Absolute quantification of Bovine Viral Diarrhea Virus (BVDV) RNA by the digital PCR technique

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Abstract. The quality control of cell lines used in research and industry is critical to ensure confidence in experimental results and to guarantee the safety of biopharmaceuticals to consumers. The BVDV is a common adventitious agent in many cell lines. We preliminarily evaluate the use of Digital Droplet PCR (ddPCR) for the detection and enumeration of genome copies of BVDV in cell culture and on FBS. The application of a commercial Real-Time PCR kit with the ddPCR technique was successful on different matrices. The technique allowed the absolute quantification of the genome without the use of calibration standards, suggesting its promising application on the development of reference materials for quantification of nucleic acids.

1. Introduction

With the uprise of therapeutic use of biopharmaceuticals produced in cell culture, the quality control of the producing cell lines assumed paramount importance to ensure its safety for users. The Bovine Viral Diarrhea Virus (BVDV) is an adventitious agent often associated with cell lines maintained in vitro [1] being propagated in culture during handling or introduced through the use of animal raw materials (fetal bovine serum, albumin, etc.) [2]. The BVDV infection on cells is often undetected [3], as sometimes it does not induce cytopathogenic changes.

Digital PCR is an emerging analytical technique used for the detection and quantification of specific sequences of nucleic acids. It can be performed without the use of any calibrant, having the potential to be considered as a primary method [4]. According to some National Metrology Institutes (NMI), when digital PCR is applied to the quantification of gene sequences or to the certification of DNA reference materials, the measurement values can be made traceable to the International System (SI), along with its associated uncertainty [5].

In this work we made a preliminary evaluation on the use of Digital Droplet PCR (ddPCR) for quantification of BVDV genome copy numbers on a commercial detection kit control, on cell culture supernatants used for virus propagation and on raw materials (fetal bovine serum) employed for the maintenance of cells in culture.
2. Materials and Methods

2.1. BVDV samples on different matrices
For virus on cell culture, supernatant of MDBK cells infected with the BVDV cytopathogenic strain NADL was collected. This material was produced at the Veterinary School of the Federal University of Minas Gerais - UFMG. For virus on fetal bovine serum (FBS), serum of naturally infected animal was used.

2.2. Viral RNA extraction
Total RNA from 500 µL supernatant of BVDV infected cell culture and from 500 µL FBS were extracted with Trizol, according to manufacturer’s instructions (Life Technologies, USA). Concentration and overall purity of RNA was estimated, respectively, by UV spectrophotometry at 260 nm and by the ratio of readings at 260 nm and 280 nm.

2.3. Controls
Purified BVDV RNA from TaqMan BVDV and Xeno RNA Kit (Applied Biosystems, USA) was used as positive controls and Milli-Q water as negative control.

2.4. cDNA synthesis and ddPCR
For cDNA synthesis, RNA extracted from cell culture-produced BVDV (8 µL), from FBS (8 µL) and from positive control (2 µL) were processed according the instructions of the High Capacity kit (Applied Biosystems, USA). As negative controls, 8 µL of Milli-Q water was processed in parallel.

A 2 µL aliquot of the cDNA from the reverse transcription step of samples and controls were used for PCR amplification with the ddPCR Supermix for Probes kit (Bio-Rad, USA).

In all cases, the oligos used for cDNA synthesis, cDNA amplification and the FAM-labeled probes used for detection were from TaqMan BVDV kit (Applied Biosystems, USA).

3. Results and Discussion
The extraction, purification and cDNA synthesis scheme used for preparation of cDNA samples and controls proved to be valid. Also, the use of the High Capacity system with the TaqMan BVDV oligos along with ddPCR amplification with the same oligos allowed the detection of PCR products by the FAM probes included on the TaqMan BVDV system.

For the amplification step itself, given the composition differences among ddPCR Supermix and Real-Time PCR mixes, even an already standardized reaction would require further optimization. Thus, for the use of TaqMan BVDV oligos and probe on ddPCR, the optimum annealing temperature (60 °C) coincided with the one recommended by the manufacturer of the detection kit (Figure 1).

![Picture of Figure 1](image-url)

**Figure 1** – Optimization of the ddPCR annealing temperature for BVDV cDNA using oligos and TaqMan probe from TaqMan BVDV. In a 54-64°C temperature gradient, 60°C was the optimum temperature for amplification. Annealing temperature versus number of FAM-labeled copies of BVDV cDNA/µL.
Once optimized, the ddPCR was able to amplify the virus-derived cDNA in samples from cells, fetal bovine serum and on the positive control. The negative control, derived from a RT reaction with water, gave no amplification signal. In addition to detect the presence of BVDV in different matrices, the ddPCR technique could quantify the specific viral nucleic acid present in each sample (Figure 2).

![Digital Droplet PCR quantification of BVDV cDNA synthesized from various matrices. Viral RNA samples from fetal bovine serum (FBS) and from cell culture (Culture) were quantified with BVDV specific oligos and probes. As positive control, cDNA was prepared from TaqMan BVDV control RNA. Water was used as negative control. In a) number of FAM-labeled copies of BVDV cDNA/µL for tested samples and controls; in b) FAM fluorescence amplitude, which marks the difference between detection of BVDV and the lack of amplification signal for different samples and controls.](image)

The quantification of BVDV RNA through the use of oligos and probe from TaqMan BVDV kit plus ddPCR Supermix proved to be suitable to test for adventitious agents in cell cultures and in raw material used on cell cultivation.

4. Conclusion
The experiments, while preliminary, shown the feasibility of adoption of commercial Real-Time PCR detection kits on a ddPCR scheme for de quantification of pathogens based on nucleic acid detection.

The ddPCR technique is easy to perform, with the advantage that absolute quantification of nucleic acid, expressed as copy number / µL does not require standards, not always available for this type of material, and overcome the need of any method or calibration like ΔΔCq.

With the experiment described here, the ddPCR showed to be an adequate tool on the development of reference materials for nucleic acid quantification on different matrices.

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