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Comparative analysis of the performance of residual host cell DNA assays for viral vaccines produced in Vero cells

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ABSTRACT

Residual host cell DNA (rcDNA) from continuous cell lines used for manufacturing of biological medicinal products has been considered as safety risk. Historically, several analytical methods have been used for rcDNA quantitation including hybridization assay, Threshold® assay and quantitative polymerase chain reaction (qPCR). Sanofi Pasteur has a wealth of experience in the development of methods quantifying rcDNA in vaccines. Here, we compared the performance of our in-house assays for quantifying rcDNA in viral vaccines produced in Vero cells. Vero alpha-satellite sequence qPCR was compared with the hybridization and Threshold® assays in terms of specificity, sensitivity and precision. The impact of viral inactivation with β-propiolactone (BPL) on rcDNA, within the vaccine production process, was also assessed. We demonstrate that the quantity of rcDNA measured is influenced by the analytical method used. Vero cell DNA-specific qPCR assay was shown to be robust with a large dynamic range and no matrix interference on a range of products. The qPCR assay demonstrated greater sensitivity and specificity versus the hybridization and Threshold® methods. Vero alpha-satellite sequence qPCR is a specific and sensitive method for the assessment of the quantity of Vero rcDNA in the highly purified vaccines.

1. Introduction

Animal cell substrates are commonly used in the manufacture of biological products. As such, trace/low quantity of DNA originating from the cell substrate used in the production process, referred to as residual host cell DNA (rcDNA), may remain in the final manufactured product. The main risks associated with rcDNA in viral vaccines are infectivity and oncogenicity; rcDNA might be capable of transmitting viral infections if retroviral proviruses, integrated copies of DNA viruses, or extra chromosomal genomes are present (FDA, 2010; Sheng-Fowler et al., 2009), or may pose a potential risk for oncogenesis if viral or cell substrate (derived from tumors or which are tumorigenic) oncogenes are present (Sheng-Fowler et al., 2010).

The guidelines defining acceptable levels of rcDNA in biological products by various regulatory agencies have evolved in the last decades. In 1987, the World Health Organization (WHO) suggested that there was only a negligible risk associated with rcDNA in a biological product when the rcDNA content was ≤100 pg in a single parenteral dose (WHO, 1987). This limit was primarily intended to minimize the potential oncogenic risk of rcDNA from products derived from continuous cell lines. However, subsequent additional information suggested that rcDNA from continuous cell lines had a much lower oncogenic risk than previously assumed and accordingly, should be considered as a general impurity (EMEA, 1997). The WHO later increased the acceptable limit to ≤10 ng per parenteral dose (WHO, 1998). However, with the caveat that in instances where the continuous-cell-line DNA may pose a greater risk (e.g., where it may include infectious retroviral provirion sequences), then the acceptable limit should be established in consultation with the national regulatory agencies (WHO, 1998). In 2001, the FDA subsequently recommended the amount and size distribution of rcDNA be evaluated and where desirable, steps taken to reduce both the amount and size of the DNA fragments (FDA, 2001). The FDA later recommended that the DNA fragments should be reduced to below the size of a functional gene (assumed to be about 200 base pairs [bp]) (FDA, 2010). For live-attenuated vaccines delivered orally like rotavirus vaccines, rcDNA should be limited to ≤100 µg/dose as orally administered DNA is absorbed approximately 10,000-fold less efficiently than parenterally administered DNA (WHO, 2007).

To date, a case-by-case risk-based assessment is recommended by

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the WHO, US Food and Drug Administration (FDA) and European Pharmacopoeia, taking into consideration the characteristics of the cell substrate, the intended use of the biological product/vaccine and, most importantly, the effect of the manufacturing process (including inactivation steps) on the size, quantity and biological activity of rcDNA fragments (European Pharmacopoeia, General chapter 5.2.3, 2018; FDA, 2001; FDA, 2010; WHO, 2013). This case-by-case risk-based approach was advocated in recognition that the rcDNA in some products may be higher than 10 ng per dose as it might be difficult to reach that level without impairing the potency of the resulting product. In these instances or when it is not possible to reduce rcDNA content further, DNA alkylating agents such as β-propiolactone (BPL) may be used to alter rcDNA to reduce any associated risk (WHO, 2013). However, more stringent rcDNA limits, e.g not more than 10 pg per dose (European Pharmacopoeia, Monograph 1056, 2019b), might be required to assure product safety when cells with tumorigenic phenotypes or other characteristics of concern are used (EMEA, 2001; FDA, 2010).

Although rcDNA assay can be undertaken at various stages throughout the manufacturing processes using appropriate methods, there is no universally appropriate method for the quantitation of rcDNA of animal origin. The membrane hybridization and Threshold® assays have traditionally been used for the quantitation of host rcDNA in biopharmaceuticals. The hybridization method is still described in some compendial documents (Chinese Pharmacopoeia, Chapter 3407, 2015; USP, General information chapter < 1130 > , 2018). However, a collaborative study across 15 laboratories revealed that the hybridization assay was associated with a high degree of variability in determining rcDNA content (Robertson and Heath, 1992). The inherent variability combined with the relatively long assay duration has meant that the hybridization assay is no longer widely used for routine rcDNA assessment in the biotechnology industry. The Threshold® assay is limited in the detection of fragments shorter than 800 bp, and may be inhibited by high DNA concentration (> 1 ng/mL) through the consumption of reagents or generation of signals too high to accurately quantitate. The Threshold® assay is also negatively impacted by short DNA fragments (20 to 80 bp) through competitive inhibition preventing the formation of complete reaction complexes (King and Panfili, 1991). In addition, the method is not specific to the DNA source, it is relatively low throughput for industrial application, lacks robustness, and the equipment is only available from one supplier and is no longer manufactured for supply in Europe.

Polymerase chain reaction (PCR)-based technologies have gained in popularity for the detection and quantitation of rcDNA in recent years, and are generally considered the most practical and robust method for routine rcDNA quantitation as they are species/sequence specific, sensitive, quick to undertake and amenable to high-throughput formats. In addition, the equipment and reagents required are available from multiple suppliers. The choice of the targeted genome region has an impact on the assay results; selecting target DNA sequences that are found in multiple copies in the host genome would increase the likelihood of detection and accurate quantitation of rcDNA (Wang et al., 2012). However, the potential for contamination also needs to be considered. For example, targeting genes that are highly conserved among mammalian species to detect mammalian rcDNA, such as the multicopy ribosomal 18S RNA gene or Alu sequences, may be susceptible to the presence of human DNA in the environment or contamination during experimental procedure and would be difficult to control. This would limit the method sensitivity, making it inappropriate for highly purified products with the most stringent specifications.

The analytical laboratories at Sanofi Pasteur have historically used the hybridization and Threshold® methods for the measurement of rcDNA in the 1980's and 1990's, respectively. A qPCR assay targeting a 90 bp segment on the simian alpha-satellite sequence (a 171 bp sequence repeated approximately 5 million times on the simian genome) based on that described by Lebron et al. (Lebron et al., 2006) was subsequently developed to quantitate Vero rcDNA in our laboratory. Here we detail the performance of this qPCR method for the assessment of rcDNA in vaccines produced in Vero cells compared with other DNA quantitation assays that have been historically used in our laboratory.

2. Methods

Although there are a number of methods available for DNA quantitation in general, only a few are appropriate for routine rcDNA quantitation in the biotechnology industry. The following assays have been used by Sanofi Pasteur for the quantitation of rcDNA from Vero cell substrate: alpha-satellite qPCR, Threshold® and hybridization.

2.1. Vaccines

Validations of analytical methods were performed on samples at the end of the Drug Substance manufacturing process (bulk stage) for the three following viral vaccines produced on Vero cells by Sanofi Pasteur: a new rabies vaccine under development produced on Vero cells using a Raw Material of Animal Origin (RMAO)-free process, the dengue vaccine (ChimeriVax™ dengue tetravalent vaccine) and the Inactivated Poliovirus Vaccine (IPV).

The rcDNA size fragment distribution was assessed at intermediate stages of the Drug Substance of the marketed rabies vaccine produced in Vero cells (VERORAB®) from Sanofi Pasteur before and after BPL viral inactivation. The rcDNA content was assessed at intermediate stages of the Drug Substance of the new rabies vaccine under development before and after BPL viral inactivation.

Additionally, the rcDNA present in five final formulations of rabies vaccines (produced in Vero cells) for human use from competitors were compared with those in the final formulation of VERORAB®.

2.2. DNA extraction

Recognized as an appropriate method for rcDNA recovery (USP, General information chapter < 1130 > , 2018), residual Vero cell DNA was extracted from 500 µL of sample using the DNA Extractor® Kit (Wako Chemicals GmbH, Neuss, Germany) according to the manufacturer's instructions. For qPCR, Threshold® and hybridization assays, rcDNA was respectively resuspended into 50 µL in nuclease-free water (Promega, Madison, USA), 500 µL in Zero Calibrator (ZC) buffer (Molecular Devices, California, USA), and 500 µL nuclease-free water. For each series of extractions, there was a negative extraction control (500 µL of nuclease-free water) and a positive extraction control (500 µL of a vaccine with a known amount of Vero rcDNA for the qPCR assay; 500 µL of ZC spiked with 50 pg High Calibrator DNA for the Threshold® assay; 500 µL of a solution of Vero cell DNA at 200 pg/mL for the hybridization assay).

2.3. DNA standard used for qPCR and hybridization assays

The standards used for Vero DNA quantitation were prepared by extraction of nucleic acids from Vero cells used for vaccine production. After cell lysis and RNase treatment, genomic DNA was isolated from proteins and other Vero cells components using QIAamp Virus BioRobot 9604 (Qiagen, Hilden, Germany). After extraction, DNA was free of proteins, nucleases, RNA and other components or inhibitors. Quantitation of purified DNA was performed by UV spectrophotometry at 260 nm. To evaluate the purity of the Vero DNA standard, the ratio between absorbance at 260 nm and 280 nm was determined. Aliquots of the Vero DNA standard were stored at below –60 °C until use.

2.4. 800 bp DNA standard used for the Vero DNA sizing assay

The 800 bp DNA standard used for Vero DNA sizing was obtained by PCR amplification of a 800 bp region targeting the β-actin gene from

Vero cells used for vaccine production. The 800 bp PCR product was further purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) and quantitated by Picogreen assay (Invitrogen, Carlsbad, California, USA). Aliquots of the 800 bp DNA standard were stored at below –60 °C until use.

2.5. Internal controls

For the Threshold® assay, all samples and negative controls (ZC) are assayed with and without a 50 pg DNA spiking. The spiking solution (1000 pg/mL) was a 5-fold dilution of High Calibrator in ZC. It was added into the samples and the ZC before DNA extraction.

For the qPCR, the internal control (IC) consisted of a 301 bp PCR product amplified from a plasmid preparation, further purified using PureLink Quick Plasmid MiniPrep Kit (Invitrogen, Carlsbad, California, USA) and quantitated by UV spectrophotometry at 260 nm. Aliquots of the IC were stored at below –60 °C until use. The sequence of the IC was designed *in silico* in order to be distinguishable from Vero DNA sequences. The sequence, including the primers and probe binding sites is detailed in **Figure S1**.

All samples were assayed with a spike of 5×10^4 copies of the IC before DNA extraction. The IC was then quantitated in parallel to Vero DNA by qPCR using a homologous standard range to determine the recovery of the spike, individually for each sample.

2.6. Vero alpha-satellite qPCR

2.6.1. Procedure

The quantitation of residual Vero DNA from extraction samples was based on the quantitation of a target sequence derived from the alpha-satellite sequence. DNA extraction efficiency was monitored by the addition of a known amount of the IC during the extraction procedure. The extraction efficiency for each sample was calculated from the post-extraction quantitation of the IC by qPCR, from which a correction factor was determined and used to provide a corrected value for residual Vero DNA present in the sample.

The sequences of the forward and reverse primers, and the Minor Groove Binder (MGB) probe used for the quantitation of Vero cell DNA are presented in Table S1. The forward primer (αFP90) and the probe (αMGB) were the same as those designed by Lebron et al. (Lebron et al., 2006). The reverse primer (αRP90) was designed in-house to get an amplified product of 90 bp (accession number GenBank: M26844.1). The IC forward (FPMGB) and reverse primers (RPMGB) and the probe (MGBCI) are also presented in Table S1.

The qPCR assays were undertaken in 50 µL volumes in 96-well plates (Multiwell Plate 96). For the quantitation of Vero cell DNA, the qPCR mix contained: 10 µL of extracted sample; 25 µL of 2X QuantiTect Probe PCR Master Mix (Qiagen, Hilden, Germany); 0.3 µL forward (30 µM) and 0.3 µL reverse (30 µM) primer, 0.4 µL αMGB probe (25 µM) and 14 µL nuclease-free water. For the IC the qPCR mix contained: 10 µL of extracted sample; 25 µL of 2X QuantiTect Probe PCR Master Mix; 0.5 µL forward (30 µM) and 0.5 µL reverse (30 µM) primer, 1 µL MGBCI probe (5 µM) and 13 µL nuclease-free water. Nuclease-free water was used as the negative control. The standard curve for the Vero DNA standard range consisted of duplicates of 10-fold serial dilutions of standard Vero DNA, from 1000 pg/reaction down to 0.01 pg/reaction, prepared in nuclease-free water and 10 µL of each dilution tested as already described. The standard curve for IC was prepared similarly to obtain dilutions from 10^6 copies/reaction down to 10^2 copies/reaction, and tested as already described. qPCR was performed in the LightCycler® 480 Real Time PCR system (Roche Molecular Diagnostics, California, USA). The thermocycler conditions were 95 °C for, followed by 40 cycles of 95 °C for and 60 °C for.

The quantitation of residual Vero DNA by qPCR was based on the absolute quantification module of the LightCycler 480 software. The standard curves of the Vero DNA standard and the IC standard were generated by plotting the log of the DNA concentration of the known standards against the crossing point (Cp) values. Quantitation of Vero rcDNA and IC DNA in the samples was determined by extrapolation from the standard curves.

2.7. Threshold® assay

2.7.1. Procedure

The total DNA assay was performed using the Threshold® Total DNA Assay Kit according to manufacturer's instructions (Molecular Devices, California, USA). Briefly, the total DNA assay is specific for single-stranded DNA (ssDNA) obtained by denaturation at + 100 °C. The ssDNA reacts with two binding proteins: a single-stranded DNA binding protein (SSB) conjugated to biotin and a monoclonal anti-DNA antibody (Mab Anti-DNA) conjugated to urease. Streptavidin, also present, binds to the biotin on the SSB conjugate. These binding proteins form a complex with ssDNA.

The DNA complex is filtered through a biotinylated nitrocellulose membrane. The biotin on the membrane captures the ssDNA complex by the streptavidin. A wash step removes excess enzyme. Non-specific binding is avoided by the use of nitrocellulose membrane precoated with albumin. The membrane is submerged in urea solution. Urease activity changes the pH of the substrate solution, which is recorded by micro-sensor in µV/sec. The standard curve was generated by plotting the log of the DNA amount of High Calibrator (pg) from the Threshold® Total DNA Assay Kit against the urease activity (µV/sec). Quantitation of Vero rcDNA in the samples was determined by extrapolation from the standard curve. The range of the standard curve is 3.1 to 200 pg.

2.8. Hybridization assay

2.8.1. Preparation of the probe

Vero cell genomic DNA (0.15 mg) was digested in 500 µL nuclease-free water containing 50 µL reaction buffer 2 (10X) (Gibco BRL, New York, USA) and 3 µL restriction enzyme HaeIII (50 U/µL) (Gibco BRL) in a water bath at 37 °C for at least. Digested Vero cell genomic DNA (10 µg) was labeled with digoxigenin using the DIG DNA Labeling and Detection Kit according to manufacturer's instructions (Roche, Basel, Switzerland). Vero DNA labeled probe was further purified using lithium chloride precipitation and resuspended at a final concentration of 60 µg/mL in 1X Tris-EDTA (10 mM Tris; 1 mM EDTA).

2.8.2. Procedure

Briefly, the heat-denaturated extracted DNA in the test product, negative control (of nuclease-free water), positive extraction control (from 500 µL of a solution of Vero cell DNA at 200 pg/mL) and Vero DNA standards (10,000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 100 pg/mL and 50 pg/mL) were slot-blotted (100 µL) onto the same nylon membrane (Amersham, Amersham, UK) under vacuum. DNA was fixed onto the membrane for 2 hours at + 80 °C. Membrane-bound DNA was hybridized to the Vero DNA labeled probe using DIG DNA Labeling and Detection Kit according to manufacturer's instructions (Roche, Basel, Switzerland). The concentration of Vero cell DNA in the sample was determined by visual comparison of the color intensities of the slots with those of the Vero DNA standards.

2.9. rcDNA size distribution assay

The assessment of the size distribution of residual Vero DNA from extracted test samples was based upon the amplification by qPCR of five overlapping regions of increasing sizes targeting the β-actin gene. The target gene sequence (nearly 1138 bp) is of sufficient length to allow for a set of amplicons that span a range from 59 bp to 620 bp (**Figure S2**). Primer pairs for the various sized and overlapping amplicons were designed using Primer Express 1.0 (Applied Biosystems, Foster City, California, USA) and assessed for amplification of Vero sequences

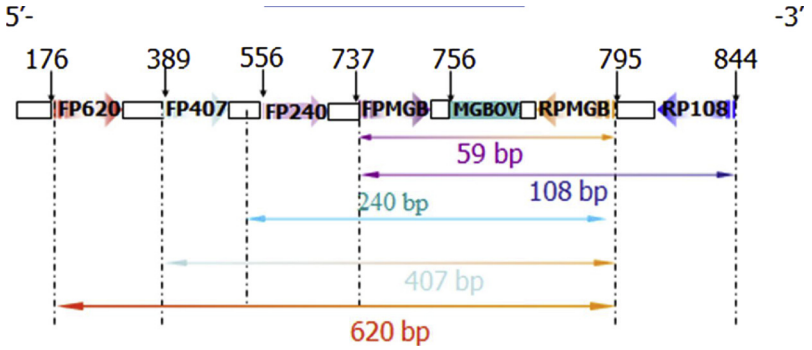

Fig. 1. β-actin gene sequence and position of the forward and reverse primers for each Vero sizing fragment.

Table 1
Details of primers and probe sequences used in the determination of residual Vero DNA size distribution.

Primers and Probe names	Direction	Sequence (5'→3')	Amplified product size
Primer			
FPMGB	Forward	CCC GAT GGC CAG GTC A	59 bp
RPMGB	Reverse	AAG AGA GCC TCA GGG CAG C	
FPMGB	Forward	CCC GAT GGC CAG GTC A	108 bp
RP108	Reverse	GGT AGT TTC ATG GAT GCC ACA G	
FP240	Forward	CCG GGA CCT GAC TGA CTA CC	240 bp
RPMGB	Reverse	AAG AGA GCC TCA GGG CAG C	
FP407	Forward	TTC AAC ACC CCA GCC ATG T	407 bp
RPMGB	Reverse	AAG AGA GCC TCA GGG CAG C	
FP620	Forward	GAT GAG GCC CAG AGC AAG AG	620 bp
RPMGB	Reverse	AAG AGA GCC TCA GGG CAG C	
Probe			
MGBOV		FAM-CCA TTG GCA ATG AGC GG-MGB-NFQ	

bp, base pairs.
FAM, 6- carboxyfluorescein label.
NFQ, NonFluorescent Quencher.
MGB, Minor Groove Binder.

(accession number GenBank: AB004047.1). Fig. 1 shows the β-actin gene sequence and the binding sites of the forward and reverse primers for each Vero DNA sizing fragment and the probe used. The sequences of the forward and reverse primers of the β-actin gene used for the five Vero DNA size fragments assessed and MGBOV probe are presented in Table 1.

DNA extraction efficiency was monitored by the addition of a known amount of the IC during the extraction procedure as described above. The positive control consisted of 500 copies/reaction of 800 bp DNA standard and was tested for each size in order to determine the actual PCR efficiencies. The Vero DNA quantities determined for each sample for all the sizes using the Vero DNA standard curve using the 59 bp amplification system were then corrected by taking into account the extraction recovery calculated for the IC and the PCR efficiency of the positive control.

The qPCR assays were undertaken in 25 µL volumes in 96-well plates (Multiwell Plate 96). The reaction mix for each of the β-actin gene fragments per well contained: 5 µL sample; 12.5 µL of 2X QuantiTect Multiplex; 0.35 µL forward (30 µM) and 0.35 µL reverse (30 µM) primer for the respective β-actin gene fragment sizes; 1 µL MGBOV probe (5 µM); and 5.8 µL nuclease-free water. For the standard curve, serial 10-fold dilutions of 800 bp DNA standard were prepared in nuclease-free water and a fixed volume (5 µL) of each dilution (10^5 to 10 copies per reaction) was tested at the 59 bp fragment size, as described for the β-actin gene above. The IC standard curve was prepared as described above. The reaction mix per well contained: 5 µL sample; 12.5 µL of 2X QuantiTect Multiplex; 0.35 µL forward (30 µM) and 0.35 µL reverse (30 µM) primer for the respective β-actin gene fragment sizes; 0.8 µL MGBCI probe (5 µM); and 6 µL nuclease-free water. Nuclease-free water (5 µL) was used as the negative control. The thermocycler conditions were 95 °C for 15 minutes, followed by 45 cycles of 94 °C for 1 minute and 60 °C for 1 minute in the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, California, USA). Quantitation of DNA in the samples assessed was determined using SDS software by extrapolation from the standard curves as described for alpha-satellite sequence qPCR. Test results provide a ratio of each amplicon relative to the shortest. This ratio is proportional to the fraction of residual Vero DNA greater than 59 bp (the value of total residual DNA) that is also greater than the size of the target amplicon.

2.10. Validation of qPCR (Vero alpha-satellite) and Threshold® assay

The accuracy, precision, repeatability, intermediate precision, specificity, quantitation limit, linearity and range of the methods for quantitative detection of rcDNA were validated according to the International Conference on Harmonisation (ICH) tripartite guidelines Q2(R1) (ICH, 2005).

3. Results

3.1. Vero alpha-satellite qPCR validation

For highly purified vaccines containing very low levels of rcDNA, the most appropriate stage to quantify impurities, including rcDNA, is the most concentrated purified stage before formulation, i.e. the drug substance. Table 2 shows the validation results for three vaccines at the drug substance stage as examples; rabies vaccine produced in a RMAO-free process, dengue vaccine and IPV. The rcDNA specification in the rabies vaccine produced in a RMAO-free process at the drug substance

Table 2
Results of validation characteristics for three vaccines assessed by Vero alpha-satellite qPCR assay.

Target	Specificity	Linearity	Linearity domain	Accuracy	Precision	Limit of quantitation
DS RMAO-free rabies vaccine	Specificity of the set of primers and probe was verified using BLASTn showing 100% identity with Vero alpha sequence DNA only. DNA from PER.C6, CHO, CEF and Vero cells were spiked at 10^5 pg/mL. The assay result is < 1 pg/mL for the PER.C6, CHO and CEF cells demonstrated a satisfactory specificity of the detection system	P (linearity) < 0.0001 P (lack of fit) > 0.07 R^2 = 09965	0.40–1084 pg/mL	The recovery is 77–90%	• 95% CI of repeatability for one measurement: x/÷ 1.28 • 95% CI of intermediate precision for one run with one measurement: x/÷ 1.46	• 0.40 pg/mL • 95% CI of repeatability for one measurement: x/÷ 1.24 • 95% CI of intermediate precision for one run with one measurement x/÷ 1.58
DS Dengue vaccine		P (linearity) < 0.0001 P (lack of fit) > 0.05 R^2 = 09937	0.50–652 pg/mL	The recovery is 83–104%	• 95% CI of repeatability for one measurement = x/÷ 1.42 • 95% CI of intermediate precision for one run with one measurement = x/÷ 1.60	• 0.50 pg/mL • 95% CI of repeatability fo one measurement = x/÷ 1.25 • 95% CI of intermediate precision for one run with one measurement = x/÷ 1.99
DS IPV		P (linearity) < 0.0001 P (lack of fit) > 0.3 R^2 = 09978	0.60–1743 pg/mL	The recovery is 94–128%	• 95% CI of repeatability for one measurement = x/: 1.3 • 95% CI of intermediate precision for one run with one measurement = x/÷ 1.57	• 0.58 pg/mL • 95% CI of repeatability fo one measurement = x/÷ 1.36 • 95% CI of intermediate precision for one run with one measurement = x/÷ 1.41

CI, Confidence Interval; DS, Drug Substance; IPV, Inactivated Poliovirus Vaccine; RMAO, Raw Material of Animal Origin; CEF, Chicken Embryo Fibroblast; CHO, Chinese Hamster Ovary; PER.C6, Human Embryonic Retinoblasts.

stage is ≤666 pg/mL (corresponding to ≤100 pg/dose). For the dengue vaccine, the rcDNA specification at the drug substance stage is ≤330 pg/mL (corresponding to ≤100 pg/dose), and that for the IPV at the drug substance stage is ≤1000 pg/mL (corresponding to ≤100 pg/dose).

3.1.1. Rabies vaccine produced in a RMAO-free process

The method was shown to be specific and linear over the range 0.40–1084 pg/mL, and accurate over the same range, with a recovery of 77–90%. The method was precise with 95% confidence intervals (CI) of repeatability and intermediate precision for one run with one measurement equal to x/÷ 1.28 and x/÷ 1.46, respectively. The limit of quantitation (LOQ) was defined as 0.40 pg/mL as the lower level at which the method was precise and accurate. The 95% CI of intermediate precision at the LOQ of 0.40 pg/mL for one run with one measurement was equal to x/÷ 1.58.

3.1.2. Dengue vaccine

The method was found to be specific and linear over the range 0.50–652 pg/mL, and accurate over the same range, with a recovery of 83–104%. The method was precise with 95% CI of repeatability and intermediate precision for one run with one measurement equal to x/÷ 1.42 and x/÷ 1.60, respectively. The LOQ was defined at 0.50 pg/mL as the lower level at which the method was precise and accurate. The 95% CI of intermediate precision at the LOQ of 0.50 pg/mL for one run with one measurement was equal to x/÷ 1.99.

3.1.3. Inactivated Poliovirus Vaccine

The method was found to be specific and linear over the range 0.60–1743 pg/mL, and accurate over the same range, with a recovery of 94–128%. The method was precise with 95% CI of repeatability and intermediate precision for one run with one measurement equal to x/÷ 1.30 and x/÷ 1.57, respectively. The LOQ was defined at 0.60 pg/mL as the lower level at which the method was precise and accurate. The 95% CI of intermediate precision at the LOQ of 0.60 pg/mL for one run with one measurement was equal to x/÷ 1.41.

3.2. Threshold® assay validation

The Threshold® assay was considered specific and linear over the range 230–1840 pg/mL for the assessment of IPV, and accurate over the same range, with an average recovery of 95% (Table 3). The method was precise with 95% CI of repeatability and intermediate precision for two runs with one measurement equal to x/÷ 1.21 and x/÷ 1.20, respectively. The LOQ was defined at 230 pg/mL as the lower level at which the method was precise and accurate. The 95% CI of intermediate precision at the LOQ of 230 pg/mL for two independent runs with one measurement was equal to x/÷ 1.31.

3.3. Comparison of the performances of Vero alpha-satellite qPCR and Threshold® assays

Table 3 compares the IPV validation results obtained with the Threshold® assay versus those obtained with Vero alpha-satellite qPCR. The upper limit of the linearity domain were validated at a similar level between the two assays, but the lower end was much lower for qPCR (about 400-fold lower) than the Threshold® assay.

3.4. Comparison between the hybridization, Threshold® and qPCR assays

Comparison of rcDNA content measured in rabies vaccines from three competitors compared with one batch of VERORAB® using the three DNA quantitation assays is summarized in Table 4. These results confirm that the quantity of rcDNA determined may be influenced by the analytical method used; in three of the five vaccines from competitors, qPCR appears to measure higher rcDNA content than the

Table 3
Inactivated Poliovirus Vaccine (IPV): a comparison of the total DNA assay by Threshold® method versus Vero alpha-satellite qPCR.

Characteristics	Total DNA assay by Threshold® Method	qPCR method
Specificity	Specific of ssDNA	Specific of Vero DNA
Accuracy	90–100%	94–128%
Precision (repeatability)	Theoretical level at 1000 pg/mL: - 95% CI of repeatability: ± 0.084, that is x/÷ 1.21 in arithmetic form	Theoretical level at 1200 pg/mL (120% of the specification) - 95% CI of repeatability for one measurement: ± 0.115 that is x/÷ 1.30 in arithmetic form
Precision (intermediate precision)	- Theoretical level at 1000 pg/mL: - 95% CI of intermediate precision for 2 runs with one measurement: ± 0.079, that is x/÷ 1.20 in arithmetic form	- Theoretical level at 1200 pg/mL: 120% of the specification - 95% CI of intermediate precision for one run with one measurement: ± 0.196 that is x/÷ 1.57 in arithmetic form
Linearity	P-linearity < 0.0001 Y = -0.180 + 1.055. X Where X = theoretical expected concentration of total DNA (log(pg/mL)) and Y = measured concentration of total DNA (log(pg/mL)) R^2 = 0.9526	P-linearity < 0.0001 Y = 0.083 + 0.967 X Where X = theoretical expected concentration of Vero DNA (log(pg/mL)) and Y = measured concentration of Vero DNA (log(pg/mL)) R^2 = 0.9978
Range (Linearity domain)	230–1840 pg/mL	0.60–1743 pg/mL
Quantitation limit	- LOQ = 230 pg/mL - Mean percent recovery at the LOQ: 91% - 95% CI of intermediate precision for 2 runs with one measurement: x/÷ 1.31	- LOQ = 0.60 pg/mL - Mean percent recovery at the LOQ: 114% - 95% CI of intermediate precision for one run with one measurement: x/÷ 1.41

LOQ, Limit Of Quantitation; qPCR, quantitative Polymerase Chain Reaction; CI, Confidence Interval; IPV, Inactivated Poliovirus Vaccine; ssDNA, single-stranded DNA.

Threshold® assay. In four of the five vaccines from competitors, qPCR measured higher rcDNA than hybridization assay, for the fifth vaccine from competitors the results were comparable. When size distribution was compared, one vaccine from the competitors was reported to have rcDNA fragments of 407 and 620 bp. The Sanofi Pasteur rabies vaccine was found to have fragmented rcDNA ranging 59–240 bp, with 81% of rcDNA less than 108 bp.

3.5. Effect of viral BPL inactivation on rcDNA content and fragment size distribution

Inactivation of Sanofi Pasteur's Vero cell rabies vaccine (VERORAB®) with BPL resulted in an average 30% reduction in rcDNA content (Table 5). Though some variability was observed among the individual harvests, there was no significant effect according to individual harvests or by batch. BPL inactivation resulted in marked reduction in rcDNA content of fragments 240 bp or larger; of note, the rcDNA content of fragments 108 bp or greater in harvests H2 and H3 were reduced by more than 50% (Table 6). Similarly, for Sanofi Pasteur's Vero cell RMAO-free rabies vaccine, inactivation with BPL resulted in an average reduction in rcDNA content of 46% (Table 7). Again, no significant batch effect was observed. It was not possible to determine any effect on RMAO-free rabies vaccine rcDNA fragments content due to the very low level of rcDNA.

Table 5
Effect of BPL inactivation on rcDNA content in intermediate stages of VERORAB® Drug Substance[a].

Product	Harvest number	Concentration of rcDNA (µg/mL)		Reduction with BPL (%)
		Before inactivation with BPL	After inactivation with BPL	
Rabies vaccine (VERORAB®) #1	H1	5.9	3.4	42
	H2	6.0	6.7	–13
	H3	5.1	4.6	12
	H4	4.2	2.5	42
	H5	1.7	1.5	12
	H6	0.9	0.7	15
Rabies vaccine (VERORAB®) #2	H1	10.1	6.5	36
	H2	13.7	8.2	40
	H3	2.7	1.8	32
	H4	2.8	1.8	35
	H5	1.0	0.1	85
	H6	0.7	0.6	17
Rabies vaccine (VERORAB®) #3	H1	7.6	6.9	9
	H2	12.2	11.2	9
	H3	8.5	5.1	40
	H4	3.2	2,6	17
	H5	1.3	1.4	–4
	H6	1.0	0.7	36

BPL, β-propiolactone.
[a] The average reduction in rcDNA content was 30% and there was no significant effect according to individual harvests or by batch.

4. Discussion

The analytical validation of our Vero alpha-satellite qPCR assay performed on three different vaccines shows that the method is robust

Table 4
Comparison of results obtained using three methods (hybridization, Threshold® and Vero alpha-satellite qPCR assays) on rabies vaccines from several manufacturers.

Product name	Hybridization	Threshold®	qPCR	rcDNA size distribution assessment by qPCR (%)				
	pg/dose	pg/dose	pg/dose	59 bp	108 bp	240 bp	407 bp	620 bp
Rabies vaccines produced on Vero cells from competitors								
Rabies Vaccine for human use (Vero cell) #1	750–3000	73,470	731,000	100	40	< 1	ND	ND
Rabies Vaccine for human use (Vero cell) #2	< 100	105,980	9,300	100	20	14	ND	ND
Rabies Vaccine for human use (Vero cell) #3	< 100	2,000–6,000	10,800	100	2	< 1	ND	ND
Rabies Vaccine for Human use (Vero cell) #4	< 100	91,130	1,200	100	27	< 8	ND	ND
Rabies Vaccine for human use (Vero cell) #5	600,000	147,450	584,000	100	82	56	MD	84
Rabies vaccine produced on Vero cells from Sanofi Pasteur								
VERORAB®	100	5,300	7,470	100	19	6	ND	ND

qPCR, quantitative Polymerase Chain Reaction; bp, base pairs; ND, Not Detected; MD, Missing Data.

Table 6
Effect of BPL inactivation on rcDNA fragment size distribution in intermediate stages of VERORAB® Drug Substance.

Harvest	Size (bp)	rcDNA size distribution	
		Before inactivation with BPL (%)	After inactivation with BPL (%)
H1	≥59	100	100
	≥108	56	53
	≥240	31	< 3
	≥407	30	< 3
	≥620	7	ND
H2	≥59	100	100
	≥108	97	43
	≥240	50	7
	≥407	22	1
	≥620	3	ND
H3	≥59	100	100
	≥108	108	34
	≥240	56	3
	≥407	55	< 1
	≥620	28	ND

BPL, β-propiolactone; ND, Not detected.

Table 7
Effect of BPL inactivation on rcDNA content in intermediate stages of RMAO-free rabies vaccine Drug Substance[a].

Product	Batch number	Concentration of rcDNA (pg/mL)		
		Before inactivation with BPL	After inactivation with BPL	Reduction with BPL (%)
Rabies vaccine produced in a RMAO-free process	1	18	11	39
	2	27	12	54
	3	50	27	46

BPL, β-propiolactone; RMAO, Raw Material of Animal Origin.
[a] The average reduction in rcDNA content was 46% and there was no significant effect according to individual harvests or by batch.

in terms of performance, sensitivity, specificity, and precision, with a large dynamic range and no matrix interference; as such it would be applicable for use on a wide range of vaccines produced on Vero cells including live vaccines and formaldehyde or BPL-inactivated vaccines. The qPCR method was specifically adapted to low DNA content in highly purified vaccines, and should be preferred over the hybridization or the Threshold® assays for the quantitation of rcDNA in vaccines produced in Vero cells. The only relative limitation of our qPCR method is that residual DNA of a length less than the amplicon size cannot be detected; nonetheless, the smallest amplicon size assessed for the alpha-satellite sequence was 90 bp and smaller fragments are not expected to pose a significant safety risk.

We recommend the use of species-specific qPCR (such as the Vero alpha-satellite sequence in our case) rather than generic qPCR methods that target genes common to cells of animal origin for the assessment of rcDNA in biopharmaceuticals produced in mammalian systems. It is of importance that the DNA standard used to elaborate the standard curve is homologous to the rcDNA present in the product, specifically when repeated sequences are targeted. Indeed, the number of repeats of the universal target genes may vary from one species to another. If the number of target sequences per genome is not the same in the DNA standard as in the rcDNA from the product, the relative quantitation will be biased.

Generic qPCR assays may also be associated with a high risk of contamination increasing the difficulty in managing negative controls, as well as high background measurements, thus reducing the sensitivity of the assay. For example, a universal qPCR described by Andre et al. was reported to have a LOQ of 5 pg per reaction (i.e. 1000 pg/mL or 500 pg/dose) (Andre et al., 2016) compared with 0.40–0.60 pg/mL with our Vero alpha-satellite qPCR assay (depending on the vaccines tested). Therefore, the former is not sufficiently sensitive for detection of rcDNA in highly purified vaccines or biologicals produced in Vero cells such as IPV for which the specification is ≤100 pg/dose (European Pharmacopoeia, Monograph 0214, 2019a). We were interested in developing the most sensitive method possible for amplification of the smallest DNA fragment targets in highly purified vaccines. To further improve the sensitivity of the assay, it is highly recommended that the rcDNA be quantitated at the most concentrated purified stage prior to final formulation, where the DNA is concentrated. Some products contain very low levels of rcDNA (e.g. less than 1 pg/mL in the Drug Substance); in these cases, it is impossible to evaluate the size of the DNA fragments with the current best performing assays such as qPCR.

The three vaccines involved in the analytical validation studies (rabies vaccine produced in a RMAO-free process, dengue vaccine and IPV), contained only small amounts of rcDNA (approximately 40 pg/mL, < 0.50 pg/mL and < 0.60 pg/mL, respectively), which is far below the limit of detection of the universal qPCR method described by Andre et al (Andre et al., 2016). It is our opinion that it would not be appropriate to select a method that is not at least as sensitive as the prescribed specifications for each specific vaccine. Even when rcDNA is detectable, the size evaluation of the DNA fragments cannot be limited to a size assessment above 200 bp and below 200 bp, as described by Andre et al. (2016). A risk-based assessment of acceptable limits for rcDNA should be made on a case-by-case basis and agreed in consultation with regulatory authorities, taking into account cell substrate phenotypic characteristics, method of intended delivery, the manufacturing process and the size, quantity and biological activity of rcDNA fragments as well as the design and performances of the analytical method used to quantitate the rcDNA (European Pharmacopoeia, General Chapter 5.2.3, 2018; WHO, 2013)

The rcDNA content measured is dependent on the analytical methods used, as clearly demonstrated by the direct comparison of the hybridization assay, the Threshold® assay and qPCR during a benchmarking assessment comparing Sanofi Pasteur's rabies vaccine (VERORAB®) with rabies vaccines from five competitors. In all cases there was poor correlation between methods (hybridization, Threshold® and qPCR). The results obtained using hybridization underestimate the rcDNA content compared to the qPCR method. The results highlight the need to select a method able to detect small fragments of DNA. Based on these limitations and previously documented lack of robustness of hybridization methods (Robertson and Heath, 1992), the waiving of this method from compendial monographs should be considered. Moreover, the rcDNA for a given vaccine was reported to be higher with qPCR than reported with the Threshold® assay. Where the rcDNA content reported with the Threshold® assay was found to be higher than with qPCR in rabies vaccines from competitors, in this particular case, complementary analyses demonstrated the presence of mycoplasma DNA in the vaccines that contributed to total DNA content measured with the Threshold® assay (data not shown). These results highlight the need for a highly specific method for assessing rcDNA from Vero cells and not DNA from other sources.

The qPCR method is also useful in documenting the performances of the purification/inactivation process as it allows the further characterization of the remaining rcDNA to assure product safety. We found that inactivation of Sanofi Pasteur's Vero cell rabies vaccines (VERORAB® and the rabies vaccine produced in a RMAO-free process) with BPL resulted in a marked reduction in rcDNA content and DNA fragments above 240 bp, suggesting that functional genes are unlikely to be present after BPL treatment. In addition, BPL alters the structure (Kubinski and Szybalski, 1975; Perrin and Morgeaux, 1995) and capability of rcDNA to be used as a template by various polymerases, further reducing the potential associated risk. The improved sensitivity achieved with qPCR allows rcDNA to be quantified and characterized in

samples from different bioprocessing stages of purification/inactivation that would otherwise be undetectable by other methods. Consequently, the qPCR method provides greater opportunity to measure rcDNA in highly purified vaccines in many cases to ≤10 pg per dose.

In summary, we have developed a qPCR assay specifically adapted for products with low amounts of fragmented rcDNA. This system uses a DNA standard purified from the Vero cell line, and is therefore representative of the actual residual DNA from these cell substrates. It is a robust method shown to be superior to previously used methods (Threshold® and hybridization assays). It is our opinion that universal PCR assays that target common generic genes should be avoided as they would be prone to high background signals, thus decreasing their sensitivity. Instead, it would be preferable to target species specific multi-copy sequences relevant to the cell substrate used as this increases sensitivity, specificity, and precision. Our proposed qPCR, targeting Vero alpha-satellite sequences, shows that it is possible to have a specific and sensitive validated method for Vero DNA across several viral vaccines, with a low LOQ.

In parallel, qPCR can be used for DNA sizing for complementary characterization of the product where applicable. Regulations and assays will continue to evolve to ensure the continued safety of viral vaccines produced on continuous cell lines. The development of new technologies including digital PCR will no doubt be a feature of this developing area.

Conflicts of interest

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2019.01.001.

References

Andre, M., Reghin, S., Boussard, E., Lempereur, L., Maisonneuve, S., 2016. Universal real-time PCR assay for quantitation and size evaluation of residual cell DNA in human viral vaccines. Biologicals 44, 139–149.

Chinese Pharmacopoeia, 2015. Chapter 3407. Determination of Residual Extraneous DNA.

EMEA, 1997. European Medicines Evaluation Agency. CPMP/BWP/382/97 – CPMP Position Statement on DNA and Host Cell Proteins (HCP) Impurities, Routine Testing Versus Validation Studies.

EMEA, 2001. European Medicines Evaluation Agency. CPMP/BWP/1143/00 - CPMP Position Statement on the Use of Tumorigenic Cells of Human Origin for the Production of Biological and Biotechnological Medicinal Products.

European Pharmacopoeia, 2018. General Chapter 5.2.3. Cell Substrates for the Production of Vaccines for Human Use. Strasbourg, France.

European Pharmacopoeia, 2019a. Monograph 0214. Poliomyelitis Vaccine (inactivated). Strasbourg, France.

European Pharmacopoeia, 2019b. Monograph 1056. Hepatitis B Vaccine (rDNA). Strasbourg, France.

FDA, 2001. Food and Drug Administration. Letter to Inform Manufacturers of the Following Interim Recommendations Pertaining to Viral Vaccine Products That Are Produced in Vero Cells and Investigated for Human Use.

FDA, 2010. Food and Drug Administration. Guidance for Industry - Characterization and Qualification of Cell Substrates and Other Biological Materials Used in the Production of Viral Vaccines for Infectious Disease Indications. U.S. Department of Health and Human Services Food and Drug Administration. Center for Biologics Evaluation and Research.

ICH, 2005. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) Harmonised Tripartite Guideline - Validation of Analytical Procedures: Text and Methodology Q2(R1).

King, R.S., Panfili, P.R., 1991. Influence of fragment size on DNA quantitation using DNA-binding proteins and a sensor-based analytical system: applications in the testing of biological products. J. Biochem. Biophys. Methods 23, 83–93.

Kubinski, H., Szybalski, E.H., 1975. Intermolecular linking and fragmentation of DNA by beta-propiolactone, a monoalkylating carcinogen. Chem. Biol. Interact. 10, 41–55.

Lebron, J.A., Troilol, P.J., Pacchione, S., Griffiths, T.G., Harper, L.B., Mixson, L.A., Jackson, B.E., Michna, L., Barnum, A.B., Denisova, L., Johnson, C.N., Maurer, K.L., Morgan-Hoffman, S., Niu, Z., Roden, D.F., Wang, Z., Wolf, J.J., Hamilton, T.R., Laux, K.M., Soper, K.A., Ledwith, B.J., 2006. Adaptation of the WHO guideline for residual DNA in parenteral vaccines produced on continuous cell lines to a limit for oral vaccines. Dev. Biol. (Basel) 123, 35–44 discussion 55-73.

Perrin, P., Morgeaux, S., 1995. Inactivation of DNA by beta-propiolactone. Biologicals 23, 207–211.

Robertson, J.S., Heath, A.B., 1992. A collaborative study to examine the sensitivity and reproducibility of assays for the detection of DNA in biologicals derived from continuous cell lines. Biologicals 20, 73–81.

Sheng-Fowler, L.L., Lewis Jr, A.M., Peden, K., 2009. Issues associated with residual cell-substrate DNA in viral vaccines. Biologicals 37, 190–195.

Sheng-Fowler, L., Cai, F., Fu, H., Zhu, Y., Orrison, B., Foseh, G., Blair, D.G., Hughes, S.H., Coffin, J.M., Lewis Jr, A.M., Peden, K., 2010. Tumors induced in mice by direct inoculation of plasmid DNA expressing both activated H-ras and c-myc. Int. J. Biol. Sci. 6, 151–162.

USP, 2018. United States pharmacopeia. General Information Chapter < 1130 > Nucleic Acid-based Techniques - Approaches for Detecting Trace Nucleic Acids (residual DNA Testing). Rockville, Maryland, USA, 41st edition. .

Wang, X., Morgan, D.M., Wang, G., Mozier, N.M., 2012. Residual DNA analysis in biologics development: review of measurement and quantitation technologies and future directions. Biotechnol. Bioeng. 109, 307–317.

WHO, 1987. World Health Organization, Acceptability of Cell Substrates for Production of Biologicals. Report of a WHO Study Group on Biologicals. Technical Report Series 747. pp. 1–29.

WHO, 1998. World Health Organization, Expert Committee on Biological Standardization. Requirements for the Use of Animal Cells As in Vitro Substrates for the Production of Biologicals. WHO Technical Report Series 878. Forty-seventh Report. Annex 1. .

WHO, 2007. World Health Organization (WHO), Guidelines To Assure The Quality, Safety And Efficacy Of Live Attenuated Rotavirus Vaccines (Oral), Technical Report Series 941. Annex 3. .

WHO, 2013. World Health Organization, Expert Committee on Biological Standardization. Recommendations for the Evaluation of Animal Cell Cultures As Substrates for the Manufacture of Biological Medicinal Products and for the Characterization of Cell Banks. Technical Report Series 978. Sixty-first Report. Annex 3. .