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Digital PCR provides absolute quantitation of viral load for an occult RNA virus

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ABSTRACT

Using a multiplexed LNA-based Taqman assay, RT-digital PCR (RT-dPCR) was performed in a prefabricated microfluidic device that monitored absolute viral load in native and immortalized cell lines, overall precision of detection, and the absolute detection limit of an occult RNA virus GB Virus Type C (GBV-C). RT-dPCR had on average a 10% lower overall coefficient of variation (CV, a measurement of precision) for viral load testing than RT-qPCR and had a higher overall detection limit, able to quantify as low as three 5′-UTR molecules of GBV-C genome. Two commercial high-yield *in vitro* transcription kits (T7 Ribomax Express by Promega and Ampliscribe T7 Flash by Epicentre) were compared to amplify GBV-C RNA genome with T7-mediated amplification. The Ampliscribe T7 Flash outperformed the T7 Ribomax Express in yield of full-length GBV-C RNA genome. THP-1 cells (a model of monocytic derived cells) were transfected with GBV-C, yielding infectious virions that replicated over a 120 h time course and could be infected directly. This study provides the first evidence of GBV-C replication in monocytic derived clonal cells. Thus far, it is the only study using a microfluidic device that measures directly viral load of mammalian RNA virus in a digital format without need for a standard curve.

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1. Introduction

Viral load testing using nucleic acids has become more prevalent over the last decade due to the advent of new technologies such as reverse transcriptase quantitative PCR or RT-qPCR (Niesters, 2001). This technology has been very useful for the diagnosis of viral disease, monitoring antiviral therapy, and providing a clean blood supply (Bustin and Mueller, 2005). This sensitivity becomes crucial for the management of many viral pathogens such as the Hepatitis C Virus and the Human Immunodeficiency Virus Type-1 (HIV-1) (Kleter et al., 1993; Chun et al., 1997). RT-qPCR is highly sensitive for viral load testing, but major problems exist with assay variability, mainly due to mass-based standard curve construction (Bustin, 2000; Freeman et al., 1999). Mass-based standard curves do not allow the absolute detection of viral load. Downstream problems include lack of normalization between sample runs, and a high coefficient of variation/standard error of the mean (CV/SEM) between replicates (Rutledge and Côté, 2003; White et al., 2009).

Digital PCR (RT-dPCR), which stems from RT-qPCR, is a limiting dilution technique that physically separates individual nucleic acid molecules on a microfluidic chip. The real-time PCR products are administered into 770 individual reaction wells (Vogelstein and Kinzler, 1999; Bhat et al., 2009). This physical partitioning allows for positive PCR amplifications to be counted directly as the number of individual molecules per well at the reaction endpoint. The limiting dilution factor is chosen such that over 50% of the chip contains no template molecules per reaction well, giving a “0” (negative) result or a “1” (positive) result indicating one molecule per positive well. A microfluidic digital PCR chip (Fluidigm) with a capacity of 48 samples and a total scale of 36,960 nanoliter chambers were used to measure viral infection. The chip-based technology allows for standard real-time PCR chemistry assays (*i.e.* Taqman) without the use of an exogenous or endogenous standard and directly measures abundance of the amplicon of interest.

Occult viruses are infections in which the patient has viremia without a clinical disease (Piroth et al., 2008). However, infection with these viruses can be associated with other clinically diseased states. For example, when HIV-1 positive patients are co-infected with GB Virus Type-C (GBV-C), there is a decrease in the temporal progression to AIDS (Williams et al., 2004). Occult viruses are often not screened with common serological tests because they are unknown or novel and establishing their association with a particular disease may require extensive investigation. RT-dPCR provides an effective means for testing the viral load for occult viruses that

Abbreviations: GBV-C, GB Virus Type C; RT-qPCR, reverse transcriptase quantitative PCR; LNA, locked nucleic acid probes; RT-dPCR, reverse transcriptase digital PCR; CV, coefficient of variation; SEM, standard error of mean.

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could easily be overlooked. It can specifically detect the presence of any viral genome of interest early in infection with prior sequence information available and bear key information needed for comprehensive patient prognosis.

GBV-C is a positive-stranded RNA lymphotropic flavivirus. It shares a 30% amino acid homology to the Hepatitis C Virus (HCV), also a member of the *Flaviviridae*, but is related more closely to the GBV-A and GBV-B viruses (Simons et al., 2000). Although 3% of the population is infected with GBV-C, blood banks do not screen for its presence since no disease state has been associated with GBV-C infection (Simons et al., 2000; Xiang et al., 2000). Several studies have shown that patients infected with HIV-1 and co-infected with GBV-C show an improved survival from AIDS related infections (Nunnari et al., 2003; Xiang et al., 2001). GBV-C fits the definition of an occult virus and its potential importance in HIV-1 infection cannot be overlooked. Thus, GBV-C provides a perfect model of an occult viral system for measurement of absolute viral load with RT-dPCR.

2. Materials and Methods

2.1. In vitro transcription of GBV-C infectious clone

The GBV-C full-length 9.2 kb viral clone was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID NIH: pAF121950 from Dr. Jinhua Xiang and Dr. Jack Stapleton (Xiang et al., 2000). Sanger sequencing using M13 forward/reverse primers was used to confirm the Iowan GBV-C strain. (Iowan strain GBV-C clone accession no. AF009606.)

Five micrograms of pCR2.1-TOPO containing the GBV-C full-length cloned plasmid DNA (pAF121950) was linearized by *Spe* I restriction enzyme and then transcribed using T7 RNA polymerase (Promega, Madison, WI) for 2.5 h at 37 °C as a standard reaction. To eliminate plasmid DNA after *in vivo* transcription, Qiagen RNase-free DNase (10,000 U/mg) (Qiagen, Valencia, CA) digestion was completed twice for 30 min at 37 °C. The resulting full-length RNA genome of GBV-C (~9.2 kb) was gel purified and quantified using a Nanodrop spectrophometer (Thermo Scientific, Wilmington, DE). This standard method did not produce enough GBV-C genomic RNA for transfection studies. The yield of RNA transcription was compared from two commercial transcription kits, the T7 Ribomax Express (Promega, Madison, WI) and Ampliscribe T7 Flash (Epicentre, Madison, WI). The GBV-C viral clone was linearized by *Spe* I digestion and transcribed *in vitro* at both 37 and 42 °C according to manufacturer instructions. Both manufacturers stated that by increasing the temperature to 42 °C, the yield of the full-length product could increase by an additional 10%. Values were measured for A_{260}/A_{260} abundance using the Nanodrop.

2.2. Cell lines and transfections

Transfected Jurkat, THP-1 and PBMCs cells were used to determine viral load and detection limit. The cells were grown to a cell density of 2×10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 2 mM of L-glutamine and 1% Penicillin/Streptomycin overnight at 37 °C in 5% CO_2. No PHA, PMA or IL-2 induction was used. Two to seven million total cells were transfected with GBV-C RNA following the transmassager's transfection protocol from Qiagen. Purified PBMCs were provided by Astarte Biologics.

2.3. RNase protection assay for infectious virus

Seven days after transfection, cell supernatants were treated with RNaseA/DNase then passaged to uninfected cells. After the first successful passage of similar cell type, the supernatant was used to infect fresh cells and sampled for viral RNA at 48, 96, and 120 h. The passaged cells were again confirmed for total viral load via RT-dPCR seven days after the initial passage. This was repeated in three independent passages.

Table 1
Primer/probe sequences with Thermocycling Parameters.

GAPDH	
Forward	5′-AAATCCCATCACCATCTTCC-3′
Reverse	5′-GGACTCCACGACGTACTCAG-3′
Probe	5′-HEX-CATCGCCCCACTTGATTTTGGA-BHQ2-3′
GBV-C 5′-UTR	
Forward	5′-TAAACCGAGCCCATTACCC-3′
Reverse	5′-ACATTGAAGGGCGACGTG-3′
Probe	ROCHE UPL #61
Thermocycling parameters	
Initial denaturation	95 °C – 3 min
Denaturation	95 °C – 15 s
Annealing/extension	60 °C – 60 s
Cycle	50

Primer/probe sequences along with thermocycling conditions 5′-UTR GBV-c UPL FAM-labeled LNA-based Taqman assay GAPDH standard Taqman HEX-labeled assay.

2.4. Total RNA isolation and cDNA synthesis

Using QIAamp® Viral RNA Mini Kit from Qiagen, total RNA from cell culture supernatants was isolated following the manufacturer's instructions (Qiagen, Valencia, CA). The total RNA was treated three times with DNase I (Stratagene, La Jolla, CA), gel-purified for high molecular weight transcript, and quality-checked for purity via an RNA Pico Chip 6000 from Agilent (Stratagene/Agilent, La Jolla, CA). All RNA used had an RIN (RNA integrity number) over 10. Any value greater or equal to seven ensures that RNA is of the highest quality. RNA was stored at −80 °C. cDNA synthesis of the total cellular RNA was carried out using random hexamers following the manufacturer's protocol for the Superscript III cDNA synthesis kit (Invitrogen, Carlsbad, CA).

2.5. UPL Taqman assay design and reaction conditions

The 5′-UTR sequence from the Iowan GBV-C clone was uploaded to the Universal Probe Library (UPL) assay designer on the Roche diagnostics website, where it was processed to indicate the viral load assay. GAPDH was used to judge the quality of the cDNA and the fill rate required for the microfluidic chip. The reaction conditions were as follows; Universal Taqman Probe Master Mix (Roche) at 1× final concentration, 300 nM forward primer, 300 nM reverse primer and 150 nM of each probe (UPL #61 Roche for 5′-UTR of GBV-C (Table 1) were mixed in a multiplex reaction. ROX normalization dye was pre-mixed to correct for any optical bias. The primer, probe sequences and the thermal cycling parameters are presented in Table 1.

2.6. RT-qPCR on the Stratagene's Mx3005P

For testing purposes, a standard GBV-C cDNA plasmid control sample was created and serially diluted for calibration. To maintain the standard over time, the pooled cDNA sample was cloned into pCR2.1 (Invitrogen) and then transformed into DH5α cells. Plasmids containing standard pooled cDNA were harvested from mid-log phase DH5α cells and further isolated using Qiagen's QIAprep Spin Miniprep kit. The resulting plasmids were digested using *Spe* I, and gel purified. Plasmid DNA containing the infectious clone was serially diluted in tenfold increments in six replicates in order to obtain a coefficient of variation values.

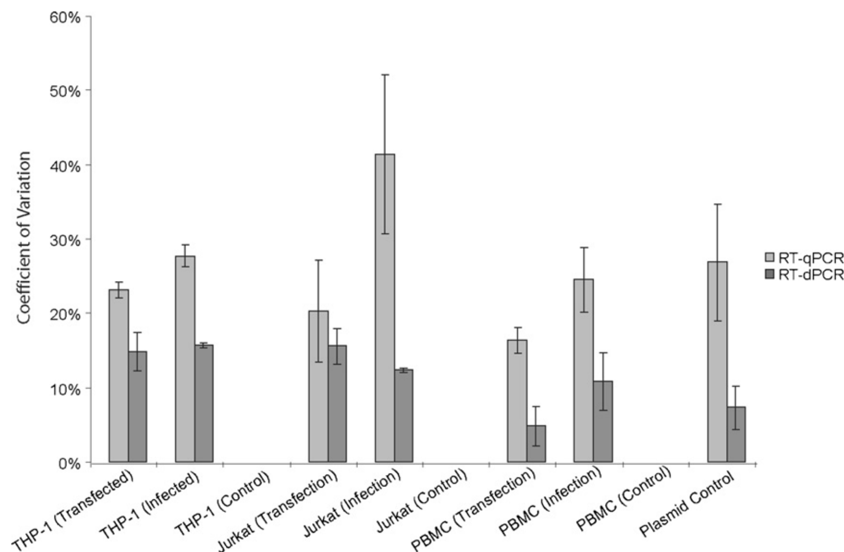

Fig. 1. Precision and reproducibility comparisons of RT-dPCR to RT-qPCR on various cell types. CV/SEM was determined with three replicates except for the plasmid control which was represented by six replicates. Viral load measurements for supernatants for various cell types are reported in Fig. 3.

2.7. RT-dPCR quantitation on Fluidigm's BioMark System

Digital PCR reaction mix containing the diluted template was loaded onto Fluidigm's 48.770 Digital Array microfluidic chip. The microfluidic chip has 48 panels and each panel contains 770 chambers. The concentration of diluted template that yielded 35–65% fill rate for amplified molecules per panel was chosen for technical replication. Six replicate panels on the digital chip were assayed in order to obtain absolute quantification of the initial concentration of sample.

3. Results

3.1. Direct measurement of GBV-C RNA viral load in multiplex Taqman reaction using a microfluidic chip

Viral load was measured for both transfected and infected GBV-C samples with RT-dPCR using a microfluidic chip. This was compared against a standard quantitative PCR reaction. The conserved 5′-untranslated region (5′-UTR) of the GBV-C genome was used to make an amplicon for RT-qPCR and RT-dPCR using 6-mer ultra-short locked-nucleic acid (LNA) Taqman chemistry from the Universal Probe Library (UPL) from Roche (Table 1). The 5′-UTR is commonly used for viral load testing of other members of the *Flaviviridae* family including HCV (Xiang et al., 2000). A standard Taqman assay, which measured GAPDH transcripts, was used as a control in each panel to ensure correct and evenly loaded samples. The GAPDH Taqman assay used a 22-mer HEX-labeled probe that allowed for multiplexing with the 5′-UTR FAM-labeled LNA-based UPL from Roche (Table 1). Both the 5′-UTR and GAPDH assay had R^2 coefficient values of 99% showing near 100% amplification efficiency. The reaction volume for the RT-dPCR chip was five-fold lower than the standard real-time PCR reaction, 4 μl as compared to the standard 20 μl used in many current quantitative PCR instruments recommended by multiple manufacturers. Processing was fast on both systems (Stratagene's Mx3005P and Fluidigm's Biomark), with an average run time of 61 min.

3.2. Digital PCR has better precision with lower coefficient of variation for viral load measurement

The coefficient of variation (CV) is a measurement of the overall precision which is an effective means of comparing qPCR and dPCR. To remove any error associated with the construction of a mass-based standard curve, digital PCR was used to calibrate the GBV-C containing plasmid-based standard curve for comparing qPCR to dPCR directly. RT-dPCR and RT-qPCR were compared for viral detection of the occult RNA virus GBV-C. The average CV/SEM for all samples combined was lower with RT-dPCR with 11.7 ± 2.2% vs. 25.8 ± 4.9% for RT-qPCR (Fig. 1). RT-dPCR was able to measure one sample (transfected PBMCs) below 5% vs. 16.4% by RT-qPCR (Fig. 1). RT-qPCR was only able to measure the plasmid control mean CV to 26.9 ± 7.8%, whereas RT-dPCR measured the same sample CV at 7.3 ± 2.9%. RT-dPCR also had lower CV/SEM values than RT-qPCR in all samples tested (Fig. 1). No GBV-C viral RNA false positives were detected in any non-transfected or non-infected control (Fig. 1).

The theoretical detection limit of the 5′-UTR assay of GBV-C is one 5′-UTR molecule. The measured absolute detection limit is between three and ten molecules per microliter of template via the limiting dilution series of the plasmid control and directly measured from a diluted infected PBMC sample (Fig. 4). RT-qPCR was unable to produce a positive signal for the same dilutions used in RT-dPCR of the plasmid control or infected PBMC sample (Fig. 4).

3.3. Viral load testing in various cell types

GBV-C tropism is thought to encompass lymphocytes and peripheral blood mononuclear cells (PBMCs), with data suggesting that $CD4^+$ T-cells are the primary site of GBV-C viral replication (George et al., 2006). Interestingly, $CD4^+$ cells and PBMCs are the same sites of replication used by HIV-1 (George et al., 2006). In this study PBMCs were transfected with the full-length GBV-C RNA genome and used for the amplification of viral stocks. The viral replication of GBV-C, like most members of *Flaviviridae* (*i.e.* HCV), is slow. GBV-C takes approximately one week in order to produce

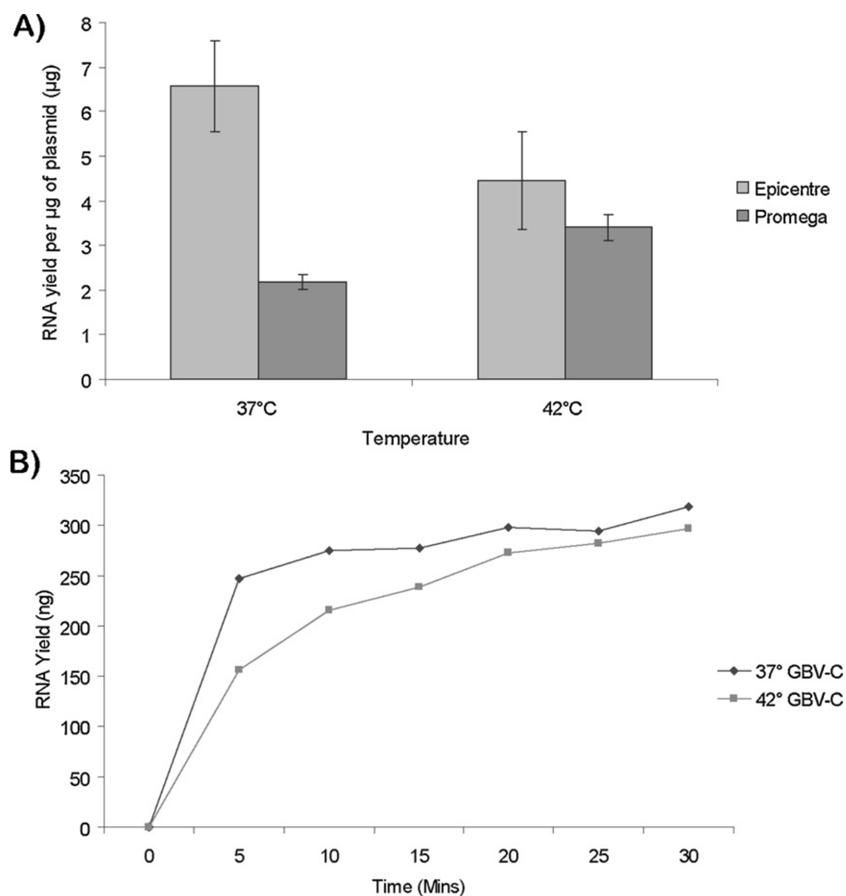

Fig. 2. *In vitro* transcription optimization of the GBV-C infectious clone. (A) Two high yield *in vitro* transcription kits (Promega T7 Ribomax and Epicentre Ampliscribe T7 flash) were compared for RNA yield per µg of starting DNA plasmid. (B) The overall yield of GBV-C RNA genome was measured using Epicentre Ampliscribe flash at both 37/42 °C for 30 min.

enough infectious virions within the cell culture medium to infect uninfected cells (Xiang et al., 2000).

In a previous study, MOLT-4 cells (a human acute lymphoblastic leukemia line) along with PBMCs were used for infection and transfection experiments (Xiang et al., 2000). In our study, Jurkat cells (an acute T-cell leukemia line) were chosen instead of MOLT-4 as Jurkat cells is a common cell line used for HIV-1 studies and since it has been purposed that GBV-C replicates in $CD4^+$ T-cells (George et al., 2006). It is unknown whether GBV-C can infect or produce infectious virions in $CD4^+$ monocytic cells such as macrophages or

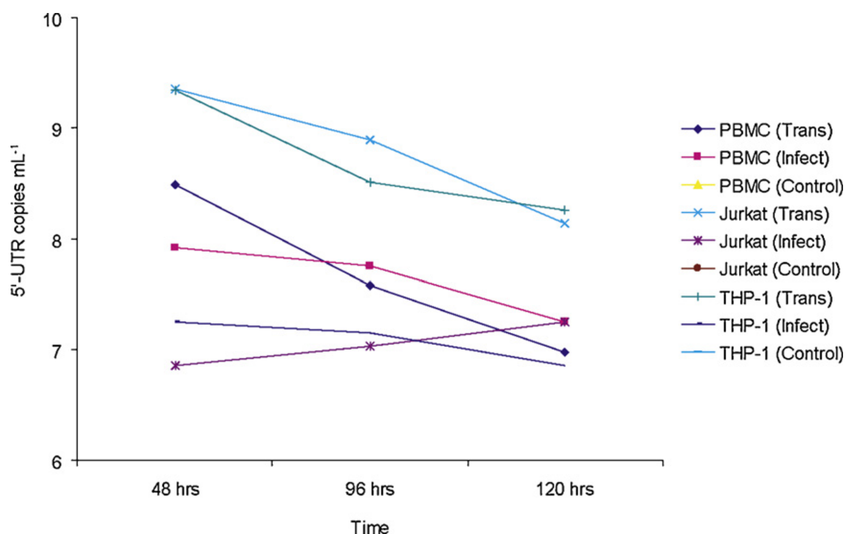

Fig. 3. Measured viral load of GBV-C via RT-dPCR. PBMCs, Jurkats, THP-1 cells were either transfected (trans), infected (infect) or control (no transfection/infection with GBV-C with total supernatant sampled at 48, 96, and 120 h following transfection.

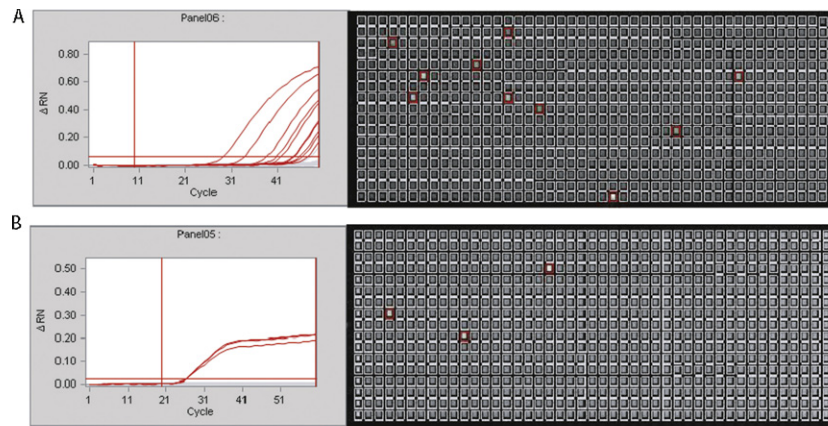

Fig. 4. Absolute measured detection limit. (A) This serial dilution series shows a final detectable dilution of a GBV-C plasmid control with a resolution of 10 amplifiable 5′-UTR molecules per microliter. (B) This was obtained directly from an infected PBMC sample at a final detectable dilution of 3 amplifiable 5′-UTR molecules per microliter.

monocyte derived dendritic cells. THP-1 cells where used in order to test whether GBV-C can replicate and produce infectious virions within monocyte derived cells.

To eliminate false positives due to DNA contaminants in RNA transcription, the plasmid containing the viral genome was digested twice with DNase I prior to transfection. For any infection, free viral RNA was removed from the supernatant with RNaseA (*i.e.* RNaseA protection assay) this means that only packaged virions could establish the infection or subsequent detection via RT-dPCR. The RNaseA-treated viral supernatant generated via transfection was sampled after one seven day passage on PBMCs then used for further infection time course studies.

The GBV-C RNA was detectable in the supernatants with RT-dPCR 48 h after initial transfection or infection and up to 120 h later (Fig. 3). In transfected cells, viral load decreased after 48 h. By contrast, infected jurkats showed a steady increase in viral load over the 120 h time period (Fig. 3). Upon infection or transfection with GBV-C, THP-1 cells showed a detectable viral load across a 120-h time period. The average drop in viral load in THP-1 was less then a half of a log unit across three separate 48-h time intervals (48, 96, 120 h).

3.4. *Optimization of high yield in vitro transcription kits for the GBV-C infectious clone*

Reverse genetics using standard T7 based linear amplification of RNA is commonly used to generate RNA genomes to create infectious viral particles for many studies including GBV-C (Xiang et al., 2000). However, this standard protocol failed to synthesize high enough yield of full-length products for standard RNA transfections. The high RNA yield was critical for engineering infectious viral particles; this step was found to be a major bottleneck in this study. Two *in vitro* transcription products were tested to achieve the high RNA requirement: the T7 Ribomax express from Promega and Ampliscribe T7 Flash from Epicentre. To compare both total RNA yield side by side, a high yielding RNA transcribing GBV-C infectious plasmid was used. The Promega T7 Ribomax and the Epicentre Ampliscribe T7 Flash kits were compared directly at both 37 °C and 42 °C to measure total RNA yield. The Epicentre Ampliscribe T7 Flash produced the most full-length GBV-C RNA genome, at 6.6 μg per μg of plasmid DNA, which was three times the amount of RNA produced by T7 Ribomax (Fig. 2A). Gel analysis revealed the products to be full-length. Epicentre suggests that 42 °C produces the highest yield, but in the case of the 13 kb plasmid, we found that reactions at 37 °C yielded more RNA within tested time limit (Fig. 2B). Due to the high yield and high percentage of full-length products, the Epicentre Ampliscribe T7 flash was used for all transfection experiments.

4. Discussion

RT-dPCR is more precise than RT-qPCR for measuring viral load. This was determined by measuring CV/SEM for the detection of GBV-C infection or transfection which has been shown previously for next generation sequencing libraries (White et al., 2009). The general precision of RT-qPCR was measured to be ±9–21% between replicates, and this study finds similar values (Rutledge and Côté, 2003). Even after removing the potential error of a mass-based standard curve using a digital PCR calibrated standard curve, RT-qPCR had on average a 10% higher CV value. RT-qPCR was unable to produce any CV lower than 10%, whereas RT-dPCR measured a sample lower than 5% CV.

RT-dPCR has a higher detection limit when compared to RT-qPCR directly using the same diluted sample. The 5′-UTR Taqman assay in a RT-dPCR was able to detect as low as three copies of the GBV-C viral genome in a diluted infected PBMC sample whereas RT-qPCR could not produce positive amplification for the same dilution. This could be explained by the fact that better PCR amplification often occurs at lower sample volume, a counter-intuitive phenomenon that should be explored in greater detail in the future.

Fill rate is an important factor in digital PCR, and refers how many wells had zero, one or two molecules via Poisson statistics. This lower CV result is likely due to the 35–65% fill rate of amplifiable molecules used in this study. A lower fill rate (10–25%) ensures that there is only one molecule per well, but it drastically increases the CV (Bhat et al., 2009). A fill rate of over 65% gives a higher probability of more than one molecule per well. This study suggests minimum of a 35% molecule fill to achieve the best precision between sample replicate measurements.

Viral load was measured in both native and immortalized cell lines, whether infected or transfected, with the occult virus GBV-C. An important finding was that GBV-C virions within the culture medium remained infectious after RNaseA/DNase treatment. This indicates that GBV-C is resistant to nuclease treatment, a capability reported for other viruses, but previously unknown in GBV-C.

THP-1 was used to determine if infectious GBV-C particles could be produced in a monocytic derived cell type. This study showed that THP-1 could be transfected with GBV-C producing infectious particles and could be re-infected with supernatant containing GBV-C. While the role of GBV-C infection in monocytic derived cells is still uncertain, this study provides the first evidence for infection and replication in this cell type, a topic which is fertile for future exploration.

Two commercial kits for high yield *in vitro* transcription were compared for long DNA transcripts (<13 kb) and for overall yield. The Ampliscribe T7 Flash from Epicentre outperformed the T7 Ribomax from Promega in both total yield and synthesis of full-length GBV-C RNA genome. These results indicate that Ampliscribe T7 Flash is the preferable method for reverse genetics of high yield and full length infectious RNA viral genome.

Uses such as single molecule sequencing (which could eliminate the need for amplification and cloning due to read capabilities), identification of somatic gene copy number (*i.e.* within tumors), and clinical identification of drug-resistant variants are just a few examples of the potential impact of dPCR (Pushkarev et al., 2009; Wang et al., 2010). The importance of RT-dPCR can be readily seen in HIV-1 infected patients undergoing HAART, in which resistance to administered antivirals develop quickly (Metzner et al., 2009). Identifying drug-resistant mutants while they are still at low frequency can have a large impact on how the patient can be treated.

In conclusion, this is the first report describing the systemic use of microfluidic RT-dPCR for the absolute quantification of a mammalian RNA virus. RT-dPCR has a lower CV/SEM and a higher absolute detection limit when compared to RT-qPCR even with removal of bias of the construction of a mass based standard curve. GBV-C can infect and replicate THP-1 cells, a common $CD4^+$ monocytic cell line, which could give further insight to the dynamics of HIV-1/GBV-C co-infection. Ampliscribe T7 Flash from Epicentre provided the highest yield of full length GBV-C infectious RNA genome in a highly effective manner.

Competing interests

SRQ is a founder, consultant, and equity holder of Fluidigm.

Authors' contributions

RAWIII acquired the data, contributed to the experimental design, data analysis/interpretation and wrote the paper. RAWIII, KRC and SRQ contributed to drafting of the manuscript and final data analysis/interpretation. All of us read and approved the final manuscript.

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References

Bhat, S., Herrmann, J., Armishaw, P., Corbisier, P., Emslie, K.R., 2009. Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. Anal. Bioanal. Chem. 394, 457–467.

Bustin, S.A., 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrinol. 25, 169–193.

Bustin, S.A., Mueller, R., 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. Clin. Sci. (Lond.) 109, 365–379.

Chun, T.W., Stuyver, L., Mizell, B.S., Ehler, A.L., Mican, A.J., Baseler, M., Lloyd, L.A., Nowak, A.M., Fauci, S.A., 1997. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci U S A. 94, 13193–13197.

Freeman, W.M., Walker, S.J., Vrana, K.E., 1999. Quantitative RT-PCR: pitfalls and potential. BioTechniques 26, 112–125.

George, S.L., Varmaz, D., Stapleton, J.T., 2006. GB virus C replicates in primary T and B lymphocytes. J. Infect. Dis. 193, 451–454.

Kleter, G.E.M., Brouwer, J.T., Heijtink, R.A., Schalm, S.W., Quint, W.G.V., 1993. Detection of hepatitis C virus RNA in patients with chronic hepatitis C virus infections during and after therapy with alpha interferon. Antimicrob. Agents Chemother. 37, 595–597.

Metzner, K.J., Giulieri, S.G., Knoepfel, S.A., Rauch, P., Burgisser, P., Yerly, S., Günthard, H.F., Cavassini, M., 2009. Minority quasispecies of drug-resistant HIV-1 that lead to early therapy failure in treatment-naive and -adherent patients. Clin. Infect. Dis. 48, 239–247.

Niesters, H.G.M., 2001. Quantitation of viral load using real-time amplification techniques. Methods 25, 419–429.

Nunnari, G., Nigro, L., Palermo, F., Attanasio, M., Berger, A., Doerr, H.W., Pomerantz, R.J., Cacopardo, B., 2003. Slower progression of HIV-1 infection in persons with GB virus C co-infection correlates with an intact T-helper 1 cytokine profile. Ann. Intern. Med. 139, 26–30.

Piroth, L., Carrat, F., Larrat, S., Goderel, I., Martha, B., Payan, C., Lunel-Fabiani, F., Bani-Sadr, F., Perronne, C., Cacoub, P., Pol, S., Morand, P., 2008. Prevalence and impact of GBV-C, SEN-V and HBV occult infections in HIV-HCV co-infected patients on HCV therapy. J. Hepatol. 49, 892–898.

Pushkarev, D., Neff, N.F., Quake, S.R., 2009. Single-molecule sequencing of an individual human genome. Nat. Biotechnol. 27, 847–852.

Rutledge, R.G., Côté, C., 2003. Mathematics of quantitative kinetic PCR and the application of standard curves. Nucleic Acids Res. 31, e93.

Simons, J.N., Desai, S.M., Mushahwar, I.K., 2000. The GB viruses. Curr. Top. Microbiol. Immunol. 242, 341–375.

Vogelstein, B., Kinzler, K.W., 1999. Digital PCR. PNAS 96, 9236–9241.

Wang, J., Ramakrishnan, R., Tang, Z., Fan, W., Kluge, A., Dowlati, A., Jones, R.C., Ma, P.C., 2010. Quantifying EGFR alternations in the lung cancer genome with nanofluidic digital PCR arrays. Clin. Chem. 56, 623–632.

White III, R.A., Blainey, P.C., Fan, H.F., Quake, S.R., 2009. Digital PCR provides sensitive and absolute calibration for high throughput sequencing. BMC Genomics 10, 116.

Williams, C.F., Klinzman, D., Yamashita, T.E., Xiang, J., Polgreen, P.M., Rinaldo, C., Liu, C., Phair, J., Margolick, J.B., Zdunek, D., Hess, G., Stapleton, J.T., 2004. Persistent GB virus C infection and survival in HIV-infected men. N. Engl. J. Med. 350, 981–990.

Xiang, J., Wunschmann, S., Schmidt, W.N., Shao, J., Stapleton, J.T., 2000. Full length GB virus C (hepatitis G virus) RNA transcripts are infectious in primary CD4-positive T cells. J. Virol. 74, 9125–9133.

Xiang, J., Wunschmann, S., Diekema, D.J., Klinzman, D., Patrick, K.D., George, S.L., Stapleton, J.T., 2001. Effect of coinfection with GB virus C (hepatitis G virus) on survival among patients with HIV infection. N. Engl. J. Med. 345, 707–714.