CELLDATA

Catalytic FFPE Nucleic Acid Isolation for Best NGS Performance

Using the RNAstorm™ Kit to Reliably Prepare Libraries for RNA-Seq

Introduction

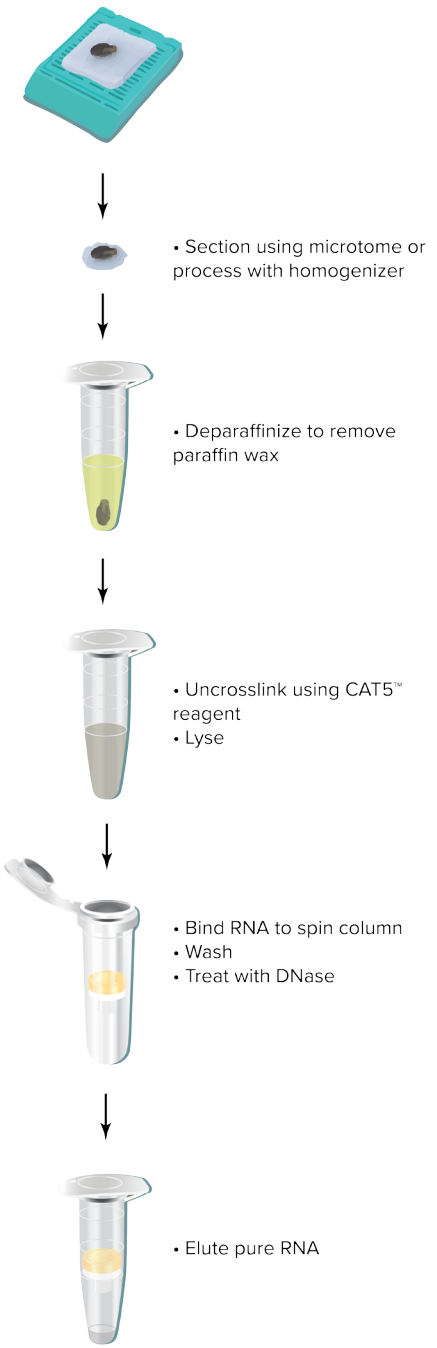
Biopsies and surgical specimens are routinely preserved by fixation with formaldehyde, in formalin-fixed paraffin-embedded (FFPE) tissue block format. While formaldehyde stabilizes tissue for storage, it also forms extensive crosslinks and adducts with nucleic acids and other biomolecules in the sample.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



Evaluation of RNA Quality Prior to Library Preparation

Success of NGS library preparation strongly depends on the quality of input nucleic acids used. Specifically, the factors that are most important for RNA-based methods are input amount, integrity and and amplifiability – all three must be acceptable for library preparation to be successful:

- Raw amount of input RNA can be measured using fluorescence-based techniques (e.g. Qubit) and/or UV/Vis spectroscopy (Nanodrop)
- RNA integrity can be measured using gel or capillary gel electrophoresis, for example on Agilent Bioanalyzer instruments, and expressed as a RIN number or DV_{200} percentage. A high DV200 has been shown to be very important for the success of NGS library preparation.[2]
- Amplifiability depends directly on the amount of RNA that is sufficiently chemically de-modified or un-cross-linked to be processed by reverse transcriptases and polymerases. This parameter is critical for NGS library preparation, because the first step of the library preparation protocol is a reverse transcription step, followed by several PCR amplification steps during library preparation. [3]

RNA Extraction

To test performance of the RNAstorm kit for next generation sequencing (RNA-seq), a normal FFPE-fixed liver sample block was chosen. Alternating identical 10 µM sections were sectioned, three using the RNAstorm™ kit, and three with a leading competitor's kit ("Competitor Q"). The resulting RNA was run on an Agilent Bioanalyzer to evaluate concentration and integrity. Average RNA concentrations were higher for the RNAstorm™ kit as shown below:

RNA Sample	RNA Concentration (ng/uL)
RNAstorm Replicate 1	309
RNAstorm Replicate 2	65
RNAstorm Replicate 3	317
Competitor Q Replicate 1	71
Competitor Q Replicate 2	127
Competitor Q Replicate 3	229

Further, significantly better integrity was observed for the RNA obtained with the RNAstorm™ kit. The variation between individual samples was also very low. In contrast, the samples purified with the Competitor Q kit had lower overall integrity and the integrity varied significantly between the three replicates. The DV_{200} values and Agilent Bioanalyzer traces for the various RNA samples are shown below and in Figure 1.

RNA Sample	DV_{200}
RNAstorm Replicate 1	79
RNAstorm Replicate 2	83
RNAstorm Replicate 3	79
Competitor Q Replicate 1	7
Competitor Q Replicate 2	74
Competitor Q Replicate 3	43

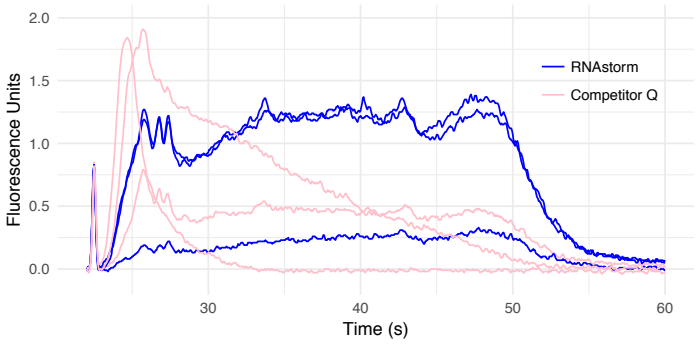


Figure 1. Comparison of RNA yield and integrity of RNA extracted either with the RNAstorm™ kit or a leading competitive commercial FFPE extraction kit ("Kit Q").

Library Preparation

RNA-seq libraries were prepared using the KAPA RNA HyperPrep kit with RiboErase (HMR) (Kapa Biosystems, Cape Town, South Africa). The amounts of input RNA were normalized for all replicates, and each library was prepared using approximately 500ng of FFPE RNA. By recommendation of the HyperPrep kit manufacturer, all FFPE RNA samples were fragmented by incubation at 65°C for 1 min. Adapter ligation was performed using KAPA Single-Indexed Adapter Set A kit

for Illumina Platforms (Kapa Biosystems, Cape Town, South Africa). All libraries were amplified for a total of 8 cycles and subsequently analyzed for sizing using the Bioanalyzer High Sensitivity DNA assay (Agilent Technologies, Santa Clara, CA) and quantified from Bioanalyzer traces as well as by qPCR using the KAPA Library Quantification kit (Kapa Biosystems, Cape Town, South Africa).

Library Characterization

Library preparation was successful for all three samples extracted with the RNAstorm™ kit, but failed entirely for one of the samples obtained with the Competitor Q kit. Higher concentrations of library were obtained for the RNAstorm™-extracted samples.

Library	Bioanalyzer (ng/uL)	Kapa Library Quant (nM)
RNAstorm Replicate 1	1.0	5.6
RNAstorm Replicate 2	1.8	5.4
RNAstorm Replicate 3	1.2	9.3
Competitor Q Replicate 1	0	0.03
Competitor Q Replicate 2	0.7	5.9
Competitor Q Replicate 3	0.7	3.5

Although no size selection was performed, the five libraries showed approximately equal insert size distributions as seen in Figure 2.

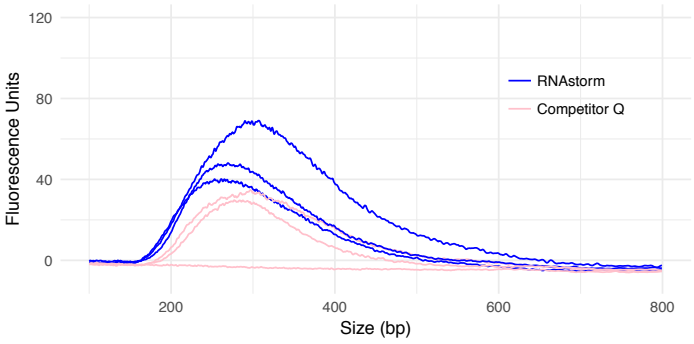


Figure 2. Library size distribution as analyzed by Agilent Bioanalyzer ranged between approximately 270 and 320 nt for all samples.

All libraries were successfully sequenced on an Illumina NextSeq 500 instrument in High Output Mode using single-end 75 bp reads. An average of 64m raw reads were obtained for each the RNAstorm-extracted samples, as compared with 58m raw reads for each of the samples obtained using the kit from Competitor Q. Excluding the first five nucleotides, median Q-scores were at or above 36 for all read positions and all samples. Reads were aligned to the hg38 human genome reference sequence using the HISAT2 aligner (v. 2.0.3). Alignment statistics are shown below:

Library	% Aligned once	% Aligned > once	% Not Aligned
RNAstorm 1	62.8%	18.5%	18.7%
RNAstorm 2	64.0%	19.6%	16.4%
RNAstorm 3	62.7%	19.9%	17.4%
Competitor Q 2	60.3%	22.8%	16.9%
Competitor Q 3	60.3%	20.8%	18.9%

A larger percentage of reads obtained with the RNAstorm™ kit aligned exactly once relative to Competitor Q. Further, a lower fraction of reads obtained with the RNAstorm™ kit aligned more than once.

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 higher RNA extraction yields, better resulting library quality, better reliability and reproducibility during library preparation, and ensures more reliable analysis of the sequencing data obtained from FFPE 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. 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
3. 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

RNAstorm™ Product Information	
Applications	RNA-seq, PCR, qPCR/RT-PCR, microarray
Kit format	Manual (spin columns); Magnetic bead kit available 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)
Type of RNA isolated	Total RNA (including miRNA)
Isolation time	50 minutes hands-on time

DNAstorm™ Product Information	
Applications	Next generation sequencing, PCR, qPCR/RT-PCR
Kit format	Manual (spin columns); Magnetic bead kit available soon
RNase treatment step	Included
Input samples	Formalin fixed samples (paraffin embedded or in fixative)
Recommended input sample amount	1-4 sections (5-10 µm each)
Type of RNA isolated	Total RNA (including miRNA)
Isolation time	50 minutes hands-on time

Orders:

RNAstorm™: Catalog No. **CD501** (50 reactions) or **CD201** (20 reaction sample kit)
DNAstorm™: Catalog No. **CD502** (50 reactions) or **CD202** (20 reaction sample kit)
Email: orders@celldatasci.com
Phone: 650.285.2376 (option 1)
Toll-free: 855.620.7300 (option 1)
Web: shop.celldatasci.com

For Research Use Only.