Interpretation of Total RNA Integrity Profiles

A successful gene expression study depends upon high quality RNA to construct an RNA-Seq library. Degraded RNA, or contaminated samples, typically result in libraries of low complexity and transcript detection bias that can negatively impact data analysis; especially if samples with varying degrees of degradation are directly compared in a gene expression study. However, the amount to which results are biased, and potentially useful, is still debated with methods for degraded or low-input samples under development\textsuperscript{1,2}. The DNACF strongly recommends each RNA sample undergo an RNA integrity check prior to library construction to reduce potential issues. Although RNA QC is relatively straightforward with the use of an automated capillary electrophoresis system such as the Advanced Analytical Fragment Analyzer, the interpretation of the results can require subjective interpretation which benefits from experience. The following comments provide a background on interpreting RNA integrity profiles, guidelines for selecting RNA samples, and examples of unique integrity profile characteristics.

**Characteristics of an Intact RNA Profile**

The RNA profile in Figure 1 illustrates high quality total RNA. Total RNA integrity assays do not directly measure messenger RNA (mRNA) but infer that the mRNA is intact by observing the integrity of the abundant ribosomal RNA. Several criteria are used to assign an RNA Quality Number (RQN) across a range of 1-10 (degraded RNA being a score of 1 and intact RNA being a 10). The main criteria are 1) the presence of discrete 18S and 28S rRNA bands, 2) the mass ratio between the 28S and 18S rRNA, 3) the absence of fragments in the pre-18S and 28S regions, and 4) absence of contaminating high molecular weight fragments.

Intact eukaryotic RNA should display sharp bands for both the 18S and 28S rRNA peaks. A mass ratio of 2:1 between the 28S and 18S rRNA is an indicator of an intact RNA since degradation is initially observed with a decline in the 28S peak. Although a ratio of 2:1 is rarely observed, an RNA sample with a calculated ratio >1.8 is considered intact.

The absence of peaks preceding the 28S and 18S peaks is also a strong indicator of intact RNA. As RNA degrades, peaks in this region will accumulate and increase as the 28S and 18S peaks...
diminish (Figure 2). Also absent should be any contaminating gDNA that is typically observed as a high molecular weight peak above the 28S peak. DNase treatment is strongly recommended as part of any extraction method. The presence of small RNA bands, typically non-coding RNA <200 nucleotides, will depend on the extraction method used. Column based methods, such as the Qiagen RNeasy kit, may exclude RNA species under 200 nucleotides. Care is necessary in selection of an extraction method to ensure inclusion of all desired RNA species.

Extraction Methods Make a Difference

Different tissues types can present a challenge to isolating intact RNA. For example, multiple factors play a role in extracting high quality RNA in sufficient quantity from plant tissue. Plant samples are typically composed of a diverse set of tissues with varying metabolite levels (e.g., polysaccharides and phenolics) as well as containing elevated levels of RNase predominately located in vacuoles. Recently reported modifications to Trizol and CTAB methods were shown effective in extracting RNA from a diverse group of plant tissues. Trial extractions are recommended to ascertain the RNA quality and yield achievable before making a final method selection for any study. Evaluation of several isolation methods from non-model plants provide practical recommendations for isolating RNA to be used in transcriptome studies.

Low RQN Score

“How low is too low for a RQN score?”, is a question often asked of core staff. The quick answer is that a RQN score of 7.0 is our facility threshold for preparing libraries. There are, however, times when scores < 7.0 occur and additional samples cannot be obtained. Samples with RQN scores between 5.0 and 7.0 (Figure 2) will produce libraries that can yield useful analysis for an experiment. Several factors should be taken into account when considering such samples. Reduced library complexity is often a concern for low RNA quality samples. A decline in mappable reads and increased duplication rates typically occur with lower RQN scores. A decline in detection of a certain portion of transcripts can also occur. It is important to consider that different transcripts are degraded at different rates. In addition, bias can exist in terms of evenness of read coverage across the length of a transcript as well as 5’-to-3’ coverage bias. Understanding these factors is important in being able to normalized samples and carry out analysis.

Finally, a highly degraded sample (RQN score < 4), such as FFPE derived RNA, does present the problem of how best to construct a library. A poly A enrichment method as used in the TruSeq mRNA stranded method will have a very pronounced bias in data as previously described. Libraries constructed with an rRNA depletion strategy have yielded improved results for low quality RNA samples. Care should be taken to choose a library construction method to match the RNA quality.

Figure 2. RNA integrity profile with an RQN score of 5.3 illustrating results observed with a partially degraded RNA sample.
Unique RNA Profiles

Insects have a unique RNA profile which may be misinterpreted as RNA degradation when first observed\(^6\). (Figure 3.a). The 28S rRNA consists of two fragments in most insects which are held together by hydrogen bonding. Under denaturing conditions, these fragments disassociate and co-migrate with the 18S peak. The resulting profile may be misinterpreted without an awareness of the unique interaction of the 28S rRNA fragments.

Plant RNA can also generate an atypical eukaryotic profile due to the unique presence of chloroplast rRNA from leaf tissues. The 16S and 23S rRNA peaks precede the 18S peak which can be perceived by the algorithm as degraded RNA. The Brassica rapa RNA sample shown in figure 3.b has an RQN score of 6.4 but the score clearly underestimates the RNA quality. The discreet 25S and 18S band and absence of a noisy baseline are indicative of high quality RNA.

References


