An Efficient Procedure for Separate Extraction of Nuclear and Cytoplasmic RNA from Cell Culture

There are increasing reports about the importance of post-transcriptional modification of mRNA (splicing) that happens in the nucleus by macromolecular ribonucleoprotein structures called splicesomes (4). Different isoforms of RNA can be generated by alternative splice sites, often tissue-specific. Moreover, several examples have been reported in which splicing errors are the molecular base of genetic diseases (1). The study of these phenomena often requires the separate isolation of nuclear and cytoplasmic RNA from cell cultures.

Our laboratory has reported a method to extract nuclear and cytoplasmic RNA from primary skin fibroblasts based on proteinase K digestion and guanidinium hydrochloride-phenol-chloroform extraction, which does not require the use of an ultracentrifuge (2). We successfully applied the same method to other primary cells, such as sternal chondrocytes. However, when we tried it in NIH 3T3 fibroblasts, we found that the nuclear RNA was contaminated by high amounts of genomic DNA that interfered with RNA migration in agarose gels. Moreover, this method is long, generally taking two separate days of work, and relatively expensive.

We found that our previously reported protocol to extract total RNA from cell culture (5) is rapid, inexpensive and leads to a good quality of DNA-free RNA suitable for Northern blot analysis, RNase protection assay and cDNA synthesis. This method is based on simultaneous DNA precipitation with potassium acetate and protein extraction with chloroform. We decided to apply the same method to extract nuclear and cytoplasmic RNA after separating the two compartments. The protocol we describe is for ten 100-mm-diameter plates of confluent NIH 3T3 fibroblasts.

After removing media, cells are washed twice with phosphate-buffered saline (PBS), scraped in 15 mL of cold PBS and spun at 200 rpm for 3 min. From this point on, all manipulations, including centrifugations, are done with the tubes at 4°C.

The pellet is re-suspended in 10 mL of RSB-Triton® X-100 (RSB = 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$) (Triton X-100 is 0.25%), transferred to a Dounce tissue homogenizer (Wheaton, Millville, NJ, USA) and homogenized with 8–10 strokes of a tightly fitting pestle. Cells are then left 5 min in ice to lyse and homogenized again with 10 strokes. At this point a sample of the solution is checked under the microscope (at 400× magnification) to verify that most of the cells are lysed. Isolated nuclei should be clearly seen, as shown in Figure 1. If not, more pestle strokes are performed until lysis is complete. After lysis, cells are spun at 2000 rpm for 5 min to pellet the nuclei.

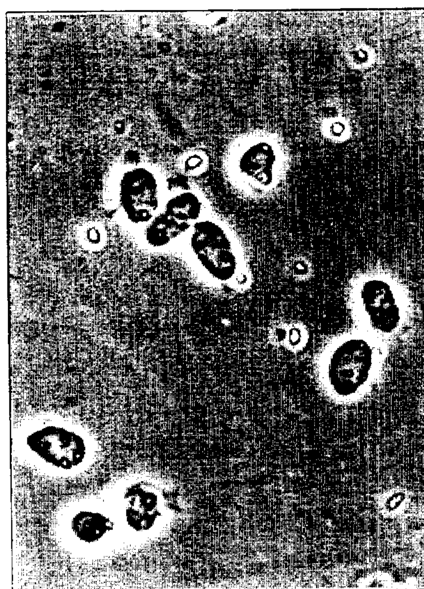

Figure 1. Phase-contrast microscopic image of nuclei extracted with the reported method at 400× magnification, following isolation from cytoplasmic compartment.

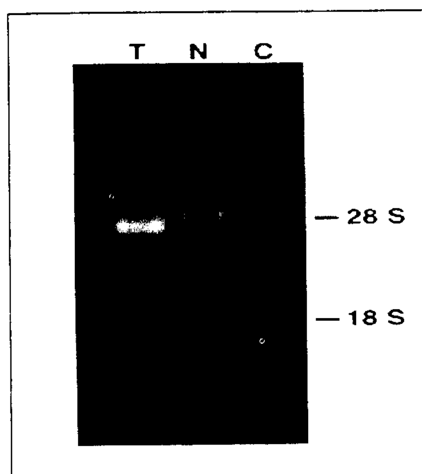

Figure 2. One percent agarose-formaldehyde gel of 5 µg of total (T), nuclear (N) and cytoplasmic (C) RNA. RNA was extracted in two separate experiments from NIH 3T3 fibroblasts using the reported technique. Nuclear and cytoplasmic RNAs were extracted from the same plate. 28S and 18S ribosomal RNA bands were visualized by staining with ethidium bromide.

The supernatant, containing the cytoplasmic compartment, is then collected and 0.22 volume of 10× SET (10% sodium dodecyl sulfate [SDS], 5 mM EDTA, 200 mM Tris-HCl, pH 7.5) is added to make the composition of the solution similar to "Solution 1" (2% SDS, 200 mM Tris-HCl, pH 7.5, 1 mM EDTA) of our published procedure (5). Then 0.6 volume of chloroform:isoamyl alcohol (24:1) and 0.33 volume of "Solution 2" (42.9 potassium acetate, 11.2 mL acetic acid and water to 100 mL) (5 M potassium acetate) are added, the solution is vortex mixed until uniformly cloudy, put in ice for 5 min and spun for 5 min at 5000 rpm. At the end of the centrifugation, a protein-DNA precipitate forms at the aqueous/organic phase interface. The aqueous phase is collected and cytoplasmic RNA is precipitated with 1 volume of isopropanol for 1 h at -20°C.

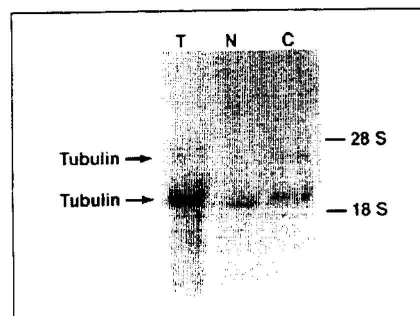

Figure 3. Northern blot hybridization with tubulin probe. RNA was extracted in two separate experiments from NIH 3T3 fibroblasts using the reported technique. Nuclear and cytoplasmic RNAs were extracted from the same plate. Total RNA was isolated by the reported technique. Eight µg of total (T), nuclear (N) and cytoplasmic (C) RNA were hybridized with a random primer labeled 1.6 kb tubulin cDNA fragment.

Benchmarks

After spinning at 5000 rpm for 20 min, the pellet is washed with 75% ethanol and resuspended in diethyl pyrocarbonate-treated H_2O.

The nuclear pellet is re-suspended in 2.0 mL of "Solution 1." Then 600 µL of ice-cold Solution 2 and 1.2 mL of chloroform-isoamyl alcohol are added, the tube is vortex mixed until cloudy, placed in ice for 5 min and nuclear RNA is extracted in the same way described for the cytoplasmic compartment.

By following this method, we extracted RNA from at least 10 plates of NIH 3T3 fibroblasts, without noticing any degradation despite the lack of "protective" agents such as guanidinium or proteinase K.

The yield is comparable to that of our original procedure described in Reference (2): from 25 to 65 µg of nuclear RNA and from 250 to 400 µg of cytoplasmic RNA. However, this modification abbreviates the time of extraction by one day. Moreover, unlike other published procedures (3), this one does not require the use of ultracentrifugation through a cesium chloride cushion.

As shown in Figure 2, 28S and 18S ribosomal RNA subunits are clearly evident in total nuclear and cytoplasmic RNA. Total RNA was extracted with the previously published method (5). In nuclear RNA preparations, we consistently observed a modest DNA contamination, which is dramatically lower than in the original guanidinium hydrochloride procedure (2) and does not interfere with the migration of the RNA in agarose gels and with its transfer and blotting. Figure 3 shows a Northern blot of nuclear and cytoplasmic RNA extracted with the new method compared with total RNA extracted using the previously published method (5). The tubulin-specific bands produced by the new method show no evidence of degradation.

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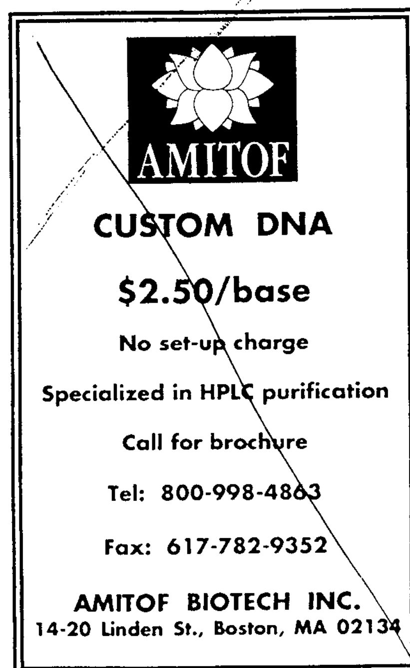

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Flexibility in the Boiling Method of Extracting Plasmid DNA Directly from Cell Culture

Plasmid vectors are routinely used for cloning/subcloning of foreign DNA. The boiling method of Holmes and Quigley (2) may be the simplest of all the existing methods for the rapid preparation of plasmid DNA. This method essentially consists of first pelleting the cells and resuspending them in a filter-sterilized buffer consisting of 8% sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 5% Triton® X-100 (STET buffer). The cells are subjected to lysozyme before being briefly exposed to boiling temperature. Recently a couple of modifications were reported to simplify this method by eliminating either the initial cell pelleting step (3) or the final steps of purifying the supernatant after the boiling step (1). These latter modifications, however, retained the use of STET buffer and lysozyme. Plasmid DNAs obtained by the above methods are usually washed with 70% ethanol before being used for analysis by various restriction enzymes. The requirement for each of the chemical components in the boiling methods (1–3) has not been tested critically nor has the possibility of further shortening the protocol been examined in detail. In this study, we systematically examined the need for various components in the boiling solution and the possibility of further shortening the protocol for preparing plasmid DNA directly from cell cultures.

The various modifications on plasmid DNA preparation (A–H) were made as given in Table 1. All preparations involved extracting recombinant plasmid (pBluescript® KS-vector [Stratagene, La Jolla, CA, USA] containing 2.3 kbp morning glory [*Pharbitis nil*] genomic DNA) from *Escherichia coli* strain XL1-Blue (Stratagene). For all preparations, except method H, 0.75 mL of bacterial culture grown overnight in LB medium was used. Method H used 0.35 mL of the same culture. Cells prepared following various modifications were kept in boiling water for 1 min. DNA pellets were vac-