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Probing fast ribozyme reactions under biological conditions with rapid quench-flow kinetics

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Abstract

Reaction kinetics on the millisecond timescale pervade the protein and RNA fields. To study such reactions, investigators often perturb the system with abiological solution conditions or substrates in order to slow the rate to timescales accessible by hand-mixing; however, such perturbations can change the rate-limiting step and obscure key folding and chemical steps that are found under biological conditions. Mechanical methods for collecting data on the millisecond timescale, which allow these perturbations to be avoided, have been developed over the last few decades. These methods are relatively simple and can be conducted on affordable and commercially available instruments. Here, we focus on using the rapid quench-flow technique to study the fast reaction kinetics of RNA enzymes, or ribozymes, which often react on the millisecond timescale under biological conditions. Rapid quench of ribozymes is completely parallel to the familiar hand-mixing approach, including the use of radiolabeled RNAs and fractionation of reactions on polyacrylamide gels. We provide tips on addressing and preventing common problems that can arise with the rapid-quench technique. Guidance is also offered on ensuring the ribozyme is properly folded and fast-reacting. We hope that this article will facilitate the broader use of rapid-quench instrumentation to study fast-reacting ribozymes under biological reaction conditions.

Keywords

Rapid-quench; RNA enzymology; Ribozyme; Ribozyme kinetics

Introduction

Mechanistic studies abound in protein and RNA enzymology. In protein enzymology, research includes, but is not limited to, understanding mechanisms utilized by transferases [1], synthases [2–4], metalloenzymes [5–7], tRNA modifying enzymes [8], regulatory

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proteins [9], and gated ion channels [10, 11]. These protein studies sometimes require anaerobic conditions, meaning that rapid-quench experiments need to be performed in the glove box. In the RNA enzymology field, mechanistic research frequently revolves around self-splicing and self-cleaving RNA enzymes, collectively referred to as ribozymes. Since ribozymes were first discovered in 1981 by the Cech and Altman labs [12, 13], much attention has focused on deducing their catalytic strategies. The discovery of ribozymes has been greatly accelerated in recent years in large part owing to keen bioinformatics insights [14, 15].

Many ribozymes react with rate constants too fast for hand mixing [16–21]. For instance, the *glmS* and twister self-cleaving ribozymes can react with half-lives of ~0.2 and 1.4 sec (unpublished), respectively, under biological conditions. It is often important to study the reaction without slowing it down by altering the solution conditions or mutating the ribozyme or substrate, as this allows for the potential of the chemical step, which is often of greatest interest, to be rate-limiting [21]. Indeed, we and others have discovered that biological-like conditions, such as the presence of molecular crowders and cellular salt concentrations, *speed up* ribozyme reactions [21–26]. To facilitate the fastest rates possible and the simplest interpretation of the data, we present strategies for obtaining a well-folded RNA as revealed by a fast, single-exponential, and complete reaction profile. Such a system makes it easier to understand the reaction and provides a greater chance for chemistry to be rate-limiting.

Various techniques exist for measuring the kinetics of fast biochemical reactions [27]. Perturbation methods such as temperature-jump and pressure-jump allow nanosecond timescale reactions to be measured [28–31], and continuous flow methods allow microsecond reactions to be measured [32]. While these methods are excellent ways to measure very fast reactions, they have the disadvantage that they are quite specialized and generally not commercially available. Many ribozyme reactions occur on the millisecond timescale and can be handled with commercially available rapid-mixing instruments. Two of the most widely used techniques, stopped-flow and rapid-quench, work by fast mixing on the millisecond timescale [33]. Stopped-flow methodologies involve the recording of data in real-time after solutions are mixed, while the rapid-quench technique involves the chemical quenching of reaction mixtures for analysis at a later time. Both methods have their advantages and disadvantages. Stopped-flow has the advantage that hundreds of time points can be collected in a few seconds and analyzed immediately. It has the disadvantage that it typically requires fluorescent labeling, which can perturb the system, and does not directly visualize RNA cleavage, miscleavage, or multiple-addition products. Rapid quench-flow has the advantage that reaction cleavage products can be directly visualized by polyacrylamide gel electrophoresis (PAGE), a method that is widely available and allows multiple products such as miscleavages or multiple additions of nucleotides, if present, to be seen. It has the disadvantage that it is more time-intensive, taking a full day to collect a few 8-point time courses.

This manuscript describes the use of rapid-quench techniques, also known as chemical-quench or rapid quench-flow (RQF), for measuring fast ribozyme kinetics. Specifically, our methodology involves the use of the KinTek RQF-3 instrument for measuring ribozyme self-

cleavage kinetics using radiolabeled RNA. Other rapid-quench instruments currently on the market include the RQF-63 from TgK Scientific and the QFM-4000 from BioLogic, which have similar capabilities (see Section VI).

Methods

I. Instrumentation and methodology

a. Overview—The rapid-quench method facilitates the study of fast ribozyme reactions by allowing for the collection of time points as short as 2 milliseconds. It also allows for acquisition of long time points through a pausing mechanism between initiation and quenching, with no upper limit on reaction time—we have collected time points out to one minute (see below). Typically, the substrate portion of the ribozyme is end-labeled with ^{32}P . Reaction samples and an appropriate quench solution are loaded into the instrument via separate syringes (Figure 1A). A drive plate, which is driven by a stepping motor, is then used to force reaction mixtures together, initiating the reaction. After a delay on the millisecond to second timescale, during which time the reaction occurs, the sample is combined with the quench solution and expelled into an Eppendorf collection tube. The quenched sample is then stored on dry ice or placed into the freezer for future analysis. After the collection of multiple time points from the same reaction in this manner, the time points are fractionated, typically by PAGE.

b. Instrumentation—The KinTek RQF-3 rapid-quench instrument is diagrammed in Figure 1A. It should be noted that rapid quench instruments purchased from other producers vary in construction but the instruments generally work according to the principles outlined below. To load solutions into the rapid-quench instrument, there are five ports located on the periphery of the instrument labeled as “Sample A (or B) Load Port” and “Drive Syringe A (or B or C) Load Port”, which facilitate loading of the samples, quench, and drive water. These ports are connected to the instrument via dedicated valves, located internal to the instrument. The instrument also contains two sample lines and eight reaction loops, with each reaction loop being a different length. Reaction samples are loaded into the sample lines on the left and right of the instrument via the Sample A and B Load Ports (Figure 1A, B). Water, which is used to push samples A and B out of the sample lines and mix them together into a single reaction loop, is loaded into Drive Syringes A and B via the Drive Syringe A and B Load Ports located on the top left and right of the instrument. Quench solution is loaded into Drive Syringe C via the Drive Syringe C Load Port located at the top right of the instrument. The three drive syringes, A-C, are then made flush with the drive plate by moving the drive plate down to meet the syringe plungers. Each reaction time point is initiated by a push of the drive plate, which forces the reactants into a mixer. For reaction times less than or equal to ~100 milliseconds, the reaction ages as it flows through the desired reaction loop at a variable flow rate. The reaction is then mixed with the quench, and the quenched solution is expelled through the exit line into a collection tube (flow of samples is depicted in Figure 1A). For reaction times longer than ~100 milliseconds, the rapid-quench instrument operates in a “push-pause-push” mode. The first push initiates the reaction; the pause allows the reaction to proceed for the desired length of time; and the second push quenches the reaction. The rapid-quench instrument can also be operated in a

third mode in which the second push introduces a third reagent, loaded into Drive Syringe C, and a third push quenches the reaction. This “push-pause-push-pause-push” format can be useful for pulse-chase reactions, which require two mixings prior to the quench. In this type of experiment, the pulse is conducted by mixing together reagents from the sample lines in the first push. The chase is carried out by introducing the third reagent in the second push, and the quench is conducted by expelling the reaction into an Eppendorf tube already containing quench. In this manuscript, we focus on the first two modes of operation, with either no pause or a single pause in “push-pause-push” mode.

The instrument components are contained within a plexiglass box as shown in Figure 1B. The box can be mounted to a benchtop or wall using a mount setup or it can be set onto the benchtop using a bench stand setup, which is depicted in Figure 1C. The rapid-quench instrument contains a highly precise stepping motor, depicted in Figure 1C, which controls movement of the drive plate. The temperature of the rapid-quench box can be varied, which allows Arrhenius parameters to be determined. To vary the temperature, an external water bath is connected to the rapid-quench box via the inlet and outlet ports shown in Figure 1C. The external water floods the interior of the box and maintains all lines and loops at a constant temperature. The manufacturer indicates that temperatures between 4°C and 70°C can be used, and the temperature is monitored with a digital thermometer, which is inserted into the box via a small port on the left side of the instrument (Figure 1C).

User control of the instrument for time point collection is performed with an associated computerized keypad (Figure 1C). The user inputs the desired reaction time via the keypad and it displays the appropriate reaction loop to achieve that reaction time point. The keypad chooses the appropriate motor speed and delay time between pushes, if necessary, to obtain the desired reaction time. The user manually turns the 8-way valve to the specified reaction loop and ensures that all five valves are in the “FIRE” position. While holding the exit line in a collection tube (described in detail below) in one hand, time point collection is initiated with the other hand via the keypad. Between every time point and after completion of an experiment, the sample lines are flushed via a vacuum pump, which removes leftover reaction samples and cleans the sample lines (Figure 2A).

Prior to using the rapid-quench instrument for data collection, a series of calibrations are performed. These calibrations are done when the instrument is first used, as well as any time tubing is replaced, and are necessary as each rapid-quench instrument varies slightly. The calibrations provide precise volumes for the two sample lines, eight reaction loops, and the exit line. The calibrations also measure the drive syringe volume delivered per step of the motor and the total number of steps necessary to expel the entire reaction sample in the loops. We note that the stepping motor on the KinTek RQF-3 is very precise, having 5,000 steps per revolution. Briefly, for these calibrations, a solution of radiolabeled ATP is used to selectively fill the various lines (or loops) of the instrument. After systematically expelling the liquid of each portion of instrument tubing into collection vials, the volume of each line (or loop) is determined through scintillation counting. The parameters obtained from the calibrations are entered into the keypad. The calibration procedure is described in detail in the KinTek RQF-3 manual. For other models, consult the appropriate user’s manual to determine if calibrations are necessary.

c. Pre-initiation sample preparation—For rapid-quench experiments, it is necessary to prepare each time point separately. Every experiment (set of ~8 time points) necessitates two reaction samples prepared at 300 μ L each. We typically work with a two-piece ribozyme system and pre-anneal radiolabeled substrate with excess enzyme, shown to be saturating in control experiments, in 1 $\times$ buffer. This solution is referred to as a “2 $\times$ RNA” solution and is described in Table 1. We then initiate the reaction with a solution containing 2 $\times$ initiation such as MgCl_2 in 1 $\times$ buffer. This solution is referred to as a “2 $\times$ Initiator” solution and is described in Table 2. Although we use this format most frequently, other ways of initiating the reaction are possible. For instance, the two reaction solutions could be 2 $\times$ enzyme/1 $\times$ buffer/1 $\times$ MgCl_2 and 2 $\times$ substrate/1 $\times$ buffer/1 $\times$ MgCl_2 . Additional formats are possible especially if the aforementioned “push-pause-push-pause-push” format is used, for example with slow binding substrates. The RNA of interest is generally 5'-end-labeled, which is done using [γ - ^{32}P]ATP and polynucleotide kinase (PNK) [34]. Reactions are prepared to allow for a zero time point, a mock time point, and eight reaction time points. The zero time point is performed outside of the rapid-quench instrument. An aliquot of the 2 $\times$ RNA solution is removed just prior to loading of the sample syringe and hand-mixed with quench. The zero time point allows the amount of reacted ribozyme prior to initiation to be calculated. In addition, a mock time point is performed inside of the rapid-quench instrument at the outset of the experiment. It is used to remove potential mixing inaccuracies induced by sample loading and is discarded.

Prior to beginning an experiment, the quench solution is prepared. Typically, the quench volume per time point is on the order of 110 μ L out of a total volume of 150 μ L. To ensure the reaction is fully and rapidly quenched, the concentration of the quench is increased. If no change in reaction rate is observed, then the quench is at an appropriate concentration. Note that the volume of quench delivered by individual instruments is not uniform due to the slight variability of the loops/lines. Therefore, the user must calculate the volume of quench delivered per reaction time point, which is determined from the final volume of sample collected and the volume of both the sample lines. For instance, in our instrument the right and left sample lines are 20 μ L and 19 μ L each while the final sample is 150 μ L, leading to a quench volume of 111 μ L.

d. Setup for collection of experiment time points—When setting up for an experiment, the following materials are needed: four 1 mL disposable, sterile polypropylene syringes for loading the two 2 $\times$ reaction samples at the Sample A and B Load Ports and for cleaning those ports, two 5 mL polypropylene syringes for loading the three drive syringes with water and quench, 500 mL beakers of water and methanol for cleaning, polypropylene collection tubes for each time point (0.65 mL and 1.5 mL both work), and powdered dry ice for freezing time point samples directly after collection. Prior to the experiment, turn on the external water bath and adjust its temperature such that the thermometer inside the box reads the desired value. Allow the system to equilibrate for approximately 30 min. Any flow rate that does not stress the box is acceptable by manufacturer standards. If the flow rate is too high, the box will make an audible noise upon initial filling of the chamber and upon continued use that could potentially damage the plastic box. Note that slower flow rates can result in a higher temperature differential between the bath and box.

Prior to an experiment, prepare the collection tubes for each time point sample. We use Eppendorf tubes with holes bored in the top, as suggested by KinTek. A hand-held pick works well for boring holes in the lid of the tube (see Figure 2B). It is also helpful to have a storage Eppendorf tube (with no hole in the lid) for each sample that contains 2× formamide loading buffer (FLB). This protects the sample from spilling out of the tube and allows direct preparation of samples for loading the gels. Once sample is added to 2× FLB, the samples are placed on powdered dry ice to assure no further reaction occurs. Fill Drive Syringes A and B with water and Drive Syringe C with quench solution. Detailed steps describing this process can be found in the KinTek manual or appropriate user's manual. After the drive syringes are filled, a flush of the instrument is performed to clean the sample lines, reaction loop, and exit line (see "Sample Loading Procedures" in the KinTek RQF-3 manual or appropriate user's manual). The instrument is now ready for sample loading and time point collection.

e. Sample Loading and time point collection—Prepare the 2× RNA and Initiator reaction samples according to Tables 1 and 2, respectively. Next, take the zero time point prior to loading the reaction samples into the instrument. To do this, manually pipet 20 µL from the 300 µL 2× RNA sample into 130 µL of quench solution and add 150 µL of 2× FLB in a sample storage tube. Draw the 2× RNA and Initiator solutions into separate disposable and sterile polypropylene 1 mL syringes and secure to the Sample Load Ports (Figure 1). The instrument is now prepared for loading of sample into the sample lines and collection of experimental time points. Detailed steps to be followed for collection of each time point can be found in the KinTek manual or appropriate user's manual.

f. Cleaning the instrument—The sample lines, reaction loop, and the exit line *must be cleaned between each time point* with water and methanol. Once an entire experiment is complete, sample lines, reaction loop, exit line, and sample load ports should be cleaned with water and methanol, and at the end of the day, the system should be cleaned with additional acid and base steps, which prevent clogging of the lines and help remove biomolecules and potential ribonucleases from the system [35]. We have found that these cleaning steps can make a large difference on the quality of data collected for ribozymes using a rapid mixing stopped-flow instrument also from KinTek. We describe here the full cleaning at the end of the day; the other cleanings are a subset of these.

To clean the instrument after completion of a set of experiments, prepare one 5 mL and five 1 mL disposable and sterile polypropylene syringes, as well as 500 mL beakers of water, methanol, 0.2 M phosphoric acid, and 0.2 M sodium hydroxide. This procedure of cleaning with acid, base, water, and methanol was adapted from the KinTek stopped-flow manual [35], which suggests utilizing even stronger acids and bases, 2 M HCl and 2 M NaOH. However, we find that 0.2 M phosphoric acid and 0.2 M NaOH work well for ribozyme work. Note that if the instrument has not been used for some time, we recommend the 0.2 M phosphoric acid and 0.2 M NaOH cleanings *before* commencing studies. If the rapid-quench instrument has been used with protein samples, cleaning with the higher concentration HCl and NaOH solutions is recommended. To fully clean the instrument, perform the following steps.

- A.** Attach the vacuum pump line to the exit line. Detach the RNA and initiation load syringes from their respective ports and discard them into radioactive waste as appropriate. If it has not been completed already, wash the sample load ports with a 1 mL syringe full of water while the vacuum is pulling (sample syringe and drive syringe valves should all be on “LOAD” for this step to allow the sample load ports to be cleaned while ensuring liquid from the drive syringes is not accidentally pulled by the vacuum as well).
- B.** Press “ESCAPE” on the keypad and when the scroll menu comes up, select Option 2 (“ADJUST POSITION”). Press “-”, and use the start key on the controller to move the drive plate up. Move the plate to within ~1 inch of the red circuit breaker depicted in the inset of Figure 1C.
- C.** Ensure the drive syringe valves are turned to the “LOAD” position. Attach the 5 mL plastic syringes that were used to load the drive syringes back onto the drive syringe load ports and push down on each drive syringe to force any leftover water and quench solution from Drive Syringes A, B, and C into the plastic syringes.
- D.** Wash the water drive syringes and quench syringe with a new 5 mL disposable syringe successively with water, 0.2 M phosphoric acid, water, 0.2 M sodium hydroxide, water, and methanol. This is done by alternating pushes on the attached 5 mL syringes and the drive or quench syringes to move the liquid into and out of the drive syringes.
- E.** Now turn all three drive syringe valves 180 degrees so as to wash the lines connecting the drive syringes to the exit line. The sample valves at the bottom must be on “FIRE” to do this step. Draw water into three 1 mL syringes and place them in the Drive Syringe A, B, and C Load Ports. Turn on the pump with the pump tubing still connected to the exit line and let the vacuum draw the water into the Drive Syringe lines. Repeat the process with 0.2 M phosphoric acid, water, 0.2 M sodium hydroxide, water, and methanol. Turn off the vacuum pump and turn the drive syringe valves back to the LOAD position.
- F.** Turn the RNA and initiation sample valves to the “LOAD” position so that the Sample Load Ports can be cleaned. Draw water into two clean 1 mL syringes and attach them to the Sample Load Ports. Turn on the pump and wash the Sample Load Ports, as well as the sample lines, reaction loops, and exit line, with water, 0.2 M phosphoric acid, water, 0.2 M sodium hydroxide, water, and methanol.
- G.** Turn the RNA and initiation valves to the “FLUSH” position to wash the sample lines, reaction loop(s), and exit line with water, 0.2 M phosphoric acid, water, 0.2 M sodium hydroxide, water, and methanol (the drive syringe valves should still be in the “LOAD” position). This is done by submersing the flush lines into the desired cleaning solvent, which allows the vacuum to pull these solvents through sample lines and reaction loop(s) out through the exit line. If more than one reaction loop was used for completion of the set of experiments, turn the 8-way valve and repeat the first portion of Step G for every other reaction loop utilized.

Before turning off the pump, turn each drive syringe valve 180 degrees from the “LOAD” position to remove any liquid that has made its way into the drive syringe lines. Finally, turn off the pump, detach the pump line from the exit line, and turn all valves back to the “FIRE” position for storage.

- H. Lastly squirt water onto a paper towel and wipe off the exterior of the instrument to prevent any crystallization of chemicals used in the experiment.
- I. At this point, the water bath, thermometer, vacuum pump, controller, and keypad can all be turned off or unplugged.

II. Validating usage and performance of the instrument

Although instruments are tested in the factory to ensure that they mix and quench according to specifications, test reactions are performed on a regular basis to ensure the instrument is calibrated and operating properly. One way to benchmark the rapid-quench instrument is to use a ribozyme reaction that is slow enough to still be performed by hand, say with a half-life of ~30 sec. Time points collected by hand and using the rapid-quench instrument can be directly compared. Figure 3 compares hand-mixed and rapid-quench reactions for the *glmS* ribozyme on the 50 sec and 15 sec timescales. We note that the rapid-quench data are somewhat noisier than the hand-mixing data. However, the differences are slight. When we compare the R^2 value of the single-exponential fit lines for each data set, we obtain values of 0.999 (Figure 3A, hand-mixed) vs. 0.949 (Figure 3A, rapid-quench), and 0.999 (Figure 3B, hand-mixed) vs. 0.961 (Figure 3B, rapid-quench). The R^2 values for the rapid-quench experiments are thus only slightly lower than those for the hand-mixed experiments. One reason the rapid-quench data may be slightly more scattered than the hand-mixed data is that every time point in the rapid-quench data requires loading of new sample, and thus every time point is in essence a separate experiment. We also investigated ten additional randomly selected datasets on the twister ribozyme from our lab to compare scatter in rapid-quench and hand-mixed experiments. We found the average R^2 values for rapid-quench and hand-mixed data to be 0.994 and 0.999, respectively, with standard deviations of 0.006 and 0.001, respectively. We thus conclude that there is little difference overall in data scatter between the two data collection methods. Also of note is that it has been reported that rates of ribozyme self-cleavage can vary by factors of two for reasons that are not understood [36–38], which could explain the slight deviation of the rapid-quench data from the hand-mixed data. Performing additional replicates will lower this type of noise.

If users cannot slow their experiments enough to conduct a hand-mixed experiment, they can make comparisons to experiments conducted by rapid-quench or other fast reaction methodologies, such as stopped-flow [27, 39, 40], or continuous flow-associated techniques [32, 41]. Alternatively, other test reactions are available such as the base-catalyzed hydrolysis of benzylidene malanitrile (BMN), and can be recommended by KinTek upon inquiry through their website.

III. Obtaining adequate amounts of RNA for experiments

Data collection via rapid-quench methods requires ~6-fold more RNA than equivalent hand-mixed reactions. This results from the necessity of performing a separate reaction for each

time point, with each time point requiring a somewhat larger volume of reaction (~40 μ L total). Performing several successive rapid-quench experiments, as is necessary for duplicating experimental conditions, can consume a moderate amount of RNA. Therefore, it is recommended to scale up RNA preparations appropriately. We find that a 2 mL transcription of the enzyme and a 2–3 $\times$ scaling up of the kinase reaction of the substrate for hand-mixed reactions are satisfactory for many days of rapid-quench studies. For obtaining large amounts of RNA, *in vitro* T7 transcriptions are recommended. T7 RNA polymerase is the most commonly utilized RNA polymerase due to its efficiency and low cost [42]. The error rate of T7 RNA polymerase is only about one error per 10^4 – 10^5 nucleobases and it can produce RNAs hundreds of nucleotides long from either plasmids or PCR products [43]. The polymerase can be purchased ready to use (e.g. Thermo Fisher Scientific) or purified from *E. coli* [44]. The RNA of interest is transcribed from DNA comprised of two strands: a hemi-duplex with a T7 promoter and template strand, or a fully double-stranded version of this DNA. The latter is helpful for highly structured DNAs and should not need to be renatured prior to transcription, although enough (50–100 mM) monovalent salt should be present to favor base pairing. To produce enough RNA for rapid-quench kinetics, either plasmid preparations or PCR of existing DNA templates are useful. Alternatively, RT-PCR can be used to prepare a dsDNA template from an *in vivo* RNA preparation. There are helpful publications in the literature that describe ways to optimize both DNA yields for transcription templates and RNA yields from transcription reactions [45–47].

Briefly, T7 transcription is an inexpensive and efficient means of producing large amounts of RNA, but not all templates transcribe similarly [42]. One of the most important considerations for RNA yield is the start sequence, and G-rich sequences are preferred [42]. For templates that transcribe poorly, it is highly recommended to use PCR or plasmid preparations of DNA template, as mentioned above, to make dsDNA. Pilot transcriptions are recommended on a case-by-case basis in which the concentrations of DNA, Mg^{2+} , NTPs, and polymerase are varied [42]. Following *in vitro* transcription, purification of the transcription mixture is required to remove NTPs, template, and aborted RNAs. The most common method of purification is through denaturing polyacrylamide gel electrophoresis (PAGE) followed by a crush and soak recovery. Electroelution can outperform crush and soak and so should be also considered for elution. Native purification methods may be preferable, as outlined in the following section.

IV. Optimization for monophasic, complete, and fast reactivity to give a well-behaved RNA system

Our lab has made extensive efforts to obtain ribozyme preparations that are well-behaved kinetically, as such behavior typically reflects a homogeneous, natively folded, and well-poised system for reaction. This makes interpretation of the data more straightforward. We attempt to obtain reaction profiles that are fast (typically meaning millisecond timescale for ribozymes), can be fit to a single exponential (as opposed to multiple exponentials that typically reflect more than one fold), and react to completion (thus minimizing the amount of inactive ribozyme). Obtaining such ribozyme preparations often means exploring diverse renaturation conditions, solution conditions, and RNA sequences. We have reported such well-behaved ribozyme preparations for the HDV [21] and the *glmS* [20] ribozymes. The

former led to rapid crystallization of the ribozyme with catalytically relevant structures [40], while the latter provided in-solution small angle X-ray scattering (SAXS) profiles that agreed with crystal structures [18, 21]. Once a well-behaved ribozyme system is obtained, the RNA can be mechanistically investigated through experiments involving thio effects, metal ion rescue, and solvent isotope effects. There are many examples of studies from our lab and others that detail these types of experiments [21, 22, 48–54].

The first consideration for rapid-quench experiments is the RNA system to be studied. We consider the reactivity of specific constructs and their propensity to misfold, as well as the need for chemical modifications, which generally require a two-piece ribozyme system. If designing a two-piece ribozyme system, it is crucial to choose a point of scission within the sequence that won't interfere with folding or reactivity and to assure that the enzyme is saturating for single-turnover transient kinetics. Furthermore, once the construct is made, its reactivity must be compared to that of the wild-type ribozyme. In addition to the construct design, one must consider whether it is best to radiolabel throughout the body of the RNA or only on the 5' end. Typically, labeling on the 5' end gives higher specific activity and is more easily quantitated to obtain kinetics data, thus serving as the more attractive candidate for rapid-quench RNA preparation.

One way to monitor a ribozyme reaction without the use of a rapid-quench instrument is to slow the reaction so that it is measurable in hand-mixed reactions. However, this approach could change the mechanism. For instance, a folding step can become rate-limiting when the Mg^{2+} concentration is lowered or if the ribozyme is not given time to fold before reaction initiation [55]. Conducting the reaction under fast, biological conditions may speed up folding and conformational changes allowing chemistry to be rate limiting [21]. This approach is useful for promoting the chemical step to be rate-limiting, although not guaranteeing it.

In order to increase the likelihood that rate constants report on the chemical step, it is important to obtain RNAs that react in a monophasic, complete, and fast manner. There are several factors that can affect reactivity. Transcribed RNA can be purified in a native or denaturing manner, which can influence folding during renaturation (Figure 4A). Typically, transcribed RNA is purified on a denaturing polyacrylamide gel followed by excision, elution into buffer, and ethanol precipitation. However, sometimes it may be desirable to keep the RNA folded just as it is folded directly after transcription [56]. Such a native purification consists of purification on a native polyacrylamide gel followed by excision, elution into buffer, and ethanol precipitation. With native purification, the RNA fold present after transcription can be preserved, which should be tested via analytical native gels.

Another factor to consider is the storage. Typically, RNA samples are stored at $-20^{\circ}C$ to minimize hydrolysis. However, with some RNA samples, storage at this temperature can result in stubborn misfolds and multimers. In addition, RNA that is purified natively may not stay natively folded if stored at $-20^{\circ}C$ necessitating use in experiments directly after purification, without storage. Alternatively, RNA can be stored at $-80^{\circ}C$ if there is suspicion that the RNA is degrading at $-20^{\circ}C$. Storage conditions should be tested on a case-by-case

basis. Frequent purity tests by PAGE are recommended to ensure that the RNA is not degraded (denaturing PAGE gels) and remains folded natively (native PAGE gels).

A major contributor to RNA kinetics is renaturation. Volume, temperature, time, and the order in which reaction components are added can all affect how the RNA folds during renaturation. We have found that lower RNA renaturation concentrations lead to lower amounts of aggregate formation, according to SAXS. Furthermore, temperature plays a large role in the folding of RNA. During renaturation, a high-temperature denaturation step is desirable to break any strong intra or intermolecular structure that the RNA has adopted during purification or storage. We have found that a renaturation procedure that caters to both secondary and tertiary structure folding conditions optimizes RNA folding [21]. For example, a natively-folded *glmS* ribozyme RNA that reacts in a monophasic, complete, and fast manner (Figure 4B) can be obtained by the following steps. The RNA is first heated at 95°C for 3 min followed by snap-cooling on ice for 10 min in buffer and monovalent salt; presumably these steps allow ample time for secondary structure to form. Upon addition of Mg^{2+} , another heating step at 55°C for 3 min followed by room temperature incubation for 10 min allows the RNA tertiary contacts to fold. Other RNA molecules might require a slightly different renaturation; however, this renaturation procedure may serve as a general guide. Our goal is reactivity in a fast, monophasic, and complete manner, as this simplifies interpretation of the data. One can test if the rate-limiting step involves chemistry, as chemical steps often have thio effects and a pH-dependence.

Another crucial consideration for ensuring well-folded RNA is the solution conditions. Temperature can have a large effect on RNA folding. Low reaction temperatures can cause misfolding or aggregation, while high reaction temperatures can induce partial unfolding or breathing of RNA structures. Thus, an intermediate reaction temperature near the *in vivo* temperature is recommended. Furthermore, divalent metal ions can significantly affect reaction rates as can molecular crowders. Suboptimal concentrations of divalent metal ions can cause incomplete formation of tertiary structure, whereas excessive concentrations can promote RNA aggregation [25]. Molecular crowders can often accelerate reaction conditions [24–26]. In addition, buffer concentration and identity, pH, and RNA concentrations are important to consider. Ensuring that all reaction components that affect the rate constant are at saturating concentrations is also necessary to simplify data interpretation (Figure 4C).

In order to confirm that the above procedures result in a monomeric and natively-folded RNA species, we also recommend testing structural properties. Heterogeneity of the preparation can be assessed by loading renatured RNA samples on native gels or testing them via fast-performance liquid chromatography (FPLC). Ideally, samples will be monodisperse, as structural homogeneity is less likely to give biphasic kinetic profiles. Dynamic light scattering (DLS) or preferably small-angle X-ray scattering (SAXS) can be used to assess aggregation and overall folding (Figure 4D). From DLS, molecular weight distributions can be obtained, while SAXS can afford this as well as overall shape, radius of gyration (R_g), maximum dimension (D_{max}), excluded particle volume, and flexibility. These can be compared to published crystal structures of the RNA. In addition, it may be useful to obtain nucleotide-resolution structural information. In-line probing tests the folding of an RNA on the nucleotide level. Through the comparison of in-line probing patterns for

different reaction conditions, folding between these different conditions can be compared to SAXS data and crystal structures [57, 58]. Once folding and reactivity of the RNA sample are optimized, reactions can be performed on the rapid-quench instrument to probe the chemical step.

Other general comments regarding working with ribozymes, or RNA in general, on the rapid-quench instrument include proper handling of the RNA samples so as to not cause degradation of the RNA. Working with gloves is essential for experimental RNA work so that RNases from skin are not transferred to RNA solutions. Ideally, the RNA should not sit for long periods of time on the benchtop while preparations are being made for an experiment. We tend to make sure the RNA is only thawed for renaturation when the instrument is set up and ready to run. Furthermore, we ensure that all syringes, tubes, and sample components that come into contact with the RNA are sterile (and usually autoclaved before use in experiments). If there is uncertainty regarding the presence of RNases on the rapid-quench instrument, which can cause RNA degradation, RNaseZap (Thermo Fisher Scientific) can be used to remove them. If the rapid-quench is being used for protein experiments in addition to RNA experiments, a sodium hydroxide or detergent wash should be performed prior to introduction of the RNA samples to the instrument.

V. Troubleshooting

a. Instrument maintenance—Algae growth can occur inside both the water bath and plexiglass rapid-quench box, which can result in blockages and decrease the visibility for sample loading. To prevent algae growth, add an algacide such as chloramine T sodium salt dehydrate to the distilled water in the bath, according to the manufacturer's specification. If the instrument will be unused for more than a week, drain the plastic box by removing the upper inlet tube. It is also prudent to change the distilled water in the bath every few months. If algae does grow in the box, add a low percentage bleach solution to the water bath and circulate for 30 min. Drain both the box and the bath and refill with distilled water and circulate.

b. Instrument Modifications for Radioactivity Safety—One of the most common ways to monitor ribozyme reactions is with RNA end-labeled with ^{32}P . All work should be conducted behind a plexiglass shield. Use the plexiglass loading port adaptor from KinTek (Figure 5A) to provide additional shielding from the radiolabeled sample. The adaptor covers a majority of the syringe and is secured to the sample loading port by a set screw.

c. Tips for loading samples—Bubble formation in the circulating water within the box is a common occurrence, especially at temperatures of $\sim 30^\circ\text{C}$ or higher. The presence of air bubbles will not directly cause problems but can be a nuisance if they form on the sample loading lines. Air bubbles that form between the sample syringe and the 8-way valve can make it particularly difficult to ascertain how much sample has been loaded, which can lead to inaccurate loading volumes. To minimize bubble formation, raise the temperature of the water bath $10\text{--}20^\circ\text{C}$ above the required experimental temperature and allowing it to remain there for some time (30 min to an hour) before bringing it back down to the experimental

temperature. The increased temperature decreases the solubility of gases and removes most of the air bubbles present before an experiment is begun.

As most solutions are colorless, it can be difficult to determine how much sample has been loaded into the sample lines even in the absence of air bubbles. We have found that placing a sheet of printer paper, white or colored, behind the rapid-quench box increases the contrast between the sample and the tubing. Some newer models of rapid-quench instrumentation do have a colored backing behind the box. We have also found that illuminating the inside of the box with a secured flashlight or a small desk light helps in monitoring the amount of sample added. If a piece of printer paper or other semi-translucent material is used as the backing, the user can shine the flashlight through the back of the box to illuminate the entire box.

With a standard 1 mL syringe, it is very easy to load too much sample into the sample loops via the sample load ports. Since injection of too much sample results in the 2× reaction solutions entering into the reaction loop prior to the start of the experiment, this mistake could prematurely mix the 2× reaction solutions together, leading to an incorrect (too high) reactivity for the time point sample. The only way to deal with a sample that has been loaded too far is to clean the loops and start again, meaning that the overloaded sample is thrown away. To prevent the loss of sample from overloading, we have adapted the following technique for loading. We have found the best way to load 2× reaction solutions is to grasp the plunger of the syringe with the thumb and index finger with these two fingers resting up against the edge of the barrel, as shown in Figure 5A. To inject the reaction samples, the thumb and index fingers are rolled towards the instrument to gently guide the plunger further into the barrel by a small amount. Resting these two fingers against the edge of the barrel allows for finer control over the amount of force used to push the plunger.

d. Sample loss prevention—As discussed above, the quench and sample are forced through the lines via a mechanical motor. This force can result in samples spraying out of the tubes. To avoid loss and spraying of radioactive samples, bend the exit line slightly, as seen in Figure 5B, using your index finger to cover the hole. Bending of the line decreases the force of the sample as it hits the collection tube, reducing the chance of the sample spraying out of the tube.

Sample loss can occur when washing out the lines after a time point. Small mistakes can result in loss of 2× Initiator and RNA solutions to the vacuum. Before turning on the vacuum, ensure that the sample load valves are in the “FLUSH” position, as any other position will expose the solutions to the vacuum. In other words, if the sample load valves are in the “LOAD” or “FIRE” positions, the vacuum will pull your unused samples into the waste. The same situation can occur when the vacuum is turned off if the sample load valves are turned back to the “LOAD” or “FIRE” positions *before* the vacuum pump connecting tube is removed from the exit line. Since these mistakes can cost the user the entirety of their 2× solutions, be hypervigilant on the sample load valve positions before and after the vacuum pump is used.

e. Signal-to-noise—As outlined in the “Pre-Initiation Sample Preparation” section, most rapid-quench time points utilize a large volume of quench, thereby lowering the RNA concentration. For our instrument, ~40 μL of reaction is diluted to 150 μL when quenched and another 2-fold when added to 2 $\times$ formamide loading buffer. Thus, each rapid-quench reaction is 7.5-fold less concentrated than an equivalent hand-mixed reaction. Signal thus tends to be lower compared to equivalent hand-mixed reactions. To counter this, freshly radiolabeled RNA will help, as will higher concentrations of limiting labeled RNA. Likewise, fluorescently labeled RNA should be at higher concentrations.

As outlined previously, performance of the instrument and user should be validated by comparison to ribozyme rates measured by other means. If rapid-quench reactions are faster than expected, ensure that the quench solution is sufficiently concentrated (see above). Similarly, for reactions that occur slower than expected, check that the RNA has not degraded. In all cases where rates deviate from expected, the calibration of the instrument should be checked according to the manual. Additionally, it is important to ensure the user selects the proper loop on the 8-way valve as incorrect loop selections can result in inaccurate reaction rates.

VI. Features of Other Commercially Available Rapid-Quench Instruments

As previously mentioned, the KinTek RQF-3 is not the only rapid-quench instrument on the market. Two additional rapid quench systems that are available include the RQF-63 from TgK Scientific and the QFM-4000 from BioLogic. Although the overall functionality of these systems is the same, there are several differences between the systems that may make one system more preferable to an individual user.

For example, TgK Scientific’s RQF-63 features a reaction loop system where the reactions loops are physically removed and replaced by the user to vary the reaction times. This is different from the KinTek RQF-3 where the reactions loops are encased in the plexiglass box and changed via the 8-way valve. The system has been used with several enzymatic systems as highlighted in various studies listed on the TgK website.

Similarly, BioLogic’s QFM-4000 has several features that differentiate it from the previously mentioned systems. For example, the QFM-4000 utilizes a single reaction loop to conduct reactions with a time range from 4 ms to 10 seconds [59]. Additionally, the reactions require only 10 to 20 μL of sample per reaction. Similar to the KinTek RQF-3, the BioLogic QFM-4000 system uses a water bath circulator to control the temperature of the drive syringes and mixing chambers. The user can independently control the temperature for each reaction syringe, keeping it lower for temperature-sensitive samples, and can also pre-incubate the samples at the reaction temperature for a brief time prior to the start of the experiment. This ensures that temperature-sensitive samples are at the proper reaction temperature during initiation while preventing degradation that would result from excessive incubation. Also the QFM-4000 system can be converted to the SFM-4000, a stop-flow system, with additional parts that are purchasable from BioLogic. Conversely, the SFM-4000 can also be converted to the QFM-4000 with additional parts purchasable from BioLogic. All three rapid-quench instruments can be moved to the glove box for anaerobic conditions if necessary.

Conclusions

This article discussed approaches for measuring ribozyme reactions on the millisecond to second timescale using the rapid-quench instrument. Methods for designing the experiment, acquiring data, and interpreting results were provided. RNA-specific approaches for cleaning the instrument and obtaining large amounts of well-behaved RNA constructs were provided, as were approaches for benchmarking the reactions and troubleshooting problems. With proper attention to detail, it is straightforward to obtain high quality ribozyme kinetics data on affordable, commercially available rapid-quench instruments. This opens the door to determining ribozyme reaction mechanisms under fast-reacting biologically relevant reaction conditions.

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- Rapid-quench is a useful technique for probing fast (<5 s) ribozyme reactions.
- Probing the reaction with rapid quench requires a well-behaved RNA system.
- Guidelines and considerations are provided for sample preparation and experiment.
- Troubleshooting for general rapid-quench procedures and issues are given.

Figure 1.

KinTek RQF-3 instrument. (A) Schematic highlighting connections of components. Colors denote which reagents pass through the lines during the course of an experiment including H₂O (light blue), quench (red), reaction mixtures (dark blue and yellow), combined reaction mixtures (green) and the quenched reaction (brown). Reaction loops, which are different than sample lines, are located in the 8-way valve (see panel B). Sample Load valves are three-way valves, with the top portion of the “T” perpendicular to the line shown. Adapted from Chemical-Quench-Flow Model RQF-3 and the Model RPL-3 Rapid Photolysis Chamber Instruction Manual [60]. (B) Photo of the box that houses the drive syringes and sample lines. (C) Photo of instrument mounted on a bench top set-up with other components.

Figure 2. Instrument cleaning setup and collection tube preparation. (A) Vacuum pump connected to the exit line for flushing sample loops and for general cleaning procedures. (B) Example of a sample collection tube with a hole bored in the top. Also shown is the boring tool, a hand-held pick. Gloves should be worn at all times, both for the sake of working with radioactivity and working with RNA.

Figure 3.

Instrument performance validation. Benchmark experiments with time points collected in a hand-mixed experiment (black) and on the rapid-quench instrument (blue) for the *glmS* ribozyme under (A) slower and (B) faster self-cleavage conditions. Time points agree suggesting that instrument collection times are reliable.

Figure 4.

Ensuring a well-behaved ribozyme system. (A) Denaturing and native gel analysis of ribozyme enzyme strand species purified either on a denaturing gel (denaturing-purified enzyme, 'E') or native gel (natively-purified monomer, 'M', and natively-purified dimer, 'D'). (B) Reactivity of a ribozyme construct where conditions were optimized to give monophasic, complete, and fast reactivity. Data were collected by rapid-quench technique. (C) Control experiments showing that the small molecule GlcN6P is saturating (left) and that the enzyme concentration chosen for experiments (indicated by a black arrow) is high enough to bind all the substrate (right). Data were collected by rapid-quench technique. (D) Small-angle X-ray scattering experiments (SAXS) show that the ribozyme shape in reaction conditions (blue envelope) matches the shape of the ribozyme crystal structure well (PDB 2NZ4). Data presented in this figure are adapted from a previous publication from our lab [21].

Figure 5. Miscellaneous rapid-quench instrument modifications. (A) Plexiglass loading port adaptor. Note the technique for holding the plunger of the 1mL syringe holding the 2× radiolabeled RNA. (B) Exit line of the rapid-quench instrument placed into a sample collection tube via a hole (not visible) in the cover of the tube. The exit line is slightly bent to prevent sample from spraying out of the collection tube.

Table 1

2× RNA reaction solution example

Reagent	Concentration in 2×-reaction solution	Final reaction concentration
1× NaCl	100 mM	100 mM
1× Buffer ^I	30 mM	30 mM
2× Ribozyme enzyme strand	200 nM	100 nM
2× Radiolabeled ribozyme substrate strand	0.5 nM	0.25 nM

^IIncludes Good buffers such as MES, HEPES, TRIS, and CHES.

Table 2

2× Initiator reaction solution example

Reagent	Concentration in 2× reaction solution	Final reaction concentration
1× NaCl	100 mM	100 mM
1× Buffer ^I	20 mM	30 mM
2× MgCl ₂	30 mM	10 mM

^IIncludes Good buffers such as MES, HEPES, TRIS, and CHES.