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White Paper

UNIQUE FREEZING PRINCIPLE OF THE EM ICE HIGH PRESSURE FREEZER

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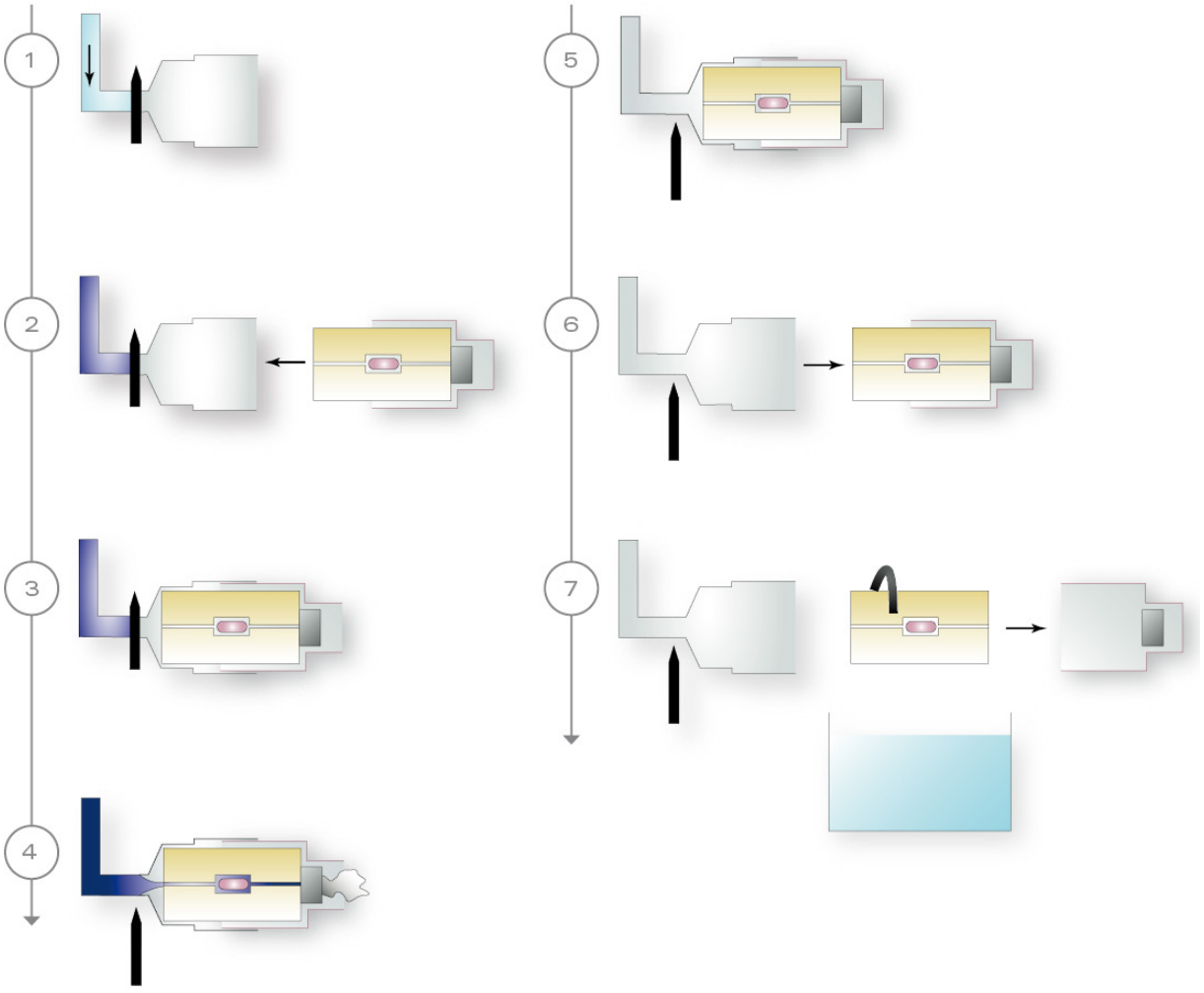
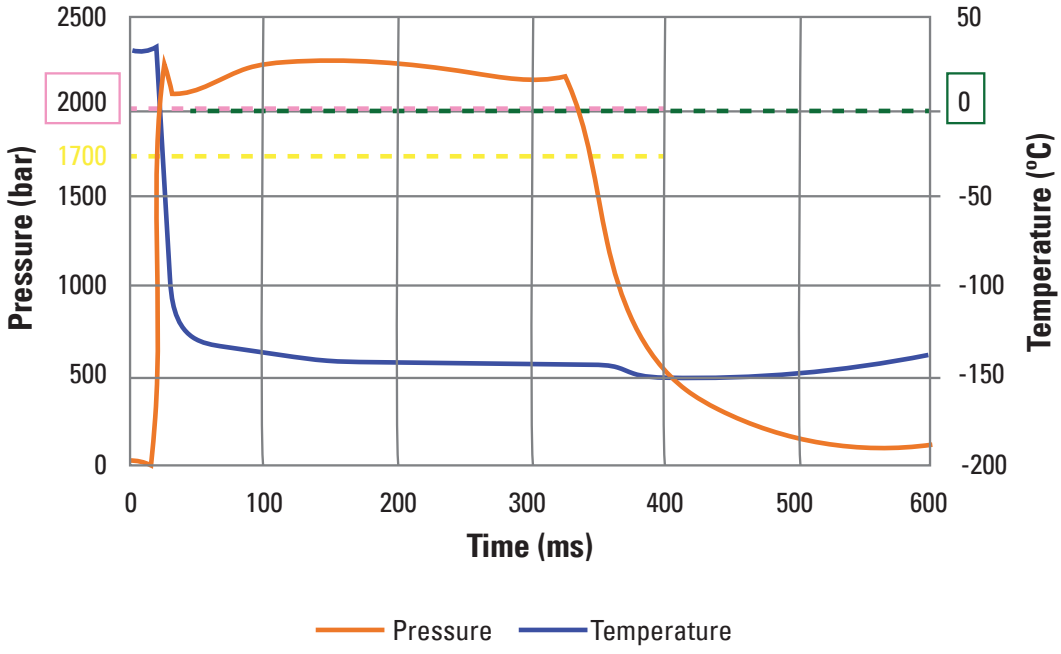
The EM ICE is a high-pressure freezer with a unique freezing principle. In this white paper we will explain in depth how the EM ICE works and how the freezing parameters are interdependent. The EM ICE functions with a single pressurized cooling liquid: liquified nitrogen (LN2). Therefore, a pressure increase and temperature decrease on the sample are directly dependent on each other. Vitrification with the EM ICE is illustrated in the following steps:



1. Sample inside the cartridge moves into the pressure chamber.
2. Liquid nitrogen gets pressurized in front of the needle valve.
3. Needle valve opens. Pressurized LN2 heads into the pressure chamber and freezes the sample. LN2 leaves the pressure chamber via the outlet nozzle. The small nozzle diameter serves to maintain the pressure in the flowing liquid inside the pressure chamber.
4. After depletion of pressurized LN2, the cold sample cartridge stays in the chamber during pressure relief.
5. Sample cartridge is moved out of the pressure chamber.
6. Sample cartridge is pulled from the transport arm, falls into the LN2 sample Dewar, and then separates.
7. Release of cartridge into the Dewar.

A standard outcome pressure and temperature curve looks like this:



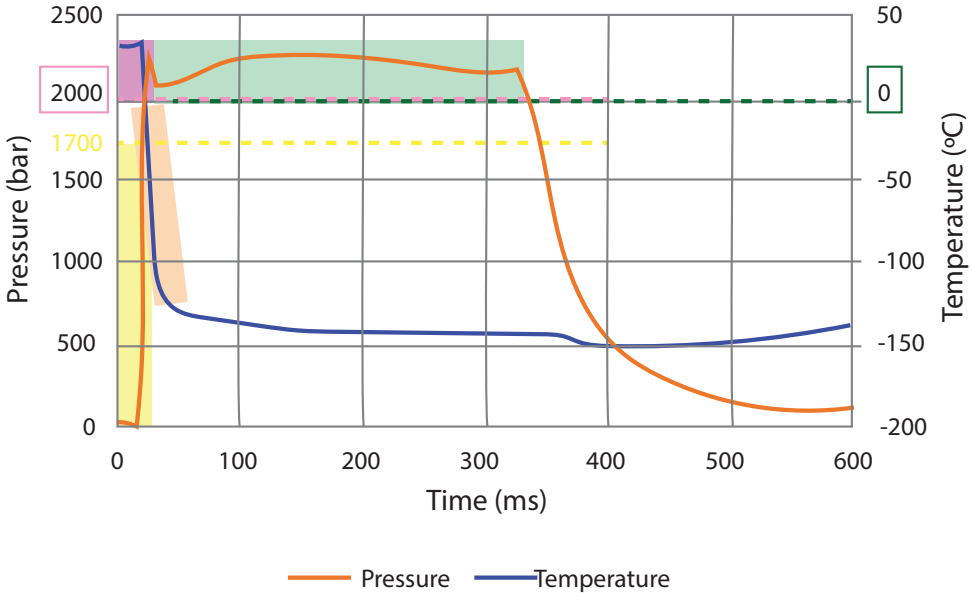
The pressure and temperature curves are measured by sensors located in front of the sample position. Reference measurements show that the pressure build up occurs at approximately the same time throughout the chamber, while the cooling of the sample is delayed due to the sensor indication. It must be noted that neither the pressure nor the temperature of the sample itself are accessible to measurements. The rate of temperature change of a sample volume element depends on the carriers used (material, form, thickness) and on the distance of the volume element from the cooled carrier surface. The pressurization may vary in magnitude to a minor extent, because of flow turbulences and pressure transduction through the carriers.

Because the temperature is dependent on the pressure, temperature alone can't be manipulated. However, the pressure curve can be adjusted with three parameters:

1. The applied pilot pressure in front of the needle valve (Fig. 1, step 2)
   > This influences the final pressure in the pressure chamber. The higher the pilot pressure, the higher the final pressure in the pressure chamber
2. The opening speed of the needle valve (Fig. 1, step 3)
   > This influences the rise time of the pressure chamber. The faster the needle valve opens, the faster the pressure in the chamber increases
3. LN2 outlet via nozzle (Fig. 1, steps 3 and 4)
   > This influences the dwell time of the pressure. The bigger the LN2 outlet, the shorter the dwell time

During production different parameters of the EM ICE are checked to ensure successful vitrification. Successful vitrification with these parameters was tested using different sample types and proven with our installed base.

The systems are checked for the following parameters (also indicated in the following graph):



- The pressure peak shall not exceed 2400 bar (pink background)
- Pressure stays above 2000 bar after the pressure increase (green background)
  - 150 ms after pressure initiation, it should be between 2100 and 2350 bar
  - The small valley after the pressure increase (indicated as critical) should be between 2000 and 2050 bar, as this pressure drop is critical to preserve fine structures like double membranes
- The dwell time of the pressure must be 300 – 400 ms (blue background)
  - The dwell time is measured as the time from when the pressure first reaches 1700 bar until it drops again below 1700 bar
  - The dwell time is set by the opening of the outlet nozzle of 0.4 mm in diameter
  - The system works fine with a dwell time as low as 250 ms.
    For the production test the limit is 300 ms, because the dwell time can reduce over time
- The rise time of the pressure curve needs to be shorter than 10 ms (yellow background)
  - The rise time is calculated for a pressure increase between 64 and 1700 bar.
  - A short rise time is critical to avoid cooling of the sample without a pressure change, which would lead to ice crystal formation

- Cooling rate must be between 15000 and 25000 K/s (orange background)
  - The cooling rate cannot be measured directly, but is determined by the temperature drop of a sensor in front of the sample. The cooling rate critically depends on the sensor heat capacity which can vary because of production reasons. Therefore, for each system, the sensor indication is calibrated to lie between 15000 and 25000 K/s. This indication may change with time as the sensor can be eroded by the turbulent LN2 stream. If it deviates significantly from this range (factor 2 or higher), this may indicate an issue with the flow of pressurized LN2.
- The p/T shift must be positive (red vertical lines indicate two points where time is measured)
  - The time for the p/T shift is measured between these two points
    1. When the pressure sensor reaches 1700 bar
    2. When the temperature sensor measures 0°C

    Note: 1700 bar is chosen here because it is known that the pressure indicated by the transducer is somewhat lagging behind the true pressure value. Furthermore, this point in the curve is influenced by other measuring artifacts known from signal transduction theory (e.g. oscillations at sharp edges)

General remark regarding the intersection point of the pressure and temperature curves:

- The intersection point cannot be directly manipulated by varying pressure or temperature parameters separately. It depends on the physical properties of the pressurized liquid nitrogen.
- The p/T shift ensures that the temperature curve crosses 0°C between 1700 and 2000 bar
  - Note: This does not reflect the situation at the sample itself. The sample will hit the 0°C mark with a time shift (around 5 ms later, but not accessible to direct measurement). Taking the p/T shift and rise time into account, the sample will cross 0°C above 2000 bar.
- However, with a faster opening of the needle valve, the pressure increase occurs faster.
  - This could push the intersection point closer to or beyond 2000 bar.
  - However, this bears also the following risks:
    - Faster pressure build up leads to a higher mechanical stress on the system → could influence the sample integrity
    - There are ways to indicate resonance waves, which increase with faster pressure build up, as they can damage sapphire sample carriers
- Increasing the pilot pressure influences the form of the pressure curve at around 2000 bar with the following consequences:
  - The p/T shift increases
  - The indicated pressure at the time when the temperature reaches 0°C increases
  - The total system pressure increases
  - The dwell time is reduced, because of faster LN2 flow
- This bears the following risks:
  - Higher pressure load on the sample which influences the sample integrity, especially for fine structures like membranes
  - Higher stress on the system with the same consequences as indicated above for the faster rise time
  - The phase diagram shows that the starting point of solidification rises again for higher pressures with another crystalline phase taking over. It is not yet clear how this influences the cryo-fixation quality.

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