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(56) Related Art
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(54) **Title**: EXCIPIENTS FOR STABILISING VIRAL PARTICLES, POLYPEPTIDES OR BIOLOGICAL MATERIAL

(57) **Abstract**: A sterile pharmaceutically acceptable aqueous solution, which solution is provided in a sealed container and comprises: a pharmaceutically acceptable aqueous solvent; viral particles or a physiologically active polypeptide; an excipient selected from a polyethyleneimine; a compound of formula (I) or a physiologically acceptable salt or ester thereof; or a compound of formula (II) or a physiologically acceptable salt or ester thereof; and optionally, one or more sugars.

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Field of the Invention

The present invention is concerned with storage-stable formulations of viruses.

Background to the Invention

Some biological molecules are sufficiently stable that they can be isolated, purified and then stored in solution at room temperature. However, this is not possible for many materials and techniques involving storage at low temperature, addition of stabilisers, freeze-drying, vacuum formation and air-drying have been tried to ensure shelf preservation. Despite the availability of these techniques, some biological materials still show unsatisfactory levels of stability during storage and some techniques lead to added cost and inconvenience. For example, refrigerated transportation and storage is expensive. Further, refrigerated transport is often not available for the transport of medicines such as vaccines in countries in the developing world.

In particular, the stresses of freeze-drying or lyophilisation can be very damaging to some biological materials. Freeze drying of biopharmaceuticals involves freezing solutions or suspensions of thermosensitive biomaterials, followed by primary and secondary drying. The technique is based on sublimation of water at subzero temperature under vacuum without the solution melting. Freeze-drying represents a key step for manufacturing solid protein and vaccine pharmaceuticals. The rate of water vapour diffusion from the frozen biomaterial is very low and therefore the process is time-consuming. Additionally, both the freezing and drying stages introduce stresses that are capable of unfolding or denaturing proteins.

Proteins are molecules with defined primary, secondary, tertiary and in some instances quaternary structures. The structure plays an important role in giving a protein its specific biological function. Unfortunately, the structural complexity of biological pharmaceuticals such as proteins makes them susceptible to various

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processes that result in structural and functional instability. Conformational integrity and functional groups must be protected from degradation

Instability can be a consequence of a variety of covalent and non-covalent reactions or modifications in solution. Degradation is generally classified into two main categories: firstly physical degradation or non-covalent pathway degradation and secondly the covalent degradation pathway.

Proteins can degrade via physical processes such as interfacial adsorption and aggregation which can significantly reduce a protein drug's potency and stability. A second consequence is that unfolding mediated by adsorption at an interface can often be an initiating step for irreversible aggregation of the protein in solution. Exposure of the protein's core at a hydrophobic surface can result in adsorption as a consequence of agitation, temperature or pH induced stresses; all of which can lead to aggregation.

Proteins may be subject to chemical modification such as oxidation, isomerisation, hydrolysis, disulfide scrambling, beta elimination, deamidation, and adduct formation. The principal hydrolytic mechanisms of degradation include peptide bond hydrolysis, deamidation of asparagine and glutamine and the isomerisation of aspartic acid. A common feature of the hydrolytic degradation pathway is that one significant formulation variable, with respect of the rates of the reactions is the pH.

As protein stability can significantly affect the safety and efficacy of a therapeutic, the composition of components in a biopharmaceutical formulation can affect the extent of protein degradation. The method of formulation of a biopharmaceutical also can impact the ease and frequency of administration.

Due to problems with instability and aggregation, most current stable formulations of proteins are not liquid formulations. Typically proteins are freeze dried (lyophilised) to provide stable formulations of the proteins. A bulking agent is often present in the formulations. The freeze dried formulations are distributed and stored in dried form, typically as a powder, in a sealed vial, ampoule or syringe. For

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example, WO 97/04801 describes stable lyophilised formulations of anti-IgE antibodies which have to be reconstituted immediately prior to use.

WO-A-2006/0850082 reports a desiccated or preserved product comprising a sugar, a charged material such as a histone protein and a dessication- or thermo-sensitive biological component. The sugar forms an amorphous solid matrix. However, the histone may have immunological consequences if the preserved biological component is administered to a human or animal.

WO 2008/114021 describes a method for preserving viral particles. The method comprises drying an aqueous solution of one or more sugars, a polyethyleneimine and the viral particles to form an amorphous solid matrix comprising the viral particles. The aqueous solution contains the polyethyleneimine at a concentration of 15μM or less based on the number-average molar mass (M_n) of the polyethyleneimine and the sugar concentration or, if more than one sugar is present, total sugar concentration is greater than 0.1M.

WO 2010/035001describes a method for preserving a polypeptide in which an aqueous solution of the polypeptide is dried, for example freeze dried, in the presence of one or more sugars and a polyethyleneimine (PEI). The resulting dried composition is typically provided as a stable dry powder in a sealed vial, ampoule or syringe. A solution is reconstituted from the powder in order to administer the polypeptide to a patient e.g. by injection.

Drying and especially freeze drying are however costly and time-consuming processes. It would be advantageous if their use could be avoided. Biologically active materials often suffer a loss of activity following heating and drying. Additionally, the need to reconstitute a freeze dried powder in a solvent before use of the polypeptide is an inconvenience. Indeed, it can carry risks for the patient or medical professional who performs the reconstitution step if the procedure is not carried out correctly.

It is thus advantageous to provide liquid virus and protein formulations that do not require reconstitution in order to be used. Consequently, there is a demand for

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stable liquid injectable virus and protein formulations. There is a demand for highly concentrated stable liquid injectable antibody formulations.

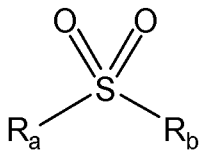
Summary of the Invention

It has now surprisingly been found that storage-stable ready-to-use aqueous solutions of viral particles can be provided by use of certain excipients and optionally one, two or more sugars. These formulations retain long term stability. They can be prepared without a drying or freeze drying step. They circumvent the need to reconstitute a solution from a freeze dried powder prior to use. It has also been found that these excipients and optionally one, two or more sugars can preserve viral particles during manufacture. Further, it has been found that these excipients and optionally one, two or more sugars can preserve samples taken from a human or animal.

The present invention provides a process for the preparation of a ready-to-use, storage-stable aqueous solution which is provided in a sealed container and which contains live viral particles, which process comprises:

(a) providing a solution comprising:

- water or physiological saline, which is optionally buffered with a physiologically acceptable buffer;
- live viral particles from *Adenoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae*, *Poxviridae or Flaviviridae*;
- an excipient which is an N,N-di(C_{1-6} alkyl)-glycine or N,N,N-tri(C_{1-6} alkyl)-glycine, or a physiologically acceptable salt or ester thereof;
- optionally one or more sugars; and
- optionally a sulfone compound of formula (IIC):



(IIC)

wherein R_a and R_b independently represent C_{1-6} alkyl; and

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(b) sealing the solution in a container.

In one aspect of the process of the invention the viral particles are from an adenovirus, vaccinia virus, influenza virus or measles virus.

In another aspect of the process of the invention the excipient is N,N-dimethylglycine or N,N,N-trimethylglycine, or a physiologically acceptable salt or ester thereof.

In a further aspect of the process of the invention the excipient is N,N-dimethylglycine or a physiologically acceptable salt or ester thereof.

In a further aspect of the process of the invention the one or more sugars comprise sucrose or mannitol.

In a further aspect of the process of the invention the one or more sugars comprise sucrose and raffinose.

In a further aspect of the process of the invention the sulfone compound of formula (IIC) is methylsulfonylmethane.

In a further aspect of the process of the invention the solution further comprises:

(a) an adjuvant;

(b) a tonicity adjustment agent; and/or

(c) a preservative.

In a further aspect of the process of the invention the solution is isotonic.

In a further aspect of the process of the invention the solution is provided in a sealed container under nitrogen.

In a further aspect of the process of the invention the sealed contained is a sealed vial, ampoule, syringe, cartridge, flexible bag or glass bottle.

In a further aspect of the process of the invention the solution is passed through a sterilising filter in step (a).

The present invention also provides a ready-to-use, storage-stable aqueous solution containing live viral particles which is provided in a sealed container, said aqueous solution being obtainable by a processes of the invention.

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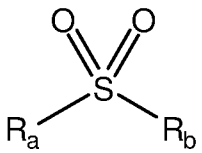
In one embodiment there is provided a process for the preparation of a ready-to-use, storage-stable aqueous solution which is provided in a sealed container and which contains live viral particles, which process comprises:

(a) providing a solution comprising:

- water or physiological saline, which is optionally buffered with a physiologically acceptable buffer;
- live viral particles from Adenoviridae, Orthomyxoviridae, Paramyxoviridae, Parvoviridae, Picornaviridae, Poxviridae or Flaviviridae;
- an excipient which is an N,N-di(C_{1-6} alkyl)-glycine or N,N,N-tri(C_{1-6} alkyl)-glycine, or a physiologically acceptable salt or ester thereof;
- optionally one or more sugars; and
- optionally a sulfone compound of formula (IIC):



(IIC)

wherein R_a and R_b independently represent C_{1-6} alkyl; and

(b) sealing the solution in a container.

Brief Description of the Figures

Figure 1a shows the effect of test formulations on the recovered activity of formulations of adenovirus held at 4°C for one week. Grey and white bars represent test formulations. Figures on the x-axis refer to concentration in M. Black bars represent control samples. "Starting" = titre of input virus for storage, "PBS" = formulation containing no further excipient, "sugars" = formulation comprising 1M sucrose, 100mM raffinose. Error bars = standard of the mean, n = 3.

Figure 1b shows the effect of test formulations on the recovered activity of formulations of adenovirus containing sugars (1M sucrose, 100mM raffinose) held at 4°C for one week. Grey and white bars represent test formulations. The Figures on the x-axis refer to concentrations in M. Black bars represent control samples, "Starting" = titre of input virus before storage, "PBS" – formulation containing no further excipients, "Sugars" = formulation comprising 1M sucrose, 100mM raffinose. Error bars = standard error of the mean, n = 3.

Figure 1c shows the effect of test formulations on the recovered activity of formulations of adenovirus containing no sugars held at 37°C for one week. Grey and white bars represent test formulations. Figures on x-axis refer to concentration in M. Black bars represent control samples, "Starting" = titre of input virus before storage, "PBS" = formulation containing no further excipients, "Sugars" = formulation comprising 1M sucrose, 100mM raffinose, Error bars = standard error of the mean, n = 3.

Figure 1d shows the effect of test formulations on the recovered activity of formulations of adenovirus containing sugars (1M sucrose, 100mM raffinose), held at 37°C for one week. Grey and white bars represent test formulations. Figures on x-axis refer to concentration in M. Black bars represent control samples, "Starting" = titre of input virus before storage, "PBS" = formulation containing no further excipients, "Sugars" = formulation comprising 1M sucrose, 100mM raffinose, Error bars = standard error of the mean, n = 3.

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Figure 2 shows the results obtained in Example 2 in which the ability of eleven formulations to stabilise adenovirus against thermal challenge was assessed following 7 days at 37°C.

Figure 3 shows the results obtained in Example 3 in which the ability of eleven formulations to stabilise MVA against thermal challenge at 37°C for 7 days was assessed.

Figure 4 shows a representation of the design space in Example 4. Numbered circles represent formulations within the design space that are tested. This design is a CCF RSM design. Numbers in circles refer to sample I.D.s in Table 3.

Figure 5 summarises the statistics of the model used to represent the data in Example 4.

Figure 6 shows terms retained in the model after fine-tuning in Example 4. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 7 is a surface response plot of the predicted recovered viral titre in formulations of TMG and mannitol in Example 4.

Figure 8 is a screen capture of settings and outputs from the optimum predictions based on the model of the data in Example 4, generated using Monte-Carlo simulations. Highlighted formulation (line 4) is the optimum identified.

Figure 9 shows a 3D representation of the design space in Example 5. Spheres represent formulations within the design space that are tested. This design is a Doehlert RSM design.

Figure 10 summarises statistics of the model used to represent the data in Example 5.

Figure 11 shows terms retained in the model in Example 5 after fine tuning. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 12 is a surface response plot of predicted viral titre using the model of Example 5 in formulations of DMG and sucrose at three different levels of raffinose, namely: “Low” = raffinose at 0mM, “Mid” = raffinose at 150mM, “High” = raffinose at 300mM.

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Figure 13 shows a screen capture of settings and outputs from the optimum predictions based on the model of the data in Example 5, generated using Monte-Carlo simulations. The predicted optima highlighted is sucrose concentration of 0.5M, DMG concentration 0.4M and raffinose concentration of 272.5mM.

Figure 14 shows an optimum region plot using the model derived from Example 5. Figure 14A is a contour plot where a cross marks the predicted optimum. Colouring indicates level of variable. Figure 14B is an graph highlighting region of model where predicted recovered viral activity is greater than or equal to that input.

Figure 15 shows the recovered viral activity from various formulations after 6 months storage at +4°C in Example 6.

Figure 16 shows recovered viral activity for the 'best' formulation in Example 6 comprising 1M sucrose, 100mM raffinose and 0.7M DMG at each time point and thermal challenge.

Figure 17 shows reduction in recovered viral activity over time at +37°C thermal challenge in various formulations in Example 6.

Figure 18 shows a representation of the design space in Example 7. Numbered circles represent formulations within the design space that were tested. This design is a CCF RSM design.

Figure 19 summarises the statistics of the model used to represent the data in Example 7.

Figure 20 shows terms retained in the model in Example 7 after fine tuning. Error bars not crossing the origin indicate a significant factor at the 95% C.I.

Figure 21 shows a contour plot of the predicted recovered viral titre in formulations of DMG and mannitol in Example 7.

Detailed Description of the Invention

Summary

Stable aqueous solutions of viral particles are provided according to the invention. The solutions are sterile pharmaceutically acceptable liquids that can be

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administered to a patient without having to be reconstituted from e.g. a dried powder immediately prior to use.

In one embodiment, the present invention relates to the preservation of viral particles by a N-alkylated glycine derivative or a salt or ester thereof, and a sulfone compound of formula (IIC). The N-alkylated glycine derivative and the sulfone compound can interact synergistically to stabilise the viral particles in a liquid setting.

The solutions may take the form of small-volume parenterals of 100ml or less or large-volume parenterals of 100ml or more. The solutions are sterile pharmaceutically acceptable liquids that can be administered to a patient without having to be reconstituted from e.g. a dried powder immediately prior to use.

The solutions are capable of exhibiting long term storage stability. They can therefore be stored for 6 to 18 months or longer in a refrigerator, i.e. at temperatures of from 2 to 8°C. In some instances, the solutions can be stored at room temperature for such periods of time. The solutions thus possess sufficient stability to enable them to be manufactured in a factory, distributed e.g. to pharmaceutical wholesalers and pharmacies, and stored prior to use without an unacceptable level of degradation occurring.

Typically, the solutions are provided as clear liquids. The solutions are usually colourless. They may additionally comprise a physiologically acceptable buffer and/or a tonicity adjustment agent and/or a preservative. The solutions may thus be isotonic. The solutions are sealed in an appropriate container in a vial, ampoule, syringe, cartridge, flexible bag or glass bottle. They are thus manufactured in ready-to-use form in a factory. They have not therefore been reconstituted from a solid composition such as a lyophilisate immediately prior to use.

The excipients of the invention can additionally preserve virus particles during manufacture of solutions of said virus particles.

Viral particles

The viral particles used in the present invention may be whole viruses such as live viruses, killed viruses, live attenuated viruses, inactivated viruses such as

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chemically inactivated viruses or virulent or non-virulent viruses. A live virus is capable of infecting and replicating within the host cell. A killed virus is inactivated and is unable to replicate within the host cell. The particles may be virus-like particles (VLPs) or nucleocapsids. The virus may be infectious to prokaryotic or eukaryotic cells. The virus may be a human or animal virus.

The viral particle may be, or may be derived from, a dsDNA virus, a ssDNA virus, a dsRNA virus, a (+)ssRNA virus, a (-)ssRNA virus, a ssRNA-RT virus or a dsDNA-RT virus. As an example but not intended to be limiting, the viral particle can be, or can be derived from, a virus of the following families:

Adenoviridae such as human adenovirus A, B, C, D, E or F including human Ad5, Ad2, Ad4, Ad6, Ad24, Ad35, Ad36 serotypes;

Flaviviridae such as yellow fever virus, west nile virus, dengue virus, hepatitis C virus;

Orthomyxoviridae such as influenzavirus A, B, C including but not limited to influenza A virus serotypes H1N1, H2N2, H3N2, H5N1, H7N7, H1N2, H9H2, H7N2, H7N3 and N10N7;

Paramyxoviridae such as human parainfluenza virus 1, measles virus and mumps virus;

Parvoviridae such as adeno-associated virus;

Picornaviridae such as human poliovirus, foot and mouth disease virus (including serotypes O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1); and

Poxviridae such as vaccinia virus, variola virus and avian poxvirus (fowlpox).

In a preferred embodiment, the viral particle can be or can be derived from an *Adenoviridae*, *Orthomyxoviridae*, *Paramyxoviridae*, *Parvoviridae*, *Picornaviridae* or *Poxviridae* virus. In a particularly preferred embodiment, the viral particle can be or can be derived from an adenovirus, vaccinia virus, influenza virus, or measles virus.

Virus-like particles (VLPs) include viral proteins derived from the structural proteins of a virus, but lack viral nucleic acid. When overexpressed, these viral structural proteins spontaneously self-assemble into particles. VLPs are replication incompetent. In some embodiments, the VLPs are viral proteins embedded within a

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lipid bilayer. Examples of VLPs includes phage-derived VLPs, human papillomavirus (HPV) L1 major capsid protein VLPs, Norwalk virus capsid protein VLPs and VLPs assembled from influenza virus structural proteins such as M1 protein, HA hemagglutinin protein and N1 neuraminidase protein.

Viral particles can be prepared using standard techniques well known to those skilled in the art. For example, a virus may be prepared by infecting cultured host cells with the virus strain that is to be used, allowing infection to progress such that the virus replicates in the cultured cells and can be released by standard methods known in the art for harvesting and purifying viruses.

Glycine derivatives

The excipient is a N,N-di(C_1-C_6 alkyl)- or N,N,N-tri(C_1-C_6 alkyl)-glycine or a physiologically acceptable salt or ester thereof. The alkyl group is typically a C_{1-4} alkyl group. Preferred alkyl groups are selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl and tert-butyl. Methyl and ethyl are particularly preferred.

Preferred glycine derivatives for use in the invention are N,N-dimethylglycine, N,N,N-trimethylglycine and physiologically acceptable salts and esters ester thereof. N, N-Dimethylglycine is also termed dimethylglycine (DMG) or 2-(dimethylamino)-acetic acid. N,N,N-trimethylglycine is termed trimethylglycine (TMG) for short and has been mentioned above as a betaine compound.

The salt is typically a salt with a physiologically acceptable acid and thus includes those formed with an inorganic acid such as hydrochloric or sulphuric acid or an organic acid such as citric, tartaric, malic, maleic, mandelic, fumaric or methanesulphonic acid. The hydrochloride salt is preferred.

The ester is typically a C_{1-6} alkyl ester, preferably a C_{1-4} alkyl ester. The ester may therefore be the methyl, ethyl, propyl, isopropyl, butyl, isobutyl or tert-butyl ester. The ethyl ester is preferred.

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Solutions containing N-alkylated glycine derivatives and sulfone compounds

1. N-alkylated glycine derivatives

The N-alkylated glycine derivative is an N,N-di(C_{1-6} alkyl)- or N,N,N-tri(C_{1-6} alkyl)-glycine. The alkyl group is typically a C_{1-4} alkyl group. Preferred alkyl groups are selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl and tert-butyl. Methyl and ethyl are particularly preferred.

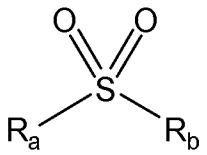
Preferred glycine derivatives for use in the invention are N,N-dimethylglycine, N,N,N-trimethylglycine. N,N-Dimethylglycine is also termed dimethylglycine (DMG) or 2-(dimethylamino)-acetic acid. N,N,N-trimethylglycine is termed trimethylglycine (TMG).

A physiologically acceptable salt or ester of a N-alkylated glycine derivative may be employed. Thus:

- The salt is typically a salt with a physiologically acceptable acid and thus includes those formed with an inorganic acid such as hydrochloric or sulphuric acid or an organic acid such as citric, tartaric, malic, maleic, mandelic, fumaric or methanesulphonic acid. The hydrochloride salt is preferred.
- The ester is typically a C_{1-6} alkyl ester, preferably a C_{1-4} alkyl ester. The ester may therefore be the methyl, ethyl, propyl, isopropyl, butyl, isobutyl or tert-butyl ester. The ethyl ester is preferred.

2. Sulfone compounds

The sulfone compound is a compound of formula (IIC):



(IIC)

wherein R_a and R_b independently represent C_{1-6} alkyl, for example C_{1-4} alkyl. Preferred alkyl groups are selected from methyl, ethyl, propyl, isopropyl, butyl, isobutyl and tert-butyl. Methyl and ethyl are particularly preferred. A preferred

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sulfone compound is methylsulfonylmethane (MSM), which is also known as dimethylsulfone ($DMSO_2$).

Sugars

Sugars suitable for use in the present invention include reducing sugars such as glucose, fructose, glyceraldehydes, lactose, arabinose and maltose; and preferably non-reducing sugars such as sucrose and raffinose. The sugar may be a monosaccharide, disaccharide, trisaccharide, or other oligosaccharides. The term *"sugar"* includes sugar alcohols.

Monosaccharides such as galactose and mannose; dissaccharides such as sucrose, lactose and maltose; trisaccharides such as raffinose; and tetrasaccharides such as stachyose are envisaged. Trehalose, umbelliferose, verbascose, isomaltose, cellobiose, maltulose, turanose, melezitose and melibiose are also suitable for use in the present invention. A suitable sugar alcohol is mannitol.

Preservation of viral activity is particularly effective when two or more sugars are used. Two, three or four sugars may be used. Preferably, the two sugars sucrose and raffinose are used. Sucrose is a disaccharide of glucose and fructose. Raffinose is a trisaccharide composed of galactose, fructose and glucose.

Aqueous Solvent

The aqueous solvent is generally water. Pure water such as water for injections is generally used. Alternatively, physiological saline may be used.

Other Components

The aqueous solution may be buffered. Any suitable physiologically acceptable buffer may be used such as a phosphate buffer. Typically, the pH will be adjusted to from 4 to 9, preferably between 5 and 8 and especially from about pH 6.5 to 7.5. The exact pH will depend, for example, on the stability in aqueous solution of the viral particles.

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For stability purposes, the solutions of the present invention should be protected from microbial contamination and growth. A preservative may therefore be present, for example in an amount of from 0.001 to 1% by weight. Examples of pharmaceutically acceptable anti-microbial agents that can be used in the formulation include:

- quaternary ammonium compounds (e.g. benzalkonium chloride, benzethonium chloride, cetrimide and cetylpyridinium chloride);
- mercurial agents (e.g. phenylmercuric nitrate, phenylmercuric acetate and thimerosal);
- alcoholic agents (e.g. chlorobutanol, phenylethyl alcohol and benzyl alcohol);
- antibacterial esters (e.g. esters of para-hydroxybenzoic acid);
- chelating agents such as disodium edentate (EDTA); and
- other anti-microbial agents such as chlorhexidine, chlorocresol, sorbic acid and its salts and polymyxin.

The presence of a tonicity adjustment agent is sometimes desirable to achieve isotonicity with body fluids resulting in reduced levels of irritancy on administration to a patient. Examples of suitable tonicity adjustment agents are sodium chloride, dextrose and calcium chloride. The isotonicity adjustment agent will desirably be added in a sufficient quantity to achieve this function. Preferably the tonicity adjustment agent is present in an amount of between 0.1 and 10% by weight.

Other additives may be present too such as co-solubilising agents and adjuvants. An adjuvant is generally present when a solution of the invention is used as a vaccine. The adjuvant is used in order to increase potency of the vaccine and/or modulate humoral and cellular immune responses.

Suitable adjuvants include, but are not limited to, mineral salts (e.g., alumuninium hydroxide ("alum"), aluminium phosphate, calcium phosphate), particulate adjuvants (e.g., virosomes, ISCOMS (structured complex of saponins and lipids)), microbial derivatives (e.g., MPL(monophosphoryl lipid A), CpG motifs, modified toxins including TLR adjuvants such as flagellin), plant derivatives (e.g.,

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saponins (QS-21)) and endogenous immunostimulatory adjuvants (e.g., cytokines and any other substances that act as immunostimulating agents to enhance the effectiveness of the vaccine).

Production of Solutions of the Invention

Solutions of the invention can be prepared by admixing the viral particles and other ingredients in any convenient order in the selected aqueous solvent. The viral particles are provided in the required amount, for example in a unit dosage amount. A pharmaceutically effective amount of the viral particles can thus be provided in the solution.

Generally, a preparation of the viral particles is admixed with an aqueous solution of the excipient(s) and optionally one or more sugars. The components of the solution may be admixed under sterile conditions. Alternatively, the components of the solution may be first admixed and the resulting solution sterilised. For example, the excipient(s) and/or optional sugars may be added during manufacture of viral particles, so that viral particles are stabilised during manufacture as well as in the final product. In some cases, however, it may be desirable to remove the excipient(s) and/or optional sugars in a purification step prior to formulation of the final product.

The solution with which the viral particles are admixed may be buffered or the solution may be buffered after admixture with the viral particles. It may be a HEPES, phosphate-buffered, Tris-buffered or pure water solution. The pH may be adjusted as desired. Typically, a solution will have a pH of from 4 to 9, preferably from 5 to 8 and especially about pH 6.5 to 7.5.

The excipient and, optionally, one or more sugars are present at concentrations which provide solutions of the required storage stability. The excipient may be an excipient of the invention as herein defined. Suitable concentrations can be determined and optimised by routine experimentation. The concentrations used in a particular instance will depend on a number of factors including:

- the particular viral particles;

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- the excipient that is being used;
- whether one or more sugars is present and, if so, the identity of the or each sugar.

The excipient and sugar(s) can be present in amounts that result in synergy interactions between the excipient and the sugar(s). For example, synergistic interactions may arise between (a) sulfones such as MSM and raffinose, and (b) N,N-dialkylglcycines such as DMG and sucrose. Suitable concentrations can be determined and optimised by routine experimentation.

The concentration of the glycine derivative compound or physiologically acceptable salt or ester thereof or compound of formula (IIC) or physiologically acceptable salt or ester thereof in the aqueous solution is generally in the range of from 0.001M to 2.5M and more especially from 0.01M to 2.5M. For example, the concentration range may be from 0.1M to 2.5M. The particular concentration of the glycine derivative compound or physiologically acceptable salt or ester thereof or compound of formula (IIC) or physiologically acceptable salt or ester thereof that is employed will depend on several factors including the viral particles; the particular glycine derivative compound or physiologically acceptable salt or ester thereof or compound of formula (IIC) or physiologically acceptable salt or ester thereof being used; whether one, two or more sugars are present and the identity of the sugar(s).

The concentration of an N,N-dialkyl- or N,N,N-trialkyl-glycine or a physiologically acceptable salt or ester thereof in the aqueous solution is generally in the range of 0.1mM to 3M or from 1mM to 2M. The concentration may be from 1mM to 1.5M or from 5mM to 1M. Preferred concentrations are from 7mM to 1.5M or from 0.07M to 0.7M. The particular concentration of an N,N-dialkyl- or N,N,N-trialkyl-glycine or a physiologically acceptable salt or ester thereof that is employed will depend on a number of factors including the viral particles; whether one or more sugars is used and, if so, the particular type of sugar(s) used. Thus:

- Preferred concentrations of the N,N-dialkyl- or N,N,N-trialkyl-glycine or a physiologically acceptable salt or ester thereof when no

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sugar is present are from 5mM to 1.5M or from 7mM to 1M or to 0.7M. More preferred concentrations are from 0.023M to 0.7M, or from 0.07M to 0.7M, such as about 0.07M.

- Preferred concentrations of an N,N-dialkyl- or N,N,N-trialkyl-glycine or a physiologically acceptable salt or ester thereof when one or more sugars are present are generally lower and in the range of from 1mM to 1M or from 5mM to 1M. More preferred concentrations are from 0.007M to 0.7M such as about 0.007M.

The components are present at concentrations which provide solutions of the required storage stability. Suitable concentrations can be determined and optimised by routine experimentation. The N-alkylated glycine derivative or salt or ester thereof and the sulfone compound of formula (IIC) can thus be present in amounts that result in synergy. The concentrations used in a particular instance will depend on a number of factors including:

- the particular viral particles to be stabilised;
- the excipient that is being used;
- whether one or more sugars is present and, if so, the identity of the or each sugar.

In particular:

- the concentration of the N-alkylated glycine derivative or salt or ester thereof in the aqueous solution for drying is generally in the range of 0.1mM to 3M or from 1mM to 2M. The concentration may be from 1mM to 1.5M or from 5mM to 1M. Preferred concentrations are from 7mM to 1.5M, from 0.07M to 0.7M, 0.1M to 1.5M or from 0.5M to 1.25M, and/or
- the concentration of the sulfone compound of formula (IIC) in the aqueous solution for drying is generally in the range of 0.1mM to 3M, from 1mM to 2M or from 0.2mM to 1M such as from 0.35mM to 1M, from 3.5mM to 0.5M, from 0.035M to 0.5M or from 0.035M to

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0.25M. The concentration may be from 0.1M to 1.5M or from 0.5M to 1.25M.

When present in the solutions of the invention, the concentration of sugar or the total concentration of sugars is at least 0.01M, typically up to saturation. Generally the sugar concentration is at least 0.1M, at least 0.2M or at least 0.5M up to saturation e.g. saturation at room temperature or up to 3M, 2.5M or 2M. The sugar concentration may therefore range from, for example, 0.1M to 3M or 0.2M to 2M. Alternatively, the sugar concentration or the total sugar concentration if more than one sugar is present may therefore range from 0.08M to 3M, from 0.15M to 2M or from 0.2M to 1M. A suitable range is from 0.05 to 1M.

When more than one sugar is present in the solutions of the invention, preferably one of those sugars is sucrose. The sucrose may be present at a concentration of from 0.05M, 0.1M, 0.25M or 0.5M up to saturation e.g. saturation at room temperature or up to 3M, 2.5M or 2M.

The ratio of the molar concentration of sucrose relative to the molar concentration of the other sugar(s) is typically from 1:1 to 20:1 such as from 5:1 to 15:1. In the case when two sugars are present and in particular when sucrose and raffinose are present, therefore, the ratio of molar concentrations of sucrose is typically from 1:1 to 20:1 such as from 5:1 to 15:1 and preferably about 10:1.

Particularly preferred solutions contain the following components:

- Sucrose at a concentration of 0.8M to 1.2M, for example about 1M; TMG at a concentration of 0.8 to 1.2M, for example about 1M; and/or raffinose at a concentration of 200 to 400 mM, for example about 300mM. Typically such a solution comprises MVA.
- Sucrose at a concentration of 0.8M to 1.2M, for example about 1M; and/or MSM at a concentration of 0.75 to 1.15M, for example about 0.95M. At these concentrations a synergistic interaction between MSM and sucrose may arise. Typically such a solution comprises adenovirus.

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- Sucrose at a concentration of 0.3M to 0.7M, for example about 0.5M; DMG at a concentration of 0.2 to 0.6M, for example about 0.4M; and/or raffinose at a concentration of 200 to 400mM, for example about 275mM. At these concentrations a synergistic interaction between DMG and raffinose may arise. Typically such a solution comprises adenovirus.

The pH of a solution of the invention may be adjusted as desired. Typically, a solution will have a pH of from 4 to 9, preferably from 5 to 8 and especially about pH 6.5 to 7.5.

A solution of the invention is pyrogen-free. The solution is thus sterilised. A solution can be sterilised by passing it through a sterilising filter. The sterilised solution can then be introduced into containers, such as vials, which are then hermetically sealed. Alternatively, sterilisation can take place e.g. by autoclaving after the solution has been sealed in a container.

The solution can thus be provided in a sealed vial, ampoule, syringe, cartridge, flexible bag or glass bottle. As a small volume parenteral (SVP), it may be provided in a disposable cartridge, disposable syringe, vial, ampoule or flexible bag. As a large volume parenteral (LVP), it may be provided in a vial, flexible bag, glass bottle or, in some cases, as a disposable syringe.

Preferably the containers are vials with non-reactive stoppers. The stopper may be Teflon ™-coated or –faced. Silicone rubber stoppers or other non-reactive stoppers are contemplated.

Cartridges, syringes, vials and ampoules are usually composed of Type I or II glass, or polypropylene. Flexible bags are typically constructed with multilayered plastic. Stoppers and septa in cartridges, syringes, and vials are typically composed of elastomeric materials. The input (medication) and output (administration) ports for flexible bags may be plastic and/or elastomeric materials. An overwrap may be used with flexible bags to retard solvent loss and to protect the flexible packaging system from rough handling.

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The solutions of the invention can be used as desired, depending upon the viral particles in solution. The solution can be withdrawn from a sealed container e.g. by a syringe and injected into a patient by a suitable route. The solution may thus be administered by subcutaneous, intramuscular, intravenous or intraperitoneal injection. A solution may alternatively be administered by infusion. The solution may be diluted prior to administration.

Preservation of viral particles during manufacture

In some circumstances, it may be desirable to use the excipient of the invention during manufacturing of a solution of viral particles, in order that the viral particles are preserved or stabilised during the manufacturing process. This can increase the yield of the process.

Typically, the excipient of the invention will be retained in the solution of viral particles and thereby in the final product. This can be advantageous since the excipient of the invention will continue to stabilise the viral particles in the final product.

Alternatively, there may be some situations in which it is preferable to remove the excipient of the invention in a purification step. Such removal can be carried out by any suitable purification technique known to those skilled in the art, such as chromatography. The exact purification method will depend on the excipient being used and suitable techniques can be readily selected by those skilled in the art.

Once the excipient has been removed, the solution of viral particles is typically sealed in a container, such as vial, ampoule, syringe, cartridge, flexible bag or glass bottle.

Preferably the solution is sterilised, for example by passing the solution through a sterilising filter, prior to introducing the solution into the container. Alternatively, it may be preferable to perform the manufacturing process and purification under sterile conditions, such that the end product is sterile.

The concentration of the excipient of the invention is preferably as set out above under “Production of Solutions of the Invention” above. The concentration of

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the sugar(s), where present, is also preferably as set out under "Production of Solutions of the Invention" above.

Measuring viral particle preservation

Preservation in relation to viral particles refers to resistance of the viral particle to physical or chemical degradation and/or loss of biological activity.

Methods of assaying for viral activity such as infectivity and/or immunogenicity are well known to those skilled in the art and include but are not limited to growth of a virus in a cell culture, detection of virus-specific antibody in blood, ability to elicit T and/or B cell responses, detection of viral antigens, detection of virus encoded DNA or RNA, or observation of virus particles using a microscope.

Further, the presence of a virus gives rise to morphological changes in the host cell, which can be measured to give an indication of viral activity. Detectable changes such as these in the host cell due to viral infection are known as cytopathic effect. Cytopathic effects may consist of cell rounding, disorientation, swelling or shrinking, death and detachment from the surface. Many viruses induce apoptosis (programmed cell death) in infected cells, measurable by techniques such as the TUNEL (Terminal uridine deoxynucleotidyl transferase dUTP nick end labelling) assay and other techniques well known to those skilled in the art.

Viruses may also affect the regulation of expression of the host cell genes and these genes can be analysed to give an indication of whether viral activity is present or not. Such techniques may involve the addition of reagents to the cell culture to complete an enzymatic or chemical reaction with a viral expression product. Furthermore, the viral genome may be modified in order to enhance detection of viral infectivity. For example, the viral genome may be genetically modified to express a marker that can be readily detected by phase contrast microscopy, fluorescence microscopy or by radioimaging. The marker may be an expressed fluorescent protein such as GFP (Green Fluorescent Protein) or an expressed enzyme that may be involved in a colourimetric or radiolabelling reaction. The marker could also be a gene product that interrupts or inhibits a particular function of the cells being tested.

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An assay for plaque-forming units can be used to measure viral infectivity and to indicate viral titre. In this assay, suitable host cells are grown on a flat surface until they form a monolayer of cells covering a plastic bottle or dish. The selection of a particular host cell will depend on the type of virus. Examples of suitable host cells include but are not limited to CHO, BHK, MDCK, 10T1/2, WEHI cells, COS, BSC 1, BSC 40, BMT 10, VERO, WI38, MRC5, A549, HT1080, 293, B-50, 3T3, NIH3T3, HepG2, Saos-2, Huh7, HEK293 and HeLa cells. The monolayer of host cells is then infected with the viral particles. The liquid medium is replaced with a semi-solid one so that any virus particles produced, as the result of an infection cannot move far from the site of their production. A plaque is produced when a virus particle infects a cell, replicates, and then kills that cell. A plaque refers to an area of cells in the monolayer which display a cytopathic effect, e.g. appearing round and darker than other cells under the microscope, or as white spots when visualized by eye; the plaque center may lack cells due to virus-induced lysis. The newly replicated virus infects surrounding cells and they too are killed. This process may be repeated several times. The cells are then stained with a dye such as methylene blue, which stains only living cells. The dead cells in the plaque do not stain and appear as unstained areas on a coloured background.

Each plaque is the result of infection of one cell by one virus followed by replication and spreading of that virus. However, viruses that do not kill cells may not produce plaques. A plaque refers to an area of cells in a monolayer which display a cytopathic effect, e.g. appearing round and darker than other cells under the microscope, or as white spots when visualized by eye; the plaque center may lack cells due to virus-induced lysis. An indication of viral titre is given by measuring "*plaque-forming units*" (PFU). Levels of viral infectivity can be measured in a sample of biological material preserved according to the present invention and compared to control samples such as freshly harvested virus or samples subjected to desiccation and/or thermal variation without addition of the preservation mixture of the present invention.

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Some types of viral particles of the invention, such as viral proteins, VLPs, or some inactivated viruses do not have the ability to form plaques in the plaque assay. In this case, preservation can be measured by other methods such as methods for determining immunogenicity which are well known to those skilled in the art. For example, *in vivo* and *in vitro* assays for measuring antibody or cell-mediated host immune responses are known in the art and suitable for use in the present invention. For example, an antibody based immune response may be measured by comparing the amount, avidity and isotype distribution of serum antibodies in an animal model, before and after immunization using the preserved viral particle of the invention.

Uses of the preserved viral particles of the invention

The solutions of the invention can be used as desired. The solution can be withdrawn from a sealed container e.g. by a syringe and injected into a patient by a suitable route. The solution may thus be administered by subcutaneous, intramuscular, intravenous or intraperitoneal injection. A solution may alternatively be administered by infusion. The solution may be diluted prior to administration.

Vaccines

The solutions of the present invention may find use as vaccines. For example, solutions containing whole killed virus, live attenuated virus, chemically inactivated virus, VLPs or live viral vectors are suitable for use as vaccines. As a vaccine the viral particles may be used as antigens or to encode antigens such as viral proteins for the treatment or prevention of a number of conditions including but not limited to viral infection, sequelae of viral infection including but not limited to viral-induced toxicity, cancer and allergies. Such antigens contain one or more epitopes that will stimulate a host's immune system to generate a humoral and/or cellular antigen-specific response.

A vaccine of the invention may be used to prevent or treat infection by viruses such as human papilloma viruses (HPV), HIV, HSV2/HSV1, influenza virus (types A, B and C), para influenza virus, polio virus, RSV virus, rhinoviruses, rotaviruses,

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hepaptitis A virus, norwalk virus, enteroviruses, astroviruses, measles virus, mumps virus, varicella-zoster virus, cytomegalovirus, epstein-barr virus, adenoviruses, rubella virus, human T-cell lymphoma type I virus (HTLV-I), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis D virus, poxvirus and vaccinia virus. The vaccine may further be used to provide a suitable immune response against numerous veterinary diseases, such as foot and mouth disease (including serotypes O, A, C, SAT-1, SAT-2, SAT-3 and Asia-1), coronavirus, bluetongue, feline leukaemia virus, avian influenza, hendra and nipah virus, pestivirus, canine parvovirus and bovine viral diarrhoea virus. In one embodiment, the vaccine is a subunit, conjugate or multivalent vaccine. For example, the vaccine of the invention may be used to treat infection by two or more different types of virus such as measles, mumps and rubella (e.g. MMR vaccine).

To measure the preservation of stability of a vaccine prepared in accordance with the present invention, the potency of the vaccine can be measured using techniques well known to those skilled in the art. For example, the generation of a cellular or humoral immune response can be tested in an appropriate animal model by monitoring the generation of antibodies or immune cell responses to the vaccine. The ability of vaccine samples to trigger an immune response may be compared with vaccines not subjected to the same preservation technique.

Viral vectors

A virus or viral vector can be used according to the present invention to transfer a heterologous gene or other nucleic acid sequence to target cells. Suitably, the heterologous sequence (i.e. transgene) encodes a protein or gene product which is capable of being expressed in the target cell. Suitable transgenes include desirable reporter genes, therapeutic genes and genes encoding immunogenic polypeptides (for use as vaccines). Gene therapy, an approach for treatment or prevention of diseases associated with defective gene expression, involves the insertion of a therapeutic gene into cells, followed by expression and production of the required proteins. This approach enables replacement of damaged genes or inhibition of expression of

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undesired genes. In particular, the virus or viral vector may be used in gene therapy to transfer a therapeutic transgene or gene encoding immunogenic polypeptides to a patient.

In a preferred embodiment, the viral particle is a live viral vector. By "live viral vector" is meant a live viral vector that is non-pathogenic or of low pathogenicity for the target species and in which has been inserted one or more genes encoding antigens that stimulate an immune response protective against other viruses or microorganisms, a reporter gene or a therapeutic protein. In particular, nucleic acid is introduced into the viral vector in such a way that it is still able to replicate thereby expressing a polypeptide encoded by the inserted nucleic acid sequence and in the case of a vaccine, eliciting an immune response in the infected host animal. In one embodiment, the live viral vector is an attenuated live viral vector i.e. is modified to be less virulent (disease-causing) than wildtype virus.

The basis of using recombinant viruses as potential vaccines involves the incorporation of specific genes from a pathogenic organism into the genome of a nonpathogenic or attenuated virus. The recombinant virus can then infect specific eukaryotic cells either in vivo or in vitro, and cause them to express the recombinant protein.

Live viral vector vaccines derived by the insertion of genes encoding sequences from disease organisms may be preferred over live attenuated vaccines, inactivated vaccines, subunit or DNA approaches. One of the most important safety features of live viral vectors is that the recipients may be immunized against specific antigens from pathogenic organisms without exposure to the disease agent itself. Safety is further regulated by the selection of a viral vector that is either attenuated for the host or unable to replicate in the host although still able to express the heterologous antigen of interest. A vaccine strain that has a history of safety in the target species offers an additional safety feature. Several systems have been developed in which the vector is deleted of essential genes and preparation of the vaccine is carried out in cell systems that provide the missing function.

A variety of vectors such as retroviral, lentiviral, herpes virus, poxvirus,

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adenoviral and adeno-associated viral vectors can be used for the delivery of heterologous genes to target cells. The heterologous gene of interest may be inserted into the viral vector. The viral vectors of the invention may comprise for example a virus vector provided with an origin of replication, optionally a promoter for the expression of the heterologous gene and optionally a regulator of the promoter. For example, adenoviruses useful in the practice of the present invention can have deletions in the E1 and/or E3 and /or E4 region, or can otherwise be maximized for receiving heterologous DNA.

The viral vector may comprise a constitutive promoter such as a cytomegalovirus (CMV) promoter, SV40 large T antigen promoter, mouse mammary tumour virus LTR promoter, adenovirus major late promoter (MLP), the mouse mammary tumour virus LTR promoter, the SV40 early promoter, adenovirus promoters such as the adenovirus major late promoter (Ad MLP), HSV promoters (such as the HSV IE promoters), HPV promoters such as the HPV upstream regulatory region (URR) or rous sarcoma virus promoter together with other viral nucleic acid sequences operably linked to the heterologous gene of interest. Tissue-specific or inducible promoters can also be used to control expression of the heterologous gene of interest. Promoters may also be selected to be compatible with the host cell for which expression is designed.

The viral vector may also comprise other transcriptional modulator elements such as enhancers. Enhancers are broadly defined as a cis-acting agent, which when operably linked to a promoter/gene sequence, will increase transcription of that gene sequence. Enhancers can function from positions that are much further away from a sequence of interest than other expression control elements (e.g. promoters) and may operate when positioned in either orientation relative to the sequence of interest. Enhancers have been identified from a number of viral sources, including polyoma virus, BK virus, cytomegalovirus (CMV), adenovirus, simian virus 40 (SV40), Moloney sarcoma virus, bovine papilloma virus and Rous sarcoma virus. Examples of suitable enhancers include the SV40 early gene enhancer, the enhancer/promoter derived from the long terminal repeat (LTR) of the Rous Sarcoma Virus, and elements

derived from human or murine CMV, for example, elements included in the CMV intron A sequence.

The viral vector containing a heterologous gene of interest may then be preserved according to the method of the invention before storage, subjecting to further preservation techniques such as lyophilisation, or administration to a patient or host cell.

Nucleic acids encoding for polypeptides known to display antiviral activity, immunomodulatory molecules such as cytokines (e.g. TNF-alpha, interleukins such as IL-6, and IL-2, interferons, colony stimulating factors such as GM-CSF), adjuvants and co-stimulatory and accessory molecules may be included in the viral vector of the invention. Alternatively, such polypeptides may be provided separately, for example in the preservation mixture of the invention or may be administrated simultaneously, sequentially or separately with viral vectors of the invention.

Preferably, the preserved viral vector of the invention may be introduced into suitable host cells using a variety of viral techniques that are known in the art, such as for example infection with recombinant viral vectors such as retroviruses, herpes simplex virus and adenoviruses. Preferably, administration of the preserved viral vector of the invention containing a gene of interest is mediated by viral infection of a target cell.

A number of viral based systems have been developed for transfecting mammalian cells.

For example, a selected recombinant nucleic acid molecule can be inserted into a vector and packaged as retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to cells of the subject either *in vivo* or *ex vivo*. Retroviral vectors may be based upon the Moloney murine leukaemia virus (Mo-MLV). In a retroviral vector, one or more of the viral genes (gag, pol & env) are generally replaced with the gene of interest.

A number of adenovirus vectors are known. Adenovirus subgroup C serotypes 2 and 5 are commonly used as vectors. The adenovirus may be a human or

non-human adenovirus. The wild type adenovirus genome is approximately 35kb of which up to 30kb can be replaced with foreign DNA.

There are four early transcriptional units (E1, E2, E3 & E4), which have regulatory functions, and a late transcript, which codes for structural proteins. Adenovirus vectors may have the E1 and/or E3 gene inactivated. The missing gene(s) may then be supplied in trans either by a helper virus, plasmid or integrated into a helper cell genome. Adenovirus vectors may use an E2a temperature sensitive mutant or an E4 deletion. Minimal adenovirus vectors may contain only the inverted terminal repeats (ITRs) & a packaging sequence around the transgene, all the necessary viral genes being provided in trans by a helper virus. Suitable adenoviral vectors thus include Ad4, Ad5, Ad7, Ad11, Ad14, Ad26, Ad35 and Ad36 vectors and simian adenovirus vectors, preferably Ad4, Ad5, Ad7, Ad35 and Ad36 vectors. Ad5 is most commonly used.

Viral vectors may also be derived from the pox family of viruses, including vaccinia viruses and avian poxvirus such as fowlpox vaccines. For example, modified vaccinia virus Ankara (MVA) is a strain of vaccinia virus which does not replicate in most cell types, including normal human tissues. A recombinant MVA vector may therefore be used to deliver a polypeptide.

Addition types of virus such as adeno-associated virus (AAV) and herpes simplex virus (HSV) may also be used to develop suitable vector systems

Administration

Solutions according to the present invention may be administered to a subject *in vivo* using a variety of known routes and techniques. The solutions are suitable for parenteral administration. For example, the vaccines can be provided as an injectable solution, suspension or emulsion and administered via parenteral, subcutaneous, oral, epidermal, intradermal, intramuscular, interarterial, intraperitoneal, intravenous injection using a conventional needle and syringe, or using a liquid jet injection system. Vaccines may be administered topically to skin or mucosal tissue, such as

nasally, intratrachealy, intestinal, sublingually, rectally or vaginally, or provided as a finely divided spray suitable for respiratory or pulmonary administration.

The following Examples illustrate the invention.

Statistics

In some of the Examples, the following statistical values were calculated:

R^2 = coefficient of determination. A measure of goodness of fit. R^2<0.5=low model significance.

Q^2 = estimate of prediction precision. A measure of goodness of prediction. Q^2 should be >0.1 for a significant model. Q^2 should be >0.5 for a good model. R^2-Q^2 < 0.2 to 0.3

Model validity (MV)= "a test of diverse model problems". Model validity < 0.25 = indicator of statistically significant model problems e.g. outliers, incorrect model / transformation.

Reproducibility (Rep) = measure of variation between replicates compared to overall variability. Reproducibility > 0.5 implies significance.

The following materials, equipment and techniques were employed unless stated otherwise in Examples 1 to 4:

Materials

HEK-293 cells (ECACC 85120602)

DMSO (Sigma D1435, Lot 118K1455)

Sucrose (Sigma 16104, Lot 70040)

Raffinose (Sigma R0250, Lot 039K0016)

PBS (Sigma D8662, Lot 118K2339)

Water (Sigma W3500, Lot 8M0411)

5ml glass vials (Adelphi Tubes VCD005)

2ml glass vials (Adelphi Tubes VCDIN2R)

14mm freeze drying stoppers (Adelphi Tubes FDIA14WG/B)
14mm caps (Adelphi Tubes CWPP14)
Adenovirus GFP (Vector Biolabs cat. 1060)
Glycine (Sigma, G7126, 118K00181)
N,N-DMG (Sigma D1156, Lot 077K1856)
SMM (Sigma, 64382, 1339210)
TMG (Sigma, B2629, 1089K1201)

Equipment

Modulyo D Freeze Dryer (Thermofisher)
HERA safe class II cabinet (Thermofisher)
Binder CO_2 Incubator (Binder)
Binder APT line TM MK thermocycling test chamber (Binder)
Thermo Scientific MaxQ 4450 Incubator (Thermofisher)
KERN EW220-3NM balance (VWR)
Elcold -45°C freezer (VWR)
Forma 900 series –80°C freezer (Thermofisher)
Synergy HT microplate reader (Biotek)

Example 1

Virus Formulation

Recombinant adenovirus (Vector Biolabs) expressing enhanced GFP (Green Fluorescent Protein) under a CMV promoter was used for ease of detection during assay.

In this study four glycinergic compounds and one thetin were each tested for efficacy as a preservative (of adenovirus) at a final concentration of 0.07-0.70M, both in co-formulations with sugars (1M Sucrose, 100mM Raffinose) and in their absence. The glycinergic compounds tested were Glycine, Sarcosine (mono-methyl glycine), DMG (Di-methyl glycine), TMG (Tri-Methyl glycine). The Thetin tested was SMM

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(S-methyl-methionine). Virus was formulated with excipient mixtures in order to test their efficacy in preserving viral activity through a period of thermal challenge. Each mixture of excipients plus virus (see Table 1 below) was made up as a stock in PBS and 300µl added to appropriately labelled 5ml glass vials.

Table 1 – Summary of treatments, each setup in triplicate. Y=Yes, N= No. Where sugars present concentration = 1M Sucrose, 100mM Raffinose.

Excipient	Conc. (M)	Sugars?	Thermal Challenge (°C)	Excipient	Conc. (M)	Sugars?	Thermal Challenge (°C)
DMG	0.70	Y	4	DMG	0.7	Y	37
DMG	0.23	Y	4	DMG	0.23	Y	37
DMG	0.07	Y	4	DMG	0.07	Y	37
DMG	0.70	N	4	DMG	0.70	N	37
DMG	0.23	N	4	DMG	0.23	N	37
DMG	0.07	N	4	DMG	0.07	N	37
Glycine	0.70	Y	4	Glycine	0.70	Y	37
Glycine	0.23	Y	4	Glycine	0.23	Y	37
Glycine	0.07	Y	4	Glycine	0.07	Y	37
Glycine	0.70	N	4	Glycine	0.70	N	37
Glycine	0.23	N	4	Glycine	0.23	N	37
Glycine	0.07	N	4	Glycine	0.07	N	37
Sarcosine	0.70	Y	4	Sarcosine	0.70	Y	37
Sarcosine	0.23	Y	4	Sarcosine	0.23	Y	37
Sarcosine	0.07	Y	4	Sarcosine	0.07	Y	37
Sarcosine	0.70	N	4	Sarcosine	0.70	N	37
Sarcosine	0.23	N	4	Sarcosine	0.23	N	37
Sarcosine	0.07	N	4	Sarcosine	0.07	N	37
TMG	0.70	Y	4	TMG	0.70	N	37

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TMG	0.23	Y	4	TMG	0.23	Y	37
TMG	0.07	Y	4	TMG	0.07	Y	37
TMG	0.70	N	4	TMG	0.70	N	37
TMG	0.23	N	4	TMG	0.23	N	37
TMG	0.07	N	4	TMG	0.07	N	37
SMM	0.70	Y	4	SMM	0.70	Y	37
SMM	0.23	Y	4	SMM	0.23	Y	37
SMM	0.07	Y	4	SMM	0.07	Y	37
SMM	0.70	N	4	SMM	0.70	N	37
SMM	0.23	N	4	SMM	0.23	N	37
SMM	0.07	N	4	SMM	0.07	N	37
None	0.00	Y	4	None	0.00	Y	37
None	0.00	N	4	None	0.00	N	37

Thermal Challenge

Three replicates of each treatment were placed at 4°C, and a further 3 at 37°C, for a period of 7 days. At this time all samples were placed at 4°C until it was practical to assay them.

Adenovirus assay

Cells permissive to the Adenovirus (HEK 293, ECACC 85120602) were seeded into 96-well-flat-bottomed cell culture dishes (VWR, UK) at 10^5 cells per ml (100µl per well) and maintained at 37°C with 5% CO_2. After achieving 90% confluence, vials containing the adenovirus plus excipient were removed from the fridge and 1 in 10, and 1 in 100 dilutions produced by serial dilution in DMEM. 100µl of each of the resultant dilutions (1 in 10 and 1 in 100) was then added wells of the plate containing HEK 293 cells. Additionally, a further sample of adenovirus, from the same source and with the same titre (on storage at –80°C) used in the excipient treatments, was thawed and used to produce a 1 in 10 dilution series (in DMEM). Dilutions ranging from 1 in 10 to 1 in 10^6 were also added to individual wells containing HEK 293s. At 48 hours, post inoculation the number of GFP (Green

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Fluorescent Protein) cells per well were counted using fluorescent microscopy, and this was subsequently converted to pfu/ml of the treated samples taking into account the volume applied and dilution of the inoculum.

Results and discussion

Recovered Viral Activity after 1 week at 4°C (Figure 1a & b) (glycinergics and SMM WITHOUT added sugar or WITH added sugar respectively)

After one week at 4°C adenoviral samples formulated in PBS alone, recovered viral activity was at 46% of the original titre. However, formulation together with sugars (1M Sucrose, 100mM Raffinose) resulted in an enhancement of recovery to 69%. All formulations of adenovirus together with glycinergics or SMM and in the absence of sugars resulted in a recovery of 52-71%. Although, this represented a significant improvement over PBS, even the best glycinergic or SMM formulations yield recovery that is only equivalent to that of sugars (see Figure 1a). When the glycinergics or SMM were formulated together with sugars, recovered viral activity was not further enhanced (50-72%). In both cases (i.e. glycinergics or SMM in the presence or absence of sugars) there was no clear dose dependency, that is to say no clear correlation between recovered viral activity and concentration of glycinergic or SMM (see Figure 1b).

Recovered Viral Activity after 1 week at 37°C (Figure 1c & d) (glycinergics and SMM WITHOUT added sugar or WITH added sugar respectively)

After one week at 37°C adenoviral samples formulated in PBS alone, recovered viral activity was 25% of the original titre. Formulation together with sugars (1M Sucrose, 100mM Raffinose) enhanced recovery to 38%.

Use of glycinergics at the higher end of the tested concentration range and as the sole excipient resulted in improved efficacy over PBS alone, and in each case a strong positive correlation was observed between glycinergic concentration and recovered viral activity. Over the concentration range tested (0.07 to 0.70 M) activity

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was enhanced from 29% to 45% in the case of glycine, from 29% to 49% with sarcosine, from 27% to 41% with DMG, and from 21 to 45 % with TMG. With each glycinergic in the concentration range tested the best results were achieved with the highest concentration tested and it was possible to recover viral activity as high or greater than when using sugars as the sole formulant (see Figure 1c).

Use of the following glycinergics, Glycine, Sarcosine, and DMG, across the full tested concentration range together with sugars resulted in improved efficacy over PBS alone. With the exception of the lowest sarcosine and DMG concentrations recovery was also superior to sugars alone. In each case a strong positive correlation was observed between glycinergic concentration and recovered viral activity. Over the concentration range tested (0.07 to 0.70 M) activity was enhanced from 41% to 54% in the case of glycine, from 37% to 56% with sarcosine, and from 37% to 52% with DMG (see Figure 1d).

An exception was that when TMG was co-formulated with sugars some kind of antagonistic effect was observed. This resulted in a negative correlation between TMG concentration and recovered activity. Activity varied with TMG concentration from 45% at 0.07M to 38.7% at 0.70M. This data suggests that sugars alter the optimum concentration of TMG, since a positive interaction between TMG and sugars is observed at 0.07M and a negative one at 0.70M, but the recovered activity never exceeds what has been observed as possible with TMG alone (see Figure 1d). Finally, SMM preserves adenoviral activity when used as the sole formulant in the range 0.07M to 0.23M (recovered activity is 33% and 43% respectively).

Example 2 - Stablisation of adenovirus

The following materials, equipment and techniques were employed unless stated otherwise in Example 2 and Example 3:

Materials

Chemical

Dimethylglycine (DMG) (Sigma D1156, Lot 077K1856)

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Dimethylsulfone (MSM)	(Sigma M81705, Lot 0001452516)
Dulbecco's Modified Eagles Medium (DMEM)	(Sigma D5796, Lot RNBB1139)
Foetal Bovine Serum (FBS)	(Sigma F7524, Lot 109K3395)
Penicillin Streptomycin (PS)	(Sigma P4458, Lot 0409M00393)
Saline Sodium Citrate (SSC)	(Sigma S6639, Lot 020M8404)
Sucrose	(Sigma 16104, Lot SZB90120)
Water	(Sigma W3500, Lot RNBB1139)

Biological

Adenovirus	(Vector Biolabs cat. 1060)
BHK-21 cell line	(ECCAC CB2857)
HEK 293	(ECACC 85120602)

Other

5ml glass vials	(Adelphi Tubes VCD005)
14mm freeze-drying stoppers	(Adelphi Tubes FDIA14WG/B)
14mm caps	(Adelphi Tubes CWPP14)

Equipment

HERA safe class II cabinet	(Thermo Fisher, EQP# 011 & 012)
DMIL LED Inverted Microscope	(Leica, EQP#062)
Binder CO_2 Incubator	(Binder, EQP#014)
Forma 900 series -80°C freezer	(Thermofisher, EQP#015)
ATL-84-1 Atlion Balance	(Acculab, EQP#088)
IP250 37°C Incubator	(LTE, EQP#016)

Preparation of liquid virus preparations

Recombinant human adenovirus Ad5 (Vector Biolabs) expressing enhanced GFP (Green Fluorescent Protein) under a CMV promoter, and with a titre (pre-freeze) of $6.7x10^5$ pfu/ml in SSC, was removed from storage at -80°C and allowed to thaw.

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50µl aliquots were added to 5 ml glass vials. To these 50µl virus samples was added 250µl of a formulation mixture composed of DMG, MSM and optionally sucrose. Each formulation mixture was made up in SSC. The concentration of DMG, MSM and sucrose in each formulation after addition to the virus sample is shown in Table 2:

Table 2 – Tested formulations

Formulation	Sucrose (M)	MSM (M)	DMG (M)
1	0.00	0.10	0.10
2	0.15	0.10	0.10
3	0.00	1.00	0.10
4	0.15	1.00	0.10
5	0.08	0.55	0.55
6	0.08	0.55	0.55
7	0.08	0.55	0.55
8	0.00	0.10	1.00
9	0.15	0.10	1.00
10	0.00	1.00	1.00
11	0.15	1.00	1.00

The vials were stoppered and capped (screw cap) before being placed at 37˚C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to 4˚C until it was practical to assay them.

Assay for infectious adenovirus

96 flat bottomed cell culture dishes (VWR, UK) were seeded with HEK 293 (ECACC 85120602) cells at 10^5 cells per ml (100µl per well) and maintained at 37ºC with 5% CO_2. After achieving 90% confluence, cells were inoculated.

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Vials containing adenovirus plus excipient were reconstituted in 300µl SSC. A 1 in 10 dilution step was then taken by taking 20µl from the reconstituted vial and adding to 180µl of Dulbecco's Modified Eagle Medium (DMEM). A further 1 in 100 dilution (of the original sample) was performed by taking 20µl of the 1 in 10 dilution and adding it to 180µl of DMEM. 100µl of each of the resultant dilution (1 in 10 and 1 in 100) was then added to wells of the plate containing HEK 293 cells.

Additionally, a further sample of adenovirus, from the same source and with the same titre (on storage at -80˚C) used in the excipient treatments, was thawed and used to produce a 1 in 10 dilution series (in DMEM + 10% FBS). Dilutions ranging from 1 in 10 to 1 in 10^6 were also added to individual wells containing HEK 293s. At 48 hours post inoculation, the number GFP (Green Fluorescent Protein) cells per well were counted using fluorescent microscopy, and this was subsequently converted to pfu/ml of the treated samples taking into account the volume applied and dilution of the inoculum.

Results

The results are shown in Figure 2. When the data was analysed by multiple linear regression (MLR) analysis using the MODDE 9.0 programme (Umetrics, Sweden), a synergistic effect was observed when MSM and DMG where used in combination

Example 3: Stablisation of MVA

Preparation of liquid virus preparations

MVA was recovered from storage at –80ºC and thawed. 50µl aliquots were added to 5 ml glass vials. To these vials was added 250µl of a formulation mixture listed in Table 1 above. The vials were stoppered and screw caps tightened to seal. The vials were immediately placed at 37˚C for thermal challenge. Thermal challenge was for 7 days, after which all the vials were returned to 4˚C until it was practical to assay them.

Assay for infectious MVA

Assay plates (96 wells) were seeded with BHK-21 cells (100µl per well, 10^5 cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed at +37°C, + 5% CO_2 for 1 to 2 hours.

Meanwhile, a dilution series of the formulated MVA samples was prepared (in the same growth media) ranging from 10^{-1} to 10^{-4}. Each dilution series was prepared 4 times. 35µl of each dilution was applied to individual wells containing BHK-21 cells and the wells were topped up with a further 65µl of media.

On day 6 after inoculation, the wells were scored for presence or absence of cytopathic effect (CPE) and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

Results

The results are shown in Figure 3.

Example 4

Materials

Chemical

	Supplier	**Product Code**	**Lot No.**
20x SSC	Sigma	S6639	020M8404
Dulbeccos Modified Eagles Medium	Sigma	D5796	RNBB1139
Foetal Bovine Serum	Sigma	F7524	109K3395
Penicillin Streptomycin	Sigma	P4458	0409M0093
Trimethyl glycine	Sigma	B2629	069K1514
Water	Sigma	W3500	8M0411

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Biological

	Supplier	**Product Code**
BHK-21 cell line	ECACC	CB2857
MVA	ATCC	VR-1508

Other

	Manufacturer	**Product Code**
5ml glass vials	Adelphi Tubes	VCD005
14mm freeze drying stoppers	Adelphi Tubes	FDIA14WG/B
14mm caps	Adelphi Tubes	CWPP14

Equipment

	Manufacturer	Equipment No.
HERA safe class II cabinet	Thermo Fisher	EQP# 011 & 012
DMIL LED Inverted Microscope	Leica	EQP#062
Binder CO_2 Incubator	Binder	EQP#014
Forma 900 series -80°C freezer	Thermofisher	EQP#015
ATL-84-1 Atlion Balance	Acculab	EQP#088
IP250 37°C Incubator	LTE	EQP#016

Methods

Design of Experiment

MODDE 9.0 was used to generate a Central Composite Face-Centred (CCF) design (see Figure 4). CCF designs are a form of Response Surface Modelling (RSM) design that tests only three levels of each factor but still supports a quadratic model. Unlike regular formulation designs non-significant factors can be eliminated from the analysis and so do not become a confounding factor.

Preparation of and thermal challenge of formulated MVA in a liquid setting

MVA was recovered from storage at -80°C and thawed. 50µl aliquots of the MVA were added to 15, 5ml, glass vials. Subsequently, 50µl aliquots of virus were added to 15, 5ml, glass vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 3 and were made up in SSC.

Table 3

Formulation No.	Sucrose (M)	Raffinose (mM)	TMG (M)	Titre (pfu/ml)
1	0.25	150.0	0.13	7.6E+04
2	0.75	150.0	0.13	3.0E+05
3	0.5	272.5	0.42	3.0E+05
4	0.25	27.5	0.71	3.0E+05
5	0.75	27.5	0.71	4.8E+05
6	0	150.0	1.00	1.9E+05
7	0.5	150.0	1.00	4.8E+05
8	0.5	150.0	1.00	4.8E+05
9	0.5	150.0	1.00	3.0E+05
10	1	150.0	1.00	3.0E+05
11	0.25	272.5	1.29	4.8E+05
12	0.75	272.5	1.29	7.6E+05
13	0.5	27.5	1.58	3.0E+05
14	0.25	150.0	1.87	4.8E+05
15	0.75	150.0	1.87	4.8E+05

The vials were stoppered and capped (screw cap) before being placed at +37°C for 1 week of thermochallenge and later placed at +4°C until it was practical to assay them.

Assay of MVA

Assay plates (96 well) were seeded with BHK-21 cells (100µl per well, 10^5 cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed at +37°C, + 5% CO_2 for 1-2 hours.

A 10 fold dilution series of the formulated MVA samples was prepared (in the same growth media) ranging from 1 in 10 to 1 in 10,000. Each dilution series was

prepared 5 times. 100µl of each dilution was applied to individual wells containing BHK-21 cells (described above).

On 6 d p.i. the wells were scored for presence or absence of CPE and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

Subsequently, a 2 fold dilution series of the formulated MVA samples was prepared ranging from 1 in 2,000 to 1 in 32,000. These dilutions were assayed separately but as before.

Results

Raw data collected in this investigation are shown in Table 3 above. Responses ranged from $7.6x10^4$ to $7.6x10^5$ $TCID_{50}$/ml, or 7.4-74.0% of starting titre (see Table 3). The model generated from this data scored reasonably on all four model assessment parameters (R^2 = 0.79, Q^2=0.49, Model Validity =0.90, Reproducibility=0.59) (see Figure 5).

Sucrose, TMG and raffinose were all predicted to have 1st order positive effects on viral recovery over the concentration range tested. Although, the raffinose effect was only significant at the 90% C.I. it was retained in the model as it improved the strength of the model and was required to preserve the model hierarchy. This was required because an interaction of TMG and raffinose was also predicted. Finally, a 2nd order non-linear effect of sucrose was observed. See Figure 6 for a summary of retained coefficients in the model.

Figure 7 is of a 4D contour plot that illustrates the interactions clearly. The optimum Sucrose concentration can be seen to be consistently between 0.6 and 0.8M, no other excipients significantly alter this. In general the higher the TMG concentration the greater the recovery of viral activity.

Monte-Carlo simulations (shown in Figure 8) point to the extreme of the tested range for an optimum (1M Sucrose, 1M TMG, 300mM Raffinose). This suggests that the optimum formulation is not covered by the tested range. However, the simulations

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predict that formulations close to this optimum should yield recovered viral activity of 94% starting titre.

Example 5

Materials

Chemical

	Supplier	Product Code	Lot No.
20x SSC	Sigma	S6639	020M8404
Dimethyl glycine	Sigma	D1156	077K1856
Dulbeccos Modified Eagles Medium	Sigma	D5796	RNBB1139
Foetal Bovine Serum	Sigma	F7524	109K3395
Penicillin Streptomycin	Sigma	P4458	0409M0093
Raffinose	Sigma	R0250	050M0053
Sucrose	Sigma	16104	SZB90120
Water	Sigma	W3500	8M0411

Biological

	Supplier	Product Code
Adenovirus	Vector Biolabs	Ad-CMV-GFP
HEK 293	ECACC	85120602

Other

	Manufacturer	Product Code
5ml glass vials	Adelphi Tubes	VCD005
14mm freeze drying stoppers	Adelphi Tubes	FDIA14WG/B
14mm caps	Adelphi Tubes	CWPP14

Equipment

	Manufacturer	Equipment No.
HERA safe class II cabinet	Thermo Fisher	EQP# 011 & 012
DMIL LED Inverted Microscope	Leica	EQP#062
Binder CO_2 Incubator	Binder	EQP#014
Forma 900 series -80°C freezer	Thermofisher	EQP#015
ATL-84-1 Atlion Balance	Acculab	EQP#088
IP250 37°C Incubator	LTE	EQP#016

Methods

Design of Experiment

MODDE 9.0 (Umetrics) was used to generate a Doehlert experimental design (see Figure 9). Doehlert designs are response surface modelling designs constructed from regular simplexes. They are easily extendable in different directions and new factors can be added to an existing design. Unlike regular formulation designs non-significant factors can be eliminated from the analysis and so do not become a confounding factor.

Furthermore, different factors within the design are tested at a different number of levels, so it is possible to allocate more test levels to factors that we suspect are of greater importance. Thus, DMG was tested at seven levels, whilst sucrose was tested at five and raffinose three. This model retains the ability to model for second order effects and interactions. The design included three factors and three replicate centre-points resulting in fifteen test samples.

Sucrose was tested between 0 and 1M. Raffinose was tested over a range of 0 to 300mM although the nature of the Doehlert design meant that tested levels did not include 0mM. Instead the following ranges were tested: 27.5, 150.0, and 272.5mM. DMG was tested over a linear range of 0 to 2M.

Stability of adenovirus in a liquid setting

Recombinant Adenovirus expressing enhanced GFP under a CMV promoter,

with a titre (pre-freeze) of $6.7x10^5$ pfu/ml in SSC, was removed from storage at -80°C and allowed to thaw. Subsequently, 50µl aliquots of virus were added to 15, 5ml, glass vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 4 and were made up in SSC.

Table 4

Formulation No.	Sucrose (M)	Raffinose (mM)	DMG (M)	Titre (pfu/ml)
1	0.25	150.0	0.13	1.1E+05
2	0.75	150.0	0.13	2.2E+05
3	0.5	272.5	0.42	3.8E+05
4	0.25	27.5	0.71	2.0E+05
5	0.75	27.5	0.71	2.6E+05
6	0	150.0	1.00	2.2E+05
7	0.5	150.0	1.00	2.2E+05*
8	0.5	150.0	1.00	3.1E+05
9	0.5	150.0	1.00	3.4E+05
10	1	150.0	1.00	4.1E+05
11	0.25	272.5	1.29	2.5E+05
12	0.75	272.5	1.29	3.7E+05
13	0.5	27.5	1.58	3.7E+05
14	0.25	150.0	1.87	2.5E+05
15	0.75	150.0	1.87	3.3E+05

* indicates an outlier eliminated from the model

The vials were stoppered and capped (screw cap) before being placed at +37°C for 1 week of thermochallenge and later placed at +4°C until it was practical to assay them. The adenovirus assay was as described below.

Assay of adenovirus

HEK 293 cells were prepared in 96 well flat bottomed cell culture dishes for inoculation by seeding at 10^5 cells per ml (100µl per well) and maintained at 37°C with 5% CO_2. After 2 hours cells were inoculated as follows.

Thermo-challenged virus samples were diluted 1 in 10, and 1 in 100 in DMEM +10% FBS +1% PS. 100µl of each of the resulting diluted virus samples were then added to individual wells of the assay plate. Additionally, a second aliquot of the original adenovirus in SSC was thawed from -80°C and a 10 fold dilution series (from 1 in 10 to 1 in 100,000) also prepared in DMEM +10% FBS +1% PS. The positive control dilution series was inoculated in duplicate to each 96 well plate used. After a further 48 hours, the number of GFP cells per well were counted using fluorescent microscopy.

Results

A very strong model was generated from this data (see Table 4). The model scored highly with all four indicators (R^2=0.97, Q^2=0.90, Model Validity=0.89, Reproducability=0.96) (see Figure 10). The model was enhanced during fine tuning by the elimination of one replicate. This formulation was flagged by the software as an obvious outlier.

All three excipients in the formulation were shown to be significant factors in the model (see Figure 11). Sucrose and raffinose only had 1st order effects whereas DMG had both 1st and 2nd order effects. In addition, there was an interaction between raffinose and DMG.

Figure 12 shows that the optimum sucrose concentration is beyond that tested. However, it is unlikely the sucrose concentration would be significantly increased due to constraints on the osmolarity of the product. At some levels DMG enhances the protective effect of the formulation, and raffinose alters the optimum DMG concentration for this purpose.

Monte-Carlo simulations were used to predict an optimum formulation (see Figure 13). The program was set to maximise recovered viral activity to a limit of $4.3x10^5$ pfu/ml (the titre of a positive control). The predicted optimum formulation was 0.5M Sucrose, 0.4M DMG, 272nM Raffinose and this was predicted to yield a titre of $3.6x10^5$pfu/ml or 84% of starting titre (based on the positive control).

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Figure 14a shows an alternative way of looking at the data. A contour plot shows DMG concentration plotted against sucrose concentration at a number of different raffinose concentrations. The plot shows the darker region (higher recovery of virus activity) moves down the Y-axis (DMG concentration) as raffinose is increased. A black cross marks the predicted optimum formulation. Figure 14b shows the region where recovery is predicted to be 100% or greater.

Example 6

Materials

Chemical

	Supplier	**Product Code**	**Lot No.**
Dimethyl glycine	Sigma	D1156	077K1856
Dulbeccos Modified Eagles Medium	Sigma	D5796	RNBB1139
Foetal Bovine Serum	Sigma	F7524	109K3395
Raffinose	Sigma	R0250	050M0053
Sucrose	Sigma	16104	SZB90120
Water	Sigma	W3500	8M0411

Biological

	Supplier	**Product Code**
Adenovirus	Vector Biolabs	Ad-CMV-GFP
HEK 293	ECACC	85120602

Other

	Supplier	**Product Code**
5ml glass vials	Adelphi Tubes	VCD005
14mm freeze drying stoppers	Adelphi Tubes	FDIA14WG/B
14mm caps	Adelphi Tubes	CWPP14

Equipment

	Manufacturer	Equipment No.
Dryer		
HERA safe class II cabinet	Thermo Fisher	EQP# 011 & 012
DMIL LED Inverted Microscope	Leica	EQP#062
Binder CO_2 Incubator	Binder	EQP#014
Forma 900 series -80°C freezer	Thermofisher	EQP#015
ATL-84-1 Atlion Balance	Acculab	EQP#088
IP250 37°C Incubator	LTE	EQP#016

Methods

Recombinant adenovirus expressing enhanced GFP under a CMV promoter, with a titre (after thawing) of $10.2x10^5$ pfu/ml in PBS, was removed from storage at -80°C and allowed to thaw. Subsequently, 50µl aliquots of virus were added to 15, 5ml, glass vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 5 and were made up in PBS.

Table 5

Formulation Name	Buffer	Raffinose (mM)	Sucrose (M)	DMG (M)
Buffer	PBS	0	0	0
Raffinose	PBS	100	0	0
Sucrose	PBS	0	1	0
Sugars	PBS	100	1	0
NE	PBS	0	0	0.7
Best	PBS	100	1	0.7

From this point onward the following treatment names are used:

- "Buffer" = PBS buffer only no excipients
- "Sucrose" = 1M Sucrose in PBS
- "Raffinose" = 100mM Raffinose in PBS

- "Sugars" = 1M Sucrose, 100mM Raffinose in PBS,
- "NE" = 0.7M DMG in PBS,
- "Best" = 1M Sucrose, 100mM Raffinose, 0.7M DMG in PBS.

The vials were stoppered and capped (screw cap) before being thermally challenged under the conditions set out in Table 6. At appropriate time points, an adenovirus assay was carried out as described in Example 5.

Results

Many data points gathered were below the detection threshold of the assay (see Table 6).

Table 6

Thermal Challenge		Formulation					
Temperature	Duration	Best	Sucrose	Raffinose	Sugars	NE	Buffer
+4°C	6 months	2.0E+06	1.8E+06	1.1E+06	1.7E+06	1.3E+06	1.0E+06
+25°C	1 month	5.2E+05	1.9E+05	1.6E+05	2.0E+05	2.3E+05	7.2E+04
	6 months	1.9E+05	6.0E+02*	6.0E+02*	1.0E+05	3.0E+04	6.0E+02*
+37°C	1	2.3E+04	3.0E+03	6.0E+02*	4.2E+03	6.0E+02*	6.0E+02*
	8	1.7E+04	6.0E+02*	6.0E+02*	6.0E+02*	6.0E+02*	6.0E+02*
	12	3.6E+03	6.0E+02*	6.0E+02*	6.0E+02*	6.0E+02*	6.0E+02*

*data-point below detection threshold

For convenience these data points have been assigned the threshold value as this is the maximum possible value they could have. It is likely that this will have little effect on the interpretation of the results as any formulation yielding such low recovery of viral activity is of little practical use as anything other than a comparator. The detection threshold for this assay is 600 pfu/ml which equates to 0.03% recovered activity.

Only one time point was tested for samples held at +4°C. The yield of virus activity after 6 months at this temperature can be seen in Figure 15. The buffer only treatment gave recovery of 50.1% starting titre and a clear indication that adeneovirus used is inherently reasonably stable in this liquid setting. This finding also shows the need for accelerated stability studies.

The "sucrose" treatment recovered 92.1% activity which after 6 months is a major improvement on buffer alone. "Raffinose" in contrast only yielded 55.5%, a slight improvement on "Buffer Alone" but worse than "Sucrose". The combined "Sugars" treatment gave a recovered virus activity of only 86.4%.

The DMG only treatment ("NE") preserved only 65.1% or virus activity, but when used in concert with the sugars a recovered activity of 99.3% was observed. This finding is close to zero loss of adenovirus at +4°C after 6 months.

The recovered viral activity in this "Best" formulation at each time-point and temperature challenge is shown in Figure 16. As previously discussed after 6 months at +4°C there is close to zero loss.

The results (see Table 6 and Figure 17) at the +25°C and +37°C thermo-challenges demonstrate that the "Best" formulation is more effective in stabilising the adenovirus than its constituent components.

Example 7

Materials

Chemical

	Supplier	**Product Code**	**Lot No.**
20x SSC	Sigma	S6639	020M8404
Dimethyl glycine	Sigma	D1156	077K1856
Dulbeccos Modified Eagles Medium	Sigma	D5796	RNBB1139
Foetal Bovine Serum	Sigma	F7524	109K3395

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Penicillin Streptomycin	Sigma	P4458	0409M0093
Water	Sigma	W3500	8M0411

Biological

	Supplier	**Product Code**
BHK-21 cell line	ECACC	CB2857
MVA	ATCC	VR-1508

Other

	Manufacturer	**Product Code**
2ml glass vials	Adelphi Tubes	VCDIN2R
13mm freeze drying stoppers	Adelphi Tubes	FDW13
Crimps	Adelphi Tubes	COTW13

Equipment

	Manufacturer	**Equipment No.**
HERA safe class II cabinet	Thermo Fisher	EQP# 011 & 012
DMIL LED Inverted Microscope	Leica	EQP#062
Binder CO_2 Incubator	Binder	EQP#014
Forma 900 series -80°C freezer	Thermofisher	EQP#015
ATL-84-1 Atlion Balance	Acculab	EQP#088
IP250 37°C Incubator	LTE	EQP#016

Methods

Design of Experiment

MODDE 9.0 was used to generate a Central Composite Face-Centred (CCF) design. CCF designs are a form of Response Surface Modelling (RSM) design that tests only 3 levels of each factor but still supports a quadratic model (see Figure 18).

Unlike regular formulation designs non-significant factors can be eliminated from the analysis and so do not become a confounding factor.

Preparation of and thermal challenge of formulated MVA in a Liquid Setting

MVA was recovered from storage at -80°C and thawed. Subsequently, 50µl aliquots of virus were added to 15, 5ml, glass vials. To each vial 250µl of an excipient blend was admixed. The excipient blend formulations once mixed with virus are described in Table 7 below and were made up in SSC. The vials were stoppered under vacuum, and capped (screw cap) before being placed at +37°C for 1 week of thermochallenge and later placed at +4°C until it was practical to assay them.

Assay of MVA

Assay plates (96 well) were seeded with BHK-21 cells (100µl per well, 10^5 cells/ml). Cells were diluted in DMEM supplemented with 10% FBS, and 1% PS. The plates were placed at +37°C, + 5% CO_2 for 1-2 hours.

Meanwhile, a 10 fold dilution series of the formulated MVA samples was prepared (in the same growth media) ranging from 1 in 10 to 1 in 10,000. Each dilution series was prepared as 5 replicates. 100µl of each dilution was applied to individual wells containing BHK-21 cells (described above).

On 6 d.p.i. the wells were scored for presence or absence of CPE and $TCID_{50}$ calculated. These were then used to estimate the concentration of infectious MVA per ml in the thermo-challenged vials.

Results

The crude $TCID_{50}$ data from this study is shown in Table 7.

Table 7

Sample I.D.	DMG (M)	Mannitol (mM)	Titre ($TCID_{50}$/ml)
1	0	6	1.90E+5
2	2	6	4.80E+3
3	0	600	3.00E+4
4	2	600	4.80E+5
5	0	303	3.00E+5*
6	2	303	3.00E+5
7	1	6	3.00E+4
8	1	600	3.00E+5
9	1	303	7.60E+5*
10	1	303	1.90E+5
11	1	303	1.90E+5

** Data point excluded from model during fine tuning as an obvious outlier*

Responses varied from 0.5 to 74.1% of starting titre. The model predicts 1st order effects of both excipients (see Figure 19 and 20). In the case of both DMG and mannitol, viral preservation is increased as concentration increases. This is clearly illustrated by the contour plot shown in Figure 21. Additionally, an interaction between DMG and Mannitol was identified.

Monte-Carlo simulations suggest that with the high concentration of each excipient tested it can be expected to achieve over 66% of starting titre after thermal challenge at +37°C for 1 week, this represents less than a 0.2 LOG loss.

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CLAIMS

1. A process for the preparation of a ready-to-use, storage-stable aqueous solution which is provided in a sealed container and which contains live viral particles, which process comprises:

(a) providing a solution comprising:

- water or physiological saline, which is optionally buffered with a physiologically acceptable buffer;
- live viral particles from Adenoviridae, Orthomyxoviridae, Paramyxoviridae, Parvoviridae, Picornaviridae, Poxviridae or Flaviviridae;
- an excipient which is an N,N-di(C_{1-6} alkyl)-glycine or N,N,N-tri(C_{1-6} alkyl)-glycine, or a physiologically acceptable salt or ester thereof; and

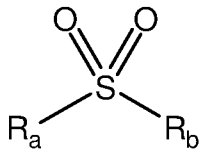
(b) sealing the solution in a container.

2. The process according to claim 1, in which the solution further comprises one or more sugars.

3. The process according to claim 2, in which the one or more sugars comprise sucrose or mannitol.

4. The process according to claim 3, in which the one or more sugars comprise sucrose and raffinose.

5. The process according to any one of the preceding claims, in which the solution further comprises a sulfone compound of formula (IIC):



(IIC)

wherein R_a and R_b independently represent C_{1-6} alkyl.

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6. The process according to claim 5 in which the sulfone compound of formula (IIC) is methylsulfonylmethane.

7. The process according to any one of the preceding claims, in which the viral particles are from an adenovirus, vaccinia virus, influenza virus or measles virus.

8. The process according to any one of the preceding claims in which the excipient is N,N-dimethylglycine or N,N,N-trimethylglycine, or a physiologically acceptable salt or ester thereof.

9. The process according to claim 8, in which the excipient is N,N-dimethylglycine or a physiologically acceptable salt or ester thereof.

10. The process according to any one of the preceding claims, in which the solution further comprises an adjuvant.

11. The process according to any one of the preceding claims, in which the solution further comprises a tonicity adjustment agent.

12. The process according to any one of the preceding claims, in which the solution further comprises a preservative.

13. The process according to any one of the preceding claims, in which the solution is isotonic.

14. The process according to any one of the preceding claims, in which the solution is provided in a sealed container under nitrogen.

15. The process according to any one of the preceding claims, in which the sealed contained is a sealed vial, ampoule, syringe, cartridge, flexible bag or glass bottle.

16. The process according to any one of the preceding claims, in which the solution is passed through a sterilising filter in step (a).

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17. A ready-to-use, storage-stable aqueous solution containing Adenoviridae, Orthomyxoviridae, Paramyxoviridae, Parvoviridae, Picornaviridae, Poxviridae or Flaviviridae live viral particles which is provided in a sealed container, said live viral particle containing aqueous solution being obtainable by a process as defined in any one of the preceding claims.

Stabilitech Ltd.

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

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Figure 1a

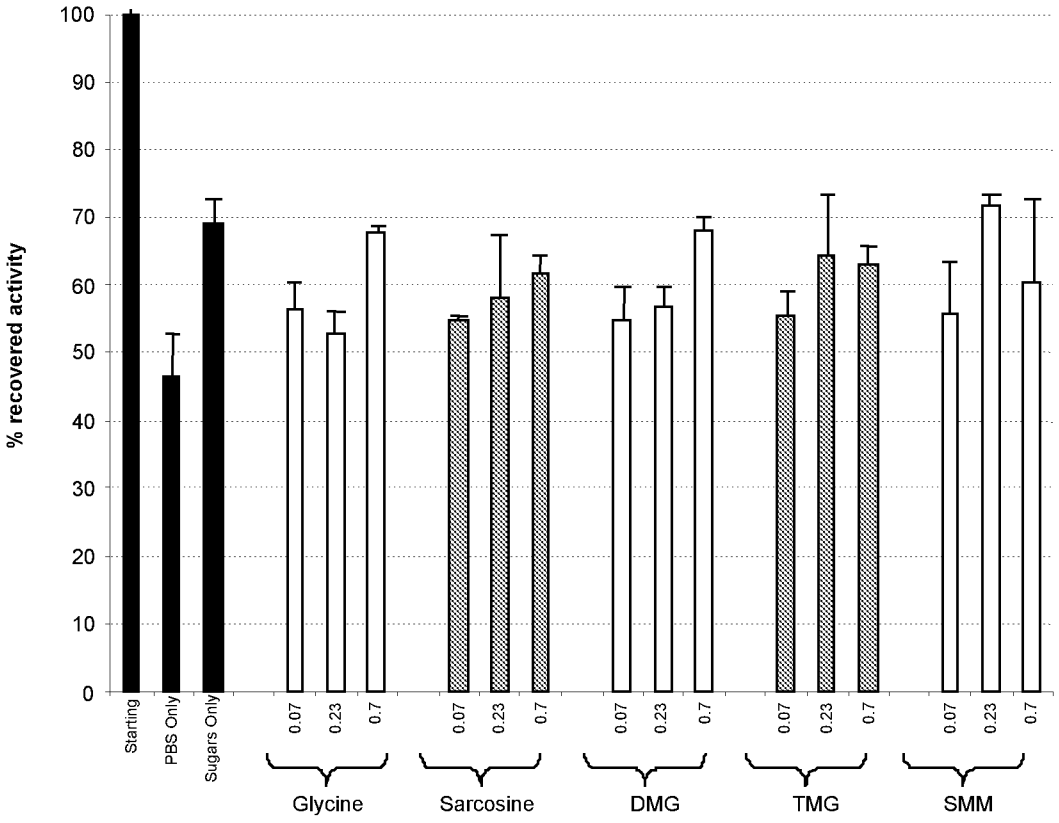

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Figure 1b

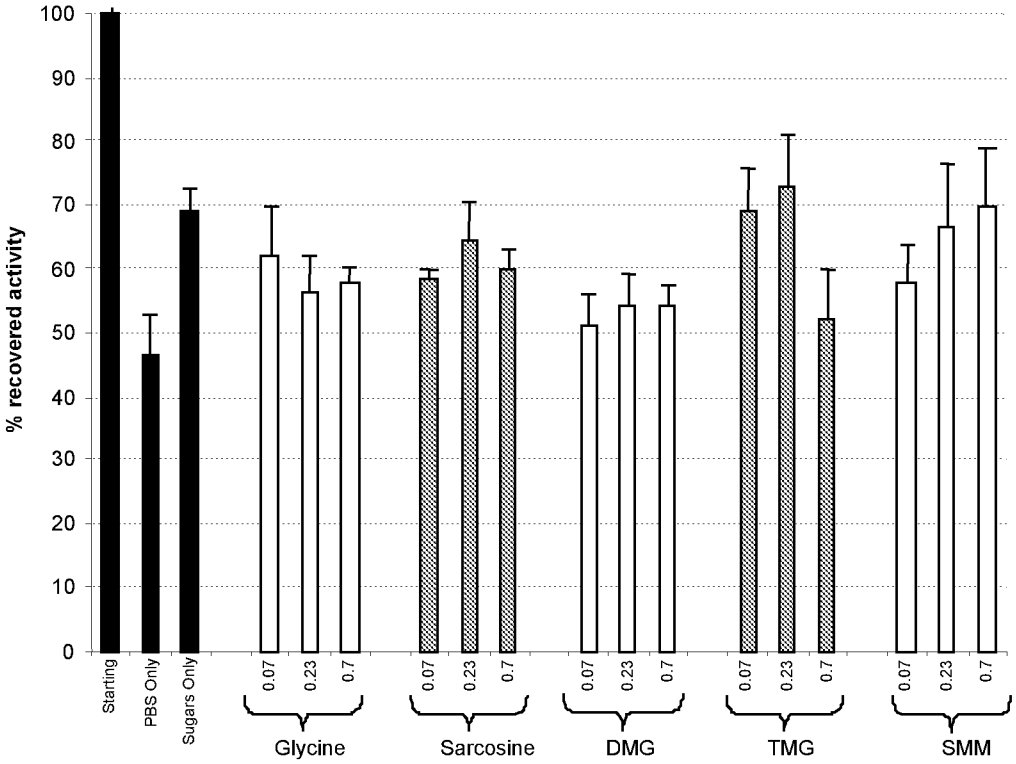

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Figure 1c

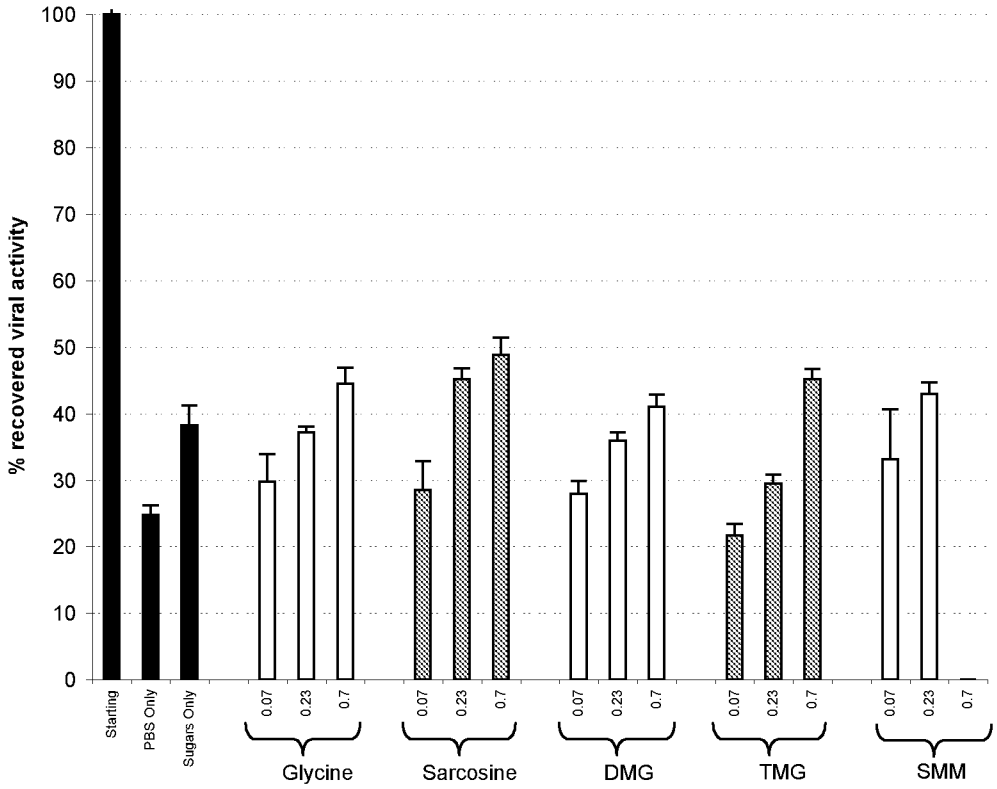

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Figure 1d

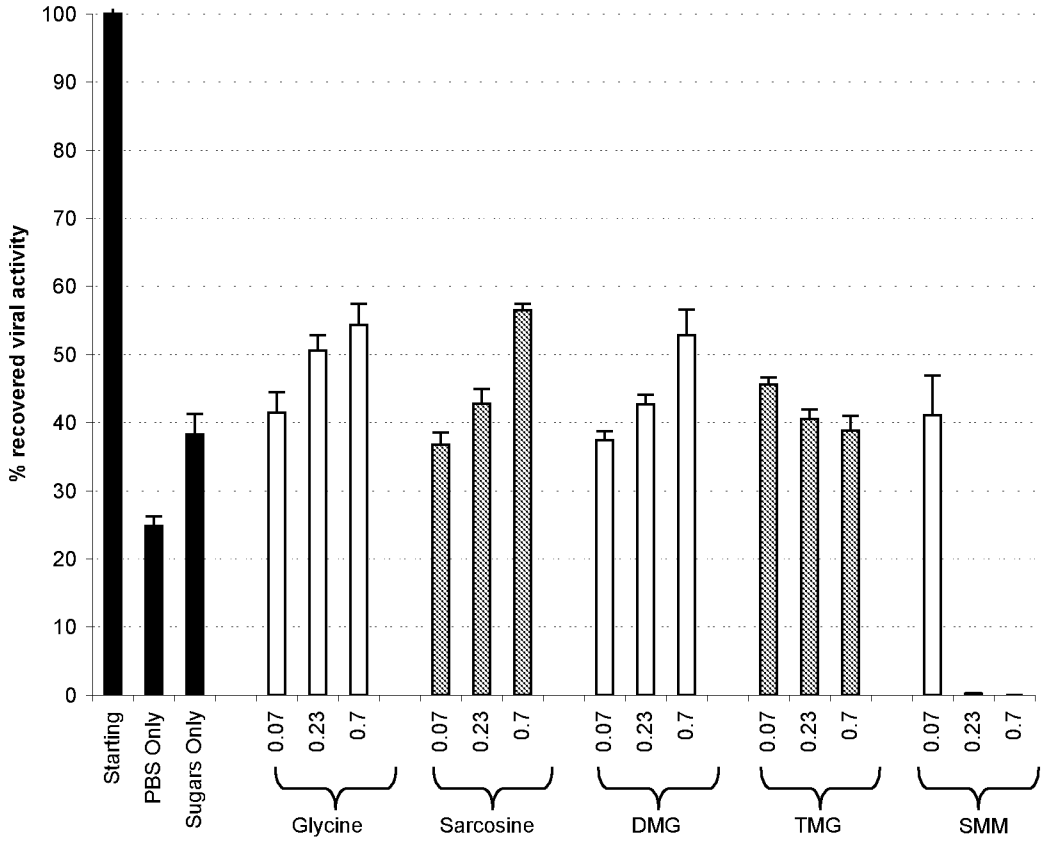

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Figure 2

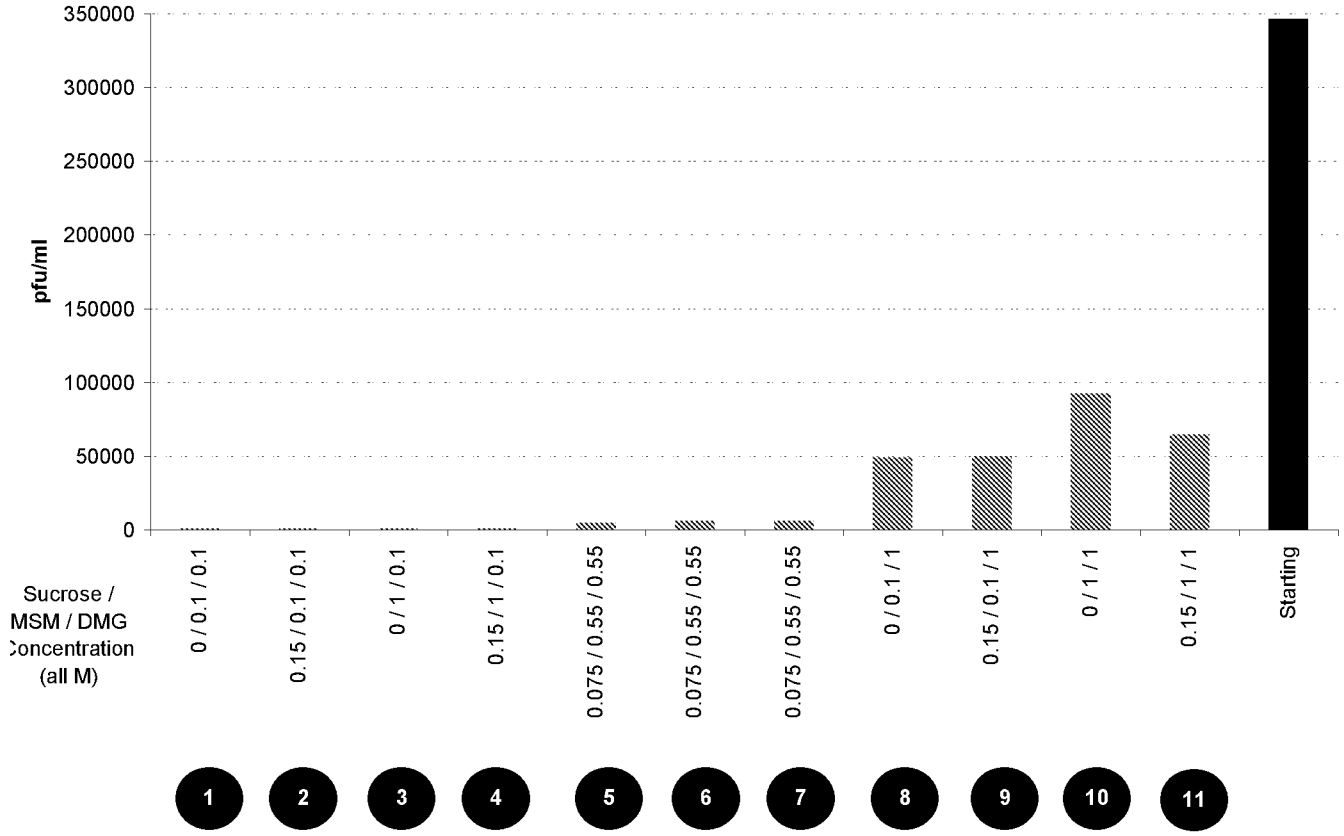

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Figure 3

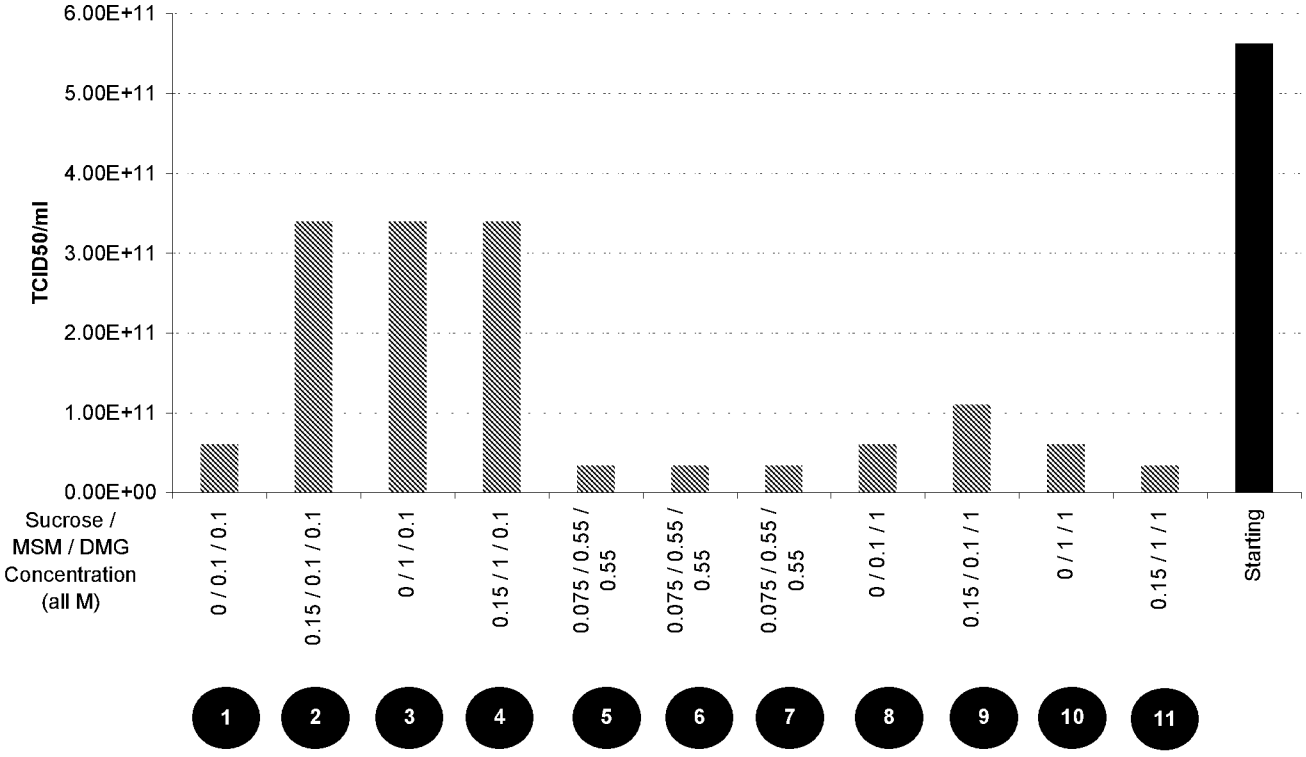

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Figure 4

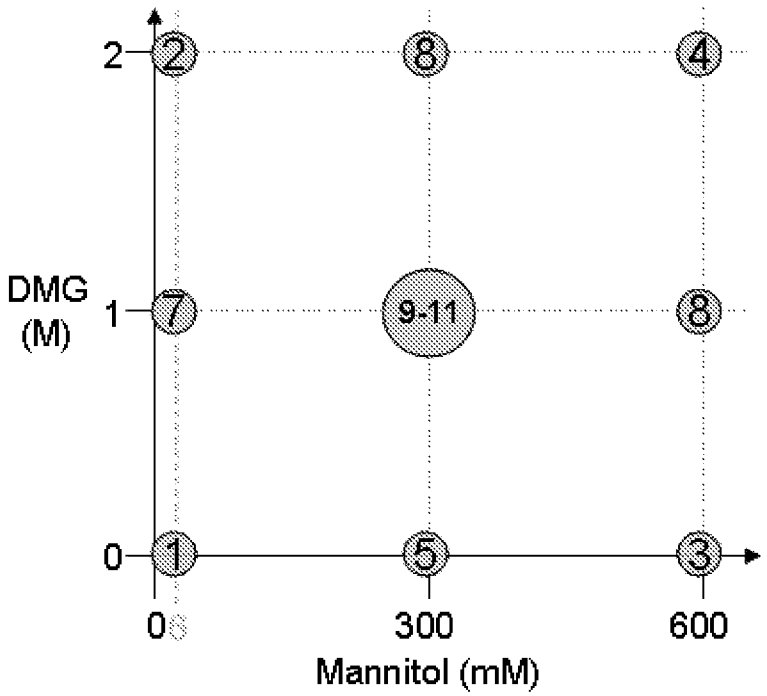

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Figure 5

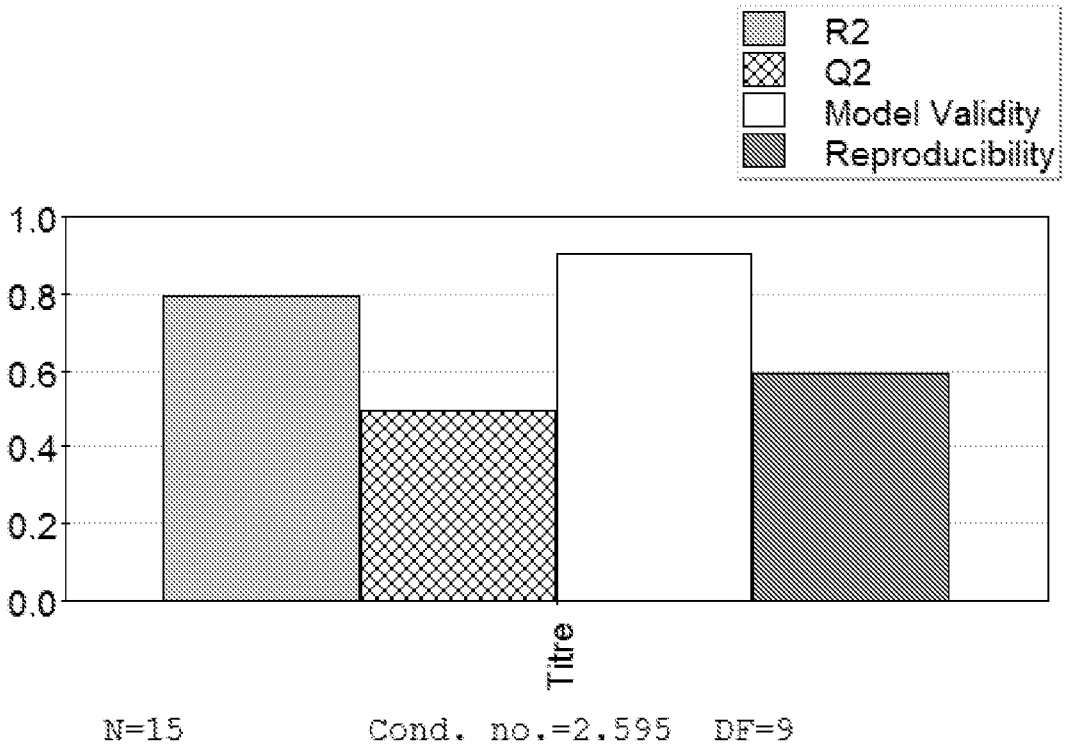

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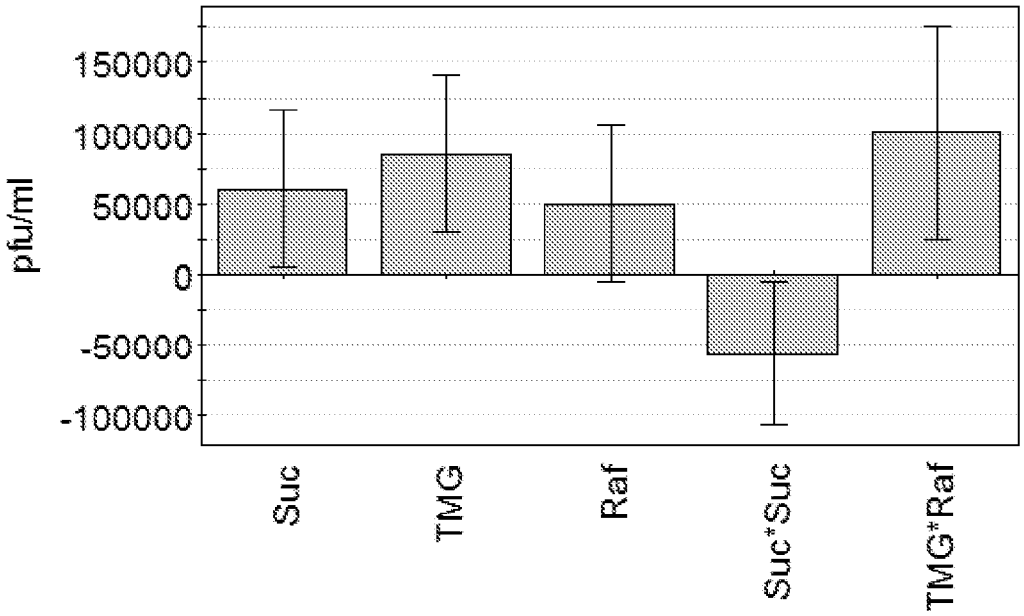
Figure 6



N=15 R2=0.794 RSD=9.226e+004
DF=9 Q2=0.494 Conf. lev.=0.95

Figure 7

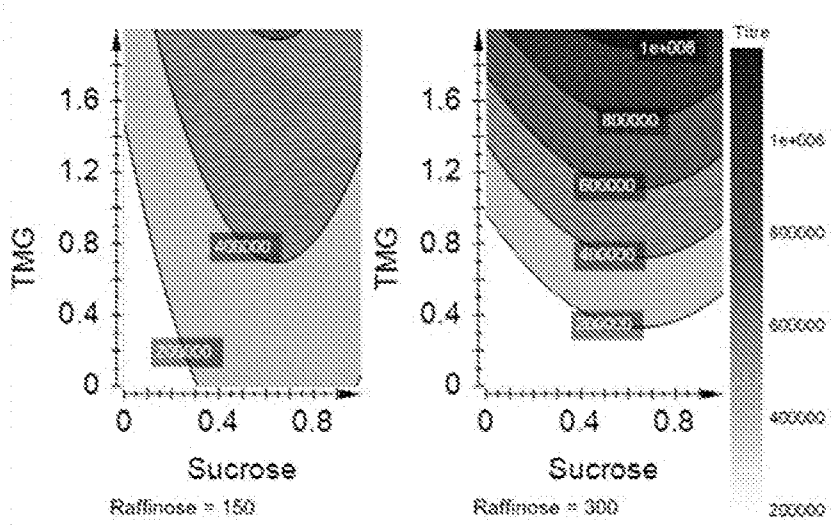


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Figure 8

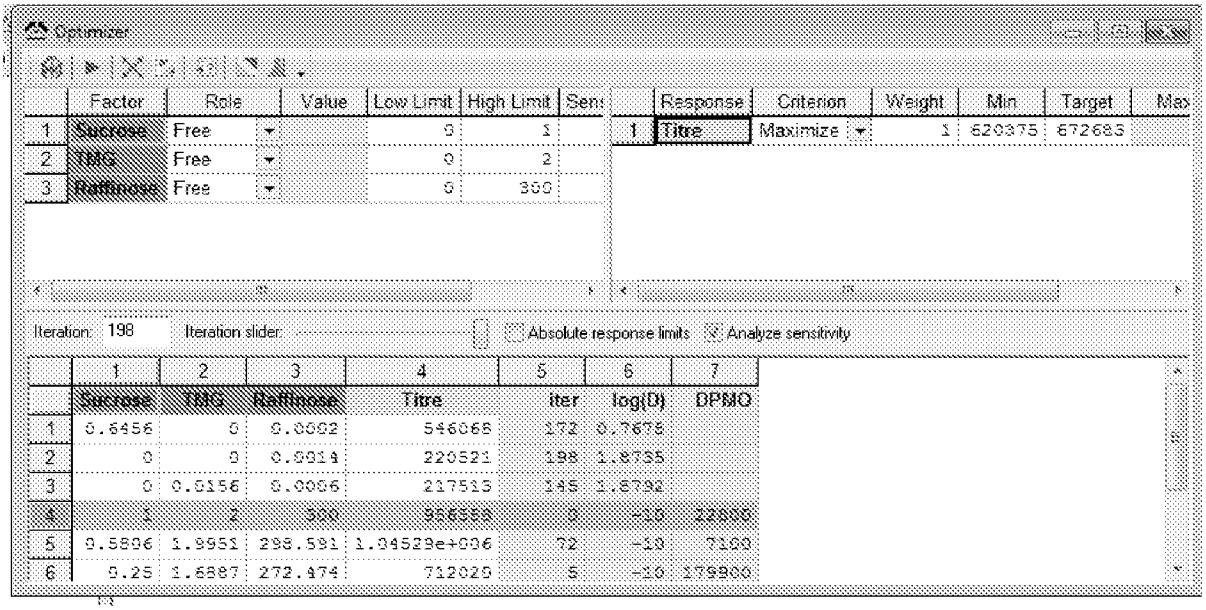

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Figure 9

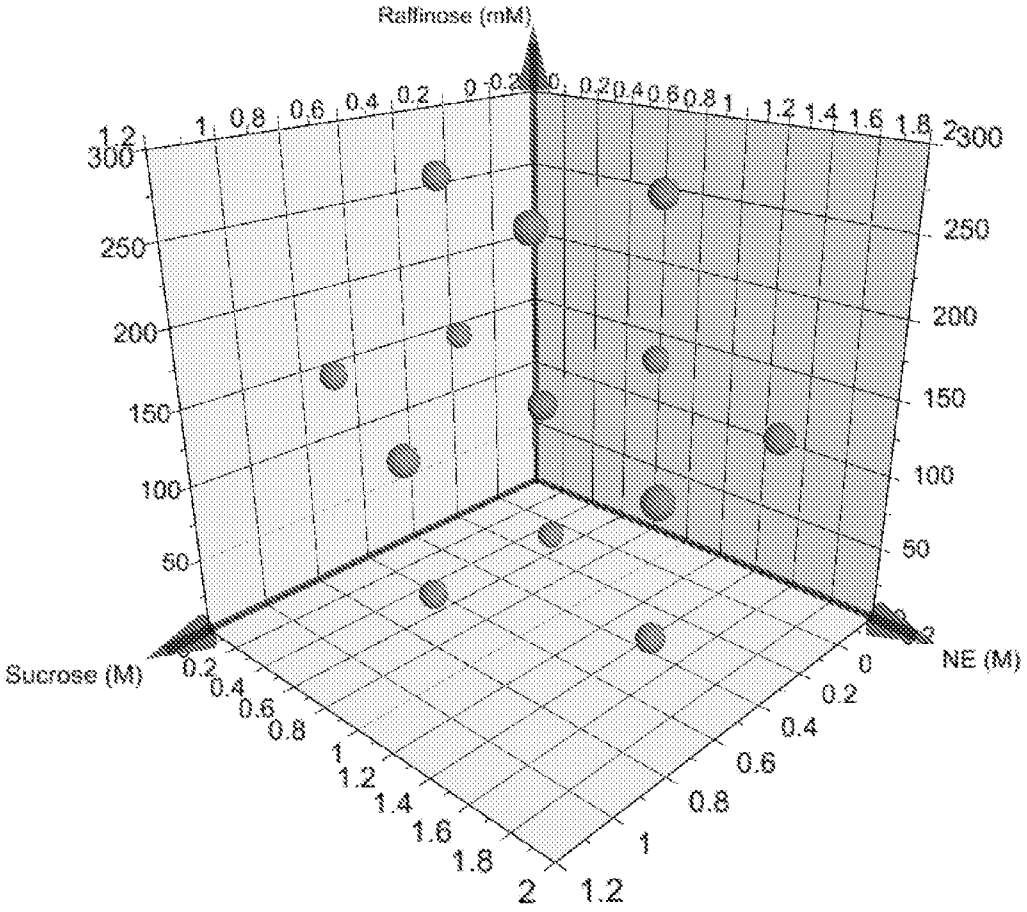

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Figure 10

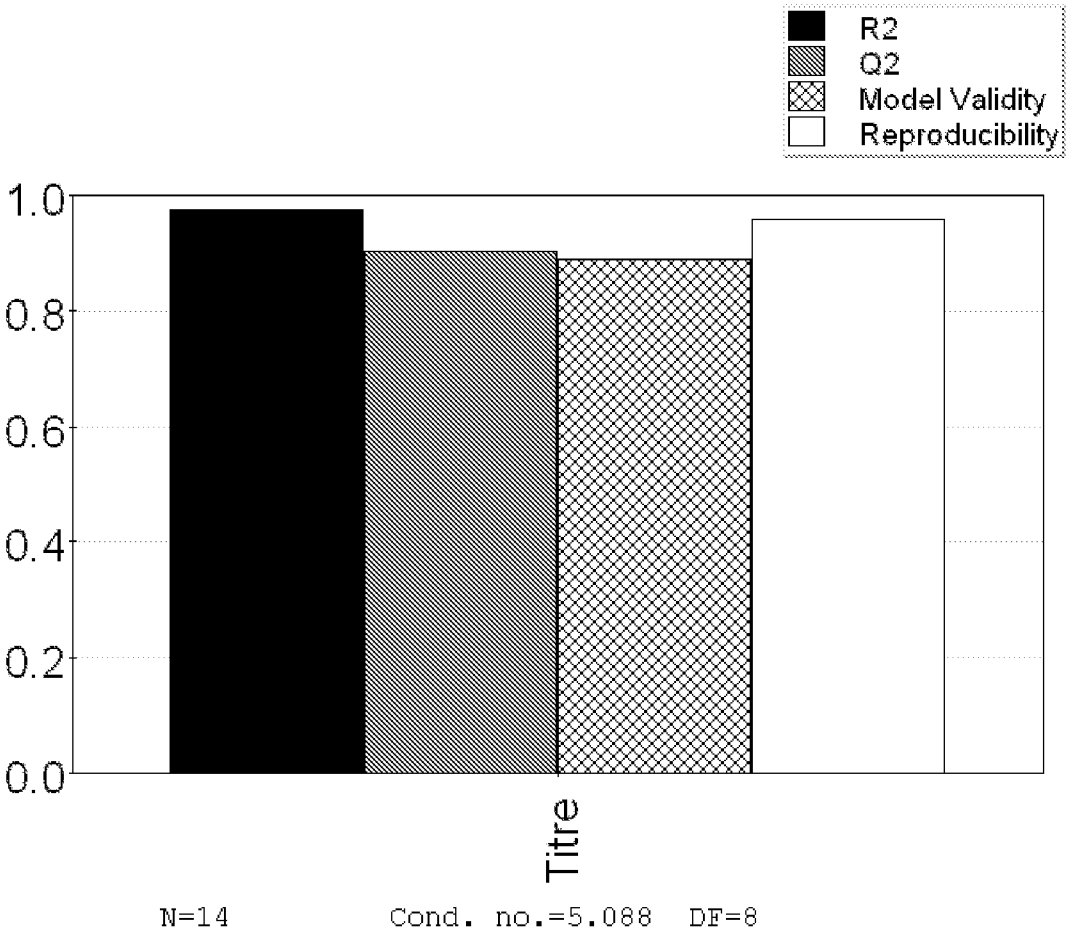

Figure 11

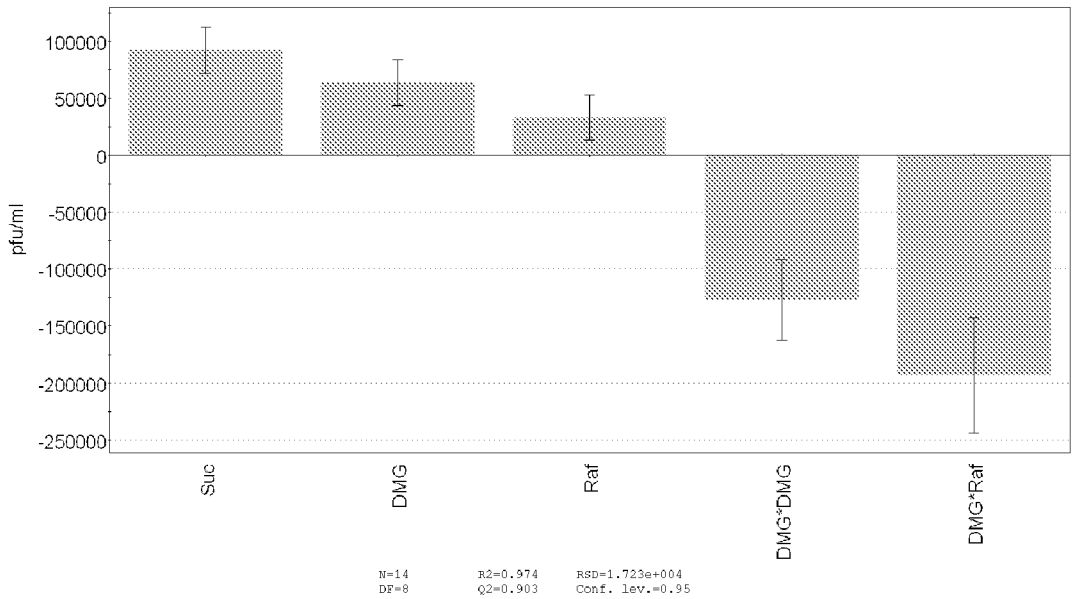

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Figure 12

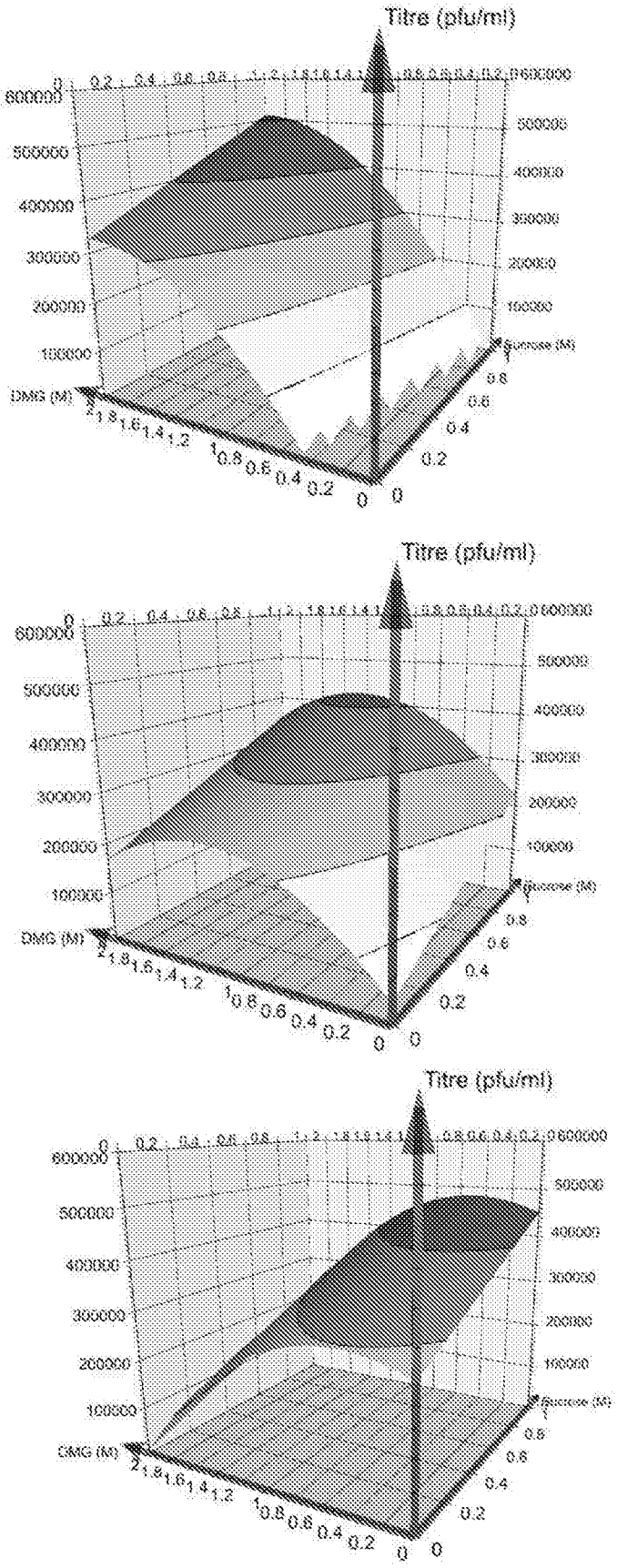

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Figure 13

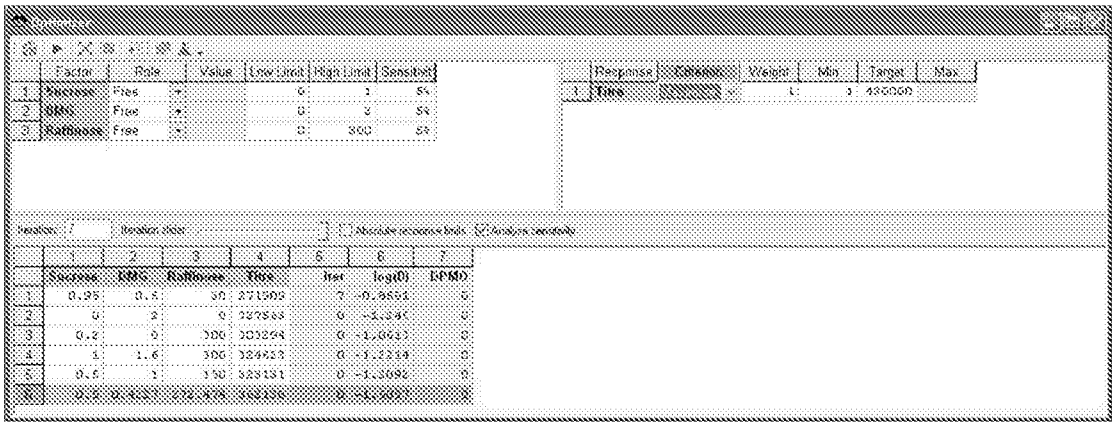
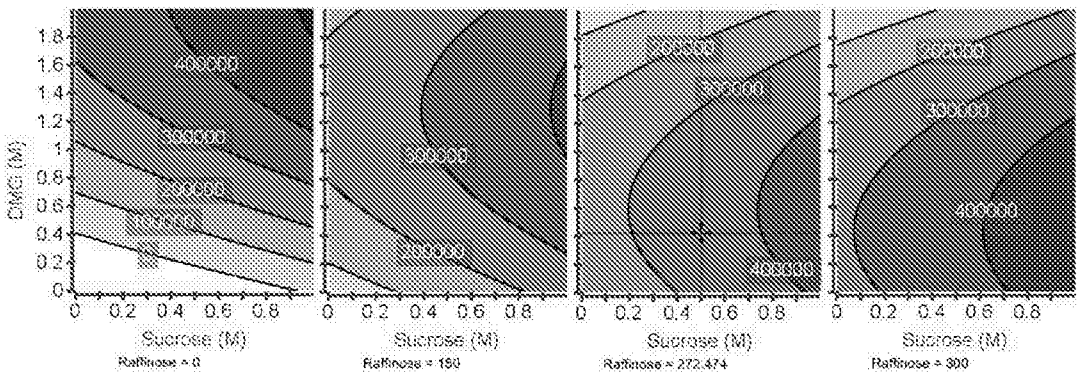


Figure 14

A



B

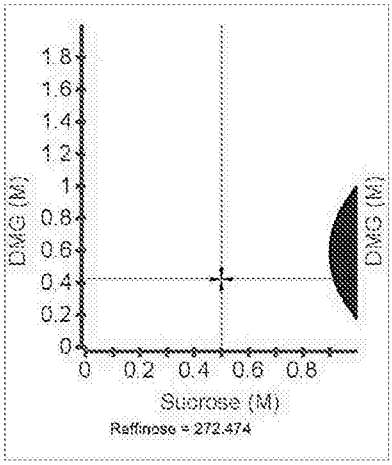


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Figure 15

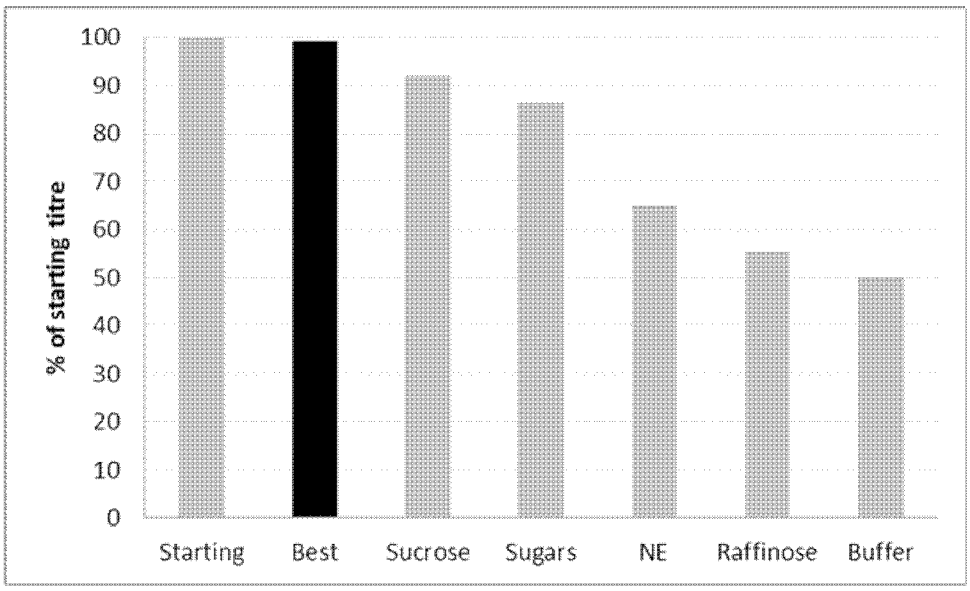

Figure 16

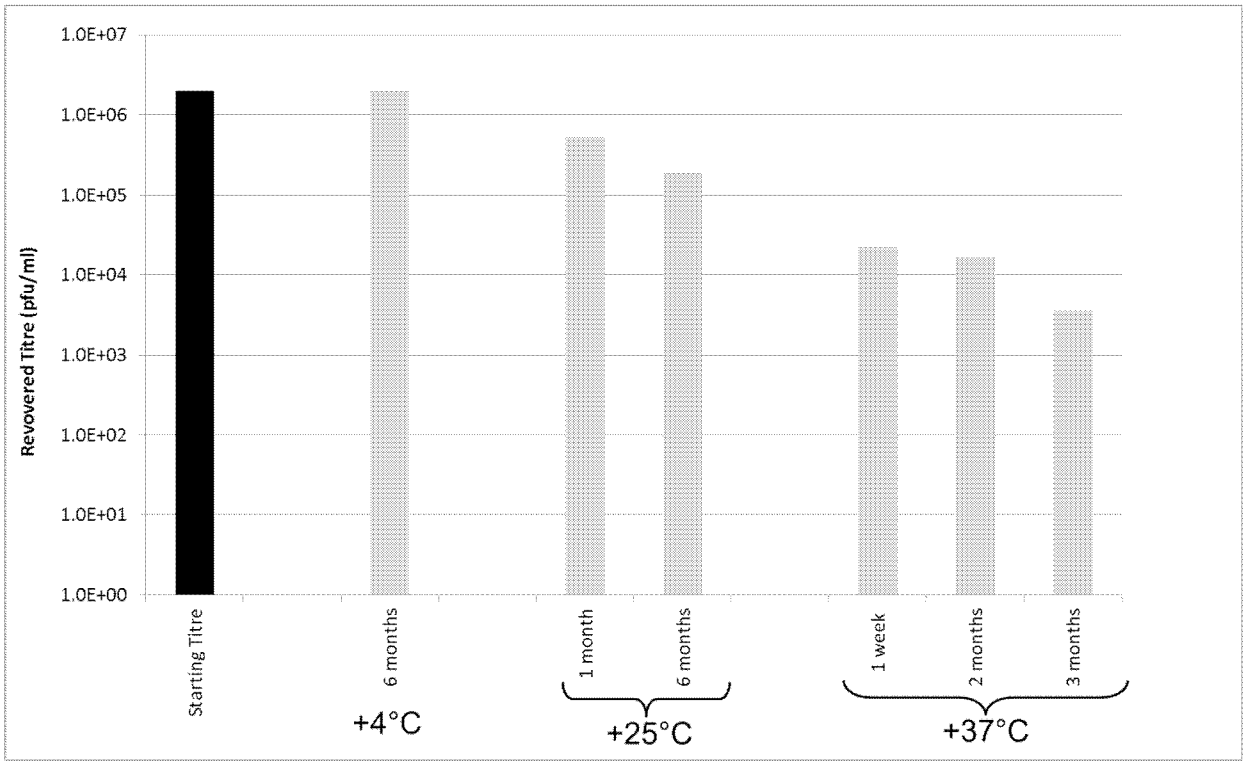

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Figure 17

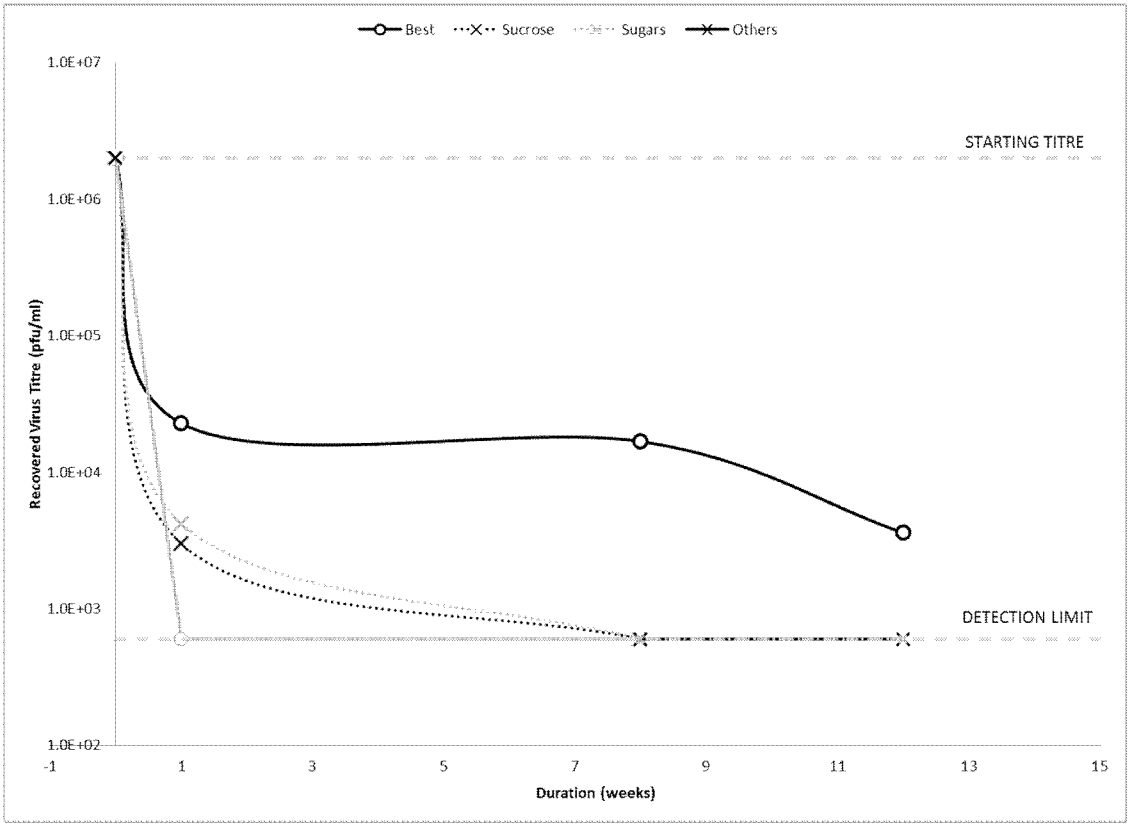

Figure 18

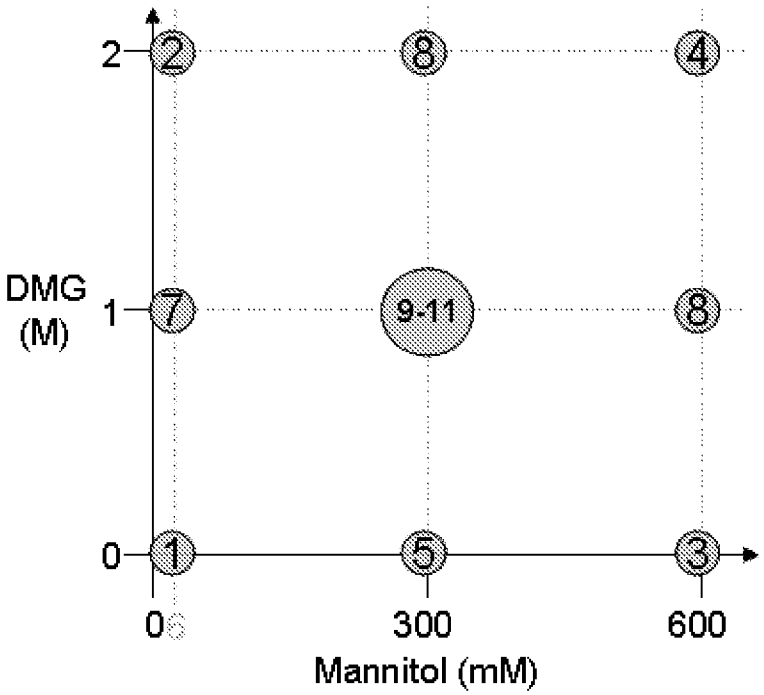

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Figure 19

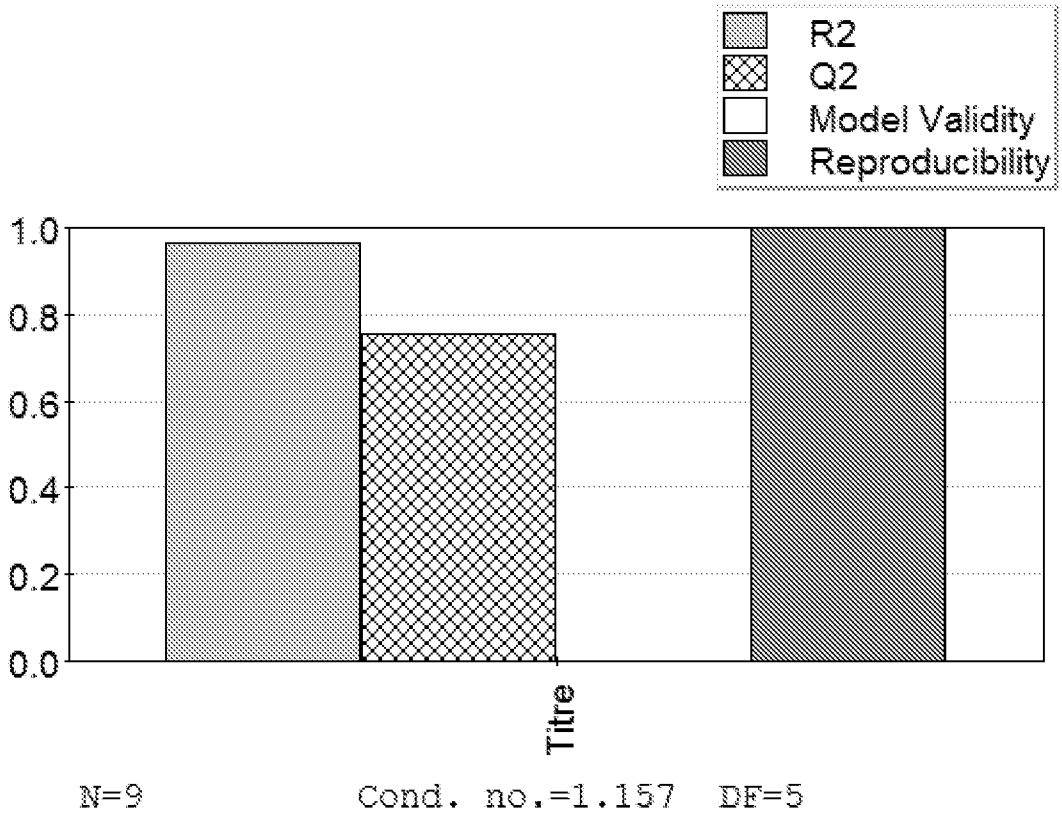

Figure 20

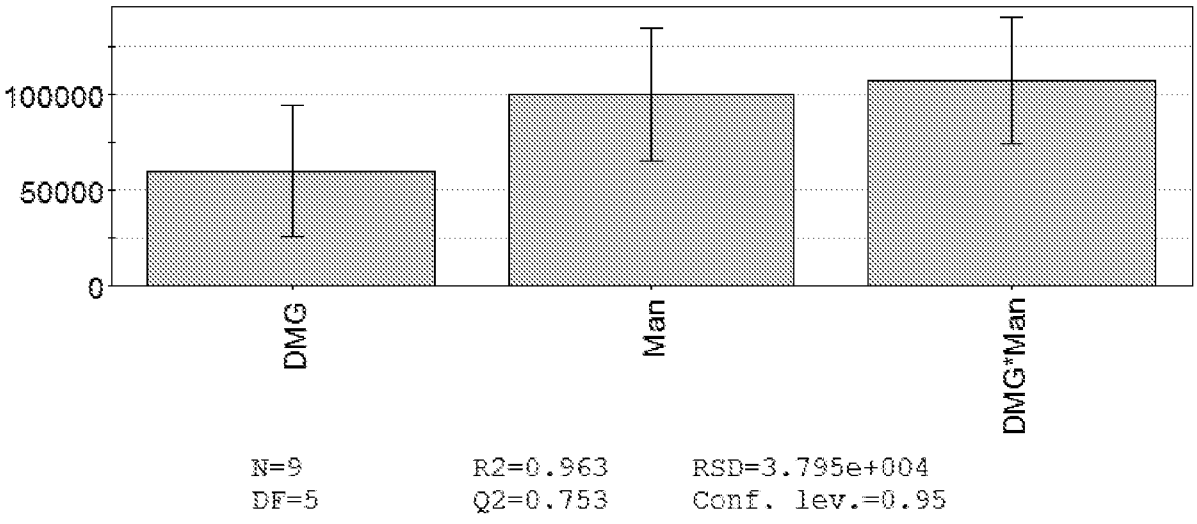

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Figure 21