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Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution

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Selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) interrogates local backbone flexibility in RNA at single-nucleotide resolution under diverse solution environments. Flexible RNA nucleotides preferentially sample local conformations that enhance the nucleophilic reactivity of 2′-hydroxyl groups toward electrophiles, such as N-methylisatoic anhydride (NMIA). Modified sites are detected as stops in an optimized primer extension reaction, followed by electrophoretic fragment separation. SHAPE chemistry scores local nucleotide flexibility at all four ribonucleotides in a single experiment and discriminates between base-paired versus unconstrained or flexible residues with a dynamic range of 20-fold or greater. Quantitative SHAPE reactivity information can be used to establish the secondary structure of an RNA, to improve the accuracy of structure prediction algorithms, to monitor structural differences between related RNAs or a single RNA in different states, and to detect ligand binding sites. SHAPE chemistry rarely needs significant optimization and requires two days to complete for an RNA of 100–200 nucleotides.

INTRODUCTION

Most RNAs function correctly only after they fold back upon themselves to form three-dimensional structures, which are difficult to predict. RNA also undergoes conformational changes in response to folding and ribonucleoprotein assembly reactions, changes in solution conditions, and binding by small molecule and protein ligands[1]. As an RNA forms a preferred secondary or tertiary structure, a characteristic set of nucleotides becomes conformationally constrained by base pairing and higher-order interactions. Determining the local flexibility as a function of nucleotide position provides important information that enables the sequence and structure of an RNA to be related to its biological function.

Local nucleotide structure can be monitored using well-established approaches that involve treating an RNA with chemical and enzymatic reagents[2]. These methods are widely used and give fair-to-excellent results, depending on the RNA. Results are typically better when multiple reagents are used together or when chemical modification information is interpreted in the context of phylogenetic covariation information[3]. In addition, reagents in current use exhibit widely varying nucleotide and structural selectivities, such that quantitative reactivity information cannot be readily compared for the different nucleotide bases or between reagents. These limitations are overcome by RNA SHAPE chemistry.

SHAPE chemistry is based on the discovery that the nucleophilicity of the ribose 2′-position is exquisitely sensitive to the electronic influence of the adjacent 3′-phosphodiester group[4,5]. Unconstrained nucleotides sample more conformations that enhance the nucleophilicity of the 2′-hydroxyl group than do base-paired or otherwise constrained nucleotides. Therefore, hydroxyl-selective electrophiles such as NMIA form stable 2′-*O*-adducts[5,6] (**Fig. 1a**) more rapidly with flexible RNA nucleotides. Local nucleotide flexibility is interrogated simultaneously at all positions in an RNA in a single experiment because all RNA nucleotides (except a few cellular RNAs carrying post-transcriptional modifications) have a 2′-hydroxyl group. Absolute SHAPE reactivities can be compared across all positions in an RNA because 2′-hydroxyl reactivity is insensitive to base identity. In principle, it is also possible that a nucleotide might be reactive because it is constrained in a conformation that enhances the nucleophilicity of a specific 2′-hydroxyl. This class of nucleotide is expected to be rare, would involve a non-canonical local geometry, and would likely be scored correctly as an unpaired position.

The NMIA reagent is also consumed by a competing hydrolysis reaction (**Fig. 1b**), which advantageously causes the reaction to be self-limiting[5]. The researcher therefore need only adjust the initial NMIA concentration to achieve an appropriate level of 2′-*O*-adduct

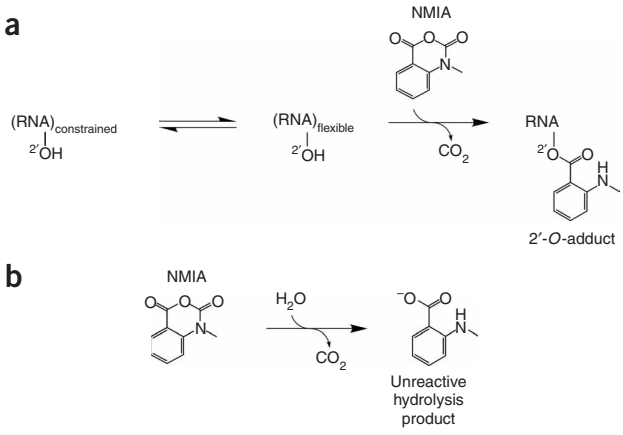

Figure 1 | Simplified mechanisms for RNA SHAPE chemistry[5]. (**a**) NMIA reacts preferentially with the 2′-hydroxyl group in flexible nucleotides to form a stable 2′-*O*-ester adduct. (**b**) Inactivation of NMIA via hydrolysis.

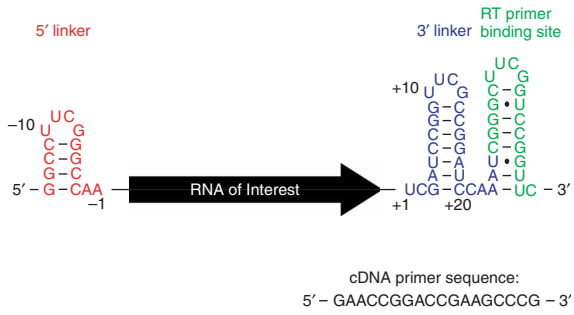

Figure 2 | RNA structure cassette to facilitate analysis of all nucleotides within an RNA of interest. These sequences contain UUCG tetraloops to enforce the designed hairpin folds and also include short dinucleotide linkers to reduce stacking of the hairpins on the internal RNA structure. DNA templates appropriate for transcribing an RNA imbedded within the structure cassette are conveniently prepared by PCR[5,7].

formation and then wait until the NMIA hydrolyzes completely; no explicit quench step is required. Once the reaction is complete, a 5′-(radio)labeled cDNA is annealed to the modified RNA, and sites of 2′-*O*-adduct formation are identified as stops to primer extension by reverse transcriptase. cDNAs are separated by standard high-resolution gel electrophoresis. Absolute NMIA reactivity at each nucleotide is then determined by comparing band intensities from the modification reaction to those of a no NMIA control. One or more dideoxy sequencing lanes are used to assign bands within the NMIA reaction and control lanes. Structural information can typically be read for ~100–150 nucleotides 5′ to the DNA primer.

The material requirements for a SHAPE experiment are modest. A complete experiment requires 3–4 pmol of RNA: 2 pmol are required for the SHAPE chemistry itself and 1 pmol is required for each sequencing experiment used for band assignment. One or two sequencing experiments are usually sufficient. RNAs of any length are appropriate substrates for SHAPE, as long as the RNA has no post-transcriptional modifications or unusually stable secondary structures that prevent its functioning as a template for primer extension. NMIA modification works well under a wide variety of solution conditions, ionic strengths and temperatures. Good results are obtained at 0–200 mM monovalent ion (NaCl, KCl or potassium acetate), 0–40 mM $MgCl_2$ and 20–75 °C.

In addition, an RNA may be modified in the presence of protein or other small and large biological ligands. Solution components that react directly with NMIA as well as organic co-solvents, including formamide and DMSO, are well tolerated but may require that reagent concentrations be adjusted. NMIA reactivity is strongly dependent on pH; thus, the major experimental restriction is that the pH be maintained close to 8.0. In practice, pH values of 7.5–8.2 work well. The dynamic range that differentiates the most reactive (flexible) and least reactive (constrained) nucleotides typically spans a factor of 20–50.

An important application of a SHAPE experiment is to obtain constraints sufficient to establish or confirm the secondary structure model of an arbitrary RNA[5–9]. SHAPE chemistry is also ideally suited to map structural variations among homologous RNAs and the structural consequences of a suite of mutations[7,10,11]. Other important applications of SHAPE include monitoring thermal melting of an RNA at single-nucleotide resolution[6], identifying regions of an RNA that do not fold to a single well-defined structure[7,8] and mapping equilibrium conformational changes that accompany an RNA folding reaction[8–10].

This protocol focuses on using NMIA to modify RNA in a structure-selective manner and then quantifying 2′-*O*-adduct formation using 5′-[^{32}P]-labeled DNA primers in the primer extension step.

RNA design

Because SHAPE reactivities are assessed in a primer extension reaction, information is lost at both the 5′ end and near the primer binding site of an RNA. Typically, the 10–20 nucleotides adjacent to the primer binding site cannot be quantified due to the presence of cDNA fragments that reflect pausing by the reverse transcriptase enzyme during the initiation phase of primer extension. The 8–10 positions at the 5′ end of the RNA also cannot be visualized because of the presence of the intense band corresponding to the full-length extension product.

To monitor SHAPE reactivities at the 5′ and 3′ ends of a sequence of interest, the RNA can either be embedded within a larger fragment of the native sequence or placed between strongly folding RNA sequences that contain a unique primer binding site[5]. We have designed a structure cassette (**Fig. 2**) that contains 5′ and 3′ flanking sequences of 14 and 43 nts and allows all positions within the RNA of interest to be evaluated in a sequencing gel. Both 5′ and 3′ extensions fold into stable hairpin structures that do not appear to interfere with folding of diverse internal RNAs[5,7]. The primer binding site of this cassette efficiently binds to a cDNA primer (**Fig. 2**). The sequence of any 5′ and 3′ structure cassette elements should be checked to ensure that they are not prone to forming stable base pairing interactions with the internal sequence.

RNA folding

The SHAPE experiment is most commonly performed with RNAs that have been generated by *in vitro* transcription[5,7,12]. These RNAs require purification by denaturing gel electrophoresis and then must be renatured to achieve a biologically relevant conformation. This protocol describes a simple approach that works well for renaturing many RNAs. However, any procedure that folds the RNA to the desired conformation at pH 8 can be substituted. The RNA is first heated and snap cooled in a low ionic strength buffer to eliminate multimeric forms. A folding solution is then added to allow the RNA to achieve an appropriate conformation and to prepare it for NMIA modification. The RNA is folded in a single reaction and is later separated into (+) and (–) NMIA reactions.

RNA modification

NMIA is added to the folded RNA to yield 2′-*O*-adducts at flexible nucleotide positions. The reaction is then incubated until essentially all of the NMIA has either reacted with the RNA or has degraded due to hydrolysis with water (**Fig. 1**). No specific quench step is required. The modified RNA is precipitated

with ethanol in order to purify the RNA from reaction products and buffer components that may be detrimental to the primer extension reaction.

Primer extension

Although analysis of RNA adducts by primer extension is widely considered to be problematic, we find that the optimized experiment described here works well for most RNAs. Key innovations include use of an optimized primer binding site (**Fig. 2**), thermostable reverse transcriptase enzyme, low [$MgCl_2$], elevated temperature, and short extension times. It is also essential to use intact, non-degraded RNA, free of reaction byproducts and other small molecule contaminants. 5′-radiolabeled DNA primers are annealed to the RNA and then extended to sites of modification in the presence of dNTPs by the activity of reverse transcriptase. The RNA component of the resulting RNA-cDNA hybrids is degraded by treatment with base. The cDNA fragments are resolved on a polyacrylamide sequencing gel.

Sequencing

Sequencing lanes generated by dideoxy nucleotide incorporation are used to assign bands in the (+) and (–) NMIA lanes. One or two sequencing reactions is usually sufficient to infer the entire sequence. These steps are conveniently performed concurrently with the primer extension reactions for the (+) and (–) NMIA tubes.

For experienced users of SHAPE technology, a simplified benchtop outline of the complete experiment is provided in **Figure 3**.

Benchtop Outline of SHAPE Experiment

(+) and (–) NMIA reactions

- ❑ Start with 2 µl 1 µM RNA.
- ❑ Add 10 µl 0.5x TE.
- ❑ Heat 95 °C, 3 min, quench on ice.
- ❑ Add 6 µl RNA folding mix.
- ❑ Incubate at desired temperature.
- ❑ Distribute 9 µl into 2 tubes [(–)NMIA and (+) NMIA].
- ❑ Add 1 µl of NMIA in DMSO or neat DMSO.
- ❑ Incubate 5 half-lives.
- ❑ Ethanol precipitate samples.
- ❑ Redissolve in 10 µl 0.5x TE.
- ❑ Add 3 µl radiolabeled primer.
- ❑ Heat 65 °C, 5 min, 35 °C, 5 min, ice 1 min.
- ❑ Add 6 µl SHAPE enzyme mix.
- ❑ Preheat 52 °C, 1 min.
- ❑ Add 1 µl Superscript III.
- ❑ Incubate 52 °C, 10 min.
- ❑ Add 1 µl 4 M NaOH.
- ❑ Incubate 95 °C, 5 min.
- ❑ Add 29 µl acid stop mix.
- ❑ Incubate 95 °C, 5 min.
- ❑ Load 2 µl on gel.

Sequencing

- ❑ Start with 1 µl 1 µM RNA.
- ❑ Add 8 µl 0.5x TE.
- ❑ Add 3 µl radiolabeled primer.
- ❑ Heat 65 °C, 5 min, 35 °C, 5 min, ice 1 min.
- ❑ Add 6 µl SHAPE enzyme mix.
- ❑ Add 1-2 µl ddNTP stock.
- ❑ Preheat 52 °C, 1 min.
- ❑ Add 1 µl Superscript III.
- ❑ Incubate 52 °C, 10 min.
- ❑ Add 1 µl 4 M NaOH.
- ❑ Incubate 95 °C, 5 min.
- ❑ Add 29 µl acid stop mix.
- ❑ Incubate 95 °C, 5 min.
- ❑ Load 2 µl on gel.

Figure 3 | Benchtop outline of the SHAPE experiment.

MATERIALS

REAGENTS

- RNA at a concentration of 0.2 µM or greater, 3 pmol minimum.
  ▲ CRITICAL All reagents as well as reaction tubes and equipment must be maintained free of RNase contamination. For best results, all chemicals should be purchased at the highest quality available and reserved for RNA use only.
- Deoxyadenosine, deoxycytidine and deoxythymidine triphosphates (dATP, dCTP, dTTP), 100 mM (GE Healthcare, cat. no. 27-2050-01, 27-2060-01, 27-2080-01)
- Deoxyinosine triphosphate (dITP), 100 mM (Trilink Biotechnologies, cat. No. N2012). Use of dITP reduces band compression in the sequencing gel.
- Dideoxyadenosine, dideoxycytidine, dideoxythymidine and dideoxyguanosine triphosphates (ddATP, ddCTP, ddTTP, ddGTP), 100 mM (Trilink Biotechnologies, cat. No. N4001-1, N005-1, N4004-1, N4002-1)
- Glycogen, 20 mg/ml (Invitrogen, cat. no. 10814-010)
- EDTA, 100 mM (Ambion, cat. no. 9260G)
- Formamide (Fluka, cat. no. 47671)
- DMSO, molecular biology grade (Sigma Aldrich, cat. no. 494429-1L)
  ▲ CRITICAL bottle must be stored in a desiccator
- N-methylisatoic anhydride (NMIA) (Invitrogen, cat. no. M-25)
  ▲ CRITICAL bottle must be stored in a desiccator at >4 °C
- Superscript III reverse transcriptase (Invitrogen, 18080-093)
- 5× SSIII FS buffer [250 mM Tris (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] (Invitrogen, cat. no. 18080-093)
- 0.1 M DTT (Invitrogen, cat. no. 18080-093)
- T4 polynucleotide kinase (New England Biolabs, cat. no. M0201S)
- 10× PNK buffer (New England Biolabs, cat. no. M0201S)
- DNA primer: use primers ~18–20 nt in length that form a 3′ G–C base pair with the target RNA.
- 4 M NaOH
- γ-[^{32}P]-ATP (6 × 10^6 Ci/mol, 10 Ci/l, Perkin Elmer cat. no. BLU502Z)
- 10 mM solutions of ddATP, ddCTP, ddTTP and/or 0.25 mM ddGTP
  ▲ CRITICAL These solutions are stable for months at –20 °C but are intolerant of freeze-thaw cycles. Maintaining small aliquots at –20 °C is recommended.
- Sequencing reagents for high-resolution polyacrylamide gel electrophoresis, including 29:1 acrylamide:bis-acrylamide, 1× TBE and urea.

REAGENT SETUP

Acid stop mix 4:25 (v/v) mixture of 1 M unbuffered Tris-HCl and stop dye (85% formamide, 1/2× TBE, 50 mM EDTA, pH 8.0, containing bromophenol blue and xylene cyanol tracking dyes)

3.3× RNA folding mix (333 mM HEPES, pH 8.0, 20 mM $MgCl_2$, 333 mM NaCl). Other conditions that are known to stabilize the functional structure of the RNA under study can be used as well. Both buffering component and ionic strength can be varied. In the modification reaction, buffer concentration should be at least twice the NMIA concentration and adjusted to pH 8.

10× NMIA in DMSO The recommended concentration of this solution varies with RNA length. For RNA reads of 100, 200 and 300 nucleotides, 10× NMIA concentrations of 130, 65 and 30 mM, respectively, work well. Due to the solubility of the reagent, the stock concentration of NMIA should not be greater than 130 mM.

SHAPE enzyme mix (250 mM KCl, 167 mM Tris HCl, pH 8.3, 1.67 mM each dNTP, 17 mM DTT, 10 mM $MgCl_2$). The enzyme mix may be prepared by combining four parts SSIII FS buffer, one part 0.1 M DTT and one part 10 mM dNTP mix (10 mM in each deoxynucleotide).
▲ CRITICAL This solution is stable for months at –20 °C but is intolerant of freeze-thaw cycles. Maintaining small aliquots at –20 °C is recommended.

5′-[^{32}P]-labeled primers These are prepared by performing the following steps: (i) Mix the following well: 1 µl 60 µM DNA primer, 16 µl γ-[^{32}P]-ATP, 2 µl 10× PNK buffer and 1 µl T4 polynucleotide kinase. (ii) Incubate at 37 °C for 30 min. (iii) Purify on 20% denaturing polyacrylamide gel (1× TBE, 7 M urea). Use autoradiography to visualize and excise the band corresponding to the

radiolabeled DNA primer. (iv) Passive elute overnight into water and remove small pieces of acrylamide from the RNA using a centrifugal filter device. (v) Recover radiolabeled DNA by ethanol precipitation (see Step 8 of the PROCEDURE but omit glycogen). (vi) Dissolve the pellet in 100 µl 1 mM HEPES, pH 8.0. The final primer solution concentration is ~0.3 µM.

EQUIPMENT

- Amicon micropure-EZ centrifugal filter devices (Millipore, cat. no. 42533)
- –80 and –20 °C freezers
- Microfuge for 1.5 ml tubes at 4 °C
- Phosphorimaging instrument and screen
- Sequencing apparatus for high-resolution polyacrylamide gel electrophoresis. We recommend using a vertical denaturing gel of dimensions 0.75 mm × 31 cm (width) × 38.5 cm (height) that is 10–12% (29:1) acrylamide, 90 mM Tris-borate, 2 mM EDTA and 7 M urea.
- Programmable incubator or heat block. We recommend an incubator with a heated top of the type typically used for performing PCR.

PROCEDURE

RNA folding

1| Add 2 pmol RNA in 12 µl 0.5× TE to a 200 µl thin-walled PCR tube.

2| Heat the RNA to 95 °C for 2 min; then immediately place the RNA on ice for 2 min.

3| Add 6 µl folding mix and mix solutions by gentle repetitive pipetting.

4| Remove the tube from ice and incubate at the desired reaction temperature for 20 min in a programmable incubator. 37 °C is a good initial choice.

5| While the tube is incubating, remove 9 µl and place it in a second tube. One tube will be used for the (+) NMIA reaction, the other for the (–) NMIA reaction.

RNA modification

▲ **CRITICAL** Condensation of reactions on the interior lid of the reaction tubes must be kept at a minimum. If a simple heat block is used, condensation must be spun down before NMIA modification and before adding Superscript III.

6| Add 1 µl of NMIA in DMSO to the (+) NMIA tube and 1 µl of neat DMSO to the (–) NMIA tube and mix well. Some initial turbidity in the tube is normal, especially when working at high NMIA concentrations.

7| Incubate the reaction for five NMIA hydrolysis half-lives. To estimate the NMIA half-life between 15 and 75 °C , use the empirical equation:

$$\text{half-life}(\text{min}) = 360 \times \exp[-0.102 \times \text{temperature}(^{\circ}\text{C})]$$

At 37 °C, NMIA has a half-life of 8.3 min; therefore, at 37 °C, the reaction should be incubated for ~45 min.

8| After the reaction has gone to completion, transfer reactions to 1.5-ml centrifuge tubes, and recover the modified RNA by ethanol precipitation: first add 90 µl water, 4 µl 5 M NaCl, 1 µl 20 mg/ml glycogen, 2 µl 100 mM EDTA pH 8, and 350 µl absolute ethanol, then incubate at –80 °C for 30 min. Sediment the RNA by spinning at maximum speed in a microfuge at 4 °C for 30 min. Perform Step 11 during this centrifugation.

9| Remove ethanol supernatant; redissolve RNA in 10 µl of 0.5× TE and transfer samples to 200 µl thin-walled PCR tubes.

■ **PAUSE POINT** The modified RNA can be stored frozen for up to one month at –20 °C.

Primer extension and RNA sequencing

10| Add 3 µl radiolabeled primer solution to the (+) and (–) NMIA tubes. Mix by repetitive pipetting.

11| To sequence the RNA for assigning bands in the (+) and (–) NMIA samples, add 3 µl of primer solution to 1 pmol of RNA in 8 µl 0.5× TE.

12| Anneal the primer to the RNA by incubating tubes at 65 °C for 5 min and then at 35 °C for 5 min, then place on ice for 1 min.

13| Add 6 µl of SHAPE enzyme mix to the (+) and (–) NMIA reactions and to the sequencing reactions. To each sequencing experiment, add 1–2 µl of one ddNTP solution as well.

14| Heat the tubes to 52 °C for 1 min.

▲ **CRITICAL STEP** Omitting this step results in poor primer extension.

15| Add 1 µl of Superscript III to each tube. Mix well by gentle repetitive pipetting. Immediately return the tube to the heat block.

16| Immediately incubate tubes at 52 °C for 10 min.

17| Add 1 µl 4 M NaOH. This degrades the RNA but does not damage DNA.

18| Heat the samples to 95 °C for 5 min.

19| Add 29 µl of acid stop mix.

20| Incubate at 95 °C for 5 min.

■ **PAUSE POINT** Samples can be stored at –20 °C for up to one month.

cDNA fragment analysis by gel electrophoresis

21| Load (+) NMIA, (−) NMIA and sequencing reactions in individual lanes of a polyacrylamide sequencing gel (29:1 acrylamide:bis acrylamide, 1× TBE, 7 M urea). Load ~2 µl per lane. For extensions of 100 or fewer nucleotides, perform electrophoresis for 150 min at 70 W. To visualize RNA extension reactions spanning more than 100 nucleotides, re-load samples after 150 min in unoccupied lanes on the gel and continue electrophoresis for an additional 150 min at 70 W. The sample loaded first will have been subjected to electrophoresis for ~300 min, yielding well-resolved positions near the 5′ end of the RNA.

22| Expose the gel overnight to a phosphor screen and quantify scanned bands using a phosphorimaging instrument. Quantify the intensity of every well-defined band in the gel for the (+) and (−) NMIA lanes by two-dimensional densitometry. This step is conveniently performed using the SAFA (Semi-Automated Footprinting Analysis) program[13].

23| Calculate absolute NMIA reactivity at each position in the RNA by subtracting (−) NMIA intensities from (+) NMIA intensities. (+) and (−) NMIA intensities should be normalized to each other by assuming the low intensity (unreactive) positions in each experiment have the same value. This calculation is equivalent to assuming that at least a few nucleotides will be unreactive in most RNAs. In assigning the SHAPE band positions, the cDNA markers generated by dideoxy sequencing are exactly 1 nucleotide longer than the corresponding (+) and (−) NMIA cDNAs.

? TROUBLESHOOTING

Please refer to **Table 1** for troubleshooting advice.

TABLE 1 | Troubleshooting table.

Problem	Reason	Solution
Bright bands present in the (−) NMIA lane.	RNase contamination; this is, by far, the most common problem encountered for new practitioners of SHAPE technology.	Identify contaminated solution by running mock SHAPE experiments with [^{32}P]-labeled RNA.
	Structure-induced pausing by the reverse transcriptase enzyme.	Heat RNA in 0.5× TE to 95 °C for 3 min, cool on ice for 3 min before adding primer. Increase the time of extension to ~30 min. Try primer extension reactions at different temperatures (±3 °C).
Very low signal in the (+) NMIA lane but an intense full-length product is observed.	Insufficient modification of RNA.	Perform modification using a 2-fold higher concentration of NMIA.
No full-length product in the (+) NMIA lane or intense bands that disappear rapidly with read length.	Excessive modification of RNA.	Perform modification using a 2-fold lower concentration of NMIA.
No full-length product in any lane.	Poor or incomplete primer extension.	The reverse transcriptase enzyme is very sensitive to $MgCl_2$ concentration. Make sure final solution conditions are 3 mM in $MgCl_2$. Enzyme is also sensitive to freezing. Retry the experiment carefully with fresh enzyme.
Smearing near the full-length extension band.	Incomplete degradation of the RNA strand or re-extension of the 3′-end of the RNA in the RNA-DNA hybrid.	Decrease extension time; incubate at 95 °C with a higher concentration of NaOH.
No sequencing bands in ddNTP sequencing lane(s).	ddNTPs were incorporated in the wrong proportion.	Adjust the ddNTP concentrations upwards or downwards by a factor of 2.
Extra bands in the ddNTP sequencing lane, not seen in (−) NMIA lane.	Pauses in extension due to misfolding.	Use a (−) NMIA reaction as the template for sequencing.
No extension in (+) or (−) NMIA lanes, but extension in sequencing lanes.	RNA lost during modification/ precipitation.	Perform the ethanol precipitation step in the presence of 20 µg glycogen as a co-precipitant.
The faintest bands in the (+) NMIA lane have significantly different intensity as compared to the corresponding bands in the (−) NMIA lanes.	Random error involved with volume measurement; gel loaded unevenly.	Small differences in background intensity can be treated by mathematical normalization (see Step 23). If intensities are significantly different, the gel should be re-run by loading samples with similar amounts of cDNA fragments in each lane.

● TIMING

Steps 1–5: 40 min

Steps 6–9: 1 h 10 min plus incubation time

Steps 10–20: 1 h

Step 21: ~3–5 h

Steps 22, 23: 3 h plus time necessary to expose screen

ANTICIPATED RESULTS

A minimal SHAPE experiment consists of three or four lanes resolved in a sequencing gel (**Fig. 4a**). This representative experiment was performed using an *in vitro* transcript corresponding to yeast $tRNA^{Asp}$ embedded within the structure cassette shown in **Figure 2**. Two sequencing lanes were used to assign the SHAPE reactivities observed in the (–) and (+) NMIA reagent lanes. The bright bands at the top of the gel correspond to the relatively abundant full-length extension product. Bands corresponding to the unextended DNA primer and to short extension products, caused by pausing of reverse transcriptase during initiation of primer extension, are too short to be observed in this gel image. Approximately 90 RNA nucleotides are sufficiently well resolved that their absolute SHAPE reactivities can be quantified.

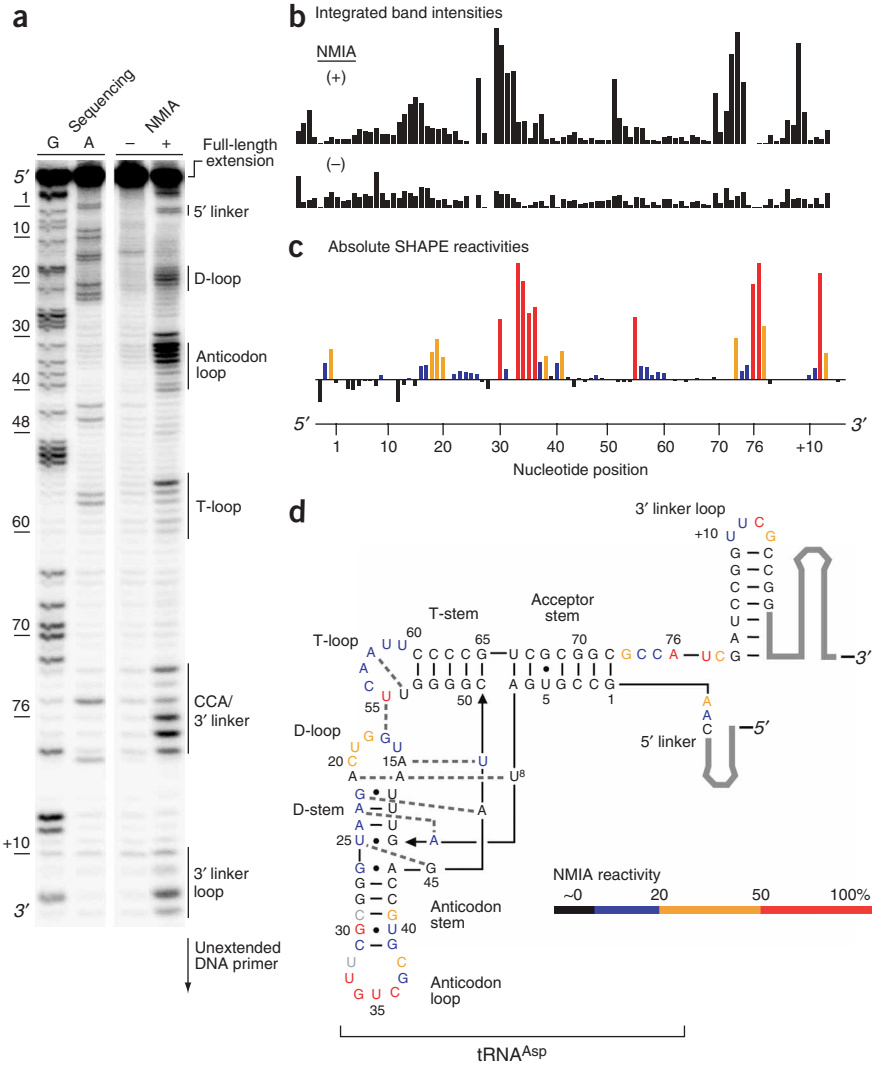

Figure 4 | Representative SHAPE analysis for yeast $tRNA^{Asp}$, embedded within a structure cassette. (**a**) Typical results from a SHAPE experiment. G and A are sequencing reactions performed using cytosine and thymidine dideoxy terminating nucleotides. These marker lanes are exactly 1 nucleotide longer than the corresponding NMIA lanes. Nucleotide numbers (at left) correspond to the (–) and (+) reagent lanes. (–) and (+) NMIA lanes represent reactions differing only in the presence of the reagent. Regions expected to be unconstrained in the yeast $tRNA^{Asp}$ construct are emphasized with bars at the right of the gel image. (**b**) Band intensities for the (+) and (–) NMIA lanes from panel **a**. (**c**) Absolute SHAPE reactivities as a function of nucleotide position. (**d**) Superposition of absolute NMIA reactivities on a secondary structure model for yeast $tRNA^{Asp}$. The same coloring scheme is used to identify reactive (and therefore unconstrained) nucleotides in panels **c** and **d**. Nucleotides that are not analyzable because they are close to the 5′ end or primer binding site or because of band compression are represented as gray lines or letters. By convention, the 75-nt $tRNA^{Asp}$ is numbered 1–76 with nt 47 omitted. Portions of this figure are reproduced with permission from *J. Am. Chem. Soc.* **127**:4659 (Copyright 2005 American Chemical Society).

Positions in which SHAPE reactivity is significantly higher in the (+) NMIA reaction as compared to the no reagent (–) control are emphasized with vertical bars and correspond precisely to hairpin loops and unconstrained linker regions in the $tRNA^{Asp}$ construct (**Fig. 4a**). Band intensities can be quantified (**Fig. 4b**) and absolute SHAPE reactivities at almost every position within the RNA are obtained by subtracting the (–) control intensities from the (+) NMIA intensities (**Fig. 4c**). Superposition of absolute band intensities on a secondary structure model for the $tRNA^{Asp}$ construct yields very precise information regarding the pattern of base pairing and the formation of non-canonical tertiary interactions in this RNA (**Fig. 4d**). Almost all base paired positions in $tRNA^{Asp}$ are unreactive; whereas nucleotides in the T-, D- and anticodon loops are reactive. 5′ and 3′ flanking nucleotides have reactivities consistent with the design of the structure cassette. SHAPE chemistry also correctly reports that most positions involved in tertiary interactions have low local nucleotide flexibility. For example, nucleotides in the linking loops (residues U8–A9 and G45–C50) that form idiosyncratic tertiary interactions with the D-stem are uniformly unreactive (**Fig. 4d**).

One important application of this technology is that SHAPE reactivities can be used to constrain the output of secondary structure prediction algorithms. For RNAs that do not contain pseudoknots, we find the RNAstructure program[14] to be extremely

useful for obtaining well-defined and experimentally constrained secondary structure models[7,9]. Heuristically, nucleotides whose reactivities are at least 50% of the most reactive positions are typically single-stranded; whereas nucleotides with reactivities that are 20–50% of this maximum reactivity are typically either single-stranded or adjacent to a single-stranded, bulged or mismatched nucleotide (this class of reactivity is implemented as the Chemical Modification constraint in the RNAstructure program[14]). SHAPE information is then typically sufficient to determine or strongly constrain possible secondary structure models for many RNAs[5–7,9,10].

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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