TOTAL RNA SAMPLE QC

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SUMMARY

Before shipping your RNA sample(s), please be sure to follow the JGI sample preparation and sample submission guidelines available at http://jgi.doe.gov/collaborate-with-jgi/pmo-overview/project-materials-submission-overview/

This protocol describes how to perform quality control of RNA samples to evaluate the quantity (using Qubit Fluorometer), quality (using the Agilent 2100 BioAnalyzer), and purity (with NanoDrop Spectrophotometer). We recommend all RNA samples to be evaluated with this protocol prior to shipping to JGI for sequencing.

MATERIALS

Materials	Vendor	Part Number
Disposables		
Pipette tips	Many available	n/a
Qubit assay tubes	Life Technologies	Q32856
TempAssure PCR 8-tube flex-free strips (or equivalent)	USA Scientific	1402-4700
Microcentrifuge tubes (nuclease-free)	Many available	n/a
Kimwipes	Many available	n/a
Compressed Air Canister (with “straw” to direct)	Many available	n/a
Reagents		
70% Isopropanol	Many available	n/a
Qubit® RNA BR Assay Kit	Life Technologies	Q10211
Qubit® RNA HS Assay Kit	Life Technologies	Q32855
RNAseZap®	Life Technologies	AM9780
DNA *AWAY*™ (Surface Decontaminant)	Thermo Scientific™	7010
Nuclease-free water	GrowCells.com	NUPW-0125
Equipment		
Pipettes	Many available	n/a
Qubit Fluorometer 2.0	Life Technologies	Q32866
Microcentrifuge (able to spin at 13,000rcf)	Many available	n/a
Vortex	Many available	n/a
Thermal Cycler Instrument (able to hold at 70 ° C for 2min)	Many available	n/a
2100 BioAnalyzer Electrophoresis Instrument (w/ accessories)	Agilent	G2939AA
Agilent RNA 6000 Nano Kit (includes chips)	Agilent	5067-1511

Agilent RNA 6000 Pico Kit (includes chips)	Agilent	5067-1512
NanoDrop Spectrophotometer	Thermo Scientific	ND-1000

SAFETY INFORMATION

Safety glasses, lab coat, and nitrile gloves should be worn at all times while performing this protocol. For more information, please contact the manufacturer and consult the appropriate MSDS.

PROCEDURE

1. Collect total RNA samples for QC. Until ready to begin lab work, store RNA in a -80°C freezer.
2. Prepare lab bench for use with RNA samples. Wipe lab bench, tube racks, pipettes, and other equipment with DNA AWAY™, RNAseZap® and 70% isopropanol (in that order).
3. After removing RNA samples from the freezer, immediately transfer to ice. Thaw all RNA samples on ice.
4. Briefly centrifuge tubes to collect droplets from the tube wall and lid.
5. Pipette-measure one RNA sample at a time until the exact volume is determined. Record the volumes.

SAMPLE QUANTITATION

Note:

1. *JGI uses a microplate reader and the Life Technologies Quant-iT™ RNA Assay Kit for quantitation. A three point (50ng/µL, 400ng/µL and 1000ng/µL) standard curve along with a blank is used for the assay. If a microplate reader is not available for determining concentration, then Qubit assay for quantitation is recommended.*
2. *If the quantitation by Qubit is performed, then it is recommended to keep Qubit kit reagents bundled in their original lot; that is, dye, buffer and standards should ideally be of the same lot when evaluating a particular sample/ set of samples.*

1. Gently vortex RNA samples (~4 seconds), centrifuge briefly to collect the sample to the bottom.
2. Set up the required number of Qubit assay tubes for standards and samples. Label the tube lids appropriately.
3. Make sufficient Qubit working solution for the total number of reactions (standards and samples), by combining 1µl Qubit RNA reagent dye to 199µl Qubit RNA buffer for each reaction. For example, for 5 reactions, use the volumes for 5X below:

# reactions	1X	5X
RNA Buffer	199	995
RNA Reagent dye	1	5
Total (µl)	200	1000

4. Add 190µl of Qubit working solution to the assay tubes for the standards. Add 10µl of each Qubit standard to the appropriate tube, and mix by vortexing ~4 seconds. Centrifuge briefly.
5. Add 198µl Qubit working solution to the assay tubes for samples. Add 2µl RNA sample and mix by vortexing ~ 4 seconds. Centrifuge briefly.
6. Incubate all tubes at room temperature for 2 minutes.
7. On the Qubit 2.0 fluorometer, select your assay ("RNA" (this is High Sensitivity) or "RNA Broad Range"), press YES to run a new calibration, and then insert the tube containing Standard #1. Close the lid, and press READ.

8. Insert the tube containing Standard #2, close the lid, and press READ. Calibration of Qubit is now complete.
9. Insert the tube containing RNA sample, close the lid, and press READ. Select calculate concentration, select the volume (2µl), select the measurement units as ng/µl, and record your concentration. Repeat for each RNA sample to test.
    a. If the sample concentration is too low, then use RNA HS Assay kit.
    b. If the sample concentration is too high, then set up serial dilutions of your samples and repeat the assay using RNA BR Assay kit.

Important!
We recommend using 2µl of Standard #2 to be treated as a sample to verify the Qubit was calibrated correctly. If the concentration of this standard is >10% of the expected concentration, then please recalibrate the Qubit.

Important!
Review JGI Sample requirements. Please concentrate samples if they are too dilute.

SAMPLE QUALITY

1. Prepare and store ladder for Nano and/or Pico assay beforehand, as needed (described in Appendix B).
2. Decide which assay (eukaryote, prokaryote or mRNA) and sensitivity is appropriate for samples—the RNA 6000 Pico kit has a range of 50pg/µL to 5ng/µL, while the RNA 6000 Nano kit has a range of 25ng/µL to 500ng/µL; samples that measure greater than 25ng/µL should be diluted to be within the range for Pico; samples measuring greater than 500ng/µL should be diluted to be within range of Nano (Qubit readings are to be used here).
3. Take out appropriate kit(s) (BioAnalyzer Nano and/or Pico, depending on what is needed) from their 2-8^{o}C storage and allow to equilibrate for 30 minutes.

NOTE: ALL pipetting steps for gel matrix or gel-dye solution should employ reverse pipetting

4. Prepare gel by pipetting 550µL gel matrix into a filter tube (provided) & centrifuging for 10min at 1500 rcf (± 20%). Store this filtered gel at 4^{o}C and use within one month of preparation.
5. Vortex (10 seconds), spin & pipette 65uL of filtered gel and 1uL dye solution (from kit) into an RNAse-free 1.5mL microcentrifuge tube (or one of the 0.5mL tubes provided in kit). Vortex & spin for 10min at 13,000 rcf.
6. Dispense into TempAssure PCR 8-tube flex-free strips (or equivalent) 1.2µL or 1.0µL of sample (use 1.2µL of samples for which no dilution will be needed).
7. Place samples on thermal cycler block and denature for 2min at 70^{o}C; remove samples from block and place on ice immediately after this 2min denaturation.
8. Dispense 9µL gel-dye mixture into well just above bottom right corner well of assay chip; pressurize gel using the syringe of the chip priming station (with the lever having been set to the highest level, for 30 seconds); release lever, wait 5 seconds and then manually pull syringe plunger back up to the 1mL mark. Further instructions for the setup of the chip priming station can be found in Appendix B.
9. Dispense 9µL gel-dye mixture into two wells directly above the pressurized gel well, then 9µL conditioning solution (**ONLY** if using RNA 6000 Pico) to "CS" well and Marker solution to all remaining wells, including ladder well (use 5µL Marker for wells that will have sample or H_2O, and 6µL for all blank or unused wells of chip).
10. While preparing chip, also clean electrode pin set by placing in a cleaning chip of 400µL 0.5X RNAseZap® for 1min, followed by a cleaning chip of 400µL H_2O for 1min, followed by a separate chip of 400µL H_2O for ≥ 2min. The desired assay (eukaryote, prokaryote or mRNA) and sensitivity (Nano or Pico) can also be selected at this point, and sample names can be added, if desired (or can be inserted after run is completed).

11. Dispense 1µL thawed (and previously denatured) ladder solution to the well that is marked with the ladder icon.
12. Dilute samples with appropriate amount (depending on the dilution factor) with nuclease-free water.
13. Load 1µL of each sample onto chip (up to 12 per Nano chip or 11 per Pico chip), keeping track of their order; whenever possible, also run 1µL H_2O (of the same nuclease-free water used for dilutions) and one Blank well (only containing 6µL of Marker), so as to verify that both the water and the Marker solution are free of contaminants.
14. Once chip is fully loaded, place it in the chip adapter of an IKA MS3 basic vortex mixer (included with the purchase of 2100 BioAnalyzer Electrophoresis Instrument) and vortex for 1min at 2200rpm (**run chip in ≤ 5min**).
15. Meanwhile, open the electrode cartridge so as to allow the electrode pin set to dry for the 1min of vortexing.
16. Spray (using "straw" to direct air) the cartridge pin set with compressed air to ensure that pins are complete dry.
17. Insert assay chip to be analyzed, close electrode cartridge, verify that sample names align with the order in which they were dispensed (and that correct assay is selected) and click the "Start" button to begin the run.
18. Once the run has completed (≤24min for a full Nano or Pico total chip, though longer for the mRNA assay), open the electrode cartridge, remove the assay chip and clean the electrodes as before: clean electrode pin set by placing in a cleaning chip of 400µL 0.5X RNAseZap® for 1min, followed by a cleaning chip of 400µL H_2O for 1min, followed by a separate chip of 400µL H_2O for ≥ 2min. Open the electrode cartridge, allow pin set to dry for 1min and spray dry with compressed air. **NOTE: this should <u>also</u> be done in between chip runs, if multiple.**
19. Analyze data from lab computer (or desk computer, if software is installed) and assess for RNA quality.

<u>Quality Assessment from BioAnalyzer Traces</u>

- First, verify that all 6 peaks of the ladder appear on its trace, whether for the Nano assay (Figure A) or for the Pico assay (Figure B); if there are problems with the ladder (such as missing peaks, for example), the sizing of the sample RNA will be inaccurate.

Figure A

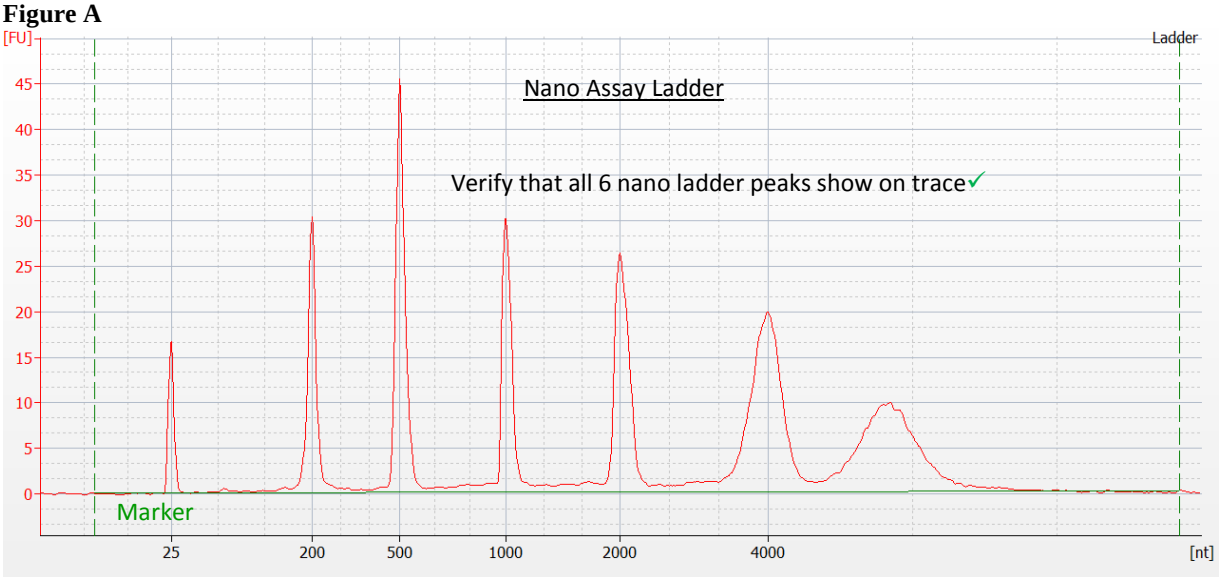
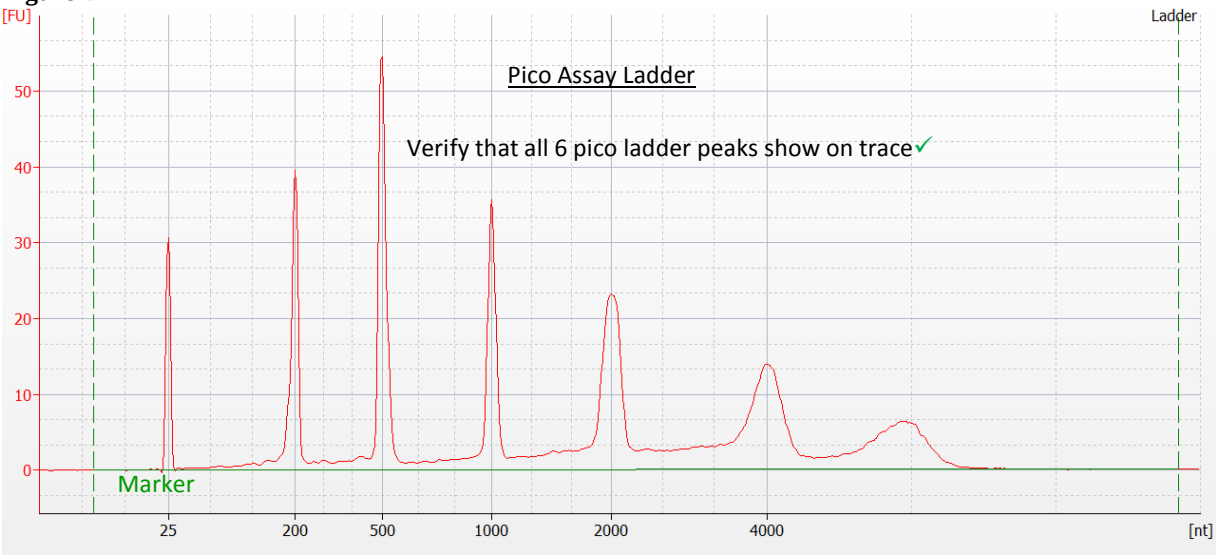
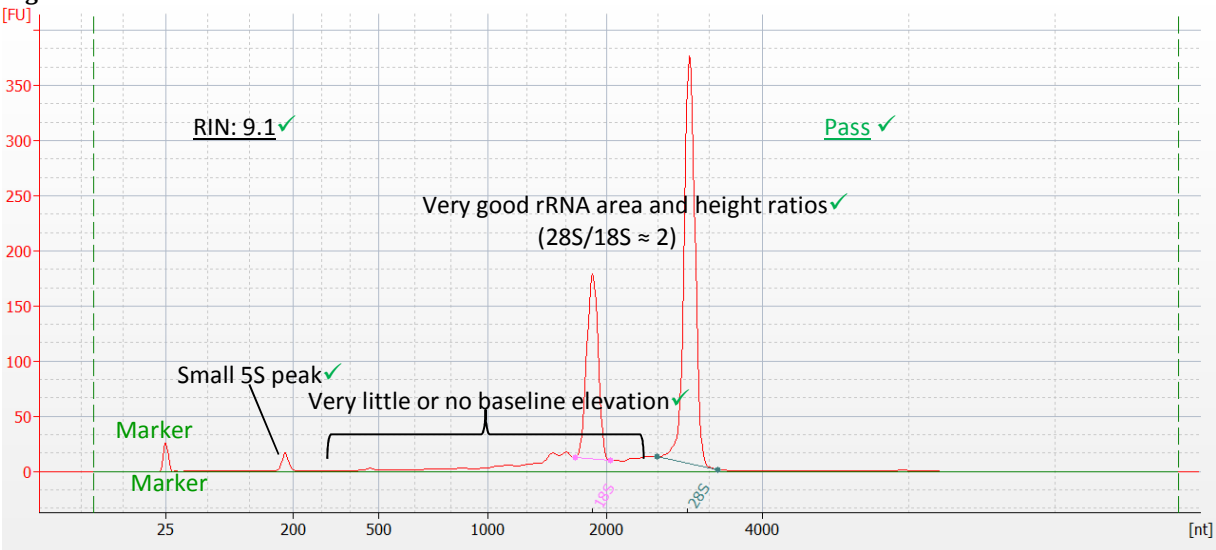


Figure B



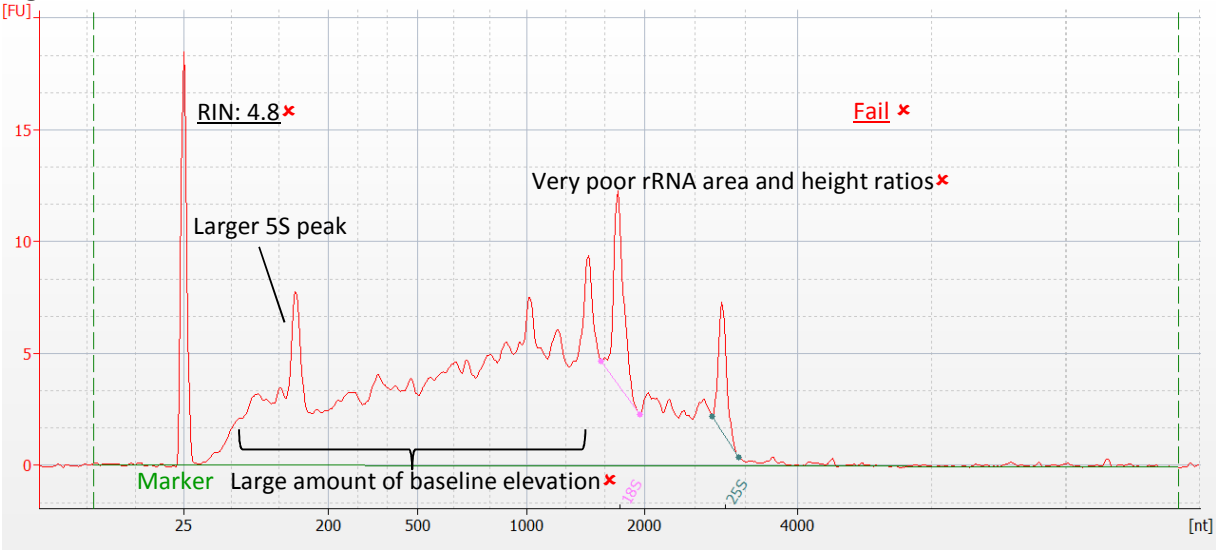
- Next, assess the quality of each RNA sample based on such criteria as: RIN value (max = 10), small 5S RNA peak, little or no baseline elevation, and good rRNA area and height ratios (very good = 1.5 to 2.0+); Figure C below shows an example of a very good quality RNA trace (and elements that demonstrate this).

Figure C



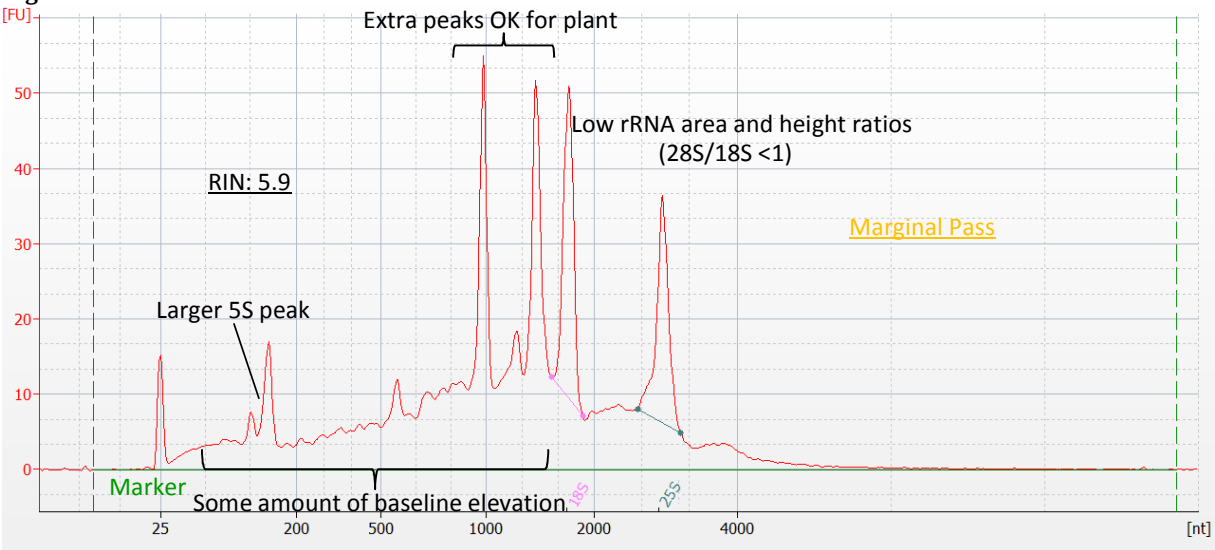
- Note all of the following characteristics that suggest poor total RNA quality: low RIN value (<6.0), larger 5S peak, baseline elevation and low rRNA area and height ratios (<0.8); samples that show these characteristics (such as in Figure D below) are of low total RNA quality and should not be sent to the JGI.

Figure D



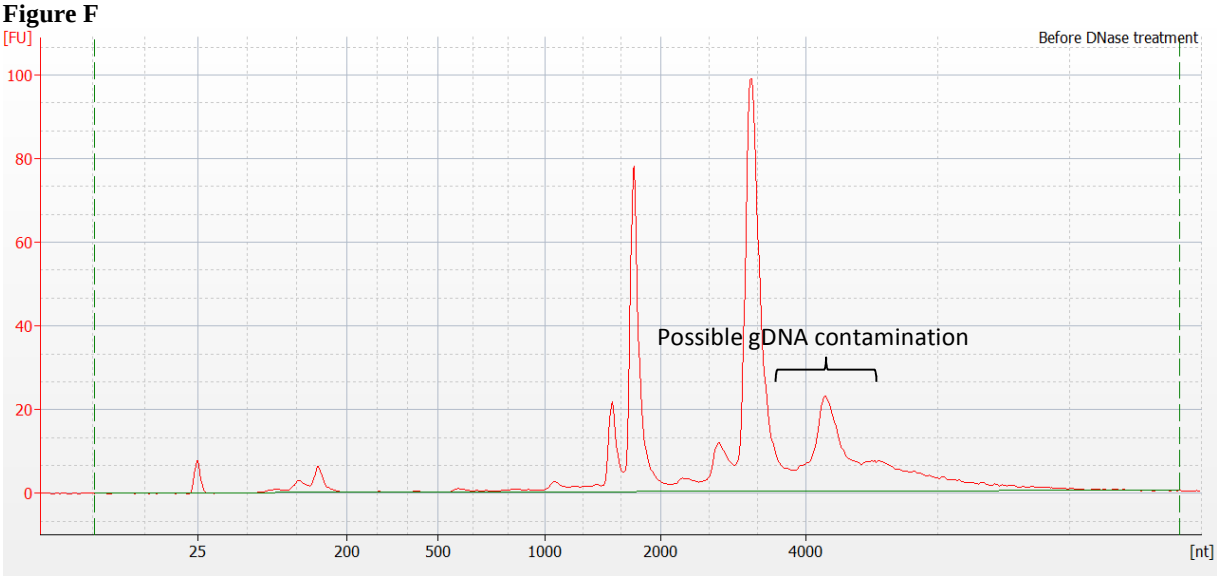
- Note also characteristics that suggest marginal RNA quality: somewhat low RIN (5.9 to 6.9), somewhat large 5S peak, extra peaks (expected for plant samples, though should generally not be greater than the rRNA peaks), some baseline elevation and lower rRNA height and area ratios (0.9 to 1.3), as in Figure E. b

Figure E



- Samples with extra peaks (especially broad peaks and in the post-4000nt range, as shown in Figure F below) should have a 1µg aliquot taken out and tested using a DNAse treatment (see “Appendix A”).

Figure F



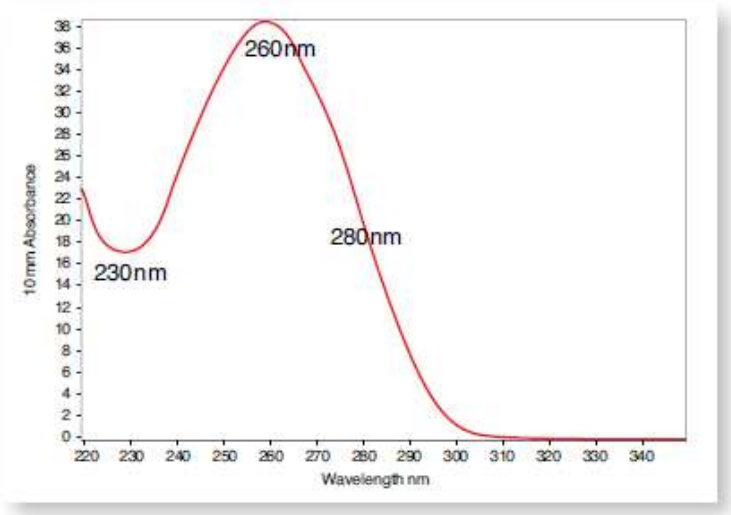
Important!
We highly recommend sending good quality RNA for sequencing [Figure C]. Degraded RNA can affect library construction and sequencing quality [Figure D]. RNA contaminated with gDNA will not be accepted by the JGI for sequencing [Figure F], and RNA should ALWAYS be DNAse treated during the extraction process.

SAMPLE PURITY

Note:

1. *We do not recommend using Nanodrop to determine sample concentration.*
2. *We recommend submitting purified RNA samples for sequencing. Data from Nanodrop helps in troubleshooting whether the contaminant present in the sample has an effect on the quality.*

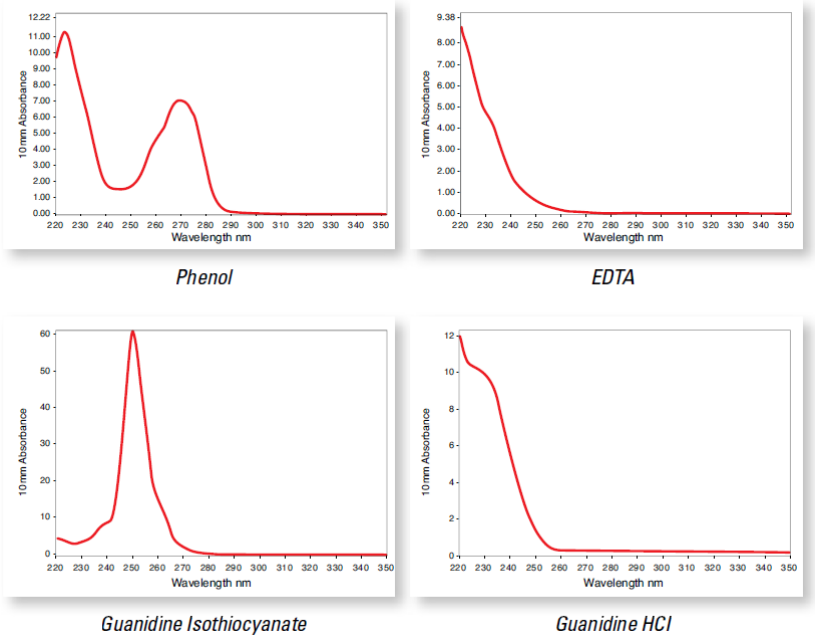
1. Clean pedestal and sampling arm with nuclease-free water and Kimwipes.
2. Pipette 1.5µl of nuclease-free water directly onto the pedestal.
3. Lower the sampling arm and select Initialize from the NanoDrop software. Surface tension is used to hold samples in place between two optical fibers.
4. When the measurement is complete, raise the sampling arm and wipe the water from the pedestal and arm.
5. Select the Nucleic Acid application, and the appropriate Sample Type.
6. Pipette 1.5µl of nuclease-free H_2O or appropriate buffer (if sample was not eluted in nuclease-free H_2O) directly onto the measurement pedestal.
7. Lower the sampling arm and select Blank.
8. When the measurement is complete, raise the sampling arm and wipe the buffer from the pedestal and arm.
9. Pipette 1.5µl of the RNA sample onto the pedestal, and close the sampling arm. Select Measure, and when complete record and save the A260/A280 and A260/A230 readings.
10. Between and after all sample measurements, clean the pedestal and arm with nuclease-free water and Kimwipes.



Typical Nucleic Acid Spectrum

11. Review the spectral image and the absorbance ratios to assess the purity of the sample. Use the following guidelines to perform analysis of the spectrum and absorbance ratios:

- The absorbance maxima for nucleic acids is at 260nm while the absorbance maxima for proteins is at 280nm
- A260/A280 ratio of ~2.0 is generally accepted as "pure" for RNA.
- Absorbance at 230 nm is accepted as being the result of other contamination (see images below); therefore the ratio of A260/A230 is frequently also calculated.. The 260/230 values for "pure" nucleic acid are often higher than the respective 260/280 values. Expected 260/230 values are commonly in the range of 2.0-2.2.
- A low 260/280 ratio may be the result of a contaminant such as protein, or a reagent such as phenol (see image below), that is absorbing at 280 nm or less. It could also be due to issues with measurement. Nanodrop QC readings should be repeated to rule out issue with measurement as the cause of this. High 260/280 purity ratios are generally not indicative of an issue.
- A low A260/A230 ratio may be the result of contaminant absorbing at 230 nm or less. Such contaminants include carbohydrates, residual phenol, residual guanidine and/or glycogen. On the other hand, a high A260/A230 ratio may be the result of either making a blank measurement on a dirty pedestal or using a blank solution that is not of a similar ionic strength as the sample solution
- Wavelength of the trough in sample spectrum should be at ~230 nm, and wavelength of the peak in sample spectrum should be at 260 nm.



APPENDIX A: TROUBLESHOOTING

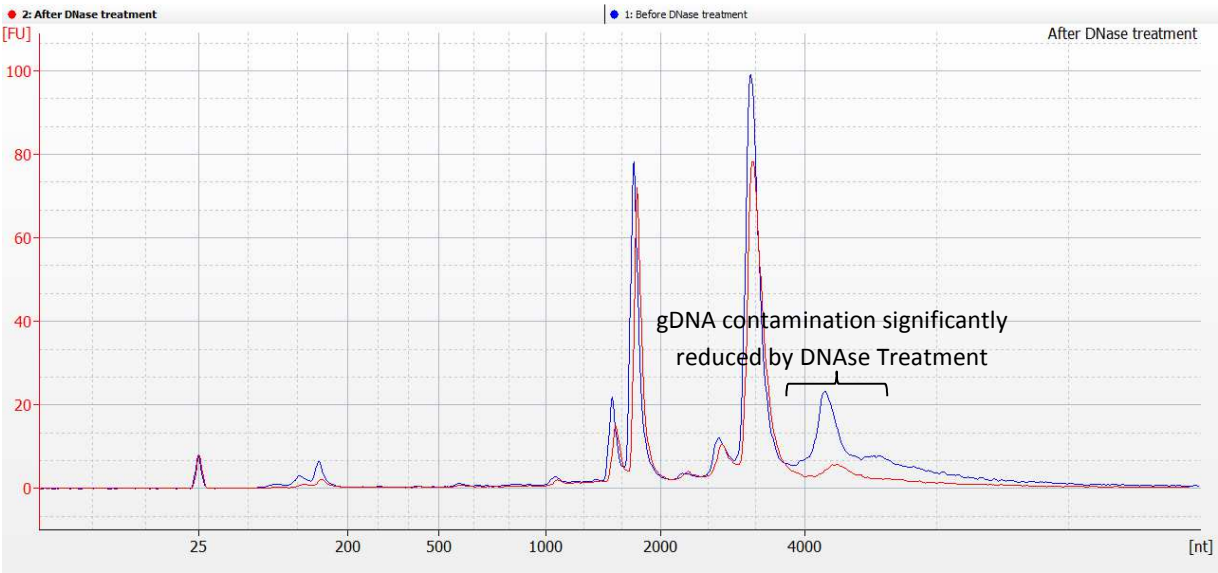
Troubleshooting Qubit readings: Ensure the following practices are followed when performing qubit analysis:

- Ensure bubbles are not introduced into the sample at the time of the reading as this can affect the results. Slight tapping on the tube wall or brief centrifugation will often help dissipate bubbles.
- If a concentration value as “too high” or “too low” is observed, this means that the sample is out of range. As needed, use a sample that is more concentrated or use a lower dilution.
- Ethanol precipitation or speed-vac can be used to concentrate samples. We recommended that the concentration for these samples be checked after the samples have been concentrated.
- The assay should be performed at room temperature and the assay tubes must be at room temperature at the time the reading is taken. The assay tubes should not be held in one’s hand for too long while in the process of reading the samples.

Troubleshooting possible Genomic DNA (gDNA) contamination (from BioAnalzyer trace):

- When there is suspected gDNA contamination, a 1µg aliquot of the RNA sample can be taken out and treated individually with DNAse to confirm (use RNAse-Free DNAse from Qiagen—Cat. No. 79254).
- Dilute the 1µg aliquot to 90µL total volume (using the H_2O that comes in the DNAse kit) in an RNAse-free 1.5mL microcentrifuge tube (such as the kind listed above for RNA QC materials).
- Take out 2.5µL from the 90µL, store in an RNAs-free tube and use as the “before DNAse” RNA portion.
- To the remaining 87.5µL, add 10µL RDD Buffer and 2.5µL DNAse (both supplied in the DNAse kit; the DNAse, once resuspended, can be stored at -20^{o}C in portions corresponding to typical needs).
- Vortex tube gently, spin down with a benchtop centrifuge and incubate for 10min at room temperature.

- After this DNAse treatment, use Qiagen's RNeasy MinElute Cleanup Kit (Cat. No. 74204) to re-purify the RNA sample from the enzymatic reaction by taking the following steps:
- Add 350µL buffer RLT (supplied in kit), mix well; add 250µL 100% ethanol (such as 111000200CSPP, from Capitol Scientific); vortex gently, spin tube and transfer all volume to filter tube (from RNeasy kit).
- Balance and spin filter tube at >10,000rcf for 15sec, transfer filter to new collection tube (CT) and discard the eluate from the filtration; add 500µL buffer RPE (from RNeasy kit; prepared by adding ethanol).
- Balance and spin filter tube at >10,000rcf for 15sec, transfer filter to new collection tube (CT) and discard the eluate from the filtration; add 500µL 100% ethanol
- Balance and spin filter tube at >10,000rcf for 2min, transfer filter to new collection tube (CT) and discard the eluate from the filtration; open filter tube
- Balance and spin filter tube at >10,000rcf for 5min, transfer filter to new RNAse-free 1.5mL microcentrifuge tube and discard the eluate from the filtration
- Dispense 14µL H_2O (from DNAse kit) directly to center of filter, close filter tube and incubate for 1min.
- Balance and spin filter tube at >10,000rcf for 1min
- Discard the filter tube and **keep the eluate (as this is the RNA)**; use as "after DNAse" RNA portion and compare to the "before DNAse" portion in a BioAnalyzer run (as described in "Sample Quality" section).
- It is recommended that the "before DNAse" RNA solution be diluted 1:2 and the "after DNAse" RNA solution be diluted 1:10 on the BioAnalyzer Pico assay (so as to be of comparable concentrations).
- If the "after DNAse" RNA portion shows complete elimination or significant reduction of peak (or broad hump), while rRNA peaks stay comparable in height and area—as below—it is likely that there is gDNA.



- Note that if there is such evidence of gDNA contamination, RNA sample(s) should not be sent to JGI, as this would not lead to successful QC, library creation, sequencing or assembly; troubleshooting of the RNA extraction process (and its DNAse step in particular) should be performed in such a case.

Troubleshooting the purity of the sample determined by Nanodrop:

- Dirty sample pedestal can lead to erroneous absorbance readings. Ensure that sample surface have been completely cleaned before starting the readings and also between samples.

Refer to Appendix C for further details from the references.

APPENDIX B

Preparing the RNA Ladder:

1. Spin the ladder down and pipette in an RNase- free vial.
2. Heat-denature the ladder for 2 min at 70 °C.
3. Immediately cool the vial on ice.
4. Prepare aliquots in 0.5 mL RNase- free vials (or in TempAssure PCR 8-tube flex-free strips) with the required amount for typical daily use.
5. Store aliquots at -70 °C. After initial heat denaturation, denaturation should not be repeated.
6. Before use, thaw ladder aliquots on ice (avoid extensive warming).

Setting Up the Chip Priming Station:

1. Replace the syringe:
    a. Unscrew the old syringe from the lid of the chip priming station.
    b. Release the old syringe from the clip. Discard the old syringe.
    c. Remove the plastic cap of the new syringe and insert it into the clip.
    d. Slide it into the hole of the luer lock adapter and screw it tightly to the chip priming station.
2. Adjust the base plate:
    a. Open the chip priming station by pulling the latch.
    b. Using a screwdriver, open the screw at the underside of the base plate.
    c. Lift the base plate and insert it again in position C. Retighten the screw.
3. Adjust the syringe clip:
    a. Release the lever of the clip and slide it up to the top position.

APPENDIX C: REFERENCES

1. Qubit:
    - (https://tools.lifetechnologies.com/content/sfs/manuals/mp32852.pdf)
    - (https://tools.lifetechnologies.com/content/sfs/manuals/mp10210.pdf) to become familiar with the protocol before setting up the assay.

2. Nanodrop :
    - T042 Technical Bulletin – Assessment of Nucleic Acid Purity: http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf
    - User Guide and further troubleshooting: http://www.nanodrop.com/library/nd-1000-v3.7-users-manual-8.5x11.pdf
3. Life Technology Quant-iT RNA BR Assay:
    - https://tools.lifetechnologies.com/content/sfs/manuals/mp10213.pdf