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# Human and Animal Viruses

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## **Background**

### **Historical Perspective**

Shackell's successful treatment of rabies virus in 1909 represents the first published account of preservation of an animal virus using a process that was an early version of freeze-drying (Shackell, 1909). Prior to this, viruses were maintained by passage in animals. However, freeze-drying provided a way to maintain virally infected material over long periods of time with relative ease when compared to serial passage in a susceptible animal host. The first experiment to demonstrate that this method could provide long-term stability was reported for a bovine virus that had been freeze-dried in 1916 and was shown to be viable after being maintained at room temperature for 30 years (Fasquelle and Barbier, 1950). Later, as mechanical freezers became more available, freezing viruses at  $-25^{\circ}\text{C}$  or less was adopted as a practical and reliable method of virus preservation. Influenza virus-infected mouse lung tissue and yellow fever virus were successfully frozen at  $-76^{\circ}\text{C}$  by Turner (1938), and Horsfall (1940), using Turner's methods, demonstrated successful storage of influenza, mouse pneumonitis, and canine distemper viruses. Following these early successes, viral repositories, diagnostic and research laboratories, and vaccine manufacturing facilities have relied on freezing and freeze-drying for preservation of viruses and viral vaccines.

The reasons for preserving viruses are similar to those for the preservation of other microorganisms and cells: successful preservation allows for long-term maintenance of consistent stocks and for reproducibility in the testing of viral samples and manufacture of vaccines. For example, field and clinical isolates need to be preserved for transport to testing laboratories; harvested virus from animals or cell cultures may not be processed immediately and require preservation until used. Viruses held to a low number of passages in animals or cell cultures represent a viral population that is similar to that found in nature, and freezing these pools guards against genetic mutations that may occur during subsequent passage. Aliquots of viral stocks frozen at a designated passage level can then be used for multiple and repeatable experiments with the same viral population. Furthermore, it is extremely important that consistency be maintained during the production of viral vaccines; new lots of final product can be prepared with frozen viral seed stocks that consistently reproduce the desired immunogenic and attenuation characteristics. For example, the World Health Organization (WHO) maintains aliquots of the original attenuated poliovirus strains developed by Dr. Albert Sabin in the 1950s. These aliquots are frozen and, when amplified properly and kept to the prescribed low passage level, should produce a vaccine today with the same properties that characterized the original Sabin vaccine. Similarly, viruses that are currently under development for use as vectors for gene therapy must be highly characterized and homogeneous for safe and effective use and need to be maintained as frozen stocks.

In order to better appreciate the requirements for freezing and freeze-drying of human and animal viruses, some consideration must first be given to understanding the structural and functional organization of this diverse group of microorganisms. The classification of viruses is based on morphological and physiochemical properties. Thus viruses are divided into those with DNA or RNA genomes and further subdivided into families based on size and structural properties. There are 6 families of DNA viruses and 13 families of RNA viruses (see Table 1). The genomes of viruses within a given family are similar in terms of their polarity, genome organization, and method of replication and have morphologic features in common, including size, shape, nucleocapsid symmetry, and presence or absence of an envelope. Other characteristics that help to classify viruses include sensitivity to low pH, heat, and lipid solvents. Sensitivity to pH is determined by subjecting a virus to a pH of 3.0. Loss of titer greater than one  $\log_{10}$  indicates that the virus is pH sensitive and acid labile. Heat treatment of a virus for 30 min at 50°C is a test of thermal stability. If the virus survives this treatment, with no more than a one  $\log_{10}$  loss of infectivity, it is considered to be thermostable. Treatment with lipid solvents, commonly ether and

**TABLE 1**  
Properties of the Families of Viruses

Family	Example	Genome <sup>a</sup>	Virion	
			Shape	Size (nm)
DNA viruses				
Hepadnaviridae	Hepatitis B virus	ds, circular	Spherical	42
Papoviridae	Papillomavirus	ds, circular	Icosohedral (72) <sup>b</sup>	45–55
Adenoviridae	Adenovirus	ds, linear	Icosohedral (252)	70–90
Herpesviridae	Herpes simplex, EBV	ds, linear	Icosohedral (162), enveloped	120–200 (envelopes), 100–110 (capsid)
Poxviridae	Vaccinia	ds, linear	Brick-shaped, enveloped	300 × 240 × 100
Parvoviridae	Parvovirus B19	ss, linear	Icosohedral (32)	18–26
RNA viruses				
Picornaviridae	Poliovirus	ss, 1, +	Icosohedral	25–30
Caliciviridae	Norwalk virus?	ss, 1, +	Icosohedral	35–40
Togaviridae	Rubella	ss, 1, +	Spherical, envelope	60–70
Flaviviridae	Dengue virus	ss, 1, +	Spherical, envelope	40–50
Orthomyxoviridae	Influenza virus	ss, 8, –	Spherical envelope	80–120
Paramyxoviridae	Measles virus	ss, 1, –	Spherical, envelope	150–300
Coronaviridae	Coronavirus	ss, 1, +	Spherical, envelope	60–220
Arenaviridae	Lassa fever virus	ss, 2, –	Spherical, envelope	50–300
Bunyaviridae	Hantaan virus	ss, 3, –	Spherical, envelope	90–100
Retroviridae	HIV-1	ss, 1, +	Spherical, envelope	80–110
Rhabdoviridae	Rabies virus	ss, 1, –	Bullet-shaped, envelope	180 × 75
Filoviridae	Ebola virus	ss, 1, –	Filamentous, envelope	800 × 80
Reoviridae	Rotavirus	ds, 10–12	Icosohedral	60–80

<sup>a</sup> ds, Double stranded; ss, single stranded; +, positive stranded RNA genome; –, negative stranded RNA genome.

<sup>b</sup> Number of capsomeres per virion.

chloroform, determines whether a virus has a lipid-containing envelope. Despite the similarities among the physical properties of these families of viruses, relatively few general rules for freezing or freeze-drying viruses can be made. For example, it is common to use membrane-stabilizing agents to preserve viruses with a lipid envelope. Picornaviruses, which lack a lipid membrane, maintain infectivity in the presence of  $MgCl_2$ . However, the optimal conditions for freezing specific viruses need to be determined empirically.

### Industrial Importance

Significant reduction of morbidity and mortality for many viral infections has been accomplished by the successful application of viral vaccines. In the case of smallpox, eradication of this important human pathogen has been realized. Polio has now been targeted as the next viral disease that can be eradicated by immunization. Vaccine production requires the use of the "seed lot system" for consistent reproduction and quality control of vaccine lots. Vaccine virus seeds are usually frozen at two passage levels prior to vaccine lot production and these stocks of virus are designated the master seed and the production or working seed, respectively. Multiple aliquots of these seeds are prepared for reference as well as for characterization and standardized testing for possible contaminants. Finally, it is also important to distribute the storage of the master virus seed and working seed among multiple freezers in order to be assured of preserving part of the stock in the event of a mechanical failure. The seed lot system establishes a viral stock that can be standardized and provides a source of viruses so that vaccine lots manufactured over decades will be closely related to the original seed lot. This is especially important for live-attenuated vaccines that in most cases have been derived by multiple serial passage of a clinical isolate of a virus. Deviation from the attenuated passage number may result in undesirable phenotypic changes of the attenuated vaccine virus. This was the case with yellow fever vaccine when uncontrolled passage of this virus resulted in a vaccine with a higher than normal incidence of side reactions (Fox *et al.*, 1942).

Presently, researchers are using the tools of molecular biology to produce a new class of recombinant viral agents that will be used for immunization or genetic therapy. For example, mammalian retroviruses that integrate into the host cell genome are being developed as vectors for delivery of human DNA sequences. The newly acquired DNA is expressed in the form of protein(s) that may be deficient in the host cell or may require *de novo* synthesis. This form of genetic therapy has been demonstrated routinely *in vitro* and *in vivo* in animal experiments and is being proposed as therapy

for some human genetic diseases (for review, see Roemer and Friedmann, 1992). In addition, recombinant viral vectors are also paving the way for a novel type of therapy in which cancer cells can be specifically targeted for infection by a recombinant virus; following infection the inserted gene is expressed and the gene product selectively makes the infected cancer cells more susceptible to subsequent chemotherapy or to natural immune surveillance in the host (Collins *et al.*, 1992; Culver *et al.*, 1992). Alternatively, recombinant vectors may express antisense RNA that can bind to and block the activity of the host gene sequences. Successful experiments of this kind require viral vectors that are thoroughly characterized and safe. Seed stocks of the recombinant viral vectors constructed to express the desired nonviral gene sequences must be frozen or freeze-dried in order to be preserved for future experiments and clinical trials.

A somewhat similar approach is being developed for vaccination using viral vectors as delivery vehicles for genes important for immunization. For example, one approach uses a highly attenuated vaccinia virus that carries, as part of its genome, various immunogenic proteins that would evoke protection against infection (Moss and Flexner, 1987). Other viral vectors used similarly include adenovirus and canarypox.

Antigens used for viral diagnostic purposes must also be quality controlled and, in general, would also be produced from a virus seed lot system. Variation of quality or quantity of viral antigens in diagnostic kits and reagents may directly influence results in the diagnostic laboratory. Regulation of the manufacture of diagnostics is similar to those for vaccine manufacture. Regulations concerning the manufacture of biological products are published by the United States Public Health Service (21 CFR 600) and outline the requirements for control of reagents and raw materials in the manufacture of diagnostics to screen blood and blood products. For these purposes, it is essential to use viral seed stocks that are appropriately characterized and preserved by freezing.

### **Characterization**

Viability of viruses is usually determined by the use of infectivity assays. These include assays whereby viral cytopathology is measured in cell cultures or, when this is not possible, potency may be determined by inoculation of animals or eggs. By far the most accepted and accurate quantitative measure of viral infectivity is the plaque assay. Most viruses form plaques in susceptible host cells. Generally, after virus inoculation on suitably sensitive cell monolayers, an agar overlay is applied so that viral cytopathology is localized. After an incubation period, viable cells are stained with a vital dye, revealing areas of cell death, the plaque-forming unit. Most viral

freezing and recovery experiments use the plaque assay to titer virus before and after treatment as a measure of viral activity. In this way, one can determine the efficiency of the freezing or freeze-drying process. Electron microscopy (EM) has also been used on occasion to visualize the effects of freezing and freeze-drying. These EM experiments are limited by the purity and titer of the viral preparation as well as the subjective interpretation of observations.

## Methods for Preservation

### Harvesting and Preparing Viruses

The success of freezing or freeze-drying of viruses is dependent on the proper preparation of the harvested virus. The source of virus may be animal tissues or organs, animal sera, virus-infected cell cultures, and, on occasion, egg allantoic or amniotic fluids.

The harvested tissue will depend on the particular host-range of the virus being grown. Harvesting methods are also dependent on the properties of viral replication. Some viruses, e.g., herpes viruses, stay cell associated whereas other are readily released from the cell into the culture fluids. In general, viruses grown after passage *in vivo* require disruption of the infected animal tissue or organ by homogenizers or aerosol-controlled blenders. Diluent consisting of buffer or cell culture media is added for release of the virus into the aqueous phase during cell disruption. Depending on the virus, serum may be required as an additive to the diluent for optimal freezing (Table 2). Viruses that are found in blood may be harvested and frozen directly in the serum after separation from blood cells or by cocultivating infected peripheral blood lymphocytes (PBLs) with susceptible cell lines. These preparations may be frozen without further treatment, although it is generally recommended that serum or PBLs be frozen and thawed only once to achieve optimal virus recovery. Similarly, cell culture harvests may require disruption of the cell monolayer as a first step. Cells may be scraped from the culture vessel surface into the culture fluids or these fluids may be removed and the cells treated separately. In either case, cells are disrupted and virus is released. In certain cases, virus-infected cells are harvested for a seed preparation. Gentle scraping and harvesting of these cells into the culture fluids or into fresh buffer or cell culture medium is required. The specific details for optimal harvesting procedures for individual viruses may be found in the literature.

Usually animal serum is used as a component of cell culture medium. Fetal bovine serum (FBS) is the most frequently used additive by cell

**TABLE 2**

Optimal Conditions for Freezing and/or Freeze-Drying Some Mammalian Viruses

Virus	Stabilizers	Storage temperature	References
Herpes	Serum, 7% DMSO in culture medium	-70° frozen	Wallis and Melnick (1968)
Herpes	Sodium glutamate	-20°C freeze-dried	Scott and Woodside (1976)
Varicella-zoster	Sucrose, sodium glutamate, albumin, KPO <sub>4</sub>	-70°C frozen	Grose (1981)
Dengue	20-50% fetal bovine serum, 0.75-2.0% bovine albumin	-70° frozen	Shope and Sather (1979)
Sindbis	20-50% fetal bovine serum, 0.75-2.0% bovine albumin	-70°C frozen	Shope and Sather (1979)
Measles	Serum, DMSO	-70°C frozen	Wallis and Melnick (1968)
Vesicular Stomatitis	Serum, DMSO	-70°C frozen	Wallis and Melnick (1968)
Respiratory syncytial	MgSo <sub>4</sub> and HEPES buffer	-70° C frozen	Fernie and Gerin (1980)
Adenovirus	Culture medium	-70°C frozen	Wallis and Melnick (1968)
Poliovirus	Culture medium	-70°C frozen	Grieff and Rightsel (1967)
Poliovirus	Dialyze out salts	-20°C freeze-dried	Berge <i>et al.</i> (1971)
Vaccinia	Culture medium	-70°C frozen	Wallis and Melnick (1968)
Influenza	Serum	-70°C frozen	Dowdle <i>et al.</i> (1979)
Influenza	0.5% gelatin	-20-4°C freeze-dried	Dowdle <i>et al.</i> (1979)
Parainfluenza	0.5% bovine albumin, serum	-60°C or greater	Chanock (1979)
Mumps	Chicken amniotic fluid	-70°C	Hopps and Parkman (1979)
Rabies	0.75% bovine albumin, serum	-70°C	Johnson (1979)
Hepatitis A	2-40% stool suspension	-70°C	Feinstone <i>et al.</i> (1979)
Hepatitis A	Culture medium	-70°C	Feinstone <i>et al.</i> (1979)



culturists and virologists because it is nontoxic to most cells and the protective effect during freezing is consistent. Therefore, FBS doubles for cell nutrition as well as a cryoprotectant for freezing and freeze-drying. Concentrations used for cell culturing and growing viruses range from 0.5 to 5% in cell culture medium. In many cases the cell culture medium containing serum constitutes the cyroprotective diluent that will be used for freezing. However, further addition of serum is required for many viruses (see Table 2). Infectious materials should be handled according to the biosafety guidelines that apply for each virus.

## Freezing

### TEMPERATURE

The rate of freezing and the final temperature must be considered for virus preparations being frozen for long-term storage. To maintain viral infectivity throughout this process, the integrity of the viral capsid, the viral envelope (if there is one), and the viral nucleic acid must be preserved. Dimmock (1967) demonstrated that viral infectivity may be reduced by heat damage to both viral proteins and nucleic acid. He showed that the stability of these components varies with elevated temperature so that inactivation at a particular temperature takes place through whichever component is least stable at that temperature. In general, most viruses may be "snap frozen" in a dry ice/ethanol bath prior to storage at temperatures  $\leq -70^{\circ}\text{C}$ . It may be preferable to freeze some viruses, such as cytomegalovirus or varicella-zoster virus, as viable infected cells. In this case, a slow controlled rate of freezing is recommended: cooling should be done at  $1^{\circ}\text{C}/\text{min}$  to  $-40^{\circ}\text{C}$  and then at  $10^{\circ}\text{C}/\text{min}$  to  $-90^{\circ}\text{C}$  (Simione and Brown, 1991). It has also been reported that influenza and measles virus may benefit from this two-step freezing process (Rowe and Snowman, 1976).

Storage temperature is also critical for some viruses with envelopes, such as influenza virus, respiratory syncytial virus, and measles virus. These all require storage at  $-65^{\circ}\text{C}$  or lower for optimal recovery after long-term storage (Rightsel and Greiff, 1967; Greiff *et al.*, 1964; Law and Hull, 1968).

It is common practice for today's virologist to store frozen viruses in ultralow-temperature freezers at temperatures of  $-65$  to  $-85^{\circ}\text{C}$ . Freezing in liquid nitrogen or its vapor phase, a common practice for preservation of mammalian cells, is not necessary for most viruses. Liquid nitrogen storage may be used as a "backup" system for viral seed repositories. No known conventional virus requires  $-196^{\circ}\text{C}$  (liquid) or  $-150^{\circ}\text{C}$  (vapor) nitrogen for short- or long-term storage.

### CRYOPROTECTANTS

In one of the first comprehensive studies of freezing and freeze-drying with representative members of most of the RNA and DNA virus families,

Rightsel and Greiff (1967) demonstrated the importance of the suspending medium used for freezing and freeze-drying. Medium containing skim milk, calcium lactobionate, normal serum albumin, dimethyl sulfoxide (DMSO), glycerol, or magnesium chloride promoted better freezing and freeze-drying recovery with a range of viruses, especially enveloped RNA viruses. Wallis and Melnick (1968) further demonstrated that DMSO or serum acted as a cryoprotectant for four different enveloped viruses, herpes virus, measles virus, Sindbis virus, and vesicular stomatitis virus. Under the conditions described DMSO performed somewhat better than serum as a cryoprotectant. The nonenveloped viruses, vaccinia, adenovirus, and poliovirus, did not require DMSO or serum for retention of viability during freezing. The authors concluded that enveloped viruses were similar to mammalian cells and that DMSO stabilizes the viral envelope much as it stabilizes the mammalian plasma membrane. However, another member of the herpes virus family, varicella-zoster (VZ), is especially sensitive to freezing and freeze-drying. Grose *et al.*, (1981) used sucrose, potassium phosphate, sodium glutamate, and albumin in a cryoprotective “cocktail” for VZ virus. For both cell-associated and cell-free virus, the cocktail was fully protective, with 100% recovery of infectivity after freezing to  $-70^{\circ}\text{C}$  and freeze-drying. Additionally, these authors found that glutamate and albumin could be removed from the cocktail without loss of protection. In contrast, Scott and Woodside (1976) showed that sodium glutamate was essential for the stability of freeze-dried herpes virus.

Certain viruses are stabilized by the addition of divalent cations. For example, respiratory syncytial virus was found to be stable for up to 1 month at  $4^{\circ}\text{C}$  following the addition of magnesium sulfate, and the titer of virus was maintained through several cycles of freezing and thawing when  $\text{MgSO}_4$  was added (Ferne and Gerin, 1980). Likewise, poliovirus was stabilized in the presence of  $\text{MgCl}_2$  (Melnick *et al.*, 1963). Alternatively, 300 mM sodium phosphate at pH 6.7 stabilized poliovirus at  $0-6^{\circ}\text{C}$  for up to 12 months (Mauler and Gruschkau, 1978).

Cryoprotectants and the rate of freezing minimize the formation of ice crystals that can damage the viruses; similarly, it is common practice to rapidly thaw frozen viral stocks at a previously determined optimal temperature to minimize ice crystal formation.

## Freeze-Drying

### FREEZE-DRYING CYCLES

Freeze-drying, or lyophilization, is a method for preserving viruses and other biological materials by removing water from frozen samples via sublimation. The result is a dried preparation that is usually more stable than

wet-frozen preparations at various temperatures and for longer periods. The process is usually divided into three stages: prefreezing, sublimation drying under vacuum (primary drying), and desorption or secondary drying. Prefreezing can be accomplished in special freezers or in the freeze-dryer itself. A freeze-dryer has a vacuum pump that removes air from a chamber that holds vials of the material to be freeze-dried. Water vapor generated through this process is condensed into a refrigerated trap and is not allowed to enter the vacuum pump. The rate of sublimation can be controlled by regulating the heat transfer to the frozen material. After final drying, inert gas is used to fill the vials and protect the freeze-dried material from the deleterious effects of oxygen. The freeze-drying cycle requires optimization for each virus.

A typical cycle that has been used successfully for dengue live-attenuated virus vaccines (all four serotypes) consisted of freezing to  $-40^{\circ}\text{C}$  for 2 hours and allowing the condenser to reach a temperature of  $-60^{\circ}\text{C}$  (K. Eckels, unpublished data). Vacuum was drawn to  $100\ \mu\text{m}$  of Hg, at which time heat was applied at a controlled rate overnight (approximately 18 hours). Final drying occurred at  $20\text{--}23^{\circ}\text{C}$  for 2 hours followed by backfilling the vials with dry, sterile nitrogen and capping. Vaccine vials were finally stored at  $-30^{\circ}\text{C}$ .

Another cycle that preserves viral infectivity has been used for dengue candidate vaccines; this consisted of freezing to  $-60^{\circ}\text{C}$  overnight followed by raising the temperature to  $-20$ ,  $0$ , and finally to  $10^{\circ}\text{C}$  over a 2-day period while vacuum was applied (K. Eckels, unpublished data). In both cases, care was taken to avoid subjecting this thermolabile virus to prolonged periods of high temperatures that could result in inactivation of viral infectivity. Greiff and Rightsel (1967) have successfully freeze-dried influenza and measles virus with cycles extending over a 48-hour period with shelf and product temperatures not exceeding  $0^{\circ}\text{C}$ . Greiff (1991) has outlined an approach for the development of a successful freeze-drying cycle. Similarly, by varying drying time, shelf temperature, and vacuum pressure an optimal lyophilization cycle for varicella-zoster vaccine was determined (Bennett *et al.*, 1991). Freeze-drying methods and cycles used by the American Type Culture Collection (ATCC) for their virus stocks can be found in Simone and Brown (1991).

#### STABILIZERS AND ADDITIVES

Stabilizers added to the viral suspension medium for freeze-drying are many of the same used for freezing. These additives promote preservation of viral infectivity by acting as antioxidants and by providing "bulk" to the virus preparation to allow easier reconstitution. Also, they should be nonhygroscopic to avoid moisture contamination of the freeze-dried mate-

rial. For vaccines, the stabilizers must also be nonimmunogenic and nonreactive in the vaccine recipient. One of the first stabilizers used for viral freeze-drying was 2.5% gum acacia to preserve the infectivity of vaccinia virus (Rivers and Ward, 1935). Peptone and normal horse serum were also successfully used for freeze-drying of vaccinia virus (Collier, 1951; Sparkes and Fenje, 1972). Calcium lactobionate and human serum albumin were cryoprotective for measles virus during freeze-drying (Greiff and Rightsel, 1967), whereas cell culture medium alone was less protective. A combination of sucrose, phosphate, sodium glutamate, and albumin was used for freeze-drying pseudorabies and herpes viruses (Scott and Woodside, 1976; Calnek *et al.*, 1970). Varicella-zoster virus was also freeze-dried successfully in this combination of stabilizers, but Grose (1981) found that he could eliminate glutamate and albumin from the mixture and still retain 100% recovery of this very labile virus. DMSO cannot be used for stabilization in freeze-dried preparations because it becomes concentrated to toxic levels (Greiff and Rightsel, 1967).

A group of viruses that requires special attention to stabilizing media is the enteroviruses, for example, poliovirus and hepatitis A virus. Early work with poliovirus demonstrated that it had a high degree of lability when freeze-dried, even with stabilizers. The lability is due to the presence of inorganic salts that may be present in the culture medium. When these are removed by dialysis prior to freeze-drying, losses do not occur (Berge *et al.*, 1971). The ATCC uses ultrafiltration, a more rapid type of dialysis to remove salts prior to freeze-drying of enteroviruses (Simione and Brown, 1991).

Vaccines that require freeze-drying have special stabilizer requirements that allow them to be used safely in humans. Animal serum cannot be used as a stabilizer and must be completely removed from the vaccine prior to bottling. When a protein substitute is required, human serum albumin has been used successfully for many vaccines. More recently, hydrolyzed gelatin has been used as a replacement for albumin (Table 3). Both of these stabilizers are acceptable for human vaccines because they usually do not stimulate deleterious immune responses in the vaccinee. Sugars such as sucrose, lactose, and sorbitol are also used either alone or in combination with a protein stabilizer. Cell culture medium is often used as a buffer because many live vaccines are harvested in their culture medium without further processing.

#### MOISTURE CONTENT

Optimal moisture content of the freeze-dried product needs to be empirically determined for each viral preparation. A good freeze-drying cycle can attain a moisture content of 1% or less in the final product.

**TABLE 3**

Optimal Conditions for Stabilization and Storage of Licensed and Experimental Viral Vaccines

Vaccine	Stabilization	Storage
Smallpox (Dryvax; Wyeth Laboratories)	No stabilizers added to the freeze-dried calf lymph	2–8°C
Measles (Attenuvax; Merck, Sharp, and Dohme)	Sorbitol, hydrolyzed gelatin	2–8°C
Mumps (Mumpsvax; Merck, Sharp, and Dohme)	Sorbitol, hydrolyzed gelatin	2–8°C
Rubella (Meruvax II; Merck, Sharp, and Dohme)	Sorbitol, hydrolyzed galatin	2–8°C
Polio, live oral (Orimmune; Lederle Laboratories)	Sorbitol stabilizer used in this non-freeze-dried vaccine	<0°C (frozen)
Yellow fever (YF-Vax; Squibb/Connaught)	Sorbitol, gelatin	0°C or less (frozen)
Dengue (experimental)	Lactose, human serum albumin, or hydrolyzed gelatin	–20°C or less (frozen)
Rabies (Imovax; Institut Merieux)	No stabilizer used	2–8°C
Influenza(Fluzone; Squibb/Connaught)	No stabilizer used in this formalin-inactivated, non-freeze-dried vaccine	2–8°C

However, depending on the virus, a higher moisture content may be desirable. A varicella virus vaccine with 6–8% moisture following freeze-drying was found to be more stable than vaccines containing less moisture (Bennett *et al.*, 1991). Various viruses have been freeze-dried to approximately 1% moisture and shown to be stable over periods of time at 4°C, but better long-term stability can be achieved by storage at –20°C.

### STABILITY

Assays for retained infectivity following freeze-drying are done on rehydrated vials of freeze-dried virus. A volume of sterile, distilled water is used to reconstitute the vial, with titration of the virus done immediately in the appropriate plaque or infectivity assay. The best control for baseline infectivity would be the virus harvest that was assayed prior to freeze-drying. Often, frozen and thawed specimens are used for baseline titers. Freeze-dried viruses are normally stored at 4°C or –20°C. Accelerated stability studies can be done to test thermostability for shorter periods at elevated temperatures. Often an ambient temperature of 25°C or temperatures of 35–37°C are used for accelerated studies. These temperatures are chosen to study thermostability for viruses, mainly vaccines, that may not have continuous refrigeration available up to the time of use. This is a problem for many live viral vaccines that are being used in developing countries (Widdus *et al.*, 1989).

The largest application of freeze-drying of viruses occur for live-attenuated viral vaccines that are thermolabile. The first viral vaccine to be freeze-dried was yellow fever vaccine (Penna, 1956). Recent advances have resulted in more stable, freeze-dried yellow fever vaccines (Robin *et al.*, 1971; Burfoot *et al.*, 1977). The WHO standard for stability of this vaccine is no more than one log<sub>10</sub> loss of potency held at 37°C for 2 weeks. A recent collaborative study of yellow fever vaccines from 12 manufacturers demonstrated that 7 of 12 vaccines met this requirement. Similar WHO stability standards are in place for live, freeze-dried measles vaccine. A method to freeze-dry and stabilize trivalent, live, attenuated, oral polio vaccine is being sought so that this vaccine can be delivered and used in developing countries without significant loss in potency. It has been proposed that thermoresistant mutants of polioviruses might serve as the prototype for the development of heat-stable vaccine strains (Kew, 1989). Alternatively, organic compounds such as WIN 51711 and R 78206, which bind to poliovirus VP1, have been found to increase the half-life of poliovirus antigen 50- to 250-fold at temperatures up to 48°C (Rombaut *et al.*, 1991). These drugs stabilize the conformation of the viral capsid and exhibit a potent antiviral effect that would not make them suitable as stabilizers for a live virus vaccine. In the future, similar compounds could be designed to

stabilize but not inhibit the replication of live poliovirus strains. Other licensed, freeze-dried live-viral vaccines are those for mumps and rubella, whereas vaccines for varicella (chicken pox) and cytomegaloviruses are being developed that will require stabilization by freeze-drying. Table 3 lists licensed vaccines as well as those still being tested and the available published data on freeze-drying of these vaccines.

## **Virus Repositories**

American Type Culture Collection

12301 Parklawn Drive

Rockville, Maryland 20852

Telephone: 1-800-638-6597 (United States and Canada, only) or 1-301-881-2600

FAX: 1-301-231-5826

Request for international reference materials should be made with a statement of intent from the investigator.

## **International Laboratories for Biological Standards**

National Institute for Biological Standards and Control

Blanch Lane, South Mimms

Potters Bar, Hertfordshire EN6 3QG

England

Telephone: 707-54753/54763

FAX: 707-46730

Contact: Dr. Timothy Forsey

Staten Seruminstitut

80 Amager Boulevard

2300 Copenhagen Street

Copenhagen

Denmark

Telephone: 45-32-95-28-17 or 45-32-68-34-66

FAX: 45-32-68-38-68 or 45-32-68-31-50

Contact: Dr. Jorn Lyng or Gert Albert Hansen

## **Other Laboratories**

Anti-Viral Research Branch

National Institutes of Allergy and Infectious Disease

National Institute of Health

Bethesda, Maryland 20892

Telephone: 301-496-8285

Contact: Thelma Gaither for catalogue of available reagents

## **Centers for Disease Control**

Atlanta, Georgia 30333

Telephone: 301-404-639-3311/3355

FAX: 301-404-639-3037/3296

**Rijksinstituut voor Volksgezondheid en  
Milieuhygiene Postbus 1**

3720 BA  
Bilthoven, The Netherlands  
Telephone: 30-749111  
FAX: 30-742971

**Influenza Viruses and Reagents**

Commonwealth Serum Laboratories  
45 Poplar Road  
Parkville, Victoria  
Australia 3052  
Telephone: 61 3 389 1340  
FAX: 61 3 388 2063  
Contact: Alan W. Hampson

National Institute for Biological Standards and Control  
Blanche Lane, South Mims  
Potters Bar, Herts. EN6 3QG  
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WHO Collaborating Center for Influenza  
Centers for Disease Control  
Influenza Branch G-16  
1600 Clifton Road  
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Food and Drug Administration  
29A/1D10  
8800 Rockville Pike  
Bethesda, Maryland 20892  
Telephone: 301-496-6828  
FAX: 301-496-1810  
Contact: Michael Williams or Roland Levandowski, MD

**HIV**

AIDS Research and Reference Reagent Program  
National Institute of Allergy and Infectious Disease  
National Institutes of Health  
Bethesda, Maryland 20892  
Telephone: 301-340-0245



FAX: 310-340-9245  
Contact: Ogden Bioservices Corp.

### **Arbovirus Collections**

Centers for Disease Control  
Division of Vector Borne Infectious Diseases  
P.O. Box 287  
Fort Collins, Colorado 80525  
Telephone: 970-221-6425  
Contact: Dr. Nick Karabatos

Department of Pathology  
University of Texas Medical Branch  
Galveston, Texas 77555  
Telephone: 409-772-6662  
Contact: Dr. Robert Shope

Epidemiology and Public Health  
Yale University School of Medicine  
New Haven Connecticut 06520  
Telephone: 203-785-6976  
Contact: Dr. Rebecca Rico-Hesse

### **Polioviruses/WHO Repository**

ATCC for Division of Viral Products  
Center for Biologics Evaluation and Research  
Food and Drug Administration  
8800 Rockville Pike  
Bethesda, Maryland 20892  
Telephone: 301-496-5041/301-443-8411  
FAX: 301-496-1810  
Contact: Dr. Ronald Lundquist or Dr. Jacqueline Muller

### **Respiratory Syncytial Virus**

WHO Reagent Bank for RSV and PIV3  
Division of Viral Products  
Center for Biologics Evaluation and Research  
Food and Drug Administration  
8800 Rockville Pike  
Bethesda, Maryland 20892  
Telephone: 301-402-0414/0415  
FAX: 301-496-1810  
Contact: Judy Beeler, MD

### **Measles Virus**

WHO Reagent Bank for Measles Virus  
Insitut Pasteur de Lyon

Unite d'Immunologie et Strategie Vaccinale  
 Avenue Tony-Garnier  
 69365  
 Lyon, Cedex 97  
 France  
 Contact: Dr. T. Fabian Wilde

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