

# Storage of Human Biospecimens: Selection of the Optimal Storage Temperature

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Millions of biological samples are currently kept at low temperatures in cryobanks/biorepositories for long-term storage. The quality of the biospecimen when thawed, however, is not only determined by processing of the biospecimen but the storage conditions as well. The overall objective of this article is to describe the scientific basis for selecting a storage temperature for a biospecimen based on current scientific understanding. To that end, this article reviews some physical basics of the temperature, nucleation, and ice crystal growth present in biological samples stored at low temperatures ( $-20^{\circ}\text{C}$  to  $-196^{\circ}\text{C}$ ), and our current understanding of the role of temperature on the activity of degradative molecules present in biospecimens. The scientific literature relevant to the stability of specific biomarkers in human fluid, cell, and tissue biospecimens is also summarized for the range of temperatures between  $-20^{\circ}\text{C}$  to  $-196^{\circ}\text{C}$ . These studies demonstrate the importance of storage temperature on the stability of critical biomarkers for fluid, cell, and tissue biospecimens.

## Introduction

**B**IOSPECIMENS INCLUDE TISSUES, CELLS, and bodily fluids (and their constituent macromolecules). It is common for biospecimens to be collected for use at a later time and different location. The critical biological properties of the biospecimen must be preserved during processing, transport, and storage. The most common method of preserving biospecimens is freezing and storage of samples at low temperatures in order to inhibit degradation. Other stabilization methods include chemical fixation,<sup>1</sup> plastination,<sup>2</sup> drying,<sup>3</sup> lyophilization,<sup>4</sup> ionic liquids,<sup>5</sup> dry state storage,<sup>6, 7</sup> and confinement.<sup>8</sup> The focus of this review will be on long-term, low temperature storage of human biospecimens. Issues associated with ischemia or storage studies of non-human samples will not be included in this review. It is noteworthy that many of the biophysical and biological events that occur during freezing will influence the stability of any biospecimen, regardless of origin.

## Physics of Biospecimens at Low Temperatures

As water is necessary for most biochemical reactions (including those that degrade molecules), understanding the mobility of water during freezing is important. The mobility (and therefore ability of the water to participate in reactions) differs between water that is liquid, solid (e.g., ice), and glass (highly viscous liquid). The use of low temperature storage to stabilize biological specimens reflects the desire to control, on some level, the behavior of water in the system (see Ref. 9 for review) and specifically to reduce mo-

bility of water by freezing or vitrification. In order to understand what happens to water during freezing, a brief tutorial of the physics at low temperature is useful.

Most fluid biospecimens are complex mixtures containing at minimum water, salt, and proteins, and therefore they do not solidify at a single temperature, but freeze over a range of temperatures. The thermodynamic states of multicomponent solutions can be described by phase diagrams<sup>10</sup> and by time-dependent temperature-time-transition (TTT) diagrams.<sup>11</sup> Specifically, the fraction of water that has been solidified and the corresponding concentration of the unfrozen fraction of solution can be determined based on temperature, for a given initial concentration and pressure.<sup>12–16</sup> Water and all other components (e.g., lipids within the biological sample) undergo phase transition and can be described by phase diagrams as well.<sup>17</sup>

Unlike most materials, water can undercool significantly below its equilibrium freezing temperature.<sup>18</sup> Pure water can freeze at temperatures ranging from  $0^{\circ}\text{C}$  to  $-40^{\circ}\text{C}$ .<sup>19</sup> It is not uncommon for biospecimens to first form ice at  $-5^{\circ}\text{C}$  to  $-15^{\circ}\text{C}$ . The onset of freezing is called nucleation and can result from water molecules forming stable ice crystals aided by irregularities in the sides of the container or particles in the sample. The undercooling of the sample (where the sample nucleates at temperatures below the melting temperature) is an important parameter for post thaw recovery of cellular biospecimens and is frequently controlled using a variety of methods.<sup>20</sup> After nucleation of a sample occurs, water is removed from the sample in the form of ice. Solutes

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(e.g., proteins and salts) are not incorporated into the solid ice crystal. This results in a partitioning of the sample into a solid component and the unfrozen liquid component whose solute concentration increases as the temperature of the sample decreases. Cells, proteins, lipids, and other components are sequestered in the gap of unfrozen liquid between adjacent ice crystals.<sup>21, 22</sup> When much of the water has been removed in the form of ice, the remaining unfrozen liquid is highly concentrated<sup>23</sup> and the molecules, cells, and tissues present are subjected to solute concentrations of  $\sim 10,000$  mOsm.<sup>24</sup> The distribution of molecules may not be uniform in these partially frozen solutions. Microsegregation of protein solutions has been imaged during freezing using Raman spectroscopy<sup>25</sup> and demonstrated that even in gaps as small as  $1\ \mu\text{m}$ , the distribution of protein was not uniform and the protein exhibited aggregation near the ice–water interface.

The binary phase diagram of NaCl–water is often used as a model of the freezing behavior of biological systems in the absence of cryoprotective agents.<sup>10</sup> For example, when isotonic saline solutions [0.9% (w/w) NaCl] freeze slowly, the gap between adjacent ice crystals will decrease until the sample fully solidifies (at the eutectic temperature) or vitrifies (the glass transition temperature,  $T_g$ ). For an isotonic saline solution, the eutectic concentration is 23.3% (w/w) NaCl and occurs at  $-21.2^\circ\text{C}$ .

For cellular biospecimens and proteins stabilized for pharmaceutical applications, it is common for stabilizing agents to be added (dimethylsulfoxide and glycerol for cellular biospecimens<sup>26</sup> and sugars and sugar alcohols for proteins<sup>27</sup>), resulting in multicomponent solutions. Empirical equations have been used to estimate the glass transition temperature for multicomponent mixtures:

$$T_g(\text{mixtures}) = T_{g1} \cdot (1 - x) + T_{g2} \cdot x + k \cdot x \cdot (1 - x) \quad < \text{Eq.1} >$$

with the glass transition temperatures  $T_{g,i}$  ( $i=1,2$ ) of the components, the weight fraction  $x$  of the cryoprotective agent and an interaction parameter  $k$ . The glass transition temperature of a 10% (w/w) dimethylsulfoxide (DMSO) solution with  $T_{g1} = -135.2^\circ\text{C}$  for water,  $T_{g2} = -122.2^\circ\text{C}$  for DMSO,  $x=0.1$ , and  $k=16$  can be calculated to be  $-132.58^\circ\text{C}$ .<sup>28</sup> Compared to aqueous DMSO solutions, the glass transition temperature of the commonly used cryoprotective agent trehalose is comparatively higher ( $T_g = -113.9^\circ\text{C}$ ).<sup>13</sup> Below the glass transition temperature, the mobility of molecules within the sample is reduced due to an increased viscosity of  $10^{13}$  Pa·s. Angell<sup>29</sup> reported that NaCl solutions cannot vitrify under “normal” cooling procedures. However, the crystallization of isotonic systems can be prevented when applying very fast cooling rates.<sup>30</sup> The glass transition temperature is dependent upon several parameters, including concentration, storage temperature, and storage time.<sup>16,31</sup> More information on the thermodynamic principals of the phenomena described above (nucleation, crystal growth, and solidification) can be found in Ref. 32.

Solidification of water in the system is also influenced by the presence of extracellular matrix present in tissue biospecimens. Water interacts strongly with collagen and other matrix components and these interactions influence solidification in tissues.<sup>33</sup> Specifically, ice crystals form preferentially in blood vessels (for vascularized tissues)<sup>34</sup> and propagate down the vessel as the sample cools. For avascular tissues (e.g., cartilage, cornea), ice crystals form at different

locations fed by water flow down very narrow channels that form in the tissue.<sup>33</sup> These large ice crystals (or ice lenses) can result in significant disruption/reorganization of extracellular matrix.<sup>35</sup> Therefore, the presence of extracellular matrix may also influence the solidification process.

As described above, biospecimens are complex solutions that freeze over a range of temperatures. During freezing, water is removed in the form of ice; solute, proteins, and even cells are partitioned from the ice. The sample is not fully solidified until a eutectic or glass forms in the remaining unfrozen fraction of liquid. The presence of extracellular matrix also influences the solidification of water. Therefore, storage of samples at temperatures in which liquid water is still present (versus frozen or vitrified water) will result in reduced stability.

## Characterizing Samples at Low Temperatures

Various modalities exist that allow for the investigation of specimens at low temperatures. These methods permit researchers to obtain detailed knowledge about the effects of temperatures on the biological sample during cooling and rewarming, and thereby contribute to improvements in the cooling and rewarming protocols. Several modalities have been adapted for low temperature measurements, including: spectroscopy, calorimetry, tomography, and microscopy. Fourier transform infrared (FTIR) and Raman spectroscopy are used to detect molecular vibrations of the sample, which can deliver information about water transport and ice crystal growth.<sup>36,37</sup> Commonly used calorimetric methods include differential scanning calorimetry (DSC) and differential thermal analysis (DTA); these techniques are used to create phase diagrams, quantify water transport through the plasma membranes,<sup>38</sup> and detect ice crystal growth within cells.<sup>39</sup> Magnetic resonance imaging (MRI)<sup>40</sup> and computer tomography (CT)<sup>41,42</sup> have been used to visualize three-dimensional ice crystal growth and permeation of cryoprotective agents within spatially extended specimens. Microscopic methods include cryomicroscopy,<sup>43</sup> directional solidification,<sup>44</sup> cryo electron microscopy,<sup>45</sup> and multiphoton microscopy.<sup>46</sup> Some of these different modalities have been used in combinations, for example, cryomicroscopy with DSC<sup>47</sup> or Raman spectroscopy.<sup>25</sup>

## Biological Activity at Low Temperature

All biological specimens contain degradative molecules. Lipases, carbohydrases, proteases, and nucleases may be present in fluid and/or tissue biospecimens. Proteases such as matrix metalloproteinases can act as both biomarkers as well as to degrade biomarkers in a sample.<sup>48</sup> Enzyme activity is a function of the dynamics of the specific protein.<sup>49</sup> Temperature has a strong influence on protein dynamics; reduced temperatures result in reduced protein dynamics/activity. The reduction in protein activity with decreasing temperature is one mechanism by which biospecimens are stabilized at low temperatures. The temperature dependence of protein activity with temperature follows an Arrhenius relationship,<sup>50</sup> whereby the rate constant  $k$  of a chemical reaction is dependent on the absolute temperature  $T$ :

$$k = A \cdot e^{-Ea/(R \cdot T)} \quad < \text{Eq.2} >$$

with the pre-exponential factor  $A$ , the activation energy  $Ea$ , and the universal gas constant  $R$ . Therefore, low

temperatures result in a low rate constant  $k$  of chemical reactions.

Optimal storage temperatures should be selected below the threshold temperature for activity of the protein. Distinct changes have been observed in the dynamic properties of different proteins near  $-53^{\circ}\text{C}$ .<sup>51–54</sup> The temperature dependent behavior of ribonuclease A (RNase A) was studied by Rasmussen and colleagues.<sup>55</sup> They determined that the substrate failed to bind the enzyme at 215 K ( $-58^{\circ}\text{C}$ ). Similar results were observed by Tilton et al.<sup>56</sup> in which RNase A was observed to change behavior at 180–200 K ( $-93$  to  $-73^{\circ}\text{C}$ ). In contrast, More and colleagues observed activity of  $\beta$ -glucosidase at temperatures as low as  $-70^{\circ}\text{C}$ .<sup>22</sup>

These studies demonstrate that for the limited number of proteins studied, protein activity may persist at very low temperatures ( $< -80^{\circ}\text{C}$ ). Therefore, storage at temperatures at which water is still mobile and proteins are still active will result in degradation of the biospecimen.

### Identifying Suboptimal Storage Conditions

Measuring the mobility of water and activity of proteins in actual biospecimens is not practical in most situations. Most biobanks will have to rely on strong and effective quality control programs to identify suboptimal storage conditions.<sup>57</sup> One approach would be to collect a small number of biospecimens for quality control testing (not distribution). These samples could be monitored prefreeze, postfreeze, and as a function of time for prevalence of biomarkers of interest. Degradation of biomarkers with time in storage suggests that the specimen is not being stored optimally and should be stored at lower temperatures. A proper quality control program will also permit determination of biomarkers that respond poorly to cryopreservation (versus those that are not stable for a given storage condition). For example, lactate dehydrogenase loses all functional activity upon freezing,<sup>58</sup> so its stability with storage will appear poor only because it has been damaged simply by freezing. Therefore, a robust quality control program will facilitate determination of biomarkers that respond poorly to cryopreservation versus those that are not stable in storage. Biospecimens containing biomarkers that respond poorly to cryopreservation must be stored using alternative modalities (e.g., dry state storage, liquid storage) and biospecimens containing biomarkers that are not stable with time in storage need to be stored at lower temperatures. It is noteworthy that for some biospecimens, specifically cells, damage to the biospecimen may be manifest in post thaw apoptosis,<sup>59</sup> which evolves over time.<sup>60–65</sup> Therefore, post thaw assessment of cellular biospecimens must accommodate the time to evolution of this damage mechanism. Monitoring biospecimens for damage resulting from freezing, stability in storage, and proper post thaw assessment methods will be critical for the continuous improvement in quality for biobanking.

### Literature Review of Storage Studies

The effect of storage conditions on the stability of biomarkers has been studied for a variety of systems. As indicated previously, the following sections will describe studies to date on the stability of human protein, cell, and tissue biospecimens. Additional references outside of the scope of this review can be found in the ISBER Biospeci-

men Sciences Working Group literature compilation found on the ISBER website at ([http://c.y.mcdn.com/sites/www.isber.org/resource/resmgr/Files/ISBER\\_BIOSPECIMEN\\_SCIENCE\\_LI.pdf](http://c.y.mcdn.com/sites/www.isber.org/resource/resmgr/Files/ISBER_BIOSPECIMEN_SCIENCE_LI.pdf)) and a working review outlining translating cryobiology principles into biobanking practice.<sup>66</sup>

### Storage of Proteins and Purified Nucleic Acids

Fluid biospecimens (e.g., serum, plasma, urine, cerebral spinal fluid, etc.) are common sources of protein biomarkers and the influence of storage temperature on the stability of a range of biomarkers is summarized in Table 1. Millions of fluid biospecimens are currently stored in biobanks around the world. The ability to use these biospecimens for downstream applications depends strongly upon the processing and storage conditions for the biospecimens,<sup>67</sup> leading to uncertainty as to the interpretation of biomarker studies.<sup>68</sup>

For instance, a recent study followed the concentration of two serum cancer markers (cancer antigens CA125 and CA15-3) over a 10-year period and observed an increase of 15% in prevalence of these biomarkers as a function of time in storage.<sup>69</sup> In another study, Kisand et al.<sup>70</sup> reported that vascular endothelial growth factor (VEGF) levels decreased dramatically after serum was repeatedly frozen and thawed while stored at either  $-20^{\circ}\text{C}$  or  $-75^{\circ}\text{C}$ . Another serum component, matrix metalloproteinase-7 (MMP-7), was stable after 5 years of storage at  $-20^{\circ}\text{C}$ , and would be stable for 100 years at  $-75^{\circ}\text{C}$  as calculated using the Arrhenius equation.<sup>70</sup> Overall, these investigators concluded that storage of serum at  $-20^{\circ}\text{C}$  is unsuitable for biomarkers.<sup>70</sup> Matrix metalloproteinase-9 (MMP-9), a biomarker for cardiovascular risk detection in clinical studies, was found to degrade over time in storage at  $-80^{\circ}\text{C}$ . After 24 months, MMP-9 levels in plasma dropped to 65%, and after 43 months the level decreased to only 1%.<sup>71</sup>

The stability of free prostate-specific antigen (PSA) in serum under different storage conditions was tested by Woodrum et al.,<sup>72</sup> who found that free PSA levels decreased over time. Storage at  $-20^{\circ}\text{C}$  led to a 0.9% loss, while storage at  $-70^{\circ}\text{C}$  led to a 0.4% loss for each month in storage.<sup>72</sup> Schiele et al.<sup>73</sup> measured the stability of apolipoprotein concentration in human serum after up to 4 years at different temperatures ( $-20^{\circ}\text{C}$  and  $-80^{\circ}\text{C}$ ). They found that apolipoprotein concentrations were not significantly affected following storage for 3 months at  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ , or storage for 4 years at  $-80^{\circ}\text{C}$ . Qvist et al.<sup>74</sup> studied the stability of C-telopeptides of type I collagen (CTX), a bone resorption marker, in blood and serum samples. After 3 years of storage at different temperatures ( $-20^{\circ}\text{C}$ ,  $-80^{\circ}\text{C}$ , and  $-150^{\circ}\text{C}$ ), CTX was reported to be stable only when frozen (i.e., at  $-20^{\circ}\text{C}$  and lower temperatures).<sup>74</sup>

Karlsen et al. found that vitamin C (ascorbic acid and dehydroascorbic acid) degenerated within plasma after only 1 day at  $-20^{\circ}\text{C}$ , leading the authors to recommend storage of these samples at  $-70^{\circ}\text{C}$ .<sup>75</sup> Rai et al.<sup>76</sup> studied pre-analytical factors by storing plasma at different temperatures ( $-80^{\circ}\text{C}$ ,  $-196^{\circ}\text{C}$ ) and performing proteomic analysis (SDS-PAGE and SELDI-TOF). They concluded that plasma should be stored in liquid nitrogen.<sup>76</sup>

Interestingly, some analytes were found to increase in concentration over time during low temperature storage. For example, Männistö et al.<sup>77</sup> evaluated thyrotropin (TPO), thyroid hormones (TSH, fT4, fT3), and thyroid autoantibody

TABLE 1. COMPILATION OF STUDIES DESCRIBING THE STABILITY OF SPECIFIC BIOMARKERS FOR BIOSPECIMENS (BIOFLUIDS, CELLS, TISSUES) AS A FUNCTION OF STORAGE TEMPERATURE/DURATION

| Biospecimen   | Fluid biospecimens        |                       |   | Tracked analyte        | Stability <sup>†</sup> | Reference |
|---|---------------------------|-----------------------|---|------------------------|------------------------|-----------|
|   | Duration                  | Storage temperature   | Tracked analyte   |                        |                        |           |
| Biological fluids   |                           |                       |   |                        |                        |           |
| Blood   | 60 min                    | -80°C, -120°C         | Creatinine  | M                      | 103                    |           |
| Blood   | 2 to 96 hours             | -80°C                 | C-reactive protein, retinol, ferritin, folic acid, fatty acids  | M                      | 104                    |           |
| Blood   | 10 min to 8 hours         | -4.3°C                | Heparin   | S                      | 105                    |           |
| Blood   | 120 hours                 | -20°C                 | Hormones in plasma and serum  | M                      | 106                    |           |
| Blood   | 1 year                    | 20 to -196°C          | Hemoglobin  | M                      | 107                    |           |
| Blood, plasma   | 24 hours                  | -80°C                 | Cell-free DNA   | N                      | 81                     |           |
| Blood, plasma   | 80 days                   | -70°C                 | Ascorbic acid   | N                      | 75                     |           |
| Blood, plasma   | 14 days                   | -6°C                  | Vitamin B <sub>12</sub> , folate  | M                      | 79                     |           |
| Blood, plasma   | 3 to 48 months            | -20°C                 | Vitamin A, B <sub>1</sub> , B <sub>2</sub> , B <sub>6</sub> , B <sub>12</sub> , E, niacin   | M                      | 108                    |           |
| Blood, plasma   | 1,3,4,5 months            | -20°C                 | Anti-dengue IgM and IgG antibodies  | N                      | 109                    |           |
| Blood, serum  | 1 year                    | -80°C                 | Matrix metalloproteinase-9  | N                      | 71                     |           |
| Plasma  | 2 year                    | -20°C, -80°C          | Triglyceride, high-density lipoprotein, cholesterol   | N                      | 110                    |           |
| Plasma  | 37 months                 | -38°C to -42°C        | Coagulation factors FV, FVIII:C and FXI; major inhibitor antithrombin III (AT III)  | S                      | 111                    |           |
| Plasma  | 15 min to 8 hours         | -80°C, -196°C         | Lithium-heparin, sodium-citrate   | M                      | 76                     |           |
| Plasma  | 24 months                 | -24°C, -74°C          | Clotting assays for factors II, V, VII, VIII, IX, X, XI and XII   | M                      | 112                    |           |
| Plasma and urine  | 1 year                    | -20°C, -70°C          | Catecholamines (CA)   | M                      | 113                    |           |
| Serum   | 5 years                   | -20°C                 | Matrix metalloproteinase-7  | M                      | 70                     |           |
| Serum   | 18 days                   | -20°C, -70°C          | Folate  | N                      | 114                    |           |
| Serum   | 7 days                    | -20°C, -70°C          | Free prostate-specific antigen  | M                      | 72                     |           |
| Serum   | 1 to 24 hours             | -80°C                 | ACT-PSA   | M                      | 115                    |           |
| Serum   | 7 days                    | -20°C                 | PSA, tPSA, cPSA, tPSA   | M                      | 116                    |           |
| Serum   | 1 week, 3 months          | -20°C, -80°C          | Serum apo E   | M                      | 73                     |           |
| Serum   | 10 days, 3 months         | -20°C                 | Lipid, lipoprotein, apolipoprotein  | M                      | 117                    |           |
| Serum   | 10 years                  | -20°C, -80°C          | Cancer antigens, CA-125 and CA-15-3   | N                      | 118                    |           |
| Serum fatty acids   | 12 to 24 months           | -2°C, -80°C           | Docosapentaenoic acid, docosahexaenoic acid   | M                      | 119                    |           |
| Serum, plasma   | 3 years                   | -20°C, -80°C, -150°C  | C-telopeptides of type I collagen (CTX)   | M                      | 74                     |           |
| Serum   | 0.5 to 23 years           | -25°C                 | Thyrotropin, thyroid hormones, and thyroid autoantibodies (TSH, FT4, TPO-Ab, or TG-Ab)  | S                      | 77                     |           |
| Serum   | 8 to 11 years             | -80°C                 | Thyroid-stimulating hormone (TSH)   | N                      | 78                     |           |
| Specimens for gastrin   | 2 weeks                   | -70°C                 | Cholylglycine, cortisol, digoxin, ferritin, follitropin, immunoglobulin E, lutropin, prolactin, thyroxin (also blood-spot thyroxin), triiodothyronine | M                      | 48                     |           |
| Urine   | 3 to 24 months            | -20°C                 | Urinary albumin   | M                      | 120                    |           |
| Urine   | 160 days                  | -20°C, -70°C          | Urinary albumin   | S                      | 121                    |           |
| <i>Cells</i>  |                           |                       |   |                        |                        |           |
| Biospecimen   | Duration                  | Storage temperature   | Tracked analyte   | Stability <sup>†</sup> | Reference              |           |
| Bone marrow cells   | 40 to 42 months           | -85°C, -140°C, -190°C | Cell number and granulocyte-monocyte colony – forming cell (CFU-c)  | M                      | 91                     |           |
| Bone marrow cells, peripheral blood mononuclear cells (PBMCs) | 48 hours, 26 to 78 months | -90°C                 | Cell recovery, viability, colony-forming unit-granulocyte macrophage (CFU-GM), clonogenic potential of autologous hematopoietic progenitor cells      | M                      | 92                     |           |

(continued)

TABLE 1. (CONTINUED)

|  |                       | Cells                |  |                        |           |
|--|-----------------------|----------------------|--|------------------------|-----------|
| Biospecimen  | Duration              | Storage temperature  | Tracked analyte  | Stability <sup>‡</sup> | Reference |
| Erythrocytes   | 37 years              | -10°C to -75°C,      | Freeze-thaw-wash recovery, hemolysis, ATP, 2,3-DPG and P50 levels, and 60% of normal RBC K + levels  | S                      | 84        |
| Haematopoietic progenitor cells                        | 1 to 31 months        | -80°C                | Cell recovery, viability, colony-forming unit-granulocyte macrophage (CFU-GM),   | M                      | 87        |
| Hemopoietic stem cells (HSCs)                          | 5 to 14 years         | -196°C               | Viability, colony formation  | S                      | 122       |
| Hemopoietic stem cells (HSCs)                          | 12 to 24 months       | -40°C, -80°C, -130°C | Viability, colony formation  | M                      | 123       |
| Hemopoietic stem cells (HSCs)                          | 11 to 19 years        | -180°C               | Viability, colony formation  | S                      | 124       |
| Liver cell spheroids (alginate-encapsulated)           | 1 to 12 months        | -80°C, -196°C        | Hepatospecific protein enzyme-linked immunosorbent assay   | N                      | 95        |
| Peripheral blood progenitor cells (PBPCs)              | 10 to 12 months       | -140°C               | Nucleated cells count, multilineage colony-forming assay, long-term culture-initiating cell assay and erythroid burst-forming assay                        | S                      | 90        |
| Peripheral blood progenitor cells (PBPCs)              | 1 to 2 years          | -80°C, -196°C        | Membrane integrity, colony formation   | M                      | 89        |
| Peripheral blood progenitor cells (PBPCs)              | 7 weeks               | -80°C                | CD34+, Colony-forming units granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E)   | M                      | 88        |
| Peripheral blood progenitor cells (PBPCs)              | 30 days               | -150°C, -80°C, -30°C | Membrane integrity, apoptosis, necrosis  | M                      | 125       |
| Peripheral blood stem cells (PBSCs)                    | 5 years               | -80°C, -135°C        | Total nucleated cell, cell viability (using acridine orange and propidium iodide), CD34 + cell content, colony-forming unit-granulocyte-macrophage content | M                      | 94        |
| Peripheral blood stem cells (PBSCs)                    | 1 to 61 months        | -80°C                | Colony-forming units granulocyte-macrophage (CFU-GM), burst-forming units erythroid (BFU-E)  | M                      | 93        |
| Platelets  | 9 days                | -80°C                | Septin 2   | M                      | 126       |
| Sperm  | 7 days, 3 months      | -70°C, -196°C        | Motility, morphology   | M                      | 127       |
| <i>Tissue biospecimens</i>                             |                       |                      |  |                        |           |
| Biospecimen  | Duration              | Storage temperature  | Tracked analyte  | Stability <sup>‡</sup> | Reference |
| Breast tumor   | 100 days              | -20°C, -196°C        | Specific estrogen receptor   | S                      | 101       |
| Heart valves   | ~3 months, 1, 2 years | -80°C, -135°C        | Tritiated-glycine  | M                      | 102       |
| Prostatic tissue extracts, plasma                      | 45 days               | -20°C, -90°C         | Creatine kinase  | M                      | 128       |
| Reproductive tract                                     | 2 to 8 weeks          | -196°C               | Estrogen and progesterone receptor   | S                      | 100       |
| Skin   | 1 to 12 months        | -196°C               | Chromosome   | S                      | 99        |
| Breast, colon, liver, lung, ovary, endometrium, cervix | 5 days                | -70°C                | DNA, RNA   | M                      | 97        |
| Tumor tissue, tumor cells                              | 1 year                | -80°C                | Cell cycle phases  | M                      | 80        |

<sup>‡</sup>Notation for the stability of the tracked analyte: M, multiple outcome; N, nonstable; S, stable.

concentrations (TPO-Ab, TG-Ab) in human serum after storage at  $-25^{\circ}\text{C}$  for up to 23 years. TSH and fT3 concentrations were not affected by  $-25^{\circ}\text{C}$  storage. However, fT4 showed significant concentration fluctuations over time. TPO-Ab and TG-Ab levels increased over time.<sup>77</sup> In another study, Panesar et al.<sup>78</sup> measured the levels of thyroid hormones (TSH, fT4, and fT3) after 8–11 years of storage at  $-80^{\circ}\text{C}$  and found a decrease in TSH and an increase in fT3 and fT4 concentrations with storage times.

Kubasik et al.<sup>79</sup> examined the effects of duration and temperature of storage on 14 different analytes in serum which are often used for radioimmunoassay procedures. They found that the analytes cholyglycine, cortisol, digoxin, ferritin, follitropin, immunoglobulin E, lutropin, prolactin, thyroxin, and triiodothyronine were stable for 2 weeks at room temperature. However, insulin and gastrin were less stable and needed to be refrigerated or stored at  $-70^{\circ}\text{C}$ , respectively.<sup>79</sup>

The stability of purified and nonpurified forms of nucleic acids has also been studied. Vindeløv et al.<sup>80</sup> investigated the effects of storage for 1 year at  $-80^{\circ}\text{C}$  on DNA extracted from JB-1 tumor cells and fine-needle aspirates of solid tumor tissue. Cells and fine-needle aspirates in cryovials were preserved by immersion in dry ice and 99% ethanol at  $-80^{\circ}\text{C}$ . Their DNA deconvolution results revealed no significant changes after one year of storage at  $-80^{\circ}\text{C}$ .<sup>80</sup> Chan et al.<sup>81</sup> studied pre-analytical factors including freeze-thaw of plasma on the integrity of circulating cell-free DNA. In their study, the plasma was stored at  $-80^{\circ}\text{C}$  for 24 h, followed by repetitive freeze-thaw cycles, which led to DNA fragmentation.<sup>81</sup>

The stability of purified mRNA is strongly dependent upon the method of storage. Riesgo et al.<sup>82</sup> compared two preservation methods: (a) a flash-freezing method using liquid nitrogen and subsequent storage at  $-80^{\circ}\text{C}$ , and (b) a fixation method that involves immersion in RNeasy<sup>®</sup> for 1 hour at  $4^{\circ}\text{C}$ , overnight storage at  $-20^{\circ}\text{C}$ , and then storage at  $-80^{\circ}\text{C}$ . After 1 month of storage, the flash freezing method delivered better results (measuring the A260/230 ratio).<sup>82</sup> These examples show that many but not all subcellular components can be stored in a  $-20^{\circ}\text{C}$  freezer and some of them degrade at storage temperatures of  $-80^{\circ}\text{C}$ .

### Storage of Cryopreserved Cells

The influence of storage conditions on cryopreserved cells has been studied extensively since the function of cells following thawing is critical for many applications. The scientific literature suggests that optimal storage conditions are a function of both the cell type and the preservation medium used. A summary of relevant studies related to storage of cells can be found in Table 1.

Blood cells (e.g., red blood cells, hematopoietic progenitors, platelets) that have been used therapeutically for decades have been studied most extensively.<sup>83</sup> When frozen in 40% (w/v) glycerol, the stability of red blood cells stored at  $-80^{\circ}\text{C}$  for up to 37 years has been demonstrated by Valeri and colleagues.<sup>84</sup> Red blood cells have also been frozen in solutions of 24% (w/w) hydroxyethyl starch (HES) and then stored at temperatures ranging from  $-10^{\circ}\text{C}$  to  $-75^{\circ}\text{C}$ .<sup>85</sup> The storage temperatures had a strong effect on hemolysis of the cells, and the authors proposed an exponential law describing the measured hemolysis over time for

a given storage temperature.<sup>85</sup> Furthermore, they proposed that devitrification and denaturation of subcellular components were the damaging factors.<sup>85</sup>

Human hematopoietic progenitor cells (HPCs) are commonly preserved using two different solutions: 5% DMSO with 6% (w/w) HES, or 10% DMSO.<sup>86</sup> The stability of HPCs frozen in these solutions has been determined as a function of storage times and DMSO concentrations at  $-80^{\circ}\text{C}$ .<sup>87</sup> While there was no difference in viability after thawing between the different DMSO concentrations, the viability changed dramatically over time. Specifically, the viability decreased steadily from about 80% after 1 month to 32% after 31 months.<sup>87</sup> Halle et al.<sup>88</sup> cryopreserved peripheral blood progenitor cells (PBPCs) in 3.5% DMSO using uncontrolled-rate freezing. After about 7 weeks in storage at  $-80^{\circ}\text{C}$ , the nucleated cells, CD34+ cells, colony-forming units granulocyte-macrophages (CFU-GM), and burst-forming units erythroids (BFU-E) had acceptable recoveries of 60.8%, 79.6%, 35.6%, and 32.6%, respectively.<sup>88</sup> In another freezing study<sup>89</sup>, autologous PBPCs were cooled to  $-90^{\circ}\text{C}$  at  $1.5^{\circ}\text{C}/\text{min}$  and stored for 1–2 years either at  $-80^{\circ}\text{C}$  or below  $-170^{\circ}\text{C}$ . No significant difference in cell count was observed between the two storage temperatures, however, only the cells stored at  $-80^{\circ}\text{C}$  had a significant reduction in membrane integrity and clonogenic potential. The authors stated that it was unclear if this reduction was solely because of the relatively high storage temperature of  $-80^{\circ}\text{C}$  or was also caused by the formation of intracellular ice.<sup>89</sup> Cilloni et al.<sup>90</sup> cryopreserved PBPCs with 10% DMSO and 10% human serum albumin (HSA) to  $-140^{\circ}\text{C}$  using an uncontrolled-rate freezing technique. The samples were stored at  $-140^{\circ}\text{C}$  for 10–12 months. Upon thawing, the absolute number of nucleated cells was reduced. Also, a multilineage colony-forming assay showed a reduction in recoveries. The assay's long-term culture-initiating cell units and BFU-E demonstrated a significant loss.<sup>90</sup>

Parker et al.<sup>91</sup> loaded bone marrow cells with 10% DMSO and then stored them at  $-85^{\circ}\text{C}$ ,  $-140^{\circ}\text{C}$ , or  $-190^{\circ}\text{C}$  for 40–42 months. Cell number and granulocyte-monocyte colony-forming cell (CFU-c) assays were performed before and after cryopreservation. While dilution and washing steps were associated with quality decrease, the CFU-c measurements showed that storage in the vapor phase of liquid nitrogen is adequate for the long-term storage of human bone marrow cells.<sup>91</sup> Also, bone marrow cells and peripheral blood mononuclear cells (PBMCs) were frozen by Ayello et al.<sup>92</sup> to  $-90^{\circ}\text{C}$  using 5% DMSO and 6% HES. These investigators tested *in vitro* cell recovery, CFU-GM, clonogenic potential of autologous HPCs and cell viability. They found no significant change and concluded that it is feasible to store these cells for more than three years at  $-90^{\circ}\text{C}$ .<sup>92</sup>

Katayama et al.<sup>93</sup> loaded human peripheral blood stem cells (PBSCs) with 10% DMSO and 8% HSA and then used a non rate-controlled freezing method for cooling to  $-80^{\circ}\text{C}$ . Samples were stored for 1–61 months to study the thawing process and the effect of long-term cryopreservation.<sup>93</sup> CFU-GM and BFU-E assays were used as quality tests. Recovery rates of CFU-GM and BFU-E showed no significant difference after 5 years of storage. The authors concluded that this cryopreservation procedure is useful to store PBSCs for clinical applications.<sup>93</sup> McCullough et al.<sup>94</sup> froze

human PBSCs under different combinations of 10% DMSO, 5% DMSO, and 6% HES with subsequent storage temperatures of  $-80^{\circ}\text{C}$  or  $-135^{\circ}\text{C}$ . After 5 years of storage, sample quality was determined by quantifying the total number of nucleated cells, cell viability, CD34+cell content, and CFU-GM content. Most of the quality tests revealed no significant differences; only the total nucleated cell count for samples stored for 24 hours at  $1^{\circ}\text{C}$ – $6^{\circ}\text{C}$  before cryopreservation declined over time.<sup>94</sup>

Massie et al.<sup>95</sup> investigated the effects of storage temperature on viability of alginate-encapsulated liver cell spheroids. Spheroids were stored at either  $-80^{\circ}\text{C}$  or  $\sim -170^{\circ}\text{C}$  for up to 12 months. The viable cell count and the function of the liver spheroids stored at  $-80^{\circ}\text{C}$  decreased substantially after 1 month and continued to degrade during the 12-month storage period. In contrast, liver cell spheroids stored for 12 months at  $-170^{\circ}\text{C}$  maintained viability.<sup>95</sup>

These examples for the storage of cellular samples show that some cell types were successfully stored at  $-80^{\circ}\text{C}$  and retained their viability over time. However, other cell types were found to lose their viability even after short periods of time at  $-80^{\circ}\text{C}$  and thus require lower storage temperatures (e.g.,  $-170^{\circ}\text{C}$ ).

### Storage of Tissues

Tissue biospecimens are important resources for diagnosis of disease, selection of therapy, and monitoring response to treatment, and can be used for treatment as well (i.e., transplantation of islets of Langerhans to treat diabetes). Studies have demonstrated that rapid changes in the expression of biomarkers occur after ligation of the blood supply.<sup>96</sup> Freezing of tissues is used to arrest the degradation of biomarkers. Long-term stability of tissue-based biomarkers depends upon long-term storage conditions. A summary of scientific studies describing the stability of tissue-based biomarkers in storage can be found in Table 1.

Jewell et al.<sup>97</sup> analyzed the stability of DNA and RNA isolated from human tissues (from breast, colon, liver, lung, ovary, endometrium, and cervix) which were frozen at  $-80^{\circ}\text{C}$  for 0 to  $\geq 12$  months. They used several methods for analysis, including electrophoresis, reverse-transcriptase polymerase chain reaction, and Northern blot. They found the DNA to be of good quality in 80% of the tissues, while the RNA was of good quality in only 60% of the tissues; suggesting that tissue DNA was more stable than RNA.

Mato et al.<sup>98</sup> found that storing of postmortem human tissue at  $-25^{\circ}\text{C}$  strongly affected the receptor density and response of the G-protein [ $^{35}\text{S}$ ]GTP $\gamma$ S, a marker that is used to analyze cannabimimetic drugs for the treatment of numerous nervous disorders. After 12–24 months of storage at  $-25^{\circ}\text{C}$ , a 50% reduction of [ $^{35}\text{S}$ ]GTP $\gamma$ S was measured within the tissues.<sup>98</sup>

In another study,<sup>99</sup> 1-mm square pieces of foreskin tissue were placed into a solution of 0.8% DMSO and serum, and then these samples were cooled in the gas phase of liquid nitrogen and stored at  $-196^{\circ}\text{C}$  for 1, 3, 6, 9, and 12 months. After thawing, the tissues were minced into smaller fragments to allow cell culture for chromosomal, morphological, and enzymatic analyses. No chromosomal damage or rearrangement was measured in any of the cryopreserved tissue samples. No significant morphological changes were revealed upon microscopic analysis of the cells. Furthermore,

the six lysosomal acid hydrolases that were tested all remained within the normal range. The authors recommend this tissue cryopreservation procedure, “when it is impossible or impractical to initiate a cell culture immediately”.<sup>99</sup>

Estrogen receptors and progesterone receptors are used as protein biomarkers in the prognosis of gynecologic carcinomas. Toppila et al.<sup>100</sup> investigated the level of estrogen and progesterone receptors in human female reproductive tract tissues after excision. Tissues were analyzed after 2, 4, 6, and 8 weeks of storage at  $-196^{\circ}\text{C}$  and in the presence or absence of sodium molybdate. The freezing process caused a 30% loss in the steroid receptors, but once the tissues were kept at  $-196^{\circ}\text{C}$  no further loss was found over the 2–8 week time period.<sup>100</sup>

Crawford et al.<sup>101</sup> compared the storage of human breast tumor tissues at  $-196^{\circ}\text{C}$  to storage in sucrose and glycerol at  $-20^{\circ}\text{C}$  for up to 100 days. They reported that progesterone in these tissues was stable for 1 month at  $-20^{\circ}\text{C}$ . They performed Scatchard analysis to investigate time-dependent estrogen receptor levels, which are used to predict outcome following therapy in breast cancer. They concluded that estrogen receptors were stable under these conditions at  $-20^{\circ}\text{C}$ , which is sufficient for any routine purpose.<sup>101</sup>

A series of studies demonstrate that storage below  $-135^{\circ}\text{C}$  is necessary for long-term storage of tissues. Brockbank et al.<sup>102</sup> studied human allograft heart valve leaflets to evaluate the effect of different storage temperatures ( $-80^{\circ}\text{C}$  and below  $-135^{\circ}\text{C}$ ) on viability of fibroblasts embedded in the valve. Samples stored at  $-80^{\circ}\text{C}$  revealed a time-dependent loss of fibroblast viability. After 6 and 24 weeks, a significant decline in tissue function was observed; only 15% of the tissue valves survived with full functionality. In contrast, short-term ( $< 3$  months) and long-term (1–2 years) storage of the tissue samples in liquid nitrogen (below  $-135^{\circ}\text{C}$ ) sustained their cellular function (i.e., fibroblast protein synthesis).<sup>102</sup>

These examples demonstrate that some tissue biomarkers are stable at relatively high temperatures (e.g., even at  $-20^{\circ}\text{C}$ ).<sup>101</sup> However, storage temperatures below  $-135^{\circ}\text{C}$  appear to be necessary in order to preserve a wider variety of biomarkers (including viability).

### Conclusion

Biospecimens are an important reagent in biomedical research and suboptimal preservation and long-term storage conditions strongly influence their quality. This review describes critical events during the freezing and storage processes. Any biospecimen will contain water plus a biological system (e.g., proteins, cells, tissues). As water is critical for many biological reactions, the physical state of water in the solution (solid, liquid, or glass) is important in understanding and controlling degradation of biological molecules. Storing the solution at low enough temperatures to reduce mobility of water by forming a solid or glass, reduces the potential for water acting to degrade the biological molecules in solution.

In addition, biospecimens contain degradative molecules (e.g., proteases, lipases, nucleases) whose activities are strongly influenced by temperature. Storage conditions need to be selected such that these molecules are inactive so that biomarkers will be preserved. Optimal storage temperatures for biological cells are a function of solution composition

and biospecimen type. Implementation of proper quality control systems for monitoring stability of biomarkers in long-term storage will permit determination of suboptimal storage conditions and continuous improvement in biospecimen quality.

### Author Disclosure Statement

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