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3       **Replication efficiency of rolling-circle replicon-based**

4 **plasmids derived from porcine circovirus 2 in eukaryotic**

5 **cells**

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

### **Abstract**

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

### **Keywords**

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

## 1. Introduction

Replicon-based plasmids derived from *Herpesviruses* (e.g. Epstein-Barr virus), *Polyomaviruses* (e.g. BK virus, Simian virus 40), *Papillomaviruses* (e.g. bovine papilloma virus 1), or *Geminiviruses* (e.g. bean yellow dwarf virus) have been developed recently for gene expression and gene therapy (Kim et al., 2006; Mor et al., 2003; Shibata et al., 2005; Van Craenenbroeck et al., 2000b). These plasmids contain a viral origin of replication and the viral genes necessary for replication. Replicon-based plasmids have proved to be highly valuable tools for: (1) producing high levels of proteins of interest in plants (Hefferon and Fan, 2004; Mor et al., 2003); (2) delivering of therapeutic or complementing gene products (Kim et al., 2006); (3) studying the regulation of replication (Takeda et al., 2005); and (4), potentially, enhancing immune responses induced by DNA-based vaccines.

The porcine circovirus type 2 (PCV2), a member of the genus *Circovirus*, family *Circoviridae*, contains a circular, single-stranded, positive-sense DNA genome which is about 1.8 kb in size. The intergenic region containing the origin of replication has a stem-loop structure, which includes an octanucleotide sequence flanked by palindromes, and is bordered by two open reading frames, ORF1 and ORF2. ORF1 is located on the positive strand and encodes the Rep and Rep' proteins involved in replication initiation. ORF2 is located on the negative strand of the intermediate double-stranded PCV2 genome that forms during replication and it encodes the Cap protein, the capsid protein in PCV2 (Mankertz et al., 2004). Owing to their ability to produce high levels of proteins in a short time, PCV2-based plasmids may be valuable vectors for vaccine purposes compared to latent virus-based plasmids.

DNA replication of replicon-based plasmids in eukaryotic cells can be measured. The replicated plasmid molecules can be distinguished from the initially transfected, input plasmid molecules by their resistance to the *DpnI* restriction enzyme, which can only cleave DNA that has been dam-methylated in bacteria. Thus, replicated plasmids can be detected by Southern

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 highly sensitive and quantitative method for detecting DNA replication and is significantly faster (Taylor and Morgan, 2003).

For DNA-vaccine purposes, it is important to use a replicon-based plasmid — a so-called replicative plasmid — that replicates rapidly to produce high quantities of vaccinating proteins in a short time. However, little is known about the replication efficiency of replicative plasmids. Replication efficiency can be estimated by comparing replicated plasmids to the total number of extracted plasmids. In plasmids that undergo theta replication, replication efficiency has been measured by comparing the number of plasmids after incubation with and without DpnI (Takeda et al., 2005).

The difference between rolling-circle replication and theta replication is that rolling-circle replicons replicate via a nicked-DNA intermediate and produce single-stranded DNA (Faurez et al., 2009). The aim of the present study was to characterise a method to estimate the efficiency of DNA replication of different rolling-circle, replicon-based plasmids using qPCR. This information will be used to determine the replication rates of replicating plasmids and to evaluate whether different primer pairs or different quantitative PCR protocols influence the estimation of replication rates. PCV2 replicon-based plasmids were used as the rolling-circle model.

## 2. Materials and methods

### 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% CO<sub>2</sub> at 37°C and split 1:10 twice a week.

### 2.2 Replicative and non-replicative plasmid constructs

The pcDNA3 plasmid and the cloning vectors pCR4 and pBlueScript KS+ were obtained from Invitrogen (Carlsbad, CA, USA). Five fragments were inserted into the plasmids (Figure 1a): (1) the enhanced green fluorescent protein (eGFP) reporter gene (GenBank accession no. U57609.1); (2) the porcine circovirus type 2 (PCV2) OriRep fragment (GenBank accession no. AF201311) that contains the Rep promoter, the origin of replication and ORF1 (Fig. 2); (3) the cytomegalovirus immediate-early promoter (pCMV) of pcDNA3; (4) a PCR amplicon obtained with oGVB2115 primers (Table 1) that contains a 1.3 kb fragment of the pBlueScript KS+ vector flanked by a *KpnI* site; (5) a PCR amplicon from pcDNA3 obtained with oGVB2117 primers (Table 1) that contains the beginning of the neomycin gene flanked by *XmaI* and *MluI* sites.

In all, eleven plasmids were constructed for specific purposes (Fig. 1a). Two plasmids were constructed to optimise the determination of the replication rate. A non-replicative plasmid was obtained by inserting the pCMV and the eGFP reporter gene into pCR4 (pCR4-GFP). A replicative plasmid was obtained by inserting PCV2 OriRep. The PCV2 OriRep fragment was amplified by PCR from PCV2 (Fenaux et al., 2002) using the oGVB2100 primer pair (Table 1) and inserted into pCR4 (pCR4-Orirep). Then, to test the optimised determination method, one non-replicative plasmid and three replicative plasmids were constructed. The non-

replicative plasmid was obtained by inserting the eGFP gene into pcDNA3 (pcDNA3-GFP). For the replicative plasmids, the OriRep fragment was inserted at three different distances from the CMV promoter in the pcDNA3 plasmid. The PCV2 OriRep fragment was amplified by PCR from PCV2 using the oGVB2101 or the oGVB2106 primer pair (Table 1) and inserted into pCR4. Each OriRep fragment was isolated from the pCR4 backbone by digestion with specific restriction endonucleases, as indicated in Figure 1a, and inserted into the pcDNA3-eGFP plasmid at 0.2kb (pOrirep0.2-GFP), 1.2kb (pOrirep1.2-GFP) or 3.2kb (pOrirep3.2-GFP) from the CMV promoter. To study the influence of the promoter and plasmid size on replication, five additional plasmids were constructed. Three of them were derived from deletions within the replicative plasmid pOrirep0.2-GFP: deletion of the *SpeI*-pCMV fragment (Fig. 1a pOrirep0.2-GFP CMV<sup>-</sup>); deletion of the *PsiI*-pSV40 neomycin fragment (Fig. 1a pOrirep0.2-GFP SV40<sup>-</sup>); deletion of the *SpeI*-pCMV fragment and the *PsiI*-pSV40 neomycin fragment (Fig. 1a pOrirep0.2-GFP CMV<sup>-</sup>/SV40<sup>-</sup>5.2kb). The latter plasmid, which contained neither CMV nor SV40 promoters, was used to construct a plasmid with a PCR amplicon obtained using oGVB2115 primers on pBluescript KS+ (pOrirep0.2-GFP CMV<sup>-</sup>/SV40<sup>-</sup>6.5kb) . The fifth plasmid was a pBluescript KS+ plasmid with an *EcoRI*-OriRep fragment from pCR4-Orirep, a *SpeI*-pCMV fragment from pcDNA3-GFP and a PCR amplicon obtained using oGVB2117 primers on pcDNA3-GFP and cloned into the open reading frame of pCMV (pKSOrirepCMVNeo) (Table 1).

*Escherichia coli* DH5α strain was transformed with each of the eleven plasmids. Plasmids were purified using the NucleoSpin® plasmid kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions and sequenced.

### 2.3 Replication and replication-defective booster

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

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

Twenty-four hours before transfection,  $4.5 \times 10^5$  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 reverse-transcription PCR (RT-PCR), 1 µg of plasmids were transfected with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.



## 2.5 Extraction of plasmids from transfected PK15 cells

Twenty-four hours after transfection the cells were scraped into 200  $\mu$ L of PBS 1X and lysed in protease and the lysis buffer provided with the QIAamp MinElute Virus Spin Kit (QIAGEN, Valencia, CA, USA) for 1 h at 56°C. Low-molecular-weight DNA was extracted with the QIAamp MinElute Virus Spin kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. This kit was used because it has been described as being suitable for purifying single-stranded DNA (Ng et al., 2009). The DNA was resuspended in 100  $\mu$ L of H<sub>2</sub>O. Plasmid extractions were performed under a DNA hood (Biocap™ DNA, Captair® bio, Erlab Biocapt).

## 2.6 Quantitative real-time PCR-based replication assay

The quantitative real-time PCR-based replication assay was based on the methylation status of plasmids transfected into eukaryotic cells. Methylation status depends on whether or not replication has occurred within eukaryotic cells. Dam-methylation of the GATC site occurs in input plasmids (i.e. generated in prokaryotic (bacterial) cells), whereas the GATC site is not methylated in plasmids that replicate in eukaryotic cells. Therefore, to differentiate replicated from non-replicated plasmids, low-molecular-weight DNA extracted from PK15 cells was incubated either with *DpnI* that cuts the dam-methylated GATC site or with *MboI* that, on the contrary, cuts the non-methylated GATC site. To reduce the background level that is observed in quantitative real-time PCR due to incompletely digested DNA, Exonuclease III (ExoIII) was added to digest any incompletely cut DNA (Taylor and Morgan, 2003). ExoIII is an exodeoxyribonuclease that does not act on intact circular plasmids, but rather digests one 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;

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

In preliminary experiments, TaqMan and SYBR Green real-time PCR assays were compared. The SYBR Green protocol was used in for the rest of the study. Primer pairs and probes indicated in Table 1 were designed using Primer Express™ Software (Applied Biosystems, Foster City, USA). Quantitative PCR was performed in an ABI Prism 7000 SDS (Applied Biosystems, Foster City, USA) in a 25 µL total volume containing 1 X Universal Master Mix or 1 X SYBR Green Master Mix (Applied Biosystems, Foster City, USA), 300 nM each primer, 200 nM probe for the TaqMan protocol and 2 µL of digested DNA. After 2 min at 50°C and incubation for 10 min at 94°C, 40 PCR cycles consisting of a 15 sec denaturing step 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<sup>8</sup> to 10<sup>1</sup> plasmid copies. The PCR experiments were performed twice using nine replicates for statistical analysis or twice in duplicate in the rest of the study. Data were analysed using Sequence Detection Software version 1.2.3 (Applied Biosystems, Foster City, USA).

## **2.7 RNA extraction and reverse-transcription PCR**

Twenty-four hours after transfection, total RNA was extracted from PK15 cells using the TRIzol® reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Plasmids were present in the same phase as RNA. To eliminate plasmids, 5 µL of total RNA was incubated with RNase-free DNase as indicated by the manufacturer (QIAGEN, Valencia, CA, USA). Then, 15 µL of DNA-free RNA was reverse-transcribed to cDNA with random primers using the high-capacity cDNA archive kit according to the manufacturer's instructions (Applied Biosystems, Foster City, USA). The cDNA was used immediately or 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 Rep cDNA and Rep' cDNA, respectively.  $\beta$ -actin cDNA was used as an internal control. The PCR reaction was performed in a total volume of 50  $\mu$ L containing 10  $\mu$ L of cDNA, 10  $\mu$ L of 5X Green GoTaq buffer, 0.2  $\mu$ M 98\_F and oGVB2118\_R primers, 0.2  $\mu$ M each dNTP and 1 unit of GoTaq® Flexi DNA polymerase (Promega, Southampton, UK) using the G-STORM GS1 system (Genetic Research Instrumentation, Braintree, Essex, UK). Thermal cycle conditions were as follows: 94°C for 5 min followed by 30 cycles at 94°C for 30 sec, 59°C for 30 sec, 72°C for 1 min with a final extension of 72°C for 10 min. Amplicons were visualised by 2% agarose gel electrophoresis and ethidium bromide staining.

## 2.8 Statistics

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

In this study, the replication rate was defined as the percentage of replicated plasmids at 24 h post-transfection. To evaluate the replication rate of plasmids, a quantitative real-time PCR-based assay was developed to quantify input plasmids or replicated plasmids, but not the cotransfected pcDNA3.1-Rep replication booster. To do so, the PCR primers targeted a GATC site and this site is not present in pcDNA3.1-Rep. In theory, the quantity of plasmids after incubation with ExoIII (qE) should be equal to the sum of the quantity of plasmids incubated with *DpnI* and ExoIII and the quantity of plasmids incubated with *MboI* and ExoIII (qDE+qME). Consequently, the replication rate can be calculated using qE or qDE+qME as the denominator.

To test this assumption, the number of plasmid copies of pCR4-GFP and of pCR4-Orirep were quantified. Visually, qE did not appear different from qDE+qME (Fig. 3). In the case of pCR4-GFP, statistics showed that qE and qDE+qME were not significantly different ( $p=0.766$ ). However, these two groups were not shown to be similar, because the Type II error was greater than 20%. To conclude on similarity at a Type II error of 20%, quantitative PCR must be performed 1526 times for each type of plasmid. In the case of pCR4-Orirep, statistical tests showed that qE and qDE+qME were significantly different ( $p=0.017$ ). Therefore, the assumption could not be validated for replicative plasmids. The replication rate of replicative plasmids incubated with *MboI* and ExoIII exceeded 100% when using qE as the denominator in the calculation (data not shown). qME and qDE had the same conditions of digestion; thus the replication rate was calculated using  $qDE/(qME+qDE)$ .

### 3.2 Detection of Rep and Rep' mRNA production

Reverse-transcription PCR was used to analyse the transcription of Rep and Rep' proteins encoded by pOrirep0.2-GFP or by pcDNA3.1-Rep. Reverse-transcription PCR amplified two cDNA fragments corresponding to the Rep mRNA (774pb) and to the spliced mRNA of Rep' (391pb) for pOrirep0.2-GFP and for pcDNA3.1-Rep (Fig. 4). These bands were not present in the cDNA controls of PK15 cells that were not transfected or that were transfected by non-replicative pcDNA3. Thus, the expression of both Rep and Rep' could be detected.

### 3.3 Necessity of cotransfection with pcDNA3.1-Rep to show the replication rate in vitro

In preliminary experiments pCR4-GFP or pCR4-Orirep were transfected in PK15 cells without the replication booster pcDNA3.1-Rep. The mean replication rate of pCR4-Orirep was similar to that of pCR4-GFP (data not shown). The cotransfection of pcDNA3.1-Rep resulted in a replication rate of 49% for pCR4-Orirep (Fig. 5). If the Rep gene of pcDNA3.1-Rep was mutated, the replication rate of pCR4-Orirep returned to the background level. No replication was observed for pCR4-GFP, even in the presence of pcDNA3.1-Rep.

### 3.3 Replication rates determined with two different quantitative real-time PCR methods and for two different amplification targets

Plasmid replication rates were compared for two primer pairs associated with a TaqMan probe or with the SYBR Green dye. The quantitative real-time PCR-based replication assays were tested with pcDNA3-GFP and pOrirep0.2-GFP (Fig. 1a); each plasmid was cotransfected with pcDNA3.1-Rep. One primer pair was situated in the neomycin gene with a GATC site located in the middle of the amplicon, and the other primer pair was placed at the junction of the GFP gene and the pcDNA3 backbone with a GATC site located in the reverse primer (Fig. 6a). Neither the quantity of plasmids evaluated by the formula  $q_{DE}+q_{ME}$  (Fig. 6b) nor the

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

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

PCV2-based replicons were cotransfected with pcDNA3.1-Rep into PK15 cells. The non-replicative plasmids have a mean background replication rate below 5% (Fig. 8). pCR4-Orirep showed a mean replication rate of  $63\% \pm 8\%$ . The mean replication rates of the three replicative plasmids encoding the eGFP gene were lower, i.e.  $14\% \pm 2\%$ ,  $13\% \pm 3\%$  and  $12\% \pm 2\%$  for pOrirep1.2-GFP, pOrirep0.2-GFP and pOrirep3.2-GFP, respectively. Compared to the three replicative eGFP encoding plasmids, pCR4-Orirep was smaller (5.2kb vs. 7.4kb) and did not contain strong eukaryotic promoters such as pCMV and pSV40.

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

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

#### 4. Discussion

In this study, a rapid, quantitative real-time PCR-based assay was developed to measure the replication rates of PCV2-based replicons. The replication rate was defined as the percentage at 24 h post-transfection of the number of replicated plasmids compared to the total number of plasmids present in the cells (i.e. replicated and initial input plasmids). The choice of the *Escherichia coli* strain in which replicon-based plasmids were produced was important. For example, the *E. coli* TOP10 strain has more mutations in the system of methylation and restriction (hsdRMS gene) than the DH5 $\alpha$  strain; this difference caused an increase in the replication rate (data not shown). Primers for short PCR amplicons containing a GATC sequence were designed to discriminate between replicated and non-replicated plasmids after incubation in eukaryotic cells. A plasmid encoding the PCV2 Rep protein (pcDNA3.1-Rep) was needed to boost and to detect the in vitro replication of the PCV2-based replicons. Comparable replication rates were determined using two different amplification targets. The use of a specific TaqMan probe and amplicon detection using SYBR Green dye also resulted in comparable replication rates.

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



shown that the Rep gene and Rep promoter had not mutated and that the Rep gene was indeed transcribed. However, dysfunction may be caused by the low production of Rep and Rep' or down-regulation of the Rep promoter. It has been reported that transcriptional interference with Epstein-Barr virus-derived vectors depends on the thymidine kinase promoter in the episomal vector (Van Craenenbroeck et al., 2003). However, in eukaryotic cells, the Rep promoter could not be perturbed by the presence of the immediate early CMV promoter or the SV40 promoter in the plasmid because pCR4-Orirep did not contain these eukaryotic promoters. Furthermore, the Rep promoter may not be exchanged with another promoter because it is down-regulated by the Rep protein (Mankertz and Hillenbrand, 2002) which limits the toxicity of Rep on cells. This down-regulation may rapidly decrease the production of proteins and also replication.

pCR4-Orirep seemed to replicate more rapidly than pOrirep0.2-GFP, pOrirep1.2-GFP and pOrirep3.2-GFP. The differences between replicative plasmids were (1) the size of the plasmids, with 5.2 kb for pCR4-Orirep and 7.3 or 7.4 kb for pOrirep0.2-GFP, pOrirep1.2-GFP and pOrirep3.2-GFP and (2) although pOrirep0.2-GFP, pOrirep1.2-GFP and pOrirep3.2-GFP contained strong eukaryotic promoters, pCR4-Orirep did not. Strong promoter-enhancers such as the CMV immediate-early gene enhancer interfere with the origin of replication of SV40 (Chen et al., 2000) and oversized vectors, such as BKV replicon plasmids, are more subject to recombination events which may lead to defective replication (Van Craenenbroeck et al., 2000a). The present study showed that size had an impact on the replication rate of rolling-circle replicon-based plasmids, since plasmids with a size of around 5.2 kb replicated at a high rate and plasmids with larger sizes replicated at lower rates. Prokaryotic rolling-circle replicons generate high-molecular-weight DNA during replication in prokaryotic cells when a fragment is inserted into the replicon (Dabert et al., 1992; Gruss and Ehrlich, 1988). It is 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.

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

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## Figure captions, tables, figures, schemes

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).

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

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.

Figure 3: Quantitation of plasmids by real-time PCR-based replication assay. Non-replicative plasmid, pCR4-GFP; replicative plasmid, pCR4-Orirep. Plasmids were cotransfected with pcDNA3.1-Rep. Total plasmids, quantity of plasmids incubated with ExoIII; quantity of non-replicated plasmids + replicated plasmids, quantity of plasmids incubated with *DpnI* and ExoIII plus quantity of plasmids incubated with *MboI* and ExoIII. The real-time PCR experiments were carried out twice using nine replicates ; standard error bars are shown.

Figure 4: Detection of Rep and Rep' mRNAs 24 h post-transfection in a non-replicative plasmid, a replicative plasmid and a plasmid encoding the Rep gene. Lane 1: non-transfected PK15 cells; lane 2: PK15 cells transfected by pcDNA3-GFP; lane 3: PK15 cells transfected by pOrirep0.2-GFP; lane 4: PK15 cells transfected by pcDNA3.1-Rep. Results for  $\beta$ -actin mRNA, used as a positive control, are shown.

Figure 5: Need for a plasmid which expresses Rep protein at high levels. pCR4-GFP or pCR4-Orirep were cotransfected into PK15 cells with a pcDN3.1-Rep, or they were cotransfected with pcDN3.1-Rep mutated. The replication rates were calculated as described above. The real time-PCR experiments were carried out twice in duplicate and the standard error bars are shown.

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

Figure 7: Influence of the real time-PCR method on the determination of the replication rates. The real-time-PCR-based replication assays were tested with TaqMan probe 2111 or SYBR Green dye. pcDNA3-GFP and pOrirep0.2-GFP, both cotransfected with pcDN3.1-Rep, were 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

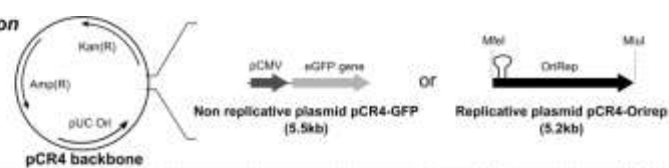
539 Figure 8: Mean replication rates for PCV2 replicons 24 h post-transfection. Plasmids were  
540 cotransfected into PK15 cells with pcDN3.1-Rep. Replication rates were calculated as  
541 described in 3.1. The quantitative real-time PCR experiments were carried out twice in  
542 duplicate and the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

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	sequence
oGV82100_F	cloning	MfeI CAATTG	1231pb	3'-CAATTGCGGGTGTGAAGATGCCATT-3'
oGV82100_R	cloning	MluI ACGCGT		3'-ACGCGTCCCCACTTAACCTTAATGA-3'
oGV82101_F	cloning	MluI ACGCGT		3'-ACGCGTGGGTGTGAAGATGCCATT-3'
oGV82101_R	cloning	MfeI CAATTG		3'-CAATTGCCCCACTTAACCTTAATGA-3'
oGV82107_F	cloning	DraIII CACGTAGTG	1231pb	3'-CACGTAGTGCCACTTAACCTTAATGAAT-3'
oGV82106_R	cloning	DraIII CACGTAGTG		3'-CACTACGTGTGTTGAAGATGCCATTTTTC-3'
oGV82115_F	cloning	KpnI GGTAAC in the end of ampicillin gene	1273pb	3'-GGTACCGTCTATTCTGTCATCCATAG-3'
oGV82115_R	cloning	KpnI GGTAAC in the middle of f1 origin		3'-GGTACCTCAAGCTCAAATCGGG-3'
oGV82117_F	cloning	XbaI CCCGGG in the beginning of neomycin gene	430pb	3'-CCCGGGCATGATTGAACAAGATG-3'
oGV82117_R	cloning	MluI ACGCGT in the middle of neomycin gene		3'-ACGCGTAGTACGTGCTCCTCGAT-3'
oGV82112_F	mutagenesis	mutations Y <sub>88</sub> in F <sub>28</sub> and S <sub>88</sub> in A <sub>88</sub>	/	3'-ACTCCATCAGTAAGTTGCTCTTTAGCGCAAAATCTTTATTCTGTGATCTGTCC-3'
oGV82112_R	mutagenesis	mutations Y <sub>88</sub> in F <sub>28</sub> and S <sub>88</sub> in A <sub>88</sub>	amplicon of 93pb and DpnI site in the middle	3'-GGAACAGATCAGCAGATAAAGAAATTTGCGCTAAAGAAAGCAACTACTGATGGAGT-3'
oGV82111_F	qPCR assay	in the GFP gene		3'-GCATGGACGAGCTGTACAAGTAA-3'
oGV82111_R	qPCR assay	in the backbone of pCDNA3	amplicon of 32pb DpnI site	3'-ATCAGCGAGCTCTAGCATTTAGG-3'
oGV82113_F	qPCR assay	in the neomycin gene		3'-GCTCCTGCCGAGAAAGTATCC-3'
oGV82113_R	qPCR assay	in the neomycin gene	in reverse primer	3'-TTCGCTTGGTGGTCCGAATG-3'
88_F	RT PCR Rep'	in the beginning of Rep gene	amplicon of Rep = 774pb	3'-GTGGGTGTTCACTCTGAATAA-3'
oGV82118_R	RT PCR Rep'	after the end of intron of Rep gene	amplicon of Rep' = 391pb	3'-AGAGCTTCTACAGCTGGGACA-3'
probe 2111	probe qPCR assay	FAM-MGB	/	3'-CTCGAGCATGCATCTA-3'
Bactine forward	RT PCR Bactine	/	64pb	3'-GATCGTGCGGACATCAAG-3'
Bactine reverse	RT PCR Bactine	/		3'-GGCCATCTCTGCTCGAA-3'



# Constructs for optimization



# Constructs for example of replication rate



# Constructs for the study of the influence of the promoter on replication



# Constructs for the study of the influence of the size on replication



Figure 1a

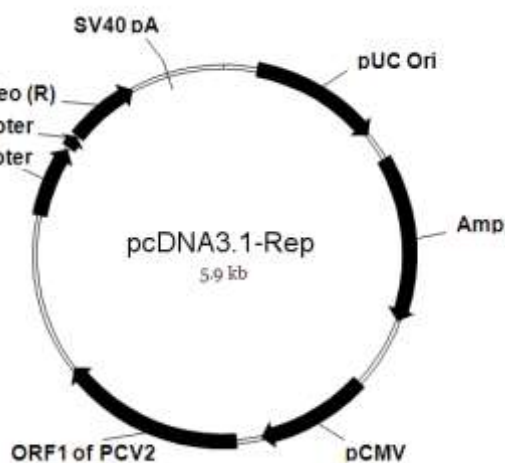


Figure 1b

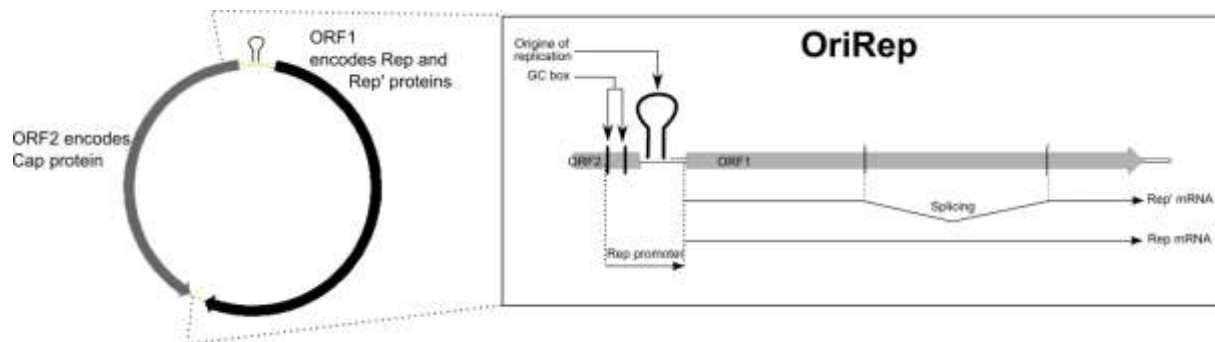


Figure 2

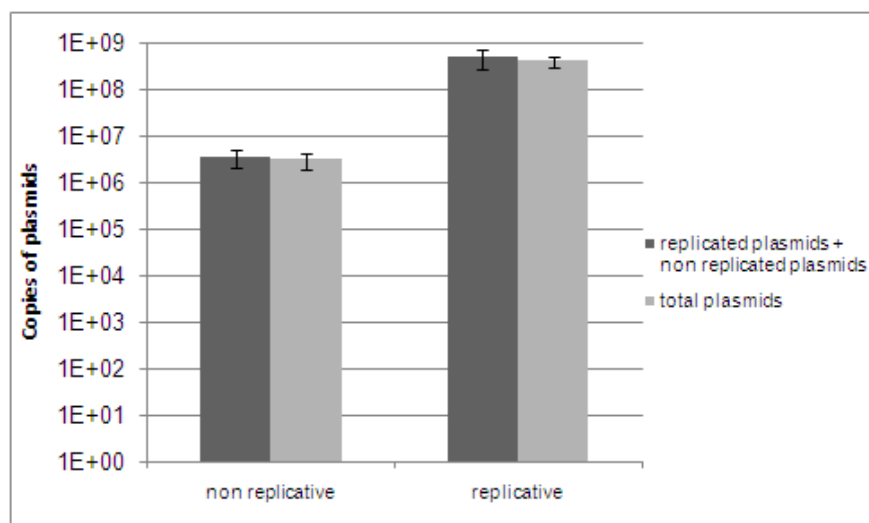


Figure 3

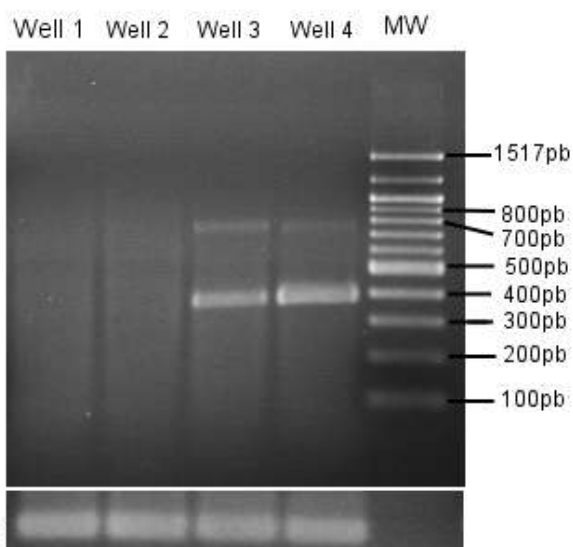


Figure 4

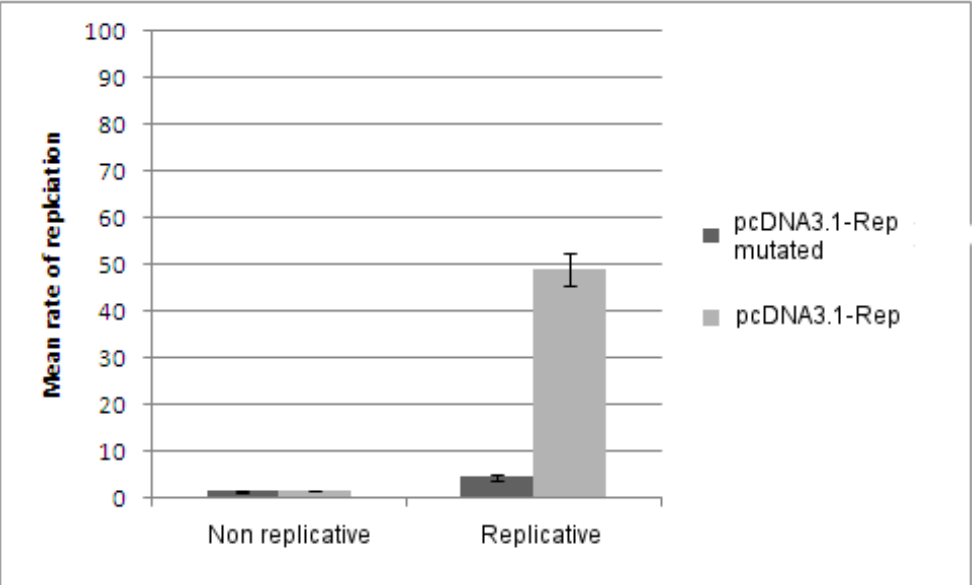


Figure 5

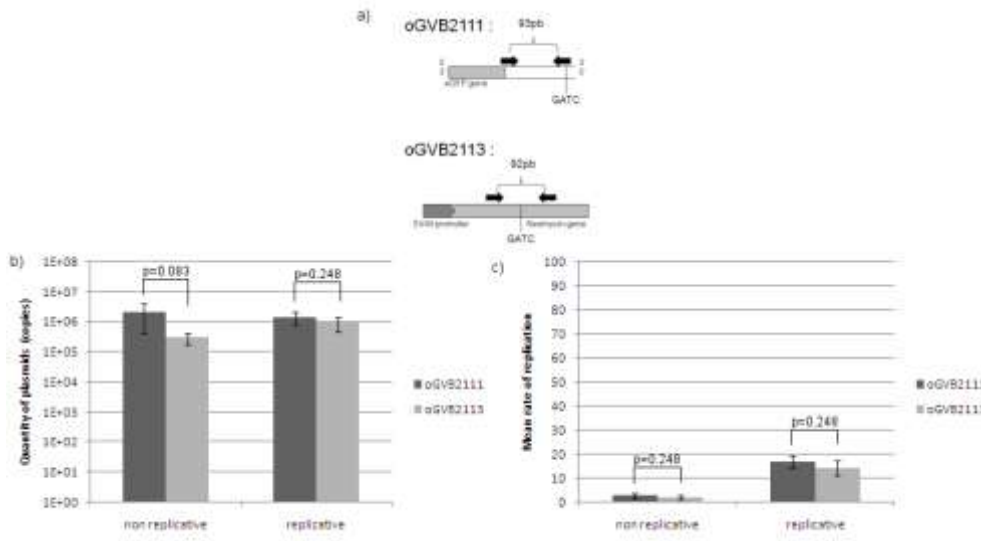


Figure 6

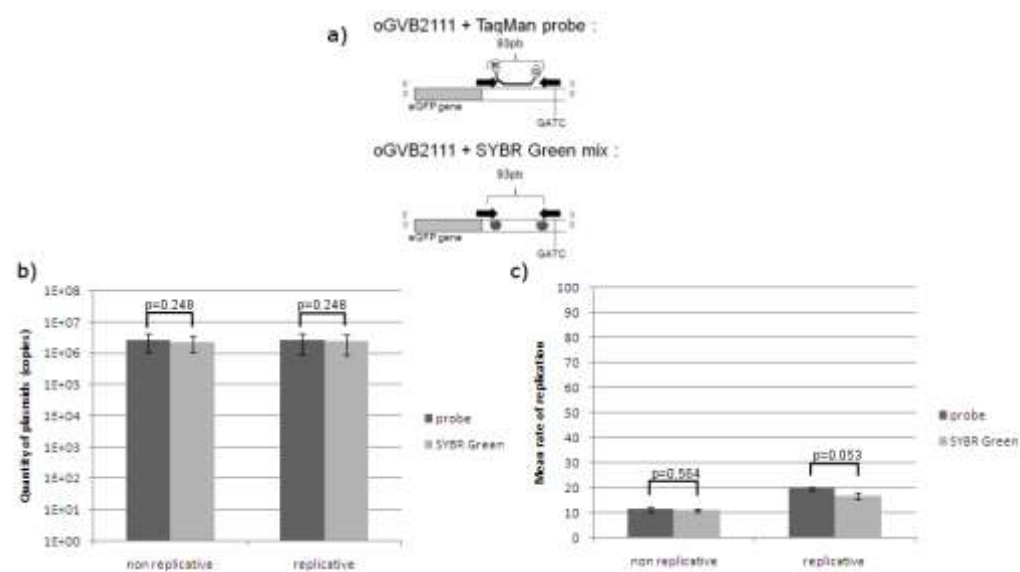


Figure 7

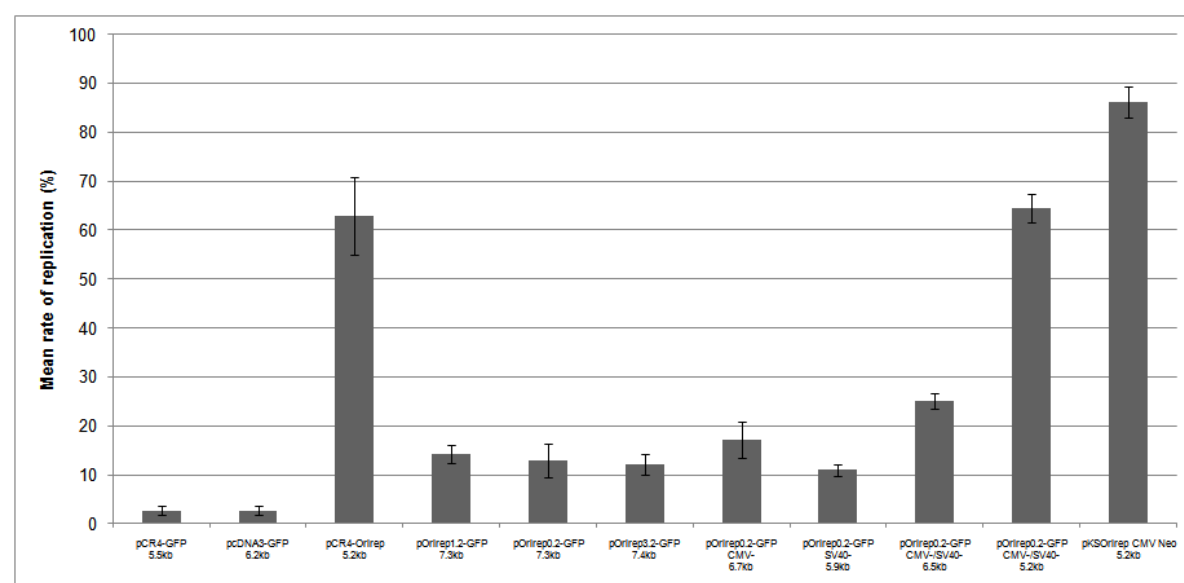


Figure 8