BIO-SAMPLES THERMAL PROTECTION:
THERMAL EXCURSIONS OF CRYOGENICALLY FROZEN VIALS IN VARIOUS CRYOBOX CONFIGURATIONS DURING TRANSIENT WARMING EVENTS AND BEST PRACTICES TO STAY BELOW Tg,H2O
INTRODUCTION

Many biological samples contained inside cryoboxes are stored in LN2 vapor-phase freezers at temperatures below -150°C to preserve their viability. The underlying assumption is that biological samples show highly reduced degradation and metabolic activity while below Tg (the glass transition temperature). However, every time a cryobox is manipulated or temporarily removed from a LN2 freezer the enclosed samples experience thermal excursions. They could be harmed by inadvertently crossing the Tg threshold or by exposure to excessive thermal excursions. Understanding sample warming is further complicated because individual vials inside a cryobox do not warm equally and at the same rate. Their temperature variations and rates of change depend on several factors, such as their location inside the cryobox, proximity to surrounding vials, vial size and volume of the sample.

The aim of this work is to provide supporting data to characterize the thermal excursions experienced by H2O-filled vials contained inside various typologies of cryoboxes in various configurations during typical transient temperature events. Specifically, we focused our attention on the frequent cold chain steps consisting of accessing single vials from a cryobox in long-term storage and transporting the whole cryobox at ambient temperature or in a dry ice (-80°C) environment.

We will also discuss thermal inertia effects contributing to vial temperature increase after the cryobox is placed back in the LN2 freezer environment.

MATERIALS AND METHODS

- Unless specified, all tested vials were filled with H2O at their max. working volume conditions
- Vials and cryoboxes were cooled inside customized LN2 vapor-phase freezers that allow for precise temperature and sample handling control conditions. Freezers temperatures were maintained at either -186°C or -175°C. Here, we use the term "cryobox" to describe any type of vials container that was tested
- When extracted from the freezers, the samples were kept inside the cryoboxes without being directly manipulated. The cryoboxes were then either introduced in a 20-22°C environment or inside a container partially filled with dry ice. Cryoboxes were handled using cryo-gloves and transferred in less than 10 seconds
- Temperature measurements were obtained using Omega type T 32-gauge thermocouple wires. The tip of the thermocouple was positioned to the outer surface of the vial, 2mm below the H2O level, and secured using aluminum tape. The data acquisition frequency was 5s
- Fig.1 and 2 exemplify the positions of the instrumented vials within a sparsely and a fully populated FluidX cryobox

RESULTS

- Table 1 reports the natural thaw rates (linearized in the -186 to 130°C temperature range) measured for vials extracted from LN2 freezers
- Major influencing variables are: the presence of the cover, the position of the vials inside the cryobox, and the cryobox population.

<table>
<thead>
<tr>
<th>H2O filled tube: From storage at -175°C to lab bench [21°C]</th>
<th>Natural thaw rates [°C/min]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Flexible 2.0ml tube*</td>
<td>6.8 - 22.3</td>
<td>5.9 - 13.5</td>
</tr>
<tr>
<td>Flexible 2.0ml tube*</td>
<td>18.2 - 30.2</td>
<td>16.8 - 21.6</td>
</tr>
<tr>
<td>Beckman 2.0ml tube*</td>
<td>13.0 - 38.9</td>
<td>13.0 - 19.8</td>
</tr>
<tr>
<td>Wheaton 2.0ml tube*</td>
<td>36.2 - 50.4</td>
<td>38.5 - 66.7</td>
</tr>
</tbody>
</table>

* Linearized thaw rate in the -175°C to -130°C range
| 96 Well Format Sample Storage Tube With Screw Cap; Tubes filled with 0.73ml of H2O; Perforated rack underside |
| 9x9 Sample Storage Tube With Screw Cap; Tubes filled with 1.8ml of H2O; Not-perforated rack underside |
| 9x9 TruCool® Hinged Cryobox with LN2 drain holes; Tubes filled with 1.8ml of H2O |
| KeepIT-100 Freezer Box; Tubes filled with 1.8ml of H2O; Perforated rack underside |

Table 1: Natural thaw rates of typical vials
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**Fig. 2** exemplifies the effect of the cryobox cover when samples are pulled out from a LN2 freezer.
- Removed cover: the vials’ natural thaw rates can increase by as much as 2Xa
- This increase is descriptive of all the cryoboxes considered in this work

*Natural air convection conditions. In forced air convection conditions, the vials thaw rates can substantially exceed the reported values.*

**Fig. 2**: Sensitivity to the presence of the cryobox cover

**Fig. 3** shows the dependence of vials’ thermal excursions on the position and number of vials in a cryobox.

- **Sparsely populated cryobox:**
  - Vials experience faster natural thaw rates (2-3X); Weak dependence on vial position
- **Fully populated cryobox:**
  - Corner vials experience the greatest thaw rates while central vials the lowest
  - Variations up to a factor 3X in vials’ thaw rates were measured

**Fig. 3**: Sensitivity to the cryobox population

**Fig. 4** shows natural thaw rates of various vials inserted inside different cryoboxes (cover off):

- 1.0ml vials warm faster than 2.0ml vials
- **Sparsely populated conditions:**
  - Presence of full walls in-between vials ensure lower natural thaw rates
  - It takes 40s to 4.2min to warm vials from -175°C to Tg,H2O
- **Fully populated conditions:**
  - Underside-perforated cryoboxes show decreased vial-to-vial thaw rate variance but increased averaged values
  - It takes 1.1min to 7.6min to warm vials from -175°C to Tg,H2O
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Fig. 4: Sensitivity to the type of cryobox and vial: -175°C to RT

Fig. 5 shows vials thermal excursions when transferred from a LN2 freezer at -175°C to either a dry ice bed or a room temperature lab bench.

- Counter intuitively, below Tg,H2O the thaw rates experienced by vials placed on a dry ice bed are greater than those placed at room temperature. The speculation is that the sublimation of dry ice produces convective motion of gaseous CO2 responsible for augmented heat transfer to the vials.
- The stronger effect was observed when using perforated cryoboxes.

Fig. 5: Sensitivity to the surrounding temperature

Fig. 6 shows the effect of vial position and cryobox population on the vials’ natural thaw rates in a confined dry ice environment.

- Sparse vials require about 5min to warm from -175°C to dry ice temperature and as low as 30s to cross Tg,H2O.
- In a fully populated cryobox, vials require between 7 and 20min to equilibrate at dry ice temperature and as low as 30 seconds to cross Tg,H2O.
- Vials located at the center of the cryobox experience the lowest thaw rates.

Fig. 6: Thaw in dry ice bed
Fig. 7 shows the thermal excursions experienced by a single vial (in a cryobox) and by a cryobox when placed inside a LN2 freezer rack, extracted for 3min from the LN2 freezer at -186°C, and then re-introduced inside.

- The single vial (@top shelf) reaches -142°C in less than 3min.
- The vial continues to warm for about 1.5min after being re-introduced inside the freezer.
- It then takes about 1hr for the vials to cool below -184°C.
- The cryobox in shelf 3 experiences a reduced thermal excursion compared to the single vial in shelf 1. However, it takes about 2 hrs for the cryobox to cool below -184°C when re-introduced inside the freezer.

![Image: Single cryobox pull out from LN2 freezer](image1)

Fig. 7: Single cryobox pull out from LN2 freezer.

Fig. 8 reports the thermal excursions experienced by 2.0ml Wheaton vials filled with 1.0ml of H2O when repeatedly pulled in and out from a LN2 vapour freezer (-186°C top temperature) for a 3min duration every 10min. The cases of a single vial inside a cryobox and a fully populated cryobox were investigated.

- Single vials exceeded Tg,H2O after the first pull out.
- Corner vials inside the fully populated cryobox required nine cycles to cross Tg,H2O.
- An equilibrium condition was reached after about 20 pull out cycles where the single vial repeatedly crossed the Tg,H2O threshold. The vial in the fully populated cryobox remained steadily above Tg,H2O.

![Image: Repeated cryobox pull out from LN2 freezer](image2)

Fig. 8: Repeated cryobox pull out from LN2 freezer.

Thermal inertia plays an important role when re-introducing vials and cryoboxes inside a LN2 vapor-phase freezer.

- Vials and cryoboxes continue to increase in temperature for a limited time after being re-introduced into the freezer. Subsequently, they undergo a slow rate recooling process in the order of hours.

CONCLUSIONS

- At temperatures colder than -130°C, the vials natural thaw rates inside cryoboxes range between 5 and 70°C/min.
- A sample can warm from -180°C to above -135°C in less than 40 seconds.
- If located favorably, a sample can avoid warming above -135°C for as much as 8-10 minutes.
- Major contributors to thaw rate: absence of cryobox cover and number of vials inside the cryobox.
- Handling of vials in a cold and controlled environment is recommended to minimize thermal excursions.
- The pull out frequency of innocent samples plays an important role on their thermal excursions. In certain conditions, a single pull out for 2 minutes is sufficient to increase the innocent sample temperature above -135°C.
- A controlled approach or device for the access of cryo-stored vials is recommended to guarantee complete thermal control of the samples.
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