COMPARISON OF RNA PRESERVATION TECHNIQUES

By

MIRIAM MALAK SOLIMAN

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Approved by:

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David T. Harris, Ph.D.

Department of Immunobiology

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Abstract:

RNA is a crucial aspect of life. The extraction of RNA allows for one to be able to code one's genetic code, therefore obtaining a great deal of information about how life functions and what composes life. The extraction of RNA from human blood samples was performed using the Direct-zol RNA MiniPrep kit, which provided all of the reagents and instructions for the proper extraction of RNA from a sample of bodily fluid. Three reagents, DNA/RNA Shield, TRIzol, and RNAlater were compared in terms of which provided the highest concentration and purtiest sample of RNA being extracted from a single blood. From the data obtained, it was found that there is no significant difference between the results obtained from the three reagents. Therefore, when conducting a larger extraction, any one of the reagents could be used for storage and extraction and will result in statistically similar results when comparing concentration of RNA as well as its purity.

Introduction:

RNA isolation from human samples allows for a great deal of genetic information to be identified and studied. RNA is crucial for life due to it being the intermediate step between DNA (which contains the genetic code of life) and proteins (the functional units of life). Although the isolation and extraction of RNA has been done for many years, new and efficient ways of doing such have recently emerged. The reagent DNA/RNA Shield is a new solution which is stated by its manufacturer to be compatible to many RNA extraction kits, yet its effects have not yet been extensively tested. TRIzol, the primary reagent used for the isolation of RNA from cells functions by inhibiting the actions of RNAse, while simultaneously disrupting the cell membrane and other components within the cell that isolate the RNA present[1]. TRIzol is the most frequently used due to the fact that it can isolate many different forms of RNA (viral RNA, microRNA, mRNA, etc.)[2,3]. The second most commonly used reagent for the extraction of RNA from cells is RNAlater. RNAlater allows for the preservation of the gene-expression code which RNA is composed of from degradation due to prolonged storage[4,5]. Although RNAlater can preserve RNA from degradation, it cannot isolate it and release it from within the cellular membrane and other organelles within the cell. Therefore, when running an extraction, samples which have been stored in RNAlater must be also placed into a solution containing TRIzol in order to disrupt the membrane of the cell. Due to DNA/RNA Shield's effects and chemical makeup being relatively unknown; it was further studied within this experiment.

This experiment utilized the three different blood storage reagents mentioned above: TRIzol, RNAlater, and DNA/RNA Shield to test which reagent would result in the greatest quantity (concentration) and purest quality (least amount of DNA and protein contamination) of RNA extracted from blood samples obtained from anonymous donors. The null hypothesis of this experiment was that there is no significant difference in the RNA obtained (when comparing the quantity and purity of the product) despite the different reagents the sample was stored in.

Methods:

Overall

RNA extraction from human blood samples was conducted twice a week for eight weeks. In the first two weeks of extraction, half of the blood sample stored in its respective reagent was used. In the remaining six weeks, the full blood sample stored in its respective reagent was used for the extraction of RNA. Each sample was labeled with the date of collection and the date of processing. All other data about the patient was kept anonymous.

Blood Preparation

Blood was supplied by the University of Arizona Health Sciences Biorepository. The blood samples were stored in a refrigerator at 4°C for up to 24 hours prior to storing the blood in the various reagents (TRIzol, RNAlater, and DNA/RNA Shield). 200 µl of blood from a single sample was added to 800 µl of TRIzol; the solution was then vortexed. 200 µl of blood from a single sample was added to 800 µl of RNAlater and then vortexed. 500 µl of blood from a single sample was added to 500 µl of DNA/RNA Shield and then vortexed. If the blood was left for longer than 24 hours in the refrigerator prior to being stored in the reagents, it was not used. To ensure consistent results, the RNA was extracted immediately after suspension in the nucleic acid prep solutions. After being stored in the respective reagents, the samples were stored in a -70°C freezer until they were needed for the extraction of RNA.

RNA Extraction

The Direct-zol RNA MiniPrep instructional manual was followed for the extraction of RNA. In the first two weeks of the experiment, for samples that were originally stored in TRIzol, 500 µl of the sample was used for the extraction, with an extra 200 µl of TRIzol added to each vial. For the samples originally stored in RNAlater, 500 µl of sample was placed into separate RNAse-free vials and a full volume (500 µl) of DPBS (Dulbecco's phosphate-buffered saline) was added into the vial in order to aid in pelleting the sample. The vial was then placed in a centrifuge and was spun for about 2 minutes. The same was done for the DNA/RNA Shield samples. In the remaining 6 weeks of the experiment, the full sample which was either stored in TRIzol, RNAlater, and/or DNA/RNA Shield was used for the extraction. The amount of DPBS which was added to each sample was then altered (1,000 µl was added to each RNAlater and DNA/RNA Shield sample).

After the samples stored in RNAlater and DNA/RNA Shield were spun for 2 minutes, a pellet of about 100 µl was present at the bottom of each vial. The supernatant was removed from each vial and 500 µl of TRIzol was added to each vial to resuspend the pellet into solution. Such resulted in a 5:1 ratio of TRIzol:sample for all three of the samples stored in the different reagents.

To each of the three samples, a full volume (600 µl) of 100% Ethanol was added and the solution was vortexed for about 1 minute. The addition of Ethanol allowed for an increase in adhesion of the RNA to the Column[11]. The mixtures were transferred to Zymo-Spin IIC Columns in a Collection Tube[11] which were provided by the Direct-zol RNA MiniPrep kit[11]. Each sample was centrifuged for about 2 minutes. The flowthrough within the Collection Tube was removed and 400 µl of RNA Wash Buffer (present in the Direct-zol RNA Miniprep kit) was added to each Column, and centrifuged for 1 minute. The remainder of the steps which were conducted can be found in the Direct-zol RNA MiniPrep instructions manual[11].

After the extraction of the RNA from the blood samples, the RNA was either stored in a -70°C freezer for about 1-5 days, or immediately measured using a Nanospectrometer. 5 µl of DNAse/RNAse-Free Water was used to clear the Nanospectrometer between measurements. 5 µl of the extracted sample was measured by the Nanospectrometer, which resulted in measurements of concentration of RNA present in the sample (measured at 260 nm). DNA or protein contamination ratios were analyzed through absorbance at 230 and 280 nm. The remainder of the sample which was not measured was stored in a -70°C freezer until it was needed for future use.

Statistical Analysis

The analysis compared the concentration and purity of RNA from three different nucleic acid storage reagents. The null hypothesis was that there is no difference in quality or quantity between the three reagents. The null hypothesis was evaluated using $\alpha = 0.05$, meaning it would be rejected if p was less than 0.05.

The effectiveness of the three reagents were compared statistically using analysis of variance (ANOVA) with adjustment for individual donor variation on the quantity of RNA extracted. This adjustment called for a mixed model ANOVA which slightly decreases degrees of freedom, but also decreases the amount of variation caused by natural differences between donors. These differences can negatively affect the analysis because different donors may have different true means and can push the results toward the null hypothesis, therefore skewing the results.

Since the quality of RNA is solely affected by the reagent and extraction method, ANOVA without subject adjustment was performed to evaluate the difference between reagents. If any of the comparisons are found to be statistically significant according to the defined α, a pairwise t test was performed.

Results:

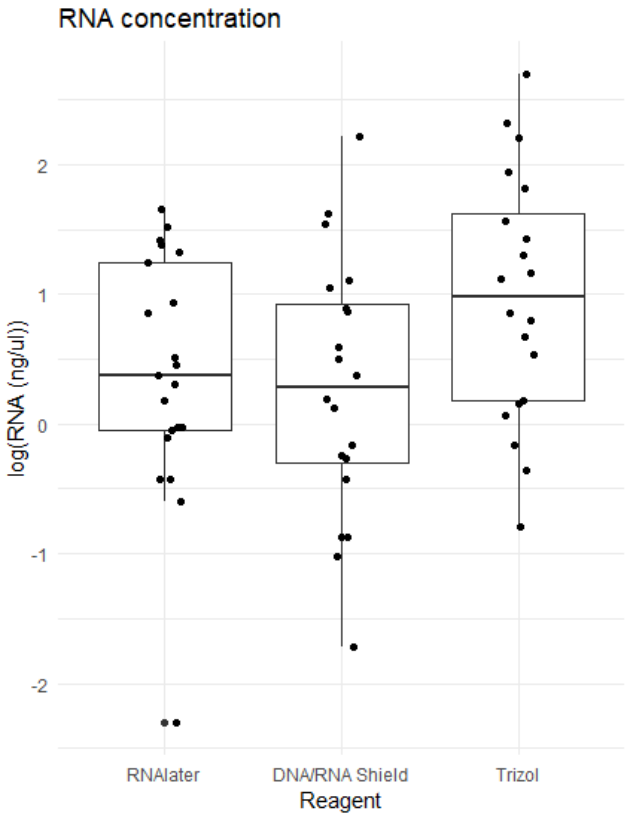

Figure 1 RNA Concentration Comparison With Log Transformation: The boxplot shows there is no significant difference between the three RNA reagents. By having no significant difference, all of the reagents had similar variance from the mean. The concentration of RNA was measured using a Nanospectrometer which measured nucleic acid concentrations at 260 nm.

According to *Figure 1*, there was no significant difference between the different reagents when analyzing the concentration of RNA which was extracted from each sample. *Figure 1* expresses the log transformation in order to correct the skewness of the data which may have been present due to using different samples from different

donors (natural aspects of the blood affecting the results). The absorbance of RNA concentration is generally measured using a Nanospectrometer, which measures RNA concentration at 260 nm.

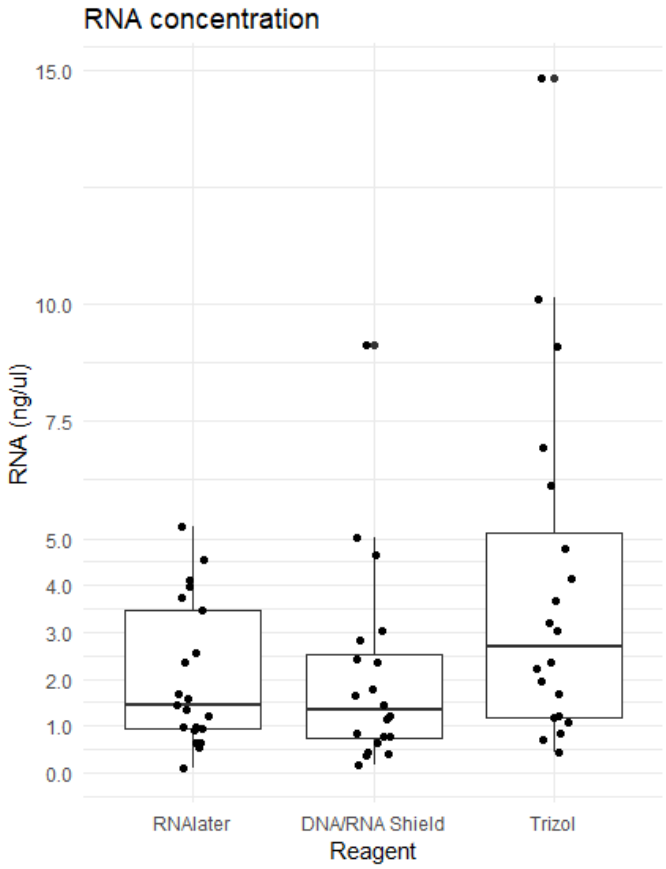

Figure 2 RNA Concentration Comparison Without Log Transformation: There was no significant difference in the concentration of RNA extracted from the different reagents found. The data within this figure was collected using Nano Spectrometry which measured the concentration of RNA within a 5 µl sample. Although all of the samples did not vary from the mean as much, it was found that TRIzol slightly varied from them mean, yet such was not statistically significant.

The data in *Figure 2* was not corrected using a log transformation, therefore TRIzol can be observed to be slightly right-skewed. There was an increase in concentration of RNA towards the end of the experiment (due to using a new Direct-zol RNA MiniPrep kit), which may have altered the data to result in the skewed data. Concentration was measured in both *Figures 1* and *2*, with the greatest concentration of RNA present within a sample was found to be the most favorable. Using a mixed ANOVA, the differences between RNA quantities were found to be approaching significance ($p = 0.159$). Yet due to the p value being larger than 0.05, the results were not found to be significant.

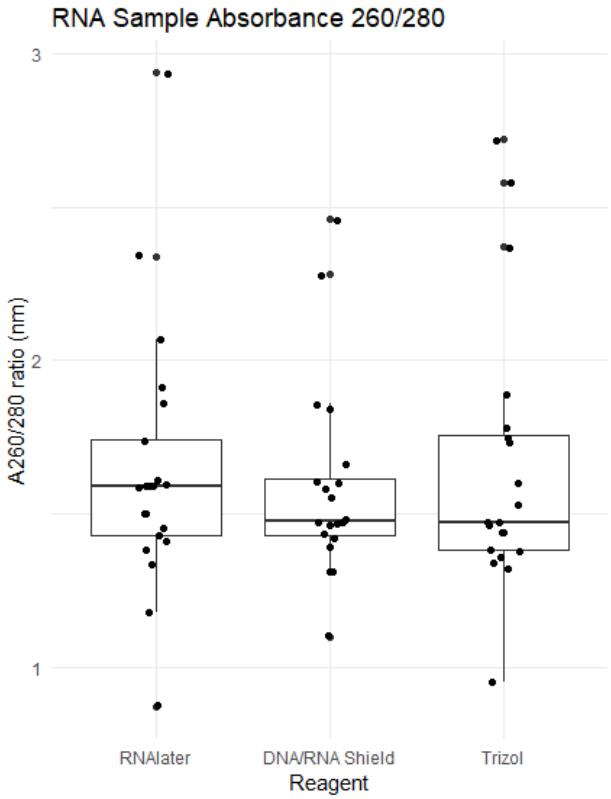

Figure 3 A260/280 nm Reagent Comparison: Using ANOVA, it was found that there was no significant difference in the absorption ratio of the RNA extracted from the three different reagents. The absorption ratio of 260/280 signifies the purity of the nucleic acid being observed. A ratio of about 2 is found to be most favorable for RNA purity[7,8].

Figure 3 expresses the absorbance of RNA at 260/280. The 260/280 measurement was tested in order to signify the purity of the RNA sample; the contaminant being the amount of DNA that is also present within the sample and other proteins (such as remnants of the proteins present in the buffers used for extraction) which can be seen at 280 nm. There was found to be no significant difference between the three reagents when comparing the purity of the RNA extracted (amount of DNA which was traced). The optimal ratio which quantifies a “pure” RNA sample is about 2[7,8].

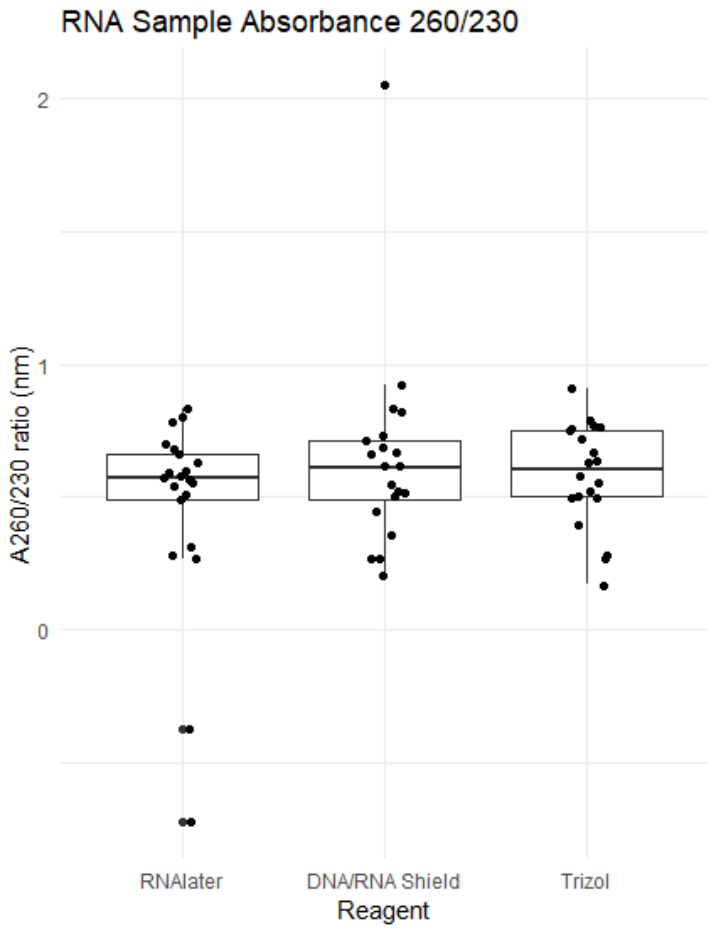

Figure 4 A260/230 nm Reagent Comparison: As stated in the statistical analysis, there was no significant difference in the 260/230 absorption ratio of the RNA which was stored in the three different reagents. The 260/230 ratio also signifies the purity of the nucleic acid being observed. For nucleic acids, a ratio of approximately 2.0-2.2 is generally found to be favorable[7,8].

Figure 4 expresses the absorbance of RNA at 230/280. A 260/230 ratio reading is used as a secondary measure of the purity of an RNA sample. Certain proteins can be seen at an absorbance wavelength of 230 nm. There was found to be no significant difference between the reagents. These results were found through running 5 µl of

each sample through the Nanospectrometer. A 260/230 ratio measurement lower than 2-2.2 indicated that there was either protein or DNA contamination within the sample[7,8].

The difference in quality, which was done by comparing absorbance ratios, were also not found to be significant. The comparison of the A260/280 ratio produced a p value of 0.870, while the A260/230 ratio gave 0.224. This indicates that the quality of RNA extracted is not affected by the storage reagent used.

Effects of Storage Reagents

Due to p being greater than 0.05, there is failure to reject the null hypothesis. By failing to reject the null hypothesis, there was found to be no statistical difference between the three storage reagents (TRIzol, RNAlater, and DNA/RNA Shield) when it came to the concentration and purity of the samples measured.

Discussion:

Discussion of Methods

Each blood sample that was obtained from the Biorepository was stored with either a: 4:1 ratio of TRIzol:sample, RNAlater:sample, or a 1:1 ratio of DNA/RNA Shield:sample. These ratios were in accordance to the storage system which is implemented by the Biorepository. It was found that if the blood sample was left in the refrigerator for longer than 24 hours after retrieval from the anonymous donor, without being placed within its proper reagent, the RNA within the blood would no longer be viable/the concentrations upon extraction would be severely reduced.

In order to extract the RNA from the blood samples, a lysing agent (such as TRIzol) was needed in order to disrupt the cell membrane (for samples stored in RNAlater and DNA/RNA Shield). Such is needed because RNAlater preserves the RNA within a sample by simply disrupting RNAse[10], which allows for the RNA within the sample to remain viable and avoid degradation with long term storage. RNAlater does not lysis the cell membrane, making the RNA inaccessible when extraction is needed. The effects of DNA/RNA Shield are relatively unknown, which was one of the main incentives behind the conduction of this experiment. It was observed that when a sample was placed in DNA/RNA Shield, at times the sample would be lysed, yet at other times it did not seem to be lysed. TRIzol was therefore added to samples stored

in both reagents in order to insure the proper lysis of all membranes and organelles within the cell. The addition of DPBS (Dulbecco's phosphate-buffered saline) was added to each sample to aid in the pelleting of the cells prior to the addition of TRIzol; this aided in the removal of excess reagent.

The Direct-zol RNA MiniPrep kit was used since the kit is compatible with all three of the different reagents. According to the kit, any sample that was not stored in TRIzol, must have been pelleted out and stored in a 3:1 ratio of TRIzol to sample. Prior to collecting data, it was found that a 3:1 ratio of TRIzol:sample did not result in the greatest concentration of RNA being extracted using the Direct-zol RNA MiniPrep kit. Samples stored in a 3:1 ratio, 4:1 ratio, 5:1 ratio, 6:1 ratio, 7:1 ratio, and 8:1 ratio were all tested to find which ratio of TRIzol:sample resulted in the greatest concentration. It was found that the maximum amount of RNA measure was at a 5:1 ratio, while it began to drop off at a 7:1 ratio. Therefore, data was collected using a 5:1 ratio of TRIzol:sample prior to transferring the solution into the column.

Upon extraction, a single sample of a total of 1,000 µl (stored in any one of the three reagents) was split in half and was used for separate extractions for the first 2 weeks of the experiment. After collecting data for about 2 weeks, it was found that using a full vial of 1,000 µl of sample resulted in a greater concentration of RNA being extracted.

Discussion of Statistical Analysis:

One of the assumptions when using ANOVA was that the data was sampled from a normally distributed population. The distribution of RNA quantity was right-skewed, so a log transformation was used to correct the skewness. The differences between RNA quantities were found to be approaching significance ($p = 0.159$) using a mixed model ANOVA. However, using our previously assigned α value, the result was not significant ($p > 0.05$). Although it was not found to be significant, the results were approaching significance, which may indicate that TRIzol may have resulted in the greatest concentration of RNA being extracted from a single sample. These results can be confirmed using the boxplot provided comparing log mean RNA concentration (ng/µl) by RNA storage reagent. As the results of the experiment stated, there was no significant difference between any one of the reagents. Although there was a bit of variance within the concentration of RNA being extracted from samples stored in TRIzol, it was not significant enough to state that there was a statistical difference between TRIzol in comparison to the other reagents which were used.

Discussion of Results:

According to the statistical analysis, there was found to be no significant difference ($p = 0.159$) between the three reagents when it came to measuring the

concentrations of RNA of the sample of RNA which was extracted. Therefore, one fails to reject the null hypothesis in that there is no significant difference between the three reagents when measuring the concentrations of RNA extracted from the three different reagents.

An analysis of the purity of the sample of RNA which was extracted was defined by both the 260/280 ratio and the 260/230 ratio. RNA is usually measured at an absorbance wavelength of 260 nm, therefore a value of about 2 for the ratio of 260/280 is defined to be pure for a sample of RNA[7]. If this value is found the be lower than 2 or abnormal, it can be said that the sample has been contaminated by protein (often times, some of the proteins which are used for the extraction of RNA)[7]. Proteins and other contaminants often measure at an absorbance of 280 nm, therefore a lower ratio indicates a great deal of contamination[7,8]. A ratio of 260/230 is taken as a secondary measurement to test the purity of the sample. An RNA sample is considered to be pure if the 260/230 sample is larger than 2[7,8]. If the ratio is found to be lower than 2, then this indicates that there is contamination from a substance that absorbs at 230 nm[7,8]. According to the statistical analysis, it was found that for purity of the RNA samples when comparing the three reagents, there was no significant difference (the comparison of the A260/280 ratio produced a p value of 0.870, while the A260/230 ratio gave 0.224). This indicates that the quality of RNA extracted is not affected by the

storage reagent used. Therefore, one fails to reject the null hypothesis that there is no difference between the three reagents when measuring the purity of a sample.

In order to insure the consistent results from the extraction of RNA from the three different reagents, the reagents all should be isolated for RNA extraction only. All surfaces must be well sanitized, and blood samples should ideally be stored in the chosen reagent no later than one hour after receiving the sample and storing into the refrigerator. In this specific experiment, it was hard to control all such aspects due to the extractions being conducted two times per week. Some of the samples may have been left in the refrigerator for longer than one hour prior to storing in its proper reagent. Such aspects were controlled for in the statistical analysis, yet in order to insure the best possible results, such details must be considered. RNA is incredibly sensitive to any foreign interaction; it must be handled with a great deal of caution and precision.

Conclusion:

The results of this experiment are significant in the further study of genetic material and genetic sequencing. The use of TRIzol, RNAlater, and DNA/RNA Shield (which is relatively new and unknown) aided in identifying which reagent may result in the purest and largest quantity of RNA for the least amount of sample being used. Due to DNA/RNA Shield still being new, it was beneficial to test its effects and possible uses. By extracting the greatest amount of RNA from a sample with the greatest purity, such can be used to further analyze the makeup of RNA, and conduct genetic research. Through the conclusions reached by this experiment, any one of the three different reagents (TRIzol, RNAlater, and DNA/RNA Shield) can be used to extract RNA for further research; all of which will most likely result in the same amount of RNA being extracted which is of the same purity.

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