World Health Organization

WHO/DRAFT/JAN 2019
ENGLISH ONLY

Guidelines on the quality, safety and efficacy of human Respiratory Syncytial Virus vaccines

NOTE:

This document has been prepared for the purpose of inviting comments and suggestions on the proposals contained therein, which will then be considered by the Expert Committee on Biological Standardization (ECBS). Publication of this draft is to provide information about the proposed *Guidelines on the quality, safety and efficacy of human Respiratory Syncytial Virus vaccines* and is intended to improve transparency of the consultation process.

The text in its present form does not necessarily represent the final conclusions of the Expert Committee. Written comments proposing modifications to this text MUST be received by 28 February 2019 entered in the Comment Form (available separately), and should be addressed to the World Health Organization, 1211 Geneva 27, Switzerland, attention: Department of Essential Medicines and Health Products (EMP).

Comments may be submitted electronically to the Responsible Officer: **Dr Tiequn Zhou** at email: **zhout@who.int.**

The outcome of the deliberations of the ECBS will be published in the WHO Technical Report Series. The final agreed formulation of the document will be edited to be in conformity with the second edition of the WHO style guide (KMS/WHP/13.1).

Contents

Guidelines published by the World Health Organization (WHO) are intended to be scientific and advisory in nature. Each of the following sections constitutes guidance for national regulatory authorities (NRAs) and for manufacturers of biological products. If an NRA so desires, these WHO Guidelines may be adopted as definitive national requirements, or modifications may be justified and made by the NRA. It is recommended that modifications to these Guidelines are made only on condition that such modifications ensure that the product is at least as safe and efficacious as that prepared in accordance with the guidance set out below.

Abbreviations

$CCID_{50}$	Cell culture infectious dose 50%
ECBS	Expert Committee on Biological Standardization
EOP	End of production cells
ERD	Enhanced respiratory disease
FI-RSV	Formalin inactivated RSV vaccine
GMO	Genetically modified organism
ICP	Immune correlate of protection
MCB	Master cell bank
MPL	3-*O*-desacyl-4'-monophosphoryl lipid A
MVA	Modified Vaccinia Ankara
NAT	Nucleic acid amplification technique
NRA	National regulatory authority
NP	Nasopharyngeal [swab or aspirate]
NS	Nasal swab
LMIC	Low- and middle-income countries
LRTI	Lower respiratory tract infections
PDVAC	Product Development for Vaccines Advisory Committee
PFU	Plaque Forming Unit
RDAI	Respiratory distress assessment instrument
RDT	Rapid diagnostic test
RSV	Respiratory syncytial virus
RSV-F	Respiratory syncytial virus fusion protein
RSV-G	Respiratory syncytial virus G or attachment protein
RSV-preF	Respiratory syncytial virus fusion protein in the prefusion conformation
RT-PCR	Reverse transcription-polymerase chain reaction

Th1	Refers to $CD4^+$ T helper cell responses that secrete cytokines such as IL-2 and IFN-gamma
Th2	Refers to $CD4^+$ T helper cells responses that secrete cytokines such as IL-5 and IL-13
T_{RM}	Resident memory T cells
VMS	Virus master seed
VWS	Virus working seed
WCB	Working cell bank

Introduction

Respiratory syncytial virus (RSV) is a globally prevalent cause of lower respiratory tract infection (LRTI) in all age groups. In neonates and young infants, the first infection may cause severe bronchiolitis, which is sometimes fatal. In older children and adults repeated upper respiratory tract infections are common; these infections may not be associated with clinical signs and symptoms or may cause mild disease.

In addition to the paediatric burden, RSV is increasingly recognized as an important pathogen in adults ≥65 years of age, leading to hospitalization and causing a mortality rate approaching that of influenza. The risk of severe disease in the older adults is increased by the presence of underlying chronic pulmonary disease, circulatory conditions, and functional disability, and is associated with higher RSV loads (1-4).

In the absence of safe and effective antiviral agents to treat RSV, there is a great unmet need for vaccines against RSV. In recent years, increased understanding of RSV biology and advances in technologies has resulted in the advancement of multiple vaccine candidates into clinical development, some of which may receive regulatory approval in the near future. The WHO Product Development for Vaccines Advisory Committee considers it a priority to ensure that emerging RSV vaccines are suitable for licensure and meet policy decision-making needs to support optimal use in low- and middle-income countries (LMIC) in addition to high-income countries (5-7).

There is a need for harmonized technical expectations to guide and facilitate the international development and assessment of candidate RSV vaccines. In response to this, WHO convened a series of consultations with experts from academic institutes, industry, regulatory authorities and other stakeholders, to review and discuss all aspects of RSV vaccine development (8,9). Furthermore, WHO convened a group of experts to prepare draft guidelines on the quality, safety and efficacy of RSV vaccines and the first draft was reviewed in an informal consultation meeting during 18-19 September 2018 by experts from academia institutions, industry, regulatory authorities and other stakeholders.

These Guidelines are developed based on experience gained so far from RSV vaccines development and outcomes from above international consultations and will need to be updated as new data become available and as vaccines are licensed. This document provides information and guidance on the production, quality control and the nonclinical and clinical evaluation of candidate RSV vaccines in the form of WHO Guidelines rather than WHO Recommendations. This format allows for greater flexibility with respect to the expected future of RSV vaccine development, production, quality control and evaluation.

Scope

These WHO Guidelines provide guidance for national regulatory authorities (NRAs) and vaccine manufacturers on the quality, nonclinical and clinical aspects of human RSV vaccines to assure their quality, safety and efficacy. The scope of the present document encompasses the leading technologies that are currently used to develop prophylactic RSV vaccines which are at clinical development stage, e.g. live attenuated including genetically modified organism (GMO) such as chimeric virus vaccines, recombinant viral and other vectored systems, protein-based including subunit and nanoparticle RSV vaccines with and without adjuvants.

Although there are many areas of overlap, there are some unique aspects to consider when describing the quality, safety and clinical testing of RSV monoclonal antibodies and a separate Guideline will be needed to focus on these products.

This document should be read in conjunction with other relevant WHO guidance, especially those on the nonclinical (10,11) and clinical (12) evaluation of vaccines, as well as relevant documents that describe the minimum requirements for an effective National Pharmacovigilance System (13). Other WHO guidance, such as that on the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14), should also be consulted as appropriate. This document covers many technology platforms, however, since it is limited in scope, the other WHO guidance documents may be referred as relevant, such as those for the manufacture of biologicals using pathogen-free, embryonated-eggs (15) if RSV vaccine is produced using embryonated hen's eggs, guidance describing the manufacture, quality control and release of Bacillus Calmette-Guerin (BCG) vaccines (16) in the case of BCG-vectored RSV vaccines.

It should be noted that there remain knowledge gaps in the scientific understanding of RSV vaccines which are being addressed by ongoing research and development. This document has been developed in the light of the available knowledge to date, and with regard to the currently most advanced candidate RSV vaccines.

Terminology

The definitions below apply to the terms as used in this Guideline. They may have different meanings in other contexts.

Adjuvant: a substance or a combination of substances used in conjunction with a vaccine antigen to enhance (for example, increase, accelerate, prolong and/or possibly target) the specific immune response to the vaccine antigen and the clinical effectiveness of the vaccine.

Adsorbed monovalent antigen bulk: a batch of purified monovalent antigen bulk adsorbed on adjuvant. Different batches of adsorbed monovalent antigen bulks may be pooled before collection into a single vessel. If a novel adjuvant is used that does not involve adsorption of the antigen to the adjuvant, the term "adjuvanted monovalent bulk" may be used.

Adventitious agents: contaminating microorganisms of the cell culture or source materials including bacteria, fungi, mycoplasma/spiroplasmas, mycobacteria, rickettsia, protozoa, parasites, transmissible spongiform encephalopathy (TSE) agents and viruses that have been unintentionally introduced into the manufacturing process of a biological product.

Candidate vaccine: an investigational vaccine which is in research and clinical development stages and has not yet been granted marketing authorization or licensure by a regulatory agency.

Cell bank: a collection of appropriate containers of cells whose contents are of uniform composition stored under defined conditions. Each container represents an aliquot of a single pool of cells.

Cell culture infectious dose 50% ($CCID_{50}$): the amount of virus sufficient to cause a cytopathic effect in 50% of inoculated replicate cell cultures, as determined in an end-point dilution assay in monolayer cell culture.

Cell substrate: cells used for the production of a vaccine.

Chimeric RSV vaccine: live-attenuated recombinant RSV vaccines integrating one or more RSV genes into either viral or bacterial vectors. Examples may include Sendai virus, parainfluenza virus, bovine RSV, measles virus or BCG as well as other constructs.

Cytopathic effect: a degenerative change in the appearance of cells, especially in tissue culture when exposed to viruses, toxic agents or non-viral infections.

Drug product: a pharmaceutical product type in a defined container closure system that contains a drug substance, in general formulated with excipients.

Drug substance: the active pharmaceutical ingredient and associated molecules. The Drug Substance is sometimes also called the final bulk. The Drug Substance may be manufactured

from one or more harvest pools and may be subsequently formulated with excipients to produce the Drug Product.

End of production (EOP) cells: cells cultured under conditions comparable to those used for production and derived from the MCB or WCB to a passage level or population doubling level comparable to or beyond the highest level reached for production.

Enhanced respiratory disease (ERD): the observation of severe lower respiratory tract disease occurring with increased frequency in infants and children (and calves) infected with wild-type RSV (or bovine RSV) following immunization with a candidate formalin-inactivated whole-virus RSV vaccine. This term has also been used to refer to histopathological evidence of inflammation observed in the lungs of cotton rats and calves immunized with formalin-inactivated whole-virus RSV vaccine prior to live RSV experimental challenge and infection.

Expression construct: defined as the expression vector containing the coding sequence of the recombinant protein.

Expression system: the host cell with its expression construct and the cell culture process that is capable of expressing protein encoded by the expression construct. Expression systems may include bacterial-based, baculo-cell-based, mammalian-based or yeast-based.

Final bulk (also known as Drug Substance): the finished vaccine prepared from one or more harvest pools and that may include excipients.

Final lot (also known as Drug Product): a collection of sealed final containers of finished vaccine that is homogeneous with respect to the risk of contamination during filling and freeze-drying. All final containers should, therefore, have been filled from one vessel of final bulk and if freeze-dried, processed under standardized conditions in a common chamber in one working session.

F protein nanoparticles: non-infectious, non-replicating, non-enveloped nanoparticles that resemble protein-protein micelles of purified native RSV F protein. Conformational epitopes are retained in this formation.

Genetically modified organism (GMO): an organism in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination.

Harvest: the material collected from cell cultures that is used to prepare the vaccine. The material may be culture supernatant, cells (which are often disrupted) or some combination thereof.

Heterologous gene: a transgene from the disease-causing organism that is integrated into the genomic sequence of the viral vector.

Immune correlate of protection (ICP): an ICP is most commonly defined as a type and amount of immunological response that correlates with vaccine-induced protection against an infectious disease and that is considered predictive of clinical efficacy (12).

Immunogenicity: the capacity of a vaccine to elicit a measurable immune response.

Infant: children less than one year old.

Live-attenuated RSV vaccine: vaccines derived either using conventional methods of attenuation such as serial passage with or without chemical mutagenesis of RSV or using recombinant methods to engineer an RSV strain recovered from plasmid cDNA(s). Such a RSV vaccine is capable of initiating an immune response following a mild infection lacking disease symptoms.

Master cell bank (MCB): a quantity of well-characterized cells of animal or other origin, derived from a cell seed at specific population doubling level or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as the vapor or liquid phase of liquid nitrogen in aliquots of uniform composition (14).

Monovalent vaccine: a vaccine containing an antigen or gene/genes encoding an antigen(s) derived from a single RSV strain or subtype and manufactured using a single harvest or using material pooled from one or more harvests of the identical antigen(s) or virus strain.

Multivalent vaccine: a vaccine containing antigens derived from more than one RSV strain or subtype. Typically, these products are manufactured by pooling individual harvests for each antigen, each virus strain or subtype.

Older adults: individuals 50 years of age and older.

Parental virus: a virus that has been manipulated in some manner to generate a viral seed with characteristics needed for production.

Particle-based or subunit RSV vaccine: RSV vaccines that only contain certain antigens or subunits of RSV. They may be produced by different expression systems.

Plaque-forming units (PFU): the amount of virus sufficient to lyse host cells and cause a single visible plaque or focus of infection due to cytopathic effect in a cell culture monolayer after proper staining of cells.

Platform technology: a production technology with which different viral-vectored vaccines are produced by incorporating heterologous genes for different proteins into an identical viral vector backbone.

Pooled harvest: a homogeneous pool of two or more single production harvests.

Premature infant: an infant born at ≤37 weeks gestation.

Purified bulk: a batch of purified antigen of a single RSV subtype. Different batches of purified monovalent antigen bulks may be pooled into a single vessel.

Qualification: determination of the suitability of a cell substrate for manufacturing based on its characterization.

RSV-naïve: subjects who have not yet been exposed to or infected with RSV.

RSV-non naïve/experienced: subjects who have experienced RSV infection in the past. Prior infection may be based on a previous clinical episode in which RSV was proven to be the aetiologic agent (e.g. the subject had a positive culture, antigen detection or RT-PCR test for RSV in the context of an RSV illness). Alternatively or in addition, subjects may have immunologic evidence of prior RSV infection. In infants with persisting maternal antibody, evidence of prior infection may be based on finding RSV-specific IgA or IgM in serum or secretions, or evidence of immune memory (e.g., a B cell or T cell memory response detected by ELISPOT). In general, passively-acquired maternal anti-RSV neutralizing or IgG antibodies are not known to persist in infants past their first birthday and most infants lose these maternal antibodies much earlier. Therefore, prior RSV infection in subjects at least 12 months of age may be identified by a single positive serology test for anti-RSV neutralizing or IgG binding antibodies.

RSV G and RSV F protein: the two major surface glycoproteins of RSV, the attachment (G) protein and the fusion (F) protein, present as a metastable pre-fusion F protein and a stable post-fusion F protein. These glycoproteins are the primary targets of neutralizing antibodies.

Seed lot system: a system according to which successive batches of virus or viral-vectored vaccine are derived from the same Virus Master Seed (VMS) lot at a given passage level. For routine production, a Virus Working Seed (VWS) lot is prepared from the VMS lot. The final product is derived from the VWS lot and has not undergone more passages from the VMS lot than the vaccine shown to be safe and effective in clinical studies. In the case of bacterial-vectored vaccines, a seed lot system based on bacterial master seed is prepared from which a bacterial working seed is derived.

Vaccine efficacy: measures protection induced by immunization in the vaccinated population sample. Vaccine efficacy is a measure of the reduction in disease attack rate (AR) between the control group that did not receive vaccination against the disease under study (ARU) and the vaccinated group (ARV). Vaccine efficacy is expressed as a percentage and is calculated from the relative risk (RR= ARV/ARU) of the disease comparing the vaccinated group to the unvaccinated control group as [(ARU-ARV)/ARU] x 100 or [1-RR] x 100. This estimate may be referred to as absolute vaccine efficacy. Alternatively, vaccine efficacy may be defined as a measure of the proportionate reduction in disease AR between a control group that is vaccinated against the infectious disease under study and the group vaccinated with the candidate vaccine. This estimate is referred to as relative vaccine efficacy (12).

Viral clearance: the combination of physical removal of virus particles and reduction of infectivity through inactivation.

Viral-vectored RSV vaccine: replication-deficient recombinant RSV vaccines that use viral expression systems such as adenovirus or modified virus Ankara to produce antigens of RSV.

Virus/bacterial master seed (master seed lot): a quantity of viral or bacterial material that has been derived from the same pre-master seed lot, has been processed as a single lot, and has a uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of viral or bacterial material of defined passage from which the working seed is derived.

Viral/bacterial working seed (working seed lot): for routine production, a working seed lot is prepared from the master seed lot under defined conditions and used to initiate production lot-by-lot. In the case of viral-vectored or live-attenuated vaccines, the final vaccine lot is derived from the virus working seed lot and has not undergone more passages from the virus master seed lot than the vaccine shown to be safe and effective in clinical studies. See Seed Lot System above.

Virus pre-master seed (virus pre-seed): a single pool of virus or virus vector particles of defined passage and from which the virus master seed is derived.

Virus working seed: a collection of appropriate containers whose contents are of uniform composition, stored under defined conditions. Each container represents an aliquot of a single pool of virus vector particles of defined passage derived directly from the virus master seed lot and which is the starting material for individual manufacturing batches of viral-vectored vaccine product.

Working cell bank (Manufacturer's Working Cell Bank, MWCB): a quantity of well-characterized cells of animal or other origin, derived from the master cell bank at a specific population doubling level or passage level, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions, such as in the vapour or liquid phase of liquid nitrogen in aliquots of uniform composition. The working cell bank is prepared from a single homogeneously mixed pool of cells. One or more of the working cell bank containers is used for each production culture.

General considerations

Respiratory syncytial virus (RSV)

RSV belongs to the Genus *Orthopneumovirus*, within the Family *Pneumoviridae*, and Order *Mononegavirales*. The members of this genus are human RSV, bovine RSV, ovine RSV, and pneumonia virus of mice. The RSV virion consists of a nucleocapsid packaged in a lipid envelope derived from the host cell plasma membrane (17). RSV is a single-stranded, non-segmented negative-sense RNA genome with 15,191–15,288 nucleotides (17,18).

The RSV envelope contains three viral transmembrane surface glycoproteins: the large glycoprotein G, the fusion protein F, and the small hydrophobic SH protein. The non-glycosylated matrix M protein is present on the inner face of the envelope. The RSV F and G glycoproteins are the only targets of neutralizing antibodies and are the major protective antigens (17). The 574-amino acid F protein forms a homotrimer that directs viral penetration and syncytium formation. The large RSV-G glycoprotein is thought to form trimers and/or tetramers and is involved in attachment; a secreted form of this protein helps RSV evade host immunity (17). The most extensive antigenic and genetic diversity is found in the attachment glycoprotein, G (17,19).

There are two major antigenic groups of human RSV, A and B that exhibit genome-wide sequence divergence, and additional antigenic variability occurs within each group (19).

Epidemiology

RSV is a leading cause of respiratory disease globally. The virus causes infections at all ages, but young infants have the highest incidence of severe disease, peaking at 1–3 months of age. By 2 years of age, virtually all children will have been infected. Globally it is estimated that RSV causes >30 million acute LRTI in young children annually, with over 3 million severe cases requiring hospitalization, making it the most common cause of hospitalization in children under 5 years of age. The global mortality attributed to RSV-acute lower respiratory infection (ALRI) in young children is as high as 118 200 per annum (20).

RSV infection does not elicit long-lasting protective immunity and repeated upper respiratory tract infections are common throughout life. Infections in adults can range from asymptomatic to life-threatening, especially among adults >65 years of age and in those with underlying heart and lung problems (21). RSV transmission follows a marked seasonal pattern in temperate areas with mid-winter epidemics but may occur during rainy seasons or year-round in the tropics.

The two major RSV subtypes, A and B, and multiple genotypes of each can either dominate or co-circulate during RSV epidemic seasons every year; the association between disease severity and a specific RSV subtype or genotype is variable with no consistent pattern discerned (22).

Disease and diagnosis

The incubation period for RSV is usually 3 - 6 days (range from 2 to 8 days). Virus enters the body usually through the eye or nose, rarely through the mouth. Virus then spreads along the epithelium of the respiratory tract, mostly by cell-to-cell transfer. As virus spreads to the lower respiratory tract, it may produce bronchiolitis and/or pneumonia. Resolution of these pathological changes may take up to 3 weeks or more. Primary infections are often symptomatic but may vary from a mild common cold to a life-threatening LRTI. The course of the illness is variable, lasting from one to several weeks. Most infants show signs of improvement within 3 or 4 days after the onset of lower respiratory tract disease (23).

RSV infection may be diagnosed by cell culture techniques, or by the direct identification of viral antigen or virus genome through rapid diagnostic techniques. Diagnosis may be supported by serological testing. However, since both acute and convalescent serum samples are needed, additional time is required before serology results are available.

Immune response to natural RSV infection

Innate and adaptive immune responses contribute not only to the control and prevention of RSV infection but also to the pathogenesis of RSV disease. The repertoire of immune responses may vary substantially over the course of a lifetime. A careful and complete description of the ontogeny and subsequent modulation of the human immune response against RSV in neonates, infants, children and adults remains an area of active investigation and a thorough summary of the many known parameters is beyond the scope of this guideline but may be found in several reviews (24-28). A few of the immune responses associated with protection or potential pathology following RSV infection are discussed in brief here including: virus neutralizing antibodies, IgG and IgA antibodies in serum and on mucosal surfaces, including epitope-specific IgG responses, and cell-mediated immunity involving RSV-specific $CD8^+$ cytotoxic T cell and $CD4^+$ T-helper cell responses.

Whilst there is no established correlate of protection it appears that high concentrations of serum anti-RSV neutralizing antibodies are associated with a substantial decrease in the risk of severe lower respiratory tract disease following infection. It is now known that the majority of serum neutralizing antibodies elicited in response to natural RSV infection are directed against the prefusion conformation of RSV-F protein with smaller amounts directed against post-fusion RSV-F and against RSV-G (29, 30). Antibodies that bind specific epitopes present on prefusion RSV-F trimer tend to be highly potent neutralizers relative to activity seen with antibodies directed against post-fusion RSV-F. Many of the antibodies directed against RSV-F are broadly neutralizing and cross-reactive with both RSV-F_A and -F_B proteins; however, subtype-specific epitopes have also been identified on both prefusion and post-fusion RSV-F (31, 32).

Most post-infection human serum samples contain IgG antibodies to the central conserved region within the RSV-G protein, a region that mediates virus binding to the cellular receptor, CX3CR1 (33-35). Antibodies that bind to this region of RSV-G react with both RSV subtypes and have been associated with protection against RSV infection *in vivo* and broad neutralizing activity *in vitro*. Antibody responses against RSV-G protein may also be subtype-specific for RSV-G_A or -G_B protein with specificity determined by substantial genetic variability within the mucin-like C-terminal domains of this protein. The C-terminal RSV-G domain can bind glycosaminoglycans and C-type lectins that may facilitate virus infection and/or alter dendritic cell signaling (36).

Mucosal anti-RSV IgA antibodies correlated with a decrease in the severity of disease in adults experimentally infected with RSV and in children with natural RSV infection (37,38).

While antibodies may prevent RSV infection, cytotoxic $CD8^+$ T cells are needed to clear virus-infected cells (39). Cytotoxic $CD8^+$ T-cells may be elicited following natural RSV infection

and following immunization if antigenic peptides are expressed in the context of MHC Class I. $CD8^+$ T cells have been detected in bronchial alveolar lavage fluids and peripheral blood of infants and children after RSV infection (40,41). Resident memory $CD8^+$ T-cells (T_{RM}) with a $CD3^+$ $CD8^+$ $CD103^+$ $CD69^+$ phenotype were recovered from lower airways using bronchoscopy in adults immediately after experimental RSV challenge, identified following re-stimulation with synthetic peptides representing sequences within RSV-N, -M and -NS2 proteins and confirmed using tetramer staining (38). In this study, T_{RM} were also detected in peripheral blood of adults 10 days after RSV challenge but were lower in frequency than the numbers recovered by bronchoscopy.

$CD4^+$ T-helper and T-regulatory (T_{REG}) cells modulate B and/or T-cell proliferation. $CD4^+$ T-helper cells in infants less than 6 months of age are epigenetically programmed to have a dominant Th2 type cytokine response that may be antigen specific (42, 43). Th2-type CD4+ T-helper cell responses are associated with cytokines that can lead to allergic inflammation; these responses have been associated with severe disease in RSV-infected infants in some studies suggesting that a dominant Th2 type cytokine response following RSV exposure is not desirable in young infants (44). During infancy, cytokine responses may also be skewed in favour of Th2-type responses as a result of down-regulation of Th1 responses. For example, a recent study identified a specific subset of neonatal regulatory B (nBreg) cells that produce anti-inflammatory IL-10 that downregulates Th1 cell responses when infected with RSV via the B cell receptor (BCR] and CX3CR1. Neonates with severe RSV-bronchiolitis had high numbers of RSV-infected nBreg cells that correlated directly with an increase in viral load and decrease in the frequency of memory Th1 cells (45).

RSV vaccine development

RSV vaccine development started in 1960s with an unsuccessful formalin-inactivated RSV vaccine (FI-RSV) (46) that induced a severe and, in two cases, lethal lung inflammatory response during the first natural RSV infection after vaccination of RSV-naïve infants. The concerns over FI-RSV vaccine hindered development of alternative RSV vaccines for many years. However, a number of candidate vaccines have been proposed over the last decade, some with promising results (47-49) and multiple observations support the feasibility of vaccination against RSV (50). Numerous efforts targeting the RSV F protein with vaccines have been based on the protective efficacy seen in infants given a neutralizing anti-F monoclonal antibody (mAb) (palivizumab). As the understanding of RSV biology has improved, the other major envelope protein, RSV-G, has emerged as another potential target reflecting its critical role in mediating infection of bronchial epithelial cells and in altering the host immune response (51).

Currently, there are no vaccines licensed for the prevention of RSV disease in any age group. Several candidates are at various stages of development with the most advanced candidate(s) in phase III clinical efficacy trials. These vaccines are mainly monovalent vaccines. Future developments might include multivalent vaccines containing antigens derived from more than one RSV strain or subtype. For this group of vaccines, special considerations might apply.

Nevertheless, due to the prior experience with the FI-RSV, a cautious approach has been taken in vaccine development, especially with regard to candidates aimed at eliciting active immunity in RSV-naïve infants.

It is important that RSV vaccine candidates with properties similar to FI-RSV, such as inactivated virus antigens and subunit protein or particle-based vaccines, are carefully studied using animal models in order to assess the potential risk of vaccine-associated enhancement of respiratory disease (ERD), prior to testing in an RSV-naïve infant population. Due to the lack of adequate animal models that mimic human RSV disease, several semi-permissive animal models are discussed in this Guideline (see Part B). Great weight is placed on histopathological finding in the lungs of challenged animals as well as comprehensive characterization of the induced immune responses, including the functionality of antibodies elicited in response to the candidate vaccine, the balance of type 1 and type 2 $CD4^+$ T-helper cell cytokine profiles, and presence or absence of CD^+8 cytotoxic T cells.

Part A. Guidelines on development, manufacturing and control of human RSV vaccines

A.1 Definitions

A.1.1 International name and proper name

Although there is no licensed RSV vaccine, the provision of a suggested international name will aid harmonization of nomenclature after licensure. The international name for Respiratory Syncytial Virus Vaccine should be “Respiratory Syncytial Virus Vaccine”, depending on the construct of the antigen, this should be further specified (e.g. live attenuated, recombinant), including the use of words such as “adjuvanted” and/or “adsorbed”, if relevant. The proper name should be the equivalent of the international name in the country of origin followed in parentheses by the virus subtype (where applicable) and name of the recombinant protein/proteins when applicable.

A.1.2 Descriptive definition name

A live-attenuated RSV vaccine which has been derived either with conventional attenuation of RSV or with recombinant biological methods should express antigens of RSV. The full proper name should identify the subtype of the parental virus from which it was derived and include gene-by-gene notations to identify deletions, insertions, mutations, and changes in gene order relevant to the attenuation phenotype. The vaccine may be presented as a sterile aqueous suspension or as freeze-dried material. Likewise, a chimeric live-attenuated RSV vaccine (for example, recombinant bovine parainfluenza RSV chimera, recombinant Sendai-RSV chimera or recombinant BCG-RSV chimera) should contain the gene(s) for the RSV antigen/antigens. These chimeric vaccines are produced by recombinant DNA technology. The vaccine may be presented as a sterile aqueous suspension or as freeze-dried material.

A particle-based or subunit vaccine should identify the RSV antigen/antigens which are included in the vaccine and produced by recombinant DNA technology. Particle-based RSV

vaccines may form nanoparticles. A particle-based or subunit vaccine might be formulated with a suitable adjuvant. The vaccine may be presented as a sterile liquid suspension.

A replication-deficient viral-vectored RSV vaccine derived from a platform technology, such as adenovirus or Modified Vaccinia Ankara (MVA), is produced by recombinant DNA technology and should identify the RSV antigen/antigens expressed by the vector. The vectored vaccine may be presented as a sterile liquid suspension or as freeze-dried material.

Such vaccines are for prophylactic use.

A.1.3 International reference materials

As the prospective vaccines differ in type, no international reference material for the various vaccine candidates is currently available.

However, the 1st WHO International Standard for antiserum to Respiratory Syncytial Virus, has been established by the WHO Expert Committee on Biological Standardization (ECBS) with assigned unitage of 1000 IU/ampoule, to be used for standardization of virus neutralization methods to measure antibody levels against RSV/A in human sera (52). This allows standardization of RSV neutralization assays independently from the assay format, and eases comparability of immunogenicity of RSV vaccine candidates.

The standard is available from the National Institute for Biological Standards and Control, Potters Bar, the United Kingdom. For the latest list of appropriate WHO international standards and reference materials, the WHO Catalogue of International Reference Preparations (53) should be consulted.

A.1.3.1 Expression of dose related to vaccine potency

In the case of live-virus and chimeric virus vaccines, potency is typically expressed in terms of the number of infectious units of virus contained in a human dose, using a specified tissue culture substrate and based on the results of clinical trials.

International standards and reference reagents for the control of RSV vaccine antigen content and potency are not available. Therefore, product-specific reference preparations may be used. The dose related to vaccine potency should be calculated against a product-specific standard. Until then, alternatively, plaque-forming units (PFUs), the median cell culture infectious dose ($CCID_{50}$) or other relevant product-specific assays can be used to express the potency and dose of the vaccine. The dose should also serve as the basis for establishment of parameters for stability and expiry date.

A.2 General manufacturing guidelines

The general manufacturing requirements contained in WHO good manufacturing practices for biological products and pharmaceutical products should apply to the design, establishment, operation, control and maintenance of manufacturing facilities for the respective type of vaccine (54,55). Manufacturing areas may be used on a campaign basis

with adequate cleaning between campaigns to ensure that cross-contamination does not occur.

Production steps involving manipulations of recombinant types which might involve live viruses should be conducted at a biosafety level consistent with the production of recombinant microorganisms, according to the principles of the WHO`s Laboratory biosafety manual (56). The basis for this is a microbiological risk assessment which results in classification in different biosafety levels. The respective classification level should be approved by the relevant authority from the country/region where the manufacturing facility is located.

A.2.1 Considerations on manufacturing of RSV vaccines

As there is currently no licensed RSV vaccine available, the following provision should be considered.

During early clinical trials, it is unlikely that there will be data from sufficient batches to validate/qualify product manufacture. However, as development progresses, data should be obtained from subsequent manufacture and should be used in support of an eventual application for commercial supply of the product.

In addition to control during manufacture, each product should be adequately characterized by the stage of development. These attributes facilitate understanding of the biology of the candidate vaccine and assessment of the impact of any changes in manufacturing that are introduced as development advances, or in a post-licensure setting. The immunogenicity of the product, when relevant and available, should also be included in the characterization programme (e.g. as part of the nonclinical pharmacodynamic evaluation). When available, and in agreement with NRA, data from platform technology could be supportive and leveraged.

By the time of submitting a marketing authorization application, the manufacturing process should be adequately validated by demonstrating that at least three consecutively produced commercial-scale batches can be manufactured consistently. Adequate control of the manufacturing process may be demonstrated by showing that each lot meets predetermined, in-process controls, critical process parameters and lot release specifications. Whenever important changes are made to the manufacturing process along the development of the vaccine, a comparability exercise should be performed between batches manufactured according to the different manufacturing processes following the ICH Q5E guideline (57). This is extremely important if changes are introduced between the Phase III pivotal study batches and future commercial batches. Any materials added during the purification process should be documented, and their removal should be adequately validated or residual amounts tested for, as appropriate. Validation should also demonstrate that the manufacturing facility and equipment have been qualified, cleaning of product contact surfaces is adequate, and critical process steps such as sterile filtrations and aseptic operations have been validated.

A.3 Control of source materials

A.3.1 Cell culture

RSV vaccine candidates have been produced in human cells (e.g. Human Embryonic Kidney cells - HEK 293), mammalian cells (e.g. Chinese Hamster Ovary cells CHO-K1, African Green Monkey Vero cells), in primary chick embryo cells, embryonated chicken eggs, and in insect cells (e.g. Sf9 derived from *Spodoptera frugiperda,* Hi-5 Rix4446 cells derived from *Trichoplusia ni*).

The use of a cell line should be based on a cell bank system (14). Sufficient information on the provenance of the cell bank should be provided. A maximum number of passages or population doublings level (PDL) should be established, if applicable. This should be established from the Master Cell bank (MCB), Working Cell bank (WCB) and the cells used for production. The cell bank or seed should be approved by the NRA.

Additional tests may include but are not limited to propagation of the master cell bank or working cell bank to or beyond the maximum *in vitro* age for production (end of production cells), examination for the presence of retroviruses and tumorigenicity in an animal test system (59). The MCB, WCB and EOP cells should be tested as described under cell substrates (see A.3.5.2.2, Continuous cell lines).

If primary cells or eggs are used, they should be based on a controlled system (for primary cells, see A.3.5.2.1; for eggs, see A.4.2.2 of WHO Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (15).

A.3.2 Source materials used for cell culture and virus propagation

Only substances that have been approved by the NRA may be added.

If serum is used for the propagation of cells, it should be tested to demonstrate absence of bacteria, fungi and mycoplasmas – as specified in the requirements of the WHO General requirements for the sterility of biological substances (60) – and freedom from adventitious viruses.

Detailed guidelines for detecting bovine viruses in serum for establishing MCB and WCB are given in Appendix 1 of WHO's Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14) and should be applied as appropriate. The guidelines for detecting bovine viruses in serum for establishing cell banks may also be applicable to production cell cultures. As an additional monitor of quality, sera may be examined for endotoxin. Gamma irradiation may be used to inactivate potential contaminant viruses, recognizing that some viruses are relatively resistant to gamma irradiation. Whatever the process used, the validation study should determine the consistency and effectiveness of the viral inactivation process while maintaining serum performance. The use of non-inactivated serum should be

strongly justified. The non-inactivated serum must meet the same criteria as the inactivated serum when tested for sterility and absence of mycoplasma and viral contaminants.

The source(s) of animal components used in culture medium (or used to produce culture medium components) should be approved by the NRA. These components should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (61).

Bovine or porcine trypsin used for preparing cell cultures (or used to prepare culture medium components) should be tested and found free of bacteria, fungi, mycoplasmas and adventitious viruses, as appropriate. The methods used to ensure this should be approved by the NRA. The source(s) of trypsin of bovine origin, if used, should be approved by the NRA and should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (61).

In some countries, irradiation is used to inactivate potential contaminant viruses in trypsin. If irradiation is used, it is important to ensure that a reproducible dose is delivered to all batches and the component units of each batch. The irradiation dose must be low enough so that the biological properties of the reagents are retained while being high enough to reduce virological risk. Consequently, irradiation cannot be considered a sterilizing process. The irradiation method should be validated by the manufacturer and approved by the NRA.

Recombinant trypsin is available and should be considered; however, it should not be assumed to be free of risk of contamination and should be subject to the usual considerations for any reagent of biological origin (14).

Human serum should not be used.

If human serum albumin derived from human plasma is used at any stage of product manufacture, the NRA should be consulted regarding the requirements, as these may differ from country to country. At a minimum, it should meet the WHO Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives (62). In addition, human albumin and materials of animal origin should comply with the current WHO guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products (61).

Penicillin and other beta-lactams should not be used at any stage of the manufacture because they are highly sensitizing substances. Other antibiotics may be used in the manufacture provided that the quantity present in the final lot is acceptable to the NRA.

Non-toxic pH indicators may be added (e.g. phenol red at a concentration of 0.002%).

A.3.3 Control of source materials for live attenuated/chimeric RSV vaccines

A.3.3.1 Virus seed/chimeric seed

A.3.3.1.1 Vaccine virus strains/chimeric strains

Strains of live RSV, attenuated biologically, chemically or by recombinant DNA technology, should be thoroughly characterized. This should include information on the origin of the strain, cell culture passage history, method of attenuation (e.g., by serial passages in animal species such as mice and chimpanzees), results of preclinical and clinical studies to prove attenuation, and whether the strains have been modified biologically, chemically, or by molecular biological methods before generation of the master seed. Furthermore, information on the complete genome sequence, and the passage level of the material used in clinical trials should be indicated. The respective strains should be approved by the NRA.

Strains of RSV recombinant viruses used for master and working seeds to produce vaccine candidates should comply with the additional specifications given in section A.3.3.1.1.1.

For chimeric RSV vaccines like bovine parainfluenza RSV chimera or recombinant Sendai RSV chimera, the provisions laid down in **Error! Reference source not found.** apply.

A.3.3.1.1.1 Strains derived by molecular methods

In some countries, if a vaccine strain derived by recombinant DNA technology is used, and due to the fact that it is a live-attenuated vaccine, the candidate vaccine is considered to be a GMO and should comply with the regulations of the producing and recipient countries regarding GMOs.

The entire nucleotide sequence of any complementary DNA (cDNA) clone used to generate vaccine-virus stocks should be determined prior to any further nonclinical study or clinical trial. The cell substrate used for transfection to generate the virus should be appropriate for human vaccine production and should be approved by the NRA.

A.3.3.1.2 Seed lot system

The production of the RSV vaccine should be based on a virus-seed lot system, to minimize the number of tissue culture passages needed for vaccine production. This includes a master seed and a working seed. Seed lots should be prepared in the same type of cells using similar conditions for virus growth as those used for production of final vaccine.

The virus working seed should have a defined relationship to the virus master seed with respect to passage level and method of preparation, such that the virus working seed retains the *in vitro* phenotypes and the genetic character of the virus master seed. Once the passage level of the virus working seed with respect to the virus master seed is established, it should not be changed without approval from the NRA.

The maximum passage level of master seed and working seed should be approved by the NRA. The inoculum for infecting cells used in the production of vaccine should be from a

virus working seed without intervening passages in order to ensure that no vaccine is manufactured that is more than one passage level from the working seed.

The virus master seed and working seed lots used to produce live-attenuated RSV vaccine should be demonstrated to be safe and immunogenic using appropriate laboratory tests. Virus seed lots should be stored in a dedicated temperature-monitored freezer that ensures stability upon storage. It is recommended that a large virus working seed lot should be set aside as the basic material for use by the manufacturer for the preparation of each batch of vaccine.

Likewise, the production of chimeric BCG/RSV vaccine should be based on a seed lot system. The provisions laid down in section A.3 of the recommendations to assure the quality, safety and efficacy of BCG vaccines apply (16).

A.3.3.2 Control of cell cultures for virus seeds

In agreement with the NRAs, tests on control cell cultures may be required and performed as described in section A.4.1.

A.3.3.3 Control of virus seeds

The following tests should be performed on virus master and working seed lots.

A.3.3.3.1 Identity test

Each virus master and working seed lot should be identified as RSV vaccine seed virus by immunological assay or by molecular methods approved by the NRA.

A.3.3.3.2 Genetic/ phenotypic characterization

Each seed should be characterized by full-length consensus nucleotide sequence determination and by other relevant laboratory and animal tests, which will provide information on the consistency of each virus seed. These tests are required to compare the new vaccine strain with the wild type and/or parent seed.

Mutations introduced during the derivation of each vaccine strain should be maintained in the consensus sequence, unless spontaneous mutations induced during tissue culture passage were shown to be without effect in nonclinical and small-scale clinical trials. Some variations in the nucleotide sequence of the virus population during passaging are to be expected, but the determination of what is acceptable should be based on experience in production and clinical use.

Genetic stability of the vaccine seed to a passage level comparable to final virus bulk and preferably beyond the anticipated maximum passage level should be demonstrated. Phenotypic characterization should focus on the markers for attenuation/modification and expression of the heterologous antigens.

For any new master seed and working seed, it is recommended that the first three consecutive bulk vaccine lots should be analyzed for consensus genome sequence changes from the virus master seed. The nucleotide sequence results should be used to demonstrate the consistency of the production process.

A.3.3.3.3 Tests for bacteria, fungi, mycoplasmas and mycobacteria

Each virus master and working seed lot should be shown to be free from bacterial, fungal and mycoplasmal contamination by appropriate tests as specified in the General requirements for the sterility of biological substances (60, 63). Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, may be used as an alternative to one or both of the compendial mycoplasma detection methods after suitable validation and agreement with the NRA (14).

A.3.3.3.4 Tests for adventitious agents

Each virus master and working seed lot should be tested in cell culture for adventitious agents relevant to the passage history of the seed virus. Where antisera are used to neutralize the recombinant RSV virus, the antigen used to generate the antiserum should be produced in cell culture from species different from that used for the production of the vaccine, and it should be free from extraneous agents. Suitable indicator cells should be selected to enable the detection of viruses. The decision on the choice of the indicator cells should be guided by the species and legacy of the production cell substrate, taking into consideration the types of viruses to which the cell substrate could potentially have been exposed. Infection with potential viruses should then be detected by a suitable assay method. For test details, refer to section B.11 of WHO's recommendation for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14). Additional testing for adventitious viruses may be performed using validated nucleic acid amplification techniques.

Each virus seed lot is tested in suckling mice if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package. Inoculate no fewer than 20 suckling mice, each less than 24 h old, intracerebrally with 0.01 mL and intraperitoneally with at least 0.1 mL of the virus seed lot. Observe the suckling mice daily for at least 4 weeks. Carry out an autopsy of all suckling mice that die after the first 24 h of the test or that show signs of illness and examine for evidence of viral infection by direct macroscopical observation. The virus seed lot passes the test if no suckling mice show evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated suckling mice survive the observation period.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection. These methods include: (i) degenerate nucleic acid amplification techniques for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (ii) nucleic acid amplification techniques with random primers followed by analysis of the amplicons on large oligonucleotide micro-arrays of conserved viral sequencing or digital subtraction of expressed sequences; and (iii) high throughput sequencing. These methods may be used in the future to supplement existing methods or as alternative methods to both *in vivo* and *in vitro* tests after appropriate validation and agreement from the NRA.

A.3.3.3.5 Tests in experimental animals

As outlined in the nonclinical part, studies should be performed in animals to determine that the master virus seed lots have attenuating features which are maintained throughout subsequent vaccine process steps. For certain vaccine candidates, it may be required to test, at least once during nonclinical development, for these features in a relevant animal model. For a master virus seed lot to be identified as attenuated, identification of attenuation should be clearly defined.

The NRA may decide that such testing does not need to be repeated each time a novel working seed lot is derived.

A.3.3.3.6 Virus titration for infectivity

The infectivity of each virus master seed and working seed lot should be established using an assay that is acceptable to the NRA. Manufacturers should determine the appropriate titre necessary to produce vaccine consistently. Depending on the results obtained in preclinical studies, plaque assays, $CCID_{50}$ assays, immunofocus-forming unit assays or $CCID_{50}$ with different read-outs may be used. All assays should be validated.

A.3.3.3.7 Control of bacterial seeds

For control of bacterial seeds, the provisions laid down in WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (16), apply.

A.3.4 Control of source material for subunit/particle-based RSV vaccines

A.3.4.1 Cells for antigen production

A.3.4.1.1 Recombinant yeast and bacteria cells

The characteristics of the parental cells and the recombinant strain (parental cell transformed with the recombinant expression construct) should be fully described and information should be given on the testing for adventitious agents and on gene homogeneity for the MCB and WCB. A full description of the biological characteristics of the host cell and expression vectors should be given. This should include genetic markers of the host cell, the construction, genetics and structure of the expression vector, and the origin and identification of the gene that is being cloned. Some techniques (e.g. deep sequencing) allow for the entire construct to be examined, while others (e.g. restriction enzyme analysis) allow for assessment of segments (64, 65). The molecular and physiological measures used to promote and control the expression of the cloned gene in the host cell should be described in detail (65).

The nucleotide sequence of the gene insert and the adjacent segments of the vector and restriction-enzyme mapping of the vector containing the gene insert should be provided as required by the NRA.

Cells must be maintained in a frozen state that allows for recovery of viable cells without alteration of genotype. The cells should be recovered from the frozen state, if necessary in selective media, such that the genotype and phenotype consistent with the recombinant

(modified) host and vector are maintained clearly identifiable. Cell banks must be identified and characterized by appropriate tests.

Data – for example on plasmid restriction enzyme mapping, nutritional requirements or antibiotic resistance (if applicable) – that demonstrate the genetic stability of the expression system during passage of the recombinant WCB up to beyond the passage level used for production should be provided to and approved by the NRA. Any instability of the expression system occurring in the seed culture during expansion or after a production-scale run should be documented. Stability studies should also be performed to confirm cell viability after retrieval from storage, maintenance of the expression system, etc. These studies may be performed as part of their routine use in production or may include samples specifically for that purpose.

A.3.4.1.1.1 Tests of recombinant yeast and bacterial MCB and WCB

MCBs and WCBs should be tested for the absence of bacterial and fungal contamination by appropriate tests, as specified in the WHO General requirements for the sterility of biological substances (63), or by a method approved by the NRA, to demonstrate that only the bacteria or yeast production strain is present and that the MCB and WCB are not contaminated with other bacteria and fungi.

A.3.4.1.2 Recombinant mammalian cells

If mammalian cells are used, the cell substrates and cell banks should conform with the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (65), and should be approved by the NRA.

A.3.4.1.3 Insect cells

If insect cells are used for expression of the RSV vaccine antigen with a baculo-based expression vector, cell substrates and cell banks should conform with the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14), as appropriate to insect cells, and should be approved by the NRA.

The MCB is made in sufficient quantities and stored in a secure environment, and is used as the source material for making the manufacturer's WCB. In normal practice a MCB is expanded by serial subculture up to a passage number (or population doubling, as appropriate) selected by the manufacturer and approved by the NRA, at which point the cells are combined to give a single pool which is distributed into ampoules and preserved cryogenically to form the WCB. WCBs of insect cells may be used for recombinant baculovirus seed lot production and antigen expression.

A.3.4.1.3.1 Tests on insect MCB and WCB

Tests of the MCB and WCB should be performed in accordance with the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological products and for the characterization cell banks (14). It is important to show that the cell banks are free from bacteria, fungi, mycoplasmas, mycobacterium species, and adventitious agents relevant to the species that may be present in raw materials used in its derivation. For insect cells, special emphasis is put on potential insect-borne human pathogens (e.g. arboviruses).

Insect viruses have not been well characterized compared with other potential adventitious agents, and thus, less information about them is available, especially about their infectivity, replicative life-cycles and pathogenicity, if any. It should be kept in mind that infection of insect cells with some insect viruses may occur without showing cytopathic effect. Testing may include specific nucleic acid amplification techniques (NAT) such as polymerase chain reaction (PCR) and other non-specific tests such as co-cultivation. The specificity and sensitivity of assays should be determined by the manufacturer and approved by the NRA.

Full characterization may be performed on either the MCB or the WCB, with more limited testing on the other, depending on the strategy chosen for testing (14). Scientific advice on the testing strategy should be sought from the NRA.

A.3.4.2 Recombinant baculovirus master and working seed virus

The recombinant baculovirus expression vector used in the production of RSV vaccine contains the coding sequence of the respective RSV antigen/antigens and should be identified by historical records. The historical records will include information on the origin and identity of the gene being cloned, and on the construction, genetics and structure of the baculovirus expression vector.

The production of vaccine should be based on the recombinant baculovirus master and working seed lot system. Recombinant baculovirus seed lots should be stored in a dedicated temperature-monitored refrigerator at a temperature that ensures stability and security.

Only recombinant baculovirus seed lots that are approved by the NRA should be used. The recombinant baculovirus master seed lot should be made in sufficient quantities to last the lifetime of the vaccine product and should be stored in a secure environment. The master seed lot is used as the source material for making the manufacturer`s recombinant baculovirus working seed lot. Either the virus master seed lots or the virus working seed lots should be fully characterized and tested extensively for adventitious agents, while the other may be more-limited testing. The testing strategy and seed lots should be approved by the NRA.

The manufacturer`s recombinant baculovirus working seed lot is used in the production of inoculum intermediates and single harvests and is prepared from the master recombinant baculovirus seed lot. It is recommended that a large lot of recombinant baculovirus working seed should be set aside as the basic material that the manufacturer should use for the preparation of each batch of the vaccine. The recombinant baculovirus working seed lot should be prepared by a defined number of passages from the recombinant baculovirus

master seed lot using a method and a passage level from the original virus seed approved by the NRA. Once the acceptable level of the working seed lot is established, it may not be changed in making future lots of working seed without approval from the NRA.

A.3.4.2.1 Tests on recombinant baculovirus master and working seed lots

The expression construct should be analyzed using NAT in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the expressed RSV antigens. The genetic stability and stability of expression of the expression construct should be demonstrated from the baculovirus master seed up to at least the highest passage level used in production, but preferably beyond this level (64, 65).

A.3.4.2.1.1 Identity

Each baculovirus master and working seed lot should be identified for the inserted RSV gene using an appropriate molecular method approved by the NRA.

A.3.4.2.1.2 Sterility test for bacteria, fungi and mycoplasmas

The provisions laid down in section A.3.3.3.3 apply.

A.3.4.2.1.3 Tests for adventitious agents

Each recombinant baculovirus seed should be tested in cell cultures for adventitious agents appropriate to the origin and the passage history of the seed baculovirus. For tests on recombinant baculovirus-permissive indicator cells, the neutralization of baculovirus is necessary. Antisera used for this purpose should be free from antibodies that may neutralize adventitious agents and should preferably be generated by the immunization of specific pathogen-free animals with an antigen from a source (other than the production cell line) which has itself been tested for freedom from adventitious agents. The inoculated indicator cells should be examined microscopically for cytopathic changes. At the end of the examination period, the cells should also be tested for haemadsorbing viruses (see section A.4.1.1).

It should be noted that infection of indicator cells with insect viruses may not reveal any cytopathic effect. Additional tests such as PCR, electron microscopy and co-cultivation may therefore be performed. It is important to show that recombinant baculovirus seed are free of adventitious agents relevant to the species used in their derivation with a special emphasis on potential insect-borne human pathogens (e.g. arboviruses). The specificity and sensitivity of assays should be determined by the manufacturer and approved by the NRA.

In general, recombinant baculovirus seeds should be assessed for absence of adventitious agents that may have been introduced during their production, including those that may be present in the source materials used at each production stages of the master and working virus seed lots. Each virus master or working seed should also be tested in animals. For details on these tests, see the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14). However, for ethical reasons, it is desirable to apply the 3R principles

(Replacement, Reduction, Refinement) to the use of animals, where scientifically appropriate (66).

New molecular methods with broad detection capabilities are being developed for adventitious agent detection may also be used in the future to supplement existing methods or as alternative methods to both *in vivo* and *in vitro* tests after appropriate validation and agreement from the NRA (seeA.3.3.3.4).

A.3.4.2.1.4 Test for mycobacterium spp

Each recombinant seed lot should be tested for mycobacterium spp. The test method and specifications should be approved by the NRA.

A.3.4.2.1.5 Test of control cells used for production of seeds

Tests on control cell cultures should be undertaken as described in section A.4.1.

A.3.4.2.1.6 Recombinant baculovirus concentration

Each recombinant baculovirus seed should be assayed for infectivity in a sensitive assay in an insect culture system. The detailed procedures for carrying out tests and interpreting results should be those approved by the NRA.

A.3.5 Control of source materials for viral-vectored vaccines

A.3.5.1 Virus vector master and working seed

The use of any viral vector should be based on a seed lot system, analogous to the cell banking system used for production cells described below.

The rationale behind the development of the viral-vectored vaccine should be described. The origin of all genetic components of the vaccine and their function should be specified; overall, this should allow a clear understanding of the functionality of the vaccine and how it is attenuated or made replication-incompetent by genetic engineering. All intended and unintended genetic modifications such as site-specific mutations, insertions, deletions and/or rearrangements to any component should be detailed in comparison with their natural counterparts. For a vaccine construct that incorporated genetic elements to control the expression of a transgene in, for example, a tissue-specific manner, evidence should be provided on product characterization and control to demonstrate such specificity. RNA editing should be discussed if relevant.

All steps from derivation of material that ultimately resulted in the candidate vaccine to the virus master seed level should be described. A diagrammatic description of the components used during vaccine development should be provided and annotated. The method of construction of the viral-vectored vaccine should be described and the final construct should be genetically characterized according to the principles discussed in this section.

The cloning strategy should ensure that if any antibiotic resistance genes are used during the development of the initial genetic construct, these are absent from the viral vaccine seed.

The nucleotide sequence of the gene insert and of adjacent segments of the vector, and restriction-enzyme mapping of the vector containing the gene insert, should be provided. Genetic stability of the vector with the recombinant construct should be demonstrated. The stability of a recombinant vector should be assessed by comparing the sequence of the vector at the level of a virus pre-master seed or virus master seed to its sequence at, or preferably beyond, the anticipated maximum passage level. The sequence should ensure that no changes occur to regions involved in attenuation (where known) or replication deficiency. Any modifications to the sequence of the heterologous insert should be investigated and demonstrated to have no impact on the resulting amino acid sequence (i.e. it should be a conservative change) or to the antigenic characteristics of the vaccine.

A.3.5.1.1 Tests on virus master seed and working seed

The virus master seed should be characterized as fully as possible. If this characterization is limited (e.g. because of limited quantities of material), the virus working seed should be fully characterized in addition to the limited characterization of the virus master seed. It should be noted that it would not be feasible to manufacture from the virus master seed in these circumstances.

Virus master seed characterization will include a description of the genetic and phenotypic properties of the vaccine vector. This should include at least a theoretical comparison with the parental viral vector/virus and is particularly important where vector modification might affect attenuation or replication competency, pathogenicity, and tissue tropism or species specificity of the vaccine vector compared with the parental vector.

Genetic characterization will involve nucleotide sequence analysis of the vaccine vector. Restriction mapping, southern blotting, PCR analysis or DNA fingerprinting will also be useful. Individual elements involved in expression of the heterologous gene(s) (including relevant junction regions) should be described and delineated.

Genetic stability of the vaccine seed to a passage level comparable to final virus bulk and preferably beyond the anticipated maximum passage level should be demonstrated.

Phenotypic characterization should focus on the markers for attenuation/modification and expression of the heterologous antigen(s), and should generally be performed in vitro under conditions that allow detection of revertants (including the emergence of replication-competent vectors from replication-incompetent vectors during passage). However, other studies including antigenic analysis, infectivity titre, ratio of genome copies to infectious units (for replicating vaccines) and in vitro yield should form part of the characterization. For replicating vectors, in vivo growth characteristics in a suitable animal model may also be informative and should be performed if justified. For some vectors (e.g. adenoviral vectors), the particle number should be measured in addition to the infectivity titre.

A subset of the above studies should be applied to the virus working seed lot and justification for the chosen subset should be provided.

Information should be given on the testing for adventitious agents.

A.3.5.2 Cell substrates

The cell substrate for the manufacture of a viral-vectored RSV vaccine should be based on controlled primary cells or a cell banking system.

A.3.5.2.1 Primary cells

Primary cells are used within the first passage after establishment from the original tissue, so it is not possible to carry out extensive characterization of the cells prior to their use. Therefore, additional emphasis is placed on the origin of the tissues from which the cell line is derived. Tissues should be derived from healthy animals/embryonated eggs subjected to veterinary and laboratory monitoring to certify the absence of pathogenic agents. Whenever possible, donor animals/embryonated eggs should be obtained from closed, specific pathogen-free colonies or flocks. Animals used as tissue donors should not have been used previously for experimental studies. Birds/animals should be adequately quarantined for an appropriate period of time prior to use for the preparation of cells.

Information on materials and components used for the preparation of primary cell substrates should be provided, including the identity and source of all reagents of human or animal origin. A description of testing performed on components of animal origin to certify the absence of detectable contaminants and adventitious agents should be included.

The methods used for the isolation of cells from tissue, establishment of primary cell cultures and maintenance of cultures should be described.

A.3.5.2.1.1 Tests on primary cells

The nature of primary cells precludes extensive testing and characterization before use. Testing to demonstrate the absence of adventitious agents (bacteria, fungi, mycoplasma, mycobacteria, and viruses) is therefore conducted concurrently and should include, where relevant, the observation of control (uninfected) cultures during parallel fermentations to the production runs. The inoculation of culture fluid from production and (where available) control cultures into various susceptible indicator cell cultures capable of detecting a wide range of relevant viruses, followed by examination for cytopathic changes and testing for the presence of hemadsorbing viruses, should also be performed routinely for batch release, in addition to pharmacopoeial testing for bacterial, fungi and mycoplasma in the control (if relevant) and production cultures. Mycoplasma and specific viruses of notable concern may also be tested for by additional methods such as PCR.

In the specific case of chick embryo fibroblasts (CEFs), the tissue should be sourced from specific pathogen-free (SPF) eggs. After preparation, the CEF cells should be tested for bacterial, fungal, mycobacterial, and mycoplasma contamination, for viral adventitious agents by in vitro assay using three cell lines, including avian and human cells (such as CEF, MRC-5 and Vero cells), and for viral adventitious agents by in vivo assay using mice and embryonated eggs, for Avian Leucosis Virus contamination and for the presence of retroviruses by measuring the reverse transcriptase (RT) activity. Testing should take into consideration that CEF cells are expected to be positive for RT activity due to the presence of

endogenous avian retroviral elements not associated with infectious retroviruses. Use of an amplification strategy (e.g., co-culturing of RT positive fluids on a RT-negative, retrovirus sensitive cell line) to determine whether a positive RT result can be attributed to the presence of an infectious retroviral agent may be necessary.

A.3.5.2.2 Continuous cell lines: Master and working cell banks

The cell banks should conform to the Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14).

An appropriate history of the cell bank should be provided. This should include the origin, identification, developmental manipulations and characteristics for the purposes of the vaccine. Full details of the construction of packaging cell lines should be given, including the nature and identity of the helper viral nucleic acid and its encoded proteins/functions. If available, information on the chromosomal location of the helper viral nucleic acid should also be provided.

Prior to licensure, genetic stability of the cell lines should be demonstrated. The stability of a production cell line should be assessed by comparing the critical regions of the cell line (and flanking regions) at the level of a pre-cell or master cell to its sequence at or beyond the anticipated maximum passage level. Stability studies should also be performed to confirm cell viability after retrieval from storage, maintenance of the expression system, etc. These studies may be performed as part of routine use in production or may include samples taken specifically for this purpose.

With regard to cell cultures, the maximum number of passages (or population doublings) allowable from the MCB through the WCB, and through production in cells, should be defined on the basis of the stability data generated above, and should be approved by the NRA.

A.3.5.2.2.1 Tests on MCB and WCB

MCBs and WCBs should be tested for the absence of bacterial, fungal, mycobacterial. mycoplasmal and viral contamination by appropriate tests, as specified in Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14), or by an alternative method approved by the NRA to demonstrate that the MCB and WCB are not contaminated with adventitious agents.

Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the pharmacopoeial mycoplasma detection methods after suitable validation and the agreement of the NRA (14).

The cell bank should be tested for tumorigenicity if it is of mammalian origin, as described in Section B of the Recommendations for the evaluation of animal cell cultures as substrates for

the manufacture of biological medicinal products and for the characterization of cell banks (14). The tumorigenic potential of the cell bank(s) should be described and strategies to mitigate risks that might be associated with this biological property should be described and justified.

A.4 Control of production for live-attenuated/chimeric RSV vaccines

A.4.1 Control of production cell cultures

In case a mammalian or animal cell line is used for propagation of the vaccine which has been thoroughly characterized and has been used for the production of other vaccines, the NRA might decide that no control cells might be necessary.

In case mammalian or animal cells are used for propagation of the vaccine and the NRA requires the use of control cells, the following procedures should be followed. From the cells used to prepare cultures for production of vaccine, a fraction equivalent to at least 5% of the total of 500 ml of cell suspension, or 100 million cells, should be used to prepare uninfected control cell cultures.

These control cultures should be observed microscopically for cytopathic and morphological changes attributable to the presence of adventitious agents for at least 14 days at the respective temperature used for the production cell culture after the day of inoculation of the production cultures, or until the time of final virus harvest, whichever comes first. At the end of the observation period, supernatant fluids collected from the control culture should be tested for adventitious agents as described below. Samples that are not tested immediately should be stored at -60°C or lower, until such tests can be conducted.

If adventitious agent testing of control cultures yields a positive result, the harvest of virus from the parallel vaccine virus-infected cultures should not be used for vaccine production. For the test to be valid, 20% or fewer of the control culture flasks should have been discarded, for any reason, by the end of the test period.

A.4.1.1 Test for haemadsorbing viruses

At the end of the observation period, a fraction of control cells comprising not less than 25% of the total should be tested for the presence of haemadsorbing viruses, using guinea-pig red blood cells. If the guinea-pig red blood cells have been stored prior to use in the haemadsorption assay, the duration of storage should not have exceeded seven days, and the storage temperature should have been in the range of 2-8°C.

In some countries, the NRA requires that additional tests for haemadsorbing viruses should be performed using red blood cells from other species, including those from human (blood group O), monkeys, and chickens (or other avian species). For all tests, readings should be taken after incubation for 30 minutes at 0-4°C, and again after a further incubation for 30 minutes at 20-25°C. The test for monkey red cells should be read once more after additional incubation for 30 minutes at 34-37°C.

For the test to be valid, 20% or fewer of the control culture flasks should have been discarded, for any reason, by the end of the test period.

A.4.1.2 Test for adventitious agents in control cell-culture fluids

Supernatant culture fluids from each of the control cell culture vessels should be tested for adventitious agents. A 10-ml sample of the pool should be tested in the same cell substrate, but not the same cell batch as that used for vaccine production, and an additional 10 ml samples in relevant cell systems.

Each sample should be inoculated into cell cultures in such a way that the dilution of the pooled fluid in the nutrient medium does not exceed 1:4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. A least one bottle of each kind of cell culture should not be inoculated to serve as a control.

The inoculated cultures should be incubated at a temperature of 35-37°C and should be examined at intervals for cytopathic effects over a period of at least 14 days.

Some NRAs require that, at the end of this observation period, a subculture is made in the same culture system and observed for a least an additional seven days. Furthermore, some NRAs require that these cells should be tested for the presence of haemadsorbing viruses.

The tests are satisfactory if no cytopathic changes attributable to adventitious agents are detected in the test sample. For the test to be valid, 20% or less of the culture vessels should have been discarded for nonspecific accidental reasons at the end of the test.

A.4.1.3 Identity of cells

Depending on the type of cells used at the production level, the cells –especially those propagated from the working cell bank – should be identified by means of tests approved by the NRA.

Suitable methods include but are not limited to biochemical tests (e.g. isoenzyme analyses), immunological tests (e.g. major histocompatibility complex assays), cytogenetic tests (e.g. for chromosomal markers) or tests for genetic markers (e.g. DNA fingerprinting).

A.4.2 Production and harvest of monovalent virus

A.4.2.1 Cells used for virus inoculation

On the day of inoculation with the seed virus, each production cell culture flask (or bottle) or control cell culture flask should be examined for cytopathic effects potentially caused by infectious agents. If the examination shows evidence of an adventitious agent, all cell cultures should be discarded.

If animal serum is used in the growth medium, the medium should be removed from the cell culture either before or after inoculation with the virus working seed. The cell cultures should be rinsed, and the growth medium replaced with serum-free maintenance medium.

Penicillin and other beta-lactam antibiotics should not be used during any stage of manufacturing. Minimal concentrations of other suitable antibiotics may be used if approved by the NRA.

A.4.2.1.1 Virus inoculation

Cell cultures are inoculated with virus working seed at a defined optimal multiplicity of infection (MOI). After viral adsorption, cell cultures are fed with maintenance medium, and are incubated at a temperature within a defined range and for a defined period.

The MOI, temperature range and duration of incubation depends on the vaccine strain and the production method, and specifications should be validated by each manufacturer.

A.4.2.2 Monovalent virus harvest pools

Vaccine virus is harvested within a defined period post-inoculation. A monovalent harvest may be the result of one of more single harvests or multiple parallel harvests. Samples of monovalent harvest pools should be taken for testing and should be stored at a temperature of -60°C or below. The manufacturer should submit data to support the conditions chosen for these procedures.

The monovalent harvest pool may be clarified or filtered to remove cell debris and stored at a temperature that ensures stability before being used to prepare the final bulk for filling. The sponsor should provide data to support the stability of the bulk throughout the duration of the chosen storage conditions, as well as to support the choice of storage temperature.

Harvests derived from continuous cell lines should be subjected to further purification to minimize the amount of cellular DNA, and/or treatment with DNase to reduce size of the DNA.

A.4.2.3 Tests on single virus harvest pools

A.4.2.3.1 Identity

A test for identity should be performed if this has not been done on the virus pool or on the bulks material.

A.4.2.3.2 Tests for bacteria, fungi, mycoplasmas and mycobacteria

A sample of each single-harvest or virus-culture supernatant should be tested for bacterial, fungal and mycoplasmal sterility as specified in General requirements for the sterility of biological substances (60,63), or by an alternative method approved by the NRA.

Nucleic acid amplification techniques alone or in combination with cell culture, with an appropriate detection method, might be used as an alternative to one or both of the pharmacopoeial mycoplasma detection methods after suitable validation and the agreement of the NRA (14).

The method for testing mycobacteria should be approved by the NRA. Nucleic acid amplification techniques might be used as an alternative to the microbiological culture method for mycobacteria after validation by the manufacturer and agreement by the NRA.

A.4.2.3.3 Tests for adventitious agents

If the single harvests are not pooled the same day, a test for adventitious agents should be performed on each single harvest.

A.4.2.3.4 Virus titration for infectivity

In the case of pooling of viral harvests, the virus content of each single harvest should be tested with an infectivity assay. Minimum acceptable titres should be established for use of a single harvest in the preparation of a virus pool or final bulk, and to confirm the consistency of production.

A.4.2.3.5 Residual bovine serum albumin content

If bovine serum is used during production, then residual bovine serum albumin (BSA) content should be measured and a maximum permitted concentration should be set and approved by the NRA.

In some countries, tests are carried out to estimate the amount of residual animal serum in the purified bulk or in the final vaccine. Other serum proteins may also be measured.

A.4.2.3.6 Test for consistency of virus characteristics

Recombinant RSV candidate vaccine lots should be tested and compared to the Master seed, working seed, or another suitable comparator, to ensure that the vaccine virus has not undergone critical changes during its multiplication in the production culture system.

Relevant assays should be identified in nonclinical studies and may include e.g. virus yield in cell culture, growth in primary human bronchial epithelial cells or plaque morphology. Other identifying characteristics may also be applicable.

Assays for the attenuation of recombinant RSV virus should also be conducted and compared to the control results.

The test for consistency may be omitted as a routine test once the consistency of the production process has been demonstrated on a significant number of batches in agreement with the NRA. Where there is a significant change in the manufacturing process, the test should be reintroduced.

A.4.3 Final bulk

A.4.3.1 Preparation of virus pool or bulk material

Only virus harvests meeting the recommendations for sterility and virus content should be pooled. The operations necessary for preparing the final bulk should be conducted in a manner that avoids contamination of the product.

In preparing the final bulk, any excipients (such as diluent or stabilizer) that are added to the product should have been shown not to impair the safety and efficacy of the vaccine in the concentration used, as approved by the respective NRA.

A.4.3.2 Tests on virus pool or bulk material

A.4.3.2.1 Test for residual materials

Each manufacturer should demonstrate, by testing each virus pool or by validating the manufacturing process that any residual materials used in the manufacturing – such as animal serum, antibiotics, residual cellular DNA and DNase – are consistently reduced to a level acceptable to the NRA.

The host-cell protein profile should be examined as part of the characterization studies (14).

For viruses grown in continuous cell-line cells, purified bulk material should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should be not more than the upper limit agreed by the NRA. If this is technically feasible, the size distribution of the DNA should be examined as a characterization test, taking into account the amount of DNA detectable using state of the art methods approved by the NRA.

A.4.3.2.2 Sterility

Except where it is subject to in-line sterile filtration as part of the filling process, the final bulk suspension should be tested for bacterial and fungal sterility according to the General requirements for sterility of biological substances (60,63), or by an alternative method approved by the NRA.

A.4.3.2.3 Storage

Prior to filling, the final bulk suspension should be stored under conditions shown by the manufacturer to allow the final bulk to retain the desired viral potency.

A.4.4 Control of production of chimeric RSV vaccines

For chimeric RSV vaccines grown on cell culture, the provisions laid down in sections A.4.1 to A.4.3 apply.

For chimeric RSV vaccines grown in eggs, most of the provisions laid down in Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (15) apply.

For chimeric BCG/RSV vaccines, most of the provisions laid down in sections A.4 of the recommendations to assure the quality safety and efficacy of BCG vaccines (16) apply.

A.5 Control of production for subunit/particle-based RSV vaccines

A.5.1 Production up to single antigen harvest

A.5.1.1 Microbial purity

Microbial purity in each fermentation vessel should be monitored at the end of the production run by methods approved by the NRA.

Any agent added to the fermenter or bioreactor with the intention to feed cells or to induce or increase cell density should be approved by the NRA. Penicillin and other beta-lactams

should not be used at any stage of manufacture because of their nature as highly sensitizing substances in humans. Other antibiotics may be used at any stage of manufacture, provided that the quantity present in the final products is acceptable to the NRA.

A.5.1.2 Production of antigen if mammalian or insect cells are used

Some mammalian cell lines have been generated which constitutively express the desired antigen.

In other technologies, cell cultures are expanded to an appropriate scale and are inoculated with the respective expression vector (e.g. recombinant baculovirus) at a defined MOI. After adsorption, the cell cultures are fed with maintenance medium and incubated within a defined temperature range and for a defined period of time.

The range of MOI, temperature, pH and incubation period will depend on the cell substrate and the specific characteristics of the expression vector. A defined range should be established by the manufacturer and approved by the NRA.

A single harvest is obtained within a defined time period post-inoculation. Several antigen harvests may be pooled. If multiple antigen harvests are pooled, each single antigen harvest should be sampled for testing, stabilized and stored under suitable conditions until pooling. Penicillin and other beta-lactams should not be used at any stage of manufacture because of their nature as highly sensitizing substances in humans. Other antibiotics may be used at any stage of manufacture, provided that the quantity present in the final product is acceptable to the NRA. Samples of single harvest pools should be taken for testing and stored at a temperature of -60°C or below.

A.5.1.3 Tests of control cell cultures

The provisions laid down in section A.4.1 apply. However, it should be noted that the control cell cultures should be incubated under conditions that are essentially similar to those used for the production cultures, with the agreement of the NRA. For insect cells, the incubation time of at least 14 days might not apply because of the specifics of cells cultivated in suspension but it should not be less than the time of collection of the single antigen harvest.

A.5.1.3.1 Tests for haemadsorbing viruses

The provision laid down in section A.4.1.1 applies. However, for cells cultivated in suspension, the test for presence of haemadsorbing viruses is not technically feasible. A test for presence of haemagglutinating agents using guinea-pig red blood cells is therefore required with spent control cell culture fluid.

A.5.1.3.2 Tests for other adventitious agents

The provisions laid down in section A.4.1 apply.

A.5.1.3.3 Test for identity of cells

The provisions laid down in section A.4.1.3 apply.

A.5.2 Purified antigen bulk

The purification process can be applied to a single antigen harvest, part of a single antigen harvest or a pool of single antigen harvests, and should be approved by the NRA. The maximum number of harvests that may be pooled should be defined by the manufacturer and approved by the NRA. Adequate purification may require several purification steps based on different biophysical and/or biochemical principles and may involve disassembly and reassembly of nanoparticles. The entire process used for the purification of the antigen should be appropriately validated and should be approved by the NRA. Any reagents added during the purification processes (such as DNAase) should be documented.

The purified monovalent antigen bulk can be stored under conditions shown by the manufacturer to allow it to retain the desired biological activity. Intermediate hold times should be approved by the NRA.

A.5.2.1 Tests on the purified antigen bulk

All quality-control release tests for the purified antigen bulk should be validated and should be shown to be suitable for the intended purpose. Assay validation or qualification should be appropriate for the stage of the development life cycle. Additional tests on intermediates during the purification process may be used to monitor for consistency and safety.

A.5.2.1.1 Identity

A test for identity should be performed by a suitable method.

A.5.2.1.2 Purity

The degree of purity of the antigen bulk and levels of residual host cell proteins should be assessed by suitable methods.

A.5.2.1.3 Protein content

Each purified antigen bulk should be tested for the total protein content using a suitable method. Alternatively, the total protein content may be calculated from measurement of an earlier process intermediate.

A.5.2.1.4 Antigen content

The antigen content may be measured on the purified monovalent antigen bulk or the adsorbed monovalent antigen bulk by an appropriate method.

The ratio of antigen content to protein content may be calculated and monitored for each purified antigen bulk.

International Standards and Reference Reagents for the control of RSV vaccine antigen are not available. Therefore, product-specific reference preparations may be used.

A.5.2.1.5 Sterility test for bacteria and fungi

The purified antigen bulk should be tested for bacterial and fungal sterility, as specified in the WHO general requirements for the sterility of biological substances (60,63), or by an alternative method approved by the NRA.

Alternatively, if the antigen is adsorbed onto an adjuvant and if the purified bulk is not stored prior to adsorption, the test can be performed on the related adsorbed antigen bulk.

A.5.2.1.6 Percentage of intact RSV antigens

If the integrity of certain RSV proteins (e.g. the F protein) is a critical quality parameter, this should be carefully monitored. The percentage of intact RSV protein should be assessed by a suitable method. The purity assay may also serve to assess the integrity of the respective conformation. Here, the percentage of e.g. intact F trimer is the ratio of intact F trimer to total F protein expressed as a percentage.

A.5.2.1.7 Nanoparticle size and structure

In the case of particle-based vaccines such as F protein nanoparticles, the size and structure of the nanoparticles are to be established and monitored. This test may be omitted for routine lot release once consistency of production has been established, in agreement of the NRA.

Suitable methods for assessing nanoparticle size and structure include dynamic light scattering, size-exclusion chromatography – high performance liquid chromatography (SEC-HPLC), transmission electron microscopy (TEM) and Disc Centrifugation Size analysis (DCS). DCS allows determination of the hydrodynamic radius of particles which sediment in a sucrose gradient in reference to spherical particles of known sizes.

A.5.2.1.8 Tests for reagents used during production or other phases of manufacture

A test should be carried out to detect the presence of any potentially hazardous reagents used during manufacture using methods approved by the NRA. This test may be omitted for routine lot release upon demonstration that the process consistency eliminates the reagent from the purified monovalent antigen bulks.

A.5.2.1.9 Tests for residual DNA derived from the expression system

The amount of residual host cell DNA derived from the expression system should be determined in the purified antigen bulk by suitable sensitive methods. The level of host cell DNA should not exceed the maximum level agreed with the NRA, taking into consideration issues such as those discussed in The WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14).

These tests may be omitted for routine lot release upon demonstration that the process consistently inactivated the biological activity of residual DNA or reduces the amount and size of the contaminating residual DNA present in the purified antigen bulk, as agreed upon with the NRA.

A.5.2.1.10 Test for residual bovine serum albumin content

If bovine serum is used during production, then residual BSA content should be measured and a maximum permitted concentration should be set and approved by the NRA.

A.5.2.1.11 Test for viral clearance

When a cell substrate is used for the production of RSV antigens, the production process should be validated in terms of its capacity to remove and/or inactivate adventitious viruses – as described in the Q5A guidelines (67). This testing is performed during manufacturing development or as part of process validation and is not intended as an assessment for lot release.

If a replicating viral vector such as baculovirus is used, then the production process should be validated for its capacity to eliminate (by removal and/or inactivation) residual recombinant virus. The provisions as listed in WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14) should be considered.

A.5.2.2 Addition of adjuvant

The purified antigens may be adsorbed onto an adjuvant such as an aluminum salt, in which case the adjuvant and the concentration used should be approved by the NRA. If an alternative adjuvant or additional adjuvant is used, this should also be approved by the NRA.

If a novel adjuvant is used that does not involve adsorption of the antigens to the adjuvant, the term “adjuvanted antigen bulk” may be used.

A.5.2.2.1 Storage

Until the adsorbed antigen bulk is formulated into the final bulk, the suspension should be stored under conditions shown by the manufacturer to allow it to retain the desired biological activity (if applicable). Hold times should be approved by the NRA.

A.5.2.2.2 Tests of adsorbed antigen bulk

All tests and specifications for adsorbed antigen bulk, unless otherwise justified, should be approved by the NRA (if applicable).

A.5.2.2.2.1 Sterility tests for bacteria and fungi

Each adsorbed monovalent antigen bulk should be tested for bacterial and fungal sterility (if applicable), as specified in the WHO General requirements for the sterility of biological substances (60,63), or by an alternative method approved by the NRA.

A.5.2.2.2.2 Bacterial endotoxins

The adsorbed antigen bulk should be tested for bacterial endotoxins using a method approved by the NRA (if applicable).

If it is inappropriate to test the adsorbed antigen bulk, the test should be performed on the purified antigen bulk prior to adsorption and should be approved by the NRA.

A.5.2.2.2.3 Identity

The adsorbed monovalent antigen bulk should be identified as the correct RSV antigen by a suitable method (for example an immunological assay), if applicable. The test for antigen may also serve as identity test.

A.5.2.2.2.4 Adjuvant concentration

Adsorbed antigen bulk may be assayed for adjuvant content until production consistency is demonstrated, if applicable.

A.5.2.2.2.5 Degree of adsorption

The degree of adsorption (completeness of adsorption) of the adsorbed antigen bulk should be assessed, if applicable. This test may be omitted upon demonstration of process consistency and should be approved by the NRA.

A.5.2.2.2.6 pH

The pH value of the adsorbed antigen bulk may be monitored until production consistency is demonstrated and should be approved by the NRA (if applicable).

A.5.2.2.2.7 Antigen content

The antigen content of the adsorbed antigen bulk should be measured using appropriate methods, if applicable. If this test is conducted on purified antigen bulk, it may be omitted from the testing of the adsorbed antigen bulk.

International standards and reference reagents for the control of RSV-F antigen content and conformation are not available. Therefore, product-specific reference preparations may be used.

A.6 Control of production of viral-vectored RSV vaccines

The manufacture of monovalent vaccine vectors starts with the amplification of the vaccine vector seed stock in a suitable cell line. The number of passages between the virus working seed lot and final viral-vectored vaccine product should be kept to a minimum and should not exceed the number used for production of the vaccine shown in clinical studies to be satisfactory, unless otherwise justified and authorized.

After harvesting of the culture product, the purification procedure can be applied to a single harvest or to a pool of single monovalent harvests. The maximum number of single harvests that may be pooled should be defined on the basis of validation studies.

If applicable to the vector platform, a control cell culture should be maintained simultaneously and in parallel to the production cell culture. Cells should be derived from the same expansion series but no virus vector should be added to the control cells. Growth medium and supplements used in culturing should be identical to the production cell culture. All other manipulations should be as similar as possible

A.6.1 Tests on control cell cultures (if applicable)

When control cells are included in the manufacturing process due to limitations on the testing of primary cells or viral harvests, or is required by the NRA, the procedures as described in section A.4.1 should be followed.

A.6.1.1 Tests for haemadsorbing viruses

The same provisions as laid down in section A.4.1.1 apply.

A.6.1.2 Tests for other adventitious agents

At the end of the observation period, a sample of the pooled fluid and/or cell lysate from each group of control cell cultures should be tested for adventitious agents. For this purpose, an aliquot of each pool should be tested in cells of the same species as used for the production of virus, but not cultures derived directly from the production cell expansion series for the respective batch. If primary cells are used for production, a different batch of that primary cell type should be used for the test than was used for production. Samples of each pool should also be tested in human cells and in a simian kidney cell line. At least one culture vessel of each kind of cell culture should remain uninoculated as a control.

The inoculated cultures should be incubated at the appropriate growth temperature and should be observed for cytopathic effects for a period of at least 14 days.

Some NRAs require that, at the end of this observation period, a subculture is made in the same culture system and observed for at least an additional 7 days. Furthermore, some NRAs require that these cells should be tested for the presence of haemadsorbing viruses.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for any reason by the end of the test period.

A.6.2 Single virus harvest

The method of harvesting the vaccine vector should be described and the titre of virus ascertained. A reference preparation should be included to validate the titration assay. Minimum acceptable titres should be established for single virus harvest or pooled single harvests.

The integrity of the integrated heterologous gene should be confirmed. An expression assay method should be described and should be performed on production harvest material or downstream (e.g. purified final bulk). For example, a Western blot analysis or other methods to confirm that the integrated gene is present and expressed should be included in the testing of every batch.

A.6.2.1 Control tests on single virus harvest

Unless otherwise justified, an identity test should be performed on each harvested virus pool. This should include identity of the expressed heterologous antigen and of the vector virus.

Tests for adventitious agents should be performed on each single harvest according to relevant parts of WHO's Recommendations for the evaluation of animal cells as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14). Additional testing for adventitious viruses may be performed using validated NATs.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection may also be used in the future to supplement existing methods or

as alternative methods to both *in vivo* and *in vitro* tests after appropriate validation and agreement from the NRA (see A.3.3.3.4).

Single or pooled virus harvests should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, as specified in the WHO's General requirements for the sterility of biological substances (60,63), or by an alternative method approved by the NRA.

For viral-vectored vaccines, due to the very high titres of the single harvests, alternatives to the classical testing for adventitious agents may be applied with the approval of the NRA.

Provided cell banks and viral seed stocks have been comprehensively tested and released, demonstrating they are free of adventitious agents, it could be discussed (and should be agreed with the NRA) whether to evaluate the possibility of delaying *in vitro* testing for adventitious agents (viral pathogens and mycoplasmas) at the cell harvest or bulk substance stages or replacing it with validated PCR tests. The method of production should be taken into account when deciding the nature of any specified viruses being sought.

Additional considerations for this approach are that no animal-derived raw materials are used during manufacture, and that the manufacturing facility operates under a GMP certificate (where applicable) with assurances that prevention of cross-contamination is well controlled in the facility. Samples should be retained for testing at a later date if required.

A.6.3 Pooled monovalent virus harvest

Single virus harvests may be pooled to form virus pools and from which the final bulk vaccine will be prepared. The strategy for pooling single virus harvests should be described. Minimum acceptable titres should be established for use of a single virus harvest in the preparation of a virus pool or final bulk. All processing of the virus pool should be described in detail.

A.6.3.1 Control tests on pooled virus harvests

Virus pools should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, as specified in the WHO's General requirements for the sterility of biological substances (60,63). Alternatively, if single virus harvests have been tested to demonstrate freedom from bacteria, fungi and mycoplasmas, these tests may be omitted on the pooled virus harvests.

A.6.4 Monovalent bulk vaccine

The monovalent bulk vaccine can be prepared from one or several virus pools with the same antigen, or it may be derived from a single virus harvest. Substances such as diluents or stabilizers or any other excipients added during preparation of the monovalent bulk or the final bulk vaccine should have been shown not to impair the potency and safety of the vaccine in the concentrations employed.

Penicillin and other beta-lactams should not be used at any stage of manufacture because of their nature as highly sensitizing substances in humans. Other antibiotics may be used at any

stage of manufacture, provided that the quantity present in the final product is acceptable to the NRA.

A.6.4.1 Control tests on monovalent bulk

The monovalent bulk vaccine should be tested, and consideration should be given to using the tests listed below, as appropriate for the individual products. Alternatively, if the monovalent bulk will be held for a short period of time and if appropriate, some of the tests listed below could be performed on the final bulk or final lot instead. If sufficiently justified, some tests may be performed on an earlier intermediate instead of on the monovalent bulk. All quality-control release tests for monovalent bulk should be validated and should be shown to be suitable for the intended purpose. Assay validation or qualification should be appropriate for the stage of the development life cycle. Additional tests on intermediates during the purification process may be used to monitor for consistency and safety.

A.6.4.1.1 Purity

The degree of purity of each monovalent bulk vaccine should be assessed using suitable methods. The purity of the bulk should be ascertained for fragments, aggregates or empty particles of the product, as well as for contamination by residual cellular proteins. Residual cellular DNA levels should also be assessed when non-primary cell substrates are used for production. The content and size of host cell DNA should not exceed the maximum level agreed with the NRA, taking into consideration issues such as those discussed in the WHO Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks (14).

Process additives should also be controlled. In particular, if any antibiotics are added during vaccine production, the residual antibiotic content should be determined and should be within limits approved by the NRA.

These tests may be omitted for routine lot release upon demonstration that the process consistently clears the residuals from the monovalent bulk vaccine, subject to the agreement of the NRA.

A.6.4.1.2 Potency

Each monovalent bulk vaccine should be tested for potency measured by a combination of the following methods.

A.6.4.1.2.1 Particle number

For relevant vectors (e.g. adenovirus vectors), the total number of virus particles per milliliter, quantitated by a technique such as qPCR or HPLC, should be provided for each batch of monovalent bulk.

A.6.4.1.2.2 Infectivity

The infectious virus titre as a measure of active product should be tested for each batch of monovalent bulk. Direct methods such as a plaque-forming assay, or indirect methods such as qPCR if suitably correlated with a direct measure of infectivity, could be considered. The particle/infectivity ratio should also be specified.

A.6.4.1.2.3 Expression of the heterologous antigen in vitro

The ability of the viral particles to express the heterologous gene should be demonstrated e.g. by a suitable method using an antigen-specific antibody (and/or conformation-specific antibody if detecting RSV prefusion F antigen] after growth of the vector in a suitable cell line.

A.6.4.1.3 Identity

Tests used for assessing relevant properties of the viral vector – such as antigen expression, restriction analysis, PCR with a specific probe or sequencing – will generally be suitable for assessing the identity of the product.

A.6.4.1.4 Sterility or bioburden tests for bacteria and fungi

Each monovalent bulk should be tested for bacterial and fungal bioburden or sterility. Bioburden testing should be justified in terms of product safety. Sterility testing should be as specified in the WHO General requirements for the sterility of biological substances (63), or by methods approved by the NRA.

A.6.4.1.5 Bacterial endotoxins

Each monovalent bulk should be tested for bacterial endotoxins. At the concentration of the final formulation of the vaccine, the total amount of residual endotoxins should not exceed that found in vaccine lots shown to be safe in clinical trials or the amount found in other lots used to support licensing. The test may be omitted once production consistency has been demonstrated after agreement from the NRA.

A.6.4.1.6 Reversion to replication competency or loss of attenuation

The viral-vectored RSV vaccines under development are either replication-incompetent in human cells or adequately attenuated to prevent disease symptoms related to the viral vector backbone. Although manufacturers generally provide theoretical justifications for why reversion to competency or virulence is unlikely to occur, low levels of viral particles may emerge that have gained the complementing gene from the production cell line by an unknown or poorly characterized mechanism. These viral particles are considered to be an impurity; it is not known whether they represent a safety concern. Consequently, it should be shown that the product is still replication-incompetent or fully attenuated (whichever is relevant) in initial batches of the product.

After demonstrating this, it may be possible to omit such tests in future batches provided a sufficient justification is made (which should include the demonstration of replication incompetence/attenuation as well as a discussion of why reversion to competency or loss of attenuation is prevented in future batches).

A.6.4.1.7 Preservative content

The monovalent bulk may be tested for the presence of preservative, if added. The method used and the permitted concentration should be approved by the NRA.

A.6.5 Final bulk vaccine

Appropriate quantities of different monovalent bulk vaccines should be pooled, mixed and formulated (if required) to form a homogeneous solution to manufacture the final bulk vaccine. The final bulk can be made up of one or more batches of a single monovalent vaccine, to give a monovalent vaccine product.

For multi-dose preparations, the need for effective antimicrobial preservation should be evaluated, taking into account possible contamination during use and the maximum recommended period of use after opening the container or reconstitution of the vaccine. If an antimicrobial preservative is used, it should not impair the safety or potency of the vaccine; the intended concentration of the preservative should be justified and its effectiveness should be validated (68).

A.6.5.1 Control tests on final bulk vaccine

The following tests should be performed on the final bulk vaccine unless it can be demonstrated that they are not necessary, such as if filling operations are performed immediately after manufacture of the final bulk, and on the same site.

A.6.5.1.1 Identity

See A.6.14.1.3.

A.6.5.1.2 Preservative

Where applicable, the amount of antimicrobial preservative should be determined by a suitable method.

A.6.5.1.3 Sterility tests for bacteria and fungi

Each monovalent bulk should be tested for bacterial and fungal bioburden or sterility. Bioburden testing should be justified in terms of product safety. Sterility testing should be as specified in the WHO General requirements for the sterility of biological substances (60,63), or by methods approved by the NRA.

A.7 Filling and containers

The requirements concerning good manufacturing practices for biological products (55) should apply to the RSV vaccine filled in the final form (54,55).

Care should be taken to ensure that the materials from which the container and, if applicable, the closure are made do not adversely affect the quality of the vaccine under the recommended conditions of storage. To this end, a container closure integrity test and assessment of extractables and/or leachables for the final container closure system are generally required for qualification of containers and may be needed as part of stability assessments. Assessment of extractables and leachables might also be required for container systems used for long-term storage of bulks and formulated bulks.

If multi-dose vaccine vials are used and these vaccines do not contain preservative, their use should be time-restricted, as is the case for reconstituted vaccines such as BCG and measles-containing vaccines (68). In addition, the multi-dose container should prevent microbial

contamination of the contents after opening. The extractable volume of multi-dose vials should be validated.

The manufacturer should provide the NRA with adequate data to prove that the product is stable under appropriate conditions of storage and shipping.

A.8 Control tests on final lot

A.8.1 Inspection of final containers

Each final container (also known as the Drug Product) in each final lot should be inspected visually, and those showing abnormalities should be discarded.

A.8.2 Identity

An identity test should be performed on a least one final, labelled container from each filling lot - in case of freeze-dried vaccines after reconstitution according to the manufacturer`s instructions for preparing the vaccine for human administration. However, it is not necessary to perform the genetic identity test on the final lot.

For multivalent vaccine each antigen component should be identified.

A.8.3 Appearance (if applicable)

The appearance of the liquid or freeze-dried vaccine should be described with respect to form and color. In the case of freeze-dried vaccines, a visual inspection should be performed or the freeze-dried vaccine, the diluent, and the reconstituted vaccine.

A.8.4 pH

The pH of the final lot should be tested in a pool of final containers, and an appropriate limit should be set to guarantee virus stability. In the case of freeze-dried vaccines, the pH should be measured after reconstitution of the vaccine with the diluent.

A.8.5 Osmolality (if applicable)

The osmolality of the final bulk may be tested, if appropriate. The osmolality test may be omitted if performed on the final bulk. Alternative tests (e.g. freezing point) may be used as surrogate measures for ionic strength/osmolality.

A.8.6 Sterility for bacteria and fungi (if applicable)

Each final lot should be tested for bacterial and fungal sterility, as specified in the WHO General requirements for the sterility of biological substances (60,63), or by a method approved by the NRA.

A.8.7 Bacterial and fungal contamination (if applicable)

For chimeric BCG/RSV vaccines, samples from each final lot should be tested for bacterial and fungal contamination by appropriate tests a specified in Part A section 5.2 of the General requirements for the sterility of biological substances (63), or by a validated method approved by the NRA.

A.8.8 Preservative (if applicable)

Each final lot should be tested for the presence of preservatives, if added.

A.8.9 Residual moisture (if applicable)

The residual moisture in a representative sample of each freeze-dried lot should be determined by a method approved by the NRA. The upper limit for moisture content should be approved by the NRA using results from stability testing. Moisture levels of 3% or lower are generally considered acceptable.

A.8.10 Pyrogenic substances

Each final lot should be tested for pyrogenic substances, if appropriate. Tests for endotoxin [for example the limulus amebocyte lysate (LAL) test] should be performed. However, where there is interference in the test – e.g. because of the addition of an immunostimulant such as MPL – a test for pyrogens in rabbits should be performed. A suitable validated monocyte-activation test may also be considered as an alternative to the rabbit pyrogen test.

The test is conducted until consistency of production is demonstrated and should be approved by the NRA.

A.8.11 Adjuvant content (if applicable)

Each final lot should be assayed for adjuvant content, if added. Where aluminum compounds are used, the amount of aluminum should not exceed 1.25 mg per human dose.

A.8.12 Protein content (if applicable)

The protein content should be determined, if appropriate. Alternatively, this may be calculated from an earlier process intermediate.

A.8.13 Degree of adsorption (if applicable)

The degree of adsorption to the adjuvant (completeness of adsorption) of each antigen present in the final bulk should be assessed, if applicable (e.g. if the adjuvant is aluminum salts), and the limit should be approved by the NRA.

This test may be omitted for routine lot release upon demonstration of process consistency, subject to the approval of the NRA.

A.8.14 Potency

An appropriate quantitative test for potency by an *in vivo* or *vitro* method should be performed using samples that are representative of each final vaccine lot. In case of freeze-dried vaccines, the potency content should be determined after the freeze-dried product has been reconstituted with the approved diluent. The method and the analysis of data from potency tests should be approved by the NRA. Vaccine potency should be compared with that of a reference preparation, and the limits of potency should be agreed with the NRA. The reference preparations used should be approved by the NRA. If an *in vivo* potency test is used to test the final lot, this test may be omitted on the final bulk.

As long as no international standards for the potency of RSV vaccines are available, manufacturers should establish a product-specific reference preparation that is traceable to a

lot of vaccine, or bulks used in the production of such a lot, which has been shown to be efficacious in clinical trials. The performance of this reference vaccine should be monitored by trend analysis using relevant test parameters and the reference vaccine should be replaced when necessary. An acceptable procedure for replacing reference vaccines should be in place (69).

For multivalent vaccines it may be necessary to perform this test on the monovalent bulk instead if analytical methods cannot distinguish between the different monovalent vaccines in the final lot.

A.8.15 Purity (if applicable)

If the RSV vaccine is a viral-vectored, testing for purity should be performed unless it is performed on the monovalent bulk or final bulk vaccine. However, limited purity testing of the final lot may be required even if purity is tested on the final bulk vaccine if, after taking the manufacturing process and nature of the vector into consideration, it is considered possible that the purity may have changed. This should be considered on a case-by-case basis.

A.8.16 Bacterial concentration (if applicable)

If appropriate, in the case of chimeric BCG/RSV vaccines, the total bacterial content of the reconstituted vaccine should be estimated for each lot by a validated method approved by the NRA and should have a value range approved by the NRA. The estimate of total bacterial content may be made either directly, by determining the dry weight of organism, or indirectly by an opacity method that has been calibrated in relation to the dry weight of the organism.

A.8.17 Extractable volume

It should be demonstrated that the nominal volume on the label can consistently be extracted from the containers.

A.8.18 Aggregates/particle size (if applicable)

If the RSV vaccine consists of nanoparticles which might be susceptible to aggregation, each final lot should be examined for particle size/aggregate content at lot release and across the shelf-life, unless it can be shown that the test is not necessary.

A.8.19 Viability (if applicable)

If appropriate, in the case of chimeric BCG/RSV vaccine, the number of culturable particles of each final lot should be determined by an appropriate method approved by the NRA (see section A.6.7 of Recommendations to assure the quality, safety and efficacy of BCG vaccines) (16).

A.8.20 Thermal stability (if applicable)

If appropriate a thermal stability test should be performed. The purpose of the thermal stability test is to demonstrate the consistency of production. Additional guidance on the

evaluation of vaccine stability is provided in the Guidelines on stability evaluation of vaccines (70).

For live-attenuated/and/or viral vectored vaccines, at least three containers of each final vaccine lot should be incubated at the appropriate temperature for the appropriate time (e.g. 37°C for seven days). The geometric mean titre (GMT) of infectious virus in the containers should not have decreased during the period of exposure by more than a specified amount (e.g. 1 $\log_{10}$) that has been justified by the production data and approved by the NRA. Titration of non-exposed and exposed containers should be carried out in parallel. A reagent for intra-assay validity control should be included in each assay.

For chimeric BCG/RSV vaccines, each final lot should be tested for thermal stability by a validated method approved by the NRA. If the production consistency is demonstrated, this test may be omitted on the final lot subject to NRA approval. If performed, the test should involve the determination of the number of culturable particles before and after the samples have been held at appropriate temperatures and for appropriate periods. For example, the thermal stability test may be carried out by taking samples of the vaccine and incubating them at 37 °C for 28 days (16). The percentage decrease in the number of culturable particles is then compared with that of samples of the same vaccine lot stored at 2 – 8 °C. The absolute value should be approved by the NRA.

A.8.21 Residual antibiotics (if applicable)

If any antibiotics were added during production, the content of the residual antibiotics should be determined, and this should be within the limits approved by the NRA.

A.8.22 Diluent (if applicable)

The recommendations given in WHO´s Good manufacturing practices for pharmaceutical products: main principles (54) should apply to the manufacturing and control of diluents used to reconstitute freeze-dried RSV vaccines. An expiry date should be established for the diluent on the basis of stability data. For lot release of the diluent, tests should be done for identity, appearance, pH, extractable volume, sterility, endotoxin, and the content of key components.

A.8.23 Safety test (if applicable)

If appropriate, for chimeric BCG/RSV vaccines, tests for absence of virulent mycobacteria and a test for excessive dermal activity have to be performed (see section A.6.4 of WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines) (16).

A.9 Records

The recommendations of Good manufacturing practices for pharmaceutical products (54) should apply, as appropriate to the level of development of the candidate vaccine.

A.10 Retained samples

A sufficient number of samples should be retained for future studies and needs. Vaccine lots that are to be used for clinical trials may serve as a reference material in the future, and a sufficient number of vials should be reserved and stored appropriately for that purpose.

A.11 Labelling

The recommendations provided of good manufacturing practices for biological products (55) should apply, as appropriate for a candidate vaccine, with the addition of the following:

The label of the carton enclosing one or more final containers, or the leaflet accompanying the container, should include:

- the name of the vaccine;
- in case of live-attenuated vaccines, a statement on the nature of the preparation, specifying the strain of RSV or recombinant RSV contained in the live-attenuated RSV vaccine that the vaccine has been prepared from.
- in case of live-attenuated/chimeric vaccines, the minimum number of infective units per human dose, the nature of any cellular systems used for the production of the vaccine, and whether the vaccine strain was derived by molecular methods.
- in case of subunit, particle-based, and viral-vectored vaccines, a statement that specifies the nature of the cells and/or any expression system used for production of the vaccine.
- in case of particle-based, subunit and viral-vectored vaccines, the volume of one recommended human dose, and the amount of active substance(s) contained in one recommended human dose.
- the immunization schedule, and the recommended routes of administration;
- the number of doses, if the product is issued in a multiple-dose container;
- a statement that contact with disinfectants should be avoided;
- a statement concerning the photosensitivity of the vaccine, based on photostability data;
- if applicable, a statement indicating the volume and nature of diluent to be added to reconstitute the vaccine, and specifying that the diluent to be used is that supplied by the manufacturer and a statement that after the vaccine has been reconstituted, the vaccine should be used without delay or, if not used immediately, stored at 2-8°C and protected from light for a maximum period defined by stability studies.
- the name and concentration of any preservative added;
- a statement of the nature and quantity, or upper limit, of any antibiotics present in the vaccine;
- the temperature recommended during storage and transport;
- the expiry/retest date;
- any special dosing schedules;
- contraindications, warnings and precautions, concomitant vaccine use, adverse events.

A.12 Distribution and transport

The recommendations in Good manufacturing practices for biological products (55) appropriate for a candidate vaccine should apply.

Shipments should be maintained within specified temperature ranges and packages should contain cold-chain monitors (71).

A.13 Stability testing, storage and expiry date

The recommendations given in Good manufacturing practices for biological products (55) and in the Guidelines on stability evaluation of vaccines (70) appropriate for the respective RSV vaccine should apply. The statements concerning storage temperature and expiry date that appear on the primary and secondary packaging should be based on experimental evidence, and should be submitted to the NRA for approval.

A.13.1 Stability testing

Adequate stability studies form an essential part of vaccine development. Guidance on the evaluation of vaccine stability is provided in the WHO Guidelines on stability evaluation of vaccines (70). Stability testing should be performed at different stages of production – namely on stored intermediates (including single harvests, purified bulk and final bulk) and the final lot. Stability-indicating parameters should be defined or selected appropriately according to the stage of production. It is advisable to assign a shelf-life to all in-process materials during vaccine production, particularly to stored intermediates such as single harvests, purified bulk and final bulk.

Accelerated thermal stability tests may be undertaken on each final lot to give additional information on the overall characteristics of the vaccine, and may also be useful in assessing comparability when the manufacturer plans to make changes to manufacturing.

For vaccine licensure, the stability of the vaccine and its final container and at the recommended storage temperatures should be demonstrated to the satisfaction of the NRA on at least three lots of the final product (or, in the case of adsorbed vaccine, on the adsorbed antigen bulks). During clinical trials, fewer data are likely to be available. However, the stability of the vaccine under the proposed storage conditions should be demonstrated for at least the expected duration of the product in the clinical trial and information should be supplemented and updated when it becomes available (72).

Following licensure, ongoing monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile (70). Data should be provided to the NRA according to local regulatory requirements.

The final stability-testing program should be approved by the NRA and should include an agreed set of stability-indicating parameters, procedures for the ongoing collection and sharing of stability data, and criteria for rejecting vaccines(s).

In-use stability should also be specified and justified with adequate data under real-time conditions.

The formulation of vaccine and adjuvant (if used) should be stable throughout its shelf-life. Acceptable limits for stability should be agreed with the NRAs.

A.13.2 Storage conditions

Before being distributed by the manufacturing establishment or before being issued from a storage site, the vaccine should be stored at a temperature shown by the manufacturer to be compatible with a minimal loss of titre (for live virus vaccine candidates) or potency (for particle based/subunit or vector-based vaccine candidates). The maximum duration of storage should be fixed with the approval of the NRA and should be such as to ensure that all quality specifications for the final product, including the minimum titre/potency specified on the label of the container (or package), will still be maintained until the end of shelf life. During clinical trials, this period should ideally be at least equal to the expected duration of vaccine administration in the clinical trial.

A.13.3 Expiry date

The expiry date should be defined on the basis of shelf-life and should be supported by the stability studies with the approval of the NRA. If the vaccine is stored at cold storage and intended for release without re-assay the expiry date is calculated from the date of removal from cold storage. This should be supported by stability studies. The expiry dates for the vaccine and the diluent (if used) may differ.

Where an *in vivo* potency test is used, the date of the potency test is the date on which the test animals are inoculated.

A.13.4 Expiry of reconstituted vaccine

In the case of for single dose containers of freeze-dried vaccines which require reconstitution, the reconstituted vaccine should be used immediately. Multi-dose containers should be kept in the dark at 2-8°C and the expiry time for use of an opened container should be defined by stability studies approved by the NRA, but should generally be not more than six hours.

Part B. Nonclinical evaluation of human RSV vaccines

B.1 General remarks

Nonclinical evaluation of RSV vaccines includes all *in vivo* and *in vitro* testing prior to and during the clinical development. Consideration should be given to the number and types of preclinical pharmacological studies to be conducted, with expectation to streamline and limit testing to those that provide results directly supportive of the proposed clinical program. Sponsors may consult NRAs to identify the most relevant studies for their regulatory submission.

Before proceeding to humans, there should be adequate information suggestive of the safety and potential for efficacy of vaccine, including product characterization, immunogenicity

studies, and toxicity and safety testing in animals. Continuation of some nonclinical testing would be expected for maintaining current good manufacturing practices and to support further clinical development.

The following sections describe the types of nonclinical information that are required for supporting initiation of a specific clinical study, or being submitted in marketing authorization application. Details on the design, conduct, and analysis of nonclinical studies are available in WHO guidelines on nonclinical evaluation of vaccines (10) that should be consulted.

B.2 Process development and product characterization

General principles described in WHO guidelines (10) for vaccine production, testing and stability are broadly applicable to RSV vaccines. The production process should be adequately controlled at critical steps to ensure consistency of manufacture. Vaccine antigens and the end-product should be well defined and thoroughly characterized to ascertain that vaccine lots used in nonclinical studies are qualified.

Vaccine lots used in nonclinical studies may be at research grade or manufactured under the GMP. Ideally, the lots tested are clinical lots. If this is not feasible, they should at least be comparable to clinical lots with respect to the concurrent clinical lot specificication.

For recombinant DNA-derived antigens one intrinsic aspect is to demonstrate the stability of their conformation(s) using suitable methods, such as negative staining and electron microscopy and/or direct antibody binding assay, ideally, using a standardized panel of monoclonal antibodies with well-defined epitope specificities. Any instability of the expressed proteins occurring during storage or after a scale-production run, should be documented. Serological investigation based on antibody-competition assay may also provide some informative data regarding the presence and stability of antigenic sites exposed in a given conformational state.

For live attenuated vaccines, the suitability of an attenuated vaccine strain needs continuous careful review to ascertain attenuation and phenotypic stability. A complete genetic sequence should be obtained to document the attenuating mutations within the virus genome that may correlate with its attenuated phenotype. Since each virus passage may introduce new mutations, studies should determine if the genetic basis of attenuation is stable over the entire manufacturing process and during replication in vaccinees. These studies should also define the phenotypes of vaccine strain as far as is practical. The critical phenotypic markers, including replication efficiency in nonhuman primates and/or primary human bronchial epithelial cells and in some cases temperature sensitivity, are considered useful for detecting reversion events.

Vaccine candidates based on live recombinant vectored vaccines are associated with similar safety issues including degree of attenuation *in vivo* and replication *in vitro*, genetic stability of the virus, and the potential risk of reversion to virulence, and should be characterized accordingly. Neurovirulence testing is not normally needed for the live attenuated or vectored

RSV vaccines, unless vaccine constructs with gene delections or modifications of the vector are suspected to have potential for neurovirulence.

The guidance on the general principles of the nonclinical assessment of vaccine adjuvants can be found in WHO Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines (11).

B.3 Nonclinical immunogenicity and protective activity

There is no animal model that precisely mimics RSV disease in humans. It is therefore acceptable to support use of RSV vaccines based on immunogenicity of vaccine in animals. Assessment of immunogenicity in animals should consider the construct designed or the type of vaccine. For certain vaccines including the protein-based, it is generally recommended that the serum antibodies with RSV-neutralizing activity be assessed in immunogenicity studies, because antibodies directed against the virus fusion (F) or attachment (G) glycoprotein of RSV neutralize the virus *in vitro* and have been associated with protective effect in animal models and/or in humans. Consideration should be given to choice of RSV subtype (i.e. A or B) as well as of cell type to be used when a vaccine construct is specifically designed to target G protein alone.

However, vaccine candidates based on recombinant viruses may by design elicit cellular immunity, such as $CD8^+$ cytotoxic T lymphocytes (CTLs) and/or type 1 $CD4^+$ T helper cells. Induction of an effective mucosal immune response may be an intended mechanism of protection for a vaccine adminstered by the intranasal route, e.g., a live attenuated or replication-competent vectored vaccine. To this end, a product-specific approach should be considered.

Prior to the initiation of clinical trials in humans, data should be in place to demonstrate a functional immune response post-vaccination in RSV-naïve animals.

For vaccines that include an adjuvant, information to support the selection and use in vaccine formulation should be provided, such as demonstrated adjuvant activity and the beneficial effect assessed by the magnitude, and/or the type, broadness, and duration of functional immune response induced (11). There is no need to perform immunogenicity studies in pregnant animals to generate a proof of transplacental antibody transfer. However, passive transfer of antibodies, generated in response to vaccination, to RSV naïve animals that are subsequently challenged with RSV virus, can provide evidence for the antibody-mediated protection and may be explored. For that, early conversation with national NRA is recommended.

For a multivalent candidate RSV vaccine, the immune responses (including any potential immunological interference between strains/subtypes) and protective activity with respect to each of the strains/subtypes targeted should be assessed.

Careful characterization of vaccine-induced immune responses in animal models is recommended, whenever feasible, during the assessment of vaccine-associated ERD anticipated for certain vaccines (see B.5.2).

The protective activity in challenged animals may be evaluated during the assessment of ERD risk. However, experience has shown that such data, especially those derived from rodents, are not necessarily predictive of immune protection in humans.

B.4 Pharmacokinetic studies

Studies to determine serum concentrations of antigens are not needed. Specific studies such as local deposition studies at the site of injection, distribution studies or viral shedding studies may be necessary, especially in case of novel adjuvants, new formulations or alternative route of administration (for example, intranasal route).

For recombinant vaccine viruses with which no prior experiments have been done, biodistribution should be studied in a full set of tissues and organs including the brain. Such study is unnecessary if supportive data generated for the same vector but using different gene insert(s) are in place and in case that the construction of recombinant virus is not suspected to result in altered tissue tropism. One species is considered sufficient if scientifically justified. Crossing the blood-brain-barrier might be an indication of potential neurovirulence (73).

B.5 Nonclinical toxicity and safety testing

B.5.1 Preclinical toxicity

Toxicity studies for RSV vaccines should be undertaken based on guidance on the general principles of toxicity assessment provided in the WHO guidelines on the nonclinical evaluation of vaccines (10). Toxicological testing should aim to identify the untoward effect by careful analysis of all major organs as well as tissues near to and distal from the site of administration, associated with vaccine dose or as a consequence of replication and tissue tropism of vaccine virus for a replicating vaccine. Toxicity studies should support the safety of the starting dose, dosing schedule, route of administration, and proposed rate of dose escalation.

Where new adjuvant is included in formulation of vaccine, for which no experience exists in relation to human use, it is advisable that the adjuvant alone be characterized, according to the WHO guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines (11).

If a vaccine candidate is intended for immunization of pregnant women or women of child-bearing age, a single developmental and reproductive toxicity study in one relevant species should be performed. Timing of submission of such data varies by geographic region or countries. Some NRAs require to exclude women of childbearing potential from large-scale clinical trials, prior to completion of developmental and reproductive toxicity study, other

NRAs can allow to recruit women of childbearing potential into early clinical trials if highly effective birth control methods are used by trial participants.

Any change introduced into manufacture or formulation of vaccine during the product development, when judged as significant, may require partial or full re-evaluation in preclinical toxicity testing (10, 74,75).

B.5.2 Preclinical safety

Studies with live-attenuated and recombinant viral-vectored vaccines entail the identification of markers of attenuation that can assist in monitoring the results during clinical evaluation phases. The primary purpose of such studies is to demonstrate that the vaccine is less virulent in animal host than comparable wild-type viruses, and that the vaccine does not exhibit any unexpected harmful tissue tropism and damage. (see B.2 and B.4).

The utility of animal models for the assessment of potential risk of ERD is still debating, due to the uncertainty and controversy of the ERD models. Nevertheless, it is generally agreed that the development of a RSV vaccine for use in RSV naïve infants should be undertaken with a great caution. The ERD animal models developed thus far may reproduce some immunopathogenic features of human ERD and may be used to support the preclinical safety assessment brief review of some representative animal models is provided below. For certain vaccines that would be biologically more likely to induce a Th2-biased T-cell response, such as the protein-based vaccines, the assessment of potential risk of ERD in animal models may be required by the NRA, prior to testing in RSV-naïve infants. Live attenuated vaccines are at low risk of ERD, based on existing evidence. Pre-clinical safety assessments for ERD are not needed prior to testing vaccines in RSV experinced/non-naive populations since these individuals are not at risk for this adverse event.

The design of preclinical safety studies should include FI-RSV vaccine as a positive control, when appropriate, and a group given RSV infection as a negative control. In certain circumstances (e.g. cotton rats, nonhuman primates), it may be important to address inflammatory responses raised by host cell proteins present in vaccine and/or RSV challenge virus preparations (76). Another important consideration is to choose the optimized vaccine dose for animal immunization, in order to allow adequate viral replication in the setting of vaccine-induced neutralizing antibodies. It may be necessary to examine serum antibody responses and lung histopathology after RSV challenge over a range of vaccine doses. Since surrogate readouts of vaccine-associated disease exacerbation vary by animal model, discussions given above should be taken into account. Measurement of viral titers in the lungs of affected animals does not predict enhanced pulmonary pathology, although this parameter is broadly suited for assessment of the protective effect of the candidate vaccine.

Irrespective of the animal model used for RSV challenge prior to ERD assessment, lung sections should be scored by a pathologist/person blinded to group assignment; the method used to summarize and compare lung histopathology scores should be adequately described.

B.5.2.1 Mouse model

Mice are relatively resistant to human RSV infection and require high challenge inocula for significant lung pathology (e.g., above 10^6 pfu) (77). The small airway epithelium of mice is not as extensively infected as it is in humans, and most virus replication occurs in Type 1 pneumocytes. Notwithstanding these limitations, a mouse model is attractive because of the relative low cost and the availability of extensive molecular tools. Certain strains, such as BALB/c mouse, have been extensively used to explore mechanisms underlying FI-RSV-enhanced disease, such as a pattern of $CD4^+$ type 2 T-cells primed by vaccine and boosted following RSV challenge, evaluation of pulmonary eosinophilia and induction or absence of RSV-specific $CD8^+$ CTLs. Other informative parameters may include body weight loss, illness, and changes in respiratory physiology, as displayed by the challenged mice.

In addition, there are a number of genetically-modified mice that may provide unique insights into pathogenesis.

B.5.2.2 Cotton rat model

Cotton rats are more susceptible to human RSV infection than mice and have been widely used to characterize vaccine-enhanced disease (78). In this model, virus replication in the lower airway is primarily limited to bronchiolar epithelium, closely resembling human infection. Several key histologic features of disease exacerbation have been reproduced in cotton rats, including neutrophilic alveolitis and peribronchiolitis consisting primarily of lymphocytes. In addition, interstitial pneumonitis appears to be another marker specific for vaccine enhancement.

B.5.2.3 Nonhuman primate model

African green monkeys are the most studied nonhuman primate (NHP) species used to model FI-RSV-enhanced disease. Enhanced pulmonary pathology has been demonstrated in this model, as manifested by severe infiltration of lymphocytes, macrophage, eosinophils, and polymorphonuclear into parenchyma and the peribronchiolar areas of the lung. However, clinical disease presentation in vaccinated monkeys is of limited comparability to humans (79).

Similarly, cynomolgus macaques display lung eosinophilia and production of type 2 cytokines after FI-RSV immunization and RSV challenge. Although fatal outcomes may occur in FI-RSV-immunized macaques, the histologic presentation observed in fatal human cases is not duplicated, as there are no inflammatory lesions in lungs at the moment of animal death (80).

NHPs, though sharing high similarity in immune system with humans, do not reproduce all immunological features as seen in humans, as significant RSV-neutralizing antibody response can be induced in FI-RSV-immunized monkeys. The limited availability, high cost and ethical considerations further present practical limitations. It is also worth noting that human RSV is semi-permissive in NHPs and the inoculum needs to be very large, even challenge

viruses matched to their hosts and able to appropriately inhibit Type 1 interferon and accomplish all other immune evasion strategy. Usually several milliliters of high titer virus stock need to be given in each nostril and sometimes intratracheally, which does not represent the type of transmission that occurs in humans.

B.5.2.4 Calf model

Calves are a natural host for bovine RSV (bRSV) and efficiently replicate the virus in the upper and lower respiratory tract. bRSV infection in calves causes a spectrum of clear clinical disease overlapping with the disease observed in RSV infected human infants, such as fever, nasal discharge, cough, and tachypnea with chest retractions, wheezing, hypercapnia and hypoxemia (81). Severe lower respiratory tract disease occurs mostly in calves less than 6 months of age. Studies to model FI-RSV disease exacerbation in calves have demonstrated a similar clinical and histopathological presentation to what has been observed in the original human trials, including detection of poorly neutralizing antibodies. The features unique to enhanced pulmonary pathology include proliferative alveolitis, alveolar syncytium and septal fibrosis.

Since bovine RSV and human RSV share enough homology in the fusion ectodomain and other conserved viral proteins, the calf model challenged with bRSV may have particular value for demonstrating protective efficacy of a vaccine based on human RSV F protein and for informing the key features of vaccine-enhanced illness.

Disadvantage of this model includes use of a different (i.e. non-human RSV) virus, a need for very large inoculum, and a need for expertise to work with large animals. Due to large size of the lung and patchy pathology, there is also concern that sampling error could interfere with an accurate evaluation of pathology.

B.6 Environmental risk assessment

Types of inactivated or protein-based RSV vaccines are unlikely to result in significant risk to the environment and thus exempted from specific environment risk assessment (ERA) studies. However, live vaccines attenuated by genetic modification or live recombinant vaccine viruses pose potential risk of spread to the third party, i.e. unvaccinated humans and/or domestic animals. For them, an ERA may be required as part of the preclinical evaluation. Data on the phenotype of live attenuated or recombinant vaccine virus, including the degree of attenuation and replication, its genetic stability, the potential to revert to a virulent virus, and the possible shedding following administration, contribute at least in part to the ERA.

In addition, the risk of recombination with wild-type virus strains should be addressed for all live attenuated vaccine virus and all live recombinant vaccine viruses.

Part C. Clinical evaluation of RSV vaccines

C.1 Introduction

Clinical studies for RSV vaccines should be conducted in accordance with the principles described in the WHO Guidelines for good clinical practice (GCP) for trials on pharmaceutical products (72) and the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (12). This section focuses only on issues that are most relevant or specific to the clinical evaluation of RSV vaccines, regardless of the vaccine construct. Guidance is provided on assays for measurement of immune responses to vaccination and for laboratory confirmation of clinical cases of RSV disease in efficacy trials. The discussion of clinical programmes is generally applicable across age and population groups, but specific attention is given to trials that evaluate the safety, immunogenicity and efficacy of vaccines intended for:

- Active immunisation of infants and toddlers (aged 28 days to 23 months), including those who were born prematurely;
- Active immunisation of pregnant women, with the primary aim of protecting the infant in the first months of life;
- Active immunisation of older adults (e.g. aged ≥ 50), including subjects with co-morbidities.

Sponsors may wish to investigate the use of RSV vaccines in other populations. These may include neonates (0-27 days), children from 2 years and adults and/or subjects with co-morbidities or immunodeficiencies predisposing to development of RSV disease. Safety and immunogenicity data should be obtained in each target population in accordance with sections C.2 and C.4. Section 6 of the WHO Guideline on clinical evaluation of vaccines; regulatory expectations (12) considers the possible need for efficacy trials and the extrapolation of results of vaccine efficacy trials between populations.

C.2 Immunogenicity trials

C.2.1 Assays

General guidance on the use and validation of assays for immune responses is provided in the WHO Guideline on clinical evaluation of vaccines; regulatory expectations (8). This section provides some guidance on assays of relevance to the investigation of immune responses to RSV vaccines, some of which may be selected for use in individual clinical development programmes according to the vaccine construct.

C.2.1.1 Humoral immunity

Neutralizing antibodies

Serum RSV neutralization assays occur in a multitude of formats (52, 82). Sponsors should provide detailed information about the identity of the cell substrate, virus challenge strain and whether neutralization is modulated by complement, stating the type and concentration if used

in the assay. Neutralization assays may use laboratory-adapted strains representative of RSV-A (such as A2, Long, or Tracy) and RSV-B subtypes (such as 18537, 9320, or B1) and/or contemporary RSV isolates like RSV/A/Ontario/2010 (ON1) and RSV/B/BA viruses of the Buenos Aires (BA) lineage (83-87) or other contemporary strains as they become available. The use of both RSV-A and –B viruses will help to verify the ability of a vaccine to elicit antibodies capable of neutralizing RSV in a subtype-specific manner.

The readout for the assay (e.g. cytopathic effect, plaque counts, fluorescence, luminescence, gene copy number) should be described. Adequate controls should be used to define a valid test and justify pooling of data across assay runs. The final serum dilution used for determining the endpoint for neutralization should include the dilution that occurs following addition of the challenge virus. The method used to calculate endpoint titres should be provided. Generally, it is recommended that the endpoint should be derived from the linear portion of the titration curve.

The results should be reported in International Unitage along with information about the performance of the International Standard (52,53).

RSV-binding antibodies

Enzyme immunoassays (EIAs) that measure anti-RSV IgG antibodies are commercially available. Sponsors may also develop in-house tests suited to the individual vaccine. If commercial assays are used it is recommended that kits are derived from the same manufacturing lot or otherwise qualified by the appropriate bridging studies in order to minimize variability in results.

- EIA to measure anti-RSV F IgG: It is recommended that RSV-F antigens used to capture anti-F binding antibodies be of high quality, with a well-characterized conformation and proven stability. During assay development, the appropriate antibody reagents should be used to confirm the predominant conformation of RSV-F antigen present by assessing the ability to bind antibodies specific for epitopes on pre-fusion and/or post-fusion conformation of RSV-F. Some pre-fusion F epitopes are specific for RSV subtype A or subtype B pre-F protein. Therefore, in some cases it may be necessary to test for IgG antibodies that bind pre-F antigens in a subtype specific manner (32, 88-90).
- Purified recombinant proteins or synthetic peptides may be used in EIAs to detect antibody responses against antigens such as RSV-G_A and -G_B proteins. Antibody responses against RSV proteins not included in the vaccine may support surveillance for RSV exposures/infections during follow-up (91).

Antibody responses to a specific protein or epitope may be detected using competitive binding studies based on EIA formats or biosensor technology wherein the antigen is applied to a surface (plate or chip), and antibody binding in the test sample measured in the presence and absence of a competitor (92).

C.2.1.2 Cell mediated immunity

CD8⁺ T cell responses

Ideally, CD8⁺ T-cells are collected at 7-14 days after a vaccine dose in adults for determination of sensitisation by *in vitro* stimulation with RSV antigens (38,93). A similar sampling window may apply to infants and children based on a finding that CD8⁺ T cell responses in peripheral blood peaked between 11 and 15 days after onset of symptoms in RSV-infected infants (41). The optimal sampling time for detecting CD8⁺ T-cells may vary by vaccine platform; a broader window may be considered if supported by data for the vaccine under study.

CD4⁺ T-cell responses

CD4⁺ T-cells in infants less than 6 months of age are epigenetically programmed to have a dominant Th2 type cytokine response that may be antigen-specific (42,43). In some cases, it may be appropriate to evaluate CD4⁺ T-cell responses in RSV-naïve infants in early phase clinical testing to determine the ratio of Th2 cytokines (such as IL-4, IL-5 and IL-13) versus Th1 type cytokines (such as IL-2 and IFN-γ) following re-stimulation with overlapping peptides representative of vaccine antigens.

C.2.2 Trial population and design

Regardless of the target population(s) for a candidate RSV vaccine, the first trials are expected to be conducted in healthy adults to provide data on safety and immunogenicity in RSV-experienced male and non-pregnant female subjects.

C.2.2.1 Infants and toddlers

It is recommended that the first trials are conducted in RSV-experienced toddlers. Depending on the vaccine construct, the nonclinical data and accumulated scientific knowledge, it may sometimes be appropriate to consider conducting a safety and immunogenicity trial in RSV non-naïve infants before moving to RSV-naive subjects. A definition of RSV naïve and -experienced subjects should be established in the protocol. For example, RSV-naïve could be based on having no documented history of RSV disease and no immunological evidence of prior exposure to RSV (based on one or more of pre-vaccination neutralizing antibody titres, anti-RSV IgA or IgM).

It is recommended that immunogenicity trials that include RSV-naïve subjects should complete follow-up for RSV disease for at least one season (or equivalent in non-seasonal regions) to provide a preliminary assessment of the risk of enhanced RSV disease based on a comparison of the severity of cases. This assessment should be completed before exposing larger numbers of RSV-naïve subjects.

The potential for maternal antibody to interfere with the immune response to active immunisation of infants should be assessed from the relationship between pre- and post-vaccination immune parameters. If maternal antibody has a negative effect on the infant immune response, consideration could be given to administering an additional dose (e.g. after 6-12 months has elapsed since completion of the primary series) and comparing the response

with that to a single dose administered to unvaccinated subjects of the same age to assess whether vaccinated infants were primed.

C.2.2.2 Pregnant women

Data obtained in non-pregnant women of childbearing potential should be used to select the initial dose regimen(s) to be tested in pregnant women. Follow-up of these non-pregnant women for neutralising antibody decay and documentation of the safety and immunogenicity of revaccination should be considered to provide an early indication of the possible need for revaccination of women in consecutive pregnancies.

Dose regimens for pregnant women may aim to maximize the difference in RSV neutralising antibody titres in cord blood between infants born to vaccinated and unvaccinated mothers whilst maintaining an acceptable safety profile. Analysis of cord blood antibody levels in infants by time elapsed between maternal vaccination and delivery may assist in determining the optimal number of doses and timing of maternal vaccination. Documenting the RSV neutralizing antibody decay curve in infants may give an early indication of how long a benefit of maternal vaccination may persist.

Unless otherwise justified, trials involving maternal vaccination should follow up infants for RSV disease until it is predicted that they will have no or negligible maternal antibody before initiating the next trial. This will allow for data on RSV disease and its severity to be collected and reviewed to assess whether there is any signal for enhanced disease in infants born to vaccinated vs. unvaccinated mothers.

Documenting the RSV neutralizing antibody decay curve in vaccinated women during and following delivery (e.g. for 6 months) may give an early indication of the need to re-vaccinate women during each pregnancy. Consideration should be given to investigating the safety and immunogenicity of revaccination during a subsequent pregnancy whenever the opportunity arises in the post-approval period.

C.2.2.3 Older adults

It is important that data are obtained from all age sub-groups (e.g. <65, 65-74, 75-84 and ≥ 85 years) within the target population in safety and immunogenicity trials to assess whether age sub-group-specific regimens may be needed. Unless otherwise justified, it is recommended that trials should document the safety and immunogenicity of additional doses administered at intervals (e.g. after 1-2 years since the primary dose[s]) to randomised subsets of subjects. This information can be used to support a booster strategy if this is later concluded to be appropriate from results of efficacy trials.

C.3 Efficacy trials

In the absence of RSV vaccines licensed and widely recommended for use in the target population of a candidate RSV vaccine, vaccine efficacy trials should compare rates of RSV disease meeting the primary case definition (see C.3.2) between groups randomised to the candidate vaccine or to no vaccination against RSV. At least one trial should be conducted in each target population proposed for the candidate vaccine (e.g. in infants ± toddlers, in pregnant women and/or in older adults), depending on the perceived suitability of the candidate vaccine for these sub-populations.

The WHO Guideline on clinical evaluation of vaccines; regulatory expectations (12) provides guidance on the need for, and design of, efficacy trials when there is a licensed vaccine available and widely recommended for use in the target population for a candidate vaccine. It also discusses situations in which efficacy may be inferred from immunogenicity data.

Before commencing efficacy trials in target populations, sponsors may consider the possible value of conducting a human challenge study.

In accordance with ICH E9- Statistical principles for clinical trials (94), consideration should be given to stratification of subjects at randomisation by important known or suspected prognostic factors measured at baseline. Factors on which randomisation has been stratified should be accounted for in the analysis.

C.3.1 Trial populations

C.3.1.1 Infants and toddlers

Selection criteria should include the minimum gestational age at birth and the minimum and maximum ages at the time of enrolment. Protocols should clarify the eligibility of infants born at ≤ 35-36 weeks of gestation.

It is not expected to be feasible to determine baseline serostatus prior to enrolment into efficacy trials. However, it is recommended that baseline blood samples are obtained at least from a randomised subset of subjects and preferably from all subjects to allow for an exploration of vaccine efficacy in RSV-naïve and experienced subsets (see section C.2.2.1 on establishing definitions). Furthermore, it is recommended that post-vaccination blood samples are obtained at least from a randomised subset of subjects, and preferably from all subjects, at a fixed point in time to allow for an exploration of vaccine efficacy according to immune status and possible identification of an immune correlate of protection.

C.3.1.2 Pregnant women

The minimum and maximum gestational stage and the method used for estimating this should be specified in the protocol and applied across all trial sites. The minimum gestational age for

vaccination should be determined from the safety and immunogenicity studies, depending on the number of doses and dose interval needed to optimise the immune response.

Protocols should state whether pregnant women with any evidence of placental insufficiency are eligible for enrolment. If there are cord blood data to suggest that vaccination increases the anti-RSV neutralising antibody transferred to the fetus despite placental insufficiency, it may be appropriate to include these women.

C.3.1.3 Older adults

To support use of a candidate RSV vaccine without an upper age limit, trials should aim to ensure that the trial population covers a wide age range. For example, it may be reasonable to aim for at least 25% of the total population to be aged >75 years. It is recommended that exclusion criteria are kept to a minimum to ensure that subjects have a range of comorbidities.

C.3.2 Efficacy trial endpoints

C.3.2.1 Primary case definition

The primary case definition should require both clinical and laboratory criteria to be met.

Clinical criteria

The primary case definition could be any RSV disease or could be confined to RSV LRTI and severe RSV disease.

The list of clinical signs and symptoms and the number to be met must be tailored to the age range of the trial population (e.g. a definition applicable to infants would not be appropriate for older adults). Information on clinical presentations from epidemiological studies of RSV and/or from completed clinical trials may be helpful when selecting the minimum signs and symptoms to be met.

Sponsors are advised to take account of proposals for classifying RSV disease severity in different age groups that come from well-recognised public health or professional bodies. For example, the WHO has published suggested clinical criteria for defining severe RSV disease in infants and toddlers (8). Published clinical scores suitable for application to RSV disease could also be considered.

Laboratory criteria

Laboratory confirmation of a case may be based on a protocol-defined commercially available rapid diagnostic test (RDT) for RSV. These tests may be based on amplification of RSV nucleic acid sequences, such as a nucleic acid amplification test (NAT) or reverse transcription polymerase chain reaction (RT-PCR) (95,96). It is recommended that the exact same RDT (e.g. a NAT from a single manufacturer that can detect low levels of virus) is used at all sites if

multiple testing sites are permitted for early phase studies. In pivotal clinical trials it is recommended that testing is conducted in a central laboratory using a single validated RDT.

The sponsor should justify the RDT(s) chosen based on their performance characteristics (sensitivity and specificity). RDTs should be able to discriminate between RSV-A and –B strains. The test method should be able to detect a low copy number (e.g. $<10^3$ gene copies per mL or fewer than 50 gene copies per reaction) of the target RSV sequence. In clinical trials involving live-attenuated RSV vaccines, a NAT test should have the ability to differentiate between vaccine and wild-type RSV strains. For example, some RSV live-attenuated strains used in candidate vaccines are gene-deletion mutants so that amplification of a target sequence from within the deleted gene can be used to distinguish vaccine strains from wild-type viruses.

During clinical trials, arrangements should be in place to collect samples from suspected cases as early as possible after onset of clinical features suggesting a possible RSV infection. Licensed test kits specify the type of sample to be collected and most frequently recommend the use of nasal swab and/or nasal wash samples. Other samples such as nasal secretions (mucus), sputum, tracheal aspirates, bronchial alveolar lavage samples and post-mortem lung tissues may be used for virus detection if the test method is modified and validated for this use. In most cases, collection of nasal swabs (NS), nasopharyngeal swabs (NP) or nasal wash aspirates (NW) will be acceptable to trial subjects. Whilst NS may be more sensitive in detecting RSV shedding (97), NW aspirates may be better at detecting virus when quantities are low (98-101). The protocol for sample collection should provide the details of the collection method, including issues such as type of swab (which may be very important for some assays) and swabbing site/action so that the protocol is applied consistently across all study sites and all trials in any one clinical development programme. Training of site personnel in sample collection may be required.

Negative controls (e.g. collection medium blanks) should be processed and tested with clinical samples to ensure that no cross-contamination occurs. A cellular DNA target sequence, such as GAPDH, may be used as an internal control to monitor the quality of the collected samples. Alternatively, upon thawing and prior to further processing, NS or NW samples may be spiked with a barcode-tagged RNA sequence to serve as a unique sample identifier and internal control to monitor efficiency of RNA extraction.

C.3.2.2 Secondary case definitions

Alternative case definitions should be defined as necessary for the purposes of the secondary analyses.

C.3.3 Case ascertainment

It is generally recommended that active surveillance (12) is used to identify cases meeting the primary and other case definitions. The method of case ascertainment should be tailored to the geographical distribution of trial sites and should include instructions to subjects and care-

givers on trigger signs and symptoms for possible RSV and presentation to site staff and/or participating healthcare facilities.

C.3.4 Analysis of efficacy

If the primary analysis is based on all RSV disease (i.e. regardless of severity), secondary analyses should be conducted based on RSV LRTI, severe RSV disease and/or other case definitions. Furthermore, in efficacy trials that enroll RSV-naïve subjects it is essential that information on case severity is captured so that the clinical presentations of cases that occur in vaccinated and unvaccinated cohorts can be compared (whether in the primary or secondary analyses) to assess the risk of vaccine-associated enhanced disease. See section C.2.3.

Some additional considerations for population sub-groups are included below.

Infants and toddlers

The primary analysis may be confined to RSV cases with onset after a time point defined by completion of the primary series. If this is the case, it is important that a secondary analysis compares numbers of cases that occur at any time from randomisation. Additional secondary analyses may compare numbers of cases that occur after each dose.

A secondary analysis should address the time between the last vaccination (scheduled or completed) and the onset of disease.

There is interest in evaluating whether vaccination impacts on the rate of asthma and symptomatic wheezing in children, which could be investigated in the post-licensure period. This would require a clear definition of symptomatic wheezing (e.g. including pulmonary function criteria in children old enough to undergo testing) along with long-term structured follow-up to maintain high retention of the original clinical trial population to determine whether there is any detectable benefit and its duration.

Pregnant women

The primary analysis may be confined to infants born a minimum number of weeks after their mothers were vaccinated. If this is the case, a secondary analysis of efficacy should be conducted in all infants regardless of the time elapsed between maternal vaccination and delivery. Some infants may be eligible for routine use of an anti-RSV monoclonal antibody according to local guidance, in which case it would be appropriate to exclude them from the primary analysis of efficacy if they have received such a monoclonal. Cases of RSV disease in these subjects should be captured and included in a secondary analysis.

It is recommended that infants born to vaccinated mothers are followed for RSV disease until it is predicted from data collected during prior immunogenicity trials that geometric mean neutralising antibody titres are similar between infants born to vaccinated and unvaccinated mothers.

Older adults

A secondary analysis should address the time between the last vaccination of the subjects (scheduled or completed) and the onset of disease.

It is recommended that, unless an immune correlate of protection has been established, subjects should continue to be followed for RSV disease to assess the potential need for re-vaccination and the intervals at which this may be required to maintain protection. One approach may be to sub-randomise subjects initially allocated to the vaccine group to receive or not receive a booster dose and follow these cohorts further for RSV cases. Data to support advice on revaccination may not be available until after first licensure and may be modified as additional data emerge.

C.4 Safety aspects

C.4.1 Infants and toddlers

Safety data obtained from trials in RSV-experienced subjects may be poorly predictive of the safety profile in RSV-naïve subjects. Therefore, a cautious approach is recommended for the commencement of trials in infants and toddlers.

Historical data indicate that the potential risk of vaccine-associated disease enhancement is highest (and perhaps confined to) RSV-naïve infants. Therefore, it is particularly important that there is a large representation of infants known or expected (e.g. from epidemiology data) to be RSV-naïve in the safety database.

If there is any vaccine-associated disease enhancement, it is expected to occur with the first natural RSV infection after completion of vaccination. The total duration of follow-up for RSV disease in clinical trials to address this issue should be decided from knowledge of the rate of RSV seroconversion as a measure of natural exposure in the regions where trials are conducted. To support the adequacy of follow-up to assess the risk of enhanced disease, a (preferably randomised) subset of subjects in the placebo group could be assessed for serological evidence of natural infection at one or more pre-defined tome points. If a signal for enhanced RSV disease has not been observed in the vaccinated group up to a time by which serology indicates a high natural exposure rate in the unvaccinated control group, it is very unlikely that it would be detected during additional follow-up.

An additional safety consideration for trials of live-attenuated RSV vaccines in RSV-naïve infants includes the need to assess the duration and magnitude of virus shedding and transmission to susceptible (RSV-naïve) close contacts. Unless the attenuation phenotype of the vaccine candidate virus has been confirmed, adequate precautions should be in place to minimize the risk of transmission of the vaccine virus from vaccinees during the period of virus shedding to contacts who are less than 1 year old or are immunocompromised.

C.4.2 Pregnant women

The threshold for determining tolerability of a vaccine during pregnancy is usually lower than that applicable to non-pregnant adults. The risk of local and systemic reactions, including fever, to vaccination should be assessed in non-pregnant women before proceeding to vaccinate pregnant women. The rates of premature delivery, complications of pregnancy or labour and the condition of infants at birth should be compared between the vaccinated and unvaccinated groups. Routine safety assessments of infants should be conducted for 6-12 months after birth.

C.4.3 Older adults

The tolerability of a vaccine may differ between subgroups of older persons by age sub-group and degree of frailty. Therefore, it is important that there are safety data obtained from all age subgroups that are to be included in the target population for routine use. If post-licensure data indicate that re-vaccination at intervals may be required, the safety profile of repeated dosing should be documented (see section C.2.2.3).

Part D. Guidelines for NRAs

D.1 General

The general recommendations for control laboratories given in the WHO Guidelines for national authorities on quality assurance for biological products (102) and WHO Guidelines for independent lot release of vaccines by regulatory authorities (103) should apply after the vaccine product has been granted a marketing authorization. These recommendations specify that no new biological substance should be released until consistency of batch manufacturing and quality has been established and demonstrated. The recommendations do not apply to material for clinical trials.

The detailed production and control procedures, as well as any significant changes in them that may affect the quality, safety and efficacy of the RSV vaccines, should be discussed with and approved by the NRA.

The NRA may obtain the product-specific working reference from the manufacturer to be used for lot release until the international or national standard preparation is established.

Consistency of production has been recognized as an essential component in the quality assurance of vaccines. In particular, during review of the marketing authorization dossier, the NRA should carefully monitor production records and quality control test results for clinical lots, as well as for a series of consecutive lots of the vaccine, produced using the procedures and control methods that will be used for the marketed vaccine.

D.2 Release and certification

A vaccine lot should be released to the market only if it fulfils all national requirements and/or satisfies Part A of these WHO Guidelines (103). A protocol for the manufacturing and

control of human RSV vaccines, based on the model protocol provided in Appendix 1 (to be developed), and signed by the responsible official of the manufacturing establishment, should be prepared and submitted to the NRA in support of a request for the release of a vaccine for use.

A Lot Release Certificate signed by the appropriate NRA official should then be provided if requested by a manufacturing establishment, and should certify whether or not the lot of vaccine in question meets all national requirements, as well as Part A of these WHO Guidelines. The purpose of this official national release certificate is to facilitate the exchange of vaccines between countries, and should be provided to importers of the vaccines. A model NRA Lot Release Certificate is provided below in Appendix 2.

Authors and acknowledgements

The **first draft** of these guidelines was prepared by Dr J. Beeler (Center for Biologics Evaluation and Research, Food & Drug Administration, United States of America), Dr E. Kretzschmar (Paul-Ehrlich Institut, Germany), Dr M. Powell (Medicines and Healthcare products Regulatory Agency, United Kingdom), Dr Y. Sun ((Paul-Ehrlich Institut, Germany) and Dr T.Q. Zhou (Technologies Standards and Norms, Essential Medicines and Health Products, Access to Medicines, Vaccines and Pharmaceuticals, World Health Organization, Geneva, Switzerland). The first draft was reviewed at the Informal consultation on WHO guidelines on the quality, safety and efficacy of Respiratory Syncytial Virus (RSV) vaccines, held in Domaine de Penthes, Geneva, Switzerland, 18 -19 September 2018, and attended by the following participants: Dr J. Beeler (Center for Biologics Evaluation and Research, Food & Drug Administration, United States of America); Dr O.G. Engelhardt, National Institute for Biological Standards and Control, MHRA, United Kingdom; Dr B.S. Graham, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States of America; Dr D. Higgins, RSV Vaccine Project, PATH's Center for Vaccine Innovation and Access, United States of America; Dr B.L. Innis, PATH's Center for Vaccine Innovation and Access, United States of America; Dr J. Korimbocus, L'Agence nationale de sécurité du médicament et des produits de santé (ANSM), France; Dr E. Kretzschmar (Paul-Ehrlich Institut, Germany); Dr C. Li, National Institutes for Food and Drug Control (NIFDC), People's Republic of China; Dr M. Powell, Medicines and Healthcare products Regulatory Agency, United Kingdom; Dr M. Nunes, University of Witwatersrand, South Africa & Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa; Dr P.A. Piedra, Baylor College of Medicine, United States of America; Dr R. Pless, Biologics and Genetic Therapies Directorate, Health Canada, Canada; Dr T. Ruckwardt, Vaccine Research Center, NIAID, NIH, United States of America; Mr B.K. Samantaray, Central Drugs Standard Control Organization, India; Dr K. Shirato, National Institute of Infectious Diseases, Japan; Dr Y. Sun, Paul-Ehrlich Institut, Germany; Dr N. Wairagkar, The Bill and Melinda Gates Foundation, United States of America; and Representatives of international federation of pharmaceutical manufacturers & associations

(IFPMA): Dr I. Dieussaert, GSK Vaccines; Dr P. Dormitzer, Pfizer Vaccines; Dr D. Guris, Merck & Co, USA; Dr S.S.S. Sesay, Sanofi Pasteur; Dr F. Takeshita, Daiichi Sankyo Co., Ltd., Japan; Dr M. Widjojoatmodjo, Janssen Vaccines; representatives of developing country vaccine manufacturers network (DCVMN): Ms C. Ma, Lanzhou Institute of Biological Products Co., Ltd., CNBG, China; Dr K. Wu, BravoVax, China; and representatives of other industries: Dr Amy B. Fix and Dr L. Fries, NOVAVAX, USA; Dr C. Heery, Bavarian Nordic, Inc., USA; WHO Headquarters: Dr M. Alali, Prequalification team (PQT), Regulation of Medicines and other Health Technologies (RHT) Unit, Essential Medicines and Health Products (EMP) Department, Access to Medicines, Vaccines and Pharmaceuticals (MVP) Cluster; Dr D. Feikin, Initiative for Vaccine Research (IVR), Immunization, Vaccines and Biologicals (IVB) Department, Family, Women's and Children's Health (FWC) Cluster; Dr S. Hirve, Global Influenza Preparedness & Response; Dr I. Knezevic, Technologies Standards and Norms (TSN) Team, Regulation of Medicines and other Health Technologies (RHT) Unit, Essential Medicines and Health Products (EMP) Department, Access to Medicines, Vaccines and Pharmaceuticals (MVP) Cluster; Dr O.C. Lapujade, Prequalification team (PQT), Regulation of Medicines and other Health Technologies (RHT) Unit, Essential Medicines and Health Products (EMP) Department, Access to Medicines, Vaccines and Pharmaceuticals (MVP) Cluster; Dr E. Sparrow, Universal Health Coverage and Health Systems and Innovation, Dr T.Q. Zhou, Technologies Standards and Norms (TSN) Team, Regulation of Medicines and other Health Technologies (RHT) Unit, Essential Medicines and Health Products (EMP) Department, Access to Medicines, Vaccines and Pharmaceuticals (MVP) Cluster.

Comments were also received post the consultation meeting during September and October 2018 from following experts: Dr M. Alali, WHO/RHT/PQ; Dr I. Dieussaert, GSK Vaccines; Dr D. Feikin, WHO/IVB/IVR; Dr L. Fries, Novavax; Dr T. Fumihiko, Daiichisankyo, Japan; Dr D. Guris, Merck & Co, USA; Drs B. Graham and Dr T. Ruckwardt, NIAID/NIH, USA; Drs D. Higgins and B. Innis, PATH, USA; Dr W.V. Molle, Sciensano, Belgium; Dr S.S.S. Sesay, Sanofi Pasteur; Dr E. Sparrow, WHO; Dr M. Widjojoatmodjo, Janssen Vaccines.

Based on above comments, the **second draft** was prepared by Dr J. Beeler (Center for Biologics Evaluation and Research, Food & Drug Administration, USA), Dr E. Kretzschmar (Paul-Ehrlich Institut, Germany), Dr M. Powell (Medicines and Healthcare products Regulatory Agency, UK), Dr Y. Sun ((Paul-Ehrlich Institut, Germany) and Dr T.Q. Zhou (WHO/RHT/TSN).

References

1. Falsey AR et al. Respiratory syncytial virus and other respiratory viral infections in older adults with moderate to severe influenza-like illness. J Infect Dis. 2014 Jun 15;209 (12):1873-81.

2. Openshaw PJM, Chiu C Culley FJ, Johansson C. Protective and Harmful Immunity to RSV Infection. Annu Rev Immunol. 2017 Apr 26;35:501-532.

3. van Asten L et al. Mortality attributable to 9 common infections: significant effect of influenza A, respiratory syncytial virus, influenza B, norovirus, and parainfluenza in elderly persons. J Infect Dis. 2012 Sep 1;206(5):628-39.

4. Fleming DM et al. Modelling estimates of the burden of Respiratory Syncytial virus infection in adults and the elderly in the United Kingdom. BMC Infect Dis. 2015 Oct 23;15:443.

5. Giersing BK, Modjarrad K, Kaslow DC, Moorthy VS, WHO Product Development for Vaccines Advisory Committee. Report from the World Health Organization's Product Development for Vaccines Advisory Committee (PDVAC) meeting, Geneva, 7–9th Sep 2015. Vaccine. 2016 Jun 3;34(26):2865–9. doi:10.1016/j.vaccine.2016.02.078. 6.

6. Giersing BK, Modjarrad K, Kaslow DC, Okwo-Bele JM, Moorthy VS. The 2016 Vaccine Development Pipeline: A special issue from the World Health Organization Product Development for Vaccine Advisory Committee (PDVAC). Vaccine. 2016 Jun. 3;34(26):2863–4. doi:10.1016/j.vaccine.2016.04.041.

7. WHO. RSV Vaccine Research and Development Technology. ROADMAP. 2017. WHO/IVB/17.12. http://apps.who.int/iris/bitstream/10665/258706/1/WHO-IVB-17.12-eng.pdf?ua=1. Accessed 16 May 2018.

8. Modjarrad K, Giersing B, Kaslow DC, et al. WHO RSV Vaccine Consultation Expert Group. WHO consultation on Respiratory Syncytial Virus Vaccine Development Report from a World Health Organization Meeting held on 23-24 March 2015. Vaccine. 2016;34:190-7. http://dx.doi.org/10.1016/j.vaccine.2015.05.093.

9. Giersing BK, Karron RA, Vekemans J, Kaslow DC, Moorthy VS. et al. Meeting report: WHO consultation on Respiratory Syncytial Virus (RSV) vaccine development, Geneva, 25-26 April 2016. Vaccine. 2017 Mar 14. pii: S0264-410X(17)30293-1. doi: 10.1016/j.vaccine.2017.02.068.

10. WHO guidelines on nonclinical evaluation of vaccines. In: WHO Expert Committee on Biological Standardization: fifty-fourth report. Geneva: World Health Organization; 2005: Annex 1 (WHO Technical Report Series, No. 927; http://www.who.int/biologicals/publications/trs/areas/vaccines/nonclinical_evaluation/ANNEX%201Nonclinical.P31-63.pdf?ua=1, accessed 24 July 2018).

11. Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines. In: WHO Expert Committee on Biological Standardization: sixty-fourth report. Geneva: World Health Organization; 2014: Annex 2 (WHO Technical Report Series, No. 987; http://www.who.int/biologicals/areas/vaccines/TRS_987_Annex2.pdf?ua=1, accessed 24 July 2018).

12. Guidelines on clinical evaluation of vaccines: regulatory expectations. In: WHO Expert Committee on Biological Standardization: sixty-seventh report. Geneva: World Health Organization; 2017: Annex 9 (WHO Technical Report Series, No. 1004; http://www.who.int/entity/biologicals/expert_committee/WHO_TRS_1004_web_Annex_9.pdf?ua=1, accessed 24 July 2018).

13. Global vaccine safety blueprint. Geneva: World Health Organization; 2012 (WHO/IVB/12.07; http://extranet.who.int/iris/restricted/bitstream/10665/70919/1/WHO_IVB_12.07_eng.pdf?ua=1, accessed 24 July 2018).

14. Recommendations for the evaluation of animal cell cultures as substrates for the manufacture of biological medicinal products and for the characterization of cell banks. In: WHO Expert Committee on Biological Standardization: sixty-first report. Geneva: World Health Organization; 2013: Annex 3 (WHO Technical Report Series, No. 978; http://www.who.int/biologicals/vaccines/TRS_978_Annex_3.pdf, accessed 24 July 2018).

15. Recommendations to assure the quality safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration. Annex 4 of WHO Technical Report Series No. 977, 2013. http://apps.who.int/medicinedocs/documents/s21091en/s21091en.pdf. Accessed 7 Jan 2019.

16. Recommendations to assure the quality, safety and efficacy of BCG vaccines, Annex 3, Technical Report Series 979. https://www.who.int/biologicals/expert_committee/TRS_979_62nd_report.pdf?ua=1. Accessed 7 Jan 2019.

17. Collins P, Fearns R, Graham B. Respiratory syncytial virus: virology, reverse genetics, and pathogenesis of disease. Curr Top Microbiol Immunol 2013;372:3-38. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4794264/pdf/nihms765735.pdf. Accessed 13 June 2018.

18. Lee, Wan-Ji; Kim, You-jin; Kim, Dae-Won; Lee, Han Saem; Lee, Ho Yeon; Kim, Kisoon. "Complete Genome Sequence of Human Respiratory Syncytial Virus Genotype A with a 72-Nucleotide Duplication in the Attachment Protein G Gene". Journal of Virology. 2012. 86 (24): 13810–1. doi:10.1128/JVI.02571-12.

19. Wayne M. Sullender. Respiratory Syncytial Virus Genetic and Antigenic Diversity. Clin Microbiol Rev. 2000 Jan; 13(1): 1–15. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC88930/pdf/cm000001.pdf, accessed 13 June 2018.

20. Ting Shi, David A McAllister, Katherine L O'Brien, Eric A F Simoes, Shabir A Madhi, et al. Global, regional, and national disease burden estimates of acute lower respiratory infections due to respiratory syncytial virus in young children in 2015: a

systematic review and modelling study. Lancet. 2017 Sep 2; 390(10098): 946–958. doi: 10.1016/S0140-6736(17)30938-8.

21. Thompson WW et al., Mortality Associated with Influenza and Respiratory Syncytial Virus in the United States, JAMA,2003, 289:179-186.

22. Silvia Vandini, Carlotta Biagi, and Marcello Lanari. Review Respiratory Syncytial Virus: The Influence of Serotype and Genotype Variability on Clinical Course of Infection. Int. J. Mol. Sci. 2017, 18, 1717; doi:10.3390/ijms18081717. Accessed 13 June 2018.

23. Epidemiology of Respiratory Syncytial Virus Infections. http://virology-online.com/viruses/RSV2.htm. Accessed 24 July 2018.

24. Russell, CDm Unger SA, Walton, m, Schwarze J, The human immune response to Respiratory Syncytial Virus Infection, Clin Microbiol Rev, 30:481-502. https://doi.org/10.1128/CMR.00090-16.

25. Lambert L, Sagfora AM, Openshaw PJM, Culley FJ, Immunity to RSV in early life, Frontier in Immunology (2014), 5:a466. https://doi.org/10.3389/fimmu.2014.00466.

26. Schmidt ME and Varga SM, Modulation of the host immune response by respiratory syncytial virus proteins. Journal of Microbiology, 2017, 55:161-171. https://doi.org/10.1007/s12275-017-7045-8.

27. Jorquera PA, Andrson L, Tripp RA, Understanding respiratory syncytial virus (RSV) vaccine development and aspects of disease pathogenesis. Expert Review of Vaccines, 2016, 15:173-187. http://dx.doi.org/10.1586/14760584.2016.1115353.

28. Miyairi I and DeVincenzo JP Human genetic FAcotrs and Respiratory Syncytail Virus Disease Severity, Clinical Microbiology Reviews, 200821:686-703. http://doi:10.1128/CMR.00017-08.

29. Ngwuta, J O et al, Prefusion F specific antibodies determine the magnitude of the RSV neutralizing activity in human sera Science and Translational medicine. Vol. 7, Issue 309, pp. 309ra162, 2015. doi:10.1126scitranslmed.aac4241.

30. Palomo C., et al, Influence of RSV F glycoprotein conformation on induction of protective immune responses, J of Virology, 90:5485-5498. doi:10.1128/JVI.00338-16.

31. Beeler JA, van Wyke Coelingh K. Neutralization epitopes of the F glycoprotein of respiratory syncytial virus: effect of mutation upon fusion function. J of Virology, 1989, 63:2941-2950.

32. Tian D, Battles MB, Moin SM, Chen M, Modjarrad K, Kumar A, et al. Structural basis of respiratory syncytial virus subtype-dependent neutralization by an antibody targeting the fusion glycoprotein. Nat Commun. 2017;8(1):1877.

33. Lee, J, Klenow, L, Coyle E., et al., Protective antigenic sites in RSV G attachement protein outside the central conserved and cystein noose domains. Plos Pathogens, 14(8) e1007262, Https://doi.org/10.1371/journal.ppat.1007262.

34. Fedechkin SO, George NL, Wolff, JT et al., Structures of RSV F antigen bound to broadly neutrlizing antibodies Sci. Immunol. 3, eaar3534(2018).

35. Jones, HG, Ritschel T., Pascual G, et al., Structural basis for recognition of the central conserved region of RSV G by neutrlizing human antibodies. PlosPAthogens 14(3) e1006935 https://doi.org/10.1371/jopurnal.ppt.1006935.

36. Schmidt, ME and Varga SM, Modulation of the host immune response by RSV proteins. Journal of Microbiology, 2017, 55:161-171, doi: 10.1007/s12275-017-7045-8.

37. Habibi MS, Jozwik A, Makris S, Dunning J, Paras A, DeVincenzo JP, et al. Impaired Antibody-mediated Protection and Defective IgA B-Cell Memory in Experimental Infection of Adults with Respiratory Syncytial Virus. Am J Respir Crit Care Med. 2015;191(9):1040-9.

38. Jozwik A, Habibi MS, Paras A, Zhu J, Guvenel A, Dhariwal J, et al. RSV-specific airway resident memory CD8+ T cells and differential disease severity after experimental human infection. Nat Commun. 2015;6:10224.

39. Ascough S, Paterson S, Chiu C. Induction and Subversion of Human Protective Immunity: Contrasting Influenza and Respiratory Syncytial Virus. Front Immunol. 2018;9:323.

40. Heidema J, Lukens MV, van Maren WW, van Dijk ME, Otten HG, van Vught AJ, et al. CD8+ T cell responses in bronchoalveolar lavage fluid and peripheral blood mononuclear cells of infants with severe primary respiratory syncytial virus infections. J Immunol. 2007;179(12):8410-7.

41. Lukens MV, van de Pol AC, Coenjaerts FE, Jansen NJ, Kamp VM, Kimpen JL, et al. A systemic neutrophil response precedes robust CD8(+) T-cell activation during natural respiratory syncytial virus infection in infants. J Virol. 2010;84(5):2374-83.

42. PrabhuDas M, Adkins B, Gans H, King C, Levy O, Ramilo O, et al. Challenges in infant immunity: implications for responses to infection and vaccines. Nature Immunology. 2011;12(3):189-94.

43. PrabhuDas M, Bonney E, Caron K, Dey S, Erlebacher A, Fazleabas A, et al. Immune mechanisms at the maternal-fetal interface: perspectives and challenges. Nat Immunol. 2015;16(4):328-34.

44. Mariani, TJ, Qiu, X, Chi, CY, Wang, L, Thaker J, Holden-Wiltse J, et al. Association of Dynamic Changes in the CD4 T-cell transcriptome with Disease Severity During Pirmary

Respriatory Syncytial Virus Infection in Young Infants. JID 2017:216: 1027-1037. DOI: 10.1093/infdis/jix400.

45. Zhivaki D, Lemoine S, Lim A, Morva A, Vidalain PO, et al. Respiratory Syncytial Virus Infects Regulatory B Cells in Human Neonates via Chemokine Receptor CX3CR1 and Promotes Lung Disease Severity. Immunity. 2017 Feb 21;46(2):301-314. doi: 10.1016/j.immuni.2017.01.010.

46. Kapikian A.Z., Mitchell R.H., Chanock R.M., Shvedoff R.A., Stewart C.E. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Am. J. Epidemiol. 1969;89:405–421. doi: 10.1093/oxfordjournals.aje.a120954.

47. Neuzil K.M. Progress toward a Respiratory Syncytial Virus Vaccine. Clin. Vaccine Immunol. 2016;23:186–188. doi: 10.1128/CVI.00037-16.

48. Graham B.S. Vaccines against respiratory syncytial virus: The time has finally come. Vaccine. 2016;34:3535–3541. doi: 10.1016/j.vaccine.2016.04.083.

49. Green C.A., Scarselli E., Voysey M., Capone S., Vitelli A., et al. Safety and immunogenicity of novel respiratory syncytial virus (RSV) vaccines based on the RSV viral proteins, F, N and M2–1 encoded by simian adenovirus (PanAd3-RSV) and MVA (MVA-RSV); protocol for an open-label, dose-escalation, single-centre, phase 1 clinical trial in healthy adults. BMJ Open. 2015;5:e008748.

50. Higgins D., Trujillo C., Keech C. Advances in RSV vaccine research and development- A global agenda. Vaccine. 2016;34:2870–2875. doi: 10.1016/j.vaccine.2016.03.109.

51. Ralph A. Tripp, Ultan F. Power, Peter J. M. Openshaw and Lawrence M. Kauvar. Respiratory Syncytial Virus (RSV): Targeting the G Protein Provides a New Approach for an Old Problem. JVI Accepted Manuscript Posted Online 8 November 2017 J. Virol. doi:10.1128/JVI.01302-17. http://jvi.asm.org/content/early/2017/11/02/JVI.01302-17.full.pdf+html. Accessed 13 June 2018.

52. World Health Organization. Report on the WHO collaborative study to establish the 1st International Standard for antiserum to Respiratory Syncytial Virus, WHO/BS/2017.2318. McDonald, Jacqueline, Rigsby, Peter, Dougall, Thomas, Engelhardt, Othmar G, Study Participants. http://apps.who.int/iris/bitstream/handle/10665/260488/W?sequence=1. Accessed 8 Jan 2019.

53. WHO Catalogue of International Reference Preparations. Blood products and related biologicals [website]. Geneva: World Health Organization. (http://www.who.int/bloodproducts/catalogue/en/, accessed 24 July 2018).

54. WHO good manufacturing practices for pharmaceutical products: main principles. In: WHO Expert Committee on Specifications for Pharmaceutical Preparations: forty-eighth

report. Geneva: World Health Organization; 2014: Annex 2 (WHO Technical Report Series, No. 986; http://www.who.int/medicines/areas/quality_safety/quality_assurance/TRS986annex2.pdf?ua=1, accessed 17 December 2017).

55. WHO good manufacturing practices for biological products. In: WHO Expert Committee on Biological Standardization: sixty-sixth report. Geneva: World Health Organization; 2016: Annex 2 (WHO Technical Report Series, No. 999; http://www.who.int/biologicals/areas/vaccines/Annex_2_WHO_Good_manufacturing_practices_for_biological_products.pdf?ua=1, accessed 17 December 2017).

56. World Health Organization. Laboratory Biosafety Manual, Third edition; 2004.ISBN: 92 4 154650 6. https://www.who.int/csr/resources/publications/biosafety/Biosafety7.pdf. Accessed 8 Jan 2019.

57. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Comparability of biotechnological/biological products subject to changes in their manufacturing process Q5E. Step 4. 2004. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5E/Step4/Q5E_Guideline.pdf. Accessed 8 Jan 2019.

58. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Derivation and characterisation of cell substrates used for production of biotechnological/biological products Q5D. Step 4. 1997. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q5D/Step4/Q5D_Guideline.pdf. Accessed 8 Jan 2019.

59. Recommendations to assure the quality, safety and efficacy of Japanese encephalitis vaccines (live, attenuated) for human use. In: WHO Expert Committee on Biological Standardization, editor. Sixty-third report. Geneva; 2012. (WHO technical report series; No.980, Annex 7). https://www.who.int/biologicals/vaccines/JE-Recommendations_TRS_980_Annex_7.pdf?ua=1. Accessed 8 Jan 2019.

60. 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: World Health Organization; 1998: Annex 3 (WHO Technical Report Series, No. 872; P69-74. http://www.who.int/biologicals/publications/trs/areas/vaccines/sterility/WHO_TRS_872_A3.pdf. Accessed 18 December 2017.

61. World Health Organisation. WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products. 2003. https://www.who.int/immunization_standards/vaccine_regulation/BLOOD_TSE_2003.pdf. Accessed 8 Jan 2019.

62. Requirements for the collection, processing and quality control of blood, blood components and plasma derivatives. In: WHO Expert Committee for Biological Standardization, editor. Forty-third report; 1994. (Technical Report Series; vol. 840; Annex 2). https://www.who.int/bloodproducts/publications/WHO_TRS_840_A2.pdf. Accessed 8 Jan 2019.

63. General requirements for the sterility of biological substances, (Requirements for Biological Substances No. 6, revised 1973). In: WHO Expert Committee on Biological Standardization, editor. Twenty-fifth report. Geneva; 1973. p. 41–61. (Technical Report Series; vol. 872, Annex 4).

64. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Quality of Biotechnological Products: Analysis of the Expression Construct in Cells Used for Production of r-DNA derived Protein Products, Q5B (Adopted by CPMP, December 1995, issued as CPMP/ICH/139/95). Step 5. 1995.

65. Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology. In: WHO Expert Committee for Biological Standardization, editor. Sixty-fourth report; 2014. (Technical Report Series; vol. 987, Annex 4). https://www.who.int/biologicals/biotherapeutics/TRS_987_Annex4.pdf?ua=1. Accessed 8 Jan 2019.

66. Shin J, Lei D, Conrad C, Knezevic I, Wood D. International regulatory requirements for vaccine safety and potency testing: a WHO perspective. Procedia in Vaccinology. 2011;5:164–70.

67. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Viral safety evaluation of biotechnology products products derived from cell lines of human or animal origin, Q5A(R1) (Adopted by CPMP, April 1997, issued as CPMP/ICH/295/95). Step 5. 1995.

68. World Health Organisation. WHO Policy Statement: Multi-dose Vial Policy (MDVP). Handling of multi-dose vaccine vials after opening, WHO/IVB/14.07. Revision 2014. Available from: http://apps.who.int/iris/bitstream/handle/10665/135972/WHO_IVB_14.07_eng.pdf. Accessed 8 Jan 2019.

69. World Health Organisation. WHO manual for the establishment of national and other secondary standards for vaccines. Available from: http://apps.who.int/iris/bitstream/handle/10665/70669/WHO_IVB_11.03_eng.pdf?sequence=1&isAllowed=y.

70. Guidelines on stability evaluation of vaccines. In: World Health Organisation, editor. WHO Expert Committee on Biological Standardization, Fifty-seventh report. Geneva: World Health Organization; 2011. 173-200. (WHO technical report series; vol. 962, Annex 3).

71. Model guidance for the storage and transport of time-and temperature-sensitive pharmaceutical products. In: Organization WH, editor. WHO Expert Committee on Specifications for Pharmaceutical Preparations Forty-fifth Report. Geneva: World Health Organization; 2011. p. 324–72. (WHO technical report series; vol. 961, Annex 9).

72. Guidelines for good clinical practice (GCP) for trials on pharmaceutical products. In: WHO Expert Committee on the Use of Essential Drugs: sixth report. Geneva: World Health Organization; 1995: Annex 3 (WHO Technical Report Series, No. 850; http://apps.who.int/medicinedocs/pdf/whozip13e/whozip13e.pdf, Accessed 8 Jan 2019.)

73. Committee for Medicinal Product for Human Use (CHMP). Guideline on quality, non-clinical and clinical aspects of live recombinant viral vectored vaccines. EMA/CHMP/VWP/141697/2009. https://www.ema.europa.eu/documents/scientific-guideline/guideline-quality-non-clinical-clinical-aspects-live-recombinant-viral-vectored-vaccines_en.pdf. Accessed 8 Jan 2019.

74. EMA. Note for guidance on comparability of medicinal products containing biotechnology-derived proteins as drug substance; nonclinical and clinical issues. (CPMP/3097/02), 2003. https://www.ema.europa.eu/documents/scientific-guideline/guideline-comparability-medicinal-products-containing-biotechnology-derived-proteins-active/bmwp/101695/06_en.pdf. Accessed 8 Jan 2019.

75. WHO. Guidelines on Procedures and Data Requirements for Changes to Approved Vaccines. WHO Expert Committee on Biological Standardization, Sixty-fifth Report - WHO Technical Report Series, No. 993, Annex 4. 2015. http://apps.who.int/medicinedocs/documents/s22021en/s22021en.pdf. Accessed 8 Jan 2019.

76. Shaw CA, Galarneau JR, Bowenkamp KE, Swanson KA, Palmer GA, Palladino G, Markovits JE, Valiante NM, Dormitzer PR, Otten GR. The role of non-viral antigens in the cotton rat model of respiratory syncytial virus vaccine-enhanced disease. Vaccine. 2013 Jan 2;31(2):306-12. doi: 10.1016/j.vaccine.2012.11.006. Epub 2012 Nov 12. https://www.sciencedirect.com/science/article/pii/S0264410X1201599X?via%3Dihub. Accessed 8 Jan 2019.

77. Waris M, Tsou C, Erdman DD, Zaki SR, and Anderson LJ. Respiratory syncytial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. J. Virol. 1996; 70(5): 2852-2860.

78. Prince GA, Curtis SJ, Yim KC and Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with lot 100 or a newly prepared reference vaccine. J General Virol. 2001; 82: 2881-2888.

79. de Waal L, Power UF, Yüksel S, van Amerongen G, Nguyen TN, Niesters HGM, de Swart RL, and Osterhaus ADME. Evaluation of BBG2Na in infant macaques: specific immune responses after vaccination and RSV challenge. Vaccine 2004; 22: 915-922.

80. de Swart RL, Kuiken T, Timmerman HH, et al., Immunization of Macaques with Formalin-Inactivated Respiratory Syncytial Virus (RSV) Induces Interleukin-13-Associated Hypersensitivity to Subsequent RSV Infection. J Virology 2002, Vol. 76, No. 22: 11561-11569. DOI: 10.1128/JVI.76.22.11561–11569.2002. https://jvi.asm.org/content/76/22/11561. Accessed 8 Jan 2019.

81. Gershwin LJ, Schelegle ES, Gunther RA, Anderson ML, Woolums AR, Larochelle DR, Boyle GA, Friebertshauser KE, and Singer RS. A bovine model of vaccine enhanced respiratory syncytial virus pathophysiology. Vaccine 1998; 16: 1225-1236

82. Hosken N, Plikaytis B, Trujillo C, Mahmood K, Higgins D, Participating Laboratories Working G. A multi-laboratory study of diverse RSV neutralization assays indicates feasibility for harmonization with an international standard. Vaccine. 2017;35(23):3082-8.

83. Kulkarni PS, Hurwitz JL, Simoes EAF, Piedra PA. Establishing Correlates of Protection for Vaccine Development: Considerations for the Respiratory Syncytial Virus Vaccine Field. Viral Immunol. 2018;31(2):195-203.

84. Laham FR, Mansbach JM, Piedra PA, Hasegawa K, Sullivan AF, Espinola JA, et al. Clinical Profiles of Respiratory Syncytial Virus Subtypes A AND B Among Children Hospitalized with Bronchiolitis. Pediatr Infect Dis J. 2017;36(8):808-10.

85. Rodriguez-Fernandez R, Tapia LI, Yang CF, Torres JP, Chavez-Bueno S, Garcia C, et al. Respiratory Syncytial Virus Genotypes, Host Immune Profiles, and Disease Severity in Young Children Hospitalized With Bronchiolitis. J Infect Dis. 2017;217(1):24-34.

86. Kim L, Rha B, Abramson JS, Anderson LJ, Byington CL, Chen GL, et al. Identifying Gaps in Respiratory Syncytial Virus Disease Epidemiology in the United States Prior to the Introduction of Vaccines. Clin Infect Dis. 2017;65(6):1020-5.

87. McLellan JS. Neutralizing epitopes on the respiratory syncytial virus fusion glycoprotein. Current Opinion in Virology. 2015(11):70-5.

88. Ngwuta JO, Chen M, Modjarrad K, Joyce MG, Kanekiyo M, Kumar A, et al. Prefusion F-specific antibodies determine the magnitude of RSV neutralizing activity in human sera. Sci Transl Med. 2015;7(309):309ra162.

89. Fries L, Shinde V, Stoddard JJ, Thomas DN, Kpamegan E, Lu H, et al. Immunogenicity and safety of a respiratory syncytial virus fusion protein (RSV F) nanoparticle vaccine in older adults. Immun Ageing. 2017;14:8.

90. Capella C, Chaiwatpongsakorn S, Gorrell E, Risch ZA, Ye F, Mertz SE, et al. Prefusion F, Postfusion F, G Antibodies, and Disease Severity in Infants and Young Children With Acute Respiratory Syncytial Virus Infection. J Infect Dis. 2017;216(11):1398-406.

91. Maifeld SV, Ro B, Mok H, Chu M, Yu L, Yamagata R, et al. Development of Electrochemiluminescent Serology Assays to Measure the Humoral Response to Antigens of Respiratory Syncytial Virus. PLoS One. 2016;11(4):e0153019.

92. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. Nat Med. 2009;15(1):34-41.

93. Green CA, Scarselli E, Sande CJ, Thompson AJ, de Lara CM, Taylor KS, et al. Chimpanzee adenovirus- and MVA-vectored respiratory syncytial virus vaccine is safe and immunogenic in adults. Sci Transl Med. 2015;7(300):300ra126.

94. ICH Harmonised tripartite guideline. Statistical principles for clinical trials, E9. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E9/Step4/E9_Guideline.pdf. Accessed 8 Jan 2019.

95. Modjarrad K, Giersing B, Kaslow DC, Smith PG, Moorthy VS, Group WRVCE. WHO consultation on Respiratory Syncytial Virus Vaccine Development Report from a World Health Organization Meeting held on 23-24 March 2015. Vaccine. 2016;34(2):190-7.

96. Giersing BK, Karron RA, Vekemans J, Kaslow DC, Moorthy VS. Meeting report: WHO consultation on Respiratory Syncytial Virus (RSV) vaccine development, Geneva, 25-26 April 2016. Vaccine. 2017.

97. Munywoki PK, Hamid F, Mutunga M, Welch S, Cane P, Nokes DJ. Improved detection of respiratory viruses in pediatric outpatients with acute respiratory illness by real-time PCR using nasopharyngeal flocked swabs. J Clin Microbiol. 2011;49(9):3365-7.

98. Meerhoff TJ, Fleming D, Smith A, Mosnier A, van Gageldonk-Lafeber AB, Paget WJ, et al. Surveillance recommendations based on an exploratory analysis of respiratory syncytial virus reports derived from the European Influenza Surveillance System. BMC Infect Dis. 2006;6:128.

99. Meerhoff TJ, Houben ML, Coenjaerts FE, Kimpen JL, Hofland RW, Schellevis F, et al. Detection of multiple respiratory pathogens during primary respiratory infection: nasal swab versus nasopharyngeal aspirate using real-time polymerase chain reaction. Eur J Clin Microbiol Infect Dis. 2010;29(4):365-71.

100. Meerhoff TJ, MacKay WG, Meijer A, Paget WJ, Niesters HG, Kimpen JL, et al. The impact of laboratory characteristics on molecular detection of respiratory syncytial virus in a European multicentre quality control study. Clin Microbiol Infect. 2008;14(12):1173-6.

101. Meerhoff TJ, Mosnier A, Schellevis F, Paget WJ, Group ERT. Progress in the surveillance of respiratory syncytial virus (RSV) in Europe: 2001-2008. Euro Surveill. 2009;14(40).

102. Guidelines for national authorities on quality assurance for biological products. In: WHO Expert Committee on Biological Standardization: forty-second report. Geneva: World Health Organization; 1992: Annex 2 (WHO Technical Report Series, No. 822; http://www.who.int/biologicals/publications/trs/areas/biological_products/WHO_TRS_822_A2.pdf. accessed 17 May 2018).

103. Guidelines for independent lot release of vaccines by regulatory authorities. In: WHO Expert Committee on Biological Standardization: sixty-first report. Geneva: World Health Organization; 2013: Annex 2 (WHO Technical Report Series, No. 978; http://www.who.int/biologicals/TRS_978_Annex_2.pdf?ua=1, accessed 17 May 2018).

Appendix 1. Model summary protocol for the manufacturing and control of human RSV vaccines

The following provisional protocol is intended for guidance. It indicates the information that should be provided as a minimum by the manufacturer to the NRA after the vaccine product has been granted a marketing authorization. The protocol is not intended to apply to material intended for clinical trials.

Since the development of these vaccines is incomplete at the time of writing this document, detailed requirements are not yet finalized. Consequently only the essential requirements are provided in this appendix. Information and tests may be added or omitted (if adequate justification is provided) as necessary to be in line with the marketing authorization approved by the NRA. It is therefore possible that a protocol for a specific product will differ from the model provided here. The essential point is that all relevant details demonstrating compliance with the license and with the relevant WHO Guidelines on a particular product should be given in the protocol submitted.

The section concerning the final product should be accompanied by a sample of the label and a copy of the leaflet that accompanies the vaccine container. If the protocol is submitted in support of a request to permit importation, it should also be accompanied by a Lot Release Certificate from the NRA of the country in which the vaccine was produced and/or released stating that the product meets national requirements as well as Part A of these WHO Guidelines.

1. Summary information on finished product (final vaccine lot)

- International name:
- Trade name/commercial name:
- Product licence (marketing authorization) number:
- Country:
- Name and address of manufacturer:
- Name and address of product licence-holder, if different:
- Vector(s) (if applicable):
- RSV virus strain(s) (if applicable):
- Batch number(s):
- Type of container:
- Number of filled containers in this final lot:
- Number of doses per container:
- Preservative and nominal concentration (if applicable):
- Summary of the composition (summary of qualitative and quantitative composition of the vaccine, including any adjuvant and other excipients):
- Target group:
- Shelf life approved (months):
- Expiry date:

- Storage conditions:

2. Control of source material

2.1 Virus and viral/bacterial vector seeds (where applicable)

2.1.1 Seed banking system

- Name and identification of virus or viral/bacterial vector:
- Origin of all genetic components (if applicable):
- Construction of virus or viral/bacterial vector:
- Nucleotide sequence of the transgene and flanking regions:
- Antigenic analysis, infectivity titre, yield (in vitro/in vivo):
- Comparison of genetic and phenotypic properties with parental vector:
- Seed bank genealogy with dates of preparation, passage number and date of coming into operation:
- Tests performed for detection of adventitious agents at all stages of development:
- Tests for bacteria, fungi, mycoplasm, mycobacteria (for virus and viral vector seeds):
- Virus titration for infectivity (for live-attenuated RSV vaccines)
- Freedom from TSE agents:
- Details of animal or human components of any reagents used in the manufacture of seed banks, including culture medium:
- Genetic stability at the level of a virus/bacterial pre-master seed or viral/bacterial master seed to its sequence at, or preferably beyond, the anticipated maximum passage level:
- Confirmation of approval for use by manufacturer, and the basis for that approval:

2.1.2 Tests on working seed lot production (for chimeric BCG/RSV-vaccines)

- Antimicrobial sensitivity:
- Delayed hypersensitivity (if applicable):
- Identity:
- Bacterial and fungal contamination:
- Absence of virulent mycobacteria:
- Excessive dermal reactivity (if applicable):

2.2 Cell cultures (where applicable)

2.2.1 Cell banking system

- Name and identification of cell substrate:
- Origin and history of cell substrate:
- Details of any manipulations (including genetic manipulations) performed on the parental cell line in the preparation of the production cell line:
- Cell bank genealogy with dates of preparation, passage number and date of coming into operation:
- Confirmation of approval for use by manufacturer, and the basis for that approval:
- Tests performed for detection of adventitious agents at all stages of development:

- Test for absence of bacterial and fungal contamination (if of yeast and bacterial origin):
- Sterility test (bacteria, fungi, mycoplasmas, virus):
- Details of animal or human components of any reagents used in manufacture of cell banks, including culture medium:
- Freedom from TSE agents:
- Genetic stability (if genetically manipulated):

2.2.2 Primary cells (if generated)

- Source of animals and veterinary control (for example, specify if animals or eggs are sourced from closed, pathogen-free colonies):
- Name, species and identification of primary cell batches:
- Details of animal or human components of any reagents used in manufacture of cells:
- Methods of isolation of the cells:
- Tests performed for detection of adventitious agents during manufacture (may be performed on control cells if necessary):
- Freedom from TSE agents:

3. Control of vaccine production

3.1 Control of production cell cultures/control cells (where applicable)

3.1.1 Information on preparation

- Lot number of master cell bank:
- Lot number of working cell bank:
- Date of thawing ampoule of working cell bank:
- Passage number of production cells:
- Date of preparation of control cell cultures:
- Result of microscopic examination:

3.1.2 Tests on cell cultures or control cells

- Identity:
- Haemadsorbing viruses:
- Adventitious agents:
- Sterility (bacteria, fungi, mycoplasmas):

3.2 Control of purified antigen bulk (where applicable)

- Identity:
- Purity:
- Protein content:
- Antigen content:
- Sterility (bacteria and fungi):
- Percentage of intact RSV antigens:
- Nanoparticle size and structure:

- Reagents during production of other phases of manufacture:
- Residual DNA derived from the expression system (if applicable):
- Residual bovine serum antigen content:
- Viral clearance (during manufacturing development):

3.3 Control of adsorbed antigen bulk (where applicable)

- Lot number of adsorbed antigen bulk:
- Date of adsorption:
- Volume, storage temperature, storage time and approved storage period:
- Sterility (bacteria and fungi)
- Bacterial endotoxin:
- Identity:
- Adjuvant:
- Degree of adsorption:
- pH:
- Antigen content:

3.4 Control of virus and viral/bacterial vector harvests or pooled harvests (where applicable)

3.4.1 Information on manufacture

- Batch number(s):
- Date of inoculation:
- Date of harvesting:
- Lot number of virus/bacterial master seed lot:
- Lot number of virus/bacterial working seed lot:
- Passage level from virus/bacterial working seed lot:
- Methods, date of purification if relevant:
- Volume(s), storage temperature, storage time and approved storage period:

3.4.2 Tests

- Identity:
- Sterility (bacteria, fungi, mycoplasmas and mycobacteria) (if applicable):
- Adventitious virus tests:
- Bacteria/fungi/mycoplasmas (for recombinant BCG/RSV vaccines):
- Virus titration for infectivity (if applicable):
- Residual bovine serum albumin (if applicable):
- Tests for consistency of virus characteristics (if applicable):
- Determination of attenuation (if appropriate):

3.5 Control of monovalent virus/viral vector bulk (where applicable)

3.5.1 Information on manufacture

- Batch number(s):
- Date of formulation:
- Total volume of monovalent bulk formulated:

- Virus pools used for formulation:
- Lot number/volume added:
- Virus concentration:
- Name and concentration of added substances (for example, diluent,
- stabilizer if relevant):
- Volume(s), storage temperature, storage time and approved storage
- period:

3.5.2 Tests

- Identity:
- Purity:
- Residual HCP:
- Residual HC DNA (if non-primary cell lines):
- Potency:
  - Particle number (if relevant e.g. for adenovirus):
  - Infectious virus titre and particle-to-infectivity ratio (if relevant e.g. for adenovirus):
  - Expression of heterologous antigen in vitro:
- Replication competence (if relevant e.g. for adenovirus):
- pH:
- Preservative content (if applicable):
- Endotoxin:
- Sterility or bioburden:

3.6 Control of final virus or viral/bacterial vector bulk (where applicable)

3.6.1 Information on manufacture

- Batch number(s):
- Date of formulation:
- Total volume of final bulk formulated:
- Monovalent virus pools used for formulation:
- Volume(s), storage temperature, storage time and approved storage period:
- Lot number/volume added:
- Virus concentration:
- Name and concentration of added substances (for example, diluent, stabilizer if relevant):

3.6.2 Tests on virus or viral vector bulk

- Identity (if applicable):
- Sterility or bioburden (if applicable):
- Concentration of antimicrobial agent, if relevant:
- Total protein:
- Residual DNA (for cell-culture vaccine):
- Ovalbumin (for egg-based vaccine):

3.6.3 Tests on bacterial vector bulk

- Bacterial and fungal contamination:
- Absence of virulent mycobacteria (if not performed on final lot):
- Bacterial concentration:
- Number of culturable particles:

4. Filling and containers

- Lot number:
- Date of filling:
- Type of container:
- Volume of final bulk filled:
- Filling volume per container:
- Number of containers filled (gross):
- Number of containers rejected during inspection:
- Number of containers sampled:
- Total number of containers (net):
- Maximum period of storage approved:
- Storage temperature and period:

5. Control tests on final vaccine lot

- Inspection of final containers:
- Identity:
- Appearance (if applicable):
- pH (if applicable):
- Osmolality (if applicable):
- Sterility (if applicable):
- Bacterial and fungal contamination (for chimeric BCG/RSV vaccines):
- Preservative (if applicable):
- Residual moisture content (for freeze-dried product):
- Reconstitution time (for freeze-dried product):
- Pyrogenic substances (if applicable):
- Adjuvant content (if applicable):
- Protein content (if applicable):
- Degree of adsorption (if applicable)
- Potency:
- Infectivity (if appropriate):
- Purity (if applicable):
- Bacterial concentration (for chimeric BCG/RSV vaccines):
- Tests for viability (for chimeric BCG/RSV vaccines):
- Extractable volume (if applicable):
- Thermal stability test (if applicable):
- Residual antibiotics (if relevant):

- Diluent (if applicable):
- Safety (for chimeric BCG/RSV vaccines):

6. Certification by the manufacturer

Name of Head of Production (typed)_____

Certification by the person from the control laboratory of the manufacturing company taking overall responsibility for the production and control of the vaccine.

I certify that lot no. _____of RSV vaccine, whose number appears on the label of the final containers, meets all national requirements and satisfies Part A[1] of the *WHO Guidelines on the quality, safety and efficacy of RSV vaccines*[2] (if applicable).

Name (typed)_____

Signature_____

Date_____

7. Certification by the NRA

If the vaccine is to be exported, attach the NRA Lot Release Certificate (as shown in Appendix 2), a label from a final container and an instruction leaflet for users.

[1] With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
[2] WHO Technical Report Series, No. 1011, Annex 4.

Appendix 2. Model lot release certificate for human RSV vaccines by NRAs

Certificate no.

This certificate is to be provided by the NRA of the country where the vaccine has been manufactured, on request by the manufacturer.

The following lot(s) of RSV vaccine produced by_____[3] in _____ [4] whose lot numbers appear on the labels of the final containers, complies with the relevant specification in the marketing authorization and provisions for the release of biological products[5] and Part A[6] of the *WHO Guidelines on the quality, safety and efficacy of human Respiratory Syncytial Virus vaccines*[7] and comply with *WHO good manufacturing practices for pharmaceutical products: main principles*[8], *WHO good manufacturing practices for biological products*[9], and *Guidelines for independent lot release of vaccines by regulatory authorities*[10].

The release decision is based on _____[11]

The certificate may include the following information:

- name and address of manufacturer;
- site(s) of manufacturing;
- trade name and common name of product;
- marketing authorization number;
- lot number(s) (including sub-lot numbers and packaging lot numbers if necessary);
- type of container used;
- number of doses per container;
- number of containers or lot size;
- date of start of period of validity (for example, manufacturing date) and expiry date;
- storage conditions;
- signature and function of the person authorized to issue the certificate;
- date of issue of certificate;
- certificate number.

The Director of the NRA (or other authority as appropriate):

---

[3] Name of manufacturer.
[4] Country of origin.
[5] If any national requirements are not met, specify which one(s) and indicate why release of the lots has nevertheless been authorized by the NRA.
[6] With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.
[7] WHO Technical Report Series, *to be determined*.
[8] WHO Technical Report Series, No. 986, Annex 2.
[9] WHO Technical Report Series, No. 999, Annex 2.
[10] WHO Technical Report Series, No. 978, Annex 2.
[11] Evaluation of product-specific summary protocol, independent laboratory testing, and/or specific procedures laid down in a defined document, etc., as appropriate.

Name (typed) _____

Signature _____

Date _____