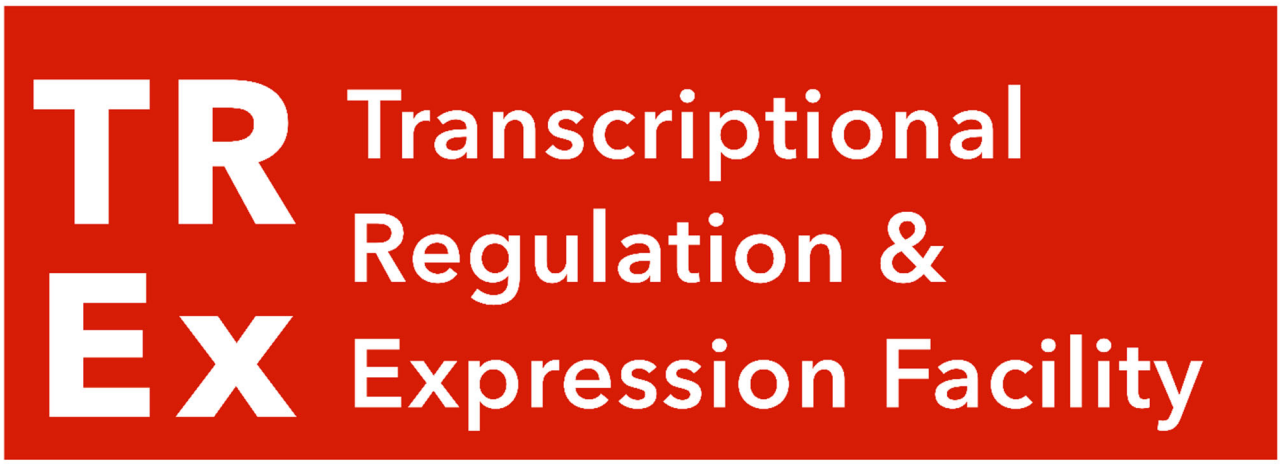
TREx Guide: Fragment Analyzer RNA QC

VERSION 1.2 (6/2020)

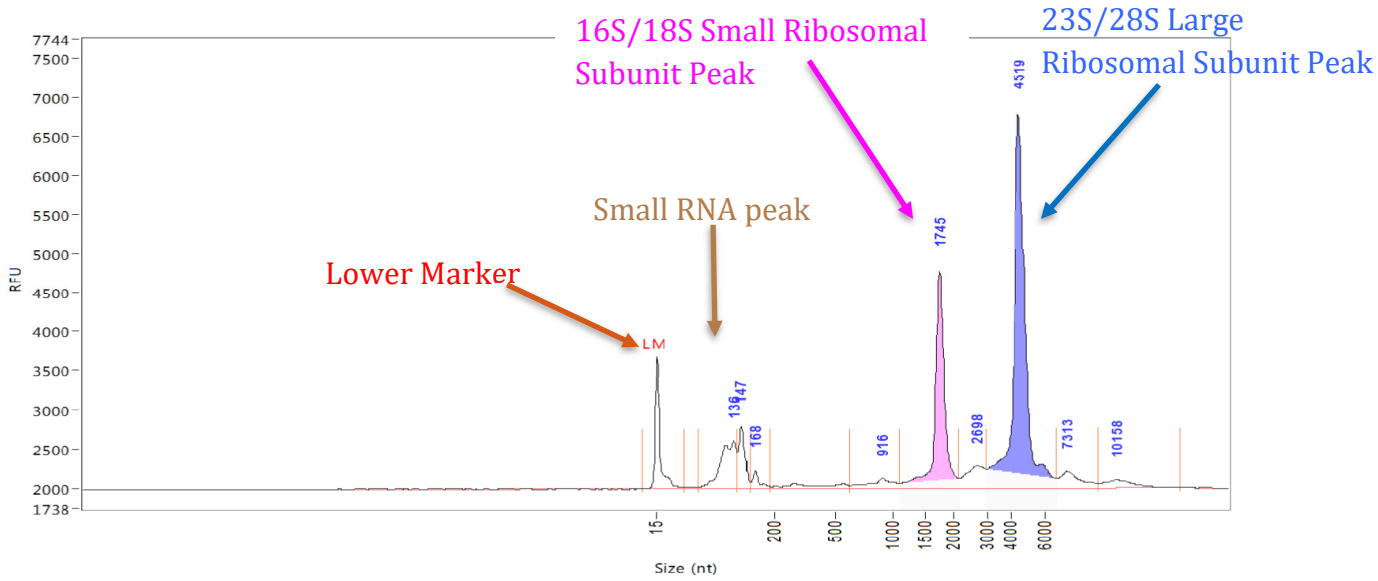


Background

Checking the integrity, or intactness, of RNA is an important step in determining how to proceed with an RNAseq experiment. At TREx, we use the Fragment Analyzer, through the BRC Genomics Core, in order to visualize RNA integrity. The Fragment Analyzer is a capillary gel electrophoresis instrument that generates an electropherogram showing both RNA fragment size and relative abundance. It also generates an RNA Quality Number (RQN) as a quantitative indication of RNA integrity.

Reading the QC Report:

RNA QC Trace (electropherogram)



- The X-axis reflects the size distribution of the RNA sample. The red line is the instrument baseline, and the black line (with peaks) is the sample.
- The y-axis reflects RFU: Relative Fluorescence Units, indicating the amount of RNA detected. RFU may change from run to run and should not be used as a comparison across multiple samples.
- The **LM (lower maker)** is spiked in for sizing purposes, but is not part of your sample.
- Distinct peaks or **'bumps' below 200 bases** are usually comprised of **small RNA**, mostly tRNA. Some methods of RNA isolation do not retain small RNA, so you may not see a bump. A large amount of material in this region, combined with a raised sample baseline, can indicate degradation (see "Degraded RNA").
- The **pink and blue peaks** represent the **small and large ribosomal subunit RNA** peaks.
- Vertical red lines delineate peaks identified by the software, which are quantified in the Peak Table. The blue numbers are the peak sizes (maximum RFU within the peak range).

Peak Table: Provides the quantitative measurements obtained from a Trace image.

Peak	Size (nt)	Conc. (ng/uL)	From (nt)	To (nt)	RFU	Rel. Conc. % (ng/uL)	Molarity (nmole/L)
1	15 (LM)	0.8982	0	61	611		151.5153
2	157	26.1357	82	225	397	19.0	556.6465
3	1927	29.7901	1635	2461	1391	21.7	47.6051
4	3313	6.9502	2461	3532	158	5.1	7.2129
5	5412	70.1255	3532	7813	2605	51.0	40.2614
6	8508	4.3940	7813	10282	89	3.2	1.5512

TIC:	137.3955	ng/uL
TIM:	653.2771	nmole/L
Total Conc.:	155.4798	ng/uL
28S/18S:	2.3	
RQN	9.7	

Peak Table Terms:

- **Peak:** Numbers the peaks in the electropherogram from left to right, including the LM
- **Size:** tallest point of the peak, **not necessarily** the average size of the peak.
- **Conc.:** Concentration in ng/uL per peak. Note: use this number with caution! May be useful as a relative quantification but is not reliable for absolute quantification.
- **From and To:** The start and end nucleotide length per peak.
- **RFU: R**elative **F**luorescence **U**nit per peak. RFU's change from run to run and should not be used as a comparison across samples.
- **Relative Conc.:** Relative concentration of that peak number, represented as a percentage of the total concentration.
- **Molarity:** Molarity of the area under the curve per peak. May be useful as a relative quantification but is not reliable for absolute quantification.
- **TIC: T**otal **I**ntegrated **C**oncentration in ng/µL. Concentration of all detected peaks, excluding the LM. *Note: we do not consider this number an accurate estimate.*
- **TIM: T**otal **I**ntegrated **M**olarity in nmole/L. Molarity of all detected peaks, excluding the LM. *Note: we do not consider this number an accurate estimate.*
- **Total conc.:** Total concentration in ng/µL of sample, excuding the LM. This estimate does include regions between peaks. Useful as a relative quantification.
- **28S/18S** or **16S/23S:** Ratio of small and large ribosomal peaks (indicated in pink and blue).
- **RQN:** (**R**NA **Q**uality **N**umber) is an indication of the integrity of the RNA on a scale from 1-10, with 10 being totally intact RNA and 1 highly degraded RNA.

*******The RNA Sequencing Core recommends that you use the QC file primarily as an indication of RNA integrity and relative size distribution. The absolute concentrations given by this instrument **may not be accurate**. The total concentration of an RNA sample is best determined using a **Nandrop or a Qubit** assay.*******

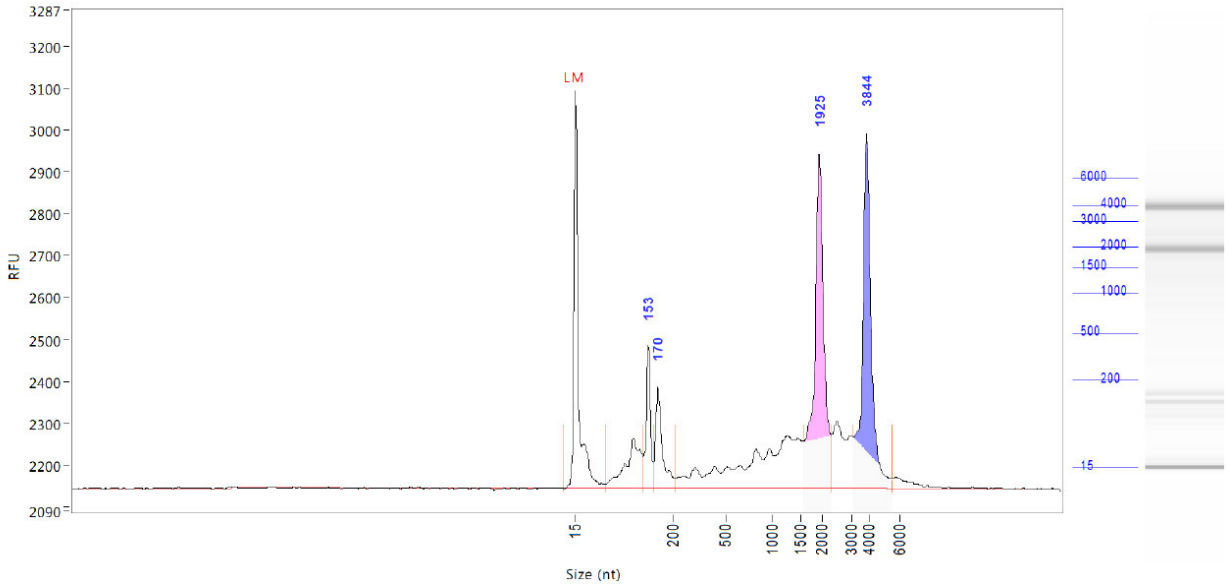
Implications of RNA QC Results

Based on what is seen in the RNA QC trace, we can determine how to proceed with RNAseq. Specifically, it informs which method to choose for sample enrichment: poly-A selection or ribosomal depletion.

Poly-A Selection

Poly-A selection enriches for the mRNA by binding the Poly-A tail of mRNA and washing away everything else. This method **requires intact RNA,** as any fragments that break off from the poly-A tail will be washed away leaving only the 3' end. A sequencing library made using Poly-A selection on a degraded sample will therefore have significant 3' bias, or may fail entirely.
We define intact RNA on the Fragment Analyzer as RNA with an RQN higher than 7.

Example Trace for Intact RNA



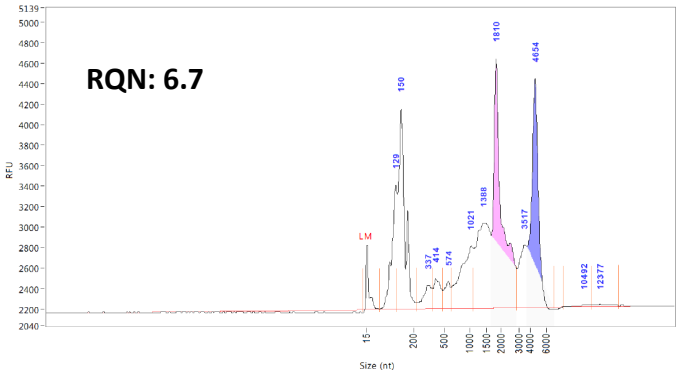
Elements of Intact RNA

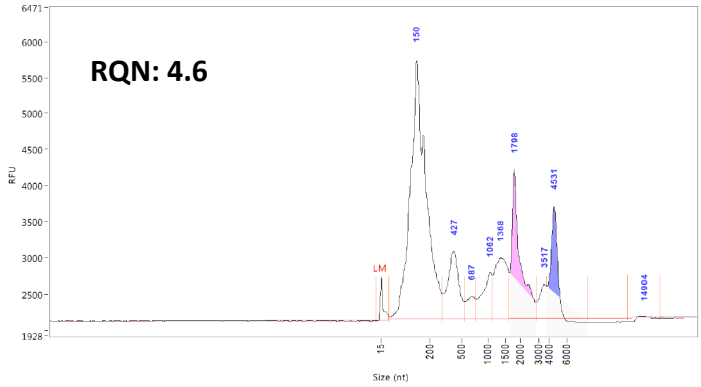
- Sample baseline (black line between the small RNA peak and the rRNA peaks) is low, close to the instrument baseline (red)
- Small and Large Ribosomal subunits form sharp spikey peaks
- Area under small RNA peaks is relatively small compared to ribosomal peaks

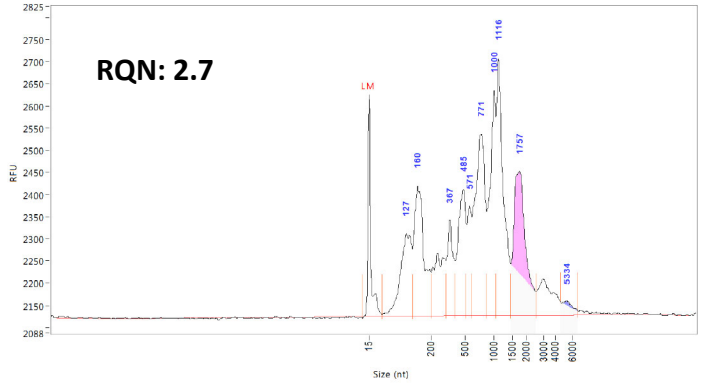
Ribosomal Depletion

Ribosomal Depletion removes ribosomal RNA by binding single stranded DNA probes to rRNA sequences and removing them. This process, while not as efficient at removing rRNA as the Poly-A method, is better suited for RNA that is degraded as it does not introduce a 3' bias. Ribosomal depleted RNAseq libraries typically contain non-coding RNAs as well as mRNAs (and residual rRNA). It is important to note that this method is highly species specific, so it is important to check if there is a depletion kit for your organism of interest before proceeding. **TREx recommends using a ribosomal depletion method for samples with an RQN<7.**

Examples of degraded RNA:







Elements of Degraded RNA:

- Raised sample baseline
- Small and Large Ribosomal Subunits form rounded peaks, often with shoulders on the low side.
- Area under small RNA peaks is large compared to ribosomal peaks
- In highly degraded samples, small and large ribosomal subunits may become unrecognizable

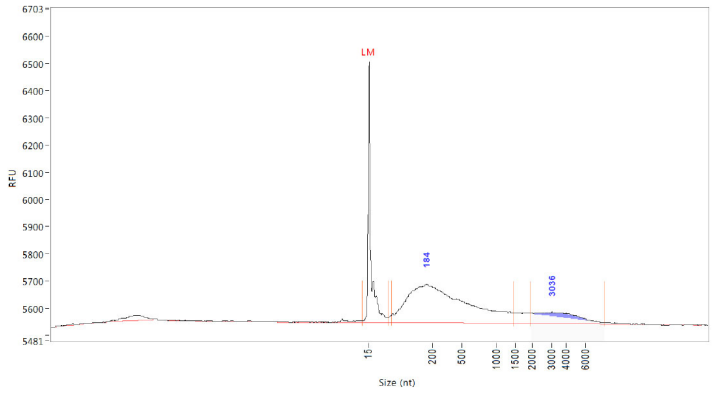
Troubleshooting: Unique Sample Types

While most Fragment Analyzer traces will look something like the ones you see above, certain types of samples tend to perform differently. We have detailed a few of the most common examples below

FFPE Samples:

Samples extracted from FFPE are known to be highly degraded and tend to have very low RQN scores. DNA contamination cannot easily be distinguished, as it will also be degraded.

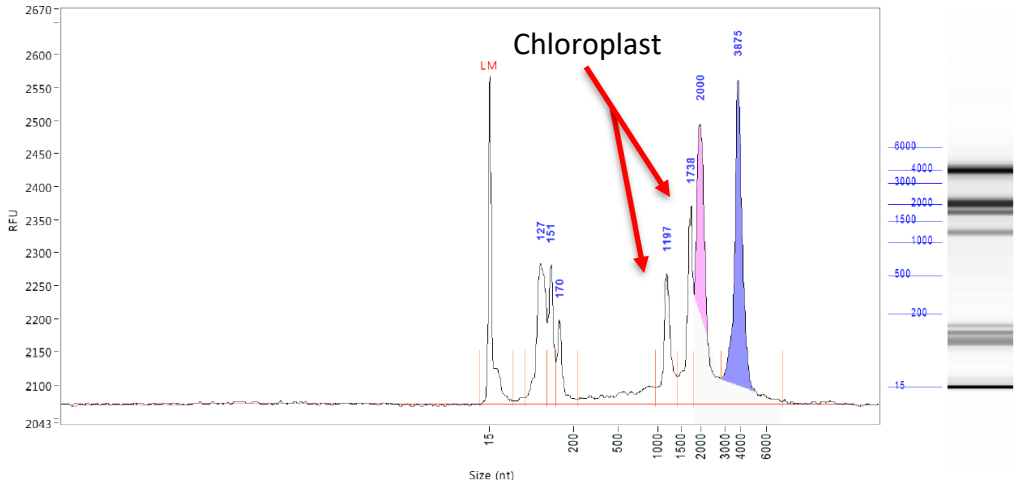
Example FFPE Sample:



Plant Samples:

Plant chloroplast RNA can show up in RNA traces and, if present, will falsely decrease RQN.

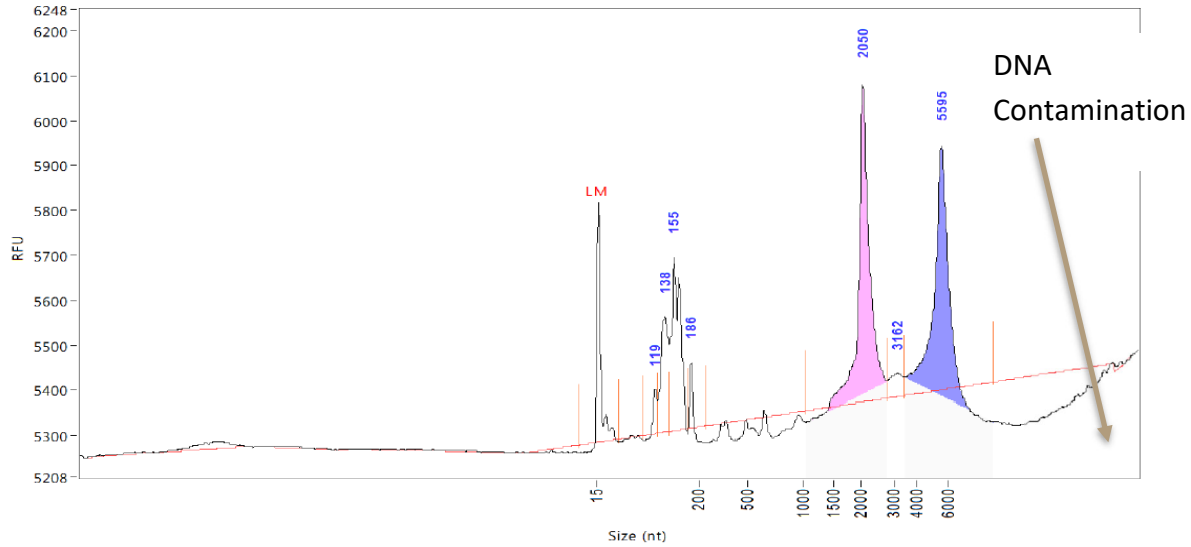
Example Plant RNA



Troubleshooting: DNA Contamination

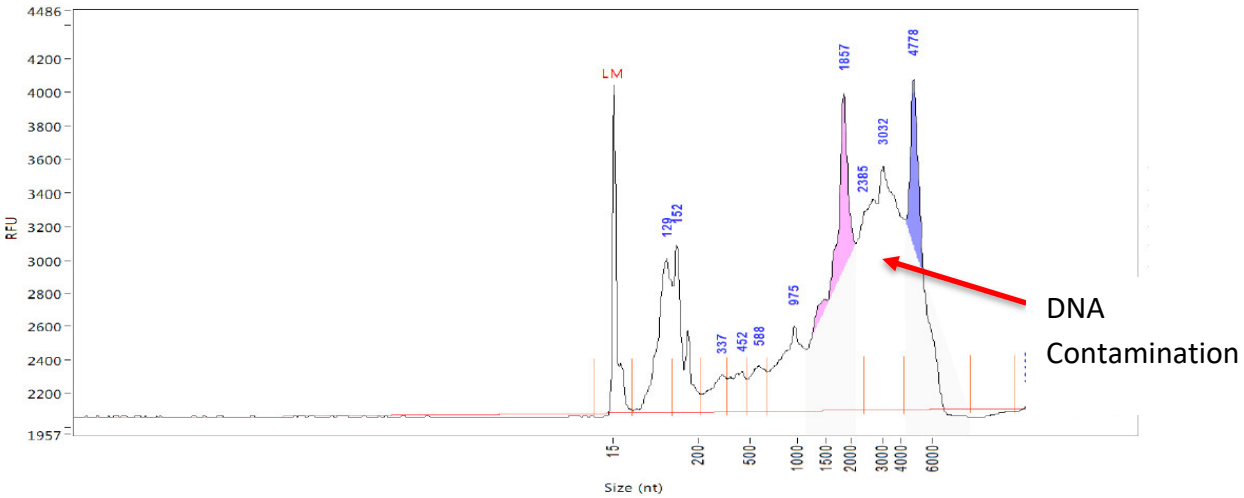
Genomic DNA is a common contaminant in RNA preps and it can often be detected in the QC trace. In eukaryotic RNA samples, gDNA appears as a smooth bump near or above 6000bp.

Example Genomic DNA in Eukaryotic RNA sample



In prokaryotic RNA samples, gDNA may appear between the small and large ribosomal subunits.

Example Genomic DNA in Prokaryotic RNA sample



If there is DNA in your RNA sample it **WILL** be carried through the RNAseq library prep and ultimately sequenced, which contributes to 'background' reads. We recommend a **DNAse treatment** to remove any DNA contamination before starting RNAseq library prep (may require cleanup or buffer exchange).

Troubleshooting: Low Signal and Machine Errors

While the Fragment Analyzer generally runs correctly, occasionally something will happen with the machine that requires a sample re-run. We have detailed the most common reasons for this to happen below.

No Lower Marker (LM):

Occasionally the LM will not be recognized by the machine or will not appear. This can throw off the sizing of a sample and a re-run should be requested.

No Signal:

If no signal is seen, it is possible that the sample was not added into the well and a re-run should be requested. If the re-run also fails, it is possible that the sample was not quantified correctly and quantification should be checked.

Very Low Signal:

If very low signal is seen, the accuracy of the RQN will be thrown off. If the sample was run on a standard sensitivity run, it should be diluted into the range of the high sensitivity run (1-5ng/ul) and submitted for a re-run. If it was already run in high sensitivity mode, the sample can be assessed on a Femto QC run.

Bubbles:

The Fragment Analyzer is a capillary instrument, and sometimes bubbles form. If you see a baseline that is not completely horizontal, or has ‘steps’ in it, a re-run should be requested if the bubble interferes with sample quality interpretation.

Positive Control:

Occasionally QC runs can give false results (indicating degradation) because of bad kit reagents. When a ‘ladder’ is run in parallel with your samples, the quality of the ladder may be useful as a positive control. Alternatively, samples run in parallel with high RQN can act as a positive control for kit reagents and run quality. If all samples (and ladder) in a run show degradation, the QC trace may not indicate the quality of the primary sample because of instrument problems or bad reagents.