

# Chapter 3

## Kinetic analysis of ribozyme cleavage

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### 1 Introduction and scope

This chapter provides an introduction to kinetic analysis of catalytic RNAs (ribozymes). We discuss choosing a ribozyme and designing experiments to test hypotheses. Practical issues are emphasized including preparing an active and uniform RNA population (*Protocol 1*), deriving kinetics equations (*Protocol 2*), and fitting and simulating data (*Protocol 3*). Later sections provide protocols for designing and preparing RNA (*Protocol 4*), and for kinetics experiments (*Protocol 5*). We conclude with a brief overview of other kinetics methods.

We assume the reader is familiar with basic biochemical and molecular biology techniques such as preparing buffers and gels, and carrying out PCR and cloning; in cases where standard protocols are available, references are given. Protocols and discussion provided then are intended to guide the reader towards useful and perhaps less common methods. The protocols are in no way comprehensive, but hopefully provide some guidelines to aid design of experiments to address the myriad of questions and mechanisms that arise. We anticipate potential users of this chapter to include those interested in working on ribozymes but lacking a background in preparing and handling RNA or in designing and interpreting kinetics experiments. Lastly, since this is a practical chapter, space prevents us from referencing many appropriate and interesting papers. Also, an emphasis is placed on work from the authors' laboratory since this is most familiar. We apologize to our colleagues whose work was not referenced.

## 2 Initial considerations for the system and experiments

### 2.1 Choosing and preparing the system

There is an interesting array of catalytic nucleic acids known. These include a limited number of naturally occurring ribozymes, the hairpin, hammerhead, hepatitis delta virus (HDV), VS, group I and II introns, and RNase P (1). The number of ribozymes has been increased by *in vitro* evolution to include ligases, polymerases, and nucleases; evolved ribozymes can catalyze organic reactions including Diels–Alder reactions and porphyrin metallations (2). RNA appears to be the catalytic portion of the ribosome and spliceosome (3, 4). Also, various catalytic DNA molecules have been evolved (2). Mechanisms are studied for fundamental interest and therapeutic potential.

#### 2.1.1 Single versus multiple-turnover reactions

It is important to recognize differences in the terms ‘catalytic RNA’ and ‘ribozyme’. Strictly speaking, a catalyst accelerates a multiple-turnover reaction without being changed itself. A few catalytic RNAs have this property, for example, RNase P and 23S rRNA; however, most ribozymes, in natural or evolved form, do not. For example, hairpin, HDV, VS1, and group I and group II introns are changed during single-turnover reactions. However, multiple-turnover enzymes can be engineered. An early example was the L-19 version of the *Tetrahymena* group I intron by Zaug and Cech (5). The first 19 nt of the ribozyme were removed, leaving the G-rich 3'-strand of the P1 stem-loop—a so-called ‘guide sequence’—at the start of the ribozyme. P1 was reconstituted *in trans* by adding an oligomer containing the 5'-portion of the strand and the scissile phosphate. This recombinant approach is general and can transform single-turnover ribozymes into true enzymes that turnover substrate. Therapeutic applications are then possible; for example, the substrate could be an undesired nucleic acid (e.g. a pathogenic mRNA). Detailed mechanistic study of multiple-turnover systems have yielded elementary rate constants for substrate binding, cleavage, conformational changes, and product dissociation (6–8). Although the majority of ribozymes can be engineered to turnover, it also remains of biophysical and medical interest to study them in their natural single-turnover form as well.

A fundamental choice then is whether to study a single-or multiple-turnover reaction. Choice impacts later decisions including initiation of kinetics and renaturation of RNA. Choice should be based on interest. If the investigator wants a therapeutic, then a two-piece system is probably best; but if interest is in how RNA folds and cleaves co-transcriptionally, then a one-piece system is better. In other cases, the choice may be less clear. For example, if interest is in a chemical step or a conformational change, then it may be possible to use either approach assuming the reaction is initiated properly and the correct step is isolated.

### 2.1.2 Obtaining a uniform and active ribozyme population: design

An important prerequisite for ribozyme characterization is obtaining an active population of molecules that is as uniform as possible. One factor that often helps is to design the RNA to avoid misfolds. Many of the RNA species prone to misfolding have native pseudoknots (9, 10). Pseudoknots involve pairings between single stranded nucleotides within a stem-loop and nucleotides outside the stem. However, the stem-loop can sequester the pseudoknot-forming strand in alternative pairings. One can try to avoid misfolds by weakening alternative pairings, strengthening native pairings, or both. Increasingly, it is found that such mutants can fold faster than wild-type (9–11). Fast folding is desired since focus can shift to tertiary folding and the intrinsic rate of bond cleavage.

Predictions of alternative RNA folds can be made by a variety of programs (see <http://bioinfo.math.rpi.edu/%7Ezukunft/rna/for> links). We commonly use *mfold* v3.1 by Zuker and Turner (<http://bioinfo.math.rpi.edu/~mfold/rna>) (12, 13). A set of optimal and suboptimal secondary structures is generated based on free energy minimization. There are ‘window’ and ‘percent suboptimality’ parameters, which modify the nature and number of suboptimal structures. The window size has a default setting that increases with RNA length; however, a smaller value, 0 being the smallest, allows for computation of more, structurally similar, suboptimal structures. Percent suboptimality sets a percentage away from the minimum free energy for viewing structures. The *mfold* algorithm does not calculate pseudoknots, which is an advantage in spotting alternative pairings. Importantly, structure prediction accuracy of *mfold* v3.1 can be increased by including experimental constraints to force or prevent base pairs.

The *mfold* package can also be used to assign promiscuity to any nucleotide within a user defined energy window, and structures can be colour coded with this information (14). P-num describes the promiscuity of a nucleotide, which is the number of *different* base pairs for a given nucleotide in all foldings within the window. S-num describes the propensity of a base to be single stranded. A base is *well-determined* if S-num is close to 1 (high likelihood of being single-stranded), or if S-num is close to 0 (high likelihood of being double-stranded) *and* P-num is low.

One should strive for a well-determined structure based on S-and P-num values, and make rational nucleotide replacements *in silico* to do this. Also, a list of suboptimal structures should be computed, and misfolds that sequester important nucleotides should be mutated *in silico* to disfavour alternative folds or to favour the native state. Often, useful mutations involve changes to A or C, which are less promiscuous than G or U; small deletions and insertions can help as well. A goal is to maximize the  $\Delta\Delta G_{37}^0$  between the optimal (desired) and suboptimal structures ( $\geq 4.5$  kcal/mol, a population difference of  $\approx 1000$ ). Using this general approach we have designed HDV ribozymes that cleave nearly 300-fold faster than wild-type (10).

### 2.1.3 Obtaining a uniform and active ribozyme population: preparation

Once the RNA has been designed, one can try several renaturation (refolding) conditions. During purification, the RNA is put through extensive unfolding, refolding, and misfolding conditions (see Section 3 and reference (15)); consequently, RNA, which is typically stored at  $-20^{\circ}\text{C}$ , should be renatured before *each* experiment. In our lab, we have seen non-renatured RNA (including RNA that was once renatured, frozen, and thawed without re-renaturation) give irreproducible kinetics and UV melting curves. Reasons for this behaviour are not always understood, but may reflect multimerization during precipitation and freezing, and kinetic trapping in inactive secondary structures. If this seems surprising, recall that local interactions in RNA are strong, and  $37.5\%$  ( $=\frac{6}{16}$ ) of the base combinations lead to stable pairs.

## Protocol 1

### Preparation of an active and uniform ribozyme population

- 1 Heat radiolabelled RNA to  $37^{\circ}\text{C}$ ,  $50^{\circ}\text{C}$ ,  $65^{\circ}\text{C}$ ,  $80^{\circ}\text{C}$ , or  $90^{\circ}\text{C}$ , and cool. Vary heating time; use shorter times for higher temperatures (e.g. 30 min at  $37^{\circ}\text{C}$ ; 2 min at  $90^{\circ}\text{C}$ ). To avoid concentrating of sample and salts, use a volume  $\geq 20\ \mu\text{l}$  in a 0.65 or 1.7 ml microcentrifuge tube. At high temperatures, we use a homemade apparatus with a central threaded rod ( $\approx 8$  in. long) housing a lower metal plate with eight holes to accommodate tubes; another plate is placed over the first and tightened with a wing nut. Advantages are that the caps cannot pop open, and condensation on the cap is minimized. Cool RNA by one of several approaches: place on bench for 10 min; turn off heat block and allow cooling over several hours; or snap cool on ice. Typically, we cool on the bench for 10 min (no spin to avoid rapid cooling), followed by a brief 2 s spin in the microcentrifuge to collect droplets.
- 2 Try several salt conditions. To avoid hydrolysis, buffer the solution at  $\text{pH} \leq 7.5$ . Likewise, renaturations above  $80^{\circ}\text{C}$  should be without added divalent ions and should contain ethylenediaminetetraacetic acid (EDTA)  $\geq 0.1\ \text{mM}$  to sequester trace metal ions. For a single-turnover reaction, avoid catalysis during renaturation; one approach is keeping the RNA in a trapped state (see below).
- 3 Pay careful attention to reproducing minute details: volume of RNA heated; time on bench; time and speed of spin to collect droplets; order of addition; age of RNA at  $-20^{\circ}\text{C}$ , etc. For reasons that are often unclear, some ribozymes give reproducible kinetics and others do not.
- 4 Try a UV melt. The above list is extensive, especially when combinations are considered; thus, one needs an idea of where to start. A suggestion is to perform a UV-melt of the ribozyme in several salts. An excellent protocol for melting is available (16).

**Protocol 1** continued

Melt from 10°C to 95°C to 10°C to 95°C, *without* prior renaturation. Compare reverse and second forward melts; these should be similar if melting is reversible. Compare first forward and other melts and look for anomalous transitions; these may represent alternate structures or higher order complexes formed during preparation or storage. Try renaturation temperatures above anomalous transitions. Also, count reversible transitions and try renaturations temperatures above a transition. Low temperature reversible transitions typically represent tertiary structure and pseudoknots, while high temperature transitions represent strong secondary structure. Quite often, effective renaturations can be obtained at 50°C or 65°C in  $\text{Mg}^{2+}$  to melt or accelerate tertiary structure transitions, and accelerate secondary structure refolding.

- 5 Examine the RNA to see if folding is uniform. A simple experiment involves a native gel, which means no urea, modest voltage ( $\approx 300$  V), and a thermostatted apparatus (or a cold room) to avoid Joule heating (17). Use the salt concentration of interest or a somewhat lower one (e.g. 10 mM  $\text{MgCl}_2$  and 50 mM KCl) in the gel and buffer to avoid heating. Renature the radiolabelled RNA for various times and temperatures and load the gel (include a no renaturation control). Run gel for 3–8 h; re-circulate or replenish buffer when the pH deviates by one unit; judge by turning off power and dipping pH paper into buffer reservoirs. Look for a single sharp band on the native gel—a necessary but not sufficient condition for a uniform population. Often, RNA has faster migration after renaturation; this could reflect dissociation of a dimer or formation of a more compact tertiary fold (18), both of which are typically desired. Run markers on the gel; double-stranded DNA (dsDNA) markers are readily available from numerous vendors (e.g. New England Biolabs), and double-stranded RNA (dsRNA) can be prepared if desired by transcription of top and bottom strands using PCR to introduce the promoter as appropriate. One does not expect an exact correlation between sample and marker because of shape differences, but an approximate correlation should be obtained.

Another approach to look for uniform folding is equilibrium dialysis (19). Substrate oligomer is radiolabelled and allowed to bind to the ribozyme. A Scatchard plot is done, and the  $x$ -intercept is evaluated. A value of unity is consistent with all of the ribozyme being active; a value less than unity is consistent with a misfolded population. While this approach is more cumbersome than the native gel, it is a true equilibrium approach.

For single-turnover ribozymes, a successful renaturation should result in monophasic kinetics with >90% completion. Compare to fast rate constants in the literature (if available) and confirm that comparable values are obtained. Observation of a lag would suggest the need for different renaturations, perhaps with longer time (20). For multiple-turnover systems, perform an active site titration with a substrate with extensive base pairing (6, 7, 20). Under these conditions, product dissociation is typically rate limiting and the stoichiometry of the burst gives the fraction of active ribozyme. If the reaction is fast enough that hand mixing gives <50% of the transient, then rapid mixing can be used.

The reaction can be initiated by several approaches. One possibility route is addition of  $Mg^{2+}$ , which is often used to follow tertiary structure formation. However, this is not desirable for probing catalysis since a lag may be observed; in these cases, renature in the presence of  $Mg^{2+}$ . Multiple-turnover reactions are often initiated by addition of oligomer substrate(s). Single-turnover reactions can be initiated by addition of an antisense (AS) oligomer (DNA is cost effective) that removes a naturally occurring or engineered misfold in the RNA (21). These two cases are similar in that an oligomer is added *in trans*, so a bimolecular association must be considered. For single-turnover reactions, the dependence on AS oligomer concentration is typically hyperbolic, revealing conditions where association is not rate-limiting. A large concentration profile should be obtained, however, to assure oligomer inhibition is not occurring due to oligomer binding at a second site.

#### 2.1.4 Structure–function relationships in ribozymes

In all cases, having a relationship between structure and function is critical. Structural information can be high resolution, such as X-ray crystal or nuclear magnetic resonance (NMR) structures, or low resolution, such as structure mapping by enzymatic and chemical probing (22). In each case, a strong sense of scepticism must be maintained. There are cases where the crystal structure is of an inactive conformer (e.g. hammerhead ribozyme), or where the enzyme-probed structure is of an inactive conformer (e.g. –30/99 HDV ribozyme (21)). Structure can be probed simply by direct cleavage of end-labelled RNA with enzymes and chemicals, or by reverse transcription to detect chemical modification of the Watson–Crick face. Commercial enzymatic kits for RNA sequencing and structure mapping are currently (2001) available (Glycobiology & Enzyme Technology).

#### 2.1.5 Powerful mutagenesis experiments are available for ribozymes

In many cases, structural insight can be obtained through site-directed mutagenesis. Of particular importance are double mutants in which a base pair, in the ground or transition state, is probed by a compensatory change (e.g. a G–C to C–G change). One checks if the individual mutations (e.g. G–G or C–C mismatches) have impaired activity and if the double has restored activity. Mutagenesis experiments involving such a ‘rescue’ are more directly interpretable than mutants that impair activity. RNA mutagenesis is done with commercial kits (e.g. ‘QuikChange’ from Stratagene), or PCR subcloning (see *Protocol 4*). In addition, single functional groups can be modified (e.g. 2′-hydroxyl, deaza bases, etc.) using nucleotide analogue interference modification (NAIM), developed by Strobel and co-workers (23).

## 2.2 Choosing the experiment

Once a system with a uniform population is ready, it is necessary to choose experiments. There is a vast ribozyme literature in which chemistry, folding,

and binding kinetics have been elucidated. Issues of metal-ligand interaction, pH-dependence, real versus kinetic  $pK_a$ 's, small molecule rescue and proton inventory have been examined. In general, one wants to identify the rate-limiting step, and address whether covalent chemistry and folding contribute to the observed rate constant. These fascinating and complex issues are discussed in numerous reviews on ribozymes (1, 24, 25) and other chapters of this book. In this section, we provide an overview of selected issues that may be of general interest and are perhaps not readily addressed in the literature; these include points on experimental design, general approaches for deriving simpler rate equations, and methods for simulating complex rate laws.

### 2.2.1 Which species should be labelled?

Some observable is required to monitor the reaction. In many cases, there is more than one species that can be labelled. In the case of multiple-turnover enzymes with two substrates, the label should be placed on the limiting substrate, to allow the full course of the reaction to be observed. If the limiting substrate is placed in only slight excess of the ribozyme (typically 5- to 10-fold), then one can monitor the active site titration and obtain  $k_{cat}$  for multiple-turnover ribozymes (6, 7, 20). Quite often, transient kinetics experiments are performed with enzyme in excess over labelled substrate ( $=k_{cat}/K_M$  conditions); these conditions allow association and dissociation rate constants and rates for chemistry to be obtained without product release being rate-limiting (6, 7). The situation for single-turnover ribozymes is similar; neither product release nor substrate binding is a concern, and a rate-limiting chemistry step can be obtained (26).

### 2.2.2 Work with ratios whenever possible

RNA samples should be end-labelled (see Section 3) rather than internally labelled so that cpm are proportional to moles of substrate and product. If the RNA must be body-labelled, then a factor proportional to moles can be obtained for each species by dividing cpm by the number of labelled bases. Labelled RNA is fractionated on a denaturing gel and cpm in substrate and precursor bands are obtained on a digital imager such as a PhosphorImager (Molecular Dynamics) (see *Protocol 5*). Ideally, *fraction of product* ( $=P/(P + S)$ ) cleaved is calculated rather than concentration to correct for loading differences, which can vary by 10% or more.

## 2.3 Deriving kinetic equations

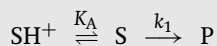
Reviews with solutions to kinetic equations are available, which are handy for many situations. Nevertheless, countless cases are either not addressed or not readily found. Furthermore, in cases where equations are available, they are often not in fraction format and must be transposed. The following protocol provides sample derivations for simple and moderately complex mechanisms.

## Protocol 2

### Deriving a rate equation using 'fraction' format

#### A. Simple mechanism

Consider a case in which the pH-profile for a single-turnover ribozyme has been obtained and the  $\log k_{\text{obs}}$  is found to vary linearly with pH, with the rate levelling off at high pH (Figure 1). A possible mechanism is shown (Scheme 1), in which precursor, S, forms product, P, with rate constant  $k_1$ , and S is inhibited upon protonation of some residue according to acid dissociation constant,  $K_A$ .



**Scheme 1**

We want a rate equation that will allow  $K_A$  and  $k_1$  to be obtained from fraction product versus time plots. (For simplicity, concentration symbols '['] are omitted in the rate equations below.)

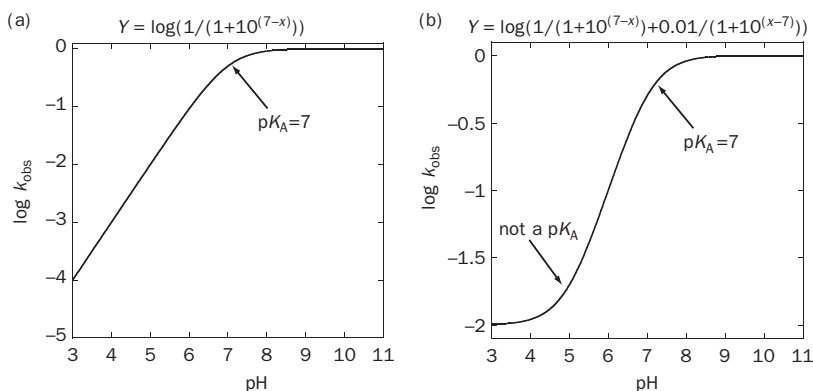
- 1 Write down the rate law.

$$\frac{dP}{dt} = -\frac{dS_{\text{tot}}}{dt} = k_1 S. \quad (1)$$

This law equates loss of  $S_{\text{tot}}$  to reactivity of S, the amount of unprotonated precursor; it is assumed that the protonated precursor ribozyme is completely non-reactive. (See Protocol 2B for a case in which multiple forms of the precursor are reactive.)

- 2 Put S in terms of  $S_{\text{tot}}$ ,  $H^+$ , and  $K_A$ , so the rate equation can be integrated.

$$S_{\text{tot}} = S + \text{SH}^+ = S(1 + H^+/K_A). \quad (2)$$



**Figure 1** (a) Graph of  $\log[\text{Equation (8)}]$ , plotting  $\log[k_1/(1 + 10^{pK_A - \text{pH}})]$  versus pH. In this example,  $k_1 = 1$  and  $pK_A = 7$ . Plot was made using the function option in KaleidaGraph v. 3.5 (Synergy Software). (b) Graph of  $\log[\text{Equation (13)}]$ , plotting  $\log[(k_1/(1 + 10^{pK_A - \text{pH}})) + (k_2/(1 + 10^{\text{pH} - pK_A}))]$  versus pH. In this example,  $k_1 = 1$ ,  $k_2 = 0.01$ , and  $pK_A = 7$ .



**Protocol 2** continued

We used the relationship  $K_A = (S)(H^+)/ (SH^+)$ . In general, it is easiest to work with dissociation constants because units are M, and they offer a straightforward graphical interpretation. One could also use the King-Altman approach to write down S in terms of  $S_{tot}$ ; this is useful when very complex models are considered (27, 28). In Equation (2), we ended up with a relationship in which  $S = f_S S_{tot}$  (Equation (3)), where  $f_S$  is the fraction of unprotonated precursor. Therefore,

$$f_S = 1/(1 + H^+/K_A). \quad (4)$$

- 3 Derive an expression for  $k_{obs}$ , the observed rate constant. In general,

$$-dS_{tot}/dt = k_{obs} S_{tot}. \quad (5)$$

Combining Equations (1), (2), (4),

$$k_{obs} = k_1/(1 + H^+/K_A). \quad (6)$$

$k_{obs}$  directly reflects the fraction of the ribozyme in the functional form,  $k_{obs} = k_1 f_S$ . Equation (5) has the solution

$$S_{tot} = (S_{tot} + P_{tot}) \exp(-k_{obs} t).$$

Since the  $SH^+$  and S are indistinguishable on a denaturing gel, we use the following expression,  $f_{cleaved} = P_{tot}/(S_{tot} + P_{tot})$ .

Thus,

$$f_{cleaved} = 1 - \exp(-k_{obs} t). \quad (7a)$$

$S_{tot}$  and  $P_{tot}$  are proportional to the cpm for each band, and the proportionality constant involves the total ribozyme. Since total ribozyme loaded is the same for  $S_{tot}$  and  $P_{tot}$  for a lane, the proportionality constants cancel in  $f_{cleaved}$ , and loading differences are not important. Thus,

$$f_{cleaved} = \text{cpm } P_{tot} / (\text{cpm } S_{tot} + \text{cpm } P_{tot}). \quad (7b)$$

- 4 An intuitive way to plot this data is with pH as the x-axis. This format allows the  $pK_A$  to be obtained both graphically and directly from the fit. Equation (6) is rearranged to give

$$k_{obs} = k_1 / (1 + 10^{pK_A - pH}). \quad (8)$$

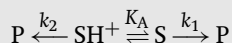
Obtain  $k_{obs}$  from time courses at various pH values using Equations (7a) and (b) and a nonlinear least squares program such as KaleidaGraph (Synergy Software). Space time points by factors of 1.5–2.0, and collect data to approximately five half-lives, or 97% complete. If data are single exponential, then 12 points are adequate; but, if data are double exponential, then 18 points should be acquired. Look to see that residuals lie

**Protocol 2** continued

above and below 0 at random spacing; if not, try an extra exponential term in the fit. Next, plot  $\log(k_{\text{obs}})$  versus pH, and fit  $y$  to  $\log[k_1/(1 + 10^{\text{pK}_A - \text{pH}})]$  using KaleidaGraph. Check that the errors in  $k_1$  and  $\text{pK}_A$  are not larger than values. Try decreasing the allowable error and test if identical fits occur.

**B. More complex mechanism**

Consider a case in which the protonated form of the precursor ribozyme also reacts.

**Scheme 2****1**

$$\frac{d\text{P}_{\text{tot}}}{dt} = -\frac{d\text{S}_{\text{tot}}}{dt} = k_1\text{S} + k_2\text{SH}^+. \quad (9)$$

We added rates of formation of P from the protonated and unprotonated S channels to give the rate for  $\text{P}_{\text{tot}}$ . This assumes that P formed from either channel is indistinguishable

**2** The value for S is from Equation (2). The value for  $\text{SH}^+$  can be obtained as follows.

$$\text{SH}^+ = \text{S}_{\text{tot}} - \text{S} = \text{S}_{\text{tot}}(\text{H}^+/\text{K}_A)/(1 + \text{H}^+/\text{K}_A). \quad (10)$$

**3** Using an approach identical to Scheme 1,

$$k_{\text{obs}} = (k_1 + k_2\text{H}^+/\text{K}_A)/(1 + \text{H}^+/\text{K}_A). \quad (11)$$

This can be written as

$$k_{\text{obs}} = [k_1/(1 + \text{H}^+/\text{K}_A)] + [k_2/(1 + \text{K}_A/\text{H}^+)] \quad (12)$$

(Note:  $k_{\text{obs}} = k_1f_{\text{S}} + k_2f_{\text{SH}^+}$ , which is generally true for a multi-channel mechanism (29).)

**4** Equation (12) is rearranged

$$k_{\text{obs}} = k_1/(1 + 10^{\text{pK}_A - \text{pH}}) + k_2/(1 + 10^{\text{pH} - \text{pK}_A}). \quad (13)$$

Again,  $k_{\text{obs}}$  is obtained from time courses at various pH values using Equation (7b);  $\log(k_{\text{obs}})$  of the data is taken, plotted versus pH, and  $y$  is fit to  $\log[\text{Equation (13)}]$ , using nonlinear least squares.

A final note: it is worth plotting the equation itself to see if it is reasonable (Figure 1); this can be done readily with v3.5 of KaleidaGraph. Scheme 2 is an interesting case as its plot looks deceptively like a double  $\text{pK}_A$  mechanism, even though it has only one  $\text{pK}_A$ .

## 2.4 Simulating data

The cases discussed in Section 2.3 were simple enough that analytical expressions could be derived. However, complex mechanisms arise for which analytical solutions cannot be readily obtained; also, one may wish to simulate data for a mechanism without deriving rate equations. Because rate laws can be written down quickly for any mechanism (see Equations (1) and (9)), it is worth having a numerical approach for simulation. Programs are available to aid this process, notably Kinsim and Fitsim, and the final chapter of this volume is devoted to computer simulation and global analysis of data fitting. Here we present a brief protocol using Microsoft Excel to simulate data. We use an iterative approach in which concentrations are adjusted by small changes from the product of rate and time. Using Excel to simulate data has several useful features:

- (a) Multiple plots can be displayed (e.g. S, P, and intermediates versus time), and instantly updated upon changing a kinetic constant.
- (b) Rate of transcription can evolve with time according to any function.
- (c) Data for any reactive species can be exported to KaleidaGraph and fit to an equation.

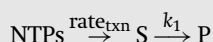
### Protocol 3

#### Simulating kinetic data using Excel

##### A. Simple mechanism

Consider a mechanism in which precursor, S, forms product, P, with rate constant,  $k_1$ , while S is being synthesized co-transcriptionally at rate,  $\text{rate}_{\text{txn}}$ .

- 1 Write down the mechanism.



**Scheme 3**

- 2 List concentrations to monitor, and any rate and equilibrium constants to input.
  - (a) [S], [P]: the concentrations of S and P at time, t;
  - (b)  $\text{rate}_{\text{txn}}$  and  $k_1$ : rate for transcription and rate constant for cleavage.
- 3 Write down the rate laws.

$$\frac{dS}{dt} = \text{rate}_{\text{txn}} - k_1 S \quad (14)$$

$$\frac{dP}{dt} = k_1 S \quad (15)$$

$\text{rate}_{\text{txn}}$  is the *rate* at which S is synthesized, and can have a complex form (see below).

**Protocol 3** continued

- 4 Write equations for concentrations at time,  $t$ , according to contribution from rate and preceding time point,  $t-i$ . 'i' is a small time increment  $\leq \frac{1}{10}$  of the shortest  $t_{\frac{1}{2}}$ .

$$S_t = S_{t-i} + \left( \frac{dS}{dt} \right) \Big|_{t-i} \times dt \quad (16)$$

$$P_t = P_{t-i} + \left( \frac{dP}{dt} \right) \Big|_{t-i} \times dt \quad (17)$$

- 5 Design an Excel spreadsheet with the necessary variables, using above equations. Figure 2 shows a typical spreadsheet with hints for determining values for cells.

- (a) Use fill command to translate data down a few rows. Confirm there are no errors.
- (b) Determine number of points desired and fill. This example uses 3600 points. With a time increment of 0.01 min, this simulates a reaction of 36 min. One should confirm that enough time points are present by increasing the number and verifying there is no effect on the shape of the simulated data or the values obtained from any fits.
- (c) Enter different values for time increment,  $\text{rate}_{\text{txn}}$ , and  $k_1$ . Use the Chart Wizard function to graph the simulated data in the same worksheet to easily monitor changes. A plot of simulated  $f_{\text{uncleaved}}$  data versus time is provided (Figure 3, points). Plots can show multiple simulations and can be used to overlay simulated and experimental data.

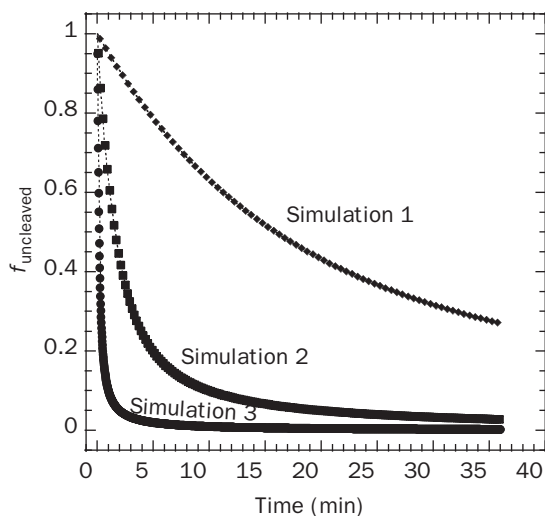
(a)

	A	B	C	D	E	F	G	H	I
1	Time	S	P	dS/dt	dP/dt	Total RNA	$f_{\text{uncleaved}}$	Time	0.01
2	0	0	0	$\text{rate}_{\text{txn}} - k_1 * B2$	$k_1 * B2$	$C2 + B2$	0	$\text{rate}_{\text{txn}}$	100
3	A2 + Time	B2 + D2*Time	C2+E2*Time	$\text{rate}_{\text{txn}} - k_1 * B3$	$k_1 * B3$	$C3 + B3$	$(B3)/(C3+B3)$	$k_1$	1
4									

(b)

	A	B	C	D	E	F	G	H	I
1	Time	S	P	dS/dt	dP/dt	Total RNA	$f_{\text{uncleaved}}$	Time	0.01
2	0	0	0	100	0	0	0	$\text{rate}_{\text{txn}}$	100
3	0.01	1	0	99.9	0.1	1	1	$k_1$	0.1
4	0.02	1.999	0.001	99.8001	0.1999	2	0.9995		
5	0.03	2.997001	0.002999	99.7002999	0.2997001	3	0.999000333		
3601	35.99	972.6981469	2626.301853	2.730185308	97.26981469	3599	0.270269004		
3602	36	972.7254488	2627.274551	2.727455123	97.27254488	3600	0.270201514		

**Figure 2** Creating an Excel spreadsheet to simulate data. (a) Include rows for all variables: time (col A); [S] and [P] (col B, C); dS/dt and dP/dt (col D, E); [RNA]<sub>tot</sub> (col F);  $f_{\text{uncleaved}}$  (col G). Concentrations are calculated according to Equations (14)–(17). Col I is for kinetic constants (as 'named cells' in the spreadsheet to immediately give changes in the simulated data (col A–G)). 'Time' represents the increment between points. For  $t = 0$ , enter zeros; use equations in subsequent rows to calculate values. (b) Representative rows at top and bottom of spreadsheet after filling out to 36 min.



**Figure 3** KaleidaGraph plot of  $f_{\text{uncleaved}}$  versus time for three sets of simulated data according to *Scheme 3*:  $k_1 = 0.1, 1.0, \text{ and } 10 \text{ min}^{-1}$ . The  $\text{rate}_{\text{txn}}$  was 100 concentration units/min; varying  $\text{rate}_{\text{txn}}$  did not affect the simulated data or the fit, in accordance with the model used to derive Equation (18) (Reference 30). The simulated data were well fit by Equation (18), and returned the simulated  $k_1$  values. Only 2%, 5%, and 50% of the actual data points are plotted for  $k_1 = 0.1, 1.0, \text{ and } 10 \text{ min}^{-1}$ , respectively, to aid visualization of the fitted line.

### Protocol 3 continued

- 6 Simulated data can be exported to Kaleidagraph for nonlinear fitting to analytical equations. For example, the data simulated above were shown to fit well to Equation (18) for co-transcriptional ribozyme cleavage (30), (Figure 3, line).

$$y = \frac{(1 - e^{-k_1 t})}{k_1 t} \quad (18)$$

## B. More complex mechanism

- 1 Simulation using a changing  $\text{rate}_{\text{txn}}$ : A unique feature of Excel is the ability to vary a rate according to a desired function. To incorporate this, it is necessary to dictate when slowing begins by an 'IF' statement in Excel:

$$= \text{IF}(\text{logical\_test}, \text{value\_if\_true}, \text{value\_if\_false})$$

For example, to simulate the  $\text{rate}_{\text{txn}}$  slowing by some increment starting at time = 30, the following column must be added to the spreadsheet to represent the  $\text{rate}_{\text{txn}}$ :

$$= \text{IF}(\text{time} < 30, \text{rate}_{\text{txn}}, \text{rate}_{\text{txn}} - \text{value})$$

**Protocol 3** continued

*Note:* time here is the total time of the reaction. This statement means if time <30, rate<sub>txn</sub> is fixed at the constant rate<sub>txn</sub>; if time ≥30, rate<sub>txn</sub> is decremented by 'value' (value should be added to column I as a named cell). If 'value' is input as 0.1, then rate<sub>txn</sub> decreases to 99.9 at time = 30.01, 99.8 at time = 30.02 and so on.

- 2 Simulating a more complex reaction mechanism: It is often the case that the simplest mechanism possible will not reproduce the data. Fortunately, complexity is easily incorporated into data simulation through addition of terms to the rate law. For the running example of this protocol, consider the case in which some of the RNA transcribed is incompetent for self-cleavage. This partitioning in the mechanism translates into additional terms in the rate law, and a third term in the simulation.

### 3 Kinetic analysis of ribozymes using radiolabelled RNA

#### 3.1 Introduction: advantages and disadvantages of radiolabelled methods

The most common label for ribozyme kinetics is radioactivity. Optical techniques provide complementary approaches and are discussed briefly in Section 4. Typically, the RNA is end-labelled with <sup>32</sup>P and the fraction reacted is determined on a digital imager (see *Protocol 5*). Advantages of radiolabelling are that the RNA is minimally perturbed, degradation and side reactions can be monitored, the reaction can be readily quantitated, and the reactant and product forms of the ribozyme can be *directly* visualized. This last feature is particularly important if multiple product species are generated. Disadvantages include handling radioactive material and waste, and having to label samples approximately every 6 weeks (the half-life of <sup>32</sup>P is 2 weeks); in addition, each time point is collected and quenched separately, making data acquisition tedious and time consuming, especially when one does not have a good initial idea of the timescale of the experiment. (Compare optical methods in which hundreds of time points are collected in real time (Section 4).) Nevertheless, radioactive detection allows the distinct advantage that one is confident that covalent chemistry is being observed. Also, most of the equipment is available in a standard biochemistry lab.

#### 3.2 Preparing ribozymes and RNA substrates

In general, RNA for *in vitro* work is prepared in one of three ways: by transcription using T7 RNA polymerase and a DNA template, by chemical synthesis, or by a combination of the two. The next three subsections discuss these approaches and their advantages and disadvantages.

### 3.2.1 RNA by T7 transcription

The most common method of preparing RNA is *in vitro* 'run-off' transcription using phage T7 RNA polymerase. Reviews have been written that describe expressing and purifying T7 polymerase, as well as transcription conditions (31). Note that no one set of conditions is optimal for all sequences, so pilot reactions should be used to optimize yield. Detailed protocols for many of the steps discussed below are available (32). Here, we provide an overview of approaches and recent developments.

There are several advantages to using T7 transcription: (a) It provides the most economical way to make RNA, especially RNA >40 nt; (b) T7 polymerase can be overexpressed in the lab, with clones available; and (c) Multi-milligram amounts of RNA can be readily prepared. Expressing and purifying your own T7 polymerase is the method of choice if substrate quantities of ribozyme are needed for biophysical experiments such as stopped flow, calorimetry, X-ray crystallography, or NMR. Alternatively, reasonable amounts (20–50 µg/20 µl reaction) of RNA, an amount sufficient to carry out radiolabelled RNA kinetics, can be prepared using commercial kits (Ambion and Epicentre), with T3 and SP6 polymerase kits available as well.

Disadvantages to T7 transcription include transcript heterogeneity at the 3'- and 5'-ends, unwanted nucleotides at the 3'-end originating from cloning sites, and the need for G residues at the first few positions of the transcript. 3'-end heterogeneity is common and usually consists of a non-templated nucleotide, referred to as ' $n + 1$ ', added randomly at the end of the transcript. 5'-end heterogeneity is less common and some start sequences should be avoided (33, 34).

Approaches have been developed to address each of these disadvantages. Of particular use is fusing self-cleaving ribozymes—hammerhead, hairpin, HDV, or VS ribozymes—upstream or downstream of the transcript (35, 36). The precursor transcript self-cleaves, often during transcription, and the appropriate band is purified by denaturing polyacrylamide gel electrophoresis (PAGE). If the transcript does not cleave to completion during transcription, then thermal cycling may help by refolding trapped species, although temperatures higher than 65°C should be avoided in the presence of  $Mg^{2+}$ . By engineering the appropriate self-cleaving ribozyme into the construct, one can have the processed transcript start with any sequence (i.e. not necessarily G-rich), eliminate the effect of stuttering at the very 5'-end of the transcript, and obtain a homogeneous 3'-end. Another advantage is that the self-cleaving ribozymes leave a 5'-hydroxyl on the processed transcript, allowing it to be radiolabelled without an extra dephosphorylation step (see Section 3.4). One obvious disadvantage of fusing self-cleaving ribozymes is that nucleotides are wasted in producing the self-cleaving ribozyme by-product; this can be especially costly for isotopically labelled transcripts for NMR. It should be noted that  $n + 1$  addition can also be minimized by using

2'-methoxy nucleotides at the last and penultimate positions of the template strand (37).

Undesired nucleotides that come from the recognition site for the restriction enzyme for run-off transcription can be avoided by using a non-palindromic type IIS restriction enzyme such as *Bsa* I for the run-off site (New England Biolabs). Type IIS restriction enzymes cut the DNA a fixed number of nucleotides *away* from an asymmetric recognition sequence. By placing the recognition sequence downstream of the desired run-off site, any sequence can be engineered into the 3'-end of the RNA (see *Protocol 4* for cloning strategies).

The DNA template for transcription is prepared by one of three ways: (a) from a cloned plasmid with a T7 promoter linearized at a downstream restriction site; (b) from a PCR fragment with a T7 promoter; or (c) from a hemi-duplex consisting of a template strand annealed to a 23-nt T7 top strand oligomer. Method (c) has the advantage that the RNA can be prepared rapidly and cheaply. This method involves obtaining a DNA oligomer, gel purifying it (optional for shorter DNA), annealing it to the top strand oligomer, and carrying out transcription (31). In addition, 2'-methoxy residues can easily be incorporated at the last two positions of the template strand to limit addition of  $n + 1$  nucleotides (see above). Disadvantages of method (c) are that it is limited to shorter RNA, typically less than 50 nt, and lower yields are obtained than in methods (a) and (b). Method (b) typically affords higher yields and is helpful for transcribing RNA with strong secondary structure. Transcribing from the PCR product can be helpful for troubleshooting if cloning for method (a) turns out to be troublesome. If desired, 2'-methoxy residues can be placed at the 5'-end of the bottom strand PCR primer so they end up at the last two positions of the template strand. The disadvantage of this method is that the DNA is not cloned, and so PCR errors could go unnoticed.

Method (a) offers the advantage that the insert is cloned and can therefore be sequenced to check for mutations. A high copy number plasmid such as pUC19 is a good choice since multi-milligram quantities of plasmid can be obtained from Qiagen plasmid kits; this is helpful if large quantities of RNA are needed for biophysical experiments. A word of warning: Qiagen kits use RNase A to remove RNA, so extreme caution must be exercised to prevent contamination of equipment and stocks. Good practice is to use aerosol-resistant tips and a set of dedicated buffers, water, and pipetman on a cart away from the lab bench. Another advantage of method (a) is that mutants (point, insertion, and deletion) can be readily generated using a kit such as QuikChange from Stratagene. In certain cases, we have found it best to culture the cells at 30°C until the mutation is established. It is also advisable to have the plasmid sequenced after each plasmid preparation to control for mutations and revertants; in general, sequencing is no longer expensive or time consuming with the use of a good sequencing centre. *Protocol 4* provides a general approach for preparing RNA 'from scratch' (i.e. any RNA of any sequence using only DNA oligomers). Specific cloning sites and ribozyme fusions are for illustrative purposes only. Multiple manufacturers are available for most reagents.



## Protocol 4

### Designing and preparing a representative RNA from DNA oligomers

#### Equipment and reagents

- 50× Advantage cDNA Polymerase Mix; 10 × cDNA PCR buffer (Clontech)
- 50 mM dNTP (12.5 mM each dATP, dCTP, dGTP, dTTP) (Amersham Pharmacia Biotech)
- T4 DNA Ligase; 10× T4 DNA Ligation Buffer (Promega)
- Alkaline Phosphatase, Calf Intestinal (CIP, New England BioLabs)
- Low melting agarose (BioWhittaker Molecular Applications)

#### Method

##### A. Design of dsDNA insert for PCR

- 1 Design two regions of four G's or C's (e.g. GCC) at each end of the PCR fragment to assist restriction enzyme cutting. These regions will be referred to as N4.
- 2 Next to each N4, place a unique cloning site, choosing from the polylinker of pUC19.
- 3 For the top strand:
  - (a) Follow the 5' cloning site with the T7 promoter: 5'TAATACGACTCACTATA.
  - (b) Insert sequence for a hammerhead ribozyme with an annealing region that is complementary to the start of the desired transcript (35, 36).
  - (c) Place the sequence for the desired transcript 3' to the cleavage site of the hammerhead ribozyme.
  - (d) Place NGAGACC after the last desired transcript nucleotide as the recognition site for *Bsa* I to ultimately yield template DNA that ends without vector sequence. (Other type IIS enzymes besides *Bsa* I can be used.) This is followed by the downstream cloning site and N4.
- 4 Divide the designed insert into top and bottom strand DNA oligomers that overlap by ≈20 nt and purchase. It is best to start and end the overlapping region with G–C pairs.

##### B. Experimental steps

- 1 Anneal PAGE-purified oligomers and extend using thermocycling. Into a 200 µl PCR tube, pipette volumes of the top and the bottom strand oligomers that give 0.5 µM of each in a 50 µl reaction. Add 5 µl 10 × cDNA PCR buffer, 1 µl 50 mM dNTP, and 1 µl 50× Advantage cDNA Polymerase Mix. Place in thermocycler and melt DNA at 94°C for 5 min. Run 5 cycles of 94°C, 1 min and 68°C, 1 min. (These temperatures should be optimized on a per case basis. Use of a gradient thermal cycler helps greatly with optimization.) Finish with 10 min at 72°C. Run 1 µl aliquots on a 2% agarose gel to monitor extension.

**Protocol 4** continued

- 2** Perform a phenol/chloroform extraction followed by an ethanol precipitation to pellet the insert. Dissolve in 25  $\mu$ l ddH<sub>2</sub>O. Double digest extended insert with restriction endonucleases for cloning. (Do not use *Bsa* I here.)
- 3** Double digest  $\approx$ 500 ng pUC19 in 100  $\mu$ l with same restriction endonucleases as in previous step. Add 2  $\mu$ l (10 U/ $\mu$ l) calf intestinal phosphatase (CIP) to the reaction to prevent self-ligation. (CIP works well in most buffers.) Incubate at 37°C for 30 min, then heat inactivate CIP at 75°C for 10 min. Gel purify plasmid on a 1% low melting agarose gel (BioWhittaker Molecular Applications). Excise the band and approximate vector content of gel slice.
- 4** Ligate insert into vector and transform into competent *E. coli* strain using in-gel methods. (BioWhittaker Molecular Applications, <http://www.bmaproducts.com>.) Miniprep and sequence DNA (32).
- 5** Purify plasmid by a Qiagen Maxi kit, re-sequence, and linearize with *Bsa* I. Run a 2  $\mu$ l aliquot on a 2% agarose gel to confirm complete digestion.
- 6** Extract with phenol/chloroform and ethanol precipitate. Dissolve pellet in 50  $\mu$ l H<sub>2</sub>O and perform a transcription (Ambion or Epicentre).

### 3.2.2 RNA by chemical synthesis

RNA can also be prepared by chemical synthesis. If a DNA synthesizer is available, then one can use phosphoramidites synthesized in the lab or obtained commercially from several sources such as Glen Research and Chemgenes. This approach has the advantage that unusual and novel oligomers can be prepared, and many novel phosphoramidites are available commercially. Of course, this approach requires a lab with a strong organic synthesis component.

A relatively new and exciting development in RNA chemistry is the commercial availability of high quality and reasonably affordable synthetic RNA oligomers. Companies such as Dharmacon will prepare RNA up to 80 nt; moreover, an increasing number of modifications are available at the sugar, phosphate, and base moieties. When short oligomers substrates ( $\approx$ 2–20mers) are required, commercial synthesis is the clear choice. No investment in a synthesizer or organic synthesis is required, and the RNA is of high quality and purity. Advantages are the versatility of sequences and chemical modifications available, and that the ends are homogeneous. Limitations include the size of the RNA and cost. If demand for RNA-based experiments continues to increase, then one may anticipate a likely decrease in associated costs and an increased number of modifications available.

### 3.2.3 RNA by semi-synthetic approaches

Enzymatically and chemically synthesized RNA molecules can be attached to afford atomic modifications inside long transcripts, allowing specific chemical

questions to be addressed. The method involves T4-DNA ligase mediated ligation of the strands, with an  $\approx 30$ mer DNA splint used to align 3'-OH and 5'-phosphate termini (38). Ligase can be prepared from a (His)<sub>6</sub> clone (23) or purchased from a commercial source (Promega is guaranteed RNase-free). If large amounts of RNA are required, then expressing and purifying the ligase clone is best.

Optimal conditions for ligations vary widely, but success appears to depend largely on forming the desired three-strand assembly over self-structure. It has been found that disrupter oligomers that hybridize with and unfold the large RNA piece away from the site of ligation can sometimes improve yield. Pilot reactions are useful, and one should vary the ratio of the three strands (start with 1 : 1 : 1), the annealing temperature, and the order of addition of the three strands. We have found in some instances, especially with GC-rich sequence near the reactive termini, that 37°C works best despite manufacturer's recommendations of 16°C. The reason may be that end fraying is less problematic with these sequences, and higher temperature may lead to faster ligation or refolding of the RNA with extensive self-structure.

Another method for introducing modifications into a T7 transcript is to prime the transcription with a GTP-related molecule (31). For example, the transcript can begin with GMP, with 5'-thiophosphate GMP, and with dinucleotides such as ApG and modified ApG dimers. Oligomers longer than dinucleotides do not work. The modified guanine is usually added in  $\approx 10$ -fold excess over GTP, and typically is incorporated at the 5'-end of  $\approx 90\%$  of the transcripts. This method can be useful for attaching a fluorescent base or probe to the 5'-end of the RNA.

### 3.3 A few guidelines for purifying and handling RNA

Much has been written on the handling of RNA. It is our opinion that for the experiments discussed here one needs to take precautions, but extraordinary efforts are not generally required. For example, older protocols suggest acid washing and baking glassware, using glass distilled water, and treating water with DEPC. We will not suggest that these precautions are unnecessary or that they be eliminated if already in practice; however, if one is preparing RNA by *in vitro* transcription, a few simple precautions appear sufficient.

- (a) Wear latex gloves at all times since the hands contain ribonucleases. Once wearing gloves, do not touch materials that are handled with bare hands or touch the face, etc.
- (b) Clean glassware thoroughly using distilled water rinses. Cover dry glassware with foil.
- (c) Use disposable RNase-free plasticware whenever possible.
- (d) Use RNase-free reagents; test for RNase contamination by incubating a radiolabelled RNA with new reagents.
- (e) Once a kinetic assay is working well, aliquot buffers and freeze at  $-20^{\circ}\text{C}$ ; if problems arise, discard old solutions and start with fresh tubes.

- (f) Use a reliable source of purified water. We use a MilliQ system from Millipore.
- (g) Avoid touching the barrel of the pipetman inside of a buffer; do not breathe on or talk over buffers and solutions; use aerosol-resistant tips whenever handling RNases.
- (h) Store RNA at  $-20^{\circ}\text{C}$  in a weakly buffered solution ( $\text{pH} < 7.5$ ) with 0.1 mM EDTA to avoid degradation. RNA has been found to keep several years with little or no degradation under these conditions. Store RNA in small aliquots to avoid repeated freeze-thaw cycles.
- (i) With each preparation of the RNA, assure that the preparation has the same kinetic properties as previous ones, for example,  $k_{\text{cat}}$ ,  $K_{\text{M}}$ , etc.

Here we present a few general suggestions and warnings for analysing RNA samples. In general, RNA is fractionated by denaturing PAGE as a first step to purification or analysis. RNA is the most thermally stable nucleic acid; thus, a few factors are worth noting about the ability of gels to denature RNA.

- (a) Urea hydrolyzes slowly with time and gels lose their denaturing power. Store acrylamide/urea solutions in brown bottles at  $4^{\circ}\text{C}$ , and prepare freshly every 2 months. Also, 8.3M Urea (50% w/v) is significantly more denaturing than 7M Urea.
- (b) Heat significantly increases the ability of gels to denature RNA. Pre-run gels until they are hot to the touch ( $\approx 60^{\circ}\text{C}$  at the glass surface); turn back on immediately after loading.
- (c) In extreme cases, RNA that is suspected to not denature under the above conditions (based on anomalously fast migration relative to markers) should be run on formamide/urea-containing gels (32). These gels have the disadvantage that they must be soaked prior to drying.
- (d) Alternatively, RNA can be denatured prior to electrophoresis by treatment with glyoxal in 50% dimethyl sulfoxide (DMSO); glyoxal modifies the Watson-Crick base pairing face of guanines, and DMSO denatures RNA (39). This reaction is robust and can be extremely helpful. The RNA can be deglyoxylated if desired; however, we typically do this experiment on a small scale and discard the RNA when finished. Once one is sure that the RNA is of the correct size, gels can be run without glyoxal or formamide to simplify kinetic assays.
- (e) If a dsRNA is present that cannot be readily denatured, one can add excess unlabelled strand of the same sequence as the labelled strand and denature to displace it (40).

### 3.4 Radiolabelling ribozymes

As discussed in Section 2.2.2, it is most convenient to work with end-labelled RNA since cpm are proportional to concentration. For 5'-end labelling, the RNA

must have a 5'-hydroxyl. If prepared by transcription, the 5'-triphosphate can be removed by treatment with CIP from New England Biolabs. It is important that all CIP be removed from the reaction; a phenol/chloroform extraction and heat inactivation at 75°C for 10 min is advised. Dephosphorylated RNA can be labelled using T4 polynucleotide kinase (PNK) and [ $\gamma$ - $^{32}\text{P}$ ] ATP (from New England Biolabs and New England Nuclear). Typically a 5  $\mu\text{l}$  reaction is done at 37°C for 1 h using 1  $\mu\text{l}$  of 25  $\mu\text{M}$  dephosphorylated RNA, 1  $\mu\text{l}$  of 25  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP, 1  $\mu\text{l}$  of 10 U/ $\mu\text{l}$  PNK, and 2  $\mu\text{l}$  of a 2.5 $\times$  PNK buffer, diluted from the supplied 10 $\times$  buffer (New England Biolabs). If poor yields are obtained, this may be attributable to a recessed 5'-end. In some cases, adding 10% DMSO to the reaction and renaturing at 90°C before adding PNK helps; alternatively, one can add a DNA disruptor oligomer capped with a 5'-phosphate, and renature prior to PNK addition. RNA can also be labelled at the 3'-end by several approaches including p\* $\text{Cp}$  with T4 RNA ligase, although uridine-free sequences are optimal (41); or extension of the transcript using [ $\alpha$ - $^{32}\text{P}$ ] ATP, Klenow fragment, and a template to the 3'-end that adds one or two extra nucleotides (42). In general, the specific activity of  $\alpha$ ATP is less than  $\gamma$ ATP, making this approach less desirable.

Radiolabelled RNA can be purified by several approaches. One possibility is to remove unreacted radioactive ATP by a G-10 or G-25 spin column (Boehringer Mannheim). However, if the RNA is partially degraded after the spin column, then the labelled strand must be gel purified. Care must be taken in handling these gels, as the buffer and gels can become quite high in counts. RNA is eluted by crush and soak, concentrated by ethanol precipitation, desalted with a 70% ethanol wash, dried in a speed-vac, dissolved in TE, and concentration determined by scintillation counting.

### 3.5 Slow (hand-mixing) kinetics

The general approach to carrying out kinetics using hand-mixing techniques involves adding all components but one and initiating with the last component. In *Protocol 5*, a typical kinetics experiment is provided. A few items are worth special emphasis. We have found that silanized tubes (available from many suppliers) give the most reproducible results and should be used at all steps. Of course, accurate and consistent pipetting is critical to success. Pipetman should be checked constantly for accuracy. Graduated tips and tubes should be used, and one should be vigilant for volume accuracy. A simple test for problems is to pipette 10  $\mu\text{l}$  of water into a P20 (use similar ratios for larger volume pipettes), then submerge the tip just below the surface of the water and watch for any leakage back into the solution over the course of  $\approx 10$  s. If leakage is detected, seals and O-rings can be replaced cheaply in the lab. In general, pipette only volumes  $\geq 2$   $\mu\text{l}$  for maximum accuracy; if necessary, a stock solution should be diluted. When initiating the reaction, the volume of the last reagent should be  $\geq 20\%$  of the total volume; in this way, one can vigorously and repeatedly pipette the last solution to obtain good mixing.

Microcentrifuge tubes with quench solution should be prepared in advance. Typically, 2–4  $\mu\text{l}$  of quench is sufficient. An aliquot is removed from the reaction,

added to an equal volume of the quench with a few pipette strokes, and frozen on powdered dry ice. The quench typically is a 95% formamide, 20 mM EDTA solution with tracking dyes. However, the details of the quench must be adapted to the reaction at hand. In particular, since EDTA forms a 1 : 1 complex with metal ions, it is important that the EDTA concentration be  $2\times$  the divalent metal ion concentration to assure excess. Nevertheless, this is sometimes not enough EDTA since EDTA binds  $\text{Mg}^{2+}$  somewhat weakly, especially at low pH. This becomes problematic if one is interested in no-or very low-divalent ion catalysis as a function of pH. The only solution known for these problems is to work with metal-buffered solutions (29, 43). An important control is to add the quench before initiating the reaction, then check the longest time point for no reaction. This should be done at several EDTA concentrations and no reaction should be observed. Another useful technique is to use small amounts of EDTA in the presence of  $\text{Mg}^{2+}$  to control for contaminating heavy metals. For example, use 10.5 mM  $\text{Mg}^{2+}$  with 0.5 mM EDTA; since EDTA has considerably higher affinity for most other metal ions, this will help reduce any spurious reactions with contaminating ions.

## Protocol 5

### A sample ribozyme kinetics experiment

#### Equipment and reagents

Kinetics experiment:

- 58  $\mu\text{l}$  of  $^{32}\text{P}$  RNA (total counts  $\geq 5000$  cpm) containing everything but initiator reagent
- 6  $\mu\text{l}$  of  $10\times$  initiator reagent (divalent metal, antisense oligomer, etc.)
- 13 silanized tubes containing 4  $\mu\text{l}$  of formamide  $2\times$  quench solution (20 mM EDTA
- pH 8.0/0.25% bromophenol blue/0.25% xylene cyanol made up in formamide)
- water bath or heat block
- bucket of dry ice, crushed to a fine powder

Gel electrophoresis and kinetic analysis:

- 23 cm (vertical)  $\times$  29 cm (horizontal)  $\times$  0.1 cm (thick) polyacrylamide gel (typically 5% PAGE) containing 7M Urea and 1  $\times$  tris-borate-EDTA (TBE) buffer
- gel electrophoresis apparatus connected to a power supply
- 300 ml of  $1\times$  TBE gel running buffer
- Imaging hardware and software (we use a Molecular Dynamics PhosphorImager and ImageQuant software for quantitation)
- Kaleidagraph or similar graphing program for data analysis

**Protocol 5** continued**Method****A. Before starting the kinetics experiment**

- 1 Prepare a 5% polyacrylamide gel.
- 2 Prepare 13 tubes each containing 4  $\mu$ l of 2 $\times$  quench solution (1 tube for each time point).
- 3 Renature radiolabelled RNA (see *Protocol 1* for renaturation suggestions).

**B. Performing the kinetics experiment**

- 1 Warm the tubes containing RNA and initiator reagent at the reaction temperature for 10 min.
- 2 Take a 0 time point by removing 4  $\mu$ l of RNA and adding it to the quench solution and store on dry ice. (Also, make sure to run a quench control as mentioned above.)
- 3 Start the reaction by adding 6  $\mu$ l of 10 $\times$  initiating reagent, and mix well by pipetting the solution up and down several times.
- 4 At each time point, remove a 4  $\mu$ l aliquot and terminate by adding it to the 2 $\times$  quench buffer. For time points taken within the first several minutes, leave the cap off; for intermediate time points (10–60 min), periodically spin down tube to collect condensation from the sides; for long time points (>1 h), sink the tubes in the bath to prevent condensation from occurring.
- 5 Pre-run gel at 35 W for 30–60 min or until the gel becomes warm/slightly hot to touch.
- 6 Denature samples by heating to 90°C for 2 min and load 8  $\mu$ l immediately on the heated gel.
- 7 Run gel at 35 W until dyes are well separated. See reference (32) for suggestions on run time and gel percentage.
- 8 Dry the gel, expose to a PhosphorImager screen for several hours before scanning the image. (Time of exposure depends on the counts loaded on the gel.)

**C. Analysing the kinetics experiment**

- 1 Using the box drawing feature of ImageQuant (or equivalent quantitation software), create an outline around each of the bands corresponding to the precursor and product species (upper and lower bands on the gel, respectively). It is best to draw one box and then use the 'copy' and 'paste' features to create all subsequent boxes so that they are identical in size.
- 2 Correct for 0 time point by pasting the same box in the location where the product band would appear for that lane. Correct for background counts by pasting four boxes randomly on the image.

**Protocol 5** continued

- 3** Determine kinetic rate constants by graphing fraction ribozyme cleaved versus time:
  - (a) Determine the pixel intensity for each of the bands using ImageQuant.
  - (b) Subtract background counts from the precursor and product bands.
  - (c) Determine  $f_{\text{cleaved}}$  as  $\text{cpm product}/(\text{product} + \text{precursor})$ .
  - (d) Correct for 0% cleavage at time = 0 s by subtracting  $f_{\text{cleaved}}$  at  $t = 0$  s from all product bands.
  - (e) Fit data according to *Protocol 2*.

### 3.6 Fast (quench-flow mixing) kinetics

By hand-mixing kinetics, the fastest time point that can be taken reliably is 10–15 s. In some cases, appreciable reaction occurs under 10 s; for example, we have isolated fast-folding mutants of the HDV ribozyme that have a half-life of 0.8 s. Even if one can capture the last 50% of a reaction with hand-mixing, it may still be worth investigating the first 50% since the reaction could have burst or lag phases. In these cases, one can use a rapid quench apparatus to obtain the faster time points. KinTec makes a thermostatted apparatus that allows for time points as short as 2 ms. The overall approach is quite similar to that described in Section 3.5. The details of performing rapid quench experiments are described in Chapters 4, 9, and 11, and are available from the manufacturer; however, a few points about the rapid quench are worth noting. In general, we use a high concentration of EDTA in the quench (final concentration of 0.3M) without formamide. More sample is required for quench-flow than hand-mixing experiments; each time point requires  $\approx 30 \mu\text{l}$ , or  $\approx 300 \mu\text{l}$  per time course.  $3 \mu\text{l}$  of the time point is mixed with formamide loading buffer, and fractionated by PAGE.

## 4 Kinetic analysis of ribozymes: overview of optical techniques

There are several optical (non-radioactive) methods available for following ribozyme reactions. Several protocols have been written for preparing modified RNAs and for following and interpreting their binding kinetics (44, 45) (see Chapters 5 and 6). Since the natural nucleobases are not themselves appreciably fluorescent, RNA labelled with a fluorescent probe has been useful for following the dynamics of reactions such as substrate binding, folding, and conformational changes. RNA can be labelled at the termini using pendant probes, or internally using modified nucleobases such as 2-aminopurine, now available from Dharmacon. Pendant probes can be placed at appropriate sites so that fluorescence resonance energy transfer (FRET) can be used and distance changes can be monitored. Also, changes in extinction coefficient can be monitored by



absorbance changes. Although this is not always useful in associating the change with a specific stretch of nucleotides, some information can be obtained by the 260/280 nm of the change. The change at 260 nm is primarily due to AU pairs, while the change at 280 nm is primarily due to GC (46).

Advantages of spectroscopic methods are that they are ideally suited to following fast processes, with real-time detection used in stopped flow mixing; they are sensitive to non-covalent processes such as binding and conformational changes; and the label can be moved to different sites. Thus, it is possible to directly observe distinct species that have not undergone covalent changes, including folding intermediates and various protonation states. By radiolabelled methods, these species would all migrate the same distance by PAGE. Optical methods may be best thought of as complementary to radiolabelled RNA techniques, which are most useful for following covalent chemistry. In some cases, fluorescent transients that follow covalent chemistry have been isolated, although a radioactive assay was required for confirmation (47).

Interesting new approaches in ribozyme kinetics have appeared in the last few years, including monitoring folding and reactions of single ribozyme molecules using FRET (48). Single molecule reactions are most useful for removing the averaging of important kinetic processes. New technologies can lead to new insights, and it should prove fascinating to watch the field of ribozyme folding and catalysis evolve in the near future.

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