

Technical Note: RNA Quality Control

Introduction

The increasing popularity of microarrays and real-time PCR for gene expression profiling has created the need to isolate high quality total RNA from a wide variety of clinical and basic research samples. While methods for the physical isolation of total RNA have evolved significantly over the last two decades, there has been limited advancement in the methodology used for comprehensively assessing RNA quality. It is therefore important for investigators to understand what the currently used quality control methods actually measure, the limitations of these techniques and the ramifications that limited RNA quality assessment has on these gene expression analysis methods.

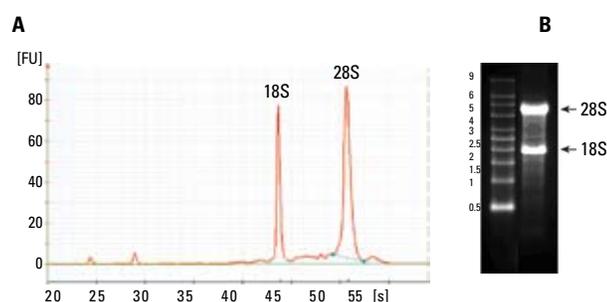


Figure 1: RNA Integrity. An RNA preparation from human breast cancer cell line MDA231 was separated on an Agilent BioAnalyzer [Panel A] and on an agarose gel [Panel B]. Crisp 28S and 18S rRNA peaks or bands indicate intact RNA. A 2:1 intensity or mass ratio between 28S and 18S rRNA is considered a benchmark for intact RNA.

Integrity: Size of ribosomal RNA

Strand integrity, as measured by size, is still as critical to a successful expression analysis by microarray or PCR as it was for Northern blot analysis. This characteristic is easily evaluated with gel-based RNA size fractionation. However, gel analysis requires 1- to 5- μ g quantities of total RNA, an extraordinary amount of material to be consumed for just a quality assessment. In many cases, this amount would be the majority or entirety of the RNA yield from a single sample.

Methodology for assessing RNA integrity has made significant progress, especially in the scale of material required to perform the analysis. Microcapillary electrophoresis using instruments such as the Agilent BioAnalyzer is becoming more commonplace since it has dramatically decreased the amount of RNA needed to evaluate integrity down to the sub-microgram scale. As long as the 18S and 28S rRNA bands produce well-defined bands or peaks without any smearing towards a smaller size, the sample is considered acceptable for further analysis (Figure 1).

A260/A280 Ratio: Protein Contamination

The most common reason for an RNA sample to fail the integrity criterion is contamination with RNase. A contaminated sample can result from either an inefficient nucleic acid extraction or contamination from an exogenous source or both. Since RNase is a protein, ultraviolet (UV) spectrophotometry at the 280 nm wavelength, based on the absorbance by the side chains of certain amino acids such as tyrosine and tryptophan, has long been used as a criterion for assessing contamination of RNA samples throughout the development of molecular biology.

This measurement may be taken at the same time as the quantification of RNA at the 260 nm wavelength. However while 280 nm is the absorbance peak for protein, the full UV spectrum of protein absorbance still shows a significant absorption at 260 nm, and the reverse is true for RNA. Therefore, general protein contamination, not just RNase, can add to the apparent absorbance of the RNA sample inflating its concentration as calculated from the 260 nm absorbance measurement. Investigators using A260 measurements alone may be misled into believing that they have higher RNA yields.

Thus, a ratio of the absorbance at 260 nm vs. 280 nm provides an essential quality control measurement accounting for spectral cross-contamination between the two biological materials. A 260 nm absorbance value that is twice the magnitude of the 280 nm absorbance indicates that there is no detectable protein contamination of the RNA sample.

A260/A230: Other contaminants

Microarrays and PCR measure signal generated by a reaction of mRNA with one or more enzymes and their substrates. Enzymatic manipulation greatly increases or amplifies the number of copies of the original mRNA to a point where the signal is easily detected. Processing RNA samples under identical enzymatic conditions is critical to the accuracy of these gene expression profiling methods. However, the presence of undetected contaminants can easily affect these reactions.

There are a wide variety of contaminants, from both endogenous and exogenous sources, that can have one of two deleterious effects on the enzyme activities utilized by microarrays and real-time PCR. First, contaminants can directly impede or inhibit the enzymatic activities used by these analysis techniques thus decreasing the signals being generated. Alternatively, the contaminating compounds may also absorb UV light artificially increasing the RNA sample's apparent concentration and yield.

A simple gene-specific normalization of the final expression analysis data cannot adequately account for all the differences in signal affected by enzyme inhibition. Recognizing that some of these contaminants also absorb light in the UV spectrum, the ratio of the absorbance at the 260 and the 230 nm wavelengths is now often being used to further assess RNA quality. A clean RNA sample should yield a value of ideally around 2.0 or generally at least greater than 1.7 (Figure 2). However, unlike the A260/A280 ratio which is principally effected by proteins and nucleic acids, the A260/A230 ratio is subject to wider fluctuations depending on the contaminant and the spectrophotometer used. Therefore, the A260/A230 ratio is a less precise parameter than the A260/A280 ratio.

In this way, UV spectrophotometry has become the principle method for assessing potential nuclease or other contamination as well as the amount of RNA within the samples.

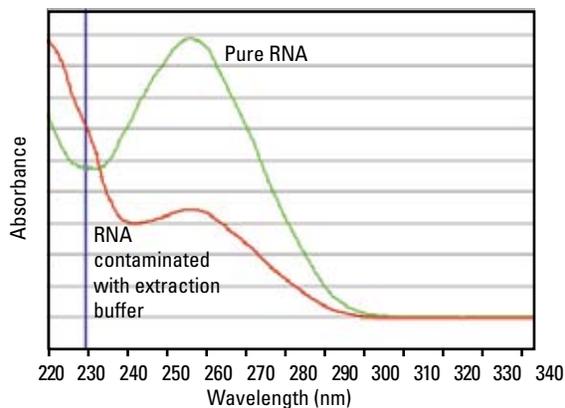


Figure 2: RNA Quality Assessment by Spectrophotometer. The absorbance spectra of a pure RNA sample and an RNA sample intentionally spiked with extraction buffer were analyzed on a UV spectrophotometer. The spectrum for the contaminated RNA sample shows a higher absorbance at A230 characteristic of contamination with guanidinium-based extraction buffers.

Contamination: Sources and their removal

Contaminants from endogenous sources can include DNA, porphyrin heme, the amino acid derivative melanin and some carbohydrates with structures that mimic a nucleic acid backbone. These contaminants co-purify with RNA to varying extents during isolation procedures and their levels differ among different tissues or cell types. Their presence can also be enhanced by inefficient extractions that result when the amount of biological material added to the lysis buffer exceeds the capacity of the buffer volume used. Re-extracting the RNA using a different isolation technique, in the former case, or the same procedure, in the latter case, often solve these common contamination problems.

Exogenous sources of contamination are generally introduced to the RNA sample as a component of the RNA isolation procedure and can include phenol, guanidinium ions, diethylpyrocarbonate (DEPC) or its contaminants from DEPC-treated water as well as excess salts and other chemicals. Since these compounds are intentionally introduced into the sample, a properly executed isolation protocol using RNase-free water instead should remove them from the sample by the end of the protocol.

The list of contaminants presented here is by no means a complete list making the development of a more comprehensive RNA quality control and assessment protocol difficult. After assessing whether your RNA samples pass the integrity and quality checks listed here, we suggest proceeding to the expression analysis method of your choice. Note that in the case of microarray systems, quality and yield can be further assessed at the level of the labeled nucleic acid before proceeding with microarray hybridization. The performance of your RNA or labeled nucleic acid in the actual gene expression assay can serve as another indicator of RNA quality. Built-in housekeeping gene controls are one indicator used in gene expression assays for RNA quality assessment.

Conclusion:

RNA samples being analyzed in any gene expression analysis system must be of equivalent quality and must be processed equally efficiently in every step of the procedure. This is a critical key to any gene expression analysis system. If this assumption is not met, gene expression changes reported by the system may result from handling, storage and processing of the sample, rather than from the experimental or physiological variables under study. It is therefore of the utmost importance, given the sensitivity of RNA to degradation, that samples be handled and isolated in an identical manner from the moment that they are first removed from their biological matrix whether that be from piece of tissue, blood from an animal, or cells from their media.

While virtually all investigators take extreme care to ensure that the microarray or real-time PCR phases of their experiments are conducted identically, many often overlook the need for that same level of care at the sample harvesting and preparation steps. While there is growing attention within the scientific community to the importance of high quality RNA handling procedures, a universally comprehensive RNA quality assessment to determine whether a standard has been met by every sample has not yet been formulated.