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## Using In Vitro and In-cell SHAPE to Investigate Small Molecule induced pre-mRNA Structural Change --Manuscript Draft--

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**TITLE:**

Using *In Vitro* and In-cell SHAPE to Investigate Small Molecule Induced Pre-mRNA Structural Changes

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**KEYWORDS:**

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**SUMMARY:**

Detailed protocols for both *in vitro* and in-cell selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) experiments to determine the secondary structure of pre-mRNA sequences of interest in the presence of an RNA-targeting small molecule are presented in this article.

**ABSTRACT:**

In the process of drug development of RNA-targeting small molecules, elucidating the structural changes upon their interactions with target RNA sequences is desired. We herein provide a detailed *in vitro* and in-cell selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) protocol to study the RNA structural change in the presence of an experimental drug for spinal muscular atrophy (SMA), survival of motor neuron (SMN)-C2, and in exon 7 of the pre-mRNA of the SMN2 gene. In *in vitro* SHAPE, an RNA sequence of 140 nucleotides containing SMN2 exon 7 is transcribed by T7 RNA polymerase, folded in the presence of SMN-C2, and subsequently modified by a mild 2'-OH acylation reagent, 2-methylnicotinic acid imidazolide (NAI). This 2'-OH-NAI adduct is further probed by a <sup>32</sup>P-labeled primer extension and resolved by polyacrylamide gel electrophoresis (PAGE). Conversely, 2'-OH acylation in in-cell SHAPE takes place *in situ* with SMN-C2 bound cellular RNA in living cells. The pre-mRNA sequence of exon 7 in the SMN2 gene, along with SHAPE-induced mutations in the primer extension, was then amplified by PCR and subject to next-generation sequencing. Comparing the two methodologies, *in vitro* SHAPE is a more cost-effective method and does not require computational power to visualize results. However, the *in vitro* SHAPE-derived RNA model sometimes deviates from the secondary structure in a cellular context, likely due to the loss of all interactions with RNA-binding proteins. In-cell SHAPE does not need a radioactive material workplace and yields a more accurate RNA

secondary structure in the cellular context. Furthermore, in-cell SHAPE is usually applicable for a larger range of RNA sequences (~1,000 nucleotides) by utilizing next-generation sequencing, compared to *in vitro* SHAPE (~200 nucleotides) that usually relies on PAGE analysis. In case of exon 7 in SMN2 pre-mRNA, the *in vitro* and in-cell SHAPE derived RNA models are similar to each other.

## INTRODUCTION:

Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) is a method of measuring the kinetics of each nucleotide in an RNA sequence of interest and elucidating the secondary structure at single-nucleotide resolution<sup>1</sup>. SHAPE methodologies, both in *in vitro* conditions<sup>2-4</sup> (purified RNA in a defined buffer system) and in living mammalian cells<sup>5,6</sup>, have been developed to investigate the secondary structure of medium length RNA sequences (typically <1,000 nucleotides for in-cell SHAPE and <200 nucleotides for *in vitro* SHAPE). It is particularly useful to evaluate structural changes in receptor RNA upon binding to RNA-interacting small molecule metabolites<sup>2,4,7,8</sup> and to study mechanistic actions of RNA-targeting molecules during drug development<sup>9,10</sup>.

RNA-targeting drug discovery has recently drawn attention in academic laboratories and the pharmaceutical industry<sup>11,12</sup> via different approaches and strategies<sup>13-16</sup>. Recent examples of RNA-targeting small molecules for clinical use include two structurally distinct experimental drugs, LMI-070<sup>17</sup> and RG-7916<sup>18,19</sup>, for spinal muscular atrophy (SMA), which showed promising results in phase II clinical trials<sup>20</sup>. Both molecules were demonstrated to target survival of motor neuron (SMN) 2 pre-mRNA and regulate the splicing process of the SMN2 gene<sup>6,17,21</sup>. We previously demonstrated the application of *in vitro* and in-cell SHAPE in an examination of the target RNA structural changes in the presence of an analog of RG-7916 known as SMN-C2<sup>6</sup>.

In principle, SHAPE measures the 2'-OH acylation rate of each nucleotide of an RNA sequence in the presence of excess amounts of a self-quenching acylation reagent in an unbiased manner. The acylation reagent is not stable in water, with a short half-life of (*e.g.*,  $T_{1/2}$  = 17 s for 1-methyl-7-nitroisatoic anhydride; or 1M7, ~20 min for 2-methylnicotinic acid imidazolide, or NAI)<sup>22</sup> and insensitivity to the identity of the bases<sup>23</sup>. This results in a more favorable acylation of the 2'-OH groups of flexible bases, which can be transformed into an accurate assessment of the dynamics of each nucleotide. Specifically, a nucleotide in a base-pair is usually less reactive than an unpaired one to a 2'-OH modifying reagent, such as NAI and 1M7.

Looking at the source of the RNA template and where 2'-OH acylation takes place, SHAPE can generally be categorized into *in vitro* and in-cell SHAPE. *In vitro* SHAPE uses purified T7 transcribed RNA and lacks a cellular context in experimental designs. In in-cell SHAPE, both the RNA template transcription and 2'-OH acylation occur within living cells; therefore, the results can recapitulate the RNA structural model in a cellular context. In-cell SHAPE has been referred to as *in vivo* SHAPE for the SHAPE carried in living cells in the literature<sup>24</sup>. Since this experiment is not performed in an animal, we termed this experiment as in-cell SHAPE for accuracy.

The strategies for the primer extension stage of *in vitro* and in-cell SHAPE are also different. In *in vitro* SHAPE, reverse transcription stops at the 2'-OH acylation position in the presence of Mg<sup>2+</sup>. A <sup>32</sup>P-labeled primer extension therefore appears as a band in polyacrylamide gel electrophoresis (PAGE) and the intensity of the band is proportional to the acylation rate<sup>1</sup>. In in-cell SHAPE, reverse transcription generates random mutations at the 2'-OH adduct position in the presence of Mn<sup>2+</sup>. The mutational rate of each nucleotide can be captured by in depth next-generation sequencing, and the SHAPE reactivity at single-nucleotide resolution can then be calculated.

A potential problem for in-cell SHAPE is the low signal-to-noise ratio (*i.e.*, a majority of the 2'-OH groups is unmodified, while the unmodified sequences occupy most of the read in next-generation sequencing). Recently, a method to enrich the 2'-OH modified RNA, referred to as *in vivo* click SHAPE (icSHAPE), was developed by the Chang laboratory<sup>25</sup>. This enrichment method may be advantageous in studying weak small molecules such as RNA interactions, especially in a transcriptome-wide interrogation.

## PROTOCOL:

### 1. *In Vitro* SHAPE

NOTE: The protocol is modified from the published protocol<sup>1</sup>.

#### 1.1 Preparing the RNA template

NOTE: The template for T7 transcription was ordered as a synthetic double-stranded DNA (dsDNA) and amplified by either insertion into an *E. coli* vector that carries a unique pair of EcoRI/BamHI restriction endonuclease sites, such as pET28a, or by PCR. The protocol for PCR amplification is illustrated below.

1.1.1 Mix the following material: 50 µL of PCR master mix (see **Table of Materials**), 2 µL for each primer in 10 µM (see **Table 1** for sequences), 200 ng of dsDNA template in 2 µL and 3 µL of dimethyl sulfoxide (DMSO), and 41 µL of H<sub>2</sub>O. Aliquot into two PCR tubes (each tube contains 50 µL of reaction mixture).

1.1.2 Set up the thermal cycler as follows: stage 1) 95 °C for 30 s; stage 2) 95 °C for 20 s; stage 3) 56 °C for 20 s; stage 4) 72 °C for 20 s; stage 5) loop back to stage 2 for 30 cycles; stage 6) 72 °C for 3 min; and stage 7) infinite holding at 4 °C until the following step.

1.1.3 Purify the PCR amplicon by extraction of gel slices (see **Table of Materials** and use the manufacturer's protocol). Elute the product DNA with 35 µL of Tris-EDTA (TE) buffer, containing 10 mM Tris (pH 8.0) and 1 mM EDTA, to yield a 0.1–0.5 µg/µL template. Normalize the purified DNA template into 0.10 µg/µL.

NOTE: Optionally, the quality of the template may be analyzed on a 1.8% agarose gel electrophoresis with 0.10 µg of DNA. A single band of desired template DNA is expected on the gel.

1.1.4 Set up the T7 transcription reaction (total volume 40 µL) by adding the following materials into a PCR tube: 4 µL of 10x reaction buffer, 4 µL of ATP, 4 µL of CTP, 4 µL of GTP, 4 µL of UTP, 4 µL of T7 enzyme (see **Table of Materials**), 4 µL of purified PCR amplicon from step 1.1.3 (0.4 µg), and 12 µL of diethyl pyrocarbonate (DEPC)-treated water. Mix by pipetting up and down 4x. Incubate at 37 °C for 4–16 h in a thermal cycler or in a 37 °C incubator.

NOTE: Start to set up step 1.1.6 while the reaction in step 1.1.4 is ongoing.

1.1.5 Add 2 µL of DNase, mix by pipetting up and down 4x, and incubate at 37 °C for 15 min. Mix with equal volume of 2x Tris-borate-EDTA (TBE)-urea sample buffer (see **Table of Materials**). Heat at 70 °C for 3 min to inactivate.

1.1.6 In an RNase-free bottle, mix 75 mL of 40% acrylamide/bisacrylamide solution (29:1, see **Table of Materials**), 180.2 g of urea, and 50 mL of 10x TBE buffer (RNase-free, see **Table of Materials**). Put the bottle on an orbital shaker (250 rpm) until all crystals of urea are dissolved (may take up to 2 h). Adjust the volume to 500 mL with DEPC-treated water. Filter the solution with a 0.2 µm membrane (see **Table of Materials**).

NOTE: In this process, avoid using specular and stir-bar to prevent RNase contamination. The solution can be stored at 4 °C for up to 1 year.

1.1.7 Clean two electrophoresis glass plates of a proper size (16.5 cm x 24 cm) with deionized water and 70% EtOH using a piece of wiping paper. Align the plates with a pair of 2.0 mm spacers (the plate with a siliconized surface is facing inward) and seal the edge of the plates with tape. Double-tape the corner of the plate to prevent leaking.

1.1.8 In a beaker, mix 200 mL of acrylamide solution from step 1.1.6, 2.0 mL of 10% ammonium persulfate solution, and 100 µL of tetramethylethylenediamine (TEMED) with an RNase-free pipette tip by stirring rapidly for 30 s. Tilt the plates on a piece of benchtop cover and pour the solution from the gap of the plates. Tap the plate to lift any bubbles and lay the plate flat on the benchtop cover. Immediately insert the comb and let the gel polymerize for at least 1 h.

NOTE: If the gel is to be used within the next day, cover the top of the gel with a piece of wet paper towel and wrap up with a larger piece of plastic wrap. Place it lying flat at 4 °C.

1.1.9 Remove the tape and clamp the plate in an electrophoresis stand. Remove the comb and add adequate 1x TBE buffer at the top and bottom of the reservoir. Pre-run the gel for 30 min at 60 W.

NOTE: Optionally, if the content of the desired RNA is ~90% or more, a mini-gel can be used in substitution of a preparative gel.

1.1.10 Wash each of the loading wells by pipetting up and down twice with the liquid in the reservoir. Add crude RNA from step 1.1.5 into the wells. Run the gel at 60 W until the xylene cyanol FF dye (light blue) passes about two-thirds of the gel's length (~60 min).

NOTE: Ensure to load no more than one-third of the height of the well (use multiple wells if necessary).

1.1.11 Immerse the gel in 1x TBE buffer with 1:10,000 dilution of the safe dye (see **Table of Materials**) in a container large enough for the gel. Put the container on an orbital shaker for 5 min at 80 rpm.

1.1.12 Under UV light, identify the desired RNA band and swiftly cut a thin band of the gel using a clean razor blade. Place 1–2 gel slices in a 1.5 mL tube.

1.1.13 Recover the RNA from the gel slice by passive elution. Add adequate elution/precipitation mix (~0.3 mL per slice) to each tube: 0.3 M NaOAc (pH 5.2), 2.5 M LiCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate (SDS) in DEPC-treated water. Rotate the tube that contains the gel splice(s) at 4 °C for 16 h.

NOTE: A typical recovery rate is ~75% in this step. To increase the yield, remove and collect the eluent and add the same volume of elution buffer, then repeat this step.

1.1.14 Combine the eluent, add 2.5x ice-cold EtOH, invert the tubes six times to mix the liquid, and place the tubes at -80 °C for at least 1 h.

1.1.15 Centrifuge for 10 min at 10,000 x g and 4 °C. Carefully remove the supernatant by pipetting. Wash the RNA pellet by adding 80% EtOH (1 mL per tube), vortex for 15 s, and centrifuge for 10 min at 10,000 x g and 4 °C.

1.1.16 Remove the supernatant and air-dry the RNA pellet for 5 min. Add 50 µL of RNase-free water and mix by pipetting up and down 10 times. Normalize the RNA concentration to 0.10 µg/µL. Store the purified RNA at -80 °C.

NOTE: Do not over-dry the RNA pellet.

1.1.17 Optionally, to analyze the quality of the RNA, dilute 1 µL (100 ng) of RNA from step 1.1.16 in 5 µL of water and mix with 5 µL of 2x TBE-urea sample buffer. Then, heat at 70 °C for 3 min and load on a 6% TBE-urea mini-gel.

1.1.17.1 Run the gel at 180 V for 1 h. Perform the staining as done in step 1.1.11. Visualize the gel with UV in an imaging system. A single band is expected at the desired length.

## 1.2 Preparing the $^{32}\text{P}$ -labeled primer

1.2.1 Set up the reaction by mixing the following materials: 10  $\mu\text{L}$  of  $\gamma$ - $^{32}\text{P}$ -ATP (~1.5 mCi, ~25  $\mu\text{M}$ , see **Table of Materials**), 2  $\mu\text{L}$  of reverse transcription (RT) primer (100  $\mu\text{M}$ , for sequence see **Table 1**), 2  $\mu\text{L}$  of 10x T4 polynucleotide kinase (PNK) reaction buffer, 1  $\mu\text{L}$  of T4 PNK (see **Table of Materials**), and 5  $\mu\text{L}$  of RNase-free water. Incubate at 37  $^{\circ}\text{C}$  for 1 h.

1.2.2 Mix with 25  $\mu\text{L}$  of 2x TBE-urea sample buffer. Heat-inactivate for 20 min at 65  $^{\circ}\text{C}$ .

NOTE: Store the labeled crude product at -20  $^{\circ}\text{C}$  if it is not to be immediately purified.

1.2.3 Run a 20% acrylamide gel at 60 W for 1 h.

CAUTION: Load the  $^{32}\text{P}$ -labeled primer from step 1.2.1 onto the gel and avoid bleeding the radioactivity into the top reservoir. Do not load more than one-third of the height of the well to ensure a thin band on the gel (use multiple wells if necessary). The liquid in the bottom reservoir can be highly radioactive.

1.2.4 Remove the glass plates of the gel cassette and lay the gel on a piece of plastic wrap. Top the gel with another piece of plastic wrap to make an air-tight sandwich. Expose the gel to a phosphor storage screen for 1 h.

1.2.5 After imaging, align and cut a thin band of the gel using a clean razor blade. Place 1 to 2 gel slices in a 1.5 mL tube. Make sure the alignment is correct by exposing the leftover gel to the phosphorus storage screen and image again.

1.2.6 Recover the  $^{32}\text{P}$ -labeled primer by following steps 1.1.13 to 1.1.16. Take 1.0  $\mu\text{L}$  of solution into a 0.4 mL tube and dilute the primer with 1x TE buffer to obtain the radioactivity of 1.0  $\mu\text{L}$  at ~100,000 cpm. Store the purified  $^{32}\text{P}$ -labeled primer at -80  $^{\circ}\text{C}$  until RNA 2'-OH modification reaction but for no longer than 4 weeks.

## 1.3 Small molecule binding and RNA 2'-OH modification

1.3.1 To prepare four samples, add 8 pmol RNA in 32  $\mu\text{L}$  of 0.5x TE buffer to a 1.5 mL tube, heat at 80  $^{\circ}\text{C}$  for 2 min, and snap-cool on ice for at least 1 min.

NOTE: Use the following equation to calculate the approximate molecular weight of RNA:  $\text{MW} = (\text{\# of bases}) \times 340 \text{ Da}$ .

1.3.2 Add 8  $\mu\text{L}$  of 5x folding mix containing 500 mM HEPES (pH 8.0), 20 mM  $\text{MgCl}_2$ , and 500 mM NaCl. Aliquot 9  $\mu\text{L}$  of the RNA mixture into each of the four PCR tubes. Add 1  $\mu\text{L}$  of 10% DMSO into the #1 tube, 1  $\mu\text{L}$  of concentrated small molecule solution (10% DMSO) into #2, 1  $\mu\text{L}$  of diluted small molecule solution (10% DMSO) into #3, and 1  $\mu\text{L}$  of water into #4.

1.3.3 Incubate the PCR tubes at 37 °C in a thermal cycler for 30 min.

1.3.4 Right before the 2'-OH modification reaction, dilute 1 µL of NAI stock solution (2 M) with 3 µL of DEPC-treated water to yield a 0.5 M working solution. Without delay, add 1 µL of NAI working solution into tubes #1, #2, and #3, and 1 µL of 25% DMSO into #4. Incubate at 37 °C in a thermal cycler for 15 min.

1.3.5 Add 100 µL of elution/precipitation mix (see step 1.1.13 for recipe), 2 µL of glycogen (15 mg/mL), and transfer all the liquid in each PCR tube into a 1.5 mL tube. Add 0.34 mL of ice-cold EtOH (3x volume) into each tube. Mix by vortexing for 5 s and place the tubes in a -80 °C freezer for at least 1 h.

NOTE: During this period, start setting up the sequencing gel to be used in step 1.5.1.

1.3.6 Centrifuge for 15 min at 20,000 x *g* and 4 °C. Remove the supernatant without disturbing the RNA pellet. Wash the RNA pellet by adding 80% EtOH (0.5 mL per tube), vortex for 15 s, and centrifuge for 15 min at 20,000 x *g* and 4 °C. Remove the supernatant again.

NOTE: Optionally, use a Geiger counter to ensure the radioactive pellet retaining in the tube.

1.3.7 Air-dry the RNA pellet for 5 min. Add 9 µL of RNase-free water and mix by pipetting up and down 10 times.

NOTE: Do not over-dry the RNA pellet. All precipitation is expected to be dissolved at the end of this step.

#### 1.4 Marker preparation and primer extension

1.4.1 Add 2 pmol of RNA in 7 µL of DEPC-treated water in each of the four 1.5 mL tubes and label them #5–#8. Add 2 µL of radiolabeled primer (step 1.2.6) in all eight tubes. Heat to anneal at 65 °C for 5 min, then immediately place the tubes on ice.

1.4.2 Add 4 µL of 5x RT buffer (see **Table of Materials**), 1 µL of dithiothreitol (DTT, 0.1 M), and 1 µL of dNTP mix (10 mM each) to each of the tubes.

1.4.3 Add 2 µL of DEPC-treated H<sub>2</sub>O to tubes #1–#4. Add 4 µL of ddATP (5 mM), 4 µL of ddTTP (5 mM), and 4 µL of ddCTP (5 mM) into tubes #5–#7, respectively. Add 1 µL of ddGTP (5 mM) and 3 µL of DEPC-treated water to tube #8. Mix by flicking each tube four times.

1.4.4 Heat at 52 °C for 1 min. Add 1 µL of reverse transcriptase (see **Table of Materials**), then mix by pipetting up and down four times. Incubate the reaction at 52 °C for 20 min.



1.4.5 Add 1  $\mu$ L of 5 M NaOH into each of the tubes to hydrolyze the RNA. Heat the tubes at 95 °C for 5 min.

1.4.6 Add 5  $\mu$ L of 1 M HCl and repeat the ethanol precipitation (steps 1.3.5 and 1.3.6), except omit the glycogen and liquid transfer.

NOTE: Omitting step 1.4.6 results in a blurring region in the middle of the gel known as the “salt front”.

1.4.7 Air-dry the DNA pellet for 5 min. Add 10  $\mu$ L of H<sub>2</sub>O and 10  $\mu$ L of 2x TBE-urea sample loading buffer and pipette up and down 10 times to redissolve. Heat at 70 °C for 5 min. Place the tubes on ice before loading onto the gel.

## 1.5 PAGE analysis

1.5.1 To prepare an 8% polyacrylamide sequencing gel, follow steps 1.1.6 to 1.1.10 with the following modifications: use 100 mL of 40% acrylamide/bisacrylamide solution (29:1) to prepare an 8% acrylamide solution, and use sequencing gel glass plates (*e.g.*, 46 x 57 cm) with a 0.4 mm spacer.

1.5.2 Load 10  $\mu$ L of the sample from step 1.4.7. Run the gel at 200 W for 3 h.

NOTE: Store the rest of the samples at -80 °C for repeating if needed.

1.5.3 Remove the top siliconized glass plate by removing the spacer and gently inserting a specular. Place a piece of large filter paper to cover the gel and press gently to allow the gel to adhere to the filter paper. Starting from the bottom end, lift the filter paper and remove the gel from the glass plate together.

1.5.4 Place the gel together with the filter paper on a piece of plastic wrap that is large enough to cover the whole gel (filter paper facing up). Transfer the filter paper/gel/plastic wrap sandwich to a gel drier with the filter paper side facing down.

CAUTION: To avoid radioactive material contamination, use 3 to 4 more pieces of large filter paper between the gel sandwich and gel drier.

1.5.5 Dry the gel at 70 °C for 1 h with a vacuum. Transfer the gel sandwich onto a photo-bleached phosphor storage screen cassette. Expose the radioactivity of the gel to the screen for 4–16 h.

1.5.6 Place the phosphor storage screen on an imaging device and scan with medium resolution (~30 min).

NOTE: If a smile-like artifact is present on the gel image, use correcting software (*e.g.*, SAFA) to process the image to a publishable quality. Keep in mind that the standard marker lane is 1 nucleotide longer than the 2'-OH modification lanes.

## 2. In-cell SHAPE

### 2.1 Primer design

NOTE: Unlike *in vitro* SHAPE, in-cell SHAPE does not need to design an expression cassette. However, an optimal primer set should be used and must be evaluated before SHAPE experiment.

2.1.1 Generate at least three unique primer pairs by a BLAST-based primer design tool (*e.g.*, Primer-BLAST). To study pre-mRNA in human cells, select the human genome (not transcriptome) as a reference database in Primer Pair Specificity Checking Parameters. Pick the size of amplicon in the range of 200–1,000 base-pair (bp).

2.1.2 Extract the genomic DNA from  $\sim 10^6$  cells of interest with a spin column-based method with the treatment of RNase A and protease K (see **Table of Materials** and use the manufacturer's protocol). Normalize the purified genomic DNA in 0.1  $\mu\text{g}/\mu\text{L}$  TE buffer.

2.1.3 Test PCR with the protocol performed in steps 1.1.1 and 1.1.2.

NOTE: The desired primer set should yield a single band on a 1.8% agarose gel.

### 2.2 Cell preparation and 2'-OH modification

NOTE: To increase the abundance of the pre-mRNA of interest, a minigene with the correct sequence under cytomegalovirus (CMV) promoter for constitutive expression is transfected into the host cells.

2.2.1 Pre-warm the culture medium containing 10% fetal bovine serum (FBS) and 1x antibiotic mixture in Dulbecco's Modified Eagle Medium (DMEM) at 37 °C. Seed 293T cells at 50% confluency 24 h prior to the transfection in culture medium. Change the medium into 2% FBS in DMEM without antibiotics  $\sim 1$  h prior to the transfection.

2.2.2 Add 10  $\mu\text{g}$  of minigene plasmid into 100  $\mu\text{L}$  of reduced serum media (see **Table of Materials**) in 1 well of a 96-well plate. Pipette the solution up and down four times to mix.

2.2.3 Add 40  $\mu\text{L}$  of transfection reagent (see **Table of Materials**) into 100  $\mu\text{L}$  of reduced serum media in another well of the plate. Pipette up and down four times to mix. Incubate at room temperature for 5 min.

2.2.4 Add the entire plasmid solution to the transfection reagent suspension. Pipette up and down four times to mix. Incubate at room temperature for 30 min.

2.2.5 Add the entire DNA/ transfection reagent mixture dropwise on the surface of the medium throughout the dish. Incubate at 37 °C for 6–12 h.

2.2.6 Aspirate the medium and gently wash once with 5 mL of phosphate-buffered saline (PBS, pH 7.4, without CaCl<sub>2</sub> and MgCl<sub>2</sub>, see **Table of Materials**). Trypsinize the cells with 3 mL of trypsin solution. Incubate at room temperature for 5 min.

2.2.7 Knock the dish gently to dissociate the cells from the bottom of the dish. Neutralize trypsin with 6 mL of culture medium. Centrifuge at 300 x *g* for 4 min and remove the medium.

2.2.8 Resuspend the ~10<sup>6</sup> cells for each sample in 199 µL of reaction medium (1% FBS in DMEM) and transfer the cell suspension into a 96-well plate. Incubate the cells with 1 µL of compound working solution or 1 µL of DMSO in all three controls for 30 min at 37 °C.

NOTE: Three control samples should be included: DMSO control with NAI, denatured RNA with NAI, and NAI negative control.

2.2.9 Add 10 µL of 2 M NAI to each sample, except for the denaturing control (DC) RNA and NAI negative controls. Pipette up and down four times to mix. Incubate at 37 °C for 15 min. Gently shake the plates to re-suspend the cells every 5 min.

2.2.10 Transfer the cells into 1.5 mL tubes and centrifuge at 400 x *g* for 2 min without delay. Remove the supernatant without disturbing the cell pellet.

2.2.11 Add 0.4 mL of guanidinium thiocyanate (see **Table of Materials**). Vortex for 15 s to homogenize. Incubate the samples at room temperature for 5 min.

NOTE: The samples can be stored at -20 °C until proceeding to the next step.

## **2.3 RNA extraction**

2.3.1 Add 0.2 mL of chloroform to each of the guanidinium thiocyanate homogenized samples. Vortex for 15 s. Incubate at room temperature for 2 min.

2.3.2 Centrifuge for 5 min at 12,000 x *g* and 4 °C. The top colorless aqueous layer contains RNA. Transfer ~380 µL of aqueous solution into a new 1.5 mL tube.

CAUTION: Do not disturb the interphase to avoid contamination.

2.3.3 Add 1.5x volume of EtOH (~570 µL). Vortex for 15 s to mix.

2.3.4 Transfer 600  $\mu$ L of RNA mixture into an RNA isolation spin-column (see **Table of Materials**). Centrifuge at 12,000  $\times g$  for 15 s. Discard the eluent. Repeat this step to pass through all liquid into the same spin column.

2.3.5 Wash the column with 0.35 mL of RW1 buffer (see **Table of Materials**) by spinning for 15 s. Add 80  $\mu$ L of RNase-free DNase I in RDD buffer (see **Table of Materials**). Incubate at room temperature for 20 min.

NOTE: It is critical to remove all DNA contamination.

2.3.6 Subsequently wash the column with 0.35 mL of RW1 buffer and 0.6 mL of 2x RPE buffer (see **Table of Materials**) by spinning for 15 s at each step. Dry the column by centrifuging the spin column with an empty collection tube at 12,000  $\times g$  for 1 min.

2.3.7 Add 32  $\mu$ L of RNase-free water to the center of the column. Incubate for 1 min. Centrifuge at 12,000  $\times g$  for 30 s to obtain  $\sim$ 30  $\mu$ L of purified RNA solution. Put the samples at -20  $^{\circ}$ C while processing the denatured sample.

## **2.4 Denatured RNA control preparation**

2.4.1 Place 30  $\mu$ L RNA sample for DC in a 1.5 mL plastic tube on ice. Add 50  $\mu$ L of formamide and 15  $\mu$ L of DC buffer containing 300 mM HEPES (pH 8.0) and 25 mM EDTA into the tube.

2.4.2 Heat to denature the RNA at 95  $^{\circ}$ C for 1 min. Quickly add 5  $\mu$ L of NAI stock solution (2 M), then flick twice to mix and put the tube back on to the heating block. Incubate at 95  $^{\circ}$ C for an additional 1 min then immediately put the tube on ice.

2.4.3 Add 0.4 mL of guanidinium thiocyanate. Repeat steps 2.3.1–2.3.4.

2.4.4 Wash the column with 0.6 mL of 2x RPE buffer (see **Table of Materials**) by spinning for 15 s at each step. Dry the column by centrifuging the spin column with an empty collection tube at 12,000  $\times g$  for 1 min.

2.4.5 Add 32  $\mu$ L of RNase-free water to the center of the column. Incubate for 1 min. Centrifuge at 12,000  $\times g$  for 30 s to obtain  $\sim$ 30  $\mu$ L of recovered DC RNA solution.

## **2.5 Primer extension**

2.5.1 Normalize the RNA solution from 2.3.7 and 2.4.5 into 0.5  $\mu$ g/ $\mu$ L with RNase-free water. Freshly prepare 30 mM MnCl<sub>2</sub> solution from MnCl<sub>2</sub>·4H<sub>2</sub>O powder.

2.5.2 Transfer 9  $\mu$ L of RNA solution for each sample into a PCR strip. Add 1  $\mu$ L of 10 mM dNTP and 1  $\mu$ L of 2  $\mu$ M gene-specific primer (GSP) in each tube. Pipette up and down four times to mix. Incubate at 65  $^{\circ}$ C for 5 min in a thermal cycler and put on ice for >1 min.

NOTE: Alternatively, 1  $\mu$ L of a random nonamer can be used in substitution of GSP.

2.5.3 In each PCR tube, add a mixture of 2  $\mu$ L of 10x RT buffer (see **Table of Materials**), 4  $\mu$ L of 30 mM  $\text{MnCl}_2$ , and 2  $\mu$ L of 100 mM DTT. Pipette up and down 4x to mix. Incubate at 42 °C for 2 min.

2.5.4 Add 1  $\mu$ L of reverse transcriptase (see **Table of Materials**). Pipette up and down 4x to mix. Incubate at 42 °C in a thermal cycler for 3 h.

## 2.6 Library preparation and next-generation sequencing

2.6.1 Set up a PCR reaction by adding 1  $\mu$ L of DNA polymerase, 5  $\mu$ L of 10x DNA polymerase buffer (see **Table of Materials**), 2  $\mu$ L of DMSO, 1.5  $\mu$ L for each of the primers (10  $\mu$ M), 34  $\mu$ L of  $\text{H}_2\text{O}$ , and 5  $\mu$ L of cDNA from step 2.5.4.

2.6.2 Set up the thermal cycler as follows: stage 1) 95 °C for 2 min; stage 2) 95 °C for 30 s; stage 3) 60 °C for 30 s; stage 4) 68 °C for 30 s; stage 5) loop back to stage 2 for 30 cycles; stage 6) 68 °C for 3 min; and stage 7) infinite holding at 4 °C until the following step.

2.6.3 Purify the amplicon by using 1.8% agarose gel.

NOTE: The PCR band should appear as a single band on the gel.

2.6.4 Construct the library by using standard fragmentation and ligation methods established in the literature<sup>6,26</sup>.

2.6.5 Sequence on a next-generation sequencing equipment using 1 x 75 single-end reads to generate approximately 10 million reads per sample.

2.6.6 Analyze the results using ShapeMapper and SuperFold software packages developed by the Weeks group<sup>26</sup>.

## REPRESENTATIVE RESULTS:

We previously demonstrated that an RNA splicing modulator, SMN-C2, interacts with AGGAAG motif on exon 7 of the SMN2 gene's pre-mRNA, and used SHAPE to assess the RNA structural changes in the presence of SMN-C2<sup>6</sup>. The binding site of SMN-C2 is distinct from the FDA-approved antisense oligonucleotide (ASO) for SMA, nusinersen, which binds and blocks the intronic splicing silencer (ISS) on intron 7<sup>27,28</sup> (**Figure 1A**). Most known splicing regulators of SMN2 exon 7 are within the ~100 nucleotide range of exon 7 in the pre-mRNA<sup>29</sup>; therefore, a 140 and 276 nucleotide-long RNA sequences were used for *in vitro* and in-cell SHAPE, representatively, which covers exon 7 and the adjacent intron region (**Figure 1A**).

In this representative *in vitro* SHAPE analysis, the RNA sequence of interest is embedded into an optimized cassette developed by the Weeks laboratory, which is compatible for most RNA

sequences<sup>1</sup>. Occasionally, the sequence of interest interacts or interferes with this cassette. In these cases, a modified cassette can be used with the following three characteristics: i) a 3'-end specific primer binding site with a more efficient hybridization affinity for the primer than any part of the RNA sequence, ii) a highly structured hairpin loop located directly upstream of the primer binding site that will show specific and reproducible SHAPE signal (this will act as both an internal control for the experiment and method to align the signal from experiment to experiment), and iii) a 5'-end hairpin structure element, which indicates the end of the SHAPE signal. The SHAPE cassette is further flanked with self-cleaving ribozyme sequences at both 5'- and 3'-ends in order to generate a homogenous RNA<sup>30</sup>. We found that hammerhead and hepatitis delta virus ribozymes are compatible to the SHAPE cassette and usually give high yield of the desired RNA. The resulting template RNA has a 3'-end of 2',3'-cyclic phosphate and a 5'-end of hydroxyl group, which do not interfere with primer extension. A 140-nucleoside long sequence covering exon 7 of SMN2 and adjacent region in the pre-mRNA was synthesized within SHAPE and ribozyme cassette as illustrated in **Scheme 1**.

In general, the sequence of interest ligated in the expression cassette should be long enough to cover the potential secondary or higher order of structure. In the case of SMN2 exon 7, a 140-nucleotide region contains two stem-loop structures<sup>6,31</sup>. The structure of the region of interest varies case-by-case and should be evaluated by trial-and-error.

Polyacrylamide sequencing gel with <sup>32</sup>P-labeled primer extension products was chosen to visualize the *in vitro* SHAPE profile in this representative experiment. An alternative visualization method is to use capillary electrophoresis with a fluorescently labeled DNA primer<sup>32</sup>. In polyacrylamide sequencing gels, ~20 nucleosides near the 5'-end and ~10 nucleotides near the 3'-end of the RNA sequence of interest will not be visualized quantitatively, due to occasionally stops at the initiation steps of reverse transcription and intense bands on PAGE gels for full-length transcripts<sup>1</sup>.

An alternative way for preparative gel recovery of an RNA template (steps 1.1.6 to 1.1.12) is to overload a mini-gel if the yield of desired RNA template is >90%. It is advised to remove the excess NTP from the RNA product by desalting the column and eluting the RNA in 50 µL of TE buffer. Measure the concentration of the RNA and load 5.0 µg in each well. During the purification step of the T7 transcribed RNA template, stop the PAGE when the xylene cyanol FF dye (light blue) passed two-thirds of the 6% denatured TBE-urea gel. The self-cleavage RNA fragment (<80 nucleosides) passed through the gel, which left the desired RNA as the only major band in the gel upon staining (**Figure 1B**).

SMN-C2 (**Figure 1C**) was synthesized according to the published procedure<sup>33</sup> and dissolved in 10 mM DMSO stock solution. The stock solution is further diluted into 500 and 50 µM in 10% DMSO solution to achieve final concentrations of 50 and 5 µM, respectively. Snap-cooled RNA refolded to its equilibrium stage in the presence of DMSO or SMN-C2 within 30 min at 37 °C. A longer incubation time did not change the outcome of the experiment. Two experimental samples (50 and 5 µM SMN-C2), two controls (DMSO and NAI- controls), and four markers (A, T, G, C) were treated for primer extension. After exposing the gel to phosphor storage screen, a successful

SHAPE experiment will show: i) a single and most-intense band at the top of the gel and ii) bands throughout the gel at single-nucleotide resolution without smear (**Figure 1D**). A common problem in PAGE analysis is that a smear region known as the “salt front” may appear in the middle of the gel (**Figure 1E**). This is probably due to high concentration of salt, DMSO, or other unwanted substance in the loading sample that can be removed by ethanol precipitation.

In *in vitro* SHAPE, a pure RNA template is the key for a successful experiment. An impure RNA template is usually the cause of undesired results. If PAGE analysis clearly shows a pattern of 2 sets of markers, it indicates that the RNA template is not homogenous and needs to be repurified by preparative TBE-urea gel.

For in-cell SHAPE, a key to a successful experiment is to design an optimized primer set for amplification. With 0.10 µg of genomic DNA-free cDNA template, a single band should be observed within 25 PCR cycles in agarose gel analysis. The repetitive intron sequences should therefore be avoided. To study the structural impact of SMN-C2 on SMN2 pre-mRNA, three primer sets (**Table 2**) was tested, and all were satisfactory (**Figure 2A**).

A low copy number of a target RNA sequence is generally a problem for in-cell SHAPE. To enrich the RNA of interest, a minigene that contains the RNA sequence of interest under a strong CMV promoter was transfected into 293T cells. Because the splicing pattern of the SMN2 minigene recapitulates that of endogenous SMN2 with or without SMN-C2<sup>19,34</sup>, we envision that the structure of SMN-C2 interacting RNA in the overexpressed SMN2 pre-mRNA likely was the same as the endogenous gene product. While the EC<sub>50</sub> of SMN-C3 in the splicing assay was ~0.1 µM<sup>6,19</sup>, a concentration at the higher end (20 µM) was used to ensure the binding state of the small molecule and target RNA sequence.

Upon isolation of the RNA, both gene-specific and random primers can be used for extension. In the case of exon 7 of SMN2, we found that SMN2E7-338-RV (**Table 2**) yields a higher copy of desired cDNA than a random nonamer, evidenced by a more intense band in the PCR amplification with SMN2E7-276 primer set (**Table 2**) after 25 cycles. In the 2'-OH modification step, a 91 µM final NAI concentration was used for an incubation period of 15 min. If a different cell type or medium is used, the incubation time must be re-optimized. If the incubation time is too long, agarose gel analysis of the amplicon will sometimes fail to show a band.

A python-based program, ShapeMapper, developed by the Weeks laboratory<sup>35</sup> was used to analyze the data generated by next-generation sequencing [for raw data of SMN-C2 treated in-cell SHAPE of SMN2 exon 7 pre-mRNA, refer to the Sequence Read Archive (SRA) database]. Throughout the amplicon, the SHAPE reactivity did not significantly change (>1), which indicated that the secondary structure remains in the presence of SMN-C2 (**Figure 2B**). This is also confirmed by the arc plots generated by SuperFold<sup>35</sup>. The connection lines indicate the possible base pairing based on SHAPE activity of each nucleotide. SMN-C2- and DMSO-treated RNA modeling is essentially the same (**Figure 2C**). Differential *in-cell* SHAPE reactivity was calculated for each nucleotide (**Figure 2B**), and the most reactivity change occurred at TSL-1 (5'-end of exon

7) but not TSL-2 (3'-end of exon 7). This result agrees with the *in vitro* SHAPE; although, the base pairing shifted from *in vitro* SHAPE RNA modeling in the in-cell SHAPE analyses (**Figure 2D**).

A common problem of in-cell SHAPE is the low mutation rate throughout the amplicon. This is usually due to genomic DNA contamination. DNA does not contain 2'-OH group; therefore, no acylation product is formed with NAI. PCR amplification thus merely reflects the low mutation rate of the DNA polymerase. Isolation of RNA by guanidinium thiocyanate followed by on-column DNase digestion is sufficient to remove all DNA in most cases. If DNA contamination persists, repeat step 2.3 for RNA isolation.

#### FIGURE AND TABLE LEGENDS:

**Scheme 1: DNA sequence for the template of RNA transcription.** The 12 bp sequence within the Hammerhead virus ribozyme sequence in red is the reverse complement of the first 12 bp of the 5'-cassette. The RNA sequence of interest presented herein is a 140 bp pre-mRNA sequence contains exon 7 of human SMN2 gene.

**Figure 1:** Experimental design and result of *in vitro* SHAPE for SMN2 exon 7 pre-mRNA in the presence of SMN-C2. **(A)** Overview of the sequence of interest for *in vitro* and in-cell SHAPE studies of the SMN2 gene. SMN-C2 binds to the AGGAAG motif on exon 7, a distinct location from the nusinersen binding site. **(B)** Purification of the T7 transcription product. Each of the three lanes contains 5.0 µg of crude RNA. The TBE-urea gel was stained with SYBR-Safe (1:10,000) for 5 min in 1x TBE buffer. Red dashed box indicates the edge of excision for RNA recovery. **(C)** The structure of SMN-C2. **(D)** *In vitro* SHAPE experiment with NAI and a 140 nucleotide-long RNA template containing exon 7. 1 = DMSO; 2 = SMN-C2 (50 µM); 3 = SMN-C2 (5 µM); 4 = lacking of NAI; 5–8 = ladders generated by addition of ddATP, ddTTP, ddCTP, and ddGTP during primer extension. PAGE was carried out on a TBE-urea sequencing gel at 60 W for 3 h. Red asterisks indicate increased band intensity with 50 µM SMN-C2<sup>6</sup>. **(E)** An example of a “salt front” region in the dashed red box from a separate experiment.

**Figure 2:** In-cell SHAPE derived RNA modeling for SMN2 exon 7 pre-mRNA. **(A)** PCR amplification with all three primer sets (**Table 2**) yielded a single band in agarose gel analysis. 1 = SMN2E7-338, 2 = SMN2E7-276, 3 = SMN2E7-251, 4 = DNA ladder. **(B)** Differential in-cell SHAPE reactivity in SMN2 minigene-transfected 293T cells for 10 µM SMN-C2 and DMSO in TSL1. SHAPE reactivity at single-nucleotide resolution. Its standard deviation was calculated by ShapeMapper software<sup>35</sup>. Green asterisks indicate significant SHAPE reactivity change induced by 10 µM SMN-C2. The numbering of the nucleotides in the 276 bp amplicon shown on x-axis. Error bars were estimated by ShapeMapper software<sup>26</sup>. **(C)** Arc plot generated by SuperFold<sup>35</sup> for the most plausible RNA secondary structure modeling by in-cell SHAPE data. **(D)** *In vitro* and in-cell SHAPE-directed modeling of exon 7 and adjacent regions. For *in vitro* RNA model, SHAPE stabilizing cassette (orange) and nucleotides 1–19 (blue) are shown in sketch. For in-cell RNA model, nucleotide numbering is aligned with *in vitro* SHAPE template. Nucleotides 1–18 and 120–140 were omitted. Significant reactivity changes are indicated in red and green asterisks for *in vitro* and in-cell SHAPE,



respectively. The secondary structures that were previously named TSL1 and TSL2<sup>31</sup> are enclosed in blue boxes.

**Table 1: The primer sequences used in the representative experiment.**

**Table 2: The primer sets for amplification of the pre-mRNA sequence of interest.** All three primer sets yield a single amplicon in a PCR reaction with genomic DNA-free cDNA template.

## DISCUSSION:

In *in vitro* SHAPE, it is critical to use high quality homogeneous RNA template. T7 transcription, however, often yields heterogeneous sequences<sup>36</sup>. Especially, sequences with  $\pm 1$  nucleotide at the 3'-terminus with non-negligible yields<sup>36</sup> are usually difficult to be removed by polyacrylamide gel purification. Heterogeneous RNA template can result in more than one set of the signal in the sequencing gel profiling of the primer extension product, which sometimes makes it difficult to interpret the result. The ribozyme at both 5'- and 3'-ends of the RNA template expression cassette will make both ends homogenous.

For both *in vitro* and in-cell SHAPE, the incubation time of 2'-OH modification reagents is another critical factor. It was suggested by the Weeks group that at least five times the half-life of the water-quenching 2'-OH acylation reagent should be used<sup>1</sup>. In our hands, incubating with NAI ( $T_{1/2} = \sim 20$  min) for >30 min in both *in vitro* and in-cell SHAPE results in an overreacted result. As shown in **Figure 1D**, a good *in vitro* SHAPE profiling should have >50% total signal on top of the gel as full-length transcript, ensuring that most of the modified RNA is only acylated once. Overreaction will render the double-acylated product non-negligible and the profiling biased to the enriched short length extension products. In in-cell SHAPE, the amplicon for the library construction should appear as a single band in agarose gel analysis (step 2.6.3). Smear of the band indicates an overreaction, and the incubate time should be reduced. In the representative experiments, a 15 min incubation time at 37 °C was optimal for *in vitro* and in-cell SHAPE. This should be used as a starting point for NAI modification in similar applications.

There are other 2'-OH modification reagents<sup>22</sup> widely used for SHAPE, such as 1M7. Compared to NAI, 1M7 has a better reactivity to 2'-OH group and shorter half-life in water<sup>22</sup>. In our hands, 1M7 formed massive amount of yellow precipitation in cell culture media in an in-cell SHAPE experiment, which complicated the RNA isolation. For a comparison reason, both *in vitro* and in-cell SHAPE used NAI as the 2'-OH modification reagent for a study of SMN-C2 and SMN2 pre-mRNA interactions. If only *in vitro* SHAPE is required, 1M7 is an alternative option as demonstrated by various studies in riboswitch structural determination<sup>7,8</sup>.

In general, in-cell SHAPE over *in vitro* SHAPE is preferred, especially if the molecule is presumably acting in the nucleus of a eukaryotic cell. RNA-binding proteins in the nucleus are abundant, and it is almost impossible to recapitulate the cellular context under *in vitro* conditions.

In the past decade, SHAPE becomes the standard method for studying the secondary structure of RNA. Compared to traditional RNA footprinting with RNase<sup>37</sup>, it is more suitable to study small

molecule-RNA interactions, as these interaction can sometimes be weak and insensitive to RNase challenges.

The major limitation of using SHAPE to study small molecule-induced RNA structural changes is that its results do not reveal the binding site. In *in vitro* and in-cell SHAPE for SMN-C2 bound pre-mRNA, the SHAPE reactivity did not change at the putative binding site (**Figure 1D, Figure 2B**); rather, the reactivity at 2 to 3 remote sites at the loop or budge region were altered. SMN-C2 presumably binds to an RNA double-helix region (**Figure 1D, Figure 2D**), which usually has a low SHAPE reactivity. Therefore, further stabilizing the structure to decrease SHAPE reactivity would probably not be observable. To generate a putative binding site, other methods such as ChemCLIP<sup>38</sup> should be used, in which a crosslinking chemical probe is involved.

A common alternative approach to map RNA secondary or higher structure and nucleotide dynamics is NMR spectrometry<sup>39,40</sup>. It has been demonstrated that RNA dynamics derived from quantitative NMR analysis strongly correlate with SHAPE activity<sup>39</sup>. In the context of small molecule binding, chemical shift perturbations can reveal the interacting nucleotides<sup>21</sup>.

As RNA-targeting small molecules become a new modality of drug development<sup>11</sup>, we envision that SHAPE will be established as a standard methodology to evaluate the structural impacts of target RNA in the presence of small molecules. In the future, a transcriptome-wide interrogation method for RNA-targeting small molecule drugs is desired.

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#### DISCLOSURES:

The authors declare no conflict of interest.

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Scheme 1

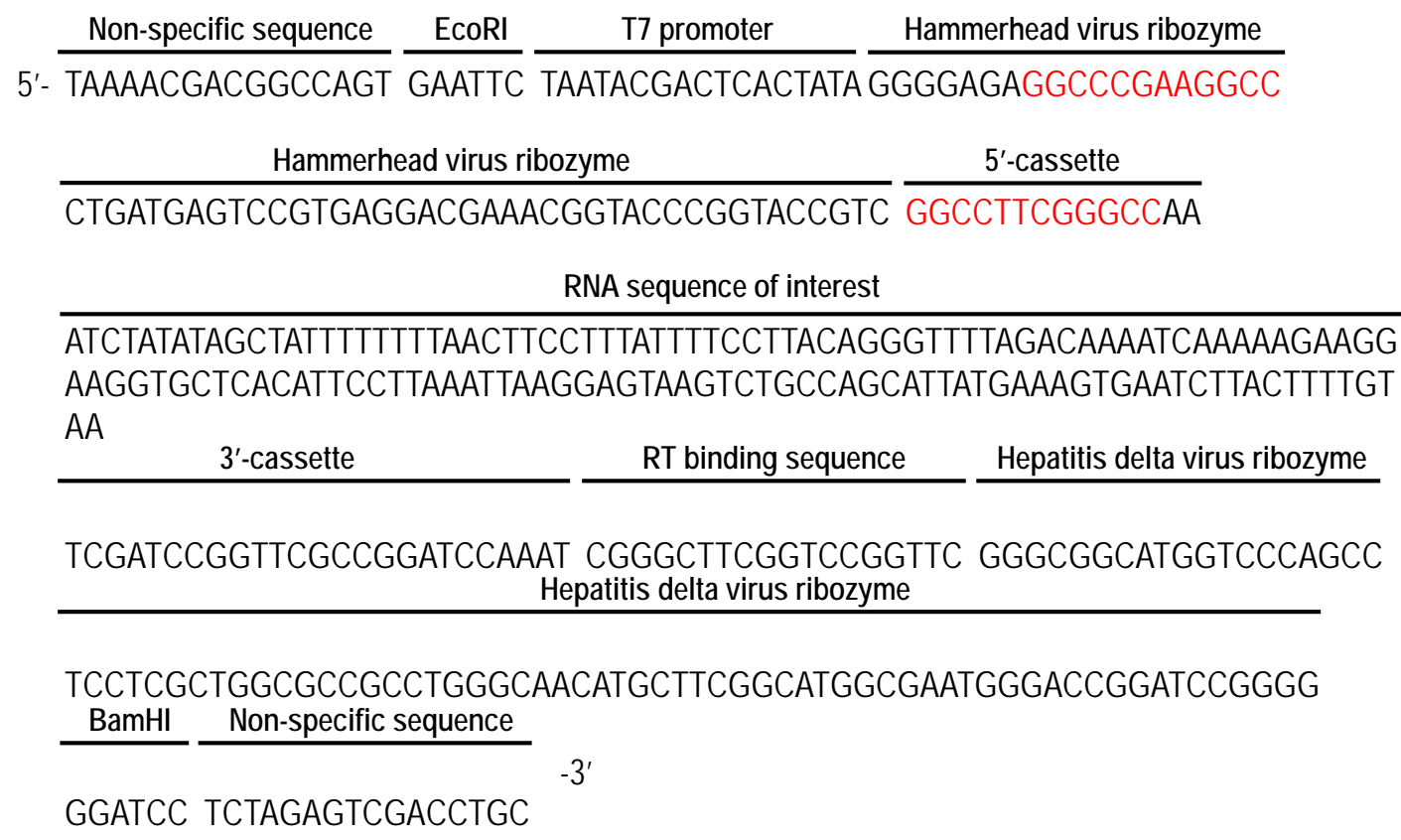


Fig 1

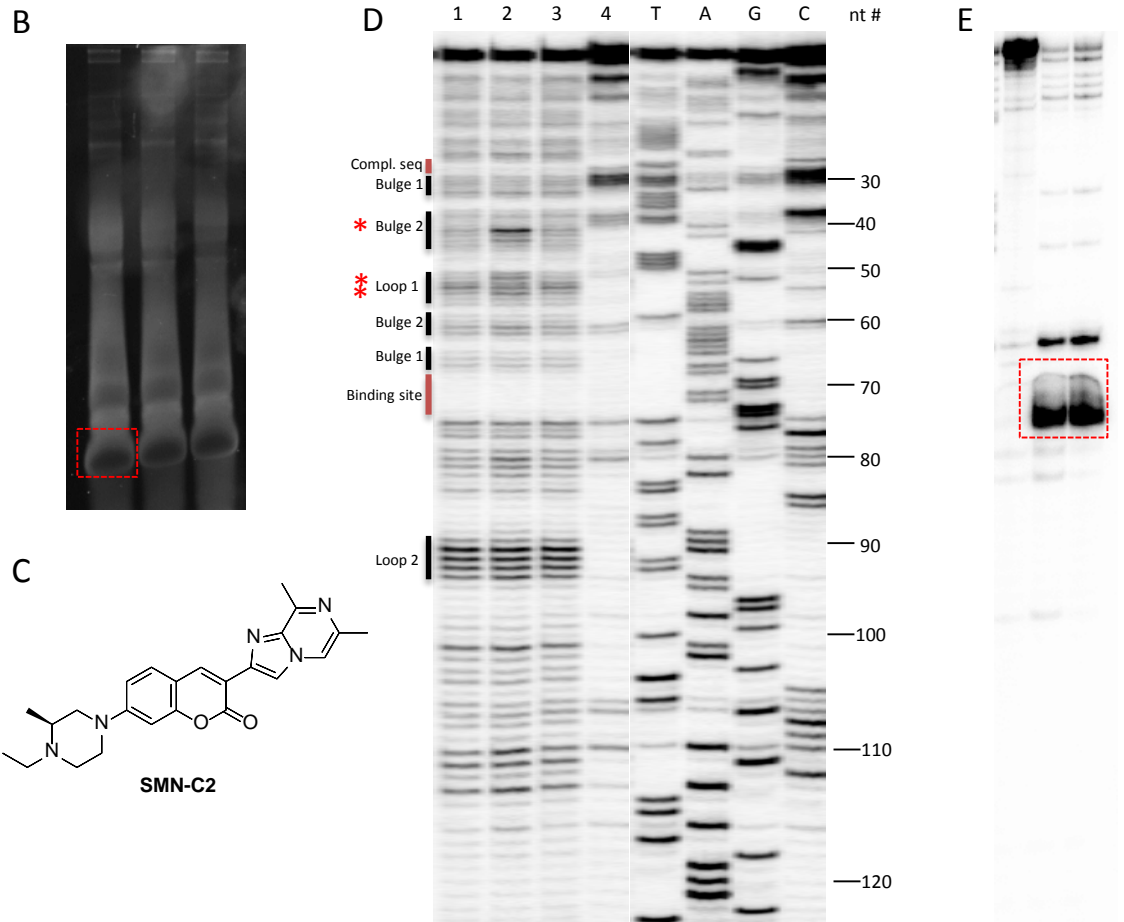
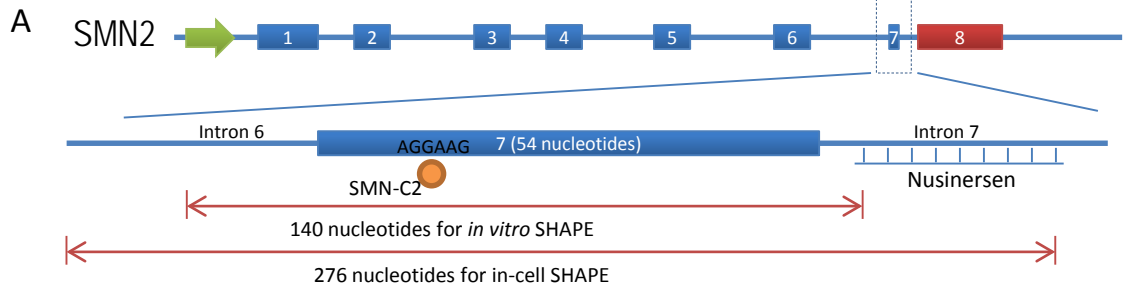


Fig 2

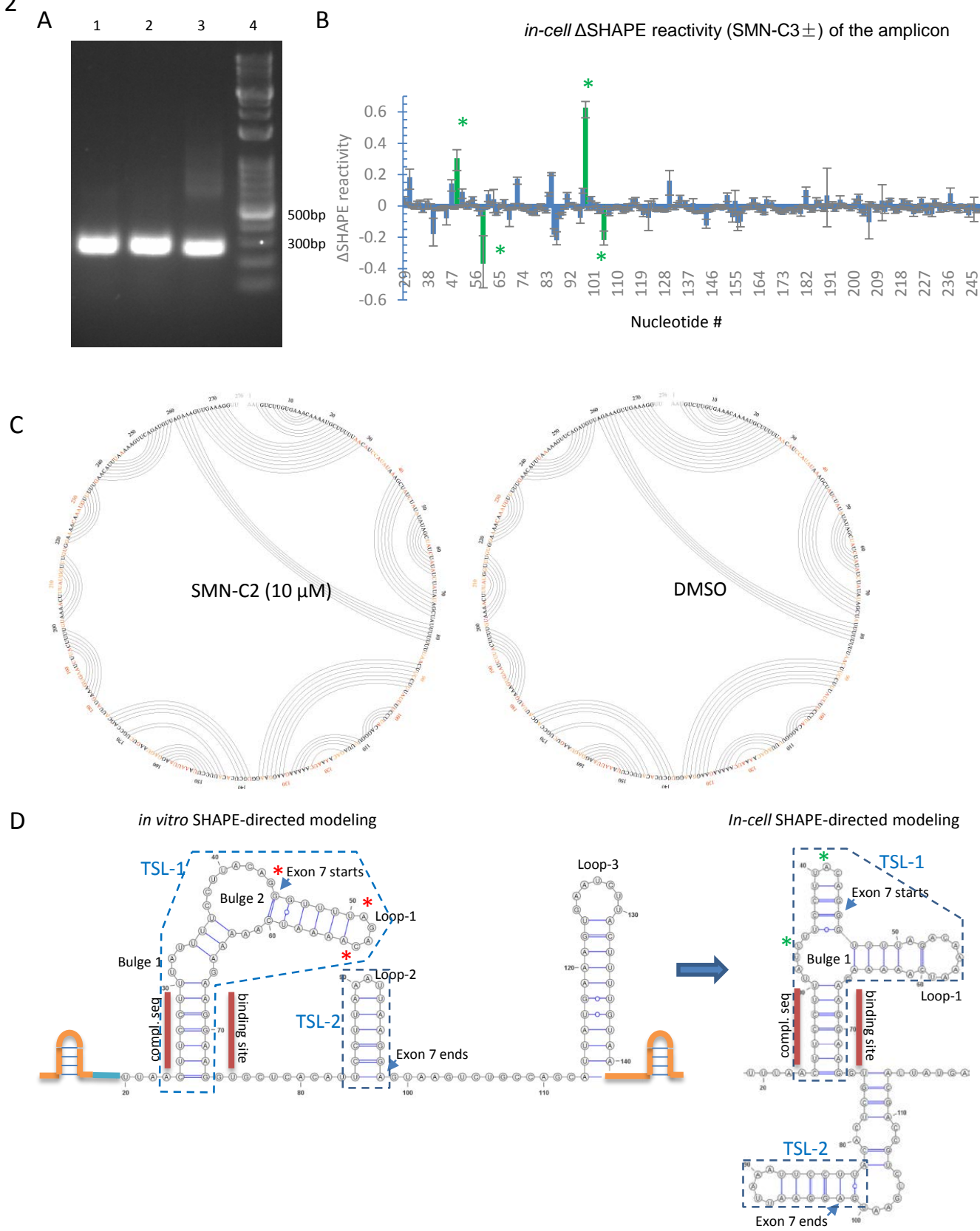


Table 1

Primer name	5'-3' Sequence
Ribozyme-FW	TAAAACGACGGCCAGTGAAT
Ribozyme-RV	GCAGGTCGACTCTAGAGGAT
RT primer	GAACCGGACCGAAGCCCG



Table 2

Name	Sequence (5'->3')
SMN2E7-338-FW	AAAGACTATCAACTTAATTTCTGA
SMN2E7-338-RV	TGTTTTACATTAACCTTTCAACT
SMN2E7-276-FW	AATGTCTTGTGAAACAAAATGCT
SMN2E7-276-RV	AACCTTTCAACTTTCTAACATCT
SMN2E7-251-FW	TGAAACAAAATGCTTTTAACATCC
SMN2E7-251-RV	TCAACTTTCTAACATCTGAACTTTT

<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
0.2 µM membrane	Thermo Fisher	166-0045
10X TBE buffer	Thermo Fisher	15581044
1X Pen-Strep	Thermo Fisher	15140122
40% acrylamide/ bisacrylamide solution	Bio-Rad	1610146
5X RT buffer	Thermo Fisher	18090010
6 % TBE-urea mini-gel	Thermo Fisher	EC6865BOX
AccuPrime pfx DNA polymerase	Thermo Fisher	12344024
Amersham Typhoon	GE Healthcare	
ChemiDoc	Bio-Rad	
ChemiDoc	Bio-Rad	
ddATP (5 mM)	Sigma	GE27-2045-01
ddNTP set (5mM)	Sigma	GE27-2045-01
Deionized formamide	Thermo Fisher	AM9342
DEPC-treated water	Thermo Fisher	750023
DNA oligonucleotide	IDT	
dNTP mix (10mM each)	Thermo Fisher	R0192
DPBS without Ca/ Mg	Thermo Fisher	14190250
Fetal bovine serum	Thermo Fisher	10438026
filter paper	Whatman	1001-917
formamide	Thermo Fisher	AM9342
FuGene HD	Promega	E2311
Gel dryer	Hoefer	GD 2000
GlycoBlue	Thermo Fisher	AM9515
Heat inactivated FBS	Thermo Fisher	10438026
Kimwipe	Kimberly-Clark	34133
large filter paper	Whatman	1001-917
MegaScript T7 transcription kit	Thermo Fisher	AM1333
MnCl <sub>2</sub> ·4H <sub>2</sub> O	Sigma	M3634
NAI stock solution, 2 M	Millipore	03-310
Nalgene Rapid-Flow™ Filter Unit	Thermo Fisher	166-0045
NextSeq500	Illumina	
NucAway column	Thermo Fisher	AM10070

Opti-MEM I	Thermo Fisher	31985062
Pen-Strep	Thermo Fisher	15140122
phosphor storage screen	Molecular Dynamics	BAS-IP MS 2025 E
Phusion Green Hot Start II High-Fidelity PCR Master Mix	Thermo Fisher	F566S
QIAamp DNA Blood Mini Kit	Qiagen	51104
random nonamer	Sigma	R7647
RNase-Free DNase Set	Qiagen	79254
RNeasy mini column	Qiagen	74104
SMN2 minigene34	Addgene	72287
spin-column	Takara	740609.5
SuperScript First-Strand Synthesis System	Thermo Fisher	11904-018
SuperScript IV Reverse Transcriptase	Thermo Fisher	18090010
SYBR-Safe dye	Thermo Fisher	S33102
T4 PNK reaction	NEB	M0201S
TBE-urea sample buffer	Thermo Fisher	LC6876
TEMED	Thermo Fisher	17919
TRIzol	Thermo Fisher	15596018
TrpLE	Thermo Fisher	12605010
$\gamma$ -32P-ATP	Perkin Elmer	NEG035C005MC

## Comments/Description

RNase Free

Representative examples

gBlock for > 200 bp DNA synthesis

Representative examples

Contains 10X reaction buffer, T7 enzyme, NTP and Turbo DNase

Representative examples

Representative examples

Also contains RNase A and protease K

Contains DNase I and RDD buffer

Also contains RW1, RPE buffer

Representative examples

Contains 10X RT buffer, SuperScript II reverse transcriptase

Contains 5X RT buffer, SuperScript IV



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Author(s):

Jingxin Wang, John Hammond, Kristen A. Johnson

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**Dear Editor,**

Enclosed is a revised version of the manuscript entitled, "Using In Vitro and In-cell SHAPE to Investigate Small Molecule induced pre-mRNA Structural Change" by Wang et al. We appreciate the thoughtful editorial comments of the journal, and based upon the feedback, we have improved and revised the manuscript text, the figures and the figure legends.

A direct thank you to Dr. Cao also for the time spent by phone and emails to help us make the decisions to focus upon the key aspects of the methodology. We agree to focus the filming upon the *in vitro* SHAPE and have make the changes in the highlighting of the manuscript accordingly. Specific responses to each comment are detailed below.

**Editorial comments:**

Changes to be made by the Author(s) regarding the written manuscript:

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 review all the highlighted steps to ensure that they are continuous and tell a complete story of the Protocol. Please include all relevant details that are required to perform the step in the highlighting. For example, the filmed content currently starts with recovering the RNA from the gel slice (step 1.1.13) without introducing how the gel slice is prepared. Similarly, the highlighted step 1.5.2 uses sample prepared in step 1.4.8, which is not highlighted for filming. It does not matter whether preparation of a polyacrylamide gel is a routine technique for a common molecular biology laboratory. It has to be mentioned/filmed if the gel/sample is going to be used in a later highlighted step. Otherwise, the viewers will have no clue where these are coming from when watching the video.

**Response:** The authors agree with the editor and now the highlighting area has been focused on 1. *In vitro* SHAPE. All highlighting area for in-cell SHAPE has been removed.

Should the unit be W or V? Please check.

**Response:** We used Watts-constant mode and therefore W is the correct unit.

Best,



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