METROLOGICAL ASSESSMENT OF MOLECULAR APPROACHES IN DIAGNOSTICS OF HUMAN CYTOMEGALOVIRUS

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Doctoral Dissertation
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MEROSLOVNO OVREDNOTENJE MOLEKULARNIH PRISTOPOV V DIAGNOSTIKI ČLOVEŠKEGA VIRUSA CITOMEGALIJE

Doktorska disertacija

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To Nastja Cinzia and my family

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Abstract

Human cytomegalovirus (HCMV) is a ubiquitous and latent human virus that is transmitted by body fluids and causes severe morbidity and mortality in transplant patients, neonates and AIDS-infected patients. Pre-emptive treatment of HCMV-infected transplant patients is based on monitoring viral kinetics and is followed by prompt changes in antiviral administration, hence it diminishes harmful effects of toxic antivirotics, reduces treatment cost and decreases the risk for antivirotic resistance. Molecular methods, such as real-time PCR (qPCR) and digital PCR (dPCR), allow for sensitive quantification of virus-DNA copies and are important tools for monitoring of viral load. Since reliable clinical diagnosis depends greatly on the comprehensive knowledge on performance parameters of DNA extraction, qPCR and dPCR, more information is required to better characterize these methods and thus improve reliability of clinical measurements. Additionally, the establishment of metrological framework for nucleic-acid tests is expected to increase the degree of standardisation of qPCR-based clinical measurements between laboratories, with dPCR as a promising candidate for a reference measurement procedure and for characterization of certified reference materials (CRMs). In this work, two metrological assessments were conducted to extend the knowledge on performance parameters of methods for manual HCMV DNA extraction (kits from Roche, Cepheid and Qiagen) and for quantification of HCMV DNA (qPCR, droplet-based and chip-based dPCR). Additionally, inter-laboratory study was performed to assess the reproducibility of dPCR for quantification of HCMV, while its suitability for direct quantification of HCMV without DNA extraction was also investigated. Each metrological study has improved the knowledge on processes involved in DNA extraction and DNA quantification. Broadened information on analytical parameters, such as DNA recovery (DNA extraction methods), precision (DNA extraction and quantification methods), analytical sensitivity, linearity of dynamic range and robustness (quantification approaches), has better defined the contribution of each method to measurement variability and bias. DNA extraction methods from Roche and Qiagen, and qPCR were shown as more suitable for routine clinical testing than others, as they are more repeatable in quantification of clinically important concentrations of virus in body fluids. In contrast to qPCR, dPCR demonstrated higher reproducibility, accuracy and robustness with higher DNA concentrations and appears suitable candidate for implementation in metrological framework. Additionally, direct quantification by dPCR provided less variable and more accurate measurements of DNA concentration than quantification of extracted DNA, and could enable a more accurate characterization of CRM for viruses.

Povzetek

Humani virus citomegalije (angleško human cytomegalovirus; HCMV) je vseprisoten latentni virus. HCMV se prenaša prek telesnih tekočin in je pogost vzrok obolevnosti in umrljivosti pri ljudeh s presajenimi organi, novorojenčkih in bolnikih z AIDS sindromom. Predbolezensko zdravljenje bolnikov s presajenim organom temelji na opazovanju kinetike virusa HCMV, kar omogoči takojšnje spremembe v zdravljenju s protivirusnimi zdravili še pred nastankom vidnih bolezenskih znakov. Molekularni metodi, kot sta verižna reakcija s polimerazo v realnem času (qPCR) in digitalna PCR (dPCR), omogočata zelo občutljivo kvantifikacijo virusne DNA in sta zato pomembni orodji pri spremljanju kinetike virusa HCMV. Ker je zanesljiva kvantifikacija virusa HCMV odvisna od zanesljivosti metod qPCR in dPCR ter metode za izolacijo DNA, je potrebno povečati poznavanje dejavnikov, ki vplivajo na njihovo delovanje. Poleg tega bi vzpostavitev meroslovnega okvirja za nukleinske kisline dvignila stopnjo poenotenja kliničnih rezultatov meritev količine HCMV virusa v krvi. Po dosedanjih raziskavah je digitalna PCR lahko primerna za referenčno merilno metodo in za ovrednotenje certificiranih referenčnih materialov znotraj prej omenjenega meroslovnega okvirja. V doktorski disertaciji smo meroslovno ovrednotili tri metode za ročno izolacijo DNA (proizvajalcev Roche, Qiagen in Cepheid) in tri metode za kvantifikacijo DNA (qPCR in dve različni dPCR). Poleg tega smo preverili uporabnost digitalne PCR za neposredno kvantifikacijo celih virusnih delcev virusa HCMV brez predhodne izolacije DNA. Naredili smo tudi medlaboratorijsko študijo, da bi ugotovili, kakšna je ponovljivost digitalne PCR znotraj in med laboratoriji pri kvantifikaciji DNA virusa HCMV. Obe meroslovni študiji sta izboljšali poznavanje procesov in dejavnikov, vključenih v izolacijo in kvantifikacijo virusne DNA. Povečali smo znanje o izkoristku izolacije DNA in občutljivosti ter ponovljivosti metod za kvantifikacijo DNA. Zaradi boljših rezultatov pri klinično pomembnih koncentracijah virusa HCMV sta metodi za izolacijo DNA (proizvajalcev Roche in Qiagen) in qPCR primernejši od ostalih analiziranih metod pri rutinski uporabi v klinični diagnostiki virusa HCMV. Pri višjih koncentracijah virusa HCMV pa je dPCR pokazala boljšo ponovljivost, natančnost in robustnost od qPCR, zaradi česar je primernejša za vzpostavitev meroslovnega okvirja. Neposredna kvantifikacija celih virusnih delcev HCMV brez predhodne izolacije DNA se je izkazala za bolj ponovljivo in natančnejšo od posredne kvantifikacije izolirane DNA. dPCR bi tako lahko s pomočjo neposredne kvantifikacije omogočila boljše ovrednotenje certificiranih referenčnih virusnih materialov.

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Abbreviations

A/H1N1	. . .	Influenza A, virus subtype H1N1
BAC	. . .	bacterial artificial chromosome
Biomark 12.765 array	. . .	12.765 Digital Array Integrated Fluidic Circuit
Biomark 37K array	. . .	qdPCR 37K Integrated Fluidic Circuit
BHQ	. . .	Black Hole Quencher
cDNA	. . .	complementary deoxyribonucleic acid
cp	. . .	copies
Cq	. . .	quantification cycle in quantitative polymerase chain reaction
CRM	. . .	certified reference material
CV	. . .	coefficient of variation
DNA	. . .	deoxyribonucleic acid
Digital MIQE guidelines	. . .	minimum information for publication of digital PCR experiments
dPCR	. . .	digital polymerase chain reaction
dsDNA	. . .	double stranded deoxyribonucleic acid
dsRNA	. . .	double stranded ribonucleic acid
E	. . .	amplification efficiency
EBV	. . .	Epstein-Barr virus
EDTA	. . .	Ethylenediaminetetraacetic acid
ELISA	. . .	Enzyme Linked Immune-Sorbent Assay
EQAS	. . .	External Quality Assurance Schemes
FAM	. . .	6-carboxyfluorescein
Fast master mix	. . .	TaqMan Fast Virus 1-Step Master Mix
FRET	. . .	fluorescence resonance energy transfer
gDNA	. . .	genomic deoxyribonucleic acid
gDNA1	. . .	extracted deoxyribonucleic acid for assessment of three quantification methods
gDNA2	. . .	centrally prepared DNA material for interlaboratory study
Gene master mix	. . .	TaqMan Gene Expression Master Mix
HCMV	. . .	Human cytomegalovirus
HEX	. . .	hexachloro-6-carboxyfluorescein
HIV	. . .	human immunodeficiency virus
IFC	. . .	Integrated Fluidic Circuit
INFECT-MET	. . .	international project named Metrology to support infectious disease diagnostics and anti-microbial resistance monitoring
INSTAND	. . .	Society for Promoting Quality Assurance in Medical Laboratories
INSTAND material 365029	. . .	Whole-virus material with the nominal concentration of 77,000 cp/mL from Society for Promoting Quality Assurance in Medical Laboratories
INSTAND material 365032	. . .	Whole-virus material with the nominal concentration of 20,000 cp/mL from Society for Promoting Quality Assurance in Medical Laboratories

IRMM	...	Joint Research Centre, Institute of Reference Materials and Measurements
IU	...	International Unit
JOE	...	Carboxy-4',5'-dichloro-2',7'-dimethoxyfluorescin
LAMP	...	loop mediated isothermal amplification
LGC	...	Laboratory of Government Chemist
LOD	...	limit of detection
LOQ	...	limit of quantification
MIQE guidelines	...	minimum information for publication of quantitative real time PCR experiments
MRC-5	...	Medical Research Council cell strain 5
NIB	...	National Institute of Biology
NIBSC	...	National Institute for Biological Standards and Control
NIST	...	National Institute of Standards and Technology
NGS	...	Next-Generation Sequencing
NTC	...	no template control
ORF	...	open reading frame
PBS	...	phosphate buffer saline
PCR	...	polymerase chain reaction
pp65	...	65 kDa matrix phosphoprotein of human cytomgalovirus
PPP	...	the concentration of oligonucleotide primers and probes
qPCR	...	quantitative polymerase chain reaction
QX100 system	...	QX100 Droplet Digital PCR System
QX200 system	...	QX200 Droplet Digital PCR System
RNA	...	ribonucleic acid
Roche master mix	...	FastStart Universal Probe Master Mix (Rox)
ROX	...	carboxy-X-rhodamine
SD	...	standard deviation
ssDNA	...	single stranded deoxyribonucleic acid
ssRNA	...	single stranded ribonucleic acid
TUBITAK	...	National Metrology Institute of Turkey
UL54	...	HCMV gene for DNA polymerase
UL83	...	HCMV gene for phosphoprotein pp65
Universal master mix	...	TaqMan Universal PCR Master Mix
WHO	...	World Health Organisation
WHO-10K	...	HCMV material with nominal concentration of 10,000 international units per mL
WHO-100K	...	HCMV material with nominal concentration of 100,000 international units per mL
WHO-1000K	...	HCMV material with nominal concentration of 1,000,000 international units per mL
WHO-1M	...	HCMV material with nominal concentration of 1,000,000 international units per mL
WHO-3K	...	HCMV material with nominal concentration of 3000 international units per mL
WHO-50K	...	HCMV material with nominal concentration of 50,000 international units per mL
WHO-500K	...	HCMV material with nominal concentration of 500,000 international units per mL
WHO-60K	...	HCMV material with nominal concentration of 5,000,000 international units per mL

Symbols

°C	=	degrees of Celsius
C	=	The number of estimated copies per 1 µL sample
D	=	dilution factor
log	=	logarithm with the base 10
λ	=	average number of nucleic-acid copies per partition
N		The number of estimated copies per mean effective reaction size
nL	=	nanolitre
M		The number of estimated copies per total reaction volume
mL	=	millilitre
$MS_{between}$	=	mean square value between groups
MS_{within}	=	mean square value within groups
MU	=	measurement uncertainty
µL	=	microliter
P	=	number of positive partitions
pH	=	potential of hydrogen
R	=	Number of analysed partitions
R2	=	Coefficient of determination
V_p	=	Partition volume
T_d	=	Number of nucleic acids per microlitre
u_r	=	variability related to intra-experiment variability
u_{ip}	=	variability related to intermediate precision
σ	=	standard deviation

Chapter 1

Introduction

1.1 Overview of Viruses

Viruses are small particles that can replicate only inside cells of other living organisms [1], [2]. They are ubiquitous and can be found in soil, water and air [3], [4]. Different viruses are able to infect almost every living organism, from prokaryotes such as archea and bacteria, to eukaryotic organisms such as fungi, plants, invertebrates and vertebrates [3], [5]–[9]. In this PhD thesis the focus will be on the methods used for clinical testing of a human cytomegalovirus (HCMV), therefore the introduction section is mostly limited to human viruses and some animal viruses that can infect a human.

1.1.1 Structure of viruses

Viruses come in diverse shapes and sizes, however they are mostly icosahedral or helical in shape with diameter between 20 nm and 2000 nm [1], [9]–[11]. They are composed of two main components, a nucleic acid that carries the structural information for replication of new viruses, and a protein coat (capsid) that covers the nucleic acid and protects it from degradation. Some viruses are also covered with envelope, a membrane with lipid bilayer, lipoproteins and glycoproteins, which is acquired during virus budding from the host cell [10], [12]. Nucleic acids found in viruses could be single- and double-stranded deoxyribonucleic acid (ssDNA; dsDNA) or single-and double stranded ribonucleic acid (ssRNA, dsRNA) [2], [9], [10]. Their genomes are of different sizes and can code from four to 200 proteins [1], [9].

1.1.2 Infection of a host cell

The majority of human viruses have a very limited host range, since their attachment to the host cell surface is driven by specific interactions between proteins receptors on the surface of the host cell and capsid or envelope proteins [2]. After entering the host cell, the viral nucleic acids usually exploit host-cell machinery for construction of new virus components (nucleic acids, capsid proteins) and their assembly into new viruses [2], [13]. This is then followed by their release from the host cell, which results in a destruction of a host cell and gradually depletes the fitness of a host organism [2].

1.1.3 Transmission of human and animal viruses

Human and animal viruses are found on every continent and can survive in many different environments for weeks and months, therefore multiple routes of their transmission exist [14]–[16]. Viruses could be transmitted directly from the infected organism to the healthy organism of the same species (by touch, blood, breast milk, saliva and air) [17]–[19] or indirectly by contaminated food or feed [19]–[21], animal vectors such as mosquitoes that transfer a virus from the infected organism to the healthy one [21], [22], animals that transmit the virus through their bite [23], contaminated fomites, and by sewage and surface waters that contain viruses derived from human and animal feces [15], [24]. Additionally, the majority of

animal and human viruses can switch the host and become lethal for other animal species or humans [25].

1.1.4 Burden of human viruses

Human viruses have a significant impact on world's economy and society, as they not only result in huge financial expenses related to healthcare but also account for high mortality and morbidity of human population worldwide. There are more than 200 virus species that infect humans, of which more than two thirds can infect other animal species, mostly mammals. Due to abundant routes of transmission and their stability, it is assumed that 60 % of infectious diseases are caused by viruses. Despite some viruses cause only minor health problems, others account for a high mortality and can also spread very fast. One notorious example of such a virus is the Ebola virus in west Africa, where a single outbreak killed more than 10,000 people in two years, with economic loss of around 7 billion $ for the healthcare related expenses, transmission control and vaccine development and deployment [26], [27]. However, Rabies virus, Yellow fever virus, Rotaviruses and HIV are just several examples of viruses that are constantly present in the population and the surrounding environment, and annually kill 55,000, 60,000, 453,000 and several million people [22], [23], [28], [29]. With numerous other human viruses that can cause similar health-related and economic problems, a lot of attention has been devoted in the last decades to reduce their impact on human society. In addition to human viruses, some animal viruses are also capable to switch their host range and eventually become lethal for humans [25]. The most notorious examples of such viruses are HIV, SARS coronavirus and Avian influenza virus, which in turn caused high mortality on humans and huge economic losses [25], [29].

1.1.5 Transmission control

Due to all detrimental effects that animal and human viruses pose to human health, a lot of effort is put into establishing the effective control over virus transmission to the healthy organisms [30]–[32]. Vaccination of healthy organisms is one of the most effective ways to protect them against virus infection and thus prevent further spreading of a virus either within human population or from animals to humans by reduction of possible reservoirs [33]–[35]. It is estimated that vaccination itself has eliminated many viral diseases and therefore prevented many death cases and increased life expectancy of human population [34], [35]. However, there are many newly emerging viruses and already known viruses that cause high morbidity and mortality, and for which the effective vaccine has still not been developed [17], [21], [34]. Additionally, due to extensive cost of vaccination, in developed countries many human age groups are vaccinated only in special circumstances or only against the most common regional viruses, whereas in less developed countries there are not enough vaccines available even for the most endangered age groups [34], [36]. The alternative strategy for reducing the risk of viral transmission across population is to prevent its transfer through fomites, feed, food, water, vector animals and other environmental reservoirs. This could be achieved by disinfections of fomites and surfaces, increased environmental and personal hygiene, protection against animal vectors or control over their reproduction. Additionally, monitoring the food, feed, water and environmental samples for virus presence by means of different diagnostic methods is of paramount importance for transmission control [15], [22], [37]–[39]. Humans and animals that are suspected of being infected should also be inspected for virus infection to promptly allow i) culling of livestock animals before the virus can spread to other animals or humans, ii) a quarantine isolation of the infected animal or human during an infectious period, or iii) antiviral treatment which could in turn result in partial or complete recovery of the infected organism [17], [25], [40], [41]. Special attention has to be devoted to the prevention of virus transmission in hospitals, where many patients are infected with different viruses. Therefore, high hygiene standards have to be maintained in hospitals. Another route of virus transmission in hospitals are blood and organ donations of infected donors. To avoid that, a thorough inspection of blood- and organ donors is a prerequisite for reducing the risk of virus transmission to the patients awaiting transfusion or transplantation [17].

1.2 Human Cytomegalovirus

1.2.1 Classification and prevalence

Human Herpesvirus 5, better known as Human cytomegalovirus (HCMV), is a human virus from family Herpesviridae. Although it is a very common human pathogen, as more than 50% of world adult population is supposed to be seropositive to it [42], [43], it seldom causes symptoms on healthy individuals since only immunocompromised patients and neonatals are facing morbidity or mortality because of HCMV primary or secondary infection [17], [44]. As a member of subfamily β-herpesviridae, it shares some mutual characteristics with Herpes viruses 6 and 7, such as narrow host range, long reproductive cycle and the ability to establish latency [45].

1.2.2 Structure and genetic variability

HCMV from the β-herpesviridae subfamily is the largest member of the Herpesviridae family, as its genome consists of around 230 kbp long linear dsDNA with more than 165 open reading frames (ORFs) [17]. The DNA is packed into the proteinaceous capsid that is enclosed into a lipid bilayer with glycoproteins, contributing to virion size around 100-200 nm [46]. HCMV genome is composed of two major segments, unique long repeat and unique short repeat, both of them flanked by inverted repeats. Most of the ORFs encode proteins but some of them code noncoding RNAs, including 14 micro RNAs which regulate about 30 % of gene transcription [47]. Nowadays, there are many different HCMV strains distributed around the world and there is no evidence of connection between viral genotype and its limitation to a particular geographical area [48].

1.2.3 Transmission and infection

The virus is transmitted by body fluids. It can be detected in tears, blood, mononuclear peripheral cells, leucocytes, breast milk and saliva. After entering the human body HCMV causes the primary infection, which is usually unapparent with healthy individuals and is coupled with mild symptoms (fever, malaise). Nevertheless, acquisition of HCMV primary infection by receiving infected transplant is a common cause of morbidity and mortality in immunocompromised patients, while primary infection in pregnant mothers can cause severe damage in neonates. After primary infection of healthy individuals is over, the virus enters into the dormant stage where it can stay for a lifetime. However, in case the immune system collapses, which could be caused prior to organ transplantation or manifestation of HIV into AIDS disease, HCMV can re-enter into the replication stage which causes a very harmful secondary infection that can lead to organ rejection, HCMV syndrome, retinitis, colitis, pneumonitis, increased rates of fungal and bacterial infections, graft dysfunction and other life threatening diseases [17], [46], [49].

1.3 Viral Diagnostics

Virus diagnostics is performed by different approaches. Considering their target range, these approaches can be very specific and target only one virus species, or they can be less specific and target a wider range of viruses. According to their principle, these approaches can be clustered into several groups: 1) methods that focus on infectivity of viruses; 2) microscopy-based methods that base on morphology of viruses; 3) serological methods that focus on immunological properties of viral proteins; and 4) molecular approaches that base on nucleic-acid sequences of viruses.

1.3.1 Methods based on infectivity of viruses

The main principle of these methods is to infect an organism or cell culture with a sample suspected for virus contamination/infection, and monitor it for virus-specific symptoms that confirm the virus presence in suspected sample. The major benefit of

infectivity-based methods is that they can exclusively detect a vital virus and determine its capability of infecting a living host or host cells. Their drawbacks are long time until the result and dependency on other approaches [50], [51].

1.3.2 Methods based on morphology of viruses

These methods depend on distinctive virus morphology. Since viruses are too small to be visible under the light microscope, the transmission electron microscopy is used. This method was often used for virus diagnostic in the past, however nowadays it is mostly used for initial identification of unknown viruses in outbreaks. The biggest advantages of this method are simplicity to perform, speed and independency of specific viral markers, such as antigen or specific nucleic acid sequences, therefore it can be used for detection of undiscovered and unsuspected viruses, and viruses that lack proper antigens and nucleic acid sequences for serological and molecular methods. Beside low specificity, other drawbacks are low reproducibility, lower sensitivity and inability to distinguish between active and inactive viruses [52], [53].

1.3.3 Serological methods based on protein properties of viruses

Serological methods are 50 years old and are widely used in diagnostics of viruses. The main principle of serological methods is interaction between monoclonal or polyclonal antibodies and specific targets (epitopes) on virus proteins (antigens) [54]. In general, the advantage of serological methods is that several of them are already standardized, commercially available, and easy to perform. Some of them, such as Enzyme linked immune-sorbent assay (ELISA) and immunofluorescent microscopy are often used for detection of animal and human viruses [55], [56].

In addition, the immunoelectron microscopy is a combination of serological method and electronic microscopy, as it uses transmission electron microscope to detect differently labeled antibodies bound to virus epitopes. This approach can also be applied for detection of human viruses, although it is mostly used for research purposes [52], [57].

1.3.4 Molecular approaches that base on nucleic-acid sequences of viruses

Molecular approaches that base on nucleic-acid sequences are often used for diagnostics of viruses [54], [58], [59]. With these molecular approaches, a sequence of a particular fragment of virus DNA or RNA is leveraged for virus detection. In general, a nucleic-acid extraction prior to analysis is a prerequisite for the success of subsequent detection, as it facilitates the separation of nucleic acids from other particles like proteins, fatty acids and sugars [60]. According to the protocol that follows, molecular methods could be classified into sequencing-based methods, hybridization-based methods (microarrays), isothermal-amplification-based methods and polymerase chain reaction (PCR)-based methods. Sequencing-based methods, such as next generation sequencing (NGS), are very useful for the detection of all viruses and for the identification of newly discovered viruses, since predetermined nucleic-acid sequences are not required for successful detection. On the other hand, they are expensive, time consuming and are currently not very useful for virus quantification, however with rapid advances in technology a routine use of NGS for quantification might be achieved very soon [61]. Hybridisation-based methods (e.g. microchip) facilitate simultaneous detection of different viruses, however they are much slower than amplification-based methods and therefore seldom used in diagnostics of viruses [62], [63]. Isothermal-based amplification tests, such as loop mediated isothermal amplification (LAMP), allow for very fast detection of suspected viruses without DNA extraction [64]. Additionally, LAMP technology can be packed into small remote devices to allow rapid and reliable on-site detection on fields and distant areas. Their major drawback compared to the most advanced PCR-based methods is narrower linear range and weak quantifiability of target nucleic acids [65]. PCR-based methods allow for very specific and sensitive detection and quantification of viruses. The most common PCR-based method for virus diagnostics is a quantitative real-time PCR (qPCR), while another version of PCR, namely digital PCR (dPCR), is also becoming an important method for virus quantification [66]. Owing to the focus of this PhD, the forthcoming text will be focused exclusively on qPCR and dPCR.

1.3.4.1 DNA and RNA of viruses

DNA and RNA are polymeric molecules that carry all virus genetic information. Both types of nucleic acids can be found in a single strand that consists of a different sequence of nucleotides, or in a double stranded form, with two homologous strands paired due to hydrogen bonds between nucleotides [67]. A unique sequence of RNA or DNA strand not only provides the information for replication of new viruses, but also allows for a specific determination of virus species through the use of molecular methods [59], [61], [67].

1.3.4.2 DNA and RNA extraction

Obtaining a pure and non-degraded nucleic acid is a prerequisite for its successful detection, as damaged DNA and the presence of other cell components result in improper detection or quantification with molecular methods. Extractions of nucleic acids are usually composed of several steps, which can be performed either manually using in-house protocols and commercial kits, or using automated devices [68]–[70]. First, lysis of the host cells is performed by different physical and chemical methods to expose naked virus nucleic-acids that were present inside them. This is followed by the addition of surfactants and detergents to degrade membrane lipids in host cell membranes or intact virus envelopes, and proteases that destroy protein complexes of host cells and virus capsids. In some cases, nucleases are also added to cut host-cell and virus DNA or RNA if only one type of RNA or DNA virus is targeted in the analysis. At the end, ethanol precipitation, phenol-chloroform extraction or mini-column purification are performed in at least two steps to first eliminate the enzymes, degraded proteins and lipids, salts, detergents and other reagents added in previous steps, and finally to precipitate or elute the targeted nucleic acid and allow its further analysis by molecular approaches. [60], [71], [72]. The recovery of nucleic acids could be impaired in several ways. Some substances that are present in the sample can prevent the cell lysis during nucleic acid extraction. Furthermore, some substances facilitate degradation of nucleic acids or even strengthen their capture on membranes of commercial columns, which results in impaired elution of nucleic acids [73], [74]. Since DNA extractions have a profound impact on subsequent amplification of extracted nucleic acids by molecular methods, it is of paramount importance to assure the selection of the suitable extraction methods.

1.3.5 Quantitative real-time PCR (qPCR)

qPCR has become the most commonly used molecular method in virus diagnostics, as it offers fast, simple, sensitive and specific detection and quantification [75]. Its principle is based on classical PCR, however it is the ability to monitor DNA amount in real time that characterizes qPCR as a very useful method for virus detection and quantification.

1.3.5.1 Effects on efficiency of qPCR amplification

In theory, each amplicon is duplicated in every cycle, contributing to 100 % amplification efficiency. However, several different factors can decrease the efficiency. Many substances present in the environment, tissues of living organisms, substances used for storage of samples or even reagents for DNA extraction cannot only affect extraction of nucleic acids (Chapter 1.3.4.2), but also directly influence the amplification through hindrance of DNA polymerase [73], [76]. Such inhibitory substances are usually eliminated during the nucleic-acid extraction procedure, however sometimes they are extracted with nucleic acids and interfere the qPCR reaction. A possible consequence of inhibition is either a complete inhibition that causes no DNA amplification or partial inhibition that results in lower DNA-amplification efficiency and therefore higher quantification cycle (Cq) values. Inhibition can be monitored using internal controls added to sample before nucleic-acid extraction, or by performing several dilutions of sample to check if the non-diluted sample had lower amplification efficiency than the more diluted ones [74], [77]. Another reason for lower efficiency could be degradation of nucleic acid that took place inside the target-amplicon sequence, which can occur during extraction, sample storage, pipetting or thermal cycling. Physical obstruction of primer-, probe- or DNA polymerase-alignment site on the target amplicon due to specific nucleic-acid structure under certain conditions is another reason for lower efficiency [78].

1.3.5.2 Quality control of qPCR-based detection and quantification

Due to different factors that influence the final detection or quantification of qPCR, quality controls need to be in place during the performance of analyses to allow reliable detection and quantification. First, a proper characterization of the extraction method and qPCR method is required, comprising of in-house validation or even inter-laboratory assessment, which confirms the method reliability. Then, a thorough control over the extraction and amplification process is of paramount importance in every routine analysis. This could be achieved by using 1) a positive template control to check the risk of false-negative results due to amplification problems, 2) negative template control, which allows to check the risk of false positives owing to contamination with DNA molecules, and 3) a standard curve that is composed of several dilutions of well characterized material with identical amplicons as the amplicon of interest (e.g. standard reference material). Standard curve is used to check the amplification efficiency of particular qPCR throughout a certain DNA-concentration range and to enable relative DNA quantification. This is performed by comparison of the Cq values of sample of interest with Cq values of standard-curve dilutions of reference materials [77], [79].

1.3.6 Digital PCR (dPCR)

dPCR is a PCR-based technology that overcomes the qPCR dependency on an external calibrant and provides an accurate, precise and robust quantification of nucleic-acid copy numbers. Despite a relatively novel technology, dPCR has already been used for different applications, including quantification of viruses and bacteria [59], [80], [81], conventional and direct quantification of reference materials for viruses [82], [83], and value assignment of certified reference materials (CRMs) containing plasmid DNA [84]–[86].

1.3.6.1 Basic principle

In its basic principle the dPCR is almost identical to qPCR since a similar PCR mixture (master mix, thermal cycling conditions and DNA template) can be used. The only difference, however, is partitioning of the PCR mixture into hundreds or thousands of partitions followed by multiple PCR cycles in each of them. The fluorescence intensity is measured in every partition in the same way as with qPCR. High fluorescence measured in any partition indicates the presence of amplified amplicons, whereas none or very small fluorescence is an indicator of absence of amplified amplicons. To simplify the analysis, all partitions with increased fluorescence are called positive and are assumed to contain at least one amplifiable amplicon after the partitioning phase, whereas partitions with low or no fluorescence are called negative as speculated to lack the amplifiable amplicon before the PCR began. It is then the ratio between positive and negative partitions which enables accurate estimation of initial nucleic-acid concentration in the analysed sample. The formula used for such estimation is based on Poisson distribution (Equation 1-1):

$$T_d = \frac{-D \times 1000}{V_p} ln\left(1 - \left(\frac{P}{R}\right)\right) \quad (1\text{-}1)$$

where T_d is the number of nucleic-acid copy number per µL, D is the dilution factor, Vp is the partition volume, P is the number of positive partitions and R the number of analysed partitions (positives and negatives together). Such estimations can be accurate only within a specified dynamic range. Too high nucleic-acid concentration in a reaction mixture exceeds the upper limit of the dynamic range and results in a lack of negative partitions, whereas an extremely low concentration goes beyond the lower limit of the dynamic range and results in a very small number of positive partitions [87]. With every dPCR instrument, increase in the number of analysed partitions extends the upper limit of the dynamic range, whereas enlargement of the volume of the analysed sample broadens the lower limit of the dynamic range and hence increases the sensitivity. Additionally, it is possible to perform a preliminary estimation of nucleic-acid concentration by other molecular approaches to enable proper

dilution of very concentrated sample. Alternatively, several dilutions of the initial sample can be simultaneously analysed and thus facilitate the presence of at least one dilution with concentration inside the required dynamic range [87].

1.3.6.2 Advantages of dPCR

The major benefit of dPCR compared with qPCR is its independence from the standard curve, which simplifies the quantification of target DNA or RNA copies [88]. It has also been shown that dPCR is more resistant to PCR inhibitors than qPCR, since due to digital format partitions with partial inhibition of amplification efficiency can still be distinguished from negative partitions [74], [89]. Additionally, several studies suggested dPCR allows for more robust, repeatable and precise nucleic-acid quantification than qPCR [66], [90]–[92].

1.3.6.3 Disadvantages compared to qPCR

At present, dPCR platforms are generally more expensive than those for qPCR [88]. Additionally, with dPCR handling time, total turnaround time and cost of sample analysis are currently higher than with qPCR [93]–[95]. However, dPCR platforms usually offer lower sensitivity compared with qPCR, which is caused by a restricted sample volume that is analyzed [82], [90], [96].

1.3.6.4 Available dPCR technologies

Two partitioning formats are known, dividing the sample into thousands of droplets (droplet-based dPCR) or from hundreds to thousands of chambers on a single array or chip (microfluidic-based dPCR), with each format being utilized in several commercial dPCR platforms from different manufacturers.

1.3.6.4.1 Droplet-based dPCR

With droplet-based technology, the reaction mixture containing nucleic acid and master mix is mixed with special oil using a droplet-generation equipment, which results in creation of emulsion with very small droplets of reaction mixture surrounded by the oil. These droplets are uniform in size and volume (e.g. 0.834 μL) and are further stabilized by components present or added in the master mix to retain their shape and volume during thermal cycling. Each droplet can either contain from zero to several target nucleic acids. This emulsion is then transferred to regular PCR plate and put to thermal cycler where PCR reaction is simultaneously performed inside every droplet. The PCR plate is then transferred to droplet reader where each sample emulsion is analyzed separately. The emulsion is aspirated and transported within the capillary to reach the fluorescence detector. Each droplet reaches the detector and is monitored for its end-point fluorescence individually since the capillary is so narrow that it allows only one droplet to pass the detector.

In the last decade, droplet-based technology was commercially implemented by Bio-Rad Laboratories and RainDance Technologies. The QX100 and QX200 systems from Bio-Rad allow the analysis of 96 samples per run, with each sample distributed into 20.000 droplets. The QX100 system allows the simultaneous detection of FAM and VIC fluorophores, whereas the QX200 additionally detects EVA green. The main drawback of Bio-Rad platforms in comparison to other platforms is longer time to complete the analysis (six hours) and the dependence on the proprietary master mix. Despite these two obstacles, Bio-Rad platforms are the most commonly used dPCR platforms, as they enable high throughput analysis, allow for the accurate estimation of nucleic-acid concentration and a wide dynamic range due to a relatively high amount of droplets, and have been released on the market earlier than some other platforms [88], [97].

Raindrop system from Rain Dance Technologies allows the analysis of eight samples per run, with each sample partitioned into one to 10 million droplets. It enables the detection of 10 different dyes, of which two of them can be used simultaneously in one run. The advantage of RainDance compared to any other dPCR platform is the number of droplets, which allows more accurate estimations and a very wide dynamic range. However, the expensive instrumentation and the longest turnaround time (8 hours) are the main two reasons why RainDance is less commonly used in laboratories than Bio-Rad [88], [98].

Recently, another droplet-based Chrystal Digital PCR technology named Naica System has been developed by Stilla Technologies. In contrast to other two droplet-based dPCR technologies, Naica System is performed on the array. In one run, 12 samples can be analysed, each distributed into 25,000 to 30,000 droplets within a single array. The biggest advantage of Naica system is the short time it needs for analysis (2 hours) [99].

1.3.6.4.2 Microfluidic-based dPCR

Different principles are used in microfluidic-based dPCR platforms from different manufacturers.

In case of Fluidigm, each reaction mixture is pipetted into one well on the array. Then, using the under pressure reaction mixtures are aspirated through small capillaries and deposited into hundreds of chambers of dedicated panel on the same array, which are physically separated by plastic walls. Then, the array is placed into the Biomark instrument that enables simultaneous thermal cycling and fluorescence monitoring in real-time. Currently, Fluidigm offers two different Biomark arrays for dPCR, the 12.765 Digital Array Integrated Fluidic Circuit (IFC) (hereinafter denoted as Biomark 12.765 array, which allows the analysis of 12 samples per run, and qdPCR 37K IFC (hereinafter denoted as Biomark 37K array) that enables the analysis of 48 samples per run. Fluidigm is currently the most commonly used microfludic-based dPCR platform, however it is less used than Bio-Rad systems.

The main advantage of the Biomark system is that it enables real-time detection of the fluorescence from many different fluorophores (three per reaction) and the use of many different master mixes. However, the drawbacks are the expensive instrument and arrays, narrow dynamic range and smaller accuracy of estimation due to the small number of partitions, and lower sensitivity compared with the Bio-Rad instrument owing to a smaller number of partitions [88], [100].

The QuantStudio 3D instrument from Thermo Scientific is one of more recent versions of dPCR platforms. It enables the distribution of a single reaction mixture into 20,000 chambers on a single array. After injection of one sample, an array is sealed and transferred to qPCR cycler where the PCR reaction takes place. Each array contains only one sample, however a simultaneous thermal cycling of 24 arrays is possible. At the end, each array is individually put into a reader where the fluorescence of up to two fluorophores inside each of 20,000 chambers is measured in 30 seconds. The benefit of QuantStudio 3D is a low cost of instrument and arrays, and shorter time of analysis than with Fluidigm and Bio-Rad. However, its drawback is the use of proprietary master mix and a relatively small throughput [101].

Recently developed Constellation dPCR from Formulatrix is another microfluidic-based dPCR platform. The samples (reaction mixture) are first pipetted into the proprietary 96-well plate and sealed. Then, the plate is placed into the Constellation drawer where each sample is distributed into 496 chambers. The plate is then put into any PCR cycler. After the completion of thermal cycling, the plate is transferred to the Constellation instrument where the fluorescence of every chamber is measured. The advantages of this method are short analysis time, high throughput analysis, and cheaper instrument price compared to the majority of other platforms. However, the drawbacks are smaller sensitivity due to small reaction volume, narrow dynamic range and smaller accuracy of estimation due to a small number of chambers [102].

Another recently developed microfluidic-based dPCR platform is Clarity Digital PCR Technology from jnmedsys. The arrays with 10,000 compartments are packed in each of eight 0.2 mL tubes on a single tube-strip. Using the loading device, the partitioning of eight samples is simultaneously performed in one minute, followed by tube sealing and transfer of tube-strips into the PCR cycler, where 96 tubes can be amplified at the same time. Strips are then put into reader where concurrent reading of eight tubes is performed within one minute. The benefits of this method are speed, low cost, small benchtop devices, high throughput and a relatively high dynamic range and precision owing to a higher number of partitions. However, a small sample probably prevents high sensitivity [103].

1.3.7 Performance parameters of qPCR and dPCR

With both methods, several performance parameters must be assessed and taken into account to evaluate the fitness for the purpose of both technologies. Based on the MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) and digital MIQE guidelines (minimum information for publication of quantitative digital PCR Experiments), these parameters must be determined and reported with every publication [66], [77].

Accuracy is defined as the difference between the measured and actual nucleic-acid copy-number concentration [77], [104].

Analytical sensitivity is defined by the limit of detection (LOD), which is the lowest nucleic-acid copy-number concentration per reaction that can still be reliably detected with 95% of confidence [77], [104], [105].

Analytical specificity is the frequency of mismatches, which are followed by amplification of non-targeted nucleic-acid sequences and could cause false positive results [77], [104].

Amplification efficiency (E) is defined as the ability of primer pair to amplify a target amplicon to the theoretical maximum of two-fold increase per every PCR cycle. It is calculated from the slope of standard curve by the formula: E=10(-1/slope) [77], [104], [105]. Since efficiency is derived from the standard curve, it can be measured for qPCR only.

Limit of quantification (LOQ) is the lowest nucleic acid copy-number concentration in a reaction that can be quantified reliably with a more or less constant coefficient of variation (CV) [77], [104]. Some guidelines recommend the LOQ to be set at the lowest concentration that yields CV value below 25 % [106].

Linear dynamic range is a range of nucleic acid copy-number concentrations that yield quantifiable results [77], [104].

Linearity of the dynamic range describes the agreement between every measurement of the standard curve and the calculated regression line throughout the whole linear dynamic range. It is expressed as a coefficient of determination (R^2) and should usually be above 0.975 [105], [107].

Precision is defined as the agreement between several measured results and is expressed as either CV or measurement uncertainty. Intra-experiment variability indicates the degree of agreement between measurements performed in the same run (experiment). Intermediate precision (inter-experiment variability) indicates the agreement between measurements performed on the same instrument using the same assay (master mix, oligonucleotide primers and probes, and cycling conditions) but carried out in different experiments. Reproducibility expresses variability between results measured in different laboratories on different instruments using same or various assays [77], [104].

Robustness of the assay denotes the ability of the assay to yield reproducible results despite slightly changed experimental conditions (different laboratories, instruments, primers and probes, their concentrations and cycling conditions). Reproducibility between laboratories is one of the components of robustness [105].

1.4 qPCR-Based Methods in Human Clinical Virology

Infectious diseases caused by viruses account for morbidity and mortality of human population. In the last decades, with the aim of providing the best public-health protection, huge emphasis has been placed on the development of methods that will allow for accurate and rapid detection and quantification of viruses, and monitoring microbe transmission across

communities [56], [58], [108]. Molecular approaches, such as qPCR, are complementing conventional microbiological methods, such as serology and culture, as they allow for a faster, more accurate and more sensitive diagnosis of viruses [56], [75], [108], [109]. Infectious disease testing market provides laboratories and clinics with diverse nucleic-acid extraction methods, qPCR instruments and chemistries from different manufacturers, leading to a broad range of results that can be obtained using disparate methodologies inside or between laboratories. This variability can have a negative effect on the detection and especially on quantitative measurements that are required for diagnostics of human cytomegalovirus (HCMV) and some other DNA viruses, such as Epstein-Barr virus and BK virus [58], [110].

1.4.1 Qualitative measurements

Qualitative qPCR is used when discrimination between the presence and the absence of a suspected virus is required. Confirmation of a virus presence is usually followed by antiviral treatment, which could, if prompt enough, decrease the health burden of virus infection. In contrast, the absence of a suspected virus infection could lead to termination or avoidance of otherwise harmful antiviral treatment. With the majority of human viruses, qualitative measurements enable sufficient viral detection and therefore facilitate prompt antiviral treatment [58], [111], [112].

With qualitative tests, analytical specificity and analytical sensitivity are of paramount importance to reduce the probability of false positive and false negative results, respectively [58]. Abundance of disparate approaches used in different clinical laboratories results in major sensitivity discordances between them. Recent external quality assurance schemes (EQAS) indicated that more than 20% of participating clinical laboratories were unable to detect some influenza A samples, indicating sensitivity problems due to suboptimal methodology [113]. Furthermore, an inter-laboratory study that compared two qPCR methods reported that more than 60% of clinical samples with pandemic influenza A/H1N1 found positive with one qPCR methods were determined as negative with another qPCR method [114]. Such lack of sensitivity and/or specificity noted with some laboratories/qPCR methods could result in misdiagnosis of disease and subsequently in insufficient prevention of disease progress [58].

1.4.2 Quantitative measurements

Quantitative measurements are commonly used with all viruses that establish latency in human body and their detection by qualitative methods does not give any relevant information. It is either the increase in virus concentration that indicates its entry into the replication phase, which could in turn result in morbidity and mortality, or the decrease of virus concentration which denotes successful termination of virus replication caused by antivirotics. In both cases, sensitive, reliable and repeatable quantitative diagnostics is a prerequisite for either prompt initiation of antiviral therapy that could reduce the health burden of disease even before the occurrence of visual symptoms, or termination of either immunosuppressive therapy or harmful antiviral therapy as soon as their detrimental effect on virus replication has been confirmed. The most common example of such a virus is HCMV, however some other viruses, such as the Epstein-Barr virus, HIV and BK virus, also require quantification for successful treatment [56], [58], [115]–[117].

1.4.3 Human cytomegalovirus – clinical activities and qPCR-based diagnostics

1.4.3.1 Prevention and treatment of disease with immunocompromised patients

The lack of effective vaccines and high prevalence of HCMV increase the need for effective antiviral treatment. Currently there are several antiviral drugs available for treatment. Ganciclovir and Valganciclovir are acyclic analogs of the nucleoside guanosine, whereas Cidofovir and Foscarnet are direct inhibitors of HCMV DNA polymerase. All four antivirotics were found to cause several harmful side effects, such as hyposperima, neutropenia and nephrotoxicity, in addition to observed genotoxic and cancerogenic effects on tested animals. Mutations in viral DNA polymerase gene could result in resistance to all four antiviral drugs[17], [118].

Due to harmful side effects, prudent use of antivirotics is required. There are two main approaches, prophylaxis and pre-emptive treatment. Universal prophylaxis involves administration of specified antivirotic dose in regular time intervals, and is mostly used with high-risk and medium-risk cohorts (the degree of risk depends on several factors, including the initial presence or absence of HCMV in both patient and donor). On the other hand, pre-emptive treatment includes monitoring of a recipient that underwent solid organ or hematopoietic stem cell transplantation, followed by potential initiation or termination of the antiviral treatment based on detected changes in the viral load. Pre-emptive treatment is usually applied with patients that have a lower risk of developing HCMV infection. Benefits of pre-emptive treatment compared to prophylaxis are lower treatment cost, reduced risk of antivirotic resistance and smaller harmful effects of antivirotics on patients. However, prophylaxis results in fewer opportunistic infections, less common graft rejection, improved graft and patient survival and lower monitoring costs [49].

1.4.3.2 Pre-emptive treatment

The pre-emptive treatment is becoming more and more common technique since it reduces the costs and harmful effects of antivirotics, and minimizes the probability of antiviral resistance. Clinical application of qPCR allows for more sensitive and precise monitoring of low viral loads than classically used immunofluorescence based pp65 antigenemia tests [56]. This increase in the sensitivity enables an insight into early HCMV replication which, linked with an immediate decision on initiation or termination of pre-emptive treatment, can play a crucial role in successful prevention of HCMV disease. Generally accepted criteria for decision on initiation and termination of pre-emptive treatment have not been defined yet, however, two major approaches have been established in the last decade, approach that monitors viral kinetics and the approach considering the threshold value [56]. The most common body fluids used for pre-emptive treatment monitoring are human plasma and whole blood [119]–[123].

1.4.3.2.1 Monitoring viral kinetics

One approach is based on monitoring the viral kinetics before deciding on the initiation or termination of pre-emptive treatment. According to some publications, the accepted international consensus proposes initiation of the treatment when more than a certain increase in viral loads between two sequential samples from one patient is measured using the same assay (including extraction and qPCR). Based on current guidelines, for viral load values < 1 000 copies/mL changes have to be at least 5-fold (0.7 $\log_{10}$) to be of clinical significance, and at least 3-fold (0.5 $\log_{10}$) for viral load values > 1 000 copies/mL [124]. The reliability of this approach might be hindered by intra- and inter-experiment variability, as comprehensive metrological support for qPCR-based measurements is lacking [58].

1.4.3.2.2 Threshold value

Setting the threshold, based on predetermined HCMV DNA concentration cut-off value, is the second approach to help decide regarding therapy initiation or termination. The threshold is defined by comparing the initial titre in patients with symptoms in contrast to those without [121]. With patients that underwent either solid organ transplantation or allogeneic hematopoietic stem cell transplantation, the observed concentration thresholds from different laboratories vary for more than 1 log (more than 10-fold differences) for each, human plasma and whole blood samples [119]–[123], [125]–[128]. Due to huge discrepancies between threshold values for samples that use the same matrix (human plasma or whole blood) and derive from the same transplantation group (solid organ or stem cells), generally accepted universal concentration threshold is currently not achievable [58]. As a consequence, each laboratory must individually perform clinical observations of correlation between viral load and symptom occurrence.

1.5 Sources of Variability in Human Clinical Virology for Quantification of HCMV and Other DNA Viruses

There are many different factors that decrease the agreement of measurements within or between clinical laboratories and hospitals that use different PCR-based methods. Those with the highest impact on variability are sample type, method for nucleic-acid extraction, calibration material for standard curve and PCR reaction components.

Sample type has an important impact on the final result due to a different amount of virus in different sample types (matrices), such as whole blood, human plasma, urine, peripheral mononuclear cells and others, and due to a different matrix effect on nucleic-acid extraction efficiency. With some viruses, such as the HCMV and BK virus, more than a 10-fold difference in measured viral load was obtained from different sample types [49], [116], [124], [129].

Method for nucleic-acid extraction can significantly increase the variability between quantitative and qualitative measurements owing to various nucleic-acid recoveries and carryover of inhibitory substances that hamper the amplification efficiency. Nucleic acids can be separated from other components by the help of their solubility between two liquid phases (phenol-chloroform extraction) or their binding to solid particles, such as magnetic beads, silica membranes, glass particles etc. With liquid-phase extractions different in-house protocols have been developed. On the other hand, with solid-phase approaches numerous versions are produced by different manufacturers, differing in extraction protocols, solid particles, buffer components etc. Additionally, commercial kits for nucleic-acid extraction approaches are designed for manual or automated extraction of nucleic acids [60], [110], [130]. With HCMV, different nucleic-acid extraction approaches (manual or automated) from different manufacturers used in the same laboratory can cause more than a 5-fold difference in measured viral load [72], [131].

Calibration material has a profound effect on the quantification of viral load, since it is used for generation of calibration curve against which the virus concentration from the clinical sample is calculated. Diverse in-house and commercial calibration materials, which are composed of different analytes (plasmid DNA, genomic DNA or whole viruses), are mostly characterised for their concentration using approaches for relative quantification that could therefore not provide very accurate quantity of the analyte [132]. Moreover, these calibration materials may exhibit low commutability, meaning that they might not be amplified equally as viral DNA from clinical samples. As observed with HCMV, Eppstein-Barr virus (EBV) and BK virus, variable commutability of different calibration materials lead to over- or underestimation of viral load [110], [133]. This in turn results in more than 3-fold differences in measured viral loads among laboratories [110].

PCR reaction components can also decrease the agreement between different methodologies or laboratories for several folds due to their impact on amplification efficiency. For every virus, there are many different oligonucleotide primer pairs and probes available, which target different genes or different amplicons of the same gene. Additionally, various master mixes also influence amplification efficiency [110], [134], [135].

1.6 Reference Measurement Framework

The reference measurement framework is a well organised scheme which allows user measurements to be traceable to well defined base unit of certain measurable quantity in the International System of Units (e.g. base units of mass and length are kilogram and meter). Development of a reference measurement framework for nucleic acid measurements of human viruses is expected to circumvent several problems related to measurement variability between laboratories. Reference measurement framework could be achieved by establishing CRMs and reference measurement procedures, which could in turn allow metrological

traceability for every clinical measurement and therefore increase the comparability between them regardless the geographical location or measurement approach used [136].

1.6.1.1 Certified reference materials (CRMs)

CRMs are tools for standardization, process characterization and quality control. They carry well characterized and enumerated analyte values that are traceable to the International System of Units (SI) or some other internationally accepted unit (IU). CRMs are a key aspect of improving the comparability and reducing the variability of quantitative viral load testing (11) by enabling traceable definition of secondary reference materials which would eventually be used for calibration of in-house or commercial calibrators utilized for routine quantification. The availability of well characterized CRMs will also enable improved evaluation of factors influencing the performance of molecular tests, such as amplification and extraction method, choice of gene target and matrix effects linked to the clinical sample type. This would in turn result in better definition of performance limits for virus diagnostic tests with qualitative and quantitative outputs [58], [133]. The CRMs in clinical studies are expected to provide traceability and facilitate long-term comparison of method performance, leading to improvements in the robustness and accuracy of diagnostic measurements.

World Health Organization (WHO) has introduced international reference materials for several human viruses. These reference materials are composed of homogenous batches of whole viruses in order to allow a complete control over a quantification process, from DNA extraction to DNA amplification [110], [132]. However, the introduction of international reference materials for HCMV and EBV, for which quantification is also important, did not significantly improve the comparability of results between laboratories [58]. Therefore, in addition to traceable definition of the concentration of the analyte and demonstration of homogeneity between vials and stability, it is necessary to demonstrate their commutability with clinical samples in order to ensure that each reference material behaves similarly to clinical samples when processed using different routine approaches [133], [137], [138]. As CRMs require commutability across a wide range of different quantification approaches to improve the comparability of results, additional work is needed to demonstrate a sufficient commutability of international reference materials for HCMV and EBV [133], [137], [138]. Additional concern with WHO international reference materials is the value assignment of viral quantity. This is usually performed in inter-laboratory assessments using different DNA extractions and qPCR-based methods [132]. The consensus viral quantity, which is derived from the arithmetical average of viral-DNA measurements (transformed into arbitrary unit, IU), is therefore not an absolute number of virus particles but just an approximation influenced by DNA loss during DNA extraction and previously described drawbacks related to qPCR quantification.

1.6.1.2 Reference measurements procedures

Reference measurement procedures are expected to enable robust, precise and reliable enumeration of viral particles and genome copy-numbers. They are also intended for providing a reliable assessment of other measurement procedures, through assessing their performance parameters for quantitative and qualitative measurements of the same analyte [139]. In addition, the introduction of reference measurement procedures is a prerequisite for development of well characterized CRMs [140], as they will allow for more precise and robust enumeration of CRMs than with currently available quantification-based methods, such as qPCR. Consequently, this will increase the traceability of values assigned to CRMs and therefore improve the definition of the international unit (IU). Additionally, due to better robustness and higher precision they will enable better comparison between batches of CRMs [58], [139], [140].

Currently, a well-established reference measurement procedure for quantification of nucleic acids of any type (bacterial, virus and eukaryotic DNA or RNA) is absent [58]. Therefore, the majority of reference-material characterizations and DNA-extraction assessments are performed by qPCR methods, which depend on calibration material and are therefore not a suitable candidate for a reference measurement procedure [132]. On the other hand, dPCR allows for quantification that is not dependent on external calibrators and might therefore be a more appropriate candidate for the reference measurement procedure [58], [66].

Chapter 2

Aims, Objectives, Hypothesis

The main aim of this work was to investigate processes involved in different molecular approaches/methods for quantification of HCMV and subsequently to improve its diagnostics. More specifically, the aim was to perform a detailed metrological assessment of different methods for DNA extraction and DNA quantification of HCMV. The focus was on investigating the advantages and drawbacks of the two most commonly used platforms of dPCR technology, droplet-based QX100 system (Bio-Rad) and microfluidic-based Biomark system (Fluidigm), and to compare them with qPCR, which is currently the most established approach for virus quantification.

Objectives:

1) Metrological assessment of three methods for manual extraction of HCMV DNA from different matrices (human plasma, cell culture), with the emphasis on DNA recovery and variability of extraction replicates within experiments and between experiments. The best-performing method was in turn selected for further inter-laboratory assessments.

2) Metrological evaluation of three molecular methods (qPCR, droplet-based dPCR and microfluidic-based dPCR) for quantification of HCMV. The emphasis was on investigating the influence of PCR components on quantification of DNA. Furthermore, with each platform several performance parameters were assessed, such as dynamic range, analytical sensitivity and precision. The best-performing methods were selected for subsequent inter-laboratory assessment.

3) Evaluation of two dPCR methods (droplet-based dPCR and microfluidic-based dPCR) for their applicability in direct quantification of HCMV without DNA extraction. The emphasis was on measured HCMV DNA concentration and measurement precision. Additionally, dPCR-based direct quantification was compared to dPCR-based quantification on isolated DNA.

4) Inter-laboratory assessment of three molecular methods (droplet-based dPCR and two microfluidic-based dPCRs) for quantification of HCMV. All participating laboratories followed the same protocols and used the same materials. The emphasis was on precision within laboratory and reproducibility between laboratories.

All aims were accomplished, with some results eventually published in international papers [92], [141], [142].

We have tested the following four hypotheses:

1) Metrological assessment of three methods for manual extraction of HCMV DNA from different matrices will give an indication on the best method for DNA-extraction method in terms of DNA recovery and precision. This will extend our knowledge on the DNA extraction

process, provide evidence on variability within and between methods for DNA extraction, and facilitate the selection of the most suitable method for manual DNA extraction for an inter-laboratory study. Furthermore, the results might be helpful when measurement variability of a certain extraction method of pre-emptive treatment would be considered.

2) Metrological evaluation of three molecular methods for quantification of HCMV will broaden our knowledge on the processes involved in the quantification of HCMV DNA, indicate the performance characteristics of qPCR, droplet-based dPCR and microfluidic-based dPCR, and facilitate the selection of the most suitable method(s) for an inter-laboratory assessment. Additionally, the results will delineate the most suitable quantification method for routine analysis of HCMV and shine a light on the most appropriate candidate for the reference measurement procedure for virus-DNA testing.

3) Evaluation of droplet-based dPCR and microfluidic-based dPCR for direct quantification of HCMV without DNA extraction will demonstrate if dPCR-based direct quantification allows for more robust and precise measurements than regular dPCR-based quantification of isolated DNA with high recovery. If yes, this would indicate dPCR-based direct quantification might be a more suitable approach for characterization and enumeration of CRMs than currently used approaches based on extracted DNA.

4) Inter-laboratory assessment of three molecular methods (droplet-based dPCR and two microfluidic-based dPCRs) for quantification of HCMV will elucidate the precision of several different dPCR platforms and estimate the reproducibility of dPCR. These results might also facilitate the selection of the reference measurement procedure for virus-DNA testing and provide additional supporting information for the candidate method for characterization and enumeration of CRMs.

This PhD is composed of four studies that were each performed according to four objectives. Therefore, the methods, results and discussion section are each compartmented into four sections, which each presents one study or objective.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Materials for quantification and extraction of HCMV DNA

- 1st WHO International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques (National Institute for Biological Standards and Control [NIBSC], code 09/162).

  Each vial of international standard comprised the lyophilised equivalent of 1 mL of whole-virus preparation of the HCMV 'Merlin' strain, diluted in 10 mM Tris-HCl buffer (pH 7.4) and 0.5 % human serum albumin. The material has been assigned a nominal concentration of 5 $\times 10^6$ international units (IU)/mL, when reconstituted in 1 mL nuclease-free water, based on data from an international collaborative study. The uncertainty of the vial content was determined by the manufacturer to be ±0.23 %. The material was shown to be stable during shipment at the ambient temperature [143]. Prior to opening, each vial was stored at -80 °C. After opening the vial, the content was reconstituted in 1 mL nuclease-free water. Hereinafter, the reconstituted material is denoted as WHO-5M material.
- Samples no. 365029 and 365032 (Society for Promoting Quality Assurance in Medical Laboratories [INSTAND]).

  The INSTAND materials presented different dilutions of cell lysates from MRC-5 cell cultures infected with HCMV. Nominal concentrations of each material, which were estimated by the means of qPCR from INSTAND Target Value Laboratories, were 77,000 cp/mL for sample no. 365029 and 20,000 cp/mL for sample no. 365032. After opening, each material was reconstituted in 1 mL double-distilled water to reach its nominal concentration.
- Standard Reference Material 2366 Cytomegalovirus (National Institute of Standards and Technology, [NIST]).

  It was composed of HCMV TowneΔ147 BAC [84]. 150 µL of component C (nominal concentration of 19641 cp/mL) were diluted in 1.35 mL TE buffer (Tris-EDTA), aliquoted and stored at -80 ºC [82]. As NIST material is composed of purified plasmid DNA, no further DNA extraction was needed prior to amplification. Due to plasmid instability reports from the manufacturer, aliquots were tested for concentration and stability on QX100 system using *UL54* assay. Average aliquot concentration with standard error was estimated to 1050±30 cp/µL.

3.1.2 Matrices and buffers for dilution of materials

- Phosphate Buffer Saline (PBS), pH 7.4
  - 137 mM NaCl (Merck, cat. no. 106404)
  - 2.7 mM KCl (Merck, cat. no. 104936)
  - 8 mM Na2HPO4 (Merck, cat. no. 10658)
  - 2 mM KH2PO4 (Merck, cat. no. 104873)

- Tris-EDTA buffer (Sigma, cat. no. T-5941)
- Acrometrix EDTA Plasma Dilution Matrix (Life Technologies, cat. no. S2284)

3.1.3 Kits and reagents for extraction of HCMV DNA

- High Pure Viral Nucleic Acid kits (Roche Diagnostics, cat. no. 11 858 874 001) [hereinafter denoted as Roche extraction kit]
- QIAamp DNA Mini Kit (Qiagen, cat. no. 51304) [henceforth denoted as Qiagen extraction kit]
- affigene DNA Extraction kit (Cepheid, cat. no. 71000942) [hereinafter denoted as Cepheid extraction kit]
- 100% ethanol (Merck, cat. no. 100983)

3.1.4 Master mixes for quantification of HCMV DNA

- ddPCR Supermix for Probes (Bio-Rad, cat. no. 1863010)
- TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, cat. no. 4444434) [hereinafter mentioned as Fast master mix]
- TaqMan Universal PCR Master Mix (Applied Biosystems, cat. no. 4304437) [henceforth denoted as Universal master mix]
- TaqMan Gene Expression Master Mix (Applied Biosystems, cat. no. 4369016) [hereinafter denoted as Gene master mix]
- FastStart Universal Probe Master Mix (Rox) (Roche Diagnostics, cat. no. 04913939001)[hereinafter denoted as Roche master mix]
- QuantStudio 3D Digital PCR Master Mix kit (Applied Biosystems, cat. no. 4482710)

3.1.5 PCR assays for quantification of HCMV DNA

- Oligonucleotide probes and primers for *UL54* assay (Eurofins) [144] (Table 3.1)
  The oligonucleotide probe of *UL54* assay contained a FAM molecule on the 5' end and BHQ1 molecule on the 3' end.
- Oligonucleotide probes and primers for *UL83* assay (Eurofins) [145] (Table 3.1)
  The oligonucleotide probe of *UL83* assay comprised a HEX molecule on the 5' end and BHQ1 molecule on the 3' end

Table 3.1: PCR assays.

Assay name	Sequence accession number	Amplicon length (bp)	Type of oligonucleotide	Sequence (5'->3')
UL54	AY422361	72	Forward primer	GGCCGTTACTGTCTGCAGGA
			Reverse primer	GGCCTCGTAGTGAAAATTAATGGT
			Probe	CCGTATTGGTGCGCGATCTGTTCAA
UL83	HM142972	159	Forward primer	GCAGCCACGGGATCGTACT
			Reverse primer	GGCTACCTCACACGAGCATT
			Probe	CGCGAGACCGTGGAACTGCG

3.1.6 Instruments and consumables for qPCR

- MicroAmp EnduraPlate Optical 384-Well Clear Reaction Plates with Barcode (Life Technologies, cat. no. 4483273)
- MicroAmp Optical Adhesive Film (File Technologies, cat. no. 4311971)
- Viia 7 Real-Time PCR System (Life Technologies)

3.1.7 Instruments, consumables and reagents for droplet-based dPCR (the QX100 system)

- QX100 Droplet Generator (Bio-Rad)
- DG8 Cartridges and Gaskets (Bio-Rad, cat. no. 1864007)
- Droplet Digital PCR Oil (Bio-Rad, cat. no. 1863005)
- Piercable Foil Heat Seal (Bio-Rad, cat. no. 181-4040)
- QX100 Droplet Digital PCR System (Bio-Rad)
- Twin Tec PCR plate 96, semi skirted (Eppendorf, cat. no. 951020362)
- Eppendorf heat sealer for PCR plates (Eppendorf, cat. no. Z605689)

3.1.8 Instruments, consumables and reagents for microfluidic-based dPCR (the Biomark system)

- 12.765 Digital Array Integrated Fluidic Circuit (Fluidigm)
- qdPCR 37K Integrated Fluidic Circuit (Fluidigm)
- Biomark HD system (Fluidigm)
- IFC Controller MX (Fluidigm)
- 20x GE sample loading reagent (Fluidigm, cat. no. 85000746)
- Fluidigm Control Line Fluid (Fludigm, cat. no. 89000020)

3.1.9 Instruments, consumables and reagents for microfluidic-based dPCR (the QuantStudio 3D system)

- QuantStudio 3D Chip Kit v1 (Applied Biosystems, cat. no. A26316)
- QuantStudio 3D Digital PCR System (Applied Biosystems)
- QuantStudio 3D Digital PCR Chip Loader (Applied Biosystems, cat. no. 4482592)
- Dual Flat Block GeneAmp PCR System 9700 (cat. no. 4428234)

3.1.10 General laboratory equipment

- Centrifuge MiniSpin (Eppendorf, cat. no. 022620100)
- Vortex 3 (Eppendorf, cat. no. 3340001)
- Automated pipettes (Eppendorf)
- Automated pipettes (Mettler Toledo)
- Tips for automated pipettes (Mettler Toledo)
- GeneAmp PCR System 9700 (Applied Biosystems, cat. no. ED000650)
- Eppendorf microtubes (Eppendorf)
- pH meter (Mettler Toledo)
- Heating block (Bio-Rad)

3.1.11 Software for statistical calculations

- Viia 7 Software version 1.2.4 (Applied Biosystems)
- QuantaSoft Software version 1.3.2.0 (Bio-Rad)
- Biomark HD Data Collection Software version 3.1.4 (Fluidigm)
- QuantStudio 3D AnalysisSuite Software (Applied Biosystems)
- Microsoft Excel 2007
- R studio version 0.98.977
- Probit software

3.2 Methods

3.2.1 Metrological assessment of three methods for manual extraction of HCMV DNA from different matrices

3.2.1.1 Overview of the study

With this study, four different whole-virus materials (WHO-60K, WHO-3K, INSTAND sample 365029 and INSTAND sample 365032; Chapters 3.1.1) were prepared and extracted for DNA using three different extraction kits (Roche, Cepheid and Qiagen; Chapter 3.1.3) (Figure 3.1). WHO-60K material was extracted in six extraction replicates in each of three consecutive experiments, making the total number of 18 extraction replicates. On the other hand, WHO-3K material and both INSTAND materials were extracted in one experiment in six (WHO-3K) or four extraction replicates (both INSTAND materials). For every material, one negative extraction control composed of pure human plasma was used in every experiment. To evaluate the recovery and variability of each extraction kit, every extraction replicate was then tested for DNA copy numbers using dPCR on the QX100 system (all four materials) or additionally qPCR on Viia 7 (WHO-60K). On dPCR, each extraction replicate was tested in duplicate (WHO-60K and INSTAND material 365029) or in triplicate (WHO-3K and INSTAND material 365032), whereas on qPCR three different dilutions of every extraction replicate were used, each tested in duplicate.

To assess DNA recovery, the measured DNA concentrations were recalculated to DNA copies/mL of the initial sample before DNA extraction was performed. Additionally, with every extraction kit the expanded MU was calculated.

To comprehensively investigate the intra-experiment and inter-experiment variability of every DNA extraction kit, two statistical approaches were used. One was focused on differences between extraction replicates (hereinafter denoted as extraction variability) (e.g. Figure 3.1, six extraction replicates of WHO-3K), whereas the other one combined the variability caused by DNA extraction and quantification method (hereinafter mentioned as measurement variability) (e.g. Figure 3.1, 18 measurement replicates, three per each of six extraction replicates of WHO-3K material). Every measured DNA copy-number concentration was also transformed into logarithmic values to facilitate the comparison with clinical measurements. Since SD values of measurements capture only 68.2% of all measurements (within $\pm 1\sigma$), for each material and extraction kit the difference between the maximum and minimum logarithmic value was also calculated to simulate the worst-case scenario in clinical testing where two consecutive measurements are compared to each other [56], [124].

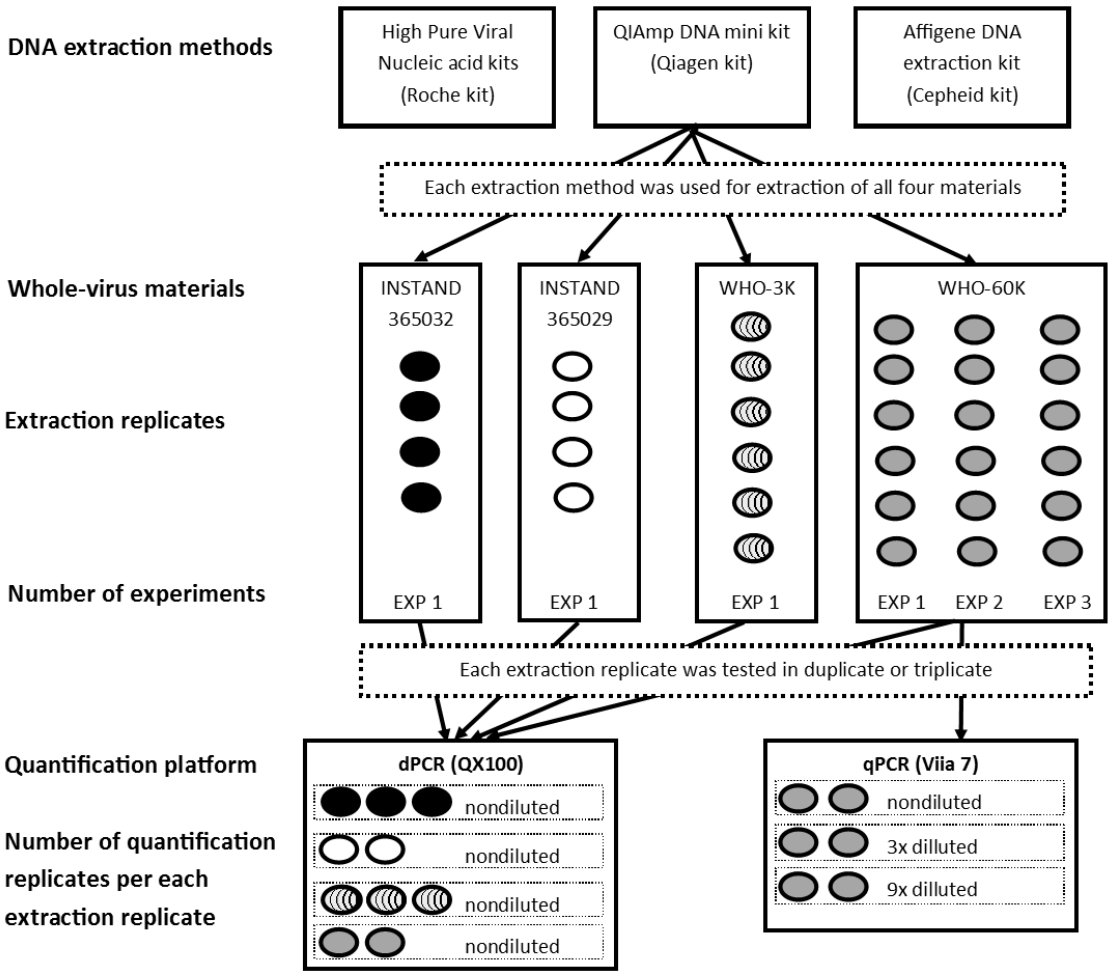


Figure 3.1: Schematical workflow of metrological assessment of three methods for manual extraction of HCMV DNA from different matrices.

3.2.1.2 Materials

Four different whole-virus materials were used in this study: two of them derived from differently diluted WHO reference material and two were INSTAND materials (nos. 365029 and 365032) (Chapter 3.1.1).

3.2.1.2.1 Preparation of two WHO materials (WHO-60K and WHO-3K)

Reconstituted WHO-5M (Chapter 3.1.1) material was additionally diluted in human plasma to allow assessment at two lower quantities of viral particles. In case of WHO-60K material, 180 µL of WHO-5M material were additionally diluted in 15 mL of human plasma to reach the nominal concentration of 60.000 IU/mL. This was then partitioned into 60 tubes with 200 µL mixture with human plasma and WHO material and stored at -80 °C until the DNA extraction. Material WHO-3K was prepared by combining 200 µL of WHO-5M material and 3.8 mL human plasma. After brief mixing, 180 µL of said mixture was additionally diluted in 14.82 mL human plasma to reach the nominal concentration of 3000 IU/ml. WHO-3K material was then partitioned into tubes containing 200 µL mixtures and underwent DNA extraction on the same day.

3.2.1.3 DNA extractions

3.2.1.3.1 DNA extractions using High Pure Viral Nucleic Acid Kit

Manual DNA extractions using High Pire Viral Nucleic Acid kit from Roche (Roche extraction kit) were performed according to manufacturer instructions. 200-µL samples contained different concentrations of each of two whole virus reference materials. Samples were composed of human plasma (both WHO materials) or reference-material's proprietary buffer (both INSTAND samples). Eluted DNA was then stored at -20°C until the subsequent DNA quantification.

3.2.1.3.2 DNA extractions using QIAamp DNA Mini Kit

Manual DNA extractions using QIAamp DNA Mini Kit from Qiagen (Qiagen extraction kit) were mostly performed according to the manufacturer's instructions, with some exceptions. 200-µL samples comprised different concentrations of each of two whole-virus reference materials. Whole virus materials were diluted either in human plasma (both WHO materials) or reference-material's proprietary buffer (both INSTAND materials). Since samples nominally contained less than 1 µg of DNA, elution in 50 µL TE buffer was carried out. No carrier DNA was added. Eluted DNA was then stored at -20°C until the subsequent DNA quantification.

3.2.1.3.3 DNA extractions using affigene DNA Extraction kit

Manual DNA extractions using affigene DNA Extraction Kit from Cepheid (Cepheid extraction kit) were performed according to manufacturer instructions. 200 µL samples contained different concentrations of each of two whole virus reference materials. Whole viruses were contained in human plasma (both WHO materials) or reference-material's proprietary buffer (both INSTAND materials). Eluted DNA was then stored at -20°C until the subsequent DNA quantification.

3.2.1.4 DNA quantification

3.2.1.4.1 Setup of PCR reaction

UL54 assay was used, targeting genomic regions within UL54 gene (Chapter 3.1.5). Oligonucleotide primers and probes of *UL54* assay were reconstituted in the amount of double-distilled water, based on manufacturer's instructions, to the intended concentration of 100 pmol/µL. Each vial of reconstituted oligonucleotides was then thoroughly mixed and centrifuged. Additionally, vial containing oligonucleotide probes were covered in aluminum foil to prevent bleaching of probes. Then, certain volumes of forward oligonucleotide primer, reverse oligonucleotide primer, oligonucleotide probe and double distilled water were combined, thoroughly mixed and centrifuged to achieve 20-fold concentrated assay mix, which resulted in final concentration of 600 nM primers and 100 nM probe (hereinafter denoted as *UL54-6/6-1* mix). This assay mix was then stored at -20°C until used for DNA quantification.

3.2.1.4.2 qPCR detection

The qPCR was carried out on the Viia 7 Real-Time PCR System. Each 20 µL-reaction was composed of 1 µL *UL54*-6/6/1 assay, 8 µL sample, 6 µL double-distilled water and 5 µL 4× concentrated TaqMan Fast Virus 1-Step Master Mix. Each component of the reaction mix was thawed, vortexed, centrifuged and diluted (if applicable) on the day of analysis before being pipetted into the 1.5 µL microcentrifuge. Samples with eluted DNA were equilibrated to the ambient temperature before being 1×, 3× and 9× diluted in double-distilled water and pipetted into the reaction mix. The material used for standard curve was a plasmid material that was 1×, 5× and 25× diluted in double-distilled water. After the addition of all components, the reaction mixture was vigorously mixed for 15 s and centrifuged. Then, a certain volume of the reaction mixture was pipetted into one well of the MicroAmp EnduraPlate Optical 384-Well Clear Reaction Plates with Barcode. After the addition of DNA into the individual wells the plate was sealed with MicroAmp Optical Adhesive Film to prevent cross contamination and loss due to evaporation. The 384-well plate was then centrifuged at 1000 g for 2 min at ambient temperature and placed into the Viia 7 Real-Time PCR System. The qPCR reactions were conducted under the universal conditions of 2 min at 50 °C, followed by 10 min at 95 °C and 45 cycles of 15 s at 95 °C and 1 min at 60 °C. The data analysis was carried out with Viia 7 Software version 1.2.4. The analysis of exported raw data was then carried out in Microsoft Excel 2010. With each reaction mix, at least one no-template control (NTC) was analyzed.

Standard curve was generated to allow relative quantification of DNA concentration in samples of interest.

3.2.1.4.3 Quantification of DNA concentration measured by qPCR

Quantification was performed by first calculating the slope and the intercept of the standard curve that comprised three different dilutions with defined DNA concentration. Then, using the calculated slope and intercept of the standard curve, raw Cq values of every measurement of the sample of interest were transformed to number of DNA cp/mL by taking into account equal amplification efficiencies of DNA templates from standard curve and from the sample of interest. For each extraction replicate, the measured DNA concentration was calculated as the average DNA concentration of every dilution that contributed to acceptable slope and with no evidence for amplification inhibition. All calculations were carried out in Excel 2007.

3.2.1.4.4 Droplet-based dPCR quantification

The droplet-based dPCR was performed on the QX100 Droplet Digital PCR System. Each 20 µL-reaction was composed of 1 µL 20-fold concentrated *UL54*-6/6/1 assay mix, 8 µL sample with extracted HCMV DNA, 1 µL double-distilled water and 10 µL ddPCR Supermix for Probes. After preparation, 20-µL reaction mixtures were pipetted into the designated sample wells on the DG8 Cartridges. Then, 70 µL Droplet Digital PCR Oil was pipetted into designated oil inlets on the same cartridge. Both types of inlets were then covered with DG8 Gaskets and transferred to QX100 Droplet Generator where droplets were generated by mixing oil and samples. Then, 40-µL content of each of eight droplet inlets on the same cartridge was simultaneously and carefully pipetted into eight wells on the Twin Tec PCR plate 96. After the PCR plate was loaded with all samples it was covered with Piercable Foil Heat Seal and sealed onto the plate by the use of Eppendorf heat sealer for PCR plates. Sealed plates were centrifuged for 2 min at 1000 g on the centrifuge and transferred to the GeneAmp PCR System 9700. There, PCR reactions were conducted simultaneously in each well using the universal qPCR conditions, with addition of 10 min at 98° C at the very end of the amplification. The plate was then inserted into the QX100 Droplet Digital PCR System where analysis of the droplets from each well was performed using QuantaSoft Software version 1.3.2.0. Fluorescence of reactions was measured for FAM channel. The fluorescence thresholds were set manually to facilitate the optimal distinction between the negative and positive droplets. Only the samples with more than 10,000 accepted droplets were considered for the analysis. The analysis of exported raw data was then carried out in Microsoft Excel 2010. With every reaction mix, at least one NTC was tested.

3.2.1.4.5 Estimation of DNA concentration measured by dPCR

Several equations were used in Excel 2007 for the estimation of DNA concentration that was measured with each dPCR platform.
The mean number of copies per partition (λ) was calculated according to Equation (3-1) [66], where k stands for the number of positive partitions and n means the number of partitions analysed:

$$\lambda = -\ln\left(1 - \frac{k}{n}\right) \tag{3-1}$$

The total reaction volume is the complete volume of the reaction mix that is transferred into the inlet or the well. The number of estimated copies per total reaction volume (M) was calculated according to Equation **Error! Reference source not found.**:

$$M = \lambda \frac{\text{total reaction volume (µL)}}{\text{partition volume (µL)}} \tag{3-2}$$

The effective reaction size is a certain portion of the total reaction volume that is transferred into the partitions analysed (chambers or analysed droplets), followed by use of

the analysis software, while the rest of the total reaction volume was excluded from the analysis. The effective reaction size is the product of the number of the partitions analysed and their volume [66].

The number of estimated copies per mean effective reaction size (*N*) was calculated accordingly as the product of mean number of copies per partition (λ) and number of analysed partitions (*n*).

The number of estimated copies per 1 µL sample (*C*) was calculated according to Equation **Error! Reference source not found.**, with d denoting the dilution of the sample prior to the analysis.

$$C = d\frac{M}{\text{sample input volume (µL)}} \tag{3-3}$$

3.2.1.5 Statistical analysis

3.2.1.5.1 Coefficient of variation (CV)

CV was calculated in Excel 2007 by dividing standard deviation (SD) of selected measurements with their average value.

3.2.1.5.2 Statistical significance of differences

Statistical significance of differences between measurements sets was calculated using Student's t-test in Excel 2007 (two tailed, two-sample equal variance), single-factor analysis of variance (ANOVA) in Excel 2007 and Tukey's test in R studio.

3.2.1.5.3 Standard and extended measurement uncertainty (MU)

With each quantification method, standard MU was calculated based on the bottom-up approach in equation [146]. MU was calculated as a combined measurement uncertainty according to Equation **Error! Reference source not found.**, where u_r is variability related to intra-experiment repeatability and u_{ip} is variability related to intermediate precision.

$$\mathrm{MU} = \sqrt{u_r^2 + u_{ip}^2} \tag{3-4}$$

u_r and u_{ip} were calculated using Equations **Error! Reference source not found.**, **Error! Reference source not found.** and **Error! Reference source not found.**, where symbol *n* is the number of independent replicates per experiment, *N* depicts the number of experiments performed on one platform, MS_{within} stands for the mean square value within groups and $MS_{between}$ represents the mean square value between groups.

$$u_r = \frac{\sqrt{\mathrm{MS}_{\mathrm{within}}}}{\sqrt{n}} \tag{3-5}$$

$$u_{ip} = \sqrt{\frac{\mathrm{MS}_{\mathrm{between}} - \mathrm{MS}_{\mathrm{within}}}{n \times N}} \tag{3-6}$$

$$u_{ip2} = \frac{\sqrt{\frac{MS_{between}}{n}} \times \sqrt[4]{\frac{2}{N \times (n-1)}}}{\sqrt{N}} \quad (3\text{-}7)$$

Both mean squares were calculated using ANOVA in Microsoft Excel 2007. If $MS_{between}$ was bigger than MS_{within}, the Equation **Error! Reference source not found.** was used to calculate the intermediate precision. In contrast, if $MS_{between}$ was smaller than MS_{within}, intermediate precision was calculated by Equation **Error! Reference source not found.**. To obtain the expanded MU, the coverage factor (*k*) was applied to it. The value of coverage factor was chosen at 95% level of confidence based on degrees of freedom.

3.2.2 Metrological evaluation of three molecular methods (qPCR, droplet-based dPCR and microfluidic-based dPCR) for quantification of HCMV

3.2.2.1 Overview of the study

Hereby, qPCR (Viia 7) and two dPCR platforms (the QX100 system and the Biomark system) were assessed using clinically relevant concentrations of two reference materials for HCMV, one containing linear genomic DNA 1 (gDNA1) and one with circular plasmid DNA (Chapter 3.1.1, Figure 3.2). This study was composed of two major assessments. In the first assessment, the influence of different PCR components (master mixes, PCR assays, oligonucleotide primer and oligonucleotide probe concentrations) on estimation of DNA concentration was evaluated for both dPCR platforms. With the second assessment, qPCR and each of two dPCR platforms were assessed for the linearity, LOD, LOQ, and precision. Two different perspectives were used, one considering the total-reaction volume and another using the effective reaction size.

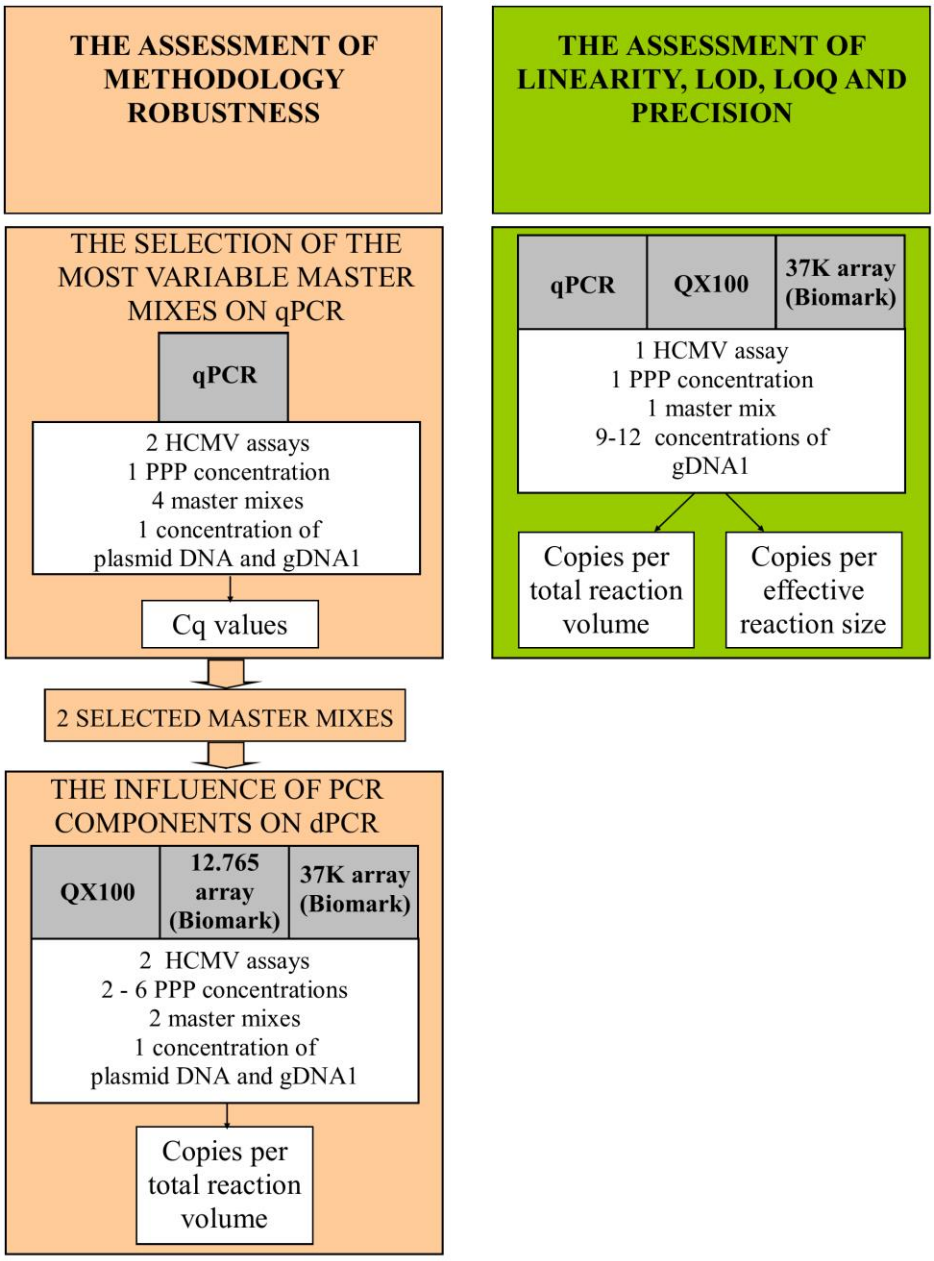


Figure 3.2: Schematical presentation of the metrological evaluation of three molecular methods for quantification of HCMV. PPP concentration denotes the concentration of oligonucleotide primers and probes.

3.2.2.1.1 The assessment of robustness of two dPCR based quantification methods

The aim of this study was to assess the influence of various PCR components on the estimation of DNA concentration measured by the Biomark system (using two different arrays 12.765 and 37K) and QX100 system. To facilitate the assessment, a preliminary analysis was initially conducted on qPCR to select a) one master mix that causes the biggest variability in Cq values between two assays, and b) two master mixes that cause the highest difference in the

result when the same assay and the same material are used. To achieve this, combinations of four different master mixes (Fast master mix, Universal master mix, Gene master mix and Roche master mix; Chapter 3.1.4), two HCMV assays (*UL54* assay and *UL83* assay, Chapter 3.1.5) with one concentration of primers and probes and two HCMV materials (plasmid DNA and gDNA1; Chapter 3.1.1) with a single DNA concentration were tested in duplicates on qPCR. Two selected master mixes from the preliminary study were then used for the subsequent metrological assessment of the QX100 system and both Biomark arrays, together with single concentration of each of two DNA materials (plasmid DNA and gDNA1) and two HCMV assays with six concentrations of oligonucleotide primers and probes (Figure 3.2). On each dPCR platform and every Biomark chip each combination of PCR components and HCMV material was tested in duplicate.

3.2.2.1.2 Assessment of linearity, LOD, LOQ and precision for three quantification methods

To assess linearity, LOD, LOQ and precision (intra-experiment variability and intermediate precision), with each platform only one PCR assay (*UL54*-6/6/2), one material (gDNA1) and a single master mix were used. The assessment was performed by one operator in two consecutive experiments with five replicates of each gDNA1 dilution per experiment. Dilutions series of gDNA1 were freshly prepared on the day of analysis. In order to eliminate bias caused by possible unequal pipetting volumes during the sample mixing with reaction mix, larger volumes of reaction mix and sample were mixed together, followed by separation of mixture into five wells or inlets. To assess qPCR and the QX100 system, 12 different dilutions of gDNA1 in double distilled water (3.8×, 7.5×, 18.8×, 75.2×, 188×, 376×, 940×, 1880×, 3760×, 9400×, 18,800× and 188,800x) were used, while the Biomark 37K array was tested with nine different concentrations of gDNA1 diluted in double distilled water (1×, 1.9×, 3.7×, 9.4×, 18.5×, 47.7×, 91×, 200× and 1000× dilution). Additionally, on each platform three to five NTCs were tested in every experiment.

3.2.2.2 Materials

Two different materials were used, plasmid DNA from NIST (Chapter 3.1.1) and gDNA1. If needed, each material was further diluted in double distilled water before the quantification analysis.

3.2.2.2.1 Genomic DNA 1

To prepare gDNA1, reconstituted WHO-5M material (Chapter 3.1.1) was additionally diluted 5× in PBS. Twenty-two 200 µL aliquots were prepared and extracted on the same day using Roche extraction kit according to manufacturer's instructions. Extracted gDNA1 was then merged, mixed, aliquoted and stored at -20 ºC. Their concentration and stability were evaluated on QX100 system using *UL54* assay. Average concentration of gDNA1 was found to be 2580±60 cp/µL.

3.2.2.3 DNA extractions

gDNA1 material was extracted using the Roche extraction kit as described in Chapter 3.2.1.3.1.

3.2.2.4 DNA quantification

gDNA1 material was extracted using the Roche extraction kit as described in Chapter 3.2.1.3.1.

3.2.2.4.1 Setup of PCR reaction

To assess the influence of different PCR components on dPCR platforms, with each of two HCMV assays (*UL54* and *UL83*) six different concentrations of oligonucleotide primers and probes were prepared using the same experimental procedure as in Chapter 3.2.1.4.1. For each assay mix, the intended final concentration of oligonucleotide primers and probes when mixed with sample and master mix into a reaction are shown in Table 3.2.

Table 3.2: Final concentration of different assay mixes.

20× assay mix	Final concentration in reaction mix [nM]		
	Forward primer	Reverese primer	Probe
UL54-3/3/1	300	300	100
UL54-3/3/2	300	300	200
UL54-6/6/1	600	600	100
UL54-6/6/2	600	600	200
UL54-9/9/1	900	900	100
UL54-9/9/2	900	900	200
UL83-3/3/1	300	300	100
UL83-3/3/2	300	300	200
UL83-6/6/1	600	600	100
UL83-6/6/2	600	600	200
UL83-9/9/1	900	900	100
UL83-9/9/2	900	900	200

3.2.2.4.2 qPCR detection

The platform and procedure were similar to those mentioned in Chapter 3.2.1.4.2, however there were some changes considering reaction volume and reaction components.
With preliminary assessment of master mixes, 10-µL reaction mixtures were composed of 0.5 µL assay (*UL54*-6/6/1 or *UL83*-6/6/1), 3.5 µL DNA sample (1× diluted plasmid DNA or 2.5× diluted gDNA1), and either of 3.5 µL double-distilled water and 2.5 µL 4× concentrated Fast master mix, or 1 µL double-distilled water and 5 µL 2× concentrated master mix (Universal master mix, Gene master mix and Roche master mix). No standard curve was performed.
With assessment of linearity, LOD, LOQ and precision, 20-µL reactions consisted of 8 µL DNA sample (different dilutions of gDNA1), 1 µL *UL54*-6/6/2 assay, 5 µL TaqMan Fast Virus 1-Step Master Mix and 6 µL double-distilled water.

3.2.2.4.3 Quantification of DNA concentration measured by qPCR

With preliminary assessment of master mixes, average Cq values obtained for every combination of assay and master mix were compared between each other.
With assessment of linearity, LOD, LOQ and precision, same procedure was used for estimation of DNA concentration as described in Chapter 3.2.1.4.3, with exception that dilutions from both experiments were also used as a calibration curve, since they all had pre-determined concentrations on the QX100 system. In addition, average DNA concentration was calculated from five or ten measurement replicates.

3.2.2.4.4 Droplet-based dPCR quantification

With both assessments, the same platform, experimental procedure, reaction volume and PCR components volume were used as in Chapter 3.2.1.4.4, however various PCR components were used and the fluorescence of *UL83* assays was measured using the HEX channel.

With assessment of the influence of PCR components on the final quantification, a 5× diluted plasmid DNA or a 12.5× diluted gDNA1 was used, and HCMV assay was each of 12 assays from the Table 3.2. NTCs were used for each combination of reaction components.
With assessment of linearity LOD, LOQ and precision, the DNA sample was one of 13 dilutions of gDNA1 and HCMV assay was *UL54*-6/2/2.

3.2.2.4.5 Microfluidic-based dPCR quantification

The microfluidic-based dPCR was carried out on the Biomark HD system. Two different arrays were used, Biomark 12.765 array, which allows simultaneous analysis of 12 samples, and Biomark 37K array that allows for the analysis of 48 samples in a single experiment. Every reaction mixture comprised 20-fold concentrated assay mix, sample with HCMV DNA, double-distilled water, 20x GE sample loading reagent and one of four different master mixes. On the Biomark 12.765 array, 8-µL reaction mixtures were used, whereas on the Biomark 37K array 4-µL reactions were used. On the same day before the analysis, every component of the reaction

mix was thawed, briefly mixed, centrifuged and diluted (if needed), followed by pipetting into the 1.5 µL microcentrifuge. Each reaction mixture was additionally vortexed and centrifuged before being added onto the array. Each array type was filled with 300 µL Fluidigm Control Line Fluid into both accumulators of the array using proprietary syringes. The filled array was then transferred to IFC Controller MX and primed within one hour using the proprietary software. After priming, each reaction mixture was individually pipetted into sample inlets with special care to avoid air bubbles. On every array, all sample inlets were filled with a reaction mixture, either with or without sample, to allow undisrupted monitoring of the fluorescence signal over all partitions on the array. Within one hour after priming occurred, filled arrays were transferred to IFC Controller MX and loaded using the proprietary software. Loaded arrays were then inserted into the Biomark HD system (Fluidigm), where amplification was performed under the same universal conditions as with qPCR. The data was then analysed using the Biomark HD Data Collection Software version 3.1.4, with manual determination of threshold, quality threshold (0.2) and the accepted quantification cycle in qPCR range between Cq values of 15 and 45. Fluorescence of reactions was measured for FAM and/or HEX channels. With each reaction mix, at least one NTC was analyzed. The analysis of exported raw data was then carried out in Microsoft Excel 2010.

For assessment of the influence of PCR components on the final quantification, both Biomark arrays were used. On the Biomark 12.765 array 8-µL reactions comprised 3.2 µL sample (4.5× diluted plasmid DNA or 10× diluted gDNA1), 0.4 µL 20× assay (either *UL54*-3/3/1, *UL54*-6/6/2, *UL54*-9/9/2, *UL83*-3/3/1, *UL83*-6/6/2 or *UL83*-9/9/2), 0.5 µL 20× GE Sample Loading Reagent and either 4 µL 2× Universal master mix or 2 µL 4× TaqMan Fast master mix and 2 µL double-distilled water. With Biomark 37K array, 4-µL reactions were composed of 1.4 µL sample (4.5× diluted plasmid DNA or 10× diluted gDNA1), 0.2 µL assay (either *UL54*-3/3/1, *UL54*-6/6/2, *UL83*-3/3/1 or *UL83*-6/6/2), 0.4 µL 20× GE Sample Loading Reagent, and either 2 µL 2× Universal master mix or 1 µL 4× Fast master mix and 1 µL double-distilled water.

With assessment of the influence of PCR components on the final quantification, only Biomark 37K array was used, with similar component volumes as mentioned above for the other assessment. However, only one master mix (Fast master mix), one DNA sample (9 dilutions of gDNA1) and one assay (*UL54*-6/6/2) were used.

3.2.2.4.6 Estimation of DNA concentration measured on each dPCR platform

On QX100 system and on Biomark system, the estimation of DNA concentration was similar to that in Chapter 3.2.1.4.5.

3.2.2.5 Statistical analysis

For statistical analysis, similar approaches were used as in Chapters 3.2.1.5.1 (Coefficient of variation) and 3.2.1.5.2 (Statistical significance of differences), with addition of several new ones as described further on.

3.2.2.5.1 Linearity of dynamic range

Linearity of dynamic range was analysed by means of Pearson's correlation coefficients in R studio.

3.2.2.5.2 Limit of detection (LOD)

LOD with 95% confidence interval was determined with Probit software [147].

3.2.2.5.3 Limit of quantification (LOQ)

LOQ was determined as the lowest nominal DNA concentration where the CV was still below 25 % [106].

3.2.2.5.4 Estimated Poisson error

The predicted Poisson error, expressed as the theoretical CV, was calculated according to Equation **Error! Reference source not found.** based on Poisson statistics [148]:

$$\mathrm{CV\ (theoretical)} = \sqrt{\left(\frac{1-e^{-\lambda}}{n\lambda^2 e^{-\lambda}}\right)} \quad (3\text{-}8)$$

3.2.2.5.5 Detection of outliers

Where appropriate, outliers were determined for several assessments using Grubbs test in R studio (package "outliers") and excluded from further analysis.

3.2.2.5.6 Determination of the effective reaction size and the total reaction volume

For every dPCR platform, the linearity, LOD, LOQ and precision (intra-experiment variability and intermediate precision) were assessed in two ways, considering either the effective reaction size or the total-reaction volume. To determine the effective reaction size, the number of analysed partitions and their volume had to be defined. On the QX100 system, the number of analysed partitions varies between each analysed inlet, thus it should be determined individually using information from QuantaSoft Analysis software. In this study, the number of accepted droplets ranged from 10,300 to 14,700, with an average of 13,600. Considering their 0.834 nL volume [85], the average effective reaction size was estimated to 11.3±0.8 µL. Conversely, the Biomark 37K array has defined the number of partitions and their volume. Each panel on the 37K array contains 770 0.84 nL chambers, thus the effective reaction size is around 0.647 µL. On the QX100 system, the total-reaction volume is 20 µL, on the Biomark 37K array it is 4 µL, while on qPCR the effective reaction size is 20 µL and is equal to the total-reaction volume.

3.2.2.5.7 Influence of variability on clinical measurements

To assess the influence of observed variability on clinical measurements, with all three quantification platforms all measured values (cp/total reaction value) were recalculated to the number of viral DNA copies per mL of human plasma. This was carried out by taking into account 100% DNA recovery of 200-µL sample extracted with Roche extraction kit into 50-µL eluted sample, followed by the quantification using 8 µL (qPCR and QX100 system) or 1.4 µL (Biomark 37K array) of eluted sample. Only values considering the total-reaction volume were taken into account. All recalculated values were transformed into logarithmic values. Then, SD and the difference between maximum and minimum value were assessed, in a same way as described in Chapter 3.2.1.1.

3.2.3 Evaluation of two molecular methods (droplet-based dPCR and microfluidic-based dPCR) for direct quantification of HCMV without DNA extraction

3.2.3.1 Overview of the study

In this evaluation, two dPCR approaches, the QX100 system and both Biomark arrays, were tested for direct quantification of two HCMV whole-virus materials, WHO material and INSTAND material, without the use of DNA extraction. This evaluation was composed of two parts (Figure 3.3). In the first part, each whole-virus material was also extracted for DNA and subsequently quantified on each dPCR platform to facilitate the comparison between direct quantification and quantification of extracted DNA. In the second part, WHO material was diluted in differently concentrated solutions of human plasma to investigate the suitability of direct quantification for clinical samples.

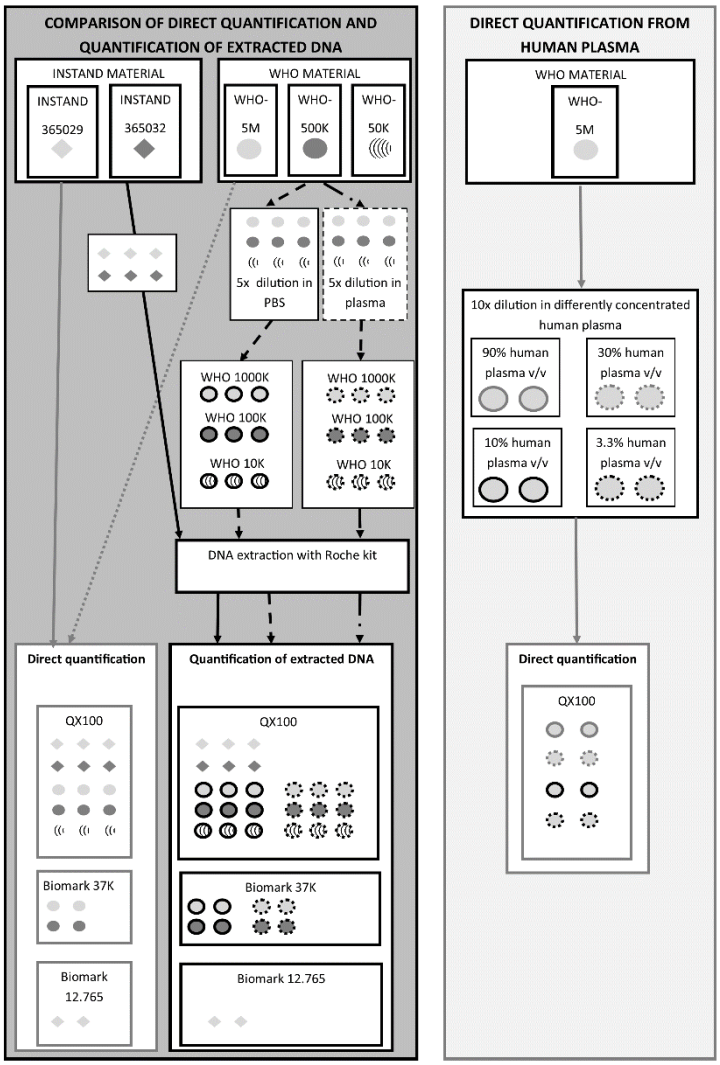


Figure 3.3: Schematical overview of evaluation of droplet-based dPCR and microfluidic-based dPCR for direct quantification of HCMV without DNA extraction.

3.2.3.1.1 Direct quantification of whole-virus samples and quantification of extracted HCMV DNA on each dPCR platform

In this comparison, dPCR-based direct quantification that does not require DNA extraction of whole viruses was compared to dPCR-based quantification of extracted DNA. Both quantification approaches were assessed for DNA recovery and variability.

For direct quantification, different concentrations of INSTAND material (365029 and 365032, Chapter 3.1.1) and WHO material (WHO-5M, WHO-500K, WHO-50K; Chapter 3.1.1) were used. With each of five materials, three replicates that did not undergo DNA extraction were directly used for quantification on the QX100 system. Additionally, WHO-5M and WHO-500K materials were directly quantified in duplicate on Biomark 37K array, and INSTAND 365029 sample was directly quantified in duplicate on the Biomark 12.765 array (Figure 3.3).

For quantification of extracted HCMV DNA, the same five materials were used as for direct quantification. However, each WHO material was additionally fivefold diluted in either PBS or human plasma prior to DNA extraction. Diluted WHO materials (WHO-1000K, WHO-100K, WHO-10K) and both INSTAND materials were then extracted in triplicate using the Roche kit. On the QX100 system with each of five materials, all three extraction triplicates

were tested each in one quantification replicate. In contrast, on each Biomark array two randomly selected extraction triplicates were measured in one measurement (WHO-1000K and WHO-100K on the Biomark 37K array; INSTAND 365029 sample on the Biomark 12.765 array) (Figure 3.3).
All ten materials (extracted and non-extracted) were quantified on the same day as their reconstitution, dilution and/or DNA extraction was performed.

3.2.3.1.2 Direct quantification from human plasma

Here, the ability of dPCR-based direct quantification for clinical applications was assessed. WHO-5M materials were tenfold diluted in human plasma to produce samples with various concentrations of human plasma (90% v/v, 30% v/v, 10% v/v and 3.3% v/v). Then, each plasma concentration was directly quantified in duplicate on the QX100 system (Figure 3.3).

3.2.3.2 Materials

In this study, two INSTAND materials (nos. 365029 and 365032) and WHO-5 material were used (Chapter 3.1.1), either undiluted (all three materials) or diluted in different matrices prior to DNA extraction and/or quantification.

3.2.3.2.1 Dilution of WHO-5M material in double-distilled water and PBS buffer or human plasma prior to DNA extraction (WHO-1000K, WHO-100K and WHO-10K material)

The WHO-5M material was first additionally diluted 10× and 100× in double-distilled water, for nominal HCMV concentrations of 5×10^5 (WHO-500K) and 5×10^4 IU/mL (WHO-50K). Then, undiluted WHO-5M and both diluted materials were further diluted five-fold either in in-house PBS buffer or human plasma to reach nominal concentrations of 1×10^6 (WHO-1000K), 1×10^5 (WHO-100K) and 1×10^4 IU/mL (WHO-10K). All six materials underwent DNA extraction and quantification (direct or that of extracted DNA) on the same day.

3.2.3.2.2 Dilution of WHO material in human plasma prior to direct quantification of whole HCMV

To directly quantify samples containing a clinically relevant matrix, the WHO-5M material was volumetrically diluted an additional 10× in 100%, 33.3%, 11.1% and 3.7% human-plasma solutions, which were beforehand prepared by volumetrically diluting in double-distilled water. This resulted in samples containing the nominal concentration of 5×10^5 IU/mL and either 90%, 30%, 10% or 3.3% (v/v) human plasma. Each of these samples was then directly transferred to the QX100 system to perform the direct virus quantification.

3.2.3.3 DNA extractions

All materials were extracted using Roche kit as described in Chapter 3.2.1.3.1. Samples were composed of human plasma (WHO materials), PBS buffer (WHO materials) or proprietary buffer (both INSTAND samples).

3.2.3.4 DNA quantification

3.2.3.4.1 Droplet-based dPCR on the QX100 system and the Biomark system

Direct quantification and quantification of extracted DNA were mostly performed in the same way as described in Chapter 3.2.1.4.4 (the QX100 system) and 3.2.2.4.5 (the Biomark system). The only difference was that *UL54*-6/6/2 assay and sometimes samples with whole viruses instead of extracted DNA were used.

3.2.3.4.2 Estimation of DNA concentration measured on both dPCR platforms

On the QX100 system and the Biomark system, the estimation of DNA concentration was similar to that in Chapter 3.2.1.4.5. All measured DNA concentrations were multiplied by the dilution factors to reach the initial DNA concentration per mL of reference material.

3.2.3.5 Statistical analysis

For statistical analysis, similar approaches were used as in Chapters 3.2.1.5.1 (Coefficient of variation) and 3.2.1.5.2 (Statistical significance of differences).

3.2.4 Inter-laboratory assessment of three molecular methods (droplet-based dPCR and two microfluidic-based dPCRs) for quantification of HCMV

3.2.4.1 Overview of the study

In inter-laboratory assessment, the comparison of different dPCR platforms was conducted to assess their intermediate precision and reproducibility for quantification of genomic DNA extracted from international reference material for HCMV. Two HCMV test materials were used in four National Metrology Institutes (NMIs): whole-virus material (WHO-1M; purchased from the manufacturer individually by each laboratory for subsequent 'local' DNA extraction of each WHO-1M Unit or Unit W), and genomic DNA (gDNA2, 'centrally' extracted from one WHO-5M unit in the National Institute of Biology [NIB] laboratory, merged, partitioned into gDNA2 units and distributed to collaborating laboratories, henceforth mentioned as centrally prepared gDNA2 Units or Unit G). The complete experimental workflow is shown schematically in Figure 3.4.

3.2.4.1.1 Detailed experimental workflow

Each of the collaborative laboratories purchased three whole-virus units (WHO-5M Units) from manufacturer that were later diluted into WHO-1M Units (e.g. Figure 3.4a, Units W1-W9), and also received three gDNA2 Units (e.g. Figure 3.4b, Units G1-G9) from Laboratory 1. Two vials with mixed primers and probes were also received by each collaborative laboratory (from Laboratory 1) on dry ice. In every participating laboratory, DNA quantification of locally extracted WHO-1M Units and centrally prepared gDNA2 Units was performed using various dPCR platforms: the QX100 system and two chip-based dPCR systems (the Biomark 37K array and the QuantStudio 3D Digital PCR). To determine the inter-experiment variability (i.e., intermediate precision) and inter-unit variability within each platform, in each laboratory and on every dPCR platform three WHO-1M Units and/or three gDNA2 Units were tested. For each dPCR platform, three experiments were performed on different days within a short period of time. In each experiment, three aliquots were tested simultaneously, each derived from a different WHO-1M or gDNA2 Unit s (e.g., Figure 3.4b, Experiment 1, aliquot 1 from Unit G1, aliquot 1 from Unit G2, aliquot 1 from Unit G3). Laboratories 1 and 2 examined both test materials in the same experiment. Additionally, to determine the reproducibility of the dPCR, and thus the potential for characterisation and value assignment of reference materials, for each test material the mean HCMV DNA concentration and corresponding measurement uncertainty were estimated using the data from all five dPCR platforms.

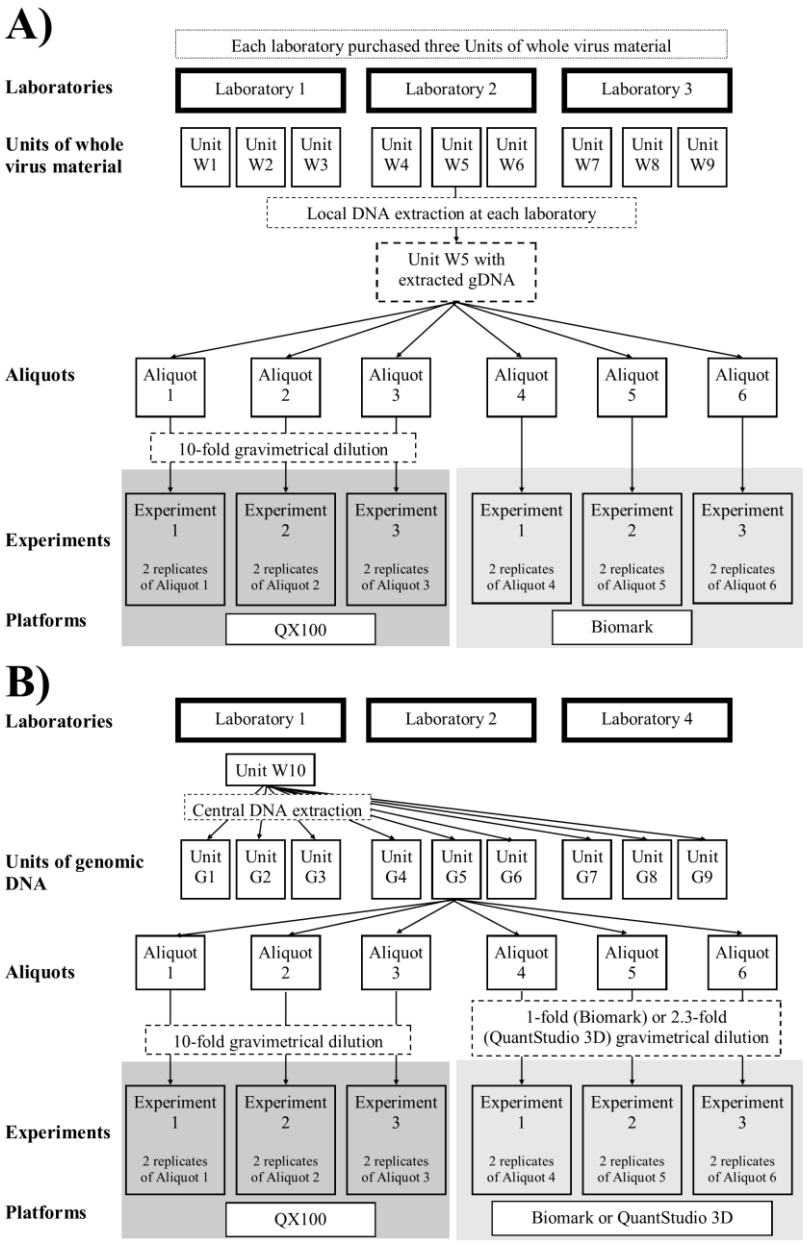


Figure 3.4: Schematical overview of the inter-laboratory assessment of three molecular methods for quantification of (a) WHO-1M material and (b) gDNA2 material. With both materials, the procedure presented for Units W5 and G5 was also applied to all other units. For every material, within each laboratory at least one of the two presented platforms was used.

3.2.4.1.2 Participants and dPCR platforms

Four NMIs participated in the collaborative study. The full names of participating laboratories are: National Institute of Biology (NIB), Slovenia; European Commission, Joint Research Centre, Institute of Reference Materials and Measurements (IRMM), European Union; Laboratory of Government Chemist (LGC), United Kingdom; National Metrology Institute of Turkey (TÜBITAK), Turkey. The participating laboratories, dPCR platforms used and materials tested are shown in Table 3.3.

Table 3.3: List of participants, dPCR platforms and test materials analysed.

Laboratory	Institute	Country	dPCR platform (manufacturer)	Material analysed
1	NIB[a]	Slovenia	QX100 Biomark	WHO-1M and gDNA2
2	IRMM[b]	European Union	QX100 Biomark	WHO-1M and gDNA2
3	LGC[c]	United Kingdom	Biomark	WHO-1M
4	TUBITAK[d]	Turkey	QuantStudio 3D	gDNA2

[a]NIB, National Institute of Biology
[b]IRMM, Joint Research Centre, European Commission
[c]LGC (formerly Laboratory of the Government Chemist)
[d]TUBITAK UME National Metrology Institute of Turkey

3.2.4.2 Materials

In this study, gDNA2 material (gDNA2 units) and WHO-1M material (WHO-1M units) were used.

3.2.4.2.1 Preparation of WHO-1M material and WHO-1M units

In Laboratory 1 and upon arrival at the collaborating laboratories (Laboratories 2 and 3), 200 µL of every vial of WHO-5M material (Chapter 3.1.1) was additionally diluted 5-fold in PBS (each collaborating laboratory used either the same PBS as Laboratory 1 Units, or purchased PBS from a manufacturer), to reach the nominal HCMV concentration of 1×10^6 IU/mL (WHO-1M unit; e.g. Figure 3.4a, Units W1-W9).

For each diluted WHO-1M unit, DNA extraction was performed using Roche extraction kit. Immediately after the elution of the DNA, each of three tubes with 50-µL eluted DNA was aliquoted as described in a chapter below. Additionally, in each participating laboratory (Laboratories 1, 2 and 3), 200 µL negative extraction control (40 µL double-distilled diluted in 160 µL PBS) was extracted using the Roche extraction kit (Chapter 3.2.1.3.1) simultaneously with extractions of WHO-1M units. Upon extraction, negative extraction control was aliquoted into nine 5 µL aliquots for subsequent analyses.

3.2.4.2.2 Preparation of gDNA2 units

Each gDNA2 Unit was centrally prepared at Laboratory 1 (Figure 3.4b, Table 3.3) by extraction of gDNA2 from the WHO-5M Unit, followed by circulation of prepared gDNA2 Units to the participating laboratories. To prepare gDNA2 Units, a WHO-5M Unit (e.g. Figure 3.4b, Unit W10) was 5-fold diluted in PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). This material was then divided into 22 200-µL aliquots, from where the gDNA2 material was extracted on the same day using Qiagen extraction kits. The extracted gDNA2 material was then pooled, mixed, and aliquoted into 50-µL aliquots (henceforth referred to as the gDNA2 Units). gDNA2 Units were stored at -20 °C for 4 months. Six of these gDNA2 Units were then sent on dry ice to two collaborative laboratories (Figure 3.4b, Table 3.3, Laboratories 2 and 4), three gDNA2 Units to each laboratory, while three of these gDNA2 Units remained at Laboratory 1.

3.2.4.2.3 Aliquot preparation and dilution of test materials

In every laboratory, from every locally extracted WHO-1M Unit (50-µL eluted volume) and each centrally prepared 50-µL gDNA2 Unit six aliquots were prepared (e.g., Figure 3.4a,

aliquots 1-6 from Unit W5) by dividing 50-µL volume into six low-binding microcentrifuge tubes (aliquot volumes used for dPCR platforms; QX100~6 µL, Biomark~9 µL, QuantStudio 3D~9 µL). All of the aliquots were then stored at -20 °C. For each dPCR platform, three aliquots from the same WHO-1M Unit or gDNA2 Unit were each analysed in one of three consecutive experiments on the same dPCR platform, within a short time interval. Each aliquot was thawed, gravimetrically diluted in double-distilled water, and analysed on the same day.

For quantification of the aliquots (derived from both the WHO-1M Units and the gDNA2 Units) on the QX100 system, 10-fold gravimetric dilutions of these aliquots were carried out on the day of the analysis. For the analyses on the Biomark system and the QuantStudio 3D system, undiluted aliquots derived from the WHO-1M Units and the gDNA2 Units were used.

3.2.4.3 DNA extractions

WHO-1M units were extracted using the Roche extraction kit as described in Chapter 3.2.1.3.1. To prepare gDNA2 Units, WHO-5M material was extracted using Qiagen extraction kit as described in Chapter 3.2.1.3.2.

3.2.4.4 DNA quantification

3.2.4.4.1 Droplet-based dPCR and microfluidic-based dPCR on Biomark

Quantification was mostly performed in the same way as described in Chapter 3.2.1.4.4. (the QX100 system) and 3.2.2.4.5 (the Biomark system), with the exception that the *UL54*-6/6/2 assay was used.

3.2.4.4.2 Microfluidic-based QuantStudio 3D System

The microfluidic-based dPCR was performed at Laboratory 4 on the QuantStudio 3D Digital PCR System. Fifteen-microliter-reaction mixtures were composed of 0.75 µL 20× concentrated *UL54*-6/6/2 assay mix, 6.75 µL sample with HCMV DNA and 7.5 µL 2× QuantStudio 3D Digital PCR Master Mix kit. Prepared reaction mixes were loaded into the QuantStudio 3D Chip Kit v1, which was sealed afterwards using the QuantStudio 3D Digital PCR Chip Loader. Each array contained only one reaction mixture. Sealed array was then placed into the Dual Flat Block GeneAmp PCR System where amplification was performed under universal qPCR conditions. Then each array was individually placed into the QuantStudio 3D Digital PCR System where the fluorescence of each chamber was monitored over the FAM channel using the QuantStudio 3D AnalysisSuite Software. The quality threshold was manually set to 0.5 in “colour by quality mode”, automatically calculated threshold was used in the “colour by calls” mode and confidence level was set to 95% with desired precision of 10%. The analysis of exported raw data was then carried out in Microsoft Excel 2010.

3.2.4.4.3 Estimation of DNA concentration measured by dPCR

On all three dPCR systems, the estimation of DNA concentration was similar to that in Chapter 3.2.1.4.5.

3.2.4.5 Statistical analysis

For statistical analysis, same approaches were used as in Chapter 3.2.1.5.1 (Coefficient of variation), Chapter 3.2.1.5.2 (Statistical significance of differences), Chapter 3.2.1.5.3 (Standard and extended measurement uncertainty) and 3.2.2.5.5 (Determination of outliers). In addition, one new approach was used.

3.2.4.5.1 Estimation of the mean DNA concentration and corresponding MU measured with different dPCR platforms

For both materials tested in inter-laboratory assessment with all five platforms from three laboratories, the mean DNA concentration and corresponding MU ($MU_{interlaboratory}$) were measured based on CCQM guidance note [149]. To select the most appropriate estimator, Grubbs test for outliers (R studio), preliminary graphical inspection to check for over-dispersion of mean values, mutual consistency check (chi-square test in Excel 2007) [149] and

calculation of Birge ratio [149] were performed. With both materials, the estimation of the mean DNA concentration and corresponding measurement uncertainty was performed using the Vangel-Ruhkin estimator in the R studio (package “metrology”), which was characterised as the most appropriate estimation method based on CCQM guidelines [149].

Chapter 4

Results

4.1 Metrological Assessment of Three Methods for Manual Extraction of HCMV DNA from Different Matrices

Three different methods for manual DNA extraction, namely Roche kit, Qiagen kit and Cepheid kit, were metrologically assessed for DNA recovery of four whole-virus materials (Figure 3.1, Chapter 3.2.1). WHO-3K and each INSTAND material were extracted in six (WHO-3K) and four extraction replicates (both INSTAND materials) in a single experiment, whereas WHO-60K material was extracted in three consecutive experiments, with six extraction replicates per experiment. For every extraction kit and material, intra-experiment and inter-experiment variability were also evaluated.

4.1.1 Assessment of DNA recovery

4.1.1.1 Extraction of whole virus materials in one experiment

With all three whole-virus materials that were extracted in a single experiment, pronounced differences between extraction kits in terms of DNA recovery were noted, with Roche kit recovering the highest amount of DNA, followed by Cepheid and Qiagen (Figure 4.1). With Roche kit, mean DNA recovery of 69%-80% was observed regardless of the material used. Lower mean DNA recoveries were observed with Qiagen (31%-53%) and Cepheid DNA extraction kits (49%-70%), which were additionally more influenced by the material used. The highest differences between extraction kits were observed with WHO-3K (3000 IU/mL) and INSTAND material no. 365029 (20,000 cp/mL), with Roche kit recovering up to 0.35 log (2.2-fold) and 0.15 log (1.4-fold) higher DNA amount than Qiagen and Cepheid, respectively. However, with the INSTAND material no. 365032 (910 cp/mL) the least pronounced differences between extraction approaches were observed, although the log difference between Roche and Qiagen kits still reached 0.2 log (1.6-fold).

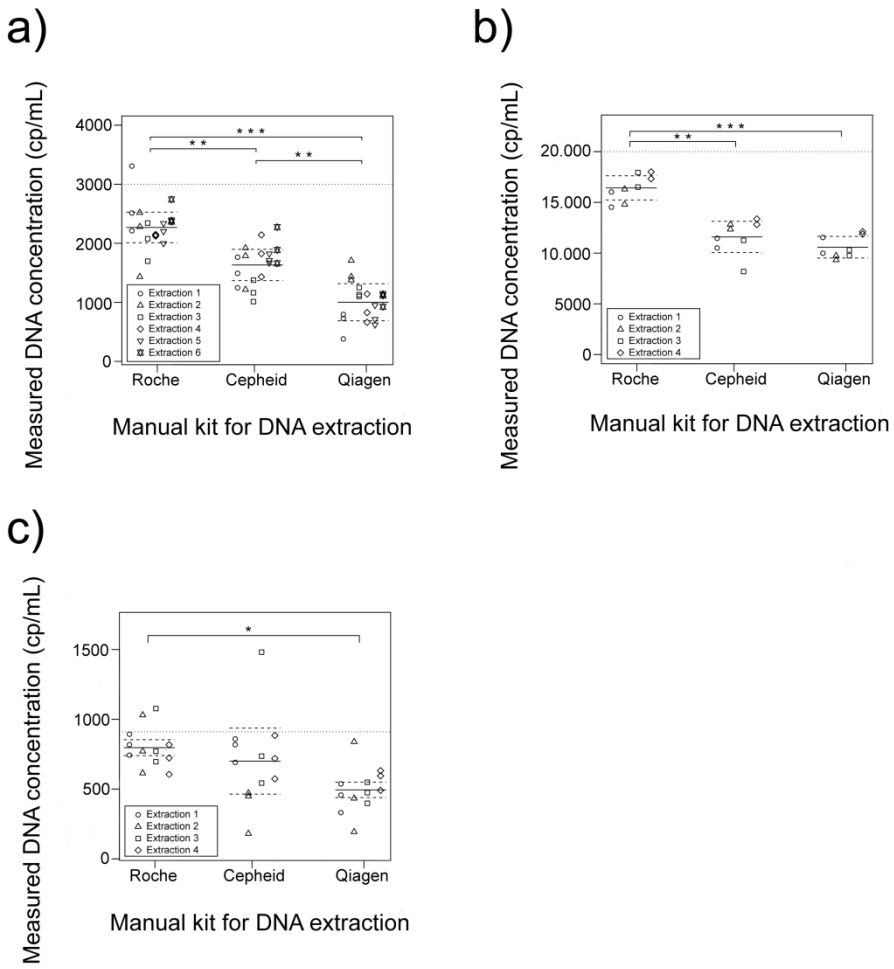


Figure 4.1: DNA recovery of whole-virus materials with different kits for DNA extraction performed in a single experiment. Three materials were used, WHO-3K (a), INSTAND sample no. 365029 (b) and INSTAND sample no. 365032 (c). On each chart, every symbol denotes a single measurement on the QX100 system. The dotted line represents nominal HCMV particle concentration in IU/mL. Full lines denote the mean measured DNA copy-number concentration extracted using a certain extraction approach. Two dashed lines above or below the full line represent one SD from the mean DNA copy-number concentration of every extraction approach, and were calculated considering average values of each extraction replicate. Symbols ***, ** and * above the bars that link two extraction kits denote statistical significance of $p < 0.001$, $p < 0.01$ and $p < 0.05$ (ANOVA, followed by Tukey posthoc test)

4.1.1.2 Extraction of whole-virus material in consecutive experiments

With WHO-60K material, only minor differences in terms of DNA recovery were noted between extraction kits (Figure 4.2). With all three kits, the mean concentration of the recovered DNA ranged from 63% to 73% of the nominal HCMV concentration stated by the manufacturer (expressed in IU/mL). With the QX100 system, a minor but statistically significant difference between Cepheid and Qiagen kit was observed (max logarithmical difference of 0.06 log), however this was not the case when qPCR was used for DNA quantification (max logarithmical difference of 0.05 log). Additionally, with every extraction kit from 11% (0.04 log) to 7% (0.03 log) lower mean DNA concentrations were measured with qPCR than with dPCR.

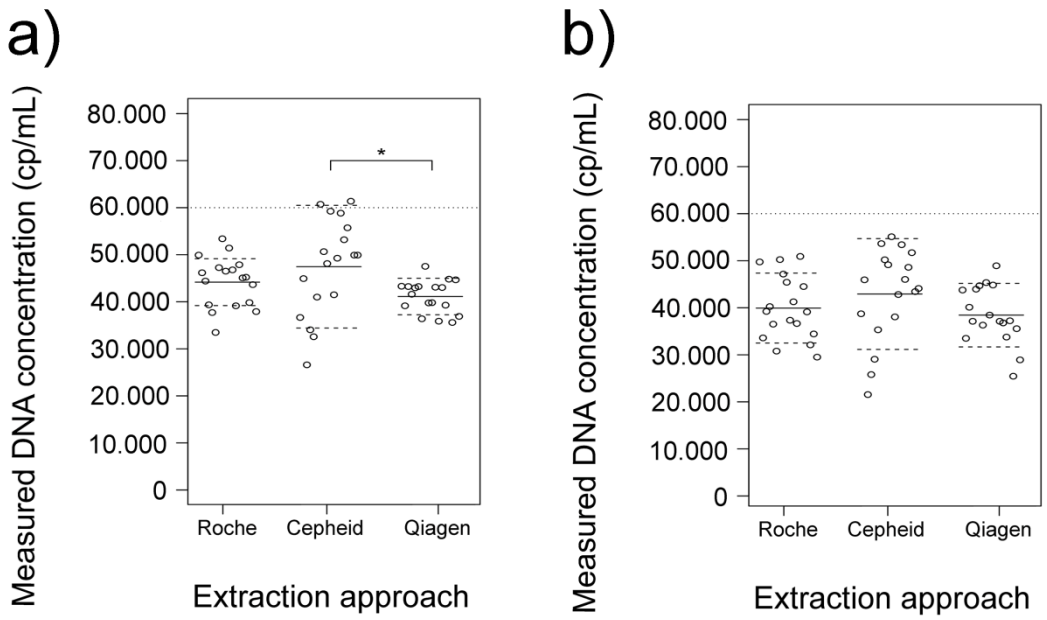


Figure 4.2: DNA recovery of WHO-60K material with different kits for DNA extraction. Extracted DNA concentrations were analysed with the QX100 system (a) and qPCR (b). On each chart, every symbol denotes an average DNA copy-number concentration measured in replicate reactions. Dotted line represents the nominal HCMV particle concentration expressed in IU/mL. Full lines denote the mean measured DNA copy-numbers of a certain extraction kit. Two dashed lines above or below the full line represent the expanded MU, which was calculated considering average values of each extraction replicate. Symbol * above the bar that links two extraction kits denote statistical significance of $p<0.05$ (ANOVA, followed by Tukey posthoc test).

4.1.2 Assessment of variability

With all four materials and for all three DNA extraction kits, intra-experiment variability was assessed by taking into account extraction replicates that were extracted in a single experiment. Additionally, with WHO-60K material, inter-experiment variability was investigated with three consecutive experiments taken into account. With each variability type, two statistical approaches were used. One was focused on differences between extraction replicates (hereinafter denoted as extraction variability), whereas the other one combined the variability caused by DNA extraction and quantification approach (hereinafter mentioned as measurement variability) (Chapter 3.2.1.1).

4.1.2.1 Intra-experiment variability

For each DNA-extraction kit, intra-experiment variability was estimated by considering extraction replicates from a single experiment, which were measured with the QX100 system (Chapter 3.2.1.1).

Roche kit demonstrated very repeatable DNA extraction (Table 4.1, Figure 4.1 and Figure 4.3), with all SD values below 0.06 log. With Qiagen extraction kit, similarly high repeatability was observed as with Roche kit, with exception of WHO-3K material, where the difference between the maximum and minimum DNA concentration was around 0.38 log (2.4 fold) and the SD was 0.14 log. With the Cepheid kit, higher variability was observed than with other two extraction kits, however differences between maximum and minimum extracted DNA concentration never exceeded 0.4 log (2.5-fold) or SD of 0.2 log (1.6-fold).

Intra-experiment measurement variability, which includes four or six extraction replicates per experiment, each analysed in duplicate or triplicate on the QX100 system, was also assessed. With all three extraction kits, variability was inversely correlated with DNA concentration (Table 4.1, Figure 4.1 and Figure 4.3). The smallest differences were once more

noted with the Roche kit, with maximum difference below 0.4 log (2.5 fold) and SD up to 0.08 log. However, with other two extraction kits, maximum differences reached 0.65 log (SD=0.16 log) and 0.91 log (SD=0.22 log), which corresponds to 4-fold and 8-fold difference, respectively.

Table 4.1: Difference between maximum and minimum DNA recovery using same DNA extraction kit in a single experiment. All DNA concentrations were transformed into logarithmic values. The number of extraction replicates is denoted as n. In case of extraction replicates, average DNA concentration of duplicate or triplicate measurements was taken into account. Values in square brackets [] denote SD of logarithmic values of all extractions or measurements.

Material	Nominal DNA concentration [IU/mL] (log value)	Max log difference between extraction replicates within one experiment [SD]				Max log difference between individual dPCR measurements within one experiment [SD]			
		n[a]	Roche	Cepheid	Qiagen	*n*[b]	Roche	Cepheid	Qiagen
WHO-60K	60.000 (4.78)	6	0.17 [0.06]	0.23 [0.08]	0.12 [0.05]	12	0.18 [0.07]	0.25 [0.08]	0.19 [0.06]
INSTAND365029	20.000 (4.30)	4	0.06 [0.03]	0.13 [0.06]	0.10 [0.04]	8	0.09 [0.04]	0.21 [0.07]	0.11 [0.04]
WHO-3K	3000 (3.48)	6	0.12 [0.05]	0.21 [0.08]	0.38 [0.14]	18	0.36 [0.08]	0.35 [0.10]	0.65 [0.16]
INSTAND365032	910 (2.96)	4	0.07 [0.03]	0.40 [0.18]	0.11 [0.05]	12	0.25 [0.08]	0.91 [0.22]	0.64 [0.16]

[a] Number of extraction replicates per experiment

[b] Number of all quantification replicates

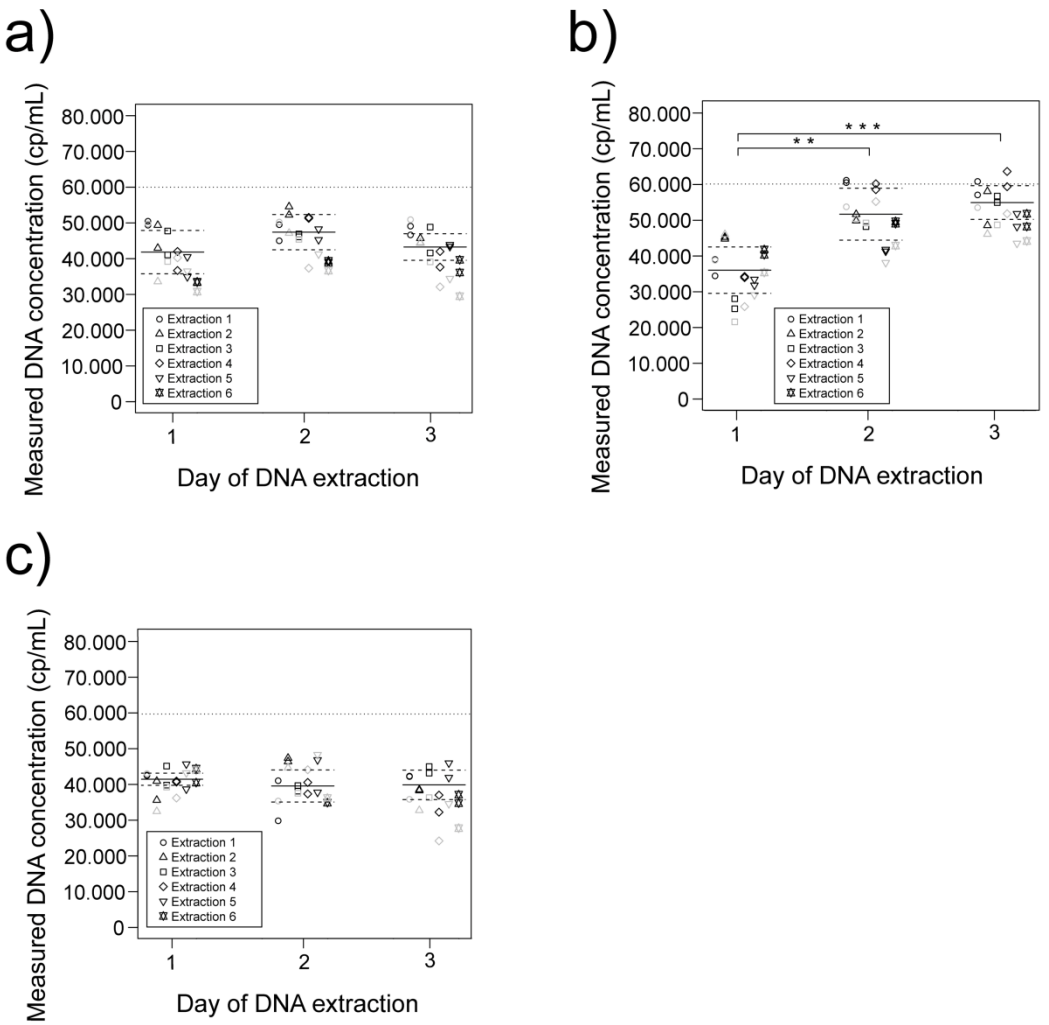


Figure 4.3: DNA recovery, and intra- and inter-experiment variability of three DNA extraction kits. Roche kit (a), Cepheid kit (b) and Qiagen kit (c) are shown. On each chart, every black symbol denotes a single measurement on the QX100 system, whereas every grey symbol denotes the average DNA copy-number concentration of six qPCR measurements. The dotted line represents the nominal HCMV particle concentration expressed in IU/mL. Full lines denote the mean DNA concentration obtained from a single experiment, measured on the QX100 system. Two dashed lines above or below the full line represent one SD from the mean DNA copy-number concentration of each experiment, and were calculated considering the average values of each extraction replicate measured on the QX100 system. Symbols *** and ** above the bars that link two extraction kits denote statistical significance of $p<0.001$ and $p<0.01$, respectively (ANOVA, followed by Tukey posthoc test).

4.1.2.2 Inter-experiment variability

For each DNA extraction kit, inter-experiment variability was estimated using combined data from all three experiments of WHO-60K DNA extractions. With results obtained from the QX100 system, the extraction variability and measurement variability were assessed. In contrast, with qPCR-based results only the extraction variability was assessed.

Cepheid kit was once again found to be the most variable kit for DNA extraction, with maximum differences between recovered DNA concentrations around 0.4 log (2.5-fold) and SD 0.1 log (Table 4.2, Figure 4.3). In contrast, Roche and Qiagen kits demonstrated repeatable

DNA extraction, with maximum differences between extraction replicates or even between single measurements not exceeding 0.3 log (2-fold difference) and SD below 0.07 log. The same pattern was observed with expanded MU, with Qiagen approach demonstrating the smallest one (9 % with QX100 system and 18 % with qPCR), followed by Roche (11 % with QX100 system and 19% with qPCR) and Cepheid (27% with QX100 system and qPCR). Additionally, qPCR was shown to be a more variable quantification method than dPCR on the QX100 system, as it resulted in higher MUs and higher differences between the measured maximum and minimum DNA concentration.

Table 4.2: Difference between the maximum and the minimum DNA recovery obtained from three experiments of DNA extraction. All DNA concentrations were transformed into logarithmic values. In case of extraction replicates, the average DNA concentration of duplicate QX100-measurements was taken into account. Values in brackets denote SD of logarithmic values of all extractions or measurements.

Quantification approach	Max log difference between extraction replicates [SD]			Max log difference between individual measurements [SD]		
	Roche	Cepheid	Qiagen	Roche	Cepheid	Qiagen
QX100 system	0.20 [0.05]	0.36 [0.10]	0.13 [0.04]	0.21 [0.06]	0.40 [0.10]	0.19 [0.04]
qPCR	0.24 [0.07]	0.41 [0.12]	0.28 [0.07]	/	/	/

4.2 Metrological Evaluation of Three Molecular Methods (qPCR, Droplet-based dPCR and Microfluidic-based dPCR) for Quantification of HCMV

Here, two dPCR platforms were assessed for the robustness of both dPCR platforms. Additionally, for qPCR and both dPCR platforms, linearity, LOD, LOQ and precision were evaluated (Figure 3.2).

4.2.1 Robustness of two dPCR platforms

Here, the preliminary selection of the most variable master mixes was carried out on qPCR. Then, the influence of various PCR components (two master mixes that caused the highest variability with qPCR, two various HCMV assays with six different primer and probe concentrations, and two different HCMV DNA materials) was assessed for the Biomark 12.756 array, Biomark 37K array and the QX100 system.

4.2.1.1 Preliminary selection of the most variable master mixes on qPCR

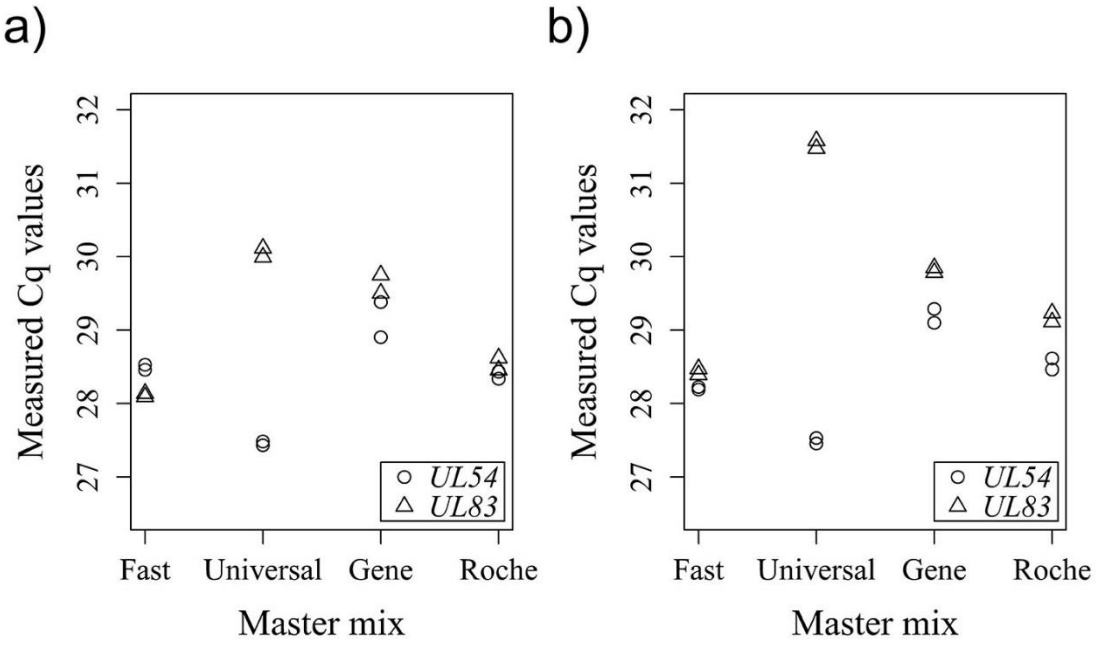
Of four master mixes tested by qPCR, the biggest differences in Cq values between both assays were observed using Universal master mix. With plasmid DNA, *UL54*-6/6/1 assay reached the threshold values 2.5 cycles before *UL83*-6/6/1 assay (Figure 4.4a), while with gDNA1 four Cq values difference between both assays was noted (Figure 4.4b). In contrast, with other three master mixes, differences between both assays ranged from 0.7 to 0.1 Cq values (Figure 4.4).

When *UL83*-6/6/1 assay was used, Fast master mix and Universal master mix generated the largest difference in Cq values between each other (3.1 Cq for gDNA1 and 1.9 Cq for plasmid DNA), whereas with *UL54*-6/6/1 assay the biggest difference was observed between Universal master mix and Gene master mix (1.7 Cq for gDNA1 and 1.7 Cq for plasmid DNA) (Figure 4.4).

Since it is highly likely that differences in Cq values are caused by disparate amplification efficiencies, the Fast master mix and Universal master mix were selected for the

next phase of assessment on the Biomark system, as they generated the biggest differences between each other and/or between both assays.

Figure 4.4: Effects of different HCMV assays and master mixes on the Cq values for qPCR.



Plasmid DNA (a) and gDNA1 (b) were used. In a single run, each DNA-template type was amplified with every combination of HCMV assay and master mix, in duplicates. As the label on y-axis, “Fast” denotes TaqMan Fast Virus 1-Step Master Mix, “Universal” stands for TaqMan Universal PCR Master Mix, “Gene” means TaqMan Gene Expression Master Mix and “Roche” represents FastStart Universal Probe Master Mix (Rox).

4.2.1.2 Influence of different PCR components on DNA quantification by dPCR

On the Biomark system, low susceptibility to the effect of different reaction components was observed (Figure 4.5). On the Biomark 12.765 array, with plasmid DNA the use of Universal master mix resulted in 12% higher estimations of DNA concentration compared to Fast master mix, regardless of the assay or PPP concentration ($p<0.01$) (Figure 4.5a). On the same array, differences were also observed with gDNA1, as *UL54* assay gave 15% higher estimations than *UL83* assay, disregarding the master mix or the concentration of oligonucleotide primers and probes ($p<0.001$) (Figure 4.5b). On the Biomark 37K array with gDNA1 tested in combination with Fast master mix, a significant 37% higher copy number estimations were observed with *UL54*-3/3/1 and *UL83*-3/3/1 when compared with those obtained with *UL54*-6/6/2 and *UL83*-6/6/2 ($p<0.001$) (Figure 4.5d). On each Biomark array, all other combinations of templates, master mixes, assays and concentrations of oligonucleotide primers and probes did not show any effect on the final estimation of DNA concentration ($p>0.05$), despite twofold differences in the fluorescence intensities between different concentrations of oligonucleotide primers and probes, and up to 2.5 Cq differences between various combinations of PCR assays and master mixes (Figure 4.6 and Figure 4.7),

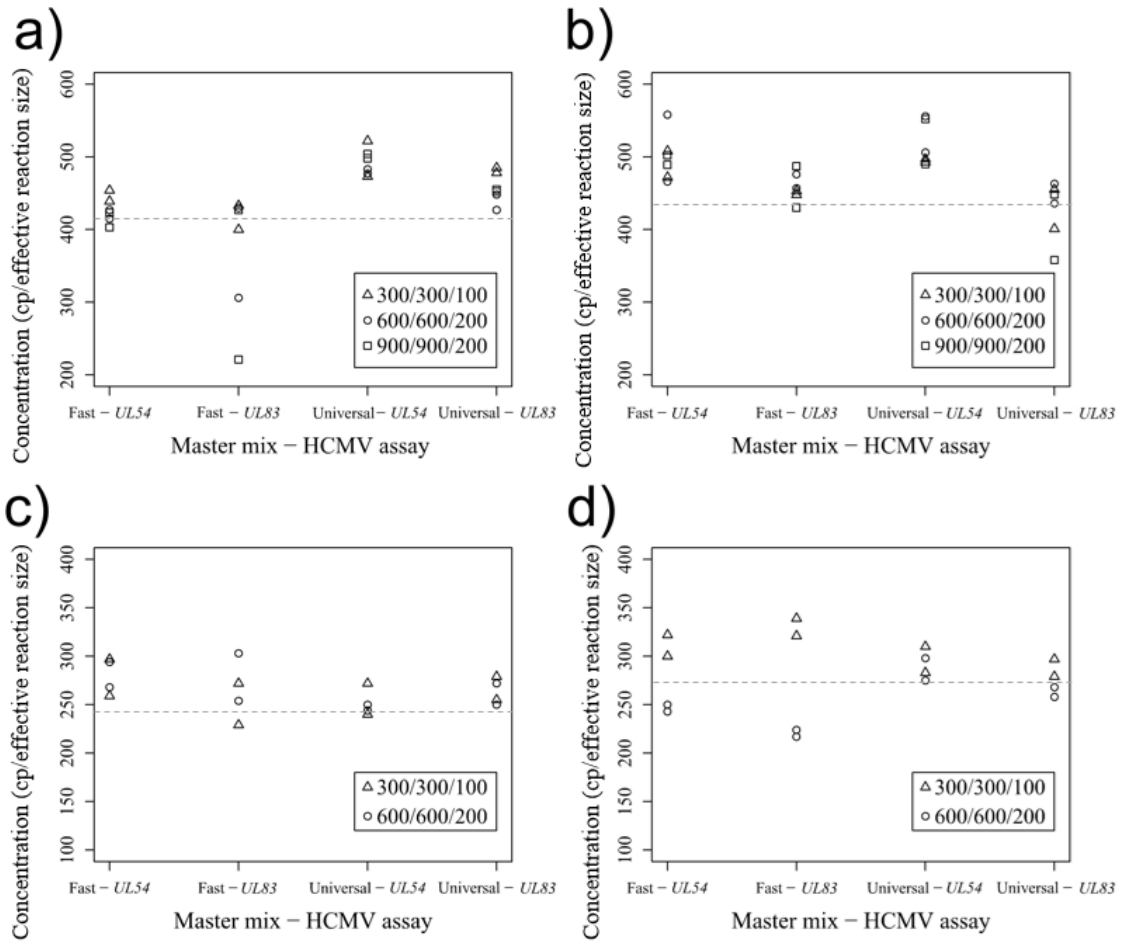


Figure 4.5: Influence of the different PCR components on the estimated DNA concentrations measured on the Biomark system. The Biomark 12.765 array (a, b) and the Biomark 37K array (c, d) were assessed using plasmid DNA (a, c) and gDNA1 (b, d). For each combination of HCMV assays and a master mix, three (a, b) and two (c, d) different concentrations of oligonucleotide primers and probes were used (e.g. PPP concentration of assay *UL54*-3/3/1 is in the figure denoted as 300/300/100). Grey dotted line denotes nominal DNA concentration based on preliminary measurements on the QX100 system using *UL54* assay.

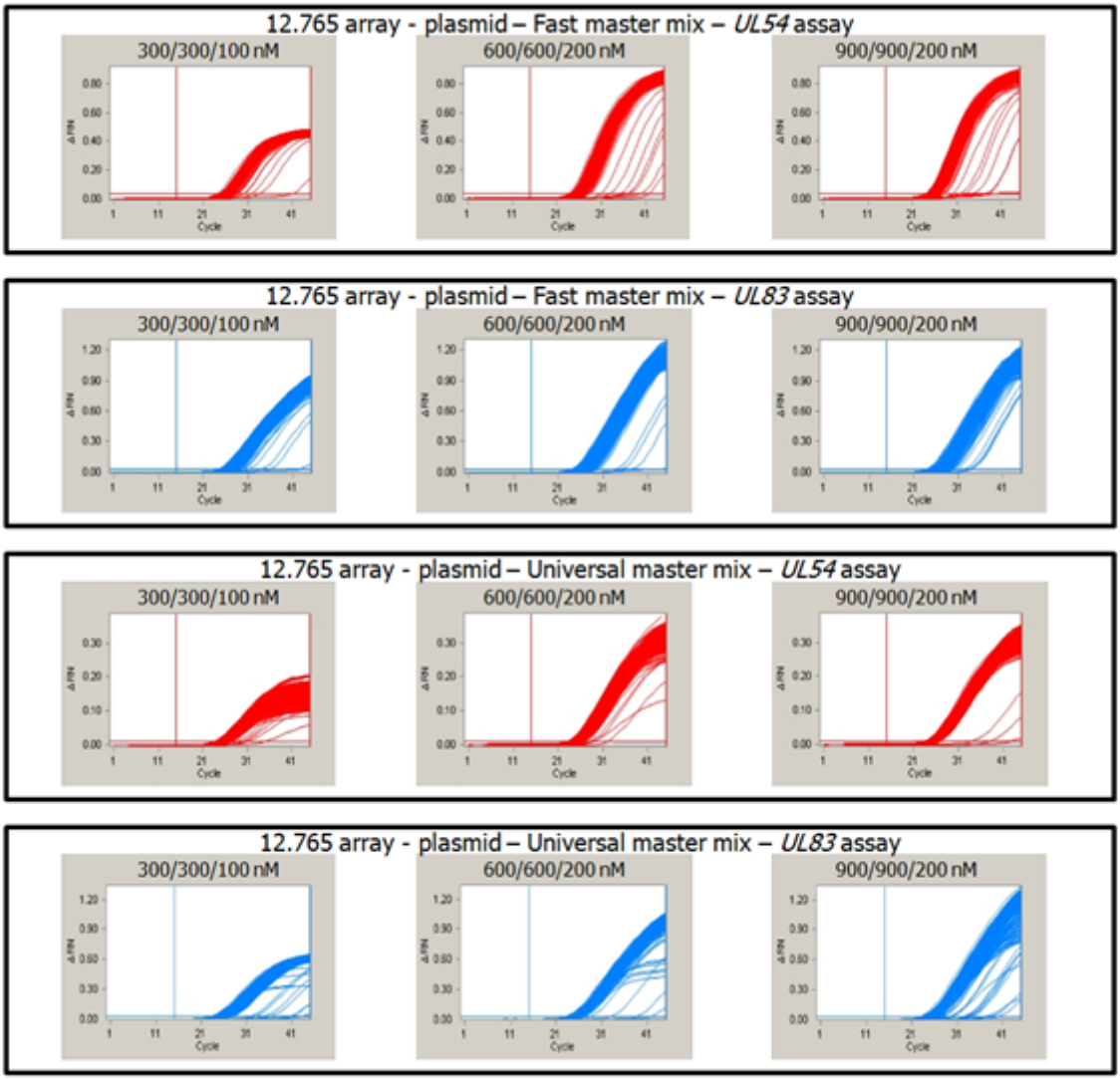


Figure 4.6: Detailed dPCR data from the assessment of influence of different PCR components on quantification of plasmid DNA using the Biomark 12.765 array. qPCR charts obtained on the Biomark 12.765 array using undiluted plasmid DNA are shown. Above each group of three qPCR charts, the array type, the template type, the master mix type and the HCMV assay type are depicted. Numbers above each chart depict different nM concentrations of forward primer, reverse primer and probe. The fluorescence threshold was determined manually for each combination of reaction components, while the quality threshold (0.2) and the accepted Cq range (15-45 Cq) were equal for all samples.

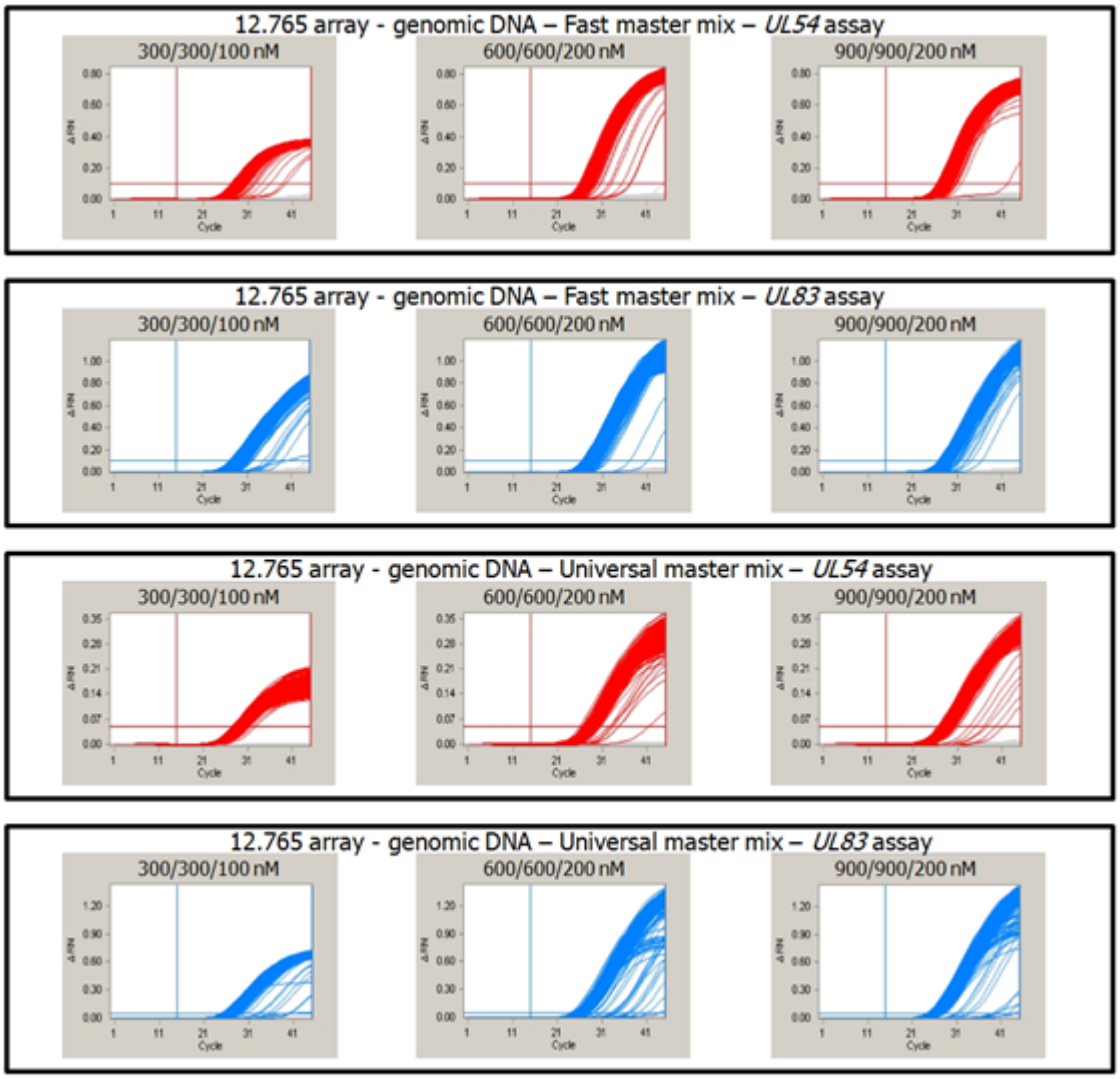


Figure 4.7: Detailed dPCR data from the assessment of influence of different PCR components on quantification of gDNA1 using the Biomark 12.765 array. qPCR charts obtained on the Biomark 12.765 array using twofold diluted gDNA1 are shown. Above each group of three qPCR charts, the array type, the template type, the master mix type and the HCMV assay type are depicted. Numbers above each chart depict different nM concentrations of forward primer, reverse primer and probe. The fluorescence threshold was determined manually for each combination of reaction components, while the quality threshold (0.2) and the accepted Cq range (15-45 Cq) were equal for all samples.

On the QX100 system, with plasmid DNA 5% higher estimations of DNA concentration were observed with *UL54* assay in comparison to *UL83* assay, regardless of the concentration of oligonucleotide primers and probes ($p<0.01$) (Figure 4.8a), whereas with gDNA1 these differences were increased to 20% ($p<0.001$) (Figure 4.8b). With both materials, higher fluorescence intensity was observed with *UL54* assay compared to that of *UL83* assay, however both assays allowed simple manual threshold settings. With different concentrations of oligonucleotide primers and probes, almost twofold differences in fluorescence intensity of droplets were observed (Figure 4.9 and Figure 4.10), with no statistically significant influence on the final estimation of DNA concentration ($p>0.05$).

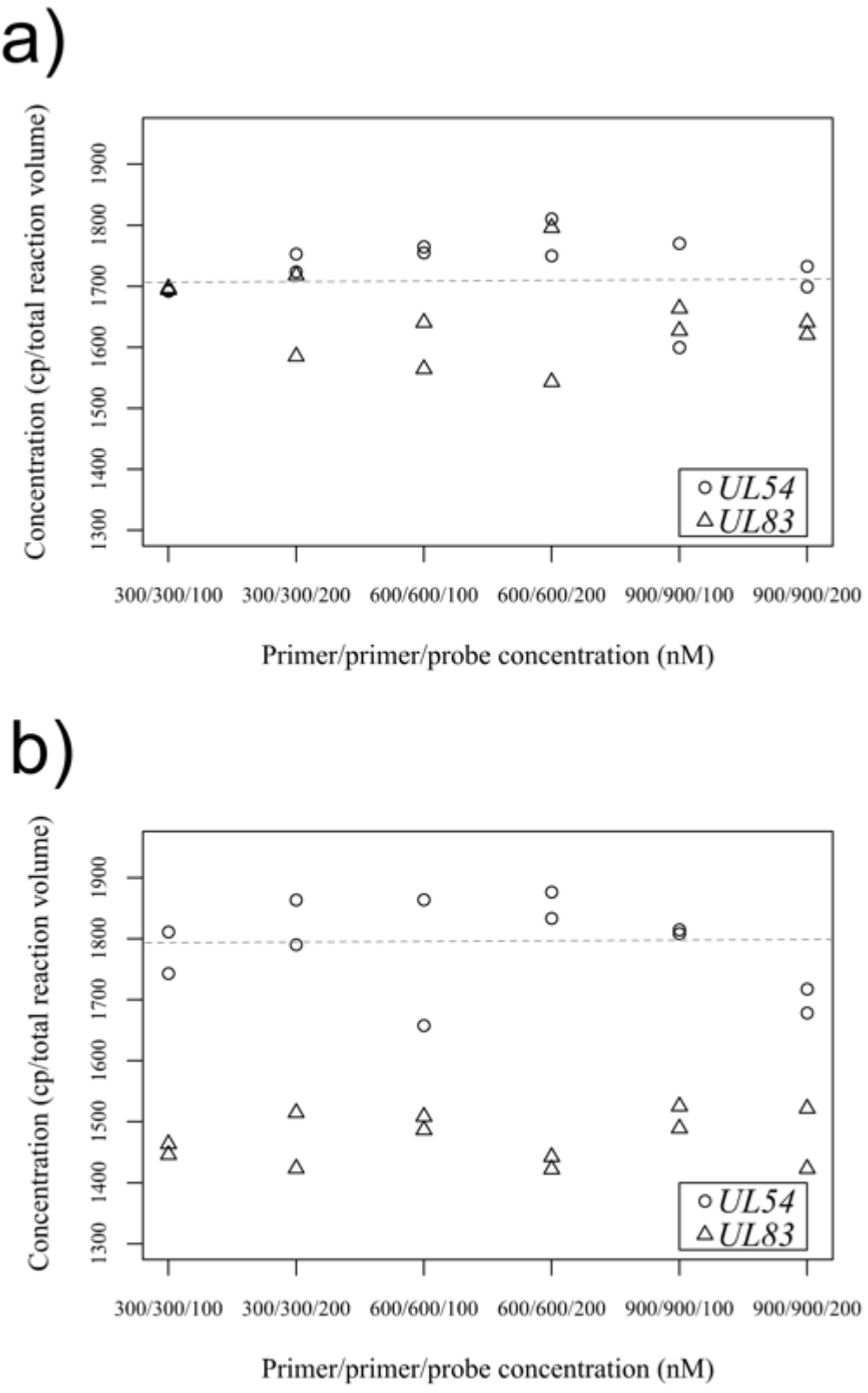


Figure 4.8: Influence of the different PCR components on the estimation of DNA concentration measured on the QX100 system. Plasmid DNA (a) and gDNA1 (b) were used. Grey dotted line denots nominal DNA concentration based on preliminary measurements on the QX100 system using *UL54* assay.

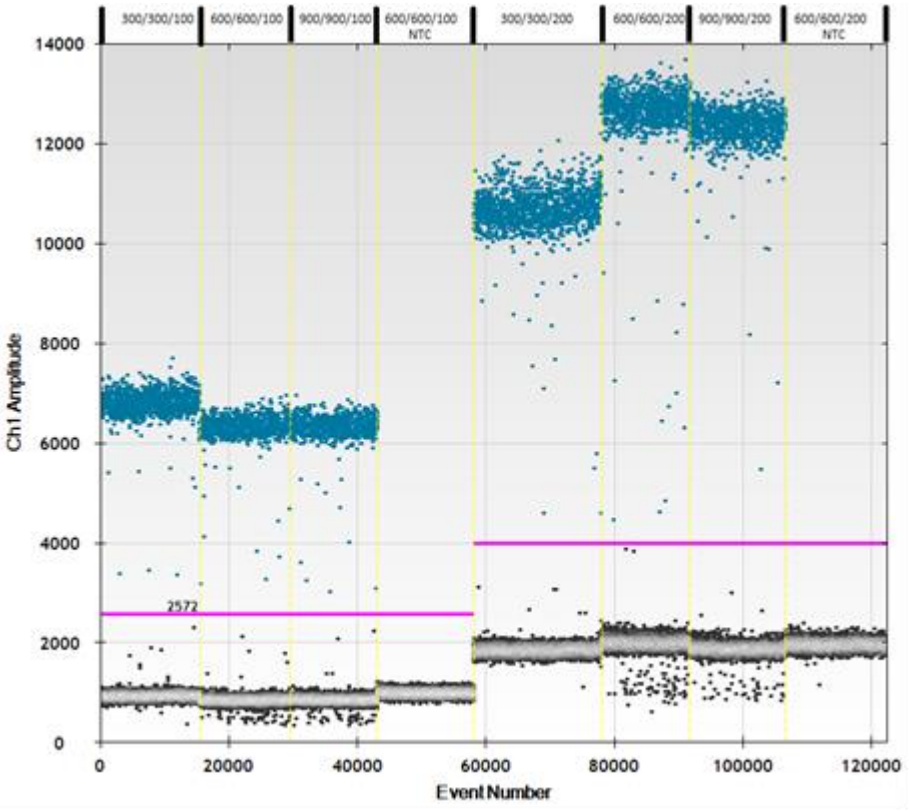


Figure 4.9: Detailed data from the assessment of influence of different PCR components on DNA quantification using the UL54 assay on the QX100 system. Each dot represents the fluorescence intensity for a particular droplet. Numbers above each column depict different nM concentrations of a forward oligonucleotide primer, reverse oligonucleotide primer and oligonucleotide probe. A two-fold diluted gDNA1 was used. The threshold was set manually.

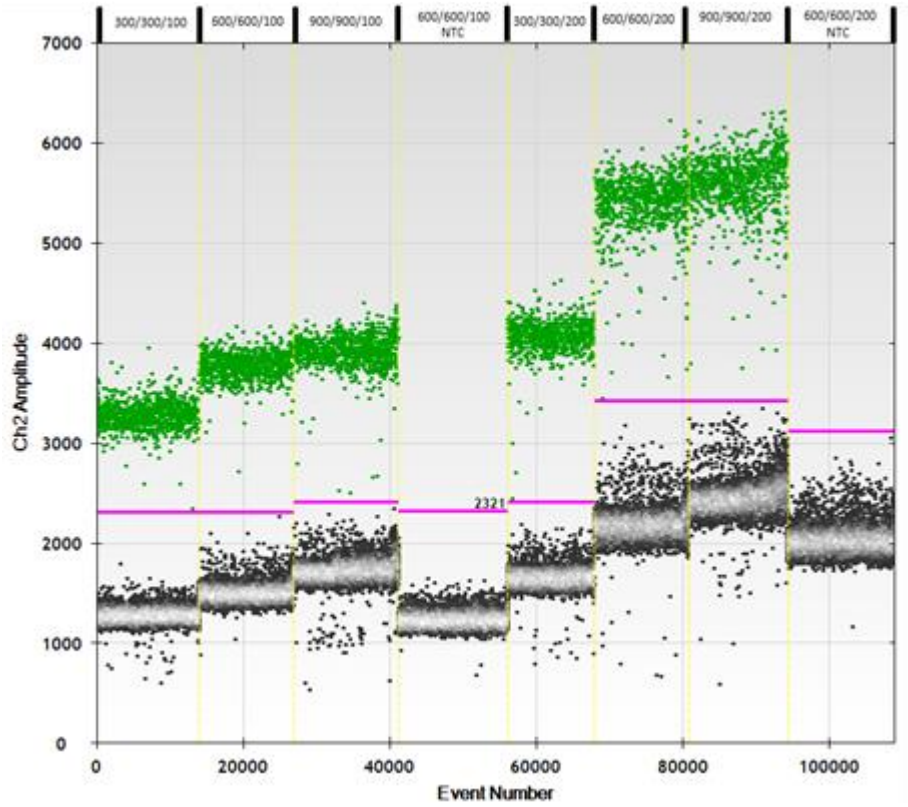


Figure 4.10: Detailed data from the assessment of influence of different PCR components on DNA quantification using the UL83 assay on the QX100 system. Each dot represents the fluorescence intensity for a particular droplet. Numbers above each column depict different nM concentrations of a forward oligonucleotide primer, reverse oligonucleotide primer and oligonucleotide probe. A two-fold diluted gDNA1 was used. The threshold was set manually.

4.2.2 Assessment of linearity, LOD, LOQ and precision for three quantification methods

Here, with each of three quantification methods (qPCR on Viia 7, the QX100 system and the Biomark 37K array), a dilution series of gDNA1 material was tested in two consecutive experiments, with five replicates per experiment. For each method, *UL54*-6/6/2 assay and one master mix were used. For every dilution tested on each quantification method, nominal and measured average M, N and λ were calculated (Table 4.3, Table 4.4, Table 4.5 and Table S1, Table S2, Table S3 from supplementary material). With qPCR and the QX100, higher concordance with nominal values was observed in comparison to the Biomark 37K array (Table 4.3, Table 4.4, Table 4.5).

Table 4.3: List of nominal and measured DNA concentrations of gDNA1 obtained with qPCR.

Nominal DNA copy numbers [a]	Measured DNA copy numbers [b]	
M ±SE [c]	M ± SE [c]	Bias (%) [d]
5,490 ±125	4,730 ±210	-14 (p <0.01)
2,740 ±60	2,740 ±55	0
1,095 ±25	1,115 ±15	2
275 ±6	305 ±7	11 (p <0.01)
110 ±2.5	110 ±7	3
55 ±1	65 ±4	19 (p <0.05)
22 ±0.5	22 ±1.5	0
11 ±0.25	10 ±1	-6
5 ±0.1	2.3 ±0.7	-55 (p <0.001)
2 ±0.05	ndf[e]	ndf[e]
1 ±0.02	ndf[e]	ndf[e]
0.1 ±0.002	ndf[e]	ndf[e]

[a] Based on initial estimations of gDNA1 on the QX100 system.
[b] Based on 10 replicates measured in two consecutive experiments.
[c] Number of DNA copies per total-reaction volume with standard error (SE). This was equal to the number of DNA copies per effective reaction size.
[d] The concordance between measured and nominal DNA concentration. Statistically insignificant bias (p > 0.05) are not given in the table.
[e] Not defined due to concentration below LOD

Table 4.4: List of nominal and measured DNA concentrations of gDNA1 obtained on the Biomark 37K array.

Nominal DNA copy numbers [a]		Measured DNA copy numbers [b]			
M ±SE [c]	N ±SE [d]	M ±SE [c]	N ±SE [d]	λ [e]	Bias (%) [f]
6,190 ±140	1,001 ±23	7,530 ±90	1,215 ±15	1.6	22 (p <0.001)
3,300 ±75	535 ±12	3,935 ±50	635 ±8.5	0.8	19 (p <0.001)
1,655 ±40	267 ±7	2049 ±45	330 ±7.5	0.4	24 (p <0.001)
660 ±15	107 ±2.5	790 ±30	130 ±5	0.2	19 (p <0.001)
330 ±8	54 ±1.2	418 ±17	68 ±3	0.08	26 (p <0.001)
132 ±3	21 ±0.5	165 ±13	26 ±2	0.03	24 (p <0.05)
66 ±1.5	11±0.2	85 ±8	13 ±1	0.02	26
33 ±0.8	5 ±0.1	50 ±5	8 ±0.7	0.01	45 (p <0.05)
6.6 ±0.1	1 ±0.02	ndf[g]	ndf[g]	ndf[g]	ndf[g]

[a] Based on initial estimations of gDNA1 on the QX100 system.
[b] Based on 10 replicates measured on two consecutive runs.
[c] Number of DNA copies per total reaction volume with standard error (SE).
[d] Number of DNA copies per effective reaction size with standard error (SE).
[e] Mean DNA copy number per partition.
[f] The concordance between measured and nominal DNA concentration. Statistically insignificant bias (p >0.05) are not given in the table.
[g] Not defined due to concentration below LOD

Table 4.5: List of nominal and measured DNA copy-number concentrations of gDNA1 obtained on the QX100 system.

Nominal DNA copy numbers [a]		Measured DNA copy numbers [b]			
M ±SE [c]	N ±SE [d]	M ±SE [c]	N ±SE [d]	λ [e]	Bias (%) [f]
5,490 ±125	3,110 ±170	5,800 ±90	3,285 ±55	0.24	6 ($p < 0.05$)
2,740 ±60	1,555 ±85	3,010 ±45	1,705 ±25	0.12	10 ($p < 0.001$)
1,095 ±25	620 ±35	1,185 ±22	700 ±13	0.05	13 ($p < 0.05$)
275 ±6	155 ±8	275 ±7	155 ±5	0.01	0
110 ±2.5	62 ±3.5	120 ±6	70 ±3	0.005	9
55 ±1	31 ±1.7	48 ±4	27 ±2	0.002	-12
22 ±0.5	12 ±0.7	19 ±2	11 ±1.2	0.001	-12
11 ±0.25	6 ±0.4	10 ±1.5	6 ±0.8	0.0004	-11
5 ±0.1	3 ±0.2	6 ±1	3 ±0.5	0.0002	8
2 ±0.05	1 ±0.06	ndf[g]	ndf[g]	0.00010	ndf[g]
1 ±0.02	0.6 ±0.03	ndf[g]	ndf[g]	0.00005	ndf[g]
0.1 ±0.002	0.06 ±0.03	ndf[g]	ndf[g]	0.000005	ndf[g]

[a] Based on initial estimations of gDNA1 on the QX100 system.
[b] Based on 10 replicates measured in two consecutive runs.
[c] Number of DNA copies per total reaction volume with standard error (SE).
[d] Number of DNA copies per effective reaction size with standard error (SE).
[e] Mean DNA copy number per partition.
[f] The concordance between measured and nominal DNA concentration. Statistically insignificant bias ($p > 0.05$) are not given in the table.
[g] Not defined due to concentration below LOD

4.2.2.1 LOD, LOQ and precision (intra-experiment variability and intermediate precision)

With every platform, the results were evaluated using two approaches, one considering the total-reaction volume and another with effective reaction size taken into account. When the total-reaction volume was considered, the QX100 system and qPCR showed lower intra-experiment variability and higher intermediate precision compared to the Biomark 37K array (Figure 4.11). Furthermore, on qPCR for DNA concentrations below 55 copies, less variable results were observed than on the QX100 system. Consequently, on qPCR the lowest LOD and the lowest LOQ were obtained, as analysis indicated reliable detection and quantification of three and 11 - 22 copies, respectively (Table 4.6). On the QX100 system, the LOD was approximately six and LOQ approximately 55 copies, whereas on the Biomark 37K array the highest LOD (14 copies) and LOQ (140 - 210 copies) were observed (Table 4.6). In contrast, when considering the effective reaction size, for the concentrations above 55 copies both dPCR platforms performed with equal or with even better intermediate precision than qPCR. On all three platforms a similar LOD was found (around three copies).

On both dPCR platforms, CV values and LOQ for either intra-experiment measurements or inter-experiment measurements were higher than the theoretical ones based on Poisson error (Figure 4.11, Equation 3-8). In contrast, all three platforms had the LOD equal or lower than the theoretical one (Table 4.6).

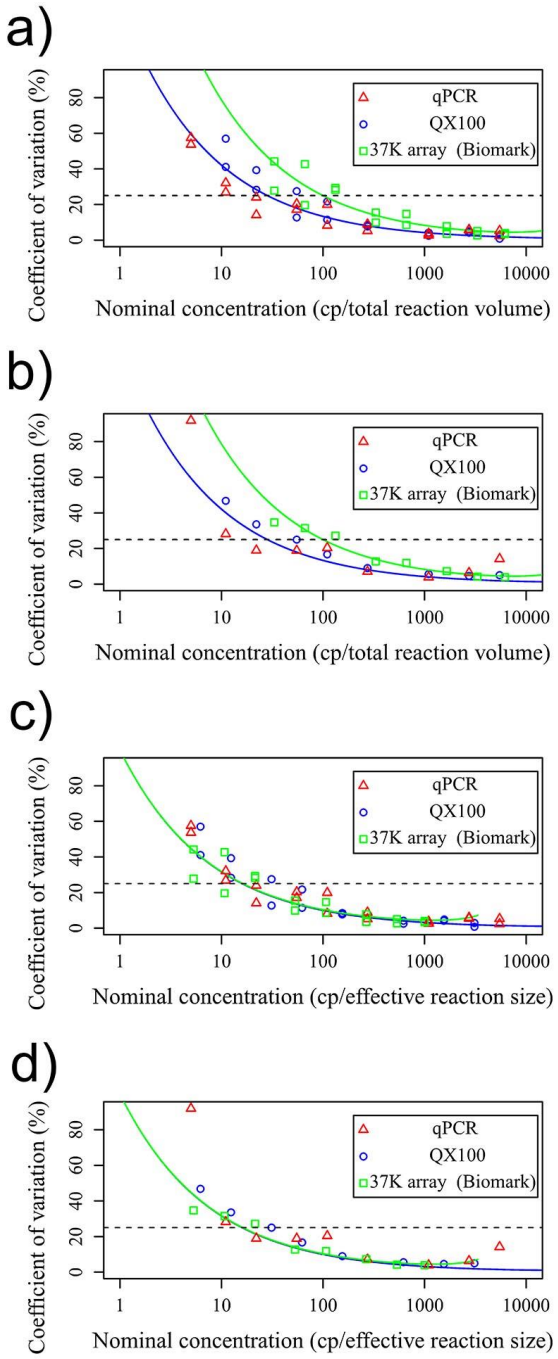


Figure 4.11: Intra-experiment variability (a, c) and intermediate precision (b, d) for each of three platforms according to the total-reaction volume (a, b) and the effective reaction size (c, d). Data are CVs for five measurements in each experiment (a, c), or for 10 combined measurements from two experiments (b, d). For each DNA concentration of gDNA1, five replicates were measured in two consecutive experiments. Blue line, theoretical CV (Poisson error) for the QX100 system (using 14,000 accepted droplets); green line, theoretical CV for the Biomark 37K array (using 770 chambers); dashed line, criterion for the limit of quantification, set at CV of 25%.

Table 4.6: Limit of detection (LOD) and limit of quantification (LOQ) for qPCR, the QX100 system and the Biomark 37K array according to two different approaches for the definition of a reaction volume. Only the intermediate precision was taken into account. LOD was calculated using the Probit software. LOQ was determined as the lowest DNA copy-number concentration for which the CV was below 25%. The expected LODs and LOQs are in the brackets

Analysis approach	DNA copy-number concentration according to analysis platform					
	qPCR		QX100		Biomark	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
Total-reaction volume	2.8 (3)	11-22	5.7 (5.3)	55 (28)	14 (18)	140-210 (100)
Effective reaction size	2.8 (3)	11-22	3.2 (3)	30 (16)	2.4 (3)	14 (18)

4.2.2.2 Linearity

All three quantification platforms showed good linearity above the LOQ, while the upper dynamic range was not determined due to clinical insignificance of high viral loads. However, on each dPCR platform slightly better linearity was observed than with qPCR, as they showed better correlation with nominal concentration (R2>0.998) than qPCR (R2>0.990) (Figure 4.12).

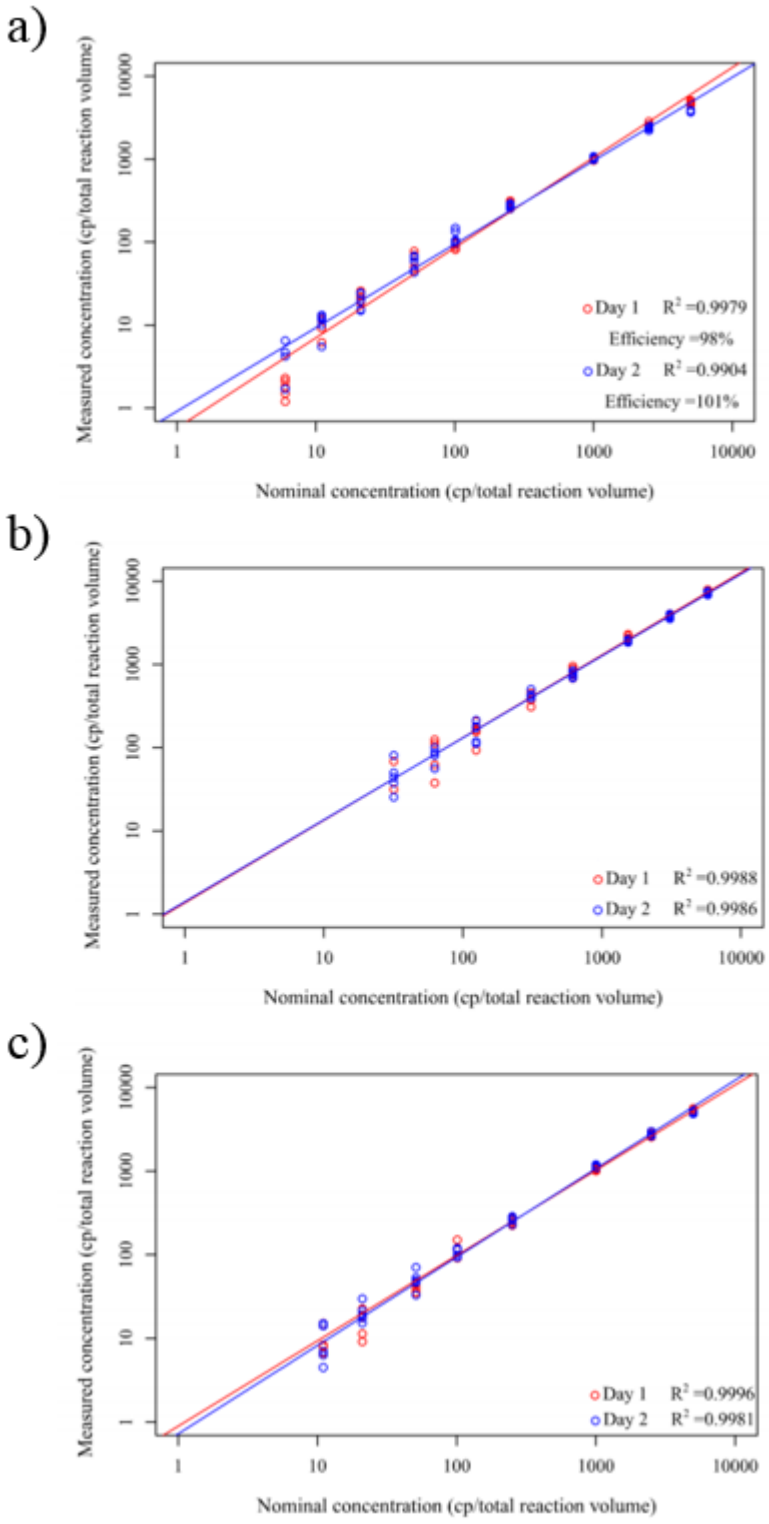


Figure 4.12: Linearity for qPCR (a), the QX100 system (b) and the Biomark 37K array (c) using data from each experiments. DNA concentrations below the LODs were omitted from the plot. In every experiment, each DNA concentration of gDNA1 was measured in five replicates.

4.2.2.3 Influence of variability on clinical applicability of the method

All measured values (cp/total reaction value) were recalculated to the number of viral DNA copies per mL of human plasma, and additionally transformed into logarithmic values. Then, SD and the difference between maximum and minimum value were assessed to facilitate the comparison between variability caused by extraction methods (4.1.2) and quantification methods.

With qPCR, the smallest SD values and the smallest differences between maximum and minimum measurement were observed with DNA concentrations below 1700 cp/mL of human plasma, as maximum differences never exceeded 0.4 log (2.5-fold) (Table 4.7). With both, qPCR and the QX100 system, maximum differences were below 0.15 log with DNA concentrations equal or above 8500 cp/mL. In contrast, with Biomark 37K array maximum differences below 0.5 log (3-fold) were noted only with DNA copy-number concentrations equal or above 25,000 cp/mL, with maximum differences below 0.15 log only for DNA concentrations above 120,000 cp/mL (Table 4.8).

Table 4.7: Difference between maximum and minimum estimated DNA concentration within and between experiments conducted with qPCR or the QX100 system. All measured DNA concentrations were first recalculated to the nominal HCMV concentration per mL of human plasma, by taking into account 100% DNA recovery of 200-µL sample extracted into 50-µL eluted sample, followed by quantification using 8 µL of eluted sample. Then, all the values were transformed into logarithmic values. The number of measurement replicates was five with intra-experiment variability and 10 with inter-experiment variability. Values in brackets denote SD of logarithmic values of five or 10 measurements.

Nominal value [cp/mL of human plasma]	Logarithmic value of nominal value	Difference between max and min logarithmic value within experiment (SD)		Difference between max and min logarithmic value between experiments (SD)	
		qPCR	QX100	qPCR	QX100
170,000	5.23	0.05 (0.02)	0.03 (0.01)	0.14 (0.06)	0.06 (0.02)
85,000	4.93	0.06 (0.02)	0.06 (0.02)	0.11 (0.03)	0.07 (0.02)
35,000	4.53	0.04 (0.02)	0.04 (0.02)	0.05 (0.01)	0.07 (0.02)
8500	3.93	0.10 (0.04)	0.10 (0.04)	0.10 (0.03)	0.11 (0.04)
3400	3.53	0.19 (0.08)	0.21 (0.09)	0.26 (0.08)	0.21 (0.07)
1700	3.23	0.20 (0.09)	0.34 (0.11)	0.26 (0.09)	0.34 (0.10)
690	2.83	0.23 (0.11)	0.42 (0.19)	0.25 (0.09)	0.54 (0.16)
350	2.53	0.38 (0.16)	0.60 (0.26)	0.38 (0.14)	0.60 (0.21)

Table 4.8: Difference between maximum and minimum estimated DNA concentration within and between experiments performed on the Biomark 37K array. All measured DNA concentrations were first recalculated to the nominal HCMV concentration per mL of human plasma, by taking into account 100% DNA recovery of 200-µL sample extracted into 50-µL eluted sample, followed by quantification using 1.4 µL of eluted sample. Then, all the values were transformed into logarithmic values. The number of measurement replicates was five with intra-experiment variability and 10 with inter-experiment variability. Values in brackets denote SD of logarithmic values of five or 10 measurements.

Nominal value	Logarithmic	Difference between max	Difference between max

[cp/mL of human plasma]	value of nominal value	and min logarithmic value within experiment (SD)	and min logarithmic value between experiments (SD)
1,100,000	6.04	0.04 (0.02)	0.06 (0.02)
590,000	5.77	0.06 (0.02)	0.06 (0.02)
300,000	5.47	0.08 (0.04)	0.10 (0.03)
120,000	5.07	0.15 (0.07)	0.15 (0.05)
60,000	4.77	0.17 (0.07)	0.20 (0.06)
25,000	4.37	0.36 (0.15)	0.37 (0.13)
12,000	4.07	0.53 (0.21)	0.53 (0.16)
6000	3.77	0.52 (0.15)	0.52 (0.19)

4.3 Evaluation of Two Molecular Methods (Droplet-based dPCR and Microfluidic-based dPCR) for Direct Quantification of HCMV Without DNA Extraction

Here, two dPCR methods (the QX100 system and the Biomark system) were assessed for direct quantification of two whole-virus materials (WHO material and INSTAND materials) (Figure 3.3). Direct quantification was also compared to quantification performed on extracted DNA. In addition, WHO material diluted in human plasma was quantified with direct quantification on the QX100 system to investigate its suitability for clinical samples.

4.3.1 Direct quantification of whole-virus samples and quantification of extracted HCMV DNA on each dPCR platform

4.3.1.1 Comparison of both quantification approaches on two dPCR platforms

Direct quantification and quantification of extracted DNA were each used for enumeration of WHO- and INSTAND whole-virus materials on the QX100 system and Biomark arrays. On both platforms with WHO material, the use of direct quantification resulted in a statistically significant increase in measured DNA concentration, compared to quantification of extracted DNA from human plasma or PBS buffer. The largest differences were noted with the Biomark system, with a 26% increase compared to DNA extractions from human plasma and 53% increase compared to DNA extractions from PBS. Moreover, on the QX100 system 18% (compared to DNA extractions from human plasma) and 35% increase (compared to DNA extractions from PBS buffer) in DNA concentration was observed (Figure 4.13). The same pattern was observed with INSTAND sample N° 365029 analysed using the Biomark system and INSTAND sample N° 365032 analysed using the QX100 system, where direct quantification resulted in 35% and 39% increase in DNA concentration, respectively, when compared to DNA extraction (Figure 4.14). However, with INSTAND sample N° 365029 analysed on the QX100 system, no statistically significant differences were noted between either of the quantification approaches (Figure 4.14).

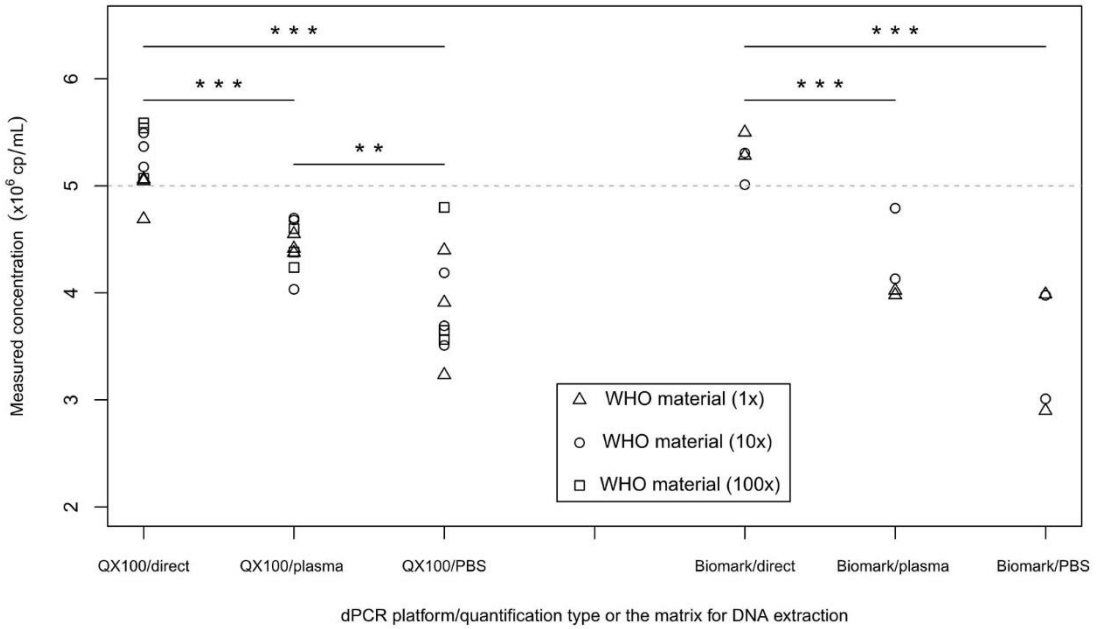


Figure 4.13: Comparison of direct quantification of DNA from whole-virus materials and quantification of extracted viral DNA using the WHO material on each of the dPCR platforms. The nominal predicted concentration is shown as a grey dotted line. Each data point represents a single dPCR measurement. All measured DNA concentrations were then multiplied by the dilution factors to reach the initial DNA concentration per mL of reference material. In the legend, the initial concentration of the WHO material is related to triangles, circles or squares which denote WHO-5M, WHO-500K or WHO-50K material. However, for the DNA extraction, an additional 5× dilution in human plasma or PBS buffer was performed for each dilution of the WHO material, therefore triangles represent WHO-1M, circles WHO-1000K and squares WHO-10K. ***, **, p <0.001 and p <0.01, respectively, between the two groups for DNA copy numbers (Student's t-tests).

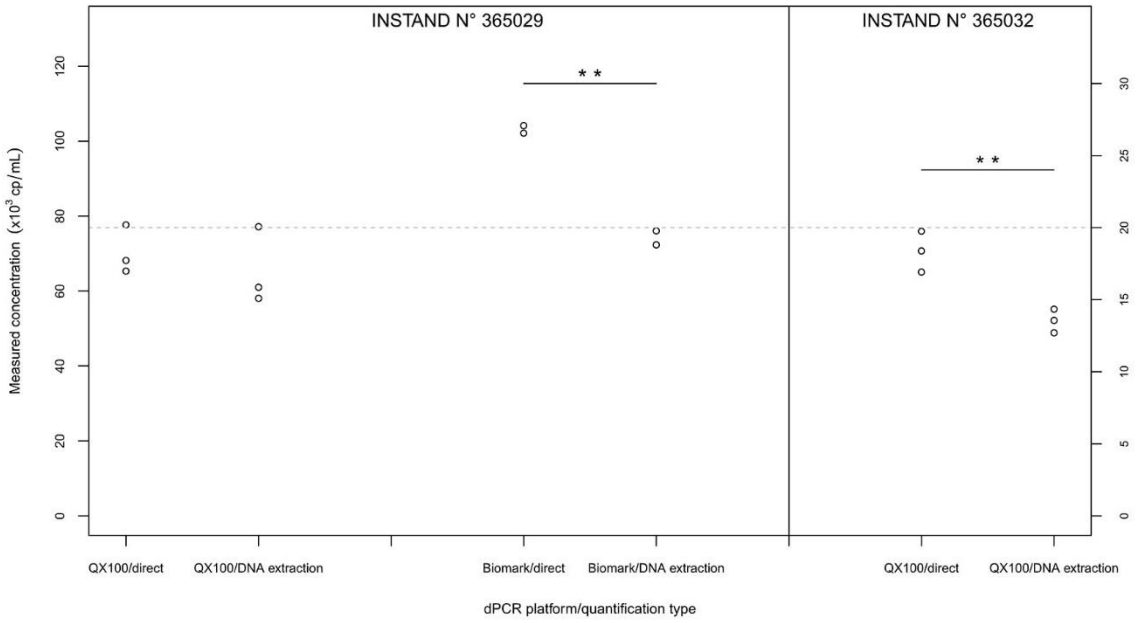


Figure 4.14: Comparison of DNA-concentration estimations between the direct quantification of DNA from whole viruses and the quantification of extracted viral DNA using the INSTAND material on each of the dPCR platforms. The nominal predicted concentration is shown as a grey dotted line. Each data point represents a single dPCR measurement. **, p <0.01, between the two groups for DNA copy numbers (Student's t-tests).

4.3.1.2 Comparison of DNA extractions from human plasma and PBS

All three concentrations of the WHO material were diluted either in human plasma or PBS buffer and subsequently extracted in triplicate. On the QX100 system, statistically significant difference was noted between both dilution matrices, with human plasma resulting in 15% higher DNA recovery than PBS ($p<0.01$). In contrast, on the Biomark system no statistically significant difference was observed between the two matrices, although 22% higher DNA recovery was noted from human plasma compared to that of PBS (Figure 4.13).

4.3.1.3 Assessment of variability of both approaches

The variability of direct quantification was found smaller or equal to that obtained by quantification of extracted DNA (Table 4.9, Table 4.10). This pattern was noted on each dPCR platform and with both whole-virus materials. In general, on the Biomark system higher variability was observed than with the QX100 system.

Table 4.9: Variability of the direct quantification of DNA from whole viruses and quantification of extracted viral DNA using different materials on the QX100 Droplet Digital PCR system. DNA extractions were performed using either WHO material diluted in human plasma and PBS, or INSTAND samples with viruses in cell lysates.

Material	Direct quantification			Quantification of extracted DNA[a]							
	Nominal viral Load[a] (IU/mL or cp/mL)	λ[b]	CV[c] (%)	Nominal viral load[a] (IU/mL or cp/mL)	Human plasma[d]		PBS[d]		Cell lysate	
					λ[b]	CV[c] (%)	λ[b]	CV[c] (%)	λ[b]	CV[c] (%)
WHO-5M	5×10^6	1.6	4	1×10^6	1.18	2	1	15	/	/
WHO-500K	5×10^5	0.17	3	1×10^5	0.12	8	0.1	9	/	/
WHO-50K	5×10^4	0.017	5	1×10^4	0.01	8	0.01	17	/	/
N° 365029[e]	7.7×10^4	0.022	9	7.7×10^4	/	/	/	/	0.08	20
N° 365032[e]	2×10^4	0.006	8	2×10^4	/	/	/	/	0.17	10

[a] Nominal viral concentration of the sample before quantification, based on the nominal concentration of each of the whole virus materials and their volumetric dilutions in double-distilled water, human plasma or PBS

[b] Mean DNA copy number per partition

[c] Coefficient of variability that was calculated on the basis of three measurements (n =3) on the QX100 system

[d] Prior to DNA extraction, WHO samples were five-fold diluted in human plasma or PBS buffer, therefore WHO-5M represents WHO-1000K, WHO-500K represents WHO-100K and WHO-50K represents WHO-10K

[e] INSTAND external quality assurance scheme sample [150]

Table 4.10: Variability of the direct quantification of DNA from whole viruses and quantification of extracted viral DNA using different materials on the Biomark HD system. DNA extractions were performed using either WHO material diluted in human plasma or PBS, or INSTAND samples with viruses in cell lysates. WHO (100×) material and INSTAND sample N° 365032 were not analysed due to inadequate nominal DNA concentrations (<40 copies/effective reaction size).

Material	Direct quantification			Quantification of extracted DNA[a]							
	Nominal viral load[a] (IU/mL or cp/mL)	λ[b]	CV[c] (%)	Nominal viral load[a] (IU/mL or cp/mL)	Human plasmad		PBS[d]		Cell lysate	
					λ[b]	CV[c] (%)	λ[b]	CV[c] (%)	λ[b]	CV[c] (%)
WHO-5M	5×10^6	1.6	2	1×10^6	1.2	3	1	25	/	/
WHO-500K	5×10^5	0.2	5	1×10^5	0.1	16	0.1	29	/	/
N° 365029 [e]	7.7×10^4	0.3	0	7.7×10^4	/	/	/	/	0.1	5

[a] Nominal viral concentration of the sample before quantification, based on the nominal concentration of each of the whole virus materials and their volumetric dilutions in double-distilled water, human plasma or PBS

[b] Mean DNA copy number per partition

[c] Coefficient of variability that was calculated on the basis of three measurements (n =3) on the QX100 system

[d] Prior to DNA extraction, WHO samples were five-fold diluted in human plasma or PBS buffer, therefore WHO-5M represents WHO-1000K and WHO-500K represents WHO-100K

[e] INSTAND external quality assurance scheme sample [150]

4.3.2 Direct quantification from human plasma

Direct quantification from human plasma was performed with WHO samples spiked in different concentrations of human plasma, which were quantified using the direct quantification on the QX100 system. With samples containing different concentrations of human plasma, various effects on the direct quantification were observed (Figure 4.15). For the samples that contained 3.3% and 10% (v/v) human plasma, the estimated DNA concentration was only 31% and 4% of the nominal concentrations, respectively. Nevertheless, the variability was low, with CV values below 11% for both pairs of duplicates. In contrast, with samples that comprised more than 30% (v/v) human plasma, no amplification occurred. Furthermore, negative effects on the droplets were noted, as only around 4600±200 droplets (30% human plasma) and 8300±900 droplets (90% human plasma) were accepted for the analysis. On the other hand, with the lowest two concentrations of human plasma no such negative influence was noted, as around 16,000 droplets were analysed. Additionally, the inclusion of human plasma influenced the intensity of the fluorescence, since in the positive droplets the fluorescence intensity was inversely proportional to the concentration of the matrix, whereas in the negative droplets the opposite effect was seen.

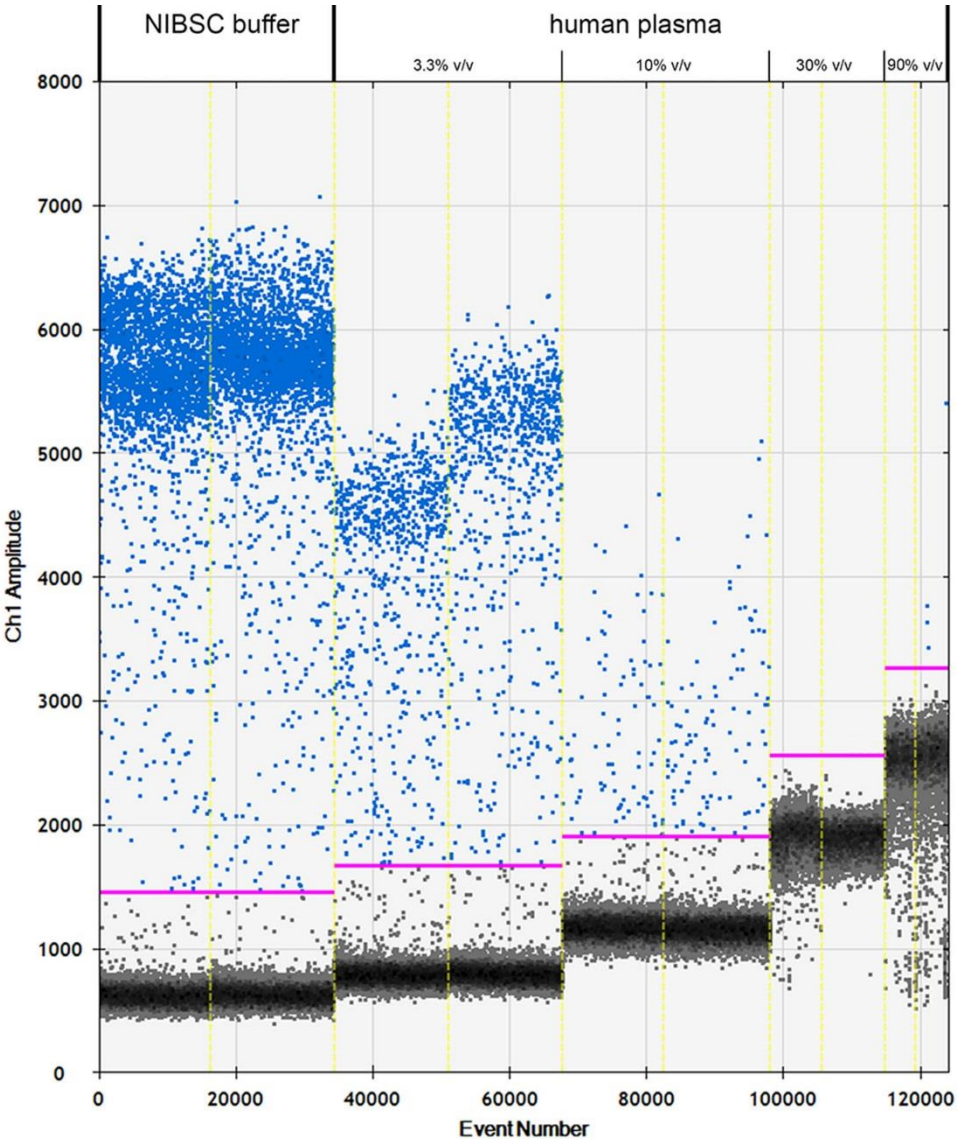


Figure 4.15: Assessment of direct quantification of viral DNA from different matrices. The left column represents the direct quantification of the WHO-500K reference material diluted in double distilled water. The columns on the right represent the WHO-5M material spiked into different concentrations of human plasma (3.3%, 10%, 30%, and 90%; all v/v), which resulted in a 10× dilution of the initial material.

4.4 Inter-Laboratory Assessment of Three Molecular Methods (Droplet-based dPCR and two microfluidic-based dPCRs) for Quantification of HCMV

In this study, the assessment of three different dPCR platforms (the QX100 system, the Biomark 37K array and the QuantStudio 3D system) was performed in four collaborative laboratories to evaluate their intermediate precision and reproducibility for quantification of two different HCMV materials, genomic DNA (centrally prepared gDNA2) and whole virus material (locally extracted WHO-1M) (Figure 3.4).

4.4.1 Intra-experiment variability and intermediate precision

On every dPCR platform three experiments were performed, with three different units analysed in each experiment in duplicates (n=6) (e.g. in each experiment units G1, G2 and G3 were tested in duplicate, Figure 3.4 and Tables S4-S9 from Supplementary material). On every platform, outliers were determined individually and excluded from further analysis.

With both materials, very small CV values related to intra-experiment variability were observed in all three dPCR platforms, as a majority of duplicates (technical replicates) had CV values below 10%, with some CV values between 10% and 40% mostly found on the Biomark 37K arrays.

With all dPCR platforms low CV values related to intermediate precision were noted, as the maximum difference between the average DNA copy-numbers of experiments was below 31%, with most of differences below 25% (Figure 4.16, Table 4.11). Furthermore, with each material and dPCR platform no statistically significant differences in the average DNA copy-numbers were observed between three consecutive experiments (Figure 4.16).

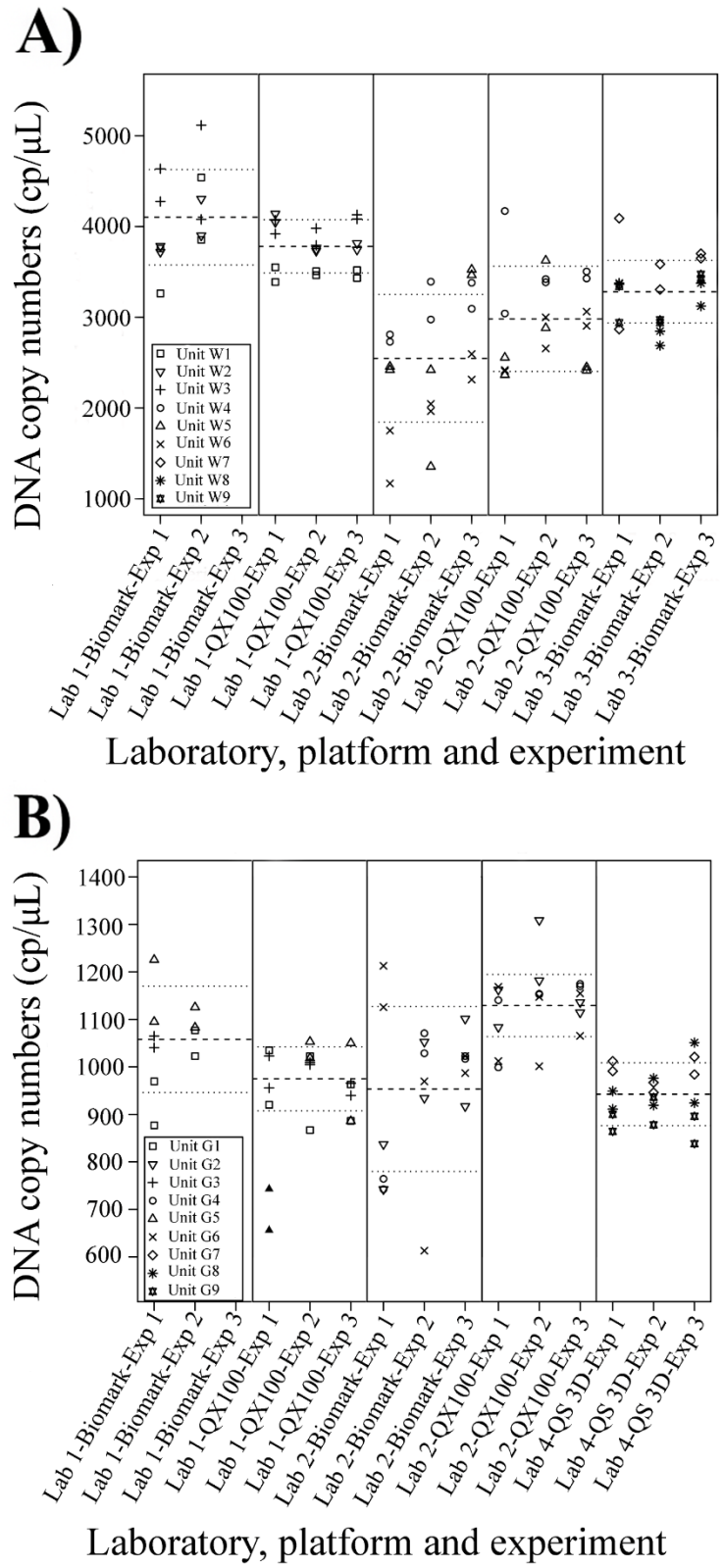


Figure 4.16: Intermediate precision for each of five dPCR platforms involved in quantification of (a) WHO-1M and (b) gDNA2. Each symbol denotes a single measurement of dPCR platform, whereas black triangles represent outliers. The average DNA copy-number obtained on each dPCR platform is shown with a dashed line, while dotted lines depict expanded MUs (Lab 1-Biomark, k=2.2; all other platforms, k=2.11).

Table 4.11: The average DNA copy-number and coefficient of variability for experiments measured on different platforms.

Material	Laboratory/Platform	Experiment 1		Experiment 2		Experiment 3	
		Average DNA copy-number (cp/µL)	CV (%)	Average DNA copy-number (cp/µL)	CV (%)	Average DNA copy-number (cp/µL)	CV (%)
WHO-1M	1 / Biomark	3906	12	4298	11	/	/
WHO-1M	1 / QX100	3852	8	3703	5	3786	8
WHO-1M	2 / Biomark	2223	29	2358	31	3062	16
WHO-1M	2 / QX100	2826	25	3161	12	2960	16
WHO-1M	3 / Biomark	3330	13	3056	11	3458	6
gDNA2	1 / Biomark	1045	11	1077	4	/	/
gDNA2	1 / QX100	983	6	996	7	948	6
gDNA2	2 / Biomark	904	23	945	18	1011	6
gDNA2	2 / QX100	1094	7	1157	8	1136	4
gDNA2	4 / QuantStudio 3D	938	6	937	4	952	8

4.4.2 Inter-unit variability

With gDNA2, three laboratories each measured three different gDNA2 units that were all extracted and aliquoted at laboratory 1. For WHO-1M, in each of three laboratories DNA was extracted from three WHO-1M units. Both materials were then quantified in duplicate on every dPCR platform over three consecutive experiments (n=6).

With gDNA2, only small CV values related to inter-unit variability were observed with each dPCR platform, as small (difference between units G <14%) and mostly statistically insignificant differences in the average DNA copy-numbers were measured between units (Table 4.12). On the other hand, with WHO-1M higher CV values related to inter-unit variability were observed compared to gDNA2 (difference between units W <55%) (Table 4.12).

Table 4.12: Mean DNA copy-numbers and coefficients of variability for individual units of HCMV test material measured on different platforms. In each laboratory, three WHO-1M units and three gDNA2 units were tested. On each platform and with every unit of HCMV test material, three aliquots were analysed, each in one of three experiments. With WHO-1M material, the first unit denotes units W1 (Laboratory 1), W4 (Laboratory 2) and W7 (Laboratory 3), the second unit denotes units W2 (Laboratory 1), W5 (Laboratory 2) and W8 (Laboratory 3) and the third unit denotes units W3 (Laboratory 1), W6 (Laboratory 2) and W9 (Laboratory 3). With gDNA2 test material, the first unit denotes units G1 (Laboratory 1), G4 (Laboratory 2) and G7 (Laboratory 4), the second unit denotes units G2 (Laboratory 1), G5 (Laboratory 2) and G8 (Laboratory 4) and the third unit denotes units G3 (Laboratory 1), G6 (Laboratory 2) and G9 (Laboratory 4).

HCMV test material	Laboratory	Platform	First unit		Second unit		Third unit		ANOVA
			Mean DNA copy number (cp/µL)	CV (%)	Mean DNA copy number (cp/µL)	CV (%)	Mean DNA copy number (cp/µL)	CV (%)	p-value
WHO-1M	1	Biomark	3853	14	3926	7	4526	10	0.10
WHO-1M	1	QX100	3476	2	3870	5	3995	3	<0.001
WHO-1M	2	Biomark	3063	9	2606	31	1973	25	0.02
WHO-1M	2	QX100	3491	11	2715	18	2741	11	0.005
WHO-1M	3	Biomark	3533	12	3129	10	3182	8	0.10
gDNA2	1	Biomark	986	9	1132	6	1053	2	0.060
gDNA2	1	QX100	949	7	1002	8	983	3	0.40
gDNA2	2	Biomark	941	14	931	13	988	19	0.81
gDNA2	2	QX100	1132	6	1164	7	1091	7	0.26
gDNA2	4	QuantStudio 3D	987	3	955	6	885	4	0.002

4.4.3 MU of each dPCR platform

With every material, for each of five dPCR platforms the average DNA copy-number and corresponding expanded MU were calculated by taking into account three units measured in three experiments. For each dPCR platform, small expanded MUs (<18%) were obtained with gDNA2, whereas with a more complex WHO-1M that requires DNA extraction higher expanded MUs (<28%) were noted (Table 4.13).

Table 4.13: The average DNA copy-number and relative expanded MUs obtained on each dPCR platform.

Laboratory / platform	Whole-virus material		Genomic DNA material	
	Average DNA copy-number (cp/µL)	Relative expanded MU (%)[a]	Average DNA copy-number (cp/µL)	Relative expanded MU (%)[a]
Laboratory 1/Biomark	4101	13	1058	11
Laboratory 1/QX100	3780	8	974	7
Laboratory 2/Biomark	2547	28	953	18
Laboratory 2/QX100	2982	20	1129	6
Laboratory 3/Biomark	3281	10	/	/
Laboratory 4/QuantStudio 3D	/	/	942	7

[a] with Biomark from laboratory 1 (k=2.2), with all other platforms (k=2.11)

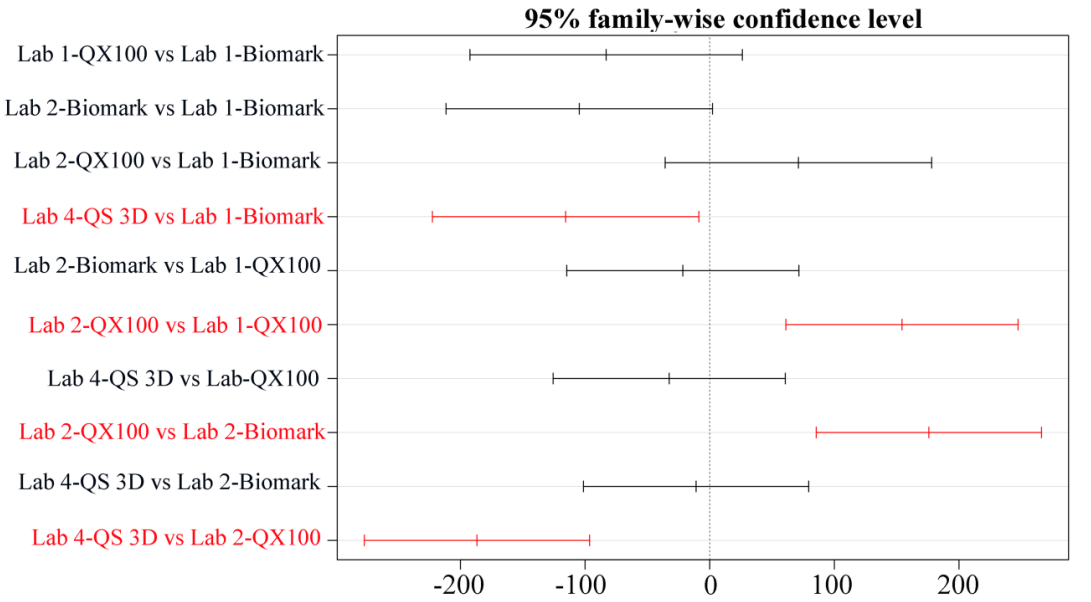
4.4.4 Intra-laboratory concordance between platforms

In laboratories 1 and 2, two different dPCR platforms were used. For both materials tested on the Biomark system in laboratory 1, the average DNA copy-number was around 8% higher than the one measured on the QX100 system (Table 4.13). The opposite was noted in laboratory 2, where both materials showed 17% smaller average DNA copy-number when measured on the Biomark system compared with those on the QX100 system.

4.4.5 Inter-laboratory variability (reproducibility) and estimation of the mean DNA copy-number with correspondent MU

The mean DNA copy-number of each material was estimated by combining the measurements from each dPCR platform (two QX100 systems, two Biomark systems and one QuantStudio 3D) from three laboratories. With gDNA2, the difference between laboratories did not exceed differences within one laboratory, since the maximum difference between dPCR platforms from two laboratories was less than 20% (e.g. Table 4.13, difference between the average DNA copy number from Laboratory 2/Biomark and Laboratory 4/Quant Studio 3D). Furthermore, no statistically significant differences were observed between the majority of platform pairs (Figure 4.17a). Conversely, with more complex DNA material, such as WHO-1M, 62% difference in the average DNA copy-number was noted between two Biomark platforms from different laboratories (e.g. Table 4.13, difference between the average DNA copy number from Laboratory 1/Biomark and Laboratory 2/Biomark), with statistically significant differences between most of the platform pairs (Figure 4.17b). Furthermore, in the Laboratory 1 approximately 40% higher average DNA copy-number was noted when compared to Laboratory 2 (Figure 4.16).

A)



B)

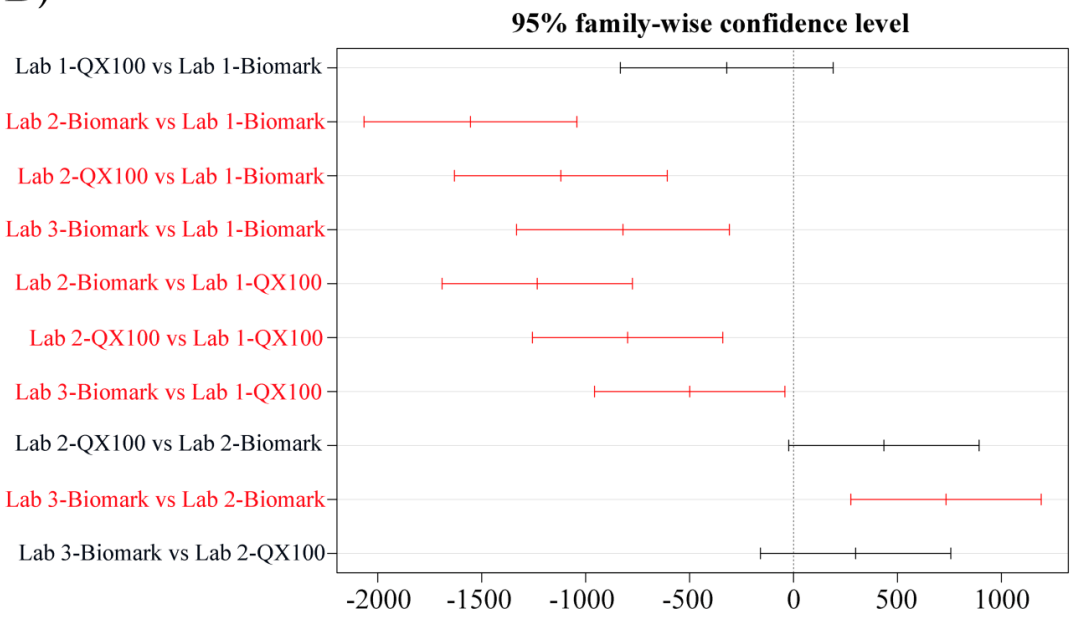


Figure 4.17: Tukey test for statistically significant differences between the mean DNA copy-numbers obtained from different dPCR platforms. Statistically significant differences obtained with (a) gDNA2 and (b) WHO-1M are shown. With platform pairs marked with a red colour, statistically significant differences in the mean DNA copy-number were observed between particular platforms.

To additionally demonstrate the suitability of dPCR as a candidate for a reference measurement procedure, its applicability for the estimation of the consensus value of different virus reference materials was assessed. With both materials, reported results from all five dPCR platforms were within a narrow expanded MU (WHO-1M, 15%; gDNA2, 6%) of the corresponding mean estimated DNA copy-numbers (Figure 4.18).

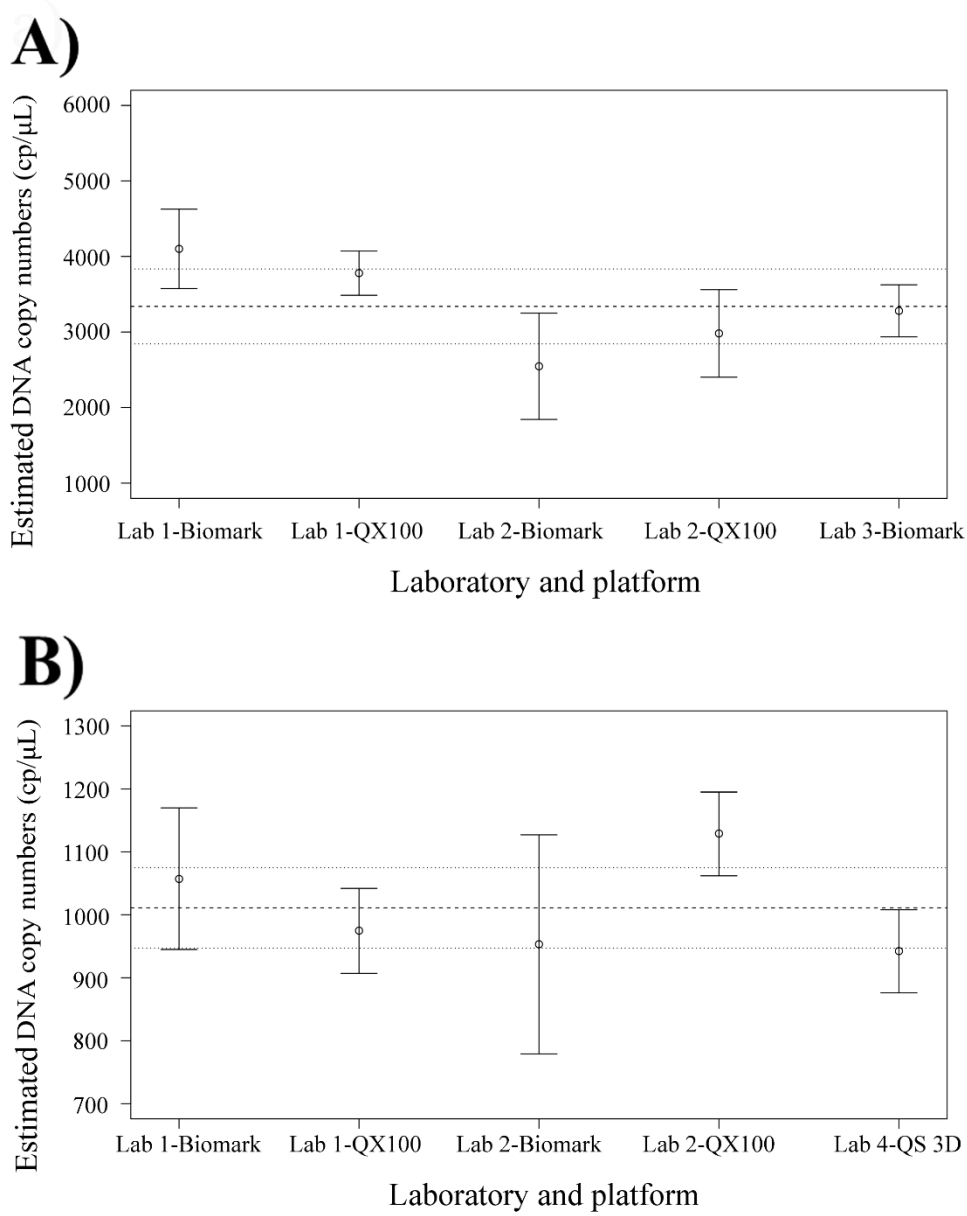


Figure 4.18: The mean estimated DNA copy-number concentration and corresponding expanded MU for (a) WHO-1M and (b) gDNA2. Each dot represents the average DNA copy-number concentration estimated with one dPCR platform, while vertical bars depict their corresponding expanded MUs (Lab 1-Biomark, k=2.2; all other platforms, k=2.11). Dashed line shows the mean estimated DNA copy-number according to Vangel-Ruhkin estimator, whereas two dotted lines denote the corresponding expanded MU.

Chapter 5

Discussion

The clinical diagnostics of human viruses became dependent on molecular methods such as qPCR [75], [114], [151]. With HCMV, reliability of clinical diagnostics is hampered by disparity of extraction kits, commercial standard materials, qPCR reagents and qPCR assays used for quantification of viral load, as well as by the lack of well characterised and commutable primary reference materials and reference measurement procedures [58], [83], [110], [133], [135], [150], [152]. This methodological shortcomings are considered to reduce the agreement between laboratories and consequently prevent the establishment of universal threshold for clinically significant viral load, which would in turn improve the pre-emptive treatment of HCMV disease [58], [110], [124]. Additionally, with the pre-emptive approach based on monitoring the viral kinetics, the accepted consensus proposes 3-fold differences (0.5 $\log_{10}$; for viral concentrations > 1 000 copies/mL) or 5-fold differences (0.7 $\log_{10}$; for viral concentrations < 1 000 copies/mL) in viral load between two consecutive measurements as an indication for viral multiplication or reduction [124]. However, the reliability of this approach is questionable since intra- and inter-experiment variabilities of measurements are not well determined [58].

In this PhD thesis, different methods for DNA extraction and DNA quantification (qPCR and dPCR) of HCMV were metrologically assessed to investigate their performance parameters, which could help set the criteria for qPCR-based pre-emptive treatment of HCMV. In addition, dPCR technology was comprehensively assessed for reproducibility and capability of direct quantification of whole viruses, to shine a light on its suitability for implementation in metrological framework.

5.1 Metrological Assessment of Three Methods for Manual Extraction of HCMV DNA from Different Matrices

DNA extractions are a prerequisite for qPCR-based or dPCR-based detection and quantification of viruses and bacteria in hospitals and clinical laboratories [75]. For quantification of HCMV and other human viruses, a variety of manual and automated DNA extraction approaches are used [153]. The use of different extraction approaches is considered to reduce the agreement between laboratories due to variable DNA recovery and removal of inhibitory substances, hence hampering the establishment of the universal concentration threshold which would improve pre-emptive treatment of HCMV disease [58]. Additionally, DNA extraction is considered as an important contributor to intra- and inter-experiment variabilities, which result in questionable reliability of current criteria for monitoring the viral kinetics [124], [154]. The majority of reports have focused mostly on assessment of different automated methods for quantification of viral DNA, with the emphasis on differences in DNA recovery between them. In this study, the assessment of three commonly used methods for manual extraction was conducted using two different matrices with a whole-virus material of HCMV. The goal of this study was to investigate the differences in DNA recovery between DNA-extraction kits, and to observe the influence of different matrices and viral concentrations. Additionally, the contribution of DNA extraction to the final intra- and inter-

experiment variability was assessed to investigate the suitability of current guidelines on initiation or termination of pre-emptive treatment against HCMV based on differences in viral load between consecutive measurements.

5.1.1 DNA recovery

In this study, between three DNA extraction kits intermediate differences in terms of DNA recovery have been observed, as the majority of differences did not exceed 0.2 log (1.5-fold) regardless the matrix or HCMV concentration, with the highest difference at approximately 0.35 log (2.2-fold)(Chapter 4.1.1.1). Such difference between extraction kits in terms of DNA recovery is concordant with a previous report on two automated methods for extraction of HCMV DNA (0.2 log) as evaluated by qPCR [123]. However, in other reports on methods for manual and automated extraction of HCMV DNA from human plasma, larger differences were noted, ranging from 0.35 log up to 0.75 log (5.5-fold) [72], [131], [154]–[157]. Since with all reports, including our study, the same matrix was used, high differences between estimated DNA recovery might be due to actual differences in DNA recovery or different rate of elimination of inhibitory substances that subsequently hamper the qPCR efficiency and lead to underestimation of DNA copy-numbers. Although different carry-over of inhibitors has been reported for methods for HCMV-DNA extraction from urine and methods for extraction of cell-free DNA from human plasma [158], [159], no such observation has been reported for methods for extraction of HCMV DNA from human plasma. Moreover, in the present study almost similar DNA recovery was measured with inhibition prone qPCR and inhibition resilient QX100 system, with constant ratio between dPCR-based and qPCR-based results obtained with each extraction kit (Figure 4.3, Table 4.2). Both observations indicate that every extraction kit eliminated inhibitory substances with a similar efficiency of removal. In addition, in the aforementioned report with the highest difference between DNA recovery (0.75 log or 5.5-fold), for all assessed extraction methods no carry-over of inhibitors was detected [72]. Therefore, it is reasonable to assume different chemistries and mechanisms involved in the extraction of HCMV DNA could cause different DNA loss during DNA extraction, which results in variable DNA recoveries between DNA-extraction kits. Hence the selection of DNA-extraction method (manual or automated) is of paramount importance when good comparability between laboratories is required.

5.1.2 Intra-experiment and inter-experiment variability of DNA extraction efficiency

Different variabilities in extraction efficiency have been observed between DNA-extraction kits. Considering variability of extraction efficiency, with HCMV concentration above 20,000 IU/mL, low variabilities were noted with Roche and Qiagen extraction approaches, whereas with Cepheid higher variability was observed (Table 4.1 and Table 4.2). However, with lower and clinically more relevant viral concentrations for testing of human plasma (3000 IU/mL and 910 cp/mL) [121], [126], [127], the lowest variability was observed with Roche extraction kit, followed by Qiagen and Cepheid extraction kits (Table 4.1). To the best of our knowledge, the reports on variability of extraction methods for HCMV from human plasma are scarce. One such assessment demonstrated slightly higher inter-experiment extraction variability of manual Qiagen approach (0.15 - 0.22 log; 1.4 fold - 1.7-fold) than observed in this study [154]. Based on information gathered from this study and aforementioned assessment, the investigated DNA extraction kits for manual extraction of HCMV DNA have generally minor to intermediate contribution to overall intra- and inter-experiment variability. However, to simulate the worst-case scenario in clinical testing, the difference between maximum and minimum logarithmic concentration should be considered instead of SD, which covers only 68.2% of all measurements. Based on results from this study the same extraction approach could result in 0.38 log difference (2.3-fold) between two consecutive DNA extractions (Table 4.1). Moreover, if only one measurement is conducted on qPCR for the purpose of HCMV quantification in a routine clinical testing, the maximum difference between two individual qPCR measurements, which use extracted DNA from two extractions, should be considered. In this case, with low HCMV concentrations the difference between two consecutive measurements of Qiagen and Cepheid was greater than currently suggested consensus differences of 0.5 log and 0.7 log (3- and 5- fold) (Table 4.2), which, when exceeded, lead to an immediate initiation or termination of antiviral therapy [124]. Based on observations in this

study, such effect of variability can be reduced by performing duplicate extractions and/or duplicate measurements (Table 4.1 and Table 4.2). Furthermore, deeper metrological assessments of methods for extraction of HCMV DNA from clinical samples would expand our knowledge on their variability, consequently allowing clinicians to use the least variable approaches for extraction of HCMV DNA. This would in turn contribute to smaller overall measurement variability in clinical testing of HCMV and assure better reliability of the pre-emptive approach. Moreover, the implementation of more repeatable extraction methods could, with additional comprehensive metrological support, further reduce the difference of 0.5 and 0.7 log (3- and 5- fold), which are currently suggested as triggers for changes in antiviral treatment. This would result in earlier detection of changes in viral kinetics and allow more prompt decisions on changes in antiviral treatment.

5.2 Metrological Evaluation of three molecular methods (qPCR, Droplet-based dPCR and Microfluidic-based dPCR) for Quantification of HCMV

With dPCR becoming an important technology for nucleic-acid quantification and detection for a myriad of applications, more information about analytical parameters is needed to facilitate further improvements in analytical performance of dPCR, which would in turn lead to a more reliable and accurate quantification of nucleic acids. In this particular study, two dPCR platforms were investigated for their resilience to different PCR reaction components for target DNA quantification. Additionally, both dPCR platforms were assessed together with qPCR in terms of linearity, LOD, LOQ, and precision.

5.2.1 Influence of different PCR components on estimation of DNA concentration using two dPCR-based methods

Various PCR components have already been shown to cause significant differences between amplification efficiencies on qPCR, which results in different Cq values [160]. However, owing to the end-point measurement of dPCR only small or no variations are expected when different reaction components are used [90]. To investigate this, two dPCR platforms were assessed with reaction components that showed large influence on efficiency on qPCR (Figure 4.4). Eventually, on each dPCR platform only a minor susceptibility to different PCR components was observed (Figure 4.5, Figure 4.8).

On the Biomark system, although some minor effects of different assays, master mixes and concentrations of oligonucleotide primers and probes were observed to influence the final estimation of DNA concentration, these effects were never consistent with both templates nor array types (Figure 4.5). Furthermore, no correlation between low assay efficiency and low estimated DNA concentration was observed, indicating other causes for observed differences (Figure 4.4, Figure 4.5). Low influence of different assays has already been reported from previous researches conducted on HCMV [148], [161] and could be explained as a consequence of molecular dropout, whose rates vary between different assays [90], [162], [163]. In case of molecular dropout, the target sequence on the DNA template becomes inaccessible to DNA polymerase, oligonucleotide primers or probes, due to DNA damage or special DNA structure that prevents those molecules to reach the target sequence [90], [162]. Additionally, an agreement in measured DNA concentration between different master mixes is concordant with other reports, where less than 50% difference was observed between master mixes [148], [163]. It is reasonable to assume various master mixes have a different influence on molecular dropout or linkages between DNA templates. Consequently, this might result in increased number of partitions that contain more than one template, which results in reduced final estimation of DNA copy numbers [90].

On the QX100 system, reduced influence of different assays was observed in agreement with previous report (Figure 4.8) [164]. Nevertheless, minor impact of various assays might be still caused by variable rates of molecular dropout, since this has already been noted in this particular assessment on the Biomark 12.765 array and in another report [148]. As the amplicon size reversely correlates with the rate of molecular dropout [134], it is reasonable to assume a twofold difference in amplicon size between both assays used in this

study might be a major reason for speculated differences in molecular dropout. This could be explained as a consequence of degradation of DNA templates, with bigger amplicons having higher probability of being inaccessible to oligonucleotide primers and probes compared with the smaller ones. With both assays higher concentrations of probes resulted in higher fluorescence intensity in positive droplets compared to assay with lower probe concentration (Table 4.9 and Table 4.10). This indicates higher probe concentration would probably enable better assay sensitivity and therefore better overall performance compared to assay with lower probe concentration, when HCMV DNA from more complex samples with more inhibition would be required.

The QX100 system has already been demonstrated to offer high robustness, as it was effectively used for multiplexing with varying assay concentrations of oligonucleotide primers and probes, with no negative effects on quantification repeatability [165]. However, high robustness observed in this study is not in agreement with another research, where 66% increase was observed when lower concentrations of oligonucleotide primer and probes were compared to the higher ones [166]. The main reason for lower robustness of that experiment might be the high amount of droplets with intermediate fluorescence (i.e. the rain), which was probably the result either of differences in PCR efficiency of the particular assay or delayed amplification (i.e. Monte Carlo effect) [81]. High proportion of rain may hamper manual or automatic discrimination between negative and positive droplets, thus leading to overestimation or underestimation of DNA concentration, and further increase the variability between different concentrations of oligonucleotide primers and probes. In contrast, with both assays in this study high increase in fluorescence intensities of positive droplets and small amount of rain resulted in a straightforward setting of the threshold, which indicates very good PCR efficiencies of each assay (Figure 4.9, Figure 4.10). Therefore, it is reasonable to assume PCR efficiency has an important impact on distinction between positive and negative droplets and therefore plays an important role in robustness of dPCR [81].

5.2.2 Linearity, LOD, LOQ, and precision of each of three quantification methods

High agreement in estimated DNA concentration between all three platforms was observed, although different dPCR platforms and even different PCR-based quantification techniques (dPCR and qPCR) were compared. Between qPCR and the QX100 system the highest concordance was noticed (Table 4.3, Table 4.5). In theory, the estimation accuracy of qPCR and a consequential concordance between estimations on dPCR and qPCR depends i) on the selection of a quantification method used for characterisation of the calibration material, and ii) the commutability between the calibrant and testing samples [133]. Some authors have already showed high over- or underestimation (>50%) of qPCR when compared to dPCR [82], [161], [167]. It is reasonable to assume that in this study, the agreement between qPCR and the QX100 system was owing to the use of i) gDNA1 that had been quantified on the same QX100 system that was later compared to, ii) and that this particular gDNA1 was on qPCR used as the calibration material and the testing material, thus reducing any reasons for low commutability. High concordance was also observed between both dPCR platforms, with the QX100 system constantly underestimating the DNA copy number compared with the Biomark system (Table 4.4, Table 4.5). The same pattern was reported from two previous assessments that compared the QX200 system and the Biomark 12.765 array, with 8% and 30% higher estimations noted on the latter when compared to the former [85], [148]. However, it is currently not possible to determine whether the Biomark system overestimates or QX100 underestimates the true DNA quantity, thus both options should be considered. With the Biomark system, overestimation of DNA concentration could be due either to occurrence of single-stranded DNA molecules during partitioning or underestimation of chamber volumes on the Biomark 37K array [90], [163], [168], since the average chamber volume is inversely related to the number of estimated DNA copies. On the other hand, overestimation of droplet volume is probably the main reason for possible underestimation of the QX100 system, since with the recent report smaller droplet volumes (0.80 µL and 0.77 µL) have been observed [169] compared to those used in this study (0.834 µL). Furthermore, the master mix used with the QX100 system might cause a higher rate of molecular dropout compared to that used with the Biomark system, since master mix was shown to influence the quantification (Figure 4.5, Figure 4.8).

In this study, higher sensitivity was observed on the qPCR compared to both dPCR platforms (Table 4.6), which is also concordant with a majority of other reports and was explained as restricted sample volume input of each dPCR platform [81], [167], [170]. Nevertheless, restricted sample volume could not be the only reason for lower analytical sensitivity of dPCR platforms, since equal sample volumes (20 µL) were used with the QX100 system and qPCR. Considering the ideal specificity of the assay, the criteria for the theoretical LOD (Poisson error with 95% probability) is met when there are on average at least three DNA copies in the analysed sample [77]. With each dPCR platform, there is a volumetric difference between the inserted sample volume (i.e., total-reaction volume) and the average analysed sample volume (i.e., effective reaction size). This could explain why higher LODs were obtained with both dPCRs when the total-reaction volume was taken into account, although similar LODs were noted with all three platforms when the effective reaction size was considered (Table 4.6).

To the best of our knowledge, this is the first comparison of the QX100 system and the Biomark 37K array in terms of linearity, analytical sensitivity and LOQ. When considering the total-reaction volume, dominant performance of the QX100 system over 37K array in terms of analytical sensitivity and precision was observed when low DNA concentrations were used (<1000 cp/total-reaction volume) (Figure 4.11, Table 4.6). Conversely, on both dPCR platforms similar performance characteristics were observed when the effective reaction size was taken into account, despite 15-fold higher number of partitions analysed with the QX100 system. Moreover, similar performance is concordant with theoretical CV values of both dPCR platforms. These findings strongly suggest that with low DNA concentrations, the variability of dPCR platform, when considering the total-reaction volume, is not influenced by the number of analysed partitions but by the ratio between the effective reaction size and the total reaction volume.

In other reports, each dPCR platform has been reported to show better precision through the entire dynamic range when compared to qPCR [161], [164], [170], [171]. However, such trend was not observed in this study. The theoretical variability of each dPCR platform can be mathematically predicted, since it based solely on the Poisson distribution. Conversely, the theoretical variability of qPCR is less predictable and is consequently higher than that of dPCR, since it additionally depends on the assay amplification efficiency [66], [77]. Nevertheless, in the present study the variability of qPCR was almost the same as the theoretical variability of dPCR when considering effective reaction size (Figure 4.11). This indicates low inhibition and low effects on amplification efficiency, and therefore it is not a surprise that variability of qPCR was sometimes even lower than those of dPCR platforms when the total reaction volume was considered. Moreover, with both dPCR platforms a slightly higher variability was observed than theoretically predicted. This might be owing to an additional round of sampling, which occurs on each dPCR platform. With every dPCR platform and qPCR, the first stochastic event occurs when a certain volume of sample is pipetted from the tube to the sample inlet. However, with both dPCR platforms an additional second stochastic event takes place when only a certain part of previously pipetted sample volume is either loaded into the chambers (the Biomark system) or analysed as accepted droplets (the QX100 system). It could be assumed that the second stochastic event increases the variability of dPCR-based quantification, especially when samples with a low DNA copy-number concentration are quantified. Sensitivity and repeatability of dPCR-based quantification could be improved by using replicate reactions, which would double the effective reaction size [77]. Additionally, since currently only 60% to 75% of droplets are accepted with the QX100 system [164], [170], [171], improvements in pipetting techniques during droplet transfer may increase the number of accepted droplets and thus diminish the influence of the second stochastic event [77].

To additionally assess all three platforms for their suitability for routine clinical testing of HCMV viral loads, the difference between maximum and minimum logarithmic DNA concentration was considered. Current guidelines suggest differences of 0.5 log (3-fold difference; HCMV DNA concentrations >1000 cp/mL) and 0.7 log (5-fold difference, HCMV DNA concentrations <1000 cp/mL) as a trigger for changes in pre-emptive treatment [124]. Therefore, the difference between maximum and minimum logarithmic DNA concentration obtained with the same quantification platform should preferentially be less than 0.3 log, since DNA extraction also contributes to some variability as seen Chapter 4.1.2 and other reports

[154]. In this study, the highest variability was noted on the Biomark 37K array, as only for concentrations above 60,000 cp/mL the difference between maximum and minimum logarithmic DNA concentration was below 0.3 log (Table 4.8). Therefore, the Biomark 37K array looks inappropriate for routine clinical testing, since all reported concentration thresholds for human plasma and the majority of reported concentration thresholds for whole blood are below 60,000 cp/mL [119], [120], [122], [123], [125], [127]. On the QX100 system, with concentrations above 3500 cp/mL differences between maximum and minimum logarithmic DNA concentration were already below 0.3 log (Table 4.7). Therefore, the QX100 system could be appropriate for routine testing of HCMV from whole blood and from human plasma when concentration thresholds above 4000 cp/mL are used [119], [120], [122], [123]. However, in some reports on human plasma and whole blood even lower cut off values were determined [121], [125], [126], [172]. Contrary to both dPCR platforms, qPCR demonstrated variability below 0.3 log even with DNA concentrations below 700 cp/mL (Table 4.7). Although at a first glance it looks reasonable to keep qPCR as the method of choice for routine monitoring of HCMV, especially when low concentration thresholds (<1700 cp/mL) are used by hospitals [121], [125], [127], such conclusions should be taken after testing of appropriate amount of clinical samples. According to some other reports, the QX100 system provided more repeatable quantification of low HCMV viral loads than qPCR [170]. Furthermore, since the LOD, LOQ and precision of qPCR-based quantification are very dependent on amplification efficiency and robustness of particular assay, it is therefore farfetched to estimate the suitability of qPCR for routine clinical applications solely based on the good performance of the qPCR assay from this study. Therefore, it could be assumed that the QX100 system already looks appropriate for implementation in routine quantification of viral loads, at least as a complementary approach to qPCR, as with some assays dPCR allows for more precise and robust quantification throughout the majority of dynamic range of HCMV viral load [59]. Moreover, the forthcoming advances in dPCR technology, such as increased effective reaction size and more concentrated master mixes, would probably improve the sensitivity and precision at lower concentrations of DNA, which could together with higher throughput and process automatization result in even higher applicability of dPCR for clinical analyses.

5.3 Evaluation of Two Molecular Methods (Droplet-based dPCR and Microfluidic-based dPCR) for Direct Quantification of HCMV Without DNA Extraction

DNA extractions are currently a prerequisite for reliable quantification of viral DNA by the means of qPCR, and are widely used for routine testing and characterisation of reference materials [75]. However, DNA extractions cause reduced DNA quantities and increase the variability of final qPCR/dPCR measurements (Chapter 5.1.1 and 5.1.2), which hampers the characterisation of reference materials using qPCR. dPCR is believed to allow for accurate quantification and is more tolerant to inhibition than qPCR, and is therefore a promising candidate for characterisation of CRMs [74], [171]. However, variability and reduced DNA recovery caused by DNA extraction would still hamper accurate characterisation of reference materials. In this study, the assessment of the QX100 and the Biomark system was conducted to investigate their suitability for direct quantification of whole-virus materials for HCMV with no DNA extraction needed. Additionally, direct quantification of HCMV from human plasma was tested to investigate the applicability of dPCR-based direct quantification for a routine quantification of HCMV.

To our knowledge, this is the first assessment of direct quantification of whole-virus material that was conducted on dPCR platform. It seems that the incubation at 95 °C before and between every PCR cycle results in degradation of coat proteins of HCMV and consecutive release of HCMV DNA, which then becomes available for oligonucleotide primers and probes. This has already been used with human viruses such as hepatitis B, human adenovirus and HCMV [173]–[175], however since the quantification was carried out by qPCR, its susceptibility to inhibitors of PCR efficiency and reliance on calibration material almost certainly hampered the reliability and accuracy of such measurements [74], [77]. Moreover, the commutability of calibration material used with qPCR-based direct quantification of clinical samples would most likely account for an additional problem, since the calibration materials do not always behave

in the same way as whole-virus-derived DNA from different matrices, resulting in overestimation or underestimation of the tested viral load [133].

5.3.1 Direct quantification of whole-virus material and quantification of extracted HCMV DNA on two dPCR platforms

To evaluate the suitability of dPCR-based direct quantification, a comparison with dPCR-based quantification of extracted DNA was conducted. With a direct quantification approach on each dPCR platform, higher DNA concentrations were estimated than with the approach using extracted DNA (Figure 4.13). Moreover, this pattern was observed with two concentrations of two different whole-virus materials. Since dPCR is not dependent on calibration material and avoids qPCR-related commutability problems [90], and is more resilient to inhibition and reduced amplification efficiency than qPCR, it is reasonable to expect that only minor over- or underestimation could occur with the direct quantification approach. The differences in estimated DNA concentration observed between direct quantification and approach using extracted DNA are probably mostly owing to reduced DNA recovery of the latter approach. Therefore, estimations of DNA concentration obtained by direct quantification approach are most likely in a closer agreement with the actual concentration of HCMV particles than those obtained with quantification of extracted DNA.

In the majority of cases good concordance between the nominal whole-virus concentrations and estimated concentrations by direct quantification was observed (Figure 4.13). However, this does not necessarily confirm the high accuracy of direct quantification, since both whole-virus materials were characterized and enumerated in inter-laboratory assessments using various DNA extractions followed by different qPCR assay [132], [150]. Potential disagreement between values estimated by direct quantification and the actual amount of virus particles present in tested material is not very likely being caused by overestimation of direct quantification [90], since both single-stranded DNA strands, which derive from the same virus, are packed inside the virus capsid when partitioning occurs. Conversely, there are several possible sources of underestimation. In contrast to extracted DNA, with direct quantification the DNA template is surrounded with virus-coat proteins and other substances present in matrices of the whole-virus material, which could exacerbate the accessibility of DNA and consequently increase the dropout rate [90], [162], [163]. However, simultaneous use of two or more assays, which target different regions on the DNA strand, should reduce the dropout rate and therefore enhance the accuracy of the direct quantification approach [162]. Non-homogeneous distribution of virus particles is another possible reason for underestimation, since virus particles tend to aggregate [175]. However, to our knowledge there is no evidence that HCMV aggregates. If aggregation is a probable reason for underestimation, use of different master mixes or matrices would change the solution pH and concentration of cations and anions, which are prerequisite for aggregation of virus particles [175]. Furthermore, adjustment of pH or addition of cations and anions into the master mix is also a possible way to prevent the aggregation. The presence of amplification inhibitors is another possible reason for underestimation.

With quantification of extracted DNA, a discordance was noted between dPCR platforms, as significantly higher DNA recovery from human plasma compared with PBS buffer was visible only on the QX100 system (Figure 4.13). The absence of statistical differences noted on the Biomark system could be caused by a smaller number of measurements conducted on the Biomark system (two dilutions measured in duplicate) compared to the QX100 system (three dilutions measured in triplicate), and higher CV values related to the variability of those measurements (Table 4.9, Table 4.10).

Therefore, with Roche DNA extraction kit, which is exclusively designed for extraction of viral DNA from human body fluids, such as human plasma or whole blood, higher DNA recovery and repeatability observed for DNA extractions from human plasma is expected. Human plasma may contribute specific molecules that facilitate DNA recovery when combined with buffers from the DNA extraction kit, or even reduce the DNA damage. This could mean that the matrix does not influence only the carry-over rate of inhibitors but also alters the rate of DNA recovery, with the latter probably accounting for the majority of observed discrepancies between matrices, since they were noted on dPCR that is resilient to several inhibitors [74],

[89], [176]. Therefore, it is likely that the INSTAND material, which was composed of lysates of HCMV-infected MRC-5 cell cultures, resulted in lower DNA recoveries compared to the WHO material in human plasma, since with the INSTAND material mostly higher discrepancies were observed between the two quantification approaches. However, this was not the case with INSTAND sample N° 365029, where similar DNA copy numbers were measured with both approaches. With a statistically significant difference between the QX100 system and the Biomark system noted only with the direct quantification approach and not with the quantification of extracted DNA, it is likely that different combinations of matrix/master-mix effects have a different influence on direct quantification, resulting either in overestimation of the Biomark system or in the underestimation of the QX100 system. Moreover, using only the extracted DNA (of INSTAND sample N° 365029) with no original matrix present, between both dPCR platforms no statistically significant differences were found, which further strengthens the aforementioned assumption of the matrix influence on direct quantification. A 4-fold more diluted matrix might explain why no such matrix/master mix effects were seen with QX100-based direct quantification of INSTAND sample no. 365032 [138]. Therefore, it is reasonable to assume that the specific matrix present in INSTAND samples has a more negative influence on direct quantification of the QX100 system compared to the Biomark system.

The repeatability of direct quantification was lower or equal to that of quantification of extracted DNA (Table 4.9; Table 4.10). It is therefore likely that the presence of a viral coat and matrices contained in WHO or INSTAND material has a mostly lower effect on variability of quantification measurements compared to that of DNA-extraction replicates. This strongly indicates that direct quantification on dPCR platforms allows for more repeatable quantification of HCMV reference materials than the approach based on DNA extraction.

5.3.2 Direct quantification from human plasma

Although dPCR has been reportedly more resilient to inhibition than qPCR [74], [89], [176], and demonstrate good performance for direct quantification of HCMV reference materials (Chapter 5.3.1), different inhibitory effects were noted on the QX100-based direct quantification from human plasma (Figure 4.15). Higher concentrations of human plasma probably hampered the proper droplet formation, since the number of droplets accepted by the software was inversely proportional to the concentration of human plasma in the samples. Furthermore, in all accepted droplets a higher base fluorescence intensity was noted in negative droplets, which suggests the presence of human plasma increases the mean volume of droplets (Figure 4.15). Conversely, in positive droplets lower fluorescence intensity was observed. This finding coincides with lower estimated DNA concentrations from human plasma compared to the WHO material. Both observations suggest that human plasma not only hampers the droplet formation but also reduces the amplification efficiency and consequently leads to underestimation of viral concentration. This is partially in agreement with the previous study on extracted HCMV DNA quantified on the Biomark system, where the repeatable 10-fold decrease in DNA copy-number concentration was observed with samples containing 25% (v/v) human plasma [176]. However, higher decrease in DNA concentration observed in our study compared to the aforementioned one may be due to the presence of an anticoagulant, 50 mM Na2EDTA, which has already been demonstrated to inhibit dPCR-based amplification [89], [176].

It seems that direct quantification using a dPCR platform (the QX100 system or the Biomark system) is not suitable for a routine quantification of HCMV from human plasma due to partial or complete inhibition that leads to underestimation of DNA concentrations. However, a successful qPCR-based quantification of HCMV from urine samples and hepatitis B from human serum without DNA extraction indicates that dPCR-based direct quantification might be suitable for quantification of HCMV and other viruses from these two clinical matrices [173], [174].

5.4 Inter-Laboratory Assessment of Three Molecular Methods (Droplet-based dPCR and two microfluidic-based dPCRs) for Quantification of HCMV

Lack of standardisation between clinical laboratories in nucleic-acid-amplification based detection and quantification is a substantial problem. The establishment of a metrological framework is expected to facilitate the standardisation of DNA-based measurements in this field [58]. Accurate and robust reference measurement procedure is of paramount importance to underpin the metrological framework, however the most appropriate methodology to do that has not been elucidated yet. dPCR has so far demonstrated high resilience to inhibitors and different reaction components, high intra-experiment repeatability and intermediate precision. Although some reports have already assessed reproducibility of dPCR in inter-laboratory studies conducted on plasmid and bacterial DNA, to the best of our knowledge such metrological assessment performed on viral DNA is lacking. Therefore, in this study the inter-laboratory assessment was conducted on three different dPCR platforms to investigate the intermediate precision and reproducibility of dPCR-based platforms for quantification of HCMV materials.

5.4.1 Intra experiment variability and intermediate precision

As a part of the inter-laboratory study, for each dPCR platform the inter-experiment variability and the intermediate precision were assessed. The small intra-experiment variability of the QX100 system and the Biomark 37K array were noted (Chapter 4.4.1), which is concordant with other studies using HCMV DNA (Chapter 5.2.2) and bacterial DNA [148]. The higher CV values observed with the Biomark system, compared with the other two dPCR platforms, might be due to the more than 25-fold smaller number of analysed partitions and/or pipetting errors, related to smaller sample volumes [148]. Moreover, with all dPCR platforms in this study high intermediate precision was observed regardless of the material used (Figure 4.16). This observation is in agreement with previous studies from individual-laboratory results on genomic HCMV DNA (Chapter 5.2.2) and different bacterial-DNA template types [177]. Low CV values related to intermediate precision of dPCR additionally underpin indications that it might be a suitable candidate as a reference measurement procedure, since it allows very repeatable quantification of viral DNA within and between experiments.

5.4.2 Inter-unit variability

With both materials, different inter-unit variabilities have been observed between three tested units of WHO-1M (e.g. Figure 3.4a, Laboratory 1, units W1-W3) or gDNA2 (e.g. Figure 3.4b, Laboratory 2, units G4-G6) (Table 4.12). The small CV values related to inter-unit variability of centrally prepared gDNA2 confirm its high homogeneity and are owing to the fact that the DNA did not have to be additionally extracted in each laboratory. This low inter-unit variability of gDNA2 measurements is also in agreement with other reports where simple DNA templates, which do not require DNA extraction (genomic DNA or plasmid DNA) were used [177], [178]. Despite the occurrence of statistically significant differences between units tested on the QuantStudio 3D, low intra-experiment variability (CV for duplicates below 10%) and good intermediate precision (CV for three experiments below 1%) were demonstrated, which could explain statistical significance of less than 12% difference between units (Table 4.12). With WHO-1M in laboratories 1 and 2, most of the differences between WHO-1M units were constant as they were observed with both dPCR platforms. With a low filling-volume uncertainty and high stability of the WHO material claimed by the manufacturer, it could be assumed that inter-unit variability was not introduced during diluting of the WHO material but during the DNA extraction, since DNA was locally extracted from each individual WHO-1M unit. This is in agreement with previous studies where up to 50% difference was noted between DNA-extraction replicates quantified by the same qPCR assay [154], [179]. Moreover, in a metrological assessment of manual DNA extraction kits conducted in this work, similar differences up to 50% (0.17 log) were noted between extraction replicates when the same DNA extraction kit (Roche) was used as with this study. As it is not very likely that extraction

variability was caused by insufficient removal of qPCR inhibitors during DNA extraction, as previously discussed in Chapter 5.1.1, it is likely that the difference in measured DNA concentration between units was mostly caused by variable DNA recovery. With the QX100 system, differences between units had a higher statistical significance in comparison to the Biomark system (Table 4.12). This finding suggests the QX100 system allows for more precise discrimination between units. This might be owing to lower intra-experiment variability and higher intermediate precision of the QX100 system compared with the Biomark system, as previously discussed in Chapter 5.2.2.

5.4.3 MU of each dPCR platform

With gDNA2 material, lower MUs were observed compared to the more complex WHO-1M material (Table 4.13), which is concordant with a previous inter-laboratory study on bacterial DNA [177]. Since intermediate precision and inter-unit variability both account for final MU, it is reasonable to assume that higher MU of the WHO-1M material compared with the gDNA2 material is mostly due to the additional DNA extraction step that was omitted with the gDNA2 material. Additionally, in two other assessments using simple and well defined DNA templates, even lower expanded MUs (< 6%) were observed with other dPCR platforms [178], [180] than observed in this study with the gDNA2 material. Hence dPCR offers a very accurate and precise estimation of the DNA copy-number concentration (high intermediate precision and low intra-experiment variability). However, the final MU is dependent on the complexity of the DNA material, with DNA extraction adding additional uncertainty components, i.e. leading to higher MU.

5.4.4 Intra-laboratory concordance between dPCR platforms

A high intra-laboratory concordance between the QX100 system and the Biomark 37K array that was observed in this study (Table 4.13) is in agreement with the previous study [148]. Additionally, low discrepancies were observed between other dPCR platforms [85], [180]. Within laboratories 1 and 2, the tendency to difference between the QX100 system and the Biomark system was very consistent, as differences in DNA concentration between both platforms were similar regardless of the material used. Similar consistency between these two platforms has already been noted with three different types of bacterial DNA [177].

However, between laboratories 1 and 2 a disagreement was noted, as on the QX100 system higher (Laboratory 2) or lower (Laboratory 1) DNA concentrations were measured compared to the Biomark system. Similar inconsistent results have already been reported in the previous report on plasmid DNA [177], which suggests the discrepancies between platforms are not always systematic but random, and the reasons for such random discrepancies have not yet been completely understood. Since the same assay, materials and cycling conditions were used in all laboratories, over- and underestimation of DNA concentration, and discrepancies between laboratories could be due either to the use of different master mixes or to incorrectly assigned partition volumes [90], [148]. For both platforms, various lot numbers of particular master mixes were used in different laboratories, which might partially contribute to observed discrepancies between platforms. With the QX100 system and the Biomark 12.765 array, more than a 10% difference in partition volume has been reported from independent assessment conducted in several laboratories [85], [87], [169], [180]. Furthermore, with the Biomark 12.765 array around 7% difference in chamber volume was found between two arrays measured in the same laboratory [181]. Therefore, it is reasonable to assume that discrepancy between Biomark 37K array and the QX100 system observed in this study might be owing to variable partition volumes of different Biomark 37K array lots and/or difference between droplet volumes generated and analysed at the QX100 systems from different laboratories.

5.4.5 Inter-laboratory variability (reproducibility) and estimation of the mean DNA concentration with correspondent MU

With gDNA2, a good agreement between laboratories was observed (Table 4.13). The reasons for minor discrepancies between platforms and laboratories are still not well understood, however they are probably primarily caused by each individual dPCR platform due

to over- or underestimation of DNA concentration (Chapter 5.2.2), and are not directly influenced by factors related to different laboratories. In contrast to the gDNA2 material, higher variability was observed with the WHO-1M material, which required local DNA extraction before DNA quantification. As already discussed in Chapters 5.1.2 and 5.4.4, and in some other reports, DNA extraction could account for variability owing to differences in DNA recoveries between extraction columns of the same manual extraction kit used by one operator within one laboratory [154], [179]. To the best of our knowledge, no inter-laboratory assessment of the same DNA extraction method for quantification of viral DNA has been performed. However, it could be speculated that different operators from different laboratories could contribute to the variability. Another source of variability might be differences in composition between the suggested in-house PBS (laboratory 1) and purchased commercial PBS (laboratories 2, 3 and 4), since the matrix was shown to have an impact on DNA recovery and variability of DNA extraction (Chapter 5.3.1). Therefore, it could be assumed that in this study local DNA extraction of WHO-1M material contributed to the higher discordance between laboratories and platforms than observed with gDNA2.

For both materials, very low expanded MUs for corresponding mean DNA concentrations were observed (Table 4.13). Since this is in agreement with other inter-laboratory assessments that used bacteriophage DNA, plasmid DNA and different types of bacterial DNA [91], [177], [178], it can be concluded that dPCR offers high reproducibility for the quantification of DNA of different complexity (plasmid DNA, genomic DNA, whole bacteria and viruses) and from different sources (viruses, bacteria, bacteriophages).

5.4.6 Suitability of dPCR as a reference measurement procedure and for characterisation of CRMs

To assess the suitability of dPCR as reference measurement procedures and for characterisation of suitable CRMs, the performance of dPCR should be compared to that of qPCR method, which is currently used for characterisation of virus reference materials [132]. The use of qPCR in several inter-laboratory studies for quantification of HCMV resulted in more than a 100-fold difference between laboratories in terms of DNA concentration [110], [132]. The main reason for such variability is most probably the disparity in quantification procedures between participating laboratories, as different assays, DNA extraction methods and variable calibration procedures can dramatically reduce the agreement between laboratories in terms of estimated DNA concentration. In contrast to qPCR, several dPCR platforms have already been shown to be resilient to inhibitors and resistant to the influence of different PCR components, hence allowing for a more accurate and robust quantification of DNA concentration than qPCR [74], [148]. The variability caused by DNA extraction method in particular should receive special attention when considering dPCR as a candidate for a reference measurement procedure and for characterisation of reference materials. The accuracy and robustness of dPCR-based DNA quantification could be further improved by the preliminary selection of the DNA extraction method with the highest DNA recovery and precision, while extraction replicates would probably reduce the influence of inter-column variability (Chapters 5.1.1 and 5.1.2). Furthermore, dPCR-based direct quantification could bypass most mentioned problems including inter-laboratory variability caused by different operators of the extraction and clean up procedures, as it does not require DNA extraction and has demonstrated accurate and repeatable quantification of DNA derived from whole-virus reference materials (Chapter **Error! Reference source not found.**).

Chapter 6

Conclusions

In this work, various methods for DNA extraction and quantification (qPCR, droplet-based dPCR and microfluidic-based dPCR) of HCMV have been comprehensively assessed to broaden our understanding of processes involved in quantification of HCMV and enlarge our knowledge of their performance characteristics. The main goal was to delineate those extraction and quantification methods that exhibit more potential either for routine quantification of HCMV in clinical laboratories or for implementation in measurement framework of nucleic acid amplification-based tests, whose establishment is expected to improve the preparation of certified reference materials and facilitate the routine quantification of HCMV.

The metrological assessment of methods for manual extraction of HCMV DNA has demonstrated that the use of various DNA extraction kits may result in variable DNA recoveries, which probably contribute to disparity in reported concentration thresholds and therefore prevent the establishment of a generally accepted threshold for preemptive treatment of HCMV. Furthermore, the intra- and inter-experiment variabilities observed with some DNA extraction kits could be further exacerbated with variability derived from DNA quantification, which may in turn lead to incorrect conclusions on virus kinetics and subsequent improper clinical decisions on administration of antivirotics. This could be improved by introduction of extraction and/or quantification replicates, while more metrological assessments may facilitate the selection of the most precise and comparable methods for DNA extraction, which could eventually enhance HCMV diagnostics.

The metrological evaluation of qPCR, droplet-based dPCR and microfluidic-based dPCR broadened our knowledge on their analytical characteristics. Owing to good sensitivity and precision with low DNA concentrations, qPCR still seems to be the appropriate method for a routine quantification of HCMV. However, the advances in dPCR technology would probably facilitate its implementation in routine diagnostics. In addition, thanks to high resilience to inhibition and better repeatability with high DNA concentrations compared with qPCR, dPCR appears to be a more appropriate candidate for the reference measurement procedure and for characterization of reference materials than qPCR.

The evaluation of two dPCR platforms for direct quantification of whole-virus materials for HCMV has indicated better performance of dPCR-based direct quantification compared to the dPCR-based quantification of extracted DNA. This finding suggests that direct quantification may leverage the resilience of dPCR to inhibitors present in whole-virus reference materials and might thus facilitate more precise value assignment of whole-virus reference materials than currently possible using DNA extraction followed by dPCR- or qPCR quantification. However, dPCR-based direct quantification does not look suitable for a routine diagnostic of HCMV from human plasma.

Inter-laboratory assessment of several dPCR platforms indicated dPCR offers a reproducible and robust quantification of HCMV materials of different complexity. This finding additionally underpins the suitability of dPCR as a candidate for a reference measurement procedure and for value assignment of virus reference materials. However, owing to variability caused by DNA extraction and some minor discrepancies between dPCR platforms, the proper validation of DNA extraction and dPCR-based quantification should be considered as a prerequisite for implementation of dPCR in a metrological framework.

Appendix A

Supplementary Material

A.1 Supplementary material for metrological evaluation of three molecular methods (qPCR, Droplet-based dPCR and Microfluidic-based dPCR) for quantification of HCMV

Table S1: Detailed information on qPCR measurements performed on Viia 7 as a part of assessment of linearity, LOD, LOQ and precision of qPCR.

Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Measured Cq value	Measured DNA copies per total reaction volumne	Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Measured Cq value	Measured DNA copies per total reaction volumne
3.8×	5490 ±125	1	25.948	5577	940×	22 ±0.5	1	33.952	23
3.8×	5490 ±125	1	25.999	5385	940×	22 ±0.5	1	33.814	25
3.8×	5490 ±125	1	25.934	5631	940×	22 ±0.5	1	34.192	20
3.8×	5490 ±125	1	26.113	4980	940×	22 ±0.5	1	33.700	27
3.8×	5490 ±125	1	26.075	5112	940×	22 ±0.5	1	34.118	21
3.8×	5490 ±125	2	26.932	4297	940×	22 ±0.5	2	34.903	25
3.8×	5490 ±125	2	26.977	4071	940×	22 ±0.5	2	34.967	16
3.8×	5490 ±125	2	26.983	4057	940×	22 ±0.5	2	35.983	21
3.8×	5490 ±125	2	27.005	4090	940×	22 ±0.5	2	35.325	15
3.8×	5490 ±125	2	26.797	4087	940×	22 ±0.5	2	34.928	26
7.5×	2740 ±60	1	26.93	2840	1880	11 ±0.25	1	34.90	12
7.5×	2740 ±60	1	26.98	2753	1880	11 ±0.25	1	34.97	11
7.5×	2740 ±60	1	26.98	2742	1880	11 ±0.25	1	35.98	6
7.5×	2740 ±60	1	27.00	2701	1880	11 ±0.25	1	35.33	9
7.5×	2740 ±60	1	26.80	3115	1880	11 ±0.25	1	34.93	12
7.5×	2740 ±60	2	26.941	2822	1880	11 ±0.25	2	34.913	12
7.5×	2740 ±60	2	27.021	2671	1880	11 ±0.25	2	35.168	10
7.5×	2740 ±60	2	27.152	2442	1880	11 ±0.25	2	34.817	13
7.5×	2740 ±60	2	27.072	2580	1880	11 ±0.25	2	36.194	5
7.5×	2740 ±60	2	26.992	2725	1880	11 ±0.25	2	34.741	13
18.8×	1095 ±25	1	28.30	1114	3760	5 ±0.1	1	40.82	0
18.8×	1095 ±25	1	28.37	1060	3760	5 ±0.1	1	38.03	1
18.8×	1095 ±25	1	28.28	1127	3760	5 ±0.1	1	38.23	1
18.8×	1095 ±25	1	28.35	1071	3760	5 ±0.1	1	39.41	1
18.8×	1095 ±25	1	28.34	1077	3760	5 ±0.1	1	38.74	1
18.8×	1095 ±25	2	28.203	1187	3760	5 ±0.1	2	36.715	3
18.8×	1095 ±25	2	28.231	1165	3760	5 ±0.1	2	38.949	1
18.8×	1095 ±25	2	28.337	1083	3760	5 ±0.1	2	35.914	6
18.8×	1095 ±25	2	28.296	1114	3760	5 ±0.1	2	35.902	6
18.8×	1095 ±25	2	28.240	1158	3760	5 ±0.1	2	36.489	4
75.2×	275 ±6	1	30.21	300	9400	2 ±0.05	1	38.37	1
75.2×	275 ±6	1	30.00	346	9400	2 ±0.05	1	38.38	1
75.2×	275 ±6	1	30.08	328	9400	2 ±0.05	1	42.50	0
75.2×	275 ±6	1	30.34	275	9400	2 ±0.05	1	38.51	1
75.2×	275 ±6	1	30.20	303	9400	2 ±0.05	1	Undeter	Undetermined
75.2×	275 ±6	2	30.323	277	9400	2 ±0.05	2	39.318	1
75.2×	275 ±6	2	30.178	306	9400	2 ±0.05	2	39.335	1
75.2×	275 ±6	2	30.199	302	9400	2 ±0.05	2	Undeter	Undetermined
75.2×	275 ±6	2	30.115	320	9400	2 ±0.05	2	40.318	0
75.2×	275 ±6	2	30.236	320	9400	2 ±0.05	2	41.783	0
188×	110 ±2.5	1	31.90	294	1880	1 ±0.02	1	Undeter	0
188×	110 ±2.5	1	31.71	94	1880	1 ±0.02	1	Undeter	Undetermined
188×	110 ±2.5	1	31.99	107	1880	1 ±0.02	1	Undeter	Undetermined
188×	110 ±2.5	1	31.78	88	1880	1 ±0.02	1	39.47	Undetermined
188×	110 ±2.5	1	31.72	102	1880	1 ±0.02	1	39.69	1
188×	110 ±2.5	2	31.104	107	1880	1 ±0.02	2	Undeter	0
188×	110 ±2.5	2	31.607	162	1880	1 ±0.02	2	42.520	Undetermined
188×	110 ±2.5	2	31.257	115	1880	1 ±0.02	2	40.296	0
188×	110 ±2.5	2	31.759	146	1880	1 ±0.02	2	39.352	0
188×	110 ±2.5	2	31.663	104	1880	1 ±0.02	2	43.174	1
376×	55 ±1	1	32.49	111	1880	0.1 ±0.002	1	Undeter	Undetermined
376×	55 ±1	1	32.72	63	1880	0.1 ±0.002	1	Undeter	Undetermined
376×	55 ±1	1	32.23	53	1880	0.1 ±0.002	1	Undeter	Undetermined
376×	55 ±1	1	32.06	75	1880	0.1 ±0.002	1	Undeter	Undetermined
376×	55 ±1	1	32.29	84	1880	0.1 ±0.002	1	Undeter	Undetermined
376×	55 ±1	2	32.835	72	1880	0.1 ±0.002	2	Undeter	Undetermined
376×	55 ±1	2	32.258	50	1880	0.1 ±0.002	2	Undeter	Undetermined
376×	55 ±1	2	32.932	74	1880	0.1 ±0.002	2	Undeter	Undetermined
376×	55 ±1	2	32.314	46	1880	0.1 ±0.002	2	Undeter	Undetermined
376×	55 ±1	2	32.463	71	1880	0.1 ±0.002	2	Undeter	Undetermined

Table S2: Detailed information on dPCR measurements performed on the QX100 system as a part of assessment of linearity, LOD, LOQ and precision of qPCR.

Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Number of positive droplets	Number of analysed droplets	Measured DNA copies per total reaction volume	Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Number of positive droplets	Number of analysed droplets	Measured DNA copies per total reaction volume
3.8×	5490 ±125	1	3218	14306	6111	940×	22 ±0.5	1	13	13222	24
3.8×	5490 ±125	1	3220	14488	6028	940×	22 ±0.5	1	10	12944	19
3.8×	5490 ±125	1	2894	12899	6093	940×	22 ±0.5	1	5	13459	9
3.8×	5490 ±125	1	2990	13491	6008	940×	22 ±0.5	1	7	14817	11
3.8×	5490 ±125	1	3131	14081	6031	940×	22 ±0.5	1	15	15491	23
3.8×	5490 ±125	2	2932	13966	5651	940×	22 ±0.5	2	9	13850	16
3.8×	5490 ±125	2	3139	14788	5722	940×	22 ±0.5	2	12	14325	20
3.8×	5490 ±125	2	2682	13105	5491	940×	22 ±0.5	2	18	13788	31
3.8×	5490 ±125	2	2992	15079	5304	940×	22 ±0.5	2	13	13911	22
3.8×	5490 ±125	2	2714	13155	5541	940×	22 ±0.5	2	10	13268	18
7.5×	2740 ±60	1	1728	14221	3107	1880×	11 ±0.25	1	4	12608	8
7.5×	2740 ±60	1	1584	14376	2800	1880×	11 ±0.25	1	9	13921	16
7.5×	2740 ±60	1	1540	12788	3077	1880×	11 ±0.25	1	8	13344	14
7.5×	2740 ±60	1	1762	15321	2930	1880×	11 ±0.25	1	4	15319	6
7.5×	2740 ±60	1	1736	15133	2922	1880×	11 ±0.25	1	5	14949	8
7.5×	2740 ±60	2	1729	14662	3009	1880×	11 ±0.25	2	2	12554	4
7.5×	2740 ±60	2	1486	13264	2849	1880×	11 ±0.25	2	8	13387	14
7.5×	2740 ±60	2	1929	16089	3063	1880×	11 ±0.25	2	8	12437	15
7.5×	2740 ±60	2	1896	14865	3272	1880×	11 ±0.25	2	3	12356	6
7.5×	2740 ±60	2	1658	13897	3047	1880×	11 ±0.25	2	4	14665	7
18.8×	1095 ±25	1	706	15134	1146	3760×	5 ±0.1	1	4	12959	7
18.8×	1095 ±25	1	663	14290	1139	3760×	5 ±0.1	1	3	13984	5
18.8×	1095 ±25	1	617	13737	1102	3760×	5 ±0.1	1	4	13284	7
18.8×	1095 ±25	1	612	13307	1129	3760×	5 ±0.1	1	4	13336	7
18.8×	1095 ±25	1	684	14245	1180	3760×	5 ±0.1	1	5	12138	10
18.8×	1095 ±25	2	694	14300	1193	3760×	5 ±0.1	2	0	13623	0
18.8×	1095 ±25	2	658	12903	1255	3760×	5 ±0.1	2	2	14153	3
18.8×	1095 ±25	2	656	13332	1210	3760×	5 ±0.1	2	3	12423	6
18.8×	1095 ±25	2	676	12718	1310	3760×	5 ±0.1	2	5	12609	10
18.8×	1095 ±25	2	556	8133	1698	3760×	5 ±0.1	2	2	12533	0
75.2×	275 ±6	1	168	13806	294	9400×	2 ±0.05	1	0	14023	4
75.2×	275 ±6	1	132	12821	248	9400×	2 ±0.05	1	2	12995	2
75.2×	275 ±6	1	135	13307	245	9400×	2 ±0.05	1	1	12095	2
75.2×	275 ±6	1	139	13073	256	9400×	2 ±0.05	1	1	13150	0
75.2×	275 ±6	1	174	15601	269	9400×	2 ±0.05	1	0	14369	2
75.2×	275 ±6	2	193	15601	312	9400×	2 ±0.05	2	1	13608	2
75.2×	275 ±6	2	164	14910	281	9400×	2 ±0.05	2	1	14458	2
75.2×	275 ±6	2	163	14067	304	9400×	2 ±0.05	2	1	12986	4
75.2×	275 ±6	2	131	12931	249	9400×	2 ±0.05	2	2	13068	2
75.2×	275 ±6	2	156	13014	286	9400×	2 ±0.05	2	1	14157	0
188×	110 ±2.5	1	81	14939	130	18800×	1 ±0.02	1	0	13801	0
188×	110 ±2.5	1	51	12381	99	18800×	1 ±0.02	1	1	13424	2
188×	110 ±2.5	1	63	12294	123	18800×	1 ±0.02	1	0	13027	0
188×	110 ±2.5	1	56	13605	99	18800×	1 ±0.02	1	0	13202	0
188×	110 ±2.5	1	73	11423	154	18800×	1 ±0.02	1	0	12112	0
188×	110 ±2.5	2	69	15521	107	18800×	1 ±0.02	2	0	13562	0
188×	110 ±2.5	2	68	13153	124	18800×	1 ±0.02	2	0	13265	0
188×	110 ±2.5	2	52	12423	101	18800×	1 ±0.02	2	0	14049	0
188×	110 ±2.5	2	63	11685	130	18800×	1 ±0.02	2	0	14050	0
188×	110 ±2.5	2	73	13610	129	18800×	1 ±0.02	2	1	13608	0
376×	55 ±1	1	22	14231	37	188000	0.1 ±0.002	1	0	14828	0
376×	55 ±1	1	20	11324	42	188000	0.1 ±0.002	1	0	14814	0
376×	55 ±1	1	28	13904	48	188000	0.1 ±0.002	1	0	14823	0
376×	55 ±1	1	23	14658	38	188000	0.1 ±0.002	1	0	14901	0
376×	55 ±1	1	29	14498	48	188000	0.1 ±0.002	1	0	14884	2
376×	55 ±1	2	32	14698	52	188000	0.1 ±0.002	2	0	13873	0
376×	55 ±1	2	34	14320	57	188000	0.1 ±0.002	2	1	13268	0
376×	55 ±1	2	31	14897	50	188000	0.1 ±0.002	2	0	12759	0
376×	55 ±1	2	20	13739	35	188000	0.1 ±0.002	2	0	13614	0
376×	55 ±1	2	44	13873	76	188000	0.1 ±0.002	2	0	12273	0

Table S3: Detailed information on dPCR measurements performed on the Biomark 37K array as a part of assessment of linearity, LOD, LOQ and precision of qPCR.

Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Number of positive chambers	Number of analysed chambers	Measured DNA copies per total reaction volume	Dilution of gDNA1	Nominal DNA copies per total reaction volume	Experiment	Number of positive chambers	Number of analysed chambers	Measured DNA copies per total reaction volume
1×	6190 ±140	1	616	770	7663	47.7×	132 ±3	1	33	770	209
1×	6190 ±140	1	628	770	8050	47.7×	132 ±3	1	15	770	94
1×	6190 ±140	1	605	770	7335	47.7×	132 ±3	1	25	770	157
1×	6190 ±140	1	614	770	7602	47.7×	132 ±3	1	26	770	164
1×	6190 ±140	1	614	770	7602	47.7×	132 ±3	1	33	770	209
1×	6190 ±140	2	617	770	7694	47.7×	132 ±3	2	34	770	215
1×	6190 ±140	2	591	770	6947	47.7×	132 ±3	2	19	770	119
1×	6190 ±140	2	611	770	7511	47.7×	132 ±3	2	28	770	176
1×	6190 ±140	2	603	770	7278	47.7×	132 ±3	2	29	770	183
1×	6190 ±140	2	614	770	7602	47.7×	132 ±3	2	18	770	113
1.9×	3300 ±75	1	438	770	4006	91×	66 ±1.5	1	6	770	37
1.9×	3300 ±75	1	445	770	4107	91×	66 ±1.5	1	18	770	113
1.9×	3300 ±75	1	431	770	3906	91×	66 ±1.5	1	10	770	62
1.9×	3300 ±75	1	430	770	3892	91×	66 ±1.5	1	20	770	125
1.9×	3300 ±75	1	446	770	4122	91×	66 ±1.5	1	13	770	81
1.9×	3300 ±75	2	421	770	3768	91×	66 ±1.5	2	13	770	81
1.9×	3300 ±75	2	429	770	3878	91×	66 ±1.5	2	14	770	87
1.9×	3300 ±75	2	437	770	3991	91×	66 ±1.5	2	16	770	100
1.9×	3300 ±75	2	445	770	4107	91×	66 ±1.5	2	9	770	56
1.9×	3300 ±75	2	408	770	3594	91×	66 ±1.5	2	14	770	87
3.7×	1655 ±40	1	298	770	2330	200×	33 ±0.8	1	5	770	31
3.7×	1655 ±40	1	291	770	2260	200×	33 ±0.8	1	7	770	43
3.7×	1655 ±40	1	265	770	2009	200×	33 ±0.8	1	11	770	69
3.7×	1655 ±40	1	257	770	1934	200×	33 ±0.8	1	8	770	50
3.7×	1655 ±40	1	275	770	2104	200×	33 ±0.8	1	8	770	50
3.7×	1655 ±40	2	257	770	1934	200×	33 ±0.8	2	6	770	37
3.7×	1655 ±40	2	250	770	1869	200×	33 ±0.8	2	4	770	25
3.7×	1655 ±40	2	268	770	2037	200×	33 ±0.8	2	13	770	81
3.7×	1655 ±40	2	266	770	2018	200×	33 ±0.8	2	7	770	43
3.7×	1655 ±40	2	257	770	1934	200×	33 ±0.8	2	8	770	50
9.4×	660 ±15	1	108	770	720	1000×	6.6 ±0.1	1	0	770	0
9.4×	660 ±15	1	142	770	971	1000×	6.6 ±0.1	1	1	770	6
9.4×	660 ±15	1	104	770	691	1000×	6.6 ±0.1	1	1	770	6
9.4×	660 ±15	1	116	770	777	1000×	6.6 ±0.1	1	0	770	0
9.4×	660 ±15	1	132	770	895	1000×	6.6 ±0.1	1	1	770	6
9.4×	660 ±15	2	126	770	851	1000×	6.6 ±0.1	2	1	770	6
9.4×	660 ±15	2	114	770	763	1000×	6.6 ±0.1	2	1	770	6
9.4×	660 ±15	2	105	770	698	1000×	6.6 ±0.1	2	2	770	12
9.4×	660 ±15	2	120	770	807	1000×	6.6 ±0.1	2	2	770	12
9.4×	660 ±15	2	107	770	712	1000×	6.6 ±0.1	2	0	770	0
18.5×	330 ±8	1	59	770	380						
18.5×	330 ±8	1	71	770	461						
18.5×	330 ±8	1	71	770	461						
18.5×	330 ±8	1	49	770	313						
18.5×	330 ±8	1	62	770	400						
18.5×	330 ±8	2	67	770	433						
18.5×	330 ±8	2	66	770	427						
18.5×	330 ±8	2	78	770	509						
18.5×	330 ±8	2	62	770	400						
18.5×	330 ±8	2	62	770	400						

A.2 Supplementary material for inter-laboratory assessment of three molecular methods (droplet-based dPCR and two microfluidic-based dPCRs) for quantification of HCMV

Table S4: Detailed information for the Biomark 37K array from Laboratory 1.

Material	Experiment number[a]	Unit	Gravimetric dilution	Number of positive chambers	Number of chambers analysed	Mean partition occupancy	Copy number (cp/µL)
WHO-1M	1	W1	1×	475	770	0.96	3262
WHO-1M	1	W1	1×	515	770	1.11	3758
WHO-1M	1	W2	1×	512	770	1.09	3718
WHO-1M	1	W2	1×	517	770	1.11	3785
WHO-1M	1	W3	1×	573	770	1.36	4635
WHO-1M	1	W3	1×	551	770	1.26	4275
WHO-1M	2	W1	2×[b]	375	770	0.67	4539
WHO-1M	2	W1	2×[b]	333	770	0.57	3852
WHO-1M	2	W2	2×[b]	336	770	0.57	3899
WHO-1M	2	W2	2×[b]	361	770	0.63	4303
WHO-1M	2	W3	1.97×[b]	410	770	0.76	5115
WHO-1M	2	W3	1.97×[b]	350	770	0.61	4078
gDNA2	1	G1	1×	175	770	0.26	877
gDNA2	1	G1	1×	191	770	0.29	969
gDNA2	1	G2	1×	233	770	0.36	1225
gDNA2	1	G2	1×	212	770	0.32	1095
gDNA2	1	G3	1×	203	770	0.31	1041
gDNA2	1	G3	1×	207	770	0.31	1065
gDNA2	2	G1	1×	209	770	0.32	1077
gDNA2	2	G1	1×	200	770	0.30	1023
gDNA2	2	G2	1×	217	770	0.33	1126
gDNA2	2	G2	1×	210	770	0.32	1083
gDNA2	2	G3	/[c]	/[c]	/[c]	/[c]	/[c]
gDNA2	2	G3	/[c]	/[c]	/[c]	/[c]	/[c]

[a] Only two experiments were performed due to technical problems with experiment 3.

[b] Dilutions were performed due to initial lost of certain aliquot volumes.

[c] Due to an error in preparation of a specific aliquot, these data were not valid.

Table S5: Detailed information for the QX100 system from Laboratory 1.

Material	Experiment number	Unit	Gravimetric dilution	Number of positive cdroplets	Number of droplets analysed	Mean partition occupancy	Copy number (cp/µL)
WHO-1M	1	W1	11.00×	1674	16402	0.11	3550
WHO-1M	1	W1	11.00×	1546	15835	0.10	3387
WHO-1M	1	W2	10.88×	1677	14377	0.12	4047
WHO-1M	1	W2	10.88×	1825	15319	0.13	4139
WHO-1M	1	W3	9.94×	1680	13642	0.13	3919
WHO-1M	1	W3	9.94×	1766	13843	0.14	4070
WHO-1M	2	W1	9.77×	1782	15985	0.12	3463
WHO-1M	2	W1	9.77×	1735	15380	0.12	3507
WHO-1M	2	W2	10.02×	1611	13803	0.12	3730
WHO-1M	2	W2	10.02×	1637	13963	0.12	3748
WHO-1M	2	W3	9.56×	1998	15410	0.14	3980
WHO-1M	2	W3	9.56×	1754	14153	0.13	3792
WHO-1M	3	W1	10.07×	1694	15403	0.12	3519
WHO-1M	3	W1	10.07×	1629	15173	0.11	3431
WHO-1M	3	W2	11.08×	1583	14859	0.11	3743
WHO-1M	3	W2	11.08×	1572	14498	0.11	3814
WHO-1M	3	W3	10.54×	1926	15895	0.13	4081
WHO-1M	3	W3	10.54×	1812	14793	0.13	4129
gDNA2	1	G1	7.80×	650	15029	0.04	1035
gDNA2	1	G1	7.80×	552	14317	0.04	920
gDNA2	1	G2	8.40×	417	14333	0.03	743
gDNA2	1	G2	8.40×	354	13749	0.03	657
gDNA2	1	G3	9.80×	444	13872	0.03	956
gDNA2	1	G3	9.80×	483	14115	0.03	1023
gDNA2	2	G1	9.77×	464	13529	0.03	1023
gDNA2	2	G1	9.77×	438	15027	0.03	867
gDNA2	2	G2	9.77×	496	14524	0.03	1018
gDNA2	2	G2	9.77×	527	14929	0.04	1053
gDNA2	2	G3	9.92×	451	13521	0.03	1009
gDNA2	2	G3	9.92×	557	16773	0.03	1005
gDNA2	3	G1	10.02×	467	14811	0.03	963
gDNA2	3	G1	10.02×	393	13523	0.03	886
gDNA2	3	G2	10.83×	385	14308	0.03	886
gDNA2	3	G2	10.83×	455	14299	0.03	1050
gDNA2	3	G3	10.28×	473	15346	0.03	966
gDNA2	3	G3	10.28×	476	15862	0.03	940

Table S6: Detailed information for the Biomark 37K array from Laboratory 2.

Material	Experiment number	Unit	Gravimetric dilution	Number of positive chambers	Number of chambers analysed	Mean partition occupancy	Copy number (cp/µL)
WHO-1M	1	W4	1×	433	770	0.83	2810
WHO-1M	1	W4	1×	425	770	0.80	2730
WHO-1M	1	W5	1×	392	770	0.71	2419
WHO-1M	1	W5	1×	396	770	0.72	2455
WHO-1M	1	W6	1×	224	770	0.34	1169
WHO-1M	1	W6	1×	310	770	0.52	1752
WHO-1M	2	W4	1×	449	770	0.87	2975
WHO-1M	2	W4	1×	486	770	1.00	3392
WHO-1M	2	W5	1×	392	770	0.71	2419
WHO-1M	2	W5	1×	253	770	0.40	1355
WHO-1M	2	W6	1×	338	770	0.58	1965
WHO-1M	2	W6	1×	348	770	0.60	2045
WHO-1M	3	W4	1×	485	770	0.99	3380
WHO-1M	3	W4	1×	460	770	0.91	3094
WHO-1M	3	W5	1×	497	770	1.04	3526
WHO-1M	3	W5	1×	492	770	1.02	3464
WHO-1M	3	W6	1×	411	770	0.76	2595
WHO-1M	3	W6	1×	380	770	0.68	2313
gDNA2	1	G4	1×	151	770	0.22	742
gDNA2	1	G4	1×	155	770	0.22	764
gDNA2	1	G5	1×	151	770	0.22	742
gDNA2	1	G5	1×	168	770	0.25	837
gDNA2	1	G6	1×	231	770	0.36	1213
gDNA2	1	G6	1×	217	770	0.33	1126
gDNA2	2	G4	1×	208	770	0.31	1071
gDNA2	2	G4	1×	201	770	0.30	1029
gDNA2	2	G5	1×	205	770	0.31	1053
gDNA2	2	G5	1×	185	770	0.27	934
gDNA2	2	G6	1×	191	770	0.29	969
gDNA2	2	G6	1×	127	770	0.18	613
gDNA2	3	G4	1×	199	770	0.30	1017
gDNA2	3	G4	1×	200	770	0.30	1023
gDNA2	3	G5	1×	213	770	0.32	1101
gDNA2	3	G5	1×	182	770	0.27	917
gDNA2	3	G6	1×	200	770	0.30	1023
gDNA2	3	G6	1×	194	770	0.29	987

Table S7: Detailed information for the QX100 system from Laboratory 2.

Material	Experiment number	Unit	Gravimetric dilution	Number of positive dropltes	Number of droplets nalysed	Mean partition occupancy	Copy number (cp/µL)
WHO-1M	1	W4	10.19×	1655	17462	0.10	3042
WHO-1M	1	W4	10.19×	1620	12693	0.14	4171
WHO-1M	1	W5	10.50×	1231	15788	0.08	2555
WHO-1M	1	W5	10.50×	1093	15103	0.08	2364
WHO-1M	1	W6	10.41×	1183	15947	0.08	2405
WHO-1M	1	W6	10.41×	1083	14517	0.08	2419
WHO-1M	2	W4	10.08×	1683	15881	0.11	3384
WHO-1M	2	W4	10.08×	1665	15556	0.11	3420
WHO-1M	2	W5	10.56×	1350	15518	0.09	2881
WHO-1M	2	W5	10.56×	1253	11580	0.11	3625
WHO-1M	2	W6	10.61×	1217	13526	0.09	2998
WHO-1M	2	W6	10.61×	1162	14504	0.08	2655
WHO-1M	3	W4	12.12×	1323	14699	0.09	3427
WHO-1M	3	W4	12.12×	1394	15162	0.10	3504
WHO-1M	3	W5	10.61×	1138	15355	0.08	2449
WHO-1M	3	W5	10.61×	1082	14802	0.08	2414
WHO-1M	3	W6	10.35×	1345	14312	0.10	3062
WHO-1M	3	W6	10.35×	1336	14947	0.09	2905
gDNA2	1	G4	10.08×	529	16269	0.03	999
gDNA2	1	G4	10.08×	486	13122	0.04	1140
gDNA2	1	G5	9.61×	602	16308	0.04	1084
gDNA2	1	G5	9.61×	516	13049	0.04	1162
gDNA2	1	G6	10.45×	589	16086	0.04	1169
gDNA2	1	G6	10.45×	502	15788	0.03	1012
gDNA2	2	G4	10.21×	470	12720	0.04	1152
gDNA2	2	G4	10.21×	553	14948	0.04	1153
gDNA2	2	G5	10.64×	480	11941	0.04	1308
gDNA2	2	G5	10.64×	591	16251	0.04	1181
gDNA2	2	G6	10.42×	566	15695	0.04	1147
gDNA2	2	G6	10.42×	495	15690	0.03	1001
gDNA2	3	G4	10.74×	588	16411	0.04	1175
gDNA2	3	G4	10.74×	569	15948	0.04	1170
gDNA2	3	G5	10.07×	561	15480	0.04	1114
gDNA2	3	G5	10.07×	566	15325	0.04	1136
gDNA2	3	G6	9.86×	620	16185	0.04	1154
gDNA2	3	G6	9.86×	583	16469	0.04	1065

Table S8: Detailed information for the Biomark 37K array from Laboratory 3.

Material	Experiment number	Unit	Gravimetric dilution	Number of positive chambers	Number of chambers analysed	Mean partition occupancy	Copy number (cp/µL)
WHO-1M	1	W7	1×	540	770	1.21	4090
WHO-1M	1	W7	1×	440	770	0.85	2868
WHO-1M	1	W8	1×	485	770	0.99	3364
WHO-1M	1	W8	1×	486	770	1.00	3376
WHO-1M	1	W9	1×	483	770	0.99	3340
WHO-1M	1	W9	1×	447	770	0.87	2940
WHO-1M	2	W7	1×	503	770	1.06	3585
WHO-1M	2	W7	1×	480	770	0.98	3305
WHO-1M	2	W8	1×	422	770	0.79	2688
WHO-1M	2	W8	1×	438	770	0.84	2847
WHO-1M	2	W9	1×	450	770	0.88	2972
WHO-1M	2	W9	1×	447	770	0.87	2940
WHO-1M	3	W7	1×	508	770	1.08	3649
WHO-1M	3	W7	1×	512	770	1.09	3701
WHO-1M	3	W8	1×	464	770	0.92	3123
WHO-1M	3	W8	1×	486	770	1.00	3376
WHO-1M	3	W9	1×	494	770	1.03	3473
WHO-1M	3	W9	1×	490	770	1.01	3424

Table S9: Detailed information for the QuantStudio 3D system from Laboratory 4.

Material	Experiment number[a]	Unit	Gravimetric dilution	Number of positive chambers	Number of accepted chambers[a]	Total number of accepted chambers	Meean partition occupancy	Copy number (cp/µL)
gDNA2	1	G7	2.32×	5371	18353	18565	0.35	991
gDNA2	1	G7	2.25×	5424	17809	17988	0.36	1012
gDNA2	1	G8	2.28×	5167	18701	18823	0.32	911
gDNA2	1	G8	2.24×	5386	18532	18634	0.34	949
gDNA2	1	G9	2.24×	4905	18314	18923	0.31	864
gDNA2	1	G9	2.25×	5017	18166	18225	0.32	900
gDNA2	2	G7	2.27×	5465	18722	18770	0.35	967
gDNA2	2	G7	2.26×	5268	18319	18422	0.34	946
gDNA2	2	G8	2.29×	4998	17994	18702	0.33	919
gDNA2	2	G8	2.24×	5318	17915	17975	0.35	976
gDNA2	2	G9	2.25×	5130	17937	17983	0.34	936
gDNA2	2	G9	2.27×	5098	18961	19382	0.31	878
gDNA2	3	G7	2.27×	5609	18422	18558	0.36	1021
gDNA2	3	G7	2.26×	5524	18610	18678	0.35	984
gDNA2	3	G8	2.28×	5219	18700	18867	0.33	924
gDNA2	3	G8	2.28×	5698	18278	18410	0.37	1051
gDNA2	3	G9	2.28×	4873	18914	19579	0.30	838
gDNA2	3	G9	2.29×	5190	19105	19229	0.32	896

[a] after application of 'colour by quality' threshold

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Publications Related to the Thesis

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Biography

Jernej Pavšič was born in 1986 in Šempeter pri Gorici. He was always interested in research and nature. After he finished high school in Nova Gorica he enrolled to Biology program at Biotechnical Faculty in University of Ljubljana.

In 2011, he finished his graduation thesis at NIB under mentorship of prof. dr. Marina Dermastia, discovering the genomic diversity of fruit-tree phytplasma.

In 2012, he became a young researcher at NIB, with dr. Jana Žel as his mentor and dr. Mojca Milavec as his co-mentor. At the same time, he was enrolled to doctoral study programme Nanosciences and Nanotechnologies at International Postgraduate School Jožef Stefan. During his 3.5-year spell at NIB, Jernej was mostly involved in two European metrological projects called INFECT-MET and BioSitrace, He assessed different technologies and approaches for detection and quantification of human pathogens using cutting edge technology such as real time PCR and digital PCR. In 2014, he went on a secondment to LGC in London, where he conducted a 3-month research on techniques for facilitation of genome sequencing of bacteria under the supervision of dr. Jim Huggett. Beside his contribution at numerous domestic and international congresses that he attended during his time at NIB and LGC, he also published several scientific papers related to standardization in clinical diagnostics of microbes and molecular methods for quantification of DNA such as dPCR and real time PCR.

In the first quarter of 2016, he switched from academic sphere to industry, as he got employed at Biofarmacevtika Mengeš, Lek d.d., Sandoz group, Novartis, where he is engaged into development of generic biological drug.