Chapter 5

How to Design U1 snRNA Molecules for Splicing Rescue

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Abstract

Mutations affecting constitutive splice donor sites (5′ss) are among the most frequent genetic defects that disrupt the normal splicing process. Pre-mRNA splicing requires the correct identification of a number of *cis*-acting elements in an ordered fashion. By disrupting the complementarity of the 5′ss with the endogenous small nuclear RNA U1 (U1 snRNA), the key component of the spliceosomal U1 ribonucleoprotein, 5′ss mutations may result in exon skipping, intron retention or activation of cryptic splice sites. Engineered modification of the U1 snRNA seemed to be a logical method to overcome the effect of those mutations. In fact, over the last years, a number of in vitro studies on the use of those modified U1 snRNAs to correct a variety of splicing defects have demonstrated the feasibility of this approach. Furthermore, recent reports on its applicability in vivo are adding up to the principle that engineered modification of U1 snRNAs represents a valuable approach and prompting further studies to demonstrate the clinical translatability of this strategy.

Here, we outline the design and generation of U1 snRNAs with different degrees of complementarity to mutated 5′ss. Using the *HGSNAT* gene as an example, we describe the methods for a proper evaluation of their efficacy in vitro, taking advantage of our experience to share a number of tips on how to design U1 snRNA molecules for splicing rescue.

Key words U1 snRNA-based therapy, Splicing modulation, 5′ss mutations, Aberrant exon skipping, Modified U1 snRNA, Mucopolysaccharidosis IIIC

1 Introduction

The U1 small nuclear ribonucleoprotein (U1 snRNP) is a key molecule involved in an early event of the splicing process. Like other snRNPs involved in the overall splicing regulation process, it contains a small RNA complexed with several proteins, namely seven Smith antigen (Sm) proteins and three U1-specific proteins (U1A, U1C, and U170K) [1]. U1 snRNA, the RNA component of the U1 snRNP is a 164 nucleotides-long molecule whose 5′ end interacts by complementarity with the 5′ splice donor site (5′ss). That interaction between the single stranded 5′ tail of the U1 snRNA molecule and the moderately conserved stretch of nucleotides that constitutes the 5′ss (CAG/GURAGU, where R is a

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purine) marks the exon-intron boundary and initiates spliceosome assembly [2]. About 40%, 22%, and 5% of normal 5′ss contain two, three, or four mismatches towards the U1 snRNA, respectively [3, 4]. This variable degree of degeneration is among the major factors that significantly contribute to hinder a clear prediction of the effect of mutations flanking the canonical GU site. Furthermore, there is a number of additional elements, which may influence the splice site selection and need to be taken into account such as splicing silencer and enhancer motifs, the presence of alternative splice sites, secondary structures, and regulatory proteins [5]. Therefore, a straightforward prediction of the effect of mutations flanking the canonical GU site without a direct assessment of the mature mRNA produced can be quite challenging. Interestingly, however, it is also the variable degree of degeneration of 5′ss and the surprising heterogeneity existing among human spliceosomal snRNA, which allows for splicing correction using modified exogenous U1 snRNAs.

Overall, the rationale on the use of modified U1 snRNAs to correct splicing defects is as simple as it can be: as 5′ss mutations alter the 5′ss recognition by the endogenous U1 snRNA, exogenous U1 snRNAs may be engineered through complementary base pairing in order to correctly recognize the mutated allele and initiate spliceosome assembly, thus suppressing the mutation effect.

So far, the effects of modified U1 snRNAs have been tested in vitro in a number of cellular platforms from patient-derived cells to model cell lines overexpressing the splicing defects under study, and their potential to either fully or partially correct those mutations was demonstrated for a number of different diseases [5, 6]. Importantly, the application of this sort of modified U1 snRNAs in animal models has also been addressed in recent studies, with a few promising results reported to date [7–10] (*see* **Note 1**).

Globally, mutations affecting constitutive 5′ss represent roughly 8% of all known genetic disease-causing variants. Their pathogenicity derives from the reduced complementarity of the U1 snRNA to the 5′ss. 5′ss mutations mostly result in exon skipping but their effect over splicing may vary. Currently, there are a number of in silico tools that may help predict disease-causing effects, but cDNA analysis remains mandatory for a proper assessment of their consequence over splicing. For example, mutations affecting RNA splicing represent more than 20% of the mutant alleles in Mucopolysaccharidosis type IIIC (MPS IIIC; *HGSNAT* gene), a rare lysosomal storage disorder that causes severe neurodegeneration. Many of these mutations are located in the conserved splice donor or acceptor sites, while few are found in the nearby nucleotides. For three mutations that affect the donor site, we have previously developed different modified U1 snRNAs with compensatory changes that may allow for proper recognition of the mutated 5′ss, in an attempt to rescue the normal splicing process.

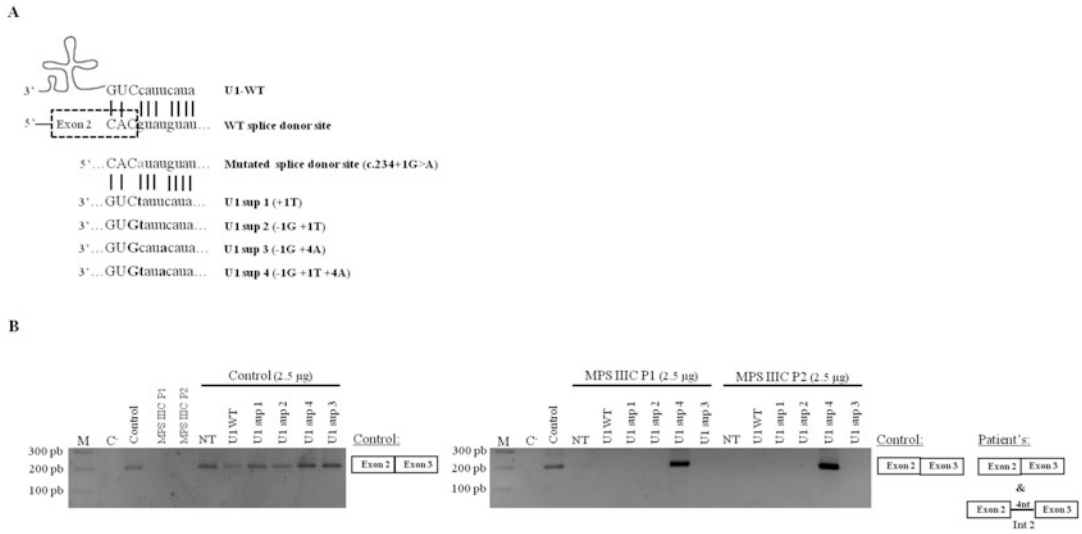

Fig. 1 Modified U1 snRNA therapeutic approach to correct the pathogenic effect of a 5′ splice site mutation on the *HGSNAT* gene. (**a**) Schematic illustration of base pairing between the wild-type U1 (U1-WT) and the 5′ss of wild-type and mutant exon 2 of the *HGSNAT* gene. The mutation position in the 5′ss is marked in grey and it is in italics. The different U1 snRNAs used for the mutated 5′ss of *HGSNAT* (designated as U1-sup, for suppressor) are also shown. The U1 sequence modifications are illustrated in bold. (**b**) RT-PCR analysis of the endogenous splicing pattern of control and MPS IIIC patients derived fibroblasts after transfection with different U1 isoforms. The constitutive splicing of exon 2 of the *HGSNAT gene* was not altered in control fibroblasts after overexpression of U1-WT or any of the modified U1 constructs. In the MPS IIIC patients 1 (MPS IIIC P1) and 2 (MPS IIIC P2), bearing the homozygous mutation c.234+1G>A, only the fully adapted U1 (U1-sup4) resulted in partial correction of exon 2 skipping

For the c.234+1G>A mutation, a totally complementary U1 snRNA allowed for partial correction of exon 2 aberrant splicing in patients' fibroblasts (Fig. 1) [11]. Here, we take advantage of our experience on the development of modified U1 snRNAs to compensate for those *HGSNAT* mutations, to present a practical overview on how to design U1 snRNA molecules for splicing rescue.

In summary, we present an overview of the experimental design for in vitro testing the potential of modified U1 snRNA vectors to correct aberrant splicing caused by 5′ss mutations. Briefly, we show: (a) how to design in silico U1's with different degrees of complementarity to each mutated 5′ss by introducing a number of sequence changes, and (b) how the different U1 vectors harboring those alterations are obtained by site-directed mutagenesis of the original wild-type (WT) human U1 snRNA-harboring pG3U1 vector [12], a derivative of pHU1 [13]. We also describe how these molecules are transfected into patients' fibroblasts and how their effectiveness on splicing redirection can be assessed by post-transfection cDNA analysis and sequencing. Finally, we elaborate on the relevance of further addressing the treatment's effect at protein level.

2 Materials

2.1 Generating Modified U1 snRNA Vectors Adapted to the 5′ss of Interest

1. The *Homo sapiens* U1 snRNA gene sequence is required to design primers for site-directed mutagenesis PCR and can be found in the Ensembl database (ENSG00000104852).
2. The sequence of the 5′ss of interest for splicing rescue can be found in Ensembl or other reference sequence databases (in this particular chapter we used the *Homo sapiens HGSNAT* gene sequence, ENSG00000165102).
3. pG3U1 vector [12] a derivative of pHU1 [13] (*see* **Note 2**).
4. Sense and antisense mutagenic primers.
5. PCR mutagenesis kit.
6. PCR thermocycler.
7. Chemically *Escherichia coli* competent cells (Homemade or commercial; usually are included in the PCR mutagenesis kits).
8. Water bath.
9. Thermomixer.
10. Ice.
11. Super optimal broth with catabolite repression (SOC) medium (commercially available).
12. Luria-Bertani (LB) agar medium (commercially available; sterilize by autoclaving) plates with selection antibiotic (100 μg/mL, ampicillin; *see* **Note 3**).
13. Sterile bacterial cell spreaders.
14. Plasmid DNA miniprep purification kit.
15. LB liquid medium (commercially available; sterilize by autoclaving).
16. Ampicillin.
17. 15 mL conical centrifuge tubes.
18. Sterile tips.
19. Orbital shaking incubator.
20. pG3U1 forward primer (U1-seq Fw—5′ CACGAAG GAGTTCCCGTG 3′).
21. Sterile flasks (1 L).
22. Endotoxin-free maxiprep plasmid DNA purification kit.
23. 40% Glycerol (sterilize by autoclaving).
24. 2 mL polypropylene conical tubes.

2.2 In Vitro Therapeutic Evaluation of Modified U1 snRNA Vectors in Human Fibroblasts

2.2.1 Transfection of Modified U1 snRNA Vectors in Human Fibroblasts

1. Human Dermal Fibroblasts from patients harboring the mutation under analysis (e.g. fibroblasts from patients' with MPS IIIC, carrying the c.234+1G>A mutation in homozigosity) and WT Human Dermal Fibroblasts to use as control.
2. Dulbecco's Modified Eagle's Medium (DMEM) + Glutamax supplemented with 10% Fetal Bovine Serum (FBS), 5% penicillin/streptomycin (PenStrep) antibiotics, and 5% amphotericin B (Fungizone®).
3. Phosphate buffered saline 1x (PBS).
4. Trypsin-EDTA.
5. CO_2 incubator.
6. 15 mL conical centrifuge tubes.
7. Refrigerated centrifuge.
8. Neubauer chamber (hemocytometer).
9. Inverted Microscope.
10. Hand cell counter.
11. T-75 cm^2 cell culture flasks.
12. 6-well cell culture plates.
13. Opti-MEM™ Reduced Serum Medium.
14. Transfection reagent.
15. 1.5 and 2 mL polypropylene conical tubes.
16. Modified U1 snRNA constructs (*see* Subheading 2.1).

2.2.2 Analysis of Splicing Rescue by RT-PCR

1. RNA isolation kit.
2. Refrigerated centrifuge.
3. 1.5 mL polypropylene conical tubes.
4. Spectrophotometer for nucleic acids measurement.
5. cDNA synthesis kit.
6. Taq DNA polymerase.
7. Oligo$(dT)_{18}$ primer mix (if required).
8. Gene-specific primers (e.g. *HGSNAT* primers—Exon 2 Fw: 5′ ACATGCAGAGCTGAAGATGGA 3′; Exon 3 Rv: 5′ GATA GATCCGTGCTGGGTG 3′).
9. Ice.
10. RNase free water.
11. PCR thermocycler.
12. Agarose gel with ethidium bromide for electrophoresis.

13. DNA Ladder (molecular weight size marker).
14. UV transilluminator.
15. Sterile scalpel blades.
16. PCR products purification kits.

3 Methods

3.1 Generating the Modified U1 snRNA Vectors

To design the primers for producing the desired modified human U1 snRNA vectors, it is first necessary to know the sequences of the 5′ss under study, both WT and mutant. Then, it is necessary to analyze the complementarity of those sequences with that of U1 snRNA. Next, several modified U1 snRNA vectors can be designed and constructed to have different complementarities to the target sequences (Fig. 2). To generate those constructs, the plasmid pG3U1 [12] (kindly provided by Prof. Dr. Belén Pérez) a derivative of pHU1 [13], containing the coding sequence of the human U1 can be used as template for site-directed mutagenesis PCR reactions (*see* **Note 2**). Depending on the number of mutations to insert in the U1 snRNA vector sequence, different mutagenic primer pairs need to be designed.

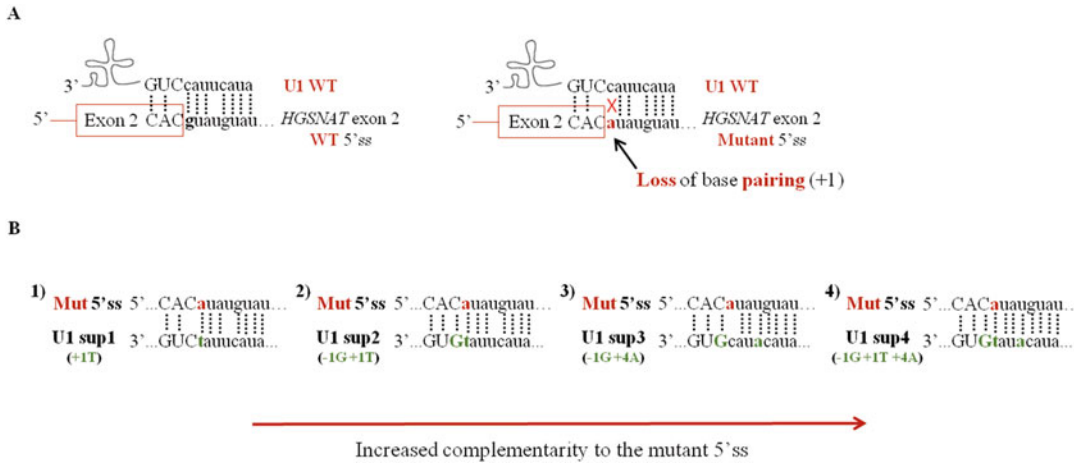

Fig. 2 Design and construction of modified U1 snRNA vectors. (**a**) Schematic representation of base pair interactions between the U1 snRNA and the wild-type and mutant 5′ss of *HGSNAT* exon 2, respectively. (**b**) Illustration of the strategy followed to increase the complementarity of U1 snRNA with the mutated 5′ss of *HGSNAT* gene. U1 complementarity was increased stepwise, and to try to compensate for the *HGSNAT* mutation at +1 position, four different U1-adaptations were designed [U1 sup1 (+1T); U1 sup2 (−1G +1T); U1 sup3 (−1G +4A); U1 sup4 (−1G +1T +4A)]. Upper case letters show exonic nucleotides, whereas the lower case letters denote intronic nucleotides. Base pairing is indicated by vertical lines and its loss by an *X*. The mutant nucleotide is highlighted in red and the changed nucleotides in the U1 sequence are illustrated in green

3.1.1 Engineering Modified U1 snRNA Vectors Adapted to the 5′ss of Interest

1. According to the different modifications to be introduced in the U1 snRNA vector sequence, design sense and antisense primers with the desired mutation(s) to be introduced by site-directed mutagenesis (*see* **Note 4**).
2. Using the mutagenic primers, perform the site-directed mutagenesis of the WT U1 snRNA vector using the mutagenesis kit (*see* **Note 5**). Briefly, mix the U1 snRNA plasmid DNA (~40 ng) with primers, buffer, dNTPs (according to the kit), apyrogenic water, a High Fidelity Taq polymerase and subject the mixture to recommended PCR conditions from the mutagenesis kit. The number of PCR cycles varies according to the type of the desired mutation(s) (*see* **Note 6)**; and the number (*n*) of min of the PCR extension step depends on the plasmid length, *n* is calculated as 1 min/kb; *see* **Note 7**. After the PCR reaction is completed, add 1 μL (10 U) of *Dpn*I restriction enzyme to the amplified products and incubate for 1 h at 37 °C to digest the parental dsDNA.
3. Use 1–4 μL of the *Dpn*I treated DNA reaction to transform *E. coli* competent cells. Briefly, thaw on ice a 50 μL aliquot of competent cells and add 1–4 μL of the digested reaction. Swirl the tube gently to mix and incubate on ice for 30 min. In a water bath or dry thermomixer, heat pulse the tube at 42 °C for 45 s and then place the reaction tube on ice for 2 min. Add room temperature SOC medium (5× the volume of competent cells) and incubate for 1 h with shaking at 600 rpm in a dry thermomixer (*see* **Note 8**). After incubation spread the appropriate volume (*see* **Note 9**) of transformation reaction on pre-warmed (37 °C) LB-agar plates containing ampicillin (100 μg/mL) and incubate at 37 °C for 16–18 h (*see* **Note 10**).
4. To obtain plasmid DNA minipreps, prepare minicultures of selected bacterial colonies to allow their growth. Add 3 mL (*see* **Note 11**) of LB medium containing ampicillin (100 μg/mL) to a 15 mL tube and using a sterilized pipette tip pick a colony and add it into the medium by pipetting up and down (or, simply, place the pipet tip into the medium). Repeat the procedure for 3–5 colonies. Incubate the tubes in an orbital shaking incubator at 220 rpm and 37 °C for 16–18 h. To purify the plasmid DNA prepare DNA minipreps using a plasmid miniprep purification kit (*see* **Note 12**). Select the mutant (s) U1 snRNA plasmid(s) by Sanger sequencing analysis (U1-seq Fw primer) using ~100 ng of purified miniprep.
5. Once the desired modified U1 snRNA construct(s) are selected, propagate them in maxicultures to obtain a high quantity of the modified construct(s) that can be used for transfection. First, prepare a miniculture of each case according to **step 4** (*see* **Note 13**). Then add 100–150 mL of LB medium

containing ampicillin (100 μg/mL) to a sterilized flask(s) (*see* **Note 11**) and innoculate all the bacterial growth from the miniculture(s). Incubate the flask(s) in an orbital shaking incubator at 37 °C and 220 rpm for 16–18 h. Using an endotoxin-free maxiprep plasmid DNA purification kit, maxiprep the plasmid(s) containing the modified U1 snRNA construct (s) and perform its sequencing analysis for validation.

3.2 In Vitro Therapeutic Evaluation of Modified U1 snRNA Vectors in Human Fibroblasts

Even though we must always find a balance between the best possible experimental design and the resources available, adequate controls may never be forgotten. Still, there is a minimum standard for cell culture experiments that must always be met if we want to draw strong conclusions out of them. Therefore, adequate controls to the variables under test should always be included (*see* **Note 14**).

3.2.1 Modified U1 snRNA Vectors Transfection in Human Fibroblasts

1. Grow both WT control and patient fibroblasts in T-75 flasks with DMEM + Glutamax medium supplemented with 10% FBS, 5% antibiotics, and 5% amphotericin B, in an incubator at 37 °C with 95% humidity and 5% CO_2 following standard cell culture procedures.
2. On the day before transfection, detach the cells by trypsinization. Briefly, discard the growth medium and wash cells with 3 mL of PBS buffer. Then, discard the PBS and add 2 mL of trypsin-EDTA. Subsequently, incubate cells with the solution for 5 min at 37 °C. After this period, check in an inverted microscope that cells are detached and add 4 mL of fresh medium to inactivate trypsin-EDTA action.
3. Harvest the cells to a 15 mL tube and centrifuge at 500 × *g* for 5 min to eliminate any traces of trypsin.
4. Discard the supernatant and resuspend cells in 4 mL of fresh medium.
5. Count cells in suspension with an hemocytometer (Neubauer chamber). Pipette a small volume of cell suspension (approximately 15 μL) to both hemocytometer chambers and count the cells present in all four external quadrants of each chamber by observing it in an inverted microscope. Considering the dimensions of the chamber (1 mm × 1 mm × 0.1 mm), each quadrant has a total volume of 0.1 mm^3, which equals 10^{-4} mL. Therefore, the total number of cells in the original suspension can be calculated with the following equation:

$$N = \frac{\sum n}{8} \times 10^4$$

where N is the total number of cells per milliliter, n is the number of cells counted in each quadrant of the Neubauer chamber and the 10^4 factor allows for the correction of the total number of cells in 1 mL of cell suspension.

6. For modified U1 snRNA vectors transfection, seed a total of ~2.5–3 × 10^5 fibroblast cells into 6-well plates and grow cells in DMEM + Glutamax medium supplemented with 10% FBS, 5% antibiotics and 5% amphotericin B, in an incubator at 37 °C with 95% humidity and 5% CO_2.
7. On the next day (cells at 80–90% confluence), transfect the cells with quantities between 1 and 3.5 μg of the modified U1 snRNA constructs using a transfection reagent according to the manufacturer's protocol (*see* **Notes 15** and **16**).
8. 24–48 h after transfection, harvest cells by trypsinization. Discard the growth medium of each plate well and wash cells with 1 mL of PBS buffer. Discard the PBS, add 500 μL of trypsin-EDTA to each well and incubate for 5 min at 37 °C. Then, check by microscopy that cells are rounding up and add 1 mL of DMEM + Glutamax medium to inactivate trypsin-EDTA. Harvest cells to 2 mL tubes and centrifuge at 500 × *g* for 5 min at 4 °C; discard the supernatant; wash cells with 1 mL of PBS buffer and centrifuge again. Proceed to RNA extraction or store the pellet(s) at −80 °C for future use.

3.2.2 Analysis of Splicing Rescue by RT-PCR

1. Extract total RNA from the transfected human fibroblasts using a RNA extraction kit according to the manufacturer's protocol. Then, perform RNA quantification using a spectrophotometer.
2. For reverse transcription, use a cDNA synthesis kit following the manufacturer's protocol, and start with 1–2 μg of total RNA. The cDNA synthesis reaction can be stored at −20 °C or used immediately for PCR amplification.
3. Perform a PCR in standard conditions using a Taq polymerase supplemented with its buffer, dNTPs, gene-specific primers for a final concentration of 0.4 μM each (e.g. *HGSNAT* primers), 2 μL of cDNA, and RNase free water to a final volume of 50 μL.
4. To evaluate the splicing rescue, analyze the amplification products through agarose gel electrophoresis in an agarose gel stained with 5 μL of ethidium bromide (*see* **Note 17**). Choose a DNA ladder according to the size of the amplified band. After separation, visualize the gel using an UV transilluminator. As an example, Fig. 1 shows the results of the partial correction of *HGSNAT* exon 2 splicing after expression of a modified U1 snRNA (totally complementary to the 5′ss of exon 2) in patients' fibroblasts.
5. Assess the identity of the obtained band(s) by sequencing analysis (*see* **Note 18**). For this purpose, purify the PCR products directly with a PCR clean-up kit if there is only one amplified band or when multiple bands are present excise each band from the gel and purify them using a gel band

purification kit. Whatever the case, follow the indications present in the manufacturer's protocol.

6. Subject the purified bands to standard automated sequencing using gene-specific primers for the amplification (e.g. *HGSNAT* primers). Compare the obtained sequence (s) with the reference sequence of the gene of interest (retrieved from the Ensembl database) using the Clustal Omega bioinformatic tool (https://www.ebi.ac.uk/Tools/msa/clustalo/), in order to analyze the effect of the modified U1 snRNA's in rescuing the normal splicing pattern.

3.2.3 Assessment of the Effect of U1 snRNA-Induced Splicing Rescue at Protein Level

While not included in this chapter, for it is case-specific, the effect of modified U1 snRNAs-treatment at protein level is mandatory whenever we want to proceed to in vivo studies in order to address the true therapeutic potential of a given U1 snRNA molecule.

Ideally, as soon as we get an RT-PCR pattern that confirms splicing correction to some extent, and that rescue is confirmed by band excision and Sanger sequencing, the overall effect of that rescue at protein level should also be checked. There is a variety of methods we can choose in order to address this issue, from the direct quantification of enzymatic activity (whenever the gene product under analysis has a catalytic activity) to that of the protein itself (through Western blot).

Usually, the method of choice depends on two major factors: the protein itself and the assays available *in house* to assess it. Virtually every method from Western blot to immunofluorescence may be informative and provide extra support to the conclusions drawn from the RT-PCR. Therefore, as a take-home message, we would recommend that, whenever designing U1 snRNA molecules for splicing rescue, the effect should be checked not only at cDNA level, but also at protein level.

4 Notes

1. This chapter is exclusively focused on mutation-adapted U1 snRNAs. Nevertheless, it is important to refer that there is a novel, second generation, of engineered U1 snRNAs, which may be used for therapeutic purposes: the so-called Exon-Specific U1 snRNAs (ExSpeU1). These ExSpeU1s are complementary to non-conserved sequences downstream of mutant 5′ss. In theory, ExSpeU1 is expected to decrease the potential of off-target effects of U1 snRNA-based therapies, while allowing for a single ExSpeU1 to rescue multiple splicing defects that affect a single exon [4–6].
2. The pG3U1 vector [12] {Susani, 2004, TCIRG1-dependent recessive osteopetrosis: mutation analysis, functional

identification of the splicing defects, and in vitro rescue by U1 snRNA} was used, but the human U1 snRNA sequence can be cloned in other standard expression vector(s).

3. Store LB-agar plates with antibiotics at 4 °C in the dark.
4. Mutagenic primers can be designed using the web-based QuikChange Primer Design Program, available online at www.agilent.com/store/primerDesignProgram.jsp (we recommend to read the "help" section of the program). However, it is important to take into account a number of considerations:
   (a) both mutagenic primers must contain the desired mutation(s) and anneal to the same sequence on opposite strands of the plasmid;
   (b) each primer should have between 25 and 45 bases in length with a melting temperature (Tm) of ≥78 °C;
   (c) the desired mutation(s) should be located in the middle of the primer (~12–15 nucleotides of the correct sequence on both sides);
   (d) the primers should have a minimum GC content of 40% and should terminate in one or more C or G bases;
   (e) the primers do not need to be 5′ phosphorylated and purification may either be performed by liquid chromatography (HPLC) or by polyacrylamide gel electrophoresis (PAGE).
5. To modify the pG3U1 we recommend to use the QuikChange™ II mutagenesis kit (Agilent). However, other site-directed mutagenesis commercial kits can be used. The kit should be chosen according to the plasmid length and the type of mutations to introduce.
6. According to the type of mutation(s) to be inserted in the U1 snRNA WT sequence, the number of PCR cycles varies. For point mutations (1 nucleotide change) use 12 cycles; for single aminoacid changes (3 nucleotides) use 16 cycles and for multiple amino acid deletions or insertions (≥4 nucleotides) use 18 cycles.
7. The number (*n*) of min of PCR extension step recommended is usually 1 min/kb. However, using the QuikChange™ II mutagenesis kit (Agilent) we usually increment the time for 2 min/kb. For the pG3U1 plasmid length, 8 min should work, but from our experience adding one more min to this step (in this case 9 min for extension) gives the best results.
8. If a thermomixer is not available, follow the site-directed mutagenesis kit manufacturer's recommendations concerning shaking of transformation reactions.

9. The entire volume of transformation reaction can be plated on a single LB-agar plate. However, depending on the transformation efficiency this may originate a huge number of colonies which are then difficult to select. Therefore, we recommend to use more than one plate and spread different volumes to increase the probability to obtain individualized colonies (e.g. 200 and 100 μL). When plaquing lower volumes a small quantity (1:1) of SOC medium can be added to the transformation reaction to dilute and help to spread the transformation product.
10. If colonies cannot be selected immediately, store plate(s) at 4 °C.
11. The total volume of the tube should allow a volume of air that is 5× the volume of LB medium (e.g. 3 mL of LB medium in a 15 mL tube; 5 mL of LB medium in a 25 mL tube, etc.).
12. Before starting the miniprep(s) procedure, a sample of bacterial culture can be preserved in a "glycerolate" for future use. For a final volume of 1 mL, add a part of bacterial culture and a part of sterilized glycerol to a 2 mL tube for a final concentration of ~10–15% of glycerol. Vortex immediately and store at −80 °C.
13. To avoid the need to pick another bacterial colony from an LB-agar plate, the glycerolate(s) (*see* **Note 12**) can be used to prepare a new miniculture. Briefly, defrost the glycerolate on ice, scrape it lightly with a pipette tip or aspirate few microliters and pipet them up and down into a tube containing the desired volume of LB medium and ampicillin (100 μg/mL). Incubate the tube(s) in an orbital shaking incubator as recommended in **step 4** of Subheading 3.1.1.
14. In the transfection experiments here referred (*see* **step 7** of Subheading 3.2.1) we included two negative controls: one where only the transfection reagent was added to the cells and other where the minigene expressing the WT U1 sequence was transfected on cells.
15. For liposome-based transfection of fibroblasts, Lipofectamine® 2000 (Invitrogen) or other commercial lipofection reagent can also be tested. To further increase transfection efficacy, the modified U1's can also be inserted into the cells by the electroporation technique. For both methods we recommend to optimize the amount of transfection reagent according to the quantity of modified U1 and number of cells to transfect.
16. To assess transfection efficiency, transfect fibroblasts with a control plasmid encoding GFP or RFP and monitor fluorescence by microscopy. Also, the cell uptake of the modified U1's can be confirmed by PCR with specific primers (U1 Fw—5′ A TCGAAATTAATACGACTCA 3′ and U1 Rv—5′ CTGGGA AAACCACCTTCGT 3′). Otherwise, clone the WT human U1

snRNA cassette from pG3U1 vector in a plasmid encoding GFP and monitor fluorescence and U1 expression simultaneously.

17. Adjust the agarose gel percentage according to the molecular weight of the target amplified products.
18. In RT-PCR analysis after U1 snRNA's transfection, the size of the amplified band(s) seen on the agarose gel can give an idea of whether the aberrantly spliced exon under study is included in the cDNA or not. However, it is necessary to sequence the amplified band(s) from control and patient fibroblasts treated with the different modified U1 snRNAs, to confirm the correct splicing pattern.

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