Incorporation of Digital PCR in The Development Process of Diagnostics Tests

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Abstract

Introduction. The recent availability of multiple digital PCR (dPCR) platforms has led to an increased interest in the development of clinical diagnostic applications where precise target quantification or testing of clinical samples with low copy numbers is required. This study is aimed on evaluation of dPCR for two areas in the development of laboratory-developed tests (LDTs): assessment of titer accuracy of viral and bacterial reference materials (RM) used for determination of analytical sensitivity and use as a comparator method for results verification.
Methods. TaqMan assays were developed for detection of *Streptococcus agalactiae* (GBS), *Streptococcus pyogenes* (SPY), *Fusobacterium necrophorum* (FSN), Epstein-Barr virus (EBV), human herpesvirus 6A (HHV-6A) and human herpesvirus 6B (HHV-6B). Commercially available titered viral and bacterial stocks of EBV, HHV-6A, HHV-6B, GBS and SPY (Zeptometrix) were used. FSN was not titered (ATCC). Bacterial and viral nucleic acids were extracted, serially diluted and tested with qPCR first to determine the three concentrations to be tested by dPCR. dPCR was also used as a comparator method for fifteen clinical specimens with low positive borderline negative signal for *Neisseria gonorrhea* (NGO) using TEM-PCR™ sexually transmitted disease panel. dPCR was performed on the OpenArray® platform with the QuantStudio™ 12K Flex (Thermo Scientific) using 768 partitions per sample. The results were analyzed using Applied Biosystems™ DigitalSuite™
Results. The copy numbers estimated by dPCR for all tested viruses were lower than reported by vendor, respectively: EBV- 2150 copies/mL vs 1E5 copies/mL; HHV-6B-2230 copies/mL vs 1E5 copies/mL; HHV-6A 1750 copies/mL vs 1E4 copies/mL. dPCR estimated an increase in GBS concentration; GBS- 3920 copies/mL vs 1E3 CFU/mL. While the FSN concentration estimated by Nanodrop and by dPCR were comparable, dPCR estimated a much lower SPY concentration of 3E7 copies/mL compared to 1.65E9 cfu/mL reported by vendor. The testing of clinical specimens confirmed NGO detection in fourteen out of fifteen samples tested with lowest estimated concentration of NGO of 25.4 copies/mL.
Conclusions. The results of this study show that dPCR can be considered for quantification of RM utilized in molecular diagnostics assay development, as concentration estimated by vendor may not be accurate and overestimated, especially for viruses. It also can be a preferred method for repeat sample testing, especially when the target is present at a low concentration and sampling error affects test repeatability. Thus, dPCR has practical utility in the development of molecular diagnostics tests at the steps which require precise quantification and repeatability.

Introduction

- dPCR is increasingly being used for quantification of DNA molecules in applications where either precise target molecule quantification or detection of targets at lower concentration is required.[1,2]
- dPCR provides absolute quantification by counting the number of partitions with detected/non detected amplification and applies Poisson correction for absolute quantification.
- This study was aimed on evaluation of dPCR for two applications: 1) accurate quantification of reference materials used in development of LDTs and 2) confirmation of the results of end-point PCR used for testing clinical samples with low pathogen concentration.

Materials & Methods

Specimens. Titered reference materials (Table 1) were used for titer verification study. Seventy organisms obtained from various sources (ATCC, BEI Resources, IRR and Zeptometrix) diluted to 1E7 cfu/mL or 0.5 McFarland standard were used for NGO specificity study (Table 2). Genitourinary swabs collected from symptomatic patients by physicians with borderline NGO detection signal were used for NGO confirmation study.

Extraction. DNA was extracted using an automated KingFisher™ Flex (Thermo Fisher Scientific, Waltham, MA) magnetic bead based system with in-house developed protocols.

Real-time PCR and dPCR were performed on a QuantStudio™ 12K Flex thermocycler (Thermo Fisher Scientific, Waltham, MA). NGO TaqMan™ assay (Ba046462) is included on OpenArray® Vaginal Microbiota Panel and assay specificity was tested with organisms listed in Table 2 according manufacturer's instructions. *Neisseria gonorrhoeae, (*Zeptometrix P/N 081482) was used as a positive control.

Table 1. The List of Reference Material Used in dPCR Verification Study

Pathogen	Vendor	Catalogue number	Starting Concentration*	Gene target
Chlamydophila pneumoniae (CPN)	ATCC	VR-1360	1E5	major outer membrane protein (*ompA*)
Fusobacterium necrophorum (FSN)	ATCC	51357	1E6	leukotoxin (*lktA*)
Legionella pneumophila (LPN)	Zeptometrix	0801645	1E6	macrophage infectivity potentiator (*mip*)
Streptococcus agalactiae (GBS)	Zeptometrix	0801545	1E5	peptidoglycan hydrolase (*pcsB*)
Streptococcus pyogenes (SPY)	Zeptometrix	0801512	1E6	pyrogenic exotoxin B (*speB*)
Epstein-Barr virus (EBV)	Zeptometrix	0810008CF	1E7	Serine/threonine-protein kinase (*BGLF4*)
Human Herpesvirus 6A (HHV 6A)	Zeptometrix	0810529CF	1E6	U83
Human Herpesvirus 6B (HHV 6B)	Zeptometrix	0810072CF	1E7	U83

* cfu/mL (bacteria); pfu/mL (viruses); copies/mL (FSN)

Materials & Methods *(continued)*

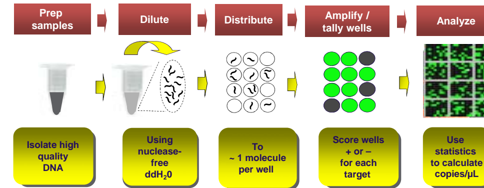

Figure 1. dPCR workflow.

The starting DNA concentration for reference material titer verification study was chosen based on results of real-time PCR. DNA template producing Cq of 27-28 was diluted 1:10 and 1:100 and tested on OpenArray® digital plates (P/N 4458070). Each diluted template, along with a no template control, were tested on 768 reaction wells (12 subarrays) on the same OpenArray® plate. DNA extracted from clinical samples was not diluted and it was tested with NGO TaqMan™ assay on 768 reaction wells. The target-specific DNA copy number was calculated using DigitalSuite™ software v 1.0 (Thermo Fisher Scientific, Waltham, MA).

Table 2. The List of Organisms Tested for Specificity of NGO (Ba046462) TaqMan™ Assay.

Acinetobacter baumannii	*Chlamydophila pneumophilia*	*Haemophilus influenzae*	*Neisseria elongata nitroreducens*	*Staphylococcus aureus*
Acinetobacter calcoaceticus	*Chlamydia trachomatis*	Herpes simplex virus 1	*Neisseria flava*	*Staphylococcus epidermidis*
Adenovirus 4	*Citrobacter freundii*	Human herpesvirus 6B	*Neisseria flavescens*	*Staphylococcus saprophyticus*
Adenovirus 40	*Clostridium difficile*	*Kingella denitrificans*	*Neisseria lactamica*	*Stenotrophomonas maltophilia*
Bacteroides fragilis	*Clostridium perfringens*	*Kingella kingae*	*Neisseria meningitidis*	*Streptococcus agalactiae*
Campylobacter jejuni	*Corynebacterium sp.*	*Klebsiella oxytoca*	*Neisseria perflava*	*Streptococcus bovis*
Candida albicans	Coxsackievirus A	*Klebsiella pneumoniae*	*Neisseria sicca*	*Streptococcus mitis*
Candida dubliniensis	Coxsackievirus B	*Lactobacillus acidophilus*	*Neisseria subflava*	*Streptococcus pneumoniae*
Candida glabrata	Cytomegalovirus	*Listeria monocytogenes*	*Proteus mirabilis*	*Streptococcus pyogenes*
Candida guilliermondii	*Enterobacter sakazakii*	*Micrococcus luteus*	*Proteus vulgaris*	*Streptococcus salivarius*
Candida kefyr	*Enterococcus faecalis*	*Moraxella catarrhalis*	*Providencia stuartii*	*Streptococcus sanguinis*
Candida krusei	*Enterococcus faecium*	*Moraxella osloensis*	*Pseudomonas aeruginosa*	*Trichomonas vaginalis*
Candida lusitaniae	Epstein-Barr virus	*Morganella morganii*	*Pseudomonas putida*	Varicella zoster virus
Candida parapsilosis	*Escherichia coli*	*Neisseria cinerea*	*Salmonella enterica typhimurium*	*Veillonella parvula*
Candida tropicalis	*Fusobacterium nucleatum*	*Neisseria elongata elongata*	*Serratia marcescens*	*Yersinia enterocolitica*

Results

dPCR As A Comparator Method for Detection of *Neisseria gonorrhoea*

Table 3. Confirmation of NGO Detection in Clinical Specimens. Three specimens and one no template control (NTC) (n=768 per sample) were tested per dPCR OpenArray® plate. Specimens were previously tested with end-point PCR followed by hybridization on microarrays with NGO assay threshold at 33780 RFU.

Plate ID	Sample	RFU	dPCR result	copies/uL	copies/mL of specimen
WFJ26	1	17740	detected	0.8	271
	2	3318	detected	3.5	1111
	3	27078	detected	88.7	28376
	NTC	N/A	not detected	N/A	
WFJ30	4	24840	not detected	N/A	
	5	29314	detected	0.1	25
	6	18972	detected	2.9	942
	NTC	N/A	not detected		
WFJ34	7	26643	detected	117.2	37501
	8	9347	detected	53.8	17229
	9	6002	detected	87.2	27902
	NTC	N/A	not detected	N/A	
WFJ39	10	30380	detected	N/A	
	11	9967	detected	26.4	8434
	12	27713	detected	10.4	3313
	NTC	N/A	not detected	N/A	
WFJ31	13	17784	detected	1.0	335
	14	6434	detected	13.6	4345
	15	19859	detected	0.2	51
	NTC	N/A	not detected	N/A	

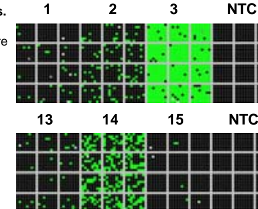

Figure 2. Examples of OpenArray® Images. Clinical specimens were tested with dPCR and end-point PCR (Table 3) and results for selected specimens are shown. 3072 partitions are shown per OpenArray® plate. Green-amplification detected per partition, black-amplification not detected, white-partition was excluded from the analysis.

Results *(continued)*

dPCR For Titer Verification/Characterization of Reference Material

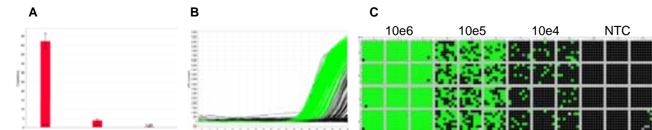

Figure 3. Estimation of titer for *Legionella pneumophila*.
DNA extracted from *L. pneumophila* at 1e9 cfu/mL was diluted and used at 1e6, 1e5 and 1e4 cfu/mL on dPCR.
A. Data produced by DigitalSuite™ software with serially diluted DNA extracted from *L. pneumophila*.
B. Amplification curves. C. Digital image of OpenArray®. 768 partitions were used per each tested *L. pneumophila* concentration.

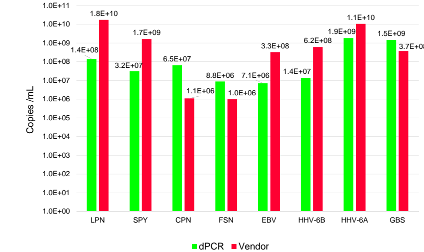

Figure 4. Comparison of titer provided by vendor versus titer measured by dPCR.
DNA was diluted to the starting concentration listed in Table 1, then 1:10 and 1:100. The untitered FSN suspension was used for DNA extraction followed by measurement of DNA concentration on NanoDrop (Thermo Fisher Scientific) and calculation of copies/mL. 768 partitions on OpenArray® were used per each tested concentration.

Conclusions

- We assessed the utility of dPCR for two different applications in the development and validation of LDTs, such as accurate quantification of nucleic acids in commercially available reference materials and confirmation method for analysis of low template specimens.
- Fourteen out of fifteen samples with elevated NGO signal (but below cutoff established by end-point PCR) were confirmed as positive for NGO with concentration ranging from 25 copies/mL to 3.8 E4 copies/mL.
- Five bacterial (three aerobes, one anaerobe and one obligate intracellular aerobe) and three viral reference materials were tested with dPCR using serially diluted DNA.
- Lower titer was detected by dPCR for all tested viral reference material, EBV, HHV-6A and HHV-6B, and for two bacteria, *L. pneumophila* and *S. pyogenes*. Elevated titer ranging from 4-fold to 59-fold compared to values provided by vendor was detected for *S. agalactiae*, and *C. pneumoniae respectively*.
- While lower titer values can be explained by differences in efficiency of DNA extraction procedure for tested viral and bacterial targets, the discrepancies for *C. pneumoniae* and *F. necrophorum* titer reflects the challenges associated with obtaining accurate titer values for culturing fastidious bacteria and anaerobes.
- The existing dPCR protocols on QuantStudio™ are limited to DNA amplification. The addition of RT-PCR would allow the expansion of dPCR applications, especially for testing of RNA virus reference materials.

Acknowledgements

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References

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2. JF Huggett, CA Foy, V Benes et al. The digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. 2013. Clinical Chemistry, 59:892-902.

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