A Review of the Issues, Evidence, and Opportunities

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Context.—Molecular diagnostic applications that use microarrays to analyze large numbers of genes simultaneously require high-quality mRNA. As these genome-wide expression assays become more commonly used in medical practice, pathologists and oncologists will benefit from understanding the importance of obtaining high-quality RNA in order to generate reliable diagnostic and prognostic information, especially as these relate to cancer.

Objective.—To review the effects that different tissue preservation techniques have on RNA quality and to provide practical advice on changes in tissue acquisition and handling that may soon be needed for certain clinical situations.

Data Sources.—A review of recent literature on RNA quality, tissue fixation, cancer diagnosis, and gene expression analysis.

Conclusions.—Studies have consistently shown that frozen tissue yields more intact RNA than formalin-fixed, paraffin-embedded tissue. The chemical modification, cross-linking, and fragmentation caused by formalin fixation often render RNA unsuitable for microarray analysis. Thus, when expression analysis involving hundreds or more than 1000 gene markers is contemplated, pathologists should consider freezing a specimen within half an hour (preferably within minutes) of surgical resection and storing it at −80°C or below. In coming years, pathologists will need to work closely with oncologists and other clinicians to determine when saving frozen tissue for microarray expression analysis is both practical and necessary. In select cases, the benefit of implementing a few extra tissue-handling steps may improve diagnostic and prognostic capability.

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The sequencing of the human genome, together with advances in the design, manufacturing, standardization, and performance of microarrays, has opened the door for routine use of gene expression profiling in clinical practice. As microarrays have become more powerful and reliable, researchers and clinicians have begun applying full-genome expression analysis in a variety of challenging clinical situations. One of the earliest applications in oncology involved the profiling of diffuse large B-cell lymphomas into a germinal-center B-like group and an activated B-like group, with patients in the latter group having significantly lower 5-year survival rates. A third category, primary mediastinal diffuse large B-cell lymphoma, was subsequently identified, with microarrays again able to predict survival after chemotherapy. Another line of research used genome-wide expression profiles to distinguish acute lymphoblastic leukemia (ALL) from acute myelocytic leukemia (AML)—admittedly a straightforward typing feat for the pathologist using a microscope—but then went further to identify a new subclass of leukemia dubbed mixed-lineage leukemia (MLL) based on selective expression of genes. Similarly, other research groups have now used genome-wide expression analysis to identify previously unrecognized subsets of cutaneous melanomas, breast carcinomas, colorectal cancer, and adult soft tissue sarcoma. Microarrays also have now been used to make multiclass distinctions among highly related tumor types, such as the adenocarcinomas, to stratify patients with cancer into subgroups with distinct clinical manifestations and different responses to therapy and to predict chemotherapy response.

As the final technical and informatic barriers to genome-wide expression testing have been removed, several microarray-based cancer tests are moving toward the laboratory marketplace, and the focus is shifting to the practical procedural variables that will influence the accuracy of results. In particular, the quality of mRNA in the tissue sample handled by the pathologist has emerged as a crucial factor in obtaining the best diagnostic and prognostic information from these powerful new genomic tests.

Frozen tissues are widely acknowledged to provide a better source of intact RNA and DNA than formalin-fixed tissues for genome-wide microarray expression analysis or other assays involving nucleic acids. Preservation of frozen tissue is fairly common in certain well-defined situations in many larger pathology laboratories when mo-
lecular analysis is required. For evaluations of lymphoid tissue, for example, some high-volume cancer centers prefer to save or freeze extra tissue for immunoglobulin and T-cell receptor (TCR) gene rearrangement studies to avoid the fragmentation of nucleic acids known to be caused by formalin. In Southern blot analysis, for example, non-fragmented DNA may be preferable for improved detection of immunoglobulin and TCR rearrangements.

In most oncology situations, however, pathology departments still rely on formalin-fixed, paraffin-embedded (FFPE) tissue as their standard method of preservation. Fortuitously, these FFPE tissues can also be analyzed for expression of a limited number of select genes using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) or other specialized techniques. Indeed, for applications that require relatively limited numbers of genes (eg, <100), archived FFPE tissues have proven to be a viable source of expression data for retrospective studies and for the development of clinical assays such as the Oncotype DX assay (Genomic Health, Inc, Redwood City, Calif). Recent studies have also reported on partial successes using a novel technique to extract RNA from formalin-fixed tissues for microarray analysis. Notwithstanding this ability to extract and analyze mRNA from some archived paraffin blocks, this article will present data showing that the mRNA in FFPE tissue is often highly degraded compared with the mRNA in frozen tissue. The literature reviewed in this article strongly suggests that degraded mRNA is an inferior starting point for the new generation of genome-wide expression tests, especially those that will be applied in complex diagnostic situations where relatively subtle alterations in hundreds or thousands of expressed gene markers may need to be analyzed simultaneously. Indeed, the only marketed microarray-based gene expression assay to date is approved for use only with fresh frozen tissue.

Thus, pathologists may need to consider setting aside frozen tissue from solid tumors, as they already do for lymphomas, for potential genome-wide expression analysis. For clinicians in institutions with access to proper equipment for freezing specimens, recognizing those special situations when such tissue acquisition and handling steps are prudent will be critical. In coming years, pathologists will need to work closely with their oncology colleagues to monitor a range of issues related to how tissue handling has an impact on genomic expression profiling.

This article reviews these issues with a focus on the growing need to capture adequate mRNA at the time of the initial biopsy procedure. After an overview of the objectives for genome-wide expression profiling, the effect of formalin on the integrity of mRNA and on expression test results will be summarized. Situations in which the quality of the tissue mRNA will be critical to genome-wide expression analysis, and to patient management and outcomes, will also be described.

**GENOME-WIDE EXPRESSION ANALYSIS IN CLINICAL ONCOLOGY: WHY MICROARRAYS REQUIRE HIGH-QUALITY RNA**

The early rapid evolution in microarray technology was accompanied by a lack of coherency and standards among the different platforms. While this created some doubts about the reliability of results, many studies have now confirmed the reliability of microarray platforms. For example, the US Food and Drug Administration–initiated MicroArray Quality Control study recently demonstrated that microarray results show good reproducibility within and between test sites, as well as good comparability between different platforms.

Although current expression microarrays vary widely in design, the essential reaction at the heart of all microarray technology is identical: the hybridization of patient-derived mRNA or copy mRNA to complementary DNA on a solid support. Thus, as outlined next, any degradation of the patient’s input mRNA will tend to obscure the overall expression profile. The impact of the degradation will depend in part on the design of the assay. For example, microarrays using oligo-dT primers for reverse transcription may be more sensitive than non–oligo-dT methods to the loss of certain full-transcript RNA messages. Also, those platforms that do not rely on either amplification or reverse transcription (such as the DNA branch method) are likely to have entirely different requirements in terms of nucleic acid quality in the starting material.

Information obtainable by techniques such as immunohistochemistry (IHC) or expression analysis of a limited number of genes by RT-PCR is composed of only a limited number of data dimensions; however, the information sought with microarray-based expression analysis is much more complex and highly multidimensional. This goal of “high multiplexy” with the microarray explains why higher-quality input RNA is essential. Simply put, more data points are required to evaluate the numerous clinical scenarios and outcomes under consideration. Although real-time RT-PCR techniques have proven capable of salvaging an mRNA signal from heavily degraded FFPE tissues, these techniques are generally limited to analysis of a panel of 10 to 200 carefully selected genes. This RT-PCR approach can, in fact, be extremely valuable in certain clinical situations. The Oncotype DX Assay illustrates one strategy wherein microarray was used to discover some of the relevant genes, and then the RT-PCR–based commercial assay is developed for use on FFPE tissues.

A similar strategy has been used to identify a 2-gene ratio (ratio of homeobox 13 [HOXB13] to interleukin 1B receptor [IL17BR]) that may predict recurrence and survival in women receiving adjuvant tamoxifen. Given the ready availability of FFPE specimens, the practicality of such a 2-step approach to test development is clear; however, the complexities and potential limitations of this approach, including a possible sacrifice of sensitivity and accuracy, have also been described. One of the chief technical limitations of the RT-PCR technique involves the need to create primers and probes specific for all of the mRNAs of interest. This requirement places a logistical limit on the number and type of expressed genes that can be analyzed. Such a narrow analysis of expression may be perfectly adequate in clinical situations in which only simple and specific binary classifications are required (eg, as with the Oncotype DX Assay that asks, “What is the likelihood of systemic disease recurrence in node-negative estrogen receptor–positive breast cancer?”). However, only a genome-wide expression microarray can measure several hundreds or thousands of genes simultaneously to answer the intrinsically more complex, multiclass questions that are common in oncology. For these genome-wide microarrays to perform optimally, the highest-quality input mRNA will likely be required and, as discussed
in the next section, the standard formalin-fixed tissue is often inadequate.

**EFFECT OF FORMALIN FIXATION ON mRNA QUALITY**

Formaldehyde was first used to fix tissues in the 1890s. Unlike alcohol fixatives, it produced only slight shrinkage and distortion of tissues.61,62 Today, formaldehyde as 10% neutral-buffered formalin is still the most common fixative used by pathologists. Unfortunately, the chemical properties that make formalin an ideal fixative for preserving tissue structure for visual or antigenic evaluation also cause it to degrade mRNA macromolecules in a severe and often random manner.25,26,83 The main impacts involve the addition of monomethylol groups to RNA bases, cross-linkage of nucleic acids to proteins, and fragmentation (Figure 1). These formalin-induced impacts are discussed after the following brief discussion of the potential for mRNA degradation due to delay in tissue preservation.

The Danger of Delay: Enzymatic Degradation

Messenger RNA is, partly by design, an ephemeral molecule. Regulation of cytoplasmic RNA degradation is one of the cell’s mechanisms for controlling gene expression, and it can be triggered by alterations such as changes in nutrient or hormone levels, warm ischemia, or tissue hypoxia.84–87 Although relatively stable thermodynamically, RNA is rapidly digested by RNase enzymes, which are ubiquitous in most tissues. This is why mRNA begins to degrade within the first hour after surgical excision of tissue if the tissue is not frozen.87,88 The RNA loss is greatest in tissues harboring high levels of endogenous RNases and proteases, such as the pancreas, gall bladder, and skin.89 Many eukaryotic mRNAs have half-lives of 30 minutes or less.90 Certain mRNAs, such as those for cytokines and proto-oncogenes, contain sequences that render them highly labile to enzymatic degradation—again, a likely regulatory mechanism for governing expression in the natural cell environment.91,92 For the pathologist, prevention of this degradation of RNA by RNases is the prime goal of rapid temperature reduction after tissue collection.

The extensive degradation of mRNA that can occur with a delay in fixation or preservation has been well documented. In one study of decades-old archived tissue blocks obtained at autopsy, Mizuno et al.93 found that the integrity of the mRNA in liver and thyroid tissue was diminished in those cases with the longest postmortem time before collection. Several other studies have correlated the length of time before completion of tissue fixation and embedding with the amount of enzymatic degradation in the RNA.33,34,83,88,94,95 In one of these studies, Florell et al.83 directly evaluated the impact of alternative tissue handling procedures on the quality of RNA. Freshly excised normal skin was taken from patients during Mohs surgery. One portion of the tissue was placed immediately into RNA-later (Ambion, Inc, Austin, Tex) for 24 hours and then stored at 4°C for 2 to 6 weeks. Another portion of the tissue was held for 30 minutes at room temperature during dissection in surgical pathology before being flash frozen at −20°C. Purification and analysis of the total RNA in these specimens showed that the tissues preserved immediately after biopsy produced distinct ribosomal bands of high-molecular-weight RNA, whereas the tissues held at room temperature resulted in smearing of lower-mo-

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**Figure 1.** How formalin fixation damages mRNA. The timing and method of tissue preservation have a direct impact on mRNA quality and, hence, the potential for more accurate diagnosis and prognosis based on genome-wide expression analysis.
The authors attribute the degradation to the 30-minute delay before freezing.

The Nature of the Formalin Damage: Methylation, Cross-linking, Fragmentation

Formalin preserves tissue structure mainly by creating cross-links between proteins. While protein-protein cross-links are the predominant reaction, extensive intermolecular cross-linking of proteins with nucleic acids also occurs.82 Specifically, formaldehyde reacts with the nucleic acid bases to form an N-hydroxymethyl (methylol) group (–CH2OH), followed by a slower electrophilic attack of this N-methylol group on amino acid bases to form methylene bridges between two amino groups.80 While this action makes neutral-buffered formalin (10%) ideal for hardening tissues for later microscopic and IHC analysis, the hydroxymethylene cross-linking between proteins and nucleic acids56,97 also makes it difficult to extract intact mRNA from formalin-fixed tissues.98–101

The adverse effect on nucleic acid quality is dramatic. In one study, nearly 40% of the adenine bases in synthetic RNA acquired monomethylol additions after fixation in formalin for several days.27 Proteinase K digestion can relieve some elements of cross-linking, and heating with guanidinium can disrupt some of the noncovalent interactions that prevent RNA solubilization and isolation; both methods are used routinely to isolate RNA,27,28,36 but neither can correct the other major impact of fixation (ie, fragmentation). RNA extracted from FFPE tissue is most often present in fragments less than 200 or 300 bases in length.27,34,36,100

The results from one animal study clearly illustrate the detrimental impact of formalin fixation on RNA quantity and transcript size. While the yield of total RNA from frozen liver was 625 μg/100 mg tissue, the yield in formalin-fixed tissue was only 30 μg/100 mg.102 Further, analysis of RNA length showed that the formalin-fixed tissue reflected the expected distribution of sizes, including the classic peaks at the ribosomal 18S and 28S equivalents (Figure 2, A), whereas the formalin-fixed tissue was extremely degraded, with the major peak reflecting a predominant molecular size of only 200 bases (Figure 2, B). In addition, RT-PCR analysis showed a significant decrease in the amount of β-actin mRNA in formalin-fixed and Carnoy-fixed tissues but not in frozen samples (Figure 3). A more recent study showed that an optimized RNA extraction technique could improve the RNA yield from FFPE tissue to 30% of the amount of RNA extracted from fresh tissue.103 Although pretreatment with RNAlater be-

**Figure 2.** Preservation of total RNA: freezing versus formalin fixation. Electropherograms from an Agilent 2100 Bioanalyzer of total RNA extracted from frozen (A) and formalin-fixed (B) rat liver tissue stored for 3 months after sample acquisition. Note the presence of ribosomal 18S and 28S peaks and the full range of RNA sizes in frozen tissue; note shift to left (smaller molecular weight with major peak at 200 bases) in formalin-fixed tissues. Adapted from Benchekroun et al102 with permission from Lippincott Williams & Wilkins, copyright 2004.

**Figure 3.** Freezing versus 3 methods of fixation. Percent template detection for β-actin transcript (244–base pair amplicon) in commercial rat liver reference. Analysis by quantitative polymerase chain reaction assays shows significantly decreased template detection in Carnoy- and formalin-fixed samples versus the reference. NA, not applicable; P values not calculated because fewer than 3 observations.

**Figure 4.** Fragmentation of RNA increases with storage time in formalin-fixed tissue. Analysis of RNA (with Agilent 2100 Bioanalyzer, RNA 6000 Nanochip) from 12 breast cancer tissue specimens collected during a 17-year period in one institution using a consistent formalin fixation protocol. Note larger molecular weights of RNA in tissue archived for just 1 year versus sizes seen in those archived for 6 or 17 years, suggesting time-dependent fragmentation of formalin-fixed, paraffin-embedded tissue even after dehydration and embedding in wax. (Lanes M1 and M2 contain reference RNA.) Adapted from Cronin et al102 with permission from the American Society for Investigative Pathology, copyright 2004.

NS, not significant (as reported; no value given); P values as reported. Adapted from Benchekroun et al102 with permission from Lippincott Williams & Wilkins, copyright 2004.
fore fixation further boosted the yield to approximately 80% of that in fresh tissue, the quality of the RNA in that pretreated FFPE remained poor (i.e., highly fragmented).

### The Extent of the Damage

How much mRNA is lost by chemical fixation? This depends primarily on the methods used for tissue preservation and RNA isolation. Total RNA quality can be assessed by determining the size distribution of the extracted RNA with agarose gel electrophoresis or an automated microcapillary electrophoretic analyzer (e.g., Agilent 2100 Bioanalyzer, Agilent Technologies, Quantum Analytics, Foster City, Calif). The quantity and quality of mRNA can be estimated by attempting to detect expression levels of known transcripts of various sizes with RT-PCR. For example, a study of fixative effect on RNA integrity in endometrial tissues on which RT-PCR was performed for various amplicon sizes of 2 genes (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] and β-globulin) showed that longer mRNA fragments were present in much lower quantities than shorter mRNA fragments in fixed tissues. The investigators found that the RNA fragmentation was much more extensive in formalin-fixed tissues than in tissues that were fixed in acetone or RNAlater (Table 1).

The reduced ability to detect changes in expression of key genes in fixed tissues has also been documented in studies that used oligonucleotide assays to monitor expression. For example, shown, that formalin-fixed tissues allowed detection of about half as many genes as could be detected with matched frozen tissues. A more recent microarray-based analysis focused on the detection of genes regulated by p38 inhibitor treatment to gauge the impact of preservation methods on expression analysis. In the frozen samples, a total of 799 genes exhibited at least a 1.5-fold change, whereas in the formalin-fixed samples, a total of only 356 genes were detected with a 1.5-fold change. Laser capture microdissection and/or novel RNA purification techniques have very recently been shown to improve the quality of the input RNA from FFPE tissues, but even in these studies the RNA quality was lower than that in frozen specimens. Coudry and coworkers reported a significant concordance between expressed genes in FFPE and frozen samples, with only a slight loss of sensitivity in the formalin-fixed specimens. However, in the Penland et al study of specially processed FFPE specimens, only 24% were suitable for microarray analysis; this low yield is unacceptable for any microarray-based diagnostic test intended for clinical use. These authors also observed a significant loss of gene nature information in the FFPE specimens, particularly among the rare or unstable transcripts.

Thus, using both overall and gene-specific measures of RNA quality, several investigators have documented the relative impact of various tissue fixation methods. Practically all published studies indicate that formalin fixation produces the greatest degree of RNA degradation (Table 2). While non-cross-linking preservatives improve RNA quality relative to that obtained with formalin fixation, many of them contain solvents that may also destroy the integrity of certain tissues and thereby preclude further histologic analysis of the tissue. At present, although formalin remains the unquestionable gold standard for most diagnostic human histopathology, flash freezing by immersion in liquid nitrogen seems to produce the highest mRNA quality for certain microarray-based analyses of gene expression.

### The Implications of mRNA Damage

Many factors affect the extent of the formalin damage to RNA. The degree of formalin-induced modification may, for example, vary with the concentration, temperature, pH, and penetration time of the formalin. The types, amount, and shape of the tissue will also affect overall fixation results, whereas the duration of fixation and the choice of embedding method, including the choice of buffer in the formalin, may directly affect RNA preservation.

Some of these factors, such as prefixation delay, fixation period, and storage time, are somewhat predictable and generalized in their impact on the RNA content. For example, Figure 4 shows increased RNA fragmentation with increased archive storage time. In addition, Cronin et al reported that the loss of average real-time RT-PCR signal was approximately 90% between samples collected in 2001 and those collected in 1985 (data not shown). The mechanism of this RNA loss in older blocks is unknown. Although the amount of mRNA lost was extensive, the investigators still found that they could quantitate relative levels of mRNA expression with RT-PCR by normalizing the expression level of the gene of interest to a reference set of genes. Even when the FFPE samples showed a 75% loss of intact amplicon template compared with paired frozen samples, use of reference genes to correct for the deterioration produced a good correlation in expression profiles for 42 test genes.

Although sample-handling variables that lead to RNA degradation can be ameliorated with normalization techniques, this assumes that the fixative has an equivalent impact on all mRNA species. However, several groups have shown that fixation can disproportionately increase or decrease the level of an mRNA of interest relative to a so-called housekeeping gene mRNA that is being used to normalize the data. The apparent variable sensitivity of different mRNA species to fixation implies that quantitation of mRNA expression from fixed tissue may provide erroneous results and provides further evidence for the importance of obtaining frozen tissue if performance of genome-wide expression analysis is anticipated.

It must be emphasized, however, that all expression assays will require their own process of validation before they can be applied clinically. If a test is designed based

### Table 1. Extent of Fragmentation in Tissues Fixed in Formalin, Acetone, or RNAlater, Measured by Relative Losses in Ability to Detect Longer Transcripts

<table>
<thead>
<tr>
<th>RNA Quality</th>
<th>GAPDH 225</th>
<th>GAPDH 406</th>
<th>β-Globulin 166</th>
<th>β-Globulin 310</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin</td>
<td>7×</td>
<td>6×</td>
<td>3×</td>
<td>3×</td>
</tr>
<tr>
<td>Acetone</td>
<td>401×</td>
<td>976×</td>
<td>15×</td>
<td>23×</td>
</tr>
<tr>
<td>RNAlater</td>
<td>4096×</td>
<td>1096×</td>
<td>35×</td>
<td>109×</td>
</tr>
</tbody>
</table>

*Table shows ratio of long transcripts to shortest glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript (121 bp) in 3 fixatives as detected by real-time polymerase chain reaction; note that higher ratio indicates more fragmentation. Adapted from Paska et al with permission from Lippincott Williams & Wilkins, copyright 2004.

expression test results, the loss of any mRNA due to degradation conserving transcript abundance is critical to experiments. In microarray procedures, any formalin damage to preserves in their relative distribution—meaning that the changes for misleading data are high. Freezing of specimens soon after biopsy may help deliver the higher-quality RNA that is required for genome-wide expression analysis.

PRACTICAL ISSUES AND NEW OPPORTUNITIES

What do these facts about the comparative quality of mRNA in formalin-fixed versus frozen tissues mean for the pathologist and the oncologist? On a very practical level, they suggest a compelling reason to consider saving extra or separate tissue samples for genome-wide expression analysis. In addition, several of the studies just reviewed indicate the types of clinical situations where these relatively simple changes in sample acquisition and handling may lead to new opportunities for improved diagnosis and prognosis.

Recommendations on Freezing Tissue for Expression Analysis

With growing use of genome-wide microarrays in the pathology laboratory, pathologists and oncologists will increasingly need to consider saving separate samples during certain biopsy procedures: one fixed for standard morphology and IHC, and the other frozen for expression analysis. While such an extra step can easily be accomplished in high-volume cancer centers and other institutions with the proper facilities for freezing specimens (e.g., liquid nitrogen), oncologists and pathologists in small offices or community practice may admittedly have difficulty implementing this recommendation. Other practical issues related to this recommendation to freeze tissue for microarray analysis involve biopsy timing and methods,
preservation methods, and overall staff communication and protocols.

In terms of biopsy timing, in cases where genome-wide expression analysis is considered, the separate or split samples may need to be taken during the initial biopsy procedure. Capturing both samples during the initial biopsy allows for future access to the tissue (assuming adequate quantities are available), is easier on the patient, and also avoids the delay and expense of a possible follow-up biopsy. Of course, in some cases the frozen sample may not need to be evaluated, and this needs to be considered in workflow and cost-effectiveness analyses. Overall, however, saving 2 tissue samples during the initial biopsy may be worth the minimal up-front effort and expense because it keeps the door open for later genomewide expression analysis. For pathologists, the practical challenge will be remaining alert for cases in which microarray-based testing might have an impact on patient management.

Based on this review of the literature, immediate freezing in liquid nitrogen appears to be the current best choice for preserving tissue for genomic analysis. Specifically, to prevent tissue ischemia and hypoxia and the related RNA degradation, researchers have recommended that approximately 0.1 cm³ of the tissue specimen, which should yield sufficient mRNA for most studies, be snap frozen in liquid nitrogen within half an hour (preferably within minutes) of surgical resection and stored at −80°C or below. This conclusion was also reached by the Tumor Analysis Best Practices Group, a group of investigators using Affymetrix oligonucleotide microarrays, the commercial platform with the widest usage in current clinical trials. In terms of tissue handling, the Tumor Analysis Best Practices Group recommends the following:

All tissue samples should be flash frozen within minutes of surgery and stored at −80°C or below. Samples should also be kept in small, airtight containers and kept from drying out during frozen storage by placing fragments of ice in with the sample.

While freezing of tissue within minutes of biopsy is the preferred method, this admittedly may not be practical in every setting. In particular, as already mentioned, smaller office practices may not have access to adequate freezing facilities. Clinicians in such settings will undoubtedly need to weigh the clinical value of cancer microarray results from larger academic centers to determine whether adding freezing facilities and changing biopsy protocols for certain clinical situations are justifiable. In the interim, compromise solutions to preserve quality mRNA may be considered. For example, since brief transport of tissue on ice before fixation and processing appears to have minimal impact on RNA quality and expression, a practical approach in the clinical setting where patients are often physically dispersed is for clinicians to send the samples by routine iced transport for centralized processing. A recent study showed that tumor samples can be frozen and thawed at least 3 times without compromising the RNA integrity and genetic expression profile. Another potential compromise solution for settings without access to adequate freezers is, as discussed below, saving a portion of the biopsy in an RNA-friendly preservative.

For those cancer centers where freezing of specimens is already routine, other questions will need to be addressed. There are, of course, many different protocols for freezing tissues, with subtle and not-so-subtle variations in parameters (eg, the delay before exposure to freezing temperature, the possible addition of a nonformalin preservative or additive, the sizing of tissue, the storage container, the speed of freezing, and the final temperature). The impacts of these variables on mRNA quality and expression results are not yet clear. The need for standardization in tissue collection and handling has been emphasized by the many regulatory, scientific, and commercial groups with interests in full-genome expression testing (eg, US Food and Drug Administration, National Cancer Institute, Affymetrix, Inc). While specific recommendations are still evolving, there is already strong consensus that warmer storage environments permit RNA degradation and that samples slated for nucleic acid microarray analysis should be rapidly frozen and then stored at −80°C in a mechanical freezer or in liquid nitrogen.

Based on such consensus, most clinicians planning to use expression analysis are developing simple institutional protocols that call for rapid freezing of more tissue samples. At the Mayo Clinic, for example, the protocol for freezing solid tumors will likely be similar to that already employed for lymphoid tissues. Currently, when a diagnosis of lymphoma is clinically suspected, one portion of the tissue is frozen in the cryostat at −20°C using an embedding medium for frozen tissue as the mounting medium, and another portion of the tissue is kept as fresh as possible. If lymphoma is still a possible diagnosis after frozen section evaluation, part of the fresh tissue is snap frozen in liquid nitrogen and stored at −80°C. This frozen tissue is then retrieved if gene rearrangement studies are warranted to confirm the diagnosis. If only small amounts of tissue are available, tissue is transferred from the cryostat to the −80°C freezer. The other portion of the fresh tissue is then placed in formalin or other fixatives, such as B5 in the case of lymphoid neoplasms, for later processing and morphologic and IHC evaluations.

Based on experience in this type of workup for lymphomas, many pathology laboratories affiliated with larger cancer centers appear to have adequate training and facilities to implement a similar tissue handling protocol for solid tumors. The indication to freeze tissue from solid tumors will need to be discussed with the clinician but will surely evolve with experience. Cases where a biopsy is performed in an office setting may present an initial challenge. In some of these cases, where typically the entire tissue specimen is placed immediately in formalin, discussion and planning will be necessary to ensure that a frozen section is requested by the clinician. These will be carefully selected cases in which gene expression analysis or any molecular technique requiring high quality nucleic acids is anticipated. As mentioned above, iced transport to an available freezer location may be one practical option for handling the office biopsy.

Alternative Methods for RNA-Friendly Tissue Preservation

What about alternative "RNA-friendly" preservative methods, such as RNAlater and the organic solvents and alcoholic fixatives? As reviewed in the previous section, several of these alternative fixatives deliver better results than formalin in terms of RNA quality or detection of specific expression markers. In some cases, these alternative fixatives may even allow pathologists to conserve a single tissue sample for combined morphologic, histologic, and molecular analysis. This may be an attractive alternative
to saving separate samples, as discussed above, especially when access to appropriate freezer facilities is limited.

In particular, RNAAlter has undergone scrutiny as a possible alternative to freezing or formalin fixation in expression studies. This solution precipitates out RNases into an aqueous sulfate salt solution and thereby preserves the intact RNA. Although RNA yields and specific gene RNA abundance with RNAAlter are generally comparable with those seen with frozen tissue, some studies indicate that freezing still may be preferred over RNAAlter for RNA preservation. Also, because of the critical importance of RNA quality to quantification of less abundant transcripts, more studies are needed that compare snap-frozen tumors with tumors in RNAAlter in genome-wide expression microarrays. In terms of preserving tissue for histology and IHC, results with RNAAlter have been mixed.

Other alternative fixatives that avoid the cross-linking associated with formalin include ethanol, methanol, Carnoy fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid), and methacarn solution (substituting 60% methanol for the ethanol in Carnoy). While all these non-formalin methods may eventually prove to be useful RNA-friendly alternatives to freezing, validation in microarray-based genome-wide expression studies will be required before they can be put into routine use.

### Other Issues to Consider

In addition to considering the preservation method, pathologists interested in genomics-based testing will need to monitor several other tissue-handling issues that will impinge, either directly or indirectly, on sample quality and hence on the clinical reliability of the expression result. Examples of such issues include the potential value of frozen specimens in traditional IHC analysis (eg, when traditionally processed tissue is nondiagnostic); the cost effectiveness of collecting and storing frozen specimens in selected clinical situations; the impacts of variations in tissue handling techniques (eg, timing of collection and preservation, use of various buffers in fixatives, method of RNA extraction, quantity of tissue); the best methods for checking RNA quantity and quality (which may differ for RT-PCR vs microarray assays); and the implications of using fine-needle aspiration to obtain the specimen.

These issues are beyond the scope of this review but deserve future study. Fine-needle aspiration, for example, has been used with success in some gene expression profiling studies, but acquiring enough fine-needle aspiration tissue to perform both the standard pathologic tests and the new expression tests may remain a challenge for certain tumors. Similarly, adequate portions of other biopsies (eg, breast, pancreatic, renal, adrenal) may be unavailable for frozen storage and eventual expression testing because of the understandable priority in using unfrozen tissue to make the initial histologic diagnosis. It may also be challenging to identify areas of tumor within prostatic tissue using gross examination of the biopsy, a key issue for microarray analysis since a high percentage of normal cells can prejudice the analysis. Minimum standards for volume and percentage of tumor tissue will need to be developed and validated for each sample collection method used in expression analysis. At the institutional level, decisions on how or when to best capture a specimen with RNA quality suitable for full-genome expression testing will need to be made by a team of specially trained pathologists, surgeons, and internists who understand the demands and limitations of biopsy in each organ system. The long-term stability of mRNA signatures in frozen or fixed specimens is another issue worthy of additional study. In particular, studies need to address the potential value of using RNAAlter in frozen specimens and of freezing isolated RNA. Factors such as fixative buffers and storage temperatures (eg, −80°C vs liquid nitrogen) must also be investigated. In some studies, even storage at −80°C has been inadequate in completely preventing RNA degradation, and thus any evaluations of expression tests based on frozen samples should also verify the reproducibility of results with samples of different vintage. In one recent analysis performed as part of the clinical validation studies for a microarray-based test for tumor tissue of origin, 64 frozen specimens stored at −80°C for 2 years or less demonstrated accuracy of results equivalent to that seen with 34 frozen specimens stored under similar conditions for 5 to 8 years (C.T.R., oral communication, April 2007). Although such findings are encouraging, especially given the documented decline in mRNA quality in stored FFPE specimens (eg, Figure 4), clinicians need to remain acutely aware that the performance of any expression test will depend critically on the specificity of the gene markers included in the test panel. If the markers chosen are inherently unstable, either during the brief period of ischemia/warmth before freezing or a long period of frozen storage after preservation, then test performance will suffer. This reinforces the need for carefully designed studies to validate test performance under a variety of demanding clinical conditions and with specimens of varying quality.

### Major Opportunities for Improved Patient Care

While many questions remain about the practicality and logistical details of collecting a frozen specimen for expression analysis, the potential benefits to patients of what, for many laboratories, is a simple tissue-handling step are clear. As discussed earlier, genome-wide expression analysis will probably provide the highest clinical value in those settings in which a clinician needs to weigh multiple variables in order to answer an inherently complex question involving diagnosis, classification, or outcome. It is in these complex situations where a gene-by-gene or IHC approach leading to a simple binary assay result will often fall short. Based on preliminary studies, the settings where genome-wide expression testing will be preferred will likely include: the classification of certain lymphomas, leukemias, and tumors of unknown origin; the identification of previously unrecognized subsets of cutaneous melanomas, breast carcinomas, colorectal cancers, and adult soft tissue sarcomas; and the stratification of patients with cancer into fine-level subgroups with distinct clinical manifestations and specific prognoses or predicted responses to therapies.

For example, based on seminal studies using microarrays and frozen tissues, researchers recently reported on a microarray-based tissue of origin assay that quantifies the similarity of tumor biopsy specimens across 15 known sites of origin. If the preliminary results can be confirmed in clinical studies, then many more patients with poorly differentiated tumors or cancers of unknown origin, which represent anywhere from 2% to 5% of all diagnosed cancers, depending on the definition and popu-
lution considered,125,144–147 will be more likely to receive disease-specific treatment.

Another setting in which genome-wide expression analysis is likely to be adopted involves prediction of response to chemotherapy. Because each patient's oncogenic state typically involves deregulation of multiple cell signaling pathways, full-genome expression analysis may provide the clearest window into the inherently complex nature of cancer.143,144 The high degree of tumor complexity and heterogeneity explains why a particular chemotherapy can be so effective in one patient but spectacularly ineffective or toxic in another. While progress has been made in selecting appropriate targeted therapies for individual patients based on biomarkers or single-gene analyses, only recently have researchers begun analyzing the simultaneous expression of thousands of genomic markers to predict a patient's response to the older agents and, importantly, to commonly used combination regimens. This new pharmacogenomic approach has now been tested, with promising results in several oncology settings,14–24,149,150 and it may eventually lead to improved prediction of clinical response to various chemotherapy regimens. But, as emphasized throughout this review, achieving this next level of sensitivity and accuracy in expression analysis results may require procedural changes at the time of specimen collection and handling. The first evidence of this evolving requirement can be seen in the recent approval—in fresh-frozen or RNA-preserved breast tissue—of a microarray-based expression test to assess risk of distant metastasis.1 More diagnostic tests of this nature are in development. As recently suggested in a commentary on attempts to use formalin-fixed tissues for expression-based prognostic in breast cancer, “It is unrealistic to expect that optimal molecular measurements should always be obtained from formalin-fixed paraffin embedded blocks . . . we are obliged to reconsider our methods of handling clinical tissue samples.”76(p959) The author’s conclusion parallels our own: “It is long accepted that to characterize lymphoma, one must obtain extra samples specifically for molecular testing. We should learn to do likewise to accurately assess prognosis and select treatment for . . . cancer.”

SUMMARY

RNA quality is a critical determinant in genome-wide analysis of gene expression. Many studies have confirmed that frozen tissues yield more intact RNA than FFPE tissues and hence allow a far more comprehensive and clinically insightful interrogation of tissue expression. As commercially produced high-density microarrays become a common platform for expression profiling in the clinical laboratory, pathologists will increasingly need to collect frozen tissues or other RNA-stabilized samples along with the standard tissues taken for morphologic and histopathologic study. Ideally, when expression analysis is contemplated, a tumor specimen would be frozen in liquid nitrogen within half an hour (preferably within minutes) of surgical resection and stored at −80°C or below. While more studies are needed to determine the ideal method and timing of tissue extraction and processing for genome-wide expression profiling, it is clear that the pathologist's current tissue handling strategy of choice (i.e., FFPE) is often incapable of tapping the full potential of genomic expression tests. In many clinical situations, the minimal amount of upfront time and cost required to collect and freeze tissue for expression analysis will pay dividends in terms of improved diagnosis and prognosis.

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**CAP ‘08 ABSTRACT PROGRAM**

Abstract and case study submissions for the upcoming CAP ‘08 meeting will be accepted beginning on February 1, 2008 through March 28, 2008. Accepted submissions will be published in the September 2008 issue of the *Archives*. 

1816 Arch Pathol Lab Med—Vol 131, December 2007 Tissue Handling for Gene Expression Analysis—Medeiros et al