

Preservation of Fine-Needle Aspiration Specimens for Future Use in RNA-Based Molecular Testing

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BACKGROUND: The application of ancillary molecular testing is becoming more important for the diagnosis and classification of disease. The use of fine-needle aspiration (FNA) biopsy as the means of sampling tumors in conjunction with molecular testing could be a powerful combination. FNA is minimally invasive, cost effective, and usually demonstrates accuracy comparable to diagnoses based on excisional biopsies. Quality control (QC) and test validation requirements for development of molecular tests impose a need for access to pre-existing clinical samples. Tissue banking of excisional biopsy specimens is frequently performed at large research institutions, but few have developed protocols for preservation of cytologic specimens. This study aimed to evaluate cryopreservation of FNA specimens as a method of maintaining cellular morphology and ribonucleic acid (RNA) integrity in banked tissues. **METHODS:** FNA specimens were obtained from fresh tumor resections, processed by using a cryopreservation protocol, and stored for up to 27 weeks. Upon retrieval, samples were made into slides for morphological evaluation, and RNA was extracted and assessed for integrity by using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, Calif). **RESULTS:** Cryopreserved specimens showed good cell morphology and, in many cases, yielded intact RNA. Cases showing moderate or severe RNA degradation could generally be associated with prolonged specimen handling or sampling of necrotic areas. **CONCLUSIONS:** FNA specimens can be stored in a manner that maintains cellular morphology and RNA integrity necessary for studies of gene expression. In addition to addressing quality control (QC) and test validation needs, cytology banks will be an invaluable resource for future molecular morphologic and diagnostic research studies. *Cancer (Cancer Cytopathol)* 2011;000:000-000. © 2011 American Cancer Society.

KEY WORDS: cryopreservation, FNA, fine-needle aspiration, RNA, biorepository, tissue bank, gene expression, molecular morphology.

Fine-needle aspiration (FNA) biopsy is a rapid, minimally invasive, and cost-effective procedure in comparison to open surgical biopsy. FNA can be performed on either superficial palpable lesions or on deep tissue lesions in combination with imaging tools such as computed tomography (CT) scans, ultrasound, and magnetic resonance imaging (MRI). Currently at most health care institutions, FNA material not used in

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fresh-tissue slide preparation is preserved in the cytology laboratory via standard histological formalin-fixed, paraffin-embedded (FFPE) processing, ie, cell blocks. Although this method of sample preservation maintains superior morphologic detail, cells are no longer viable, and nucleic acid integrity is severely compromised. As molecular testing is increasingly used as a clinical diagnostic tool, the need to maintain both morphologic and nucleic acid integrity in all patient specimens is becoming increasingly important.

Cryopreservation is a commonly used method for preservation of hematopoietic and other single-cell suspension sample types. It has major advantages over other preservation methods because it can maintain nucleic acid integrity, cell viability, and morphologic detail. Cells are isolated from blood, body fluids, or tissue-culture environments and then concentrated and frozen at temperatures approaching -200°C . The use of cryoprotectants and controlled-rate freezing are imperative to the success of the process.¹

Investigation of the literature revealed that there is little information concerning the success of cryopreservation of cells collected via FNA. Hematopoietic cells have been collected by bone marrow aspiration and cryopreserved for future transplantation for many years, and, in reproductive medicine, oocytes have been successfully collected by FNA and cryopreserved for subsequent in vitro fertilization,² but there are no reports that detail the cryopreservation of primary human tumor cells obtained from FNA. Our main objective was to assess the potential usefulness of cryopreservation of FNA material as a means of preserving such samples for ancillary RNA-based testing. We simulated collection of clinical FNA biopsies by performing aspirations on resected tissues that were received in surgical pathology, and then cryopreserved them by using a modified version of standard methods as described herein. Criteria used to assess suitability of the method were maintenance of morphologic detail and nucleic acid integrity in the thawed specimens.

MATERIALS AND METHODS

Human Tissue Specimens

Approval from the Virginia Commonwealth University Institutional Review Board was obtained for collection of

Table 1. FNA Tumor Types and Diagnoses

	No. of Specimens by Preservation Method	
	Cryopreserved	RNAlater
Adenocarcinoma		
Colon	1	0
Lung	3	0
Pancreatic	0	1
Endometrial	0	2
Breast	1	3
Cholangiocarcinoma	2	0
Hepatocellular	0	1
Gastric	0	1
Renal cell carcinoma	6	1
Thyroid carcinoma (papillary)	2	0
Squamous cell carcinoma (lymph node metastases)	2	1
Bone and soft tissue tumor		
Hemangiopericytoma	1	0
Pleomorphic sarcoma	2	1
Fibromyxoid sarcoma	1	0
Osteosarcoma	1	0
Melanoma	1	0
Germ cell tumor (embryonal carcinoma)	0	1
Pheochromocytoma	1	0
Benign lymph node	1	1
Total	25	13

all samples used in this study. Thirty-eight FNA specimens were obtained by either a board-certified pathologist (E. D.) or trained pathologists' assistants (T. L. and J. P.) from clinical surgical resections submitted for routine pathological review at our institution. Table 1 identifies the tumor type and/or diagnosis for each specimen. Tissues used for specimen collection were kept at 4°C for 0-27 hours before FNA.

Cell Collection and Preservation Methods

Using a 23-gauge needle, we obtained 1 or 2 aspirates from the central portion of the tumor and deposited them into a cryovial containing either 1.0 mL RNAlater (Applied Biosystems/Ambion, Dallas, Tex) or 0.9 mL of cryopreservation media (80% fetal bovine serum [FBS] plus 20% Roswell Park Memorial Institute [RPMI] media 1640; Invitrogen, Carlsbad, Calif). RNAlater specimens ($n = 13$) were stored for less than 1 month at 4°C before RNA extraction. One hundred μL of dimethyl sulfoxide (DMSO) were added to each specimen collected in cryopreservation media ($n = 25$) immediately before freezing.

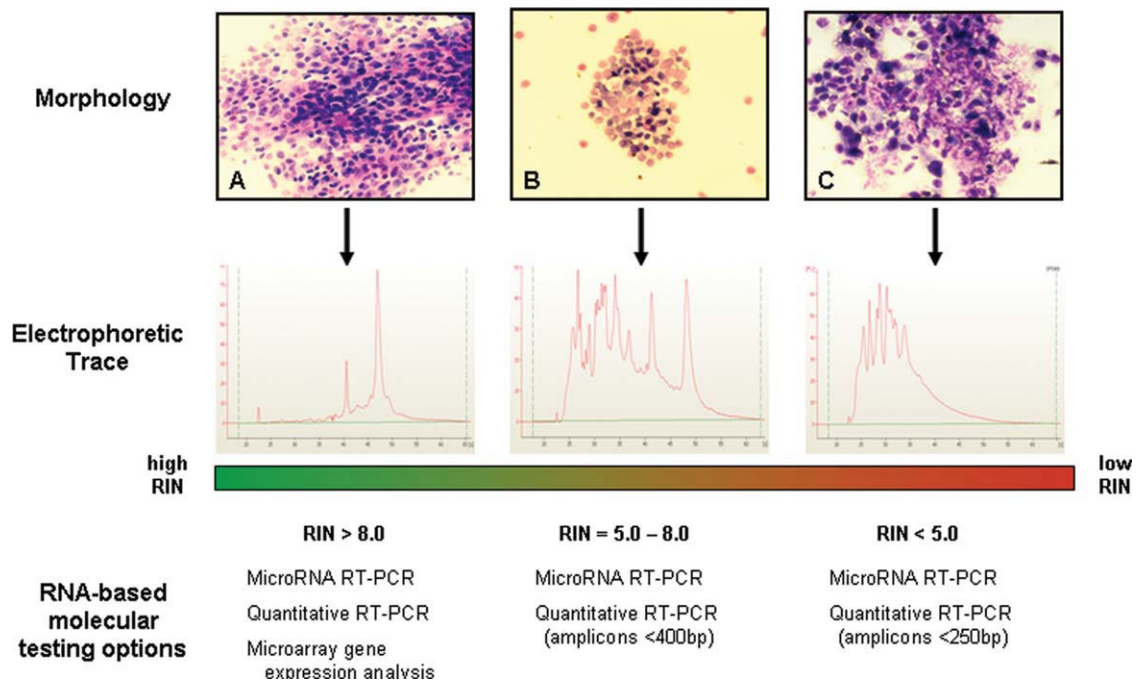


FIGURE 1. (A) Renal cell carcinoma (sample #8 in Table 2) was cryopreserved for 20 weeks and shows good morphology and intact RNA. (B) Thyroid papillary carcinoma (sample #10 in Table 2) was cryopreserved for 24 weeks and shows good morphology and moderate degradation of RNA. (C) Renal cell carcinoma (sample #7 in Table 2) was cryopreserved for 24 weeks and shows good morphology but with background necrosis and degradation of RNA.

The samples were then gently mixed by inversion and placed in an isopropanol-filled cryo 1°C freezing container (Nalgene Labware/Thermo Fisher Scientific, Waltham, Mass) at -80°C. After 12-24 hours, samples were moved to the vapor phase of a liquid nitrogen tank for long-term storage (1-27 weeks).

At varying time intervals, cryopreserved FNA specimens were quickly thawed by submersion in a 37°C water bath. Samples were agitated until there was only a very small portion remaining as ice. The samples were then immediately pipetted into a conical tube containing 10 mL cold RPMI and pelleted by centrifugation.

Morphological Evaluation

A portion of each thawed specimen was used to prepare a Diff Quik (Thermo Fisher Scientific, Waltham, Mass) stained cytopsin. In addition, a small portion of each FNA that was collected for deposition into RNAlater was also used to make a Diff Quik-stained cytopsin. These slides were prepared immediately upon aspiration from the tissue and before RNAlater exposure. All cytopsin

were reviewed by a pathologist (E. D. or E. O.) and evaluated for various morphological characteristics including: tumor cell type, percentage of necrosis, and for the cryopreserved samples, percentage of intact cells. The reported percentage of intact cells was determined by estimating the percentage of cells with intact nuclei.

RNA Extraction and Quality Control

The cells remaining after cytopsin preparation from both cryopreserved and RNAlater sample types were placed in lysis buffer, and RNA was extracted by using the Ambion RNAqueous-Micro extraction kit (Ambion, Austin, Tex). RNA integrity and yield were evaluated by analysis on the Agilent 2100 bioanalyzer RNA 6000 Pico Chip kit using the RNA Integrity Number (RIN) software (Agilent Technologies, Santa Clara, Calif). This software computes a score based on the full electrophoretic trace generated during analysis of the RNA species as opposed to focusing solely on the 18s and 28s rRNA bands. Scores computed by using the RIN algorithm can range from 1 (low RNA integrity) to 10 (high RNA integrity) (Fig. 1).

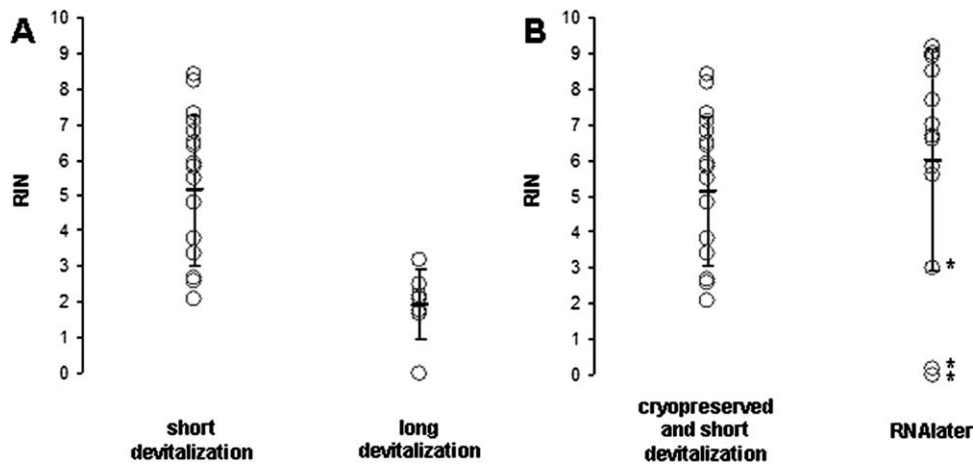


FIGURE 2. (A) Impact of devitalization time on RNA integrity is shown. FNA specimens collected from tissues with short devitalization times ($T_d \leq 6$ hours) have significantly higher average RIN ($P < .001$) compared with those with long devitalization times ($T_d > 19$ hours). (B) RIN scores are shown for cryopreserved versus RNAlater FNA samples. FNA specimens collected and immediately deposited in the RNA stabilizing reagent RNAlater and cryopreserved FNA specimens with short devitalization times do not have significantly different average RIN scores. *indicates $\geq 50\%$ cellular necrosis or lysis.

Statistics

Pearson correlation coefficients were calculated between the RIN and the following variables to assess the components of the protocol that had an effect on RNA integrity: 1) devitalization time (T_d), the time interval from surgical resection to FNA collection; 2) processing time (T_p), the time interval from FNA collection to freezing; 3) time frozen (T_f), the amount of time each sample remained frozen at liquid nitrogen temperatures; and 4) percentage necrotic cells.

RESULTS

Factors Affecting RNA Integrity

Twenty-five FNA specimens were collected from surgically resected human tissues for cryopreservation. These specimens were received in surgical pathology at our institution for the purpose of clinical diagnosis and staging and are typically stored at 4°C for <24 hours before gross examination. Specimens that were suspicious for cancer, as identified by the pathologists' assistants, were selected for FNA. Tumor cells were aspirated and immediately deposited into a media filled cryovial. Eighteen aspirations were collected from tissues with short T_d (0.2-6.0 hours postsurgical resection), and the remaining 7 samples were collected from tissues with long T_d (19-27 hours postsurgical resection). In previous work, we documented that

$T_d \leq 2$ hours did not result in a significant decrease in RNA integrity from surgical specimens.³ In these experiments, we extended the T_d to include samples up to 27 hours postsurgical resection. As shown in Figure 2A, we did see a significant decrease in RNA integrity, measured by average RIN values, between short and long T_d sample groups ($P < .001$). Average RIN scores for these 2 groups were 5.14 (SD = 2.1) and 1.93 (SD = 1.0), respectively. Of note, the RIN scores for the long T_d samples were uniformly poor. The highest score attained in the latter group of samples was 3.2, indicating that the RNA had been severely compromised.

Although many of the FNA samples collected during the first 6 hours of devitalization demonstrated acceptable RNA integrity, this was not true for all short T_d samples (Fig. 2). However, the variability in RIN scores for the short T_d samples was not significantly correlated with devitalization duration ($r = -0.14$). There were RIN scores as high as 6.4 and 8.2 at 6-hour and 4-hour postsurgical collection times, respectively, but there were also 2 samples with RIN scores of 3.8 and 3.4 that had been collected within 1 hour of surgical resection. A possible explanation for the low RIN scores is the presence of necrosis. In 5 of 8 specimens with short T_d and RIN < 5.0 , necrosis was identified during morphological evaluation of the FNA (Table 2). The average percentage of necrosis for short T_d samples with RIN < 5.0 was 29%, whereas for samples with RIN > 5.0 , the average

Table 2. Cryopreserved FNA Sample Characteristics

Sample Number	Tissue Type/Diagnosis	Devitalization Time	Processing Time	% Intact Cells	% Necrosis	RIN
1	Squamous cell carcinoma	0.2	0.3	70	30 ^a	3.8
2	Colon adenocarcinoma	2.3	0.8	100	10	6.8
3	Renal cell carcinoma	2.3	0.3	100	0	5.9
4	Pleomorphic sarcoma	3.0	0.3	<5	0	5.5
5	Hemangiopericytoma	22.3 ^a	0.3	>50	0	2.1
6	Renal cell carcinoma	3.5	0.3	>80	0	2.6
7	Renal cell carcinoma	22.0 ^a	1.3 ^a	40	30 ^a	1.8
8	Renal cell carcinoma	4.0	1.0	50	0	8.2
9	Pheochromocytoma	20.5 ^a	0.8	90	0	1.7
10	Thyroid carcinoma (papillary)	1.5	0.5	75	0	4.8
11	Renal cell carcinoma	4.8	0.8	20 ^a	0	2.6
12	Cholangiocarcinoma	2.0	1.5 ^a	50	15	2.1
13	Lung adenocarcinoma	2.3	2.8 ^a	0 ^a	50 ^a	2.6
14	Pleomorphic sarcoma	22 ^a	1.33 ^a	NA	NA	0
15	Melanoma	26.8 ^a	0.8	<5 ^a	0	3.2
16	Breast adenocarcinoma	1.0	0.1	90	0	7.1
17	Squamous cell carcinoma (benign lymph node)	2.5	0.3	30	50 ^a	2.7
18	Squamous cell carcinoma (tumor lymph node)	2.5	0.3	30	0	5.8
19	Lung adenocarcinoma	0.7	0.2	1 ^a	90 ^a	3.4
20	Lung adenocarcinoma	0.5	0.2	90	0	8.4
21	Fibromyxoid sarcoma	0.5	0.2	45	0	7.3
22	Cholangiocarcinoma	0.3	0.3	80	5	6.5
23	Thyroid carcinoma (papillary)	6.0	0.8	80	0	6.4
24	Renal cell carcinoma	19.25 ^a	2.25 ^a	NA	NA	2.5
25	Osteosarcoma	24.5 ^a	0.5	20 ^a	0	2.2

^aValues are outside the range of recommended acceptable values for each category.

percentage of necrosis was only 1.5%. In fact, all samples regardless of T_d with $\geq 30\%$ necrosis ($n = 5$) had severe RNA degradation ($RIN \leq 3.8$). In addition, in our independent data set comprising 13 FNA specimens collected and immediately deposited in RNAlater, the only cases with low RIN scores ($n = 3$; Fig. 2B *starred samples) were those associated with either $\geq 50\%$ cellular necrosis or significant cell lysis.

Factors With Minimal to No Effect on RNA Integrity

It is not always practicable in the clinical context of FNA collection to be able to immediately process samples for cryopreservation. In many hospitals, FNAs are performed in outpatient clinics, at the bedside, and in radiology, not in FNA clinics within pathology departments. Thus, there may be a significant lag time between aspiration and return to the cytopathology laboratory for completion of sample processing. To assess this variable in our data set, we recorded the amount of time aspirated samples

remained at 25°C before freezing (T_p). The range for T_p was 0.10-2.75 hours. Excluding samples that had $T_d \geq 19$ hours, we did not observe a significant correlation between T_p and RIN in our remaining samples. We did see a trend toward an inverse correlation between T_p and RIN ($r = -0.37$), but the significance level was low ($P = .07$). This may be partly explained by the finding that most of our samples were processed within 1 hour of collection. Only 2 samples (12 and 13; see Table 2) had $T_p > 1$ hour, and both of these samples had poor RIN scores. However, there was no correlation between T_p and RIN scores from samples processed within 1 hour of collection ($r = .02$). In fact, in 1 sample collected at the 1-hour point, we measured an RIN of 8.2 (Fig. 3A).

We also examined RNA integrity of cryopreserved FNA specimens as a function of the time the cells were kept frozen in liquid nitrogen (T_f). T_f ranged from 1 to 27 weeks, and as shown in Figure 3B, we did not observe any correlation ($r = 0.04$) between T_f and RIN scores from samples with short T_d and T_p (≤ 6 hours and 1 hour, respectively). There were a range of RIN scores across the

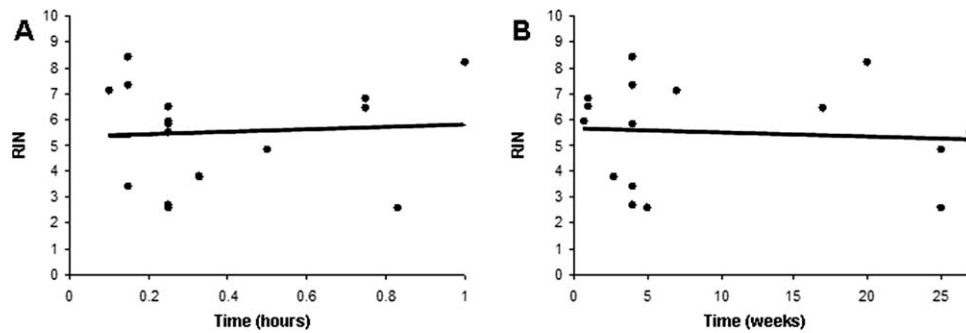


FIGURE 3. (A) RNA integrity versus processing time (T_p): FNA-sample RIN scores do not correlate with lag time between aspiration and freezing time (T_p) when specimens are processed for cryopreservation within 1 hour of aspiration. (B) RNA integrity versus frozen time duration (T_f): FNA-sample RIN scores do not correlate with total frozen time (T_f).

sample group. One of the highest RIN scores (8.2) recorded from any sample was from an FNA that was cryopreserved for 20 weeks. These data suggest that extended cryopreservation of FNA samples does not affect RNA integrity.

To evaluate whether the overall process of cryopreservation contributed to a measurable decrease in RNA integrity, we compared the RIN values from cryopreserved FNA specimens to RIN values from an independent set of 13 FNA specimens that were collected and immediately deposited in RNAlater. The RNAlater samples all had short T_d and T_p (<2 hours and 0.25 hours, respectively) and were not frozen. Removing from the analysis the cryopreserved FNA specimens with devitalization times (T_d) >19 hours and processing times (T_p) >1 hour (FNA short T_d and T_p samples; $n = 16$), we saw no significant difference between the average RIN scores of either sample group ($P = .6$). Cryopreserved FNA short T_d and T_p samples had an average RIN score of 5.5, whereas the average score for the RNAlater samples was 6.0 (Fig. 2B). Overall, 70% of cryopreserved FNA short T_d and T_p samples had RIN scores higher than 5.0 compared with 77% of RNAlater samples. In addition, there was no significant difference in RNA yield between the FNA specimens due to preservation method ($P > .05$). Average total RNA yields were 4.1 ng and 6.1 ng for cryopreserved and RNAlater specimens, respectively.

Morphological Assessments on Cryopreserved FNAs

The utility of cryopreservation as a method for banking of FNA material for future molecular testing depends not

only on the preservation of RNA but also on the maintenance of morphologic detail for post-thaw evaluation by a pathologist. Although the addition of cryoprotective agents and the use of controlled rate freezing procedures generally protect cellular integrity, the process of cryopreservation may still cause some cellular damage and/or morphological artifact. To determine whether these had occurred, we evaluated the cellular morphology of a variety of cryopreserved tumor types including sarcomas, carcinomas, and various other neoplasms from diverse organ sites including breast, lung, kidney, and thyroid among others (Table 1).

The cryopreserved samples demonstrated good tumor cell morphology in specimens with intact cells (19 of 25 samples). Diagnostic morphologic features such as high nuclear:cytoplasmic ratio, irregular nuclear contours, intranuclear inclusions, nuclear grooves, and/or prominent nucleoli were apparent in the malignant cells in the cryopreserved specimens. Necrosis greater than or equal to 30% was identified in 5 of 25 cases, and these cases yielded degraded RNA (average RIN = 3.5). Degenerating cells and/or damaged cells were identified in 6 cases. In the 6 cases with a high percentage of cellular damage ($\leq 20\%$ intact cells), there was also severe RNA degradation (average RIN = 3.2; Table 2). Rarely, artifacts including eosinophilic cytoplasm and vacuolated-appearing cytoplasm were identified, and these could possibly be related to cryopreservation.

DISCUSSION

Information gleaned from molecular research of human cancers is resulting in the rapid development of targeted

pharmaceutical therapies⁴⁻⁶ and novel biomarker detection assays that aid in diagnostic, prognostic, and treatment decisions.⁷⁻⁹ The continued successful development of this type of testing is contingent on access to clinically annotated primary human tissue samples for experimental and test-validation purposes. Pathology departments are increasingly finding that they have become crucial players in this effort and must undertake a leadership role in their institutions' efforts to establish translational research centers and practice cutting edge clinical care.

Cytology specimens, particularly FNAs, have received little attention as a source of material for clinical molecular testing, with the notable exception of human papilloma virus (HPV) detection in cervical cells. Recently, a number of studies have shown promising results for the use of FNA specimens in ancillary molecular testing. Zhang et al. described the use of lymph node FNAs for polymerase chain reaction (PCR) confirmation of T-cell-receptor oligoclonality.¹⁰ For aid in treatment decisions in nonsmall cell lung cancer (NSCLC), the presence of epidermal growth factor receptor (EGFR) mutations was successfully evaluated by using PCR from tissue procured by image-guided FNA.¹¹ In addition, researchers at the University of Pittsburgh Medical Center analyzed FNA specimens with the morphological diagnosis of "follicular lesion of undetermined significance/atypia of undetermined significance" for BRAF and RAS point mutations and RET/PTC and PAX8/PPAR γ gene rearrangements by using PCR and reverse transcription PCR (RT-PCR), respectively. Positive test results, ie, PCR detection of mutation(s), were associated with a morphological diagnosis of carcinoma in the corresponding excisional biopsy in 100% of cases.¹² These same molecular techniques could potentially be applied to other FNA sampled tissues (liver, breast, pancreas) to aid clinical decisions.

Although some of the above tests can be performed on the FFPE tissue blocks that exist in many pathology archives, these are suboptimal materials for molecular testing because of nucleic acid degradation and cross-linking that result from formalin fixation that, in turn, can lead to ambiguous test results. Working with fresh tissues is optimal but often impractical given the time constraints of laboratory workflow. As a result, many institutions have created centralized frozen tissue biorepositories that are staffed with dedicated, trained personnel. Specimen handling and storage in these facilities is designed specifically

to preserve the integrity of biomolecules useful in downstream molecular assays. Underscoring the importance of such a resource, the National Cancer Institute (NCI) created the Office of Biorepositories and Biospecimen Research. Its activities include the publication of the NCI Best Practices for Biospecimen Resources, which contains guidelines for the development of biorepositories including advice on how to best maintain the integrity of these important specimens¹³ (http://www.allirelandnci.com/pdf/NCI_Best_Practices_060507.pdf).

Although frozen-tissue banks are now recognized as crucial resources for the future growth of clinical molecular test development, the feasibility of prospectively banking cytology specimens has not been evaluated. The major utility of developing such a resource would be the preservation of diseased cell types that would not be available for research or clinical testing by other means. Certain conditions and cell types, such as metastatic disease and premalignant tumors, may only ever be sampled via cytology, as surgical excision is not always the best option for the patient's clinical care. Translational research and molecular test development in these areas would be greatly facilitated if there were protocols set in place for banking FNA specimens.

Our goal in the present study was to test whether cryopreservation of patient FNA specimens could serve as a suitable method for banking diagnostic material for future nucleic acid—based molecular testing. We primarily focused on the quality of RNA that could be recovered from such specimens because RNA is the nucleic acid most susceptible to degradation. To maximize the number of molecular testing applications using RNA, a high level of RNA integrity is most desirable (Fig. 1).¹⁴⁻¹⁷ DNA integrity was not evaluated here, but in general, it is much more resistant to degradation than RNA. Our findings can be summarized as follows. To maximize RNA integrity (high quality RNA), devitalization time (T_d) should be kept to 6 hours or less and postaspiration processing time (T_p) to 1 hour or less. For the majority of clinically obtained FNA samples, this would seem to be quite feasible. Conversely, the amount of time samples are frozen (tested up to 27 weeks) does not seem to affect the quality of the RNA recovered. Third, the presence of cellular necrosis associated with the sample appears to decrease the overall quality of RNA recovered. Although we had too few samples exhibiting high levels of necrosis

to reach statistical significance using univariate analysis, the percentage of necrosis did appear to be a statistically significant variable negatively affecting RIN when examined across all samples by using multivariate linear regression analysis ($P < .05$; data not shown). In further support of this interpretation, we did observe that all samples with $>30\%$ necrosis had associated RNA degradation (Table 2). A limitation of our study, because of the diversity of specimens, was that we were not able to make any judgment regarding to the effect of cryopreservation based on tissue type. It may be that the intrinsic characteristics of different cells will render them more or less susceptible to rupture by osmotic pressure during the freezing and/or thawing process. Finally, our evaluation of the morphological characteristics of cryopreserved FNAs showed that while there may occasionally be some artifactual changes in morphology, the ability to identify diseased versus non-diseased cell types is maintained. This is important because it permits the use of purification processes that depend on visual identification of cells, such as laser-capture microdissection.

Cryopreservation is not the only option available for preservation of nucleic acids. Reagents based on chaotropic salts and phenol (TRIzol, Invitrogen; TRI Reagent, Ambion) inactivate nucleases but destroy morphology. A few other methods using ethanol and other proprietary reagents (RNAlater, Ambion; PreservCyt, Hologic, Bedford, Mass; FineFIX,¹⁸ Milestone, Kalamazoo, Mich) stabilize nucleic acids and, to some degree, preserve morphology. However, with cryopreservation, there is not only preservation of morphology and nucleic acids, but there is the potential additional benefit of preserving viability as well. The cryopreservation procedure described here is one that we routinely use for preservation of neoplastic hematopoietic cells; these specimens routinely demonstrate $>80\%$ viability upon thawing. Maintaining tumor cell viability would allow for the use of these specimens in downstream functional assays including, conceivably, drug toxicity assays.

In summary, molecular marker detection is becoming an increasingly important ancillary tool for diagnosis and clinical management. It is important that pathology departments be prepared for the development, validation, and implementation of new molecular diagnostic assays. Banking of cryopreserved cytology specimens could be an important cost-effective asset in this development.

CONFLICT OF INTEREST DISCLOSURES

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