciphering the combinatorial control executed by various *cis*-elements (12). However, readout generated from minigenes cannot be always extrapolated to understand the role of a given *cis*-element in the context of an endogenous gene. Use of an antisense oligonucleotide (ASO)-based approach addresses this issue as it can be employed to assess the impact of a given splicing *cis*-element in the context of both the endogenous and the minigene systems (13,14). The ASO-based strategy is also useful for uncovering the position-specific role of residues associated with long-distance RNA–RNA interactions (15,16).

Disruption of splicing regulators can lead to aberrant splicing causing a disease (17–24). It has been estimated that ~4% of synonymous mutations are deleterious, since they result in aberrant splicing due to disruption of exonic splicing enhancers (25). Another study showed that ~10% of exonic disease-associated alleles disrupt splicing (26). Furthermore, it was proposed that ~22% of disease alleles that were originally classified as missense mutation may in fact affect splicing (27). Comparable portion (~25%) of missense and nonsense mutations that cause human inherited diseases, was predicted to change splicing due to alterations they create in functional splicing signals (28). These numbers combined with the number of known splicing mutations indicate that up to one-third of all disease-causing mutations might change pre-mRNA splicing (27,28). It has been shown that using an ASO-based approach one can manipulate the outcome of splicing, so that a particular exon can be selectively removed or included (29,30). Therefore, ASOs represent an invaluable tool that could be used for therapeutic purposes to treat a human disease caused by aberrant splicing.

Here we describe an example of ASO-mediated splicing correction in Spinal Muscular Atrophy (SMA), a leading genetic cause of infant mortality (31–33). SMA is caused by the loss of the *Survival Motor Neuron 1 (SMN1)* gene (34). *SMN2*, a nearly identical copy of *SMN1*, fails to compensate for the loss of *SMN1* due to predominant skipping of exon 7 during *SMN2* pre-mRNA splicing (35). Our earlier discovery of an intronic splicing silencer N1 (ISS-N1) produced the leading therapeutic target for an ASO-mediated restoration of *SMN2* exon 7 inclusion (13,36–41). ISS-N1 is a complex regulatory element that harbors two hnRNP A1/A2 motifs, is engaged in long-distance interactions, and sequesters a putative cryptic 5' splice site (15,16,36–42). Mechanism by which ISS-N1 regulate splicing is described elsewhere (43–46). Importantly, ISS-N1 discovery culminated in nusinersen, the first Food and Drug Administration (FDA)-approved drug for SMA treatment and the first antisense drug to restore expression of a full-length functional protein via splicing correction (47,48). Therefore, nusinersen represents a glowing example of an ASO success story.

In the method we describe here, ASOs with different chemistries were used to correct splicing of *SMN2* exon 7 in primary SMA-patient-derived fibroblasts and HeLa cells. This method could be easily adapted for an ASO-based splicing modulation in other cell types. Complementing this method, several recent reports describe ASO-based methods of splicing correction in mouse models of SMA (36–41). To render ASOs resistant to degradation within cells, our reported approaches have used the phosphorothioate backbone (PS) and 2'-*O*-methyl (2'-OMe) modifications (13–16,49). These non-proprietary modifications remain one of the most frequently used oligonucleotide chemistries for in vivo applications. ASOs with phosphorothioate modifications, where one of the nonbridging oxygen atoms is substituted with a sulfur atom, maintain the negatively charged backbones. Therefore, oligonucleotides with this chemistry can be efficiently delivered across cellular membrane using cationic liposomes. For the ease of explanation, we refer to all ASOs with PS and 2'-OMe modifications as 2OMe ASOs. Here we describe usage of Lipofectamine 2000 for lipoplexes-mediated 2OMe ASO transfection of primary SMA patient fibroblasts. Lipofectamine 2000 was also used to deliver 2OMe ASOs together with p*SMN2 I6* minigene into HeLa cells. A minigene system is very useful for initial screening of ASOs, particularly when relevant patient cells are not available. To manipulate *SMN2* exon 7 splicing we also used what is known as third-generation ASOs, namely phosphorodiamidate morpholino ASOs (PMOs). In these oligonucleotides the ribose sugar and the phosphodiester bond are replaced with a morpholino ring and a phosphorodiamidate linkage, respectively (50). Due to the latter chemical modification, PMOs are uncharged, hence, Lipofectamine 2000 cannot be used to deliver PMOs into cells. Instead, for the delivery of PMOs into cells we used nucleofection procedure. Overall, the methods of splicing manipulation we describe here can be applied to any gene, given that proper cis-element targets for ASOs have been identified. The approach could also be used for novel target discovery and pre-clinical development of ASOs in a cell culture-based model.

2. Materials

2.1 Components for Cell Culture

1. SMA-patient-derived primary fibroblasts (GM03813, Coriell Cell Repositories).
2. HeLa cells (American Type Culture Collection, ATCC).
3. Minimum Essential Medium with nonessential Amino Acids, without Glutamine (MEM, Life Technologies).
4. Dulbecco's Modified Eagle Medium, high glucose (DMEM, Life Technologies).
5. GlutaMAX-I (Life Technologies) or equivalent supplement of L-glutamine.
6. Fetal Bovine Serum (FBS, Life Technologies).

2.2 Cell Culture-related Equipment and Supplies

1. Nikon Eclipse TS100 inverted microscope or equivalent.
2. Nikon Intensilight C-HGFI fluorescence illuminator.
3. NAPCO Series 8000 WJ CO₂ Cell Incubator (Thermo Scientific) or equivalent.

4. Biological Safety cabinet (Thermo Scientific 1300 Series A2) or equivalent.
5. Hemocytometer.
6. Tissue culture supplies (tissue-culture-treated 100 mm dishes, T-75 flasks, 6-well plates, serological pipettes, 50 mL sterile tubes, cell lifters, etc).

2.3 Components and Equipment for Antisense Transfection

1. Opti-MEM I Reduced Serum Medium (Opti-MEM, Life Technologies).
2. Dulbecco's Phosphate-Buffered Saline (DPBS, Life Technologies).
3. 0.25% trypsin-EDTA solution (Life Technologies).
4. 2'-O-methyl modified and phosphorothioate backbone-containing Antisense Oligonucleotides (2OMe ASOs) (Dharmacon or TriLink) (*see Note 1*).
5. Phosphoroamidate Morpholino Antisense Oligonucleotides (PMOs) (Sarepta) (*see Note 1*).
6. p*SMN2* *I6* minigene.
7. Lipofectamine 2000 (Life Technologies).
8. Amaxa P2 primary cell 4D-Nucleofector X kit (32 reactions) supplied with the Nucleofector solution, the Supplement, a positive control pmaxGFP vector and a 16-well Nucleocuvette strip (Lonza).
9. Lonza 4D-Nucleofector System (4D-Nucleofector Core unit and 4D-Nucleofector X unit).
10. Hermle Z300 microcentrifuge with a swing-out rotor or equivalent.
11. Thermo Scientific Sorvall Legend RT Plus centrifuge or equivalent.
12. 1.5 mL microcentrifuge tubes (USA Scientific).
13. 50 mL sterile centrifuge tubes.
14. Tissue culture supplies (tissue-culture-treated 6-well plates, serological pipettes, etc).

2.4 Components for Total RNA Isolation

1. TRIzol Reagent (Life Technologies) (*see Note 2*).
2. Chloroform (*see Note 2*).

¹For optimal results, we recommend to use HPLC-purified ASOs. ASOs were dissolved in RNase-free water to a final concentration of 0.5 mM. ASO concentration was determined using the spectrophotometer by measuring absorbance at 260 nm and calculating the molarity based on extinction coefficient provided by the manufacturer. 2OMe ASOs were aliquoted and stored at -20 °C. PMOs were stored at +4 °C. Vials with fluorescent-dye-conjugated 2OMe ASOs were stored wrapped in aluminum foil to prevent photobleaching. When designing an ASO, one should consider that, at least in the case of 2OMe ASOs, the longer ones might produce greater off target effect and display lesser target specificity as we observed for a 20-nt-long ASO, Anti-ISS-N1, as compared to an 8-nt-long ASO, 3up8 (14).

²When working with the hazardous volatile reagents, use a chemical fume hood. Always wear lab coats and gloves, and use eye protection. When working with radioactive material, use proper shielding recommended by your institution. Dispose of all hazardous materials as per rules established by your institution.

3. Isopropanol.
4. Ethanol, 200 proof for molecular biology (Sigma-Aldrich).
5. RQ1 RNase-free DNase (Promega).
6. Phenol-chloroform (1:1), equilibrated with Tris-EDTA (TE) buffer, pH 8.0 (*see* Note 2).
7. 3 M potassium acetate, molecular biology grade (pH 5.5, Ambion).
8. GlycoBlue (Ambion).
9. Dry ice.
10. Hermle Z233 M-2 microcentrifuge with an angle-rotor or equivalent.
11. BioMate 3 (Thermo Scientific) or similar spectrophotometer.
12. A source of RNase-free ultrapure water. We use Cascada Bio Water Purification System.
13. 1.5 mL microcentrifuge tubes, RNase free.

2.5 Components for RT-PCR and Imaging

1. SuperScript III Reverse transcriptase kit (Invitrogen), Oligo(dT)_{12–18} primer (Invitrogen, 0.5 µg/µL), a gene-specific primer (Integrated DNA Technologies, IDT, 2 µM), dNTP mixture (10 mM each, Sigma-Aldrich).
2. *Taq* DNA polymerase (5 U/µL) with 10x reaction buffer and 25 mM MgCl₂ solution (New England Biolabs, NEB), forward and reverse primers (IDT, *see* Note 3).
3. DdeI (NEB).
4. [γ -³²P]-ATP (6,000 Ci/mmol) (PerkinElmer, *see* Note 2).
5. [α -³²P]-dATP (3,000 Ci/mmol) (PerkinElmer, *see* Note 2).
6. 10X Tris-borate EDTA (TBE) electrophoresis buffer (*see* Note 4). Dilute 10 times to make working solution of 1x.
7. 6x DNA loading buffer (*see* Note 5).

³.To monitor the effect of ASOs on *SMN* exon 7 splicing we used the following primer pairs. For the p*SMN2* *I6* minigene, the forward and reverse primer anneal to the vector backbone (P1, 5'-CGA CTC ACT ATA GGC TAG CC-3') and exon 8 (P2, 5'-GCA TGC AAG CTT CCT TTT TTC TTT CCC AAC AC-3'), respectively. The PCR product size is 333 bp when exon 7 is included, and 279 bp when exon 7 is skipped (51). For the endogenous *SMN* gene, the primers anneal to exon 6 (N-24, 5'-CCA GAT TCT CTT GAT GAT GCT GAT GCT TTG GG-3') and exon 8 (P2). The PCR product size is 281 bp when exon 7 is included, and 227 bp when exon 7 is skipped (13). To be able to distinguish between transcripts generated from the endogenous *SMN1* and *SMN2* genes based on the absence/presence of DdeI cleavage site, primer N-24 was used with primer P26 (5'-GTA CAA TGA ACA GCC ATG TC-3') (52). To monitor splicing of multiple *SMN* exons in a single PCR reaction we also used the forward and reverse primer that anneal to exon 2b (5'-hSMN-E2b, 5'-GAA TAC TGC AGC TTC CTT ACA ACA G-3') and exon 8 (P2- 2, 5'-CTT CCT TTT TTC TTT CCC AAC AC-3'), respectively. The size of the PCR product that corresponds to the full-length SMN transcript is 774 bp (49).

⁴.To make 10x TBE, dissolve 108 g Trizma base, 55 g boric acid, and 9.3 g EDTA in ultrapure water (total volume 1 L). Confirm that pH is equal to 8.3.

⁵.6X DNA loading buffer contains 0.05% (w/v) Bromophenol Blue, 0.05% (w/v) Xylene Cyanol, and 30% (v/v) glycerol. To make 10 mL of 6X loading dye dissolve Bromophenol Blue and Xylene Cyanol (5 mg each) in a mixture containing 1 mL of ultrapure water and 6 mL of 10X TBE buffer, add 3 mL of glycerol and mix well. Store as 1 mL aliquots at -20 °C.

8. 40% acrylamide/bis-acrylamide solution (29:1, Thermo Fisher Scientific), 10% (w/v) ammonium persulfate (APS) solution, 10% (w/v) sodium dodecyl sulfate (SDS) solution and *N,N,N',N'*-tetramethylethylenediamine (TEMED).
9. RNase-free water.
10. Sigmacote (Sigma-Aldrich).
11. Thermo cycler.
12. Micro Bio-Spin Columns P-30 Tris Chromatography columns (Bio-Rad).
13. Hermle Z233 M-2 microcentrifuge with an angle-rotor or equivalent.
14. Native polyacrylamide gel of medium size (dimensions of 17.3 cm x 16 cm x 0.15 cm) (*see* Note 6).
15. A midi-gel casting system.
16. Gel electrophoresis systems.
17. Power supply.
18. AB15 pH meter (Fisher Scientific) or equivalent.
19. Model 583 Gel Dryer (Bio-Rad) or equivalent.
20. FLA-5000 Image Reader (Phosphorimager) with Multi Gauge Software (Fuji Photo Film Inc) or similar system.
21. Phosphorimager screen (cassette).
22. Chromatography Paper 3MM Chr (Whatman).
23. 0.2 mL thin-walled tubes, RNase free.
24. 1.5 mL microcentrifuge tubes, RNase free.
25. Plastic wrap.
26. Paper towels.

2.6 Components for Western Blotting and Imaging

1. Radioimmunoprecipitation assay (RIPA) buffer (Boston BioProducts) (*see* Note 7).
2. 100x Halt Protease Inhibitor Single-Use cocktail (Thermo Scientific) (*see* Note 7).

⁶.To make a medium size 1.5 mm thick 6% native polyacrylamide gel, prepare 60 mL of the following solution. Combine 44.52 mL of ultrapure water, 9 mL of 40% (w/v) acrylamide-bis-acrylamide solution (29:1) and 6 mL of 10X TBE. To start polymerization, add 400 μ L of 10% (w/v) APS and 80 μ L of TEMED. Of note, if the gel will be used for running PCR products generated with a 5' end-³²P-labeled primer, first filter the above solution to remove any solid particles and then initiate gel polymerization. Pour the mixture into a gel-casting cassette, insert a comb and allow the gel to polymerize for at least 30 min. For a 5% gel decrease the volume of acrylamide- bis-acrylamide solution to 7.5 mL.

⁷.Immediately before usage supplement RIPA buffer with 100 $\times$ Halt Protease Inhibitor Single-Use cocktail. Use 10 μ L of the cocktail per 1 mL of RIPA buffer.

3. BCA protein assay kit (Thermo Scientific) or Bio-Rad protein assay dye reagent concentrate.
4. 2 M Tris-HCl (pH 8.8), 1 M Tris-HCl (pH 7.6), 1 M Tris-HCl (pH 6.8), 5 M NaCl, Tween 20.
5. Tris-Glycine sodium dodecyl sulfate (SDS) running buffer (10x, Boston BioProducts): 250 mM Tris Base, 1.92 M glycine, 1% SDS, pH 8.3. Dilute 10 times to make working solution of 1x.
6. 2x Laemmli Sample Buffer (Bio-Rad, *see* Note 8).
7. Color Prestained Protein Standard, Broad range (11–245 kDa) or equivalent.
8. Tris buffered saline with Tween 20 (10x TBST): mix 195 mL water, 500 mL 1 M Tris-HCl (pH 7.6), 300 mL 5 M NaCl and 5 mL Tween 20. Dilute 10 times to make working solution of 1x.
9. Blocking solution: 5% (w/v) nonfat milk in 1x TBST (*see* Note 9).
10. Trans-blot Turbo RTA Transfer Kit (includes pre-cut mini-size LF PVDF membranes, mini-size transfer stacks, and 5x Trans-Blot Turbo transfer buffer). Dilute the buffer 5 times to make working solution of 1X.
11. Primary antibodies: mouse monoclonal anti-SMN antibody (BD Transduction Laboratories) and rabbit polyclonal anti- β -actin antibody (Sigma-Aldrich).
12. Secondary antibodies: horseradish peroxidase (HRP)-conjugated anti-mouse goat antibody (Jackson ImmunoResearch), and (HRP)-conjugated anti-rabbit donkey antibody (GE Healthcare).
13. Restore™ Western Blot Stripping Buffer (Thermo Scientific).
14. SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).
15. SDS-PAGE gels of mini size (dimensions 8.2 cm x 7.3 cm x 0.15 cm) (*see* Note 10).
16. Gel casting system.
17. Gel electrophoresis system.

⁸. Immediately before usage supplement 2x Laemmli Sample Buffer with β -mercaptoethanol (add 50 μ L of β -mercaptoethanol to 950 μ L of the buffer as per the manufacturer's instructions).

⁹. Blocking Solution can be reused several times.

¹⁰. To cast a SDS-PAGE gel, start with making a separating gel. For one 1.5 mm thick mini size gel prepare 10 mL of the separating gel solution. To make 10 mL of a 10% polyacrylamide separating gel solution, combine 5.4 mL of ultrapure water, 2.5 mL of 40% (w/v) acrylamide-bis-acrylamide solution (29:1), 1.875 mL of 2 M Tris-HCl (pH 8.8), 100 μ L of 10% (w/v) SDS, 100 μ L of 10% (w/v) APS, and 25 μ L of TEMED. For a 12% polyacrylamide separating gel, increase the volume of 40% acrylamide-bis-acrylamide solution to 3 mL. Mix the solution and pour it into a gel-casting cassette (filling ~3/4 of the space), layer the solution with alcohol (ethanol or isopropanol), and allow the gel to polymerize for at least 30 min. After the gel is polymerized, pour alcohol out and rinse the gel-casting cassette with distilled water to get rid of any traces of alcohol. Dry the inside of the cassette with paper towels. Prepare the stacking gel solution. To make 4 mL of a 5% polyacrylamide stacking gels solution, combine 2.912 mL high purity water, 0.5 mL of 40% (w/v) acrylamide-bis-acrylamide solution (29:1), 0.5 mL of 1 M Tris-HCl (pH 6.8), 40 μ L of 10% (w/v) SDS, 40 μ L of 10% (w/v) APS, and 8 μ L of TEMED. Mix the solution and pour it on top of the polymerized separating gel. Insert a comb and allow the stacking gel to polymerize for at least 30 min.

18. Power supply.
19. 96-well plates U-bottom with lids (Greiner Bio-one).
20. Microplate reader spectrophotometer (Spectra Max) or similar instrument for measuring protein concentration.
21. Trans-blot Turbo Transfer Cell (Bio-Rad).
22. Rocking Platform (VWR) or equivalent.
23. Standard Analog Shaker (VWR) or equivalent.
24. Hermle Z233 M-2 microcentrifuge with an angle-rotor or equivalent.
25. UVP BioSpectrum AC imaging System (UVP).
26. Plastic trays/containers.
27. 1.5 mL microcentrifuge tubes.

3. Method

3.1 Delivery of ASOs into GM03813 Cells

3.1.1 Transient transfection of GM03813 cells with 2OMe ASOs—Cells used for transfection should be regularly passaged after reaching ~90% confluency.

(The procedure is described for a single transfection in one well of a 6-well plate.)

1. Pre-plate ~ 1.3 to 1.4×10^5 GM03813 cells per one well of a 6-well plate in a total volume of 2 mL of MEM supplemented with GlutaMax-I and FBS (see Notes 11–13). Incubate cells at 37°C in a CO₂ incubator (set at 5% CO₂) for ~24 hours (see Note 14).
2. Transfect cells using the following procedure (see Notes 15 and 16). In a 1.5 mL microcentrifuge tube gently mix 6 μ L of Lipofectamine 2000 with 244 μ L of Opti-MEM I and incubate at room temperature for 5 min. Meanwhile in a separate tube mix 1 μ L of 100 μ M stock solution of an 2OMe ASO with 249 μ L of Opti-MEM I. After the 5-minutes incubation combine Lipofectamine 2000-Opti-MEM and 2OMe ASO-Opti-MEM solutions (total volume becomes 500 μ L), mix and continue incubation for another 20 to 30 min. Add 500 μ L of the 2OMe ASO Lipofectamine complex drop wise to the pre-plated GM03813 cells

¹¹.To prepare growth medium for GM03813 fibroblasts, mix 500 mL of MEM with 75 mL of FBS and 5 mL of 100X GlutaMAX-I. To prepare growth medium for HeLa cells, mix 500 mL of DMEM with 50 mL of FBS.

¹³.If in addition to splicing pattern the effect of ASO on protein levels will be analyzed as well, increase the number of transfected cells by either using more wells of 6-well plates per given ASO or by switching to larger dishes. For GM03813 cells we recommend using at least one 100 mm dish per each ASO. If using 100 mm dishes, preplate ~ 8 to 9×10^5 GM03813 cells per one dish in a total volume of 8 mL.

¹⁴.When using Lipofectamine 2000, cells should be ~90% confluent at the time of transfection.

¹⁵.GM03813 cell can be efficiently transfected using a broad range of ASO:Lipofectamine 2000 ratios. For example, we use 1:8 ratio to transfect GM03813 cells in 6-well plates and 100 mm dishes. To transfect HeLa cells in 24-well plates, we use 1:2.5 ratio recommended by the manufacturer.

¹⁶.To monitor transfection efficiency, an ASO labeled with a fluorophore can be used. For example, ASOs with Cy3 attached to their 5' or 3' end are commercially available. GM03813 cells have relatively large nuclei. Therefore, transfected cells could be readily identified by fluorescing nuclei several hours after the transfection took place.

(from Subheading 3.1.1, **step 1**). Rock the plate gently back and forth. The final 2OMe ASO concentration becomes 40 nM. Return the cells to a CO₂ incubator (see Note 17). Perform parallel transfection experiments with a positive and a negative control 2OMe ASOs. Also, create a mock transfection control, where the 2OMe ASO is replaced with water. In case of multiple transfections with 2OMe ASOs of different length, maintain the total amount of nucleic acids used per each transfection constant by adding a carrier, for example, a negative control 2OMe ASO. The same principle applies when transfecting different amounts of the same 2OMe ASO.

3. To analyze splicing pattern only, 24 h post transfection, remove the culture medium by aspiration, wash the cells with DPBS, and add 1 mL of TRIzol directly to the well to lyse the cells. Pipette the lysate up and down several times and transfer it to a 1.5 ml microcentrifuge tube.
4. To analyze the effect of an 2OMe ASO on splicing as well as on protein levels, 48 h post transfection remove the culture medium by aspiration and wash the cells three times with ice-cold DPBS. Following washes, add ice-cold DPBS directly to the well/dish (see Notes 13 and 18). Collect cells by scraping and make appropriate aliquots for immediate use in Subheading 3.3, **step 1** and/or Subheading 3.4, **step 1**.

3.1.2 Nucleofection of GM03813 cells with PMOs—Cells used for nucleofection should be regularly passaged after reaching ~ 90% confluency. As per the manufacturer's recommendations, cells should be passaged 2 to 4 days prior to nucleofection.

(The procedure is described for 16 simultaneous nucleofections. One nucleofection is then used for seeding a single well of a 6-well plate.)

1. Fill 16 wells of 6-well plates with 2 mL of GM03813 growth medium (see Note 11) and place the plates in a CO₂ incubator for equilibration.
2. Add the Nucleofector Supplement to the Nucleofector Solution and mix (see Note 19).
3. Place the desired amount of each PMO in 1.5 mL microcentrifuge tubes (at a maximum 2 µL volume per tube; see Note 20) using the appropriate stock solution(s). For example, if 2 µL of 100 µM PMO stock is used, the final concentration of this PMO during the nucleofection procedure will be 10 µM. Include the positive control, for example, a PMO known to promote *SMN2* exon 7 inclusion (also see Note 21). As a negative control use a scrambled PMO as well as substitute water for a PMO. Close and set the tubes aside.

17. We recommend replacing culture medium with fresh one 6 to 8 h after transfection.

18. Use ~1.2 mL of DPBS per one 100 mm tissue-culture dish.

19. Once the Nucleofector Supplement is added to the Nucleofector Solution, the mixture is stable for 3 months at 4 °C.

20. The volume of PMO solution should not exceed 10% of a nucleofection reaction, the total volume of which is 20 µL.

21. Amara P2 primary cell 4D-Nucleofector X kit contains a pmaxGFP expression vector that could be used as a positive control for nucleofection efficiency.

4. Proceed with harvesting GM03813 cells. Use four to five 100 mm plates of these cells. Start with aspiration of media from the plates and wash cells once with DPBS using 10 ml per one plate (*see* Note 22). Add 1ml of trypsin solution to each plate and start the incubation (*see* Note 23). When most cells become detached, stop trypsinization by adding 5 mL of GM03813 growth medium (*see* Note 11) to each plate. Gently pipette cell suspension up and down multiple times until there are no cell clumps left (presence of clumps in suspension should be monitored using a microscope). Pool cell suspension from all plates into a single 50 mL tube and mix.
5. Using a hemocytometer count the number of cells per 1 mL of cell suspension. Considering that 2.4×10^5 GM03813 cells is used per one nucleofection, determine the volume of cell suspension that contains the required number of cells (2.4×10^5 cells $\times$ 17 nucleofections = 40.8×10^5 cells.) Increase the volume of this cell suspension to 40 mL by adding a fresh growth medium, gently mix and precipitate the cells by centrifugation at $90 \times g$ for 10 min at room temperature. For this purpose, we use a swinging bucket rotor. Remove supernatant, resuspend the cell pellet in 40 mL of fresh growth medium and repeat centrifugation. Remove as much supernatant as possible making sure not to disrupt the cell pellet.
6. Gently and carefully resuspend the cell pellet in 306 μ L (18 μ L $\times$ 17 nucleofections) of the Nucleofector Solution prepared at Subheading 3.1.2, **step 2**. Add 18 μ L of this cell suspension to each 1.5 mL microcentrifuge tube containing 2 μ L of PMO solution and mix. Total volume of each sample now is 20 μ L.
7. Transfer the content of each tube to individual wells of a 16-well Nucleocuvette strip (*see* Note 24). Gently tap the Nucleocuvette strip to ensure that there are no air bubbles and samples cover the bottom of the wells.
8. Place the Nucleocuvette strip with cells into the 4D-Nucleofector X Unit of the pre-started 4D-Nucleofector System. For GM03813 cells nucleofection select the program “EN 150”. Start nucleofection process by pressing the “Start” on the display.
9. After run is complete, incubate the Nucleocuvette strip at room temperature for 10 min followed by addition of 80 μ L of fresh growth medium (*see* Note 22) to each well of the Nucleocuvette strip. Resuspend the cells. After that move the entire content of each well to individual wells of 6-well plates prepared at Subheading 3.1.2, **step 1**.

²². All nucleofection solutions should be prewarmed to room temperature. Tissue culture medium and DPBS should be prewarmed to 37 °C.

²³. Due to a small volume of trypsin used, make sure that all cells are covered with the solution to prevent cell drying and death. The duration of incubation with trypsin depends on a cell line and the “potency” of a trypsin solution. ~5 min in a 37 °C incubator is a good starting point. Light tapping of a plate facilitates cell detachment.

²⁴. Since prolong exposure of cells to the Nucleofector Solution might have a negative effect on their viability and nucleofection efficiency, from this step on work as quickly as possible.

10. To analyze splicing pattern only, 24 h post nucleofection remove the culture medium by aspiration, wash the cells with DPBS and add 1 mL of TRIzol directly to each well to lyse the cells. Pipette the lysates up and down several times and transfer them to 1.5 mL microcentrifuge tubes.

3.2 Transient co-transfection of HeLa cells with 2OMe ASOs and the pSMN2 I6 minigene

Cells used for transfection should be regularly passaged after reaching ~70% to 90% confluency.

(Described for a single transfection in a well of a 24-well plate.)

1. Pre-plate $\sim 1.1 \times 10^5$ HeLa cells in 0.5 mL of DMEM (*see* Notes 11 and 12). Incubate cells at 37°C in a CO₂ incubator (similarly as in Subheading 3.1.1, **step 3**). Use cells for transfection the next day (*see* Note 14).
2. Transfect cells using the following procedure. Gently mix 2 µl of Lipofectamine 2000 with 48 µL of Opti-MEM I and incubate at room temperature for 5 min. Meanwhile mix 0.6 µl of 50 µM stock solution of a 2OMe ASO plus 1 µl of 0.1 µg/µL pSMN2 I6 minigene solution with 48.4 µL of Opti-MEM I. After the 5-minutes incubation combine Lipofectamine 2000-Opti-MEM and nucleic acids-Opti-MEM solutions (total volume becomes 100 µL), mix and continue incubation for 20 to 30 minutes. Add the 100 µL nucleic acids-Lipofectamine complex drop wise to pre-plated HeLa cells (from Subheading 3.2.1, **step 1**). Rock the plate gently back and forth. The final 2OMe ASO concentration becomes 50 nM. Return the cells to a CO₂ incubator (*see* Note 17). Perform parallel transfection experiments with a control 2OMe ASO. Also, create a mock transfection control, where a 2OMe ASO is replaced with water. In case of multiple transfections when different amount of a given 2OMe ASO is used with 0.1 µg of the plasmid, continue to maintain the total amount of the nucleic acids constant by adding a control 2OMe ASO. Similar principle applies when co-transfecting a minigene of interest with 2OMe ASOs of different length.
3. To analyze the effect of a 2OMe ASO on splicing of pSMN2 I6 minigene, 24 h post transfection remove the culture medium by aspiration and wash the cells with DPBS. Add 1 mL of TRIzol directly to the well to lyse the cells. Pipette the lysate up and down several times and transfer it to a 1.5 mL Micro centrifuge tube.

3.3 Isolation of total RNA, RT-PCR and Imaging

1. If using cells from Subheading 3.1.1, **step 4**, start with their precipitation using one-fourth of cell suspension collected at this step. Pellet the cells by spinning them at 2,000x *g* for 1 min at 4°C. Aspirate DPBS and resuspend the pellet in 1 mL of TRIzol to lyse the cells. Pipet the lysate up and down several times and leave it at room temperature for 5 min to insure the complete dissociation of the

¹².To count cells, use a hemocytometer.

nucleic acid:protein complexes (*see* Note 25). The same 5-minutes incubation applies to the samples in TRIzol generated at Subheading 3.1.1, **step 3**, Subheading 3.1.2, **step 10**, and Subheading 3.2, **step 3**. Following 5 min incubation, add 200 μ L of chloroform per 1 mL of TRIzol and hand-shake the sample for 20 s. Incubate at room temperature for up to 5 min. Spin the sample at $12000 \times g$ for 15 min at 4 °C. Transfer ~450 μ L of aqueous (upper colorless) phase to a fresh 1.5 mL microcentrifuge tube, add equal volume of isopropanol, mix and incubate for 10 min at room temperature. To precipitate total RNA, spin the sample at $12000 \times g$ for 10 min at 4 °C. Discard the supernatant and wash the RNA pellet with 75% (v/v) ethanol. To do so, add 1 mL of ethanol solution to the pellet, close and invert the tube several times (*see* Note 26). Spin down the sample at maximum speed for 5 min at room temperature. Discard 75% ethanol making sure not to lose the pellet, briefly spin the sample and carefully remove residual ethanol collected at the bottom of the tube using a pipette. Air-dry the pellet at room temperature (*see* Note 27). Dissolve the pellet in an appropriate volume of RNase-free water (*see* Note 28). To determine the yield of total RNA, measure RNA concentration using a spectrophotometer.

2. Perform DNase treatment of total RNA prepared in Subheading 3.3, **step 1** as follows: in a 50 μ L reaction combine RNA sample, RNase-free water, 5 μ L of 10 $\times$ RQ1 DNase buffer, and the required amount of RQ1 RNase-free DNase (*see* Note 29). Mix and incubate the reaction at 37 °C for 30 min (*see* Note 30). To terminate the reaction, add to the tube 50 μ L of water, 10 μ L (1/10th volume) of 3 M potassium acetate (pH 5.5) and 100 μ L of phenol-chloroform mixture and vortex. If the starting amount of total RNA is small, also add 0.5 μ L of GlycoBlue (*see* Note 31). Spin the mixture at 13,000 rpm for 5 min at room temperature. The mixture will separate into lower organic and upper aqueous phases. Transfer 96 μ L of aqueous phase to a fresh 1.5 mL microcentrifuge tube.
3. To precipitate total RNA from the aqueous phase collected in Subheading 3.3, **step 2**, add to the sample 2.5 $\times$ volume of 100% ice-cold ethanol. Close the tube and invert it several times to mix all the components. Place the tube either on dry ice for 5 min or in a freezer (–80 °C for 30 min or –20 °C for at least 1 h). To precipitate the RNA, spin the cold mixture at 13,000 rpm for 30 min at 4 °C. Wash the RNA pellet with 1 mL of 70% (v/v) ethanol similarly as described in

²⁵. At this point the procedure can be interrupted. According to the manufacturer samples can be stored in TRIzol for up to 1 year at –20 °C.

²⁶. At this point the procedure can be interrupted. The RNA pellets can be stored in 75% ethanol for at least a year at –20 °C.

²⁷. The duration of drying depends on the size of an RNA pellet. We find drying RNA pellets for 1 min at room temperature works well.

²⁸. To dissolve total RNA prepared from GM03813 fibroblasts collected from one well of a 6-well plate and from HeLa cells collected from one well of a 24-well plate, we recommend using ~10 μ L of water.

²⁹. As per the manufacturer's instructions we use 1 unit (which correspond to 1 μ L) of RNase-free DNase per 1 μ g of total RNA. The volume of DNase should not exceed 10% of the reaction volume. Therefore, when treating more than 5 μ g of total RNA increase the volume of the reaction.

³⁰. Use filter tips when pipetting solutions containing DNase.

³¹. GlycoBlue facilitates RNA recovery and makes small RNA pellets more visible due to their blue color. As per manufacturer's instructions a final concentration of GlycoBlue is 50–150 μ g/mL.

Subheading 3.3, **step 1**. Dissolve the pellet in an appropriate volume of RNase-free water. Measure RNA concentration using a spectrophotometer.

4. To generate first-strand cDNA, assemble a reverse transcription (RT) reaction. Depending on how much cDNA is required for downstream usage, the total volume of an RT reaction can vary from 5 to 20 μL . We used 10 μL reaction to be sufficient enough for multiple cDNA applications. To assemble and run 10 μL RT reaction, begin with combining the RNA (see Note 32) and RNase-free water in a total volume of 6.0 μL in a 0.2 mL thin-walled tube (see Note 33). Add 0.5 μL of Oligo(dT)_{12–18} (see Note 34) and 0.5 μL of dNTP and mix by pipetting up and down. In a thermocycler incubate the tube at 65 °C for 5 min and place it on wet ice for up to 5 min. Add to the reaction mixture 2 μL of 5 $\times$ first-strand buffer, 0.5 μL of 0.1 M DTT and 0.5 μL SuperScript III Reverse Transcriptase (see Note 35), mix by pipetting (the total volume now is 10 μL). Incubate the reaction at 50 °C for 50 min. To terminate the reaction, heat it at 70 °C for 15 min. Use the RT reaction directly as a template in the following PCR step. If not used immediately, the RT reaction should be stored at –20 °C.
5. Radioactive PCR is performed using either a trace amount of [α -P³²]-dATP or a 5'-end P³²-labeled primer. To label the primer, assemble a T4 polynucleotide kinase (PNK) reaction (all the components except the enzyme) in a 0.2 mL thin-walled tube. The volume of a PNK reaction depends on the amount of a labeled primer required for PCR amplification (see Note 36). Incubate the reaction mixture at 94 °C for 3 min and place it on wet ice for 5 min. Briefly spin the tube before opening it and adding T4 polynucleotide kinase to the reaction mixture (see Note 37). Pipet the reaction mixture up and down several times. Close the tube and return it for incubation at 37 °C for 30 min to 1 h. Inactivate the enzyme by heating the reaction at 65 °C for 20 min (see Note 38).
6. To remove unincorporated [γ -³²P]-ATP from the PNK reaction mixture use a Micro Bio-Spin column. Start with spinning the column at 1000 $\times g$ for 2 min at room temperature to remove the column packaging buffer. Next, exchange water

³².To amplify endogenous *SMN* transcripts we generally use 1.5 μg of total RNA per 10 μL of RT reaction. To analyze splicing of transcripts generated from the p*SMN2* *I6* minigene, the amount of total RNA can be decreased. For example, we use 0.5 μg of total RNA per 10 μL of an RT reaction. If the amount of recovered total RNA is small, either reduce the amount of RNA used per 10 μL of an RT reaction or decrease the volume of an RT reaction to 5 μL .

³³.If the total volume of an RT reaction is 5 μL , combine the RNA and water in a total volume of 2.75 μL . Decrease the volume of all other components, except SuperScript III enzyme (see Note 35), two times.

³⁴.We also used gene-specific primers to generate cDNA. When using a gene-specific primer, substitute 0.5 μL of Oligo(dT)_{12–18} with 0.5 μL of 2 μM primer. In addition, perform the reaction at 55 °C instead of at 50 °C.

³⁵.If the total volume of an RT reaction is 5 μL , use 0.5 μL of SuperScript III enzyme diluted 2 times immediately before usage (e.g., using RNase-free water).

³⁶.For example, to label 25 pmol of a primer, combine 16.5 μL water, 4 μL of 10 $\times$ T4 PNK buffer, 2.5 μL of 10 μM primer, and 16 μL [γ -³²P]-ATP. After the enzyme addition (see below), 1 μL of this reaction will contain 0.625 pmol of the labeled primer. Maintain the volume of a PNK reaction as small as reasonably possible, since after desalting the reaction will be used directly as a “primer source” for PCR.

³⁷.We use 1.0 μL of the enzyme per 40 μL of a PNK reaction.

³⁸.As an alternative, a PNK reaction can be terminated using phenol-chloroform extraction, particularly when labelling is done in a large volume reaction (100 μL and more). For this add phenol-chloroform mixture to the reaction (The volume of phenol-chloroform mixture should be equal to the volume of the reaction.) and vortex. Spin the mixture at 13,000 rpm in a microcentrifuge for 5 min at room temperature. The mixture will separate into lower organic and upper aqueous phases. Transfer ~90% of aqueous phase to a fresh 1.5 mL microcentrifuge tube. This aqueous phase is now ready to be loaded on a desalting Micro Bio-Spin column(s) for removing unincorporated [γ -P³²]-ATP.

for Tris buffer in which the gel in the column is suspended. To do so, add 500 μL of ultrapure water to the column and spin it at $1000 \times g$ for 1 min at room temperature. Discard the flow-through. Repeat the step of adding water and spinning the column three more times. Next, place the column in a clean 1.5 mL microcentrifuge tube and add water (*see* Note 39) directly to the center of the column. Spin the column for 4 min at $1000 \times g$, repeat this step of adding water and spinning the column until the volume of the eluate is equal to the volume loaded on the column (*see* Note 39). Place the column in a clean 1.5 mL microcentrifuge tube. Carefully apply the heat-inactivated PNK reaction from Subheading 3.3, **step 5** (also *see* Note 38) directly to the center of the column. Spin the column at $1000 \times g$ for 4 min at room temperature. The eluted 5'-end-labeled primer is now in water and is ready to be used in PCR (*see* Note 40).

7. When using the 5'-end labeled primer, for a 50 μL PCR reaction combine the required volume of water, 5 μL of $10\times$ *Taq* reaction buffer, 3 μL of 25 mM MgCl_2 , 1 μL of dNTPs, 1 μL (10 pmol) of 10 μM forward primer (*see* Note 3), 0.5 μL (5 pmol) of 10 μM unlabeled reverse primer (*see* Note 3), the PNK reaction from Subheading 3.3, **step 6** (the volume used should contain 5 pmol of the ^{32}P -labeled reverse primer, *see* Note 36), 4 μL of the RT reaction from Subheading 3.3, **step 4** and 0.5 μL of *Taq* DNA polymerase. Mix all the reaction components by pipetting up and down. Place the tube (0.2 mL thin-walled) with the reaction mixture in a Thermocycler (*see* Note 41).
8. When using trace amount of radioactivity, for a 50 μL PCR reaction combine 34.2 μL of water, 5 μL of $10\times$ *Taq* reaction buffer, 3 μL of 25 mM MgCl_2 , 1 μL of dNTPs, 1 μL (10 pmol) of 10 μM forward primer (*see* Note 3), 1 μL (10 pmol) of 10 μM reverse primer (*see* Note 3), 4 μL of RT reaction from Subheading 3.3, **step 4**, 0.3 μL of $[\alpha\text{-}^{32}\text{P}]\text{-dATP}$ and 0.5 μL of *Taq* DNA polymerase. Mix all the reaction components by pipetting up and down. Place the tube (0.2 mL thin-walled) with the reaction mixture in a Thermocycler (*see* Note 41).
9. PCR profile we use consists of initial denaturation at 95 $^{\circ}\text{C}$ for 3 min, 16 to 22 cycle of amplification and a final extension at 68 $^{\circ}\text{C}$ for 7 min. The annealing temperature depends on a primer pair we use for PCR, the extension time, on the size of the expected PCR products (*see* Note 3).
10. To distinguish between splice products generated from the endogenous *SMN1* and *SMN2* genes, perform PCR in the presence of trace amount of radioactivity (*see* Subheading 3.3, **step 8** and Note 3). Each sample should be amplified in a minimum 25 μL reaction. If 25 μL volume is used, after PCR is complete, transfer the reaction to a 1.5 mL microcentrifuge tube, add to the sample 75 μL

³⁹The volume of water used at this step should be equal to the volume of a PNK reaction that will be subjected to cleanup. The recommended sample application volume should not exceed 75 μL . Therefore, if primer labelling was done in more than a 75 μL PNK reaction, split the reaction between multiple columns accordingly.

⁴⁰Due to radiolysis, we do not recommend storing the labeled primer. It should be used as soon as possible.

⁴¹The volume of a radioactive PCR reaction for one sample can be decreased to 10 μL , since only 0.5 to 2 μL of the reaction are needed for analysis. The relative amount of an unlabeled vs end-labeled primer can be adjusted if the signal in an image is too strong (*see* Subheading 3.3, **step 14**).

of water, 10 μ L of 3 M potassium acetate (pH 5.5), 0.5 μ L of GlycoBlue and 110 μ L of phenol-chloroform and vortex. Spin the mixture at 13000 rpm for 5 min at room temperature. The mixture will separate into lower organic and upper aqueous phases. Transfer 96 μ L of aqueous phase to a fresh 1.5 mL microcentrifuge tube and precipitate DNA similarly as described for total RNA precipitation (*see* Subheading 3.3, **step 3**), except use 2 $\times$ volumes of ice-cold ethanol. Dissolve DNA palette in water and use the entire sample for overnight DdeI digestion (in a 50 or 100 μ L reaction volume). Next day stop the restriction digestion by phenol:chloroform extraction followed by ethanol precipitation (*see* above). Dissolved DNA palette in 8 μ L of water and use for analysis.

11. To analyze the splicing pattern of exon 7, directly load up to 2 μ L of PCR sample (from Subheading 3.3, **step 7** or **8**) or 2 μ L of the restriction digestion sample (from Subheading 3.3, **step 10**) mixed with 6X DNA loading buffer, on a native polyacrylamide gel (medium size). We routinely use 5% or 6% native polyacrylamide gels (*see* Note 6).
12. Run gel electrophoresis in 1 $\times$ TBE running buffer at ~220 V at room temperature. Stop the run based on the size of PCR products of interest and the desired degree of their separation (*see* Note 42). Upon electrophoresis completion, transfer the gel to chromatography paper and cover it with plastic wrap (*see* Note 43).
13. Dry the gel using Gel Dryer apparatus at 80 $^{\circ}$ C. Expose the dried gel to a phosphorimager screen. The duration of exposure varies. For best results determine what time is suitable for your experiments.
14. Analyze and quantify the results using an Image Reader FLA- 5000 and Multi Gauge software.

3.4 Western Blotting

1. Spin ~3/4 of the cell suspension collected at Subheading 3.1.1, **step 4** at 2000 $\times g$ for 1 min at 4 $^{\circ}$ C and remove the supernatant. Repeat the spin briefly and remove as much of residual DPBS as possible by pipetting. Take care not to disturb the cell pellet. Immediately resuspend the pellet in ice-cold RIPA buffer supplemented with Halt Protease Inhibitor Single-Use cocktail (use ~2 $\times$ volumes of cell pellet, *see* Note 7). Incubate the suspension on wet ice for 30 min gently mixing it from time to time. Spin the lysate at 13,000 rpm for 15 min at 4 $^{\circ}$ C to remove cell debris. Collect the supernatant and measure protein concentration

42. For example, if using a 5% gel, ~1 h 30 min run at 220 V is sufficient enough to separate *SMN* exon7-included and exon 7-skipped products amplified with the majority of primer pairs annealing to exon 6 and exon 8. Use the position of Xylene Cyanol and Bromophenol Blue in the gel for guidance. For example, in a 5% native polyacrylamide gel Xylene Cyanol and Bromophenol Blue migrate with a 260 bp and 65 bp band, respectively (53).

43. To transfer a gel to a piece of paper, first separate the plates (the gel will remain attached to a larger plate if a smaller plate is Sigmacote-treated). Tilt the plate with the gel vertically and tap it gently on a paper towel to remove the excess of liquid from the gel. Return the plate on the bench with the gel facing up and cover the gel with a piece of chromatography paper. 5% and 6% gels will stick to the paper. Lift the paper with the attached gel from the plate and cover the gel with a plastic wrap. Cut excess of paper and plastic (to the size of the gel). Now you have a "sandwich" consisting of the piece of paper at the bottom, the gel in the middle and a plastic wrap on top, ready to be dried.

using either BCA protein assay kit or Bio-Rad protein assay dye reagent concentrate.

2. Mix the required volume of cell lysate (*see* Note 44) with the equal volume of 2× Laemmli Sample Buffer (*see* Note 8), boil the sample for 5 min and spin it at 13,000 rpm for 5 min at room temperature. Load the sample and a prestained protein marker on a SDS-PAGE gel (mini size) and proceed with electrophoresis to separate proteins. We routinely use 10% and 12% gels (*see* Note 10).
3. Initially run gel electrophoresis at 50 V using 1× Tris-glycine-SDS running buffer. As soon as the prestained protein standard moves into the separating gel (*see* Note 45), increase the voltage to 100 V.
4. Upon electrophoresis completion transfer proteins from the gel to a PVDF membrane using a Trans-blot Turbo RTA Transfer Kit and a Trans-blot Turbo Transfer Cell (*see* Note 46).
5. Block the PVDF membrane in Blocking solution (50 mL) at either room temperature for 1 h or at 4 °C overnight using a rocking platform or a shaker (*see* Note 47).
6. Transfer the membrane into the primary antibody solution (mouse monoclonal anti-SMN, 1:5000 dilution). Incubate the membrane with shaking at room temperature for 1 h using a rocking platform (*see* Note 47).
7. Wash the membrane three times with 1× TBST, 15 min each (*see* Note 48).
8. Incubate the membrane with secondary antibody (HRP- conjugated anti-mouse, 1:5000 dilution) with shaking at room temperature for 1 h using a rocking platform (*see* Note 47).
9. Wash the membrane three times in 1× TBST, 15 min each wash (*see* Note 48).
10. To detect the protein signal, develop the blot using either SuperSignal West Dura Extended Duration Substrate or SuperSignal West Femto Maximum Sensitivity Substrate at room temperature for 5 min as instructed by the manufacturer.
11. Scan the membrane using a UVP BioSpectrum AC Imaging System.
12. To reprobe the membrane for another protein (e.g., β -actin that would serve as a loading control, *see* Note 49), strip the membrane at room temperature for 15 min using Western Blot Stripping Buffer (*see* Note 50).

⁴⁴. Load 10 to 20 μ g of protein lysate per well on a SDS-PAGE gel.

⁴⁵. Prestained protein standard appears as a single band as it runs through a stacking gel. As soon as it crosses into a separating gel, prestained protein standard appears as a set of bands that separate more and more as the standard continues to move through the gel.

⁴⁶. Follow the manufacturer's recommendations in preparing the components and assembling them in a "transfer sandwich." We find that the preprogrammed (LIST) protocol "1.5 mm Gel" works well for efficient transfer of proteins from 1.5 mm thick gels.

⁴⁷. We find that membrane blocking and subsequent incubation with primary and secondary antibodies can also be done for 30 min at 37 °C.

⁴⁸. Use ~200 mL 1× TBST for each washing step.

⁴⁹. For β -actin use rabbit polyclonal anti- β -actin antibody (1:2000 dilution) and HRP-conjugated anti-rabbit donkey antibody (1:2000 dilution).

⁵⁰. Western Blot Stripping Buffer can be reused multiple times. As it gets "older" increase the duration of stripping.

13. Wash the membrane multiple times with 1× TBST, 2 to 5 min each wash, to get rid of any traces of stripping buffer.
14. Repeat **steps 5** through **11** of this section.

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