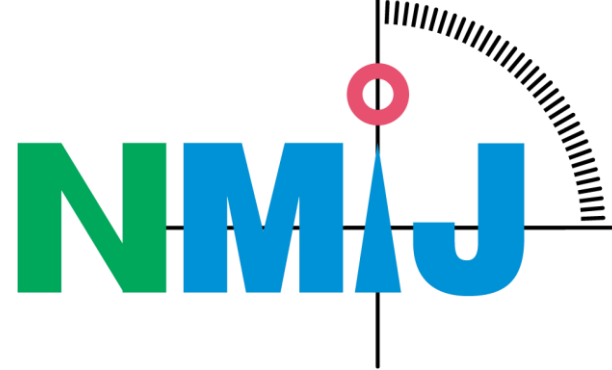


Investigating Factors Influencing RNA Enumeration: Trilateral Evaluation and Benchmarking Using One- and Two-Step Reverse Transcription-Digital PCR with a Certified Reference Material

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Introduction

Measurements of ribonucleic acid (RNA) are key to viral pathogen diagnostics, vaccine manufacturing, development of gene and cell therapies and monitoring of cancer treatment (Figure 1) but often lack traceability. Reverse transcription-digital PCR (RT-dPCR) is a potential reference methodology for sequence-specific enumeration of RNA, however a better understanding of sources of bias and measurement uncertainty would support its application as an SI-traceable technique.

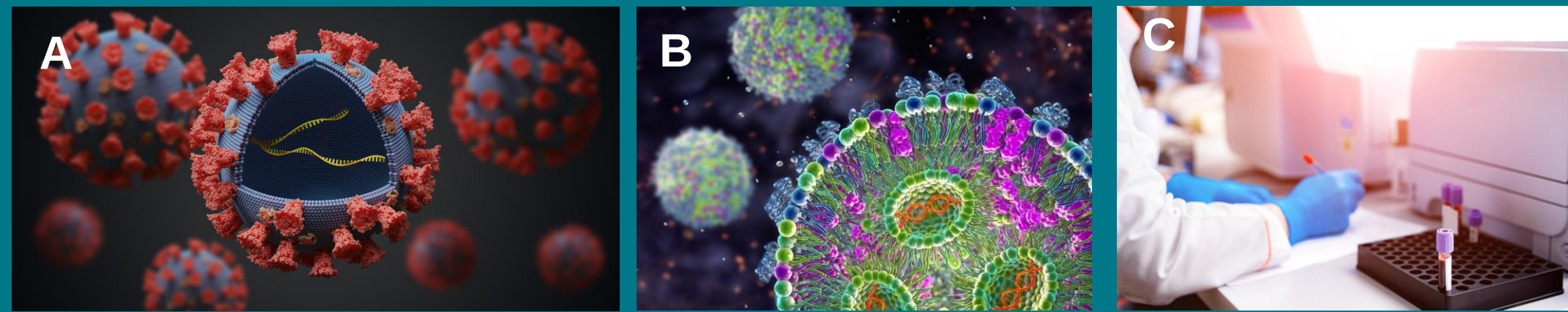


Figure 1. A. SARS-CoV-2 virus containing RNA genome; B. RNA lipid nanoparticle; C. Cancer blood test

NMIJ CRM 6204-b

The features of NMIJ CRM 6204-b (Fig. 2A)

- SI-traceable quantification by LC-IDMS* and ICP-MS* (Figure 2B)
- Five types of single-strand RNA with different lengths (533 or 1033 bases) and sequences
- Artificial sequences making it suitable as an internal control
- Uniform base composition: GC content is approximately 50 %

The production scheme of NMIJ CRM 6204-b

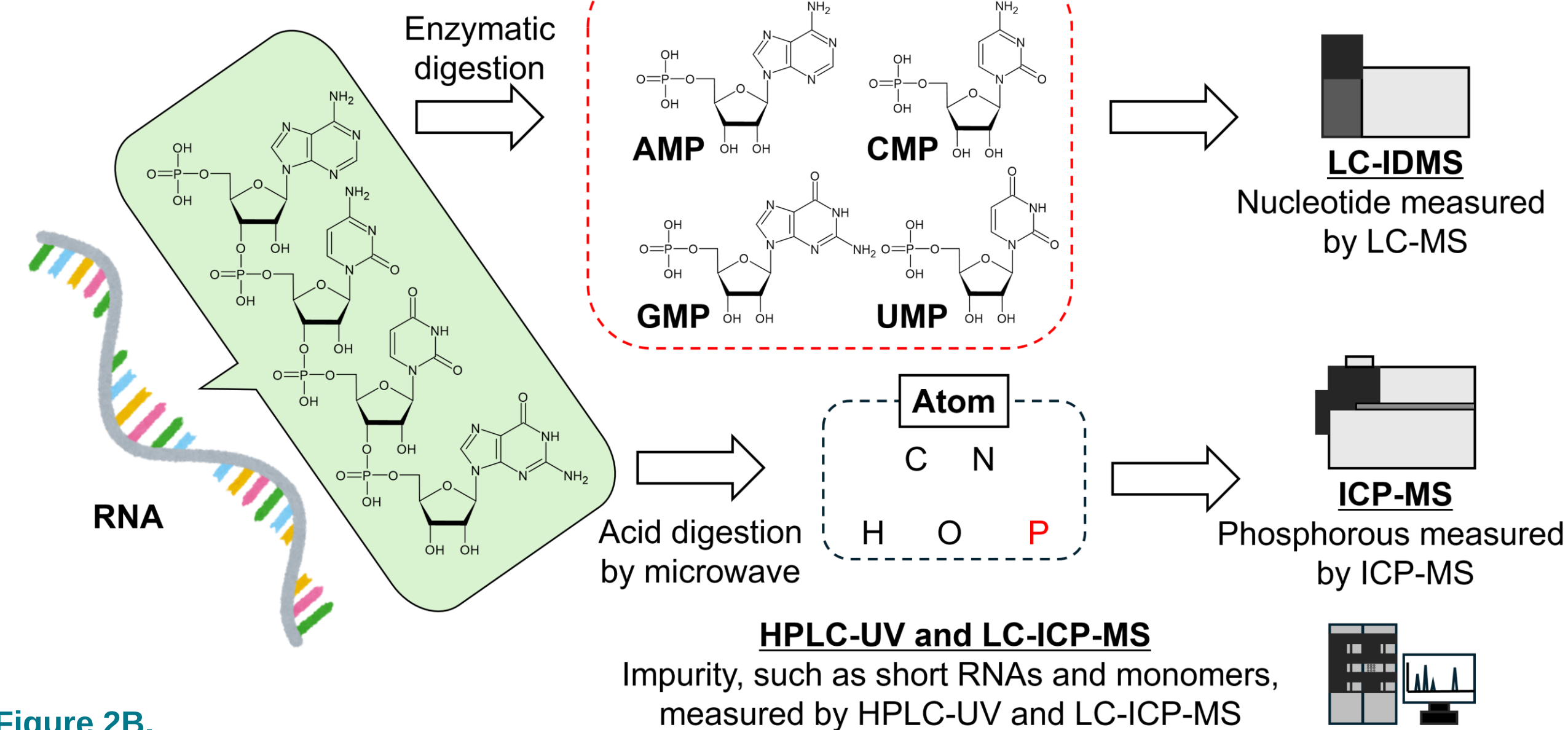


Figure 2B.

As a purity analysis, the presence of impurities (short RNAs and monomers) in the RNA solutions were checked by HPLC-UV and LC-ICP-MS (Figure 2C). The chromatograms with a single peak and no impurity peaks were obtained, indicating that these RNA solutions are very high purity. CRM homogeneity and stability were evaluated using same methods.

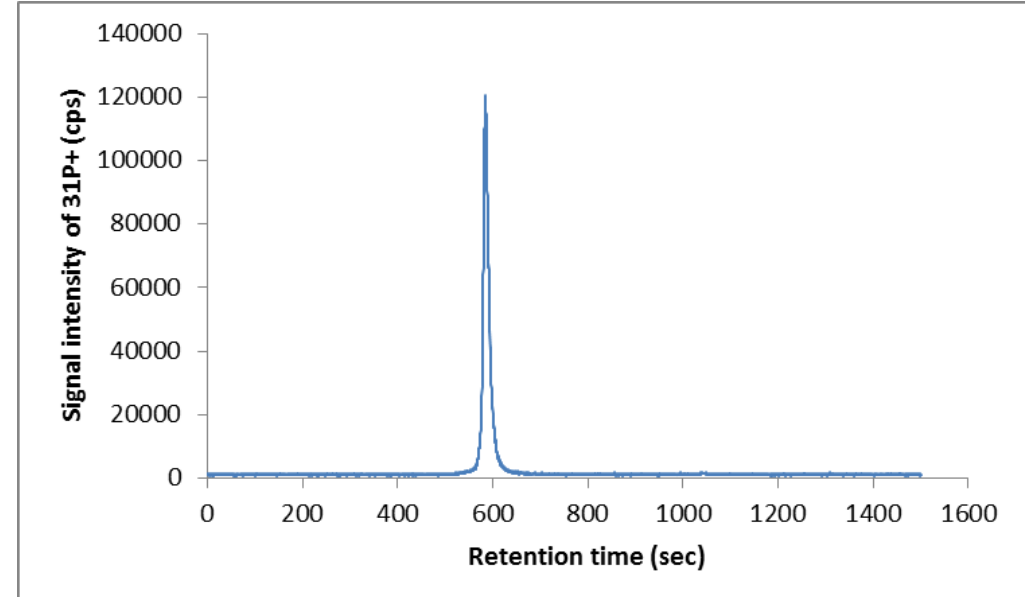
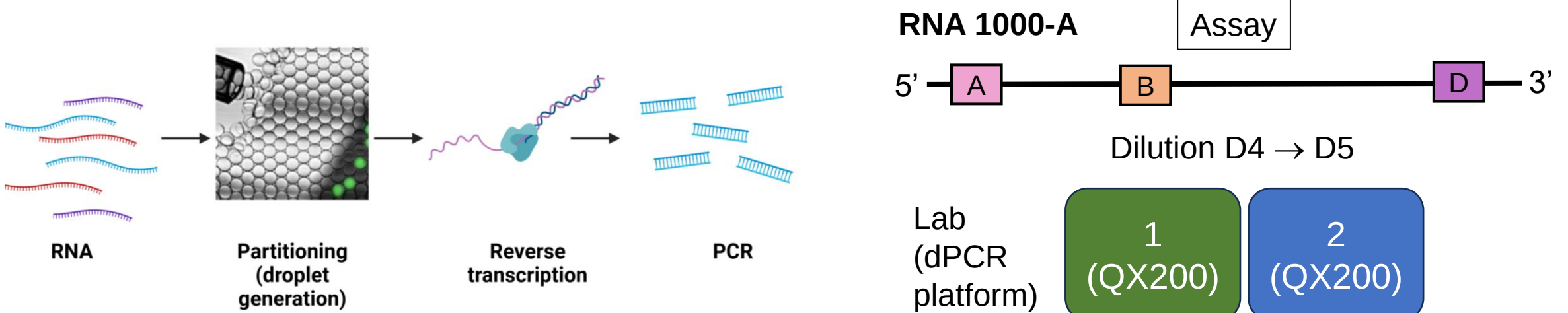


Figure 2C. The chromatogram of LC-ICP-MS

Methods

One-step RT-dPCR interlaboratory comparison



Two-step RT-dPCR interlaboratory comparison

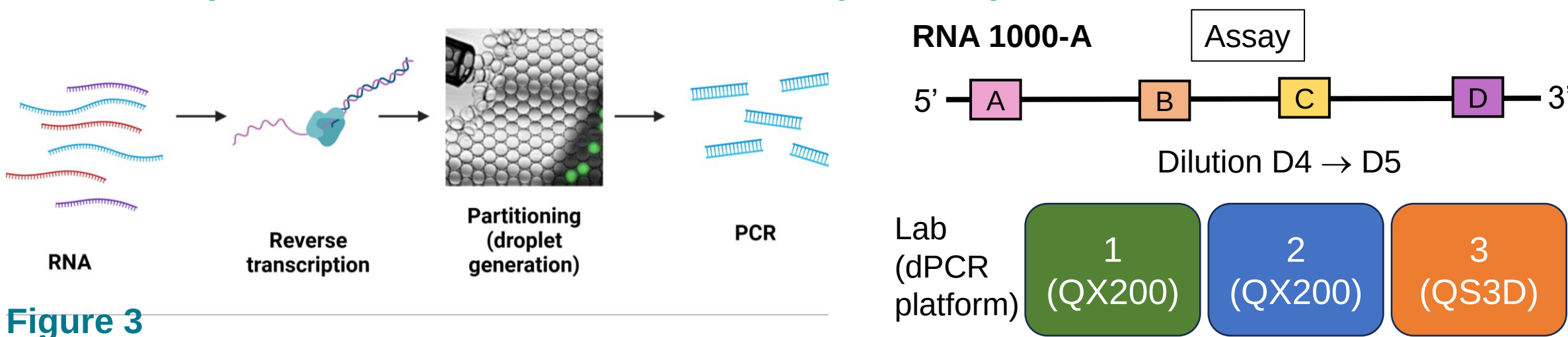


Figure 3

Results

- Results between two analyte concentrations D4 (mean RNA copies/dPCR partition (lambda) 1.46) and D5 (mean lambda 0.15) were in close agreement (Figure 4A).
- Interlaboratory variation was observed (7%) (Figure 4B, Table 1) but within- and between dPCR platform variation was similar.
- For the one-step RT-dPCR comparison, pre-selection of the three best performing assay resulted in smaller variation (3%) compared to two-step comparison, where all assays were included (Figure 4A/Table 1).
- Standard uncertainties for a single measurement encompassing all sources of random variation are calculated in Table 1.

Table 1: (right). Analysis of RT-dPCR sources of uncertainty

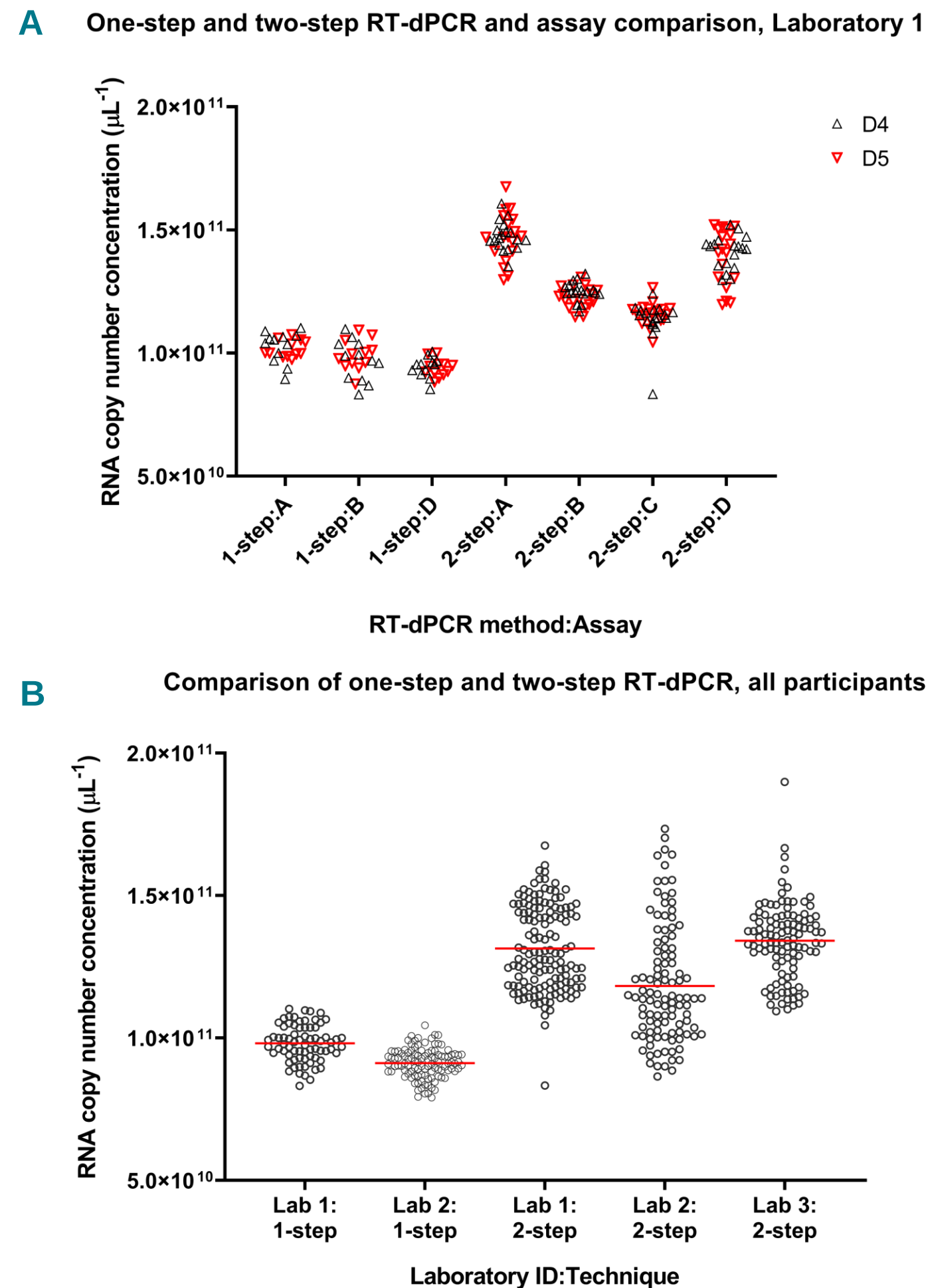


Figure 4. A. Intra-laboratory and B. Inter-laboratory sources of variation in RT-dPCR. (B) Red line, mean.

Source of random error	Relative standard uncertainty
Assay (one-step; 3 assays)	3.0%
Assay (two-step; 4 assays)	11.5%
Interlaboratory	7.1%
Between-experiment	4.7%
Residual (repeatability)	6.5%
Unit (homogeneity)	0.9%
Combined (min. assay variation)	11.2%
Combined (max. assay variation)	15.7%

Comparison with CRM assigned value

Significant differences were found between one- and two-step RT-dPCR methods, with two-step results in agreement with the assigned value and uncertainty of the CRM, whilst one-step results were ~27% lower (Figure 5).

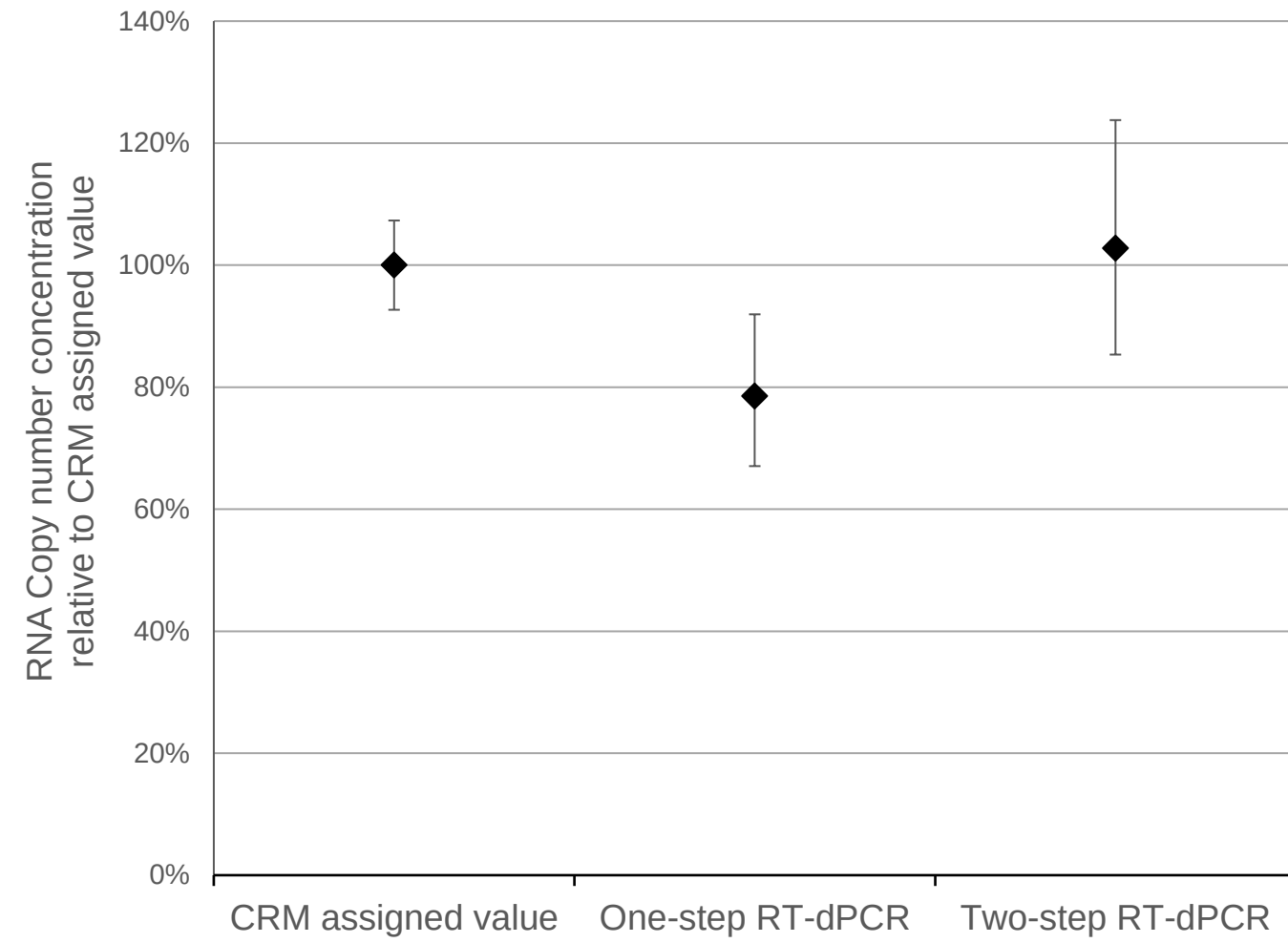


Figure 5: Comparison between CRM assigned value and RT-dPCR-based estimates. Error bars show expanded uncertainty ($k = 2$ (CRM), $k = 3.18$ (One-step RT-dPCR), $k = 2.57$ (Two-step RT-dPCR))

Conclusions and future work

- This study helps to clarify sources of bias and uncertainty which have been observed as interlaboratory variation CCQM-NAWG studies – P199¹ and P199.b² (%CV 15%-31%).
- By employing a CRM with concentration values assigned by orthogonal techniques (such as ID-MS and ICP-MS), RT-dPCR systems can be qualified and sources of bias defined.
- Further work to evaluate sequence-related variation in RT efficiency and mechanisms to correct for bias which consider RNA sequence diversity are needed.
- In addition, techniques to ensure the sequence and fragment size (molecular weight) purity of RMs (Figure 5A,B) are critical for underpinning the traceability of enumeration methods, which count full length sequences, through orthogonal methods, which detect their constituent building blocks (e.g. nucleotides).
- These approaches will support the application of RT-dPCR as a reference method for sequence-specific quantification (Figure 5C) in a range of sectors including laboratory diagnostics, biotechnology and synthetic biology.

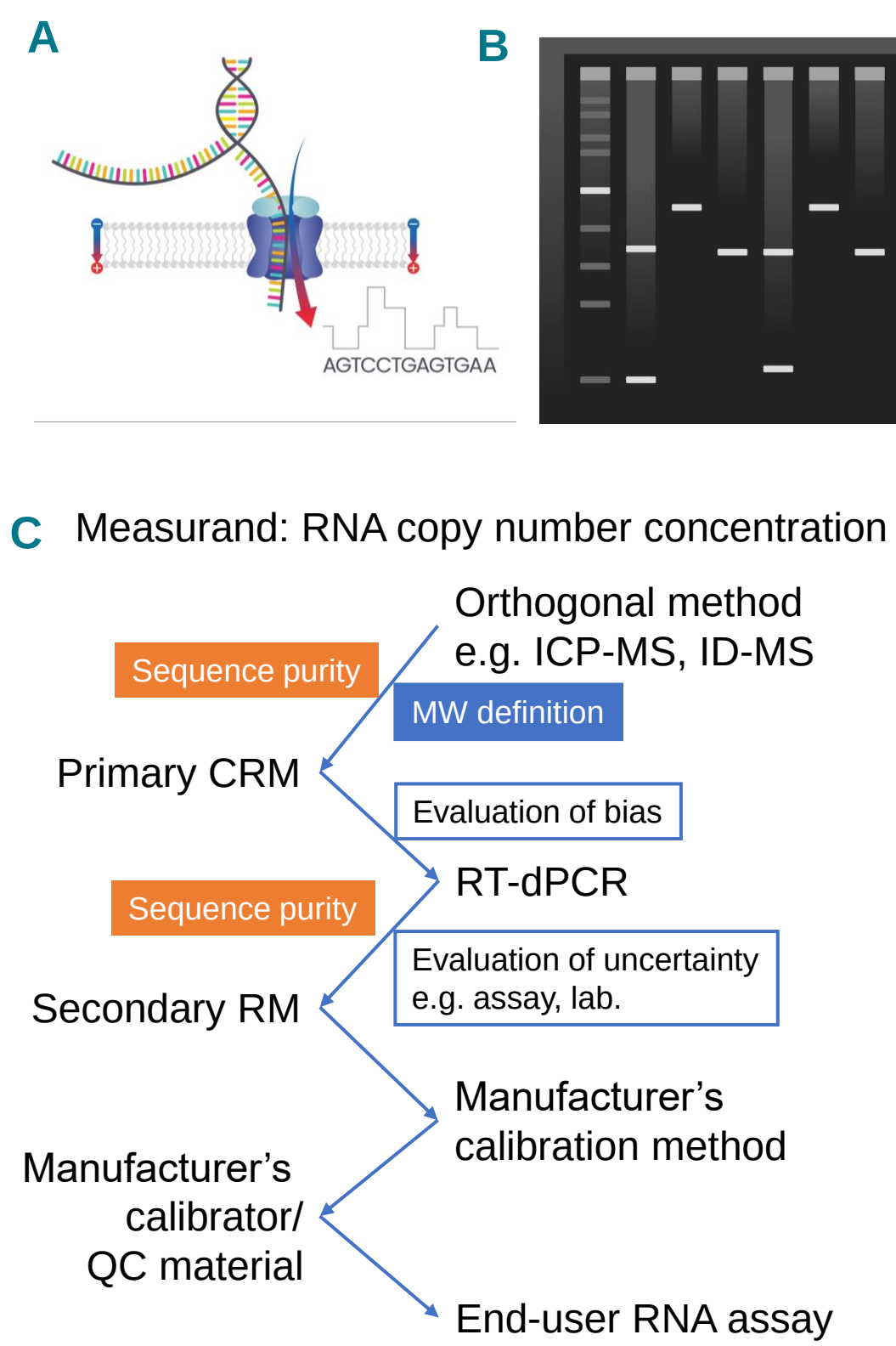


Figure 5. Elements of an SI-traceable measurement hierarchy for RNA copy number concentration. A. Methods for determination of sequence purity e.g. nanopore sequencing. B. Fragment size/molecular weight, e.g. gel electrophoresis. C. Model calibration hierarchy.

*Abbreviations: HPLC-UV, high performance liquid chromatography-ultraviolet spectrophotometry; ICP-MS, inductively coupled plasma-mass spectrometry; ID-MS, isotope dilution mass spectrometry; LC, liquid chromatography; QS3D, Quantstudio 3D (Thermo Fisher Scientific); QX200, QX200 Droplet Digital PCR System (Bio-Rad).

References

1. Devonshire et al. (2024). bioRxiv. doi: <https://doi.org/10.1101/2024.04.12.589043>
2. Devonshire et al. (2024). bioRxiv. doi: <https://doi.org/10.1101/2024.03.27.584106>

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