Bacteria Freeze Drying Protocol

Freeze drying bacteria is a useful method for long-term preservation. Freeze drying bacteria is a multistep process which involves culturing the microbes, suspending them in a lyophilization medium/buffer, subjecting them to the freeze drying process, and then subsequently storing them properly. For a more in-depth discussion on the freeze drying process, see Bacterial Lyophilization: An Overview. The following general protocol is aimed at providing a guide for individuals who are new to lyophilizing bacteria. Every lab has different tools and instruments, thus suggestions on how to adapt available materials to the standard protocol are provided.

Key Considerations

- No one method will work for all microorganisms. A method developed for one freeze dryer will not necessarily translate to a different make or model.

- Not all microbes can be successfully freeze dried. Certain strains, such as mutants with deficient membranes, may not survive the process or may die off rapidly once freeze dried.

- Lyoprotective media, such as skim milk solution, sucrose solution, or other freeze drying buffers can dramatically impact survival rates.

- Vials used for freeze drying should always be made of glass. Atmospheric water can diffuse into plastic tubes and damage freeze dried samples. Furthermore, samples are safest when flame sealed under vacuum in glass ampoules or tubes.

- Freeze drying protocols should be tested before committing to large scale projects. Evaluation can take 1-2 months and yield results that indicate cells will be stable for long durations.

- The liquid used to rehydrate freeze dried bacteria can impact viability. Culture broth, e.g., TSA or LB, is
Preparation

Like any process, the preparation can take longer than the hands-on portion of the freeze drying itself. It is important that all solutions, vials, stoppers, glass wool, etc., are sterilized before starting.

Documentation - It is vital to keep accurate records of lyophilized bacteria as well as using dependable techniques for labeling individual samples. We recommend using a hard copy, freeze drying log which can be used to record each batch of samples processed. The labeling of tubes/vials is also critical and should be done using a durable label. Tube writing pens and hand labeling are options as long as the ink is indelible. A clever labeling technique can be done by placing small, sterile paper labels inside the sample tube (usually lodged against the walls so it doesn't actually touch the culture) which is subsequently sealed in the tube along with the sample.

Lyophilization Medium - Bacteria need a lyoprotectant which helps them survive the freeze drying process. This medium can be very simple, such as 10% skim milk, or complicated such as those that use animal sera. Good media have two main components: the lyoprotectant that stabilizes the cells when water is removed, and matrix agent that allows the entire sample to retain its shape during and after processing. Disaccharides such as sucrose and trehalose are excellent lyoprotectants. Matrix forming additives, often referred to as excipients, include mannitol, BSA, serum, and skim milk. Following are several common media used for bacterial lyophilization:

- **10% Skim Milk** - To prepare, mix 10 gm dry skim milk with 100 ml deionized water and sterilize by autoclaving. This medium is not overly effective but it is inexpensive and easy to prepare. Bacteria which are lyophilized in skim milk have 10% or less viability as compared to some of the better formulations listed below.
- **10% Sucrose** - Dissolve 10 gm of sucrose in 100 ml deionized water and sterilize by autoclaving. Sucrose is a lyoprotectant and provides good viability, especially when compared to skim milk. However, bacteria preserved in sucrose must be kept cold during lyophilization to prevent melting and collapse of the sample.

Reagent 18 - This ATCC formulation is more involved to prepare, but the results are well worth it. Add 0.75 gm Trypticase Soy Broth, 10 gm sucrose, and 5 gm BSA Fraction V to 100 ml deionized water. BSA will denature and clump if autoclaved, thus sterilize by filtration using a 0.2 micron filter. Microbial Freeze Drying Buffer (OPS Diagnostics, Lebanon, NJ) - This commercial formulation is very similar to Reagent 18, but it lacks animal protein (i.e., there is no BSA). For large scale projects, it is more cost effective than using BSA which tends to be very expensive.

Lyophilization Vials/Tubes - Vials and tubes used for lyophilizing bacteria (or anything for that matter) are made of glass. Plastic doesn't work, especially microfuge tubes, for long-term storage (water can pass through plastic!). The choice of vial or tube is very important for long-term survival of freeze dried bacteria. The difference between vials and tubes (ampoules being considered as a tube) is that vials are sealed with a stopper while tubes/ampoules are sealed with a flame. Following are more details on vials and tubes.

Vials are designed for freeze drying in a shelf lyophilizer which is equipped with a stoppering plate. Vials are filled and then fitted with a split stopper, i.e., a stopper which has a notch that allows gas flow while sitting loosely in the vial opening. After freeze drying, the stoppering plate is lowered and pushes the stoppers into the vial under vacuum. When the vacuum is released, atmospheric pressure secures the stopper and the vacuum within the vial. After removing from the lyophilizer, stoppers are further secured with an aluminum band which is crimped in place. Vials are very convenient and easy to use, but they can leak during long-term storage. For the short-term, they are very good.

Tubes, including ampoules, are the best container for long-term storage of freeze dried bacteria. These are most commonly attached to a manifold that holds multiple tubes. Tubes and ampoules are frozen using a freezer or dry ice bath and then quickly connected to the manifold before they melt. After the samples are dry, the neck of the tube or ampoule is sealed off using a propane or acetylene torch. Tubes and ampoules sealed under vacuum are impervious to moisture (assuming there are no pinhole leaks). The downside is that they require much more labor in comparison to vials.

There are many different configurations of tubes, the most practical which will be described here.

- Virtually any borosilicate glass test tube or tubing can be used for freeze drying. Borosilicate glass is more difficult to seal than soda-lime tubes, but it is more durable. In using tubes, the culture medium is added to a sterile tube which is then loosely plugged with sterile glass wool. The sample is frozen, hooked to the vacuum, and processed. Once dry, a torch is used to seal the tube between the sample and vacuum manifold.
- Ampoules are the easiest container to seal with a flame due to their design. As above, the cell suspension is added to a sterile ampoule, usually with a Pasteur pipette or very narrow micropipette tip. The ampoule is then loosely plugged with sterile glass wool. Following processing, the ampoule is sealed using a flame. As
the ampoule has a very thin neck, it is much easier to seal. Ampoules can also be purchases pre-scored which makes cracking them open very easy as compared to tubes.

Culturing and Preparing Bacteria

Using good aseptic culturing technique, grow cells in liquid culture or on agar plates until solid cultures are dense or liquid cultures are in early stationary phase. In practice, this would be equivalent of shaking a bacterial culture overnight or letting cells grow on a plate for 1-2 days. For agar cultures, growing cells on slants (glass tubes) may be more practical as it omits the centrifugation step below. However, liquid cultures normally yield a greater number of viable cells.

For liquid cultures, the cells are centrifuged, the culture broth is removed, and the pellet is suspended in an equal volume of lyophilization medium. We recommend Reagent 18 or the Microbial Freeze Drying Buffer, though skim milk and sucrose will work. For agar cultures, flood the plate/tube with 5-10 ml of lyophilization medium. Using a sterile pipette, flush the medium over the colonies to dislodge the cells. Transfer the cell suspension to a sterile tube. We recommend that a cell count is performed which can be compared to cells following freeze drying. A simple dilution to extinction protocol is available here.

Aliquot the cell suspension into sterile vials or tubes. Only 250-500 µl is needed per vial as this represents around 10^9 bacteria. Place split stoppers on the vials or loosely plug tubes with glass wool. Vials with split stoppers are technically open to the air, thus at risk of contamination. In practice we have not found this to be an issue. If a vial contains upwards of a billion bacteria, one or two contaminating microbes become insignificant when that culture is rehydrated and streaked. If there is fear of contamination, or containment, then glass wool or cotton can be placed under the stopper to prevent contamination.

Freeze Drying Process - Shelf Lyophilizer

Turn on the lyophilizer and start the condenser. If there is an external condenser using a dry ice/ethanol mixture then prepare this as well. The shelf can be set to 4°C.

Center the vials on the shelf. This placement is important so that the stoppering plate can evenly press on the stops following freeze drying.

Using either manual or programmed controls, freeze the samples down to -40°C. This step should take approximately 30-60 minutes and is very dependent upon the instrument. If the rate of freezing can be controlled, then a drop of 1°C per minute is a practical rate. Once the samples reach temperature, they should be visibly frozen (clear liquid turn opaque and skim milk appears solid).

Allow the sample to sit at -40°C for 1 hr to ensure complete freezing. Vials at the center of a cluster may freeze more slowly than those on the outside.

Turn on the vacuum pump. Within 10-20 minutes, the vacuum should be under 200 millitorr (mtoorr). Depending on the instrument, pressure may be reported in mtoorr, mbar, Pascal, or "inches Hg" on a vacuum gauge. For reference, 100 mtoorr = 0.133 mbar = 13.3 Pascal = 29.9° Hg = 0.000132 atm = 99.99% vacuum.

Once the vacuum is below 200 mtoorr, increase the temperature of the shelf for primary drying, the phase associated with water sublimation. The temperature of the shelf is dependent upon the lyophilization medium. For sucrose, keep the shelf temperature at -25°C. For Reagent 18 or Microbial Freeze Drying Buffer, the shelf can be as high as -15°C. In any case, the greater the difference in temperature between the shelf and the condenser/ice trap, the more efficient the primary drying process will be.

If melting of the samples occurs, then it might be necessary to empirically determine a shelf temperature. A practical means to do this involves placing a sample of the lyoprotective medium on a shelf temperature and incrementally lowering the temperature every 15 minutes. At some point the sample freezes. Under vacuum with a cold trap, your sample will be safe and will remain frozen. This is a practical method and is certainly not necessarily the most efficient primary drying temperature, but it should work well enough.

Primary drying is the longest phase of the freeze drying process. The idea is to keep the sample colder than condenser (or ice trap) but still sufficiently warm so that water sublimes rapidly. The temperature of the shelf can be raised to above the melting temperature as long as the sublimation process removes the heat flowing into the sample sufficiently fast to prevent melting and sample collapse (where the matrix literally caves in). The time for primary drying will also depend upon the volume of the sample. For bacteria, samples rarely need to be large and typically are 0.25 to 0.5 ml. A limited number of samples (10-20) in a shelf dryer can be completed in just a couple of hours. A fully loaded dryer with several hundred samples may take longer. Safely, a primary drying period which is overnight should work, but test this first before you attempt to freeze dry large numbers of vials. As a standard guide, freeze dry overnight.

Samples still contain moisture following primary drying. The amount is debatable, but it somewhere between 2 and 4%. This moisture level needs to be reduced and that is done by pumping heat into the sample during the secondary drying phase. This phase is relatively short, lasting 1 to 2 hours, but important for long-term viability. However, over drying of the bacteria can be detrimental as well. Once again, based on the idiosyncrasies of your lyophilizer
and samples, the ideal time for secondary drying needs to be determined experimentally. Generally, raise the shelf temperature to 20°C and dry for 2 hours.

With the vacuum in place, stopper the vials using the stoppering plate/mechanism. Release the vacuum, remove the vials, and further secure the rubber bungs/stoppers with foil crimp seals. It is best to store the vials at 4°C in the dark.

Test the freeze dried bacteria for viability as compared to the original culture (see link). Additionally, monitor the stability/viability of the freeze dried cultures by testing at 30, 90, 180 and 365 days. A good protocol will yield nearly 100% viable cells. Anything above 50% is considered acceptable by many labs. Skim milk will yield 10-20%. However, % viable after freeze drying is not as important as the number viable following storage. If viability starts to decline rapidly by a log or more per month, then modification of the protocol is probably necessary. Note that some strains are simply very difficult to freeze dry and no matter what, these may die off quickly following lyophilization.

Freeze Drying using a Manifold

Once bacteria have been dispensed into vials or tubes, freeze in a -80°C freezer or equivalent. Flash freezing can be done in a dry ice/ethanol bath, but such samples tend to dry slower. Keep the samples frozen (use dry ice if necessary) until they are connected to the manifold.

Turn on the lyophilizer and condenser/cold trap. The manifold valves should be turned off and the vacuum turned on. Allow the vacuum to pull down to 200 mtorr or less.

 Expediently connect a vial/tube to the manifold and open the valve. The vacuum will immediately start the sublimation process and pull heat from the sample. In turn, hook up the remaining samples. The vacuum will increase each time a valve is open, but it should begin to lower immediately. If the vacuum doesn't drop after a tube is attached, there might be a leak in that connection thus shut that valve and proceed to the others.

Freeze drying with a manifold relies on ambient heat to drive the sublimation of the water. As the available water decreases, the temperature will gradually climb to ambient. This may take 2-3 hours. Often frost that formed on the outside of the tubes will dissipate once the sample is done.

Using an acetylene torch (propane will work but it takes longer), seal each vial or tube. Wear safety glasses to protect your eyes from shattering glass and gloves (such as cotton gardening gloves) to protect your hands from the hot glass.

Sealed vials should be stored at 4°C in the dark.

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