Final Publishable JRP Summary for SIB54 Bio-SITrace
Traceability for biologically relevant molecules and entities

Overview

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, 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.

Need for the project

Currently there is a lack of reference methods and materials for the counting of biological molecules (such as nucleic acids and proteins) or cells which is a major hindrance for measurement traceability and comparability. This in turn impacts accreditation and the ability to ensure regulatory compliance.

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

These 3 cases represented clinical importance, but also gave a spread of biological factors from whole cells and proteins to DNA molecules.

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 development of reference measurement methods and reference materials for counting cells, DNA and proteins needs to be done in close collaboration with relevant stakeholders, so that the benefits can be translated directly into the provision of useful reference measurement values. Technical outputs also need to be disseminated to appropriate standardisation bodies associated with the European In Vitro Diagnostic Medical Device (IVD) Directive 98/79/EC and ISO 17511, the in vitro diagnostic medical devices standard which includes requirements for purity determination of primary calibrators/reference materials.

EMRP
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Scientific and technical objectives

In order to develop an SI-traceable reference measurement system for biological entity measurements the project's objectives were to:

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).

Results

Identify and develop approaches to the treatment of uncertainties in enumeration

The project successfully developed a conceptual framework for achieving traceability of biological molecules (nucleic acids and proteins) and entities (cells) to the SI using state of the art counting and purity assessments. Definitions for "measurand" and "purity" in the context of biologically relevant molecules and entities were also proposed and traceability chains appropriate to bio-measurement developed. Experimental strategies and model systems were established and technical work on developing the reference methodologies, materials and measurement target definition and characterisation were carried out. This has now allowed the demonstration of SI traceability through counting, for example nucleic acid and cell measurements, underpinning the development of reference materials and compliance with IVD regulation and standards.

Develop traceable nucleic acid, protein and cell measurement methods based on enumeration technologies.

Nucleic Acids

In consultation with key stakeholders, an analytically challenging model system was selected to measure gene mutations in a non-invasive, cell-free DNA diagnostic model (i.e. measurement of small amounts of tumour DNA shed into the blood) reflecting both clinical need (colorectal cancer) and a lack of reference materials. This model can be used to guide treatment of certain cancers and is representative of other cancer mutations as well as genetic measurements associated with foetal abnormalities, donor organ rejection and antimicrobial drug resistance.

PCR is a technique used to amplify a single segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. This objective used digital PCR, a modified version of conventional PCR that gives an absolute quantification of nucleic acids by measuring individual molecules. Materials consisting of DNA of varying sizes containing the target mutant sequence were produced to mimic relevant circulating fragments in blood. Assays to detect and quantify the gene mutation in a large background by digital PCR were designed and optimised. The project also systematically investigated the principal experimental factors that affect quantitative measurements by digital PCR i.e. assay chemistries, DNA template size and different dPCR platforms based on different technologies and reaction volumes.

The project successfully developed a digital PCR method for enumeration of a DNA mutation in the cancer gene KRAS. The method showed good quantitative reproducibility in two large inter-laboratory studies incorporating 7 NMIs and 21 end-user laboratories.

Proteins

The potential of Electrospray-Differential Mobility Analysis (ES-DMA) as a primary reference method for Low-density lipoprotein particle determination and enumeration of the main lipoprotein sub-classes was assessed. This is important for monitoring lipoproteins as a precursor to development of cardiovascular disease. A complementary method atomic force microscopy (AFM) was also successfully implemented to perform confirmation measurements. Results showed that although ES-DMA can provide SI-traceable results, measurement uncertainties need to be improved if it is to be recognised as a primary reference method that could be used in the calibration sector. The first international study comparing all of the advanced lipoprotein methods currently used (ES-DMA, Nuclear magnetic resonance, Liquid Chromatography–Mass Spectrometry and Apolipoprotein B) was orgnised by the project and results showed good comparability between the methods. An External Quality Assessment scheme was also organised in which candiate reference materials produced by the project were used to assess the accuracy of methods used to measure LDL cholesterol in 118 routine medical laboratories across Europe.

The project successfully developed the first technology platform in Europe for lipoprotein enumeration by ES-DMA that can be applied by the in vitro diagnostic sector. For the first time, AFM was also used to visualise lipoproteins in 3D, measuring their size and discriminating the different lipoprotein subclasses. The first candidate reference materials which can be used in advanced lipoprotein testing were also produced by the project as well as the first international study comparing advanced lipoprotein techniques.

Cells

Primary reference methods based on microscopy and flow cytometry were developed and used to determine cell concentrations in a range of haematological and immunological model systems. Comparison of the flow cytometery amd microscopy methods yielded good agreement and SI traceability of cell concentration measurements in blood, control blood, cerebrospinal fluid and suspensions prepared from cell cultures was established. In addition, a secondary reference procedure based on relative enumeration was also developed and used to determine platelet concentrations in blood and achieved accuracies below 0.5 %. Finally, the flow cytometry method developed was applied to a circulating tumour cell model system, where target cells could be detected at a dilution ratio of at least 1:1000 with a relative uncertainty of around 10 %.

The project developed a prototype reference flow cytometer and transferred it to a laboratory commissioned by the German Society of Clinical Chemistry and Laboratory medicine. This can now be used to provide reference measurement values for external quality assurance schemes.

Develop methods for purity characterisation of pure calibration standards for biological measurement

To meet the ISO 17511 requirements for purity determination of primary calibrators/reference materials in the hierarchy of SI traceability, methods had to be developed for the materials being studied in this project.

Nucleic Acids

The potential of NGS for the quantitative assessment of sequence purity of the gene mutation materials from objective 2 was investigated. Results showed that although NGS was capable of measuring impurities from non-target sequences (e.g. bacterial contamination), the background was too high to measure rare sequence impurities and further work is needed to improve the sensitivity of NGS to assign purity to nucleic acid reference materials. In parallel, physicochemical methods (e.g. capillary electrophoresis and inductively coupled plasma mass spectrometry) were used by the project to assess the purity of the same materials.

A novel statistical model was also developed and successfully used to assign traceable numerical values and associated uncertainties to the purity of the materials by combining information from physicochemical (e.g. chromatography) and biological measurement methods (e.g. digital PCR).

Proteins

Advanced lipoprotein testing methods were used to confirm lipoprotein purity/identity of counted objects with orthogonal techniques such as Coherent Anti-Stokes Raman Scattering microscopy, apolipoprotein profiling by isotope dilution mass spectrometry, gel electrophoresis and ES-DMA.

Cells

Cell sorting was used to determine the purity of blood samples. Results showed that assigning reference measurement values independent from the type of cell detection is only possible in certain sample types (e.g. fresh blood).

For the first time, a statistical approach was developed by the project and shown to produce purity estimates and associated uncertainties from combined sources of knowledge such as differences between experimental methods (e.g. digital PCR, chromatography). Assigning measurement uncertainty to the counting approach is required for establishing the SI traceability. In conclusion, the model developed can be used by reference material producers to give more reliable purity values to their materials.

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.

List of publications

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