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Chapter 3.4

RNA ISOLATION USING CsCl GRADIENTS

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Through the years, different methods have been developed to extract high quality RNA from Lotus japonicus that can be used for downstream applications such as northern blot analysis, RT-PCR, and cDNA probe synthesis for microarray analysis. Several companies have developed fast and simple RNA extraction kits (Qiagene, Nucleobond, etc.) suitable for extraction from small amounts of tissue (from 100-200 mg). However, RNA prepared with kits is commonly contaminated with genomic DNA, proteins, and polysaccharides. Here we describe a RNA extraction method that yields high quality RNA from different tissue types of Lotus japonicus (Sambrook et al. 1989).

PROCEDURE FOR LYSIS AND ULTRACENTRIFUGATION

The method involves two steps. In the first step, cellular lysis is promoted by the presence of the ionic detergent Sarcosyl in the extraction buffer. Sarcosyl together with the chelating agent EDTA and the strong reductant, β-mercaptoethanol inactivates ribonucleases (RNases) by disrupting protein tertiary folding. During the first step, RNA is liberated together with other cellular components such as genomic DNA and mitochondrial DNA.

The second step includes an isopycnic ultracentrifugation through a caesium chloride (CsCl) gradient that separates RNA from DNA and proteins based on differences in their buoyant densities.

Solutions for lysis and ultracentrifugation

For all solutions, use Diethyl pyrocarbonate (DEPC)-treated H_2O or, in the case of EDTA and NaCl, treat the entire solution with DEPC (Sambrook et al. 1989). Always use freshly made extraction buffer.

Extraction buffer

- 100 mM Tris-HCl, pH 7.5

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- 50 mM EDTA, pH 8.0
- 500 mM NaCl
- 5% β-Mercaptoethanol

CsCl Cushion

- 96% CsCl
- 100 mM EDTA, pH 8.0

Sarcosyl (N-Lauryl-sarcosine) solution

- 14% Sarcosyl

NaCl solution

- 1M NaCl

Lysis and ultracentrifugation procedure

The following method is based on 0.5 g of starting material but it can be applied to larger tissue samples by increasing buffer volumes and the size of the ultracentrifuge tube.

1. Homogenize 0.5 g of tissue in liquid nitrogen and transfer the resulting powder to a 15 ml centrifuge tube.
2. Immediately add 3.33 ml of extraction buffer and 1 ml of 14% Sarcocyl, vortex briefly.
3. Add 0.65 g of CsCl powder, mix well, and incubate at 65°C for 15 min.
4. Centrifuge at 4°C for 20 min at 9500 rpm (Kontron A8.24 rotor).
5. Filter the supernatant through Miracloth (Calbiochem) and store on ice while preparing the CsCl cushion.
6. Add 1.6 ml of CsCl to a 5 ml ultracentrifuge tube.
7. Apply 3.4 ml of the filtered supernatant carefully to the top of the cushion
8. Centrifuge at 20°C for 20 hours at 40,000 rpm
9. The centrifugation results in two phases. Genomic DNA is located at the interface whereas RNA is located at the bottom of the tube below the cushion phase. Remove 1/3 of the upper phase and carefully add a similar volume of DEPC H_2O to the tube (referred to subsequently as a wash step).
10. Repeat this washing step 2 times removing more of the upper phase each time without disturbing the cushion. Make sure that the genomic DNA at the interface is completely removed to limit contamination of RNA.
11. Remove ½ of the cushion and wash with DEPC H_2O

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12. Remove the second half of the cushion, re-suspend the RNA in 133 µl DEPC H_2O and transfer to a 1.5 ml eppendorf tube
13. Add 2.5 vol absolute ethanol (EtOH), mix, and incubate at -20°C for a minimum of 2 hours.
14. Centrifuge at 4°C for 30 min at 10.000 rpm in a microcentrifuge.
15. Wash the RNA pellet in 0.5 ml 70% EtOH. Repeat the centrifugation and re-suspend the pellet in 133 µl DEPC H_2O
16. Precipitate over-night by adding 1/10 vol of 1 M NaCl and 2.5 vol of absolute EtOH.
17. Centrifuge at 4°C for 30 min at 10.000 rpm, wash with 0.5 ml 70 % EtOH, air-dry at room temperature and re-suspend the pellet in 30-50 µl of DEPC H_2O
18. Store the RNA at -80°C

The concentration and quality of the RNA is determined by measuring the absorbance at both 260 nm and 280 nm. The purity of RNA is estimated by the ratio of absorbance at 260 to 280 nm with RNA absorbing at 260 nm and proteins absorbing at 280 nm. A ratio of 1.8-2.0 indicates high purity.

DENATURING GEL-ELECTROPHORESIS OF RNA

For downstream applications such as northern blot or simply for checking the quality of the RNA (besides the spectrophotometric measurement) analysis of the RNA on a denaturing gel is appropriate. Intact RNA is usually observed as two distinct bands on the gel representing the 28S and 18S ribosomal RNA. Smearing of bands indicates degradation of the RNA. Different electrophoresis methods have been developed, but in this section, we describe the use of a formaldehyde procedure.

Solutions for electrophoresis

RNA-gel

- 1.5% agarose
- 1x gel-running buffer
- 17% formaldehyde

10x gel-running buffer

- 0.2 M MOPS
- 50 mM Sodium Acetate
- 10 mM EDTA
- Adjust pH to 7.0 using NaOH. Do not autoclave the running buffer but use sterile DEPC treated H_2O for preparing the buffer.

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RNA-loading buffer

- 20% Ficoll 400
- 0.1225% Bromophenol blue
- 0.1225% Xylene cyanol FF
- 4% Ethidium bromide

Dissolve in TE-buffer made with DEPC treated H_2O.

Electrophoresis procedure

The agarose is melted in H_2O, whereupon the running buffer and formaldehyde is added and the gel solidified. Due to the toxicity of the formaldehyde, all procedures after addition of formaldehyde are performed in a chemical hood.

The RNA samples are prepared by mixing the following:

- 10-20µg RNA (max volume 40 µl)
- 12 µl Formaldehyde
- 40 µl Formamide
- 8 µl 10x running buffer

The RNA samples are incubated at 56°C for 10 min and 10 µl of RNA loading buffer added before the samples are loaded onto the gel submerged in 1 x running buffer. The gel is run at 3-4 V/cm for 3-4 hours with circulation of the buffer.

The RNA is evaluated by UV illumination of the gel.

REFERENCES

Sambrook J, Fritsch EF, and Maniatis T. (1989). **Molecular cloning**: **A Laboratory Manual**, Ed.2. Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.