Primer Extension Analysis Using AMV Reverse Transcriptase

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[Abstract] Primer extension analysis is a useful method to determine the transcription start point or a processing site on an RNA molecule. It can also allow a quantitative measurement of an RNA species.

Materials and Reagents

1. AMV reverse transcriptase from the avian myeloblastosis virus (Finnzyme, catalog number: F570) (other reverse transcriptases can also be used, by adapting the reaction buffer)
2. T4 polynucleotide kinase + 10x reaction buffer (Biolabs, catalog number: M0201)
3. RNasin Plus RNase inhibitor (Promega Corporation, catalog number: N2611)
4. Glycogen (Acros Organics, catalog number: 422950010)
5. NaCl
6. Tris HCl (pH 7.5)
7. EDTA
8. $MgCl_2$
9. EtOH
10. dATP, dCTP, dGTP, dTTP (Promega Corporation, catalog number: U120D, U121D, U122D, U123D)
11. γ-^{32}P ATP (3,000 Ci/mmole, 10 µCi/µl) (PerkinElmer, catalog number: BLU502A)
12. DTT (Promega Corporation, catalog number: P117B)
13. 5x ss-Hybridization buffer (see Recipes)
14. 1.25x RT buffer (see Recipes)

Equipment

1. Incubator

Procedure

A. Primer labelling
   1. Labelling mix (total volume 10 µl):
      a. 1 µl oligonucleotide (20-25 mer, 10 pmoles/µl)
      b. 3.5 µl γ^{32}P ATP (10 µCi/µl)
      c. 1 µl T4 DNA polynucleotide kinase
      d. 1 µl 10x polynucleotide kinase reaction buffer
      e. 3.5 µl H_2O
   2. Incubate 30 min at 37 °C, stop reaction on ice.

B. Precipitation
   1. Add 1/10 vol 10 M LiCl.
   2. Add 3 vol EtOH (96 %).
   3. Incubate 30 min at -80 °C.
   4. Centrifuge 20 min at >10,000 *x g* at room temperature (RT), take off supernatant, don't wash.
   5. Air-dry pellet and take up in 10 µl (consider concentration to be 0.5 pmoles/µl).

C. Annealing step
   1. Annealing mix (total volume 10 µl):
      a. 10 - 20 µg total RNA
      b. 10 U RNasin RNase inhibitor
      c. 0.5 pmole of 5' labelled primer (1 µl)
      d. 2 µl 5x ss-hybridization buffer
      e. Add water to 10 µl

D. Extension step
   1. Add directly to the annealing mix:
      a. 40 µl 1.25x RT buffer (pre-warmed at 50 °C)
      b. 10 U AMV transcriptase
      c. 10 U RNasin inhibitor
   2. Incubate 30 min at 50 °C (extension).

E. Extension termination
   1. Add to the reaction mix:
      1 µl EDTA (0.5 M)

6 µl NaOH (1 M)

2. Incubate 10 min at 55 °C
3. Add 6 µl HCl (1 M) (neutralization).

F. Precipitation

1. Add 1/10 vol 10 M LiCl
2. Add 25 µg glycogen
3. 2.5 vol EtOH
4. Incubate at -20 °C for > 1 h.
5. Centrifuge 10 min at >10,000 *x g* at room temperature, wash pellet 1x with 70% EtOH.
6. Air-dry pellet and take up in 15 µl of classic DNA Loading dye buffer.

G. Separation of reaction products on denaturing polyacrylamide gel.

Recipes

1. 5x ss-Hybridization buffer
   1.5 M NaCl
   50 mM Tris HCl (pH 7.5)
   10 mM EDTA
2. 1.25x RT buffer
   1.25 mM dATP
   1.25 mM dCTP
   1.25 mM dGTP
   1.25 mM dTTP
   12.5 mM DTT
   12.5 mM Tris HCl (pH 8)
   7.5 mM $MgCl_2$

Acknowledgments

This laboratory protocol is a free adaption of various published and unpublished protocols and has evolved over time. We acknowledge the support by funds from the CNRS (UPR 9073) and Univ Paris Diderot, Sorbonne Paris Cite.

References

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