Molecular Characterization of DNA, mRNA and miRNA Biomarkers in Preoperative Fine Needle Aspirates Collected in RNA*Retain*® Pre-Analytical Solution

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SUMMARY

- RNA*Retain*® is a pre-analytical solution* manufactured under cGMP that protects nucleic acids in freshly collected tissues or cells**
- RNA*Retain*® is compatible with the collection, storage and shipping of preoperative pancreatic or thyroid FNAs for direct extraction of total RNA or total nucleic acid**
- Analysis of specific DNA, mRNA or miRNA biomarkers in RNA*Retain*®-preserved FNAs might in the future facilitate the molecular characterization and preoperative differential diagnosis of suspicious cancer FNAs

INTRODUCTION

Cytopathological diagnosis of fine needle aspirates (FNA) is challenging and can be inconclusive. Molecular testing for relevant markers associated with specific cancers can improve the preoperative diagnosis of FNAs. For example, we previously showed that a 2-miRNA classifier, initially developed and validated on FFPE tissue, can help identify benign versus malignant pancreatic FNAs (Szafranska et al. Oncogene 2007 and Clin. Chem. 2008). Testing for the presence of various DNA (RAS, BRAF) or RNA (PAX8/PPAR, RET/PTC) biomarkers has also be shown to improve the diagnosis of thyroid nodule FNA biopsies.

RNA*Retain*® is a solution* that preserves nucleic acids in tissues or cells (Figure 1) allowing nucleic acid extraction and molecular analysis at a later time and/or different location**. RNA*Retain*® is manufactured under cGMP and is cleared in the US as an accessory to the Agendia's MammaPrint® test. Three additional product configurations (1, 5 or 6 mL) are available in Europe as CE-marked IVD pre-analytical devices. Here we evaluated the compatibility of a research use RNA*Retain*® 1 mL formulation for the preservation of nucleic acids in preoperative FNAs and downstream analysis of specific DNA, mRNA or miRNA biomarkers.

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	3 Years	1.7	1.99

Figure 1. Stability of RNA*Retain*®-preserved specimens. MCF-7 cultured cells (0.5 million) collected in 1 mL of RNARetain were stored at various temperatures in triplicate. The total RNA extracted from each specimen after removal of the solution was shown to be 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 3 year at -15 to -30°C (study further ongoing).

MATERIALS & METHODS

RNA*Retain*® (RUO*) was formulated as 1 mL aliquots in 2 mL single-use vials allowing collection, storage, shipment, and recovery of 1 to 3 FNA passes per vial. Ultrasonography (US)-guided FNAS were collected at various sites, immersed in RNARetain and processed as described in Figure 2. All human specimens in this study were de-identified and evaluated according to protocols approved by their respective institutions. Purified nucleic acids were subjected to several quality controls including concentration/purity (spectrophotometry) and integrity (Agilent Bioanalyzer). Expression of specific biomarkers was determined using simplex quantitative real-time PCR assays based on TaqMan® technology (performed on an ABI 7500 or 7900 instrument) or multiplex qualitative end-point PCR assays based on Signature® technology (performed on a Luminex 200 instrument) .

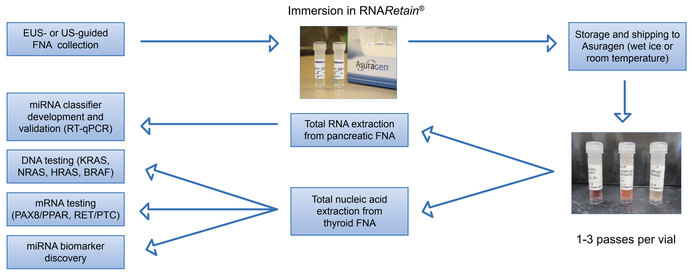


Figure 2. Study overview. FNAs were collected at various sites (see acknowledgement section) and immediately immersed in RNA*Retain*®. One to 3 passes were collected per 2 mL vial containing 1 mL of solution. The pancreatic endoscopic ultrasonography (EUS)-guided FNAs were shipped on wet ice to Asuragen where total RNA was extracted directly from the vial using a laboratory-validated extraction method. The total RNA was then used to assess miRNA expression by real-time RT-PCR (see Figure 4). The thyroid ultrasonography (US)-guided FNAs were shipped at room temperature to Asuragen where total nucleic acid was extracted directly from the vial using a proprietary extraction method developed by Asuragen. The total nucleic acid was then used to test for known DNA and RNA biomarkers and to evaluate miRNA expression for novel biomarker discovery (see Figure 5 and 6).

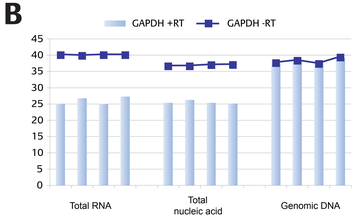
RESULTS

Nucleic Acid Extraction from RNA*Retain*®

A

	Sample ID	ng/ul	A260/280
Total RNA	Control	19.0	2.0
		16.1	2.1
	RNA*Retain*	30.2	1.9
		27.9	2.0
Total nucleic acid	Control	264.2	2.0
		118.7	2.0
	RNA*Retain*	695.2	2.0
		730.3	2.0
Genomic DNA	Control	28.1	1.9
		23.2	1.9
	RNA*Retain*	37.1	1.9
		37.6	1.9

B



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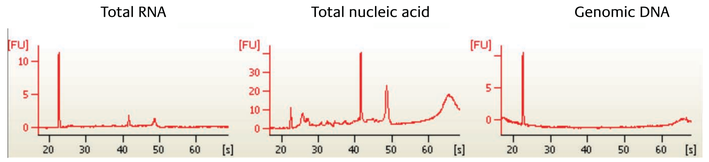
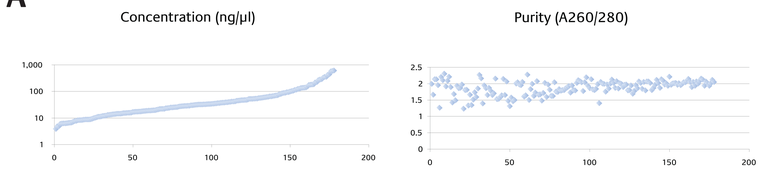


Figure 3. Direct extraction of total nucleic acid from RNA*Retain*®. (A) Total RNA, genomic DNA or total nucleic acid (DNA+RNA) can be extracted directly from RNA*Retain*®. Model FNA specimens were spiked in 1 mL of RNA*Retain*® then subjected to 3 different nucleic acid extraction protocols without prior removal of the solution. These procedures were developed so that the cell-free nucleic acids released during the FNA collection process could be recovered together with the cellular nucleic acids. Results for control extractions in the absence of RNA*Retain*® are also shown. **(B)** Representative example of quantitative real-time RT-PCR analysis for 3 types of nucleic acid fractions (10 ng input) extracted from the same sample. The graph shows the cycle threshold (Ct) for the GAPDH transcript with (+RT) or without (-RT) reverse transcription step. **(C)** Representative example of Bioanalyzer traces for 3 types of nucleic acid fractions extracted from the same sample.

Pancreatic EUS-FNAs

A



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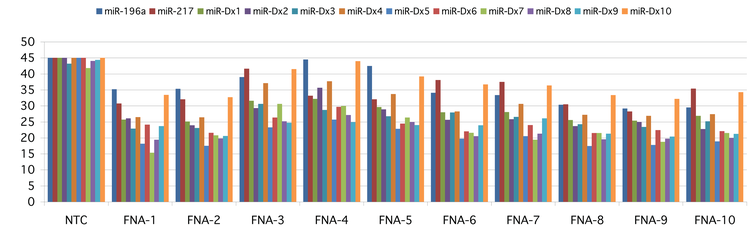
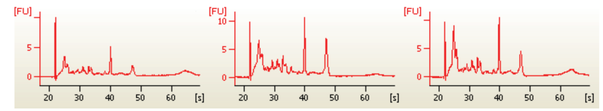


Figure 4: Recovery and analysis of total RNA from pancreatic FNAs. (A) Concentration (ng/µL) and purity (A260/280) of total RNA extracted from 178 pancreatic EUS-FNAs. The total yield varied from 0.4 to 60 µg (6.5 µg on average). The A260/280 ratio varied from 1.2 to 2.3 (1.9 on average). The highest variability in purity was observed for the samples with the lowest total RNA concentration. **(B)** Representative example of quantitative RT-PCR results with 30 ng of total RNA extracted from 10 FNAs. The graph shows the cycle threshold (Ct) obtained for miR-196a, miR-217 and 10 additional miRNAs. This approach is currently used at Asuragen to develop an extended miRNA classifier for the identification of benign lesions versus pancreatic ductal adenocarcinoma in specimens with low percent tumor and FNAs. The goal is to further improve the miR-196a/miR-217 classifier that was previously developed and validated for specimens with greater than 60% tumor content.

Thyroid US-FNAs

A



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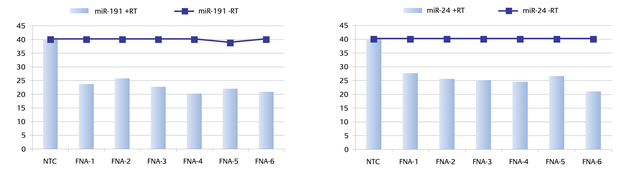

Figure 5. Recovery and analysis of total nucleic acid from thyroid FNAs. (A) Representative examples of Bioanalyzer traces for total nucleic acids extracted from 3 independent FNAs. Extraction from 36 FNAs resulted in an average yield of 0.8 µg (0.1-3.3 µg) with an average purity ratio of 1.7 (A260/280, 1.3-2.2). **(B)** Representative example of quantitative RT-PCR results with 5 ng of total nucleic acid extracted from 6 FNAs. The graph shows the cycle threshold (Ct) obtained for miR-191 or miR-24 with (+RT) or without (-RT) reverse transcription step. This approach is currently used at Asuragen to identify novel sets of miRNAs that may be able to distinguish between benign hyperplastic thyroid nodules and neoplastic thyroid lesions (see poster ST08 for more information on novel thyroid miRNA biomarkers).

Signature® KRAS/BRAF Mutations (DNA)

	G12R	G12V	G13D	G12D	G12A	G12C	G12S	EC	G13S	G13R	G13C	G13V	G13A	V600E	EC
Neg Control	248	153	114	85	97	34	239	7484	50	36	49	78	42	9746	6067
Pooled Pos	2751	2157	1975	3671	3005	1730	3156	7309	3489	5441	3866	5988	3643	9881	6582
FNA-1	140	183	66	14	173	87	102	5853	3	75	66	37	37	34	4307
FNA-2	229	134	86	35	61	74	189	10006	16	80	53	110	47	138	8005
FNA-3	139	176	134	70	49	66	187	9605	59	0	52	98	17	72	8306
FNA-4	187	118	61	38	149	99	141	10335	76	73	40	177	63	35	8509
FNA-5	243	118	127	92	62	65	154	10108	28	27	42	120	66	41	8837
FNA-6	240	83	141	112	78	57	202	9973	54	46	40	171	39	88	8539

HRAS/NRAS assay (DNA)

	H-G12V	H-Q61L	H-Q61R	N-Q61K	N-Q61R	N-Q61L	EC
Neg Control	56	55	46	65	18	78	12772
Pooled Pos	8740	7316	8533	4579	4773	6534	9951
FNA-1	107	104	94	120	33	72	7509
FNA-2	93	55	87	69	68	43	9927
FNA-3	67	116	81	90	59	43	10097
FNA-4	82	80	75	8502	72	53	10172
FNA-5	133	102	107	78	91	32	9670
FNA-6	126	104	189	69	68	51	10214

Fusion transcripts assay (RNA)

	PAX8/PPAR	RET/PTC1	RET/PTC3	EC
NTC	36	25	41	81
Pooled Pos	7070	8124	9578	5850
FNA-1	53	81	101	4808
FNA-2	67	57	14	4593
FNA-3	10	67	85	5340
FNA-4	5	40	34	6946
FNA-5	13	57	83	8523
FNA-6	71	47	47	5108

Figure 6. Analysis of known papillary or follicular thyroid cancer biomarkers in total nucleic acid from thyroid FNAs.** Representative example of results with 6 total nucleic acid samples tested at 20 or 40 ng input per DNA- or RNA-based assay, respectively. BRAF V600E and 12 KRAS mutations in codon 12 and 13 were assessed with the Signature® KRAS/BRAF Mutations (RUO*) kit. Prototype assays based on the same Signature® technology were used to test either for 6 NRAS/HRAS codon 12 or 61 mutations or for 3 fusion transcripts. One FNA was found positive for NRAS Q61K mutation and confirmed by an independent method. The risk for potential false positive or false negative results was controlled by the inclusion of batch controls in every run and the co-amplification and co-detection of an endogenous control (EC) in every reaction. (see poster ST45 for more information on the Signature® technology platform, including preliminary analytical sensitivity).

CONCLUSIONS

These results demonstrate that a 1 mL research use formulation of RNA*Retain*® is compatible with the collection, storage, and transport of preoperative FNAs from different tissue types*. Total nucleic acid was efficiently recovered from the pre-analytical solution and successfully tested for known DNA and RNA biomarkers as well as novel miRNA candidates**. This process could in the future complement the cytological diagnosis of challenging neoplastic diseases such as pancreatic and thyroid cancers. Ultimately, validation of standardized collection and testing protocols might improve the differential diagnosis of suspicious cancer FNAs and facilitate preoperative decision making.

Acknowledgments

FNAs were collected at various institutions worldwide. The authors would like to acknowledge the contribution from multiple collaborators at Austin Diagnostics (Austin, TX), Bonne Sante (Warsaw, Poland), Brigham and Women's Hospital (Boston, MA), Dartmouth's Hitchcock Medical Center (Lebanon, NH), H. Lee Moffitt Cancer Center (Tampa, FL), Texas Diabetes and Endocrinology (Austin , TX), University of Pittsburgh Medical Center (Pittsburgh, PA), and the University of Sherbrooke (Quebec, Canada).

*For research use only. Not for use in diagnostic procedures.
**Preliminary research data. The performance characteristics of this reagent have not yet been established.