



The Development of Live, Attenuated Rotavirus Vaccines

A Manufacturer's Resource Guide

Developed by PATH's Rotavirus Vaccine
Program—An Accelerated Development and
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About PATH

PATH is an international, nonprofit organization that creates sustainable, culturally relevant solutions, enabling communities worldwide to break longstanding cycles of poor health. By collaborating with diverse public- and private-sector partners, we help provide appropriate health technologies and vital strategies that change the way people think and act. www.path.org



About the GAVI Alliance

An alliance of all the major stakeholders in immunization, the GAVI Alliance includes among its partners developing country and donor governments, the World Health Organization (WHO), UNICEF, the World Bank, the vaccine industry in both industrialized and developing countries, research and technical agencies, NGOs, and the Bill & Melinda Gates Foundation. It is estimated that more than 1.7 million early deaths will have been prevented as a result of support by GAVI up to the end of 2005.



GAVI's efforts are critical to achieving the Millennium Development Goal on child health, which calls for reducing childhood mortality by two-thirds by 2015. Of the more than 10 million children who die before reaching their fifth birthday every year, 2.5 million die from diseases that could be prevented with currently available or new vaccines. www.gavialliance.org

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About this guide

This guide is a comprehensive compilation of publicly available information intended to serve as a resource for vaccine manufacturers interested in developing and registering an attenuated, oral rotavirus vaccine. It highlights various decisions to be made during development; provides examples of preclinical studies, manufacturing processes, assay procedures, and clinical trial strategies; and presents the advantages and disadvantages of multiple scenarios at each development milestone.

Limitations

The information provided is generic to the development of a live-virus, tissue culture-produced vaccine, potentially applicable to a broad spectrum of rotavirus vaccine candidates; however, processes, procedures, and protocols will almost certainly need to be modified to meet the specific needs of a particular vaccine development program.

While the information contained in this guide is considered to be in the public domain, it remains the responsibility of each manufacturer to investigate the intellectual property status of all technologies and reagents used.

This guide intends only to educate the manufacturer in seeking the appropriate information and guidance from national control authorities. It is not a regulatory document and cannot be used to replace or supplement official guidance documents from national control authorities.

Other resources

In addition to this guide, the World Health Organization (WHO) will soon publish the *WHO Guidelines to Assure the Quality, Safety and Efficacy of Live Attenuated Rotavirus Vaccines (Oral)* in 2006. These WHO guidelines provide a definitive series of recommendations for any manufacturer interested in rotavirus vaccines.

A list of consultants available to assist in the development of rotavirus vaccines can be obtained by contacting PATH (see title page for contact information).

Tell us what you think

Given that the science of rotavirus vaccine development is continually advancing, this guide will be updated periodically—if manufacturers find it useful. Please contact us to provide comments on the guide (see title page for contact information).

An overview of rotavirus and development of a vaccine

The high incidence of rotavirus disease around the world has prompted international agencies, including the World Health Organization (WHO), the Global Alliance for Vaccines and Immunization (GAVI), and PATH, to identify the development of a rotavirus vaccine as a high priority. These organizations are helping establish regional rotavirus surveillance networks in the countries most affected by rotavirus disease. The wealth of data being generated by these surveillance networks will help vaccine manufacturers design optimal vaccines and, most importantly, set the political and economic framework for distributing vaccine in the countries most in need—in effect, “priming” these countries for when the vaccine becomes available.

This section describes the rotavirus disease burden, the virus, and the status of vaccine development.

Disease burden

Rotavirus is the most common cause of severe gastroenteritis in infants worldwide. It causes severe diarrhea and vomiting, resulting in the death of one infant every minute due to dehydration, according to the most recent mortality estimates reported at the 2004 Sixth International Rotavirus Symposium in Mexico City.¹ Worldwide, rotavirus is associated with 5 percent of deaths in children younger than 5 years. The highest incidence of deaths is found in developing nations of Asia, Africa, and the Americas.^{2,3}

The incidence of disease is staggering. Each year, rotavirus causes approximately 111 million episodes of gastroenteritis requiring home care, 25 million clinic visits, 2 million hospitalizations, and approximately 500,000 deaths.^{4,5}

All children around the world, regardless of economic or geographic boundaries, become infected with rotavirus in the first few years of life. The disease in the majority of children is mild to moderate and self-limited. Once infected, natural immunity generally prevents children from acquiring subsequent episodes of severe rotavirus diarrhea, but asymptomatic or mildly symptomatic re-infection is common. However in 10 to 20 percent of the infected children, the diarrhea will be

¹ Wuethrich B, de Quadros CA, Glass RI, Andrus JK, Santos JI, Steele D. *Rotavirus and Rotavirus Vaccines: Proceedings of the Sixth International Rotavirus Symposium*, July 7–9, 2004, Mexico City. Washington, DC: Albert B. Sabin Vaccine Institute; 2005. Available at: http://www.rotavirusvaccine.org/documents/MexicoCityProceedings_000.pdf.

² Glass RI, Bresee JS, Parashar U, et al. Rotavirus vaccines: past, present, and future. *Archives of Pediatrics*. 2005;12(6):844–847.

³ Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases*. 2003;9(5):565–572.

⁴ Widdowson MA, Bresee JS, Gentsch JR, Glass RI. Rotavirus disease and its prevention. *Current Opinion in Gastroenterology*. 2005;21(1):26–31.

⁵ Parashar UD, Hummelman EG, Bresee JS, Miller MA, Glass RI. Global illness and deaths caused by rotavirus disease in children. *Emerging Infectious Diseases*. 2003;9(5):565–572.

severe enough to require medical attention. When available, oral rehydration therapy is usually effective in treating acute watery diarrhea, but in some cases of severe dehydration, hospitalization with intravenous rehydration may be required.

The serotypes

The majority of rotavirus disease has been caused by five distinct serotypes of rotavirus, designated G1, G2, G3, G4, and G9 based on the molecular composition of the outer coat viral glycoprotein (VP7). In the last decade intense rotavirus surveillance and better laboratory assay methods have resulted in the detection of additional G serotypes that play a role in human disease. Although serotypes G1 to G4 and G9 are still the predominate serotypes around the world, serotypes G5 and G8 have been isolated in Latin America, India, and Africa and are beginning to cause a higher percentage of disease.

Rotavirus serotype is also determined by a second protein on the viral surface (VP4), known as the P-type. This protein is less diverse than VP7 among human strains and may account in part for the heterotypic immunity observed following most primary rotavirus infections.

Status of vaccine development (as of February 2006)

The prevalence of multiple and different rotavirus serotypes throughout the world complicates vaccine development strategies. Fortunately the two major vaccines currently in clinical trials by Merck (RotaTeq[®]) and GlaxoSmithKline (Rotarix[®]), based on entirely different approaches, will provide data to help shape decisions on future vaccine compositions.

Merck and GSK vaccines

The Merck bovine-human reassortant vaccine contains five antigens (G1 to G4 and P1), whereas the GSK vaccine contains a single, attenuated human rotavirus serotype, G1P1. Both vaccines have been shown to have similar efficacy against any rotavirus gastroenteritis, to have up to 90 to 100 percent efficacy against severe rotavirus gastroenteritis, and to have heterotypic protection against multiple virus serotypes.^{6,7} Recent licensure of Rotarix[®] in predominately Latin American countries and of RotaTeq[®] in the US will provide additional postmarketing effectiveness data against nonvaccine serotypes. Both vaccines are also in clinical trials for efficacy in developing nations.

Other vaccine strategies

Some other vaccine strategies being pursued include additional bovine virus-based human reassortants developed at the National Institutes of Health in Washington, DC; a rhesus rotavirus-based human reassortant (Rotashield[®]); and vaccines based on attenuated strains of rotavirus that

⁶ Ruiz-Palacios, GM, Perez-Schael, I, Velazquez, RF, et al. Safety and efficacy of an attenuated vaccine against severe rotavirus gastroenteritis. *New England Journal of Medicine*. 2006;354:11–21. Available at: <http://content.nejm.org/cgi/content/full/354/1/11>.

⁷ Vesikari, T, Matson, D, Dennehy, P, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *New England Journal of Medicine*. 2006;354:23–33. Available at: <http://content.nejm.org/cgi/content/full/354/1/23>.

enter newborn nurseries in hospitals—known as neonatal strains—being developed in Australia and India.

The story of Rotashield®

The development of any new rotavirus vaccine cannot ignore the events that led to the withdrawal of Wyeth’s rhesus rotavirus vaccine, RotaShield®, from the marketplace. RotaShield® was introduced in the US in October 1998 and was withdrawn in less than a year when epidemiologists established a temporal relationship between RotaShield® administration and intussusception. Preliminary estimates suggested a risk of one case per 2,500 children immunized, but more extensive analysis estimated this rate to be approximately one case per 10,000 children or fewer. Recent re-analysis of the CDC case-control study that examined intussusception and RotaShield® revealed that intussusception was linked to the age of the child at vaccination.⁸

The majority of intussusceptions occurred in children 3 months of age or older, which did not conform to the manufacturer’s recommendation that the first dose of the vaccine be given at 2 months of age. Estimates of attributable risk of intussusception were higher in older infants, but the risk of intussusception had not increased when infants had received the first dose of RotaShield® prior to 60 days of age.⁹ The detailed pathogenic mechanisms for intussusception are not defined but are very likely complex. Age-specific risk factors for intussusception suggest that early delivery of the first dose of vaccine could be desirable.

Going forward

The production processes and test methods for the manufacture of rotavirus vaccines are well understood, and compared to many production processes, they are straightforward and have been used previously to make other vaccines. Consequently, global vaccination against rotavirus disease should not be hampered by lack of vaccine candidates, lack of safety and efficacy experience, lack of acceptance in developing countries, nor lack of manufacturing expertise. Rather, the limiting factor will be lack of global manufacturing and distribution capacity required for worldwide vaccination.

Because the costs of vaccines are inversely proportional to the volume of vaccine produced, it will be incumbent on the countries in need to establish manufacturing capability. Keeping the manufacturing process, formulation, and delivery devices simple and easy to transfer around the globe will be essential to achieving global vaccination. The remaining sections of this guide will address ways to achieve this goal.

⁸ Simonsen L, Viboud C, Elixhauser A, Taylor RJ, Kapikian AZ. More on RotaShield and intussusception: the role of age at the time of vaccination. *The Journal of Infectious Diseases*. 2005;192(Suppl 1):S36–S43.

⁹ Simonsen L, Viboud C, Elixhauser A, Taylor RJ, Kapikian AZ. More on RotaShield and intussusception: the role of age at the time of vaccination. *The Journal of Infectious Diseases*. 2005;192(Suppl 1):S36–S43.

Manufacturing a rotavirus drug substance and product

Manufacturers of live-virus vaccines face challenges that are unique to the biopharmaceutical industry. The need to maintain virus viability throughout the production process, limited ability for clearance of potential adventitious agents, the need to maintain genetic stability of the vaccine virus, the difficulties of characterizing a complex biological, and the need to maximize yield have presented challenges to the industry. Nevertheless, due especially to continued advances in the science and technology, a large number of live-virus vaccines have been successfully developed and commercialized by many pharmaceutical companies.

There are many production processes, formulations, container closure systems, registration strategies, and clinical development strategies that can be followed in the development of a rotavirus vaccine. The decision to choose one process over another or one registration pathway over another is often driven by regulatory or compliance standards or economics, and the right decision can substantially accelerate vaccine registration.

For example, the yield or amount of virus produced per milliliter of cell culture medium versus the amount of virus required in a dose of final vaccine is critical. A virus-cell culture system that produces 100 to 1000 times the amount of virus required in the final dose will substantially drive down the cost and complexity of manufacturing facility and process. The stability of the vaccine and the delivery system chosen for the final product and antacid can easily contribute 50 percent of the cost to produce the vaccine. Assays selected for characterization and lot release can require large investment in equipment and expertise.

This section highlights advances in vaccine development that can expedite the approval process and reduce the manufacturing cost of the vaccine.

Good vaccine development practices

Vaccine manufacturers that may not have the luxury of prior experience in global licensure of vaccines can benefit from the lessons of others. As essential as good manufacturing practices, or GMPs, are for commercial vaccine manufacturing, establishing good vaccine *development* practices is equally important in managing the regulatory process toward product registration. Good vaccine development practices may include the following steps:

Characterize the vaccine early and often in the development process

The more data a company has on a new vaccine from the first preclinical batch produced in a non-GMP environment to consistency or process-validation batches, the easier a product will advance through the process. For example, manufacturing changes will occur during process development. Data on significant quality attributes of the vaccine, such as process recovery, yield, and impurity profiles, can be used to assess the impact of future process changes. Stability-indicating assays can be identified early that will drive formulation development. Developing assays to characterize a vaccine will often lead to lot-release assays that have specific relevance to the product or possibly to an in vitro correlate of vaccine efficacy.

Validate assays and develop reference standards early in the development process

Data collected during the development phase are only as valid as the assays used to generate the data. A common mistake is to base process development decisions on data from invalid assays, then find that the data are different after the assay has been validated. Reference standards from vaccine lots used to establish safety and efficacy and reagents used to measure critical quality attributes of the vaccine should be identified early in the process. This will allow time to plan stability programs for these standards and identify long-term supply of the standards.

Define the manufacturing processes before entering into phase 3 efficacy studies

Time spent developing a robust and scalable process before production of phase 3 vaccine will reduce the likelihood of making process changes late in vaccine development. The consequence that a process change may modify a critical quality attribute of the vaccine can be devastating. Regulatory agencies understand that a process will need to be scaled from the one used to produce phase 1 clinical supplies to the one selected to manufacture consistency or process-validation batches. The key is to select a process that can be expanded to commercial scale without the need for different technology (for example, changing from flasks to microcarriers) or changes in cell substrate or passage level of the virus.

Collect data from pre-approved protocols

The vaccine manufacturing process should be developed and data compiled through the execution of pre-approved protocols. For example, when experiments are performed to define the optimum concentration of trypsin needed for virus seed activation, these experiments should be run under an approved protocol. Data from this protocol can be summarized in a report that can be used (in 2 to 3 years) to define the operating range (that is, the minimum and maximum concentration of trypsin) for this step. These data will ultimately be needed for process validation, so it is wise to document early.

Preclinical vaccine development milestones

Once a rotavirus vaccine development program has been initiated, a well thought out vaccine development strategy should be documented—both to help organize the individuals involved and to identify weaknesses in the program, so they can be mitigated. The strategy should define tasks that need to be completed and milestones that indicate progress toward goals, the first of which would be to enter into a phase 1 safety evaluation in humans. Several important steps for achieving the first goal are described below.

Develop a process for manufacture and production of clinical vaccine supplies

The emphasis initially is to establish a process with sufficient virus yield, purity, and stability to begin a phase 1 safety study. Virus yield and impurity levels are important. The virus yield will determine whether the formulation of a final product is even possible and whether the overall program is viable, as extremely poor yields can doom the program from the beginning. Because the phase 1 trials are safety trials, the level of impurities will help define a “not to exceed” specification for phase 2 and 3 trials.

Another important step is selecting a manufacturing location that is compliant with good manufacturing practices and that has the quality assurance systems required for producing live-virus

vaccine for clinical trials. Although the manufacturing processes will be further developed as the project matures, the design, construction, and validation of a new manufacturing facility or the identification of a contract facility for commercial production is often a rate-limiting stage in obtaining product registration. Therefore, the earlier a final manufacturing process is defined, the better.

Develop a stable vaccine formulation and buffer system

A final product formulation that ensures stability of the vaccine virus until completion of the phase 1 trial is needed. Vaccine made from process-development studies is used for development of the early formulation, although the formulation does not have to be the one selected for later clinical trials. A method of packaging and shipping the vaccine to the clinical sites needs to be identified. Most often, live-virus vaccines used for phase 1 studies are stored as a liquid, frozen at $<-60^{\circ}\text{C}$, but this is rarely or ever the final storage or shipping condition of the commercial product.

For an orally administered, acid-labile, live-virus rotavirus vaccine, an acid-neutralizing buffer formulation should be chosen early in the clinical evaluation of the vaccine. In this case the final formulation should be as close as possible to the one intended for registration, because the method used for acid neutralization may affect vaccine take, safety, immunogenicity, and potency.

Conduct preclinical studies and develop lot-release assays

Before initiating phase 1 trials, the manufacturer will need sufficient data on the vaccine to ensure the safety of the virus seed, cell substrate, and final vaccine. A testing facility can be contracted or established in-house to perform the necessary studies.

At a minimum the *in vivo* and *in vitro* tests should be performed as well as preclinical toxicology studies as required by the national control authority. Since there are no widely accepted and readily available standard *in vitro* or *in vivo* models to assess rotavirus vaccine safety, attenuation, immunogenicity, or efficacy, this guide provides alternative testing strategies. Additional testing requirements will be defined in pre-phase 1 meetings with the control authority.

Develop clinical study protocol

Efforts to design and obtain regulatory approval of a phase 1 safety trial should begin at least nine months before the start of the trial. Key elements include preparing clinical protocols, identifying clinical investigators, obtaining approvals by institutional review boards, and meeting to discuss clinical protocol with the control authority in the country where the clinical trial will be performed. In addition, the quantity of vaccine required to conduct the study should be known and good clinical practice oversight should be in place.

Manufacturing process

Figures 1 and 2 depict the significant elements common to the production of most live-virus vaccines. The production process for bulk drug substance is repeated for each virus serotype or strain planned to be used in the final formulation and is generally known as the single virus harvest pool.

Multiple batches of single viral harvests of the same serotype can be processed either individually or pooled with other single viral harvests (produced from the same production run of cells) to produce a single virus harvest pool before downstream processing. Each single virus harvest has a

Figure 1. Overview of rotavirus vaccine manufacturing process for production of single virus harvest.

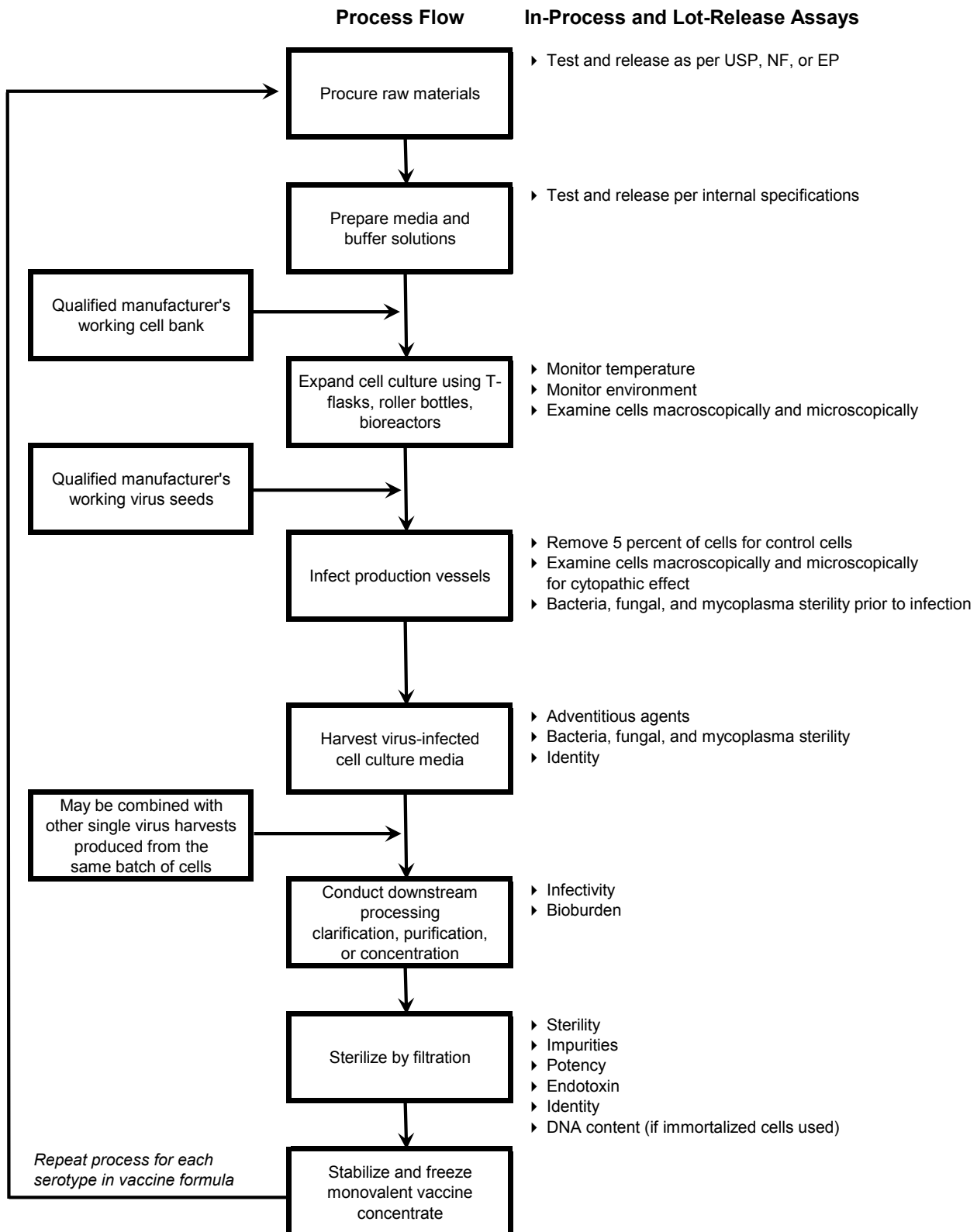
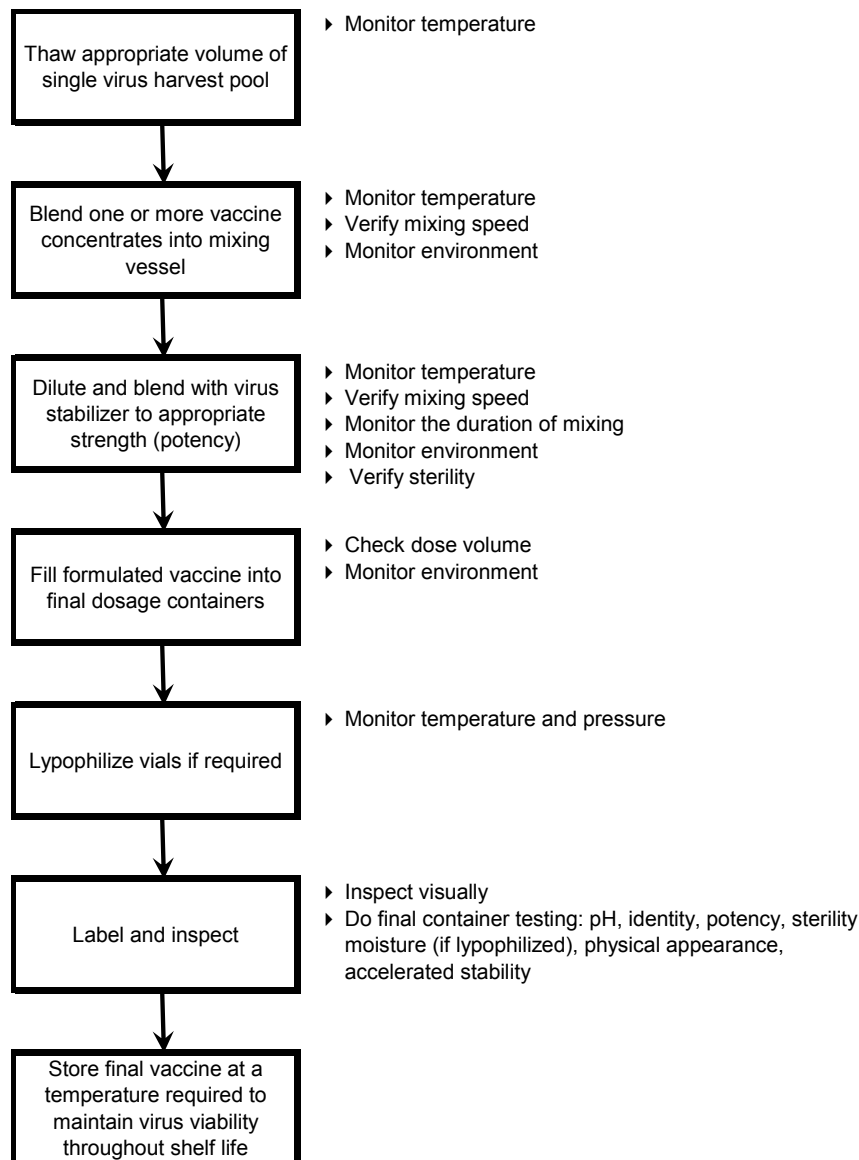


Figure 2. Overview of rotavirus vaccine manufacturing process production of drug product



corresponding batch of control cells that are tested. Each single virus harvest pool is tested individually and stored frozen at low temperatures until formulated into the final product. Stability data on the vaccine concentrates normally permit storage from 18 to 36 months—sufficient time to stockpile an inventory well in advance of formulating the final product for initiation of clinical trials or commercial distribution.

Raw materials and components

The extent to which raw materials are purchased and used for the production of buffer and media solutions for cell and virus growth, purification processes, and formulation depends on the company’s ability to manufacture high-quality and consistent buffers and media solutions within their own facility. If contracting production to a second party, systems for procuring raw materials and storing and testing them become less important.

The production facility should contain an area with controlled access and environmental controls (temperature and humidity) for sampling and storing incoming raw material. Raw materials are tested and released according to European or United States pharmacopeias or assay methods developed by the company. Where two assay methods exist for the same compound, the manufacturer should compare them and choose the more stringent method, to satisfy both regulatory bodies.

It is often more convenient and cost-effective to contract the testing of raw materials. The assays are generally chemical, are compendial, and will likely have already been validated by the contract laboratory. Once a number of lots have been received from the vendor and tested by the company, the vendor can be “qualified,” and release of the raw material can be based on certificates of analysis and compliance from the vendor along with routine auditing of the vendor.

Serum used for the propagation of cells should be tested to demonstrate freedom from bacteria, fungi, mycoplasmas, and infectious viruses. Validated molecular tests for bovine viruses may replace the cell culture tests of bovine sera. Irradiation may be used to inactivate potential contaminant viruses.

Trypsin used for preparing cell cultures and aiding in virus infection should also be tested and found free of cultivable bacteria, fungi, mycoplasmas, and infectious viruses—especially bovine or porcine parvoviruses, as appropriate. The trypsin should be gamma-irradiated if possible.

For other processing aids used in manufacturing, such as sterilizing filters, cell culture support matrices, pipettes, tubing, vials, stoppers, and storage containers, purchasing pre-sterilized and disposable components will make cleaning, validation, and processing much simpler. As with purchased raw materials, incoming specifications should be developed and quality-assured before the materials reach the loading dock. Generally, laboratory aids are released on physical measurements and vendor certificates of analysis.

Production of media and buffer solutions

The decision about whether to produce the media and buffers in-house is dependent on the facilities and resources available to the manufacturer. Generally, these solutions will have sufficient stability to allow production by a contract manufacturer and long-term storage at the vaccine production facility before they are needed. For process development and production of phase 1 clinical trial material, contract manufacturing is cost-effective and faster than in-house production. It allows internal resources to be devoted to other vaccine development activities. In the long term, in-house produced solutions tend to be less expensive while giving the manufacturer greater control over the quality of the solutions.

The decision to use media and solution that are free of animal-derived raw materials should be made early in the development program and should be evaluated during the initial development of the manufacturing process and formulation. If the decision is made to use animal-free materials, all production processes—back as far as cell and virus seed development—should use animal-free materials. Elimination of animal-derived raw materials removes the need for extensive testing for animal virus contaminants and quality control over the supplier of the raw materials.

There have been significant advancements made by suppliers of media and components for vaccine manufacturing that are free of animal-derived proteins. The serum-free media have been shown to

support cell growth and virus replication in a number of culture conditions. Proteins typically used to stabilize live-virus vaccines, such as albumin, can now be purchased as recombinant protein produced by bacteria or yeast fermentation.

Quality assurance of the raw materials and the media and buffers cannot be over emphasized. Variability in process yields, impurity levels, and virus stability are often traced to changes made in production of the raw materials, media, and buffer solutions. A testing plan and specifications for the solutions should be agreed on before establishing a business agreement with the contract facility. Depending on the complexity and number of solutions required, it is not uncommon to dedicate one full-time person to audit these suppliers.

Manufacturers should identify stability-indicating assays for each media and buffer and establish a stability program early. The more stability data that are available, the easier a contract facility or in-house manufacturer will be able to adjust to a rotavirus vaccine production schedule later in the program.

More information

The following documents provide more information on the production of media and buffer solution.

WHO. Good Manufacturing Practices for Pharmaceutical Products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations Thirty-second report*. Geneva: WHO; 1992. Annex 1 WHO Technical Report Series No. 823.

WHO. *Guidelines on Transmissible Spongiform Encephalopathies in relation to Biological and Pharmaceutical Products*. Geneva: WHO; 2003. Available on the internet at <http://www.who.int/biologicals>.

Cell substrates

The selection of a cell substrate used to produce rotavirus vaccine is fairly limited. Two well characterized cell substrates—the fetal rhesus diploid cell line, or FRhL-2 cell line, and African green monkey kidney heteroploid line, or Vero—are most commonly used. Both are available from the American Type Culture Collection (PO Box 1549, Manassas, Virginia, USA). These substrates have the advantage of being approved by various regulatory agencies for production of live-virus vaccines and therefore are well characterized.

In instances of inadequate virus growth using a banked cell system, rotavirus vaccines may also be produced in primary cells (for example, monkey kidney or chicken embryo) obtained directly from trypsinized tissue of normal, healthy animals. The quality control issues surrounding the use of primary cells require special attention. Animals should be of a species approved by the national control authority and not previously used for experimental purposes. Regardless of the cell substrate chosen, the manufacturer should verify that the virus strain selected for vaccine production produces sufficient yield in the substrate at the appropriate passage level to make the vaccine commercially viable. Time spent on cell substrate selection can improve the chance of vaccine success.

Cell cultures prepared directly from animals show a high frequency of viral contamination. The number of isolated viruses and frequency of isolation depend on many factors, including methods of isolation, test cell systems used, number of passages, length of incubation and co-cultivation and are directly proportional to the duration of the incubation period of the cultures.

The frequency of contaminated cell cultures can be reduced by carefully screening the animals for the absence of antibodies to the relevant viruses. Animals, especially those that are pathogen-free, should be bred in a carefully controlled colony. In addition, the use of secondary or tertiary cells on which testing for adventitious agents could be performed will reduce the frequency of contaminated production cell cultures.

Production of uninfected control cultures and the extensive testing required for relevant adventitious agents become more challenging when producing live-virus vaccines on primary cells. The WHO guidelines on the production and control of oral poliomyelitis vaccine provide guidance on the use of primary cell substrates. Regardless of the cell substrate chosen, manufacturers should verify that the virus strain selected for vaccine production produces sufficient yield in the substrate at the appropriate passage level to make the vaccine commercially viable. Time spent on cell substrate selection can improve the chance of vaccine success.

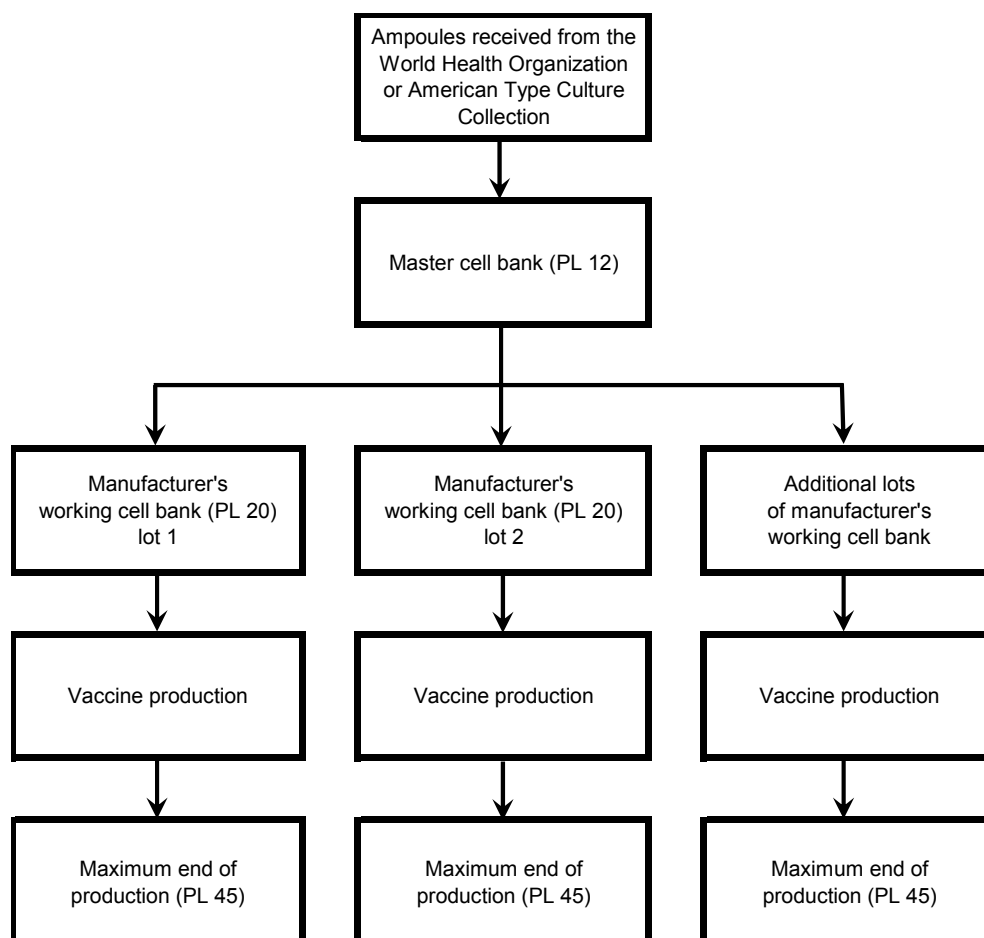
Generally 3 to 5 ampoules of the cell substrate are procured from either the American Type Culture Collection, the World Health Organization, or a private source. Two or more ampoules are pooled and the cells passaged in tissue culture flasks enough times to accumulate an adequate amount of cells in which to pool and establish a master cell bank. Considerable time and money will be spent qualifying the master cell bank; therefore, sufficient ampoules of the master cell bank should be stored to allow 50 to 75 years of vaccine production. The master cells are stored at -70°C or below (for example, in the vapor phase of liquid nitrogen) in **multiple** secure locations, preferably at the manufacturing site and remote locations. *Note: Manufacturers should confirm that the freezing and cell stabilization techniques are appropriate and successful before establishing the master cell bank. The master cells become the cornerstone of the rotavirus vaccine production program.*

Ampoules from the master cell bank are propagated to a passage level sufficient to produce a quantity of cells for the preparation of a manufacturer's working cell bank. One batch of the working cell bank (300 to 500 ampoules) may be sufficient for 2 to 3 years of vaccine production using 1 to 3 ampoules of cells per production batch. Vaccine will then be produced from ampoules of the manufacturer's working cell bank. When the batch is depleted, ampoules of master cell bank are again propagated to make additional batches of the manufacturer's working cell bank. **Figure 3** is an example of a cell banking system.

Regardless of the cell substrate selected, the qualification of a diploid or heteroploid cell substrate is similar. It is important to show that the cell banks are free of adventitious agents relevant to the animal species used in derivation. Cell banks should be assessed for the absence of adventitious agents that may have been present during production, including those that may be present in the raw materials used at each of these stages. **Table 1** lists some tests that may be required for qualifying a cell substrate for vaccine production. Some tests may be added or deleted depending on the origin of the cell and the raw materials that have come into contact with the cell during its passage history. The majority of the tests performed are for detecting the presence of extraneous viral and microbial contamination. The specific tests required for qualification of the master cell bank will be specified by the relevant national regulatory agency.

An important characteristic of the cell substrate to establish is the maximum end of production (MEOP) for the particular process being developed. This is expressed as either population-doubling level (PDL) or passage level (PL) beyond the level of the working cell bank at which vaccine production cannot occur.

Figure 3. A cell banking system



A PDL or PL is used to document and quantify the passage of cells. A population doubling is determined by performing a viable cell count at each passage of cells where cells may undergo 2 or 3 doublings at each passage. A passage level is defined by the manufacturer, usually as one “splitting of cells” and passage from one container into a larger container or multiple containers under a well defined production process.

The MEOP is the maximum cell passage used for vaccine production, where a number of the tests to qualify the cell substrate are performed. Sufficient PL or PDL is established between the master working cell bank and MEOP to produce enough cells to infect a single virus harvest or multiple harvests. Keeping the PL of the master working cell bank low gives the opportunity for many population doublings until MEOP is reached. For example, the master working cells of a diploid cell bank may be at PL 20 with the MEOP qualified at PL 40 to 50 and the master working cells of a heteroploid cell line (Vero) may be at PL 134 and qualified at PL 148.

If the cell substrate has been developed in media containing fetal bovine serum, now is the time to adapt the cells to a serum-free medium before making the master cell bank, if desired. Heteroploid cells such as Vero adapt well to serum-free media, whereas diploid cells tend to be more fastidious.

Table 1. Qualification of cell substrates

Qualification Tests	Amplification	Endpoint
Bovine leukemia virus	None	PCR
Bovine polyoma	None	PCR
Bovine immunodeficiency virus	None	PCR
Bovine adventitious viruses (9CFR 113.53)	BT, Vero	CPE, FA, Had
HIV types 1 & 2	Molt 4	PCR
Human T-cell leukemia virus	Molt 4	PCR
Hepatitis A virus	None	PCR
Hepatitis B virus	None	PCR
Hepatitis C virus	None	PCR
Epstein Barr virus	None	PCR
Murine retrovirus	<i>Mus dunni</i>	FA
Murine agents (Map)	Mice	FA, CF, ELISA
Porcine adventitious viruses (9CFR 113.53)	PK	CPE, FA, Had
Simian immunodeficiency virus	174 x CEM	PCR
Simian retrovirus1& 2	None	PCR
Simian foamy virus	Vero	PCR
Simian T-cell leukemia virus	None	PCR
Simian virus 40	None	PCR
Simian cytomegalovirus	None	PCR
Mycoplasma	Cell culture	FA
	Growth media	Growth
Bacteria and fungi	Bacteriological media	Growth
<i>M. tuberculosis</i>	Bacteriological media	Growth
Tissue culture safety	AGMK, WI-38, FRhL-2, MDBK, PRK, PHA	CPE, Had
Animal safety	Adult and suckling mice, rabbits, guinea pigs	Survival
Reverse transcriptase	Induced & noninduced; PCR	Enzyme activity
Electron microscopy	Induced & noninduced	Virus-like particles
Karyology	None	Diploid karyotype
Tumorigenicity at MEOP	Nude mice	Tumor formation
	Soft agar	Growth
Life span	None	Senescence

Abbreviations: AGMK, African green monkey; BT, bovine turbonate; CF, complement fixation; CPE, cytopathic effect; FA, fluorescent antibody; Had, hemadsorption; MDBK, Madin Darby bovine kidney; PCR, polymerase chain reaction; PHA, primary human amnion; PK, porcine kidney; PRK, primary rabbit kidney.

Use of serum-free media for production of rotavirus vaccine is especially significant given the amount of trypsin required for virus propagation. Presence of serum will neutralize trypsin activity, and a serum-free medium eliminates this concern.

There are advantages and disadvantages associated with the use of primary diploid cell lines (which have a finite life span) and heteroploid cell lines (which have an infinite life span). Although some strains of rotavirus may grow to higher titers in primary cells, the level of quality control and testing for adventitious agents needed can outweigh the benefits of using primary cells. Primary and diploid cells are more susceptible to the effects of trypsin and thus tend to detach more readily during virus infection when using roller bottles and bioreactors, limiting the options for production processes. The finite life span of diploid cells can limit the passage level at MEOP and thus the batch size.

The regulatory acceptance of Vero cells for vaccine production has expanded the manufacturing options available to manufacturers. However, the tumorigenicity potential of heteroploid cells makes qualification and production-control issues (for example, passage level and nucleic acid content in final product) more demanding than with diploid cells.

More information

The following documents provide more information on cell substrates:

Asaad F, Furesz J, Grachev V, et al. Annex 3: requirements for continuous cell lines used for biologicals production. In: *WHO Expert Committee on Biological Standardization Thirty-sixth Report*. WHO: Geneva; 1987:93–107. WHO Technical Report Series No.745. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_745.pdf.

Council of Europe. Cell substrates for the production of vaccines for human use. In: *European Pharmacopoeia*. Strasbourg: Council of Europe; 2002:415–418. European Treaty Series No. 50.

ICH Q5D quality of biotechnological/biological products: derivation and characterization of cell substrates used for production of biotechnological/biological products. *Federal Register*. 1998;63(82):50245–50249.

United States Department of Health and Human Services (HHS), United States Food and Drug Administration (FDA), United States Center for Biologics Evaluation and Research (CBER), Center for Drug Evaluation and Research (CDER). *Guidance for Industry: Q5A Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin*. Rockville, MD: CDER; 1998. Guidance for Industry Series.

WHO. Recommendations for the production and control of poliomyelitis vaccine (oral). In: *WHO Expert Committee on Biological Standardization Fifty-first Report*. Geneva: WHO; 2002. WHO Technical Report Series No.904.

WHO. Requirements for the use of animal cells as in vitro substrates for the production of biologicals (Addendum 2003). In: *WHO Expert Committee on Biological Standardization. Fifty-fourth report*. Geneva: WHO; 2005. Annex 4 WHO Technical Report Series No. 927.

WHO. Requirements for the use of animal cells as in vitro substrates for the production of biologicals. In: *WHO Expert Committee on Biological Standardization Forty-seventh report*. Geneva: WHO; 1998. Annex 1 WHO Technical Report Series No. 878.

Virus seed

The rotavirus seed virus used for vaccine production is selected based on the specific vaccine being evaluated, the serotypes desired in the final product (G1, G2, G3, G4, G9, and others) the method of attenuation, and the amount of virus produced relative to an immunizing dose. A vaccine must provide broad protection against existing and emerging rotavirus serotypes. Whether this means that the vaccine will need to contain multiple serotypes is currently not known.

One method of attenuation is to exploit the host range restriction properties of animal rotaviruses (Jennerian) by genetic reassortment with human rotavirus with the desired serotypes. Alternatively, insertion of multigenic mutations, either by serial passage in cell culture or by chemical mutation, will attenuate rotaviruses. Finally the virus must be able to grow to sufficient titer to make the vaccine commercially acceptable.

Once the virus seed is identified and is believed to be suitably attenuated, the virus is biologically cloned by limiting dilution on the production cell substrate, and an individual virus clone is chosen as the starting material for expansion. Cloning is done to enhance genetic homogeneity as well as to remove potential adventitious agents that may have been present from the initial virus isolation or derived from the attenuation process. Finally a master virus seed and manufacturer's working virus seed are produced by the same banking system as for the cell substrate (**Figure 4**). That is, cells are grown on a suitable, flat matrix (in tissue culture flasks or roller bottles) or microcarriers and infected with trypsin-activated virus; the virus-infected cell culture media is clarified by low-speed centrifugation or filtration; and stabilizers are added and frozen at less than -60°C in multiple locations.

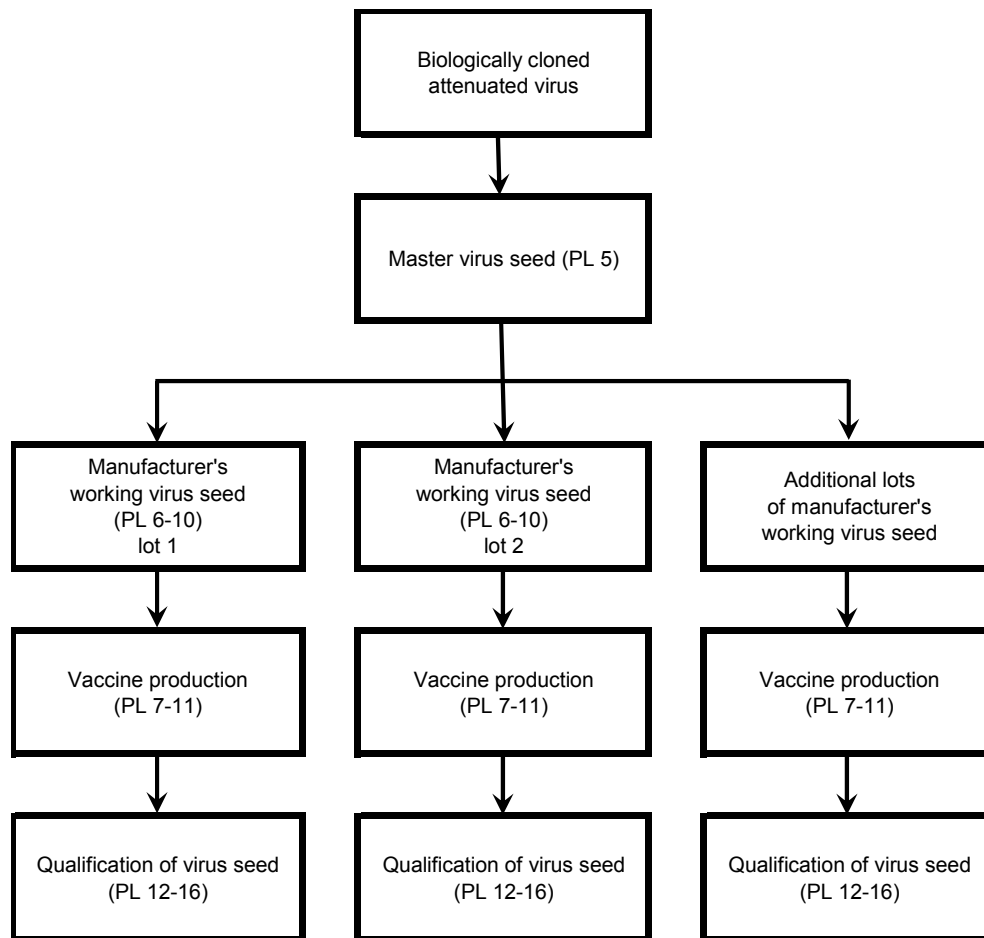
The process of producing the virus seed stocks will be the same as that used for production of the vaccine except at a smaller scale, with purification generally limited to sterile filtration. It may take multiple virus passages from the master virus seed to the working virus seed in order to build sufficient volume of the working virus seed. Once the passage number is established, it should not change. Sufficient ampoules of virus are frozen (for example, 100 for the master virus seed and 300 to 500 for the working virus) for many years of production.

Control over the passage level of virus seed is important. Tests performed on the virus seed will confirm the stability of the viral genome and absence of adventitious agents over a defined passage range. Other *in vivo* and *in vitro* assays may be performed to verify attenuation and other phenotypic characteristics of the virus—all benchmarked to the passage level of virus. The passage level used to qualify the virus seed is generally 5 to 10 passages beyond that used for vaccine production.

Qualification

The virus seed is qualified by passage of the manufacturer's working virus seed under conditions identical to that used for manufacturing to a passage level equivalent to that used for vaccine production and a minimum of five passages beyond the production level. As with the cell substrates, the virus seeds are qualified by performing a variety of tests for adventitious agents appropriate to the passage history of the virus seed. These tests are also specified by the appropriate regulatory authority. **Table 2** is a list of tests frequently used to qualify a virus seed. A stability program should be established as soon as the master virus seed and working virus seed are produced to monitor the infectivity (viability) over time.

Figure 4. Banking system for virus seed



In vitro assays to assess the safety and verify the attenuation phenotype of rotavirus vaccines are not readily available. Consequently, genetic characterization of the viruses has played a major role in the development of the vaccines currently licensed or nearing licensure. The integrity or stability of the virus genome has been studied as it is passaged through the cell substrate to produce the master virus seed, working virus seed, vaccine production lot, and—ultimately—virus isolated in the stools of vaccinees.

Genetic characterization of the virus seeds has included determination of the nucleotide sequence of the complete viral genome, analysis of the molecular basis of the attenuated phenotype, and determination of the genetic stability of the virus seed. Typically the entire genomic sequence of all 11 gene segments of the mater virus seed is sequenced and compared with the sequence of the working virus seed, vaccine production lots, and virus shed in stools of vaccinees. Given the error frequency of the RNA replicating polymerase, base changes will likely be found. Attempts can be made to evaluate the impact of the change (for example, changes in silent or protein-coding regions) or the source of this microheterogeneity (possibly the virus seed stock).

If the attenuation loci are known, sequencing around these sites can be performed instead of the entire genome. Vaccine virus seeds that have been attenuated by genetic reassortment with animal rotaviruses have an easier path. The presence or absence of entire gene segments derived from the animal virus can be determined by RNA hybridization or electropherotyping against known

standards. However, it should be noted that the molecular basis of attenuation of the two vaccines currently closest to widespread use (RotaTeq[®] and Rotarix[®]) is unknown.

Table 2: Qualification of master rotavirus seed

Qualification Tests	Amplification	Endpoint
Human T-cell leukemia virus	Molt 4	PCR
Hepatitis A virus	None	PCR
Hepatitis B virus	None	PCR
Hepatitis C virus	None	PCR
Epstein Barr virus	None	PCR
Simian immunodeficiency virus (MAC)	174 x CEM	PCR
Simian immunodeficiency virus (AGMK)	Molt 4	PCR
Simian retrovirus 1 & 2 (D-type retrovirus)	None	PCR
Simian foamy virus	Vero	PCR
Simian T-cell leukemia virus	None	PCR
Simian virus 40	None	PCR
Simian cytomegalovirus	None	PCR
Reverse transcriptase	PCR	Enzyme activity
Bovine leukemia virus	None	PCR
Bovine polyoma virus	None	PCR
Bovine immunodeficiency virus	None	PCR
Bovine adventitious viruses (9CFR 113.53)	BT, Vero	CPE, FA, Had
Murine retrovirus	<i>Mus dunni</i>	FA
Murine agents (Map)	Mice	FA, CF, ELISA
Porcine adventitious viruses (9CFR 113.53)	PK	CPE, FA, Had
Animal safety	Adult and suckling mice, rabbits, guinea pigs	Survival
Tissue culture safety	AGMK, WI-38, FRhL-2, MDBK, PRK, PHA	CPE, Had
Mycoplasma	Cell culture	FA
	Growth media	Growth
Bacteria and fungi	Bacteriological media	Growth
<i>M. tuberculosis</i>	Bacteriological media	Growth

Abbreviations: BT, bovine turbonate; CF, complement fixation; CPE, cytopathic effect; FA, fluorescent antibody; Had, hemadsorption; PCR, polymerase chain reaction; PK, porcine kidney.

More information

The following documents provide more information on virus seeds:

Council of Europe. Mycoplasmas. In: *European Pharmacopoeia Supplement 2000 to 3rd Edition*. Strasbourg: Council of Europe; 1997:45. European Treaty Series No. 50.

Council of Europe. Tests for extraneous agents in viral vaccines for human use (General method N 2.6.16). In: *European Pharmacopoeia 4th edition*; 2002: 148–149.

WHO. General requirements for the sterility of biological substances (Requirements for Biological Substances No. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization Forty-sixth report*. Geneva: WHO; 1995. Annex 3 WHO Technical Report Series No. 872.

Expansion of cell substrate

The manufacturing process begins with the expansion of the cell substrate from 1 to 3 ampoules of the master working cell bank to a sufficient number of cells to produce one or more single harvests. There are four technologies (support matrices) that can be used to expand anchorage-dependent cells like Vero and FRhL-2 cells, each with advantages and disadvantages, ranked in order of least to most complex:

- Traditional 162 cm² plastic T-flasks.
- Cell factories consisting of multiple layers of flat plastic.
- Roller bottles.
- Microcarriers.

Before the production method is selected, small-scale experiments (1 to 5 liters) can be run to determine the yield of virus produced per milliliter using the selected cell and virus system. The best system is the simplest and most cost-effective that produces sufficient virus to meet the anticipated demand for the vaccine at an estimated potency. Assume a 50 percent downstream processing loss and another 25 percent loss throughout the shelf life of the vaccine.

Bovine or rhesus human reassortant rotaviruses typically grow to $1 \times 10^{7.5}$ PFU/ml in tissue culture fluid harvests using Vero or FRhL-2 cells grown in static cultures (flat matrix surfaces) and 1×10^8 or more using microcarriers in bioreactors due to higher cell densities achievable with this system. Rotaviruses of human origin (neonatal strains) frequently grow 0.5 log lower.

If a manual process uses T-flasks, validation of aseptic processing can be problematic. Some manufacturers elect to process flasks with robotics or use cell factories that require less manipulation. Flat surfaces can be used to grow either diploid or heteroploid cells, whereas roller bottles and microcarriers are better suited for heteroploid cells (Vero). The use of bioreactor technology would be preferred if the utility systems, capital equipment, and expertise are readily available to the company. Using bioreactor technology improves control of the process, increases virus yields, decreases production cycle time, and makes validation easier.

The size of the individual batches of monovalent vaccine is determined by many factors, including the time required to process one batch of vaccine, storage capabilities, testing costs versus risk of

losing an entire batch, and the population doubling level (PDL) qualified for the cell substrate. The latter will determine the maximum number of cells that can be produced for any given batch.

The size of the bioreactor or the number of flasks, roller bottles, or cell factories that can be manipulated in a day is also a limiting factor. Generally a single virus harvest of rotavirus-infected cell culture media ranges from 50 to 100 liters, and a virus harvest pool could be as large as 500 liters. Multiple virus harvests from the same batch of infected cells can be obtained if the virus does not cause significant cytopathic effect. An ample supply of cells is continually being produced to allow production of 50 to 100 liters of virus-infected cell culture media per week during commercial manufacturing of rotavirus vaccine.

Virus infection

Cells that have reached confluency or an appropriate cell density are ready to be infected with trypsin-activated manufacturer's working virus seed. Before infection, 5 percent of the total number of cells to be infected are removed and designated as "control cells." The control cells are manipulated and incubated in a manner that is identical to that of the production lot. The cells and spent tissue-culture media are tested for adventitious agents, hemadsorbing virus, and cell identity.

The intent of the infection process is to amplify a small volume of rotavirus (approximately 2 to 5 ml of the working virus seed stock) to approximately 100 liters of virus with an equivalent infectivity titer to that of the virus seed. The amount of trypsin and time required to activate the working virus seed (cleave the VP4 protein) should be determined experimentally. The smallest volume of activated virus seed that will keep the cells covered with growth media is added. If bovine serum was used for cell growth, the cells are washed in serum-free media or buffered salt solution sufficiently to remove all traces of the serum before virus infection.

The multiplicity of infection (MOI), or the number of infective particles per cell needed to achieve maximum infectious virus titer, should also be determined by experimentation. The MOI can alter the proportion of infectious to noninfectious (defective) virus particles; therefore, an MOI chosen for initial clinical trials should be maintained throughout vaccine development. Although the biological significance of noninfectious particles present in rotavirus vaccines is unknown, the amount of infectious (triple-shelled) to noninfectious (double-shelled) particles is used as a measure of manufacturing consistency and quality of the final product. The lowest MOI that produces sufficient amplification for production should be chosen to minimize the production of noninfectious particles. Methods used to measure infectious to noninfectious particles include infectivity titer to total viral protein, infectivity titer to total particle count (electron microscopy), and separation of double- and triple-shelled particles on cesium chloride gradients.

After the virus has been allowed to infect the cells (1 to 2 hours), the virus inoculum is removed and fresh growth medium is added containing additional porcine trypsin to support virus replication.

The amount of trypsin in the maintenance media should also be determined experimentally, sufficient to produce the maximum virus titer in the harvest fluid. Some rotaviruses cause complete cell lysis in 2 to 4 days, whereas others are much less cytopathic. Daily microscopic examination of the cells is required to ascertain when complete cytopathic effect has been observed.

Once the process is fully developed and virus growth kinetics established, the optimal period of time to harvest will become standardized. The virus-infected culture medium is removed for further

processing. Some serotypes of rotavirus tend to be cell-associated; thus, a freeze-thaw step or mild sonication of the cell culture mixture before harvesting the infected culture media can improve virus yield. In other cases, viruses can be released from the cell—methods to disrupt cells are not required for optimal harvest. Here as well, preliminary studies will indicate whether the specific strain of rotavirus is cell-associated and whether a freeze-thaw step increases the viral yield.

A hold step can be incorporated into the process at this stage. Multiple single virus harvests can be individually sampled for testing and stored frozen. Two or more single virus harvests of the same serotype derived from the same ampoule(s) of master working cell bank, each with a batch of control cells, can be combined at a later date into a single virus harvest pool for further processing.

Purification

There are many factors that should be considered in developing a downstream processing strategy, with the understanding that any processing step included will result in some loss of viable virus. The goals of downstream processing should be to:

- Remove cellular debris from the vaccine harvest.
- Remove or reduce the levels of impurities.
- Concentrate the virus.
- Add a virus stabilizer.
- Sterilize the virus preparation.
- Achieve some level of clearance from adventitious agents (applicability for live-virus vaccines is generally limited).

Removal of cellular debris can be achieved by clarification filtration or low-speed centrifugation. For Vero cell–produced vaccine, nucleic acid can be reduced in size by treatment with benzonase then removed by ultrafiltration using a 50,000 MW membrane or removed by ion-exchange chromatography. It is not necessary to incorporate steps to remove nucleic acid from vaccine produced on diploid cells.

WHO currently recommends a limit of 100 micrograms of cellular DNA for oral rotavirus vaccines. The quantity and size of cellular DNA can also be measured for process characterization and manufacturing consistency. Concentration of the virus would be needed if the titer in the virus harvest were too low to formulate a final product, to remove water to reduce storage volume, or to assist in final vaccine. Concentration can be achieved by ultrafiltration or buoyant density isopycnic ultracentrifugation. A virus-stabilizing solution usually containing, at a minimum, sucrose would be added to stabilize the virus during subsequent storage at -60°C as a monovalent vaccine concentrate. Finally, the vaccine preparation should be rendered sterile by filtration.

At the conclusion of purification, the monovalent vaccine concentrate is placed in suitable containers (for example, FEP containers) of a size convenient for final vaccine formulation and stored frozen at $<-60^{\circ}\text{C}$ until needed for final vaccine formulation. Storage could be possible for up to five years without deleterious effects to the virus. For some viruses, the rate at which the monovalent concentrate is frozen (degrees reduced per hour) can be important in maintaining virus viability. Experiments can be performed using equipment that controls the rate of freezing by assessing virus titer before and after freezing at various rates of freezing.

Virus recovery at each of these process steps should be evaluated by infectivity titration and operating parameters established to minimize loss. For example, the rate at which the vaccine temperature is lowered to <-60°C for storage, the type of membrane filters chosen for concentration, and filtration operation can affect virus viability. It is not uncommon to lose 50 percent or 0.3 log or more of virus during downstream processing.

Small batches of monovalent vaccine from single viral harvests (1 to 5 liters) are usually produced during vaccine development to reduce costs, but, more importantly, to acquire data on multiple smaller batches to evaluate the robustness and consistency of the process. As the process becomes developed and the product well characterized, production scale should increase when pivotal efficacy studies are planned. Ultimately, consistency of manufacturing and process-validation batches will be performed at full commercial scale.

More information

The following documents provide more information on purification:

CPMP. *Note for Guidance on Virus Validation Studies the Design, Contribution, and Interpretation of Studies Validating the Inactivation and Removal of Viruses*. CPMP/BWP/268/95. London: EMEA; 1996. Available at: <http://www.emea.eu.int/pdfs/human/bwp/026895en.pdf>.

Grachev VP, Magrath DI. Annex 1: good manufacturing practices for biological products. In: *WHO Expert Committee on Biological Standardization Forty-second Report*. Geneva: WHO; 1992:20–30. WHO Technical Report Series No.822. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_822.pdf.

Final dosage formulation

A successful rotavirus vaccine is one that meets the needs of the intended customer. A vaccine of equal quality, safety, and efficacy can be formulated for a developed nation in one delivery system and formulated in an entirely different manner to meet the needs of a developing nation. Whereas speed and convenience of vaccination are important to a private physician in a developed nation, cost and storage conditions may be of paramount importance for vaccination in a developing country. Once the individual monovalent vaccines are stored frozen awaiting formulation into the final product, many options are available to the manufacturer to tailor the vaccine to its intended market.

Development of a final dosage formulation can be a long process, because most decisions are based on real-time stability tests that can take 12 months or more. An optimal dosage formulation should be developed in parallel with the process development.

Changes in the process can affect stability of the final product. Determining the stability, or rate at which each of the vaccine virus components degrade over time, is essential using as many different container closure systems and dosage formulations (for example, liquid, frozen, or lyophilization) that are available to the company. A “matrix” evaluation, where multiple variables are studied in various combinations, can be a good screening tool.

Assessing the stability of vaccine formulations exposed to higher temperatures for a shorter duration can also cut down formulation development time. By “screening” multiple formulations by exposure to higher temperatures, a few candidate formulations can be selected for longer studies at the

recommended storage conditions. Ultimately, the stability profile of each virus component, together with the manufacturing losses that occur during formulation and filling, can be determined. To compensate for these losses, an “overage” will be added at the time of formulation. Typically for a live virus vaccine, approximately 0.5 log₁₀ PFU/dose overage is added, provided human safety data is available at the higher titer.

Some rotavirus vaccines may require neutralization of stomach acidity prior to vaccination. Experiments to determine the optimal antacid formulation can be run using simulated gastric fluid (34.8 mEq HCL per liter solution simulates infant stomach acid at pH 3.2) mixed with antacid formulations and virus to assess virus viability over time.

This same experiment can be used to evaluate and optimize buffering systems for the new vaccine. The gastric fluid volume of a two-month-old infant is approximately 20.0 ml. One can evaluate various combinations of carbonate, citrate, and succinate salts, and even buffered milk can be used to raise the pH to neutrality. This buffer solution must either be incorporated into the vaccine formulation or provided separately—for example to reconstitute a lyophilized powder. Ideally the vaccine and buffer should be administered simultaneously, but this is not essential for vaccine efficacy. Given the simplicity of the buffer formulation and manufacturing process, identifying a contract manufacturer for the buffer should be possible.

Deciding on whether to add the antacid to the vaccine at formulation or just before vaccination will depend on the stability profile of the virus in the presence of the antacid. Ideally the buffer used to neutralize the stomach acid should be added at formulation, although rotavirus tends not to be stable in the presence of various buffer salts and antacids and thus must be mixed at vaccination or administered separately. Current rotavirus vaccines under development use both approaches. Some mix the antacid with the vaccine just before vaccination, which is more expensive to the manufacturer (a sterile vaccine and sterile antacid in one package) and cumbersome for the health care provider to use. Or, if the vaccine virus is stable, the antacid and vaccine are together in a single container, which is less expensive to produce and easier to administer.

At formulation, the individual virus serotypes (if more than one strain is included) are aseptically added to a formulation vessel to a strength (infectivity titer) sufficient to ensure that the titer following formulation, filling, and processing (for example, lyophilization and freezing) will meet the potency specification at product-release and throughout the product shelf life. The diluent used to dilute the serotypes to the appropriate strength contains the stabilizers needed to keep the virus viable throughout the shelf life. The liquid blend can then be filled into single- or multidose vials or filled into single-dose dispensing devices that can serve both to store the vaccine and be used for vaccination. Multidose containers will require a preservative to guard against bacterial growth after opening, and the effects of the preservative on virus stability should be evaluated.

If the liquid blend is stable without further processing, the vials can be inspected for particulates and container or closure defects, then labeled and stored at freezer or refrigerator temperatures until shipped to the customer. Alternatively, the vials can be lyophilized to improve stability at refrigeration temperatures; however, lyophilization is costly, requires a reconstitution diluent, and requires considerable process development time and expertise.

The ultimate rotavirus formulation can vary significantly but might be one that consists of a single-dose liquid preparation containing vaccine and buffer, packaged into a device that would serve as the instrument of vaccination and could be stored at refrigeration temperatures. Although nonenveloped

viruses such as rotavirus are relatively stable, achieving this ultimate formulation may not be possible. Options need to be carefully considered when choosing the less-than-ideal formulation—the cost of manufacturing can begin to escalate.

For example, a multidose liquid refrigeration or frozen stable vaccine requiring minimal processing steps is much less expensive to produce than single-dose vials requiring lyophilization and a reconstitution diluent. In general, single- or multidose vaccines that are stable as liquid (or frozen) are the least expensive formulations to produce, but the most difficult to develop. The volume of vaccine administered per dose—0.1 ml, 0.5 ml or 1.0 ml—will also impact cost and convenience to the end user. The volume per dose will depend on the concentration of virus in the monovalent bulk and the total amount of virus in the final dose (including overage) required to ensure adequate virus titer at the end of shelf life. The decision to pursue a single- or multidose container will also depend upon the desire to mix the virus with antacid before vaccination, as the stability of the virus in the presence of antacid for an extended period must be shown.

An alternative to the above formulation and filling processes would involve bulk lyophilization of the individual monovalent vaccine concentrates before storage. The bulk vaccine is then stored as a powder rather than a liquid. At formulation, appropriate amounts of dried power are blended with antacid buffer salts to the required strength. (Note: vaccine may be monovalent.) The powder can then be either filled into single- or multidose vials or compressed into tablets for distribution. (Tablets are dissolved in water before vaccination.) Other options may include vitrification, spray-drying, or microencapsulation technologies.

Aseptic formulation and filling (and lyophilization, if needed) requires expensive facilities (more than \$1000 per square foot), high operating costs, and extensive validation and expertise. Contract manufacturers are available for this activity, but identifying a contractor that can process live virus is difficult. The need for virus containment, aseptic processing, and multiple product processing using the same equipment are significant challenges for the potential contract manufacturer.

More information

The following documents provide more information:

Ayres J, Benmansour A, Breschkin A, et al. Annex 2: guidelines for national authorities on quality assurance for biological products. In: *WHO Expert Committee on Biological Standardization Forty-second Report*. Geneva: WHO; 1992:31–46. WHO Technical Report Series No.822. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_822.pdf.

WHO. Annex 1: good manufacturing practices for pharmaceutical products. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations Thirty-second Report*. Geneva: WHO; 1992:14–79. WHO Technical Report Series No.823. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_823.pdf.

WHO. Annex 4: good manufacturing practices for pharmaceutical products main principles. In: *WHO Expert Committee on Specifications for Pharmaceutical Preparations Thirty-seventh Report*. Geneva: WHO; 2003:36–89. WHO Technical Report Series No.908. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_908.pdf.

Product characterization and lot-release testing

Assays that are performed throughout the development of rotavirus vaccine fall into four main categories: lot-release assays, in-process controls, preclinical safety assays, and characterization assays. When initiating a rotavirus vaccine development program, an equal amount of effort should be devoted to assay development as that used for process development and production of clinical trial materials. The data obtained from these assays shape the decisions made for the entire program. Decisions based on no data or from data supported by poor assays (for example, from untrained operators, unqualified assays) can lead to devastating consequences later, during vaccine registration.

Lot-release assays

Lot-release assays are identified early in the program and are used to test vaccine and buffers produced for phase 1 clinical studies. **Table 3** lists typical lot-release assays that, at a minimum, would be required to release rotavirus vaccines. The assays and corresponding specifications are derived from various regulatory documents as well as product-specific assays developed by the company (for example, potency and identity assays).

The most current description of proposed release assays for rotavirus vaccines can be found in the recently published WHO guidance document, *Guidelines to Assure the Quality, Safety and Efficacy of Live Attenuated Rotavirus Vaccines (Oral)*. In some cases however, assays listed under specific product monographs or related regulatory documents are applicable to rotavirus vaccines as well—for example, *Points to Consider on the Development of Live Attenuated Influenza Vaccines*.¹⁰

The quality assurance department will release the batch against approved specifications. As the product matures through development, assays will be added and subtracted from the initial list based on interactions with control authorities and experience in applying the assays to the specific product. Limited assay validation or reference standard qualification is conducted for assays used to release phase 1 vaccine. For live-virus vaccines, there is an emphasis on safety assays, and assay qualification is usually limited to evaluating whether the vaccine interferes with the performance of the assay (“matrix effect”). Before phase 3 vaccine is produced, all assays should be validated and standards qualified.

Specifications are initially established based on data obtained during the development of the manufacturing process and those defined in various regulatory documents. Product monographs prepared by the manufacturer serve as a repository and documentation system for all assays.

The monographs will identify each assay that is performed, where the sample originates, the method (standard operating procedure) used for testing, and the specifications for product release for each assay. Specifications for quantitative assays are usually broad at the beginning of the development program and tighten as more data are accumulated from lots used in preclinical and clinical studies.

¹⁰ Committee for Proprietary Medicinal Products (CPMP). *Points to Consider on the Development of Live Attenuated Influenza Vaccines*. EMEA/CPMP/BWP/2289/01. London: European Agency for the Evaluation of Medicinal Products (EMA); 2003. Available at: <http://www.emea.eu.int/pdfs/human/bwp/228901en.pdf>

Table 3. Minimal lot-release assays for rotavirus vaccines

Sample Location	Assay	Purpose	Method
Production cells before infection	Identity	Verify correct cell substrate	Isoenzyme
	Mycoplasma	Detection of mycoplasma	Cell culture, agar and broth with PCR, growth or fluorescent staining as endpoints
	Sterility	Evidence of microbial or fungal contamination	Broth with visual growth as endpoint
Viral harvest, pre-purification	Adventitious agents	Verify absence of viral contamination	Tissue culture amplification with CPE and Hemadsorption endpoints
	Mycoplasma	Detection of mycoplasma	Cell culture, agar and broth with PCR, growth or fluorescent staining as endpoints
	Sterility	Evidence of microbial or fungal contamination	Broth with visual growth as endpoint
	Potency	Quantitate the amount of infectious virus	Tissue culture with plaque or TCID ₅₀ endpoints; RNA quantitation
Viral harvest, post-purification	Potency	Quantitate the amount of infectious virus	Tissue culture with plaque or TCID ₅₀ endpoints, RNA quantitation
	Reverse transcriptase	Look for any evidence of retroviral contamination	Dual template reverse transcriptase assay or PERT.
	Identity	Correct virus serotype	Electropherotyping, plaque neutralization, RNA hybridization, PCR
	Residual DNA	Assess amount of cellular DNA	Picogreen
	Sterility	Evidence of microbial or fungal contamination	Broth with visual growth as endpoint
Control cells	Adventitious agents	Verify absence of viral contamination	Tissue culture amplification with CPE and Had endpoints
	Mycoplasma	Detection of mycoplasma	Cell culture, agar and broth with PCR, growth or fluorescent staining as endpoints
	Sterility	Evidence of microbial or fungal contamination	Broth with visual growth as endpoint
	Hemadsorption	Look for any evidence of contaminating hemadsorbing virus	Hemadsorption with red blood cells

Table 3. Minimal lot-release assays for rotavirus vaccines (continued)

Sample Location	Assay	Purpose	Method
Filled containers	Potency	Quantitate amount of live virus. If more than one strain, each strain done separately.	Tissue culture plaque or focus assay using serotype specific monoclonal antibodies, RNA quantitation
	pH	pH within acceptable range	pH meter
	Identity	Presence of rotavirus serotypes	FFA, IPA, PCR
	Physical appearance	Gross product defects	Visual observation
	Sterility	Look for any evidence of microbial or fungal contamination	Broth with visual growth as endpoint
	Accelerated degradation	Acceptable virus loss over time compared to control	Heated and unheated samples for a defined period of time

Abbreviations: CPE, cytopathic effect; FFA, fluorescent focus assay; IPA, immunoperoxidase assays; PCR, polymerase chain reaction; PERT, PCR-enhanced reverse transcriptase.

The monographs serve as a means of tracking changes to the specifications as the product and corresponding assays mature.

Adventitious agents

The processing of live rotavirus vaccines, like many other live viral vaccines, involves cell disruption and, if any, incomplete purification of the virus. In-process adventitious agent inactivation steps are not included for live viral vaccines, because these steps may compromise the live nature of the vaccine itself. As a result, validation of clearance of any adventitious agents may not be possible. For these reasons comprehensive testing for adventitious agents and qualification of the vaccine source materials are essential as part of vaccine safety control.

As with any viral vaccines, production of rotavirus vaccines also involves cells, virus seed, and biological reagents (such as growth supplements, serum, trypsin, and any virus stabilizers used in the final product). Hence, each of these components must be tested to ensure that they are free from adventitious agents. The full passage history of the seed material used for vaccine development is needed to identify all substrates through which the seed materials have been passed, to aid the development of appropriate adventitious agents testing programmes. The early passage history for the candidate vaccines varies but may include monkey kidney cells, primary dog kidney cells, or a combination of these and other substrates. It will be essential to show that the virus seeds are free of adventitious agents relevant to the animal species used or free of the substrates used in the derivation of the seeds.

Potency assays

There are no reliable animal models for testing the immunogenicity or estimating the efficacy (potency) of rotavirus vaccines. The amount of live virus that is present in the vaccine is demonstrated, through clinical trials, to correlate with the efficacy of the vaccine. Thus, the rotavirus infectivity assay is used to determine the potency (evaluation of infectious units) of virus seeds, monovalent vaccine concentrates, and filled containers of monovalent or multivalent vaccines. A robust potency assay is necessary for all subsequent vaccine development activities. Infectivity assays that are performed on monovalent vaccine samples will likely differ from the assays performed on multivalent samples. Test methods include:

- Tissue culture plaque assay (PFU/ml).
- Cell culture infectious dose 50 (CCID₅₀/ml).
- Focus forming assay (FFU/ml).
- Infectious units (IU/ml).

All assays rely on the ability to detect virus replication on a cell substrate.

A conventional **plaque assay** is based on the use of continuous African green monkey kidney cells (MA-104) or Vero cells as the cell substrate. MA-104 cells are generally better for plaquing rotavirus. Test sample dilutions are inoculated onto confluent monolayers and incubated for one hour at 37°C. The inoculum is then removed and an agar overlay containing acetylated trypsin is added. The plates are incubated at 37°C in a CO₂ incubator for four days, after which a second agar overlay, containing neutral red, a vital stain, is added. The plates are incubated for an additional day, then plaques are observed and counted. The titer calculated is expressed in plaque-forming units per milliliter (PFU/ml) or per single dose.

A rotavirus reference standard is included in each assay to establish that the conditions of the assay are correct and that the results obtained for test samples in that assay are accurate. If a 50 percent endpoint is desired (**CCID₅₀**), infected wells are counted as either exhibiting cytopathic effect or not. The inverse of the dilution containing 50 percent of the wells infected is the endpoint dilution, and the titer can be calculated using the Spearman-Kärber method. This same assay can be adapted to an ELISA method to detect viral growth if cytopathic effect is not extensive.

The potency of multivalent vaccines using plaque or CCID₅₀ assays requires the use of multiple serotype-specific neutralizing monoclonal antibodies in various combinations. Since each monoclonal antibody can neutralize the infectivity of its homologous virus, a pool of any three of the four selectively neutralizes three of the viruses in the tetravalent vaccine (for example) while permitting only the fourth one to grow. The titer of each serotype in the vaccine is then determined.

The **focus forming assay** is ideal for multivalent vaccines where specific antibodies that are not cross-reactive are available. Instead of eliminating three of four viruses with pools of neutralizing monoclonal antibodies, the test employs serotype-specific monoclonal antibodies as primary antibodies and fluorescein- or horseradish peroxidase-tagged goat or sheep anti-mouse immunoglobulin as detection antibodies. Sample dilutions are inoculated onto confluent monolayers of MA-104 or Vero cells and incubated for one hour at 37°C. The inoculum is then removed and fresh media applied. One or more rounds of virus replication are allowed to occur, and the infected cells are identified and counted by either fluorescein- or immunoperoxidase-coupled specific antibody. The assay permits direct estimation of a single virus titer within the vaccine. This

technique is commonly used to monitor virus infectivity and offers several advantages as a method for identifying and quantifying individual viruses within a mixed population within vaccine formulations.

The virus content (**infectious units**) may also be determined by molecular quantitation—for example, quantitative real-time PCR, in which the viral RNA is quantitated over time following infection of a cell monolayer with the test article and a reference standard. The rate of RNA production over background is compared to a virus standard whose infectious titer has been determined. This method can be used as individual or multiplex reactions. Primers made to unique regions of genome of each serotype being assayed provide the specificity required.

Identity assays

Identity by monoclonal antibody. The availability of serotype-specific monoclonal antibodies permits the development of two identification tests that can be used as lot-release tests. The first assay is based on plaque reduction observed when a suitable dilution of a multivalent vaccine is incubated in the presence (and absence) of various combinations of monoclonal antibody pools. In the example of a tetravalent vaccine, five pools are made (P0, P1, P2, P3, and P4), each containing three of the four monoclonal antibodies except for the one designated P0, which consisted of all four. Thus, the assay procedure permits a determination of the identity of the virus permitted to replicate in the absence of the relevant monoclonal antibody. For a monovalent vaccine, the test sample is shown to be neutralized by the homologous antibody and not heterologous ones.

Identity by immunostaining. A second identity assay is similar in concept to the plaque reduction neutralization test in the use of serotype-specific antibodies to permit identification of an individual virus within a mixed population. By treating infected monolayers with serotype-specific monoclonal antibodies as primary antibodies and fluorescein-tagged goat antimouse immunoglobulins as detection antibodies, it is possible to determine the identity of the individual virus.

Identity by electrophoretotyping. The rotavirus genome consists of 11 double-stranded RNA molecules numbered 1 to 11 in order of their increasing electrophoretic mobility on polyacrylamide gels. A molecular identity test can also be employed for monovalent preparations and has long been the “gold standard” for identifying rotaviruses. Electrophoretotyping can be used as a means by which to identify gross alterations in genomic differences between the parent wild-type viruses and/or animal donor virus (if employed) and the vaccine strains. The identity of the vaccine virus is confirmed by comparing the electrophoretic profile of the vaccine virus to the RNA electrophoretic profile of a known human rotavirus serotype.

Identity by hybridization and PCR. Other molecular identity tests are RNA/RNA hybridization and enzyme restriction maps of PCR-amplified VP7 gene segments.

Identity by sequencing. The ultimate identity test involves full genomic sequencing with comparison to a reference strain. This analysis would not be done as a routine release assay, but as a characterization assay.

The decision to perform all or a portion of these assays in-house versus using contract laboratories is an important one. Many problems that occur during the manufacturer of live-virus vaccines are assay-related and include invalid assays, false positive results, reference reagents that do not perform as expected, matrix issues, lack of virus neutralization and assay variability. Performing assays in-house gives added control when investigating invalid assays and out-of-specification results.

However, the use of contract laboratories will provide expertise and facilities that may not be immediately available to the manufacturer.

The use of contract testing services, like use of contract manufacturers of media and buffers, will expedite initiation of phase 1 and 2 clinical trials. Before phase 3, a decision on the testing location should be made. Usually the effort is split: routine compendial assays such as mycoplasma, tissue culture safety, and sterility, are performed by a contract laboratory, whereas product-specific assays, such as potency, identity, and genotyping are performed by the company. Furthermore, the latter assays are more likely to be transferred to a control authority for lot-release purposes.

Reagents and reference standards

Reagents and standards that will be needed for lot-release testing of rotavirus vaccine are as follows:

- Polyclonal or monoclonal virus neutralizing antisera (nonserotype-specific) for use in tissue culture safety tests capable of neutralizing the titer of virus typically in the viral harvests (approx 1×10^8 PFU/ml).
- Serotype-specific, virus-neutralizing antisera—generally monoclonal—used in serotype-specific identity assays such plaque neutralization, immunoblot identity tests, and potency assays.
- Mycoplasma reference standards for cell culture and growth media assays.
- Reference viruses used in tissue culture adventitious agents assay and Had assays—for example, PIV-3 and poliovirus.
- Reference standard for monovalent potency test for assaying infectivity of individual vaccine virus serotypes. Usually obtained as a gradient-purified virus from a viral harvest.
- Reference standard for multivalent potency test for assaying infectivity of individual vaccine virus serotypes if final formulation is multivalent.
- Virus stocks for electropherotyping from animal (if used) and human rotavirus used for preparing virus seed.

Reagents and reference standards should be developed in concert with the development of the assays. Reference standards are usually used to confirm assay validity—that is, to demonstrate that each assay is performing within acceptable limits. Reference standards are used to confirm that the cell culture or biological growth media used in detecting contamination can in fact support the growth of biological agents. Reference standards can be used in determining identity and potency of the vaccine by comparing the infectivity or genotype of the test article to a standard of known infectivity or genetic profile. Live monovalent virus reference standards for infectivity, electropherotyping, and identity assays can be purified from viral harvests by the manufacturer. The virus preparations are “qualified” by assigning an infectivity titer and confirming the serotype. The reference is diluted to an appropriate strength, aliquoted in an appropriate volume, and stored frozen. When new reference standards are made they are standardized against the existing reference standard.

Certain test procedures require the use monoclonal, polyclonal, or polyvalent antisera. Classical tests for adventitious viruses, for example, require that the vaccine viruses be “neutralized” or otherwise eliminated to prevent interference with procedures designed to detect other viral or microbial agents.

To prepare a sufficient quantity (generally 3 to 5 liters) of high titered antisera for use in this and other tests, goats or sheep are often chosen as an appropriate animal because they can be acquired rotavirus-free and can produce a sufficient quantity of antisera. Animals are immunized repeatedly with each vaccine virus to generate neutralizing antisera.

To obtain antisera of high titer and to eliminate the creation of antibodies against components of the cell substrate in which the vaccine viruses are propagated, each virus is propagated in cell substrate different than that used for vaccine production, and it is then purified by cesium chloride or sucrose gradient centrifugation.

After a suitable neutralizing antibody titer is developed, antisera is obtained and tested for the ability to neutralize homologous virus at a titer equivalent to that commonly observed in the viral harvests. The antisera must be sterile filtered, free of mycoplasma, and unable to neutralize common human viral pathogen—that is, the pathogen for which the adventitious agents test is attempting to detect. Consequently, the virus-neutralizing capability is evaluated against 10 to 15 model human and animal viruses. Model viruses may include poliovirus, measles, mumps, and rubella viruses, respiratory syncytial virus, herpes simplex virus, adenovirus, varicella zoster virus, canine parainfluenza virus, cytomegalovirus, parainfluenza virus, and influenza virus. The polyvalent antisera can also be used in a variety of other applications such as ELISA, western blotting, and immunofluorescence staining.

For quality control assays where virus-specific quantitation or neutralization is required, serotype-specific monoclonal antibodies directed against each component of the vaccine are needed. Many, but not all, monoclonal antibodies are directed against either of the two outer capsid proteins of rotavirus, VP4 and VP7, and are capable of neutralizing viral infectivity. Contract laboratories or research institutions are available to produce either polyclonal or monoclonal virus-neutralizing antisera and may take up to six months to produce and qualify these reagents.

More information

The following documents provide more information:

Dotzel M. International conference on harmonization guidance (ICH) on Q6A specifications. Test procedures and acceptance criteria for new drug substances and new drug products: chemical substances. *Federal Register*. 2000;65(251):83041–83063. See also www.ich.org.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q2B Validation of Analytical Procedures Methodology*. Rockville, MD: CDER; 1996. Guidance for Industry Series. See also www.ich.org.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q6B specifications Test Procedures and Acceptance Criteria for Biotechnological/Biological Products*. Rockville, MD: CDER; 1999. Guidance for Industry Series. See also www.ich.org.

HHS, FDA, CDER. *Guideline for Industry: Q2A Text on Validation of Analytical Procedures*. Rockville, MD: CDER; 1995. Guideline for Industry Series. See also www.ich.org.

Lebron JA, Troilo PJ, Pacchione S et al. Adaptation of the WHO guideline for residual DNA in parenteral vaccines on continuous cells lines to a limit for oral vaccines. *Developments in Biologicals*. 2005: volume 123 (in press).

In-process control tests

In-process control tests are assays performed during vaccine manufacturing to monitor the quality of the processes being used. Data can be used to make immediate judgment on the vaccine being processed. Examples include:

Equipment monitoring

- Temperature and humidity of incubators.
- Temperature of water baths.
- Dose-volume checks of filling equipment.
- Vacuum or pressure level, product temperature, and shelf temperature of lyophilizer.
- Temperature and other process controls on process vessels.
- Pressure and flow rates during filtration and concentration procedures.

Specifications are established during the performance qualification validation of each piece of equipment.

Environmental monitoring

- Viable and nonviable particulate monitoring of processing spaces.
- Air velocities.
- Pressure differentials between manufacturing spaces.

Specifications are established based on the intended design and performance qualification validation of the facility.

Process monitoring

- Macroscopic and microscopic examination of cells.
- Hemadsorption tests on control cells.
- Infectivity and bioburden assays.

Specifications are usually set forth by the company or in regulatory documents.

Preclinical safety studies

Preclinical safety studies are performed to demonstrate that the vaccine is safe for testing in humans. Preclinical studies can also be used to identify safety-related risks and can be included in the clinical protocols and in investigational drug brochures. These studies (for example, specific assays for adventitious agents and acute toxicity studies) are usually performed one time only on virus seeds, cells, and the vaccine. WHO and EMEA documents (listed below) provide guidance on testing that might be required as well as consultation with the appropriate control authority.

In general, guidance and recommendations that apply to any live, attenuated virus vaccines do also apply to rotavirus vaccines. The majority of preclinical assays will be those that are used to test for specific adventitious agents on the cell and virus banks and raw materials. If the materials used to produce the rotavirus vaccine (virus seed, cell substrate, and raw materials) are free of adventitious agents, the vaccine produced in a controlled environment from these materials will not require significant testing.

Assays are targeted toward agents that might be present as a result of the passage history or source of the test article. If mouse ascites fluid were used to produce monoclonal antibodies used in the strain selection process, assays for mouse adventitious agent are designed. Other tests are used to evaluate the safety of the vaccine virus itself. Animal models are of very limited if any utility for evaluating the safety, attenuation, and transmission of rotavirus vaccines. The traditional animal safety or abnormal toxicity test will be performed as a routine release test on the final product.

One issue often raised with live, attenuated vaccines is the potential reversion to virulence and the possible transmission and exchange of genetic material with wild type or other microorganisms. If attenuation is a result of site-specific mutations, these loci can be mapped. The presence of the donor genes can be verified if the vaccine is attenuated through genetic reassortment with animal viruses.

Without the aid of an animal model to demonstrate the potential reversibility of virulence of attenuated virus strains, the “stability” of the genetic sequence should be evaluated. The manufacturer may be asked to compare the consensus genetic sequence of the input virus seed to the virus in the final product. The stability of the genetic sequence through cell culture passage and to vaccine virus shed from vaccinees provides a level of quality assurance that attributes of the final vaccine (for example, attenuation and key epitopes for antigenicity) remain unchanged.

Rotavirus is not known to be neurotropic; therefore, the classical neurovirulence model would not be appropriate. Finally, the level of process residuals or impurities can be measured. Although rotavirus vaccine is an oral product and the effect of most impurities on vaccine safety is unknown, knowing the amount of residual endotoxin, fetal bovine sera, cellular protein, and nucleic acid in batches of vaccine used in clinical trials provide data on quality attributes that can be used to evaluate manufacturing consistency and process changes.

Toxicology. Toxicity studies in animals may be needed to assess the potential toxic effect of the virus or excipients on target organs, immune system, or systemic toxicity. Nonclinical evaluation of rotavirus vaccines should be based on existing WHO guidelines; however, there are rotavirus-specific issues that should be considered. For animal studies, a full human dose of vaccine with antacid given orally that corresponds to that intended for use in clinical trials should be used. The design of toxicity tests for rotavirus vaccines will likely include acute, single- and repeat-dose, oral vaccination studies administered in mice or rats (strains moderately susceptible to human rotavirus). The manufacturer should undertake an environmental risk assessment based on a survey of the scientific literature as it relates to the particular strain of virus used. Finally, the pathogenic mechanisms of intussusception associated with oral rotavirus vaccination are currently unknown, and no animal model readily exists to evaluate the risk of intussusception. The manufacturer should keep abreast of the evolving scientific knowledge and plan preclinical and clinical vaccine evaluation strategy accordingly.

More information

The following documents provide more information on preclinical safety studies:

Committee for Medicinal Products for Human Use (CHMP). *Concept Paper on the Development of a Guideline on Viral Safety Evaluation of Biotechnological Products to be Used in Clinical Trials*. EMEA/CHMP/BWP/124447/2004. London: EMEA; 2004. Available at: <http://www.emea.eu.int/pdfs/human/bwp/12444704en.pdf>.

Council of Europe. Test for neurovirulence of live-virus vaccines. In: *European Pharmacopoeia*. Strasbourg: Council of Europe; 1997:99. European Treaty Series No. 50.

CPMP. *Note for Guidance on Preclinical Pharmacological and Toxicological Testing of Vaccines*. CPMP/SWP/465/95. London: EMEA; 1997. Available at: <http://www.emea.eu.int/pdfs/human/swp/046595en.pdf>.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q3B(R) Impurities in New Drug Products*. Rev ed 1. Rockville, MD: CDER; 2003. Guidance for Industry Series.

WHO. Annex 3: general requirements for the sterility of biological substances (requirements for biological substances no. 6, revised 1973, amendment 1995). In: *WHO Expert Committee on Biological Standardization Forty-sixth Report*. Geneva: WHO; 1998:69–74. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_872.pdf.

WHO. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: *WHO Expert Committee on Biological Standardization Fifty-second report*. Geneva: WHO; 2002. Annex 1 WHO Technical Report Series No. 924.

WHO. Guidelines on nonclinical evaluation of vaccines. In: *WHO Expert Committee on Biological Standardization Fifty-fourth report*. Geneva: WHO; 2005. Annex WHO Technical Report Series No. 927.

Wood DJ, Furesz J, Torlak MB, et al. Annex 1: recommendations for the Production and Control of Poliomyelitis Vaccine (Oral). In: *WHO Expert Committee on Biological Standardization Fiftieth-report*. Geneva: WHO; 2002. WHO Technical Report Series, No. 904. Available at: http://whqlibdoc.who.int/trs/WHO_TRS_904.pdf.

Characterization assays

Characterization tests are product-specific assays used to gain a better understanding of the physicochemical and biological properties of the vaccine. Data from these assays are helpful in establishing a benchmark profile of the vaccine (for example, genetic sequence, ratio of live to dead particles, and impurity profile) to be used to assess changes introduced during vaccine development.

Characterization assays are performed for a number of reasons, including to:

- Define a measurable attribute in the vaccine that can be linked to safety, immunogenicity, or efficacy in human trials.
- Provide quantitative data that can be used to demonstrate manufacturing consistency.

- Provide a scientific basis to justify the acceptability of process, facility, or formulation changes.
- Apply new assay technology that may replace existing or outdated methods.

Assays developed to characterize the vaccine and the starting materials often are incorporated into or replace lot-release assays. The significance of these assays in measuring critical quality attributes such as impurities, potency, identity, safety, and attenuation become apparent after evaluating many lots of vaccine. The data from the in vitro assays may be correlated to human safety and efficacy data. Below is a listing of some typical vaccine characterization assays that a manufacturer might consider implementing:

- Ratio of live to dead or defective particles.
- Ratio of double to triple layered particles.
- Total cellular protein.
- Total viral protein.
- Process residuals (fetal bovine serum) and impurities (endotoxin).
- Quantitative assays for viral proteins (VP7, VP6, and VP4).
- Real-time quantitative PCR to determine the number of genomic copies of virus.

More information

The following documents provide more information on characterization assays:

HHS, FDA, CBER, CDER. *Guidance for Industry: Q3A(R) Impurities in New Drug Substances*. Rev ed. Rockville, MD: CDER; 2002. Guidance for Industry Series.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q3B(R) Impurities in New Drug Products*. Rev ed 1. Rockville, MD: CDER; 2003. Guidance for Industry Series.

Stability evaluation

Stability studies are conducted before initiating phase 1 clinical trials to provide preliminary indication that the potency of the vaccine will remain within acceptable limits from the time the vaccine is filled and potency tested at release until completion of all vaccinations. These studies need to be performed using the same formulation, storage temperature, and container or closure system that will be used in clinical trials. This formulation is generally never the final formulation or delivery system that will be used for later clinical studies and commercial production. For example, the United States Food and Drug Administration requires three months of real-time stability data at the recommended storage temperature before clinical evaluation can begin. A degradation rate or amount of infectious virus lost over time can be calculated and a prediction made as to how long the virus concentration will remain within specification. Each batch of vaccine produced for clinical study should be placed on long-term stability (at least 2 years, with time point every 3 or 6 months). These data will begin to shape an understanding of the vaccine stability profile.

The most significant stability-indicating assay for any live-virus vaccine is the amount of infectious virus present in the product. This has been shown in human clinical studies where a response has been correlated with amount of live virus in the vaccine and in animal studies where biological activity can be nullified when the vaccine virus is inactivated. Other stability-indicating markers can be a pH shift or a change in physical appearance.

Stability studies on the final containers are critical for determining the shelf life of the vaccine and establishing the potency at release and at the end of shelf life. The virus degradation rate will be used to calculate how much virus must be placed in the final container at the beginning of storage to ensure there will be sufficient virus at the end of storage.

Stability studies are initiated for a variety of reasons:

- To establish the expiration date of the final product or monovalent bulk concentrate.
- To qualify a container-closure system if product contact parts change.
- To change the composition of the final formulation.
- To evaluate the vaccine after reconstitution (if lyophilized) or thawing (if frozen).
- To evaluate the effects of shipping or storage conditions if the product is held outside the recommended storage temperatures.
- To evaluate the stability of the final vaccine that has been formulated using monovalent vaccine that is at the end of its expiration period.
- To establish how long the vaccine can be stored outside of recommended storage temperature before vaccination.
- To determine stability when vaccine is removed from recommended temperature, placed at room temperature, and then returned to recommended storage temperature.
- To determine stability of final product that was formulated from monovalent bulk concentrate after one or multiple freezes and thaws. (This is very helpful in order to use remnants of monovalent vaccine bulks after formulation).

- To assess the impact of a process change or raw material change on the stability of the final and monovalent product.

Typically a minimum of three lots of vaccine are placed on stability study for 3 to 6 months beyond estimated time of expiration. Final vaccine should be formulated from different batches of monovalent bulk concentrate. In the case of buffers and solution, each lot would be produced from different batches or vendors of raw materials.

During vaccine development and before registration, stability protocols should be written and executed on the following materials:

- Manufacturer's working virus seed and cell bank at storage temperatures.
- Monovalent vaccine concentrates at storage temperatures.
- Final filled product and buffer diluent at storage and accelerated temperatures.
- Media and solutions used in production at storage temperatures.
- Reagents and standards used in critical assays at storage temperatures.

Stability-indicating assays should be justified in the stability protocols written for each study. Infectivity, pH, physical appearance, moisture (if lyophilized), and sterility are examples of assays used to study virus-containing materials. Cell viability or growth curves can be used for cell banks. Concentration of specific salts or proteins (for example, trypsin), pH, and physical appearance can be used to study buffers and solutions. Some companies conduct functional tests (for example, how well did the test article perform for its intended use?) in their stability programs for media and buffers. It is important to store the materials under study in the correct volume, container-closure systems, and temperature ranges that will be used when conducting clinical studies or when the vaccine is on the commercial market.

Real-time stability studies where product is stored at the recommended temperature and tested for the duration of the intended storage time provide the data to support expiration dating of these materials. However, accelerated studies are often performed where the virus materials are placed and assayed for shorter time intervals at higher temperatures—25°C, 30°C, 37°C, and 42°C. These studies are often performed when a number of different formulations of the vaccine need to be evaluated quickly or to evaluate the stability of the vaccine under adverse shipping and handling conditions. Although not very reliable for live-virus vaccines, virus degradation at these accelerated conditions have been used to predict the long-term stability at lower temperatures. (Arrhenius distribution is an analysis whereby the stability of a vaccine preparation at a specific storage temperature [for example, 2°C to 8°C] is predicted based on real-time degradation rates obtained when the vaccine is stored at elevated temperatures.)

More information

The following documents provide more information on stability evaluation:

HHS, FDA, CBER, CDER, ICH. *Guidance for Industry: Q1A(R2) Stability Testing of New Drug Substances and Products*. Rev ed 2. Rockville, MD: CDER; 2003. Guidance for Industry Series.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q1C Stability Testing for New Dosage Forms*. Rockville, MD: CDER; 1996. Guidance for Industry Series.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q1D Bracketing and Matrixing Designs for Stability Testing of New Drug Substances and Products*. Rockville, MD: CDER; 2003. Guidance for Industry Series.

HHS, FDA, CBER, CDER. *Guidance for Industry: Q1E Evaluation of Stability Data*. Rockville, MD: CDER; 2004. Guidance for Industry Series. Available at: <http://www.fda.gov/cder/guidance/5531fnl.pdf>.

HHS, FDA, CDER. *Guideline for Industry: Q5C Quality of Biotechnological Products Stability Testing of Biotechnological/Biological Products*. Rockville, MD: CDER; 1996. Guidance for Industry Series.

Documentation

All the documentation required for manufacturing clinical trial material fall into one of the 27 quality system elements (QSE) listed below. It is not the intention of this guide to provide guidance or examples of documentation required to satisfy each element. There are consulting firms that provide expertise in a number of these areas, and they would be willing to establish or audit quality systems.

QSE 1	Corporate Policies and Procedures
QSE 3	Audits
QSE 4	ADER and Physical Complaints
QSE 5	Investigations
QSE 6	Material Review Board
QSE 7	Problem Batch Resolution
QSE 8	Product Release
QSE 9	Change Control
QSE 10	Components and Raw Materials
QSE 11	Laboratory Operations
QSE 12	Calibration
QSE 13	Maintenance
QSE 14	Facilities, Equipment, Critical Systems
QSE 15	Validation/Qualification
QSE 16	Environmental Monitoring/Contamination Control/Aseptic Processing
QSE 17	Reprocess/Rework
QSE 18	Manufacturing/Packaging/Labeling
QSE 19	Quality Unit/Notification to Management
QSE 20	Training
QSE 21	Warehouse Distribution
QSE 22	Return and Salvage
QSE 23	Computer Systems
QSE 24	Contract Manufacturing and Testing
QSE 25	Recall
QSE 26	Stability
QSE 27	Technology Transfer

There are some procedures that are uniquely important to consider when manufacturing and testing a live-virus vaccine. Product changeover and cleaning procedures are important to be able to use one set of processing suites for campaign manufacturing of multiple serotypes of rotavirus. Rotavirus inactivation studies are needed for treatment of waste, spill cleanup, and selection of the appropriate cleaning and disinfecting agent. The environmental quality standards used during vaccine production that are required for an orally administered vaccine need to be discussed with the control authority.

Facilities

A discussion on the design and quality standards for a live-virus vaccine manufacturing or development facility is difficult since the process chosen will dictate the facility to be constructed or the contract facility sought. There are some general design considerations that could serve as a guide:

- Live-virus processing areas should have negative pressure differentials to surrounding space and dedicated heating ventilation and air conditioning. They should be physically segregated from other killed vaccines or other biopharmaceutical product manufacturing. The exception might be if the live-virus processing procedures use an entirely closed system.
- Personnel, product, and material flow should be unidirectional from clean or sterile environment toward dirty or contaminated areas.
- As the vaccine progresses from harvest, purification, formulation, and filling, the environmental standards and process controls get more restrictive.
- Open processes, if required, are performed under laminar flow hoods (cells) or biosafety class III cabinets (virus) with class A, ICH class 8, or class 100 environmental standards.
- Personnel and material flow into and out of processing areas are controlled by appropriate air locks and gowning facilities.
- The quality control and assay or process-development labs are segregated from the manufacturing areas unless well controlled, validated, cleaning and changeover procedures are in place.
- Pass through autoclaves, washers, and lyophilizers should be used to maintain acceptable product and material flow.

Clinical evaluation

Clinical trials in humans are classified into phases: phase 1 (safety), phase 2 (dose ranging, immunogenicity, efficacy), phase 3 (large-scale pivotal efficacy studies) and phase 4 (postmarketing). In most countries, prior approval from the national control authority is required before initiating these studies (IND/CTX), and ethical clearance is required in all countries in accordance with the Helsinki Declaration.

The WHO guidelines on regulatory expectations for clinical evaluation are appropriate for development of rotavirus vaccines with consideration given to some rotavirus-specific issues.

Live rotavirus vaccines have been developed based on evidence that natural rotavirus infections elicit protective immune responses, particularly against severe rotavirus disease. Immune responses, specifically rotavirus antibody responses, have been routinely measured during all rotavirus vaccine trials. This has been done to both use these responses as markers for vaccine “take” and to identify surrogate markers of protection or “correlates of immunity.” However, in all of the rotavirus clinical trials conducted to date, there has emerged no reliable immune correlate of protection for rotavirus vaccines. Secretory IgA is the most satisfactory laboratory parameter currently available, and, for a period after vaccination, much of the specific serum IgA is of this type, so that serum IgA levels can act as measure of seroconversion. Serum and stool rotavirus antibody responses have been effectively used as measures of vaccine “take” with all candidate live-virus vaccines.

There are some specific issues that may be considered when designing rotavirus vaccine clinical studies. These include age of vaccine administration, severity of rotavirus illness used as an endpoint for assessment, seasonality, the need for studies in diverse geographical regions (developed and developing country settings), interference health factors, concomitant administration of rotavirus vaccine with oral poliovirus vaccine, number of doses administered, monovalent versus polyvalent vaccine formulations, and the wild-type rotavirus serotypes circulating in a population.

First, the age at which the oral rotavirus vaccine should be given is an important issue. The vaccine should be given early enough to prevent illness, which is very frequent in the first year of life in developing countries and sufficiently early (<3 months of age) in order to prevent a possible association of the vaccine with intussusception.

Second, results to date show that rotavirus vaccine is more efficacious against severe illness. In consequence, standard definitions of (a) a diarrhea episode and (b) the severity of illness (recommended as the primary endpoint of efficacy studies) are critical to facilitate objective comparison of efficacy trials. Hospitalization may not be appropriate as an endpoint because this is a context-sensitive situation that may not reflect, in all cases, the severity of illness.

Third, the design of clinical trials might consider the seasonality of natural rotavirus infections. Rotavirus disease is seasonal in temperate climates where peak incidence of disease occurs in the winter months; whereas, in countries with the highest rotavirus mortality, the tropical climate promotes year-round infection. This seasonality may affect vaccine efficacy. In countries where the virus circulates year-round (tropical settings), vaccine efficacy may be independent of the time of vaccine administration.

Fourth, the efficacy of rotavirus vaccine should be studied in diverse geographical regions. Vaccine efficacy has been observed to be lower in poor developing country settings in comparison with developed countries; therefore, it is necessary to study the performance of rotavirus vaccines in very poor countries of Africa or Asia, where the risk of rotavirus mortality is the highest.

Finally, in designing efficacy trials for rotavirus vaccine, consideration should be given to control for possible interference by factors such as other vaccinations, malnutrition, vitamin and mineral deficiencies, enteric infections (multiple infections), malaria, parasites, HIV, hepatitis, immunodeficiencies, and breast feeding.

There are likely to be many factors that are responsible for this difference in immunogenicity and protective efficacy of the rotavirus vaccines in diverse geographical settings. In designing trials for rotavirus vaccine (pre- or postlicensure), consideration should be given to control for possible interference by factors such as other vaccinations, malnutrition, vitamin and mineral deficiencies, multiple enteric infections, malaria, parasitic infections, HIV, hepatitis, immunodeficiencies, maternal vertically-passed antibodies, and breast feeding.

Two or three doses may be advantageous in broadening the spectrum of heterotypic protection and overcoming the interference with maternal antibodies and concomitant administration of oral polio vaccine and other vaccines.

After marketing, large-scale surveillance studies will be needed once vaccines are approved. In the case of rotavirus, effective execution of these studies will require knowledge of strain distribution, establishment of expertise specific to the laboratory country, and programs to educate physicians and policymakers. Postmarketing surveillance will be needed to assess vaccine effectiveness against emerging strains of rotavirus, monitor the risk of intussusception, ensure early delivery of the first dose of vaccine, establish costs and benefits of vaccination, and provide guidance to health care providers on the disease burden and need to vaccinate. WHO is preparing a guidance document on the development of standardized protocols for postmarketing surveillance of rotavirus vaccines.

More information

The following documents provide more information about clinical evaluation:

CPMP. *Note for Guidance on Clinical Evaluation of New Vaccines*. CPMP/EWP/463/97. London: EMEA; 1999. Available at: <http://www.emea.eu.int/pdfs/human/ewp/046397EN.pdf>.

Protocols and regulatory guidelines for designing and conducting clinical trials can be found on the National Institute for Allergy and Infectious Diseases website, Division of Microbiology and Infectious Diseases, at <http://www.niaid.nih.gov/dmid/clinresearch/>.

Voordouw B, Kawano M, Knezevic I. Annex 1: guidelines on clinical evaluation of vaccines regulatory expectations. In: *WHO Expert Committee on Biological Standardization Fifty-second Report*. Geneva: WHO; 2004. WHO Technical Report Series, No.924. Available at: http://www.who.int/biologicals/publications/trs/areas/vaccines/clinical_evaluation/035-101.pdf.

Regulatory documents

The type and amount of documentation and the level of interaction with the control authority will increase as the rotavirus development program advances toward regulatory approval. Preliminary meetings with the appropriate regulatory agency will help the manufacturer gain an understanding of the preclinical studies that will be required to enter phase 1 human safety studies and to discuss the design of the phase 1 studies.

Before the initial meeting, the manufacturer should provide the agency with a briefing document that outlines the vaccine development strategy and highlights the issues that the manufacturer must have clarified in order to proceed.

The initial interactions with regulators establish a framework for all future development activities and usually combine representatives from manufacturing, quality, and clinical development areas. Subsequent meetings address registration strategies for specific functional areas such as manufacturing and toxicology. Some areas that might require regulatory agency input would include:

Clinical development

- Design of phase 1, 2, pivotal efficacy, and transmission trials
- Postmarketing studies
- Design of clinical bridging studies
- Safety or efficacy issues as they develop
- Design of clinical consistency lot trials

Process and assay development

- Preclinical safety studies required for phase 1 studies
- Qualification requirements for cell substrates and virus seeds
- Acceptability of using animal-derived raw materials in the process
- Agreement on a comparability protocol to justify a process or facility change
- Testing strategy and proposed specifications
- Alternate testing methods

Manufacturing

- Adequacy of technology transfer from the research and development units to manufacturing business units
- Method for demonstration of manufacturing consistency
- Design and quality standards of the manufacturing facility

Regulatory affairs

- Agreement on the registration strategy and timelines
- Agreement on format of Common Technical Document
- Identification of lot-release laboratories

Acronyms

CFR	Code of Federal Regulations
CPE	cytopathic effect
GMP	current good manufacturing practices
Had	hemadsorption
IND/CTX	investigation new drug/clinical trial exemption
MEOP	maximum end of production
MOI	multiplicity of infection
PDL	population doubling level
PFU	plaque-forming unit
PL	passage level
QSE	quality system elements
TCID ₅₀	tissue culture infectious dose 50
VP4	viral protein 4
VP7	viral protein 7
WHO	World Health Organization

Glossary

active ingredient	A substance that is intended to furnish pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment, or prevention of disease.
Code of Federal Regulations	United States regulations governing all aspects of drug manufacturing and distribution within the United States.
current good manufacturing practices, or GMP	Current Good Manufacturing Practices.CFR 21—Parts 210 and 211, and Parts 600 and 610 (US) and Commission Directive 91/356/EEC (EU).
cytopathic effect	Morphological changes observed on cells infected with virus.
downstream processing	One or more processing steps performed on a viral harvest for the purpose of purifying, concentrating, or sterilizing the active ingredient in the viral harvest.
excipient	Substances other than the active drug that have been appropriately evaluated for safety and are included in the drug product to either aid the processing of the drug product during manufacture, protect or enhance stability, or improve patient acceptability.
hemadsorption	The ability of a virus to cause infected cells to adsorb to red blood cells. Also a test involving red blood cells performed to determine the presence of hemadsorbing viruses in cell culture.
inactive ingredient	Any component deliberately included in the drug product other than an active ingredient.
IND/CTX	Investigation new drug/clinical trial exemption—a regulatory document requesting approval to perform clinical investigation of an unapproved vaccine.
manufacturer's working cell bank	A quantity of uniform cells frozen in aliquots, one or more of which would be used for production purposes. In normal practice, a master cell bank is expanded by serial subculture up to a passage level (or population doubling, as appropriate) at which point the cells are combined to give a single pool distributed into ampoules and preserved cryogenically to form the manufacturer's working cell bank.
manufacturer's working virus seed	A quantity of virus of uniform composition, fully characterized and derived from a master seed. The working seed lot is used for the production of candidate vaccine lots.
master batch record	Documentation that provides the history of a batch from the raw material stage to completion of the batch lot.

master cell bank	A quantity of fully characterized cells of human, animal, or other origin stored frozen in aliquots of uniform composition, one or more of which would be used for the production of a manufacturer's working cell bank.
master virus seed	A quantity of virus derived from an original isolate, processed at the same time to assure a uniform composition and having been characterized to the extent necessary to support developing the working seed lot. The characterized master seed lot is used for preparation of working seed lots.
maximum end of production	The maximum passage or population doubling level for which virus infection may occur. Tests are performed on cells that reach maximum end of production to demonstrate that no changes have occurred during passage from the manufacturer's working cell bank passage level to the end of production.
multiplicity of infection	Number of viable virus particles per cell to be infected.
oral rehydration solution	A balanced salt solution given to infants with severe diarrhea.
passage level	A unit of measure that quantifies the passage of cells in culture as the passage of cells from one container to a second or into multiple number containers.
plaque-forming unit	The smallest quantity of virus suspension that will produce a plaque in monolayer cell cultures.
population doubling level	A unit of measure that quantifies the passage of cells in culture as the doubling of cell viable number.
process validation	A batch of product used to establish documented evidence that provides a high degree of assurance that a specific process will consistently produce a product meeting its predetermined specifications and quality characteristics.
quality control	The testing of materials, intermediates, and product and support resources (critical utilities) to ensure that they meet established standards or specifications.
quality system elements	The programs, activities, and procedures that are designed to assure that quality standards are routinely met.
reverse transcriptase	An enzyme present in all retroviruses.
specifications	The quality parameters to which the products or materials must conform and that serve as a basis for quality evaluation.
stability	The continued conformance of the active ingredient/inactive ingredient or drug product to their specifications under various environmental conditions.

standard operating procedures	A written, authorized set of instructions for performing operations.
T-flask	A tissue culture flask; a stationary cell culture system for propagating cells on a flat surface.
TCID ₅₀	The quantity of a virus suspension that will infect 50 percent of cell cultures that have been inoculated.
validation	Documentation providing a high degree of assurance that any procedure, process, equipment, material, or activity consistently leads to the expected results.
viral protein 4	One of two proteins constituting the outer shell of the rotavirus particle. Antibody to viral protein 4 neutralizes virus infection in cell culture. It is the determinant protein in defining the P serotype.
viral protein 7	One of two proteins constituting the outer shell of the rotavirus particle. A glycoprotein in which antibody to viral protein 7 neutralizes virus infection in cell culture. It is the determinant protein in defining the group-specific (G) serotype.