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Optimization of a Protocol for *Escherichia coli* RNA Extraction and Visualization

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We attempted to improve a protocol to allow for the consistent extraction of total RNA from prokaryotic cells. Total RNA was isolated from *Escherichia coli* B23 and yeast cells using Trizol. In the comparison of two different protocols it was found that performing the phase separation independent from the bead bashing results in increased yield and purity of the RNA. We also attempted to improve resolution of the low molecular weight RNA molecules by comparing samples of RNA run on agarose and polyacrylamide gels. The polyacrylamide gels proved superior in resolving 5S, 5.8S rRNA and tRNA. Degassing significantly improved the well formation, resulting in more distinct bands. A comparison of glyoxal and formaldehyde denaturants was inconclusive, but preliminary findings suggest that formaldehyde has better potential. The use of yeast as an internal standard for prokaryotic RNA extraction was unsuccessful, but shows promise for future investigation.

A wide range of methodologies exists for both the isolation and visualization of ribonucleic acids (RNA). While different protocols have been purported to give the best yield and purity, no single method has been proven superior. An improvement to the single step guanidine isothiocyanate phenol-chloroform extraction protocol, first demonstrated by Chomczynski and Sacchi (3), has been developed by Life Technologies. Trizol, the main reagent, is effective in maintaining the integrity of RNA during cell disruption. Guanidinium thiocyanate is an effective protein denaturant which acts to inhibit ribonuclease (RNase; 2). While detailed protocols exist for its use in eukaryotic RNA extraction, the company admits that no in-house data has been generated demonstrating its effectiveness for prokaryotic RNA extraction (Invitrogen, personal communication).

The majority of current protocols favour the use of agarose gel electrophoresis for visualization of RNA. Agarose gels used in total RNA extraction tend to allow for good resolution of both high and low molecular weight RNA molecules. However, the length of the gels necessary to allow for good resolution of the low molecular weight RNA may be prohibitively large for use in some laboratories. It has been demonstrated, and confirmed herein, that resolution of low molecular weight RNA molecules can be more effectively obtained on polyacrylamide gels (4).

Variability in RNA yield has been problematic in quantifying the concentration of RNA within the cell. In the absence of a reliable method for determining the efficiency of individual RNA isolations, it can be difficult to determine whether changes in the amounts of RNA isolated are due to changing cellular levels of RNA or RNA lost during the isolation. The introduction of an internal standard would resolve this problem.

We set out to establish a more effective method to isolate and visualize total RNA. This included optimizing the Trizol extraction protocol, as well as comparing both agarose and polyacrylamide gels for their effectiveness in resolving total RNA. We investigated the potential use of eukaryotic RNA, specifically yeast RNA, as an internal control.

MATERIALS AND METHODS

Equipment notes. Glassware was treated to eliminate RNase by baking at 180°C for at least 2 h. Deionized water was treated with 0.1% diethyl pyrocarbonate (DEPC) at 37°C in a rotating water bath for 18 h, then autoclaved for 15 min. Fresh, nuclease-free bags of microfuge tubes and micropipettor tips were used throughout the experiment.

Cell Pellets. Bacterial Control – An overnight culture of *Escherichia coli* B23 cells (supplied by Dr. Ramey, University of British Columbia), growing in M9 minimal salts media with 0.1% glucose (1), was diluted in fresh media to obtain an optical density of 0.062 at 460 nm. The culture was grown in a shaking water bath set at 220 rpm and 37° C. When the OD_{460} reached 0.246 (time zero), a 2 ml sample was taken for processing, and subsequent samples were obtained at 1 and 2 h. The samples were centrifuged at 12000 x g for 5 min and the cell pellet was stored on ice. Yeast control – *Saccharomyces cerevisiae* (supplied by Brian McClure, UBC) was grown overnight in YPD (1) media in a shaking water bath set at 100 rpm and 30°C. One ml of the culture was centrifuged at 12000 x g for 5 min and the cell pellet was stored on ice. Bacteria plus Yeast – At each time interval, 0.1 ml of yeast was added to 2 ml of the bacteria culture. The volume of yeast added was standardized to approximate the volume of cytoplasm in 2 ml of bacterial cells. The culture was centrifuged at 12000 x g for 5 min and the cell pellet was stored on ice.

Cell homogenization. Total RNA was extracted from the cells using the Trizol reagent (guanidine isothiocyanate phenol-chloroform method) as described by the Invitrogen Life Technologies protocol. Several modifications were made to this protocol, as such, we will refer to them as Methods 1 and 2.

Method 1 – The cell pellet was resuspended in 1 ml of Trizol reagent and transferred to a 2 ml screw cap centrifuge tube containing 1 ml of 0.1 mm diameter Biospec glass beads. A small air space was maintained for the addition of chloroform after the homogenization.

Method 2 – The cell pellet was resuspended in 1 ml of Trizol reagent and transferred to a 2 ml screw cap microfuge tube containing 1.5 ml Biospec glass beads. The volume of beads was increased so that minimal air space existed once the beads were added.

The cells were mechanically disrupted by bead bashing the suspension in the Mini Bead-Beater 1 (Biospec, Bartlesville). The cells were bead bashed for 1 min at 4800 rpm then placed on ice for 1 minute. Cells were again bead bashed for 1 min at 4800 rpm then allowed to incubate at room temperature (15 – 30°C) for 5 min.

Method 1 – After cell homogenization, 200 µl of chloroform was added directly to the bead bashing tube. Then, 300 µl of the aqueous phase was transferred to a fresh microfuge tube, leaving behind approximately 200 µl of the aqueous phase.

Method 2 – The sample was not centrifuged at this stage; 500 µl of the homogenized sample supernatant was transferred to a fresh microfuge tube and 200 µl of chloroform was added. The remainder of the phase separation was performed as indicated in the Life Technologies protocol.

Following the RNA precipitation and wash with 75% ethanol (RNase free), the samples were allowed to air dry for 10 min. The RNA pellets were redissolved in 50 µl of DEPC-treated water and incubated at 60°C for 10 min. The RNA samples were stored at -20°C for 3 weeks then transferred to -80°C in light of concerns regarding RNase activity.

Determination of Purity and Yield. The RNA was evaluated by measuring the sample absorbance spectrum between 240 and 300 nm. The A_{270} served as a measure of phenol contamination (9), while the concentration of RNA was calculated from the A_{260} and A_{280} values.

Agarose Gels. Agarose gels were prepared to 1.2% using 1× TBE buffer. The samples were prepared by diluting the RNA in the appropriate amount of DEPC treated water to obtain a total of 500 ng RNA in either 12 µl or 15µl. Either 2 or 3 µl of 6× loading buffer was added to all samples. The samples were not heat-treated.

Polyacrylamide Gels. The recipe for 8% separating and 4% stacking polyacrylamide gels were obtained from the Short Protocols in Molecular Biology (1), with several modifications. These modifications included increasing the concentration of ammonium persulfate from 70 to 300 µl, TEMED from 4.6 to 30 µl and degassing the stacking gel for 30 min at –550 mmHg. The concentration of the RNA was standardized to either 500 ng or 1 µg/lane.

Gel staining. After electrophoresis, gels were stained using SYBR Green (Cambrex, Rockland). For the staining of non-denaturing gels, SYBR Green was diluted to 1/10000 in a solution of 1× TBE, whereas the glyoxal and formaldehyde gels required a 1/5000 dilution. Agarose gels were stained for 20 min on a rotating platform at approximately 30 rpm. Polyacrylamide gels were stained for 15 min at the same rotational speed.

Denatured RNA sample preparation. For glyoxal treatment, the RNA was denatured using the protocol described by Farrell (6) to obtain a final concentration of 1 M glyoxal, 50% dimethylsulfoxide, and 1× TBE substituted for sodium phosphate buffer (pH 7.0). For formaldehyde treatment, the RNA was denatured using the protocol described by Farrell (6) to obtain a final concentration of 0.6 M formaldehyde, and 0.5× TBE substituted for MOPS buffer.

RESULTS AND DISCUSSION

RNA Extraction. The first and second cultures were grown on M9 minimal medium and the RNA extractions were done using the Trizol/bead bashing technique. The first RNA extraction (Method 1) used the bead bashing tube for phase separation instead of transferring the homogenized cell supernatant into a fresh tube as in the second RNA extraction (Method 2). This required leaving an air space in the top of the tube during the bead bashing step that was not present in the second RNA extraction. Air pockets in the bead bashing tube may lead to inefficient cell lysis, thus decreasing the extraction efficiency (Dr. W. Ramey, personal communication). The loss in efficiency was expected to be compensated for by allowing for an increase in the retrievable aqueous phase. The theory is that by performing phase separation in the bead bashing tube it would allow for retrieval of all possible RNA that could be lost among the beads. The chloroform in method 1 was able to mix with the entire 1 ml Trizol sample, whereas method 2 only allowed the chloroform to mix with 0.5 ml of the Trizol, as the other half of the sample was trapped in the bead matrix. This resulted in an aqueous phase of approximately 500 µl in method 1 (300 µl of which was utilized in the extraction) and approximately 200 µl of aqueous phase in method 2. This indicates that it should be possible to extract twice as much RNA using method 1. The relative absorbance of each of the cultures indicate that there was up to 3× the amount of cells used in method 1 (Tables 1 and 2), therefore one would expect much greater yields. However, the data clearly demonstrates that method 2 is superior.

Method 1 was also less consistent in extracting RNA and it is possible that variation in the size of the air pocket during bead bashing leads to variability in cell lysis. Method 2 did not have an air space, which may account for the higher RNA yields obtained. Furthermore, method 1 showed higher phenol contamination (Table 1 and 2, A_{270}), a factor that would likely be aggravated by attempts to increase yield through taking more of the aqueous phase.

The initial aim of this experiment was to compare the effect of antibiotic treatment on the concentration of the different RNA species within the bacterial cell. After the cultures were split, chloramphenicol was added to a final concentration of 40 µg/ml, kanamycin to 100 µg/ml. This approach was abandoned due to inconsistent results.

Table I: Method 1 - RNA extraction from *Escherichia coli* B23

Timepoint	Sample	Culture concentration (OD_{460})	Concentration of isolated RNA				Concentration of RNA (ng/μl)
			A_{260}	A_{270}	A_{280}	A_{260}/A_{280}	
pre-split	pre-split	0.7	0.3	0.6	0.3	1.3	12
1 h post-split	control	1.8	8.7	ND	5.1	1.7	363
	chloramphenicol	1.1	3.4	ND	2.0	1.7	145
	kanamycin	1.0	9.5	ND	5.6	1.6	400
2 h post-split	control	1.8	1.0	1.4	1.0	1.1	31
	chloramphenicol	1.1	0.3	0.5	0.3	1.0	8
	kanamycin	1.0	0.4	0.5	0.3	1.2	12

Table II: Method 2 - RNA extraction from *Escherichia coli* B23 and *Saccharomyces cereviseae*

Timepoint	Sample	Culture concentration (OD_{460})	Concentration of isolated RNA				Concentration of RNA (ng/μl)
			A_{260}	A_{270}	A_{280}	A_{260}/A_{280}	
pre-split	pre-split	0.2	1.4	1.2	0.6	2.3	68
1 h post-split	control	1.2	5.6	4.7	2.7	2.1	255
	chloramphenicol	0.3	ND	ND	ND	ND	ND
	kanamycin	0.2	2.0	1.7	0.9	2.2	92
2 h post-split	control	1.5	11	9.2	4.9	2.3	545
	chloramphenicol	0.3	4.0	3.3	2.1	1.9	177
	kanamycin	0.2	0.8	0.7	0.3	2.6	40

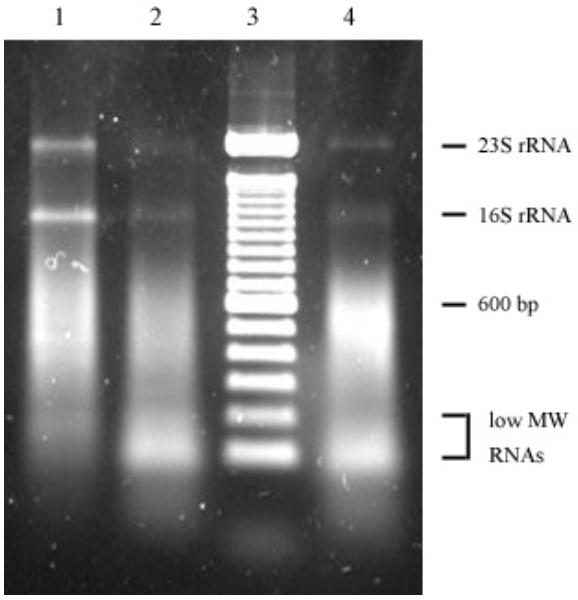


Figure 1. RNA samples isolated from *E. coli* B23 electrophoresed on a 1.2% agarose gel for 1 h at 100 V. Lane 1 contains RNA isolated from untreated cells, while lanes 2 and 4 contain RNA from cells treated with antibiotics. Lane 3 contains a 100 bp DNA ladder.

Overall, method 2 is the preferable technique for obtaining increased purity and consistency while still providing adequate RNA yields.

RNA Storage. The RNA samples in this experiment were stored at –20°C, which has been reported to cause extensive single- and double-stranded breaks in nucleic acids (5). Significant ambiguity exists in the literature as to the proper long-term storage of nucleic acids. Some sources suggest that 5°C is sufficient for storage (5), others suggest that –20°C is adequate, while others claim that –70°C or lower is essential to maintain the integrity of an RNA sample (5).

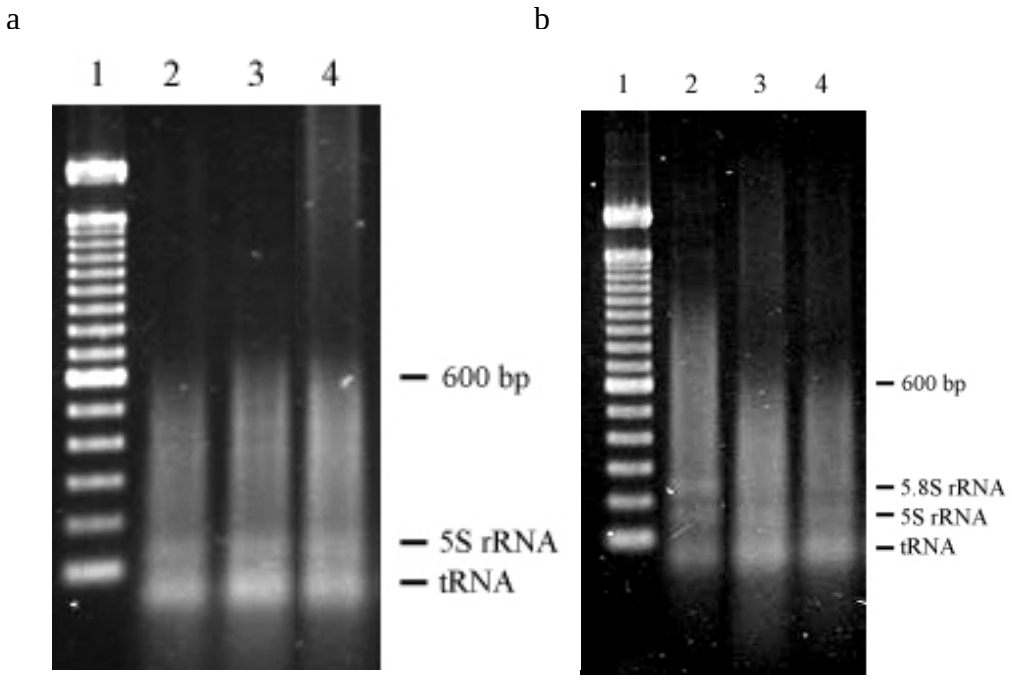


Figure 2. RNA samples isolated from *E. coli* B23 electrophoresed on a 1.2% agarose gel for 40 min at 100 V and for 1 h 40 min at 120 V. Lane 1 in each gel contains a 100 bp DNA ladder. a) Lanes 2-4 contain only bacterial RNA. b) Lanes contain yeast (2) or bacterial and yeast RNA (3 and 4).

Agarose Gel Electrophoresis. Agarose gel electrophoresis of total RNA isolated using method 1 allowed for the resolution of the high molecular weight rRNA (Fig. 1). The resolution of the low molecular weight RNA was poor with only a diffuse band observed around the 100 bp DNA standard. Smearing throughout the lane indicated degradation of the RNA by RNase despite attempts made to limit RNase contamination.

The high molecular weight rRNA molecules from the total RNA isolated using method 2 could not be visualized by agarose gel electrophoresis (Fig. 2a and 2b). There was an absence of bands around 2900 and 1500 bp where the 23S rRNA and the 16S RNA would be expected to run.

Although the diffuse low molecular weight bands are still visible around the 100 bp DNA standard, smearing was observed throughout the lanes indicating that a portion of the RNA had been subject to degradation by RNase. The absence of the larger rRNA bands suggests possible preferential degradation of the large rRNA molecules. The increased degradation noted in the RNA sample isolated by method 2 may be partially attributed to extended storage at -20°C, repeated freezing and thawing and more opportunities for RNase contamination due to increased handling of these samples (5). In an effort to improve the resolution of the low molecular weight RNA molecules isolated during method 2, polyacrylamide gel electrophoresis was performed and several modifications were made to optimize the procedure.

Polyacrylamide Gel Assembly. Initially, when the RNA was run on polyacrylamide in the absence of a stacking gel, inadequate polymerization led to the wells filling with different levels with polyacrylamide solution. This resulted in the samples entering the gel matrix at varying heights.

In order to resolve these problems, a stacking gel was added where the concentrations of TEMED and ammonium persulfate were increased. A marked increase in the strength and stability of the well walls was observed, as well as the depth and flatness of the bottoms. However, significant amounts of unpolymerized polyacrylamide solution were still present in the wells and needed to be removed using a Hamilton syringe.

In an attempt to further improve upon these results, we used a vacuum chamber to remove oxygen from the stacking gel. As molecular oxygen is known to inhibit the polymerization of polyacrylamde, the stacking gels were

degassed, after pouring, by placing the entire casting apparatus in a vacuum chamber and applying a negative pressure of –550 mmHg for 30 min. Comparing the stability and shape of the wells in gels that were degassed versus those that were not confirms that degassing significantly improves well formation, resulting in deeper wells with much flatter bottoms. Flat-bottomed wells are important in the absence of a stacking gel, as it ensures that the samples will enter the gel in a uniform manner. Uneven well bottoms lead to wavy bands in the gel, as observed in the first non-denaturing gels (Fig. 3a and 3b). Using a stacking gel reduces the necessity for perfectly formed wells as the interface between the stacking and separating gels will concentrate the molecules into a tight band.

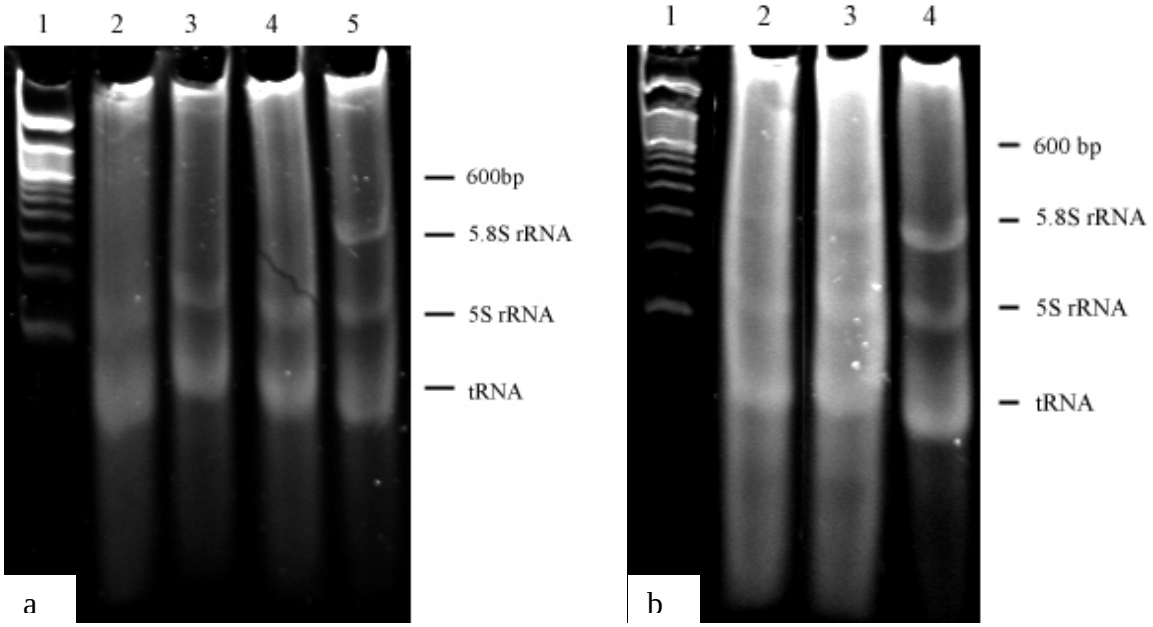
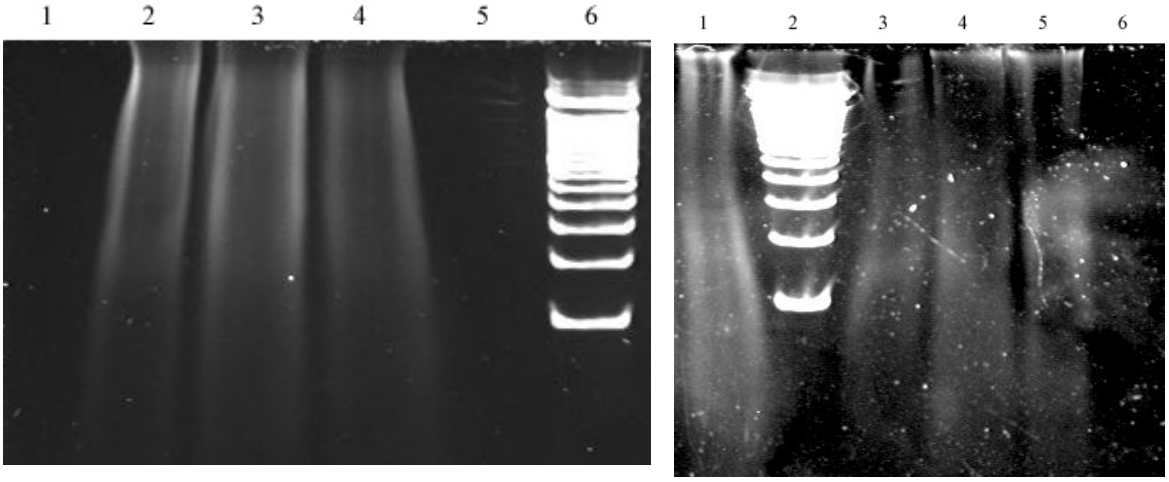


Figure 3. RNA samples isolated from *E. coli* B23 electrophoresed on a 8% polyacrylamide gel at 100 V for 40 min. Lane 1 in both gels contains a 100 bp DNA ladder. a) Lanes contain bacterial RNA (2-4) or yeast RNA (lane 5). b) Lanes contain bacterial and yeast RNA combined (2 and 3) or yeast RNA (lane 4).

RNA Denaturing. A comparison between two accepted methods of denaturing RNA, glyoxal versus formaldehyde, indicates that formaldehyde may be more suited for visualization of low molecular weight RNA molecules on polyacrylamide gels. There was a complete absence of bands on the gel in which RNA samples were treated with glyoxal, however a broad smear was noted along the edges of the lanes (Fig. 4a). This observation suggests that the lack of bands was not due to a failure of the SYBR Green staining solution, but rather to degradation of the RNA. This may have resulted from the oxidation of glyoxal, as oxidized glyoxal is known to cause RNA degradation (8). The fanning out of the lanes containing the RNA towards the empty lanes underlines the importance of having equal charge across the gel (Dr. W. Ramey, personal communication). In an effort to eliminate this fanning out, blanks containing all reagents except the isolated RNA were loaded in extra lanes (Fig.4b). This significantly improved the situation; however the lack of salt in the DNA standard caused the adjacent lanes to fan out toward the DNA ladder (Fig.4b). The presence of the ladder suggests that degradation is to blame for the lack of RNA bands, as opposed to the sample running off the gel. Glyoxal treatment is known to slow the rate at which nucleotides travel through gels (8), which suggests that any non-degraded pieces of RNA present in the samples would appear above the last band of the DNA ladder. We suggest that the ready-to-load RNA/DNA ladders are not useful in these situations and recommend that the standard fragments be treated using the same denaturation protocol that is used for the RNA samples.

The RNA samples treated with formaldehyde show bright distinct bands representing the 5.8S, 5S rRNA and tRNA; however, there is significant fanning out of the lanes (Fig.5a). When the gel was repeated and blanks were substituted into the extra lanes, significant improvement is noted; however, all the RNA is degraded and no bands are visible (Fig.5b). It is noted in the literature that when the pH of the formaldehyde drops below 3.5, degradation

of the RNA occurs (6). Measurement of the formaldehyde utilized in the sample preparations indicates that the pH was 2.9. It's unlikely that the pH would have changed in the 24 h period between sample preparations, and therefore a possible explanation could be that the reagents were added in a different order.



a b

Figure 4. Glyoxal denatured RNA samples isolated from *E. coli* B23 electrophoresed on an 8% polyacrylamide gel for (a) 45 min or (b) 1 h at 100 V. a) Lanes 2-4 contain RNA, 6 contains a 100 bp DNA ladder; 1 and 5 were left empty. b) The separating gel was overlayed with a 4% stacking gel. Denaturant was added to the empty lane 6.

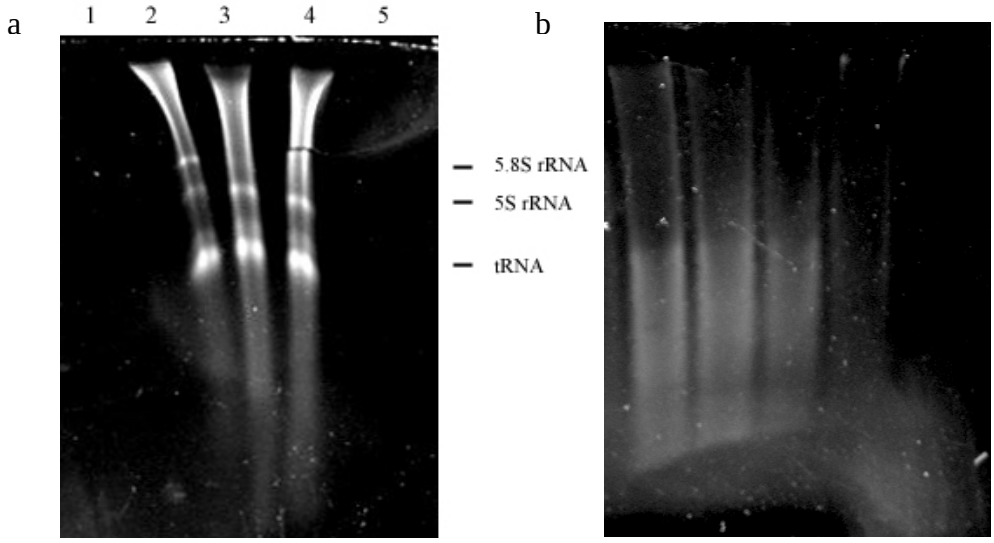


Figure 5. Formaldehyde denatured RNA samples isolated from *E. coli* B23 electrophoresed on an 8% polyacrylamide gel for (a) 1 h 10 min at 100 V and (b) 2 h at 150 V. a) Lanes contain yeast RNA (2), bacterial RNA (3) and yeast and bacterial RNA (4). Lanes 1 and 5 were left empty. b) Samples were loaded into the 4% stacking gel, with empty lanes containing denaturant.

Resolution of Yeast 5.8S rRNA. The major difference between prokaryotic and eukaryotic low molecular weight RNA is the presence of a 5.8S rRNA in eukaryotic organisms. The objective was to try to resolve the 5.8S and 5S rRNA molecules. This is visible in Figure 3a, which shows good resolution of low molecular weight RNA species.

The lanes containing the bacterial RNA samples show the characteristic 5S rRNA band and a wide band below representing the various species of tRNA. The lane containing the yeast RNA shows both the 5S rRNA and tRNA bands running alongside the bacterial bands of the same type, as well as the 5.8S rRNA running above. Similar results can be seen in Figure 3b, in which lanes 2 and 3 contain samples of bacteria and yeast combined, with lane 4 containing pure yeast.

Figure 6 incorporated some of the improvements mentioned previously, namely by adding a stacking gel and degassing. This resulted in significantly tighter bands than those seen in Figures 3a and 3b. However, some variation in the migration pattern was still observed, as shown by the slower migration of the yeast samples in lane 4 (the 5S rRNA of the yeast sample did not run as far as the 5S rRNA from the bacterial sample in lane 3).

The RNA ladder not only served as a molecular weight marker, but also as a control for degradation. The significant amount of degradation of the RNA ladder suggests that RNase contamination is present in either the sample loading buffer or at least one ingredient in the polyacrylamide gel itself.

The heavy staining seen within the stacking gel at the top of the figure is likely due to aggregation of the low molecular weight RNA which were then trapped in the stacking gel (6).

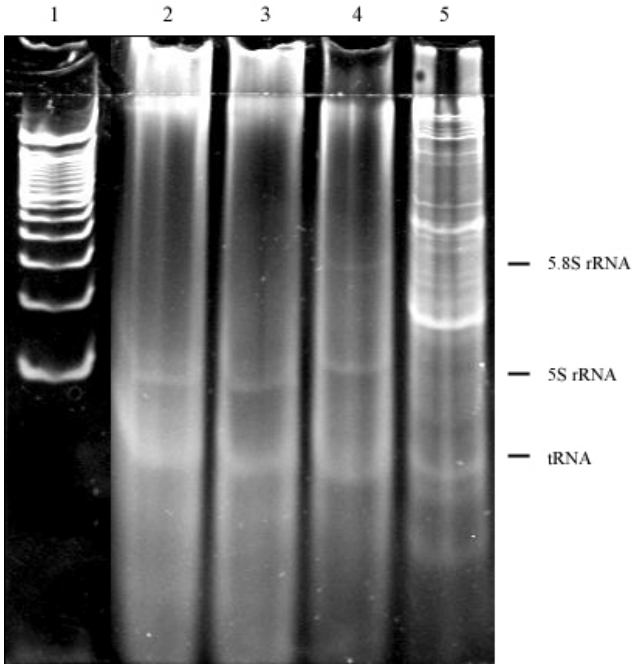


Figure 6. Non-denatured RNA samples isolated from *E. coli* B23 electrophoresed on an 8% polyacrylamide gel overlayed with a 4% stacking gel for 1 h at 100 V. Sample lanes contain yeast and bacterial RNA (2), bacterial RNA (3) and yeast RNA (4). Lanes 1 and 5 contain a DNA and RNA ladder, respectively.

Internal extraction standard. Attempts to use yeast as an internal standard in bacterial RNA isolation proved to be ineffective in the current protocol. High background in the lanes containing both yeast and bacteria, combined with and low interference in the yeast only lanes, resulted in difficulty in performing accurate pixel counts. However, with improvements to the protocol it may show promise for future use. While the 5S and 5.8S rRNA bands were easily resolved, the bacterial and yeast 5S bands were found to co-migrate and were visible only as a single band (Fig.2b). This does not allow for direct determination of the amount of bacterial RNA within the combined yeast and bacteria RNA lane. By obtaining a numerical value for the strength of the 5.8S band and the 5S band in the yeast only lane, it would be possible to determine a ratio of their strength. By applying this ratio to the 5.8S band in the yeast and bacteria combined lane, it would be possible to calculate a theoretical value for the strength of the 5S band due to yeast. Subtraction of this number from the pixel count values for the 5S band in the yeast and bacteria lane would allow one to quantify the signal strength attributable to the bacterial 5S rRNA in that lane.

Conclusion. In comparing the two methods used for RNA isolation, it was determined that method 2 resulted in better purity and yield. It was also shown that polyacrylamide gels are more effective at separating low molecular

weight molecules than agarose gels. Degassing is valuable for improving the polymerization of polyacrylamide gels. In comparing the two denaturation methods, formaldehyde was found to be more effective than glyoxal. When electrophoresing denatured samples in polyacrylamide gels, it is important to treat all lanes equally to ensure that lanes run evenly.

FUTURE EXPERIMENTS

An internal standard is a useful tool when quantifying components within a cell. This enables one to ensure that different levels of RNA are due to cell differences, not differences in applying the protocol to each sample. The current experiment showed the potential use of yeast RNA for this purpose, though several additional parameters would increase the chances of success. In an effort to prevent RNA degradation, the use of pipettes specifically designated for RNA is suggested. The gel box and comb should be treated with 0.5% sodium dodecyl sulfate, washed with DEPC-treated water and rinsed with ethanol. When denaturing samples, the pH of the formaldehyde should be greater than 4 as a lower pH will result in degradation. Together these measures would decrease the background interference and allow for accurate pixel counts. The use of urea as a denaturant could also be explored as an alternative to formaldehyde and glyoxal. The importance of storing the RNA at -80°C and minimizing the time between isolation and electrophoresis should also be tested.

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