

Time-resolved RNA SHAPE chemistry: quantitative RNA structure analysis in one-second snapshots and at single-nucleotide resolution

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Published online 10 September 2009; doi:10.1038/nprot.2009.126

RNA selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry exploits the discovery that conformationally dynamic nucleotides preferentially adopt configurations that facilitate reaction between the 2'-OH group and a hydroxyl-selective electrophile, such as benzoyl cyanide (BzCN), to form a 2'-O-adduct. BzCN is ideally suited for quantitative, time-resolved analysis of RNA folding and ribonucleoprotein (RNP) assembly mechanisms because this reagent both reacts with flexible RNA nucleotides and also undergoes auto-inactivating hydrolysis with a half-life of 0.25 s at 37 °C. RNA folding is initiated by addition of Mg²⁺ or protein, or other change in solution conditions, and nucleotide resolution structural images are obtained by adding aliquots of the evolving reaction to BzCN and then 'waiting' for 1 second. Sites of the 2'-O-adduct formation are subsequently scored as stops to primer extension using reverse transcriptase. This time-resolved SHAPE protocol makes it possible to obtain 1-second structural snapshots in time-resolved kinetic studies for RNAs of arbitrary length and complexity in a straightforward and concise experiment.

INTRODUCTION

RNA structural transitions are central to the ability of RNA to function inside the cell^{1–3}. A full understanding of RNA function therefore requires a nucleotide resolution view of the time-resolved mechanisms by which RNA molecules fold, interconvert between distinct states, and function in higher order assemblies. RNA folding events occur on a wide range of timescales, ranging from microseconds (μ s) to minutes, but many rate-determining steps occur on timescales spanning tens of seconds to minutes^{4–7}. These critical events might, in principle, be conveniently monitored using simple benchtop kinetics.

Current, well-established and highly useful approaches for probing the kinetics of RNA folding at nucleotide resolution include dimethyl sulfate (DMS) and hydroxyl radical footprinting^{8–11}. In practice, both time-resolved DMS and hydroxyl radical footprinting are experimentally challenging to perform and require significant experiment-specific optimization. DMS reactivity probes a subset of nucleotides, largely adenosine, and requires that the reagent be quenched in a separate inactivation step⁸. Hydroxyl radical footprinting is a powerful approach for probing the solvent accessibility of the RNA backbone, but either requires the use of a synchrotron¹⁰ or adding a separate quench step¹². To date, these experimental challenges have limited the interest in evaluating folding states and the associated folding pathways for large RNAs at nucleotide resolution. Time-resolved RNA selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry addresses these limitations and makes possible experimentally straightforward single-nucleotide resolution and simultaneous analysis of local structure at nearly every position in an RNA with 1-second time resolution¹³.

SHAPE chemistry exploits the discovery that the nucleophilic reactivity of the ribose 2'-hydroxyl position is strongly gated by the underlying local nucleotide flexibility (Fig. 1a)^{14–16}. Flexible nucleotides preferentially adopt conformations that react with a hydroxyl-selective electrophile, such as benzoyl cyanide (BzCN), to form a 2'-O-adduct. In contrast, base paired or otherwise

conformationally constrained nucleotides are unreactive. As almost all ribonucleotides possess a free 2'-hydroxyl, local nucleotide flexibility at each position in an RNA is interrogated simultaneously in a single experiment^{14,17}. Because RNA folding and ribonucleoprotein (RNP) assembly reactions typically involve large changes in the local environment at many nucleotides, SHAPE represents an ideal approach for monitoring these changes, comprehensively and at single-nucleotide resolution.

During the RNA structure-probing reaction, SHAPE electrophiles are concurrently inactivated by hydrolysis. Thus, a specific quench step is not required, provided the adduct-forming reaction with RNA is allowed to continue until all reagent is

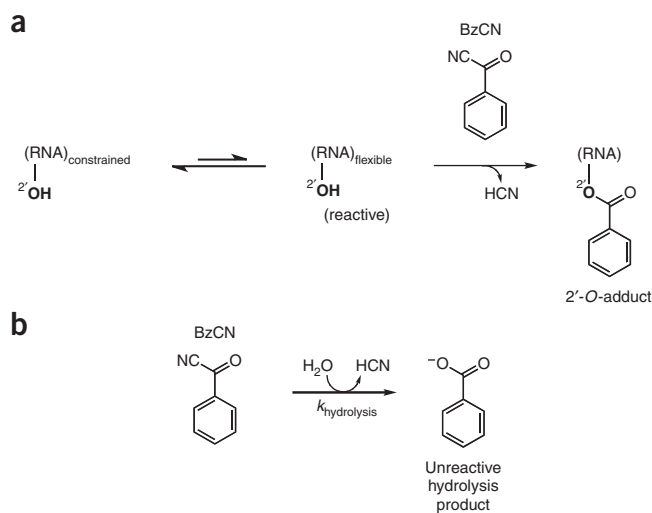


Figure 1 | The mechanism of RNA SHAPE chemistry with BzCN. (a) BzCN reacts with 2'-hydroxyl groups at conformationally flexible positions to form a 2'-O-adduct. (b) Parallel BzCN inactivation by hydrolysis.

consumed (Fig. 1b). BzCN undergoes hydrolysis with a half-life of 0.25 s at 37 °C: the reaction between BzCN and RNA is therefore complete in ~1 s.

Once the reaction is complete, sites of 2'-O-adduct formation are identified by annealing a 5'-labeled DNA primer to the modified RNA and scoring stops to primer extension using reverse transcriptase. The length and amount of a cDNA transcript correlates with the position and degree of modification at each position in the RNA. The length of each cDNA is assigned by comparison with a dideoxy sequencing reaction^{13,18}.

There are two potential limitations of time-resolved SHAPE as described in this protocol. SHAPE primarily measures local nucleotide flexibility. In general, this information gives good coverage of both secondary and tertiary structure interactions during an RNA folding or RNP assembly reaction^{15,19–21}. However, there may be some instances in which analysis of backbone solvent accessibility is essential or can provide critical additional information. In these cases, fast hydroxyl radical cleavage is a good alternative^{10,11}. The method outlined here emphasizes using time-resolved SHAPE in an experimentally straightforward benchtop approach that ultimately yields ~1-s structural images of RNA structure, taken 5–15 s apart. SHAPE can be adapted to faster timescales, in a more complex experiment, using a rapid mixing device and quenching the BzCN reagent with dithiothreitol¹³.

Time-resolved SHAPE with BzCN was initially used to study the time-resolved folding of the specificity domain of the RNase P RNA¹³. We anticipate that further application of this approach will make possible facile kinetic studies of many RNA in ~1-s snapshots. Time-resolved SHAPE holds broad potential for understanding structural biogenesis and the conformational interconversions essential for the function of complex RNA molecules at single-nucleotide resolution.

Experimental design

Time-resolved SHAPE takes advantage of the intrinsic feature of BzCN that it degrades completely in water in ~1 s. Thus, the experiment is as simple as adding a solution of an RNA folding reaction to a small aliquot of BzCN, 'waiting' for 1 s, and then analyzing the results by primer extension at a later point. Experimental conditions should be adjusted so that roughly 1 in 100–300 nucleotides (nts) are modified before BzCN inactivation. It is not necessary that exactly one modification occurs per RNA, but only that the level of modification is sufficiently sparse that sites of 2'-O-adduct formation are uncorrelated. Higher levels of modification produce stronger peak signals, whereas lower reagent concentrations yield longer read lengths for analysis by capillary electrophoresis. As BzCN reacts rapidly with trace amounts of water, it is important to take care in setting up the experiment to work with dry BzCN and DMSO. The concentration of buffer (e.g., HEPES) should be higher than the final concentration of BzCN to prevent acidification of the solution upon reagent hydrolysis (Fig. 1b). The reaction is largely insensitive to the presence of other solution components that might react with BzCN, including amines, carbohydrates and proteins.

MATERIALS

REAGENTS

- RNA at a concentration of ~1 μM, ~3 pmol per time point, dissolved in 0.5× TE buffer (5 mM Tris, 0.5 mM EDTA, pH 8.0).
- Deoxyadenosine, deoxycytidine and deoxythymidine triphosphates (dATP, dCTP and dTTP), 100 mM (Invitrogen, cat. nos. 55082, 55083, 55085)

RNA folding. RNA folding reactions can be initiated in many ways including changes in temperature, ionic strength or addition of a protein or small-molecule ligand. This protocol focuses on analyzing the formation of RNA tertiary structure initiated by addition of Mg²⁺ to a solution that otherwise contains all components necessary to stabilize the native structure. However, this approach is readily applied to most other methods of initiating an RNA folding or RNP assembly reaction and then makes it possible to analyze the resulting RNA conformational changes on the 1-s timescale.

Primer extension. Sites of time-dependent 2'-O-adduct formation can be analyzed using DNA primers containing either a 5'-radiolabel or a 5'-fluorescent tag. This protocol focuses on quantifying sites of 2'-O-adduct formation in a high-throughput way using fluorescently labeled primers in the primer extension step and then rapidly resolving and quantifying cDNA products by capillary electrophoresis^{13,18}. To use 5'-[³²P]-labeled DNA primers and conventional sequencing gel electrophoresis, the procedure below should be followed up to Step 17. An alternate protocol for analyzing radiolabeled cDNAs by conventional gel electrophoresis has been described earlier (and replaces Steps 18–24 below)¹⁷. In general, the quality of the quantitative nucleotide reactivity information is far superior using capillary electrophoresis; however, use of radiolabeled primers can be more sensitive in cases where RNA is limiting. Using either primer extension approach, no reactivity information is obtained at the sequence to which the primer binds, nor 20–50 nts 5' of the reverse transcriptase start site^{14,17}. Thus, the DNA primer-binding site can either be a natural sequence 3' of the RNA sequence of interest or an appended 'structure cassette' sequence¹⁴ that allows the entire structure of the RNA to be evaluated.

Analysis of time-progress curves. We typically normalize SHAPE data to a scale that spans 0 to ~2. Zero corresponds to an unreactive position and 1.0 is defined as the average intensity of highly reactive nucleotides^{13,15,18}. On this scale, time-dependent changes in reactivity are generally significant and quantifiable if they are ≥0.2 SHAPE units (Fig. 2a). The quality and reproducibility of the time-resolved SHAPE profiles are typically quite high and it is often possible to distinguish processes characterized by either a single

$$I = A + (1 - A)e^{-k_2t} \quad (1)$$

or double

$$I = A + (1 - A - B)e^{-k_1t} + Be^{-k_2t} \quad (2)$$

exponential (Fig. 2b)¹³, or in terms of two irreversible consecutive steps⁴:

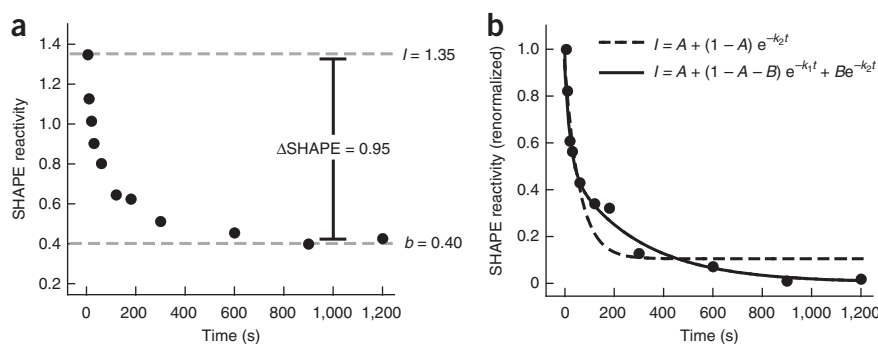
$$I = A(k_1 - k_2) - 1[k_2e^{(-k_1t)} - k_1e^{(-k_2t)}] + (1 - A) \quad (3)$$

More complex kinetic schemes can also be handled by other approaches²².

- Deoxyinosine triphosphate (dTTP), 100 mM (Trilink Biotechnologies, cat. no. N2012). Use of dTTP reduces band compression at G residues and increases resolution of primer extension products by capillary electrophoresis.
- Dideoxyadenosine, dideoxycytidine, dideoxythymidine and dideoxyguanosine triphosphates (ddATP, ddCTP, ddTTP and ddGTP),



Figure 2 | Analysis of time-progress curves. Nucleotide G217 of the RNase P specificity domain is shown. (a) Representative change in SHAPE reactivity as a function of time. Y-axis data are shown using absolute SHAPE reactivities that have been normalized to a scale that spans 0 to ~1.5, where 1.0 is defined as the average intensity of highly reactive positions. The absolute change in SHAPE reactivity at this nucleotide is 0.95, which means the time-progress curve can be quantified at a high level of accuracy and significance. (b) Quantitative analysis of time-resolved changes in local nucleotide flexibility. Data from a have been renormalized on the y axis such that reactivity at time = 0 is defined as 1.0 and is obtained by dividing the initial maximum intensity ($I = 1.35$), after first subtracting the plateau SHAPE reactivity ($b = 0.40$). Most nucleotides tend to follow simple kinetic behavior and are well fit using straightforward kinetic equations. A double exponential (solid line, equation (2)) is the best choice for these data.



(Trilink Biotechnologies, cat. nos. N-4001, N-4005, N-4004 and N-4002); prepare 10 mM solutions of ddATP, ddCTP and ddTTP and/or 0.25 mM of ddGTP by diluting in sterile water. ▲ **CRITICAL** Nucleotide solutions are stable for months at $-20\text{ }^{\circ}\text{C}$ but are intolerant of freeze-thaw cycles. It is recommended to maintain small aliquots at $-20\text{ }^{\circ}\text{C}$.

- Glycogen, 20 mg ml⁻¹ (Invitrogen, cat. no. 10814-010)
- EDTA, 0.5 M (Ambion, cat. no. 9260G)
- Deionized formamide (Applied Biosystems, cat. no. 4311320)
- DMSO, molecular biology grade (Sigma-Aldrich, cat. no. D8418). ▲ **CRITICAL** DMSO bottle should be stored in a desiccator at room temperature ($\sim 22\text{ }^{\circ}\text{C}$).
- BzCN (Sigma-Aldrich, cat. no. 115959). ▲ **CRITICAL** BzCN bottle should be stored in a desiccator at room temperature.
- SuperScript III Reverse Transcriptase (Invitrogen, cat. no. 18080-093)
- 5× SSIII FS buffer (250 mM Tris (pH 8.3), 375 mM KCl, 15 mM MgCl₂) (Invitrogen, cat. no. 18080-093)
- 0.1 M of DTT (Invitrogen, cat. no. 18080-093)
- DNA primer: use a primer ~ 18 to 20 nt in length and that forms a 3' GC base pair with the target RNA.
- 0.65 and 1.5 ml RNase-free polypropylene (Eppendorf) reaction tubes.

EQUIPMENT

- -80 and $-20\text{ }^{\circ}\text{C}$ freezers
- Microfuge for 1.5-ml reaction tubes at $4\text{ }^{\circ}\text{C}$
- Automated capillary electrophoresis instrument. We use instruments typically sold for DNA sequencing applications and have obtained excellent results with instruments from both Beckman^{15,18} and Applied Biosystems (ABI)^{13,23}. It is easier to obtain fluorescently labeled DNA primers for ABI instruments.
- Programmable incubator or heat block. We recommend an incubator with a heated top of the type typically used for carrying out PCR.
- ShapeFinder software system (freely available to academic researchers at <http://bioinfo.unc.edu/Downloads>).

PROCEDURE

RNA folding ● **TIMING 20 min**

1| Add 78 pmol of RNA (3 pmol per each (+) and (-) reagent time point) in 144 μl of sterile H₂O to a 0.65-ml reaction tube. This procedure yields 13 total data points, including a no-Mg²⁺ reference.

? TROUBLESHOOTING

- 2| Heat the RNA solution to $95\text{ }^{\circ}\text{C}$ for 2 min and then place the RNA on ice for 1 min.
- 3| Add 72 μl of 3.3× no-Mg²⁺ folding buffer and mix thoroughly.
- 4| Incubate the tube at the desired reaction temperature (25 or $37\text{ }^{\circ}\text{C}$) for 5–10 min.
- 5| Pre-incubate a 0.65-ml reaction tube containing 22 μl of 10× MgCl₂ solution at the same temperature as the RNA solution.

REAGENT SETUP

Enzyme stop mix 50 mM EDTA (pH 8.0). It can be stored at room temperature and is stable for many months.

TE buffer 10 mM Tris, 1 mM EDTA (pH 8.0). It can be stored at room temperature and is stable for many months.

3.3× no-Mg²⁺ RNA folding solution 333 mM HEPES (pH 8.0) and 333 mM NaCl. It can be stored at room temperature and is stable for many months. These are the conditions under which the RNA is equilibrated before initiating the folding reaction. Buffer, ions and ionic strength can be varied. In general, reaction with BzCN is tolerant of wide variations in experimental conditions. The primary critical requirement is that the final concentration of buffer should be greater than the BzCN concentration during the modification reaction.

10× MgCl₂ 100 mM MgCl₂. It can be stored at room temperature and is stable for many months. Any concentration of MgCl₂ sufficient to yield the biologically active or target RNA structure can be used.

10× BzCN in DMSO The optimal concentration can vary with RNA length. The useful range is 100–800 mM. For longer RNAs, use the lower end of these BzCN concentrations. A good starting concentration is 400 mM of BzCN (40 mM, final concentration). ▲ **CRITICAL** The BzCN solution should be prepared fresh for every experiment and can be stored at room temperature for 1 d if kept in a desiccator.

SuperScript enzyme mix 250 mM KCl, 167 mM Tris-HCl (pH 8.3), 1.67 mM each dNTP, 17 mM DTT and 10 mM MgCl₂. The enzyme mix can be prepared by combining 4 parts of SSIII FS buffer, 1 part of 0.1 M DTT and 1 part of 10 mM dNTP mix (10 mM in each deoxynucleotide). The enzyme mix must be stored at $-20\text{ }^{\circ}\text{C}$ and is sensitive to freeze-thaw cycles. It is recommended to maintain small aliquots.

5'-fluorescently labeled primers These can be ordered from several companies including Applied Biosystems, IDT and Trilink Biotechnologies. At least three, preferentially four, DNA primers, each labeled with a different fluorophore, are needed. Compatible dye sets depend on the sequencing instrument used. Fluorescent-labeled primers must be stored at $-20\text{ }^{\circ}\text{C}$ away from light and are sensitive to freeze-thaw cycles. It is recommended to maintain small aliquots.

PROTOCOL

RNA modification ● TIMING length of time course plus 1 h 30 min

6| Aliquot 1 μl of $10\times$ BzCN in DMSO and 1 μl of neat DMSO into each of 13 0.65-ml reaction tubes; these are the (+) and (–) BzCN tubes, respectively.

7| Take a no- Mg^{2+} initial reference time point by transferring two 9- μl aliquots of the RNA solution into reaction tubes containing 1 μl of BzCN in DMSO or 1 μl of DMSO. This point can also be taken as a time = 0 reference.

8| Initiate RNA folding by adding the remaining 198 μl solution of re-folded RNA to the tube containing $10\times$ MgCl_2 (~220 μl of total final volume). Mix thoroughly and start timing.

9| At desired time points (up to 12 total), remove 9 μl and add to a 0.65-ml reaction tube containing 1 μl of pre-aliquoted $10\times$ BzCN in DMSO. Mix thoroughly by rapid pipetting 2–3 times. Repeat by adding 9 μl to a tube containing 1 μl neat DMSO and mix thoroughly (each time point requires in total 18 μl of the folded RNA solution). Reaction with BzCN is complete in 3 and 1 s at 25 and 37 $^\circ\text{C}$, respectively; no explicit quench step is required. Obtaining time points at 15-s intervals is straightforward. With practice, time points at 5–7-s intervals are possible.

? TROUBLESHOOTING

10| At the end of the time course, when all time points have been obtained, recover the modified RNA solution by ethanol precipitation. To each tube (26 total), add 90 μl of sterile H_2O , 5 μl of 4 M NaCl, 1 μl of 20 mg ml^{-1} glycogen, 400 μl of 100% ethanol, mix and incubate at -80°C for 30 min. Sediment the RNA solution by spinning at $\geq 10,000$ r.p.m. ($\geq 9,000g$) speed in a microfuge at 4 $^\circ\text{C}$ for 15 min.

11| Remove the supernatant and re-dissolve RNA solution in 10 μl of $0.5\times$ TE buffer.

■ PAUSE POINT Modified RNA solution can be stored overnight at -20°C .

Primer extension and RNA sequencing ● TIMING 2 h

12| Add 3 μl of 0.3 μM fluorescently or radiolabeled primers to the (+) and (–) BzCN reactions.

? TROUBLESHOOTING

13| For each required sequencing reaction, add 3 μl of 0.3 μM fluorescently or radiolabeled primer to 3 pmol of RNA in 8 μl of $0.5\times$ TE buffer. Each time point requires its own sequencing reaction; hence, multiply by the number of time points (one reference, plus 12 time points, as described here).

▲ CRITICAL STEP For resolution by a capillary electrophoresis instrument, each reaction (including (+) and (–) reagent and sequencing) will be monitored using a DNA primer labeled with a different color-coded fluorophore. Several compatible fluorescent dye sets are possible. For resolution on an ABI instrument, we typically use dye sets consisting of FAM, VIC, NED and PET (ABI G5 dye set); FAM, TET, HEX and NED; or FAM, JOE, TAMRA and ROX.

14| Anneal the primer to the RNA solution by heating at 65 $^\circ\text{C}$ for 5 min and placing on ice for 1 min.

15| Add 6 μl of SuperScript enzyme mix to the (+) and (–) BzCN reactions and to the sequencing reactions. To each sequencing reaction, also add 1 μl of ddNTP solution.

? TROUBLESHOOTING

16| Add 1 μl of SuperScript III to each tube. Mix well and incubate at 45 $^\circ\text{C}$.

17| Incubate at 45 $^\circ\text{C}$ for 1 min, 52 $^\circ\text{C}$ for 25 min, 65 $^\circ\text{C}$ for 5 min and then place on ice.

▲ CRITICAL STEP Omitting the 45 $^\circ\text{C}$ step can lead to poor primer extension, especially for longer RNAs.

▲ CRITICAL STEP For radiolabeled primers, omit Steps 18–24 below and replace with Steps 17–23 as described in reference 17.

? TROUBLESHOOTING

18| Add 4 μl of 50 mM EDTA (pH 8.0) to each tube to quench the extension reaction and place on ice.

19| For each time point, combine the (+) and (–) BzCN reactions and a sequencing reaction (22 μl each, total 66–88 μl) into a 1.5-ml reaction tube and recover the cDNAs by ethanol precipitation: add 240 μl of 100% ethanol to each tube (13 total) and incubate at -80°C for 15 min. Sediment the cDNA by spinning at ≥ 10000 r.p.m. ($\geq 9,000g$) in a microfuge at 4 $^\circ\text{C}$ for 15 min.

20| Remove the supernatant and add 800 μl of 70% ethanol. Invert the tube to dislodge the pellet and spin at maximum speed in a microfuge at 4 $^\circ\text{C}$ for 2 min.

Figure 3 | Electropherogram analysis using ShapeFinder²⁴. (a) Unprocessed capillary electrophoresis electropherogram exported from an ABI sequencer (no matrixing step required). (b) Resulting trace after application of the Fitted Baseline Adjust and Mobility Shift:Cubic tools. (c) Application of the Align and Integrate tool to quantify all peaks in the (+) and (−) reagent channels by whole-trace Gaussian integration. Channels are split for clarity and ease of analysis; the aligned RNA sequence is at the bottom of the window.

21| Repeat Step 20.

▲ **CRITICAL STEP** Omitting this step leaves residual salt in the primer extension reaction solutions and can lead to poor resolution during capillary electrophoresis.

? **TROUBLESHOOTING**

22| Dry the pellet by vacuum for 10 min and resuspend in 10 μl of deionized formamide (this volume will vary depending on the capillary electrophoresis instrument used).

■ **PAUSE POINT** The resuspended pellets can be stored at −20 °C away from light for an extended period of time. For best results, run samples immediately.

cDNA analysis by capillary electrophoresis ● **TIMING 45 min**

23| Load each 10 μl sample into separate wells on a capillary electrophoresis DNA sequencing instrument and run.

? **TROUBLESHOOTING**

24| Export the resulting raw traces into ShapeFinder²⁴ (Fig. 3a), process the data by applying (i) a baseline adjustment, (ii) a mobility shift appropriate for the dye set used²⁴ and (iii) a multiplicative scaling factor, if necessary, to the (+) and (−) BzCN traces (Figs. 3b and 4a; see Table 1 and ref. 24 for details). Quantify the intensity of all peaks in the (+) and (−) BzCN reactions by whole-trace Gaussian integration using the Align and Integrate tool in ShapeFinder (Fig. 3c). ShapeFinder outputs a text file containing the integrated areas for each peak in the electropherogram, the net reactivity after subtraction of the (−) reagent background and the normalized SHAPE reactivities using a scale in which 1.0 is defined as the average intensity of highly reactive positions (Fig. 4b). These steps can be fine-tuned in a spreadsheet application.

? **TROUBLESHOOTING**

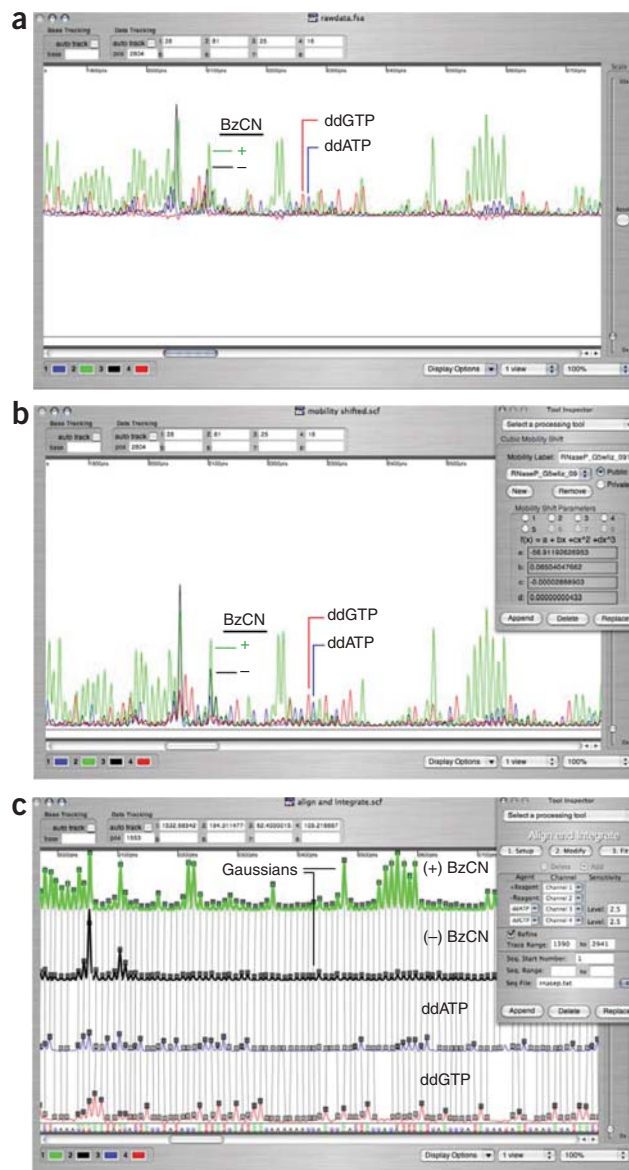
Data analysis ● **TIMING 1 or more days**

25| Plot the normalized SHAPE reactivities as a function of time for each nucleotide in the RNA. Inspect the individual curves for their kinetic behavior. Typically, one-half of the nucleotides in an RNA will show no change. This is the expected result because the local environment at some positions does not change with folding. At other nucleotides, the SHAPE reactivities will decrease (largest number of positions) or increase (a few positions). In both cases, a significant change in the local nucleotide flexibility during folding is one with $\Delta\text{SHAPE} \geq 0.2$ (Fig. 2a).

? **TROUBLESHOOTING**

26| We generally interpret time–progress curves in terms of net, normalized intensity plots at each nucleotide position: (i) perform an initial exponential fit to determine the plateau value (*b*) at long times; (ii) subtract this value from all data points; and (iii) re-normalize intensities to a scale spanning 0–1.0 by dividing the intensity of the first time point (*I*) (Fig. 2a).

27| Individually fit time-dependent changes in SHAPE reactivity for each nucleotide to an appropriate kinetic equation. For most nucleotides, a single or double exponential or an equation describing two consecutive steps is generally sufficient (Fig. 2b



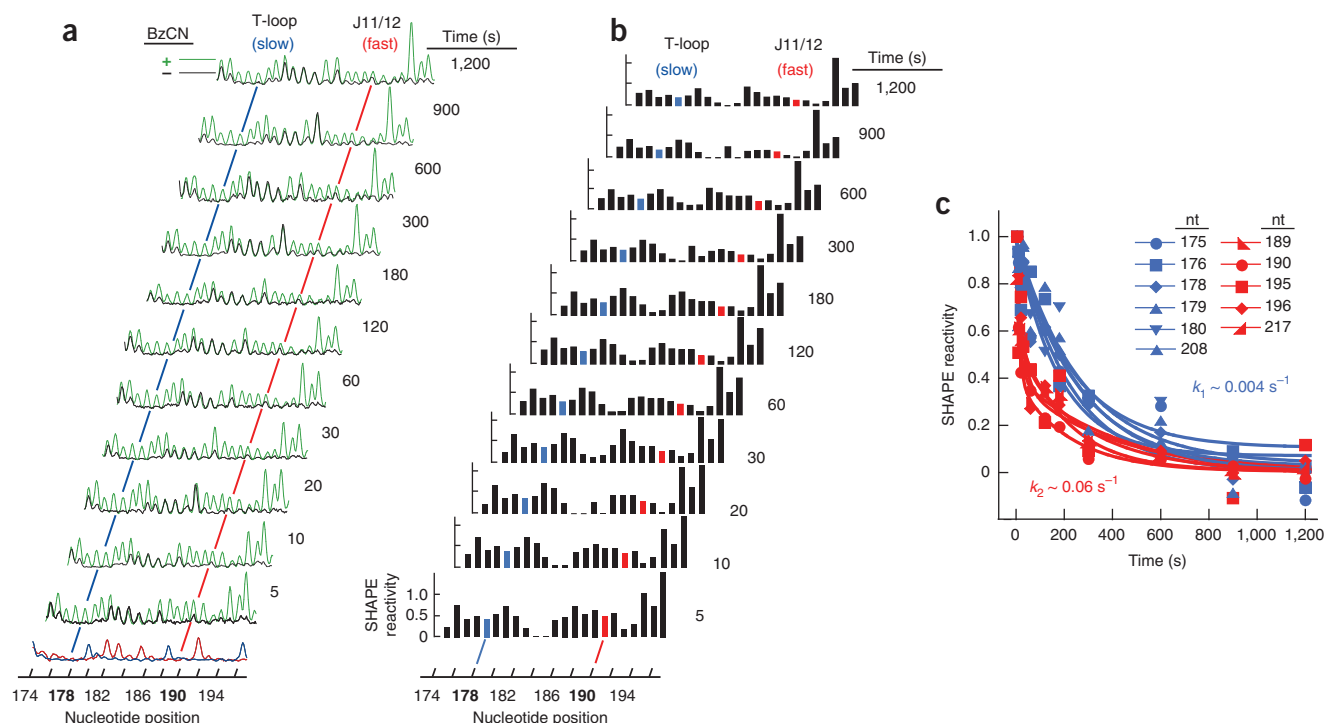


Figure 4 | Representative time-resolved SHAPE analysis of the RNase P specificity domain RNA. **(a)** Processed capillary electrophoresis traces obtained for the reaction of BzCN with the RNase P specificity domain for folding times spanning 5 s–20 min. RNA folding was initiated by the addition of Mg²⁺ to RNA pre-equilibrated in buffer and NaCl. (+) and (–) reagent channels are shown in green and black; sequencing channels (at bottom) were used to assign peak position in ShapeFinder²⁴. The difference in peak intensity between the (+) and (–) BzCN channels reports the extent of the 2'-O-adduct formation at each point. Nucleotides involved in tertiary interactions show a decrease in reactivity over time. For example, nucleotide 178 becomes protected from reaction with BzCN on a slow timescale, whereas nucleotide 190 undergoes this transition more rapidly (blue and red lines, respectively). **(b)** Absolute SHAPE reactivities as a function of nucleotide position and time. **(c)** Representative time-progress curves monitored by time-resolved SHAPE. For the RNase P RNA, many nucleotides show a decrease in SHAPE reactivity as a function of time, indicating a significant increase in structural constraints as the RNA forms tertiary interactions. Each time-dependent profile was fit individually to either a single or double exponential (equation (1) or (2) in the main text). All curves found to fit a single exponential (in blue) were characterized by the same rate constant, at 0.004 s⁻¹. Nucleotides best fit by a double exponential (in red) were characterized by a fast phase at 0.06 s⁻¹ and a slow phase at 0.004 s⁻¹. Adapted in part from reference 13.

and see equations (1–3)). For each time-progress curve, the equation that best corresponds to the time-resolved SHAPE data can be distinguished either by observation or using Pearson's *r*-value. Group nucleotides according to rate constants that are less than twofold apart (**Fig. 4c**).

? TROUBLESHOOTING

28 | Superimpose kinetic data on a structural model of the RNA. Develop a model for the RNA folding or RNP assembly mechanism based on clustering nucleotides that fold with similar rates. Use the number of observed rate constants to estimate the number of kinetically significant structural intermediates (**Fig. 5**).

● TIMING

- Steps 1–5, RNA folding: 20 min
- Steps 6–11, RNA modification: length of time course plus 1 h 30 min
- Steps 12–22, Primer extension and RNA sequencing: 2 h
- Step 23, cDNA analysis by capillary electrophoresis: 45 min
- Steps 24–28, Data analysis: 1 or more days depending on the nature and depth of analysis

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Reason	Solution
1,12	Low-fluorescence signal in electropherogram	Not sufficient primer or RNA in primer extension reactions	Increase the amount of primer and/or RNA (to 5–8 pmol)
1–21	Intense peaks present in the (–) BzCN capillary electrophoresis trace	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 5′-fluorescently or [³² P]-labeled RNA
9	Very low signal in the (+) BzCN channel but an intense full-length product is observed	Insufficient modification of RNA	Perform modification using a twofold higher concentration of BzCN
	No signal in the (+) BzCN trace	No modification of RNA	BzCN is very sensitive to trace amounts of water. Make sure the BzCN/DMSO stock is kept dry and use fresh solutions for each experiment
	No full-length product in the (+) BzCN trace or intense bands that disappear rapidly with read length	Excessive modification of RNA	Perform modification using a twofold lower concentration of BzCN
12–17	Intense peaks present in the (–) BzCN capillary electrophoresis trace	Structure-induced pausing by the reverse transcriptase enzyme	Heat the RNA solution in 0.5× TE buffer to 95 °C for 3 min, cool on ice for 3 min before adding primer Increase the time of extension at both 52 °C and 65 °C
	No full-length product in any channel in the capillary electrophoresis electropherogram	Poor or incomplete primer extension	The reverse transcriptase enzyme is very sensitive to MgCl ₂ concentration. Ensure final solution conditions are 3 mM in MgCl ₂ . Enzyme is also sensitive to freezing. Retry the experiment with fresh enzyme
15	No sequencing bands in ddNTP sequencing trace(s)	ddNTPs were incorporated in the wrong proportion	Adjust the ddNTP concentrations upward or downward twofold
23	Poor resolution or broad peaks in the electropherogram	Too much residual salt present in the sample when loaded onto the capillary electrophoresis instrument	Perform additional 70% ethanol wash before drying and resuspending the pellet in formamide
23,24	The faintest bands in the (+) BzCN channel have significantly different intensity compared with the corresponding peaks in the (–) BzCN channel	Random error involved with volume measurement; difference in the quantum yield of different fluorophores	Re-normalize traces using the Scale Factor tool in ShapeFinder. Run primers labeled with each fluorophore to determine the multiplicative factor that yields equal intensities across fluorophores
	Sharp or negative peaks are present in the unprocessed or processed electropherogram	Improper automatic matrixing of dyes	Ensure overall dye intensity is roughly the same in all lanes. Use primer concentrations that yield similar intensities for each fluorescent signal
25–27	Time–progress curves do not clearly fit a simple exponential equation	Multiple RNA folding populations or transitions	Ensure that the RNA is in a single defined conformation upon initiation of the folding reaction. Perform equilibrium SHAPE experiments on the RNA under initial folding conditions and evaluate whether data are consistent with a single conformation

Portions of this table are adapted from reference 17.



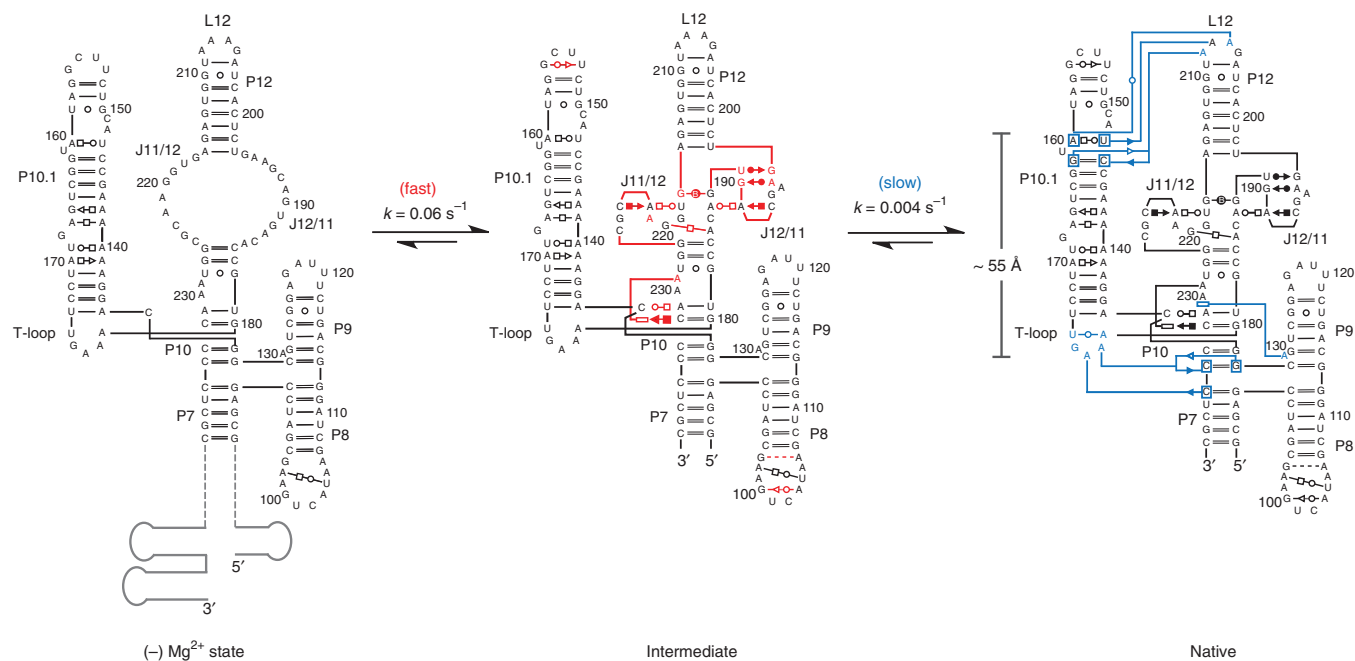


Figure 5 | Mechanism for folding of the RNase P specificity domain determined by time-resolved SHAPE. Secondary structure is drawn to approximate the arrangement of RNA helices and tertiary interactions as they occur in three dimensions². Fast-forming interactions are emphasized in red. These are interpreted as reflecting formation of a distinct folding intermediate. Interactions that form in the slow, rate-determining, step are in blue. Structure cassette sequences are shown schematically in gray in the first panel. Reprinted with permission from reference 13.

ANTICIPATED RESULTS

Time-resolved SHAPE provides 1-s snapshots of RNA structure for RNAs of nearly arbitrary complexity over time periods spanning a few seconds to many minutes. The fluorescently encoded data for each individual time point are resolved in three or four channels in a single capillary using capillary electrophoresis instrument (Fig. 3a). Time-progress curves can also be monitored using [³²P]-labeled DNA primers and resolved on sequencing gels¹⁷, but data are generally of much higher quality using capillary electrophoresis.

The representative experiment outlined here was performed using an *in vitro* transcript corresponding to the *Bacillus subtilis* RNase P specificity domain²⁵ embedded within a structure cassette that facilitates analysis of the very 5' and 3' ends of the RNA¹⁴. Tertiary folding of the RNase P RNA was initiated by the addition of Mg²⁺ (to 10 mM) to a pre-equilibrated solution containing RNA, buffer and 100 mM of NaCl. Each capillary electrophoresis electropherogram (Fig. 4a) contains reactivity data for the entire RNase P RNA (~150 nts). Peaks in the processed capillary electrophoresis traces are quantified by subtracting background intensities in the no-reaction control from the (+) BzCN reaction. After normalization, absolute SHAPE reactivities were obtained at almost every position within the RNA over a dense sampling of time points (Fig. 4b). Nucleotides that show significant changes in SHAPE reactivity typically fall naturally into a smaller set of kinetically distinct behaviors. In the RNase P RNA, nucleotides partition into two distinct categories, both in terms of the observed rate constant and in terms of whether each curve was best fit by a single or double exponential. Approximately half the nucleotides in the RNase P RNA folded slowly ($k_1 = 0.004 \text{ s}^{-1}$) in a transition characterized by a single exponential (Fig. 4c, blue).

The remaining nucleotides exhibiting significant time-dependent change in SHAPE reactivity were well described by fitting to a double exponential (Fig. 4c, red). The slower rate constant, 0.004 s^{-1} , was identical to that observed for the positions characterized by a single exponential, whereas the faster rate constant, at 0.06 s^{-1} , represents an additional kinetically significant step.

Time-resolved SHAPE data then make possible modeling of kinetic intermediates in the folding pathway for an RNA at nucleotide resolution. For the RNase P specificity domain RNA, time-resolved SHAPE clearly identifies two kinetically significant steps. A good first step is typically to superimpose the observed kinetic behavior on a secondary structure model for the RNA. Consistent with the two categories of reactivity changes, these changes cluster within two secondary (and tertiary) structure regions of the RNA. The fast folding step involves nucleotides in the tertiary structure module involving the J11/12 and J12/11 loops (residues 185–196 and 217–225 nts) and also in the interaction of A229 with C134 and C232 (in red, Fig. 5).

Nucleotides characterized by the slower folding step involve the formation of three distinct sets of long-range interactions: docking of the T-loop (U175–A179) at P7–P10, stacking of A230 on A130 in P9, and the docking of the GAAA tetraloop (P12) into its receptor in P10.1 (in blue, **Fig. 5**).

A comprehensive folding mechanism for the RNase P specificity domain was also developed before our study using equilibrium and kinetic approaches^{26,27}. In general, nucleotide resolution information was obtained for folding intermediates under equilibrium conditions using chemical mapping experiments²⁶, and time-resolved information was obtained for the overall folding of the whole RNA domain, including for relatively fast processes that are not accessible by benchtop time-resolved SHAPE²⁷. These approaches yielded a model that shares major features with that determined by time-resolved SHAPE, including that short-range tertiary interactions form prior to longer-range tertiary interactions and that the tetraloop-receptor and T-loop interactions form simultaneously in the rate determining step (**Fig. 5**). A useful feature of time-resolved SHAPE was that sufficient information required to create a nucleotide-resolution model for the predominant RNA folding pathway could be obtained in a single, concise set of experiments. Time-resolved SHAPE also revealed the distinct rates for each folding transition and the specific nucleotides involved in each step.

We envision that time-resolved SHAPE with BzCN will make possible facile analysis of the pathways for formation of individual sets of tertiary interactions in complex RNAs and RNPs on timescales as short as a few seconds using very simple benchtop kinetics.

ACKNOWLEDGMENTS This work was supported by a grant from the National Science Foundation (MCB-0416941 to K.M.W.).

AUTHOR CONTRIBUTIONS S.A.M. and K.M.W. collaborated on all aspects of the conception, design, presentation and writing of this paper.

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1. Tinoco, I. Jr. & Bustamante, C. How RNA folds. *J. Mol. Biol.* **293**, 271–281 (1999).
2. Leontis, N.B. & Westhof, E. Analysis of RNA motifs. *Curr. Opin. Struct. Biol.* **13**, 300–308 (2003).
3. Gesteland, R.F., Cech, T.R. & Atkins, J.F. (eds.). *The RNA World* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2004).
4. Webb, A.E. & Weeks, K.M. A collapsed state functions to self-chaperone RNA folding into a native ribonucleoprotein complex. *Nat. Struct. Biol.* **8**, 135–140 (2001).
5. Furtig, B. *et al.* Time-resolved NMR studies of RNA folding. *Biopolymers* **86**, 360–383 (2007).
6. Williamson, J.R. Biophysical studies of bacterial ribosome assembly. *Curr. Opin. Struct. Biol.* **18**, 299–304 (2008).
7. Woodson, S.A. RNA folding and ribosome assembly. *Curr. Opin. Chem. Biol.* **12**, 667–673 (2008).
8. Tijerina, P., Mohr, S. & Russell, R. DMS footprinting of structured RNAs and RNA-protein complexes. *Nat. Protoc.* **2**, 2608–2623 (2007).
9. Hennelly, S.P. *et al.* A time-resolved investigation of ribosomal subunit association. *J. Mol. Biol.* **346**, 1243–1258 (2005).
10. Sclavi, B., Woodson, S., Sullivan, M., Chance, M. & Brenowitz, M. Following the folding of RNA with time-resolved synchrotron X-ray footprinting. *Methods Enzymol.* **295**, 379–402 (1998).
11. Shcherbakova, I. & Brenowitz, M. Monitoring structural changes in nucleic acids with single residue spatial and millisecond time resolution by quantitative hydroxyl radical footprinting. *Nat. Protoc.* **3**, 288–302 (2008).
12. Brenowitz, M., Chance, M.R., Dhavan, G. & Takamoto, K. Probing the structural dynamics of nucleic acids by quantitative time-resolved and equilibrium hydroxy radical ‘footprinting’. *Curr. Opin. Struct. Biol.* **12**, 648–653 (2002).
13. Mortimer, S.A. & Weeks, K.M. Time-resolved RNA SHAPE chemistry. *J. Am. Chem. Soc.* **130**, 16178–16180 (2008).

14. Merino, E.J., Wilkinson, K.A., Coughlan, J.L. & Weeks, K.M. RNA structure analysis at single nucleotide resolution by selective 2'-hydroxyl acylation and primer extension (SHAPE). *J. Am. Chem. Soc.* **127**, 4223–4231 (2005).
15. Mortimer, S.A. & Weeks, K.M. A fast-acting reagent for accurate analysis of RNA secondary and tertiary structure by SHAPE chemistry. *J. Am. Chem. Soc.* **129**, 4144–4145 (2007).
16. Gherghe, C.M., Shajani, Z., Wilkinson, K.A., Varani, G. & Weeks, K.M. Strong correlation between SHAPE chemistry and the generalized NMR order parameter (S2) in RNA. *J. Am. Chem. Soc.* **130**, 12244–12245 (2008).
17. Wilkinson, K.A., Merino, E.J. & Weeks, K.M. Selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution. *Nat. Protoc.* **1**, 1610–1616 (2006).
18. Wilkinson, K.A. *et al.* High-throughput SHAPE analysis reveals structures in HIV-1 genomic RNA strongly conserved across distinct biological states. *PLoS Biol.* **6**, e96 (2008).
19. Wilkinson, K.A., Merino, E.J. & Weeks, K.M. RNA SHAPE chemistry reveals nonhierarchical interactions dominate equilibrium structural transitions in tRNA^{Asp} transcripts. *J. Am. Chem. Soc.* **127**, 4659–4667 (2005).
20. Wang, B., Wilkinson, K.A. & Weeks, K.M. Complex ligand-induced conformational changes in tRNA^{Asp} revealed by single-nucleotide resolution SHAPE chemistry. *Biochemistry* **47**, 3454–3461 (2008).
21. Duncan, C.D.S. & Weeks, K.M. SHAPE analysis of long-range interactions reveals extensive and thermodynamically preferred misfolding in a fragile group I intron RNA. *Biochemistry* **47**, 8504–8513 (2008).
22. Leaderach, A., Shcherbakova, I., Liang, M.P., Brenowitz, M. & Altman, R.B. Local kinetic measures of macromolecular structure reveal partitioning among multiple parallel pathways from the earliest steps in the folding of a large RNA molecule. *J. Mol. Biol.* **358**, 1179–1190 (2006).
23. Gherghe, C.M., Mortimer, S.A., Krahn, J.M., Thompson, N.L. & Weeks, K.M. Slow conformational dynamics at C2'-endo nucleotides in RNA. *J. Am. Chem. Soc.* **130**, 8884–8885 (2008).
24. Vasa, S.M., Guex, N., Wilkinson, K.A., Weeks, K.M. & Giddings, M.C. ShapeFinder: a software system for high-throughput quantitative analysis of nucleic acid reactivity information resolved by capillary electrophoresis. *RNA* **14**, 1979–1990 (2008).
25. Krasilnikov, A.S., Yang, X., Pan, T. & Mondragon, A. Crystal structure of the specificity domain of ribonuclease P. *Nature* **421**, 760–764 (2003).
26. Baird, N.J., Westhof, E., Qin, H., Pan, T. & Sosnick, T.R. Structure of a folding intermediate reveals the interplay between core and peripheral elements in RNA folding. *J. Mol. Biol.* **352**, 712–722 (2005).
27. Baird, N.J., Fang, X., Sridivya, N., Pan, T. & Sosnick, T.R. Folding of a universal ribozyme: the ribonuclease P RNA. *Q. Rev. Biophys.* **40**, 113–161 (2007).

