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1 **Generation of recombinant pestiviruses using a full-genome amplification**
2 **strategy**

3

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22 **Abstract**

23 Complete genome amplification of viral RNA provides a new tool for the
24 generation of modified viruses. We have recently reported a full-genome
25 amplification strategy for recovery of pestiviruses (Rasmussen et al., 2008). A
26 full-length cDNA amplicon corresponding to the Border disease virus-Gifhorn
27 genome was generated by long RT-PCR and then RNA transcripts derived from
28 this amplicon were used to rescue infectious virus. Here, we have now used this
29 full-genome amplification strategy for efficient and robust amplification of three
30 additional pestivirus strains: the vaccine strain C and the virulent Paderborn
31 strain of Classical swine fever virus plus the CP7 strain of Bovine viral diarrhoea
32 virus. The amplicons were cloned directly into a stable single-copy bacterial
33 artificial chromosome generating full-length pestivirus DNAs from which
34 infectious RNA transcripts could be also derived.

35

36 Keywords: *Pestivirus*, *Flaviviridae*, long RT-PCR, full-genome amplicon,
37 infectious clone, pBeloBAC11

38

39 **Introduction**

40 The genus *Pestivirus* is part of the family *Flaviviridae* and contains economically
41 important animal viruses such as Classical swine fever virus (CSFV), Bovine
42 viral diarrhoea virus (BVDV) and Border disease virus (BDV). Pestivirus virions
43 are enveloped and contain a positive-stranded RNA genome of about 12.3 kb
44 which has a single long open reading frame flanked by non-translated regions
45 (5'NTR and 3'NTR), which are important for viral replication. Generation of
46 pestivirus cDNA clones is a general prerequisite for genetic manipulation of
47 their genomes, a process that can be both lengthy and laborious. Several
48 infectious cDNA clones containing complete pestivirus genomes have been
49 described (Meyers et al., 1996; Moormann et al., 1996; Ruggli et al., 1996;
50 Vassilev et al., 1997; Fan and Bird, 2008). These cDNA clones have provided
51 new options for directed genetic manipulations of pestiviruses. However,
52 construction of these infectious cDNA clones has been hampered due to the
53 relatively large size of the viral RNA genome and because of genetic instability
54 of the cloned cDNA in combination with the plasmid vectors in the bacterial
55 host. Therefore, new strategies are needed to facilitate construction of stable
56 infectious cDNA clones from a wider range of strains.

57 Generation of infectious cDNA clones can be facilitated by using
58 long RT-PCR for full-genome amplification. This approach has been applied
59 with success to a number of RNA viruses from the *Flaviviridae* (Tellier et al.,
60 1996; Gritsun and Gould, 1998; Zhang et al., 2001). For pestiviruses, the
61 methodology has been used to obtain the entire open reading frame for
62 sequencing studies (Jones et al., 2006) as well as for virus rescue (Rasmussen

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63 et al., 2008). Full-length genome amplification facilitates, in addition to virus
64 rescue, direct full-length sequence analysis and the amplicons can also be
65 directly inserted into suitable cloning vectors.

66 In the present study, we developed an optimized, efficient and
67 streamlined method for the robust amplification of full-length cDNAs of four
68 strains (BVDV-CP7, BDV-Gifhorn, CSFV-C and CSFV-Paderborn). This
69 procedure was followed by the insertion of the BDV and CSFV amplicons into
70 the stable single-copy vector pBeloBAC11 (Wang et al., 1997). This bacterial
71 artificial chromosome (BAC) has been shown to successfully maintain the full-
72 length cDNAs of Japanese encephalitis virus (Yun et al., 2003) and BVDV-SD1
73 (Fan and Bird, 2008). Using this procedure, we have generated new full-length
74 cDNA clones of BDV and CSFV, which enlarges the range of cloned pestivirus
75 genomes that can be manipulated.

76

77 **Materials & Methods**

78 The different viruses used in this study were; BVDV-CP7 from bovine KOP-R
79 cells (10^6 TCID₅₀/ml); CSFV-Paderborn from porcine PK15 cells (10^7 TCID₅₀/ml);
80 vaccine strain CSFV-C from porcine PK15 cells ($10^{4.75}$ TCID₅₀/ml); and BDV-
81 Gifhorn from ovine SFT cells (10^6 TCID₅₀/ml). Infected cells were grown in
82 Dulbecco's Modified Eagle Medium supplemented with 10% BVDV-free foetal
83 bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. The total
84 RNA was extracted from the cells using a combined Trizol/RNeasy protocol.
85 This protocol was used to obtain high-quality full-length genomic RNA. Briefly,
86 total RNA was extracted from 1 ml cell supernatant or cell lysate with 3 ml Trizol

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87 LS reagent (Invitrogen) and 0.8 ml chloroform. After phase separation the
88 aqueous phase was mixed with an equal volume of 75% ethanol and 0.7 ml of
89 the resulting mix was transferred sequentially to the same RNeasy column
90 (Qiagen). The column was washed according to the manufacturer's protocol.
91 Total RNA was eluted from the column with 4 times 30 μ l nuclease free water
92 and stored at -80°C until use. Pestivirus genomes were amplified from total
93 RNA preparations using long RT-PCR. It was found that the third RNA eluate
94 empirically performed the best in long RT-PCR, presumable due to a higher
95 proportion of full-length genomic RNA in this fraction.

96 The total RNA was reverse transcribed at 50°C for 90 minutes
97 using SuperScript III reverse transcriptase kit (Invitrogen) and specific cDNA
98 primers (Table 1). For improved long RT-PCR efficiency, the resulting cDNA
99 was subsequently treated with RNase H to remove the RNA template. Full-
100 length PCR amplification was performed using primers specific for the 5'NTR
101 and the 3'NTR of each strain (Table 1) with the Accuprime High Fidelity kit
102 (Invitrogen) which consists of a mixture of *Taq* and proofreading *Pyrococcus*
103 *GB-D* DNA polymerases. Reactions containing 2 μ l cDNA were then amplified
104 in a final volume of 50 μ l using 94°C for 30 seconds followed by 35 cycles of
105 94°C for 15 seconds, 65°C for 30 seconds and 68°C for 12 minutes. Samples
106 (1-10 μ l) of each PCR reaction were analysed on a 1 % agarose gel in TBE
107 buffer alongside a 1kb DNA ladder (Fermentas).

108 Standard techniques were used for cloning of the full-length
109 amplicons into pBeloBAC11 (Invitrogen). Briefly, pBeloBAC11 (7507 bp) was
110 digested with *NotI* and afterwards treated with Antarctic phosphatase (NEB).

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111 The full-length amplicons of about 12.3 kb were also digested with *NotI* and
112 purified using the Nucleotide removal kit (Qiagen). Equal amounts of vector and
113 amplicons were mixed and ligated in 25 μ l reactions overnight at 16°C using
114 DNA ligase for long fragments (Takara Bio) according to the manufacturer's
115 recommendations. The products (1 μ l) were electroporated into
116 electrocompetent DH10B T1 phage resistant *E.coli* (Invitrogen) and
117 transformants harbouring the cloned amplicons were isolated from selective LB
118 plates containing 12.5 μ g/ml chloramphenicol. Small scale preparations of DNA
119 from selected transformants were obtained using the BAC miniprep protocol
120 previously described (Warming et al., 2005) and the presence of the full-length
121 pestivirus cDNA was determined following restriction analysis using *NotI*.
122 Selected BAC DNAs were digested with *NotI*, *in vitro* transcribed using T7 RNA
123 polymerase and the RNA transcripts were tested for infectivity as previously
124 described (Reimann et al., 2004; Rasmussen et al., 2007). Immunostaining of
125 electroporated porcine SK6 cells (CCLV RIE262) was performed with the pan-
126 pestivirus antibody (C16) targeting the non-structural NS3 proteins (Reimann et
127 al., 2004).

128

129 **Results**

130 Full-length cDNAs corresponding to four different pestivirus strains were
131 successfully amplified in single long RT-PCRs using an optimized version of the
132 full-genome amplification strategy described by Rasmussen et al. (2008). The
133 original protocol was modified by substituting the Elongase enzyme mix with the
134 Accuprime High Fidelity kit. This polymerase mix, in combination with the

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135 Superscript III reverse transcriptase, efficiently produced, from each of the
136 different viral RNAs, amplicons of approximately 12.3 kb, corresponding to the
137 full-length genomes (Fig. 1). The Accuprime High Fidelity polymerase mix
138 increased the specificity and efficiency of the long RT-PCR compared to the
139 Elongase enzyme mix and produced distinct products of the expected size
140 without smears and aberrant amplification products (Fig. 1). High yields (several
141 micrograms) of DNA were obtained in each long RT-PCR. For amplification of
142 complete genomes in a single RT-PCR, specific primers were designed based
143 on nucleotide sequences of the terminal 5'NTR- and 3'NTR- genomic ends
144 (Table 1). The primers for amplification of BDV-Gifhorn were described in
145 Rasmussen et al. (2008). The primers for BVDV-CP7 (Meyers et al., 1996,
146 Accession number BVU63479), and for the CSFV-C (Accession number
147 AY259122) were based on published sequences. Similarly, the 5'NTR primer
148 for CSFV-Paderborn was designed from Accession number AY072924
149 (Oleksiewicz et al., 2003) but this sequence lacks approximately 75 nucleotides
150 from the terminus of the 3'NTR. Therefore, the 3'Paderborn reverse primer was
151 designed using a nucleotide alignment of known full-genome CSFV sequences.
152 Generally, knowledge of less than 50 nucleotides of the terminal sequences
153 from each of the strains was needed for successful amplification of the whole
154 genome.

155 By incorporating unique *NotI* restriction sites within the primers
156 (Table 1), the amplicons were designed for subsequent insertion into
157 pBeloBAC11. Full-length amplicons of the two strains CSFV-C and CSFV-
158 Paderborn, and also the BDV-Gifhorn were obtained using the respective *NotI*

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159 primers (Fig. 2). Amplification and cloning of BVDV-CP7 was not attempted by
160 this strategy due to an internal *NotI* site in the cDNA sequence.

161 The amplified genomes were digested with *NotI* and ligated directly
162 into the *NotI* site in pBeloBAC11. Electroporation of DH10B *E.coli* cells with the
163 ligated products yielded small and large bacterial colonies (from a few to
164 several hundreds) after incubation for 24 hours at 37°C. Both small and large
165 colonies were tested for the presence of full-genome amplicons. Generally, two
166 thirds of the tested transformants carried a BAC containing an insert of the
167 expected size of approximately 12.3 kb (Fig. 3). One third of the transformants
168 either carried BACs with smaller inserts, empty BACs or no visible BAC vector.
169 There was no correlation between the size of the BAC inserts and the size of
170 the bacterial colonies.

171 Testing of the BAC clones for their ability to generate virus
172 infectivity in cells is ongoing in our laboratories. The preliminary results of this
173 evaluation show that infectious pestiviruses can be rescued from the DNAs
174 generated by direct insertion of full-length genomes into the BAC vector. For
175 example, selected BAC clones harbouring the CSFV-Paderborn full-length
176 cDNA were *NotI* digested, *in vitro* transcribed and the RNA transcripts were
177 tested for infectivity by electroporation into porcine cells. RNA derived from one
178 out of two tested BAC clones (pBeloPader10) was shown to efficiently replicate
179 in porcine cells. The cells electroporated with RNA transcripts from
180 pBeloPader10 showed a high number of NS3-positive cells (as judged by
181 immunofluorescence, Fig. 4) 24 hours later and infectious virus could be
182 recovered after 1st and 2nd cell passages. In addition, RNA transcripts derived

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183 from three BAC clones harbouring BDV-Gifhorn full-length cDNA have been
184 tested for infectivity in ovine cells. Following electroporation, viral RNA derived
185 from each of these three BAC clones was found to be replicating in the cells
186 (data not shown). However, only rather few cells were scored as positive after
187 the 1st cell passage indicating a possible growth defect in these constructs.

188

189 **Discussion**

190 The use of the full-length genome amplification strategy for amplification of
191 pestivirus genomes has several advantages; (i) the same optimized protocol
192 can be used for amplification of a wide selection of strains; (ii) only limited
193 terminal nucleotide sequence information of a given strain is needed for
194 amplification of the whole genome; (iii) multiple cloning steps can be
195 circumvented since full-length amplicons can be directly *in vitro* transcribed or
196 inserted into plasmid vectors; and (iv) construction of genetically modified
197 viruses can be facilitated using stable cDNA clones.

198 In the present study, four different pestiviruses representing each of
199 three main types (CSFV, BVDV and BDV) were amplified by long RT-PCR. This
200 demonstrates that the recently described full-length genome amplification
201 strategy (Rasmussen et al., 2008) is of a generic nature since it can be utilized
202 for amplification of diverse pestivirus strains. Furthermore, the direct cloning of
203 the amplified genomes into the stable single-copy BAC vector, pBeloBAC11,
204 shows that we have created an efficient, streamlined strategy for direct
205 preparation of new pestivirus cDNA clones. Full-length cDNAs corresponding to
206 other members of the *Flaviviridae*, cloned in pBeloBAC11, have previously been

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207 shown to be stable after multiple generations in *E.coli* cells (Yun et al., 2003;
208 Fan and Bird, 2008) and we anticipate that our constructs will display similar
209 stability in the bacterial host but this does need to be determined.

210 Our new strategy allows construction of stable infectious BAC
211 DNAs from a single full-length PCR product, which gives an increased flexibility
212 in the design of new genetically modified pestiviruses. Since long RT-PCR
213 preserves the genetic variants present in a virus population (Chumakov, 1996)
214 many independent full-length cDNA clones can be obtained and tested which
215 increases the probability of finding infectious constructs. For targeted design of
216 genetically modified pestiviruses, the efforts can now be expedited and focused
217 on, in principal, any pestiviral strain and is hence not limited by the availability of
218 existing infectious cDNAs. Furthermore, our full-genome amplification protocol
219 obviates the time-consuming and costly process of construction and screening
220 of infectious clones by traditional methods. Therefore, the full-genome
221 amplification strategy significantly simplifies and streamlines the workflow for
222 the generation of new recombinant pestiviruses and also facilitates direct full-
223 length sequence analysis.

224

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230 and Production Sciences (DRCTPS grant 274-07-0198).

231 **Conflict of interest statement**

232 None

233

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286
287

288 Table 1: Primers used for long RT-PCR^a

289 **Primers for CSFV-C and CSFV-Paderborn**

290	5'Paderborn_T7 (forward)	<u>TAATACGACTCACTATAGTATACGAGGTTAGCTCGTCCTCGTGACAAACATT</u>
291	5'Paderborn_T7_NotI (forward)	ATATGCGGCCGCT <u>AATACGACTCACTATAGTATACGAGGTTAGCTCGTCCTCGTGACAAACATT</u>
292	3'Paderborn (reverse)	GGGCCGTTAGGAAATTACCTTAGTCCAACCTGTGGA
293	3'Paderborn-NotI (reverse)	ATATGCGGCCGCGC GGGCCGTTAGGAAATTACCTTAGTCCAACCTGTGGA
294	3'Paderborn_cDNA (reverse)	GGGCCGTTAGGAAATTACCTTAGT
295	5'C-strain_T7 (forward)	<u>TAATACGACTCACTATAGTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATC</u>
296	5'C-strain_T7_NotI (forward)	ATATGCGGCCGCT <u>AATACGACTCACTATAGTATACGAGGTTAGTTCATTCTCGTATACACGATTGGACAAATC</u>

297

298 **Primers for BVDV-CP7**

299	5'BVDV CP7_T7 (forward)	TAATACGACTCACTATAGTATACGAGAATTAGAAAAGGCAC
300	3'BVDV CP7 (reverse) ^b	GGGTGACGTCGGGTGTACCCTCATAC

301

302 **Primers for BDV-Gifhorn**

303	5'T7Gif-NotI (forward)	ATATGCGGCCGCT <u>AATACGACTCACTATAGTATACGAGAGTAGTTCAGGCTCGTATGCAAAATTGGGTGTTTC</u>
304	3'Gif-NotI (forward)	ATATGCGGCCGCGGGGCTGTTAGGGTTTTTCCCTTAATCCAACCTATGGACTTCAG

305 ^a T7 promoter is underlined, and NotI sites are shown in italics.

306 ^b Same primer was also used as cDNA primer.

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307 Fig. 1. Full-genome amplification of pestiviruses by long RT-PCR using T7-
308 5'NTR and 3'NTR primers. The pictures show agarose gel electrophoresis of
309 the full-length amplicons of BDV-Gifhorn (A, 1 μ l), BVDV-CP7 (B, 1 μ l), CSFV-C
310 (C, 10 μ l) and CSFV-Paderborn (D, 10 μ l).

311

312 Fig. 2. Full-genome amplification of pestiviruses by long RT-PCR using T7-
313 5'NTR and 3'NTR primers containing *NotI* restrictions sites. The picture shows
314 agarose gel electrophoresis of the full-length amplicons of BDV-Gifhorn (A, 10
315 μ l), CSFV-C (B, 10 μ l) and CSFV-Paderborn (C, 10 μ l). The non-specific bands
316 in A and C are most likely caused by the increased complexity of the primers
317 (extra *NotI* sites).

318

319 Fig. 3. *NotI* digestion of BAC transformants. DNA preparations from BAC
320 transformants were digested with *NotI* and analysed by agarose gel
321 electrophoresis. Four have the correct size insert (A-C, E), whereas one (D) has
322 a smaller insert, and another (F) contained no detectable BAC vector.

323

324 Fig. 4. Recovery of infectious CSFV-Paderborn derived from *in vitro* transcribed
325 pBeloPader10 RNA electroporated into porcine SK6 cells. The pictures show
326 immunofluorescence (IF) staining of cells expressing the NS3 proteins after the
327 1st (Panel A) and 2nd (Panel B) cell culture passage. Panels C and D are control
328 cells electroporated without any RNA transcripts.

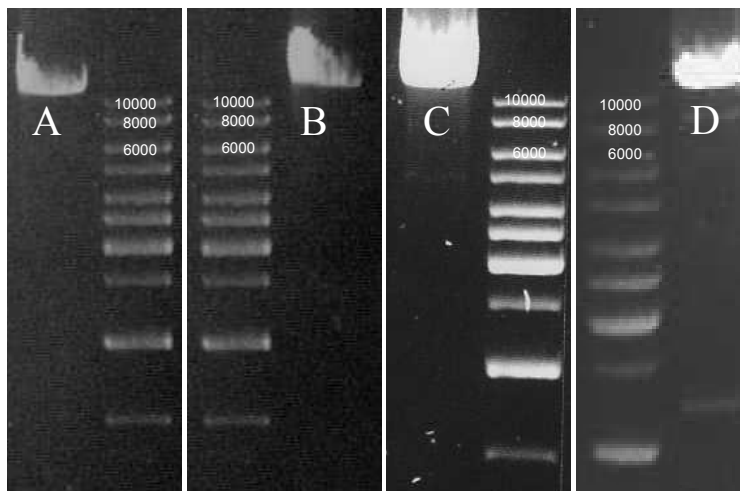
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332 Figure 1.

333



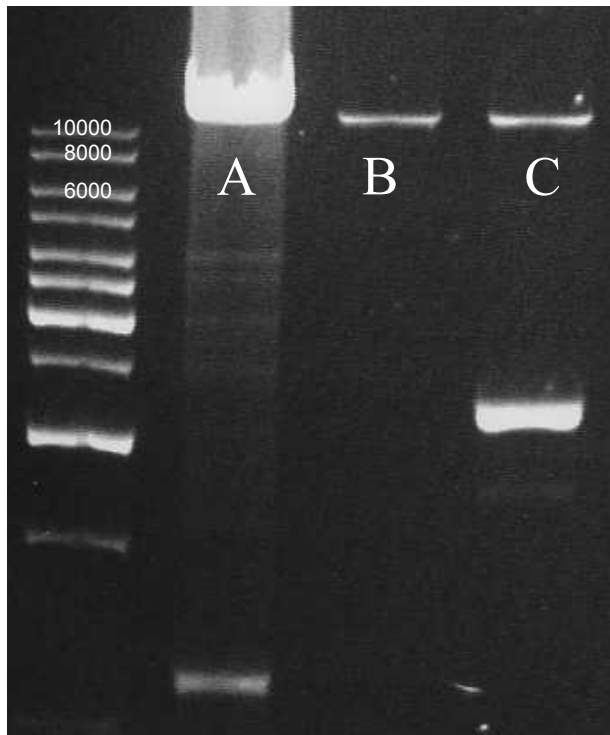
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337 Figure 2.

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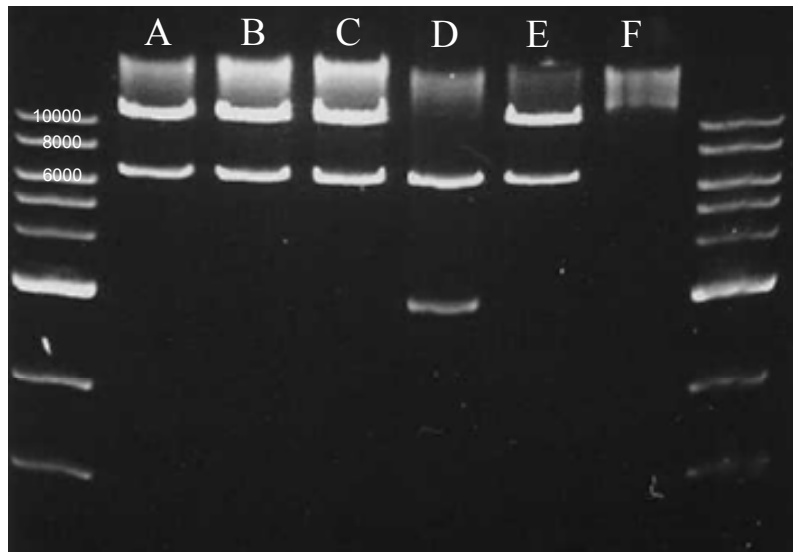


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341 Figure 3.

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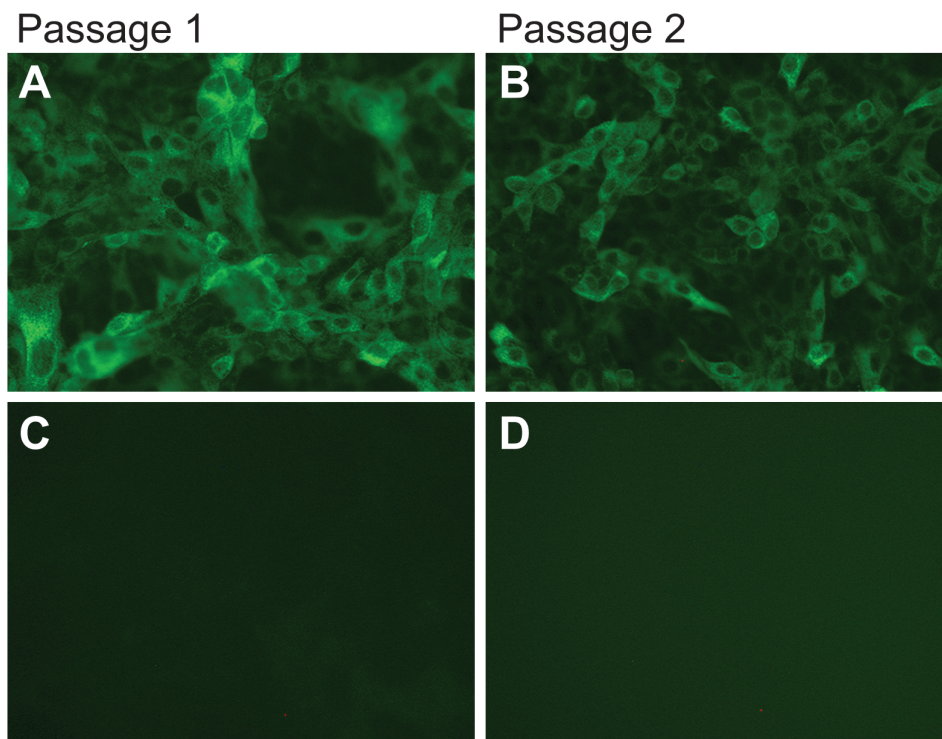
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345 Figure 4.

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