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Three-Detergent Method for the Extraction of RNA from Several Bacteria

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Recent trends in molecular bacteriology have highlighted the importance of examining and comparing gene expression in different species in many cases. Also, studies with a number of different bacterial strains may be required when working on their ecology or population biology. In all such cases, high-efficiency protocols applicable to a variety of bacteria are relevant. A potential hurdle in the isolation of intact

RNA from bacteria is the relatively short half-life of the messenger RNA. Hence, the rapidity of cellular lysis and complete inhibition of RNases is of particular importance in such protocols.

A mixture of detergents at low pH was previously shown to be efficient for cellular lysis for mycobacteria (4). On this basis, we have developed a three-detergent method for the isolation of RNA from several gram-negative bacterial species. In our method, cellular lysis is achieved through a combination of SDS, Tween® 20 and Triton® X-100 while genomic DNA contamination is reduced through acid depurination-cum-deproteination through the use of citrate-buffered phenol (pH 4.0). The three detergents are readily available: SDS is

anionic and the other two are neutral. We have tested this method on several gram-negative bacterial genera from different habitats, including *Pseudomonas*, *Burkholderia*, *Agrobacterium*, *Escherichia* and *Edwardsiella* and on the gram-positive *Bacillus*. In each case, clean intact RNA (A_{260}/A_{280} nm ratio of 1.80–2.09) was obtained easily.

Several methods generally have been used to isolate RNA from both gram-negative and gram-positive bacterial species: hot acid phenol lysis (1,2,5,9) or the commercially available RNeasy® kit (Qiagen, Valencia, CA, USA) (3,6,8). Unfortunately, hot phenol is corrosive, and the toxic, noxious fumes produced require that the work be performed in the fume hood. Al-

Figure 1. Separation and analysis of RNA. (A) Ethidium bromide-stained 1.4% TBE agarose gel showing RNA extracted from *P. putida* 39169 using different methods. Lane 1, 2% SDS; lane 2, 5% SDS; lane 3, three-detergent technique; lane 4, LiCl, 1 h; lane 5, LiCl, 3 h; lane 6, LiCl, overnight; lane 7, DNaseI treatment; lane 8, RNeasy kit; lane 9, TRIZOL reagent. (B) Methylene blue-stained membrane showing total RNA prepared from various species electrophoresed in a 1.3% MOPS-formaldehyde agarose gel. Lane M, RNA Ladder (Life Technologies); lane 1, *B. cereus* 14579 (ATCC); lane 2, *B. subtilis* 6051 (ATCC); lane 3, *P. putida* 39169; lane 4, *A. tumefaciens* AGL1; lane 5, *E. tarda* PPD130/91; lane 6, *B. cepacia* 53267 (ATCC); lane 7, *P. aeruginosa* BO267; lane 8, *E. coli* XL1-Blue. (C) Northern blot analysis of total RNA from *P. putida* 39169 wild-type (lane 1) and a Tn5-*gus* single-insertion mutant M5 (lane 2) with a ³²P-labeled *tetA* probe. Lane M, RNA ladder. (D) RT-PCR amplification products of DNase-treated total RNA (method 4) from three *P. putida* Tn5-*gus* single-insertion mutants M5, M7 and M17. Lane M, GeneRuler™ 100 bp DNA Ladder Plus (MBI Fermentas, Amherst, NY, USA); lanes 1–3, mutants M5, M7 and M17, respectively; lane 4, positive control with M17 genomic DNA as starting template; lane 5, negative control with M17 RNA as starting template but omitting reverse transcriptase.

though the RNeasy kit has been used by researchers, its cost may be prohibitive for those who work with a large number of samples. Additionally, not many reports of its use with gram-negative bacteria are available.

The TRIzol[®] Reagent (Life Technologies, Gaithersburg, MD, USA) is a monophasic solution of phenol and guanidine isothiocyanate. Bacterial cells are first lysed in this reagent; then the addition of chloroform is followed by centrifugation, which separates the solution into an aqueous phase and an organic phase. The RNA, which partitions into the aqueous phase, is recovered by precipitation with isopropyl alcohol. We have successfully used this TRIzol reagent method for the isolation of RNA from various gram-negative bacterial species. Unfortunately, we found this method to be better suited for small-scale preparations (0.5–1 mL cultures), and there was still genomic DNA carry-over.

The method described here works efficiently with various gram-negative bacterial species, i.e., *Pseudomonas* spp., *Burkholderia cepacia*, *Escherichia coli*, *Edwardsiella tarda* and *Agrobacterium tumefaciens*. Fifty milliliter cultures (A_{600} of between 1.2–1.6) were harvested by centrifugation at $6000\times g$ for 15 min; the supernatant was decanted, and the cell pellet was resuspended in the remaining drops of supernatant (<1 mL). Twenty milliliters of STT buffer (10 mM Tris-HCl, pH 8.0, 20 mM EDTA, 2% SDS, 1% Tween 20, 1% Triton X-100) were added, and the suspension was vortex mixed for 1 min. We added a 1/40 volume of 1 M HCl, and the mixture was incubated at room temperature for 5 min. Two milliliters of 2 M sodium acetate (pH 4.0) were then added. The cell lysate was extracted twice with an equal volume of citrate-buffered phenol (pH 4.0):chloroform (4:1) at room temperature.

The phases were separated by centrifugation at $6000\times g$ for 15 min, and this was followed by a single or occasionally two chloroform extractions. Deproteinized RNA was precipitated with an equal volume of isopropanol for 1 h at -20°C , and the RNA was pelleted by centrifugation at $6000\times g$ for 30 min (designated method 1). For the gram-positive *Bacillus* strains, the cell

pellet was first resuspended in 2 mL of TE containing lysozyme (5 mg/mL) and incubated at 37°C for 15 min. The subsequent steps in the extraction procedure were as described above (designated method 2). Alternatively, RNA could be precipitated with 0.5 volume of 6 M LiCl, incubated at 4°C for several hours and then pelleted by centrifugation at $6000\times g$ for 30 min (designated method 3). RNA pellets were washed with 10 mL of 70% ethanol and resuspended in either formamide/EDTA (9 parts formamide:1 part 0.5M EDTA) or 0.05% SDS.

In cases in which the RNA was intended for use in RT-PCR or primer extension studies, the RNA obtained after isopropanol precipitation was resuspended in RNase-free water. Chromosomal DNA persisting in the preparation was digested with 100 U of DNaseI (Roche Molecular Biochemical, Mannheim, Germany) followed by phenol/chloroform extraction and reprecipitation with isopropanol. The RNA pellet was then dissolved in either formamide alone (to prevent enzymatic inhibition by the high EDTA concentrations) or in RNase-free water (designated method 4). It should be noted that DNaseI treatment could be performed on LiCl-precipitated RNA samples as well, although the yields would be lower. Therefore, we chose to DNase-treat the isopropanol-precipitated RNA samples.

RNA was also extracted from *P. putida* (39169; ATCC, Rockville, MD, USA) with the TRIzol reagent in accordance with the manufacturer's protocol. Briefly, 0.5 mL of cells was pelleted and resuspended in 1 mL of TRIzol. The suspension was incubated for 5 min at room temperature, then 270 μL of chloroform were added. The samples were vortex mixed and incubated for 10 min at room temperature. Phases were separated by centrifugation at $12000\times g$ for 15 min at 4°C . RNA was precipitated from the upper aqueous phase by the addition of 2/3 volume of isopropanol, incubated for 10 min at room temperature and followed by centrifugation at $12000\times g$ for 10 min at 4°C . The RNA pellet was washed with 1 mL of 70% ethanol, dried and then resuspended in 100 μL of formamide/EDTA.

Table 1 shows the quality and quan-

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tity of the RNA obtained. The RNA yields ranged between 21.8 and 47.2 µg RNA/mL starting culture, and the A_{260}/A_{280} nm ratios were between 1.80 and 2.09. Figure 1A shows the gel profile of total RNA obtained from *P. putida* wild-type using different methods. A non-denaturing gel was used because it shows more clearly both the RNA quantity and quality and the degree of persisting DNA. Figure 1, lane 3 shows that the quantity of RNA isolated using the three-detergent technique was significantly higher than when a single detergent was used (Figure 1, lanes 1 and 2, 2% and 5% SDS, respectively).

Having established that this three-detergent method was the most efficient, we then proceeded to optimize the reduction of chromosomal DNA carry-over. The persisting DNA and RNA yields obtained from LiCl precipitation for 1 h, 3 h and overnight are shown in Figure 1, lanes 4–6, respectively. Total yields are reduced, but so are the persisting DNA. Lane 7 shows

Table 1. Average, Based on Three Experiments, RNA Recovery from Different Bacterial Strains

Strains	Method ^a	A_{260}/A_{280}	Yield (µg RNA/mL Starting Culture)
<i>P. putida</i> 39169	1	2.05 ± 0.13	47.2 ± 3.2
<i>P. putida</i> 39169	3	1.921 ± 1.936	8.1 ± 22.1
<i>P. putida</i> 39169	4	1.89 ± 0.06	25.1 ± 2.8
<i>P. putida</i> 39169	TRIZOL	2.08 ± 0.10	34.5 ± 2.7
<i>Epicurian coli</i> Ø XL1-Blue	1	2.09 ± 0.11	35.7 ± 2.0
<i>P. aeruginosa</i> BO267	1	1.95 ± 0.14	46.9 ± 3.3
<i>E. tarda</i> PPD 130/91	1	2.08 ± 0.08	25.3 ± 2.4
<i>B. cepacia</i> 53267	1	1.91 ± 0.05	21.8 ± 2.0
<i>A. tumefaciens</i> AGL1	1	1.80 ± 0.04	24.4 ± 1.7
<i>B. cereus</i> 14579	2	2.01 ± 0.14	37.4 ± 2.4 (24) ^b
<i>B. subtilis</i> 6051	2	2.05 ± 0.16	39.2 ± 1.9 (9) ^b

^aMethod 1: Cells were lysed in 20 mL of STT extraction buffer, and RNA was precipitated with a 1 vol of isopropanol; method 2: as in method 1, but with an additional lysozyme treatment prior to cell lysis with STT; method 3: RNA was precipitated with LiCl for either 1 h, 3 h or overnight; method 4: RNA was first precipitated with isopropanol and then DNase-treated.

^bRNA yields obtained if lysozyme treatment was omitted.

the RNA obtained from isopropanol precipitation followed by DNase I treatment. The contaminating DNA is fully removed, and the RNA yields are still higher (1.1- to 3.1-fold) than that obtained from LiCl precipitation.

RNA was also isolated using two commercial kits, RNeasy and TRIzol reagent, lanes 8 and 9, respectively. Only the TRIzol reagent gave good quality RNA (Table 1), but there was still genomic DNA contamination (Figure 1A). Based on the manufacturer's recommendations, the RNeasy kit yielded little RNA with large DNA contamination. Two modifications were subsequently found to significantly improve RNA quality using the kit. These were the use of lysozyme at 0.5 mg/mL followed by a 15 min incubation period at 37°C (data not shown). However, there was still genomic DNA carryover, and the yields were lower. This poor yield could possibly be due to the soil bacteria that produced more exopolysaccharides, which makes cellular lysis more difficult. Figure 1B illustrates the use of this

method to isolate RNA from several other gram-negative bacterial species. In each case, the two ribosomal bands were clear and distinct.

Figure 1C shows the suitability of the RNA isolated by this method for the detection of single-copy gene expression by northern blot analysis. Total RNA from *P. putida* 39169 wild-type and a Tn5-*gus* single-insertion mutant M5 prepared using method 1 was electrophoresed in a 1.4% MOPS-formaldehyde agarose gel and transferred onto a HybondTM-N nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, Bucks, England, UK) under standard conditions (7). The 1.23 kb tetracycline-resistance marker on the transposon (*tetA*) transcript was detected with a ³²P-labeled *tetA* probe generated using the RediprimeTM DNA Labelling System (Amersham Pharmacia Biotech).

The RNA was also found to be suitable for the detection of gene expression by RT-PCR (Figure 1D). The RNA was first DNase-treated to remove any

contaminating chromosomal DNA (method 4) and the *tetA* transcript was amplified using gene-specific primers. First-strand cDNA synthesis was performed with SUPERSCRIPT™ II Reverse Transcriptase (Life Technologies) following the manufacturer's recommendations. PCRs on the cDNA were then performed with 2 U of BIOTOOLS™ DNA Polymerase (Biotechnological & Medical Laboratories, Madrid, Spain) according to the manufacturer's recommended conditions. A band of the expected size (937 bp) was obtained (Figure 1D), which demonstrated the suitability of the RNA for amplification.

In summary, we present a three-detergent method that provides a simple and rapid method for the isolation of RNA from several gram-negative bacterial species. The detergents helped in higher yields, and the acidification with 1 M HCl was observed to reduce the amount of chromosomal DNA carry-over, possibly by enhancing the depurination of DNA and its subsequent partitioning into the acid phenol. This procedure requires few solutions, thus minimizing contamination with RNases. Dissolution of the RNA pellet in formamide/EDTA or 0.05% SDS would serve to inhibit residual RNase activity (if any). In cases in which the RNA is used only for northern blot analysis, LiCl precipitation might be the method of choice. The amount of contaminating DNA is sufficiently reduced while it still maintains a decent yield of RNA. Under the more exacting requirements of RT-PCR or primer extension, the extra step of DNaseI treatment would then be a necessity.

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