Protocol

Analysis of RNA by Primer Extension

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For mapping the 5' termini of mRNA molecules, primer extension is the method of choice. A purified oligonucleotide is end-labeled using polynucleotide kinase. The probe and a population of mRNA are allowed to hybridize, and the primers and template are used to carry out reverse transcription using an enzyme cloned from the Moloney murine leukemia virus. The primer extension products are separated on a denaturing polyacrylamide gel and analyzed by radiography.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Ammonium acetate (10 M)
[γ -³²P]ATP (10 mCi/mL, 7000 Ci/mmol)
Carrier RNA (yeast tRNA)
Chloroform
Denaturing polyacrylamide gel containing 8 M urea

*In many cases, a miniprotein gel apparatus (e.g., Bio-Rad Mini-PROTEAN 13 cm × 13 cm × 0.75 mm) can be used to analyze radiolabeled primer extension products (see Tables 1 and 2 in Protocol: **Mapping RNA with Nuclease S1** [Green and Sambrook 2021a] and the note to the entry for Polyacrylamide gel electrophoresis in the Materials list of Protocol: **Mapping RNA with Nuclease S1** [Green and Sambrook 2021a]). The method used to prepare a minidenaturing polyacrylamide gel is described in Step 1 of Protocol: **Mapping RNA with Nuclease S1** (Green and Sambrook 2021a). The preparation of larger gels is described in Protocol: **Separation of RNA according to Size: Electrophoresis of RNA through Denaturing Urea Polyacrylamide Gels** (Green and Sambrook 2021b).*

Dithiothreitol (1 M)
DNA markers, radiolabeled, for gel electrophoresis

*See Introduction: **Mapping RNA** (Green and Sambrook 2021c) (the section Optimizing Primer Extension Reactions).*

Ethanol
Formamide loading buffer for RNA gels <R>
Input RNA to be analyzed

Preparations of poly(A)⁺ RNA are preferred, especially when setting up primer extension reactions for the first time or when preparations of total RNA produce extension products of different lengths.

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KCl (1.25 M)

Oligonucleotide primer

These primers should be 20–30 nt in length and preferentially purified through Sep-Pak chromatography and by gel electrophoresis. Crude preparations of oligonucleotides give rise to higher backgrounds on the autoradiogram, especially in the area of the film corresponding to the low-molecular-weight region of the polyacrylamide gel. Resuspend the purified oligonucleotide at a concentration of ~60 ng/μL (5–7 pmol/μL) in TE (pH 7.6).

Phenol

Polynucleotide kinase

Primer extension mix for RNA analysis <R>

Protein inhibitor of RNase

*These inhibitors are sold by several manufacturers under various trade names (e.g., RNasin from Promega, PRIME Inhibitor, 5 Prime → 3 Prime). For more details, see the section Inhibitors of RNases in Introduction: **How to Win the Battle with RNase** (Green and Sambrook 2019).*

Reverse transcriptase

A cloned version of reverse transcriptase encoded by the Moloney murine leukemia virus (Mo-MLV) is the enzyme of choice in this protocol. Mutants of the enzyme that lack RNase H activity (e.g., StrataScript, Stratagene) have some advantages over the wild-type enzyme because they produce higher yields of full-length extension product and work equally well at both 47°C and 37°C (for review, see Gerard et al. 1997).

Reverse transcriptase supplied by different manufacturers varies in its activity per unit. When using a new batch of enzyme, set up a series of extension reactions containing equal amounts of poly(A)⁺ RNA and oligonucleotide primer, and different amounts of enzyme. If possible, the primer should be specific for an mRNA present at moderate abundance in the preparation of poly(A)⁺ RNA. Assay the products of each reaction by gel electrophoresis as described in this protocol. Use the minimal amount of enzyme required to produce the maximum yield of extension product. The units used in this protocol work well with most batches of StrataScript.

Sodium acetate (3 M, pH 5.2)

TE buffer, 10× <R>

Prepare TE (pH 7.6).

Trichloroacetic acid (1% and 10% TCA)

Dilute 100% stock solution 1/10 and 1/100 just before use. Chill the working solutions in ice.

Water baths preset to 42°C and 95°C, and the appropriate annealing temperature (see Step 12)
Whatman 3MM filter paper (or equivalent)

Equipment

METHOD

*Prepare all reagents used in this protocol with DEPC-treated H₂O (see Introduction: **How to Win the Battle with RNase** [Green and Sambrook 2019]).*

Preparation of the Oligonucleotide Probe

1. Phosphorylate the oligonucleotide primer in a reaction containing:

Oligonucleotide primer (5–7 pmol or 60 ng)	1 μL
Distilled deionized H ₂ O	6.5 μL
Kinase buffer (10×)	1.5 μL
Polynucleotide kinase (~10 units)	1 μL
[γ-32P]ATP (7000 Ci/mmol)	2 μL

Incubate the reaction for 60 min at 37°C.

The final concentration of radiolabeled ATP in the reaction should be ~30 nM.

2. Stop the kinase reaction with the addition of 500 μL of TE (pH 7.6). Add 25 μg of carrier RNA.

3. Add 400 μL of equilibrated phenol (pH 8.0) and 400 μL of chloroform (or 800 μL of commercial phenol:chloroform [1:1]). Vortex vigorously for 20 sec. Separate the aqueous and organic phases by centrifugation for 2 min in a microcentrifuge.
4. Transfer the aqueous layer to a fresh sterile microcentrifuge tube and extract with 800 μL of chloroform. Vortex vigorously for 20 sec. Separate the aqueous and organic phases by centrifugation for 2 min in a microcentrifuge. Again transfer the aqueous layer to a fresh sterile microcentrifuge tube.
5. Repeat Step 4.
6. Add 55 μL of sterile 3 M sodium acetate (pH 5.2) and 1 mL of ethanol to the aqueous layer from Step 5. Mix by vortexing, and store the solution for at least 1 h at -70°C .
7. Collect the precipitated oligonucleotide primer by centrifugation at maximum speed for 15 min at 4°C in a microcentrifuge. Remove and discard the radioactive supernatant. Wash the pellet in 70% ethanol and centrifuge again. Discard the supernatant and dry the precipitate in the air. Dissolve the precipitate in 500 μL of TE (pH 7.6).
8. Count 2 μL of radiolabeled oligonucleotide primer in 10 mL of scintillation fluid in a liquid scintillation counter. Calculate the specific activity of the radiolabeled primer assuming 80% recovery. The specific activity should be $\sim 2 \times 10^6$ cpm/pmol of primer.

Hybridization and Extension of the Oligonucleotide Primer

9. Mix 10^4 to 10^5 cpm (20–40 fmol) of the DNA primer with 0.5–150 μg of the RNA to be analyzed. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ethanol. Store the solution for 60 min at -70°C , and then recover the RNA by centrifugation at maximum speed for 10 min at 4°C in a microcentrifuge. Wash the pellet with 70% ethanol and centrifuge again. Carefully remove all of the ethanol, and store the pellet at room temperature until the last visible traces of ethanol have evaporated.

*The primer should be in ~ 10 -fold molar excess over the template RNA (see the discussion on Optimizing Primer Extension Reactions in Introduction: **Mapping RNA** [Green and Sambrook 2021c]).*

10. Resuspend the pellets in 8 μL of TE (pH 7.6) per tube. Pipette the samples up and down several times to dissolve the pellets.
11. Add 2.2 μL of 1.25 M KCl. Vortex the samples gently, and then deposit the fluid in the base of the tubes by centrifuging for 2 sec in a microcentrifuge.
12. Place the oligonucleotide/RNA mixtures in a water bath set at the appropriate annealing temperature. Incubate the samples for 15 min at the optimum temperature, as determined in preliminary experiments (see the discussion on Optimizing Primer Extension Reactions in Introduction: **Mapping RNA** [Green and Sambrook 2021c]).

The kinetics of annealing between the oligonucleotide primer and the mRNA template are remarkably rapid under typical primer extension conditions, in which the primer is in excess of the target mRNA. For this reason, the time of annealing in Step 12 can be limited to 15 min. Some protocols include elaborate heating and cooling routines at this step, but in our hands, these Byzantine variations are rarely necessary.

13. While the oligonucleotide and RNA are annealing, supplement an aliquot of primer extension mix with dithiothreitol and reverse transcriptase as follows: Thaw a 300- μL aliquot of primer extension mix on ice and then add 3 μL of 1 M dithiothreitol and reverse transcriptase to a concentration of 1–2 units/ μL . Add 0.1 unit/ μL of protein inhibitor of RNase, gently mix by inverting the tube several times, and store it on ice.

It is possible to establish the DNA sequence of a primer-extended product by including dideoxynucleotides (terminators) in the reaction mix. In a 5'-end mapping experiment, knowing the exact sequence of the primer-extended product allows precise positioning of the end in the 5'-flanking region of the gene. This approach has been successful in many laboratories when examining mRNAs that are relatively abundant in the cell (class I antigens, rat liver steroid 5α -reductase mRNA, and yeast alcohol dehydrogenase 2 mRNA). For protocols on primer extension sequencing, see Geliebter et al. (1986) and Hahn et al. (1989).

14. Remove the tubes containing the oligonucleotide primer and RNA from the water bath, and deposit the fluid in the bases of the tubes by centrifuging for 2 sec in a microcentrifuge.
15. Add 24 μ L of supplemented primer extension mix to each tube. Gently mix the solution in the tubes, and again deposit the liquid at the tube bottoms by centrifugation.
16. Incubate the tubes for 1 h at 42°C to allow the primer extension reaction to proceed.
17. Terminate the primer extension reactions by the addition of 200 μ L of TE (pH 7.6), 100 μ L of equilibrated phenol (pH 8.0), and 100 μ L of chloroform. Vortex for 20 sec. Separate aqueous and organic phases by centrifugation for 4 min at room temperature in a microcentrifuge.

There is often a considerable amount of radioactivity remaining in the well of the polyacrylamide gel after electrophoresis (see Step 22 below). In our hands, there is rarely a correlation between the amount of this radioactivity in the well and the amount of the desired primer extension product. The aggregated material in the well may represent longer extension products derived by spurious priming of the oligonucleotide on nontarget mRNAs or contaminating genomic DNA templates. Rarely, this background can represent aggregates of the desired primer extension product with molecules of RNA.

If there is a significant amount of radioactivity trapped in the well of the gel, try treating the primer-extended products after Step 16 with RNase: Add 1 μ L of 0.5 M EDTA (pH 8.0) and 1 μ L of DNase-free pancreatic RNase (5 mg/mL) to each tube, and incubate the reactions for 30 min at 37°C. Add 150 μ L of TE (pH 7.6) containing 0.1 M NaCl and 200 μ L of phenol:chloroform. Vortex for 30 sec, and centrifuge at maximum speed for 5 min at room temperature in a microcentrifuge. Continue the protocol at Step 18. Alternatively, the primer-extended products from Step 16 can be treated with NaOH to hydrolyze the RNA template before electrophoresis: Add 1.0 μ L of 10 N NaOH to the solution, and incubate for 10 min at room temperature. Neutralize the NaOH by the addition of 1/10 volume of 3 M sodium acetate (pH 5.2), and continue the protocol at Step 17.

18. Precipitate the nucleic acids by the addition of 50 μ L of 10 M ammonium acetate and 700 μ L of ethanol. Mix well by vortexing, and incubate the ethanol precipitations for at least 1 h at –70°C.

Purification and Analysis of the Primer Extension Products

19. Collect the precipitated nucleic acids by centrifugation for 10 min at 4°C in a microcentrifuge. Carefully rinse the pellets with 400 μ L of 70% ethanol. Centrifuge again for 5 min at 4°C, and remove the 70% ethanol rinse with a pipette. Store the open tubes at room temperature until all visible traces of ethanol have evaporated.
20. Dissolve the nucleic acid precipitates in 10 μ L of formamide loading buffer. Pipette the samples up and down to assist resuspension.
21. Heat the samples for 8 min at 95°C. Then plunge the tubes into an ice-water bath, and immediately analyze the primer extension products by electrophoresis through a denaturing polyacrylamide gel.

*End-labeled DNA fragments of known size should be used as molecular-weight markers on the gel (see the discussion on Optimizing Primer Extension Reactions in Introduction: **Mapping RNA** [Green and Sambrook 2021c]).*

22. After the tracking dyes have migrated an appropriate distance through the gel (Table 2 of Protocol: **Mapping RNA with Nuclease S1** [Green and Sambrook 2021a]), turn off the power supply and dismantle the electrophoresis setup. Gently pry up one edge of the larger glass plate and slowly remove the plate from the gel. Cut off one corner of the gel for orientation purposes.

Wear eye protection when prying the glass plates apart.

23. If a polyacrylamide gel 1.0 mm in thickness was used, fix the gel in TCA. Transfer the glass plate containing the gel to a tray containing an excess of 10% TCA. Gently rock or rotate the tray for 10 min at room temperature.

The gel will usually float off the glass plate during this incubation. Do not allow the gel to fold up on itself.

This step is not necessary if a thin gel (0.4 mm thick) was used. In this case, proceed to Step 26.

24. Pour off the 10% TCA solution, and replace it with an excess of 1% TCA. Gently rock or rotate the tray for 5 min at room temperature.
25. Pour off the 1% TCA solution, and briefly rinse the fixed gel with distilled deionized H₂O. Lift the glass plate together with the gel out of the tray and place them on a flat benchtop. Apply paper towels or KimWipes to the sides of the gel to remove excess H₂O.
Do not place towels on top of gel.
26. Cut a piece of Whatman 3MM filter paper (or equivalent) that is 1 cm larger than the gel on all sides. Transfer the gel to the filter paper by laying the paper on top of the gel and inverting the glass plate.
27. Remove the plate, and dry the gel on a heat-assisted vacuum-driven gel dryer for 1.0–1.5 h at 60°C.
28. Establish an image of the gel using autoradiography or phosphorimaging.

RELATED INFORMATION

For additional details on primer extension assays, see Introduction: **Mapping RNA** (Green and Sambrook 2021c) (specifically the sections Primer Extension and Optimizing Primer Extension Reactions).

RECIPES

Formamide Loading Buffer for RNA Gels

Deionized formamide	80% (w/v)
EDTA (pH 8.0)	10 mM
Xylene cyanol FF	1 mg/mL
Bromophenol blue	1 mg/mL

Purchase a distilled deionized preparation of formamide and store in small aliquots under nitrogen at –20°C.

Alternatively, deionize reagent-grade formamide by stirring on a magnetic stirrer with Dowex XG8 mixed bed resin for 1 h and filtering it twice through Whatman No. 1 paper. Store deionized formamide in small aliquots under nitrogen at –70°C.

Primer Extension Mix for RNA Analysis

Reagent	Quantity (for 10 mL)	Final concentration
Tris-Cl (1 M, pH 8.4, at room temperature)	200 µL	20 mM
MgCl ₂ (1 M)	100 µL	10 mM
dNTP solution containing all four dNTPs (100 mM)	160 µL	1.6 mM
Actinomycin D (5 mg/mL)	100 µL	50 µg/mL

Dissolve actinomycin D in methanol at a concentration of 5 mg/mL before supplementing the primer extension mix. Store the actinomycin D stock solution at –20°C in the dark.

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TE Buffer, 10×

100 mM Tris-Cl (desired pH)

10 mM EDTA (pH 8.0)

Sterilize solutions by autoclaving for 20 min at 15 psi (1.05 kg/cm²) on liquid cycle. Store the buffer at room temperature.

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