Difficult to Lyophilize Diagnostic Assay Formulations

Stabilizing Diagnostic Assay Formulations

A major challenge in the development of new laboratory diagnostic tests is in stabilizing the formulation. The formulations used in diagnostic testing are complex, containing active components like proteins, enzymes, antibodies, and even magnetic particles. In addition to these active ingredients, other components like buffers, salts, co-solvents, stabilizers, and excipients are commonly included to optimize performance and provide stability to the active ingredients. Despite the best efforts of Research and Development scientists, the long-term stability of these formulations is almost always limited. As such, organizations looking to scale production of diagnostic products have a few choices:

Provide the diagnostic test as a kit with several reagent components where mixing is required by the end user.

- Utilize Use cold-chain shipping option (at temperatures of -20 C and below) to preserve sensitive components.
- Lyophilize the formulation into single-dose form where the product only needs to be rehydrated at the point-of-use.

Challenges in Lyophilizing Assay Diagnostic Formulations

Lyophilization is a popular choice for diagnostic products, as the process is user friendly, and eliminates the need for expensive cold-chain shipping. A limitation of lyophilization is that it requires substantial expertise to design and scale a product. Formulations designed for lyophilization must take into account the functional requirements of the assay, while also optimizing the lyophilization process, not an easy task. Lyophilization is always a complicated process, and challenges are faced in each industry and application that it is used. When applied to diagnostics, lyophilization can be even more difficult than usual, as the formulations tend to be the most complex.

This article will focus on a number of challenges and the solutions that are specific to lyophilizing diagnostic assays.

Complications from Glycerol

Maintaining the stability of proteins, antibodies, and other active ingredients is the primary goal for lyophilized diagnostic assays. When supplied in a liquid form, proteins and antibodies are frequently held in storage buffers that contain 25-50% glycerol as glycerol plays a key role in protecting proteins in liquid and frozen states.[1] However, glycerol has a negative impact on the lyophilization process.

One of the critical parameters for lyophilization is the glass transition temperature of the reagent mix. A product being lyophilized should be kept below this temperature during primary drying. The glass transition temperature for a complete diagnostic formulation will be related to the weighted average of the individual components in the formulation. Glycerol has a very low glass transition temperature, so it will drastically lower the glass transition temperature of the overall mixture.[2] Thus, it needs to be present in as low a quantity as possible.

There are several strategies that can be employed to remove glycerol from reagents. However, often low concentrations will remain present in the assay despite the removal technique, and even low concentrations of glycerol can be problematic. While formulations with low concentrations of glycerol can technically be lyophilized, frequently the resulting product will contain an unpredictable glassy structure. This causes poor appearance, poor reconstitution, and can impact stability.

One strategy to improve the quality of products that contain glycerol, is to simply dilute the reagent which contains glycerol, relative to the other components in the product. Table 1 provides a simple example. Consider a product containing an active ingredient with 5% glycerol residual and an excipient. When provided in a 10 uL dose, the amount of glycerol in the final formulation might be 0.5%. However, it is possible to double the reaction volume without altering the rest of the formulation. Both product A and B contain 10 ug of active ingredient per dose, both have a 10% excipient concentration, but product B has *half* the glycerol. Though a simple strategy, in some cases this can drastically improve the quality of a lyophilized product containing glycerol without affecting product performance.

Table 1: Diluting glycerol containing reagents improves lyophilization characteristics.

	10 mg/mL reagent mix containing 5% glycerol contamination	20% excipient stock solution	Water	Total reaction volume	Glycerol concentration
Product A	1 uL	5 uL	4 uL	10 uL	0.5%
Product B	1 uL	10 uL	9 uL	20 uL	0.25%

Ultra-cold Processing Temperatures

As alluded to earlier, one aspect that makes glycerol incompatible with lyophilization is that it has a low glass transition temperature, which is the temperature below which a product needs to be kept during lyophilization. Along with glycerol, there are several components frequently found in diagnostic products which cause very low glass transition temperatures, below those that are typically found in other industries/applications. Buffers, salts, and potentially other co-solvents often cause much lower glass transition temperatures.

Needing to keep a product at low temperature during lyophilization is only a problem because the colder a product is during lyophilization, the slower water will sublimate. The relationship between sublimation rate and product temperature is exponential. Meaning, even small reductions in product

temperature can drastically increase the processing time required to lyophilize a product. Thus, it is optimal to freeze dry products at as high a temperature as possible (i.e., just below their glass transition temperature) to keep production efficient.

Small Dose Size Unlocks Low Temperature Processing

In many classical applications, a few milliliters of product are filled into a vial and then these vials are freeze dried. This means that a few *grams* of water need to be sublimated from each unit (in this case, a vial) for the lyophilization process to be done. Diagnostic products, on the other hand, are often lyophilized in single dose formats. Sometimes they are lyophilized directly into cartridges used at point-of-need or point-of care. Another possibility is lyobeads; a single or multi analyte assay into one single sphere of lyophilized material containing all the reagent needed for a single reaction. The amount of product required for a single diagnostic reaction is often on the order of a few microliters, meaning that per unit, only *milligrams* of water need to be sublimated during the lyophilization process. This changes the paradigm in a way that allows for processing at lower temperatures and provides a solution for dealing with products with low glass transition temperatures, such as formulations with substantial glycerol content.

Consider the case study from table 2, which describes a hypothetical formulation containing 10% solids. Product C is lyophilized traditionally, in vials containing 5 mL of a diagnostic formulation. Meanwhile, Product D is the same diagnostic formulation lyophilized in single dose lyobead format. In this example, we are processing the formulation at a shelf temperature of -42 C and a chamber pressure of 30 mTorr, to accommodate the product's low glass transition temperature. The overall sublimation rate for a vial will be larger, due to the large surface area from which sublimation occurs. However, lyobeads benefit from direct contact with the freeze dryer shelf or tray, and their tiny dimensions. It takes less time to lyophilize a lyobead with a diameter of 2-3 millimeters, than a vial which has a fill height of potentially a centimeter or more.

Table 2: Smaller units lead to shorter lyophilization time, allowing low lyophilization temperatures to remain economically viable.

	Format	Amount of water per unit	Shelf Temperature	Chamber Pressure	Primary Drying Time
Product C	**5 mL** of formulation in a vial	4.5 g	-42 C	30 mTorr	Days to Weeks
Product D	**20 uL** of formulation in single dose lyobead	18 mg	-42 C	30 mTorr	Hours to Days

Given the above example, many industries would consider a cut-off of around -40 C when it comes to lyophilization. Below this, lyophilization happens too slowly to be viable. Diagnostic products which frequently have glass transition temperatures below -40 C, necessitate processing below this cutoff. Lyophilizing in individual small doses can enable this possibility.

Lyobeads Containing Glycerol: A Case Study

The relative impact of reducing glycerol content, and decreasing lyophilization temperature, is demonstrated in figure 1. The figure shows lyobeads made from formulations which are identical, bar slightly different glycerol contents. Figure 1a demonstrates the characteristic rough glassy surface related to products containing glycerol. Figure 1b has the same glycerol content but is freeze dried on a much colder cycle. Not only does it appear better cosmetically; the lack of rough glassy surface also corresponds to better dissolution properties, and likely better long-term stability.

Figure 1c also shows improved appearance, but this time it is from reducing the glycerol content and keeping the same warmer freeze dry cycle as figure 1a. Again, this will improve functionality along with cosmetic appearance. Figure 1d combines both techniques and has the best appearance overall. Which technique is right for a given application will ultimately depend on the formulation and other product specific characteristics.

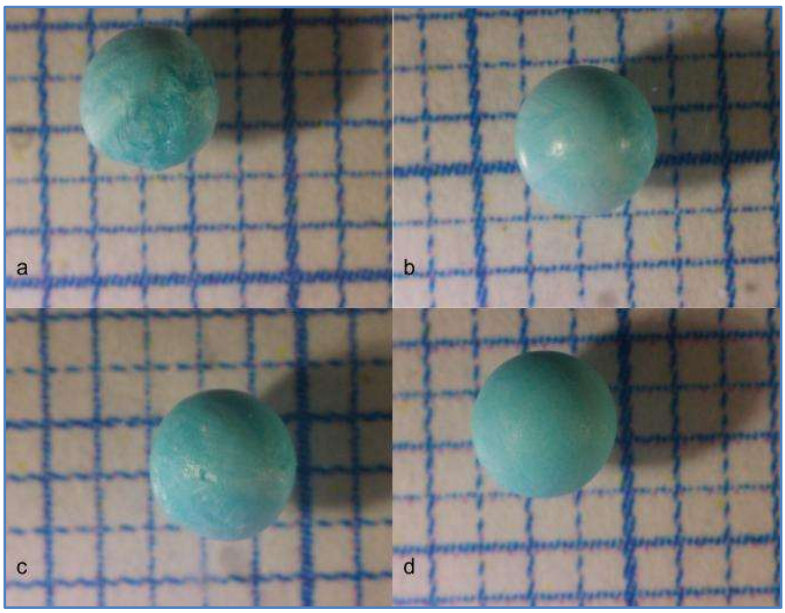

Figure 1: Lyobeads containing glycerol. 1a) Contains 0.4% glycerol and is freeze-dried on a warm cycle. 1b) Contains 0.4% glycerol and is lyophilized on a cold cycle. 1c) Contains 0.2% glycerol and is freeze-dried on a warm cycle. 1d) Contains 0.2% glycerol and is freeze-dried on a cold cycle.

Low Temperature Batch Size Considerations

When lyophilizing at ultra-low temperatures, a few new considerations need to be made when moving from pilot batches to full scale production. The most important thing to optimize ~~for~~ when designing a lyophilization protocol for a new product is primary drying rate. A fast primary drying rate means short primary drying times. Since primary drying is the most time-consuming part of the process, a lot of development effort revolves around determining primary drying efficiencies.

One of the equations which governs the primary drying process states that sublimation rate (*dm/dt*) is proportional to the difference between the vapor pressure of ice (P_i), and the partial pressure of water in the chamber (P_{H2O}).[3] The vapor pressure of ice is fixed and is determined by the product temperature. The partial pressure of water in the chamber is the portion of the chamber pressure that is contributed by water. The partial pressure of water can vary based on load size, which becomes important to development scientists working with low temperature diagnostic formulations.

The partial vapor pressure of water in the chamber comes from water which has sublimated from the product and is traveling to the condenser. Small batch sizes may not produce enough vapor to saturate the chamber, meaning the partial pressure will be below the total chamber pressure. The rest of the pressure is contributed by inert gas or air. Large production batches, on the other hand, do produce enough water vapor to completely saturate the chamber. A consequence of higher water pressure (P_{H2O}) is that batch size can have a significant impact on sublimation rate, as it causes the partial pressure of water in the chamber to approach the vapor pressure of ice.

Table 3 provides a case study in the effect of batch size at low temperatures. Product E and Product F are lyophilized with the same shelf temperature and chamber pressure, which in this case leads to a product temperature of -46 C. The vapor pressure of ice at this temperature is 48 mTorr. Product E has a load size that is too small to fully saturate the chamber, leading to a partial pressure of only 10 mTorr. Product F is a full-scale production batch that saturates the 30 mTorr chamber pressure. The difference between P_i and P_{H2O} is shown in the column to the right. The difference between these values is much higher for Product E, the pilot-size batch, which means it will have a significantly higher sublimation rate, and thus a shorter primary drying time.

Table 3: Small batch sizes correspond to shorter primary drying times for low temperature lyophilization cycles.

	Batch size	Shelf Temperature	Chamber Pressure P_C	Product Temperature T_P	Vapor Pressure at Product Temperature P_i	Partial Pressure of Water in Chamber P_{H2O}	P_i-P_{H2O}
Product E	Pilot batch of **1000 lyobeads**	-42 C	30 mTorr	-46 C	48 mTorr	10 mTorr	**38 mTorr**
Product F	Production batch of **25000 lyobeads**	-42 C	30 mTorr	-46 C	48 mTorr	30 mTorr	**18 mTorr**

When designing lyophilization cycles at more traditional temperature ranges, the effect of the chamber water pressure is much less apparent. This is because P_i grows exponentially with temperature. A product temperature of -20 C corresponds to a P_i of more than 700 mTorr. This value is so large that small differences in, P_{H2O} become less noticeable in the traditional temperature range for lyophilization. Normally development scientists don't need to consider the implication that a batch size has on cycle length. When working with diagnostic products at ultra-low temperatures, batch size becomes a factor that needs to be considered during the development cycle, and scientists should account for the extra cycle length accordingly.

DMSO and other Organic Solvents Pose Development Challenges

Organic solvents, such as dimethyl sulfoxide (DMSO), are another class of tough-to-lyophilize components that frequently end up in the formulations for diagnostic assays. Organic solvents can perform a few different functions in diagnostic products. They can serve as co-solvents which help solubilize certain molecules. Besides, DMSO in particular, can have cryoprotective properties and is sometimes used to protect cells and proteins from freeze-thaw cycles.

DMSO poses three main development challenges. First, its vapors can be corrosive to freeze-dryers. Second, when mixed with water it often has low eutectic temperatures which necessitate low lyophilization temperatures. Third, it has a low vapor pressure, making it challenging to completely remove during lyophilization.

The first problem is solvable with careful awareness. DMSO won't harm metal surfaces, but it can damage the acrylic, plastic, and rubber parts which are found in the chambers and condensers of freeze dryers. Handling formulations with DMSO in dedicated freeze dryers and increasing the maintenance or replacement frequency for sensitive components like rubber gaskets are good ideas. The second problem is also manageable. DMSO may form a eutectic mixture with water that has a low freezing point. As previously discussed above in detail, diagnostic assay formulations can often be processed at surprisingly low temperatures, which makes handling products with DMSO less challenging than in other industries.

The final challenge of incomplete removal of DMSO is an issue of which development scientists should be aware. This arises due to the lower vapor pressure of DMSO, as compared to water. A lower vapor pressure for DMSO means that its sublimation will be slower and require lower chamber pressures. This means increasing cycle length, and/or needing to settle for higher residual amounts of DMSO in the finished product. DMSO, like glycerol, is a plasticizer so it may adversely affect the long-term stability of a diagnostic assay. Though, important to note the same strategy that earlier was discussed for glycerol can be applied here. Diluting the amount of DMSO relative to other components will reduce the plasticizing effect it has on the finished product which will improve the product's long-term stability prospects.

Summary

- Diagnostic assay formulations face unique challenges that can make developing lyophilized products more difficult and time consuming than in other industries.
- Processing at extremely low temperatures (below -40 C) causes slow drying times that may not be feasible for typical lyophilization products.
- The small dose size of many diagnostic products means that when lyophilized as single dose units, like lyobeads, lyophilization times can be reasonable even under extremely cold conditions.
- Lyobeads containing glycerol are improved by reducing glycerol content, along with drying at colder temperatures.

- At very low temperatures, new considerations need to be made to account for longer freeze-drying times for large batch sizes.
- The ability to process diagnostic products at lower than usual temperatures allow for greater capacity to tolerate other difficult to lyophilize substances, like DMSO.

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