Promega

Technical Bulletin

Primer Extension System–AMV Reverse Transcriptase

INSTRUCTIONS FOR USE OF PRODUCT E3030.

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Primer Extension System – AMV Reverse Transcriptase

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1. Description

Primer extension analysis is used to determine the location and to quantitate the amount of 5´-end of specific RNAs. An end-labeled oligonucleotide is hybridized to RNA and is utilized as a primer by reverse transcriptase in the presence of deoxynucleotides. The RNA is reverse transcribed into cDNA and is analyzed on a denaturing polyacrylamide gel. The length of the cDNA reflects the number of bases between the labeled nucleotide of the primer and the 5´-end of the RNA; the quantity of cDNA product is proportional to the amount of targeted RNA (1).

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2. Product Components and Storage Conditions

Product	Size	Cat.#
Primer Extension System—AMV Reverse Transcriptase	40 reactions	E3030

Includes:

- 500µl AMV Primer Extension 2X Buffer
- 75µl Primer Extension Sodium Pyrophosphate, 40mM
- 100ng 1.2kb Kanamycin Positive Control RNA
- 50pmol Primer Extension Control Primer (5pmol/µl)
- 50u AMV Reverse Transcriptase (AMV RT)
- 500ng ϕX174 DNA/*Hin*f I Dephosphorylated Markers
- 100u T4 Polynucleotide Kinase (T4 PNK)
- 100µl T4 Polynucleotide Kinase (T4 PNK) 10X Buffer
- 1ml Loading Dye
- 1.25ml Nuclease-Free Water

Storage Conditions: The buffers, enzymes and DNA markers should be stored at –20°C. Store the Control RNA at –70°C.

3. Primer and DNA Marker Labeling

This protocol requires a phosphorylation reaction to end-label the primer for use in subsequent primer extension analysis. DNA markers used for size determination can also be prepared by the end-labeling method (see Section 3.B).

Materials to Be Supplied by the User

- sterile microcentrifuge tubes
- primer specific for sample RNA
- [γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml)
- Whatman® DE81 2.3cm circular filters
- heat lamp
- scintillation counter
- 0.5M sodium phosphate buffer (pH 6.8)

Note: [γ-^{33}P]ATP (3,000Ci/mmol, 10mCi/ml) may be substituted in this protocol. See the Note in Section 5.

3.A. Primer Labeling

1. Assemble the following reaction in a sterile microcentrifuge tube:

Control Primer or a specific primer (10pmol; see Table 1)	2µl
T4 PNK 10X Buffer	1µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml)	3µl
T4 Polynucleotide Kinase (5–10u/µl)	10u
Nuclease-Free Water to a final volume of	**10µl**

   Note: For quantitation of rare messages, the specific activity of the primer can be increased by adding additional [γ-^{32}P]ATP to the kinase reactions.

2. Incubate at 37°C for 10 minutes.

3. Heat to 90°C for 2 minutes to inactivate the T4 PNK, then centrifuge briefly in a microcentrifuge. Bring the final concentration of the Control Primer to 100fmol/µl by adding 90µl of Nuclease-Free Water. Store at –20°C.

   Note: The final concentration of the specific end-labeled primer can be adjusted at this point by adding an appropriate amount of Nuclease-Free Water (see Section 4.B, Step 1, Note).

4. Determine the percent incorporation:

   a. Add 4µl of phosphorylated primer to 12µl of water and mix. Spot 3µl of the diluted, phosphorylated primer onto each of four Whatman® DE81 2.3cm circular filters.

   b. Dry the filters briefly under a heat lamp. Set two filters aside for the determination of total cpm in the sample ("total" filters).

   c. Wash the other 2 filters ("incorporated" filters) twice in 50ml of 0.5M sodium phosphate buffer (pH 6.8), for 5 minutes each, to remove the unincorporated [γ-^{32}P]ATP.

   d. Dry the washed filters under a heat lamp.

   e. Place the filters in individual vials, add appropriate scintillation fluid and count in a scintillation counter.

   f. Calculate the average cpm for the "total" and "incorporated" filters: Percent incorporation = (cpm incorporated/cpm total) × 100.

      The expected incorporation is at least 30%, and incorporation lower than this may result in less than optimal primer extension, particularly with rare messages.

Table 1. Amount of Primer Needed to Equal 10pmol.

Primer Length	ng of Primer Equal to 10pmol
15mer	50ng
16mer	53ng
17mer	56ng
18mer	59ng
19mer	63ng
20mer	66ng
24mer	79ng
In general: ng of primer = pmol of primer × 0.33 × N, where N = length of primer in bases.	

3.B. DNA Marker Labeling

1. Assemble the following reaction in a sterile microcentrifuge tube:

ϕX174 DNA/*Hin*f I Dephosphorylated Markers (250ng)	5µl
T4 PNK 10X Buffer	1µl
[γ-^{32}P]ATP (3,000Ci/mmol, 10mCi/ml)	3µl
T4 Polynucleotide Kinase (5–10u/µl)	10u
Final Volume	**10µl**

2. Incubate at 37°C for 10 minutes.

3. Heat to 90°C for 2 minutes to inactivate the T4 PNK. Centrifuge briefly in a microcentrifuge and add 190µl of Nuclease-Free Water. Store at –20°C. Loading 1µl of the diluted phosphorylated markers onto a gel will provide dark bands on an autoradiogram following overnight exposure of the gel to film. Figure 1 shows the banding pattern of the ϕX174 DNA/*Hin*f I Dephosphorylated Markers (Cat.# E3511) on a denaturing polyacrylamide gel.

   Note: For accurate sizing of single-stranded denatured DNA or RNA, the markers must be denatured before loading onto the gel. Before loading, add the phosphorylated markers directly to the Loading Dye, heat at 90°C for 10 minutes and then immediately load the markers onto the denaturing gel (see Section 5).

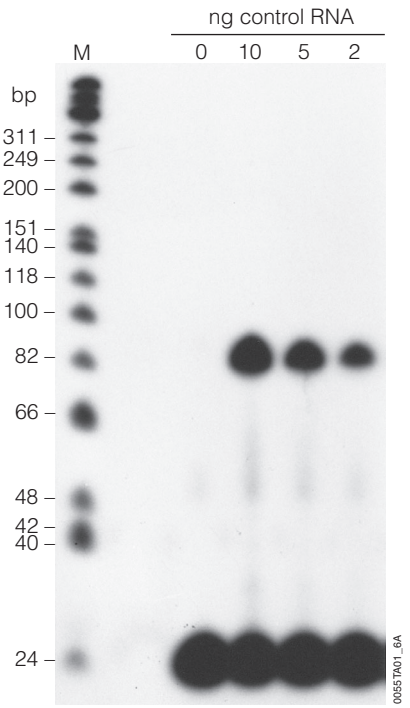

Figure 1. Gel analysis of ^{32}P-labeled DNA markers and Control RNA primer extension products. ϕX174 DNA/*Hin*f I Dephosphorylated Markers (lane M) and the products from primer extension reactions using Control RNA were separated by electrophoresis on an 8% denaturing polyacrylamide gel (19:1 acrylamide:bis). Zero, 10, 5 or 2ng of Control RNA were used in the reaction, and one-half of each reaction was loaded onto the gel. The reactions and gel electrophoresis were performed as described in the protocol with the exception that the film was exposed overnight at room temperature with no intensifying screen. The length in base pairs for each *Hin*f I fragment in the marker lane is indicated next to the corresponding band on the autoradiogram. In the lanes containing Control RNA, the upper band (87 bases) represents the major cDNA product of the Control RNA, and the lower band (25 bases) represents the ^{32}P-labeled Control Primer.

4. Primer Extension Reaction

To assay the levels of a specific RNA, cDNA is synthesized using an end-labeled primer complementary to the target RNA. The amount of cDNA obtained is a measure of the amount of target RNA, and the size of the cDNA reflects the distance from the primer to the 5´-end of the RNA.

For effective primer extension, the length of the primer and the distance of the primer from the 5´-end of the RNA should be considered. A primer of at least 20 nucleotides will allow for a high degree of specificity when used with a mixed population of RNA (2). Ideally, the primer should hybridize approximately 100 bases downstream from the 5´-end of the RNA and should not self-anneal (3).

Materials to Be Supplied by the User

- sterile microcentrifuge tubes
- sample RNA
- labeled primer specific for sample RNA
- waterbaths, heat block or incubator at 58°, 42° and 90°C

4.A. Experimental Design Considerations for Sample and Control RNA

Control RNA

The 1.2kb Kanamycin Positive Control RNA (Control RNA) is an in vitro transcription product. It is provided at a concentration of 10ng/µl. One nanogram of Control RNA is approximately 2.5fmol. The amount of Control RNA recommended for the primer extension reaction depends upon the intensity of the autoradiographic signal required. For example, using room temperature exposure of the gel to film, 10ng of Control RNA per reaction provides a strong signal after a 3-hour exposure; 5ng of Control RNA gives an intermediate signal after 3 hours but a strong signal after overnight exposure; 2ng of Control RNA also provides a good signal after an overnight exposure.

Sample RNA

Target RNA preparations should be checked prior to performing primer extension analysis to verify the integrity of the RNA. For example, assess RNA sample integrity by gel analysis for the presence of 18S and 28S ribosomal bands in total RNA samples. The presence of a specific RNA can be confirmed prior to primer extension by Northern blot or RT-PCR analysis.

The optimal amount of RNA and primer to use per assay depends on the type of RNA analyzed (total RNA or poly(A)+) and the relative abundance of the RNA of interest. When using total RNA, 10µg of RNA per reaction is a good starting point (1), but it may be necessary to use as much as 100µg per reaction for analysis of rare messages. Poly(A)+ RNA samples will require 0.1–1µg of RNA per reaction, depending upon the relative enrichment of poly(A)+ RNA over total RNA and the relative amount of the specific RNA to be assayed.

4.B. Protocol for Primer Extension of Control and Sample RNA

1. Combine the components for primer annealing in microcentrifuge tubes. Mix by gently pipetting. Dilute the control RNA in Nuclease-Free Water to provide the desired amount of RNA per 5µl aliquot (see Section 4.A, Control RNA). The Sample RNA may or may not need to be diluted to obtain the desired amount of RNA per 5µl aliquot.

   Note: For accurate quantitation of a particular RNA, the primer should be in excess of the RNA. For example, 100fmol of Control Primer is in excess of the amount of Control RNA in the reaction listed previously. However, a large excess of primer can lead to the appearance of nonspecific primer extension products. The amount of a specific primer may need to be adjusted for each type of transcript in order to optimize the ratio between primer and RNA in the primer extension reaction.

	Primer Extension	**no RNA**
Control RNA or Sample RNA	5µl	—
Nuclease-Free Water	—	5µl
^{32}P labeled Control Primer or specific labeled primer	1µl	1µl
AMV Primer Extension 2X Buffer	5µl	5µl
Final Volume	**11µl**	**11µl**

   If the same primer is used for several samples, a primer mixture can be prepared by combining AMV Primer Extension 2X Buffer and the primer in a tube and dispensing 6µl of the mixture into each reaction tube. For example, if there are 5 reactions, the primer mixture can be prepared for 6 tubes, thus ensuring adequate volume for 5 aliquots.

2. Anneal the primer and RNA by heating the tubes at 58°C for 20 minutes followed by cooling at room temperature for 10 minutes.

   The 58°C temperature is optimal for annealing the Control Primer and Control RNA. The optimal temperature for other primers and templates may be different. In general, the optimal annealing temperature should be at or near the melting temperature (T_m) for the primer (1). Time may be extended from 1 hour to overnight.

   Note: Use of a denaturing hybridization solution may be necessary to maximize annealing of the primer to complex mixtures of RNA. The following solution is recommended: 40mM PIPES (pH 6.4), 1mM EDTA, 0.4M NaCl and 80% deionized formamide (1). Anneal the primer and RNA by heating the tubes at **45°C** when using the denaturing hybridization solution. Formamide and salts in the hybridization solution must be eliminated before the final primer extension reaction. After annealing the primer and RNA, perform an ethanol precipitation to recover the annealed RNA. Suspend in 6µl water and 5µl of the AMV Primer Extension 2X Buffer before continuing with Step 3 of the protocol.

4.B. Protocol for Primer Extension of Control and Sample RNA (continued)

3. Prewarm the 40mM Sodium Pyrophosphate, Nuclease-Free Water and AMV Primer Extension 2X Buffer to room temperature or 37°C. (Sodium pyrophosphate may precipitate in solutions that are cooler than room temperature.) Combine the components for a "master" reverse transcriptase (RT) extension mix. For example, to ensure sufficient mix for 5 reactions, prepare a master RT mix for 6 tubes. Add the AMV RT last and mix by pipetting and by gently inverting the tube. Do not vortex. Prepare the following:

	Per Reaction	6 Reactions
AMV Primer Extension 2X Buffer	5µl	30µl
Sodium Pyrophosphate, 40mM	1.4µl	8.4µl
AMV RT	1u	6u
Nuclease-Free Water to a final volume of	**9µl**	**54µl**

   Immediately dispense 9µl of the RT mix into each reaction tube containing annealed primer/RNA.

4. Incubate at 41–42°C for 30 minutes.

5. Add 20µl of Loading Dye to each tube. Heat the tubes (including the markers) at 90°C for 10 minutes and load samples directly onto a gel. A typical volume loaded is 20µl of the 40µl volume. Store the remainder of the samples at –20°C.

Caution: Loading Dye contains formamide, which is a teratogen.

Note: For convenience, we have eliminated extractions and precipitations after the primer extension reaction. However, if desired, the entire reaction can be loaded onto the gel by concentrating the products of the entire primer extension reaction with an ethanol precipitation step performed immediately following the 30-minute incubation at 42°C. We have observed losses of 5–15% of the products under these conditions.

5. Gel Analysis

We typically analyze primer extension products on a 16 × 18cm denaturing polyacrylamide gel containing 8% acrylamide (19:1 acrylamide:bis), 7M urea and TBE 1X buffer. Include labeled DNA markers on the gel to determine the size of primer extension products. Run the gel at 250 volts in TBE 1X buffer until the bromophenol blue dye is at most 2cm from the bottom of the gel. Prepare the labeled DNA markers as described in Section 3.B. Add 10µl of loading dye to a tube containing 1µl of labeled marker and 9µl of TE. Heat to 90°C for 10 minutes.

Wrap the gel in plastic wrap and expose it to X-ray film with or without an intensifying screen, overnight at –70°C. Orientation of both the gel and film are necessary if the primer extension products are to be quantitated.

Note: When using ^{33}P, exposure times should be optimized (longer exposure required compared to ^{32}P). The sensitivity of the assay using ^{33}P is comparable to ^{35}S but less sensitive than ^{32}P. The decision to use ^{32}P or ^{33}P should be based on the expected abundance of the products. Because the amount of quenching for low emitters such as ^{33}P can be affected by the thickness of the gels, membrane blotting is recomended over gel drying when ^{33}P is used (4).

For quantitation, excise the appropriate bands from the gel and place into scintillation vials. Add scintillation fluid to the vials and count using a program appropriate for measuring ^{32}P decay. The band corresponding to the kinased primer alone, from the "No RNA" reactions, should also be excised and counted. Alternatively, the gel can be analyzed using phosphorimaging instrumentation.

The primer extension product of the Control RNA is 87 bases in length. It is common to see some smaller, minor bands with the Control or sample RNA. Smaller primer extension products can arise if secondary structure in the RNA, between the 5´-end and the oligonucleotide priming site, stops the reverse transcriptase during the primer extension reaction. If the ratio of primer to template or the annealing temperature is not optimized, nonspecific primer extension products can also be generated.

6. Calculations

The amount of RNA converted to cDNA can be determined by counting the cpm in the primer extension product and the "No RNA" bands. The primer extension products and the primers from the "No RNA" reactions are individually excised from the gel and counted in scintillation fluid, thereby equalizing the effects of quenching by polyacrylamide and of ^{32}P decay. Alternatively, the "cpm" equivalents can be determined using a phosphorimaging instrument and the appropriate software.

The following example describes how to calculate the femtomoles of RNA converted to cDNA. In this example, one-half of the sample primer extension product (20µl of 40µl) was loaded onto a gel. The primer alone from the "No RNA" reaction contained 100fmol of primer, and one-half of this reaction was also loaded onto the gel. The counts in the excised bands were:

Sample: 3,912cpm
Primer: 142,359cpm

First, calculate the cpm/fmol primer:

cpm/fmol = 142,359cpm/[100fmol × (20/40)] = 2,847cpm/fmol

The 20/40 factor corrects for the amount of primer loaded onto the gel. If a different fraction of the reaction is loaded onto the gel, this factor should be adjusted accordingly.

Next, calculate the fmol of primer extension product, or cDNA:

The cpm in the primer extension product is converted to fmol based upon the cpm/fmol of the primer. This assumes each cDNA was synthesized from a single primer that hybridized to the target RNA.

$$\text{fmol cDNA} = \frac{\text{cpm in cDNA product}}{\text{cpm/fmol} \times \text{fraction of reaction loaded}}$$

Sample: 3,912cpm/[2,847cpm/fmol × (20/40)] = 2.75fmol cDNA

These values represent the amount of cDNA synthesized in the primer extension reaction.

7. Composition of Buffers and Solutions

T4 Polynucleotide Kinase 10X Buffer

500mM	Tris-HCl (pH 7.5)
100mM	$MgCl_2$
50mM	DTT
1mM	spermidine

AMV Primer Extension 2X Buffer

100mM	Tris-HCl (pH 8.3 at 42°C)
100mM	KCl
20mM	$MgCl_2$
20mM	DTT
2mM	each dNTP
1mM	spermidine

Loading Dye

98%	formamide
10mM	EDTA
0.1%	xylene cyanol
0.1%	bromophenol blue

0.5M sodium phosphate buffer (pH 6.8)

86.18g	$Na_2HPO_4 \cdot 7H_2O$
24.63g	$NaH_2PO_4 \cdot H_2O$

Bring to a final volume of 1L with water; adjust pH if neccessary.

TBE 1X buffer

89mM	Tris base
89mM	boric acid
1.9mM	EDTA

8. References

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