

Annex 2

Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines

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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 respiratory syncytial virus (RSV) vaccines. 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

| | |
|--------------------|--|
| bRSV | bovine respiratory syncytial virus |
| BSA | bovine serum albumin |
| CCID ₅₀ | cell culture infectious dose 50% |
| EIA | enzyme immunoassay |
| EOP | end of production (cells) |
| ERA | environmental risk assessment |
| ERD | enhanced respiratory disease |
| FI-RSV | formalin inactivated RSV |
| GMO | genetically modified organism |
| GMP | good manufacturing practice(s) |
| HPLC | high-performance liquid chromatography |
| ICP | immune correlate of protection |
| IgG | immunoglobulin G |
| LRTI | lower respiratory tract infection(s) |
| MCB | master cell bank |
| MOI | multiplicity of infection |
| MS | master seed |
| MVA | modified vaccinia Ankara |
| NAT | nucleic acid amplification technique |
| NRA | national regulatory authority |
| NP | nasopharyngeal (swab or aspirate) |
| NS | nasal swab |
| NW | nasal wash (aspirate) |
| PCR | polymerase chain reaction |
| PFU | plaque-forming unit(s) |
| qPCR | quantitative polymerase chain reaction |
| RDT | rapid diagnostic test |
| RSV | respiratory syncytial virus |

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|-----------------|--|
| RSV-F | respiratory syncytial virus fusion protein F |
| RSV-G | respiratory syncytial virus attachment protein G |
| RT-PCR | reverse transcription polymerase chain reaction |
| Th1 | type 1 T-helper (cell) |
| Th2 | type 2 T-helper (cell) |
| T _{RM} | resident memory T-cells |
| URTI | upper respiratory tract infection(s) |
| WS | working seed |
| WCB | working cell bank |

Introduction

Human respiratory syncytial virus (RSV) is a globally prevalent cause of lower respiratory tract infection (LRTI) in all age groups. In infants and young children the first infection may cause severe bronchiolitis that can sometimes be fatal. In older children and adults without comorbidities, repeated upper respiratory tract infections (URTIs) are common and range from subclinical infection to symptomatic upper respiratory tract disease.

In addition to the paediatric burden of disease, RSV is increasingly being recognized as an important pathogen in older adults, with infection leading to an increase in hospitalization rates among those aged 65 years and over, and to increased mortality rates among the frail elderly that approach the rates seen with influenza. The risk of severe disease in adults is increased by the presence of underlying chronic pulmonary disease, circulatory conditions and functional disability, and is associated with higher viral loads (1–6). RSV is also a nosocomial threat both to young infants and among immunocompromised and vulnerable individuals (7). High mortality rates have been observed in those infected with RSV following bone marrow or lung transplantation.

In the absence of safe and effective antiviral agents to treat RSV infection there is an unmet need for RSV vaccines. In recent years, increased understanding of the biology of RSV and associated technological advances have resulted in the entry of multiple candidate vaccines into clinical development, some of which may receive regulatory approval in the near future. The WHO Product Development for Vaccines Advisory Committee has highlighted the importance of ensuring that emerging RSV vaccines are suitable for licensure (8, 9) and meet policy decision-making needs to allow for their optimal use in low- and middle-income countries, in addition to high-income countries. A corresponding WHO roadmap has also been published (10).

There is therefore a recognized need for harmonized technical expectations to guide and facilitate the international development and assessment of candidate RSV vaccines. In response to this need, 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 (11, 12). Following this process of consultation, WHO brought together a group of experts to prepare draft WHO Guidelines on the quality, safety and efficacy of human RSV vaccines. In September 2018, WHO organized the first of a series of informal expert consultations attended by a wide range of stakeholders to further develop and refine the draft document. Inputs were also received from several rounds of public consultation following the posting of the draft document on the WHO Biologicals website during the course of 2018–2019. In May 2019, WHO organized a second informal consultation attended by experts and stakeholder representatives to review the

latest draft of the Guidelines and to propose further improvements prior to the submission of the Guidelines to the WHO Expert Committee on Biological Standardization.

The resulting current document has therefore been developed based on the experience gained to date in RSV vaccine development and on the contributions and outcomes of the international consultations described above. Unless otherwise specified, these WHO Guidelines are concerned only with human RSV strains and human RSV vaccines. The information provided may need to be updated as new data become available and as vaccines are licensed. The document therefore provides information and guidance on the production, quality control, nonclinical and clinical evaluation of candidate human RSV vaccines in the form of WHO Guidelines rather than WHO Recommendations as this format will allow for greater flexibility in response to future developments.

Purpose and scope

These WHO Guidelines provide guidance to national regulatory authorities (NRAs) and vaccine manufacturers on the manufacturing processes and nonclinical and clinical evaluation of human RSV vaccines required to assure their quality, safety and efficacy. The scope of the present document encompasses the leading technologies currently being used to develop prophylactic RSV vaccines at the clinical development stage (13). These include live-attenuated vaccines (including those based on genetically modified organisms (GMOs) such as chimeric virus vaccines), vaccines produced using recombinant viral and other vectored systems, and protein-based vaccines (including subunit and nanoparticle formulations with and without adjuvants). Some principles contained herein may also be applicable to vaccines manufactured using other platforms.

Despite possible overlaps, the quality, safety and clinical testing of RSV monoclonal antibody products involves a number of unique considerations and separate guidance will be needed with a specific focus on these products.

This document should be read in conjunction with other relevant WHO guidance, especially on the nonclinical (14, 15) and clinical (16) evaluation of vaccines, as well as relevant documents on the minimum requirements for an effective national pharmacovigilance system (17). Other WHO guidance should also be consulted as appropriate, including 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 (18). In addition, despite covering a number of different technology platforms, the current document is limited in its scope and other WHO guidance documents should be consulted as relevant. This may include WHO guidance relevant to the manufacture of biologicals using pathogen-free embryonated eggs (19) if human RSV vaccines were to be produced in this way, or guidance on the

manufacture, quality control and release of bacille Calmette-Guérin (BCG) vaccines (20) 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 human RSV vaccines.

Terminology

The definitions given below apply to the terms as used in these WHO Guidelines. These terms may have different meanings in other contexts.

Adjuvant: a substance or 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, mycoplasmas/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 that is at the research and clinical development stage, and that 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 bank system: a system that consists of cell banks of defined population doubling or passage levels that generally include the **master cell bank** (MCB) derived from a cell seed and a **working cell bank** (WCB) derived from the MCB.

Cell culture infectious dose 50% (CCID₅₀): 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 to manufacture a biological product. The cells may be primary cells or continuous cell lines and may be grown in monolayer or suspension culture conditions.

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

Chimeric RSV vaccine: a live-attenuated recombinant RSV vaccine expressing one or more RSV proteins in the context of the replication of viral or bacterial vectors. Examples of such vectors include, but are not limited to, Sendai virus, parainfluenza virus, bovine RSV, measles virus and BCG.

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 and sealed container-closure system that contains a **drug substance** typically formulated with excipients and prepared in the final dosage form and packaged for use. The collection of all vials of the drug product resulting from one working session constitutes the **final lot**.

Drug substance: the active pharmaceutical ingredient and associated molecules.

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.

Expression construct: a vector (plasmid or virus) capable of promoting the expression of the coding sequence(s) of recombinant protein(s) after introduction into host cells.

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 be bacterial-cell-based, baculovirus-insect-cell-based, mammalian-cell-based or yeast-cell-based.

Final bulk: a formulated vaccine preparation from which the final containers are filled. The final bulk may be prepared from one or more lots of purified **drug substance** formulated to contain all excipients and homogeneous with respect to composition.

Final lot: a collection of sealed final containers of the **drug product** that is homogeneous with respect to the risk of contamination during filling and freeze-drying. All final containers must, therefore, have been filled from a single vessel of final bulk in one working session, and if freeze-dried, processed under standardized conditions in a common chamber in one working session.

Formalin-inactivated respiratory syncytial virus (FI-RSV) vaccine: a formalin-inactivated whole-virion respiratory syncytial virus vaccine manufactured using the Bernett strain of RSV grown in African green monkey kidney cell cultures. Historically, the alum-adjuvanted product was causally related to vaccine-associated enhanced respiratory disease (ERD) noted in

vaccinated infants upon subsequent exposure to RSV during clinical trials conducted in the 1960s.

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 and used to prepare the vaccine. The material may be culture supernatant, cells (which are often disrupted) or some combination of the two.

Heterologous gene: (a) in the context of an **expression construct** this term refers to the transgene from the disease-causing organism that is integrated into the backbone genomic sequence of the vector; (b) in the context of genes derived from RSV subtype A or B strains this term may be used to refer to the gene associated with the other subtype as in: “the gene for RSV-G_A was expressed as well as the heterologous gene for subtype B viruses, RSV-G_B”.

Immune correlate of protection (ICP): 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 (16).

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

Infant: a child less than one year old.

Live-attenuated RSV vaccine: a vaccine 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 complementary DNA (cDNA). Such a 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 vapour or liquid phase of liquid nitrogen) in aliquots of uniform composition (18).

Master seed (MS); see also **seed lot system** below: 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 (WS)** is derived.

Monovalent bulk vaccine: a quantity of vaccine derived either using a single harvest or using material pooled from one or more harvests and processed in a single production run.

Monovalent vaccine: a vaccine containing antigen or gene(s) encoding antigen derived from a single RSV strain or subtype.

Multivalent vaccine: a vaccine containing antigens or genes encoding antigens derived from more than one RSV strain or subtype.

Nanoparticle vaccine: a vaccine which contains or is manufactured using material(s) in the nanoscale range (1–100 nm) or engineered to have properties related to its structure or dimensions ranging in size from 1 to 1000 nm (21). Some nano-sized vaccines consist of self-assembled proteins or self-assembled virus-like particles. Others are prepared using technologies such as synthetic polymers, inorganic materials, liposomes or immunostimulating complexes, which are assembled with the respective antigen or antigens.

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

Particle-based or subunit RSV vaccine: an RSV vaccine that only contains certain antigens or subunits of RSV. Such vaccines may be produced by different **expression systems**.

Plaque-forming unit (PFU): the smallest amount of virus sufficient to lyse host cells and cause a single visible focus of infection in a cell culture monolayer after proper staining of cells.

Platform technology: a standard method used for the manufacture of vaccines based on the use of heterologous gene inserts for different proteins either in an identical vector backbone or expressed from a recombinant cell line.

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

Pre-master seed; see also **seed lot system** below: a single pool of virus or viral/or bacterial-vectored particles of defined passage from which the **master seed** is derived.

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.

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

RSV-naive: denoting subjects who have not yet been exposed to RSV antigen by infection or vaccination.

RSV-non naive/experienced: denoting 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 etiological agent (for example, the subject had a positive culture, antigen detection or reverse transcription polymerase chain reaction (RT-PCR) test for RSV in the context of an RSV illness). Alternatively, or in addition, subjects may have immunological evidence

of prior RSV infection. In infants with persisting maternal antibody, evidence of prior infection may be based on finding RSV-specific immunoglobulin A (IgA) or immunoglobulin M (IgM) in serum or secretions, or evidence of immune memory (for example, a B-cell or T-cell memory response detected by ELISPOT). However, these serology tests may lack sensitivity and, if used alone, may underestimate the RSV-exposed population (22). In general, passively acquired maternal anti-RSV neutralizing or immunoglobulin G (IgG) binding 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.

Seed lot system: a system in which successive batches of vaccine are derived from the same **master seed** (MS) lot at a given passage level. For routine production, a **working seed** (WS) lot is prepared from the MS lot. The final product is derived from the WS lot and has not undergone more passages from the MS lot than the vaccine shown to be safe and effective in clinical studies. The seed lot system is usually based on the use of a **pre-master seed**, MS and WS.

Single harvest: a quantity or suspension derived from a batch of production cells inoculated with the same seed lot and processed together in a single production run.

TSE-relevant animal species: animals such as cattle, sheep, goats and other animals naturally susceptible to infection with transmissible spongiform encephalopathy (TSE) agents via the oral route.

Vaccine-associated enhanced respiratory disease (ERD): severe lower respiratory tract disease following infection with wild-type RSV that occurs at a higher frequency in infants and children following immunization.

Vaccine efficacy: a measure of the 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 $[(ARU - ARV)/ARU] \times 100$ or $[1 - RR] \times 100$. This estimate may be referred to as absolute vaccine efficacy.

Viral clearance: an evaluation of the manufacturing process to determine and measure the effects of removal of virus particles and/or reduction of their infectivity through inactivation.

Viral-vectored RSV vaccine: a recombinant replication-deficient or conditionally replicating RSV vaccine that uses viral expression systems such as adenovirus or modified vaccinia Ankara (MVA) to express one or more antigens of RSV.

Working cell bank (WCB): a quantity of well-characterized cells of animal or other origin, derived from the MCB, dispensed into multiple containers, cryopreserved and stored frozen under defined conditions (such as in the vapour or liquid phase of liquid nitrogen) in aliquots. One or more of the WCB containers is used for each production culture.

Working seed (WS); see also **seed lot system** above: for routine production, a WS lot is prepared from the MS 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 WS lot and has not undergone more passages from the virus MS lot than the vaccine shown to be safe and effective in clinical studies. In the case of bacterial-vectored vaccines, a bacterial WS is derived from the bacterial MS.

General considerations

Respiratory syncytial virus (RSV)

RSV belongs to the genus *Orthopneumovirus* within the family Pneumoviridae and order Mononegavirales. Members of this genus include human RSV, bovine RSV (bRSV) and murine pneumonia virus. The RSV virion consists of a nucleocapsid packaged in a lipo-protein envelope derived from the host cell plasma membrane (23). RSV has a single-stranded, non-segmented negative-sense RNA genome consisting of between 15 191 and 15 288 nucleotides (23, 24).

The RSV envelope contains three viral transmembrane surface glycoproteins: the putative attachment glycoprotein G (RSV-G), the fusion glycoprotein F (RSV-F) and the small hydrophobic glycoprotein (RSV-SH). The non-glycosylated matrix M protein is present on the inner face of the envelope. RSV-F and RSV-G are the major targets of neutralizing antibodies and are the major protective antigens (23). The 574 amino acid RSV-F is a class I fusion protein that is cleaved into F2 and F1 fragments that form a trimer of heterodimers that mediates viral entry and syncytium formation. RSV-F on the virion surface exists in a metastable pre-fusion conformation that transitions to a stable post-fusion conformation spontaneously and during membrane fusion. There are at least five defined antigenic sites associated with neutralization on RSV-F. The 300 amino acid RSV-G is thought to form oligomers but whether dimeric or tetrameric forms are the dominant structures on the virus is not known; a monomeric secreted form of this protein is involved in immunomodulation and potentially acts as a decoy antigen that helps RSV evade host immunity (23). RSV-G can interact with CX3CR1 and other proteins but it is not required for virus entry and propagation in vitro and so its precise functional role in cell attachment is still the subject of debate. RSV-G is heavily glycosylated and has mucin-like domains on each end of the molecule surrounding a central conserved domain

that is a target for neutralizing antibodies. The most extensive genetic diversity is found in the mucin domains of RSV-G (23, 25). The 65 amino acid RSV-SH is a pentameric ion channel and is analogous to the M2 protein in influenza viruses. Although it is not a target for neutralizing antibodies, anti-SH-specific antibodies can protect through antibody-Fc-mediated mechanisms (26).

There are two major antigenic subtypes of human RSV (RSV/A and RSV/B) determined largely by antigenic drift and duplications in RSV-G sequences, but accompanied by genome-wide sequence divergence, including within RSV-F (25, 27–29).

Epidemiology

Human RSV is a leading cause of respiratory disease globally. The virus causes infections at all ages. Young infants, including healthy full-term infants as well as those born prematurely, and those with chronic lung disease and congenital heart defects, have the highest incidence of severe disease, peaking at 1–3 months of age. By 2 years of age, almost 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 in young children is estimated to be as high as 150 000 per annum (30). In addition to the toll associated with acute RSV infection, the burden attributed to chronic disease (such as recurrent wheezing and asthma later in childhood) may be quite high. It is not precisely known if there is a direct causal relationship between early and severe RSV infection in infancy with asthma later in life or if symptomatic LRTI with RSV simply identifies those who are genetically predisposed to wheezing and/or asthma. Numerous factors may contribute to, and be involved in, the association between RSV bronchiolitis and wheezing illnesses later in childhood (31). In one multi-centre, randomized, placebo-controlled double-blind study it was demonstrated that monthly treatment of preterm infants with anti-RSV-F monoclonal antibody (palivizumab) during the RSV season decreased the number of parent-reported wheezing days and episodes during the first year of life even after treatment ended when compared to the number of days and episodes reported for the control group (32).

RSV infection does not elicit long-lasting sterilizing immunity and repeated URTI are common throughout life. Infections in adults can range from asymptomatic to life threatening, with severe infections more common in adults > 65 years of age and in those with underlying heart and lung problems (33). RSV transmission follows a marked seasonal pattern in temperate areas (with winter epidemics) but may occur during rainy seasons or all year round in the tropics (34–37).

The two major RSV subtypes (RSV/A and RSV/B) and multiple genotypes of each can either dominate or co-circulate during RSV epidemic seasons each year. The association between disease severity and a specific RSV subtype or genotype is variable with no consistent pattern having yet been discerned (38).

Disease and diagnosis

The incubation period for RSV is usually 3–6 days (ranging from 2 to 8 days). The virus typically enters the body through the eye or nose, or rarely through the mouth. The virus then spreads along the epithelium of the respiratory tract, primarily by cell-to-cell transfer. As the virus spreads to the lower respiratory tract it may produce bronchiolitis and/or pneumonia. Primary infections are often symptomatic and can range from mild URTI 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 3–4 days after the onset of lower respiratory tract disease (39). RSV infection also occurs in adults where it is often a mild upper respiratory tract illness. However, in adults – particularly those > 65 years of age and those with comorbidities such as congestive heart failure or chronic obstructive pulmonary disease or immunocompromised individuals – severe illness may result (40–42). Fever and constitutional symptoms are less common than they are in influenza infection. Upper respiratory illness progresses over several days to lower respiratory symptoms of cough, new or increased sputum production, wheezing and shortness of breath. Abnormal breath sounds and/or radiographic pneumonia occur in 25–30% of cases. In patients with comorbidities, mortality ranges from 6.5% to 10% (43, 44).

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 this requires both acute and convalescent serum samples, serological diagnosis is not immediate.

Immune response to natural RSV infection

Innate and adaptive immune responses can 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. Providing 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. Although a detailed account of all the many known parameters is beyond the scope of these Guidelines a number of in-depth reviews are available (45–49). In addition, a number of immune responses associated with protection or

potential pathology following RSV infection are discussed briefly below. These include: (a) virus neutralizing antibodies; (b) IgG and IgA antibodies in serum and on mucosal surfaces (including epitope-specific IgG responses); and (c) cell-mediated immunity involving RSV-specific CD8+ cytotoxic T-cell and CD4+ T-helper cell responses.

While there is no established immune correlate of protection (ICP), 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. This finding is based on the results of studies involving passively administered polyclonal or monoclonal antibodies, and on the clinical trials that led to the licensure of the monoclonal antibody palivizumab. The majority of neutralizing activity elicited in response to natural RSV infection in most individuals is directed against antigenic sites found exclusively on the pre-fusion conformation of RSV-F. There are antigenic sites on the post-fusion conformation of RSV-F that are shared with sites on the pre-fusion conformation and a smaller fraction of neutralizing activity is directed against those shared sites and against RSV-G (50, 51). This can vary between individuals and can be influenced by the way in which neutralization is measured. For example, using immortalized cells (Vero and HEp-2) to measure virus neutralization in vitro may underestimate the contribution made by anti-RSV-G antibodies in blocking virus attachment to cells mediated by RSV-G binding to its cognate receptor CX3CR1. In contrast, using primary human airway epithelial cells that express CX3CR1 but relatively low amounts of heparan sulfate on apical surfaces may provide a more sensitive and biologically relevant in vitro system for detecting anti-RSV-G-specific neutralizing antibodies. Some antibodies that bind specific epitopes present on the pre-fusion RSV-F trimer exhibit highly potent neutralizing activity relative to activity seen with antibodies directed against the shared epitopes retained on 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, some anti-RSV-F antibodies bind epitopes and neutralize RSV in a subtype-specific manner (52, 53).

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 (54–56). 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 domains of this protein. RSV-G can bind glycosaminoglycans and C-type lectins and these interactions may facilitate virus infection and/or alter dendritic cell signaling (57).

Mucosal anti-RSV IgA antibodies have been demonstrated to correlate with protection against experimental challenge with wild-type RSV in adults (58, 59).

While antibodies may prevent RSV infection, cytotoxic CD8+ T-cells are involved in the clearance of virus-infected cells based on studies in animals and in immunocompromised individuals (60–62). Cytotoxic CD8+ T-cells may be elicited following natural RSV infection or following immunization if antigenic peptides are expressed in context with major histocompatibility complex class I proteins. CD8+ T-cells have been detected in bronchial alveolar lavage fluids and in the peripheral blood of infants and children following RSV infection (63, 64). Resident memory CD8+ T-cells (T_{RM}) with a CD3+ CD8+ CD103+ CD69+ phenotype have been recovered from lower airways using bronchoscopy in adults immediately following experimental RSV challenge, identified by re-stimulation with synthetic peptides representing sequences from RSV-N, -M and -NS2 proteins and confirmed using tetramer staining (60). In this study, T_{RM} were also detected in the peripheral blood of adults 10 days after RSV challenge but at a lower frequency than those recovered by bronchoscopy. The presence of CD8+ T_{RM} in adult lungs after RSV challenge was associated with reduced respiratory symptoms and lower viral loads (60).

CD4+ T-helper and T-regulatory (T_{REG}) cells modulate B and/or T-cell function. CD4+ T-helper cells in infants under 6 months of age are epigenetically programmed to have a dominant type 2 T-helper (Th2) cell cytokine response that may be antigen specific (65, 66). CD4+ Th2 cell responses are associated with cytokines that can lead to allergic inflammation. Such responses have been associated with severe disease in RSV-infected infants in some studies – suggesting that a dominant Th2 cell cytokine response following RSV exposure is not desirable in young infants (67). This is supported by the finding that genetic polymorphisms associated with clinically severe RSV disease are located in cytokine and cytokine receptor genes associated with Th2 cell responses (49). It has been suggested that cytokine responses during infancy may be skewed in favour of Th2 cell responses as a result of the down-regulation of type 1 T-helper (Th1) cell responses mediated by anti-inflammatory cytokines such as IL-10 (68). In the study involved, a specific subset of neonatal regulatory B (nBreg) cells produced anti-inflammatory IL-10 when infected with RSV via the B-cell receptor 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 (68).

History of RSV vaccine development

RSV vaccine development began in the 1960s with an unsuccessful formalin-inactivated RSV (FI-RSV) vaccine (69) that induced a severe – and in two cases lethal – lung inflammatory response during the first natural RSV infection after

vaccination of RSV-naive infants. This response to natural RSV infection has been referred to as vaccine-associated enhanced respiratory disease (ERD). The concerns over the FI-RSV vaccine hindered the development of alternative RSV vaccines for many years. Two major characteristics of vaccine-associated ERD have been defined and can be summarized as follows:

- Firstly, serological analyses of sera from the youngest infants with the most severe disease showed that these vaccinees had exhibited good induction of anti-RSV binding antibodies (as determined by complement fixation and ELISA) but weak induction of antibodies with neutralizing and fusion-inhibiting activities (70–74). Tissue sections from the lungs of the two vaccinees who died of RSV infection showed evidence of immune complex deposition and complement activation in small airways (75). These data suggest that weakly neutralizing antibodies induced by the FI-RSV vaccine left these infants vulnerable to infection and may have contributed to the risk of severe disease in vaccinees subsequently infected with RSV.
- Secondly, an allergic inflammation characterized by Th2-biased CD4+ T-helper cells producing IL-4, IL-5 and IL-13 associated with pulmonary eosinophilia, mucus production and neutrophilic alveolitis has been observed to various degrees in mice, cotton rats, calves and non-human primates immunized with FI-RSV or a similarly prepared antigen prior to challenge. The lung histopathology seen in the infants who died during the original FI-RSV vaccine trial showed similar neutrophilic alveolitis and pulmonary eosinophils in peribronchiolar infiltrates, suggesting that an overly exuberant allergic inflammatory response to the vaccine contributed to the complications seen thereafter.

However, a number of candidate vaccines have been proposed and evaluated over the last decade; some with promising results (76–80), and a number of observations have supported the feasibility of vaccination against RSV (81, 82). 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 of these in Phase III clinical efficacy trials (13). The vaccine construct and/or safety profile generated during nonclinical testing may help to determine acceptability for specific target populations (see Part B below).

Understandably, prior experience with FI-RSV vaccine dictates the cautious approach that has been taken in vaccine development, especially regarding candidate vaccines designed to elicit active immunity in RSV-naive infants. It is widely recognized that safety data derived from clinical testing in RSV-experienced individuals (including adults, older children and toddlers) will not predict the risk of vaccine-associated ERD. It is also agreed that the

risk of vaccine-associated ERD among RSV-naive infants may vary according to the specific vaccine under consideration. For example, post-immunization surveillance of 175 very young infants given intranasal live-attenuated RSV vaccines did not identify a significant increase in risk of vaccine-associated ERD (83).

However, other candidate vaccines proposed for testing and use in RSV-naive infants should have a strong justification based on data derived from nonclinical testing that will discriminate the properties of the new candidate vaccine from those properties associated with FI-RSV vaccine. When evaluated in nonclinical tests, a candidate vaccine for RSV-naive infants should: (a) induce anti-RSV neutralizing antibodies; (b) avoid induction of non-neutralizing RSV antibodies and have a relatively low anti-RSV-F IgG ELISA binding-to-neutralizing antibody ratio; (c) avoid induction of allergic inflammation characterized by a Th2-biased CD4+ T-cell response (IL-4, IL-5, IL13 and/or mucus production); and (d) should not induce alveolitis after a valid, live RSV challenge. Evidence of the ability to elicit CD8+ T-cells in nonclinical testing may also be desirable to help distinguish the candidate vaccine from FI-RSV which does not elicit this response; while RSV-specific CD8+ T-cells may facilitate clearance of RSV-infected cells and promote Th1 responses, it is not known if this response is necessary for the prevention of vaccine-associated ERD. Pulmonary eosinophilia, while not thought to be causally related to vaccine-associated ERD, can be a marker of a dominant Th2 type cytokine response, and the presence of pulmonary eosinophils in animals after challenge should be heeded (84). In addition, immune complex deposition in the lungs of mice immunized with FI-RSV prior to a live RSV challenge was directly linked mechanistically to the pathology seen in lung tissues from the two fatal cases observed during the original FI-RSV vaccine trials (75). The exact predictive value of these animal models for determining the true risk of vaccine-associated ERD in humans will only be determined once these candidate vaccines proceed into clinical trials in RSV-naive infants. Nonclinical testing needs to be designed to control for potential confounding factors and results interpreted cautiously so as not to inadvertently dismiss vaccines with the potential to safely provide protection for very young and vulnerable infants.

For the reasons given above, and despite the fact that the current animal models do not accurately mimic all aspects of either human RSV disease or vaccine-associated ERD, it is expected that candidate vaccines with the immunopathological properties of FI-RSV will be evaluated in one or more animal models with appropriate positive and negative controls prior to testing in an RSV-naive infant population in order to demonstrate that the candidate vaccine meets the requirements (a)–(d) outlined above as applicable to each candidate vaccine. Several semi-permissive animal models that may be used for this safety assessment are discussed in Part B below. While no preference

is given to one animal model over the other, careful thought should be given to identify the model or models most compatible with the candidate vaccine under consideration. For example, it is known that tissue culture components in some vaccine preparations may provoke lung inflammatory responses characteristic of vaccine-associated ERD in rodent models following challenge (85–88). This problem can be avoided by using the neonatal calf model (89, 90). End-points for this safety analysis may include lung histopathology (to include an assessment of neutrophilic alveolitis and mucus production), pulmonary virus load (using infectious virus, genome copy number and/or reporter gene read-out) and measurement of vaccine-induced immune responses (to include neutralizing antibodies against RSV A and RSV B strains, cytokine secretion profile and phenotype of pulmonary T-cells post-challenge (including the presence or absence of CD8+ cytotoxic T-cells).

International reference materials

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

However, a First WHO International Standard for antiserum to respiratory syncytial virus was established by the WHO Expert Committee on Biological Standardization, with an assigned unitage of 1000 IU/vial (91). This reference material is intended to be used in the standardization of virus neutralization methods for measuring antibody levels against RSV/A in human sera. It was subsequently shown that this reference material could also be used to measure antibody levels against RSV/B in human sera, with an assigned unitage of 1000 IU/vial (92). The use of the reference material will thus allow for the standardization of RSV neutralization assays independent of assay format and will facilitate comparability of immunogenicity among candidate RSV vaccines.

The WHO international 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 (93) should be consulted.

Expression of dose related to vaccine potency

In the case of live-virus and chimeric viral or bacterial RSV vaccines, potency is typically expressed in terms of the number of infectious units of virus or culturable particles of bacteria contained in a human dose, using a specified tissue culture substrate or by inoculation on a solid medium, and based on the results of clinical trials.

In the case of subunit/particle-based RSV vaccines, potency is expressed using a suitable *in vitro* or *in vivo* method, which should be developed by the manufacturer. In the case of viral-vectored vaccines, potency is usually expressed using a combination of different methods.

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. Alternatively, until international reference preparations become available, assays based on plaque-forming units (PFU), the cell culture infectious dose 50% (CCID₅₀), colony forming units (CFU) 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 the establishment of parameters for stability and expiry date.

Part A. Guidelines on the development, manufacture and control of 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 help in the harmonization of nomenclature after licensure. The international name should be “respiratory syncytial virus vaccine”. Depending on the construct of the antigen this should be further qualified (for example, live-attenuated, recombinant) including through 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 language of the country of origin, followed in parentheses by the virus subtype (where applicable) and name of the recombinant protein(s) when applicable.

A.1.2 Descriptive definition

A live-attenuated RSV vaccine which has been derived either through conventional attenuation of RSV or through 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 solution, 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. These

chimeric vaccines are produced by recombinant DNA technology. The vaccine may be presented as a sterile aqueous suspension or as freeze-dried material.

The description of a particle-based or subunit vaccine should identify the RSV antigen produced by recombinant DNA technology that is included in the vaccine. 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 MVA) is produced by recombinant DNA technology and the RSV antigen expressed by the vector should be identified. The vectored vaccine may be presented as a sterile liquid suspension or as freeze-dried material.

All of the above types of RSV vaccines are for prophylactic use.

A.2 General manufacturing guidelines

The general manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (94) and WHO good manufacturing practices for biological products (95) should apply to the design, establishment, operation, control and maintenance of manufacturing facilities for each type of RSV vaccine. Manufacturing areas may be used on a campaign basis with adequate cleaning and changeover procedures 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 *Laboratory biosafety manual* (96). The basis for this is a microbiological risk assessment which results in the classification of activities into different biosafety levels. The respective classification level should be approved by the relevant authority of the country/region in which the manufacturing facility is located. The assessment should take into account both the backbone and the targeted RSV antigen involved.

Moreover, whenever in vivo tests are performed during vaccine development or manufacturing, it is desirable for ethical reasons to apply the 3Rs principles (Replacement, Reduction, Refinement) to minimize the use of animals where scientifically appropriate (97).

A.2.1 Considerations in the manufacturing of RSV vaccines

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

During early clinical trials it is unlikely that data from sufficient batches will be available 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 the commercial supply of the product.

In addition to control during manufacture, each product should be adequately characterized at each stage of its development. The resulting attributes will 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 (for example, as part of the nonclinical pharmacodynamic evaluation). When available, and in agreement with the NRA, platform technology data could be supportive and leveraged.

Prior to submitting a marketing authorization application, the manufacturing process should be adequately validated by demonstrating that at least three consecutively produced commercial-scale drug substance and drug product batches can be manufactured consistently. Drug product batches should be produced from individual drug substance batches. 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 during vaccine development, a comparability exercise should be performed between batches manufactured according to the different manufacturing processes following the ICH Q5E guideline (98). 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**

This section addresses the control of source materials for: (a) cell lines used as substrates (section A.3.1); (b) cell culture and virus propagation (section A.3.2); (c) live-attenuated/chimeric RSV vaccines (section A.3.3); (d) subunit/particle-based RSV vaccines (section A.3.4); and (e) viral-vectored RSV vaccines (section A.3.5).

A.3.1 **Control of source materials for cell lines used as substrates**

Candidate RSV vaccines have been produced in: (a) human cell lines (for example, human embryonic kidney cells – HEK 293, PERC6); (b) mammalian

cell lines (for example, Chinese hamster ovary cells (CHO-K1), African green monkey Vero cells); (c) primary chick embryo cells and embryonated chicken eggs; and (d) insect cell lines (for example, Sf9 derived from *Spodoptera frugiperda* and Hi-5 Rix4446 cells derived from *Trichoplusia ni*).

The use of a cell line should be based on a cell bank system (18). Sufficient information on the provenance of the cell bank should be recorded. A maximum number of passages or maximum population doubling level should be established, if applicable. This should be established for the MCB, WCB and the cells used for production. The cell bank or seed 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 WCB. In normal practice the MCB is expanded by serial subculture up to a passage number (or population doubling level, 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.

Additional tests may include, but are not limited to, propagation of the MCB or WCB to or beyond the maximum in vitro age for production (end of production (EOP) cells), and examination for the presence of retroviruses, other adventitious agents and tumorigenicity when relevant (18). The MCB, WCB and EOP cells should be tested as described 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 (18).

If primary cells or eggs are used they should be produced using a controlled system (18). In the case of eggs, further guidance is available in section A.4.2.2 of the WHO Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (19).

A.3.2 Control of source materials for cell culture and virus propagation

Only substances that have been approved by the NRA may be added. Whenever possible the use of materials of animal origin should be avoided.

If serum is used for the propagation of cells it should be tested to demonstrate the absence of bacteria, fungi and mycoplasmas – as specified in the 1995 amendment (99) to the WHO General requirements for the sterility of biological substances (100) – and freedom from adventitious viruses. Bovine serum should comply with the current *WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (101).

Detailed guidance on detecting bovine viruses in serum that is being considered for use in establishing an MCB and WCB are given in Appendix 1

of 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 (18) and should be applied as appropriate. This same guidance may also apply 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, while recognizing that some viruses are relatively resistant to gamma irradiation. Whatever process is 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 mycoplasmal and viral contaminants.

The source(s) of animal components used in the culture medium (or used to produce culture medium components) should be approved by the NRA. Components derived from TSE-relevant animal species should comply with the current *WHO Guidelines on transmissible spongiform encephalopathies in relation to biological and pharmaceutical products* (101).

Bovine or porcine trypsin used for preparing cell cultures (or used to prepare culture medium components) should be tested and found to be 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* (101).

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 to 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 to be 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 the risk of contamination and should be subject to the usual considerations for any reagent of biological origin (18).

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 relevant 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 (102). 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* (101).

Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Acceptable residual levels should be approved by the NRA (95).

Non-toxic pH indicators may be added (for example, 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 **Control of 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 (for example, 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 (MS). Furthermore, information on the complete genome sequence and on the passage level of the material used in clinical trials should be indicated. The respective strains should be approved by the NRA.

The strains of recombinant RSV used for the MS and working seed (WS) used to produce candidate vaccines should comply with the additional specifications given in section A.3.3.1.2 below.

For chimeric RSV vaccines (such as bovine parainfluenza RSV chimera or recombinant Sendai RSV chimera) the provisions laid down below in section A.3.5.1 apply.

A.3.3.1.2 *Strains derived by molecular methods*

In some countries, if a live-attenuated vaccine strain derived by recombinant DNA technology is used 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 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.3.3.1.3 *Seed lot system*

The production of RSV vaccine should be based on a virus seed lot system to minimize the number of tissue culture passages needed for vaccine production. This will involve the use of an MS and a WS. Seed lots should be prepared in the same type of cells using similar conditions for virus growth as those used for production of the final vaccine.

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

The maximum passage level of the MS and WS should be approved by the NRA. The inoculum for infecting cells used in the production of vaccine should be from a virus WS with as few as possible intervening passages in order to ensure that the characteristics of the vaccine remain consistent with the lots used in clinical trials.

Virus seed lots should be stored in a dedicated temperature-monitored freezer that ensures stability upon storage. The duration of stability should be monitored by controlled testing at the selected storage temperature and conditions. It is recommended that a large virus WS lot 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. For such vaccines the provisions laid down in section A.3 of the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines apply (20).

A.3.3.2 **Control of cell cultures for virus seeds**

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

A.3.3.3 **Control of virus seed lots**

The following tests should be performed on virus MS and WS lots.

A.3.3.3.1 *Identity*

Each virus MS and WS 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 nucleotide sequence determination and by other relevant laboratory and animal tests in order to provide information on the consistency of each virus seed. Molecular markers of

attenuation shall be identified and defined during the establishment of the viral seed (see Part B below). These tests are required to compare the new vaccine strain with the wild-type and/or parent virus. The sequence of the MS defines the consensus sequence of a vaccine strain.

Mutations introduced during the derivation of each vaccine strain should be maintained in the consensus nucleotide 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.

The genetic stability of the vaccine seed to a passage level comparable to final 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 RSV antigens. For example, if attenuation is associated with temperature sensitivity, cold adaptation, plaque-size or host-range restriction, the phenotype of the candidate vaccine virus associated with attenuation should be shown to be conserved at passage levels required for manufacture and ideally beyond.

For any new MS and WS it is recommended that the first three consecutive bulk vaccine lots should be analyzed for consistency of manufacturing and for identity of the active substance based on relevant quality parameters.

A.3.3.3.3 *Sterility tests for bacteria, fungi, mycoplasmas and mycobacteria*

Each virus MS and WS lot should be shown to be free from bacterial, fungal, mycoplasmal (or spiroplasmal if insect cells are used) and mycobacterial contamination using appropriate tests as specified in the WHO General requirements for the sterility of biological substances (99, 100). Nucleic acid amplification techniques (NATs), either alone or in combination with cell culture and 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 (15).

A.3.3.3.4 *Tests for adventitious agents*

Each virus MS and WS 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 RSV or the vector virus the antigen used to generate the antiserum should be produced in cell culture from species different from that used for production of the vaccine and should be free from adventitious agents. Suitable indicator cells should be selected to enable the detection of viruses. The choice of 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 such viruses should then be tested for using a suitable assay method. For test details, refer to section B.11 of 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 (18).

Each virus MS lot should also be tested in animals if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package (103). The animals used might include guinea-pigs, adult mice and suckling mice. For test details, refer to section B.11 of 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 (18). For ethical reasons it is desirable to apply the 3Rs principles (Replacement, Reduction, Refinement) to minimize the use of animals where scientifically appropriate (97).

New molecular methods with broad detection capabilities are available for adventitious agent detection. These methods include: (a) degenerate NAT for whole virus families with analysis of the amplicons by hybridization, sequencing or mass spectrometry; (b) NAT with random primers followed by analysis of the amplicons on large oligonucleotide microarrays of conserved viral sequencing or digital subtraction of expressed sequences; and (c) high-throughput sequencing. These methods may be used 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 Part B below, studies should, when relevant, be performed in animals to determine that the MS virus displays attenuating features which are maintained throughout subsequent vaccine process steps. For certain candidate vaccines it may be required to test at least once during nonclinical development for these features in a relevant animal model. For an MS virus to be identified as attenuated the criteria for determining attenuation should be clearly defined.

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

A.3.3.3.6 *Virus titration for infectivity*

The infectivity of each virus MS and WS lot should be established using an assay 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, immunofocus assays or CCID₅₀ with read-outs such as quantitative PCR (qPCR) may be used. All assays should be validated.

A.3.3.4 Control of bacterial seeds

For the control of bacterial seeds, the provisions laid down in the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (20) 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 recorded on the testing carried out for adventitious agents and on the genetic homogeneity of 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 being cloned. Some techniques (for example, sequencing) allow for the entire construct to be examined, while others (for example, restriction-enzyme mapping) allow for assessment of segments of respective plasmids (104, 105). 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 (105).

The nucleotide sequence of the gene insert and adjacent segments of the vector, along with restriction-enzyme mapping data for the vector containing the gene insert, should be provided, as required, to 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 and 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 or 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 should also be monitored to confirm cell viability after retrieval from storage, and to confirm maintenance of the expression system. These studies may be performed as part of the routine use of the expression system in production or may include samples specifically taken for such a purpose.

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

Such 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 (99, 100), or by an alternative 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 or with fungi.

A.3.4.1.2 *Recombinant mammalian cells*

If recombinant 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 (18) and the WHO Guidelines on the quality, safety and efficacy of biotherapeutic protein products prepared by recombinant DNA technology (105) and should be approved by the NRA.

A.3.4.1.3 *Insect cells*

WCBs of insect cells may be used for recombinant baculovirus seed lot production and antigen expression. If insect cells are used for expression of the RSV vaccine antigen with a baculovirus-based expression vector, 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 (18), as appropriate to insect cells, and should be approved by the NRA.

A.3.4.1.3.1 Tests on insect MCB and WCB

Testing of insect MCBs and WCBs should be performed in accordance 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 (18). 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 their derivation. For insect cells a special emphasis is placed on potential insect-borne human pathogens (for example, arboviruses). Moreover, the risk from insect cell lines inherently contaminated with viral agents should be assessed (106, 107).

Insect viruses have not been well characterized compared with other potential adventitious agents, and less information about them is therefore available, especially on 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 involve specific NAT-based assays such as PCR and other nonspecific 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 (18). Scientific advice on the testing strategy should be sought from the NRA.

A.3.4.2 Recombinant baculovirus MS and WS lots

The recombinant baculovirus expression vector used in the production of RSV vaccine contains the coding sequence of the respective RSV antigen and should be identified by historical records. The historical records will include information on the origin and identity of the gene being cloned, details on the method of construction, and the nucleotide sequence of the selected baculovirus expression vector in the context of the final construction.

The production of vaccine should be based on a seed lot system. Recombinant baculovirus seed lots should be stored in a dedicated temperature-monitored refrigerator or freezer at a temperature shown formally by the manufacturer to ensure stability for the duration of the planned period of storage.

Only recombinant baculovirus seed lots that are approved by the NRA should be used. The recombinant baculovirus MS lot should be made in sufficient quantities to meet anticipated needs for long-term production and should be stored in a secure environment. The MS lot is used as the source material for making the manufacturer's recombinant baculovirus WS lot. Either the MS or the WS should be fully characterized and tested extensively for adventitious agents, while the other may be subjected to more limited testing. The testing strategy and seed lots should be approved by the NRA.

It is recommended that a large lot of recombinant baculovirus WS should be set aside as the basic material for use by the manufacturer in the preparation of each batch of the vaccine. The recombinant baculovirus WS lot should be prepared based on a defined number of passages from the recombinant baculovirus MS lot using a method and a passage level from the MS lot approved by the NRA. Once the acceptable passage level of the WS is established it may not be changed for future lots of WS without approval from the NRA.

A.3.4.2.1 Tests on recombinant baculovirus MS and WS lots

The expression construct should be analyzed using NAT-based assays in conjunction with other tests performed on the purified recombinant protein for assuring the quality and consistency of the expressed RSV antigen. The genetic stability and stability of expression of the expression construct should be demonstrated from the baculovirus MS up to at least the highest passage level used in production, but preferably beyond this level (104, 105).

A.3.4.2.1.1 Identity

Each baculovirus MS and WS 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 tests for bacteria, fungi, mycoplasmas and mycobacteria

The provisions laid down in section A.3.3.3.3 above 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 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 below).

It should be noted that the 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 seeds are free of adventitious agents relevant to the insect species used in their derivation with a special emphasis placed on potential insect-borne human pathogens (for example, arboviruses). The specificity and sensitivity of the assays used should be determined by the manufacturer and approved by the NRA.

In general, recombinant baculovirus seeds should be assessed for the presence of adventitious agents that may have been introduced during their production, including those that may have been present in the source materials used at each production stage of the MS and WS lots. 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 (18). Whenever *in vivo* tests are performed it is desirable for ethical reasons to apply the 3Rs principles (Replacement, Reduction, Refinement) to minimize the use of animals where scientifically appropriate (97).

New molecular methods with broad detection capabilities are being developed for adventitious agent detection. Such methods may be used at the discretion of the NRA 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 section A.3.3.3.4 above).

A.3.4.2.1.4 Test of control cells used for production of seeds

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

A.3.4.2.1.5 Virus titration for infectivity

Each recombinant baculovirus seed should be assayed for its infectivity in the insect cell line chosen for antigen manufacture using a sensitive assay. The detailed procedures used to carry out the tests and interpret their results should be approved by the NRA.

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

A.3.5.1 Virus vector MS and WS

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

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 for 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 incorporates genetic elements to control the expression of a transgene (for example, in 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 the derivation of the material that ultimately resulted in the candidate vaccine to the virus MS 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 complete nucleotide sequence of the gene insert and of the vector should be provided and may be supplemented by restriction-enzyme mapping of the vector containing the gene insert. The genetic stability of the vector with the recombinant construct during amplification associated with manufacture 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 MS to its sequence at, or preferably beyond, the anticipated maximum passage level. Any modifications to the sequence of the heterologous insert should be demonstrated to have no impact on the resulting antigenic characteristics of the vaccine.

A.3.5.1.1 *Tests on virus MS and WS*

A.3.5.1.1.1 **Identity**

Both the vector and the RSV-specific genetic components of the virus MS and WS should be identified by immunological assay or by molecular methods acceptable to the NRA.

A.3.5.1.1.2 **Genetic and phenotypic characterization**

The virus MS should be characterized as fully as possible. If this characterization is limited (for example, because of limited quantities of material) the virus WS should be fully characterized in addition to the limited characterization of the MS. It should be noted that it would not be feasible to manufacture the vaccine directly from the virus MS in these circumstances.

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

Genetic characterization will involve a complete nucleotide sequence analysis of the vaccine vector which might be supplemented by restriction-enzyme mapping, southern blotting, PCR analysis or DNA fingerprinting. Promoter elements involved in expression of the RSV-derived gene(s) (including relevant junction regions) should be described and delineated.

The genetic stability of the vaccine seed to a passage level comparable to final vaccine 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, and should be performed *in vitro* under conditions that allow for the detection of revertants (including the emergence of replication-competent vectors from replication-incompetent vectors during passage) and of changes to the stability of the heterologous gene insert during replication of the recombinant vector. However, other studies – including antigenic analysis, infectious titre, ratio of genome copies to infectious units (for replicating vaccines) and *in vitro* yield – should also 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 (for example, 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 WS lot and justification for the chosen subset should be provided.

A.3.5.1.1.3 Sterility tests for bacteria, fungi, mycoplasmas and mycobacteria

The provisions laid down in section A.3.3.3.3 above apply.

A.3.5.1.1.4 Tests for adventitious agents

Information should be given on the testing for adventitious agents, as outlined in section A.3.3.3.4 above. The methods used and results obtained should be acceptable to the NRA.

A.3.5.1.1.5 Virus titration for infectivity

The infectivity of each virus MS and WS lot should be established as outlined in section A.3.3.3.6 above.

A.3.5.2 Cell substrates

The cell substrate used for the manufacture of a viral-vectored RSV vaccine should be based on controlled primary cells or a cell banking system using continuous cell lines as outlined 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 (18). See section A.3.1 above for further information.

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

A.4.1 Control of cell cultures

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

In cases where mammalian or other 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 the production of vaccine a fraction equivalent to at least 5% of the total cell suspension, or 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 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 is the longer. At the end of the observation period, supernatant fluids collected from the control culture should be pooled and 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, no more than 20% 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 7 days and the storage temperature should have been in the range $2-8^{\circ}\text{C}$.

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 humans (blood group O), monkeys and chickens (or other avian species). For all tests, readings should be taken after incubation for 30 minutes at $2-8^{\circ}\text{C}$, and again after further incubation for 30 minutes at $20-25^{\circ}\text{C}$. The test using monkey red blood cells should be read once more after additional incubation for 30 minutes at $34-37^{\circ}\text{C}$.

For the test to be valid, no more than 20% 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 additional 10 mL samples tested 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 type of cell culture should not be inoculated in order to serve as a control.

The inoculated cultures should be incubated at a temperature of $35-37^{\circ}\text{C}$ 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 at least an additional 7 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, no more than 20% of the culture flasks should have been discarded, for any reason, by the end of the test period.

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 WCB – should be identified by means of tests approved by the NRA.

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

A.4.2 Production and harvest of monovalent bulk vaccine

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 WS. 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 at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Acceptable residual levels should be approved by the NRA (95).

A.4.2.2 Virus inoculation

Cell cultures are inoculated with virus WS 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 will depend on the vaccine strain and the production method, and specifications should be validated by each manufacturer.

A.4.2.3 Monovalent bulk vaccine

Monovalent bulk vaccine is harvested within a defined period post-inoculation. A monovalent bulk vaccine may be the result of one or 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 manufacturing 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 treatment with DNase to reduce the size of retained host cell DNA is also recommended.

A.4.2.4 Tests on monovalent bulk vaccine

A.4.2.4.1 Identity

A test for identity should be performed if this has not been done on the single harvest or pooled harvest.

A.4.2.4.2 Sterility tests for bacteria, fungi, mycoplasmas and mycobacteria

A sample of each monovalent bulk or virus culture supernatant should be tested for bacterial, fungal, mycoplasmal and mycobacterial sterility as specified in the WHO General requirements for the sterility of biological substances (99, 100) or by an alternative method approved by the NRA.

NAT-based assays, alone or in combination with cell culture and 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 with the agreement of the NRA (18).

The method used for testing for mycobacteria should be approved by the NRA. NAT-based assays might be used as an alternative to the microbiological culture method for mycobacteria after validation by the manufacturer and with the agreement of the NRA.

A.4.2.4.3 *Tests for adventitious agents*

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

A.4.2.4.4 *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 the use of a single harvest in the preparation of a virus pool or final bulk, and to confirm the consistency of production. A reference preparation should be included to validate the titration assay.

A.4.2.4.5 *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.4.2.4.6 *Test for consistency of virus characteristics*

Recombinant RSV candidate vaccine lots should be tested and compared to the MS, WS or other 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, for example, virus yield in cell culture, growth in primary human bronchial epithelial cells or plaque morphology. Other identifying characteristics may also be applicable.

Assays for assessing the attenuation of recombinant RSV should also be conducted and the results 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 and 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 final bulk**

Only monovalent bulk vaccine meeting the recommendations for sterility, freedom from adventitious agents 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, to the satisfaction of the NRA, not to impair the safety and efficacy of the vaccine in the concentration used.

A.4.3.2 Tests on final bulk

A.4.3.2.1 Test for residual materials

The manufacturer should demonstrate by testing each final bulk or by validating the manufacturing process that any residual materials used in the manufacturing process – 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 (105).

For viruses grown in continuous-cell-line cells, the final bulk material should be tested for the amount of residual cellular DNA, and the total amount of cell DNA per dose of vaccine should not be more than the upper limit agreed by the NRA. Where 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 Bacterial and fungal 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 WHO General requirements for the sterility of biological substances (99, 100) 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–A.4.3 above apply.

For chimeric RSV vaccines grown in eggs, most of the provisions laid down in the WHO Recommendations to assure the quality, safety and efficacy of influenza vaccines (human, live attenuated) for intranasal administration (19) apply.

For chimeric BCG/RSV vaccines, most of the provisions laid down in sections A.4 of the WHO Recommendations to assure the quality safety and efficacy of BCG vaccines (20) 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 **Production of antigen if recombinant yeast or bacteria are used**

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 increase cell density should be approved by the NRA. Penicillin and other beta-lactam antibiotics should not be used at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Acceptable residual levels should be approved by the NRA (95).

Genetic integrity and stability of the expression vector during the process of vaccine manufacture shall be confirmed by appropriate methods in order to ensure consistency of vector-based protein expression.

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 (for example, 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-lactam antibiotics should not be used at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Acceptable residual levels should be approved by the NRA (95).

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 (if applicable)**

When control cells are included in the manufacturing process, the provisions laid down in section A.4.1 above 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 above 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 above apply.

A.5.1.3.3 *Identity of cells*

The provisions laid down in section A.4.1.3 above 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 DNase) should be documented.

The purified monovalent antigen bulk should be stored under conditions shown by the manufacturer to allow it to retain the desired biological activity. Intermediate hold times should be validated by the manufacturer and 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 consistency and safety.

A.5.2.1.1 Identity

A test for identity should be performed using 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. In the case of yeast-derived products these tests may be omitted for routine lot release upon demonstration that the purification process consistently eliminates the residual components from the monovalent bulks to the satisfaction of the NRA.

A.5.2.1.3 Protein content

Each purified antigen bulk should be tested for 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 Bacterial and fungal sterility

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 (99, 100) or by an alternative method approved by the NRA.

Alternatively, if the antigen is directly adsorbed onto an adjuvant and no samples can be drawn, the test can be performed on the related adsorbed antigen bulk, if properly justified.

A.5.2.1.6 *Percentage of intact RSV antigens*

If the integrity of certain RSV proteins (for example, the F protein) is a critical quality parameter, this should be carefully monitored. The percentage of intact RSV protein trimer should be assessed in comparison to a reference standard. Using a suitable panel of monoclonal antibodies, the percentages of F protein that exist in the pre-fusion state and post-fusion state could be individually assessed. Such assays could be used to assure consistency of manufacture.

A.5.2.1.7 *Nanoparticle size and structure*

In the case of particle-based vaccines such as F protein nanoparticle vaccines, 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 with 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 and disc centrifugation size analysis. Disc centrifugation size analysis allows for the determination of the hydrodynamic radius of particles which sediment in a sucrose gradient when referenced against 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 process consistency eliminates the reagent from the purified monovalent antigen bulks, subject to the agreement of the NRA.

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 (18).

These tests may be omitted for routine lot release upon demonstration that the process consistently inactivates 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 the residual BSA content should be measured and a maximum permitted concentration should be set and approved by the NRA.

A.5.2.1.11 *Viral clearance study*

When a cell substrate is used for the production of RSV antigens then the production process should be validated in terms of its capacity to remove and/or inactivate adventitious viruses – as described in the Q5A guidelines (108). This validation 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 listed 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 (18) should be taken into consideration.

A.5.2.2 **Purified adjuvanted bulk**

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 then 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*

If applicable, all tests and specifications for adsorbed antigen bulk should, unless otherwise justified, be approved by the NRA.

A.5.2.2.2.1 **Bacterial and fungal sterility**

Each adsorbed 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 (99, 100) or by an alternative method approved by the NRA.

A.5.2.2.2.2 Bacterial endotoxins

For a vaccine intended to be administered parenterally the adsorbed antigen bulk should be tested for bacterial endotoxins using a method approved by the NRA. The total amount of residual bacterial 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.

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 antigen bulk should be identified as the correct RSV antigen by a suitable method (for example, an immunological assay), if applicable.

A.5.2.2.2.4 Adjuvant concentration

Adsorbed antigen bulk should 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

If applicable, the pH value of the adsorbed antigen bulk may be monitored until production consistency is demonstrated, and should be approved by the NRA.

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 for viral-vectored RSV vaccines

The manufacture of vaccine vectors starts with the amplification of the vaccine vector seed stock in a suitable cell line. The number of passages between the virus WS lot and final viral-vectored vaccine product should be kept to a

minimum and should not exceed the number of passages used for production of the vaccine shown in clinical studies to be satisfactory, unless otherwise justified by the manufacturer and authorized by the NRA. A maximum number of passages should be defined for which the identity of the vaccine has been demonstrated.

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. The growth medium and supplements used in culturing should be identical to those used for 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 described in section A.4.1 above should be followed.

A.6.1.1 Tests for haemadsorbing viruses

The provision laid down in section A.4.1.1 above applies.

A.6.1.2 Tests for other adventitious agents

The provisions laid down in section A.4.1.2 above apply.

A.6.2 Single virus harvest

The method used to harvest the vaccine vector should be described and the virus titre ascertained. A reference preparation should be included to validate the titration assay. Minimum acceptable virus titres should be established for both single virus harvests and for 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 (for example, on purified final bulk). For example, a Western blot analysis (or other method 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 crude or purified single harvest, whichever is the most appropriate. This should include the identity of the expressed heterologous antigen and of the vector virus.

Tests for adventitious agents should be performed on each single harvest according to the relevant parts of 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 (18). Additional testing for adventitious viruses may be performed using validated NAT-based assays or other methods such as next generation sequencing.

New molecular methods with broad detection capabilities are being developed for adventitious agent detection and may also be used once validated 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 section A.3.3.3.4 above).

Single virus harvests should be tested to demonstrate freedom from bacteria, fungi and mycoplasmas, as specified in the WHO General requirements for the sterility of biological substances (99, 100) or by an alternative method approved by the NRA.

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

Provided that cell banks and viral seed stocks have been comprehensively tested to demonstrate freedom from adventitious agents, 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 or other NAT-based methods such as next generation sequencing, could be discussed and agreed upon with the NRA. The method of production should be taken into account when deciding upon the 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 good manufacturing practices (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 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 the

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, mycobacteria (if applicable) and mycoplasmas, as specified in the WHO General requirements for the sterility of biological substances (99, 100). Alternatively, if single virus harvests have been tested to demonstrate freedom from bacteria, fungi, mycobacteria (if applicable) and mycoplasmas then 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-lactam antibiotics should not be used at any stage of manufacture because they are highly sensitizing substances in humans. Other antibiotics may be used during early stages of production. In this case, the use of antibiotics should be well justified, and they should be cleared from the manufacturing process at the stage specified in the marketing authorization. Acceptable residual levels should be approved by the NRA (95).

A.6.4.1 **Control tests on monovalent bulk**

The monovalent bulk vaccine should be tested and consideration given to using the tests listed below, as appropriate for each individual product. Alternatively, if the monovalent bulk will be held for a short period of time then some of the tests listed below could, if appropriate, 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 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.

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 (18).

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 (for example, adenovirus vectors) the total number of virus particles per mL (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. If the particle number can be determined, the particle/infectivity ratio should also be specified.

A.6.4.1.2.3 **Expression of the heterologous antigen in vitro**

If not otherwise justified, the ability of the viral particles to express the heterologous gene should be demonstrated using a suitable method, for example based on the use of an antigen-specific antibody (and/or conformation-specific antibody if detecting RSV pre-fusion 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-enzyme mapping, PCR with a specific probe or sequencing – will generally be suitable for assessing the identity of the product.

A.6.4.1.4 *Bacterial and fungal bioburden or sterility*

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 carried out as specified in the WHO General requirements for the sterility of biological substances (99, 100) or by an alternative method approved by the NRA.

A.6.4.1.5 *Bacterial endotoxins*

For vaccine intended to be administered parenterally, each monovalent bulk should be tested for bacterial endotoxins using a method approved by the NRA. 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. It is not known whether such viral particles represent a safety concern. Consequently, it should be shown that the vaccine virus is still replication-incompetent or fully attenuated (whichever is relevant) in initial batches of the monovalent bulk.

After demonstrating this, it may be possible to omit such tests in future batches provided a sufficient justification is made (which should include discussion of why reversion to competency or loss of attenuation would be unlikely in future batches).

A.6.5 **Final bulk vaccine**

Appropriate quantities of 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 the final vaccine product.

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 (109).

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, for example where filling operations are performed immediately after manufacture of the final bulk, and on the same site:

A.6.5.1.1 *Identity*

See section A.6.4.1.3 above.

A.6.5.1.2 *Preservative*

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

A.6.5.1.3 *Bacterial and fungal sterility*

Each final bulk should be tested for bacterial and fungal sterility. Sterility testing should be carried out as specified in the WHO General requirements for the sterility of biological substances (99, 100) or by an alternative method approved by the NRA.

A.7 **Filling and containers**

The relevant manufacturing recommendations contained in WHO good manufacturing practices for pharmaceutical products: main principles (94) and WHO good manufacturing practices for biological products (95) should apply to the RSV vaccine filled in the final form.

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 storage conditions. 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 the qualification of containers, and may be needed as part of stability assessments. Assessment of extractables and/or 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 then the vaccine may contain preservative; the use of which should be compliant with the WHO Policy Statement: multi-dose vial policy (109), as is the case for reconstituted vaccines such as BCG and measles vaccines. 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 storage and shipping conditions (see section A.13 below).

A.8 Control tests on final lot

Where applicable or appropriate, the following tests should be performed on the final lot unless otherwise justified and agreed with the NRA. All tests and specifications should be approved by the NRA. The specifications should be defined on the basis of the results of tests on lots that have been shown to have acceptable performance in clinical studies.

A.8.1 Inspection of final containers

Every final container in each final lot should be inspected visually and/or in an automated manner, and those showing abnormalities (for example, improper sealing, clumping or the presence of particles) should be discarded and recorded for each relevant abnormality. A maximum limit should be established for the percentage of containers that can be rejected before triggering investigation of the cause, potentially resulting in batch failure.

A.8.2 Identity

An identity test should be performed on at least one final labelled container from each filling lot – in the 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 vaccines each antigen component should be identified.

A.8.3 Appearance

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

A.8.4 pH

The pH of the final lot should be tested 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

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

A.8.6 **Bacterial and fungal sterility**

Each final lot should be tested for bacterial and fungal sterility, as specified in the WHO General requirements for the sterility of biological substances (99, 100) or by an alternative method approved by the NRA.

A.8.7 **Bacterial and fungal contamination**

For chimeric BCG/RSV vaccines, samples from each final lot should be tested for bacterial and fungal contamination by appropriate tests as specified in Part A section 5.2 of the WHO General requirements for the sterility of biological substances (100) or by an alternative method approved by the NRA.

A.8.8 **Preservative**

Each final lot should be tested for the concentration of preservative, if added.

A.8.9 **Residual moisture**

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 based on the results of stability testing. Moisture levels of 3% or less are generally considered to be acceptable.

A.8.10 **Pyrogenic substances**

Each final lot should be tested for pyrogenic substances, if appropriate. Tests for bacterial endotoxin (for example, the limulus amoebocyte lysate (LAL) test) should be performed. However, if there is interference in the test – for example, because of the addition of an immunostimulant such as 3-*O*-desacyl-4'-monophosphoryl lipid A – a test for pyrogens should be performed. The classical rabbit pyrogen test should now be replaced by a validated monocyte-activation test approved by the NRA.

A.8.11 **Adjuvant content**

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**

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

A.8.13 Degree of adsorption

The degree of adsorption to the adjuvant (completeness of adsorption) of each antigen present in the final bulk should be assessed, if applicable (for example, if the adjuvant is aluminum salts) and the lower 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 *in vitro* or *in vivo* quantitative test for potency should be performed using samples representative of each final vaccine lot. In the case of freeze-dried vaccines, potency should be determined after the freeze-dried product has been reconstituted with the approved diluent.

The potency test used and method of data analysis 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.

Until international standards for the potency of RSV vaccines become available, manufacturers should establish a product-specific reference preparation that is traceable to a lot of vaccine, or to bulks used in the production of such a lot, shown to be efficacious in clinical trials. The performance of this reference preparation should be monitored by trend analysis using relevant test parameters and the reference preparation should be replaced when necessary. An acceptable procedure for replacing reference preparations should be in place (110).

For multivalent vaccines it may be necessary to perform potency tests on the monovalent bulks if analytical methods cannot distinguish between the different monovalent vaccines in the final lot.

A.8.15 Purity

Testing for purity should be 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 vaccine 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 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 the 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 the RSV vaccine consists of nanoparticles which might be susceptible to aggregation then each final lot should be examined for particle size/aggregate content at lot release and across the shelf-life. This test may be omitted for routine lot release upon demonstration of process consistency, subject to the approval of the NRA.

A.8.19 **Viability**

If appropriate, in the case of chimeric BCG/RSV vaccines, 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 the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (20).

A.8.20 **Thermal stability**

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 WHO Guidelines on stability evaluation of vaccines (111).

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 (for example, 37 °C for 7 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 (for example, 1 log₁₀) 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. After production consistency has been demonstrated, this test may be omitted on subsequent final lots 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 (20). The percentage decrease in the number of culturable particles is then compared with that of samples of the same vaccine lot stored at the recommended temperature. An upper limit on the acceptable percentage decrease in culturable particles compared to the untreated vaccine should be approved by the NRA.

A.8.21 **Residual antibiotics**

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

A.8.22 **Diluent**

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (94) 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 carried out for identity, appearance, pH, extractable volume, sterility, endotoxin and the content of key components.

A.8.23 **Safety test**

If appropriate, for chimeric BCG/RSV vaccines, tests to confirm the absence of virulent mycobacteria and a test for excessive dermal activity should be performed – see section A.6.4 of the WHO Recommendations to assure the quality, safety and efficacy of BCG vaccines (20).

A.9 **Records**

The recommendations given in WHO good manufacturing practices for pharmaceutical products: main principles (94) 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 labelling recommendations provided in WHO good manufacturing practices for biological products (95) should be followed as appropriate. 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 the case of live-attenuated vaccines, a statement on the nature of the preparation, specifying the strain of RSV or recombinant RSV that the vaccine has been prepared from;
- in the 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 the 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 the production of the vaccine;
- in the case of subunit and particle-based vaccines, a statement that specifies the nature and content of adjuvant contained in one human dose;
- in the case of subunit, particle-based 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 route(s) of administration;
- the number of doses if the product is issued in a multi-dose container;
- a statement to the effect that product 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, specifying that the diluent to be used is that supplied by the manufacturer – and a statement to the effect that after the vaccine has been reconstituted it should be used without delay or, if not used immediately, stored under conditions of time and temperature formally shown not to affect stability, and protected from light for a maximum period defined by stability studies;
- the name and concentration of any preservative added;
- a statement on 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, and information on concomitant vaccine use and on potential adverse events.

A.12 **Distribution and transport**

The requirements given in WHO good manufacturing practices for pharmaceutical products: main principles (94) and WHO good manufacturing practices for biological products (95) should apply. Further guidance is provided in the WHO Model guidance for the storage and transport of time- and temperature-sensitive pharmaceutical products (112).

A.13 **Stability testing, storage and expiry date**

The recommendations given in WHO good manufacturing practices for biological products (95) and WHO Guidelines on stability evaluation of vaccines (111) appropriate for the respective RSV vaccine should apply. Furthermore, the WHO Guidelines on the stability evaluation of vaccines for use under extended controlled temperature conditions might apply (113). 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 (111). Stability testing should be performed at different stages of production – namely on stored intermediates (including single harvests, monovalent bulk vaccine and final bulk) and on 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 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 more data become available (114).

Following licensure, ongoing monitoring of vaccine stability is recommended to support shelf-life specifications and to refine the stability profile (111). Data should be provided to the NRA according to local regulatory requirements.

The final stability-testing programme 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. Moreover, the stability of the antigen-adjuvant adsorption (if specified) should be demonstrated for the duration of the shelf-life. Acceptable limits for stability should be agreed with the NRA.

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 for no longer than a fixed length of time and at a temperature shown by the manufacturer to be compatible with a minimal loss of potency. The maximum duration of storage should be fixed with the approval of the NRA based on the results of stability studies, and should be such as to ensure that all quality specifications for the final product, including the minimum potency specified on the container or package, are 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.

A.13.3 **Expiry date**

The expiry date should be defined on the basis of shelf-life in the final container and should be supported by stability studies approved by the NRA. The expiry date should be based on the date of blending of the final bulk, the date of filling, the date of the first valid potency test on the final lot, or the date of removal from the freezer, as appropriate, and agreed with the NRA.

Additional stability studies are needed during development to support a determination of the shelf-life of the vaccine after thawing prior to administration, both before and after the addition of diluent, if relevant. The shelf-life 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.13.3.1 Expiry of reconstituted vaccine

In the case of 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 comply with the WHO Policy Statement: multi-dose vial policy (109). If a preservative is used, data supporting the stability and efficacy of antimicrobial preservation should be generated and approved by the NRA.

Part B. Nonclinical evaluation of RSV vaccines

B.1 General remarks

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

Before proceeding to human testing, there should be adequate information suggestive of the safety and potential efficacy of the vaccine, including product characterization, immunogenicity studies, and toxicity and safety testing in animals. The continuation of some nonclinical testing would be expected in order to maintain adherence to current GMP and to support further clinical development (14, 115).

The following sections describe the type of nonclinical information required to support the initiation of a specific clinical study, or that should be submitted in a marketing authorization application. Guidance on the designing, conducting and analysis of nonclinical studies is available in the WHO guidelines on nonclinical evaluation of vaccines (14) which should be consulted.

B.2 Process development and product characterization

The general principles described in the WHO guidelines on nonclinical evaluation of vaccines (14) regarding 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 confirm that vaccine lots used in nonclinical studies are qualified.

Vaccine lots used in nonclinical studies may be at research grade or manufactured under 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 specification.

For recombinant DNA-derived antigens one intrinsic aspect is to demonstrate the stability of their conformation(s) using suitable methods. These methods include negative staining and electron microscopy and/or a 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 production-scale run should be documented. Serological investigation based on antibody-competition assays using post-immunization sera may also provide 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 phenotype of the vaccine strain as far as is practical. The critical phenotypic markers, including replication efficiency in animal models and/or primary human bronchial epithelial cells or other relevant cultured cells, temperature sensitivity and/or cold adaptation *in vitro*, are useful for detecting reversion events.

Candidate vaccines based on live viral-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 vectored RSV vaccines unless vaccine constructs with gene deletions or modifications of the vector are suspected to have the potential for neurovirulence.

Guidance on the general principles of the nonclinical assessment of vaccine adjuvants can be found in the WHO Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines (15).

B.3 Nonclinical immunogenicity and protective activity

There is no animal model that precisely mimics RSV disease in humans. Despite this, it is acceptable to demonstrate vaccine immunogenicity in animal models as the rationale to support advancing a candidate vaccine to the clinical setting. Assessment of immunogenicity in animals should consider the construct designed or the type of vaccine. For certain vaccines (including protein-

based ones) it is generally recommended that the serum antibodies with RSV-neutralizing activity be assessed in immunogenicity studies because antibodies directed against RSV-F or RSV-G neutralize the virus in vitro and have been associated with a protective effect in animal models and/or in humans. Consideration should be given to the choice of RSV subtype (A or B) as well as to the cell type to be used for assessing neutralizing antibody responses when a vaccine construct is specifically designed to target RSV-G alone.

Candidate vaccines may be designed to elicit cellular immunity. The cellular response is typically assessed by evaluating the effect of the vaccine on the number or functional specificity of CD8+ cytotoxic T-lymphocytes and/or type 1 CD4+ T-helper (Th1) cells. For other products, induction of an effective mucosal immune response may be an intended mechanism of protection – for example, for a vaccine administered by the intranasal route. This is the case for some live-attenuated or replication-competent vectored vaccines. Therefore, a product-specific approach to the evaluation of candidate vaccines should be taken.

For vaccines that include an adjuvant, information to support the adjuvant selection and its inclusion in the vaccine formulation should be provided, based for example on demonstrated adjuvant activity and the beneficial effect assessed in terms of the magnitude and/or the type, broadness and duration of the functional immune response induced (15). The passive transfer of antibodies, generated in response to vaccination, to RSV-naive animals that are subsequently challenged with RSV can provide evidence for antibody-mediated protection and may be explored. In such cases, early discussion with the NRA is recommended.

For a multivalent candidate RSV vaccine the immune responses to each of the vaccine antigens 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 **General considerations** above).

Protective activity in challenged animals may be evaluated during the assessment of vaccine-associated ERD risk (see section B.5.2 below). 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 the case of novel adjuvants, new formulations or alternative route of administration (for example, intranasal route).

For live viral-vectored vaccines for which no prior experiments have been done, biodistribution should be studied in a full set of tissues and organs, including the brain. Such a study is unnecessary if supportive data generated for the same vector but using different gene insert(s) are available and in cases where the construction of the vector is not suspected to result in altered tissue tropism. Testing in one species is considered sufficient if scientifically justified. Crossing of the blood–brain barrier might be an indication of potential neurovirulence and should trigger additional safety testing (115).

B.5 Nonclinical toxicity and safety testing

B.5.1 Preclinical toxicology

Toxicology studies for RSV vaccines should be undertaken based on the guidance on the general principles of toxicity assessment provided in the WHO guidelines on nonclinical evaluation of vaccines (14). Toxicological testing should aim to identify any untoward effect associated with a vaccine dose, or as a consequence of replication and tissue tropism of vaccine virus in the case of a replicating vaccine, by careful analysis of all major organs as well as tissues near to and distal from the site of administration. Toxicology studies should support the safety of the starting dose, dosing schedule, route of administration and proposed rate of dose escalation.

When a new adjuvant for which no experience exists in relation to human use is included in the formulation of a vaccine it is advisable that the adjuvant alone be characterized in accordance with the WHO Guidelines on the nonclinical evaluation of vaccine adjuvants and adjuvanted vaccines (15).

If a candidate vaccine is intended to be used for the immunization of pregnant women or women of childbearing age, a single developmental and reproductive toxicity study in one relevant species should be performed. The timing of submission of such data varies by geographical region or country. Some NRAs require the exclusion of women of childbearing potential from large-scale clinical trials prior to the completion of developmental and reproductive toxicity study. In other cases, NRAs can allow the recruitment of women of childbearing potential into early clinical trials if highly effective birth control methods are used by trial participants.

Any change introduced into the manufacturing or formulation of a vaccine during product development, when judged to be significant, may require partial or full re-evaluation in preclinical toxicity testing (14, 116, 117).

B.5.2 Preclinical safety

Studies with live-attenuated and live viral-vectored vaccines entail the identification of markers of attenuation that can assist during the clinical evaluation phases. The primary purpose of such studies is to demonstrate that

the vaccine is less virulent in the animal host than comparable wild-type viruses, and that the vaccine does not exhibit any unexpected harmful tissue tropism and damage (see sections B.2 and B.4 above).

The need for potential vaccine-associated ERD risk assessment in animal models is determined by the type of RSV vaccine and/or the target population(s) and should be considered on a case-by-case basis. For example, for candidate vaccines with immunological characteristics similar to that of FI-RSV and developed for the active immunization of RSV-naïve infants, a preliminary assessment of the vaccine-associated ERD risk is crucial (see **General considerations** above). Such investigations may not be needed for live-attenuated RSV vaccines based on existing experience and if agreeable to the relevant NRAs. For live chimeric RSV vaccines experience is more limited and for many new candidate vaccines does not exist at the time of writing these Guidelines, and a more cautious approach should therefore be taken. This testing is not required for RSV vaccines indicated for use in RSV-experienced/non-naïve populations.

When the conducting of such a study is justified, the inclusion of adequate controls will have to be considered – for example, a group given intranasal RSV infection needs to be included as a negative control, and when appropriate another group should receive FI-RSV at a dose level shown to cause ERD as a positive control. Furthermore, RSV disease enhancement has been noted to occur in the absence of RSV antigen, thus raising doubts regarding the validity of data resulting from the use of these models. Therefore, it may be important to address inflammatory responses that may be due to host cell proteins or components of the cell culture medium used to produce the vaccine and/or the RSV challenge virus (85). Another important consideration is the choice of the vaccine dose. It may be necessary to examine serum antibody responses and lung histopathology after RSV challenge over a range of vaccine doses. In some cases, the dose may need to be optimized so that it is capable of inducing a measurable immune response to the vaccine while also permitting some degree of viral replication in the lungs of vaccinated animals after challenge. Since surrogate read-outs of vaccine-associated disease exacerbation vary by animal model, the weight given to each of the factors discussed above for consideration should be tailored according to the animal model used – for example, the confounding effect of cell culture serum has mainly been reported in rodent models. Although the measurement of viral titres in the lungs of affected animals does not predict enhanced pulmonary pathology, this parameter is broadly suited to assessment of the protective effect of the candidate vaccine. To enhance regulatory acceptance, it is recommended that the relevant NRA be engaged in discussion of the design of preclinical testing for vaccine-associated ERD risk at an early stage of product development.

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

A brief review of some representative animal models is provided below. It is important to note that the mechanism of action for human vaccine-associated ERD is not fully understood, and that current small-animal models primarily reproduce some immunopathological features of human ERD. Accordingly, the interpretation of these data should be undertaken with extreme caution.

B.5.2.1 Mouse model

Mice are relatively resistant to human RSV infection and require high titres of challenge inocula for significant lung pathology (for example, above 10^6 PFU) (118). 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, the mouse model is attractive because of the relatively low cost and the availability of extensive molecular tools. Certain strains, such as BALB/c mice, have been extensively used to explore the mechanisms underlying FI-RSV-associated ERD, such as patterns of CD4+ Th2 activities after vaccination and RSV challenge, immune complex deposition, pulmonary eosinophilia and induction or absence of RSV-specific CD8+ cytotoxic T-lymphocytes (89, 118–120). Other informative parameters displayed by the challenged mice may include body weight loss, illness and changes in respiratory physiology.

In addition, there are a number of models based on the use 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-associated ERD (121). In this model, virus replication in the lower airway is primarily limited to bronchiolar epithelium, closely resembling human infection. Several key histological features of disease exacerbation have been reproduced in cotton rats, including neutrophilic alveolitis and peribronchiolitis primarily caused by lymphocyte infiltration. In addition, interstitial pneumonitis appears to be another marker specific to the enhanced pulmonary pathology.

B.5.2.3 Non-human primate model

African green monkeys are one of the non-human primate species that has been used to model FI-RSV-associated ERD. Enhanced pulmonary pathology that closely resembles ERD in humans has been demonstrated in this model, as

manifested by severe infiltration of lymphocytes, macrophage, eosinophils and polymorphonuclear cells into parenchyma and the peribronchiolar areas of the lung. However, data on this model are limited and clinical disease presentation in vaccinated monkeys is of limited comparability to humans (122).

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 histological presentation observed in fatal human cases is not duplicated as there are no inflammatory lesions in the lungs at necropsy (123).

Despite sharing a high degree of similarity with the human immune system, non-human primates do not reproduce all of the immunological features seen in humans, as significant RSV-neutralizing antibody responses can be induced in FI-RSV-immunized monkeys. In addition, their limited availability, high cost and ethical considerations further present practical limitations on the use of non-human primates. It is also worth noting that human RSV is semi-permissive in non-human primates and the inoculum needs to be very large, with challenge viruses matched to their hosts and able to appropriately inhibit type 1 interferon and accomplish all other immune-evasion strategies. Typically, several mL of high-titre virus stock need to be given in each nostril and sometimes intratracheally – which does not reflect the type of transmission that occurs in humans.

B.5.2.4 Calf model

Calves are a natural host for bRSV and efficiently replicate the virus in the upper and lower respiratory tract. Infection with bRSV in calves causes a spectrum of clinical disease resembling the disease observed in RSV-infected human infants, such as fever, nasal discharge, cough, and tachypnea with chest retractions, wheezing, hypercapnia and hypoxemia (124). 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 that 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. However, these outcomes could not be consistently reproduced in all studies reported (89).

Since the fusion protein ectodomains of bRSV and human RSV share significant homology and since other viral proteins are highly conserved across strains, the calf model challenged with bRSV may have value in demonstrating the protective efficacy of a vaccine based on human RSV-F and for assessing the risk of vaccine-associated ERD. However, an absence of protection in this model may not be predictive of the human situation.

Disadvantages of this model include the need to use a different (that is, non-human) RSV strain, the need for very large inocula and the need for expertise in working with large animals. Due to the large size of the lung and the potentially unequal distribution of signs of disease within it, there are also concerns that sampling errors could interfere with an accurate evaluation of pathology.

B.6 Environmental risk assessment

Inactivated or protein-based RSV vaccines are unlikely to result in significant risk to the environment and thus are exempted from specific environmental risk assessment (ERA) studies. However, live vaccines attenuated by genetic modification or live viral vectors pose a potential risk of spread to a third party – that is, unvaccinated humans and/or animals. For such vaccines, an ERA may be required as part of the preclinical evaluation. Data on the phenotype of live-attenuated vaccine virus or live viral vectors (including their degree of attenuation and replication, their genetic stability, the potential for reversion to a virulent virus and the possibility of shedding following vaccine administration) will contribute at least in part to the ERA.

In addition, the issue of the vector's potential for recombination with other infectious agents that might coincidentally occur in vaccinees should be addressed as appropriate. For live-attenuated RSV vaccines, one study has suggested that the rate of RSV recombination can be very low and may not pose a concern for vaccine safety (125).

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 (114) and the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (16). 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 the 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 immunization of infants and toddlers (aged 28 days to 23 months), including those who were born prematurely;

- active immunization of pregnant women, with the primary aim of protecting the infant in the first months of life;
- active immunization of older adults (for example, aged ≥ 50), including subjects with comorbidities.

Sponsors may wish to investigate the use of RSV vaccines in other populations. These may include neonates (0–27 days), children from 2 years of age and adults and/or subjects with comorbidities or immunodeficiencies predisposing to the development of RSV disease. Safety and immunogenicity data should be obtained in each target population in accordance with sections C.2 and C.4 below. Section 6 of the WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (16) considers the possible need for efficacy trials and the extrapolation of results of vaccine efficacy trials between populations.

At the time of preparing the current guidance, no vaccines against RSV had been approved. Subjects who have already received any RSV vaccine should not be enrolled into clinical trials intended to be confined to RSV-naïve subjects. Furthermore, depending on the trial objectives, it may be necessary to exclude subjects who have received any RSV vaccine from trials intended to be confined to RSV-experienced subjects.

C.2 Immunogenicity trials

C.2.1 Assays

General guidance on the use and validation of assays for measuring immune responses is provided in the WHO Guidelines on clinical evaluation of vaccines; regulatory expectations (16). This section provides specific 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

C.2.1.1.1 *Neutralizing antibodies*

Serum RSV neutralization assays occur in a multitude of formats (91, 126). Sponsors should provide detailed information on 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 (127, 128). 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 lineage (73, 129–132) or other contemporary strains as they become available. The use of both RSV/A and RSV/B viruses will help to

verify the ability of a vaccine to elicit antibodies capable of broadly neutralizing RSV strains irrespective of subtype.

The read-out for the assay (for example, cytopathic effect, plaque counts, fluorescence, luminescence or gene copy number) should be described. Adequate controls should be used to define a valid test and to justify the pooling of data across assay runs. When calculating neutralization end-points, the final serum dilution should take into account the addition of the challenge virus. The method used to calculate end-point titres should be provided. Generally, it is recommended that the end-point should be derived from the linear portion of the titration curve.

The results should be reported in International Units (IU) along with information on the performance of the international standard. The First WHO International Standard for antiserum to respiratory syncytial virus (91–93) has been established to facilitate comparison of RSV neutralizing antibody responses across different neutralization assay formats, thereby permitting a closer comparison of the responses elicited by various candidate vaccines. To date, the international standard has been validated for the harmonization of RSV neutralization assays using post-infection adult and paediatric sera, as well as adult vaccinee sera representing three candidate RSV-F vaccines with similar antigen conformations assayed against RSV/A and RSV/B strains. The international standard harmonizes output when RSV/A and RSV/B strains are used, with or without the inclusion of complement in the assays.

C.2.1.1.2 *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 appropriate bridging studies in order to minimize variability in results.

- Use of 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 the pre-fusion and/or post-fusion conformations of RSV-F, with appropriate bridging studies performed to confirm the suitability of each new lot of RSV-F antigen prior to use. Some pre-fusion RSV-F epitopes are specific to RSV/A or RSV/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 (53, 74, 133, 134).

- Purified recombinant proteins or synthetic peptides may be used in EIAs to detect antibody responses against antigens such as RSV-G/A and RSV-G/B proteins. Antibody responses against RSV proteins not included in the vaccine may support surveillance for RSV exposures/infections during follow-up (135).

Antibody responses to a specific protein or epitope may be detected using competitive binding studies based on EIA formats or biosensor technology wherein antibody binding to the antigen of interest is evaluated in the presence of a competitor (136).

C.2.1.2 Cell-mediated immunity

C.2.1.2.1 CD8+ T-cell responses

Ideally, CD8+ T-cells are collected at 7–14 days after a vaccine dose in adults for determination of sensitization by *in vitro* stimulation with RSV antigens (60, 137). A similar sampling window may apply to infants and children based on the finding that CD8+ T-cell responses in peripheral blood peaked between 11 and 15 days after onset of symptoms in RSV-infected infants (64). The optimal sampling time for detecting CD8+ T-cells may vary by vaccine platform and a broader window may be considered if supported by data for the vaccine under study.

C.2.1.2.2 CD4+ T-cell responses

CD4+ T-cells in infants less than 6 months of age are epigenetically programmed to have a dominant Th2 cytokine response that may be antigen specific (65, 66). 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) to Th1 cytokines (such as IL-2 and IFN- γ) following *in vitro* re-stimulation (using overlapping peptides representative of vaccine antigens, inactivated virus or purified protein antigen). CD4+ T-cell memory response in RSV-naïve infants given a priming dose of vaccine may be detected as early as 10–14 days after this first dose but these memory responses should persist and may also be detected using samples collected at later time points.

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. For live viral-vectored vaccines these initial studies should provide an indication of whether pre-existing or vaccine-associated immune responses to the vector

have an impact on responses to the RSV antigen and whether such issues need to be explored in sequential trials in target populations.

C.2.2.1 **Infants and toddlers**

Unless justified based on accumulated evidence specific or relevant to the candidate vaccine, a safety and immunogenicity trial should be conducted in RSV-experienced subjects before considering a trial in RSV-naive subjects. A definition of RSV-naive and RSV-experienced subjects should be included in the protocol. For example, RSV-naive subjects could be defined as having no documented history of RSV disease and no immunological evidence of prior exposure to RSV. It may also be possible to apply an age cut-off based on relevant epidemiological data to minimize the risk that RSV-naive subjects are falsely determined to be RSV-experienced.

The potential for maternal antibody to interfere with the immune response to active immunization 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 (for example, 6 months after 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. Dose regimens for pregnant women may aim to maximize the difference in RSV neutralizing 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 timing of maternal vaccination.

Documenting the RSV neutralizing antibody decay curve in vaccinated women following delivery (for example, for 3–6 months) may give an early indication of the need to revaccinate women during each pregnancy. Consideration should be given to investigating the safety and immunogenicity of revaccination post-delivery or during a subsequent pregnancy whenever the opportunity arises in the post-approval period.

Documenting the RSV neutralizing antibody decay curve in infants until titres are below the limit of quantitation of the assay may give an early indication of the maximum duration of protection that might be expected.

C.2.2.3 Older adults

It is important that data are obtained from all age subgroups (for example, < 65, 65–74, 75–84 and ≥ 85 years) within the target population in safety and immunogenicity trials to explore the possibility that age-subgroup-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 – for example, after 1–2 years since the primary dose(s) – to randomized subsets of subjects. This information can be used to support a revaccination strategy if this is later concluded to be appropriate from the 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 between groups randomized to the candidate vaccine or to no vaccination against RSV. At least one trial should be conducted in each target population subgroup proposed for the candidate vaccine (for example, in infants \pm toddlers, in pregnant women and/or in older adults) depending on the perceived suitability of the candidate vaccine for these population subgroups.

The WHO Guidelines on clinical evaluation of vaccines: regulatory expectations (16) provide guidance on the need for, and design of, efficacy trials when there is a licensed vaccine available that is widely recommended for use in the target population for a candidate vaccine. The Guidelines also discuss 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 Statistical principles for clinical trials, ICH Topic E9 (138), consideration should be given to the stratification of subjects at randomization by important known or suspected prognostic factors measured at baseline. The factors on which randomization has been stratified should be accounted for in the analysis.

Efficacy trials in infants and toddlers and in older adults may aim to recruit subjects just before the expected RSV season (where seasonality occurs) to accumulate the required number of cases for the primary analysis as quickly as possible.

It is recommended that efficacy trials that evaluate passive protection of infants born to mothers vaccinated during pregnancy should recruit women who are expected to deliver shortly before, or in the early weeks of, the RSV season. In this way, their infants can be followed for efficacy through one RSV season during which maternal antibody levels will decay. See also section C.4.2 below.

It is recommended that post-vaccination blood samples are obtained at least from a randomized subset of subjects, and preferably from all subjects, at a fixed point in time to allow for an exploration of vaccine efficacy according to immunological parameters and possible identification of an ICP.

Depending on the population(s) in which efficacy is to be evaluated, sponsors should consider the range of clinical and laboratory parameters that could be captured in addition to those to be included in the primary case definition (see section C.3.2 below). Data on additional parameters could be used to support secondary or exploratory analyses of efficacy in subgroups defined by the presence or absence of specific clinical and/or laboratory findings, including cases caused by RSV/A compared to RSV/B. In this regard, the efficacy trials will not be powered to determine efficacy against RSV/A or RSV/B and the numbers of cases due to each subtype is expected to vary depending on the regions in which trials are conducted and the seasons. Therefore, data demonstrating the ability of the vaccine to elicit broadly comparable immune responses to subtypes A and B will be important in supporting an expectation of efficacy regardless of RSV type.

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.

It is not expected to be feasible to determine baseline RSV serostatus prior to enrolment into efficacy trials. To allow for a retrospective analysis of vaccine efficacy according to RSV-naive and RSV-experienced status, it is recommended that baseline blood samples are obtained at least from a randomized subset of subjects and preferably from all subjects.

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.

Consideration should be given to information expected to be available on placental insufficiency at trial sites. If such data are expected to be widely available, protocols should state whether pregnant women with any evidence of placental insufficiency are eligible for enrolment. For example, if there are cord blood data to suggest that vaccination increases the anti-RSV neutralizing 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 trial 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 end-points

C.3.2.1 Primary case definition

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

C.3.2.1.1 Clinical criteria

The primary case definition could be any RSV disease or could be confined to RSV LRTI, which could also be defined by severity.

The list of clinical signs and symptoms and the number to be met must be tailored to the age range of the trial population (for example, 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. For example, in an efficacy trial in infants and young children, information could be captured on respiratory rates, oxygen saturation, lower chest wall indrawing, new-onset apnoea, acute ventilatory failure and inability to feed. For objective measurements, such as respiratory rate and oxygen saturation, the actual values should be recorded at intervals during the illness.

Sponsors are advised to take account of proposals for classifying RSV disease severity in different age groups that come from well-recognized public health or professional bodies. For example, WHO has published suggested clinical criteria for defining severe RSV disease in infants and toddlers (11). Published clinical scores suitable for application to RSV disease could also be considered.

C.3.2.1.2 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 the amplification of RSV nucleic acid sequences – for example, RT-PCR (11, 12). It is recommended that the same RDT (for example, a NAT-based assay 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 (see also section C.3.3 below).

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 RSV/B strains. The test method should be able to detect a low copy number (for example, < 103 gene copies per mL or < 50 gene copies per reaction) of the target RSV sequence. In clinical trials involving live-attenuated RSV vaccines, a NAT-based assay should have the ability to differentiate between vaccine and wild-type RSV strains. For example, some live-attenuated RSV 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 or nasopharyngeal swab and/or nasal wash samples. Other samples such as nasal secretions (mucus), sputum, tracheal aspirates, bronchial alveolar lavage samples and postmortem 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 (NP) swabs or nasal wash (NW) aspirates will be acceptable to trial subjects. Whilst NS may be more sensitive in detecting RSV shedding (139), NW aspirates may be better at detecting virus when quantities are low (140–143). 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 (for example, collection medium blanks) should be processed and tested with clinical samples to ensure that no cross-contamination occurs. A human 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 is used to identify cases meeting the primary and other case definitions (16). The method of case ascertainment should be tailored to the geographical distribution of trial sites

and should include instructions to subjects and caregivers on trigger signs and symptoms of possible RSV disease and on presentation to site staff and/or participating health care facilities.

Nevertheless, some trial subjects who develop RSV disease may present at health care facilities not participating in the trial, where confirmation of the diagnosis of RSV may occur using different laboratory tests to those listed in the trial protocols. It is recommended that sponsors plan prospectively for these occurrences. Every effort should be made to capture these cases and to obtain and record clinical and laboratory data from the non-participating health care facilities. The protocol and statistical analysis plan (SAP) should clarify how these cases may be included in the primary or any predefined secondary analyses (for example, it may be appropriate that only those cases for which certain data elements are available should be included in certain analyses).

C.3.4 Analysis of efficacy

If the primary analysis is based on a primary end-point defined as all RSV disease (that is, regardless of severity – see section C.3.2.1 above) then secondary analyses should be conducted based on RSV LRTI, severe RSV disease and/or other case definitions.

In infants and toddlers and in older adults, the primary analysis may be confined to RSV cases with onset after a stated minimum number of days after completion of the assigned dose(s). In such cases, it is important that a secondary analysis compares numbers of cases that occur at any time from randomization. Additionally, a secondary analysis should address the time between the last assigned dose (scheduled or completed) and the onset of disease.

In trials that evaluate protection afforded by maternal antibody, 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 that elapsed between maternal vaccination and delivery.

Some additional considerations for population subgroups are provided below.

C.3.4.1 Infants and toddlers

In efficacy trials that enrol RSV-naive subjects it is essential that detailed 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 ERD (see section C.4.1 below).

There is interest in evaluating whether RSV vaccination impacts on the rate of asthma and symptomatic wheezing in children. This could be investigated

in the post-licensure period. This would require a clear definition of symptomatic wheezing (for example, 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 if so its duration.

C.3.4.2 **Pregnant women**

Some infants born to mothers who were vaccinated during pregnancy may be eligible for routine use of an anti-RSV monoclonal antibody in line with local guidance, in which case it may be appropriate to exclude them from the primary analysis of efficacy if they have received such a monoclonal. If excluded from the primary analysis, cases of RSV disease in these subjects should be captured and included in a secondary analysis of efficacy in the all-randomized population.

C.3.4.3 **Older adults**

It is recommended that, unless an ICP has been established, subjects should continue to be followed for RSV disease to assess the potential need for revaccination and the intervals at which this may be required to maintain protection. One approach may be to sub-randomize subjects initially allocated to the vaccine group to receive or not receive revaccination and to 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 (for example, on local and systemic reactogenicity) obtained from trials in RSV-experienced subjects of various ages may be poorly predictive of the safety profile in RSV-naive subjects. Therefore, a cautious approach is recommended when commencing trials that enrol RSV-naive subjects.

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

Any vaccine-associated ERD would be expected to occur with the first natural RSV infection after completion of vaccination. Unless otherwise justified based on evidence relevant to the candidate vaccine construct, it is recommended that the duration of follow-up for RSV disease in all trials that include RSV-naive subjects should be sufficient to maximize the likelihood that

subjects will have been exposed to wild-type RSV. The duration of follow-up for RSV disease to address this issue in any one trial should be decided upon based on knowledge of the rate of natural exposure with increasing age in the region(s) in which the trial is conducted. The assessment of risk in any one trial that includes RSV-naïve subjects – even if this is a preliminary evaluation conducted as part of a relatively small immunogenicity trial – should be completed before initiating the next trial(s) in which RSV-naïve subjects will be exposed to the candidate vaccine.

Additional safety considerations for trials with live-attenuated RSV vaccines in RSV-naïve infants include the need to assess the duration and magnitude of virus shedding. Depending on the results, consideration should be given to conducting a study to assess the risk of transmission to RSV-naïve close contacts. Until the risk has been assessed and/or based on results, precautions should be put in place to minimize the risk of transmission of the vaccine virus from vaccinees during the period of virus shedding to contacts who are under 1 year of age and/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 to vaccination (including fever) should be assessed in non-pregnant women before proceeding to the vaccination of 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.

As discussed in section C.3 above, trials involving maternal vaccination should follow-up infants to assess protection against RSV disease through one season (or the equivalent period in non-seasonal regions). The data collected on the severity of cases of RSV disease should be reviewed to assess whether there is any signal for vaccine-associated ERD in infants born to vaccinated mothers compared to those born to unvaccinated mothers.

C.4.3 **Older adults**

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

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 (144) and WHO Guidelines for independent lot release of vaccines by regulatory authorities (145) 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 (145). A summary protocol for the manufacturing and control of RSV vaccines, based on the model summary protocol provided in Appendix 1 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 vaccine. A model NRA Lot Release Certificate is provided below in Appendix 2.

Authors and acknowledgements

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Taking into consideration the comments received, a second draft was prepared by Dr J. Beeler, United States Food and Drug Administration, the USA, Dr E. Kretzschmar, Paul-Ehrlich-Institut, Germany), Dr M. Powell, Health Products Regulatory Authority, Ireland, Dr Y. Sun, Paul-Ehrlich-Institut, Germany and Dr T.Q. Zhou, World Health Organization, Switzerland. The second draft was posted on the WHO Biologicals website for a first round of public consultation from 22 January to 28 February 2019 and comments were received from: Dr M.C. Annequin, Agence nationale de sécurité du médicament et des produits de santé, France; Dr P. Dormitzer, Pfizer Vaccines, the USA; Dr I. Feavers, National Institute for Biological Standards and Control, the United Kingdom; Dr D. Feikin, World Health Organization, Switzerland; Dr C. Heery, Bavarian Nordic, Inc., the USA; Dr D. Higgins, PATH, the USA; Dr N. Hosken, PATH, the USA; Dr J.L. Hurwitz, St. Jude Children's Research Hospital, the USA; Dr D. Kapse, Serum Institute of India Pvt. Ltd, India; Dr J. Korimbocus, Agence nationale de sécurité du médicament et des produits de santé, France; Dr C. Li, National Institutes for Food and Drug Control, China; Dr D.L. Skea, Sanofi Pasteur, France (*on behalf of IFPMA*); Dr K. Smits, Federal Agency for Medicines and Health Products, Belgium; Dr W. Van Molle, Sciensano, Belgium; and Dr L.R. Yeolekar, Serum Institute of India Pvt. Ltd, India.

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Based on the outcomes of the above informal consultation, a fourth draft was prepared by Dr J. Beeler, United States Food and Drug Administration, the USA, Dr E. Kretzschmar, Paul-Ehrlich-Institut, Germany, Dr M. Powell, Health Products Regulatory Authority, Ireland, Dr Y. Sun, Paul-Ehrlich-Institut, Germany and Dr T.Q. Zhou, World Health Organization, Switzerland. The resulting document WHO/BS/2019.2355 was then posted on the WHO Biologicals website for a second round of public consultation from June to September 2019 and written comments received from: Dr I. Feavers, National Institute for Biological Standards and Control, the United Kingdom; Dr D. Higgins, PATH, the USA; Dr J. Korimbocus, Agence nationale de sécurité du médicament et des produits de santé, France; Dr J. Southern, Advisor to the South African Health Products Regulatory Authority; Dr W. Van Molle, Sciensano, Belgium; Dr L. Yeolekar, Serum Institute of India Pvt. Ltd, India; and Dr W. Weston (*on behalf of IFPMA provided the consolidated comments of Daiichi Sankyo, Japan; GSK Vaccines, Belgium; Janssen Vaccines & Prevention B.V., Netherlands; Pfizer Vaccines, the USA; and Sanofi Pasteur, France*). Special input was also sought on scientific issues from: Dr B.S. Graham, National Institutes of Health, the USA; and Dr R.A. Karron, Johns Hopkins University, the USA.

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Appendix 1

Model summary protocol for the manufacturing and control of 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):
- 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, mycoplasma, 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 vaccine bulk (where applicable)**

3.5.1 **Information on manufacture**

- Batch number(s):
- Date of formulation:
- Total volume of monovalent bulk prepared:
- Virus/bacteria harvest used for formulation:
- Lot number/volume added:
- Virus/bacteria 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 (if applicable):
- Residual host cell protein (if not done on final bulk or final product):
- Residual host cell DNA (if non-primary cell lines; if not done on final bulk or final product):
- Potency:
- Particle number (if relevant, for example for adenovirus):
- Infectious virus titre and particle-to-infectivity ratio (if relevant, for example for adenovirus):
- Expression of heterologous antigen in vitro:
- Replication competence (if relevant, for example for adenovirus):
- pH:
- Preservative content (if applicable):
- Endotoxin:
- Sterility or bioburden:

3.6 Control of final vaccine bulk (where applicable)

3.6.1 Information on manufacture

- Batch number(s):
- Date of formulation:
- Total volume of final bulk formulated:
- Monovalent bulk vaccine used for formulation:
- Volume(s), storage temperature, storage time and approved storage period:
- Lot number/volume added:
- Virus/bacteria 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 doses, if the product is presented in a multiple-dose 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:
- 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):
- Pyrogenic substances (if applicable):
- Adjuvant content (if applicable):
- Protein content (if applicable):
- Degree of adsorption (if applicable)
- Potency:
- Particle number (if relevant, for example for adenovirus):
- Infectious virus titre and particle-to-infectivity ratio (if relevant, for example for adenovirus):
- Expression of heterologous antigen in vitro:
- Purity (if applicable):
- Bacterial concentration (for chimeric BCG/RSV vaccines):
- Tests for viability (for chimeric BCG/RSV vaccines):
- Extractable volume (if applicable):
- Aggregates/particle size (if applicable)

- Viability (for chimeric BCG/RSV vaccines)
- 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 A¹ of the WHO Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines.²

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. 1024, Annex 2.

Appendix 2

Model NRA Lot Release Certificate for RSV vaccines

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

Certificate no. _____

The following lot(s) of RSV vaccine produced by _____¹ in _____,² whose lot numbers appear on the labels of the final containers, meet all national requirements³ and Part A⁴ of the WHO Guidelines on the quality, safety and efficacy of respiratory syncytial virus vaccines⁵ and comply with WHO good manufacturing practices for pharmaceutical products: main principles,⁶ WHO good manufacturing practices for biological products,⁷ and Guidelines for independent lot release of vaccines by regulatory authorities.⁸

The release decision is based on _____⁹

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;

¹ Name of manufacturer.

² Country of origin.

³ If any national requirements have not been met, specify which one(s) and indicate why the release of the lot(s) has nevertheless been authorized by the NRA.

⁴ With the exception of provisions on distribution and shipping, which the NRA may not be in a position to assess.

⁵ WHO Technical Report Series, No. 1024, Annex 2.

⁶ WHO Technical Report Series, No. 986, Annex 2.

⁷ WHO Technical Report Series, No. 999, Annex 2.

⁸ WHO Technical Report Series, No. 978, Annex 2.

⁹ Evaluation of the product-specific summary protocol, independent laboratory testing and/or specific procedures laid down in a defined document, and so on as appropriate.

- 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 appropriate authority)

Name (typed) _____

Signature _____

Date _____