Troubleshooting Forum

Molecular Biology Techniques Q&A

RNA Isolation: Improving Quality

This month's questions from the Molecular Biology Forums (online at molecularbiology.forums.biotechniques.com) come from the "RNA Methods" section. Entries have been edited for concision and clarity. Mentions of specific products and manufacturers have been retained from the original posts, but do not represent endorsements by, or the opinions of, *BioTechniques*.

How can I improve the quality of my RNA? (Thread 28637)

Q I isolated RNA from peripheral blood mononuclear cells (PBMCs) using the TRIzol method. NanoDrop and Bioanalyzer analyses indicated that I had excellent RNA. The 260/280 values were usually ~1.8 and the RIN was between 7.5 and 9.9. But the 260/230 values were <1, with the most common reading being ~0.5.

I want to use this RNA for Affymetrix microarray analysis. The director of the microarray facility told me that the poor 260/230 values might be caused by TRIzol contamination, which can be resolved by performing a purification step after extraction with TRIzol, using either phenol-chloroform with sodium acetate reprecipitation in isopropanol or a kit with spin columns.

When I tested these methods with unimportant RNA, I lost 90% of my RNA in my first phenol-chloroform extractions. I changed every reagent possible, including getting new phenol and trying only water-buffered. I got a new bottle of chloroform and made fresh sodium acetate and diethylpyrocarbonate (DEPC)-treated H₂O. Following these changes, I got yields of ~70% and RNA looked fine when checked by NanoDrop. But with the Bioanalyzer, I got RIN values of 4.4 and no bands showed up in a gel, indicating that the RNA was degraded. I tried a commercial kit from Norgen, from which I recovered ~60% of the RNA, but it was also degraded and had horrible NanoDrop values. What else can I do to clean up my RNA and get higher 260/230 values?

A I use the Qiagen RNeasy mini kit for RNA cleanup after TRIzol extraction and add the step for on-filter DNase digestion to remove any genomic DNA. With this method, the purified RNA is good enough for microarray work.

A I'm having very similar problems using TRI Reagent for extractions from skin punches. I read that using butanol helps remove the phenol, which is usually responsible for low 260/230 ratios, but I haven't tried it yet.

Q I read that butanol is supposed to remove salts and concentrate the RNA by pulling away the water, leaving a clean, concentrated sample. I'm going to try the Qiagen kit next, but if it doesn't solve the problem, I'll try butanol and let you know how it works.

Why do the NanoDrop values seem perfect when the Bioanalyzer shows degradation?

A Chloroform is better than butanol for removing phenol. You should not see so much RNA loss. Was your phenol-chloroform water saturated? When extracting small volumes, you can do a back-extraction after collecting the upper phase by adding more water to the organic phase. Vortex this briefly, spin, and then add the supernatant to the previously separated supernatant to collect anything left behind during the first extraction. Be certain that you don't take any of the organic phase, since this is likely the source of the contamination.

One possible explanation for RNA loss during extraction and precipitation is that the sample has already degraded. Tiny pieces of nucleic acid don't precipitate efficiently. After the RNA is out of the chaotropic guanidium thiocyanate (GSCN) solution, it is vulnerable to nucleases. At that point, I always keep it on ice and get it into the freezer as soon as possible.

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Another possibility is that your RNA is overdried. After precipitation, overly dry RNA can become insoluble, which will show up as a lower spec ratio. Invitrogen recommends spinning at $<12000\times g$, $8000\times g$ for the wash, and drying the RNA as little as possible. I often wash the sample, pour off most of the liquid, and then spin again to move all of the liquid to the bottom of the tube for easier removal. I remove any drips of liquid not dangerously near the pellet with a tip and suction. To dry the RNA, I just leave the tubes at room temperature for 10 min at the most. Even if my resulting pellet is slightly moist, enough liquid has been removed that it can be resuspended in water.

The NanoDrop tells you the optical density, which can't tell you the quality of the RNA. OD only indicates if protein is contaminated with nucleic acid, and with less reliability if nucleic acid is contaminated with something else. The OD does not tell you about degradation since degraded nucleic acids will have similar ratios to

intact ones. dNTPs themselves absorb even more strongly, if I remember correctly.

What causes low 260/230 ratios? (Thread 27622)

Q I am trying to obtain RNA for a microarray study using the Illumina system at my University's core facility. I extracted RNA from mouse T cells using the Qiagen RNeasy mini kit and stored it in 10 mM Tris pH 7.4 at -80°C . I sent samples of the RNA to the core facility, but the 260/230 ratios were really low. They would like the ratio to be between 1.8 and 2. Some of my samples were ~ 1.5 , but the majority of them were ~ 0.5 as measured on the NanoDrop.

I tried cleaning the RNA using the Qiagen kit's clean up protocol, but that drastically decreased my already low ratio. I also tried precipitating with 0.1 volume 3 M sodium acetate and 2.5 volumes 100% EtOH, but this had no effect on this ratio.

What caused this problem and how can I fix it?

A I found that adjusting the isopropanol extraction does an excellent job of reducing 260/230 ratios and increasing 260/280 ratios for monophasic lysis reagents. You might try making the RNA extraction solution with 1.2 M NaCl and 0.8 M sodium citrate in DEPC H_2O . Add 0.25 volumes of isopropanol and 0.25 volumes of RNA extraction solution to the RNA. Mix thoroughly and incubate for 10 min at room temperature. Spin down the tubes for 10 min at 4°C . Wash the pellets twice with 75% ethanol, spin, dry, and resuspend the pellet in DEPC H_2O .

A Are you sure that's not 0.5 volumes? There is a modified protocol in the TRIzol packaging insert that has worked for me. It is for RNA in chaotropic guanidinium/phenol solution instead of water. Start with 1 mL TRIzol to lyse the cells. Add the chloroform to force the phenol into the organic phase and remove the supernatant. You should recover ~ 0.5 – 0.6 mL supernatant. Add 0.25 mL isopropanol and 0.25 mL high-salt solution (1.2 M NaCl and 0.8 M sodium citrate) to the supernatant. Mix, spin, and recover the pellet. An additional precipitation of the original lysate can help to remove solid matter or floating oil.

That protocol appears to suggest adding 0.5 volumes of isopropanol and salt solution (or 0.25 volumes of the original lysate) to the RNA. The protocol above is indicated for RNA in water. I don't know how that changes it, but check if that was an error.

A When your RNA is in chaotropic guanidinium/phenol solution, the protocol that accompanies the TRIzol reagent would be the solution. For RNA in water, just add 0.25 volumes of RNA extraction solution and 0.25 volumes of isopropanol and mix them properly. Keep the tubes at room temperature for 10 min.

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