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Protocols

Real-world performance of the NeuMoDx™ HCV Quant Test for quantification of hepatitis C virus (HCV)-RNA

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ABSTRACT

Quantification of hepatitis C virus (HCV)-RNA in serum or plasma samples is an essential parameter in HCV diagnostics. Here, the NeuMoDx™Molecular System (Qiagen) was tested for the most common HCV genotypes and compared to the cobas c6800 system (Roche).

HCV-RNA from 131 plasma/serum samples from chronically infected patients was determined in parallel on the NeuMoDx and c6800 systems. Linearity was analysed using the four most common HCV genotypes (1−4) in our cohort. The coefficient of variation (CV) within (intra-assay) and between (inter-assay) runs was calculated based on HCV-RNA concentration. Quantitative HCV-RNA results were highly correlated on both test systems (R^2 = 0.7947; y = 0.94 x + 0.37). On average, the NeuMoDx and c6800 HCV RNA levels showed a mean difference of only 0.05 log10 IU/mL but with a broad distribution (±1.2 2 x SD). The NeuMoDx demonstrated very good linearity across all HCV genotypes tested at concentrations between 1.7 and 6.2 log10 IU/mL (R^2 range: 0.9257–0.9991) with the highest mean coefficient of determination for genotype 1 (R^2 = 0.9909). The mean intra- and inter-assay CV for both serum and plasma samples was <5 %. The NeuMoDx HCV-RNA Assay demonstrates high subtype-independent comparability, linearity, and reproducibility for the quantification of HCV-RNA in serum and plasma samples from chronically infected patients.

1. Introduction

The World Health Organization (WHO) estimates that 58 million people are infected with the hepatitis C virus (HCV) and are at risk of liver damage and liver cancer (WHO, 2017). With the availability of novel direct acting antivirals (DAAs), high sustained viral response (SVR) rates > 95 % are achieved in most patients (European Association for the Study of the Liver, 2020; Panel, 2018; Sarrazin et al., 2018). The most recent therapies are well-tolerated and have pangenotypic activity (EMA, 2017a, 2017b) with treatment duration between 8 and 12 weeks (Aghemo and Colombo, 2018; Brown et al., 2020; Chahine et al., 2017; Puoti et al., 2018). In 2016, this therapeutic success led the WHO to defining new targets for the elimination of HCV in 2030. The goal is to diagnose 90 % of people with chronic hepatitis C infection and treat 80 % of them (WHO, 2016).

Detection and quantification of HCV-RNA in serum or plasma samples is an important parameter in HCV diagnostics and essential for the diagnosis of an active HCV infection. HCV-RNA quantification is necessary for monitoring treatment responses to detect viral rebound or reinfections and/or the rare possibility of antiviral resistance (European Association for the Study of the Liver, 2020; Panel, 2018; Sarrazin et al., 2018). The quantitative assay of HCV-RNA is available on automated platforms, including more recently fully automated, closed systems with random access that cover the entire molecular diagnostic process from sample to result. In contrast to prior systems that work with batches of samples, random access systems allow continuous sample loading which further optimizes the analysis workflow and reduces hands-on time. One such random access system is the NeuMoDx™ 288 Molecular System (NeuMoDx; QIAGEN Sciences (Waltham, MA, USA)), launched in 2019.

Despite the availability of international reference standards,

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comparative studies of different diagnostic platforms and assays have sometimes shown discrepancies between quantitative test results when clinical samples were tested (Braun et al., 2017; Margariti et al., 2016; Wiesmann et al., 2015; Wirden et al., 2017). Thus, the aim of the current study was to investigate the new real-time PCR assay for quantitative HCV-RNA determination on the NeuMoDx platform, the NeuMoDx™ HCV Quant Test. For this purpose, a comparative analysis of the quantitative HCV-RNA results of the NeuMoDx and the widely used cobas c6800 system (Roche) was performed using patient specimens. To exclude assay-specific variations depending on the HCV-RNA assay itself and/or the different HCV genotypes, the linearity and reproducibility of the results were evaluated.

2. Materials and methods

2.1. Patient samples and HCV characterization

The serum and plasma samples from chronically HCV-infected persons used in this study were collected after written informed consent within the PEPSI study (Study for Therapy Optimisation of HCV Infection; ethics vote study ID: 4071). Baseline HCV-RNA levels were determined using the cobas 6800 HCV assay (Roche) as routine diagnostic testing. HCV genotyping was performed by sequencing the *NS5A* gene region of HCV with subsequent subtyping using the geno2pheno[hcv] tool (Kalaghatgi et al., 2016; Walker et al., 2019). The HCV samples covered the HCV genotypes 1–4 mostly prevalent in Europe.

2.2. NeuMoDx™ HCV Quant assay

The NeuMoDx™Molecular System is a fully automated molecular diagnostic system that integrates the extraction, purification, quantification, and results interpretation of infectious diseases pathogens with a turnaround time of about 75 minutes for the HCV assay. The HCV Quant assay is CE-IVD approved and targets highly conserved sequences in the 5' untranslated region in the HCV genome. The dynamic ranges of quantification for HCV assay is 7.7–1.6×10^8 IU/mL (0.9–8.2 log IU/mL, according to the manufacturer's instructions) across genotypes, respectively. The required input volume is 700 µL including 150 µl dead volume depending on sample tube.

2.3. Method comparison to the cobas® HCV Test

HCV-RNA quantification using the NeuMoDx™ HCV Quant assay was compared to the cobas HCV test on the 6800 platform, which is routinely used in our laboratory for diagnostics. As indicated by the manufacturer, the HCV assay has a linear quantitative range of 15–10^7 IU/mL, with a limit of detection (LOD) of 10.65 IU/mL in EDTA-plasma and 12.43 IU/mL in serum. For method comparison, 131 HCV residual plasma (n=88) and serum samples (n=43) obtained during routine diagnostics of patients with a chronic HCV-infection with viral loads determined on the c6800 reference system (HCV-RNA range: 27–11,300,000 IU/mL) were compared to results of the HCV Assay on the NeuMoDx™ platform.

2.4. Linearity

Subtype-specific linearity was analyzed using 19 HCV serum (n=8) or plasma samples (n=11) from patients harboring one of the four most common HCV genotypes (1−4) in our cohort. The selection criterion for the HCV-RNA serum or plasma samples was the initial HCV-RNA concentration, which had to allow at least four dilution levels for the analysis of the linearity and a comparable quantity of different matrices. Accordingly, in order to cover a broad range of the assay's quantitation spectrum, high titer plasma or serum samples were used. Dilution series in log dilutions were prepared with plasma from HCV negative donors and tested in duplicate with the NeuMoDx HCV assay according to the manufacturer's instructions.

2.5. Reproducibility

For intra- and inter-assay precision analysis, 7 plasma and 7 serum samples with different HCV-RNA concentrations (range 0–1000,000 IU/mL; provided by Qiagen) were quantified in triplicates per run in 10 different runs. The HCV samples were defined as HCV genotypes 1a and 3 as well as the Accuplex™ recombinant virus material for HCV (SeraCare). The coefficient of variation (CV) within (intra-assay) and between runs (inter-assay) was calculated based the HCV-RNA concentration (log10 IU/mL). For the determination of intra-assay variability, the triplicate analyses of the first runs were used for both serum and plasma samples. For the inter-assay analysis, the first result from the ten different runs was used for each sample. The tests were performed independently on five different days for serum and plasma samples.

2.6. Statistical analysis

Statistical analyses were performed using either R, version 4.3.1 including the mcr package (R Core Team, 2023; Potapov et al., 2023) or GraphPad Prism software, version 9.2.0 (GraphPad Software, San Diego, California USA). The overall correlation of HCV-RNA levels for samples quantified by the cobas6800 and NeuMoDx HCV assays was analyzed by simple linear regression and Passing-Bablok regression analysis (Passing and Bablok, 1983). Bland-Altman analysis was used for calculation of mean difference and standard deviation (SD) of assay agreement. Genotype-specific analysis of linearity was assessed by simple linear regression. Coefficient of variation (CV) was calculated for analysis of the intra- and inter-assay variability of the HCV assay. $P<0.05$ was considered statistically significant.

3. Results

To investigate the performance of the NeuMoDx HCV test, the HCV-RNA concentration of a total of 131 samples from our routine diagnostic with sufficient sample volume initially quantified on the cobas6800 instrument was quantified in addition on the NeuModx platform using the HCV Quant Test. The samples were selected to cover a broad range of viral loads (range 27–11,300,000 IU/mL). Comparing paired HCV-RNA values with the c6800 as the reference and NeuMoDx as the comparison group, we observed a high correlation ($R^2= 0.7947$; $y = 0.94\ x + 0.37$), (Fig. 1A, B). Viral loads determined with the NeuModx HCV assay were on average 1.6-fold higher than those determined with the cobas HCV assay, with only a mean difference (bias) of 0.05 log10 IU/mL, but with a broad distribution of the results (± 1.2 2 x SD, according to Passing–Bablok regression regression analysis), (Fig. 1C). However, in 93 % (122/131) of the samples tested, the difference between the assays was within the ± 1.2 2 x SD of the mean bias. Of the remaining samples 3.8 % (5/131) were higher and 3.1 % (4/131) lower with the NeuMoDx HCV assay with an average of 1.5 log levels (range −1.73–1.6 log IU/mL HCV RNA, Table 1). The samples also harboured a broad range of HCV genotypes, so the influence of genotype and/or subtype can be largely ruled out. However, a possible influence of the matrix could be observed in these outliers. While all samples with lower quantification the NeuMoDx HCV assay were plasma samples, the samples with higher quantification were exclusively serum samples. To further characterise these samples, they were additionally quantified using the Alinity m HCV assay. A large variation of 0.86 log levels (range −0.73–2.91) was also observed in comparison to the Alinity HCV-RNA assay. However, since such a large variation was also observed in comparison to the c6800 HCV-RNA assay with an average deviation of 1.09 log levels (range −1.62–1.64), an assay-specific effect can most probably be excluded.

Although by definition both a viral load change of more than 0.5 log or ± 2 SD indicates a significant clinical difference (Bonner et al., 2014;

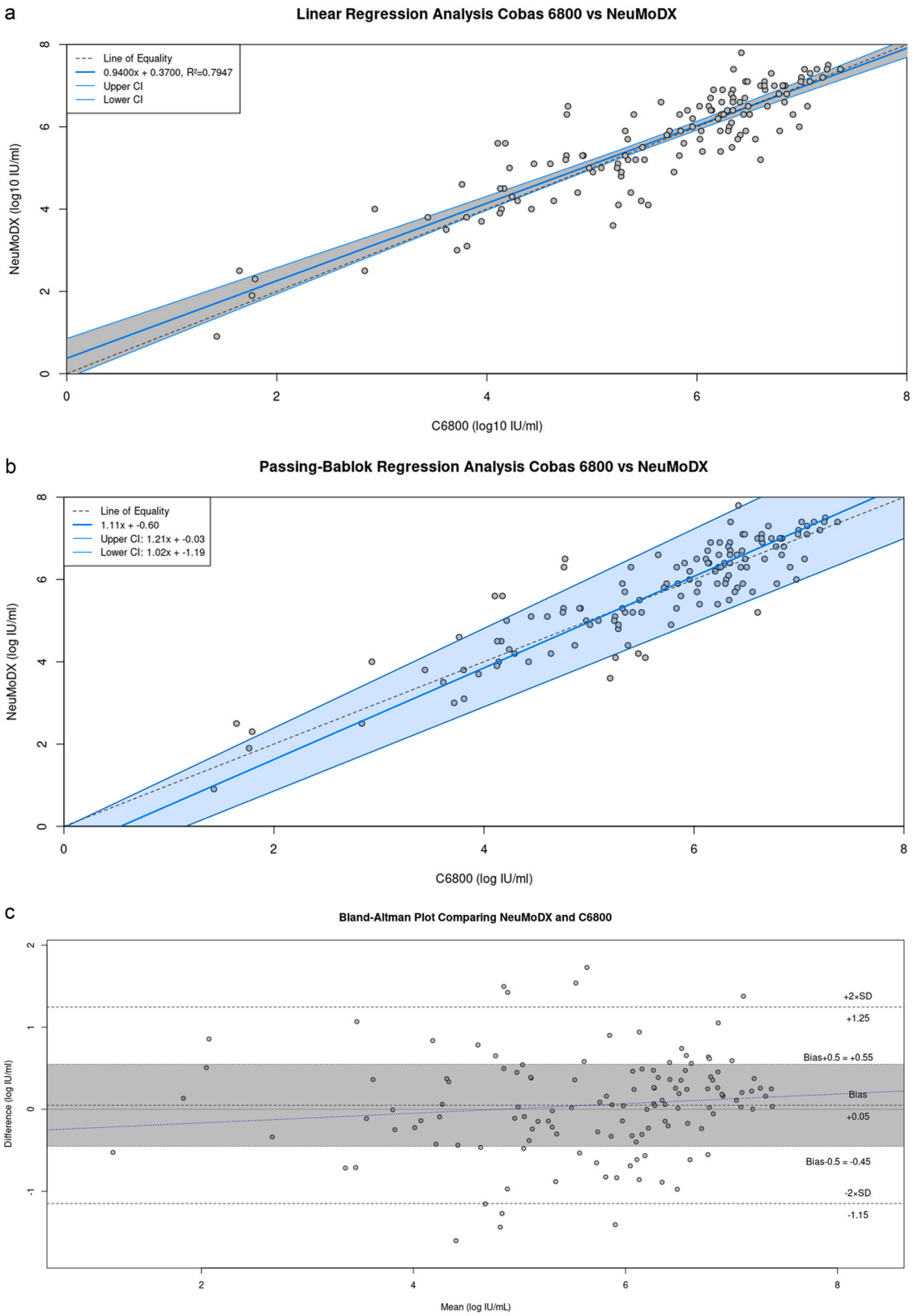

Fig. 1. Comparison of HCV-RNA concentrations detected with the NeuMoDx and the c6800 quantitative HCV-RNA assays. The HCV-RNA concentration of a total of 131 routine diagnostic samples was quantified in parallel on both the NeuModx and the cobas6800 instrument. A: Linear regression analysis. B: Passing-Bablok regression analysis. The dashed lines represent the lines of equality, and the thick blue lines show the regression lines and represent the line of best fit. The thin blue lines indicate the 95 % confidence intervals (CI) of the corresponding regression line calculated from the sample data. C: Bland-Altman analysis: The middle horizontal dashed line indicates the mean titer difference values of HCV-RNA concentration (log10 IU/mL) detected with the NeuMoDx and the c6800 system, the upper and lower dashed lines represent the ± 2 x SD limits from the means, and the upper and lower dotted lines represent the ± 0.5 log cut-offs of the mean values, the range of clinically tolerable deviations is highlighted in grey. The diagonal solid line (blue) presents the linear regression line.

Table 1
Samples with significant variation quantified by NeuMoDx, cobas and Alinity HCV RNA assays.

Samples +/- 2 SD			HCV-RNA log10 IU/mL		
sample ID	HCV genotype	matrix	NMDx	c6800	Alinity
10	1b	plasma	4.10	5.54	5.81
32	3	plasma	4.20	5.47	7.11
93	1a	plasma	5.20	6.61	4.99
94	3a	plasma	3.60	5.20	4.04
58	1a	serum	6.50	4.77	5.80
61	1a	serum	6.30	4.76	5.98
84	1b	serum	5.60	4.18	4.87
100	1a	serum	7.80	6.42	7.33
118	4r	serum	5.60	4.10	5.34

NMDx, NeuMoDx; c6800, cobas 6800

Saag et al., 1996), we detected a higher frequency of significant differences between the assays when we chose the 0.5 log cut-off (Fig. 1C). In total, 15.3 % of the samples (20/131) showed differences greater than 0.5 log and 22.1 % of the samples (29/131) showed differences less than 0.5 log. Here, a direct association with the matrix could not be observed, suggesting that there is no systematic over- or under-quantification due to the matrix, but rather a random observation.

To analyse the genotype-specific linearity of the NeuMoDx HCV assay, a panel of 19 different serum and plasma samples from patients with varying viral loads was tested. The panel included the genotypes 1–4 and a total of nine different HCV subtypes and was tested in serial dilutions. The regression analysis of the test results showed excellent linearity for all HCV genotypes tested (R^2 range: 0.9257–0.9991, Fig. 2, supplemental Table 1) with the highest mean determination coefficient for genotype 1 (R^2= 0.9909) followed by genotype 3a (R^2= 0.9791).

To investigate the intra- and inter-assay variability of the NeuMoDx HCV test, 7 serum and 7 plasma samples with a high range of HCV-RNA concentrations (range 0–1,000,000 IU/mL) were tested for reproducibility. The samples were tested in triplicates in 10 different runs on 5 consecutive days. Analysis of each triplicate of the same run showed low intra-assay variation with a coefficient of variation of 3.46 (range 1.69–6.93 %) for plasma and 4.20 % (range 0.99–10.58 %) for serum samples (Table 2). The inter-assay coefficient of variation of 10 independent runs was in average 4.70 % (range 1.96–8.91 %) for plasma and 3.46 % (range 0.31–12.79 %) for serum samples (Table 2). Overall, an increase in variability was observed with decreasing HCV RNA concentration, with the highest variability observed below 2 log10 IU/mL HCV RNA levels near to lower limit of detection (LLOD).

Across all analyses, the NeuMoDx HCV test showed a very low failure rate. Only 2.1 % (15/719) samples were assessed as “indeterminate” or “unresolved” indicating a failure in the amplification of the internal control or a failure in sample processing.

4. Discussion

The detection and quantification of HCV-RNA as a diagnostic marker is crucial for the detection and monitoring of chronic HCV infection. For this purpose, several assays are available on fully automated platforms. One of the latest systems is the NeuMoDx system from Qiagen. This study aimed to investigate the suitability of the newest quantitative HCV assay for routine diagnosis.

In a direct comparative analysis of quantitative HCV-RNA assays conducted on the NeuMoDx and cobas 6800 systems, a strong correlation between the results obtained from both assays was observed. However, it is worth noting that although only a small mean bias was observed in the method comparison (0.05 log10 IU/mL), the results of the HCV-RNA quantification showed a large variation of up to 2.4 log10 IU/mL, with a total of 37.4 % of the samples deviating more than 0.5 log levels defined as the cut-off for clinical significance. Not only the comparison between the HCV RNA tests on the NeuMoDx and the c6800 showed large variations in our analysis, but also the direct comparison of the outliers with the HCV RNA results of the Alinity assay, both with the results on the NeuMoDx, but also with the c6800 HCV-RNA assay.

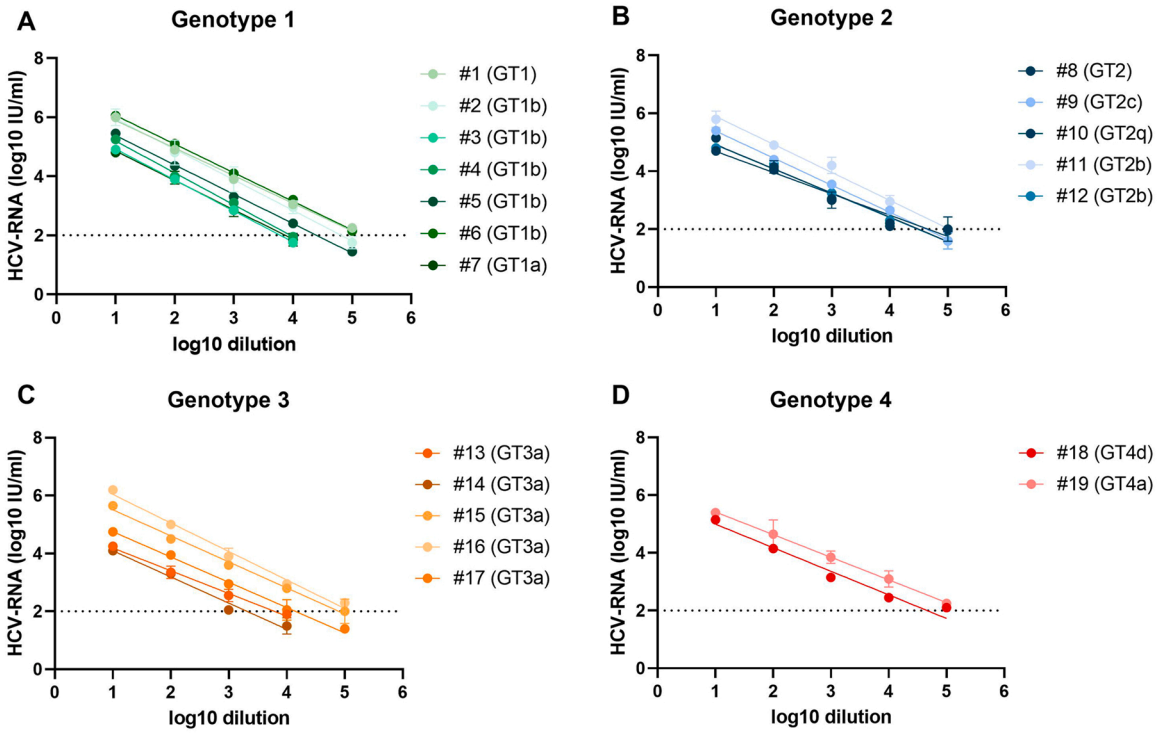

Fig. 2. Linearity analysis of HCV patient sample dilutions across the HCV genotypes 1–4 with the NeuMoDx HCV test. The linearity of the NeuModx HCV assay was evaluated using 19 HCV serum or plasma samples from patients with one of the four most common HCV genotypes (1−4) in our cohort. Dilution series in logarithmic dilutions were prepared and tested in duplicate. The dotted line indicates the expected limit of linearity (LOL) at 100 IU/mL HCV-RNA.

Table 2
Intra- and inter-assay reproducibility of the NeuMoDx HCV assay using plasma and serum samples.

Sample ID	HCV genotype	HCV-RNA (log10 IU/mL)	Plasma samples Intra-assay (n=3)			Inter-assay (n=10)			Serum samples Intra-assay (n=3)			Inter-assay (n=10)		
			Mean HCV-RNA (log10 IU/mL)	SD	CV (%)	Mean HCV-RNA (log10 IU/mL)	SD	CV (%)	Mean HCV-RNA (log10 IU/mL)	SD	CV (%)	Mean HCV-RNA (log10 IU/mL)	SD	CV (%)
B	3	1.35	1.67	0.12	6.93	1.50	0.13	8.91	1.70	0.10	5.88	1.68	0.21	12.79
E	1a	2	2.40	0.10	4.17	2.24	0.18	8.20	1.97	0.21	10.58	2.14	0.06	2.67
C	3	3	3.00	0.10	3.33	3.04	0.14	4.46	2.83	0.12	4.08	2.95	0.07	2.30
F	1a	4	4.30	0.10	2.33	4.32	0.11	2.60	4.27	0.06	1.35	4.21	0.05	1.16
D	3	5	5.03	0.12	2.29	5.01	0.10	1.96	4.93	0.12	2.34	4.95	0.07	1.50
G	Accuplex™ not specified	6	5.90	0.10	1.69	6.01	0.12	2.08	5.83	0.06	0.99	5.81	0.02	0.31
Total					**3.46**			**4.70**			**4.20**			**3.46**

SD, standard deviation; CV, coefficient of variation; Accuplex™, recombinant virus material for HCV (SeraCare)

Overall, these results demonstrate that there is no clear trend towards over- or under-quantification in the NeuMoDx HCV assay, but large variation between the different HCV-RNA assays. However, these assay-specific variations in quantitative HCV RNA results were also observed in other studies, which also showed an excellent correlation between the systems, but also differences in the absolute test results (Wiesmann et al., 2015; Wirden et al., 2017; Chevaliez et al., 2020; Mouna et al., 2020; Mourik et al., 2023). Thus, despite the existence of an international reference standard for quantifying HCV RNA, quantitative differences among HCV quantitative assays exist.

These results clearly indicate that any change in HCV RNA quantification assay for monitoring should take into account these assay-dependent variations in test results. It should also be noted that HCV RNA levels can be subject to large spontaneous variations in individual patients (Deutsch et al., 2013; Fierro et al., 2015). Thus, clinical decisions should not be made on the basis of small quantitative changes.

In addition to the direct comparative analysis with other HCV assays, the linearity of the NeuMoDx HCV assay was also analyzed as a function of HCV genotype. In this study, 19 different patient samples with HCV genotypes 1–4 available in our cohort were analyzed. All the samples harbouring different genotypes and subtypes showed a good linearity across all dilution levels, confirming the study of Besombes and colleagues (Besombes et al., 2021). It should be emphasised that our analyses were performed with patient material and the results are therefore representative of the use of the NeuMoDx HCV assay in routine diagnostics.

Not only linearity but also reproducibility is important for use of quantitative assays in virological diagnostics. Therefore, another aim of our investigations was the analysis of the intra- and inter-assay variability of the HCV assay. For this purpose, a logarithmic dilution series was measured in triplicates in 10 different assays on 5 consecutive days using serum and plasma samples. A very low intra-assay variation of less than 5 % was observed for both plasma and serum samples in triplicate analyses, which is comparable to, and in some cases better than other HCV Real-Time assays with variations of up to 34 % (Wiesmann et al., 2015; Park et al., 2022; Vermehren et al., 2017). The analysis of the inter-assay variation also showed only a small variation of less than 5 %, irrespective of the material used. The greatest relative intra- and inter-assay variation was observed for very low viral loads close to the assay-specific quantification limits, similar to observations in other studies (Wiesmann et al., 2015; Vermehren et al., 2017) and is most likely due to stochastic phenomena rather than inherent problem of an individual assay. Overall, however, very high reproducibility with low variation was demonstrated for HCV RNA results without matrix influence and a failure rate of only 2.1 % comparable to a failure rate of 2.3 % found in another study (Besombes et al., 2021).

With a sample volume of 550 µl plus dead volume, which is comparable to other assays on automated systems (Park et al., 2022; Vermehren et al., 2017), the NeuMoDx HCV test has an LOD of 8 IU/ml. In addition to this protocol, the NeuMoDx also offers the option of using only 200 µL sample volume, which increases the LOD to 30 IU/ml, but offers the possibility of testing samples with a low sample volume.

There are some limitations to this study. (I) There was a small sample size in the comparative analysis due to the high sample volume requirements for HCV quantification on different platforms, which were typically not available in archived routine diagnostic samples. (II) The HCV genotypes 2 and 4 were under-represented in our linearity analysis and HCV genotypes 5 and 6 were not tested as these genotypes are also under-represented in clinical routine diagnostic samples in Germany. (III) The upper limit of quantification was not reached in all samples, partly due to the fact that some HCV genotypes, e.g. the genotypes 2 and 3, are characterized by lower HCV-RNA concentrations (Jamalidoust et al., 2017; Riaz et al., 2016; Rong et al., 2012). (IV) Due to the limitations of the sample volume, the analysis of the genotype specific linearity could not be compared to another assay.

5. Conclusions

In conclusion, this study demonstrated high correlation of the NeuMoDx HCV assay with the established cobas 6800 HCV assay for the detection and monitoring of active HCV infection but also a broad distribution of the quantitative results. The results also indicate a high level of linearity across different genotypes and demonstrate precise performance of the NeuMoDx HCV-RNA assay. Taken together, the NeuMoDx HCV assay complements existing routine diagnostic platforms.

CRediT authorship contribution statement

Nadine Lübke: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Andreas Walker:** Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Martin Obermeier:** Writing – review & editing, Formal analysis. **Jennifer Camdereli:** Writing – review & editing, Visualization, Validation, Resources, Methodology. **Martha Paluschinski:** Writing – review & editing, Visualization, Validation, Methodology. **Lara Walotka:** Writing – review & editing, Visualization, Validation, Methodology. **Anna-Kathrin Schupp:** Writing – review & editing, Validation, Supervision, Methodology. **Inga Tometten:** Writing – review & editing, Visualization, Validation, Methodology. **Sandra Hauka:** Writing – review & editing, Visualization, Validation, Methodology. **Eva Heger:** Methodology, Writing – review & editing. **Jörg Timm:** Writing – review & editing, Writing – original draft, Supervision, Investigation,

Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2024.114937.

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