The Undergraduate's Guide to RNA Isolation

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TRIzol: An Overview

TRIzol is usually ordered from Invitrogen (commercial) or made right here in the lab. The recipe can be found in the appendix of this manual.

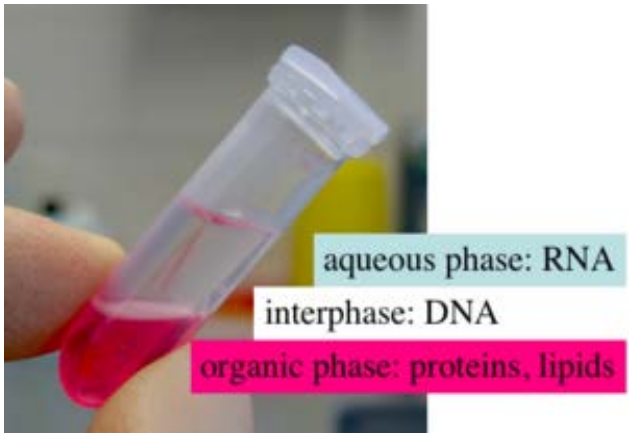
Invitrogen: <http://products.invitrogen.com/ivgn/product/15596026#>
SKU# 15596-018 (200mL) - $274.37 MSRP
15596-026 (100mL) - $156.78 MSRP

The prices above are the manufacturer's listed prices for TRIzol, however we do have a purchase order code to get discounts from Invitrogen. Please see Yong-Jin, the lab manager.

TRIzol is a solution of organic solvents and denaturing agents which can solubilize biological material as well as denature proteins. This prevents the RNA from degradation by inhibiting RNases and simultaneously disrupting cell components during sample homogenization. This method of RNA isolation was developed by Chomczynski and Sacchi in 1987. With TRIzol, one is able to isolate RNA, DNA, and proteins, from the same sample.

1 mL of TRIzol can dissolve ~10^6 cells. For larger amounts, scale up accordingly. Overloading the TRIzol may result in partial RNA degradation. After the addition of chloroform, the solution will split into three portions:

- Aqueous – contains exclusively RNA, due to the acidity of the solution.
- Interphase – contains DNA and proteins.
- Organic Phase – contains proteins, lipids, and some DNA.



Courtesy of Openwetware.org

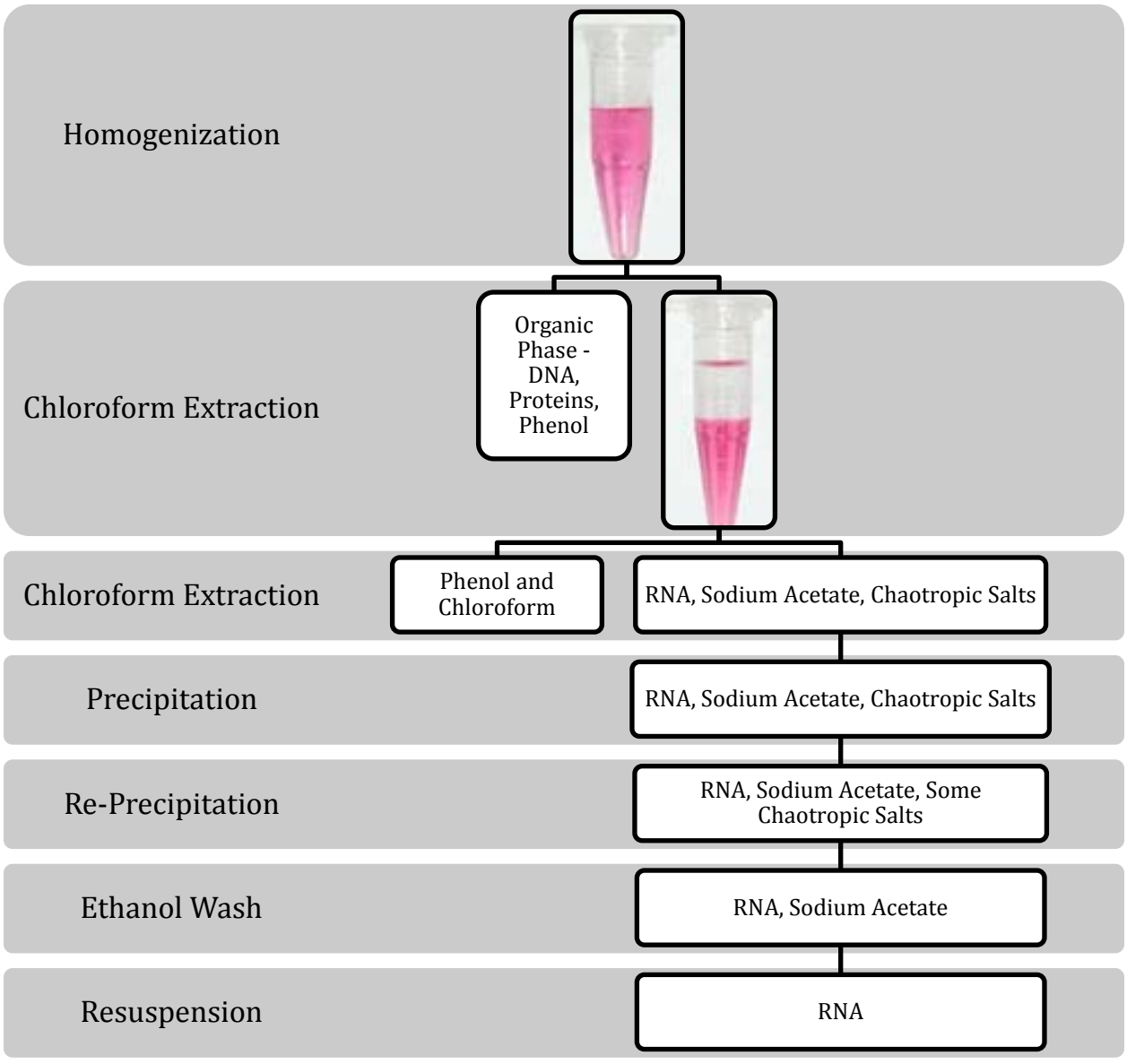
RNA is then precipitated using isopropanol. However, due to the high salt content of TRIzol, a reprecipitiation step was added, thanks to Donald C. Rio, his co-authors, and their book, *RNA: A Laboratory Manual*. After reprecipitation, the RNA is washed again, followed by resuspension in RNase-free water. Quantification is determined by Nanodrop ND-1000 and qualification by gel electrophoresis/northern blotting.

RNA isolated using this protocol can be used for reverse-transcription and real-time, quantitative polymerase chain reaction (RT-PCR/QPCR).

RNases have now become your worst enemy. Their sole purpose: to seek and degrade your RNA. They are everywhere, in your saliva, dust particles, hands, jeans, you name it. How will you fend off these nasty intruders? Say hello to your new best friend and weapon, Sodium Dodecyl Sulfate (SDS). SDS is a detergent, which can act as a mild inhibitor of RNases. Be sure to wear gloves at all times and thoroughly wash the pipettes with SDS before you begin your isolation.

What is TRIzol Made of?

- Phenol – This organic solvent must be acidic in order to exclusively isolate RNA; desired pH is ~4.7. For DNA isolation from TRIzol, the pH is ~7.9.
- Guanidinium Thiocyanate and Ammonium Thiocyanate – Both salts act as protein denaturants because they are chaotropic reagents. They prevent the degradation of RNA by inhibiting DNases and RNases.
- Sodium Acetate – Sodium ions will come into contact with the negatively-charged, phosphate backbone of RNA via Van Der Waal's forces, essentially depolarizing the nucleic acid. Once isopropanol is added, the RNA will precipitate out of solution due to the great difference in polarity between the RNA and H_2O.



Materials Needed

Please consult the Material Safety Data Sheet before continuing with the protocol: https://tools.invitrogen.com/content/sfs/msds/2011/15596026_MTR-NAIV_EN.pdf

RNA Isolation and Quantification:

- Chloroform
  - Caution: Fumes are toxic, use chemical hood.
- 100% Isopropyl Alcohol (Isopropanol)
- 3M Sodium Acetate (NaOAc), pH 5.2
- 100% Ethanol
- 75% Ethanol
- RNase-Free Water
- Centrifuge and rotor capable of reaching 12,000*x g*
- Polypropylene microcentrifuge tubes

RNA Qualification via Gel Electrophoresis

- Agarose
- 10x MOPS Buffer
- 2x RNA Loading Dye with Ethidium Bromide
  - Caution: Contains both Formamide and Ethidium Bromide, both of which are highly toxic and carcinogenic.
- Formaldehyde, 37% vol/vol
  - Caution: Toxic and carcinogenic, use chemical hood.

Protocol:

Do NOT risk RNA contamination and/or degradation: Before beginning RNA isolation, wipe down the pipettes and table with 0.1-0.5% SDS. Make sure you properly allocate your time for RNA isolation; it takes roughly 5 hours (split between two days) to complete. Remember, there is a double-chloroform extraction to remove any excess phenol; please be very careful during the separation.

Phase Separation

Trying doing this portion of the protocol in the fume hood. As stated earlier, both phenol and chloroform are highly toxic, so protect yourself by performing the phase separation in the chemical hood.

1. After the samples have thawed, homogenize the sample by vortexing briefly and spin down. Add 200µL of chloroform per 1mL of TRIzol to each tube.
2. Shake the samples vigorously, then vortex for 2 minutes. When 1 minute has elapsed, stop and invert the samples and continue vortexing for another minute.
3. Incubate at room temperature for 3 minutes to allow phase separation.
4. Centrifuge the samples at 4°C (in the Cold Room) for 15 minutes at 12,000*x g.*
5. Remove up to 475µL of the aqueous (upper, clear) phase and put into new tube. If you cannot remove 475µL, remove as much as possible *without* disturbing the inter- or lower-phase.
6. Add 475µL of chloroform to the aqueous phase; a 1:1 ratio of aqueous:chloroform.
7. Shake the samples vigorously then vortex for 1 minute. When 30 seconds has elapsed, stop and invert the samples and continue vortexing for another 30 seconds.
8. Incubate at room temperature for 3 minutes to allow phase separation.
9. Centrifuge the samples at 4°C for 15 minutes at 12,000*x g.*
10. Remove up to 400µL of the aqueous (upper) phase and transfer into new tube. If you cannot remove 400µL, remove as much as possible *without* disturbing the lower-phase.

Precipitation

11. Add 250µL of 100% isopropyl alcohol to the aqueous phase and shake vigorously.
    a. Some people like to incubate their RNA in the 4°C refrigerator to allow for maximum precipitation (up to 60 minutes), others will incubate at room temperature for fear of excessive salt precipitation (10 minutes).
12. Centrifuge the samples at 4°C for 10 minutes at 12,000*x g*.
13. Identify the pellet and carefully pour out the supernatant. Spin the residual liquid down and remove the excess by using long-tipped pipette tips.
14. Add 100µL RNase-free water, 10µL 3M NaOAc (0.3M total), and 275µL 100% ethanol (2.5x total volume) to the sample. Leave samples in -20°C or -80°C overnight.

Re-Precipitation

(If you left your samples in the -80°C: Allow the samples to thaw at room temperature for 2-5 minutes.)

15. Centrifuge the samples at 4°C for 15 minutes at maximum speed.
16. Identify the pellet and pour out the supernatant.
17. Add 1mL (1000µL) of 75% Ethanol to each tube and vortex briefly. Ensure the pellet is now floating.
18. Centrifuge the samples at 4°C for 5 minutes at 7,500*x g*.
19. Identify the pellet and carefully pour out the supernatant. Spin the sample down and remove the excess supernatant by using the long-tipped pipette tips.
20. Allow the pellet to dry. The size of the pellet will determine the length of drying, which is normally 5 to 10 minutes. Avoid over-drying by checking on the samples often.
21. Resuspend the samples in RNase-free H_2O, depending on the size of the pellet. Normally, this is in the range of 20 to 50µL. Put samples on ice if continuing onto the next steps.

Quantification and Qualification

To determine the quantity of RNA in a sample, we will be measuring UV absorbance using a spectrophotometer, the NanoDrop ND-1000 by ThermoScientific. The NanoDrop is located in the King Lab, just down the hall (the doorcode is 351). *Note: The NanoDrop only tells you about the quantity and purity of your RNA in your sample, whether it is degraded or not. It does NOT tell you anything about the quality of your RNA and if it is or not degraded.*

To determine the quality of your samples, we will be using gel electrophoresis, which will run the RNA on a denaturing (meaning it contains chemicals which denature proteins, i.e. RNases etc.), 1.2% agarose gel. Next, we visualize the gel with 302nm UV light, using the presence and sharpness of the bands as an indicator of the quality of the RNA.

There is a troubleshooting section at the end of this chapter which includes pictures and ways to fix purity problems with your RNA.

Quantification Using UV Absoprtion

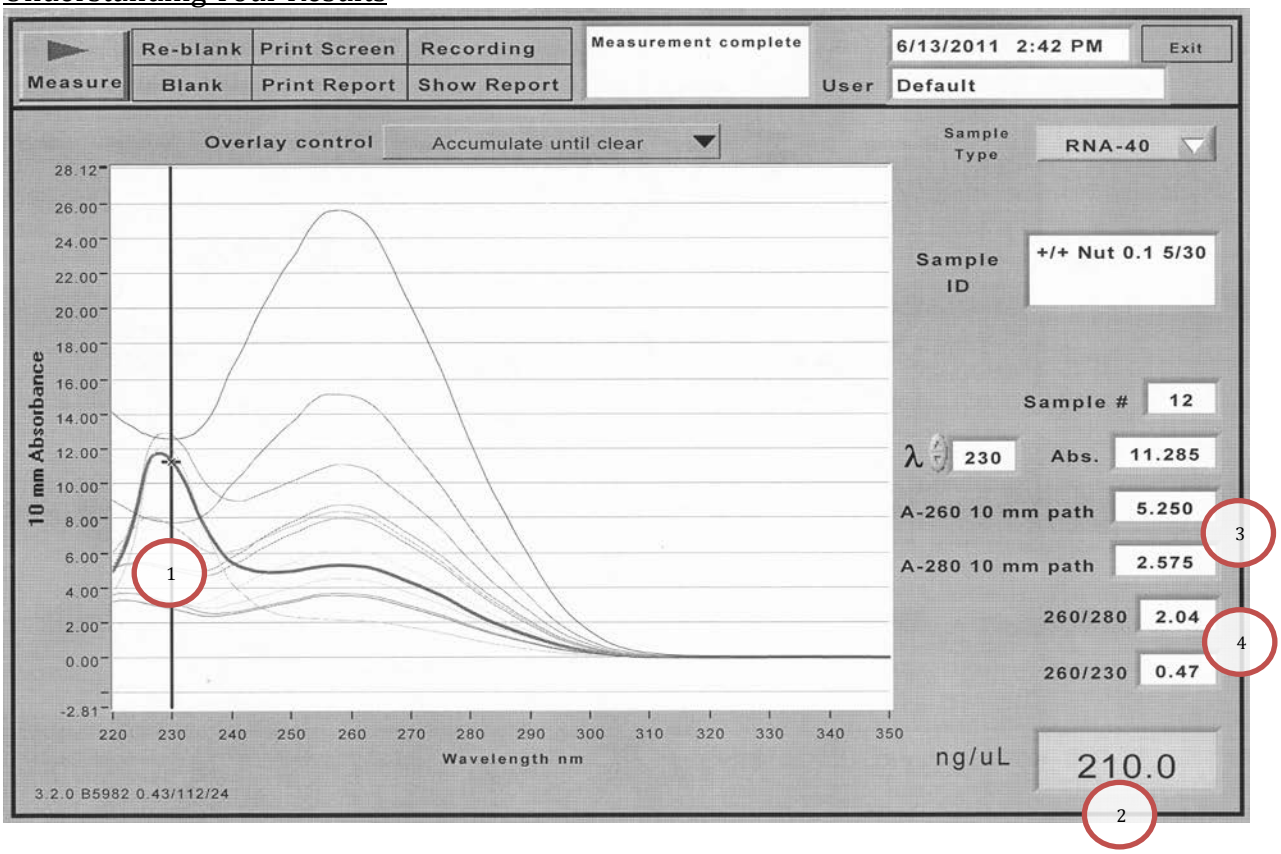
1. Take 1-1.5µL of each of the samples and a blank to measure the concentration on the Nanodrop ND-1000 (in the King Lab).
2. Open the program ND-1000.exe.
3. Click on 'Nucleic Acids.'
4. Before initializing the Nanodrop, sand down the measurement pedestals (both top and bottom) with a Kimwipe to remove any residual liquids.
5. Initialize the instrument by loading 1-1.5µL of RNase-free H_2O.
6. Click OK and wait as the Nanodrop initializes.
7. DON'T WIPE the sample off, rather, pat the measurement pedestal down several times with a Kimwipe. Wiping the sample will cause it to smear everywhere and possibly contaminate future measurements. Now it is time to load your samples. Load your blank first. Remember to select 'RNA-40' when measuring your samples, including the blank. Click 'Blank' and let the spectrophotometer measure the background.



8. Pat the measurement pedestal down several times with a Kimwipe before loading your sample. If you wish, you can label your samples with the program.

9. Click 'Measure' to quantify your sample. Repeat this step several times until you have completed all your samples.
10. If you wish to have a copy of your graphs with your readings, go to File > Include Graphs in Report.

Understanding Your Results



Here is what your graph will look like once you measure your samples. I have highlighted some key areas which you should know how to interpret. As an example, we will be using the data from the measured sample, as indicated by the bold line on the graph.

1: This shows the UV absorbance from 220-350nm of our sample. The shape of this graph is crucial to determining the quality of your sample for downstream applications.

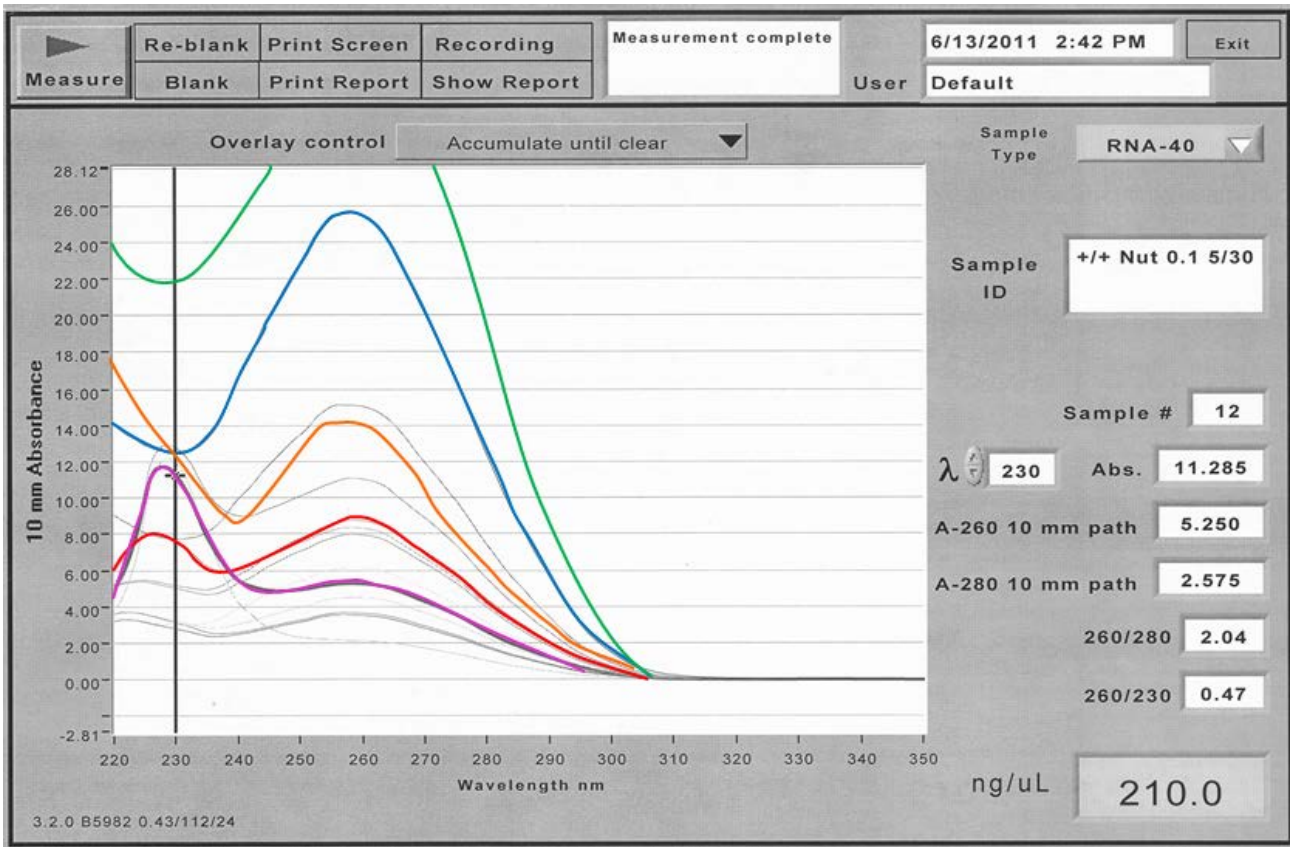
2: This shows the concentration in ng/µL of your sample. Multiply this number by the total amount of solvent used to dissolve your RNA to get your total RNA. This number can be skewed if your RNA is impure.

3: The pair of numbers is simply an indicator of how the concentration of your sample was calculated. The Nanodrop uses the Lambert-Beer Law which states $C = \frac{A_{260}}{\varepsilon l}$, where C is the concentration, ε is the specific absorption constant (0.025 $\frac{mL \times cm}{\mu g}$), and l is the length (1cm).

4: These two ratios are used to assess the quality of your RNA, whether it's pure or contaminated with components of TRIzol. For purified RNA, you want an A_{260}:A_{280} and an A_{260}:A_{230} ratio of ~2.0. The unpaired bases in RNA absorb more UV light than the base pairs in duplex DNA, thus when your ratio is <2.0, it will usually indicate some DNA contamination.

Here are some general guidelines when trying to make sense of your graph:

- RNA has a maximum absorption at 260nm, which can be used reliably to quantify the sample. However, if the RNA is contaminated with DNA, protein, phenol, and/or chaotropic salts, the measurement will be off, as these contaminants slightly absorb UV at 260nm.
- Protein usually absorbs at 280nm, while phenol and TRIzol usually absorb between 220-230nm and 270nm.
- Chaotropic salts will often absorb at ~230nm and ~260nm.
- The NanoDrop is accurate up to approximately 3000ng/µL for RNA. When your sample is between 2-100ng/µL, the upper/lower limit is ±2ng/µL, while for >100ng/µL, it is ±2%.

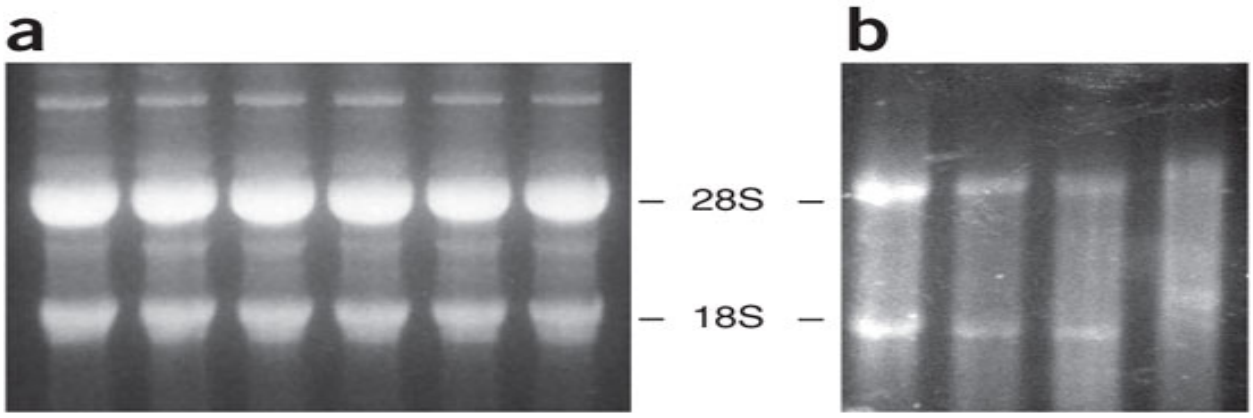


Red – Yuck, you somehow managed to get phenol in your sample despite *two* chloroform extractions. Do a chloroform wash and re-precipitate.
Orange – Shame on you for contaminating your sample with protein. This is probably because during the first chloroform extraction, you got too close to the interphase or your tip was too close to the eppendorf's wall, which drew in some proteins. Usually you can continue without any consequences, but some prefer to re-extract with TRIzol again.
Green – Hey, not bad, you just overwhelmed the NanoDrop because your RNA has oversaturated the stage. This happens when your concentration is >2,500ng/µL. Dilute your RNA and requantify.
Purple – Looks like some of those nasty, chaotropic salts (which will denature enzymes) like your RNA a little too much. Re-precipitate your RNA to get rid of these suckers.
Blue – Time to gloat and brag…don't you wish you had perfect curves like this?

<u>Qualification Using Gel Electrophoresis</u>

1. In a new PCR-strip, add 2x RNA Loading Dye with Ethidium Bromide (found in the 4°C refrigerator) and 300-500ng of RNA, up to a volume of 20µL.
2. Run the program '75HEAT' or '75.' This will denature and linearize the RNA. The program has a calculated measurement of temperature, with 10 minutes at 75°C, followed by a cool down to 4°C forever (until you are ready to load the gel).
3. Thoroughly clean the gel apparatus (buffer tub, lid, plate, combs, etc.) with 0.1-0.5% SDS followed by a rinse with Milli-Q H_2O. On a lining of paper towels, allow the gel apparatus to dry in the chemical fume hood.

4. As the RNA denatures and the gel apparatus dries, it is time to make the gel. Find a clean 250mL Erlenmeyer flask and rinse it once with the Milli-Q H_2O (which should be RNase-free).
5. Add 1.8g of agarose and 130.5mL of Milli-Q H_2O and mix briefly.
6. Heat the mixture for 2 minutes at 90% power.
7. Once the agarose has fully dissolved into the water, allow the solution to cool down to ~60°C (where you can touch the bottom and not be burned by it) and add 15mL of 10x MOPS and 4.5mL of Formaldehyde (37% vol/vol).
8. Pour the gel into the proper casting and allow it to solidify for 40-50 minutes in the chemical fume hood.
9. For making the running buffer, add 450mL of Milli-Q (RNase-free) H_2O to 50mL of 10 MOPS buffer and mix thoroughly.
10. Load the samples and run the gel for 1 hour at 70V.
11. Visualize the gel using UV spectroscopy at 302nm wavelength. There will be two distinct, clear bands. The upper band is the 28S, while the bottom is 18S. If you're RNA is really good, then you will see the 5S band at the bottom. Check the picture below, adapted from from *Nature Protocol*.



(a) RNA gel electrophoresis. Six samples, 20µg per lane of different pancreatic cancer cell lines were run on an agarose gel. The clear appearance of the 28S and 18S bands with no smearing indicates high quality of RNA. **(b)** An example of degraded RNA. *Note: For gels of total RNA, the 28S and 18S ribosomal subunits are visible and act as convenient markers (~5 and 2 kb, respectively). The quality of RNA is detected by the appearance of sharp ribosomal RNA bands without smear and the 28S band should be twice as bright as the 18S band (the theoretical ratio of 28S to 18S is ~2.7:1). (Streit et al., 2008)*

Recipes

Homemade TRIzol
Face it, we are still in a recession, and grant money is short.

To prepare 1L of TRIzol:

Chemical	Amount	Final Concentration
Ammonium Thiocyanate	30.45g	0.4M
Guanidine Thiocyanate	94.53g	0.8M
Sodium Acetate	13.6g	0.1M
Glycerol	50mL	5%
Phenol	380mL	38%

Adjust the volume and pH to 900mL with H_2O, and then re-adjust to 1000mL. The final pH should be 4.40. TRIzol is light-sensitive, so aliquot into the brown bottles and store them in the 4°C.
To test the pH:
Add 20% chloroform to a small sample and centrifuge at 4°C for 15 minutes at 12,000*x g*. Remove the aqueous phase and measure its pH with pH paper.

10x MOPS Buffer
MOPS stands for 3-[N-Morpholino]-propanesulfonic acid. According to Invitrogen, MOPS is a "zwitterionic buffer frequently in the electrophoresis of RNA on agarose gels." MOPS is chosen as the preferred electrolyte for this activity because it reduces heat generation while allowing separation to occur.

To prepare 1L of 10x MOPS Buffer:

Chemical	Amount	Final Concentration
MOPS pH 7.0	41.85g	0.2M
Sodium Acetate	4.1g	50mM
EDTA	3.7g	10mM

Adjust volume with H_2O to 900mL and pH to 7.0, then re-adjust to 1000mL. MOPS is light-sensitive, so wrap the bottle in aluminum foil and label appropriately. It can be stored at room temperature.

2x RNA Loading Dye
Here is how to make the RNA loading dye you will use for quantifying RNA. Thank you Mr. Erich Sabio for the recipe.

95% formamide,
0.025% bromophenol blue,
0.025% xylene cyanol FF,
0.025% ethidium bromide,
0.5 mM EDTA

Appendix

TRIzol Material Safety and Data Sheets
https://tools.invitrogen.com/content/sfs/msds/2012/15596026_MTR-NALT_EN.pdf

Nanodrop ND-1000 User Manual
http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf

References

Chomczynski, Piotr and Sacchi, Nicoletta, "Single-Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction", Analytical Biochemistry, Volume 162, Issue 1, April 1987, Pages 156-159, ISSN 0003-2697, 10.1016/0003-2697(87)90021-2. <http://www.sciencedirect.com/science/article/pii/0003269787900212>

Fermentas, "2x RNA Loading Dye." < http://www.fermentas.com/templates/files/tiny_mce/coa_pdf/coa_r0641.pdf>

Invitrogen, "TRIzol Reagent and Isolation Protocol." <http://tools.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf>

Invitrogen, "TRIzol MSDS." <https://tools.invitrogen.com/content/sfs/msds/2011/15596026_MTR-NAIV_EN.pdf>

Invitrogen, "MOPS." <http://tools.invitrogen.com/content/sfs/manuals/sp_9570.pdf>

Rio, Donald C., Gregory J. Hannon, Manuel Ares, Jr., and Timothy W. Nilsen. *RNA: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 2011. Print.

Streit, Sylvia, Christoph W. Michalski, Mert Erkan, Jörg Kleeff, and Helmut Friess. "Northern Blot Analysis for Detection and Quantification of RNA in Pancreatic Cancer Cells and Tissues." Nature Protocols 4 (2008): 37-43. 11 Dec. 2008. <http://www.nature.com/nprot/journal/v4/n1/full/nprot.2008.216.html>

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.

Vanherle, Bieke, Timmer, Erika, and Wackers, Paul, "RNA Quality Control," Biomedical Genomics, 2007. <http://biomedicalgenomics.org/RNA_quality_control.html>