Published in final edited form as:

Methods Mol Biol. 2015 ; 1297: 187–196. doi:10.1007/978-1-4939-2562-9_13.

A High-Throughput Screening Assay for the Functional Delivery of Splice-Switching Oligonucleotides in Human Melanoma Cells

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Abstract

Since the conception of RNA nanotechnology (Cell, 94:147, 1998), there has been tremendous interest in its application for the functional delivery of RNA into cells. Splice-switching oligonucleotides (SSOs) are an emerging antisense drug class with the ability to therapeutically modify gene expression. A wide variety of chemical modifications have been devised to try to increase the activity and stability of SSOs. Also, as with most nucleic acid therapeutics, delivery into the cell is the major hurdle for in vivo and clinical applications. As a result, various RNA nanoparticles are being constructed for targeted delivery of therapeutics. However, it is difficult to find a practical assay to measure splice-switching activity. Here, we describe a model delivery system that can be used as a convenient, high-throughput assay to quantitatively measure the functional delivery and splicing redirection in a live human melanoma cell line.

Keywords

Transfection; Alternative splicing; Splice-switching oligonucleotides (SSOs); Delivery; Nanomaterials; Luciferase

1 Introduction

Antisense oligonucleotides show great therapeutic promise, in particular a class of antisense oligos called splice-switching oligo-nucleotides (SSOs). This is illustrated by the large gap between the number of gene products and the number of human genes which is largely due to alternative splicing. In fact, over 90 % of multiexon genes exhibit splicing variants [1]. Alternative splicing is also prevalent in genetic diseases with up to 50 % of all genetic mutation in disease affecting mRNA splicing [2]. Therefore, SSOs have the ability to alter gene expression through splicing and possibly correct aberrant splicing patterns in genetic disorders rather than only knocking down protein expression as with siRNA. In particular, SSOs have shown great success to alter the splicing patterns of Bcl proteins in cancer therapy and as a possible therapeutic for genetic disorders such as Duchenne's muscular dystrophy [3–5]. Modification of splicing by SSOs is achieved by binding to a pre-mRNA target involved in splicing, thus forming a duplex and preventing the access of splicing factors to the target site.

A variety of chemical base modifications have been utilized in attempts to increase the stability, efficacy, potency, binding affinity, and uptake of SSOs. Unlike other antisense

RNA, which relies on RNase H-mediated cleavage of the mRNA target, SSOs require that the RNA/SSO complex remain intact. This is most commonly achieved through base modifications that do not support RNase H cleavage of the target, the most common being 2'-O-Methyl (2'-OMe) [6]. However, a variety of other modifications can also be used to block RNase H activity such as phosphorodiamidate morpholinos, locked nucleic acids, peptide nucleic acids, and 2'-O-methoxyethyl. On top of granting the SSOs RNase H resistance, all of the above modifications are also known to increase the binding affinity of the oligo to its target [7]. Most SSOs also have a chemically modified backbone in which one of the oxygens in the phosphodiester bond is replaced with sulfur. This phosphorothioate (PS) backbone modification provides resistance to nuclease degradation, a common problem with therapeutic RNA, therefore providing higher bioavailability [8]. Chemical modification may also affect the uptake of SSOs into cells. For example, phosphorothioate modifications have been shown to increase the uptake of SSOs via absorption by cell surface proteins, while phosphorodiamidate morpholino and other modifications leading to non-charged oligos may prevent the SSO from interacting with the cell surface and prevent uptake [6].

SSOs alone do not have the penetrating power to pass through the cell membrane and the nuclear envelope to reach the site of pre-mRNA in the nucleus. RNA nanotechnology is an emerging field stemming from the discovery that RNA nanoparticles can be assembled from reengineered RNA fragments via hand-in-hand interactions using bottom-up assembly [9]. The field is gaining momentum rapidly and has clear advantages over other traditional nano-delivery systems [10]. RNA nanotechnology is therefore poised to make a tremendous impact on the more challenging problem of SSO delivery. With such an overabundance of possible approaches to constructing RNA/SSO nanoparticles, there is a need for a simple, efficient, and high-throughput assay to screen for functionality and delivery efficiency. This chapter outlines the protocol for such a system. In this well-characterized, genetically engineered human melanoma cell line the successful functional delivery of a SSO results in the corrected splicing of an aberrantly inserted human β -globin intron in a luciferase reporter gene (Fig. 1) [11]. Thus, delivery of SSO results in the upregulated expression of functional luciferase that can easily be measured using a luminometer. For a more fine-tuned measurement of SSO delivery, an RT-PCR assay can easily be performed to confirm the SSO specific changes in expression as a function of the delivered RNA nanoparticle. This new molecular cell biology technique is amenable to high-throughput screening and a variety of potential applications [12–15] and represents an exciting new tool in the advancement and study of RNA nanotechnology.

2 Materials

2.1 A375 pLuc Cell Culture and SSO Transfection

1. A375/pLuc cell line gifted by Dr. RL Juliano of the UNC School of Pharmacy, and constructed as described by Kang S, et al. [11].
2. Cell culture medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS and 1 % penicillin–streptomycin–neomycin solution (Sigma).

3. Transfection medium: indicator-free DMEM.
4. Phosphate buffered saline (PBS).
5. 96-well cell culture plate.
6. Trypan blue solution 0.4 % (Sigma).
7. ON-705 SSO with PS and 2'-OMe modifications: 3'-CCUCUUACCUCAGUUACA-5' (IDT) (*see* Note 1).
8. Lipofectamine 2000 (Invitrogen).

2.2 Luciferase Assay

1. Opaque 96-well plate.
2. Luciferase Cell Culture Lysis 5× Reagent (Promega) (*see* Note 2).
3. Luciferase Assay system (Promega).
4. SpectraMax luminometer (Molecular Devices) (*see* Note 3).

2.3 RT-PCR Assay

1. 6-well cell culture plate.
2. TRIzol (Life Technologies) (*see* Note 4).
3. RNeasy Mini Kit (Qiagen).
4. β-mercaptoethanol.
5. 100 % ethanol.
6. 20-gauge needle and syringe (*see* Note 5).
7. Microcentrifuge.
8. RNase-Free DNase Set (Qiagen).
9. AMV Reverse Transcriptase (Promega).
10. RNase inhibitor, Human Placenta (New England Biolabs).
11. dNTPs (Promega) (*see* Note 6).
12. PTC-200 Thermocycler (MJ Research).

¹Spin down tube in a microcentrifuge to ensure all of the lyophilized oligo is at the bottom of the tube. Add the appropriate amount of water to make a 20 μM stock solution.

²This stock reagent needs to be diluted with water to achieve a 1× working concentration before use. The concentrated lysis reagent is very viscous, especially at low temperatures. Therefore it is recommended to let the reagent reach room temperature before pipetting and to pipette very slowly to ensure the desired amount of reagent is drawn into the pipette.

³This luminometer has a reagent injection system. This saves time and also allows each well to be read directly after injection ensuring that luciferase activity is at its peak. Reagent injection can be performed manually; however, it is important to be aware that luciferase activity will decline within minutes after addition of reagent making it difficult to read a large number of samples accurately.

⁴TRIzol is light sensitive, so be sure to store it in a dark space and to cover any container that it is stored in foil.

⁵A variety of techniques can be used to homogenize the cells before RNA isolation but we have found that this technique works well. The needles and syringe are sterilized before use by repeatedly passing 100 % ethanol through the syringe.

⁶The dNTPs come in separate tubes and therefore need to be combined to make a working dNTP stock mix. In order to make a 10 mM dNTP stock add 10 μl of each 100 mM dNTP into a tube and add 60 μl of nuclease free water.

13. PCR Tubes.
14. 50 mM MgCl₂.
15. Oligo dTVN (*see* Note 7).
16. Luciferase PCR Primers. Forward: TTGATATGTGGATTTTCGAGTCGTC.
Reverse: TGTCAATCAGAGTGCTTTTGGCG (*see* Note 8).
17. GoTaq Green Master Mix (Promega).
18. NanoDrop 2000 (Thermo Scientific).
19. 100 bp DNA ladder (New England Biolabs).

3 Methods

3.1 A375 pLuc Cell Culture and SSO Transfection (See Note 9)

1. In 200 µl of DMEM with 10 % FBS and 1 % penicillin–streptomycin–neomycin solution, seed 10,000 A375/pLuc cells per well of a 96-well plate 1 day prior to transfection. Maintain cells under standard incubation conditions: humidified, 37 °C, and 5 % CO₂.
2. Prepare two mixtures in separate microcentrifuge tubes. In the first add 2.5 µl of Lipofectamine and bring to a final volume of 125 µl with indicator-free DMEM. In the second tube add 10 µl of stock SSO solution and bring to a total volume of 125 µl.
3. After letting the tubes incubate at room temperature for 5 min they are combined and allowed to incubate for another 20 min, allowing Lipofectamine–SSO complexes to form. Dilute the contents to a final volume of 1 ml using indicator-free DMEM.
4. Aspirate the media from the plated cells, wash with PBS, and replace with 200 µl of the SSO/Lipofectamine solution.
5. Incubate the cells under standard conditions for 8 h. Aspirate off the transfection media and add 200 µl of DMEM with 10 % FBS and 1 % penicillin–streptomycin–neomycin solution. Allow the cells to recover for 36 h before moving on to the luciferase assay in Subheading 3.2.

3.2 Luciferase Assay

1. Aspirate the cell culture media from the treated cells and wash each well with 200 µl of PBS.

⁷Spin down tube to ensure that all of the lyophilized primer is at the bottom of the tube and add 40 µl of nuclease free water to the 10 µg of oligo to make the stock concentration of 0.25 µg/µl.

⁸Again, spin down the lyophilized primer and then add the appropriate amount of nuclease free water to make a working concentration of 20 µM.

⁹The following protocol is for making a master solution to treat 5 wells of the 96-well plate with a final in well volume of 200 µl and a final in well SSO concentration of 200 nM. This protocol only goes over how to set up a positive control treatment. However, any sort of treatment complex can be tested with the SSO as long as the final in well SSO volume remains at 200 nM. 200 nM is the optimized concentration for the splicing system. Concentrations higher than 200 nM do not significantly increase RLU's.

2. Aspirate off the PBS and add 25 μ l of 1 $\times$ Luciferase Cell Culture Lysis Reagent to each well, mix by pipetting five times, and let incubate at room temperature for 15 min.
3. Transfer the 25 μ l of lysates in each well to an opaque 96-well plate.
4. To prepare the luciferase assay reagent, add the supplied Luciferase Assay Buffer to the vial containing the lyophilized Luciferase Assay Substrate.
5. Program the luminometer to inject 100 μ l of Luciferase Assay Reagent into each well and to read each well with an integration time of 20 s. The resulting reading is reported in relative light units (RLUs).
6. RLU results are plotted in an XY scatter plot as shown in Fig. 2.

3.3 RNA Isolation and RT-PCR (See Note 10)

1. Seed A375/pLuc cells in 2 ml of DMEM with 10 % FBS and 1 % penicillin–streptomycin–neomycin solution in a six well plate. Incubate cells under standard conditions until 80 % confluent.
2. Transfect cells as described above ensuring that the final SSO concentration in well is 200 nM.
3. Aspirate the media, trypsinize the cells, and transfer the cell solution to a new conical tube. Spin down the cells for 1 min at 1,000 $\times g$ and aspirate off the supernatant. Resuspend the cells in 100 μ l of TRIzol.
4. Perform the RNA isolation with DNase treatment using the Qiagen RNeasy Mini Kit and RNase-Free DNase Set according to the manufacturer's instructions. Make sure to add β -mercaptoethanol to the buffer RLT (10 μ l β -mercaptoethanol for every 1 ml RLT) prior to cell lysis to inhibit RNase activity. Also, use the 20-gauge needles to perform the homogenization step as described in the manufacturer's protocol.
5. Isolated RNA is quantified on a NanoDrop 2000 and diluted to 1 μ g/ μ l using RNase-free water.
6. For one RT reaction combine the following reagents listed below. A stock solution is recommended if several RT reactions are to be performed (see Note 11).

¹⁰RNA is notoriously unstable and prone to degradation while RNases are very stable and have high activity. Aseptic techniques should always be taken when handling RNA and gloves should be changed regularly to reduce the chances of RNase contamination. If possible chose a low traffic area away from any air vents to perform the procedure in order to further reduce the possibility for RNase contamination.

¹¹If yield is low and a 1 μ g/ μ l RNA solution could not be achieved from isolation, it is important that enough solution is added so that there is 2 μ g of total RNA in the RT reaction. The amount of RNase-free water added to the solution can be adjusted so that the total volume remains 20 μ l.

Reagents	Reaction volume (μl)
5× AMV Buffer	4.0
50 mM MgCl ₂	2.0
10 mM dNTPs	2.0
RNase inhibitor	1.0
AMV RT	1.0
Oligo dTVN	2.0
RNase-free water	6.0
Isolated RNA	2.0
Total volume	20.0

7. Run samples on the thermocycler using the following program: Step 1: 42 °C for 25 min Step 2: 99 °C for 5 min Step 3: hold at 4 °C.
8. After RT reaction dilute the cDNA with 20 μl of nuclease-free water.
9. Set up the one PCR reaction by combining the following reagents. A stock solution is recommended if several PCR reactions are to be performed.

Reagents	Reaction volume (μl)
GoTaq Green Master Mix (2×)	12.5
Forward Luciferase Primer	0.5
Reverse Luciferase Primer	0.5
cDNA	2.0
Nuclease-free water	9.5
Total volume	25.0

10. Run samples on the thermocycler using the following program:
 - Step 1: 95 °C for 5 min
 - Step 2: 95 °C for 45 s
 - Step 3: 53 °C for 45 s
 - Step 4: 72 °C for 1 min and 45 s
 - Step 5: Go to Step 2, repeat 32 times
 - Step 6: 72 °C for 5 min
 - Step 7: hold at 4 °C
11. Run samples on a 2 % agarose gel with a 100 bp ladder as shown in Fig. 3. Use densitometry using ImageJ software to further quantify splicing correction as shown in Fig. 4.

References

1. Wang ET, Sandberg R, Luo S, et al. Alternative isoform regulation in human tissue transcriptomes. *Nature*. 2008; 456:470–476. [PubMed: 18978772]
2. Lopez-Bigas N, Audit B, Ouzounis C, et al. Are splicing mutations the most frequent cause of hereditary disease? *FEBS Lett*. 2005; 579:1900–1903. [PubMed: 15792793]
3. Bauman J, Li S, Yang A, et al. Anti-tumor activity of splice-switching oligonucleotides. *Nucleic Acids Res*. 2010; 38(22):8348–8356. [PubMed: 20719743]
4. van Deutekom J, Janson A, Ginjaar I, et al. Local dystrophin restoration with anti-sense oligonucleotide. *N Engl J Med*. 2007; 357:2677–2686. [PubMed: 18160687]
5. Mann C, Homeyman K, Cheng A, et al. Antisense-induced exon skipping and synthesis of dystrophin in the mdx mouse. *Proc Natl Acad Sci U S A*. 2000; 98(1):42–47.
6. Chan J, Lim S, Wong W. Antisense oligonucleotides: from design to therapeutic application. *Clin Exp Pharmacol Physiol*. 2006; 33:533–540. [PubMed: 16700890]
7. Bauman J, Jearawiriyapaisarn N, Kole R. Therapeutic potential of splice-switching oligonucleotides. *Oligonucleotides*. 2009; 19(1):1–13. [PubMed: 19125639]
8. Kurreck J. Antisense technologies: improvement through novel chemical modifications. *Eur J Biochem*. 2003; 270:1628–1644. [PubMed: 12694176]
9. Guo P, Zhang C, Chen C, et al. Inter- RNA interaction of phage ϕ 29 pRNA to form a hexameric complex for viral DNA transportation. *Mol Cell*. 1998; 2:149–155. [PubMed: 9702202]
10. Shu Y, Pi F, Sharma A, et al. Stable RNA nanoparticles as potential new generation drugs for cancer therapy. *Adv Drug Deliv Rev*. 2014; 66:74–89. [PubMed: 24270010]
11. Kang S, Cho M, Kole R. Up-regulation of luciferase gene expression with antisense oligonucleotides: implications and applications in functional assay development. *Biochemistry*. 1998; 37(18):6235–6239. [PubMed: 9572837]
12. Mäe M, Samir E, Lundin P, et al. A stearylated CPP for delivery of splice correcting oligonucleotides using a non-covalent co-incubation strategy. *J Control Release*. 2009; 134:221–227. [PubMed: 19105971]
13. Kotula J, Pratico E, Ming X, et al. Aptamer-mediated delivery of splice-switching oligonucleotides to the nuclei of cancer cells. *Nucleic Acid Ther*. 2012; 22:187–195. [PubMed: 22703281]
14. Abes S, Williams D, Prevot P, et al. Endosome trapping limits the efficiency of splicing correction by PNA-oligolysine conjugates. *J Control Release*. 2006; 110:595–604. [PubMed: 16377019]
15. El-Andaloussi S, Johansson H, Lundberg P, et al. Induction of splice correction by cell-penetrating peptide nucleic acids. *J Gene Med*. 2006; 8:1262–1273. [PubMed: 16900561]

Fig. 1. Schematic illustrating how SSO delivery blocks the aberrant splice site and therefore inducing functional luciferase expression

Fig. 2. Scatter plot showing the luminescence of untreated A375/pLuc cells and those treated with 200 nM of SSO. *** p -value <0.0001

Fig. 3. Results of RT-PCR analysis of luciferase mRNA splicing correction in A375/pLuc cells untreated and treated with SSO. Aberrantly spliced mRNA containing an intron shows a band at 268 bp while the correctly spliced mRNA shows a band at 142 bp. L = ladder. - = non-treatment control. + = cells treated with SSO

Fig. 4.

Densitometry was performed using ImageJ software on the image in Fig. 3 to quantify splicing correction. The percentage of correctly splicing band intensity of total band intensity in each lane is shown. In untreated A375/pLuc cells, the correctly spliced band accounted for approximately 3 % of total intensity. However, in the SSO treated A375/pLuc cells, the correct band accounted for approximately 15 % of total intensity