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Replication efficiency of rolling-circle replicon-based plasmids derived from porcine circovirus 2 in eukaryotic cells

25

26 Abstract

27 In this study, a method was developed to measure replication rates of rolling-circle replicon-28 based plasmids in eukaryotic cells. This method is based on the discriminative quantitation of 29 *Mbo*I-resistant, non-replicated input plasmids and *Dpn*I-resistant, replicated plasmids. To do 30 so, porcine circovirus type 2 (PCV2) replicon-based plasmids were constructed. These 31 plasmids contained the PCV2 origin of replication, the PCV2 Rep promoter and the PCV2 32 Rep gene. The results show that the replication rate depends on the length of the PCV2 33 replicon-based plasmid and not on the respective position of the Rep promoter and the 34 promoter of the gene of interest that encodes the enhanced green fluorescent protein (eGFP). 35 In all cases, it was necessary to add the Rep gene encoded by a plasmid and cotransfected as a 36 replication booster. This method can evaluate the replication potential of replicon-based 37 plasmids quickly and is thereby a promising tool for the development of plasmids for vaccine 38 purposes.

39

40 Keywords

41 Porcine Circovirus type 2 (PCV2); rolling-circle; replicon-based plasmid; quantitative PCR
42 assay; replication efficiency.

44 **1. Introduction**

45 Replicon-based plasmids derived from *Herpesviruses* (e.g. Epstein-Barr virus),

46 Polyomaviruses (e.g. BK virus, Simian virus 40), Papillomaviruses (e.g. bovine papilloma

47 virus 1), or *Geminiviruses* (e.g. bean yellow dwarf virus) have been developed recently for

48 gene expression and gene therapy (Kim et al., 2006; Mor et al., 2003; Shibata et al., 2005;

49 Van Craenenbroeck et al., 2000b). These plasmids contain a viral origin of replication and the

50 viral genes necessary for replication. Replicon-based plasmids have proved to be highly

51 valuable tools for: (1) producing high levels of proteins of interest in plants (Hefferon and

52 Fan, 2004; Mor et al., 2003); (2) delivering of therapeutic or complementing gene products

53 (Kim et al., 2006); (3) studying the regulation of replication (Takeda et al., 2005); and (4),

54 potentially, enhancing immune responses induced by DNA-based vaccines.

55 The porcine circovirus type 2 (PCV2), a member of the genus *Circovirus*, family

56 Circoviridae, contains a circular, single-stranded, positive-sense DNA genome which is about

57 1.8 kb in size. The intergenic region containing the origin of replication has a stem-loop

58 structure, which includes an octanucleotide sequence flanked by palindromes, and is bordered

59 by two open reading frames, ORF1 and ORF2. ORF1 is located on the positive strand and

60 encodes the Rep and Rep' proteins involved in replication initiation. ORF2 is located on the

61 negative strand of the intermediate double-stranded PCV2 genome that forms during

62 replication and it encodes the Cap protein, the capsid protein in PCV2 (Mankertz et al., 2004).

63 Owing to their ability to produce high levels of proteins in a short time, PCV2-based plasmids

64 may be valuable vectors for vaccine purposes compared to latent virus-based plasmids.

65 DNA replication of replicon-based plasmids in eukaryotic cells can be measured. The

66 replicated plasmid molecules can be distinguished from the initially transfected, input plasmid

67 molecules by their resistance to the *Dpn*I restriction enzyme, which can only cleave DNA that

has been dam-methylated in bacteria. Thus, replicated plasmids can be detected by Southern

69 blotting (Mankertz et al., 1997) or by quantitative PCR (qPCR) (Baxter and McBride, 2005; Taylor and Morgan, 2003). In comparison with Southern blotting, quantitative PCR is a 70 71 highly sensitive and quantitative method for detecting DNA replication and is significantly 72 faster (Taylor and Morgan, 2003). 73 For DNA-vaccine purposes, it is important to use a replicon-based plasmid — a so-called 74 replicative plasmid — that replicates rapidly to produce high quantities of vaccinating 75 proteins in a short time. However, little is known about the replication efficiency of 76 replicative plasmids. Replication efficiency can be estimated by comparing replicated 77 plasmids to the total number of extracted plasmids. In plasmids that undergo theta replication, 78 replication efficiency has been measured by comparing the number of plasmids after 79 incubation with and without DpnI (Takeda et al., 2005). 80 The difference between rolling-circle replication and theta replication is that rolling-circle 81 replicons replicate via a nicked-DNA intermediate and produce single-stranded DNA (Faurez 82 et al., 2009). The aim of the present study was to characterise a method to estimate the 83 efficiency of DNA replication of different rolling-circle, replicon-based plasmids using qPCR. 84 This information will be used to determine the replication rates of replicating plasmids and to 85 evaluate whether different primer pairs or different quantitative PCR protocols influence the 86 estimation of replication rates. PCV2 replicon-based plasmids were used as the rolling-circle 87 model.

2. Materials and methods

90 2.1 Culture of PK15 cells

Porcine circovirus type 1 (PCV1)-free porcine kidney (PK15) cells were grown in Eagle
minimal essential medium supplemented with 5% foetal bovine serum and 1% penicillin
(10 000 u/mL)-streptomycin (10 000 µg/mL) (Gibco, Invitrogen, Carlsbad, CA, USA) in 5%
CO₂ at 37°C and split 1:10 twice a week.

95

96 **2.2 Replicative and non-replicative plasmid constructs**

97 The pcDNA3 plasmid and the cloning vectors pCR4 and pBlueScript KS+ were obtained 98 from Invitrogen (Carlsbad, CA, USA). Five fragments were inserted into the plasmids (Figure 99 1a): (1) the enhanced green fluorescent protein (eGFP) reporter gene (GenBank accession no. 100 U57609.1); (2) the porcine circovirus type 2 (PCV2) OriRep fragment (GenBank accession 101 no. **AF201311**) that contains the Rep promoter, the origin of replication and ORF1 (Fig. 2); 102 (3) the cytomegalovirus immediate-early promoter (pCMV) of pcDNA3; (4) a PCR amplicon 103 obtained with oGVB2115 primers (Table 1) that contains a 1.3 kb fragment of the pBlueScript 104 KS+ vector flanked by a KpnI site; (5) a PCR amplicon from pcDNA3obtained with 105 oGVB2117 primers (Table 1) that contains the beginning of the neomycin gene flanked by 106 XmaI and MluI sites. 107 In all, eleven plasmids were constructed for specific purposes (Fig. 1a). Two plasmids were 108 constructed to optimise the determination of the replication rate. A non-replicative plasmid 109 was obtained by inserting the pCMV and the eGFP reporter gene into pCR4 (pCR4-GFP). A 110 replicative plasmid was obtained by inserting PCV2 OriRep. The PCV2 OriRep fragment was 111 amplified by PCR from PCV2 (Fenaux et al., 2002) using the oGVB2100 primer pair (Table 112 1) and inserted into pCR4 (pCR4-Orirep). Then, to test the optimised determination method,

113 one non-replicative plasmid and three replicative plasmids were constructed. The non-

| 114 | replicative plasmid was obtained by inserting the eGFP gene into pcDNA3 (pcDNA3-GFP). |
|-----|---|
| 115 | For the replicative plasmids, the OriRep fragment was inserted at three different distances |
| 116 | from the CMV promoter in the pcDNA3 plasmid. The PCV2 OriRep fragment was amplified |
| 117 | by PCR from PCV2 using the oGVB2101 or the oGVB2106 primer pair (Table 1) and |
| 118 | inserted into pCR4. Each OriRep fragment was isolated from the pCR4 backbone by digestion |
| 119 | with specific restriction endonucleases, as indicated in Figure 1a, and inserted into the |
| 120 | pcDNA3-eGFP plasmid at 0.2kb (pOrirep0.2-GFP), 1.2kb (pOrirep1.2-GFP) or 3.2kb |
| 121 | (pOrirep3.2-GFP) from the CMV promoter. To study the influence of the promoter and |
| 122 | plasmid size on replication, five additional plasmids were constructed. Three of them were |
| 123 | derived from deletions within the replicative plasmid pOrirep0.2-GFP: deletion of the SpeI- |
| 124 | pCMV fragment (Fig. 1a pOrirep0.2-GFP CMV ⁻); deletion of the <i>PsiI</i> -pSV40 neomycin |
| 125 | fragment (Fig. 1a pOrirep0.2-GFP SV40 ⁻); deletion of the SpeI-pCMV fragment and the PsiI- |
| 126 | pSV40 neomycin fragment (Fig. 1a pOrirep0.2-GFP CMV ⁻ /SV40 ⁻ 5.2kb). The latter plasmid, |
| 127 | which contained neither CMV nor SV40 promoters, was used to construct a plasmid with a |
| 128 | PCR amplicon obtained using oGVB2115 primers on pBluescript KS+ (pOrirep0.2-GFP |
| 129 | CMV ⁻ /SV40 ⁻ 6.5kb) . The fifth plasmid was a pBluescript KS+ plasmid with an <i>EcoRI</i> -OriRep |
| 130 | fragment from pCR4-Orirep, a SpeI-pCMV fragment from pcDNA3-GFP and a PCR |
| 131 | amplicon obtained using oGVB2117 primers on pcDNA3-GFP and cloned into the open |
| 132 | reading frame of pCMV (pKSOrirepCMVNeo) (Table 1). |
| 133 | <i>Escherichia coli</i> DH5 α strain was transformed with each of the eleven plasmids. Plasmids |
| 134 | were purified using the NucleoSpin® plasmid kit (Macherey-Nagel, Düren, Germany) |
| 135 | according to the manufacturer's instructions and sequenced. |

2.3 Replication and replication-defective booster

138 A pcDNA3.1 plasmid encoding the PCV2 ORF1 (Rep) called pcDNA3.1-Rep (Fig. 1b), 139 previously described and characterised in the AFSSA laboratory (Blanchard et al., 2003) was 140 used as a replication booster for the replicative plasmids. Replication of the PCV2 genome is 141 aborted if the tyrosine-96 of motif III of the Rep protein is substituted with phenylalanine-96. 142 However, the mutated Rep protein can still down-regulate the Rep promoter (Mankertz and 143 Hillenbrand, 2002; Steinfeldt et al., 2007). The mutation in motif III of the Rep protein was 144 introduced with the QuikChange XL site-directed mutagenesis kit (Stratagene-Agilent 145 Technologies, Waldbronn, Germany) according to the manufacturer's protocol using 146 pcDNA3.1-Rep as the template. The oGVB2112 primers used for mutagenesis are given in 147 Table 1. The mutation of the tyrosine-96 in the motif III sequence inhibits the digestion of 148 DNA by PstI. The constructs were thus characterised by enzymatic restriction with PstI and 149 constructs, which could not be digested by this enzyme, were sequenced to confirm the 150 sequence of Rep.

151

152 2.4 Transfection of PK15 cells with PCV2-based replicative and non-replicative plasmids

Twenty-four hours before transfection, 4.5×10^5 of PK15 cells were plated onto 6-well tissue culture plates. The final volume was 2 mL. For the quantitative real-time PCR-based replication assay, 10 ng of plasmids were transfected to optimise the method and 10^9 copies of plasmids were transfected to study the replication of different plasmids. The plasmids were cotransfected with 1 µg of pcDNA3.1-Rep or pcDNA3.1-Rep mutated and, for reversetranscription PCR (RT-PCR), 1 µg of plasmids were transfected with LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

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162 **2.5 Extraction of plasmids from transfected PK15 cells**

163 Twenty-four hours after transfection the cells were scraped into 200 µL of PBS 1X and lysed 164 in protease and the lysis buffer provided with the QIAamp MinElute Virus Spin Kit 165 (QIAGEN, Valencia, CA, USA) for 1 h at 56°C. Low-molecular-weight DNA was extracted 166 with the QIAamp MinElute Virus Spin kit (QIAGEN, Valencia, CA, USA) according to the 167 manufacturer's instructions. This kit was used because it has been described as being suitable 168 for purifying single-stranded DNA (Ng et al., 2009). The DNA was resuspended in 100 µL of 169 H₂O. Plasmid extractions were performed under a DNA hood (BiocapTM DNA, Captair[®] bio, 170 Erlab Biocapt).

171

172 **2.6 Quantitative real-time PCR-based replication assay**

173 The quantitative real-time PCR-based replication assay was based on the methylation status of 174 plasmids transfected into eukaryotic cells. Methylation status depends on whether or not 175 replication has occurred within eukaryotic cells. Dam-methylation of the GATC site occurs in 176 input plasmids (i.e. generated in prokaryotic (bacterial) cells), whereas the GATC site is not 177 methylated in plasmids that replicate in eukaryotic cells. Therefore, to differentiate replicated 178 from non-replicated plasmids, low-molecular-weight DNA extracted from PK15 cells was 179 incubated either with DpnI that cuts the dam-methylated GATC site or with MboI that, on the 180 contrary, cuts the non-methylated GATC site. To reduce the background level that is observed 181 in quantitative real-time PCR due to incompletely digested DNA, Exonuclease III (ExoIII) 182 was added to digest any incompletely cut DNA (Taylor and Morgan, 2003). ExoIII is an 183 exodeoxyribonuclease that does not act on intact circular plasmids, but rather digests one 184 strand of duplex DNA at a blunt end, at a 5' overhang or at internal nicks. The enzyme acts in a $3' \rightarrow 5'$ direction and produces stretches of single-stranded DNA (Rogers and Weiss, 1980; 185

186 Weiss, 1976). The conditions of digestion used were those described previously (Morgan and 187 Taylor, 2005).

188 In preliminary experiments, TaqMan and SYBR Green real-time PCR assays were compared. 189 The SYBR Green protocol was used in for the rest of the study. Primer pairs and probes 190 indicated in Table 1 were designed using Primer ExpressTM Software (Applied Biosystems, 191 Foster City, USA). Quantitative PCR was performed in an ABI Prism 7000 SDS (Applied 192 Biosystems, Foster City, USA) in a 25 µL total volume containing 1 X Universal Master Mix 193 or 1 X SYBR Green Master Mix (Applied Biosystems, Foster City, USA), 300 nM each 194 primer, 200 nM probe for the TaqMan protocol and 2 µL of digested DNA. After 2 min at 195 50°C and incubation for 10 min at 94°C, 40 PCR cycles consisting of a 15 sec denaturing step 196 at 94°C and a 1 min annealing/extension step at 60°C were performed. Plasmid copy numbers were determined by analysing a standard DNA curve ranging from 10^8 to 10^1 plasmid copies. 197 198 The PCR experiments were performed twice using nine replicates for statistical analysis or 199 twice in duplicate in the rest of the study. Data were analysed using Sequence Detection 200 Software version 1.2.3 (Applied Biosystems, Foster City, USA).

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2.7 RNA extraction and reverse-transcription PCR

Twenty-four hours after transfection, total RNA was extracted from PK15 cells using the

204 TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA,

205 USA). Plasmids were present in the same phase as RNA. To eliminate plasmids, 5 µL of total

206 RNA was incubated with RNase-free DNase as indicated by the manufacturer (QIAGEN,

- 207 Valencia, CA, USA). Then, 15 µL of DNA-free RNA was reverse-transcribed to cDNA with
- 208 random primers using the high-capacity cDNA archive kit according to the manufacturer's
- 209 instructions (Applied Biosystems, Foster City, USA). The cDNA was used immediately or
- 210 stored at -20°C until use. To differentiate Rep RNA from Rep' RNA, intron-flanking primers

were used (Table 1: 98_F and oGVB2118_R). The amplicon size was 774pb and 391pb for 211 212 Rep cDNA and Rep' cDNA, respectively. β-actin cDNA was used as an internal control. The 213 PCR reaction was performed in a total volume of 50 µL containing 10 µL of cDNA, 10 µL of 214 5X Green GoTaq buffer, 0.2 µM 98_F and oGVB2118_R primers, 0.2 µM each dNTP and 1 215 unit of GoTaq® Flexi DNA polymerase (Promega, Southampton, UK) using the G-STORM 216 GS1 system (Genetic Research Instrumentation, Braintree, Essex, UK). Thermal cycle 217 conditions were as follows: 94°C for 5 min followed by 30 cycles at 94°C for 30 sec, 59°C for 218 30 sec, 72°C for 1 min with a final extension of 72°C for 10 min. Amplicons were visualised 219 by 2% agarose gel electrophoresis and ethidium bromide staining.

220

221 2.8 Statistics

222 The aim of the statistical analysis was to test in each population, i.e. non-replicative and 223 replicative plasmids, the difference between the quantity of DNA incubated with ExoIII only 224 (qE) and the sum of the quantity of DNA incubated with *Dpn*I and ExoIII (qDE) and *Mbo*I 225 and ExoIII (qME). Two-tailed Student *t*-tests were used and computed using Systat 9.0 226 software. The associated power of the Student *t*-tests was computed with the POWER 227 procedure of SAS software (SAS, 2004). The quantities of plasmids in digested DNA samples 228 were considered to be different if the Type I error was less than 5% and considered to be 229 similar if the Type II error was less than 20%, i.e. a power higher greater than 80%, for an 230 associated Type I error at 5% (Jenny, 2001; Melot, 2003). The Mann-Whitney test was used 231 for comparisons of differences in the results of real-time PCR on two different amplification 232 targets and in the results of real-time PCR using the TaqMan probe or SYBR Green.

3. Results

3.1 Optimisation of the replication rate calculation

235 In this study, the replication rate was defined as the percentage of replicated plasmids at 24 h 236 post-transfection. To evaluate the replication rate of plasmids, a quantitative real-time PCR-237 based assay was developed to quantify input plasmids or replicated plasmids, but not the 238 cotransfected pcDNA3.1-Rep replication booster. To do so, the PCR primers targeted a 239 GATC site and this site is not present in pcDNA3.1-Rep. In theory, the quantity of plasmids 240 after incubation with ExoIII (qE) should be equal to the sum of the quantity of plasmids 241 incubated with *Dpn*I and ExoIII and the quantity of plasmids incubated with *Mbo*I and ExoIII 242 (qDE+qME). Consequently, the replication rate can be calculated using qE or qDE+qME as 243 the denominator. 244 To test this assumption, the number of plasmid copies of pCR4-GFP and of pCR4-Orirep 245 were quantified. Visually, qE did not appear different from qDE+qME (Fig. 3). In the case of 246 pCR4-GFP, statistics showed that qE and qDE+qME were not significantly different 247 (p=0.766). However, these two groups were not shown to be similar, because the Type II 248 error was greater than 20%. To conclude on similarity at a Type II error of 20%, quantitative 249 PCR must be performed 1526 times for each type of plasmid. In the case of pCR4-Orirep, 250 statistical tests showed that qE and qDE+qME were significantly different (p=0.017). 251 Therefore, the assumption could not be validated for replicative plasmids. The replication rate 252 of replicative plasmids incubated with MboI and ExoIII exceeded 100% when using qE as the 253 denominator in the calculation (data not shown). qME and qDE had the same conditions of 254 digestion; thus the replication rate was calculated using qDE/(qME+qDE).

| 256 | 3.2 Detection of Rep and Rep' mRNA production |
|------------|--|
| 257 | Reverse-transcription PCR was used to analyse the transcription of Rep and Rep' proteins |
| 258 | encoded by pOrirep0.2-GFP or by pcDNA3.1-Rep. Reverse-transcription PCR amplified two |
| 259 | cDNA fragments corresponding to the Rep mRNA (774pb) and to the spliced mRNA of Rep' |
| 260 | (391pb) for pOrirep0.2-GFP and for pcDNA3.1-Rep (Fig. 4). These bands were not present in |
| 261 | the cDNA controls of PK15 cells that were not transfected or that were transfected by non- |
| 262 | replicative pcDNA3. Thus, the expression of both Rep and Rep' could be detected. |
| 263 | |
| 264 265 | 3.3 Necessity of cotransfection with pcDNA3.1-Rep to show the replication rate in vitro |
| 266 | In preliminary experiments pCR4-GFP or pCR4-Orirep were transfected in PK15 cells |
| 267 | without the replication booster pcDNA3.1-Rep. The mean replication rate of pCR4-Orirep |
| 268 | was similar to that of pCR4-GFP (data not shown). The cotransfection of pcDNA3.1-Rep |
| 269 | resulted in a replication rate of 49% for pCR4-Orirep (Fig. 5). If the Rep gene of pcDNA3.1- |
| 270 | Rep was mutated, the replication rate of pCR4-Orirep returned to the background level. No |
| 271 | replication was observed for pCR4-GFP, even in the presence of pcDNA3.1-Rep. |
| 272 | |
| 273 274 | 3.3 Replication rates determined with two different quantitative real-time PCR methods and for two different amplification targets |
| 275 | Plasmid replication rates were compared for two primer pairs associated with a TaqMan probe |
| 276 | or with the SYBR Green dye. The quantitative real-time PCR-based replication assays were |
| 277 | tested with pcDNA3-GFP and pOrirep0.2-GFP (Fig. 1a); each plasmid was cotransfected with |
| 278 | pcDNA3.1-Rep. One primer pair was situated in the neomycin gene with a GATC site located |
| 279 | in the middle of the amplicon, and the other primer pair was placed at the junction of the GFP |
| 280 | gene and the pcDNA3 backbone with a GATC site located in the reverse primer (Fig. 6a). |
| 281 | Neither the quantity of plasmids evaluated by the formula qDE+qME (Fig. 6b) nor the |

282 replication rates (Fig. 6c) were significantly different, whatever the primer pair. This was 283 verified for pcDNA3-GFP and pOrirep0.2-GFP (Fig. 6b and 6c). Moreover, the use of a 284 TaqMan probe or a SYBR Green dye (Fig. 7a) did not introduce significant variation in the 285 quantity of plasmids measured (Fig. 7b) or in the mean replication rates (Fig. 7c) of either 286 construct.

- 287
- 288 289

3.4 Example application: mean replication rate of PCV2-based replicons in PK15 cells 24 h post-transfection

290 PCV2-based replicons were cotransfected with pcDNA3.1-Rep into PK15 cells. The non-

291 replicative plasmids have a mean background replication rate below 5% (Fig. 8). pCR4-

292 Orirep showed a mean replication rate of $63\% \pm 8\%$. The mean replication rates of the three

293 replicative plasmids encoding the eGFP gene were lower, i.e. $14\% \pm 2\%$, $13\% \pm 3\%$ and 12%

294 \pm 2% for pOrirep1.2-GFP, pOrirep0.2-GFP and pOrirep3.2-GFP, respectively. Compared to

295 the three replicative eGFP encoding plasmids, pCR4-Orirep was smaller (5.2kb vs. 7.4kb) and

296 did not contain strong eukaryotic promoters such as pCMV and pSV40.

297

3.5 Mean replication rate depended on plasmid size, but not on the presence of 298 strong eukaryotic promoters

299 To determine whether the size of the plasmid or the presence of strong eukaryotic promoters 300 could modify the mean replication rates of the PCV2-based replicons, five additional plasmids 301 were constructed and tested. Plasmids with or without eukaryotic promoters and greater than 302 5.9 kb in size still had low mean replication rates. On the contrary, 5.2 kb plasmids with or 303 without eukaryotic promoters had replication rates of about the same magnitude as pCR4-304 Orirep (Fig. 8).

4. Discussion

307 In this study, a rapid, quantitative real-time PCR-based assay was developed to measure the 308 replication rates of PCV2-based replicons. The replication rate was defined as the percentage 309 at 24 h post-transfection of the number of replicated plasmids compared to the total number of 310 plasmids present in the cells (i.e. replicated and initial input plasmids). The choice of the 311 Escherichia coli strain in which replicon-based plasmids were produced was important. For 312 example, the E. coli TOP10 strain has more mutations in the system of methylation and 313 restriction (hsdRMS gene) than the DH5a strain; this difference caused an increase in the 314 replication rate (data not shown). Primers for short PCR amplicons containing a GATC 315 sequence were designed to discriminate between replicated and non-replicated plasmids after 316 incubation in eukaryotic cells. A plasmid encoding the PCV2 Rep protein (pcDNA3.1-Rep) 317 was needed to boost and to detect the in vitro replication of the PCV2-based replicons. 318 Comparable replication rates were determined using two different amplification targets. The 319 use of a specific TaqMan probe and amplicon detection using SYBR Green dye also resulted 320 in comparable replication rates.

321 As in theta replication, rolling-circle replication is a semi-conservative replication system. 322 However, a rolling-circle replicon replicates via a nicked DNA intermediate and produce 323 single-stranded DNA (Faurez et al., 2009). The present study showed that the quantity of 324 plasmids after incubation with ExoIII (qE) and the sum of the quantity of plasmids after 325 incubation with DpnI and ExoIII plus the quantity of plasmids after incubation with MboI and 326 ExoIII (qDE+qME) were significantly different in replicative plasmids. This difference may 327 be due to the products of rolling-circle replication, namely single-stranded DNA and nicked 328 DNA. In contrast to viruses that replicate via rolling-circle replication, rolling-circle replicon-329 based plasmids do not encode the capsid protein which up-regulates the amount of single-330 stranded DNA by protecting viral DNA from host proteins (Padidam et al., 1999).

331 Consequently single-stranded DNA probably did not contribute to the difference between qE 332 and qDE+qME. On the contrary, nicked plasmids may have an impact on the number of 333 plasmid copies detected by quantitative real-time PCR. The efficiency of ExoIII digestion 334 may vary with the quantity of nicked DNA and digested DNA (Hoheisel, 1993). The qME to 335 qE ratio exceeded 100%: more DNA was detected when DNA was incubated with MboI and 336 ExoIII than with ExoIII only. The difference in the quantity of plasmids detected by 337 quantitative real-time PCR may be due to the complete digestion of nicked DNA in qE but not 338 in qME. ExoIII may digest all the double-stranded plasmids and produce single-stranded 339 DNA, thereby decreasing the number of plasmid copies detected by quantitative real-time 340 PCR. Because qDE and qME had the same conditions of digestion, qDE+qME was used to 341 calculate the replication rate instead of qE. Hemi-methylated plasmids are cleaved by DpnI 342 under conditions of enzyme excess, long digestion times or small quantities of DNA template 343 (Lu et al., 2002) but are not cleaved by MboI (Stancheva et al., 1999). Thus, hemi-methylated 344 plasmids are not amplified by PCR, resulting in a possible underestimation of the replication 345 rate. To be detected, replication of plasmids had to be sufficient for detection by real-time 346 PCR assays.

347 This method made it possible to differentiate the replication efficiency of different plasmids. 348 In this study, non-replicative plasmids showed a background level lower than 10% of the 349 mean replication rate in all the experiments. This background level could be explained by a 350 percentage of input DNA that becomes DpnI-resistant (Takeda et al., 2005). In this study, 351 mean replication rates could only be determined when the replicative plasmids were 352 cotransfected with pcDNA3.1-Rep. There are two possible explanations for this result: (1) the 353 replicative plasmids could not replicate because Rep and/or Rep' proteins were not produced, 354 or (2) the replication rate was too low to be detected because only low quantities of Rep 355 and/or Rep' proteins were produced. The first explanation can be discounted because it was

356 shown that the Rep gene and Rep promoter had not mutated and that the Rep gene was indeed 357 transcribed. However, dysfunction may be caused by the low production of Rep and Rep' or 358 down-regulation of the Rep promoter. It has been reported that transcriptional interference 359 with Epstein-Barr virus-derived vectors depends on the thymidine kinase promoter in the 360 episomal vector (Van Craenenbroeck et al., 2003). However, in eukaryotic cells, the Rep 361 promoter could not be perturbed by the presence of the immediate early CMV promoter or the 362 SV40 promoter in the plasmid because pCR4-Orirep did not contain these eukaryotic 363 promoters. Furthermore, the Rep promoter may not be exchanged with another promoter 364 because it is down-regulated by the Rep protein (Mankertz and Hillenbrand, 2002) which 365 limits the toxicity of Rep on cells. This down-regulation may rapidly decrease the production 366 of proteins and also replication. 367 pCR4-Orirep seemed to replicate more rapidly than pOrirep0.2-GFP, pOrirep1.2-GFP and 368 pOrirep3.2-GFP. The differences between replicative plasmids were (1) the size of the 369 plasmids, with 5.2 kb for pCR4-Orirep and 7.3 or 7.4 kb for pOrirep0.2-GFP, pOrirep1.2-GFP 370 and pOrirep3.2-GFP and (2) although pOrirep0.2-GFP, pOrirep1.2-GFP and pOrirep3.2-GFP 371 contained strong eukaryotic promoters, pCR4-Orirep did not. Strong promoter-enhancers such 372 as the CMV immediate-early gene enhancer interfere with the origin of replication of SV40 373 (Chen et al., 2000) and oversized vectors, such as BKV replicon plasmids, are more subject to 374 recombination events which may lead to defective replication (Van Craenenbroeck et al., 375 2000a). The present study showed that size had an impact on the replication rate of rolling-376 circle replicon-based plasmids, since plasmids with a size of around 5.2 kb replicated at a high 377 rate and plasmids with larger sizes replicated at lower rates. Prokaryotic rolling-circle 378 replicons generate high-molecular-weight DNA during replication in prokaryotic cells when a 379 fragment is inserted into the replicon (Dabert et al., 1992; Gruss and Ehrlich, 1988). It is 380 possible that replication of PCV2 replicon-based plasmids was initiated, but that replication

could not be terminated due to the large plasmid size, thereby affecting the replication rate. In
this study, the strong eukaryotic promoters pCMV and pSV40 did not affect the replication
rates.

384 In conclusion, the method described in this study made it possible to calculate plasmid 385 replication rates in eukaryotic cells and to compare different rolling-circle replicons. Using 386 this method, the influence of plasmid size, the presence of strong eukaryotic promoters and 387 the location of the OriRep sequence in the plasmid were studied. This method should be 388 useful for rolling-circle or theta replicons, and in particular for studying the regulation of 389 replicon or virus replication. The quantitative real-time PCR assay reported here could also 390 advantageously be used to evaluate the replication potential of replicon-based plasmids 391 developed for vaccine purposes. Unlike gene therapy, the replicon-based plasmids have to 392 replicate rapidly to produce large amounts of the encoded proteins of interest in a short time. 393

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487 **Figure captions, tables, figures, schemes**

488

Figure 1a: Representation of replicative and non-replicative plasmids. From left to right: backbones, non-replicative plasmids and replicative plasmids. Light grey arrow: eGFP reporter gene; black arrow with a stem-loop: PCV2 OriRep; dark grey arrow: IE CMV promoter; black arrow: PCR amplicon obtained with oGVB2115 primers (Table 1); white arrow: PCR amplicon obtained with oGVB2117 primers (Table 1).

494

495 Figure 1b: Representation of the replication booster used in this study, pcDNA3.1-Rep. The496 backbone is pcDNA3.1 zeo+.

497

Figure 2: PCV2 fragment used to render plasmids replicative. The PCV2 genome is
represented on the left. It contains ORF1 (in black), ORF2 (in grey) and an origin of
replication (stem loop). The OriRep fragment is boxed.

501

502 Figure 3: Quantitation of plasmids by real-time PCR-based replication assay. Non-replicative

503 plasmid, pCR4-GFP; replicative plasmid, pCR4-Orirep. Plasmids were cotransfected with

504 pcDNA3.1-Rep. Total plasmids, quantity of plasmids incubated with ExoIII; quantity of non-

- replicated plasmids + replicated plasmids, quantity of plasmids incubated with *Dpn*I and
- 506 ExoIII plus quantity of plasmids incubated with *Mbo*I and ExoIII. The real-time PCR
- 507 experiments were carried out twice using nine replicates ; standard error bars are shown.508
- 509 Figure 4: Detection of Rep and Rep' mRNAs 24 h post-transfection in a non-replicative
- 510 plasmid, a replicative plasmid and a plasmid encoding the Rep gene. Lane 1: non-transfected
- 511 PK15 cells; lane 2: PK15 cells transfected by pcDNA3-GFP; lane 3: PK15 cells transfected
- 512 by pOrirep0.2-GFP; lane 4: PK15 cells transfected by pcDNA3.1-Rep. Results for β-actin
- 513 mRNA, used as a positive control, are shown.
- 514

515 Figure 5: Need for a plasmid which expresses Rep protein at high levels. pCR4-GFP or

516 pCR4-Orirep were cotransfected into PK15 cells with a pcDN3.1-Rep, or they were

517 cotransfected with pcDN3.1-Rep mutated. The replication rates were calculated as described 518 above. The real time-PCR experiments were carried out twice in duplicate and the standard

- 519 error bars are shown.
- 520

521 Figure 6: Influence of the two primer pairs designed for the quantitative real time-PCR on the 522 determination of the replication rates. The real-time-PCR-based replication assays were tested 523 with two primer pairs and pcDNA3-GFP and pOrirep0.2-GFP were both cotransfected with 524 pcDN3.1-Rep. a) Representation of the primer pairs b) Quantity of plasmids depending on the 525 primers used. c) Mean replication rate for non-replicative and replicative plasmids for each 526 primer pairs. The real time-PCR experiments were carried out twice in duplicate and the 527 standard error bars are given. The Mann-Whitney test was used to compare differences

- 528 between results.
- 529

530 Figure 7: Influence of the real time-PCR method on the determination of the replication rates.

531 The real-time-PCR-based replication assays were tested with TaqMan probe 2111 or SYBR

- 532 Green dye. pcDNA3-GFP and pOrirep0.2-GFP, both cotransfected with pcDN3.1-Rep, were
- 533 used. a) Representation of the two quantitative real-time PCR methods b) Quantity of
- plasmids after real time-PCR with the TaqMan probe or the SYBR Green dye. c) Mean rate of
- replication after real time-PCR with the TaqMan probe or the SYBR Green dye. The real-time

- 536 PCR experiments were carried out twice in duplicate and the standard error bars are shown.
- 537 The Mann-Whitney test was used to compare differences between results.
- 538
- 539 Figure 8: Mean replication rates for PCV2 replicons 24 h post-transfection. Plasmids were
- 540 cotransfected into PK15 cells with pcDN3.1-Rep. Replication rates were calculated as
- 541 described in 3.1. The quantitative real-time PCR experiments were carried out twice in
- 542 duplicate and the standard error bars are shown.
- 543

546 547 Table 1: Primer and probe sequences for construction of plasmids, for real-time PCR and for reverse transcriptase PCR

| name | function | Specificity | size of amplicon | tequence |
|-----------------|------------------|--|----------------------------|---|
| 06V82100_F | claning | Mfei CAATTG | anatala (| 5' LAATTGCGGGTGTTGAAGATGCCATT 3' |
| oGV92100_R | cloning | MILLI ACGCGT | 123100 | 5' ACGCGTCCCCACTTAACCCTTAATGA 3' |
| 0GV92101_F | claning | Miul ACGCGT | 19991221 | 3' ACGCGTEGGG7GTTGAAGATGCCATT 3' |
| oGV82101_R | cloning | Mfoi CAATTG | 153750 | 5' CAATTGEECCACTTAAECETTAATGA 3' |
| 0GV82107_F | claning | Draill CACOTAGTG | 1777.00 | 3' CACGTAGTGCCACTTAATGAAT ¥ |
| oGV82106_R | cloning | Draili CACGTAGTG | 113100 | 5' CACTACGTGTGTGAAGATGCCATTTTTC 3' |
| nGV82115_F | claning | Kpni GGTACC in the end of ampicillin gene | SYTEMA . | 5' GGTACCG1CTATTTCGTTCATCCATAG 3' |
| oGV82115_R | cloning | Kpni GGTACC in the middle of f1 origin | 147200 | 5' BETACETCAASETCTAAATCEBES 3' |
| 06V82117_F | cloning | Xmal CCCGGG in the beginning of neomycin gene | (Séan | 3' CCCG6666CATGATTGAACAAGATG 3' |
| o6V82117_R | cloning | Miur ACGCGT in the middle of neorwysin gene | +ouhn | 5' ACGCGTAGTACGTGCTCGCTCGAT 3' |
| 06V82112_F | mutagenesis | mutations Y_{aa} in F_{aa} and S_{aa} in Δ_{aa} | V | 5'-ACTCCATCAGTAAGT1GCCTTCTTTAGCGCAAAATTCTTTATTCTGCTGATCTGTTCC-3' |
| oGV82112_R | mutagenesis | mutations Yae in Fat and Saa in App | | 5'-668AACAGATCAGCAGAATAAAGAATTTTGCGCTAAAGAAGGCAACTTACTGATGGAGT-3' |
| 06V82111_F | qPCR aseay | in the GRP gene | amplicon of 93pb and Doni | 5' GCATGGACGAGCTGTACAAGTAA 3' |
| oGV82111_R | qPCR essay | in the backbone of pcDNA3 | site in the midle | 5' ATCAGCGAGCTCTAGCATTTAGG 3' |
| oGV92113_F | GPCR assay | in the reamycin gene | amplicon of 92pb Opni site | 5' GCTCCTGCCGAGAAAGTATCC # |
| oGV82113_fl | qPCR assay | in the neomycin gene | in reverse primer. | S' TTCOCTTOSTOSTCOAATO 3' |
| 98_F | RT PCR Bep! | in the beginning of Repigene | amplicon of Rep = 774pb | 5' GTGGGTGTTCACTCTGAATAA 3' |
| 00V82118_R | IT PCR Rep' | after the end of intron of Rep gene | amplicon of Rep' = 331pb | 5' AGABETTETACAGETBGGACA 3' |
| probe 2111 | probe gPCR assay | FAM-MGB | 1 | 5' CTCGAGCATGCATCTA 5 |
| pactine forward | RT PCR Bactine | 1 | 64pb | 5'-GATCGTGCGDGACATCAAG-3' |
| Bactine reverse | RT PCR Bactine | 1 | | 5'-66CCATCTCCT6CTCGAA-3' |

549







