Digital PCR: a reference measurement procedure for infectious disease molecular testing

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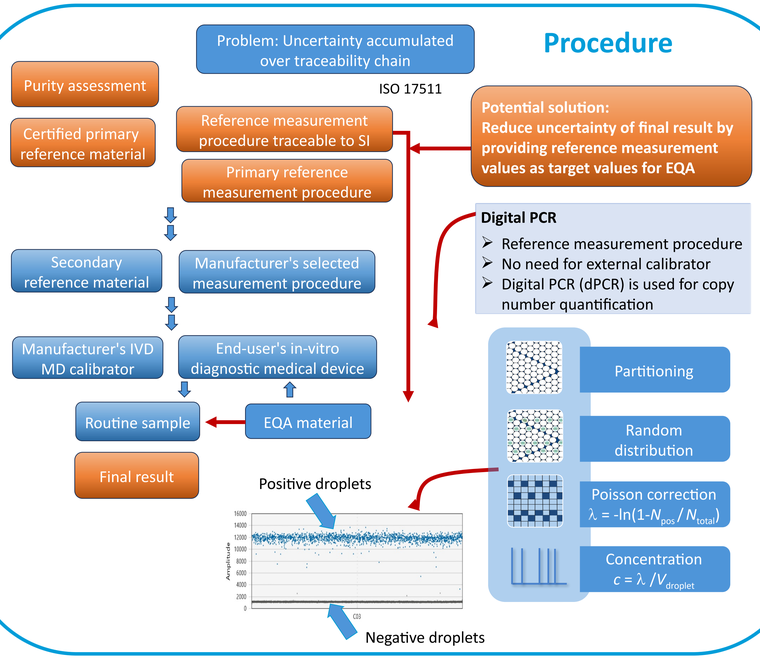
Introduction

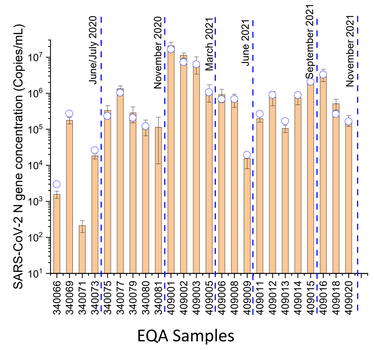
Metrology facilitates the advancement of accurate, SI-traceable measurements. Standardization of methods promotes measurement accuracy and reproducibility and facilitates comparison of quantitative data where a lack of standardization can leads to variability in results. Polymerase chain reaction (PCR) based techniques represent a diagnostic solution for infectious pathogens and are deployed to detect the genomes (DNA/RNA) of infectious agents as they can be quickly designed and demonstrate high sensitivity and specificity. Digital PCR (dPCR) is a single molecule enumeration technique that can reproducibly quantify nucleic acid sequences without using calibration. dPCR offers a route to provide reference values to support PCR testing and diagnostics. dPCR has been used for quantitative detection of pathogens and has been established to serve as a potential reference measurement procedure (RMP) for the accurate quantification of nucleic acids from pathogens such as *Mycobacterium tuberculosis*, human cytomegalovirus[1], human immunodeficiency virus-1[2], some of which are already listed as reference methods in the JCTLM database (https://www.jctlmdb.org/#/app/home). Recently, three National Metrology Institutes/Designated Institutes (NMIs/DIs) developed a potential reverse transcription-dPCR (RT-dPCR)-based RMP for SARS-CoV-2 RNA[3] and support external quality assurance (EQA) by assigning metrologically traceable values to whole viral SARS-CoV-2 EQA material. This poster will describe some of the recent work on dPCR-based RMPs on SARS-CoV-2 and monkeypox (Mpox) and show how this can be applied to improve nucleic acid analysis measurements in the EQA schemes, clinical laboratories and harmonization studies for infectious disease diagnostics.

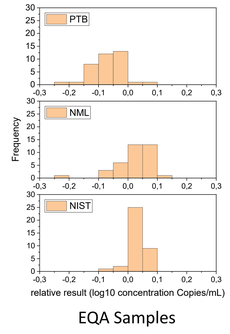
Procedure



Results

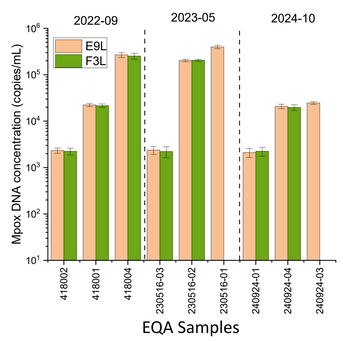
dPCR SARS-CoV-2

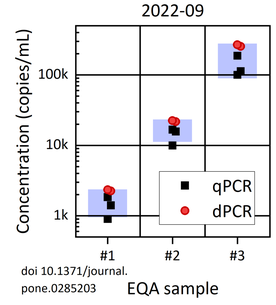




Left: Bars show mean RNA copy number concentration for SARS-CoV-2 in the six EQA schemes between 2020 and 2021 estimated as reference values by three NMI´s. The error bars show the expanded uncertainty with 95 % confidence intervals for whole virus SARS-CoV-2 EQA material. Open circles give the target concentration in copies/mL as consensus values from all quantitative EQA results. Right: Reproducibility and measurement bias from six EQA schemes. Data were log transformed ($\log_{10}$ concentration in copies/mL). Histogram showing the deviation of individual measurement laboratory dPCR result. No systematic deviations indicating bias are observed.

dPCR Mpox





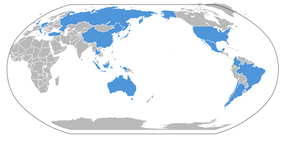
Left: Bars show mean DNA copy number concentration for Mpox virus in the three EQA schemes between 2022 to 2024 estimated as reference values by PTB. Error bars show the expanded uncertainty with 95 % confidence intervals for whole virus Mpox INSTAND material. Right: Quantitative results of EQA participants for Mpox virus from 2022[4]. Quantitative values were reported by only a few laboratories in the EQA schemes. The shaded area indicates the 95% confidence interval for the mean value.

Note: Concentrations are given in copies/mL, but for higher order materials and methods the concentration can be given in 1/mL or IU/mL.

Materials and Methods

- SARS-CoV-2**:** inactivated whole virus dilution panels from INSTAND e.V. (genome detection of coronaviruses) with different genotypes and subtypes.
- One-step reverse transcription digital PCR (RT-dPCR) method was applied using the QX200™ (BioRad) with RNA extraction using QIAamp viral RNA mini kit (Qiagen).
- The assay targeted the nucleocapsid (N) gene.
- Participation in the EQA was for the period of two years (04/06/11-2020 and 04/06/09/11 2021).
- The EQA datasets were statistically analysed by NML, LGC. The reference value was estimated using either linear mixed or generalised linear models.
- Mpox virus: inactivated whole virus dilution panels from INSTAND e.V. (genome detection of monkeypox virus). dPCR method was used with DNA extraction using High Pure Viral Nucleic Acid Kit (Roche). The EQA participation covered three years (2022 – 2024).

CCQM Nucleic Acid Analysis Working Group (NAWG)
(A) To promote metrological traceability of measurement results for the nucleic acid analysis.
(B) To enable measurements to be made with confidence.



Current studies: CCQM-P232 fire drill (influenza), CCQM-K190/CCQM-P249 (SARS-CoV-2 Whole Virus) Key comparison and Pilot Studies

Conclusions

- dPCR is highly reproducible as has been demonstrated in studies as well as CCQM-NAWG studies such as P199 (HIV-1), P199-b (SARS-CoV-2) etc.
- Our findings demonstrate that dPCR can be used as a RMP to support quantitative and qualitative EQA schemes.
- The further development of SI-traceability of RNA and DNA measurements can be supported by comparison with alternative techniques such as ID-MS and single molecule flow cytometry. dPCR concentration values closely agree with the values from other methods and can support the development of SI-traceability for dPCR.
- dPCR can complement other molecular methods such as qPCR and is a route for standardization of molecular infectious disease testing.
- CCQM NAWG international pilot and key comparison studies support standardization of nucleic acids measurement such as P199 (HIV-1), P199-b (SARS-CoV-2 and P232 (influenza) RNA quantification.

References

(1) *Methods* (2022); 201:65–73, (https://doi.org/10.1016/j.ymeth.2021.03.016).
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(4) PLOS One (2022); (https://doi.org/10.1371/journal.pone.0285203).

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