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EMRP
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TABLE OF CONTENTS

1 Executive Summary

Introduction

Accurate counting of biologically relevant molecules (nucleic acids and proteins) and entities (cells) underpins many sectors including the food, healthcare, and biotechnology sectors. For example, viral load monitoring in patients, circulating tumour cells in cancer or presence of GMOs (genetically modified organisms), lend themselves to description in terms of number of discrete entities such as DNA copies or number of cells. There is, however, a lack of higher order reference methods and materials to facilitate traceability and measurement comparability which impacts upon accreditation and regulatory compliance.

This project improved the state of the art in two key areas for providing higher order SI-traceability in biological measurement: (1) the application of purified calibration materials and (2) the use of enumeration (counting) technologies. The project developed methods and protocols for metrologically sound characterisation of pure biological materials to be used as calibration materials, and developed new counting methods.

The Problem

Previous attempts to assign SI values to nucleic acid reference materials have proved difficult. However, recent developments in absolute molecular measurement approaches such as digital polymerase chain reaction (PCR) and next generation sequencing (NGS) offer the potential for performing higher order measurements. Digital dPCR has potential as a highly sensitive, precise and reproducible method for the molecular quantification of DNA targets, however, it relies on a number of assumptions which are hard to validate without independently measured reference standards with low uncertainty. NGS relies on ultra-high throughput sequencing of many millions of individual fragments of DNA, giving a count proportional to the numbers of copies of each sequence present. However, NGS technologies have, to date, mostly been applied to qualitative studies.

The Solution

In response to this problem, Bio-SITrace developed a framework for achieving traceability of bio-molecules (nucleic acids and proteins) and bio-entities (cells) to the SI using higher order enumeration and purity assessments.

The project focussed on the need for characterisation, calibration and reference materials for three key biological molecules and entities:

- Counting of circulating tumour cells and DNA biomarkers in monitoring for minimal residual disease
- Counting of lipoprotein particle assemblies associated with forming fatty deposits in arteries and coronary heart disease risk
- Blood cell counting for haematological and immunological diagnosis and therapy control

Impact

The results from this project enable the demonstration of SI traceability through counting for nucleic acid and cell measurements. This underpins the development of reference materials and supports compliance with the IVD Directive 98/79/EC, and international standards such as ISO 17511. The technical outputs from this project have made a significant contribution to the development of international standards.

In total, 9 papers describing the project's scientific achievements have been published in peer-reviewed journals. Further dissemination has included focussed stakeholder workshops and presentations (43 in total) at significant stakeholder conferences including the International Federation of Clinical Chemistry (IFCC) WORLDLAB Congress, IFCC & European Federation of Laboratory Medicine EUROMEDLAB, IEEE

International Symposium on Medical Measurement and Applications (MeMeA) and the Joint Committee for Traceability in Laboratory Medicine (JCTLM) Members' and Stakeholder's Meeting.

The project successfully developed methods and materials which can be used to count biological molecules accurately. Key outputs of the project included proof of concept papers and traceability chains for accurate and traceable quantification of nucleic acids and cells which are currently being incorporated into two international standards being developed under ISO TC276 (Biotechnology) and revision of ISO 17511, the in vitro diagnostic medical devices standard.

2 Project context, rationale and objectives

Context

The 2011 BIPM report “Study of Measurement Service and Comparison Needs for an International Measurement Infrastructure for the Biosciences and Biotechnology” clearly identified the measurement services, international comparisons and collaborative R&D needed to underpin the comparability of bio-measurement, based on identified needs for metrology support from industry and regulators. These needs included the key requirement of “Support for fundamental metrology, aimed at making bio-measurements traceable to the SI”.

The principles of EU Directive 98/79/EC with regard to the importance of traceability of calibrators were further elaborated under an EU harmonised Standard, ISO 17511:2003, In vitro diagnostic medical devices – Measurement of quantities in biological samples – Metrological traceability of values assigned to calibrators and control materials. In ISO 17511:2003 it is explained that standardisation among different routine measurement procedures should ideally be achieved according to fundamental metrological principles with full traceability to SI units, supported with higher order reference measurement procedures and reference materials.

Objectives

Bio-SITrace aimed to develop methods and protocols for metrologically sound characterisation of pure biological materials for calibration. It also aimed to develop new counting approaches including methods of verification and measurement uncertainty evaluation that permit their use as reference methods and provide the basis for a substantial increase in the range of biological measurements that can rely on traceability to the SI.

The project focussed on three main objectives:

1. *Identify and develop approaches to the treatment of uncertainties in enumeration*.
   The project considered the uncertainty of the counting, the uncertainty of selecting a sample and the uncertainty of identifying the correct biological entities. By developing methods to count individual molecules or entities with a reliable uncertainty budget the project aimed to enable traceability of molecular measurements to SI.
2. *Develop traceable nucleic acid, protein and cell measurement methods based on enumeration technologies*.
   Establish procedures and uncertainty budgets suitable for primary reference measurements of the amount of nucleic acid based on single-molecule detection and counting, in particular using digital PCR. Develop and evaluate reference measurement procedures for the counting of cells across a range of techniques.
3. *Develop methods for purity characterisation of pure calibration standards for biological measurement.*
   Assess methods for the characterisation of purity for reference materials intended for use as higher order calibrators in SI traceable biological measurements, including the application of NGS methods as well as conventional methods (e.g. chromatography).

3 Research results

Objective 1: Identify and develop approaches to the treatment of uncertainties in enumeration

The main aim of this part of the project was to develop a conceptual framework for achieving traceability of bio-molecules (nucleic acids and proteins) and bio-entities (cells) to the SI using higher order enumeration methods such as digital PCR, next generation sequencing and flow cytometry.

Project work in this area included the development of a model for establishing metrological traceability for counting through chain of calibrations and the definition of an experimental strategy for higher order enumeration and purity measurements.

Establishing a model for bio-molecular and bio-entity traceability

A general model for traceability, based on the traditional use of primary and intermediate calibrators was established for cell enumeration, lipoprotein measurement and nucleic acid measurement by PTB, LNE and LGC respectively. In each case, the measurand was identified, the primary measurements necessary to establish SI traceability specified and a summary traceability chain developed. An example for blood cell measurement is illustrated in Figure 1. The resulting models were disseminated within the project for further use, and after further refinement and development within the project are being incorporated into relevant International Standards.

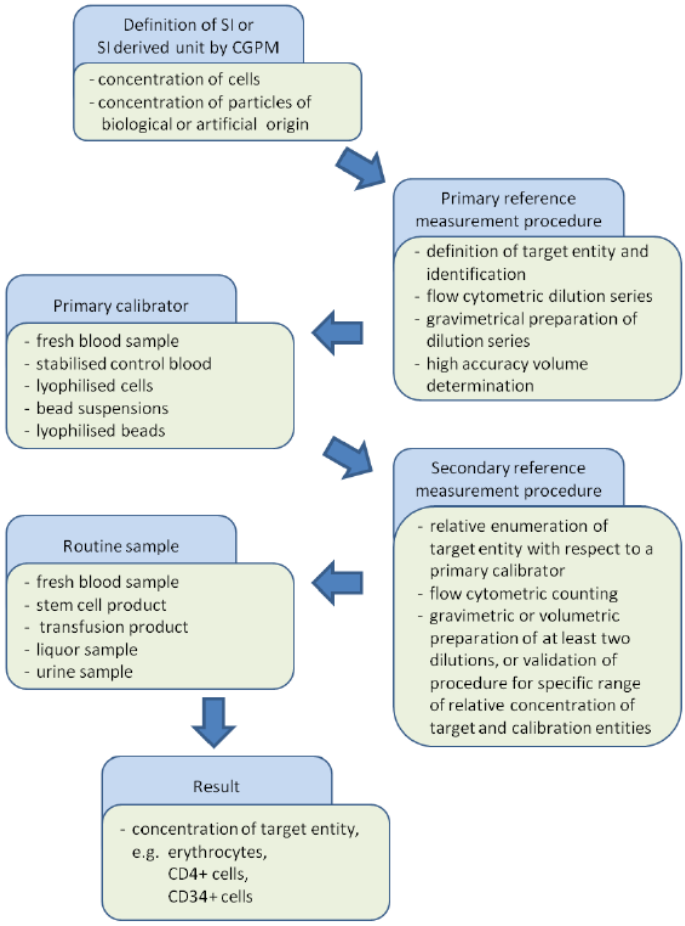

Figure 1: A model for metrological traceability based on enumeration – Example: Calibration hierarchy for measurement of (blood) cells based on ISO 17511.

Defining the experimental strategy for development of higher order enumeration and purity measurements

A detailed review of experimental strategies for enumeration and purity measurements applied to nucleic acids, lipoproteins, cells and proteins (see Objective 3 for further work on protein purity) was completed at LGC, PTB and LNE respectively with input from NIB and TUBITAK. Primary measurement techniques included flow cytometric measurement of cell concentrations, microscopic cell counting, nucleic acid counting using PCR and a range of candidate methods for proteins. The experimental strategies were implemented in later stages of the project; in particular to achieve Objective 2 relating to enumeration strategies and Objective 3 for characterisation and use of pure materials.

Uncertainty evaluation for enumeration

Models for uncertainty evaluation in enumeration, using digital PCR as a model system were developed. The work demonstrated that cell volume variation not only added to the uncertainty, as expected, but also led to consistent bias in digital PCR. This is an important finding for the emerging technology of 'droplet' digital PCR, in which the reaction cell volume is not directly controlled. A detailed mathematical foundation developed at LGC within a previous EMRP project NEW04 (Uncertainty), provided an opportunity to develop an advanced model for the assessment of uncertainties relating to possible failure of the Poisson distribution assumption underlying current digital PCR estimation methods, providing adjusted values and more realistic uncertainty estimates for this key technology. Further work in the project (together with detailed experimental work contributing to Objective 3) established the principal factors affecting results in enumeration as a primary method.

Key research outputs and conclusions

The project successfully achieved this scientific and technical objective. In summary:

- General models for establishing higher order metrological traceability for biological measurements using enumeration and use of pure materials were developed. These models were applied to work in the technical phases of the project and disseminated through published work on stem cell enumeration.
- Experimental strategies were defined to implement the general models above that have been demonstrated using model systems in the course of delivery of Objectives 2 and 3.
- Advanced models for uncertainty evaluation in enumeration with particular attention to high accuracy nucleic acid measurement were developed.
- Models for cell and nucleic acid traceability chains and uncertainty evaluation will be incorporated into developing ISO standards including TC 276 Biotechnology WG3 – ISO 20395, and TC 212 WG2 – ISO17511.

Objective 2: Develop traceable nucleic acid, protein and cell measurement methods based on enumeration technologies

Enumeration methods for nucleic acids

The main aim of this part of the project was to establish procedures suitable for primary reference measurements of amount of nucleic acid based on single-molecule detection and counting (enumeration) and assignment of SI traceability to DNA control samples, calibrators and reference materials. The principal experimental factors and sample characteristics that affect quantitative measurement using digital PCR (dPCR) were investigated and results reported to assist validation of digital PCR measurement systems including the associated uncertainties.

Selection of Model Systems

At LGC and in consultation with key stakeholders, an analytically challenging model system was selected to measure mutations in the *KRAS* (Kirsten rat sarcoma viral oncogene homolog) gene in a non-invasive, cell-free DNA diagnostic model (i.e. measurement of small amounts of tumour DNA shed into the blood – "liquid biopsies") reflecting both clinical need (colorectal cancer) and a lack of reference materials. This target is used to guide treatment of certain cancers and the model is representative of other cancer associated mutations as well as genetic measurements associated with foetal abnormalities, donor organ rejection and antimicrobial drug resistance. The analytical method chosen was digital PCR (dPCR), a modified version of conventional PCR that affords absolute quantification of nucleic acids by measuring individual molecules.

Preparation of test materials

Plasmid constructs were designed at LGC and synthesised by a commercial supplier which contained a genomic DNA fragment of *KRAS* exon 2 that is the location of seven frequently observed pathogenic single nucleotide variant (SNV) mutations. An additional plasmid containing the wild type (WT) *KRAS* sequence would be used as a negative control. One of the mutant SNVs for colorectal cancer (G12D) management was subsequently chosen from the plasmids containing mutant sequences.

Plasmid preparations of G12D and WT *KRAS* were used at LGC for restriction enzyme digestion to generate varying fragment lengths containing the *KRAS* SNV, from very short fragments (<200 bp) to larger fragments (several kilobases). Following further stakeholder consultation and literature review, the plasmid digestion strategy producing a 186 bp fragment containing the mutant SNV was chosen to be most relevant for a cell-free DNA model.

Experimental factors affecting quantitation by dPCR

The following sources of bias affecting dPCR were assessed by LGC and NIB in a previous EMRP project HLT08 (INFECT-MET):

- PCR mastermix
- Template concentration (so called λ value (template copies per dPCR partition)
- Template type (plasmid, intact and fragmented genomic DNA)

For enumeration of DNA templates differing by a single base pair, different levels of specificity may be required depending on whether the material being analysed is a 'pure' reference material where purity is interrogated independently to quantification, or a mixed sample (e.g. suitable for clinical proficiency training schemes). A number of assay chemistries (Intercalating Dyes, Hydrolysis probes, Competitive Allele-specific TaqMan (CAST) PCR were compared which were/were not able to discriminate between mutant and wild-type SNVs. In addition, to investigate the magnitude of other sources of bias such as partition volume which may contribute to the uncertainty associated with dPCR measurements, dPCR was compared with an orthogonal method - inductively coupled plasma mass spectrometry (ICP-MS). While PCR can measure both pure and mixed samples the orthogonal method can only measure the former.

Assessing the accuracy of dPCR by comparison with an orthogonal method (ICP-MS)

The concentrated stock of the 186 bp *KRAS* fragment was quantified at LGC using digital PCR, ICP-MS and the Qubit Fluorometer. The three quantification methods were orthologous i.e. each method used different techniques to quantify the material and so were not linked in any way.

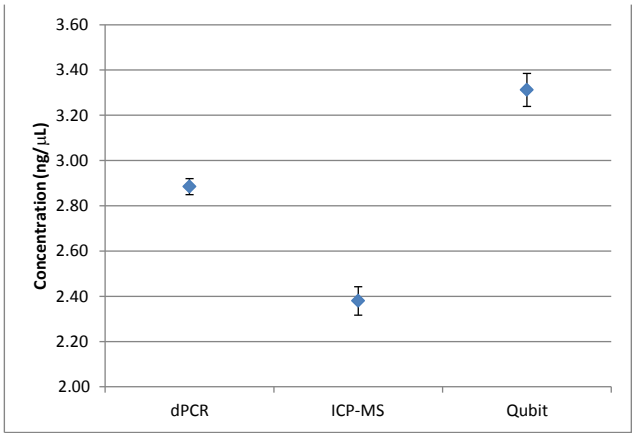

Figure 2:.Measurement comparison of the G12D fragment as measured by dPCR, ICP-MS and Qubit. Error bars represent the standard deviation between the triplicate measurements.

Results showed a 21% difference in concentration between the dPCR and ICP-MS results (Figure 2). The Qubit method measured a 14% higher concentration than that measured by dPCR that was consistent with previous studies. Good concordance was observed between dPCR and ICP-MS measurements. A much more highly concentrated stock of plasmid fragment DNA was used for the ICP-MS measurements compared to the high dilution applied to the dPCR measurements which may be attributable for the ~20 % difference observed. Work is ongoing (post-project) to establish full uncertainty budgets for dPCR and ICP-MS measurements.

Effect of test material characteristics on quantitation by dPCR

The aim of this part of the project was to assess the influence of the total DNA fragment size on simultaneous dPCR-based quantification of the WT and mutant *KRAS* templates.

The WT and G12D plasmids were digested to produce different fragment sizes:

- EcoRI restriction enzyme (fragment size with target sequence, 2634 bp)
- Combination of EcoRI and NsiI restriction enzymes (fragment size with target sequence, 373 bp)
- Combination of AfIII and NsiI restriction (fragment size with target sequence, 186 bp)

Samples containing different ratios of mutant to WT (1:1, 1:10, 1:100, 1:1000, 1:2000) copy numbers were then prepared and quantified using the QX100™ Droplet Digital™ PCR system at LGC and NIB.

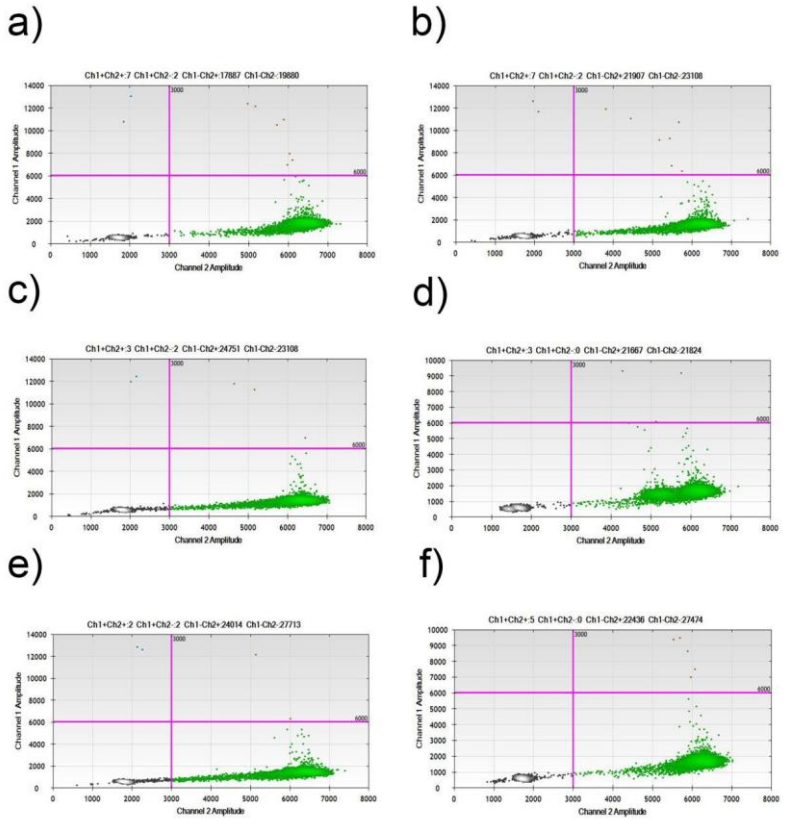

Figure 3: The occurrence of false-positive droplets in samples of wild-type plasmid DNA and without G12D plasmid DNA. 2D plots are shown, Ch1 – G12D assay (threshold of 6000), Ch2 – WT assay (threshold of 3000). Samples with EcoRI- linearised plasmids tested in experiments 1 (A) and 2 (B), samples with EcoRI/NsiI- fragmented plasmids analysed in experiments 1 (C) and 2 (D), and samples containing AflIII/NsiI-fragmented plasmids tested in experiments 1 (E) and 2 (F) are shown.

Results suggested a negative correlation between the size of the fragment containing the target sequence and the assay performance in terms of cross-reactions (number of false-positive droplets in G12D assay when amplifying WT target DNA only) and subsequent false positive rate. At ratios above 1:100, all three fragment sizes allowed repeatable determination of the G12D and WT DNA. However, false-positive droplets diminished the sensitivity of the G12D assay in particular for samples containing low G12D DNA copy numbers in high WT background, which is the type of sample most relevant to the clinical application of the assay. Further optimisation of the concentrations of primers and probes and/or reaction amplification conditions would be needed to improve the applicability of the assay.

Reproducibility of digital PCR detection chemistries

KRAS G12D mutant plasmid or a mixture of mutant and WT were evaluated using five different assays with three different detection chemistries at LGC. Samples were analysed using the QX100™ Droplet Digital™ PCR system. The detection chemistries investigated were:

- TaqMan® MGB hydrolysis probes using a previously published assay (Taly et al 2013) and a commercial pre-validated assay (PrimePCR™ ddPCR™ mutation detection assay, Bio-Rad).
- A Scorpion hybridisation probe designed during the study.
- Two non-discriminating assays ‘EvaGreen 80 bp’ and ‘EvaGreen 164 bp’ used the double stranded DNA binding (dsDNA) dye with two amplicon sizes. This method was able to quantify the pure sequences but if sequences were mixed with WT.

The detection chemistries were compared when measuring a variety of target concentrations.

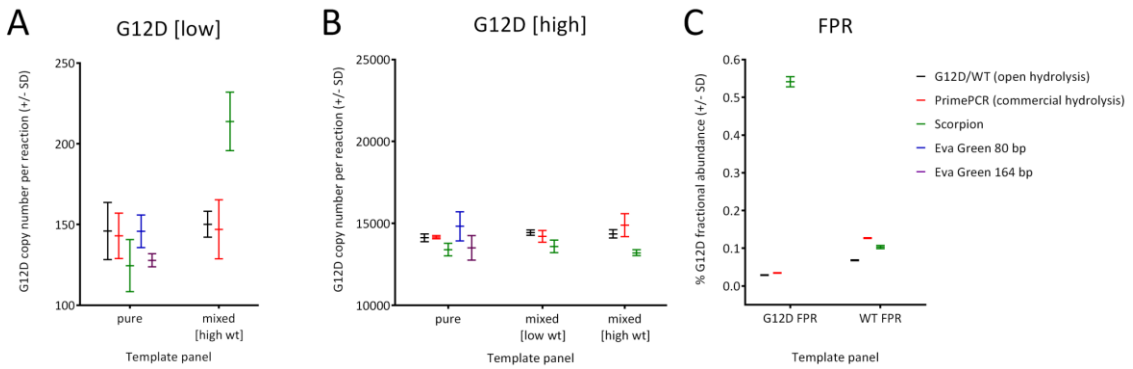

Figure 4: Comparison of dPCR assay chemistries. Quantification of samples containing *KRAS* G12D molecules at (A) low (nominally 100 copies/reaction) or (B) high (nominally 10^4 copies/reaction) concentration. Samples were either pure or mixed (contained *KRAS wt* molecules). (C) False positive rate (FPR) of the assays using the high concentration pure samples. Error bars represent the standard deviation between experiments.

Results showed that when using mixed samples, which are more comparable to *in vivo* situations, the Eva Green approaches could not discriminate between WT and mutant. In addition, when the mutant sequence was low relative to the WT, the Scorpion chemistry showed poor specificity due to cross reactivity with the more abundant WT sequence. These data demonstrate that dPCR chemistries show good reproducibility when measuring pure sequences and offer a potential reference method for value assignment of nucleic acid reference materials. Furthermore the specific of the hydrolysis probes when measuring the mixed samples open the possibility of it being used as a reference method and/or diagnostic method on more complex clinical samples.

Reproducibility of digital PCR instruments

KRAS G12D mutant plasmid or a mixture of mutant and WT were evaluated at LGC on five different dPCR instruments using the TaqMan® MGB hydrolysis probes using a previously published assay (Taly et al 2013). The instruments investigated were:

- QX200 (Bio-Rad)
- Raindrop (Raindance Technologies)
- Quantstudio 3d (Thermo Fischer Scientific)
- Biomark (Fluidigm)
- Constellation digital PCR system (Formulatrix)

The instruments were compared when measuring a variety of target concentrations.

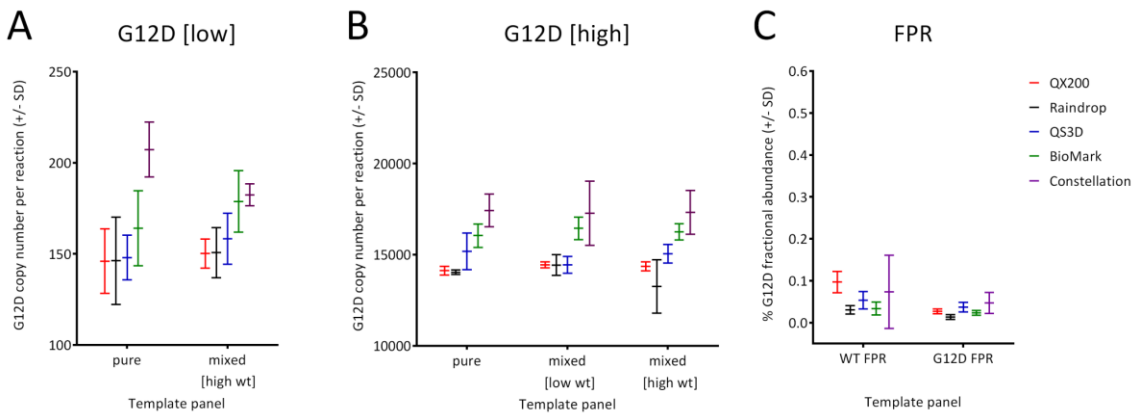

Figure 5: Digital PCR platform comparison. Quantification of samples of containing *KRAS* G12D molecules at (A) low or (B) high concentration using five different dPCR platforms. (C) False positive rate (FPR) of the dPCR platforms. Error bars represent the standard deviation between experiments. Details of the template panel are given in the legend of Figure 4.

Results showed that concentration estimates from the dPCR platforms were within 20% of each other when measuring pure samples. These data demonstrate that the dPCR instruments investigated show good reproducibility when measuring pure and mixed sequences with the hydrolysis probes offering a potential reference method for value assignment of nucleic acid reference materials and in clinical samples.

Inter-laboratory reproducibility (National Measurement Institutes/Designated Institutes and expert laboratories)

To investigate dPCR platform inter-laboratory reproducibility, a comparison study was organised by LGC. Seven NMIs/DIs (LGC, NIB, TUBITAK, INRIM, NIST, JRC and KRISS) and one expert laboratory (Paris Descartes University) using three different dPCR platforms (QX100/QX200, Quantstudio and Raindrop) participated in the study. This study enabled assessment of the reproducibility in a situation akin to a reference material provider value assigning different reference materials. The *KRAS* G12D and WT 186 bp fragments produced by LGC were used for this study.

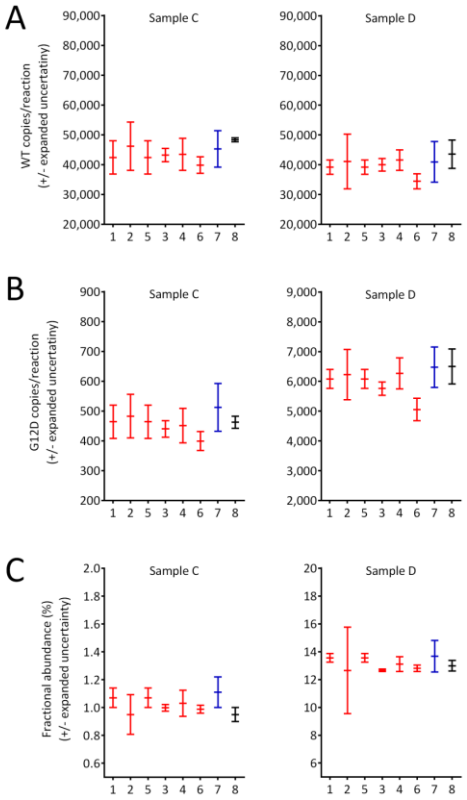

Figure 6: Inter-laboratory results when measuring mixed samples *KRAS* and WT sequence A) *KRAS* WT concentration, (B) G12D concentration and (C) % G12D fractional abundance. The dPCR platform used were QX200 (red bars), Quantstudio (blue bars) and Raindance (Black bars). Error bars represent the expanded uncertainty.

Different laboratory results showed good agreement for both targets with relative uncertainties of ≤15% reported for the G12D fractional abundance with no differences between the platforms.

Inter-laboratory reproducibility (end user laboratories)

To investigate dPCR inter-laboratory reproducibility in a format more similar to an end user (i.e. clinical) laboratory, 21 laboratories using the QX100/QX200 took part in a comparison study, which was organised by LGC in close collaboration with Bio-Rad. The *KRAS* G12D and WT 186 bp fragment materials that were produced by the project were used for this study.

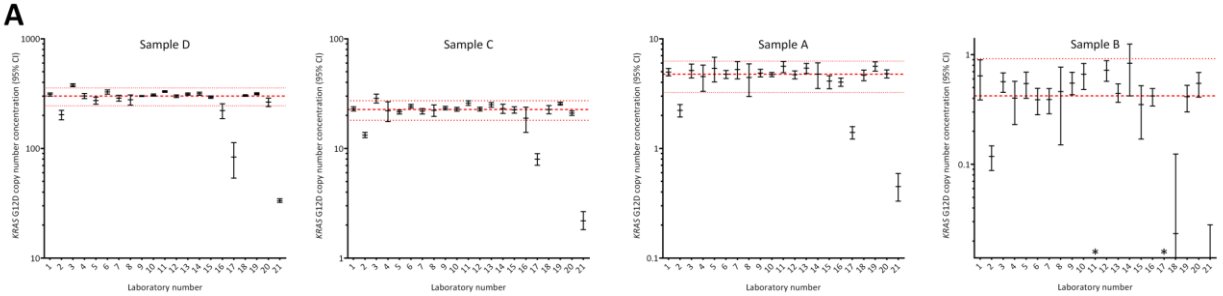

Figure 7: Inter-laboratory comparison. The originally submitted values from all twenty-one laboratories are shown for

Samples A-D in descending *KRAS* G12D copy number concentration from left to right. For each graph the anonymised laboratory number is shown in the x-axis with the *KRAS* G12D. For each participant, the submitted mean value is plotted as a short black horizontal line together with the 95% confidence interval based on triplicate measurements of three units (n=9). The red horizontal dashed line represents the median value across labs and the red horizontal dotted lines represent the upper and lower MAD_E intervals with 99% confidence. For Sample B, the lower confidence interval is not shown as it is approximately zero. Asterisks (*) just above the x-axis indicate laboratories that reported either a zero value or values below the range of the y-axis. All graphs show two orders of magnitude, shown on the log_{10} scale, though the range varies according to the sample.

This study has confirmed that dPCR can perform highly reproducible absolute quantification of an SNV between laboratories differing in their reported value by <12%. These findings suggest dPCR could have an important role in enabling reproducible measurements as a reference method to value assign secondary reference materials in end user laboratories.

Key research outputs and conclusions

The project successfully achieved this part of the scientific and technical objective. dPCR was evaluated as a method that could provide an SI traceable reference method for value assigning DNA reference materials to the number 1. In this work the *KRAS* SNV was investigated and dPCR evaluated for:

- Accuracy by comparison to the orthogonal method ICP-MS.
- Sources of potential bias were investigated by comparing different target sizes, chemistries and instruments.
- Reproducibility was evaluated using expert laboratories and those more akin to an end user clinical testing laboratory.

These findings support the hypothesis that dPCR could be an SI traceable reference method to the unit 1 when value assigning DNA reference materials. The dPCR method for KRAS developed at LGC was transferred to six NMIs (NIB, TUBITAK, INRIM, NIST, JRC and KRISS) and one expert laboratory (Paris Descartes University) for the inter-laboratory study on reproducibility.

Enumeration methods for large molecular structures: application to lipoprotein testing

It has been shown that Cardiovascular Disease (CVD) is mainly caused by atherosclerosis, a pathology that is mostly induced and fed by hypercholesterolemia and especially by elevated Low-density lipoprotein-cholesterol concentration (LDL-C). However, many clinical trials indicate that LDL Particle number (LDL-P) and non-HDL Particle number (non-HDL-P) are better predicators of the risk to develop cardiovascular disease than LDL-C and a valuable target for therapy. It has also been demonstrated that the smaller the lipoproteins, the higher the risk. Therefore, it is useful to measure lipoprotein size in addition to concentration, to obtain the most comprehensive data on CVD risk. To address the need for traceability chains in the field of advanced lipoprotein testing, appropriate standards and reference methods capable of finely depicting the lipoprotein profile and provide new insight into CVD risk assessment are necessary.

The main aim of this part of the project was to develop a platform for lipoprotein analysis by Electrospray-Differential Mobility Analysis (ES-DMA) with the objective to assess the potential of this technique to be recognised as a primary reference method for absolute enumeration of lipoproteins. In addition to providing lipoprotein particle number concentration, this technique also provides full size distribution of the particles. This information is clinically relevant as since the cut-off between large LDLs and small LDLs is very narrow, it is important to have information about the uncertainty of the measurement.

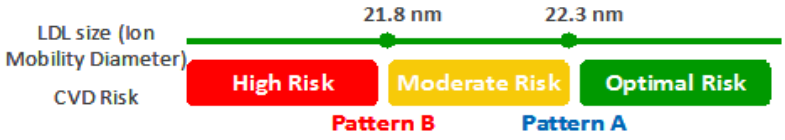

Figure 8: Classification of the patient's risk profile according to the LDL particles diameters (Pattern A = large LDLs and low CVD risk; Pattern B = small LDLs and high CVD Risk).

Development of instrumentation

LNE successfully developed an experimental set-up composed of an electrospray source and a Differential Mobility Analysing System (DMAS or SMPS for Scanning Mobility Particle Sizer, ISO 15900).

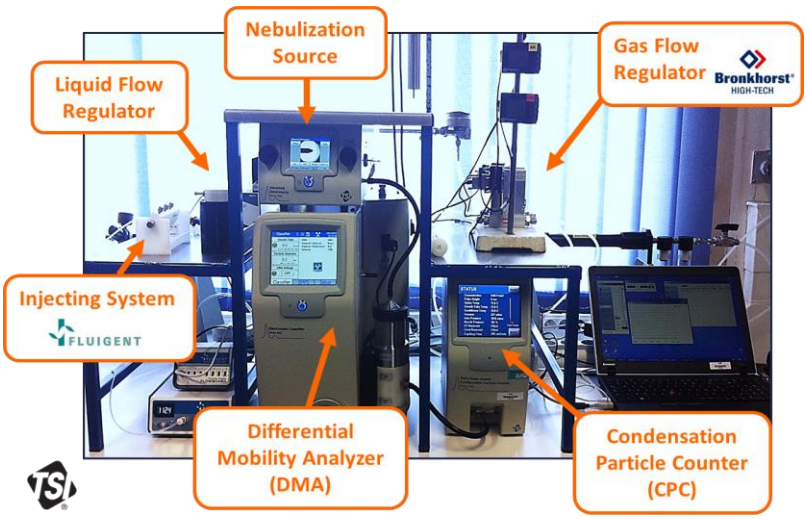

Figure 9: LNE's ES-DMA platform.

ES-DMA set-up was composed of three parts; an electrospray (ES) system containing a soft X-ray neutralization source and a differential mobility analyzer (DMA) coupled to a condensation particle counter (CPC). The ES-DMA platform was greatly optimised by LNE during the project. The electrospray (ES) generator was improved by the manufacturer by coupling an X-Ray neutralization source directly at the exit of the ES chamber. This resulted in a more stable aerosol and increased the efficiency and sensitivity of the system. A new injection system was also implemented which increased stability and enabled precise control of the liquid flow-rate injected. This modification also allowed liquid flow rate measurement using a nano-flowmeter which was calibrated in order to obtain SI-Traceable nano-flows.

Lipoprotein preparation

Since plasma proteins interfere with the signal of High-density lipoproteins (HDLs), LNE aimed to develop purification techniques to isolate lipoproteins. It was initially envisioned to compare different purification techniques such as ultracentrifugation (UC), Field flow fractionation (FFF) and affinity separation but as lipoproteins are classified according to their density and UC is the only method available to separate lipoproteins according to this parameter, this was ultimately chosen as the main method of choice. However, UC is sample consuming and suffers from a lack of reproducibility because it involves manual pipetting steps. It was therefore decided to eliminate the Lipoprotein purification step from the workflow because direct analysis of atherogenic particles in full serum samples by ES-DMA was possible.

In comparison to Low-density lipoproteins (LDLs), the signal associated with HDL particles is greatly interfered by plasma proteins and determination of HDL-P is not possible without sample preparation. As the main objective of this project was to determine particle number concentration of atherogenic lipoproteins (LDL-P and/or non-HDL-P), it was decided that analyses would be performed by diluting serum samples. Working at higher dilutions is advantageous as it decreases the probability for two particles ending up in the same droplet. Consequently, the rate of non-specific aggregation generated by the ES generator is greatly diminished leading to clearer number size distributions.

Assessment of lipoprotein purity

During the course of this project, different types of potential calibrators were produced at LNE using different purification approaches and characterised using a large variety of analytical methods.

Primary calibrators consisting of purified lipoproteins

Solutions of purified lipoproteins were produced by LNE and analysed by ES-DMA, Tube Gel Electrophoresis and Size exclusion chromatography-Fast protein liquid chromatography (SEC-FPLC) at LNE, Flow cytometry at PTB and AFM (Atomic Force Microscopy) and Multimodal NLO (CARS) at INRIM.

1) Frozen solutions of purified lipoproteins

Work showed that the stability of purified lipoproteins was limited. The signal loss over time that was observed by ES-DMA at LNE was confirmed by the observation of lipoprotein debris by imaging at INRIM and abnormal fluorescence signals by Flow cytometry at PTB. This confirmed that freezing and/or storage time was associated with the loss of the structural integrity of lipoproteins. As a consequence, frozen solutions of purified lipoproteins cannot be considered as a reliable anchor in a traceability chain.

2) Fresh solutions of purified lipoproteins

Preparing fresh solutions of purified lipoproteins was shown to not be a reliable option because of the variability associated with the purification methods. Fresh solutions of purified lipoproteins have limited stability and preparing international standards in a reproducible manner would require important efforts without being certain that the successive batches will have similar compositions and behaviour. This could introduce significant batch to batch variations.

3) Stabilised solutions of purified lipoproteins

Analysis of WHO SP3-08 (Apolipoprotein B), the reference material used for calibration, by ES-DMA showed a systematic bias that was related to the matrix and the sample's conductivity. It was also found that good accuracy could only be reached with the WHO-SP3-08 reference reagent for lipoprotein testing, a material of similar matrix and conductivity to a serum sample. Therefore, using stabilised solutions of purified lipoproteins as calibrators, even though it would seem the most adapted calibration strategy, would result in matrix related biases and would not appear to be a reliable approach. As a consequence, using calibrators consisting of matrix materials represents the most promising approach for lipoprotein enumeration by ES-DMA.

Calibrators consisting of matrix materials

Lipoprotein measurements by ES-DMA on full serum samples are made difficult by the presence of other particles which signal interfering substances with thus leading could lead to overestimating lipoprotein concentration.

The major sources of interference in a serum sample being proteins, purity assessment of lipoproteins imply identifying and quantifying all proteins having the same size as lipoproteins. Identification of proteins present in a serum sample is easily achievable using High Resolution Mass Spectrometry (HRMS). After performing a trypsic digestion of the sample, an in silico analysis of results can be conducted to identify which proteins correspond to the peptides detected by mass spectrometry. Once the proteins present in the sample have been identified, a first screening step can be done by considering the size of the identified proteins: only those having a size between 7 and 30 nm can potentially interfere with lipoproteins signal in ES-DMA. In other words, small proteins having a diameter of less than 7nm should not be considered as possible interfering particles. However, some small proteins being able to form oligomers which size will be larger than 7nm, it is necessary to measure pure proteins standards by ES-DMA, to check which one(s) could interfere with the relevant diameter range. This approach will would make it possible to identify which proteins should be considered as impurities, either in their monomeric form or in an oligomerized state.

After having identified proteins that should be considered as impurities, the next step would consist in quantifying these. Although protein absolute quantification by mass spectrometry is certainly possible, the associated measurement uncertainty is quite high (usually 10-15%, not less than 5%), which means that uncertainty associated with the purity of the calibrator would likely be incompatible with its use as international standard in a traceability chain.

Lipoprotein analysis by ES-DMA

Liquid flow-rate was measured with the calibrated nano-flowmeter and the pressure corresponding to a 100 nL/min liquid flow in the capillary determined. A blank number size distribution was then measured in buffer. In order to limit the passivation phenomena that induce errors in the particle concentration measurements, the capillary is passivated by injecting a Quality Control (QC) sample (undiluted serum) at high flow rates. LDL-P concentration measurement for this QC was verified using the control map and acceptance criteria set in place following a repeatability and intermediate precision study. The WHO SP3-08 calibrator and real patient samples were then run randomly in the sequence in order to minimize carry-over effects.

All data was recorded using the manufacturer's software and then processed using software developed at LNE to calculate the final concentration and its associated uncertainty. This software was divided into two distinct parts. The first part determined the uncertainties associated to the particle concentration measurement in the aerosol phase and the second part calculated the particle concentration (and its associated uncertainties) in the liquid sample.

Uncertainty calculation for lipoprotein particle number concentrations by ES-DMA

The uncertainties associated to the particle concentration were calculated according to the uncertainty propagation model from the Guide of Uncertainty Measurements (GUM). The estimated expanded uncertainty (k=2) represented about 11% of the measured non-HDL-P. The parameter-relative contributions to this variance are summarised in Figure 10. The most influent parameters were electrospray efficiency, total peak integration and liquid flow-rate. These results are in agreement with what was expected. Indeed, the uncertainty on peak integration is directly related to particle number concentration measurements, i.e. to the inner variability, repeatability and reproducibility of the ES-DMA system. Similarly, the influence of the liquid flow-rate was expected but the addition of the calibrated nano-flowmeter greatly reduced its contribution to the overall uncertainty.

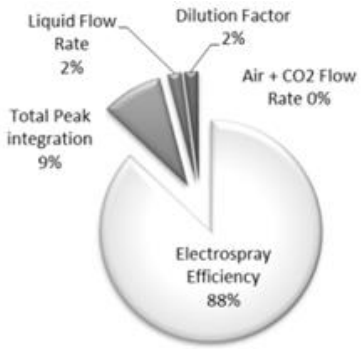

Figure 10: Example of the uncertainty budget for non-HDL-P measurement by ES-DMA.

As electrospray efficiency is a correcting factor, its impact on the final concentration and on its uncertainty is major. However, this is again consistent with what was expected as electrospray efficiency is highly variable and depends on many experimental parameters such as flow rate, capillary inner diameter, applied voltage, sample matrix, conductivity and analysed particles. As a consequence, even though LNE developed a calibrated ES-DMA system intended to account for these variations of electrospray efficiency, using this corrective factor implies inducing an important error in the calculation.

In addition, it is to keep in mind that although WHO SP3-08 is a reference material, it is not a certified reference material traceable to the SI. Indeed, its attributed concentration was determined by a consensus reference method (apoB - immuno-nephelometry) for which the measurand is different from ES-DMA's. This residual bias could therefore not reflect an accuracy bias but the evidence of this measurand difference.

Application to the measurement of real patient samples

Table 1 shows a summary of the LDL particles diameter measured for three patient samples by ES-DMA. The measurements and their associated standard deviations were translated in the corresponding risk profile for the patient according to the classification presented in Figure 8.

	Diameter (nm)		Type
	Mean	SD	
CS 020	22,49	0,05	Type A
CS 001	21,70	0,04	Type B
CS 003	21,70	0,01	Type B

Table 1: Measured diameters and associated risk profiles for the three native patient samples.

After further processing of the data with LNE's software, the peak areas and corresponding particle concentrations in solution were calculated with their associated uncertainties. Results are shown in Table 2.

	non-HDL-P $_{ES\text{-}DMA}$ [mg/dL]		Reference apoB [mg/dL]		Bias [%]
	Mean	U(k=2)	Mean	U(k=2)	
CS 020	52,5	8,0	54,6	0,6	-3,8%
CS 001	100,6	12,9	101,3	1,1	-0,8%
CS 003	133,6	18,7	118,3	4,1	12,9%

Table 2: non-HDL-P calculated concentrations for 3 patient samples.

The bias calculated for the non-HDL-P concentration (Table 2) measured with the calibrated ES-DMA platform was acceptable but calculating the non-HDL-P concentration using the WHO SP3-08 reference material led to large uncertainties (around 15%) that do not meet the requirements for a reference method at the top of the traceability chain.

The approach using single point calibration with the WHO SP3-08 material as a calibrator for non-HDL particle concentration measurement in full serum samples provided consistent results with apoB measured by the immuno-nephelometry designated reference method. As far as particle diameter is concerned, the small uncertainties associated allowed LNE to classify patients according to their risk profile based on their mean LDL particle size which is one of ES-DMA's major advantages.

Comparison of different methods for non-HDL-P measurement

Although ES-DMA cannot currently be considered as a primary reference method due to the need for regular calibration and too large measurements uncertainties, it remains a valuable method to assess CVD risk in clinical practice. As lipoprotein particle number can also be measured by Nuclear Magnetic Resonance (NMR), ApoB quantification by Isotope Dilution Mass Spectrometry or immuno-nephelometry/turbidimetry, LNE organised the largest ever cross-platform comparison between advanced lipoprotein testing methods in order to assess their comparability. Methods that do not count lipoproteins but still provide additional information on samples characteristics such as lipoprotein size, apolipoprotein content, cholesterol content and distribution across the different lipoprotein sub-classes, TG concentration were included in the study - apolipoprotein profiling by LC/MS/MS, analytical ultracentrifugation, Tube Gel Electrophoresis, Gradient Gel Electrophoresis and routine lipid measurement methods (TCh/LDL-C/HDL-C/TG assays). Participants included industrial partners (Quest Diagnostics, Quantimetrix, Atherotech, LabCorp) and expert research laboratories (NIH, CDC, Univ Washington, Univ Leiden, CHORI).

Results confirm that agreement between the different lipoprotein enumeration methods is perfectible (RSD = 12%) and that standardisation efforts are needed to improve results comparability and ensure coherent diagnostics and treatment-decision making worldwide. LNE have produced candidate calibrators that could be the basis of new traceability chains but a consensus is still needed to decide whether standardisation is possible in advanced lipoprotein testing to improve comparability of results or through harmonization (e.g. in conjunction with the AACC harmonization initiative). This work will be pursued in the context of a new IFCC working group on apolipoproteins post-project.

Key research outputs and conclusions

- LNE successfully developed a platform for lipoprotein absolute enumeration by ES-DMA, a method that separates particles according to their size and directly counts them.
- After validating the size calibration of the ES-DMA platform, lipoprotein size distributions were measured in lipoprotein fractions purified by ultracentrifugation and serum samples. The mean and modal diameters of the atherogenic particles (i.e. LDLs) with their associated uncertainties were measured with good precision and accuracy. Size measurements confirmation was conducted on INRIM's AFM platform. SI traceability of particle size was assured through reference materials consisting in certified nanospheres.
- The ES-DMA's imprecision of around 15% appeared to be inappropriate for a reference method used to value assign calibrators and Certified Reference Materials. Establishment of the uncertainty budget using software developed by LNE revealed that the main uncertainty source was the estimation of the electrospray transmission efficiency, a highly variable parameter that is strongly affected by sample composition and especially its conductivity/salinity. ES-DMA therefore cannot free itself from regular calibration and that calibrators should consist of materials with a composition similar to that of the measured samples.
- The ES-DMA platform was used to measure the concentrations of different lipoprotein classes and subclasses in patient samples. Lipoprotein number concentrations could be made traceable to the SI by establishing traceability of the different input parameters. For the first time, absolute enumeration of lipoproteins was achieved, providing a new measurement capability that was not available before the project. This capability could potentially be applied to the absolute enumeration of any other colloidal suspension of nano(bio)particles having a diameter between 5 and 100 nm (e.g. viruses, manufactured nanoparticles).
- LNE organised the largest ever cross-platform comparison between advanced lipoprotein testing methods to assess their comparability. Results show that agreement between the different lipoprotein enumeration methods is perfectible and that standardization efforts are needed to improve results comparability and ensure coherent diagnostics and treatment-decision making worldwide. Reference materials consisting of patient samples and serum pools will be made commercially available by LNE to help manufacturers validate and calibrate their methods to obtain comparable and accurate results.

Establishing traceability for cell concentration measurements

The aim of this part of the project was to develop reference methods for high accuracy cell concentrations in suspensions to assign traceable values to specific target cell populations in blood samples or model systems. Different approaches were used to establish primary methods based on flow cytometry and microscopy which allow the direct determination of cell concentrations with the lowest possible uncertainties. Furthermore, to allow end users to obtain traceable cell concentration measurements, flow cytometric enumeration of target cells relative to calibration beads was investigated and uncertainties derived.

Selection of Model Systems

Besides the investigation of fresh blood samples and cerebrospinal fluid (CSF), haematological and immunological model systems were selected by LGC and PTB. The first model consisted of a T-helper cell line which expressed CD4 (cluster of differentiation 4) receptor (CD4+) mixed with a background cell line which was CD4 negative. CD4+ is the most common way to assess anti-viral drug intervention time for HIV treatment as a loss of these cells and cell marker is an indicator of disease progression. The primary reference procedure developed later in the project was validated using fresh EDTA (Ethylenediaminetetraacetic acid) stabilised blood for the enumeration of red blood cells (RBCs) and adapted for this model.

To develop high accuracy methods for rare cell detection, a circulating tumour cell in blood model was developed consisting of epithelial cells from an adenocarcinoma cell line in a background of white blood cells (T-lymphocytes).

Primary reference procedures

Flow cytometric and the microscopically based primary methods are characterised by:

- Identification of target cells by unique physical or biological properties (the latter identified by specific or unspecific labelling).
- Direct measurement of the volume of a diluted sample.
- Gravimetrical preparation of the dilution, if a dilution step is needed.

In microscopy, spatial overlapping cells are accessible in the images and generally the cell number can be corrected accordingly. In flow cytometry, however, temporal coincidence can lead to counting losses of more than 10-20%. To identify, quantify and correct for such counting loss due to random coincidences, the flow cytometric primary reference procedures shall include the preparation of a dilution series consisting of at last four dilutions and enumeration of target cells in each dilution with a sufficient statistical relevance (i.e. typically >40000 events for statistical uncertainties <0.5%) .

Since such coincidences depend on instrumental characteristics (i.e. time for signal processing and digitisation) as well as on rheological properties (i.e. flow) of the cells. Calibration of the instrument is not possible and dilution series have to be prepared for each blood sample.

All these primary methods rely on the enumeration of the cell number N and the measurement of the volume V. Hence, the concentration is determined according to

$$C = \frac{N}{V} \quad . \tag{3.1}$$

As described in DIN 58932-3 (Haematology - Determination of the concentration of blood corpuscles in blood - Part 3: Reference method for the determination of the concentration of erythrocytes), the coincidence corrected number N is derived by extrapolation of a dilution series (Annex A2) or alternatively from one measurement using the approximation (Annex A3)

$$N \approx \frac{N_{ri}}{\phi_i} \cdot \left(\frac{1}{1 - N_{ri} \cdot \tau / t} \right) \tag{3.2}$$

The symbols N_{ri}, ϕ_i, τ and t indicate the number of recorded events for the dilution i, the volume fraction of the original sample in the measurement suspension, the (average) pulse width of the signals and the total measurement time.

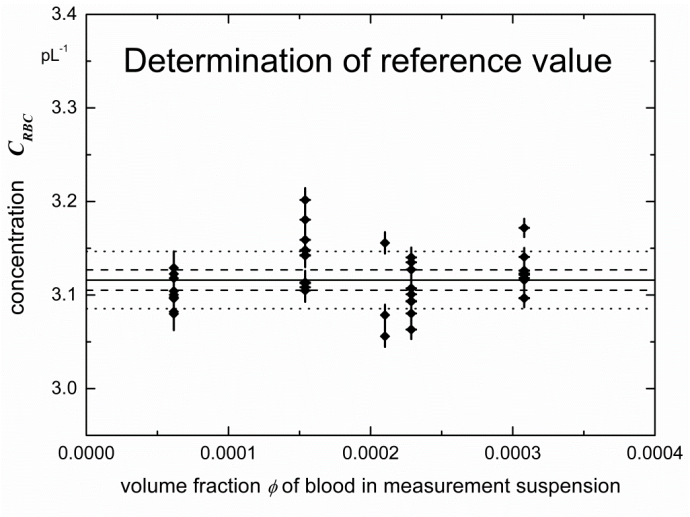

Figure 11: Coincidence corrected erythrocyte concentrations calculated using equation 3.2 for different dilutions. For each dilution, several repeat measurements were accomplished, each of which is represented by a black symbol. The weighted average amounts to C_{RBC} = 3.116 10^6 µL^{-1}. The combined uncertainty of the average $u(C_{RBC})$ = 0.011 10^6 µL^{-1} corresponding to 0.35% is included as dashed lines. The standard deviation of 0.031 10^6 µL^{-1} (dotted lines) indicates the uncertainty for a single repeat measurement.

To determine high accuracy reference values for cell concentrations and to reliably estimate the combined uncertainty, the influencing quantities listed in Table 3 were characterised at PTB. Depending on the specific target cells and the sample, the influencing quantities needed to be controlled in each reference measurement or estimated from experiments using model systems. As an example, Figure 12 shows a scatter plot of a control blood sample. Besides the clusters of erythrocytes and platelets, so called erythrocyte 'ghosts' (red blood cells which have lost haemoglobin) were identified. The appearance of such ghosts is characteristic for control blood samples, since the stabilisation of fresh blood causes erythrocyte membranes to disrupt and reassemble. In impedance based routine instruments, such ghosts are counted as intact RBCs while in optical haematology analysers, RBCs are assigned as platelets. For the determination of erythrocyte concentrations, systematic deviations of up to 1% might be acceptable. However, assigning the 1% population of RBC ghosts as platelets, their concentrations would be significantly overestimated and deviations up to 25% would be observed. The appearance of such additional populations in the sample could be interpreted as impurities, since such objects are not found in blood samples from healthy people.

Influencing quantity	Effect on measurements	Procedure to quantify change in concentration
Adhesion	Loss of cells on container walls	- Determination of concentration immediately and 30 min after preparation
Agglomeration	Reduced cell count and / or misclassification	- Analysis of pulse height distributions in histograms or 2 dimensional scatter plots - control of pulse width of individual events
Carry over	Increased count due to cells released from tubing	- Background determination between different series of measurements
Sedimentation	Increase or decrease in count rate depending on the position of the needle used to withdraw the suspension	- Stirring during measurement if target cells are not destroyed or modified - Determination of time dependence of the count rate
Red blood cell (RBC) ghosts	Reduced cell count and / or misclassification	- Application of different methods for target object identification, i.e. impedance change, light scatter and antibody staining (CD235a for RBCs)

Lysis	Destruction of target cells (leukocytes) and / or perturbations due to agglomeration of cell fragments (of RBCs)	- Comparison experiments using various reagents for lysis - Application of lysis free methods
Evaporation	Increase in cell concentration	- Determination of evaporation rate and correcting for the corresponding volume change during the measurement

Table 3: Overview of relevant influencing quantities, the corresponding effect and the strategy to correct corresponding systematic deviations.

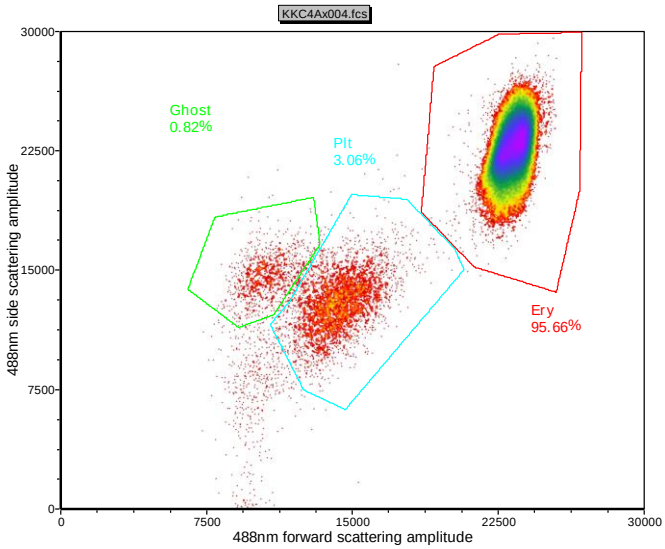

Figure 12: Analysis of a control blood sample used for external quality assurance of analytical haematology laboratories by the home built flow cytometer with direct sample injection. For each event, the amplitude of the 488nm forward scattering signal is plotted against the amplitude of the 488nm side scattering signal. Clusters corresponding to three populations are observed: intact erythrocytes, erythrocyte ghosts and platelets. The appearance of so called erythrocyte ghosts is typical for such control samples and results in systematic deviations.

Secondary reference procedures

Secondary reference procedures are based on the relative enumeration of the target cells with respect to the number of other cells in the sample or with respect to particles of artificial origin added to the sample. When using suspensions of cells or particles as calibrator, the volume V in equation 3.1 is replaced by the ratio N^{cal}/C^{cal} of the number of counted calibrator particles and their concentration. Consequently, the target cell concentration is derived from the number of target cells and the number of calibrator beads according to

$$C = \frac{N}{N^{cal}} \cdot C^{cal} \quad . \qquad (3.3)$$

Development of instrumentation

To evaluate the reference procedures, three flow cytometers were set-up or modified to meet the requirement for high accuracy measurements. In addition, a microscopic slide scanner was used for the development of reference procedures. In total four instruments were applied to determine reference values for cell concentrations:

- A home built laser flow cytometer (see Figure 13) with direct sample injection utilising a gravimetrically calibrated syringe
- A modified commercial laser flow cytometers (Sysmex Partec CyFlow ML, CUBE 8) with pressure driven sample injection
- Impedance based cell counting using a balance for volume determination
- Slide scanning microscopy (Zeiss Axio Observer Z1) in combination with counting chambers to enumerate cells

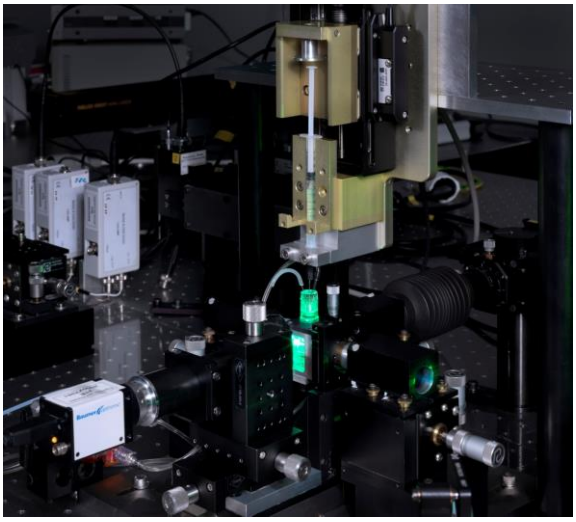

Figure 13: Part of the optical set-up of the home built flow cytometer and the motor driven syringe for direct injection of the sample in the flow cell. The flow cell appears in green colour caused by reflections and scattering of the laser beam of the 532nm laser.

Microscopic cell characterisation

In addition to standard contrast methods such as bright field, phase contrast and fluorescence microscopy, fluorescence lifetime imaging (FLIM) and multimodal two-photon excitation fluorescence (TPEF) in combination with coherent anti-Stokes Raman scattering (CARS) were applied as alternative modalities for microscopic cell characterisation at INRIM. The advantage of fluorescence lifetime imaging is the high sensitivity allowing single molecule detection, contrast enhancement and the possibility to distinguish the target molecule from autofluorescence.

Imaging modalities based on non-linear effects such as TPEF and CARS allow optical sectioning and can be used to determine volumes of cells or to identify overlapping objects. In addition, complementary (contrast) information is obtained because of different selection rules for two photon microscopy and CARS compared to single photon fluorescence excitation. An example for TPEF and CARS is shown in Figure 14, where both methods were applied to analyse a sample containing a mixture of CSF cells and non-stained polystyrene microspheres mimicking an impurity. Results showed that both methods are sensitive to different molecules. Cells were visible by TPEF (Figure 14A) and the polystyrene beads were successfully identified by CARS (Figure 14B). In order to compare both images, the edges of the cells are indicated by white lines in Figure 3.B. Whereas identification of the artificial polystyrene beads was shown to be straightforward, detection and differentiation of cells was difficult and further work (post-project) would be required.

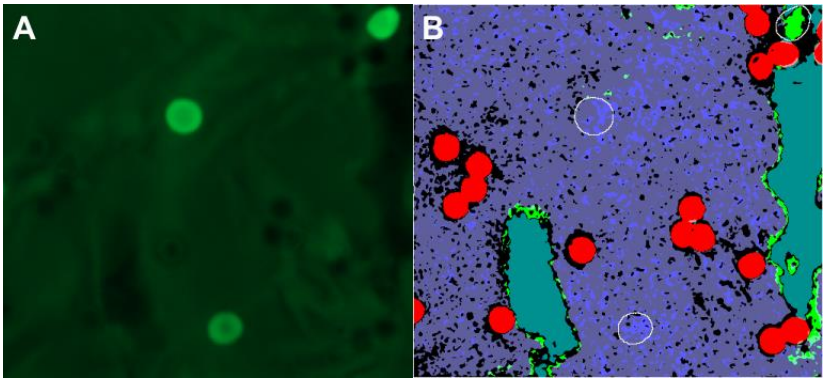

Figure 14: Simultaneous measurement of two photon exited fluorescence (A) and CARS (B) for a CSF sample spiked with polystyrene micro beads. In (B), the reconstructed image of the sample is depicted in false colours. Different colours represent different classes of molecules with similar CARS spectra present in the sample. Polystyrene particles are indicated in red. The edges of the cells in (A) are shown in (B) as white lines.

Cross-platform comparison of primary and secondary procedures

Enumeration of cells was performed using flow cytometry and microscopy. The model system used for this comparison was a T-helper cell line which expressed CD4 receptor (CD4+). Two suspensions of cells at concentrations of 500 μL^{-1} and 50 μL^{-1} were prepared by LGC, stained with anti-CD4-PE and sent to PTB and INRIM. For the relative measurements of these cells with respect to calibrator beads according to the secondary reference procedure, PTB prepared aliquots of a suspension of yellow green fluorescent beads and assigned reference values of the concentration of these particles with an accuracy of 2.3% for a single repeat measurement. The target cells and calibrator particles were distinguished by observing the fluorescence in two optical wavelength band passes adapted to FITC (typically 535nm ± 25 nm) and PE (typically 575nm ± 15 nm).

The results of the comparison experiments are summarised in Table 4. The different approaches to derive reference values by a primary method (measurement series #1 to #3) result in non-weighted average values of C_{high} = (411 ± 26) μL^{-1} and C_{low} = (45 ± 4) μL^{-1}, the uncertainties refer to combined standard uncertainties. The corresponding relative uncertainties amount to about 6% for C_{high} and 9% for C_{low} , respectively. For the low concentration sample the values agree within their uncertainties. On the other hand, for the high concentration the differences are larger relative to the uncertainties, indicating that systematic deviations occur.

measurement series	JRP partner	instrument	procedure	number of measurement series	C_{high} μL^{-1}	$u(C_{high})$ / μL^{-1} μL^{-1}	C_{low} μL^{-1}	$u(low)$ μL^{-1}
1	PTB	microscopy, Nicon Eclipse 90i, slide scan	primary	4, 6	395.6	14.6	43.6	-1.4
2	INRIM	microscopy, Zeiss Axio Observer Z1, Fuchs-Rosenthal chamber	primary	6	429.6	12.4	46.7	-0.4
3	PTB	self build flow cytometry, direct sample injection	primary	15	408.4	3.1	45.0	0.6
4	LGC	flow cytometry, BD FACS CANTO II	secondary	12	589.5	5.7	62.3	1.6
5	PTB	self build flow cytometry, direct sample injection	secondary	15	424.8	10.0	46.8	1.4

Table 4: Comparison of concentrations of two preparations of CD4+ cells applying microscopic and flow cytometric cell enumeration. The standard uncertainties of the respective average are included.

For both samples analysed, the values derived by flow cytometry are close to the average and lie in between the respective two microscopic results measured at PTB and INRIM. Results show that flow cytometry is to be preferred because of easier sample preparation and better statistics. Further effort would be required to obtain a closer agreement and to identify the origin of the observed differences.

Measurement series #4 and #5 represented results obtained with the secondary procedure. The results of series #5 are in good agreement with the results obtained using the primary procedures whereas for series #4, significant deviations were observed. These differences were identified as a significant reduction in the concentration of calibrator beads due to adhesion loss during transportation, resulting in an increased concentration for both samples. Hence, when applying the secondary procedure, influences correlated with the calibrator (i.e. inhomogeneous distribution of beads in the calibrator solution, variation when preparing aliquots of the calibrator solution and adhesion loss in the containers and tubing) need to be controlled and if possible, corrected for.

Key research outputs and conclusions

The project successfully achieved this part of scientific and technical objective. In summary, with respect to the enumeration of cells the project:

- Established SI Traceability of cell concentration measurements in blood, control blood, cerebrospinal fluid and suspensions prepared from cell cultures.
- Developed flow cytometric and microscopic primary procedures, the comparison of which yielded good agreement.
- Achieved accuracies below 0.5% for flow cytometric concentration measurements of erythrocyte concentrations in blood.
- The flow cytometric primary procedure developed was successfully applied to assign reference values to calibrators (i.e. number of calibrator beads or concentrations of beads in suspension) needed for the secondary method.
- Relative enumeration of cells with respect to calibrator beads was demonstrated to be suited as secondary method and applicable to clinical environments.
- For the first time, a dedicated reference flow cytometer is commercially available, modified according to the requirements of the project and defined in a revised (national) standard DIN 58932-3, to implement primary procedures.
- In cooperation with stakeholders engaged in the DIN committee “NAMed Haematology”, a revised version of the standard describing the flow cytometric primary procedure for the enumeration of red blood cells in blood was published and two drafts specifying the primary procedure for the determination of CD4 positive cells and the requirements for the secondary procedure, i.e. the relative measurement of cell concentrations with respect to calibrator particles, were developed.

Objective 3: Develop strategies for purity characterisation of pure calibration standards for biological measurement

The aims of this part of the project were:

- To develop a clear, agreed, understanding of the role of pure and purified biological materials in the establishment of metrological traceability to the SI.
- Based on this understanding, to develop and assess measurement methods for the characterisation of purity for biological materials intended for use as higher order calibrators in SI traceable biological measurements.

Defining the concept of "purity" for bio-molecules and bio-entities

An initial key activity involving all project partners reviewed the role of pure materials in establishing traceability. The activity generated an agreed set of concepts and definitions to guide work on the establishment of metrological traceability through the use of purified biological materials as calibrators. The concepts and definitions were provided to all project participants for reference. In addition, LGC provided the resulting report to an IUPAC committee developing guidelines for the preparation of pure reference materials for higher order calibration, and has contributed directly to the development of international guidelines for pure reference material certification.

Development of Next Generation Sequencing (NGS) for the quantitative assessment of sequence purity of nucleic acid reference materials

The potential of Next Generation Sequencing (NGS) for the quantitative assessment of sequence purity of the *KRAS* materials from Objective 2 was investigated at LGC. Samples were prepared containing *KRAS* materials mixed at different ratios (100% WT, 100% G12D, 0.1% G12D in WT and 0.01% G12D in WT) and sequenced with the MiSeq NGS platform. As a direct platform comparison, the same samples were analysed by dPCR using the QX200 platform.

Sample	**A**		**B**		**C**		**D**	
Target	WT	G12D	WT	G12D	WT	G12D	WT	G12D
Expected	100%	0%	0%	100%	99.90%	0.1	99.99%	0.01
dPCR	99.99%	0.01%	0.42%	99.58%	99.90%	0.103%	99.99%	0.011%
NGS	99.62%	0.27%	6.46%	93.09%	99.56%	0.34%	99.50%	0.38%

Table 5: Evaluation of the sensitivity of dPCR and NGS when measuring mixtures of mutant and wild type variants.

Results showed that, despite sequencing only a short fragment containing the region of interest without initial PCR amplification, detecting rare mutations at 0.1 and 0.01% was beyond the capabilities of a standard NGS sequencing strategy with the MiSeq platform, as the underlying error rate was at a similar or higher level than which the mutations were present in the samples. In contrast, dPCR achieved superior sensitivity and was able to accurately measure the proportion of G12D fragment present in a background of WT.

Physicochemical methods for nucleic acid purity

Physicochemical characterisation relies on the use of a range of different measurement techniques that are sensitive to different potential contaminants. Inorganic and non-biological organics need to be characterised when estimating nucleic acid content through, for example, phosphorus or nitrogen determination, both of which can routinely be measured with clear traceability to the SI. When measuring nucleic acids directly, for example by fluorescence of intercalating dyes (below), nucleic acid contaminants must be separated and measured so that their contribution to the fluorescent signal can be corrected. Similarly, sequence variants must be characterised and allowed for in assigning a value for nucleic acid purity.

To establish a consistent strategy for physicochemical methods, the project developed a checklist of methodologies appropriate to each contaminant type. The project also further developed a specific methodology for fluorescence detection. The total amount of DNA can be measured easily by fluorescent intercalating dyes such as PicoGreen, however DNA integrity is critical in downstream characterisation and quantification studies. Contamination of the *KRAS* materials produced with other DNA fragments was possible and would affect the results of further analysis such as NGS and dPCR. It was therefore important to develop a sensitive method to investigate structural impurity content of DNA. High Performance Liquid Chromatography (HPLC) was used at TUBITAK to evaluate the integrity of DNA in the *KRAS* fragment samples produced in Objective 2 by LGC. The chromatograms of each sample are shown below:

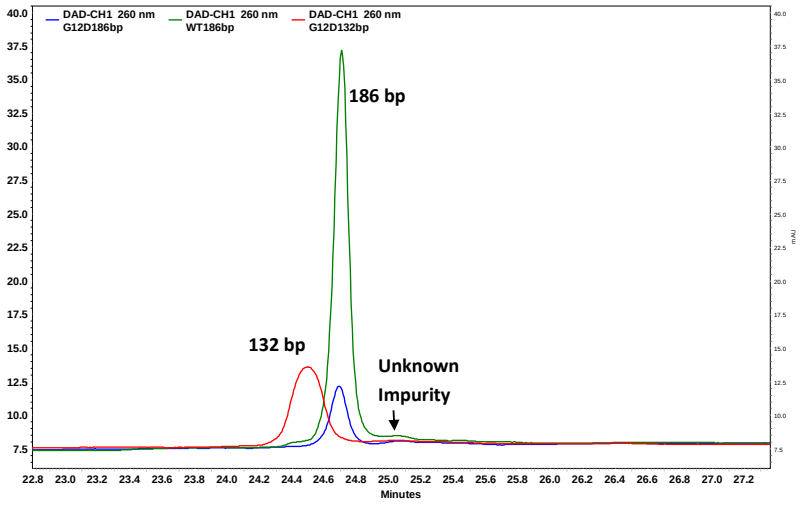

Figure 15: Chromatograms of *KRAS* and WT Samples. Samples of G12D 186 bp (blue), WT 186 bp (green) and G12D 132 bp (red) were run separately and chromatograms overlapped.

Results showed that the 132 bp and 186 bp DNA fragments were well separated from each other. The WT 186 bp DNA sample contained a very small 132 bp DNA fragment in addition to an unknown impurity peak. The ratio of 132 bp DNA peak area to the 186 bp DNA peak area was calculated as 1.88 ± 0.09% and the unknown DNA impurity peak constituted 5.32 ± 0.28% of the total DNA.

This approach provides a method to characterise and define non target sequence impurities when developing cell free DNA like fragments for subsequent molecular analysis.

Model for purity assessment

The aim of this part of the project was to establish a model for assigning a metrologically traceable numerical value and associated uncertainty to the purity of the *KRAS* materials developed in Objective 2 combining information from the physicochemical (chromatography) and biological measurement methods (dPCR) used.

At LGC, a Bayesian approach was used to infer the purity of DNA (i.e. the G12D mutation or WT *KRAS*) in a nominally 100% sample from the data produced by several techniques that provide information about the amounts of different DNA constituents of the sample. Bayesian statistics is a methodology that is increasingly being seen by metrologists as the future of measurement uncertainty evaluation.

The purity in this case is defined as the proportion of either WT or G12D *KRAS* mutant DNA by mass with respect to the total mass of DNA. The experiments contributing to the analysis include measurements of G12D and WT concentrations using dPCR performed by LGC and measurements of the relative concentrations of DNA of different lengths using HPLC, which were carried out by TUBITAK.

A Bayesian model for purity assignment combines information from experiments (or other sources, if available) with any available prior information to provide a quantitative estimate of purity together with a statement of the uncertainty associated with the assigned value. For the *KRAS* model system, the overall purity is the mass proportion of a particular target, G12D DNA, out of the total DNA comprising G12D, wild type (WT) and any uncharacterised DNA species with different length to the 186 bp G12D and WT). The total amount of the known contributors could be measured using digital PCR. This information was combined with chromatographic data that provided information on the amount of DNA present with different sequence lengths. In addition, a standard prior assumption (Jeffryes prior, which relates the likelihood of an experimental observation to the estimated uncertainty) was used to compensate for the absence of information on the absolute sizes of the efficiencies. The result is a statistical model that needs to be solved in order to obtain purity values and their uncertainties.

Here, the model required a numerical solution as it is not amenable to an exact algebraic solution. The software 'OpenBugs' was used to simulate from the posterior distribution using Markov Chain Monte Carlo. This only requires the above model, information on the assumed prior and the data to be specified and avoids the need to derive the posterior distribution by algebraic manipulation. The model showed good convergence indicating successful solution. The results are summarised as a density plot (Figure 16) that shows the distribution of purity values that could reasonably be assigned. In addition, mean values and 'credible intervals' (broadly similar to the traditional 95% confidence interval) can be calculated; these are given in Table 6. Since the model includes data on other quantities, such as the G12D and WT content, updated estimates of these are also provided.

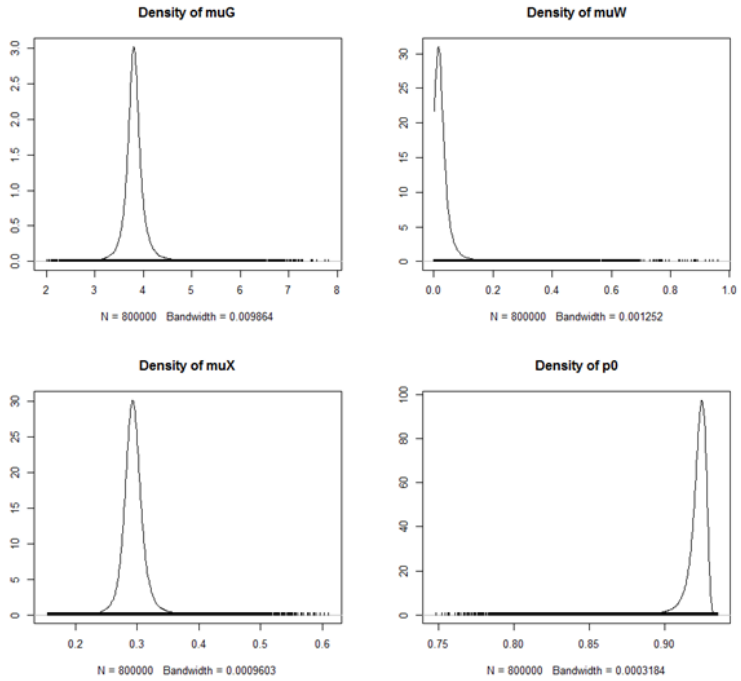

Figure 16: Density plots of posterior MCMC sample for main parameters and p_0.

Parameter	Mean	s. d.	95% Credible Interval	
			Lower (2.5%)	Upper (97.5%)
G12D content	3.823	0.246	3.400	4.341
WT content	0.030	0.036	0.001	0.107
Uncharacterised DNA content (total)	0.295	0.020	0.260	0.337
Purity p_0	0.922	0.008	0.903	0.930

Table 6: Sample estimates for posterior parameter means, standard deviations and 95% credible intervals for a nucleic acid purity determination. Nucleic acid content is in ng/uL; p_0 is dimensionless.

The distribution and the purity interval show 'negative skew', that is, an asymmetric distribution with a longer 'tail' to the left. This arises because the model shows some possibility of contamination leading to low purity,

while purity over 1.0 is not physically possible. This is therefore a much more realistic distribution than might be inferred by traditional uncertainty evaluation methods, which typically assign a symmetric uncertainty interval.

For this calculation, the nucleic acid purity for G12D – G12D content as a proportion of total DNA content - has a mean of 0.922 ng/uL and a standard deviation of 0.008 ng/uL; because the intervals are asymmetric, the equivalent of an expanded uncertainty interval is from 0.903 to 0.930.

Definition of the use of pure reference materials for protein quantitation

LGC and LNE organised a joint EMRP/CCQM workshop on the topic calibration of protein measurements using pure materials which was hosted by BIPM. Approximately 60 delegates attended, including leading experts from industry, academia and national measurement institutes. Calibration against pure materials certified or verified by a technically competent body is a widely applied strategy for calibration in measurements for the molecular sciences. For proteins, this strategy presents particular challenges related to the complexity of protein structure. The workshop considered the advantages, limitations and technical requirements associated with the use of purified proteins for calibration.

The workshop concluded that pure materials were considered to be a potentially effective strategy for achieving SI traceability in at least a useful proportion of protein measurements. The use of pure materials already appeared to be within reach for smaller peptides, but the complexity of proteins made considerable further development necessary. It was agreed that the steps necessary for development and use of pure protein reference materials would need to include:

- Clear identification of the molecular species and the measurand of interest
- Confirmation of identity of the material, including primary sequence confirmation and any required higher order structure
- Establishment of sufficient purity using an appropriate range of techniques

In considering ‘sufficient’ purity, it was clear that absolute purity was not the key issue; rather, it was crucial that the level of purity, and the particular impurities, were sufficiently well characterised for a particular end use. In identifying purities, no single technique was considered sufficient. In addition to careful considerations of likely impurities, it was invariably necessary to apply as wide a range of methods as possible to identify a full range of unexpected impurities. At present, techniques available for characterising the purity of proteins were capable of identifying a very comprehensive range of impurities and modifications. However, much work is still needed to improve the quantitative assessment of purity, especially for larger molecules.

Key research outputs and conclusions

The project successfully achieved this scientific and technical objective. The work on establishing metrological traceability through the use of pure and purified calibration materials has:

- Developed and disseminated a set of concepts and definitions to guide work on the establishment of metrological traceability through the use of purified biological materials as calibrators, which has contributed to the development of international guidelines for purity assessment and certification.
- Assessed the use of next generation sequencing (NGS) as a technique for the establishment of purity for nucleic acid reference materials, concluding that NGS was not, at the present stage of development, capable of sufficiently low detection limits for the characterisation of high purity materials.
- Established a checklist of physicochemical methods for use in the characterisation of purified nucleic acid reference materials and applied this to the determination of purity in a model system relevant to clinical cancer diagnosis.
- Developed a formal Bayesian approach for the estimation of purity and its uncertainty that takes into account information from a range of measurement techniques, and demonstrated this approach on a nucleic acid material.

- Organised an international metrology workshop on the use of pure materials for protein calibration, which set out clear requirements for future work on the use of pure materials for achieving SI traceability in protein measurement.

The value of the joint research in this project allowed partners to benefit from the different metrology expertise and perspective of other partners and allowed for the proposal of a consensus approach to the problems, more likely to achieve international acceptance for the new concepts for establishing bio-measurement traceability that have been developed.

4 Actual and potential impact

The results from this project enable the demonstration of SI traceability through counting for nucleic acid and cell measurements. This underpins the development of reference materials and supports compliance with the IVD Directive 98/79/EC, and international standards such as ISO 17511.

Contribution to standards

The technical outputs from this project have made a significant contribution to the development of international standards and guidance through the following routes:

- the development of the German Institute for Standardisation (DIN) Haematology standard 58932-3. “Reference measurement procedure for the determination of red blood cell concentration in blood”.
- contributing to the drafting of ISO 20395 “Biotechnology: Guidelines for evaluating the performance of targeted nucleic acid quantification methods“ publication expected by June 2019.
- the drafting of ISO/WD 20391-1 "Biotechnology – Cell counting: Part 1: General guidance on cell counting methods” and “Biotechnology – Cell Characterisation: Part 1” which will incorporate the project’s outcomes on the use of flow cytometry for cell counting and cell characterisation.
- revisions to ISO 17511 (In vitro diagnostic medical devices - requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples) which will include a new section on traceability of biomolecular counting using the cell and nucleic acid traceability chains developed by the project as examples.
- A modified reference exemplifying counting of copies of a particular nucleic acid sequence amplified by digital PCR with formal traceability to the SI established through appropriate, validated measurement procedures will be included in the proposed revision of the SI brochure.

Dissemination

In total, 9 papers describing the project’s scientific achievements have been published in peer-reviewed journals. Further dissemination has included focussed stakeholder workshops and presentations (43 in total) at significant stakeholder conferences including the International Federation of Clinical Chemistry (IFCC) WORLDLAB Congress, IFCC & European Federation of Laboratory Medicine EUROMEDLAB, IEEE International Symposium on Medical Measurement and Applications (MeMeA) and the Joint Committee for Traceability in Laboratory Medicine (JCTLM) Members' and Stakeholder's Meeting.

Early impact

Examples of the project’s outputs being taken up by relevant communities include:

- The results of the interlaboratory study in objective 2 have been published and are significant in demonstrating good laboratory analytical performance with calibrator independent digitial PCR. Guidance notes from the project on digital PCR validation has enabled participants to optimise and improve their performance.
- The digital PCR method developed in objective 2 was used to count cell free DNA Reference Standards from a UK genomics company adding confidence in their application for assessing the performance of high throughput analytical platforms such as NGS and Quantitative PCR for low level tumour DNA analysis. This also demonstrates the potential for NMI’s to develop measurement

services for industrial stakeholders to provide traceability to the SI for their IVD products in line with regulatory requirements.

- Outputs from the lipoprotein enumeration work in objective 2 were taken up by 5 major IVD manufacturers: TSI, Quest Diagnostics, Quantimetrix, Labcorp, Atherotech. TSI (an ES-DMA manufacturer) and Quest Diagnostics (a major ES-DMA user in clinical practice) both benefited from the characterisation that was performed of the ES-DMA platform. Quantimetrix benefitted from the multi-centre comparison that was organised between themselves, partner LNE and the Centers for Disease Control and Prevention. Labcorp used the results of the cross-platform comparison to recalibrate their ES-DMA method and obtain better agreement with the Immuno-nephelometry designated reference method. Atherotech compared the performance of their new assay for Low-density lipoprotein measurement with the other methods used in the project's comparison.
- As part of objective 2 a prototype reference flow cytometer was developed and transferred to the Reference Institute for Bioanalytic at the Klinikum Karlsruhe in Germany (which is accredited to DIN) to provide reference measurement values, and SI traceability through accurate cell counting for external quality assurance schemes.
- As part of objective 2, the biotechnology company Sysmex Partec will produce a flow cytometer modified according to the requirements defined by the project, for the determination of reference values for cell concentrations.
- As part of objective 3, the model developed for purity assignment has been communicated to International Union of Pure and Applied Chemistry project on characterisation of pure certified reference materials. Since the model is applicable to a wide range of materials in addition to the biological materials studied in the project, this will provide internationally recognised tools for realistic uncertainty evaluation for purity certification and in turn lead to more reliable purity values.

Future and wider impact

As part of objective 3, the project hosted an international workshop on the use of pure protein materials for achieving SI traceability in protein measurement, in which clear requirements for future work on the use of pure materials for achieving SI traceability in protein measurement were identified.

The project's reference method for the quantification of gene mutations can be used by Reference Material producers and to form the basis of calibration services provided to nucleic acid-based IVD manufacturers. The guidelines for digital PCR method validation and uncertainty calculation will also enable the development of reference methods for other cancer targets and clinically relevant targets.

Efforts to standardise lipoprotein measurements to improve comparability of results and ensure coherent diagnostics and treatment decision making worldwide will be pursued in the context of a new IFCC working group on apolipoproteins.

Reference to and clarification of metrological traceability for important biomeasurements involving cell and nucleic acids in significant new ISO Standards will allow the biotechnology and IVD industry to comply with emerging regulations e.g. the new IVD EU regulations and help ensure greater measurement comparability and consumer and regulator confidence.

The project successfully developed methods and materials which can be used to count biological molecules accurately. Key outputs of the project included proof of concept papers and traceability chains for accurate and traceable quantification of nucleic acids and cells which are currently being incorporated into two international standards being developed under ISO TC276 (Biotechnology) and revision of ISO 17511, the in vitro diagnostic medical devices standard.

5 Website address and contact details

A public website was created to keep end-users informed about project events and publications: http://biositrace.lgcgroup.com/

The contact person for general questions about the project is Helen Parkes (Helen.Parkes@lgcgroup.com).

6 List of publications

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