4

Lyophilization of Proteins

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Summary

This chapter describes the methods that can be applied to successfully freeze-dry proteins. Laboratory applications are given at small scale, typified by the purification of a protein intermediate as part of the analytical characterization of a protein, and at intermediate scale, as illustrated by the pilot development of a lyophilized protein reference material such as for use in bioassay or immunoassay. Advice on common problems with freeze-drying of proteins is also given.

Key Words: Lyophilization; freeze-drying; thermal analysis; freeze-drying microscopy; stabilizer; glass transition temperature; formulation.

1. Introduction

Lyophilization, the removal of the majority of the water in a sample under conditions of low temperature and vacuum, is a widely used technique in the areas of protein purification, protein reagent preparation, and the manufacture of protein biomolecules for therapeutic and diagnostic applications. It can be performed on a sample in order to:

1. Increase stability by minimizing the available water residual in the sample, which facilitates degradative processes such as peptide bond hydrolysis, deamidation, and the like.
2. Reduce the volume of the sample so facilitating a further processing step in a purification process.
3. Store a protein sample in a convenient format requiring less storage space and permitting storage under ambient conditions.
4. Simplify transport of the protein material with minimal loss of activity between geographic locations—obviating the need for cold chain shippage.
5. Remove volatile buffer/solvent components facilitating reformulation.

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Proteins were some of the earliest biomolecules to be lyophilized—indeed the need for bulk preparations of plasma proteins in the second World War was a driving force in the development of lyophilization as an industrial process. Proteins are highly diverse and whereas a single-chain protein with a highly ordered tertiary structure may freeze-dry with little difficulty, a multimeric protein with multiple domains and hydrophobic proteins (such as a membrane receptor complex) will pose a far more demanding challenge to achieve successful lyophilization.

1.1. Theory of Freeze-Drying

The theoretical basis of freeze-drying is discussed elsewhere in this volume (*see* Chapter 2) and so space will not be given here to reiterate. Freeze-drying is split into three process stages, freezing, primary drying at lower temperatures when most of the water is removed, and secondary drying at ambient or higher temperatures to minimize the final unbound water content. To maintain their biological activity during freeze-drying, conservation of the higher order structure of proteins is vital. Proteins found in aqueous solution are hydrated and so removal of the water shell during lyophilization will have serious thermodynamic implications for the maintenance of their correctly folded structure when that water shell is in part or wholly removed by desiccation. The optimum residual water content for proteins should be determined on a case-by-case basis, but several publications have illustrated that an optimum water content is required to maintain the activity of proteins under study and that drying beyond that level may also be detrimental ***(1)***, whereas high water content may well result in excessive degradative changes on storage ***(2)***.

1.2. Impact of Freezing on Proteins

During the freezing process, water crystallizes to ice and the excluded excipient salts concentrate to local concentrations far higher than those in the original liquid state. This may in itself have implications for the stability of the protein(s) present, which may be destabilized and denature because of the change in ionic strength. In addition, if buffer salts, such as mixed phosphate buffer are present, the selective crystallization or precipitation of one of these salts at a higher temperature to the other may result in localized pH shifts, which again may induce denaturation of the proteins ***(3)***. Such denaturation may lead to exposure of normally buried residues and an increase in aggregation, some of which may be irreversible on reconstitution. Other proteins may be satisfactorily immobilized in the lyophilized state but undergo changes that result in aggregation on reconstitution ***(4)***. Membrane-bound proteins may pose special problems when undergoing lyophilization, cell membranes are particularly prone to disruption during the dehydration process and so membrane-associated proteins will also be at risk.

In order to successfully lyophilize proteins to yield a product of cosmetically acceptable appearance, all of the water in the sample must first be fully immobilized, either as ice crystals or bound with the nonvolatile and amorphous components of the sample, in the superviscous state known as a glass. The temperature to which samples must be cooled to ensure this amorphous state is discussed elsewhere (*see* Chapters 2 and 3). Practically, the glass transition (Tg′) or collapse temperature (Tc) for the formulation should be determined and temperature probes used in the samples to ensure that this temperature is not exceeded in the product during drying.

Finally, it must not be assumed that, although slower, degradative reactions will be entirely absent in a reduced water environment, and they should be considered and assessed during short or longer term storage in the lyophilized states ***(5)***. Saccharides are often chosen as stabilizers for lyophilization processes, but covalent modification of proteins in the presence of reducing sugars is well known, especially at elevated temperatures ***(6)*** and the need to control this should be considered when choosing excipients and storage conditions. All of these issues must be addressed by the selection of suitable formulation and processing conditions if stable lyophilized protein preparations, which retain full biological activity, are to be achieved.

1.3. Formulation/Stabilizer Choice for Proteins

When considering freeze-drying of proteins, their formulation is particularly important, for instance:

1.3.1. pH/Buffers

Buffers should be kept to a minimum concentration to maintain pH in the desired application and in particular, mixed buffer systems, such as phosphate, should be used cautiously as pH shifts can occur on freezing, histidine, citrate, or Tris buffers being preferable.

1.3.2. Salts

Inorganic salts reduce the glass transition temperature (Tg′) of the formulation and so limit the temperature at which primary drying can be performed. Therefore, salt concentrations should be reduced to a minimum and where alternative osmotic modulators can be selected that have less impact on the Tg′ should be preferred.

1.3.3. Stabilizers

Hydrogen bond-forming sugars, such as trehalose or sucrose, are well documented as excellent stabilizers for freeze-drying of proteins. Other stabilizers may be more specific in their effects (such as metal ions, e.g., zinc ***[7]***), whereas

others, such as mannitol or glycine, are valuable as matrix formers without offering the stabilizing benefits of sucrose/trehalose.

1.3.4. Dry Weight

In order to produce a robust lyophilized cake it is important that the dry weight of nonvolatiles be sufficient (for example, 10 mg/container).

1.3.5. Protein Concentration

Proteins themselves are often common choices as stabilizers for use in formulating for freeze-drying. Albumin has a high Tg′, is a good cake-former, and also blocks nonspecific binding of proteins to glass surfaces, making it an ideal choice of stabilizer for low concentrations of recombinant proteins.

2. Materials

2.1. Protocol 1: Lab-Scale Lyophilization on Reverse-Phase Fractions From High-Performance Liquid Chromatography Run

1. A lab-scale freeze-dryer (e.g., Edwards Modulyo, Virtis Advantage, or similar unit) (**Fig. 1**) (*see* **Notes 1** and **2**).
2. Suitable containers for freeze-drying the product, e.g., round-bottomed glass flasks, glass vials, plastic microcentrifuge tubes, microtiter plates (e.g., from VWR Ltd., Poole, UK) (*see* **Note 3**).
3. Parafilm™.
4. Freezing system, e.g., liquid nitrogen or dry-ice acetone, or a –70°C freezer.

2.2. Protocol 2: Pilot Scale Freeze-Drying

2.2.1. Freeze-Drying of a Dilute Concentration of a Purified Protein as a Reference Preparation for Laboratory Assay

1. Ampoules or vials (preferably type I glass formed from cylindrical tubes (suppliers, e.g., Adelphi Tubes, Haywards Health, UK).
2. Closures (e.g., Adelphi Tubes).
3. Hot air oven or autoclave.
4. Pipets (e.g., Gilson, Anachem, Luton, UK) or autodispensing syringe (M-Lab, Hamilton Bonaduz, Switzerland).

2.3. Differential Scanning Calorimetry

1. Modulated differential scanning calorimeter with cooling accessory (e.g., TA Instruments (Surrey or Perkin Elmer [Beaconsfield, Crawley, UK]).
2. Sample pans (e.g., part no. 900825.902 TA Instruments) and crimper.
3. Dry nitrogen gas.
4. Liquid nitrogen for cooling accessory.

2.4. Freeze-Drying Microscopy

1. A commercially available freeze-drying microscope is available from Biopharma (Biopharma Technology Limited, Winchester, UK), or Linkam (Epsom Downs, UK).

Fig.1. Typical benchtop freeze-dryer suitable for drying from flasks via the manifold or from trays of vials placed on the chamber shelf. (Photo Courtesy of Biopharma, UK.)

2. Quartz glass crucible, 13-mm glass cover slips, and semicircular metal shims (Linkam).
3. Ethanol.
4. Liquid nitrogen.

3. Methods

Sodium azide must *not* be present in samples to be dried on the majority of freeze-dryers because of the risk of forming explosively reactive metal azides at brazed tubing connections, though some dryers are marketed as "azide friendly." Although samples need not necessarily be fully solubilized, a homogeneous suspension should be produced before freezing commences.

The process of freeze-drying may be divided into the following stages:

1. Freezing. Lab-scale freeze-drying is often performed in general laboratory glassware items such as round-bottomed glass flasks, glass vials, plastic microcentrifuge tubes, or even microtiter plates (*see* **Note 3**). Containers must not be more than one-third filled or sudden volume and thermal changes may result in the container shattering, with catastrophic loss or cross-contamination of the valuable samples.
2. Drying temperature. Simple units are different to production freeze-dryers in that usually there is no control of the product temperature and the product warms to ambient temperature in an uncontrolled manner over several hours. Conversely, the condenser is temperature controlled usually at –60°C or less and a vacuum

maintained typically at 10–100 µbar. Lyophilization is rapid, usually completed overnight, it can be even hours dependent on the drying system and solvent being removed.

Product is usually removed by breaking vacuum to air or inert gas. Individual samples may be removed from a manifold without losing vacuum on the system as a whole, although some laboratory systems may offer precooled sample shelves in which case samples may only be removed at one time with back fill with air/inert gas. Simple stoppering mechanisms may be available, allowing more sophisticated lyophilization conditions to be modeled with individual vials being fully sealed by lyophilization closures prior to the system being opened to atmosphere. No stoppering is possible for samples lyophilized in flasks on a manifold (*see* **Note 4**).

3.1. Lab-Scale Lyophilization of High-Performance Liquid Chromatography Protein Fractions

The following protocol describes a method for freeze-drying protein fractions from a reverse-phase high-performance liquid chromatography fractionation, prior to further analysis for example by peptide mapping.

1. Switch on condenser and vacuum pump on the benchtop freeze-dryer, assemble the manifold or place samples on shelf, and ensure that a good vacuum (100 µbar or less is achieved) and that the condenser is down to –50°C or lower.
2. Dispense fractions into microcentrifuge tubes or other suitable containers ensuring that the microcentrifuge tubes are not more than 33% full. Pierce holes in the lid or remove lid and replace with Parafilm pierced with several holes.
3. Snap freeze microcentrifuge tubes by dipping until partially submersed in liquid nitrogen or dry/acetone (beware cold hazard), alternatively freezing in a suitable spark-proof deep freezer (–40°C or lower).
4. Place frozen microcentrifuge tubes into a Quickfit style round-bottom flask (neck wide enough to allow easy loading and removal of the tubes). Freeze outside of glass to –60°C or below and attach on manifold. Apply vacuum by opening the manifold valve so as to connect the flask to the condenser. To avoid shattering of the flasks place nylon meshing around the glass spheres. Alternatively, place the sample containers in a suitable tray within the chamber of the dryer (for the Modulyo type) or within the chamber on the shelf (for a unit such as the Virtis Advantage). Check to ensure that the vacuum achieved returns to below 100 µbar.
5. Allow the samples to completely warm to room temperature (usually overnight [16 h] but may be less for volatile solvents).
6. Carefully allow the vacuum to be released by switching the manifold valve slowly to prevent material ablating from the microcentrifuge tubes.
7. The system can be left on and fractions can be dried over several days before the condenser needs thawing out. If multiple flasks on a manifold are used, different flasks can be removed at different times depending on when they have completed drying.

A number of common problems may arise during freeze-drying and **Notes 5–10** give some helpful tips for trouble-shooting and quality control (*see* **Note 11**).

3.2. Pilot Scale Freeze-Drying

3.2.1. Freeze-Drying of a Dilute Concentration of Purified Protein as a Reference Preparation for In-House Standard

This protocol describes the lyophilization of a purified protein as a small batch (50–500 vials), suitably formulated so as to stabilize the biological activity, prevent the degradative processes possible in the liquid state, to facilitate storage as an in-house reference material, and to help distribution to other laboratories for analysis. Pilot scale freeze-drying is applicable to formulation development and product development environments, where a more rigorous and yet still easily affordable lyophilization solution is required and where multiple containers are required, of identical product composition and concentration, for stability study, repeat assay, or distribution.

1. Selection and preparation of glassware and closures. Glassware (preferably type I glass formed from cylindrical tubes) should be washed without detergents and baked/autoclaves to sterilize if low bioburden is required (*see* **Note 12**). To prepare such a protein it is most convenient to use small stoppered or screw-cap vials (usually glass) or to use ampoules that can be flame sealed after lyophilization. Closures could be siliconized to ease stoppering and should be baked to reduce residual moisture content (e.g., 16 h at 116°C) as otherwise this moisture may transfer to the lyophilized product on storage.
2. Formulation choice. A range of formulations should be assessed to allow evaluation of the choice of buffers and the impact of freezing and of dehydration on the target protein, based on the points outlined (*see* **Subheading 1.3.**).
3. Dispensing of sample. The method of dispensing the product will depend on the number of containers to be filled and the accuracy and consistency of fill volume required. Options range from a simple pipet, an autodispensing syringe, to a peristaltic pump.
4. Freezing. The timescale of freezing may be important (*see* **Note 13**). Freezing temperature can be derived from study of the critical glass transition or collapse temperatures by thermal analysis or freeze-drying microscopy experiments (respectively), and the length of the freezing step should be such to ensure that the contents of all of the containers have frozen completely.
5. Lyophilization cycle design. For primary drying, conditions of vacuum and temperature must be set so as to permit drying without collapse of the cake structure, but at a sufficiently warm temperature that sublimation occurs quickly to achieve drying in a practical time period (*see* **Note 14**).
6. Temperature probes to monitor process. Setting a simple thermocouple into the solution to be dried before freezing will allow the freezing and drying process to be monitored. Although the data acquired will apply to the container monitored

alone, and the probe will itself introduce differences to the nucleation point and tell little about the process in neighboring containers, it is still a useful and widely applied measure of the progress of a freeze-drying cycle.

7. Back-filling/stoppering. Once lyophilization is completed product containers can be stoppered under vacuum, or under an inert atmosphere depending on the design specification of the product (*see* **Note 15**).
8. Assessment of product parameters. The lyophilized product should be assessed against frozen but undried material (frozen baseline) taken from the fill in order to assess the recovery of functional activity. Other parameters, such as appearance and residual moisture content, should be assessed.

3.3. Experimental Determination of Glass Transition/Collapse Temperature

3.3.1. Determination of Glass Transition Temperature by Modulated Differential Scanning Calorimetry

The principle of modulated differential scanning calorimetry and its application to developing freeze-drying conditions is described elsewhere ***(8)***.

1. Aliquots of the sample in the intended lyophilization buffer or a range of buffers are prepared and an 80-µL aliquot is dispensed per pan and the pans crimped with an appropriate crimper.
2. The sample pan and an empty crimped reference pan are placed within the oven of the modulated differential scanning calorimetry machine.
3. Apply a programmed freeze to –70°C and rewarming to ambient temperature using proprietary software, recording the heat flow profile, and the reversing heat flow profile. Use a range of heating rates between 0.5 and 3°C/min with a modulating heating rate such that the overall thermal trend is heating only.
4. Data analysis: from the profile note any glass transition (an inverse sigmoidal event in the reversing heat flow profile in the thermal region below the melting temperature. Proprietary software will help to set this and identify the glass transition temperature.

Repeat analyses are recommended to establish robustness of the Tg′ determination.

Temperature calibration may be made using materials of well-characterized melting points such as indium (156.6°C) and *n*-decane (–29.6°C).

3.3.2. Determination of Collapse Temperature by Freeze-Drying Microscopy

1. Assemble the sample by placing the metal shim onto a cleaned quartz glass crucible. Dispense an aliquot (5 µL) of the sample within the shim and place a 13-mm cover slip over the sample.
2. Insert the crucible into the metal sample holder and place this into the microscope cryostage.

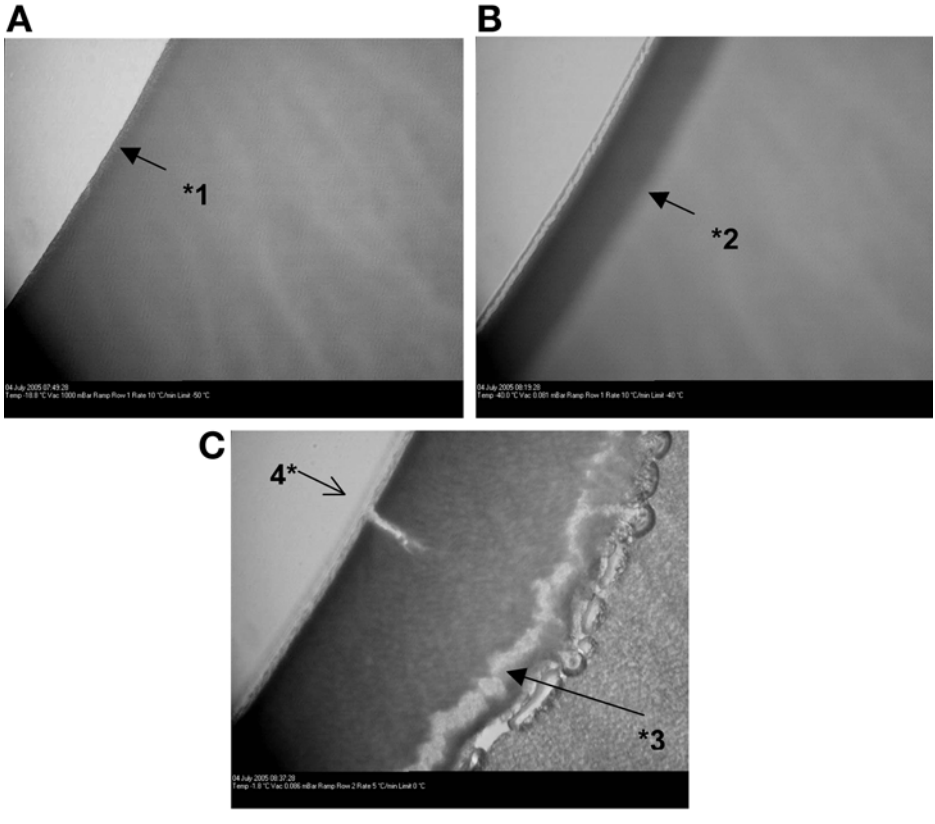

Fig. 2. Freeze-drying microscopy profile of an albumin solution showing **(A)** sample frozen with air/liquid interface (*1), **(B)** primary drying under vacuum with drying front advancing (*2), and finally **(C)** collapse of freeze-drying front (*3) and rupture of sample skin at surface (*4) as temperature was raised past the collapse temperature. Data produced by Ms. Kiran Malik, NIBSC.

3. Focus the microscope on the interface between the edge of the liquid sample and air using a ×4 or ×10 objective. Polarized light and differential interference techniques significantly improve the quality of the image obtained.
4. Freeze the sample to –50°C at 5°C/min.
5. Hold unit frozen then apply vacuum to a selected vacuum level (25–100 μbar).
6. Ramp the temperature at a rate of 1–5°C/min and monitor physical appearance during the warming phase. Once collapse has been observed the temperature may be reduced again, the frozen state restored, and the collapse event revisited with a slower ramp rate to refine the collapse temperature evaluation.
7. Once the collapse temperature is established, raise the temperature to ambient and clean the cell and slide thoroughly, removing any biological materials with neat ethanol.

8. The collapse temperature is determined by repeated cycling through the temperature range that results in structural collapse of the ordered drying front (*see* **Fig. 2**). The calibration of the stage temperature probe can be checked by performing an initial study on each day of operation using a sample with well-characterized glass transition or eutectic (e.g., sucrose at –32°C or NaCl at –21.5°C).

4. Notes

1. Lyophilization is a process that is widely used in biotechnology in a range of different situations. It may be performed on a small benchtop dryer with little process control or in a multimillion pound industrial lyophilizer whose operation is carefully monitored and controlled by computer systems and described in detailed written operating protocols. Laboratory-scale lyophilization differs from the production process commonly applied to the industrial preparation of biological/biotechnology materials in the scale of the operation, the degree of process and parameter control available, and the requirements in terms of the properties of the lyophilized product.
2. A lab-scale freeze-dryer may be of simple design where solvent is removed from products frozen in round-bottom flasks or in containers such as microcentrifuge tubes contained within such flasks. There is no product temperature control or monitoring, no controlled shelf temperature, and the drying process is driven merely by the uncontrolled warming of the sample to ambient temperature over a period of hours while the condenser remains below –50°C to trap the subliming water vapor.
3. Containers used should be capable of withstanding rapid snap freezing (either by immersion in liquid nitrogen or dry-ice acetone. Beware cryogenic liquid and asphyxiant hazards) or freezing within a freezer at –70°C without cracking or shattering. The containers must then also be capable of withstanding exposure to deep vacuum conditions. Plastic microcentrifuge tubes, frozen by careful incubation in cryogenic liquid, are particularly suited for column fractions such as those obtained from preparative peptide mapping, although microtiter plates may be equally useful providing cross-contamination is avoided, in such cases freezing on the shelf of a freezer would be more practicable. The caps must be pierced with a syringe needle or other suitable means, to allow escape of subliming water vapor.

   Such samples may well dry down to leave no visible solid material but reconstitute, often in only a few microliters of diluent, to produce samples suitable for MALDI–mass spectrometry or further enzymatic degradation procedures.

   Standard glass vials designed for lyophilization are ideally suited for operation within such a lab-scale machine providing either a shelf or sample plate is available. More complex laboratory dryers may allow for these vials to be part-stoppered with rubber closures and then be fully stoppered at the end of lyophilization. In small numbers they can be placed suitably sealed (with Parafilm or equivalent material, again carefully punctured in several places to allow subliming vapors to escape) within larger glass flasks.

Larger volumes (up to 100 mL) may be lyophilized on simple lab-scale dryers by freezing in flasks at not more than one-quarter filled, frozen by immersion in a cryogenic bath (beware star-cracking of reused flasks that may implode when placed under vacuum) and attached to a manifold (*see* **Fig. 1**). Such flasks should be contained within plastic netting to minimize risk were the flask to implode under vacuum.

4. Laboratory-scale dryers have proven themselves to be very versatile equipment, and adaptations have allowed stoppering to be achieved using a manual screw mechanism, manifolds that can accommodate six or more quick-fit flasks of up to 500 mL, specialist adaptors that allow for several dozens of ampoules to be dried and then flame sealed individually for the drying of microbial cultures and the like.
5. Poor appearance: Though appearance is not usually a critical factor in laboratory-scale applications, on occasions an oily or sticky residue may result after freeze-drying rather than a dry powder. This is due to product temperature exceeding the glass transition or collapse temperature. The sample may need to be frozen to a lower temperature, or salts may need to be further diluted or the samples reformulated, especially if excipients such as glycerol are present, which will not readily undergo sublimation. For larger scale experiments in vials/ampoules the product should have an acceptable appearance, homogeneous across the batch. Poor appearance (collapse) may be caused by shelf temperature being too high during primary drying resulting in the Tg' being exceeded. Wispy cakes result from insufficient dry weight mass and so this should be addressed by use of bulking agents (glycine or mannitol are common examples, but other proteins, sugars and polymers such as dextran or polyvinyl pyrollidone have been used). Products that are removed from the dryer with a satisfactory appearance but which show collapse of structure or transformation to a glassy gel on storage may have too high a residual water content. Discoloration on storage at ambient or higher temperatures of lyophilized product formulated with reducing sugars such as glucose or hydrolyzable sugars, may be because of browning reactions whereby amino groups in the product undergo a Schiff base-type reaction with the carbonyl group on the sugar.
6. Product boil-out. For microcentrifuge tubes ensure that caps are secure and do not overfill the containers. Release vacuum gently to prevent disturbing fine powders. Product loss from vials/ampoule is less common, but cannot be assumed not to occur to some degree. It is worse where the product cake is less robust.
7. Incomplete removal of liquid. Samples that return to a liquid or gelatinous state after removal have not undergone sufficient drying and the period on the dryer should be extended or samples reformulated as in **Note 5**.
8. Cracking of container. Star cracks in round-bottom flasks will prevent formation of a good vacuum and may result in catastrophic implosion of the flask, with subsequent loss of valuable sample and possible injury. Glass flasks should always be contained within plastic netting to prevent such accidental injury.
9. Loss of bioactivity, where activity is lost on drying, may be a result of pH or salt concentration shifts during freezing resulting in loss of biological activity and

protein denaturation. If this is the case, trial freeze/thaw the material and see if activity is lost. If activity remains on freeze-thawing but is lost on freeze-drying then it is more likely that the material can not withstand dehydration, to the degree to which the sample has been dried. Try lyophilizing for a shorter period to remove less water and/or add stabilizers that are known to be lyoprotecting, such as sucrose or trehalose ***(9,10)***. Loss of activity on storage may be because of too high a residual moisture content, as many degradative reactions of proteins require water.

10. Vacuum pumps and maintenance. Inability to draw a sufficiently low vacuum may be because of problems with the door seal on the chamber and/or condenser, which require regular inspection and cleaning if contaminated. Another possible cause is contamination of the vacuum pump oil or leaks within the pump tubing, fittings, or equipment.
11. Quality control:
    a. Vial-to-vial variability. Visually inspect all vials in the batch and assess the appearance and biological activity across the fill. Heterogeneity should be mapped to see whether it correlates particular location. Appearance is more important in large-scale freeze-drying than for laboratory applications as above. However, heterogeneity should be avoided and poor appearance may indicate suboptimal drying.
    b. Moisture determination. It may be necessary to make some assessment of this so as to predict suitability for longer term storage. Commonly used methodology includes colourometric Karl Fischer and thermogravimetric analysis ***(11)***.
    c. Storage stability analysis. Stress studies using elevated temperatures and humidities over time can indicate the likely storage stability of the lyophilized material. The trends in activity loss can be analyzed using Arrhenius or similar kinetic models to predict the stability expected at storage temperature ***(12)***.
    d. Activity recovery. The recovered biological activity should be measured against controls taken before lyophilization, so the choice of formulation and stabilizer that gives best activity recovery can be identified and then applied at scale up.
12. Closures are available in a variety of formats and compositions ***(13)*** and selection should be based on the specific application.
13. Some have recommended rapid freezing rates, especially if the target protein loses activity rapidly in the liquid state or if the selected formulation results in marked pH shifts during freezing and denaturation. However, rapid freezing may result in small ice crystals and slower drying times; hence if rapid freezing is necessary then an annealing (thermal tempering) step ***(14)*** may be needed to correct the effects.
14. Vacuum conditions can be set by selecting the atmospheric pressure based on the vapor pressure of ice at the shelf temperature ***(3)***, and then experimenting with different shelf temperatures until the highest temperature is identified that does not result in the critical collapse temperature being exceeded in the product.

    During secondary drying, shelf temperature is set to ambient or higher temperature with hard vacuum applied to maximize the loss of remaining water.

The typical length of the step has been approximated at one-third of the primary drying ***(15)*** but should be sufficient but not exceed that which yields product of the target residual moisture content.

15. Sealing under vacuum will allow reconstitution fluid to be drawn in at a greater rate and may aid dissolution of lyophilized products. If an atmosphere is required, then inert gas is preferable to minimize oxidation of the proteins over prolonged storage. Moisture content of the product may increase over time, with water equilibrating from that residual in the closure, and for this reason closures should be pretreated to minimize residual water content.

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