A neutral pH thermal hydrolysis method for quantification of structured RNAs

STEPHEN C. WILSON, DANIEL T. COHEN, XIN C. WANG, and MING C. HAMMOND

ABSTRACT
Riboswitch aptamers adopt diverse and complex tertiary structural folds that contain both single-stranded and double-stranded regions. We observe that this high degree of secondary structure leads to an appreciable hypochromicity that is not accounted for in the standard method to calculate extinction coefficients using nearest-neighbor effects, which results in a systematic underestimation of RNA concentrations. Here we present a practical method for quantifying riboswitch RNAs using thermal hydrolysis to generate the corresponding pool of mononucleotides, for which precise extinction coefficients have been measured. Thermal hydrolysis can be performed at neutral pH without reaction quenching, avoids the use of nucleases or expensive fluorescent dyes, and does not require generation of calibration curves. The accuracy of this method for determining RNA concentrations has been validated using quantitative 31P-NMR calibrated to an external standard. We expect that this simple procedure will be generally useful for the accurate quantification of any sequence-defined RNA sample, which is often a critical parameter for in vitro binding and kinetic assays.

INTRODUCTION
In vitro biochemical analysis often requires an accurate method for measuring the sample concentration of single-stranded RNAs with some degree of secondary structure, such as riboswitches and other regulatory elements in mRNAs, RNA components of ribonucleoprotein complexes, and long noncoding RNAs. One general and convenient method to quantify nucleic acid concentrations is to measure the UV absorbance at 260 nm and apply Beer’s law. However, the extinction coefficient for an intact oligonucleotide with variable degrees of base-pairing is difficult to determine exactly. For example, extinction coefficients calculated using the nearest-neighbor method, which takes into account hypochromicity from base stacking but not from pairing interactions, were overestimated by up to 20% for single-stranded DNA oligonucleotides, depending on base-pairing propensities (Kallansrud and Ward 1996). Similarly, we have determined that the nearest-neighbor method overestimates extinction coefficients for riboswitch aptamers, causing a systematic underestimation of RNA concentrations by up to 30%. This result is noteworthy because popular online calculators of nucleic acid properties indiscriminately apply this algorithm toward the determination of sample RNA concentrations from UV absorbance measurements.

Nucleic acids have been quantified using various approaches including enzymatic hydrolysis, inorganic phosphate determination, and fluorescence-based assays. These methods suffer from specific drawbacks that limit their convenience. RNA treatment with snake venom phosphodiesterase and bacterial alkaline phosphatase requires extraction prior to quantitation as well as careful handling and storage in order to prevent laboratory contamination (Kallansrud and Ward 1996). Incubation times of 8–24 h also are not uncommon, making same-day experimentation difficult (Andrus and Kuimelis 2001). Inorganic phosphate determination entails RNA strand digestion and oxidation of the phosphate monoesters into orthophosphate. The amount of orthophosphate is subsequently determined spectrophotometrically with the aid of a colorimetric reagent. In addition to having multiple steps and requiring calibration curves, this method is susceptible to inflated concentrations if other phosphate-containing species are present (Murphy and Trapane 1996; Runquist 2005). Fluorescence-based assays have the advantage of being highly sensitive with some dyes allowing RNA
detection down to 1 pg/μL (Le Pecq and Paoletti 1966; Jones et al. 1998; Gallagher 2000). However, since these methods rely on dye intercalation, they are inherently an indirect method of detection. The extent of single and double strandedness of the analyte as well as the presence of other contaminants in the sample must be carefully considered before constructing the calibration curve for the assay.

While base hydrolysis as a means for quantifying nucleic acids has been reported in the literature (Katz and Comb 1963; Werner 2005), methodologies vary dramatically with little to no experimental corroboration regarding concentration, temperature, buffer, and duration. Here we describe a standardized protocol for thermal hydrolysis of RNA samples that is inexpensive, uses minimal amounts of material, completes within 2 h, minimizes error associated with intermolecular hypochromicity, and can even be performed under neutral pH conditions. Overall, this method for quantification of sequence-defined RNA samples is convenient and accurate, eliminates the potential for nuclease contamination, and does not require reaction quenching, calibration curves, or expensive reagents.

RESULTS AND DISCUSSION

Observation of an appreciable hypochromic effect for riboswitch aptamers

The riboswitch aptamers that bind to the metabolite ligands flavin mononucleotide (FMN), S-adenosylmethionine (SAM), and cyclic di-GMP (c-di-GMP) have distinct structural folds as indicated by their secondary structure maps (Winkler et al. 2002, 2003; Sudarsan et al. 2008), but in each case we observe substantial (20%–32%) increases in \( A_{260} \) values upon sample heating up to 95°C (Fig. 1). Increases in UV absorbance also have been observed for other riboswitches upon heating (Wickiser et al. 2005; Yamauchi et al. 2005; Santner et al. 2012). The change in UV absorbance is reversible upon cooling, and so corresponds to thermal denaturation of duplex and other types of nucleobase interactions that reduce UV absorbance of the folded RNA. The slight discrepancy between the initial \( A_{260} \) value before heating and the final value after cooling is most likely due to some hydrolysis of the RNA during the 140-min time course of the thermal denaturation experiment (Fig. 1B).

The hypochromic effect is observed in the absence of ligand, which is consistent with riboswitch aptamers having some apo structure even in the absence of Mg\(^{2+}\) (Yamauchi et al. 2005). Interestingly, the UV melt curves for the FMN, c-di-GMP, and Bs SAM-I riboswitch aptamers do not level off at 95°C, which also appears to be due to some hydrolysis of the RNA. In support of this hypothesis, the presence of 2 mM Mg\(^{2+}\) leads to increased hydrolysis, as observed by the UV absorbance (Fig. 1A). This effect has previously been quantified (Li and Breaker 1999). Based on these data, we conclude that thermal denaturation of riboswitch RNAs is

FIGURE 1. The UV absorbance of riboswitch aptamers exhibit the hypochromic effect. Secondary structures and UV melt or cooling curves for (A) the FMN riboswitch aptamer upstream of the impX gene from Fusobacterium nucleatum, (B) the SAM-I riboswitch aptamer upstream of the yif gene from Bacillus subtilis (Bs), and (C) the c-di-GMP/GEMM-I riboswitch aptamer upstream of the floX gene from Vibrio cholerae. Data shown are independent absorbance measurements of RNA samples from a single transcription reaction.
not a convenient or reliable method for determining the UV absorbance without the hypochromic effect from base-pairing interactions. At high temperatures, partial hydrolysis may occur, whereas at moderate temperatures, complete denaturation is not assured. In addition, the extinction coefficients of nucleic acids are affected by temperature, which makes it difficult to accurately extrapolate the RNA concentration (Adler et al. 1967).

**Evaluation of conditions to avoid incomplete and side reactions in thermal hydrolysis of RNA**

Complete hydrolysis of the RNA to the constituent mononucleotides would eliminate the hypochromicity associated with both secondary structure and nearest-neighbor effects. Since the RNA sequence is known and the extinction coefficients of mononucleotides have been determined with high accuracy (Cavaluzi and Borer 2004), the measured UV absorbance of a hydrolyzed sample aliquot could be used to back-calculate the stock RNA concentration. However, incomplete hydrolysis or side reactions would decrease the accuracy of the procedure, so we set out to evaluate the conditions for thermal hydrolysis that would minimize these problems.

As monitored by the change in UV absorbance, an RNA sample with starting \( A_{260} \) value of \( \sim 1 \) AU is fully hydrolyzed within 90 min at 95°C and pH 9, whereas at lower temperatures the reaction takes longer to complete (Fig. 2A). Polyacrylamide gel analysis of the \(^{32}P\) body-labeled FMN riboswitch aptamer is consistent with the UV results, showing partial hydrolysis at 15 min, increased hydrolysis at 60 min, and complete hydrolysis by 90 min (Fig. 2B). While lower concentrations of sample are hydrolyzed faster, the error associated with the microvolume UV/Vis spectrometer is \( \sim 6\% \) at 1 AU and increases linearly below this absorbance value (Thermo Scientific 2010). Analysis of more dilute RNA samples can be performed using a standard UV/Vis spectrometer, but this in turn would require larger sample volumes. In practice, we find that stock concentrations of in vitro transcribed RNAs have to be diluted in order to reach starting \( A_{260} \) values of \( \sim 1 \) AU.

In addition to requiring extended times for reaction completion, more highly concentrated samples may exhibit a hypochromic effect even after hydrolysis due to the homo-association and hetero-association of mononucleotides. For example, a previous study showed that the molar absorptivity of \( 5'\)-AMP is inversely correlated to its concentration in solution, causing its extinction coefficient to range between 11 and 16 \( M^{-1} \text{cm}^{-1} \) for sample concentrations between 1 mM and 10 \( \mu \)M, respectively (Morcillo et al. 1987). Hetero-association of nucleotides also leads to hypochromic effects at millimolar concentrations (Peral et al. 1986). Assuming equimolar amounts of each nucleotide, 1 AU is equivalent to 90 \( \mu \)M of total NMPs and 22 \( \mu \)M of AMP. Thus, we have observed that performing the thermal hydrolysis on RNA samples with starting \( A_{260} \) values in the range of 0.85–1.2 AU allows for complete hydrolysis of the sample within 90 min and minimizes any intermolecular hypochromic effects that would be observed after hydrolysis.

Incubation of nucleic acids at nonphysiological pH and high temperatures in aqueous solution may lead to side reactions that may change the identities of the nucleobases and the associated extinction coefficients. Dephosphorylation, depurination, and depyrimidination of nucleobases have
been shown to occur predominantly at acidic pH (Oivanen et al. 1998). In contrast, cytosine deamination to uridine does occur at both acidic and very basic pH and would result in a 37% increase in the A$_{260}$ value (Lindahl and Nyberg 1974; Oivanen and Lonnberg 1990; Cavaluzzi and Borer 2004). Under the mildly basic reaction conditions used for RNA hydrolysis (pH 9 for 120 min at 95°C), however, no appreciable change in UV absorbance was observed for the model substrate 2′-3′ cyclic CMP (Fig. 2A). This result is consistent with a prior study demonstrating that the rate of cytosine deamination in DNA at 100°C is slowest between the pH range of 8–8.5 and exhibits only a small dependence on pH in the range of 6–9 (Lindahl and Nyberg 1974).

Furthermore, we found that the rate of phosphodiester cleavage by thermal hydrolysis is similar between the pH range of 7–9 under the buffer conditions utilized (Fig. 2C). PAGE analysis further confirms that RNA hydrolysis to the nucleotide monophosphates at 95°C and pH 7 was completed within 90 min (Fig. 2D). This result is consistent with a prior study that described neutral thermal hydrolysis of RNA probes as a factor that may complicate the interpretation of hybridization assays (Tenhunen 1989).

For the purpose of determining RNA concentrations, the observation of neutral thermal hydrolysis is noteworthy because none of the side reactions described above is expected to occur appreciably at neutral pH, even at elevated temperatures, on the timescale of the procedure. For example, dephosphorylation half-lives for 2′ or 3′ UMP and 2′ or 3′ AMP are between 20 and 200 h at pH 7 and 90°C (Oivanen and Lonnberg 1989, 1990). Both depurination and depyrimidination of DNA have been found to occur on a timescale on the order of years ($t_{1/2}$ ∼ 5–20 yr) at physiological pH and 70°C and 80°C, respectively (Lindahl and Nyberg 1972; Han et al. 2000). By comparison, our work indicates that RNA hydrolysis to mononucleotides occurs with a half-life of ∼40 min at pH 7 and 95°C.

**Evaluation of the neutral thermal hydrolysis method to determine RNA concentrations**

Thermal hydrolysis performed at basic pH requires a step to neutralize the reaction prior to UV absorbance measurement, as the extinction coefficients of the mononucleotides are influenced by pH (Voet et al. 1963). Thermal hydrolysis performed at neutral pH is more convenient because it does not require this quenching step. Recently, the extinction coefficients at 260 nm for the nucleoside 5′ monophosphates at pH 7 were redetermined using NMR spectroscopy to accurately measure sample concentrations (Cavaluzzi and Borer 2004). While RNA hydrolysis results in an interconverting mixture of nucleoside 3′ monophosphates and 2′ monophosphates, the extinction coefficients for these species are closely approximated by the values for the 5′ monophosphates (Supplemental Table S1), as the location of the phosphate group has little effect (Katz and Comb 1963; Abrash et al. 1967; Puglisi and Tinoco 1989; Cavaluzzi and Borer 2004).

Using the standardized protocol, we performed neutral thermal hydrolysis on samples of the Bs SAM-I, FMN, and cyclic di-GMP riboswitch aptamers in order to determine the stock RNA concentrations (Fig. 3A; Table 1). We also validated the accuracy of thermal hydrolysis on the same constructs as well as the P$_i$ SAM-I riboswitch aptamer (Karns et al. 2013) using quantitative $^{31}$P-NMR. Quantitative $^{31}$P-NMR data was acquired on each of the four riboswitch samples along with an external standard of 5′ AMP (Fig. 3B; Table 2). As shown, the results between the two methods are in very good agreement (no more than 7% error between the two values) for all four riboswitches, which validates the accuracy of our method.

The error associated with the NMR analysis is ∼6% and includes error from preparation of the external standard, 90° pulse width calibration, and signal-to-noise of the spectrum. However, NMR quantification errors of <1% can be achieved (Cavaluzzi and Borer 2004). By comparison, we estimate the error associated with the thermal hydrolysis assay to be <7%, which includes error associated with the microvolume UV/Vis instrument and micropipetting. While the above estimate used the stated measurement errors from the instrument manufacturer (2.5%–4% for absorbance measurements in the range of 1.5–2 AU), we have found in our study that error from replicate measurements using 2 µL of sample is <2%. It is likely that the small absorbance fluctuations at the endpoint of hydrolysis are the result of the aforementioned sources of error compounded. These fluctuations amount to <5% changes in absorbance once the endpoint is achieved and can be accounted for by averaging the endpoint absorbances from a hydrolysis time course (Fig. 3A).

It should be noted that the NMR quantitation experiment required a standard sample for calibration and 0.2 mL of sample with micromolar concentrations of RNA in order to obtain a high signal-to-noise ratio of 53 for the P$_i$ sample, for example. While a more sensitive NMR instrument would reduce the amount of material and time required for this experiment, the neutral pH thermal hydrolysis method still is much more operationally simple and sparing of RNA samples. It also should be preferable to hydrolysis procedures that use enzymes, which require lengthy incubation periods and extraction prior to quantitation (Andrus and Kuimelis 2001). Fluorescence-based assays using intercalating dyes for RNA quantitation can be highly sensitive (detection limits as low as 1 pg/µL) (Le Pecq and Paoletti 1966; Jones et al. 1998); however, they require the generation of calibration curves and use of a fluorometer instrument, which may require larger sample volumes. In addition, enzymes or fluorescent intercalating dyes are relatively expensive and specialized reagents compared with the hydrolysis buffer we describe.

Proper interpretation of the data from assays for RNA–ligand interactions such as ITC (Gilbert and Batey 2009), fluorescence quenching (Davies and Arenz 2008), fluorescence
anisotropy (Luedtke et al. 2003), or surface plasmon resonance (Hendrix et al. 1997) require the accurate determination of parameters that include the concentration of the RNA. In this study, we show that application of Beer’s law using the algorithms currently utilized by online calculators (http://www.idtdna.com/Analyzer/Applications/Instructions/Default.aspx?AnalyzerDefinitions=true) result in an underestimation of sample concentrations due to the hypochromic effect for structured RNAs like riboswitches (Table 1). The % error between estimated RNA concentrations and the RNA concentrations measured by our neutral hydrolysis method ranges from 25% to 32% for these RNAs, which are 46%–55% base-paired. We expect that there would be a general correlation between the degree of underestimation and extent of RNA structure. However, apo samples also are expected to be heterogeneous in folded and unfolded structures, especially in water without magnesium, which may lead to variability in the absorbance values for intact RNAs. Thus, our data cannot be fully extrapolated to estimate the % error for a given % base-paired, but the trend should be that more base-pairing leads to higher % error.

Another common method is to use a standard conversion factor of 40 ng/μL for 1 AU (Werner 2005). Variations in the value of the conversion factor have been reported ranging from 37 ng/μL to 51 ng/μL, depending on the salt form and identity of the RNA (Werner 2005). As expected, the exact conversion factors for each of the four riboswitch aptamers are higher than the standard value of 40 ng/μL, which would lead to an underestimation of sample concentrations of up to 13% (Table 1).

Interestingly, accurate assessments of the total RNA concentration have revealed that the active or competently folded fraction of structured RNA samples is not always 100% (Supplemental Fig. 1). However, it is possible to experimentally determine the fraction of active RNA, or one should state the implicit assumption that the active RNA concentration is equal to the total RNA concentration. Errors associated with inaccurate determination of RNA concentrations due to the hypochromic effect and/or indeterminate fraction of active RNA in the sample could explain the discrepancies often observed between dissociation constants and other parameters measured by different assay methods.

In conclusion, for any sequence-defined RNA sample such as the product of in vitro transcription or chemical synthesis, the neutral pH thermal hydrolysis method is a convenient method of choice for the determination of RNA concentrations. The benefits of this method are its simplicity, low cost, limited resource consumption, and accuracy. As more interesting structured RNAs continue to be discovered in all domains of life, it will be important to accurately measure their biophysical properties in order to determine their function.

MATERIALS AND METHODS

Oligonucleotides and reagents

DNA oligonucleotides, including the full riboswitch aptamer sequences, were purchased from IDT and Elim BioPharmaceuticals.
(see Supplemental Data section). After PCR amplification, the templates were cloned into the pCR2.1-TOPO vector following the manufacturer’s protocol and confirmed by sequencing. Acetic acid, Na2CO3, and EDTA were obtained from Fisher Scientific. 2’–3’ cyclic CMP and 5’-AMP were purchased from Sigma-Aldrich, and [α-32P]GTP was procured from PerkinElmer.

**In vitro transcription**

DNA templates were made through PCR amplification using Phusion DNA polymerase with primers that added the T7 polymerase promoter sequence, and subsequently purified using a QiaQuick PCR Purification kit. Templates (7–10 μg) were transcribed in a 1-mL reaction for 3 h at 37°C using T7 RNA polymerase (2500 units) in 40 mM Tris-HCl at pH 8, 6 mM MgCl2, 10 mM DTT, 2 mM spermidine, 8 mM rNTPs (2 mM each), Superasin (100 units), and inorganic pyrophosphatase (5 units). RNA was purified in a denaturing (7.5 M urea) 6% polyacrylamide gel and was extracted from gel pieces overnight using CrushSoak buffer (10 mM Tris-HCl at pH 7.5, 200 mM NaCl, 1 mM EDTA). RNAs were precipitated overnight in ethanol (2.5:1 volume of ethanol to supernatant) at −20°C, pelleted at 13,000 rpm at 4°C for 25 min, and the supernatant was removed. RNAs were dried using a spin-vac for ~30 min, then resuspended in water or 50 mM Tris (pH 7.4).

To make the 32P-body-labeled FMN riboswitch aptamer, template (2 μg) was transcribed in a 300-L reaction for 3 h using T7 RNA polymerase (3200 units) in 40 mM Tris-HCl at pH 8, 6 mM MgCl2, 10 mM DTT, 2 mM spermidine, 8 mM rNTPs (2 mM each), and 34 μCi of [α-32P]GTP (3000 Ci/mmol). After 1 h, the reaction was centrifuged briefly to pellet the pyrophosphate byproduct, and an additional 1 μmol of MgCl2 was added to the reaction.

**UV denaturation and renaturation curves**

Riboswitch aptamer RNAs were dissolved in degassed buffer (50 mM sodium cacodylate, 100 mM KCl at pH 7). Absorbance measurements at 260 nm (Varian Cary 100 Bio UV-Vis spectrophotometer, 1-cm path length cuvettes) were measured from 25°C to 95°C, or vice versa, at a temperature interval of 0.5°C min−1 for denaturation or 10°C min−1 for renaturation.

**Determination of RNA concentration by thermal hydrolysis**

To a 2 μL aliquot of the RNA sample diluted to a starting A260 of ~10 AU was added 2 μL of 10X hydrolysis buffer (500 mM Na2CO3, 100 mM EDTA at pH 7–9) and 16 μL of sterile water in an Eppendorf tube with a Safe-Lock lid. The reaction mixture (20 μL final volume) was incubated at 95°C for 90 min in a dry-heat block. The tubes were cooled to room temperature and briefly centrifuged prior to opening. Reactions performed at pH 8 or 9 were neutralized using 0.1 M acetic acid prior to analysis. The control hydrolysis reaction of 180 μM 2’–3’ cCMP was performed as described at pH 9.

The UV absorbance at 260 nm for a 2-μL aliquot of the reaction mixture was measured using a Nanodrop-8000 microvolume UV/Vis spectrometer, and the value was multiplied by the dilution factor in order to obtain the A260, hydrolyzed value. Typically, measurements were taken for three aliquots to account for sampling error. The concentration of the stock RNA sample was calculated using the following equation:

\[
[A_{RNA}] = \frac{A_{260, hydrolyzed}}{\sum_{i} n_i \varepsilon_{i,260}},
\]

where \(b\) is the path length, \(i\) is the nucleotide identity (A, C, U, or G), \(n_i\) is the number of nucleotide \(i\) present in the sequence, and \(\varepsilon_i\) is the literature extinction coefficient for the given mononucleotide. The following extinction coefficients for the 5’ mononucleotides were used to approximate the extinction coefficients for the hydrolysis products, which are 3’ mononucleotides or 2’–3’ cyclic

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**TABLE 1.** Comparison of RNA concentration estimates by measuring UV absorbance before and after hydrolysis

| Riboswitch aptamer | Avg. A260 before hydrolysis (n = 3) | Stock [RNA] by IDT calculator (μM) (n = 3) | Calc. ε260 valuec | Dilution factor | Stock [RNA] by neutral thermal hydrolysis (μM) (n = 3) | Conversion factor for 1 AU (ng/μL) |
|-------------------|----------------------------------|------------------------------------------|------------------|----------------|--------------------------------|-------------------------------|
| Bs SAM-I          | 1.14 ± 0.01                      | 1,211,500                                | 37.7 ± 0.4       | 1.86 ± 0.02    | 1,431,850                      | 52.0 ± 0.4                   |
| Pi SAM-I          | 1.01 ± 0.01                      | 1,169,400                                | 86.2 ± 0.9       | 1.77 ± 0.02    | 1,402,910                      | 126 ± 2                      |
| Fn FMN            | 0.88 ± 0.02                      | 1,128,300                                | 44.8 ± 0.9       | 1.406 ± 0.004  | 1,348,300                      | 60.0 ± 0.2                   |
| Vc2 CEMM-I        | 1.17 ± 0.01                      | 1,084,100                                | 53.8 ± 0.5       | 1.89 ± 0.02    | 1,278,970                      | 74.0 ± 0.7                   |

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**TABLE 2.** Comparison of RNA concentration measurements by thermal hydrolysis and quantitative NMR

| Riboswitch aptamer | Stock [RNA] by neutral thermal hydrolysis (μM) (n = 3) | Stock [RNA] by quantitative NMR (μM) | NMR signal-to-noise | % Difference |
|-------------------|----------------------------------|-------------------------------------|---------------------|-------------|
| Bs SAM-I          | 179 ± 7a                          | 188                                 | 86                  | 5           |
| Pi SAM-I          | 126 ± 2b                          | 136                                 | 53                  | 7           |
| Fn FMN            | 320 ± 10b                         | 328                                 | 161                 | 2           |
| Vc2 CEMM-I        | 248 ± 18c                         | 268                                 | 127                 | 7           |

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\(a\) Error from technical replicates of the spectrometer reading.

\(b\) Error from independent replicates of the hydrolysis experiment.

\(c\) Determined using IDT online calculator (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer/).

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\(\varepsilon_{i,260}\) is the nucleotide identity (A, C, U, or G), \(n_i\) is the number of nucleotide \(i\) present in the sequence, and \(\varepsilon_i\) is the literature extinction coefficient for the given mononucleotide. The following extinction coefficients for the 5’ mononucleotides were used to approximate the extinction coefficients for the hydrolysis products, which are 3’ mononucleotides or 2’–3’ cyclic
mononucleotides: $pA = 15,020$ M$^{-1}$ cm$^{-1}$, $pC = 7070$ M$^{-1}$ cm$^{-1}$, $pG = 12,080$ M$^{-1}$ cm$^{-1}$, $pU = 9660$ M$^{-1}$ cm$^{-1}$ (Cavaluzzi and Borer 2004).

**Polyacrylamide gel electrophoresis (PAGE) analysis of thermal hydrolysis reactions**

Different timepoints of a thermal hydrolysis reaction of $^{32}$P-radiolabeled FMN riboswitch aptamer RNA were analyzed alongside unlabeled FMN riboswitch aptamer RNA were analyzed alongside unhybridized RNA, $[\alpha-^{32}P]GTP$, and $[^{31}P]GMP$ (from hydrolysis of $[\alpha-^{32}P]GTP$) on a 10% PAGE gel (25 W for 1.5 h). The gel was dried prior to exposure to a PhosphorImager cassette, which was imaged using a Typhoon 9410 laser scanning imager.

**RNA quantitation by $^{31}P$-qNMR using an external standard**

All NMR experiments were performed on a Bruker Avance II 600 MHz spectrometer equipped with a 5-mm broadband probe and Z-axis gradient. An external standard of 5 MHz spectrometer equipped with a 5-mm broadband probe and

$Z$-axis gradient. An external standard of 5 MHz spectrometer equipped with a 5-mm broadband probe and

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