Isolating RNA is easy and fun.

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Recommended Citation
Chambers, J. A., and V.E. Russo (1986) "Isolating RNA is easy and fun.," Fungal Genetics Reports: Vol. 33, Article 2. https://doi.org/10.4148/1941-4765.1573

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Abstract
Isolating RNA is easy and fun.

This regular paper is available in Fungal Genetics Reports: https://newprairiepress.org/fgr/vol33/iss1/2
We reported previously the development of a general method for cloning Neurospora nuclear genes by sib selection, using a library of N. crassa genomic DNA fragments in plasmid pRAL1 (Akins and Lambowitz Mol. Cell. Biol. 5:2272-2278, 1985). Fig. 1 is a revised map of plasmid pRAL1.

The size of the plasmid is measured more accurately as 4.7 kb, rather than 4.4 kb reported previously. In addition, the position of the EcoR1 site in the qa-2^+ gene was indicated incorrectly in the previous map. The sizes of the EcoR1 fragments are 2.8 and 1.85 kb.

We and others have now cloned at least ten genes using the pRAL1 library: nic-1 and inl (Akins and Lambowitz, Molec. Cell. Biol. 5:2272-2278, 1985), cyt-18 (Akins and Lambowitz, unpubl.), cyt-(297-24) Kuiper, de Vries, Akins and Lambowitz, unpubl.), cyt-4 (Serizawa, Akins and Lambowitz, unpubl.), cyt-(289-4) (Kubelik and Lambowitz, unpubl.), his-2 (Akins, Lambowitz and Kinsey, unpubl.), van (Mann, Metzenberg, Akins and Lambowitz, unpubl.), cys-3 (Paletta, Marzluf, Akins and Lambowitz, unpubl.) and met-7 (Dr. M. Case, University of Georgia, personal communication). The library is available to all investigators. This work supported by NIH grant GM23961. - - - Dept. of Biochemistry, St. Louis University School of Medicine, St. Louis, MO 63104
2. Pulverize the frozen mycelium under liquid nitrogen in a mortar and pestle.

3. Resuspend the mycelium in 7-10 volumes of 6M urea, 3M LiCl. This may freeze, but this is unimportant, because it will thaw fairly quickly.

4. Pour the suspension, which should be fairly viscous, into the homogenizer vessel (50 ml vessel of the Sorvall omni-mixer) and wash the mortar out with an equal volume of urea/LiCl.

5. Homogenize the mycelium. In the 50 ml vessel of the Sorvall Om-Mixer, we use 2 x 30-60 s on ice to homogenize effectively. Any conditions that produce a useful homogenate for other extractions should do.

6. Centrifuge the homogenate. We use 10,000 rpm (16,000 g max) in the Sorvall HB4 rotor for 20 min, 4° C. It is better to use a swinging bucket rotor because the resulting pellet is more compact and firmer. A clean separation between pellet end supernatant is very important. Ignore any lipid layer on the supernatant.

7. Decant the supernatant and store at -20° C overnight. This may freeze, but it does not seem to affect yields.

8. Centrifuge as before. Discard the supernatant and drain the pellet thoroughly.

9. Resuspend the pellet by vigorous vortexing with 5 ml 0.5% SDS, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA. The suspension is often milky and viscous. If the pellet does not break up effectively, the suspension can be centrifuged (10,000 rpm, 16,000 g max 10 min 4° C, Sorvall HB4 rotor) and the pellet resuspended and extracted. This appears to give some improvement in the A260/A230 ratio (a measure of carbohydrate contamination).

10. Extract the suspension or pooled supernatants once with an equal volume of chloroform/isoamyl alcohol. It is important to obtain a good emulsion. Vortex vigorously 4 to 5 times for 15-30 s. Centrifuge (5000 rpm, 4000 g max, 10 min 4° C, Sorvall HB4 rotor) to separate the phases.

11. Remove the aqueous phase, add ammonium acetate to 2.5 M and two volumes of ethanol to the aqueous phase. Precipitate the nucleic acids for 1 h at -70° C. Recover the precipitate by centrifugation (10,000 rpm, 20 min, 4° C, Sorvall HB4 rotor), wash the pellet once or twice with 70% ethanol and dry under vacuum.

12. Redissolve the pellet in the appropriate buffer for your next step.

**Oligo (dT) cellulose chromatography**

We have used protocols described by Schleif and Wensink (Practical Methods in Molecular Biology, Springer Verlag, 1981, pp. 168-170) and by Maniatis et al. (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1982, pp. 197-198) to purify polyA\(^+\) mRNA and find that they work equally well. It is important not to load more than 10 mg of total RNA per gram of oligo (dT) cellulose. If you find a poor yield of poly A\(^+\) RNA (less than 1% of total RNA) it is possible that the column was overloaded. In that case we find that the non-binding (poly A\(-\)) fraction can be reapplied to a column after washing the column with alkali and buffer as described in the references cited above and more poly A\(^+\) RNA recovered from it. As judged by stimulation of in vitro protein synthesis and the pattern of translation products this second (and even third) extraction is as good as that obtained on the first pass. With reasonable yields of poly A\(^+\) RNA (.20 ug at 4-5 ug ml\(^-1\)) we do not need to add carrier for ethanol precipitation. With smaller quantities we use Sigma tRNA, type V, and about 100 ug ml\(^-1\) as carrier.

In general, we find that the RNA from step 12 has an A260/A280 of 2.2.1 and A260/A230 of 2.2. The poly A\(^+\) content is about 1.5% of total. There is very little DNA as judged by agarose gel-electrophoresis. As judged by methyl mercury agarose gel electrophoresis, the RNA is up to 4000 nucleotides long with little evidence of degradation of the ribosomal RNAs. Using rabbit reticulocyte lysate supplied by Amersham for in vitro translation (Cat. No. N90, 0.2 ug poly A\(^+\) in 2 ul double distilled H2O + 10 ul Reticulocytes) we typically get a 40-fold stimulation of incorporation with polypeptides in excess of 200 kd being made. Depending upon the protocol used, we find that cDNA with median lengths between 700 and 1300 nucleotides is made.
A rider to be added is that we are isolating RNA from cultures growing under rather benign conditions. If cultures are grown under conditions likely to induce high levels of nucleases (e.g. phosphate or nitrogen limitation) then there may be problems with nucleases. In that case we stress the rapid freezing in liquid nitrogen and suggest homogenising the mycelium in a larger volume of urea/lithium chloride (say 25-40 vols) to dilute the nucleases.

Acknowledgements. We thank Uta Marchfelder for excellent technical assistance. This work supported by the Deutsche Forschungsgemeinschaft - - - Max Planck Institut fur Molekulare Genetik, Ihnstrasse 73, D-1000 Berlin 33, Federal Republic of Germany

Debets, A.J.M., and C.J. Bos

Isolation of small protoplasts from Aspergillus niger

For fusion or transformation experiments with Aspergillus nidulans, we usually prepare protoplasts from conidiospores with Oerskovia lytic-enzymes (Bos and Slakhorst, 1981 Can. J. Microbiol. 400-407). The main advantage of conidial protoplasts is that they are very homogeneous and contain only one or two nuclei depending on the incubation time during the isolation procedure, whereas protoplasts from mycelium vary strongly in size and in number of nuclei. The protoplasts were suitable for fusion and transformation experiments (Bos et al. 1983 Experientia Suppl. 45:298-299; Wernars et al. 1985 Current Genetics 9:361-368).

Although it was possible to isolate protoplasts from conidiospores of some A. niger strains in the same way, it was less successful with some other strains. In these cases only osmotically stable spheroplasts were obtained. Therefore, we searched for improvement of the methods for isolation of protoplasts both from conidiospores as well as from young hyphae with the aim to obtain a homogenous suspension of small protoplasts.

Protoplasts from conidiospores.

With the strains which produce spheroplast in the usual way (see previous paragraph), protoplasts could be released from the conidiospores by careful manipulation of the osmotic strength of the lytic medium. Protoplasts were released by a gradual reduction of the osmotic strength to less than 1.0 osmol (0.4 M KCl). In the swollen state the protoplasts are very fragile and the osmotic strength had to be increased again slowly to about 1.5 osmol (0.7 M KCl). This treatment is critical and sometimes a considerable loss of protoplasts has been observed. Another disadvantage of this procedure is that the Oerskovia enzymes which are used for the lytic treatment are not available commercially.

Protoplasts from young hyphae

Owing to the problems in preparing conidial protoplasts of some A. niger strains, we modified the procedure of Musilkova and Fencl (1968 Folia Microbiol. 13:235-239) so that we obtained homogeneous protoplast suspensions from young hyphae. A. niger strains were grown for 16 h at 30°C after inoculation of 200 ml CP medium (Bos and Slakhorst 1981 l.c.) in 1000 ml Erlenmeyer flasks on a rotary shaker at about 100 rpm. The medium was inoculated with conidiospores from a 4 or 5 day old culture to a final concentration of 10^6 spores per ml. Resulting hyphae were harvested on Miracloth on a Buchner funnel and were resuspended in 20 ml lytic medium containing per ml 10 mg Novozym 234, 0.7 M NaCl, 0.2 M CaCl2 (osmotic strength about 1.8 osmol). Incubation was at 30°C for 2.5 h. The high osmotic strength of this lytic medium provides a slow release of the protoplasts from the young hyphal tips. Microscopically, the protoplasts appeared quite homogenous in size and they were stable in further experiments as long as the osmotic strength was kept above 1.2 osmol (0.6 M KCl) and below 2.3 osmol.

Using this method, up to 10^8 protoplasts could be prepared. Mycelial debris was removed by filtration over a glass wool plug. Protoplasts were concentrated by centrifugation at about 1100 x g, washed with and resuspended in a medium of suitable osmotic strength (1.5 osmol, e.g., 0.7 M KCl or 1.2 M sorbitol + 50 mM CaCl2). The protoplasts have been used successfully in fusion and in transformation experiments. The regeneration frequency was usually above 30%. - - - Dept. of Genetics, Agricultural University, 53 Gen. Foulkesweg, 6703 BM, Wageningen, The Netherlands