



## RESEARCH ARTICLE

# REVISED maxRatio improves the detection of samples with abnormal amplification profiles on QIAgen's artus HIV-1 qPCR assay [version 3; peer review: 2 approved]

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## Abstract

**Background:** Accurate viral load (VL) determination is paramount to determine the efficacy of anti-HIV-1 therapy. The conventional method used, fit-point (FP), assumes an equal efficiency in the polymerase chain reaction (PCR) among samples that might not hold for low-input templates. An alternative approach, maxRatio, was introduced to compensate for inhibition in PCR.

**Methods:** Herein, we assessed whether maxRatio could improve VL quantification using 2,544 QIAgen artus HI virus-1 RT-PCR reactions. The assay's standard dilutions were used to build external standard curves with either FP or maxRatio that re-calculated the VLs.

**Results:** FP and maxRatio were highly comparable (Pearson's  $\rho=0.994$ , Cohen's  $\kappa=0.885$ ), and the combination of the two methods identified samples ( $n=41$ ) with aberrant amplification profiles.

**Conclusions:** The combination of maxRatio and FP could improve the predictive value of the assay.

## Keywords

HIV, qPCR, quantification, maxRatio, viral load.



This article is included in the Pathogens gateway.

## Open Peer Review

Approval Status

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1. **Emiliano Panieri** , Sapienza University of Rome, Rome, Italy
2. **Joel Tellinghuisen** , Vanderbilt University, Nashville, USA

Any reports and responses or comments on the article can be found at the end of the article.

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**Author roles:** **Marongiu L:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Shain EB:** Conceptualization, Formal Analysis, Software, Validation; **Martinelli M:** Formal Analysis, Validation; **Pagliari M:** Formal Analysis, Validation; **Allgayer H:** Funding Acquisition, Project Administration, Supervision

**Competing interests:** No competing interests were disclosed.

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**REVISED Amendments from Version 2**

1. We re-arranged the acronyms in the paper to make the labelling clearer, specifically by clarifying the use of internal control (IC) in the method section.
2. We corrected a wrong proportion to 45.5%.
3. We encountered no issues in downloading fully functional comma-separated files. We recommend using the "original file format" option provided by Harvard Dataverse when downloading the datasets. We added a dictionary file explaining the fields' names used in the datasets associated with the present work.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Infection with HIV-1 accounts for a global prevalence of 38 million cases and a one million deaths yearly<sup>1</sup>. An accurate viral load (VL), typically carried out by quantitative polymerase chain reaction (qPCR), is pivotal for addressing the efficacy of antiviral therapies<sup>2</sup>. The threshold level of detection for the HIV-1 VL has been reported in the range 20–44 viral genomic copies per milliliter (c/mL)<sup>3,4</sup>.

qPCR data are usually analyzed by the fit-point (FP) method, which assumes equal amplification efficiency between samples<sup>5</sup>. However, anomalies in the background fluorescence at low template input, can affect the quantification<sup>6–8</sup>. An alternative method, maxRatio, was introduced to overcome these issues<sup>9</sup>. It has been reported that maxRatio conferred a marginal increase in assay accuracy over FP<sup>10,11</sup>.

FP provides only a quantification cycle (Cq) value, which is then used to calculate VL. MaxRatio, instead, gives two parameters: one associated with the reaction's efficiency (MR) and one equivalent to, albeit distinct from, Cq. These two parameters can be linked to bestow a quantitative cycle (FCNA) compensated for inhibition.

In the present work, we aimed to determine whether maxRatio could improve the determination of HIV-1 VL. We compared the quantification of HIV-1 VL computed by FP and maxRatio on a dataset generated with the QIAgen artus HIV assay, which has a reported limit of detection of 35.5 c/mL, and we showed that maxRatio could pinpoint samples with abnormal amplification profiles.

## Methods

### Dataset

The amplification data (see *Underlying data*<sup>12</sup>) obtained with the QIAgen artus HI Virus-1 RT-PCR kit were collected by the Public Health England Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Hills Road, Cambridge CB2 0QW, UK, during the year 2016. All data were anonymized before use. The reactions were subdivided into clinical samples, control dilutions (CDs), and non-template

controls (NTCs). The CDs were based on known dilutions of *in vitro* transcribed HIV-1 RNA provided by the artus kit, corresponding to 405, 4,050, 40,500, and 405,000 c/mL. Each reaction also contained a primer set targeting an internal control (IC) to assess the proper extraction of the samples.

### Data analysis

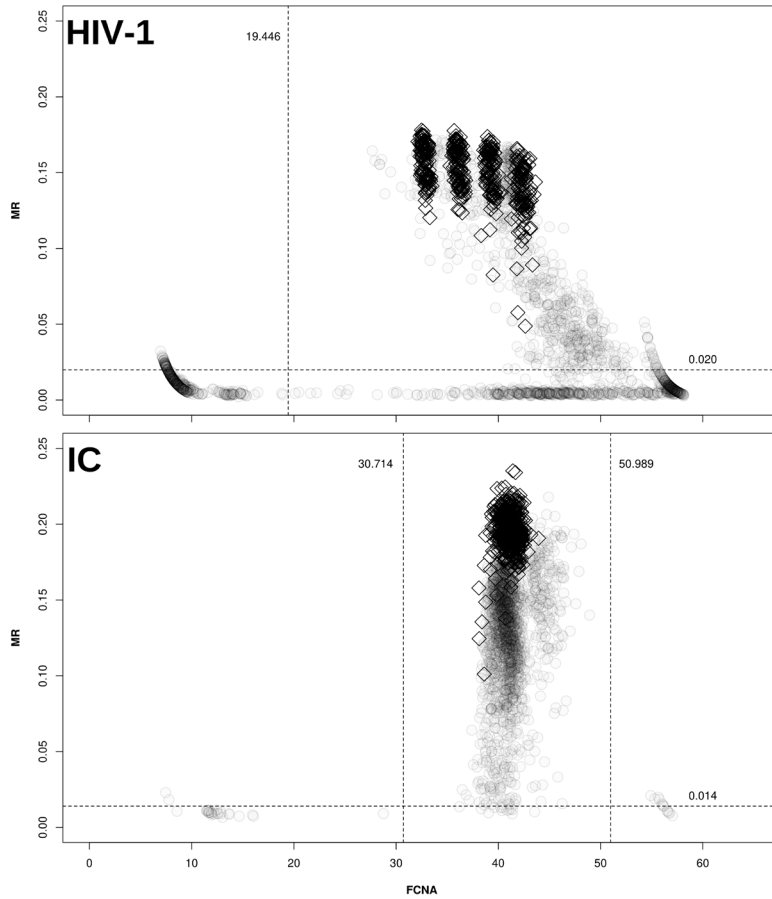
The FP method generated the Cq by registering the fractional cycle where the fluorescence passed the threshold of 0.2 units. The maxRatio transform of the amplification data and determination of the cut-offs were computed as previously described<sup>9,10</sup>. Different operators visually inspected the reaction's profiles and classified each reaction as either passed or failed. Using R v.3.6, linear models (standard curves, SC) were built on the CDs and applied to calculate the copy numbers according to the formula  $10^{(x-b)/m}$  where  $x$  is the quantitative cycle (either Cq or FCNA),  $b$  and  $m$  are the intercept and slope, respectively, of the linear models<sup>13</sup>. Testing the difference between the expected and the calculated copy numbers was carried out with an unpaired t-test. VL correlation was obtained with the Pearson product-moment coefficient  $\rho$ <sup>14</sup> and agreement between methods was tested with the Cohen's  $\kappa$ <sup>15</sup>; both are reported with their 95% confidence interval (CI).

## Results

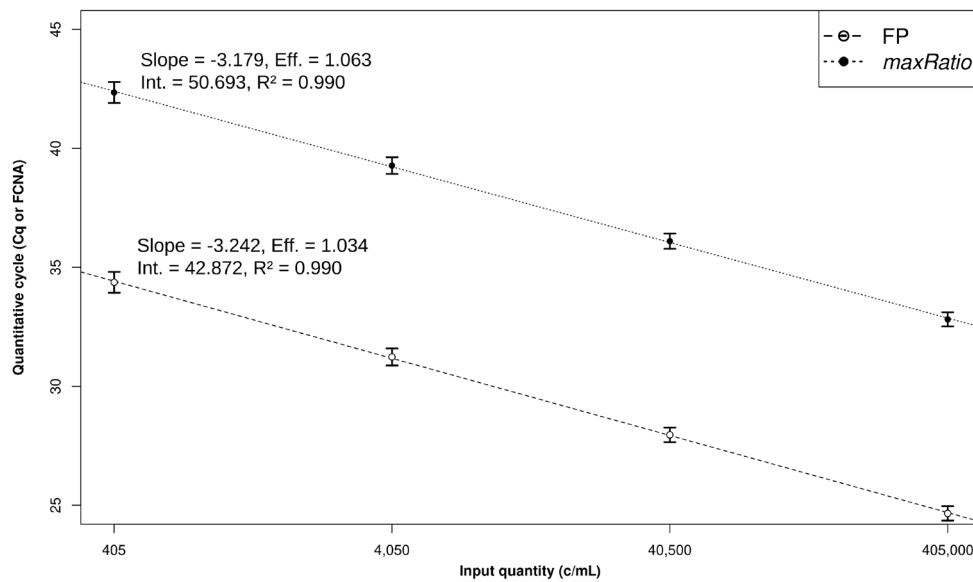
The present dataset was derived from 122 individual *artus* HIV-1 runs, corresponding to 2,544 reactions (480 CDs, 122 NTCs and 1,931 clinical samples). The cut-offs obtained by expectation-maximization analysis were multiplied by 2.7 to generate the values used to filter the *maxRatio* data, as depicted in [Figure 1](#).

The CDs were used to build SCs ([Figure 2](#)) that quantified both the CDs ([Table 1](#) and [Figure 3](#)) and the clinical samples ([Figure 4](#)). Overall, the VLs obtained with the two methods were very strongly correlated ( $\rho = 0.994$ , 95% CI: 0.993-0.994) and the agreement in the stratification of the reactions into reactive and non-reactive was noticeably robust ( $\kappa = 0.885$ , 95% CI: 0.863-0.907). Both methods identified 307 (15.9%) and 28 (1.5%) samples within and above the quantification range 405–405,000 c/mL ( $\rho = 0.988$ , 95% CI: 0.985-0.991 and  $\rho = 0.992$ , 95% CI: 0.982-0.996, respectively), and 1,571 (81.3%) below this range ( $\rho = 0.844$ , 95% CI: 0.829-0.858).

FP quantified 22 reactions below the detection limit of 20 c/mL that were interpreted as non-reactive by maxRatio. Conversely, maxRatio identified 18 reactions below 20 c/mL while FP quantified them above this level. Visual inspection of the amplification profiles of the reactions failed by FP showed that 10 of them (45.5%) had a proper sigmoid shape for the IC signal that, however, was discarded by the FP (as exemplified in [Figure 5A](#)). In contrast, the others had a low signal for either HIV-1 or IC recovered by maxRatio ([Figure 5B](#)). Conversely, 15 (83.3%) of the reactions failed by maxRatio showed either a low IC or HIV-1 input ([Figure 6A](#)), whereas the FCNA of the remaining reactions produced fractional VL that were rounded to 0 c/mL ([Figure 6B](#)).



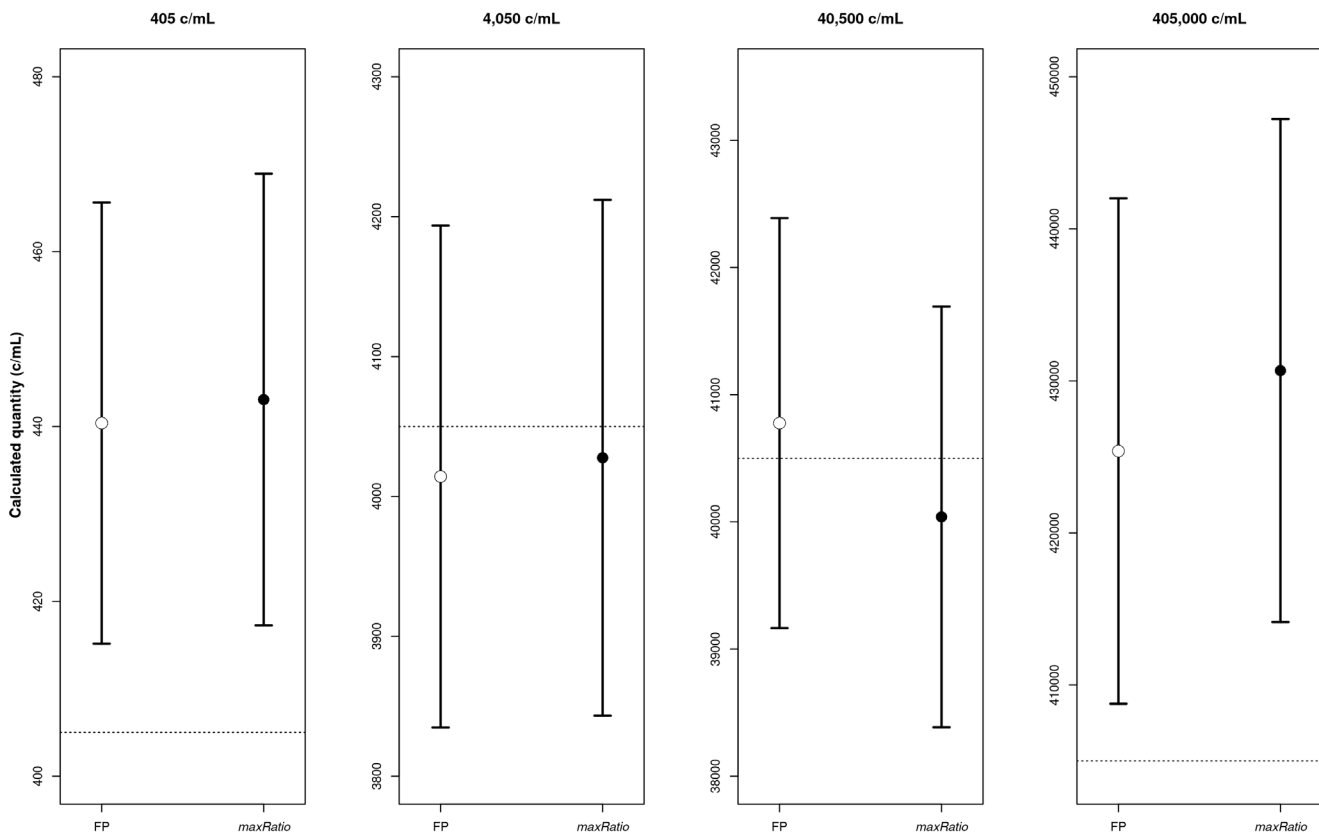
**Figure 1. Cut-offs for maxRatio.** Clinical samples (○) and CDs (◇) are plotted on the maxRatio plane; the numbers report the obtained cut-offs for MR (horizontal lines) and FCNA (vertical lines). Upper panel: MR/FCNA pairs for the HIV-1 target. To note how the CDs form four distinct clusters, corresponding to the different standard dilutions. Lower panel: MR/FCNA pairs for the IC target. To note that the data form a single cloud because the IC input was virtually the same for all reactions. The presence of two outlier groups at low and high FCNA values required to instantiate two cut-offs.



**Figure 2. Linear models and CD quantification.** Development of the linear models. The Cq (○) and FCNA (●) were used to build SCs for FP (dashed line) and maxRatio (dotted line). The dots and bars represent the mean and standard deviation of the data, respectively. The characteristics of the models are reported.

**Table 1. Comparison of copy numbers for the control dilutions.** The mean VL is reported together with the 95% CI (calculated), the difference between the calculated and the expected concentration (difference), and the result of the t-test (p-value). Statistical significance is represented by \* and \*\* for values below 0.05 and 0.01, respectively. The copy numbers are given in c/mL.

Dilution	FP			maxRatio		
Expected	Calculated	Difference	p-value	Calculated	Difference	p-value
405	440 (415-466)	35	0.006**	443 (417-469)	38	0.004**
4,050	4,014 (3,835-4,194)	36	0.694	4,028 (3,843-4,212)	22	0.811
40,500	40,777 (39,164-42,389)	277	0.735	40,039 (38,385-41,693)	-461	0.582
405,000	425,384 (408,765-442,002)	20,383	0.017*	430,673 (414,132-447,215)	25,673	0.003**

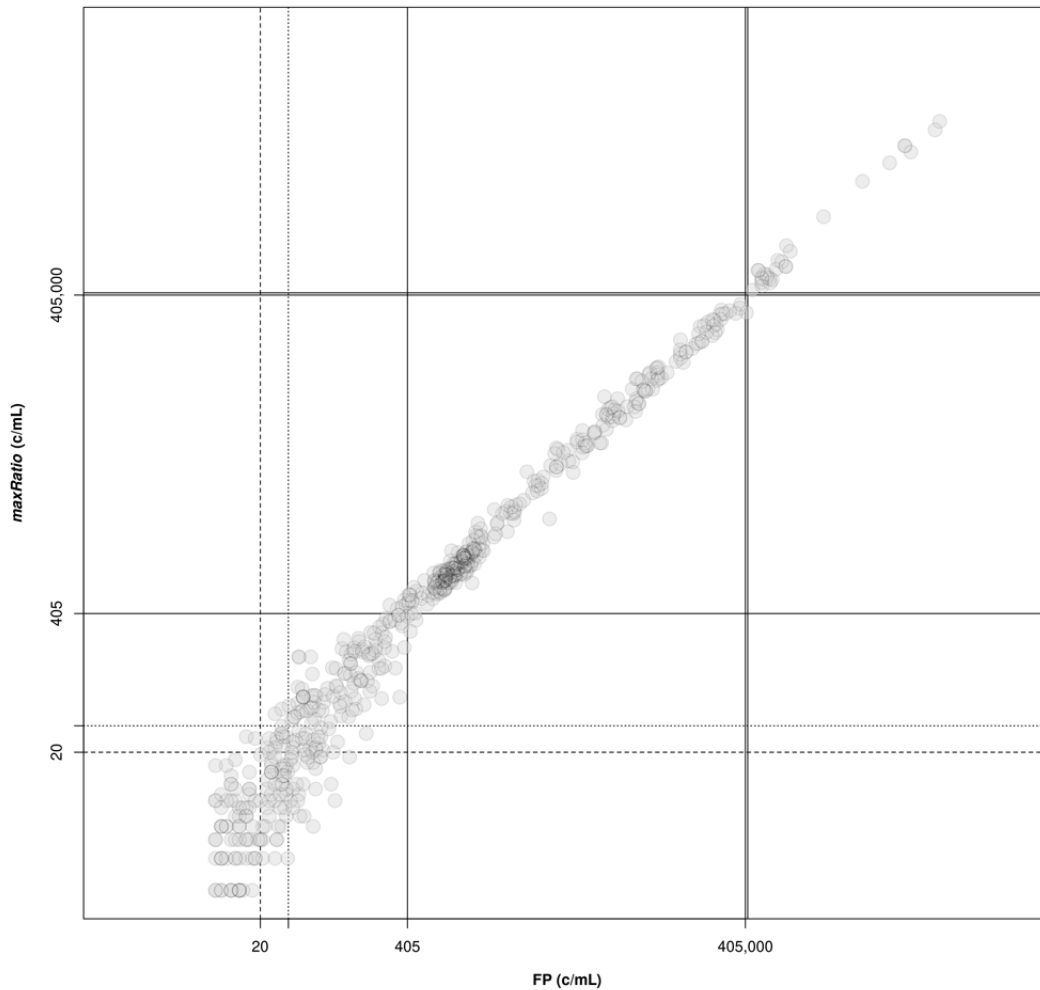


**Figure 3. CD quantification.** The four panels represent the calculated copy numbers obtained using FP (○) or maxRatio (●) for each dilution. The dots and bars represent the mean and the 95% CI of the data, respectively.

### Discussion

The purpose of the present work was to assess the potential benefits of maxRatio in determining HIV-1 VL given its inherent compensation of PCR inhibition. Contrary to our expectation, the SCs we built with either FP or maxRatio were virtually

the same. Both methods gave VLs significantly divergent from the expected copy numbers at the lower and upper CDs, and maxRatio was, in general, more discrepant from the expected copy numbers than FP. Even concerning the samples' quantification, the two methods produced essentially the same VLs.

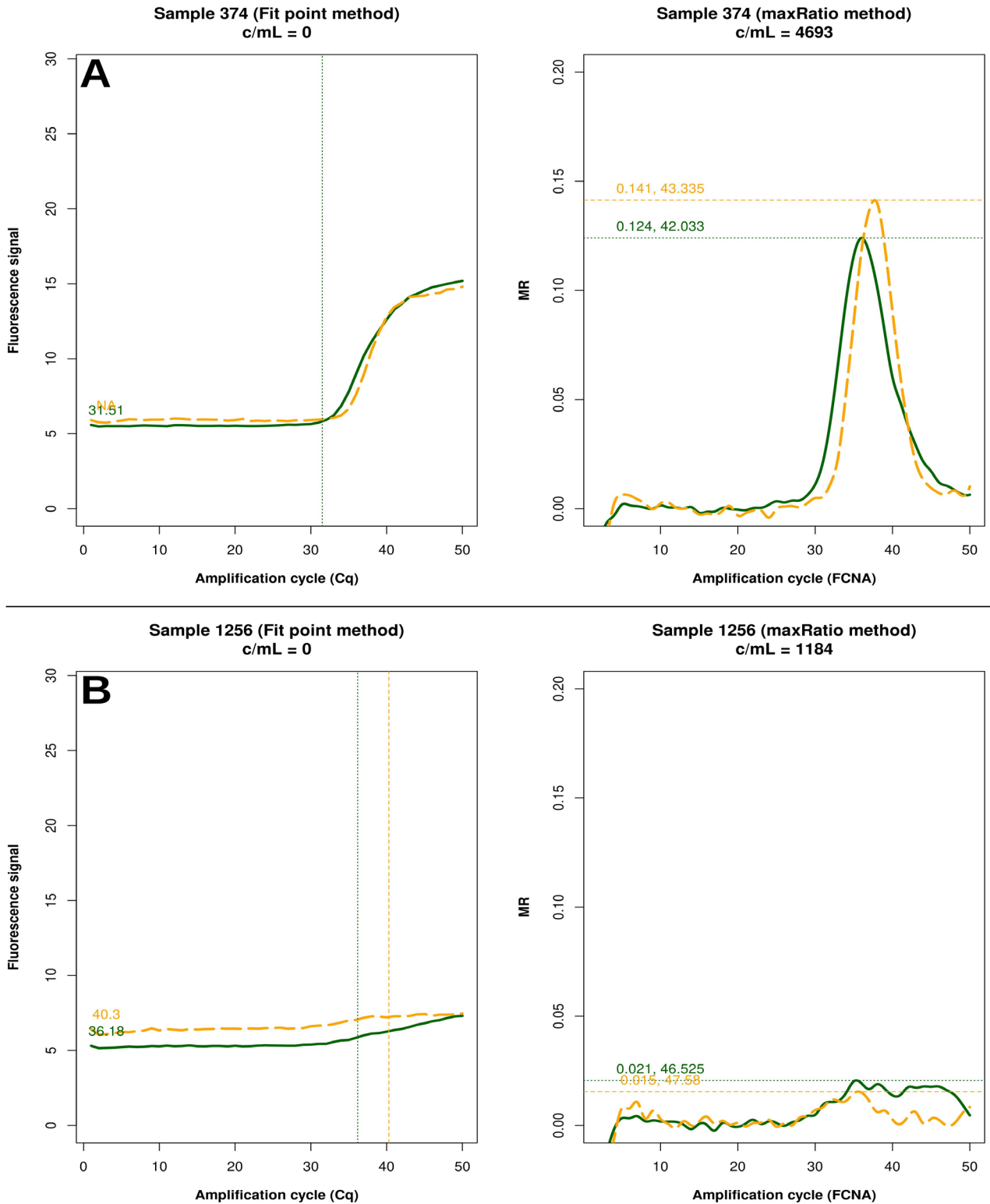


**Figure 4. Correlation of VL obtained by FP and maxRatio.** The external standard curves were used to calculate the VL for the clinical samples. The following thresholds are depicted: lower (solid lines) and upper (double solid lines) limits of the quantification range; limit of detection of HIV-1 diagnostic in general (dashed line) and artus HI Virus-1 assay in particular (dotted line).

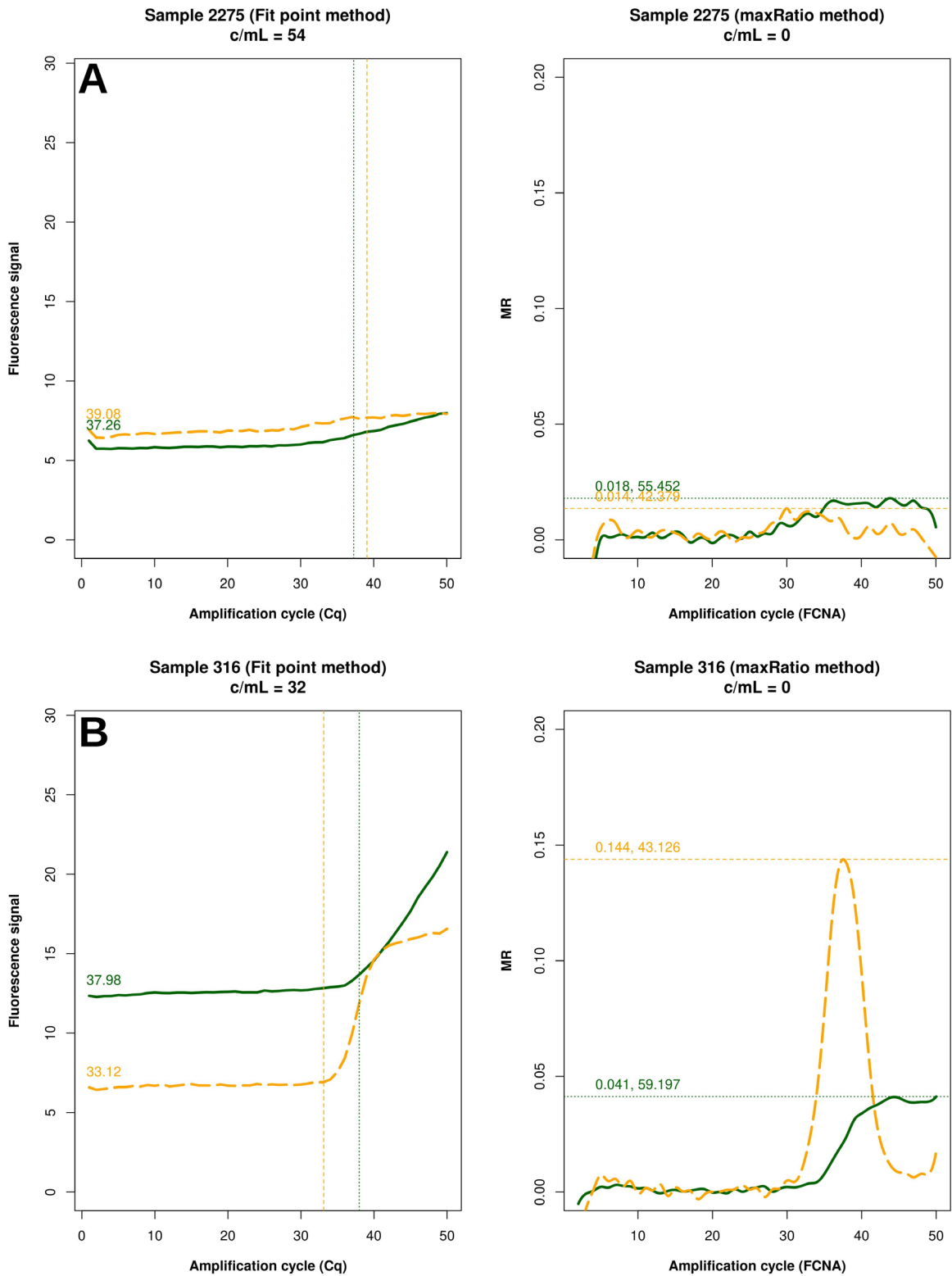
The main difference between the two methods was in terms of sample's reactivity. By accepting the reactions identified as non-reactive by FP, but reactive by maxRatio, there would have been 18 false-negative results. Conversely, 15 samples identified as non-reactive by maxRatio showed aberrant IC that raised quality control, rather than false-positive, issues overlooked by FP.

The current use of maxRatio is to confirm the reactivity determined by FP on the Abbott m2000rt platform. Our data supported this combination as the most effective approach for screening purposes. Samples in disagreement between FP and maxRatio would require further assessment that could reduce the workload involved in issuing the results and minimize the risk of providing false results.

The present work had some limitations. Firstly, the sample size was small. Since the correlation between the two methods was high (99.4% overall and 84.4% below the quantification limit of 405 c/mL), a more extensive sample set would provide only a marginal improvement in comparing the two algorithms. However, more samples will provide more instances of amplification profiles that are processed differently by FP and maxRatio. In the present study, 40 reactions out of 1931 samples (2.07%) showed discrepancies in quantification at the clinical threshold of 20 c/mL. Expanding such a subset of discrepant reactions to, say, 4000 could provide a database of profiles that can facilitate (perhaps using machine learning approaches) identifying the characteristics that led to the failure in quantification. Moreover, analysis of qPCR chemistries other than *artus HI virus-1* might determine whether such



**Figure 5. Samples not quantified only by FP.** Example of raw amplification profiles for the samples whose FP did not provide a VL. The panels display the FP (left) the maxRatio (right) transforms of the amplification data. The solid line represents the HIV-1 template and the dashed line the IC template. **(A)** The majority of the samples had a proper IC profile but the output was undetermined. **(B)** The minority of the samples showed low target inputs.



**Figure 6. Samples not quantified only by maxRatio.** Example of raw amplification profiles for the samples whose maxRatio did not provide a VL. The panels display the FP (left) the maxRatio (right) transforms of the amplification data. The solid line represents the HIV-1 template and the dashed line the IC template. **(A)** The majority of the samples had abnormal IC profiles that were overlooked by FP. **(B)** The minority of the samples showed proper amplification profiles, but the calculation provided fractional VLs that were rounded to zero.



characteristics are common to all reactions or peculiar to the kit used herein. Secondly, the CDs were prepared by diluting the control samples provided in the kit, but the actual concentration was not measured. Finally, we did not have access to the actual issued results; thus, we could not confirm the official VL values.

In conclusion, we compared FP and maxRatio in providing HIV-1 VL. Contrary to our expectations, maxRatio did not give a better quantification than FP, but combining the two methods could minimize issuing false results.

## Data availability

### Underlying data

Harvard Dataverse: artusHIV\_amplificationData. <https://doi.org/10.7910/DVN/0QQNPF>

This project contains the following underlying data:

- amplificationDataRaw.tab. (Raw amplification data, file is comma-delimited.)
- viralLoads.tab. (Raw viral loads data, file is comma-delimited.)

- dictionary.tab (explanation of the fields' names used in the other files, file is comma-delimited.)

Data are available under the terms of the [Creative Commons Zero "No rights reserved" data waiver](#) (CC0 1.0 Public domain dedication).

## Acknowledgements

We would like to thank Dr. Martin D. Curran (Public Health England, Clinical Microbiology and Public Health Laboratory, Addenbrooke's Hospital, Cambridge UK) for providing the amplification data.

We would also like to thank Prof. Clementina Elvezia Cocuzza (University of Milano Bicocca, Department of Medicine and Surgery, Monza, Italy) and Dr. Maria Serena Beato ('Istituto Zooprofilattico Sperimentale delle Venezie', Department of Diagnostics in Animal Health, Legnaro, Italy) for their moral support.

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# Open Peer Review

Current Peer Review Status:  

Version 3

Reviewer Report 14 September 2021

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Joel Tellinghuisen 

Department of Chemistry, Vanderbilt University, Nashville, TN, USA

With minor exceptions, I accept the author's explanations and clarifications. Regarding the data, I wish I had saved what I downloaded initially but I did not. I recall clicking somewhere on the left side of the download page, and in two attempts, I got nothing sensible. I *was* able to get the data now, though they seem to be space-separated rather than csv; and the file would be much smaller if the fluorescence data didn't have 13 figures after the decimal point!

My suggestion of just specifying a minimal *plateau* level was based on the observation that most of the questionable rxs never actually grow, and those that grow only a little for late cycles ultimately give 0 copies anyway. I did not intend this to mean anything about threshold levels.

I agree that my thinking was flawed when I suggested that the MR cycle would roughly equal the FDM: Maximum first difference indeed does not equal maximum ratio. My comment about instrument dependence was also wrong, as indeed the ratios will not change with scaling. On the other hand, two identical growth profiles superimposed on different baselines will give different MRs, and that would seem to make their FCNAs different. In this connection, is the conversion from FCN to FCNA used here as given in eq 2 in ref 9? That eqn is  $FCNA = FCN - \log_2(MR)$ . When I apply that, for example, to the green curve in Fig. 5B (MR), I get  $FCNA \sim 36 - \log_2(0.021) = 41.6$ , which is much smaller than the given 46.525. Should that eqn perhaps have a factor of 2 with the  $\log_2$  term?

**Competing Interests:** No competing interests were disclosed.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

Reviewer Report 01 September 2021

<https://doi.org/10.5256/f1000research.58856.r93096>

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**Emiliano Panieri** 

Department of Physiology and Pharmacology, Faculty of Pharmacy and Medicine, Sapienza University of Rome, Rome, Italy

I have no further comments

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Oncology, cellular and molecular biology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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### Version 2

Reviewer Report 13 July 2021

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**Joel Tellinghuisen** 

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The authors use a method apparently first described in their ref 9 (2008), which was coauthored by the 2<sup>nd</sup> author of this work. This maxRatio method seems to be a useful tool for automated screening of very large numbers of qPCR reaction profiles for non-detects, though it is not clear to me that it is much better than, for example, just setting a low limit on acceptable plateau levels, perhaps combined with an upper limit on acceptable  $C_q$  values, beyond which the template number would round to zero. I did not see any comparisons of this sort either here or in ref 9. And I am not well enough acquainted with the various instruments to know what other possible tools there are for this kind of discrimination. However, it does appear to me that the title is not quite a truthful description of the work, since the authors note in Results that the MR method and the “fixed point” (FP) method with which they compare it gave nearly identical “stratification of the reactions into reactive and non-reactive.” And then early in the Discussion (and again in the final paragraph of the Conclusion), they acknowledge that the two methods gave virtually identical standard curves (SC). On the other hand, it is not clear to me how the discrimination was done in the FP method. This may have been by manual inspection (bottom of 1

<sup>st</sup> column, p 3), in which case the MR method would still be advantageous. (But, as noted, the “inspection” could also be automated.)

The near-agreement in the 2 standard curves is not surprising, because the FCNA is essentially a first-derivative maximum (FDM), shifted slightly from the use of finite differences and even more by the correction  $-\log_2(\text{MR})$  (which is not mentioned here but is given in eq 2 in ref 9). Incidentally, this correction must only apply for a particular instrument and its fluorescence axis scale, because it clearly changes as the data are scaled. In any event, both  $C_t$  (used here for  $C_q$  in the FP method) and the FDM are legitimate  $C_q$  markers, so they should indeed give similar SCs. In fact,  $C_t$  is arguably the poorest  $C_q$  marker, for reasons Spiess and I have explained, most recently in *Biomol. Detect. Quantif.* 17 (2019) 100084.<sup>1</sup> Thus, the use of, for example, a *relative* threshold obtained from a fit model that includes the baseline function, should give even better FP results.

I wanted to check some of the authors' results but unfortunately could not easily get the data from their referenced source (p 9 and ref 12). The Excel files seem not to be .csv, as labeled; the .tab files are text, but the application for opening them is not given (GitHub perhaps?). Nor is their content explained. I did attempt to reproduce their SCs in Figure 2, by digitizing the plotted data (WebPlotDigitizer, <https://apps.automeris.io/wpd/>). My results more or less confirmed theirs with one exception: My uncertainties for the back-calculated  $N_0$  values for FP in Table 1 were ~10% smaller than theirs for the first 3 concentrations but ~40% larger for the highest. The relative errors here depend on only the structure of the data, so the last should also have been small by ~10% (this discrepancy from digitization limitations). The larger error makes the discrepancy  $< 2s$  for this case, removing one of the two values judged to be discordant.

Some specific problems:

1. Some labels are not defined: EM (1<sup>st</sup> para of Results), SD (5<sup>th</sup> line of Discussion). And IC appears in Fig. 1 but isn't defined until several paragraphs after Fig. 1 is mentioned.
2. I don't understand the introductory sentence in the last para of Results. And if the 10 which had sigmoidal appearance were of 22, that would be 45.4%, not 43.4%.
3. While it is clear that the reaction profiles in Figs 5B and 6A should not give results, I don't understand why those in 5A do not.

## References

1. Tellinghuisen J, Spiess AN: qPCR data analysis: Better results through iconoclasm. *Biomol Detect Quantif.* 2019; **17**: 100084 [PubMed Abstract](#) | [Publisher Full Text](#)

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

No

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** statistical data analysis

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

Author Response 19 Jul 2021

### **Luigi Marongiu**

Eric B. Shain is indeed the developer of maxRatio, as reported in Ref. 9. Shain and Marongiu collaborated to apply maxRatio in qPCR classification as either reactive or non-reactive in other works. The purpose of the present study was to assess whether maxRatio could provide more accurate copy numbers than the classical FP due to maxRatio's inherent capability to compensate for qPCR inhibition. Contrary to our expectations, maxRatio did not provide a significant improvement over FP. Hence we considered our results as "grey," and we embraced the F1000Research policy of welcoming "confirmatory and negative results, as well as null studies... irrespective of the perceived level of interest or novelty".

Our title reflects the fact that maxRatio helped identify reactions that were classified as non-reactive by FP. Unsurprisingly, the classification of the majority of the reactions was similar between the two methods. The focus of the analysis was instead on the few disagreed reactions, which are also those where an operator might have more difficulties inspect. About half of the reactions classified as non-reactive by FP had a proper amplification profile, suggesting false-negative results. Conversely, this ratio was only about a fifth using maxRatio. Despite the small sample set, we believe that when applied to the vast volumes characteristic of diagnostic laboratories, such a minor improvement could still be beneficial.

As reported in the Method section, the amplification profile of the individual reactions was visually inspected by a trained operator, and the operator classified each reaction as either reactive or non-reactive. Conversely, classification by maxRatio was done directly by mathematical methods. The evaluation of the amplification profiles generated by maxRatio was done post hoc purposely to compare the two methods.

For the sake of brevity, we did not report the details of the FCNA's calculation. However, we referred to the original paper cited on maxRatio, as correctly noted by the reviewer, including the details of the expectation-maximization (EM) step reported in the first

paragraph of the results section. We agree with the reviewer that the slope of the standard curves built with either FP or maxRatio should be similar. The focus of the work was not on the subtle differences between the standard curves based on the two methods but their applications.

The driving force of this and the previous works on the application of maxRatio done by Shain and Marongiu was to avoid the definition of a baseline threshold level. Firstly, the guidelines for the threshold value are too generic to give universal outcomes. Secondly, the threshold value might not be the same for each PCR assay. Thirdly, in our experience, the threshold level is set without much mathematical consideration. Conversely, maxRatio provides an objective way to quantify the reactions that dispense the threshold level altogether. As the reviewer remarked in his cited paper (Biomol Detect Quantif 2019;17:100084) the threshold level does not hold throughout the PCR while "most workers have taken [it] as a level near baseline where it is hoped that eq. 2 [associating the copy number to the efficiency of the reaction] remains valid". We agree with the conclusion of the author in his paper -- that is, that the absolute threshold is a poor choice -- because we believe that maxRatio allows overcoming its use. We disagree with the reviewer when he stated, "it is not clear to me that [maxRatio] is much better than, for example, just setting a low limit on acceptable plateau levels". If the reviewer meant to lower the threshold, there would be firstly the problem mentioned above of where to set the cut-off level, and secondly, there would be a trade-off between picking up more true positives at the expense of more false positives. This trade-off would require further determination of the impact of the analytical method on the sensitivity and specificity of the assay that (a) was beyond the scope of our work and (b) is skipped altogether by using maxRatio.

Furthermore, we disagree with the reviewer when he remarked, "FCNA is essentially a first-derivative maximum (FDM), shifted slightly from the use of finite differences and even more by the correction  $-\log_2(\text{MR})$ ". In fact, maxRatio is not the same as FDM. maxRatio does not use differences. maxRatio consistently identifies a cycle number earlier and closer to the exponential portion of the amplification curve. In addition, maxRatio provides a second measure (MR) which is highly useful in evaluating reactive-nonreactive status. FCNA helps correct the quantitation of PCR results where the response is partially inhibited. FDM cannot do this. We also disagree with his statement, "This correction must only apply for a particular instrument and its fluorescence axis scale because it clearly changes as the data are scaled." maxRatio works by calculating ratios. It is entirely unaffected by multiplicative scaling effects.

We loaded the datasets following the Harvard Dataverse's guidelines, essentially uploading a comma-delimited table. We quickly downloaded the original data simply by clicking on the "download" icon and selecting the download option. On downloading the data saved on Harvard Dataverse, the comma-separated values original file format" can be opened directly with a spreadsheet application (in our case: LibreOffice Calc).

On downloading the alternative version "tab-delimited", Harvard Dataverse automatically adds the extension .tab, but the file's layout remains comma-delimited, implying that the spreadsheet applications do not appropriately recognize such file format. We recommend downloading the dataset only in its original format. Nevertheless, the procedure of data downloading is under the domain of Harvard Dataverse.

We believe that the names of the fields in the datasets were self-explicatory, but we have now included a dictionary to supply more information.

We assume that the discrepant back-calculated copy numbers might be due to the different approaches taken (actual calculation based on the quantification thresholds in our paper, digitalization applied by the reviewer).

**Competing Interests:** None

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The authors properly addressed all my previous requests. I am satisfied with their reply and I have no additional issues.

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly



**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Oncology, cellular and molecular biology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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Version 1

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**General comment:**

The paper from Marongiu *et al.* presents an alternative approach to the conventional use of fit-point (FP) or MaxRatio methods, which can be used to accurately determine the viral load of HIV-1 patients under experimental conditions wherein the amplification efficiency might vary across the samples, as in the case of low-copy number of viral RNA or in presence of PCR inhibitors. This is a clinically relevant issue since the VLs widely differ among HIV-1 infected individuals, potentially leading to issues of false-positive/negative results when the viremia is very low or the efficiency of PCR reactions is not uniform. It is therefore of utmost importance to improve not only the analytical sensitivity of qPCR methods but also their predictive value to avoid that a substantial fraction of HIV-1 patients will be incorrectly diagnosed, causing a negative impact on their prognosis and treatment monitoring. In this regard, the approach proposed by the authors, based on both FP and MaxRatio is straightforward and when correctly applied can represent a cost-effective alternative to the time-consuming and labor-intensive work required to validate false positive and false negative results. In general, the manuscript is well written, concise and presented in a clear, scientifically-sound way. The data are quite exhaustive, corroborated by appropriate statistical analysis and firmly support the authors' conclusions.

However, some minor changes will be required to improve the MS quality, which are listed below:

- The authors correctly indicate that the small-size of the tested samples represents a limitation to their study. Can also the authors discuss whether increasing the sample size is



expected to proportionally increase the accuracy of the proposed method? In other words, in the authors' opinion, is the number of tested samples expected to influence the predictive value of the FP + MaxRatio use? It might be useful to further elaborate this aspect in the discussion to help the readers to gain insights on this limitation.

- The authors should indicate which kind of t-test was used to assess the statistical significance of the data contained in Table 1 (i.e. paired or unpaired, with or without other corrections such as Welch's or Bonferroni's) and which software was used to perform the statistical analysis.
- In the Results paragraph it is suggested to mitigate the following statement: "...the agreement in the stratification of the reactions into reactive and non-reactive was almost perfect ( $\kappa = 0.885$ , 95% CI:0.863-0.907).". Instead, "noticeably robust" can be a good alternative.
- Few lines below it is suggested to slightly rearrange the sentence "...the FP identified 22 reactions above the limit of detection of 20 c/mL, while maxRatio failed the quantification" (i.e. while Max Ratio was unsuccessful).
- Few lines below it is suggested to substitute "sigmoid outlook" with "sigmoidal shape".
- Few lines below it is suggested to change "...of the remainder of the reactions" into "...of the remaining reactions".
- In the Figure 1 legend, the authors describe a panel A (MR/FCNA pairs for the HIV-1 target) and a panel B (MR/FCNA pairs for the IC) which are however absent in the Figure 1. Please add the correct indication or either refer to the panels as upper and lower.
- In the Figure 5-6 legend, it should be corrected "the panels displays" with "the panels display".
- In the last three lines of the Discussion section please rephrase the sentence: "...maxRatio did not give a better quantification than FP, but combining the two methods could minimize issuing false results" into "maxRatio did not result in a better quantification than FP but when combined together these two methods could..."

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Oncology, cellular and molecular biology

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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