# High Pressure Cryo-Cooler for X-Ray Crystallography

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**Abstract.** High Pressure cryocooling was developed as an alternative method for cryopreservation of macromolecular crystals and successfully applied for various technical and scientific studies by Cornell University's researchers and scientist [1]. The method requires the preservation of crystal hydration as the crystal is pressurized with dry helium gas. Previously, crystal hydration was maintained either by coating crystals with a mineral oil or by enclosing crystals in a capillary which was filled with crystallization mother liquor. These methods are not well suited to weakly diffracting crystals because of the relatively high background scattering from the hydrating materials.

The method involves mounting protein crystals in a cryoloop with a thin coating of oil, pressurizing the crystal up to 200 MPa (2000 atm) in He gas, cooling the crystal under pressure, and then releasing the pressure. This process results in dramatic improvement in diffraction quality in terms of diffraction resolution and crystal mosaicity. In this publication we describe the design and control aspect of this instrument.


**FIGURE 1.** ADC High Pressure Cryo-Cooler

# INTRODUCTION

Pharmaceutical companies currently use x-ray crystallography to determine exactly how drug lead compounds and their protein targets interact. To date, x-ray crystallography is the most effective technique in the field of structural biology; out of the approximately 35,000 protein structures solved, x-ray crystallography is responsible for about 29,000. The promise of structural biology to improve human health is great, and any method or device that can speed the solving of protein structures will contribute to fulfilling that promise.

Cornell University developed a novel method to cryo-cool protein crystals without the need for penetrative cryoprotectants. The method involves mounting protein crystals in a cryoloop with a droplet of oil, pressurizing the crystal up to 200 MPa (2000 atm) in He gas, cooling the crystal under pressure, and then releasing the pressure. This process results in dramatic improvement in diffraction quality in terms of resolution and mosaicity. Figure 1 shows an actual high pressure cryo-cooler.

## Design Features

This devices fundamental design is based on a process developed and patented by Cornell University scientists Prof. Sol M. Gruner (http://bigbro.biophys.cornell.edu/) and Dr. Chae Un Kim; US Patent No. 8,030,449. This exciting new technology enables the simultaneous capture of both amplitude and phase information from a single anomalous diffraction (SAD) of a cryo-cooled protein crystal, thereby providing sufficient data to solve the crystal structure of a protein with a previously unknown structure. Flash-freezing at atmospheric pressure requires the use of cryoprotectants. Finding the right cyroprotectant for each sample type can be a long, trial-and-error process. The High Pressure Cryo-Cooler eliminates the need to use cryoprotectants and produces superior results. Figure 2 shows actual diffraction data obtained at the Cornell High Energy Synchrotron Source (CHESS).

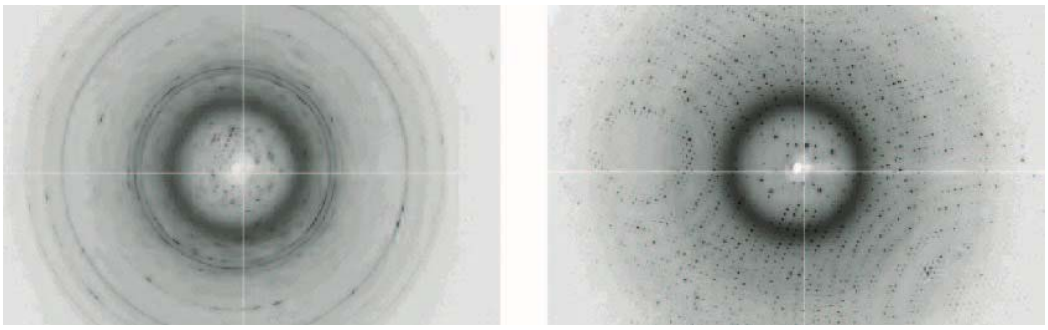


**FIGURE 2.** Diffraction data obtained at CHESS facility

The project was first funded by The National Institutes of Health. The National Institutes of Health (NIH), through its National Institute of General Medical Sciences (NIGMS), funds MacCHESS for two purposes: core research as motivated by the important biomedical problems and support to all structural biologists making use of the CHESS facility for crystallographic and small-angle X-ray scattering experiments, as well as for novel experiments requiring special equipment and staff assistance not readily available at other synchrotron sources.

## Process

Once a protein crystal has been picked up using a MicroMount™ or similar device, the crystal is analyzed using x-rays. A protein structure is shown in Figure 3. Unfortunately, a typical protein crystal at room temperature survives only a fraction of the x-ray dose required for a complete high resolution data set before it becomes irrevocably radiation-damaged. To inhibit the radiation damage, protein crystals are typically flash cooled at atmospheric pressure by plunging them into liquid nitrogen (77° K or -196° C). Cryocooling also reduces the thermal motion within the crystal, enabling the collection of higher quality data.

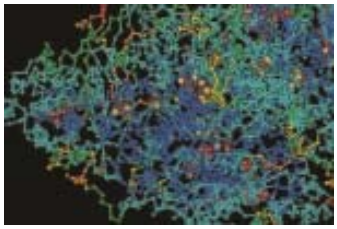


**FIGURE 3.** Protein Structure

Freezing protein crystals successfully and without damage, however, is a tricky business. Proteins crystals form in an aqueous solution and can contain 50 percent, or more, water by weight. As anyone knows who is lucky enough to be by a northern lake on a frigid night early in the winter, as the ice noisily heaves and cracks, freezing water expands with great force—more than enough to damage the crystals one is trying to protect. The goal of flash freezing is for the water to form amorphous ice rather than crystalline ice, which expands with such force. Cryoprotectants are typically added to promote this result. Unfortunately, since each protein is unique, a specific cryoprotectant must be formulated for each, a task that proves difficult or impossible in many cases.

This innovation has eliminated the need for cryoprotectants, increasing cryocooled protein crystal quality. Instead of freezing protein crystals at atmospheric pressure, they have decided to cryo-cool their protein crystals under high pressure. Under these conditions, the water turns into a high density amorphous solid, similar to a glass, which minimizes crystal disruption. This process is simple in concept—pressurize a protein crystal in helium at room temperature up to between 100 and 400 MPa (about 14,500 to 58,000 psi), cryocool the crystal to 77º K, then release the pressure while maintaining the low temperature—but complicated in practice, due to the high pressures involved.

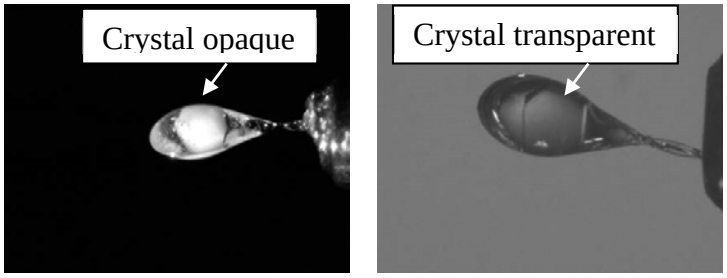
Protein crystal structure is solved by determining the repetitive three-dimensional electron density distribution of protein molecules in a crystalline arrangement. Each crystal reflection has an intensity (amplitude) and phase, and both are needed to generate a protein structure. The information obtained from x-ray diffraction is not sufficient alone to solve the structure of a protein crystal, since the method provides only intensity data. In a further innovation to their basic method, Gruner and Kim combined a technique developed by others with their high pressure cryocooling method to obtain both intensity and phase information from a single protein crystal, enabling them to solve the protein's crystal structure. To accomplish this, they added a second pressurization step in krypton or xenon gas, instead of helium. During this additional step, krypton or xenon atoms bind to specific locations in the protein [2]. By taking advantage of the diffraction from these distinct atoms, complete phasing information is derived. Kim proved the efficacy of this method by solving the structure of porcine pancreas elastase. He used only phase and amplitude data obtained by diffraction of a single crystal cryocooled with Cornell's new process.

## Proposed Mechanism

Cornell University successfully pressure cooled more than a dozen different proteins. It was observed in almost all cases that water vitrification in protein crystals was achieved without any penetrative cryoprotectants. Furthermore, in many cases, improvement in diffraction quality was observed. These effects involve the dynamics of water under pressure and the formation of high density amorphous (HDA) ice.

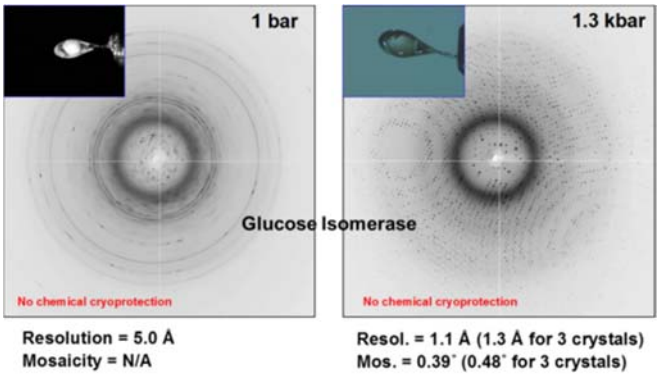
## Water Vitrification

Protein crystals are frozen in droplets of water at room pressure and 1,300 atomspheres pressure; the crystalline ice in Figure 4 (a) is cloudy, while the amorphous ice in Figure 4 (b) is transparent. The bottom two images are the x-ray diffraction patterns captured from the corresponding protein crystal. The rings on the pattern on the lower left photo arise from ice crystals. More importantly, the quality of the diffraction obtained by the pressure-frozen crystal, Figure 5 (b), is superior to the diffraction from a crystal frozen at room pressure, Figure 5 (a).




(a) (b)

**FIGURE 4.** (a) Crystalline ice photo, (b) Amorphous ice photo




(a) (b)

**FIGURE 5.** (a) Crystal frozen at room pressure, (b) Pressure-frozen crystal

## Diffraction improvement

When protein crystals are cooled at room pressure, the unit cell volume usually shrinks by ~ 5%. But liquid water inside the crystals turns into either ice I or LDA (Low Density Amorphous) ice which expands by ~ 6.7 % ( 0.94 g/cm3 at 77 K, 0.1 MPa).This volume discrepancy leads to disorder in the crystal. When rapidly cooled at high pressure, however, liquid water may freeze into HDA (High Density Amorphous) ice and, once formed, it stays metastable in the HDA state when the pressure is removed, as long as the temperature is kept below 120 K 9). HDA ice has higher density (1.17 g/cm3 at 77 K, 0.1 MPa) and this mitigates the volume related crystal disruption [3].

## HPC-201 Specification

The high-pressure cryo-cooler is designed to hold 3 samples at a time. Crystal samples are picked up using a standard cryoloop. Cryoloops are mounted to heavy duty stainless steel tubing in the unit and are then ready to be pressurized and cooled. A high pressure oil pump provides helium gas to the samples. External controls allow the sample to be first pressurized and then cooled by an $LN_2$ bath. Once pressure is released the samples can be removed and handled like any other samples prepared by the conventional flash freezing.

**TABLE 1.** Features

| | |
|---|---|
| Pressurizing Gas | Helium or compatible inert gas |
| Working Pressure | Up to 200 MPa |
| Cooling Fluid | LN2 |
| Cryo Cooling Temp | 77 K (-196 °C) |
| Sample Capacity (per pressure & cooling cycle) | 3 |
| Process Time | < 10 min (2 min for pump operation; ~5 min under pressure; 1 min freezing) |
| ZEISS Microscope | SteREO Discovery. V8 |
| LN2 Dewar | Taylor-Wharton HC34 |

**TABLE 2.** Connection Data

| | |
|---|---|
| Voltages, frequencies | 50/60 Hz |
| Power input, approximately | 100 to 230 VAC |
| Oil pump pressure | 200 MPa |
| LN2 Dewar holding | 200 Days |

## Experimental Results

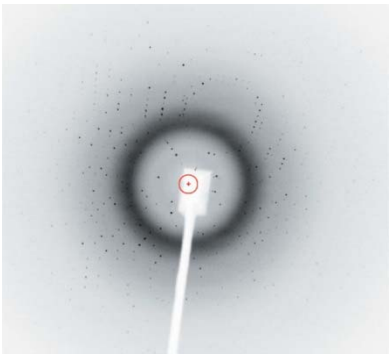


**FIGURE 6.** High-pressure cryocooled RNase A crystal

Figure 6 displays an x-ray diffraction pattern of a high-pressure cryocooled RNase A crystal, grown in the presence of 10%(v/v) glycerol inside a polycarbonate capillary. The crystal diffracted beyond 2 A ˚ and the mosaicity is 0.4º. Note the absence of sharp crystalline ice lines, which indicates successful cryocooling. (From Journal of Applied Crystallography; Yi-Fan Chen, Mark W. Tate and Sol M. Gruner; 2009 42, 525-530)

Two cases with dramatic improvement in diffraction quality are shown below for glucose isomerase and thaumatin. (Contribution from: Chae Un Kim & Sol M.Gruner; Cornell University)

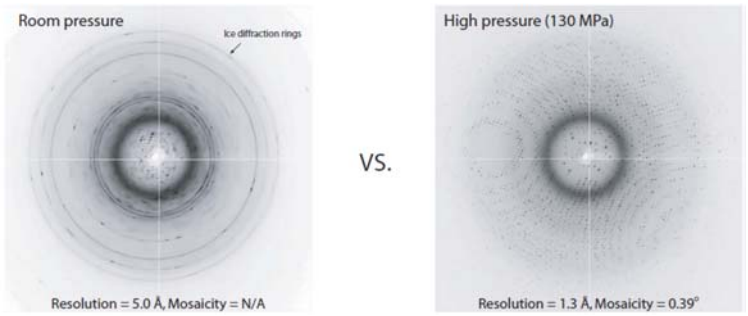



**FIGURE 7.** Glucose Isomerase

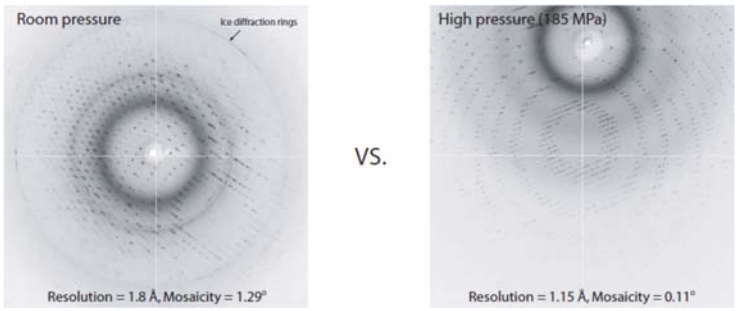



**FIGURE 8.** Thaumatin

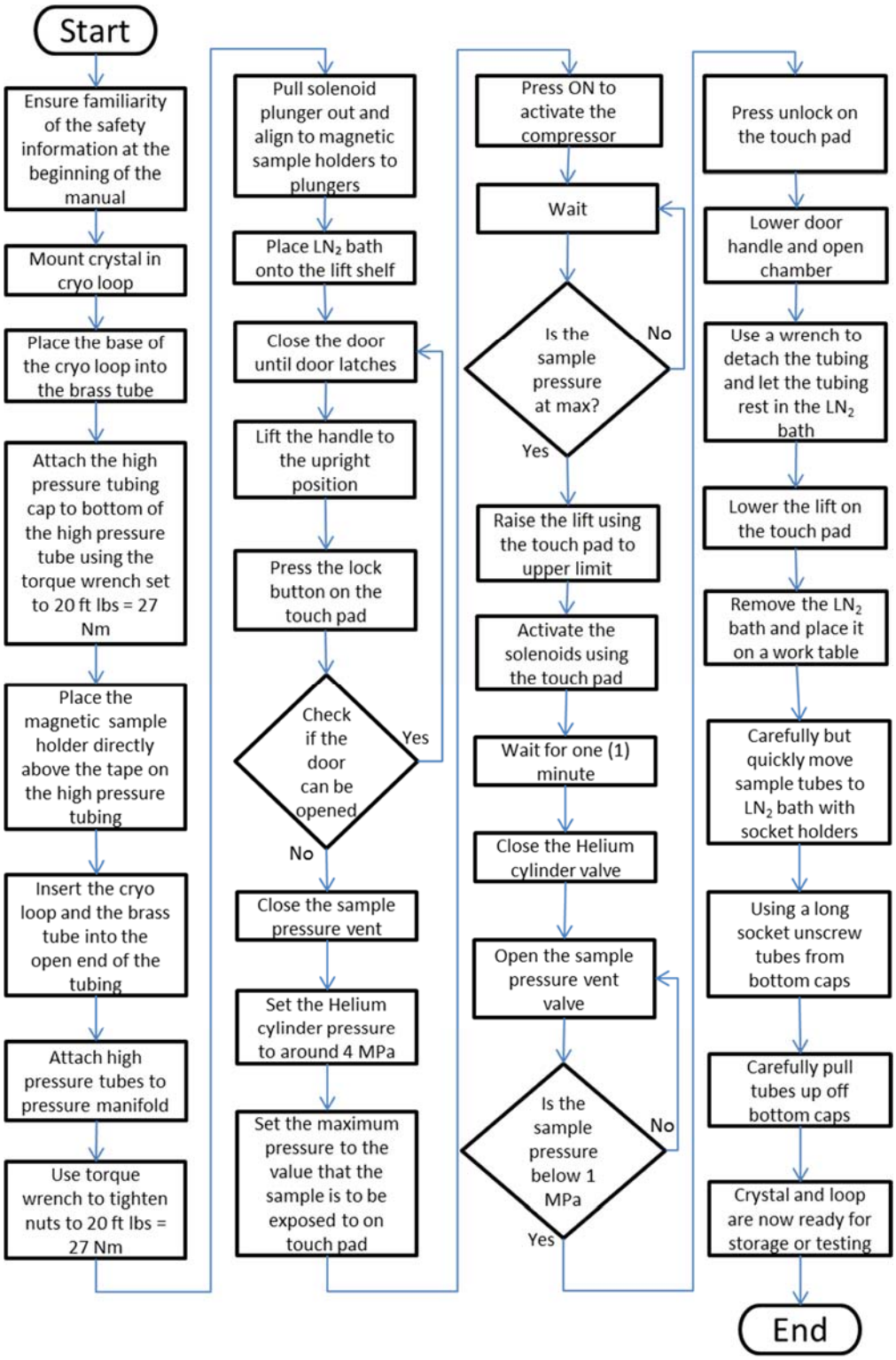



**FIGURE 9.** HPC-201 Process Flow Chart

## Summary

Cryocooling was developed as an alternative method for cryopreservation of macromolecular crystals and successfully applied for various technical and scientific studies. Using this patented technology, ADC developed and commercialized a turn-key user friendly and safe system for use in major research facilities around the world.

## References

1. High-pressure cooling of protein crystals without cryoprotectants; Chae Un Kim, Raphael Kapfer, and Sol M. Gruner; Acta Crystallographica Section D, Biological Crystallography, ISSN 0907-4449
2. Cryogenic x-ray diffraction microscopy utilizing high-pressure cryopreservation; Enju Lima, Yuriy Chushkin, Peter van der Linden, Chae Un Kim, Federico Zontone, Philippe Carpentier, Sol M. Gruner, and Petra Pernot; PHYSICAL REVIEW E 90, 042713 (2014)
3. A high-pressure cryocooling method for protein crystals and biological samples with reduced background X-ray scatter: Chae Un Kim, Jennifer L. Wierman, Richard Gillilan,a Enju Limac and Sol M.