

RNA LEXICON

CHAPTER #5

DNase: To Treat or Not To Treat



In an RNA sequencing experiment, the quality of the RNA used for library generation is of the utmost importance. Put another way, high quality RNA will have the best chance of leaving the researcher with high quality data. There are many instances where researchers may find themselves in a place where high quality, high purity RNA starting material is impossible, such as working with FFPE samples (this will be covered in a future Lexicon chapter). Thus, we can think of this through yet another lens: *getting the most from what you have*. Whether you are working with FFPE samples or cell lines, the goal is to get the most from what your starting material can offer. In many cases, DNase treatment is a key step in maximizing the quality of the RNA samples used for sequencing.



With few exceptions, any RNA sample will contain some amount of contaminating genomic DNA (gDNA). It is not surprising then, that many RNA extraction kits recommend, or even require DNase treatment of RNA samples before proceeding to challenging downstream applications, such as transcriptome analysis.

In RNA-Seq applications, random primers or short oligo(dT) primers cannot distinguish between RNA and DNA, and will also hybridize to residual gDNA. In addition, reverse transcriptases are promiscuous enzymes which are able to use DNA as template molecule. As a result, unwanted gDNA will be channeled through the entire RNA-Seq workflow, which can cause biases and quantification issues during the final data analysis steps. Therefore, it is critical to remove any residual gDNA to obtain the best quality data.

1. Genomic DNA Removal Methods

The most common means of DNA removal is by DNase digestion. DNase, short for Deoxyribonuclease, is a DNA-specific endonuclease that cleaves single- and double-stranded DNA, leaving behind 5' phosphorylated oligonucleotide products. Because of this versatility it is used in a wide range of biological applications. It is important to note that DNase should be removed afterwards as residual DNase may also affect downstream reactions in the library preparation.

1) On-column DNase Treatment (during Extraction)

On-column digestions are commonly used during the RNA extraction procedure. In these methods, the lysate is loaded onto a column where a filter substrate binds the RNA and contaminant DNA. A series of wash steps and an incubation step with a DNase containing solution are used to digest genomic DNA leaving behind your intact RNA. The RNA is then washed before it is finally eluted off the column.

Column digestions are among the most commonly used methods, though they are not without their pitfalls. The two main drawbacks – on-column digestion increases the time requirement of the RNA extraction and can decrease the RNA quality in case contaminants are not efficiently removed prior to the DNase treatment step. Performing the treatment on column-bound DNA can also result in residual gDNA carry-over. Despite these downsides, column digestions are frequently performed due to their ease of use and accessibility.

2) Acid Phenol Extraction of RNA

Acidic buffered phenol / chloroform extraction is an established method used commonly to minimize carry-over of gDNA. It is used in labs all around the world as it yields high-quality, high-purity RNA. For many samples, no additional DNA digestion is needed after acid phenol extraction. For more details see our previous chapter on RNA Extraction.

3) DNase I Digest after RNA Extraction

DNase treatment can also be done in solution following RNA extraction. As opposed to the column-based method described above, DNase I digest in solution is commonly accepted as a more efficient and thorough way to eliminate gDNA from an RNA sample. Routinely, extracted RNA is mixed with DNase and reaction buffer and incubated either at room temperature or 37 °C for 15 min to 1 hour. Following incubation, the DNase is inactivated or removed by one of the clean-up methods described below to stop the reaction and purify the RNA for further processing.

2. DNase Clean-up Methods

Remaining DNase should be removed from the sample before the experiment proceeds. If DNase is carried over into library preparation, primers initiating reverse transcription may be degraded ultimately affecting the efficiency of the library generation. The method of DNase clean-up is perhaps just as important for the sample quality as the use of DNase in the first place. To minimize DNase presence in the final RNA sample there are a number of options available, each with their own strengths and weaknesses.

1) Purification

Column- or bead-based purifications and ethanol precipitation are widely used to remove not only DNase itself but also the reaction components. Using a clean-up leaves you with the pure RNA sample eluted in water or buffered TRIS solution. This allows further processing of the sample without any interference from the reaction components used in the previous DNase digest.

Column-based clean-up is quick and easy method to purify nucleic acids after enzymatic reactions. The reaction is mixed with binding buffer and directly loaded to the column. The intact RNA is bound to the filter substrate while proteins, i.e., DNase, short DNA oligonucleotides and residual buffer components are washed away before the RNA is finally eluted.

When working with many samples, bead-based purification can be used to increase the number of samples that can be handled at a time or even automate the clean-up. In this method, nucleic acids are bound to the surface of specific magnetic beads, while short fragments, proteins and buffer components remain in the supernatant and are removed. The beads can be collected using magnets, and are washed before the nucleic acids, in this case the cleaned-up RNA, is eluted.

Ethanol precipitation is another common clean-up method: The reaction mix is precipitated in presence of ethanol and salt (commonly LiCl is used for RNA precipitation) and if needed a carrier is added, e.g., glycogen. The pellet is washed to remove reaction components before it is resuspended in buffer or water.

Ethanol precipitation is often used for precious samples as it preserves the valuable sample by minimizing material loss.

Even though purification methods are the clear choice for obtaining the cleanest possible sample, other methods of DNase inactivation are often preferred due to convenience, cost- and time-savings.

2) Heat inactivation

Using a brief incubation at elevated temperatures is yet another popular method of inactivating DNase. While heat inactivation does not remove reaction components, the simplicity of the method is its major benefit, requiring only 5 minutes at ~75 °C. However, due to the temperature and buffer conditions in the reaction, the RNA can also be fragmented easily. As RNA integrity is of utmost importance for RNA-Seq, heat inactivation should be avoided especially when working with lower quality material.

3) EDTA Chelation

The RNA fragmentation associated with heat inactivation as described above can be mitigated by the addition of EDTA. While this sounds good at surface value, special care should be taken with the amount of EDTA added to the reaction. Too much EDTA can chelate the divalent metal ions required for enzyme activity in reverse transcription, a crucial step in RNA-Seq library preparation.

4) Proteinase K

An additional method for DNase inactivation is Proteinase K treatment. While Proteinase K is particularly effective at digesting proteins and thus deactivating DNase, Proteinase K itself needs to be removed to ensure that the enzymes required for subsequent reactions are not inactivated by Proteinase K carry-over.

3. Detecting gDNA in your Sample

When is DNase treatment required for your RNA sample and how can you decide if the gDNA really was efficiently removed? As you can imagine, due to the similarities between these nucleic acids, detecting gDNA in an RNA sample is difficult. But there are a few methods that can be used to determine whether or not gDNA is present:

- 1 Optical Density (OD) measurements using a spectrophotometer and comparing emissions at different wave lengths can give you an indication of the purity of your RNA sample, e.g., an OD 260/280 value below 2 can indicate DNA contamination.
- 2 Some spectrophotometric assays using fluorescent dyes specific for either RNA or DNA can distinguish between RNA and DNA within a sample.
- 3 Agarose gel electrophoresis can also show gDNA in the high molecular weight region of the gel (Fig. 1A).
- 4 Running the extracted RNA on a Fragment Analyzer using extended run time settings can reveal gDNA contamination in a similar way to that of the gel above: a high molecular weight “bump” in the trace indicates the presence of gDNA in the sample (Fig. 1B).

Most of these methods only detect rather high levels of gDNA presence in a sample. Unfortunately, RNA-Seq is so sensitive to gDNA contamination that the amount required to negatively impact the process falls below the detection threshold of some of the methods described above. This is a primary contributing factor to the popularity of DNase treatments in RNA-Seq workflows.

The best approach to detect residual gDNA and ensure the RNA preparation is indeed fully DNA-free is to run a PCR or qPCR with primer pairs for a specific set of marker genes. Commonly referred to as house-keeping genes, there are some popular genes such as GAPDH, genes encoding RNA-polymerase subunits, actin, rDNA loci, and others. As PCR can detect trace amounts of DNA from as low as one molecule, PCR is the method of choice to exclude gDNA contamination in workflows that require highly pure RNA that needs to be absolutely free of DNA.

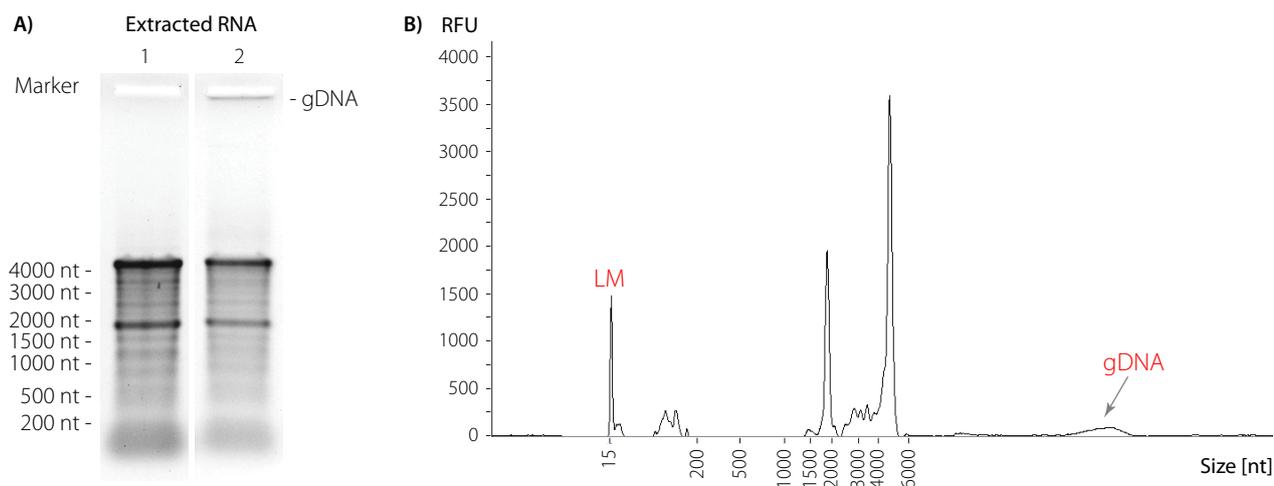


Figure 1 | Assessing your RNA sample for genomic DNA (gDNA). A) Stained agarose gel assessing RNA extracted using an RNA extraction method with (lane 1) and without gDNA removal (lane 2). A high molecular weight band is visible in lane 2, indicating the presence of genomic DNA. B) Fragment Analyzer trace with extended runtime assessing RNA extracted from white blood cells. A high molecular weight “bump” indicates gDNA contamination of the sample.

4. To Treat or Not to Treat

The question remains, when is it necessary to treat the RNA preparation with DNase, and when is it unnecessary? In general, DNase treatment should always be considered for the following circumstances:

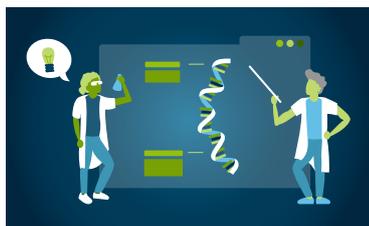
- ✓ When working with blood, DNase treatment is essential. Unlike other eukaryotic cells, e.g., cell lines, blood cells contain more DNA than RNA. This makes gDNA carry-over highly likely, even when using RNA extraction methods such as acidic phenol / chloroform extraction.
- ✓ Degraded RNA samples are very likely to contain fragmented pieces of DNA with lower molecular weight than that of intact gDNA. These short DNA sequences can be co-isolated with RNA and thus a DNase treatment is recommended.
- ✓ In FFPE samples, as degradation increases, so does the occurrence of cross-links between macromolecules and thus DNA carry-over. A DNase treatment is recommended.
- ✓ Mechanical disruption is very harsh on samples and commonly, gDNA carry-over is observed due to sheering / fragmentation of the gDNA in the process. Here, we also recommend DNase treatment.
- ✓ Finally, sample types with large quantities of short, extra-chromosomal DNA should be treated with DNase. This includes bacteria, which can carry plasmids that reach high copy numbers. These copies increase the DNA content and shift the DNA:RNA ratio more heavily in favor of DNA, making very difficult to avoid carry-over during RNA extraction. DNase treatment is therefore also recommended for bacterial samples.

When can DNase treatment be omitted?

As you have seen, some RNA extraction procedures are more suitable to minimize gDNA carry-over than others, especially acidic phenol / chloroform extraction can remove gDNA from many standard sample types. Also, 3' mRNA-Seq library preps tend to pick up less gDNA background than random primed whole transcriptome library preps, due to the fact that 3'-Seq methods rely on poly(A) stretches for priming. When both are used in combination and the RNA is of high quality and derived from an unproblematic sample type, the risk of compromising your RNA-Seq experiment with DNA contamination is minimal.

Those running targeted sequencing experiments can breathe a sigh of relief, as it were. DNase treatment is not needed for most RNA-Seq applications using targeted primers for specific genes of interest. The risk of contamination with accessible gDNA for exactly this location is low and genomic loci for primer binding are often far from the respective regions of the transcript (e.g., due to the presence of introns), and thus DNase treatment is not required.

Curious to learn more?



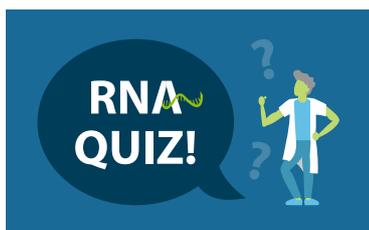
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