Best practices for RNA-seq success, part I: Optimizing sample prep

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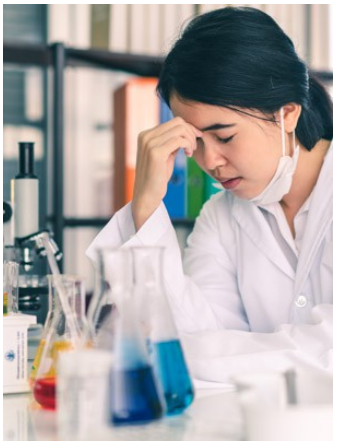
BIO VIEW

To all NGS newbies and sequencing savants alike...welcome! In this article, you'll learn tips and tricks on how to achieve robust and reliable RNA-seq data, starting with RNA sample prep. How do I prevent my RNA from degrading after I collect it? What is the best purification kit for my research application? When is it necessary to perform rRNA depletion prior to cDNA synthesis? All these questions and more will be answered in our blog! Written in collaboration with our Technical Support Scientists, Drs. Marianne Rivkin and Bakhyt Zhumabayeva. Special thanks to them and Dr. Peiyong Huang, manager of the Technical Support team.

> "Research is so unpredictable. There are periods when nothing works and all your experiments are a disaster and all your hypotheses are wrong."
>
> —Francis Collins, former director of the NHGRI and project head of the Human Genome Project

Let's face it. Research really *is* unpredictable. A scientist may spend hours planning experiments and adopting different analysis strategies depending on the organism being studied and their research goals… only to start all over again if even *one* thing goes wrong. RNA-seq is certainly no exception.



There is no optimal, "one-size-fits-all" experimental template for the variety of different applications and analysis scenarios in which RNA-seq can be used. Every RNA-seq experiment could potentially have different optimization methods for transcript quantification, normalization, and ultimately differential expression analysis. However, all RNA-seq workflows can essentially be boiled down to 3 steps: RNA extraction and sample preparation, cDNA synthesis and library preparation, and finally, data analysis.

While each step of the experimental setup is of critical importance to get the most reliable results possible, sample prep stands at a pivotal point in experimental success.

1 RNA sample extraction and stabilization

While RNA extraction is a notoriously daunting and oftentimes frustrating task, it is not impossible. As RNA can be unstable and highly susceptible to degradation, **the number one priority is to get the RNA out and stabilized as quickly as possible** (ideally at the time of collection). Once that has happened, downstream storage and processing are much more manageable. Common methods for sample stabilization include snap freezing with liquid nitrogen, dry-ice ethanol baths, immediate storage in a -80°C freezer, or submerging the sample in a stabilization reagent meant to inactivate nucleases. However, these approaches have drawbacks, such as freeze-thaw damage of nucleic acids or inhibiting/interfering with cDNA synthesis. We instead recommend immediate solubilization in a lysis buffer that contains RNase inhibitors (i.e. the RNA Lysis Buffer included in our purification kits).

2 RNA sample purification

The wide variety of RNA purification methods available can make it difficult to decide which one to use. Two very important points to keep in mind while making your selection are 1) choose the most suitable kit for your starting material (quantity and source, e.g., plant, tissue, mammalian cells), and 2) make sure your kit is compatible with downstream cDNA synthesis and sequencing.

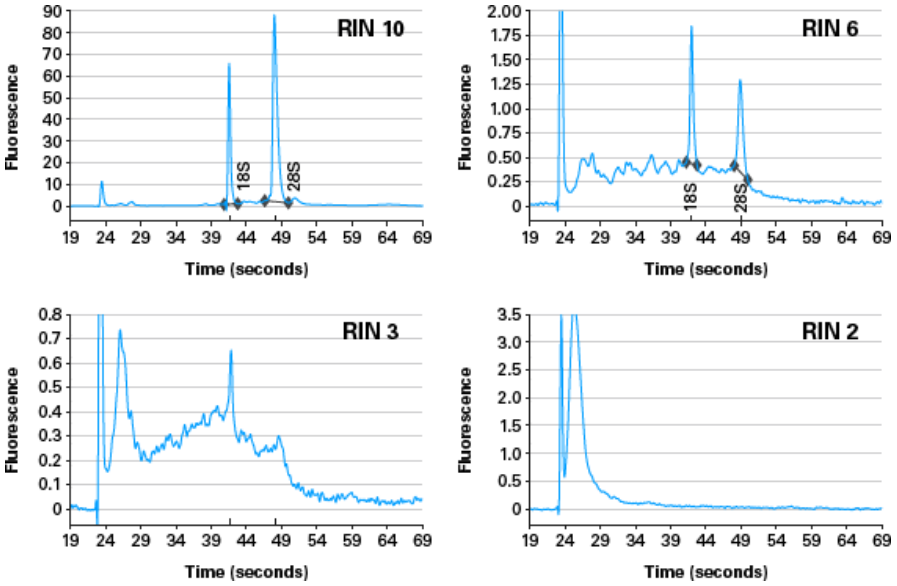
Choosing the right kit for your starting material is extremely important as certain cell types are harder to lyse than others (e.g. cells with cell walls, such as plant cells). Check out our RNA purification kit finder to help make your selection easier.

NOTE: RNA isolated from compromised cells may have higher DNA contamination, including single-stranded DNA that may not be removed completely during bulk RNA purification – therefore compromised RNA may require additional DNase treatment followed by column purification. The use of carrier RNA or co-precipitants is **not** recommended since it will interfere with downstream cDNA synthesis.

3 RNA sample quantity and integrity analysis

It is extremely important to accurately quantify and assess the integrity of your RNA sample prior to library construction as it helps to inform your primer selection and downstream strategy. We recommend determining the RNA integrity using the RNA Integrity Number (RIN) estimated by the Agilent 2100 Bioanalyzer system.

Randomly-primed cDNA synthesis kits are compatible with RNAs of various RINs (as instructed in the respective user manuals of each kit). However, if opting for a kit that uses oligo(dT) priming to initiate first-strand synthesis, the RNA sample should have a RIN value of ≥8 to ensure selective and efficient full-length cDNA synthesis. High-quality mammalian total RNA should show very distinct 28S and 18S ribosomal bands corresponding roughly to 4.2 kb and 2.0 kb, respectively.



Example Bioanalyzer electropherograms of RNA samples with different RINs, from highest (RIN 10) to lowest integrity (RIN 2). Source: Agilent Technologies application note: RNA Integrity Number (RIN)-Standardization of RNA Quality Control.

RNA concentration can be determined via the Agilent 2100 Bioanalyzer using one of the following assay kits below, depending on the volume of total RNA or mRNA sample available for measurement:

- Bioanalyzer RNA 6000 Pico Kit — for sample volumes as little as 50 pg of total RNA/mRNA
- Bioanalyzer RNA 6000 Nano Kit — for sample volumes as little as 5 ng of total RNA/mRNA

RNA concentration can also be determined either via an equivalent microfluidic device such as a fluorometer (e.g. Qubit) or a UV/Vis spectrophotometer (e.g. Nanodrop). Both work slightly differently and have their own strengths and weaknesses that can be summarized in the table below.

Qubit fluorometer	Nanodrop UV/Vis spectrophotometer
Prior preparation with compatible reagents is required, meaning each sample read will come at a small cost	All measurements are performed on a pedestal; no reagent preparation or the use of standards is required, saving time and money
Measures nucleic acid concentration	Can measure both nucleic acid concentration and purity. The 260/280 and 260/230 ratios give an indication of how pure the sample is from protein and salt contaminants
Sensitivity - can detect minute concentrations of nucleic acids (≥10 pg/µL); uses sample volumes from 1 – 20 µL	**Sensitivity** - covers a sample concentration range of 2 ng/µL to 15 µg/µL; uses 0.5–2 µL of sample
Specificity - reagents enable specific binding to the nucleic acid of interest, meaning concentrations are not affected by the presence of other contaminants (best for mixed RNA/DNA samples)	**Specificity** - sample readings are very prone to the presence of contaminants. For example, both DNA and RNA absorb at 260 nm, therefore having both in the same sample can overestimate concentration readings

4 Primer selection

To initiate reverse transcription for cDNA synthesis, reverse transcriptases require a short DNA oligonucleotide called a primer to anneal to the RNA template and serve as a starting point for the synthesis of a complementary cDNA strand. Depending on the RNA template integrity and the downstream applications, you have two choices available: oligo(dT) primers and random primers. The differences between both can be summarized in the table below.

While a random priming approach of first-strand cDNA synthesis allows for the analysis of both coding and non-coding RNAs, an oligo(dT) priming approach captures only polyadenylated RNAs, representing primarily coding RNAs. Oligo(dT) priming is not ideal for compromised or degraded RNA samples (as compromised RNA often lacks the poly(A) tail), or in the case of isolating non-polyadenylated transcripts such as lncRNAs. For compromised RNA, a random priming approach is more suitable; however, it requires the removal of rRNA sequences, as rRNA can be randomly primed and incorporated into the final library, which is undesirable (see step 5, "rRNA depletion").

Random primers	Oligo(dT) primers
The primer mixture consists of oligonucleotides with random base sequences	The primer mixture usually consists of a stretch of 12-18 deoxythymidines (dTs) and may contain up to 30 dTs
DNA synthesis using reverse transcription of high-integrity eukaryotic and prokaryotic coding and non-coding RNAs, as well as compromised RNAs	First-strand cDNA synthesis is performed by the reverse transcriptase
No template specificity	Specificity for poly(A) tails of eukaryotic mRNA
Anneals to any RNA species in the sample	Anneals to polyadenylated RNA sequences
Reverse transcription of any RNA template; regardless of polyadenylation/integrity	Reverse transcription of only polyadenylated RNA

5 rRNA depletion

Ribosomal RNA (rRNA) is a type of non-coding RNA which is the predominant form of RNA found in most cells; it makes up to 90% of cellular RNA despite never being translated into proteins itself. Ribosomal depletion allows for efficient detection of functionally relevant coding as well as non-coding transcripts through the removal of highly abundant rRNA species while leaving other transcripts of interest intact. We currently offer a variety of options for specific depletion of mammalian rRNA.

For 10–100 ng samples of mammalian total RNA, we recommend using the RiboGone - Mammalian kit for rRNA depletion. This kit can be used in combination with the SMARTer Stranded RNA-Seq Kit or SMARTer Universal Low Input RNA Kit. We also offer kits incorporating rRNA removal into the main RNA-seq workflow: the SMARTer Stranded Total RNA Sample Prep Kit - HI Mammalian for RiboGone-based rRNA removal, as well as the SMARTer Stranded Total RNA-Seq Kits - Pico Input Mammalian for the removal of rRNA sequences from pre-amplified cDNA.

Once complete, rRNA depletion may be validated on an Agilent 2100 Bioanalyzer system (the 18S and 28S peaks should no longer be visible).

NOTE: As mentioned previously, this is a requisite step only if you decide to use random primers for cDNA synthesis or have compromised

RNA as your sample input. This is not a required step if you decided to take the oligo dT priming route.

Next steps

Okay, you have isolated and purified your input RNA, and have ensured its quality and integrity – now what? Well, sample prep is only a third of the battle. Lucky for you, we are here to help you throughout your entire RNA-seq experiment. In the next part of this blog series, we discuss cDNA synthesis and library preparation. Sign up to be notified of when we publish the next installment!

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