Freeze-drying – a manufacturing approach to increase the stability of *in vitro* diagnostic (IVD) assays

Freeze-drying (lyophilisation) is becoming increasingly popular with IVD test manufacturers looking to simplify assay handling and processing. Biofortuna is a leading supplier of freeze-dried tests, and has a wealth of expertise in transforming molecular diagnostic reagents into instantly soluble freeze-dried pellets. The Company has a strong track record in contract development and manufacturing, utilising its proprietary freeze-drying know how to help customers optimise assay kit formulation. This white paper outlines the key considerations when developing a freeze-dried IVD test and discusses some of the strategies which can be used to ensure rapid, successful and cost-effective assay development.
Introduction

The main benefits of freeze-drying for IVD assay kits are eliminating the need for cold storage of reagents, increasing the shelf life of products at ambient temperatures and enabling ‘one tube’ assay protocols. These attributes are particularly useful for point-of-care or field testing applications, but can be equally beneficial in a laboratory setting, simplifying storage and reducing hands-on time. However, an in-depth understanding of the lyophilisation process is essential to ensure that the freeze-dried assay maintains the activity and performance of the pre-lyophilised ‘wet’ assay. This white paper outlines the various steps of the lyophilisation process, the potential challenges, and the strategies which can be used to overcome them.
What is lyophilisation?

Lyophilisation is the process of removing water from a product to the point where it is no longer biologically or chemically active. Lyophilisation of an assay prevents degradation of its components such as enzymes, antibodies, proteins, DNA or oligonucleotides, allowing them to be safely transported and stored at room temperature with no loss in performance. The lyophilisation process is technically complex and requires significant expertise to achieve consistent and reliable results, but is essentially comprised of three steps:

1. **Freezing** – solidification of an assay mix
2. **Primary drying** – low temperature water removal by sublimation (converting frozen water from a solid directly to a gas).
3. **Secondary drying** – desorption of residual water

Understanding your formulation

There is no such thing as a ‘one-size-fits-all’ freeze-drying protocol, and the success of the lyophilisation process in both physical and economic terms relies on careful formulation and analysis of your product. It is therefore vital to understand the thermodynamic properties of your formulation before designing a freeze-drying protocol, to both preserve assay activity and maximise its stability post-lyophilisation. Typically, a frozen protein formulation consists of two phases; approximately 80 per cent is ice crystals, and the remaining 20 per cent is an amorphous or a partially crystalline phase that contains excipients and proteins. It is the latter of these phases that forms the freeze-dried cake or pellet, containing pores where the ice crystals have been removed. The contents of this amorphous or crystalline phase also determines the critical temperatures of a formulation, which must be determined in order to design an optimal and formulation-specific freeze-drying protocol. Various analytical techniques, including freeze-drying microscopy (FDM) and differential scanning calorimetry (DSC), should be used to determine the characteristics and critical temperatures of your formulation in order to design an optimal, efficient and cost-effective freeze-drying protocol.

Freeze-drying microscopy

FDM is used to determine visible changes in a formulation. This technique subjects a sample of the formulation to the lyophilisation process on a small scale, which can be observed using light microscopy. As with a full-scale lyophilisation, the sample is frozen to a specified temperature and a vacuum is applied. The sublimation front can be observed moving from the outside of the sample inwards, and the structure of the material yet to be lyophilised can be observed. Once sublimation begins, the temperature is raised gradually to the point that the softened lyophilised material can no longer support its own structure, known as the collapse temperature ($T_c$).
Differential scanning calorimetry

DSC is a thermoanalytical technique used to measure the amount of energy required to heat a sample compared with a reference. For completely amorphous formulations, DSC is used to determine the glass transition temperature ($T_g$); the temperature at which the formulation transitions from a brittle “glassy” state into a viscous “rubbery” state. For partially crystalline formulations, DSC also determines the eutectic temperature ($T_{eu}$); the temperature at which the super-lattice releases all of its components into a liquid mixture at once. DSC can also be used to analyse dry, post-lyophilisation samples, helping to determine the residual moisture content and establish recommended storage temperatures to maximise stability.

Achieving the right formulation

Optimisation of the lyophilisation formulation can be used to both preserve assay performance and ensure that the freeze-drying process is as cost-effective as possible. There are a number of additives which can be incorporated into a lyophilisation mix, which can be broadly divided into three categories: stabilisers, bulking agents and critical temperature modifiers.

Stabilisers

The freeze-drying process exposes the components of a diagnostic assay to extremes of temperature, pressure and moisture content. In many cases, this could potentially lead to the damage or denaturation of sensitive reagents, affecting assay performance. Stabilisers provide protection during freezing (cryoprotectants) or drying (lyoprotectants) and include a range of buffers, sugars (disaccharides/polsaccharides), polymers and proteins. Stabilisers are particularly important when freeze-drying biological components, such as enzymes or antibodies, but the choice and concentration of these protectants is critical, as these additives must not interfere or inhibit assay performance. Simple screening of potential stabilisers using the wet formulation (prior to freeze-drying) can determine whether assay sensitivity, performance or detection is likely to be affected. FDM and DSC are used to identify any resulting changes in the $T_g$ or $T_{eu}$ and to help determine the optimal concentration for the additive.

Bulking agents

Bulking agents are generally used to cosmetically enhance the appearance of the final product. They are generally inert, having no functional impact on the product itself, although some bulking agents (for example, sugars or polymers) aid reconstitution of the assay components at the point of use.
The temperatures used for the freezing and primary drying steps can have a huge impact on the speed and cost of lyophilisation processes. For example, raising the freezing temperature by 5ºC can halve the primary drying time, by increasing the vapour transfer rate and accelerating the sublimation process. This leads to shorter cycle times and increased throughput, making the overall lyophilisation process far more economical. There are a number of additives which can be used to raise the $T_g$ to ensure cost-effective drying, including glycine, mannitol and polyvinylpyrrolidone (PVP).

Critical temperature modifiers

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Additional design challenges

The huge number of lyophilisation additives available makes a structured approach to formulation development essential. It is vital to understand the potential effects of individual additives on the behaviour and performance of each diagnostic test and there are a number of other critical factors to consider when developing the formulation, including the glycerol and salt concentrations, the reaction volume and the vessel type. The optimal formulation will vary significantly depending on the assay design and intended use, as well as the analyte being tested. For example, the stability requirements of an assay kit designed for occasional point-of-care use in outreach clinics will be very different from an assay intended for high volume testing in a hospital laboratory. The performance requirements of the final assay, including the need for long-term stability and rapid reconstitution, must therefore be considered from the outset. There may also be additional challenges specific to the assay type. For example, bulking agents commonly used for immunoassays may interfere with PCR reactions and, therefore, may not be suitable for molecular diagnostic applications.
The idea of freezing is to cool the assay mix to the point where all biological or chemical activity stops. As the morphology of the resulting ice crystals determines both mass and heat transfer rates through the dry layer, the freezing parameters have a strong influence on the primary and secondary drying steps. With uncontrolled freezing, ice nucleation occurs randomly, leading to variable drying rates. Ice nucleation can be controlled by cooling a formulation to a temperature below equilibrium freezing point but above the temperature at which spontaneous nucleation occurs. This process results in larger, more uniform ice crystals, increasing vial-to-vial homogeneity and reducing variability in drying behaviour. As protein aggregation occurs on the surface of ice, decreasing the available surface area with controlled nucleation also has a positive effect on activity recovery. Partially crystalline formulations characteristically display incomplete crystallisation during freezing, lowering the $T_g'$ and leading to longer drying times. In these instances, an annealing or thermal cycling step may be beneficial. This involves warming the formulation to above its $T_g'$ (but below its $T_{eu}$), then re-cooling it to increase crystallisation efficiency.

**Designing a freeze-drying protocol**

**Freezing**

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Primary drying

The drying process can begin once the assay mixture is fully frozen. First, the frozen product is subjected to a strong vacuum, with the exact pressure depending on the primary drying temperature. This results in a difference between the ice vapour pressure and the chamber pressure, resulting in sublimation. The formulation is then heated to speed up sublimation and generally needs to occur above -40°C for lyophilisation to be economically viable. For formulations with low protein concentrations, the primary drying temperature is usually kept below \( T_g \) as, at this temperature, the assay mix has a completely solid structure with no movement or flex, allowing subsequent removal of water without damaging the assay components. However, formulations with high protein concentrations can sometimes be dried at temperatures above the \( T_g \) but below the \( T_c \) to accelerate drying.

Insufficient primary drying will cause the product structure to collapse, leading to poor rehydration characteristics, loss of assay performance and reduced long-term stability. As mentioned above, stabilisers, bulking agents and critical temperature modifiers can be used to both improve lyophilisation parameters and create a more cost and time-effective process, as well as to improve post-lyophilisation protein recovery.

Secondary drying

Following primary drying, up to eight percent of the initial moisture content can still be present in the pellet, usually directly bound to the assay components. The aim of secondary drying is therefore to remove this residual water, ideally to less than one percent. However, the optimal value will vary according to the formulation, product stability and long-term storage conditions. To achieve secondary drying, still under vacuum, the product is warmed to 20-40°C and held for several hours. For amorphous formulations, the temperature ramp rate to the secondary drying temperature needs to be approximately 0.1-0.3°C/min to avoid exceeding the glass transition of the lyophilised cake, which would result in shrinkage. This highlights the importance of understanding the physical characteristics of your formulation prior to protocol development.

Post-lyophilisation analysis

Characterisation of the freeze-dried cake or pellet is critical for understanding the impact of the lyophilisation process. In addition to DSC analysis, Karl-Fischer titration (KF) is an effective quality control technique to verify the reproducibility of the lyophilisation protocol. It can be used to rapidly and accurately quantify the residual moisture content in lyophilised samples, providing a rapid assessment of the suitability of the freeze-drying protocol. Although lower moisture content does not necessarily guarantee long term stability, it is a good indicator that a minimum level of moisture removal has been achieved.

Experience is key

The complexities and numerous pitfalls associated with optimising an assay for lyophilisation can make the process seem more like a 'black art' than science. However, working with a knowledgeable and experienced partner can help to significantly accelerate formulation development, ensure a cost-effective freeze-drying process and reduce the overall cost of bringing an assay to the market. While there are a large number of companies with experience in freeze-drying reagents and biochemical tests, lyophilisation of molecular diagnostics kits presents a number of unique challenges. Choosing an R&D partner that has a strong track record in the molecular diagnostics sector – such as Biofortuna – and taking advantage of their expertise in the early stages of assay development, can maximise the chances of successful product development.
Experience is key (cont)

This knowhow can also be important in identifying assay reagents or additives which are not suitable for lyophilisation, enabling alternatives to be selected or allowing the design of assay protocols combining both lyophilised and non-lyophilised components, without wasting time and resources. The potential challenges don’t simply stop with the completion of R&D, as lyophilisation processes need to be translated into a cGMP environment. This is critical to the successful scale-up and transfer of freeze-dried assays to manufacturing and can be simplified by selecting a lyophilisation partner that has experience of working to ISO 13485 and FDA CFR21 part 820 compliant quality systems, as well as offering dispensing, assembly and packaging of diagnostic assay kits in a controlled environment, ready for shipment to your customers.

Conclusions

The primary aim of any freeze-drying protocol should be to maintain, or even enhance the stability of pre-lyophilised ‘wet’ assays. However, the complexity of the process makes it very difficult to develop a reliable, reproducible and cost-effective protocol without extensive knowledge and experience. While the lyophilisation of diagnostic reagents is now relatively commonplace, with a wealth of literature and knowhow available to support product development, the growing popularity of molecular diagnostics in a clinical environment has thrown up new challenges for assay kit manufacturers.

Working with a knowledgeable lyophilisation partner that understands the demands of both molecular testing workflows and manufacturing for regulated environments, such as Biofortuna, can help to ensure cost-effective lyophilisation of assays without affecting performance. Whether you’re looking to simplify testing workflows, improve assay stability or reduce transport and storage costs, freeze-drying can simplify the end-user experience of an IVD product, enhancing your reputation in a highly competitive market.

For further information, email services@biofortuna.com or visit www.biofortuna.com.

About Biofortuna

Biofortuna is an international IVD contract development and manufacturing business. We provide assay development and manufacturing services, with a wealth of expertise in immunoassay and molecular diagnostic assay stabilisation. Our FDA registered and ISO 13485 certified facilities provide small scale pilot batch manufacture up to full commercial production of finished kits. Biofortuna also offers a portfolio of molecular diagnostic products for applications including oncology, autoimmune conditions and infectious disease.