Polyadenylated RNA Sampling

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[Abstract] Polyadenylation is a post-transcriptional modification of RNA occurring in prokaryotes, eukaryotes and organelles. However, the function and extent of bacterial polyadenylation are in marked contrast to those of eukaryotic poly(A) tails. In fact, the long poly(A) tails of eukaryotic mRNAs play an important role in their exportation to the cytoplasm and promote mRNA stability and translation, whereas the short bacterial tails facilitate RNA decay. One of the obstacles encountered by investigators studying bacterial polyadenylation is the scarcity of polyadenylated RNAs. The method described here allows reverse transcription and PCR amplification of the whole population of polyadenylated RNAs provided that the poly(A) tails are long enough to hybridize to oligo dT30 sequence. To this end utilization of exoribonucleases deficient strains may be useful.

Materials and Reagents

1. Kit SMART cDNA (BD Biosciences, catalog number: PT3041-1)
2. Sfi I restriction enzyme
3. pDNR-LIB (BD Biosciences, catalog number: PT3508-5)
4. Jet sorb kit (Genomed)
5. *Escherichia coli* XLI Blue cells
6. Wild-type bacteria or bacteria deficient in 3'-->5' exoribonucleases (see Reference 1)
7. Phenol (MP) chloroform (Merck KGaA)

8. T4 DNA ligase (New England Biolabs)
9. ATP (Promega Corporation)
10. Powerscript reverse transcriptase
11. Ethanol
12. Potassium chloride
13. LB medium
14. Buffer 1 (see Recipes)
15. Buffer 2 (see Recipes)

Equipment

1. Beckman centrifuges
2. JA20 rotor

Procedure

A. RNA extraction

1. Bacteria were grown in LB medium.
2. At OD_{650}=0.4 10 ml aliquots were rapidly mixed with an equal volume of 100% ethanol preequilibrated at -70 °C.
3. Cells were pelleted for 10 min at 4,300 *x g* and 4 °C in the JA20 rotor of a Beckman centrifuge.
4. Cells were resuspended in 1.5 ml 4 °C preequilibrated buffer 1.
5. Mix with 1.5 ml of buffer 2 lysis buffer prewarmed at 95 °C.
6. Cells were lysed for 2 min at 95 °C.
7. RNAs were extracted twice at 65 °C with 3 ml of phenol saturated with water.
8. RNAs were extracted once with chloroform at room temperature.

9. RNAs were then precipitated with 2.5 volumes ethanol in the presence of 0.3 M NaCl, washed with 70% ethanol, and dissolved in water.
10. RNA concentration was determined using a Nanovue.

B. RT-PCR

1. 1 µg total RNA was incubated with 10 pmol CDSIII/3'PCR (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30NN-3') and 10 pmol d'oligo SMART IV (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3') oligos (kit SMART cDNA, BD Biosciences) dissolved in water.
2. Incubate for 2 min at 72 °C then 2 min in ice.
3. Add 10 units PowerScript reverse transcriptase in 2 mM DTT, 2 mM dNTP, 1x buffer from the supplier).
4. Incubate for 1 h at 42 °C.
5. Amplify cDNA with 20 pmol 5'PCR Primer (5'-AAGCAGTGGTATCAACGCAGAGT-3'), 20 pmol CDSIII/3'PCR oligo, 0,2 mM dNTP, 2 µl Avantage 2 Polymerase Mix 1x. Incubate 1 min at 92 °C, then perform 22 cycles (15 sec at 95 °C, 6 min at 68 °C).
6. PCR products were analysed on agarose 1% and purified according to their size if required.

C. Cloning and sequencing

1. Digest cDNAs with 200 units Sfi I for 3 h at 37 °C.
2. Digest pDNR-LIB plasmid with 30 units Sfi I for 3 h at 37 °C.
3. Purify the linear plasmid on agarose gel and elute the DNA by using the Jetsorb kit.
4. Precipitate both cDNAs and plasmid and resuspend in water.
5. Ligate cDNAs and linear plasmid with 600 units T4 DNA ligase, 1 mM ATP, in recommended buffer for the night at 16 °C for 24 h.
6. 150 µl supercompetent *Escherichia coli* XLI Blue were transformed with 5 µl ligation mix.

Recipes

1. Buffer 1

   10 mM Tris-HCl (pH 7.3)

   10 mM potassium chloride

   5 mM magnesium chloride buffer

2. Buffer 2

   20 mM Tris-HCl (pH 7.9)

   200 mM NaCl

   40 mM EDTA

   1% SDS

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References

1. BD SMART ™ PCR cDNA Synthesis Kit User Manual.
2. Maes, A., Gracia, C., Hajnsdorf, E. and Regnier, P. (2012). Search for poly(A) polymerase targets in *E. coli* reveals its implication in surveillance of Glu tRNA processing and degradation of stable RNAs. *Mol Microbiol* 83(2): 436-451.

