Baculovirus stability in serum-free lyophilized and wet storage conditions

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Abstract

The baculovirus expression vector system (BEVS) is an effective way to produce recombinant proteins for biopharmaceuticals. However baculovirus stocks are stored in subzero temperatures to maintain virus stability, and fetal bovine serum is commonly used in the storage solution. In an effort to lower transportation and storage costs, a storage formulation that can effectively store the baculovirus in above frozen temperatures without the use of FBS would be beneficial. In this study, DMSO, ethylene glycol, glycerol, sucrose, sorbitol, sucrose-phosphate, and sucrose-phosphate-glutamate were added to baculovirus stock at various concentrations to determine the most effective stabilizer for virus storage at 4°C. Of the seven additives studied, 1 M sorbitol most effectively preserved baculovirus stock over a period of 47 weeks stored in 4°C. Formulations that include sucrose, L-arginine, and Pluronic F68 were created to determine their effectiveness on virus stability in a freeze-dried state stored at room temperature. In a lyophilized state, 0.5 M sucrose maintained baculovirus stock stability after 5 weeks of storage. Lyophilized stocks not containing sucrose were no longer infective after 5 weeks.

Dedication

Dedicated to my parents, Michael and Elizabeth Colandro

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Chapter 1: Introduction and Objectives

The *Baculoviridae* are rod shaped double stranded DNA viruses with a genome of 80-180 kbp in size that infect only arthropods (van Oers, 2011). The *Baculoviridae* are divided into two genera, the nuclear polyhedrosis viruses (NPVs) and the granulosis viruses (GVs) (Funk et al., 1997). NPVs contain many virions within large polyhedron-shaped structures, called polyhedrin, while GVs produce small granules that normally include one virion (Funk et al., 1997). NPVs are studied more thoroughly and used in the laboratory more often. The *Autographia califronica* multiple capsid nucleopolyhedrovirus (AcMNPV) is the most widely used baculovirus in research (Possee, 1997). The baculovirus infects insects and can be used in the lab to produce recombinant proteins.

There are many advantages to using the baculovirus for protein expression. The system produces high quantities of proteins in a short amount of time (O'Reilly et al., 1994; Bedard et al., 1994; Shuler et al., 1995). Baculoviruses do not pose a risk to human health because they only infect arthropods and they also carry out posttranslational modifications similar to mammalian cells (Jarvis, 1997; Shuler et al., 1995; King and Possee, 1992). The baculovirus genome can be readily modified to express recombinant proteins due to commercially available kits such as the BaculoGold™ (Drugmand et al., 2012). Although the baculovirus only infects arthropods, the virus can be manipulated to enter mammalian cells, broadening the range of proteins the virus can successfully produce (Monsma et al., 1996).

There are limitations to the BEVS, however, due to the stability of the virus over time in warmer temperatures (Jorio et al., 2006; Chen et al., 2012). Storage of the virus stock is energy intensive and the stability of the virus decreases over time when stored in

temperatures below freezing (Jorio et al., 2006; Chen et al., 2012). Cryoprotectants, such as sugars, can be used to preserve biological material from freezing damage (Chen et al., 2012; Ohtake et al., 2010; Bakaltacheva et al., 2007). Dimethyl sulfoxide (DMSO), sucrose, and glycerol have shown to help preserve cells and virus particles for long-term storage in subzero temperatures (Jorio et al., 2006). Fetal bovine serum (FBS) has been typically used in the past, usually at concentrations of 10%, in storage solutions (O'Reilly et al., 1994). FBS has many disadvantages including economical and ethical issues, and it would be advantageous to find a storage solution without FBS (Brunner et al., 2010).

It is desirable to store baculovirus stock in a transportable solution that can be shipped at warmer conditions to lower transportation costs substantially. The baculovirus maybe shipped to other factories once a new recombinant baculovirus is engineered or moved from one site to another in industry. The objective of this research is to increase the stability of baculoviruses stored for an extended period of time without using fetal bovine serum in the formulation. To accomplish this goal, baculoviruses will be stored in different solutions where it will be determined which additive is most effective. Formulations will be stored at 4°C and room temperature in both wet storage and lyophilized storage. Baculoviruses titer will be determined throughout the storage period to discover the best storage solution.

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Chapter 2: Literature Review

Abbreviations:

BEVS: Baculovirus expression vector system
NPVs: Nuclear polyhedrosis viruses
GVs: Granulosis viruses
ODV: Occlusion-derived virus
BV: Budded virus
AcMNPV: *Autographia californica* multiple capsid nucleopholyhedrovirus
VLP: Virus-like particle
PTM: Post translational modification
FBS: Fetal bovine serum
DMSO: Dimethyl sulfoxide
EG: Ethylene glycol
SP: Sucrose-phosphate
SPG: Sucrose-phosphate-glutamate

The baculovirus expression vector system (BEVS) is a very powerful system in biomanufacturing (O'Reilly et al., 1994; Bedard et al., 1994; Drugmand et al., 2012). The expression system can produce high concentrations of recombinant proteins that more closely resemble higher eukaryotic proteins than those produced by prokaryotic expression systems. Currently, Ceravix, a vaccine against HPV, is produced using this expression system (Sokolenko et al., 2012; Drugmand et al., 2012). The baculovirus may be stored in 10% (v/v) heat-inactivated FBS for a year at 4°C in the dark, but there is a need for serum-free conditions (O'Reilly et al., 1994; Jarvis et al., 1994). Cryoprotectants and lyoprotectants can be used to stabilize the baculovirus for longer periods of time in colder temperatures and in a freeze-dried state (Jorio et al., 2006).

Baculovirus Characteristics

Baculovirus is a rod shaped, double stranded DNA virus that infects and replicates only in arthropods (Funk et al., 1997). Depending on the virus species, baculovirus genomes range

from 80 to 180 kbp in size (van Oers, 2011). There are two genera of the *Baculoviridae*, the nuclear polyhedrosis viruses (NPVs) and the granulosis viruses (GVs). NPVs contain many virions within large polyhedron-shaped structures, called polyhedrin, while GVs produce small granules that normally include one virion (Funk et al., 1997). The NPVs are used more in the laboratory and are studied more thoroughly.

During a single infection, there are two forms of virus, the occlusion-derived virus (ODV) and the budded virus (BV). ODVs are composed of a matrix made mostly of polyhedrin in NPVS or granulin in GVs (Funk et al., 1997). The envelopes of the two forms differ due to their roles during infection (Funk et al., 1997). The occluded form is designed to withstand harsh environmental conditions, but break down in insect midgut, while the budded form must move and infect cells within the insect (Funk et al., 1997).

The Baculovirus Life Cycle

The occluded form of the nuclear polyhedrosis virus is ingested by the host, for example a widely studied host, lepidopteron, and is dissolved in the insect midgut with a high pH environment, releasing the occlusion virions (Funk et al., 1997). The occlusion virions are passed across the peritrophic membrane (a protective lining secreted in the midgut that surrounds ingested items) (Federici, 1997). The virion envelope interacts with the microvillar membrane of the midgut epithelial cells and enters through fusion of the virion envelope with the microvillar membrane (Federici, 1997). The nucleocapids, which is a protein structure enclosing nucleic acid, travel from the membrane with the microvillus via the microtubules to the nucleus through the nuclear pore (Federici, 1997). The nucleocapsid releases the viral DNA and new DNA is detected within 6 hours post infection (Federici, 1997 and Lu et al., 1997a). Nucleocapids begin to form near the nuclear membrane and then pass through the membrane, acquiring an envelope (Federici, 1997).

The envelope is shed in the cytoplasm and the nucleocapids bud through the plasma membrane, at the base of the midgut cell (basal lamina side), acquiring a new envelope studded with viral proteins, such as gp64 (Federici, 1997). The budded virus enters the hemolymph to produce a systemic infection (Funk et al., 1997).

The budded form of the virus enters the cell through endocytosis, where the virion gp64 envelope fusion protein interacts with host receptors (Funk et al., 1997; Federici, 1997). The plasma membrane of the host cell engulfs the enveloped virion, where the endosomal membrane fuses with the viral membrane through acidification, releasing the nucelocapsid into the cytoplasm (Funk et al., 1997). More budded virions are produced early in the infections and peaks at about 12-16 hours, the production then shifts to occluded virions (Federici , 1997).

Late in the infection, the occluded form of the virus is produced in the nuclei of the insect cell (Funk et al., 1997). High polyhedron production levels are produced when the *polh* gene is activated (Federici, 1997). With the help of the viral p10 protein that triggers nuclear lysis and other proteases, the occlusion bodies are released (Federici, 1997). During occluded virion build up, chitinase, encoded by the virus, is produced, and it hydrolyzes chitin in the lepidopteron (Federici, 1997). Eventually the insect dies and vast amounts of polyhedra are released (Federici, 1997). The occluded virions undergo harsh environmental conditions and are later ingested by another insect and the cycle begins again (Funk et al., 1997).

Baculovirus protein expression vector

The baculovirus expression vector system (BEVS) is a recombinant baculovirus that has been genetically modified by inserting a foreign double-stranded DNA into the genome (Jarvis, 1997). The basis of the BEVS is the ability of the baculovirus to produce high

quantities of the polyhedrin (*polh)* and *p10* genes during late gene expression (Jarvis, 1997; Jurio et al., 2006). The *polh* gene is not necessary in insect cell culture, and is therefore the site of insertion of recombinant DNA that is regulated by the *polh* promoter (Jarvis, 1997). The most commonly used baculovirus is the *Autographia californica* multiple capsid nucleopolyhedrovirus (AcMNPV) (Possee, 1997). The entire genome of AcMNPV has been identified and is currently the most well studied model (Schultz and Friesen, 2009). The AcMNPV can easily infect *Spodoptera frugiperda* (Sf), *Trichoplusia ni* (Tn), *Mamestra brassicae* and *Estigmene acrea* insect cell lines, where protein expression occurs in the early, late, and very late stages of infection (Possee, 1997).

BEVS Advantages and Disadvantages

Advantages

The BEVS can produce large quantities of functional recombinant proteins, similar to prokaryotic systems (Jarvis, 1997). It is possible for the BEVS to produce 100-800 mg/liter of recombinant protein, and in some cases 20-250 times greater levels than that of mammalian cells (Shuler et al., 1995). The recombinant protein concentration is often a significant proportion of total proteins in the host cell (Jarvis, 1997). In addition to high levels of protein, the eukaryotic system has processing abilities, allowing the recombinant protein to appear more genuine (Jarvis, 1997; Shuler et al., 1995; King and Possee, 1992). Eukaryotic expression systems can fold proteins, create disulfide bonds and perform post-translational modifications better than prokaryotic expression systems. Specifically, insect cells can post-transnationally modify proteins by phosphorylation, glycosylation, proteolyic processing, correct single peptide cleavage, myristylation, and palmitylation (Shuler et al., 1995).

Another major advantage to using the BEVS is that the virus can only infect arthropods and therefore eliminates the possibility of infecting humans (Jarvis, 1997; Shuler et al., 1995; King and Possee, 1992). This quality allows for easier industrial production and genetic manipulation. BEVS products are produced much more quickly and easier than mammalian expression systems (Shuler et al., 1995). Insect cells in subculture will not be transformed, unlike mammalian cell cultures, where the host cell maybe transformed upon viral infection, which requires purification procedures to remove nucleic acids (Shuler et al., 1995). Transformed mammalian cell lines are feared to be cancer-promoting to the recipient if nucleic acids are not removed correctly (Shuler et al., 1995). Mammalian viruses also have a danger of reverting back to its original pathogenicity and could ultimately infect the recipient of the product with a harmful virus (Shuler et al., 1995). Lastly, insect cells can be easily scaled up to large-scale volumes for recombinant protein production (King and Possee, 1992).

Baculoviruses are genetically stable because they have a relatively small genome and large foreign DNA can be inserted into the genome without affecting normal infection and replication (King and Possee, 1992; Drugmand et al., 2012). Constructing recombinant baculoviruses is relatively simple and rapid due to commercially available kits (Drugmand et al., 2012).

Disadvantages

Although there are many advantages of the BEVS, there are also some limitations. A major disadvantage is that the system uses a virus to infect a host cell, which ultimately leads to the death of the host organism, decreasing efficiency and kinetics of recombinant protein production (Jarvis, 1997; King and Possee, 1992). Another limitation is that the recombinant protein expression levels vary, where membrane-bound and secreted

glycoproteins are typically expressed at lower levels than active proteins (Jarvis, 1997; Shuler et al., 1995). Although the recombinant proteins are post-transnationally modified, the proteins are not expressed identically to those from higher eukaryotes resulting in different modifications (Jarvis, 1997). A major difference in post-translational modification (PTM) is glycosylation: insect cells produce simple, un-branched sugar-side chains with high mannose content, resulting in a lower molecular weight than the proteins produced in their natural host (King and Possee, 1992). These differences in post-translational processing may influence the antigenicity of some proteins, but this may be advantageous because small changes in structure may be appropriate for some vaccines (Shuler et al., 1995).

Infection of Mammalian Cell Culture

The virus can be modified to enter mammalian cells by endocytosis using the glycoprotein gp64 on the baculoviral envelope (Monsma et al., 1996). Not only are baculoviruses used for protein expression, the foreign peptides and proteins can be displayed on the viral surface (Kost et al., 2005). The peptides and proteins are immunogens that can improve transduction of mammalian cells (Kost et al. 2005).

Current Applications in BEVS Expressed Proteins

The baculovirus system can produce subunit vaccines through the expression of antigen proteins or producing virus-like particles (VLPs) (Drugmand et al., 2012). A VLP does not contain any genetic material, but contains recombinant surface proteins from different pathogenic viruses (Drugmand et al., 2012). The first vaccine produced using this system, approved in 2000, was to protect pigs against the swine fever virus (Drugmand et al., 2012). The first human vaccine produced from the BEVS was Cervarix, a VLP vaccine to protect against human papilloma types 16 and 18 (Sokolenko et al., 2012; Drugmand et al., 2012).

There are many human therapeutics in development, such as a vaccine against human influenza that could be useful for seasonal flu shots (Jin et al., 2008; Lin et al., 2008; Drugmand et al., 2012). A vaccine against HIV-AIDS is also in development (Drugmand et al., 2012). The insect cell-baculovirus expression vector system could produce many important vaccines in the future, so further research of the BEVS is important.

The BEVS could also be used as a means of gene therapy and gene delivery (Drugmand et al., 2012). Baculoviruses that can effectively deliver genes to mammalian cells are a safer substitute than mammalian virus vectors (Drugmand et al., 2012). More research must be done on this system, but baculoviruses have been manipulated to deliver genes into insect cells (Drugmand et al., 2012). Tests for infectious agents, such as viruses, protozoa, bacteria and also human autoantibodies and cancer markers have been developed using recombinant proteins produced in a BEVS (Jarvis, 1997).

The original use of baculoviruses by humans was using them as bioinsecticides, mainly towards larvae control (Wood, 1995). Using recombinant proteins, the baculovirus insecticide can be enhanced to kill pests faster than previous wild-type baculovirus systems (Wood, 1995). Chemical pesticides can be harmful to the environment and therefore this is a way to biologically control insects. There are, however, environmental issues with regard to large distribution of recombinant baculoviruses and ecological studies must assess the effects it may cause on the environment and human health (Wood, 1995).

Large Scale use of Baculovirus-Protein Expression System

Current Storage Conditions of the Baculovirus

It may be desirable to store recombinant baculovirus stocks for a long period of time or transport stocks over long distances. Baculovirus stocks may be stored at 4°C, in TNM-FH

medium supplemented with 10% (v/v) FBS for several months to a year and remain relatively stable (Jarvis, 1997). The most important factor that contributed to the decline in stability was exposure to light (Jarvis, 1997). Storage at -80°C yields even higher stability over a year (Jorio et al., 2006). Damage occurs when biological material is stored at low temperatures because of intracellular ice formation that could lead to cell or virus death (Mandumpal et al., 2010). Because the baculovirus is enveloped, as a result of budding through cellular membranes, freezing and thawing may affect both cells and virus similarly (Howell and Miller, 1983). The formation of ice outside and inside the membrane, ice recrystallization, and change in pH and solute concentration can cause degradation of virus (Howell and Miller, 1983).

Protein structure may alter during storage due to physical and chemical changes. Proteins may change chemically by deamination of asparagine residues, hydrolysis of the polypeptide backbone, oxidation of cysteine and methionine residues (Burke et al., 1999). They may alter physically by conformational changes that could lead to unfolding of the protein or expose hydrophobic amino acid side chains; these changes may lead to protein aggregation or precipitation. Both physical and chemical modifications can lead to denaturation of the protein and loss of biological activity (Burke et al., 1999). Adding an excipient to a solution can protect proteins from damage during long-term storage.

Disadvantages

Temperature

Industrially, storing and transporting large stocks in low temperatures may be extremely expensive. Therefore, it would be advantageous to find a storage formula that will keep the baculovirus stock stable at a temperature higher than 0°C.

Serum

Currently, insect cultures are supplemented with serum, usually fetal bovine serum (FBS). Serum has many functions and is a complex mixture that is ill defined (Brunner et al., 2010). Serum functions in cell culture media to provide growth factors and hormones, binding and transport proteins, protease-inhibitors, fatty acids and lipids, attachment and spreading factors, additional amino acids, vitamins, and trace elements (Brunner et al., 2010). The serum also helps maintain osmotic pressure, reduce shear stress and provide detoxification (Brunner et al., 2010). Although FBS provides many advantages to cell culture, there are also some drawbacks. Because serum supplements are ill defined, there is some variation in the composition and may result in variability of viral growth within cell cultures (Brunner et al., 2010; Price and Gregory, 1982). Serum may also contain biological contaminates such as viruses, fungi, bacteria, mycoplasma, or prions or introduce endotoxins, haemoglobin or other factors into the culture media (Brunner et al., 2010; Dermont, 1999; Wessman and Levings, 1999; Even et al., 2006). Along with variation in content, there are ethical concerns to consider. FBS is obtained from bovine fetuses that are removed from pregnant cows during slaughter, the fetuses are then punctured without anesthesia and maybe conscious for the duration of the bleeding procedure, which takes between 5 and 35 min (Jochems et al., 2002). The demand of FBS today requires at least 1,000,000 bovine fetuses to be harvested each year to meet the growing demand of 500,000 liters per year (Brunner et al., 2010; Jochems et al., 2002; Shah, 1999). FBS is expensive because the supply is directly correlated to the beef packing industry, not just on the demand of the product (Brunner et al., 2010). The beef industry is currently declining as more people worldwide are slowing down their consumption of red meat and switching to other protein sources (Brunner et al., 2010). Therefore as the FBS demand grows world wide, due to an increase biopharmaceutical production, the supply is decreasing. Another major concern with using

animal sera is the effect on downstream purification of a biopharmaceutical product. Because the serum is not well defined, elements within the solution may co-elute with the protein product in a chromatography column (Jayme, 2011; Brunner et al., 2010; Even et al., 2006). At an industrial scale, using FBS may be extremely expensive and therefore would be advantageous to find a different storage solution that keeps the baculovirus stable.

Lyophilization

Lyophilization, or freeze-drying, is a unit operation that is frequently used as a way to lengthen the stability of viruses at ambient temperatures (Croyle et al., 2001a). There are three phases of the process, freezing, primary drying, and secondary drying (Burke et al., 1999; Croyle et al., 2001a). Samples are frozen between -40 and -50°C initially and then once completely frozen, the pressure is decreased and the temperature is increased (Burket et al., 1999; Croyle et al., 2001a). These conditions cause the water in the sample to sublimate, where water changes from ice directly to vapor (Burke et al., 1999; Croyle et al., 2001a). After the primary drying period, any additional water that remains in the sample must be removed by adding heat until the sample is completely dry (Burke et al., 1999; Croyle et al., 2001a).

Many viruses, such as the influenza virus and adenovirus, have been effectively preserved using this process (Croyle et al., 2001b; de Jonge et al., 2007; Wilshut et al., 2007). After lyophilization, the item may be stored at room temperature if the conditions are correct. The process however can be complicated and the parameters must be adjusted properly, or the virus may oxidize or degrade (Cryole et al. 1998). Croyle et al. (2001a) found that samples lyophilized in 1M sucrose lost the least amount of adenovirus titer. Factors that may influence virus stability include glass transition temperature, final moisture content, and virus concentration. The glass transition temperature, Tg' is "the temperature at the

maximum freeze concentration where, in a frozen solution, the residual non-ice phase forms a glass" (Croyle et al., 2001a). The Tg' is an upper temperature limit, because below the Tg', the water will be rapidly removed and not disturb the virus that is formed when freezing, and thus not disrupt the virus stability (Croyle et al., 2001a). Lower molecular weight molecules lower the Tg', while higher molecular weights raise the Tg' (Burke et al., 1999). The final moisture content can be very important on maintaining the virus titer; it was found that lower final moisture content yielded a higher virus titer (Croyle et al., 2001a). However the sample should also not be over-dried because this will decrease the titer. Over-drying is thought to aggregate the viral particles, which makes the virus particles not virulent (Croyle et al., 2001a). The adenovirus was found to have optimal moisture content of 1.3-1.5% final moisture content to maintain a high virus titer (Croyle et al., 2001a). The virus concentration was also an important factor in virus viability; a 10 fold higher original virus titer yielded less of a loss than a lower original virus titer when stored at 4°C (Croyle et al., 2001a).

When freeze-drying protein for storage, there is no straightforward formulation. However, there are three components that should be considered. A crystallizing agent, surfactant, and amorphous protectant together create a stabilizing solution for freeze-dried proteins (Liu et al., 2005; Osterberg et al., 1997). The crystallizing bulking agent enhances lyophilization properties and the stability of the protein. A bulking agent creates a matrix for the virus particles to be dispersed when lyophilized (Liu et al., 2005). Mannitol, glycine, albumin, and sodium chloride are examples of bulking agents that can aid in protein stabilization when freeze-dried (Osterberg et al., 1997). Adding a surfactant offers additional stabilization and also minimizes protein degradation by adsorption to surfaces of the protein (Liu et al., 2005). An amorphous solution, also known as a lyoprotectant or cryoprotectant, does not interact with the protein and prevents denaturation (Osterberg et al., 1997). The

baculovirus is composed of a capsid composed of proteins, surrounded by an envelope studded with proteins, a surfactant may help with virus stabilization.

In a pervious study, a formula of 0.5M sucrose and 20% FBS was found to effectively store the baculovirus after either lyophilization or atmospheric drying, at room temperature for short term transportation (Chen et al., 2012). It is important to see if there is a formulation that stabilizes the virus the same, if not better, than using FBS.

Cryoprotectants and Lyoprotectants

Additives are added to the virus storage solution to enhance virus stability (Chen et al., 2012, Jorio et al., 2006, Croyle et al., 2001a). Naturally, microorganisms can adjust their macromolecules to enhance survival during extreme environmental conditions such as change in temperature or pH (Burke et al., 1999). For example, in thermophilic conditions, organisms respond by producing heat-stable enzymes that alter an amino-acid sequence slightly that will increase rigidity (Burke et al., 1999). Microorganisms accumulate sugars to protect them from dehydration. Sugars protect biological material by serving as water substitutes, so they maintain lipid bilayers and native protein structure by fulfilling hydrogen-bonding interactions with proteins and lipid bilayers (Burke et al., 1999). Without a hydrogen-bonding substitution, intra- and inter-protein interactions may lead to denaturation and aggregation (Arakawa et al., 1991). Sugars help with stability during dehydration because they form a glass state that prevents biochemical reactions from occurring because diffusion occurs in the order of micrometers per year (Burke et. al, 1999). The preservative must be able to cross the lipid bilayer so that it can provide protection on both sides of the membrane to maintain biological activity (Burke et al., 1999). Sucrose, sorbitol, DMSO, ethylene glycol, glycerol, mannitol, and gelatin have been previously used in the storage of baculovirus at various concentrations (Chen et al., 2012

and Jorio et al., 2006). These chemicals are commonly used as cryoprotectants and lyoprotectants. Polyhydric sugars and alcohols preserve protein structure by increasing the transition temperature of proteins in aqueous solutions and decrease hydrogen bond rupturing by the induction of water, which stabilizes proteins within the virus (Arakawa and Timasheff, 1982; Gerlsma, 1970). Cryoprotectants and lyoprotectants should not interact with the protein or virus, and therefore will not interfere with the native folding of the protein when stored for periods of time (Osterberg et al., 1997).

Sugars and sugar alcohols, such as sucrose and sorbitol, have shown to be helpful in cryopreservation (Chen et al., 2012; Ohtake et al., 2010; Bakaltacheva et al., 2007; Jorio et al., 2006; Liu et al., 2005; Croyle et al., 2001a). Sugars and amino acids cause hydration of proteins, which stabilizes the inherent conformation of the protein (Osterberg et al., 1997; Burke et al., 1999). Sorbitol is a common cryoprotectant and commonly used to store herpesvirus at -70°C. Sorbitol can be toxic to some cell cultures at high concentrations (Johnson, 1990; Howell and Miller, 1983; Medearis, 1964). Sucrose is often used as a storage protectant. Sucrose, at a concentration of 44.5% could store respiratory syncytial virus for 2 years in -70°C (Law and Hull, 1968). Jorio et al., (2006) found that glycerol, DMSO, and sucrose equally protected a purified baculovirus n storage in -80°C or liquid nitrogen.

Dimethylsulfoxide (DMSO) and glycerol have been used in cryopreservation of enveloped viruses in the past (Burke et al., 1999). Incorporating DMSO into a solution widens the glass transition of water towards both lower and higher temperatures, which increases thermodynamic stability; therefore stabilizing the glass state, which reduces the nucleation and consequent crystallization of water (Mandumpal et al., 2011). Ethylene glycol (EG) is

not the best glass former, but it can penetrate cell membranes. EG is relatively non-toxic and is commonly used as a cryoprotectant (Kuleshova, et al., 1999).

Sucrose-phosphate-glutamate (SPG) has been shown to stabilize influenza for at least a year in -20°C degree storage (Yannarell et al., 2002). SPG contains 0.218 M sucrose, 0.0071 M dipotassium phosphate, 0.00376 M monopotassium phosphate, and 0.0049 M potassium glutamate (Yannarell et al., 2002). SPG has been successfully used to transport herpesvirus on wet ice (Johnson, 1990). A study by Johnson (1990) concluded that SPG is equal or better than Eagle MEM containing 2% FBS for 3 days in 4 or 23°C incubators. Sucrose-phosphate (SP) is similar to SPG, but it contains no glutamate. 0.2 M SP is composed of 74.62g/L sucrose, 1.22g/L dipotassium phosphate, and 0.52g/L monopotassium phosphate (Howell and Miller, 1983). Herpesvirus, rickettsiae, chlamydiae, and mycoplasma have been successfully stored in SP (Miller and Howell, 1983; Larew and Myers, 1982; Smith et al., 1977; Bovarnick et al., 1950).

Pluronic F68 is a nonionic block copolymer surfactant and is used to control shear forces within a suspension culture (Murhammer and Goochee, 1990). Pluronic F68 has a molecular weight of 8500 and consists of triblock copolymers with a central block that is a hydrophobic poly(propylene oxide) with a hydrophilic poly(ethylene oxide) block on either side (Kabanov et al., 2002). Pluronic F68 (Poloxamer 188) has a high safety rating, and FDA approved it for medical and pharmaceutical uses (FDA, 2012). Pluronic F68 has effectively been used as a surfactant in freeze-drying in pervious studies (Lee and Lin, 2011; Croyle et al., 2001b). Pluronic F68 is thought to be effective in freeze-drying formulations because it inhibits protein-protein interactions and aggregation (Lee and Lin, 2011; Hamada et al., 2009).

Some amino acids, such as lysine, arginine, histidine, and glycine, act as crystalline bulking agents in freeze dried solutions (Mattern et al., 1999). However only, lysine, arginine, histidine, and citrulline exhibited amorphous solids after lyophilization (Mattern et al., 1999). L-arginine has been used as a stabilizer because of its ability to inhibit protein aggregation by increasing the solubility of proteins (Hamada et al., 2009; Arakawa et al., 2007; Shiraki et al., 2002).

Summary

There are many advantages to using the BEVS in both insect cells and mammalian cells to express recombinant proteins. Many vaccines are in development, and a vaccine has been successfully produced, and FDA approved using this system for protein expression. There are, however, improvements needed for baculovirus storage and stability over time. Serum-free storage techniques need to be established as the supply of FBS decreases and the demand increases. Although long-term storage in lower temperature environments appears to be quite stable, there is room for improvement, specifically in storage at room temperature. If improvements can be made at ambient temperatures, shipping of vaccines will be cheaper and more efficient.

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Chapter 3

Abstract

The baculovirus expression vector system (BEVS) is an effective way to produce recombinant proteins for biopharmaceuticals. However baculovirus stocks are stored in subzero temperatures to maintain virus stability, and fetal bovine serum is commonly used in the storage solution. In an effort to lower transportation and storage costs, a storage formulation that can effectively store the baculovirus in above frozen temperatures without the use of FBS would be beneficial. In this study, DMSO, ethylene glycol, glycerol, sucrose, sorbitol, sucrose-phosphate, and sucrose-phosphate-glutamate were added to baculovirus stock at various concentrations to determine the most effective stabilizer for virus storage at 4°C. Of the seven additives studied, 1 M sorbitol most effectively preserved baculovirus stock over a period of 47 weeks stored in 4°C. Formulations that include sucrose, L-arginine, and Pluronic F68 were created to determine their effectiveness on virus stability in a freeze-dried state stored at room temperature. In a lyophilized state, 0.5 M sucrose maintained baculovirus stock stability after 5 weeks of storage. Lyophilized stocks not containing sucrose were no longer infective after 5 weeks.

Keywords: baculovirus protein expression system, stability, storage, lyophilization

Abbreviations:

AcMNPV: *Autographia californica* multiple capsid nucleopholyhedrovirus
BEVS: Baculovirus expression vector system
FBS: Fetal Bovine Serum
DMSO: Dimethyl sulfoxide
SP: Sucrose-phosphate
SPG: sucrose-phosphate-glutamate

Introduction

The baculovirus insect cell protein expression system (BEVS) produces high concentrations of recombinant proteins for biopharmaceutical use (Shuler et al., 1995; Jarvis, 1997). The baculovirus is a rod shaped, double-stranded DNA virus with a genome of 80-180 kbp in size (Funk et al., 1997; van Oers, 2011). The BEVS system produces the VLP vaccine, Ceravix, for the protection against HPV types 16 and 18 (Drugmand et al., 2012; Sokolenko et al., 2012), and many other vaccines are in development (Jin et al., 2008; Lin et al., 2008; Drugmand et al., 2012).

The BEVS is a useful expression system because the baculovirus only infects arthropods, meaning human infection is not a threat (Funk et al., 1997). The BEVS modifies proteins more similar to mammalian cells than yeast and prokaryotic systems; post translational modifications such as phosphorylation, glycosylation, proteolytic processing occur within the insect cell (King and Possee, 1992; Shuler et al., 1995; Jarvis, 1997). The BEVS produces large quantities of recombinant proteins, 20-250 times greater than mammalian systems (Shuler et al., 1995). The baculovirus is genetically stable due to its relatively small genome and easy to construct recombinant systems due to commercially available kits (Drugmand et al., 2012).

Fetal bovine serum (FBS) is frequently used as a supplement in insect cell cultures to provide growth factors and hormones, additional amino acids, vitamins, and trace elements, protease-inhibitors, fatty acids and lipids, attachment and spreading factors, binding and transport proteins (Brunner et al., 2010). Serum is a complex ill-defined mixture that may vary in composition resulting in variability (Price and Gregory, 1982; Brunner et al., 2010). FBS may introduce viruses, prions, or fungi into a culture (Dormont, 1999; Wessman and Levings, 1999; Even et al., 2006; Brunner et al., 2010). There are economical and ethical

issues associated with the use of FBS. Currently the supply of FBS is declining, as the demand for beef is declining, resulting in an increase in price (Brunner et al., 2010). Ethically, FBS is harvested from fetal cows when pregnant mothers are slaughtered, where the fetuses are punctured without anesthesia and may remain conscious during the bleeding procedure, which takes 5-35 minutes (Jochems et al., 2002). In downstream processing of protein purification, animal sera may cause contamination issues and co-elude with the protein product in a chromatography column (Even et al., 2006; Brunner et al., 2010; Jayme, 2011). As a result of these issues, it would be advantageous to diverge from the use of FBS in insect cell culture and baculovirus storage.

Over time, baculovirus stability declines when stored in warmer temperatures (Jorio et al., 2006; Chen et al., 2012). It would be beneficial to find a formulation that would store the virus in warmer temperatures to lower transportation costs and long-term storage costs. Jorio et al., (2006) reported a 1.5 log loss in baculovirus supernatant after 10 months in 4°C conditions. Non-supplemented baculovirus stock lost all of its infectivity after 4 weeks post-dehydration (Chen et al., 2012). Adjuvants are added to biological materials when undergoing changes in environmental conditions (Croyle et al., 2001a; Jorio et al., 2006; Chen et al., 2012). Some microorganisms have the ability to express enzymes to help maintain protein structure and function when in extreme conditions, such as temperature or pH (Burke et al., 1999). In extreme temperatures, microorganisms accumulate sugars to protect from dehydration (Burke et al., 1999); this principle can be applied to storage solutions that undergo freezing and thawing conditions.

Lyophilization is a common way to preserve biological materials at ambient temperatures (Croyle et al., 2001a). When viruses and proteins are lyophilized for storage, three components have been proposed- crystallizing bulking agent, surfactant, and an amorphous

protectant (Osterberg et al., 1997; Liu et al., 2005). Crystallizing bulking agents are used to create a matrix to disperse the virus within a solution (Liu et al., 2005). Surfactants add stability and minimize protein degradation by adsorption to surfaces of the protein (Liu et al., 2005). In the present paper, L-arginine was selected as the crystallizing bulking agent and Pluronic F68 served as a surfactant. Sugars and sugar alcohols, such as sucrose, are commonly selected as lyoprotectants, amorphous solids, to protect viruses when lyophilized in the past (Bakaltacheva et al., 2007; Ohtake et al., 2010; Chen et al., 2012). In a previous study, 0.5 M sucrose and 20% FBS effectively preserved the baculovirus when freeze-dried; with a 70% transduction efficiency when stored for 3 weeks after dehydration (Chen et al., 2012). Many additives, such as DMSO, glucose, sorbitol, trehalose, mannitol, gelatin and glycerol, were tested to determine the optimal solution (Chen et al., 2012). Additionally, L-arginine is effective in preserving freeze-dried proteins because the agent increases the solubility of proteins and thus preventing protein aggregation (Shiraki et al., 2002; Arakawa et al., 2007; Hamada et al., 2009). Pluronic F68 is a surfactant that has been previously shown to be effective in freeze-drying formulations because of their ability to inhibit protein-protein interactions and aggregation (Croyle et al., 2001b; Hamada et al., 2009; Lee and Lin, 2011).

It is advantageous to determine a storage formulation, without FBS, that optimizes the stabilization of the baculovirus over a long period of time at 4°C and room temperature storage. Sucrose, sorbitol, DMSO, ethylene glycol (EG), sucrose-phosphate (SP), sucrose-phosphate-glutamate (SPG), and glycerol were added in a storage solution at two concentrations to determine a suitable solution to store baculovirus stock at 4°C over 10 months. Sucrose, L-arginine, and Pluronic F68 were used to stabilize baculovirus stock in a lyophilized state for a month.

Materials and Methods

Materials

Recombinant AcMNPV containing the EGFP gene was obtained from Dr. X.J. Meng (Department of Biomedical Sciences and Pathobiology, College of Veterinary Medicine, Virginia Tech). The recombinant baculovirus was constructed by inserting the EGFP gene into BaculoGold DNA from Clontech and stock was created using *Spodoptera frugiperda* (Sf9) cells. Grace's Insect Medium, supplemented; Sf-900™ III SFM (1X); and Pluronic F68 solution were purchased from Gibco (Grand Island, NY, USA). Heat inactivated, fetal bovine serum and antibiotic-antimycotic were provided by Dr. X.J. Meng's lab. Ethylene glycol; glutamate, Corning© Costar © cell culture plates, 96 well, flat bottom, tissue-culture treated, and corning © cell culture flasks, surface area 75cm^2, angled neck were purchased from Sigma Aldrich (St. Louis, MO, USA). Sorbitol, DMSO, glycerol, arginine, monopotassium phosphate, and dipotassium phosphate were purchased from Fisher Scientific (Pittsburgh, PA, USA). Sucrose was obtained from Amresco (Solon, OH, USA).

Culturing Sf9 Cells

Sf9 cells were taken from a liquid nitrogen storage tank and placed in 37°C for a rapid thaw. Cells were washed with Grace's Media supplemented with 10% FBS and centrifuged (ThermoScientific Sorrall Legend XTR) at 500G for 5 min at 10°C (DMSO removed from storage solution). Media was decanted and 10 mL of cells were placed in a 75 cm^2 T-flask containing 10 mL of Grace's Medium supplemented with 10% FBS. The flasks were placed in a 27°C incubator. Cultures were split every 3-4 days, once the monolayer was confluent on the bottom of the 75 cm^2 T-flask.

Serum-free suspension cultures were grown using SF-900 III SFM and split ever 3-4 days to a fresh cell density of $0.8*10^6$ cells/mL. Trypan blue dye exclusion was used to determine

cell viability. A haemocytometer slide was used to determine cell density and monitor viability (Improved Neubauer Levy Ultra Plane; Hausser & Son Philadelphia, PA, USA). Cells were examined under a Nikon eclipse TE300 microscope.

Creating virus stock

Once the virus has been amplified, the virus-insect cell culture was centrifuged at 1000 ×*g* for 15 minutes at 10°C. The supernatant was decanted into a sterile bottle and was covered in aluminum foil and stored at 4°C until aliquoted into storage solutions for time checks.

4°C Storage

Storage solutions were sterile filtered using a 0.22 µL syringe filter. The virus stock was diluted at a ratio of "virus solution: added solution" of 1:1, in PBS and cryoprotectant. Final concentrations of solutions of DMSO, EG, and glycerol were 5% and 10%; sucrose, sorbitol, and SP were 0.5 M and 1 M; and SPG was 0.25 M and 0.5 M. 50% PBS with 50% virus stock was used as a control. These samples were placed in 4°C in the dark and tested to determine change in virus titer over time.

Lyophilization Storage

Solutions were sterile filtered through a 0.22 µm syringe filter. The virus stock was diluted at a ratio of "virus solution: added solution" of 1:1 in PBS with cryoprotectants. Final concentrations were shown in Table 1. Solutions were dried in 0.5 mL vials with a fill volume of 0.150 mL. Solutions were frozen for 36 hours in -80°C freezer and lyophilized for 26 hours using a Labconco FreeZone 4.5 (Kansas City, MO). Solutions were then stored at room temperature in the dark.

Analytical Methods

Quantification of Virus Titer

To determine the virus titer, serial dilutions were made in a 96 well plate. 110 µL of virus and media supplemented with FBS were serially diluted. 100 µL of Sf9 cells were added to each well, aliquoting 6.5 $*10^4$ Sf9 cells per well. The plates were sealed using parafilm and placed in an incubator at 27°C for 48 hours. The wells were counted to determine virus titer using a Nikon Eclipse TE300 fluorescent microscope. Units are in GFP expressing units per mL.

Statistical Analysis

Statistical analysis was conducted in JMP using a one-way ANOVA model with a P value <0.05 and a 95% confidence interval. All results were completed in triplicates, with three vials per storage solution per time point, where each vial was measured three times to determine virus titer.

Results

Effect of long term storage on baculovirus stocks stored at 4°C

Baculovirus supernatent titer stored at 4°C decreased over 10 months. Without an effective preservative, virus titer decreased more significantly (Figure 1). 4 different solutions were selected to demonstrate titer deterioration over time. 1 M sorbitol and 10% DMSO were chosen because they maintained the highest virus titer throughout the 47 week period. 1 M sucrose was used, as it is one of the most commonly used preservatives, and lastly, virus was supplemented with PBS alone to show the need for an additive. Figure 1 demonstrates that virus stability was significantly higher (p<.001) when supplemented with 1 M sorbitol. 10% DMSO also helped stabilize the virus (with the exception of week 18), but it was not as

effective as sorbitol. 1 M sucrose did not stabilize the titer of the virus as effectively as some of the other additives. Baculovirus supplement with just PBS had the lowest overall titer throughout. After 30 weeks of storage, virus stability began to decrease more quickly, losing at least 0.5-log titer. The standard deviation varies considerably because conditions within the assay may have varied from well to well. Although the same volume of insect cells and virus stock were added to each well, it may have been slightly different, resulting in a difference in titer. Well location may also be a factor in the differences of virus titer. When using the multichannel pipette, every volume added appeared to be the same but in reality the volume may have varied. Lastly, human error may have contributed to the differences in positive GFP cells. GFP expressing insect cells were counted by hand in a fluorescent microscope; when there were many fluorescing cells, the count may not have been exact.

After 47 weeks, virus supplement with 10% glycerol, 1 M sucrose-phosphate, 10% ethylene glycol, 0.5 M sucrose, 10% DMSO, and 1 M sorbitol were significantly higher than virus stored solely in PBS at the 0.05% significance level (Figure 2). Of all additives used at 2 concentrations, 1 M sorbitol maintained the highest virus titer, with 10% DMSO maintaining the second highest virus titer.

Without additives, the virus titer decreased and had the lowest virus titer when only PBS was used in the storage solution. 10% serum had the second lowest virus titer over 47 weeks and was consistently low over the ten-month period (data not shown); evidently the virus needs an additive to be stored for long periods of time at 4°C.

Baculovirus titer was better stabilized in higher concentrations of additive (Figure 3). Two concentrations were selected for each additive to determine whether concentration made a significant difference. Higher concentrations were 10% glycerol, ethylene glycol, and DMSO, 1 M for sucrose, sorbitol, SP, and 0.5 M for SPG where the mean virus titer per testing period

was determined. Lower concentrations were calculated in the same manner. Throughout 47 weeks, a higher concentration of excipient was significantly better than the lower concentration, except in week 18 ($p<0.05$).

Effect of lyophilized baculovirus stocks stored at room temperature

Virus titer decreased quickly in a lyophilized state stored at room temperature (Figure 4) over 5 weeks. The result showed that sucrose is necessary to store the virus dried at room temperature. Within 12 days, the virus lost more than a 3-log titer when supplemented with FBS and PBS. After 5 weeks, baculovirus with 10% serum and PBS was completely nonviable, a 6-log decrease, meanwhile stock stored in 0.5 M sucrose only lost 2-logs in titer. Stocks containing L-arginine and Pluronic F68 at concentrations of 4% and 0.06% respectively did not significantly affect stability when combined with sucrose and were not tested on their own.

Discussion

In 4°C storage the baculovirus supernatant stock, in various chemical solutions, was monitored over a period of 10 months. Some additives were more beneficial to virus titer than others, specifically 1 M sorbitol and 10% DMSO helped maintain a higher virus titer. In a previous study, glycerol (2.5%, 5%, 10%), DMSO (2.5%, 5%, 10%), and sucrose (0.25 M, 0.5 M, 1 M) were found to be equally effective in baculovirus storage at -80°C (Jorio et al., 2006), but the results from this study show that the additives do not stabilize the virus uniformly at 4°C. There might be a cut off concentration where the virus titer is affected negatively, which may be explored in the future. FBS has previously been shown to effectively store the baculovirus for up to a year (Jarvis and Garcia, 1994; O'Reilly et al., 1994), but other additives are shown to be more effective in preserving virus titer; in addition the chemicals used in this study are much cheaper than FBS and do not have nearly

as many shortcomings as FBS does. A higher concentration of excipients are more capable of stabilizing the baculovirus because there is more sugar excipient to react with amino groups within the virus, stabilizing proteins in a liquid state by creating carbohydrate adducts (Wang, 2005). Purifying the baculovirus supernatant may be beneficial (Jorio et al., 2006); simply centrifuging the baculovirus-insect cell culture and using the supernatant combined with an additive is a quick and easy lab procedure. In 4°C storage, the baculovirus supernatent was more stable than diafiltered concentration in a pervious study; in contrast in liquid nitrogen, the diafiltered concentrate upheld the stabilization of the baculovirus better (Jorio et al., 2006).

Aggregation is the most likely reason for virus titer deterioration because over time virus particles collide, causing a clumping effect (Jorio et al., 2006). Virus particles stored in subzero conditions do not move in solution, reducing the chance to collide. An additive is still needed at subzero temperatures to protect viral viability during freezing and thawing processes (Burke et al., 1999). Because the solution is a liquid at 4°C, other chemicals added might improve stability by inhibiting interactions. Sugars, amino acids, surfactants, salts, polymers, and polyols have been shown to inhibit aggregation (Wang, 2005); supplementing virus stocks with a combination of these excipients may be beneficial in stabilizing baculovirus at 4°C. Trial and error is the common route in finding which excipients work best for virus stabilization (Wang, 2005).

The data show that freeze-dried baculovirus stock stored at room temperature lost titer in a short period of time. The baculovirus is unstable without a lyoprotectant and decreases in viable particles dramatically. 0.5 M sucrose solution effectively stabilized the virus for 1 month. Pluronic F68 and L-arginine were not shown to significantly preserve virus titer when freeze-dried with sucrose. It maybe beneficial to determine their effects on virus titer

without sucrose added. In a previous study, a combination of 0.5 M sucrose and 20% FBS protected the lyophilized baculovirus the best (Chen et al., 2012). There may be other combinations of chemicals that work better without FBS; L-arginine and Pluronic F68 were chosen because they worked effectively in a study on measles preservation (Ohtake et al., 2010). However a different crystallizing buffering agent and surfactant may work better on lyophilized baculovirus. Other conditions may also increase or decrease the overall virus stability. Factors such as osmotic pressure, pH, initial virus concentration, final water content, and factors in freeze-drying may all be optimized for the best solution. L-arginine and Pluronic F68 did not appear to be helpful in the stabilization of baculovirus; a different excipient may work better, such as 1 M sorbitol that was found to optimally stabilize the virus at 4°C.

Economically, FBS adds a significant cost to the storage solution. If 20 L of baculovirus stock was stored, it would cost $1,035 more to supplement the storage solution with 10% (v/v) FBS than using sorbitol (prices obtained from Gibco and Fisher Scientific). The other storage additives are similar in price to sorbitol. Therefore, not only does FBS alone not stabilize the baculovirus as well as other additives, the use also adds a significant expense.

In the future, the stability of the baculovirus should be studied in more detail to determine the major cause of virus deterioration. A few excipients should be selected and an end-point dilution or plaque assay should be used to determine virus titer more accurately. The method performed in this paper was chosen to quickly monitor many different storage solutions, but produced much more variable titers than expected. A formulation of excipients maybe studied to determine a perfect solution to store the baculovirus over time. TEM images would be beneficial to see what is happening to the viral particles during storage. Finding a mixture that would keep the virus stable in higher temperatures would

be beneficial to lower energy and shipping costs. In a lyophilized state, it would be beneficial to optimize freeze-drying conditions by altering shelf temperature, condenser temperature, pressure, and time.

Conclusions

In this study, the baculovirus supernatant stored in 1 M sorbitol in a 1:1 "PBS+excipient:virus stock" solution was found to maintain the highest virus titer when stored for at least 10 months at 4°C. We believe when shipping the baculovirus short distances, a 0.5 M sucrose mixture is suitable to stabilize lyophilized baculovirus. This mixture dramatically lowers shipping cost, as the temperature is above 0°C and is also serum-free, adding many advantages to the perseverance of BEVS.

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Tables

Table 1. Formulation components for lyophilized AcMNPV.

Components	Formulation				
	1	2	3	4	5
Sucrose (M)	0	0.5	0.5	0.5	0.5
L-Arginine (%, w/v)	0	0	4	4	0
Pluronic F68 (%, v/v)	0	0	0.06	0	0.06
Fetal Bovine Serum (%, v/v)	10	0	0	0	0

Figures

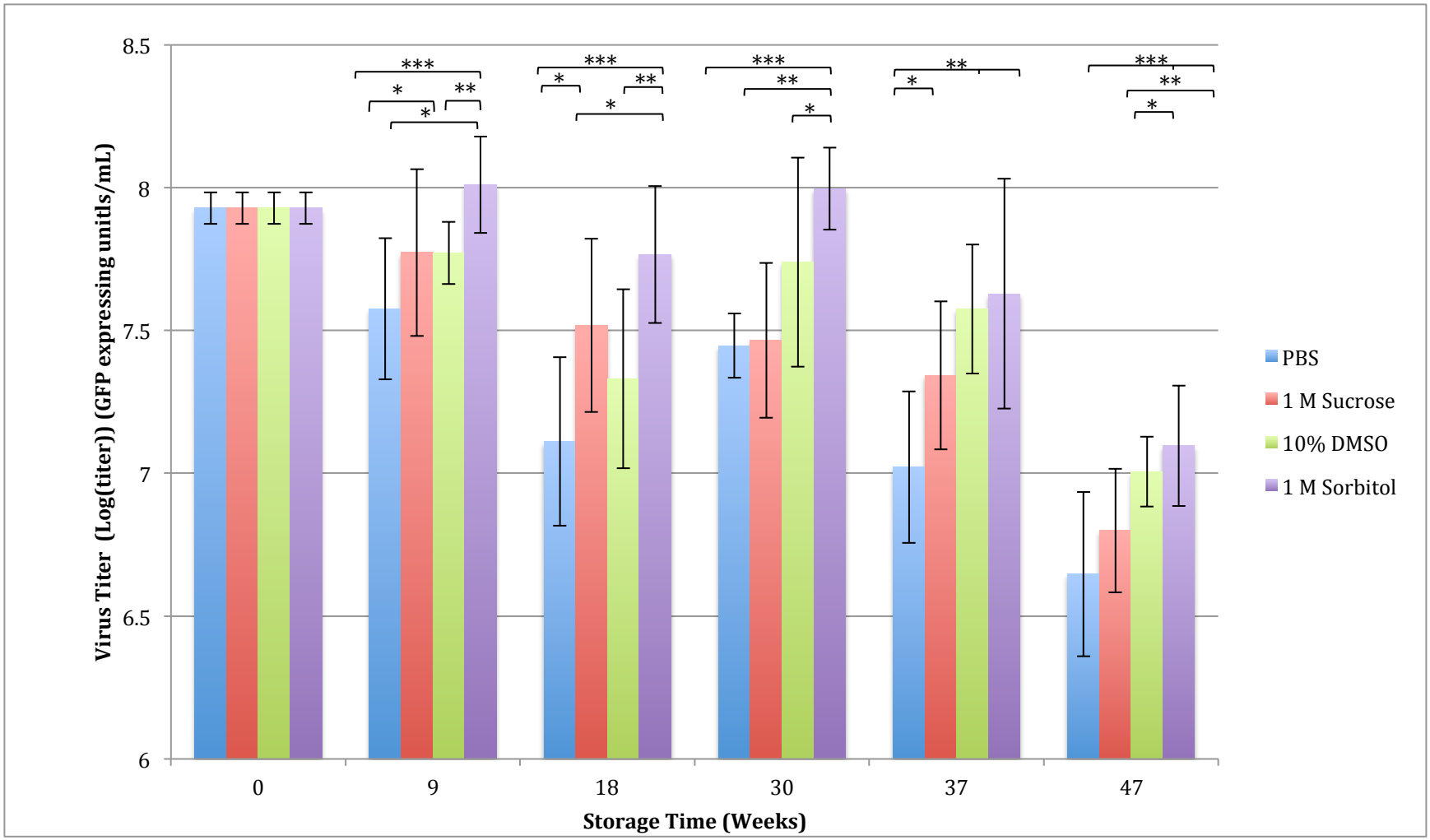

Figure 1. Effect of selected excipients on baculovirus stability stored at 4°C over time.

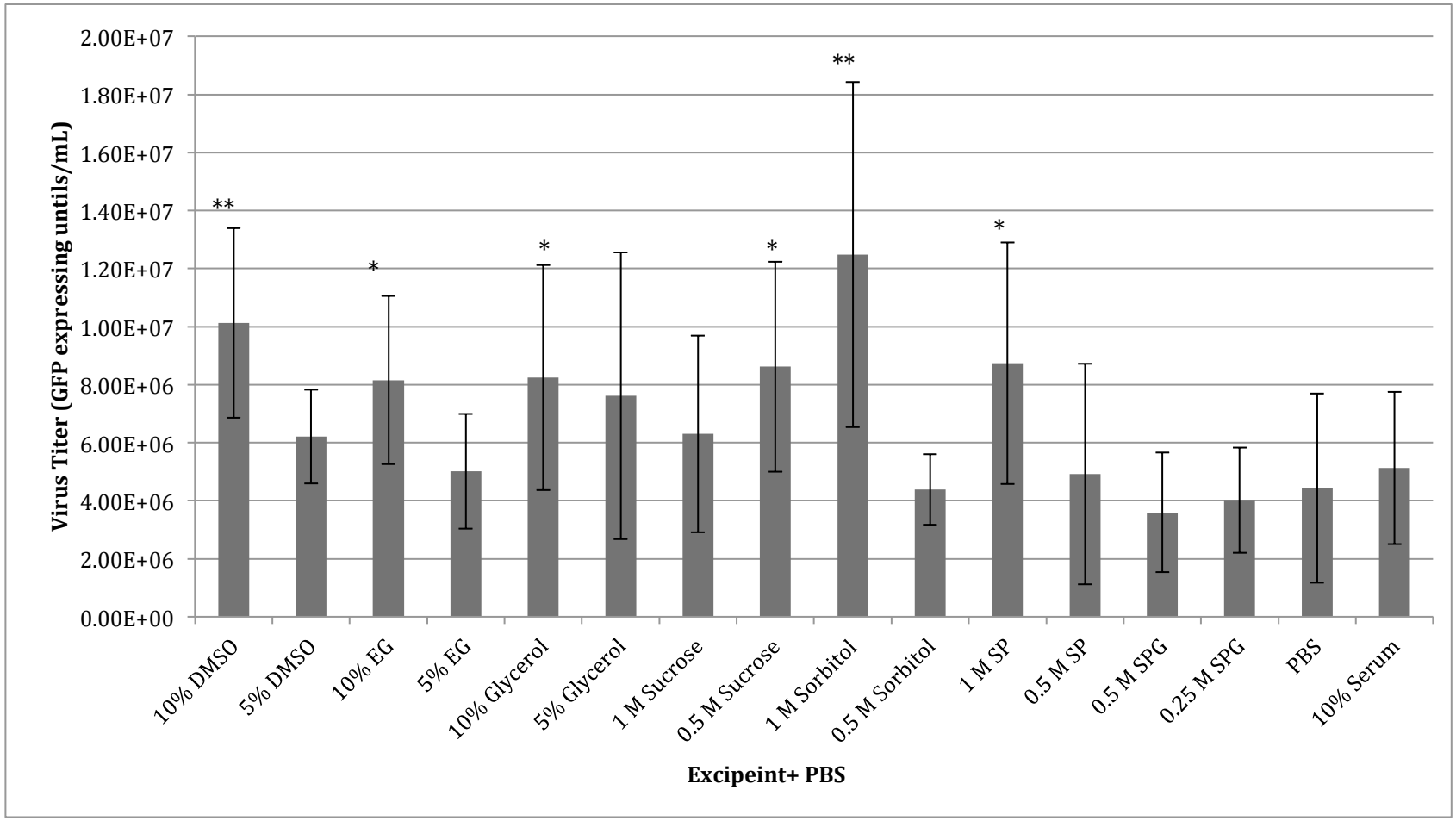

Figure 2. Virus titer in 4°C wet storage after 47 weeks with different additives.

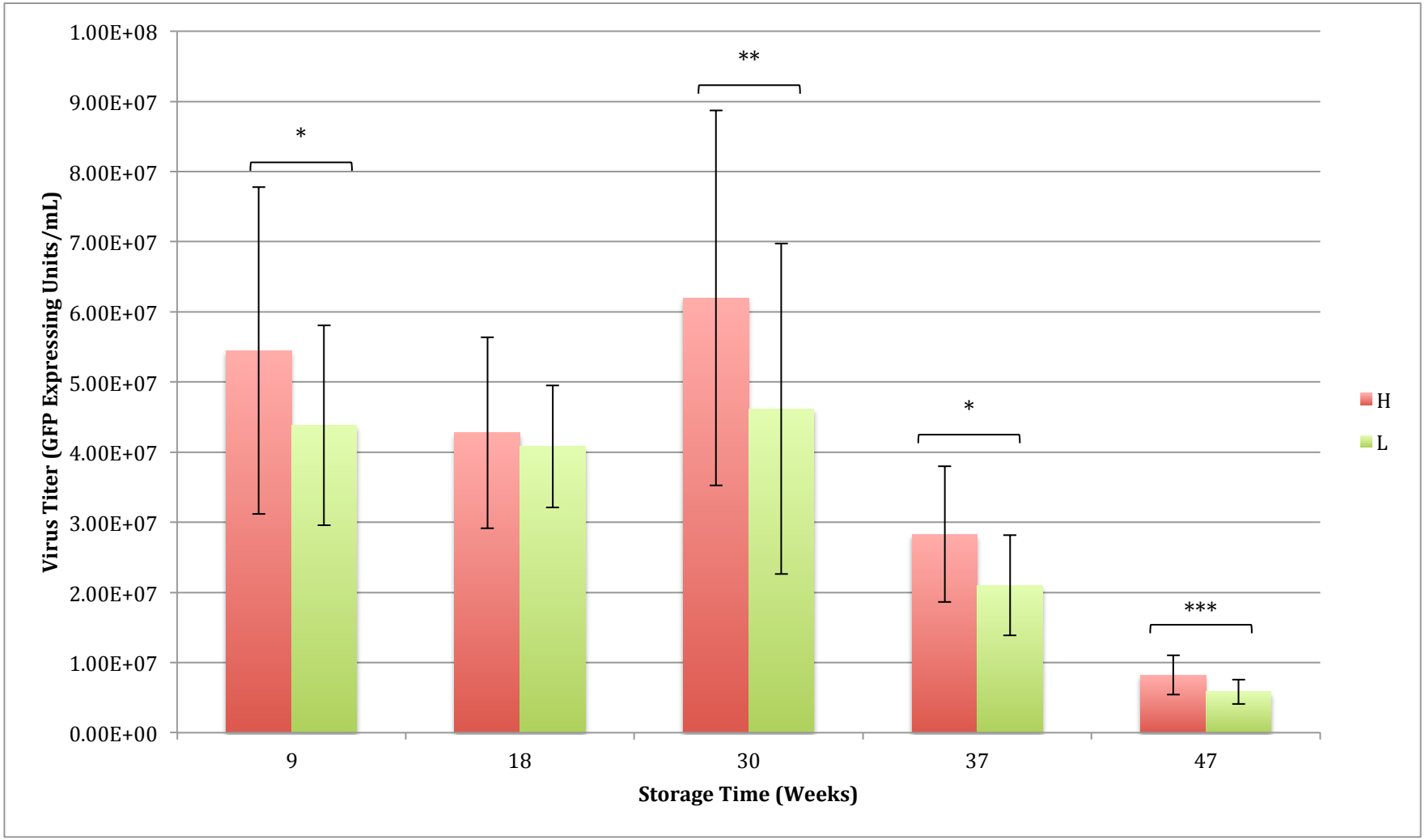

Figure 3. Effect of high and low excipients concentration on virus titer stored at 4°C.

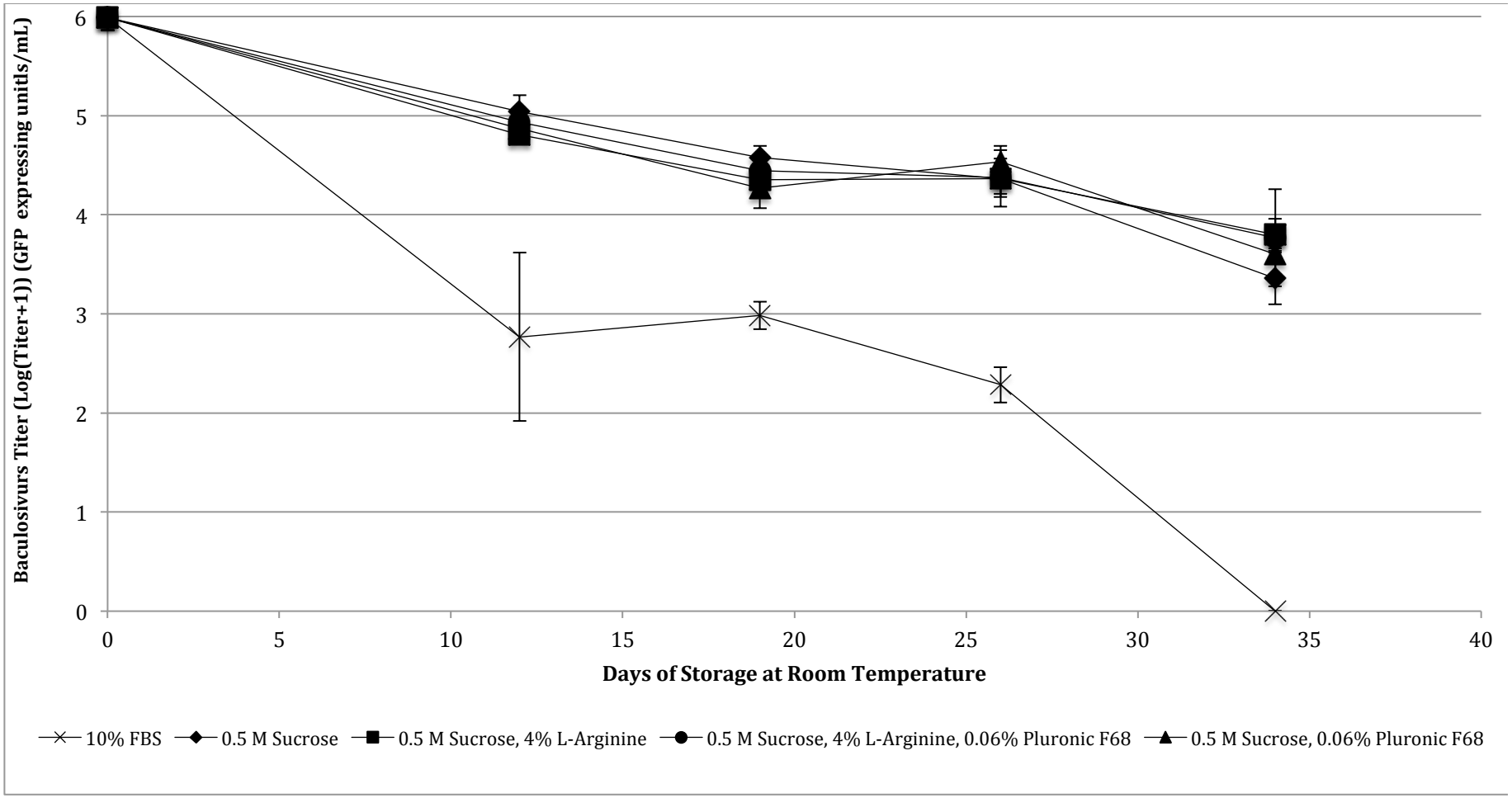

Figure 4. Lyophilized baculovirus stored at room temperature.

Legends for figures

Figure 1.

Wet storage at 4°C of AcMNPV supernatant over 47 weeks. Selected excipients were 1 M sorbitol, 10% DMSO, 1 M sucrose, and PBS. The data is expressed in means ± SD, where * denotes *p<0.05, **p<0.01, ***p<0.001 that are statistically significant.

Figure 2

Wet storage of AcMNPV supernatant after 47 weeks. The data is expressed in means ± SD, statistical significant between PBS and excipients was determined by one-way ANOVA analysis, where *p<0.05, and **p<0.01.

Figure 3.

Effect of high and low excipient concentration on virus titer stored at 4°C. Means of high and low excipient concentrations were combined. The data is expressed in means ± SD, statistical significant was determined by one-way ANOVA analysis, where *p<0.05, **p<0.01, and ***p<0.001.

Figure 4.

Lyophilized baculovirus stored at room temperature. The symbols correspond to: (✕) 10% FBS; (◆) 0.5 M Sucrose; (■) 0.5 M Sucrose, 4% L-Arginine; (●) 0.5 M Sucrose, 4% L-Arginine, 0.06% Pluronic F68; (▲) 0.5 M Sucrose, 0.06% Pluronic F68. 0 days is before freeze-drying has taken place.

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Appendix A: Raw Data

The virus titer varied between time periods. Theoretically, the baculovirus titer must decrease over time because the virus cannot replicate without cells. The virus titer may have increased due to differences in cell viability at each time point or possibly one- or two-hour difference in incubation may have lead to a higher titer. Meaning the uptake of the virus may have been favored at some points and not at other due to the insect cells health. Sf9 cells were added to each well at the same concentration each week, but there is variability in this. Cell concentration was determined using a haemocytometer, adding inconsistency to the assay. Figure A-1 below shows the variability of virus titer over 47 weeks.

Student's two-tailed t test and two-way ANOVA placed statistical significance using all data over 47 weeks (Table A-1).

Table A-1. Statistical significance of each treatment over 47 weeks

Treatment	Least Square Means*
1 M Sorbitol	0.348^{A}
10% DMSO	$0.314^{A,B}$
10% Ethylene Glycol	0.308^{B}
10% Glycerol	$0.300^{B,C}$
1 M SP	$0.293^{B,C,D}$
5% Glycerol	$0.268^{C,D,E}$
5% DMSO	$0.262^{D,E}$
0.25 M SPG	$0.256^{D,E}$
0.5 M Sucrose	0.253^{E}
1 M Sucrose	0.252^{E}
0.5 M Sorbitol	0.247^{E}
0.5 M SP	0.244^{E}
5% Ethylene Glycol	$0.234^{E,F}$
0.5 M SPG	$0.234^{E,F}$
10% Serum	$0.232^{E,F}$
PBS	0.198^{F}

*LSMeans followed by the same level are not significantly different at the 0.05 level using Student's T test (JMP)

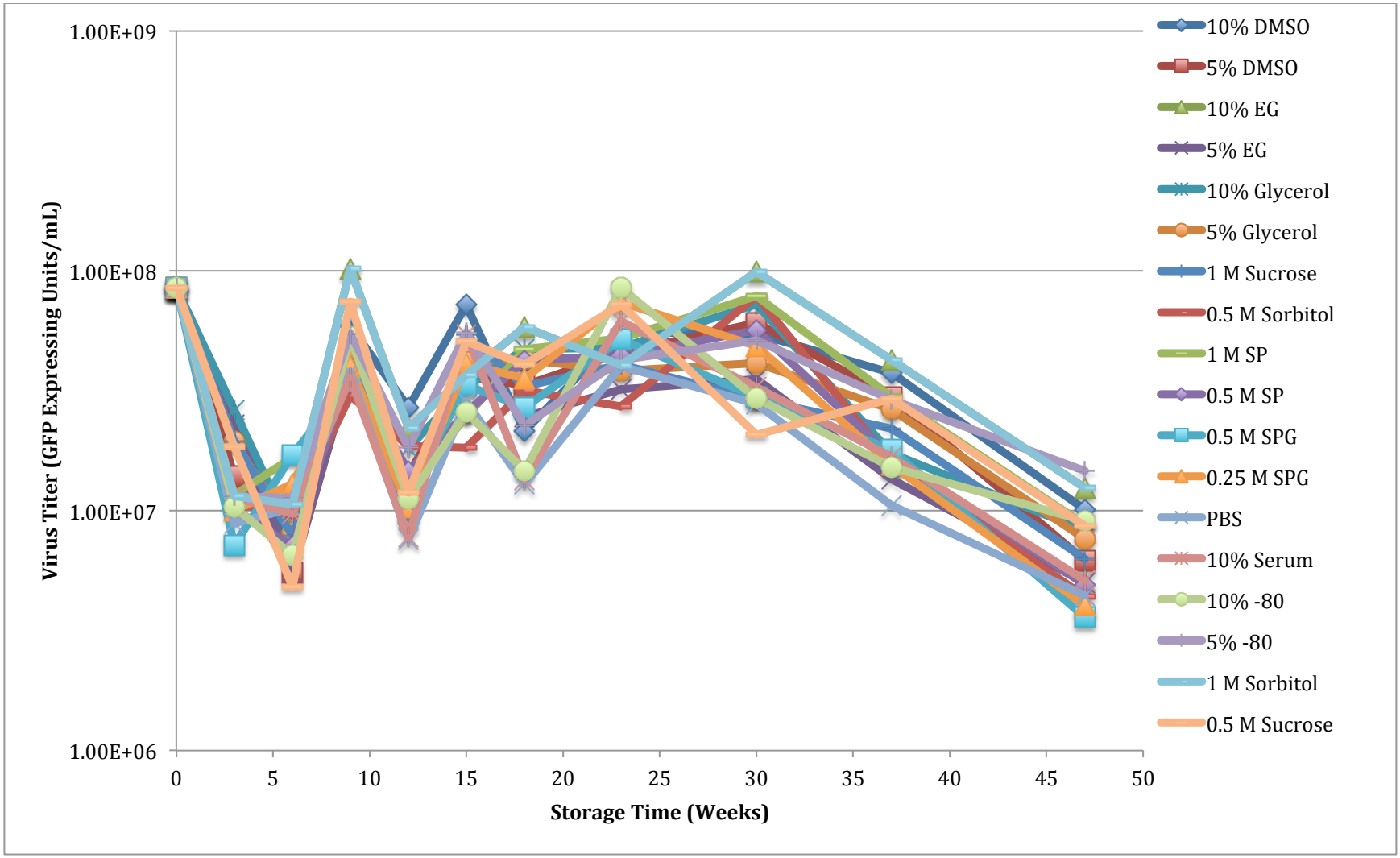

Figure A-1. Raw data of baculovirus stored at 4°C