Validation and Performance Characteristics of RNA*Retain*™ Pre-analytical RNA Stabilization Solution for Collection and Storage of Human Cellular and Tissue Specimens

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SUMMARY POINTS:

- RNA*Retain*™ is a versatile, non-toxic, non-organic solution contained within a single-use specimen receptacle manufactured under cGMP.*
- In Europe, RNA*Retain*™ is intended for the collection, storage and transport of clinical human cellular and solid tissue specimens and stabilization of intracellular RNA within these specimens for subsequent nucleic acid extraction and molecular analysis.
- High quality total RNA compatible with downstream molecular analysis is recovered from normal breast tissues and cultured breast cancer cells after storage for up to 3 days at 35-39°C, up to 7 days at 18-25°C, up to 30 days at 2-8°C and up to a year at -15 to -30°C.

INTRODUCTION

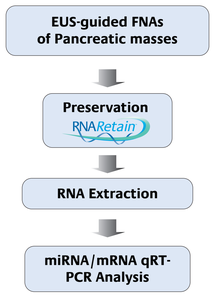
Stabilization of RNA in biological samples for subsequent nucleic acid extraction and molecular analysis is necessary to prevent specific and nonspecific RNA degradation from occurring. RNA*Retain*™ Pre-analytical RNA Stabilization Solution is a proprietary formulation contained within a single-use specimen receptacle manufactured under cGMP. RNA*Retain*™ rapidly permeates most cell and tissue types to protect cellular RNA from ribonucleases present in freshly collected specimens. The use of RNA*Retain*™ in conjunction with the MammaPrint® assay, a 70 gene expression signature that predicts the risk of distant metastases in breast cancer patients, was cleared by the FDA in 2007. RNA*Retain*™ is also available as a CE-labeled in vitro diagnostic device in Europe and as a custom research-use-only product in the USA. Here, we evaluated the use of RNA*Retain*™ for collection, storage and RNA stabilization of fine-needle aspirates (FNAs) and validated its performance characteristics using cultured breast cancer cells and normal breast tissues.

MATERIALS AND METHODS

Frozen and endoscopic ultrasound-guided FNA (EUS-FNA) pancreatic specimens were collected with approved protocols as part of standard clinical care and considered to be remnant material unnecessary for patient treatment. Specimens were processed as described in Szafranska *et al*. (Clin. Chem. 2008). Normal human breast tissue specimens were collected as shown in Figure 6 from commercial sources in compliance with 45 CFR 46 and other regulatory guidance. MCF-7 human breast adenocarcinoma cells were cultured and collected under standard tissue cell culture methods. Total RNA from breast and cell specimens was extracted using the *mir*Vana™ miRNA Isolation Kit protocol (Ambion, Austin, TX). RNA concentration and purity were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA integrity was assessed with an Agilent 2100 Bioanalyzer and the RNA 6000 LabChip kit (Agilent Technologies, Palo Alto, CA). microRNA and mRNA real-time quantitative RT-PCR analyses were performed with TaqMan® assays and the TaqMan 7900 system (Applied Biosystems, Foster City, CA, USA).

RESULTS

Evaluation of Pancreatic EUS-FNAs



FNA	RNA Yield (µg)	RNA Purity (A260/280)
FNA-1	2.0	1.56
FNA-2	0.8	1.69
FNA-3	1.3	1.87
FNA-4	1.9	1.51
FNA-5	9.0	1.96
FNA-6	1.0	1.78
FNA-7	0.6	1.87
FNA-8	1.7	1.9
FNA-9	10.1	1.8
FNA-11	1.1	1.72
FNA-12	1.4	1.97
FNA-13	9.5	1.95
FNA-14	20.5	1.99
FNA-15	8.2	1.98
Average	4.9	1.83

Figure 1: Recovery of RNA from pancreatic FNA specimens preserved in RNA*Retain*™. Two to three FNAs from 14 cancerous or normal pancreas specimens were collected and stored in 1 mL of RNA*Retain*™. After incubation overnight at 4°C, specimens were frozen and RNA was isolated 1 to 3 months later. Due to the cellular heterogeneity of the FNA specimens, RNA yield ranged from about 1 to 20 µg per specimen.

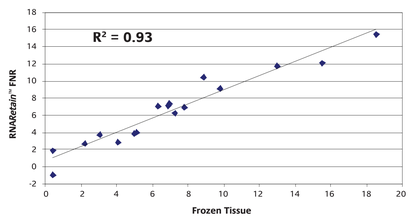


Figure 2: Comparison of mRNA and miRNA expression levels in non-paired frozen tissue sections and FNAs collected in RNA*Retain*™. Expression levels of 12 miRNAs (miR-196a, -217, -130b, -145, -143, -148a, -375, -210, -223, -155, -31, -96, and -205) and 4 mRNAs (BIRC5, CEACAM6, MUC4 and UPAR) were measured by real-time quantitative RT-PCR in 18 pancreatic ductal adenocarcinoma specimens (8 frozen tissues and 10 FNAs collected in RNA*Retain*™).

For mRNAs, only 7 FNA specimens were analyzed due to the limiting amount of purified RNA. Data were normalized to miR-24 or GAPDH expression levels for miRNAs and mRNAs, respectively. Mean normalized expression levels for each of the 16 targets in FNA specimens were plotted against the values obtained in frozen specimens. The overall correlation was 93% indicating that expression levels of mRNAs and miRNAs are highly similar between RNA*Retain*™-preserved and frozen specimens.

Temperature and Time of Storage Study

Temp (°C)	Time	Integrity (28S:18S)	Purity (A260/A280)
35 to 39	3 Days	1.3	1.94
18 to 25	7 Days	1.6	2.02
2 to 8	30 Days	1.2	1.98
-15 to -30	1 Year	1.9	1.93

Figure 3: Purity and integrity of total RNA within RNA*Retain*™-preserved specimens is maintained over multiple temperatures and time. MCF-7 cell line specimens collected in RNA*Retain*™ (0.5 million cells) were stored at the indicated temperatures for a time course study. At various time points, total RNA was purified from triplicate specimens. As a process control, RNA was also purified from flash-frozen cell specimens stored below -75°C (data not shown) for each time point. The average integrity and purity of the RNA*Retain*™-preserved specimens show that the RNA was of acceptable quality for downstream molecular analyses for up to 3 days at 35-39°C, up to 7 days at 18-25°C, up to 30 days at 2-8°C and up to a year at -15 to -30°C (study further ongoing).

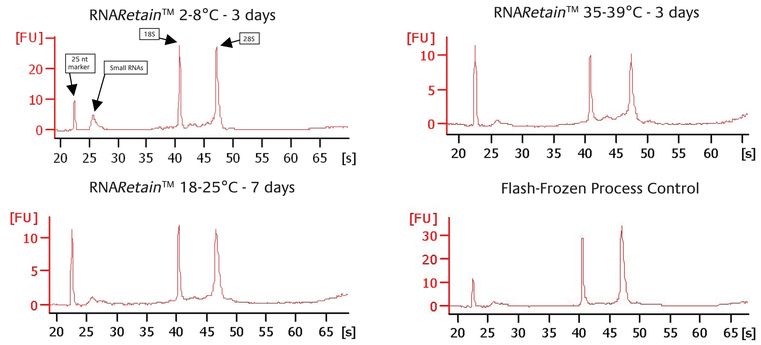


Figure 4: Representative Agilent Bioanalyzer electropherogram traces. Total RNA extracted from RNA*Retain*™-preserved cell line replicates stored at various temperatures have a similar profile as total RNA extracted from flash-frozen cells stored below -75°C for the same period of time.

Days	Temp (°C)	Storage	Replicates	Integrity (28S:18S) AVG	Integrity (28S:18S) SD	Integrity (28S:18S) %CV	Purity (A260:A280) AVG	Purity (A260:A280) SD	Purity (A260:A280) %CV
0	N/A	N/A	6	1.8	0.1	2.9	1.95	0.04	2.19
3	18 to 25	RNA*Retain*™	6	1.6	0.1	6.5	1.96	0.01	0.42
	< -75	Flash-Frozen	6	1.7	0.1	6.0	1.99	0.01	0.59
7	2 to 8	RNA*Retain*™	6	1.6	0.1	4.8	2.01	0.02	1.09
	<-75	Flash-Frozen	6	1.6	0.1	5.2	1.98	0.04	1.77

Figure 5: Purity and integrity of total RNA within RNA*Retain*™-preserved specimen replicates. RNA*Retain*™-preserved replicates (n=6) were stored at 2-8°C for 7 days or 18-25°C for 3 days (post 2-8° hold overnight) prior to RNA isolation. RNA was also isolated from fresh and flash-frozen replicates (n=6) as process controls. No significant differences were observed between the fresh, RNA*Retain*™-preserved or flash-frozen replicates at both storage temperatures.

Normal Breast Tissue Study

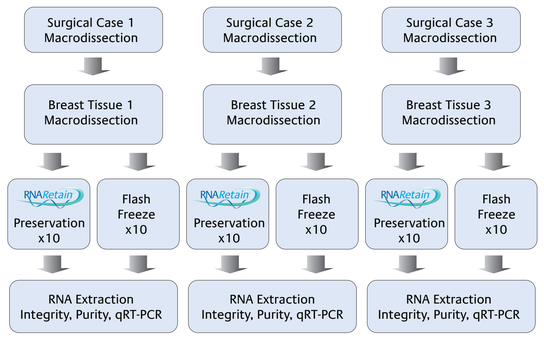


Figure 6: Experimental design. Three normal female human breast tissue specimens (~15 g each) were each divided into 10 equal sections (~1 cc each). Each of the 10 sections was then further bisected into 2 equal halves (~0.5 cc each). One half was placed into a 5 mL vial of RNA*Retain*™ and the other half was flash frozen using liquid nitrogen (day 1). Each of the respective sample halves were processed concurrently to maintain spatial (with respect to collection from contiguous areas of macrodissected tissues) and temporal (with respect to equivalent RNA*Retain*™ immersion or snap freezing time points) consistency between sample pairs. After overnight storage at 4°C, the 30 RNA*Retain*™-preserved specimens were shipped on wet ice (day 2) using a standard overnight carrier to a second facility for RNA isolation. The 30 frozen specimen were shipped in parallel on dry ice. RNA isolation was performed upon receipt by a single operator (day 3).

B

Biological Replicates	Storage	Technical Replicates	Integrity (28S:18S) AVG	Integrity (28S:18S) SD	Integrity (28S:18S) %CV	Purity (A260:A280) AVG	Purity (A260:A280) SD	Purity (A260:A280) %CV	GAPDH qRT-PCR (Ct) AVG	GAPDH qRT-PCR (Ct) SD	GAPDH qRT-PCR (Ct) %CV
1	RNA*Retain*™	10	1.5	0.3	18.8	1.97	0.03	1.51	21.01	0.41	1.95
	Flash-Frozen	10	1.6	0.2	13.6	1.92	0.06	2.92	21.94	0.19	0.87
2	RNA*Retain*™	10	1.6	0.2	11.9	2.03	0.04	2.01	21.38	0.28	1.33
	Flash-Frozen	10	1.5	0.2	15.3	2.01	0.11	5.42	21.70	0.30	1.37
3	RNA*Retain*™	10	1.5	0.2	10.3	1.96	0.14	6.92	22.01	0.87	3.97
	Flash-Frozen	10	1.5	0.2	8.6	1.96	0.09	4.70	21.59	0.69	3.19

Figure 7: RNA integrity, purity and mRNA expression levels are equivalent for RNA*Retain*™-preserved and flash-frozen human breast tissues. (A) For each sample, RNA quality and purity was assessed by measuring the 28S and 18S ribosomal RNA (rRNA) ratio on the Agilent Bioanalyzer (top panels) and the A260 to A280 ratio on the NanoDrop (middle panels). Purified RNA was also tested by real-time quantitative RT-PCR using a TaqMan® assay specific for GAPDH mRNA and 50 ng RNA input. A single reverse transcription reaction was performed per replicate then split into triplicate PCR reactions. The average GAPDH expression level (raw cycle threshold units) was plotted for each pair and each surgical case (bottom panels). **(B)** No statistically significant difference was observed between the 10 tissue pairs collected in RNA*Retain*™ or flash frozen for each of the 3 surgical cases.

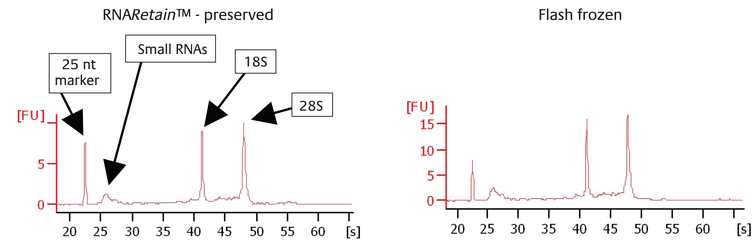


Figure 8: Representative Agilent Bioanalyzer electropherogram traces. Total RNA samples extracted from RNA*Retain*™-preserved or flash frozen human breast tissue pairs (surgical case #3, replicate #5) have a similar profile and 28S:18S rRNA ratio (~1.5).

CONCLUSION

RNA*Retain*™ eliminates the need to immediately process specimens, allowing nucleic acid extraction and molecular analysis at a later time and/or different location. It also eliminates the need to both flash-freeze specimens, a process that involves manipulation of potentially hazardous agents such as liquid nitrogen, and to keep specimens frozen throughout storage and transport. High quality total RNA was reproducibly recovered from 0.5 cc human breast biopsies after 3 days of storage in RNA*Retain*™. Further validation experiments are ongoing to determine the performance of RNA*Retain*™ with additional cellular or tissue types and extend its use to other clinically relevant human specimens such as fine needle aspirates.

* **RNA*Retain*™** is a CE-Marked IVD and is cleared in the U.S. for use with the MammaPrint® assay only.