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

Background

Accurate counting of biological entities underpins many sectors including healthcare, security, environment, biotechnology and food. Examples include viral load monitoring in patients, haematology blood cell counts, circulating tumour cells in cancer, presence of GMOs, allergens and pathogens in foods, host cell contamination in vaccines and biopharmaceuticals and QC of advanced cell therapy products. These biomeasurements lend themselves to description in terms of number of discrete entities such as DNA copies or number of cells. However, a lack of higher order reference methods and materials is a major hindrance for deriving traceability and measurement comparability and impacts upon accreditation and regulatory compliance.

Bio-SITrace aims to improve the state of the art in two key areas for providing higher order/SI traceability in biological measurement: the application of purified calibration materials and the use of enumeration technologies.

Need for the project

The 2011 BIPM report “Study of Measurement Service and Comparison Needs for an International Measurement Infrastructure for the Biosciences and Biotechnology” clearly identified that 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” in accordance with ISO 17511.

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

Scientific and technical objectives

The Bio-SITrace project addresses the following scientific and technical objectives:

1. Identify and develop approaches to the treatment of uncertainties in enumeration
2. Develop traceable nucleic acid, protein and cell measurement methods based on enumeration technologies
3. Develop strategies for purity characterisation of pure calibration standards for biological measurement

Bio-SITrace will improve the state of the art by:

- Undertaking a thorough assessment of the use of pure materials and enumeration techniques in the traceability chain in order to provide a clear framework for deploying these techniques usefully in practical applications of biomeasurement
- Establishing procedures and uncertainty budgets suitable for primary reference measurements of amount of nucleic acid based on single-molecule detection and counting (enumeration), in particular employing digital PCR. The principal experimental factors and sample characteristics that affect quantitative measurement using digital PCR will be investigated and general guidance for validation

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and uncertainty evaluation for new digital PCR measurement systems will be prepared. The focus will be on “liquid biopsy “ measurement for cancer diagnosis, therapy and monitoring, where low copy number enumeration of specific target nucleic acid & cell biomarkers is of increasing importance

- Developing and evaluating reference measurement procedures for the enumeration of cells. Particular attention will be paid to key contributions to uncertainty, including identification, selection, and counting. Reference systems, secondary procedures and calibrators will be developed for model systems chosen for practical importance, including haemoglobin variants and circulating tumour cells for cancer monitoring
- Developing a novel application of Scanning Mobility Particle Size spectrometry for the enumeration of biological particles. The application proposed is the development of a primary method for the enumeration of the main atherogenic particle subclasses responsible for atherosclerosis, in particular lipoprotein assemblies
- Assessing measurement methods for the characterisation of purity for nucleic acid materials intended for use as higher order calibrators in SI traceable biological measurements, including the application of next generation sequencing (NGS) as well as traditional physicochemical methods. Particular attention will be paid to the assessment and minimisation of uncertainties, resulting in procedures suitable for characterisation of primary calibrators

These technical activities will be carried out using model systems chosen for stakeholder relevance and feasibility.

Combining these activities in a single project provides important synergies and efficiency gains. Development of systems for purity characterisation will provide well characterised materials for verification of enumeration techniques. Enumeration techniques will in turn provide quantitative measures of possible interferents in pure materials. Uncertainty evaluation for enumeration techniques applicable to molecular, cell and particulate materials will allow development of consistent, state of the art approaches to measurement uncertainty evaluation for enumeration.

Expected results and potential impact

Bio-SITrace aims to reduce some of the most important barriers to adoption of SI traceability for biological measurements. This will help to widen the applicability of SI traceability for a wide range of biological measurements. Wider application of the SI will in turn:

- Reduce reliance on consensus values for primary certified materials which may change on replacement, improving the long term consistency of biological measurements
- Provide reference measurement capabilities which can reduce the use of assays based on biological activity in laboratory animals
- Enable the production of a wider range of SI-traceable reference materials for calibration and validation of biological measurement systems in the field
- Allow National Measurement Institutes and Designated Institutes to provide more reliable reference values for proficiency schemes, in the long term reducing disagreements between assay kit manufacturers and improving agreement between different laboratories worldwide
- Simplify approval of reference measurement systems and reference materials by the Joint Committee of Traceability in Laboratory Medicine (JCTLM) by providing clear traceability to the SI
- Facilitate conversion of relevant values from International Units (IU) to SI, an aim of IVD manufacturers that is also supported by discussion with NIBSC and other WHO laboratories

To date, Bio-SITrace has addressed the scientific and technical objectives as follows:

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

The project successfully achieved this scientific and technical objective. A conceptual 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 was developed. Definitions for “measurand” and “purity” in the context of biologically relevant molecules and entities were 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 started.

2. Development of traceable nucleic acid, protein and cell measurement methods based on enumeration technologies

Nucleic Acids

Work to develop molecular enumeration approaches has continued and is progressing well. An analytically challenging model system was selected that measures mutations in the KRAS (Kirsten rat sarcoma viral oncogene homolog) cancer gene in a non-invasive, cell-free DNA diagnostic model (i.e. measurement of trace levels of tumour DNA shed into the blood). The analytical method chosen was digital PCR, a modified version of conventional PCR that affords absolute quantification of nucleic acids by measuring individual molecules. Stakeholder consultation identified a single mutation in the KRAS gene (G12D) as the most relevant to be measured by the enumeration approaches being developed. G12D mutation is the most frequent KRAS mutation in colorectal cancer and is associated with reduced efficacy of relevant therapies. Materials, consisting of DNA fragments of varying sizes containing the target G12D sequence have now been produced to mimic relevant circulating fragments in blood. Assays to detect and quantify the KRAS mutation in a large background of wild-type nucleic acid sequences by digital PCR were designed and optimised. The principal experimental factors that affect quantitative measurements by digital PCR have also been systematically investigated, namely assay chemistries, DNA template size and dPCR platforms which are based on different technologies and reaction volumes. The reproducibility of the digital PCR method developed is being evaluated through an inter-laboratory study between project partners and external laboratories.

Proteins

Work to develop advanced analysis methods to profile and count lipoprotein particles of relevance to cardiovascular disease monitoring has continued and is making good progress. Electrospray-Differential Mobility Analysis (ESI-DMA) is being assessed as a primary reference method for Low-density lipoprotein particle (LDL-P) determination and enumeration of the main lipoprotein groups. Lipoprotein purification techniques have also been developed to prevent High-density lipoprotein (HDL) measurement interference by plasma proteins. A large international cross-platform comparison between advanced lipoprotein testing methods has been carried out to determine if a reference method can be proposed for lipoprotein enumeration (i.e. standardisation), or if a harmonisation initiative would be more relevant. An EQA scheme was organised in which commutable certified reference materials were used to assess the accuracy of field methods used to measure LDL-C in 118 routine medical laboratories. Bias ranged from -0.5 to +14.5% with an average of +5.2%. Positively biased LDL-C results imply needlessly giving costly treatments to patients.

Cells

Work to develop and evaluate reference measurement procedures for the enumeration of cells and determination of their concentrations in a number of haematological and immunological model systems has continued and is progressing well. A microscopically based primary reference measurement procedure has been developed and applied to detect white blood cells in cerebrospinal fluid (CSF). In addition, an advanced microscopy platform has also been implemented to detect a mixture of labelled and non-labelled cells from CSF allowing non-invasive discrimination between different cells. A cell material consisting of CD4+ cells (immune cells infected by HIV, monitoring the levels of which plays an important role in determining disease progression) has been prepared and used in a cross-platform comparison of microscopic and flow cytometric counting methods. Results determined with the primary procedures based on microscopy as well as flow cytometry are in good agreement and demonstrate the robustness of the primary procedures.

3. Development of strategies for purity characterisation of pure calibration standards for biological measurement

Work has now started to develop a novel Bayesian staitistical model to assign traceable numerical values and associated uncertainties to the purity of the KRAS materials from objective 2 combining information from physicochemical (e.g. chromatography) and biological measurement methods (e.g PCR, sequencing).

Dissemination

Technical outputs from this project continue to inform development of international standards and guidance: Specific project contributions to date include:

- Contributed to the development of DIN Haematology standard standard 58932-3. “Reference measurement procedure for the determination of red blood cell concentration in blood”.
- Lead in developing ISO/PWI 20395: Quality considerations for targeted nucleic acid quantification methods – this is now being drafted as a NWIP within ISO TC276 (biotechnology) WG3 Analytical methods. The NWIP and outline working draft (WD) were tabled and discussed at the last ISO TC276 WG3 meeting and a formal NWIP is currently being drafted by LGC (using knowledge accrued & guidance outputs from Bio-SITrace prior to submission for electonic vote in March 2016.
- Project input has also been made into the ongoing drafting of ISO TC276 WG3 series of cell analysis standards ISO/WD 20391-1 "Biotechnology – Cell counting: Part 1: General guidance on cell counting methods” and “Biotechnology – Cell Characterisation: Part 1”. These standards are progressing well and incorporating JRP input specifically with respect to flow cytometry for cell counting, and cell characterisation methods.
- Input to TC212 WG2 “Reference Systems”: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). Specific JRP draft contribution to informative annex proposed, describing examples of traceability chains for cells and nucleic acids.
- Recommendations and text for revision of the SI brochure, based on Bio-SITrace activity has been proposed to the CCU revision drafting committee for inclusion in the SI brochure revision. A modified reference exemplifying counting of “a number of cellular or biomolecular entities (e.g. copies of a particular nucleic acid sequence), …..a formal traceability to the SI can be established through appropriate, validated measurement procedures.” has now been included in the 9th revision of the SI brochure which is currently open to consultation.

Dissemination is also being achieved through active stakeholder engagement achieved through direct collaborative interaction with relevant instrument manufacturers, reference laboratories, and relevant professional interest bodies.

Further dissemination has included focussed stakeholder workshops and presentations at significant stakeholder conferences including the International Federation of Clinical Chemistry WORLDLAB Congress, IFCC & European Federation of Laboratory Medicine EUROMEDLAB, IEEE International Symposium on Medical Measurement and Applications (MeMeA) and JCTLM Members' and Stakeholder's Meeting.

Publications include:

- Standardisation considerations for circulating cfDNA analysis (Anal. Bioanal.Chem, 2014)
- Considerations for digital PCR as an accurate molecular diagnostic tool (Clin. Chem, 2014)
- Evaluation of digital PCR for absolute RNA quantification (PLoS One, 2013)
- Multiple hypothesis testing for metrology applications (Accred Qual Assur, 2013)

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