Small 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 small RNA samples to evaluate the quantity (using Qubit Fluorometer), quality (using electropherograms) and purity (using NanoDrop Spectrophotometer). We recommend all small 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 Small RNA Analysis Kit	Agilent (AATI)	DNF-470-0275
Bioanalyzer Small RNA Kit	Agilent	5067-1511
Equipment		
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 small 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 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 Quantiflouor 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 ±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

3.1. Fragment Analyzer small RNA kit or Bioanalyzer small RNA kit

Note: Qualitative sensitivity of small RNA kit is 25 – 2,500 pg/µL at microRNA region for Fragment Analyzer and 50 – 2,000 pg/µL for Bioanalyzer. A concentration higher than the range should be diluted down before loading.

3.1.1. Prepare the appropriate kit according to the manufacturer's guide (refer Appendix A)
3.1.2. Denature 2 µL of samples at 70°C for 10 min (or 1.6 µL of samples and 2 min denature for Bioanalyzer kit) on a thermal cycler or a heat block
3.1.3. Immediately store the denatured samples and ladder on ice
3.1.4. Mix 2 µL of samples or ladder with 18 µL of Diluent Marker in the Sample Plate (or 1 µL of samples load on Bioanalyzer chip)
3.1.5. Prepare Fragment Analyzer or Bioanalyzer according to the manufacturer's guide and run the Sample Plate or the chip
3.1.6. Review the data and assess RNA quality for each sample
- Figure 1 shows a typical small RNA electropherogram. The region of microRNAs (miRNA) is sometimes overlaid by degraded ribosomal RNAs (rRNAs) and the prominent transfer RNAs (tRNAs) peak is a good indicator of miRNA existence in the small RNA (sRNA) sample.
- Fragment Analyzer will show the size and concentration of both these sRNA (10-200 nt) and the miRNA regions (10-40 nt) as well as the % miRNA of the sRNA region in the Small RNA Summary tables as in Figure 2.

- Figures 3-5 show the various quality of total RNA run on Bioanalyzer small RNA kit and resulted smRNA-seq libraries.

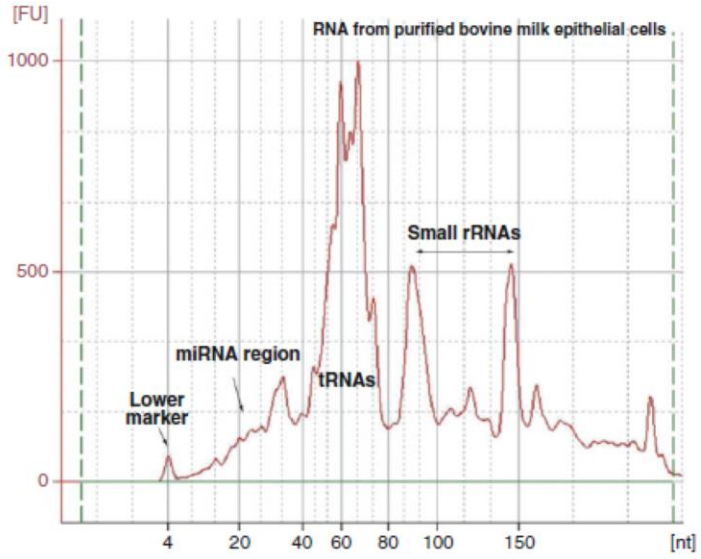

Figure 1. A diagram of small RNA electropherogram. The prominent transfer RNA peak (tRNAs) is a good indicator of miRNA existence.

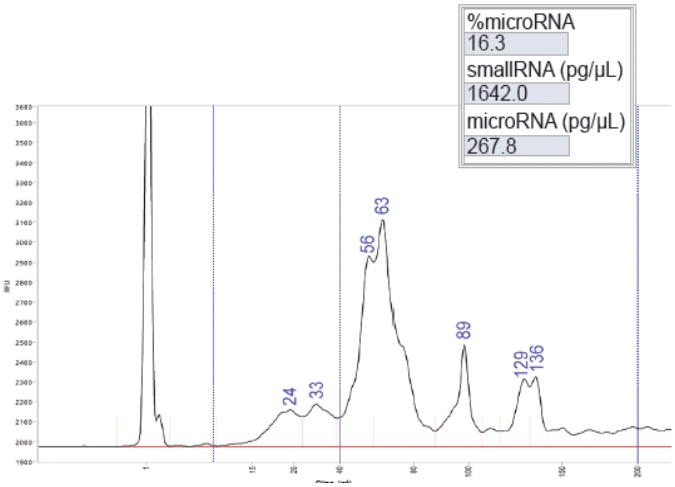

Figure 2. A typical good quality small RNA with 16.3% microRNA region.

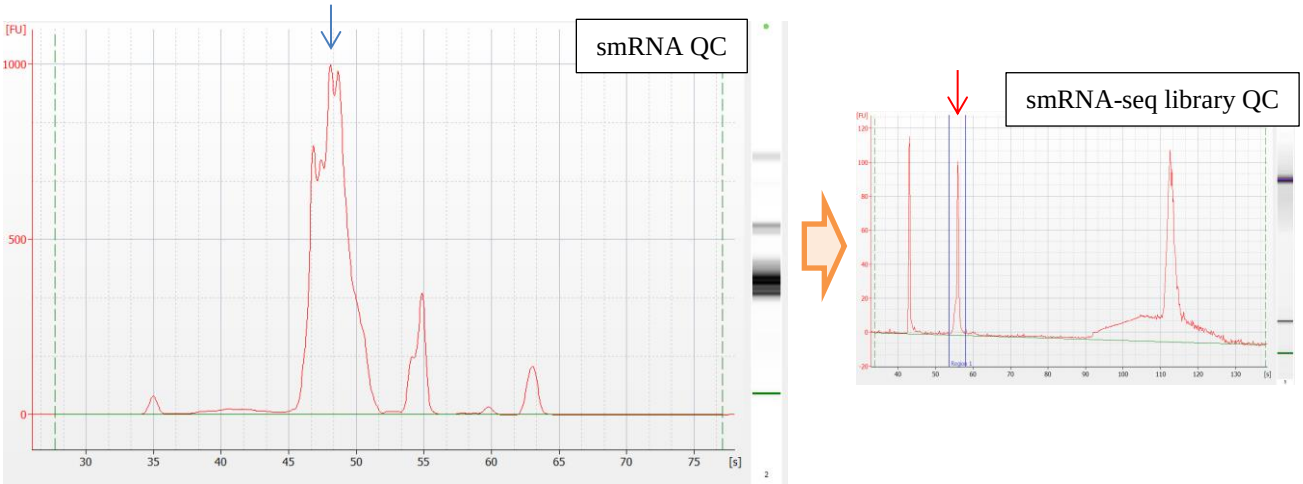

Figure 3. A good quality small RNA sample with prominent tranfer RNA peak (blue arrow) produced a good quality of smRNA-seq library at 142 bp (red arrow).

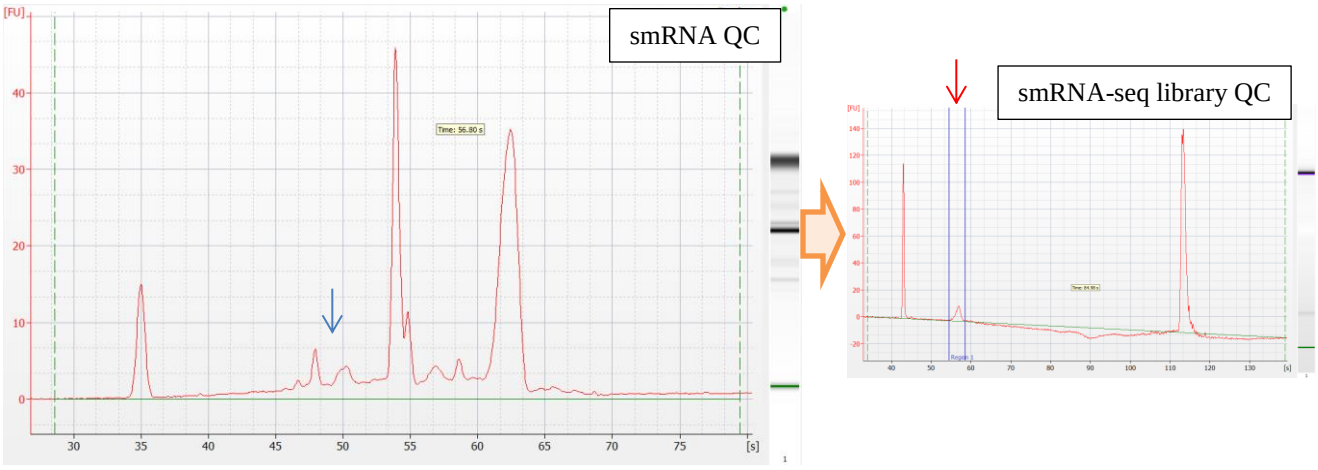

Figure 4. RNA sample lost transfer RNA peak (blue arrow) did not produce a successful smRNA-seq library (red arrow).

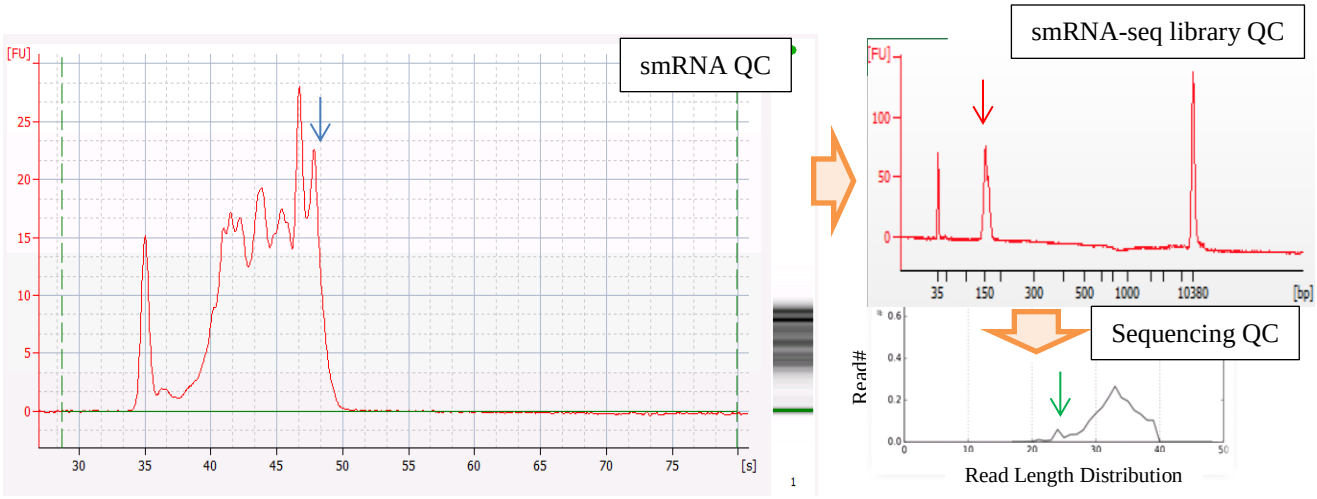

Figure 5. RNA sample showing a severe degradation with neither transfer RNA peak (blue arrow) nor rRNA peaks produced smRNA-seq library with high yield (red arrow). However, sequencing revealed that the library contained a small population of microRNA (green arrow) and majority degraded rRNA.

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 be 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 µL 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 µL 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 µL 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: 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:
   - Small RNA Kit: https://www.aati-us.com/documents/quick-start-guides/dnf-470/dnf-470-quick-start-guide-12-capillary-01-06-2016.pdf
   - Small RNA brochure: https://www.aati-us.com/documents/brochures/small-rna-brochure.pdf

5. Bionalayzer Small RNA Kit:
   https://www.agilent.com/cs/library/usermanuals/Public/G2938-90093_SmallRNA_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