1 A high cell density perfusion process for MVA virus production:

2 process integration with inline DNA digestion and cost analysis

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21 Running title (no more than 45 characters):

22 Integrated viral vector production at HCD

23

24 **Abstract:**

25 By integrating continuous cell cultures with continuous purification methods, process yields and product

26 quality attributes were improved over the last 10 years for recombinant protein production. However, for

27 the production of viral vectors such as Modified Vaccinia virus Ankara (MVA), no such studies have been

28 reported although there is an increasing need to meet the requirements for a rising number of clinical

29 trials against infectious or neoplastic diseases. Here, we present for the first time a scalable suspension

30 cell (AGE1.CR.pIX cells) culture-based perfusion process in bioreactors integrating continuous virus

31 harvesting through an acoustic settler with semi-continuous chromatographic purification. This allowed to

32 obtain purified MVA particles with a space-time yield >600% higher for the integrated perfusion process

33 (1.05×10^{11} $TCID_{50}/L_{bioreactor}$/day) compared to the integrated batch process. Without further optimization,

34 purification by membrane-based steric exclusion chromatography resulted in an overall product recovery

35 of 50.5%. To decrease the level of host cell DNA prior to chromatography, a novel inline continuous DNA

36 digestion step was integrated into the process train. A detailed cost analysis comparing integrated

37 production in batch versus production in perfusion mode showed that the cost per dose for MVA was

38 reduced by nearly one third using this intensified small-scale process.

39

1 Introduction

To date, the implementation of an integrated perfusion process is one option to decrease manufacturing costs and to potentially increase the quality of a product (Bielser, Wolf, Souquet, Broly, & Morbidelli, 2018; Walther et al., 2015; Walther et al., 2019; Xu & Chen, 2016). A considerable amount of research has been conducted on integrated continuous production of recombinant proteins such as monoclonal antibodies (Godawat, Konstantinov, Rohani, & Warikoo, 2015; Karst, Steinebach, & Morbidelli, 2018; Karst, Steinebach, Soos, & Morbidelli, 2017; Konstantinov & Cooney, 2015; Pinto, Napoli, & Brower, 2019; Warikoo et al., 2012). However, to our knowledge, options for an integrated viral vector production in perfusion mode have not been reported in the literature, although it seems evident that this could offer improvements in biopharmaceutical product quality (Allison et al., 2015).

Similar to recombinant protein manufacturing, process intensification for viral vectors could be a solution to lower production costs and space requirements for culture vessels. Process intensification may also help to satisfy the increasing demand for viral vectors at high concentrations for R&D, clinical trials, and commercialization (Kaemmerer, 2018; van der Loo & Wright, 2015). Intensification can be achieved with bioreactors coupled to devices for harvesting of infectious units with subsequent continuous purification. A techno-economic analysis could provide insights about costs differences between a batch and a perfusion process for viral vector production (Cameau, Pedregal, & Glover, 2019; Gränicher, Tapia, et al., 2020; Pearson, 2020), similarly to recombinant protein production (Klutz, Holtmann, Lobedann, & Schembecker, 2016; Lim, Washbrook, Titchener-Hooker, & Farid, 2006; Pleitt, Somasundaram, Johnson, Shave, & Lua, 2019; Pollock, Ho, & Farid, 2013). This could allow identifying key factors and bottlenecks allowing cost-savings. To our knowledge, only a few studies in bioprocess economics related to viral vector manufacturing for gene therapy were performed, so far (Cameau et al., 2019; Comisel et al., 2020). Up to now, no studies evaluated the costs of virus production using a perfusion system linked to a suspension cell culture.

The establishment of integrated perfusion processes requires the use of cell retention systems that allow high process robustness, scalability, and continuous virus harvesting. In addition to cell retention as a

66 preemptive processing step, continuous virus harvesting could also result in higher production yields and

67 better product quality (Gränicher, Coronel, et al., 2020; Manceur et al., 2017; Petiot et al., 2011).

68 To date, membrane-based alternating tangential flow (ATF) perfusion is the most preferred technology for

69 biopharmaceuticals production (Bielser et al., 2018). Surprisingly, however, product retention has been

70 observed even with membranes that have pore sizes five times larger than the virus diameter (Genzel et

71 al., 2014; Gränicher, Tapia, et al., 2020; Nikolay, Leon, Schwamborn, Genzel, & Reichl, 2018). Similar

72 effects have been observed in perfusion processes for recombinant proteins but could be avoided by

73 using membranes with pore sizes larger than 2 µm (Pinto et al., 2019; S. B. Wang, Godfrey, Radoniqi,

74 Lin, & Coffman, 2019).

75 The acoustic settler technology is an alternative to ATF for continuous virus harvesting and can be scaled

76 to a perfusion flow rate of 1000 L/day (Gränicher, Coronel, et al., 2020; Gränicher, Tapia, et al., 2020;

77 Manceur et al., 2017). Such a device circumvents the pore size complications of the ATF, but changes in

78 temperature in the acoustic flow chamber need to be controlled during operation to maintain virus

79 stability.

80 The use of an online probe measuring the electric capacitance allows to monitor cell concentration, cell

81 size, metabolic state, apoptosis and viral infection (Justice et al., 2011; Nikolay et al., 2018; Petiot,

82 Ansorge, Rosa-Calatrava, & Kamen, 2017; Vazquez-Ramirez, Jordan, Sandig, Genzel, & Reichl, 2019).

83 In addition, it was shown that capacitance sensors can be used to determine other key process

84 parameters, i.e. the optimal time of virus harvesting (Grein et al., 2018; Negrete, Esteban, & Kotin, 2007).

85 Here, we present for the first time a fully integrated cell culture-based perfusion system allowing an end-

86 to-end viral vector production at high cell densities. The avian suspension cell line AGE1.CR.pIX was

87 used to produce MVA, which is a promising vector for vaccination against various infectious diseases and

88 certain forms of cancers (https://ClinicalTrials.gov). An acoustic filter was utilized for continuous

89 harvesting of MVA. In addition, a capacitance sensor was used to monitor cell growth, control the

90 perfusion rate, and decide on the time of virus harvesting. For viral vector purification, a semi-continuous

91 method using membrane-based steric exclusion chromatography (SXC) was directly linked to the

92 continuous virus harvest stream. Achieving a maximum viable cell concentration (VCC) of up to 37×10^6

93 cells/mL during virus production (dilution step at 12 hpi), the space-time yield (STY) of purified MVA

94 particles for the process established was $1.05 \times 10^{11} TCID_{50}/L_{bioreactor}$/day. The production setup allowed an

95 overall recovery of virus particles of 50.5%, with a concentration of host cell DNA per dose below the

96 limits typically set for human vaccines by regulatory authorities. Furthermore, we could reduce

97 significantly the host cell DNA level by integrating a DNA digestion step in continuous mode prior to

98 chromatography. The collected data allowed then for an academic techno-economic analysis between

99 batch and perfusion.

100 ## 2   Materials and methods

101 ### 2.1   Cells, virus, and media

102 An immortalized Muscovy duck retina suspension AGE1.CR.pIX cell line was used as a host for

103 production of MVA-CR19.GFP (infectious titer: $4.1 \times 10^8 TCID_{50}$/mL), which contains a green-fluorescent-

104 protein insertion cassette (Jordan et al., 2020). Chemically-defined CD-U5 medium (Biochrom-Merck,

105 Darmstadt, Germany) supplemented with 2 mM L-glutamine (Sigma-Aldrich, St. Louis, USA) and 10

106 ng/mL recombinant insulin-like growth factor (LONG-R^3 IGF, 91590C; Sigma-Aldrich, St. Louis, USA) was

107 used for cell growth. Shake flasks were used for cells maintenance and for inoculation of the bioreactors

108 as described earlier (Gränicher, Tapia, et al., 2020; Jordan et al., 2011).

109 ### 2.2   Bioreactor cultivation

110 In order to compare MVA production in batch or in perfusion mode, the cells were cultivated in bioreactors

111 in batch or in perfusion mode.

112 ### 2.2.1   Batch cultivations

113 DASGIP bioreactors (1 L maximum working volume; Eppendorf AG, Hamburg, Germany) were inoculated

114 at a VCC of 1.0×10^6 cells/mL (initially,

115 working volume (V_w) = 500 mL). A stirring speed of 145 rpm using a pitched-blade impeller was chosen.

116 The pH was maintained at 7.2 through CO_2 sparging and NaOH (0.55 M) base addition. The dissolved

117 oxygen level (DO) was maintained at 40% air saturation using a drilled-hole L-sparger. Temperature was

118 maintained at 37°C. Once the VCC reached 4×10^6 cells/mL, the V_w was doubled from 500 mL to 1000

119 mL by addition of fresh medium and cells were infected at a multiplicity of infection (MOI) of 0.05

120 infectious units/cells ($TCID_{50}$ assay). Seed virus (MVA-CR19.GFP; section 2.1) was treated for 1 min in a

121 sonication water bath at 45 kHz prior to usage. Virus production in batch mode was done according to the

122 optimized method described by Lohr et al. (Lohr, 2014). The integrated virus production in batch mode

123 was performed in triplicate with three parallelized bioreactors using the same cell culture seed train.

2.2.2 Perfusion cultivation

125 A Biostat bioreactor (1 L maximum V_w; Sartorius AG, Göttingen, Germany) was used to cultivate the cells.

126 DO was set to 40% using a drilled-hole L-sparger, using pure oxygen. The pH was kept to 7.2 using CO_2

127 and temperature was controlled at 37°C. The system was agitated using a pitched-blade impeller at 180

128 rpm. The perfusion bioreactor was inoculated at a VCC of 1×10^6 cells/mL (V_w = 550 mL) and perfusion

129 was started at a VCC of 6×10^6 cells/mL. The same medium used in batch mode was used for the

130 perfusion process (section 2.2.1).

131 An acoustic settler device with a power of 2 W and a frequency of 2.1 MHz was used for cell retention.

132 The parameters of the acoustic settler were set as reported in a previous publication (Gränicher, Tapia, et

133 al., 2020). A constant recirculation flow rate of five reactor volumes per day (day^{-1}) was applied for the

134 acoustic settler system. Before infection, a cell-specific perfusion rate (CSPR) of 50 pL/cell/day was

135 chosen. An online capacitance probe operating in a frequency range of 1 to 10 MHz connected to a

136 controller (ArcView Controller 265, Hamilton, Bonaduz, Switzerland) was used to adjust a Watson-Marlow

137 harvest pump as described previously (Nikolay et al., 2020). The signal from the capacitance probe was

138 recorded every 12 min. The perfusion rate was adjusted according to the VCC in the bioreactor based on

139 the permittivity signal, leading to a constant CSPR. Therefore, during the cell growth phase, the

140 correlation between the VCC and the permittivity signal was determined by linear regression (the resulting

141 slope corresponds to the "cell factor").

142 The bioreactor cell culture medium was replaced using a perfusion rate of 8–12 day^{-1} for 2 to 3 h before

143 infection. The cells were then infected at a VCC of 50×10^6 cells/mL with MVA-CR19.GFP at a MOI of

144　0.05 infectious units/cell ($TCID_{50}$ assay), as described in section 2.2.1. The V_w was increased from 550

145　mL to 1000 mL at 12 hours post infection (hpi) (similar to the process described by Vasquez-Ramirez et

146　al. (2019)). From 0 to 12 hpi, the perfusion was stopped. Afterwards, a constant perfusion rate of 1.75

147　day^{-1} was maintained. The V_w was decreased from 1000 mL to 800 mL at 36 hpi to reduce medium

148　consumption. Accordingly, the harvest pump flow rate had to be decreased from 66.7 mL/h to 54 mL/h to

149　maintain the perfusion rate. Virus release in the cell culture supernatant was monitored using the

150　maximum permittivity signal $\Delta\varepsilon_{max}$, in pF/cm. The cell membrane capacitance (C_m; in $\mu F/cm^2$) and

151　intracellular conductivity (σ_i; in mS/cm) were calculated as previously described (Petiot et al., 2017). With

152　the onset of virus release (about 40 hpi), the harvesting line was directed to a harvest bottle (bottle B1 in

153　Figure 1) kept at 4°C, and later purified. Harvesting of MVA particles released was initiated 10.6 h after

154　the maximum permittivity signal was reached. This corresponded to the time when about 8 to 10% of the

155　total number of infectious virions (Vir_{tot}, bioreactor and harvest vessel, section 2.4) was released from the

156　infected cells (see section 3.3). This definition was chosen to ensure high titers in the harvesting line

157　($> 10^8$ $TCID_{50}$/mL) and to avoid any product concentration step before chromatography. Samples during

158　cell culture were taken every 8 to 14 h.

159　**2.3　Process integration of MVA production**

160　To match the scenarios for integrated virus production, both batch and perfusion cultivations were

161　performed in bioreactors (section 2.2). MVA bioreactor harvests were purified as described below. In the

162　special case of the perfusion strategy, MVA particles were continuously harvested and semi-continuously

163　purified as illustrated in Figure 1. A picture of the experimental setup is shown in the Appendices (Figure

164　A.1).

165　Due to the lytic nature of MVA replication, the upstream process was not fully continuous but split into two

166　phases: a cell growth phase, in which cells were cultivated from 1 x 10^6 cells/mL to 50 x 10^6 cells/mL, and

167　a virus production phase, initiated when cells were infected with MVA and continued with a dilution step at

168　12 hpi (inducing host cell death visible from 75 hpi onwards) (Tapia, Vazquez-Ramirez, Genzel, & Reichl,

169　2016). The continuously harvested virus raw material was semi-continuously purified. Therefore, virus

170   particles that passed the cell retention device were continuously filtered and treated with endonuclease

171   before purification by SXC in bind-elute mode using an ÄKTA Pure system (Figure 1).

172   **2.3.1   Harvest and clarification**

173   *Batch*

174   Once the viability of the infected culture dropped to 70%, harvesting was initiated and 95.3% of the V_w

175   was first clarified using the acoustic settler (10 L acoustic chamber version; SonoSep Technologies,

176   Hinterbrühl, Austria) with an acoustic power of 3 W and a frequency of 2.1 MHz, at a flow rate of 252 mL/

177   h. Sodium azide 0.05% v/v was added to the harvest to reduce contamination risk. Then 700 mM salt

178   (NaCl, NaBr and KCl) was added and the supernatant was depth filtered using a polypropylene filter (PP3

179   Sartopure, 0.45 µm pore size, 120 cm^2 (5051306P4--OO--B) or 4.5 cm^2 (5055306PV--LX--C) filtration

180   surface; Sartorius AG, Göttingen, Germany), at a constant flow rate of 0.45 mL/min/cm^2 and a filtration

181   capacity of at least 126 L/m^2. The clarified cell culture supernatant was subsequently treated with

182   endonuclease and further clarified as described in section 2.3.2.

183   *Perfusion*

184   As described in section 2.2.2, the bioreactor was continuously harvested at 40 hpi onwards. The cell

185   culture harvest from the acoustic settler was not suitable for direct purification using SXC as the

186   contamination level of cells and cell debris passing through the cell retention device was too high.

187   Therefore, the cell culture harvest was first collected in bottle B1 and salts were continuously added to

188   reach 700 mM of NaCl, NaBr and KCl mixture, (as illustrated in Figure 1) to avoid virus interaction with

189   the depth filter, to stabilize virus particles and to facilitate endonuclease treatment (Table 1). Sodium

190   azide was also continuously added to bottle B1 (Table 1). The harvest was then clarified using a

191   polypropylene depth filter with a pore size of 0.45 µm (filtration capacity of 240 L/m^2; Sartopure PP3, 120

192   cm^2 filtration area), transferred to bottle B2 (Figure 1) for DNA digestion and microfiltration as described in

193   section 2.3.2.

194   **2.3.2   DNA digestion and microfiltration**

195   *Batch*

196 DNA in the supernatant was digested using endonuclease at a final activity of 35 U/mL (DENARASE®,

197 enzyme activity > 250 U/µL determined by the manufacturer, 20804-100k; c-Lecta, Leipzig, Germany),

198 mixed with 3 mM $MgCl_2$. The cell culture supernatant was incubated in a glass bottle for 4 h at 37°C and

199 stirred at 100 rpm using a magnetic agitator. The endonuclease step was optimized by decreasing the

200 amount of endonuclease needed to achieve DNA depletion up to 1000-fold within 4 h. In a scouting

201 experiment, the stability of infectious virions at 37°C was demonstrated for a period of at least 12 h

202 (Gränicher, Tapia, et al., 2020). Finally, the treated cell culture supernatant was filtered using 0.45 µm

203 cellulose acetate filters (Minisart NML Syringe Filter, 6.2 cm^2 total filtration area, 16555-K; Sartorius AG,

204 Göttingen, Germany) at a flow rate of 8 mL/min/cm^2 and a filtration capacity of 175 L/m^2. The treated cell

205 culture supernatant was either stored at -80°C or directly purified through SXC, as described in section

206 2.3.3.

207 *Perfusion*

208 The clarified cell culture broth was continuously treated in bottle B2 with 37 U/mL endonuclease

209 (DENARASE®) and with 4 mM $MgCl_2$ (Table 1). After bottle B2, the material was continuously transferred

210 to a coiled silicone tube (3.2 mm inner diameter, 32.5 m length, GESSULTRA-C-125-2H; VWR, Radnor,

211 USA) with a retention time of 4 h at 37°C in an incubator. The product was collected continuously into

212 bottle B3. The harvest from bottle B3 was filtered using 0.45 µm cellulose acetate filters (filtration capacity

213 of 290 L/m^2; Minisart NML Syringe Filter, 6 x 6.2 cm^2 total filtration area). The filtered product was then

214 collected in bottle B4, and stored at 4°C before the chromatography step (described in section 2.3.3). As

215 the process was operated continuously, the V_w of bottles B1, B2, B3 and B4 were kept constant at 180,

216 120, 60 and 120 mL, respectively.

217 **2.3.3   Purification through steric exclusion chromatography**

218 Membrane-based SXC was performed using an ÄKTA Pure 25 system (Cytiva, Uppsala, Sweden) as

219 described previously (Marichal-Gallardo, Pieler, Wolff, & Reichl, 2017), using PBS with NaCl, NaBr and

220 KCl (700 mM final salt concentration) as elution buffer and polyethylene glycol (PEG, 81260-1KG; MW

221 6000, dissolved in PBS + 700 mM NaCl, NaBr and KCl; Sigma-Aldrich, St-Louis, USA) as equilibration

222 buffer. A total surface of 70 cm^2 of regenerated cellulose (14 x 25 mm stacked membranes, 1 µm pore

223 size, 10410014; GE, now Cytiva, Uppsala, Sweden) was used. Optimized purification settings

224 (Appendices) were determined as following: PEG concentration = 7.2% w/v, flow rate = 8.2 mL/min.

225 UV was monitored at a wavelength of 280 nm and 360 nm. The column was operated at 27 to 75%

226 breakthrough of the dynamic binding capacity of the column. This allowed purifying 45 mL sample per

227 cycle, lasting 40 min in total, including column regeneration time. The column (XX3002500; EMD

228 Millipore, Burlington, USA) was regenerated each time by flushing 25 mL of 2 M NaCl in 1 M NaOH. The

229 membranes of the column were replaced every 4 cycles. Consecutive series of bind-elute steps allowed

230 the purification of 67.5 mL/h of cell culture supernatant. The SXC protocol used for purification was

231 identical for both batch and perfusion cultures.

2.4 Analytics and yield calculations

233 The VCC and percentage cell viability were determined using a Vi-CELL XR (Beckman-Coulter, Brea, CA,

234 USA). Glucose, glutamine, lactate and ammonium concentrations were measured using a Bioprofile 100

235 plus (Nova biomedical, Waltham, MA, USA).

236 For titration of the MVA-CR19.GFP strain in the supernatant, a median tissue culture infectious doses

237 ($TCID_{50}$) assay with serial 2-fold dilutions instead of 10-fold dilutions (as described by Nikolay et al.

238 (2020)) were performed, resulting in a standard deviation of $\pm$ 0.077 $\log_{10}(TCID_{50}/mL)$ (standard deviation

239 of a sample measured by 3 operators, performed for each in triplicate). Samples purified by SXC were

240 sonicated with a VialTweeter (UP200St, Power = 160 W, Amplitude = 100%, Pulse = 30%; Hielscher

241 Ultrasound Technology, Teltow, Germany) to dissolve virus aggregates before measurements. The total

242 number of infectious virions measured in the harvest vessel and in the bioreactor (Vir_{tot}, based on $TCID_{50}$),

243 the concentration of infectious virions produced ($C_{vir, tot}$, $TCID_{50}/mL$), the volumetric virus productivity (P_v,

244 $TCID_{50}/L/day$), and the cell-specific infectious virus yield (CSVY, $TCID_{50}/cell$) were calculated as described

245 previously by Gränicher, Tapia, et al. (2020). The space-time yield of purified MVA was calculated based

246 on the bioreactor V_w (STY, $TCID_{50}/L_{bioreactor}/day$). On a linear scale, the $TCID_{50}$ assay contributes an error of

247 +19.4/-16.3% to $C_{vir, tot}$, Vir_{tot}, CSVY, P_v, and STY. For perfusion, the recovery (in %) of each filtration or

248 DNA digestion step was calculated as the ratio of the average titer after and before the step as shown in

249 Table 2.

250 For batch cultures, the recovery was calculated stepwise as the ratio between the total number of

251 infectious virions after and before the filtration or DNA digestion step. The average was calculated as the

252 average recovery of three integrated batch bioreactor runs.

253 Recovery of SXC (in %) was calculated for each purification cycle as described earlier (Marichal-Gallardo

254 et al., 2017). The average SXC recovery of perfusion was the mean of all cycles performed for one

255 integrated process. To reduce the consumption of spin tubes, buffers and regenerated cellulose, the SXC

256 was operated 23% of the period during the virus production phase, always with 3 to 4 consecutive cycles

257 (intervals < 9 h). The average SXC recovery for the batch process was calculated based on the 4 x 3

258 purification cycles (the SXC column is replaced after four purification cycles) performed for each triplicate.

259 The concentration of host cell DNA was measured through a qPCR assay (Rotor-Gene Q real-time PCR

260 cycler; Qiagen, Hilden, Germany), correlated with a standard host cell DNA concentration of lysed

261 AGE1.CR.pIX cells measured through a Picogreen assay as described earlier (Marichal-Gallardo et al.,

262 2017). The total protein concentration was measured with a Bradford assay (Marichal-Gallardo et al.,

263 2017). The host cell DNA and total protein per dose was calculated as previously described (Gränicher,

264 Coronel, et al., 2020). One dose was considered here as equal to 10^8 plaque forming units (PFU) (Wyatt,

265 Earl, Eller, & Moss, 2004), which is equivalent to 1.43 x 10^8 $TCID_{50}$ (ATCC, 2012).

266 **2.5   Economic analysis**

267 To estimate the impact on cost per dose for an end-to-end MVA production of a batch and a perfusion

268 system, the process simulation software SuperPro Designer v10 (Intelligen Inc., Scotch Plains, USA) was

269 used. All data of upstream processing (USP) and downstream processing (DSP) relate to cost of goods

270 collected at the Department of Bioprocess Engineering (Max Planck Institute, Magdeburg, Germany) for 1

271 L bioreactor scale in an academic environment. Key assumptions used to compare batch and perfusion

272 processes: i) Production runs over 47 weeks per year and the seed train process 65% of the time (31

273 weeks per year). ii) Fill & finish costs and duration are considered the same for batch and perfusion. iii)

274 MVA preparations of both processes are assumed to have the same product quality. iv) All bioreactors

275 are assumed to operate at maximum volume capacity. v) Indirect costs relevant for cost of goods

276 evaluation such as waste disposal (similarly to other cost analysis publication for viral vector production

277   (Comisel, Kara, Fiesser, & Farid, 2020)) and depreciation maintenance and plant depreciation were not

278   considered for both systems. vi) Costs related to QA/QC, operation of the facility, and labor were taken

279   from default values given by the software.

280   **3   Results**

281   **3.1   Intensified cell culture for MVA production.**

282   First, to establish a robust process that allows for continuous harvesting and high MVA yields, two

283   perfusion experiments using the acoustic settler (runs 1–2) were performed. As a control, a batch process

284   was operated in triplicate (runs A-C). Achieving maximal VCCs of 36.9–38.0 x 10^6 cells/mL during virus

285   production (Figure 2A) (dilution at 12 hpi), an average recovery of 107 ± 18% was observed for the virus

286   material collected after the settler (volume = 2.8–2.9 L; Figure 2B). The Vir_{tot} produced in the harvest and

287   in the bioreactor vessel was 20.4 x 10^{11} and 9.1 x 10^{11} $TCID_{50}$ for run 1 and 2, respectively (Figure 2B).

288   For the batch runs, an average Vir_{tot} of 2.4 ± 0.6 x 10^{11} $TCID_{50}$ was measured. For the perfusion runs, the

289   CSVY was 24.0 and 55.4 $TCID_{50}$/cell, and the P_v 1.43 and 2.53 x 10^{10} $TCID_{50}$/L/day for run 1 and 2,

290   respectively. As a comparison, an average CSVY of 46.9 ± 13.2 $TCID_{50}$/cell and an average P_v of

291   3.82 ± 0.93 x 10^{10} $TCID_{50}$/L/day were obtained for the triplicate batch runs A-C (Figure 2C-D).

292   **3.2   Process integration for viral vectors production.**

293   Compared to the batch processes A-C, similar recovery yields and impurity levels were obtained during

294   purification (Figure 3; DSP as in section 2.3). Total recovery for batch (runs A to C) and perfusion (run 1)

295   was equal to 54.7% and 50.5%, respectively (Figure 3A). Recovery for depth filtration was 59.8–81.6%.

296   The DNA digestion step allowed for the perfusion and batch process an about 3 $\log_{10}$ depletion of host

297   cell DNA per dose, reaching <10 ng host cell DNA/dose (assuming a MVA dose input of 1.43 x 10^8

298    $TCID_{50}$; section 2.4). Compared to the raw material in the bioreactor, the total protein amount per dose

299   decreased by a factor of 18.3 for the perfusion and 2.2 for the batch system after purification by SXC

300   (final value: 11–37 µg total protein/dose; Figure 3C). When performing a two-sample t-test, the decrease

301   of host cell DNA per dose and the decrease of total protein per dose was found to be statistically

302   significant (p value <0.05) for the perfusion and batch systems, respectively. The large error observed for

303 host cell DNA per dose (perfusion mode) after the depth filtration step (Figure 3B) was probably due to

304 partial host cell DNA digestion as endonuclease was added in bottle B2 (Figure 1) and sampling times

305 were different. A STY of 10.5 x 10^{10} $TCID_{50}$/$L_{bioreactor}$/day for the perfusion and 1.7 ± 0.3 x $10^{10}TCID_{50}$/

306 $L_{bioreactor}$/day for the batch processes were obtained. This comparison is relevant in order to assess the

307 impact of the bioreactor footprint on the productivity and the potential of perfusion considering all the

308 aspects from USP to DSP.

3.3   Control of perfusion rate and evaluation of MVA harvesting time based on online
309
capacitance probe measurements
310

311 The perfusion rate during the cell growth phase could be successfully controlled using a capacitance

312 probe for run 1. No offset between the offline VCC and online VCC was observed (Figure 4). A CSPR of

313 48.0 pL/cell/day was kept constant during at least three days before virus infection. The first CSPR value

314 obtained 96 h before infection was estimated too high due to a pump calibration error. No limitation in

315 glucose concentration was observed during the whole run (data not shown).

316 During the virus production phase, the trends of the offline VCC followed the same dynamics as for the

317 $\Delta\varepsilon_{max}$ signal (Figure 5A and Figure 5C), except that the values were given every 8 to 14 h for the offline

318 VCC and every 0.2 h for the online permittivity signal. A correlation between the VCC or the $\Delta\varepsilon_{max}$ signal

319 was observed with the onset of MVA release (defined in section 2.2.2). For all runs including the batch

320 run, the expected time of MVA release in the supernatant (based on the permittivity signal decrease time

321 point plus 10.6 h; corresponding to the time when about 8 to 10% of Vir_{tot} was released from the infected

322 cells) seemed to correlate with the increase of the virus titer in the bioreactor supernatant, reaching a titer

323 in the range of 0.5–1.0 x 10^8 $TCID_{50}$/mL at that time point (Figure 5). By harvesting the perfusion

324 bioreactor 10.6 h after the maximum $\Delta\varepsilon_{max}$ signal or maximum offline VCC was achieved (illustrated by the

325 vertical line in Figure 5), 81–95% of the produced infectious virions could be harvested (Appendices,

326 Table A.2). Note: This time interval (10.6 h) is an average from run 1, run 2, the control run (data from run

327 *4* of Gränicher, Tapia, et al. (2020)) and a batch run (run C) (Table A.2, illustrated in Figure 5). For batch

328 run C, a delay of the virus release and the cell death was observed (Figure 5C-D). Overall, the maximum

329 permittivity signal was determined between 24–48 hpi (Figure 5). We therefore suggest that this could

330 help to decide on harvesting time.

3.4   Economic analysis: Batch versus perfusion

332 To allow for an economic analysis, data for cost of goods from end-to-end MVA production in batch

333 (average values for runs A, B and C) were compared to an end-to-end MVA production in perfusion

334 mode. Data from the perfusion cultivations 1 and 2 were used to estimate the average Vir_{tot}, and the

335 process time for the USP part (referred to as "Seed train" and "Cell culture" in Figure 6C). The data from

336 run 1 was used to estimate the costs regarding the DSP part (referred to as "Filtration and DNA digestion"

337 and "Chromatography" in Figure 6C), as only run 1 was integrating USP with DSP.

338 The capital expenditures (CAPEX), was 10% higher for the perfusion than for the batch process for the 1

339 L bioreactor scale (Figure 6A). Concerning the operating expenditures (OPEX), the value for the perfusion

340 process was overall 26% higher than for the batch process, which can be attributed to higher labor costs

341 required for operation of the perfusion system (Figure 6A). More specifically, for both batch and perfusion

342 systems, the highest costs came from the endonuclease used for DNA digestion (30–32%), followed by

343 costs for cell culture media (21–25%) and seed virus (13–27%) (Figure 6B). Costs for filters and SXC

344 membranes were between 5–17%. Overall, for the different production steps from the seed train to the

345 SXC, the batch and perfusion systems had similar cost per dose at the 1 L bioreactor scale, except for the

346 seed train and cell culture step: here, costs for the batch system were about 5.2 and 3.5-fold higher,

347 respectively (Figure 6C). At the 1 L bioreactor scale, the perfusion process allowed to produce about 3.5-

348 fold more doses per year than the batch system (Figure 6D). This resulted in a 2.8-fold decrease of cost

349 per dose. At the 1000 L scale, 42 and 147 millions of doses are projected yearly in the batch and

350 perfusion systems, respectively. Targeting a defined number of doses per year, the perfusion system and

351 the batch system showed similar costs per dose (Figure 6E). Nevertheless, for the same bioreactor scale,

352 the operation of a perfusion system is always advantageous in terms of cost per dose (Figure 6D). At the

353 200 L scale, the cost per dose for a perfusion system is still 1.8-fold lower than for a batch process. More

354 details about the economic analysis are available in the Appendices.

4 Discussion

Cell growth, CSVY and P_v (Figure 2) of the presented perfusion system were in the same range as in previous experiments (Gränicher, Tapia, et al., 2020), with maximum values of 50 x 10^6 cells/mL, 55.4 $TCID_{50}$/cell, and 2.53 x 10^{10} $TCID_{50}$/L/day.

The total recovery for the perfusion and batch systems were similar (50.5% and 54.7%, respectively; Figure 4A), showing that the intensified perfusion process did not have a negative impact on cell clarification, host cell DNA removal and SXC. A total recovery of about 50–55% is in accordance with results reported from other groups using other DSP processes. Recoveries of 61–63% for adenovirus (Fernandes et al., 2013; Moleirinho, Silva, Alves, Carrondo, & Peixoto, 2020), 41% for MVA (Leon et al., 2016), 52% for influenza virus (Kalbfuss, Wolff, Morenweiser, & Reichl, 2007) and 20–60% for AAV production (Moleirinho et al., 2020; Terova, Soltys, Hermans, De Rooij, & Detmers, 2018) were reported. Successful application of membrane-based SXC for influenza virus, yellow fever virus, AAV, baculovirus, hepatitis C virus, and Orf virus purifications have been reported (Lothert, Offersgaard, et al., 2020; Lothert, Pagallies, Feger, Amann, & Wolff, 2020; Lothert, Sprick, et al., 2020; Marichal-Gallardo, 2019; Marichal-Gallardo et al., 2021). This suggests that the integrated process established here may also be transferrable to other virus manufacturing processes (Bissinger et al., 2021). The short purification cycles of the SXC method (of about 40 min) allowed to greatly simplify the semi-continuous purification process. In addition, the less complex bind-elute steps in single-column SXC requires fewer optimizations than conventional multi-column chromatography trains (Gerstweiler, Bi, & Middelberg, 2021; Patil & Walther, 2018).

Clarification steps are particularly challenging due to the large size of MVA virions (250–350 nm). Here, a depth filtration efficiency of 59.8–81.6% (Figure 2) was observed for batch and perfusion systems, and depth filtration was the main cause for the reduction of process yields. Similar findings were reported for large scale manufacturing of vaccinia viruses with depth filters with < 5 µm pore size (Leon et al., 2016; Ungerechts et al., 2016). Other publications reported recoveries of 85–90% when using polypropylene depth filters with pore sizes of 0.45–0.60 µm after a centrifugation step, or from the supernatant of an adherent cell culture for smaller viruses such as adenovirus (Fernandes et al., 2013), hepatitis C virus-like

particles (Xenopoulos, 2015) or influenza virus (Bernd Kalbfuss et al., 2007). Recoveries up to 74% for clarification of vaccinia virus raw material (centrifuged cell lysate and 1:5 diluted in 0.5 M ammonium sulfate and 3 M NaCl) with 0.8 μm cellulose acetate filter were also reported (Vincent, 2017). The polypropylene material used here for depth filtration seems well suited for clarification of virus-containing supernatants and is relatively inert (Besnard et al., 2016; Cherradi et al., 2018) with a surface tension energy lower than other common material such as polyethylene, polyethylene sulfone or polystyrene membranes (Fenouillot, Cassagnau, & Majesté, 2009; Kim, Rana, Matsuura, Chung, & Khulbe, 2010). In addition, this material largely prevents electrostatic interaction with virus particles (MVA carries a high negative charge at neutral pH (Michen & Graule, 2010)) in contrast to diatomaceous earth, which is a standard material used for depth filtration (lower recovery observed, data not shown) (Besnard et al., 2016; Cherradi et al., 2018). In addition, the adjustment of appropriate salt concentrations also improved yields in depth filtration (section 2.3.1, data not shown). This corresponds to previous findings that demonstrated that salt addition reduced the interaction of virus particles with cell debris and DNA (Hughes et al., 2007; Jordan et al., 2015) and suppressed the aggregation of viral vectors (Wright et al., 2005).

Host cell DNA is one of the most critical and persistent contamination in virus particle purification. An inline endonuclease treatment step efficiently reduced host cell DNA levels before subsequent SXC purification. The use of chaotropes for efficient DNA digestion was also essential, as it helped to separate DNA from the surface of viral particles (Jordan et al., 2015). A host cell DNA reduction of around 500-fold was needed for the perfusion process established here (Figure 3B) in order to meet the requirements typically set by regulatory authorities (<10 ng/dose). The establishment of this novel continuous inline DNA digestion step was inspired from the use of plug flow reactors with immobilized enzymes (Pitcher, 1978), and resulted in an over 10'000-fold reduction of DNA (Figure 3B). Unlike chemostats, plug flow reactors allow a narrow distribution of the residence time.

The perfusion rate was controlled via estimation of the VCC by an online capacitance probe (Figure 4). Similar findings were reported by Nikolay et al. (2018) for a different avian cell line. It thus seems that this technique is a versatile method (Nikolay et al., 2020; Wu et al., 2021) as long as the diameter of the cells

409 remains constant during the time course of cultivations. There are several options to correlate the

410 permittivity signal with the VCC (Cannizzaro, Gügerli, Marison, & von Stockar, 2003). For our case, a

411 simple linear regression between the permittivity signal and the offline VCC was precise enough to

412 determine the VCC during the cell growth phase. During the virus production process, the time of MVA

413 release (term defined in section 2.2.2) could be determined with $\Delta\varepsilon_{max}$ or offline VCC with a precision of

414 about ± 4 h, over four different runs in perfusion or batch mode (Figure 5). The use of $\Delta\varepsilon_{max}$ was more

415 accurate than offline VCC due its higher measurement frequency. Previous publications already used the

416 permittivity signal to correlate with the optimal time of harvest of measles virus (Grein et al., 2018) and

417 AAV in a baculovirus expression system (Negrete et al., 2007). Petiot et al. (2017) used $\Delta\varepsilon_{max}$ and critical

418 frequency (Fc) values to determine changes in C_m and σ_i values over the virus infection for different

419 enveloped (e.g. lentivirus, influenza virus) and non-enveloped viruses (reovirus) to monitor the status of

420 the virus infection phase. In our case, monitoring of Fc, C_m and σ_i did not lead to clear results

421 (Appendices). More cultivations should be performed to infer about a correlation between the permittivity

422 signal and MVA release for perfusion and batch systems (Figure 5 and Appendices, Table A.2). This is in

423 particular important for perfusion processes, were the time of significant virus accumulation needs to be

424 identified for initiation of subsequent process steps, i.e. chromatographic purification. Furthermore, it

425 would support the establishment of robust processes following the guidelines of the PAT initiative (FDA,

426 2004).

427 In order to assess the benefit of integrated perfusion processes, an economic analysis was performed

428 using SuperPro designer software. Based on the results shown in Figure 6, the cost per MVA dose could

429 be reduced by a factor of 2.8 for production of MVA in perfusion mode at the 1 L scale, compared to the

430 batch system. Advantages of perfusion systems were already shown for USP in MVA production,

431 although without cost evaluation (Gränicher, Tapia, et al. 2020). Although operation of the perfusion

432 system is more labor intensive, the cost per dose was lower as the production capacity increased by a

433 factor of 3.5 (1 L scale). Furthermore, the seed train costs were decreased as fewer bioreactor runs per

434 year need to be performed (Figure 6). Costs were mainly reduced for the seed train and USP (Figure 6C),

435 similarly to what was observed for AAV (Cameau et al., 2019) and lentivirus manufacturing (Comisel et

436 al., 2020). Higher costs for seed virus for perfusion over batch processes were estimated (Figure 6B), as

437   cultures are infected at a higher VCC and, to keep the MOI, more virus is needed (> 10-fold; section 2.2).

438   Nevertheless, process time was not drastically prolonged (Appendices). The cell culture media cost was

439   not higher for the perfusion system (Figure 6B) because the CSPR was kept to a minimum and, although

440   the perfusion cultivations need higher media volumes, more virus can be produced than in batch. As also

441   observed in AAV manufacturing, the establishment of intensified USP systems has little impact on the

442   DSP cost per dose (Cameau et al., 2019), although the chromatography method used was different for

443   the both cases. Concerning raw materials and consumables costs, the significant costs for DNA digestion

444   could be further reduced by optimizing the endonuclease treatment step in the future. Finally, the low

445   costs of the SXC purification step led to a very low contribution to the overall consumable stocks, in

446   contrast to other DSP techniques that required expensive resins or coated surfaces (Comisel et al.,

447   2020).

448   So far, few studies have addressed bioprocess economics for production of viral vectors (Cameau et al.,

449   2019; Comisel et al., 2020) or virus-like particles (Chuan, Wibowo, Lua, & Middelberg, 2014). For all of

450   them, using suspension cell culture in batch mode appeared to be the most cost-effective option. Here,

451   suspension cell culture in perfusion mode is presented as an additional option to further reduce costs.

452   Although for a fixed amount of MVA doses per year the perfusion system would not decrease the costs

453   per dose compared to batch, the CAPEX is not the same across scales for batch and perfusion systems.

454   For example, a 200 L batch bioreactor is predicted to produce as much as a 50 L perfusion bioreactor

455   ($7.6–8.4 \times 10^6$ doses per year). While the cost per dose is not reduced for the perfusion system, the

456   CAPEX is about 1.2-fold lower resulting in a faster return of investments (Appendices). In addition, the

457   use of perfusion systems is always advantageous for the same bioreactor scale (Figure 6), which might

458   be of interest for modification of existing virus manufacturing plants towards an increase of product

459   output.

460   As an outlook, the recovery of the integrated process could be further increased by optimizing the first

461   depth filtration step, as this resulted in the most significant drop in virus titers (Figure 3A). For instance,

462   depth filters with larger pore sizes could be added before the used depth filter, in order to remove more

463   efficiently large cell debris without product retention. The concentration of the salt used as a chaotropic

464   agent could also be re-evaluated. Indeed, an increase in the ionic strength might decrease the zeta

465   potential of the membrane below a critical value. As a result, the electrostatic repulsion between the feed

466   and the membrane could be decreased resulting in membrane fouling, unspecific product adsorption or

467   aggregation of flocs that may also contain virus particles (Breite, Went, Prager, & Schulze, 2016; Lukasik,

468   Scott, Andryshak, & Farrah, 2000).

469   In conclusion, an integrated perfusion process for MVA production has been established with a minimum

470   of clarification and purification steps. An overall product recovery of 50.5% was achieved, allowing to

471   increase the STY by 600% compared to a batch system operated at the same scale. This was mainly due

472   to the fact, that the virus production phase could be kept constant for both processes. Furthermore, the

473   observed purification performance of membrane-based SXC was not hampered due to cell culture

474   process intensification. The use of an online capacitance probe allowed the control of the perfusion rate

475   during the cell growth phase and indicated the time of MVA release to initiate subsequent processing

476   steps. Finally, a detailed cost analysis, based on several runs performed in batch and perfusion mode,

477   indicated that the cost per dose in MVA production would be decreased by a factor of 2.8 if the system

478   would be operated in perfusion mode at the 1 L scale.

Author contributions

GG, PMG, IJ, YG and UR contributed to conception and design of the study. GG, SG and MB performed the experiments and process modelling. GG and MB analyzed the data. GG wrote the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

GG, MB, SG, and YG declare that they have no conflict of interest. PMG and UR are inventors of a pending patent application describing the SXC virus purification technology used in this study. IJ is an employee of ProBioGen AG, which has established the AGE1.CR.pIX cell line, the MVA-CR19.GFP and the CD-U5 medium.

References

Afshar, S., Salimi, E., Fazelkhah, A., Braasch, K., Mishra, N., Butler, M., . . . Bridges, G. E. (2019). Progression of change in membrane capacitance and cytoplasm conductivity of cells during controlled starvation using dual-frequency DEP cytometry. *Analytica Chimica Acta, 1059*, 59-67. doi:https://doi.org/10.1016/j.aca.2019.01.046

Allison, G., Cain, Y. T., Cooney, C., Garcia, T., Bizjak, T. G., Holte, O., . . . Zezza, D. (2015). Regulatory and Quality Considerations for Continuous Manufacturing May 20–21, 2014 Continuous Manufacturing Symposium. *Journal of Pharmaceutical Sciences, 104*(3), 803-812. doi:https://doi.org/10.1002/jps.24324

ATCC. (2012). FAQ's: Converting TCID[50] to plaque forming units (PFU): Is it possible to determine from the TCID[50] how many plaque forming units to expect?

Besnard, L., Fabre, V., Fettig, M., Gousseinov, E., Kawakami, Y., Laroudie, N., . . . Pattnaik, P. (2016). Clarification of vaccines: An overview of filter based technology trends and best practices. *Biotechnology Advances, 34*(1), 1-13. doi:https://doi.org/10.1016/j.biotechadv.2015.11.005

Bielser, J. M., Wolf, M., Souquet, J., Broly, H., & Morbidelli, M. (2018). Perfusion mammalian cell culture for recombinant protein manufacturing - A critical review. *Biotechnol Adv, 36*(4), 1328-1340. doi:10.1016/j.biotechadv.2018.04.011

Bissinger, T., Wu, Y., Marichal-Gallardo, P., Riedel, D., Liu, X., Genzel, Y., . . . Reichl, U. (2021). Integrated production of an influenza A vaccine candidate with MDCK suspension cells. *Authorea*. doi:10.22541/au.161476429.90376721/v1

Breite, D., Went, M., Prager, A., & Schulze, A. (2016). The critical zeta potential of polymer membranes: how electrolytes impact membrane fouling. *RSC Advances, 6*(100), 98180-98189. doi:10.1039/C6RA19239D

Cameau, E., Pedregal, A., & Glover, C. (2019). Cost modelling comparison of adherent multi-trays with suspension and fixed-bed bioreactors for the manufacturing of gene therapy products. *Cell and Gene Therapy Insights, 5*(11), 1663-1674. doi:10.18609/cgti.2019.175

Cannizzaro, C., Gügerli, R., Marison, I., & von Stockar, U. (2003). On-line biomass monitoring of CHO perfusion culture with scanning dielectric spectroscopy. *Biotechnology and Bioengineering, 84*(5), 597-610. doi:10.1002/bit.10809

Cherradi, Y., Le Merdy, S., Sim, L.-J., Ito, T., Pattanaik, P., Haas, J., & Boumlic, A. (2018). Filter-Based Clarification of Viral Vaccines and Vectors. *BioProcess International, 16*(4), 48-53.

Chuan, Y. P., Wibowo, N., Lua, L. H. L., & Middelberg, A. P. J. (2014). The economics of virus-like particle and capsomere vaccines. *Biochemical Engineering Journal, 90*, 255-263. doi:https://doi.org/10.1016/j.bej.2014.06.005

Comisel, R.-M., Kara, B., Fiesser, F. H., & Farid, S. S. (2020). Lentiviral vector bioprocess economics for cell and gene therapy commercialisation. *Biochemical Engineering Journal*, 107868. doi:https://doi.org/10.1016/j.bej.2020.107868

FDA, F. a. D. A. (2004). *Guidance for industry PAT - a framework for innovative pharmaceutical development, manufacturing and quality assurance*. Rockville

Fenouillot, F., Cassagnau, P., & Majesté, J. C. (2009). Uneven distribution of nanoparticles in immiscible fluids: Morphology development in polymer blends. *Polymer, 50*(6), 1333-1350. doi:https://doi.org/10.1016/j.polymer.2008.12.029

Fernandes, P., Peixoto, C., Santiago, V. M., Kremer, E. J., Coroadinha, A. S., & Alves, P. M. (2013). Bioprocess development for canine adenovirus type 2 vectors. *Gene Therapy, 20*(4), 353-360. doi:10.1038/gt.2012.52

Genzel, Y., Vogel, T., Buck, J., Behrendt, I., Ramirez, D. V., Schiedner, G., . . . Reichl, U. (2014). High cell density cultivations by alternating tangential flow (ATF) perfusion for influenza A virus production using suspension cells. *Vaccine, 32*(24), 2770-2781. doi:10.1016/j.vaccine.2014.02.016

Gerstweiler, L., Bi, J., & Middelberg, A. P. J. (2021). Continuous downstream bioprocessing for intensified manufacture of biopharmaceuticals and antibodies. *Chemical Engineering Science, 231*, 116272. doi:https://doi.org/10.1016/j.ces.2020.116272

Godawat, R., Konstantinov, K., Rohani, M., & Warikoo, V. (2015). End-to-end integrated fully continuous production of recombinant monoclonal antibodies. *Journal of Biotechnology, 213*, 13-19. doi:https://doi.org/10.1016/j.jbiotec.2015.06.393

Gränicher, G., Coronel, J., Trampler, F., Jordan, I., Genzel, Y., & Reichl, U. (2020). Performance of an acoustic settler versus a hollow fiber-based ATF technology for influenza virus production in perfusion. *Appl Microbiol Biotechnol, 104*, 4877–4888. doi:10.1007/s00253-020-10596-x

Gränicher, G., Tapia, F., Behrendt, I., Jordan, I., Genzel, Y., & Reichl, U. (2020). Production of Modified Vaccinia Ankara Virus by Intensified Cell Cultures: A Comparison of Platform Technologies for Viral Vector Production. *Biotechnology Journal*, 2000024. doi:10.1002/biot.202000024

Grein, T. A., Loewe, D., Dieken, H., Salzig, D., Weidner, T., & Czermak, P. (2018). High titer oncolytic measles virus production process by integration of dielectric spectroscopy as online monitoring system. *Biotechnology and Bioengineering, 115*(5), 1186-1194. doi:10.1002/bit.26538

Hughes, K., Zachertowska, A., Wan, S., Li, L., Klimaszewski, D., Euloth, M., & Hatchette, T. F. (2007). Yield increases in intact influenza vaccine virus from chicken allantoic fluid through isolation from insoluble allantoic debris. *Vaccine, 25*(22), 4456-4463. doi:https://doi.org/10.1016/j.vaccine.2007.03.017

Johnson, L., Gupta, A. K., Ghafoor, A., Akin, D., & Bashir, R. (2006). Characterization of vaccinia virus particles using microscale silicon cantilever resonators and atomic force microscopy. *Sensors and Actuators B: Chemical, 115*(1), 189-197. doi:https://doi.org/10.1016/j.snb.2005.08.047

Jordan, I., Horn, D., Thiele, K., Haag, L., Fiddeke, K., & Sandig, V. (2020). A Deleted Deletion Site in a New Vector Strain and Exceptional Genomic Stability of Plaque-Purified Modified Vaccinia Ankara (MVA). *Virologica Sinica, 35*(1), 212-226. doi:10.1007/s12250-019-00176-3

Jordan, I., Northoff, S., Thiele, M., Hartmann, S., Horn, D., Höwing, K., . . . Sandig, V. (2011). A chemically defined production process for highly attenuated poxviruses. *Biologicals, 39*(1), 50-58. doi:https://doi.org/10.1016/j.biologicals.2010.11.005

Jordan, I., Weimer, D., Iarusso, S., Bernhardt, H., Lohr, V., & Sandig, V. (2015). Purification of modified vaccinia virus Ankara from suspension cell culture. *BMC Proceedings, 9*(Suppl 9), O13-O13. doi:10.1186/1753-6561-9-S9-O13

Justice, C., Brix, A., Freimark, D., Kraume, M., Pfromm, P., Eichenmueller, B., & Czermak, P. (2011). Process control in cell culture technology using dielectric spectroscopy. *Biotechnology Advances, 29*(4), 391-401. doi:https://doi.org/10.1016/j.biotechadv.2011.03.002

Kaemmerer, W. F. (2018). How will the field of gene therapy survive its success? *Bioeng Transl Med, 3*(2), 166-177. doi:10.1002/btm2.10090

Kalbfuss, B., Genzel, Y., Wolff, M., Zimmermann, A., Morenweiser, R., & Reichl, U. (2007). Harvesting and concentration of human influenza A virus produced in serum-free mammalian cell culture for the production of vaccines. *Biotechnology and Bioengineering, 97*(1), 73-85. doi:10.1002/bit.21139

Kalbfuss, B., Wolff, M., Morenweiser, R., & Reichl, U. (2007). Purification of cell culture-derived human influenza A virus by size-exclusion and anion-exchange chromatography. *Biotechnol Bioeng, 96*(5), 932-944. doi:10.1002/bit.21109

Karst, D. J., Steinebach, F., & Morbidelli, M. (2018). Continuous integrated manufacturing of therapeutic proteins. *Curr Opin Biotechnol, 53*, 76-84. doi:10.1016/j.copbio.2017.12.015

Karst, D. J., Steinebach, F., Soos, M., & Morbidelli, M. (2017). Process performance and product quality in an integrated continuous antibody production process. *Biotechnol Bioeng, 114*(2), 298-307. doi:10.1002/bit.26069

Kim, Y., Rana, D., Matsuura, T., Chung, W.-J., & Khulbe, K. C. (2010). Relationship between surface structure and separation performance of poly(ether sulfone) ultra-filtration membranes blended with surface modifying macromolecules. *Separation and Purification Technology, 72*(2), 123-132. doi:https://doi.org/10.1016/j.seppur.2010.01.006

Klutz, S., Holtmann, L., Lobedann, M., & Schembecker, G. (2016). Cost evaluation of antibody production processes in different operation modes. *Chemical Engineering Science, 141*, 63-74. doi:https://doi.org/10.1016/j.ces.2015.10.029

Konstantinov, K. B., & Cooney, C. L. (2015). White Paper on Continuous Bioprocessing May 20–21 2014 Continuous Manufacturing Symposium. *Journal of Pharmaceutical Sciences, 104*(3), 813-820. doi:10.1002/jps.24268

Labeed, F. H., Coley, H. M., & Hughes, M. P. (2006). Differences in the biophysical properties of membrane and cytoplasm of apoptotic cells revealed using dielectrophoresis. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1760*(6), 922-929. doi:https://doi.org/10.1016/j.bbagen.2006.01.018

Leon, A., David, A. L., Madeline, B., Guianvarc'h, L., Dureau, E., Champion-Arnaud, P., . . . Schwamborn, K. (2016). The EB66(R) cell line as a valuable cell substrate for MVA-based vaccines production. *Vaccine, 34*(48), 5878-5885. doi:10.1016/j.vaccine.2016.10.043

Lim, A. C., Washbrook, J., Titchener-Hooker, N. J., & Farid, S. S. (2006). A computer-aided approach to compare the production economics of fed-batch and perfusion culture under uncertainty. *Biotechnology and Bioengineering, 93*(4), 687-697. doi:10.1002/bit.20757

Lohr, V. (2014). *Characterization of the avian designer cells AGE1.CR and AGE1.CR.pIX considering growth, metabolism and production of influenza virus and Modified Vaccinia Virus Ankara (MVA), Dissertation.* (PhD Thesis). Otto-von-Guericke Universität, Magdeburg. (http://hdl.handle.net/11858/00-001M-0000-0024-3EDE-2)

Lothert, K., Offersgaard, A. F., Pihl, A. F., Mathiesen, C. K., Jensen, T. B., Alzua, G. P., . . . Wolff, M. W. (2020). Development of a downstream process for the production of an inactivated whole hepatitis C virus vaccine. *Scientific Reports, 10*(1), 16261. doi:10.1038/s41598-020-72328-5

Lothert, K., Pagallies, F., Feger, T., Amann, R., & Wolff, M. W. (2020). Selection of chromatographic methods for the purification of cell culture-derived Orf virus for its application as a vaccine or viral vector. *Journal of Biotechnology, 323*, 62-72. doi:https://doi.org/10.1016/j.jbiotec.2020.07.023

Lothert, K., Sprick, G., Beyer, F., Lauria, G., Czermak, P., & Wolff, M. W. (2020). Membrane-based steric exclusion chromatography for the purification of a recombinant baculovirus and its application for cell therapy. *Journal of Virological Methods, 275*, 113756. doi:https://doi.org/10.1016/j.jviromet.2019.113756

Lukasik, J., Scott, T. M., Andryshak, D., & Farrah, S. R. (2000). Influence of Salts on Virus Adsorption to Microporous Filters. *Applied and Environmental Microbiology, 66*(7), 2914-2920. doi:10.1128/aem.66.7.2914-2920.2000

Manceur, A. P., Kim, H., Misic, V., Andreev, N., Dorion-Thibaudeau, J., Lanthier, S., . . . Ansorge, S. (2017). Scalable Lentiviral Vector Production Using Stable HEK293SF Producer Cell Lines. *Hum Gene Ther Methods, 28*(6), 330-339. doi:10.1089/hgtb.2017.086

Marichal-Gallardo, P. (2019). *Chromatographic purification of biological macromolecules by their capture on hydrophilic surfaces with the aid of non-ionic polymers.* (PhD Thesis). Otto-von-Guericke-Universität, Magdeburg. (https://pure.mpg.de/rest/items/item_3248240/component/file_3249620/content)

Marichal-Gallardo, P., Börner, K., Pieler, M. M., Sonntag-Buck, V., Obr, M., Bejarano, D., . . . Grimm, D. (2021). Single-use capture purification of adeno-associated viral gene transfer vectors by membrane-based steric exclusion chromatography. *Human Gene Therapy*. doi:10.1089/hum.2019.284

Marichal-Gallardo, P., Pieler, M. M., Wolff, M. W., & Reichl, U. (2017). Steric exclusion chromatography for purification of cell culture-derived influenza A virus using regenerated cellulose membranes and polyethylene glycol. *Journal of Chromatography A, 1483*, 110-119. doi:10.1016/j.chroma.2016.12.076

Michen, B., & Graule, T. (2010). Isoelectric points of viruses. *Journal of Applied Microbiology, 109*(2), 388-397. doi:10.1111/j.1365-2672.2010.04663.x

Moleirinho, M. G., Silva, R. J. S., Alves, P. M., Carrondo, M. J. T., & Peixoto, C. (2020). Current challenges in biotherapeutic particles manufacturing. *Expert Opinion on Biological Therapy, 20*(5), 451-465. doi:10.1080/14712598.2020.1693541

Negrete, A., Esteban, G., & Kotin, R. M. (2007). Process optimization of large-scale production of recombinant adeno-associated vectors using dielectric spectroscopy. *Applied Microbiology and Biotechnology, 76*(4), 761-772. doi:10.1007/s00253-007-1030-9

Niklas, J., Schräder, E., Sandig, V., Noll, T., & Heinzle, E. (2011). Quantitative characterization of metabolism and metabolic shifts during growth of the new human cell line AGE1.HN using time resolved metabolic flux analysis. *Bioprocess and biosystems engineering, 34*(5), 533-545. doi:10.1007/s00449-010-0502-y

657 Nikolay, A., Bissinger, T., Gränicher, G., Wu, Y., Genzel, Y., & Reichl, U. (2020). Perfusion control for
658   high cell density cultivation and viral vaccine production. In R. Pörtner (Ed.), *Animal cell*
659   *biotechnology* (Vol. 2095, pp. 141-168): Humana Press, New York, NY.
660 Nikolay, A., Leon, A., Schwamborn, K., Genzel, Y., & Reichl, U. (2018). Process intensification of
661   EB66(R) cell cultivations leads to high-yield yellow fever and Zika virus production. *Appl Microbiol*
662   *Biotechnol, 102*(1), 8725-8737. doi:10.1007/s00253-018-9275-z
663 Patil, R., & Walther, J. (2018). Continuous Manufacturing of Recombinant Therapeutic Proteins:
664   Upstream and Downstream Technologies. In B. Kiss, U. Gottschalk, & M. Pohlscheidt (Eds.),
665   *New Bioprocessing Strategies: Development and Manufacturing of Recombinant Antibodies and*
666   *Proteins* (pp. 277-322). Cham: Springer International Publishing.
667 Pearson, S. (2020). Process Intensification of Viral-Based Vaccines. Where Are the Bottlenecks?
668   *BioProcess International, 18*(6), 68-70.
669 Petiot, E., Ansorge, S., Rosa-Calatrava, M., & Kamen, A. (2017). Critical phases of viral production
670   processes monitored by capacitance. *J Biotechnol, 242*, 19-29. doi:10.1016/j.jbiotec.2016.11.010
671 Petiot, E., Jacob, D., Lanthier, S., Lohr, V., Ansorge, S., & Kamen, A. A. (2011). Metabolic and Kinetic
672   analyses of influenza production in perfusion HEK293 cell culture. *BMC Biotechnology, 11*(84), 1-
673   12. doi:10.1186/1472-6750-11-84
674 Pinto, N. D. S., Napoli, W. N., & Brower, M. (2019). Impact of micro and macroporous TFF membranes on
675   product sieving and chromatography loading for perfusion cell culture. *Biotechnol Bioeng*.
676   doi:10.1002/bit.27192
677 Pitcher, W. H. (1978). *Design and operation of immobilized enzyme reactors*, Berlin, Heidelberg.
678 Pleitt, K., Somasundaram, B., Johnson, B., Shave, E., & Lua, L. H. L. (2019). Evaluation of process
679   simulation as a decisional tool for biopharmaceutical contract development and manufacturing
680   organizations. *Biochemical Engineering Journal, 150*, 107252.
681   doi:https://doi.org/10.1016/j.bej.2019.107252
682 Pollock, J., Ho, S. V., & Farid, S. S. (2013). Fed-batch and perfusion culture processes: Economic,
683   environmental, and operational feasibility under uncertainty. *Biotechnology and Bioengineering,*
684   *110*(1), 206-219. doi:10.1002/bit.24608
685 Tapia, F., Vazquez-Ramirez, D., Genzel, Y., & Reichl, U. (2016). Bioreactors for high cell density and
686   continuous multi-stage cultivations: options for process intensification in cell culture-based viral
687   vaccine production. *Appl Microbiol Biotechnol, 100*(5), 2121-2132. doi:10.1007/s00253-015-7267-
688   9
689 Terova, O., Soltys, S., Hermans, P., De Rooij, J., & Detmers, F. (2018). Overcoming downstream
690   purification challenges for viral vector manufacturing: enabling advancement of gene therapies in
691   the clinic. *Cell. Gen. Therapy Insights, 4*(2), 101-111. doi:10.18609/CGTI.2018.017
692 Ungerechts, G., Bossow, S., Leuchs, B., Holm, P. S., Rommelaere, J., Coffey, M., . . . Nettelbeck, D. M.
693   (2016). Moving oncolytic viruses into the clinic: clinical-grade production, purification, and
694   characterization of diverse oncolytic viruses. *Molecular Therapy - Methods & Clinical*
695   *Development, 3*, 16018. doi:https://doi.org/10.1038/mtm.2016.18
696 van der Loo, J. C. M., & Wright, J. F. (2015). Progress and challenges in viral vector manufacturing.
697   *Human Molecular Genetics, 25*(R1), R42-R52. doi:10.1093/hmg/ddv451
698 Vazquez-Ramirez, D., Jordan, I., Sandig, V., Genzel, Y., & Reichl, U. (2019). High titer MVA and
699   influenza A virus production using a hybrid fed-batch/perfusion strategy with an ATF system. *Appl*
700   *Microbiol Biotechnol, 103*(1), 3025-3035. doi:10.1007/s00253-019-09694-2
701 Vincent, D. I. W. (2017). *Purification of Recombinant Vaccinia Virus for Oncolytic and Immunotherapeutic*
702   *Applications using Monolithic Column Technology.* University College London, London. Retrieved
703   from https://discovery.ucl.ac.uk/id/eprint/10038211
704 Walther, J., Godawat, R., Hwang, C., Abe, Y., Sinclair, A., & Konstantinov, K. (2015). The business
705   impact of an integrated continuous biomanufacturing platform for recombinant protein production.
706   *J Biotechnol, 213*(1), 3-12. doi:10.1016/j.jbiotec.2015.05.010
707 Walther, J., Lu, J., Hollenbach, M., Yu, M., Hwang, C., McLarty, J., & Brower, K. (2019). Perfusion Cell
708   Culture Decreases Process and Product Heterogeneity in a Head-to-Head Comparison With Fed-
709   Batch. *Biotechnology Journal, 14*(2), 1-10. doi:10.1002/biot.201700733
710 Wang, S., Godfrey, S., Ravikrishnan, J., Lin, H., Vogel, J., & Coffman, J. (2017). Shear contributions to
711   cell culture performance and product recovery in ATF and TFF perfusion systems. *J Biotechnol,*
712   *246*, 52-60. doi:10.1016/j.jbiotec.2017.01.020

713 Wang, S. B., Godfrey, S., Radoniqi, F., Lin, H., & Coffman, J. (2019). Larger Pore Size Hollow Fiber
714   Membranes as a Solution to the Product Retention Issue in Filtration-Based Perfusion
715   Bioreactors. *Biotechnology Journal, 14*(2), 1800137. doi:10.1002/biot.201800137
716 Warikoo, V., Godawat, R., Brower, K., Jain, S., Cummings, D., Simons, E., . . . Konstantinov, K. (2012).
717   Integrated continuous production of recombinant therapeutic proteins. *Biotechnology and*
718   *Bioengineering, 109*(12), 3018-3029. doi:10.1002/bit.24584
719 Wright, J. F., Le, T., Prado, J., Bahr-Davidson, J., Smith, P. H., Zhen, Z., . . . Qu, G. (2005). Identification
720   of factors that contribute to recombinant AAV2 particle aggregation and methods to prevent its
721   occurrence during vector purification and formulation. *Molecular Therapy, 12*(1), 171-178.
722   doi:https://doi.org/10.1016/j.ymthe.2005.02.021
723 Wu, Y., Bissinger, T., Genzel, Y., Liu, X., Reichl, U., & Tan, W.-S. (2021). High cell density perfusion
724   process for high yield of influenza A virus production using MDCK suspension cells. *Applied*
725   *Microbiology and Biotechnology, 105*(4), 1421-1434. doi:10.1007/s00253-020-11050-8
726 Wyatt, L. S., Earl, P. L., Eller, L. A., & Moss, B. (2004). Highly attenuated smallpox vaccine protects mice
727   with and without immune deficiencies against pathogenic vaccinia virus challenge. *Proceedings*
728   *of the National Academy of Sciences, 101*(13), 4590-4595. doi:10.1073/pnas.0401165101
729 Xenopoulos, A. (2015). Production and Purification of Hepatitis C Virus-like Particles [Webinar]. *EMD*
730   *Millipore Webinar Series*.
731 Xu, S., & Chen, H. (2016). High-density mammalian cell cultures in stirred-tank bioreactor without external
732   pH control. *Journal of Biotechnology, 231*, 149-159.
733   doi:https://doi.org/10.1016/j.jbiotec.2016.06.019
734 Zimmermann, D., Zhou, A., Kiesel, M., Feldbauer, K., Terpitz, U., Haase, W., . . . Sukhorukov, V. L.
735   (2008). Effects on capacitance by overexpression of membrane proteins. *Biochemical and*
736   *Biophysical Research Communications, 369*(4), 1022-1026.
737   doi:https://doi.org/10.1016/j.bbrc.2008.02.153

738

739

740 **Tables**

741 **Table 1:** Process parameters used for continuous clarification and DNA digestion of bioreactor harvests after the
742 acoustic settler.

Parameter	Cell culture harvest	NaCl, NaBr and KCl	NaN_3	DENARASE®, diluted in PBS + 5% sucrose	$MgCl_2$
Initial concentration	-	6000 mM	6.2% v/v	1628 U/mL	176 mM
Final concentration	-	700 mM	0.08% v/v	37 U/mL	4 mM
Point of addition [a]	B1	B1	B1	B2	B2
Flow rate$_{in}$ [mL/h]	54.0	7.5	0.8	1.5	1.5
Flow rate$_{out}$ [mL/h]	62.3	62.3	62.3	65.3	65.3

743 [a] Bottle names according to the scheme shown in Figure 1.

744

745 **Table 2:** Parameters used to calculate the recovery of each filtration or DNA digestion step in perfusion mode.

Recovery [%] [a]	Average infectious virus titer between t_{n-1} and t_n before the step	Average infectious virus titer between t_{n-1} and t_n after the step
Acoustic settler filtration [b]	Bioreactor supernatant	Bottle B1 [c]
Depth filtration	Bottle B1 [c]	Bottle B2 [c]
DNA digestion	Bottle B2 [c]	Bottle B3 [c]
Final filtration	Bottle B3 [c]	Bottle B4 [c]

746 [a] The recovery is calculated as the ratio of the average titer after and before the step.
747 [b] Ratio for the settler filtration recovery calculated similarly to the sieving coefficient calculated for recombinant protein perfusion
748 cultures (S. Wang et al., 2017).
749 [c] Bottle names according to the scheme shown in Figure 1.

750

Figure legends

Figure 1: Scheme of an integrated process for cell culture-based virus production in perfusion mode. The integrated MVA production is separated in three main steps, separated by grey vertical dotted lines: 1) Virus production in perfusion mode using an acoustic filter, 2) cell clarification and DNA digestion, and 3) steric exclusion chromatography (SXC) as a series of bind-elute steps. MVA is produced using AGE1.CR.pIX cells grown in suspension in a stirred tank bioreactor. To achieve high cell concentrations, the cells are retained in the bioreactor while cell free medium is continuously harvested through the acoustic chamber controlled by the SonoSep control unit (acoustic filter as perfusion system). To allow a constant bioreactor working volume and weight, fresh medium is added into the bioreactor through a peristaltic pump controlled by a scale. During the cell growth phase, the harvest flow rate is controlled based on the estimation of the viable cell concentration using a capacitance sensor. After infection, a decrease in the permittivity signal indicates virus particle release, and initiates cell clarification and subsequent chromatography steps. The harvest containing MVA (which was first cell clarified through the acoustic settler) is collected into bottle B1. Salt and sodium azide (NaN_3) are added to bottle B1 as well. The virus harvest is then continuously filtered through a polypropylene depth filter with 0.45 µm pore size (Filter 1). For continuous endonuclease digestion (addition of endonuclease and magnesium chloride ($MgCl_2$) in bottle B2), the harvest is incubated into a plug-flow reactor (indicated with the coiled red tube) at 37°C with a residence time of 4 h. The endonuclease-digested product is continuously collected into bottle B3. After another filtration step using cellulose acetate depth filter with 0.45 µm pore size (Filter 2), the harvest is collected into bottle B4 at 4°C. An ÄKTA Pure 25 system is used to purify the virus harvest using membrane-based SXC operated in a semi-continuous bind-elute mode; the composition of buffer solutions (including buffer solution with PEG) used in purification are described in section 2.3.3. Finally, purified MVA is collected into 50 mL tubes (not illustrated). The color of the horizontal arrow going from red to green illustrates the stepwise purification of the MVA and the removal of contaminating host cell DNA.

Figure 2: MVA production in AGE1.CR.pIX cells in perfusion and in batch mode (stirred tank bioreactor, CD-U5 medium). (A) Viable cell concentration (●) and cell viability (▲), (B) total number of infectious virions produced (●) and recovery coefficient (from the acoustic settler filtration step) (▲), (C) cell-specific infectious virus yield and (D) volumetric virus productivity (for infectious virions). The black, red and blue colors correspond to run 1, run 2 (one replicate) and the batch runs (average from runs A-C, in triplicate), respectively. The error bars on graphs C and D correspond to the standard deviation of the batch runs performed in triplicate.

Figure 3: Product recovery and impurity removal of the different purification steps for the integrated batch or perfusion processes. (A) Percentage recovery of the total number of infectious virions of individual process steps, (B) level of host cell DNA per dose, and (C) level of total protein per dose of the integrated batch processes (grey) and the integrated perfusion run 1 (red). To estimate contamination levels, a MVA dose input of 1.43×10^8 $TCID_{50}$ was assumed (see section 2.4). The MVA raw material for steric exclusion chromatography was purified in semi-continuous mode, as described in section 2.3.3. Error bars of the batch process correspond to the standard deviation of triplicate runs, as described in section 2.4. Error bars of run 1 correspond to the standard deviation of the yields for continuous harvesting between 36 and 87 hpi (time intervals between samples < 14 h), as described in section 2.4.

Figure 4: Online monitoring of cell concentrations using a capacitance probe for process automation and control during the growth phase of run 1 (AGE1.CR.pIX cells grown in perfusion mode using an acoustic filter). Offline (●) and online (black line) viable cell concentration, cell-specific perfusion rate (▲). The cell factor (described in section 2.2.2) converting the permittivity signal to a viable cell concentration was equal to 0.57.

Figure 5: Online monitoring of a capacitance probe for process automation and control during MVA production using suspension AGE1.CR.pIX cells. (A) Maximum permittivity signal ($\Delta\varepsilon_{max}$; solid line) and offline viable cell concentration

798 (●) for three cultivations in perfusion mode (run 1= black, run 2= red, data from run *4* of Gränicher, Tapia, et al.
799 (2020) = grey). (B) Infectious virus titer in the bioreactor supernatant for three cultivations in perfusion mode (run 1=
800 black, run 2= red, data from run *4* of Gränicher, Tapia, et al. (2020) = grey). (C) Maximum permittivity signal ($\Delta\varepsilon_{max}$;
801 solid line) and offline viable cell concentration (●) for one cultivation in batch (run C). (D) Infectious virus titer in the
802 bioreactor supernatant for one cultivation in batch mode (run C). The vertical lines (for each run in the respective
803 color) correspond to the expected time of MVA release in the supernatant, which is on average 10.6 h after the
804 maximum permittivity signal (between 12 and 36 h post infection for perfusion and between 24 and 48 h post infection
805 for batch). This time interval of 10.6 h was determined based on the optimal time of virus harvesting for a perfusion
806 process (which is the time of MVA release, corresponding to the time when about 8 to 10% of the total number of
807 infectious virions was released from the infected cells), as described in section 2.2.2. The cell factor (described in
808 section 2.2.2) used to convert the permittivity signal to an online viable cell concentration was equal to 0.57, 0.65 and
809 0.44 for run 1, run 2 and the perfusion control run, respectively.

810

811 **Figure 6:** Economic analysis for an end-to-end production of MVA using AGE1.CR.pIX cells cultivated either in batch
812 or in perfusion mode. (A) CAPEX and OPEX of a batch or a perfusion process at the 1 L scale operated over 47
813 weeks per year. (B) Raw material and consumables costs in batch and perfusion at the 1 L bioreactor scale. (C)
814 Contribution of the seed train, cell culture, filtration plus DNA digestion and chromatography (SXC) steps on the cost
815 per dose for batch (grey) or perfusion (red), at the 1 L scale (full) or at the 10 L scale (dashed). (D) Cost per dose
816 (solid line) and number of annually produced doses (dotted line) as a function of the bioreactor scale (1, 10, 50, 200
817 and 1000 L working volume) for the batch (black) or the perfusion process (red). (E) Cost per dose as a function of
818 the number of annually produced doses. A MVA dose input of 1.43×10^{8} $TCID_{50}$ was considered for graphs C, D and
819 E. For the economic analysis, the SuperPro designer software was used (section 2.5). Average data from runs A-C
820 were used to estimate the costs for an integrated batch process. Average data from runs 1-2 were used to estimate
821 the costs regarding the "Seed train" and the "Cell culture" (C) for the perfusion process. Finally, the data from run 1
822 were used to estimate the costs of DSP, i.e. "Filtration and DNA digestion" and "Chromatography" (C), as only run 1
823 was integrating USP with DSP for the perfusion mode.

824

825

Appendices

Material and methods: illustration of an integrated perfusion process

Figure A.1: Picture of the process set-up, separated into three parts by red dashed vertical lines as shown in the scheme of Figure 1: 1) Virus production in the bioreactor (Biostat system coupled to an acoustic settler), 2) cell clarification and DNA digestion and 3) steric exclusion chromatography with an ÄKTA Pure 25 system operated in bind-elute mode. The plug flow reactor used for continuous DNA digestion at 37°C with a retention time of 4 h is located in the incubator, indicated by the orange square on the left side of the picture. The yellow color of the bioreactor and the bottles is due to the GFP protein expressed by the AGE1.CR.pIX cells after infection with the recombinant MVA.

SXC

SXC purification was optimized through a design of experiment (DoE) approach (Table A.1). Based on 10 randomized runs, a surface response was generated. The starting material consisted of a pooled harvest of a perfusion run (*run 4* from a previous study (Gränicher, Tapia, et al., 2020); performed as described in section 2.2.2), that was clarified and endonuclease treated as described in section 2.3. The infectious virus titer of the pooled harvest was equal to 4.05×10^8 $TCID_{50}$/mL. The endonuclease treatment step was required as preliminary results showed a strong co-elution of host cell DNA with virus particles and a decrease in recovery (data not shown). As a result, the DNA content was far beyond the 10 ng/dose limit imposed by the authorities. The PEG molecular weight, the membrane surface, the membrane material and the salt concentration were not modified.

Table A.1: Design space for optimization of steric exclusion chromatography.

Parameter	Lower range	Middle range	Upper range
PEG concentration [% w/v]	6	8	10
System flow rate [mL/min]	2	5	8

Capacitance probe measurements

852 **Figure A.2:** Additional capacitance probe data for process automation and control during MVA production using
853 AGE1.CR.pIX cells in perfusion mode. (A) Critical frequency (Fc), (B) membrane capacitance (C_m; solid line) and
854 intracellular conductivity (σ_i; dotted line) and (C) viable cell diameter for three cultivations in perfusion mode (run 1=
855 black, run 2= red, data from run 4 of Gränicher, Tapia, et al. (2020) = grey).). The vertical lines (for each run in the
856 respective color) correspond to the expected time of MVA release in the supernatant, which is on average 10.6 h
857 after the maximum permittivity signal (between 12 and 36 h post infection). This time interval of 10.6 h was
858 determined based on the optimal time of virus harvesting for a perfusion process (which is the time of MVA release,
859 corresponding to the time when about 8 to 10% of the total number of infectious virions was released from the
860 infected cells), as described in section 2.2.2. The cell factor (described in section 2.2.2) converting the permittivity
861 signal to the viable cell concentration was equal to 0.57, 0.65 and 0.44 for run 1, run 2 and the perfusion control run,
862 respectively.

863

864 A range of 0.8–1.8 $\mu F/cm^2$ for C_m and 3.5–11.0 mS/cm for σ_i were obtained (Figure A.2), which

865 corresponds to the range of other C_m and σ_i values reported for human cell lines or CHO cells (Afshar et

866 al., 2019; Labeed, Coley, & Hughes, 2006; Petiot et al., 2017; Zimmermann et al., 2008). Higher Fc

867 values were observed for the batch system (Figure A.3) compared to the perfusion system (Figure A.2C),

868 which could be eventually explained by a different geometry of the bioreactor, leading to a closer

869 proximity of the capacitance probe with the metallic part of the bioreactor.

870 **Figure A.3:** Additional capacitance probe data for process automation and control during MVA production using
871 AGE1.CR.pIX cells in batch mode. Critical frequency (Fc) for one cultivation in batch mode (run C).). The vertical
872 lines (for each run in the respective color) correspond to the expected time of MVA release in the supernatant, which
873 is on average 10.6 h after the maximum permittivity signal (between 12 and 36 h post infection for perfusion and
874 between 24 and 48 h post infection for batch). This time interval of 10.6 h was determined based on the optimal time
875 of virus harvesting for a perfusion process (which is the time of MVA release, corresponding to the time when about 8
876 to 10% of the total number of infectious virions was released from the infected cells), as described in section
877 2.2.2.

878

879 **Table A.2:** Critical time points used to predict the onset of virus release, using the capacitance probe.

Parameters	Run 1	Run 2	Control run	Batch run C
Time of maximum $\Delta\varepsilon_{max}$ [hpi] [a]	25.4	34.2	22.1	46.9
Time of expected virus release [hpi] [b]	36.0	44.8	32.7	57.5
Time of measured virus release [hpi] [c]	40.0	41.0	35.5	54.5
Calculated percentage of harvested virions after the expected virus release [%]	93.4	81.1	94.8	84.8 [d]

880 hpi: Hours post infection, $\Delta\varepsilon_{max}$: Maximum permittivity signal, Fc: Critical frequency.
881 [a] Value considered between 12 and 36 hpi for runs 1−2 and the control run. Due to the major delay in term of virus release and cell
882 death, the range was set between 24 and 48 hpi for the batch run C.
883 [b] Value considering the maximum permittivity signal plus 10.6 h.
884 [c] Considering the frequency of virus sampling (section 2.2), the time of virus release was rounded up to every 0.5 h.
885 [d] For the batch run C, the totality of the batch was harvested at the end of the run (as there is no continuous harvest). The
886 presented value indicates here the percentage of virus released in the supernatant after the expected virus time release.

887

888

889 *Economic analysis*

890 The detailed economic report for MVA production is shown for each bioreactor scale (from 1 to 1000 L;

891 Tables A.3–5) in batch or in perfusion mode. In addition, the economic report for the seed train generation

892 is also shown here (Tables A.6–7).

893 **Table A.3:** Material and consumable costs per unit.

Material or consumable	Unit	Price per unit [$]	Source
AGE1.CR.pIX cells: Seed cells for 1 L bioreactor [a]	mg of dry weight	66.70	Table A.7
AGE1.CR.pIX cells: Seed cells for 10 L bioreactor [a]	mg of dry weight	12.24	Table A.7
AGE1.CR.pIX cells: Seed cells for 50 L bioreactor [a]	mg of dry weight	2.44	Table A.7
AGE1.CR.pIX cells: Seed cells for 200 L bioreactor [a]	mg of dry weight	0.92	Table A.7
AGE1.CR.pIX cells: Seed cells for 1000 L bioreactor [a]	mg of dry weight	0.30	Table A.7
NaOH 0.55 M (liquid)	kg	0.27	Molbase.com
Air	kg	0.01 [b]	SuperPro Designer
Carbone dioxide	kg	2.00	Molbase.com
Oxygen	kg	0.01 [b]	SuperPro Designer
CD-U5 medium (liquid)	kg	44.00	Biochrom-Merck
Seed virus (MVA.CR19-GFP)	mg of virus [c]	35444.00	Model on SuperPro Designer
PBS (liquid)	kg	0.12	Molbase.com
KCl (solid salt)	kg	3.00	Molbase.com
NaBr (solid salt)	kg	3.00	Molbase.com
NaCl (solid salt)	kg	8.00	Molbase.com
NaOH (solid salt)	kg	1.26	Molbase.com
Sodium azide 6.2% (liquid)	kg	0.15	Molbase.com
$MgCl_2$ (solid salt)	kg	0.08	Molbase.com
DENARASE (endonuclease in liquid)	mg [d]	499.50	c-Lecta
Water (liquid)	kg	0.10	SuperPro Designer
PEG 7.2% w/v in PBS (liquid)	kg	1.07	Sigma
Depth filter	cm^2	0.15	Sartorius AG
Microfilter	cm^2	0.45	Sartorius AG
Cellulose membrane for chromatography	cm^2	0.09	GE Healthcare
Shake flask (maximum 1000 mL working volume)	1 item	1.80	SuperPro Designer

894 [a] These costs were included in the "Seed train" costs of Figure 7C, and reported according to Table A.7

895 [b] Negligible costs as air and oxygen gas supply already included in the plant (CAPEX costs).

896 [c] One $TCID_{50}$ was considered as one infectious MVA particle, which has a mass of about 7.9 fg (Johnson, Gupta, Ghafoor, Akin, &
897 Bashir, 2006).

898 [d] 1 mg approximated to a volume of 1 mL, with an activity of 250 endonuclease U/μL according to manufacturer (https://www.c-
899 lecta.com/products-services/products/denarase/)

900

901

902 **Table A.4:** Economic analysis for MVA production at the 1, 10, 50, 200, and 1000 L bioreactor scale in batch mode.

Parameters	1 L	10 L	50 L	200 L	1000 L
Batches per year [Batch/year]	53	53	53	53	53
Yearly produced doses [10^4 Doses/year] [a]	4	43	215	842	4210
Cost per dose [$/dose] [a]	63.60	7.66	1.87	0.77	0.45
Operating time for one integrated run [h]	173	173	173	173	173
Capital expenditure					
Bioreactor and gas system [k$] [b]	576	576	576	614	750
Acoustic settler [k$]	14	14	150	150	600
Filtration equipment [b]	50	50	50	50	59
Chromatography equipment [b]	186	208	337	698	2706
Other equipment such as intermediate tanks [b]	-	-	-	-	-
Direct fixed capital costs [k$] [b] [c]	4948	5043	6522	8366	21292
Capital expenditure (Total) [k$] [b]	**5341**	**5447**	**7035**	**9078**	**23232**
Operating expenditure					
Seed train, dry weight AGE1.CR.pIX [k$/year]	523	961	957	1417	2310
NaOH 0.55 M (liquid) [$/year]	1	3	15	58	291
Air [$/year]	1	1	1	1	1
Carbone dioxide [$/year]	4	37	185	727	3635
Oxygen [$/year]	1	1	1	1	1
CD-U5 medium (liquid) [$/year]	2332	23320	116600	457600	2288000
Seed virus (MVA.CR19-GFP) [$/year]	1484	1480	74202	291208	1456040
PBS (liquid) [$/year]	18	177	885	3451	17244
KCl / NaBr / NaCl (solid salt) [$/year]	12	113	561	2203	11014
Sodium azide 6.2 % (liquid) [$/year]	0	0	0	0	0
NaOH (solid salt) [$/year]	58	581	2904	11282	56355
$MgCl_2$ (solid salt) [$/year]	1	2	10	40	197
DENARASE (endonuclease in liquid) [$/year]	3647	36474	182368	715709	3578546
Water (liquid) [$/year]	9	93	464	1802	9003
PEG 7.2% w/v in PBS (liquid) [$/year]	44	437	2185	8490	42408
Depth filter [$/year]	586	5863	29315	115046	575229
Microfilter [$/year]	1338	13376	66879	262470	1312352
Cellulose membrane for chromatography [$/year]	1836	18365	91823	356756	1781980
Labor costs [k$/year] [b] [d]	1098	1098	1101	1090	1139
Facility-dependent costs [k$/year] [b]	932	950	1228	1577	4017
Quality control / Quality assurance (QC/QA) costs [k$/year] [b]	165	165	165	163	171
Operating expenditure (Total) [k$/year]	**2729**	**3287**	**4021**	**6476**	**18779**

903 [a] One MVA dose is equal to 1.43 x 10^8 $TCID_{50}$.
904 [b] Calculated with SuperPro Designer.
905 [c] Includes: Total plant direct costs (Equipment purchase, installation, process piping, instrumentation, insulation, electrical,
906 buildings, yard improvement, auxiliary facilities), Total plant indirect costs (Engineering, construction) and contractor's fee &
907 contingency.
908 [d] Labor costs were determined with an average operator salary of 69$/hour.
909

910

911 **Table A.5:** Economic analysis for MVA production at the 1, 10, 50, 200, and 1000 L bioreactor scale in perfusion
912 mode.

Parameters	1 L	10 L	50 L	200 L	1000 L
Batches per year [Batch/year]	31	31	31	31	31
Yearly produced doses [10^4 Doses/year] [a]	15	152	758	3033	14674
Cost per dose [$/dose] [a]	22.63	2.72	0.80	0.43	0.33
Operating time for one integrated run [h]	274	274	274	274	274
	Capital expenditure				
Bioreactor and gas system [k$] [b]	576	576	576	613	748
Acoustic settler [k$] [b]	14	14	150	150	600
Filtration equipment [b]	50	50	50	50	88
Chromatography equipment [b]	182	203	332	698	2744
Other equipment such as intermediate tanks [b]	77	77	77	77	77
Direct fixed capital costs [k$] [b) c]	5392	5487	6915	8773	21804
Capital expenditure (Total) [k$] [b]	**5874**	**6003**	**7639**	**10082**	**26384**
	Operating expenditure				
Seed train, dry weight AGE1.CR.pIX [k$/year]	354	649	647	976	1539
NaOH 0.55 M (liquid) [$/year]	0	0	0	0	0
Air [$/year]	1	1	1	1	1
Carbone dioxide [$/year]	786	7865	39324	157295	761104
Oxygen [$/year]	1	1	1	1	1
CD-U5 medium (liquid) [$/year]	10432	104318	521589	2086356	10095723
Seed virus (MVA.CR19-GFP) [$/year]	11295	112953	564765	2259059	10930930
PBS (liquid) [$/year]	23	221	1102	4407	21236
KCl / NaBr / NaCl (solid salt) [$/year]	29	293	1467	5866	28384
Sodium azide	1	2	10	41	197
NaOH (solid salt) [$/year]	122	1220	6099	24396	117575
$MgCl_2$ (solid salt) [$/year]	1	2	10	40	197
DENARASE (endonuclease in liquid) [$/year]	12759	127589	637943	2551770	12347275
Water (liquid) [$/year]	19	195	974	3898	18784
PEG 7.2% w/v in PBS (liquid) [$/year]	211	2107	10533	42130	203501
Depth filter [$/year]	558	5582	27908	111633	540162
Microfilter [$/year]	1418	14179	70895	283579	1372158
Cellulose membrane for chromatography [$/year]	3857	38572	192859	771435	3717819
Labor costs [k$/year] [b) d]	1756	1756	1757	1758	1711
Facility-dependent costs [k$/year] [b]	1016	1034	1303	1654	4116
Quality control / Quality assurance (QC/QA) costs [k$/year] [b]	263	263	263	264	257
Operating expenditure (Total) [k$/year]	**3431**	**4117**	**6046**	**12957**	**47793**

913 [a] One MVA dose is equal to 1.43×10^8 $TCID_{50}$.
914 [b] Calculated with SuperPro Designer.
915 [c] Includes: Total plant direct costs (Equipment purchase, installation, process piping, instrumentation, insulation, electrical,
916 buildings, yard improvement, auxiliary facilities), Total plant indirect costs (Engineering, construction) and contractor's fee &
917 contingency.
918 [d] Labor costs were determined with an average operator salary of 69$/hour.
919

920

921 **Table A.6:** Economic analysis for the AGE1.CR.pIX N-1 seed train generation across the scales.

Parameters	N-1 for 1 L bio. [a]	N-1 for 2 L bio. [a]	N-1 for 5 L bio. [a]	N-1 for 10 L bio.	N-1 for 20 L bio.	N-1 for 50 L bio.	N-1 for 100 L bio.	N-1 for 200 L bio.	N-1 for 1000 L bio.
Cell culture working volume [L]	0.1	0.2	0.5	1.0	2.0	5.0	10.0	20.0	100.0
Batches per year [Batch/year]	53	53	53	53	53	53	53	53	53
Yearly produced cells [kg of dry weight/year] [b]	15	30	74	148	296	741	1482	2964	14819
Cost per mg of cells [$/mg of dry weight]	66.70	33.36	13.36	5.57	2.79	1.33	0.68	0.36	0.11
Operating time for one run [h]	96	96	96	96	96	96	96	96	96
Capital expenditure									
Bioreactor and gas system [k$]	100	100	100	100	100	233	241	283	666
Direct fixed capital costs [k$] [c] [d]	622	622	622	622	622	1407	1459	1703	3977
Capital expenditure (Total) [k$] [c]	**740**	**740**	**740**	**740**	**740**	**1566**	**1622**	**1882**	**4296**
Operating expenditure [d] [e]									
Air [$/year]	-	-	-	-	-	1	1	1	1
Carbone dioxide [$/year]	1	1	2	4	8	19	37	74	370
Oxygen [$/year]	-	-	-	-	-	-	-	-	-
CD-U5 medium (liquid) [$/year]	233	466	1166	2332	4664	11660	23320	46640	233200
Shake flasks [$/year]	10	10	10	10	20	-	-	-	-
Labor costs [k$/year] [c] [f]	614	614	614	614	614	614	615	615	616
Facility-dependent costs [k$/year] [c]	117	117	117	117	117	265	275	321	749
Quality control / Quality assurance (QC/QA) costs [k$/year] [c]	92	92	92	92	92	92	92	92	92
Operating expenditure (Total) [k$/year]	**989**	**989**	**990**	**826**	**828**	**983**	**1005**	**1074**	**1692**

922 [a] As the N-1 step for inoculation is at a low volume scale (100–500 mL), the costs before the N-1 were included in the OPEX by an
923 increase of +20% of the actual calculated operational expenditure.
924 [b] Calculated following the measured cell volume (measured with a ViCell XR), converted to dry weight using the volumetric weight
925 of 0.25 g dry weight / 1 mL cell volume. Value measured for a similar cell line, AGE1.HN (Niklas, Schräder, Sandig, Noll, & Heinzle,
926 2011).
927 [c] Calculated with SuperPro Designer.
928 [d] Includes: Total plant direct costs (Equipment purchase, installation, process piping, instrumentation, insulation, electrical,
929 buildings, yard improvement, auxiliary facilities), Total plant indirect costs (Engineering, construction) and contractor's fee &
930 contingency.
931 [e] For the calculation of the operating expenditure, the AGE1.CR.pIX cell culture seed train was here not taken into account but will
932 be taken into account in Table A.7 in order to consider the whole seed train from the shake flask to the final production bioreactor (at
933 the final scale of 1, 10, 50, 200 or 1000 L).
934 [f] Labor costs were determined with an average operator salary of 69$/hour.

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936

937 **Table A.7:** Assumptions made for the generation of the full seed trains for AGE1.CR.pIX cells to inoculate the MVA
938 production bioreactor at a 1, 10, 50, 200, or 1000 L scale.

Seed train step	Parameter	1 L	10 L	50 L	200 L	1000 L
N-4	Cell culture working volume at the N-4 step [L]	-	-	-	-	0.1
	mg of cell dry weight needed at N-4 to generate 1 mg at final scale [mg of dry weight]	-	-	-	-	0.001
	Costs at the N-4 step to generate 1 mg at final scale [$/mg of dry weight at final scale]	-	-	-	-	0.06
N-3	Cell culture working volume at the N-3 step [L]	-	-	-	0.2	1.0
	mg of cell dry weight needed at N-3 to generate 1 mg at final scale [mg of dry weight]	-	-	-	0.01	0.01
	Costs at the N-3 step to generate 1 mg at final scale [$/mg of dry weight at final scale]	-	-	-	0.28	0.06
N-2	Cell culture working volume at the N-2 step [L]	-	0.1	0.5	2.0	10.0
	mg of cell dry weight needed at N-2 to generate 1 mg at final scale [mg of dry weight]	-	0.1	0.1	0.1	0.1
	Costs at the N-2 step to generate 1 mg at final scale [$/mg of dry weight at final scale]	-	6.67	1.11	0.28	0.07
N-1	Cell culture working volume at the N-1 step [L]	0.1	1.0	5.0	20.0	100.0
	mg of cell dry weight needed at N-1 to generate 1 mg at final scale [mg of dry weight]	1	1	1	1	1
	Costs at the N-1 step to generate 1 mg at final scale [$/mg of dry weight at final scale]	66.70	5.57	1.33	0.36	0.11
From N-4 to N stage	**Total costs of the seed train [$/mg of dry weight at final scale]** [a]	**66.70**	**12.24**	**2.44**	**0.92**	**0.30**

939 [a] These costs were used in Table A.2.
940