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REQUIREMENTS FOR PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED GUIDELINE

**VIRAL SAFETY EVALUATION OF BIOTECHNOLOGY
PRODUCTS DERIVED FROM CELL LINES OF HUMAN OR
ANIMAL ORIGIN
Q5A(R2)**

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ICH Consensus Guideline

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1. INTRODUCTION

This guideline concerns the testing and evaluation of the viral safety of biotechnology products, and it outlines what data should be submitted in marketing application and registration packages for those products. Biotechnology products include biotherapeutics and certain biological products derived from cell cultures initiated from characterised cell banks of human or animal origin (e.g., mammalian, avian, insect). In this document, the term “virus” excludes non-conventional transmissible agents like those associated with mammalian prions (e.g., bovine spongiform encephalopathy, scrapie). Applicants are encouraged to discuss bovine spongiform encephalopathy-associated issues with the appropriate regulatory authorities.

This document covers products produced from *in vitro* cell culture using recombinant DNA technologies such as interferons, monoclonal antibodies, and recombinant subunit vaccines. It also covers products derived from hybridoma cells grown *in vivo* as ascites: special considerations apply for these products, and Annex 1 contains additional information on testing cells propagated *in vivo*. The document also applies to certain genetically-engineered viral vectors and viral vector-derived products, which can undergo virus clearance without a negative impact on the product. These products may include viral vectors produced using transient transfection or from a stable cell line, or by infection using a recombinant virus. It also includes viral vector-derived recombinant proteins, for example, baculovirus-expressed Virus-Like Particles (VLPs), protein subunits and nanoparticle-based vaccines and therapeutics. Furthermore, the scope includes Adeno-Associated Virus (AAV) gene therapy vectors that depend on helper viruses such as baculovirus, herpes simplex virus or adenovirus for their production. Specific guidance on genetically engineered viral vectors and viral vector-derived products is provided in Annex 7. Inactivated viral vaccines and live attenuated viral vaccines containing self-replicating agents are excluded from the scope of this document.

The risk of viral contamination is a feature common to all biotechnology products derived from cell lines. Such contamination could have serious clinical consequences and can arise from the contamination of the source cell lines themselves (cell substrates) or from exogenous introduction of adventitious virus during production. To date, however, biotechnology products derived from cell lines have not been implicated in the transmission of viruses. Nevertheless, the safety of these products with regards to viral contamination can be reasonably ensured only by applying a comprehensive virus testing program and assessing virus removal and

32 inactivation achieved by the manufacturing process, as outlined below. Three principal,
33 complementary approaches have evolved to control the potential viral contamination of
34 biotechnology products:

- 35 • Selecting and testing cell lines and other raw materials, including media components,
36 for the absence of undesirable infectious viruses;
- 37 • Assessing the capacity of the production processes to clear infectious viruses; and
- 38 • Testing the product at appropriate steps of production for the absence of contaminating
39 infectious viruses.

40 Some virus clearance steps used during production of genetically engineered viral vectors and
41 viral vector-derived products may not be as effective as when used for recombinant proteins. In
42 such cases, considerations for further risk reduction (e.g., treatment of raw materials, extensive
43 testing for broad virus detection) can be applied (see Annex 7).

44 For statistical reasons, a quantitative virus assay's ability to detect low viral concentrations
45 depends on sample size. Therefore, establishing that an infectious virus contaminant is absent
46 from a product will depend not just on direct testing for the presence of a contaminant, but also
47 on demonstrating that the purification regimen can remove or inactivate the viruses.

48 The type and extent of viral tests and viral clearance studies required at different steps of
49 production will depend on various factors and should be considered on a case-by-case and step-
50 by-step basis. The factors that should be considered include the extent of cell bank
51 characterisation and qualification; the nature of any viruses detected, culture medium
52 constituents, culture methods, facility and equipment design; the results of viral tests after cell
53 culture; the ability of the process to clear viruses; and the type of product and its intended
54 clinical use. The purpose of this document is to provide a general framework for virus testing,
55 experiments for the assessment of viral clearance, and a recommended approach for the design
56 of viral tests and viral clearance studies.

57 Manufacturers should adjust the recommendations presented here to their specific product and
58 its production process. The approach used by manufacturers to ensure viral safety should be
59 explained and justified. In addition to the detailed data that is provided, an overall summary of
60 the viral safety assessment would be useful to regulatory reviewers. This summary should

61 contain a brief description of all aspects of the viral safety studies and strategies used to prevent
62 virus contamination.

63 **2. POTENTIAL SOURCES OF VIRUS CONTAMINATION**

64 Virus contamination of biotechnology products may arise from the original source of the cell
65 lines or from adventitious introduction of virus during production processes, including
66 generation of a recombinant production cell line and/or cell line banking. Introduction of
67 potential adventitious viruses from a Master Virus Seed (MVS) or Working Virus Seed (WVS)
68 is discussed in Annex 7. Use of well characterised banks and MVS or WVS can reduce the risk
69 of virus contamination. Furthermore, helper viruses used for the production of recombinant
70 proteins, VLPs, or gene therapy viral vector products are also considered as process-related
71 viral contaminants (see Annex 7).

72 **2.1 Viruses that Could Occur in the Master Cell Bank**

73 Cells may have latent or persistent virus (e.g., herpesvirus) and endogenous retrovirus, and
74 those viruses can be transmitted vertically from one cell generation to the next. In such cases,
75 the virus may be constitutively expressed or may unexpectedly become expressed as an
76 infectious virus.

77 Viruses may be introduced in the Master Cell Bank (MCB) by several routes such as 1)
78 derivation of the cell line from an infected animal, 2) use of a virus to establish the cell line, 3)
79 use of contaminated biological reagents (e.g., antibodies for selection) or raw materials for cell
80 culturing (e.g., animal or human serum and porcine trypsin), or 4) contamination during cell
81 handling and banking processes.

82 **2.2 Adventitious Viruses that Could be Introduced During Production**

83 Adventitious viruses may contaminate the production process by several routes including, but
84 not limited to, 1) the use of contaminated biological raw materials or reagents such as animal
85 serum components during cell culture, 2) the use of a virus or viral vector (including helper
86 viruses used in their production) to induce expression of specific genes encoding a desired
87 protein (see Annex 7), 3) the use of a contaminated raw material or reagent used during
88 downstream purification, such as a monoclonal antibody coupled affinity resin for product
89 selection or purification, 4) the use of a contaminated excipient during formulation, and 5)
90 contamination from the environment, including storage of non-biological raw materials or

91 during cell culture and medium handling.

92 Monitoring cell culture parameters can be helpful in the early detection of potential adventitious
93 viral contamination. Manufacturers should avoid using human- and animal-derived raw
94 materials (e.g., human serum, bovine serum, porcine trypsin) in their manufacturing processes
95 when possible. When this is not possible, the use of animal-derived raw materials should be
96 supported by the relevant documentation or qualification of the material, commensurate with
97 risk. Information such as the country of origin, tissue of origin, virus inactivation or removal
98 steps applied during the manufacturing process of the material, and the types of virus testing
99 that have been performed on the raw material should be provided.

100 When possible, cell culture media or media supplement treatments such as gamma irradiation,
101 virus filtration, high temperature short time processing, or ultraviolet C irradiation can be used
102 as additional virus risk mitigation measures.

103 **3. CELL LINE QUALIFICATION: TESTING FOR VIRUSES**

104 An important part of qualifying a cell line for use in the production of a biotechnology product is
105 the appropriate testing for the presence of viruses.

106 **3.1 Suggested Virus Tests for Master Cell Bank, Working Cell Bank, and Cells at the** 107 **Limit of *In Vitro* Cell Age Used for Production**

108 Table 1 shows an example of virus tests to be performed only once at various cell levels, including
109 MCB, WCB, and cells at the Limit of *In Vitro* Cell Age (LIVCA) that are used for production.

110 ***3.1.1 Master Cell Bank***

111 Extensive screening for both endogenous and adventitious viral contamination should be
112 performed on the MCB. For heterohybrid cell lines in which one or more partners are human or
113 non-human primate in origin, tests should be performed to detect viruses of human or non-human
114 primate origin because viral contamination arising from these cells may pose a particular hazard.

115 Testing for adventitious viruses should include both broad and specific virus detection assays as
116 described in Table 1. Introduction of new methodologies for detecting a broad range of
117 adventitious viruses is encouraged. To ensure detection of contaminating viruses, the testing
118 approach should be based on the origin and history of the cell line and the potential exposure to
119 materials of human or animal origin during cell line generation and MCB expansion.

120 3.1.2 Working Cell Bank

121 Each WCB should be tested for adventitious viruses as described in Table 1. When appropriate,
122 if adventitious virus tests have been performed on the MCB, and cells cultured up to or beyond the
123 LIVCA have been derived from the WCB and used to test for the presence of adventitious viruses
124 then similar tests may be omitted on the initial WCB. Antibody production tests are usually not
125 recommended for the WCB. An alternative approach in which complete testing is carried out on
126 each WCB rather than on the MCB would also be acceptable.

127 3.1.3 Cells at the Limit of In Vitro Cell Age Used for Production

128 The LIVCA established for production should be based on data derived from production cells
129 expanded under pilot plant scale or commercial scale conditions to the proposed *in vitro* cell age
130 or beyond. Generally, the production cells are obtained by expansion of the WCB; the MCB could
131 also be used to prepare the production cells. Cells at the LIVCA should be evaluated once for those
132 endogenous viruses that may have been undetected in the MCB. Cells at the LIVCA are also
133 referred to as end of production cells. The performance of suitable tests (as outlined in Table 1)
134 at least once on cells at the LIVCA used for production would provide further assurance that the
135 production process does not lead to activation of endogenous viruses or amplification of
136 adventitious viruses, including slow-growing viruses. If any adventitious viruses are detected at
137 this stage, the process should be checked carefully to determine the source of the contamination.

138 3.2 Recommended Virus Detection and Identification Assays

139 A number of assays can detect endogenous and adventitious viruses. Table 2 lists examples of
140 such assays. These assays are recommended, but the list is not all-inclusive nor definitive. The
141 most appropriate techniques may change with scientific progress; proposals for alternative
142 techniques should be accompanied by adequate supporting data. Manufacturers are encouraged
143 to discuss these alternatives with the appropriate regulatory authorities. A comprehensive
144 testing strategy includes consideration of the cell line origin; the passage history; and the raw
145 materials and reagents used for cell line generation, cell bank preparation, and production. The
146 strategy should include additional assays as appropriate based on risk assessments of the cell
147 substrate, raw materials, and reagents used. For example, if there is a relatively high possibility
148 of the presence of a particular virus, specific tests or other approaches for detection of that virus
149 should be included unless otherwise justified. Appropriate controls should be included to
150 demonstrate adequate assay sensitivity and specificity.

151 Next Generation Sequencing (NGS) and Nucleic Acid Amplification Techniques (NATs) such
152 as Polymerase Chain Reaction (PCR) may be appropriate for broad and specific virus detection,
153 respectively. The introduction of these tests may be done without a systematic head-to-head
154 comparison with the currently recommended *in vitro* and *in vivo* assays. In particular, a head-
155 to-head comparison is not recommended for *in vivo* assays to meet the intent of the global
156 objective to replace, remove, and refine the use of animals. Because of the assay sensitivity and
157 breadth of virus detection, NGS may also be used to replace cell-based infectivity assays, to
158 overcome potential assay limitations, or to detect viruses without visible phenotypes in the
159 assay system. Positive results should be investigated to determine whether detected nucleic
160 acids are associated with an infectious virus.

161 The following is a brief description of a general framework that the manufacturer should use to
162 develop a comprehensive viral testing scheme that is specific (or appropriate) to the product
163 and manufacturing process. The testing plan or strategy should be accompanied with
164 appropriate justification for the approach.

165 **3.2.1 Tests for Retroviruses**

166 Tests for retroviruses should be performed for the MCB and for cells cultured up to or beyond
167 the LIVCA used for production. These tests include infectivity assays by direct inoculation or
168 co-cultivation, assays for Reverse Transcriptase (RT) activity, and evaluation of particles by
169 Transmission Electron Microscopy (TEM).

170 If the cell line is not known to produce retroviral particles, TEM should be performed on cells
171 and a PCR-based RT assay (e.g., the product-enhanced RT assay) should be carried out on
172 clarified supernatant. The PCR-based RT assay is particularly useful because it can detect the
173 RT activity of all retroviruses; however, the RT activity can be associated with an infectious or
174 non-infectious retrovirus. Because some cellular DNA polymerases can cross-react and lead to
175 a positive RT result, confirmation of the RT activity (as a result of a retrovirus contamination)
176 or a positive TEM result should be followed by an assay to detect infectious retroviruses in
177 permissible cells, including a human cell line and a sensitive readout assay for retrovirus
178 detection.

179 If a cell line is known to constitutively produce retroviral particles (as occurs in some cell lines
180 derived from rodent, insect, and avian species), RT activity is expected and therefore a PCR-
181 based RT assay may not be needed. TEM should be performed to examine the type of retroviral

182 particles (e.g., type-A and type-C) present. To determine whether the endogenous retroviral
183 particles are infectious, infectivity assays should be performed using relevant permissive cells
184 (e.g., *Mus dunni* and SC-1 cells for rodent retroviruses) with sensitive readout assays for
185 retrovirus detection (e.g., a product-enhanced Reverse Transcriptase (RT) assay, a Sarcoma-
186 Positive, Leukemia-Negative (S⁺L⁻) assay, or an XC plaque assay or a broad molecular assay).

187 Retroviral testing results should be interpreted considering all available data. Cell lines
188 expressing endogenous retrovirus particles are not precluded from use in manufacturing based
189 on risk evaluation as discussed in Section 3.3 and Section 5.

190 Induction studies have not been found to be useful for cell lines that have been well
191 characterised for endogenous retroviruses (e.g., Chinese Hamster Ovary (CHO), NS0, and
192 Sp2/0). However, such studies may help to evaluate a new cell substrate for the presence of
193 unknown endogenous retroviruses. Furthermore, induction studies for latent DNA viruses (e.g.,
194 herpesvirus in human cells) and latent RNA viruses (e.g., nodavirus in insect cells) may also be
195 appropriate based on risk assessment. These studies may help inform the virus testing and
196 clearance strategy for products derived from a new cell substrate.

197 **3.2.2 *In Vitro* Cell Culture Infectivity Assays**

198 *In vitro* tests are carried out by inoculating a test article (see Table 2) into various susceptible
199 indicator cell cultures capable of detecting a wide range of human and relevant animal viruses.
200 The choice of cells used in the test should be based on a risk assessment considering the species
201 of origin of the cell substrate to be tested. The panel of cell lines should include a cell line of the
202 species of origin and a human and a non-human primate cell line susceptible to human viruses.

203 The nature of the infectivity assay and the sample to be tested are governed by the type of virus
204 that may be present based on the origin or handling of the cells. For cell line qualification, the
205 test should be performed as a 14-day initial cell culture followed by a secondary passage with
206 a 14-day duration followed by observation for both cytopathogenic and
207 hemadsorbing/hemagglutinating viruses.

208 Alternatively, molecular virus detection methods may be used to supplement (e.g., when
209 required to address certain limitations such as test article-mediated interference or toxicity) or
210 replace the cell culture assays.

211 **3.2.3 *In Vivo* Assays**

212 NGS is encouraged as a replacement for *in vivo* assays because of the breadth of viruses it
213 detects and because its use promotes the global objective to replace, reduce, and refine the use
214 of animal testing. Use of NGS to replace *in vivo* assays may be justified by submitting a
215 validation package. Based on risk assessment and on the overall testing strategy, the use of the
216 *in vivo* assay may include inoculation of test article (see Table 2) into suckling mice, adult mice,
217 and embryonated eggs. Additional animal species may be used depending on the nature and
218 source of the cell lines being tested. The health of the animals should be monitored, and any
219 abnormality should be investigated to establish the cause.

220 **3.2.4 Antibody Production Tests**

221 Antibody production tests should be performed when the potential exists for exposure to viruses
222 of a specific animal species. For example, the presence of rodent viruses in cell lines of rodent
223 origin, or generated by passage through rodents and the use of reagents that may have been
224 derived from rodent materials, can be detected by inoculation of the test article (see Table 2)
225 into Specific-Pathogen Free (SPF) animals, such as mice, rats, and hamsters, that are
226 subsequently tested for antibodies to specific agents. Examples of such tests are the Mouse
227 Antibody Production (MAP) test, Rat Antibody Production (RAP) test, and Hamster Antibody
228 Production (HAP) test. The viruses currently screened for in the antibody production assays are
229 discussed in Table 3.

230 Virus-specific PCR or targeted molecular methods can be used as a replacement assay for the
231 animal testing described in Table 3.

232 **3.2.5 Molecular Methods**

233 Molecular methods can be used to supplement or replace *in vitro* cell culture-based and *in vivo*
234 animal assays.

235 **3.2.5.1 Nucleic Acid Amplification Techniques**

236 Nucleic Acid Amplification Techniques (NATs) such as PCR-based methods are typically used
237 singly or in a multiplex format to detect virus sequences from known viruses or known closely
238 related virus families. Targeted NGS methods may also apply for sensitive detection of known
239 viruses. These molecular methods can be used to supplement cell culture assays when there are
240 limitations as a result of assay interference, and they are effective tools for specific virus
241 detection when such viruses cannot be readily grown in cell culture for detection by infectivity
242 assays. NAT methods also have the capacity to be adapted for more broad range virus detection

243 (e.g., degenerate PCR), but specificity may be reduced. Because of the assay specificity,
244 multiple virus-specific PCR assays may be needed to detect the breadth of viruses that would
245 be detected by a single more general biological assay. NAT assays should be appropriately
246 qualified or validated for their intended use.

247 **3.2.5.2 Next Generation Sequencing**

248 New advanced molecular methods such as NGS (also known as high-throughput sequencing)
249 are available with demonstrated capabilities for broad virus detection. NGS can provide defined
250 sensitivity and breadth of virus detection and can reduce animal use and testing time. For any
251 NGS method used, a validation package should be provided to support its use for the
252 application. This includes the method validation and assay or matrix-specific qualification, as
253 suitable. Based on the potential safety concerns, the bioinformatic analysis can be targeted to
254 specific viruses or can be agnostic for broad virus detection. NGS can replace the *in vivo* tests
255 with broad virus detection for unknown or unexpected virus species. NGS can also supplement
256 or replace the *in vitro* cell culture assays for detection of known and unknown or unexpected
257 virus species. Furthermore, the assay may also be used for the detection of known viruses, and
258 it can replace the HAP, MAP, and RAP tests and other virus-specific PCR assays.

259 Use of NGS should be considered particularly for characterisation or testing of a cell substrate
260 and cell bank, for detection of known and unknown viruses, and in a viral seed or harvest if
261 there is assay interference as a result of lack of effective neutralisation of the vector virus (see
262 Annex 7) or toxicity due to the product or media components. In such applications, NGS can
263 be used to detect viral sequences present in the cell DNA (genomics) or expressed as RNA in
264 cells (transcriptomics), or it can be used to detect viral genome present in particles (viromics).
265 The rationale for selecting these different strategies should be provided.

266 When applying NGS for sensitive detection of known viruses and/or broad detection of novel
267 viruses, applicants should consider several critical steps in the NGS workflow. These include 1)
268 sample treatment (when performed) and processing based on the type of sample material, 2)
269 efficient viral nucleic acid extraction (including enveloped and nonenveloped particles) and
270 library preparation, 3) selection of a suitable sequencing platform, and 4) comprehensive
271 bioinformatics analysis against a database with diverse representation of viral sequences of
272 different viral families. Steps for sample treatment and processing can be carried out to
273 maximize virus detection.

274 Suitable standards or reference materials should be used for assay qualification and validation
275 to evaluate performance of the different steps involved in the methodology and to demonstrate
276 sensitivity, specificity, and breadth of virus detection. This can include using currently available
277 reference virus reagents with distinct physical (size, enveloped and non-enveloped), chemical
278 (low, medium, and high resistance), and genomic (DNA, RNA, double- and single-stranded,
279 linear, circular) characteristics to evaluate the performance of the entire NGS workflow or
280 specific steps; a comprehensive viral database should be used with diverse viral sequences for
281 broad virus detection. Furthermore, other standard types may be used to evaluate the specific
282 technical and bioinformatic steps. Since NGS has a complex workflow, manufacturers
283 are encouraged to have discussions with the appropriate regulatory authorities regarding
284 expectations for method validation and data submission.

285 **3.3 Acceptability of Cell Lines**

286 Some cell lines used to manufacture a product will contain endogenous retroviruses, other
287 viruses, or viral sequences that may become reactivated as infectious viruses. In such
288 circumstances, the action plan recommended for manufacture is described in Section 5. The
289 acceptability of cell lines containing viruses other than endogenous retroviruses will be
290 considered on an individual basis by the appropriate regulatory authorities, considering a risk-
291 benefit analysis based on the benefit of the product and its intended clinical use, the nature of
292 the contaminating viruses, their potential for infecting humans or for causing disease in humans,
293 the purification process for the product (e.g., viral clearance evaluation data), and the extent of
294 the virus tests conducted on the purified bulk.

295 **4. TESTING FOR VIRUSES IN UNPROCESSED BULK**

296 It is recommended that manufacturers develop programs to continuously assess adventitious
297 viruses in production batches. The scope and extent of virus testing on the unprocessed bulk
298 should be determined by considering several points including the nature of the cell lines used
299 to produce the desired products, the results and extent of virus tests performed during the
300 qualification of the cell lines, the cultivation method, the raw material and reagent sources, and
301 the results of viral clearance studies.

302 The unprocessed bulk constitutes one or multiple pooled harvests of cells and culture media. A
303 representative sample of the unprocessed bulk, removed from the production reactor before further
304 processing, represents one of the most suitable levels at which the possibility of adventitious virus

305 contamination can be determined with a high probability of detection. Appropriate testing for
306 viruses should be performed on the unprocessed bulk. For perfusion or continuous manufacturing
307 processes, cells may not be readily accessible (e.g., due to use of hollow fiber or similar
308 microfiltration systems). In such cases, the unprocessed bulk would constitute fluids harvested
309 from the bioreactor. The potential influence of cell separation technology and progressive filter
310 fouling on the representativeness of these unprocessed bulk test samples should be considered.
311 If unprocessed bulk is toxic in test cell cultures, initial partial processing (e.g., minimal sample
312 dilution or alternative testing assays) can be considered (see Section 3.2). In certain instances, it
313 may be more appropriate to test a mixture of both intact and disrupted cells and their cell culture
314 supernatants that were removed from the production reactor before further processing. For
315 processes that involve continuous harvest, the sampling strategy (including periodicity and
316 composition of the samples) should be justified because adventitious viruses and endogenous
317 virus particles can variate along the cell culture duration (see Section 7).

318 Adventitious virus testing should be routinely applied to each unprocessed bulk. This may include
319 *in vitro* screening assays using several cell lines or broad molecular virus detection methods such
320 as NGS (see Section 3.2). Based on the risk assessment (considering the cell substrate, use of
321 animal-derived raw materials or reagents, and level of virus clearance of the process), the indicator
322 cell cultures should be observed for at least 2 weeks. Detection for specific viruses or families
323 of viruses may also be appropriate to include based on risk assessment (e.g., Minute virus of
324 mice). When appropriate, a PCR or other molecular method may also be selected as rapid test
325 methods can facilitate real-time decision making.

326 If any adventitious viruses are detected at the unprocessed bulk stage, the harvest should not be
327 used for product manufacture unless justified. (See Section 5 for guidance on the use of material
328 in which an adventitious virus has been detected in the harvest material.) The process should be
329 carefully checked to determine the root cause and extent of the contamination, and appropriate
330 actions should be taken. For continuous manufacturing processes, release of a final subplot
331 requires documented absence of viral contamination for the period during which cultivation
332 fluids were harvested for manufacture of that subplot. If an adventitious virus is detected, a
333 procedure to segregate potentially contaminated material should be considered to mitigate
334 wider production impact.

335 **5. RATIONALE AND ACTION PLAN FOR VIRAL CLEARANCE STUDIES AND** 336 **VIRUS TESTS ON PURIFIED BULK**

337 It is important to design the most relevant and rational protocol for virus tests from the MCB
338 level, through the various steps of drug production, and to the final product including evaluation
339 and characterisation of viral clearance from unprocessed bulk. The evaluation and
340 characterisation of viral clearance plays a critical role in this scheme. The goal should be to
341 obtain the best reasonable assurance that the product is free of virus contamination.

342 In selecting viruses to use for a clearance study, it is useful to distinguish between the need to
343 evaluate processes for their ability to clear viruses that are known to be present and the desire
344 to estimate the robustness of the process by characterising the clearance of non-specific
345 “model” viruses (described later). Definitions of relevant, specific, and non-specific “model”
346 viruses are given in the glossary. Process evaluation requires knowledge of how much virus
347 may be present in the process, such as in the unprocessed bulk, and how much can be cleared,
348 to assess product safety. Knowledge of the time dependence for inactivation procedures is
349 helpful in ensuring the effectiveness of the inactivation process. When evaluating clearance of
350 known contaminants, in-depth time-dependent inactivation studies, demonstration of
351 reproducibility of inactivation or removal, and evaluation of process parameters should be
352 performed. When a manufacturing process is characterised for robustness of clearance using
353 non-specific “model” viruses, particular attention should be paid to non-enveloped viruses in
354 the study design. The extent of viral clearance in characterisation studies may be influenced by
355 the results of tests on cell lines and unprocessed bulk. These studies should be performed as
356 described below (see Section 6).

357 Table 4 presents an example of an action plan used in response to the results of virus tests on
358 cells or unprocessed bulk. The plan includes the process evaluation and the characterisation of
359 viral clearance and virus tests on purified bulk. Various cases are presented in the table and are
360 described below. In all cases, characterisation of clearance using non-specific “model” viruses
361 should be performed. The most common situations are Cases A and B. Production systems
362 contaminated with a virus other than a rodent retrovirus normally are not used. When there are
363 well-justified reasons for drug production using a cell line from Cases C, D, or E, these should
364 be discussed with the appropriate regulatory authorities. In Cases C, D, and E, it is important to
365 have validated and effective steps to inactivate or remove the virus in question from the
366 manufacturing process.

367 **Case A:** When no virus, virus-like particle, or retrovirus-like particle has been demonstrated in the
368 cells or the unprocessed bulk, virus removal and inactivation studies should be performed with

369 non-specific “model” viruses, as previously stated.

370 **Case B:** In rodent cell lines, if only a rodent retrovirus (or a retrovirus-like particle that is believed
371 to be non-pathogenic, such as rodent A- and R-type particles) is present, the process evaluation
372 using a specific “model” virus (such as a murine leukemia virus) should be performed. Purified
373 bulk should be tested using suitable methods with high specificity and sensitivity for the detection
374 of the virus in question. For marketing authorisation, data from at least 3 lots of purified bulk at
375 pilot plant scale or commercial scale should be provided. Cell lines such as Chinese Hamster Ovary
376 (CHO), C127, BHK and murine hybridoma cell lines have frequently been used as substrates for
377 drug production with no reported safety problems related to viral contamination of the products.
378 For these cell lines in which the endogenous particles have been extensively characterised and
379 clearance has been demonstrated, it usually is not recommended to test for the presence of the non-
380 infectious particles in the purified bulk or drug substance. Studies with non-specific “model”
381 viruses, as in Case A, are appropriate. A similar approach may be relevant for insect cell lines (e.g.,
382 Sf9) that produce endogenous retroviral-like particles that have been extensively characterised.

383 **Case C:** When the cells or unprocessed bulk are known to contain a virus (other than a rodent
384 retrovirus) for which there is no evidence of infectivity to humans (e.g., Sf9 rhabdovirus (such as
385 those identified in Table 3, footnote 2 except rodent retroviruses (Case B)), virus removal and
386 inactivation evaluation studies should use the identified virus. If it is not possible to use the
387 identified virus, “relevant” or specific “model” viruses should be used to demonstrate acceptable
388 clearance. Time-dependent inactivation for identified (or “relevant” or specific “model”) viruses
389 at the critical inactivation steps should be obtained as part of the process evaluation for these
390 viruses. Purified bulk should be tested using suitable methods with high specificity and sensitivity
391 for detecting the virus in question. For the purpose of marketing authorisation, data from at least 3
392 lots of purified bulk manufactured at pilot plant scale or commercial scale should be provided.

393 **Case D:** If a known virus is infectious to humans (such as those viruses indicated in Table 3,
394 footnote 1), is identified, the product should be acceptable only under exceptional circumstances.
395 In such instances, the identified virus should be used for virus removal and inactivation evaluation
396 studies and specific methods with high specificity and sensitivity for the detection of the virus in
397 question should be used. If it is not possible to use the identified virus, relevant and/or specific
398 “model” viruses (described later) should be used. The process should be shown to remove and
399 inactivate the selected viruses during the purification and inactivation processes. Time-dependent
400 inactivation data for the critical inactivation steps should be obtained as part of the process

401 evaluation. Purified bulk should be tested using suitable methods with high specificity and
402 sensitivity for the detection of the virus in question. For marketing authorisation, data from at least
403 3 lots of purified bulk manufactured at pilot plant scale or commercial scale should be provided.

404 *Case E:* When a virus that cannot be classified by currently available methodologies is detected
405 in the cells or unprocessed bulk, the product is usually considered unacceptable because the virus
406 may be pathogenic. In the rare case in which there are well-justified reasons for drug production
407 using such a cell line, this should be discussed with the appropriate regulatory authorities before
408 proceeding further.

409 *Case F:* When a helper virus is used in production, clearance of the virus should be
410 demonstrated using the helper virus itself or a specific model virus (e.g., baculovirus,
411 adenovirus, herpesvirus).

412 **6. EVALUATION AND CHARACTERISATION OF VIRAL CLEARANCE** 413 **PROCEDURES**

414 Evaluation and characterisation of the virus removal or inactivation procedures are important
415 for establishing the safety of biotechnology products. Past instances of contamination have
416 occurred with agents whose presence was not known or even suspected. Though this happened
417 to biological products derived from various source materials other than fully characterised cell
418 lines, it reinforces that assessment of viral clearance provides a measure of confidence that any
419 unknown, unsuspected, and harmful viruses may be removed. Studies should be carried out in
420 a well-documented and controlled manner.

421 The objectives of viral clearance studies are 1) to assess process steps that effectively inactivate
422 or remove viruses and 2) to estimate quantitatively the overall level of virus reduction obtained
423 by the process. These should be achieved by the deliberate addition (i.e., “spiking”) of
424 significant amounts of a virus to the crude material or to different fractions obtained during the
425 various process steps and demonstrating its removal or inactivation during the subsequent steps.
426 It is not necessary to evaluate or characterise every step of a manufacturing process if adequate
427 clearance is demonstrated by the use of fewer steps. It should be considered that other steps in
428 the process may have an indirect effect on the viral inactivation or removal achieved.
429 Manufacturers should explain and justify the approach used in studies to evaluate virus
430 clearance. In general, in order to determine the amount of endogenous virus particles that enter
431 the purification process, quantification should be performed on three cell cultures campaigns,

432 lots or batches. This data should be submitted as part of the marketing application or registration
433 package.

434 The reduction of virus infectivity may be achieved by removing virus particles or by
435 inactivating viral infectivity. For each production step assessed, the possible mechanism of loss
436 of viral infectivity should be described with regard to whether it results from inactivation or
437 removal. For inactivation steps, the study should be planned so that samples are taken at
438 different times and an inactivation curve is constructed (see Section 6.2.5).

439 Viral clearance evaluation studies are performed to 1) demonstrate the clearance of a virus
440 known to be present in the MCB, or 2) ensure that adventitious viruses that could not be
441 detected, or that might gain access to the production process, would be cleared. Reduction
442 factors are normally expressed on a logarithmic scale to show that, while residual virus
443 infectivity will never be reduced to zero, it may be greatly reduced mathematically.

444 In addition to clearance studies for viruses known to be present, studies to characterise the
445 ability to remove or inactivate other viruses should be conducted. The purpose of studies using
446 viruses with a range of unknown or unexpected biochemical and biophysical properties is to
447 characterise the robustness of the procedure rather than to achieve a specific inactivation or
448 removal goal. A demonstration of the capacity of the production process to inactivate or remove
449 viruses is desirable (see Section 6.3). Such studies are not performed to evaluate a specific
450 safety risk. Therefore, achieving a specific clearance value is not needed.

451 **6.1 The Choice of Viruses for Evaluation and Characterisation of Virus Clearance**

452 Viruses for clearance evaluation and process characterisation studies should be chosen to resemble
453 viruses which may contaminate the product and to represent a wide range of physicochemical
454 properties to test the ability of the system to eliminate viruses in general. The manufacturer should
455 justify the choice of viruses according to the aims of the evaluation and characterisation study
456 provided in this guideline.

457 **6.1.1 “Relevant” Viruses and “Model” Viruses**

458 A major issue in performing a viral clearance study is to determine which viruses should be
459 used. Such viruses fall into three categories: 1) “relevant” viruses, 2) specific “model” viruses,
460 and 3) non-specific “model” viruses.

461 “Relevant” viruses are used in the process evaluation of viral clearance studies which are the

462 identified viruses or of the same species as the viruses that are known, or likely to contaminate
463 the cell substrate or any other reagents or materials used in the production process. The process
464 for purification and/or inactivation should demonstrate the capability to remove and/or
465 inactivate such viruses. When a “relevant” virus is not available or when it is not well adapted
466 to the process evaluation of viral clearance studies (e.g., it cannot be grown *in vitro* to
467 sufficiently high titers), a specific “model” virus should be used as a substitute. An appropriate
468 specific “model” virus can be a virus which is closely related to the known or suspected virus
469 (same genus or family), having similar physical and chemical properties to the observed or
470 suspected virus.

471 Cell lines derived from rodents usually contain endogenous retrovirus particles or retrovirus-
472 like particles, which may be infectious (C-type particles) or non-infectious (cytoplasmic A- and
473 R-type particles). The capacity of the manufacturing process to remove and/or inactivate rodent
474 retroviruses from products obtained from such cells should be determined. This can be
475 accomplished by using a murine leukemia virus--a specific “model” virus in the case of cells of
476 murine origin.

477 For CHO cell-derived products, CHO-derived endogenous virus particles can also be used for
478 viral clearance experiments. There is no infectivity assay for these particles, and the detection
479 assay (e.g., molecular or biochemical) should be qualified for its use. When human cell lines
480 secreting monoclonal antibodies have been obtained by the immortalisation of B lymphocytes
481 by Epstein-Barr Virus, the ability of the manufacturing process to remove and/or inactivate a
482 herpes virus should be determined. Pseudorabies virus may also be used as a specific “model”
483 virus.

484 When the purpose is to characterise the capacity of the manufacturing process to remove and/or
485 inactivate viruses in general (i.e., to characterise the robustness of the clearance process), virus
486 clearance characterisation studies should be performed with non-specific “model” viruses with
487 differing properties. Data obtained from studies with “relevant” and/or specific “model” viruses
488 can also contribute to this assessment. It is not necessary to test all types of viruses. Preference
489 should be given to viruses that display a significant resistance to physical and/or chemical
490 treatments. The results obtained for such viruses provide useful information about the ability of
491 the production process to remove and/or inactivate viruses in general. The choice and number
492 of viruses used should be influenced by the quality and characterisation of the cell lines and the
493 production process.

494 Annex 2 and Table A-1 provide examples of useful “model” viruses representing a range of
495 physicochemical structures and examples of viruses that have been used in viral clearance
496 studies.

497 **6.1.2 Other Considerations**

498 Additional points to be considered:

- 499 • Viruses that can be grown to high titer are desirable, although this may not always be
500 possible;
- 501 • There should be an efficient and reliable assay for the detection of each virus used for
502 every stage of manufacturing that is tested; and
- 503 • The health hazard that certain viruses may pose to the personnel performing the clearance
504 studies should be considered.

505 **6.2 Design and Implications of Virus Clearance Evaluation and Characterisation** 506 **Studies**

507 **6.2.1 Facility and Staff**

508 It is inappropriate to introduce any unintended virus into a production facility because of good
509 manufacturing practice constraints. Therefore, viral clearance studies should be conducted in a
510 separate laboratory equipped for virological work and performed by staff with virological
511 expertise in conjunction with production personnel involved in designing and preparing a
512 scaled-down version of the purification process.

513 **6.2.2 Scaled-Down Production System**

514 The validity of scaling down should be demonstrated. The level of purification of the scaled-
515 down version should represent the production procedure as closely as possible. For
516 chromatographic equipment, column bed-height, linear flow-rate, flow-rate-to-bed-volume
517 ratio (i.e., contact time), buffer and gel types, pH, temperature, and concentration of protein,
518 salt, and product should all be shown to be representative of commercial-scale manufacturing.
519 A similar elution profile should result. For other procedures, similar considerations apply.
520 Unavoidable deviations should be discussed with regard to their influence on the results.

521 **6.2.3 Analysis of Step-Wise Elimination of Virus**

522 When viral clearance studies are performed, assessment of the contribution of more than one
523 production step to virus elimination should be considered. Steps that are likely to clear virus
524 should be individually assessed for their ability to remove and inactivate virus, and the exact
525 definition of an individual step should be considered. Sufficient virus should be present in the
526 material of each step to be tested so that an adequate assessment of the effectiveness of each
527 step is obtained. Generally, virus should be added to in-process material at each step to be tested.
528 In some cases, adding high titer virus to unpurified bulk and testing its concentration between
529 steps is sufficient. When virus removal results from separation procedures, it is recommended
530 that the distribution of the virus load in the different fractions be investigated, if appropriate
531 and if possible. When virucidal buffers are used in multiple steps within the manufacturing
532 process, alternative strategies such as parallel spiking in less virucidal buffers, can be carried
533 out as part of the overall process assessment. The virus titer before and after each step being
534 evaluated should be determined. Quantitative infectivity assays should have adequate
535 sensitivity and reproducibility and should be performed with sufficient replicates to ensure
536 adequate statistical validity of the result. Quantitative assays not associated with infectivity may
537 be used if justified. Appropriate virus controls should be included in all infectivity assays to
538 ensure the sensitivity of the method. Also, the statistics of sampling virus when at low
539 concentrations should be considered (see Annex 3).

540 ***6.2.4 Determining Physical Removal versus Inactivation***

541 Reduction in virus infectivity can be achieved by the removal or inactivation of virus. For each
542 production step assessed, the possible mechanism of the loss of viral infectivity should be
543 described as related to inactivation or removal. If little clearance of infectivity is achieved by
544 the production process and the clearance of virus is considered to be a major factor in the safety
545 of the product, specific or additional inactivation/removal steps should be introduced. It may
546 be necessary to distinguish between removal and inactivation for a particular step. As an
547 example, when there is a possibility that a buffer used in more than one clearance step may
548 contribute to inactivation during each step (i.e., the contribution to inactivation by a buffer
549 shared by several chromatographic steps), the removal achieved by each of these
550 chromatographic steps should be distinguished.

551 ***6.2.5 Inactivation Assessment***

552 For the assessment of viral inactivation, unprocessed crude material or intermediate material
553 should be spiked with infectious virus and the reduction factor calculated. It should be

554 recognised that virus inactivation is not a simple first order reaction and is usually more
555 complex with a fast “phase 1” and a slow “phase 2”. Therefore, the study should be planned in
556 such a way that samples are taken at different times and an inactivation curve be constructed.
557 It is recommended that studies for inactivation include at least one time point less than the
558 minimum exposure time and greater than zero, in addition to the minimum exposure time.
559 Additional data are particularly important if the virus is a “relevant” virus known to be a human
560 pathogen, and an effective inactivation process is being designed. However, for inactivation
561 studies in which non-specific “model” viruses are used or when specific “model” viruses are
562 used as surrogates for virus particles such as the CHO intracytoplasmic retrovirus-like particles,
563 reproducible clearance should be demonstrated in at least two independent studies. Whenever
564 possible, the initial virus load should be determined from the virus which can be detected in the
565 spiked starting material. If this is not possible, the initial virus load may be calculated from the
566 titer of the spiking virus preparation. When inactivation is too rapid to plot an inactivation curve
567 using process conditions, appropriate controls should be performed to demonstrate that
568 infectivity is indeed lost by inactivation.

569 ***6.2.6 Function and Regeneration of Columns***

570 Over time and after repeated use, the ability of chromatography columns and other devices used
571 in the purification scheme to clear virus may vary. Chromatography media/resin lifetime use
572 should be indicated, and critical process parameters that impact viral clearance should be
573 defined.

574 For protein A affinity capture chromatography, prior knowledge indicates that virus removal is
575 not impacted or slightly increases for used (e.g., end-of-life) chromatography media/resin.
576 Therefore product-specific studies with used resin are not expected. Prior knowledge might also
577 apply to other chromatography types involved in viral clearance (e.g., anion exchange or cation
578 exchange). Accordingly, to support repeated resin use for other chromatography types,
579 equivalent prior knowledge including in-house experience and a detailed justification should
580 be provided instead of product-specific viral clearance studies with end of lifetime resin.

581 Assurance should be provided so that any virus potentially retained by the production system
582 would be adequately destroyed or removed before reusing the system. For example, evidence may
583 be provided demonstrating that the cleaning and regeneration procedures inactivate or remove
584 virus.

585 **6.2.7 Specific Precautions**

586 The following specific precautions should be considered:

- 587 • Care should be taken in preparing the high-titer virus to avoid aggregation, which may
588 enhance physical removal and decrease inactivation thus distorting the correlation with
589 actual production;
- 590 • Consideration should be given to the minimum quantity of virus which can be reliably
591 assayed;
- 592 • The study should include parallel control assays to assess the loss of infectivity of the virus
593 due to such reasons as the dilution, concentration, filtration, or storage of samples before
594 titration;
- 595 • The virus “spike” should be added to the product in a small volume so as not to dilute or
596 change the characteristics of the product. Diluted, test-protein sample is no longer identical
597 to the product obtained at commercial scale;
- 598 • Small differences in buffers, media, or reagents (for example) can substantially affect viral
599 clearance;
- 600 • Virus inactivation is time-dependent; therefore, the amount of time a spiked product
601 remains in a particular buffer solution or on a particular chromatography column should
602 reflect the conditions of the commercial-scale process;
- 603 • Buffers and product should be evaluated independently for toxicity or interference in
604 assays used to determine the virus titer, as these components may adversely affect the
605 indicator cells. If the solutions are toxic to the indicator cells, dilution, adjustment of the
606 pH, or dialysis of the buffer containing spiked virus might be necessary. If the product
607 itself has anti-viral activity, the clearance study may need to be performed without the
608 product in a “mock” run, although omitting the product or substituting a similar protein
609 that does not have anti-viral activity could affect the behaviour of the virus in some
610 production steps. Sufficient controls to demonstrate the effect of procedures used solely to
611 prepare the sample for assay (e.g., dialysis, storage) on the removal/inactivation of the
612 spiking virus should be included;

613 • Many purification schemes use the same or similar buffers or columns repetitively. The
614 effects of this approach should be considered when analysing the data. The effectiveness
615 of virus elimination by a particular process may vary with the stage in manufacture at
616 which it is used; and

617 • Overall reduction factors may be underestimated when production conditions or buffers
618 are too cytotoxic or virucidal and should be discussed on a case-by-case basis. Overall
619 reduction factors may also be overestimated due to inherent limitations or inadequate
620 design of viral clearance studies.

621 **6.3 Interpretation of Virus Clearance Studies**

622 The object of assessing virus inactivation/removal is to evaluate and characterise process steps
623 considered effective in inactivating/removing viruses and to estimate quantitatively the overall
624 level of virus reduction obtained by the manufacturing process. For virus contaminants, as in
625 Cases B through E, it is important to show that not only is the virus eliminated or inactivated,
626 but that there is excess capacity for viral clearance built into the purification process to ensure
627 an appropriate level of safety for the final product. The amount of virus eliminated or
628 inactivated by the production process should be compared to the amount of virus which may be
629 present in unprocessed bulk.

630 To carry out this comparison, it is important to estimate the amount of virus in the unprocessed
631 bulk. This estimate should be obtained using assays for infectivity or other methods such as
632 Transmission Electron Microscopy (TEM) or a quantitative Nucleic Acid Amplification
633 Technique (NAT). The entire purification process should be able to eliminate substantially more
634 virus than is estimated to be present in a single-dose-equivalent of unprocessed bulk. See Annex 4
635 for calculation of virus reduction factors and Annex 5 for calculation of estimated particles per
636 dose. Manufacturers should recognise that clearance mechanisms may differ among virus classes.
637 A combination of factors should be considered when judging the data supporting the effectiveness
638 of virus inactivation/removal procedures. These include:

- 639 • The appropriateness of the test viruses used;
- 640 • The design of the clearance studies;
- 641 • The log reduction achieved;

- 642 • The time-dependence of inactivation;
- 643 • The potential effects of variation in process parameters on virus inactivation/removal;
- 644 • The limits of assay sensitivities; and
- 645 • The possible selectivity of inactivation/removal procedure(s) for certain classes of viruses.

646 It is recommended to design a downstream process that clears a wide range of potential virus
 647 contaminants. In this context, whenever feasible and not adversely affecting the product,
 648 implementing two distinct effective steps that complement each other in their mode of action is
 649 recommended. One of the manufacturing steps should effectively clear non-enveloped viruses.
 650 An effective virus removal step generally gives reproducible reduction of virus load in the order
 651 of 4 logs or more shown by at least two independent studies. However, it is recognised that
 652 steps giving a reproducible reduction in the order of 1 to 3 logs contribute towards viral safety
 653 and can be considered for assessment of overall virus reduction. Process steps dedicated to virus
 654 inactivation/removal such as Solvent/Detergent treatment, treatment with detergent alone, virus
 655 filtration (nanofiltration), or incubation at low pH, have been very successful in clearing a wide
 656 range of viruses. Using virus filters designed for removal of small viruses is also an effective
 657 virus clearance step for the smaller parvovirus or polyomaviruses. Finally, there is experience
 658 of efficient inactivation of Xenotropic Murine Leukemia Virus (XMuLV) and pseudorabies
 659 virus by incubation at low pH after protein A capture step for purification of monoclonal
 660 antibodies.

661 Acceptable overall clearance can be achieved by any of the following steps: multiple inactivation
 662 steps, multiple complementary separation steps, or combinations of inactivation and separation
 663 steps. Separation methods may be dependent on the extremely specific physico-chemical
 664 properties of viruses which influence their interaction with stationary phases for chromatography
 665 (e.g., resins or chromatography membranes) and precipitation properties, “model” viruses can
 666 be separated in a different manner than a target virus. Manufacturing parameters influencing
 667 separation should be properly defined and controlled. Differences may originate from changes
 668 in surface properties such as glycosylation. However, despite these potential variables, effective
 669 removal can be obtained by a combination of complementary separation steps or combinations
 670 of inactivation and separation steps. Therefore, well designed separation steps, such as
 671 chromatographic procedures, filtration steps and extractions, can be effective virus removal

672 steps provided that they are performed under appropriately controlled conditions.

673 An overall reduction factor is generally expressed as the sum of the individual factors. However,
674 reduction in virus titer of the order of 1 log₁₀ or less would be considered negligible and could
675 be ignored unless justified.

676 If little reduction of infectivity is achieved by the production process, and the removal of virus
677 is considered to be a major factor in the safety of the product, a specific, additional
678 inactivation/removal step or steps should be introduced. For all viruses, manufacturers should
679 justify the acceptability of the reduction factors obtained. The factors listed above will be
680 considered in evaluating the results.

681 **6.4 Limitations of Viral Clearance Studies**

682 Viral clearance studies are useful for contributing to the assurance that an acceptable level of
683 safety in the final product is achieved but do not by themselves establish safety. However, a
684 number of factors in the design and execution of viral clearance studies may lead to an incorrect
685 estimate of the ability of the process to remove virus infectivity. These factors include the
686 following:

- 687 • Virus preparations used in clearance studies for a production process are usually obtained
688 from specific cell cultures. The behaviour of such virus spike in a production step may
689 be different from that of the native viral contaminant from a biological raw material in
690 the cell culture medium or replicating in the manufacturing cells. For example, this could
691 include if virus particles used for spiking and native virus from a respective production
692 intermediate differ in purity or degree of aggregation;
- 693 • Inactivation of virus infectivity frequently follows a biphasic curve in which a rapid initial
694 phase is followed by a slower phase. It is possible that virus escaping a first inactivation
695 step may be more resistant to subsequent steps. For example, if the resistant fraction takes
696 the form of virus aggregates, infectivity may be resistant to a range of different chemical
697 treatments and to heating;
- 698 • The ability of the overall process to remove or inactivate virus is expressed as the sum of
699 the logarithm of the reductions at each step. The summation of the reduction factors of
700 multiple steps, particularly of steps with little reduction (e.g., below 1 log₁₀), may
701 overestimate the true potential for virus elimination. Addition of individual virus

702 reduction factors resulting from similar inactivation mechanisms during the
 703 manufacturing process may also overestimate overall virus clearance. Furthermore, if
 704 reduction values achieved by repetition of identical or near identical procedures are
 705 included, they should be justified;

706 • The expression of reduction factors as logarithmic reductions in titer implies that, while
 707 residual virus infectivity may be greatly reduced, it will never be reduced to zero. For
 708 example, a reduction in the infectivity of a preparation containing 8 log₁₀ infectious units
 709 per ml by a factor of 8 log₁₀ leaves zero log₁₀ per ml or one infectious unit per ml, taking
 710 into consideration the limit of detection of the assay; and

711 • Pilot-plant scale processing may differ from commercial-scale processing despite care
 712 taken to design the scaled-down process.

713 **6.5 Statistics**

714 The viral clearance studies should include the use of statistical analysis of the data to evaluate the
 715 results. The study results should be statistically valid to support the conclusions reached (refer to
 716 Annex 3).

717 **6.6 Application of Prior Knowledge for Evaluation of Viral Clearance**

718 As a general principle, viral clearance is evaluated by experiments when the virus is added to
 719 the product-specific in-process material of each step to be investigated. When a manufacturer
 720 is developing similar products by established and well-characterised processes (i.e., using the
 721 same platform technology), viral clearance data generated for other products might be
 722 applicable to the new product for the same processing step. However, to make use of data from
 723 such a step, the process step must be well-understood. The representativeness of the prior
 724 knowledge for the specific process step should be clearly justified. The prior knowledge
 725 comprised of external and in-house experience should cover the aspects outlined below:

- 726 • There should be an understanding of the mechanism underlying virus clearance;
- 727 • There should be an understanding of all process parameters that may affect viral
 728 clearance;
- 729 • It should be clear that interactions between virus and product do not affect viral
 730 clearance.

- 731 • The composition of a specific process intermediate may affect viral clearance. For some
732 process steps, even small differences in buffers, media, reagents, level, and profile of
733 impurities (for example) may substantially affect viral clearance. Therefore, the
734 representativeness of the composition of the process intermediate(s) from other products
735 should be justified. In addition, processing before the specific step for the new and the
736 established product(s) should follow a similar strategy unless prior knowledge indicates
737 robustness of virus clearance with respect to composition of the process intermediate;
738 and
- 739 • The general limitations of viral clearance studies as outlined in Section 6.4, should be
740 considered when applying prior knowledge to a specific product.

741 External prior knowledge (including published data) can be supportive in indicating the
742 potential of a step to inactivate/remove viruses and can provide insight to the mechanisms
743 involved. Such data can also be used to define the critical process parameters and in setting
744 worst-case limits for testing in a specific viral clearance step. Performing viral clearance studies
745 at worst-case conditions can help reduce the number of product-specific experiments. However,
746 the application of published reduction factors to a specific product should be supported by
747 demonstration of comparability of the processes across manufacture of different products
748 involved, comparability of the product intermediates, and an assurance that product-specific
749 attributes do not affect virus reduction. Therefore, published data should be carefully assessed
750 and supplemented with in-house experience (internal prior knowledge) for a given platform
751 technology.

752 The decision on the acceptability of virus clearance data without product-specific experiments
753 is made on a case-by-case basis while considering the whole viral safety concept for a medicinal
754 product, including the nature and characterisation of the cell substrate and raw materials and
755 the overall viral clearance strategy. If the data package does not sufficiently support the use of
756 prior knowledge, product-specific viral clearance studies should be performed.

757 When deriving a LRV claim using prior knowledge, the claim should be justified considering
758 all LRVs from the relevant platform data. A conservative LRV claim is advised to avoid a risk
759 for overestimating the reduction capacity of the process step.

760 Annex 6 provides cases when, according to current understanding, prior knowledge including
761 in-house experience with viral reduction data from other products could be used to claim a

762 reduction factor for a new product from the same manufacturing platform.

763 **6.7 Re-Evaluation of Viral Clearance**

764 Whenever significant changes in the production or purification process are made, the effect of
765 that change, both direct and indirect, on viral clearance should be considered and the system re-
766 evaluated, as needed. For example, changes in production processes may cause significant
767 changes in the amount of virus produced by the cell line; changes in process steps may change
768 the extent of viral clearance.

769 Changes in the manufacturing process during life-cycle management that may affect virus
770 clearance efficacy could be evaluated using internal knowledge and the platform concept. If the
771 internal knowledge (in-house experience) with other products cannot be extrapolated to specific
772 products and/or the platform concept can no longer be applied, product-specific viral clearance
773 studies must be performed.

774 **7. POINTS TO CONSIDER FOR CONTINUOUS MANUFACTURING** 775 **PROCESSES**

776 Continuous Manufacturing (CM) processes are dynamic systems consisting of integrated plural
777 unit operations in which raw materials; process intermediates; and starting materials enter the
778 manufacturing process continuously; and products are discharged throughout the
779 manufacturing process. CM can be applied to some or all unit operations. An understanding of
780 the integrated process and its dynamics, in addition to each unit operation, is essential to identify
781 and mitigate the risk to viral safety. A description of the types of CM processes for the
782 manufacture of therapeutic proteins can be found in ICH Q13 (Annex 3).

783 In terms of virus safety, technical aspects for CM may differ from those encountered in batch
784 processes including concepts of detection and removal of viruses; material traceability; system
785 dynamics; monitoring frequency start-up/shutdown; advanced process controls; process
786 validation; process models; and continuous process verification.

787 However, basic principles and expectations (such as science- and risk-based approaches and
788 their implementation to control virus risk), that are based on process understanding are the same
789 as for batch manufacturing. This also includes contamination prevention strategies (see Section
790 2.2). For example, the physical and chemical conditions to inactivate or remove viruses derived
791 from experience or prior knowledge of batch production are applicable when the target state of

792 control regarding process parameters, which are relevant for virus clearance is ensured even in
793 dynamic processes (see Section 6.6).

794 **7.1 Viral Safety in CM Processes**

795 Control of viruses in CM processes should be based on a risk assessment of potential sources
796 of contamination (e.g., the starting and raw materials and extended cell culture duration), the
797 ability of the process to remove viruses, and the testing capability to ensure absence of viruses.
798 Guidance on testing provided in Sections 3 and 4 is also considered applicable to CM. Based
799 on this assessment, a strategy should be developed to include the type and frequency of
800 adventitious virus testing undertaken to demonstrate that the process is free of contamination
801 during cell culture and other downstream steps.

802 **7.2 General Considerations for Virus Clearance in CM**

803 To design the manufacturing process and the virus clearance study, the following should be
804 considered:

- 805 • The manufacturing process may be partially run in continuous or connected mode of
806 operation and it is possible to use knowledge/experiences of virus clearance study
807 design based on batch processes for the evaluation of unit operation if suitable;
- 808 • The potential risk of each unit operation and the connection between equipment (e.g.,
809 use of a surge or mixing tank between unit operations to mitigate differences in mass
810 flow rate or inhomogeneity of input materials) should be assessed to cover any impact
811 to the virus reduction capabilities;
- 812 • There should be appropriate process monitoring and sampling strategies in place to
813 detect inadvertent disturbance or adventitious virus contamination. If conducting real
814 time decision making, this should include a procedure to determine the impact of the
815 disturbance or contamination on the output material quality and product. According to
816 the impact, the diversion of the potential non-conforming material from the product
817 stream or the disposition of the material produced should be taken into account; and
- 818 • The virus clearance study designs should consider potential impact of the following if
819 applicable:

- 820 ○ Fluctuation of input material attributes (e.g., viral load, concentration and
- 821 homogeneity of protein or impurities, and level of aggregation);
- 822 ○ Flow rate, temporal disturbance or pausing;
- 823 ○ Operational loading capacity;
- 824 ○ Multicolumn cycling.

825 CM also presents unique aspects to consider for virus safety.

826 ***7.2.1 Potential Risk Related to Longer Periods in Cell Culture Production***

827 Fluctuations in the levels of endogenous retrovirus may occur over time in the production
 828 culture so an assessment should be made of the appropriate sampling point so as not to impact
 829 the dose risk factor calculation for the drug product (see Section 4 and Considerations in Section
 830 3 for cell line qualification).

831 ***7.2.2 Approach to Virus Clearance Study***

832 Although CM is expected to maintain a state of control, the manufacturing process will include
 833 periods when the process output may vary during start-up, termination, and temporary process
 834 disturbance (e.g., potentially high virus load for a short period of time in case of a virus
 835 contamination). The risks for such periods may be addressed using best practices for clearance
 836 studies addressed elsewhere in this guideline. Considerations specific to CM would include:

- 837 • Chromatography
 - 838 ○ For the process of repeating sub-batches (e.g., multi-column), a batch process
 - 839 could serve as a scale-down model with well-justified target process
 - 840 conditions (e.g., flow rate, resin load vs column overload, resin cleanability);
 - 841 ○ Simultaneous validation of two or more connected unit operations could be
 - 842 an option according to the equipment design and system integration (e.g., bind
 - 843 and elute mode of Cation Exchange Chromatography (CIEX) and flow
 - 844 through mode of Anion Exchange Chromatography (AEX)), but only when
 - 845 all unit operations are to be validated for viral clearance. For connected unit
 - 846 operations, if the loading of the challenge material does not differ from batch
 - 847 operation, it is possible to evaluate with a conventional scale-down model;
- 848 • Low pH/solvent detergent inactivation

- 849 ○ Validation as a batch process could be appropriate with well-justified target
850 process conditions;
- 851 ○ For virus inactivation (e.g., pH and solvent/detergent) the control of relevant
852 dynamic process parameters should be ensured (e.g., pH, solvent/detergent
853 concentration, homogeneity and mixing, temperature, residence time);
- 854 ○ Care should be taken in evaluating/justifying the effect of scale (e.g.,
855 residence time distribution) when a scale-down model is applied for
856 inactivation in dynamic process;
- 857 ● Virus filtration
 - 858 ○ Validation as a batch process could be appropriate if settings of parameters
859 which have impact on virus clearance do not vary beyond ranges tested in
860 the virus clearance study (e.g., worst case setpoint); and
 - 861 ○ Process controls should be defined to allow for filter changes and post-use
862 integrity testing while maintaining viral clearance capacity. This should
863 include not interrupting the continuous process and allowing material
864 diversion in the event of a filter failure.

865 **8. SUMMARY**

866 This guideline suggests approaches for evaluating the risk of viral contamination and for the
867 removal of virus from product, thus contributing to the production of safe biotechnology
868 products derived from animal or human cell lines and emphasises the value of many strategies,
869 including:

- 870 ● Thorough characterisation/screening of cell substrate starting material to identify
871 which, if any, viral contaminants are present;
- 872 ● Assessment of potential risk by determination of the human cell tropism or
873 knowledge of human infections;
- 874 ● Establishment of an appropriate program of testing for adventitious viruses in
875 unprocessed bulk;
- 876 ● Careful design of viral clearance studies using different methods of virus inactivation
877 or removal in the same production process to achieve maximum viral clearance; and

- 878 • Performance of studies which assess virus inactivation and removal.

879 **9. GLOSSARY**

880 **Adventitious Virus**

881 See Virus.

882 **Cell Substrate**

883 Cells used to manufacture product.

884 **End of Production Cells (EOPC)**

885 Cells harvested (under conditions comparable to those used in production) from the MCB or
886 WCB cultured to a passage level or population doubling level comparable to or beyond the
887 highest level reached in production. End of production cells are cells at or beyond the LIVCA.

888 **Endogenous Virus**

889 See Virus.

890 ***In Vitro* Cell Age**

891 A measure of the period between thawing the MCB vial(s) and harvesting the production vessel
892 that is measured by elapsed chronological time in culture, population doubling level of the cells,
893 or passage level of the cells when subcultivated by a defined procedure for dilution of the
894 culture.

895 **Inactivation**

896 Reduction of virus infectivity caused by chemical or physical treatment.

897 **Master Cell Bank (MCB)**

898 An aliquot of a single pool of cells which generally has been prepared from the selected cell
899 clone under defined conditions, dispensed into multiple containers and stored under defined
900 conditions. The MCB is used to derive all working cell banks.

901 **Master Virus Seed (MVS)**

902 A master virus seed (stock, lot, or bank) is a preparation of a vaccine virus, helper virus, or viral
903 vector from which all future production will be derived.

904 **Minimum Exposure Time**

905 The shortest period for which a treatment step will be maintained.

906 **Next Generation Sequencing (NGS)**

907 Also referred to as high throughput sequencing (HTS) or massive parallel sequencing (MPS) or
908 deep sequencing, multi-step nucleic acid-based technology with broad capabilities for agnostic
909 detection of known and unknown adventitious agents. In some cases, NGS can be used for targeted
910 detection of known viruses.

911 **Platform Manufacturing (according to ICH Q11)**

912 The approach of developing a production strategy for a new drug starting from manufacturing
913 processes similar to those used by the same applicant to manufacture other drugs of the same
914 type (e.g., as in the production of monoclonal antibodies using predefined host cell, cell culture,
915 and purification processes for which considerable experience already exists).

916 **Platform Validation**

917 Throughout this guideline, this term exclusively refers to platform validation of virus clearance.

918 In this context, platform validation is defined as the use of prior knowledge including in house
919 experience with viral reduction data from other products, to claim a reduction factor for a new
920 similar product, according to current understanding.

921 **Prior Knowledge**

922 Prior knowledge refers to existing knowledge and includes internal knowledge (e.g.,
923 development and manufacturing experience), external knowledge (e.g., scientific and technical
924 publications, including vendors' data, literature, and peer-reviewed publications), or the
925 application of established scientific principles (e.g., chemistry, physics, and engineering
926 principles).

927 **Process Characterisation of Viral Clearance**

928 Viral clearance studies in which non-specific “model” viruses are used to assess the robustness
929 of the manufacturing process to remove and/or inactivate viruses.

930 **Process Evaluation Studies of Viral Clearance**

931 Viral clearance studies in which “relevant” and/or specific “model” viruses are used to
932 determine the ability of the manufacturing process to remove and/or inactivate these viruses.

933 **Process Robustness of Viral Clearance**

934 The term robustness is used to describe one of the two different characteristics. One
935 characteristic is the ability of a process or process step to tolerate variability of materials and
936 changes of the process without negative impact on clearing a virus. The other characteristic is
937 the ability to clear a wide range of specific and non-specific model viruses.

938 **Production Cells**

939 Cell substrate used to manufacture product.

940 **Supplementary Test Method**

941 A test method used to provide data to refine the conventional testing. It is a test method used to
942 overcome a limitation(s) in an existing test method, such as test article interference or toxicity.

943 **Unprocessed Bulk**

944 One or multiple pooled harvests of cells and culture media. When cells are not readily accessible,
945 the unprocessed bulk would constitute fluid harvested from the fermenter.

946 **Virus**

947 Intracellularly replicating infectious agents that are potentially pathogenic, that possess only a
948 single type of nucleic acid (either RNA or DNA), that are unable to grow and undergo binary
949 fission, and that multiply in the form of their genetic material.

950 **Adventitious Virus**

951 Unintentionally introduced contaminant viruses.

952 **Endogenous Virus**

953 Viral entity whose genome is part of the germ line of the species of origin of the cell line
954 and is covalently integrated into the genome of animal from which the parental cell line
955 was derived. In this guideline, intentionally introduced, non-integrated viruses such as
956 Epstein-Barr Virus used to immortalise cell substrates or Bovine Papilloma Virus.

957 **Helper Virus**

958 In the context of this guideline, a helper virus is a virus or a virus vector that provides a
959 function to enable expression or replication of the product.

960 **Non-Specific Model Virus**

961 A virus used for characterisation of viral clearance of the process when the purpose is to
962 characterise the capacity of the manufacturing process to remove and/or inactivate viruses
963 in general (i.e., to characterise the robustness of the purification process).

964 **Relevant Virus**

965 Virus used in the process evaluation studies that is either the identified virus, or of the same
966 species as the virus that is known, or likely to contaminate the cell substrate or any other
967 reagents or materials used in the production process.

968 **Specific Model Virus**

969 Virus which is closely related to the known or suspected virus (same genus or family),
970 having similar physical and chemical properties to those of the observed or suspected virus.

971 **Viral Clearance**

972 Elimination of target virus by removal of viral particles or inactivation of viral infectivity.

973 **Virus-Like Particles**

974 Structures visible by electron microscopy which morphologically appear to be related to known
975 viruses.

976 **Virus Removal**

977 Physical separation of virus particles from the intended product.

978 **Viral Vector**

979 A recombinant virus that may be applied *in vivo* as a medicinal product or applied *ex vivo* for other
980 advanced therapeutic applications. The genetically engineered viral vector may require a helper
981 virus for production.

982 **Viral Vector-Derived Product**

983 A product encoded and expressed by a recombinant virus. The genetically engineered viral vector
984 may require a helper virus for production.

985 **Working Cell Bank (WCB)**

986 The WCB is prepared from aliquots of a homogeneous suspension of cells obtained from culturing
987 the MCB under defined culture conditions.

988 **Working Virus Seed (WVS)**

989 A working virus seed (stock, lot, or bank) is produced from the MVS.

990 **Table 1. Virus Tests Recommended to Be Performed Once at Various Cell Levels**

| | MCB | WCB ^a | Cells at the LIVCA ^b |
|---|-----------------------------|------------------|---------------------------------|
| Tests for Retroviruses and Other Endogenous Viruses | | | |
| Infectivity | + | - | + |
| Electron microscopy ^c | + ^c | - | + ^c |
| Reverse transcriptase ^d | + ^d | - | + ^d |
| Other virus-specific tests ^e | as appropriate ^e | - | as appropriate ^e |
| Tests for Non-Endogenous or Adventitious Viruses | | | |
| <i>In vitro</i> Assays or NGS ^j | + ^f | + ^f | + ^f |
| <i>In vivo</i> Assays or NGS ^j | + ^g | - ^g | + ^g |
| Antibody production tests or specific molecular assay ^{h, j} | + ^h | - | - |
| Other virus-specific tests ⁱ | + ⁱ | - | - |

991 a. Section 3.1.2.

992 b. Cells at the limit: cells at the limit of *in vitro* cell age used for production (See Section 3.1.3).

993 c. May also detect other agents.

994 d. If a cell line is known to constitutively produce retroviral particles, the assay may not be needed.

995 e. As appropriate for cell lines that are known to have been infected by such agents.

996 f. The *in vitro* virus test is performed directly on the WCB or on LIVCA cells directly derived from this WCB.
 997 Tests for viruses using broad molecular methods (NGS) can be used as supplementary or replacement assays
 998 for the *in vitro* tests (cell culture and PCR) based on the risk assessment.

999 g. *In vivo* testing may be performed based on risk assessment. However, *in vivo* testing is not necessary for well-
 1000 characterised cell lines such as CHO, NS0 and SP2/0, based on cell line history; prior knowledge; and other
 1001 risk-based considerations. This includes prior *in vivo* virus testing or NGS testing of the parental untransfected
 1002 cell line and control of the derivation of the MCB from the parental cell bank. Prior knowledge of virus safety
 1003 testing of other MCB derived from the same parental cell bank including the method used to establish the
 1004 MCB also should be considered. The test is generally not necessary for the first WCB or subsequent WCB if
 1005 they are prepared under approved controlled conditions. For cells at the LIVCA, the test may not be necessary
 1006 based on prior knowledge and other risk-based considerations.
 1007 If residual risk remains, retention of the test or replacement with a molecular method for broad virus
 1008 detection (e.g., NGS or PCR) can be considered to detect viruses that may have been introduced during
 1009 establishment of the MCB or during culture of the cells at the LIVCA stage.

1010 h. e.g., MAP, RAP, HAP, which is usually applicable for rodent cell lines. Virus specific PCR or targeted
 1011 molecular methods can be used as a replacement assay to the animal testing. e.g., based on the origin and
 1012 history of the cell line including associated raw materials and reagents.

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- 1013 i. e.g., based on the origin and history of the cell line including associated raw materials and reagents
- 1014 j When applicable, NGS should be considered to replace the *in vivo* test and may be used to supplement or
- 1015 replace the *in vitro* and other virus specific tests based on assay suitability and risk assessment.

1016 **Table 2. Examples of the Use and Limitations of Assays Which Can Be Used to Test for Virus**

| <i>TEST</i> | <i>TEST ARTICLE</i> | <i>DETECTION CAPABILITY</i> | <i>DETECTION LIMITATION</i> |
|-----------------------------------|--|--|---|
| Antibody Production | Lysate of cells and their culture medium | Specific viral antigens | Antigens not infectious for animal test system |
| <i>in vivo</i> virus screen | Lysate of cells and their culture medium | Broad range of viruses | Viruses failing to replicate or produce diseases in the test system |
| <i>in vitro</i> virus screen for: | | Broad range of viruses | Viruses failing to replicate or produce diseases in the test system |
| 1. Cell bank characterisation | 1. Lysate of cells and their culture medium (for co-cultivation, intact cells should be in the test article) | | |
| 2. Production screen | 2. Unprocessed bulk harvest or lysate of cells and their cell culture medium from the production reactor | | |
| TEM on: | | Virus and virus-like particles | Qualitative assay with assessment of identity |
| 1. Cell substrate | 1. Viable cells | | |
| 2. Cell culture supernatant | 2. Cell-free culture supernatant | | |
| Reverse transcriptase (RT) | Cell-free culture supernatant | Retroviruses and expressed retroviral RT | Only detects enzymes with optimal activity under preferred conditions. Interpretation may be difficult due to presence of cellular enzymes; background with some concentrated samples |
| Retrovirus (RV) infectivity | Cell-free culture supernatant | Infectious retroviruses | RV failing to replicate or form discrete foci or plaques in the chosen test system |
| Cocultivation | Viable cells | Infectious retroviruses | RV failing to replicate |
| 1. Infectivity endpoint | | | 1. See above under RV infectivity |
| 2. TEM endpoint | | | 2. See above under TEM ^a |
| 3. RT endpoint | | | 3. See above under RT |
| PCR (Polymerase chain reaction) | Cells, culture fluid, and other materials | Specific virus sequences | Primer sequences must be present. Does not indicate whether virus is infectious |
| NGS | Cells, culture fluid and other materials | Broad range of viruses | Positive result does not indicate whether virus is infectious and may require further investigation |

1017 a. In addition, may be difficult to distinguish test article from indicator cells

1018

1019 **Table 3. Viruses Detected in Antibody Production Tests**

| <i>MAP⁴</i> | <i>HAP⁴</i> | <i>RAP⁴</i> |
|--|--|--|
| Ectromelia Virus ^{2,3} | Lymphocytic Choriomeningitis Virus (LCM) ^{1,3-} | Hantaan Virus ^{1,3} |
| Hantaan Virus ^{1,3} | Pneumonia Virus of Mice (PVM) ^{2,3} | Kilham Rat Virus (KRV) ^{2,3} |
| K Virus ² | Reovirus Type 3 (Reo3) ^{1,3} | Mouse Encephalomyelitis Virus (Theilers, GDVII) ² |
| Lactic Dehydrogenase Virus (LDM) ^{1,3} | Sendai Virus (SV) ^{1,3} | Pneumonia Virus of Mice (PVM) ^{2,3} |
| Lymphocytic Choriomeningitis Virus (LCM) ^{1,3,} | SV5 | Rat Coronavirus (RCV) ² |
| Minute Virus of Mice ^{2,3} | | Reovirus Type 3 (Reo3) ^{1,3} |
| Mouse Adenovirus (MAV) ^{2,3} | | Sendai Virus ^{1,3} |
| Mouse Cytomegalovirus (MCMV) ^{2,3} | | Sialodacryoadenitis Virus (SDAV) ² |
| Mouse Encephalomyelitis Virus (Theilers, GDVII) ² | | Toolan's H-1 Virus ^{2,3} |
| Mouse Hepatitis Virus (MHV) ² | | |
| Mouse Rotavirus (EDIM) ^{2,3} | | |
| Pneumonia Virus of Mice (PVM) ^{2,3} | | |
| Polyoma Virus ² | | |
| Reovirus Type 3 (Reo3) ^{1,3} | | |
| Sendai Virus ^{1,3} | | |
| Thymic Virus ² | | |

- 1020
- 1021 1. Viruses for which there is evidence of capacity for infecting humans or primates.
- 1022 2. Viruses for which there is no evidence of capacity for infecting humans.
- 1023 3. Virus capable of replicating *in vitro* in cells of human or primate origin.
- 1024 4. NAT such as PCR assays or other targeted molecular methods can be used for replacing specific rodent virus
- 1025 testing.
- 1026

1027 **Table 4. Recommended Action Plan for Process Assessment of Viral Clearance and Virus Tests**
 1028 **on Purified Bulk**

| | Case A | Case B | Case C ² | Case D ² | Case E ² | Case F |
|--|------------------|------------------|---------------------|---------------------|---------------------|------------------|
| STATUS | | | | | | |
| Presence of virus ¹ | - | - | + | + | (+) ³ | - |
| Virus-like particles ¹ | - | - | - | - | (+) ³ | - |
| Retrovirus-like particles ¹ | - | + | - | - | (+) ³ | - |
| Virus identified | not applicable | + | + | + | - | + |
| Virus infectious for humans | not applicable | - ⁴ | - ⁴ | + | unknown | (+) ⁹ |
| Presence of helper virus | - | - | - | - | - | + |
| ACTION | | | | | | |
| Process characterisation of viral clearance using non-specific “model” viruses | yes ⁵ | yes ⁵ | yes ⁵ | yes ⁵ | yes ⁷ | yes ⁵ |
| Process evaluation of viral clearance using “relevant” or specific “model” viruses | no | yes ⁶ | yes ⁶ | yes ⁶ | yes ⁷ | yes ⁹ |
| Test for virus in purified bulk | not applicable | no | yes ⁸ | yes ⁸ | yes ⁸ | yes ⁹ |

- 1029 1. Results of virus tests for the cell substrate and/or at the unprocessed bulk level. Cell cultures used for production that are
 1030 contaminated with viruses generally should not be used unless justified by specific viral clearance and risk assessment.
 1031 Endogenous viruses (such as retroviruses) or viruses that are an integral part of the MCB may be acceptable if appropriate
 1032 viral clearance evaluation procedures are followed.
- 1033 2. Source material that is contaminated with viruses, whether they are known to be infectious and/or pathogenic in humans,
 1034 should only be used under exceptional circumstances by demonstration of specific viral clearance and risk assessment.
- 1035 3. Virus has been observed by either direct or indirect methods.
- 1036 4. Believed to be non-pathogenic.
- 1037 5. Characterisation of clearance using non-specific “model” viruses should be performed.
- 1038 6. Process evaluation for “relevant” viruses or specific “model” viruses should be performed.
- 1039 7. See text under Case E.
- 1040 8. The absence of detectable virus should be confirmed for purified bulk by means of suitable methods having high specificity
 1041 and sensitivity for the detection of the virus in question. For marketing authorisation, data from at least 3 lots or batches
 1042 of purified bulk manufactured at pilot-plant scale or commercial scale should be provided.
- 1043 9. Virus may or may not be infectious for humans. Process evaluation for the helper virus (recombinant or wild type) should
 1044 be performed. If this is not possible, then a specific model virus should be used.). When utilised in production, the helper
 1045 virus is quantified in the unprocessed bulk stage using at least three cell culture campaigns to determine the target for virus
 1046 clearance. Following purification, absence of detectable helper virus is determined using an infectivity assay with relevant

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1047 permissive cell lines for sensitive virus detection. Alternatively, molecular methods may be used. Absence of the
1048 residual helper virus should be confirmed for each purified bulk.

1049 **ANNEX 1: PRODUCTS DERIVED FROM CHARACTERISED CELL BANKS**
1050 **WHICH WERE SUBSEQUENTLY GROWN *IN VIVO***

1051 For products manufactured from fluids harvested from animals inoculated with cells from
1052 characterised banks, additional information regarding the animals should be provided.

1053 Whenever possible, animals used in the manufacture of biotechnological/biological products
1054 should be obtained from well-defined, specific pathogen-free colonies. Adequate testing for
1055 appropriate viruses, such as those listed in Table 3, should be performed. Quarantine procedures
1056 for newly arrived and diseased animals should be described and assurance provided that all
1057 containment, cleaning, and decontamination methodologies employed within the facility are
1058 adequate to contain the spread of adventitious agents. This can be accomplished through the
1059 use of a sentinel program. A listing of agents for which testing is performed should also be
1060 included. Veterinary support services should be available onsite or within easy access. The
1061 extent to which the vivarium is segregated from other areas of the manufacturing facility should
1062 be described. Personnel practices should be adequate to ensure safety.

1063 Procedures for the maintenance of the animals should be fully described. These would include
1064 diet, cleaning and feeding schedules, provisions for periodic veterinary care if applicable, and
1065 details of special handling that the animals may require once inoculated. A description of the
1066 priming regimen(s) for the animals, the preparation of the inoculum and the site and their route
1067 of inoculation should also be included.

1068 The primary harvest material from animals may be considered an equivalent stage of
1069 manufacturing unprocessed bulk harvest from a bioreactor. Therefore, all testing considerations
1070 previously outlined in Section 4 of this document should apply. In addition, the manufacturer
1071 should assess the bioburden of the unprocessed bulk, determine whether the material is free of
1072 mycoplasma, and perform species-specific assay(s) as well as *in vivo* testing in adult and
1073 suckling mice.

1074 **ANNEX 2: THE CHOICE OF VIRUSES FOR VIRAL CLEARANCE STUDIES**

1075 **A. *Examples of Useful “Model” Viruses***

1076 a. Non-specific “model” viruses representing a range of physico-chemical structures:

1077 – SV40 (*Macaca mulatta polyomavirus*), animal parvovirus or some other small, non-
1078 enveloped viruses;

1079 – a parainfluenza virus or influenza virus, Sindbis virus or some other medium-to-large,
1080 enveloped, RNA viruses;

1081 – a herpes virus (e.g., HSV-1 or a pseudorabies virus), or some other medium-to-large,
1082 DNA viruses.

1083 These viruses are examples only, and their use is not mandatory.

1084 b. For cell substrates producing retroviral-like particles, murine retroviruses are commonly
1085 used as specific “model” viruses. It may be also possible to use endogenous murine or
1086 other rodent retrovirus particles.

1087 **B. *Examples of Viruses That Have Been Used in Viral Clearance Studies***

1088 Several viruses which have been used in viral clearance studies are listed in Table A-1.
1089 However, as these are merely examples, the use of any of the viruses in the table is not
1090 mandatory and manufacturers are invited to consider other viruses, especially those which may
1091 be more appropriate for their individual production processes. Generally, the process should be
1092 assessed for its ability to clear at least three different viruses with differing characteristics.

1093 **Table A-1: Examples of Viruses Which Have Been Used in Viral Clearance Studies**

| <i>Virus</i> | <i>Family</i> | <i>Genus</i> | <i>Natural host</i> | <i>Genome</i> | <i>Env</i> | <i>Size (nm)</i> | <i>Shape</i> | <i>Resistance a</i> |
|--|---------------|------------------|----------------------------|---------------|------------|------------------|--------------|---------------------|
| Vesicular Stomatitis Virus b | Rhabdo | Vesiculovirus | Equine Bovine | RNA | yes | 70x150 | Bullet | Low |
| Parainfluenza Virus | Paramyxo | Paramyxovirus | Various | RNA | yes | 100-200+ | Pleo/Sphere | Low |
| MuLV | Retro | gammaretrovirus | Mouse | RNA | yes | 80-110 | Spherical | Low |
| Sindbis Virus | Toga | Alphavirus | Human | RNA | yes | 60-70 | Spherical | Low |
| BVDV | Flavi | Pestivirus | Bovine | RNA | yes | 50-70 | Pleo-Sphere | Low |
| Pseudorabies Virus b,c | Herpes | Varicellovirus | Swine | DNA | yes | 120-200 | Spherical | Med |
| Autographa californica multiple nucleopolyhedrovirus c | Baculo | Alphabaculovirus | Insect | DNA | yes | 250-300 | Polyhedral | Med |
| Adenovirus Type 2 or Type 5 c | Adeno | Adenovirus | Human | DNA | no | 70-90 | Icosahedral | Med |
| Vesivirus 2711 | Calici | Vesivirus | | RNA | no | 27-40 | Icosahedral | Med |
| Encephalomyocarditis Virus (EMCV) | Picornia | Cardiovirus | Mouse | RNA | no | 25-30 | Icosahedral | Med |
| Reovirus 3 | Reo | Orthoreovirus | Various | RNA | no | 60-80 | Spherical | Med |
| SV40 | Papova | Polyomavirus | Monkey | DNA | no | 40-50 | Icosahedral | Very high |
| Parvoviruses (canine, murine, porcine) d | Parvo | Parvovirus | Canine Mouse Porcine | DNA | no | 18-24 | Icosahedral | Very high |

1094 a. Resistance to physicochemical treatments based on studies of production processes. Resistance is relative to the specific treatment, and it is used in the context of the understanding of the biology of the virus and the nature of the manufacturing process. Actual results will vary according to the treatment.

1097 b. Relevant model for rhabdovirus found in insect cells

1098 c. Specific models or relevant virus for helper virus used for viral vector production

1099 d. May be used as single worst-case model virus for larger spherical/icosahedral viruses and enveloped viruses at validation of virus filters.

1101 These viruses are examples only, and their use is not mandatory.

1102 **ANNEX 3: STATISTICAL CONSIDERATIONS FOR ASSESSING VIRUS AND**
1103 **VIRUS REDUCTION FACTORS**

1104 Virus titrations suffer the problems of variation common to all biological assay systems.
1105 Assessment of the accuracy of the virus titrations and reduction factors derived from them and the
1106 validity of the assays should be performed to define the reliability of a study. The objective of
1107 statistical evaluation is to establish that the study has been carried out to an acceptable level of
1108 virological competence.

1109 1. Assay methods can be either quantal or quantitative. Quantal methods include infectivity
1110 assays in animals or in Tissue-Culture-Infectious-Dose (TCID) assays, in which the animal
1111 or cell culture is scored as either infected or not. Infectivity titers are then measured by the
1112 proportion of animals or culture infected. In quantitative methods, the infectivity measured
1113 varies continuously with the virus input. Quantitative methods include molecular-based
1114 methods or plaque assays in which each plaque counted corresponds to a single infectious
1115 unit. Both quantal and quantitative assays are amenable to statistical evaluation.

1116 2. Variation can arise within an assay as a result of dilution errors, statistical effects and
1117 differences within the assay system that are either unknown or difficult to control. These
1118 effects are likely to be greater when different assay runs are compared (between-assay
1119 variation) than when results within a single assay run are compared (within-assay
1120 variation).

1121 3. The 95% confidence limits for results of within-assay variation normally should be on the
1122 order of $\pm 0.5 \log_{10}$ of the mean. Within-assay variation can be assessed by standard
1123 textbook methods. Between-assay variation can be monitored by the inclusion of a
1124 reference preparation, the estimate of whose potency should be within approximately 0.5
1125 \log_{10} of the mean estimate established in the laboratory for the assay to be acceptable.
1126 Assays with lower precision may be acceptable with appropriate justification.

1127 4. The 95% confidence limits for the reduction factor observed should be calculated wherever
1128 possible in studies of clearance of “relevant” and specific “model” viruses. If the 95%
1129 confidence limits for the viral assays of the starting material are $+s$, and for the viral assays
1130 of the material after the step are $+a$, the 95% confidence limits for the reduction factor are

1131
$$\sqrt{S^2 + a^2}$$

1132 **Probability of Detection of Viruses at Low Concentrations**

1133 At low virus concentrations (e.g., in the range of 10 to 1,000 infectious particles per liter), it is
 1134 evident that a sample of a few millilitres may or may not contain infectious particles. The
 1135 probability, p , that this sample does not contain infectious viruses is:

1136
$$p = ((V-v)/V)^n$$

1137 when V (liter) is the overall volume of the material to be tested, v (liter) is the volume of the sample
 1138 and n is the absolute number of infectious particles statistically distributed in V .

1139 If $V \gg v$, this equation can be approximated by the Poisson distribution:

1140
$$p = e^{-cv}$$

1141 when c is the concentration of infectious particles per liter.

1142 Or, $c = \ln p / -v$

1143 As an example, if a sample volume of 1 ml is tested, the probabilities p at virus concentrations
 1144 ranging from 10 to 1,000 infectious particles per liter are:

| | | | | |
|------|-----|------|------|------|
| 1145 | c | 10 | 100 | 1000 |
| 1146 | p | 0.99 | 0.90 | 0.37 |

1147 This indicates that for a concentration of 1,000 viruses per liter, in 37% of sampling, 1 ml will not
 1148 contain a virus particle.

1149 If only a portion of a sample is tested for virus and the test is negative, the amount of virus which
 1150 would have to be present in the total sample to achieve a positive result should be calculated and
 1151 this value taken into account when calculating a reduction factor. Confidence limits at 95% are
 1152 desirable. However, in some instances, this may not be practical due to material limitations.

1153 **ANNEX 4: CALCULATION OF REDUCTION FACTORS IN STUDIES TO**
1154 **DETERMINE VIRAL CLEARANCE**

1155 The virus reduction factor of an individual purification or inactivation step is defined as the
1156 \log_{10} of the ratio of the virus load in the pre-purification material and the virus load in the post-
1157 purification material that is ready for use in the next step of the process. If the following
1158 abbreviations are used:

1159 Starting material:

1160 vol v' ; titer $10^{a'}$;

1161 virus load: $(v')(10^{a'})$,

1162 Final material:

1163 vol v'' ; titer $10^{a''}$;

1164 virus load: $(v'')(10^{a''})$,

1165 the individual reduction factors R_i are calculated according to

1166
$$10^{R_i} = (v')(10^{a'}) / (v'')(10^{a''})$$

1167 This formula takes into account both the titers and volumes of the materials before and after the
1168 purification step.

1169 Because of the inherent imprecision of some virus titrations, an individual reduction factor used
1170 for the calculation of an overall reduction factor should be greater than 1.

1171 The overall reduction factor for a complete production process is the sum logarithm of the
1172 reduction factors of the individual steps. It represents the logarithm of the ratio of the virus load
1173 at the beginning of the first process clearance step and at the end of the last process clearance
1174 step. Reduction factors are normally expressed on a logarithmic scale, which implies that, while
1175 residual virus infectivity will never be reduced to zero, it may be greatly reduced
1176 mathematically.

1177 **ANNEX 5: CALCULATION OF ESTIMATED PARTICLES PER DOSE**

1178 This Annex is applicable to those viruses, such as endogenous retroviruses, for which an estimate of
1179 starting numbers can be made.

1180 Example:

1181 *I. Assumptions*

1182 Measured or estimated concentration of virus in cell culture harvest = 10^6 /ml

1183 Calculated viral clearance factor = $>10^{15}$

1184 Volume of culture harvest needed to make a dose of product = 1 litre (10^3 ml)

1185 *II. Calculation of Estimated Particles/Dose*

1186 $(10^6 \text{ virus units/ml}) \times (10^3 \text{ ml/dose})$

1187 Clearance factor $>10^{15}$

1188 = 10^9 particles/dose

1189 $\frac{10^9 \text{ particles/dose}}{\text{Clearance factor } >10^{15}}$

1190 = $<10^{-6}$ particles/dose

1191 Therefore, less than one particle per million doses would be expected.

1192 The case above is typical for the reduction of endogenous retroviruses during the manufacture of
1193 monoclonal antibodies from rodent cells (Case B). In a comprehensive risk assessment for a specific
1194 virus, additional factors should be considered, such as the host range of the virus, the pathogenicity
1195 of the virus, measures to avoid contamination, testing measures, the route of administration, and the
1196 human infectious dose.

1197 In the Case B scenario for Chinese Hamster Ovary (CHO) cells, a safety margin of $<10^{-4}$
1198 particles/dose is considered acceptable for Retroviral-Like Particles (RVLs) for recombinant
1199 proteins if *in vitro* testing fails to identify the presence of infectious retroviruses.

1200 **ANNEX 6: EXAMPLES OF PRIOR KNOWLEDGE INCLUDING IN-HOUSE**
1201 **EXPERIENCE TO REDUCE PRODUCT-SPECIFIC VALIDATION**
1202 **EFFORT**

1203 According to the general principles for a platform validation approach, robust virus clearance
1204 should be demonstrated across products from the same platform and the procedure for virus
1205 clearance should follow established and well-characterised conditions. In addition, it should be
1206 shown that the composition of the product intermediate is comparable to the intermediates used
1207 in virus clearance studies unless prior knowledge indicates robustness of virus clearance with
1208 respect to product intermediate composition.

1209 In this context, platform validation is defined as the use of prior knowledge including in-house
1210 (applicant-owned data) experience with viral reduction data from other products, to claim a
1211 reduction factor for a new similar product. In general, a virus clearance claim for a new product
1212 based on prior knowledge including in-house experience should include a discussion of all the
1213 data available and the rationale to support the platform validation approach (see Section 6.6).
1214 Part of the prior knowledge and in-house data used to reduce product-specific validation could
1215 be provided as a comparison of the new product and its manufacturing process with other in-
1216 house products, related process conditions, and product intermediates.

1217 Process steps dedicated to virus clearance (e.g., inactivation by detergent, low pH and removal
1218 by viral filtration) are suitable for a platform validation approach.

1219 Therefore, examples for application of prior knowledge to XMuLV inactivation/removal by
1220 detergent and incubation at low pH as well as virus filtration are given below.

1221 These mock examples are provided for illustrative purposes, only suggest how the platform
1222 validation approach could be applied, and should not be used as a template or the sole basis for
1223 a regulatory submission.

1224 Tables A-2 to A-4 summarize process parameters and their potential criticality for the
1225 individual process step according to the current understanding of a wide range of process
1226 conditions applied across industry. The actual impact of process parameters and intermediates
1227 on XMuLV clearance should be assessed by prior knowledge and in-house experience.

1228 Based on evolving process understanding, further process steps may be recommended for
1229 platform validation in the future.

1230 Inactivation by Solvent/Detergent (SD) or Detergent Alone

1231 Based on the mechanism of action, detergent concentration of SD reagents or detergent alone
1232 is an important process parameter.

1233 In addition, hydrophobic impurities such as lipids, cell debris, or components of cell culture
1234 media such as antifoaming agents can impact virus inactivation by challenging the detergent or
1235 SD mixture in solubilizing the virus lipid envelope and therefore should be assessed.

1236 There is, so far, no indication that the interaction between virus and a specific therapeutic
1237 protein affects inactivation by detergent. Aggregates (e.g., cell debris or aggregated virus
1238 particles) can potentially entrap and protect viral particles from detergent access. Therefore, at
1239 manufacture, the product intermediate (e.g., Harvested Cell Culture Fluid (HCCF)) should be
1240 clarified from cells/cellular debris including a filtration step of ≤ 0.2 μm nominal pore size
1241 before detergent inactivation.

1242 The following paragraph describes how to apply a platform validation approach to XMuLV
1243 inactivation using SD or Triton X-100 as an example. The approach may also be applicable to
1244 alternate detergents shown to provide robust and efficient XMuLV inactivation.

1245 Triton X-100 is a non-ionic detergent commonly used in membrane research to solubilize lipid
1246 bilayers. It inactivates enveloped viruses by solubilizing the virus lipid envelope thus rendering
1247 the virus non-infectious. Triton X-100 has been widely used for viral inactivation in
1248 manufacturing processes of plasma-derived products for many years as well as in platform
1249 purification processes for Monoclonal Antibodies (MAb) by addition to HCCF.

1250 The European Chemicals Agency included Triton X-100 in the Authorisation List (Annex XIV)
1251 due to hormone-like activity of degradation compounds in the environment. Therefore, though
1252 widely used, the pharmaceutical industry is looking into alternate detergents. Other detergents
1253 with similar physicochemical properties are commercially available and achieve efficient
1254 XMuLV inactivation.

1255 Because of the non-ionic nature of Triton X-100, its effectiveness should not be sensitive to pH,
1256 to ionic strength, or to the nature of the counter ions in HCCF. Prior experience indicates
1257 effective XMuLV inactivation in HCCF at 0.2 % Triton X-100 concentration, at 15°C, and at
1258 60-minute incubation across multiple products from platform processes covering a range of
1259 typical lipid and total protein content in HCCF. However, as indicated below, applying a Triton

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1260 concentration of 0.5% is recommended to ensure effective and reliable inactivation when
1261 omitting product-specific experiments.

1262 Table A-2 summarizes process parameters and their potential criticality for detergent-based
1263 inactivation of lipid-enveloped virus.

1264 **Table A-2: Summary of Process Parameters and Their Potential Impact for Detergent**
 1265 **Inactivation**

| <i>Process parameter</i> | <i>Potential Impact</i> | <i>Rationale</i> |
|---|-------------------------|--|
| SD or Triton X-100 concentration | High | Inactivating agent |
| Incubation time | High | Mechanism of inactivation is time-dependent |
| Temperature | High | Impact on inactivation kinetics |
| Pre-treatment by 0.2 µm filtration | High | Removal from the starting intermediate of aggregates potentially entrapping and protecting viral particles from detergent access |
| Total lipid content or surrogate parameter in HCCF | Low | Low impact observed with worse-case HCCF |
| Type of product | Low | No impact on inactivation observed for MAb, half antibody, fusion protein or recombinant protein |
| Total protein content in HCCF | Low | Low impact observed with worse-case HCCF |
| pH | Low | Triton X-100 is a non-ionic detergent |
| Ionic strength | Low | See above |
| Buffer salt in HCCF | Low | See above |
| Potential interaction between virus particle and product | Low | No impact on inactivation observed and disruption of lipid envelope lowers probability of interaction with product |

1266 Thus, consistent with current process understanding $\geq 0.5\%$ Triton X-100 treatment of clarified
 1267 HCCF for ≥ 60 minutes at $\geq 15^\circ\text{C}$ effectively inactivates XMuLV for multiple cell-culture
 1268 derived products. Treatment with 1% Triton X-100 and 0.3% Tri-N-Butylphosphate (TNBP)
 1269 for $\geq 30\text{min}$ or treatment with 1% polysorbate 80 and 0.3%TNBP for $\geq 6\text{h}$ at $\geq 23^\circ\text{C}$ effectively
 1270 inactivates retroviruses. According to current process understanding, a platform validation
 1271 approach may be applied for XMuLV inactivation by SD treatment or treatment with Triton X-
 1272 100 alone.

1273 **Incubation at Low pH**

1274 Low pH treatment inactivates enveloped viruses by denaturing proteins located in the viral
 1275 envelope, thus disrupting the lipid envelope. Low pH treatment of the capture chromatography
 1276 product pool has been widely used for retrovirus inactivation in manufacturing processes of
 1277 cell-culture-derived products such as monoclonal antibodies (MAb).

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1278 Inactivation efficiency depends on the concentration of hydrogen ions as the inactivating agent,
1279 measured as pH, incubation time and temperature, and buffer matrix. Extremely high ionic
1280 strength may impact inactivation efficiency as well.

1281 Table A-3 summarizes process parameters and their potential impact for low pH inactivation of
1282 XMuLV.

1283 **Table A-3: Summary of Process Parameters and Their Potential Impact for low pH inactivation**
 1284 **and impact on XMuLV**

| <i>Process Parameter</i> | <i>Potential Impact</i> | <i>Rationale</i> |
|---|-------------------------|---|
| pH | high | Inactivating agent |
| Incubation time | high | Mechanism of inactivation is time-dependent |
| Temperature | high | Impact on inactivation kinetics |
| Buffer matrix | high | Available data show that inactivation robustness depends on buffer matrix |
| Product concentration | low | No impact on inactivation observed |
| Type of product | low | No impact on inactivation observed for MAb, half antibody, bispecific antibody, fusion protein or recombinant protein |
| NaCl concentration (a) | low | No impact if ≤ 500 mmol/L sodium chloride |
| Potential interaction between virus particle and product | low | No impact on inactivation observed. |

1285 (a): to date, data on influence of ionic strength of other buffers is limited.

1286 Consistent with current process understanding low pH treatment at \leq pH 3.6, $\geq 15^\circ\text{C}$ for ≥ 30
 1287 min at ≤ 500 mmol/L sodium chloride concentration is effectively inactivating XMuLV.

1288 Acetate and citrate buffer are most commonly used and allow for robust XMuLV inactivation.

1289 According to the current process understanding, a platform validation approach can be applied
 1290 for XMuLV inactivation by low pH treatment.

1291 **Virus Filtration**

1292 The mechanism of action of virus filtration is size-based particle removal. In general,
 1293 volumetric throughput of the product intermediate as well as the volumetric throughput of the
 1294 buffer used for flushing filters and pressure including pressure interruptions are potentially
 1295 critical parameters in virus filtration.

1296 A potential interaction of virus particles with the product is not critical when the virus particle
 1297 size is much larger than the distribution of filter pore size. However, when the virus particle
 1298 size and pore size are similar, the influence of the potential interaction on flow dynamics and
 1299 virus retention is not fully understood.

1300 This section focuses on using prior knowledge and in-house experience in virus filtration of
 1301 other products to claim retrovirus removal by small and large virus-retentive filters.

1302 Factors that impact efficient retrovirus removal by small-virus filters are well understood with
1303 respect to variation of process parameters such as membrane type, flow- or pressure-controlled
1304 filtration mode, and pressure interruptions. Based on predictability and robustness of virus
1305 removal this process step is considered suitable for a platform validation approach.

1306 For virus removal using small virus filters, one option is to apply parvovirus log reduction
1307 values for larger spherical/icosahedral viruses and enveloped viruses. However, sometimes this
1308 could result in underestimating virus removal capacity (e.g., retrovirus removal capacity) as a
1309 result of parvovirus passage. Given the size-based mechanism of action, and industry's
1310 experience of robust complete retrovirus removal with small virus filters, companies could use
1311 their in-house data from parvovirus and retrovirus removal to build a platform retrovirus
1312 clearance claim for commonly used small virus filters.

1313 According to the size-based removal mechanism, the theoretical risk of virus passage through
1314 a small-virus retentive filter is higher for small viruses than for retroviruses.

1315 A thorough understanding of the impact of pressure interruptions, as well as volume throughput
1316 and filter flush volume reflecting good manufacturing practice conditions should be conserved.

1317 If using prior knowledge and in-house experience from other products to claim parvovirus
1318 removal, at least one confirmatory product-specific validation run using a parvovirus should be
1319 performed.

1320 The type of virus filter is important for virus reduction and its robustness with respect to impact
1321 of process parameters and should be considered when designing platform data.

1322 **Table A-4. Summary of Process Parameters and Their Potential Impact for Parvovirus**
 1323 **Clearance by Small Virus-Retentive Filters**

| <i>Process Parameter</i> | <i>Potential Impact</i> | <i>Rationale</i> |
|---|-------------------------|--|
| Volumetric throughput of product intermediate loaded on the virus filter | High | Low level parvovirus passage has been observed depending on the specific filter type |
| Volumetric throughput of the buffer used for flushing filters | High | Low level parvovirus passage has been observed |
| Pressure | high | Pressure should not exceed the upper limit for filter operation. Low pressure can be worse case for a specific membrane type. Pressure interruption (if occurring during filtration or at switching from filtration of product intermediate to filter flush) should be considered. |
| Type of product | low | No impact on virus clearance observed for MAb, half antibody, bispecific antibody, fusion protein or recombinant protein |
| Product concentration | low | No negative impact on virus clearance observed |
| pH | low | No negative impact on virus clearance due to size-based removal |
| Ionic strength | low | Limited impact on virus clearance has been observed |
| Buffer matrix | low | Limited impact on virus clearance has been observed |
| Potential interaction between virus particle and product | low | Specific interaction between virus and antibody can enhance virus retention |

1324

1325 **ANNEX 7: GENETICALLY-ENGINEERED VIRAL VECTORS AND VIRAL**
1326 **VECTOR-DERIVED PRODUCTS**

1327 **7.1 Introduction**

1328 Advances in biotechnology have led to an emergence of new and advanced production
1329 platforms expressing new product types manufactured using characterised cell banks of human
1330 or animal origin (i.e., avian, mammalian, or insect). The scope of Annex 7 includes helper-virus
1331 dependent and helper-virus independent genetically-engineered viral vectors and viral vector-
1332 derived products that are amenable to virus clearance based on considerations of the
1333 physicochemical properties of the product. These products include Virus-Like Particles (VLPs)
1334 and protein subunits that are produced using baculovirus/insect cells, nanoparticle-based
1335 vaccines, and viral-vector products such as AAV. These medicinal products may be applied *in*
1336 *vivo* or *ex vivo*.

1337 Helper-virus independent products are manufactured using stably transformed or transiently
1338 transfected cell lines or by infection with a protein-expression virus vector (e.g., recombinant
1339 baculovirus). Helper-virus dependent products require a helper virus to enable expression of the
1340 product or replication of the viral vector (e.g., adeno-associated virus or recombinant proteins
1341 that are expressed using a helper virus such as herpes simplex virus or adenovirus).

1342 The potential sources for virus contamination for a biopharmaceutical product are described in
1343 Section 2 of the main guideline. Additional contamination risks such as those introduced by the
1344 expression system and the potential for contamination with replication competent virus should
1345 be considered. The susceptibilities of the cell substrate to adventitious viruses should be
1346 carefully considered when assessing the potential for extrinsic contamination during product
1347 manufacture. The use of well-characterised cell banks and virus seeds can reduce the risk of
1348 virus contamination. Furthermore, helper viruses used for production are considered process-
1349 related viral contaminants.

1350 Viral safety and contamination controls of new product types should be assured through the
1351 application of a comprehensive program of material sourcing, virus testing at appropriate steps
1352 of manufacture and removal and/or inactivation of adventitious viruses and helper viruses by
1353 the manufacturing process. If virus clearance is limited, virus safety should focus on the testing
1354 and control of the raw materials and reagents and the manufacturing process.

1355 Accordingly, a risk-based approach should be applied for demonstrating viral safety of the

1356 product.

1357 **7.2 Testing for Viruses**

1358 Extensive testing and characterisation for both endogenous and adventitious viral
 1359 contamination should be performed at suitable stages of manufacturing to support the overall
 1360 product safety. Based on the product type and its associated risk factors, the testing scheme
 1361 should apply across the product lifecycle. Table A-5 below provides an outline of the tests to
 1362 be performed at various steps during production. The tests applied for virus seeds, vector
 1363 harvest, and drug substance are described. Although the testing and characterisation scheme
 1364 proposed for the cell substrates used for viral vector production are broadly aligned with Table
 1365 1 in the main guidance document, additional considerations may apply for these product types,
 1366 and are therefore specified within Table A-5 below for completeness.

1367 The type and extent of testing depends on the risk assessment considering the specific risk
 1368 factors associated with the cell substrate and the manufacturing process. Factors that should be
 1369 considered include the origin, passage history and characteristics of the cell substrate and viral
 1370 vector, the raw materials and reagents and culture methods used, the reliance on helper
 1371 virus(es), and the capacity of the manufacturing process to inactivate and/or remove viruses.

1372 **Table A-5: Tests that Should Be Performed at Applicable Manufacturing Stages**

| <i>Test</i> | <i>MCB, WCB, Cells at the LIVCA</i> | <i>Virus Seed^k</i> | <i>Unprocessed Bulk (Harvest)</i> | <i>Drug Substance</i> |
|---|--|-------------------------------|-----------------------------------|-----------------------|
| Test for adventitious or endogenous viruses | | | | |
| ^{a, b} <i>In vitro</i> assays or NGS | ⁱ See Table 1 of main guideline | + ^h | + ^h | - |
| ^b <i>In vivo</i> assays or NGS | | + ^h | - ^{h, l} | - |
| ^c other virus specific tests | | l | l | - |
| ^d Antibody production assays or specific molecular assay | | + ^{i, l} | - | - |
| Tests for Endogenous, Helper and Replication Competent Viruses, as applicable | | | | |
| ^e retroviruses | ⁱ See Table 1 of main guideline | + | + ^l | - |
| ^f residual helper Viruses | NA | - | + | + ^l |
| ^g replication competent viruses | + | + | (+) | (+) |

1373 ^aTesting should be performed on permissive cell lines, based on risk assessment. The indicator cells cultures should be observed
 1374 for at least 2 weeks, with a further secondary passage of 2 weeks of observation. Include testing for haemadsorbing and

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1375 hemagglutinating viruses. For products produced in insect cell lines, the testing should include a permissive cell line for
1376 arboviruses (e.g., BHK cells). If viral vectors and viral vector-derived products cannot be neutralised, a validated alternative
1377 assay can be used. Testing should be performed on the virus seed and the unprocessed bulk harvest before downstream
1378 processing. In some cases, the unprocessed bulk harvest may be the same as the drug substance.

1379 ^bWhen applicable, broad NGS should be considered to replace the *in vivo* adventitious virus tests and may be used to
1380 supplement or replace the *in vitro* tests based on assay suitability and risk assessment.

1381 ^cSpecies-specific virus detection (e.g., NAT and cell culture or targeted NGS) is determined based on risk assessment of the
1382 cell substrate, raw materials or reagents, and manufacturing process. This may include human or rodent species-specific viruses,
1383 arboviruses in insect cells, and bovine or porcine viruses if serum components or trypsin are used.

1384 ^dAntibody production test (MAP, HAP, RAP) or virus specific NAT or targeted NGS may be performed based on risk
1385 assessment of the cell substrate, raw materials, or reagents and manufacturing process.

1386 ^e The presence of retrovirus using reverse transcriptase assays at the MCB and virus seed stage should be considered. If the
1387 MCB or virus seed is positive for reverse transcriptase activity, follow-up should include quantification of potential retroviral
1388 particles in the unprocessed bulk harvest from at least three cell culture campaigns to determine the target level for virus
1389 clearance. In addition, a PCR-based RT assay (PBRT) assay, for example, the product-enhanced reverse transcriptase assay
1390 (PERT), should be performed on the Unprocessed Bulk (harvest), based on risk assessment.

1391 ^fWhen used in production, the helper virus should be quantified at the unprocessed bulk stage using at least three cell culture
1392 campaigns to determine the target for virus clearance. Following purification, absence of detectable helper virus should be
1393 determined using infectivity assay with relevant permissive cell lines for sensitive virus detection. Alternatively molecular
1394 methods may be used. Absence of residual helper virus should be confirmed for each purified bulk (Case F, Table 4).

1395 ^gReplication Competent Virus (RCV) may develop at any step during manufacturing (e.g., at initial transfection or transduction
1396 steps and through production). Current recommendations include testing for RCV at multiple stages of manufacture to detect
1397 for recombination or for the vector virus to revert to parental or wild type phenotype. The manufacturing stages and test
1398 methods are when applicable and product dependent. For example, RCV testing is performed on cells and supernatant derived
1399 from the stably-transfected vector producer or packaging MCB and LIVCA and during the qualification of the virus seed or
1400 cell bank. Tests for RCV apply during production, with testing performed on vector producing cells and supernatant from each
1401 unprocessed bulk harvest or at each drug substance/final lot, when applicable. For example, replication-competent virus testing
1402 is typically performed at unprocessed bulk harvest to ensure detectability or drug substance step for Adeno-Associated Virus
1403 (AAV) based products indicated as (+) in the table.

1404 ^h. When assay interference may occur control cells cultured in parallel are tested at the virus seed and unprocessed bulk harvest
1405 stages.

1406 ⁱ. For cell lines of insect origin tests for species-specific viruses and arboviruses should be carried out. Refer to Table 4 (Case
1407 B, C, and E) for action steps to be taken for virus detection in cell substrates used for production.

1408 ^j. Testing should be performed if cell substrate/cell bank not tested

1409 ^k. Depending on the product type, the virus seed may be used to manufacture a vaccine virus, viral vector, or helper virus. The
1410 virus seed is generated from an established cell line. Consistent with a risk-based approach, the virus testing should consider
1411 the origin of the cell line and raw materials and reagents used for preparation of the virus seed to ensure that the absence of
1412 adventitious virus may originate from the cell substrate and the absence of replication competent virus. Testing should be
1413 applied on the virus seed before processing. The Working Virus Seed (WVS) is derived directly from the MVS; a subset of
1414 adventitious agent testing therefore applies based on a risk assessment. An alternative approach in which complete testing is
1415 carried out on each WVS rather than on the MVS would also be acceptable.

1416 ^l testing based on risk assessment

1417 (+) alternative testing stage

1418 NA not applicable

1419 **7.3 Virus Clearance**

1420 The risk of contamination with adventitious viruses and residues of viruses used during

1421 production such as helper viruses and protein expression vectors should be mitigated following
1422 the general principles of this guideline to the extent possible.

1423 The virus clearance should be validated using representative and qualified scale down systems.

1424 The physicochemical characteristics of the viral vector and the viral vector-derived product will
1425 determine how virus clearance will apply within the product purification scheme. Virus-
1426 clearance validation should include model viruses representative of adventitious, endogenous,
1427 and if possible, the relevant helper virus. Sections 5 and 6 (including application of prior
1428 knowledge) as such apply, using the action plan for the selection of specific and non-specific
1429 model viruses described in Table 4. Common virus inactivation steps such as treatment with
1430 detergent or solvent/detergent may be suitable, when the product is compatible, such as non-
1431 enveloped viral vectors. Alternatively, virus filtration may be more suitable for small viral
1432 vector such as AAV or nanoparticle-based vaccines when virus removal can be based on the
1433 size exclusion. When appropriate, viral clearance studies should be performed to determine
1434 virus reduction factors for the relevant step(s) of the production process.

1435 Examples include:

- 1436 • Subunit proteins and VLPs produced using baculovirus/insect cells can be purified
1437 and high levels of virus log reduction factors can be achieved through the
1438 manufacturing process and are validated by viral clearance studies; and
- 1439 • Some viral-vector products such as AAV are amenable to robust viral clearance
1440 steps, ensuring adventitious and helper virus clearance inactivation or removal.

1441 The helper virus is considered a process-related viral contaminant. The manufacturing
1442 processes need to ensure an excess of helper virus clearance. Acceptable log-reduction factors
1443 can be based on risk assessment.

1444 Since virus clearance steps during production may not achieve the same robustness as for
1445 recombinant proteins, the viral safety of these products relies also on closed processing, testing
1446 and other preventative controls (see Sections 2.2, 3, and 4).