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Digital droplet PCR for influenza vaccine development

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Abstract

Development of influenza vaccine processes requires virus quantification to optimize conditions in cell culture or in the associated downstream purification steps. Modern methods include qPCR, which utilizes TaqMan chemistry to detect and quantify viral RNA by comparison of a RNA standard of known concentration. Digital droplet PCR (ddPCR) is similar to qPCR in that it shares the same chemistry for nucleic acid detection. However, in ddPCR, the sample is diluted into partitions ('droplets') in order to separate and isolate single molecules. Upon PCR amplification, the droplet's fluorescent intensity depends on the presence or absence of the target; as such, positive and negative droplets are identified, which allows for absolute quantification of the viral genomes. The digital approach has enabled several key advantages. First, a standard is no longer required. Second, efficiency of the reverse transcription and the kinetics of the amplification, principles in qPCR, have no impact on the final digital PCR quantification. For this reason, the extracted RNA does not need to be purified from the reagents needed to lyse the virus. Also, viral associated RNA released by infected cells can be measured directly, further improving the quality of the data generated. Additional improvements to the approach include duplexing with a second assay that measures host cell DNA concentration. The method has been successfully implemented with automation in support of multiple upstream and downstream process development efforts for influenza vaccine manufacturing.

Selection and peer-review under responsibility of the 8th Vaccine Conference Organizing Committee.

Keywords: influenza; virus; vaccine; ddPCR; assay; development

1. Introduction

Viral titers are commonly determined by quantification of viral genomic fragments[1]. One such protocol, qPCR, requires that viral RNA be purified and concentrated by both column, centrifugal methods and extracted from cell

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harvest[2]. The recovered RNA is then retro-transcribed to cDNA and amplified by PCR. qPCR measures the cycle number at which the fluorescence, produced by a TaqMan probe, crosses a threshold identified as 'crossing point' or Ct value. A standard curve is generated by an independent serial dilution of known in vitro transcribed RNA (quantification of the standard RNA is based on optical density). The RNA concentration of the sample is extrapolated by comparing the Ct value of the sample and the Ct of the standard[3]. The precision of the assay is limited by the nature of the Ct determination (where precision is defined by ± one PCR cycle); the resulting value has a relative standard deviation that can exceed 50%. In addition, the assay workflow is complex with several steps introducing possibility for error including sample preparation, concentration, column RNA purification, and use of the RNA standard. Moreover, results obtained over time, using different instruments and standards are not comparable.

Digital droplet PCR (ddPCR) is similar to qPCR in that the approach is based on the detection of the amount of cDNA with a TaqMan probe, but the amplification is detected by a different method. The sample is diluted into partitions ('droplets') in order to separate and isolate single molecules (Figure 1A-B). A Poisson distribution analysis is used to determine the number of template molecules in a droplet. The value of $\ln(1-p)$, where p is the fraction of positive droplets, determines the number of target copies per droplet and thus the number of copies of target molecules in a 20 µl sample. Upon PCR amplification, the partitions with a fluorescent signal indicate the presence of the target (Figure 1C-D). The signal is generated by cleavage of a TaqMan probe, which has been designed to target the matrix (M) gene fragment of the viral RNA[4]. The first advantage of the ddPCR approach over qPCR is that absolute quantification is possible, so a standard is no longer required. Second, ddPCR only detects the presence or absence of the target; although the signal is generated by endpoint PCR, some degree of efficiency is required in order to distinguish the positive drops from the background. Efficiency of the reserve transcription impacts absolute quantification, as a genome that fails to be reverse-transcribed does not produce a positive signal. For this reason, the extracted RNA does not need to be purified from the reagents needed to lyse the virus. As a result, the workflow is greatly simplified; a lysis buffer is added directly to the cell supernatant (Figure 2A), then the material is diluted, mixed with the PCR reaction, divided into droplets, and the starting RNA material is amplified. The 'digital' signal entails the counting of the positive droplets, performed by a fluorimeter, which effectively measures the number of RNA molecules present in the sample directly.

During the development of an influenza cell culture process, we were also interested in measuring cell lysis to determine whether other strains of influenza viruses might behave differently in cell culture at large volumes. Cellular lysis had an important impact during vaccine manufacturing in particular due to the release of genomic DNA. Large amounts of DNA caused difficulties in the downstream process and the ability to purify the antigen product from contaminants. Measuring DNA levels in the cell supernatant was challenging due to the complexity of the sample. PicoGreen has been a common technique used to develop downstream processes, but it could not be used for measuring DNA in the cell supernatant as the assay suffered from matrix interference. As part of this work, we developed and implemented a ddPCR protocol for DNA quantification in the harvest. Eukaryote genomes contain a high proportion

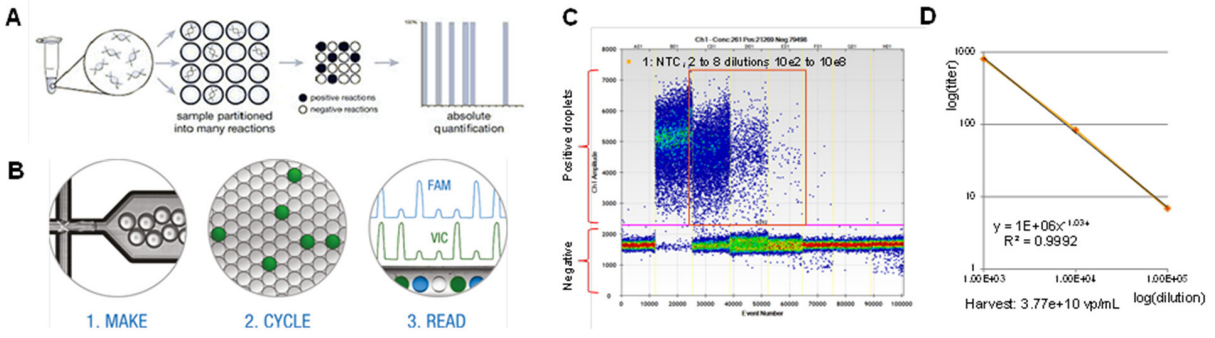

Figure 1. Digital Droplet PCR. Single molecules are isolated in separate partitions (A: Bio-Rad ddPCR system (20,000 droplets) concept in which a greater total number of droplets results in higher accuracy for Poisson-based counting, B: partitions created by emulsion, images provided courtesy of Bio-Rad Laboratories, Inc.). Upon PCR amplification, the droplets with a fluorescent signal indicate the presence of the target molecule. The 'digital' signal entails the counting of the positive and negative droplets, performed by a fluorimeter (C: single molecule detection on serially diluted samples. D: quantification from the three dilutions highlighted in C).