1 **Production of high titer attenuated poliovirus strains on the serum-free PER.C6® cell culture**
2 **platform for the generation of safe and affordable Next Generation IPV**

3 Barbara P. Sanders^{a#}, Isabel de los Rios Oakes^a, Vladimir van Hoek^a, Ying Liu^b, Wilfred Marissen^a,
4 Philip D. Minor^c, Eckard Wimmer^b, Hanneke Schuitemaker^a, Jerome H.H.V. Custers^a, Andrew
5 Macadam^c, Jeronimo Cello^b, Diana Edo-Matas^a

6
7 ^a Janssen Infectious Diseases and Vaccines, Pharmaceutical Companies of Johnson and Johnson,
8 Leiden, the Netherlands

9 ^b Department of Molecular Genetics and Microbiology, Stony Brook University, Stony Brook, USA.

10 ^c Division of Virology, National Institute for Biological Standards and Control, Potters Bar, United
11 Kingdom

12
13 #Address correspondence to: Barbara P. Sanders, bsander2@its.jnj.com

14 Postal Address: Janssen Infectious Diseases and Vaccines

15 Archimedesweg 4-6, 2333CN, Leiden, The Netherlands

16 Tel: +31 (0) 71 519 9173

17

18 **Key words:** Poliovirus, PER.C6® cells, Next Generation IPV, Sabin, rationally-attenuated strains,
19 vaccines

20 **Abbreviations**

21 cIPV: conventional Inactivated Poliovirus Vaccine, DSP: Down Stream Process, IRES: internal
22 ribosome entry site, GPEI: Global Poliomyelitis Eradication Initiative, MOI: Multiplicity of Infection, OD:
23 optical density, OPV: Oral Poliovirus Vaccine, sIPV: Sabin-based inactivated poliovirus vaccine,
24 TCID₅₀ = Tissue Culture Infectious Dose 50%, VAPP: Vaccine Associated Paralytic Poliomyelitis

25

26 **Abstract**

27 **Background:** As poliovirus eradication draws closer, alternative Inactivated Poliovirus Vaccines (IPV)
28 are needed to overcome the risks associated with continued use of the Oral Poliovirus Vaccine and of
29 neurovirulent strains used during manufacture of conventional (c)IPV. We have previously
30 demonstrated the susceptibility of the PER.C6® cell line to cIPV strains; here we investigated the
31 suspension cell culture platform for growth of attenuated poliovirus strains.

32 **Methods:** We examined attenuated Sabin strain productivity on the PER.C6® cell platform compared
33 to the conventional Vero cell platform. The suitability of the suspension cell platform for propagation of
34 rationally-attenuated poliovirus strains (stabilized Sabin type 3 S19 derivatives and genetically
35 attenuated and stabilized MonoCre(X) strains), was also assessed. Yields were quantified by
36 infectious titer determination and D-antigen ELISA using either serotype-specific polyclonal rabbit
37 sera for Sabin strains or monoclonal cIPV-strain-specific antibodies for cIPV, S19 and MonoCre(X)
38 strains.

39 **Results:** PER.C6® cells supported the replication of Sabin strains to yields of infectious titers that
40 were in the range of cIPV strains at 32.5°C. Sabin strains achieved 30-fold higher yields ($p < 0.0001$)
41 on the PER.C6® cell platform as compared to the Vero cell platform in infectious titer and D-antigen
42 content. Furthermore, Sabin strain productivity on the PER.C6® cell platform was maintained at 10L
43 scale. Yields of infectious titers of S19 and MonoCre(X) strains were 0.5-1 $\log_{10}$ lower than seen for
44 cIPV strains, whereas D-antigen yield and productivities in doses/ml using rationally-attenuated
45 strains were in line with yields reported for cIPV strains.

46 **Conclusions:** Sabin and rationally-attenuated polioviruses can be grown to high infectious titers and
47 D-antigen yields. Sabin strain infection shows increased productivity on the PER.C6® cell platform as
48 compared to the conventional Vero cell platform. Novel cell platforms with the potential for higher
49 yields could contribute to increased affordability of a next generation of IPV vaccines needed for
50 achieving and maintaining poliovirus eradication.

51 **Introduction:**

52 Since 1988, the remarkable efforts of the Global Poliomyelitis Eradication Initiative (GPEI)[1] have
53 rendered the eradication status of poliomyelitis tangibly close. However, the two trivalent vaccines that
54 have brought the combat against poliovirus (PV) this far; conventional Inactivated Poliovirus Vaccine
55 (cIPV) based on Salk strains and Oral Poliovirus Vaccine (OPV) based on Sabin strains [2, 3], are
56 considered unsuitable for achieving and maintaining poliomyelitis eradication.

57 The three live attenuated (Sabin) strains in OPV may revert to neurovirulent forms and cause Vaccine
58 Associated Paralytic Poliomyelitis (VAPP) in vaccinees or their contacts [4]. Furthermore, the Sabin
59 strains can evolve into transmissible viruses, causing outbreaks of paralytic disease [5]. Accordingly,
60 withdrawal of OPV has been recognized and is envisioned to start as early as 2016 [6].

61 cIPV is made up of formalin-inactivated, wild-type strains, which by definition cannot revert to
62 neurovirulence or cause VAPP in recipients [7, 8]. cIPV implementation is constrained in developing
63 countries with respect to availability and price [9, 10]. Furthermore, cIPV manufacturing is currently
64 limited to regions of high hygienic standards and vaccine coverage due to the bio-safety risks
65 associated to the handling of large volumes of highly concentrated, neurovirulent PVs. Escape of
66 these viruses from a manufacturing facility could cause poliomyelitis outbreaks in areas of low/no
67 immunization. Consequently, development of Next Generation IPV based on attenuated strains,
68 particularly on the Sabin strains, is actively encouraged by the World Health Organization and its
69 collaborators [11]. Recently, Sabin-based IPV (sIPV) have been licensed in Japan and China, with
70 many more in development [12-15].

71 In parallel, novel, rationally-attenuated PV strains could also replace the neurovirulent Salk strains in
72 cIPV. Potential strengths of rationally-attenuated strains lie in their increased attenuation and genetic
73 stability, which would reduce the risk of virus re-introduction into the population and potential
74 outbreaks of poliomyelitis upon escape from a manufacturing facility. In addition, these novel strains
75 contain the capsid sequences of the wild-type cIPV strains. cIPV has been used successfully in the
76 field since the 1950s; international reference standards are available and the vaccine's dosing
77 strategy and immunogenicity is well defined. Therefore, mimicking the immunogenic profile of cIPV
78 strains may facilitate clinical development.

79 To address the issues of affordability and bio-safety surrounding cIPV, we studied the suitability of the
80 PER.C6® cell platform [16] for production of attenuated PV strains. The PER.C6® cell line, derived

81 from primary human retinal cells, has the capacity to grow in suspension to high cell density, without
82 the addition of serum or micro-carriers. We have previously demonstrated the susceptibility of the
83 PER.C6® cell line to cIPV strains, where significantly increased infectious titers and D-antigen yields
84 were obtained in comparison to the conventional Vero cell platform, using infection conditions that
85 mimicked IPV production [17].

86 The work herein evaluates Sabin strain productivity on this suspension cell platform at laboratory
87 scale and in 10L bioreactors. We directly compare Sabin productivity on the PER.C6® versus the Vero
88 cell platform, the only platform currently used by sIPV manufacturers. Finally, we also test the
89 suitability of the cell culture platform for propagation of rationally-attenuated strains, namely the S19
90 strains (stabilized Sabin type 3 derivatives) and MonoCre(X) strains (attenuated and genetically
91 stabilized cIPV strains), developed at the National Institute for Biological Standards and Control
92 (NIBSC) and Stony Brook University, respectively.

93

94

95 **Material and Methods:**

96 **Viruses:**

97 Sabin 1, 2 and 3 virus stocks were generated by performing two passages of Sabin 1, 2 and 3 viruses
98 purchased at NIBSC (cat#01/528, 01/130 and 01/532, respectively) in adherent Vero cells at 32.5°C.

99 cIPV vaccine strains (Mahoney, MEF-1 and Saukett) and rationally-attenuated (S19, MonoCre and
100 MonoCreX) virus stocks were generated by transfection of synthetically derived PV genomic RNA, as
101 described previously [18, 19]. Viruses were scaled up in PER.C6® cells and all stocks were
102 sequenced to confirm identity and the presence of attenuating mutations.

103 S19 strains are based on Sabin 3 genomic sequences where domain V of the internal ribosome entry
104 site (IRES) has been genetically modified to negate reversion of the known Sabin 3 attenuating
105 mutation (nt 472), and further mutations have been incorporated to decrease thermostability and
106 increase attenuation [20]. This was achieved by exchanging strong G-C base pairs for weaker A-U.
107 Reversion is highly unlikely as viruses are required to undergo multiple reversions with an unfavorable
108 intermediate G-U bond. The capsid sequence has been replaced with the corresponding individual
109 cIPV capsids resulting in three derivative strains with the S19 modified IRES [21, 22].

110 MonoCre strains are genetically modified cIPV strains where the cis-acting replication element(cre),
111 has been inactivated by multiple point mutations and a new functioning cre has been inserted into the
112 spacer region in between the IRES and clover leaf in the 5'UTR [23]. The positioning of the cre
113 element induces attenuation, whilst deletion of the newly inserted cre from the 5'UTR is highly unlikely
114 as the cre element is a pre-requisite for virus viability [24].

115 The MonoCreX strains are identical to the MonoCre stains, except in addition, one third of the capsid
116 region is deoptimized for codon pair bias [25]. This strategy retains the protein sequence, as only
117 synonymous mutations are incorporated. The many silent mutations result in neighboring of
118 unpreferred codon pairs which further increases virus attenuation and decreases risk of reversion due
119 to the sheer number of inserted mutations.

120 Both S19 and MonoCre(X) derivatives display high attenuation in transgenic mouse models as well as
121 genetic stability of inserted mutations, whilst cIPV capsid sequences are retained ([20, 21, 24, 25],
122 unpublished data).

Cell culture:

Culture and seeding of small scale experiments (10-20ml) with suspension PER.C6® cells (1.0×10^7 cells/ml) and Vero cells ($\sim 1 \times 10^6$ cells/ml) on micro-carriers were performed as described previously[17]. Adherently grown Vero cells (for virus titer determination) were maintained in Minimal Essential Medium (MEM, Life Technologies) supplemented with 5% FBS (Life Technologies) in T175 culture flasks at 37°C and 10% CO₂.

For manufacturing scale in 10L bioreactors, cell culture and media composition was identical to prior work[17] but at larger scale. Pre-culture was performed in cultibags and scaled up to 10L bioreactors. Prior to infection, cells were diluted in Permexcis medium to a cell density of 1.25×10^7 viable cells/ml.

Assays:

Infectious titer determination was performed by TCID₅₀ (Tissue Culture Infectious Dose 50%) on Vero cells for the cIPV, Sabin and MonoCre(X) strains, as described[17] and on PER.C6® cells for the S19 strains. Briefly, Vero or PER.C6® cells were seeded in 96-multi-well plates with 1.25×10^4 or 6.5×10^4 cells/well, respectively. The next day, eleven serial 5-fold dilutions of the virus sample, using 4 or 8 replicates, were prepared in Vero medium (MEM supplemented with 5% FBS) or PER.C6® culture medium (DMEM with 10% FBS and 10mM MgCl₂) were added to the cells. Plates were incubated at 32.5–33°C (attenuated strains) or 35–37°C (cIPV strains), for 13 days. The end-point titer was calculated by method of Spearman and Kärber[26].

D-antigen content of the S19 and MonoCre(X) strains was determined using a D-antigen ELISA typically used for the quantification of cIPV D-antigen content, performed by Bilthoven Biologicals [17]. This protocol utilizes cIPV-specific monoclonal detection antibodies specific to one native epitope, for quantification of D-antigen content.

Sabin strain detection was performed using serotype-specific polyclonal pooled rabbit sera for capture and detection of D-antigen units. Briefly, 96-multi-well plates were coated with type-specific capture antibodies by incubation for 16-24 hours at 2-8°C. Plates were then incubated for 60mins at 37°C with blocking buffer (1% BSA and 2% normal rabbit serum (Tebu-bio BV)). Virus and reference standard (cIPV) were diluted in 8 serial 2-fold dilutions in blocking buffer and incubated for 90min. Detection was performed with type-specific polyclonal antibodies in Blocking buffer, and incubated for 60min at

152 37°C. ExtrAvidin-Peroxidase conjugate (Sigma) was added for 30min. After each step plates were
153 washed 4x with Wash Buffer (PBS pH 7.4 containing 0.05% Tween-20 (Calbiochem). TMB substrate
154 (Sigma) was used to determine D-antigen concentrations in reference to the standard curve,
155 measured at optical density (OD) 450nm with a reference OD of 639nm in a spectrophotometer.

156 **Statistical analysis:**

157 PER.C6® versus Vero productivity was assessed using a two-way ANOVA model with Sabin strain,
158 cell line and their interaction as fixed factors. Statistical analyses were performed using SAS version
159 9.4. Statistical significance level was set at 5%.

160

Results:

Replication kinetics of Sabin strains are comparable to cIPV strains in PER.C6® cells at 32.5°C.

To determine susceptibility of PER.C6® cells to infection by Sabin strains, single-step growth curves were determined at Multiplicity of Infection (MOI)=2 and 32.5°C, a production temperature for Sabin strains recommended for OPV manufacturing, used successfully by Thomassen *et al* [27]. Replication kinetics were compared to the cIPV strains at the same infection temperature (32.5°C, Figure 1). All three Sabin viruses showed growth curves comparable to cIPV strains with respect to kinetics and maximum titers reached. The maximum titers reached for Sabin strains at 32.5°C were between 9.5–10 Log₁₀TCID₅₀/ml, which is slightly lower (0–0.5 Log₁₀TCID₅₀/ml) than those obtained by cIPV strains at their production temperature (35°C, data not shown and [17]).

Sabin strain infection results in higher infectious titer and D-antigen yields on the PER.C6® cell platform as compared to the Vero cell platform

A direct comparison of the Sabin productivity yields of the PER.C6® and Vero cell platforms at small scale (20 ml) was performed, as previously performed with cIPV strains [17]. The different culture conditions used per cell line represented envisioned production conditions for each cell line for a direct comparison of production platforms. Vero cell cultivation mimicked as closely as possible cIPV production as described in literature [28–31]. When certain parameters were unknown, conditions were optimized (shaking speed, cell seeding density, micro-carrier type and time post seeding prior to inoculation) to obtain the highest cell density for infection. Three independent infections (n=3, of which two were tested for D-antigen content) were performed in suspension PER.C6® cells (1.0 x10⁷ cells/ml) and Vero cells on micro-carriers (1.11, 1.04 and 1.46x10⁶ cells/ml for n=1, 2 and 3, respectively, which corresponds to the maximum cell density reached during pre-culture).

Infections were performed using MOI=1, incubated at 32.5°C and harvested at 24 hours post infection (hpi) for PER.C6® cells (shown by replication kinetics, Figure 1, to be sufficient time to reach the plateau titer), and at full cytopathic effect for Vero cells (between 24–48hpi).

Sabin strains reached higher yields in PER.C6® cells as compared to Vero cells in terms of infectious titer (30-fold increase, p<0.0001) and D-antigen content. Titers obtained in PER.C6® cells ranged between 9.3–10.1 Log₁₀TCID₅₀/ml whilst the titers reached in Vero cells were between 7.9–8.5

Log₁₀TCID₅₀/ml (Figure 2A). Similarly, on a per cell basis, there was a 3.6-fold increase in infectious units produced per cell (TCID₅₀/cell, Figure 2C) in PER.C6® cells as compared to Vero cells (p<0.0001).

D-antigen units (DU) per milliliter (Figure 2B) was also 30-fold higher in PER.C6® cells than in Vero cells (p<0.0001). Average D-antigen yields were 1396, 184 and 1554 DU/ml for Sabin 1, 2 and 3 in PER.C6® cells as compared to 84, 5 and 37 DU/ml in Vero cells. On a per cell basis (Figure 2D) D-antigen yields were 3.7-fold higher on PER.C6® cells as compared to Vero cells (p=0.0004). The lower absolute D-antigen yields for serotype 2 as compared to types 1 and 3 are caused by the intrinsic nature of the virus strains and antibodies used per serotype-specific assay[32].

Sabin 1, 2 and 3 infections in PER.C6® cells can be scaled up to 10L bioreactor scale whilst maintaining productivity

To evaluate the productivity of the Sabin strains on the PER.C6® cell platform at larger scale, 10L bioreactors were infected with MOI=1 and incubated at 32.5°C with a cell density of 12.5x10⁶cells/ml. Harvests taken at 48hpi and were quantified for D-antigen content and infectious titers (Table 1). The infectious titers and D-antigen yields were in line with yields obtained as small scale (Figures 1 and 2). Infectious titers in 10L Bioreactors were between 9.2–9.9 Log₁₀ TCID₅₀/ml, and D-antigen yields were approximately 1500 DU/ml for Sabin 1 and 3, and 200 DU/ml for Sabin 2 (Table 1).

Rationally attenuated strains reach infectious titers comparable to Sabin strains and D-antigen yields in the range of cIPV strains on the PER.C6® cell culture platform.

Susceptibility and productivity of PER.C6® cells to infection with rationally-attenuated strains (S19 and MonoCre(X)'s) was examined (Table 2) at their optimal growth temperature (33°C). Strain-specific titration conditions (temperature, cell line) that provided maximum sensitivity of the titration assay were used. For the S19 strains titration on Vero cells versus titration of the same virus sample on PER.C6® cells resulted in up to 1 log₁₀ lower end-point titers (data not shown). All attenuated strains showed some degree of temperature sensitivity, therefore 33°C was used for the titration incubation temperature.

The infectious titers obtained for rationally-attenuated strains in PER.C6® cells ranged between 9.2–9.9 log₁₀ TCID₅₀/ml, which is approximately 0.5 log lower than titers obtained with the cIPV strains at

217 production conditions (35°C), but comparable to what was observed for the Sabin strains (Figures 1-2,
218 Table 1). Reported infectious titers of the S19 strains were approximately 7.7 Log₁₀TCID₅₀/ml in Vero
219 cells [21] and the MonoCre(X) strains yielded between 7.5 – 8.3 Plaque Forming Units (Log₁₀PFU)/ml
220 in Hela cells[24].

221

222 Variation in D-antigen content was considerable, especially for serotypes 1 and 3. However, yields for
223 the rationally-attenuated strains on PER.C6® cells were in the range obtained with cIPV strains.

224 Productivities in doses/ml (harvest based on cIPV dosing of 40:8:32 DU/dose for types 1, 2 and 3,
225 respectively, and an assumed overall recovery of 40% (as previously described [31, 33, 34]) are in
226 line with the yields with cIPV strains, and as observed with cIPV strains at 2L bioreactor scale
227 [17](Table 2).

228 **Discussion:**

229 To achieve and maintain poliomyelitis eradication, vaccination may have to continue for many years,
230 and perhaps even indefinitely [35, 36]. OPV use must cease, and due to the biosafety risks
231 associated with the use of cIPV, an IPV based on attenuated strains is preferred. The recent
232 accidental escape of wild-type PV from a manufacturing facility in Belgium [37] underlines this risk. To
233 adhere to the WHO goal for the manufacture of high capacity, low cost, immunogenic, IPV based on
234 attenuated strains, our aim was to study the suitability of a suspension cell culture platform for the
235 high yield production of such alternative vaccine strains.

236 Here, we established that PER.C6® cells are susceptible to Sabin strain infection at 32.5°C with
237 growth kinetics and maximum titers close to those obtained by cIPV strains. Moreover, we
238 demonstrated an increased productivity of Sabin strains on the PER.C6® cell platform with respect to
239 infectious titer and D-antigen yields, when directly compared to the Vero cell platform at small scale.
240 Although all effort was made to create optimal conditions for the Vero cell line, we cannot exclude that
241 the titers and D-antigen values obtained are sub-optimal. Nonetheless, yields obtained on the Vero
242 cell platform are similar to the titers described in literature [27]. Furthermore, a similar increase in
243 productivity was observed during a direct comparison of Vero and PER.C6® cell platforms using cIPV
244 strains [17]. The higher productivity of the suspension cell culture platform is mostly (but not only)
245 explained by the 10-fold higher cell density achieved with PER.C6® cell cultures as compared to Vero,
246 where the necessity for micro-carriers limits maximum cell densities. A higher Sabin productivity on
247 PER.C6® versus Vero cells was also observed on a per cell basis, which we had also previously
248 identified for the cIPV strains [17].

249 Production of Sabin strains was scaled up to 10L bioreactors whilst maintaining high productivity. At
250 this scale PER.C6® culture harvest averages of 9.9, 9.2 and 9.5 Log₁₀TCID₅₀/ml and 1618, 216 and
251 1550 DU/ml were obtained for Sabin 1, 2 and 3, respectively. By contrast, at 350L manufacturing
252 scale, Vero culture harvests produce 8.7, 7.8, and 8.4 Log₁₀TCID₅₀/ml and 120, 25 and 50 DU/ml for
253 types 1, 2 and 3 respectively [27]. Although the different assays used for D-antigen quantification do
254 not allow a direct comparison with published data, our yields obtained on Vero cells (84, 5 and 37
255 DU/ml) are in the range of those previously reported for the Vero cell platform [27]. Taken together;
256 these data suggest more vaccine doses could be produced per volumetric unit of PER.C6® culture

harvest as compared to Vero culture harvest, assuming similar recoveries after purification and inactivation for the two cell platforms. IPV production using the conventional Vero cell line utilizes an established and validated purification and inactivation process which has already been devised and explored (albeit for cIPV strains) since the 1980s [30]. Such an optimized process is yet to be put in place for PER.C6® derived poliovirus harvests. Nonetheless, preliminary data showed PER.C6®-based sIPV to be immunogenic in the Wistar Rat Potency model for *in vivo* immunogenicity after applying a purification and inactivation process for cIPV strains, a non-optimized process for Sabin strains (data not shown). The recoveries after purification and inactivation of PER.C6®-derived Sabin bioreactor harvests were acceptable and in line with those published by Thomassen *et al* [27]. Currently, work is ongoing, supported by the Bill and Melinda Gates Foundation, to show proof-of-concept in clinical trials of PER.C6®-cell-based sIPV and to further optimize the production process to achieve significantly reduced Cost of Goods.

Due to the intrinsic differences in antigenic profiles of Sabin and cIPV viruses, the dosing strategy of a sIPV vaccine is complicated by the fact that each manufacturer utilizes different D-antigen ELISA's without an available international reference standard, which impedes standardization of sIPV dosing. Rationally-attenuated strains which contain identical cIPV capsid sequences, and thus identical antigenicity, may be preferred, as they allow use of cIPV dosing and reference standards. Replication of such rationally designed attenuated strains was supported by the PER.C6® cell platform, achieving at their envisioned production temperature (33°C), maximum infectious titers in the range of Sabin strains, and close to cIPV strains. The high productivity of the rationally-attenuated strains demonstrated here, may further increase the affordability of the vaccine as compared to sIPV's, which typically require more DU per vaccine dose of serotypes 2 and 3 [14, 32, 38]. However, these novel strains require extensive further characterization as the regulatory requirements for their acceptance and path to market authorization are not yet clearly defined. Nonetheless, our data promises synergy between genetically stable attenuated strains and suspension cell lines, such as the PER.C6® cell platform, for the production of an affordable and safe IPV.

Introduction of a novel cell line for IPV manufacturing requires extensive characterization to ensure that the new platform produces a vaccine with comparable clinical safety and efficacy to the currently marketed Vero-based cIPV. Exchanging the established Vero cell platform therefore entails the

addressing of many regulatory hurdles, which has presumably limited exploration of alternative cell lines for IPV production. However, promising results have been obtained using Sabin 1 for the screening of hematopoietic cell lines to serve as novel viral vaccine substrates [39]. Using a suspension cell line may reduce the cost of goods as volumetric productivity can be significantly increased whilst avoiding the use of expensive ingredients such as serum or micro-carriers, as is the case for the PER.C6® cell platform. Furthermore, the use of a novel suspension cell line can be combined with other strategies the WHO has set in place to increase affordability of IPV [40] (i.e. alterations in dose scheduling, dose fractionation, routes of administration, and adjuvant addition). Ultimately, the use of novel suspension cell lines, such as the PER.C6® cell platform, capable of propagating multiple attenuated polioviruses to high yields, could lead to the generation of an affordable next generation IPV to support the eradication endgame.

298 **Acknowledgements**

299 We would like to thank all our colleagues for their significant contributions to the work presented here:
300 Natasa Papic, Angel Huidobro, Marloes Naarding, Annemiek Verwilligen, Aleksandra Kozyczkowska,
301 Dalinda Sánchez Vidaña, Andreas Freidig, Tiziano de Rosa, Maarten Pennings, Tobias Kamphuis,
302 Jeroen Tolboom, Maarten Santman, Richard Verhage, and Annemieke Manten.

303 We would like to give special thanks to Joanne Wolter for critical reading and reviewing of the
304 manuscript.

305 We also thank Batavia Bioservices for their services in performing the small scale Vero cell infections,
306 as well as Bilthoven Biologicals for performance of the Salk specific D-antigen ELISA's.

307 **Funding**

308 Costs associated with development of this manuscript were mainly funded by Janssen,
309 Pharmaceutical companies of Johnson & Johnson. Part of the costs associated with performance of
310 the 10L bioreactor runs were funded by the Bill and Melinda Gates Foundation grant #OPP1084240.

311

312 References:

- 313 [1] GPEI. History of Polio: <http://www.polioeradication.org/Polioandprevention/Historyofpolio.aspx>. 2015.
- 314 [2] Sabin AB. Properties and behavior of orally administered attenuated poliovirus vaccine. *JAMA*.
- 315 1957;164(11):1216-23.
- 316 [3] Salk JE, Krech U, Youngner JS, Bennett BL, Lewis LJ, Bazeley PL. Formaldehyde treatment and safety
- 317 testing of experimental poliomyelitis vaccines. *Am J Public Health Nations Health*. 1954;44:563-70.
- 318 [4] Pliaka V, Kyriakopoulou Z, Markoulatos P. Risks associated with the use of live-attenuated vaccine poliovirus
- 319 strains and the strategies for control and eradication of paralytic poliomyelitis. *Expert Rev Vaccines*. 2012;11:609-
- 320 28.
- 321 [5] Minor P. Vaccine-derived poliovirus (VDPV): Impact on poliomyelitis eradication. *Vaccine*. 2009;27:2649-52.
- 322 [6] WHO. Polio Eradication & Endgame Strategic Plan 2013-2018. Available at:
- 323 http://www.polioeradication.org/Portals/0/Document/Resources/StrategyWork/PEESP_EN_US.pdf. 2013.
- 324 [7] John TJ. Anomalous observations on IPV and OPV vaccination. *Dev Biol (Basel)*. 2001;105:197-208.
- 325 [8] Robertson SE, Traverso HP, Drucker JA, Rovira EZ, Fabre-Teste B, Sow A, et al. Clinical efficacy of a new,
- 326 enhanced-potency, inactivated poliovirus vaccine. *Lancet*. 1988;1:897-9.
- 327 [9] Heinsbroek E, Ruitenberg EJ. The global introduction of inactivated polio vaccine can circumvent the oral
- 328 polio vaccine paradox. *Vaccine*. 2010;28:3778-83.
- 329 [10] Zehrung D. Improving the affordability of inactivated poliovirus vaccines (IPV) for use in low- and middle-
- 330 income countries. An economic analysis of strategies to reduce the cost of routine IPV immunization. Seattle:
- 331 PATH; 2010.
- 332 [11] WHO. Recommendations for the production and control of poliomyelitis vaccine (inactivated). Technical
- 333 Report Series No 910 World Health Organization. Geneva2002.
- 334 [12] Bakker WA, Thomassen YE, van't Oever AG, Westdijk J, van Oijen MG, Sundermann LC, et al. Inactivated
- 335 polio vaccine development for technology transfer using attenuated Sabin poliovirus strains to shift from Salk-IPV
- 336 to Sabin-IPV. *Vaccine*. 2011;29:7188-96.
- 337 [13] Shimizu H. Development and introduction of inactivated poliovirus vaccines derived from Sabin strains in
- 338 Japan. *Vaccine*. 2014.
- 339 [14] Sun M, Ma Y, Xu Y, Yang H, Shi L, Che Y, et al. Dynamic profiles of neutralizing antibody responses elicited
- 340 in rhesus monkeys immunized with a combined tetravalent DTaP-Sabin IPV candidate vaccine. *Vaccine*.
- 341 2014;32:1100-6.
- 342 [15] Liao G, Li R, Li C, Sun M, Li Y, Chu J, et al. Safety and immunogenicity of inactivated poliovirus vaccine
- 343 made from Sabin strains: a phase II, randomized, positive-controlled trial. *J Infect Dis*. 2012;205:237-43.
- 344 [16] Fallaux FJ, Bout A, van der Velde I, van den Wollenberg DJ, Hehir KM, Keegan J, et al. New helper cells and
- 345 matched early region 1-deleted adenovirus vectors prevent generation of replication-competent adenoviruses.
- 346 *Hum Gene Ther*. 1998;9:1909-17.
- 347 [17] Sanders BP, Edo-Matas D, Custers JH, Koldijk MH, Klaren V, Turk M, et al. PER.C6((R)) cells as a serum-
- 348 free suspension cell platform for the production of high titer poliovirus: a potential low cost of goods option for
- 349 world supply of inactivated poliovirus vaccine. *Vaccine*. 2013;31:850-6.
- 350 [18] van der Werf S, Bradley J, Wimmer E, Studier FW, Dunn JJ. Synthesis of infectious poliovirus RNA by
- 351 purified T7 RNA polymerase. *Proc Natl Acad Sci U S A*. 1986;83:2330-4.
- 352 [19] Sanders BP, Liu Y, Brandjes A, van Hoek V, de Los Rios Oakes I, Lewis J, et al. Brunenders: A partially
- 353 attenuated historic poliovirus Type I vaccine strain. *J Gen Virol*. 2015.
- 354 [20] Macadam AJ, Ferguson G, Stone DM, Meredith J, Knowlson S, Auda G, et al. Rational design of genetically
- 355 stable, live-attenuated poliovirus vaccines of all three serotypes: relevance to poliomyelitis eradication. *Journal of*
- 356 *virology*. 2006;80:8653-63.
- 357 [21] Macadam A. Inactivated Poliovaccine. International Patent Number: WO 2012/090000 A1. United
- 358 Kingdom2012.
- 359 [22] Minor PD. The polio-eradication programme and issues of the end game. *J Gen Virol*. 2012;93:457-74.
- 360 [23] Yin J, Paul AV, Wimmer E, Rieder E. Functional dissection of a poliovirus cis-acting replication element [PV-
- 361 cre(2C)]: analysis of single- and dual-cre viral genomes and proteins that bind specifically to PV-cre RNA. *Journal*
- 362 *of virology*. 2003;77:5152-66.
- 363 [24] Toyoda H, Yin J, Mueller S, Wimmer E, Cello J. Oncolytic treatment and cure of neuroblastoma by a novel
- 364 attenuated poliovirus in a novel poliovirus-susceptible animal model. *Cancer research*. 2007;67:2857-64.
- 365 [25] Coleman JR, Papamichail D, Skiena S, Fletcher B, Wimmer E, Mueller S. Virus attenuation by genome-scale
- 366 changes in codon pair bias. *Science*. 2008;320:1784-7.
- 367 [26] Hamilton M, A.; Russo, R., C.; Thurston, R., V. Trimmed Spearman-Kärber Method for Estimating Median
- 368 Lethal Concentration in Toxicity Bioassays. *Environmental Science and Technology*. 1977;11:714-9.
- 369 [27] Thomassen YE, van 't Oever AG, van Oijen MG, Wijffels RH, van der Pol LA, Bakker WA. Next generation
- 370 inactivated polio vaccine manufacturing to support post polio-eradication biosafety goals. *PloS one*.
- 371 2013;8:e83374.
- 372 [28] Duchene M, Peetermans J, D'Hondt E, Harford N, Fabry L, Stephenne J. Production of poliovirus vaccines:
- 373 past, present, and future. *Viral Immunol*. 1990;3:243-72.
- 374 [29] Montagnon BJ, Fanget B, Nicolas AJ. The large-scale cultivation of VERO cells in micro-carrier culture for
- 375 virus vaccine production. Preliminary results for killed poliovirus vaccine. *Dev Biol Stand*. 1981;47:55-64.

[30] van Wezel AL, van Herwaarden JA, van de Heuvel-de Rijk EW. Large-scale concentration and purification of virus suspension from microcarrier culture for the preparation of inactivated virus vaccines. *Dev Biol Stand.* 1979;42:65-9.

[31] van Wezel AL, van Steenis G, van der Marel P, Osterhaus AD. Inactivated poliovirus vaccine: current production methods and new developments. *Rev Infect Dis.* 1984;6 Suppl 2:S335-40.

[32] Westdijk J, Brugmans D, Martin J, van't Oever A, Bakker WA, Levels L, et al. Characterization and standardization of Sabin based inactivated polio vaccine: proposal for a new antigen unit for inactivated polio vaccines. *Vaccine.* 2011;29:3390-7.

[33] Kreeftenberg H, van der Velden T, Kersten G, van der Heuvel N, de Bruijn M. Technology transfer of Sabin-IPV to new developing country markets. *Biologicals.* 2006;34:155-8.

[34] Thomassen YE, van 't Oever AG, Vinke M, Spiekstra A, Wijffels RH, van der Pol LA, et al. Scale-down of the inactivated polio vaccine production process. *Biotechnol Bioeng.* 2013;110:1354-65.

[35] Chumakov K, Ehrenfeld E, Wimmer E, Agol VI. Vaccination against polio should not be stopped. *Nat Rev Microbiol.* 2007;5:952-8.

[36] Thompson KM, Kalkowska DA, Duintjer Tebbens RJ. Managing population immunity to reduce or eliminate the risks of circulation following the importation of polioviruses. *Vaccine.* 2015.

[37] ECDC. The accidental release of 45 litres of concentrated live polio virus solution into the environment - Belgium Monitoring current threats: ECDC Communicable Disease Threats Report (CDTR), week 37/2014: European Centre for Disease Prevention and Control; 2014.

[38] Okada K, Miyazaki C, Kino Y, Ozaki T, Hirose M, Ueda K. Phase II and III Clinical Studies of Diphtheria-Tetanus-Acellular Pertussis Vaccine Containing Inactivated Polio Vaccine Derived from Sabin Strains (DTaP-sIPV). *J Infect Dis.* 2013;208:275-83.

[39] Oosterhoff D, van de Weerd G, van Eikenhorst G, de Gruijl TD, van der Pol LA, Bakker WA. Hematopoietic cancer cell lines can support replication of Sabin poliovirus type 1. *BioMed research international.* 2015;2015:358462.

[40] Okayasu H, Sutter RW, Jafari HS, Takane M, Aylward RB. Affordable inactivated poliovirus vaccine: strategies and progress. *J Infect Dis.* 2014;210 Suppl 1:S459-64.

Figure 1

Figure 2

Table 1:

10L Bioreactor yields	Average Titer $\pm$ SD (Log ₁₀ TCID ₅₀ /ml)	Average D-antigen content $\pm$ SD (DU/ml)
Sabin 1	9.9 $\pm$ 0.1	1618 $\pm$ 69
Sabin 2	9.2 $\pm$ 0.0	216 $\pm$ 18
Sabin 3	9.5 $\pm$ 0.1	1550 $\pm$ 36

Yields in terms of infectious titer (Log₁₀TCID₅₀/ml) and D-antigen content (DU/ml) for Sabin 1, 2 and 3 at 10L Bioreactor scale. Data represent an average of two (n=2) independent 10L bioreactor production runs inoculated with an MOI=1, at 32.5°C with a cell density of infection of 12.5x10⁶ cell/ml. MOI: Multiplicity of Infection expressed in TCID₅₀/cell.

Table 2:

Infectious Titer (Log ₁₀ TCID ₅₀ /ml)	Type I		N	Type II		N	Type III		N
	Average± SD			Average± SD			Average± SD		
S19 ^a	9.6 ± 0.23		3	9.2 ± 0,05		3	9.4 ± 0.13		3
MonoCre ^b	9.5 ± 0.16		3	9.9 ± 0,00		2	9.4 ± 0.00		2
MonoCre-X ^b	9.5 ± 0.09		3	9.7 ± 0,05		3	9.3 ± 0.27		3
cIPV ^b	10.2 ± 0.01		3	10.0 ± 0.06		3	9.7 ± 0.13		2

D-antigen Content	Type I			Type II			Type III		
	Average ± SD DU/ml	Average Doses/ml _c	N	Average± SD DU/ml	Average Doses/ml _c	N	Average± SD DU/ml	Average Doses/ml _c	N
S19	3211±274	32	3	584±75	29	3	1465±213	18	3
MonoCre	4577±494	46	2	507±1	25	2	973±98	12	2
MonoCre(X)	4100±504	41	3	989±55	49	3	1643±438	21	3
cIPV	3366±436	34	3	518±41	26	3	1602±379	20	2

Infectious titers (Log₁₀TCID₅₀/ml), D-antigen content (DU/ml), and Average Doses per milliliter for each of the rationally attenuated strains (S19, MonoCre and MonoCreX) as compared to cIPV strains after infection of high density suspension PER.C6® cells. Cells were infected at a cell density of 10⁷ viable cells per ml with an MOI of 1 – 2 and incubated at 33°C (attenuated strains) and 35°C (cIPV strains) and harvested at 24 – 48 hours post infection.

Titration were performed at 33°C for the attenuated strains and at 37°C for cIPV strains.

^a Titration on PER.C6® cells; ^b Titration on Vero cells. ^c Average doses per milliliter of crude harvest assuming a Down Stream Process (DSP) recovery of 40% and cIPV dosing of 40:8:32 DU/dose for types 1, 2 and 3, respectively. MOI: Multiplicity of Infection expressed in TCID₅₀/cell.