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EMRP European Metrology Research Programme
Programme of EURAMET

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1 Executive Summary (not exceeding 1 page)

Introduction

INFECT-MET successfully developed highly accurate methods (towards SI traceability) and materials to support the accurate quantification of infectious disease-causing pathogens in clinical samples. It also assigned traceable values to DNA materials used in External Quality Assessment schemes (EQAS) to support efforts to standardise quantitative detection in terms of replacing 'consensus' values with traceable reference values. Furthermore, a framework was developed to enable the scientific community to establish suitable reference systems for pathogen testing. The project also contributed to international standards development activities in this area.

The Problem

Infectious diseases account for over 20 % of human deaths globally and 25 % of all morbidity. Respiratory tract infections (RTI) such as pneumonia, influenza and tuberculosis account for almost 50 % of all pathogen associated deaths. Accurate and rapid diagnosis alongside methods for monitoring transmission and spread in the community and resistance to therapeutic agents are vital for public health protection. Molecular approaches, such as quantitative polymerase chain reaction (qPCR) and sequence analysis, offer the potential to improve management of infectious diseases, through increased speed, accuracy, sensitivity and information, when compared to conventional microbiological methods. However, issues concerning quality, comparability and traceability of measurements using emerging genomic approaches have been highlighted widely.

The Solution

In response to this problem, INFECT-MET developed novel measurement procedures and validation frameworks to support current and emerging molecular approaches for efficient, harmonised and rapid diagnosis, surveillance and monitoring of infectious diseases. The projectestablished routes for improving the accuracy, robustness, comparability and traceability of measurements within the diagnostics/epidemiological communities across Europe and linked into international standardisation initiatives in this area.

INFECT-MET successfully achieved all scientific and technical objectives. In particular, the value of the test materials and methods developed in the project and their potential as prototype reference systems for increased standardisation of molecular diagnostics, particularly through adoption in EQAS, was demonstrated.

Impact

At the end of the project there are many examples of the outputs being taken up by the relevant communities such as supporting EQAS to standardise quantitative detection of pathogens and help clinical laboratories establish new sequencing capabilities and standardised protocols. Considerable dissemination to the relevant user communities also took place during the course of the project. The results and the quality and traceability guidance for infectious disease molecular diagnostics developed in the INFECT-MET project are currently being disseminated into the official international Standards system through various working groups of ISO TC212 (laboratory medicine) and ISO TC276 (biotechnology).

In terms of future and wider impact, the development of higher order methods, a metrology framework and standards, the output of INFECT-MET will help healthcare providers and the biotechnology/diagnostic industry to demonstrate the reliability of their assays in a traceable way. The outputs will have particular potential for impact in supporting both the activity of the proposed new network of Reference Laboratories for Class D (infectious pathogens) IVDs, and the requirements for demonstrating metrological traceability, mandated by the new EU IVD regulation, which has now received EU parliament approval for a partial general approach, and is now expected to enter into force by early 2016.

2 Project context, rationale and objectives

Context

Respiratory infections are one of the major public health challenges worldwide. Classical diagnostic tools and approaches are time consuming and have become inadequate for accurate and reliable detection and quantification of the infectious agent. In recent years new technologies based on detection of nucleic acids (molecular methods) have been developed that allow faster and more sensitive diagnostics of respiratory infectious agents. Depending on the needs, methods can be qualitative (e.g. detection and identification of an infectious agent for proper treatment of patients e.g. treatment with antibiotics, quarantine) or quantitative (e.g. quantification of an infectious agent e.g. before and after treatment to determine the efficiency of the treatment). To enable reliable implementation of new technologies, they should be assessed in terms of accuracy and sensitivity. Higher order methods, with the potential for traceability to the SI or equivalent, and reference materials that would provide reference for such assessments are lacking.

Pathogens (e.g. bacteria and viruses) may be present in clinical samples at very low levels making accurate detection and measurement challenging. In many instances, tests are being used in non-commercial 'home-brew' formats of variable and undefined quality and even commercially available tests cannot always be directly compared between laboratories due to a lack of traceable reference standards and reference methodologies. This lack of consistency can lead to over- or under-diagnosis of infectious diseases, resulting in inappropriate treatment with concomitant financial/quality of life costs associated with increased morbidity, mortality, disease spread and spread of antimicrobial resistance.

Improved comparability and traceability across Europe of approaches used for the surveillance and monitoring of infectious diseases and detection of antimicrobial resistance mutations is needed. Similarly, emerging approaches for the rapid detection of infectious agents need improved metrics for quantifying performance in order to facilitate faster regulatory approval. Full confidence in molecular measurements can only be achieved if the appropriate metrology framework, standards and higher order methods which have reduced uncertainties, compared to standard approaches, are developed.

Currently, higher order methods and reference materials, which are required for compliance with the In Vitro Diagnostics (IVD) regulation and ISO clinical testing standards and which are essential for assessing performance and improving comparability, are lacking for all but a small handful of pathogens. Without the development of such methods and materials for high-priority infectious agents such as influenza and tuberculosis healthcare providers and the biotechnology/diagnostics industry will be unable to reliably demonstrate the reproducibility of their assays (tests) in a traceable and comparable manner. This is critical for the implementation of assays deployed in a wide range of healthcare settings. Higher order methods and approaches are needed for assigning traceable values to reference standards in order to improve the quality and comparability of current and emerging molecular assays.

Objectives

INFECT-MET aimed to develop novel measurement procedures and validation frameworks to support current and emerging molecular approaches for efficient, harmonised and rapid diagnosis, surveillance and monitoring of infectious diseases.

The project focussed on four main objectives:

- **Develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents, such as viruses and bacteria.** Develop and evaluate higher order methods based on enumeration (for example, digital polymerase chain reaction (PCR) and single molecule counting in flow) for accurate measurement of infectious agents with known uncertainties and investigation of sample extraction from different matrices (e.g. blood, plasma and sputum).
- **Quantitatively and comparatively evaluate new and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial**

resistance mutations. Investigate the measurement challenges associated with emerging methodologies such as next generation sequencing (NGS) for surveillance, epidemiology and antibiotic resistance screening.

- **Quantitatively and comparatively evaluate new and emerging diagnostic technologies for the rapid detection of infectious agents.** Investigate the measurement challenges associated with emerging methodologies for rapid, near-patient testing, including DNA/microfluidic surface interactions and isothermal nucleic acid amplification evaluations.
- **Develop methodologies for accurately quantifying the performance of commercially available diagnostic assays, 'in-house' clinical assays and novel emerging approaches.** Develop a reference measurement framework using higher order measurement approaches in collaboration with end-user communities to improve calibration and quality assurance of current clinical PCR approaches.

3 Research results

Objective 1: Develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents, such as viruses and bacteria

The main aim of this part of the project was to develop higher order methods for accurate measurements of infectious agents, to assign traceable values to test materials and to assess their performance and measurement uncertainty.

Selection of Model Systems

Three model systems of infectious agents were selected by LGC, JRC, NIB and UCL in consultation with key stakeholders for the development and evaluation of higher order methods. Model systems were selected based on relevance to stakeholders, clinical need and availability of materials. *Mycobacterium tuberculosis* (TB) was selected as a bacterial model, Human Cytomegalovirus (hCMV) as a viral DNA model and Influenza as a viral RNA model.

The consortium allocated 1 model system to the following partners:

- Tuberculosis (LGC, UCL)
- Human Cytomegalovirus (NIB)
- Influenza (JRC)

Selection and evaluation of nucleic acid extraction methods

Different extraction methods were selected by LGC, JRC, NIB, PTB and UCL for each of the model systems (Tuberculosis, Human Cytomegalovirus and Influenza) for evaluation. In addition to commercially available extraction kits, 'open methods' (preferred from a regulatory point of view) were also evaluated as there is a need to develop reliable extraction methods which can be reproduced by reference measurement procedure users without depending on an external producer. Reliance on commercial systems means that there is no control by the user over the method and the stability of performance of such 'black box' systems cannot be ensured over time, unless dedicated and representative standards are available and used by the extraction kit manufacturer to prove equal performance over time.

Through a series of inter-laboratory studies between LGC, JRC, NIB, PTB and UCL, the best-performing extraction methods were identified for the 3 model pathogens. The methodology for validating the extraction method is shown in Figure 1.

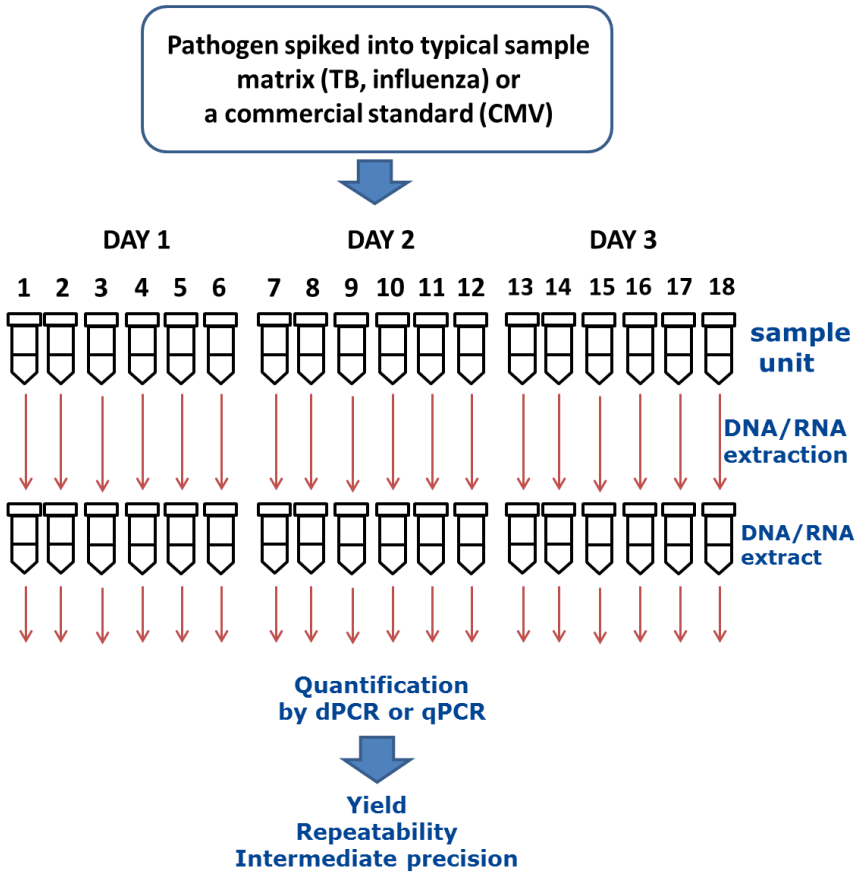


Figure 1: Validation of nucleic acid extraction methods. Several different extraction methods were compared for each model pathogen.

LGC and UCL compared the efficiency of the selected DNA extraction methods/kits for Tuberculosis in spiked sputum samples. Genomic DNA was extracted from a culture of *Mycobacterium bovis Bacillus Calmette–Guérin* (*M. bovis* BCG) in human sputum using the Cetyltrimethylammonium bromide (CTAB) NaCl method (open method) and the commercial Prepman Ultra kit (Applied Biosystems).

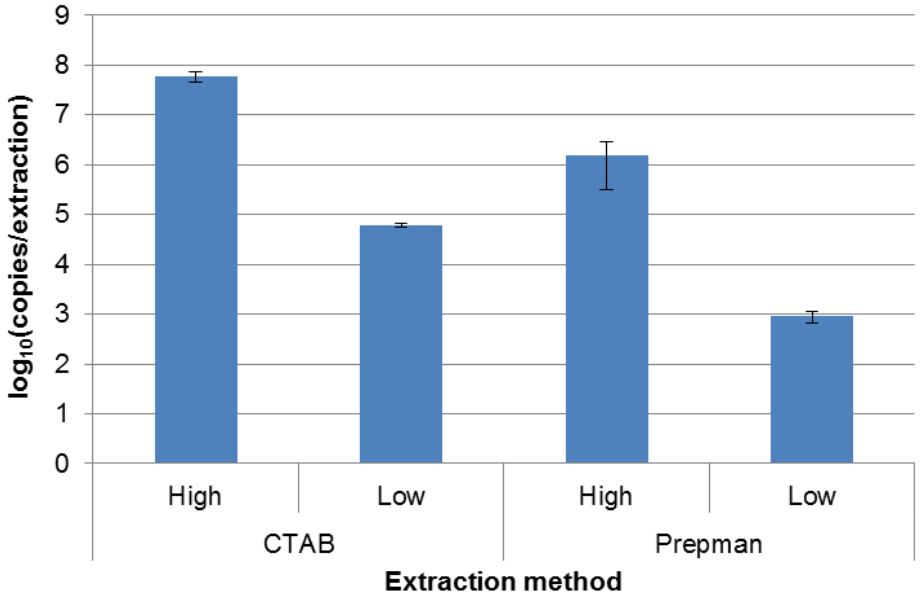


Figure 2: Comparison of genomic DNA (gDNA) yields of two methods for the extraction of *M. bovis* BCG from human sputum at two spiking levels (high, low). Mean gDNA copies ± standard deviation (SD) for each extraction method and spiking level are displayed on a log10 scale.

Results showed that the Cetyltrimethylammonium bromide (CTAB) NaCl method was the most efficient method for the extraction of DNA from spiked sputum samples based on highest yield and repeatability compared to the Prepman method (Figure 2).

NIB and PTB compared the efficiency of the selected DNA extraction methods/kits for Human Cytomegalovirus (hCMV) in buffer and human plasma. DNA extraction from Human Cytomegalovirus (hCMV) was performed using WHO International Standard material (NIBSC) diluted in human plasma (Applied Biosystems) using a High Pure Viral Nucleic Acid kit (Roche) (Figure 3) and a modified phenol-chloroform method (open method) (Figure 4).

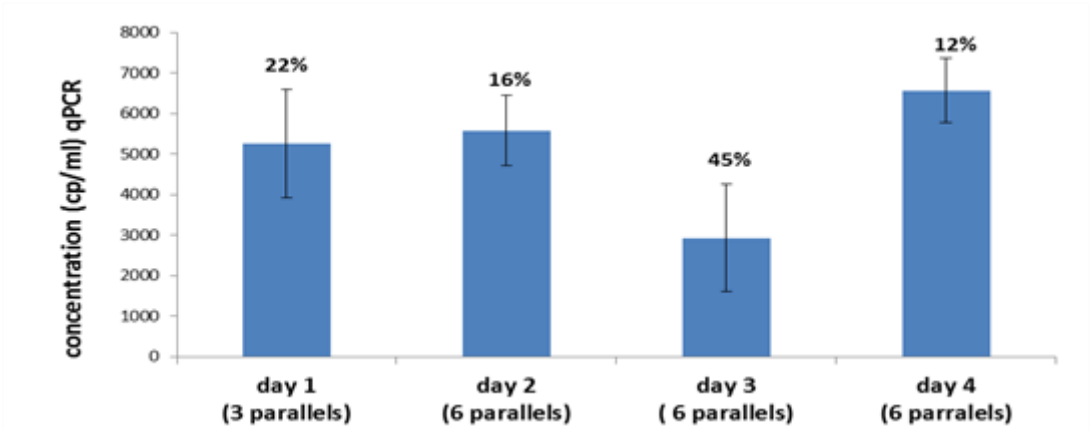


Figure 3: Comparison of gDNA yields of hCMV. Assessment of intra-experiment repeatability for High Pure Viral NA kit extraction from human EDTA plasma. 6 replicates (200 µL each) with a nominal concentration of 3000 IU/ml were extracted on 3 different days (days 2,3,4). Results of extractions of 3 samples (same calibration material, matrix and nominal concentration) obtained from the first assessment (day 1) were also included in the repeatability analysis. The error bars represent the SD, numbers above the bars depict the coefficient of variation (CV).

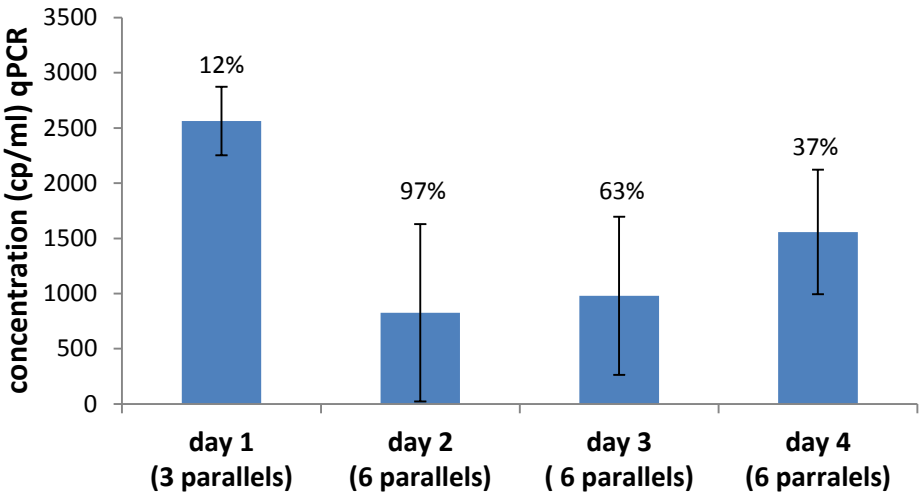


Figure 4: Comparison of gDNA yields of hCMV. Assessment of intra-experiment repeatability for phenol/chloroform extraction from human EDTA plasma. 6 replicate samples (200 µL each) with nominal concentration 3000 IU/ml were extracted on 3 different days (days 2,3,4). Results of extractions of 3 samples (same calibration material, matrix and nominal concentration) obtained from the first assessment (day 1) were also included in the repeatability analysis. The error bars represent the SD, the numbers above the bars depict CV.

Results show that the High pure Viral Nucleic Acid kit (Roche) was the most efficient method for the extraction of gDNA from plasma in terms of producing higher yields of gDNA and with better repeatability.

JRC compared the efficiency of the selected RNA extraction methods on different matrices for Influenza. Three RNA extraction methods from Influenza virus spiked into human nasal wash were evaluated: High Pure Viral RNA Kit (Roche applied Science), QIAamp® Viral RNA Mini kit (Qiagen), and TRIzol® LS Reagent (open method).

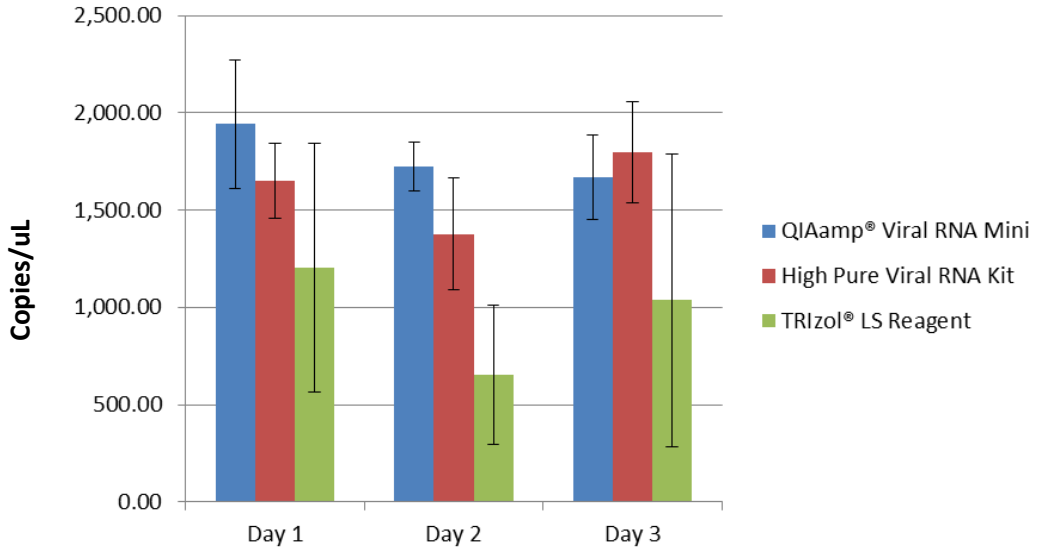


Figure 5: Repeatability and reproducibility of influenza A RNA extraction methods. Copy number yields are compared for three extraction methods performed on three separate days and displayed relative to the mean ± SD (n = 6). The precision of the extraction methods was compared in terms of repeatability and reproducibility between independent extractions. The repeatability of the methods ranged from a mean coefficient of variation (%CV) of 12.4 % for QIAamp® Viral RNA Mini, 15.7 % for the High Pure Viral RNA Kit, to as much as 60 % for TRIzol® LS Reagent.

Of the three extraction methods assessed, the commercial kits proved superior both in terms of yield and repeatability compared with the open method (Figure 5). Since the latter method involves RNA precipitation, pelleting, washing and re-suspension, its overall poorer performance could be explained by the loss of pellet during one of these steps, or by the lower homogeneity of the resulting sample.

Assessments of extraction methods for all three model organisms showed that the extraction of DNA/RNA is a major contributor to the variability of pathogen quantification results and indicated the need to further decrease their variability. Nucleic acid extraction methods show varied repeatability and intermediate precision which should be carefully considered, particularly when comparing results of diagnostic tests established in different laboratories which can potentially lead to erroneous clinical conclusions.

Although commercial kits showed better performance compared to open methods for two model systems there is a need to develop reliable extraction methods which will be fully understood in terms of influence quantities and parameters and under the full control of the user. Extractions are a critical part of reference measurement procedures and there is a need for them to be reproduced by reference measurement procedure users without depending on an external producer (so-called open methods).

Test materials

Test materials of three orders of complexity were prepared for each of the three model pathogens:

- Level 1: Nucleic acid molecule (plasmid, *in vitro*-transcribed (IVT) RNA)
- Level 2: Nucleic acid extract (total genomic DNA, total genomic RNA)
- Level 3: Whole microbe preparation

The three different levels/types of test material enabled the nucleic acid-based tests to be characterised for the infectious agents during different stages of the analytical process (extraction, amplification and measurement/detection of nucleic acids).

LGC and UCL prepared the TB DNA test materials at three orders of complexity: a plasmid construct containing two TB target genes, TB genomic DNA from the laboratory strain H37Rv and BCG spiked in artificial sputum.

NIB prepared the hCMV DNA test materials at three orders of complexity: standard reference hCMV sourced from NIST, DNA extracted from the WHO International hCMV standard and whole virus preparations.

JRC prepared the Influenza test materials at three orders of complexity: an in vitro transcribed RNA molecule containing the influenza A target gene, total genomic RNA from the influenza virus (propagated in cell culture) and whole microbe material (nasal wash spiked with the influenza virus).

Higher order methods/value assignment of test materials

Performance data was collated for the two main commercially available digital PCR (dPCR) methodologies: droplet based dPCR using the QX100 platform (BioRad) and microfluidic dPCR using the BioMark platform (Fluidigm). Digital PCR has the potential for improved metrological traceability for nucleic acid amplification-based tests as it provides an absolute quantification method which is not reliant upon quantification of standards by UV spectroscopy or fluorescence measurements, neither of which are SI-traceable. Early results of the application of dPCR to the measurement of infectious agents are encouraging in terms of the clinical sensitivities and specificities of the dPCR methods in comparison to established qPCR-based assays. Therefore dPCR-based methods were developed and assessed for potential application as higher order methods.

dPCR methods for the quantification of Tuberculosis, Human Cytomegalovirus and Influenza were developed at LGC, NIB and JRC respectively.

LGC developed a dPCR method for the quantification of Tuberculosis. PCR assays (real-time and digital) for selected target genes (16S rRNA and rpoB) were developed and assessed for dynamic range, linearity, repeatability, intermediate precision and LOD. Measurement uncertainty was assessed on representative samples (supplied by UCL) and it was shown to be fit for purpose.

NIB developed a dPCR method for the quantification of hCMV. PCR assays (real-time and digital) for the selected target gene (DNA polymerase (UL54) were developed and assessed for dynamic range, linearity, repeatability, intermediate precision and LOD. Measurement uncertainty was assessed on representative samples and it was shown to be fit for purpose.

JRC developed a reverse transcription (RT-) dPCR method for the quantification of Influenza A (H1N1) genomic RNA. The method was assessed for dynamic range, linearity, repeatability, intermediate precision and LOD. Measurement uncertainty was assessed on representative samples and it was shown to be fit for purpose.

Three separate inter-laboratory studies (1 for each model system) were completed at LGC, NIB and JRC to determine the reproducibility of the dPCR methods developed and to assign values to the corresponding panels of test materials. The inter-laboratory studies for TB, hCMV and Influenza were organised by LGC, NIB and JRC respectively. Protocols for measurement procedures were prepared and test materials were sent to all participating partners.

Results of inter-laboratory studies indicated no systemic bias connected to the different dPCR platforms used (QX100 and BioMark). The most consistent results were observed with the TB test materials, where very small differences were observed between the two platforms (<7 %). With the Influenza materials, higher values were measured with BioMark compared to QX100, while with the hCMV materials the opposite was observed.

In all three studies, the estimated measurement uncertainties obtained were very low. The lowest measurement uncertainty was observed for measurements of TB control plasmid (1.8 % for combined measurements and 17.2 % for single repeat measurements). As expected, measurement uncertainty increased with the increased complexity of the test materials. The highest measurement uncertainty was estimated for measurements of lower concentration of whole Influenza virus, where measurement uncertainty of combined measurements was 9.1 % and that of single repeat measurements was 56 %.

In addition, measurement procedures were transferred to the third platform, QuantStudio3D (Life Technologies) at TUBITAK. Limited data from the QuantStudio3D platform indicate that values obtained with this platform are higher compared to the other two platforms. However there are significant uncertainties related to this platform due to limited information on partition and consequently on reaction volume.

Transferability and reproducibility of measurement procedures was shown in the inter-laboratory comparison which included three laboratories (JRC, LGC and NIB). The assessed measurement procedures are candidates for use as reference measurement procedures. The majority of value assignments of test materials are based on consensus values. This project demonstrated the feasibility of using dPCR for the value assignment of test materials (Figure 6) which could subsequently be used as calibrators and/or for molecular method validation.

Effective cooperation was demonstrated between partners for this part of the project. NIB and JRC were supported by LGC with respect to the experimental design of the Inter-laboratory studies. TUBITAK supported LGC, NIB, and JRC by offering to perform independent measurements on the developed test materials using a different digital PCR platform.

Model system	Test material	Weighted average concentration [cp / µL]	Expanded combined uncertainty	expansion factor k	Additional (type B) uncertainty (if applicable)
Influenza A	*In vitro* transcribed (IVT) RNA	1.02E+10	5.47E+08 (5.3 %)	2.01	1.43E+09 (14.0 %)
	Total genomic RNA	9.07E+05	5.62E+04 (6.2 %)	2.01	1.54E+05 (17.0 %)
	Influenza A in nasal wash (higher concentration)	1.52E+03	9.61E+01 (6.3 %)	2.01	2.6E+02 (17.0 %)
	Influenza A in nasal wash (lower concentration)	51	5 (9.1 %)	2.01	13 (26.0 %)
hCMV	Total genomic DNA	985	44 (4.5 %)	2.01	107 (10.9 %)
	Whole virus preparation	2814	123 (4.4 %)	1.98	672 (23.9 %)
M. tuberculosis	TB Control Plasmid	1.30E+05	3.25E+03 (2.5 %)	1.98	1.21E+04 (9.3 %)
	M. tuberculosis H37Rv genomic DNA	4.24E+04	2.49E+03 (5.9 %)	1.98	8.75E+03 (20.6 %)
	M. bovis BCG in artificial sputum	5.87E+04 (1.17E+07 copies/unit)	2.18E+03 (3.7 %)	1.98	9.00E+03 (15.3 %)

Figure 6: Summary of all test materials with their assigned values and uncertainties.

Orthogonal methods

For the evaluation of the trueness of measurement procedures orthogonal methods were investigated (i.e. additional methods that provide very different selectivity to the primary method and which can be used to investigate the primary method). PTB developed a dedicated flow cytometer for DNA fragment detection by laser induced fluorescence of intercalating dyes. With enhancement of the stability of sample delivery, and of the detection sensitivity, DNA fragments with sizes down to 4316 bp could be detected in flow. The detected fluorescence showed very good linear correlation to the fragment length proving that DNA fragment length determination is possible with the current setup. For the bacterial model, the concentration of the circular plasmid pUC19TB, which was used for preparing TB test materials, was measured. Flow cytometry was also used to evaluate the trueness of the method developed to quantify TB in the bacterial model. The estimation of CFU (colony forming units) based on culture, showed good concordance with both the dPCR method developed and direct counting with flow cytometry.

Two dPCR platforms BioMark™ HD System (Fluidigm) and QX100™ Droplet Digital PCR System (BioRad) were used for direct quantification of hCMV using whole virus reference material from NIBSC (1st WHO International Standard for Human Cytomegalovirus for Nucleic Acid Amplification Techniques). Measurement of extracted DNA from two matrices (plasma and PBS) was additionally carried out to provide a comparison with direct quantification in terms of variability and estimated DNA copy number. Using the direct quantification approach DNA copy numbers were 18 % - 39 % higher compared to the measurement after the DNA was extracted.

The results from independent orthogonal methods are crucial in the context of assessing trueness and in that respect; the results from this project are encouraging and suggest that estimation of trueness should be feasible.

This project successfully achieved this scientific and technical objective. For the first time in Europe, digital PCR methods to enable absolute quantification of infectious agents with improved traceability to SI through the concept of enumeration were developed and validated for the three model systems with full uncertainty budgets. A suite of test materials was produced for each model system and traceable values assigned using the developed methods. As part of this objective, the partners NIB (Slovenia, NMI) and TUBITAK (Turkey, NMI) developed an increased capability in digital PCR as a result of the project. Such methods and materials could also be used by clinical research laboratories outside the consortium to evaluate their routine in-house assays and to facilitate comparisons to other assay formats. They could also be used by in vitro diagnostic (IVD) developers to validate their diagnostic assays and to facilitate faster regulatory approval. The methodologies could also be used for traceable value assignment of reference materials, calibrants and External Quality Assessment (EQA) materials by reference material developers and EQA providers.

Objective 2: Quantitatively and comparatively evaluate new and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations

The last decade has seen an increase in the number of higher throughput and/or more extensive analytical technologies that offer unique opportunities for infectious disease analysis for surveillance of infectious disease load, epidemiology and resistance. These approaches offer much, but come with a set of frequently unique challenges when considering standardisation of measurement. The main aim of this part of the project was to investigate new and emerging molecular approaches for surveillance, monitoring of infectious disease load, and detection of antimicrobial resistance mutations.

Selection of emerging methodologies

Following consultation with NIB, UCL and with clinical and academic collaborators (University College London Hospitals, Royal Free Hospital, St Georges Hospital, University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia), LGC selected the new and emerging technologies that will be investigated. The choice of technologies was also guided by a series of discussions with diagnostic and instrument providers (Roche, Cepheid, Biorad, Raindance, Fluidigm and Life Technologies). These will be Digital PCR, High throughput and Next Generation Sequencing (whole genome and amplicon).

Model systems

Following consultation with clinical stakeholders, the following model systems were selected to develop and evaluate methodologies to monitor antimicrobial resistance mutations in infectious diseases:

- Influenza Oseltamivir (Tamiflu) resistance was used to develop digital PCR methods to detect rare single RNA mutations
- Multi-drug resistant tuberculosis was used to develop deep sequencing approaches to detect a number of known drug resistance mutations
- Extensively drug-resistant tuberculosis was used to investigate shotgun sequencing approaches to sequence drug resistance isolates
- Chronic obstructive pulmonary disease (COPD) was used to develop deep sequencing approaches to identify the relevant pathogens involved in the disease which can involve multiple pathogens

Influenza Oseltamivir (Tamiflu) resistance

LGC, with input from NIB, developed a novel digital PCR method to detect rare single RNA mutations using Influenza Oseltamivir (Tamiflu) resistance as a model system. Initial work focussed on minority target single nucleotide polymorphism (SNP) analysis using digital PCR to identify point mutations that confer oseltamivir drug resistance in influenza. A digital PCR assay based on the routine real time PCR clinical method was developed and optimised to discriminate between wild-type (sensitive) and mutant (resistant) sequences (Figure 7).

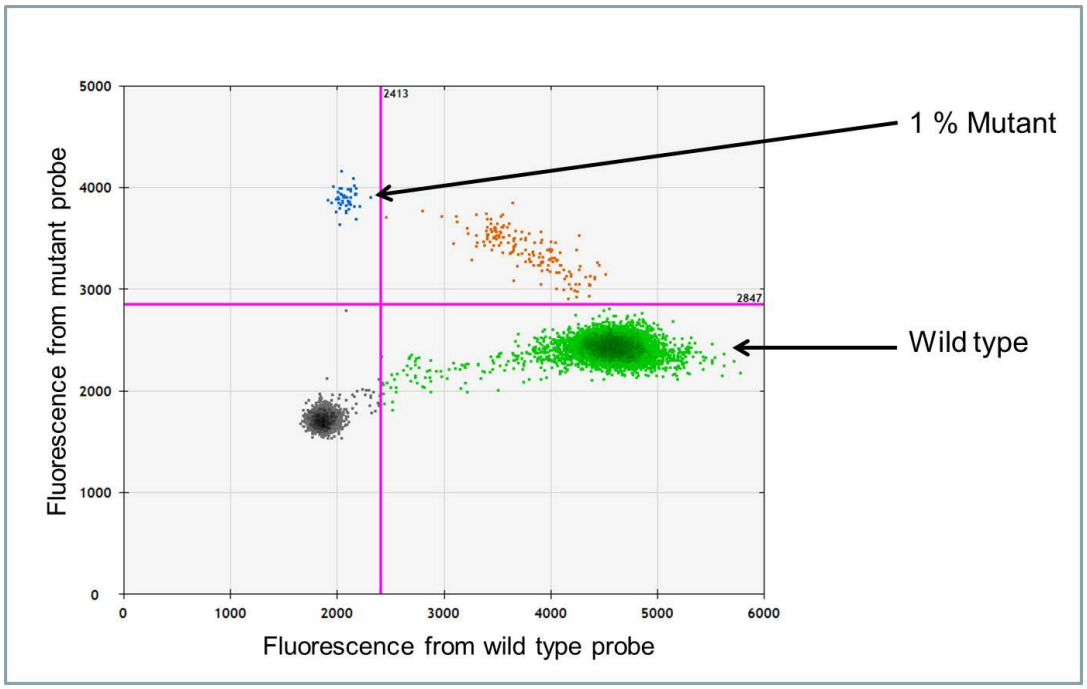


Figure 7: Figure showing the ability of dPCR to measure 1 % mutant sequence in a predominantly sensitive (wild type) sample. The conventional method routinely used in the clinic cannot detect below 5 %.

Following assay optimisation to improve discrimination between wild-type (sensitive) and mutant (resistant) sequences, the method was used to analyse 'blinded' clinical samples sourced from University College London Hospital (UCLH).

Results showed the utility of the developed method in being able to identify samples containing the resistance mutation and an improved sensitivity compared to the PCR method used clinically.

Multi-drug resistant tuberculosis (MDR-TB)

Multi-drug-resistant tuberculosis (MDR-TB) is a form of TB infection caused by bacteria that are resistant to treatment with at least two of the most powerful first-line anti-TB drugs, isoniazid and rifampicin.

LGC with input from UCL and NIB developed a novel sequencing approach to detect known DNA mutations using MDR-TB as a model system. Work focussed on amplicon (ultra deep) sequencing of the rpoB gene, which encodes the beta subunit of the bacteria's RNA polymerase and causes resistance to rifampicin. PCR assays targeting the rpoB gene were developed and optimised at LGC.

MDR-TB and wild type (drug sensitive) strains were cultured by UCL, and DNA was extracted and characterised by PCR, optical and electrophoretic methods at LGC.

A 1:1 mixture of drug sensitive and drug resistant isolates were sequenced on the Roche GS Junior and data was analysed by NIB. Results show that this sequencing approach is able to detect rare mutations present at a level of around 1 % of the mixture (Figure 8).

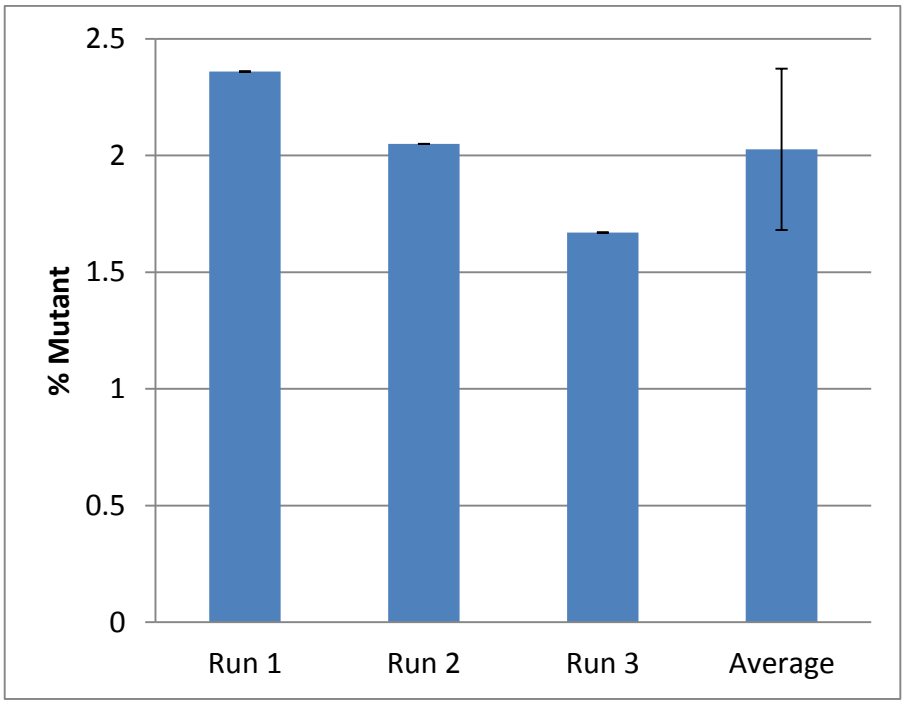


Figure 8: Graph demonstrating the variation between runs when measuring small percentages of resistance (mutant) sequence in the predominantly wild type sequence.

Extensively drug-resistant tuberculosis (XDR-TB)

Extensively drug-resistant tuberculosis (XDR-TB) is a rare form of MDR-TB caused by bacteria that are resistant to some of the most effective anti-TB drugs (i.e. XDR-TB strains are resistant to more anti-TB drugs than MDR-TB strains). XDR-TB strains, which are resistant to at least four anti-TB drugs, have arisen after the mismanagement of individuals with MDR-TB.

XDR-TB drug resistant strains, sourced from the University of Lisbon, have been selected. Samples of DNA, extracted from extensively drug resistant tuberculosis (XDR-TB) clinical isolates were then sent to LGC by the University of Lisbon.

Whole genome sequencing was performed using the Illumina platform to determine any errors that could be introduced by the sequencing process. Data was analysed by collaborators at the London School of Hygiene and Tropical Medicine and showed that the precision of the sequencing instrument was very high, with an error rate of 0.05 % when measuring 2000 DNA sequences (Figure 9).

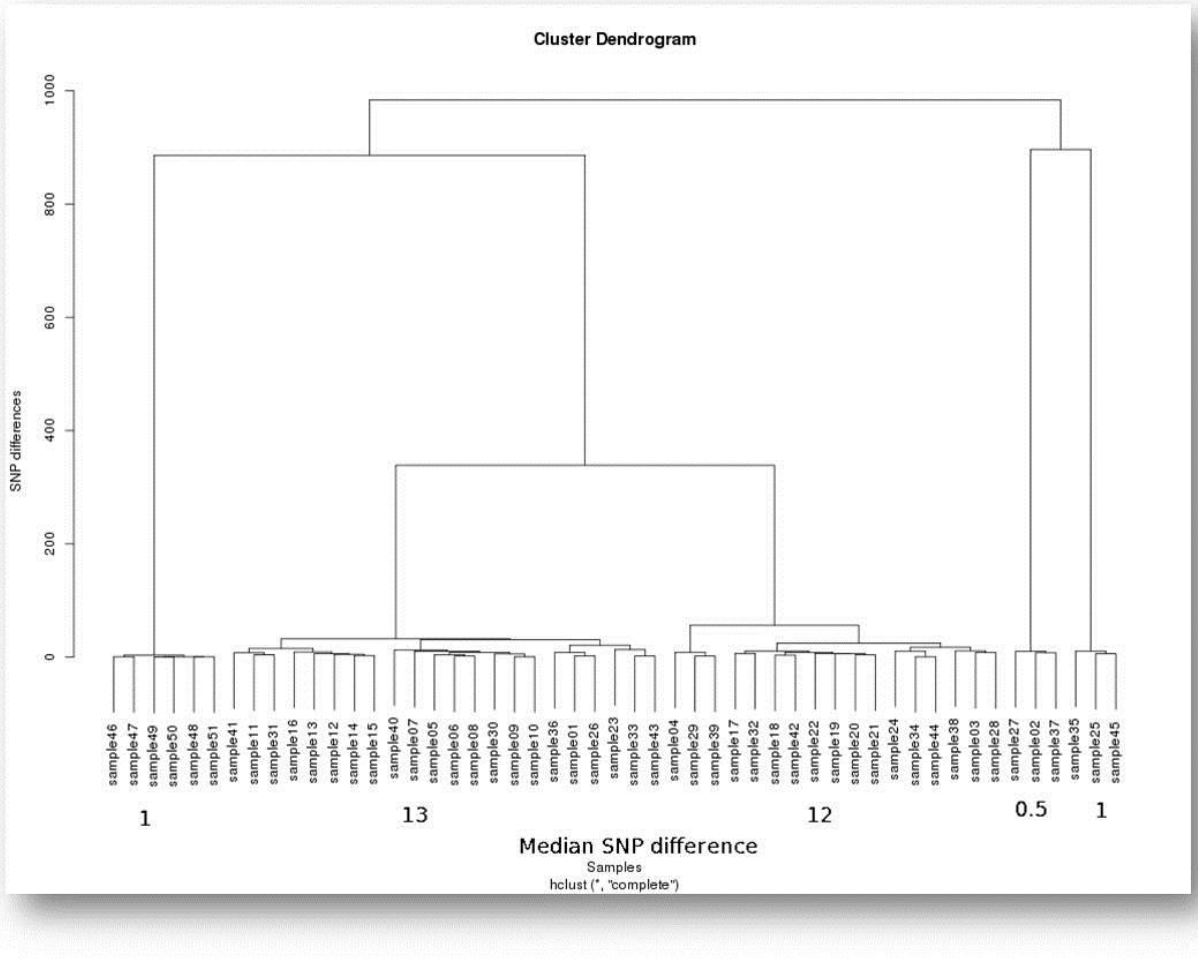


Figure 9: Dendrogram demonstrating the genetic differences between different sequencing experiments.

Chronic obstructive pulmonary disease (COPD)

LGC, in collaboration with NIB and UCG, developed Next Generation Sequencing (NGS) approaches for the surveillance and monitoring of the pathogens involved in chronic obstructive pulmonary disease (COPD).

A whole cell material (WCM) containing a mixture of 6 COPD-causing bacterial species (*Haemophilius influenza, Moraxella catarrhalis, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumonia* and *Pseudomonas aeruginosa*) at clinically relevant levels was prepared at LGC. Bacterial isolates of these species were sourced from a commercial supplier.

A high throughput quantitative PCR method for the identification and quantification of target COPD pathogens was completed at LGC, with input from NIB as part of the 3-month secondment. This method was used to successfully identify and quantify the 6 COPD causing bacterial species in extracted samples of whole cell material spiked into clinical samples. Strategies were developed to enrich the target COPD pathogens.

Work to develop NGS approaches for the surveillance and monitoring of the pathogens involved in COPD has been completed at LGC in collaboration with UCG. The WCM was spiked into clinical samples provided by UCG and DNA was extracted.

Clinical samples from COPD patients, provided by UCG, were spiked with the WCM at LGC and used to evaluate the performance of the sequencing process (including DNA extraction/purification). Results showed

that determining spike concentration was challenging when the pathogen was already present in the sample. Additional work using synthetic sputum demonstrated that it was the sequencing method rather than the extraction method that had the main impact on the data obtained and caused the most bias (Figure 10).

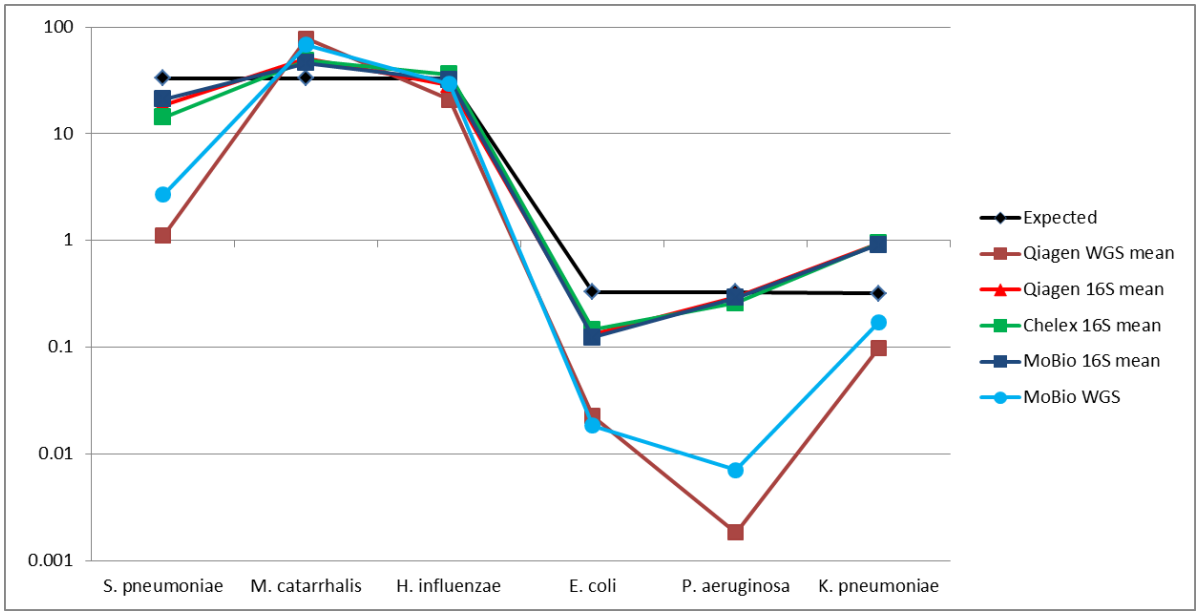


Figure 10: Graphs to show the relative proportion of six different organisms in the WCM when the expected (black line) is compared with different extraction (Qiagen, Chelex and MoBio) and sequencing (16S or WGS) methods.

The project successfully achieved this scientific and technical objective. An evaluation of novel molecular approaches (digital PCR and NGS) was completed for all of the clinical model systems. A novel digital PCR method was developed to detect drug resistance in influenza which showed an increased sensitivity over the current clinical method. New knowledge of the potential of NGS to detect low levels of drug resistant mutations and identification of pulmonary disease causing pathogens in clinical samples was also developed. These findings have helped to define the metrology support needed for emerging technologies such as NGS which can be used to direct future standardisation and metrology development work.

Objective 3: Quantitatively and comparatively evaluate new and emerging diagnostic technologies for the rapid detection of infectious agents

New methodologies were investigated for the rapid detection of infectious diseases by nucleic acid amplification. Tests were performed because of their potential to reduce costs, to achieve relevant diagnostic results in a short time and in a point-of-care setting. In particular, isothermal nucleic amplification tests were addressed, since:

- Time consuming thermo-cycling required for polymerase chain reaction (PCR) is avoided
- Specificity is expected to be higher compared to PCR
- There is robustness against influencing quantities like temperature, ion concentration and matrix variation
- Handling is easier when using freeze dried reagents
- Integration in disposable, micro fluidic devices is possible
- Development of complete, cartouche based point of care tests for specific clinical samples including DNA extraction can be achieved

- It is expected that the market share of isothermal nucleic acid tests (NATs) will increase in the near future

Rapid and near patient NATs rely on the handling of small volumes in the nL to µL range. It follows that high surface to volume ratios in reaction chambers or tubing for sample transport cannot be avoided and hence DNA/RNA loss on surfaces may significantly influence the outcome of qualitative and quantitative test. Consequently, we applied the surface sensitive X-ray methods photoelectron spectroscopy (XPS) and time of flight secondary ion mass spectroscopy (ToF-SIMS) to detect DNA adhesion on different surfaces including channels on microfluidic PCR chips. The results obtained with ToF-SIMS are compared with fluorescence microscopy and a correlation was observed.

Survey results: Clinical need for rapid, near-patient NATs

To obtain reliable information on the clinical need for rapid and near patient nucleic acid tests a survey was carried out by PTB, BAM, NIB, LGC and UCL. Analytical laboratories, regularly participating in round robin tests organised by the stakeholder INSTAND as well as manufacturers of instrumentation and kits for nucleic acid amplification tests were contacted. The survey revealed that a much shorter turnaround time is requested for selected pathogens compared to the approx. 2+ hours required when applying temperature cycling amplification, i.e. PCR. In this context, methicillin-resistant *Staphylococcus aureus* (MRSA), norovirus, influenza A and B viruses, cytomegalovirus (hCMV) as well as hepatitis B virus (HBV) and hepatitis C virus (HCV) were most frequently mentioned. 8 % of the laboratories which participated in this survey applied isothermal amplification of nucleic acids to identify infectious pathogens.

Isothermal nucleic amplification of human CMV and *Mycobacterium tuberculosis* H37Rv strain

Loop-mediated isothermal amplification (LAMP) was applied to detect human CMV (hCMV) and the H37Rv Tuberculosis strain. Limits of detection (LOD) were derived as well as the range for which quantification of the copy number is possible.

The WHO International Standard hCMV DNA (hCMV) was sourced from NIBSC. As result of a worldwide collaborative study, the assigned concentration of this material corresponds to $5x10^6$ International Units (IU) when reconstituted in 1 mL of nuclease free water.

For hCMV LAMP detection, primer sequences, published by Nixon et al. (2014) were used to amplify the UL54 target gene. Experiments were performed in the Genie II (Optigene, England) instrument at a reaction temperature of 65 °C. The limit of detection was determined to be (42 ± 12) copies per reaction corresponding to a 95 % probability of a positive result. The lower limit of quantification amounts to 100 copies per reaction, and the dynamic range for quantification is between 100 cp rx^{-1} and 10000 cp rx^{-1}. To further simplify the sample handling, a hCMV LAMP protocol based on a premixed frozen test kit was investigated. The kit was custom made by Amplex BioSystems GmbH (Gießen, Germany) and applied to detect the UL54 gene of hCMV. The premixed test for hCMV was found to have a limit of detection of LODhCMV = (85 ± 20) cp rx^{-1} and a limit of quantification amounting to LOQhCMV = 300 cp rx^{-1}. Both numbers are higher by a factor of 2 and 3, respectively, compared to the kit based on the use of various liquid reagents. Thus, the advantage of easy handling and a shorter turnaround time results in a reduced sensitivity.

A premixed, custom made kit (Amplex BioSystems GmbH, Gießen, Germany) was used to detect the H37Rv strain of Tuberculosis. In Figure 11, the amplification time, for which the fluorescence signal crosses a defined intensity level, and is hence positive, is plotted against the concentration in different matrices (Tris-EDTA buffer (TE-buffer), NaCl solution and serum).

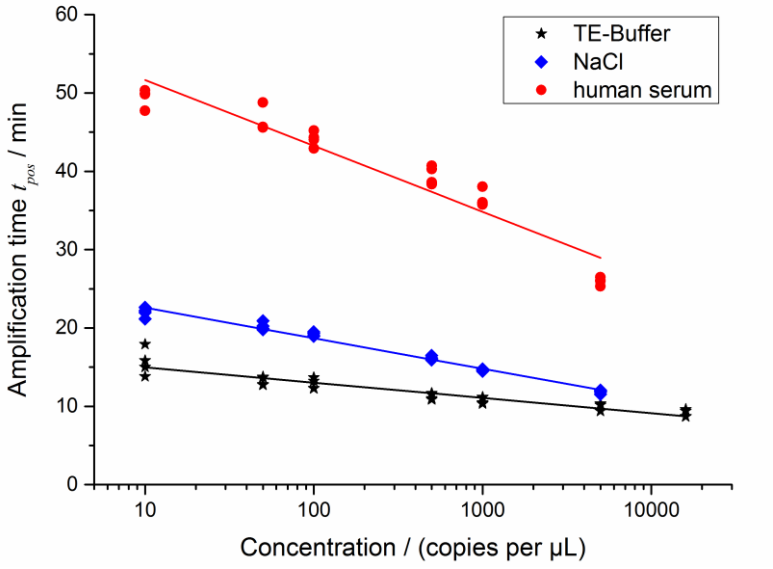


Figure 11: Amplification time versus concentration of *M. tuberculosis* in Tris-EDTA buffer (black), 0.9 % NaCl solution (blue) and in human serum (red). The lines are obtained from linear regression.

The most efficient amplification was observed in Tris-EDTA buffer. The amplification time was slightly higher in 0.9 % NaCl solution and increased significantly by a factor of approximately 3 when detecting H37Rv in human serum. The straight lines represent linear fits, indicating that in TE-buffer and in NaCl solution quantitative results can be deduced from the observed amplification time t_{pos}. It is exceptional that LAMP reliably amplifies the target sequence in high complexity matrices like human serum, since it is well known that PCR does not work in such medium. However, quantification of H37Rv in human serum is not recommended because of the poor amplification and the resulting low reproducibility in repeat measurements and the deviations from a linear dependence between amplification time and concentration.

DNA loss on surfaces

Rapid tests based on nucleic acid amplification require handling of small volumes resulting in high surface to volume ratios, which is unfavourable with respect to adhesion loss of DNA or RNA target molecules. To detect DNA adhesion loss, X-ray Photoelectron Spectroscopy (XPS)/Time-of-Flight Secondary Ion Mass Spectrometry (TOF-SIMS) (ToF-SIMS) and microscopic fluorescence imaging were applied at BAM and PTB respectively. PCR microchips were opened and adhesion of DNA on the surface of the micro fluidic channels was studied. SIMS analyses of the PCR micro channels thereby indicated a trend to increased DNA loss on the channel walls with increasing pH value. Using the SIMS surface analysis method, PDMS residues were detected in the micro channels.

To correlate the fluorescence and the SIMS measurements of the DNA, a region of interest of 500x500 µm^2 was chosen on a spot of DNA and buffer solution deposited on a glass substrate (Figure 12a). After scaling to a common raster of 11x11 pixels, the determined DNA intensity per pixel for both the fluorescence and the SIMS analyses was compared.

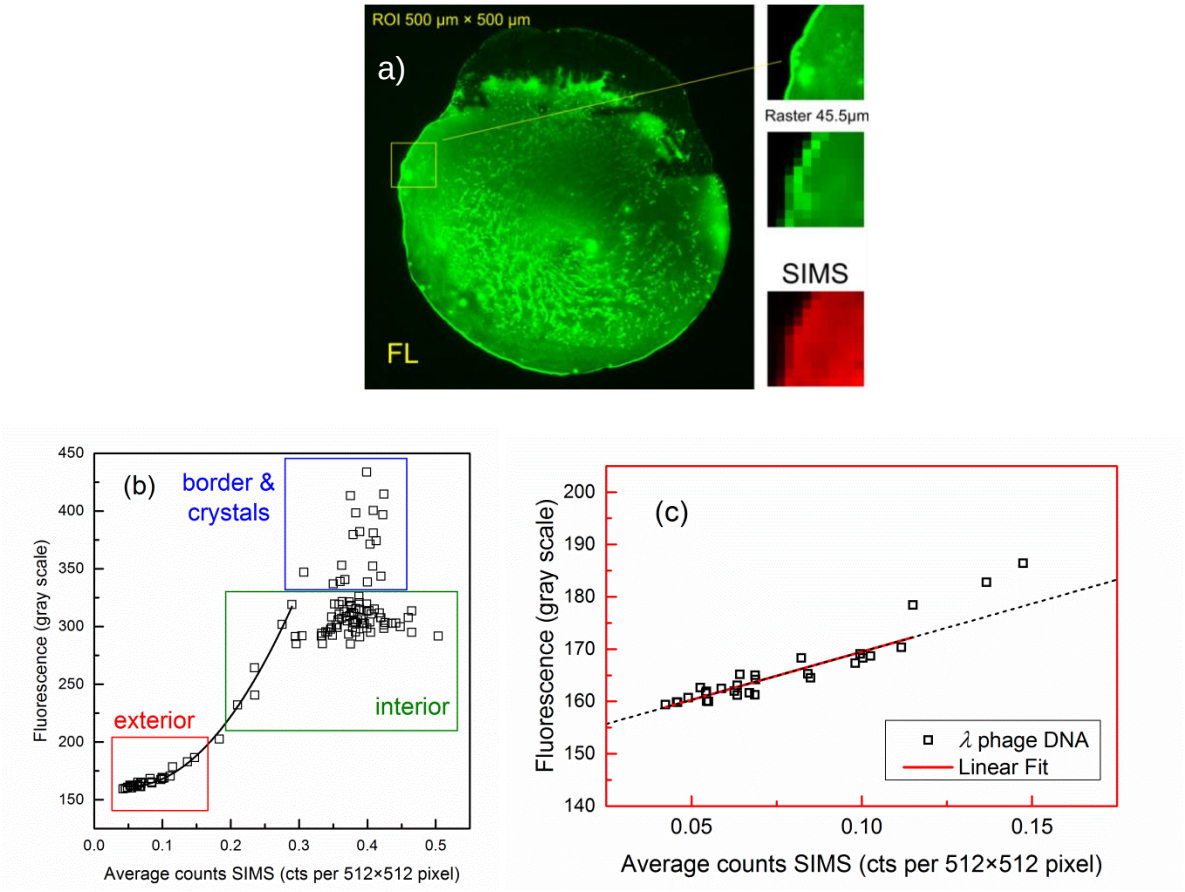


Figure 12: λ Phage Picogreen stained DNA on glass substrates. A correlation of fluorescence and SIMS data taken at different areas (exterior, interior and Border & crystals) has been established.

Three different regions were identified indicating the position of the deposited DNA as analysed by fluorescence and SIMS (Figure 12b). At low DNA concentrations, there was a linear correlation between fluorescence intensity and SIMS signal (Figure 12c). Increasing the amount of DNA resulted in a non-linear fit (Figure 12b). Deviation from a linear behaviour between DNA intensity and fluorescence values is explained by the different penetration depths of the two techniques. For SIMS, DNA is detected in a layer of typically 1-2 nm depth while fluorescence microscopy measures the whole area.

Comparative studies for performance evaluation of LAMP

To assess the performance of LAMP for hCMV detection, comparative studies of digital PCR, non-digital PCR and LAMP amplification were performed at LGC and NIB. It follows from our results that different nucleic acid amplification methods targeting a clinically important viral pathogen can readily be transferred to a commercially available chip based digital format and directly compared with their real-time counterparts. Transferring LAMP assays to a digital format improved quantification and both digital PCR and digital LAMP were more robust when challenged with selected inhibitors (e.g. plasma, EDTA and ethanol).

This project demonstrated the application of a performance metric comparable to PCR efficiency in the evaluation of quantitative LAMP (qLAMP) assays which is potentially applicable to other isothermal nucleic acid amplification (NAA) technologies. The isothermal doubling time metric described here facilitates the comparison of qLAMP assays that could assist assay development and validation activities within the developer and user communities.

Concept for the quality assurance of rapid and near-patient point of care (POC) nucleic acid amplification tests

Rapid and POC tests based on nucleic amplification tests shall be specified according to

- Its use as a qualitative or quantitative test
- The addressed target gene and the matrix
- The DNA/RNA extraction and the instrumental settings (i.e. temperature profile and reaction time)
- Limit of detection (LOD) using high purity material with values assigned by dPCR
- Linear range for quantitative tests

The limit of detection for both qualitative and quantitative tests is defined as the minimum copy number per reaction for which 95 % yield positive results. For quantitative tests it is recommended that at the concentration corresponding to the lower limit of quantification the relative standard deviation does not exceed 25 %.

Compared to routine analytical laboratories analysing more than 1000 samples per month, at present rapid and near-patient tests are applied much less frequently. Hence, it is not feasible to request a daily monitoring of the instruments' performance and the functioning of the test kit using a control sample. Instead it is recommended to use a control only on the days when the test is used to perform an internal quality assurance.

For external quality assurance, it is recommended that rapid and near-patient nuclei amplification tests shall also be included in the same external quality assurance scheme as tests applied in routine analytical laboratories. Since medical decisions based on rapid and near-patient NATs result in identical consequences concerning patients' treatment and safety, the same material should be provided for the external quality assurance of routine analytical laboratories and in POC settings. To account for the presently lower performance levels of rapid and near-patient tests, the evaluation limits for quantitative tests shall be set to allow higher variations. It is particularly important to include qualitative tests to allow assessment of the limit of detection declared by the manufacturer and to prevent false negative results. It is expected that the examination of POC tests by external quality assurance schemes will result in an improvement of their performance for the benefit of the health system.

As result of our survey of analytical laboratories, which was significantly supported by INSTAND e.V., the need for rapid and near patient nucleic acid amplification tests was documented. In particular, turnaround times of 15 min to 30 min are requested, e.g. for methicillin-resistant *Staphylococcus aureus* (MRSA), norovirus, and cytomegalovirus (hCMV).

LGC, NIB, PTB and TUBITAK evaluated two different kits for isothermal nucleic acid amplification (LAMP) of hCMV as well as digital LAMP by comparison experiments with dPCR and qPCR. Our results demonstrate that hCMV LAMP is not limited to a qualitative test as it can be applied for quantification of hCMV concentrations in a range that is dependent on the specific kit.

An isothermal LAMP test based on a reagent kit for the H37Rv strain of *M. tuberculosis* was investigated. Again, the test was found to be suited as a quantitative tool for the determination of the concentration of H37Rv DNA in TE-buffer and NaCl solution for concentrations above 10 copies per µL in the measurement solution. In addition, the superior performance of LAMP is demonstrated by reliably amplifying the target sequence in a high complexity matrix, i.e. human serum, which is not possible when applying PCR. Since amplification efficiency was significantly lower in human serum only qualitative information can be obtained.

At BAM, DNA deposited on PMMA or glass surfaces was identified by XPS and ToF-SIMS. First results were obtained revealing a correlation between the surface sensitive SIMS and fluorescence microscopy, performed at PTB. We conclude that SIMS could be developed into a valuable tool to characterise surfaces with respect to the quantification of adhesion loss of DNA, which depends on various influences like e.g. pH value.

Based on the results from all partners a concept to evaluate isothermal (LAMP) NATs and to establish internal and external quality assurance of such tests was derived.

The project successfully achieved this scientific and technical objective. New methodologies which offer a distinct advantage in terms of time and cost over standard qPCR approaches were tested and new knowledge was gained on the adhesion loss of DNA in materials used in the manufacture of microfluidic devices which can lead to inaccurate measurement results.

Objective 4: Develop methodologies for accurately quantifying the performance of commercially available diagnostic assays, 'in-house' clinical assays and novel emerging approaches

The infectious disease testing market is one of the most rapidly growing segments of the in vitro diagnostic industry, with advances in molecular diagnostic technologies being one of the main driving forces behind the expected growth. However, more effort is still needed for the transfer of these technologies around the world, with issues concerning quality, comparability and traceability of measurements being widely highlighted.

Despite advances in the molecular diagnostics of pathogens, the establishment of standards, reference methods and reference materials have lagged behind. Full confidence in molecular measurements can only be achieved if the appropriate metrology framework, standards, and higher order reference measurement procedures are developed. Reference materials which are traceable to SI or the equivalent are currently missing. The current WHO International Standards address some of the needs, but they mostly cover a limited range of viral pathogens predominantly those critical for the screening of blood donations (e.g. hepatitis A & B, HIV) and blood safety. They are expressed in international units (IU), which are often consensus values defined by the particular standard and they are therefore considered not to have an uncertainty.

The results of External Quality Assessment Schemes (EQAS) in laboratory medicine indicate that the differences between results provided by routine clinical laboratories in Proficiency Testing (PT) schemes are often as high as several orders of magnitude which reflects reproducibility issues in the preparation of standards with an arbitrarily assigned value or difficulties in calibrating the routine methods.

INFECT-MET developed validated measurement procedures and a validation framework to support current and emerging molecular approaches for efficient, harmonised and accurate diagnosis and monitoring of infectious diseases.

The suitability of the digital PCR (dPCR) methods and test materials developed earlier in the project for establishing a reference system and the existence of possible unrecognised bias was assessed by inter-laboratory trials for the bacterial Tuberculosis (TB) model and viral Cytomegalovirus (hCMV) model. The validated methods were checked for consistency between laboratories and amongst different methods on common samples. Furthermore, the project evaluated whether the developed test materials, with assigned values based on the dPCR method could be used as reliable calibrants for routine quantitative PCR (qPCR) methods. Calibration with reference materials, which have different characteristics from routine samples, leads to a calibration bias and to a break in the traceability chain leading to a lack of comparability of measurement results over time and space. Therefore it is of crucial importance to assess the commutability of the candidate reference materials or calibrators. To ensure their suitability, the candidate reference materials/calibrators should behave similarly to the relevant clinical samples and produce consistent results for typical clinical samples and calibrators/reference materials used for the calibration of the methods.

Higher order reference system for bacterial DNA

For the assessment of the potential of the dPCR method to establish a reference system and of the commutability (interchangeability) of the TB test materials, a comparison of the digital PCR (QX200, BioRad), and qPCR analysis was carried out at LGC. In addition, a semi-quantitative Cepheid Xpert MTB/RIF method was performed at UCL and external laboratories in Europe and Africa. Test materials were analysed using two assays targeting the rpoB and 16S rRNA genes conserved in the *M. tuberculosis* complex (closely related

species including *M. tuberculosis* and *M. bovis*) and three digital PCR (dPCR) platforms (Biomark, Fluidigm; QX100, BioRad; Quantstudio 3D, Life Technologies) in an earlier inter-laboratory study.

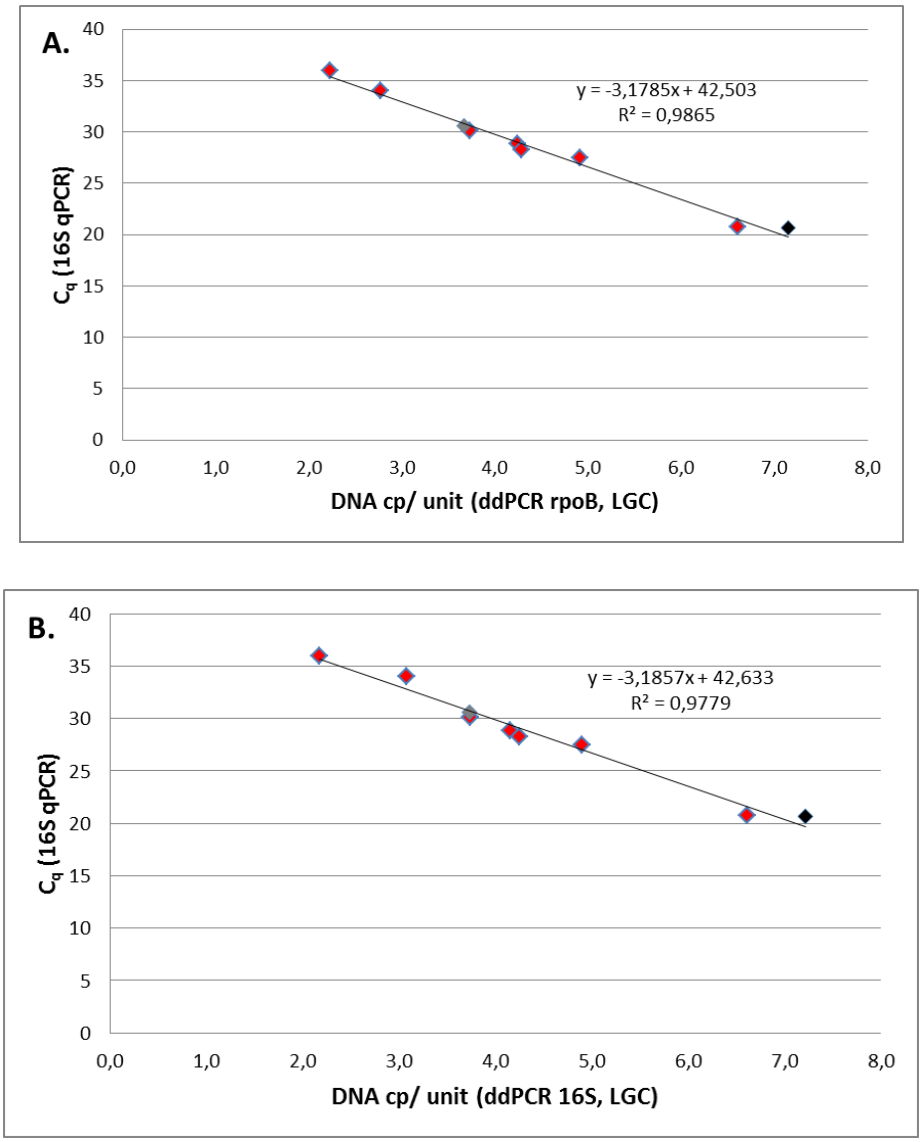


Figure 13: Commutability of the test materials for rpoB dPCR (A) and 16S dPCR (B) in comparison with qPCR output metrics (Cq) without calibration. Mean values measured for test materials 1 (T1) and 2 (T2) are indicated in grey and black, respectively.

The quantitative cycle (Cq) numbers of the 16S qPCR were compared to the rpoB (A) and 16S (B) dPCR assays (Figure 13) and a good correlation of the results of both methods was observed. Consequently, it can be concluded that the test materials (T1 and T2) could in principle be used to calibrate and standardise qPCR methods applied in this study. The slope of the correlation line is close to the theoretically expected value, which is - 3.33.

The semi-quantitative GeneXpert MTB/RIF method is not intended to be used for quantification of *Mycobacterium tuberculosis* and has consequently not been optimised for this purpose. Therefore, it is not surprising that a correlation between GeneXpert and dPCR methods for clinical samples and potential calibrators (T1 and T2) is lacking. The standardisation effect of using dPCR as a reference method and using reference materials with similar properties as samples T1 and T2 would be limited for this method.

Higher order reference system for viruses

For the assessment of the potential of the dPCR method to establish a reference system and of the commutability (interchangeability) of the hCMV test materials, an inter-laboratory study was organised by JRC in cooperation with Charité. Six native clinical CMV samples, three samples from 2 previous INSTAND EQA schemes for the detection of the hCMV genome in which JRC, NIB and LGC participated, one vial of the 1st WHO International Standard CMV DNA from NIBSC (NIBSC code: 09/162 2010) were analysed with routine quantitative PCR (qPCR) methods by 5 external INSTAND expert laboratories and with the validated digital PCR (dPCR) method applied by NIB.

As commutability is a method-specific characteristic, results of both clinical samples and test materials (1st WHO International Standard CMV DNA and the INSTAND CMV EQA samples) were evaluated for six qPCR methods, applied by five external laboratories if compared to the validated dPCR method on the one hand and among each other in a pairwise manner on the other hand. Results were presented as log10-transformed concentrations and only measures above LOD were considered.

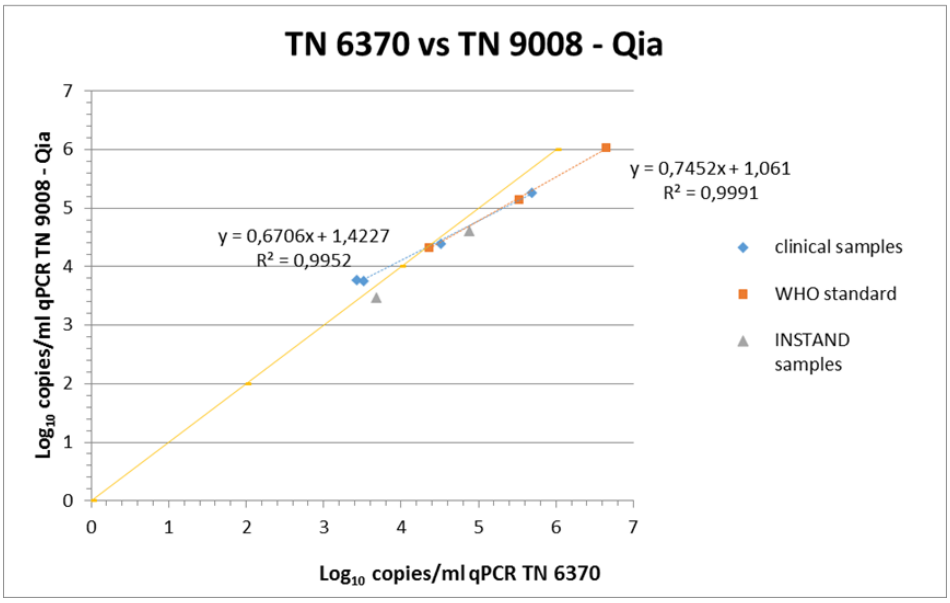


Figure 14: Commutability plot of test materials (1st WHO International Standard CMV DNA and INSTAND EQA samples) among qPCR methods used by participants TN 6370 and TN 9008. Calibration for TN 9008 is done with the quantitation standards supplied by Qiagen. Results for 4 clinical samples (viral load > LOD), a 3-point WHO-dilutions series and 2 INSTAND samples, are indicated in blue, orange and grey, respectively.

For 10 out of 15 pairs of qPCR methods, it was demonstrated that the test samples (1st WHO International Standard hCMV DNA and the INSTAND EQA samples) were commutable materials (Figure 14). It should be noted that the slope in the pairwise comparisons was not 1 and therefore elimination of inter-method bias would require recalibration with a commutable material.

However, if the validated dPCR method was used, poor correlation and an introduced bias between clinical samples and test samples of up to about 1 log10 could be seen for all measurement procedures (Figure 15). Hence the non-commutability of the test samples for dPCR versus qPCR methods would have quite dramatic consequences for a reference system and standardisation attempts. However, it should be noted that due to the limited number of samples and replicates, as well as the non-linear correlation between the clinical and test samples for most qPCR versus dPCR methods, these observations are related to a high uncertainty (i.e. ~ 40 %).

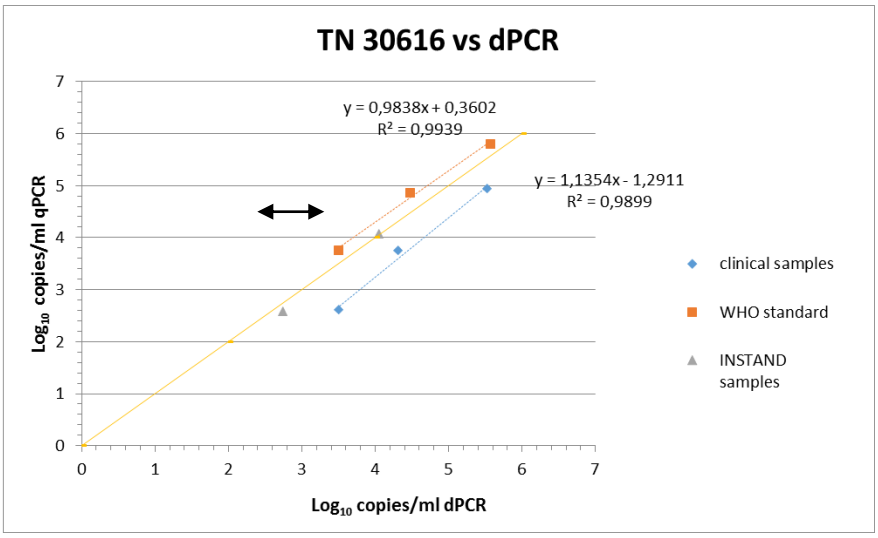


Figure 15: Commutability plot of test materials (1st WHO International Standard hCMV DNA and INSTAND EQA samples) for dPCR in comparison with qPCR data of participant TN 30616. Results of 3 clinical samples (viral load > LOD), a 3-point WHO-dilutions series and 2 INSTAND samples, are indicated in blue, orange and grey, respectively.

To estimate the between method bias introduced by calibrators or reference materials which are lacking commutability, at an acceptable level of uncertainty, a more comprehensive study would have to be performed, involving more clinical samples and more independent replicates of the test materials to be used for calibration. The methods established in INFECT-MET could eventually be used to assign values to more commutable, and hence more suitable, reference materials. The suitability of the reference materials should be experimentally assessed and not be based on assumptions.

In the absence of commutable calibrators, the validated dPCR method could be used for assigning viral load values to clinical samples, which could then in turn be used to calibrate routine assays. This approach is common practice in the in vitro diagnostics area.

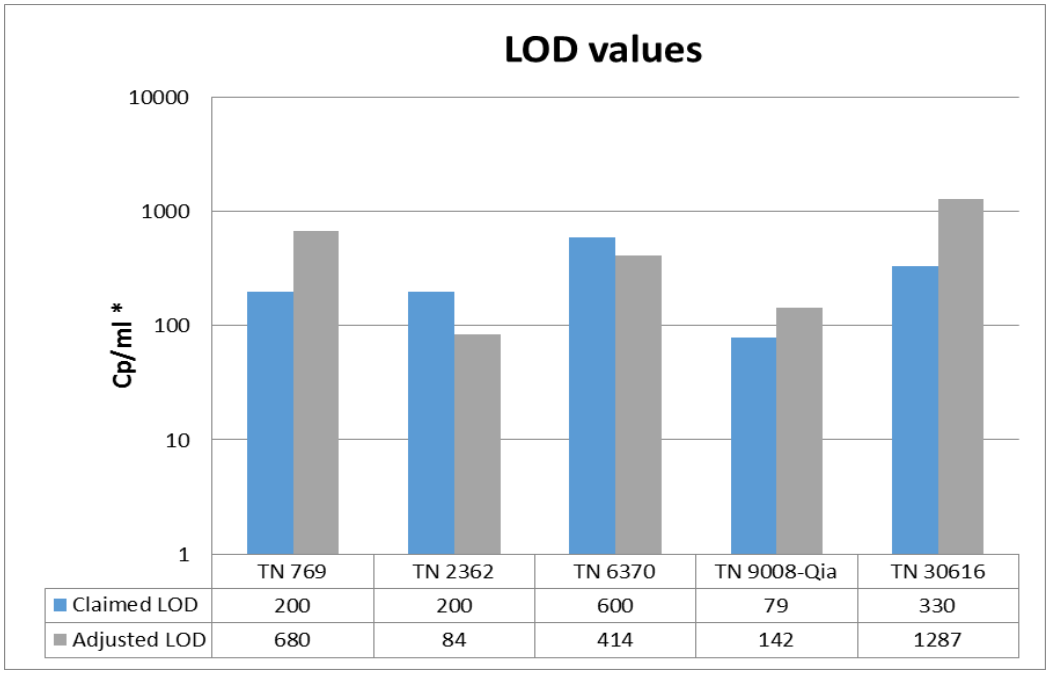
The performance of the commercially available and 'in-house' clinical assays used in this study to quantify hCMV, has been assessed by comparing the diagnostic efficiency and the analytical sensitivity (LOD) of the assay. A recalculation of the LOD was made by multiplying the claimed LOD with the slope of the linear regression line between clinical samples, which were obtained by dPCR versus routine qPCR methods.

Assuming that 1 IU/ml corresponds to 1 copy/ml, the number of samples falsely classified to fall below or above the LOD, could be reduced from nine to six after LOD correction, indicating a small improvement in the consistency between the LOD and diagnostic efficiency of the assays.

End users of diagnostic methods should be aware that diagnostic efficiency tends to be over- or under-estimated, which could lead to misdiagnosis and inappropriate treatment (Figure 16). Hence, adequate calibration could improve the determination of the LOD and increase the diagnostic efficiency of end-user methods. Both viral dynamics and the correct determination of the LOD to define the diagnostic threshold are of crucial importance for the diagnostic monitoring of patients under treatment.

Even though, this limited inter-laboratory study could not show an ideal correlation between the dPCR reference method and routine methods used for the measurement of hCMV in clinical samples, dPCR could form a solid basis for calibration and standardisation and would go well beyond the state of the art of standardisation approaches applied so far.

If a better understanding of the underlying reasons for the limited commutability and unrecognised sources of bias could be achieved, related bias could be eliminated and a robust reference system, consisting of commutable reference materials and reference methods could be set up. Even more efficient (i.e. bias free) metrological traceability could be established, which would lead to previously unachieved equivalent measurement results across methods, time and space for pathogen detection and quantification with unprecedented performance limits.



	TN 769	TN 2362	TN 6370	TN 9008-Qia	TN 30616
Claimed LOD	200	200	600	79	330
Adjusted LOD	680	84	414	142	1287

Figure 16: Graph showing the differences in claimed LOD by the different laboratories and the estimated LOD, recalculated by multiplying the claimed LOD with the slope of the linear regression line between clinical samples, which were obtained by dPCR versus routine qPCR methods. * All LOD's are reported in copies/ml, except for the laboratory TN 769, which uses IU/ml.

Recommendations for reference systems for pathogen testing

In light of the results obtained in the project, the developed methods could be used for establishing a stable and reproducible reference system with values expressed as sequence copy numbers traceable to the SI provided that all the critical parameters are taken into consideration and are under control. It is important to emphasize that this document represents a concept of the way in which the validation of molecular diagnostics assays could be performed. The findings from the studies carried out in the framework of INFECT-MET using three selected pathogens should be interpreted with caution. While the project tackled bacterial, DNA virus, and RNA virus models in a systematic manner, one cannot assume that the methods and experimental conditions selected in the study for the three models are optimal for other species belonging to these groups of pathogens.

Molecular identification/quantification of pathogens is a complex, multi-step process influenced by many factors. In addition, changing multiple parameters can lead to magnification effects. Hence, the availability of reference materials allowing for method validation, quality control, and assay calibration is of utmost importance. The project provides valuable clues particularly to reliably characterising and assigning property values to reference materials and calibration materials with molecular methods.

Efforts will be made post-project at JRC to understand the underlying reasons for outlying and discrepant results as well as for the non-commutability of calibrators which will allow so far unrecognised sources of bias

to be identified. Elimination of bias is crucial in terms of trueness of measurement results and for achieving the goal of measurement result equivalence across methods and over space and time. More comprehensive commutability studies need to be carried out for improving the statistical confidence for drawing further conclusions. Some remaining issues (like limited commutability of potential calibrators and reference materials as well as limitations in correlation between methods) are subject to further investigation (results pending). Although no commutable reference materials/standards could be identified for method pairs including the dPCR method validated by INFECT-MET the calibration bias resulting from the use of non-commutable reference materials could be avoided by using the dPCR method for assigning viral load values to clinical samples which could then in turn be used to calibrate routine assays. In the absence of commutable calibrators this approach is common practice in the in vitro diagnostics area.

INFECT-MET proved that dPCR with optimised extraction is reproducible and robust. Multiple influence parameters and quantities for dPCR and qPCR have been identified by INFECT-MET and can be controlled. Some of these influence parameters would not be expected in theory but are relevant in practice.

Although there are currently no quantitative routine methods for TB, methods and suitable materials have been identified which could be used to standardise quantitative TB measurements.

Improvements could be achieved to more objectively assess the LOD of quantitative hCMV methods and to reduce discrepancies between methods.

Even for the more tricky quantification of RNA viruses, progress was made by INFECT-MET. Although the source of discrepant results could not be identified, agreement between different laboratories applying an optimised dPCR method, including the outlying laboratory upon re-analysis, has been surprisingly good for measurements of highly unstable RNA.

INFECT-MET produced the following recommendations in a best practise guidance document for the scientific community on how a suitable reference system for pathogen detection could be established:

1. Validation of the nucleic acid extraction methods
2. To develop open nucleic acid extraction methods
3. Consideration of parameters influencing dPCR quantification results
4. Orthogonal methods should be used to evaluate the trueness of higher order reference methods
5. Digital PCR can be used to assign SI-traceable values to reference materials/calibrators
6. The performance, transferability, robustness and reproducibility of the reference method should be assessed
7. Suitability of qPCR calibrators should be evaluated on a case-by-case basis
8. Commutability studies should be carried out with candidate reference materials/calibrators to ensure their suitability

The project successfully achieved this scientific and technical objective. The value of the test materials and methods developed in this project and their potential as prototype reference systems for increased standardisation of molecular diagnostics, particularly through adoption in EQAS, was demonstrated.

Summary

- For the first time in Europe, digital PCR methods to enable absolute quantification of infectious agents with improved traceability to SI through the concept of enumeration were developed and validated for three model systems (Tuberculosis, Cytomegalovirus and Influenza) with full uncertainty budgets.
- A suite of novel test materials of differing levels of complexity (nucleic acid molecule, nucleic acid extract and whole microbe preparation) was produced for each model system and traceable values assigned using the developed methods through a series of inter-laboratory studies between project partners.
- Wider inter-laboratory comparison studies for Tuberculosis and Cytomegalovirus, involving end-user laboratories participating in External Quality Assessment schemes (EQAS), were completed, demonstrating the suitability of the digital PCR methods developed during the project for the analysis of clinical samples. The value of the test materials and digital PCR methods and their potential as prototype reference systems for increased standardisation of molecular diagnostics, particularly through adoption in EQAS, was demonstrated.
- Recommendations for the scientific community on how a suitable reference system could be established have been formulated into a best practice guidance document.
- An evaluation of novel molecular approaches (digital PCR and Next Generation Sequencing) for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations was completed.
- A novel digital PCR method was developed to detect drug resistance in influenza which showed an increased sensitivity over the current clinical method.
- New knowledge of the potential of Next Generation Sequencing to detect low levels of drug resistant mutations and identification of pulmonary disease causing pathogens in clinical samples was also developed. These findings have helped to define the metrology support needed for emerging technologies such as NGS which can be used to direct future standardisation and metrology development work.
- New methodologies which offer a distinct advantage in terms of time and cost over standard quantitative PCR (qPCR) approaches were tested and new knowledge gained into the adhesion loss of DNA in materials used in the manufacture of microfluidic devices which can lead to inaccurate measurement results.

4 Actual and potential impact

Dissemination

The results and the quality and traceability guidance for infectious disease molecular diagnostics developed in the INFECT-MET project are currently being disseminated into the official international Standards system through the following routes:

1. ISO TC212 laboratory medicine WG2 - ISO 17511 revision (JRC & LGC committee participation & drafting)-developing examples of traceability schemes for pathogen detecting in vitro diagnostic devices.
2. ISO TC212 laboratory medicine WG4 - Technical Specification© ISO 2014 – ISO/TS 17822-1:2014(E) 63 Part 1: General requirements, terms and definitions In vitro diagnostic test systems — Qualitative nucleic acid-based in vitro examination procedures for detection and identification of microbial pathogen – publications in reference list.
3. ISO TC212 laboratory medicine WG4 - Consideration of submitting a PWI (Proposed Work Item) on quantitative PCR for infectious disease diagnostics, (inputting results from INFECT-MET). This proposed work item is being drafted by LGC/UK for consideration and approval at the November 2015 meeting of TC212 WG4.

4. ISO TC276 biotechnology WG3 analytical methods (LGC participation & lead on NWIP drafting committee) – input into ISO/PWI 20395: “Quality considerations for targeted nucleic acid quantification methods”. This approved PWI (Proposed Work Item) is currently being developed into a NWIP (New Work Item Proposal) for voting at the October meeting of ISO TC276.

Considerable dissemination to the relevant user communities has taken place during the course of the project. In total, 1 book and 7 peer-reviewed papers describing INFECT-MET scientific achievements have been published in peer-reviewed journals. Wider dissemination of outputs from the project was also achieved through an article published in the November 2014 issue of Laboratory News (UK). 39 Presentations have been given at relevant international professional and clinical symposiums and the consortium has also been active in organising and participating in a number of workshop/training events with the user community.

Early impact

At the end of the project there are many examples of the outputs being taken up by the relevant communities:

- As part of the project’s objective *to develop methodologies for accurately quantifying the performance of commercially available diagnostic assays*: ‘in-house’ clinical assays and novel emerging approaches our successful participation in External Quality Assessment schemes (EQAS) for Cytomegalovirus (CMV) highlighted the utility of the digital PCR method developed in the project. All samples analysed were designated as correct and in good agreement with the target values. Results from this scheme will support efforts to standardise quantitative detection of CMV in terms of replacing ‘consensus’ values with traceable reference values. This will ultimately lead to more reliable tests and therefore more accurate/effective diagnosis. Discussions have begun with the organisers of EQAS to use the digital PCR method developed for CMV to value assign materials in future schemes.
- As part of the objective *to develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents*: clinical laboratories collaborating on the project (e.g. Bolnisnica Golnik Klinicni oddelek za pljucne bolezni in alergijo (UCG)) have benefited from the step-wise approach to assessing the performance of commercial extraction kits in order to select the best kit to use in a clinical setting. These and other clinical laboratories will be able to benefit from the guidance provided in the best practice guidance document.
- As part of the project’s objective *to develop highly accurate methodologies for the quantitative measurement of infectious agents*: the digital PCR method developed was used to value assign commercially available TB material from a microbiology-biotechnology company which develops, manufactures and markets ready-to-use PCR-based, and other, kits for the diagnosis of infectious diseases. The method used was able to provide the company with additional quantitative data on the amount of TB in their products, resulting in improved confidence in test performance.
- As part of the project’s objective *to evaluate new and emerging molecular approaches for the surveillance and monitoring of infectious disease load and detection of antimicrobial resistance mutations*: the materials developed and donated by the project in prototype format for evaluation by end-users are already starting to have an impact on the way end-user laboratories perform their measurements and quality control. For example, the whole microbe control material has been used by a clinical laboratory to establish a next generation sequencing capability. Having a material that contains quantified amounts of bacteria has ensured that their laboratory and bioinformatic workflow was consistent and re-producible. The material is also being used by UCL to evaluate new sequencing technology being developed by a UK company.

As an example of findings from the project being taken up by external laboratories, selection of the optimal TB extraction kit (part of the objective *to develop quantitative, validated and highly accurate methodologies for the measurement of infectious agents)* has helped UCL standardise their sample processing protocol in a clinical evaluation of Whole Genome Sequencing of TB directly from sputum samples.

Future and wider impact

Through the development of higher order methods, a metrology framework and standards, the output of INFECT-MET will help healthcare providers and the biotechnology/diagnostic industry to demonstrate the reliability of their assays in a traceable way. The outputs will have particular potential for impact in supporting both the activity of the proposed new network of Reference Laboratories for Class D (infectious pathogens) IVDs, and the requirements for demonstrating metrological traceability, mandated by the new EU IVD regulation, which has now received EU parliament approval for a partial general approach, and is now expected to enter into force by early 2016.

The IFCC (International Federation of Clinical Chemists and Laboratory Medicine) sub-committee on Molecular Diagnostics is currently focusing international effort on supporting increased measurement quality in infectious disease diagnostics. The Chair of the Committee has stated an interest in disseminating the project recommendations for reference systems for pathogen testing, through the IFCC member network.

The social impact of the project comes from supporting the provision of improved healthcare to citizens across Europe. The development of reference methodologies and materials with reduced uncertainties and improved traceability to the SI are now available and their uptake by commercial and clinical laboratories via routes such as External Quality Assessment schemes will lead to more robust and comparable diagnosis and monitoring of infectious diseases and antimicrobial resistance.

5 Website address and contact details

A public website was created where the main public deliverables have been made available for the end-users and to keep them informed about project meetings and events: http://infectmet.lgcgroup.com/

The contact person for general questions about the project is Dr Carole Foy (Carole.Foy@lgcgroup.com).

6 List of publications

Nixon, G., Garson, J., Grant, P., Nastouli, E., Foy, C., Huggett, J. (2014) A comparative study of sensitivity, linearity and resistance to inhibition of digital and non- digital PCR and LAMP assays for quantification of human cytomegalovirus. *Anal Chem*, 6;86(9):4387-94.

Honeybourne, I. (2014) The Molecular Bacterial Load Assay replaces solid culture for measuring early bactericidal response to anti-tuberculosis treatment. *J. Clin. Microbiol,* 52(8):3064.

Huggett, J., O'Grady, J. (2014) Molecular Diagnostics: Current Research and Applications – Book. EAN: 9781908230416 9781908230645.

Nixon, G., Svenstrup, H., Donald, C., Carder, C., Stephenson, J., Morris-Jones, S., Huggett, J., Foy, C. (2014) A novel approach for evaluating the performance of real time quantitative loop-mediated isothermal amplification-based methods. Biomolecular Detection and Quantification 2: 4–10.

Devonshire, A., Honeyborne, I., Gutteridge, A., Whale, A., Nixon, G., Wilson, P., Jones, G., McHugh, T., Foy, C., Huggett, J. (2015) Highly reproducible absolute quantification of Mycobacterium tuberculosis complex by digital PCR. *Anal Chem,* 87(7):3706-13.

Neukammer, J., Hussels, M., Kummrow, A., Devonshire, A., Foy, C., Huggett, J., Parkes, H., Zel, J., Milavec, M., Schimmel, H., Unger, W., Akgoz, M., Mchugh, T., Tomic, V., Grunert, H.P., Zeichhardt, H. (2015) Survey results on nucleic acid tests of infectious diseases: Present status and need for rapid and patient near diagnostics. *GMS Zeitschrift zur Förderung der Qualitätssicherung in medizinischen Laboratorien*, Vol. 6, ISSN 1869-4241.

Pavšič J., Devonshire A., Parkes H., Schimmel H., Foy C., Karczmarczyk M., Gutierrez Aguirre I., Honeybourne I., Huggett J., McHugh T., Milavec M., Zeichhardt H., Žel J. (2015) Standardising clinical measurements of bacteria and viruses using nucleic acid tests. J. Clin. Microbiol, Volume 53, Number 7

Luznik, D., Kosnik, M., Tomic, V. (2015) Comparison of Seeplex PneumoBacter Detection ACE assay with in-house multiplex PCR for identification of Streptococcus pneumoniae. *New Microbiol.* 38(1):51-8.