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RNA*snap*™: a rapid, quantitative and inexpensive, method for isolating total RNA from bacteria

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ABSTRACT

RNA*snap*™ is a simple and novel method that recovers all intracellular RNA quantitatively (>99%), faster (<15 min) and less expensively (~3 cents/sample) than any of the currently available RNA isolation methods. In fact, none of the bacterial RNA isolation methods, including the commercial kits, are effective in recovering all species of intracellular RNAs (76–5700 nt) with equal efficiency, which can lead to biased results in genome-wide studies involving microarray or RNAseq analysis. The RNA*snap*™ procedure yields ~60 μg of RNA from 10^8 *Escherichia coli* cells that can be used directly for northern analysis without any further purification. Based on a comparative analysis of specific transcripts ranging in size from 76 to 5700 nt, the RNA*snap*™ method provided the most accurate measure of the relative amounts of the various intracellular RNAs. Furthermore, the RNA*snap*™ RNA was successfully used in enzymatic reactions such as RNA ligation, reverse transcription, primer extension and reverse transcriptase–polymerase chain reaction, following sodium acetate/ethanol precipitation. The RNA*snap*™ method can be used to isolate RNA from a wide range of Gram-negative and Gram-positive bacteria as well as yeast.

INTRODUCTION

Historically working with bacterial RNA has been technically difficult because of its highly labile nature and the complicated procedures used for its isolation. Early RNA extractions employed guanidium isothiocyanate to lyse cells and denature proteins, while the RNA was isolated using a cesium chloride cushion and ultracentrifugation (1). Subsequently, a hot phenol isolation method replaced cesium chloride gradients (2). However, RNA extractions using hot phenol had significant problems due to both the toxicity of the phenol and because the RNA obtained was not consistently of high quality (3). Subsequently, a protocol was developed that combined guanidium isothiocyanate and phenol that yielded much more reproducible results compared to earlier methods (4).

As the interest in RNA metabolism in bacteria grew, many companies developed kits making it easier for any laboratory to isolate total RNA. These kits, which are relatively expensive, can be very useful for isolating RNA enriched for specific sizes, since the kits vary greatly in the chemistry and/or mechanics used to lyse cells, denature and remove proteins and to actually isolate the RNA. The use of detergents to promote cell lysis led to the discovery of a cationic detergent (Catrimox-14®, Iowa Biotechnology Corp., Coralville, IA, USA) that both aided cell lysis and captured RNA and DNA by precipitation (5,6). This method had the major advantage of not using phenol and provided good yields of high-quality RNA (7,8). However, shortly after Qiagen acquired the patent rights to Catrimox-14® the detergent was withdrawn from the market. Subsequently, a variant of the Catrimox-14® isolation procedure was developed using a slightly different surfactant trimethyl(tetradecyl)ammonium bromide (called Catrimide), which is a very effective and inexpensive substitute (9).

As we initiated a detailed study of rRNA processing in *Escherichia coli*, we wanted to use an RNA isolation procedure that could give us a rapid and accurate assessment of all RNA species within the cell. However, all current RNA isolation procedures contain multiple transfer steps, leading to reduced sample recovery. Furthermore, although each manufacturer provides specifications for the yield and RNA quality resulting from their procedure,

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there is no published side-by-side comparison of the various methods in terms of total RNA yield, RNA quality, size distribution of the isolated RNA molecules, time to carry out the procedure and cost per sample. In fact, upon examination of the various RNA samples we obtained using various kits and our own in-house experience with the Catrimide/LiCl method, it was apparent that none of the current RNA isolation methods provide an accurate representation of the intracellular RNA pools, since each method appears to selectively enrich for either large or small RNAs relative to the levels of medium sized species. Thus, depending on the isolation method used certain size classes of RNA were either enriched or depleted relative to the total RNA population.

We describe here a new RNA isolation procedure (called RNA*snap*™, for **S**imple **N**ucleic **A**cid **P**urification) that quantitatively recovers >99% of all RNA species in one step. The isolation method is remarkably simple, rapid, reproducible and inexpensive. With Gram-negative bacteria, it yields high-quality RNA in <15 min that can be used directly for both polyacrylamide and agarose northern analysis.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strain MG1693 (*thyA715 rph-1*) (provided by the *E. coli* Genetic Stock Center, Yale University) was grown with shaking at 37°C in Luria broth supplemented with thymine (50 μg/ml) to exactly 50 Klett units above background (No. 42 green filter or OD_{600} 0.4), which is $\sim 10^8$ cfu/ml. Other strains were generously provided by the Departments of Microbiology and Marine Sciences at the University of Georgia.

RNA*snap*™ RNA isolation method for Gram negative bacteria

One milliliter of bacterial culture (10^8 cells) was centrifuged at 16 000*g* for 30 s and the supernatant was removed by aspiration. The cell pellet was stored in dry ice until ready for extraction. Cell pellets were then resuspended in 100 μl of RNA extraction solution [18 mM EDTA, 0.025% SDS, 1% 2-mercaptoethanol, 95% formamide (RNA grade)] by vortexing vigorously. The cells were lysed by incubating the sample at 95°C in a sand bath for 7 min. The cell debris was pelleted by centrifuging the warm sample at 16 000 *g* for 5 min at room temperature. The supernatant was carefully transferred to a fresh tube without disturbing the clear gelatinous pellet.

RNA*snap*™ RNA isolation method for Gram positive bacteria and yeast

To isolate RNA from organisms with tough cell walls such as yeast (*Saccharomyces cerevisiae*) and Gram-positive bacteria (*Bacillus subtilis*), the following modification to the RNA*snap*™ was added. The pellet from 1 ml of cells (10^8) was resuspended in 100 μl of RNA extraction solution. The resuspended cells were transferred to a 0.5 ml screw cap tube containing ~200 μl of chilled zirconia beads (from Ambion Ribopure™ kit). The cells were beaten on a vortex mixer with a small tube adapter for 10 min. The samples were then treated as described above.

Catrimide/LiCl RNA isolation method

This procedure was performed similarly to the method described by Mohanty *et al*. (9), but was modified for one ml samples. Briefly, 1 ml of bacterial culture was added to 500 μl of stop buffer, which was previously frozen horizontally in a 1.5 ml microcentrifuge tube. The cells were immediately mixed by vortexing vigorously, and then pelleted by centrifugation at 5000*g* for 5 min at 4°C. The supernatant was carefully removed by aspiration, and the pellet was suspended in 200 μl of lysis buffer by vortexing. The sample was then placed into a dry-ice ethanol slurry for 90 s, and followed by 90 s of incubation in a 37°C water bath. This freeze–thaw cycle was repeated four times in total. After the fourth 37°C incubation, the sample was transferred into the dry ice–ethanol slurry in order to refreeze the solution, and 35 μl of 20 mM acetic acid was then added to the frozen solution. The sample was then placed back into the 37°C water bath, followed by addition of 200 μl of 10% Catrimide [(trimethyl (tetradecyl)ammonium bromide)] when the sample was almost completely thawed. The sample was briefly vortexed and centrifuged at 16 000*g* for 10 min at 4°C. The supernatant was carefully removed by aspiration, and the pellet was suspended in 500 μl of 2 M LiCl in 35% ethanol by vortexing vigorously. The sample was then incubated at room temperature for 5 min, followed by centrifugation at 16 000*g* for 10 min at 4°C. The supernatant was carefully removed by aspiration and the pellet was resuspended in 500 μl of 2 M LiCl in water followed by a repeat centrifugation. The pellet was briefly vortexed in 75% ethanol and centrifuged at 8000*g* for 5 min at 4°C. The ethanol was removed by aspiration, and the tube was briefly centrifuged for a second time in order to collect and remove the remaining ethanol with a pipette. The pellet was allowed to air dry at room temperature for 10 min and subsequently hydrated by the addition of 100 μl of RNase-free water and incubated at room temperature for 10 min. The tube was vigorously vortexed, centrifuged at maximum force (21 000*g*) at room temperature for 1 min to pellet cell debris, and the RNA containing supernatant was transferred to a new tube.

TRIzol® Max™ Bacteria, RNeasy® Protect Bacteria and RiboPure™ Bacteria RNA isolation methods

These RNA extraction procedures were done according to the manufacturer's recommendations and protocols specific for the number of *E. coli* cells and conditions in which they were grown. Any step described as optional, but that might improve the quality or yield of RNA was followed. No optional DNase I treatment was performed on any RNA sample used in this study. Every effort was made to ensure that the extracted RNA using each method met the manufacturer's guidelines in terms of overall RNA yield, A_{260}/A_{280} ratio and RNA quality.

Determination of RNA quantity and quality

RNA quantity and A_{260}/A_{280} ratios were determined using a Nanodrop™ 2000c (Thermo Scientific). The amount of RNA in the RNA*snap*™ supernatants was determined by A_{260}, using the RNA extraction solution as a blank. RNA quality was assessed by running 250 ng of each RNA sample, as determined by A_{260}, on a 1.2% agarose–0.5 × TBE gel with ethidium bromide, run at 5 v/cm for 1 h. RNA samples were denatured prior to loading by suspension in Gel Loading Buffer II (95% formamide, 18 mM EDTA and 0.025% each of SDS, xylene cyanol and bromophenol blue, Ambion) and heating for 5 min at 95°C. Approximately 100 ng of each RNA sample were subsequently analyzed on a Bioanalyzer RNA chip (Agilent Technologies) using the manufacturer's recommendations.

Quantitative determination of RNA recovery using the RNA*snap*™ method

In order to estimate the amount of RNA remaining in the pellet, we performed an RNA*snap*™ extraction using 10 ml of *E. coli* cells (10^8 cells/ml) using 500 µl of RNA extraction solution. After the supernatant was recovered and placed into a separate tube, an additional 500 µl of room temperature RNA extraction solution was gently added to the gelatinous pellet in order to wash the pellet of any remaining RNA containing supernatant, which could not be initially removed without disturbing the pellet. The tube was then spun at 16 000*g* for an additional 5 min and the supernatant was again removed without disturbing the pellet. The pellet was then suspended in 100 µl of RNase-free water. Subsequently, 100 µl of acidic phenol/chloroform (Ambion, 5:1 solution, pH 4.5) was added and the tube was vortexed vigorously for 30 s. The tube was then centrifuged at 16 000*g* for 5 min and the aqueous phase was transferred to a fresh tube and sodium acetate/ethanol precipitated. The precipitated RNA was hydrated in 20 µl of RNase-free water. After the RNA was fully dissolved, the total amount of RNA was determined based on A_{260} and was compared with the amount of RNA in the first 500 µl volume of RNA extraction solution recovered from the pellet.

Northern analysis

Two types of northern blots were performed in this study, 6% polyacrylamide/ 8.3 M urea 1 × TBE gels for small RNA species (*lpp, cspE,* 5S rRNA, *ryhB and pheU*/*pheV*) and 1.2% Agarose 1 × MOPS gels for larger species (*rpsJ* operon, *adhE and ompF*). Northern analysis was performed as described in Stead *et al.* (10). The RNA isolated by the RNA*snap*™ method was used directly for polyacrylamide gels after dilution to the desired loading volume in a formamide-based RNA loading dye. For agarose northerns, the RNA in the extraction solution was brought up to a total volume of 10 µl with RNA*snap*™ RNA extraction solution. Subsequently, 4 µl of loading solution (3.8 µl of any formamide-based RNA loading dye along with 0.2 µl of 37% formaldehyde) were added. The samples were heated at 65°C for 5 min and placed on ice for 1 min followed by brief centrifugation before loading onto a 1.2% Agarose 1 × MOPS gel, similar to the method of Vincze and Bowra (11). Subsequently the RNA was transferred to a positively charged nylon membrane by electroblotting (9).

The northern membranes were subsequently probed with multiple ^{32}P-labeled oligonucleotide probes such that the signals for the *lpp,* 5S rRNA and *pheU*/*V* transcripts were simultaneously visualized on a single membrane (similarly for *cspE*/*ryhB* and *adhE*/*ompF*). This approach helped to determine if loading errors could account for differences in signals between the two replicates, as the percentage difference should be the same for each of those RNA species probed in the same lanes, unless the RNA extraction method used caused non-quantitative recovery of a particular RNA species. It was also possible that a technical error during the transfer of RNA from the gel to the nitrocellulose membrane accounted for a difference between replicates, but this type of error is extraordinarily rare with polyacrylamide northerns in our hands, and occurs infrequently with agarose northerns.

Sodium acetate/ethanol precipitation method

The RNA*snap*™ RNA sample was first diluted with four volumes of water followed by addition of 1/10 volume of 3 M sodium acetate, pH 5.2 and the sample was mixed by pipetting. Three volumes of 100% ethanol were then added, the sample mixed briefly by vortexing and incubated for at least 60 min at −80°C. The tube was centrifuged at 16 000*g* for 30 min at 4°C. The supernatant was carefully removed by aspiration and the pellet was washed with 250 µl of 75% ethanol, followed by centrifugation at 8000*g* for 5 min at 4°C. The supernatant was removed via aspiration and the tube was briefly centrifuged again. Following the removal of any remaining ethanol, the pellet was air dried. The pellet was resuspended in water and centrifuged at 16 000*g* to pellet any remaining water insoluble proteins and the RNA containing supernatant was transferred to a fresh tube.

Reverse transcriptase–polymerase chain reaction

SK4390 (*rph-1 ΔrppH thyA715*Km^r) was grown with shaking at 37°C in Luria broth supplemented with thymine (50 µg/ml) and kanamycin (25 µg/ml) until 20 Klett units above background (No. 42 green filter). The culture was then shifted to 44°C for 2 h. The culture was maintained at 80 Klett units above background by making periodic dilutions with pre-warmed Luria broth. RNA was extracted using the RNA*snap*™ procedure described above or the TRIzol® Max™ method according to manufacturer's instructions (Invitrogen). Both RNA samples were subjected to sodium acetate/ethanol precipitation, DNA removal with the DNA-free kit™ (Ambion) and a final sodium acetate/ethanol precipitation. Five micrograms of each RNA sample was reverse transcribed using a *lpp* gene-specific primer (LPP538:CAGGTACTA TTACTTGGGGTAT) using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. The cDNAs were amplified using two

gene-specific primers (LPP538 and LPPPCR1:GCTACAT GGAGATTAACT) using GoTaq® Green Master Mix (Promega). The polymerase chain reaction (PCR) products were run on a 2% agarose–Tris–acetate–EDTA gel and visualized with ethidium bromide in a G-Box (Syngene). For additional confirmation that the *lpp* cDNA had been amplified, Southern blot analysis was performed by transferring the PCR products to a Nytran® SuPerCharge membrane using a Turboblotter™ (Schleicher and Schuell). The membrane was probed with ^{32}P-5′-end-labeled *lpp* specific oligonucleotide (LPP562A:CGCTTGCGTTCACGTCG) and scanned with a Phosphorimager (Storm™840, GE Healthcare) (data not shown).

Primer extension analysis

Primer extension analysis was performed as described by Stead *et al*. (10) with an oligonucleotide primer specific to the 5′-end of mature 23S rRNA, which is identical for each of the seven *E. coli* rRNA operons (5′-CGTCCTTCATC GCCTCTGACT-3′). An amount of 250 ng of total RNA (isolated using the RNA*snap*™ procedure) was used for the reverse transcription reactions. Only half of each reaction mixture was run on the gel. The sequencing ladder was derived from the *rrnB* operon.

RESULTS

Development of RNA*snap*™, a rapid and highly quantitative RNA isolation method

In most isolation methods, the amount of total RNA present is initially determined based on either absorbance at 260 nm (A_{260}) or through the use of fluorescent dyes. Although these approaches provide an accurate estimate of the RNA present in a particular sample, the relative amounts of each RNA species can vary widely depending on the distribution of each RNA species. These variations are directly related to the particular isolation method employed due to the inherent properties of the matrices used in each procedure, which are biased towards either large (rRNA or other large mRNAs) or small (tRNAs and sRNAs) RNA species (see below). In order to help address the problems of both representative and quantitative recovery, we sought to develop a one-step RNA extraction procedure that could be carried out in a single tube in which total RNA was quantitatively recovered in the supernatant and the bulk of the DNA and proteins were left in the pellet. We hypothesized that such an approach would both greatly simplify RNA isolation and would provide a more accurate overview of the actual intracellular distribution of all RNA species, since any losses associated with multiple handling steps, such as phenol/chloroform extraction, would be eliminated.

During the development of the RNA*snap*™ method, we took advantage of the fact that *E. coli* cells were easily lysed in a boiling solution, such as used in colony PCR methods. In addition, it is standard practice to denature RNA in a formamide-based loading solution prior to its separation on either polyacrylamide or agarose gels. We combined aspects of these two techniques to develop the formamide-based RNA extraction solution described here (see 'Materials and Methods' section). We observed that exponentially growing *E. coli* cells were rapidly lysed when suspended in this solution and heated at 95°C for 7 min. Following centrifugation for 5 min at 16 000*g*, the RNA was in the supernatant and the gelatinous pellet contained protein, cell debris and the majority of the DNA. The RNA was quantified based on A_{260} by first blanking a spectrophotometer with the RNA extraction solution. It was important that the RNA extraction solution was made fresh and was also used as the blank, since the A_{260} of the extraction solution itself changed over time after the addition of 2-mercaptoethanol. A one ml sample of an early exponential culture of *E. coli* (10^8 cells) yielded 60 ± 3 µg of total RNA with the entire procedure taking <15 min (Table 1).

The RNA*snap*™ isolated RNA was suitable, without any further treatment, for northern analysis using either polyacrylamide or agarose gels (Figure 2). The genomic DNA contamination in the RNA*snap*™ sample was comparable to that obtained with the other isolation methods (data not shown). However, although minor genomic DNA contamination does not interfere with northern blot analysis and some enzymatic reactions, it can interfere during experiments involving reverse transcription and RNAseq analysis. Thus, RNA*snap*™ RNA was

Table 1. Overall comparison of the various RNA isolation procedures

Parameters	RNA*snap*™	TRIzol® Max™ bacteria	RNeasy® protect bacteria	RiboPure™ bacteria	Catrimide/LiCl
Cost/sample (US dollars)[a]	0.03	4.20	8.10	7.14	0.20
Approximate yield[b] (µg)	60	27	35	15	35
Approximate time[c] (min)	15	60	40	40	60
Size range for efficient RNA isolation[d] (nt)	76 to >5700	76 to 3000	200 to >5700	300 to >5700	76 to >5700

[a]Cost data based on the list price of chemicals or extraction kits.
[b]The approximate yield is based on the average of multiple independent isolations starting with 10^8 cells. All of these methods have the ability to handle $>10^8$ cells. For example, the RiboPure™ Bacteria kit recommends using 10^9 cells.
[c]The approximate isolation time is based on the time it took from starting the isolation procedure to determining the concentration of the isolated RNA.
[d]The RNA size range data is based on the sizes of specific RNAs detected by northern analysis (Figure 2).

subject to DNase I treatment using the DNA-free™ kit (Ambion) following sodium acetate/ethanol precipitation for experiments involving primer extension and reverse transcriptase (RT)–PCR (see below and 'Materials and Methods' section).

The RNA*snap*™ method recovers >99% of all RNA species

Even though the RNA*snap*™ procedure was rapid and yielded more total RNA per cell than any other method tested (Table 1), it was important to determine how much RNA remained in the gelatinous pellet. Accordingly, we scaled up the isolation to 10 ml of culture (10^9 cells), but again carried out the protocol in a single tube. Following removal of the supernatant containing the RNA, the pellet was gently washed once with the extraction solution at room temperature. After a subsequent centrifugation, the pellet was resuspended in water and extracted using acidic phenol/chloroform (See 'Materials and Methods' section). The aqueous phase was precipitated with sodium acetate/ethanol and resuspended in water. In each of two replicates, ~2.5 µg of high-quality RNA was recovered from the re-extracted pellet, while >700 µg of RNA were found in the original supernatant, indicating that the efficiency of RNA recovery from *E. coli* using the RNA*snap*™ method was >99% (data not shown). An amount of 250 ng of RNA from both the re-extracted pellet and the original supernatant were run on an agarose gel to confirm the presence, quality and quantity of the RNA. Interestingly, the profile of the various abundant RNA species (tRNAs, 5S rRNA, sRNAs, 16S rRNA and 23S rRNA) was identical between the two RNA samples upon visual inspection of the agarose gel (data not shown).

Analysis of RNA*snap*™ isolated RNA

In an attempt to determine the size distribution of the transcripts present in the RNA isolated by the RNA*snap*™ method, we compared the RNA samples obtained using our previously optimized Catrimide/LiCl method (9) and three of the most widely used commercially available RNA isolation kits [TRIzol® Max™ Bacteria (Invitrogen), RNeasy® Protect Bacteria (Qiagen) and RiboPure™ Bacteria (Ambion)]. Each extraction method was tested using at least two independent biological replicates and two or more technical replicates per biological replicate. The quality of each RNA sample was assessed using three main criteria: purity as determined by a spectrophotometer ($A_{260/280}$ ratio); the 23S rRNA/16S rRNA ratio as determined by Bioanalyzer analysis (Agilent Technologies); and an RNA integrity number (RIN) derived from the Bioanalyzer analysis (Table 2). The RIN number (standardization of RNA quality control) was developed using total eukaryotic RNA, based on a numbering system of 1–10, with 1 being the most degraded RNA and 10 being the most intact (Agilent Technologies). It has been demonstrated that with bacterial RNAs a RIN value <7 led to significant variations in data (12).

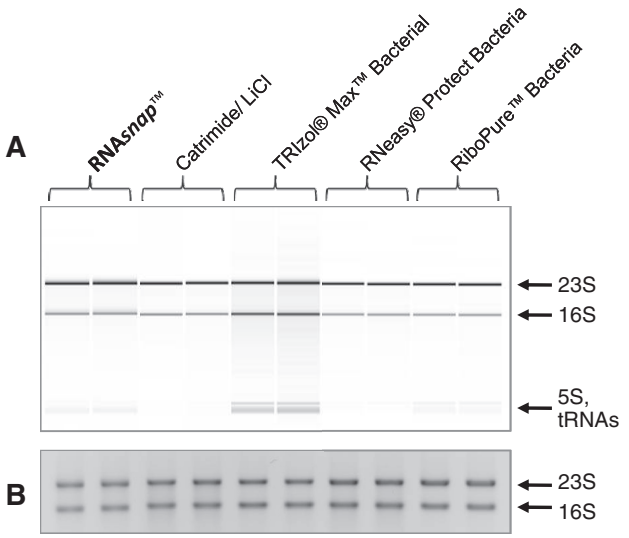

Figure 1. Quality assessment of RNA samples isolated by each method. (**A**) A representative composite bioanalyzer digital gel image using two technical replicates of each of the RNA extraction method tested (see 'Materials and Methods' section). (**B**) A representative composite image of technical replicates of 250 ng of total RNA (based on A_{260}) from each RNA extraction method electrophoresed on a 1.2% agarose–0.5× TBE gel and stained with ethidium bromide.

As shown in Figure 1, the quality of the RNA derived using the RNA*snap*™ method was as good or better than RNA obtained by the other methods tested based on both bioanalyzer analysis (Figure 1A and Table 2) and agarose gel electrophoresis (Figure 1B). The ratio of *E. coli* 23S to 16S rRNA in the samples isolated by the RNA*snap*™ method was 1.8, which came closer to the theoretical ratio of 1.88 (2904 nt/1541 nt) than any other method tested (Table 2). The $A_{260/280}$ ratio of ~2.0 for all the RNA preparations (Table 2) indicated that all of the samples were relatively pure with the possible exception of the RNA*snap*™ sample. Normally, an $A_{260/280}$ ratio of 1.8–2 is indicative of highly purified RNA when resuspended in a buffered solution like Tris-EDTA, pH 8.0. However, this ratio is highly dependent on the pH and the ionic strength of the solution (13). The pH of the RNA*snap*™ RNA sample was ~9.4. As predicted, resuspension of the RNA in RNase-free water after a sodium acetate/ethanol precipitation significantly improved the ratio (Table 2). Additionally, diluting the RNA*snap*™ RNA sample 4-fold with RNase-free water improved the $A_{260/280}$ ratio to 1.9 (data not shown), which was comparable to the other methods shown in Table 2. Thus, the low 260/280 ratio seen with the RNA*snap*™ RNA sample most likely resulted from the presence of formamide.

Interestingly, there were significant differences in terms of the amounts of the rRNAs and tRNAs present (Figure 1) as well as RIN (Table 2). The RNA*snap*™, Catrimide/LiCl, RNeasy® and Ribopure™ methods yielded comparable amounts of 16S and 23S rRNAs, which were significantly higher than what was observed with the TRIzol® Max™ Bacteria method (Table 2). In contrast, the TRIzol® Max™ Bacteria method yielded the

Table 2. RNA quality scores

Criteria	RNA*snap*™	TRIzol® Max™ bacteria	RNeasy® protect bacteria	RiboPure™ bacteria	Catrimide/LiCl	RNA*snap*™ precipitated[a]
$A_{260/280}$[b]	1.73 ± 0.01	1.97 ± 0.02	2.13 ± 0.01	2.12 ± 0.02	2.00 ± 0.01	1.92 ± 0.02
23S rRNA/16S rRNA[c]	1.80 ± 0.01	1.21 ± 0.05	2.38 ± 0.55	2.05 ± 0.16	1.73 ± 0.15	1.21 ± 0.08
RIN[d]	9.5 ± 0.00	7.9 ± 0.17	9.0 ± 0.52	9.4 ± 0.21	9.4 ± 0.29	9.5 ± 0.35

[a]RNA from original extraction solution was precipitated using sodium acetate/ethanol and resuspended in water (see 'Materials and Methods' section).
[b]Obtained using a Nanodrop 2000c.
[c]Obtained from Agilent Bioanalyzer analysis.
[d]RIN obtained from Agilent Bioanalyzer analysis. Each value is the average of at least two replicates.

highest concentrations of 5S rRNA and tRNAs, followed by the RNA*snap*™ method (Figure 1A).

The obvious differences in the distribution of RNAs among of the most abundant RNA size classes obtained from the various RNA isolation methods (Figure 1) led us to determine the relative abundances of specific RNA molecules ranging in size between 76 and 5700 nt using northern analysis. Since the RNA*snap*™ method recovered >99% of total cellular RNA, we calculated the abundance of each transcript derived from the other methods (Figure 2) relative to what was obtained with the RNA*snap*™ RNA (Table 3). Transcripts >1000 nt (*ompF, adhE* and the *rpsJ* operon) were less abundant in the TRIzol® Max™ RNA compared to any of the other methods (Table 3). In fact, the recovery of the larger transcripts decreased gradually as a function of increased size leading to very low recovery of the ~5700 nt *rpsJ* operon mRNA (the largest transcript tested). Furthermore, the variability from one isolation to another using the TRIzol® Max™ method was also very high for larger transcripts (Table 3, higher standard deviations). In contrast, all the other RNA isolation methods contained the larger species at levels that were 1.6 - to 4.4-fold higher than the RNA*snap*™ RNA.

At the lower end of the RNA size spectrum, i.e. transcripts <300 nt (*pheU*/*pheV, ryhB,* 5S rRNA), the RNeasy® Protect Bacteria, RiboPure™ and Catrimide/LiCl methods yielded significantly less RNA with up to 20-fold decreases for some species (Figure 2 and Table 3). The one exception was the *ryhB* small regulatory RNA, which was present in comparable amounts in all five RNA samples (Table 3). The TRIzol® Max™ sample consistently had between 1.4 - and 2-fold higher levels of all three small RNAs tested (Table 3). For the two species in the 300-nt range (*cspE* and *lpp*) all five methods gave comparable levels (Table 3), within experimental error.

Taken together, it is clear that each of the current RNA isolation methods has distinct biases regarding transcript size. Thus while the RNA*snap*™ method appeared to be less efficient in isolating larger transcripts compared to the RNeasy® Protect Bacteria, RiboPure™ and Catrimide/LiCl methods, the higher abundance of larger RNA molecules was accompanied by underrepresentation of the smaller molecules (Table 3). Similarly, higher levels of small RNAs (Table 3) as well as thick bands of tRNA and 5S rRNA in the TRIzol® Max™ RNA samples (Figure 1A) were at the expense of larger RNA species (Table 3).

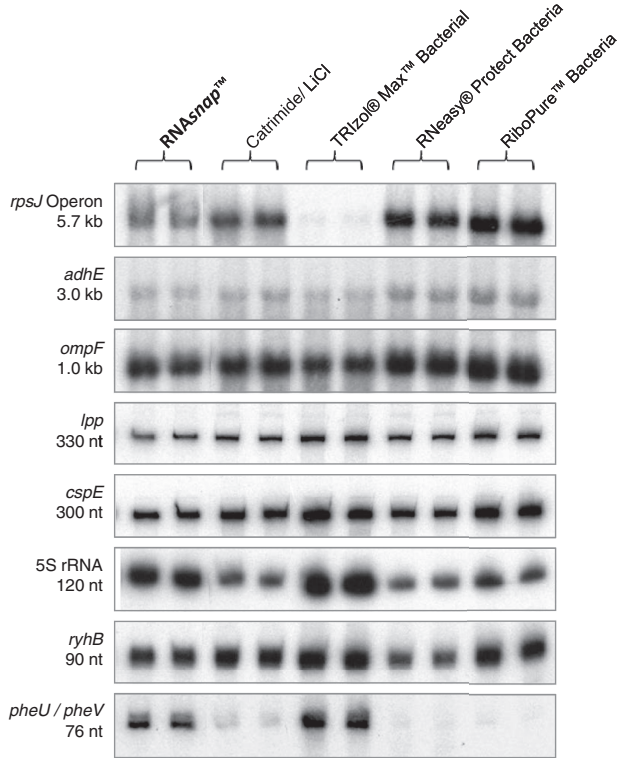

Figure 2. Northern analysis of specific RNA species using total RNA isolated by each RNA isolation method. Five micrograms of total RNA (based on A_{260} and two independent technical replicates) were used for northern analysis on each of the eight specific RNAs listed on the sides of the autoradiograms along with the approximate size of each transcript. The *rpsJ*, *adhE* and *ompF* transcripts were separated on agarose gels while the rest of the transcripts were separated on polyacrylamide gels (see 'Materials and Methods' section).

Overall, of the commonly used RNA isolation kits, TRIzol® Max™ was the best for isolating small RNAs, but it selectively lost larger RNA species (Table 3). In the case of the RNeasy® Protect Bacteria, RiboPure™ and Catrimide/LiCl RNA samples, small RNAs were either underrepresented (Catrimide/LiCl) or almost completely absent (RNeasy® Protect Bacteria and RiboPure™ Bacteria).

Table 3. Northern analysis comparison of specific transcript levels in total RNA isolate by various methods

Transcript	Size (nt)	Relative RNA Abundance in total RNA isolated by each method[a]				
		RNA*snap*™	TRIzol® Max™ bacteria	RNeasy® protect bacteria	RiboPure™ bacteria	Catrimide/LiCl
pheU/V	76	1	1.64 ± 0.11	0.09 ± 0.11	0.05 ± 0.05	0.15 ± 0.10
ryhB	90	1	1.39 ± 0.56	0.8 ± 0.26	1.68 ± 0.33	1.01 ± 0.55
5S rRNA	120	1	1.99 ± 0.13	0.35 ± 0.01	0.77 ± 0.34	0.45 ± 0.10
cspE	300	1	1.67 ± 0.0	1.04 ± 0.14	1.86 ± 0.50	0.93 ± 0.22
Lpp	330	1	2.03 ± 0.16	1.24 ± 0.08	1.64 ± 0.02	1.09 ± 0.05
ompF	1000	1	0.81 ± 0.47	1.51 ± 0.40	1.94 ± 0.11	1.40 ± 0.46
adhE	3000	1	0.53 ± 0.10	1.67 ± 0.11	2.62 ± 0.90	1.24 ± 0.44
rpsJ operon	5700	1	0.47 ± 0.36	2.60 ± 0.33	4.37 ± 0.33	1.62 ± 0.48

[a]Each transcript was probed with a specific ^{32}P-labeled oligonucleotide probe (sequences available on request) using northern blot analysis as described in 'Materials and Methods' section. Each blot was scanned using a GE Storm 840 PhosphorImager and the band corresponding to each transcript was quantified using ImageQuant TL software. The values obtained for the RNA*snap*™ RNA were set at 1 and used to normalize the other RNA samples. Each relative abundance value is the average of at least two independent replicates.

Generality of RNA*snap*™ RNA isolation method

Isolation of RNA from stationary phase cells using current methods has been difficult (9). In contrast, the RNA*snap*™ method worked equally well with either late stationary phase or exponential phase cells (data not shown). In addition, the RNA*snap*™ procedure was easily and quantitatively scaled up to handle 10 ml of culture (10^9 cells) for situations where larger amounts of RNA were needed. Furthermore, the RNA*snap*™ RNA could be used directly in both polyacrylamide/urea and agarose gels without further purification (Figure 2).

Although all the data shown here involved *E. coli* RNA, we have used the RNA*snap*™ method to successfully isolate high-quality RNA from a number of other Gram-negative bacteria including: *Alcalingenes faecalis* (ATCC 8750); *Serratia marcescens* (ATCC 14756); *Shigella flexneri* (ATCC 9199); *Pseudomonas aeruginosa* (ATCC 27853); *Salmonella enterica* (ATCC 29629); *Ruegeria pomeroyi* (ATCC 700808); and *Myxococcus xanthus* DK1622. Additionally, using a slightly modified version of the RNA*snap*™ method (see 'Materials and Methods' section) in which zirconium bead homogenization was added for lysis efficiency, high-quality RNA was obtained from two Gram-positive bacteria: *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 6538). The modified method also worked well with both *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

Using RNA*snap*™ for primer extension and RT–PCR experiments

The RNA*snap*™ isolated RNA was further tested for its functionality in commonly applied techniques such as RT–PCR, RNA ligation and primer extension analysis. It should be noted that for all applications involving enzymatic reactions, the RNA from the RNA*snap*™ method was further purified using a sodium acetate/ethanol precipitation step (see 'Materials and Methods' section). Specifically, we compared RNA samples isolated using either the RNA*snap*™ or the Trizol® Max™ RNA isolation procedures in an RT–PCR experiment that amplified the *E. coli lpp* mRNA. As shown in Figure 3, there was ~1.6-fold more *lpp* mRNA in the Trizol® Max™ isolated RNA compared to the RNA*snap*™ isolated RNA after 10 cycles, which reflected the relative abundances shown in Table 3. The PCR amplification reached a plateau after 10 cycles (Figure 3). In addition, RNA*snap*™ isolated RNA was used in determining the 5′- and 3′-ends of the *pheU* and *pheV* tRNA transcripts (Bowden,K., Mohanty,B. K. and Kushner,S.R., manuscript in preparation) by initially ligating the 5′- and 3′-ends of the transcripts (14). RNA*snap*™ isolated RNA has also been used successfully in various primer extension experiments. For example, in the experiment shown in Figure 4, we have examined the 5′-termini of 23S rRNA in *rnc-14* and wild-type strains.

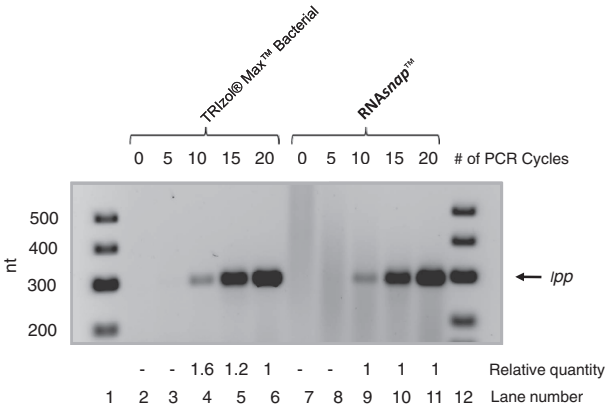

Figure 3. Comparison of RNA*snap*™ and Trizol® Max™ isolated RNA in an RT–PCR experiment. RNA isolated from SK4390 (*rph-1 ΔrppH*) was reversed transcribed with a primer specific for the *lpp* mRNA (~330 nt) and subsequently PCR amplified for either 5,10,15, or 20 cycles (see 'Materials and Methods' section). The amplified PCR products were run on a 2% agarose gel and quantitated using ImageQuant TL software (GE). The amount of PCR product at the end of a fixed number of cycles from RNA*snap*™ isolated RNA was set at 1 and compared with the amount of product obtained using Trizol® Max™ isolated RNA. Lanes 1 and 12, Gene Ruler™ Low Range DNA Ladder (Fermentas).

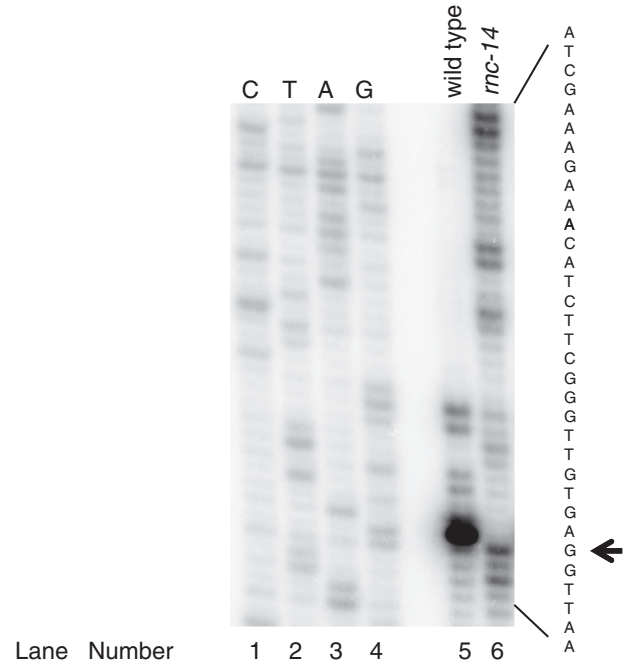

Figure 4. Primer extension analysis using RNA*snap*™ isolated RNA. The primer extension was carried out as described in 'Materials and Methods' section. Lanes 1–4, sequencing ladder derived from *rrnB* operon. Leftward arrow indicates the mature 5′-terminus of 23S rRNA (wild-type, lane 5), which is missing in the absence of RNase III (*rnc-14*, lane 6).

DISCUSSION

We have described here a simple, rapid and reproducible RNA isolation procedure (RNA*snap*™) that yields high-quality RNA from Gram-negative bacteria (Figures 1 and 2), Gram-positive bacteria and yeast that can be used for northern analysis without any further purification. As shown in Table 1, not only did the RNA*snap*™ method provide the highest total RNA yield of all five isolation procedures (1.7- to 4-fold higher), but it was also the fastest and least expensive.

Furthermore, the method ensures the isolation of the widest range of RNA species (Table 1). Using eight transcripts ranging in size between 76 and 5700 nt, we have demonstrated that the RNA*snap*™ isolation procedure is an unbiased method that likely preserves the *in vivo* distribution of all RNA species, thus providing the most accurate representation of intracellular RNA pools compared to any of the other isolation methods tested. Furthermore, it works equally well with exponential and stationary phase cultures.

For downstream applications such as primer extension analysis, RNA ligation and RT–PCR, further purification of RNA*snap*™ isolated RNA using sodium acetate/ethanol precipitation was very straightforward. A faster but significantly more expensive option was the RNeasy® kit (or similar silica-column-based extraction kit) or RiboPure™ kit, which can be used to recover the RNA from the formamide-based RNA extraction solution. Using either column-based method following the RNA*snap*™ extraction yielded extremely high-quality RNA suitable for any type of highly-sensitive RNA analysis (data not shown). However, the drawback to using a column, as demonstrated in this study (Table 3, RNeasy® Protect Bacteria and RiboPure™ Bacteria), was the non-quantitative recovery of RNA species depending on their size and possible secondary/tertiary structure of the RNA molecule.

With the advent of qRT–PCR, microarrays and next generation sequencing, genome-wide expression profiling has become an indispensible tool to decipher biological systems. However, at the heart of the most robust and sophisticated gene-expression analysis lays the quality and reproducibility of the extracted RNA pool. For example, if a research group were to use a column-based RNA extraction methodology, such as those tested in this study, to examine maturation of small RNAs <200 nt, the results of the study would be flawed due to non-quantitative recovery of RNA molecules <200 nt using the RNA extraction methods (Table 3). Alternatively, if a group were to examine the relative abundance of a 1000 nt transcript compared with a 5000 nt transcript, the ratio between the two abundances would vary considerably based on the RNA extraction methodology employed. More importantly, it is clear that no RNA isolation methodology (with the exception of the RNA*snap*™ method) is suitable for the study of all types and sizes of RNA molecules in the same experiment. Overall, the quality and representative recovery offered by RNA*snap*™ method is unmatched by the other methods tested in this study and is uniquely suited for highthroughput gene-expression analyses.

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