Catalytic FFPE Nucleic Acid Isolation for Best NGS Performance

Using the RNAstorm™ Kit to Extract RNA with Better Amplifiability and Integrity

Introduction

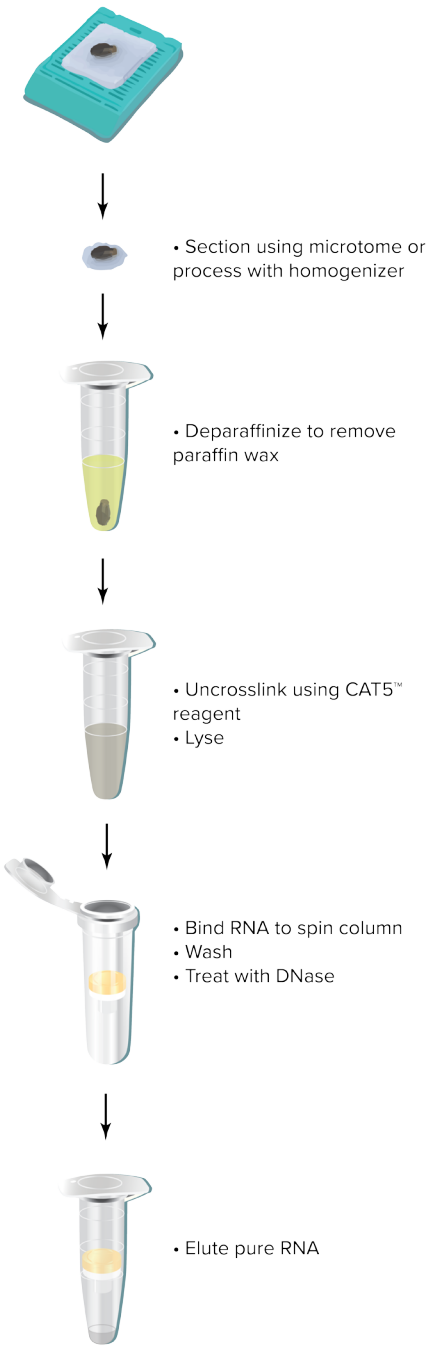
Biopsies and surgical specimens are routinely preserved by fixation with formaldehyde, in formalin-fixed paraffin-embedded (FFPE) tissue block format. While formaldehyde stabilizes tissue for storage, it also forms extensive crosslinks and adducts with nucleic acids and other biomolecules in the sample. Such modifications strongly interfere with downstream molecular analysis methods.

Existing methods rely primarily on heat to remove crosslinks and adducts, which is only partially effective and leads to additional fragmentation of labile nucleic acids such as RNA. In contrast, the catalytic CAT5™ technology developed by Cell Data Sciences and included in the RNAstorm™ kit greatly accelerates the removal of formaldehyde damage and allows the use of milder conditions, resulting in markedly improved recovery of amplifiable RNA. This greatly enhances the chances of success in recovering high yields of good quality nucleic acids suitable for a variety of applications, including RNA-seq or other next-generation sequencing (NGS), qPCR, microarray, and gene expression analysis.

The RNAstorm™ Kit

The RNAstorm™ extraction kit includes chemical catalysts that speed up demodification of formaldehyde-induced adducts, including on alkylated and crosslinked bases. This proprietary CAT5™ technology was shown to lead to RNA with higher quality and yields, better integrity, and greater amplifiability.[1]

The kit provides all necessary reagents for extraction of total RNA from FFPE tissue samples following a user-friendly protocol which can be performed in less than 1 hour of hands-on time. Following sectioning using a microtome, the tissue is first deparaffinized using a non-toxic xylene-free solution. Then, the tissue is treated using CAT5™ reagent and lysed using a protease solution. After binding to a spin column and washing, the RNA is treated with DNase I to remove contaminating genomic DNA. Finally, pure RNA is obtained after eluting with RNase-free water or a low-salt buffer.



Evaluating FFPE-derived RNA Quality and Quantity

The following techniques are commonly used to evaluate the quality of an RNA sample:

- Simple concentration measurements obtained using UV/Vis spectroscopy (e.g. Nanodrop™) or using an RNA-specific fluorescent dye (e.g. Qubit™).
- Measurements of RNA integrity using gel or capillary gel electrophoresis, for example on Agilent Bioanalyzer instruments, and expressed as a RIN number or DV_{200} percentage.
- Measurements of amplifiability, which depends directly on the amount of RNA that is sufficiently chemically de-modified or un-crosslinked to be processed by reverse transcriptases and polymerases. The method of choice is quantitative RT-PCR and the result can be expressed as a Ct number or as a relative or absolute amount of RNA. Amplifiability is the most reliable way of quantitating input nucleic acids for NGS library preparation.[2]

Quantitative RT-PCR remains the best method for judging the overall quality of an RNA sample because it depends on concentration, integrity, as well as amplifiability of the sample, and this Note therefore relies primarily on qPCR data. The other methods have significant drawbacks, especially for the degraded RNA that tends to be present in FFPE samples. Specifically, Nanodrop™ and Qubit™ measurements can be highly inaccurate.[2] Capillary electrophoresis is a useful measure of the degree of nucleic acid fragmentation, although RIN numbers do not correlate well with success in downstream assays and samples of widely different quality can have very similar RIN numbers.[3] DV_{200} (the percentage of RNA fragments > 200 bp) is a preferred measure, especially when the end-use is RNA-Seq on an Illumina platform. This metric was therefore used in this Note to estimate RNA integrity.

Testing Recovery of Amplifiable RNA from FFPE Samples

The Cell Data Sciences' commercial RNAstorm™ RNA FFPE extraction kit was tested on several FFPE tissue samples. To ensure reproducible and quantitative measurements of RNA recovery, pairwise FFPE sections were extracted in triplicate from each tissue block using both the RNAstorm™ kit and a popular commercial kit (Kit Q). Several types of both normal and tumor FFPE specimens. The oldest sample was fixed in 1976 and the newest one in 2015.

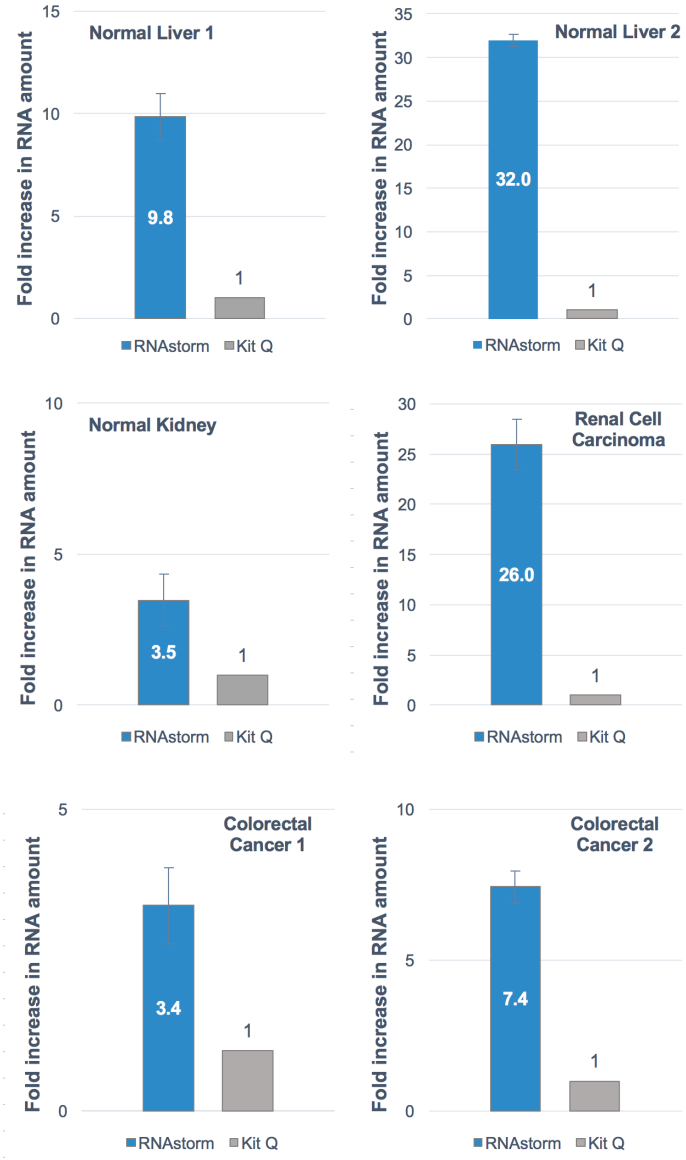


Figure 1. Comparison of RNA recovery by quantitative RT-PCR from six different FFPE tissue samples. "Kit Q" represents a market-leading competitive commercial FFPE extraction kit. Relative amounts of RNA were estimated from the Ct number observed for each sample.

Quantitative RT-PCR was used for relative quantification of amplifiable RNA. Ct values were measured in triplicate. An 83-bp amplicon from a highly expressed gene (TPT1) was used, and melt curves were analyzed to ensure specificity

of amplification. Relative amounts of RNA were estimated based on Ct values observed and plotted in Figure 1, where a Ct difference of 1 corresponds to a two-fold difference in concentration.

The RNAstorm™ Kit Greatly Increases Yields of Amplifiable RNA

For all samples tested and shown in Figure 1, significant and consistent increases were observed in the amount detected by quantitative PCR. The Ct values obtained with the RNAstorm™ kit relative to Kit Q are shown in Table 1.

Sample	Ct (RNAstorm)	Ct (Kit Q)
Normal Liver 1	29.2	32.5
Normal Liver 2	30.8	35.8
Normal Kidney	29.5	31.3
Renal Cell Carcinoma	31.4	36.1
Colorectal Cancer 1	30.4	32.1
Colorectal Cancer 2	28.1	31.0

Table 1. Fold-increase in recovery of amplifiable RNA recovery, extrapolated from Ct values in Figure 2, by quantitative RT-PCR by using the RNAstorm™ kit over Kit Q.

While RNA concentrations as measured by Qubit were also generally higher (data not shown), these concentration differences alone were not sufficient to explain the large differences in Ct values between the RNAstorm™ kit and Kit Q. This suggests that the integrity of the RNA and the extent of chemical modification and crosslinking play a significant role for the samples tested here, and in qPCR performance of FFPE samples generally.

Increased RNA Integrity is Observed Using the RNAstorm™ Kit

Increases in RNA integrity were also observed using capillary electrophoresis. Samples were processed using RNA 6000 Nano total RNA kits on an Agilent 2100 Bioanalyzer instrument, and the data was analyzed using Agilent Expert software. As an example, the traces obtained for one of the tissue samples (renal cell carcinoma) are displayed in Figure 2.

As discussed above, DV_{200} values were calculated in lieu of RIN numbers and are shown in Figure 3. A value of 30% has been previously identified by Illumina as a threshold value for allowing RNA-Seq library preparation performed with Tru-Seq reagents.[3] Here, most samples tested yielded DV_{200}

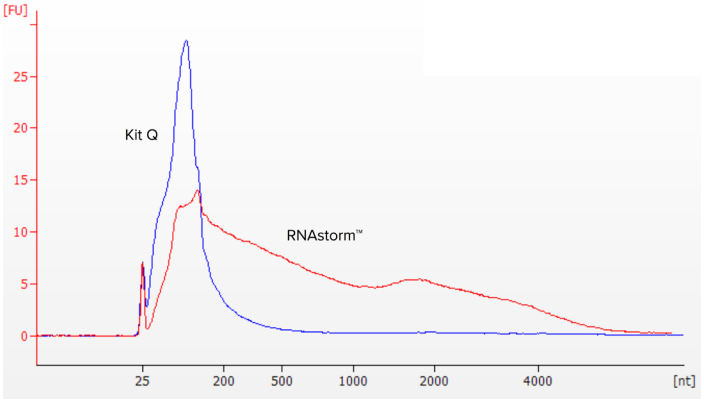


Figure 2. Analysis of RNA size distribution measurement by capillary electrophoresis (Agilent Bioanalyzer 2100) of renal cell carcinoma sample shows improved integrity of RNA obtained with the RNAstorm™ kit.

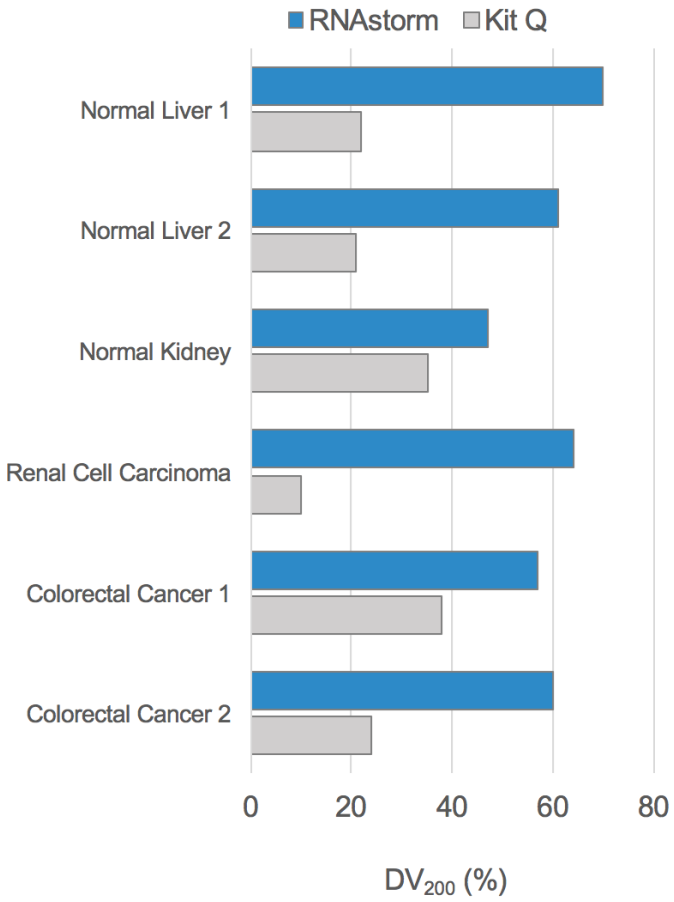


Figure 3. Comparison of RNA integrity by measurement of DV_{200} based on Agilent Bioanalyzer capillary electrophoresis results. "Kit Q" represents a market-leading competitive commercial FFPE extraction kit.

values of less than 30% when tested with a competitor's kit. In contrast, the values observed with the RNAstorm kit were significantly higher and ranged from 47 to 70%.

Conclusions

Hundreds of millions of patient FFPE samples are estimated to exist currently in biobanks world-wide, and many more millions are generated every year. The demand for analyzing this patient data will continue to increase due to the wealth of information available through next-generation sequencing methods. However, nucleic acid extraction methods have so far not been able to provide the quality of nucleic acids necessary for reliable sequencing. NGS library preparation relies on multiple enzymatic steps, including reverse transcription and amplification, which require nucleic acids with high integrity and amplifiability. The RNAstorm™ kit provides a sample processing solution specifically tailored for advanced downstream applications, and which allows end-users to reliably work with challenging yet valuable formalin-fixed samples.

Literature:

1. Karmakar et al., Organocatalytic Reversal of Formaldehyde Adducts of RNA and DNA Bases, Nature Chemistry 2015, 7, 752-758; doi:10.1038/nchem.2307
2. Arreaza et al., Pre-Analytical Considerations for Successful Next-Generation Sequencing (NGS): Challenges and Opportunities for Formalin-Fixed and Paraffin-Embedded Tumor Tissue (FFPE) Samples, Int. J. Mol. Sci. 2016, 17, 1579; doi:10.3390/ijms17091579
3. Evaluating RNA Quality from FFPE Samples: Guidelines for obtaining high-quality RNA sequencing results from degraded RNA with the TruSeq® RNA Access Library Preparation Kit, Illumina Inc., downloaded at http://www.illumina.com/documents/products/technotes/technote-truseq-rna-access.pdf

Product Information	
Applications	RNA-seq, PCR, qPCR/RT-PCR, microarray
Kit format	Manual (spin columns); Magnetic bead kit coming soon
DNAse treatment step	Included
Input samples	Formalin fixed samples (paraffin embedded or in fixative)
Recommended input sample amount	1-4 sections (5-10 µm each)
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