

METHOD FOR MANUFACTURING VIRAL VACCINES AND COMPOSITIONS THEREOF

FIELD

The present disclosure relates to method of producing clarified virus pool and manufacturing of viral vaccines, more particularly, manufacturing a lyophilized/freeze-dried live attenuated virus vaccine composition/formulation comprising of Measles, Mumps, Rubella antigens/immunogens or combinations thereof.

BACKGROUND

All publications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. The following description includes information useful in understanding the present invention. It is not an admission that any of the information provided herein is prior art or relevant to the presently claimed invention, or that any publication specifically or implicitly referenced is prior art.

Measles is an acute viral illness caused by Morbillivirus from family paramyxovirus. Measles is characterized by a prodrome of fever (as high as 105°F) and malaise, cough, coryza, and conjunctivitis, followed by a maculopapular rash. Rash spreads from head to trunk to lower extremities. Measles is usually a mild or moderately severe illness with possibility of further complications as pneumonia, encephalitis, and death. Approximately, one case of encephalitis and two to three deaths occur for every 1,000 reported measles cases.

In rare cases, Measles infection is also followed by subacute sclerosing panencephalitis (SSPE), a fatal disease of the central nervous system that generally develops 7 to 10 years after infection. Among persons who contracted measles during the resurgence in the United States (U.S.) in 1989 to 1991, the risk of SSPE was estimated to be 7 to 11 cases/100,000 cases of measles. The risk of developing SSPE be higher when measles occurs prior to the second year of life. The average incubation period for measles is 11 to 12 days, and the average interval between exposure and rash onset is 14 days, with a range of 7 to 21 days. Persons with measles are usually considered infectious from four days before until four days after onset of rash with the rash onset being considered as day zero.

Before the introduction of measles vaccine in 1963 and widespread vaccination, major epidemics occurred approximately every 2 to 3 years and measles caused an estimated 2.6 million deaths each year. More than 140 000 people died from measles in 2018 – mostly children under the age of 5 years, despite the availability of a safe and effective vaccine.

Accelerated immunization activities have had a major impact on reducing measles deaths. During 2000 to 2018, measles vaccination prevented an estimated 23.2 million deaths. Global measles deaths have decreased by 73% from an estimated 536 000 in 2000 to 142 000 in 2018. According to the World Health Organization (WHO), the largest outbreak of measles was seen in India in 2022 with reported measles cases of 12,773 making India's goal to eliminate measles by 2023 to be impractical. There was a recent outbreak of measles in Maharashtra with 3075 cases and 13 deaths. As per WHO, more than 58 000 people in 41 of the 53 Member States in the Region – straddling Europe and central Asia – were infected with measles, in 2023, resulting in thousands of hospitalizations and 10 measles-related deaths.

Measles is still common in many developing countries – particularly in parts of Africa and Asia. The overwhelming majority (more than 95%) of measles deaths occur in countries with low per capita incomes and weak health infrastructures. Measles outbreaks can be particularly deadly in countries experiencing or recovering from a natural disaster or conflict. Damage to health infrastructure and health services interrupts routine immunization and overcrowding in residential camps greatly increases the risk of infection.

Mumps is an acute viral illness caused by a paramyxovirus that typically presents as swelling of the parotid (parotitis) or another salivary gland[s]. Parotitis occurs as unilateral or bilateral and lasts from 3 to 7 days (average 5 days); most cases resolve within 10 days. In some cases, nonspecific prodromal symptoms precede parotitis by several days, including low-grade fever, lasting 3 to 4 days, myalgia, anorexia, malaise, and headache. The incubation period ranges from 12 to 25 days, but parotitis typically develops 16 to 18 days after exposure to mumps virus.

Mumps can occur in a person who is fully vaccinated, but vaccinated persons are at much lower risk for mumps and mumps complications. Mumps reinfection in patients who previously had natural infection or recurrent mumps (parotid swelling resolves and then weeks to months later occurs on the same or other side) can also occur. Mumps infection may present only with nonspecific or primarily respiratory symptoms or be asymptomatic. Among unvaccinated

people, approximately 20% of infections are asymptomatic; frequency of asymptomatic infection among vaccinated people is unknown.

Worldwide, mumps is not as well controlled as measles and rubella. From 1999 to 2019, on average, about 500,000 mumps cases were reported to the World Health Organization annually; however, global mumps incidence is challenging to estimate as mumps is not a notifiable disease in many countries. As of 2019, mumps vaccine is routinely used in 122 of 194 (63%) countries. Since the mid-2000s, mumps outbreaks have also been reported among populations with high 2-dose MMR coverage in other countries, including United Kingdom, Ireland, New Zealand, Canada, Netherlands, Spain and Norway. Despite these outbreaks, mumps incidence is still much higher in countries that do not have routine mumps vaccination.

Rubella is an acute contagious viral infection that occurs most often in children and young adults. Rubella is the leading vaccine-preventable cause of birth defects. While rubella virus infection usually causes a mild fever and rash in children and adults, infection during pregnancy, especially during the first trimester, can result in miscarriage, Foetal death, stillbirth, or infants with congenital malformations, known as congenital rubella syndrome (CRS). The rubella virus is transmitted by airborne droplets when infected people sneeze or cough. Humans are the only known host. There is no specific treatment for rubella but the disease is preventable by vaccination.

Rubella is a viral illness caused by a Togavirus of the genus Rubivirus and is characterized by a mild, maculopapular rash. The rubella rash occurs in 80% of rubella-infected persons and is sometimes misdiagnosed as measles or scarlet fever. Children usually develop few or no constitutional symptoms, but adults may experience a 1 to 5-day prodrome of low-grade fever, headache, malaise, mild coryza, and conjunctivitis. Postauricular, occipital and posterior cervical lymphadenopathy is characteristic and precedes the rash by 5 to 10 days. Arthralgia or arthritis may occur in up to 70% of adult women with rubella. Rare complications include thrombocytopenic purpura and encephalitis. Rubella is transmitted through direct or droplet contact from nasopharyngeal secretions and has an average incubation period of 17 days (range: 12 to 23 days). Persons with rubella are most infectious when rash is erupting, but they can shed virus from 7 days before to 7 days after rash onset.

Measles and rubella vaccination are an integral part of immunization programmes worldwide, contributing to progress towards achieving global immunization goals and, more broadly, the

Global Health Security Agenda and the United Nations Sustainable Development Goals (SDGs).

The Region of the Americas, which was verified to have eliminated measles in 2016, lost its measles elimination status in 2018. Globally, reported measles cases more than doubled from 2017 to 2018, from 170 000 to 350 000. This upward trend continued into 2019, with several countries experiencing large measles outbreaks. In 2019, the Democratic Republic of the Congo, Ukraine and Brazil reported 333 017, 57 282 and 18 203 confirmed cases of measles, respectively, while Chad reported more than 26 600 suspected cases. Vaccination coverage remains low or very low in several countries. In 2019, seven countries had coverage of the first dose of measles-containing vaccine (MCV1) below 50% and 23 had coverage below 70%, indicating that 30–50% of children in these countries had not received any doses of measles vaccine through routine service delivery mechanisms.

To achieve the goal of providing full immunization coverage of more than 90% and to reach the goal of universal immunization program, India has launched an Intensified Mission Indradhanush (IMI) 4.0 in phases in 2022. Although the prevention of measles and rubella is a priority, according to the recommendations of FOGSI [Federation of Obstetric and Gynecological Societies of India (FOGSI)], the combination of MMR vaccine is preferred over rubella vaccine for the purpose of routine preconception vaccination. Despite the availability of MMR vaccines, recent outbreaks of measles and a higher prevalence of mumps are alarming and warrant a broader MMR vaccination coverage in the country.

The worldwide market demand for MMR vaccines is in the order of approximately 110 million doses per year. Efficient vaccine production requires the growth of large-scale quantities of virus produced in high yields from a host system. The process and cultivation conditions under which a virus strain is grown is of great significance with respect to achieving an acceptable high yield of the strain. Thus, in order to maximize the yield of the desired virus, both the system and the cultivation conditions must be adapted specifically to provide an environment that is advantageous for the production of the desired virus. Therefore, a continuing need exists for safe and effective methods to produce viruses and antigen. Moreover, there is a need for an approach to viral propagation, employing materials that are already available and requiring a minimal number of time-consuming manipulations, wherein the selection of a combination of host cells, culture medium, growth conditions and production system is essential to achieve an efficient production process.

However, live attenuated vaccines are particularly fragile and their vaccinating action is quickly destroyed when they are exposed to temperatures above + 4 ° C and sometimes even less. Such vaccine instability is unacceptable, especially when these vaccines are used, on a large scale in tropical countries where they may accidentally be exposed to the high ambient temperatures prevailing in these countries. It is therefore necessary to have a stable and heat-resistant vaccine capable of withstanding deterioration when it is stored for long periods of time and is accidentally exposed to high temperatures.

Vaccine stability depends on various factors such as storage temperature, storage time, the vaccine composition, as well as the surrounding gas and the vial in which the vaccine is kept. Vaccines tend to lose their effectiveness over time when stored under ambient or warm temperatures. Hence there is a need to develop methods of vaccine composition which elicits a protective immune response with improvements in stability and shelf life of the vaccines.

There are various disadvantages linked to the use of serum and of animal-derived components, mainly their cost, the batch to batch variability in their composition, their association with a higher contamination risk by adventitious agents, and the subsequent difficulties encountered in downstream processing (e.g. purification to get rid of the serum- proteins or of the introduced animal-derived proteins).

Before manufacturing-scale mammalian cell cultivation process starts in a bioreactor, a seed culture inoculum is typically prepared. This involves culturing production cells in a series of single or multi-plate flasks in incubators and/or smaller bioreactors of increasing volume until enough cells are available for inoculation into the production bioreactor. The process involves transferring a cell population from one culture vessel to a larger one. Generally, a 20% to 50% dilution of the cell population is used for each transfer or subculture. In the incubator, the flasks with culture medium are stationary or clamped to a rotating platform to swirl the culture and facilitate gas transfer between the culture medium and the atmosphere in the incubators. Typically, the incubator for a mammalian cell culture process is set at 37°C with 5% carbon dioxide (CO₂) and a humidity level higher than about 80%. Similar temperatures and CO₂ levels are used for seed cultures grown in bioreactors. When the seed culture reaches a sufficient volume and cell density, it is inoculated into the production flask/ bioreactor.

After seed culture is inoculated into the bioreactor medium, parameters such as pH, temperature, and level of dissolved oxygen are controlled to the prescribed levels during the

cell cultivation process. pH is typically controlled by adding basic or acidic solutions when necessary during the process. Commonly used base solutions include sodium bicarbonate, sodium carbonate and sodium hydroxide solutions. Dissolution of carbon dioxide (CO₂) is commonly used to achieve a more acidic pH. Although other acids are available for controlling pH, the dissolved CO₂ and sodium bicarbonate combination forms a most stable and favourable buffer system for the cell culture. The preferred temperature of the culture medium or solution for mammalian cell cultivation processes is about 37°C. The desired level of dissolved oxygen in the culture medium or solution is typically achieved through air sparging using sparger installed on the bottom of the bioreactor or through headspace, along with agitation of the culture medium or solution using impellers which breakup the large air/oxygen bubbles to enhance the transfer of oxygen to the cell medium from the sparged air bubbles or using any other gaseous exchanger systems. Purging the bioreactor headspace with a cover gas provides a limited degree of surface gas exchange. Disadvantageously, air-sparging and agitation of the culture medium may result in foaming and shear damage to the mammalian cells which adversely impacts cell viability. Accumulations of foam on the surface of the culture medium also serve to further limit surface gas exchange and to reduce the available working volume of the bioreactor.

Mammalian cells are known to be sensitive to the amount of dissolved carbon dioxide in the cell culture media. Mammalian cell cultures exposed to excess carbon dioxide levels during the exponential growth phase may demonstrate reduced production of monoclonal antibodies or other desired biological products. Before inoculation, the pH of the slightly alkaline culture media has to be lowered with carbon dioxide adjusted to an optimum value. This often leads to elevated levels of dissolved carbon dioxide at the beginning of the lag phase of many mammalian cell culture processes. Dissolved carbon dioxide in mammalian cell culture bioreactors originates from chemical and biological sources. The chemical source of carbon dioxide is equilibrium chemical reactions occurring within the cell culture medium that includes a selected amount of a buffer solution containing sodium bicarbonate and/or sodium carbonate. Additionally, carbon dioxide may be directly sparged into the slightly alkaline culture medium to reduce the pH of the broth to a prescribed level, usually around 7.0, resulting in more dissolved carbon dioxide. The biological source of carbon dioxide is a product of the respiration of the mammalian cells within the bioreactor. This biological source of carbon dioxide increases with cell density and generally reaches its maximum value at about the same time that cell density within the bioreactor is maximized. However, as more carbon dioxide is

produced, the pH of the cell culture medium trends toward acidic such that additional bicarbonate is needed to keep the pH of the cell culture medium or solution within the desired range. To offset the effects of increased dissolved carbon dioxide which depresses the pH, one may add sodium bicarbonate so as to maintain the pH of the solution within the prescribed range. Both of these means to offset the effects of increased carbon dioxide have other negative consequences on the mammalian cell culture process.

The addition of sodium bicarbonate, needed to adjust the pH of the solution to offset the carbon dioxide, also increases osmolality. (Osmolality represents the number of dissolved particles per kilogram of solution and is commonly reported as mOsm/kg by freeze-point depression). The addition of sodium bicarbonate will also increase the equilibrium saturation level of dissolved carbon dioxide allowed in the solution, making carbon dioxide more difficult to be removed during the aeration process. It is known in the art that increased levels of either dissolved carbon dioxide or increased osmolality have adverse or negative impacts on cell density or yield.

Carbon dioxide dissociates into bicarbonate ions at a pH of 7 in water. Only a fraction of the carbon dioxide remains as free CO₂ in an un-dissociated state. Removing the dissolved carbon dioxide from a cell culture thus becomes difficult as most mammalian cell cultures take place at pH in the range of 6.5 to 7.5. The dissociated bicarbonate ions are not easily removed and generally must be recombined into free carbon dioxide before they can be stripped out of the solution.

The conventional method of removing or stripping dissolved carbon dioxide from a mammalian cell culture solution is by sparging the cell culture solution with air or a gas mixture of air/oxygen/nitrogen in agitated tanks. However, gas sparging in agitated tanks results in adverse effects to the cell culture process. In particular, the gas-bubble breakage at the tip of the rotating agitator is a source of high shear rate that damages mammalian cell membranes, often sufficiently to cause cell death. Even when damage is sub-lethal, cell productivity is compromised in the period that the damaged membrane is repaired.

Also, sparging air or nitrogen into the bioreactor creates gas bubbles rising to the surface of the solution within the bioreactor where the gas is released into the headspace. Gas bubble breakage at the top surface of the cell culture solution is often more damaging to the mammalian cells than the damage caused by the agitator. Restraining the agitator speed and

limiting the gas sparging rate are currently viewed as the best means to avoid such damage and increase cell viability. However, these measures reduce the amount of carbon dioxide that can be removed and the excess that cannot be removed also inhibits cell growth and viability. These disadvantages are particularly challenging to overcome in large, commercial- scale bioreactors where the shear rate goes up substantially with the diameter of the impellers. Also, the greater hydrostatic head of large-scale bioreactors tends to increase the solubility of carbon dioxide, meaning that more carbon dioxide needs to be removed to maintain dissolved CO₂ levels within an optimal range.

As per the above explained parameters, there is need of manufacturing process which can overcome the issues related to the process parameters/steps involved in the manufacturing of measles vaccine.

Accordingly, need exist for large scale, cost effective and safe method of producing viral vaccines that comprises minimum animal origin components, provide high virus yield, ensures virus structure integrity/stability preservation across manufacturing and storage.

Applicant has surprisingly found improved large scale affordable /safe manufacturing processes (encompassing cultivation, purification & formulation stages) that utilizes minimum animal origin components, provides high virus yield, ensures virus structure integrity/ stability preservation across manufacturing and storage wherein 1) Recombinant Trypsin (at least 95% pure, having molecular weight 23 to 25 kilodalton; Specific activity 2500 USP units/mg; low endotoxin content) at optimal pH (7.2 to 7.6) is used; 2) > 50% virus yield obtained (increased by 30-40 %) due to optimal pH (7.2 to 7.8), optimal concentration of sodium bicarbonate in medium (1.0 to 2.5 g/L), Low multiplicity of infection (MOI) i.e. virus to cell ratio (for measles and rubella bulk vaccine manufacturing – 1:5 to 1: 20 and for mumps bulk vaccine manufacturing – 1:20 to 1: 60), multiple harvesting; 3) use of Gamma irradiated FBS (Dose range of 20 to 50K Gy); 4) optimum virus: Stabilizer-I: Stabilizer-II ratio (80:20:10); 5) use of MLTCF-10 vented with Corning vented caps (instead of milex filter) resulting in slow dissociation of sodium bicarbonate.

OBJECTS

Some of the objects of the present disclosure, which at least one embodiment herein satisfies, are as follows:

An object of the present disclosure is to ameliorate one or more problems of the prior art or to at least provide a useful alternative.

An object of the present disclosure is to provide clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus.

Another object of the present disclosure is to provide improved method for manufacturing live attenuated lyophilized viral vaccine composition.

Another object of the present disclosure is to provide improved method for manufacturing live attenuated lyophilized viral vaccine composition comprising atleast one virus selected from a group consisting of a Measles, Mumps and Rubella.

Another object of the present disclosure is to provide easy and simple method for manufacturing live attenuated viral vaccine bulk which may contribute to the increase in the final yield of bulk vaccine and doses.

Another object of the present disclosure is to provide easy and simple method for manufacturing live attenuated lyophilized viral vaccine composition which may contribute to the increase in the final yield of bulk vaccine and doses.

Still another object of the present disclosure is to provide a method for manufacturing live attenuated lyophilized viral vaccine composition using Gamma irradiated FBS.

Still another object of the present disclosure is to provide a method for manufacturing live attenuated lyophilized viral vaccine composition using recombinant trypsin at optimal pH.

Yet another object of the present disclosure is to provide a method for manufacturing live attenuated lyophilized viral vaccine composition with high virus yield using optimal pH, optimal concentration of sodium bicarbonate in medium, low MOI i.e. virus to cell ratio and multiple harvesting.

Still another object of the present disclosure is to provide a method for manufacturing live attenuated lyophilized viral vaccine composition using MLTCF-10 vented with Corning vented caps (instead of milex filter).

Yet another object of the present disclosure is to provide a live attenuated lyophilized viral vaccine composition containing optimum virus: stabilizer ratio.

Another object of the present disclosure is to provide a live attenuated lyophilized/freeze-dried viral vaccine composition/formulation wherein, post-reconstitution the composition preserves the desired characteristics of the virus, including stability and immunogenicity.

Other objects and advantages of the present disclosure will be more apparent from the following description, which is not intended to limit the scope of the present disclosure.

SUMMARY

Applicant provides a method of producing a clarified virus pool, the method comprising: providing a cell line in a cell media and supplement; treating cells of cell line with enzyme; infecting the cell line with a virus to form an infected cell line; washing of the infected cell line with the virus media; harvesting the infected cell line in the media to obtain a harvest; optionally re-harvesting the infected cell line; adding a stabilizer to the harvest; and clarifying the harvest to obtain a clarified virus pool (CVP).

In an aspect, the present invention is directed to a method of producing a clarified virus pool, the method comprising:

- a. providing a cell line in a cell media, a buffer and a supplement;
- b. treating cells of the cell line with at least one enzyme;
- c. infecting the cell line with a virus to form an infected cell line;
- d. washing of the infected cell line with a virus media and the buffer;
- e. harvesting the infected cell line in the media to obtain a harvest; optionally re-harvesting the infected cell line one or more times;
- f. adding at least one stabilizer to the harvest; and
- g. clarifying the harvest to obtain a clarified virus pool (CVP).

optionally, wherein the cell line or the infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or

both.

In another aspect, the present invention is directed to a clarified virus pool (CVP) obtained by the method as disclosed.

In another aspect, the present invention is directed to a method of producing a measles clarified virus pool.

In another aspect, the present invention is directed to a measles clarified virus pool (CVP) obtained by the method as disclosed.

In another aspect, the present invention is directed to a method of producing a mumps clarified virus pool.

In another aspect, the present invention is directed to a mumps clarified virus pool (CVP) obtained by the method as disclosed.

In another aspect, the present invention is directed to a method of producing a rubella clarified virus pool.

In another aspect, the present invention is directed to a rubella clarified virus pool (CVP) obtained by the method as disclosed.

In another aspect, the present invention is directed to method of obtaining a lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprising one or more CVP selected from a measles CVP, a mumps CVP, a rubella CVP, or a combination thereof, blending of CVP, followed by lyophilizing the blended CVP.

In another aspect, the present invention is directed to lyophilized/ freeze-dried MMR immunogenic composition.

In another aspect, the present invention is directed to kit comprising the lyophilized/freeze-dried MMR immunogenic composition.

Applicant provides a method of producing a clarified virus pool of viruses such as measles, mumps, rubella. The enzyme treatment done for cells of cell line is recombinant trypsin. Washing of infected cell line is done with virus medium without FBS. The buffer added in cell media and virus medium is selected NaHCO_3 (sodium bicarbonate). Sodium bicarbonate in virus medium maintains the physiological pH which gives the significant outcomes. Applicant has found the improved large scale affordable/ safe manufacturing processes (encompassing cultivation, purification & formulation stages) that utilize minimum animal origin components, provide high virus yield, ensure virus structure integrity/stability preservation across

manufacturing & storage wherein 1) Recombinant Trypsin (at least 95% pure, having molecular weight 23 to 25 kilodalton; Specific activity 2500 USP units/mg; low endotoxin content) at optimal pH (7.2 to 7.6) is used; 2) > 50% virus yield obtained (increased by 30 to 40 %) due to optimal pH (7.2 to 7.8), optimal concentration of sodium bicarbonate in medium (1.0 to 2.5 g/L) Low MOI i.e. virus to cell ratio (for measles and rubella bulk vaccine manufacturing – 1:5 to 1: 20 and for mumps bulk vaccine manufacturing – 1:20 to 1: 60), multiple harvesting; 3) use of Gamma irradiated FBS (Dose range of 20 to 50KGy); 4) Optimum virus: Stabilizer-I: Stabilizer-II ratio (80:20:10); 5) use of MLTCF-10 vented with Corning vented caps (instead of millex filter) resulting in slow dissociation of sodium bicarbonate.

DETAILED DESCRIPTION OF THE DRAWING

The present invention will now be described with the help of the accompanying drawing, in which:

Figure 1 illustrates yield of Measles virus.

DESCRIPTION

Although the present disclosure may be susceptible to different embodiments, certain embodiments are shown in the drawing and following detailed discussion, with the understanding that the present disclosure can be considered an exemplification of the principles of the disclosure and is not intended to limit the scope of disclosure to that which is illustrated and disclosed in this description.

Embodiments are provided so as to thoroughly and fully convey the scope of the present disclosure to the person skilled in the art. Numerous details are set forth, relating to specific components, and processes, to provide a complete understanding of embodiments of the present disclosure. It will be apparent to the person skilled in the art that the details provided in the embodiments should not be construed to limit the scope of the present disclosure. In some embodiments, well-known composition, well-known processes, and well-known techniques are not described in detail.

The terminology used, in the present disclosure, is only for the purpose of explaining a particular embodiment and such terminology shall not be considered to limit the scope of the present disclosure. As used in the present disclosure, the forms “a”, “an”, and “the” may be intended to include the plural forms as well, unless the context clearly suggests otherwise.

The terms “comprises”, “comprising”, “including”, and “having” are open ended transitional phrases and therefore specify the presence of stated features, integers, steps, operations, elements, modules, units and/or components, but do not forbid the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof. The particular order of steps disclosed in the process of the present disclosure is not to be construed as necessarily requiring their performance as described or illustrated. It is also to be understood that additional or alternative steps may be employed.

The terms first, second, third, etc., should not be construed to limit the scope of the present disclosure as the aforementioned terms may be only used to distinguish one element, component, region, layer or section from another component, region, layer or section. Terms such as first, second, third etc., when used herein do not imply a specific sequence or order unless clearly suggested by the present disclosure.

It is understood that each feature or embodiment, or combination, described herein is a non-limiting, illustrative example of any of the aspects of the invention and, as such, is meant to be combinable with any other feature or embodiment, or combination, described herein. For example, where features are described with language such as “one embodiment”, “some embodiments”, “certain embodiments”, “further embodiment”, “specific exemplary embodiments”, and/or “another embodiment”, each of these types of embodiments is a non-limiting example of a feature that is intended to be combined with any other feature, or combination of features, described herein without having to list every possible combination. Such features or combinations of features apply to any of the aspects of the invention.

Definitions:

In order for the present disclosure to be more readily understood, certain terms are first defined below. Additional definitions for the following terms and other terms may be set forth throughout the specification.

The term “PDL” is “population doubling level” refers to the total number of times the cells in a given population have doubled during in vitro culture.

The term “working cell bank” refers to quantity of cells of uniform compositions derived from the master cell bank at a finite passage level, dispensed in aliquots into individual containers

appropriately stored in liquid nitrogen containers at -196°C, one or more of which would be used for production purposes.

The term “cell media” may be interchangeably used with the term “cell growth media”.

The term “virus media” may be interchangeably used with the term “virus growth media” and “washing media”

The terms “CF” refers to “cell factory” and “CS” refers to “cell stack”.

The terms MLTCF refers to “Multilayered Tissue culture flask”.

The term “working seed virus” refers to the virus used for infecting the cell line.

The term “freeze-drying/ freeze dried/lyophilize/ lyophilization” involves lyophilization and refers to the process by which a suspension/solution is frozen, after which the water is removed by sublimation at low pressure.

The term “sublimation” refers to a change in the physical properties of a composition, wherein the composition changes directly from a solid state to a gaseous state without becoming a liquid.

The terms “Tresivac” refers to “Measles, Mumps, Rubella Vaccine, Live Attenuated (Freeze-Dried) (MMR vaccine) of Serum Institute of India, Ltd”.

The term “Blind vaccine” refers to diluent used for dilution of virus bulk or clarified virus pool/ pools during blending or used for mixing with virus bulk or clarified virus pool/ pools during blending.

The term “bulk vaccine” refers to the solution containing the clarified virus pool of virus or the blended solution of containing the clarified virus pool of more than one virus.

The terms “bulk vaccine” or may be interchangeably used with the term “virus bulk vaccine”

The term "dose" herein is typically one administration of the vaccine of the invention, which is typically one injection. A typical human dose is 0.5mL. Of course, various doses may be administered in a vaccine administration schedule.

The present disclosure envisages a method for manufacturing viral vaccines. In a preferred embodiment, the present disclosure provides a method for manufacturing of live attenuated

lyophilized/freeze dried virus vaccine which includes one or more than one virus. The lyophilized/freeze dried virus vaccine include the bulk of one or more virus vaccine.

The present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases virus titre by addition of media in the manufacturing process.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases virus titre by addition of media post infection with virus in the manufacturing process.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases virus yield by addition of media post infection with virus in the manufacturing process.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases viral load by addition of media post infection with virus in the manufacturing process.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases final yield of bulk vaccine and doses by addition of media post infection with virus in the manufacturing process.

In a preferred embodiment, the present disclosure provides a method of producing a clarified virus pool in the manufacturing of live attenuated lyophilized/freeze dried virus vaccine which includes one or more than one virus increases virus titre, virus yield, final yield of bulk vaccine and doses by addition of media post infection with virus in the manufacturing process.

GENERAL CVP PRODUCTION METHOD

In an aspect of the present disclosure, the method in accordance with the present disclosure comprise:

- a. providing a cell line in a cell media, a buffer and a supplement;
- b. treating cells of the cell line with at least one enzyme;
- c. infecting the cell line with a virus to form an infected cell line;
- d. washing of the infected cell line with a virus media and the buffer;
- e. harvesting the infected cell line in the media to obtain a harvest; optionally re-harvesting the infected cell line;
- f. adding atleast one stabilizer to the harvest; and
- g. clarifying the harvest to obtain a clarified virus pool (CVP).

optionally, wherein the cell line or the infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with additional buffer or
- both.

In an embodiment, the method of producing the CVP is for preparation of bulk virus vaccine/ bulk virus immunogenic composition.

In another embodiment, the method for producing CVP includes:

- a) Providing a cell line depending on the virus;
- b) Revival & passaging of cells in Tissue culture flask (TCF);
- c) Preparation of cell factories/ cell stacks;
- d) Treating cells of the cell line with at least one enzyme, the enzyme including trypsin, i.e. Trypsinization of cell factories/ cell stacks;
- e) Infecting the cell line by addition of virus i.e. infection;
- f) Washing of the infected cell line using he virus media and the buffer;
- g) Harvesting the infected cell line in the media to obtain the harvest; re-harvesting the infected cell lines to obtain multiple harvests;
- h) Adding atleast one stabilizer to the harvest;
- i) Clarifying of the harvest to obtain the clarified virus pool (CVP)
- j) Storing the CVP below -20°C as a drug substance i.e. bulk virus vaccine

optionally, wherein the cell line or the infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with additional buffer or
- both.

STEP a):

In an embodiment, the method of producing the clarified virus pool includes providing the cell line in the cell media, the buffer and the supplement.

In an embodiment, the cell line is provided in a container. The container includes covered flasks, bottles, roller bottles, serrated roller bottles, cuboidal containers, round bottom containers, cell factories.

In an embodiment, the container is covered. The covering for container includes caps.

CELL LINE

In another embodiment, the cell line used as a substrate for the virus growth includes animal cell line, insect cell line, human cell line, primary cell line (Chick embryo fibroblast (CEF)), diploid cell line (including Human Lung Fibroblast (MRC-5)), continuous cell line.

In an embodiment, the cell line used as a substrate for the virus growth is selected from the different cell lines such as Rhesus monkey kidney (RhMK) cells; Primary rabbit kidney cells; Human foreskin fibroblasts; Chick embryo fibroblast (CEF); Human epidermoid carcinoma cells (HEp-2); Human lung carcinoma cells (A549); Human Cervix Epithelial (HeLa); African Green Monkey Kidney Epithelial (Vero); Human Lung Fibroblast (MRC-5); Human Lung Fibroblast (MRC-9); Mouse Embryo Fibroblast (NIH3T3); Mouse Connective Tissue Fibroblast (L929); Chinese Hamster Ovary Fibroblast (CHO); Syrian Hamster Kidney Fibroblast (BHK-21); Human embryo Kidney Epithelial (HEK-293); Human Liver Epithelial (HepG2); Bovine Aorta Endothelial (BAE-1); Human Neuroblastoma Neuronal (SH-SY5Y); Mouse Myeloma Lymphoblast (NS0); Human Hystiocytic Lymphoma Lymphoblast (U937); Human Leukemia Lymphoblast (HL60); Mouse B-cell Lymphoma Lymphoblast (WEHI231); Mouse Lymphoma Lymphoblast (YAC1); Human Myeloma Lymphoblast (U266B1); Human T-cell Leukemia Lymphoblast (Jurkat); Human Monocyte Leukemia Lymphoblast (THP-1); Human embryonic lung cells (W1-38); Madin Darby canine kidney cells (MDCK); Human embryonic retinal cells (PER.C6); Human embryonic retinoblasts (HER.911); Murine non-secreting myeloma (Sp2.0); Epithelial cells of African green monkey kidney origin (BSC-1

cells); Rhesus Monkey Kidney Epithelial Cells (LLC-MK2 cells); Cercopithecus aethiops monkey kidney cells (CV-1 cells); African green monkey kidney fibroblast-like cells (COS-cells); Crandell- Rees Feline Kidney Cells (CRFK cells); Rapidly Accelerated Fibrosarcoma cells (RAF cells); Normal Rabbit Kidney Epithelial Cells (RK-13 cells); Transformed C3H Mouse Kidney-1 (TCMK-1 cells); Pig Kidney Epithelial Cells (LLC-PK1 cells); Porcine kidney cells (PK15 cells); Rabbit kidney cell line (LLC-RK1 cells), Nonsecreting myeloma cell lines (NS-1 cells), New human male diploid cell strain (TIG-1, TIG-7); nonhuman primate diploid cell line (FRhL-2); Human foetal lung (IMR-90, IMR-91) cells; human diploid lung fibroblasts (including WI-38) and others.

In an embodiment, the cell line used as a substrate for the growth of measles virus, mumps virus, rubella virus is selected from Human Lung Fibroblast (MRC-5) cell line and Chick embryo fibroblast (CEF) cell line.

In another embodiment, MRC-5 cells at lower passage were obtained from NIBSC, UK. The master and working cells banks are prepared and stored at -196°C in liquid nitrogen. This stock of cells is used for production purposes. The cell stock has been characterized and tested according to the Ph. Eur and WHO.

In another embodiment, Chick Embryo Fibroblast (CEF) cells is prepared by using 9 to 11 days old embryo of specific pathogen free (SPF) chicken eggs. These SPF eggs are received from Lohmann Germany and Hy-Vac, USA etc.

In an embodiment, the cell line is provided with the cell media, the supplement and the buffer.

CELL MEDIA

In an embodiment, for the method of producing the CVP, the cell line is provided with a cell media (herein after interchangeably referred to as cell growth media) for growth and propagation. The cell media has nutrients including carbon sources, carbohydrates, vitamins, amino acids, minerals, growth factors, hormones, sugars, glucagon, cyclodextrin, inorganic salts, buffers or combination thereof.

The carbon source includes carbohydrates, glucose, glutamine, sucrose, dextrose, galactose, fructose or combinations thereof.

In another embodiment, the cell growth medium is selected from basal medium, enriched medium, selective and indicator medium, transport media and storage media.

In another embodiment, the cell growth medium is selected from different media such as basal culture media, cell culture media well suited for the growth of a broad spectrum of mammalian cells, Minimum Essential Medium supplemented with L- Glutamine, multipurpose media, minimum essential medium modified to contain Balanced Salt Solution (BSS) and amino acids.

In another embodiment, the Balanced salt solution includes Earle's balanced salt solution (EBSS), Gey's balanced salt solution (GBSS), Hanks' balanced salt solution (HBSS), (Dulbecco's) Phosphate buffered saline (PBS), Puck's balanced salt solution, Ringer's balanced salt solution (RBSS), Alsever's solution, Simm's balanced salt solution (SBSS), TRIS-buffered saline (TBS), Tyrode's balanced salt solution (TBSS). In a preferred embodiment, the Balanced Salt Solution is Earle's or Hanks's BSS.

In an embodiment, the cell growth medium is selected from Medium 199 (M-199), Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM); Eagle's Minimum Essential Medium (E-MEM), Hank's MEM (H-MEM), Iscove's Modified Dulbecco's Medium (IMDM), Ham's nutrient mixtures (F-10 and F-12), Leibovitz (L-15), Roswell Park Memorial Institute (RPMI)-1640; Neurobasal medium; Schneider's Drosophila medium; McCoy's 5A Medium; Dynamis medium; Essential 8 (E8) media; StemFlex culture media; Airway Epithelial Cell basal medium; alpha-modified minimum essential medium (α -MEM); StemMacs iPS-Brew media; TeSR-E8, mTeSR1, mTeSR Plus medium; GMEM(Glasgow Minimum Essential Medium); Opti-MEM I; SmGM-2; fibroblast growth media / FGM; StemPro-34 serum free growth medium; mTESR1 medium; ECGM-2 media; Williams' Medium E; Medium M254; CnT07 media; TNM-FH media; mammary epithelial cell growth basal medium (MEBM); complete skeletal muscle media and NeuroCult NS-A basal medium.

In another embodiment, the cell growth medium as synthetic cell culture medium is Minimum Essential Medium. The medium is sterilized by using sterile grade hydrophilic filters.

In a preferred embodiment, the filtration of the medium is done by 0.1 μ sterile grade hydrophilic filters.

In another embodiment, cell growth medium is supported or added with the buffer and the supplements.

In another embodiment, the cell growth medium is Minimum Essential Medium (MEM) which is prepared using powdered MEM supplemented with L-Glutamine, Foetal bovine serum (FBS) and pH buffer by mixing all the ingredients in pre-sterilized water for injection. The medium is sterilized by 0.1 μ filtration.

BUFFER

In an embodiment, for the method of producing the CVP, the buffer is used.

The buffer includes NaHCO₃, NaOH, NaCl, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) i.e. HEPES, (piperazine-N,N'-bis(2-ethanesulfonic acid)) i.e. PIPES, or (2-(N-morpholino)ethanesulfonic acid), i.e. MES.

In an embodiment, the buffer includes NaHCO₃ (sodium bicarbonate).

In a preferred embodiment, the buffer, i.e. NaHCO₃ (sodium bicarbonate) is in used in a batch in concentration range from 0.5 to 4.0 g/L, or 0.8 to 3.5 g/L or 1.0 to 3.0 g/L or 1.0 to 2.5 g/L or 1.0 to 2.3 g/L, or 1.0 to 2.1 g/L, or 1.0 to 2.0 g/L.

In a more preferred embodiment, the buffer, i.e. NaHCO₃ (sodium bicarbonate) is in used in a batch in concentration range from 1.0 to 1.9 g/L, or 1.2 to 1.9 g/L.

In another embodiment, the infected cell line is washed with the virus media and the buffer.

In another embodiment, the cell line or the infected cell line in exponential growth phase is grown with re-addition of the buffer.

In another embodiment, the cell line or the infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In another embodiment the re-addition of the buffer is performed by boosting with additional buffer, i.e. NaHCO₃ or providing additional amount of the buffer with the cell media or the virus media or both.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 5% to 40% or 5% to 35% or 5% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 10% to 30% or 15% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 0.1 g/L to 1.6 g/L or 0.1 g/L to 1.2 g/L or 0.1 g/L to 1.0 g/L or 0.1 g/L to 0.8 g/L or 0.1 g/L to 0.6 g/L.

In an embodiment, the total buffer concentration including the boosted buffer concentration for the cell media or the virus media or both is in the range of 0.6 g/L to 6.0 g/L, or 0.8 g/L to 6.0 g/L, or 1.0 g/L to 6.0 g/L, or 1.2 g/L to 6.0 g/L in the batch.

In another embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.2 g/L to 5.9 g/L, or 1.2 g/L to 5.8 g/L, or 1.2 g/L to 5.7 g/L, or 1.2 g/L to 5.6 g/L in the batch.

In preferred embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.5 g/L to 2.0 g/L in the batch.

In an embodiment, the method of preparing the CVP includes providing the cells of the cell line with the cell media, the buffer and at least one supplement.

The pH is critical process parameters for growth of cells. pH is controlled by proper venting to allow gaseous exchange and increasing the buffering capacity of cell media by adding additional quantity of the buffer in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield.

Thus, pH is one of the critical process parameters in viral vaccine manufacturing process. Exposure of the virus to extremes of pH inactivates the virus. It has been reported that virus is stable at pH in the range from 6 to 8 and a progressive inactivation of virus occurs on either side. Physical stability of virus is highly compromised in acidic environment. Even slight changes in pH affects the native confirmation of the virus.

Regulation of pH is particularly important immediately following cell seeding when a new culture is establishing and is usually achieved by one of two buffering systems; (i) a “natural” buffering system where gaseous CO₂ balances with the CO₃/ HCO₃ content of the culture medium and (ii) chemical buffering using a zwitterion called HEPES. Cultures using natural

bicarbonate /CO₂ buffering systems need to be maintained in an atmosphere of 5-10% CO₂ in air usually supplied in a CO₂ incubator. Bicarbonate/CO₂ is low cost, non-toxic and also provides other chemical benefits to the cells. HEPES has superior buffering capacity in the pH range 7.2-7.4 but is relatively expensive and can be toxic to some cell types at higher concentrations. HEPES buffered cultures do not require a controlled gaseous atmosphere.

In an embodiment, growth of cells is be controlled by adding additional quantity of NaHCO₃ in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield. pH as one of the critical process parameters for growth of cells is be controlled by proper venting to allow gaseous exchange and increasing the buffering capacity of cell media by adding additional quantity of NaHCO₃ in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield.

SUPPLEMENTS

In an embodiment, the at least one supplement includes additional amino acids, cholesterol, proteins, lactoferrin, linoleic acid, glucagon, cyclodextrin, yeast extracts, serums, antioxidants, vitamins, buffers, antibiotics, nutrients, trace elements, adherence, and extension factors.

In another embodiment, the at least one supplement includes serum, antibiotics, additional amino acid.

In another embodiment, the at least one supplement includes serum, antibiotics.

In another embodiment, the at least one supplement includes antibiotics, additional amino acid.

In another embodiment, the at least one supplement includes serum, additional amino acid.

In an embodiment, serum as supplement is bovine serum or calf serum. Serum as supplement is foetal bovine serum or foetal calf serum. Serum as supplement is foetal bovine serum.

Foetal bovine serum, (FBS) is the most widely used basal media supplement for in vitro cell culture. It contains very low level of antibodies and high concentration of growth factors such as hormones, attachment factors and transport proteins. FBS is used to keep cells alive for longer period of time. FBS contains a large number of components, like growth factors, proteins, vitamins, trace elements, hormones which are essential for the growth and maintenance of cells. FBS effectively promotes and sustains the growth of cells at low densities

used for biological manufacturing. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. FBS is obtained from foetuses harvested in abattoirs from healthy animals. Serum lots from the manufacturer contain pools of serum collected from many different animals.

Being an animal origin material, FBS has an inherent risk of being contaminated with transmissible adventitious agents. Bovine serum might be contaminated by many different bovine viruses. It is evident that each serum batch has to be tested for those viruses which are ubiquitous and of known risk. The possibility of the introduction and replication of adventitious agents during cell culture has long been recognized as a potential risk which leads to virus contaminated final product. There have been a number of instances where laboratory studies provided evidence for the presence of adventitious agents in marketed vaccines. Such risks of adventitious agents are typically mitigated by testing bovine serum for absence of adventitious agents. Most regulatory bodies allow the use of animal derived materials only when their use can be justified due to absence of viable alternative. For that, each serum lot must be tested for sterility and adventitious agents. Virus testing is typically performed in accordance with various regulatory guidelines. However, the testing methods have their own limitations and sometimes the contaminating adventitious agent may escape detection.

FBS undergo post-manufacturing treatment methods as follows;

1. Filtration: Triple 0.1 μm filtration has become the standard method of aseptic processing for various types of serum. While this methodology will remove bacteria and most mollicutes, it cannot render serum free of all viruses. Nanofiltration using viral retentive filters (20 nm nominal pore size) can be effective for removal of even small viruses such as parvoviruses. However, it suffers from scalability and flux decay with serum materials. Therefore, nanofiltration is not suitable for large-scale processing of serum product.
2. UV Treatment: Ultraviolet irradiation (typically 254 nm) has been shown to be effective for pathogen reduction. The method is not only effective for larger microbes such as bacteria and mollicutes but is also very effective for most viruses. It is commonly employed for high-volume water disinfection. UV irradiation has also demonstrated efficacy in small-scale experiments aimed at clearing adventitious agents from cell culture media. However, it has not yet been optimized to effectively handle the larger

volumes of media needed for commercial-scale bioproduction, and further technology development is needed.

3. High temperature short time (HTST) Treatment: Effective viral load reduction has been demonstrated for most viruses following HTST treatment. The most heat-resistant viruses, such as parvoviruses, require temperatures exceeding 115 °C. A significant issue with this modality is the fact that the biological activity of the serum can be seriously compromised at the relatively high temperatures required for treatment. This can potentially result in significantly reduced cell growth with various cell lines.
4. Traditional Heat Inactivation: Routine heat treatment of animal serum is performed in many cell culture labs as one of their normal procedures and is included in many biomanufacturing protocols. A wide range of temperatures ranging from 45–62 °C, and times from 15–60 minutes may be called for. The most common methodology requires the heating of serum at 56 °C for 30 minutes. Variability has been identified in the: (1) exact temperatures and exposure times used; (2) mixing of the serum in the bottles; and (3) depth of water in the water bath relative to the height of the serum bottles. These factors could negatively impact the biological activity of the serum post-treatment. For these reasons, heat inactivation of FBS is not an ISIA-recommended practice unless it has been shown to be necessary for the specific cell culture application.
5. Chemical Treatment: Serum treatments with chemicals such as ethyleneimine or beta-propiolactone (BPL) have also been used as strategies to mitigate risk from adventitious agents in specific applications. Chemical treatment processes have specific hazards that must be managed to assure safety of the operators and are not commonly used commercially on a large scale. Ozone technology (serum ozonation) is not suitable for serum treatment as it causes severe oxidation/peroxidation of serum components essential to cell culture growth, significantly reducing the serum's performance.
6. Ionizing Radiation: The various forms of ionizing radiation that have the potential to reduce microbial and viral burden in serum include: electron beam, X-irradiation.
 - a. Electron beam utilizes high energy electrons to cause inactivation of contaminants. The electron beam does not, however, have the penetrating ability necessary to irradiate large finished bottles (e.g., 500 mL and 1 L plastic bottles) of serum.
 - b. X-irradiation theoretically should combine the best characteristics of electron beam and gamma irradiation. However, the commercial application of X-irradiation for this

purpose has been limited because of the lack of facilities that can handle large batches of serum.

In an embodiment, foetal bovine serum as supplement is treated with gamma irradiation. Gamma irradiation is one of the most widely used and effective methods for the inactivation of viruses and mycoplasmas in animal sera. Gamma irradiation has both the penetrating power and ease of handling that allow it to be routinely used for pathogen reduction in finished serum bottles. Gamma irradiation is a very efficient and straight forward means for inactivating many different virus types in FBS. As FBS can be contaminated by adventitious viruses, gamma irradiation is, after routine quality control for virus detection, the best method to increase the safety of using it in the production of Biologicals. Gamma irradiation is performed on serum in the original product containers. It is typically performed at low temperatures, thereby keeping the serum quality and performance unaffected.

In an embodiment, FBS is irradiated with Gamma irradiation in the dose range of 20 to 50K Gy. Gamma irradiated FBS is used as supplement in the cell media. Gamma irradiated FBS is not used as supplement in the virus media. This cause the saving of a substantial quantity of FBS and leads to a cost-effective step.

The Foetal bovine serum (FBS) is used during formation of the clarified virus pool as well as preparation of virus bulk vaccine.

In an embodiment, the FBS is provided in the range of 5% to 15%, preferably 10% to 12.5% of the batch.

In an embodiment, antibiotic as supplement is not added at any stage of cell growth or virus growth. In an embodiment, antibiotic as supplement is added at any stage of cell growth or virus growth. In an embodiment, antibiotic as supplement added is selected from the group consisting of kanamycin, streptomycin, and neomycin; preferably neomycin. Antibiotics are commonly used in cell culture to prevent contamination, maintain aseptic conditions, or select for cells containing genetic modifications.

In an embodiment, additional amino acid as supplement include glutamine.

STEP b):

In an embodiment, the method of producing the clarified virus pool includes treating the cells of the cell line with at least one enzyme.

ENZYME

In an embodiment, for the method of producing the CVP, the cells of the cell line are treated with at least one enzyme.

In an embodiment, the enzyme is selected from trypsin, recombinant trypsin, dipase, collagenase, hyaluronidase, elastase, cysteine protease, deoxyribonuclease I and chymotrypsin.

In a preferred embodiment, the enzyme includes trypsin.

Trypsin is a proteolytic enzyme (serine protease), used as a cell dissociating agent in tissue culture techniques. As such, it becomes a critical raw material for all tissue culture-based vaccines such as, Measles, Mumps, Rubella, Rabies, Rota etc.

Trypsinization is an essential technique to be taken into consideration for Optimization of MRC-5 Cell Culture Process. For a vaccine production process, trypsinization may result in additional medium costs and increased contamination risks. On the other hand, too high trypsin activities in the virus production medium could result in cell detachment. Therefore, optimizing trypsin activity is essential.

In particular, batch to batch variations of trypsin, media, and differences in shear stress of cultivation systems need to be considered. Increase in trypsin activity may result in an earlier increase in haemagglutinin (HA) titre in serum free medium, while too low concentrations may prevent successful virus replication. Additionally, increased trypsin activity might complicate large-scale production processes; hence a single addition of trypsin may be preferred.

Porcine trypsin is used as a cell dissociation reagent in tissue culture technology. These preparations are nothing, but acetone extracts of pig pancreas and normally contain mixture of other enzymes such as chymotrypsin, elastase, caboxypeptidase, kallikriens, and insulinase, effects of which on cell culture are not completely understood. The preparations of these acetone extracts are further mixed with lactose to adjust the potencies. This trypsin has been successfully used for last many decades for commercial scale manufacturing of tissue culture

based vaccines and other biological products. However, being an animal origin material, it has a potential to introduce known or unknown adventitious agents inherent in the source material to the final product.

As many as 55 porcine virus species of human host range, from 17 different families having been stated as potential contaminating viruses. Even after extensive purification of animal-derived trypsin, however, there are contaminating activities in most preparations that can have undesirable consequences for both experimental research and pharmaceutical therapeutic protein processing. Such risks of various adventitious agents are typically mitigated by testing the porcine trypsin for absence of the extraneous agents, however the testing methods have their own limitations and sometimes the contaminating adventitious agents may escape detection.

In a more preferred embodiment, the enzyme includes recombinant trypsin.

Strict guidelines and regulations from regulatory bodies (e.g. Food and Drug Administration as well as other national and international regulatory bodies) has led to a need for pure trypsin of recombinant origin. Non-Recombinant trypsin is associated with contaminations. Rotavirus vaccine are prone to contamination with porcine circovirus DNA sequences originated from porcine trypsin that was used during development of vaccine.

EMA 2013 (European Medicines Agency), guidelines recommend use of two different cell lines to test adventitious agents that could be found in porcine trypsin. These guidelines also suggest use of animal-component free reagents, such as recombinant trypsin, in place of animal derived trypsin. Recombinant trypsin is a genetically engineered protein expressed in suitable microorganism (*E. coli*, *Pichia pastoris*) and purified by high pressure liquid chromatography.

Recombinant trypsin is safer to porcine origin trypsin and completely eliminates the risk of contamination by animal origin reagents.

In an embodiment, the trypsin used for trypsinization step is a mixture of trypsin (0.25%, recombinant) and EDTA (0.005%). Trypsin solution is sterilized by 0.1 μ filtration and similar to growth medium, trypsin is also added to TC flasks through an additional 0.1 μ filter (i.e. terminal filtration for each operation).

In an embodiment, the recombinant trypsin of at least 95% purity with low endotoxin content facilitates avoiding any adverse effects on cell line. The recombinant trypsin used here is of molecular weight 23 to 25 kilodalton; specific activity - 2500 USP units/mg.

In an embodiment, the addition of recombinant trypsin enzyme at optimum pH facilitates effective protein breakdown that dislodges the monolayer of MRC-5 cells from substrates (polystyrene, glass surfaces).

In an embodiment, cells of cell line are easily detached with little or no clumps when compared to the cells at acidic pH. When the cells are in the growing phase at optimum pH range of 7.4 to 7.8, effective trypsinization is carried out. The optimum pH range for growth of MRC-5 cells is 7.4 to 7.8 which causes improvement in trypsinization. Trypsinization is greatly improved in cultures maintained at physiological pH throughout the incubation period.

STEP c):

In an embodiment, the method of producing the clarified virus pool includes infecting the cell line with a virus to form an infected cell line.

VIRUS

In another embodiment, for the method of producing the CVP, the virus is selected from the group comprising single-stranded, positive-sense, negative-sense, enveloped, non-enveloped RNA viruses belonging to the family Picornaviridae, Caliciviridae, Togaviridae, Matonaviridae, Flaviviridae, Coronaviridae, Retroviridae, Filoviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae, Arenaviridae, Paramyxoviridae, or combinations thereof.

In another embodiment, the virus is selected from the group comprising single-stranded, positive-sense, enveloped, non-enveloped RNA viruses belonging to the family Picornaviridae, Caliciviridae, Togaviridae, Matonaviridae, Flaviviridae, Coronaviridae, Retroviridae, or combination thereof.

In another embodiment, the virus is selected from the group comprising single-stranded, negative-sense, enveloped, RNA viruses belonging to the family Filoviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae, Arenaviridae, Paramyxoviridae, or combination thereof.

In another embodiment, the virus is selected from the group comprising single-stranded, negative-sense, enveloped, RNA viruses belonging to the family Paramyxoviridae.

In another embodiment, the virus is selected from the group comprising single-stranded, positive-sense, enveloped, RNA viruses belonging to the family Matonaviridae.

In another embodiment, the virus is selected from but not limited to Adeno-associated virus, Aichi virus, lyssavirus, Banna virus, Barmah forest virus, polyomavirus, Bunyamwera virus, herpesvirus, Chandipura virus, Chikungunya virus, Cosavirus, Coxsackievirus, Dengue virus, Eastern chimpanzee simian foamy virus, Eastern equine encephalitis virus, Ebolavirus, Echovirus, Epstein-Barr virus, Hepatitis virus, Hantaan virus, Hendra virus, Horsepox virus, adenovirus, astrovirus, coronavirus, cytomegalovirus, enterovirus, Human immunodeficiency virus, Human metapneumovirus, Human papillomavirus, parainfluenza, parvovirus, respiratory syncytial virus, Human T- lymphotropic virus, Human torovirus, Influenza virus, Japanese encephalitis virus, arenavirus, Marburg virus, Langat virus, Lassa virus, Lymphocytic choriomeningitis virus, Machupo virus, Mayaro virus, Measles virus, Mokola virus, Molluscum contagiosum virus, Monkeypox virus, Mumps virus, New York virus, Nipah virus, Norwalk virus, Orf virus, Oropouche virus, Pichinde virus, Poliovirus, Punta toro phlebovirus, Puumala virus, Rabies virus, Rift valley fever virus, Rosavirus, Ross river virus, Rotavirus, Rubella virus, Sagiyama virus, Salivirus, Toscana virus, Seoul virus, Tick-borne virus, Vaccinia virus, Varicella-zoster virus, Variola virus, Vesicular stomatitis virus, Vientovirus, West Nile virus, Yellow fever virus or Zika virus.

In another embodiment, the virus is the single-stranded, negative-sense, enveloped, RNA virus belonging to the family Paramyxoviridae selected from measles and mumps virus.

In another embodiment, the virus is the single-stranded, positive-sense, enveloped, RNA virus belonging to the family Matonaviridae is rubella virus.

In another embodiment, the viral vaccine comprises three viruses selected from Measles virus, Mumps virus and Rubella virus.

In another embodiment, the viral vaccine comprises three viruses selected from Measles morbillivirus, Mumps orthorubulavirus and Rubivirus rubellae.

In another embodiment, the measles virus strain in live attenuated lyophilized/freeze dried virus vaccine composition/formulation of measles vaccine is selected from the group comprising Edmonston strain, including the Schwartz, the Edmonston-Zagreb, the Moraten strains; CAM-70; TD 97; Leningrad-16; AIK-C strain and Shanghai 191 (Ji-191) strains.

In an embodiment, the measles virus strain in live attenuated lyophilized/freeze dried virus vaccine is the Edmonston-Zagreb measles vaccine strain and obtained from Institute of Immunology, Zagreb, Croatia.

In another embodiment, the mumps virus strain in live attenuated lyophilized/freeze dried virus vaccine composition/formulation of mumps vaccine is selected from the group comprising Jeryl-Lynn, RIT 4385, Leningrad-3, Leningrad-Zagreb (L-Zagreb), Urabe Am9, Hoshino strain, Torii strain and S79 Rubini strains.

In another embodiment, the mumps virus strain in live attenuated lyophilized/freeze dried virus vaccine is the L-Zagreb MUMPS vaccine strain and obtained from Institute of Immunology, Zagreb, Croatia.

In another embodiment, the rubella virus strain in live attenuated lyophilized/freeze dried virus vaccine composition/formulation of rubella vaccine is selected from the group comprising Wister RA 27/3 strain, BRD-2 strain, Matsuba, DCRB19, Takahashi, Matsuura and TO-336 strains.

In another embodiment, the rubella virus strain in live lyophilized/freeze dried virus vaccine is the Wister RA 27/3 rubella vaccine strain and obtained from Institute of Immunology, Zagreb, Croatia.

The term "live" is used in its conventional meaning, a live virus is a virus which has not been inactivated, i.e. a virus capable of replicating on permissive cells. A live attenuated vaccine virus is a virus which does not induce the disease caused by the corresponding wild-type virus in animals or humans and which is capable of inducing a specific immune response.

MULTIPLICITY OF INFECTION (MOI)

In an embodiment, the cell line is infected with a virus to form an infected cell line. Virus infection is performed by adding virus to cell line (i.e. for virus to cell ratio) at MOI 1: 5 to

1:60. Low MOI or low MOI range is preferred to avoid the cytotoxicity observed at the higher MOI. Low MOI leads to better infectivity of virus.

STEP d):

In an embodiment, for the method of producing the clarified virus pool, the infected cell line is washed with the virus media and the buffer.

VIRUS MEDIA

In an embodiment, for the method of producing the clarified virus pool, the infected cell line is washed with the virus media and the buffer. The virus medium acts as a washing medium. The virus media includes basal media, enriched media, selective and indicator media, transport media, storage media, carbon sources.

In an embodiment, the carbon source includes carbohydrates, glucose, glutamine, sucrose, dextrose, galactose, fructose or combinations thereof.

In another embodiment, the virus media (hereinafter interchangeably referred to as virus medium) is selected from Medium 199 (M-199), Dulbecco's Modified Eagle's Medium (DMEM), Minimum Essential Medium (MEM); Eagle's Minimum Essential Medium (E-MEM), Hank's MEM (H-MEM), Iscove's Modified Dulbecco's Medium (IMDM), Ham's nutrient mixtures (F-10 and F-12), Leibovitz (L-15), Roswell Park Memorial Institute (RPMI)-1640; Neurobasal medium; Schneider's Drosophila medium; McCoy's 5A Medium; Dynamis medium; Essential 8 (E8) media; StemFlex culture media; Airway Epithelial Cell basal medium; alpha-modified minimum essential medium (α -MEM); StemMacs iPS-Brew media; TeSR-E8, mTeSR1, mTeSR Plus medium; GMEM (Glasgow Minimum Essential Medium); Opti-MEM I; SmGM-2; fibroblast growth media / FGM; StemPro-34 serum free growth medium; mTESR1 medium; ECGM-2 media; Williams' Medium E; Medium M254; CnT07 media; TNM-FH media; mammary epithelial cell growth basal medium (MEBM); complete skeletal muscle media and NeuroCult NS-A basal medium.

In another embodiment, virus media is added post infection with virus for washing of the infected cell lines in the manufacturing process.

In another embodiment, washing of the infected cell lines by the virus media is performed post infection with virus in the manufacturing process.

BUFFER

In an embodiment, for the method of producing the CVP, the buffer is used.

The buffer includes NaHCO_3 , NaOH , NaCl , (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) i.e. HEPES, (piperazine-N,N'-bis(2-ethanesulfonic acid)) i.e. PIPES, or (2-(N-morpholino)ethanesulfonic acid), i.e. MES.

In a preferred embodiment, the buffer includes NaHCO_3 (sodium bicarbonate).

In a preferred embodiment, the buffer, i.e. NaHCO_3 (sodium bicarbonate) is used in a batch in concentration range from 0.5 to 4.0 g/L, or 0.8 to 3.5 g/L or 1.0 to 3.0 g/L or 1.0 to 2.5 g/L or 1.0 to 2.3 g/L, or 1.0 to 2.1 g/L, or 1.0 to 2.0 g/L.

In a more preferred embodiment, the buffer, i.e. NaHCO_3 (sodium bicarbonate) is used in a batch in concentration range from 1.0 to 1.9 g/L, or 1.2 to 1.9 g/L.

In another embodiment, the infected cell line is washed with the virus media and the buffer.

In another embodiment, the cell line or the infected cell line in exponential growth phase is grown with re-addition of the buffer.

In another embodiment, the cell line or the infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In another embodiment the re-addition of the buffer is performed by boosting with additional buffer, i.e. NaHCO_3 or providing additional amount of the buffer with the cell media or the virus media or both.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 5% to 40% or 5% to 35% or 5% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 10% to 30% or 15% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 0.1 g/L to 1.6 g/L or 0.1 g/L to 1.2 g/L or 0.1 g/L to 1.0 g/L or 0.1 g/L to 0.8 g/L or 0.1 g/L to 0.6 g/L.

In an embodiment, the total buffer concentration including the boosted buffer concentration for the cell media or the virus media or both is in the range of 0.6 g/L to 6.0 g/L, or 0.8 g/L to 6.0 g/L, or 1.0 g/L to 6.0 g/L, or 1.2 g/L to 6.0 g/L in the batch.

In another embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.2 g/L to 5.9 g/L, or 1.2 g/L to 5.8 g/L, or 1.2 g/L to 5.7 g/L, or 1.2 g/L to 5.6 g/L in the batch.

In preferred embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.5 g/L to 2.0 g/L in the batch.

In an embodiment, the method of preparing the CVP includes providing the cells of the cell line with the cell media, the buffer and at least one supplement.

The pH is critical process parameters for growth of cells. pH is controlled by proper venting to allow gaseous exchange and increasing the buffering capacity of cell media by adding additional quantity of the buffer in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield.

In an embodiment, the pH is maintained in the range of 4.0 to 8.0. In a preferred embodiment, the pH is maintained in the range of 5.0 to 8.0, or from 6.0 to 8.0, or from 6.5 to 8.0.

In a more preferred embodiment, the pH is maintained in the range of 6.7 to 7.9, or from 6.9 to 7.9 or from 7.0 to 7.9, or from 7.1 to 7.9, or from 7.1 to 7.8.

Thus, pH is one of the critical process parameters in viral vaccine manufacturing process. Exposure of the virus to extremes of pH inactivates the virus. It has been reported that virus is stable at pH in the range from 6 to 8 and a progressive inactivation of virus occurs on either side. Physical stability of virus is highly compromised in acidic environment. Even slight changes in pH affects the native confirmation of the virus.

Regulation of pH is particularly important immediately following cell seeding when a new culture is establishing and is usually achieved by one of two buffering systems; (i) a "natural" buffering system where gaseous CO₂ balances with the CO₃/ HCO₃ content of the culture medium and (ii) chemical buffering using a zwitterion called HEPES. Cultures using natural bicarbonate /CO₂ buffering systems need to be maintained in an atmosphere of 5-10% CO₂ in

air usually supplied in a CO₂ incubator. Bicarbonate/ CO₂ is low cost, non-toxic and also provides other chemical benefits to the cells. HEPES has superior buffering capacity in the pH range 7.2-7.4 but is relatively expensive and can be toxic to some cell types at higher concentrations. HEPES buffered cultures do not require a controlled gaseous atmosphere.

In an embodiment, growth of cells is be controlled by adding additional quantity of NaHCO₃ in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield. pH as one of the critical process parameters for growth of cells is be controlled by proper venting to allow gaseous exchange and increasing the buffering capacity of cell media by adding additional quantity of NaHCO₃ in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield.

STEP e):

In an embodiment, the method of producing the clarified virus pool includes harvesting the infected cell line in the media to obtain a harvest.

In an embodiment, the method of producing the clarified virus pool includes harvesting and optionally re-harvesting the infected cell line one or more times.

In a preferred embodiment, the re-harvesting is performed at least once, or twice, or thrice, or four times, or five times, or six times, seven times, or eight times, or nine times, or ten times.

In a more preferred embodiment, the re-harvesting is performed at least twice. The re-harvesting is associated with getting > 50% virus yield. The increase in yield is about 30 to 40 % more compared to yield obtained by traditional methods.

In an embodiment, the multiple harvesting (re-harvesting or more than one harvesting) leads to > 50% virus yield by adding optimal concentration of sodium bicarbonate in medium at optimal pH ranging from 6 to 8.

OPTIONAL INACTIVATION: (FOR RABIES, ROTA VIRUSES)

In an alternate embodiment, the harvesting is followed by inactivation of the virus.

In a preferred embodiment, the virus used for infection is a polio virus, or a rabies virus. The cell line used is a vero cell line. The harvesting is followed by inactivation of the virus. The

inactivation is performed by chemical (usage of chemicals like β -propiolactone, formaldehyde) or physical (heat/ thermal inactivation) methods.

STEP f)

In an embodiment, the method of producing the clarified virus pool includes adding at least one stabilizer to the harvest.

In an embodiment, stabilizers form an important part of excipients. Consistent physico-chemical properties of the stabilizers help in maintaining the quality attributes of the final product, especially thermostability.

In an embodiment, the at least one stabilizer includes at least one carbohydrate, at least one protein, an amino acid, or a combination thereof.

In an embodiment, stabilizer is selected from the group consisting of atleast one carbohydrate, atleast one amino acid and atleast one hydrolyzed protein.

In accordance with the embodiments, atleast one carbohydrate as stabilizer is selected from a group consisting of, but not limited to, natural carbohydrates, synthetic carbohydrates, polyols, glass transition facilitating agents monosaccharides, disaccharides, trisaccharides, oligosaccharides and their corresponding sugar alcohols, polyhydroxyl compounds such as carbohydrate derivatives and chemically modified carbohydrates, hydroxyethyl starch and sugar copolymers. Both natural and synthetic carbohydrates are suitable for use. Synthetic carbohydrates include, but are not limited to, those which have the glycosidic bond replaced by a thiol or carbon bond. Both D and L forms of the carbohydrates are used. The carbohydrate is non-reducing or reducing. Where a reducing carbohydrate is used, the addition of inhibitors of the Maillard reaction is preferred. Reducing carbohydrates suitable for use in the composition are those known in the art and include, but are not limited to, glucose, sucrose, maltose, lactose, fructose, galactose, mannose, maltulose and lactulose. Non-reducing carbohydrates include, but are not limited to, non-reducing glycosides of polyhydroxyl compounds selected from sugar alcohols and other straight chain polyalcohols. Other useful carbohydrates include raffinose, stachyose, melezitose, dextran, cellibiose, mannobiose and sugar alcohols. The sugar alcohol glycosides are preferably monoglycosides, in particular the compounds obtained by reduction of disaccharides such as lactose, maltose, lactulose and maltulose. Glass forming agent is selected from the group consisting of sucrose, mannitol, trehalose, mannose, raffinose, lactitol,

lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose sorbitol, dextrose, fructose, glycerol, D-sorbitol, and fucose, or a combination thereof.

In an embodiment, the carbohydrate is sorbitol. It is used to protect the infectivity of the virus during the freeze-drying process. Typically, the sorbitol is present at a concentration range of 1-20% (w/v), preferably in the range of 1-10% (w/v), and more preferably in the range of 3-6% (w/v). Yet preferably the D-sorbitol is present at a concentration of 5% (w/v).

In an embodiment, atleast one amino acid as stabilizer is selected from the group, but not limited to, tricine, leucine, iso-leucine, L-histidine, glycine, glutamine, L- arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Histidine, Methionine, Proline, Serine, Threonine, or a combination thereof.

In an embodiment, amino acid as stabilizer is selected from the group consisting of tricine, L-arginine hydrochloride, L-histidine and L-alanine as suitable amino acids individually or in combination. The amino acid includes tricine at a concentration ranging in between 0.1% and 2% weight/volume (w/v), preferably in between 0.1-1%, more preferably in between 0.1-0.5%, most preferably equal to 0.3% (w/v). The amino acid includes L-histidine at a concentration ranging in between 0.1% to 2% (w/v), preferably in between 0.1-1%, more preferably in between 0.1-0.5%, most preferably equal to 0.21% (w/v). The amino acid includes L-alanine at a concentration ranging in between 0.01% and 1% weight/volume, preferably in between 0.05-0.5%, more preferably in between 0.08- 0.2%, most preferably equal to 0.1% (w/v). The amino acid includes L-arginine hydrochloride ranging in between 0.1% and 10% weight/volume, preferably in between 0.1-5%, more preferably in between 0.1-3%, most preferably equal to 1.6% (w/v).

In an embodiment, atleast one hydrolysed protein as stabilizer is selected from a group consisting of gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate and whey protein hydrolysate or protein such as serum albumin.

In another embodiment, hydrolyzed protein is selected from a group consisting of gelatin/ gelatine at a concentration ranging in between 0.1% and 10% weight/volume, preferably in between 0.1-5%, more preferably in between 0.1-3%, most preferably equal to 2.5% (w/v) and lactalbumin hydrolysate at a concentration ranging in between 0.1% and 2% weight/volume

(w/v), preferably in between 0.1-1%, more preferably in between 0.1-0.5%, most preferably equal to 0.35% (w/v) individually or in combination.

As used herein, the term "gelatin" means a sterile non-pyrogenic protein preparation (e.g., fractions) produced by partial acid hydrolysis (type A gelatin) or by partial alkaline hydrolysis (type B gelatin) of animal collagen, most commonly derived from cattle, pig, and fish sources. Gelatin can be obtained in varying molecular weight ranges. Hydrolysed gelatin is a protein-based stabilizer and is effective due to its interaction with viral particles as well as its ability to inhibit surface adsorption. Hydrolyzed gelatin creates and maintains desirable structure/appearance of a lyophilized vaccine cake. Hydrolysis converts high molecular weight gelatin (>100,000 Da) to low molecular weight gelatin (between 2000 and 5000 Da). Low molecular weight gelatin is less likely to stimulate gelatin-specific IgE than high molecular weight gelatin in vaccinated subjects. Recombinant sources of gelatin may also be used.

In an embodiment, the gelatin is from porcine origin procured from Europe (Germany & France).

In an embodiment, the method of preparation of Stabilizer-I i.e. gelatin-sorbitol stabilizer involves partial hydrolysis of gelatin, which can either be achieved by autoclaving at 110 to 120°C for 120 minutes. Preferably hydrolysis is carried out in a mixing vessel, where it would be possible to measure the actual temperature of the product with consistent degree of mixing and uniformness.

In an embodiment, the Stabilizer-I i.e. gelatin-sorbitol is filtered using a depth filter like Cuno filter or 0.2 micron rated membrane filter. The 0.2 micron rated membrane filter provide advantages such as possibility of testing of integrity before and after application.

In an embodiment, the Stabilizer-II comprises of L-Histidine, L- Alanine, Tricine, L-Arginine hydrochloride and Lactalbumin hydrolysate.

In an embodiment, virus harvest and stabilizers (as mentioned in Table 1) are mixed in specific ratio (v/v) as 80: 20: 10 (Harvest: Gelatin-Sorbitol stabilizer (Stabilizer-I): Stabilizer- II) and the samples of stabilized virus are collected and stored below -60°C.

Table 1: Components and their concentrations in virus bulk vaccine

Sr No	Component	Final Concentration in vaccine
1	Virus Harvest	Base

	<u>Gelatin-Sorbitol stabilizer (Stabilizer-I)</u>	
2	Gelatin, Partially hydrolyzed	0.1-5 %
3	D-sorbitol	1-10%
	<u>Stabilizer-II</u>	
4	L-Histidine	0.1-1 %
5	L- Alanine	0.01-1 %
6	Tricine	0.1-1 %
7	L-Arginine hydrochloride	0.1-5 %
8	Lactalbumin hydrolysate	0.1-10 %

STEP g)

In an embodiment, the method of producing the clarified virus pool includes clarifying the harvest to obtain a clarified virus pool (CVP).

In an embodiment, the CVP is obtained by the method of producing the clarified virus pool, wherein the clarified virus pool is obtained after the clarification of the harvest.

FILTERS

In an embodiment, the method of producing the clarified virus pool includes clarifying the harvest using at least one filter.

In an embodiment, the harvest is clarified/ clarification is performed using at least one filter.

In another embodiment, the harvest is clarified/ the clarification is performed using a combination of two or more filters.

In another embodiment, the harvest is clarified/ the clarification is performed using a combination of two or more filters arranged in series.

In another embodiment, the harvest is clarified/ the clarification is performed using a combination of two or more filters, wherein the at least one filters has same or different pore size.

In an embodiment, the at least one filter has pore size in the range of 0.1 μ to 10.0 μ .

In another embodiment, the at least one filter has pore size in the range of 0.1 μ to 9.5 μ , or in the range of 0.1 μ to 9.0 μ , or in the range of 0.1 μ to 8.5 μ , or in the range of 0.1 μ to 8.0 μ .

In another embodiment, the harvest is clarified/ the clarification is performed using a combination of two or more filters, wherein the filters have same pore size.

In another embodiment, the harvest is clarified/ the clarification is performed using at least one filter set. The filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ .

In another embodiment, the harvest is clarified/ the clarification is performed using at least two filter sets.

The first filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ . In a preferred embodiment, the first filter set includes a first filter with pore size in the range of 4 μ to 8 μ and a second filter with pore size 0.1 μ to 0.9 μ .

The second filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ . In a preferred embodiment, the first filter set includes a first filter with pore size in the range of 4 μ to 8 μ and a second filter with pore size 0.1 μ to 0.9 μ .

In an embodiment, the material of construction of one or more filters used for clarification is selected from modified PVDF, cellulose acetate, polypropylene. These filters are validated for the process.

In another embodiment, the clarification step is performed using other techniques such as ultracentrifugation, chromatography, precipitation and nanofiltration.

EXPONENTIAL GROWTH PHASE

In an embodiment, the method of producing the clarified virus pool includes growing the cell line or the infected cell line in exponential growth phase.

In an embodiment, as the cells reach the exponential growth phase, the cells become more metabolically active and each cell produces the maximum amount of carbon dioxide. This excess of carbon dioxide generated by cells increases the dissolved carbon dioxide level and cause decrease in the pH. The addition of sodium bicarbonate along with optimum ventilation or aeration at exponential growth phase helps proper gaseous exchange that facilitates the slow

dissociation of sodium bicarbonate viz a viz reduction of CO₂ and thus maintains the optimum stable pH throughout the process. This leads to no change in the virus native form and avoids the virus inactivation.

VENTILATION/ AERATION

In an embodiment, the method of producing the clarified virus pool includes growing the cell line or the infected cell line in exponential growth phase with additional ventilation or aeration.

In an embodiment, excess CO₂, can inhibit respiration reactions of the cells, and may act as an acid poison by crossing membranes and changing intracellular compartment pH.

In an embodiment, the ventilation/ aeration is provided by growing the cell line or the infected cell line in the containers having vented caps/ vented coverings/ venting caps.

In an embodiment, the ventilation/ aeration is provided by using MLTCF-10 vented with Corning vented caps (instead of milex filter used in routine process). The use of MLTCF-10 vented with Corning vented caps results in the slow dissociation of sodium bicarbonate and maintain the pH towards alkaline side.

In an embodiment, when ventilation/ aeration is provided, the pH is maintained at 6.5 to 8.0 or 7.0 to 8.0 or 7.2 to 7.8. Here the growth of cells is to be controlled by proper venting to allow gaseous exchange which does not cause fluctuation in the pH, increases the cell count and increases the yield.

In an embodiment, the use of MLTCF-10 vented with Corning vented caps provide 40 to 120% more cell yield when compared to MLTCF-10 vented with milex filters.

RE-ADDITION OF BUFFER

In an embodiment, the cell line or the infected cell line in exponential growth phase is grown with re-addition of the buffer.

In an embodiment, the cell line or the infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In a preferred embodiment, the buffer, i.e. NaHCO_3 (sodium bicarbonate) is in used in a batch in concentration range from 0.5 to 4.0 g/L, or 0.8 to 3.5 g/L or 1.0 to 3.0 g/L or 1.0 to 2.5 g/L or 1.0 to 2.3 g/L, or 1.0 to 2.1 g/L, or 1.0 to 2.0 g/L.

In a more preferred embodiment, the buffer, i.e. NaHCO_3 (sodium bicarbonate) is in used in a batch in concentration range from 1.0 to 1.9 g/L, or 1.2 to 1.9 g/L.

In another embodiment, the infected cell line is washed with the virus media and the buffer.

In another embodiment the re-addition of the buffer is performed by boosting with additional buffer, i.e. NaHCO_3 or providing additional amount of the buffer with the cell media or the virus media or both.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 5% to 40% or 5% to 35% or 5% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 10% to 30% or 15% to 30%.

In an embodiment, the boosted buffer concentration for the cell media or the virus media or both during the re-addition of the buffer is in the range of 0.1 g/L to 1.6 g/L or 0.1 g/L to 1.2 g/L or 0.1 g/L to 1.0 g/L or 0.1 g/L to 0.8 g/L or 0.1 g/L to 0.6 g/L.

In an embodiment, the total buffer concentration including the boosted buffer concentration for the cell media or the virus media or both is in the range of 0.6 g/L to 6.0 g/L, or 0.8 g/L to 6.0 g/L, or 1.0 g/L to 6.0 g/L, or 1.2 g/L to 6.0 g/L in the batch.

In another embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.2 g/L to 5.9 g/L, or 1.2 g/L to 5.8 g/L, or 1.2 g/L to 5.7 g/L, or 1.2 g/L to 5.6 g/L in the batch.

In preferred embodiment, the total buffer concentration for the cell media or the virus media or both including the boosted buffer concentration is in the range of 1.5 g/L to 2.0 g/L in the batch.

CLARIFIED VIRUS POOL (CVP)

In an embodiment, the clarified virus pool (CVP) is obtained by the method as disclosed herein.

In an embodiment, the CVP is obtained by the method of producing the clarified virus pool, wherein the clarified virus pool is obtained after the clarification of the harvest.

In an embodiment, the method of producing the clarified virus pool, wherein the clarified virus pool is obtained for a virus including Measles morbillivirus, Mumps orthorubulavirus, Rubivirus rubella, Adeno-associated virus, Aichi virus, lyssavirus, Banna virus, Barmah forest virus, polyomavirus, Bunyamwera virus, herpesvirus, Chandipura virus, Chikungunya virus, Cosavirus, Coxsackievirus, Dengue virus, Eastern chimpanzee simian foamy virus, Eastern equine encephalitis virus, Ebolavirus, Echovirus, Epstein-Barr virus, Hepatitis virus, Hantaan virus, Hendra virus, Horsepox virus, adenovirus, astrovirus, coronavirus, cytomegalovirus, enterovirus, Human immunodeficiency virus, Human metapneumovirus, Human papillomavirus, parainfluenza, parvovirus, respiratory syncytial virus, Human T- lymphotropic virus, Human torovirus, Influenza virus, Japanese encephalitis virus, arenavirus, Marburg virus, Langat virus, Lassa virus, Lymphocytic choriomeningitis virus, Machupo virus, Mayaro virus, Measles virus, Mokola virus, Molluscum contagiosum virus, Monkeypox virus, Mumps virus, New York virus, Nipah virus, Norwalk virus, Orf virus, Oropouche virus, Pichinde virus, Poliovirus, Punta toro phlebovirus, Puumala virus, Rabies virus, Rift valley fever virus, Rosavirus, Ross river virus, Rotavirus, Rubella virus, Sagiyama virus, Salivirus, Toscana virus, Seoul virus, Tick-borne virus, Vaccinia virus, Varicella-zoster virus, Variola virus, Vesicular stomatitis virus, Viento virus, West Nile virus, Yellow fever virus or Zika virus.

In an embodiment, the CVP is obtained by the method of producing the clarified virus pool, wherein the clarified virus pool is of Measles morbillivirus or measles virus.

In an embodiment, the CVP is obtained by the method of producing the clarified virus pool, wherein the clarified virus pool is of Mumps orthorubulavirus or mumps virus.

In an embodiment, the CVP is obtained by the method of producing the clarified virus pool, wherein the clarified virus pool is of Rubivirus rubellae or rubella virus.

MEASLES

In another embodiment, the method for producing a measles clarified virus pool comprises;

- a. providing the cell line in the media, the buffer and the supplement;
- b. treating cells of the cell line with the enzyme;
- c. infecting the cell line with Measles morbillivirus to form a measles infected cell line;

- d. washing of the measles infected cell line with the virus media and the buffer;
 - e. harvesting the measles infected cell line in the media to obtain a harvest; optionally re-harvesting the measles infected cell line;
 - f. adding the stabilizer to the harvest; and
 - g. clarifying the harvest to obtain the measles clarified virus pool (CVP).
- optionally, wherein the cell line or the infected cell line in exponential growth phase is
- grown with additional ventilation or aeration, or
 - provided with re-addition of the buffer or
 - both.

In an embodiment, the cell line used as a substrate for the measles virus growth is Human Lung Fibroblast (MRC-5) cell line.

In an embodiment, the enzyme is recombinant trypsin.

In an embodiment, the cell growth medium used for the measles virus growth is Minimum Essential medium.

In an embodiment, cell growth medium used for the measles virus growth is supported or added with the supplements.

In an embodiment, supplements added with cell growth medium used for the measles virus growth are glutamine, foetal bovine serum and sodium bicarbonate.

In an embodiment, the preparation of cell factories comprises of reviving MRC-5 cells preserved in the liquid nitrogen as working cell bank. While preparing working cell bank, freezing of cells is performed at population doubling level (PDL-19) in a plastic ampoule i.e. plastic disposable sterile, Nunc. Further the revived cells are cultivated in plastic disposable sterile tissue culture flask (Nunc) with the cell growth medium and cells are incubated at $36\pm 1^{\circ}\text{C}$. At each passaging step, the cell growth medium is added into the tissue culture flask through an additional 0.1μ filter (i.e., terminal filtration for each operation). Antibiotic is not added at any stage of cell or virus growth.

In an embodiment, for measles bulk vaccine preparation, the process of preparation of cell factories comprises of reviving MRC-5 cells preserved in the liquid nitrogen as working cell bank. While preparing working cell bank, freezing of cells is performed at population doubling level (PDL-19) in a plastic ampoule i.e. plastic disposable sterile, Nunc. Further the revived

cells are cultivated in plastic disposable sterile tissue culture flask (Nunc) with the cell growth medium and cells are incubated at $36\pm 1^{\circ}\text{C}$. At each passaging step, the cell growth medium is added into the tissue culture flask through an additional 0.1μ filter (i.e., terminal filtration for each operation). Antibiotic is not added at any stage of cell or virus growth.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of measles virus bulk is in the range of 5% to 15%, preferably 8% to 13% and FBS is irradiated with GAMMA irradiation at the dose range of 20 to 50KGy.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of measles virus bulk is 10% to 12.5%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of measles virus bulk is 10%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of measles virus bulk is 12.5%.

In an embodiment, the virus media used in the process of producing a measles clarified virus pool is Minimum Essential Medium with sodium bicarbonate and without glutamine, foetal bovine serum.

In an embodiment, sodium bicarbonate as buffer in virus media is in the range 1 to 2.5 g/L, 1.2 to 2.3 g/L, 1.4 to 2.1 g/L, 1.6 to 2.0 g/L and 1.8 to 2.0 g/L.

In an embodiment, the virus media contains minimum essential medium (MEM) which differs from cell medium in that does not contain foetal bovine serum (FBS). Hence it is used to replace cell medium during washing of roller bottles so as to get rid of foetal bovine serum i.e. heterologous proteins. It also acts as maintenance medium for infected MRC-5 cells in the roller bottles in which the released virus is accumulated during incubation of roller bottles containing MRC-5 cells infected with Measles virus.

In an embodiment, FBS supplemented in the cell growth medium is treated with gamma irradiation.

In an embodiment, the cell culture using Human Lung Fibroblast (MRC-5) cell line being a human diploid cell line require maintenance of pH in the physiological range or little higher

(7.4 to 7.6) is equally important since, at this pH the cells are more healthy and binding sites (CD-46) for virus would be in more conceivable form for infection. Also, the exposure of cells to low pH induces 'Early Contact Inhibition phenomenon' which affects the cell growth and ultimately virus yield.

In an embodiment, the trypsinization is performed after the formation of confluent monolayer of the cells using trypsin solution and transferred to the other tissue culture flask with the cell growth medium.

In an embodiment, the trypsin used for trypsinization step is a mixture of trypsin (0.25%, recombinant) and EDTA (0.005%). Trypsin solution is sterilized by 0.1 μ filtration and similar to growth medium, trypsin is also added to TC flasks through an additional 0.1 μ filter (i.e. terminal filtration for each operation).

In an embodiment, the recombinant trypsin used for trypsinization step is derived from a bacterium *E. coli* (Source: Biogenomics) and from a yeast *Pichia pastoris* (Source: Richcore).

The optimum pH range for growth of MRC-5 cells was found to be 7.4 to 7.8. which cause improvement in trypsinization. Trypsinization is greatly improved in cultures maintained at physiological pH throughout the incubation period. Cells are easily detached with little or no clumps as compared to the cells at acidic pH. The reason being the cells are in the growing phase and optimum pH for trypsin is the range of 7.4 to 7.8.

In an embodiment, the cells grown in TC flasks are passaged further to next bottle by trypsinizing the cells from one flask and divided into 2 flasks. In the earlier doubling (up to PDL-26-27), the confluency is achieved within about 4 days, while it takes about 6-8 days at later cell doublings (PDL-28-32). At PDL-26-27, 100% sampling of cells from flasks is carried out for mycoplasma testing and karyotyping (Identity of cells). The passaging of cells is continued up to PDL-30 with 1:2 split ratio. At PDL-30, the cells from TC flasks are seeded in cell factory (Nunc, 6320 cm²) with 4-6 TC flasks per cell factory. This step offers 2 population doublings in one stroke as against to one in TC flasks. A considerable reduction on handling TC flasks is therefore achieved. Around 80-120 million cells are used to seed one CF and with about 1.8 –2.0 L of MEM supplemented with FBS. After about 48-72 hours or after 2-3 days, one medium change is given and after 72 hours from this change, the cells are used to seed roller bottles or cell cube modules after trypsinization. The last doubling used for cell culture

used for vaccine production is PDL-33. This limit is based on the 2/3rd normal life span of MRC-5 cells, which is confirmed to be at 50 population doublings.

In an embodiment, for production of clarified virus pool of measles i.e. measles bulk vaccine preparation, the use of MLTCF-10 and MLTCF-32 is done before the infection of virus.

In an embodiment, the cells are in suspension. Virus infection is performed by adding working seed virus to each roller bottle at MOI (i.e. virus to cell ratio) of 1:5 to 1:20. The cells with virus in roller bottle are placed on roller apparatus (Bellco, USA/ RBiolab, India) and incubated at 0.4 rpm at $36\pm 1^{\circ}\text{C}$.

In an embodiment, after about 40 to 44 hours of infection, the monolayers of infected cells (in roller bottles) are washed 2 times using virus media (MEM without FBS) to remove traces of FBS and finally seeded with MEM without FBS. The rollers are then further incubated with 0.7 rpm at $32\pm 1^{\circ}\text{C}$.

During production of clarified virus pool of measles i.e. routine measles virus production, post infection, the infected cells are incubated at $36 \pm 1^{\circ}\text{C}$ for 40 to 48 hrs. till washing. At this stage, it has been observed that the pH usually drops below 7.2. This indicates that the buffering capacity of the culture media is not enough to take care of the acid produced by the infected cells and fails to maintain the pH above physiological and therefore, to increase the buffering capacity, the culture media used for Cell Pool preparation should be boosted with additional Sodium Bicarbonate (NaHCO_3). Considering the seeding density per roller (40 – 60 million), MOI (i.e. virus to cell ratio) (1:8 to 1:12) and post infection incubation period (40 to 48 hrs.), CM used for Cell Pool preparation only was added in the range selected from the group of 1 to 2.5 g/L, 1.2 to 2.3 g/L, 1.4 to 2.1 g/L, 1.6 to 2.0 g/L and 1.8 to 2.0 g/L of NaHCO_3 . The increased concentration of NaHCO_3 in CM used for Cell pool preparation boosts the buffering capacity of the medium. pH of the spent medium before washing of the infected culture bottles was evaluated and found to be not below 7.2. It was maintained between 7.4 to 7.6. The buffering capacity of cell media was increased by adding additional quantity of NaHCO_3 in the cell media which cause no fluctuation in the pH, increase the cell count i.e. increase the cell yield or % yield.

Thus, pH is one of the critical process parameters in viral vaccine manufacturing process. Exposure of the virus to extremes of pH inactivates the virus. It has been reported that measles

virus is most stable at pH 7.6 and a progressive inactivation of virus occurs on either side. Physical stability of measles virus is highly compromised in acidic environment. Even slight changes in pH affects the native confirmation of the virus. The infectivity of measles virus is also dependant on the conformational stability of viral proteins. The alkaline medium not merely neutralizes the acid produced by infected cells, but also enhances the virus yield and its stability after release into the medium. Therefore, maintenance of optimum pH throughout the process is inevitable so as to maintain its native confirmation. It has also been reported that the alkaline medium suppresses the production of defective particles.

In another embodiment, the cell line or the measles infected cell line in exponential growth phase is grown with re-addition of the buffer.

In another embodiment, the cell line or the measles infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In an embodiment, the incubation of the measles infected cells is performed at temperature ranging from 30°C to 40°C, which cause impact on the growth of virus. The growth of virus depends on the respective temperature required for the specific virus growth resulting into the high yield of virus.

In an embodiment, antibiotic is not added at any stage of cell growth or virus growth. In an embodiment, antibiotic is added at any stage of cell growth or virus growth.

In an embodiment, cell medium change is performed during the virus bulk vaccine preparation.

In another embodiment, cell medium change is not performed. Cell medium change is not practically possible as it leads to the addition in the cost and also increases number of operations, materials, unnecessary man movement in clean rooms and ultimately chance of contamination. By avoiding medium change to MLTCF-10, additional burden on Clean rooms can be lowered which will add to improvement in cGMP practices.

In an embodiment, the tissue culture flask of Nunc's i.e. MLTCF-10 has two vents (0.2 μ milex filters, 50 mm diameter). The MLTCF-10 were used in the process including cultivation of MRC-5 in cell growth medium which contains MEM Hank's supplemented with 10 % FBS and concentration of sodium bicarbonate of 2.0 g/L. The pH was adjusted to 6.9 to 7.1 by purging CO₂ gas before use. The seeding count was 100 to 160 million cells and incubation temp. and

period were $36 \pm 1^\circ \text{C}$ and 5-7 days respectively. The cell yield obtained was 400 to 500 million cells. The usual pH shifts observed during the incubation period was as high as 7.8 to 8 to as low as 7.0 or below.

The tissue culture flask of Corning's i.e. CS-10 venting caps has Standard 33 mm threaded caps having $0.2 \mu\text{m}$ pore nonwetable membranes sealed directly to the caps. The cultures were initiated from same inoculums. (100 – 120 million cells). During first 24 - 48 hrs, where the lower pH (7.0 to 7.2) is required for attachment, the vents were closed tightly by sterile tape / aluminium foil to prevent gaseous exchange. After 48 hrs, the vents were opened by removing the tape / foil to allow the gaseous exchange. It has been observed that pH remained in the range of 7.4 to 7.6 during rest of the incubation period.

It is observed that in MLTCF-10 with milex filters (routine process), pH dropped below physiological range, whereas in MLTCF-10 having vented caps, pH was maintained in the range of 7.6 to 7.78. MLTCF-10 vented with Corning Vented caps yielded 45 to 114 % more cells than with MLTCF -10 vented with milex filter. Therefore, it was found that the venting caps provides proper gaseous exchange due to which sodium bicarbonate dissociated slowly to maintain the pH towards little alkaline side as against the routinely used vents with milex filters.

In cultures with milex filters where pH dropped below non-physiological range (below 7.1), the less cell growth (count) could not have been caused by simple nutrient depletion, since the mere maintaining pH in the range of 7.6 to 7.8 yielded more cell count when vented caps are used for gaseous exchange. Hence removal of the carbon dioxide physically is not required.

In an embodiment, the adjustment of pH by gaseous exchange and extra addition of NaHCO_3 in cell media is also be applicable for other mammalian cells. (for e.g. Vero cells used for Rota & Rabies vaccines).

The fill volume per vial of final product (vaccine) of 10 dose batches was found to be reduced to 0.5 ml from 1.0 ml. The consumption of Blind Vaccine (diluent used for dilution of bulk during blending) was found to be reduced by approximately 30 %. These factors also contribute to increase in the output of the filling section.

In an embodiment, the rollers are then harvested based on the extent of infection for taking out the virus. It is usually 3 times in first 48 hours after washing, and then once in 24 hours. About

5 to 10 times, preferably 6-7 times the rollers are harvested, and the harvesting is stopped once the number of cells in roller bottles drops to 20-30%.

In an embodiment, during each harvest, grouping of rollers is carried out, each group with 30-35 rollers. Virus from each group is collected in sterile plastic disposable bags and stabilizers are added to it in a proportion as specified.

In an embodiment, the sterile plastic disposable bags are selected from ethylene vinyl acetate (EVA), ultra-low density polyethylene (ULDP) and other biologically acceptable material of construction (MOC).

In an embodiment, harvest and stabilizers are mixed in specific ratio.

In an embodiment, measles virus harvest and stabilizers (as mentioned in Table 2) are mixed in specific ratio (v/v) as 80: 20: 10 (Harvest: Gelatin-Sorbitol (Stabilizer-I): Stabilizer- II) and the samples of stabilized virus are collected and stored below -60°C.

Table 2: Components and their concentrations in measles virus bulk vaccine

Sr No	Component	Final Concentration in vaccine
1	Measles Virus Harvest	Base
	<u>Gelatin-Sorbitol (Stabilizer-I)</u>	
2	Gelatin, Partially hydrolyzed	0.1-5 %
3	D-sorbitol	1-10%
	<u>Stabilizer-II</u>	
4	L-Histidine	0.1-1 %
5	L- Alanine	0.01-1 %
6	Tricine	0.1-1 %
7	L-Arginine hydrochloride	0.1-5 %
8	Lactalbumin hydrolysate	0.1-10 %

In another embodiment, the stabilized measles virus harvest is clarified/ the clarification is performed using at least one filter set. The filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ .

In an embodiment, the stabilized measles virus harvest is then clarified using one of the following series or in combination - first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series and first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series.

In an embodiment, the stabilized measles virus harvest is then clarified using one of the following series or in combination – first filter with 6 μ and second filter with 0.45 μ filters in series and first filter with 6 μ and second filter with 1.2 μ filters in series. The filtered samples are collected in one of the following collectors - sterile disposable 10 L media bags (Stedim or Nalgene bags) as Clarified virus Pools (CVPs).

In an embodiment, the stabilized measles virus harvest is then clarified using first filter with 6 μ and second filter with 0.45 μ filters in series. The filtered samples are collected in one of the following collectors - sterile disposable 10 L media bags (Stedim or Nalgene bags) as Clarified virus Pools (CVPs).

In an embodiment, uninfected control cultures are evaluated using tests comprising of Microscopic examination; Test for presence of mycoplasma; Karyology; Test for presence of haemadsorbing extraneous agents using - Simian cells/Human cells/Human Diploid cells.

In an embodiment, measles virus infected cell line i.e. MRC-5 cells is evaluated using tests comprising of Microscopic examination; Test for presence of mycoplasma; Karyology; Test for presence of haemadsorbing extraneous agents using - Simian cells/Human cells/Human Diploid cells.

In an embodiment, measles virus pool used as harvested virus pool are evaluated using tests comprising of Sterility test, Test for presence of mycoplasma, Test for virus content, Test in cell culture of neutralized virus pool for adventitious agents using- Simian cells/Human cells/Human Diploid cells.

In an embodiment, Clarified Virus Pools (CVPs) of measles virus are evaluated using tests comprising of Sterility test, Identity test, Test for clarification, Test for virus content, Test for presence of mycoplasma and adventitious agents.

In an embodiment, the Clarified Virus Pools in sterile disposable plastic bags are stored in clean cold rooms and maintained at temperature below -20°C. These CVPs are further used to prepare final bulk for the specific virus vaccine just prior to filling and lyophilization.

In an embodiment, the Clarified Virus Pools of measles virus in sterile disposable plastic bags are stored in clean cold rooms and maintained at temperature below -20°C. These CVPs are

further used to prepare final bulk for the specific virus vaccine just prior to filling and lyophilization.

In an embodiment, in the method for producing a measles clarified virus pool as disclosed, mentions the use of optimal concentration of sodium bicarbonate in medium (1.0 to 2.5 g/L) and low MOI (i.e. virus to cell ratio) ranging from 1:5 to 1: 20 which provides the cell yields in the form of % yield or recovery of virus obtained is selected from the group more than 10%, more than 20%, more than 30%, more than 40%, more than 50% and more than 60%. As yield is increased, the doses per lot is increased from 8 to 9 million doses to 13 to 16 million doses. The annual facility requirement for bulk vaccine preparation was found to be changed from two facilities to one facility. The substantial quantity of FBS used with the cell growth media can be saved i.e. 7.0 to 8.0 L of FBS per lot is saved. Annually around 350.0 L FBS is saved.

In an embodiment, during method for producing a measles clarified virus pool which provide measles bulk vaccine, the process for pooling the number of roller bottles during the harvesting stage could be 50 roller bottles or more per group. Typically, in a lot of 500 roller bottles, either 10 groups of 50 rollers can be made or up to 2 groups for 250 roller bottles each can be made. This provides advantages such as less number of aseptic connections, and aliquoting from a well-mixed and homogenized pool as well as less number of sampling.

MUMPS

In an embodiment, the method for producing a mumps clarified virus pool comprises;

- a. providing the cell line in the media, the buffer and the supplement;
- b. treating cells of the cell line with the enzyme;
- c. infecting the cell line with the Mumps orthorubula virus to form a mumps infected cell line;
- d. washing of the mumps infected cell line with the media and the buffer;
- e. harvesting the mumps infected cell line in the media to obtain the harvest; optionally re-harvesting the mumps infected cell line;
- f. adding the stabilizer to the harvest; and
- g. clarifying the harvest to obtain the mumps clarified virus pool (CVP).

optionally, wherein the cell line or the infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or

- both.

In an embodiment, the cell line used as a substrate for the mumps virus growth is Chick embryo fibroblast (CEF) cell line.

In an embodiment, the enzyme is recombinant trypsin.

In an embodiment, the cell growth medium used for the mumps virus growth is Minimum Essential medium.

In an embodiment, cell growth medium used for the mumps virus growth is supported or added with the supplements.

In an embodiment, supplements added with cell growth medium used for the mumps virus growth are glutamine, foetal bovine serum, sodium bicarbonate and neomycin sulphate.

In an embodiment, the preparation of cell factories comprises of initially incubating the specified pathogen free (SPF) chicken eggs at $37\pm 1^{\circ}\text{C}$ for 9-11 days. The incubated eggs are candled on the day of preparation of chick embryo fibroblast (CEF). The live eggs are identified by a healthy blood venation on the inside membrane of eggs and healthy movement of embryo. The non-fertile eggs are identified by absence of any venation while the dead eggs show poor venation and no movement / development of embryos. The live and healthy eggs are selected for preparation of CEF. These eggs are surface disinfected using 70% isopropyl alcohol and taken into aseptic area. Eggs are cut open manually with the help of forceps and embryos are taken out. The head, limbs and viscera of embryos are removed, and the embryos are minced into small pieces. These pieces are initially collected in chilled ($2-8^{\circ}\text{C}$) phosphate buffered saline (PBS). Later, the embryos are washed with sufficient quantity of chilled PBS for removal of RBCs.

In an embodiment, for mumps bulk vaccine preparation, the process of preparation of cell factories comprises of initially incubating the specified pathogen free (SPF) chicken eggs at $37\pm 1^{\circ}\text{C}$ for 9-11 days. The incubated eggs are candled on the day of preparation of chick embryo fibroblast (CEF). The live eggs are identified by a healthy blood venation on the inside membrane of eggs and healthy movement of embryo. The non-fertile eggs are identified by absence of any venation while the dead eggs show poor venation and no movement / development of embryos. The live and healthy eggs are selected for preparation of CEF. These

eggs are surface disinfected using 70% isopropyl alcohol and taken into aseptic area. Eggs are cut open manually with the help of forceps and embryos are taken out. The head, limbs and viscera of embryos are removed, and the embryos are minced into small pieces. These pieces are initially collected in chilled (2-8°C) phosphate buffered saline (PBS). Later, the embryos are washed with sufficient quantity of chilled PBS for removal of RBCs.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of mumps virus bulk is in the range of 5% to 15%, preferably 8% to 13% and FBS is irradiated with GAMMA irradiation at the dose range of 20 to 50KGy.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of mumps virus bulk is 10% to 12.5%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of mumps virus bulk is 10%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of mumps virus bulk is 12.5%.

In an embodiment, the virus media used in the process of producing a mumps clarified virus pool is Minimum Essential Medium with sodium bicarbonate and without glutamine, foetal bovine serum, neomycin sulphate.

In an embodiment, sodium bicarbonate in virus media is in the range 1 to 2.5 g/L, 1.2 to 2.3 g/L, 1.4 to 2.1 g/L, 1.6 to 2.0 g/L and 1.8 to 2.0 g/L.

In an embodiment, the virus medium contains minimum essential medium (MEM) which differs from cell medium in that does not contain foetal bovine serum (FBS) and Neomycin Sulphate. Hence it is used to replace cell medium during washing of cell factories / Cell stacks so as to get rid of foetal bovine serum i.e. heterologous proteins and Neomycin Sulphate. It also acts as maintenance medium for infected CEC in the cell factories / Cell stacks in which the released virus is accumulated during incubation.

In an embodiment, the trypsinization is performed after the washing of the embryos with sufficient quantity of chilled PBS for removal of RBCs. The embryos are suspended in PBS and trypsin solution (both prewarmed in 36°C water bath) in conical trypsinization flasks containing bar magnets. The trypsinization is carried out in 36°C incubator on a magnetic

stirrer for 30 – 40 minutes. After 24 hrs of incubation all the culture vessels i.e production cultures and control cultures are replaced with fresh cell medium (MEM supplement with 5% to 15% FBS and neomycin). The chick embryo fibroblast cells after incubation is observed routinely under microscope for progress of growth.

In another embodiment, the cell line or the mumps infected cell line in exponential growth phase is grown with re-addition of the buffer.

In another embodiment, the cell line or the mumps infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In an embodiment, after 2 to 3 days of incubation of chick embryo fibroblast cells virus infection is performed by adding working seed virus to cells at MOI (i.e. virus to cell ratio) of 1:20 to 1:60. The virus is adsorbed onto the monolayer for 1 hour at $33\pm 1^{\circ}\text{C}$. After the adsorption period, the cell factories are again fed with MEM with 10% FBS and Neomycin at the concentration 50ppm. After infection, the incubation is continued at $33\pm 1^{\circ}\text{C}$.

In an embodiment, after two days or 40 to 50 hours of infection, the medium from all the cell factories is discarded and the monolayer of infected cells (in cell factories) are washed 2 times using MEM without FBS and without neomycin to remove traces of FBS and neomycin. After washing, the cell factories are re-fed using fresh MEM without FBS and neomycin. The cell factories are then further incubated at $33\pm 1^{\circ}\text{C}$.

In an embodiment, the virus accumulated in cell factories is harvested based on the extent of infection for taking out the virus. Every time after harvesting, the cell factories are again seeded using fresh MEM without FBS. The incubation of cell factories is continued at $33\pm 1^{\circ}\text{C}$. A total of about 5 to 10 times, preferably 6 times harvests are collected with a frequency of one harvest per day.

In an embodiment, during each harvest, virus from each group is collected in sterile plastic disposable bags and stabilizers are added to it in a proportion as specified.

In an embodiment, the sterile plastic disposable bags are selected from ethylene vinyl acetate (EVA), ultra-low density polyethylene (ULDPE) and other biologically acceptable material of construction (MOC).

In an embodiment, mumps virus harvest and stabilizers (as mentioned in Table 3) are mixed in specific ratio (v/v) as 80: 20: 10 (Harvest: Gelatin-Sorbitol (Stabilizer-I): Stabilizer- II) and the samples of stabilized virus are collected and stored below -60°C.

Table 3: Components and their concentrations in Mumps virus bulk vaccine

Sr No	Component	Final Concentration in vaccine
1	Mumps Virus Harvest	Base
	<u>Gelatin-Sorbitol (Stabilizer-I)</u>	
2	Gelatin, Partially hydrolyzed	0.1-5 %
3	D-sorbitol	1-10%
	<u>Stabilizer-II</u>	
4	L-Histidine	0.1-1 %
5	L- Alanine	0.01-1 %
6	Tricine	0.1-1 %
7	L-Arginine hydrochloride	0.1-5 %
8	Lactalbumin hydrolysate	0.1-10 %

In another embodiment, the stabilized mumps virus harvest is clarified/ the clarification is performed using at least one filter set. The filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ .

In an embodiment, the stabilized mumps virus harvest is then clarified using one of the following series or in combination - first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series and first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series.

In an embodiment, the stabilized mumps virus is then clarified using one of the following series or in combination - first filter with 6 μ and second filter with 0.45 μ filters in series and first filter with 6 μ and second filter with 1.2 μ filters in series. The filtered samples are collected and stored in sterile plastic disposable bags as Clarified Virus Pools (CVPs).

In an embodiment, the stabilized mumps virus harvest is then clarified using first filter with 6 μ and second filter with 1.2 μ filters in series. The filtered samples are collected and stored in sterile plastic disposable bags as Clarified Virus Pools (CVPs).

In an embodiment, uninfected control cultures for producing clarified virus pool of mumps virus is evaluated using tests comprising of Microscopic examination; Test for presence of mycoplasma; Karyology; Test for presence of haemadsorbing extraneous agents using - Simian cells/Human cells/Human Diploid cells.

In an embodiment, harvested mumps virus pool or mumps virus pool is evaluated using tests comprising of Sterility test, Test for presence of mycoplasma, Test for virus content, Test in cell culture of neutralized virus pool for adventitious agents using- Simian cells/Human cells/Human Diploid cells.

In an embodiment, Clarified Virus Pools (CVPs) of mumps virus is evaluated using tests comprising of Sterility test, Identity test, Test for clarification, Test for virus content, Test for presence of mycoplasma and adventitious agents.

In an embodiment, the Clarified Virus Pools of mumps virus in sterile disposable plastic bags are stored in clean cold rooms and maintained at temperature below -20°C. These CVPs are further used to prepare final bulk for the specific virus vaccine just prior to filling and lyophilization.

In an embodiment, in the method for producing a mumps clarified virus pool as disclosed, which provides mumps bulk vaccine, the process of incubation of SPF eggs is carried out in egg incubator with a capacity of 1400 eggs or a scaled-up version of up to 2400 eggs, the later providing an advantage of improvised production capacity.

In an embodiment, the method for producing a scale up of mumps clarified virus pool which provides mumps bulk vaccine is carried out using CF10 or CS10 and CF40 or CS40. All are sterile, disposable, gamma-radiated containers for the growth of cells and viruses, the later providing almost four times the surface area for cultivation in almost same footprint. This improvised scale up method was established without impacting the productivity and quality attributes of the product through a process validation path.

RUBELLA

In an embodiment, the method for producing a rubella clarified virus pool comprises;

- a. providing the cell line in the media, the buffer and supplement;
- b. treating cells of the cell line with enzyme;
- c. infecting the cell line with a Rubivirus rubellae virus to form a rubella infected cell line;
- d. washing of the rubella infected cell line with the media and the buffer;
- e. harvesting the rubella infected cell line in the media to obtain the harvest; optionally re-harvesting the rubella infected cell line;
- f. adding the stabilizer to the harvest; and

g. clarifying the harvest to obtain the rubella clarified virus pool (CVP).

optionally, wherein the cell line or the rubella infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or both.

In an embodiment, the cell line used as a substrate for the rubella virus growth is Human Lung Fibroblast (MRC-5) cell line.

In an embodiment, the enzyme is recombinant trypsin.

In an embodiment, the cell growth medium used for the rubella virus growth is Minimum Essential medium.

In an embodiment, cell growth medium used for the rubella virus growth is supported or added with the supplements.

In an embodiment, supplements added with cell growth medium used for the rubella virus growth are glutamine, foetal bovine serum, sodium bicarbonate, dextrose and neomycin sulphate.

In an embodiment, the preparation of cell factories comprises of reviving MRC-5 cells preserved in the liquid nitrogen as working cell bank. Freezing of cells is performed at population doubling level (PDL-19) in a plastic ampoule i.e. plastic disposable sterile, Nunc. Further the revived cells are cultivated in plastic disposable sterile tissue culture flask (Nunc) with the cell growth medium and cells are incubated at $36\pm 1^{\circ}\text{C}$. At each passaging step, the cell growth medium is added into the tissue culture flask through an additional 0.1μ filter (i.e., terminal filtration for each operation). The cell growth medium is supplemented with antibiotic which is later on washed off during washing stage.

In an embodiment, for rubella bulk vaccine preparation, the process of preparation of cell factories comprises of reviving MRC-5 cells preserved in the liquid nitrogen as working cell bank. While preparing working cell bank, freezing of cells is performed at population doubling level (PDL-19) in a plastic ampoule i.e. plastic disposable sterile, Nunc. Further the revived cells are cultivated in plastic disposable sterile tissue culture flask (Nunc) with the cell growth

medium and cells are incubated at $36\pm 1^{\circ}\text{C}$. At each passaging step, the cell growth medium is added into the tissue culture flask through an additional 0.1μ filter (i.e., terminal filtration for each operation). The cell growth medium is supplemented with antibiotic which is later on washed off during washing stage.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of rubella virus bulk is in the range of 5% to 15%, preferable 8% to 13% and FBS is irradiated with GAMMA irradiation at the dose range of 20 to 50KGy.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of rubella virus bulk is 10% to 12.5%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of rubella virus bulk is 10%.

In an embodiment, the Foetal bovine serum (FBS) added with cell growth medium for preparation of rubella virus bulk is 12.5%.

In an embodiment, the virus media is Minimum Essential Medium with sodium bicarbonate, dextrose, glutamine and without foetal bovine serum, neomycin sulphate.

In an embodiment, sodium bicarbonate in virus media is in the range 1 to 2.5 g/L, 1.2 to 2.3 g/L, 1.4 to 2.1 g/L, 1.6 to 2.0 g/L and 1.8 to 2.0 g/L.

In an embodiment, the virus medium contains minimum essential medium (MEM) which differs from cell medium in that does not contain foetal bovine serum (FBS). Hence it is used to replace cell medium during washing of roller bottles so as to get rid of foetal bovine serum i.e. heterologous proteins. It also acts as maintenance medium for infected MRC-5 cells in the cell cube system in which the released virus is accumulated during incubation of MRC-5 cells infected with Rubella virus.

In an embodiment, the trypsinization is performed after the formation of confluent monolayer of the cells using trypsin solution and transferred to the other tissue culture flask with the cell growth medium.

In an embodiment, the trypsin used for trypsinization step is a mixture of trypsin (0.25%, recombinant) and EDTA (0.005%). Trypsin solution is sterilized by 0.1μ filtration and similar

to growth medium, trypsin is also added to TC flasks through an additional 0.1 μ filter (i.e. terminal filtration for each operation).

In an embodiment, the Cell Cube system is used for rubella bulk vaccine preparation. This comprises of Cell Cube modules (sterile, plastic disposable, 85,000 cm²), an oxygenator bottle and a circulation pump. of cell cube system). The medium in Cell Cube system is continuously circulated (except after the seeding of cells) through the Cell Cube and oxygenator bottles through circulation pump. The oxygenator bottle is provided with compressed air, oxygen and carbon dioxide gases. This helps to maintain desired pH and dissolved oxygen content of the medium at the set value. Each Cell Cube module is an 85000 cm², sterile, disposable container, having 100 parallel polystyrene plates inside fused together for easy and uniform ingress, distribution and egress of the circulating medium. Four numbers of such modules are integrated in every production lot.

In another embodiment, the cell line or the rubella infected cell line in exponential growth phase is grown with re-addition of the buffer.

In another embodiment, the cell line or the rubella infected cell line in exponential growth phase is grown with additional ventilation or aeration and re-addition of the buffer.

In an embodiment, after 7-8 days of incubation, the medium from all the Cell Cube system is discarded/removed and virus infection is performed by adding working seed virus to the monolayer of cells at MOI (i.e. virus to cell ratio) of 1:5 to 1:20. The virus is adsorbed onto the monolayer for 2 hours at 31 $\pm$ 1°C. After the adsorption period, media re-circulation in the Cell Cube system is started and the incubation is continued at 31 $\pm$ 1°C.

In an embodiment, after two days of incubation virus infected cells, the monolayer of infected cells is washed with MEM without FBS to remove the traces of FBS and neomycin. After washing, the Cell Cube system is finally seeded with MEM without FBS. The Cell Cube system is then further incubated at 31 $\pm$ 1°C.

In an embodiment, the virus accumulated in Cell Cube system is harvested based on the extent of infection for taking out the virus and every time after harvesting, the Cell Cube system is again seeded with MEM without FBS. The incubation of the Cell Cube system is continued at 31 $\pm$ 1°C. A total of around 5 to 10 times, preferably 8-9 times harvests are collected in a span of 8-10 days.

In an embodiment, during each harvest, the virus from entire Cell Cube system (including oxygenator) is collected in 50 L pre-sterilized polypropylene bottles or 50 L media bag and stabilizers are added to it in a proportion as specified.

In an embodiment, rubella virus harvest and stabilizers (as mentioned in Table 4) are mixed in specific ratio (v/v) as 80: 20: 10 (Harvest: Gelatin-Sorbitol (Stabilizer-I): Stabilizer- II) and the samples of stabilized virus are collected and stored below -60°C.

Table 4: Components and their concentrations in rubella virus bulk vaccine

Sr No	Component	Final Concentration in vaccine
1	Rubella Virus Harvest	Base
	<u>Gelatin-Sorbitol (Stabilizer-I)</u>	
2	Gelatin, Partially hydrolyzed	0.1-5 %
3	D-sorbitol	1-10%
	<u>Stabilizer-II</u>	
4	L-Histidine	0.1-1 %
5	L- Alanine	0.01-1 %
6	Tricine	0.1-1 %
7	L-Arginine hydrochloride	0.1-5 %
8	Lactalbumin hydrolysate	0.1-10 %

In another embodiment, the stabilized rubella virus harvest is clarified/ the clarification is performed using at least one filter set. The filter set includes a combination two filters, wherein a first filter has pore size in the range of 3.1 μ to 10.0 μ and a second filter has pore size in the range of 0.1 μ to 3.0 μ .

In an embodiment, the stabilized rubella virus harvest is then clarified using one of the following series or in combination – first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series and first filter with 5 μ to 7 μ and second filter with 0.1 μ to 2 μ filters in series.

In an embodiment, the clarification of stabilized rubella virus harvest of rubella is carried out using a first filter of 5 μ or 6 μ with a second filter of 0.45 μ or 0.2 μ to obtain Clarified Virus Pools (CVPs). Final filtration using filter of 0.45 μ or 0.2 μ provide better sterility assurance.

In an embodiment, the clarification of stabilized rubella virus harvest of rubella is preferably carried out using a first filter of 6 μ with a second filter of 0.45 μ to obtain Clarified Virus Pools (CVPs). Final filtration using filter of 0.45 μ provide better sterility assurance.

In an embodiment, uninfected control cultures used for producing clarified virus pool of rubella virus are evaluated using tests comprising of Microscopic examination; Test for presence of mycoplasma; Karyology; Test for presence of haemadsorbing extraneous agents using - Simian cells/Human cells/Human Diploid cells.

In an embodiment, harvested rubella virus pool or rubella virus pool is evaluated using tests comprising of Sterility test, Test for presence of mycoplasma, Test for virus content, Test in cell culture of neutralized virus pool for adventitious agents using- Simian cells/Human cells/Human Diploid cells.

In an embodiment, Clarified Virus Pools (CVPs) of rubella virus are evaluated using tests comprising of Sterility test, Identity test, Test for clarification, Test for virus content, Test for presence of mycoplasma and adventitious agents.

In an embodiment, the Clarified Virus Pools of rubella virus in sterile disposable plastic bags are stored in clean cold rooms and maintained at temperature below -20°C. These CVPs are further used to prepare final bulk for the specific virus vaccine just prior to filling and lyophilization.

In an embodiment, the method for producing a rubella clarified virus pool which provide Rubella virus bulk vaccine is performed using roller bottles, cell factories or Cell Cube modules, which are all single use, disposable gamma-radiated containers. The Cell Cube provide advantages in terms of high surface area to volume ratio and hence improved yields within a near-close system that is amenable to principles of PAT. The cell cube is a parallel plate bioreactor-based process which could be scaled up by integrating 04 Cell Cube modules of 85000 cm² each to provide a total cultivation area of 3,40,000 cm² in small volume of up to 40 L, thus achieving concentration of virus during the virus harvesting stage.

In an embodiment, the method for producing a rubella clarified virus pool, the clarification of stabilized virus harvest of rubella is carried out using filter of 0.45 µ or 0.2 µ and provide better sterility assurance.

METHOD OF OBTAINING LYOPHILIZED/ FREEZE-DRIED MMR IMMUNOGENIC COMPOSITION:

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises one or more CVP selected from a measles CVP, a mumps CVP, a rubella CVP, or a combination thereof, blending of CVP, followed by lyophilizing the blended CVP.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises the blending step which is carried out when a combination of CVPs is present in the immunogenic composition.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles immunogenic composition comprises blending of measles CVP with diluent and subjecting the blended solution to the lyophilization process.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Mumps immunogenic composition comprises blending of mumps CVP with diluent and subjecting the blended solution to the lyophilization process.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Rubella immunogenic composition comprises blending of rubella CVP with diluent and subjecting the blended solution to the lyophilization process.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises blending of one or more than one bulk virus i.e. CVP with diluent and subjecting the blended solution to the lyophilization process.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises blending of measles CVP, a mumps CVP and a rubella CVP with diluent and subjecting the mixture to the lyophilization process.

In an embodiment, a method of obtaining lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises blending of measles CVP, a mumps CVP and a rubella CVP with diluent and optionally subjecting the mixture to the lyophilization process.

In an embodiment, the method of obtaining a lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises the following steps;

- a) CVPs of measles, mumps and rubella virus are kept for thawing at 30 to 35°C in incubator and mixing of the multiple components present in the CVPs and blind vaccine is done obtain a blended solution as homogenous bulk of the final product;
- b) the homogenous bulk of the final product obtained is clarified through a 0.45µ filter;
- c) the homogenous bulk of the final product is aseptically filled into sterilized vials and transferred to trays.
- d) trays are transferred to the Lyophilizer for lyophilization / freeze drying process.
- e) Freeze drying the homogenous bulk of the final product containing in the vials obtained in step d) comprising the steps of freezing, sublimation and secondary drying.

In an embodiment, the method of obtaining a lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition comprises the following steps;

- a) Thawing CVPs of measles, mumps and rubella virus at 30 to 35°C;
- b) blending the thawed measles, mumps and rubella virus CVPs and blind vaccine to obtain a blended solution;
- c) clarifying the blended solution through a 0.45µ filter to obtain a homogenous bulk;
- d) aseptically filling the homogenous bulk into sterilized vials, followed by transferring the vials to a lyophilizer/ freeze dryer.
- e) lyophilizing/ freeze drying the vials containing the homogenous bulk, wherein lyophilizing freeze-drying comprises freezing, primary drying/ sublimation and secondary drying.

In an embodiment, CVPs of measles, mumps and rubella virus are taken out from -20°C storage and kept for thawing at 30 to 35°C incubator to mix the multiple components present in the CVPs and blind vaccine. Blind vaccine comprises of diluent used for dilution of bulk during blending. CVPs and blind vaccine are mixed to prepare a homogenous bulk of the final product which is clarified through a 0.45µ filter. The blending of above components is performed in a stainless steel tank fitted magnetic stirrer and a jacket in which water is circulated at 33°C. The blended solution is aseptically filled into sterilized vials on a filling machine, a sterilized lyophilization stopper is partially inserted into the neck of the vial and the vials are transferred to trays. These trays are transferred to the Lyophilizer for lyophilization / freeze drying process.

In an embodiment, the method of lyophilizing/ freeze-drying comprises;

- a) pre-freezing shelf, loading the trays containing vials of the immunogenic composition on the shelf;
- b) freezing;
- c) primary drying/ sublimation and
- d) secondary drying

In an embodiment, the method of obtaining a lyophilizing/ freeze-drying step comprises;

- e) pre-freezing shelf comprises freezing at -35°C to -60°C .
- f) freezing step comprising freezing at -50°C to -60°C for 450 minutes to 650 minutes;
- g) primary drying/ sublimation step comprising ramping at $+0.5^{\circ}\text{C}/\text{minute}$ to $1.0^{\circ}\text{C}/\text{minute}$ at temperature of -30°C to 30°C , for 1200 minutes to 1700 minutes at 80 to 120 μbar ; and
- h) secondary drying step comprising ramping at $+0.5^{\circ}\text{C}/\text{minute}$ to $1.0^{\circ}\text{C}/\text{minute}$ to achieve a shelf temperature of 20 to 30°C , holding for 300 minutes to 500 minutes at 20 to 35 μbar .

In an embodiment, lyophilization / freeze drying process comprises the steps of freezing, sublimation and secondary drying. The vial trays are loaded on a pre-frozen shelf of -40°C . The shelf is taken down to -50°C and held till product reaches -40°C , Condenser is cooled below -60°C . After a specific period of Pre-freezing, sublimation process is initiated by applying vacuum and starting a controlled heating cycle. After sublimation, the product undergoes secondary drying at 23°C and vacuum levels of 25 to 30 μbar for 7 hours. At the end of secondary drying, the product is stoppered at approx. 25 micro bar abs. vacuum. After stoppering, the vacuum is broken using 0.2-micron filtered air, the product is unloaded for Aluminum cap sealing. The Lyophilization cycle has consistently yielded least loss of Lyophilization, constant thermo-stability value, consistent residual moisture and solubility characteristic.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises atleast one viruses; stabilizer comprising atleast one carbohydrate, atleast one amino acid and atleast one hydrolyzed protein.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises; live attenuated measles virus presents at a dose of not less than 1000 CCID₅₀ per dose (0.5 mL),

live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose (0.5 mL) and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose (0.5 mL).

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises at least one carbohydrate selected from a group consisting of natural carbohydrate, synthetic carbohydrate, monosaccharides, disaccharides, trisaccharides, oligosaccharides, reducing sugar, non-reducing sugar, sugar alcohols, polyol, polyhydroxyl compounds, chemically modified carbohydrates and glass transition facilitating agents which include sucrose, mannitol, trehalose, mannose, raffinose, lactitol, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose sorbitol, dextrose, fructose, glycerol, sorbitol, and fucose and a combination thereof; at least one carbohydrate at concentration range of 1-20% (w/v); at least one of the carbohydrate is present at a concentration of 1 to 20% (w/v), 1 to 10% (w/v), preferably 3-6% (w/v);

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises at least one amino acid selected from a group consisting of tricine, leucine, iso-leucine, L-histidine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Histidine, Methionine, Proline, Serine, Threonine and a combination thereof; at least one amino acid at concentration range of 0.01-10% (w/v); at least one of the amino acid selected from a group consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v).

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises at least one hydrolyzed protein obtained by chemical, enzymatic or thermal hydrolysis of protein from either plant or animal sources.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises at least one hydrolyzed protein selected from a group consisting of gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate and whey protein hydrolysate; at least one hydrolyzed protein at concentration range of 0.1-10% (w/v); at least one of the hydrolysed protein selected from a group consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of Measles, Mumps, Rubella antigen with the excipients mentioned in Table 5;

Table 5: Components and their concentration ranges in live attenuated lyophilized/ freeze-dried MMR immunogenic composition (Measles, Mumps, Rubella (MMR) vaccine)

Component	Quantity
Measles virus	Not less than 1000 CCID ₅₀ /dose
Mumps virus	Not less than 5000 CCID ₅₀ /dose
Rubella virus	Not less than 1000 CCID ₅₀ /dose
Gelatin (Partially hydrolysed)	0.1-5%
D-sorbitol	1-10%
L-Histidine	0.1-1%
L-Alanine	0.01-1%
Tricine	0.1-1%
Arginine	0.1-5%
Lactalbumin Hydrolysate	0.1-10%
Minimum Essential Medium (MEM)	Base

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of an adjuvant selected from a group consisting of aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate or a mixture thereof.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of an immunostimulatory component selected from a group consisting of an oil and water emulsion, MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, 3-deacylated monophosphoryl lipid A, AS01, AS03, an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, CRL-8300 adjuvant, muramyl dipeptide, TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, QS-21, ISCOMS, Chitosan, saponin combination with sterols and lipids.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of a pharmaceutically acceptable additive selected from a group consisting of transporter, excipient, binder, carrier, isotonic agent, emulsifier and humectant.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of excipient selected from a group consisting of salt including NaCl, KCl, KH₂PO₄, Na₂HPO₄.2H₂O, CaCl₂, and MgCl₂; non-ionic surfactant including polysorbate 20, polysorbate

40, polysorbate 60, polysorbate 65, polysorbate 80, polysorbate 85, nonylphenoxypolyethoxethanol, octylphenoxypolyethoxethanol, oxtoxynol 40, nonoxynol- 9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene- 660 hydroxystearate, polyoxyethylene- 35 ricinoleate, soy lecithin and a poloxamer - 0.001%-0.05%; polymers including dextran, carboxymethylcellulose, hyaluronic acid and cyclodextrin.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition is reconstituted with an aqueous solution selected from a group consisting of saline, buffer and WFI (water for injection).

In an embodiment, the buffering agent in the lyophilized/ freeze-dried MMR immunogenic composition is selected from a group consisting of HEPES, Citrate-phosphate, carbonate, phosphate, citrate, lactate, gluconate, borate, histidine buffer, succinate buffer and tartrate buffering agents, as well as more complex organic buffering agents including a phosphate buffering agent that contains sodium phosphate and/or potassium phosphate in a ratio selected to achieve the desired pH. As an amino acid, histidine act as a free radical scavenger, and also stabilize viral proteins via non-covalent histidine–protein interactions in the solid state.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises of buffer selected from a group consisting of sodium chloride, acetate, carbonate, citrate, lactate, gluconate, tartrate, phosphate buffer saline, borate, histidine buffer, succinate buffer, HEPES, TRIS and Citrate-phosphate.

In an embodiment, the dose of lyophilized/ freeze-dried MMR immunogenic composition is 0.5 mL.

In an embodiment, the final pH of the reconstituted lyophilized/ freeze-dried MMR immunogenic composition is in the range of pH 6.5 to 7.5.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition is in the form of a single dose composition and is free of preservative.

In another embodiment, the lyophilized/ freeze-dried MMR immunogenic composition is in the form of a the multi-dose composition and the multi-dose composition additionally comprise preservative selected from the group comprising of 2-phenoxyethanol, Benzethonium chloride (Phemerol), Phenol, m- cresol, Thiomersal, Formaldehyde, paraben esters (e.g. methyl-, ethyl-,

propyl- or butyl- paraben), benzalkonium chloride, benzyl alcohol, chlorobutanol, p-chlor-m-cresol, or benzyl alcohol or a combination thereof. The lyophilized/freeze-dried virus vaccine composition include material for a single immunization or include material for multiple immunizations (i.e. a 'multidose' kit). The inclusion of a preservative is preferred in multidose arrangements. As an alternative (or in addition) to including a preservative in multidose compositions, the compositions contained in a container having an aseptic adaptor for removal of material.

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises:

- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
- b) stabilizer comprising;
 - carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).

In an embodiment, the lyophilized/ freeze-dried MMR immunogenic composition comprises:

- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
- b) stabilizer comprising
 - carbohydrate consisting of sorbitol present at a concentration of 5% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.3% (w/v), L-histidine present at a concentration of 0.21% (w/v), L-alanine present at a concentration of 0.1% (w/v) and L-arginine hydrochloride present at a concentration of 1.6% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.35% (w/v).

In an embodiment, final bulk of live attenuated lyophilized/ freeze-dried MMR immunogenic composition is evaluated using tests comprising of Sterility test, Test for presence of mycoplasma, Test for virus content and Test for residual animal serum.

In an embodiment, final product of live attenuated lyophilized/ freeze-dried MMR immunogenic composition is evaluated using tests comprising of Sterility test, Test for presence of mycoplasma, Identity test, General safety test, Moisture content, Test for virus content (Potency), Thermostability (Accelerated temperature Test).

In an embodiment, methods for storage and utilization of various intermediate media and reagents is improvised, by increasing reliance on single use disposable and gamma radiation sterilized containers is accomplished, with advantages such as decreased load on validated cleaning procedures and autoclave sterilization. The examples being use of media bags of 20 L and 50 L capacities for collection and storage of cell medium and virus medium to be used for the preparation of drug substances and blind vaccine. Thus, the number of glass bottles and stainlesssteel tanks used in media preparation has been significantly reduced and replaced by bags of USP class VI plastic, without impacting the product quality attributes.

In an embodiment, the live attenuated lyophilized/ freeze-dried MMR immunogenic composition comprising of Measles, Mumps, Rubella antigen is found to be stable at -20°C, 2°C to 8°C, 25°C and 37°C for different time intervals/periods.

In an embodiment, the live attenuated lyophilized/ freeze-dried MMR immunogenic composition is administered an immunologically effective amount of the vaccine composition to a human subject via parenteral (subcutaneous or intradermal or intramuscular or intraperitoneal or intravenous administration or injectable administration or pulmonary administration, suppositories, needle-less injection, transcutaneous) or sustained release from implants or administration by eye drops or Mucosal (oral, intranasal, pulmonary, rectal or vaginal) or buccal or peroral or intragastric or perlingual, alveolar or gingival or olfactory or respiratory mucosa administration or interthecally, intralymphatically, via bladder instillation, or via scarification or any other routes of immunization.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: at least one virus (antigen) selected from a group consisting measles virus, mumps virus and rubella virus; other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: at least two virus (antigen) selected from a group consisting measles virus, mumps virus and rubella virus; other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: at least three viruses (antigens) selected from a group consisting measles virus, mumps virus and rubella virus; other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried Measles (M) immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: measles virus (antigen); other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried Measles, Rubella (MR) immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: measles and rubella viruses (antigens); other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, the final product of live attenuated lyophilized/ freeze-dried Measles, Mumps, Rubella (MMR) immunogenic composition is kept in a vaccine kit, wherein the kit comprises of:

- a) a first container containing a lyophilized/freeze-dried virus vaccine composition/formulation comprising: measles, mumps and rubella viruses (antigens); other excipients; and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) virus vaccine composition.

In an embodiment, a kit comprising the lyophilized/freeze-dried MMR immunogenic composition comprises;

- a) a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v); amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v); and

- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.

In another embodiment, a kit comprising the lyophilized/freeze-dried MMR immunogenic composition comprises;

- a) a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration 5% (w/v); amino acid consisting of tricine present at a concentration 0.3% (w/v), L-histidine present at a concentration 0.21% (w/v), L-alanine present at a concentration 0.1% (w/v) and L-arginine hydrochloride present at a concentration 1.6% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration 0.35% (w/v); and
- b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.

According to some embodiments of the present disclosure, the live attenuated lyophilized/freeze-dried MMR vaccine composition/formulation is in the form of a single dose composition and is free of preservative.

In some embodiments of the present disclosure, the live attenuated lyophilized/ freeze-dried MMR vaccine composition/formulation is in form of a multi-dose composition.

In some embodiments of the present disclosure, the live attenuated lyophilized/ freeze-dried MMR vaccine composition is formulated for administration to a human subject or children 2 years of age or below.

In an embodiment of the present disclosure, the live attenuated lyophilized/ freeze-dried MMR vaccine composition is safe, immunogenic and induces persistence of protection.

Throughout this specification the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps and can mean “includes”, “including”, and the like; “consisting essentially of” or “consists essentially” likewise is open-ended, allowing for the presence of more than that which is recited so long as basic or novel characteristics of that which is recited is not changed by the presence of more than that which is recited, but excludes prior art embodiments.

The use of the expression “one or more” or “at least one” suggests the use of one or more elements or ingredients or quantities, as the use is in the embodiment of the invention to achieve one or more of the desired objects or results. While certain embodiments of the inventions have been described, these embodiments have been presented by way of example only and are not intended to limit the scope of the inventions. Variations or modifications to the composition of this invention within the scope of the invention, may occur to those skilled in the art upon reviewing the disclosure herein. Such variations or modifications are well within the spirit of this disclosure.

The numerical values given for various physical parameters, dimensions and quantities are only approximate values and it is envisaged that the values higher than the numerical value assigned to the physical parameters, dimensions and quantities fall within the scope of the invention unless there is a statement in the specification to the contrary.

While considerable emphasis has been placed herein on the specific features of the preferred embodiment, it will be appreciated that many additional features can be added and that many changes can be made in the preferred embodiment without departing from the principles of the disclosure. These and other changes in the preferred embodiment of the disclosure will be apparent to those skilled in the art from the disclosure herein, whereby it is to be distinctly understood that the foregoing descriptive matter is to be interpreted merely as illustration of the disclosure and not as a limitation.

TECHNICAL ADVANTAGES

A Platform method/ manufacturing process of viral vaccines related to a lyophilized/freeze-dried live attenuated virus vaccine composition/formulation Monovalent, Bivalent or Trivalent

viral vaccine comprising of Measles, Mumps and Rubella antigens/immunogens of the present disclosure described herein above has several technical advantages including, but not limited to, the realization of:

1. Improved large scale affordable /safe manufacturing processes (encompassing cultivation, purification & formulation stages).
2. Utilizes minimum animal origin components, provides high virus yield & ensures virus structure integrity/stability preservation across manufacturing & storage.
3. pH as one of the critical process parameters for growth of cells is controlled by proper venting to allow gaseous exchange and increasing the buffering capacity of cell media by adding additional quantity of NaHCO_3 in the cell media which cause no fluctuation in the pH, increase the cell count and increase the yield.
4. Use of MLTCF-10 vented with Corning vented caps (instead of millex filter) resulting in slow dissociation of sodium bicarbonate.
5. Measles bulk vaccine yield is increased i.e. > 50 % approximately (increase of yield per lot from 8 to 9 million doses to 13 to 16 million doses) due to optimal pH (7.2 to 7.8); optimal concentration of sodium bicarbonate in medium (1.0 to 2.5); Low MOI (i.e. virus to cell ratio) (for measles and rubella bulk vaccine manufacturing – 1:5 to 1:20 and for mumps bulk vaccine manufacturing – 1:20 to 1:60); multiple harvesting. The increased yield of measles bulk vaccine resulted in change of annual requirement of measles bulk vaccine from two facilities to one facility.
6. During measles bulk vaccine preparation, the process for pooling the number of roller bottles during the harvesting stage by making 2 groups for 250 roller bottles each provide advantages such as less number of aseptic connections, and aliquoting from a well-mixed and homogenized pool as well as less number of sampling.
7. The Cell Cube used for Rubella bulk vaccine preparation provide advantages in terms of high surface area to volume ratio and hence improved yields within a near-close system that is amenable to principles of PAT.
8. The use of 0.2 μ filter for filtration of stabilized virus during Rubella bulk vaccine preparation provide better sterility assurance.
9. During mumps bulk vaccine preparation, the incubation of SPF eggs in egg incubator with a capacity of scaled up version of up to 2400 eggs provide improvised production capacity.

10. Scaling up of mumps bulk vaccine production using MLTCF10 or CS10 and CF40 or CS40 provide four times the surface area for cultivation in almost same footprint.
11. Use of Recombinant Trypsin (at least 95% pure, having molecular weight 23 to 25 kilodalton; specific activity- 2500 USP units/mg; low endotoxin content) at optimal pH (7.2 to 7.6).
12. Use of Gamma irradiated FBS.
13. Use of optimum virus: Stabilizer-I: Stabilizer-II ratio (80:20:10) ratio.
14. The optimum pH range for growth of MRC-5 cells is 7.4 to 7.8. which cause improvement in trypsinization. Trypsinization is greatly improved in cultures maintained at physiological pH throughout the incubation period. Cells are easily detached with little or no clumps as compared to the cells at acidic pH. The reason being the cells are in the growing phase and optimum pH for trypsin is the range of 7.4 to 7.8.
15. Substantial quantity of FBS can be saved. Currently approximately 30.0 L of FBS is used per lot of Measles. 7.0 to 8.0 L of FBS per lot (used for cell medium for medium change) can be saved. Annually around 350.0 L FBS can be saved.
16. Medium change is possible way of increasing the cell yield but is not practical as it will add to the cost. By avoiding medium change to MLTCF-10, additional burden on Clean rooms can be lowered which will add to improvement in cGMP practices.
17. Fill volume per vial of final product (vaccine) of 10 dose batches is reduced to 0.5 mL from 1.0 mL.
18. Consumption of Blind Vaccine (diluent used for dilution of bulk during blending) was found to be reduced by approximately 30 %.
19. Output of filling department will be increased.
20. The platform concept of adjusting pH by gaseous exchange and extra addition of NaHCO_3 in cell media can also be applicable for other mammalian cells too e.g. Vero cells used for Rotavirus & Rabies vaccines.

EMBODIMENTS:

The present invention is illustrated in more detail by the following embodiments and combinations of embodiments which result from the corresponding dependency references and links:

- I. A method of producing a clarified virus pool, the method comprising:
 - a. providing a cell line in a cell media, a buffer and a supplement;

- b. treating cells of the cell line with at least one enzyme;
 - c. infecting the cell line with a virus to form an infected cell line;
 - d. washing of the infected cell line with a virus media and the buffer;
 - e. harvesting the infected cell line in the media to obtain a harvest; optionally re-harvesting the infected cell line one or more times;
 - f. adding at least one stabilizer to the harvest; and
 - g. clarifying the harvest to obtain a clarified virus pool (CVP).
- optionally, wherein the cell line or the infected cell line in exponential growth phase is
- grown with additional ventilation or aeration, or
 - provided with re-addition of the buffer or
 - both.
- II. The method as described in embodiment I, wherein the virus is selected from single-stranded, positive-sense, negative-sense, enveloped, non-enveloped RNA viruses belonging to the family Picornaviridae, Caliciviridae, Togaviridae, Matonaviridae, Flaviviridae, Coronaviridae, Retroviridae, Filoviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae, Arenaviridae, Paramyxoviridae.
 - III. The method as described in any one embodiments I to II, wherein the virus is single-stranded, negative-sense, enveloped, RNA viruses belonging to the family Paramyxoviridae and single-stranded, positive-sense, enveloped, RNA viruses belonging to the family Matonaviridae.
 - IV. The method as described in any one embodiments I to III, wherein the virus is Measles morbillivirus (measles virus), Mumps orthorubulavirus (mumps virus) and Rubivirus rubellae (rubella virus).
 - V. The method as described in any one embodiments I to IV, wherein the cell line is selected from animal cell line, mammalian, avian, insect cell line, human cell line, primary cell line, diploid cell line, continuous cell line, Rhesus monkey kidney (RhMK) cells; Primary rabbit kidney cells; Human foreskin fibroblasts; Chick embryo fibroblast (CEF); Human epidermoid carcinoma cells (HEp-2); Human lung carcinoma cells (A549); Human Cervix Epithelial (HeLa); African Green Monkey Kidney Epithelial (Vero); Human Lung Fibroblast (MRC-5); Human Lung Fibroblast (MRC-9); Mouse Embryo Fibroblast (NIH3T3); Mouse Connective Tissue Fibroblast (L929); Chinese Hamster Ovary Fibroblast (CHO); Syrian Hamster Kidney Fibroblast (BHK-21); Human embryo Kidney Epithelial (HEK-293); Human Liver Epithelial (HepG2); Bovine Aorta Endothelial (BAE-1); Human

Neuroblastoma Neuronal (SH-SY5Y); Mouse Myeloma Lymphoblast (NS0); Human Histiocytic Lymphoma Lymphoblast (U937); Human Leukemia Lymphoblast (HL60); Mouse B-cell Lymphoma Lymphoblast (WEHI231); Mouse Lymphoma Lymphoblast (YAC1); Human Myeloma Lymphoblast (U266B1); Human T-cell Leukemia Lymphoblast (Jurkat); Human Monocyte Leukemia Lymphoblast (THP-1); Human embryonic lung cells (W1-38); Madin Darby canine kidney cells (MDCK); Human embryonic retinal cells (PER.C6); Human embryonic retinoblasts (HER.911); Murine non-secreting myeloma (Sp2.0); Epithelial cells of African green monkey kidney origin (BSC-1 cells); Rhesus Monkey Kidney Epithelial Cells (LLC-MK2 cells); Cercopithecus aethiops monkey kidney cells (CV-1 cells); African green monkey kidney fibroblast-like cells (COS-cells); Crandell-Rees Feline Kidney Cells (CRFK cells); Rapidly Accelerated Fibrosarcoma cells (RAF cells); Normal Rabbit Kidney Epithelial Cells (RK-13 cells); Transformed C3H Mouse Kidney-1 (TCMK-1 cells); Pig Kidney Epithelial Cells (LLC-PK1 cells); Porcine kidney cells (PK15 cells); Rabbit kidney cell line (LLC-RK1 cells), Nonsecreting myeloma cell lines (NS-1 cells), New human male diploid cell strain (TIG-1, TIG-7); nonhuman primate diploid cell line (FRhL-2); Human foetal lung (IMR-90, IMR-91) cells; human diploid lung fibroblasts and others.

- VI. The method as described in any one embodiments I to V, wherein the cell line is selected from Human Lung Fibroblast (MRC-5) cell line and Chick embryo fibroblast (CEF) cell line.
- VII. The method as described in any one embodiments I to VI, wherein the enzyme is selected from trypsin, recombinant trypsin, dipase, collagenase, hyaluronidase, elastase, cysteine protease, deoxyribonuclease I and chymotrypsin.
- VIII. The method as described in any one embodiments I to VII, wherein the cell media includes basal media, enriched media, selective and indicator media, transport media, storage media, carbon sources, or combination thereof.
- IX. The method as described in any one embodiments I to VIII, wherein the supplement includes amino acids, cholesterol, proteins, lactoferrin, linoleic acid, yeast extracts, serums, antioxidants, vitamins, antibiotics, nutrients, trace elements, adherence agents, extension factors, or combinations thereof.
- X. The method as described in any one embodiments I to IX, wherein the buffer is selected from the NaHCO_3 , NaOH, NaCl, HEPES, PIPES, MES, phosphates, carbonates, Hank's, Earl's or combinations thereof.

- XI. The method as described in any one embodiments I to X, wherein the virus media for washing the infected cell line is selected from basal media, enriched media, selective and indicator media, transport media, storage media, carbon sources, or combination thereof.
- XII. The method as described in any one embodiments I to XI, wherein the washing of the infected cell lines by the virus media is performed post infection with virus having MOI in the range of 1:5 to 1: 60 and is followed by incubation at 30 to 40°C.
- XIII. The method as described in any one embodiments I to XII, wherein the at least one stabilizer includes at least one carbohydrate, at least one protein, at least one amino acid, or a combination thereof.
- XIV. The method as described in any one embodiments I to XIII, wherein the clarification of the harvest is carried out using filters, preferably the filters are of material of construction selected from modified PVDF, cellulose acetate, polyethersulfone, polypropylene.
- XV. The method as described in any one embodiments I to XIV, wherein the clarification of the harvest is carried out using at least one filter having pore size in the range of 0.1 to 10.0 μ .
- XVI. The clarified virus pool (CVP) obtained by the method as described in any one of embodiments I to XV.
- XVII. The method as described in any one of embodiments I to XVI, for producing a measles clarified virus pool, the method comprising:
- providing the cell line in a cell media, the buffer and the supplement;
 - treating cells of the cell line with the enzyme;
 - infecting the cell line with Measles morbillivirus to form the measles infected cell line;
 - washing of the measles infected cell line with the virus media and the buffer;
 - harvesting the measles infected cell line in the media to obtain the harvest; optionally re-harvesting the measles infected cell line;
 - adding the stabilizer to the harvest; and
 - clarifying the harvest to obtain the measles clarified virus pool (CVP).
- optionally, wherein the cell line or the measles infected cell line in exponential growth phase is
- grown with additional ventilation or aeration, or
 - provided with re-addition of the buffer or
 - both.
- XVIII. The method as described in embodiment XVII, wherein the cell line is Human Lung Fibroblast (MRC-5) cell line.

- XIX. The method as described in any one of embodiments XVII or XVIII, wherein the enzyme is recombinant trypsin.
- XX. The method as described in any one of embodiments XVII to XIX, wherein the cell media is Minimum Essential Medium.
- XXI. The method as described in any one of embodiments XVII to XX, wherein the supplement includes glutamine and foetal bovine serum.
- XXII. The method as described in embodiment XXI, wherein foetal bovine serum is in the range 5% to 15%.
- XXIII. The method as described in any one of embodiments XVII to XXII, wherein the virus media is Minimum Essential Medium.
- XXIV. The method as described in any one of embodiments XVII to XXIII, wherein the buffer comprises of sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4.0 g/L to maintain optimal pH of 6 to 8.
- XXV. The method as described in any one of embodiments XVII to XXIV, wherein the measles virus includes Edmonston strain, including the Schwartz, the Edmonston-Zagreb, the Moraten strains; CAM-70; TD 97; Leningrad-16; AIK-C strain and Shanghai 191 (Ji-191) strains.
- XXVI. A measles clarified virus pool (CVP) obtained by the method as described in any one of embodiments XVII to XXV.
- XXVII. The method as described in any one of embodiments I to XVI for producing a mumps clarified virus pool, the method comprising:
- providing the cell line in the cell media, the buffer and the supplement;
 - treating cells of the cell line with the enzyme;
 - infecting the cell line with the Mumps orthorubula virus to form a mumps infected cell line;
 - washing of the mumps infected cell line with the virus media and the buffer;
 - harvesting the mumps infected cell line in the media to obtain the harvest; optionally re-harvesting the mumps infected cell line;
 - adding the stabilizer to the harvest; and
 - clarifying the harvest to obtain the mumps clarified virus pool (CVP).
- optionally, wherein the cell line or the mumps infected cell line in exponential growth phase is
- grown with additional ventilation or aeration, or

- provided with re-addition of the buffer or
 - both.
- XXVIII. The method as described in embodiment XXVII, wherein the cell line is Chick embryo fibroblast (CEF) cell line.
- XXIX. The method as described in embodiments XXVII or XXVIII, wherein the enzyme is recombinant trypsin.
- XXX. The method as described in any one of embodiments XXVII to XXIX, wherein the cell media is Minimum Essential Medium.
- XXXI. The method as described in any one of embodiments XXVII to XXX, wherein the supplement includes glutamine, foetal bovine serum, and neomycin sulphate.
- XXXII. The method as described in embodiment XXXI, wherein foetal bovine serum is in the range 5% to 15%.
- XXXIII. The method as described in any one of embodiments XXVII to XXXII, wherein the virus media is Minimum Essential Medium.
- XXXIV. The method as described in any one of embodiments XXVII to XXXIII, wherein the buffer is sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4.0 g/L to maintain optimal pH of 6 to 8.
- XXXV. The method as described in any one of embodiments XXVII to XXXIV, wherein the virus includes Jeryl-Lynn, RIT 4385, Leningrad-3, Leningrad-Zagreb (L-Zagreb), Urabe Am9, Hoshino strain, Torii strain and S79 Rubini strains.
- XXXVI. A mumps clarified virus pool (CVP) obtained by the method as described in any one of embodiments XXVII to XXXV.
- XXXVII. The method as described in any one of embodiments I to XVI for producing a rubella clarified virus pool, the method comprising:
- a. providing the cell line in the cell media and the supplement;
 - b. treating cells of the cell line with the enzyme;
 - c. infecting the cell line with the Rubivirus rubellae to form a rubella infected cell line;
 - d. washing of the rubella infected cell line with the virus media and the buffer;
 - e. harvesting the rubella infected cell line in the media to obtain the harvest; optionally re-harvesting the rubella infected cell line;
 - f. adding the stabilizer to the harvest; and
 - g. clarifying the harvest to obtain the rubella clarified virus pool (CVP).

optionally, wherein the cell line or the rubella infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or
- both.

XXXVIII. The method as described in embodiment XXXVII, wherein the cell line is Human Lung Fibroblast (MRC-5) cell line.

XXXIX. The method as described in embodiments XXXVII or XXXVIII, wherein the enzyme is recombinant trypsin.

XL. The method as described in any one of embodiments XXXVII to XXXIX, wherein the cell media is Minimum Essential Medium.

XLI. The method as described in any one of embodiments XXXVII to XL, wherein the supplement includes glutamine, foetal bovine serum, neomycin sulphate or combination thereof.

XLII. The method as described in embodiment XLI, wherein foetal bovine serum is in the range 5% to 15%.

XLIII. The method as described in any one of embodiments XXXVII to XLII, wherein the virus media is Minimum Essential Medium.

XLIV. The method as described in any one of embodiments XXXVII to XLIII, wherein the buffer is sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4 g/L to maintain optimal pH of 6 to 8.

XLV. The method as described in any one of embodiments XXXVII to XLIV, wherein the virus includes Wister RA 27/3 strain, BRD-2 strain, Matsuba, DCRB19, Takahashi, Matsuura and TO-336 strains.

XLVI. A rubella clarified virus pool (CVP) obtained by the method as described in any one of embodiments XXXVII to XLV.

XLVII. A method of obtaining a lyophilized/ freeze-dried MMR immunogenic composition comprising at least one CVP selected from a measles CVP, a mumps CVP, a rubella CVP or a combination thereof, the method comprising:

providing a measles CVP of embodiment XXVI or obtained by any one of embodiments XVII to XXV, a mumps CVP of embodiment XXXVI or obtained by any one of embodiment XXXVII to XXXV, a rubella CVP of embodiment XLVI or obtained by any one of embodiments XXXVII to XLV, or a combination thereof,

blending of the CVPs, followed by lyophilizing the blended CVPs.

- XLVIII. The method of embodiment XLVII, wherein the method includes:
- thawing at least one CVP of measles, mumps, rubella virus or combination thereof at 30 to 35°C to obtain the thawed CVP;
 - blending the thawed CVP and blind vaccine to obtain a blended solution;
 - clarifying the blended solution through a 0.45 μ filter to obtain a homogenous bulk;
 - aseptically filling the homogenous bulk into sterilized vials, followed by transferring the vials to a lyophilizer/ freeze dryer.
 - lyophilizing/ freeze drying the vials containing the homogenous bulk.
- XLIX. The method according to embodiment XLVIII, wherein the lyophilizing/ freeze-drying step comprises:
- pre-freezing shelf, loading the trays containing vials of the MMR immunogenic composition on the shelf;
 - freezing;
 - primary drying/ sublimation and
 - secondary drying
- L. A lyophilized/ freeze-dried MMR immunogenic composition obtained by the method as described in embodiment XLIX, the composition comprising:
- at least one virus;
 - stabilizer comprising at least one carbohydrate, at least one amino acid and at least one hydrolyzed protein.
- LI. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose.
- LII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of at least one carbohydrate selected from a group consisting of natural carbohydrate, synthetic carbohydrate, monosaccharides, disaccharides, trisaccharides, oligosaccharides, reducing sugar, non-reducing sugar, sugar alcohols, polyol, polyhydroxyl

compounds, chemically modified carbohydrates and glass transition facilitating agents which include sucrose, mannitol, trehalose, mannose, raffinose, lactitol, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose sorbitol, dextrose, fructose, glycerol, sorbitol, and fucose and a combination thereof.

- LIII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of atleast one amino acid selected from a group consisting of tricine, leucine, iso-leucine, L-histidine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Histidine, Methionine, Proline, Serine, Threonine and a combination thereof.
- LIV. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of atleast one hydrolyzed protein obtained by chemical, enzymatic or thermal hydrolysis of protein from either plant or animal sources.
- LV. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of at least one hydrolyzed protein selected from a group consisting of gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate and whey protein hydrolysate.
- LVI. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, wherein the composition comprises;
- a) at least one carbohydrate at concentration range of 1-20% (w/v)
 - b) at least one amino acid at concentration range of 0.01-10% (w/v);
 - c) at least one hydrolyzed protein at concentration range of 0.1-10% (w/v)
- LVII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment LVI, wherein at least one of the carbohydrate is sorbitol present at a concentration of 1 to 20% (w/v), 1 to 10% (w/v), preferably 3-6% (w/v).
- LVIII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment LVI, wherein at least one of the amino acid selected from a group consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v).
- LIX. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment LVI, wherein atleast one of the hydrolysed protein selected from a group consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).

- LX. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of an adjuvant selected from a group consisting of aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate or a mixture thereof.
- LXI. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of an immunostimulatory component selected from a group consisting of an oil and water emulsion, MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, 3-deacylated monophosphoryl lipid A, AS01, AS03, an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, CRL-8300 adjuvant, muramyl dipeptide, TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, QS-21, ISCOMS, Chitosan, saponin combination with sterols and lipids.
- LXII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, comprising of a pharmaceutically acceptable additive selected from a group consisting of transporter, excipient, binder, carrier, isotonic agent, emulsifier and humectant.
- LXIII. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, wherein the excipient is selected from a group consisting of salt including NaCl, KCl, KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, CaCl_2 , and MgCl_2 ; non-ionic surfactant including polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 80, polysorbate 85, nonylphenoxypolyethoxethanol, octylphenoxypolyethoxethanol, octoxynol 40, nonoxynol-9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene-660 hydroxystearate, polyoxyethylene-35 ricinoleate, soy lecithin and a poloxamer - 0.001%-0.05%; polymers including dextran, carboxymethylcellulose, hyaluronic acid and cyclodextrin.
- LXIV. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment L, wherein the lyophilized/freeze-dried viral vaccine composition is reconstituted with an aqueous solution selected from a group consisting of saline, buffer and WFI (water for injection).
- LXV. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment LXIV, wherein the buffer is selected from a group consisting of sodium chloride, acetate, carbonate, citrate, lactate, gluconate, tartrate, phosphate buffer saline, borate, histidine buffer, succinate buffer, HEPES, TRIS and Citrate-phosphate.

- LXVI. The lyophilized/ freeze-dried MMR immunogenic composition as described in embodiment LXIV, wherein the final pH of the reconstituted composition is in the range of pH 6.5 to 7.5.
- LXVII. The lyophilized/freeze-dried MMR immunogenic composition as described in any of the previous embodiments L to LXVI, comprising:
- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
 - b) stabilizer comprising
 - carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).
- LXVIII. The lyophilized/freeze-dried MMR immunogenic composition as described in embodiment LXVII, comprising:
- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
 - b) stabilizer comprising
 - carbohydrate consisting of sorbitol present at a concentration of 5% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.3% (w/v), L-histidine present at a concentration of 0.21% (w/v), L-alanine present at a concentration of 0.1% (w/v) and L-arginine hydrochloride present at a concentration of 1.6% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.35% (w/v).
- LXIX. The lyophilized/freeze-dried MMR immunogenic composition as described in embodiment LXVIII, wherein the lyophilized virus vaccine composition is in the form of a single dose composition or a multi-dose composition.
- LXX. The lyophilized/freeze-dried MMR immunogenic composition as described in embodiment LXIX, wherein the multi-dose composition additionally comprises preservative.

- LXXI. A kit comprising the lyophilized/freeze-dried MMR immunogenic composition as described in embodiment LXVII and LXVIII comprises;
- a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v); amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v); and
 - a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.
- LXXII. A kit comprising the lyophilized/freeze-dried MMR immunogenic composition as described in embodiment LXVII and LXVIII comprises;
- a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration 5% (w/v); amino acid consisting of tricine present at a concentration 0.3% (w/v), L-histidine present at a concentration 0.21% (w/v), L-alanine present at a concentration 0.1% (w/v) and L-arginine hydrochloride present at a concentration 1.6% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration 0.35% (w/v); and
 - a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.

EXAMPLES:

The foregoing description of the embodiments has been provided for purposes of illustration and not intended to limit the scope of the present disclosure. Individual components of a particular embodiment are generally not limited to that particular embodiment, but, are interchangeable. Such variations are not to be regarded as a departure from the present disclosure, and all such modifications are considered to be within the scope of the present disclosure.

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the compositions and techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. The present disclosure is further described in light of the following examples which are set forth for illustration purpose only and not to be construed for limiting the scope of the disclosure.

However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1:

A. Strains and their source: Strains of viruses (measles, mumps and rubella) and their sources are mentioned in below Table 6.

Table 6: Strains and their source

Virus	Strain	Source
Measles virus	Edmonston-Zagreb measles vaccine strain	Institute of Immunology, Zagreb, Croatia
Mumps virus	L-Zagreb mumps vaccine strain	Institute of Immunology, Zagreb, Croatia
Rubella virus	Wister RA 27/3 rubella vaccine strain	Institute of Immunology, Zagreb, Croatia

B. Cell lines and their source: Cell lines used for the growth of viruses are mentioned in below Table 7

Table 7: Cell lines and their source

Cell Line	Source
Human Lung Fibroblast (MRC-5) cell line	Human Lung Fibroblast (MRC-5) cells at lower passage were obtained from NIBSC, UK
Chick embryo fibroblast (CEF) cell line	Chick Embryo Fibroblast (CEF) cells is prepared by using 9 – 11 days old embryo of specific pathogen free (SPF) chicken eggs. These SPF eggs are received from Lohmann Germany and Hy-Vac, USA

C. Ingredients selected for virus vaccine (MMR vaccine) manufacturing process:

1. Foetal bovine serum, (FBS):

FBS is the most widely used basal media supplement for in vitro cell culture. It contains very low level of antibodies and high concentration of growth factors such as hormones, attachment factors and transport proteins. FBS effectively promotes and sustains the growth of cells at low densities used for biological manufacturing. Serum also adds buffering capacity to the medium and binds or neutralizes toxic components. FBS is obtained from foetuses harvested in abattoirs from healthy animals. Serum lots from the manufacturer are the pools of serum collected from many different animals.

Being an animal origin material FBS has an inherent risk of being contaminated with transmissible adventitious agents. In case of serum obtained from abattoirs, it is not usually possible to demonstrate freedom from bovine viral infections. Bovine serum might be contaminated by many different bovine viruses. It is evident that each serum batch has to be tested for those viruses which are ubiquitous and of known risk. The possibility of the introduction and replication of adventitious agents during cell culture has long been recognized as a potential risk which leads to virus contaminated final product. There have been a number of instances where laboratory studies provided evidence for the presence of adventitious agents in marketed vaccines. Such risks of adventitious agents are typically mitigated by testing bovine serum for absence of adventitious agents. Most regulatory bodies allow the use of animal derived materials only when their use can be justified because there is no viable alternative. For that, each serum lot must be tested for sterility and adventitious agents. Virus testing is typically performed in accordance with various regulatory guidelines. However, the testing methods have their own limitations and sometimes the contaminating adventitious agent may escape detection.

Gamma irradiation:

Gamma irradiation is a very efficient and straightforward means for inactivating many different virus types in FBS. As FBS can be contaminated by adventitious viruses, gamma irradiation is, after routine quality control for virus detection, the best method to increase

the safety of using it in the production of Biologicals. One of the reasons why the gamma irradiation method has been commonly used for reduction of adventitious contaminants in serum is that it may be performed on serum in the original product containers. And also, it is typically performed at low temperatures, thereby keeping the serum quality and performance unaffected. Both gamma irradiated & non-gamma irradiated FBS are tested for cell growth suitability and then used in commercial production.

FBS (from Australia/ New Zealand) is irradiated with GAMMA irradiation used in this invention at the dose range of 20 to 50 KGy.

2. Recombinant trypsin:

Trypsin is a proteolytic enzyme (serine protease), used as a cell dispersing agent in tissue culture techniques. As such, it becomes a critical raw material for all tissue culture-based vaccines such as, Measles, Mumps, Rubella, Rabies, Rota etc. Porcine trypsin is a widely used reagent in the manufacturing of biological medicinal products. It is extracted from pig pancreas and therefore, being an animal origin material, trypsin has an inherent risk of being contaminated with transmissible adventitious. As many as 55 porcine virus species of human host range, from 17 different families having been stated as potential contaminating viruses. Such risks of various adventitious agents are typically mitigated by testing the porcine trypsin for absence of the extraneous agents, however the testing methods have their own limitations and sometimes the contaminating adventitious agents may escape detection.

Regulatory guidelines are now encouraging to use of animal-component free reagents, such as recombinant trypsin, in place of animal derived trypsin. Recombinant trypsin is a genetically engineered protein expressed in suitable microorganism (*E. coli*, *Pichia pastoris*) and purified by high pressure liquid chromatography. Use of recombinant trypsin as an alternative to conventional porcine origin trypsin can completely eliminate the risk of contamination by animal origin reagents. Recombinant trypsin is from two different vendors (Biogenomics & Richcore (Laurus Bio)) for producing MMR clarified virus pool or MMR bulk vaccine.

D. Cell Media and Virus Media:

Cell lines were grown on the cell media, the buffer and the supplement. The infection of the cell lines was done and infected cell lines were grown. The infected cell line is washed with the virus media and the buffer.

Below tables (Table 8 to 13) provide details of cell media and virus media for producing a clarified virus pool of Measles virus, Mumps virus and Rubella virus.

Table 8: Cell media for producing a clarified virus pool of Measles virus (10 Litre batch size)

Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	No. of bottles (Equivalent to 10 liter of medium)	1	10.63 g/L
2.	Glutamine	gm	3.5	0.35 g/L
3.	Sodium bi carbonate	gm	15	1.5 g/L
4.	1N Hydrochloric acid	ml	15 ml / As required for pH adjustment.	1.5 ml/L
5.	Water for Injection (20°C-30°C)	Lit	q.s. to 10 lit.	q.s.
6.	Foetal Bovine Serum (FBS)*	Lit	1.0 to 1.25	0.1 to 0.125 litre

* The amount FBS is added in a variable percentage from 10% - 12.5% depending upon the make / lot no. of FBS and its growth promoting properties.

Table 9: Cell media for producing a clarified virus pool of Mumps virus (10 Litre batch size)

Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	No. of bottles (Equivalent to 10 liter of medium)	1	10.63 g/L
2.	Glutamine	gm	3.5	0.35 g/L
3.	Sodium bi carbonate	gm	15	1.5 g/L
4.	1N Hydrochloric acid	ml	15 ml / As required for pH adjustment.	1.5 ml/L
5.	Water for Injection (20°C-30°C)	Lit	q.s. to 10 lit.	q.s.
6.	Foetal Bovine Serum (FBS)*	Lit	1.0 to 1.25	0.1 to 0.125 litre
7.	Neomycin Sulphate	gm	0.55	0.055 g/L

* The amount FBS is added in a variable percentage from 10% - 12.5% depending upon the make / lot no. of FBS and its growth promoting properties.

The exact batch size will vary based on the quantity of FBS added per 10L of medium.

Table 10: Cell media for producing a clarified virus pool of Rubella virus (10 Litre batch size)

Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	No. of bottles (Equivalent to 10 liter of medium)	1	10.63 g/L
2.	Glutamine	gm	3.5	0.35 g/L
3.	Sodium bi carbonate	gm	15	0.15 g/L
4.	1N Hydrochloric acid	ml	15 ml / As required for pH adjustment.	0.15 ml/L
5.	Dextrose	gm	5	0.5 g/L
6.	Water for Injection (20-30°C)	Lit	q.s. to 10 lit.	q.s.
7.	Foetal Bovine Serum (FBS)*	Lit	1.0 to 1.25	0.1 to 0.125 litre
8.	Neomycin sulphate	gm	0.55	0.055 g/L

* The amount FBS is added in a variable percentage from 10% - 12.5% depending upon the make / lot no. of FBS and it's growth promoting properties.

The exact batch size will vary based on the quantity of FBS added per 10L of medium.

Table 11: Virus media for producing a clarified virus pool of Measles virus (10 Litre batch size)

Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	Bottle content equivalent to 10 liter virus medium	1	10.63 g/L
2.	Sodium bi carbonate	gm	7.5	0.75 g/L
3.	Water for Injection	Liter	9-9.5	0.9-0.95 g/L
4.	1N Hydrochloric acid*	ml	7.5 ml / As required for pH adjustment.	0.75 g/L
5.	Water for Injection	Lit	q.s. to 10 lit.	q.s.

*Quantity is flexible depending on activity of the ingredients

Table 12: Virus media for producing a clarified virus pool of Mumps virus (10 Litre batch size)

Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	Bottle content equivalent to 10 liter virus medium	1	10.63 g/L
2.	Sodium bi carbonate	gm	7.5	0.75 g/L
3.	Water for Injection	Liter	9-9.5	0.9-0.95 g/L
4.	1N Hydrochloric acid*	ml	7.5 ml / As required for pH adjustment.	0.75 g/L
5.	Water for Injection	Lit	q.s. to 10 lit.	q.s.

* Quantity is flexible depending on activity of the ingredients

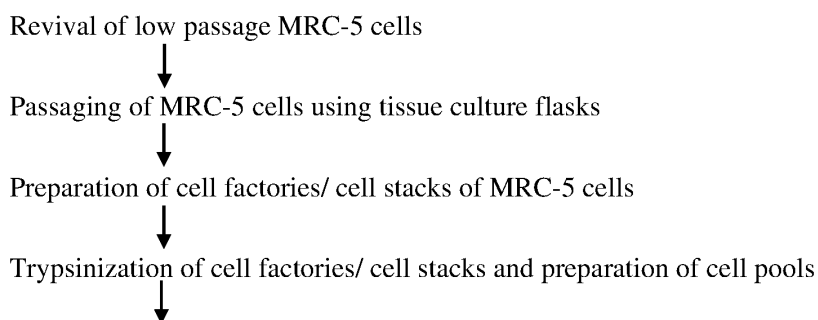
Table 13: Virus media for producing a clarified virus pool of Rubella virus (10 Litre batch size)

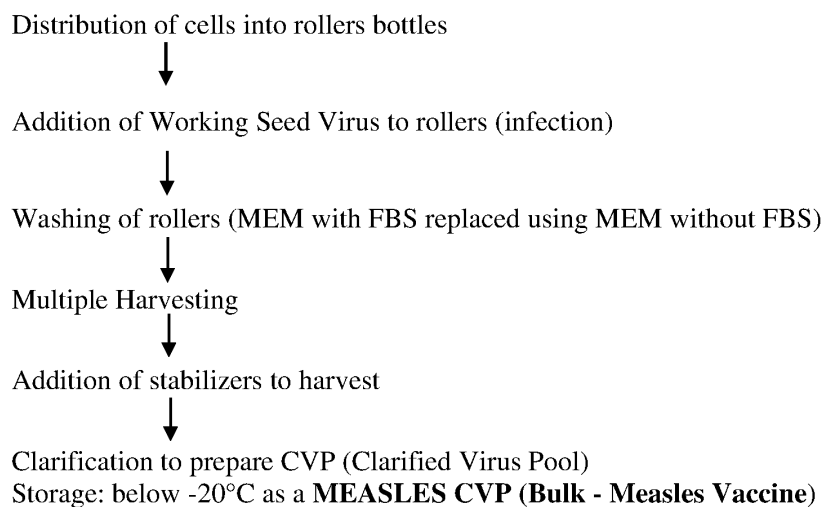
Sr. No.	Ingredient	Unit of Measurement	Quantity/ Batch or Lot	Quantity per litre
1.	MEM Powder	Bottle content equivalent to 10 liter virus medium	1	10.63 g/L
2.	Sodium bi carbonate	gm	7.5	0.75 g/L
3.	Dextrose	gm	5	0.5 g/L
4.	L-Glutamine	gm	3.5	0.35 g/L
5.	Water for Injection	Liter	9-9.5	0.9-0.95 g/L
6.	1N Hydrochloric acid*	ml	7.5 ml / As required for pH adjustment.	0.75 g/L
7.	Water for Injection	Lit	q.s. to 10 lit.	q.s.

*Quantity is flexible depending on activity of the ingredients

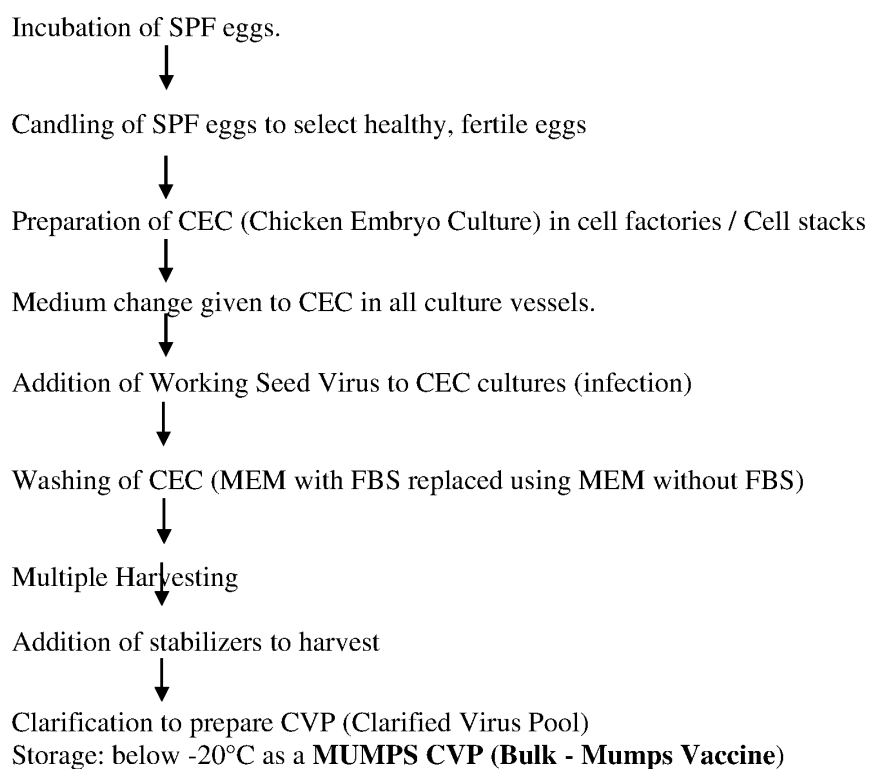
EXAMPLE 2:

FLOW CHART- MEASLES VACCINE PRODUCTION

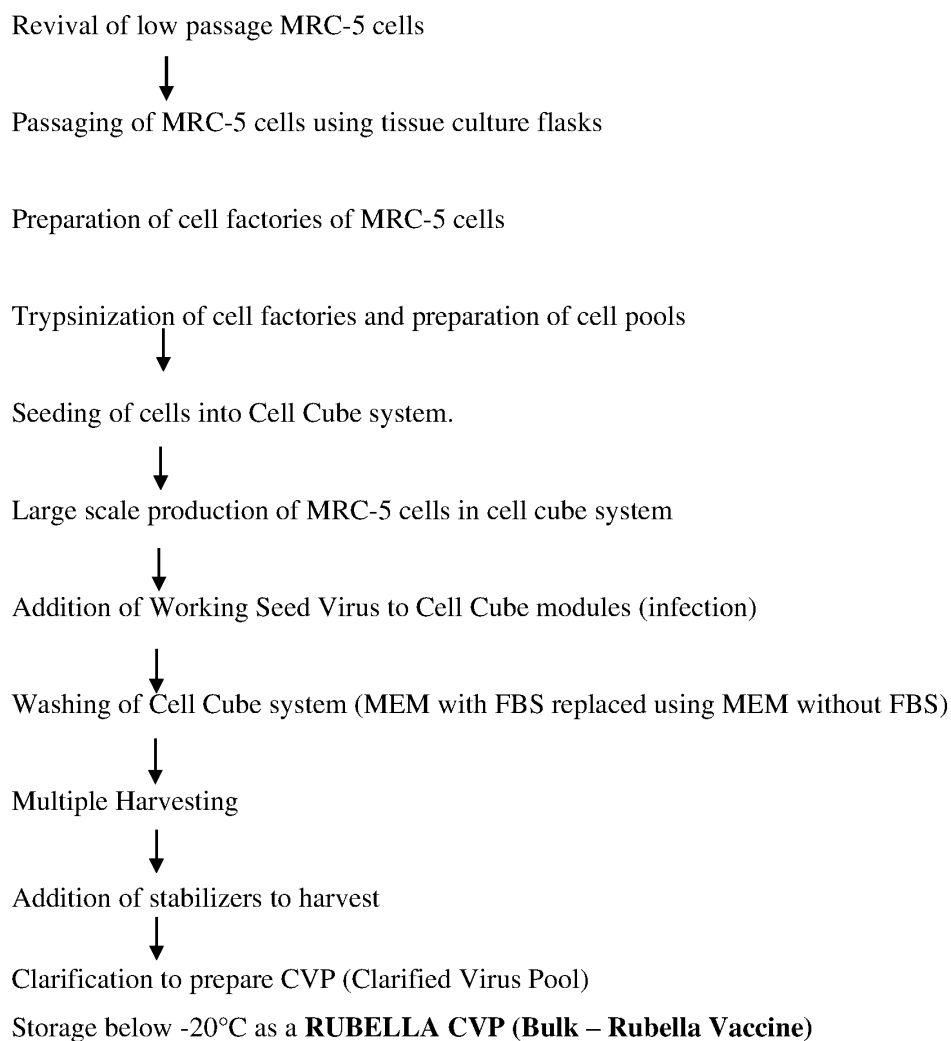
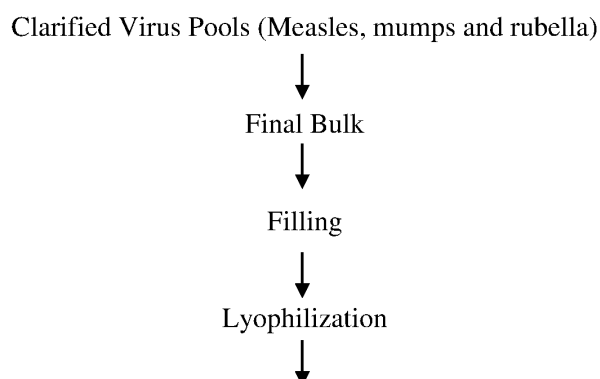




FLOW CHART: MUMPS VACCINE PRODUCTION



FLOW CHART: RUBELLA VACCINE PRODUCTION LOT

**Lyophilization:**

Final product (MMR vaccine - **Live attenuated lyophilized/
freeze-dried MMR immunogenic composition**)

EXAMPLE 3: Experiments/ Optimizations performed during production of clarified virus pool of Measles virus

A. Optimization of growth of MRC-5 cells in Cell Factories (FBS Saving):

MRC-5 cells are cultivated in cell growth medium which contains MEM Hank's supplemented with 10 % FBS, L glutamine and 1.5 g/L sodium bicarbonate. The cultures are then incubated at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 4 to 6 days.

When MRC-5 cells are cultured in cell growth medium containing CO_2 - bicarbonate buffering system, there is an initial alkaline shift, with the highest pH (about 8.0) in about 2 to 8 hrs., due to lack of CO_2 in the atmosphere, followed by progressive acidification of the medium, over a period of 48-96 hrs, to as low as pH 6.8; the rate and degree of these shifts vary with the seeding density and metabolic activity of the cultured cells. The cultures eventually become "contact inhibited" and further cell enters in stationary phase. Eagle, H. (1974), reported that maximum cell yield is obtained when the MRC-5 cells are maintained at physiological pH or little higher throughout the incubation period (i.e. 7.4 to 7.8). At this pH, the early contact inhibition of growth may not develop, and the cells may achieve population densities as much greater than those ordinarily observed.

This can be achieved by following methods;

1. Supplementation with organic buffers:

To grow cells at a reasonably constant pH, the bicarbonate of the medium (26 mM) can be supplemented with nongaseous buffers: 20-50 mM "Hepes" (N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid). It is better at maintaining physiological pH despite of changes in CO_2 concentrations. Usually it is used in *in vitro* experiments. This will be a major change in the process, hence it was not logically used and performed.

2. Medium Change / medium perfusion:

Medium changes / perfusion had a profound effect on cellular metabolism, especially on DNA and protein synthesis. As a culture approached confluency, or contact inhibition

phenomenon, DNA, RNA and protein synthesis were sequentially inhibited. On the contrary, without a medium change sequential changes in the rates of macromolecular synthesis occurred when the culture approached confluency. DNA synthesis is affected first, followed by RNA synthesis which declined very rapidly and protein synthesis which declined steadily but slowly. However, after a medium change there was a sequential stimulation of DNA, RNA and protein synthesis in the same order as they were inhibited. The inhibitory mechanism that is affected by cell crowding is obviously reversed by a medium change.

To check the possibility of medium change and whether medium change give high cell yield following experiment was performed;

Spent medium of cell media/ cell growth medium from MLTCF -10 at 48 hrs. and 120 hrs. was analyzed for components for e.g. residual glucose, lactic acid, amino acids, (arginine, cystine, etc.), etc. And percentage utilization was calculated. Decline in pH during the cell growth is also determined. The results are summarized below in Table 15 and 16;

Table 15: Utilization of cell growth medium change

Sr.no.	Component	Actual cell growth medium	Spent 48 hrs		Spent 120 hrs.	
		Conc. µg/ml	Conc. µg/ml	% utilization	Conc. µg/ml	% utilization
		(with 10% FBS)				
1	Arginine	73.827	47.345	35.870	47.146	36.14
2	Cystine	8.941	5.4745	38.771	2.984	66.63
3	Histidine	445.391	263.438	40.852	201.478	54.76
4	Isolucine	48.542	27.072	44.230	25.839	46.77
5	Lucine	49.744	28.0465	43.618	28.316	43.08
6	Lysine	56.58	34.138	39.664	41.419	26.80
7	Methionine	14.086	9.176	34.857	9.262	34.25
8	Phenyl alanine	30.038	18.926	36.993	24.157	19.58
9	Threonine	45.091	29.419	34.756	35.893	20.40
10	Tyrosine	27.388	24.138	11.867	26.044	4.91
11	Valine	48.435	30.785	36.441	31.878	34.18
12	Glutamic acid	12.6889	29.063	-129.043	69.66	-448.98
13	Serine	2.748	1.167	57.533	7.401	-169.32
14	Alanine	6.603	8.432	-27.700	35.893	-443.59
15	Proline	1.014	3.582	-253.254	14.33	-1313.21
16	Glycine	3.93	2.581	34.326	4.478	-13.94

17	Glucose	87	58	66.66	0	100 %
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Table 16: Change in pH

	pH profile				
Cell growth medium	Spent -24 hrs	Spent- 48 hrs	Spent -72 hrs	Spent -96 hrs	Spent – 120 hrs
7.2	7.95	7.48	7.25	6.9	6.8

An amino acid would become growth limiting if it is 100% utilized by the cells. It is observed that no nutrient except glucose was found to be sufficiently utilized to become growth-limiting after 96 h growth. It has been observed that in general, less than 50 % of a. acids were utilized by the cells and no amino acid was found to be sufficiently utilized to become growth-limiting even after 120 hrs., which indicates that merely m. change has not increased the cell yield.

Glucose is fully consumed by the cells at 120 hrs. Consumption pattern of glucose shows utilization being completed after 72 hrs. pH was dropped below physiological range at 72-96 hrs. Under the conditions of this experiment all amino acids were present in excess throughout the culture period. It seems that glucose as well as acidic pH might be the two growth limiting factors. However, L-glutamine serve as an alternative carbon source even if glucose is depleted from the medium and therefore acidic pH remains the only growth limiting factor. However; Medium change may help to supplement the glucose, the instant carbon source and to maintain the pH in physiological range throughout the incubation period (96 hrs.) and hence cells remains in actively metabolic stage.

Therefore, it is evident that medium change is not required and also is not practical as it will add to the cost and will increase no. of operations, materials, unnecessary man movement in clean rooms and ultimately chance of contamination.

3. Maximum cell yields without medium change

This can be achieved by following two ways;

a) Maintaining proper gaseous exchange with the surrounding environment:

MRC-5 cells were cultivated in cell growth medium which contains MEM Hank's supplemented with 10 % FBS and concentration of sodium bicarbonate of 2.0 g/L. The pH was adjusted to 6.9 to 7.1 by purging CO₂ gas before use. For MLTCF (Multilayered Tissue Culture Flask) -10 (CS-10, Corning or CF-10, Nunc's), the seeding count was 100 to 160

million cells and incubation temp. and period was $36 \pm 1^\circ \text{C}$ and 5-7 days respectively. The MLTCF has two vents (0.2 μ milex filters, 50 mm diameter). The cell yield obtained was 400 to 500 million cells. Usual pH shifts observed during the incubation period as high as 7.8 to 8 to as low as 7.0 or below.

Eagle, H. (1974), reported that maximum cell yield is obtained when the MRC-5 cells are maintained at physiological pH or little higher throughout the incubation period (i.e. 7.4 to 7.6).

An experiment was designed to achieve this. In this expt. Corning CS-10 (MLTCF-10) were used. The original venting caps were used instead of milex filters on both the ports. These venting caps are Standard 33 mm threaded caps having 0.2 μm pore nonwetable membranes sealed directly to the caps to allow gas exchange. The results were compared with routinely used MLTCF -10 having 0.2 μm milex vent filters (50 mm). The cultures were initiated from same inoculums. (100 – 120 million cells). During first 24 - 48 hrs, where the lower pH (7.0 to 7.2) is required for attachment, the vents were closed tightly by sterile tape / aluminium foil to prevent gaseous exchange. After 48 hrs, the vents were opened by removing the tape / foil to allow the gaseous exchange. It has been observed that pH remained in the range of 7.4 to 7.6 during rest of the incubation period. The details of cell yield, and pH is summarized below in Table 17.

Table 17: Comparison of Vent (milex filters) with Venting caps (Corning Inc.)

Passage no.	Dt. seeding	Seeding count (10^6) per MLTCF-10	Dt. Passaging	Vent (milex filters) on both ports		Venting caps (Corning Inc.) on both ports	% increase in Cell yield	
				Cell count (10^6)	pH spent	Cell count (10^6)	pH spent	
P27	17.06.13	120	24.06.13	550	6.98	850	7.66	54.5
P27	18.06.13	115	25.06.13	500	7.10	820	7.71	64.0
P28	24.06.13	120	01.07.13	520	7.05	880	7.68	69.2
P28	25.06.13	105	02.07.13	500	7.12	920	7.76	84.0
P29	01.07.13	110	08.07.13	485	6.90	815	7.63	68.0
P29	02.07.13	120	09.07.13	500	7.18	840	7.78	68.0
P30	08.07.13	115	15.07.13	490	7.10	710	7.71	45.0
P30	09.07.13	110	16.07.13	410	7.14	880	7.78	114.0

It is observed that in MLTCF-10 with milex filters (routine process), pH dropped below physiological, whereas in MLTCF-10 having vented caps, pH was maintained in the range of 7.6 to 7.78. MLTCF-10 vented with Corning Vented caps yielded 45 to 114 % more cells than with MLTCF -10 vented with milex filter.

Therefore, it is evident from the above data that the venting caps provides proper gaseous exchange due to which sodium bicarbonate dissociated slowly to maintain the pH towards alkaline side as against the routinely used vents with milex filters.

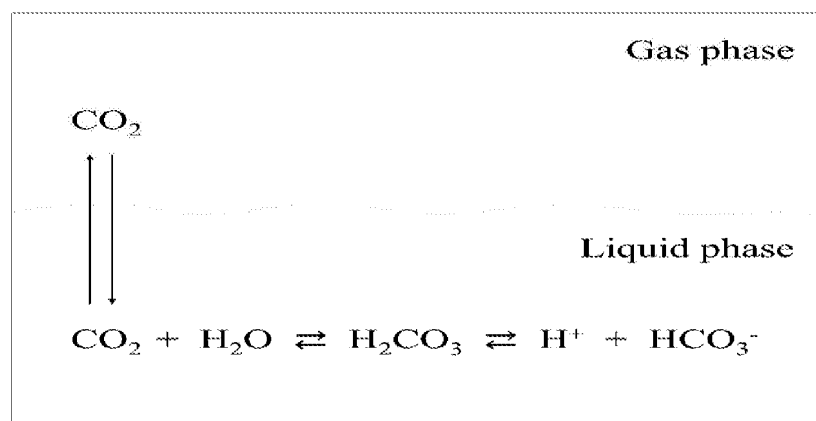
In cultures with milex filters where pH dropped below non-physiological range (below 7.1), the less cell growth (count) could not have been caused by simple nutrient depletion, since the mere maintaining pH in the range of 7.6 to 7.8 yielded more cell count when vented caps are used for gaseous exchange.

Another important observation is that trypsinization is greatly improved in cultures maintained at physiological pH throughout the incubation period. Cells are easily detached with little or no clumps as compared to the cells at acidic pH. The reason being the cells are in the growing phase and optimum pH for trypsin is the range of 7.4 to 7.8.

It is important to note that for proper gaseous exchange, Corning's MLTCF are ideal since its port size is optimum (33 mm) as compared to Nunc's MLTCF because the latter is having very narrow port size (10 mm) though, having 50 mm milex vent filter.

b) Increasing the Buffering capacity of Cell Growth Medium: by increasing concentration of Sodium Bicarbonate:

The pH control mechanism of Cell Culture Media is based on the bicarbonate buffer system. When dissolved in water, sodium bicarbonate (NaHCO_3) dissociates to form a sodium ion (Na^+) and a bicarbonate ion (HCO_3^-). The latter reacts with H^+ in solution to form carbonic acid (H_2CO_3), which dissociates into CO_2 and H_2O . These two reactions attain their respective equilibria. The CO_2 in solution also reaches equilibrium with CO_2 in the gas phase. (Refer Equation 1) As a result, increasing the concentration of gas phase CO_2 increases the amount of CO_2 that is dissolved in the culture medium, in turn raising the H_2CO_3 concentration and lowering the pH. In contrast, if the concentration of the gas phase CO_2 is lowered, then the pH rises due to the reverse reaction.



Equation 1: Dissociation of buffer

The amount of sodium bicarbonate (NaHCO_3) in the medium dictates the amount of CO_2 that should be used to maintain the desired pH. The excess carbon dioxide generated by the cells will increase the dissolved carbon dioxide level and decrease the solution pH. In order to maintain the preferred pH, additional required bicarbonate has to be added,

Currently in MMR production, NaHCO_3 at 1.5 g/L is being used in Cell Medium (CM) used for cell passaging. The MLTCF-10 /CS -10 is seeded with around 150 to 225 million cells in 2.0 L CM. After 48 hours, it is replenished with 2.0 L fresh CM (bicarbonate at 1.5 g/L). The pH profile of Cf-10 with and without medium change is computed is given below Table 18;

Table 18: Comparison of MLTCF-10 /CS-10 with No Medium change and CF-10 / CS-10 with M. Change at 48 hrs considering pH parameter.

Time	pH: MLTCF-10 /CS-10 with No Medium change	pH: MLTCF-10 / CS-10 with Medium Change at 48 hrs.
0 hrs. (CM)	7.45	7.45
24 hrs.	7.95	7.95
48 hrs.	7.35	7.37
72 hrs.	7.15	7.40
96 hrs.	6.95	7.05
120 hrs.	6.80	7.02
144 hrs.	6.80	6.90
168 hrs.	6.80	6.85

As the cells reach the exponential growth phase, they become maximally metabolically active and each cell produces its maximum carbon dioxide output.

The excess carbon dioxide generated by the cells increases the dissolved carbon dioxide level and decrease the medium pH. Therefore, acidic pH acting as growth limiting factor, was controlled and maintained at desired pH (7.5 to 7.7) throughout the incubation period by adding sodium bicarbonate into the cell media, in the range of 2.0 to 2.5 g/L.

By doing this, maximum yield of MRC-5 cells can be achieved in MLTCF-10/CS-10 without giving medium change, thereby saving substantial quantity of FBS, the costliest item used in Measles vaccine production.

B. pH maintenance:

pH is one of the critical process parameters in viral vaccine manufacturing process. Exposure of the virus to extremes of pH inactivates the virus. It has been reported the Measles Virus (MV) is most stable at pH 7.6 and a progressive inactivation of virus occurs on either side. Physical stability of MV is highly compromised in acidic environment. Even slight changes in pH affects the native conformation of the virus (Black,1959). The infectivity of MV is also dependent on the conformational stability of viral proteins. The alkaline medium not merely neutralizes the acid produced by infected cells, but enhances the virus yield and its stability after release into the medium. Therefore, maintenance of optimum pH throughout the process is inevitable so as to maintain its native conformation. Also, it has been reported that alkaline medium suppresses the production of defective particles. (Yoshino et al. Archives of Virology 31 --38 © by Springer-Verlag 1975).

Similarly, for MRC-5, being a human diploid cell line, maintenance of pH in the physiological range or little higher (7.4 to 7.6) is equally important since, at this pH the cells are more healthy and binding sites (CD-46) for MV would be in more conceivable form for infection. Also, it is known fact that exposure of cells to low pH induces 'Early Contact Inhibition phenomenon' which affects the cell growth and ultimately virus yield.

During routine MV production, post infection, the infected cells are incubated at 36 ± 1 °C for 40 to 48 hrs. till washing. At this stage, it has been observed that the pH usually drops below 7.2. This indicates that the buffering capacity of the CM is not enough to take care of the acid produced by the infected cells and fails to maintain the pH above physiological and therefore, to increase the buffering capacity, the CM used for Cell Pool preparation should be boosted with additional Sodium Bicarbonate (NaHCO_3). Currently the concentration of

NaHCO₃ is 1.5 g/L. During harvesting, since the harvesting interval is much less (maximum 24.0 hrs.), the concentration of NaHCO₃ (0.75 g/L) in Virus Medium (VM) seems to be sufficient and hence no need to change it. With all this thought process and considering the seeding density per roller (40 – 60 million), MOI (i.e. virus to cell ratio) (1:8 to 1:12) and post infection incubation period (40 to 48 hrs.), CM used for Cell Pool preparation only was added with 1.8 to 2.0 g/L of NaHCO₃. The results are tabulated below in Table 19 and depicted in Figure 1;

Table 19: Effect of NaHCO₃ on the measles virus vaccine yield (million doses)

NaHCO ₃ = 1.5 g/L		NaHCO ₃ = 1.8 g/L	
Lot no.	MD	Lot no.	MD
0660M02	9.96	0661M01	17.80
0660M03	8.63	0661M02	14.54
0660M04	7.53	0661M03	15.85
0660M05	8.02	0661M04	16.90
0660M06	7.92	0661M05	15.42
0660M07	7.70	0661M06	16.12
0660M08	7.05	0661M07	15.91
0660M09	7.42	0661M08	15.67
0660M10	6.95	0661M09	15.39
0660M11	7.13	0661M10	14.80
0660M12	6.55	0661M11	16.25
Avg.	7.72		15.88

It is observed that the increased concentration of NaHCO₃ in CM used for Cell pool preparation has boosted the buffering capacity of the medium. Before washing of the infected culture bottles, pH of the spent medium was checked and found that it was not dropped below 7.2, rather maintained in between 7.4 to 7.6. Therefore, since MEM has weak buffering capacity, pH should be controlled in order to get optimum cell growth and virus yield.

EXAMPLE 4:

Following tables (Table 20) provide components and their concentrations present in Live attenuated lyophilized/ freeze-dried MMR immunogenic composition

Table 20: Components and their concentration ranges in live attenuated lyophilized/ freeze-dried MMR immunogenic composition (Measles, Mumps, Rubella (MMR) vaccine for 0.5 mL dose

Component	Quantity
Measles virus	Not less than 1000 CCID ₅₀ / 0.5mL dose
Mumps virus	Not less than 5000 CCID ₅₀ / 0.5mL dose
Rubella virus	Not less than 1000 CCID ₅₀ / 0.5mL dose
Gelatin (Partially hydrolysed)	0.1-5%
D-sorbitol	1-10%
Histidine	0.1-1%
L-Alanine	0.01-1%
Tricine	0.1-1%
Arginine	0.1-5%
Lactalbumin Hydrolysate	0.1-10%
Minimum Essential Medium (MEM)	Base

Table 21: Components and their concentrations in live attenuated lyophilized/ freeze-dried MMR immunogenic composition (Measles, Mumps, Rubella (MMR) vaccine for 0.5 mL dose

Component	Quantity
Measles virus	Not less than 1000 CCID ₅₀ / 0.5mL dose
Mumps virus	Not less than 5000 CCID ₅₀ / 0.5mL dose
Rubella virus	Not less than 1000 CCID ₅₀ / 0.5mL dose
Gelatin (Partially hydrolysed)	2.5%
D-sorbitol	5%
Histidine	0.21%
L-Alanine	0.1%
Tricine	0.3%
Arginine	1.6%
Lactalbumin Hydrolysate	0.35%
Minimum Essential Medium (MEM)	Base

EXAMPLE 5: Tests performed on the final bulk of MMR vaccine

The tests performed on the final bulk Measles, Mumps and Rubella Vaccine are mentioned below in Table 22 and Summary results of Tests on MMR vaccine (Final Bulk – 1 Dose) are mentioned in Table 23 to 25.

Table 22: Tests performed on the final bulk Measles, Mumps and Rubella Vaccine

TEST	REQUIREMENTS	REFERENCE
Description	Yellowish Liquid.	In-House
Test for sterility	A sample of 10.0 ml from final bulk vaccine is tested for sterility by direct inoculation for each media type i.e. fluid thioglycollate and soyabean casein digest medium. The final bulk meets the requirements of the test for sterility. Specification: Direct inoculation - The results comply with the test for sterility	Ph. Eur. 5 th Edition 2005, 2.6.1.
Test for Residual Animal Serum Protein (Bovine serum Albumin)	With BSA: Not more than 50 ng per single human dose. Specification: Residual protein content: Not more than 50 ng per single human dose.	Ph. Eur. 5 th Edition 2005, 2.7.1.
Test for Mycoplasma	Sample of 20 ml from final bulk vaccine is tested by direct inoculation method. The final bulk meets the requirements of the test for Mycoplasma. Specification: Direct inoculation - Negative	Ph. Eur. 5 th Edition 2005, 2.6.7.
Test for virus content	Potency of each component is measured after neutralization of the other two components on the appropriate cell culture.	In – house

Table 23: Summary results of Tests on MMR vaccine (Final Bulk – 1 Dose)

SUMMARY RESULTS OF TESTS ON MEASLES, MUMPS AND RUBELLA VACCINE (FINAL BULK - 1 DOSE)					
BATCH NO.	Tests				
	Description	Sterility	Virus Content	Residual Animal Protein	Mycoplasma
	Yellowish liquid	No growth should be observed in any of the sterility media	For information only	Not more than 50 ng / 0.5 mL dose	Negative
1	Yellowish liquid	No growth observed	Satisfactory	2.310 ng/dose	Negative
2	Yellowish liquid	No growth observed	Complies	1.960 ng/dose	Negative
EU839	Yellowish liquid	No growth observed	Complies	3.480 ng/dose	Negative
EU840	Yellowish liquid	No growth observed	Complies	10.000 ng/dose	Negative
EU841	Yellowish liquid	No growth observed	Complies	5.560 ng/dose	Negative
EU842	Yellowish liquid	No growth observed	Complies	4.470 ng/dose	Negative
EU843	Yellowish liquid	No growth observed	Complies	6.610 ng/dose	Negative
EU844	Yellowish liquid	No growth observed	Complies	6.430 ng/dose	Negative
EU845	Yellowish liquid	No growth observed	Complies	4.020 ng/dose	Negative
EU846	Yellowish liquid	No growth observed	Complies	2.700 ng/dose	Negative
EU847	Yellowish liquid	No growth observed	Complies	1.950 ng/dose	Negative
EU848	Yellowish liquid	No growth observed	Complies	10.780 ng/dose	Negative
EU849	Yellowish liquid	No growth observed	Complies	1.830 ng/dose	Negative
EU850	Yellowish liquid	No growth observed	Complies	7.630 ng/dose	Negative
EU852	Yellowish liquid	No growth observed	Complies	5.160 ng/dose	Negative
EU853	Yellowish liquid	No growth observed	Complies	1.370 ng/dose	Negative
EU854	Yellowish liquid	No growth observed	Complies	7.070 ng/dose	Negative
EU855	Yellowish liquid	No growth observed	Complies	7.120 ng/dose	Negative
EU856	Yellowish liquid	No growth observed	Complies	2.680 ng/dose	Negative
EU858	Yellowish liquid	No growth observed	Complies	3.400 ng/dose	Negative

Table 24: Summary results of Tests on MMR vaccine (Final Bulk – 1 Dose)

SUMMARY RESULTS OF TESTS ON MEASLES, MUMPS AND RUBELLA VACCINE (FINAL BULK – 1 DOSE)					
BATCH NO.	Tests				
	Description	Sterility	Virus Content	Residual Animal Protein	Mycoplasma
	Yellowish liquid	No growth should be observed in any of the sterility media	For information only	Not more than 50 ng / 0.5 mL dose	Negative
EU916	Yellowish liquid	No growth observed	Complies	6.770 ng/dose	Negative
EU917	Yellowish liquid	No growth observed	Complies	7.970 ng/dose	Negative
EU918	Yellowish liquid	No growth observed	Complies	9.700 ng/dose	Negative
EU919	Yellowish liquid	No growth observed	Complies	6.750 ng/dose	Negative
EU920	Yellowish liquid	No growth observed	Complies	8.340 ng/dose	Negative
EU921	Yellowish liquid	No growth observed	Complies	8.340 ng/dose	Negative
EU922	Yellowish liquid	No growth observed	Complies	8.670 ng/dose	Negative
EU923	Yellowish liquid	No growth observed	Complies	8.770 ng/dose	Negative
EU924	Yellowish liquid	No growth observed	Complies	4.460 ng/dose	Negative
EU925	Yellowish liquid	No growth observed	Complies	2.960 ng/dose	Negative
EU941	Yellowish liquid	No growth observed	Complies	0.790 ng/dose	Negative
EU942	Yellowish liquid	No growth observed	Complies	9.740 ng/dose	Negative
EU943	Yellowish liquid	No growth observed	Complies	10.550 ng/dose	Negative
EU944	Yellowish liquid	No growth observed	Complies	17.210 ng/dose	Negative
EY945	Yellowish liquid	No growth observed	Complies	9.570 ng/dose	Negative
EU946	Yellowish liquid	No growth observed	Complies	6.210 ng/dose	Negative
EU947	Yellowish liquid	No growth observed	Complies	9.500 ng/dose	Negative
EU948	Yellowish liquid	No growth observed	Complies	3.300 ng/dose	Negative
EU949	Yellowish liquid	No growth observed	Complies	2.980 ng/dose	Negative
EU950	Yellowish liquid	No growth observed	Complies	0.520 ng/dose	Negative

Table 25: Summary results of Tests on MMR vaccine (Final Bulk – 1 Dose)

SUMMARY RESULTS OF TESTS ON MEASLES, MUMPS AND RUBELLA VACCINE (FINAL BULK – 1 DOSE)					
BATCH NO.	TESTS				
	Description	Sterility	Virus Content	Residual Animal Protein	Mycoplasma
	Yellowish liquid	No growth should be observed in any of the sterility media	For information only	Not more than 50 ng / 0.5 mL dose	Negative
1070	Yellowish liquid	No growth observed	Complies	10.810 ng/dose	Negative
1071	Yellowish liquid	No growth observed	Complies	16.700 ng/dose	Negative
1072	Yellowish liquid	No growth observed	Complies	12.980 ng/dose	Negative
1073	Yellowish liquid	No growth observed	Complies	12.660 ng/dose	Negative
1074	Yellowish liquid	No growth observed	Complies	15.070 ng/dose	Negative
1075	Yellowish liquid	No growth observed	Complies	9.950 ng/dose	Negative
1076	Yellowish liquid	No growth observed	Complies	13.540 ng/dose	Negative
1077	Yellowish liquid	No growth observed	Complies	15.970 ng/dose	Negative
1078	Yellowish liquid	No growth observed	Complies	8.360 ng/dose	Negative
1079	Yellowish liquid	No growth observed	Complies	8.230 ng/dose	Negative
1080	Yellowish liquid	No growth observed	Complies	15.26 ng/dose	Negative
1081	Yellowish liquid	No growth observed	Complies	5.450 ng/dose	Negative

1082	Yellowish liquid	No growth observed	Complies	25.350 ng/dose	Negative
1083	Yellowish liquid	No growth observed	Complies	3.040 ng/dose	Negative
1084	Yellowish liquid	No growth observed	Complies	11.110 ng/dose	Negative
1085	Yellowish liquid	No growth observed	Complies	7.520 ng/dose	Negative
1086	Yellowish liquid	No growth observed	Complies	10.990 ng/dose	Negative
1087	Yellowish liquid	No growth observed	Complies	9.120 ng/dose	Negative
1088	Yellowish liquid	No growth observed	Complies	19.530 ng/dose	Negative
1089	Yellowish liquid	No growth observed	Complies	14.470 ng/dose	Negative
1090	Yellowish liquid	No growth observed	Complies	13.850 ng/dose	Negative

EXAMPLE 6: Clinical study of Measles, Mumps, Rubella Vaccine, Live Attenuated (Freeze-Dried) (MMR vaccine)

Clinical study of Measles, Mumps, Rubella Vaccine, Live Attenuated (Freeze-Dried) (MMR vaccine) as mentioned in Table 26 are performed considering seroconversion parameter and observing the adverse effects for evaluating immunogenicity and safety/ reactogenicity of Measles, Mumps, Rubella Vaccine, Live Attenuated (Freeze-Dried) (MMR vaccine)

Table 26: Clinical study of Measles, Mumps, Rubella Vaccine, Live Attenuated (Freeze-Dried) (MMR vaccine)

Product & Dose	Route of administration	Age group	Seroconversion		
			Measles	Mumps	Rubella
SIPL MMR vaccine (0.5 ml)	im (intramuscular)	9-12 months	81.00%	91.50%	100.00%
SIPL MMR vaccine (0.5 ml)	sc (subcutaneous)	9 – 18 months	74.30%	90.50%	94.50%
SIPL MMR vaccine (0.5 ml)	sc (subcutaneous)	15 – 18 months	84.00%	82.00%	84.00%
SIPL MMR vaccine (0.5 ml)	sc (subcutaneous)	18 – 24 months	88.50%	91.20%	98.20%

Seroconversion is the transition from the point of viral infection to when antibodies (IgG) of the virus become present in the blood. From Table 26, it was found that IgG positivity after immunization raised in the range 70% to 90% for measles virus, 80% to 92% for mumps virus and 80% to 100% for rubella virus. No any adverse effects were observed after immunization. This indicates that the SIPL MMR vaccine (0.5 ml dose) is immunogenic and safe.

EXAMPLE 7: Stability data of Live attenuated Lyophilized MMR Vaccine at 2-8°C for 3, 6, 9, 12, 18, 24, 30 months (M)**Table 27: Batch A - Stability data**

Test	Limits/ Specification	Initial	3M	6M	9M	12M	18M	24M	30M
Description	Yellowish white friable mass	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Particulate matter ($\geq 10\mu$)	Should be less than 6000 particles per container	152 particle/ container	181 particle/ container	185 particle/ container	102 particle/ container	169 particle/ container	106 particle/ container	195 particle/ container	155 particle/ container
Particulate matter ($\geq 25\mu$)	Should be less than 600 particles per container	2 particle/ container	4 particle/ container	2 particle/ container	1 particle/ container	1 particle/ container	1 particle/ container	6 particle/ container	2 particle/ container
Residual moisture content	Should be less than 2.000% w/w	0.500 % w/w	0.532 % w/w	0.534 % w/w	0.471 % w/w	0.451 % w/w	0.507 % w/w	0.407 % w/w	0.509 % w/w
Measles virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.783 Log10 CCID50/0.5 mL	3.792 Log10 CCID50/0.5 mL	3.708 Log10 CCID50/0.5 mL	3.667 Log10 CCID50/0.5 mL	3.625 Log10 CCID50/0.5 mL	3.542 Log10 CCID50/0.5 mL	3.333 Log10 CCID50/0.5 mL	3.250 Log10 CCID50/0.5 mL
Mumps virus concentration - Potency	NLT 3.7 Log10 CCID50/0.5 mL	4.617 Log10 CCID50/0.5 mL	4.583 Log10 CCID50/0.5 mL	4.542 Log10 CCID50/0.5 mL	4.542 Log10 CCID50/0.5 mL	4.625 Log10 CCID50/0.5 mL	4.542 Log10 CCID50/0.5 mL	4.417 Log10 CCID50/0.5 mL	4.333 Log10 CCID50/0.5 mL
Rubella virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.883 Log10 CCID50/0.5 mL	3.875 Log10 CCID50/0.5 mL	3.917 Log10 CCID50/0.5 mL	3.917 Log10 CCID50/0.5 mL	3.958 Log10 CCID50/0.5 mL	3.958 Log10 CCID50/0.5 mL	3.667 Log10 CCID50/0.5 mL	3.667 Log10 CCID50/0.5 mL

Table 28: Batch B - Stability data

Test	Limits/ Specification	Initial	3M	6M	9M	12M	18M	24M	30M
Description	Yellowish white friable mass	Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Particulate matter ($\geq 10\mu$)	Should be less than 6000 particles per container	239 particulate/ container	163 particulate/ container	338 particulate/ container	203 particulate/ container	306 particulate/ container	309 particulate/ container	282 particulate/ container	261 particulate/ container
Particulate matter ($\geq 25\mu$)	Should be less than 600 particles per container	2 particulate/ container	5 particulate/ container	4 particulate/ container	7 particulate/ container	8 particulate/ container	3 particulate/ container	1 particulate/ container	0 particulate/ container
Residual moisture content	Should be less than 2.000% w/w	0.460 % w/w	0.513 % w/w	0.434 % w/w	0.406 % w/w	0.410 % w/w	0.539 % w/w	0.417 % w/w	0.404 % w/w
Measles virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.883 Log10 CCID50/0.5 mL	3.792 Log10 CCID50/0.5 mL	3.792 Log10 CCID50/0.5 mL	3.625 Log10 CCID50/0.5 mL	3.667 Log10 CCID50/0.5 mL	3.417 Log10 CCID50/0.5 mL	3.500 Log10 CCID50/0.5 mL	3.458 Log10 CCID50/0.5 mL
Mumps virus concentration - Potency	NLT 3.7 Log10 CCID50/0.5 mL	4.717 Log10 CCID50/0.5 mL	4.708 Log10 CCID50/0.5 mL	4.625 Log10 CCID50/0.5 mL	4.625 Log10 CCID50/0.5 mL	4.500 Log10 CCID50/0.5 mL	4.292 Log10 CCID50/0.5 mL	4.333 Log10 CCID50/0.5 mL	4.417 Log10 CCID50/0.5 mL
Rubella virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.983 Log10 CCID50/0.5 mL	3.917 Log10 CCID50/0.5 mL	3.958 Log10 CCID50/0.5 mL	3.875 Log10 CCID50/0.5 mL	3.917 Log10 CCID50/0.5 mL	4.000 Log10 CCID50/0.5 mL	3.875 Log10 CCID50/0.5 mL	3.792 Log10 CCID50/0.5 mL

Table 29: Batch C - Stability data

Test	Limits/ Specification	Initial	3M	6M	9M	12M	18M	24M	30M
Description		Complies	Complies	Complies	Complies	Complies	Complies	Complies	Complies
Particulate matter ($\geq 10\mu$)	Yellowish white friable mass Should be less than 6000 particles per container	224 particle/ container	210 particle/ container	267 particle/ container	210 particle/ container	446 particle/ container	436 particle/ container	201 particle/ container	215 particle/ container
Particulate matter ($\geq 25\mu$)	Should be less than 600 particles per container	3 particle/ container	3 particle/ container	1 particle/ container	3 particle/ container	9 particle/ container	11 particle/ container	3 particle/ container	3 particle/ container
Residual moisture content	Should be less than 2.000% w/w	0.426 % w/w	0.522 % w/w	0.420 % w/w	0.431 % w/w	0.459 % w/w	0.457 % w/w	0.448 % w/w	0.382 % w/w
Measles virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.683 Log10 CCID50/0.5 mL	3.625 Log10 CCID50/0.5 mL	3.875 Log10 CCID50/0.5 mL	3.583 Log10 CCID50/0.5 mL	3.542 Log10 CCID50/0.5 mL	3.708 Log10 CCID50/0.5 mL	3.458 Log10 CCID50/0.5 mL	3.208 Log10 CCID50/0.5 mL
Mumps virus concentration - Potency	NLT 3.7 Log10 CCID50/0.5 mL	4.583 Log10 CCID50/0.5 mL	4.625 Log10 CCID50/0.5 mL	4.667 Log10 CCID50/0.5 mL	4.583 Log10 CCID50/0.5 mL	4.583 Log10 CCID50/0.5 mL	4.542 Log10 CCID50/0.5 mL	4.375 Log10 CCID50/0.5 mL	4.167 Log10 CCID50/0.5 mL
Rubella virus concentration - Potency	NLT 3.0 Log10 CCID50/0.5 mL	3.767 Log10 CCID50/0.5 mL	3.708 Log10 CCID50/0.5 mL	3.708 Log10 CCID50/0.5 mL	3.833 Log10 CCID50/0.5 mL	3.792 Log10 CCID50/0.5 mL	3.708 Log10 CCID50/0.5 mL	3.667 Log10 CCID50/0.5 mL	3.542 Log10 CCID50/0.5 mL

From Tables 27, 28 and 29, it was found that Live attenuated Lyophilized MMR Vaccine is stable at 2-8°C for 3, 6, 9, 12, 18, 24, 30 months (M).

WE CLAIM:

1. A method of producing a clarified virus pool, the method comprising:
 - a. providing a cell line in a cell media, a buffer and a supplement;
 - b. treating cells of the cell line with at least one enzyme;
 - c. infecting the cell line with a virus to form an infected cell line;
 - d. washing of the infected cell line with a virus media and the buffer;
 - e. harvesting the infected cell line in the media to obtain a harvest; optionally re-harvesting the infected cell line one or more times;
 - f. adding at least one stabilizer to the harvest; and
 - g. clarifying the harvest to obtain a clarified virus pool (CVP).optionally, the cell line or the infected cell line in exponential growth phase is
 - grown with additional ventilation or aeration, or
 - provided with re-addition of the buffer or
 - both.
2. The method as claimed in claim 1, wherein the virus is selected from single-stranded, positive-sense, negative-sense, enveloped, non-enveloped RNA viruses belonging to the family Picornaviridae, Caliciviridae, Togaviridae, Matonaviridae, Flaviviridae, Coronaviridae, Retroviridae, Filoviridae, Bunyaviridae, Rhabdoviridae, Orthomyxoviridae, Arenaviridae, Paramyxoviridae.
3. The method as claimed in claim 1 or 2, wherein the virus is single-stranded, negative-sense, enveloped, RNA viruses belonging to the family Paramyxoviridae and single-stranded, positive-sense, enveloped, RNA viruses belonging to the family Matonaviridae.
4. The method as claimed in any one of the claims 1 to 3, wherein the virus is Measles morbillivirus (measles virus), Mumps orthorubulavirus (mumps virus) and Rubivirus rubellae (rubella virus).
5. The method as claimed in any one of the claims 1 to 4, wherein the cell line is selected from animal cell line, mammalian, avian, insect cell line, human cell line, primary cell line, diploid cell line, continuous cell line, Rhesus monkey kidney (RhMK) cells; Primary rabbit kidney cells; Human foreskin fibroblasts; Chick embryo fibroblast (CEF); Human epidermoid carcinoma cells (HEp-2); Human lung carcinoma cells (A549); Human Cervix Epithelial (HeLa); African Green Monkey Kidney Epithelial (Vero); Human Lung Fibroblast (MRC-5); Human Lung Fibroblast (MRC-9); Mouse Embryo Fibroblast (NIH3T3); Mouse Connective Tissue Fibroblast (L929); Chinese Hamster Ovary

Fibroblast (CHO); Syrian Hamster Kidney Fibroblast (BHK-21); Human embryo Kidney Epithelial (HEK-293); Human Liver Epithelial (HepG2); Bovine Aorta Endothelial (BAE-1); Human Neuroblastoma Neuronal (SH-SY5Y); Mouse Myeloma Lymphoblast (NS0); Human Histiocytic Lymphoma Lymphoblast (U937); Human Leukemia Lymphoblast (HL60); Mouse B-cell Lymphoma Lymphoblast (WEHI231); Mouse Lymphoma Lymphoblast (YAC1); Human Myeloma Lymphoblast (U266B1); Human T-cell Leukemia Lymphoblast (Jurkat); Human Monocyte Leukemia Lymphoblast (THP-1); Human embryonic lung cells (W1-38); Madin Darby canine kidney cells (MDCK); Human embryonic retinal cells (PER.C6); Human embryonic retinoblasts (HER.911); Murine non-secreting myeloma (Sp2.0); Epithelial cells of African green monkey kidney origin (BSC-1 cells); Rhesus Monkey Kidney Epithelial Cells (LLC-MK2 cells); Cercopithecus aethiops monkey kidney cells (CV-1 cells); African green monkey kidney fibroblast-like cells (COS-cells); Crandell- Rees Feline Kidney Cells (CRFK cells); Rapidly Accelerated Fibrosarcoma cells (RAF cells); Normal Rabbit Kidney Epithelial Cells (RK-13 cells); Transformed C3H Mouse Kidney-1 (TCMK-1 cells); Pig Kidney Epithelial Cells (LLC-PK1 cells); Porcine kidney cells (PK15 cells); Rabbit kidney cell line (LLC-RK1 cells), Nonsecreting myeloma cell lines (NS-1 cells), New human male diploid cell strain (TIG-1, TIG-7); nonhuman primate diploid cell line (FRhL-2); Human foetal lung (IMR-90, IMR-91) cells; human diploid lung fibroblasts and others.

6. The method as claimed in any one of the claims 1 to 5, wherein the cell line is selected from Human Lung Fibroblast (MRC-5) cell line and Chick embryo fibroblast (CEF) cell line.
7. The method as claimed in any one of the claims 1 to 6, wherein the enzyme is selected from trypsin, recombinant trypsin, dipase, collagenase, hyaluronidase, elastase, cysteine protease, deoxyribonuclease I and chymotrypsin.
8. The method as claimed in any one of the claims 1 to 7, wherein the cell media includes basal media, enriched media, selective and indicator media, transport media, storage media, carbon sources, or combination thereof.
9. The method as claimed in any one of the claims 1 to 8, wherein the supplement includes amino acids, cholesterol, proteins, lactoferrin, linoleic acid, yeast extracts, serums, antioxidants, vitamins, antibiotics, nutrients, trace elements, adherence agents, extension factors, or combinations thereof.

10. The method as claimed in any one of the claims 1 to 9, wherein the buffer is selected from the NaHCO_3 , NaOH , NaCl , HEPES, PIPES, MES, phosphates, carbonates, Hank's, Earl's or combinations thereof.
11. The method as claimed in any one of the claims 1 to 10, wherein the virus media for washing the infected cell line is selected from basal media, enriched media, selective and indicator media, transport media, storage media, carbon sources, or combination thereof.
12. The method as claimed in any one of the claims 1 to 11, wherein the washing of the infected cell lines by the virus media is performed post infection with virus having MOI in the range of 1:5 to 1: 60 and is followed by incubation at 30 to 40°C.
13. The method as claimed in any one of the claims 1 to 12, wherein the at least one stabilizer includes at least one carbohydrate, at least one protein, at least one amino acid, or a combination thereof.
14. The method as claimed in any one of the claims 1 to 13, wherein the clarification of the harvest is carried out using filters, preferably the filters are of material of construction selected from modified PVDF, cellulose acetate, polyethersulfone, polypropylene.
15. The method as claimed in any one of the claims 1 to 14, wherein the clarification of the harvest is carried out using at least one filter having pore size in the range of 0.1 to 10.0 μ .
16. The clarified virus pool (CVP) obtained by the method as claimed in any one of the claims 1 to 15.
17. The method as claimed in any one of the claims 1 to 16, for producing a measles clarified virus pool, the method comprising:
 - a. providing the cell line in a cell media, the buffer and the supplement;
 - b. treating cells of the cell line with the enzyme;
 - c. infecting the cell line with Measles morbillivirus to form the measles infected cell line;
 - d. washing of the measles infected cell line with the virus media and the buffer;
 - e. harvesting the measles infected cell line in the media to obtain the harvest; optionally re-harvesting the measles infected cell line;
 - f. adding the stabilizer to the harvest; and
 - g. clarifying the harvest to obtain the measles clarified virus pool (CVP).

optionally, wherein the cell line or the measles infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
 - provided with re-addition of the buffer or
 - both.
18. The method as claimed in claim 17, wherein the cell line is Human Lung Fibroblast (MRC-5) cell line.
19. The method as claimed in any one of the claims 17 or 18, wherein the enzyme is recombinant trypsin.
20. The method as claimed in any one of the claims 17 to 19, wherein the cell media is Minimum Essential Medium.
21. The method as claimed in any one of the claims 17 to 20, wherein the supplement includes glutamine and foetal bovine serum.
22. The method as claimed in claim 21, wherein foetal bovine serum is in the range 5% to 15%.
23. The method as claimed in claim any one of the claims 17 to 22, wherein the virus media is Minimum Essential Medium.
24. The method as claimed in any one of the claims 17 to 23, wherein the buffer comprises of sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4.0 g/L to maintain optimal pH of 6 to 8.
25. The method as claimed in any one of the claims 17 to 24, wherein the measles virus includes Edmonston strain, including the Schwartz, the Edmonston-Zagreb, the Moraten strains; CAM-70; TD 97; Leningrad-16; AIK-C strain and Shanghai 191 (Ji-191) strains.
26. A measles clarified virus pool (CVP) obtained by the method as claimed in any one of claims 17 to 25.
27. The method as claimed in any one of the claims 1 to 16 for producing a mumps clarified virus pool, the method comprising:
- a. providing the cell line in the cell media, the buffer and the supplement;
 - b. treating cells of the cell line with the enzyme;
 - c. infecting the cell line with the Mumps orthorubula virus to form a mumps infected cell line;
 - d. washing of the mumps infected cell line with the virus media and the buffer;
 - e. harvesting the mumps infected cell line in the media to obtain the harvest; optionally re-harvesting the mumps infected cell line;
 - f. adding the stabilizer to the harvest; and
 - g. clarifying the harvest to obtain the mumps clarified virus pool (CVP).

optionally, wherein the cell line or the mumps infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or
- both.

28. The method as claimed in claim 27, wherein the cell line is Chick embryo fibroblast (CEF) cell line.
29. The method as claimed in claim 27 or 28, wherein the enzyme is recombinant trypsin.
30. The method as claimed in any one of the claims 27 to 29, wherein the cell media is Minimum Essential Medium.
31. The method as claimed in any one of the claims 27 to 30, wherein the supplement includes glutamine, foetal bovine serum, and neomycin sulphate.
32. The method as claimed in claim 31, wherein foetal bovine serum is in the range 5% to 15%.
33. The method as claimed in any one of the claims 27 to 32, wherein the virus media is Minimum Essential Medium.
34. The method as claimed in any one of the claims 27 to 33, wherein the buffer is sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4.0 g/L to maintain optimal pH of 6 to 8.
35. The method as claimed in any one of the claims 27 to 34, wherein the virus includes Jeryl-Lynn, RIT 4385, Leningrad-3, Leningrad-Zagreb (L-Zagreb), Urabe Am9, Hoshino strain, Torii strain and S79 Rubini strains.
36. A mumps clarified virus pool (CVP) obtained by the method as claimed in any one of the claims 27 to 35.
37. The method as claimed in any one of the claims 1 to 16 for producing a rubella clarified virus pool, the method comprising:
 - a. providing the cell line in the cell media and the supplement;
 - b. treating cells of the cell line with the enzyme;
 - c. infecting the cell line with the Rubivirus rubellae to form a rubella infected cell line;
 - d. washing of the rubella infected cell line with the virus media and the buffer;
 - e. harvesting the rubella infected cell line in the media to obtain the harvest; optionally re-harvesting the rubella infected cell line;
 - f. adding the stabilizer to the harvest; and
 - g. clarifying the harvest to obtain the rubella clarified virus pool (CVP).

optionally, wherein the cell line or the rubella infected cell line in exponential growth phase is

- grown with additional ventilation or aeration, or
- provided with re-addition of the buffer or
- both.

38. The method as claimed in claim 37, wherein the cell line is Human Lung Fibroblast (MRC-5) cell line.
39. The method as claimed in claim 37 or 38, wherein the enzyme is recombinant trypsin.
40. The method as claimed in any one of claims 37 to 39, wherein the cell media is Minimum Essential Medium.
41. The method as claimed in any one of the claims 37 to 40, wherein the supplement includes glutamine, foetal bovine serum, neomycin sulphate or combination thereof.
42. The method as claimed in claim 41, wherein foetal bovine serum is in the range 5% to 15%.
43. The method as claimed in any one of the claims 37 to 42, wherein the virus media is Minimum Essential Medium.
44. The method as claimed in any one of the claims 37 to 43, wherein the buffer is sodium bicarbonate added to virus media and the buffer is in the range 0.5 to 4 g/L to maintain optimal pH of 6 to 8.
45. The method as claimed in any one of the claims 37 to 44, wherein the virus includes Wister RA 27/3 strain, BRD-2 strain, Matsuba, DCRB19, Takahashi, Matsuura and TO-336 strains.
46. A rubella clarified virus pool (CVP) obtained by the method as claimed in any one of claims 37 to 45.
47. A method of obtaining a lyophilized/ freeze-dried MMR immunogenic composition comprising at least one CVP selected from a measles CVP, a mumps CVP, a rubella CVP or a combination thereof, the method comprising:
providing a measles CVP of claim 26 or obtained by any one of claims 17 to 25, a mumps CVP of claim 36 or obtained by any one of claim 27 to 35, a rubella CVP of claim 46 or obtained by any one of claims 37 to 45, or a combination thereof,
blending of the CVPs, followed by lyophilizing the blended CVPs.
48. The method as claimed in claim 47, wherein the method includes:
a) thawing at least one CVP of measles, mumps, rubella virus or combination thereof at 30 to 35°C to obtain the thawed CVP;

- b) blending the thawed CVP and blind vaccine to obtain a blended solution;
- c) clarifying the blended solution through a 0.45 μ filter to obtain a homogenous bulk;
- d) aseptically filling the homogenous bulk into sterilized vials, followed by transferring the vials to a lyophilizer/ freeze dryer.
- e) lyophilizing/ freeze drying the vials containing the homogenous bulk.

49. The method as claimed in claim 48, wherein the lyophilizing/ freeze-drying step comprises:

- a) pre-freezing shelf, loading the trays containing vials of the MMR immunogenic composition on the shelf;
- b) freezing;
- c) primary drying/ sublimation and
- d) secondary drying

50. A lyophilized/ freeze-dried MMR immunogenic composition obtained by the method as claimed in claim 49, the composition comprising;

- a) atleast one virus;
- b) stabilizer comprising atleast one carbohydrate, atleast one amino acid and atleast one hydrolyzed protein.

51. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose.

52. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of atleast one carbohydrate selected from a group consisting of natural carbohydrate, synthetic carbohydrate, monosaccharides, disaccharides, trisaccharides, oligosaccharides, reducing sugar, non-reducing sugar, sugar alcohols, polyol, polyhydroxyl compounds, chemically modified carbohydrates and glass transition facilitating agents which include sucrose, mannitol, trehalose, mannose, raffinose, lactitol, lactobionic acid, glucose, maltulose, iso- maltulose, maltose, lactose sorbitol, dextrose, fructose, glycerol, sorbitol, and fucose and a combination thereof.

53. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of atleast one amino acid selected from a group consisting of tricine, leucine, iso-leucine, L-histidine, glycine, glutamine, L-arginine, L-arginine hydrochloride, lysine, L-alanine, Tryptophan, Phenylalanine, Tyrosine, Valine, Cysteine, Glycine, Histidine, Methionine, Proline, Serine, Threonine and a combination thereof.
54. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of atleast one hydrolyzed protein obtained by chemical, enzymatic or thermal hydrolysis of protein from either plant or animal sources.
55. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of at least one hydrolyzed protein selected from a group consisting of gelatin, lactalbumin hydrolysate, monosodium glutamate, collagen hydrolysate, keratin hydrolysate, peptides, Casein hydrolysate and whey protein hydrolysate.
56. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, wherein the composition comprises;
- d) at least one carbohydrate at concentration range of 1-20% (w/v)
 - e) at least one amino acid at concentration range of 0.01-10% (w/v);
 - f) at least one hydrolyzed protein at concentration range of 0.1-10% (w/v)
57. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 56, wherein at least one of the carbohydrate is sorbitol present at a concentration of 1 to 20% (w/v), 1 to 10% (w/v), preferably 3-6% (w/v).
58. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 56, wherein at least one of the amino acid selected from a group consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v).
59. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 56, wherein atleast one of the hydrolysed protein selected from a group consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).
60. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of an adjuvant selected from a group consisting of aluminum hydroxide, aluminum phosphate, aluminum hydroxyphosphate, and potassium aluminum sulfate or a mixture thereof.

61. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of an immunostimulatory component selected from a group consisting of an oil and water emulsion, MF-59, a liposome, a lipopolysaccharide, a saponin, lipid A, lipid A derivatives, Monophosphoryl lipid A, 3-deacylated monophosphoryl lipid A, AS01, AS03, an oligonucleotide, an oligonucleotide comprising at least one unmethylated CpG and/or a liposome, Freund's adjuvant, Freund's complete adjuvant, Freund's incomplete adjuvant, CRL-8300 adjuvant, muramyl dipeptide, TLR-4 agonists, flagellin, flagellins derived from gram negative bacteria, TLR-5 agonists, fragments of flagellins capable of binding to TLR-5 receptors, QS-21, ISCOMS, Chitosan, saponin combination with sterols and lipids.
62. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, comprising of a pharmaceutically acceptable additive selected from a group consisting of transporter, excipient, binder, carrier, isotonic agent, emulsifier and humectant.
63. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, wherein the excipient is selected from a group consisting of salt including NaCl, KCl, KH_2PO_4 , $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, CaCl_2 , and MgCl_2 ; non-ionic surfactant including polysorbate 20, polysorbate 40, polysorbate 60, polysorbate 65, polysorbate 80, polysorbate 85, nonylphenoxypolyethoxethanol, octylphenoxypolyethoxethanol, octoxynol 40, nonoxynol- 9, triethanolamine, triethanolamine polypeptide oleate, polyoxyethylene- 660 hydroxystearate, polyoxyethylene- 35 ricinoleate, soy lecithin and a poloxamer - 0.001%- 0.05%; polymers including dextran, carboxymethylcellulose, hyaluronic acid and cyclodextrin.
64. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 50, wherein the lyophilized/freeze-dried viral vaccine composition is reconstituted with an aqueous solution selected from a group consisting of saline, buffer and WFI (water for injection).
65. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 64, wherein the buffer is selected from a group consisting of sodium chloride, acetate, carbonate, citrate, lactate, gluconate, tartrate, phosphate buffer saline, borate, histidine buffer, succinate buffer, HEPES, TRIS and Citrate-phosphate.
66. The lyophilized/ freeze-dried MMR immunogenic composition as claimed in claim 64, wherein the final pH of the reconstituted composition is in the range of pH 6.5 to 7.5.
67. The lyophilized/freeze-dried MMR immunogenic composition as claimed in any one of the claims 50-66, comprising:

- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
 - b) stabilizer comprising
 - carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v).
68. The lyophilized/freeze-dried MMR immunogenic composition as claimed in claim 67, comprising:
- a) live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose;
 - b) stabilizer comprising
 - carbohydrate consisting of sorbitol present at a concentration of 5% (w/v);
 - amino acid consisting of tricine present at a concentration of 0.3% (w/v), L-histidine present at a concentration of 0.21% (w/v), L-alanine present at a concentration of 0.1% (w/v) and L-arginine hydrochloride present at a concentration of 1.6% (w/v); and
 - hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.35% (w/v).
69. The lyophilized/freeze-dried MMR immunogenic composition as claimed in claim 68, wherein the lyophilized virus vaccine composition is in the form of a single dose composition or a multi-dose composition.
70. The lyophilized/freeze-dried MMR immunogenic composition as claimed in claim 69, wherein the multi-dose composition additionally comprises preservative.
71. A kit comprising the lyophilized/freeze-dried MMR immunogenic composition as claimed in claim 67 or 68 comprises;

- a) a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration of 1 to 10% (w/v); amino acid consisting of tricine present at a concentration of 0.1% to 2% (w/v), L-histidine present at a concentration of 0.1% to 2% (w/v), L-alanine present at a concentration of 0.01% to 1% (w/v) and L-arginine hydrochloride present at a concentration of 0.1% to 5% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 0.1% to 5% (w/v) and lactalbumin hydrolysate present at a concentration of 0.1% to 2% (w/v); and
 - b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.
72. A kit comprising the lyophilized/freeze-dried MMR immunogenic composition as claimed in claim 67 or 68 comprises;
- a) a first container containing a lyophilized (freeze-dried) viral vaccine composition said composition comprising: live attenuated measles virus present at a dose of not less than 1000 CCID₅₀ per dose, live attenuated mumps virus present at a dose of not less than 5000 CCID₅₀ per dose and live attenuated rubella virus present at a dose of not less than 1000 CCID₅₀ per dose; carbohydrate consisting of sorbitol present at a concentration 5% (w/v); amino acid consisting of tricine present at a concentration 0.3% (w/v), L-histidine present at a concentration 0.21% (w/v), L-alanine present at a concentration 0.1% (w/v) and L-arginine hydrochloride present at a concentration 1.6% (w/v); and hydrolyzed protein consisting of gelatin present at a concentration of 2.5% (w/v) and lactalbumin hydrolysate present at a concentration 0.35% (w/v); and
 - b) a second container containing an aqueous solution selected from saline or water for injection (WFI) for the reconstitution of the lyophilized (freeze-dried) vaccine composition.