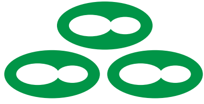
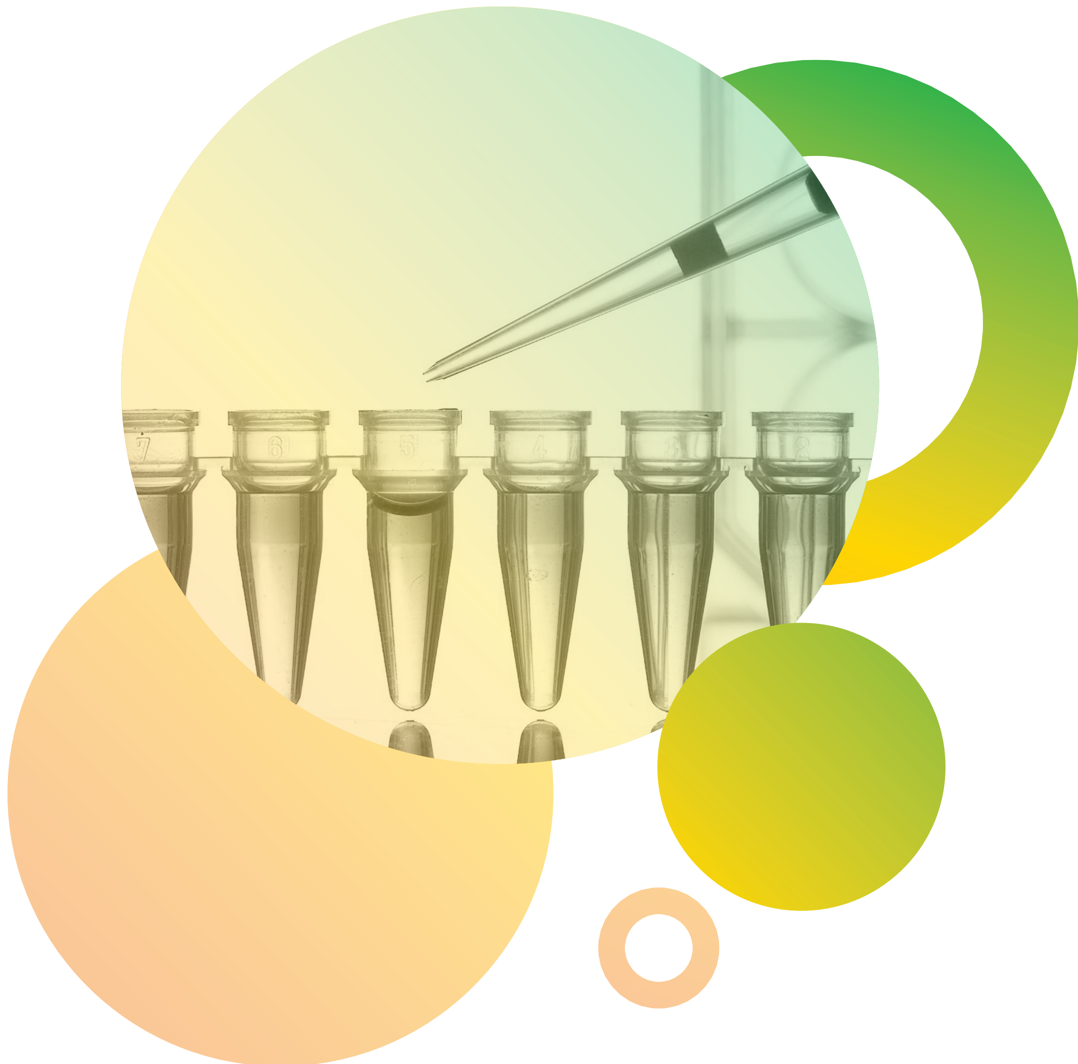



# Considerations for Maximizing the Quality of RNA-Seq Libraries and Sequencing Data



RNA-sequencing (RNA-seq) has become an increasingly popular technique for transcriptome profiling. The preparation of high-quality RNA-seq libraries is critical to the integrity and reliability of the sequencing data. In this article, we discuss some tips and tricks to help maximize the quality of RNA-seq libraries.

## Preserve RNA integrity

The integrity of the input RNA is critical for generating RNA-seq libraries that yield high-quality data. Degraded RNA can bias measurements of gene expression, provide uneven gene coverage, and prevent differentiation between alternatively spliced transcripts. Thus, it is recommended to use high-integrity RNA as input whenever possible. To achieve high-integrity RNA inputs, it is essential to reduce the activity of RNases, a class of enzymes that can degrade RNA quickly. To reduce cellular RNase activity, extract RNA from samples immediately upon collection. If temporary sample storage is needed, preserve the samples in a stabilization solution such as DNA/RNA Shield™ or TRIzol®, as the lytic properties of these products deactivate cellular RNases effectively. Use sterile filter tips during the entire workflow to minimize RNase and cross-sample contamination. Keep purified RNA on ice while in use and store at -80°C in aliquots to reduce freeze-thaw cycles.

## QC the input RNA

It is a good practice to perform quality control on the input RNA before proceeding to library preparation. Integrity and purity are two main factors to look at.

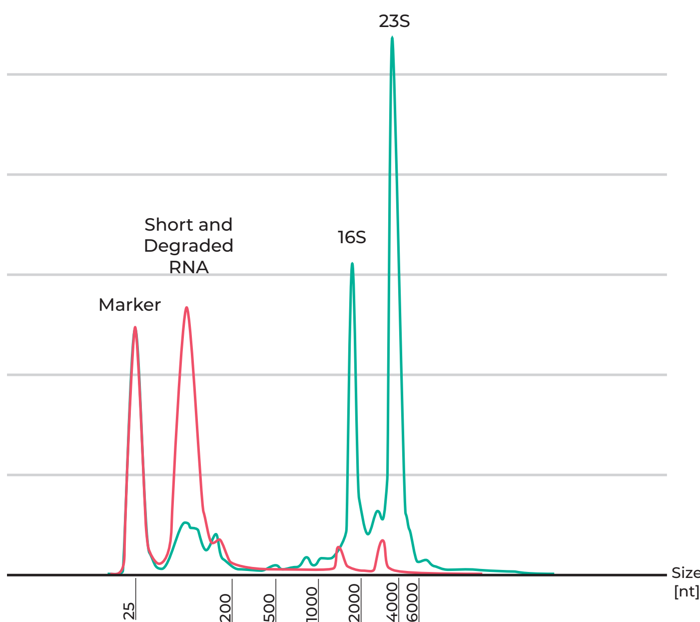



**Figure 1.** High quality RNA is indicated by the presence of two slow-moving bands (blue). In contrast, degraded RNA is indicated by an accumulation of short, fast-moving fragments (red).

The integrity of RNA can be evaluated using electrophoresis. High quality RNA will have two prominent, slow-migrating bands representing the rRNA fragments (e.g., 16S and 23S for bacteria). Smearing and a prominence of smaller fragments indicates RNA degradation (Figure 1).

Instruments like Agilent's TapeStation® make this QC check simple by calculating a metric based on the extent of degradation, called RNA Integrity Number (RIN). RIN scores range from 1 (lowest quality) to 10 (highest quality). For certain sample types, like formalin-fixed paraffin-embedded (FFPE) sections, RNA quality is inherently poor. Some kits, like Zymo-Seq RiboFree® Total RNA Library Kit, include modified protocols to accommodate degraded RNA inputs.

The purity of the input RNA can be evaluated using a UV-Vis spectrophotometer such as a NanoDrop™ to ensure the RNA is free from contaminating phenol and chaotropic salts. Ideal A260/A280 ratios for pure RNA range from 1.8-2.0, and ideal A260/A230 ratios from 2.0-2.2. However, avoid using NanoDrop™ for quantification since free nucleotides and contaminants can bias the data. Instead, quantify RNA using a more specific method, such as Qubit® fluorometric quantification, which uses dyes specific for ssDNA, dsDNA, and RNA.

Additionally, DNase I treatment of the input RNA is recommended, as the remaining DNA can interfere with RNA-seq library preparation, consume sequencing reads, and bias gene expression analysis. DNase I treatment can be performed as part of RNA extraction or afterwards as an additional cleanup with a kit such as RNA Clean & Concentrator™.

## Deplete rRNA or enrich RNA of interest

Maximize the efficiency of your sequencing run by depleting ribosomal RNA (rRNA) or enriching for the RNA species of interest. Ribosomal RNA typically makes up more than 80% of a cell's total RNA. Depending on the species, depletion can lower the presence of rRNA in final libraries to as little as <1%, while increasing protein-coding transcripts >5-fold. Virtually all RNA-seq library preparation kits on the market include steps to remove rRNA or enrich for an RNA species of interest such as mRNA.

## Perform bead clean-up carefully

Solid Phase Reversible Immobilization (SPRI) beads are commonly used in RNA-seq workflows to purify and select for certain sized fragments. Attention to detail is critical for SPRI bead clean-up steps to ensure a good recovery of anticipated products. Prior to use, equilibrate the bead solutions to room temperature for 30-60 minutes. Additionally, homogenize the

bead solutions by inverting and vortexing. These steps ensure effective size selection by maximizing bead binding capacity and improving volume aspiration accuracy.

Extra care should be taken when drying the beads. Avoid over drying the beads as it can reduce yields, while under drying the beads runs the risk of ethanol carryover which can inhibit enzymes in the subsequent steps. Optimally dried beads appear as uncracked mud without gloss (Figure 2, middle tube). Since differences in temperature and humidity between regions, labs, and seasons can influence the optimal drying duration, a test run to identify the optimal drying duration is recommended.

## Avoid excess cycles of PCR during amplification

PCR amplification is a major source of bias in RNA-seq. Though this step is unavoidable in most library preparation kits, the bias can be minimized by avoiding excess cycles. Follow the recommended PCR cycles in the library preparation protocol. When such guidance is not available, perform a titration using control inputs to pinpoint the necessary cycle numbers that produces sufficient library for sequencing.

## Perform Library QC

The size distribution and quantity of the final libraries should be determined before sequencing. Visualize the final libraries with a TapeStation®, Bioanalyzer® or traditional agarose gel. Ensure no adapter dimers are present, and if so, remove them with an additional size-selection bead clean up. Quantify the libraries using a qPCR-based method (e.g., quantification kits from NEBNext® or KAPA®) to ensure the most accurate quantification going into sequencing, as cluster density is crucial to the quality and success of sequencing.

**Figure 2.** Over-dried beads are cracked and flakey, resembling dried mud. Under-dried beads are glossy and wet, like saturated mud. Optimally dried beads appear damp, but lack gloss.

RNA-seq is a cutting-edge technology that has been gaining popularity. Just like any technique, there is plenty of room for errors when preparing a library for RNA-seq. The tips and tricks mentioned above can help minimize the chance of introducing such errors. The next time you prepare RNA-seq libraries, consider these points to maximize quality and ensure the most reliable, cost-effective data.

**To learn more about RNA sequencing, please visit**
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