A comparison of two methods for detection of norovirus RNA in environmental swab samples

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Abstract

Norovirus is the principal cause of acute gastroenteritis globally, infecting and causing disease in people of all ages. Contaminated food and water are recognised as major routes of infection, often leading to outbreaks of gastroenteritis that can spread between countries and continents. Standardised molecular methods are available for the detection of norovirus from water and food items such as shellfish and fresh produce such as leafy greens and berry fruits, which are some of the most commonly identified food products as being contaminated at source. Detection of norovirus from stool samples also relies on similar molecular methods, but some differences exist between the nucleic acid extraction, reverse transcription, and amplification strategies recommended by the ISO 15216-1:2017 and related annexes, and those typically employed in clinical diagnostic or reference laboratories. Here we conduct a direct comparison of two methods for the detection and quantitation of norovirus directly from a stool sample and from swabs artificially contaminated with a dilution of the stool sample to simulate environmental sampling. We also compare the use of the linear dsDNA standard as recommended in the ISO 15216:2017 method against an *in vitro* transcribed single stranded RNA (ssRNA) for the estimation of the norovirus genome copy number present in a sample. Our results show that the two methods have comparable sensitivity for the detection of norovirus RNA from a clinical sample or from a contaminated swab. The use of a ssRNA standard revealed that quantitation performed against a linear dsDNA standard consistently underestimated the genome copy numbers by 1.5 to 2 log, due to the relative inefficiently of the reverse transcription step. This has important implications for the estimation of the sensitivity of norovirus detection methods, comparability of results across sites and assessment of viral loads that may be clinically significant or estimated to constitute infectious doses.

Introduction

Norovirus is the commonest cause of acute gastroenteritis worldwide. In the UK, there are over 3 million norovirus infections each year (Harris et al., 2017, Tam et al., 2012). Onset of clinical illness usually begins 12-48 hours post infection, and presents as vomiting, non-bloody diarrhoea, abdominal cramps and low-grade fever, with symptoms lasting 12-72 hours, and usually self-resolving after this time.

Transmission of viruses via the food chain is an increasingly recognised public health problem (reviewed in Iturriza-Gomara and O'Brien, 2016). Norovirus transmission occurs via a faecal-oral pathway, and there are multiple potential routes of transmission, including direct person-to-person and indirect via contained food, water and the environment. The attribution of norovirus infections by these different routes of infection is poorly understood, however, it is likely that food-associated transmission of norovirus is important, and it has been estimated that between 14%-23% of norovirus infections may be food-associated (Adak et al., 2002, Verhoef et al., 2015).

Several factors likely contribute to the transmission of norovirus through the food chain: (i) the low infectious dose (Glass et al., 2000, Teunis et al., 2008); (ii) high viral shedding in stool (Atmar et al., 2008); (iii) environmental stability of the virus (D'Souza et al., 2006); and, (iv) a high rate of asymptomatic shedding (Amar et al., 2007, Phillips et al., 2010). Together, these factors mean that contamination of foods by food handlers or from surfaces contaminated with the virus in kitchen environments is likely to be an important source of transmission and outbreaks.

To better understand the epidemiology and ultimately assess the contribution made by the food chain to the burden of norovirus infections, sensitive in molecular detection of viruses in food and environmental samples is required, as well as development of tractable methods for deployment in public health laboratories. The recent validation of a methodology for detection of norovirus in shellfish and soft fruits as an international standard [ISO 15216-1:2017] (ISO, 2017, Lees and CENWG6TAG4, 2010) is significant progress in this field. However, even when laboratories apply methodologies that are compliant with ISO 15216-1:2017, there is room for differences in various of the steps of the method, and in some cases, the

recommended methods may not be universally adoptable due to constraints on the technology available within differently resourced laboratories.

Here we present a comparison of two nucleic acid extraction and two norovirus detection protocols; one representing methods widely used in diagnostic and public health laboratories for the detection of norovirus in clinical samples, and second according to standardised method described in ISO15216-1:2017 and as per recommendations in the related annexes. Further, we compare the use of in vitro transcribed single stranded RNA and linear dsDNA as external quantitation standards for estimating viral nucleic acid load by real-time PCR.

Methods

Preparation of specimens and swabs

For the purposes of method validation, two stool specimens, one each containing a GI and GII norovirus, which were prepared as a 10% suspensions in PBS, and tenfold serial dilutions of the suspension were prepared.

Aliquots of the tenfold dilutions were either extracted directly, or used to contaminate Viscose swabs (Technical Service Consultants Ltd, Heywood, UK). Swabs were contaminated in duplicates, and both aliquot and swab pairs were extracted using two different methods, as described below.

All specimens were spiked with Mengo virus (strain vMC_0) cell culture supernatant as a process control.

Extraction method A

Extraction of total nucleic acid using a manual guanidinium thiocynate (GTC)-silica method based on that described previously (Boom et al., 1990, Green et al., 1993). Briefly, either 200µl of stool suspension, or a contaminated swab tip, were immersed into 1ml L6 (Severn Biotech, Kidderminster, UK) and incubated at room temperature, after which the swab was discarded where relevant, and to both stool and swab lysates, 20µl silica extraction matrix (Severn Biotech) added, followed by incubation at room temperature with agitation for 15 minutes. Silica was pelleted by centrifugation and the pellet washed twice in 1ml L2 (Severn Biotech), twice in 70% ethanol and once in 100% acetone. Silica pellets were air-dried and suspended in 50µl molecular grade water. Total nucleic acid was eluted from the silica by incubation at 56^{o}C for 15 minutes after which silica was removed by centrifugation.

Extraction method B

Extraction of total nucleic acid using a semi-automated guanidinium isothiocynate (GTC)-silica method based on that described previously (Lees and CENWG6TAG4, 2010) and adopted as part of the recently published ISO standard 15216-1:2017 (ISO, 2017), and related annexes. Briefly, either 200µl of stool suspension, or a contaminated swab tip were immersed into 2ml NucliSENS Lysis Buffer (bioMérieux, Baisingstoke, UK) and incubated at room temperature, after which the swab was

discarded where relevant. Total nucleic acid was extracted from both stool and swab lysates using the NucliSENS extraction system (bioMérieux) operated either on the miniMAG or easyMAG system (bioMérieux) according to manufacturer's instruction. Total nucleic acid was eluted into 100µl elution buffer.

Detection method A

The RNA in the total nucleic acid was converted to randomly-primed cDNA by reverse transcription (RT) as previously described (Allen et al., 2014), and norovirus detected using genogroup-specific real-time PCR assays (qPCR) as previously described (Kageyama et al., 2003). Detection of Mengo virus was performed as previously described (Pinto et al., 2009).

Detection method B

Norovirus RNA was detected using genogroup-specific one-step combined RT-qPCR assays as described (Lowther et al., 2012). Detection of Mengo virus was performed as previously described (Pinto et al., 2009)

Production of IVT ssRNA

Partial ORF1 and complete ORF2 and ORF3 were amplified as a single amplicon from a GII.3 norovirus-positive faecal specimen and cloned into pCR2.1-TOPO vector (Thermo Fisher Scientific, Leicestershire, UK) according to manufacturer's instruction. Plasmid DNA was prepared from overnight bacterial cultures using the QIAprep Spin Miniprep Kit (Qiagen). The concentration of the eluted plasmid was measured using the Qubit® DNA BR Assay Kit (Thermo Fisher Scientific), and a dilution series of the plasmid was prepared in the range of $6x10^{7}$ copies/µL to $6x10^{-2}$ copies/µL.

Primers RNA-SC-F (5′-<u>TAATACGACTCACTATA</u>GAGGGTGAATGGATTTTT-3′) and RNA-SC-R (5′-AGGCCGGCGGCACCATCATTAGATGG-3′) were designed to produce an amplicon consisting of a T7 RNA polymerase promoter (underlined) upstream of the norovirus ORF1/ORF2 junction from this plasmid. Amplification was performed using the Expand High Fidelity System (Roche, West Sussex, UK), with 0.4µM each primer. Thermal cycling conditions were: 95°C for 5 min, followed by 3 cycles of 95°C for 1 minute, 60°C for 30 seconds (decreasing by 2°C every 3 cycles,

with an additional 23 cycles at 50°C), 72°C for 3 minutes and a final extension step of 72°C for 5 minutes.

The resulting amplicon was purified using the QIAQuick Gel-Extraction Kit (Qiagen), and this used as template for *in vitro* transcription with the MEGAscript T7 High Yield Transcription Kit (Fisher Scientific). The *in vitro* transcribed single stranded RNA (IVT ssRNA) was treated with Turbo DNase (Fisher Scientific) and precipitated using lithium chloride. Purified IVT ssRNA was suspended in 30 μL DEPC-treated water (Fisher Scientific). The concentration of IVT ssRNA and residual DNA template was measured using Qubit® RNA BR and DNA HS Assay Kits (Thermo Fisher Scientific), and subsequently the IVT ssRNA was diluted to a range of 6×10^{5} copies/μL to 6×10^{-2} copies/μL.

Validation of the IVT ssRNA and linear dsDNA external standards

Validation of the standards was performed using primers and probes as described by Kageyama et al (2003) (as detection method A, above) and used in a one-step and two-step assay format, described briefly below.

The final one-step assay included 1X Precision One-Step™ qRT-PCR Mastermix (Primerdesign, Hampshire, UK), 0.5μM each primer, 0.125μM probe, and 5μl of IVT ssRNA. Alternatively, norovirus-specific primers and probe were replaced with 1μl of the internal control (IC) primer-probe mix (as provided with kit). Thermal cycling conditions were as follows: 55°C for 10 minutes, 95°C for 8 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds.

The final two-step assay included 1X PrecisionPLUS Mastermix (Primerdesign), 0.4μM each primer, 0.1μM probe, and 5μl of plasmid DNA. Thermal cycling conditions were as follows: 95°C for 2 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 60 seconds.

The linear dsDNA standard was validated as a single standard curve in six replicates using the one-step assay, and three independent standard curves over 20 replicates using two-step assay.

The IVT ssRNA standard was validated as three independent standard curves used across 14 replicates in the one-step assay. A one-step (no RT control) and two-step assay was performed on each dilution to detect the presence of residual DNA.

For each respective standard, the mean C_q value and calculated copy number were averaged for each 10-fold dilution, the slope and y-intercept could then be calculated by linear regression analysis.

Absolute quantitation of Lenticule® Disc reference standards

Lenticule® Discs [batch ID: PHE-RMNOROG2/231415] (Public Health England, Porton Down, UK) containing GII norovirus were prepared according to manufacturer's instruction. For validation up to three Lenticule® Discs were rehydrated per experiment, and this was performed three times to give seven replicates overall. Lenticule® Disc suspensions were serially diluted from $1.1x10^4$ copies to $1.1x10^{-2}$ copies, and nucleic acid extracted as described in extraction method A (above).

Results and Discussion

Comparison of methods

Although the principles of both extraction methods are the same, there are some minor differences in relation to the extraction platforms, reagents, as well as in the reverse transcription and polymerase chain reaction (RT-PCR) reagents and conditions. The differences between the two methods are the following:

Extraction method A is a manual method whereas extraction method B is semi-automated. Both involve lysis of the virus capsid using high concentration of the chaotropic agent guanidinium isothiocyanate and detergents, followed by binding of the released virus nucleic acid to fractionated silica. Whilst extraction method B used magnetic silica as a means for recovery of the silica-bound nucleic acid, extraction method A relies on centrifugation.

For amplification of norovirus RNA, detection method A uses a two-step approach, by which RNA is reverse transcribed using random hexamers, hence producing cDNA of all the RNA present in the sample, and then this is followed by separate norovirus genogroup-specific (GI and GII) PCRs and a third PCR for amplification and detection of Mengo virus. Detection method B employs a one-step approach, in which the same primers are used for generating cDNA in reverse transcription step prior to the amplification in the same tube/well on three different reactions to detect norovirus GI or GII or Mengo virus.

Although the primers used in all norovirus-specific assays amplify the same region of the virus genome (ORF1/ORF 2 junction), there are minor differences in the sequence of some of the norovirus-specific primers and probes used (Figure 1). Primers and probes used to amplify and detect Mengo virus are identical (data not shown).

Comparison of the sensitivity of the two methods:

Tenfold serial dilutions of a suspension of a stool specimen containing a GII norovirus were used to contaminate swabs. Duplicate swabs contaminated with the GII norovirus were extracted using extraction method A and extraction method B,

and nucleic acid extracts from both extraction methods were tested in both detection method A and detection method B using a Rotor-gene Q (Qiagen) (Table 1).

The results show that extraction protocol A is marginally more sensitive than extraction protocol B, regardless of the RT-PCR method used; however, the difference is not significant. The process control (Mengo virus) was detected in all samples with either extraction/detection protocol combination (data not shown).

To compare further the sensitivity of the two detection protocols, a stool specimen containing a GI norovirus was prepared as a series of tenfold dilutions and total nucleic acid extracted and norovirus RNA detected using both protocols. The results were calibrated against a dsDNA standard curve (kindly supplied by Dr James Lowther, Centre for the environment, fisheries and aqua science [Cefas], Weymouth, UK) (Tables 2a, 2b and 2c).

For the detection of norovirus RNA directly from a stool suspension or from contaminated swabs, the results showed that both methods had an identical end point, and that the C_q value differences for each dilution were < 3 cycles (or within a log if expressed as DNA quantity), and therefore, not significantly different.

In contrast, for the detection of dsDNA detection, protocol A can detect a single norovirus copy, and suggests that this PCR protocol is marginally more sensitive than detection protocol B.

Absolute quantitation with a plasmid standard can underestimate viral load

Additionally, we examined whether differences in sensitivity were observed between use of IVT ssRNA or linear dsDNA as an external standard for estimation of viral load by qPCR.

Both IVT ssRNA and linear dsDNA were titrated at ten-fold dilutions between $3x10^6$ to $3x10^3$ copies per reaction, and tested using one-step and two-step assays, respectively (Figure 2). Comparison of the two external standards indicated that the IVT ssRNA generated a higher C_q values at an identical predicted copy number to the linear dsDNA (Figure 2)

Lenticule® Discs are standardised reference materials which contain a geometric mean of 4.04 log_{10} GII HuNoV genome copies/disc. Lenticule® Discs were diluted

and quantitated with the IVT ssRNA and linear dsDNA standards. Tenfold dilutions of the Lenticule® Disc preparation were estimated to be at 3.39±0.17, 2.78±0.14 and 1.57±0.089 log_{10} copy number/reaction against the dsDNA standard, whereas the IVT ssRNA standard estimated higher titres of 5.31±0.17, 4.72±0.13 and 3.57± 0.082 log_{10} copy number/reaction (Figure 3), a difference of between 1.5 to 2 log_{10}. and consistent with the 6-7 C_q value differences observed when both standards (dsDNA and ssRNA) were compared against each other.

Conclusions

We present data that demonstrates two different nucleic acid extraction protocols – one manual and one semi-automated – and two different norovirus qPCR detection methods – a one-step and a two-step protocol – have comparable sensitivity for the detection of GI and GII norovirus in environmental swab samples, and clinical specimens.

Comparison of viral load estimation by qPCR against external standards indicates that norovirus load may be underestimated when using a linear dsDNA standard compared to an IVT ssRNA standard. The Lenticule® Discs batch tested had, according to the manufacturer's information, an expected range of 1.65 to 4.46 $\log_{10}$ copy number/reaction, which was in close agreement with the quantitation obtained against the linear dsDNA standard; hence, it is likely that the quantity of norovirus in the Lenticule® Discs was calculated using a DNA standard. Quantification of the Lenticule® reference material against the IVT ssRNA standard yielded an increase in copy number of between 1.5 to 2 log. This can be explained by the relative poor efficiency of the RT step, as the sensitivity-limiting step in the reaction. The implications of this are that the sensitivity of RT-PCR methods commonly reported against DNA standards may be overestimated. Also, as different reverse transcription enzymes and can have different efficiencies, it is likely that the degree of overestimation will also differ depending on the enzyme/RT system used. The choice of standard and has important implications for the interpretation of data obtained using qPCR as viral load may be considered as an indicator of potential for infectiveness, or in a clinical setting for assessing weather a norovirus. This is also an important factor to consider when comparing form different laboratories and/or studies.

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References

ADAK, G. K., LONG, S. M. & O'BRIEN, S. J. 2002. Trends in indigenous foodborne disease and deaths, England and Wales: 1992 to 2000. *Gut,* 51**,** 832-41.

ALLEN, D. J., ADAMS, N. L., ALADIN, F., HARRIS, J. P. & BROWN, D. W. 2014. Emergence of the GII-4 Norovirus Sydney2012 strain in England, winter 2012-2013. *PLoS One,* 9**,** e88978.

AMAR, C. F., EAST, C. L., GRAY, J., ITURRIZA-GOMARA, M., MACLURE, E. A. & MCLAUCHLIN, J. 2007. Detection by PCR of eight groups of enteric pathogens in 4,627 faecal samples: re-examination of the English case-control Infectious Intestinal Disease Study (1993-1996). *Eur J Clin Microbiol Infect Dis,* 26**,** 311-23.

ATMAR, R. L., OPEKUN, A. R., GILGER, M. A., ESTES, M. K., CRAWFORD, S. E., NEILL, F. H. & GRAHAM, D. Y. 2008. Norwalk virus shedding after experimental human infection. *Emerg Infect Dis,* 14**,** 1553-7.

BOOM, R., SOL, C. J., SALIMANS, M. M., JANSEN, C. L., WERTHEIM-VAN DILLEN, P. M. & VAN DER NOORDAA, J. 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol,* 28**,** 495-503.

D'SOUZA, D. H., SAIR, A., WILLIAMS, K., PAPAFRAGKOU, E., JEAN, J., MOORE, C. & JAYKUS, L. 2006. Persistence of caliciviruses on environmental surfaces and their transfer to food. *Int J Food Microbiol,* 108**,** 84-91.

GLASS, R. I., NOEL, J., ANDO, T., FANKHAUSER, R., BELLIOT, G., MOUNTS, A., PARASHAR, U. D., BRESEE, J. S. & MONROE, S. S. 2000. The epidemiology of enteric caliciviruses from humans: a reassessment using new diagnostics. *J Infect Dis,* 181 Suppl 2**,** S254-61.

GREEN, J., NORCOTT, J. P., LEWIS, D., ARNOLD, C. & BROWN, D. W. 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. *J Clin Microbiol,* 31**,** 3007-12.

HARRIS, J. P., ITURRIZA-GOMARA, M. & O'BRIEN, S. J. 2017. Re-assessing the total burden of norovirus circulating in the United Kingdom population. *Vaccine,* 35**,** 853-855.

ISO 2017. Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR -- Part 1: Method for quantification [ISO 15216-1:2017].

ITURRIZA-GOMARA, M. & O'BRIEN, S. J. 2016. Foodborne viral infections. *Curr Opin Infect Dis,* 29**,** 495-501.

KAGEYAMA, T., KOJIMA, S., SHINOHARA, M., UCHIDA, K., FUKUSHI, S., HOSHINO, F. B., TAKEDA, N. & KATAYAMA, K. 2003. Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *J Clin Microbiol,* 41**,** 1548-57.

LEES, D. N. & CENWG6TAG4 2010. International standardisation of a method for detection of human pathogenic viruses in molluscan shellfish. *Food Environ. Microbiol.,* 66**,** 3241-3248.

LOWTHER, J. A., GUSTAR, N. E., POWELL, A. L., HARTNELL, R. E. & LEES, D. N. 2012. Two-year systematic study to assess norovirus contamination in oysters from commercial harvesting areas in the United Kingdom. *Appl Environ Microbiol,* 78**,** 5812-7.

PHILLIPS, G., TAM, C. C., RODRIGUES, L. C. & LOPMAN, B. 2010. Prevalence and characteristics of asymptomatic norovirus infection in the community in England. *Epidemiol Infect,* 138**,** 1454-8.

PINTO, R. M., COSTAFREDA, M. I. & BOSCH, A. 2009. Risk assessment in shellfish-borne outbreaks of hepatitis A. *Appl Environ Microbiol,* 75**,** 7350-5.

TAM, C. C., RODRIGUES, L. C., VIVIANI, L., DODDS, J. P., EVANS, M. R., HUNTER, P. R., GRAY, J. J., LETLEY, L. H., RAIT, G., TOMPKINS, D. S., O'BRIEN, S. J. & COMMITTEE, I. I. D. S. E. 2012. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. *Gut,* 61**,** 69-77.

TEUNIS, P. F., MOE, C. L., LIU, P., MILLER, S. E., LINDESMITH, L., BARIC, R. S., LE PENDU, J. & CALDERON, R. L. 2008. Norwalk virus: how infectious is it? *J Med Virol,* 80**,** 1468-76.

VERHOEF, L., HEWITT, J., BARCLAY, L., AHMED, S. M., LAKE, R., HALL, A. J., LOPMAN, B., KRONEMAN, A., VENNEMA, H., VINJE, J. & KOOPMANS, M. 2015. Norovirus genotype

profiles associated with foodborne transmission, 1999-2012. *Emerg Infect Dis,* 21**,** 592-9.

Figures and Tables

Tables

Table 1: Oligonucleotide primer and probes used in both detection method A and detection method B. Differences between the sequences are highlighted. See also Figure 1.

Table 2: Comparison of extraction protocol A vs extraction protocol B, and detection protocol A and detection protocol B.

Table 3: Sensitivity comparison of the norovirus GI one-step vs the two-step RT-PCR. (a) Using 200µl of a 10% suspension of a clinical sample. (b) Using 200µl of a 10% suspension of the same clinical sample to contaminate swabs. (c) Using the Cefas cDNA standard curve.

Figures

Figure 1: Alignment of primer and probe sequences used in detection method A (COG1F, COG1R, RING1ABTP, COG2F, COG2R, RING2P) and detection method B (QNIF4, NV1LCR, NVGG1p, QNIF2, COG2R, QINFs) real-time PCR methods against appropriate reference strains: GI using Norwalk virus (accession M87661) and GII using Lordsdale virus (accession X86557).

Figure 2: Standard curve validation of the RNA and plasmid template in one and two-step qPCR assays (Red – linear dsDNA, and Blue – IVT ssRNA). Cq – Quantification cycle (cycle threshold).

Figure 3: Quantitation of Lenticule® Disc reference materials with different standards A) linear dsDNA B) IVT ssRNA (Boxes and whiskers represent the standard error and range around the mean, respectively. The dashed lines correspond to the upper and lower expected range of the Lenticule® Disc batch).

Table 1: Comparison of extraction protocol A vs extraction protocol B, and detection protocol A and detection protocol B.

RT-PCR Method	Detection A		Detection B	
Nucleic acid extraction method	Extraction A	Extraction B	Extraction A	Extraction B
Swab contaminated with 200µl of :	C_q[1]	C_q	C_q	C_q
10% suspension	23.11	25.61	21.03	23.14
10^{-1} dilution	26.82	29.12	25.16	26.42
10^{-2} dilution	32.11	34.26	30.04	30.78
10^{-3} dilution	34.08	38.02	32.44	33.38
10^{-4} dilution	37.14	ND[2]	35.23	37.35
10^{-5} dilution	ND	ND	ND	ND
10^{-6} dilution	ND	ND	ND	ND
10^{-7} dilution	ND	ND	ND	ND
10^{-8} dilution	ND	ND	ND	ND
10^{-9} dilution	ND	ND	ND	ND
10^{-10} dilution	ND	ND	ND	ND
Negative control (PBS)	ND	ND	ND	ND

[1]C_q – Quantification cycle (cycle threshold); [2]ND – no virus RNA detected

Table 2: Sensitivity comparison of the norovirus GI one-step vs the two-step RT-PCR. (a) Using 200µl of a 10% suspension of a clinical sample. (b) Using 200µl of a 10% suspension of the same clinical sample to contaminate swabs. (c) Using the Cefas dsDNA standard curve

3a

	Detection protocol A		Detection protocol B	
Stool Sample	**C_q[1]**	**Quantity**	**C_q**	**Quantity**
10% suspension	18.1	1.8E+05	17.3	4.8E+04
10^{-1} dilution	22.4	1.0E+04	20.0	9.7E+03
10^{-2} dilution	25.5	1.4E+03	24.3	7.8E+02
10^{-3} dilution	29.7	8.8E+01	27.0	1.5E+02
10^{-4} dilution	34.2	4.8E+00	31.2	1.3E+01
10^{-5} dilution	38.4	3.0E-01	33.7	3.1E+00
10^{-6} dilution	ND[2]		ND	
10^{-7} dilution	ND		ND	
10^{-8} dilution	ND		ND	
10^{-9} dilution	ND		ND	
10^{-10} dilution	ND		ND	
Negative control (PBS)	ND		ND	

2b

Swab contaminated with:	Detection protocol A		Detection protocol B	
	C_q	Quantity	C_q	Quantity
10% suspension	21.7	1.66E+04	19.3	1.48E+04
10^{-1} dilution	25.8	1.15E+03	23.2	1.44E+03
10^{-2} dilution	29.7	8.59E+01	25.4	4.03E+02
10^{-3} dilution	34.4	4.21E+00	30.5	2.01E+01
10^{-4} dilution	37.4	5.66E-01	34.1	2.44E+00
10^{-5} dilution	39.7	1.28E-01	38.3	2.04E-01
10^{-6} dilution	ND		ND	
10^{-7} dilution	ND		ND	
10^{-8} dilution	ND		ND	
10^{-9} dilution	ND		ND	
10^{-10} dilution	ND		ND	
Negative control (PBS)	ND		ND	

2c

dsDNA standard curve dilution:	Detection protocol A		Detection protocol B	
	Mean C_q	Quantity	Mean C_q	Quantity
10-1	22.0	1.00E+04	19.75	1.00E+04
10-2	26.3	1.00E+03	24.15	1.00E+03
10-3	29.8	1.00E+02	27.8[4]	1.00E+02
10-4	33.5	1.00E+01	31.55	1.00E+01
10-5	36.1	1.00E+00	ND	-
10-6	ND[3]	-	ND	-

[1]C_q – Quantification cycle (cycle threshold)CT – Cycle threshold;

[2]ND – no virus RNA detected;

[3]One replicate omitted, one no virus RNA detected;

[4]Not mean, one replicate only as one replicate was no virus detected.

Figure 1

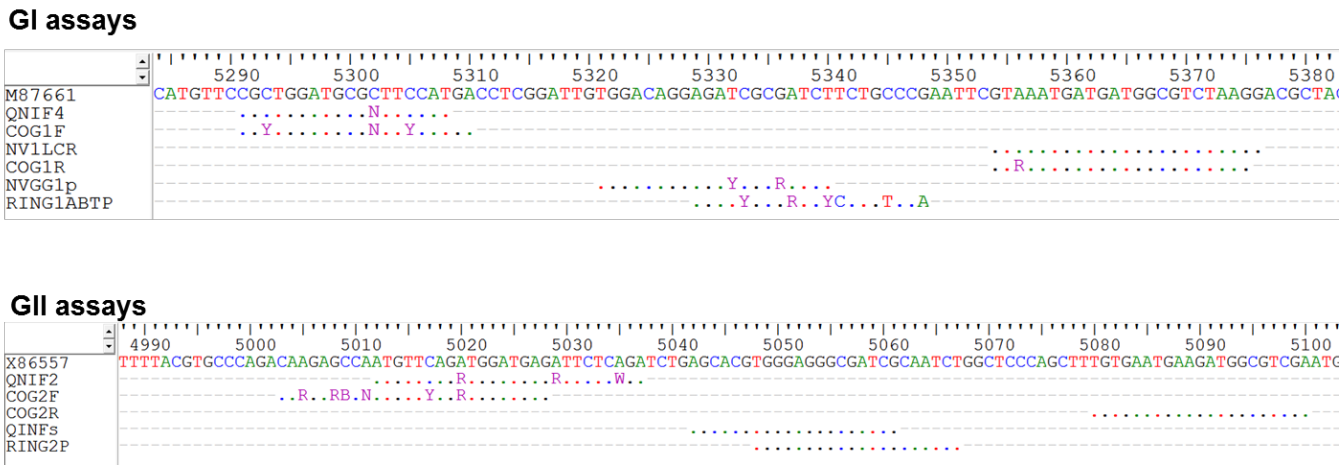


Figure 2

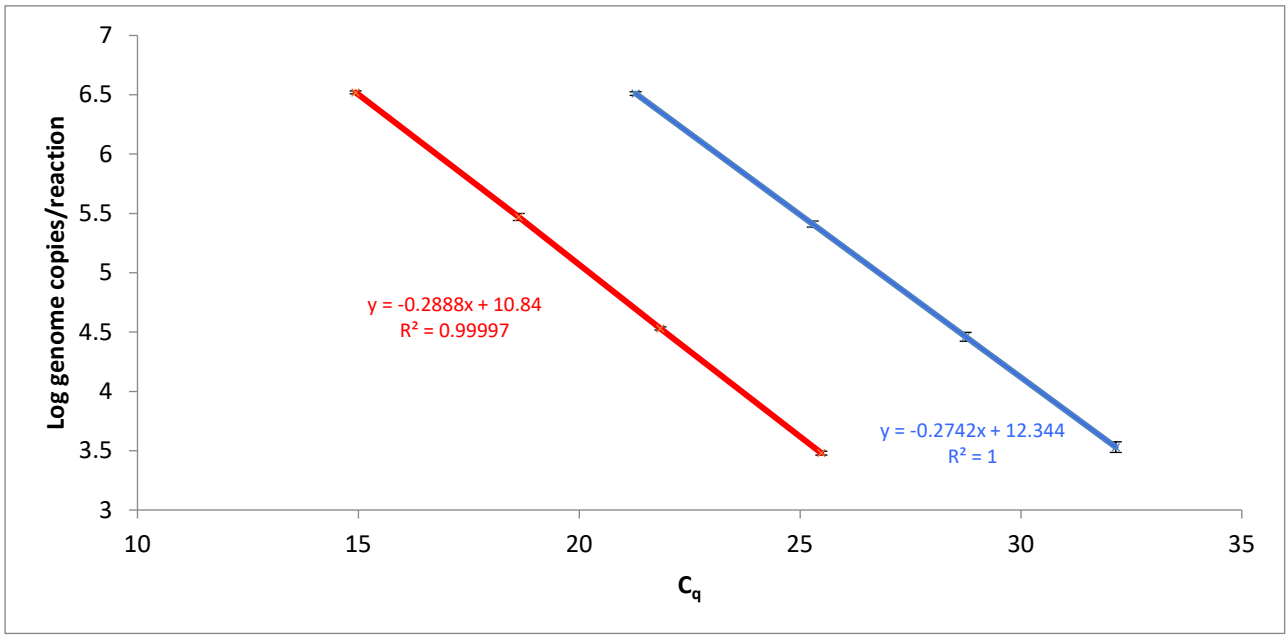
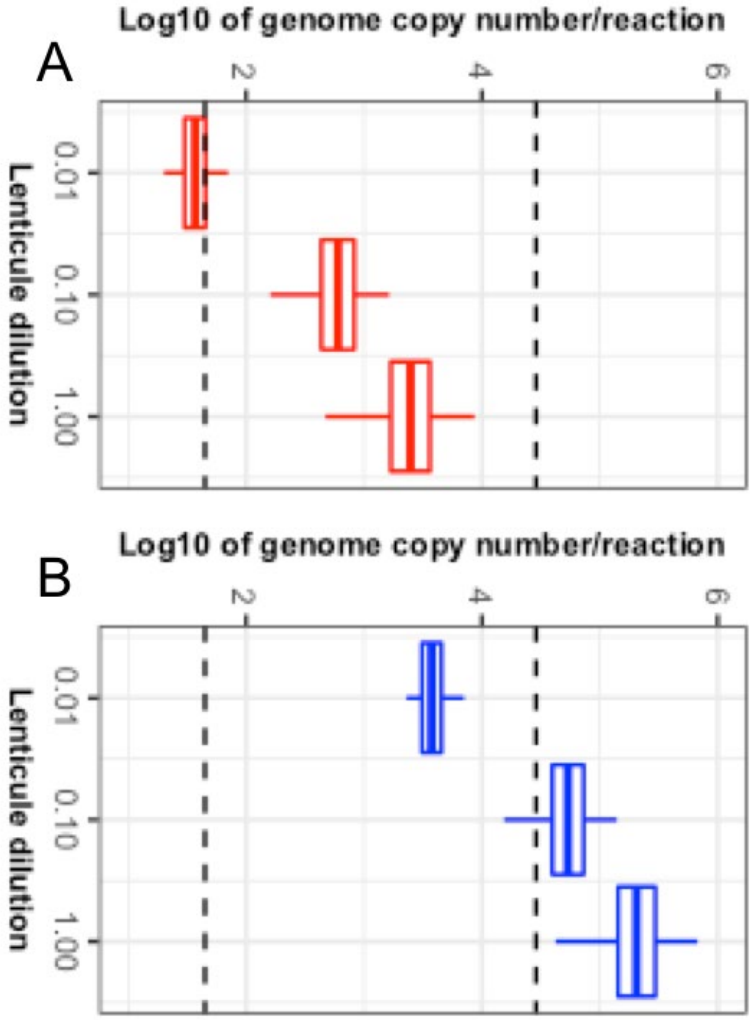


Figure 3


A
Log10 of genome copy number/reaction
2
4
6
Lenticule dilution
0.01
0.10
1.00
B
Log10 of genome copy number/reaction
2
4
6
Lenticule dilution
0.01
0.10
1.00