Time-Resolved Hydroxyl Radical Footprinting of RNA with X-Rays

This unit describes the use of a synchrotron X-ray beam to carry out hydroxyl radical footprinting of RNA. The cleavage reactions typically require 10 to 50 msec of exposure to the X-ray beam. Hence, this method is suitable for probing kinetic folding intermediates or other transient conformational states that appear on the 50-msec to 100-sec timescales. The protocols given here were developed to probe the folding pathway of the *Tetrahymena* ribozyme (Sclavi et al., 1997, 1998a), but are applicable to a variety of catalytic RNAs or RNA-protein complexes. A facility for X-ray footprinting is operated by the Center for Synchrotron Biosciences at beamline X28C of the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory.

Equilibrium experiments are conducted by exposing the RNA to the X-ray beam using a sample holder and electronic shutter (see Basic Protocol 1). Kinetic experiments are carried out using a commercial rapid mixing apparatus modified to withstand high-flux X-radiation (see Basic Protocol 2). RNA folding reactions are initiated by rapidly mixing the RNA with buffer containing $MgCl_2$. After a defined interval, the RNA is pushed into a flow cell in the X-ray beam and irradiated for a short time. Cleavage products are collected and analyzed by polyacrylamide gel electrophoresis (see Basic Protocol 3).

CAUTION: Prospective users must inform themselves of the training and safety requirements at individual synchrotron facilities. The following procedures should only be performed by personnel who have been trained in the operation of X-ray beamlines and the use of radioactive materials, and received authorization by the operating facility. Standard precautions to minimize exposure to β rays and prevent radioactive contamination of personnel and equipment should be followed at all times.

NOTE: Care must be taken to avoid introducing ribonucleases into the samples. This can usually be achieved by purchasing good-quality machine-packaged disposable plasticware, using gloves while handling samples and preparing solutions, and storing disposables in clean, dust-free containers. All solutions should be prepared with deionized water (18 MΩ) that is free of pyrogens and organics (e.g., tissue culture grade). Although less desirable, water treated with diethylpyrocarbonate (DEPC; *APPENDIX 2A*) may be used if a source of nuclease-free water is not available. Solutions should be sterilized by filtration (0.2-μm filter) or autoclaved.

NOTE: Nucleic acid or protein samples within the experimental hutch (except the sample in use) should be placed in a lead-lined container before activating the X-ray beam, to guard against degradation due to scattered radiation.

STRATEGIC PLANNING

These experiments require access to a synchrotron X-ray beamline that has been suitably modified for footprinting studies. Requirements for the beamline configuration are discussed briefly at the end of this unit and elsewhere (Sclavi et al., 1998b; Ralston et al., 2000b). Protocols in this unit describe experiments conducted at beamline X28C at the NSLS, using equipment available to beamline users. Information regarding facilities maintained by the Center for Synchrotron Biosciences at the NSLS can be obtained at *http://www.aecom.yu.edu/home/csb/*.

Contributed by Sarah A. Woodson, Michael L. Deras, and Michael Brenowitz
Current Protocols in Nucleic Acid Chemistry (2001) 11.6.1-11.6.24

Synchrotron experiments must be planned 2 to 6 months in advance to allow users sufficient time to complete safety and equipment training before beginning work. Advance planning is particularly important if radioactive materials are to be used. It is essential to ensure that the required equipment is available and, if necessary, to allow for construction or installation of additional equipment.

Before undertaking experiments at the synchrotron, it is important to determine whether the RNA or RNA-protein complex of interest can be studied effectively by hydroxyl radical footprinting. This is best determined by conducting preliminary experiments with Fe(II)-EDTA, which also generates hydroxyl radicals (*UNIT 6.5*). The cleavage patterns induced by Fe(II)-EDTA and X-rays are indistinguishable, although Fe(II)-EDTA reactions proceed more slowly. The experimental conditions (i.e., temperature, Mg^{2+} concentration, and protein concentration) should be adjusted to maximize the extent of protection. In addition, one should determine whether the transition is complete within 60 sec. Conformational changes that take longer than 60 sec are more easily probed by chemical methods (see Commentary).

It is recommended that one begins with X-ray footprinting experiments under equilibrium conditions (see Basic Protocol 1). These experiments can be used to confirm that the optimum conditions have been selected, and establish the equilibrium parameters of the transition. The required X-ray exposure time for the sample must be determined from a dose-response curve and consideration of the current in the synchrotron ring at the time of the experiment (see Support Protocol 2). This information is indispensable in planning time-resolved experiments (see Basic Protocol 2).

BASIC PROTOCOL 1

EQUILIBRIUM X-RAY FOOTPRINTING OF RNA

This protocol is useful for performing titrations with Mg^{2+}, Na^{+}, urea, or protein. A temperature-controlled aluminum block is used to hold individual samples in the X-ray beam. An electronic shutter controls the exposure time.

Materials

^{32}P end-labeled RNA in 6-µCi aliquot (see Support Protocol 1)
CE buffer, pH 7.5 (see recipe) or other appropriate buffer (see Critical Parameters)
1 M $MgCl_2$ (*APPENDIX 2A*; optional)
Precipitation cocktail (see recipe)
100% ethanol

Aluminum sample holder with electronic shutter and support stand (see Commentary, Figure 11.6.1)
Support table
Controller cable
Detector for the automatic vertical alignment device
Refrigerated recirculating bath with attachment tubes
1.5-mL and 0.5-mL microcentrifuge tubes with captive screw caps and O-ring seals (Rainin)
Linagraph paper (Kodak)
Masking tape
Temperature-controlled heating block or water bath
Lead sample box

CAUTION: Cacodylic acid is an arsenic compound and is toxic.

Set up shutter and sample holder

1. Install the shutter and sample holder assembly so that the sample tube is aligned with the X-ray beam (Figure 11.6.1). At beamline X28C, bolt the stand assembly to the support table, and connect the controller cable to the shutter. Attach the detector for the automatic vertical alignment device to the sample holder and plug in its output cable. Check that the shutter and stand are aligned with each other and horizontally aligned with the end of the beampipe.

2. Attach the tubes from a refrigerated recirculating water bath to the aluminum sample holder. Turn on the water bath and adjust the temperature as desired.

   Water should enter the bottom port on the sample block and exit the top port.

3. Extend the retractable flight tube from the end of the beampipe to the face of the shutter assembly.

   The flight tube reduces scattered X-ray radiation (see Commentary).

4. Engage the beamline interlock system, exit the hutch, and enable the X-ray beam.

   Experimental hutches at the NSLS are equipped with an interlock system to prevent accidental exposure of personnel. Procedures for enabling the beam are established by the synchrotron facility administration.

5. Perform the semi-automatic alignment protocol according to instructions on the Center for Synchrotron Biosciences website (*http://www.aecom.yu.edu/home/csb/*). Shut off the beam, and place the shutter in a closed position.

6. Confirm alignment of the beam manually by covering the sample tube hole with linagraph paper and masking tape. Activate the beam, and expose the paper for 1 sec.

   The burn mark on the paper should be centered over the hole, and should cover the area that is occupied by the sample.

Prepare RNA samples

7. Thaw one 20-µL aliquot of ^{32}P end-labeled RNA (6 µCi) at room temperature. Microcentrifuge 15 sec at maximum speed.

   RNA samples should be prepared and shipped to the beamline a day in advance (see Support Protocol 1). Store at –20°C or on dry ice in a lead container when not in use.

8. Add 30 µL of the appropriate buffer (typically CE buffer) to each aliquot, bringing the total volume per aliquot to 50 µL. Vortex the samples and microcentrifuge 15 sec at maximum speed.

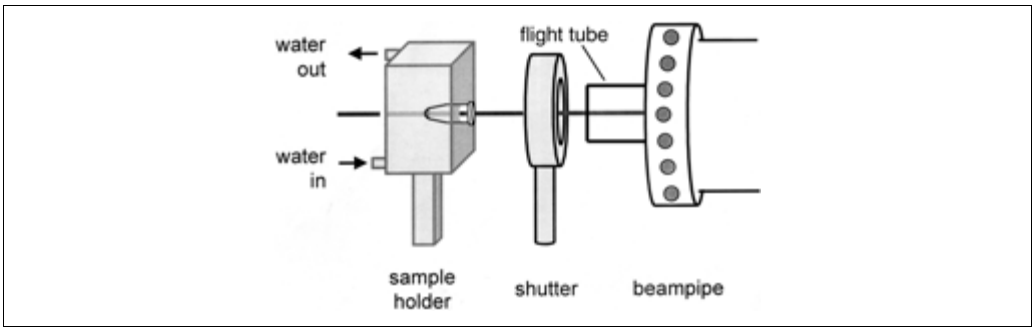

Figure 11.6.1 Stand with aluminum sample holder and automatic shutter for equilibrium footprinting experiments. Adapted from Ralston et al. (2000b) with permission from Harcourt.

9. Label twenty 0.5-mL microcentrifuge tubes. Add 2.5 µL RNA from step 8 to each tube.

10. Add the desired buffer or reagents (2.5 µL) to each tube.

    For example, to measure the Mg^{2+}-dependence of RNA folding, prepare a series of solutions containing 2× $MgCl_2$ in CE buffer. Add 2.5 µL of 2× $MgCl_2$ to eighteen samples. Add 2.5 µL of CE (no Mg^{2+}) to two samples to serve as unfolded controls.

    The total volume of each sample should not exceed 5 µL, to ensure that the entire sample remains within the cross-section of the beam.

11. Anneal the RNA by incubating the samples at the desired temperature until the folding reaction has reached equilibrium, or by heating 1 to 2 min at 85° to 95°C and cooling to the desired temperature.

    As an example, the Tetrahymena ribozyme should be incubated 20 min at 42°C or 2 to 4 hr at 30°C.

    Prolonged incubation of RNA at temperatures >50°C should be avoided to minimize hydrolysis.

Expose RNA to X-ray beam

12. Set aside one of the samples without $MgCl_2$.

    This sample will not be placed in the X-ray beam and will serve as an unexposed control.

13. Place one tube in the sample block with the large end facing the beam.

14. Interlock and exit the hutch. Turn on the X-ray beam, and then activate the shutter to expose the sample. Turn off the X-ray beam once the exposure is complete.

    Each sample should be exposed for the same time. Exposure times are typically 10 to 50 msec at X28C, depending on the beam current. The optimal time should be determined from a dose-response experiment (see Support Protocol 2).

15. Re-enter the hutch and remove the sample from the holder. Add 15 µL distilled water, 5 µL precipitation cocktail, and 75 µL of 100% ethanol. Store the sample in a lead box.

16. Repeat steps 13 to 15 until all the samples have been irradiated.

Clean up

17. Store samples at −20°C or on dry ice in a lead container.

18. Turn off and disconnect the water bath and the shutter controller.

19. Dismount the sample holder and shutter, and store the entire assembly in a secure place.

20. At the end of the experiments, complete all safety checks and disable the beamline.

BASIC PROTOCOL 2

TIME-RESOLVED X-RAY FOOTPRINTING OF RNA

This protocol describes a standard method for time-resolved X-ray-dependent hydroxyl radical cleavage of RNA. A Kin-Tek rapid mixing device is used to initiate the reaction of interest (such as Mg^{2+}-induced folding). After a programmed delay, the RNA is pushed into a flow cell and exposed to the X-ray beam. A progress curve is produced by varying the delay time. Samples are processed and analyzed as described in Basic Protocol 3.

Materials

CE buffer, pH 7.5 (see recipe) or other appropriate buffer (see Critical Parameters)
2 to 8 μCi ^{32}P end-labeled RNA, divided between two 20-μL aliquots (see Support Protocol 1)
100% ethanol
Precipitation cocktail (see recipe)
CE20 buffer (see recipe)
Additional buffers or salts as desired (see Critical Parameters)
0.6 μCi RNA for prefolded controls (see Support Protocol 1)

Rapid-quench apparatus, modified for X-ray footprinting experiments (e.g., RQF-3, Kin-Tek; see Commentary, Figure 11.6.2, and Support Protocol 3)
1-mL and 5-mL Luer-lok disposable syringes
Temperature-controlled heating block or water bath
1.5-mL microcentrifuge tubes with captive screw caps and O-ring seals (Rainin)
13-G needles
15-mL sterile disposable culture tubes
Lead-lined box
Refrigerated recirculating water bath

NOTE: It is important to become familiar with the operation of the rapid-quench apparatus (Figure 11.6.2) and the valve settings illustrated in Figure 11.6.3 (LOAD syringes, LOAD sample, FIRE, and FLUSH). For RNA folding experiments, drive syringe B is loaded with buffer (e.g., CE or CE20 buffer), and the RNA sample is placed in the bottom right sample loop. Drive syringe A and the bottom left sample loop are loaded with CE or CE20 buffer. The third Quench syringe (C) is not used in the standard footprinting protocol. The plunger for the C syringe should remain fully depressed.

CAUTION: Care must be taken to protect personnel from β radiation and prevent contamination of work area. Gloves, equipment, and work surfaces should be frequently monitored using a hand-held survey meter. Safety procedures pertaining to use of the beamline, such as the interlock system, must be observed at all times. Caution should be used in handling the waste container, sample syringe port, and exit line, which may be contaminated with radioactive material.

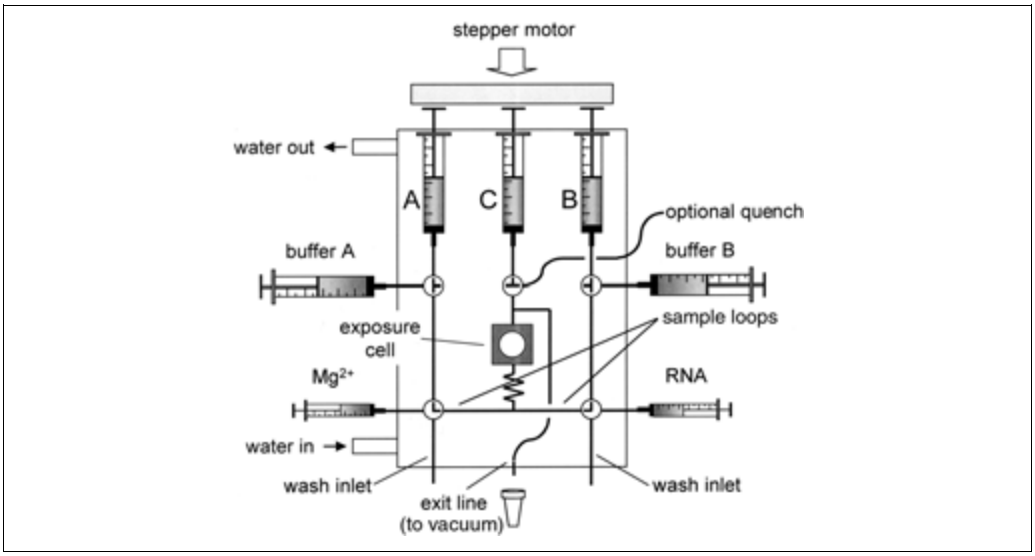

Figure 11.6.2 Modified rapid-quench apparatus for time-resolved footprinting. Adapted from Sclavi et al. (1998b) with permission from Harcourt.

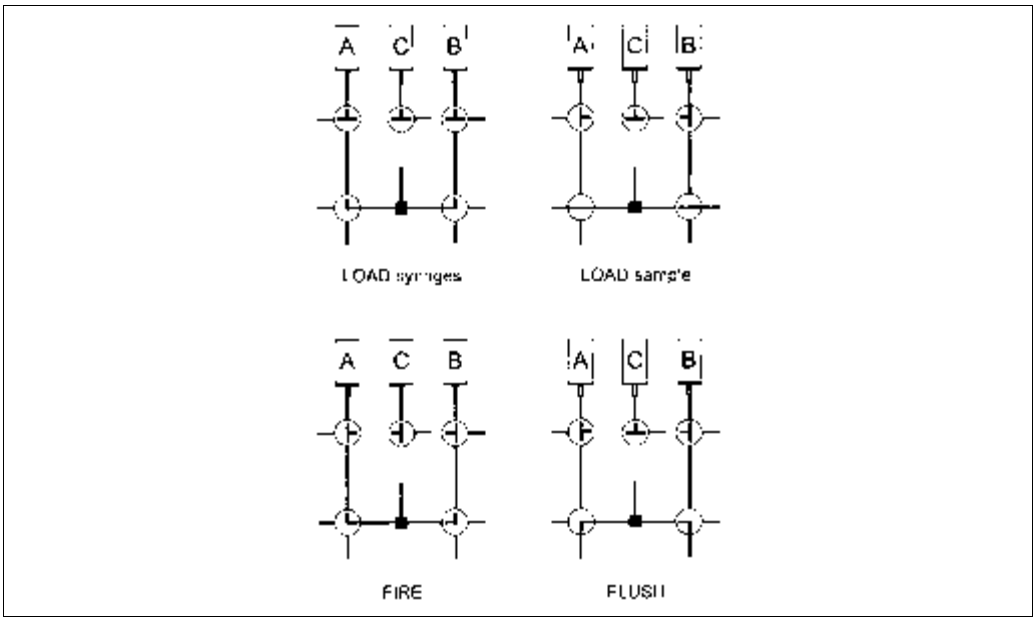

Figure 11.6.3 Valve settings for Kin-Tek rapid chemical quench. Adapted with permission from manufacturer's manual.

Adjust drive platform

1. Set up the rapid-quench apparatus as described in Support Protocol 3. Open a software routine that allows one to adjust the position of the drive platform (option 2 in the control software).

2. Use the remote actuate (small red) button on the stepper-motor to raise the platform.

   The platform should just contact the plungers of drive syringes A and B when they are completely filled. One may calibrate and mark the desired starting position for the drive platform in advance.

Load drive syringes

3. If using a Kin-Tek apparatus, turn the top row of valves to the LOAD syringe position, and inject 1× CE buffer using a 5-mL disposable syringe. Work the solution back and forth to remove bubbles. Fill the A and B drive syringes in the same manner.

4. Turn the top valves so they are aligned vertically (FIRE). Remove the 5-mL disposable syringes.

Anneal RNA

5. Thaw one 20-µL aliquot of ^{32}P end-labeled RNA (1 to 4 µCi) at room temperature. Mix gently.

6. If desired, anneal the RNA by incubating 1 min at 95°C in a heating block. Microcentrifuge 15 sec at maximum speed.

7. Add 120 µL of 1× CE buffer to the sample (140 µL final volume). Mix well (vortex) and microcentrifuge briefly at maximum speed.

   This yields enough sample for a "priming shot" and nine experimental trials.

Load sample syringes

8. Using a 1-mL disposable syringe, pull the plunger back and draw in 0.3 mL air. Then, draw up the entire diluted RNA sample prepared in step 7.

The final drop of RNA solution can be transferred to the syringe tip using a pipet. The air in the syringe will be used to push the entire sample into the sample loop of the rapid-quench apparatus.

9. Turn the two bottom sample loop valves to the LOAD sample position. Attach the RNA sample syringe to the right sample port, and fill the tubing between the port and the valve. Leave the 1-mL syringe in place, as it will be used for subsequent experiments.

   The top valves should be in the FIRE position. Be sure that the vacuum line is disconnected from the exit tube.

10. Using a 1-mL syringe, fill the left sample loop with CE buffer.

11. Turn the bottom valves to the FIRE position.

    All valves should be in the FIRE position, except for drive syringe valve C, which remains in the LOAD position.

12. Remove the cap from a spare 1.5-mL screw-cap microcentrifuge tube and punch a hole through the cap using a 13-G needle. Thread the exit line of the quench-flow through the cap (it should fit snugly). Screw an empty microcentrifuge tube onto the exit line adapter, and place it in a tube rack on the aluminum shelf under the rapid-quench apparatus.

    This tube will be used to collect waste.

    An initial shot primes the drive lines and should be performed every time the drive syringes or sample loops are filled. It also avoids a timing error that occurs when the BASIC control program is started. (Only the first shot is affected by this error.)

    CAUTION: *The sample syringe containing RNA should be covered with either leaded Plexiglas or separate Plexiglas and lead shields to protect the sample from X-rays and the investigator from β radiation.*

Load sample loops and fire a priming shot

13. Turn the upper and lower valves to LOAD sample. Advance exactly 10 µL from each sample syringe into the left and right sample loops, respectively.

    The valve and sample loop should be precalibrated and marked to indicate the proper position of the fluid meniscus. The amount of solution is determined by the volume of the sample loops (see Commentary).

14. Turn all valves to the FIRE position.

    The valve to drive syringe C should remain in the LOAD syringes position, unless a quench solution is used.

15. Open the software routine for the time-resolved footprinting experiment. Set speed and distance of the stepper motor to achieve the desired folding delay and exposure times. For the dummy shot, enter a delay time of 0.01 sec.

    Each push of the drive platform is defined by the speed (rpm) of the stepper motor and a distance (the number of turns of the motor). Refer to Table 11.6.1 for a standard "four push, one pause" routine for synchrotron hydroxyl radical footprinting using a Kin-Tek apparatus. Refer to Table 11.6.2 for setting exposure times.

16. Once the parameters are set, type "G" at the controller keyboard to fire the drive motor.

    Before firing, be sure that a microcentrifuge tube is attached to the exit line.

Table 11.6.1 Sample Parameters for Time-Resolved X-ray Footprinting at X28C

Speed (rpm)	Distance	Event[a]	Sample position
180	400	Mix	Advanced through mixing T up to exposure cell
0	0	Fold	Variable pause to allow additional folding time
Variable	600	Expose	Advanced through exposure cell Speed determines exposure time
180	500	Exit	Advanced up to the end of the exit line
50	500	Eject	Ejected into collection tube

[a]The time required for each step is given in commentary (see Background Information, discussion of instruction).

Flush mixing and exit loops

17. Turn the lower valves to FLUSH, and allow any sample remaining in the exit line to drain into the waste or sample collection tube.

    Alternatively, turn the valve under syringe C 180 degrees, and expel the sample from the exit line by blowing air through port C with an empty 1-mL syringe. Close the valve (return to LOAD syringes).

    The lines of the rapid mixing apparatus are flushed and rinsed after each shot.

18. Gently unscrew the microcentrifuge tube from the exit line.

19. Attach the vacuum to the exit line using the Luer adapter.

    If the valves are not set to the FLUSH position before attaching the vacuum, the contents of the sample syringes will be aspirated into the waste.

20. With values in the FLUSH position, dip the wash inlet (tubing with a T joint protruding from the bottom of the rapid-quench apparatus) into a 15-mL tube of deionized water for 5 sec, and then into a 15-mL tube of 100% ethanol for 5 sec. Allow the vacuum to pull air through the system for 30 sec to dry.

    The vacuum will draw the solutions through the mixing and exit loops.

21. While holding the exit line, carefully detach the vacuum. Hang the end of the vacuum line (with Luer adapter) in an empty, disposable 15-mL tube to avoid contaminating the work area. Screw the waste tube onto the exit line.

    CAUTION: *The exit line and the end of the vacuum line should be handled with care to keep them free of ribonuclease, and to avoid transferring radioactive material to gloves and work surfaces.*

Table 11.6.2 Setting Exposure Times at X28C

Beam current, 2.8 GeV (mA)	Speed (rpm)	Exposure (msec)
300-200	240	10
	160	15
200-150	120	20
	96	25
150-100	30	20
	60	40
—	48	50
	40	60

In a typical experiment (20 reactions), an unexposed control and a reaction without Mg^{2+} are acquired first (reactions 1 and 2). The left side drive syringe and the bottom left sample loop are then refilled with CE20, and 16 folding time points (reactions 3 to 18) are acquired by varying the folding delay time. Finally, two controls with prefolded RNA are performed (reactions 19 and 20).

22. Dispense 20 µL precipitation cocktail into each of 20 screw-cap microcentrifuge tubes. Label tubes 1 through 20 (or as desired). Recap tubes and hold at room temperature until needed.

    These tubes will be used to collect samples. It is important to avoid introducing nucleases or other contaminants.

Perform a "no exposure" control

23. Screw tube no. 1 onto the exit line adapter. Set the valves to LOAD sample, and inject 10 µL RNA and 10 µL CE buffer into the right and left sample loops as in steps 13 and 14.

24. Set the folding delay time to 0.01 sec, and repeat steps 15 and 16.

25. Expel any sample remaining in the exit line into the collection tube as in step 17.

26. Remove the tube from the exit line, and flush lines with deionized water and 100% ethanol as described in steps 18 through 21.

27. Add 600 µL of 100 % ethanol to the expelled sample, recap, and store at 4° to –20°C in a lead-lined box.

    It is important that the screw caps form a leak-proof seal to prevent loss of sample during shipment.

Perform a control with unfolded RNA

28. Attach tube no. 2 to the exit line. Reload the sample loops as in steps 13 and 14, except this time the interlock system should be enabled as the user exits the hutch.

29. Enter a delay of 0.01 sec in the controller software.

30. Turn on the X-ray beam when the interlock safety alarm has turned off.

31. Once the beam is on, fire the rapid-quench apparatus (type "G"). Turn the beam off as soon as the software displays a message indicating that the experiment has finished.

    The time that the beam is on should be kept to a minimum to limit X-ray damage to samples and equipment.

32. Collect the sample and flush the mixing apparatus as in steps 17 to 21. Precipitate the RNA as in step 27.

Exchange buffer in drive syringe A

33. Close the top valves to drive syringes A, B, and C (LOAD syringes). Close the bottom left valve (180° from LOAD sample) and open bottom right valve (as in LOAD sample). Carefully remove the syringe containing RNA, and set aside for future use.

    To probe Mg^{2+}-induced folding, the CE buffer in the left side of the mixing apparatus (drive syringe A) must be exchanged for CE20 buffer.

34. Attach the vacuum to the exit port and open the bottom left valve (LOAD sample). To empty drive syringes, turn the bottom and lower valves to FIRE. Slowly depress the plunger of syringe A and B until the syringe is empty. Open the lines connecting drive syringes to air (opposite of FIRE).

35. Disconnect the vacuum, and return all valves to LOAD syringes.

36. Using the control software, move the drive platform up as in steps 1 and 2.

37. Fill drive syringe A with CE20 buffer as described in steps 3 and 4. Fill drive syringe B with additional CE buffer.

38. Load the bottom left sample loop with CE20 buffer (using a fresh 1-mL syringe) as in steps 9 through 11. Replace the syringe containing RNA on the right sample port.

39. Perform a priming shot and flush the mixing and exit lines, as described in steps 13 to 21.

Expose samples with variable folding delays

40. Using collection tubes no. 3 to 18, acquire time-resolved data by repeating steps 28 to 32 for each sample. Vary the length of the folding delay by entering the appropriate value into the software before firing each shot.

    If the folding delay is longer than the time required for the interlock safety shutter to open (3 sec), begin the folding routine, then turn on the X-ray beam when 3 sec of the delay remains. A standard laboratory timer can be used to measure folding delays of ≥10 sec.

41. When the first aliquot of RNA is expended (typically after the ninth sample), prepare the second aliquot as in steps 5 to 7. Remove the right sample syringe, and replace it with a fresh 1-mL syringe of RNA (steps 8 to 9). Fire a priming shot, then repeat step 40 for the remaining reactions, no.10 through 18.

42. When the experimental reactions are complete, open the right sample syringe to the vacuum (it should point to the right), and aspirate any remaining RNA. Flush the lines as in steps 17 to 21. Remove the vacuum from the exit line, and the 1-mL syringe from the right sample port.

    Alternatively, the sample syringe can be removed and remaining RNA recovered before the system is flushed with water and 100% ethanol.

Acquire controls with prefolded RNA

43. Prepare a 0.6-µCi (50-µL) aliquot of RNA by annealing in $MgCl_2$ under conditions in which the RNA is known to fold completely.

    For the Tetrahymena ribozyme, this is done by placing 5.5 µL of 100 mM $MgCl_2$ on the inside lid of the microcentrifuge tube, incubating 1 min at 95°C, then immediately microcentrifuging 1 min.

44. Load the annealed RNA into the right sample loop, and fire a priming shot (steps 8 to 9, and steps 13 to 21).

45. Using collection tubes no. 19 and 20, perform two shots with a 0.01-sec folding delay, as in steps 28 to 32.

46. Store samples in a lead container at −20°C or on dry ice.

    Samples should be stored at −20° or −70°C until they are ready to be analyzed by polyacrylamide gel electrophoresis (see Basic Protocol 3). They should be analyzed as soon as possible after exposure, ideally within 1 week.

    Samples may be shipped to the investigator's home laboratory for analysis. Users should consult with the synchrotron facility staff regarding procedures for packaging and shipping radioactive materials.

47. When the experiment is complete, thoroughly flush and disassemble the rapid-quench apparatus (see Support Protocol 3). At the end of the day, complete all safety checks, and disable the beamline.

SAMPLE WORK-UP AND DATA ANALYSIS

BASIC PROTOCOL 3

Radiolabeled products of X-ray-induced hydroxyl radical cleavage are analyzed directly by electrophoresis on a polyacrylamide sequencing gel. The intensity of the bands is quantified using a storage phosphorescence imager (Molecular Dynamics or equivalent). Equilibrium constants or kinetic rate constants are obtained by fitting changes in the relative extent of cleavage (or protection) to appropriate models.

Materials

Irradiated ^{32}P end-labeled RNA (see Basic Protocols 1 and 2)
Unirradiated ^{32}P end-labeled RNA (see Support Protocol 1)
2× formamide loading buffer (*APPENDIX 2A*) or loading buffer containing urea
RNase T1 cocktail (see recipe)
0.5 U/mL RNase T1 (USB)
CE buffer, pH 7.5 (see recipe) or TE buffer (*APPENDIX 2A*; see Critical Parameters)
Prefolded control RNA (see Basic Protocol 2)

Imaging system with large exposure cassettes (Phosphorimager, Molecular Dynamics; or equivalent)
Densitometer
Image analysis software for personal computer (PC) or Macintosh computer (ImageQuant, Molecular Dynamics; NIH Image; or equivalent)
Spreadsheet software (Microsoft Excel or equivalent)
Graphical fitting software (KaleidaGraph, SigmaPlot, or equivalent)

Additional reagents and equipment for denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B*)

NOTE: Samples should be stored at −20° or −70°C until they are ready to be analyzed. This should be done as soon as possible after X-ray exposure, ideally within 1 week.

Prepare RNA for gel electrophoresis

1. Microcentrifuge irradiated and unirradiated ^{32}P end-labeled RNA samples 30 min at maximum speed, 4°C.

   Samples should be kept on ice or at 4°C at all times.

2. Carefully remove the supernatant using a pipet or aspirator, avoiding contact with the RNA pellet. Dispose of the supernatant as radioactive waste.

   If using a standard pipet, the solution in the pipet tip can be checked with a survey meter to make sure that the RNA pellet is not lost. If using an aspirator, it should be connected to a trap that can contain radioactive liquid.

3. Recap the tubes and microcentrifuge for an additional 5 sec at 4°C. Use a pipet tip to remove any remaining supernatant.

4. Dry the samples in a Speedvac evaporator at room temperature.

5. Redissolve each sample in 6 µL of 1× loading buffer with urea or formamide. Vortex briefly, and microcentrifuge 15 sec at maximum speed, room temperature. Hold samples at room temperature or 4°C.

Run sequencing gel

6. Cast a 33 × 42–cm sequencing gel using an appropriate concentration of polyacrylamide (*APPENDIX 3B*). Use a comb that forms separate, flat-bottomed wells ~0.8-cm wide.

   The authors have obtained good results with 6% to 10% (19:1 mono/bis) polyacrylamide gels.

7. Mount the polymerized gel on an electrophoresis apparatus. Preheat gel for 10 to 20 min at 55 W.

8. Prepare a sequencing ladder by digestion with RNase T1. Combine the following:

   7 µL RNase T1 cocktail
   3 µL ^{32}P 5′-end-labeled RNA (~100,000 cpm)
   1 µL 0.5 U/mL RNase T1.

   Incubate 12 min at 50°C. Place on ice.

   The concentration of RNase T1 should be adjusted to produce an even ladder of fragments. Samples should be loaded as soon as possible (within 10 min) to prevent overdigestion. Ribonuclease reactions can be held up to 60 min on dry ice.

9. If desired, prepare an undigested control by combining 3 µL of 5′-end-labeled RNA with 2 µL of 1× CE or TE buffer and 5 µL of 2× loading buffer.

10. Immediately before loading, heat samples 1 min at 85° to 90°C and place on ice.

11. Load samples, with the RNase T1 digest next to lanes containing prefolded controls (see Basic Protocol 2, tube no. 19 and 20). Run the gel at 55 W for the desired length of time.

    Several electrophoresis runs may be necessary to resolve all of the cleavage products if the RNA is >150 nt.

12. Disassemble the sequencing apparatus, transfer the gel to Whatman 3MM filter paper, and dry under vacuum. Place the gel in a large Phosphorimager cassette.

    Depending on the radioactivity of the samples, exposure may require 1 to 5 days.

Data analysis

13. Scan the gel on a densitometer and display the results using image analysis software.

14. Identify residues that are protected from cleavage in the prefolded RNA control lanes by comparison with the pattern of RNase T1 digestion.

    A similar pattern should be visible in experimental lanes (e.g., with increasing $MgCl_2$ concentration or longer folding times).

15. Define a rectangular area within the lane that includes a group of protected residues that behave similarly to each other over the course of the experiment. Use the copy feature of the image analysis software to place identical boxes in each lane of the gel.

    Great care must be taken to define the contours of the protected region accurately, as inclusion of adjacent bands in the area to be quantified will considerably reduce the quality of the analysis. A tilted rectangle or rhomboid can be used to account for "smiling" effects at the edge of the sequencing gel.

16. Repeat step 15 for all residues that are protected and that are resolved by the gel.

17. In the same manner, box one or more bands of constant intensity.

Cpm of protected regions

time	p4	p7	p3	p8a	j7/8	l5a	j4/5	ref
0	4468830 21	3862135 69	10337590 14	13304065	15239070 8	6709014 14	6022584 90	13935066 38
0 01	604393 93	683071 84	1905315 96	2641309 72	3674178 12	722658.3	694579.69	1765946 57
0 09	1171682 65	1326051 82	3912380 47	5219519 02	7187669 41	1477396 64	1420927 46	3665873 31
0 1	1142774 72	1185041 79	3492800 25	4813622 87	6645087.66	1357364 36	1322887 02	3563621 93
0 3	540874 84	706793 97	1927401 75	2595875 12	4182242 92	581902 72	687071 28	1354329 27
0 5	2786714 14	2728207 6	6512907 31	5510048 76	11539369 13	3584376 99	2948763 0	9570076 8
0 9	1042360 78	1256704 43	3545227 39	4748778 46	7178909 07	1061867 52	1110832 6	2614515 59
1	313265 01	366147 69	938247 17	1419712 33	2144209 44	279779 69	317185 35	697797 84
3	579551 31	706307 83	1849361 21	2508337 17	3085103 45	593075 98	661751 81	1638361 93
5	3158950 8	3283563 53	6587465 14	9955561 08	11400328 27	3687806 02	3347597 14	8439868 35
10	917256 84	1020390 41	2314020 57	3493818 82	4414195.14	944501 83	982459 55	2575548 62
15	3229746 77	3237461 24	6297165 97	11987708 88	15637561 51	3293170 94	3013726 91	9125418 81
20	634789 62	749709 14	1794089 13	2704241 43	2618132 2	629731 34	640385	1675947 88
30	3728099 07	3484174 53	6458442 82	14103563 4	3884585 78	3629248 06	3412703 63	11347052 27
60	682562 86	816947 03	1840512 74	2827056 71	15766200 67	693067 73	700375 35	1933518 97
90	623171 12	775544 04	1678384 37	2484801 93	3883205 47	625443 5	630815 22	1641994 03
120	504893 5	669301 5	1352857 82	2016784 61	3313914 34	500704 05	508434 14	1342516 02

Figure 11.6.4 Organization of data from image analysis program using Excel.

These bands, which usually correspond to nucleotides that are not protected from cleavage, will serve as a reference.

18. Integrate the volume of all of the rectangles (objects). Export the results of the integration to a spreadsheet program.

    The average background intensity may be subtracted from each pixel if desired (see Commentary).

19. Use the drag-and-drop editing features of the spreadsheet program to arrange the volume (cpm) of each band or collection of bands into a table, as illustrated in Figure 11.6.4.

    It is usually easiest to arrange the data so that each column represents a set of residues (across the lanes of the gel), and each row corresponds to an experimental variable, such as folding time or $MgCl_2$ concentration (represented by one lane). Enter the time or concentration in the first column of the spreadsheet. Label the columns with the sequence of the protected residues or reference bands.

20. Calculate the "protection ratio" for each column of data, according to

$$P = 1 - \frac{\text{cpm protected}}{\text{cpm reference}}$$

    where cpm protected is the volume integral of the protected region of interest and cpm reference is the volume integral of an appropriate reference band.

21. Export the data as tab-delimited text to a scientific graphing program, such as KaleidaGraph or SigmaPlot. Plot the protection ratio versus time (or concentration), and fit the data to the desired rate expression or binding isotherm (see Commentary for further discussion).

    It is useful to normalize the extent of protection by defining the fractional $\overline{Y}$ as

$$\overline{Y} = \frac{P - P_{\min}}{P_{\max} - P_{\min}}$$

    where P_{min} and P_{max} are the values obtained for unfolded and fully folded RNA. These should correspond to the upper and lower baselines, respectively, of the transition.

SUPPORT PROTOCOL 1

PREPARATION OF RADIOLABELED RNA

Unlabeled RNA is prepared by in vitro transcription of plasmid DNA with T7 RNA polymerase (*UNIT 11.4*), according to standard methods (Milligan et al., 1987; Milligan and Uhlenbeck, 1989). The RNA must be treated with phosphatase before labeling at the 5′ end with polynucleotide kinase. After gel purification, the labeled RNA is dispensed into aliquots (usually 10 µCi, 20 µL each) to guard against total loss of sample at the beamline. Samples are shipped in leak-proof screw-cap tubes.

Materials

10 pmol/µL RNA, treated with calf intestinal phosphatase (*UNIT 6.3*)
T4 polynucleotide kinase and 10× kinase buffer
6000 Ci/mmol (10 µCi/µL) γ-^{32}P]ATP
TE buffer, pH 7.5 (optional; *APPENDIX 2A*)
CE buffer, pH 7.5 (see recipe)
Microcentrifuge tubes with captive screw caps and O-ring seals (Rainin)

Additional reagents and equipment for phosphorylation reaction (*UNIT 6.3*), for preparing and running a preparative polyacrylamide sequencing gel (*APPENDIX 3B*), for phenol/chloroform extraction and ethanol precipitation (*APPENDIX 2A* and, e.g., *CPMB UNIT 2.1A*)

NOTE: Radioactive materials must be labeled and shipped in compliance with federal and state regulations. Consult with the radiation safety officer of your home institution and the receiving institution before planning to ship radioactive materials.

Prepare ^{32}P end-labeled RNA

1. Phosphorylate 40 pmol phosphatase-treated RNA using T4 polynucleotide kinase and 50 µCi [γ-^{32}P]ATP (*UNIT 6.3*).

   Unlabeled or 3′-end-labeled RNA may also be used.

2. Purify the labeled RNA by denaturing polyacrylamide gel electrophoresis (*APPENDIX 3B*) on a 20 × 20–cm gel.

3. Elute RNA, extract with phenol/chloroform, and precipitate with ethanol (*APPENDIX 2A* and, e.g., *CPMB UNIT 2.1A*). Dissolve the final pellet in TE or CE buffer and store up to 3 months at −20°C.

4. Measure the activity of the purified RNA by counting 1 or 2 µL of the final solution in a liquid scintillation counter (1 µCi = 2.2 × 10^6 cpm).

   Typically, 1 µL contains 10^6 cpm. The solution should minimally contain 250,000 cpm/µL or 0.1 µCi/µL.

Dispense aliquots of RNA for shipping

5. Determine the total amount of RNA that will be required for the footprinting experiments.

   Each equilibrium or time-dependent footprinting experiment requires 4 µCi, or 200,000 to 400,000 cpm, per sequencing gel lane.

   Extra solution may be left in the home laboratory to serve as a control for background hydrolysis of the RNA during shipping and handling at the beamline.

6. Dilute the required amount of RNA in 1× CE buffer at a final concentration of 3 µCi/20 µL, or 6 µCi/20 µL for equilibrium experiments. Mix thoroughly, and pipet 20-µL aliquots into leak-proof screw-cap microcentrifuge tubes.

A range of 2 to 4 μCi per aliquot, or 4 to 8 μCi per aliquot for equilibrium experiments, may be used. Higher concentrations of ^{32}P provide better signal-to-noise ratios.

7. Prepare additional aliquots for prefolded control reactions by diluting 0.6 μCi of labeled RNA (from stock solution) into a final volume of 50 μL CE buffer. Place the solution in a leak-proof screw-cap microcentrifuge tube.

   It may be necessary to make serial dilutions of the stock solution in order to accurately transfer 0.6 μCi. This is sufficient for two control reactions.

8. Prepare additional 0.6-μCi aliquots as needed (at least one aliquot per time-resolved experiment).

Package RNA samples for shipment to the beamline

9. Store samples at −20° or −70°C until ready for shipment to the beamline.

10. Package samples for shipment on dry ice, according to the specific instructions provided by beamline personnel.

    In general, a Radiation Shipping Notice must be included inside the package. The outside should be labeled with (1) a "limited quantity notification" (49 CFR 173.421), if applicable, (2) a dry ice hazard diamond, and (3) the overnight shipper's label, with shipping and return addresses. One may be required to obtain an authorization number from the recipient.

    Detailed instructions for shipping radioactive materials to beamline X28C at the NSLS should be obtained from the beamline supervisor. As these protocols are subject to change, users should verify that they are using the correct protocol before each shipment.

DETERMINE OPTIMAL EXPOSURE TIME

SUPPORT PROTOCOL 2

The optimal time that samples should be exposed to the X-ray beam is determined by measuring the fraction of RNA that is cleaved after variable exposure times. The resulting dose-response curve is used to determine how much irradiation is required to cleave 10% to 30% of the RNA strands. This should be done in the buffer used for RNA folding experiments to control for the effect of solutes.

Additional Materials *(also see Basic Protocols 1 and 3)*

1 to 2 μCi 5′-^{32}P-labeled RNA in 10 μL CE buffer

Prepare and expose samples

1. Dilute RNA with 40 μL CE buffer (final 50 μL). Mix thoroughly and briefly microcentrifuge.

   CE20 buffer can also be used, if desired.

2. Divide the sample into five 10-μL aliquots. Place aliquots in screw-cap microcentrifuge tubes with O-ring seals.

3. Set up the sample holder and shutter as described (see Basic Protocol 1, steps 1 to 7).

4. Expose four aliquots to the X-ray beam for varying times (see Basic Protocol 1, steps 14 and 15). Reserve one aliquot as an unexposed control.

   At X28C, exposure times typically range from 10 to 100 msec.

5. Store irradiated and control aliquots on dry ice if they will not be analyzed right away (store up to 3 days).

Determine extent of RNA cleavage

6. Cast and preheat a small (20-cm) 5% to 8% polyacrylamide sequencing gel.
7. Thaw aliquots of RNA (if frozen) and add 8 µL of 2× formamide loading buffer to each. Heat samples briefly (30 to 60 sec) at 85°C.
8. Load on the gel and run the gel so that the full-length RNA migrates at least one third of the way down.

   The object is to separate the full-length (uncleaved) RNA from the products.

9. Disassemble the gel and dry under vacuum. Expose the gel to a Phosphorimager screen long enough so that the full-length RNA appears as a dark band, but does not saturate the Phosphorimager screen.
10. Use the volume integration feature of the image analysis software to quantify the amount of uncut RNA remaining after cleavage. Prepare a plot of the fraction of the natural logarithm of uncut RNA versus exposure time.

    This plot should yield a straight line.

    The dose depends on the beam current (or flux) as well as exposure time. The exposure time must be adjusted to account for variations in the beam current during each 24-hr period.

SUPPORT PROTOCOL 3

SET UP RAPID-QUENCH MIXING APPARATUS

A modified Kin-Tek RQF-3 rapid-quench apparatus is used for X-ray footprinting at X28C (see Commentary). The mixing apparatus must be installed at the beginning of the experiment. Users should consult the manufacturer's literature for more detailed information on the maintenance and use of the rapid-quench device. This protocol should be adapted to suit equipment available at individual facilities.

Additional Material *(also see Basic Protocol 2)*

Detergent (e.g., Absolve, NEN)
Plastic-backed absorbent bench paper
Diaphragm vacuum pump (details)
Vacuum/vent filter, 0.2 µm (Millipore Millex 50 mm, or equivalent)
Side-arm flask with one-hole stopper and Teflon tube
Thick-walled soft tubing (e.g., Tygon, Nalgene) to fit Teflon tube
Adapter (male M6 to male Luer) to connect soft vacuum tubing with $^1/_{16}$-in.-o.d. polypropylene tubing (exit line of rapid-quench)
5-mL syringes

Install rapid-quench apparatus

1. Mount the Kin-Tek rapid-quench apparatus (in polycarbonate water jacket) on the stepper motor frame (Figure 11.6.2).
2. Use the provided bolts to attach a horizontal aluminum plate on the Unistrut frame, near the bottom of the mixing apparatus. Mount a second vertical aluminum plate on the rear of the rapid-quench box so that it rests against the first plate.

   The horizontal plate will support sample tube racks. The vertical plate shields the sample exit lines from the X-ray beam.

3. Cover all exposed surfaces of the work area with plastic-backed absorbent bench paper to prevent contamination of hard surfaces in case a radioactive sample is spilled.

4. Outside the hutch, turn on the controller and begin running the control software. If using a personal computer to interface with the controller, open the Kin-Tek software (or equivalent) in DOS and download the desired BASIC control program.

   Calibration settings and mixing parameters for individual mixers can be stored between experiments and imported into the Kin-Tek control software. The control code modified for X-ray footprinting at X28C is available from the Center for Synchrotron Biosciences.

Connect water bath and vacuum lines

5. *Optional:* Remove nucleases by soaking sample ports and internal tubing for 1 hr or overnight in detergent. Before use, remove all traces of detergent by thorough rinsing with RNase-free water followed by 100% ethanol.

6. Inside the hutch, connect the recirculating water bath to the jacket surrounding the rapid-quench apparatus. Set the temperature as desired.

7. Connect the vacuum pump to the side arm of a 0.5-L side-arm flask, through a 0.2-µm filter.

   CAUTION: *This flask will collect the waste between kinetic shots, and therefore will be contaminated with radioactive material and should be labeled accordingly. Be extremely careful when handling liquid waste containers, and be sure that secondary trap and vacuum lines are secured. Always wear gloves, and monitor them frequently using a survey meter.*

8. Attach thick-walled soft tubing to a Teflon tube in the top of the side-arm flask. Insert a male M6 to male Luer adapter into the opposite end of the tubing.

   The vacuum tubing should be stored in a clean, covered box to minimize risk of RNase contamination. The adapter should fit snugly over the $\frac{1}{16}$-in.-o.d. tubing that is used as the exit line of the rapid-quench apparatus.

9. Turn on the vacuum pump. Attach the vacuum to the exit line, and aspirate any solution from the sample loops and drive syringes.

Align mixing apparatus with X-ray beam

10. Secure the automatic alignment device to the registration pins on the back of the mixing box, and attach the output cable.

11. Enable the X-ray beam and follow the instructions for the automatic alignment procedure described on the Center for Biosciences website (*http://www.aecom.yu.edu/home/csb/*).

    If the beam is not aligned correctly with the exposure port, the extent of RNA cleavage may be drastically reduced. Although not necessary, it is prudent to check the alignment of the mixer at the end of an experimental session. This test will help troubleshoot the experiment in the event of poor results.

12. Remove the alignment device from the rapid-quench apparatus and extend the flight tube to the back of the rapid quench.

    Failure to do so will prevent the samples from being exposed to X-rays during the experiment.

13. *Optional:* Manually check the alignment by taping Kodak Linagraph paper over the exposure cell port on the rear of the rapid-quench apparatus. Expose the paper to the X-ray beam for 1 sec, and verify that the burn mark on the paper is centered in a depression caused by the outline of the exposure port.

    The apparatus is now ready for use as described in Basic Protocol 2.

Clean up

14. At the end of the experiment, discard the screw cap on the exit line as radioactive waste.
15. Attach the vacuum to the exit line, and aspirate any remaining solutions from the sample loops and drive syringes. Leave the vacuum on.
16. With a fresh 5-mL syringe of deionized water, rinse out all the syringes and sample loops. Use the vacuum to aspirate each line after rinse.
17. Repeat step 15 with a 5-mL syringe of 100% ethanol.
18. Remove the vacuum line and turn off the pump and water bath. Remove the mixing apparatus from the support stand and store in a covered plastic box.
19. Exit the control software and shut off the controller.

SUPPORT PROTOCOL 4

CALIBRATE RAPID-QUENCH MIXING APPARATUS

Each mixing apparatus should be calibrated when first installed, or when tubing is changed. The parameters for the drive platform distances can be stored in the controller software. The performance of the rapid-quench mixing apparatus is evaluated visually using a water-soluble dye such as bromphenol blue.

Additional Materials *(also see Basic Protocol 2 and Support Protocol 3)*

0.25% (w/v) bromphenol blue in water
Dental mirror
Small flashlight

1. Set up a rapid mixing apparatus (see Support Protocol 3). Load drive syringes with water or CE buffer, as described (see Basic Protocol 2, steps 1 to 4).
2. Load 0.25% bromphenol blue solution into the right sample loop (instead of RNA), as described in Basic Protocol 2, steps 8 to 11. Fill the left sample loop with water or CE buffer.
3. Set up a mock footprinting experiment with a long folding delay (>10 sec) and a very long exposure time (10 sec). Install a microcentrifuge tube at the exit tube to collect the ejected sample.
4. Exit the hutch without enabling the beam. Type "G" to go.
5. Quickly re-enter the hutch. Inspect the flow of colored solution in the tubing using a dental mirror and flashlight. If the dye and water are not mixing correctly, empirically adjust the distance values in the Kin-Tek control program.

   After the first push, the dye should advance up to the exposure cell, but should not enter it. The dye should mix uniformly with water. Once the "exposure" push starts, the dye should smoothly pass through the exposure cell and into the microcentrifuge tube.

REAGENTS AND SOLUTIONS

Use RNase-free deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2A****; for suppliers, see* ***SUPPLIERS APPENDIX****.*

CE (sodium cacodylate/EDTA) buffer, 1× (pH 7.5)

1.0 mL 1 M sodium cacodylate
20 µL 0.5 M EDTA, pH 8.3 (*APPENDIX 2A*)
Deionized water to a final volume of 100 mL

Filter sterilize through a 0.2-μm filter
Store up to 6 months at room temperature

CE20 buffer, 1×

Prepare as for 1× CE buffer (see recipe), but include 2 mL of 1 M $MgCl_2$ (*APPENDIX 2A*; final 20 mM).

Precipitation cocktail, 10×

8 mL 4 M NaCl (*APPENDIX 2A*)
0.5 mL 0.5 M EDTA, pH 8.3 (*APPENDIX 2A*)
100 μL 10 mg/mL carrier tRNA
150 μL 20 mg/mL glycogen
Bring to 10 mL with deionized water
Store in 1-mL aliquots up to 1 year at −20°C

RNase T1 cocktail

100 μL 10 M urea (see recipe)
3 μL 1 M sodium citrate, pH 3.5
1.5 μL 0.1 M EDTA, pH 8.3 (*APPENDIX 2A*)
2 μL 2% (w/v) bromphenol blue and xylene cyanol
3 μL 10 mg/mL carrier tRNA
Store in 0.2-mL aliquots up to 1 year at −20°C

Urea, 10 M

Mix 0.22 g urea in 200 μL water. Warm at 50°C and vortex to dissolve. Store up to 1 year at −20°C.

COMMENTARY

Background Information

X-ray-dependent hydroxyl radical footprinting

Hydroxyl radicals have been widely used to probe the conformation of nucleic acids and nucleic acid–protein complexes (Tullius and Dombroski, 1985; Latham and Cech, 1989; Dixon et al., 1991; Strahs and Brenowitz, 1994). Reaction of hydroxyl radical with ribose results in oxidation of the sugar and elimination of phosphate groups, leading to strand cleavage.

Cleavage is relatively insensitive to base sequence and secondary structure of the RNA (Celander and Cech, 1990). However, the susceptibility of individual nucleotides to cleavage in the presence of a hydroxyl radical correlates well with the solvent accessibility of the ribose C4′ (Latham and Cech, 1989; Cate et al., 1996; Balasubramanian et al., 1998). Nucleotides that are inaccessible to bulk solvent due to RNA tertiary structure or interactions with a protein are protected from cleavage. The resulting footprint provides information about the conformation of the nucleic acid, and can be quantified to determine the fraction of structured molecules in the population.

Chemical methods for generating hydroxyl radicals, such as the Fenton-Haber reaction (Dixon et al., 1991) or disproportionation of peroxynitrous acid (King et al., 1993), typically require several seconds or longer to cleave 20% to 30% of the RNA (Chaulk and MacMillan, 2000; Hampel and Burke, 2001). This unit describes the use of a synchrotron X-ray beam to generate hydroxyl radicals in aqueous solution. The advantage of this method is that cleavage reactions can typically be completed in 10 to 50 msec. Therefore, "X-ray footprinting" can be used to probe kinetic folding intermediates or other transient conformations.

The protocols given here were initially developed to probe the folding pathway of the *Tetrahymena* ribozyme (Sclavi et al., 1997, 1998a), but can be applied to a variety of catalytic RNAs or RNA-protein complexes. Recent improvements to the method are described in Ralston et al. (2000b) and Dhavan et al. (2001).

To carry out cleavage reactions on nucleic acids in the millisecond timescales, the beam must deliver a sufficient flux of photons to the sample so that the steady-state concentration of

hydroxyl radicals is 0.5 to 1.2 μM (Ralston et al., 2000b). Hydroxyl radicals are produced after excitation of water molecules by absorbed photons, according to the following equation (Klassen, 1987).

$$H_2O \xrightarrow{h\nu} H_2O^+ + e^-_{dry} \xrightarrow{H_2O} H_3O^+ + \bullet OH + e^-_{aq}$$

The steady-state concentration of hydroxyl radicals and the dose required to cleave the sample depend on many factors (Ralston et al., 2000b), including the energy absorbed by the sample. A bending magnet beamline at the NSLS operating at 2.8 GeV delivers 5×10^{14} photons/sec to a 10-μL sample, with an absorption maximum near 7.5 keV (Ralston et al., 2000b). Absorption of 10 keV produces 287 hydroxyl radicals (Klassen, 1987). The steady-state concentration of hydroxyl radical can be estimated from the photon flux, and is ~10^{-6} M for NSLS footprinting beamlines (Sclavi et al., 1998b). The effective concentration of hydroxyl radicals will be reduced by free radical scavengers in the sample (see Critical Parameters).

Instrumentation

The design of equipment for X-ray footprinting experiments has been described previously (Sclavi et al., 1998b; Ralston et al., 2000b; Dhavan et al., 2001). Equilibrium experiments are carried out using an aluminum sample holder and stand, which places a standard microcentrifuge tube horizontally in the path of the beam (Figure 11.6.1). The sample is vertically aligned with the beam using a photodiode detector placed behind the sample block and a motorized stage. The alignment apparatus operates with a resolution of 50 μm. This level of precision is necessary to obtain reproducible extents of RNA cleavage at X28C.

The exposure time is regulated by an electronic shutter placed between the sample block and the end of the beampipe. The electronic shutter is controlled by a microprocessor placed outside of the experimental hutch. The path of the beam between the end of the beampipe and the shutter is covered by a retractable flight tube. This reduces the amount of scattered radiation in the experimental hutch. The aluminum sample block is connected to a water bath to maintain constant temperature. This is sufficient to dissipate the small amounts of heat generated over short (<100 msec) irradiation times. The beam causes an ~0.5°C/sec rise in the temperature of the sample (Sclavi et al., 1998b).

A modified Kin-Tek stopped-flow rapid-quench apparatus has been installed by the Center for Synchrotron Biosciences staff at beamline X28C for rapid mixing experiments (Figure 11.6.2). The mixing valve present in a standard rapid-quench apparatus is replaced with a flow cell mounted on a steel plate at the back of the box. This enables the flow cell to be placed close to the end of the beampipe (Dhavan et al., 2001). The flow cell itself is constructed of Vespel to minimize damage from X-rays.

Sample and buffer is driven through the lines by syringes at the top of the apparatus. After samples (10 to 20 μL) flow through a mixing T, they are aged in a 60-μL loop before being pushed through the flow cell. If desired, a quench solution can be delivered via a third syringe. However, a chemical quench is not normally necessary, because the hydroxyl radical concentration decreases rapidly (<1 msec) as soon as the beam is turned off (Sclavi et al., 1998b).

The Kin-Tek mixers at X28C are configured for either 10 μL or 20 μL sample volumes. Larger volumes yield improved precision, but increase the mixing dead time. If the volume of the sample is larger than the capacity of the X-ray exposure cell, the exposure times will increase.

The Kin-Tek RQF-3 uses a stepper motor and platform to advance the pistons of the drive syringes. The distance traveled by the sample is determined by the number of turns of the stepper motor. The time required to advance the sample over this distance depends on the motor speed. Typical parameters for RNA footprinting at X28C are given in Table 11.6.1. The exposure time is regulated by controlling the speed at which the sample flows past the exposure port. The exposure time is given by

$$t_{xray}\ (\text{msec}) = \frac{(0.04\ \text{rev})}{(s\ \text{rpm})} \times \frac{60\ \text{sec}}{1\ \text{min}} \times 1000$$

Hence, a 10-msec exposure would require a motor speed, *s*, of 0.024 rpm.

Critical Parameters

X-ray-dependent hydroxyl radical footprinting has been used successfully to probe Mg^{2+}-dependent folding of ribozymes, urea-induced denaturation of RNA, and the formation of protein-DNA complexes (Sclavi et al., 1998a; Deras et al., 2000; Ralston et al., 2000a; Dhavan et al., 2001). Although synchrotron X-ray footprinting provides sequence-specific information about the tertiary conformation of RNAs that cannot be presently obtained by other methods, several variables will determine the likelihood of success. The critical parame-

ters discussed below include conformational stability of the RNA, optimum exposure times, factors influencing signal-to-noise ratios of product bands, and sample handling.

Conformational stability

X-ray-dependent footprinting will be successful only if the hydroxyl radical protection pattern produced by the folded RNA or RNA-protein complex is well defined. It is important that the RNA tertiary structure or protein complex of interest is stable under the final conditions of the experiment. If the RNA tertiary structure is unstable, or if the RNA folds into multiple conformations, the footprint will be faint and difficult to interpret. Nonspecific RNA-protein complexes obscure native interactions and make interpretation of the data difficult. It is important to optimize the conditions using Fe(II)-EDTA-dependent footprinting before undertaking experiments at the synchrotron beamline. Parameters that should be evaluated include buffer, pH, Mg^{2+} concentration, temperature, protein concentration, and protocols for annealing the RNA.

Hydroxyl radicals react with nucleic acid bases as well as the ribose moiety (Dizdaroglu and Bergtold, 1986; Breen and Murphy, 1995). In general, these modifications do not lead to strand scission and are not detected by the protocols described here. The most common base modifications, 8-oxo-G and 5-hydroxy-C, are not expected to drastically destabilize RNA secondary structure (Wallace, 1998). Nonetheless, it is important to be aware that specific base modifications could induce unfolding of the RNA, and this could alter the observed cleavage pattern. Similarly, rapid oxidation of amino acid side chains may destabilize RNA-protein complexes and cause them to dissociate during the X-ray exposure.

Exposure time

To interpret footprinting experiments quantitatively, it is important that the extent of cleavage be adjusted so that each molecule is cleaved no more than once, on average. This is usually achieved by limiting the extent of cleavage to 10% to 30% of the starting material (Brenowitz et al., 1986). The exposure time required to cleave 10% to 30% of the RNA depends on the energy and beam current at the time of the experiment (Sclavi et al., 1998b). It also depends on the length of the RNA (longer RNAs require shorter exposure times). Ideally, the exposure time should be determined experimentally for each substrate by acquiring a dose-response curve (see Support Protocol 2). The deadtime of the experiment will be determined largely by the exposure time required to generate the cleavage pattern.

The extent of cleavage will also be affected by the presence of free radical scavengers in the sample, such as Tris and glycerol. Inorganic buffers such as phosphate or cacodylate give excellent results and should be used when possible. Concentrations <30 mM of Tris can be tolerated, as long as the exposure time is lengthened to compensate for the reduced rate of cleavage. Carboxylic acids (such as EDTA) and urea do not interfere with hydroxyl radical cleavage (Ralston et al., 2000a).

Signal-to-noise ratio

The ability to quantify the extent of protection from a sequencing gel will depend in large part on the intensity of the bands relative to the background activity in the lane. Several factors influence the signal-to-noise ratio. First, it is essential that a sufficient amount of ^{32}P be used, so that individual bands can be detected using a phosphorescent screen or other imaging device. The authors found that, for the *Tetrahymena* ribozyme (388 nt), quantitation of the protection pattern improved as the amount of sample increased from 200,000 to 400,000 cpm per lane. By contrast, experiments using <150,000 cpm per lane failed to yield interpretable results. Since the cpm per band depends on the number of products, less radiolabeled material is required to analyze shorter RNAs.

It is important that the cross-section of the beam is large enough to cover the entire sample or flow cell. Otherwise, only part of the sample will be irradiated. Similarly, air bubbles can exclude sample from the beam, and the dissolved oxygen will alter the distribution of cleavage products. The resolution of sequencing gels is improved by using 19:1 mono/bis acrylamide, and by precipitating samples with ethanol to remove excess salt.

Background RNA cleavage

Another critical parameter for enhancing the signal-to-noise ratio is the extent of background cleavage. Samples should be protected from scattered X-radiation when the beam is on. Recent improvements at X28C (Dhavan et al., 2001) have reduced scattered radiation. The flight tube was extended so that it reaches the face of the stopped-flow apparatus, and a lead and Plexiglas housing was constructed to cover the sample-loading syringe.

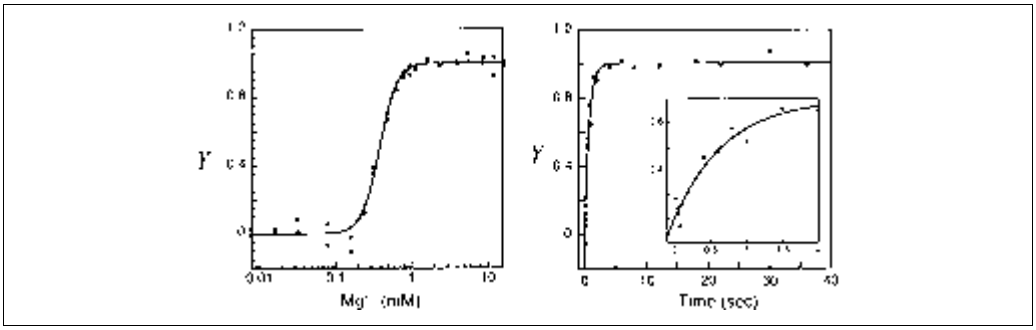

Figure 11.6.5 Results of X-ray footprinting experiments showing Mg^{2+}-dependent folding of the P4-P6 domain of the *Tetrahymena* ribozyme. Reprinted from Deras et al. (2000) with permission from the American Chemical Society.

Trace contamination by ribonucleases presents another source of background cleavage, and should be minimized to the extent possible. Even 2% cleavage by nucleases can lower the quality of the data. Besides incautious preparation of buffers and samples, common trouble spots are mishandling of the exit tube and sample-loading syringe on the Kin-Tek stopped-flow apparatus, prolonged exposure of tips and samples to airborne contaminants, and contaminated O-rings and lids in screw-cap microcentrifuge tubes. Additional control reactions can be added to the protocol to troubleshoot sources of background cleavage.

If the facility is to be used for several different applications, such as protein and nucleic acid footprinting, it is helpful to maintain separate mixing apparatuses for each type of experiment. This reduces the chance of RNase contamination in RNA folding experiments. Nucleases can be removed by soaking the sample ports and internal tubing for 1 hr or overnight in a detergent such as Absolve (NEN). The detergent must be removed by thorough rinsing with RNase-free water and ethanol.

Anticipated Results

Quantitative analysis of footprinting experiments has been discussed in detail elsewhere (Brenowitz et al., 1986). Nucleotides that become protected from hydroxyl radical cleavage because of tertiary structure or protein binding should be visible as clear regions within a ladder of bands on the gel. Comparison with a sequence ladder should permit assignment of the protected regions to particular sequences within the RNA. Interpretation of the data is improved considerably if a three-dimensional structure or model is available.

The relative extent of protection is determined by comparing the intensity of cleavage products in unfolded and fully folded controls. A detailed presentation of the integration method described in Basic Protocol 3 can be found in *CPMB UNIT 12.4*. The equilibrium folding of RNA as a function of Mg^{2+} concentration can be described by the Hill equation (Celander and Cech, 1991; Sclavi et al., 1997). The fractional saturation of a protected region, $\overline{Y}$, is fit to

$$\overline{Y} = \frac{P - P_{\min}}{P_{\max} - P_{\min}} = \frac{(K_{\text{app}}/[\text{Mg}^{2+}])^{n_{\text{H}}}}{1 + (K_{\text{app}}/[\text{Mg}^{2+}])^{n_{\text{H}}}}$$

where K_{app} is an apparent dissociation constant corresponding to the midpoint of the transition, n_{H} is the Hill coefficient, and $P_{\min}$ and $P_{\max}$ are the upper and lower baselines of the transition (see Basic Protocol 3, steps 19 and 20). An example of this type of data is given in Figure 11.6.5.

Time-dependent experiments should be fit to an appropriate rate expression, as illustrated in Figure 11.6.5. For single first-order transitions, this yields

$$\overline{Y} = \frac{P - P_{\min}}{P_{\max} - P_{\min}} = 1 - e^{-k_{\text{obs}}T}$$

The first equality holds only if the upper plateau of the kinetic transition defined by $P_{\max}$ represents the full extent of folding, as determined from equilibrium experiments.

Variations in sample loading can be controlled by normalizing the radioactivity in the bands of interest to another band in the gel whose intensity remains constant over the experiment. In general, the authors find that extreme variations in sample recovery increase the scatter of the data, even after normalization.

Transitions that occur on the 0.1- to 10-sec timescales are easily monitored by the current state of equipment at beamline X28C. Slower

transitions (1 to 10 min) can be more difficult to detect, because the long aging times in the sample loop lead to poor sample recovery. Processes occurring on timescales of <50 msec cannot be easily probed by X-ray footprinting using the current experimental protocol. It is hoped that future improvements to the beamline will shorten the deadtime of the experiment to several milliseconds.

Time Considerations

Experiments should be planned 1 to 2 months in advance. Preparation of samples for shipment to the beamline requires 3 days, and samples must be shipped at least 24 hr in advance. Careful planning and execution are essential, as experiments at the beamline are often scheduled over a 1- to 3-day period. Roughly 1 week should be allowed for gel electrophoresis and for analysis of the data.

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