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,
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 RNASnap\textsuperscript{TM}, for Simple Nucleic Acid Purification) 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 $<15\text{ min}$ 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 $37^\circ\text{C}$ in Luria broth supplemented with thymine (50 mg/ml) to exactly 50 Klett units above background (No. 42 green filter or OD\textsubscript{600} 0.4), which is $\sim10^8\text{ cfu/ml}$. Other strains were generously provided by the Departments of Microbiology and Marine Sciences at the University of Georgia.

RNASnap\textsuperscript{TM} RNA isolation method for Gram negative bacteria

One milliliter of bacterial culture ($10^8$ cells) was centrifuged at 16000g 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 \textmu{l} 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 16000g for 5 min at room temperature. The supernatant was carefully transferred to a fresh tube without disturbing the clear gelatinous pellet.

RNASnap\textsuperscript{TM} 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 RNASnap\textsuperscript{TM} was added. The pellet from 1 ml of cells ($10^8$) was resuspended in 100 \textmu{l} of RNA extraction solution. The resuspended cells were transferred to a 0.5 ml screw cap tube containing $\sim200\text{ \mu{l}}$ of chilled zirconia beads (from Ambion Ribopure\textsuperscript{TM} 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 \textmu{l} 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 5000g for 5 min at 4\°C. The supernatant was carefully removed by aspiration, and the pellet was suspended in 200 \textmu{l} 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 \textmu{l} 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 \textmu{l} of 10\% Catrimide [(trimethyl (tetradeyl)ammonium bromide)] when the sample was almost completely thawed. The sample was briefly vortexed and centrifuged at 16000g for 10 min at 4\°C. The supernatant was carefully removed by aspiration, and the pellet was suspended in 500 \textmu{l} 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 16000g for 10 min at 4\°C. The supernatant was carefully removed by aspiration and the pellet was resuspended in 500 \textmu{l} of 2 M LiCl in water followed by a repeat centrifugation. The pellet was briefly vortexed in 75\% ethanol and centrifuged at 8000g 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 \textmu{l} of RNase-free water and incubated at room temperature for 10 min. The tube was vigorously vortexed, centrifuged at maximum force (21000g) at room temperature for 1 min to pellet cell debris, and the RNA containing supernatant was transferred to a new tube.

TRizol\textsuperscript{®} Max\textsuperscript{TM} Bacteria, RNeasy\textsuperscript{®} Protect Bacteria and Ribopure\textsuperscript{TM} 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\textsubscript{260}/A\textsubscript{280} ratio and RNA quality.
Determination of RNA quantity and quality

RNA quantity and $A_{260}/A_{320}$ ratios were determined using a Nanodrop™ 2000c (Thermo Scientific). The amount of RNA in the RNA$\text{sn}ap^\text{TM}$ 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 x 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$\text{sn}ap^\text{TM}$ method

In order to estimate the amount of RNA remaining in the pellet, we performed an RNA$\text{sn}ap^\text{TM}$ extraction using 10 ml of E. coli cells (10⁶ 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 16000g for an additional 5 min and the supernatant was again removed without disturbing the pellet. The pellet was then resuspended 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 16000g 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 x TBE gels for small RNA species (lpp, cspE, SS rRNA, ryhB and pheU/pheV) and 1.2% Agarose 1 x 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$\text{sn}ap^\text{TM}$ method was used directly for polyacrylamide gels after dilution to the desired loading volume in a formamide-based RNA loading dye. For agarose northern, the RNA in the extraction solution was brought up to a total volume of 10 μl with RNA$\text{sn}ap^\text{TM}$ 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 x MOPS gel, similar to the method of Vineze 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 32P-labeled oligonucleotide probes such that the signals for the lpp, SS 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$\text{sn}ap^\text{TM}$ 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 16000 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 16000 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 thyA715Km') 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$\text{sn}ap^\text{TM}$ 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 TTACTTGGGATAT) using SuperScript® III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. The cDNAs were amplified using two
gene-specific primers (LPP538 and LPPPCR1:GCTACATGGAGATTAAC) 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 32P-5'-end-labeled lpp specific oligo-nucleotide (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 tRNA operons (5'-CGTCCCTGACGCCTCTGACT-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 rRNA 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 (A260) 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 16000g, 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 A260 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 A260 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 (108 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 d isolation (µm) | 76 to >5700 | 76 to 3000           | 200 to >5700            | 300 to >5700       | 76 to >5700    |

aCost data based on the list price of chemicals or extraction kits.

bThe approximate yield is based on the average of multiple independent isolations starting with 10⁸ cells. All of these methods have the ability to handle >10⁹ cells. For example, the RiboPure™ Bacteria kit recommends using 10⁹ cells.

cThe approximate isolation time is based on the time it took from starting the isolation procedure to determining the concentration of the isolated RNA.

dThe RNA size range data is based on the sizes of specific RNAs detected by northern analysis (Figure 2).
The RNAsnap™ method recovers >99% of all RNA species

Even though the RNAsnap™ 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 (10⁹ 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 RNAsnap™ 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 RNAsnap™ isolated RNA

In an attempt to determine the size distribution of the transcripts present in the RNA isolated by the RNAsnap™ 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), RNasey® 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 (A₂₆₀/₂₈₀ 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).
highest concentrations of 5S rRNA and tRNAs, followed by the RNA snap TM 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 TM 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 TM RNA (Table 3). Transcripts >1000 nt (ompF, adhE and the rpsJ operon) were less abundant in the TRIzol® Max TM 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 TM 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 TM 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 TM 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 TM 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 TM method appeared to be less efficient in isolating larger transcripts compared to the RNeasy® Protect Bacteria, RiboPure TM 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 TM RNA samples (Figure 1A) were at the expense of larger RNA species (Table 3).

Overall, of the commonly used RNA isolation kits, TRIzol® Max TM 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 TM and Catrimide/LiCl RNA samples, small RNAs were either underrepresented (Catrimide/LiCl) or almost completely absent (RNeasy® Protect Bacteria and RiboPure TM Bacteria).

### Table 2. RNA quality scores

| Criteria | RNA snap TM | TRIzol® Max TM | RNeasy® protect Bacteria | RiboPure TM | Catrimide/LiCl | RNA snap TM precipitated |
|----------|-------------|----------------|-------------------------|-------------|----------------|--------------------------|
| A260/280 | 1.7 ± 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 | 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 | 9.5 ± 0.00 | 7.9 ± 0.17      | 9.0 ± 0.52              | 9.4 ± 0.21  | 9.4 ± 0.29      | 9.5 ± 0.35               |

*RNA from original extraction solution was precipitated using sodium acetate/ethanol and resuspended in water (see ‘Materials and Methods’ section).*  
*Obtained using a Nanodrop 2000c.*  
*Obtained from Agilent Bioanalyzer analysis.*  
*RIN obtained from Agilent Bioanalyzer analysis. Each value is the average of at least two replicates.*
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* |
|------------|----------|---------------------------------------------------------------|
| pheUV      | 76       | TRZisol® MaxTM bacteria: 1.64 ± 0.11 | RNeasy® protect bacteria: 0.09 ± 0.11 | RiboPure®TM bacteria: 0.05 ± 0.05 | Catrimide/LiCl 0.15 ± 0.10 |
| cybB       | 90       | 1.39 ± 0.56 | 0.8 ± 0.26 | 1.68 ± 0.33 | 1.01 ± 0.55 |
| SS rRNA    | 120      | 1.99 ± 0.13 | 0.35 ± 0.01 | 0.77 ± 0.34 | 0.45 ± 0.10 |
| cspE       | 300      | 1.67 ± 0.00 | 1.04 ± 0.14 | 1.86 ± 0.50 | 0.93 ± 0.22 |
| Lpp        | 330      | 2.03 ± 0.16 | 1.24 ± 0.08 | 1.64 ± 0.02 | 1.09 ± 0.05 |
| ompF       | 1000     | 0.81 ± 0.47 | 1.51 ± 0.40 | 1.94 ± 0.11 | 1.40 ± 0.46 |
| adhE       | 3000     | 0.53 ± 0.10 | 1.67 ± 0.11 | 2.62 ± 0.90 | 1.24 ± 0.44 |
| rpsJ operon| 5700     | 0.47 ± 0.36 | 2.60 ± 0.33 | 4.37 ± 0.33 | 1.62 ± 0.48 |

*Each transcript was probed with a specific 32P-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 RNA, we have used the RNA isolated using either the RNA snap ethanol precipitation step (see ‘Materials and Methods’ section) in which zirconium.

Generality of RNA snapTM 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 (10⁷ 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: Alcaligenes 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.
methods tested in this study and is uniquely suited 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 high-throughput gene-expression analyses.

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