# An Improved Cryogen for Plunge Freezing

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## Abstract

The use of an alkane mixture that remains liquid at 77 K to freeze specimens has advantages over the use of a pure alkane that is solid at 77 K. It was found that a mixture of methane and ethane did not give a cooling rate adequate to produce vitreous ice, but a mixture of propane and ethane did result in vitreous ice. Furthermore, the latter mixture produced less damage to specimens mounted on a very thin, fragile holey carbon substrate.

## Keywords

cryogen; plunge freezing; electron cryomicroscopy; ice embedding; transmission electron microscopy; specimen preparation

## INTRODUCTION

Specimens for transmission electron cryomicroscopy are typically prepared by plunge-freezing thin aqueous films into a liquid cryogen. It is essential for preparing these specimens to have cooling rates of about $10^4$ K/s or greater, so that the specimens will be embedded in amorphous, as opposed to crystalline, ice. The cryogens of choice have been alkanes, ethane (Et) or propane (Pr), cooled to their freezing points by thermal contact with liquid nitrogen (LN). A difficulty arises, however, due to the fact that, if left in contact with LN, the alkane will freeze completely, but if isolated from the LN, the temperature of the alkane will rise, possibly to the point that the cooling rate, final temperature, or both may be inadequate to produce amorphous ice. (The liquid ranges of Et and Pr are 89.9 to 184.6 K and 83.5 to 231.0 K, respectively.) This necessitates repeated cooling or warming steps to assure proper freezing conditions. Typically, the Et or Pr are cooled until a small fraction of the alkane solidifies. Specimens are frozen until there is so much solid that damage to the specimen by collision becomes likely, then cryogen gas is blown onto the solid to melt it, after which more specimens can be frozen, and this process is repeated as necessary.

An advanced apparatus for automating the freezing process, the Vitrobot, was developed, and a cryogen holder was designed with a limited thermal contact between the LN and the cryogen (see Fig. 1A). Experience in many labs has shown that this slowed the freezing of Et or Pr, but did not eliminate it, so, as with earlier equipment, cryogen gas was used to melt the solid. A later version of the Vitrobot incorporated a new style of cryogen holder (see Fig. 1B), in which the LN and the cryogen were thermally isolated by an insulating layer of plastic foam. With this holder the cryogen never freezes during specimen preparation; however, because it is not in thermal contact with LN, the temperature can rise to the point

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that the ice is partly crystalline. To avoid this, a bridge is placed between the LN and cryogen, which conducts heat from the cryogen to the LN. The bridge is left in place until there is a little solid cryogen, then removed to continue specimen freezing.

Griffith et al. (2006) pointed out that the eutectic mixture of methane (Me) and Et remains liquid at 77 K. They did not give the composition of the eutectic, however. Wikipedia gives a formula for calculating the freezing point depression:

$$\Delta T = \frac{R(T_m)^2}{\Delta H_{fus}} X_2,$$

where $\Delta T$ is the amount the freezing point is lowered, $R$ is the gas constant, $T_m$ is the melting point of the major component, $\Delta H_{fus}$ is the heat of fusion of the major component, and $X_2$ is the mole fraction of the minor component. The values of the parameters for Me and Et in this equation were found in the *Handbook of Chemistry and Physics*, except for the heat of fusion, which was taken from Wikipedia (www.wikipedia.org). This formula is accurate only for small values of $X_2$, but it predicts that the fraction of Me necessary to depress the freezing point of Et to 77 K is 0.55.

One of us (W.F.T.) realized that a mixture of Pr and Et should be just as effective a cryogen as pure Et and would likely remain liquid at 77 K (the temperature of the liquid nitrogen bath). Because it was not known whether a Me-Et mixture would provide sufficiently rapid cooling to give amorphous ice, a similar calculation was made for a Pr-Et mixture. This calculation predicted that a 63% Pr, 37% Et mixture would freeze at 77 K. This article describes the rationale and reports our now routine use of the mixture.

## MATERIALS AND METHODS

A 50-50 mixture of Me and Et was purchased from Scott Specialty Gasses. This mixture is likely to be close to the eutectic, and if the mixture was not itself liquid at 77 K, the component in excess should freeze out, leaving a mixture whose freezing point was 77 K. Experiments were performed with a gravity-operated plunge freezer and with a Vitrobot. In each case, a few µL of a specimen of *Caulobacter crescentus* in culture medium were placed on a Quantifoil grid, the excess liquid was blotted off, leaving a layer of liquid that was a few hundred nanometers thick, and this was plunged into the cryogen. The frozen specimens were placed in LN, loaded into a Gatan 626 cryoholder, and examined on a FEI T12 electron microscope.

Pr was obtained from a local hardware store, and Et was purchased from Gilmore Liquid Air. The appropriate mixture of Pr and Et was prepared in either of two ways. The cryogen cup of the gravity-operated plunge freezer was filled to about 60% of capacity with Pr, which was allowed to solidify, then Et was added both to melt the Pr and fill the cup. Alternatively, the cryogen cup of the Vitrobot was filled to about 40% of capacity with Et, which was allowed to solidify, then Pr was added to melt the Et and fill the cup. The specimens and freezing steps were the same as for the Me-Et experiments.

Because both Me-Et and Pr-Et mixtures exist that are liquid at 77 K, a redesigned cryogen cup for the Vitrobot was constructed (see Fig. 1C,D). The main differences between this and previous designs are, first, the new design has much greater thermal contact between the LN and the cryogen, and, second, it incorporates the grid box storage and barrier features of newer Vitrobot cryogen cups with the outer insulating construction of the older Vitrobot

cups. The redesigned cryogen cup was fabricated simply by milling the central hole and the outer annulus from an aluminum cylinder.

## RESULTS

As predicted by the equation for freezing point depression, the 50-50 Me-Et mixture remained largely in the liquid state when in close thermal contact with LN. A small amount of solid did form at the metal wall of the cryogen cup on the gravity-operated plunge freezer, but no solid was observed when the mixture was liquefied in the old-style Vitrobot cup. The Pr-Et mixture, approximately 60-40, did not show any formation of solid, even when this mixture was put into direct contact with LN (see Fig. 2). In all cases, the thermal contact was maintained for at least 30 min.

Examination of the specimens frozen with the Me-Et mixture, either with the gravity-operated plunge freezer (see Fig. 3) or the Vitrobot (see Fig. 4), showed crystalline ice, but the grids frozen using the Pr-Et mixture using either the gravity plunger (see Fig. 5) or the Vitrobot (see Fig. 6) showed amorphous ice and specimens at least as well frozen as with either Et or Pr alone as the cryogen. None of the cells on any grid, whether frozen with the Me-Et or Pr-Et mixtures, showed major damage—broken membranes or displacements of parts of the cell due to ice crystal growth—but subtler forms of damage cannot be excluded. Because diffraction contrast can interfere with three-dimensional reconstructions, however, cells embedded in crystalline ice are not suitable specimens for electron cryomicroscopy.

An additional advantage of using the Pr-Et mixture was demonstrated when specimens mounted on C-flat grids were frozen. Previous experience has shown that these grids are fragile and that the carbon film was removed from most of the grid squares when the grids were plunged into Et; however, the grids frozen using the Pr-Et mixture had many more areas of intact film.

## DISCUSSION

After these experiments demonstrated that the results using a Pr-Et mixture were at least as good as those using pure Et as the cryogen, most of the personnel from this lab began to use the mixture with good results. At that time, a mixture of 63% Pr, 37% Et was purchased from Gilmore Liquid Air and has been used directly instead of making the mixture from the separate gasses, as had initially been done for the tests. The advantages of using the purchased mixture are greater purity and consistency, as well as the convenience of freezing the cryogen in one step, rather than two. Data collected from grids frozen with the commercial mixture are not presented in this article, but have been presented in Briegel et al. (2008).

Although the cryogen should be allowed to cool until its temperature is low enough to assure amorphous ice, once this is the case grids may be frozen in rapid succession without the need either to warm the cryogen or recool the cryogen. There is not a solid layer of cryogen to monitor to assure that the temperature is low enough, but the large thermal contact between the cryogen and the LN provided by the redesigned cryogen cup in the Vitrobot or the existing cup in the gravity plunger assures that more heat will be removed from the cryogen by the LN than will be added by plunging many grids in succession.

There may be a slight increase in cooling rate using a Pr-Et mixture compared to that for a pure alkane because heat conduction is proportional to the temperature difference, which is a few kelvin greater for the mixture at 77 K than for either alkane at its freezing point. It is not known why the C-flat grids retained more intact film when frozen with the mixture, but one possibility is that the viscosity of the mixture, which is above its freezing point, is less than

the viscosity of either alkane at its freezing point. We have not measured these viscosities, however.

The reason that the Me-Et mixture did not produce amorphous ice is likely to be that both Et and Pr have many more modes into which energy can be transferred than does the simpler Me. Thus, when thermal energy is transferred into the Me-Et mixture, fewer modes can accept the energy, so the cooling rate will be less. It was reported by Brüggeller and Mayer (1980) that a cooling rate of $10^3$ K/s produces crystalline ice. Because it is known that $10^4$ K/s cooling rate is adequate to produce amorphous ice, the heat transfer rate of Me-Et must be sufficiently lower than that of either pure Pr or Et that the cooling rate becomes inadequate.

## Acknowledgments

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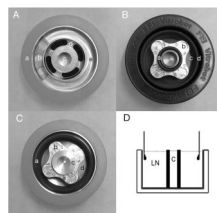
**Figure 1.**
Vitrobot cups. **A:** Old-style cup. LN is put into the outer annulus (e), which has limited thermal contact (d) with the inner cup (c). The outer insulation is labeled a, and the grid box holder is labeled b. When the apparatus has cooled, Et or Pr can be liquified in the inner cup. **B:** New-style cup. LN is put into the outer annulus (c), which is thermally isolated from the inner cup by an annulus of insulating material (a). A thermal bridge (not shown) can be inserted to cool the inner cup and allow Et or Pr to be liquified. The grid box storage table is labeled b, and the floating barrier is labeled d. **C:** Redesigned cup. Note the good thermal contact provided by the direct contact of the aluminum wall of the inner cup (c) with LN in the outer annulus (d) and the presence of the grid box storage table (b) and black plastic floating barrier (a) that are features of the new-style cup. **D:** Side view diagram of redesigned cup. Liquid nitrogen is labeled LN, and the cryogen is labeled C.

**Figure 2.**
Gravity plunger showing inner cup filled with Pr-Et and outer cup filled with LN. When the LN was filled to a level higher than the top of the inner cup, it displaced some of the Pr-Et, which floated on the LN and remained liquid even at the boundary, where it was in direct contact with LN (a). The nonpolar Pr-Et mixture remained separate from the polar LN. The edge of the inner cup is labeled b, a bubble of nitrogen gas rising to the surface of the Pr-Et is labeled c, the LN is labeled d, the edge of the outer cup is labeled e, and the edge of the grid box storage cup is labeled f.
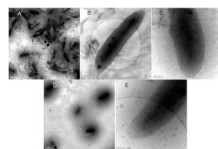
**Figure 3.**
Freezing with Me-Et mixture using gravity plunger. **A:** Overview showing extensive crystalline ice. Note that the dark lines in **A** and **B** are bend contours indicating crystallinity. **B:** Higher magnification showing bacterium embedded in crystalline ice. **C:** Bacterium from the same grid embedded in mostly amorphous ice. **D:** Overview of different grid showing mostly amorphous ice. Note that there are only a few bend contours near the upper left of the image, while most of the ice has much smaller intensity variations indicating that it is amorphous. **E:** Higher magnification showing bacterium embedded in amorphous ice, but with artifacts either in or on the surface of the ice.
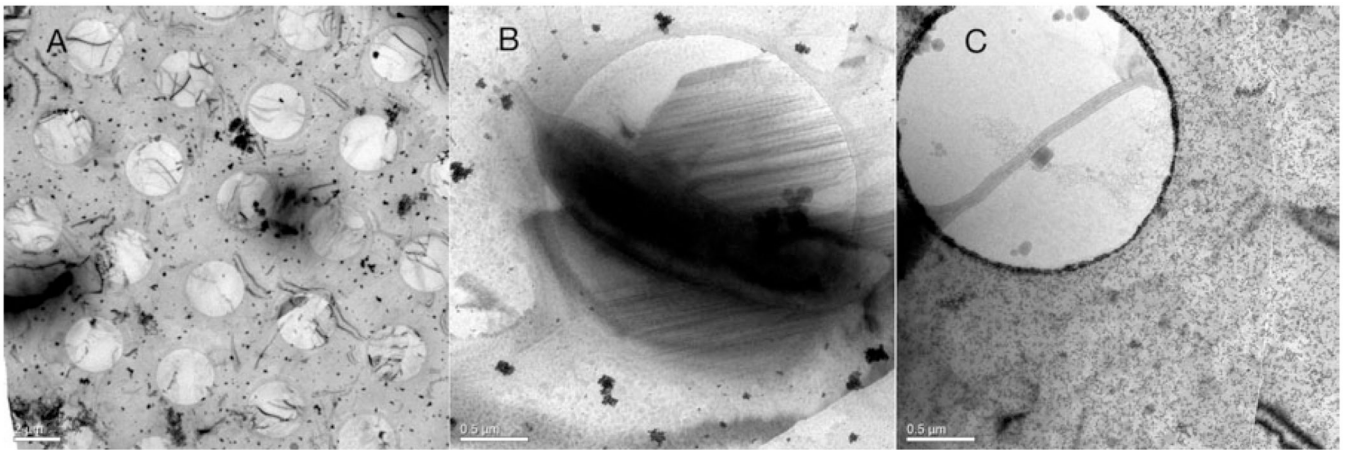
**Figure 4.**
Freezing with Me-Et using Vitrobot. **A:** Overview showing extensive crystalline ice. **B:** Higher magnification showing bacterium embedded in crystalline ice. Note that the alternating dark and light bands are thickness contours indicating that the area is crystalline. **C:** Stalked end of a bacterium from same grid embedded in amorphous ice.
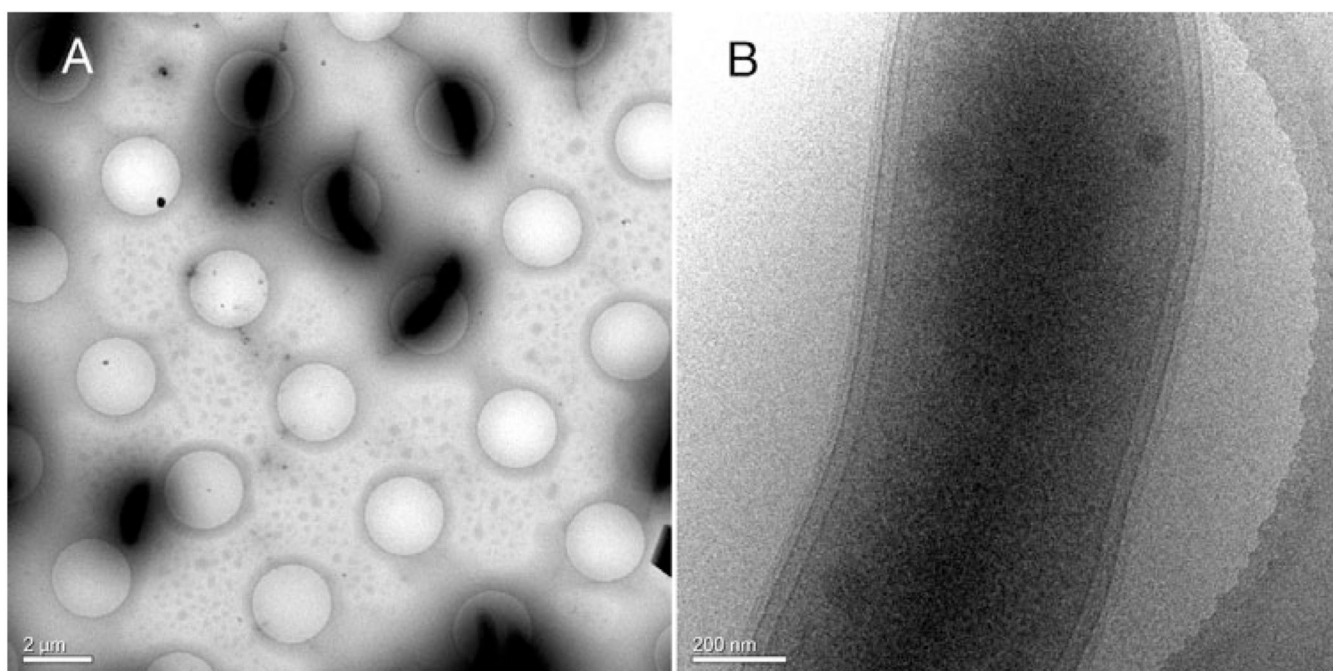
**Figure 5.**
Freezing with Pr-Et using gravity plunger. **A:** Overview showing amorphous ice. **B:** Higher magnification showing bacterium embedded in amorphous ice.
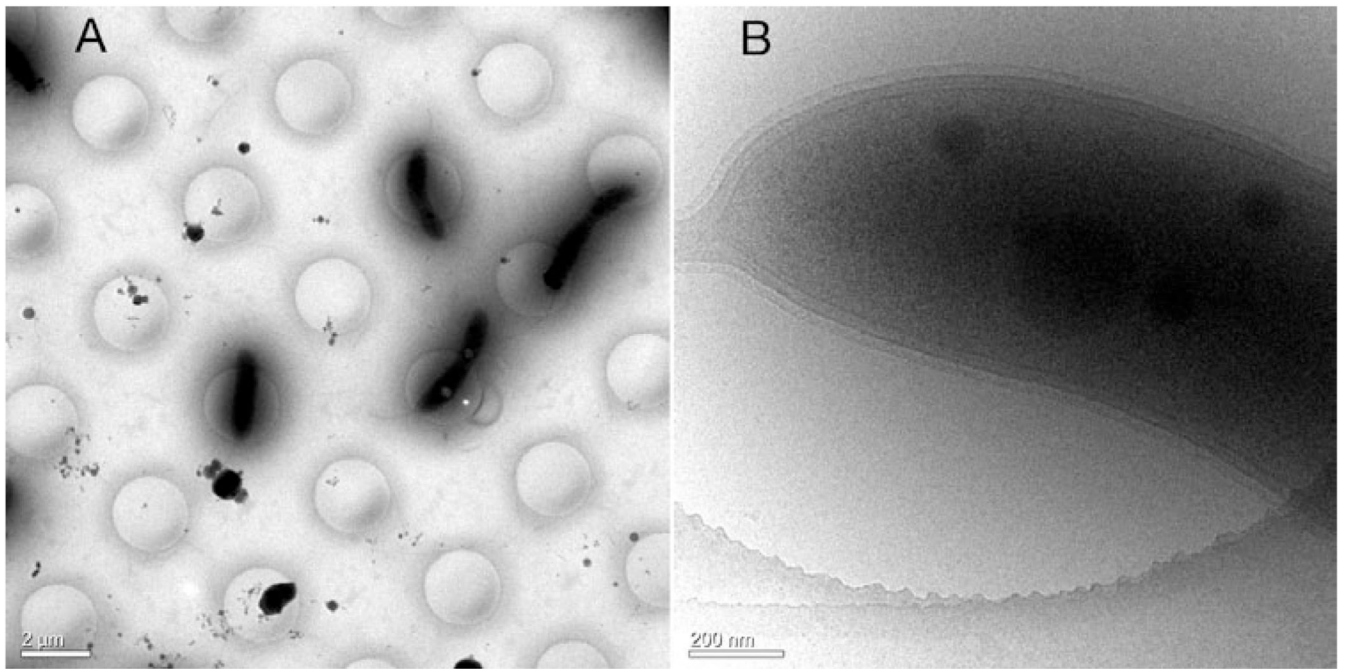
**Figure 6.**
Freezing with Pr-Et using Vitrobot. **A:** Overview showing amorphous ice. **B:** Higher magnification showing bacterium embedded in amorphous ice.