If chicken cell cultures are used, a sample of fluids pooled from the control cultures shall be tested for adenoviruses and for avian retroviruses such as avian leukosis virus, by a method approved by the national regulatory authority.

A test for retroviruses using a sensitive polymerase chain reaction (PCR)-based reverse transcriptase (Rtase) assay may be used. The results of such assays need to be interpreted with caution because Rtase activity is not unique to retroviruses and may derive from other sources, such as retrovirus-like elements that do not encode a complete genome (15). Nucleic acid amplification tests for retrovirus may also be used.

3.2.2.1.5 Identity test

At the production level, and for vaccines produced in human diploid cells or continuous cells only, the cells should be identified by means of tests approved by the national regulatory authority.

Suitable tests are isoenzyme analysis, immunological tests and cytogenetic marker tests.

3.2.2.2 Cell cultures for vaccine production

3.2.2.2.1 Tests for adventitious agents

On the day of inoculation with the virus working seed lot, each cell culture or a sample from each culture vessel should be examined for degeneration caused by infective agents. If such examination

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shows evidence of the presence in a cell culture of any adventitious agent, the culture should not be used for vaccine production.

If animal serum is used for cell cultures before the inoculation of virus, the medium should be removed and replaced with serum-free maintenance medium, after the cells have been washed with serum-free medium, if appropriate. If suspension cultures are used, testing should be conducted to assure that serum levels are reduced to acceptable levels. This testing may be performed at a later stage of production.

3.2.2.2.2. Tests for bacteria, fungi and mycoplasmas

A volume of at least 20 ml of the pooled supernatant fluids from the production cell culture should be tested for bacterial and fungal sterility and for mycoplasmas. The tests for bacterial, fungal and mycoplasmal sterility should be performed as described in the revised General Requirements for the Sterility of Biological Substances (Requirements for Biological Substances No. 6) (16).

3.2.2.3 Control of single harvests

3.2.2.3.1 Single harvest

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After inoculation of the production cells with the virus working seed lot, both inoculated and control cell cultures should at no time be at a temperature outside the range approved by the national regulatory authority for the relevant incubation periods. The optimal range for pH, multiplicity of infection, cell density and time of incubation should be established for each manufacturer, and be approved by the national regulatory authority.

The virus suspension should be harvested not later than a number of days after virus inoculation agreed by the national regulatory authority.

It is advisable that the inoculated cell cultures should be processed in such a manner that each virus suspension harvested remains identifiable as a single harvest and is kept separate from other harvests until the results of all the tests prescribed in Part A sections 3.2.2.1, 3.2.2.2 and 3.2.2.3.3, 3.2.2.3.4 and 3.2.2.3.5 have been obtained.

3.2.2.3.2 Sampling

Samples required for the testing of single harvests should be taken immediately on harvesting. If the tests for adventitious agents as described in Part A, section 3.2.2.3.3 are not performed immediately, the samples taken for these tests should be kept at a temperature of -60 ^{o}C or lower, and subjected to no more than one freeze-thaw cycle.

3.2.2.3.3 Tests of neutralized single harvests for adventitious agents

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For the purposes of the recommendations specified in this section of Part A, the volume of each single harvest taken for neutralization and testing should be at least 10 ml and should be such that at least a total of 50 ml or the equivalent of 500 doses of final vaccine, whichever is the greater, has been withheld from the corresponding bulk suspension.

The antisera used for neutralization should be of non-human origin and should have been prepared using virus cultured in cells from a species different from that used in the production of the vaccine. Samples of each virus harvest should be tested in human cells sensitive to measles and at least one other sensitive cell system.

Complete neutralisation of vaccinia virus may be difficult to achieve at high virus concentrations. If this is the case, specific tests can supplement non-specific testing by standard tissue culture tests or eggs. Specific tests could include PCR, immunochemical tests or antibody production tests in animals. The extent of testing for specific adventitious agents may vary and depends on the agents that could be present based on the nature and origin of the substrate used for vaccine production and the origin of the virus seed. The NRA should approve the test program for viral adventitious agents. For the sake of saving costly biological reagents, such as high titred vaccinia neutralising sera, testing for viral adventitious agents may not necessarily be performed at the level of the single harvest but may be performed at later stages of the manufacturing process, for example at the level of the final bulk. This option should be approved by the NRA.

The neutralized suspensions should be inoculated into bottles of these cell cultures in such a way that the dilution of the suspension in the nutrient medium does not exceed 1 in 4. The area of the cell

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sheet should be at least 3 cm^2 per ml of neutralized suspension. At least one bottle of each kind of cell culture should remain uninoculated and should serve as a control and should be maintained by nutrient medium containing the same concentration of the specific antiserum used for neutralization.

Animal serum may be used in the propagation of the cells, but the maintenance medium used after inoculation of the test material should contain no added serum other than the smallpox neutralizing antiserum.

The inoculated cultures should be incubated at a temperature of 35-37 ^{o}C and should be observed for a period of at least 14 days.

If adequately justified lower temperatures may be used.

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

If any cytopathic changes due to adventitious agents occur in any of the cultures, the virus harvest should be discarded.

3.2.2.3.4 Additional tests if chick cell cultures are used for production

A volume of each neutralised virus pool equivalent to 100 human doses of vaccine or 10ml, whichever represents the greatest volume, shall be tested in a group of fertilised chicken's eggs by the allantoic route of inoculation, and a similar sample shall be tested in a separate group of eggs by the yolk-sac route of inoculation. In both cases 0.5ml of inoculum shall be used per egg.

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The virus pool passes the test if, at the end of a three to seven day observation period, there is no evidence of the presence of any adventitious agents. If an adventitious agent is detected in the uninoculated controls, the test may be repeated.

3.2.2.3.5 Sterility tests

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given the revised Requirements for Biological Substances No. 6 (16)

3.2.3 Production In Primary Rabbit Kidney Cells

3.2.3.1 Tests of cell cultures used for vaccine production

On the day of inoculation with virus working seed lot, each cell culture should be examined for degeneration caused by an infective agent. If, in this examination, evidence is found of the presence in a cell culture of any adventitious agent, the entire group of cultures concerned should not be used for vaccine production.

On the day of inoculation with the virus working seed lot, a sample of at least 30 ml of the pooled fluid is removed from the cell cultures of the kidneys of each group of animals used to prepare the primary cell suspension The pooled fluid should be tested in primary kidney-cell cultures prepared from the same species, but not the same group of animals, as that used for vaccine production. The

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pooled fluid should be inoculated into bottles of these cell cultures in such a way that the solution of the pooled fluid in the nutrient medium does not exceed 1 in 4. The area of the cell sheet should be at least 3 cm^2 per ml of pooled fluid. At least one bottle of each primary cell culture should remain uninoculated and should serve as a control.

The cultures should be incubated at a temperature of 34-36 ^{o}C and should be observed for a total period of at least four weeks. During this observation period and after not less than two weeks incubation, from each of these cultures at least one subculture of fluid should be made in the same tissue culture system. The subculture should also be observed for at least two weeks.

Serum may be added to the original culture at the time of subculturing, provided that the serum does not contain antibody or other inhibitors to adventitious agents of the cell culture donor species.

For the tests to be valid, not more than 20% of the culture vessels should have been discarded for non-specific accidental reasons by the end of the respective test periods.

If, in these tests, evidence is found of the presence of an adventitious agent, the single harvest from the whole group of cell cultures concerned should not be used for vaccine production.

If these tests are not done immediately, the samples of pooled cell-culture fluid should be kept at a temperature of -60 ^{o}C or below

3.2.3.2 Tests of control cell cultures

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Cultures prepared on the day of inoculation with the virus working seed lot from 25%, of the cell suspension obtained from the kidneys of each group of animals used to prepare the primary cell suspension should remain uninoculated, and should serve as controls. These control cell cultures should be incubated under the same conditions as the inoculated cultures for at least two weeks, and

should be examined during this period for evidence of cytopathic changes. For the tests to be valid, not more than 20% of the control cell cultures should have been discarded for non-specific, accidental reasons. At the end of the observation period, the control cell cultures should be examined for degeneration caused by an infectious agent. If this examination or any of the tests required in this section shows evidence of the presence in a control culture of any adventitious agent, the vaccinia grown in the corresponding inoculated cultures from the same group should not be used for vaccine production.

3.2.3.2.1 Tests for haemadsorbing viruses.

At the time of harvest or not more than four days after the day of inoculation of the production cultures with the virus working seed lot, a sample of 4% of the control cell cultures should be taken and should be tested for haemadsorbing viruses. At the end of the observation period, the remaining control cell cultures should be similarly tested. The tests should be made as described in Part A, section 3.2.2.1.2.

3.2.3.2.2 Tests for other adventitious agents.

At the time of harvest, or not more than seven days after the day of inoculation of the production cultures with the working seed lot, a sample of at least 20 ml of the pooled fluid from each group of

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control cultures should be taken and tested in primary kidney-cell cultures, as described in Part A, section 3.2.3.1.

At the end of the observation period for the original control cell cultures, similar samples of the pooled fluid should be taken and the tests in primary kidney-cell cultures should be repeated, as described in Part A, section 3.2.3.1.

In some countries, fluids are collected from the control cell cultures at the time of virus harvest and at the end of the observation period. Such fluids may then be pooled before testing for adventitious agents.

3.2.3.3 Tests for neutralized single harvests in primary kidney-cell cultures.

The specifications given in part A section 3.2.2.3.3 shall apply with the addition that each neutralized single harvest should be additionally tested in primary kidney-cell cultures prepared from the same species, but not the same group of animals, as that used for vaccine production.

3.2.3.4 Sterility tests

A volume of at least 10 ml of each single harvest should be tested for bacterial, fungal and mycoplasmal sterility according to the requirements given the revised Requirements for Biological Substances No. 6 (16)

3.2.4 Production In Animal Skins

3.2.4.1 Vaccines produced in the skin of living animals

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The animals shall be freed of ectoparasites, and each animal shall be kept under veterinary supervision for at least two weeks prior to the inoculation of the seed virus. Before inoculation the animals shall be cleaned, and thereafter kept in scrupulously clean stalls until the vaccinal material is harvested.

The use of bedding, unless sterilized and changed frequently, should be avoided. The stalls, including feed boxes, should be designed so as to make cleaning easy, and dust-producing food should be avoided.

During a period of five days before inoculation and during incubation the animals shall remain under veterinary supervision, they shall remain free from any sign of disease, and daily rectal temperatures shall be recorded. If any abnormal rise in temperature occurs, or if any clinical sign of disease is observed, the production of vaccine from the group of animals concerned shall be suspended until the cause of these irregularities has been resolved. The prophylactic and diagnostic procedures adopted to exclude the presence of infectious disease shall be submitted for approval to the national control authority.

According to the species of animal used and the diseases to which that animal is liable in the country where the vaccine is being produced, the prophylactic and diagnostic procedures to be used will vary. They must exclude the possibility of transmitting diseases within the country where the vaccine is prepared, but consideration should also be given to the danger of spreading diseases to other countries or continents to which the vaccine may be shipped.

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Special attention should always be given to foot-and-mouth disease, transmissible spongiform encephalopathies, brucellosis, Q fever, tuberculosis, and dermatomycosis, but in some areas it will be necessary to consider diseases such as contagious pustular dermatitis (orf), pulpy kidney disease, sheep pox, anthrax, rinderpest, haemorrhagic septicaemia, Rift Valley fever, and many others.

The inoculation of seed virus shall be made on such parts of the animal as are not liable to be soiled by urine and faeces. The surface used for inoculation shall be so shaved and cleaned as to procure the

nearest possible approach to surgical asepsis. If any antiseptic substance deleterious to the virus is used in the cleaning process it shall be removed by thorough rinsing with sterile water prior to inoculation. During inoculation, the exposedsurface of the animal not used for inoculation shall be covered with sterile covering.

Many workers prefer to inoculate the ventral surface of female animals. If male animals are used this area is more liable to soiling by urine and faeces than the flank, which may be equally susceptible to vaccinia virus and easier to keep clean, especially since the animal tends to rest on the uninoculated side.

It is recommended that the animal be anaesthestized during the process of shaving, cleaning and inoculation.

After inoculation the area may be covered with suitable antibiotics.

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Before the collection of the vaccinal material, any antibiotic shall be removed and the inoculated area shall be subjected to a repetition of the cleaning process. The uninoculated surfaces shall be covered with sterile covering.

Before harvesting, the animal shall be killed painlessly. The animals shall be exsanguinated before harvesting to avoid heavy admixture of the vaccinal material with blood.

The vaccinal material from each animal shall be collected separately with aseptic precautions.

All animals used in the production of vaccine shall be examined by autopsy. Special attention should be given to examine the central nervous system for evidence of transmissible spongiform encepholopathy. If evidence of any generalized or systemic disease other than vaccinia is found, or encephalopathy, the vaccinal material from the entire group of animals exposed shall be discarded.

3.3 Control of bulk suspension

3.3.1 Preparation of bulk suspension

The bulk suspension should be treated to remove cell debris.

The national control authority may require the further purification of harvests derived from continuous cell lines to remove cellular DNA, and/or the use of DNAase treatment to reduce the size of DNA fragments. If the harvests are derived from human diploid or primary cell cultures, further purification is not required.

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3.3.2 Sampling

Samples of the bulk suspension prepared as described in section 3.3.1 should be taken immediately and, if not tested immediately, should be kept at a temperature of -60 °C or below until the tests described in the following sections are performed.

3.3.3 Identity test

The vaccinia virus in the bulk suspension should be identified by serological or molecular tests.

Care should be taken to ensure that the sera used are mono-specific by titrating them against homotypic and heterotypic viruses of known virus titre. The use of monoclonal antibodies may be useful in this test. Molecular tests such as restriction fragment length polymorphism or partial sequencing, especially of terminal DNA sequences which show the greatest variation between vaccinia strains, may be useful as identity tests.

3.3.4 Virus concentration

The amount of infective vaccinia virus per ml of filtered bulk suspension should be determined in the chick egg chorioallantoic membrane, or in cell cultures, in comparison with a reference preparation assayed in the same system.

The virus concentration as determined by this test should be the basis for the quantity of virus used in the neurovirulence tests in mice (Part A, section 3.3.5.1) and for preparing the final bulk (Part A,

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section 3.4). The detailed procedures for carrying out this test and for interpreting the results should be those approved by the national regulatory authority.

3.3.5 Test for consistency of virus characteristics

The vaccinia virus in the bulk suspension prepared as described in section 3.3.1 should be tested in comparison with the working seed virus with regard to certain characteristics, as described in the following below, to ensure that the vaccine virus has not undergone changes during its multiplication in the production culture system. From the results of these tests for successive batches of vaccine a critical assessment may be made of the consistency of vaccine quality (see Part B, section 2).

3.3.5.1 Test for neurovirulence

The neurovirulence of the bulk suspension should be compared to a comparator original seed (or equivalent) by intracerebral inoculation of suckling mice.

Direct inoculation of vaccinia virus into the central nervous system of suckling mice has been shown to discriminate between clonal derivatives of vaccinia. It is not a model of post-infectious, demyelinating disease since the pathology is quite distinct. The purpose of the test is to show consistency of production and that the each new filtered bulk suspension is not significantly different in neurovirulence phenotype than the comparator original seed.

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Suckling 3-5 day old CD-1 outbred mice are inoculated intracerbrally with 20ul of the filtered bulk suspension or the comparator vaccine. The target titre of the inoculum is 5.0 log10 pfu/ml. The titre of virus in the inoculum should be confirmed by titration of the residual inocula, and should be within 0.5 log10 pfu of the target. The mice are observed for up to 21 days and the mortality ratio and survival times are compared between groups. The mortality ratio of the filtered bulk suspension should not exceed that of the control comparator group by Fishers exact test, and the filtered bulk suspension should not show more rapid time to death than the comparator control, based on a log rank test.

Other test formats in mice may be used to discriminate between acceptable and unacceptable batches, and may be approved by the NRA.

The NRA may approve neurovirulence tests in other species such as rabbit.

3.3.6 Tests for cellular DNA

For viruses grown in continuous cells the bulk suspension should be tested for residual cellular DNA. By calculation the removal process should be shown to reduce consistently the level of cellular DNA to less than 10 ng per human dose. This test may be omitted, with the agreement of the national regulatory authority, if the manufacturing process is validated to achieve this specification.

The cytoplasmic replication cycle and specific structures needed for genome replication and endcapsidation of vaccinia virus make it very unlikely that host cell DNA is also encapsidated during smallpox vaccine production. Integration of foreign DNA into the vaccinia virus genome is only possible by specific encapsidation or homologous recombination, the latter process requiring a

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minimum length of homologous DNA sequences. From these observations it seems extremely unlikely that cellular DNA sequences would be integrated. However, evolution of viral genes with cellular homologs suggests that this may be able to happen, but rarely, and with selective pressure.

3.4 Final Bulk

The operations necessary for preparing the final bulk should be conducted in such a manner as to avoid contamination of the product.

The dilution and mixing procedures involved in preparing the final bulk should be those approved by the national control authority.

3.4.1 Preservatives, stabilisers and additives

Any stabilizers, preservatives or additives that may be added to the bulk suspension should have been shown to the satisfaction of the national control authority not to impair the safety or efficacy of the

vaccine and to improve the stability of the vaccine in the concentrations used. If phenol is present it's concentration should not exceed 0.5% and it should comply with pharmacopoeial specifications.

It may be advisable to consider the difficulties with human albumin as a stabiliser. This is because the expiry period of albumin may be less than the expiry period of the vaccine, especially where long-term stockpile storage of smallpox vaccines is intended. There may be difficulties too if the batch of human albumin is subject to a recall from the market.

3.4.2 Tests for bacteria and fungi

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For vaccines other than those prepared on animal skins, the final bulk should be tested for bacterial and fungal sterility in accordance with the requirements given in Part A, section 5, of the revised Requirements for Biological Substances No. 6 (Requirements for the Sterility of Biological Substances) (16). If phenol or other preservatives are used, this test should be performed on samples taken before any preservatives are added.

3.4.3 Bioburden tests

For vaccines produced on animal skins only, a volume agreed by the NCA of final bulk after the addition of preservatives should be tested for bacterial bioburden using the tests given the revised Requirements for Biological Substances No. 6 (16). From the number of colonies appearing on the plates the number of living bacteria in 1ml of final bulk shall be calculated. If this number exceeds 50, the final bulk shall be discarded. In addition the tests described in part A sections 3.4.3.1, 3.4.3.2, 3.4.3.3 and 3.4.3.4 shall also be performed.

3.4.3.1 *Test for the presence of* Escherichia coli

At least three 1-ml samples of a 1: 100 dilution of the final bulk after addition of preservatives shall be cultured on plates of a medium suitable for differentiating *E. coli* from other bacteria. The plates shall be incubated for 48 hours at 35°C to 37°C. If *E. coli* is detected, the final bulk shall be subjected to further processing or discarded.

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The need for further treatment should be an exceptional occurrence since the presence of *E.coli* in this test might indicate a heavy faecal contamination. It should be justified by a report into the manufacture of the batch. In some countries further treatment is not permitted.

3.4.3.2 Test for the presence of haemolytic streptococci, coagulase-positive staphylococci, or any other pathogenic micro-organisms which are known to be harmful if introduced into the human body by the process of vaccination

At least three 1-ml samples of a 1: 100 dilution of the final bulk after addition of preservatives shall be cultured on plates of blood agar. The plates shall be incubated for 48 hours at 35°C to 37°C and the colonies appearing shall be examined. If any of the organisms mentioned are detected, the final bulk shall be discarded.

In some countries culture of the final bulk after addition of preservatives in salt meat broth is made for the purpose of detecting staphylococci.

3.4.3.3 Test for the presence of Bacillus anthracis

Any colony seen on any of the plates used in the tests described in Part A, sections 3.4.3., 3.4.3.7., 3.4.3.2 and 3.4.3.3 which morphologically resembles *B. anthracis* shall be examined. If the organisms

contained in the colony are non-motile, further tests for the cultural character of *B. anthracis* shall be made, including pathogenicity tests in suitable animals. If *B. anthracis* is found to be present, the final bulk, and any other associated bulks, shall be discarded.

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In countries where anthrax presents a serious risk, this test should be based on tests of each single harvest. The application of molecular tests for *B anthracis* are encouraged.

3.4.3.4 Test for the presence of Clostridium tetani and other pathogenic spore-forming anaerobes

A total volume of not less than 10 ml of the final bulk after addition of preservatives, taken preferably from the depth of the bulk and not from the upper surface, shall be distributed in equal amounts into ten tubes, each containing not less than 10 ml of a medium suitable for the growth of anaerobic micro-organisms. The tubes shall be held at 65°C for one hour in order to reduce the content of non-spore-forming organisms, after which they shall be incubated for at least one week between 35°C and 37°C. From every tube showing growth, subcultures shall be made on to plates of a suitable medium which shall be incubated anaerobically at the same temperature. All anaerobic colonies shall be examined and identified and if *Cl. tetani* or other pathogenic spore-forming anaerobes are present the final bulk shall be discarded.

Organisms resembling pathogenic *Clostridia* found in the tube culture from which the subculture was made may be tested for pathogenicity by inoculation into animals as follows: Groups of not less than two guinea-pigs and five mice are used for each tube culture to be tested. 0.5 ml of the cultures is mixed with 0.1 ml of a freshly prepared 4% solution of calcium chloride and injected intramuscularly into each of the guinea-pigs; 0.2 ml of the cultures mixed with 0.1 ml of this calcium chloride solution are injected intramuscularly into each of the mice. The animals are observed for one week. If any animal develops symptoms of tetanus, or if any animal dies as a result of infection with spore-forming anaerobes, the final bulk should be discarded.

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If other methods are used for this test, they should have been demonstrated, to the satisfaction of the national control authorities, to be at least equally effective for detecting the presence of *Cl. tetani* and other pathogenic spore-forming anaerobes.

4. FILLING AND CONTAINERS

The requirements concerning filling and containers given in "Good manufacturing practices for biological products"(9) should apply to vaccine filled in the final form.

Care should be taken to ensure that the material of which the container is made does not adversely affect the virus content of the vaccine under the recommended storage conditions.

5. CONTROL TESTS ON FINAL PRODUCT

Samples should be taken from each filling lot for the tests described in the following sections.

5.1 Identity test

The vaccinia virus should be identified by an appropriate method.

Appropriate methods include serology, growth characteristics and molecular methods.

5.2 Tests for bacteria and fungi

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