How does the Bioanalyzer work?

The bioanalyzer instrument detects biomolecules by laser-induced fluorescence. Each LabChip® contains an interconnected set of microchannels that is used for separation of nucleic acid fragments based on their size as they are driven through it electrophoretically. During chip preparation, the channels of the chip are filled with the gel-dye mix. Then, twelve samples and ladder with marker are loaded in each well. During the chip run, the dye intercalates directly with the analytes (DNA and RNA assays) or with SDS-micelles (protein assays) and all bands pass the detector at different speeds. The software automatically compares the unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify the ribosomal RNA peaks.

How to Assess the Total RNA Data

1. RNA Ladder Results

The RNA 6000 ladder standard is run on every chip from a specified ladder well and is used as a reference for data analysis. The RNA 6000 ladder contains six RNA fragments ranging in size from 0.2 to 6 kb (0.2kb, 0.5kb, 1.0kb, 2.0kb, 4.0kb, and 60.kb) at a total concentration of 150ng/µl. The software automatically compares the unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify the ribosomal RNA peaks. The ladder also serves as a built-in quality control measurement of system performance under standard conditions.

Major features of a successful ladder run are:
- 6 RNA peaks (s/w calls for 5 ladder peaks only)
- 1 Marker peak
- All 7 peaks are well resolved

Note: Peak ratios and peak heights for the RNA ladder may vary from one batch of RNA 6000 ladder to the next. Assay performance will not be affected by this variation.
2. Total RNA Sample Results

The bioanalyzer electropherogram of total RNA shows two distinct ribosomal peaks corresponding to either 18S and 28S for eukaryotic RNA or 16S and 23S for prokaryotic RNA and a relatively flat baseline between the 5S and 18S ribosomal peaks.

Major features for a successful total RNA run are:

- Two ribosomal peaks: Any mRNA migrating between the ribosomes will be smooth and lack distinct peaks.
- The baseline between 29 seconds and the 18S ribosomes is relatively flat and free of small rounded peaks corresponding to smaller RNA molecule. Depending on the RNA extraction method, the small 5S, 5.8S and tRNA may be present in the electropherogram from 24-27 seconds.
- 1 marker peak

3. Ribosomal RNA ratio and baseline for the quality assessment of RNA sample

In general, the height of the 28S ribosomal peak should be twice that, or at least equal to the 18S ribosomal peak. Variability in this ratio may indicate partial degradation of the sample by ribonuclease contamination during the purification procedure. In the case of complete sample degradation these bands will disappear. However, ribosomal ratios will vary according to the species and tissue type as well according to the RNA extraction method. For a specific tissue, similar values should be found so that after establishing quality criteria for a specific sample type, the ribosomal ratio can be used as a quick check for RNA sample quality.

Beside the ratio, the baseline is a kind of trademark and could use as a secondary parameter for the RNA quality. If you have a flat baseline, your RNA is OK. The more peaks visible (and higher background) between the two ribosomal bands and below the 18S (16S) band, the worse the sample quality. In the lower part of the electropherogram around 24 to 29 seconds, 5S RNA and transfer RNA can be seen because they cannot be separated on the bioanalyzer. Typically, trizol isolations do not remove 5S and tRNA, while many column-based RNA extraction kit do remove these small RNA species. In Eukaryotic cells the 5S/tRNA should represent 5 to 10 % (maximum) of the 18S and the 28S. This population of small fragments is composed of several population from 5S to 8S and the
profile is tissue and cell specific. In some Prokaryote cells, there is 20% of messenger and 30 to 40% of 5S/tRNA (information from Agilent Technology Inc).

4. The Degraded Total RNA

RNase degradation of total RNA samples produces a shift in the RNA size distribution toward smaller fragments and a decrease in fluorescence signal and the 18S and 28S peak can no longer be identified with certainty (figure A). Degraded total RNA will lack a smooth baseline and typically contains multiple peaks that are as large or larger than the ribosomal peaks. The most highly degraded products have a migration time between 22 and 24 seconds. With more severe degradation (figure B), the spectrum shifts entirely toward early migration times.

5. Contamination in Total RNA

Example 1:
The peak seen here migrating between 24 and 29 seconds is most likely tRNA, 5S ribosomal RNA, or a combination of both.

Example 2:

The bump running just after the 28S ribosomal peak is genomic DNA contamination. It is suggested a DNase1/RNase free treatment to get rid of this genomic DNA contamination.

How to Assess the mRNA Results

1. mRNA Sample Results
   Messenger RNA should be checked for degradation as well as significant levels of ribosomal RNA contamination. Intact poly (A)+ RNA samples display broad size distributions. The 18S and 28S peak can no longer be identified with certainty.
Major features for a successful mRNA run are:

- Broad hump: Intact poly (A)+ RNA samples display broad size distributions (as described above) and the size range can be estimated by overlaying the RNA 6000 ladder containing RNA fragments of known size (0.2kb, 0.5kb, 1.0kb, 2.0kb, 4.0kb and 6.0kb)
- 1 marker peak

2. Degradation of mRNA

As with total RNA, RNase degradation of mRNA shifts the RNA size distribution toward smaller fragments. The Figure above shows that Poly (A)+ RNA (60 ng/µL) from cultured Jurkat cells was incubated for 15 minutes at room temperature with very dilute RNase A (1 x 10^-6 and 2 x 10^-6 mg/mL, resp.). Samples (1.0-µL aliquots) were analyzed using the RNA 6000 LabChip kit (Data from Agilent Technology Inc).

3. Ribosomal RNA Contamination in mRNA Preparations

Too much ribosomal contamination can artificially inflate the measured concentration of starting mRNA template. The Agilent 2100 bioanalyzer automatically calculates the percent ribosomal RNA in mRNA samples and can detect as low as five percent contamination. Ribosomal RNA contamination in mRNA preparations is easily identified by the presence of ribosomal RNA peaks.
How does RNA quantitation on the bioanalyzer compare to RNA quantitation on UV?

In an Agilent Technology application note, results from the bioanalyzer are compared to results generated with UV and ribogreen. In most of the cases, a good match is found between the 3 methods for RNA quantitation. In some cases, a deviation from the UV value was observed (i.e. > 20% difference from UV value), the bioanalyzer showed most of the time a lower value than UV. The explanation for this phenomenon is as follows: RNA preps can contain varying amounts of phenol, genomic DNA and other matrix components that also absorb at 260 nm. If a sample contains one of these components, the UV value will be higher than the value from the bioanalyzer, which only detects RNA with the intercalating dye (similarly to detecting RNA via ribogreen).