Synthesis of 5' end-labeled RNA

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[Abstract] 5' end-labeled RNA molecules are useful substrates to analyse the endo- and exonucleolytic activities of various ribonucleases. Here two protocols are given to synthesize P^{32} labeled RNAs with a 5' PPP or 5' P moiety. 5' exoribonucleases generally do not work on 5' PPP RNA and require a 5' P substrate. The activity of certain endoribonucleases like *E. coli* RNase E or *B. subtilis* RNase Y can be stimulated by a 5' P moiety.

Materials and Reagents

1. RQ1 Dnase (Promega Corporation, catalog number: M6101)
2. Calf intestinal phosphatase (F. Hoffmann-La Roche, catalog number: 713023)
3. Phenol/Chlorophorm (1:1) (MP Biomedicals, catalog number: AQUAPH01)/Carlo Erba, catalog number: 438601)
4. T7 RNA polymérase (Promega Corporation, catalog number: P207B)
5. T4 Polynucleotide Kinase (Biolabs, catalog number: M0201)
6. RNasin RNase inhibitor (Promega Corporation, catalog number: N2611)
7. DTT(Promega Corporation, catalog number: P117B)
8. NTPs (F. Hoffmann-La Roche, catalog number: 1277057)
9. γ^{32}P GTP (6,000 Ci/mmole, 10 µCi/µl) (PerkinElmer, catalog number: BLU504Z)
10. γ^{32}P ATP (3,000 Ci/mmole, 10 µCi/µl) (PerkinElmer, catalog number: BLU502A)
11. 3 M NaOAc (pH 4.7) (see Recipes)

Equipment

1. Illustra™ MicroSpin™ G-25 column (GE Healthcare, model: 27-5325-01)

Procedure

A. Synthesis of 5'-P RNA
   1. *In vitro* Transcription (30 µl)
      T7 RNA polymerase 5x buffer 6 µl

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RNasin (40 U/µl)	0.75 µl
100 mM DTT	3 µl
2.5 mM NTPs	6 µl
DNA template (PCR fragment)	400 ng
H_2O (RNase free)	to final volume (30 µl)
T7 RNA polymerase (20 U/µl)	1.5 µl

a. Incubation 1 h 30 min at 37 °C.
b. Add to the reaction mix 2 µl of RQ1 DNase (1U/µl).
c. Incubation 30 min at 37 °C (digestion of template DNA).
d. Put on ice to stop reaction.
e. Addition of 18 µl H_2O to bring volume to 50 µl.
f. Purification of RNA by Spin-G25 column (load sample on gel bed, centrifuge 2 min at 735 *x g* (~3,000 rpm in a microfuge).

2. Dephosphorylation step (50 µl)

Calf intestinal phosphatase (CIP 1 U/µl)	2.5 µl
CIP 10x buffer	5 µl
Purified RNA	32.5 µl
H_2O	10 µl

a. Incubation 30 min at 37 °C.
b. Add 1 vol of Phenol/Chlorophorm (1:1), vortex 3 min.
c. Centrifuge 3 min at >10,000 *x g* to separate phases.
d. Precipitate upper aqueous phase by adding 1/10 vol. 3 M NaOAc (pH 4.7) + 3 vol. ETOH (96 %).
e. Centrifuge 10 min at >10,000 *x g*, take off EtOH and wash pellet once with 70% EtOH by inverting tube.
f. Air-dry pellet and take up the RNA in the desired volume of H_2O.

3. Labelling reaction (20 µl)

Dephosphorylated RNA	1 µg
T4 Polynucleotide Kinase 10x buffer	2 µl
RNase inhibitor (40 U/µl)	0.25 µl
γ^{32}P ATP (10 µCi/µl)	5 µl
T4 Polynucleotide Kinase (10 U/µl)	1.25 µl
H_2O	to 20 µl final volume

a. Incubate 2 h at 37 °C.
b. Precipitation as in step 2 and take up the pellet in desired volume.

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B. Synthesis of 5'-PPP RNA

T7 RNA polymerase 5x buffer	6 µl
RNasin (40 U/µl)	0.75 µl
DTT (100 mM)	3 µl
ATP, UTP, CTP (2.5 mM)	6 µl
GTP (100 µM)	3.75 µl
PCR	400 ng
γ^{32}P GTP	7.5 µl
H_2O	to 30 µl final volume
T7 RNA polymerase (20 U/µl)	1.6 µl

1. Incubate 1 h, 30 min at 37 °C.
2. Add to the reaction mix 2 µl of RQ1 DNase (1 U/µl).
3. Incubation 30 min at 37 °C (digestion of DNA).
4. Precipitation with 1/10 vol 3 M NaOAc (pH 4.7) + 3 vol EtOH + 25 µg Glycogen.
5. Centrifuge 10 min at >10,000 *x g*, take off EtOH and wash pellet once with 70% EtOH by inverting tube.
6. Air-dry pellet and take up the RNA in the desired volume of H_2O.

Recipes

1. 3 M NaOAc (pH 4.7)
   Dissolve 40.8 g of sodium acetate. $3H_2O$ in a final volume of 100 ml, adjust pH with glacial acetic acid.

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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