SOP: Primer Extension

By

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A. Oligonucleotide labeling by phosphorylation

1. Add the following components to a 1.5-ml microcentrifuge tube:

Oligonucleotide primer (2 µM)	5 µl (10 pmol)
5× Forward Reaction Buffer	10 µl
T4 Polynucleotide Kinase (Gibco BRL)	2 µl (20 units)
[γ-^{32}P]ATP (10 µCi/µl, 3000 Ci/mmol)	5 µl (50 µCi)
Sterile water	28 µl
Total:	50 µl

Note:

a) The oligonucleotide used should be:
(i) a 30- to 40-mer to allow a reasonable stringency of hybridization conditions; and

(ii) exon I sequence, close to the predicted transcription start site (not more than 100–150 bp).

b) Instead of [α-^{32}P]dCTP, [γ-^{32}P]ATP must be used in this analysis.

2. Incubate at 37°C for 10 min.

3. Heat at 65°C for 10 min to stop the reaction.

4. Purify the oligonucleotide using the QIAquick Nucleotide Removal Kit from Qiagen. Elute the oligonucleotide with 200 µl EB buffer.

5. Count 5 µl by liquid scintillation counting.

B. Hybridization

1. Mix 2×10^4 cpm of the labeled oligonucleotide with 10 µg (up to 50 µg, depending on gene expression level) of the total RNA analyzed (Note: Overloading of primer would cause artifactual bands).

2. Add 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol (–20° C). Store the solution at –80°C for at least 1 h.

3. Centrifuge at 12,000× g for 15 min at 4°C.

4. Wash the pellet with 70% ethanol (–20° C) and re-centrifuge for 5 min at 4°C.

5. Carefully remove all of ethanol and air-dry the pellet for 5 min (Note: An over-dried pellet cannot be easily re-dissolved).

6. Redissolve the pellet in 30 µl of hybridization buffer (40 mM PIPES (pH 6.4); 1 mM EDTA (pH 8.0); 0.4 M NaCl; 80% deionized formamide) by drawing through a pipettor tip 50–100 times.

7. Denature the nucleic acids at 85°C for 10 min. Quickly transfer the hybridization mixture to a water bath set at 30–35°C. Incubate for 8–12 h or overnight (i.e. 16 h).

8. Add 170 µl sterile water and 400 µl absolute ethanol (–20°C), mix well, and place on ice for 1 h. Repeat steps 3–5.

C. Primer extension

1. Redissolve the RNA:primer hybrid in the following RT buffer:

5×First Strand Buffer	8 µl
0.1 M DTT	4 µl
50 mM dNTP mix	4 µl
RNase inhibitor or RNaseOut (Gibco, BRL)	2 µl
DEPC-treated water	20 µl

Note: Such a high dNTP concentration (i.e. 5 mM) helps minimize artifact bands.

2. Incubate at 42°C for 2 min. Add 2 µl (400 U) of SUPERSCRIT II and gently re-suspend. Incubate for 50 min at 42°C.

3. Inactivate the reaction by heating at 70°C for 15 min. Slowly cool down to room temperature.

4. Add 2 µl DNAase-free pancreatic RNAase (5 µg/ml). Incubate the reaction for 30 min at 37°C. A further 3 min-incubation at 95°C can enhance complete RNA degradation. Slowly cool down to room temperature.

6. Add 158 µl TE buffer and 200 µl phenol: chloroform:IAA (24:24:1). Vortex for 30 s, and centrifuge at full speed for 5 min at room temperature.

6. Transfer the upper, aqueous phase to a clean tube. Add 0.1 volume of 3 M sodium acetate (pH 5.2), 2.5 volumes of absolute ethanol (–20°C) and glycogen.

7. Store the solution at –80°C for at least 1 h. Centrifuge at 14,000 rpm for 15 min at 4°C. Wash the pellet with 70% ethanol (–20° C) and re-centrifuge for 5 min at 4°C. Air-dry the primer-extended product for 5 min.

8. Dissolve the pellet in 4 µl TE buffer, add 6 µl formamide loading buffer (80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue), and mix well. Store at –20°C before gel electrophoresis.

9. Heat 5 µl of the mixture for 5 min at 95°C. Immediately chill on ice.

10. Analyze the radiolabeled cDNA by co-electrophoresis with a DNA sequencing ladder generated from an appropriate genomic clone (exon I + promoter) using the same oligonucleotide of above, through a 6% denaturing polyacrylamide gel.