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Research paper

Influence of primer & probe chemistry and amplification target on reverse transcription digital PCR quantification of viral RNA

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ABSTRACT

Compared to other PCR technologies, digital PCR is a potentially highly accurate approach for the quantification of nucleic acid fragments. This study describes the impact of four experimental factors, namely primer and probe chemistry, PCR amplification target, duplexing, and template type, on the measurement results obtained by reverse transcription digital PCR (RT-dPCR) of viral RNA using *influenza A* virus as a model. Along conventional dual labelled probes (DLP), alternative primer and probe chemistries, including Zip Nucleic Acids (ZNAs), Locked Nucleic Acids (LNAs), and Scorpions®, were compared with two RNA template types: i) total genomic RNA extracted from cell cultured influenza A and ii) a synthetically prepared RNA transcript (*In vitro* transcribed RNA).

While apparently duplexing or a different PCR target choice did not have a significant influence on the estimated RNA copy numbers, the impact of the choice of primer and probe chemistry and template type differed significantly for some methods. The combined standard uncertainty of the dPCR analysis results has been assessed, taking into account both the repeatability and the intermediate precision of the procedure.

Our data highlight the importance of dPCR method optimisation and the advantage of using a more sophisticated primer and probe chemistry, which turned out to be dependent on the template type. Considerations are provided with respect to the molecular diagnostics of viral RNA pathogens, and more specifically, for precise quantification of RNA, which is of tremendous importance for the development of RNA calibration materials and the qualification of these calibrants as certified reference materials.

1. Introduction

Digital PCR (dPCR) builds on the traditional PCR amplification and fluorescence-probe based detection methods, as known from quantitative real-time PCR (qPCR). Therefore, dPCR uses the same primers and probes as the widely applied qPCR, but has been reported to be capable of higher sensitivity and precision due to the underlying principle of limiting dilution, whereby the sample is diluted and partitioned into many separate reaction partitions (chamber-based dPCR) or droplets (droplet-based dPCR) before amplification [1]. Due to another characteristic of dPCR, namely its independence from calibrants containing the DNA or RNA template subject to analysis, the method has become an attractive option for nucleic acid quantification. It has been successfully applied in the certification of reference materials used for standardising qPCR assays widely employed in clinical diagnostics and research areas [2–4]. Despite these major advantages, several studies have also reported significant bias when measuring both DNA and/or RNA using dPCR [5–7]. Such discrepancies have been attributed to the choice of the dPCR format (chamber vs droplet-based dPCR), the complexity of the nucleic acid template, detection reagents used, and pre-analytical steps such as nucleic acid extraction [3,4]. RNA poses a particular measurement challenge due to its instability and the additional step required for detection and quantification: reverse transcription (RT), during which complementary DNA (cDNA) is synthesised.

Another influencing factor could be the choice of the primer and probe system. Conventional dual labelled probes (DLP) with a fluorophore and a quencher, such as a Taqman® probe, are widely employed for (RT-) qPCR and (RT-) dPCR. In recent years, an increasing number of alternative PCR chemistries have become available. These include Locked Nucleic Acids (LNA), Zip Nucleic Acids (ZNA), and Scorpions®, among others. Several studies demonstrated that

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the use of alternative primer and/or probe chemistries may offer improved assay sensitivity [8–10]. So far, such options have been investigated as a factor influencing measurement results when using real-time PCR technology. This study evaluated the impact of the amplification target, the fluorophore, primer and probe chemistry, and duplexing on measurements by digital PCR to assess their influence on the associated bias.

2. Materials and methods

2.1. Materials

2.1.1. In vitro *transcribed (IVT) RNA*

In vitro transcribed RNA was synthesised in-house, using the pGEM®-T easy plasmid vector containing an insert, covering the entire segment 7 (M gene) of influenza A. The insert originated from RNA extracted from influenza A virus A/Aichi/2/68 (H3N2) purchased from the ATCC® Collection (LGC Standards, Molsheim Cedex, France). SuperScript® VILO™ Master Mix (Invitrogen, Carlsbad, CA, USA) was used for cDNA synthesis. The insert was generated using High Fidelity Platinum® PCR SuperMix (Invitrogen) and previously published primers Bm-M-1 and Bm-M-1027R [11]. A standard cloning procedure using pGEM®-T Easy Vector System II (Promega Benelux b.v., Leiden, The Netherlands) was carried out according to the manufacturer's recommendations. Plasmid DNA was extracted from a 5 ml Luria-Bertani (LB) broth culture (grown overnight in a shaking incubator at 37 °C) using The Wizard® Plus SV Minipreps System (Promega) and following the manufacturer's protocol. The identity of the insert was confirmed by sequencing of both DNA strands (Eurofins MWG Operon, Ebersberg, Germany). *In vitro* transcription was carried out with the RiboMAX™ Large Scale RNA Production System and T7 polymerase (Promega) using *Sal*I (Promega) – linearised plasmid as a template. The transcript was purified using SV Total RNA Isolation System (Promega) according to the producer's protocol, which included treatment with DNase I to remove unwanted DNA from the RNA preparation. The size, purity and integrity of the IVT RNA were confirmed by analysis on the Agilent 2000 Bioanalyzer using the RNA 6000 Pico kit (Agilent Technologies, Diegem, Belgium). Purified IVT RNA was diluted in the RNA Storage Solution (Ambion®, Austin, Texas, USA) and 50 μl aliquots were stored at −70 °C. The concentration of IVT RNA was assessed by spectrophotometry (NanoDrop® ND-1000, Wilmington, DE, USA) to be 1.83×10^{10} copies/μl. Based on the sequencing results and the position of the T7 promoter, the size of the expected RNA molecule was assessed to be 1147 bp.

2.1.2. *Extracted total genomic RNA*

Genomic RNA was prepared from cell cultured influenza A virus strain A/Aichi/2/68 (H3N2) propagated in St Georges Hospital, London, and extracted using QIAamp® Viral RNA Mini kit (Qiagen, Inc., Germantown, MD, USA), according to manufacturer's protocol, with minor modifications concerning the elution of the RNA. Samples were eluted in two steps, each using 120 μl RNA Storage Solution (Ambion®). Extracted RNA samples were pooled and gently mixed to obtain a homogeneous solution. Twenty and fifty μl aliquots were stored at −70 °C. The concentration of the total genomic influenza RNA was determined by dPCR using 12.765 digital arrays on the BioMark™ HD System (Fluidigm Corporation, San Francisco, CA, USA) according to the published CDC protocol targeting a conserved sequence of the influenza A matrix gene [12]. Three independent aliquots were subjected to dPCR, each measured in triplicate, resulting in an average concentration of 1.27×10^6 copies/μl and relative standard deviation (RSD) of 2.2%.

2.2. Methods

2.2.1. *Primer and probe chemistry*

In total, seven different primer and probe chemistries were applied in this study and are indicated by a specific code in Table 1. 'DLP-FAM' and 'DLP-HEX' are both assays using dual labelled probes (DLP) and primers, synthesised according to a published validated qualitative RT-qPCR method by CDC [12]. In DLP-HEX, the fluorescent dye FAM is replaced by a HEX™ fluorophore. Also the primers and probes from 2 other methods, namely 'DLP-GRAM' and 'DLP-HA gene', are based on validated methods described by the Pasteur Institute [13] and Yang et al. [14], respectively. The HA gene primers and probe target the H3-haemagglutinin (HA) gene instead of the matrix (M) gene. The primer and probe sequences of the fifth method are identical to the ones of the DLP-FAM and DLP-HEX approaches, but they have been conjugated to repeating spermine derivative cationic units to generate ZNA primers and probe. Scorpions® and LNA primers and probes were designed using the online tool OligoArchitect™ (Sigma-Aldrich, St. Louis, MO, USA). All primers and probes were synthesised and HPLC-purified by Sigma UK, except for ZNAs which were synthesised by Sigma USA. Single-use aliquots of oligonucleotides, reconstituted in nuclease-free water (Promega) were stored at −20 °C. Primer and probe information is included in Table 1 and their position on the M gene can be seen in Fig. S1.

2.2.2. *RT-dPCR*

dPCR experiments were performed using the 37K IFC Digital Arrays of the BioMark platform (Fluidigm) and the RNA UltraSense™ One-Step Quantitative RT-PCR System (Invitrogen). Quantification of the RNA was done under intermediate precision conditions (independent runs performed on different days) with samples diluted gravimetrically in RNA storage solution buffer (Ambion®) and run in triplicate (extracted gRNA) or quintuplicate (IVT RNA). The position of different experiments on the digital array was randomised over three different days. The same sample dilution was used for all the methods on individual days to allow for direct comparison. The digital array was primed and loaded according to manufacturer's protocol. Thermal cycling conditions were: 50 °C for 30 min for reverse transcription, 95 °C for 2 min for denaturation and inactivation of RTase, followed by 45 PCR cycles at 95 °C for 15 s and 55 °C for 30 s [12]. RNA storage solution buffer (Ambion®), constituting the no-template control, was included in each experiment. Also a negative control, sonicated human gDNA (Cambio) at 25 ng/μl was analysed to check for unspecific reactions. The analysis was performed utilising the Fluidigm dPCR software version 4.1.2 to assess the concentration by counting the number of positive partitions (H) out of the total number of partitions (C) from which the Poisson distribution was used to estimate the average number of DNA copies per partition (λ) via $\lambda = \ln(1\text{-}H/C)$ [15].

Six methods were applied for the analysis of influenza A IVT RNA, consisting of the established RT-PCR methods from CDC [12] and the Pasteur Institute [13], both using dual labelled primers and probe (DLP-FAM and DLP-GRAM), a method with 6-FAM™ replaced by HEX™ fluorophore (DLP-HEX), an experiment with ZNA primers (ZNA), a LNA primers and probe-based method (LNA) and finally a method using Scorpions chemistry (Scorpions®). When extracted genomic RNA (gRNA) of influenza A was used as a template, nine different methods were evaluated. In addition to the six methods mentioned above, which are all amplifying particular fragments of the M gene, the extracted gRNA was also quantified with an H3- primer and probe set targeting another gene of the influenza A virus, the haemagglutinin (HA) encoding gene (DLP-HA gene) [14]. Further, the effect of duplexing was evaluated by combining primers and probes targeting the M gene (DLP – HEX) and the HA gene (DLP – HA gene) in a single reaction by using different