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Genome Center Maastricht

Quality assessment of total RNA

- RNA quality control: NanoDrop® ND-1000
- RNA quality control: Agilent's 2100 Bioanalyzer

The invaluable help of Bieke Vanherle, Erika Timmer and Paul Wackers is acknowledges in performing the experiments described in this section.

RNA quality control using the NanoDrop ND-1000

There are three quality controls that are performed on isolated RNA. One is to determine the quantity of RNA that has been isolated, the second is the purity of RNA that has been isolated and the third is the integrity of the RNA that has been isolated.

Measuring the Quantity of RNA using the Nanodrop.

Nucleic acids are traditionally quantified using UV absorption using a spectrophotometer.
In its simplest form the absorbance is measured at 260 and 280 nm. The concentration of nucleic acid can be determined using the Beer-Lambert law, which predicts a linear change in absorbance with concentration.

An A260 reading of 1.0 is equivalent to about 40 µg/ml of RNA and the OD at 260 nm is used to determine the RNA concentration in a solution.

RNA has its absorption maximum at 260 nm and the ratio of the absorbance at 260 and 280 nm is used to assess the RNA purity of an RNA preparation. Pure RNA has an A260/A280 of 2.1. You will see in many protocols that a value of 1.8-2.0 indicates that the RNA is pure. This depends, however on how you performed the measurement and the source of putative contaminations.

Ideally, scanning spectrophotometry should be used as this makes it possible to also identify possible sources of contamination. One of the problems using conventional spectrophotometers is that the cuvettes are large making it difficult to measure low concentrations of RNA without losing an unacceptable fraction of the sometimes precious and valuable RNA sample.
The NanoDrop® ND-1000 UV-Vis Spectrophotometer enables highly accurate analyses of extremely small samples with remarkable reproducibility. The sample retention system eliminates the need for cuvettes and capillaries, which decreases the amount of sample required for the measurement.

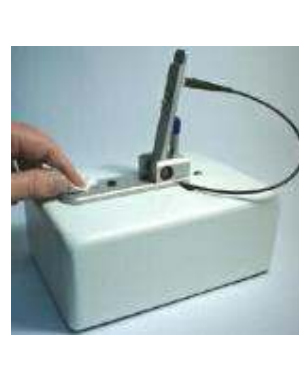
Surface tension is used to hold a column of liquid sample in place while a measurement is made. This is done by pipeting the sample (only 1 or 2 micro liters!!) directly onto one measurement pedestal. A measurement column is then drawn between the ends of two optical fibers to establish the measurement path as shown in the figure. The measurement is made, typically in less than 10 seconds, and the spectrum and its analysis is shown on the screen of the attached PC and archived on the PC. Once the measurement is complete, the sample is simply wiped from the measurement pedestals. The archived data
can be manipulated by a spreadsheet program such as MS Excel.

Using the Nanodrop will provide you with a scan of the absorbance from about 200 nm up to 350 nm, which is the relevant region for determining RNA concentration and purity.

The NanoDrop ND-1000 enables the analysis of 1 ul samples, and also eliminates the need for cuvettes and capillaries.

With the sample apparatus open, a droplet of sample is pipetted onto the measurement pedestal. When the sample apparatus is closed, the sample arm slightly compresses the droplet and a sample column is drawn. Surface tension alone holds the sample in place.

When the measurement is complete, the sample apparatus is opened and the sample is simply wiped from both the sample arm and sample pedestal using an ordinary dry laboratory wipe.



As mentioned above, RNA has its absorbance maximum at 260 nm and this absorbance is not dependent on the pH of the solution. However, the absorbance of some of the contaminants (like proteins) in the RNA solution have an absorbance that is pH-dependent. This means that although the A260 reading of the RNA solution with remain the same at different pHs, the A280 reading will differ at a pH dependent manner.

It has been shown that significant variability in the A260/A280 ratio can occur when different sources of water were used to perform the spectrophotometric determinations. Adjusting the pH of water used for spectrophotometric analysis from approximately 5.4 to a slightly alkaline pH of 7.5-8.5 significantly increased RNA A260/A280 ratios from approximately 1.5 to 2.0.

Wilfinger WW, Mackey K, Chomczynski P., Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. Biotechniques. 1997, Mar;22(3): 474-6, 478-81.

Consequently, if you measure your RNA in pure water, and you have an OD A260/A280 ratio of 1.8, your RNA quality might be pure but you will not be able to exclude that it is contaminated with DNA, protein or something else. Measuring RNA in a buffered solution like TE (pH 8.0) will result in an OD A260/A280 ratio that is more reliable. If you measure RNA in TE (pH 8.0), you should get an OD A260 reading very close to 2.0. If not, your sample is contaminated.

The same sample measured in pure water that gives you an OD of maybe 1.5-1.8 could give you an OD ratio of 2.0 in TE pH 8.0.

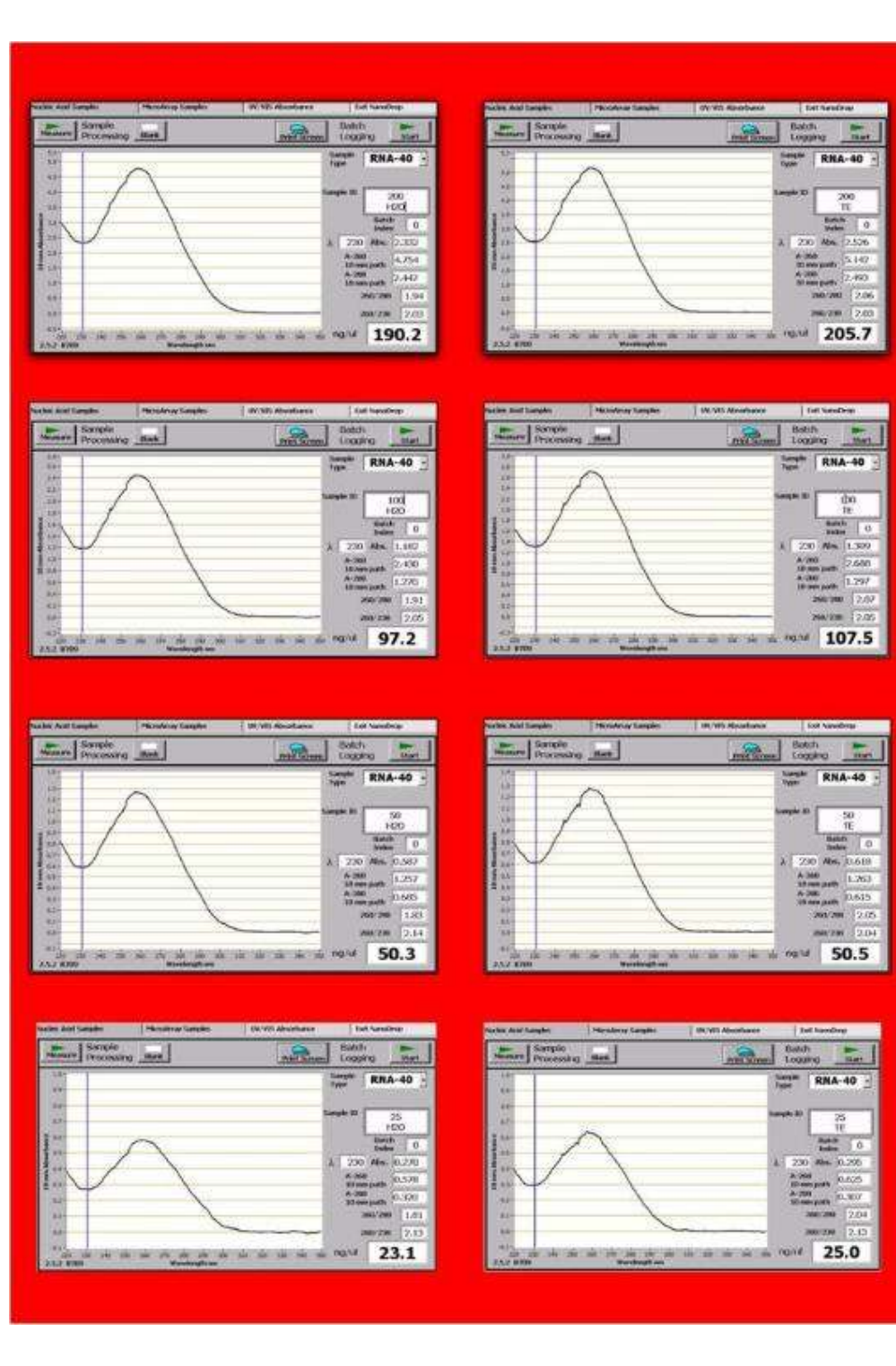
As can be seen from the figure, measuring RNA absorbance in water and diluting it twofold for several times will result in a decrease of the A260/A280 ratio. Performing the measurement in TE (pH 8.0) results in exactly the same OD A260/A280 for all the dilutions.

Besides the pH, the A260/A280 ratio is also dependent of the ionic strength of the spectrophotometric solution. Therefore, it is important to use exactly the same buffer as a diluent and as the blank, sometimes pure water is used incorrectly as a blank. This is in particular a problem if you do not know the composition of for example the elution solution that comes with a commercial RNA isolation kit.

Most protocols only mention the OD A260/A280 ratio but you will often see in the region of 230 nm a strong absorbing contaminant. Let there be no mistake, this is not RNA as pure RNA has a single peak with a maximum at 260 nm. There are three sources of contaminations that produce peaks in the 220-230 nm region, these are proteins, chaotropic salts like guanidinium isothiocyanate and phenol. All these three are either present in your tissue sample or are present in most tissue lysis solutions derived from the original Chomczynski and Sacchi protocol.

Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 1987 Apr;162(1):156-9.

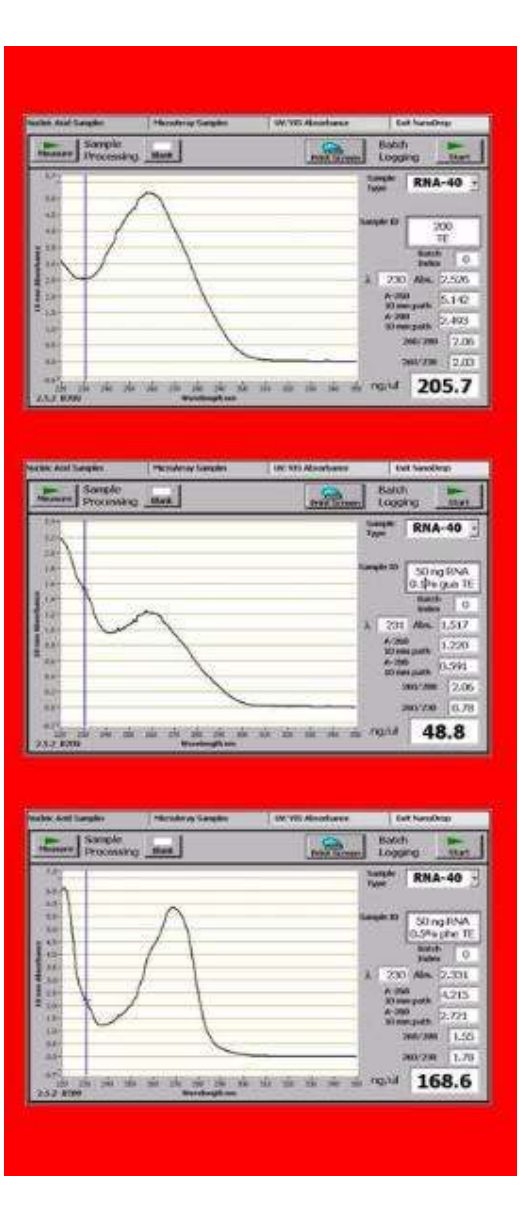
To our opinion, it is important that not only the OD A260/A280 ratio should be very close to 2.0, but that in addition, also the OD A260/A230 ratio should be very close to 2.0. Specially, when isolating low amounts of RNA the OD A260/A230 ratio drops significantly to sometimes under the 1.0. This clearly indicates, contamination with chaotropic salts or rests of phenol or protein in the RNA solution.



Serial dilution of RNA 200-20 ng/µl in pure water (left panel) or in TE pH8 (right panel). This figure shows the effect of measuring RNA absorbance in buffered TE, pH 8.0 or non-buffered solutions (pure water). The left panel shows the results of measuring in pure water. It is clear that the same RNA concentrations give comparable values in water or TE pH 8.0 as the absorbance of RNA at 260nm itself is independent of pH. However, measuring in TE pH 8.0 results in significantly higher OD 260/280 ratios as the absorbance of contaminants is pH dependent.
The OD A260/A280 for the 25 ng/µl RNA in pure water is 1.81, while it is 2.04 in TE

The Good, The Bad and the Ugly

Here below you will see some RNA absorbance spectra of RNA that is pure, and also of RNA for which you should be able to conclude that the sample is not pure. In order to help you identify possible sources of contaminations, we have spiked pure RNA with the most commonly used reagents used for the RNA extraction and purification procedures. Hopefully, this may be helpful for you to identify your source of contamination if it interferes with your specific downstream processes.



The Good
Both the OD A260/A280 as the OD A260/A230 ratio are 2.0 or more.

Perfect, you can do with this RNA whatever you like, everything should work.

The Bad
The OD A260/A280 ratio is over 2.0 but the OD A260/A230 ratio is below 1.0. Be careful! This indicates that the sample contains impurities. Some downstream procedures may work perfectly while others may give problems.

The Ugly
Don't even think of using this RNA! Just perform an extra purification step.

Effect of several frequently used reagents on the absorbance of RNA

Minor contamination problems: TRIS, ethanol and isopropanol

Effect of TRIS contamination

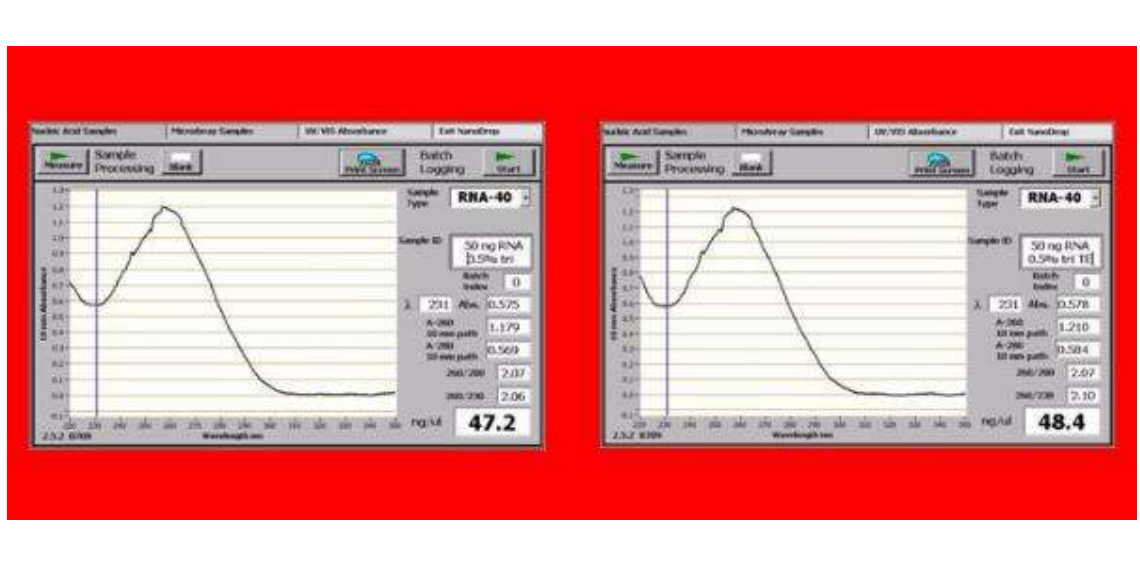


Figure 1.
This figure shows that TRIS contamination does not influence the absorbance spectrum of RNA significantly. As TRIS is a buffer by itself, in this case there is also no difference between measuring in water (left panel) or TE pH8.0 (right panel).

Effect of isopropanol contamination

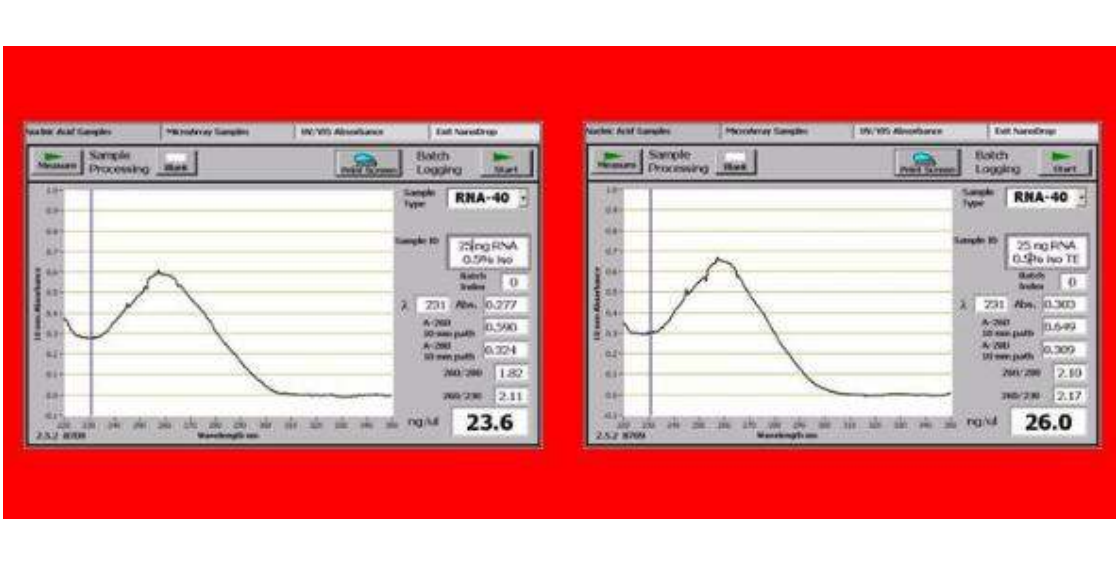


Figure 2.
This figure shows that isopropanol does not have a significant effect on the absorbance spectrum of RNA when measured in TE pH 8.0. However, when the measurement in made in pure water, the OD A260/A280 ratio of 1.82 at 0.5 % isopropanol contamination is reason for serious doubt on the quality of the RNA. In TE the OD A260/A280 ratio is 2.10 indicating high purity.

Effect of ethanol contamination

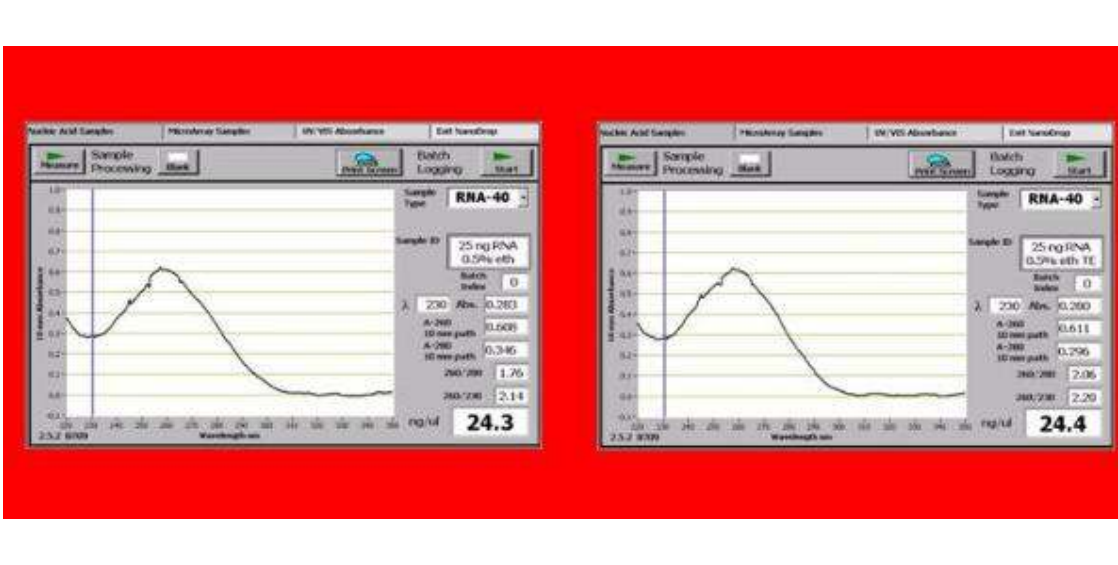


Figure 3.
This figure shows that ethanol does not have a significant effect on the absorbance spectrum of RNA when measured in TE pH 8.0. However, when the measurement in made in pure water, the OD 260/280 ratio of 1.76 at 0.5 % ethanol contamination is reason for serious doubt on the quality of the RNA. In TE the OD A260/A280 ratio is 2.06 indicating high purity.

Major contamination problems: protein, guanidine isothiocyanate and phenol

Effect of protein contamination

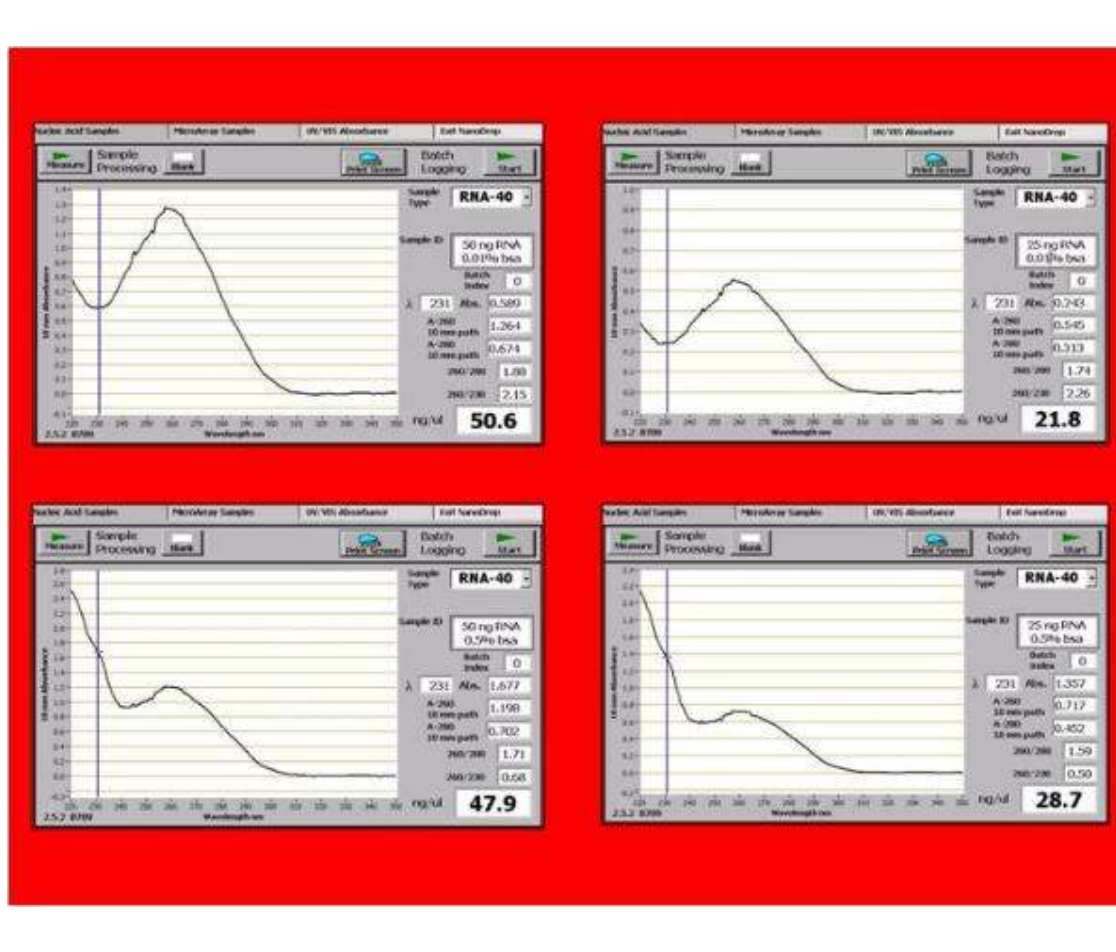


Figure 4.
In this figure the effect of protein contamination can be seen. A contamination level of 0.01 % BSA (upper panels) shows an almost normal absorbance spectrum although the OD A260/A280 ratio is below 1.9, which is a warning that something is contaminating the sample. At 0.5 % BSA (lower panels), the absorbance spectrun looks awful and this is a clear indication that there is a significant level of contamination in these samples.

Effect of guanidine isothiocyanate contamination

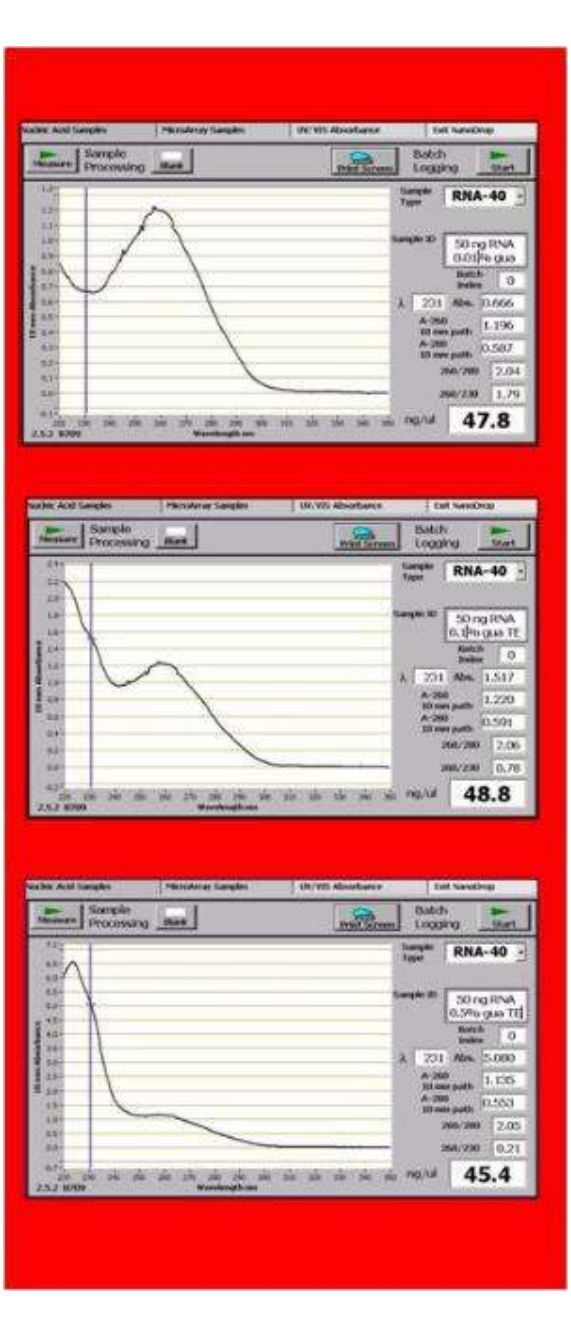


Figure 5.
This figure shows the dramatic effect that guanidine isothiocyanate has on the absorption spectrum of RNA. Note that guanidine isothiocyanate contrary to phenol hardly influences the OD A260/A280 ratio, which in these examples is still well above 2.0. Therefore, Guanidine isothiocyanate has a small effect on the quantification of RNA. Guanidine isothiocyanate on the other hand, has a very strong effect on the OD A260/A230 ratio, which at the 0.5 % contamination level drops below 0.5. An OD A260/A230 below 1 should be avoided as the level of guanidine might have a deleterious effect on downstream enzymatic reactions.

Effect of phenol contamination

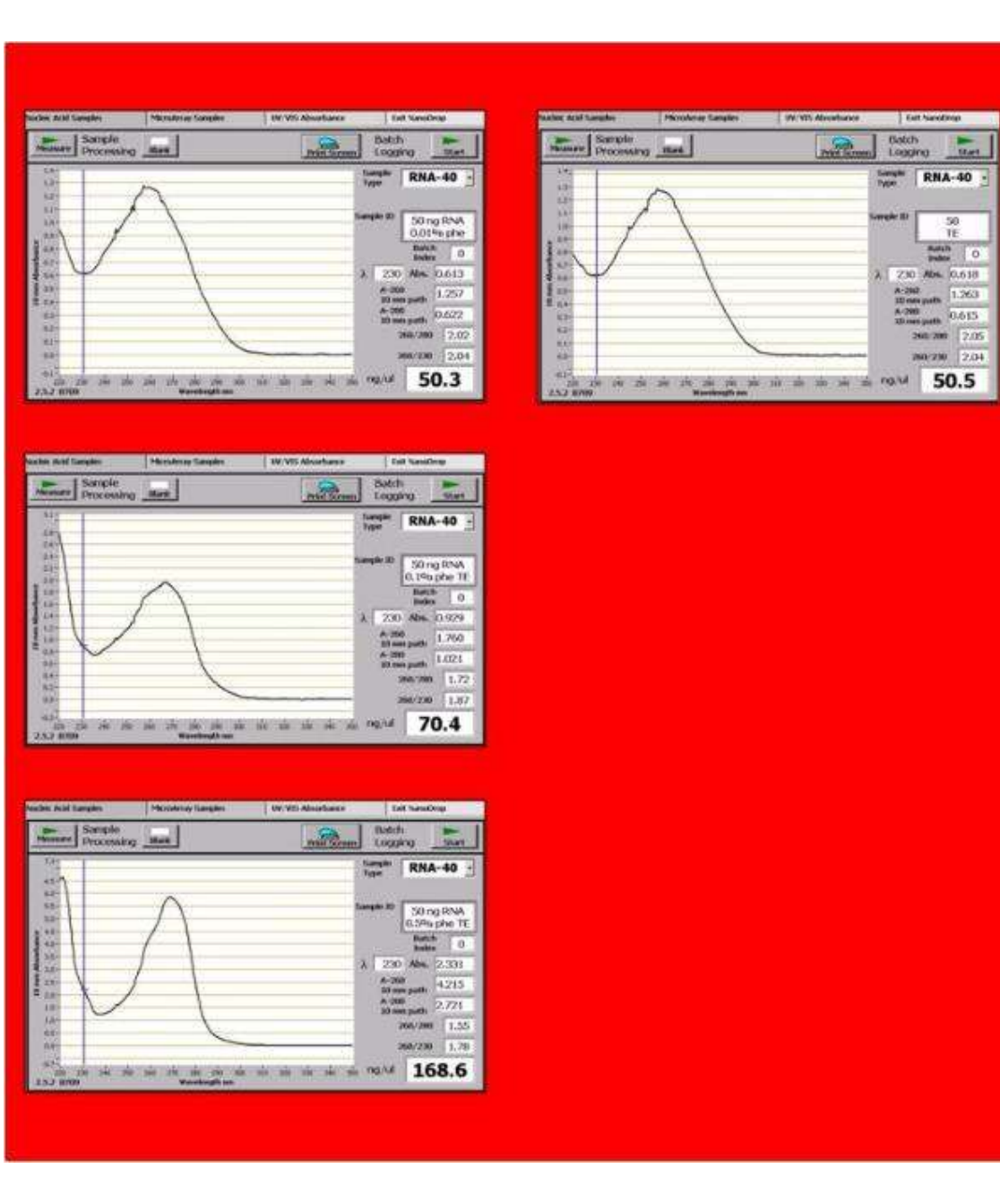


Figure 6.
This figure shows the dramatic effect that phenol can have on the absorbance spectrum of RNA. Phenol has a very strong effect on the quantification of RNA. Note that at a 0.5 % contamination level, the measured concentration is over three times as high as the actual value of 50 ng/µl. This strong disturbing effect of phenol on the quantification of RNA is due to the contaminating absorption peak at 270 nm. At very low RNA concentrations below 10 ng/microliters this contaminating peak is often even confused for RNA. It cannot be stated more clear: THE RNA ABSORBANCE PEAK IS NEVER AT 270 nm. If the peak is at 270 nm it arose from a contamination. At low RNA concentration after the RNA has been isolated using a method based on phenol extraction, the erroneous overestimation of RNA levels is a serious problem in RNA work.

Dose response effects of common contaminators on RNA absorbance

When you make an absorption spectrum of your RNA sample with the Nanodrop, you will be able to save your data and import them into an Excel spreadsheet. In this way you will be able to make composite figures and compare the results obtained from several purification procedures or the effects of contaminants on the absorbance of RNA.

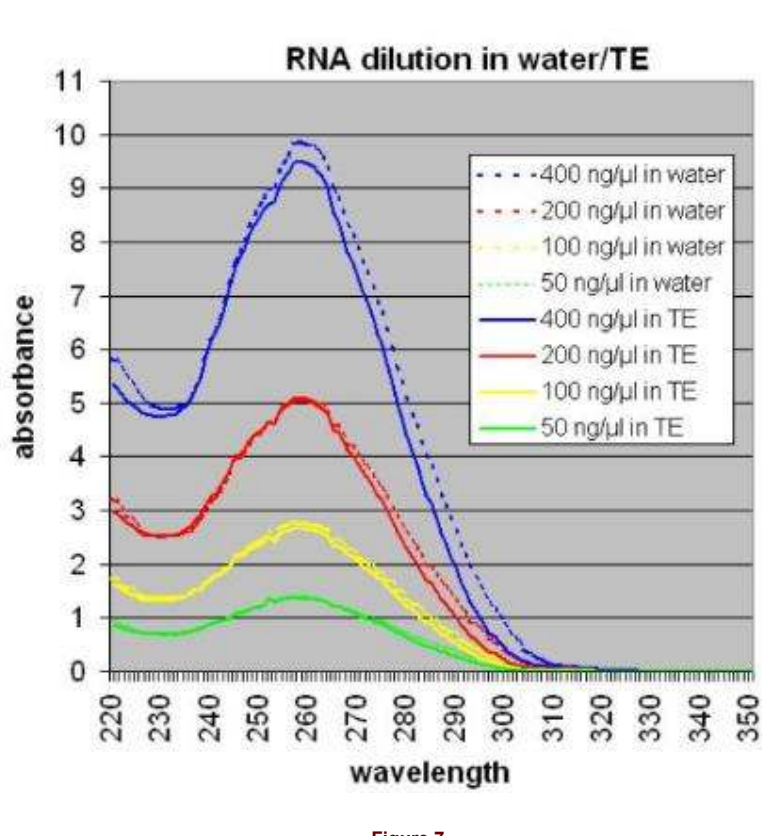


Figure 7.
This figure shows the effect of measuring absorbance of RNA in non-buffered water or in TE pH 8.0. Note the increase in absorbance at 280nm in the non-buffered solution which results in a lower OD 260/280 ratio.

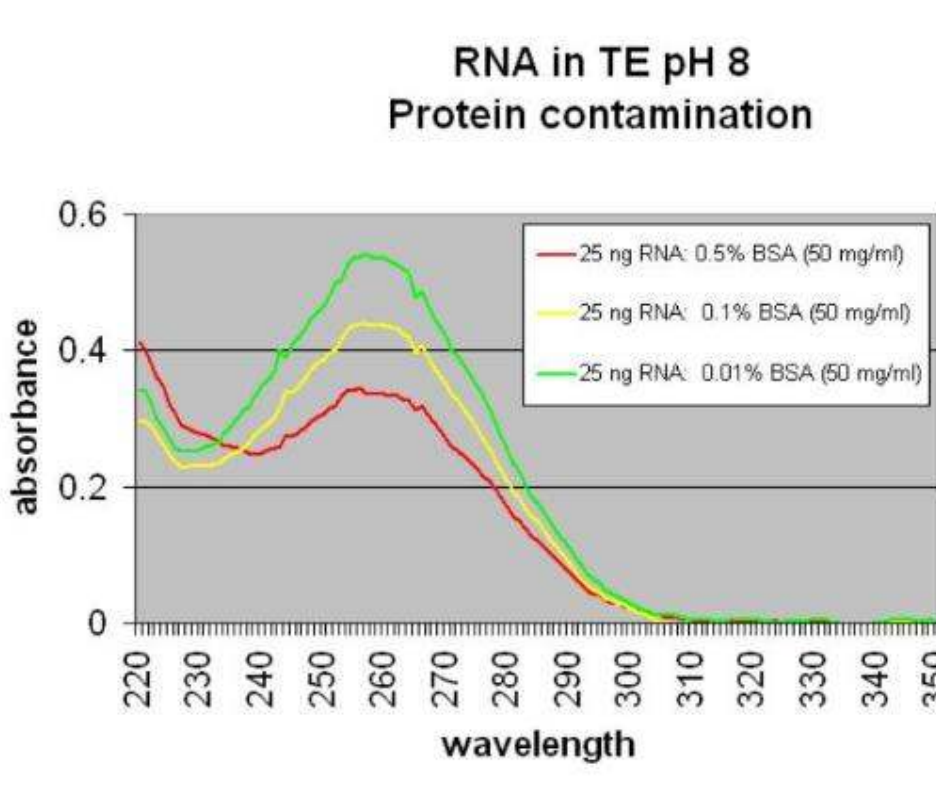


Figure 8.
This figure shows the effect of protein contamination on the RNA absorbtion. Note that protein decreases the absorption both at 260 and at 280 nm so that the net results is a decrease in the OD 260/280 ratio well below 1.9.

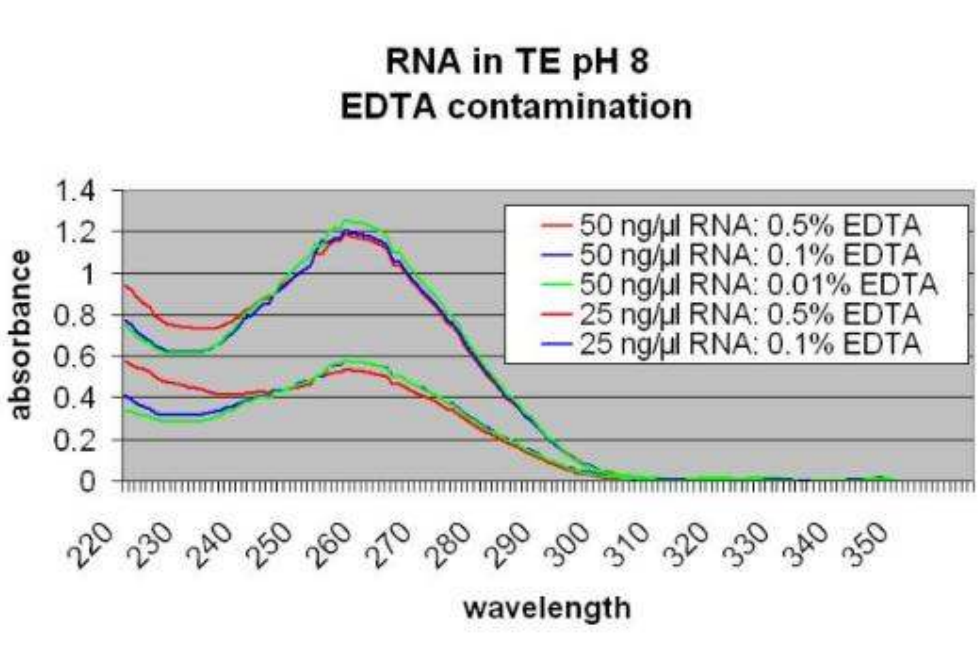


Figure 9.
This figure shows that EDTA contaminations will not significantly disturb OD measurements at 260 and 280 nm. Also alcohols like ethanol and propanol do not have significant effects at low contamination levels of a few percents (V/V).

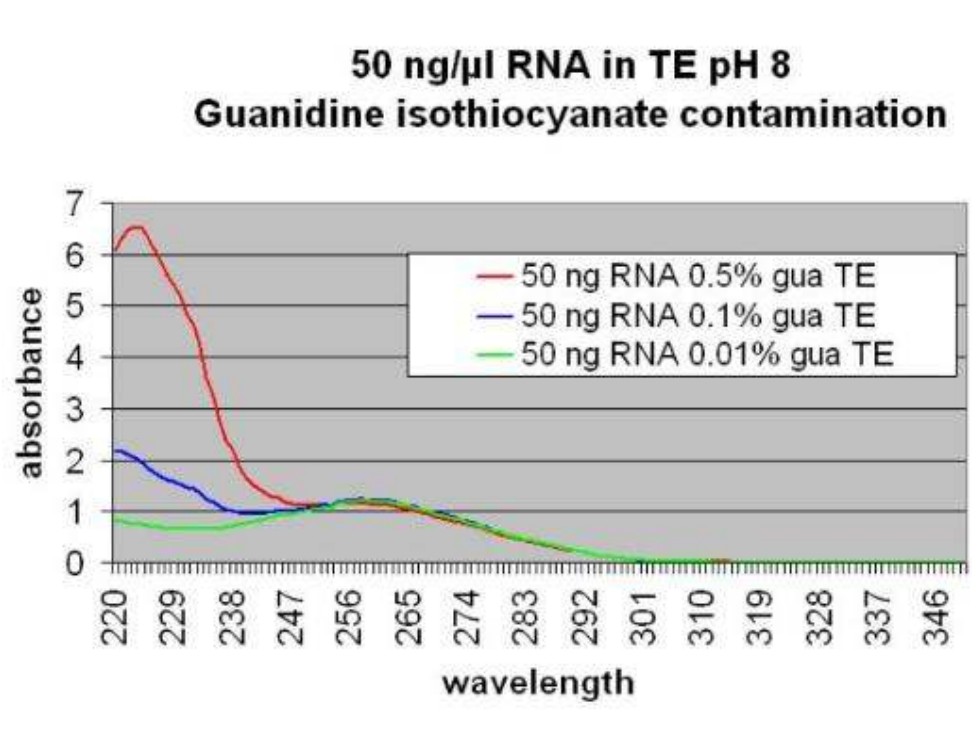


Figure 10.
This figure shows the dramatic effect of guanidine isothiocyanate on the absorbance of RNA. Note the large absorbance below 230 nm.

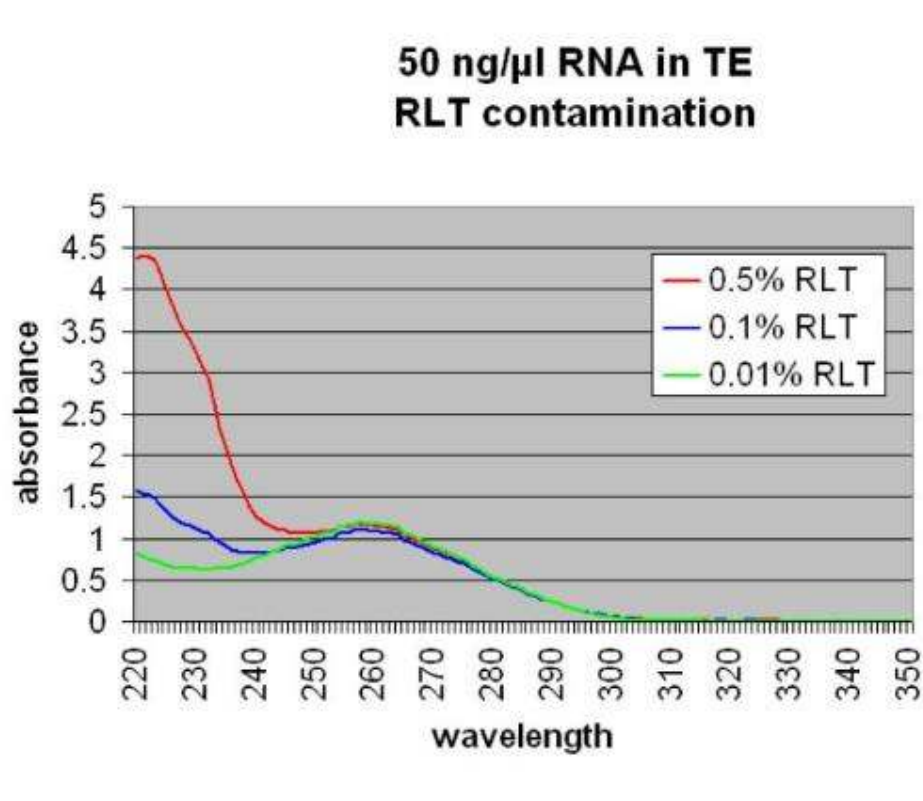


Figure 11.
This figure shows that RLT, which is a buffer used in the Qiagen RNeasy RNA isolation kits also contains a guanidine isothiocyanate-like component that may interfere significantly with RNA OD measurements. Note also for RLT the large absorbance below 230 nm.

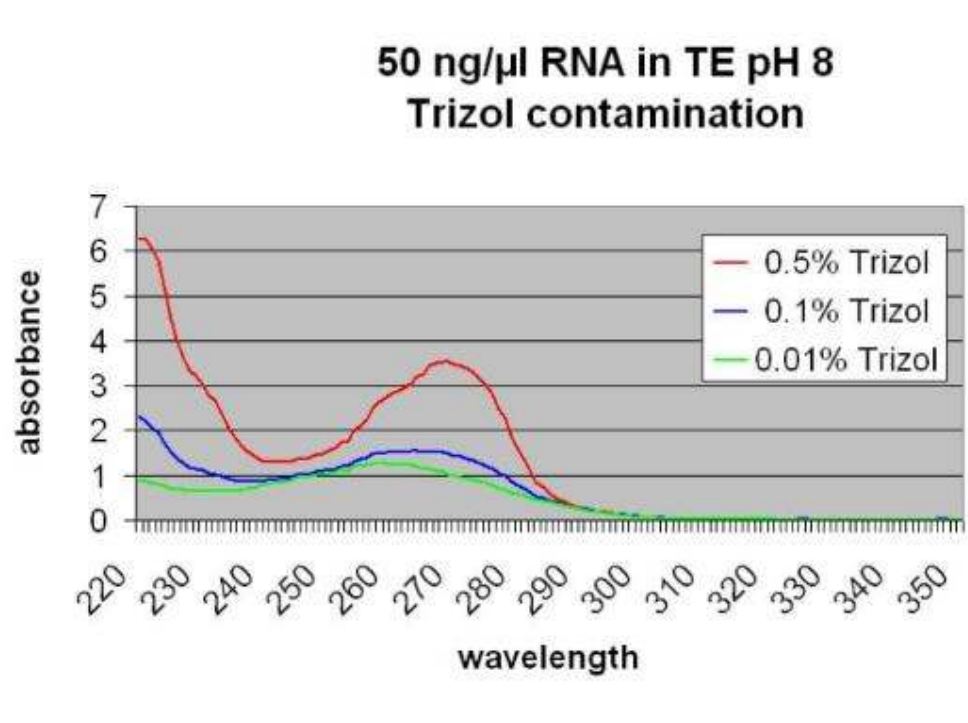


Figure 12.
This figure shows the effect of Trizol on the absorbance of RNA. Note that Trizol also contains guanidine isothiocyanate that strongly absorbs below 230 nm. In addition, Trizol contains phenol which is responsible for the strong absorption at 270 nm.

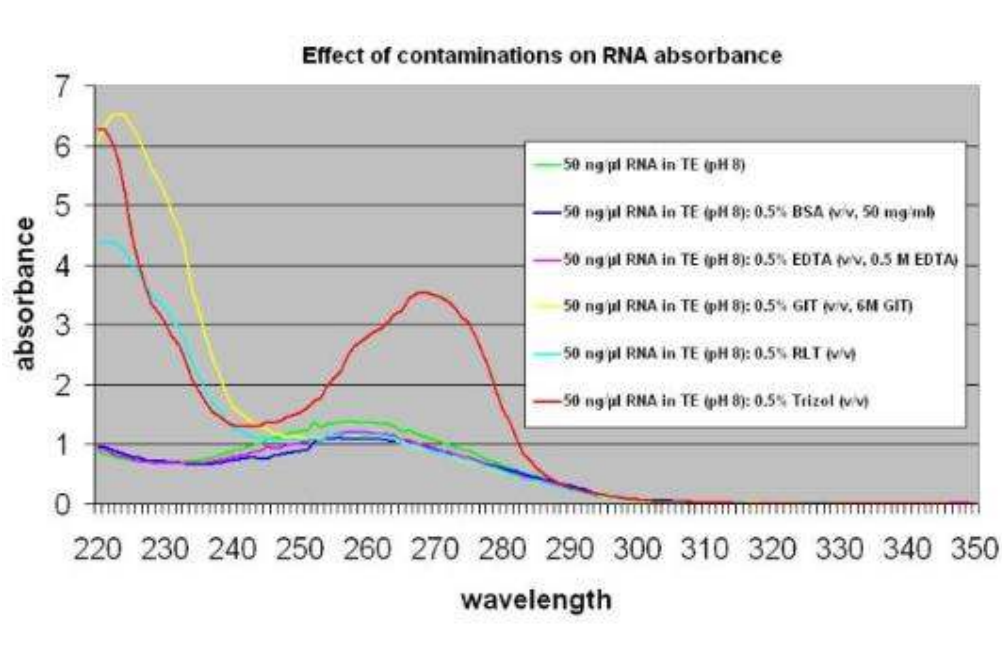


Figure 13.
A composite figure showing relative effects on frequent RNA contaminations. GIT: Guanidine isothiocyanate, RLT: buffer used in Qiagen RNeasy kits, Trizol contains both guanidine isothiocyanate and phenol. Phenol is responsible for the large absorbance at 270 nm.

Next: RNA quality control: Agilent's 2100 Bioanalyzer