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A review of room temperature storage of biospecimen tissue and nucleic acids for anatomic pathology laboratories and biorepositories

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Abstract

Frozen biospecimens are crucial for translational research and contain well preserved nucleic acids and protein. However, the risk for catastrophic freezer failure as well as space, cost, and environmental concerns argue for evaluating long-term room temperature storage alternatives. Formalin-fixed paraffin embedded (FFPE) tissues have great value but their use is limited by cross-linking and fragmentation of nucleic acids, as well as loss of enzymatic activity. Stabilization solutions can now robustly preserve fresh tissue for up to 7 days at room temperature. For longer term storage, commercial vendors of chemical matrices claim real time stability of nucleic acids of over 2 years and their accelerated aging studies to date suggest stability for 12 years for RNA and 60 years for DNA. However, anatomic pathology biorepositories store mostly frozen tissue rather than nucleic acids. Small quantities of tissue can be directly placed on some chemical matrices to stabilize DNA, however RNA and proteins are not preserved. Current lyophilization approaches can preserve histomorphology, DNA, RNA, and proteins though RNA shows moderate degradation after 1–2 years. Formalin free fixatives show improved but varying abilities to preserve nucleic acids and face validation as well as cost barriers

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CONFLICTS OF INTEREST

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in replacing FFPE specimens. The paraffin embedding process can degrade RNA. Development of robust long-term room temperature biospecimen tissue storage technology can potentially reduce costs for the biomedical community in the face of growing targeted therapy needs and decreasing budgets.

Keywords

Room temperature; tissue; biospecimen; biorepository; biobank

1. INTRODUCTION

Formalin-fixed paraffin embedded (FFPE) tissues and ultralow temperature frozen tissue (at −80°C to −190°C) are the most widely used sources of nucleic acids, protein, and histology for diagnostic and research purposes [1–3]. At ultra-low temperatures, high molecular weight nucleic acids and enzymatically active protein in tissues are preserved for many years but RNA may be more prone to degradation [4]. Equipment, space, power, maintenance, and handling costs for frozen biospecimens are substantial over the long-term. Freezer failure, even with temperature alarms, is a real concern as demonstrated by loss of one third of the specimens in a national autism brain bank [5]. Liquid nitrogen is hazardous to work with and failure to maintain adequate levels in storage containers can result in loss of specimens.

Formalin-fixed paraffin-embedded (FFPE) tissue, the ubiquitous room temperature clinical tissue biospecimen, has been evaluated as a potential source for nucleic acids and proteins. However, FFPE samples have cross-linked and fragmented nucleic acids, denatured proteins, and DNA modifications at an estimated rate of as much as one mutation per 500 bases [6]. RNA is also fragmented and RNA yield is poor though samples may be amenable for RT-PCR [6]. Enzymatic activity is lost with formalin fixation [6]. Therefore, FFPE samples are not ideal for a significant subset of molecular analyses which require better preserved nucleic acids or protein. Also, formalin is a known carcinogen. Alternative formalin-free fixatives that better preserve nucleic acids have been developed but have been slow to gain acceptance. However, traditional formalin-fixed samples are convenient to store, have high quality histology, and obviate risk of handling human material after fixation. Many laboratories have validated hundreds of clinical assays, mostly immunohistochemical stains but also in situ hybridization and PCR assays, on formalin fixed tissues.

Clearly, frozen biospecimens and the ubiquitous FFPE specimens have many advantages but also some significant drawbacks. What are alternative room temperature approaches that can provide cost and space savings yet provide high quality nucleic acids or proteins after long-term storage? Desiccated tissues of some Egyptian and other mummies have been shown to retain histologic details and yield protein and DNA after thousands of years [7,8]. Similarly, removing moisture from biospecimens significantly slows water dependent enzymatic activity including DNA and RNA nuclease activity [9]. Room temperature storage methods, which often leverage desiccation in part, have significant potential to change the current paradigm of costly yet vulnerable frozen storage. In this review, we discuss the availability, applications, and limitations of some major room temperature biospecimen storage modalities: desiccated chemical or polymer matrices, stabilization solutions, lyophilization or freeze-drying, and formalin-free fixatives (See Table 1).

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2. CURRENT ROOM TEMPERATURE STORAGE MODALITIES

2.1 Dry chemical or polymer matrices can preserve DNA only or RNA only for years and potentially for decades

To some extent, desiccated chemical matrices mimic natural extremophile biology that allows organisms such as tardigrades or brine shrimp to protect cellular systems in a dried state and later revive via rehydration- a process known as anhydrobiosis [10]. In contrast to extremophiles which inclusively preserve cellular structure, nucleic acids, and protein, currently commercial chemical matrices permit DNA-only long-term (years) room temperature storage or RNA-only storage and not both simultaneously. For most medical center biorepositories, it may not be cost effective to prospectively extract DNA and RNA from all tissue biospecimens when only a subset of specimens may be used. These matrices may be best suited for specific situations such as storage of excess extracted nucleic acids, storage of specific samples with predicted high demand for nucleic acids, or back-up of frozen biospecimens.

There is little independent data, particularly long-term data, regarding most of the commercial matrices. The one matrix with extensive data is Flinders Technology Associates (FTA) filter paper, a macroporous cellulose matrix treated with uric acid, detergent, and chelating agent. Upon application of a biosample to FTA paper, cells are lysed and DNA is released [11–13]. Cell lysis results in loss of tissue morphology but the DNA binds to the paper and is preserved. In addition to the effect of chelating agents, the DNA is dried in order to slow water-dependent enzymatic activity. FTA technology can be used to store genomic, plasmid, whole blood, or intracellular DNA; however, the manufacturer does not recommend storage of RNA. Forensic and evolutionary biology studies indicate utility of FTA paper for PCR assays after greater than 7 years [14–16]. DNA from 60 surgical specimens stored on FTA cards for 49 days at room temperature performed equally compared to freshly extracted DNA when assessed by PCR and IgH gene rearrangement tests [12]. Formal studies of the utility of FTA paper samples for whole exome and whole genome sequencing is lacking. Storage formats, testing results, reusability features, maximum load specifications, and mechanism are summarized in Table 2.

Chemical matrices use various combinations of reagents that minimize or prevent oxidation, hydrolysis, and nuclease activity. The chemical matrix is typically layered in wells of a titer plate or tube. Most of the matrices are proprietary and only limited information is available as to their precise composition (See Table 2). Nucleic acids samples are added to the matrix then dried and stored. Addition of water or more commonly a buffer can elute the nucleic acids out of the matrices for use. Forensic buccal, blood, and semen DNA stored for 45-months (3.66 years) using Biomatrica DNAstable showed limited degradation compared to −20°C controls when assessed by real-time PCR [22]. Genomic DNA stored for 1 year using Biomatrica's SampleMatrix® or at −20°C displayed equal DNA recovery and no detectable inhibition of amplification assessed by quantitative PCR, gel electrophoresis, and multiplex short tandem repeats analysis [20]. 336 RNA samples stored for 2 weeks and shipped in Biomatrica RNAstable, IntegenX GenTegra RNA, or −80°C were shown to be equally amenable to parallel RNA quality analyses and real-time PCR [28]. Room temperature DNA and RNA storage formats, testing results, reusability features, and maximum load specifications are summarized in Table 2 and Table 3 respectively. Peer reviewed studies that assess the utility of these chemical matrices for whole exome/genome sequencing and cDNA microarray analyses are highly desirable.

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2.2 Stabilization Solutions may preserve whole tissues at room temperature up to 7 days and DNA for 6 months

One stabilization solution, QIAGEN's AllProtect Tissue Reagent (QIAGEN, Valencia, CA, USA), preserves DNA, RNA, and protein in fresh tissue for up to 7 days at room temperature or one year in a refrigerator (see Table 1). The liquid reagent permeates cells, inactivates endogenous nucleases, and reduces microbial and reactive oxygen species activity but its composition is proprietary. AllProtect is of particular interest as specimens placed in AllProtect then frozen may potentially have short-term protection against freezer failure giving a few days to transfer samples safely to a working freezer. Five mice liver samples immersed in AllProtect before storage at −80°C (without snap-freezing) demonstrated better DNA/RNA quality than snap-frozen (without Allprotect) controls [29]. In contrast, 111 breast tissue samples immersed in AllProtect then snap-frozen and stored at −80°C did not show significant differences in DNA or RNA quality relative to fresh frozen controls [30]. Both studies measured DNA quality by $A_{260/280}$ outputs on Nanodrop 2000™ (Thermo scientific, Waltham, MA, USA) and 1.0% agrose gel electrophoresis, and RNA quality by Agilent 2100 Bioanalyzer® (Agilent Technologies, Inc., Santa Clara, CA, USA) [20,21]. Tissue or blood cells can also be placed directly into Biomatrica's DNAgard solution but this stabilizes DNA only though for 6 months at room temperature per the manufacturer's data (see Table 4).

2.3 Current lyophilization (Freeze-drying) approaches can preserve DNA, RNA, and protein including enzymatic activity at room temperature albeit with some nucleic acid degradation

Lyophilization, also known as freeze-drying or cryodesiccation, is a three-step dehydration process [31]. First, the lyophilizer, a device capable of manipulating temperature and pressure, freezes extracellular and intracellular water within a sample into ice. Second, pressure is lowered and temperature is raised sufficiently under a vacuum to sublimate ice directly into water vapor removing 95% of cellular moisture. Third, elevated temperatures break physicochemical bonds binding residual water molecules to tissue leaving a final desiccated sample with residual water content of about 1–4% of original tissue moisture [32–33]. In our laboratory, brain tumor samples stored for one year at room temperature in the dark and in vacuum-sealed vials displayed modest DNA and moderate RNA degradation but were suitable for histology, immunohistochemistry, PCR, RT-PCR, gene methylation, copy number variation, Western blots, and enzymatic assays [34]. In a study by Matsuo and colleagues, lyophilized rat liver tissue stored for four years at ambient room temperature in nitrogen-filled vials demonstrated minimal degradation, but RNA degradation was seen near the one year time-point of storage [35]. Despite the RNA degradation, RT-PCR was successful. They noticed, as did we, variable degrees of vacuolization in the histological sections probably related to ice crystal formation but histologic details were generally of good quality. 140 lyophilized human colon tumor, breast tumor, kidney for epithelial tumors, lymph node tumor, and skin lymphomas for lymphoid tumors samples stored in the dark at room temperature for one year exhibited no marked DNA loss as assessed by a ND-1000 Spectrophotometer and 1% agrose gel electrophoresis, or RNA loss as evaluated with a RNA 6000 NanoAssay and qRT-PCR [36]. Additional work is required to optimize lyophilization protocols and storage conditions including whether additives such as antioxidants or RNase inhibitors may enhance stability of lyophilized tissues.

2.4 Fixation with formalin-free fixatives and paraffin embedding can affect nucleic acid quality

The search for formalin substitutes has been driven by two major factors: its potential carcinogenicity [6] and the fact that formalin fixation yields fragmented and cross-linked DNA and RNA [9]. At least seven alcohol-based, non-crosslinking, formalin-free fixatives-

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UMFIX (Sakura Finetek USA, Torrance, CA), FineFIX (Leica Biosystems, Buffalo Grove, IL), HOPE (Polysciences Inc, Warrington, PA), methacarn, modified methacarn, Paxgene Tissue (Qiagen, Valencia, CA), RCL2 (Alphelys SAR, Plaisir, France) are reported to have a satisfactory quality of histology and extracted macromolecules (nucleic acids and proteins) [37–39]. Fixative composition, mechanisms, and independent testing results are detailed in Table 5. Crosslinking and non-alcoholic fixatives generally do not perform as well as non-crosslinking and alcoholic fixatives [38,39]. Non-crosslinking fixatives do not require heat-induced epitope retrieval [38]. Of the fixatives in Table 5, modified methacarn is the favored fixative in six independent evaluations, though two others suggest that UMFIX and RCL2 are equal in performance [38,40]. Paxgene Tissue induces histological artifacts that outweigh its molecular protective benefits [41,42]. A recent study also implicates high temperatures during the paraffin embedding process as a cause of reduced RNA yields and poor RNA quality in FFPE tissues [43]. It is important to note that placing fresh tissue into a formalin-free fixative alone is insufficient to preserve nucleic acid integrity. Care must be taken to ensure that the tissue processor uses ethanol in lieu of formalin during its cycles. Further study of formalin-free fixatives taking into account the embedding and processing conditions may permit a better understanding of how and whether to integrate these fixatives into daily practice.

3. Potential benefits of room temperature storage relative to frozen storage

3.1 Room temperature storage has lower fiscal, labor, and environmental costs over the long-term

After relatively modest upfront costs, room temperature storage requires minimal space and basic overhead for maintaining room temperature. Conversely, the upfront costs of freezers are substantial. Outfitting a room to store 10 freezers may require appropriate air-conditioning and installing circuitry connected to back-up generators and dedicated lines for each freezer. Costs to outfit the room may be as much as 20 – 40,000 United States Dollars (USD). Additionally, a freezer has a life span and may have to be replaced after 10–15 years. Furthermore, there are expenses associated with space needs. For example, the National Neurologic AIDS Bank, Los Angeles, CA maintains multiple of its own freezers in leased space that is subject to periodic rent increases and also rents freezer space at a commercial biobank. Annual costs for frozen storage such as warranties, maintenance, electricity, back-up power sources, back-up carbon dioxide, or liquid nitrogen tanks accumulate over the long-term. It has been estimated that the one year storage cost for a lyophilized sample is 3 euros compared to 24 euros at −80°C and 31 euros in liquid nitrogen [36]. In a vendor sponsored study at Stanford University, it was estimated from 12 pilot laboratories that converting from freezer to room temperature storage would save 1.2 – 1.4 million USD per year [62]. While we take the absolute amount of savings with a grain of salt, we do believe that cost savings do accrue particularly the longer the more specimens are retained. In addition to the financial cost, the environmental cost of freezers is substantial. In comparison to the 4000 lbs CO_2 and 15,000 lbs CO_2 of a −80 °C and −150 °C freezer respectively, a typical household refrigerator ejects approximately 1,700 lbs CO_2 annually [63]. Currently, shipping of frozen biospecimens requires the use of dry ice, specially trained personnel to package the specimens, and high freight costs related to shipping a hazard (dry ice). If the biospecimen does not arrive in time, it will thaw completely. Room temperature preservation approaches avoids these issues [17,26].

3.2 Less space is required for room temperature storage

In a vendor-sponsored study conducted at Stanford University, storage in dry multi-well plates was estimated to reduce nucleic acid storage space requirements by about 70% over frozen storage [63]. Frozen tissues are stored in tubes, which is less spatially efficient than

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storing tissue derivatives on a high density plate at room temperature. A typical 96-well plate dimensions may be 127 mm by 86 mm by 8mm thick. In comparison, 96 purified DNA samples stored within standard 1.7ml microcentrifuge tubes (9.2 mm diameter, 38.7 mm length)(Sorenson Biosciences, Salt Lake City, UT, USA) aligned adjacently occupies approximately 883 mm by 883 mm by 39 mm- a substantially larger volume. For −150°C chest freezers where compressor and other mechanical requirements are particularly high, the actual storage space for containers may be as little as 17.3% of the total freezer volume (0.23 m^3 storage capacity; 1.34 m^3 total volume- Sanyo MDF-C2156VANC, Sanyo North America, Wooddale, IL, USA). In our experience, space needs for a freezer exceed the base footprint of the freezer as there is a need for adequate spacing in between units. To cool their contents, freezers generate substantial heat and packing large numbers of freezers too closely can result in chronic mechanical failures related to the stressor of elevated local temperatures. Liquid nitrogen tanks are also inefficient space-wise due to height limitations and the need for attached liquid nitrogen supply tanks.

4. ISSUES IN IMPLEMENTING NEW ROOM TEMPERATURE STORAGE MODALITIES

4.1 Issues in replacing formalin fixation

Why haven't formalin-free fixatives been more widely accepted in the pathology community? After all, the new fixatives offer potential advantages over FFPE. The higher cost of the fixatives is one impediment. Also, many current testing modalities such as PCR or RT-PCR work reasonably well with FFPE specimens reducing the impetus for change. In the United States, intra-laboratory validation for microscopy and routine testing is a major barrier. According to College of American Pathologists (CAP) guidelines, changes in biospecimen processing that can impact testing must be validated. To switch to a new formalin-free fixative, immunohistochemistry procedures for each antibody in a laboratory's repertoire and diverse molecular assays would need to be revalidated [58]. Furthermore, formalin-free fixatives and lyophilization face the challenge of producing histology equal or superior to that of FFPE in the subjective eyes of demanding pathologists. Earlier generation formalin-free fixatives Glyo-Fixx, STF-Streck, Omnifix, Histochoice, and Histofix were reported to have a lower quality of histology [65]. RCL2 is reported to allow good morphology, though pigment deposition, degranulation, and RBC lysis can occur [39]. FineFIX can lead to partial tissue disintegration during fixation, shrinkage artifacts, degranulation, and RBC lysis [39]. One review concludes however that there are relatively minor differences that do not interfere with establishing the correct diagnostic conclusion [66]. The growth of whole exome and whole genome sequencing as well as other next-generation molecular approaches that require a higher quality of nucleic acids could tip the balance towards formalin alternatives.

4.2 Issues in replacing frozen tissues

In terms of replacing frozen tissue, no modalities exist currently that permit simple room-temperature preservation of tissue while preserving histology, DNA, RNA, and protein for the long-term (years long). Most current modalities preserve DNA or RNA alone. In addition, existing published real time stability data for the commercial matrix technologies is fairly limited except for FTA paper. Nevertheless, the experience with FTA paper and commercial accelerated aging studies of newer matrices suggest room temperature stability in the order of decades is likely to be achievable. There are large provincial or national genetic repositories that use matrices in multi-well plates for room temperature DNA storage but these laboratories also have significant resources such as robotic high-throughput nucleic extraction that lower their cost for doing so. Lyophilization has an intriguing potential to provide a means to preserve all tissue components including enzymatic activity; however,

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further research on lyophilized specimens is needed to better stabilize nucleic acids for long term storage [34].

5. CONCLUSIONS

Tissues frozen at ultra-low temperatures are the current gold standard biospecimen for translational research and can yield DNA, RNA, and protein amenable to a broad range of testing from next generation sequencing to enzymatic assays. The rapidly accelerating accrual of biospecimen in the biomedical world and decreasing biomedical funding should prompt consideration of long-term (decades) room temperature storage modalities that promise lower costs and increased efficiency. In the United States alone, there were an estimated 200 million biospecimens in the year 2000 and approximately 600 million a decade later [67]. FFPE tissue, while a room temperature storage modality and ubiquitous in pathology departments, presents challenges for modern gene expression and next-generation sequencing technologies and does not preserve enzymatic activity. Exciting advances have been made in short term (7 days) room temperature tissue storage with stabilization solutions. Although stabilization liquids are not a long term solution, the concept of storing and freezing tissue in stabilization solutions should be considered for insurance against unexpected freezer failure or temperature fluctuations. Unfortunately, long-term room temperature storage desiccated chemical matrices are currently limited to nucleic acids. We believe that these may be suited for situations where excess nucleic acids extracted for experiments need to be stored, high demand specimens can be preemptively extracted, or a compact back-up solution is required. One concern for biobankers is backing up specimens to a geographically separate location in order to mitigate the risk of water or fire damage to a freezer room. The ability to create back-up room temperature samples facilitates identification of adequate space. While very useful for some niche purposes, present room temperature technologies are not ready to replace frozen tissue or FFPE tissue. We believe that national funding institutions and pharmaceutical companies should invest substantially in developing low cost, low maintenance, room temperature biospecimens that can be used widely in the surgical pathology and biomedical fields. At many biorepositories including ours, we must maintain both FFPE and frozen biospecimens in parallel to meet clinical and research needs- a fundamentally inefficient process. A single high quality, low cost biospecimen storable over decades at room temperature, widely adoptable by pathology departments, amenable to histologic examination, and suitable for modern genetic analyses can produce critical efficiencies- directly by removing frozen specimen-associated costs and indirectly by expediting robust targeted therapy clinical trials.

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ABBREVIATIONS

FFPE	formalin-fixed paraffin embedded
RT-PCR	reverse transcription polymerase chain reaction
FTA	Flinders Technology Associates
IgH	immunoglobulin heavy chain
qRT-PCR	quantitative reverse-transcription polymerase chain reaction

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USD	United States Dollars
CAP	College of American Pathologists

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Highlights

1. Room temperature storage can lower spatial, fiscal, and environmental costs.
2. Current room temperature technologies can store purified nucleic acids for years.
3. Stabilization solutions permit room temperature whole tissue storage for 1 week.
4. Alcohol-based formalin-free fixatives yield good histology and nucleic acids.
5. Lyophilization shows promise for long-term whole tissue stabilization.

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Table 1

Overview of room temperature storage modalities

Modality	Application	Mechanism	Notes
Formalin-fixed paraffin embedded (FFPE)	Good histology and immunohistochemistry; can be used for some molecular assays that tolerate degraded nucleic acids.	Dehydrates tissue and renders inert enzymes that can degrade nucleic and proteins.	Low cost relative to non-formalin fixatives. DNA and RNA yield poor. Nucleic acids fragmented. Nucleic acids and proteins cross-linked.
Formalin-free fixed paraffin embedded	Preserves DNA and RNA and histomorphology in paraffin-embedded specimens.	Dehydrates tissue and renders inert most enzymes that can degrade nucleic acids and proteins. Avoids formalin cross-links that damage macromolecules.	Often alcohol-based. Morphology may differ slightly from FFPE tissue. To use, will need to revalidate immunostains and other tests. Costs are higher currently.
Desiccated chemically modified matrices	Protects purified DNA only or RNA only from lysed cells, tissue, or blood samples.	Chemical shielding protects macromolecules from UV light, microbial, or nuclease insult.	Cell lysis destroys tissue morphology. Protein not preserved. Current platforms store either DNA or RNA but not both for years.
Stabilization liquids	Whole tissue can be stored to preserve DNA RNA, and protein (or DNA only). No data for histology or enzymatic activity.	Liquids permeate tissue and provide chemical protection.	1–7 days preservation at room temperature; 1 year at 4 degrees Celsius.
Lyophilization	Preserves genomic DNA, RNA, proteins including enzymatic activity, and histomorphology of whole tissue samples.	Sublimation of tissue moisture through cold temperatures and pressure variations removes water which inhibits nucleases and microbes.	Investigational only. Nucleic acids and proteins preserved for 1–4 years but RNA particularly may have moderate degradation after 1 year.

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Table 2

Room temperature storage of DNA

DNA Storage	Biomatrica	IntegenX	QIAGEN **	FTA paper	
Product Name	**DNA Stable**	**GenTegra™ DNA**	**QIAsafe DNA**	**IntegenX GenPlate (FTA paper)**	**GE Healthcare Whatman FTA (FTA paper)**
Storage Formats	1.5 ml centrifuge tube; 96-well plate; 96-tubes plate; or custom	1.7 ml centrifuge tubes; 1.5ml tubes; 0.3 ml cluster tubes; or 96-well plate	96-well plates	384-well plate, 6mm diameter wells, with or without barcode, available in 3,6,12, or 24 regions.	4-well FTA elute ™ micro cards, 11mm diameter wells, without barcode, available in 25 or 100 card pack
Room Temp. Storage Time Tests	26 months real time tested. 30 years* simulated at 60°C. −20°C control.	Four months real time tested. 10 years* simulated at 76°C	More than 2 years of real time stability data. Up to 30 years* in accelerated studies	>7 years real time (serum DNA)	>17 years real time (buccal DNA)
Reusability	Up to 3 Rehydration/Dryin g Cycles	Up to 4 Rehydration/ Drying Cycles	Up to 4 Rehydration/ Drying Cycles	One time use only	One time use only
Max DNA Per Tube or Well	30 µg	25 µg	30 µg	10µl	40µl
Mechanism	Proprietary glass-like polymer SampleMatrix® "shrink-wraps" and shields DNA or RNA respectively from heat and UV radiation [17–23].	Inert inorganic mineral matrix containing reactive oxygen species scavengers minimizes oxygen diffusion to reduce natural oxidation and hydrolysis [19, 24–26].	QIAGEN utilizes Biomatrica's SampleMatrix® technology.	Macroporous cellulose matrix containing pre-applied uric acid, a weak base, anionic surfactant or detergent, and a chelating agent denatures proteins, inactivates nucleases, and slows enzymatic activity after drying [10,11].	

* Accelerated aging via storing biospecimens at elevated temperatures has been utilized to predict long-term stability with claims in the order of decades. These claims are based on the Arrhenius equation that describes the increased rate of chemical reactions as a function of increased temperature [27].

** QIAGEN's QIAsafe DNA uses Biomatrica's SampleMatrix technology

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Table 3

Room temperature storage of RNA

RNA Storage	**Biomatrica**	**IntegenX**
Product Name	**RNA stable**	**GenTegra™ RNA**
Storage Formats	1.5 ml Screw-Cap Tube; 96 tube-Well Plate	0.3 ml cluster tubes (96 tubes per rack); or 1.5 ml screw-cap tubes (96 tubes per rack); 1.7ml Flip-top tubes
Room Temperature Storage Time Tests	29 months real time tested. 12 years* simulated at 45°C–80°C controls	7 months real time tested. 14 years* simulated at 76°C
Reusability	Up to 3 Rehydration/Drying Cycles	Up to 4 Rehydration/Drying Cycles
Max RNA Per Tube or Well	100 μg	20 μg

*Accelerated aging via storing biospecimens at elevated temperatures has been utilized to predict long-term stability with claims in the order of decades. These claims are based on the Arrhenius equation that describes the increased rate of chemical reactions as a function of increased temperature [27].

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Table 4

Room temperature storage of blood-derived DNA

Collecting & Transporting Whole Blood	**Biomatrica**	**Qiagen** *	**FTA paper**	
Product Name	**DNAgard Blood**	**QIAsafe DNA Blood**	**IntegenX GenPlate (FTA paper)**	**GE Healthcare Whatman FTA (FTA paper)**
Product Formats	DNAgard reagent 10,50,100 ml (stabilizes at least 40–400ml of blood)	Blood Tubes and 48-Well Plates (50–200μl of whole blood)	10μl biosample stored in 384-well plate, 6mm diameter wells, with or without barcode, available in 3,6,12, or 24 regions.	40ul biosample stored in 4-well FTA elute ™ micro cards, 11mm diameter wells, without barcode, available in 25 or 100 card pack
Application	Immediate stabilization of DNA in blood for room temperature processing and shipping. Recovery will be comparable to a frozen sample.	Protection and stabilization of DNA in blood at room temperature. Allows recovery of up to 2 μg DNA from 50 μl blood	Stabilization of DNA only from whole blood, buccal swabs, serum, and tissue.	Stabilization of DNA only from whole blood, buccal swabs, serum, and tissue
Room Temperature Storage Time	14 months real time tested. 8 years** simulated at 50°C.	14 months real time at ambient temperature 8 years** simulated at 50°C.	>7 years real time (serum DNA)	>17 years real time (buccal DNA)

* QIAGEN's QIAsafe DNA Blood uses Biomatrica's SampleMatrix technology

** Accelerated aging via storing biospecimens at elevated temperatures has been utilized to predict long-term stability with claims in the order of decades. These claims are based on the Arrhenius equation that describes the increased rate of chemical reactions as a function of increased temperature [27].

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Table 5

Formalin and formalin-free fixatives

Fixative	Chemical composition	Mechanism	Macromolecule integrity and reported uses
Formalin	Variable; typically 10% neutral buffered formalin-3.7% formaldehyde in water with 1% methanol and sodium phosphate buffer (Non-proprietary)	Formaldehyde, a reactive electrophile, reacts with macromolecule functional groups to form a -CH2-methylene covalent bridge crosslinking macromolecules [44]. This crosslinking is theorized to fragment nucleic acids [39,40].	Nucleic acids fragmented and cross-linked. Proteins cross-linked. DNA often 400bp or less; RNA most 25-200bp; PCR, RT-PCR, CISH, FISH, IHC, Western blots, cDNA microarrays [45–49].
Methacarn	60% absolute methanol, 30% chloroform, and 10% glacial acetic acid. Modified methacarn omits chloroform. (Non-proprietary)	Methanol and chloroform stabilize helical proteins and nucleic acids by replacing water-protein hydrogen bonds and preserving hydrophobic interactions in proteins [50]. Methanol is more protic than ethanol and formaldehyde, and in theory, is superior to both in replacing water-to-nucleic acid hydrogen bonds [50].	High molecular weight DNA, rRNA, mRNA (most 400–3000bp; up to 6000bp), proteins preserved; PCR (up to 2900 bp), RT-PCR (most 300–700bp, up to1900bp), IHC, Western blots, cDNA microarrays [50–52].
UMFIX	Polyethylene glycol-based fixative (proprietary)	Ethylene glycol has two hydroxyl groups as opposed to methanol's single group. This provides superior hydrogen bonding potential than formaldehyde, methanol, and ethanol but decreases access to small binding sites due to larger size [50].	High molecular weight DNA, RNA (400–3000bp; up to 6000bp), and protein preserved; PCR, RT-PCR, IHC, CISH, FISH, mass spectroscopy, and cDNA microarrays. Tissue stable at room temperature for 8 weeks [38,52,53].
FineFIX	Patented formula diluted with ethanol to a 70% concentration (proprietary)	Mechanism of patented formula is proprietary. Ethanol extends the nonpolar chain of methanol possibly decreasing access to small binding sites and weakening hydrophobic interactions within globular proteins [50].	High molecular weight DNA, mRNA, proteins preserved; rRNA degraded; PCR, RT-PCR, IHC, CISH, FISH, Western blot [39,54].
HOPE	Hepes glutamic acid buffer mediated organic solvent protection effect fixative (proprietary)	The protection solution, central to HOPE fixation, is proprietary. Dehydration is accomplished in acetone [55].	High molecular weight DNA, RNA and protein preserved; PCR, RT-PCR, CISH, FISH, IHC, Northern blot. Long RNA strands >2kb in some cases; Good protein yields for Western blot [55–58].
RCL2	Ethanol, acetic acid, complex carbohydrate (proprietary)	Ethanol extends the nonpolar chain of methanol possibly decreasing access to small binding sites and weakening hydrophobic interactions within globular proteins [50]. Ethanol is more protic than formaldehyde, and is theoretically superior in replacing water-to-nucleic acid hydrogen bonds [50].	High molecular weight DNA, mRNA, and protein preserved; good RNA yields but rRNA degraded; PCR, RT-PCR, IHC, CISH, FISH, array CGH [39,58–61].

PCR= Polymerase chain reaction; RT-PCR= Reverse transcription polymerase chain reaction; IHC= Immunohistochemistry; CISH= Colorimetric in situ hybridization; FISH= Fluorescent in situ hybridization; CGH= Comparative genomic hybridization