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SHAPE Profiling to Probe Group II Intron Conformational Dynamics During Splicing

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Abstract

Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) is a widely used technique for studying the structure and function of RNA molecules. It characterizes the flexibility of single nucleotides in the context of the local RNA structure. Here we describe the application of SHAPE-MaP (mutational profiling) to study different conformational states of the group II intron during the self-splicing reaction.

Keywords

RNA structure; SHAPE-MaP; group II intron; chemical structure probing; next-generation sequencing

1 Introduction

Chemical probing of RNA structure was first developed in the 1970s and 1980s to determine the secondary structure of RNA [1]. It is widely used today in RNA biochemistry to gain insight into the structure and function of RNA molecules [2]. This method involves the chemical modification of RNA with a probing reagent, whose reactivity with a particular nucleotide depends on the local environment of this residue. These reactivities are encoded as a chemical adduct on each nucleotide. The position of these adducts in the sequence can then be detected by primer extension, in which the reverse transcriptase (RT) stops at modified nucleotides that have reacted with the probe. This allows for relatively accurate secondary structure determination of any given RNA. Currently, the most widely used technique for RNA chemical probing is selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) [3]. SHAPE chemical probes react with the 2'-hydroxyl of flexible nucleotides, but not conformationally-restrained nucleotides such as those found in A-form helices [4]. The major advantage of SHAPE chemistry is that it can modify all four nucleotides -- A, G, C, or U. This is in contrast to other chemical probes such as dimethyl sulfate, CMCT, and kethoxal that react with only a subset of nucleobases [5]. Since the development of the SHAPE protocol, it has become the most common technique for experimental determination of RNA secondary structures. Beyond just base-pairing information, it can also capture conformational changes in RNA, such as riboswitches, RNaseP, lncRNAs, ribosomes, and spliceosomes [6][7][8][9][10].

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Here we describe the application of SHAPE to understand the dynamics of group II intron splicing. Group II introns are structured ribozymes that perform a self-splicing reaction [11]. Both the mechanism and structure of group II introns are analogous to the eukaryotic spliceosome, suggesting that they evolved from a common ancestor [12][13]. By performing SHAPE on a group II intron [14] at different stages of splicing, we were able to demonstrate the dynamic role of junction regions in the group II intron during the second step of RNA splicing (Fig. 2C) [15]. The method described below is a modification of the SHAPE-MaP protocol developed by Smola *et al.* with adaptations to make it suitable for group II introns [16]. This method combines SHAPE modification with high-throughput sequencing to facilitate structure analysis of large RNAs (Fig. 1).

2 Materials

2.1 Constructs

1. *P.li.LSU2* wild-type group II intron (WT intron), cloned into pUC57 with a 250-nt 5’ exon and a 150 nt-3’ exon and a BamHI cut site at the end of the RNA sequence.
2. *P.li.LSU2* second-step mutant (2s mut intron), generated from Pli WT by mutating G296 (exon-binding sequence 3) to A, U622 to A, and all U’s within the first 39 nucleotides of the 3’ exon to A’s. The 2s mut can undergo the first step of splicing but is blocked from the second step of splicing due to the mutations in the 3’ splice site.

2.2 Template Preparation

1. Plasmid Maxiprep Kit
2. 3 M sodium acetate, pH 5.2
3. Isopropanol
4. 70% and 100% ethanol
5. High-fidelity BamHI with enzyme buffer
6. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v)

2.3 *In Vitro* Transcription and Non-denaturing Purification

1. 10× transcription buffer: 400 mM Tris-HCl, pH 7.5, 250 mM $MgCl_2$, 50 mM DTT, 20 mM spermidine
2. 10× low-Mg^{2+} transcription buffer: 400 mM Tris-HCl, pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 20 mM spermidine
3. 100 mM each ribonucleotide triphosphate (NTPs)
4. 20% Triton X-100
5. T7 RNA polymerase
6. Thermostable inorganic pyrophosphatase

7. 0.1 M calcium chloride
8. Turbo DNase
9. Proteinase K
10. 0.22 um syringe filters
11. 10-ml plastic syringe
12. 15 mL centrifugal filter, 100 kDa NMWL
13. RNA storage buffer: 5 mM sodium cacodylate, pH 6.5, 10 mM $MgCl_2$
14. Ca^{2+} storage buffer: 5 mM sodium cacodylate, pH 6.5, 10 mM $CaCl_2$

2.4 SHAPE Modification

1. 100 mM 1-methyl-7-nitroisatoic anhydride (1M7) dissolved in DMSO, stored in small aliquots at −20 °C.
2. Dimethyl sulfoxide (DMSO)
3. 10× denaturing control (DC) buffer: 500 mM HEPES pH 8.0, 40 mM EDTA
4. *E. histolytica* Dbr1 (generous gift of Dr. John Hart). Commercially available Dbr1 enzyme should also work, though they have not been tested by the authors.
5. 2× Dbr1 buffer: 100 mM HEPES, pH 7.0, 200 mM NaCl, 2 mM DTT
6. RNA miniprep kit

2.5 Reverse Transcription

7. Reverse transcription primer (*see* Table 1).
8. 2.5× MaP buffer: 125 mM Tris, pH 8.0, 187.5 mM KCl, 15 mM $MnCl_2$, 25 mM DTT, 1.25 mM dNTPs. Prepare fresh for every experiment with 1 volume of 5× pre-MaP buffer containing all ingredients except $MnCl_2$ and 1 volume of freshly prepared 30 mM $MnCl_2$.
9. SuperScript II (Thermo Fisher). Be sure to use this particular reverse transcriptase, as others may not have the same properties for mutational profiling.
10. DNA clean-up spin columns.

2.6 Library Preparation and Sequencing

1. 2× Q5 master mix (made in-house): 2.5× Q5 reaction buffer, 400 uM dNTPs, 0.04 U/ul Q5 Hot Start DNA polymerase. Store at 4 °C and it will work for at least a month.
2. Primers: See Table 1. Barcodes for the Uni_{Fwd} primer are from Smola et al. [16] and are the same as the barcodes from the TruSeq Small RNA Library Preparation Kit (https://support.illumina.com/content/dam/illumina-support/

documents/documentation/chemistry_documentation/experiment-design/illumina-adapter-sequences-1000000002694-09.pdf)

3. PCR purification kit (use one that elutes DNA with 10 ul volumes)
4. AMPure XP beads (Beckman Coulter)
5. Magnetic stand for 96-well plate
6. 80% ethanol, freshly prepared

2.7 Software

1. ShapeMapper 2 (https://github.com/Weeks-UNC/shapemapper2)
2. deltaSHAPE (https://weeks.chem.unc.edu/software-files/deltaSHAPE_v1.0.tar.gz)

3 Methods

3.1 Template Preparation

1. Purify plasmids from DH5α cells with WT intron or 2s mut intron with a plasmid maxiprep kit.
2. Split each maxiprep elution into three 1-ml aliquots in 2-ml microcentrifuge tubes (*see* Note 1).
3. To each tube, add 100 ul of 3 M sodium acetate, pH 5.2, 700 ul isopropanol and spin down in a microcentrifuge 30 minutes, maximum speed (>15,000 × *g*) at 4 °C.
4. Remove the isopropanol and add 500 ul 70% ethanol. Spin down at maximum speed for 10 minutes at 4 °C.
5. Remove the ethanol and air dry for 10 minutes.
6. Resuspend each pellet in 100 ul H_2O and combine the 3 tubes containing the same plasmid.
7. Measure the concentration of plasmid on a Nanodrop.
8. Add 40 ul enzyme buffer, 1 U/ug plasmid of high-fidelity BamHI, and H_2O up to 400 ul. Incubate at 37 °C for 2 hours to overnight.
9. Add 50 ul H_2O, 50 ul 3 M sodium acetate, pH 5.2, and 500 ul phenol-chloroform-isoamyl alcohol.
10. Vortex for 15 seconds, and spin down for 1 minute, maximum speed at room temperature.
11. With a P200 pipette set to 200 ul, transfer the top layer to a new tube twice, being careful not to disturb the interface between the two layers.

[1]We use 1.7-ml microcentrifuge tubes instead of 50-ml centrifuge tubes because our 50-ml centrifuge tubes are only rated for 10,000 × *g*, whereas the maxiprep kit manual for the kit we use (Sigma GenElute HP) asks to centrifuge at >15,000 × *g*.

12. Add 400 ul phenol-chloroform-isoamyl alcohol and repeat steps 10 and 11, setting the P200 to 140 ul this time instead.
13. Add 700 ul ethanol and incubate for 10 minutes in a −80 °C freezer.
14. Spin down 10 minutes, maximum speed at 4 °C.
15. Remove ethanol and wash with 400 ul 70% ethanol and spin down for 10 minutes, maximum speed.
16. Air dry for 10 minutes and resuspend in 300 ul H_2O.
17. Check the concentration of linearized plasmid on Nanodrop and adjust to 500 ng/ul by adding H_2O.

3.2 *In Vitro* Transcription and Non-denaturing Purification

1. In a 1.7-mL microcentrifuge tube, add 100 ul 10× transcription buffer, 25 ul NTPs, 100 ul linearized template, 2 ul 20% Triton X-100, 5 ul T7 RNA polymerase, 0.5 ul thermostable inorganic pyrophosphatase. Incubate at 37 °C for 3 hours to overnight. To make pre-catalytic intron, use low-Mg^{2+} 10× transcription buffer instead (*see* Note 2).
2. Add 12 ul 0.1 M $CaCl_2$ and 10 ul Turbo DNase and incubate at 37 °C for 45 minutes (*see* Note 3).
3. Add 10 ul Proteinase K and incubate at 37 °C for 1 hour (*see* Note 4).
4. For spliced and 2s mut, transfer to a 2-ml centrifuge tube and add 1 ml of 2× intron splicing buffer. Incubate at 45 °C for 20 minutes. Omit step for pre-catalytic.
5. Equilibrate tubes to room temperature for 20 minutes, then place in a 4 °C refrigerator for 1 hour to overnight.
6. Spin down 10 minutes, 4 °C at maximum speed to pellet white precipitate (*see* Note 5).
7. Transfer supernatant to 10-ml plastic syringe with a 0.22 um syringe filter attached and push down plunger to filter.
8. Transfer filtered RNA to Amicon Ultra-15 centrifugal filter and spin down 20 minutes maximum speed at room temperature in a swinging bucket rotor.
9. Add 15 ml RNA storage buffer and spin down 20 minutes again. Repeat 5 more times (*see* Note 6).

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2. The low-Mg^{2+} transcription buffer suppresses group II intron splicing during the transcription reaction. The yield is lower than with the regular transcription buffer, so we normally pool four 1-mL low-Mg^{2+} transcriptions for non-denaturing purification.
3. Turbo DNase stops the transcription by degrading the DNA template.
4. Proteinase K degrades the enzymes and any contaminating proteins.
5. White pyrophosphate precipitates usually form in large-scale transcription reactions such as the one described in this protocol. The purpose of cooling tubes and filtering is to remove the precipitate to prevent the centrifugal filter from getting clogged.
6. Repeated filtering the transcribed RNA removes the free nucleotides and amino acids leftover from the transcription workup and exchanges the buffer to the RNA storage buffer. The RNA storage buffer was chosen based on its suitability for crystallography. Use Ca^{2+} RNA storage buffer instead for the pre-catalytic intron.

10. Use a P200 to draw up the retentate from the filter and wash the filter membrane 3 times each side. Transfer into a 1.7-ml centrifuge tube.

11. RNA is stored at room temperature or 4 °C (*see* Note 7).

3.3 SHAPE Modification

1. Add RNA storage buffer to 10 pmol of RNA for 18 ul final volume.

2. Add 1 ul 100 mM 1M7 (modified) or 1 ul DMSO (untreated control) to separate PCR tubes

3. Transfer 9 ul of RNA to 1M7 and DMSO tubes and incubate at 37 °C for 1 minute, 15 seconds.

4. Place tubes on ice.

5. Add H_2O to 5 pmol of RNA for 3 ul final volume in a new PCR tube, then add 5 ul formamide and 1 ul of 10× DC buffer. Incubate at 95 °C for 1 min (*see* Note 8).

6. Add 1 ul of 100 mM 1M7 to a PCR tube.

7. Add 9 ul denatured RNA to the 1M7 tube and incubate at 95 °C for 1 minute. Place tubes on ice.

8. For spliced and 2s mut, add 11 ul 2× Dbr1 buffer and 1 ul of 5 uM Dbr1. Incubate at room temperature for 1 hour (*see* Note 9).

9. Add H_2O to make the final volume of each tube 100 ul.

10. Use an RNA purification kit to purify RNA, eluting with 30 ul H_2O.

11. Store modified RNA in a −20 °C freezer.

3.4 Reverse Transcription with Mutational Profiling Conditions

1. Add 1 ul 2 uM reverse transcription primer to PCR strips for each RNA sample.

2. Transfer 10 ul of modified RNA into PCR strips. In thermocycler, incubate at 65 °C for 5 minutes, then cool to 4 °C.

3. Add 8 ul MaP buffer and incubate at 42 °C for 2 minutes.

4. Add 1 ul of Superscript II (SSII) and incubate at 42 °C for 3 hours.

5. Heat to 70 °C for 15 minutes to inactivate SSII and degrade RNA, then hold at 4 °C.

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7. Our lab has a designated RNA workspace and we do not normally see RNase contamination. We do not add RNase inhibitors and our RNA seems to be free of degradation for at least two weeks at room temperature, as judged on a denaturing polyacrylamide gel.

8. The latest ShapeMapper 2 publication suggests the denaturing control can be omitted to save on sequencing costs, but it may be important for structured RNAs such as the group II intron to control for biases in reverse transcription due to its structure.

9. Dbr1 removes the 2'–5' lariat linkage between the first intron nucleotide and the branchpoint adenosine. The readthrough of the branchpoint adenosine may be worse without this step, and it will likely cause the reverse transcriptase to introduce spurious mutations around the branch point. It may also interfere with primer binding.

6. Transfer reverse transcription reactions to G-25 spin columns and spin down to collect purified cDNA.

7. Store cDNA at −20 °C.

3.5 Library Preparation and Sequencing

1. Combine 5 ul cDNA, 1 ul of 25 uM $Step1_{Fwd}$ primers, 1 ul of 25 uM $Step1_{Rev}$ primer, 18 ul H_2O, and 25 ul 2× Q5 master mix in 96-well PCR plate for each sample. To cover entire cDNA with two 500 bp amplicons, two different primer pairs are needed (*see* Note 10 and Tables 1 and 2).

2. Run thermocycler with the following program: 98 °C 30 seconds; 5 cycles of 98 °C 10 seconds, 65 °C 30 seconds, 72 °C 20 seconds; 72 °C 2 minutes (*see* Note 11).

3. Use PureLink PCR micro spin columns to purify PCR products, eluting with 10 ul H_2O.

4. Combine 1 ul of 25 uM barcoded Uni_{Fwd} primer, 1 ul of 25 uM Uni_{Rev} primer, 10 ul purified PCR product, 13 ul H_2O, and 25 ul 2× Q5 master mix. For each sample, use a different barcoded Uni_{Fwd} primer.

5. Run thermocycler with the following program: 98 °C 30 seconds; 25 cycles of 98 °C 10 seconds, 65 °C 30 seconds, 72 °C 20 seconds; 72 °C 2 minutes.

6. Add 45 ul AMPure XP beads that have been equilibrated to room temperature to each PCR reaction. Mix and incubate for 5 minutes at room temperature (*see* Note 12).

7. Place the plate on the magnetic stand until beads stick to the side of the tube (about 30 seconds). Pipet off the supernatant.

8. Add 200 ul 80% ethanol, incubate at 30 seconds, and pipet off ethanol.

9. Repeat step 8 two more times.

10. Air dry beads 15 minutes.

11. Take the plate off the magnetic stand, resuspend beads with 17 ul H_2O, and incubate at room temperature for 2 minutes.

12. Place the plate on the magnetic stand until beads stick to the side of the tube. Transfer 15 ul DNA to new plate or tubes.

13. Run samples on TapeStation with D1000 ScreenTape to ensure each PCR product is the correct size (*see* Note 13).

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10. For this sequencing configuration, the maximum desired amplicon size is 500 base pairs. Since the intron is 588 nucleotides, we use two overlapping primer pairs to cover the entire intron sequence.
11. For a pilot experiment, we increase the number of cycles to 25 and check the PCR product on a 2% agarose gel. For problematic PCR reactions, we have found success with touchdown PCR or adding 1 M betaine, although this may increase PCR error rates. Another troubleshooting option is to treat the cDNA with 1 ul RNase H to remove RNA from the cDNA.
12. The amount of AMPure XP in this protocol corresponds to a 0.9× AMPure XP : DNA ratio. A 0.7×–0.8× ratio may be superior in reducing carryover of small PCR artifacts.
13. Remember that the size of the DNA should be the sequence bounded by the primers plus the additional adapter sequence.

14. Quantify each sample with Qubit fluorometer (*see* Note 14).
15. Pool 0.1 pmol of each library into a 1.7-ml tube. Spin down in SpeedVac until the volume is reduced to 20 ul.
16. Extract DNA with sizes between 550 and 850 base pairs with PippinHT system using a 1.5% agarose gel cassette.
17. Check pool on TapeStation as in step 13 and measure library DNA concentration with Qubit.
18. Sequence on MiSeq with 2 × 300 bp paired-end configuration, spiking in 15% PhiX (*see* Note 15).

3.6 Data Analysis

1. Prepare a FASTA file containing the target RNA sequence (formatted as DNA with T instead of U)
2. Run ShapeMapper 2 command with the --amplicon flag, with the target sequence, primer sequences, and the adapter-trimmed FASTQ files corresponding to the modified, untreated, and denatured samples as inputs (Fig. 2A).
3. Run deltaSHAPE.py with resulting .map files as input, using -p 0 and -f 0,1 flags (*see* Note 16) (Fig. 2B).

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14. Qubit quantification is essential for accurate concentration determination. Do not substitute with spectrophotometric methods (e.g. Nanodrop).
15. Because only one amplicon is sequenced, the PhiX control helps balance the fluorescent signals to improve run quality.
16. deltaSHAPE was originally intended to detect protein-binding sites in the RNA spanning multiple nucleotides. For that purpose, it averages SHAPE signals over multiple adjacent nucleotides and detects binding sites over 3 nucleotide windows by default. Because changes in conformations in a protein-free context may not be correlated among adjacent nucleotides, these assumptions do not hold true and we changed the deltaSHAPE settings to not average over multiple nucleotides and show differences over single nucleotide positions.

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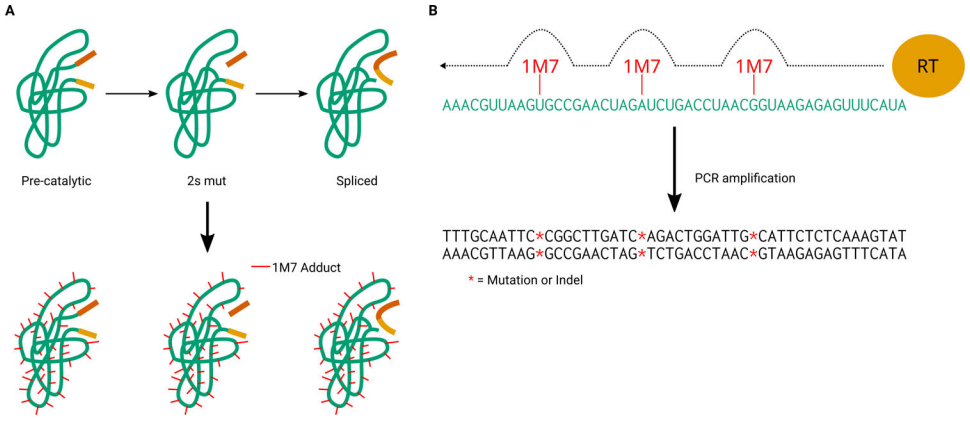

Figure 1.

Overview of SHAPE-MaP experiment on a group II intron. (A) Group II introns at different stages of splicing were probed separately. (B) Reverse transcription of modified RNA under mutational profiling conditions. The Mn^{2+} in the MaP buffer causes the reverse transcriptase to read through 1-methyl-7-nitroisatoic anhydride adducts on the RNA and introduce a mutation at that residue.

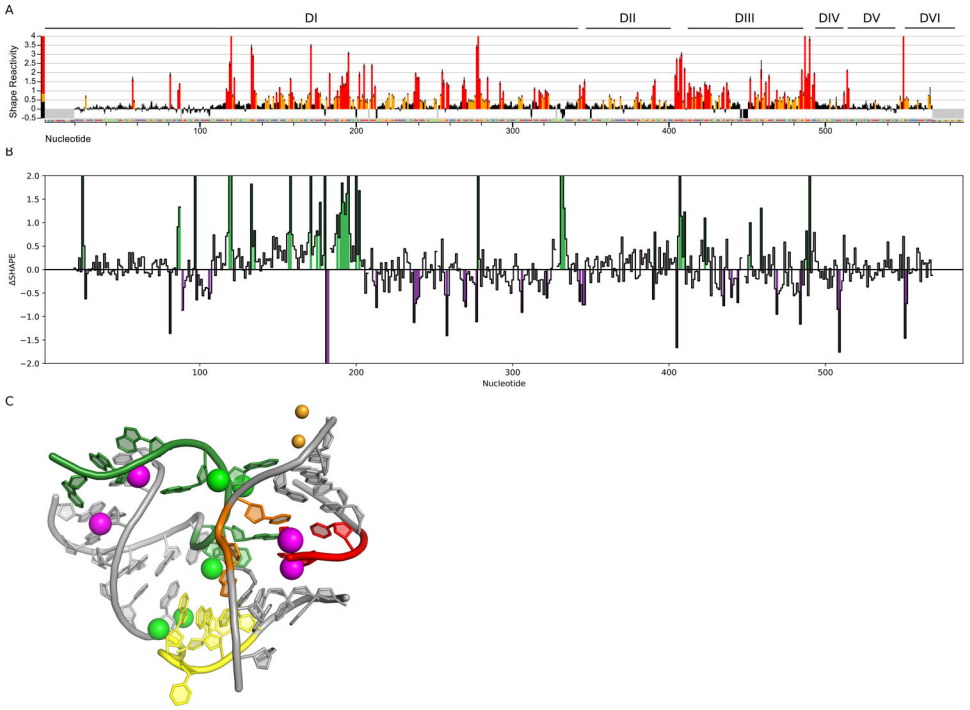

Figure 2.

SHAPE-MaP differences in the core of the group II intron (PDB 4R0D). (A) Example SHAPE reactivity profile for the spliced intron from ShapeMapper 2. Red corresponds to nucleotides with reactivities greater than 0.85 and orange corresponds to nucleotides with reactivities between 0.4 and 0.85. The six domains of the intron are also labeled. (B) Example deltaSHAPE profile showing differences between 2s mut and spliced introns. Nucleotides colored green have increased SHAPE reactivity; purple have decreased SHAPE reactivity. (C) SHAPE reactivity differences between 2s mut and spliced introns in the junction regions. Junction 2/3 is colored dark green, junction 3/4 is colored yellow, junction 4/5 is colored orange, and junction 5/6 is colored red. Green and purple spheres represent the 2'-hydroxyls of residues showing significant SHAPE reactivity increases and decreases, respectively. Light orange spheres correspond to the catalytic Mg^{2+} ions (M1/M2).

Table 1.

Primers used for sequencing.

#	**Primer name**	
1	Pli_RT_588	Agttggataggtagacgatc
2	Pli_RT_3'Exon	Catagttacagccgccgttt
3	Pli_MaP_F_89	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN Taatcgctaggtgagaagc
4	Pli_MaP_R_588	CCCTACACGACGCTCTTCCGATCTNNNNNagttggataggt Agacgatc
5	Pli_MaP_F_246	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN Accctccttttatggggtaa
6	Pli_MaP_R_3'Exon	CCCTACACGACGCTCTTCCGATCTNNNNNcatagttacagc Cgccgttt
7	Pli_MaP_F_1	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNN gtgcgacaagaagttcagg
8	Pli_MaP_R_427	CCCTACACGACGCTCTTCCGATCTNNNNNctcgatcgttacaaccaagc
9	Uni_{Fwd}	CAAGCAGAAGACGGCATACGAGAT[Barcode]GTGACT GGAGTTCAGAC
10	Uni_{Rev}	AATGATACGGCGACCACCGAGATCTACACTCTTTCCC TACACGACGCTCTTCCG

Table 2.

Step 1 Primers for different group II intron conformational states. Refer to Table 1 for numbering.

State	Sequence	Reverse transcription	$Step1_{Fwd}$	$Step1_{Rev}$
Spliced	89–588	1	3	4
	1–427	1	7	8
2s mut	246–3’Exon	2	5	6
	1–427	2	7	8
Pre-catalytic	246–3’Exon	2	5	6
	1–427	2	7	8