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Preparation of High Temperature Sample Grids for Cryo-EM

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TITLE:

Preparation of High-Temperature Sample Grids for Cryo-EM

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SUMMARY:

This paper provides a detailed protocol for preparing sample grids at temperatures as high as 70 °C, prior to plunge freezing for cryo-EM experiments.

ABSTRACT:

The sample grids for cryo-electron microscopy (cryo-EM) experiments are usually prepared at a temperature optimal for the storage of biological samples, mostly at 4 °C and occasionally at room temperature. Recently, we discovered that the protein structure solved at low temperature may not be functionally relevant, particularly for proteins from thermophilic archaea. A procedure was developed to prepare protein samples at higher temperatures (up to 70 °C) for cryo-EM analysis. We showed that the structures from samples prepared at higher temperatures are functionally relevant and temperature dependent. Here we describe a detailed protocol for preparing sample grids at high temperature, using 55 °C as an example. The experiment made use of a vitrification apparatus modified using an additional centrifuge tube, and samples were incubated at 55 °C. The detailed procedures were fine-tuned to minimize vapor condensation and obtain a thin layer of ice on the grid. Examples of successful and unsuccessful experiments are provided.

INTRODUCTION:

The cryo-EM technology for solving the structures of protein complexes has continued to improve, particularly in the direction of obtaining high-resolution structures[1,2]. In the meantime, the landscape of its application has also been expanded by varying the sample conditions such as pH or ligands prior to the vitrification process[3], which involves the preparation of sample grids followed by plunge freezing[4,5]. Another important condition is the temperature. Although cryo-

EM experiments, like X-ray crystallography, are performed at low temperatures, the structure solved by cryo-EM reflects the structure at the solution state prior to vitrification. Until recently, the majority of single particle analysis (SPA) cryo-EM studies use samples that are kept on ice (i.e., at 4 °C) prior to vitrification[6], though a number of studies use samples at around room temperature[7-10] or as high as 42 °C[11]. In a recent report, we performed temperature-dependent studies of the enzyme ketol-acid reductoisomerase (KARI) from the thermophilic archaeon *Sulfolobus solfataricus* (*Sso*) at six different temperatures from 4 °C to 70 °C[12]. Our studies suggest that it is important to prepare sample grids at functionally relevant temperatures and that cryo-EM is the only structural method that is practically feasible for solving the structure of the same protein complex at multiple temperatures.

The major difficulty for vitrification at high temperatures is to minimize vapor condensation and achieve thin ice. Here we report the detailed protocol used for preparing sample grids at high temperatures in our previous study of the *Sso*-KARI[12]. We assume that the readers or viewers are already experienced in the overall sample preparation and data processing procedures for cryo-EM experiments and emphasize the aspects relevant to high temperature.

PROTOCOL:

NOTE: This protocol aims to use a modified commercial vitrification apparatus to prepare the cryo-electron microscopy (cryo-EM) samples at specific temperatures, especially higher than 37 °C. The overall experimental setup is shown in **Figure 1**. The protocol uses 55 °C as an example. For the specific conditions at other temperatures, please refer to Supplementary Table 2 in reference[12].

1. Preparation of the vitrification apparatus

1.1 Make a 1 cm hole in a 50 mL centrifuge tube on its closed end.

1.2 Place the tube in the vitrification apparatus chamber at the ultrasonic water outlet, as shown in **Figure 2**.

NOTE: The purpose is to minimize water condensation by guiding water vapor to the heat exchanger through the tube before reaching the entire chamber.

1.3 Set up the vitrification apparatus temperature to the specified temperature (e.g., 55 °C, as shown in **Figure 3**), and allow the vitrification apparatus chamber to reach to 55 °C and 100% relative humidity. Let it stand for at least half an hour to stabilize the conditions before starting the experiment.

2. Warming up the sample and the tools

2.1 Place the water bath on a hotplate and set the hotplate to the desired temperature (here 55 °C). Check with a thermometer to ensure that the water reaches 55 °C.

2.2 Incubate the sample in the water bath and preheat the pipette tip on the edge of the hotplate for 2 min or longer before the blotting experiment.

NOTE: The highest temperature setting for the vitrification apparatus chamber is 60 °C. To prepare higher temperature grids for cyro-EM experiment (e.g., 70 °C), the sample is incubated in the water bath at 80 °C, and the average between the sample temperature and the vitrification apparatus temperature is estimated to be the actual temperature of the sample on the grid (70 °C in this case). See the **Discussion** section for further details and limitations on this estimation.

3. Preparation for the blotting experiment

3.1 Glow discharge a holey carbon-supported grid at 25 mA for 30 s, or alternative to the values depending on the device used.

3.2 Incubate the tweezers with the grid in the vitrification apparatus for 2 min or longer.

3.3 Fill the ethane container with ethane according to standard procedures. Do not let the ethane overflow.

NOTE: This step takes about 10 min and should be followed immediately with the blotting experiment to avoid freezing.

3.4 Place the vitrification filter paper into the vitrification apparatus chamber no sooner than 5 min before the blotting experiment.

NOTE: Placing the filter paper in the chamber too soon will cause it to become too wet.

4. Blotting experiment

NOTE: When holding the grid, ensure that the grid is stable and there is a minimal contact area with the tweezers (**Figure 4**). This is done to maintain the best cooling efficiency of ethane and to avoid non-vitreous ice.

4.1 Use a pipette tip to apply 7-9 μL of the sample to the grid. Then wait for 1-2 s, blot for 1-1.5 s, and quickly plunge the sample to liquid ethane.

4.2 Transfer the grid from liquid ethane to the cryo box, which is stored in liquid nitrogen.

NOTE: This step must be performed very carefully because the tweezers is still hot, so the entire cooling tank is full of vapor at this time.

5. Quality check for the grids

5.1 Clip the grids and upload them to a cryo-EM instrument.

5.2 Use the display screen of the cryo-EM instrument and the low dose function of the software to screen the ice condition on the grid and the distribution of the sample on the grid.

NOTE: Often, the grids are very dry, or the ice layer is too thick. The success rate of the grids prepared at high temperatures is substantially lower than that at room temperature or 4 °C. Representative results of good grids and non-satisfactory grids are shown in the next section.

5.3 If the quality of the resulting grid is not good, repeat the process of grid preparation with varied conditions (such as waiting time, blotting time, etc.). If the quality of the resulting grid is good, repeat the same steps to make grids at a different temperature.

6. Data collection

6.1 Transfer the good quality grids to a high resolution cryo-EM instrument.

6.2 Perform data collection and data analyses according to established procedures.

NOTE: As shown in our previous publication, the resolution of the structure is not affected by the high temperature[12].

REPRESENTATIVE RESULTS:

The low magnification overview is shown in **Figure 5A,B**. Panel A is an example of a successful grid. There is an ice gradient from top left (thicker) to bottom right (thinner or empty). Such a grid makes it easier to find an appropriate thickness of the ice layer in the middle area suitable for data collection, such as the blue and green boxes. The grid B is too dry. The squares in the grid have bright contrast, which means that the ice layer is too thin or there is no ice layer at all. Only the two squares indicated by the red arrows are suitable for data collection.

Furthermore, examples of the low-dose images from different grids are shown in **Figure 5C,D**. The image in panel C shows that most of the ice is in the crystalline form, not suitable for data collection. On the other hand, image in panel D shows that the ice layer is mostly in an amorphous state, suitable for data collection.

Please note that this is a short paper focusing on grid preparation at high temperatures. The grid only contains the sample for data collection. A good grid has a good chance, though not a definite chance, to generate good data for solving a high-resolution structure. The real cryo-EM data and final structures for the examples described in this paper are already described in the published paper[12]. In short, we have obtained grids good enough for data collection, solved the structures of two *Sso*-KARI complexes at six different temperatures each, and compared the structures from different temperatures for each complex, as well as the structures between the two complexes from the same temperature. The results indicate that the structure of each complex is temperature-dependent and that the temperature-dependent changes are different between the two complexes. Importantly, the successive structural changes correlate well with the

successive temperature changes, which is a strong indication for the success of the temperature-dependent sample grid preparation.

FIGURE LEGENDS:

Figure 1: The overall experimental setup for high-temperature cryo-EM sample preparation. The items shown include vitrification apparatus, incubator, timer, pipette tip placement, cooling tank, and tweezers.

Figure 2: Modification of the vitrification apparatus chamber. A 50 mL tube is installed at the ultrasonic spray outlet as indicated by the red arrow[12].

Figure 3: Appearance of the vitrification apparatus during the experiment. The screen shows the temperature at 55 °C and the humidity at 100%.

Figure 4: Using tweezers to grab the grid. It is recommended that the tweezers grip the gird with as little contact as possible, but it must be able to hold the grid stably during the process of the operation.

Figure 5: Representative results: Grid checking by Cryo-EM. (**A,B**) show the overall state of the grid. (**C,D**) show examples of the low-dose images from different grids.

DISCUSSION:

In step 1 of the protocol, make sure that the centrifuge tube has been installed well and does not fall when the experiment is in progress. Due to the accumulation of a large number of water droplets in the chamber, which could change the adsorption capacity of the filter paper, it is recommended that the overall time of the experiment should not exceed 30 min after the vitrification apparatus chamber reached the equilibration temperature. If the operation time exceeds 30 min, the operator needs to replace the filter paper and wait for the cabin to balance the temperature and humidity again. At step 8 of the protocol, the suggested sample volume of 7-9 µL is larger than usual since otherwise, the sample evaporates quickly at high temperature, leading to empty squares on the grid. On the other hand, it is highly recommended that the sample applied does not exceed 9 µL. Otherwise, it is very likely that the sample will drip down during the process of moving the tweezer before blotting. Overall, a key to the success of this technique is stable and fast gripping of grids and correct and stable execution of each time-limited action. Furthermore, it is recommended that each round of experiments deals with only one specific high temperature. Before proceeding to perform the experiment at another temperature, all systems must be fully recovered and reset.

Due to the high temperature and high humidity of the chamber, the window is often covered with fog leading to difficulties in launching the experiment. Use of little soapsuds is recommended to clear the window. If grids are not good, possible reasons are that the steps described above are not followed precisely and/or that the steps take too long. Try to repeat the preparation of sample grids with precision and swiftness. If the grids are still not good after repeats, then try to adjust the conditions. The more frequent problem observed in this experiment is no ice on the grid at

the high temperature. If so, try to reduce the blotting time further. On the other hand, if the ice is too thick, try to increase the blotting time.

A limitation of the high-temperature cryo-EM is that the maximum heating temperature on the vitrification apparatus is 60 ˚C. To reach to the higher temperature, the sample was heated above 60 ˚C (e.g., 80 ˚C), and the average between the sample temperature and the vitrification temperature was estimated to be the real temperature of the sample on the grid (70 ˚C, in this case). There could be some inaccuracy based on this estimation. A possible future solution is to build a thermocouple to measure the grid temperature accurately right before plunge-freezing. Another potential limitation is the stability of the protein at high temperatures. A separate experiment using circular dichroism should be performed to ensure that the protein is stable at the temperature for the planned cryo-EM experiment.

Another limitation is that only two types of commercially available vitrification apparatus can be heated to above 37 ˚C and up to 60 ˚C as mentioned above (e.g., Thermo Fischer Vitrobot and Leica EM-GP). The vitrification apparatuses from other vendors are either limited to room temperature or adjustable only between 4 ˚C and 37 ˚C. However, it is possible for research groups to build their own plunging devices with extended temperature ranges in the future.

Our protocol is modified from existing protocols[4-6], with the purpose of preparing the grids at temperatures higher than room temperature. Without making the modifications described here, the chance of success for making good high-temperature sample grids suitable for cryo-EM image collection is very small.

Two papers in 2019 have demonstrated that protein structures are temperature dependent, in correlation with the temperature dependence of protein functions, in the range of 4 °C to 42 °C for the TRP channel TRPV3[11] and 4 °C to 70 °C for *Sso*-KARI[12]. These reports are likely to encourage a change in cryo-EM research in that more future studies will be performed at functionally relevant temperatures, usually at 37 °C. The stability of the purified protein at this temperature could be a concern. However, it is required to incubate the protein sample at this temperature for only 2 min according to our protocol. Alternatively, physiological conditions can be achieved by imaging proteins in cells using tomography and sub-tomo averaging. Furthermore, cryo-EM can be used to study the mechanism and intermediates of protein unfolding at high temperatures, likely in the range of 40 °C to 80 °C. These studies will all benefit from the protocol described here.

ACKNOWLEDGMENTS:

The authors thank Dr. Hervé Remigy of Thermo Fisher Scientific for useful advice. The cryo-EM experiments were performed at the Academia Sinica Cryo-EM Facility (ASCEM). ASCEM is supported by Academia Sinica (Grant No. AS-CFII-108-110) and Taiwan Protein Project (Grant No. AS-KPQ-109-TPP2). The authors also thank Ms. Hui-Ju Huang for the assistance with the sample preparation.

DISCLOSURES:

The authors declare no competing financial interests.

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Figure 1

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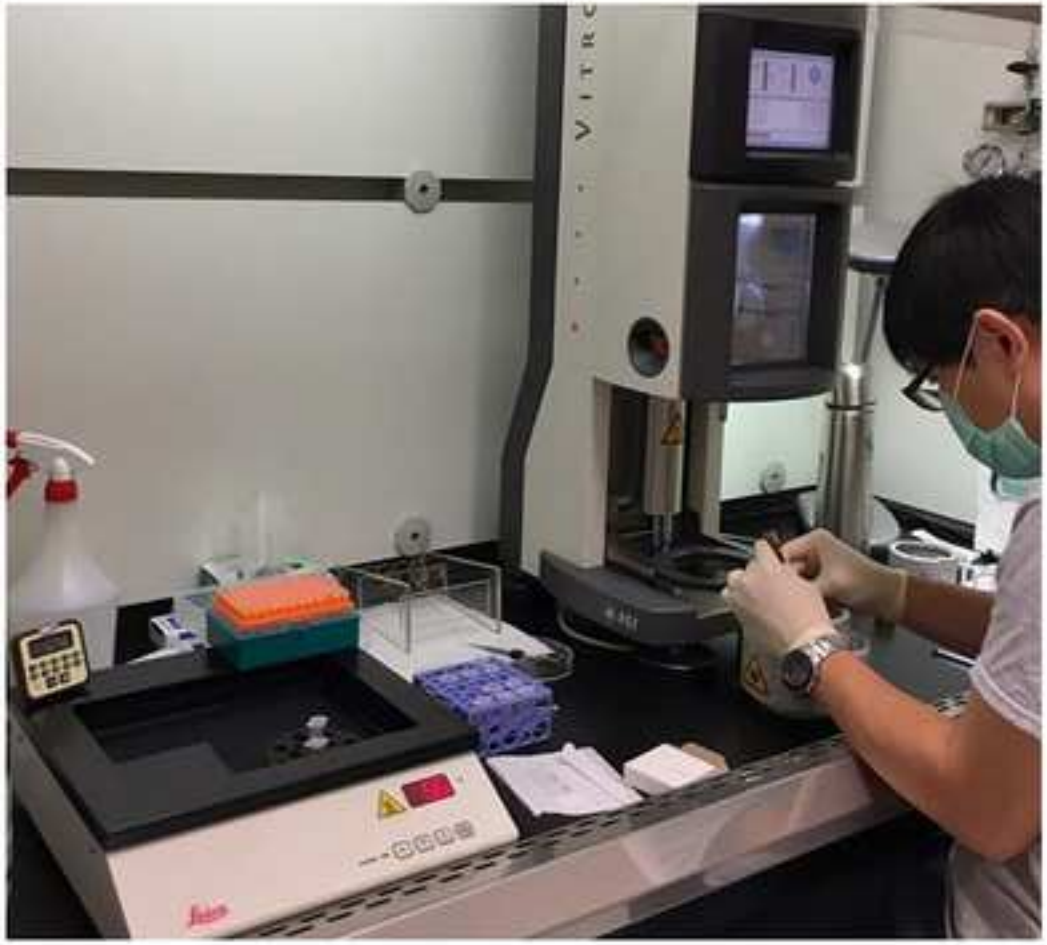

Figure 2

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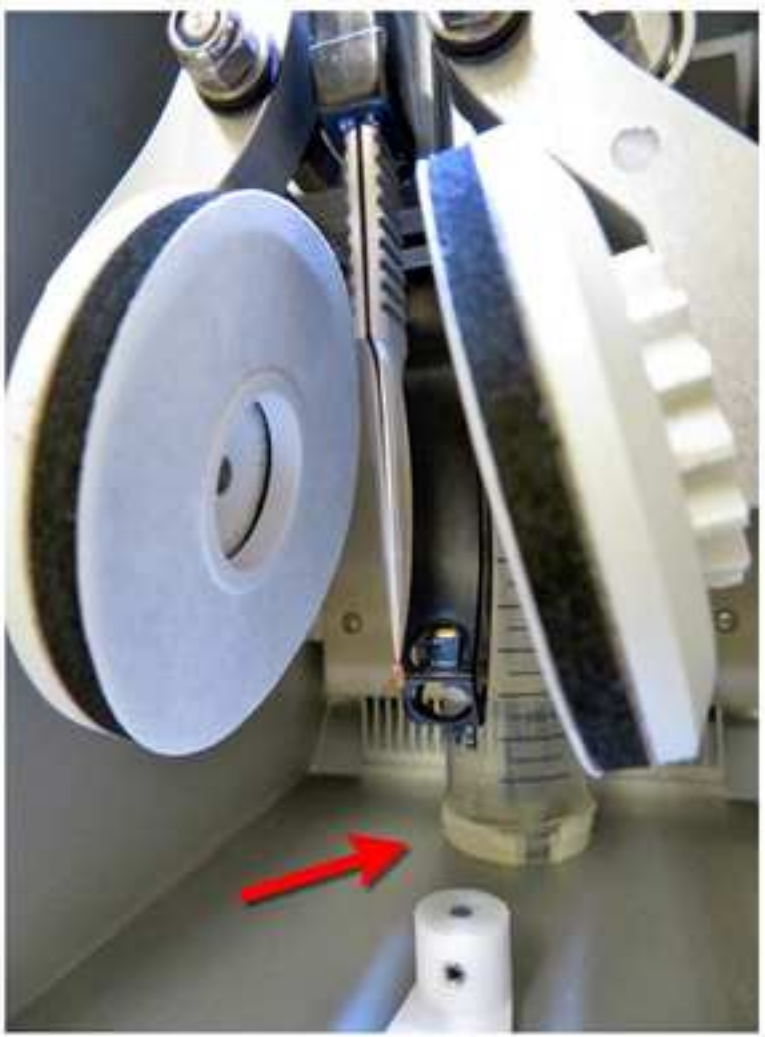

Figure 3

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Figure 4

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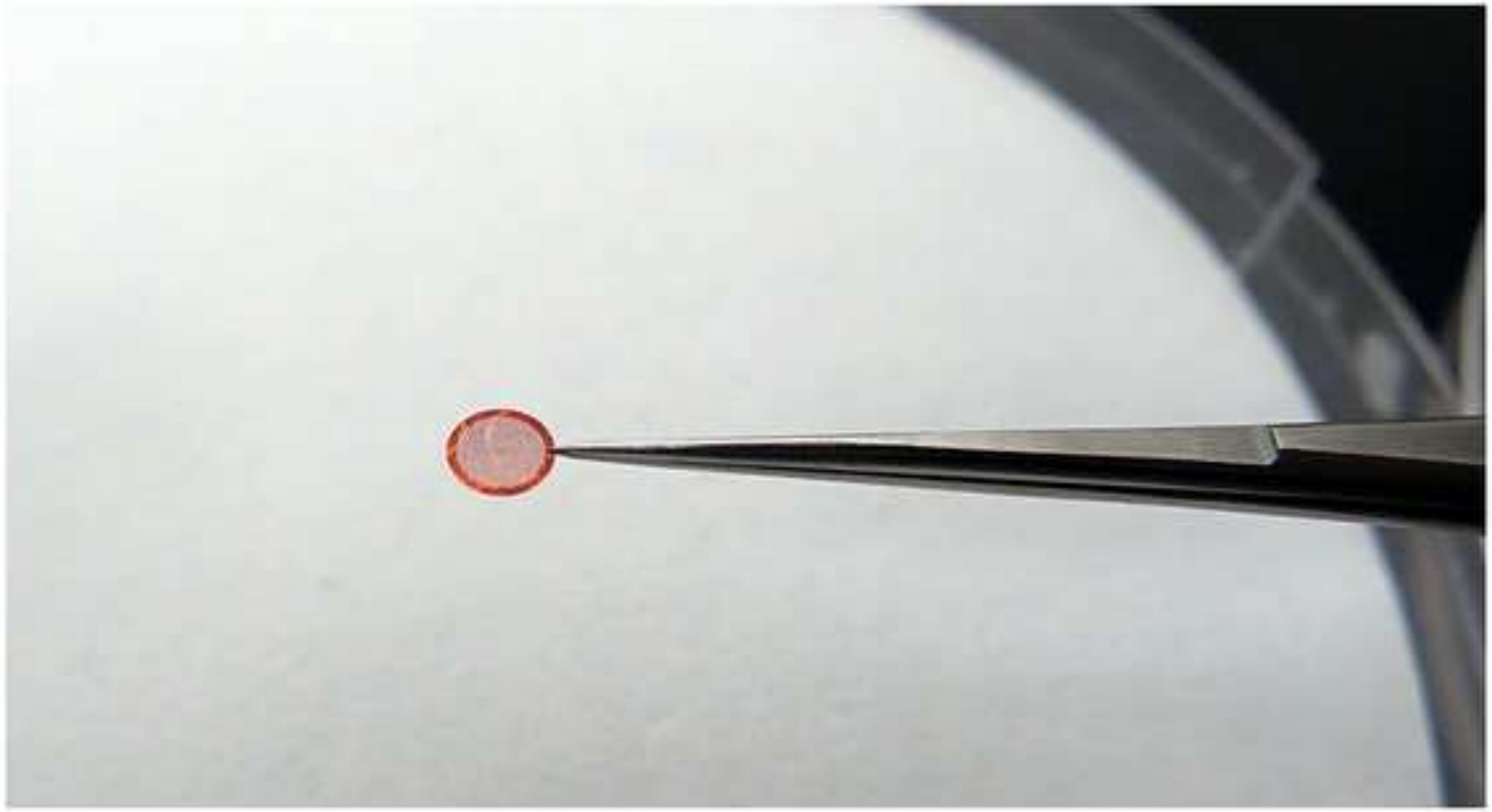
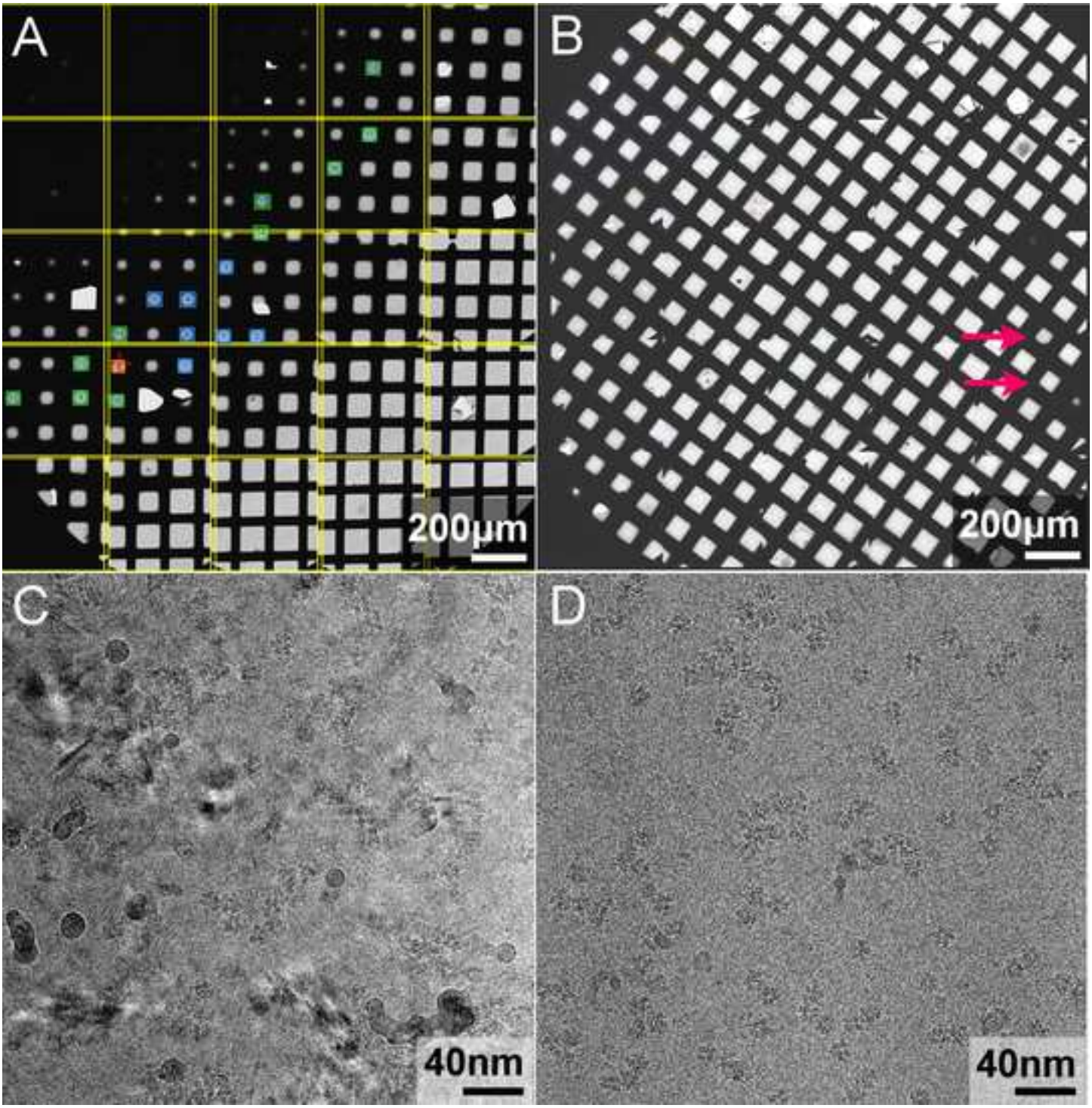


Figure 5



Materials

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Table of Materials

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Dear Editor:

Thank you for the suggestions and comments. We have thoroughly addressed the editorial comments and the reviewers' comments, as described by the point-by-point responses in blue color. Please let me know if any further information or revision is needed.

Sincerely,

Ming-Daw Tsai

The language in the manuscript is not publication grade. Please employ professional copy-editing services. The senior author has edited the writing carefully.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done
2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points. Done
3. Video is the part of JoVE publications please do not make a separate mention in the text. Done
4. Please do not include references in the abstract. Please move the hyperlink to the reference and use in text citation. Done
5. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Done
6. Please ensure the Introduction include all of the following with citation:

a) A clear statement of the overall goal of this method - Yes

b) The rationale behind the development and/or use of this technique - Yes

c) The advantages over alternative techniques with applicable references to previous studies - Yes

d) A description of the context of the technique in the wider body of literature - Yes

e) Information to help readers to determine whether the method is appropriate for their application - Added a new sentence at the end of Introduction.

7. Please make subsections in the protocol and make a cohesive story. Each subsection should contain steps to show how to perform the procedure. - Done
8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” - Done
9. The Protocol should contain only action items that direct the reader to do something. - Done
10. Please use the following numbering scheme in the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes. - Done
11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step. Each action of the step should be clearly described. - Done
12. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. – Done the best we can.
13. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. – This is a short protocol and all steps should be in the video.
14. Please discuss all figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol. Figures 1-4 are in the Protocol. Figure 5 is discussed in the Representative Results.
15. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included. We have moved the description from the legend of Figure 5 to the Representative Results.

However, please note that this is a short paper focusing on the grid preparation at high temperatures. The grid is still a sample, not the final structure. The final structure is already described in the published paper (JACS 2019). A new paragraph have been added to describe the final results and how good grids can lead to good structures.

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17. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. Done.

18. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Done.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes the protocol for preparing sample grids for high temperature cryo-EM. The topic should be of interest to many who want to use cryo-EM to study enzymatic behavior at various temperatures.

The manuscript is delightful to read. While it does need a good copy-editing, the description is clear enough for those familiar to cryo-EM and contains several tricks and tips that traditional publication lacks.

Major Concerns:

None

Minor Concerns:

1. Line 109-112 of the first revision estimates the temperature of the sample on the grid to be half-way between that at incubation and vitrification apparatus. Is there any experimental evidence of this? Given the surface area to mass ratio of the grid

plus sample, the chance for that to be so much higher than the value of the vitrification apparatus is small.

Thanks for raising this good point. Since Reviewers #2 and #4 also raised related points, we have prepared a combined response as follows:

Combined response related to the 70 °C temperature: Reviewers 1 and 2 are concerned that the estimation that the actual sample temperature is the average (70 °C) between the external water bath (80 °C) and the Vitrobot chamber temperature (60 °C) is not accurate, and that it should be very close to the latter. Reviewer 4 suggested the possibility of measuring the temperature directly by a thermocouple (which is not possible with the current apparatus). We agree that the real temperature may not be as high as 70 °C, but it should be higher than 60 °C because we use very short wait time (1-2s) and blot time (1-1.5s) at high temperature, and that our result supports higher temperature. Even though this only concerns a special situation (above 60 °C), it should not be deleted from the current manuscript since we are reporting the protocol for a paper already published (JACS 2019). Instead, we have included the concerns and suggestions in the DISCUSSION. In addition, we have changed the original step 9 of the protocol to become a NOTE instead a step.

2. Line 105-106 and Line 175. The need to apply 7-9 uL of the sample to the grid is most interesting. What was the reason for such high volume?

Response: The main reason to use larger volume is that at higher temperature the sample evaporates quickly in the chamber, leading to empty squares on the grid. We have now added a sentence in DISCUSSION to explain this point, thanks to your suggestion: “the suggested sample volume of 7-9 μL is larger than usual since otherwise the sample evaporates quickly at high temperature, leading to empty squares on the grid.” In addition, we have added another sentence at the first paragraph of PROTOCOL: “For the specific conditions at other temperatures, please refer to Supplementary Table 2 in reference 12.”

Reviewer #2:

Manuscript Summary:

The manuscript describes a method for plunge freezing macromolecules on EM grids at high temperatures, as detailed in a results paper from the same group (Chen et al 2019 JACS). The frozen grids are later used for imaging of the macromolecules using

single particle (SPA) cryo-EM. The authors use a commercial plunging device, the Vitrobot, in which the sample-handling chamber can be heated up to 60°C. They add a punctured Falcon tube to redirect the water vapor and reduce condensation in the sample-handling chamber. Additionally, examples of grids suitable and unsuitable for data collection are shown.

In general, the protocol is clearly written, detailed and efficient, except for issues raised below.

Thanks for the overall support. The detailed comments below are very insightful. We can address most of them following each specific comment. However, the issue about the 70 °C temperature has been commented at three places under "Minor Concerns", which are very good points but not straightforward to address. Since Reviewers 1 and 4 also raised similar concerns, here we provide a combined response for this issue.

Combined response related to the 70 °C temperature: Reviewers 1 and 2 are concerned that the estimation that the actual sample temperature is the average (70 °C) between the external water bath (80 °C) and the Vitrobot chamber temperature (60 °C) is not accurate, and that it should be very close to the latter. Reviewer 4 suggested the possibility of measuring the temperature directly by a thermocouple (which is not possible with the current apparatus). We agree that the real temperature may not be as high as 70 °C, but it should be higher than 60 °C because we use very short wait time (1-2s) and blot time (1-1.5s) at high temperature, and that our result supports higher temperature. Even though this only concerns a special situation (above 60 °C), it should not be deleted from the current manuscript since we are reporting the protocol for a paper already published (JACS 2019). Instead, we have included the concerns and suggestions in the DISCUSSION. In addition, we have changed the original step 9 of the protocol to become a NOTE instead a step.

Major Concerns:

The protocol is limited to plunging and quality assessment of the grids, whereas the method for viewing temperature-induced changes in macromolecules using SPA comprises additional steps. Particularly, the primary result of the method is not nice grids, but multiple atomic models that can be aligned, compared and interpreted, as the authors show in their 2019 JACS paper. Several other JoVE publications describe very similar cryo-EM plunging techniques for both SPA and tomography. However, these publications normally present a broader view of the method, including sample

preparation prior to plunging, low dose imaging, image processing, reconstruction and interpretation. A more complete view of the current technique would include, for example, a presentation of how the multiple temperature-dependent structures are resolved from the data and compared.

Response: This is an excellent point but it depends on the scope of this protocol. The earlier JoVE publications, for example, ref. 4 in our manuscript, were intended for beginners of cryo-EM. At the present time, cryo-EM is already very popular, and there are also free, openly accessible videos on the web. If we cover too much and too broadly, it could lose the main point. Our main point is temperature dependence, and only the researchers already familiar with cryo-EM will be interested in doing temperature dependence. Furthermore, the procedures for solving the structures from variable temperatures, and comparison of the structures from different temperatures, are already descried in detail in our 2019 JACS paper (ref 12). Therefore, the focus of our protocol is on the sample preparation at different temperatures. This point has been explained in the INTORDUCTION. Nonetheless, we appreciate this comment, and have added the following sentence in the last part of INTRODUCTION: "It assumes that the readers or viewers are already experienced in the overall sample preparation and data processing procedures of cryo-EM, and emphasizes the aspects relevant to high temperature."

Minor Concerns:

Line 53 - "though a small of number of studies use samples around room temperature" - the number of studies that use samples plunged at RT is hard to assess, and is likely not small at all. These include not only SPA studies, but also cell biology and soft material studies. Additionally, cooling chambers on plungers were not widely available before the automated commercial plungers became popular, so plunging at 4°C was not an easy task. Thank you for the point. We did survey many recent SPA studies to draw our conclusion, but we agree that there are earlier studies as well as cell and materials studies. In response, we have now specified SPA studies, and deleted "small" in this sentence.

Line 56 - "from 4 °C to 70 °C" - see my comment on the 70°C temperature below. Please see the above combined response related to the 70°C temperature.

Line 88 - glow discharge conditions depend on the device available. Some devices do not reach 25 mA. We have added "or alternative values depending on the device used". The name and maker of our device are listed in the Excel Table "JoVE_Materials".

Line 102 - Sample incubation time depends on the sample. Thanks for pointing out. We have checked the protocol again and confirmed that it is 2 min. The extra words

“or longer” have been deleted now.

Line 105 - (also mentioned in line 175) - I wonder why use such a large volume? Typically 2.5 ul are enough when using the Vitrobot. Additionally, when applying such a large volume the absorbed drops from consecutive grids overlap on the blotting paper. Meaning that you blot the second grid in an area of the filter paper that is wet from the previous grid. To avoid this, it is possible to run a blotting cycle (without a grid) between grids, so that the filter paper rotates away from the last blotting position. Related questions have been raised by other reviewers also. In fact, we did explain this point in the first section of DISCUSSION. The explanation has been extended, and now reads as follows: “At step 8 of the protocol, the suggested sample volume of 7-9 μL is larger than usual since otherwise the sample evaporates quickly at high temperature, leading to empty squares on the grid. On the other hand, it is highly recommended that the sample applied does not exceed 9 μL. Otherwise it is very likely that the sample will drip down during the process of moving the tweezer, before blotting.”

Lines 108-112 (step 9) - Was a temperature of 70°C measured at the grid position from the time the sample was loaded until plunging? If not, please remove this step of the protocol, as there is no reason to assume that the temperature of the sample remains constantly at 70°C, while the grid and surrounding chamber are at 60°C. In fact, I assume that since the volume of the water is so small, its temperature will instantly equilibrate to the chamber and grid temperature. This will happen even faster after blotting, when only nano liters remain on the grid. (The observation of conformational changes in the Sso-KARI complex, as described in the JACS paper, is not a proof that the sample was at 70°C within the Vitrobot chamber. For example, the enzyme may react slowly to the change from 80°C to 60°C, which may not apply to other enzymes). Please see the above combined response related to the 70°C temperature.

Line 116 - The word "smoke" is not appropriate here. Changed to “vapor”.

Line 119 - Please mention that software other than EPU can also be used for screening and imaging. Revised.

Line 125-126 (step 12) - Please elaborate here what is "not good" and how to solve typical problems. For example, if the ice is too thick, increase blotting time… etc. These issues can also be detailed in the Modifications and troubleshooting section. Thanks again. We have added additional points in the Modifications and troubleshooting section: (3) If the grids are still not good after proficient repeats, then try to adjust the conditions. The more often problem is no ice on the grid at high temperature. If so, try to further reduce the blotting time. On the other hand, if the ice is too thick, try to increase the blotting time.

Line 128-129 (step 13) - Are blotting conditions really identical for all temperatures? No. Shorter blot time was used for higher temperatures. We have now added the following sentence at the beginning paragraph of PROTOCOL: “For the specific conditions at other temperatures, please refer to Supplementary Table 2 in reference 12.”

Line 137 - "Atlas" is EPU jargon. Maybe change to "low magnification overview", or something similar. Thanks for the suggestion. “The atlas” has been changed to “the atlas of low magnification overview”.

Line 157 - In figure 5B, please avoid the colorful squares from EPU screening. They are confusing and not helpful in this case. You can unclick them. Good suggestion and corrected. Thanks.

Line 158 - Change "holes" to "squares". Corrected and thanks.

Line 161 - 165K is not a meaningful number for magnification, but is rather a magnification step in a specific microscope. Adding a number here is not essential because there is a scale bar in the figure. Thanks for the comment. We agree and have changed the sentence to “Examples of the low-dose images from different grids.

Line 184 - Are there no other reasons for unsuccessful grids? For example, inappropriate blotting time or blotting power, inappropriate buffer conditions (high glycerol, detergent) etc. Please add typical reasons or refer to other places in the text where they appear. As explained in our response to the Major Concerns, we have cited previous literature including references 4-6 which all deal with detailed procedures for grid preparation. We also assume readers and viewers for the high-temperature study already have some experience with cryo-EM. So here it is better not to get too much into general problems. Nonetheless, your suggestion is highly appreciated.

Line 188-192 - See my comment on the 70°C temperature above. I think the maximum temperature of the sample while in the plunging chamber is dictated by the temperature that can be set in the chamber. One can think of further heating the chamber, although this has to be discussed with the manufacturer and tested. Please see the above combined response related to the 70°C temperature.

Line 199 - Leica's plunger is called EM-GP. Corrected, thanks. Please also mention that groups can build their own plunging devices using published designs that are available online. Thanks for the suggestion and we have added a sentence: “However, it is possible for research groups to build their own plunging devices with extended temperature ranges in the future.”

Line 203 - The phrase "higher than room temperature" includes also "higher than ice temperature". Corrected, thanks.

Line 210 - "paradigm shift" is probably an overstatement, please change. The "paradigm shift" has been replaced by “change”.
Line 210-211 - "most future studies will be performed at functionally relevant temperatures, usually at 37°C or higher" - The reason for not working at physiological temperatures is that purified proteins are normally unstable at these conditions. On top, stabilizing them requires specialized buffers, which are again, non-physiological. Finding ways to plunge at high temperature is a step forward, but does not solve these major instability issues. Alternatively, as a point for discussion, physiological conditions can be achieved by imaging proteins in cells using tomography and sub-tomo averaging. Such experiments would require higher plunging temperatures, particularly when dealing with thermophiles. Thanks for the useful suggestions, though the protein only needs to be incubated at 37 °C for 2 min. We have added the following sentences: “Stability of the purified protein at this temperature could be a concern. However, it is required to incubate the protein sample at this temperature for only 2 min according to our protocol. Alternatively, physiological conditions can be achieved by imaging proteins in cells using tomography and sub-tomo averaging.”

Finally, I have tried the Falcon tube trick on our local Vitrobot. After a few minutes, a large water puddle appeared at the bottom of the tube around the ultrasonic water outlet. Can this puddle interfere or damage in any way the vapor system?
Response: This puddle will not damage the vapor system, but it may affect the supply of water vapor, so it is recommended that high-temperature grid preparation be completed as quickly as possible.

Reviewer #3:
Manuscript Summary:
Chang et al. present a method for the preparation of cryo-EM grids at high temperature. Overall, the method is clear and will be helpful to several researchers. Thanks for the positive comment.

Major Concerns:
My main comment is the title, as there is of course no such thing as high temperature EM, so the title should be changed to make clear that it is the (part of the) sample preparation before plunge freezing) which occurs at high temperature, not the data collection.

Thanks for the comment. The title has been revised from "**Preparation of Sample Grids for High Temperature Cryo-EM"** to "**Preparation of High Temperature Sample Grids for Cryo-EM".**

Minor Concerns:
Falcon tube -this is a brand so should have a capital (or use some generic name). However, at some point the exact brand, type, and size (maybe serial number) of tube should be put in the manuscript to help reproduction. Same with the filter papers used.

Corrected, and the detailed information for the tube and the filter paper are added to the Excel Table "JoVE_Materials". Thanks for the suggestions.

L38 "applications can also expand" --> "applications can also be expanded". Corrected. Thanks.
L91 "the cubic ice" --> "non-vitreous ice". Corrected. Thanks.
L105 "(e.g. 60 °C)" --> I guess this should be a different number? Not sure where it is. The text is (for example, 70 °C).
L112 "smoke"--> the correct work is not smoke which implies fire, I guess vapour is better? Corrected. Thanks.
L116 "the EPU" --> "EPU". Corrected. Thanks.
L136 "gradient ice" --> "ice gradient". Corrected. Thanks.
L156 describe A first, before B. Corrected. In addition, the description of C and D and also changed to be in order. However, these explanations have now been moved from the legend of Figure 5 to the section "REPRESENTATIVE RESULTS".
Table: "tedpella" --> "Ted Pella". Corrected. Thanks.

Reviewer #4:
Manuscript Summary:
This paper describes the procedure for freezing cryo-EM samples whose temperature kept high, such as 60 C degree.

Major Concerns:
The validity of this method is mainly depend on their JACS paper 2019.
Even in that paper, the sample temperature (=grid temperature) is not directly measured by other method, such as thermocouple.
So, the method for monitoring the sample temperature is desired.

If validity of this procedure is not an issue, the protocol is clearly written and the reviewer think the protocol is useful for cryo-electron microscopists who want to study molecules at higher temperatures.

Response: Thanks for raising the point of measuring the grid temperature directly by another method such as a thermocouple. However, there is a practical limitation in such measurement, since the grid needs to be plunge-frozen quickly (within seconds). The thermocouple needs to be built into the vitrification apparatus. This is an important point for future applications. Since Reviewers 1 and 2 also raise temperature-related comments, we have prepared a combined response:

Combined response related to the 70 °C temperature: Reviewers 1 and 2 are concerned that the estimation that the actual sample temperature is the average (70 °C) between the external water bath (80 °C) and the Vitrobot chamber temperature (60 °C) is not accurate, and that it should be very close to the latter. Reviewer 4 suggested the possibility of measuring the temperature directly by a thermocouple (which is not possible with the current apparatus). We agree that the real temperature may not be as high as 70 °C, but it should be higher than 60 °C because we use very short wait time (1-2s) and blot time (1-1.5s) at high temperature, and that our result supports higher temperature. Even though this only concerns a special situation (above 60 °C), it should not be deleted from the current manuscript since we are reporting the protocol for a paper already published (JACS 2019). Instead, we have included the concerns and suggestions in the DISCUSSION. In addition, we have changed the original step 9 of the protocol to become a NOTE instead a step.