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Abstract

The Biospecimen Science Group at the National Institute of Standards and Technology (NIST) Biorepository has been responsible for the curation, maintenance, and preparation of biological and environmental specimens at cryogenic temperatures since 2002. The team's expertise in cryohomogenization was leveraged to lead NIST's efforts in generating large batches of homogenous, fresh-frozen materials using a Palla VM-KT Vibrating mill. Most of these materials are part of the NIST SRM (Standard Reference Material) program, aiding NIST Technical Project Leads (TPLs), collaborators and stakeholders in verifying the accuracy of measurements, calibrating instruments, and/or supporting the development of new measurement methods. Collaborating institutions can also request cryohomogenization of matrices for their own specific use. This report outlines important considerations and required preparations prior to cryomilling and describes the procedure for operating the cryomill at the Cryogenic Reference Material Production Facility, located within the NIST Biorepository.

Keywords

biorepository; cryohomogenization; reference material.

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1. The NIST Biorepository

The National Institute of Standards and Technology (NIST) Biorepository, formally the Marine Environmental Specimen Bank, was established in 2002 at the Hollings Marine Laboratory (HML) in Charleston, South Carolina. The NIST Biorepository contains an archive of cryogenically preserved specimens, including marine sediments, fish tissues, bivalves, marine mammal tissues, and seabird eggs collected in collaboration with various external partner institutions for biomonitoring of environmental contaminants. Specimens are stored frozen in large capacity liquid nitrogen (LN_2) vapor-phase freezers (≤ -150 °C) to maintain their integrity for downstream analyses. These freezers are housed in a large ISO Class 5 and ISO Class 7 cleanroom laboratory to limit sample contamination during processing and archival activities [1].

NIST was able to leverage this large cleanroom space and access to bulk LN_2 to establish the NIST Cryogenic Reference Material Production Facility (CRMPF) within the Biorepository. A Palla VM-KT Vibrating Mill (KHD Humboldt Wedag, Cologne, Germany) was procured and connected to the NIST LN_2 bulk tank via the Biorepository's in-line LN_2 piping system [1]. Using the cryomill, large quantities of frozen material can be ground at the request of external stakeholders and for the production of NIST reference materials in collaboration with Technical Project Leads (TPLs).

NIST is charged with producing Standard Reference Materials® (SRMs), Reference Materials (RMs), and Research Grade Test Materials (RGTMs) to assist public, private, government, and academic sectors by providing higher-order calibration and validation materials to customers intending to produce metrologically traceable calibrants, to support method development and harmonization and to support measurand discovery and characterization [2]. While the majority of these products are generated in the Office of Reference Materials at NIST headquarters in Gaithersburg, MD, the unique capability provided by the CRMPF helps to support their efforts in serving stakeholders through RM processing and production.

2. Safety

When working in the NIST Biorepository and operating equipment therein, various aspects of safety must be considered. Because the Biorepository is located within the HML, a facility operated by the National Oceanic and Atmospheric Administration (NOAA), both NIST and NOAA safety policies must be followed. A Chemical Hygiene Plan and Emergency Preparedness Plan, both NOAA documents, were developed for the HML and are followed in the NIST Biorepository. In addition, a Job Hazard Analysis (NOAA) and Hazard Review (NIST) have also been completed and are regularly reviewed and maintained for the lab space and for the cryomilling procedure.

Full cleanroom coveralls, including hoods, surgical masks and shoe covers, or cleanroom shoes and socks, are worn at all times to limit contamination of the laboratory space [1]. Additional PPE (personal protective equipment) includes safety glasses and dust-free vinyl gloves, which are donned when performing work in the cleanroom.

Due to the large quantities of LN_2 utilized in the laboratory, the cleanrooms are equipped with oxygen monitors that alert occupants to oxygen limiting conditions (≤ 19.5%). Additionally, an E-Stop (emergency stop) system can be manually activated in the event of an emergency to stop the flow of LN_2 into the HML. Personal oxygen monitors are also required to be worn by anyone occupying the lab space, which is particularly important when operating the cryomill or working in and around the LN_2 freezers.

During cryomilling activities thermal insulated gloves are worn to protect users from cold temperature exposure and ear plugs may be worn for auditory comfort. Risk assessments are performed prior to beginning activities to evaluate potential biological hazards and/or exposure to aerosols or particulate matter generated during the milling process. An air particle counter can be used to help assess risk. If there are concerns regarding airborne particle exposure, face shields or N95 respirators can be worn while operating the mill after receiving appropriate approval. Safety datasheets (SDSs) are reviewed and maintained for cleaning/disinfecting agents and materials being milled, as available. To alleviate potential ergonomic stress due to standing, leaning, bending etc., staff rotate through the various positions at the cryomill during operation, as needed.

In 2023, the sound abatement enclosure surrounding the cryomill was replaced and updated to address various practical concerns related to operation, safety, and cleanup during and after milling. The updated enclosure is constructed of Lexan panels to allow for visual inspection of the equipment during operation. The doors on either end of the enclosure latch and are fitted with solenoid locking switches that automatically turn off the cryomill's vibrating motor when either door is opened. Additionally, an E-Stop button was installed on the Electrical Control Panel, which can be depressed in the event of emergency and will turn off the vibrating motor and stop the flow of LN_2 to the cryomill. Each of these safety features will be referenced in the following operation instructions.

3. Equipment Overview

Various pieces of equipment are utilized to successfully generate a fresh-frozen powder using the cryomill and each will be introduced and described in the following sections.

3.1 Palla VM-KT Vibrating Mill

The Palla VM-KT Vibrating Mill (referred to as the cryomill, herein) is designed to pulverize pieces of frozen material into a fine powder at ultra-low temperatures using LN_2 (Figure 1). The cryomill is connected to the NIST LN_2 bulk tank (Figure 2) via the Biorepository's in-line LN_2 piping system. The LN_2 and cryomill motor are powered by the Electrical Control Panel (Figure 3). When the motor is turned on the Grinding Chamber begins to oscillate, rotating a set of titanium rods located within the chamber that are responsible for pulverizing the material, which flows through the mill in the direction indicated in Figure 1. At any point during operation, the E-stop button (Figure 3, top right) located on the Electrical Control Panel can be depressed and the oscillation and flow of LN_2 will cease.

Figure 1. The Palla VM-KT Vibrating Mill

Figure 2. The NIST Biorepository LN_2 bulk tank

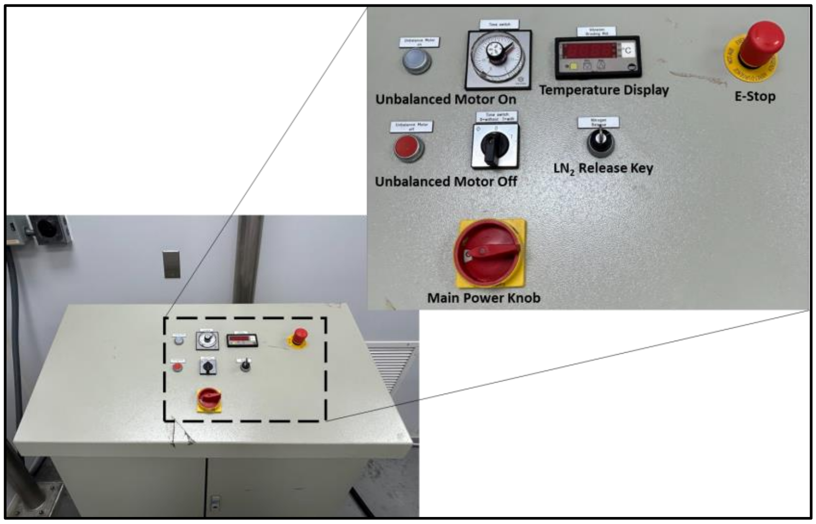


Figure 3. The Electrical Control Panel

The cryomill can be operated with or without a Vibrating Trough Conveyor, used to assist with the consistent delivery of material. Details regarding assembly and operation using this equipment will be discussed in Sections 4.1.3 and 5.2.2, respectively.

3.2 LN_2 vapor-phase freezers

Two large-mouth LN_2 vapor-phase freezers (MVE Biological Solutions, Ball Ground, GA, USA, Figure 4) are used to temporarily hold frozen material during the cryomilling process. Working Freezer #1 is located on the inlet-side of the cryomill, storing cold material to be fed into the mill, and Working Freezer #2 is located on the outlet-side of the mill and holds the ground material after it exits the mill.

Figure 4. LN_2 Working Freezers

3.3 Compressed Air Defragmentation Device (CADD)

To ensure frozen material can freely flow into the cryomill, pieces must be smaller than 2.5 cm in diameter. Larger pieces will become lodged in the inlet and inhibit consistent flow of material into the Grinding Chamber. The CADD (Figure 5) can be used to crush material into smaller pieces for ease of delivery. Compressed air is used to lift a large weight into the air and then drop the weight onto a specialized polytetrafluoroethylene (PTFE) container holding larger pieces of frozen material. The force of the weight crushes the material, generating smaller pieces which can pass through the cryomill [1]. Alternatively, frozen material may be smashed manually with a mallet, or thawed material can be chopped into smaller pieces and then refrozen prior to delivery to the cryomill.

Figure 5. The Compressed Air Defragmentation Device (CADD)

3.4 Heat Sealer

A Vertrod industrial impulse heat sealer (PAC Machinery, San Rafael, CA, USA) is also utilized in the CRMPF to create large, fluorinated ethylene propylene (FEP) bags for storing material following cryomilling (Figure 6). Large sheets of FEP are sealed along the open edges and are used to line wire baskets, which hold the fresh-frozen powder until it can be bottled. It is important the temperature of the heat sealer is set to at least 232 °C to create a strong seal.

Figure 6. The Vertrod industrial heat sealer

3.5 Labconco Steam Scrubber

The Labconco Steam Scrubber (Kansas City, MO, USA) is an industrial dish washer utilized for cleaning specific pieces of equipment used during the cryomill procedure (Figure 7). It should be moved to the CRMPF sink when needed, as it uses both deionized (DI) water and hot tap water during operation and drains through hoses placed securely in the sink to avoid spillage.

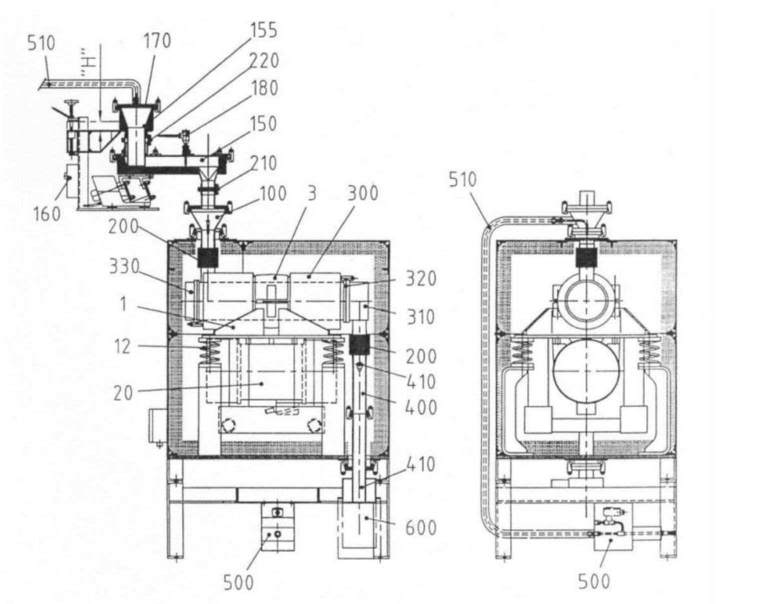
Figure 7. The Labconco Steam Scrubber

4. Preparing to cryomill

This section will outline the steps required for assembling the cryomill, both with and without the Vibrating Trough Conveyor, preparing the LN_2 vapor-phase freezers and preparing material for cryomilling.

4.1 Cryomill assembly

The cryomill must be assembled prior to use and most of the required parts are illustrated in the diagram provided by the manufacturer (Figure 8). Those parts that are not shown include securing clamps (to fasten metal components together), metal bands (to fasten PTFE bellows to metal components), titanium rods, a temperature probe, an exhaust hose/pipe, an LN_2 line connected to the Biorepository's in-line LN_2 piping system, and a thyristor control mounted to the safety enclosure. Each of these pieces of equipment will be discussed in the following sections.



Part Number	Part Name
1	Vibrating Body Mount
3	Clamping Yolk
12	Pressure Springs
20	Vibration Motor
100	Inlet Socket
150	Vibrating Trough Conveyor
155	Filling Socket
160	Thyristor Control
170	Mount Cover
180	Temperature Pick-Up
200	Inlet/Outlet Bellow
210	Mount Bellow
220	Vibrating Trough Conveyor Inlet Bellow
300	Material Grinding Chamber
310	Material Chamber Outlet Head
320	Material Chamber and Material Chamber Outlet Junction
330	Material Chamber End Cap
400	Outlet Tube
410	Temperature Pick-Up
500	Liquid Nitrogen Valve Box
510	Flexible Liquid Nitrogen Supply Line
600	Collection Basin

Figure 8. Detailed schematic of the cryomill and table of parts

4.1.1 Outlet assembly

1. Open the doors on both sides of the cryomill enclosure (Figure 9).

Figure 9. Cryomill with safety enclosure doors open

2. Attach the Material Chamber End Cap (330) to the Material Grinding Chamber (300) using six securing clamps. Tighten the nuts on the securing clamps using a wrench, ensuring that the six securing clamps are in a “star pattern” (Figure 10).

Figure 10. Material Chamber End Cap

3. Place the clean titanium rods inside the clean material grinding chamber (300, Figure 11).

Figure 11. Titanium rods in the Material Grinding Chamber

4. Attach the Material Chamber Outlet Head (310) to the Material Grinding Chamber (300) using the provided securing clamps. Tighten the nuts on the securing clamps with a wrench, ensuring that the six securing clamps are in a “star pattern” to ensure a tight seal (Figure 12).

Figure 12. Material Chamber Outlet Head

5. Insert the Outlet Tube (400) through the bottom of the cryomill body and secure using four securing clamps. Tighten clamps with a wrench and ensure the vent is positioned toward the exhaust hose (Figure 13).

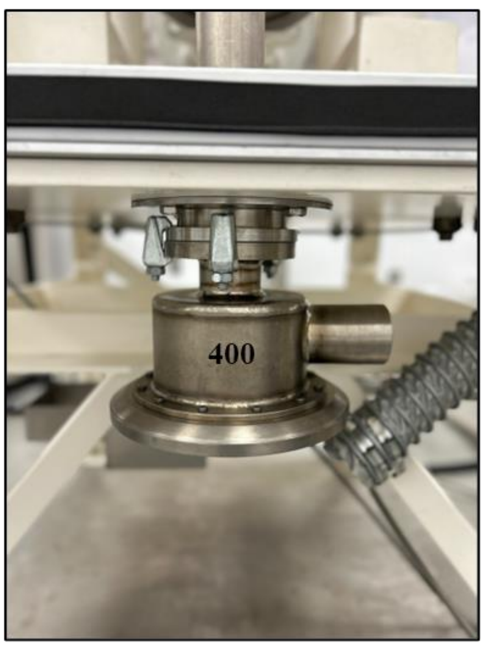


Figure 13. Outlet Tube

6. Secure the Temperature Pick-Up (410) to the Outlet Tube (400) using two securing clamps and tighten with a wrench (Figure 14).

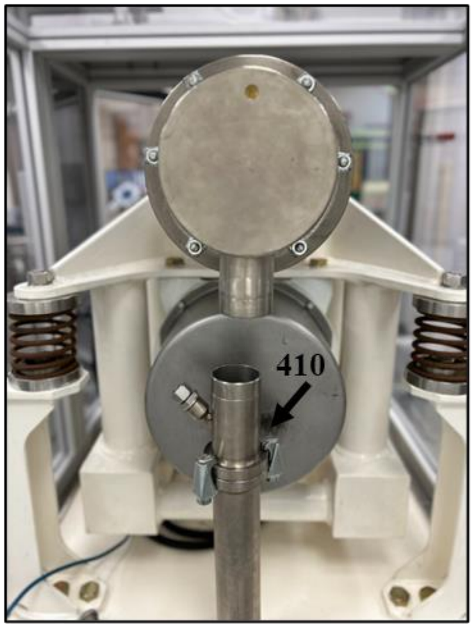


Figure 14. Temperature Pick-Up secured to the Outlet Tube

7. After ensuring the nut collar is in place, insert the temperature probe into the slot on the Temperature Pick-Up (410) and tighten the nut using a wrench (Figure 15). This probe will read the temperature at the outlet and will be displayed on the Electrical Control Panel.

Figure 15. Temperature probe (indicated by the arrow) secured to the Temperature Pick-Up

8. Place one metal band onto the lower portion of the Outlet Bellow (200) and a second metal band onto the upper portion of the Outlet Bellow. Ensure that the metal hinges of the lower and upper bands are facing away from the body of the bellow (Figure 16).

Figure 16. Metal bands (indicated by the arrows) positioned on the bellow

9. Position the Outlet Bellow between the Material Chamber Outlet Head (310) and the Temperature Pick-Up (410). The bellow may need to be compressed to position it properly. Expand the bellow until it overlaps the grooves on both the Material Chamber Outlet Head (310) and the Temperature Pick-Up (410). Tighten the screws on the metal bands using a flat head screwdriver to secure the bellow in place (Figure 17).

Figure 17. Outlet Bellow

9. Secure the exhaust hose to the vent on the Outlet Tube (400) using the two metal bands attached to the exhaust hose and tighten with a flat head screwdriver (Figure 18). The exhaust hose should be secured to the exhaust pipe installed in the cleanroom floor.

Figure 18. Exhaust hose (indicated by the arrow) connected to the Outlet Tube

4.1.2 Inlet assembly without the Vibrating Trough Conveyor

1. Set the Inlet Socket (100) on the inlet at the top of the safety enclosure, ensuring the LN_2 arm is positioned toward the existing LN_2 line, which should be connected to the Biorepository LN_2 supply piping. Secure the Inlet Socket to the inlet using four securing clamps and tighten with a wrench (Figure 19).

Figure 19. Inlet Socket

2. Place one metal band onto the lower portion of the Inlet Bellow (200) and a second metal band onto the upper portion of the Inlet Bellow. Ensure that the metal hinges of the lower and upper bands are facing away from the body of the bellow (Figure 16). Position the Inlet Bellow between the Material Chamber End Cap (330) and the Inlet Socket (100). The bellow may need to be compressed to position it properly. Expand the bellow until it overlaps the grooves on both the Material Chamber End Cap (330) and the Inlet Socket (100). Tighten the screws on the metal bands with a flat head screwdriver to secure the bellow in place (Figure 20).

Figure 20. Inlet bellow

3. Attach the Flexible LN_2 Supply Line (510) to the LN_2 arm on the Inlet Socket (100) and tighten the nuts using two wrenches (Figure 21). The other end of the Flexible LN_2 Supply Line remains affixed to the LN_2 Valve Box (500). The Biorepository's in-line piping system remains attached to the LN_2 Valve Box.

Figure 21. Flexible LN_2 supply line (indicated by the arrow)

4.1.3 Inlet assembly with the Vibrating Trough Conveyor

1. Place the inlet socket cover on top of the Inlet Socket (100) and secure four securing clamps using a wrench (Figure 22).

Figure 22. Inlet socket cover (indicated by the arrow)

2. Secure the Vibrating Trough Conveyor support stand to the mount platform on the outside of the safety enclosure using the four sets of nuts and bolts provided and tighten with a wrench (Figure 23).

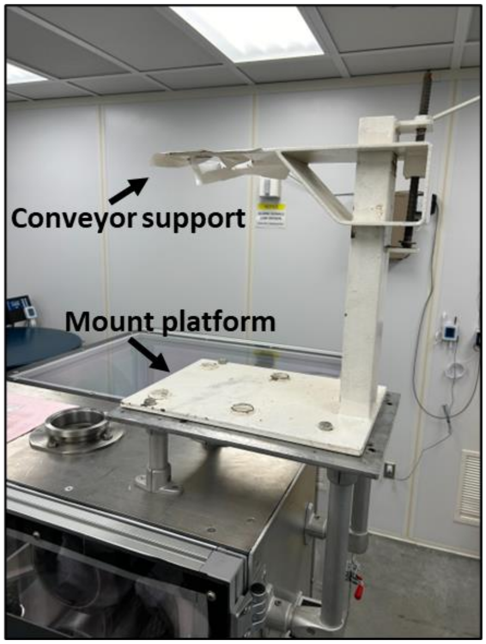


Figure 23. Vibrating Trough Conveyor support stand

3. Secure the vibrating trough conveyor lid to the vibrating trough conveyor base using ten securing clamps and tighten with a wrench. The securing clamps attach to the specific locations indicated (Figure 24).

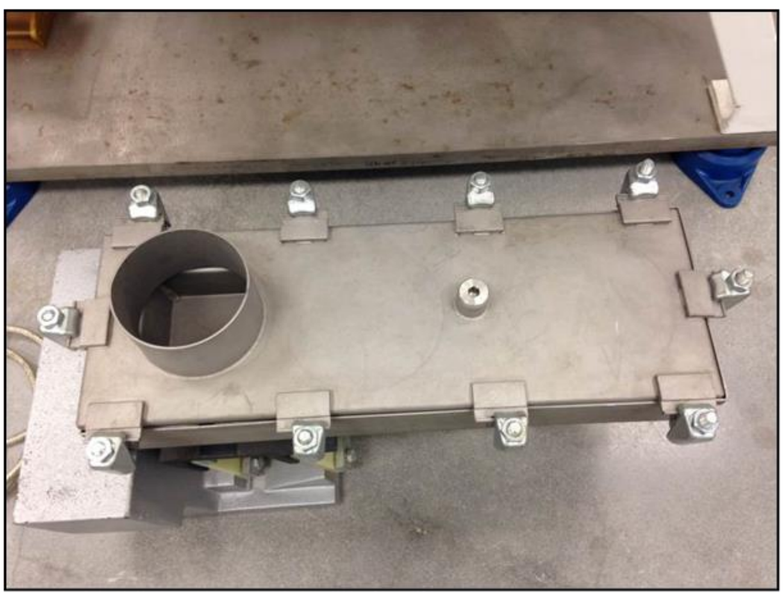


Figure 24. Vibrating Trough Conveyor with lid attached

4. Place the Vibrating Trough Conveyor (150) onto the support stand, positioning the four rubber feet into the associated grooves on the stand. **Caution: The conveyor is extremely heavy!**

5. Place one metal band onto the lower portion of the Mount Bellow (210) and a second metal band onto the upper portion of the Mount Bellow. Ensure that the metal hinges of the lower and upper bands are facing away from the body of the bellow (Figure 16). Position the Mount Bellow between the Vibrating Trough Conveyor (150) and the inlet socket cover. The bellow may need to be compressed to position it properly. Tighten the screws on the metal bands with a flat head screwdriver to secure the bellow in place (Figure 25).

Figure 25. Vibrating Trough Conveyor on the support stand

6. Place one metal band onto the lower portion of the Vibrating Trough Conveyor Inlet Bellow (220) and a second metal band onto the upper portion of the Vibrating Trough Conveyor Inlet Bellow. Ensure that the metal hinges of the lower and upper bands are facing away from the body of the bellow (Figure 16). Position the Vibrating Trough Conveyor Inlet Bellow onto the opening of the Vibrating Trough Conveyor (150).

7. Position the Filling Socket (155) through the hole in the support stand and align the opening with the opening of the Vibrating Trough Conveyor Inlet Bellow (220). Ensure there is a gap between the Filling Socket and the Vibrating Trough Conveyor (150) to prevent metal on metal contact. If no gap is present, adjust the height of the top portion of the support stand to create a gap.

8. Expand the Vibrating Trough Conveyor Inlet Bellow (220) sufficiently to connect the Vibrating Trough Conveyor (150) and the Filling Socket (155). Tighten the screws on the metal bands to secure the bellow in place (Figure 26).

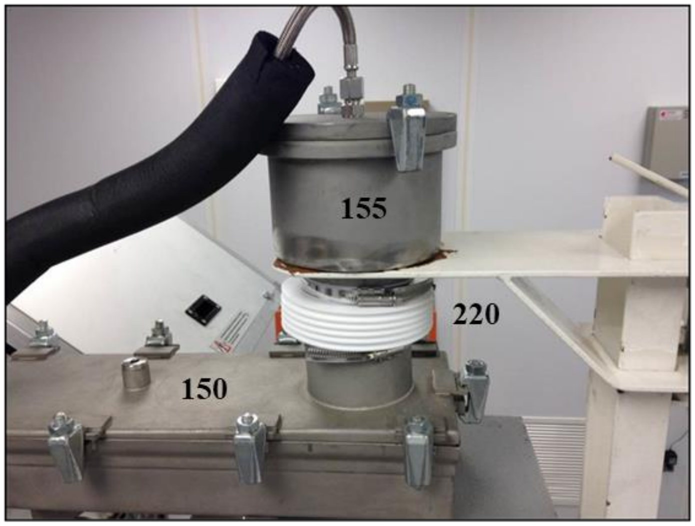


Figure 26. Filling Socket

9. Secure the Mount Cover (170) to the Filling Socket (155) using two to three securing clamps and tighten with a wrench (Figure 26).

10. Attach the Flexible LN_2 Supply Line (510) to the Mount Cover (170) and tighten the nut using a wrench. The other end of the Flexible LN_2 Supply Line remains affixed to the LN_2 Valve Box (500). The Biorepository's in-line piping system remains attached to the LN_2 Valve Box.

11. Plug the electrical cord attached to the Vibrating Trough Conveyor (150) into the Thyristor Control (160) mounted on the safety enclosure. The thyristor is used to adjust the vibrational speed of the trough conveyor to control material delivery from the Vibrating Trough Conveyor to the Material Grinding Chamber (300) (Figure 27).

Figure 27. Thyristor control

4.2 Preparing the LN_2 vapor-phase freezers

Freezers and equipment should be pre-cooled prior to beginning the cryohomogenization process. This is important to ensure that all equipment that will come in contact with the frozen material is cool enough to prevent material from thawing or sticking. Freezers should be plugged into the wall outlets and connected to the Biorepository LN_2 piping system. Round platforms or racks are typically stacked in the freezers to raise the height of the work surface off the bottom of the freezers (Figure 28). This prevents unnecessary ergonomic stress when material is being either removed from or added to the freezers, but also keeps the material low enough in the freezer to ensure it remains frozen. When the LN_2 valve is opened to start filling the freezer, care should be taken to ensure the liquid level stays below the work surface to prevent potential injury or contamination of the material.

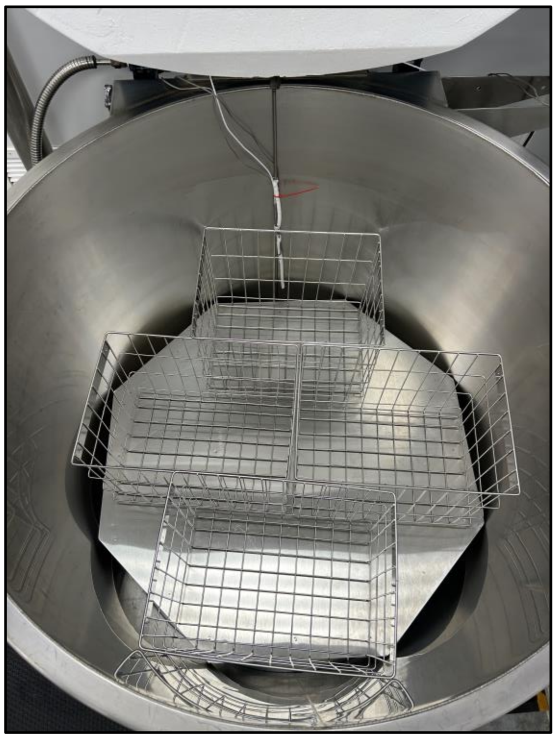


Figure 28. LN_2 vapor-phase freezer

Receptacles are prepared prior to cryomilling to catch milled material at the outlet and can be any container compatible with the material being milled and that will also withstand cryogenic temperatures. Typically, large FEP bags are made by cutting a sheet of FEP from the stock roll (approximately 1 to 1.5 m long), folding it in half, and sealing the open long edges using the heat sealer (See Section 3.4). These bags are used to line wire baskets, which will store homogenate after it passes through the cryomill. The baskets are usually double-lined with FEP bags to prevent any potential breakage or spillage, which would result in loss of material. The FEP-lined baskets are then placed in Working Freezer #2 to cool. Depending on what is being milled, the material exiting the cryomill may not need to remain frozen. However, if the material will be reintroduced to the cryomill for another round of milling, it should be pre-cooled prior to introduction.

Clean stainless steel buckets and scoops are also placed in the freezers to precool. These are typically used for scooping material into and collecting material from the cryomill during operation. The scoops are also used to distribute material between wire baskets following milling to promote further blending.

4.3 Preparing the material

Material chosen for cryogenic homogenization should be smaller than 2.5 cm in diameter to fit through the Inlet Socket (100). If material is too large it may clog the cryomill. Large pieces can be crushed using the CADD or a mallet or can be cut into smaller pieces prior to freezing. Preparation of the material largely depends on the type of material being milled, the downstream application, and the preference of the principal investigator/TPL.

5. Cryomill operation and quality assessment

This section will outline the steps for precooling and safety operating and cryomill at the CRMPF.

5.1 Pre-cooling

It is important to cool the cryomill prior to introducing material to prevent blockage or seizing of the mill components.

1. Ensure all securing clamps and metal bands on the cryomill are tightened and that the Flexible LN_2 Supply Line is securely attached to the Inlet Socket (100) or the Mount Cover (170).

2. Ensure that the knob on the LN_2 Valve Box (500) is turned all the way to the right. This valve controls the flow of LN_2 to the cryomill and is in the "CLOSED" position when completely to the right (Figure 29).

Figure 29. LN_2 Valve Box

3. Close and latch the two doors on either end of the safety enclosure. Each door is equipped with solenoid locking switches. Opening either door during operation will cause the cryomill to stop vibrating and stop the flow of LN_2 (Figure 30).

Figure 30. Solenoid locking switch

4. Place a collection container under the Outlet Tube (400) to catch any residual LN_2 that may flow out of the chamber during cooling.

5. Turn the buck boost converter for the cryomill "ON" at the wall by flipping the lever up (Figure 31) and turn the large red power knob clockwise so the "|" is visible in the knob view window. It will be necessary to wait several seconds for the control panel to run machine diagnostics (Figure 3). The lights will illuminate and display the temperature at the Temperature Pick-Up (410).

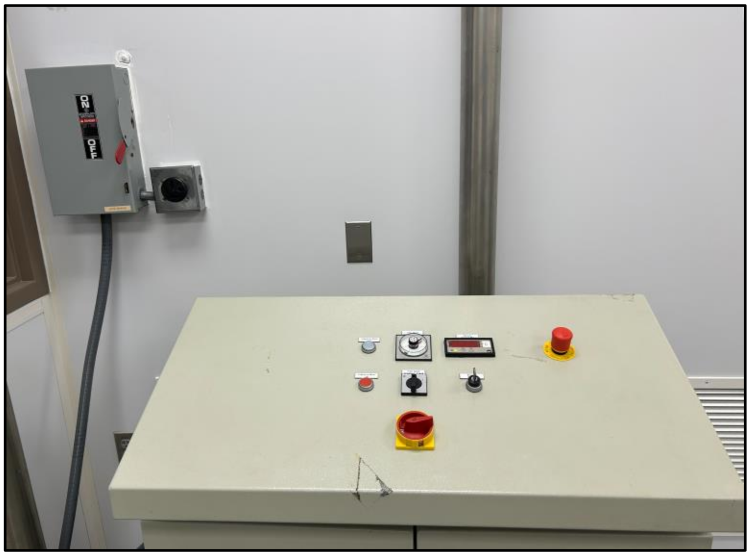


Figure 31. Buck boost converter

6. Open the LN_2 wall valve connected to the house supply line (Figure 32).

Figure 32. LN_2 wall valve

7. To start the flow of LN_2 to the cryomill, turn the nitrogen release key on the Electrical Control Panel from "0" to "|" (Figure 3) and turn the knob on the LN_2 Valve Box (500) at least five rotations counterclockwise (Figure 29). Liquid nitrogen should begin to flow from the house supply line to the LN_2 Valve Box (500), through the Flexible LN_2 Supply Line (510), then through the Material Grinding Chamber (300). If LN_2 does not begin to flow, make sure that the

house LN_2 wall valve is open and the flow rate valve on the LN_2 Valve Box (500) is opened. A preprogrammed temperature for LN_2 (−190 °C) has been set using the Electrical Control Panel temperature settings. To program a different set temperature, see the Palla Cryomill technical documentation for instruction.

8. Allow at least one hour for the cryomill to reach the desired pre-set temperature. Upon reaching the desired temperature, the LN_2 Valve Box will begin "clicking." This clicking is a regulator in the LN_2 Valve Box opening/closing to regulate the flow of LN_2 into the cryomill and maintain the set temperature.

5.2 Cryomilling

Typically, four people are needed to operate the cryomill and successfully mill frozen material: 1.) one loading material from Working Freezer #1 into stainless steel buckets, 2.) one standing atop the safety ladder and feeding material into the cryomill, 3.) one at the outlet catching milled material in a stainless steel bucket, and 4.) one receiving full buckets and emptying them into receptacles in Working Freezer #2 (Figure 33).

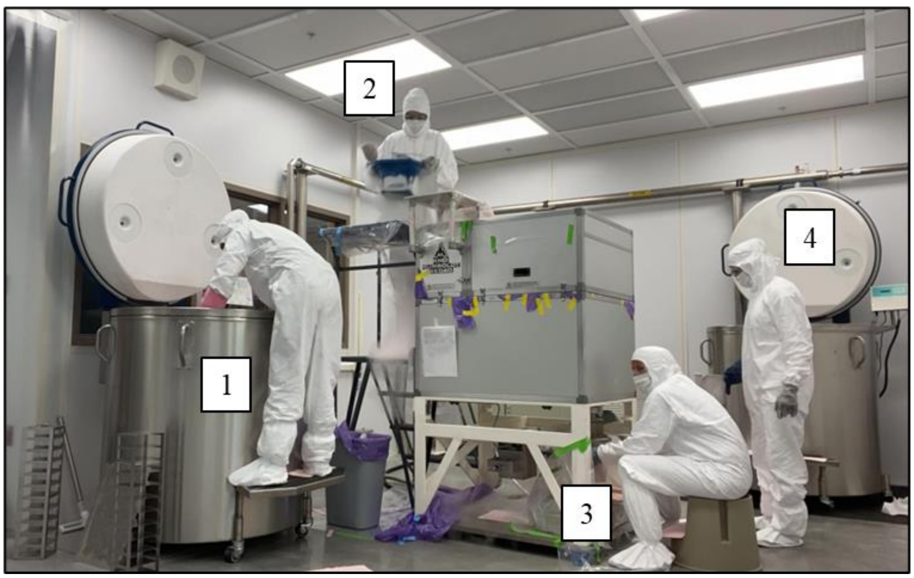


Figure 33. Positions of People Operating the Cryomill

Vibration of the Material Grinding Chamber is controlled using the white button labeled "Unbalance Motor On" on the Electrical Control Panel. This starts the Vibration Motor (20) and the titanium rods will begin rotating. When the Material Grinding Chamber is empty, operation of the mill is loud, so earplugs may be used. If the Vibration Motor does not start, ensure the doors on each end of the safety enclosure are shut and the E-Stop is not engaged. To stop the Vibration Motor at any point, depress the red "Unbalance Motor Off" button on the Electrical Control Panel. In an emergency, depress the E-Stop button to stop the Vibration Motor and the flow of LN_2 to the cryomill.

The introduction of material and the vibrating action of the mill can warm the Material Grinding Chamber, so the temperature should be monitored during operation. During the milling procedure, the temperature at the Temperature Pick-Up (410) may increase as shown on the Electrical Control Panel display. If the mill becomes too warm (> −80 °C), stop feeding material into the chamber and stop the Vibration Motor to allow LN_2 to cool the mill. If using the Vibrating Trough Conveyor, the Mount Cover (170) should be placed back on the Filling Socket (155) and the LN_2 flow can be adjusted to cool the cryomill. If the Vibrating Trough Conveyor is not in use, the LN_2 flow rate can be controlled during cryomilling by adjusting the black knob on the LN_2 control valve box (500). Increasing the flow rate during operation can also help cool the mill.

5.2.1 Cryomilling without the Vibrating Trough Conveyor

This section outlines the protocol for actively cryomilling frozen material without the Vibrating Trough Conveyor. Ensure the equipment is properly assembled before beginning (Section 4.1.1 and 4.1.2).

1. Once the cryomill has reached the desired temperature and each team member is in place, depress the “Unbalanced Motor On” button to start the Vibration Motor (20).

2. The person at Working Freezer #1 will load a bucket of frozen material into a precooled stainless steel bucket using a scoop and pass the bucket to the person on the safety ladder.

3. The person on the safety ladder will slowly feed the material into the Inlet Socket by either pouring or using a cooled scoop. The material will travel through the Inlet Bellow (200), into the Material Grinding Chamber (300), through the Outlet Bellow (300) and Outlet Tube (400) and into a precooled collection bucket held in place by the person at the outlet. This process is repeated until all material is milled (Figure 34).

Figure 34. Cryomilling without the Vibrating Trough Conveyor

4. As collection buckets are filled, the person catching the material at the outlet will pass the full bucket to the person at Working Freezer #2 and that person will simultaneously hand an empty pre-cooled bucket to the person catching at the outlet. This is done as quickly as possible to minimize loss of material at the outlet.

5. The person at Working Freezer #2 will empty the full bucket of material into a receptacle in the freezer, alternating receptacles to facilitate additional blending of the material.

6. Once cryomilling is complete, depress the “Unbalanced Motor Off” button on the Electrical Control Panel, turn the black knob on the LN_2 Valve Box (500) all the way to the right, turn the nitrogen release key on the Electrical Control Panel from “|” to “0”, and turn the large red knob counter-clockwise so the “0” is visible in the knob view window.

7. Turn the buck boost converter for the cryomill “OFF” at the wall by pulling the lever down and close the LN_2 wall valve connected to the LN_2 Supply Line (510).

5.2.2 Cryomilling with the Vibrating Trough Conveyor

This section outlines the protocol for actively cryomilling frozen material using the Vibrating Trough Conveyor. Ensure the equipment is properly assembled before beginning (Section 4.1.1 and 4.1.3).

1. Once the cryomill has reached the desired temperature and each team member is in place, stop the flow of LN_2 using the LN_2 Box Valve (500), turning the dial all the way to the right.

2. Depress the “Unbalanced Motor On” button to start the Vibration Motor (20).

3. Flip the green switch on the Thyristor Control (160) to power the conveyor and turn the black knob on the thyristor control to the desired vibration speed (Figure 27).

4. The person at Working Freezer #1 will load a bucket of frozen material into a precooled stainless steel bucket using a scoop and pass the bucket to the person on the safety ladder.

5. The person on the safety ladder will remove the Mount Cover (170) and slowly feed the material into the Filling Socket (155) by either pouring or using a cooled scoop. The material will travel through the Vibrating Trough Conveyor (150), Inlet Socket (100) and Inlet Bellow (200), into the Material Grinding Chamber (300), through the Outlet Bellow (300) and Outlet Tube (400) and into a precooled collection bucket held in place by the person at the outlet. This process is repeated until all the material is milled (Figure 35).

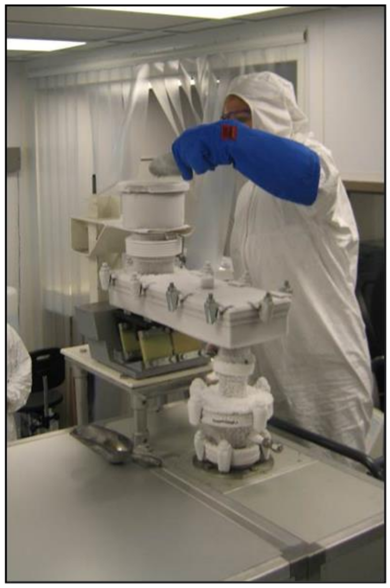


Figure 35. Cryomilling with the Vibrating Trough Conveyor

6. As collection buckets are filled, the person catching the material at the outlet will pass the full bucket to the person at Working Freezer #2 and that person will simultaneously hand an empty pre-cooled bucket to the person catching at the outlet. This is done as quickly as possible to minimize loss of material at the outlet.

7. The person at Working Freezer #2 will empty the full bucket of material into a receptacle in the freezer, alternating receptacles to facilitate additional blending of the material.

8. If the mill warms to > −80 °C, stop adding material to the conveyor, turn the black knob on the Thyristor Control (160) all the way to the right, and press the "Unbalanced Motor Off" button on the Electrical Control Panel. Attach the Mount Cover (170) to the Filling Socket (155) using at least two securing clamps, tighten with a wrench, and increase the flow of LN_2 using the LN_2 Box Valve (500) to cool. Resume at Step 1 in this section to continue milling.

9. Once cryomilling is complete, depress the "Unbalanced Motor Off" button on the Electrical Control Panel, turn the black knob on the LN_2 Valve Box (500) all the way to the right, turn the nitrogen release key on the Electrical Control Panel from "|" to "0", and turn the large red knob counter-clockwise so the "0" is visible in the knob view window.

10. Turn the buck boost converter for the cryomill "OFF" at the wall and close the LN_2 wall valve connected to the LN_2 Supply Line (510).

5.3 Particle size determination as a process indicator

To assess the effectiveness of the cryohomogenization procedure, particle size analysis is conducted using a Malvern Mastersizer 3000 (Malvern Panalytical, Malvern, United Kingdom). The instrument uses laser diffraction to determine the size distribution of particles between 0.01 to 3500 µm in diameter, which is used to make assessments of homogeneity of the milled material to help determine how many rounds of cryomilling should be conducted.

While material is being milled, a team member collects about 2 to 3 g from the Outlet Tube (400) using a small container (e.g., a perfluoroalkoxy (PFA) jar). Using established technical procedures, the particle size results from each round of cryomilling are compared to determine if the material is continuing to decrease in average size and if the size distribution of the particles is decreasing (i.e., the material is becoming more homogenous with respect to size). Once the size of the milled material is deemed acceptable, the cryomilling procedure is complete.

6. Cryomill disassembly and cleaning

The cryomill must be cleaned at the end of each day following use, particularly if the material is biological. Prior to dismantling, the mill must be allowed to partially thaw to remove the securing clamps and safely handle the equipment. Different pieces of equipment are cleaned in different ways based on their composition. Most parts of the cryomill must only be washed by hand with dish soap and water. However, the titanium rods, stainless steel buckets and scoops can be cleaned in the Labconco Steam Scrubber (see Section 6.2). After metal parts have been washed, they are sprayed with 70% isopropyl alcohol spray, such as Deconahol® (Veltek Associates Inc., Malvern, PA), and wiped using a lint-free wipe, such as a Technicloth® Cleanroom Wiper (Texwipe, Kernersville, NC), before being stored. Additionally, after a cryomilling activity is complete, the CRMPF should be thoroughly cleaned according to Biorepository Cleanroom Maintenance procedures [1].

Of note, the Lexan safety enclosure should not be cleaned with anything other than warm water and dish soap. The use of solvents, such as Deconahol®, on the Lexan panels will cloud the surface, obscuring a clear view of the equipment during operation.

6.1 Disassembling the cryomill

Once power to the cryomill has been turned off, the mill can be safely disassembled.

1. Wipe any bulk material sitting on the top of the safety enclosure or around the cryomill using a cloth and warm water.

2. Open the doors of the safety enclosure and loosen the metal bands securing the Inlet/Outlet Bellows (200) using a flat head screwdriver. Work the bellows loose from the inlet and outlet and hand-wash thoroughly with warm water and dish soap. Rinse with water and set aside to dry.

3. Using a wrench, loosen the securing clamps on the Material Chamber Outlet Head (310) and remove.

4. Loosen the nut for the temperature probe and wipe the probe clean, ensuring the nut collar is stored in a safe location.

5. Loosen the securing clamps on the Temperature Pick-Up (410) and remove.

6. Loosen the vent from the Outlet Tube (400) using a flat head screwdriver and loosen the securing clamps on the Outlet Tube using a wrench.

7. Remove the titanium rods carefully and discard any remaining material that may be in the Material Grinding Chamber (300). The rods should be rinsed in the sink with warm water before being loaded into the Steam Scrubber.

8. Loosen the securing clamps on the Material Chamber End Cap (33) and remove.

9. Disconnect the Flexible LN_2 Supply Line (510) from the Inlet Socket (100) (if used) and disassemble the remainder of the cryomill inlet set up. If using the Vibrating Trough Conveyor, disassemble the conveyor and support in the reverse order of assembly. The bellows should be hand-wash thoroughly with warm water and dish soap, then rinsed and set aside to dry.

10. The Material Grinding Chamber (300) can then be left to thaw. It may be beneficial to place absorbent pads or wipes at the ends of the chamber to minimize spillage. Once the chamber is thawed, warm water and dish soap are used for cleaning and the chamber is then wiped with Deconahol® and a lint-free wipe.

11. All securing clamps, metal bands, nuts and bolts should also be cleaned with warm water and dish soap and be allowed to air dry prior to storage.

6.2 Operating the Labconco Steam Scrubber

Non-insulated pieces of equipment used during the cryomilling procedure (e.g., titanium rods, stainless steel buckets and scoops) are cleaned in the steam scrubber. Insulated pieces of equipment should only be cleaned by hand in the sink, using warm water and dish soap. Once dry, all metal equipment should be cleaned with Deconahol® and a lint-free wipe, as previously described.

1. Move the steam scrubber to the sink located in the CRMPF.

2. Attach the distilled (DI) water line on the steam scrubber to the sink DI water faucet and attach the tap water line on the steam scrubber to the sink tap water faucet (Figure 36).

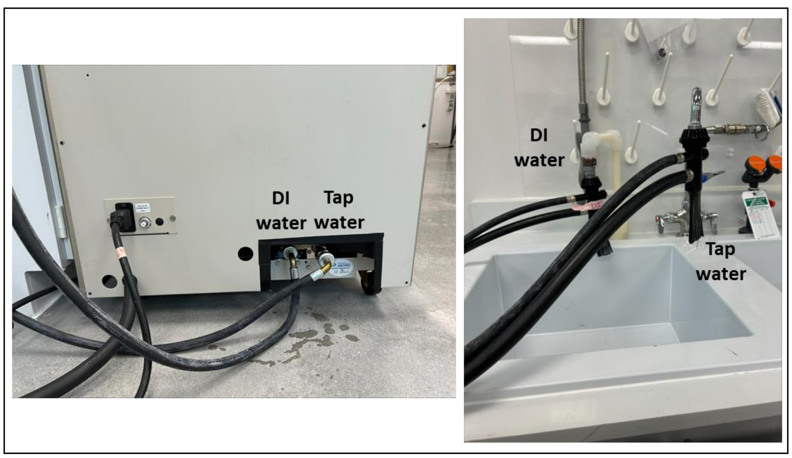


Figure 36. DI and tap water connections

3. Place all drain lines inside the sink basin then plug the steam scrubber electrical cord into an electrical outlet.

4. Load the non-insulated pieces of equipment into the steam scrubber. Add LabSolutions detergent (Labconco, Kansas City, MO) to the detergent cup, and LabSolutions neutralizing acid rinse to the neutralizing solutions port (Figure 37). Close the detergent cup and neutralizing acid rinse port.

Figure 37. Steam scrubber door

5. Close and latch the steam scrubber door securely. Items may need to be rearranged to allow the door to close.

6. Choose the Science Plus program on the steam scrubber screen (Figure 38). Turn on the DI and hot tap water from the sink and press the “Run/Cancel” button to start the wash cycle.

Figure 38. Steam scrubber screen

7. When the cycle is complete, turn off the DI and tap water at the sink, open the door to the steam scrubber and unload equipment. Unplug the steam scrubber and store.

8. Wipe each piece of washed equipment with Deconahol® and a lint-free wipe and ensure it is dry before storing.

7. Summary

The Palla VM-KT Vibrating mill at the NIST CRMPF is used to generate large batches of fresh-frozen homogenates for use in reference material production and other collaborative projects. Ensuring appropriate preparation, safe operation and thorough post-operational cleaning of the mill will allow for successful production of high-quality materials.

References

[1] Pugh, R. , Ellisor, M. , Moors, A. , Porter, B. and Becker, P. (2007), Marine Environmental Specimen Bank: Clean Room and Specimen Bank Protocols, NIST Interagency/Internal Report (NISTIR), National Institute of Standards and Technology, Gaithersburg, MD, [online], https://tsapps.nist.gov/publication/get_pdf.cfm?pub_id=832165 (Accessed January 30, 2024)

[2] Beauchamp, C. , Camara, J. , Carney, J. , Choquette, S. , Cole, K. , DeRose, P. , Duewer, D., Epstein, M. , Kline, M. , Lippa, K. , Lucon, E. , Molloy, J. , Nelson, M. , Phinney, K. , Polakoski, M. , Possolo, A. , Sander, L. , Schiel, J. , Sharpless, K. , Winchester, M. and Windover, D. (2021), Metrological Tools for the Reference Materials and Reference Instruments of the NIST Material Measurement Laboratory, Special Publication (NIST SP), National Institute of Standards and Technology, Gaithersburg, MD, [online], https://doi.org/10.6028/NIST.SP.260-136-2021, https://tsapps.nist.gov/publication/get_pdf.cfm?pub_id=933027 (Accessed January 30, 2024)

Change Log

In May 2024, the following changes were made to the report:

- Introduction – More specific details relevant to reference material production in the NIST Biorepository have been added.
- Safety – Details regarding improvements made to the sound abatement enclosure and electrical control panel have been added. Additional information regarding new safety procedures related to oxygen depletion have also been updated.
- Sections 3-6 – New images have been used to accurately depict changes made to the sound abatement enclosure and to clarify operational procedures/practices.