

Journal of Visualized Experiments

A reporter based cellular assay for monitoring splicing efficiency

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63014R1
Full Title:	A reporter based cellular assay for monitoring splicing efficiency
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Additional Information:	
Question	Response
Please specify the section of the submitted manuscript.	Biochemistry
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (\$1400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Phoenix, Arizona, United States
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TITLE:

A Reporter Based Cellular Assay for Monitoring Splicing Efficiency

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KEYWORDS:

RNA–protein interaction; pre-mRNA splicing; spliceosome; U1 snRNP; intron; exon; splice site; Dup51p reporter; exon skipping

SUMMARY:

This protocol describes a minigene reporter assay to monitor the impact of 5′-splice site mutations on splicing and develops suppressor U1 snRNA for the rescue of mutation-induced splicing inhibition. The reporter and suppressor U1 snRNA constructs are expressed in HeLa cells, and splicing is analyzed by primer extension or RT-PCR.

ABSTRACT:

During gene expression, the vital step of pre-mRNA splicing involves accurate recognition of splice sites and efficient assembly of spliceosomal complexes to join exons and remove introns prior to cytoplasmic export of the mature mRNA. Splicing efficiency can be altered by the presence of mutations at splice sites, the influence of trans-acting splicing factors, or the activity of therapeutics. Here, we describe the protocol for a cellular assay that can be applied for monitoring the splicing efficiency of any given exon. The assay uses an adaptable plasmid encoded 3-exon/2-intron minigene reporter, which can be expressed in mammalian cells by transient transfection. Post-transfection, total cellular RNA is isolated, and the efficiency of exon splicing in the reporter mRNA is determined by either primer extension or semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). We describe how the impact of disease associated 5′ splice-site mutations can be determined by introducing them in the reporter; and how the suppression of these mutations can be achieved by co-transfection with U1 small nuclear RNA (snRNA) construct carrying compensatory mutations in its 5′ region that basepairs with the 5′-splice sites at exon-intron junctions in pre-mRNAs. Thus, the reporter can be used for the design of therapeutic U1 particles to improve recognition of mutant 5′ splice-sites. Insertion of *cis*-acting regulatory sites, such as splicing enhancer or silencer sequences, into the reporter can also be used to examine the role of U1 snRNP in regulation mediated by a

specific alternative splicing factor. Finally, reporter expressing cells can be incubated with small molecules to determine the effect of potential therapeutics on constitutive pre-mRNA splicing or on exons carrying mutant 5' splice sites. Overall, the reporter assay can be applied to monitor splicing efficiency in a variety of conditions to study fundamental splicing mechanisms and splicing-associated diseases.

INTRODUCTION:

Pre-mRNA splicing is an essential processing step that removes non-coding introns and precisely ligates coding exons to form mature mRNA. Recognition of consensus sequences at exon-intron junctions, referred to as 5'-splice site and 3'-splice site, by components of the splicing machinery initiates the splicing process. The U1 small nuclear ribonucleoprotein (snRNP) recognizes the 5'-splice site by base pairing of the U1 snRNA to the pre-mRNA¹. Genetically inherited mutations that alter 5'-splice site sequences are associated with many diseases^{2,3}. It is predicted that the loss of basepairing of U1 snRNA with the mutant 5'-splice sites, causes aberrant splicing, which can compromise translation of the affected transcript. A potential therapeutic approach to correct the splicing defects involves suppression of mutations by modified U1 snRNA carrying compensatory nucleotide changes in its 5'-region that basepairs with the 5'-splice site. Such modified U1 snRNAs, also referred to as exon specific U1 snRNAs, have been found to be effective in reversing splicing defects, resulting in increased protein expression from the rescued mRNA⁴⁻⁸.

Here, we describe the U1 snRNP complementation assay, which is a reporter-based cellular splicing assay that allows assessment of the effect of 5'-ss mutations on splicing of an exon and can also be used for the development of modified U1 snRNAs to enable the rescue of exon inclusion. We also provide protocols for monitoring of the spliced reporter transcripts by primer extension and RT-PCR, and for determining the expression of modified U1 snRNAs by primer extension and RT-qPCR.

PROTOCOL:

1. Reagents and buffers

NOTE: All sterilization using vacuum filters should be performed with 0.2 µm polyethersulfone (PES) membrane in a biosafety cabinet.

1.1. Prepare RNase-free water by adding 1.0 mL of diethylpyrocarbonate (DEPC) to 1.0 L deionized water, mix for at least 1 h at room temperature (RT), autoclave, and then cool to RT before use.

1.2. Prepare Dulbecco's Modified Eagle Medium (DMEM) by mixing one packet of DMEM powder (13.4 g), 3.7 g of sodium bicarbonate, 100 mL of fetal bovine serum (FBS), and penicillin and streptomycin to ~800 mL of sterile deionized water. The final concentration of penicillin and streptomycin in DMEM should be 50 U/mL and 50 µg/mL, respectively. Adjust the pH to 7.4 and

then make up the volume to 1.0 L with sterile deionized water. Sterilize by filtration and store at 4 °C.

1.3. Prepare 10x phosphate buffered saline (10xPBS) by adding 25.6 g of disodium hydrogen heptahydrate ($\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$), 2 g of potassium dihydrogen phosphate (KH_2PO_4), 2 g of potassium chloride (KCl), and 80 g of sodium chloride (NaCl) to 800 mL of deionized water. Mix to dissolve and make up the volume to 1.0 L. Sterilize by filtration and store at 4 °C.

1.4. Prepare 0.5 M ethylene diamine tetraacetic acid (EDTA) by dissolving 186.1 g of $\text{Na}_2 \bullet \text{EDTA} \bullet 2\text{H}_2\text{O}$ into ~800 mL of deionized water. Adjust pH to 8.0 and then make up the volume to 1.0 L with sterile deionized water. Sterilize by filtration and store at 4 °C.

1.5. Prepare 1x Trypsin-EDTA solution by mixing 100 mL of 10x trypsin (2.5%), 2 mL of 0.5 M EDTA, and add 1x PBS up to 1.0 L. Sterilize by filtration and aliquot into 50 mL conical tubes. Store at 4 °C for 1-2 weeks or freeze at -20 °C if not in use.

1.6. Prepare 2x formamide DNA/RNA loading dye by mixing 14.4 mL of formamide and 0.6 mL of 0.5M EDTA for a final volume of 15 mL. Add bromophenol blue and xylene cyanol powder to a final concentration of 0.02% and store at 4 °C.

NOTE: Formamide is toxic and corrosive. Read the material safety data sheets for additional safety procedure.

1.7. Prepare 5x Tris/Borate/EDTA (5x TBE) buffer by mixing 54.0 g of tris base, 27.5 g of boric acid, and 20 mL of 0.5M EDTA into ~800 mL of deionized water. Mix to dissolve and make up the volume to 1.0 L with deionized water.

1.8. Prepare Urea-PAGE solution by mixing 200 mL of 5x TBE, 250 mL of 40% 19:1 bis/acrylamide, and 450.5 g of urea. Then add deionized water up to 1.0 L. Mix until the ingredients are completely dissolved, then sterilize by filtration, and store at 4 °C in an amber glass bottle.

NOTE: Bis/acrylamide is toxic. Read the material safety data sheets for additional safety procedures.

1.9. Prepare 10% ammonium persulfate (APS) by dissolving 1 g of APS in 10 mL of deionized water.

2. Cotransfection of HeLa cells with the reporter and U1 snRNA plasmids

NOTE: The transfection of HeLa cells must be performed under sterile conditions in a biological safety cabinet. The outer surface of all materials must be sprayed with 70% ethanol before being introduced into the biological safety cabinet.

2.1. Maintain HeLa cells in DMEM in a 37 °C incubator with 5% CO₂ by passaging every 2-3 days when the cells are about 80-90% confluent.

2.2. For passaging HeLa cells, aspirate the spent medium and then incubate cells with 3 mL of 0.25% trypsin containing 1 mM EDTA at 37 °C for 3 min.

2.3. After incubation, add 7 mL of fresh DMEM. Transfer the cell suspension to a 10 mL tube, centrifuge at 1,000 x g for 5 min.

2.4. Resuspend the cell pellet in 10 mL of fresh DMEM, and then plate the cells on a new tissue culture dish at 20% confluence.

2.5. For transient transfections, count HeLa cells with a clean hemocytometer slide and prepare a suspension with a density of 2.5×10^5 cells/mL.

2.6. Seed 1.0 mL of 2.5×10^5 cells/mL HeLa cell suspension into each well of a 12-well plate and incubate overnight at 37 °C.

2.7. The next day, aspirate the spent medium and add 0.8 mL of fresh DMEM with serum.

2.8. Prepare Solution I by adding 0.2 µg of Dup51 or Dup51p reporter plasmid, 1.8 µg of pcDNA, pNS6U1, or pNS6U1-5a plasmid, and 100 µL of transfection medium into a new 1.5 mL microcentrifuge tube.

2.9. Prepare a master mix of Solution II by adding 100 µL of transfection medium and 4 µL of transfection reagent per sample for all samples to be transfected.

2.10. Prepare the transfection mix by adding 100 µL of Solution II into each microcentrifuge tube containing Solution I.

2.11. Vortex the transfection mixes for 15 s, centrifuge in a tabletop microcentrifuge at 3,000 x g for 10 s at RT, and then incubate at RT for 5 min.

2.12. Add all 200 µL of the transfection mix into one well of the 12-well HeLa cell plate to achieve a final volume of 1.0 mL per well and incubate the plate at 37 °C for 48 h.

2.13. After incubation, extract RNA from the transfected HeLa cells with commercially available guanidine isocyanate solution.

NOTE: This reagent contains phenol, and, therefore, this step should be performed in a fume hood. The use of DEPC treated water is recommended for resuspension of extracted RNA.

2.13.1 Aspirate the spent medium and add 500 µL of the reagent into each well. Incubate at RT for 5 min.

2.13.2 Homogenize by pipetting up and down. Then transfer the solution to a new 1.5 mL microcentrifuge tube.

2.13.3 Add 100 μ L of chloroform and vortex for 15 s.

2.13.4 Centrifuge at 12,000 $\times g$ for 15 min at 4 °C.

2.13.5 Transfer 200 μ L of the RNA containing, top aqueous layer to a new 1.5 mL microcentrifuge tube.

2.13.6 Add 2 μ g of glycogen and 200 μ L of isopropanol to each RNA sample. Mix by inverting the tubes.

2.13.7 Collect the RNA precipitate by centrifugation at 12,000 $\times g$ for 10 min for 4 °C.

2.13.8 Remove and discard the supernatant without disturbing the RNA pellet.

2.13.9 Wash the pellet twice by adding 1.0 mL of 70% ethanol, inverting the tubes, and centrifuging as described in Step 2.13.4.

2.13.10 Air dry the pellet for ~ 20-30 min at RT and resuspend the RNA in 10-20 μ L of RNase-free water.

2.13.11 Determine RNA concentration by measuring absorbance at 260 nm as described by Desjardins and Conklin⁹.

2.14. Proceed with primer extension or store RNA samples in -20 °C. Isolated RNA can be stored at -20 °C for 6-12 months.

3. ³²P-labeling of oligonucleotides

NOTE: Steps involving the use of ³²P-ATP and ³²P-labeled oligonucleotides must be performed behind an acrylic shield by trained individuals with approval from authorized institutional entities. The protocol described below can be used for labeling of oligonucleotides, Dup3r and U17-26-R, and markers for urea-polyacrylamide gel electrophoresis (urea-PAGE). Use of DEPC treated water is recommended for resuspension of oligonucleotides and size exclusion beads.

3.1. To a 1.5 mL microcentrifuge tube, add oligonucleotide, T4 polynucleotide kinase (T4 PNK), T4 PNK buffer, and ³²P-ATP as described in **Table 1**. Add ³²P-ATP last to the mixture; this is important.

NOTE: For the addition of radioactive solutions, use of filter tips is recommended.

3.2. Incubate in a water bath at 37 °C for 30 min.

3.3. While the labeling reactions are incubating, resuspend the size exclusion beads with a 25 kDa molecular weight cut off by gently vortexing for ~10 s.

NOTE: The size exclusion beads should be prepared according to the manufacturer's instructions and stored as a 50% suspension in 25% ethanol at 4 °C.

3.4. Prepare columns by transferring 600 µL of the resuspended beads to a disposable mini column placed in a 1.5 mL collection tube and returning beads stock to 4 °C.

3.5. Centrifuge at 2,000 x *g* for 1 min at RT and discard the flow through.

3.6. Wash beads by adding 300 µL of RNase-free water to the column.

3.7. Repeat steps 3.5. and 3.6. twice and transfer the mini-column to a new 1.5 mL centrifuge tube.

3.8. Add the kinase reaction mix to the size-exclusion bead column and centrifuge at 5000 x *g* for 1 min at RT.

3.9. Collect and save the flow through, which has the ³²P-labeled oligonucleotide, and discard all tips and tubes into an acrylic waste box.

3.10. Add 20 µL of RNase-free water to dilute ³²P-labeled oligonucleotide to a final concentration of 2.5 µM.

NOTE: Dilute the labeled markers as needed for loading on urea-PAGE gels.

3.11. Store the labeled oligonucleotide in an acrylic microtube box at -20 °C or proceed with primer extension analysis.

4. Analysis of the spliced reporter transcripts by primer extension

NOTE: It is recommended to clean surfaces and equipment with an RNase inactivating reagent before use.

4.1. Add 2.0 µg of RNA extracted from the Hela cells into separate 200 µL microcentrifuge tubes and add RNase-free water to make up the volume to 6.55 µL.

4.2. Prepare Master Mix I with the diluted ³²P-Dup3r and dNTPs as shown in **Table 2** and add 0.9 µL of the mix to each PCR tube containing RNA samples.

4.3. Incubate the tubes, first at 65 °C for 5 min and then on ice for 1 min.

4.4. Prepare Master Mix II with 5x First Strand Buffer, dithiothreitol (DTT), RNase inhibitor, and reverse transcriptase as shown in **Table 2** and add 2.55 µL of the mix to each sample.

4.5. Add 2.55 µL of the mix to each tube containing RNA and Master Mix 1; the total volume of the reaction should be 10 µL. Keep the tubes at RT for 10 min.

4.6. Transfer the tubes to a dry bath or a thermal cycler and incubate, first at 50 °C for 60 min and then at 70 °C for 15 min.

4.7. After incubation, add 10 µL of 2x formamide RNA loading dye to each sample and store in an acrylic box at -20 °C or proceed with the separation of fragments by urea-PAGE using a 14-cm long gel and visualization of gel image as described below in Step 8.

4.8. Perform densitometric scanning of the gel image with an image analysis software and use the band intensities of included and skipped products to calculate the percentage of exon 2 inclusion as shown below.

$$\% \text{ Exon 2 Inclusion} = \frac{\text{Intensity of Included Product}}{\text{Intensity of Included Product} + \text{Intensity of Skipped Product}} \times 100\%$$

5. Analysis of the spliced reporter transcripts by fluorescent RT-PCR

NOTE: The RT-PCR analysis described below uses random hexamers for cDNA synthesis and a combination of unlabeled Dup8f and Cy5-labeled Dup3r oligonucleotides for PCR amplification of the spliced products.

5.1. For cDNA synthesis, add 2.0 µg of RNA extracted from the transfected Hela cells into separate 200 µL microcentrifuge tubes and add RNase-free water to make up the volume to 11.0 µL.

5.2. Prepare Master Mix I, containing random hexamers and dNTP as shown in **Table 3** and add 2.0 µL of the mix to each sample. Incubate, first at 65 °C for 5 min then on ice for 1 min.

5.3. Prepare Master Mix II, containing First Strand Buffer, RNase inhibitor, DTT, and reverse transcriptase as shown in **Table 3** and add 7 µL of the mix to each tube.

5.4. Keep the tubes at RT for 10 min, and then incubate at 50 °C for 60 min and 70 °C for 15 min.

NOTE: Completed cDNA reactions may be stored at -20 °C.

5.5. For PCR, dilute the cDNA reaction to yield a concentration of 100 ng/µL and transfer 1.0 µL of each cDNA sample to new PCR tubes.

5.6. Prepare a master mix consisting of Dup8f, Cy5-Dup3r, dNTPs, Taq buffer, Taq polymerase, and water as described in **Table 4** and add 10.5 μ L of the mix to each tube containing cDNA.

5.7. Perform PCR using a thermal cycler with an initial denaturation step at 94 °C for 3 min; followed by 20 cycles of denaturation (94 °C for 30 s), annealing (65 °C for 30 s), and extension (72 °C for 15 s), and a termination step at 72 °C for 5 min.

5.8. Add 12.5 μ L of 2x formamide DNA loading dye to each tube and heat at 95 °C for 5 min.

5.9. Store the PCR reaction at -20 °C or proceed with urea-PAGE as described below in Step 8.1-8.4.

5.10. After electrophoresis, remove the glass plates from the electrophoresis apparatus and scan using a fluorescence imager to visualize the gel.

5.11. Perform densitometric scanning of the gel image and use band intensity of the included and the skipped products to calculate percentage of exon 2 inclusion as described in Step 4.8.

6. Analysis of variant U1 snRNA expression by primer extension

6.1. Add 2.0 μ g of RNA extracted from the Hela cells into separate 200 μ L microcentrifuge tubes and add dATP, RNase-free water to make up the volume to 4.7 μ L as shown in Table 5.

6.2. Add 10,000 CPM of the 32 P-U1₇₋₂₆-R oligonucleotide to each tube.

NOTE: To prepare a 10,000 cpm/ μ L solution of 32 P-U1₇₋₂₆-R (from Step 3), dilute the labeled oligonucleotide (1:20 dilution), determine cpm in 1.0 μ L using a scintillation counter, and further dilute with deionized water to prepare a solution of 10,000 cpm/ μ L in a new 1.5 mL microcentrifuge tube.

6.3. Incubate at 65 °C for 5 min, and then on ice for 1 min.

6.4. Prepare a master mix with 5x First Strand Buffer, RNase inhibitor, DTT, and reverse transcriptase as shown in **Table 5** and add 1.8 μ L of the mix to each sample.

6.5. Incubate, first at RT for 10 min and then at 42 °C for 10 min.

6.6. After incubation, add 10 μ L of 2x formamide RNA loading dye into each sample and store in an acrylic box at -20 °C or proceed with separation of fragments by urea-PAGE using a 38-cm long gel (see Step 8).

7. Analysis of variant U1 snRNA expression by RT-qPCR

7.1. Dilute the cDNA stock prepared as described above in Steps 5.1 – 5.4 to a concentration of 0.2 ng/μL.

7.2. Pipette 5.0 μL of diluted cDNA into individual wells of a 96-well qPCR plate in triplicate. Add deionized water instead of cDNA for no-template control (NTC).

7.3. Prepare two separate primer mixes consisting of the forward and reverse primers for U1 and U2 snRNAs, and water as shown in **Table 6**.

NOTE: Sequences for U1 and U2 snRNA specific primers are provided in **Table 7**.

7.4. Add 5.0 μL of the U1 and U2 snRNA primer mix to the sample and NTC wells.

7.5. Add 10.0 μL of real-time PCR mix to each well.

7.6. Seal the plates with an optical film, then centrifuge at 1,000 x g for 2 min at RT to collect the reactions to the bottom of the wells.

7.7. Perform qPCR with an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of a 2-step protocol consisting of denaturation (95 °C for 15 s) and annealing/extension (62 °C for 60 s) while collecting the threshold quantification cycle (Cq) values of target amplicons.

7.8. Finish the qPCR reaction by checking for a single peak in the dissociation curve for U1 and U2 snRNA reactions.

7.9. From the quantification cycle (Cq) values, calculate delta Cq (ΔCq) for U1 and U2 snRNAs as compared to the pcDNA control.

7.10. Determine variant U1 snRNA expression as relative quantity (RQ) of U1 as compared to U2 using the ΔΔCq value as shown below for all samples.

$$\Delta Cq_{U1 \text{ or } U2} = Cq_{U1 \text{ or } U2} - Cq_{pcDNA}$$

$$\Delta\Delta Cq = \Delta Cq_{U1} - \Delta Cq_{U2}$$

$$RQ_{U1} = 2^{-\Delta\Delta Cq}$$

8. Setup and running of Urea-PAGE gels

NOTE: Assembly of glass plates and the gel running apparatus should be performed according to manufacturer's protocol. The casting of the 10% urea-PAGE gel can be performed according to a previously described protocol by Summer et al.¹⁰. Steps involving the preparation of markers and samples, and of running and visualization of gels are described below. Optionally, to prevent the gel from sticking to glass plates, the inner surface may be coated with silicone solution by adding 1 mL of the solution onto the surface and evenly spreading over the entire surface with tissue. Once dry, the plates should be washed with deionized water and dried again.

CAUTION: Unpolymerized acrylamide is neurotoxic and must be handled with protections recommended in the material safety data sheet.

8.1. Prepare the primer extension samples and markers by heating at 95 °C for 5 min and then centrifuging in a tabletop microcentrifuge at 3,000 x *g* for 5 s at RT.

8.2. Prior to loading the makers and samples, flush out the wells with 1x TBE buffer to remove the settled urea.

8.3. Load 10 µL/sample/well and run the gel at 300-500V for 2-3 h or until the xylene cyanol reaches the bottom.

NOTE: About 1,000 cpm of the ³²P-labeled marker can be loaded.

8.4. After electrophoresis, remove the glass plates from the electrophoresis apparatus.

8.5. Carefully separate the two plates so that the gel lays flat on either glass surface and transfer the gel onto a filter paper and cover it with plastic wrap.

8.6. Vacuum dry the gel on the filter paper at 80 °C for 30 min using a gel dryer.

8.7. Place the dried gel in a phosphor imaging cassette and keep at RT overnight.

NOTE: The phosphor screen should be erased using a light box prior to use.

8.8. To visualize the gel image, remove the screen and scan using phosphor imager.

REPRESENTATIVE RESULTS:

The splicing reporter Dup51, a three exon-two intron minigene, was derived from the human β -globin gene and has been described previously (**Figure 1A**)^{11,12}. We created a mutant reporter, Dup51p, by introducing Usher syndrome associated 5'-splice site mutations that occur in exon 3 of the protocadherin 15 (*PCDH15*) gene¹³. The 5'-splice site sequence at the exon 2-intron 2 junction was changed from CAG/GUUGGUAUC to AUG/GUGUGUAUC (/ is the exon-intron boundary) (**Figure 1B**)¹⁴. These sequence changes alter the predicted pattern of 5'-splice site basepairing with the endogenous U1 snRNA and also result in skipping of exon 2 in the mature transcript (**Figure 2**). The effect of these mutations could be suppressed by a compensatory change in the 5'-region of U1 snRNA. Substitution of the uracil with adenine at the 5th position of the U1 snRNA (U1-5a) reversed the effect of 5'-splice site mutations in Dup51p (**Figure 1B**).

The splicing of the Dup51 and Dup51p transcripts can be monitored by primer extension using ³²P-Dup3r or by semi quantitative RT-PCR using Dup8f and Dup3r oligonucleotides (**Figure 1**); sequences are provided in **Table 7**. When expressed in HeLa cells, the Dup51 minigene expresses the mature full-length transcript in which exon 2 is spliced-in efficiently (**Figure 2A,B**, lane 1). The

5'-splice site mutations in Dup51p reduce exon 2 inclusion from ~95% to ~30%, leading to the formation of the shorter transcript (**Figure 2A,B**, lane 5). Coexpression of Dup51p with U1-5a snRNA rescues exon 2 splicing and restores formation of full-length transcript (**Figure 2A,B**, lane 8). Coexpression with wildtype U1 (U1-WT) or U1-5g variant does not have a similar effect on Dup51p splicing (lanes 6 and 7). U1-WT, U1-5g, or U1-5a coexpression also does not alter the splicing pattern of the Dup51 reporter (lanes 2-4). Overall, these results indicate that the rescue of exon 2 splicing in Dup51p transcripts is specifically due to the U>A mutation in the U1-5a snRNA.

To monitor the expression of transfected U1 snRNAs, primer extension or RT-qPCR can be applied. Detection of U1-5a variant transcripts by primer extension uses the U1₇₋₂₆ oligonucleotide, which is 20 nucleotides long and basepairs near the adenine at the 5th position of U1 snRNA (**Figure 3A**). In the presence of dATP alone, U1₇₋₂₆ allows incorporation of 2-3 nucleotides for the endogenous wildtype U1 snRNA yielding 22 or 23 nucleotides long product (**Figure 3B**, lanes 1, 2, 5, and 6). For the U1-5a and U1-5g variants, extension terminates after addition a single adenosine producing 21 nucleotides long product (lanes 3, 4, 7, and 8). Analysis by RT-qPCR allows estimation of fold increase in expression of the variant U1 over the endogenous wildtype snRNA. Here, U1 specific primer pairs are designed so that no overlap exists with the mutations introduced into the variant snRNAs (**Figure 3A**). After normalization to U2 snRNA expression, the transfected U1-wt, and the U1-5g and U1-5a variants were found to be expressed at 3-5 folds over the endogenous snRNA (**Figure 3C**). Endogenous U1 is estimated to be present at 10⁶ copies per cell in HeLa cells¹⁵. Thus, the exogenous variant snRNAs are expressed at ~3-5 fold over the endogenous U1. The ability of the U1-5a snRNA to rescue exon 2 inclusion to ~95% demonstrates that levels of the exogenous variant snRNAs are sufficient for the splicing of the reporter pre-mRNAs.

FIGURE AND TABLE LEGENDS:

Table 1: ³²P-labeling of oligonucleotides. Reaction for preparation of a 5'-³²P-labeled primer using ³²P-ATP.

Table 2: ³²P-Dup3r primer extension. Reaction for the analysis of spliced reporter transcripts by primer extension.

Table 3: cDNA Synthesis. Reaction for the synthesis of cDNA from total RNA.

Table 4: Fluorescent RT-PCR. Reaction for the analysis of the spliced reporter transcripts by RT-PCR using Cy5-labeled primer.

Table 5: ³²P-U1-snRNA primer extension. Reaction for the analysis of variant U1 snRNA expression by primer extension.

Table 6: Quantitative RT-qPCR. Reactions for quantitative analysis of variant U1 snRNA expression by RT-qPCR.

Table 7: Sequence of oligonucleotides primers. List of primer names, sequences, and the technique the primers were used for.

Figure 1: Dup51 and Dup51p Minigene Reporters. (A) Schematic representation of the three-exon/two-intron minigene reporters, Dup51 and Dup51p. Locations of the Dup8f and Dup3r in exons 1 and 3, respectively, are indicated. (B) Predicted basepairing of the wildtype U1 snRNA and the U1-5a variant with the 5'-splice site sequences of Dup51 and Dup51p reporter transcripts.

Figure 2: Analysis of Spliced Reporter Transcripts. HeLa cells were cotransfected with the Dup51 or Dup51p minigene plasmids and pcDNA control or pNS6U1 plasmids for U1-wt, U1-5g, or U1-5a snRNAs. (A) Primer extension analysis with ³²P-labeled Dup3r primer demonstrating the splicing of the Dup51-WT and Dup51p minigene transcripts. The mRNA products are indicated to the left of the gel image. The percentage of the full-length product (\pm s.d., $n = 3$) was calculated from band intensities of the two mRNA isoforms and is represented in the graphs below. (B) RT-PCR analysis with Dup8f and Cy5-Dup3r primers on cDNA prepared from total RNA extracted from HeLa cells demonstrating splicing of the Dup51-WT or Dup51p minigene transcripts. The mRNA products are indicated to the left of the gel image. The percentage of the full-length product (\pm s.d., $n = 3$) was calculated from band intensities of the two mRNA isoforms and is represented in the graphs below.

Figure 3: Analysis of the variant U1-5a snRNA. HeLa cells were cotransfected with the Dup51 or Dup51p minigene plasmids and pcDNA control or pNS6U1 plasmids for U1-wt, U1-5g, or U1-5a snRNAs. (A) A two-dimensional representation of the U1 snRNA sequence. Position of the U1₇₋₂₆r primer, used for primer extension analysis of U1 snRNA, is indicated by the red line. Position of U1₁₇₋₃₉F and U1₅₈₋₈₀R primers, used for RT-qPCR of U1 snRNA, is indicated by the blue lines. (B) Primer extension analysis with ³²P-labeled U1₇₋₂₆ to detect the expression of the wildtype U1 snRNA, and the U1-5g and U1-5a variants. Positions of the products formed from U1-wt and the variants snRNAs are indicated. (C) RT-qPCR analysis of the levels of U1 snRNA expression in HeLa cells. The fold change in U1-snRNA expression was calculated relative to the pcDNA negative control and normalized to U2-snRNA, fold change (\pm s.d.; $n = 3$) in U1 is graphed.

DISCUSSION:

The assay can be adapted for splicing analysis in cell lines other than HeLa, however, factors affecting transfection efficiency, such as cell confluency and quantity of DNA may need to be optimized. The reporter to U1 construct ratio is another critical parameter that may need to be determined depending upon the expression levels observed in other cell types. The quality of extracted RNA is critical for splicing analysis; therefore, the use of RNase-free water and decontamination of surfaces with RNase inactivating agents is highly recommended.

Analysis of reporter transcripts by either primer extension or fluorescent RT-PCR yield similar results, hence either technique can be employed for monitoring changes in the inclusion of exon 2 in the mature transcript. The fluorescent PCR reaction must be stopped and quantified in the

exponential phase of amplification, which is dependent on the initial RNA concentration. Thus, the number of amplification cycles may have to be determined for transfections conditions employed for the assay. The ease of use due to the non-radioactive nature is an advantage of the RT-PCR assay. Although primer extension with radioactively labeled primer requires extra safety precautions, it can detect signals from a low amount of RNA. The low throughput of primer extension and RT-PCR is a limitation of this assay.

The U1 snRNP complementation assay has been employed for multiple purposes. In addition to reversing the effects of 5'-splice site mutations, U1 snRNAs carrying changes to the 5'-region have been applied for gene silencing and for studying splicing mechanisms. Fortes et al. showed that targeting U1 snRNAs to terminal exons of endogenous transcripts can efficiently inhibit gene expression¹⁶. Roca et al. used U1 complementation to study the noncanonical mechanisms of 5' splice site recognition and showed that the U1 snRNA can basepair to 5'-splice sites in an alternate register where basepairing is shifted by one nucleotide and also in "bulge registers" where bulged nucleotides are present on either the pre-mRNA or the snRNA^{17,18}. This reporter system can potentially be used to study the role of U1 snRNP in splicing modulation by small molecules and splicing regulatory proteins. By substituting exon 2 of Dup51p with a target exon, the assay can be applied for examining the mechanism of action of small molecules that are known to enhance or repress splicing of specific exons, as in the case of splice switching compounds¹⁹. By incorporating regulatory sequences for alternative splicing factors into the exons or introns, it should be possible to examine U1 dependence in splicing regulation. This concept was applied by Hamid et al. to demonstrate the involvement of the stem-loop 4 of the U1 snRNA in splicing regulation by the polypyrimidine tract binding protein 1²⁰. We have used this assay to study the functions of stem-loops 3 and 4 of the U1 snRNA. By incorporating mutations in the stem-loop of the suppressor U1-5a snRNA that activates a mutant 5' splice site in the Dup51p splicing reporter, we have identified their critical roles in the formation of cross intron contacts with the U2 snRNP specific protein SF3A1 in the early steps of spliceosome assembly^{14,21}. Thus, the three-exon two-intron Dup51 reporter serves as an adaptive tool for monitoring splicing efficiency in studies on mechanisms underlying spliceosome assembly and splicing-associated disease.

ACKNOWLEDGMENTS:

This work was supported by funds to S.S. from the National Institutes of Health (R21CA170786 and R01GM127464) and the American Cancer Society (the Institutional Research Grant 74-001-34-IRG) and to S.S. and W.M. from the Valley Research Partnership Program (P1-4009 and VRP77). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

- 1 Zhuang, Y., Weiner, A. M. A compensatory base change in U1 snRNA suppresses a 5' splice site mutation. *Cell*. **46** (6), 827-835 (1986).

571 2 Scotti, M. M., Swanson, M. S. RNA mis-splicing in disease. *Nature Review Genetics*. **17** (1),
572 19-32 (2016).

573 3 Ward, A. J., Cooper, T. A. The pathobiology of splicing. *Journal of Pathology*. **220** (2), 152-
574 163 (2010).

575 4 Scalet, D. et al. Disease-causing variants of the conserved +2T of 5' splice sites can be
576 rescued by engineered U1snRNAs. *Human Mutation*. **40** (1), 48-52 (2019).

577 5 Yamazaki, N. et al. Use of modified U1 small nuclear RNA for rescue from exon 7 skipping
578 caused by 5'-splice site mutation of human cathepsin A gene. *Gene*. **677** 41-48 (2018).

579 6 Yanaizu, M., Sakai, K., Tosaki, Y., Kino, Y., Satoh, J. I. Small nuclear RNA-mediated
580 modulation of splicing reveals a therapeutic strategy for a TREM2 mutation and its post-
581 transcriptional regulation. *Science Reports*. **8** (1), 6937 (2018).

582 7 Balestra, D. et al. Splicing mutations impairing CDKL5 expression and activity can be
583 efficiently rescued by U1snRNA-based therapy. *International Journal of Molecular Sciences*. **20**
584 (17), 20174130, (2019).

585 8 Donadon, I. et al. Exon-specific U1 snRNAs improve ELP1 exon 20 definition and rescue
586 ELP1 protein expression in a familial dysautonomia mouse model. *Human Molecular Genetics*. **27**
587 (14), 2466-2476 (2018).

588 9 Desjardins, P., Conklin, D. NanoDrop microvolume quantitation of nucleic acids. *Journal*
589 *of Visualized Experiments*. (45), e2565, (2010).

590 10 Summer, H., Gramer, R. Droge, P. Denaturing urea polyacrylamide gel electrophoresis
591 (Urea PAGE). *Journal of Visualized Experiments*. (32), e1485 (2009).

592 11 Dominski, Z., Kole, R. Selection of splice sites in pre-mRNAs with short internal exons.
593 *Molecular Cell Biology*. **11** (12), 6075-6083 (1991).

594 12 Amir-Ahmady, B., Boutz, P. L., Markovtsov, V., Phillips, M. L., Black, D. L. Exon repression
595 by polypyrimidine tract binding protein. *RNA*. **11** (5), 699-716 (2005).

596 13 Le Guedard-Mereuze, S. et al. Sequence contexts that determine the pathogenicity of
597 base substitutions at position +3 of donor splice-sites. *Human Mutation*. **30** (9), 1329-1339
598 (2009).

599 14 Sharma, S., Wongpalee, S. P., Vashisht, A., Wohlschlegel, J. A., Black, D. L. Stem-loop 4 of
600 U1 snRNA is essential for splicing and interacts with the U2 snRNP-specific SF3A1 protein during
601 spliceosome assembly. *Genes and Development*. **28** (22), 2518-2531 (2014).

602 15 Steitz, J. A. et al. Functions of the abundant U-snRNPs. *Structure and function of major*
603 *and minor small nuclear ribonucleoprotein particles*. Springer Berlin Heidelberg, Berlin,
604 Heidelberg. 115–154 (1988).

605 16 Fortes, P. et al. Inhibiting expression of specific genes in mammalian cells with 5' end-
606 mutated U1 small nuclear RNAs targeted to terminal exons of pre-mRNA. *Proceedings of the*
607 *National Academy of Sciences U. S. A.* **100** (14), 8264-8269 (2003).

608 17 Roca, X. et al. Widespread recognition of 5' splice sites by noncanonical base-pairing to
609 U1 snRNA involving bulged nucleotides. *Genes and Development*. **26** (10), 1098-1109 (2012).

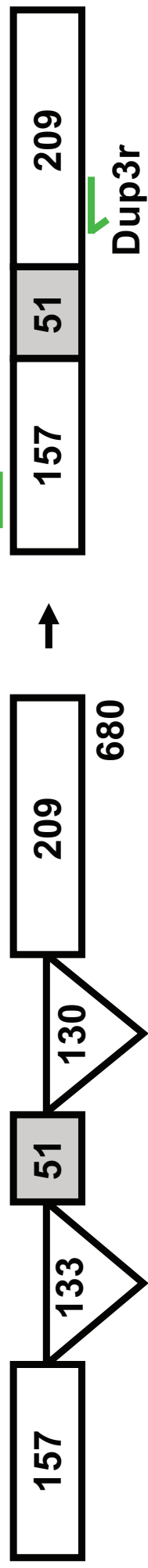
610 18 Roca, X., Krainer, A. R. Recognition of atypical 5' splice sites by shifted base-pairing to U1
611 snRNA. *Nature Structural Molecular Biology*. **16** (2), 176-182 (2009).

612 19 Taladriz-Sender, A., Campbell, E., Burley, G. A. Splice-switching small molecules: A new
613 therapeutic approach to modulate gene expression. *Methods*. **167** 134-142 (2019).

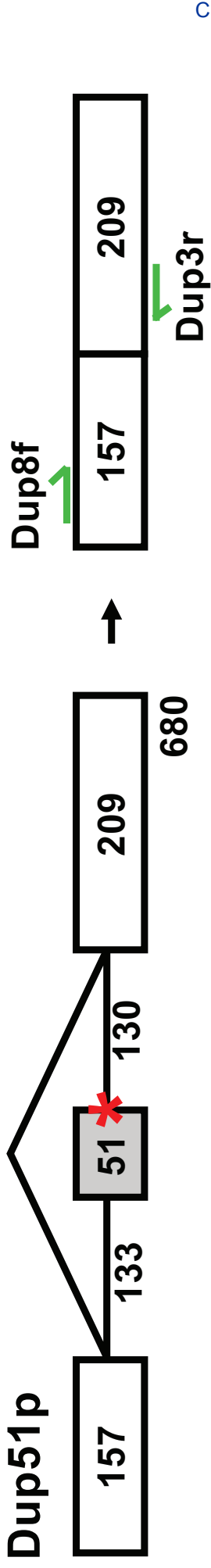
614 20 Hamid, F. M., Makeyev, E. V. A mechanism underlying position-specific regulation of
615 alternative splicing. *Nucleic Acids Research*. **45** (21), 12455-12468 (2017).
616 21 Martelly, W. et al. Synergistic roles for human U1 snRNA stem-loops in pre-mRNA splicing.
617 *RNA Biology*. 1-18 (2021).
618

A

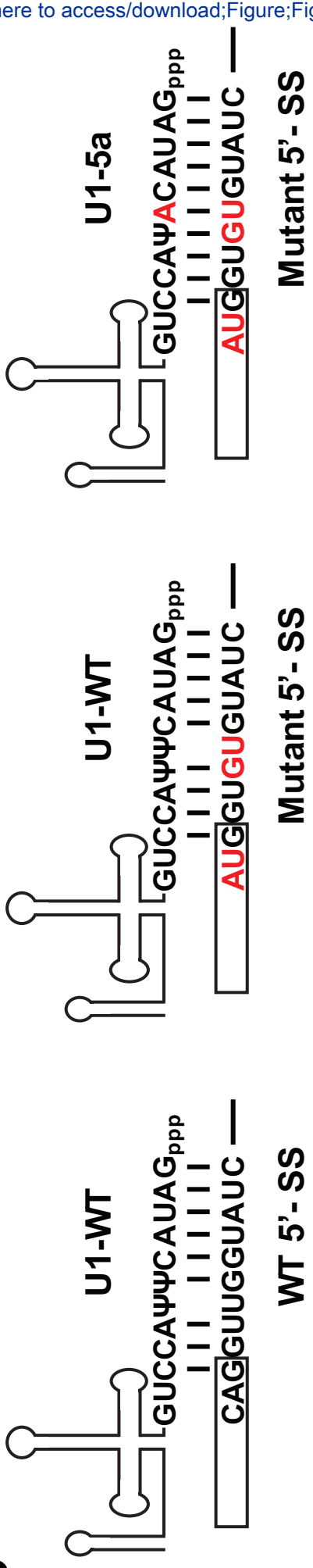
Dup51



Dup51p

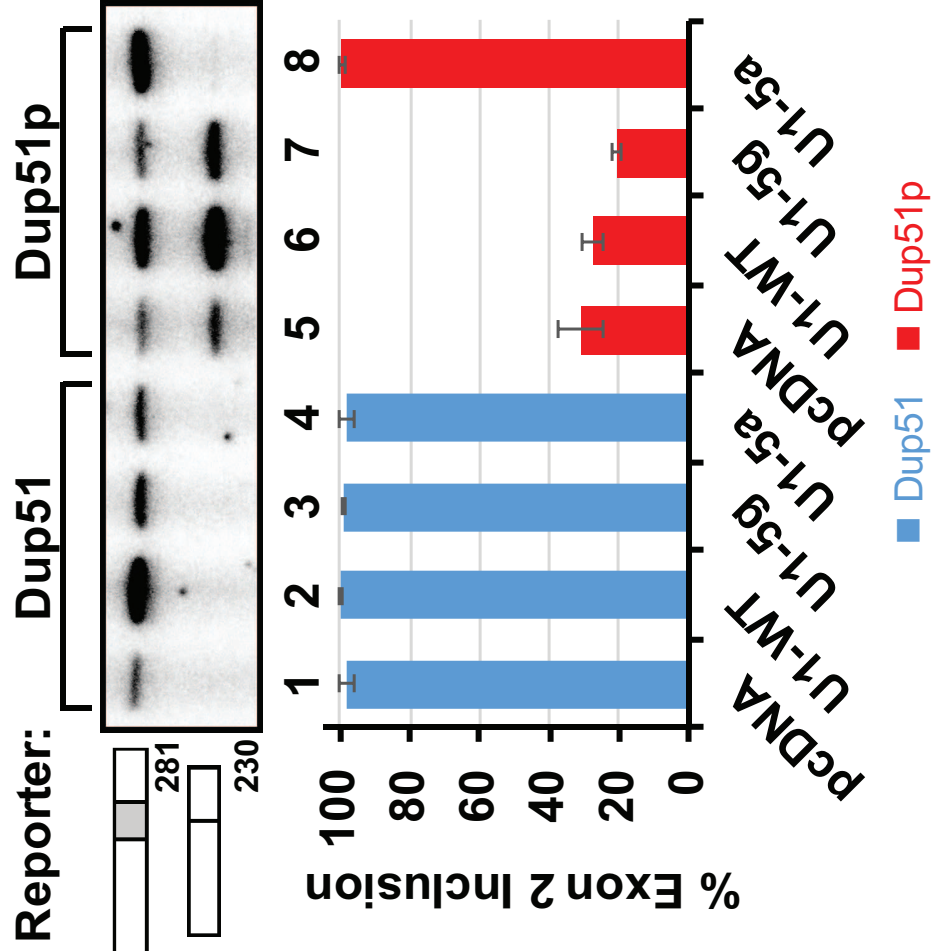


B



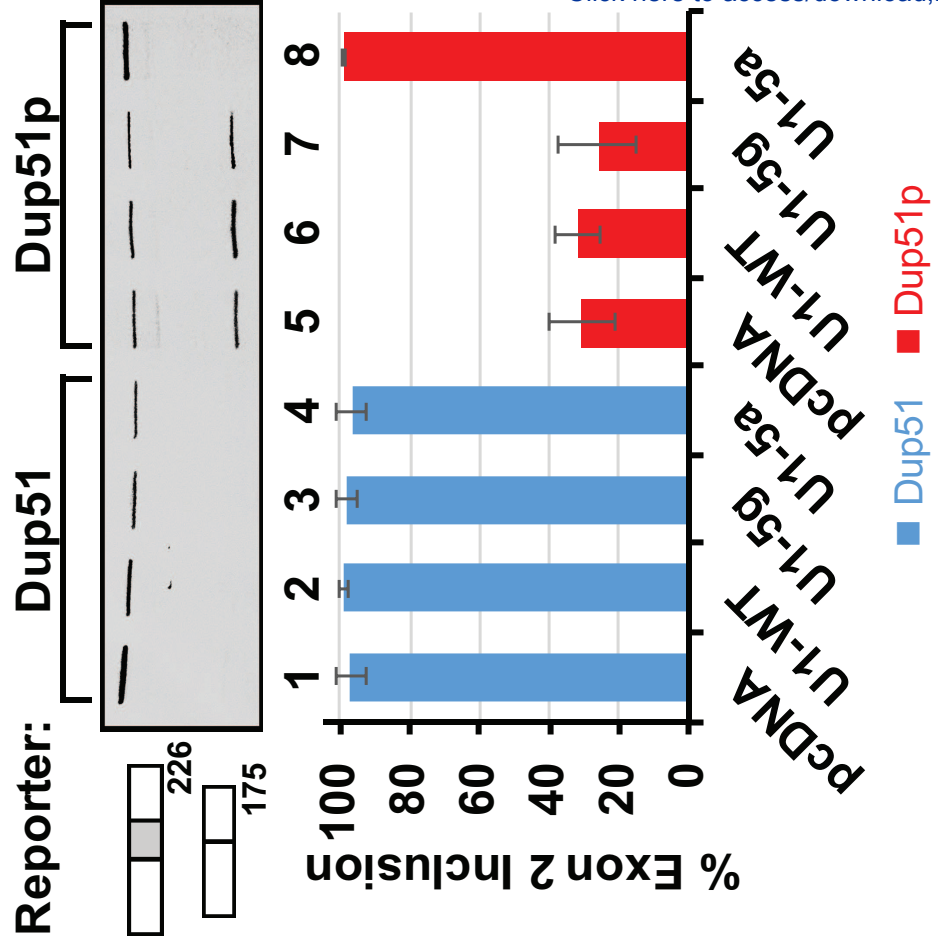
B

Primer Extension

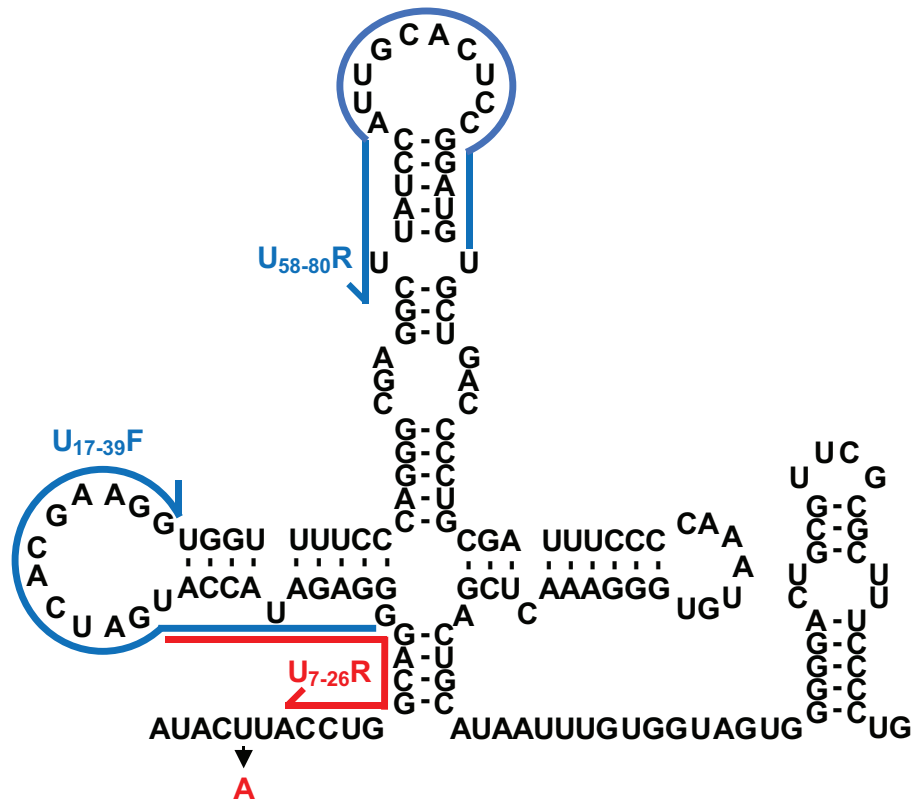


A

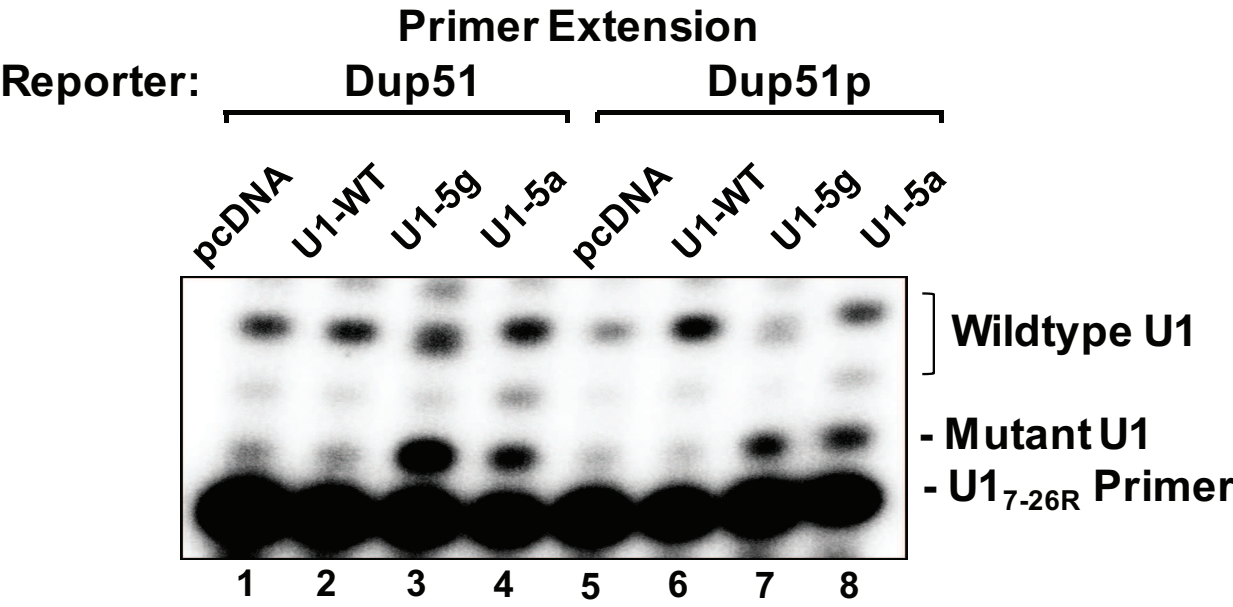
RT-PCR



A



B



C

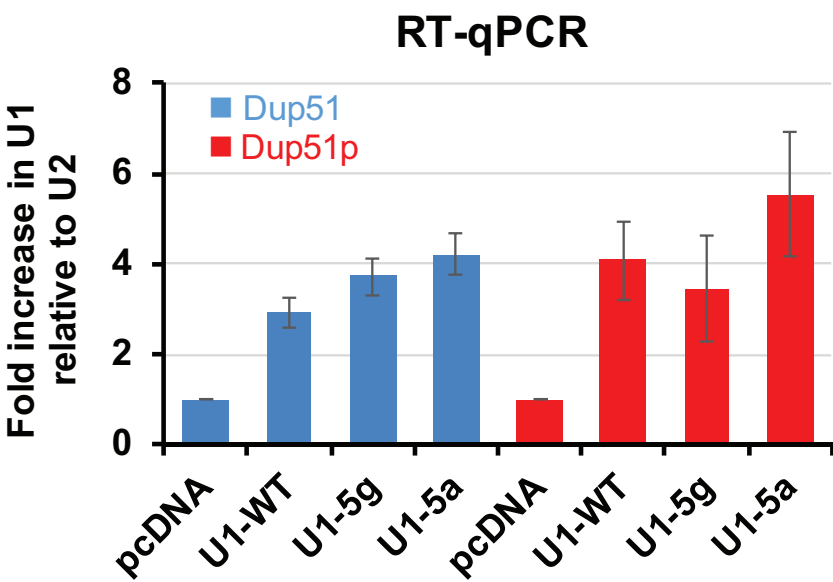


Table 1. ^{32}P -labeling of oligonucleotides. Reaction for preparation of a 5'- ^{32}P -labeled primer using ^{32}P -ATP.

Ingredients	Amount for One Reaction
PNK Buffer (10x)	2.0 μL
T4 Polynucleotide Kinase (T4 PNK) (10,000 U/mL)	1.0 μL
Oligonucleotide* (10 μM)	10.0 μL
^{32}P -ATP (10 mM)	1.0 μL
ddH ₂ O (DEPC treated)	Bring up volume to 20.0 μL with ddH ₂ O

*For labeling markers, 0.5 μL of the pBR322 DNA-Mspl Digest or the Low Molecular Weight Marker is used.

Table 2. 32 P-Dup3r Primer Extension. Reaction for analysis of spliced reporter transcripts by primer extension.

Ingredients - Master Mix I	Amount for One Reaction
RNA and ddH ₂ O (DEPC treated)	6.55 μ L
32 P-Dup3r (2.5 μ M)	0.4 μ L
dNTP (10 mM)	0.5 μ L
Ingredients – Master Mix II	Amount for One Reaction
First-Strand Buffer (5x)	2.00 μ L
RNase Inhibitor (40 U/ μ L)	0.25 μ L
DTT (0.1 M)	0.05 μ L
Reverse Transcriptase (RT) (200U/ μ L)	0.25 μ L

Table 3. cDNA Synthesis. Reaction for synthesis of cDNA from total RNA.

Ingredients - Master Mix I	Amount for One Reaction
RNA and ddH ₂ O (DEPC treated)	11.0 µL
Random Hexamer (50 ng/µL)	1.0 µL
dNTP (10 mM)	1.0 µL
Ingredients – Master Mix II	Amount for One Reaction
First-Strand Buffer (5x)	4.0 µL
RNase Inhibitor (40 U/µL)	1.0 µL
DTT (0.1 M)	1.0 µL
Reverse Transcriptase (RT) (200U/µL)	1.0 µL

Table 4. Fluorescent RT-PCR. Reaction for analysis of the spliced reporter transcripts by RT-PCR using Cy5-labeled primer.

Ingredients	Amount for One Reaction
cDNA Template (100 ng/μL)	1.00 μL
Forward Primer (Dup8f) (10 μM)	0.25 μL
Cy5 Reverse Primer (Cy5-Dup3r) (10 μM)	0.25 μL
dNTP (10 μM)	0.25 μL
Taq Buffer (10x)	1.25 μL
Taq DNA Polymerase (5000 U/mL)	0.25 μL
ddH ₂ O (DEPC treated)	Bring up volume to 12.5 μL with ddH ₂ O

Table 5. ^{32}P -U1-snRNA Primer Extension. Reaction for analysis of variant U1 snRNA expression by primer extension.

Ingredients	Amount for One Reaction
RNA and ddH ₂ O (DEPC treated)	4.325 μL
dATP (10 mM)	0.375 μL
^{32}P -U1 ₇₋₂₆ -R oligo nucleotide*	1.000 μL
Ingredients – Master Mix	Amount for One Reaction
First-Strand Buffer (5x)	1.5 μL
RNase Inhibitor (40 U/ μL)	0.1 μL
DTT (0.1 M)	0.1 μL
Reverse Transcriptase (RT) (200U/ μL)	0.1 μL

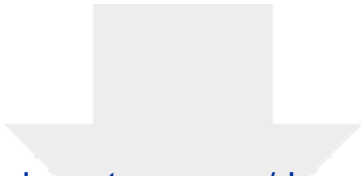
*10,000 CPM of the ^{32}P -U1₇₋₂₆-R oligonucleotide should be added to the diluted RNA (Step 6.2.).

Table 6. Quantitative RT-qPCR. Reactions for quantitative analysis of variant U1 snRNA

Ingredients	Amount for One Reaction
Real Time PCR Mix	10.0 μL
cDNA (0.2 ng/ μL)	5.0 μL
Forward Primer (10 μM)	0.2 μL
Reverse Primer (10 μM)	0.2 μL
ddH ₂ O (DEPC treated)	4.6 μL

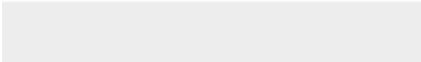
Table 7. Sequence of Oligonucleotides Primers. List of primer names, sequences, and the technical application.

Oligo Name	Sequence (5' to 3')	Technique
Dup8f	GACACCATGCATGGTGCACC	Fluorescent RT-PCR
Cy5-Dup3r	/5Cy5/AACAGCATCAGGAGTGGACAGATCCC	Fluorescent RT-PCR
Dup3r	AACAGCATCAGGAGTGGACAGATCCC	Primer Extension
U1 ₇₋₂₆ R	TGGTATCTCCCCTGCCAGGT	Primer Extension
U1/ ₁₇₋₃₉ F	GGAGATACCATGATCACGAAGG	RT-qPCR
U1/ ₅₈₋₈₀ R	CATCCGGAGTGCAATGGATAAG	RT-qPCR
U2/ ₈₋₁₉ F	CTCGGCCTTTTGGCTAAGATCA	RT-qPCR
U2/ ₆₂₋₈₄ R	TCCTCGGATAGAGGACGTATCA	RT-qPCR



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Table of Materials
Materials Table_07-22-2021.xls





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Dr. Nilanjana Saha
Review Editor
Journal of Visualized Experiments

Dear Dr. Saha:

I am submitting a revised version of our manuscript (JoVE63014) entitled "A reporter based cellular assay for monitoring splicing efficiency" for your consideration.

We sincerely appreciate the editor and the reviewers for their constructive feedback. In response to their comments, we have revised the manuscript to carefully address each of the comments. The changes to the manuscript are tracked to identify the edits and content for videography is highlighted. The details of our response and the edits to the manuscript are attached.

We hope that with these improvements, the manuscript is now acceptable for publication in *Journal of Visualized Experiments*. Please let me know if you need more information.

Thank you for your consideration!

Sincerely,

A handwritten signature in blue ink that reads 'Shalini' with a horizontal line underneath.

Shalini Sharma, PhD
Associate Professor

We thank the editors and reviewers for their careful reading of the manuscript. In response to their helpful suggestions, we have revised the text and figures, and trust that the revisions clarify and enhance the revised manuscript. The specific changes and our responses to the points raised in the critique are tracked in the revised manuscript and detailed below:

Editorial comments:

Changes to be made by the Author(s):

- 1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**

We have proofread the manuscript to check for spelling or grammar and would willingly correct any further errors.

- 2. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, a protocol is presented to ..."**

We have revised the abstract, reducing the size 50 words.

- 3. The current Abstract is over the 150-300 word limit. Please rephrase the Abstract to more clearly state the goal of the protocol.**

We checked and found that the abstract is 291 words long, and therefore, did not change it.

- 4. Please ensure that abbreviations are defined at first usage.**

We have gone through the protocol and defined abbreviations at first usage on pages 1 (line 38 and 40) and 2 (line 82) of the revised manuscript.

- 5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each Step to supplement the actions seen in the video so that viewers can easily replicate the protocol.**

We have gone through the protocol to check that we are explaining how each step is performed. However, if any of the Steps need further explanation, we would be happy to provide it.

- 6. Please add more details to your protocol steps:**

Step 1.1: What the (*) symbol denotes here?

This was an error; the symbol has been deleted.

Step 1.2/1.3/1.4/1.5: Please mention the filtration membrane size and material.

A NOTE has been added to page 2 that includes membrane size and material information.

Step 2.10/8.1: Please mention the centrifugation speed and temperature.

The centrifugation speed and temperature have been added to Steps 2.11 (2.10 in the original manuscript) and 8.1 which are on page 4 and page 10, respectively, of the revised manuscript.

Step 2.12: Please elaborate on this step in case this needs to be filmed.

Step 2.13 (2.12 in the original manuscript) is described in detail in Steps 2.13.1 – 2.13.11 on page 5 of the revised manuscript.

Step 2.13: How long can the RNA be stored?

RNA can be stored for 6-12 months. This information has been added to Step 2.14 (2.13 in the original manuscript) on page 5, line 203 of the revised manuscript.

Step 3.3: Please provide a detailed explanation for this step in case this needs to be filmed.

Step 3.3 has been elaborated further and appears in Steps 3.3 and 3.4 on pages 5 of the revised manuscript.

Step 3.4: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). Also, please mention the temperature.

In Step 3.5 (Step 3.4 in the original manuscript), centrifugation speed has been converted to centrifugal force and temperature has been added.

Step 3.7/7.6: What is the centrifugation temperature?

Centrifugation temperature has been added to Steps 3.8 (Step 3.7 in the original manuscript) and 7.6 on pages 6 and page 9, respectively of the revised manuscript.

Step 4.8: Please explain how the step is done.

An equation for the calculation of % exon 2 inclusion has been added to Step 4.8 on page 6 of the revised manuscript.

7. **Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Also, please ensure that it is in line with the title of the manuscript. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will shall be available to the reader.**

Protocol steps are separated by one-line space. The steps that need to be visualized have been highlighted.

8. **As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:**
 - a) **Critical steps within the protocol**
 - b) **Any modifications and troubleshooting of the technique**

- c) Any limitations of the technique
- d) The significance with respect to existing methods
- e) Any future applications of the technique

To cover the above points, a paragraph has been added to the Discussion on page 12 of the revised manuscript.

9. **Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).**

Figure have been prepared as .ai files.

10. **Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.**

All tables have been removed from the manuscript and prepared separately as an .xlsx file. Table titles and descriptions have been added after the Representative Results on pages 11-12 of the revised manuscript.

11. **Please spell out the journal titles in the References.**

References have been formatted in the JoVE output style using Endnote20.

Reviewer #1:

Major Concerns:

1. **In this study, the author designed the splicing site mutations associated with Usher syndrome and designed the corresponding U1 snRNA to restore the splicing abnormalities caused by this mutation. Then, in different minigenes or different types of mutations, whether a U1 snRNA can be designed to restore the effects of mutations?**

The reviewer is correct, minigenes carrying other 5'-splice site mutations can be used to design U1 snRNA carrying compensatory sequence changes to their 5'-region for rescue of splicing.

2. **The author believes that the exogenous variant snRNA may be sufficient to splice the reporter gene precursor mRNA. Please provide direct evidence to confirm it.**

We appreciate the reviewer for bringing this up. Actually, the ability of the U1-5a snRNA to rescue exon 2 inclusion to ~95% is the evidence that the variant U1-5a snRNA levels are sufficient for splicing of the Dup51p transcript. We have edited the text to include this explanation on page 11, line 464-467 of the revised manuscript.

Reviewer #2:

Minor Concerns:

I have only a few questions concerning the method and reagents described:

1. **item 1.2, please indicate the concentration of components used to prepare DMEM or the amount of each packet of DMEM (in grams) - although many labs will use DMEM from the same manufacturer, other media might also be used; also please indicate the final penicillin/streptomycin concentration.**

The amount of DMEM powder (in grams), and the final penicillin and streptomycin concentrations have been added to Step 1.2 on pages 2-3 of the revised manuscript.

2. **item 1.5, please indicate trypsin molarity to prepare the 10X solution.**

The 10x solution has 2.5% trypsin and this information has been added to Step 1.5 on page 3 of the revised manuscript.

3. **item 1.6, please indicate the final volume of the formamide DNA/RNA loading dye.**

The final volume of the formamide dye has been added to Step 1.6 on page 3 of the revised manuscript.

4. **item 2.1, is the number of passages important for this experiment? This might be important to note because different labs might have HeLa cells with variable number of passages.**

The number of HeLa cell passages is not important for these experiments.

5. **item 2.7, the medium used here is still DMEM? With or without serum?**

In Step 2.7, the medium used is DMEM with serum and the text has been edited on page 4 of the revised manuscript.

6. **item 2.12, does the RNA need to be precipitated after extraction? If so, please specify.**

Yes, the RNA needs to be precipitated. The steps involved in extraction and precipitation have been elaborated on page 5 of the revised manuscript.

7. **item 3.3, please specify the recommended exclusion size of the beads.**

The beads have a molecular weight cut off of 25 kDa. This information has been added to Step 3.3 on page 5 of the revised manuscript.

8. **item 8, is there a recommended length for the Urea-PAGE gels?**

Length of the urea-PAGE gels has been added to Steps 4.7 and 6.6 of the revised manuscript on pages 6 and 8, respectively.

9. **Tables 1-5: please specify the enzymes concentrations (T4 PNK or RT), in U.**

The enzyme concentration has been added in units to Tables 1-5.