

Total RNA Sample QC

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SUMMARY

Before shipping your RNA samples, please be sure to follow the JGI sample preparation and sample submission guidelines available at <https://jgi.doe.gov/user-program-info/pmo-overview/project-materials-submission-overview/>

This protocol describes how to perform quality control of total RNA samples to evaluate the quantity (using Qubit Fluorometer), quality (using electropherograms) and purity (using NanoDrop Spectrophotometer). We recommend all RNA samples to be evaluated with this protocol prior to shipping to JGI.

MATERIALS

Materials	Vendor	Part Number
Disposables		
Pipette tips		
RNase-free 1.5 mL microcentrifuge tubes	ThermoFisher	AM12450
Qubit assay tubes (0.5 mL)	ThermoFisher	Q32856
PCR 8-tube strip	USA Scientific	1402-4700
Reagents		
RNaseZap®	ThermoFisher	AM9780
Thermo Scientific™ DNA AWAY™ Surface Decontaminant	ThermoFisher	7010
70% Isopropanol	ThermoFisher	TX3270
Qubit RNA BR Assay Kit	ThermoFisher	Q10211
Qubit RNA HS Assay Kit	ThermoFisher	Q32855
Quant-iT™ RNA Assay Kit	ThermoFisher	Q10213
Quantifluor RNA System	Promega	E3310
Fragment Analyzer Standard Sensitivity RNA Analysis Kit	Agilent (AATI)	DNF-471-0500
Fragment Analyzer High Sensitivity RNA Analysis Kit	Agilent (AATI)	DNF-472-0500
Bioanalyzer RNA 6000 Nano Kit	Agilent	5067-1511
Bioanalyzer RNA 6000 Pico Kit	Agilent	5067-1512
RNase-Free DNase Set	Qiagen	79254
RNeasy MinElute Cleanup Kit	Qiagen	74204
100% Ethanol	ThermoFisher	111000200CSPP

<i>Equipment</i>		
Pipettes		
Microcentrifuge		
Vortex		
Qubit Fluorometer	ThermoFisher	Q32871, Q33216, Q33226
Thermal cycler or heat block (70°C)		
2100 Bioanalyzer	Agilent	G2939BA
Fragment Analyzer Automated CE System	Agilent (AATI)	FSv2-CE2F
NanoDrop Spectrophotometer	ThermoFisher	ND-1000, ND-2000, ND-3300, ND-ONE-W

SAFETY INFORMATION

- Safety glasses, lab coat, and nitrile gloves should be worn at all times while performing work in the lab during this protocol.
- Alcohols are highly flammable and irritating to the eyes. Vapors may cause drowsiness and dizziness. Keep containers closed and keep away from sources of ignition such as smoking. Avoid contact with skin and eyes. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.

PROCEDURE

1. Preparation

- 1.1. Extracted total RNA samples could be stored at -80°C until ready to begin lab work
- 1.2. Prepare lab bench for use with RNA samples by wiping area and materials with DNA AWAY™, RNAaseZap® and 70% isopropanol in the order
- 1.3. Thaw RNA samples on ice
- 1.4. Gently mix total RNA by tapping the tubes (avoid vortex) and quick spin before opening the tubes
- 1.5. Record the volumes measured by pipette

2. Quantification by Qubit Fluorometer

Note: If a microplate reader is available, ThermoFisher's Quant-iT™ RNA Assay Kit for a broad range assay and Promega's Quantifluor RNA System for a high sensitivity assay are recommended for RNA quantitation with a large number of samples. Four point standard curve including a blank could be used for the assay which is utilized in JGI quantification system (0, 50, 400, 1,000 ng/μL standard points for a broad range assay and 0, 1.5, 10, 80 ng/μL for a high sensitivity assay).

Note: NanoDrop measurements are generally not reliable as quantification and JGI does not accept the concentration measured by NanoDrop.

- 2.1. Set up the number of 0.5 mL Qubit assay tubes you will need for 2 standards and the samples

- 2.2. Label the tube lids
- 2.3. Make the Qubit working solution by diluting Qubit RNA BR reagent 1:200 in Qubit RNA BR buffer in microcentrifuge tubes (each assay tube requires ~200 μL of working solution)
- 2.4. Load 190 μL of Qubit working solution and add 10 μL of each Qubit standard, vortex 2-3 seconds and quick spin (200 μL final volume)
- 2.5. Load 198 μL of Qubit working solution and add 2 μL of your sample, vortex 2-3 seconds and quick spin (200 μL final volume)
- 2.6. Incubate at room temperature for 2 min
- 2.7. On the Qubit Fluorometer, select your assay (RNA Broad Range), press YES to run a new calibration, and then insert the tube containing Standard #1. Close the lid, and press READ
- 2.8. Insert the tube containing Standard #2, close the lid, and press READ. Calibration of Qubit is now complete
- 2.9. Insert the tube containing RNA sample, close the lid, and press READ
- 2.10. Select calculate concentration, select the volume (2 μL), select the measurement units as ng/ μL , and record your concentration. Repeat for all RNA samples
 - 2.10.1. If the sample concentration is too low, then use RNA HS Assay kit
 - 2.10.2. If the sample concentration is too high, then set up serial dilutions of your samples and repeat the assay using RNA BR Assay kit

Note: We recommend using 2 μL of each sample instead of 1 μL to increase the accuracy.

Note: We recommend using 2 μL of Standard #2 to be treated as a sample to verify the Qubit was calibrated correctly. If the concentration of this standard is above $\pm 10\%$ of the expected concentration, then please recalibrate the Qubit.

Note: Review JGI Sample requirements and concentrate samples if they are too dilute. Speedvac without applying heat is recommended for concentrating samples to minimize degradation and yield loss.

3. Quality check

Note: Described methods here are using electropherograms. Alternatively, total RNA quality could be assessed in regular (non-denaturing) 1% agarose gel in 1x TAE or 0.5x TBE using DNA ladder markers. It is recommended to denature RNA at 70°C for 2 min and store on ice before loading.

3.1. Fragment Analyzer standard or high sensitivity total RNA kit

Note: Determine sensitivity appropriate for the samples: standard kit has a desired range of concentration for 5 ng/ μL to 500 ng/ μL and high sensitivity kit for 50 pg/ μL to 5 ng/ μL . A concentration higher than the confident range should be diluted down before loading.

- 3.1.1. Prepare the appropriate kit according to the manufacturer's guide
- 3.1.2. Aliquot 2 μL of each sample and ladder into the Sample Plate
- 3.1.3. Denature at 70°C for 2 min on a thermal cycler
- 3.1.4. Immediately store the denatured samples and ladder on ice
- 3.1.5. Mix denatured 2 μL of samples or ladder with 20 μL of Diluent Marker in the Sample Plate
- 3.1.6. Prepare Fragment Analyzer according to the manufacturer's guide and run the Sample Plate
- 3.1.7. Review the data and assess RNA quality for each sample following the guides:

- Assess RQN (RNA Quality Number). The highest RQN is 10 with no degradation. Lower the number, lower the quality. JGI recommends sending RNA samples with RQN above 6.0.
- Assess 28S/18S area ratio. 28S/18S=2.0 or higher means a good quality. Lower the number, lower the quality. JGI recommends sending RNA samples with 28S/18S above 0.8.
- The following Figures 1-5 show the various quality of total RNA run on Fragment Analyzer with HS RNA kit.

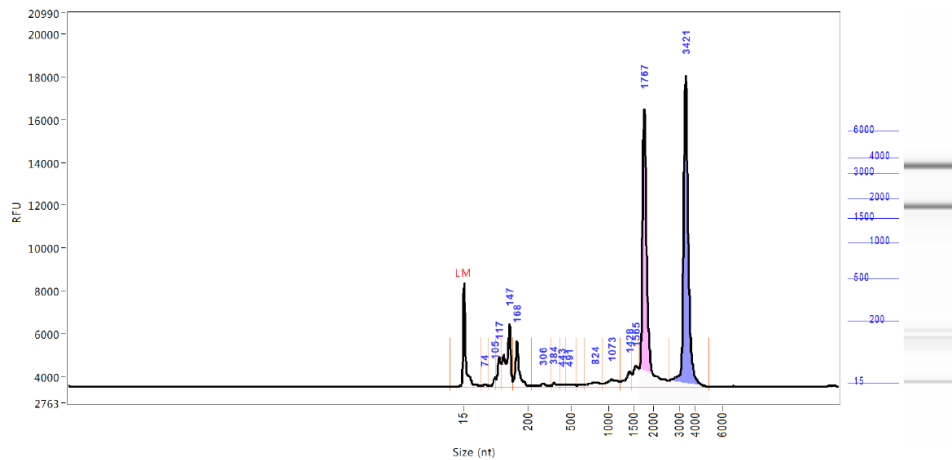


Figure 1. A typical good quality total RNA with 28S/18S=1.3, RQN=8.8.

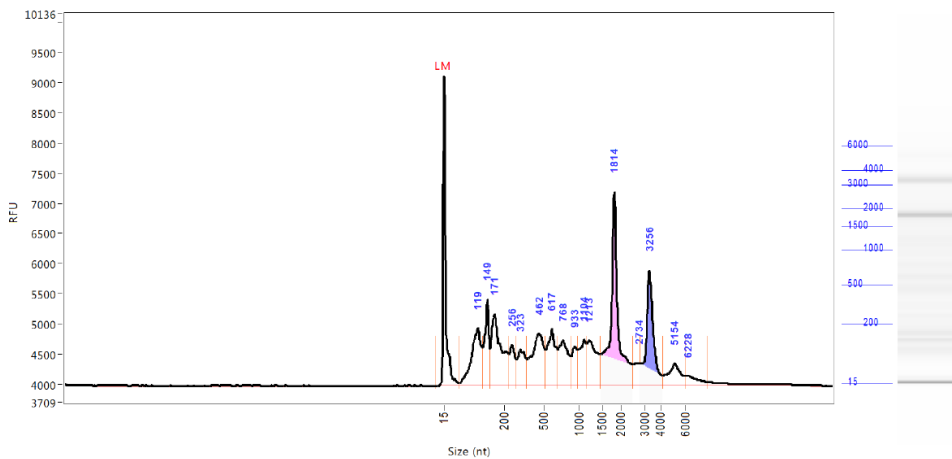


Figure 2. A marginal passed quality total RNA with 28S/18S=0.6, RQN=4.5.

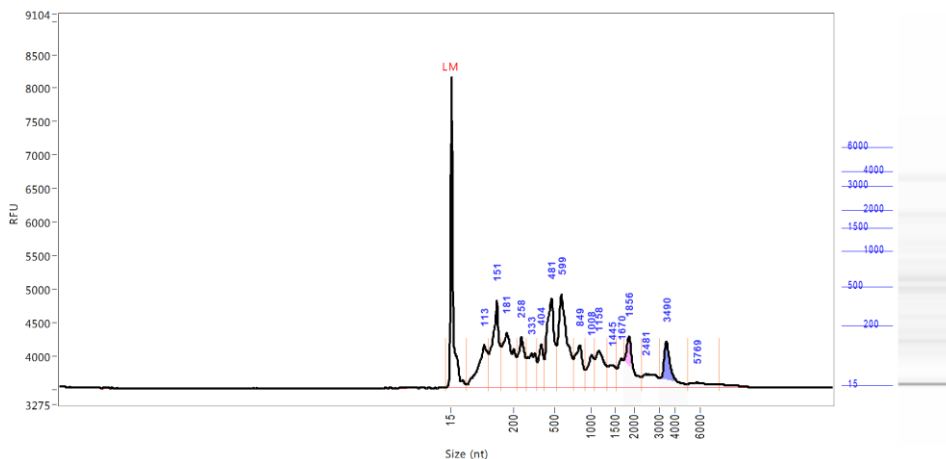


Figure 3. Failed quality total RNA with 28S/18S=1.6, RQN=2.6 with elevated baseline and lower rRNA heights which indicate degraded RNA.

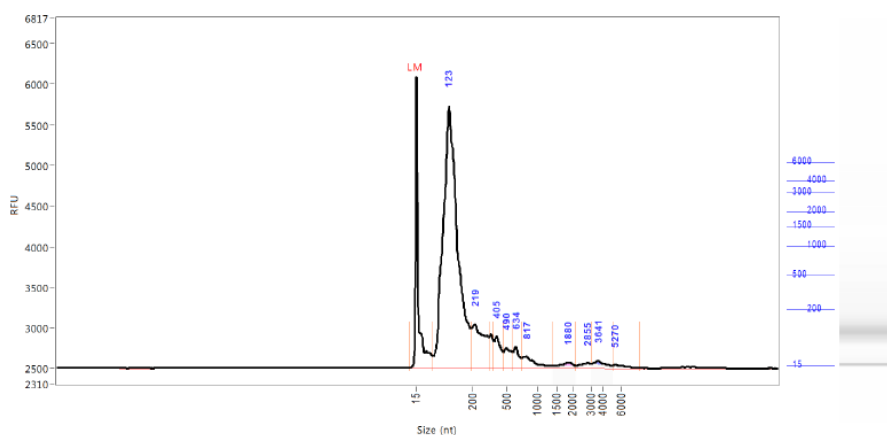


Figure 4. Fully degraded and failed total RNA with 28S/18S=0.7, RQN=1.9 showing prominent with short fragments and with no rRNA peaks.

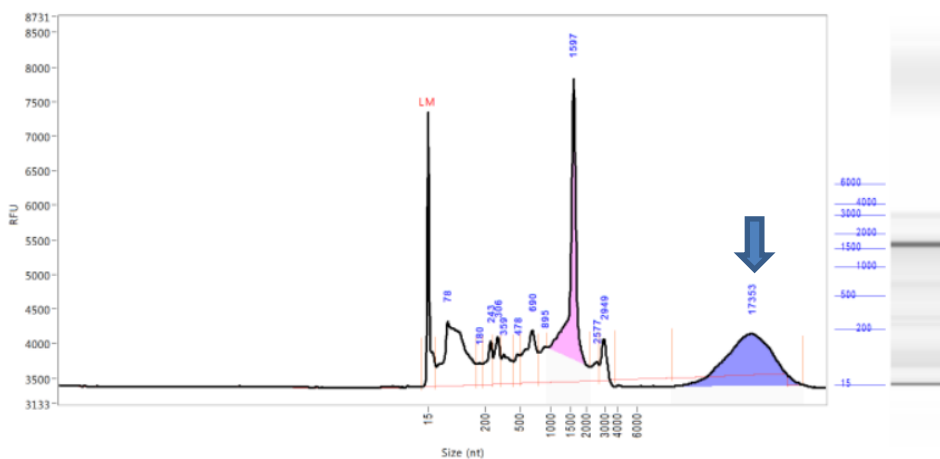


Figure 5. The example showing the suspected genomic DNA contamination which will fail regardless of total RNA quality if the area indicated by the arrow is proven to be genomic DNA by a method described in Appendix A.

3.2. Bioanalyzer RNA 6000 Nano or Pico Kit

Note: Determine assay type (eukaryote, prokaryote or mRNA) and sensitivity appropriate for the samples: RNA 6000 Nano kit has a confident range of concentration for 25 to 500 ng/μL and RNA 6000 Pico kit for 50 pg/μL to 5 ng/μL. A concentration higher than the confident range should be diluted down before loading.

- 3.2.1. Prepare the appropriate kit according to the manufacturer's guide
- 3.2.2. Aliquot 1.2 μL of each sample into PCR 8-tube strip
- 3.2.3. Denature at 70°C for 2 min on a thermal cycler or a heat block
- 3.2.4. Immediately store the denatured samples on ice
- 3.2.5. Prepare the chip according to the manufacturer's guide and add 1 μL of denatured samples
- 3.2.6. Review the data and assess RNA quality for each sample following the guides:
 - Assess RIN (RNA Integrity Number) which is the highest as 10 with no degradation. Lower number, lower the quality. JGI recommends sending RNA samples with RIN above 6.0.
 - Assess 28S/18S area ratio. 28S/18S=2.0 or higher means a good quality. Lower the number, lower the quality. JGI recommends sending RNA samples with 28S/18S above 0.8.
 - The following Figures 6-9 show the various quality of total RNA run on Bioanalyzer 6000 RNA kit.

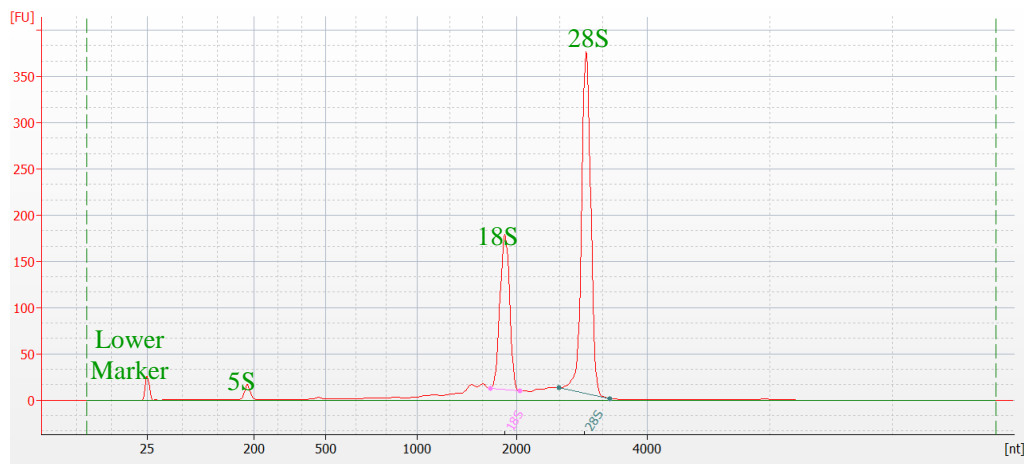


Figure 6. A typical good quality total RNA with 28S/18S=2, RIN=9.1.

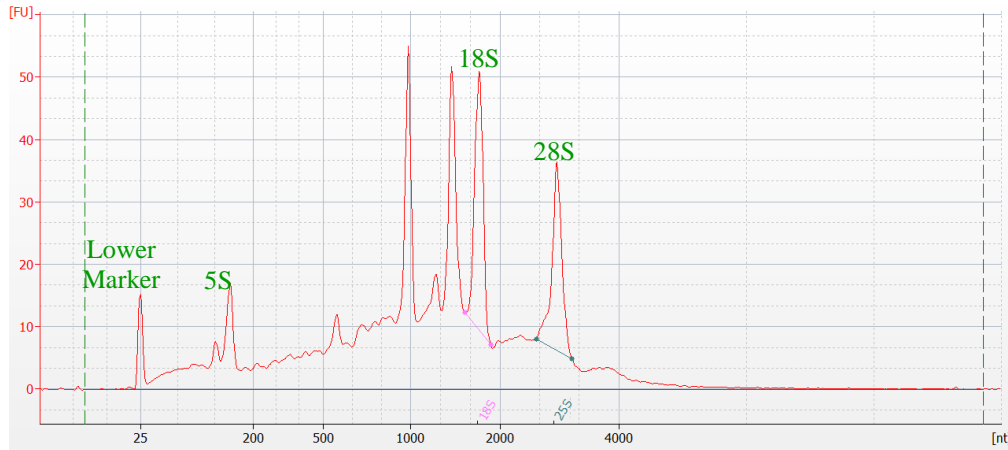


Figure 7. A marginally passed quality total RNA with 28S/18S<1, RIN=5.9. The example is from plant total RNA and it is common to have extra peaks (prokaryotic rRNA peaks) in plant material. The elevated baseline shows the degraded RNA.

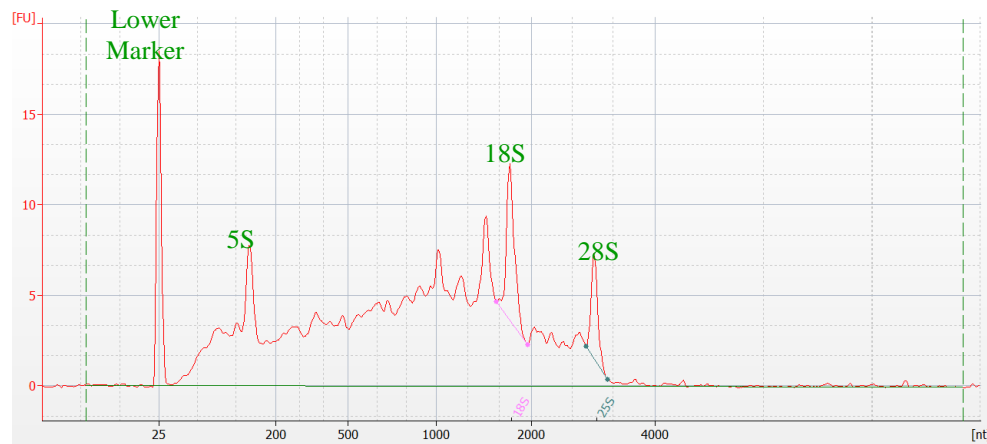


Figure 8. Failed quality total RNA with RIN=4.8 with elevated baseline and lower rRNA heights.

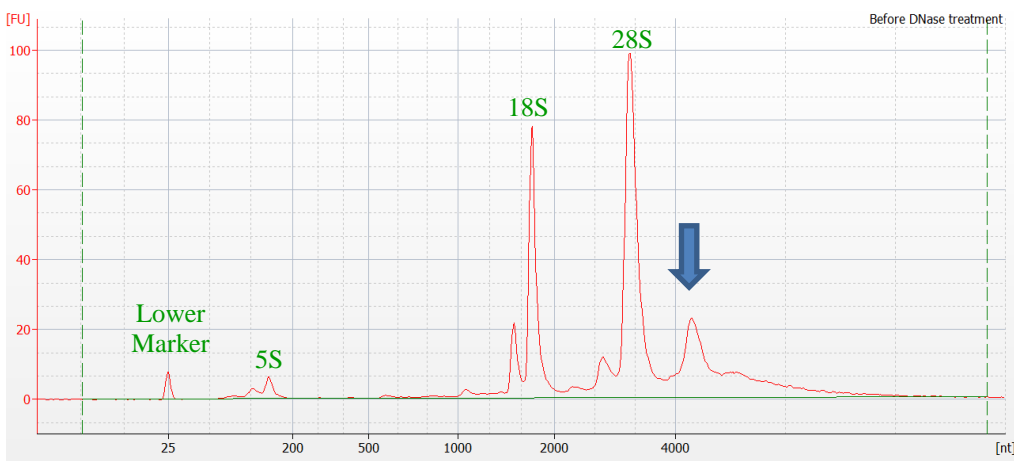


Figure 9. The example showing the suspected genomic DNA contamination which will fail regardless of total RNA quality if the area indicated by the arrow is proven to be genomic DNA by a method described in Appendix A.

4. Purity check by NanoDrop

Note: JGI uses NanoDrop to determine sample purity only. We do not recommend using NanoDrop to determine sample concentration. We recommend submitting high purity RNA samples with appropriate OD measurements. Data from Nanodrop helps in troubleshooting whether the contaminants present in the sample. Low purify samples are recommended to re-purified with suitable purification methods.

- 4.1. Clean pedestal and sampling arm with nuclease-free water and a KimWipe
- 4.2. Pipette 1.6 μ L of nuclease-free water directly onto the pedestal and lower the sampling arm
- 4.3. Surface tension is used to hold samples between two optical fibers
- 4.4. Select “Initialize” from the NanoDrop software
- 4.5. When the initialization is complete, raise the sampling arm and wipe the pedestal and the arm with a KimWipe
- 4.6. Select “Nucleic Acid” and the appropriate “Sample Type”
- 4.7. Pipette 1.6 μ L of nuclease-free water directly onto the pedestal
- 4.8. Lower the sampling arm and select “Blank”
- 4.9. When the measurement is complete, raise the sampling arm and wipe the pedestal and the arm with a KimWipe
- 4.10. Pipette 1.6 μ L of RNA sample onto the pedestal and lower the sampling arm
- 4.11. Select “Measure”
- 4.12. When the measurement is complete, record A260/A280 and A260/A230 ratios
- 4.13. Between and after all sample measurements, clean the pedestal and arm with nuclease-free water and a KimWipe
- 4.14. Review the spectral image and the absorbance ratios to assess the purity of the sample using the following guidelines:
 - The wavelength of maximum absorption for both DNA and RNA is 260nm, while the maximum absorbance for proteins is at 280 nm.
 - Very pure RNA will have an A260/A280 ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential DNA or protein contamination. A low A260/A280 ratio is likely due to mixing phases when removing the upper aqueous phase of the Trizol separation or is also more common in samples with a very low yield of RNA.
 - The A260/A230 ratio should also be above 2.0. A low A260/230 ratio indicates contamination with the wash solutions, chaotropic salts, phenols or protein. A low A260/A230 ratio is most likely due to contamination of the samples with washing buffers during the Minispin tube washes. Be more careful when handling the tubes, especially when adding wash solution or removing the spin-through. Try to gently pour out the flow-through and then carefully wipe away drops on the outer rim of the collection tube with a KimWipe.

APPENDIX A: Determination of genomic DNA contamination

Note: one quick way to check the genomic DNA contamination is to run Fragment Analyzer with High Sensitivity 50 Kb kit. If there is a significant amount of large fragment >6 kb, it suggests genomic DNA

contamination. However, this method would not identify degraded DNA and require DNase treatment as described below.

When there is suspected gDNA contamination, a 1 µg aliquot of the RNA sample could be treated individually with DNase to confirm use RNase-Free DNase (Qiagen).

1. Dilute the 1 µg aliquot to 90 µL total volume using the nuclease-free water that comes in the DNase kit in a RNase-free 1.5mL microcentrifuge tube
2. Save 2.5 µL of 90 µL in new RNase-free tube as “before DNase” control
3. Add 10 µL RDD Buffer and 2.5 µL DNase to the remaining 87.5 µL sample
4. Mix the tube gently and Quick spin
5. Incubate for 10 min at room temperature
6. After this DNase treatment, use RNeasy MinElute Cleanup Kit (Qiagen) to re-purify the RNA sample following steps:
 - a. Add 350 µL buffer RLT, mix well and quick spin
 - b. Add 250 µL 100% ethanol, mix well and quick spin
 - c. Transfer the entire volume to a filter column on a collection tube
 - d. Spin the filter column at >10,000 rcf for 15 sec
 - e. Discard the eluate and transfer the filter column to a new collection tube
 - f. Add 500 µL buffer RPE (ethanol pre-added)
 - g. Spin the filter column at >10,000 rcf for 15 sec
 - h. Discard the eluate and add 500 µL 100% ethanol
 - i. Spin the filter column at >10,000 rcf for 2 min
 - j. Discard the eluate and transfer the filter column to a new collection tube
 - k. Spin the filter column at >10,000 rcf for 5 min
 - l. Discard the eluate and transfer the filter column to new RNase-free 1.5 mL microcentrifuge tube
 - m. Add 14 µL of nuclease-free water from the kit to the center of the filter column
 - n. Incubate for 1 min
 - o. Spin the filter column at >10,000 rcf for 1 min
 - p. Discard the filter column and keep the eluate, label as “after DNase”
7. Dilute “before DNase” to 1:2 and “after DNase” to 1:10 with nuclease-free water to run on the BioAnalyzer Pico assay
8. If “after DNase” shows complete elimination or significant reduction of the suspected gDNA peak, it is likely that there is gDNA contamination in the original RNA sample

APPENDIX B: References

1. Qubit:
 - Qubit RNA BR Assay: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_RNA_BR_Assay_UG.pdf
 - Qubit RNA HS Assay: https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Qubit_RNA_HS_Assay_UG.pdf
2. Quant-iT™ RNA Assay Kit:
https://assets.thermofisher.com/TFS-Assets/LSG/manuals/Quant_iT_RNA_Assay_UG.pdf

3. Quantifluor RNA System:
<https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/quantifluor-rna-system-protocol.pdf>
4. Fragment Analyzer:
 - Standard Sensitivity RNA Analysis Kit: <https://www.aati-us.com/documents/quick-start-guides/dnf-471/dnf-471-quick-start-guide-12-capillary-11-03-2015.pdf>
 - High Sensitivity RNA Analysis Kit: <https://www.aati-us.com/documents/quick-start-guides/dnf-472/dnf-472-quick-start-guide-96-capillary-11-03-2015.pdf>
5. Bionalayzer:
 - RNA 6000 Nano Kit: https://www.agilent.com/cs/library/usermanuals/public/G2938-90034_RNA6000Nano_KitGuide.pdf
 - RNA 6000 Pico Kit: https://www.agilent.com/cs/library/usermanuals/Public/G2938-90046_RNA600Pico_KG_EN.pdf
6. NanoDrop :
 - T042 Technical Bulletin – NanoDrop 260/280 and 260/230 Ratios:
<http://www.nhm.ac.uk/content/dam/nhmwww/our-science/dpts-facilities-staff/Coreresearchlabs/nanodrop.pdf>
 - User manual for ND-1000:
<https://www.baylor.edu/bsb/doc.php/210102.pdf>
7. RNase-Free DNase Set:
<https://www.qiagen.com/us/resources/resourcedetail?id=b0ca9e5a-ff87-476e-811b-ff80e4f07b3f&lang=en>
8. RNeasy MinElute Cleanup Kit:
<https://www.qiagen.com/us/resources/resourcedetail?id=06f3d3ff-5926-4915-bffe-4adcf99266aa&lang=en>