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Permalink

Journal

Biopreservation and Biobanking, 20(6)

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Publication Date

2022-12-01

DOI

10.1089/bio.2021.0023

Peer reviewed

Assessment of 2-Year Storage Conditions on Protein, RNA, and DNA in Unstained Human Tissue Sections, Including a Novel Multiplex Digital Gene Expression Profiling Method with Implications for Biobanking

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Background: Formalin-fixed, paraffin-embedded (FFPE) tissues are a valuable resource for clinical and basic science research. Paraffin blocks and the resulting unstained sections (USS) are often stored for years before being used. Previous studies have evaluated the effects of time, temperature, humidity, and inert gases on preservation of USS; however, no study has examined all four variables together.

Methods: In the current work, we prospectively and blindly assessed time points from 0 to 24 months, room versus refrigerated temperature, and presence of a desiccant and/or nitrogen atmosphere on a variety of benign and malignant tissues from North America and Africa. End points included immunohistochemistry (IHC), *in situ* hybridization (ISH), extracted RNA and DNA quantity and quality, and messenger RNA performance in a novel, multiplexed digital gene expression profiling assay of both housekeeping and tumor-specific genes.

Results: We found that using current methods of antigen retrieval, staining, and extraction, the end points of IHC, ISH, RNA, and DNA were well preserved under the various conditions tested, with implications that pre-embedding factors contribute to variability in subsequent tissue integrity. We also document that spectrophotometric estimations of nucleic acid concentrations were in general estimated to be higher than with fluorimetric methods, which may be pertinent to end assay development. We further describe a new multiplex assay, the PlexSet digital gene expression assay, suitable for evaluating RNA quality in FFPE tissues.

Conclusion: Altogether, these results may provide helpful guidance with regard to approaches for long-term storage conditions for USS.

Keywords: tissue, storage, biorepository, immunohistochemistry, RNA, gene expression profiling

Introduction

FORMALIN-FIXED, PARAFFIN-EMBEDDED (FFPE) tissues are an invaluable resource for clinical, translational, and basic science investigation. Neutral buffered formalin is the standard fixative in most clinical settings and the most common fixative encountered in hospital archives and tissue banks. In clinical practice, after the initial diagnostic procedures needed for pathologic diagnosis are completed, the FFPE tissue blocks are retained for regulatory compliance and, at this point, may become valuable research materials. These excess diagnostic materials are generally stored at local ambient temperature and humidity.

To conserve precious and small tissues, unstained sections (USS) are often precut and stored. These thin pieces of tissue are henceforth exposed to their storage environment conditions (temperature, humidity, oxygen) and, thus, may be even more vulnerable to degradation than the tissue in the original block, which remains encased in paraffin. The optimal conditions and maximum duration of storage for USS is a topic of ongoing investigation.

Prior investigations have evaluated the effects of temperature, humidity (effects of desiccants to decrease hydrolysis), or atmospheric makeup (air or the more inert nitrogen to prevent oxidation) on stored slides.[1] In particular, both endogenous water retained in tissues from inadequate tissue processing, as well as exposure to ambient water in high humidity conditions, can together lead to protein hydrolysis and degradation, leading to reduced antigenicity.[2]

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Assessment of protein integrity on FFPE tissues is generally performed using immunohistochemistry (IHC) and slide scoring by a trained pathologist. The length of slide storage, even for 2 months, was previously reported to negatively affect IHC staining for the nuclear antigen p53 and other important breast cancer associated biomarkers.[3,4]

These storage effects on antigens are reportedly most significant for nuclear and plasma membrane markers, while cytoplasmic antigens appear to be relatively preserved, even for decades. Furthermore, antigen recovery either from deep sectioning of blocks (not possible with USS) or antigen retrieval using heat and or enzymatic approaches can often partially or fully recover antigenicity.[5–8] High primary antibody titers may also overcome lost immunoreactivity in stored sections.[9] No effect from tissue section adhesives has been reported as a factor nor does the use of a monoclonal or polyclonal primary antibody appear to play a role.[10]

Assessment of RNA and DNA can be performed directly on USS using *in situ* hybridization (ISH) techniques, colorimetric detection, and direct visualization. Or the nucleic acids can be extracted, quantified, and qualified. The effects of storage on RNA and DNA detection with ISH and visualization have been less well studied than IHC. However, at least one study indicated that the impact of storage conditions on messenger RNA (mRNA) and DNA may not be as pronounced as for protein detection with IHC, while another study suggested that storing slides at −20°C rather than room temperature may improve downstream RNA ISH signals.[8,11]

For RNA and DNA extraction techniques, a common approach to evaluate RNA or DNA integrity is to perform amplification using reverse transcription polymerase chain retraction (RT-PCR) for endogenous reference or "housekeeping" genes. Given the high level of expression of most of these genes in most cells, as well as the amplification steps for these techniques, moderate changes in mRNA or DNA abundance and integrity may not be detectable. Newer non-amplification dependent assessments of RNA or DNA are available, which may be better able to detect subtle differences in nucleic acid quantity or quality.[12]

To our knowledge, no studies to date have looked at combinations of temperature, humidity, and atmosphere, over a long period of time, in a range of tissues with subsequent evaluation of protein antigens at various cellular locations (nuclear vs. cytoplasmic or cytoplasmic membrane), as well as RNA and DNA with both *in situ* and extraction methods. In this study, we sought to evaluate all these conditions and end points. We found no consistent patterns and variations common to all tissues, suggesting that preanalytical variables are the more critical factors in the success of long-term tissue storage. We document the differences in nucleic acid quantification between spectrophotometric and fluorometric methods, as well as the utility of a multiparameter digital gene expression profiling approach to endogenous gene mRNA assessment.

Materials and Methods

Tissues

We identified nine paraffin blocks representing various anatomic sites and diagnoses. Since the laboratory is the technical core for the AIDS and Cancer Specimen Resource

(ACSR, https://acsr1.com), an HIV malignancy tissue bank, these included lymphoid and nonlymphoid benign tissues (reactive or inflammatory) and malignant diagnoses (lymphoma, carcinoma, and sarcoma) (Table 1). Six of the nine tissue blocks were from the hospital archives of the University of Arizona, Tucson, Arizona that had undergone standard clinical fixation and processing (4–24 hours in neutral buffered formalin) and have been stored as blocks for between 15 and 23 years, while three tissues were donated to the ACSR >10 years ago from Uganda (tissues from Africa comprise ∼5% of the ACSR tissue bank) with an unknown fixation procedure. This research was conducted under an Institutional Review Board approved protocol regarding protection of human subjects in research.

Study design and storage conditions

To avoid surface effects resulting from previous storage, all tissue blocks were faced and ∼20–30 μm were removed before obtaining the tissue ribbons used in the study. USS were sectioned at 4 μm thickness with the Time 0 materials immediately stained or extracted for RNA and DNA, which served as the baseline controls, while the USS for the subsequent time points were placed into their respective storage conditions and held until time of analysis. The USS were stored in four ways: room temperature with no desiccation (bench top storage) (RT), room temperature with desiccation (RT+D), refrigerated at 4°C with desiccation (4C+D), or refrigerated at 4°C, with desiccation, under a nitrogen atmosphere (4C+D+N2).

In the desiccated conditions, slides were placed inside a glass desiccator with Drierite pellets (Sigma-Aldrich, Saint Louis, MO) that were changed when the color indicator indicated increasing humidity (approximately every 2 months). For the nitrogen condition, pure nitrogen was piped into a sealed desiccator in the refrigerator with the desiccant packet placed inside. The condition of refrigeration with no desiccant was not used due to the known extreme moisture condensation within refrigerators. Samples were assessed immediately after sectioning (at Time 0 or baseline) and then at 3, 6, 12, and 24 months.

IHC and ISH

IHC was performed using the Discovery Ultra Instrument (Roche, Indianapolis, IN). All USS were subjected to the standard, on-instrument, deparaffinization protocols: Primary antibodies, probe, detection kits, and counterstaining details are detailed in Supplementary Table S1.

Slide scoring

In a blinded manner and for consistency, all IHC and ISH results were scored by a single, board certified pathologist (B.L.). The entire section was considered rather than a representative number of cells, with the following conditions: pan-cytokeratin was observed in cytoplasm of epithelial cells for the skin, tonsil, and breast carcinoma (as well as in tumor for breast carcinoma and adenocarcinoma); CD20 considered on cell membranes of lymphocytes (in tumor cells for hematologic disease and nontumor cells for nonhematologic disease); Ki67 was considered in the nuclei of the proliferating basal cells of epithelium and in

TABLE 1. TISSUES AND DIAGNOSES

Location	Diagnosis	Source
Lymph node	Diffuse large B cell lymphoma	University Hospital, Tucson, AZ
Lymph node	Hodgkin lymphoma	University Hospital, Tucson, AZ
Spleen	Hodgkin lymphoma	University Hospital, Tucson, AZ
Tonsil	Follicular lymphoma	University Hospital, Tucson, AZ
Skin	Benign lymphoid hyperplasia	University Hospital, Tucson, AZ
Breast	Adenocarcinoma	University Hospital, Tucson, AZ
Lymph node	Kaposi's sarcoma	Donated to ACSR
Skin	Kaposi's sarcoma	Donated to ACSR
Skin	Adenocarcinoma	Donated to ACSR

ACSR, AIDS and Cancer Specimen Resource; AZ, Arizona.

tumor cells for hematologic diseases; and U6 was observed in the cytoplasm as a global marker for RNA in all cells.

Since the number of positive cells and the intensity of the stain can vary with successive tissue sections or IHC/ISH batch performance, both were considered using a method previously described by Allred et al.[13] The percentage of positive cells (appropriate to the stain/cell type/disease) was scored and assigned a proportion score as follows: 0% positive = 0, ≤1% positive = 1, ≤10% positive = 2, ≤33% positive = 3, ≤66% positive = 4, and between 66% and 100% positive = 5. Intensity was graded on a range of 0 to 3+ as: absent (0+), weak (1+), moderate (2+), or strong (3+).

Recognizing the heterogeneity of protein expression that can be seen in human tissue samples, we modified the ranges of staining intensity that were recorded using the following system: uniformly negative staining = intensity score 0, uniformly weak 1+ or variable staining from weak to medium (1–2+) = intensity score 1, uniformly medium (2+) staining or variable staining from weak or medium to strong (1+/2+ to 3+) = intensity score 2, and uniformly strong (3+) staining = intensity score 3. "Allred-type" scores calculated by adding intensity score plus proportion score were calculated for each observation (all data in Supplementary Table S2).[13]

DNA and RNA

DNA and RNA extraction were performed simultaneously from the same sample using the Qiagen AllPrep DNA/RNA FFPE Extraction Kit (Qiagen, Germantown, MD) as per manufacturer's instructions. Nucleic acid quantity was measured with two methods: absorbance at 260 nm using the NanoDrop 1000 system (Thermo Fisher Scientific, Wilmington, DE) and with intercalating dyes using the Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA).

Multiplex digital expression analysis of RNA

Rather than measuring a single RNA transcript with RT-PCR, we chose a digital gene expression profiling assay with known outstanding reproducibility.[14,15] The nCounter platform (NanoString Technologies, Seattle, WA) takes advantage of solution phase hybridization (without amplification), with fluorescent bar code tags, which are hybridized to target RNA transcripts, immobilized, then digitally counted. PlexSet offers greater flexibility by pooling multiple hybridization products, which can increase the number of samples assessed per cartridge eightfold, decreasing cost.[16]

Calibration was performed using a reference sample, which was a pool of RNA from various sample types re-

sulting in moderate expression of all targets. This sample was compared across all eight "plexes" to account for variability in each hybridization reaction. The chosen probes and rationale for inclusion are listed in Table 2. The data from each set of materials were analyzed using the nSolver 4.0 software (NanoString Technologies). Normalization to β-Actin (ACTB) as the universal housekeeper was performed. The data were then exported to Excel (Microsoft, Seattle, WA) and JMP® (SAS Institute, Cary, NC) statistical software for data manipulation and visualization.

Statistical analysis

For each stain, the "Allred-type" scores were considered for all nine tissues together at baseline as the null hypothesis group, with total number of observations dependent upon tissue and disease as described in "Slide Scoring," and this grouping was repeated for each storage condition at the final time point of 24 months. For IHC and ISH stains, a two-tailed student's t-test assuming equal variances (since we use the same tissues throughout) was used to determine if any of the conditions resulted in significantly different results from baseline after 24 months (Supplementary Table S3).

For both the NanoDrop and Qubit methods, the means of the nucleic acid concentrations were calculated over all samples at baseline and the end point for each storage condition. For each nucleic acid species, these means were compared as a group between the two methods for using a one-tailed paired t-test for Qubit readings being lower than those from the NanoDrop. For the PlexSet data, the mean of the 23 normalized gene counts for each sample was calculated at baseline and at 24 months for each condition and compared using two-tailed paired t-test. All statistical tests were carried out at a 95% confidence level.

Results

Immunohistochemistry

Overall, the tissues processed in both the North American and African sites stained well at all time points and conditions. No antigens were diminished in any consistent manner according to storage condition or time point. The IHC included antigens typically found at different subcellular locations, including cytokeratins (cytoplasmic, detected with pancytokeratin cocktail), CD20 (cell membrane), and Ki67 (nuclear), and during scoring it was noted that the stains remained appropriately localized. Of interest, CD20 was a robust antigen in lymphoma tumor cells in 2 of the 3

TABLE 2. LIST AND PURPOSE OF GENES IN THE PLEXSET ASSAY

Gene name	Notes	Expected expression
ISY1	Lymphoid housekeeper	All cells
TRIM56	Lymphoid housekeeper	All cells
R3HDM1	Lymphoid housekeeper	All cells
WDR55	Lymphoid housekeeper	All cells
UBXN4	Lymphoid housekeeper	All cells
OPA1	Lymphoid housekeeper	All cells
PHF23	Lymphoid housekeeper	All cells
GIT2	Lymphoid housekeeper	All cells
DNAJB12	Lymphoid housekeeper	All cells
IK	Lymphoid housekeeper	All cells
GSK3B	Lymphoid housekeeper	All cells
VRK3	Lymphoid housekeeper	All cells
WAC	Lymphoid housekeeper	All cells
TBP	Lymphoid housekeeper	All cells
ACTB	Housekeeper	All cells
CD20	Alias MS4A1	B cells
Ki67	Alias MKI67, MIB1	Proliferating cells
CD79a	B cell marker	B and plasma cells
EBER1	EBV encoded RNA transcripts	EBV infected cells
HHV8	Human herpes virus 8	Kaposi's sarcoma
KRT5	Keratin 5, basic high molecular weight cytokeratin	Skin epithelium
KRT7	Keratin 7, basic low molecular weight cytokeratin	Adenocarcinomas, particularly those originating above the diaphragm such as lung, breast, salivary gland
KRT10	Keratin 10, acidic high molecular weight cytokeratin	Skin epithelium
KRT20	Keratin 20, acidic low molecular weight cytokeratin	Adenocarcinoma, particularly those below the diaphragm such as of gastrointestinal origin

lymphoid samples tested. However, in one sample, CD20 showed decreased staining at 6 months in the benchtop conditions (RT) and in all conditions starting at 1 year (photomicrographs in Fig. 1). That only one of the three B cell lymphoma cases demonstrated any alterations in CD20 appeared to indicate that staining alterations could be attributed to intersample variability rather than the antigen itself or variations in storage conditions. All IHC data are in Supplementary Table S2, with a summary of "Allred-type" scores in Supplementary Table S3. Based on the summary statistics, we failed to reject the null hypothesis that the scores would be the same for each condition at baseline after 24 months for every storage condition.

In situ hybridization

The U6 ISH assay was used as a global assessment of mRNA in the tissue sections. Similar to the IHC results, the tissues processed in either North America or Africa stained moderate to strongly for mRNA under varied storage conditions with no consistent trends related to storage condition. At least one case showed apparent loss of U6 mRNA staining in RT conditions. Although this case was from Africa, another African case did not show a similar pattern result suggesting that, like the one case of CD20 IHC, the poor result was tissue- rather than storage specific (see example photomicrographs in Fig. 1). All ISH data are listed in Supplementary Table S2, with a summary of "Allred-type" scores in Supplementary Table S3. As with the IHC, we failed to reject the null hypothesis that the scores would be the same for each condition at baseline after 24 months for every storage condition.

RNA and DNA quantity by NanoDrop and Qubit

RNA and DNA were recovered from tissues stored under all conditions and storage times without significant variation over the various conditions, indicating that RNA is relatively protected within the USS. For both the NanoDrop and Qubit methods, the means of the RNA and DNA concentrations were calculated for all samples at baseline and end point for all conditions. These means were compared to baseline within NanoDrop and Qubit readings and also between methods. Generally, the Qubit method for determining nucleic acid concentrations showed lower concentrations compared to the NanoDrop method (RNA NanoDrop vs. Qubit $p = 0.003$, DNA NanoDrop vs. Qubit $p = 0.031$, using a one-tailed paired t-test). All RNA and DNA data are in Supplementary Table S4, with a summary of end points and p-values in Supplementary Table S5.

PlexSet assessment

In initial titration experiments using ACTB, the most highly expressed gene, the total output measured as "counts," was linear between 25 and 100 ng of total input. At 200 ng of input, the data were no longer linear (saturation was reached). Thus, 100 ng input was deemed optimal. In triplicate runs of three separate samples, the R^2 correlation coefficients were outstanding at 0.9985, 0.9987, and 1.0000 for inputs of 25, 50, and 100 ng (data not shown).

We normalized gene expression to the commonly used ACTB gene transcript. Using this approach, upon inspection of the data we saw very little influence of storage conditions on RNA quality using the PlexSet assay. This suggests that RNA preservation includes a wide variety of individual

mRNA molecules and, as also demonstrated by the U6 mRNA staining, is fairly robust across storage conditions by this method.

For the PlexSet data, the mean of the 23 normalized to ACTB gene counts for each sample was calculated at baseline and at 24 months for each storage condition and compared using a two-tailed paired t-test, reported in Supplementary Table S6. Of interest, one sample (an adenocarcinoma from Uganda) performed poorly with a high degree of normalization required (more than a factor of 10, flagged by the nSolver software) even for the baseline, which either indicates low RNA loading or a poor-quality specimen. Since RNA was detected, we can conclude that this flag indicates a poor-quality specimen. Three other samples had one condition which was either omitted based on normalization factor or with a significant p-value; none of these results was for the same storage condition, indicating that they are likely spurious results.

Evaluation of the gene probes in specific samples, related to tissue or tumor lineage, showed variable results across the samples in expected patterns based on known tissue/tumor characteristics. For example, the CD20 gene transcript typical of B cell tumors was high in samples containing B cell tumors, while HHV8 viral transcripts were high in Kaposi sarcoma, and cytokeratins were highest in carcinomas (See Fig. 2 showing normalized, log2-transformed gene counts for four different samples at 24 months for all conditions and baseline).

Discussion

We successfully conducted a prospective study of several hundred USS over 24 months under four different storage conditions with a variety of protein, RNA, and DNA end points. As opposed to our initial assumption that the combined conditions of refrigeration, desiccation, and nitrogen atmosphere would significantly contribute to tissue preservation, no sustained trends between storage conditions were found. We attribute these positive results to the ever-increasing sophistication in commercial antigen retrieval, IHC, ISH, and nucleic acid extraction methods.

FIG. 1. Representative photomicrographs of IHC staining results (all taken at $20\times$ magnification). Antibodies and staining protocols were standardized for all tissues, conditions, and time points and are summarized in Supplementary Table S1. **(A)** Breast carcinoma; H&E stained section **(a)**, Cytokeratin at Time 0 with ATS (in tumor) = 8, ATS (in stroma) = 8 **(b)**, 24 months, room temperature with no desiccant with ATS (in tumor) = 8, ATS (in stroma) = 8 **(c)**, and 24 months, 4°C, with desiccant, N2 atmosphere with ATS (in tumor) = 8, ATS (in stroma) = 8 **(d)**. No changes are detectable in cytokeratin staining between Time 0 compared to the longest time point under the ''worst'' or ''optimal'' conditions of storage. **(B)** Diffuse Large B cell lymphoma in lymph node; H&E stained section **(a)**, CD20 at Time 0 with ATS (in tumor) = 7, ATS (in nontumor) = 8 **(b)**, CD20 at 24 months, room temperature, no desiccant with ATS (in tumor) = 6, nontumor area missing from slide **(c)**, and 24 months at 4°C, with desiccant, N2 atmosphere with ATS (in tumor) = 6, ATS (in nontumor) = 7 **(d)**. CD20 is typically strongly expressed on Diffuse Large B cell lymphoma and is 2+ and moderately punctate in this particular case and demonstrated further degeneration under all conditions to 1+ staining. Similar decline in stain performance for Ki67 and U6 was observed in this sample, and samples with other B cell malignancies showed no such decline in CD20 **(C, D)**, leading to the conclusion that the problem is inherent to the sample and not an effect of storage over time. **(C)** Diffuse Large B cell lymphoma in a spleen; H&E stained section demonstrating tumor **(a)**, CD20 at Time 0 with ATS (in tumor) = 8 **(b)**, CD20 after 24 months at room temperature without desiccant with ATS (in tumor) = 7 **(c)**, and CD20 after 12 months at 4°C, with desiccant, and N2 atmosphere with ATS (in tumor) = 8 **(d)**. Despite the fact that spleens are known for rapid autolysis, CD20 is strongly expressed at Time 0, as well as both the ''worst'' and ''optimal'' storage conditions. **(D)** Follicular B cell lymphoma grade 3 in a tonsil; H&E stained section **(a)**, CD20 at Time 0 with ATS (in tumor) = 8, ATS (in nontumor) = 8 **(b)**, CD20 after 24 months at room temperature without desiccant with ATS (in tumor) = 8, ATS (in nontumor) = 8 **(c)**, CD20 after 24 months at 4°C, with desiccant, and N2 atmosphere with ATS (in tumor) = 8, ATS (in nontumor) = 8 **(d)** indicating that CD20 is not, in itself, an unstable antigen. Similar to the case shown in **(C)**, there is no diminishment of staining over time under the different conditions indicating that variability between specimens overrides the storage conditions. **(E)** Benign lymphoid hyperplasia in the skin. H&E stained section **(a)**, Ki67 nuclear antigen at Time 0 with ATS (in epithelium) = 4 **(b)**, Ki67 after 24 months at room temperature without desiccation with ATS (in epithelium) = 4 **(c)**, Ki67 after 24 months at 4°C, with desiccant, and N2 atmosphere with ATS (in epithelium) = 5 **(d)**. This representative case demonstrated excellent preservation of Ki67 antigenicity over time and storage conditions. **(F)** Kaposi's sarcoma in skin case from Africa. H&E stained section **(a)**, Ki67 nuclear antigen at Time 0 with ATS (in tumor) = 7, ATS (in nontumor) = 5 **(b)**, Ki67 after 24 months at room temperature without desiccant with ATS (in tumor) = 7, ATS (in nontumor) = 5 **(c)**, Ki67 after 24 months at 4°C with desiccant and N2 atmosphere with ATS (in tumor) = 7, ATS (in nontumor) = 5 **(d)**. This case from Africa demonstrated excellent Ki67 antigenicity as demonstrated at Time 0 compared to the ''worst'' conditions on the bench top and the carefully optimized conditions of refrigeration, desiccation, and nitrogen. **(G)** Hodgkin lymphoma in lymph node; H&E stained section **(a)**, U6 mRNA *in situ* hybridization at Time 0 with ATS (all cells) = 7 **(b)**, U6 mRNA after 24 months at room temperature without desiccation with ATS (all cells) = 7 **(c)**, and U6 mRNA after 24 months at 4°C with desiccant and N2 atmosphere with ATS (all cells) = 7 **(d)**. This case demonstrated a slight degradation by visual inspection after 24 months on the bench top as seen in **(c)**, however, not enough to affect the ATS and likely adequate for additional *in situ* hybridization assays. The photographed field for panel c shows a particularly nodular area of the Hodgkin lymphoma with surrounding fibrosis. No degradation is seen in the optimized conditions. **(H)** African Kaposi's sarcoma in a lymph node; H&E stained section **(a)**, U6 mRNA *in situ* hybridization at Time 0 with ATS (all cells) = 7 **(b)**, U6 mRNA after 24 months at room temperature without desiccation with ATS (all cells) = 7 **(c)**, and U6 mRNA after 24 months at 4°C with desiccant and N2 atmosphere with ATS (all cells) = 7 **(d)**. As compared to **(G)**, this African case demonstrated excellent fixation suitable for *in situ* hybridization as reflected at Time 0 and representative conditions at 24 months. These results indicate that changes in staining are likely more related to preanalytical variables rather than storage conditions. ATS, ''Allred-Type'' score; H&E, hematoxylin and eosin; IHC, immunohistochemistry; mRNA, messenger RNA; N2, nitrogen; 4°C, 4 degrees centigrade; $20\times$, $20\times$ magnification.
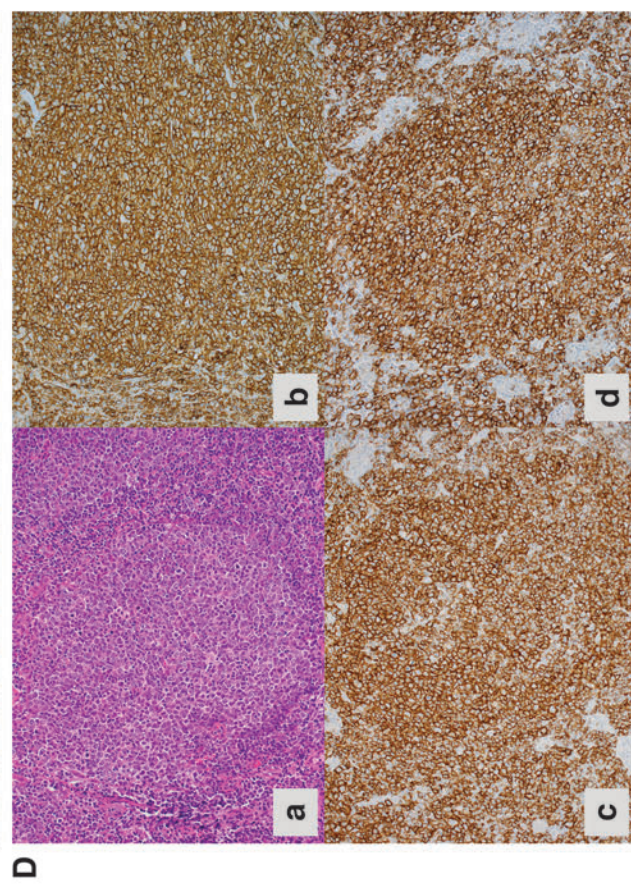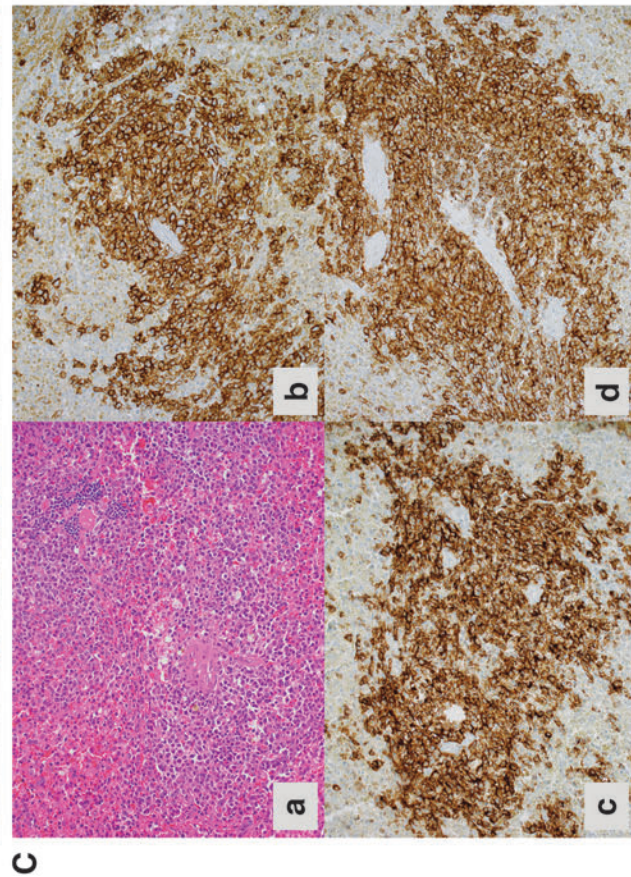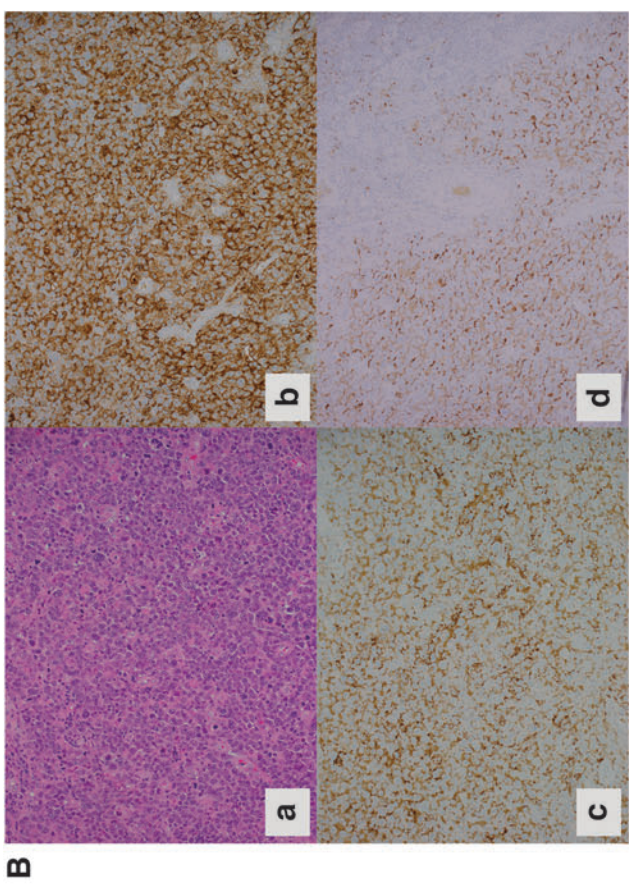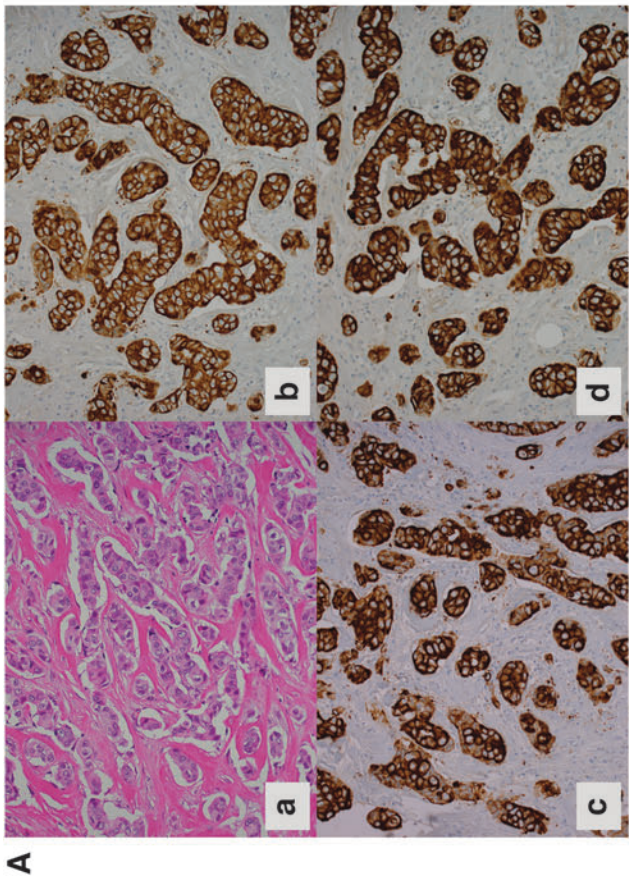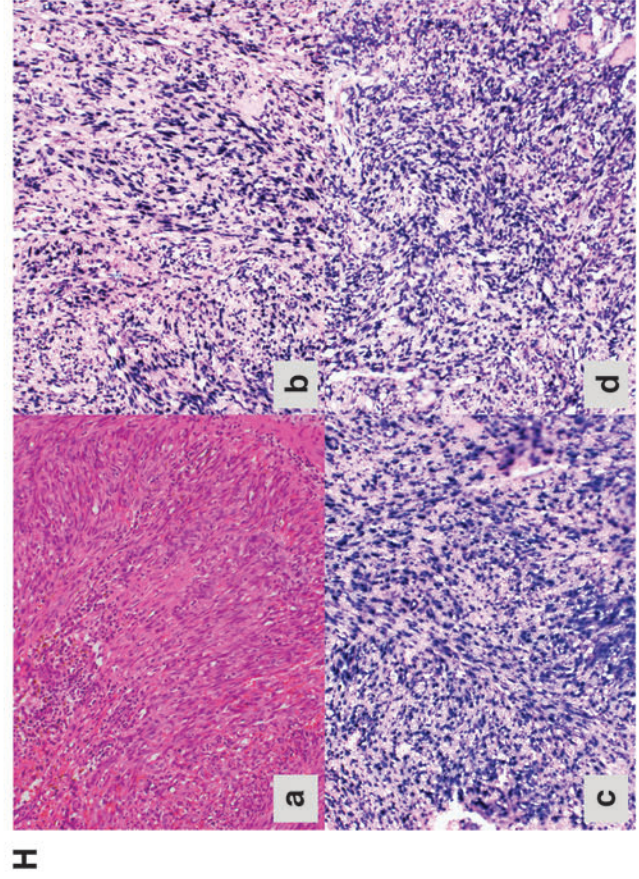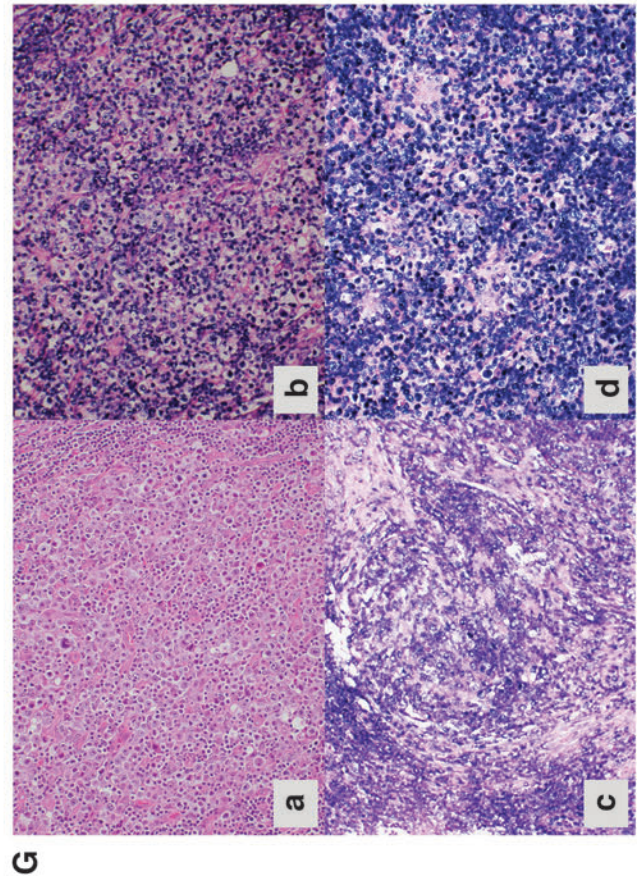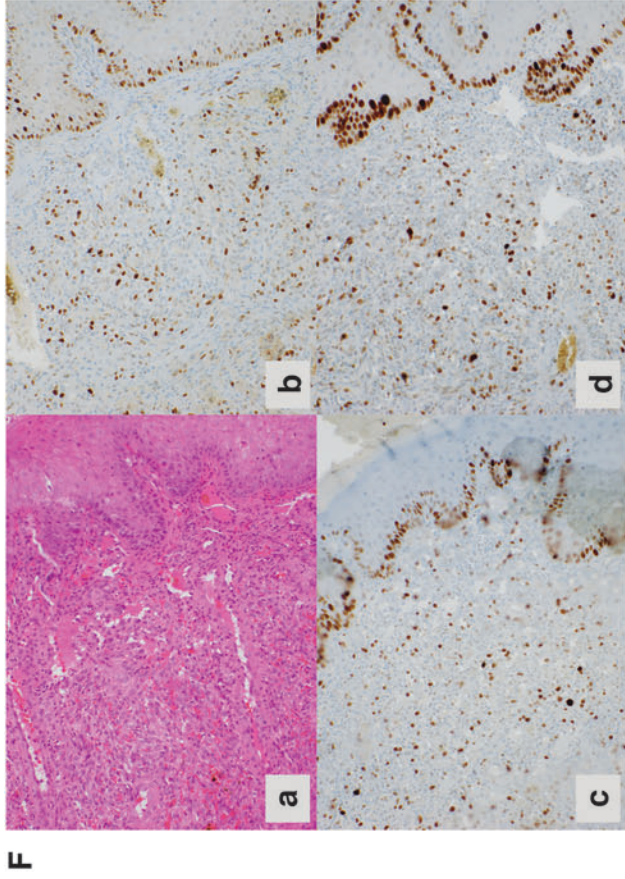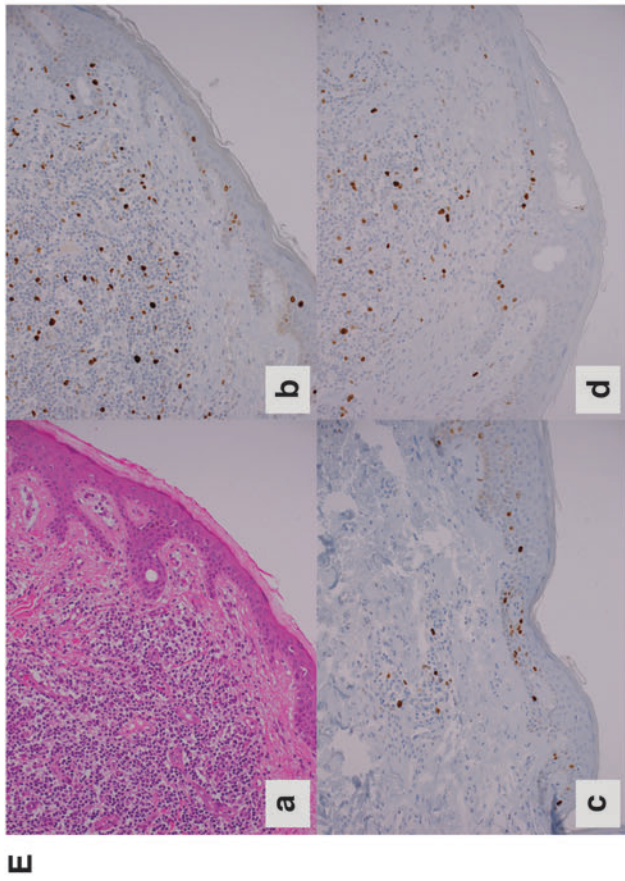
FIG. 1. (*Continued*).

Using the ''Allred-type'' score, which combines information about the intensity of the stains and percent of cells stained, ensured that we were not overly susceptible to effects of routine variances which can occur, such as changes in tissue morphologies as the block is sectioned or staining intensity when the stain is run 3, 6, 12, or 24 months after the initial baseline run. One caveat to our study is that diagnostics-grade antibodies and the staining platform and the nCounter platform were chosen for evaluation because less robust techniques/reagents would likely require rigorous revalidation at each time point, introducing unnecessary variability into the study. What variation we did observe appeared to imply that preanalytical variables are the major contributor to tissue degradation over time.

Attempts to assess RNA and DNA quality by fragment size using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) were unsuccessful, with metrics such as the RNA Integrity Number already quite low at baseline measurements (between 1 to 3 on a scale of 10). This is not completely unexpected for older paraffin-embedded tissue blocks; however, it does preclude using such a method to assess changes in the nucleic acid quality over time. In contrast, the multiplex PlexSet chemistry on the nCounter system for simultaneous assessment of multiple mRNA species was successful in all samples analyzed and implies the platform's usefulness for analysis of even heavily degraded RNA.

Optimal storage for USS is desirable for both clinical and biobanking uses. Others previously demonstrated that extreme conditions such as chemical oxidation with hydrogen peroxide, high heat (56°C), and ultraviolet A (UVA) photo-oxidation with UVA or even windowpane light exposure or fluorescent lights can all damage tissue sections and assays for specific antigens.[17,18] In general practice, therefore, it is safe to assert that storage in non-air-conditioned areas in the summer months or direct sunlight should be avoided.

Previous work has reported that storage at −80°C may preserve antigenicity of USS stained for breast cancer associated antigens, but this type of extremely cold storage is unfeasible for most laboratories. Beyond these extremes, we have endeavored to add to the increasing knowledge base of what constitutes ideal conditions for storage of USS. We observed that within temperature-controlled buildings, out of direct sunlight, antigenicity is preserved in IHC assays across tissue types and protein targets using clinical grade reagents and staining. As opposed to earlier reports on the negative effects of room temperature, humidity, or natural atmosphere, it appears that tissue degradation may be overcome with modern, standardized antigen retrieval techniques, including both heat and enzyme digestions as well as improved detection systems that are in common use. Fairly similar to our results, prior investigators have reported that cold-stored slides (4°C) were the least affected particularly for IHC for a subset of antibodies related to the nucleus or cytoplasmic membrane, while actin and keratins, which are in cytoplasmic cellular locations, showed no decay; and that the nuclear and cytoplasmic membrane markers could be ''recovered'' with heat antigen retrieval.[19] Previously, our tissue bank performed paraffin coating of slides before storage as suggested by some authors.[8,20] However, the difficulty of fully removing the paraffin coating (even after multiple by-hand and on-instrument protocols) made subsequent IHC and nucleic acid retrieval so difficult that the practice was abandoned (personal experience, data not shown).

Our observation that the preanalytic variables appeared to contribute to the overall success of antigen preservation during storage is highly concordant with previous studies. The impact of preanalytical variables such as time to fixation, type, and length of fixation and tissue processing are well described, particularly as related to IHC.[21–23] Detection of phosphorylated signaling molecules by IHC may be especially sensitive to time to fixation and type of fixation as related to inactivation of cellular phosphorylases.[10,24] We plan future work to evaluate preanalytical variables in a prospective manner using mouse tissues, which can then be freshly harvested and manipulated in different ways.

Our results for RNA and DNA are in agreement with previous studies evaluating the impact of storage conditions

FIG. 2. Representative cases showing mRNA levels measured using PlexSet technology for 23 individual genes. **(A)** ACTB normalized gene expression in breast carcinoma; graph depicts expression at baseline and at 24 months under the different storage conditions. Moderate expression of cytokeratins 5 and 7 is observed, with few to no transcripts for the B cell marker CD20 and no HHV8 viral transcripts (*blue arrows*). There is little variability in the expression levels between the different storage conditions across the various measured genes. The *dots* representing the different storage conditions are remarkably similar indicating little loss of specific mRNA transcripts. In addition, there is no particular storage condition that shows the highest expression across genes, that is, no storage condition appears to favor mRNA overall. **(B)** ACTB normalized gene expression in diffuse large B cell lymphoma in a lymph node; graph depicts expression at baseline and at 24 months under the different storage conditions. In line with known biology, high expression of the B cell associated gene CD20 is observed, with no evidence of viral HHV8 and no cytokeratin expression due to lack of epithelial elements in the tissue (*blue arrows*). There is little variability and no pattern across the different storage conditions. **(C)** ACTB normalized gene expression in a benign follicular hyperplasia of the skin; graph depicts expression at baseline and at 24 months under the different storage conditions. Again, in line with the Tissue biology, high expression of cytokeratins (KTR5 and KTR10) and moderate expression of the B cell marker CD20 but low expression of the KS marker HHV8 is observed (*blue arrows*). There is little variability and no pattern across the different storage conditions. **(D)** ACTB normalized gene expression in an African case of Kaposi sarcoma in the skin; graph depicts expression at baseline and at 24 months under the different storage conditions. Reflecting tumor biology again, low expression of the B cell marker CD20 is observed alongside high levels of the cytokeratin KRT5, and the expected positive expression of the viral gene HHV8 characteristic of this tumor (*blue arrows*). The African case demonstrated good recovery and an appropriate expression pattern of mRNA. Similar to **(A–C)**, there is little variability in expression levels of individual genes in the different storage conditions. 4C + D + N2, refrigerated, with desiccant, under nitrogen gas atmosphere; 4C + D, refrigerated, with desiccant; RT + D, room temperature, with desiccant; RT, room temperature, no desiccant.

on subsequent ISH techniques, gene copy number aberrations, chromosomal translocations, and presence of mRNA in slides stored up to 1 year with the explanation being that the FFPE tissue section provides a ''protective milieu even for mRNA molecules.''[8] For nearly all observations, the NanoDrop method demonstrated higher nucleic acid concentrations compared to the Qubit method. This agrees with our prior unpublished experience, as well as other reports, that DNA and RNA concentrations are higher (and possibly overestimated) with the spectrophotometric NanoDrop method.[25,26] A fluorometric method, such as Qubit, may therefore be a more accurate
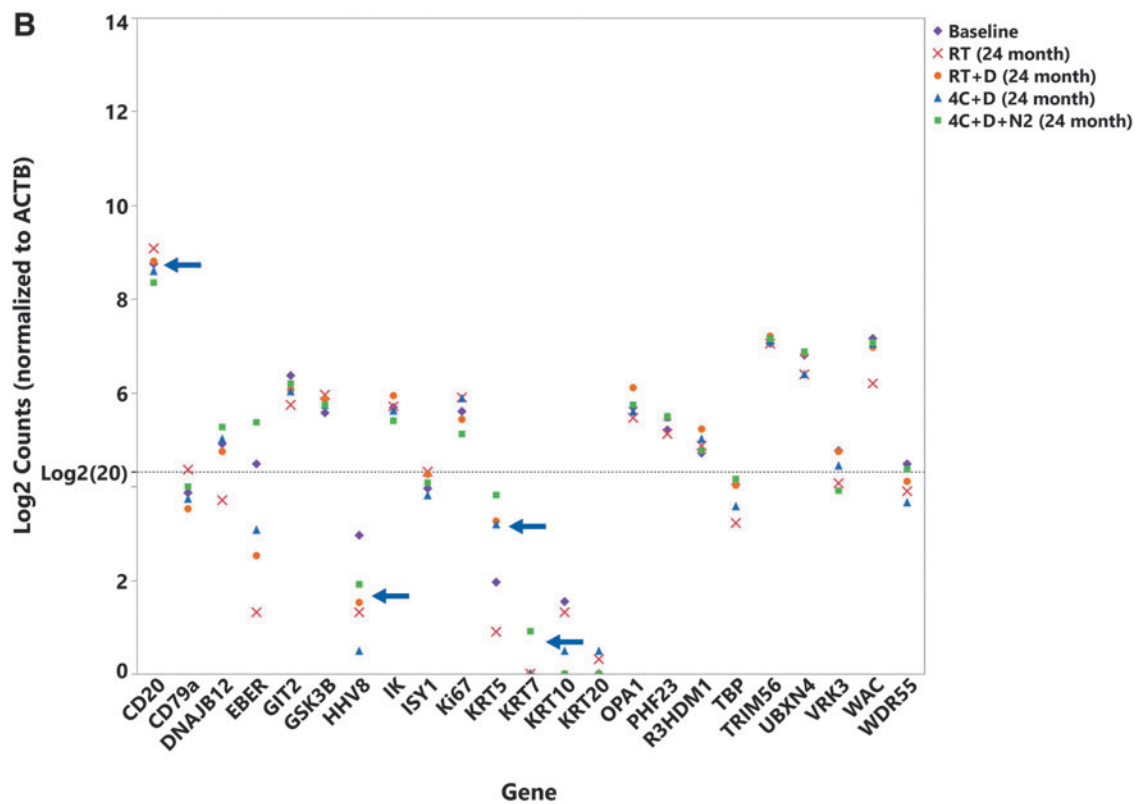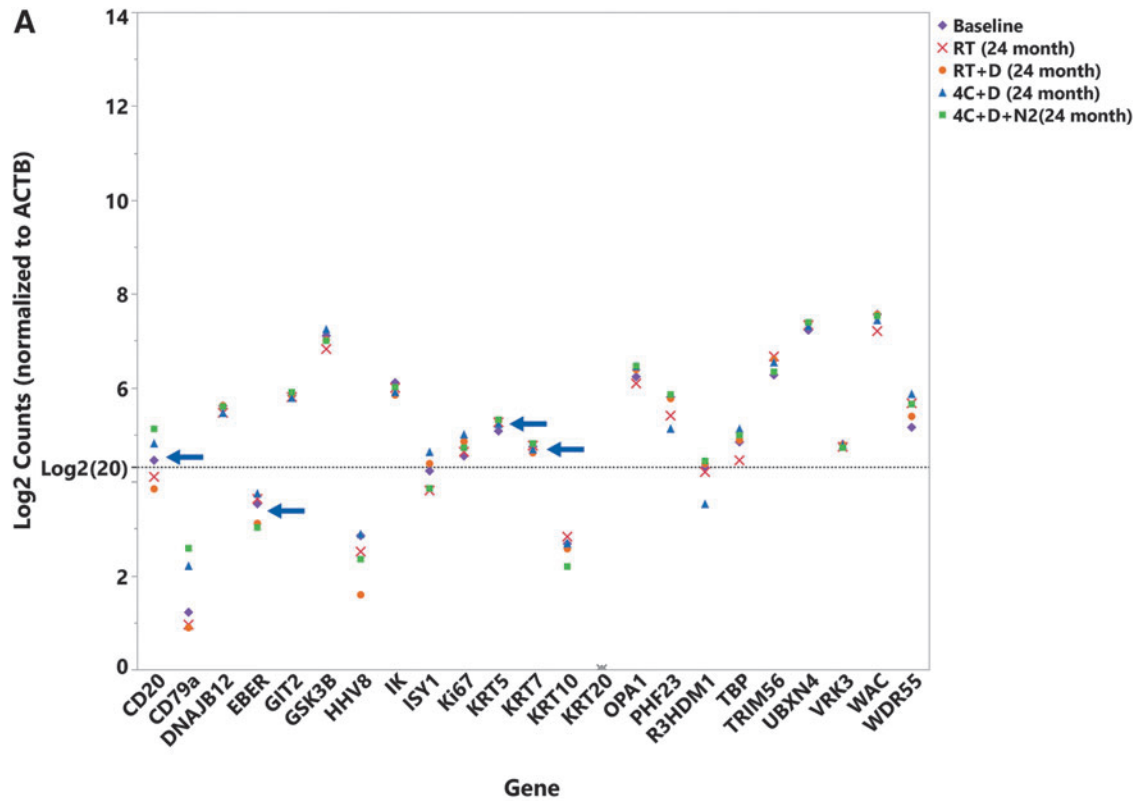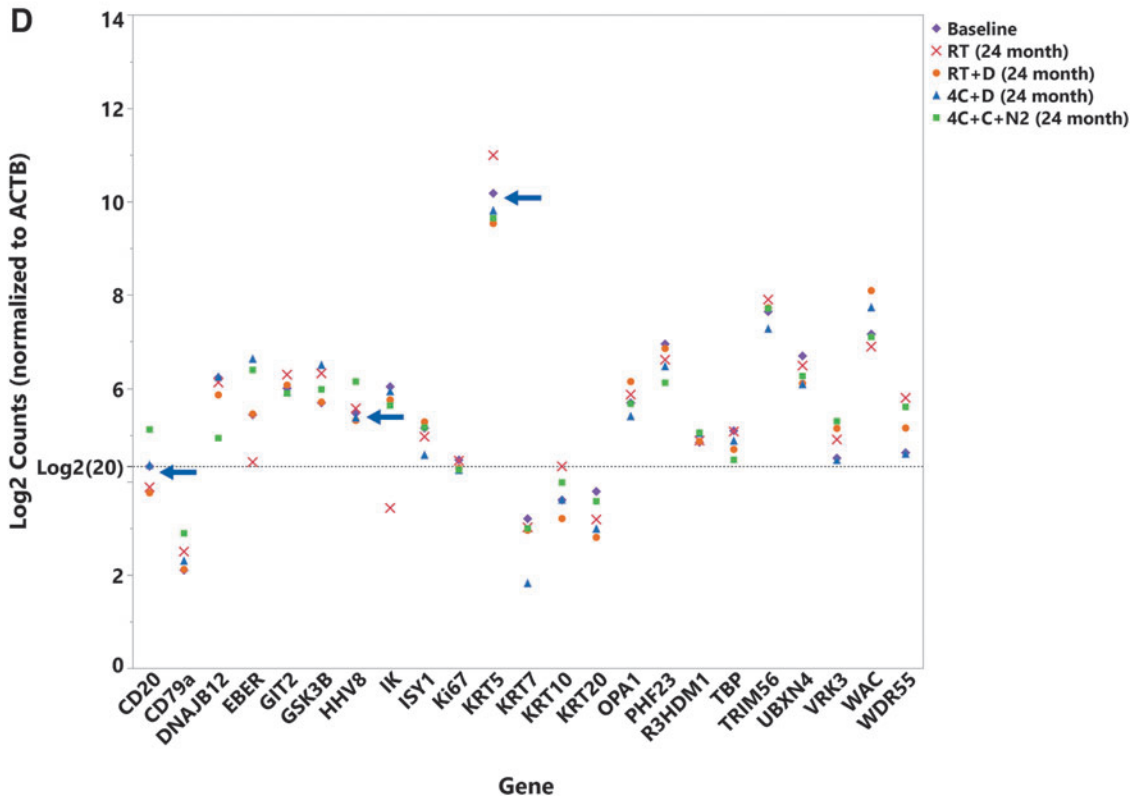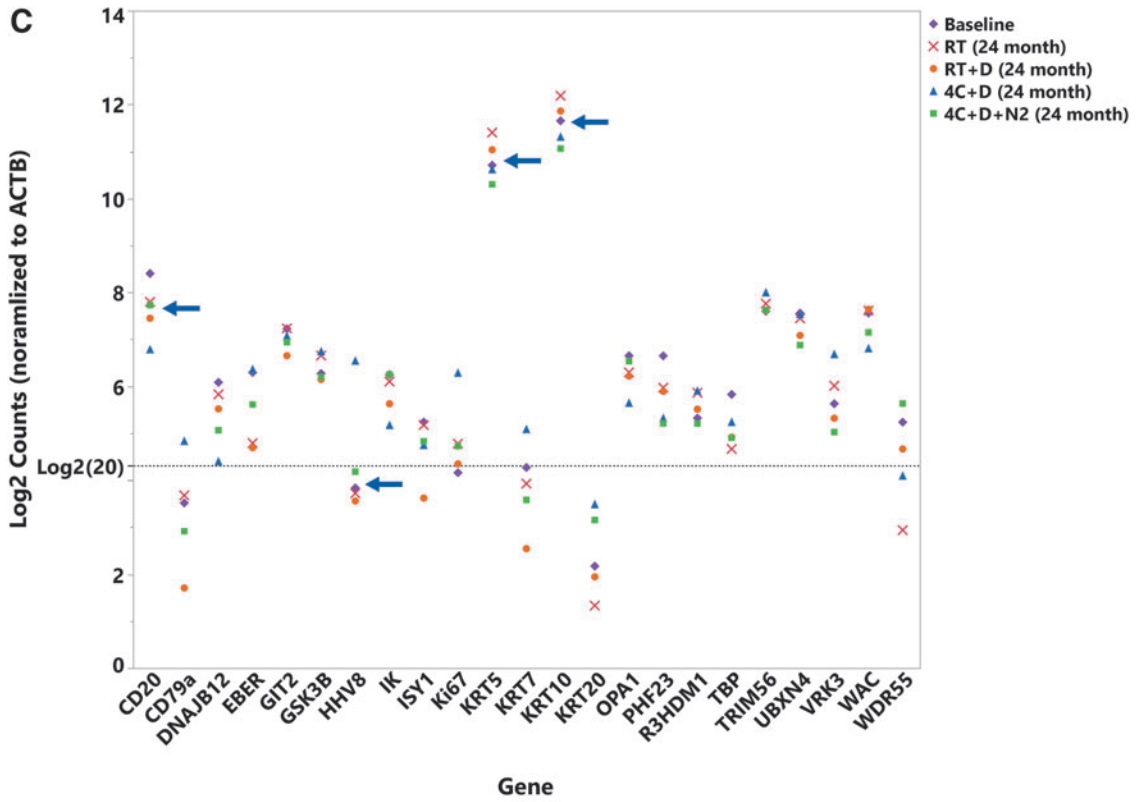
FIG. 2. (*Continued*).

measurement of nucleic acid quantity and may be particularly important for high sensitivity downstream methodologies such as DNA sequencing.

In the more thorough evaluation of RNA quality using the PlexSet approach, the individual gene expression levels were also unaffected by storage conditions and time, further displaying the stability of RNA in the FFPE tissues, and may also reflect on the robust nature of digital gene expression profiling, which relies on hybridization and does not require a high quality mRNA template as is typically

needed for amplification-based techniques. The low level of input (100 ng) and outstanding replication between runs indicate the utility and robust performance of this approach. These results agree with previous studies, which demonstrate successful RNA-seq or gene expression profiling using high density oligonucleotide arrays or digital techniques.[15,27,28]

With certain limitations as to the number and type of end points assessed and a 2-year time period, this study assessed different approaches to tissue preservation and storage to optimize tissue suitability for subsequent clinical or research studies. We found no reproducible evidence that refrigeration at 4°C, presence of a desiccant, or an oxygen-depleted atmosphere significantly improved the chosen end points over 24 months. The PlexSet system for simultaneous assessment of multiple mRNA correlated well with expected gene expression levels based on the known cell populations within the tissues and may be useful for future projects assessing RNA expression patterns.

Author Disclosure Statement

No conflicts of interest to declare.

Funding Information

Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4
Supplementary Table S5
Supplementary Table S6

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