CCQM-NAWG STRATEGY DOCUMENT 2021-2030

Version 2.0 18.09.2025

1. EXECUTIVE SUMMARY

The focus of NAWG is to support global comparability, metrological traceability and accuracy in the analysis of nucleic acid polymer sequences and their abundance. Nucleic acid analysis is used for a wide range of global measurements which underpin research and practical applications in most areas of life science [1]. The various sectors in which molecular methods can be applied to identify and characterize nucleic acids along with their versatility has meant that nucleic acid analysis is not only used for genetic based applications, but also to support broader areas of bioanalysis such as protein and cellular biology.

Since its initiation in 2015, the NAWG has primarily served the foods and feed and health sectors, although nucleic acid analysis is also important in the environmental (e.g. species/microbial surveillance) and all types of biotechnology/synthetic biology (spanning industrial, agricultural and pharmaceutical) sectors. These areas are also likely to benefit from NAWG activities in the future. The food authentication area is the most metrologically advanced (with respect to traceability and understanding of sources of uncertainty) in terms of routinely applied measurements and it is in this sector that NMIs have the most NAWG supported CMCs. While molecular testing is used in clinical diagnostics, metrology is not as widely applied in this sector as it is for certain areas of clinical chemistry; however, this is changing due to the desire of stakeholders to apply increasingly advanced high throughput and quantitative measurements to assist in patient management as well as regulation in cases requiring metrological traceability and uncertainty evaluation.

In addition to NMIs, DIs, and associated CCQM WGs (CAWG, PAWG and OAWG), key NAWG stakeholders include other reference material producers and proficiency test/external quality assurance providers and the testing communities they serve. As metrology is a relatively new concept in the field of nucleic acid measurements, considerable efforts are ongoing to inform potential stakeholders how measurement science can support their applications. This is achieved via (locally, regionally and NAWG led) workshops, publications, regional interactions and communication and cooperation between NAWG and international or regional organizations such as ISO, CEN (The European Committee for Standardization), WHO, JCTLM, IFCC, SOGAT (Standardisation of Genome Amplification Techniques), ESCMID (European Society for Clinical Microbiology and Infectious Disease) and EMN-TraceLabMed to better understand standardization challenges and to apply state-of-the-art metrology to support existing and future stakeholders. NAWG members actively participate in many of these groups and committees. In addition, joint studies with other CCQM WGs are being discussed in specific areas, such as microbial measurement (CAWG) and environmental DNA with GAWG.

The early Key Comparisons were in the food analysis space supporting measurement claims to measure DNA from maize [2], GM Rice [3] and canola [4], with the current study K86.d aimed at supporting CMCs pork in beef raising the challenge of mass based thresholds being evaluated using genetic targets. NAWG activities in the healthcare sector are increasing; molecular diagnostic tools are applied to genotype patients, determine tumour and microbial genetics as to estimate pathogenic burden, to measure transcriptional surrogate biomarkers of disease and to measure disease predictors using changes at the epigenetic level. To develop NMI measurement capabilities in these areas, a series of medically relevant pilot studies have been conducted which focused on the identification and quantification of nucleic acid sequences and key comparisons are planned. Recently this has led to the completion of two key comparisons in support of CMCs for the analysis of nucleic acid sequences involved in cancer and pathogen testing. Additional KCs and pilot studies are planned to expand on this topic to include matrix based measurements and those which evaluate modifications to nucleic acids

The ubiquity of the nucleic acid analysis methods, the molecules they target and challenges associated with their measurement are typically agnostic to sectors. Consequently, the NMIs are in a unique position to advance the measurements in one specific subject area (such as *in vitro* diagnostics) using knowledge from another (such as food and feed testing). The NAWG will need to explore how this pan sector characteristic can be capitalized on to support broader scope nucleic acid measurement capabilities with planning activities discussing potential KCs and pilots relevant to the environment, biotechnology and therapeutic sectors.

As we progress into the next decade, NAWG activities will likely continue to support food and feed and clinical associated testing with Key Comparisons reflecting additional unmet needs, covering different matrices, species and molecular challenges (such as discriminating gene edited from natural sequences). It is anticipated that food and feed associated measurements will expand to the agriculture/biotechnology sector; pilot studies to monitor crop disease are planned and we will explore how to support novel genomic technologies applied in the development of precision bred organisms. The NAWG strategy heading towards 2030 will considerably strengthen activities associated with molecular reference measurement procedures (likely necessitate improved examination of sequence structure and composition) and materials to support clinical nucleic acid analysis. These are likely to build on established capabilities for underpinning SI traceable quantitative measurements of nucleic acids copy number and copy number concentration in aqueous solutions and explore routes to apply such capacity to assist in matrix reference materials and/or reference measurement procedures on real samples; such capacity will also be of value to the other sectors including industrial biotechnology where novel methods, such as CRISPR CAS-9 based tools, are being applied.

The increasing need for genomic, transcriptomic and epigenomic analyses means that the NAWG will need to explore the development of strategies to support advanced sequencing capabilities, applied for purity analysis. The measurement challenges associated with these types of 'non-targeted' methods are likely to increase with the development of newer, simplified sequencing technologies more suitable to less specialized settings.

2. SCIENTIFIC, ECONOMIC AND SOCIAL CHALLENGES

While nucleic acids were discovered in the late 19th century, it was not until almost a century later that their role in heredity was confirmed. Nucleic acids are used by living organisms to maintain and transmit the instructions for reproducing, building and maintaining a cell/multicellular organism. In this analogy, the letters of the genetic sequence spell out genes. A key mechanism by which nucleic acids enable biological function is through the Central Dogma whereby protein coding genes are transcribed from DNA into a complementary RNA copy which is then translated into an amino acid sequence. It is within the sequence of nucleic acids monomers that the triplet code for all the protein amino acids sequences required for cellular life are written. Nucleic acid sequence analysis is used to examine genetic changes, identify the presence of species of organisms or even to determine the relatedness of organisms within a species.

A number of high accuracy chemical methods, used by NAWG members, are able to examine the presence of nucleic acid sequences and, in some cases, to identify the individual nucleotide monomers bases. However, as all living organisms essentially use the same set of nucleotide monomers to function, it is necessary to 'read' the sequence and determine the arrangement of these monomers to determine (for

example) whether the sequence in question is from a genetically modified organism, a human gene (that may contain a clinically important mutation) or that of an infecting pathogen. Molecular methods allow nucleic acid sequences to be determined, and where necessary, quantified and further evaluated for modification, such as epigenetic differences. The development of molecular methods, including complementary hybridization, nucleic acid amplification technologies (such as the polymerase chain reaction) and sequencing coupled with the ubiquity of nucleic acids in life sciences, has meant these same methods can be applied nucleic acid analysis across different specialties.

Despite this, metrology and metrological considerations (such as routes to traceability and sources of uncertainty) are not commonly investigated, with those applying nucleic acid analysis typically assuming their systems are fit for purpose in this respect. Certainly, many breakthroughs in sequence discovery, genome evaluation and routine monitoring have succeeded without the assistance of the measurement science community. Yet as these fields have pushed to offer ever more high-throughput ('omic') and precise quantitative solutions, the life science community has been met with challenges associated with standardization and traceability. Many of those working with these methods neither realize that measurement science is a field in its own right, nor that a global infrastructure exists (of which the CCQM NAWG is a part) to support them in this respect. Given the wide variety of applications in the nucleic acid analysis space the challenges associated with supporting in terms of measurement science are vast. The NAWG will continue to support the various sectors that are more metrologically established while also championing advancements in the other sectors.

In September of 2020, a questionnaire was sent to all NAWG members to determine the current and future priorities of the NAWG. Eighteen institutes responded to the survey: BVL (Germany), EXHM/GCSL-EIM (Greece), GLHK (Hong Kong), HSA (Singapore), INMC (Colombia), INMETRO (Brazil), INRIM (Italy), JRC (EU), KRISS (Korea), NIB (Slovenia), NIMC (China), NIMT (Thailand), NMIA (Australia), NMIJ (Japan), NML at LGC (UK), TUBITAK UME (Turkey), NIST (USA) and VNIIM (Russia).

The questionnaire covered the following topics:

- current and potential future subject areas in which NMIs/DIs wish to claim CMCs (for both absolute measurements and fractional abundance measurements)
- types of nucleic acids measurements (with relation to matrix)
- nucleic acids measurement procedures (sequence specific/non-specific)
- identification of measurement capabilities which require NAWG attention and support
- questions, related to NMI participation in Key/Pilot studies and leading/co-leading a study

For fractional abundance measurements of sequence (Figure 1), more than half of respondents currently hold CMCs or intend to hold CMCs in the future in food analysis (72%), *in vitro* diagnostics for infectious diseases (67%), *in vitro* diagnostics for human genetics and non-communicable diseases (67%), regenerative medicine and gene therapy (61%), and biotechnology (56%). For absolute nucleic acid sequence number concentration measurements (Figure 2), more than half of respondents currently hold CMCs or intend to hold CMCs in the futurein *in vitro* diagnostics for infectious diseases (78%), regenerative medicine and gene therapy (67%), *in vitro* diagnostics for human genetics and non-communicable diseases (61%), biotechnology (56%), and food analysis (56%).

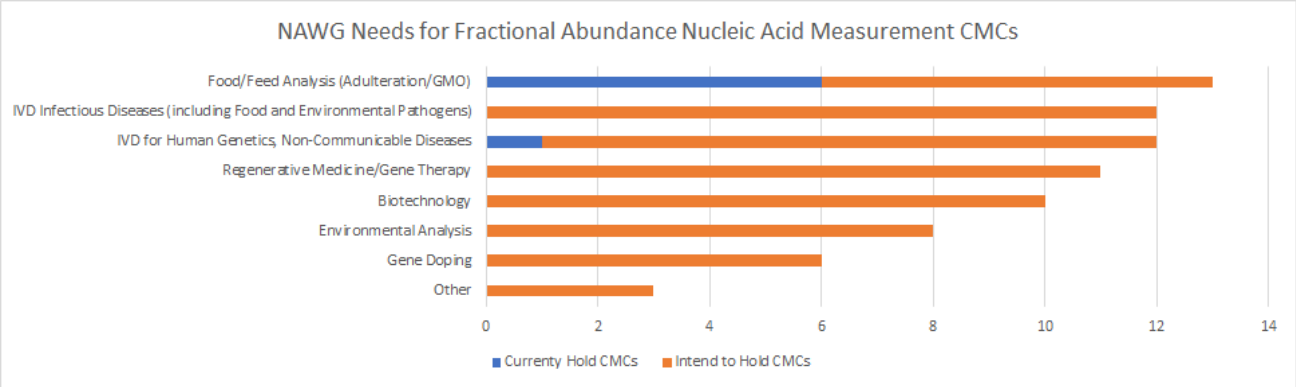

Figure 1. Subject areas in which NAWG members currently hold CMCs or intend to hold CMCs within the next 5 years for fractional abundance nucleic acid measurements.

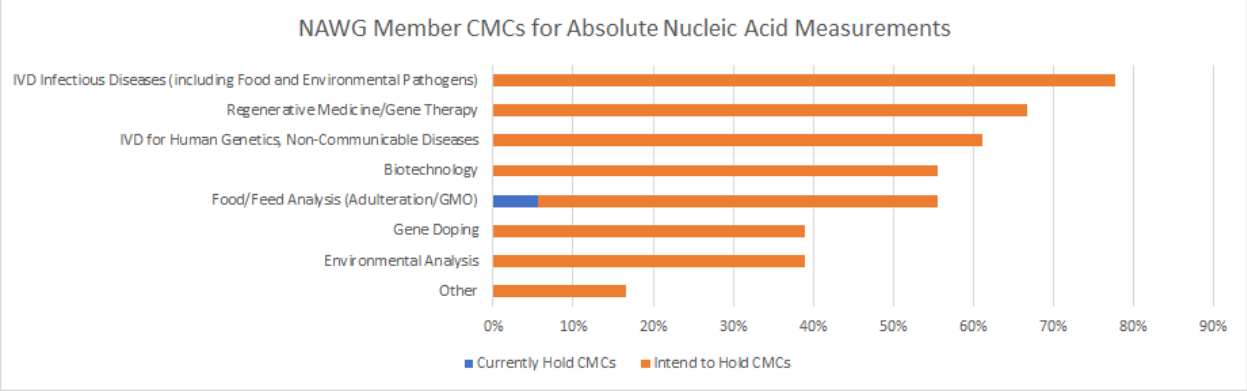

Figure 2. Subject areas in which NAWG members currently hold CMCs or intend to hold CMCs within the next 5 years for absolute quantification of nucleic acid measurements.

Member NMIs and DIs offer a wide range of products and services to their stakeholders including certified and non-certified reference materials, calibration services, and regulatory functions. The NAWG engages with its members and stakeholders through key comparisons and pilot studies, workshops with academia and industry, liaisons with other relevant working groups and committees.

As evidenced by the COVID-19 pandemic, new pathogens and clinically important variants are constantly being discovered; it may not always be possible to create certified reference materials for each new, important nucleic acid sequence in a timely fashion. The NAWG has demonstrated the potential use for candidate SI traceable reference measurement procedures for nucleic acid measurement (such as IDMS, Flow Cytometric counting and dPCR) and value-assignment of reference materials that enables a dynamic means of supporting the established reference material routes for enabling traceability.

In parallel with recognizing current and future needs in standardization and traceability, NAWG is also addressing some of the fundamental scientific challenges associated with nucleic acid analysis. Nucleic acids vary in their stability which can impact both reference materials and biobanks as well as routine testing during sample handling. In the absence of DNA digesting enzymes DNA is generally considered relatively stable; however, low mass concentrations (less than 5 ng/µL) of both DNA and RNA require the addition of stabilizing molecules (such as yeast tRNA or other background RNA/DNA) to prevent decreases in concentration over time. RNA can be less stable than DNA; both due to inherent chemical reasons and

because RNases, which can quickly degrade RNA, are both ubiquitous and stable in the environment. Reduced stability of a material (be that reference material or clinical samples) may impact on the quantity of a given sequence or the composition of its matrix, both of which may impact the measurement. Further, information on the wider target sequence composition may also be useful with storage as factors like fragment size may also be affected by stability resulting in potential discrepancies between methods.

Scientific challenges associated with the measurement of a given nucleic acid can vary due to the complexity of the nucleic acid in question (e.g. sequence, size, secondary structure, etc.) as well as the associated matrix. Consequently, sources of uncertainty and routes to traceability must frequently include volume as an additional consideration. Nucleic acid analyses are typically multi step processes requiring prior manipulation to purify the nucleic acids for analysis from the sample ('pre-analytical' steps). When conducting nucleic acid analysis, a key distinction is whether the analyte is part of a larger molecule (typically the case with genes associated with genetic modification) or whether it is contained within the sequence being measured (such as when measuring actionable genetic variants during cancer treatment). From an analytical point of view, these two categories can present distinct challenges when considering sources of uncertainty. Furthermore, the analytical sources of error can differ when the same nucleic acid sequence is measured in different matrices (aqueous solution, matrix materials or biological samples).

When considering a given measurand, the type of analysis (Figure 3) can be categorized by whether the nucleic acid analysis is a method that is 'targeted' (such as PCR or hybridization) to identify a defined sequence(s) (and potentially quantity and/or any modifications) where the intended target is specified, or whether the nucleic acid sequence(s) (and potentially quantity and/or any modifications) is unknown and the 'non targeted' (such as sequencing) measurement intends to determine this. The type of analysis also needs to consider the type of analysis (Figure 3) which can be broadly categorized into presence/absence 'nominal' examination (e.g. sequence is present or not), relative quantification/fractional abundance or absolute quantification (e.g. number concentration of sequence).

When discussing a given measurement, and associated challenges, the type of sample (including properties of the nucleic acid and its matrix) also need to be considered (Figure 3). At their simplest level of complexity nucleic acids can vary from a few tens to billions of bases (term used to describe a nucleotide monomer which is approximately 300 Daltons, consequently large DNA molecules can be >100 million kilo Daltons (Figure 4) and this may be comprised of mixtures of different genomes, further complicating the measurement with certain techniques. Further sources of error will be added when additional steps are required to prepare the nucleic acids for analysis such as purification and nucleic acid digestion (e.g. restriction enzyme treatment), adaptation (e.g. adapter ligation), modification (bisulfite conversion) or generation of complementary nucleic acids (reverse transcription). Purification (or extraction) is often required when performing nucleic acid measurements on real samples, due to the fact the matrix is typically unsuitable for the final molecular analysis steps. For example, formalin fixation of biological tissue, for preparation of formalin-fixed paraffin-embedded (FFPE) samples is widely used in pathological laboratories to preserve patient's biopsies from degradation for further investigations. These materials can be stored for decades and are often considered as a valuable source of well characterized human samples although are not ideal for storage of nucleic acids. Isolation of nucleic acids from FFPE samples is challenging due to formalin-induced crosslinking of proteins and nucleic acids, and the fragmentation of high molecular weight genomic DNA and mRNA molecules. All of these steps contribute uncertainty and must be considered when exploring routes to traceability and the most accurate measurements.

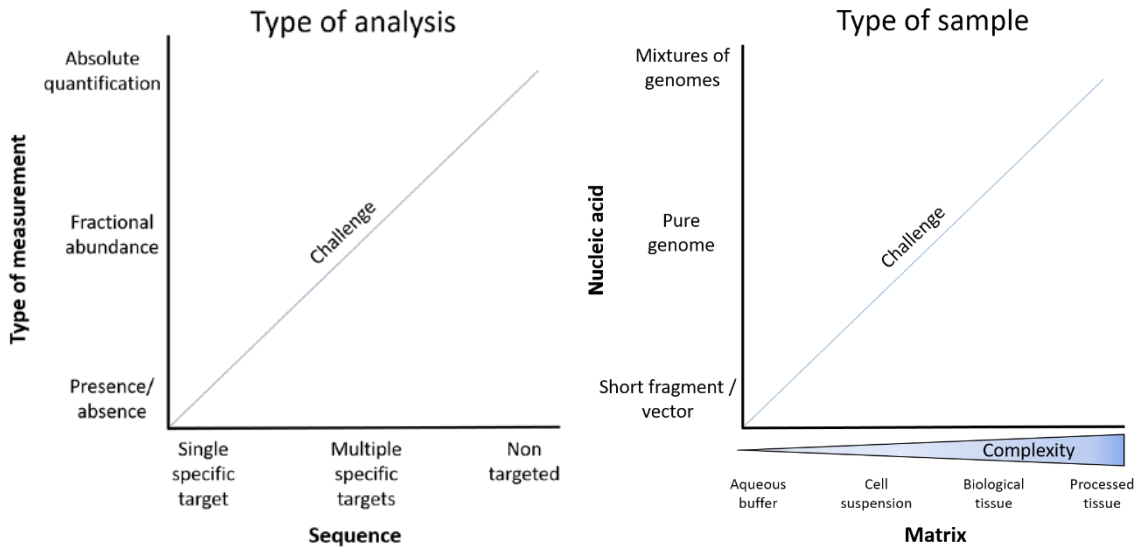

Figure 3. Type of sample and Type of analysis NAWG studies vary in the complexity of the analysis and the type of sample. The simplest type of study would be the detection of the presence or absence of a plasmid in a buffered solution. A much more complex study would be the identification of all nucleic acid species in a processed food matrix, along with the abundance of each.

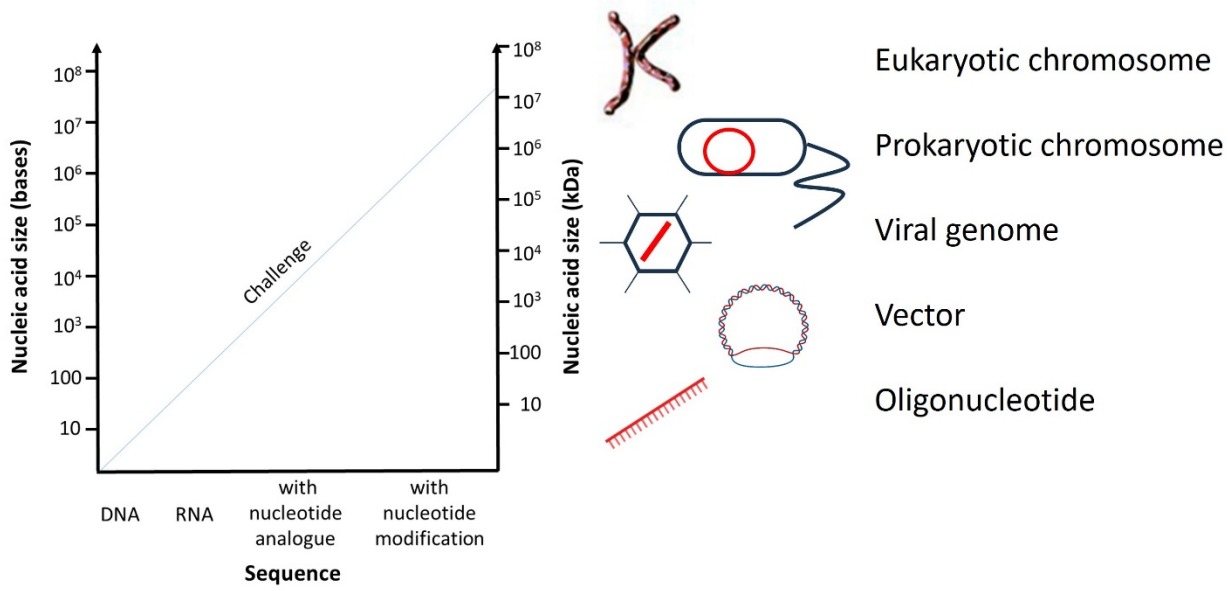

Figure 4. Nucleic acid size range (bases & kDA). NAWG studies currently consider DNA and RNA from all categories. Non molecular 'chemical' methods for measuring amount of nucleic acids, such as IDMS, have been included in some comparisons (e.g. CCQM-K181 [5]). Comparisons explicitly focusing on chemical measurement will begin with CCQM-K199 (see page 14) with more planned over the next decade.

3. VISION AND MISSION

The CCQM's vision is:

A world in which all chemical and biological measurements are made at the required level of accuracy to meet the needs of society.

The mission of the CCQM is:

To advance global comparability of chemical and biological measurement standards and capabilities, enabling member states and associates to make measurements with confidence.

4. STRATEGY

In line with the CCQM's vision and mission, the aims of the 2021 to 2030 NAWG strategy are:

To contribute to the resolution of global challenges by improving the accuracy of nucleic acid measurements. This is associated with areas such as food safety and healthcare as well as other topics such as biotechnology (including biobanking), environmental analysis, etc. and by identifying and prioritizing critical measurement issues and developing studies to compare relevant measurement methods and standards. The aim is to ensure the analyte is clearly understood, how it is relevant to what is intended to be measured and that all sources of uncertainty are considered and, where possible, quantified.

To promote the uptake of metrologically traceable chemical and biological measurements, through workshops and roundtable discussions with key stakeholder organizations, to facilitate interaction, liaison and cooperative agreements, and receive stakeholder advice on priorities for CCQM work programmes.

To progress the state of the art of chemical and biological measurement science, by investigating new and evolving technologies, measurement methods and standards and coordinating programmes to assess them. This includes the progressing from fractional abundance measurements to application of absolute SI traceable methods for nucleic acid analysis.

To improve efficiency and efficacy of the global system of comparisons for chemical and biological measurement standards conducted by the CCQM, by continuing the development of strategies for a manageable number of comparisons to cover core capabilities while also supporting capacity development in the fast advancing field of nucleic acid analysis.

To continue the evolution of CMCs to meet stakeholders' needs, incorporating the use of broad claim CMCs where applicable to cover a broader range of services and considering options to present these in a way that meets stakeholder needs and encourages greater engagement with the CMC database.

To support the development of capabilities at NMIs and DIs with emerging activities, by promoting a close working relationship with RMOs including mentoring and support for NMIs and DIs preparing to coordinate comparisons for the first time and promoting knowledge transfer activities including workshops, as well as secondments to other NMIs, DIs and the BIPM.

To maintain organizational vitality, regularly review and, if required, update the CCQM structure and strategy for it to be able to undertake its mission and best respond to the evolution of global

measurement needs, by prioritizing where new areas or issues should be addressed within the structure and evolving working group remits as required.

5. ACTIVITIES TO SUPPORT THE STRATEGY

5.1. PROGRESSING METROLOGY SCIENCE

In order to achieve its strategic goals in addressing global scientific challenges within nucleic acid analysis, the NAWG works to support members in nucleic acid RM development and calibration and measurement services, in areas where the NAWG support is desired (Figure 5). In addition, the NAWG provides an active forum to discuss investigative research which underpins nucleic acid measurement expertise.

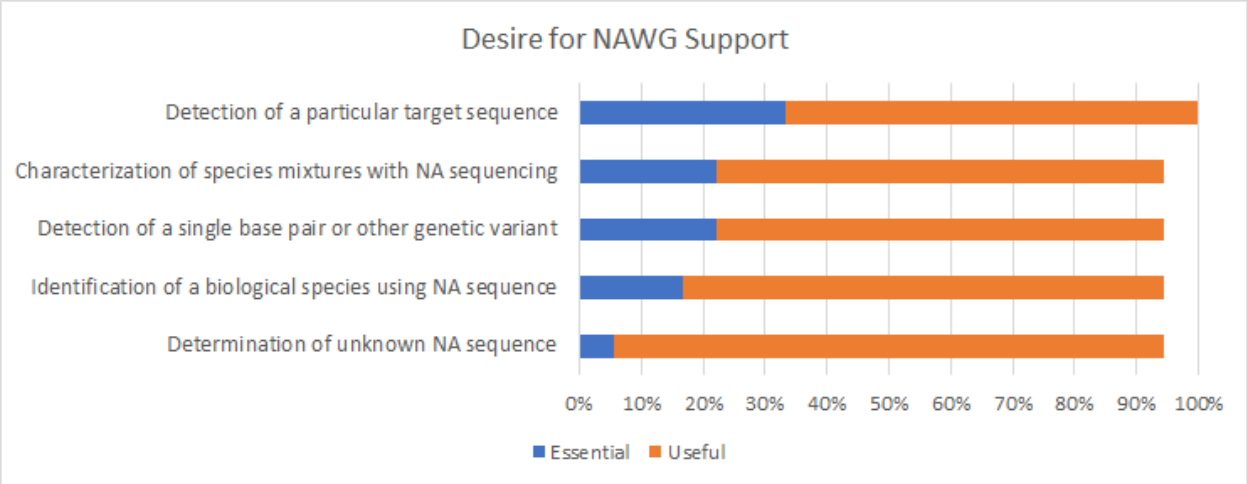

Figure 5. Areas in which NAWG's support is desired according to results from the questionnaire (2020)

A major impact that the NAWG (and its predecessor the Bioanalysis Working Group) has achieved is in supporting CMCs for wider application of capabilities that have been developed by individual, or groups of NAWG members [6-16]. A good example is in the measurement of genomic DNA from plant tissues with series of key comparisons supporting CMCs from a variety of different biological matrices [2-4]; this series is continuing with the high protein matrices in K86.d. Another considerable major technical goal of the NAWG has been to demonstrate routes for SI-traceable nucleic acid measurements with NAWG members working as a group to demonstrate the first examples of this [17-19].

The COVID-19 pandemic highlighted the need and role for reference measurement procedures in support of the development and application of diagnostic tests that were identifying the presence (and not the quantity) of nucleic acids. Such methods can be developed at a pace comparable to that of the tests in question thus providing routes to support test evaluation through value assignment of reference materials. The NAWG has conducted pilot studies (P199b [20]) and key comparisons (K181 [5]) to support CMCs for pathogen associated nucleic acids measurements and conducted the first CCQM pandemic fire drill (P232) as part of the CCQM Pandemic RoadMap [21] the report of which is currently being finalized in 2025. This pathogen related work is moving from purified nucleic acid in aqueous solutions to support CMCs when value assigning whole pathogen materials with the initiation of K190 in which NMIs will assign values to WHO and secondary standards.

In order to realize the strategic plan, the NAWG intends to pursue key comparisons and pilot studies to support nucleic acid measurements in sectors including, but not limited to, food and feed, health, environment and biotechnology (Figure 6).

Current, future and planned Studies:

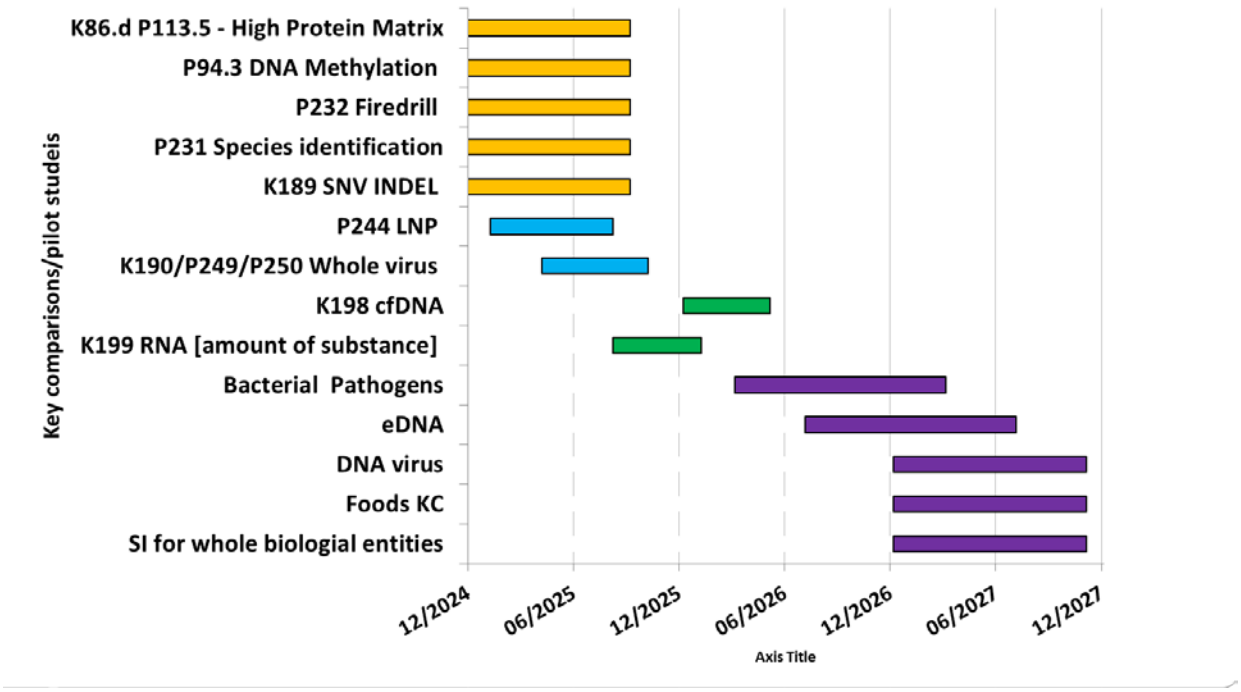

Figure 6. List of current (final report being drafted [orange], ongoing [blue] or in preparation [green]) and planned topic areas under discussion (purple)

These studies include sequence-specific RNA measurements (molecular diagnosis of RNA viruses), DNA measurements (high protein matrix, cancer biomarkers, plant pathogens, etc.), qualitative DNA identification (biological species determination) and modified DNA measurement (DNA methylation in biological matrix). By completing these planned studies, the NAWG will broaden its scope within its measurement space in terms of type of analysis (Figures 7) and type of sample (Figure 8).

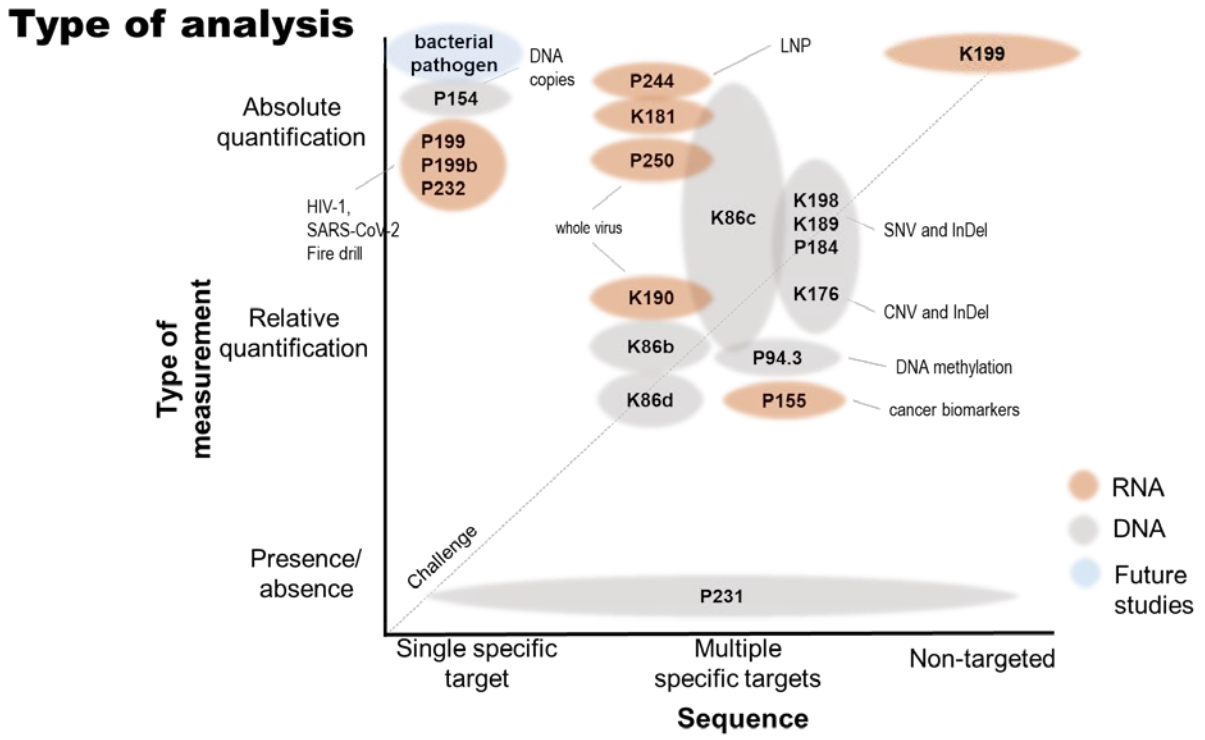

Figure 7. NAWG measurement space in type of analysis

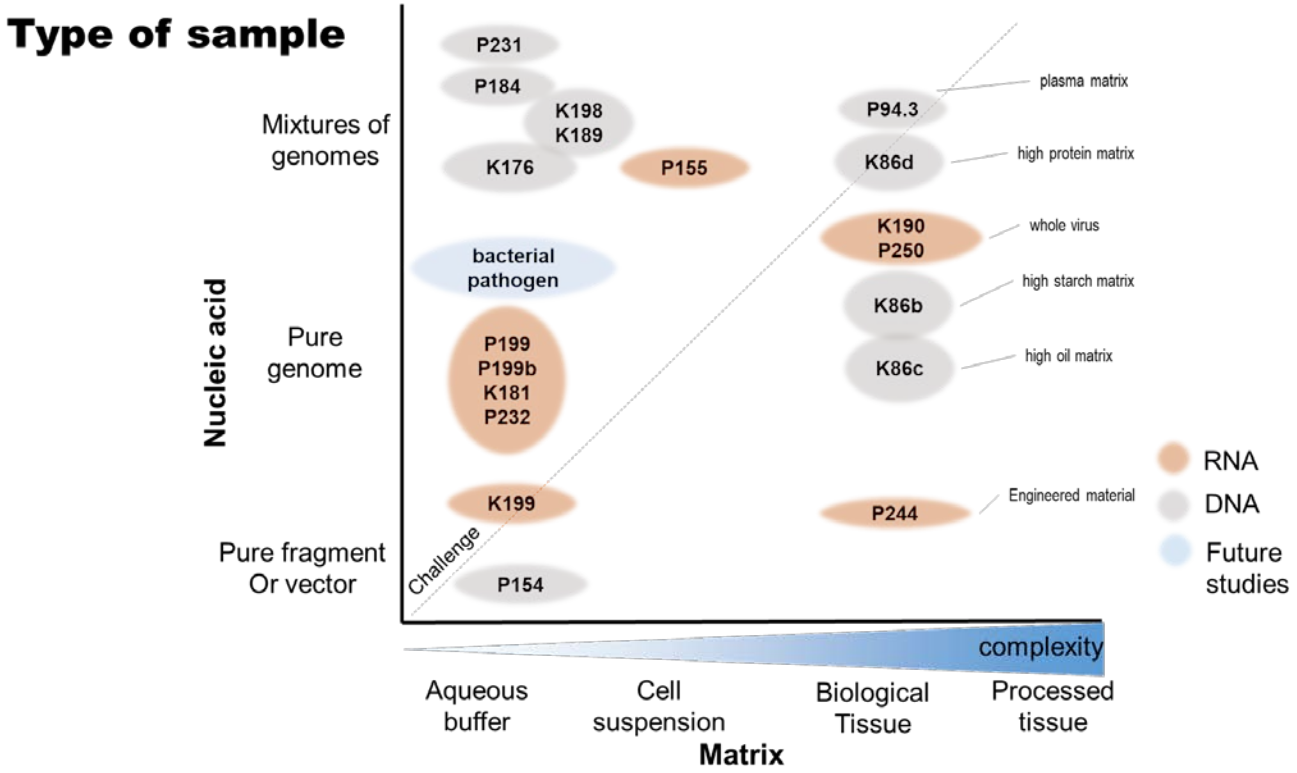

Figure 8. NAWG measurement space according to type of sample

Current and near Future NAWG Studies

Food based testing

K86d P113.5 (High Protein Matrix) aims to measure the DNA copy number ratio of pork specific DNA sequence to beef specific DNA sequence and universal genomic gene sequence of mammalian genomes, both in a matrix, relevant for processed food measurements and in solution. The study included both key comparison aspects and stand alone pilot options. In the KC portion, participants were required to perform measurements on DNA isolated from protein-rich pork/beef mixture and on pork/beef DNA in aqueous solution. In the Pilot study, participants had to report pork mass fraction in pork/beef mixture basing on results of DNA copy number ratio measurements of pork-specific gene sequence to universal genomic gene sequence, but it was at a much lower percentage than for KC portion. This study builds on previous work by the NAWG in K86b [3] (high starch matrix) and K86c [4] (high fat matrix) and the draft B is about to be completed.

P231 represents a first for the NAWG as it introduces the notion of uncertainty when considering identity while also tests participants ability to measure sequence quantity without knowing what species is present in the samples. Qualitative analysis (species-specific sequence presence/absence - 'nominal property' examination) by the results of DNA sequence analysis and quantitative analysis of animal species DNA presence using the species-specific gene sequence in a single DNA extract of unknown meat species or a mixture of samples which are the lyophilized DNA extract of unknown meat species. This included single species (chicken) and mixtures of different species (pork, goat and horse). In all cases the correct species were identified from the respective materials by all participants with the majority of laboratories providing agreed proportions of relative mixtures for the material comprising mixtures of species. The aim of P231 is to promote meat species authenticity analysis and identification of food and feed samples.

The NAWG is currently exploring future KCs in the foods space which will look to support the maintainance of existing CMCs while also considering new challenges such as the use of engineering biology in the development of precision bred organisms.

Pathogen nucleic acid detection

As a result of the COVID-19 pandemic the NAWG has built on earlier work to establish considerable pathogen related activities with the P199 series (CCQM P199 [22] and P199b [20] Studies: HIV and SARS-CoV-2 RNA Quantification) and the first CCQM Firedrill (P232) which is a CCQM Pandemic Roadmap initiative that explores how rapidly NMIs can respond to support outbreak diagnostic testing and K190 which is the first NAWG KC to utilise the WHO reference materials. Due to the relevance of viral detection in the context of the COVID-19 pandemic, other pathogenic viruses and the use of viruses as vectors (e.g. for vaccines, gene therapy, synthetic biology, etc.), the Key Comparisons K181 was conducted evaluating SARS-CoV-2 RNA sequence number concentration to support CMCs in viral genome measurement in aqueous solutions and published in 2025 [5]. This represent the first CCQM RNA sequence key comparison study.

CCQM-K190 extends this series of pathogen nucleic acid analysis building on K181 [5] (and the P199 series [20, 22]) by necessitating the measurement of RNA from whole virus and thus represents the first KC

measuring a clinically relevant nucleic acid requiring preprocessing and extraction. This KC will include an assessment of the lysis and extraction and will also consider relative measurements performed in the clinic relative to the WHO international standard material. P250 is a stand-alone pilot study aligned to K190 that considers RNA sequence number concentration per unit volume from whole viral extracts.

Quantification of genetic changes used to guide therapeutic interventions

K176 [23] investigated HER2 overexpression, caused by an amplification of the HER2 gene within cells, is important in breast cancer therapy; while treatments for HER2+ cancer are available they can have serious cardiac contraindications, so they should only be used when necessary. Consequently, accurate measurement of HER2 copy number in the genome is important to ensure those at most risk are correctly managed. The two main methods of detection HER2 overexpression are IHC (immunohistochemistry) and FISH (fluorescence in situ hybridization), which can have equivocal results. K176 investigated candidate reference measurement procedures for the quantification of HER2 gene copies. This study builds on previous NAWG studies (P154, P184) and expands participants' capabilities from SNV (single nucleotide variant) and INDEL (small insertions and deletions) to large structural variants.

The CCQM-P184 pilot study [24] investigated variant copy number concentration and allelic frequency, even when different assays and platforms were used and supported traceable measurement of the target sequence copy number concentration and copy number ratio of gene mutations (SNV and INDEL) when present in a background of wild-type DNA. CCQM-K189 builds on learnings from P184 to support the measurement of copy number concentration and fractional abundance of mixtures of the sequences variants to provide support for CMC claims when measuring variant sequences in purified genomic DNA. The variants in question represent actionable sequences the presence of which is used to guide diagnostic and treatment in lung cancer. These targets are more challenging to measure than those evaluated by K176 (see above) as they must be distinguished from closely related variants that represent the predominant human genome sequence. K189 is currently at the draft A stage. K198 is currently being planned and is expected to proceed later in 2025 and will extend the support for CMCs available from K184 to measuring smaller fragments of DNA more commonly measured in specimens like liquid biopsies.

Epigenetic analysis

CCQM-P94.3 is a continuation of the P94 series of studies (P94 in 2006, P94.1 in 2008, P94.2 in 2012) which aims to support SI-traceable measurement of DNA methylation levels in a sample which resembles a liquid biopsy. The study has supported development of competencies in analyzing DNA methylation levels and in producing relevant certified methylation reference materials. The measurand will be the methylated DNA copy number ratio [mC/(mC+C)] of target sequence. Nucleic Acid methylation occurs with the addition of a methyl groups to some nucleotides in the Nucleic Acid sequences. This addition is an example of an epigenetic modification that are used as markers for disease progression necessitating reference measurement system to support for the quantitative analysis. P94.3 underscores the importance of validated methods to support low-level DNA methylation analysis and promotes the development of SI-traceable measurement systems for epigenetic biomarkers. Such advancements are particularly relevant in support of evaluation of the performance of diagnostic tests. The final draft of CCQM P94.3 is currently being circulated and the final report is anticipated soon.

Bacterial identification and quantification

Bacterial plant pathogens are commercially relevant, as they can have devastating socioeconomic consequences and also negatively influence biodiversity. Most jurisdictions have import restrictions to prevent the spread of plant pathogens, but there is a lack of reference materials and reference methods for bacterial quarantine plant pathogens. Additionally, another type of bacteria, mycoplasma, represent common cell culture contaminants which can have a negative impact on biomedical research, biotechnology and biopharmaceutical production as well as being pathogens too. Additional important bacterial targets including human, foodborne and zoonotic pathogens may also be investigated as part of this study series. NAWG participants will measure genomic copy number concentrations of bacteria. As this study builds on the previous DNA copy number quantification studies P154 [19], P184 [24], and the K86 series, it will expand NAWG measurement capabilities to bacterial gene quantification. These activities will likely include cross working group comparisons with the CAWG, in which quantification of whole bacteria cells are included and could be further complemented through techniques such as PCR for viable cell count.

NAWG studies for therapeutic production and characterization.

P244 explores measurements associated with Lipid Nano Particle (LNP) mRNA therapeutics. The first LNP drug was approved in the US in 2018 (Onpattro). In 2020, Covid vaccines using mRNA LNP technology were given emergency use approval. Pharmaceutical companies measure various attributes for LNPs such as size, polydispersity, RNA encapsulation, and RNA content yet these measurements are in their infancy and could benefit from the development of reference measurement procedures and reference materials/standards. P244 is led by NIST, with support from NML, and will explore measurements of mRNA lipid nanoparticles and also includes SAWG and IAWG participation. Materials are currently being distributed for P244 which will allow NAWG members to explore and develop their capacity to assist the growing area of nucleic acid therapeutics including RNA number concentration and LNP encapsulation efficiency measurements. It is anticipated P244 result will be reported in September 2025

K199 Nucleic acid traceability to the amount of substance.

The analytical techniques supported by the NAWG include the use of molecular methods commonly applied by end user stakeholders such as qPCR, dPCR and sequencing where the quantitative results of which are typically reported using copy number concentration even when the final results are expressed as a ratio or other unit such as mass. To underpin the accuracy of these methods the NAWG has used methods that have established routes for SI traceability such as isotope dilution mass spectrometry (IDMS) which provides an orthogonal route traceable to the amount of substance [5, 19, 20, 22]. This has been developed within the CCQM over the last 2 decades by working groups including the OAWG. The methods typically measure the monomer content after complete hydrolysis of pure nucleic acid preparation of known sequence. While this has been used to provide alternative methods for several NAWG key comparison and pilots studies there remains no mechanism for NAWG members to support CMCs when using chemical methods traceable to the amount of substance to measure DNA or RNA which represent polymers of very large molecular weight. Digestion of the nucleic acid polymers, sources of impurity and quantitative conversion efficiency have been assumed by the field and there remain biases between methods like IDMS and dPCR which would benefit from further evaluation. K199 will initiate series of key comparisons which will investigate chemical analysis of DNA and RNA to address impact of sequence, sequence sizes, modifications, secondary structure, etc.

Environmental DNA

The analysis of environmental DNA (eDNA) represents an emerging and rapidly advancing approach for monitoring biodiversity, based on the detection and quantification of genetic material—including whole cells, extracellular DNA, and, in some cases, entire organisms—shed into the environment. Originally applied to detect elusive or endangered species, eDNA has evolved into a powerful, non-invasive tool for comprehensive ecosystem assessment. Analysis of eDNA may also be important in industry to explore the impact of microbial-communities assisting in corrosion, affecting solar panel efficiency and other aspects of technosphere-environment interactions. eDNA is detectable across a broad range of matrices, including freshwater, marine environments, snow and ice, soil, sediments, air, and targeted samples such as fecal material, biofilms, and environmental surfaces. To ensure the reliability, comparability, and regulatory acceptance of eDNA-based measurements, robust metrological support is urgently needed. This will require coordinated efforts across several CCQM working groups, including NAWG, CAWG, SAWG, and others, to develop validated reference methods, reference materials, and interlaboratory comparisons. NAWG identifies water as the most suitable initial matrix for establishing comparability and traceability through international comparisons.

Additional topics for potential activities in the near future include bacterial genome quantification, liquid biopsy analysis as well as the increasingly relevant topic of environmental DNA analysis; many of these topics offer co activities with other working groups.

Future challenges, longer term plans (Beyond 2030):

The nucleic acid analysis field is fast moving in terms of technological development and its application. The last decade has seen a transformation in massively parallel sequencing and PCR based nucleic acid quantification, with an increasing application of these approaches in applied settings. Longer term NAWG priorities will need to reflect this dynamic analytical space - although the similarity in the associated analytical challenges between sectors will enable the NMI community to be well positioned to respond to this. It is likely that the NAWG will need to expand its understanding of matrix effects on analysis, moving from the leading work in the foods space (K86 series) to more complex genomic measurements (such as sequencing) as well as other biological matrices (such as clinical and environmental samples).

To ensure the NMI community is ready for the next priority, pilot studies that allow NAWG members to develop capacity in new areas will be important to support its stakeholders. This may be the case whether there is an increase in the use of sequencing in foods and feed analysis, involved in applications as authentication, adulteration, labeling , identity, provenance or food safety, epigenetic IVD solutions, the need to identify CRISPR modifications, etc. A wider application of CRISPR, and other gene editing technologies, may require NMIs and DIs to develop analytical tools from capabilities that enable reading the genome to understanding how to write it, to identify both products or organisms as a result of engineering biology or synthetic biology and unintended modifications

Four broad areas predicted to form the subject of key comparisons and/or pilot studies (Figure 6) over the next ten years when considering nucleic acid analysis spanning food, health and environmental testing:

1) SI traceability, including matrix materials

The last decade has seen the NMI community demonstrate SI traceable DNA measurement is possible using chemical analysis of pure materials in high concentration or, when using digital PCR, more complex mixed samples capable at very low concentrations using enumeration. The NAWG will continue to explore this space with key comparisons to broaden the scope of measurement, both in terms of sequence type and complexity as well as for RNA. Reference measurement systems that include methods, as well materials, to support traceability may become increasingly important to support matrix materials, or direct clinical measurements, as more nucleic acid targets are measured. How reference measurement systems can support precise and multiple quantitative nucleic acid measurements will need to be explored for this to become a reality.

2) Nucleic Acid Characterization

Nucleic acid reference materials can differ in their composition and homogeneity in terms of sequence, fragment size and, where appropriate, matrix. This can impact on how a material may perform and characterization of these will metrics enable a better understanding of a given measurement. NAWG will explore the measurement of these characteristics using different sizing and sequencing methods to develop and compare capacity amongst NAWG members.

3) Broad scope measurement capabilities

An important aspect of the NAWG activities will be to ensure comparisons enable capabilities with broad application. While the ubiquity of essentially the same nucleic acids across life sciences lend themselves to broader scope claims, the NAWG will need to demonstrate they are appropriate. Examples might be how capabilities demonstrated in the K86d participation, which will deal with high protein food matrices, might be applied to a clinical biopsy or how many different viral pathogen genomes will need to be evaluated to claim broader capabilities in viral measurement.

4) Non-targeted NA analysis

With the development of increasingly advanced sequencing technologies a new paradigm in nucleic analysis has emerged. Instead of applied analytical approaches measuring a specific target, or targets, nucleic acid analysis is increasingly capitalizing on the ability of methods to measure what is present in an untargeted approach; such as when exploring the presence of a potential undefined change that may confer resistance to a cancer therapy or antimicrobial or unique genetic modification. As these approaches become increasingly applied it is likely the NMI community will need to consider supporting their accuracy and the NAWG will commit time to considering this challenge as needed.

5.2. IMPROVING STAKEHOLDER INVOLVEMENT

The main stakeholders of NAWG are:

- NMIs and DIs
- The food and feed industry and regulators
- Clinical medicine sector
- Industry: IVD industry, biopharmaceutical manufacturers, pharmaceutical/advanced therapy medicinal products (including gene therapy), instrument manufacturers, veterinary diagnostic manufacturers, sanitarian laboratories
- Academia: universities, regional and international (EMBL) research institutes and
- International Organizations: ISO, IFCC, JCTLM, WHO, SOGAT, ESCMID

- Public stakeholders: regulators, governmental laboratories, reference laboratories, inspection agency for IVD and biopharmaceutical registration, third-party testing agency

Due to the fact that the application of metrological concepts is relatively new to nucleic acid analysis, the NAWG is not as well established amongst stakeholders as other CCQM working groups or those from other CCs. However, NAWG members have made considerable efforts to disseminate NAWG's work, to transfer knowledge of NMI/DI activities, and to solicit feedback, both independently, regionally and as part of NAWG.

Webinars

NAWG members frequently give webinars to disseminate NMI activities. With molecular methods offering the main testing method for SARS-CoV-2 infection the NAWG is a contributor to the CCQM webinar series on 'Ensuring the Reliability of Measurements in Response to the Covid-19 Pandemic.'

As well as the usual routes of scientific interaction (such as conference presentations, peer reviewed publications, etc.) NAWG members are also active participants in numerous committees and working groups involved in standardizing and applying nucleic acid analysis. Examples include:

The leader of the Nucleic Acid Review Team of the Joint Committee for Traceability in Laboratory Medicine (JCTLM) is also a member of the NAWG. The JCTLM maintains a database of higher metrological order reference methods, materials and services. The cited nucleic acid reference measurement procedures have all built on regional and international work from NAWG members some of which benefited from NAWG led activities. With the successes of NMIs and DIs over the last ten years in developing capacity/knowledge and the resultant outputs it is likely that activities will continue to feed into the JCTLM. JCTLM Working Group on Traceability: Education and Promotion (JCTLM-TEPWG) considers nucleic acids measurements to be a rare area in laboratory medicine, where routes to explore how SI units can be better applied to afford standardization of clinical tests results, NAWG representative participate in JCTLM-TEPWG activity.

NAWG members are active participants in several ISO working groups including TC/276 Biotechnology and ISO TC/212 Clinical diagnostic testing and in vitro diagnostic test systems. Through these activities NAWG members have been instrumental in ensuring nucleic acid metrology is considered in several recent documents including ISO 20395:2019 (Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR) and ISO 17511:2020 (In vitro diagnostic medical devices — Requirements for establishing metrological traceability of values assigned to calibrators, trueness control materials and human samples). NAWG members are also part of the drafting committee of the 276/212 joint working group 6 "Quality Practice for detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) by nucleic acid amplification methods."

NAWG members participate in SoGAT (The international working group on Standardization of Genomic Amplification Techniques) which supports the development and implementation of WHO reference materials. NAWG members are also increasing activities to drive knowledge transfer around nucleic acid analysis metrology with additional stakeholder committees/group such as IFCC (Committee for molecular diagnosis) and the European Society for Clinical Microbiology and Infectious Diseases (Study Group on Genomic and Molecular Diagnostics).

5.3. PROMOTING GLOBAL COMPARABILITY

The strategic approach for selecting the NAWG comparisons continues to evolve due to the necessity of accommodating the full range and complexity of nucleic acid measurement challenges encountered by CCQM NMI/DIs in delivering services to their customers. The NAWG priorities in the latest member survey focus on medical diagnostic tests and food and feed analysis, followed by biotechnology, environmental analysis and gene doping.

NMI and DIs provide services, such as:

- CRM production and value assignment (ranging from nucleic acid variant and mutation detection, pathogen quantification to microbial metagenome sequencing)
- Digital PCR calibration services for proficiency testing, nucleic acid reference materials and external quality assessment (EQA) material value assignment
- GMO detection, identification and quantification

The NAWG provides support to member NMIs and DIs in performing these services.

The activities of the NAWG have followed on from those initiated in the Bioanalysis WG (BAWG). Within NAWG, two Track A and seven Track D (pilot) studies were organized (Table 1 and 2). All studies were Model 1.

Table 1. Description of the types of studies that the NAWG performs.

	Objectives	**NAWG examples**	**Misc.**
Track A **(Key Comparison)**	To test the core competencies for measurement services delivered to customers, and cover the range of recognized measurement capabilities required to deliver reference measurement services in nucleic acid analysis	Fractional abundance of sequences associated with genetic modification	
Track C **(Key Comparison)**	To assess emerging areas of global interest and importance	None conducted in NAWG	

Track D **(Pilot Study)**	To assess capability for new techniques and areas	targeted gene expression, absolute quantification of DNA/RNA	Number of P studies relatively is high in NAWG, these studies are used to support CMCs

The NAWG Track A comparisons test the core competencies for measurement services delivered to customers and cover the range of recognized measurement capabilities which are required to deliver reference measurement services in nucleic acid analysis. Under a Track A comparison, successful demonstration of a competency in the comparison (e.g., relative quantification of DNA/RNA fragments, fractional abundance of genetic variants) is considered to be broadly applicable to the measurement of a range of analytes in a variety of matrices, as described in the "How Far The Light Shines" (HFTLS) associated with the comparison, provided the same general measurement approach is used.

Table 2. NAWG Key Comparisons and Pilot Studies

	Description	Coordinating Laboratory	Start Date	Status (as of 03/2021)	Comments
CCQM-K86.b	Relative quantification of Bt63 in GM rice matrix sample	NIMC/GLHK	2015	Study complete. Report Draft A discussed & KCRV agreed October 2016. Draft B report agreed April 2017 submitted to KCDB following minor amendments requested on CCQM WG Chair review. Available on KCDB. Will be published in Metrologia.	Run in parallel to CCQM-P113.3
CCQM-K86.c	Relative quantification of genomic DNA fragments extracted from high oil matrix (OSR/canola)	NRC/IRMM	2016	Study complete. Preliminary results discussed April 2017.KCRV determined & Draft B report agreed. Approval for CMC review April 2018. Final report minor amendments in progress - prior to submission to KCDB.	Run in parallel to CCQM-P113.4
CCQM-	Relative	NIMC/GLHK	2015	Study complete	Run in parallel with

P113.3	quantification of Bt63 in GM rice matrix sample				CCQM-K86.b
CCQM-P113.4	Relative quantification of genomic DNA fragments extracted from high oil matrix (OSR/canola)	NRC/IRMM	2016	Study complete	Run in parallel with CCQM-K86.c
CCQM-P154	Absolute quantification of DNA	KRISS (NMIA, IRMM, NML at LGC)	2013	Study complete. Report submitted to KCDB.	Published Anal Chem [19].
CCQM-P155	Multiple cancer cell biomarker measurement	NML at LGC	2014	Study complete. Final report agreed and published on CCQM NAWG website. Scientific publication in preparation.	Reporting delays due to CIM participation issues discussed and resolved April 2017. Final report approved Oct 2017 (WIKI available).
CCQM-P184	Copy number concentration and fractional abundance of a mutation (SNV or INDEL) mixed with WT DNA	NMIA/NML at LGC	2017	Study complete. Core competence in nucleic acid mutation measurement.	Published report as pre-print [24]
CCQM-P199	Copy number concentration of HIV-1 RNA genomic sequences	NML at LGC/(NIBSC)	2018	Draft report compiled due for circulation	Published report as pre-print [22].
CCQM-P199b	Copy number concentration of SARS-CoV-2 RNA genomic sequences	NML at LGC/NIMC/NIBSC/NIST	2020	Draft report circulated amongst NAWG	Published report as pre-print [20].

CCQM K176/ P218	Breast cancer biomarker HER2 copy number variation (CNV) measurement	NIMC/ NML at LGC	2021	Published	Completed [23]
CCQM K181/ P227	SARS-CoV-2 RNA copy number quantification	NIMC/ NML at LGC/NIST/ NIBSC	2022	Published	Completed [5]
CCQM-NAWG P232	FireDrill Influenza RNA copy number quantification	NML at LGC	2022	Second draft shared on April 2025	
CCQM P94.3	Quantitative analysis of DNA methylation of a defined human genomic DNA region	UME / KRISS	2022	Draft A in process	
CCQM P231	The species specific meat composition determinatio n of DNA extracted from meat	UME/NIMT	2024	First draft shared on April 2025	
P244	Lipid Nanoparticles with Encapsulated RNA	NIST/LGC	2025	Draft A in process	
K190/ P249	SARS-CoV-2 Whole Virus Key comparison	LGC, MHRA and NIST	2025	In progress	

	and Pilot Study				
P250	SARS-CoV-2 Whole Virus Standalone Pilot Study	LGC, MHRA and NIST	2025	In progress	
K198/ P255	Cancer variant measurement in cell-free DNA	KRISS, NIM, NML@LGC	2025	Protocol being drafted	
K199/ P256	Nucleic acid traceability to the amount of substance	NMIA	2025	Protocol being drafted	

The NAWG's strategic plan for future Key comparisons and pilot studies is based on types of analyses provided in services by NMIs/DIs and more specifically informed by a participant survey to prioritize NA measurement study areas required to support services. The NAWG strategy prioritizes:

- Quantification of nucleic acids, including DNA and RNA. Specific measurands include specific sequence absolute copy number concentrations and fraction abundances, within and between different samples. Applications include GMO determination, gene expression and quantification of genes: including single nucleotide variants.
- Nucleic acid sequencing (massively paralleled generation sequencing and Sanger sequencing). There is a need for standardization in this area. Sequencing is increasingly used for identity "measurements", metagenomics, genome editing identification and characterization and sequence purity analysis, including purity determination for reference materials.
- Identification and quantification of epigenetic differences, particularly methylation.

In the short to medium term it is expected that these services will be extended, with an emerging trend towards the use of nucleic acid sequencing.

5.4. INTERACTION WITH RMO ACTIVITIES

APMP: The bioanalysis area is part of the APMP-TCQM in which there have not been any studies that are directly relevant to NAWG activities. The most relevant APMP study is APMP QM-P35 Quantification of *E. coli* in drinking water (in progress). In addition, quantification of other microorganisms in food has been considered and proposed to the APMP members. However, the methodology considered in these studies are culture-based, which is not a nucleic acid measurement. At the 40th APMP General Assembly, the

biometrology sub-committee is newly organized under TCQM along with organic, inorganic, and gas sub-committees. This biometrology sub-committee is expected to initiate focused discussions and perhaps organize comparisons within APMP.

COOMET: Since 2019 COOMET Technical Committee 1.8 “Physic-Chemistry'' includes the Bioanalysis subcommittee 1.8.6 acting in the field of nucleic acid analysis activity. In the meeting of TC 1.8 in October 2024 key areas of bioanalytical activities were discussed by participating NMIs and DIs, including development of new reference materials certified by DNA copy number concentration/copy number ratio. In 2024 the Primary Standard of DNA sequence copy number unit GET 220-2024 passed the state trials and was approved in Russian Federation. A set of certified reference materials for meat identity control was approved in the end of 2024. A partnership with agricultural sanitation laboratories was established focused on the development of metrological support for quarantine bacteria diagnostics. The attestation of secondary standards has been started to expand the areas of application of nucleic acid analysis - including organizations - manufacturers of medical devices for in vitro diagnostics, veterinary and sanitary control laboratories. NAWG members chairs the SC 1.8.6 and coordinates COOMET activity in the nucleic acid analysis field.

EURAMET:
Nucleic acid analysis is part of Bio and Organic Analysis Subcommittee under the Technical Committee of Metrology in Chemistry (TC-MC), a Joint EURAMET-Eurachem Technical Committee. While there were no EURAMET comparisons in this field yet, European NMIs and DIs work closely together and with stakeholders through projects of European Partnership for Metrology (EPM). Notable projects with relevance to nucleic acid analysis from this and previous programs include Infect-Met, AntiMicroResist, BioSITrace, SEPTIMET, GenomeMET and COMET. Furthermore, European Metrology Networks (EMNs) were established to formulate common metrology strategies including aspects such as research, infrastructure, knowledge transfer and services in different fields. NAWG members are involved in three EMN’s: EMN for Traceability in Laboratory Medicine, EMN for Safe and Sustainable Food and EMN for Pollution Monitoring.

SIM:

The Chemical Metrology Working Group (MWG 8) of SIM includes metrology in biological measurements within its scope. The aim is to provide tools that guarantee the traceability and reliability of measurement results in various areas, including bioscience and biotechnology. Regarding nucleic acids, the SIM.QM.Pilot study, which was coordinated by CENAM in 2021, aimed to quantify the DNA copy number of SARS-CoV-2 using digital PCR. This was the first attempt to develop this type of study in the region.

Several NMI and DI at the SIM have developed RM and interlaboratory studies for nucleic acids: NIST-USA for viruses, cancer biomarkers, sequencing standards and microbial pathogens, CENAM-Mexico for GMOs in crops such as maize, soybean and cotton, INM-Colombia for proficiency testing for respiratory viruses and soil crop pathogens, INMETRO-Brazil with nucleic acid measurement services for quality control of cell therapy products. Based on the 'CCQM roadmap for metrology readiness for infectious disease pandemic response' and the results of the nucleic acids fire drill study (CCQM P232), several initiatives to prepare for a possible outbreak are being developed, including a research test-grade material for influenza H5N1 to be used as QC in new diagnostic tests by NIST, and a roadmap to strengthen the quality

infrastructure for producing molecular biology supplies, particularly nucleic acids, and to ensure the quality of clinical laboratories in Colombia.

- ANNEX

1. GENERAL INFORMATION

General information and terms of reference in format used for 2017-2026 strategy
(Headings from previous template)

CC Name: Consultative Committee for Amount of Substance: Metrology in Chemistry and Biology (CCQM)
CC Working Group: Nucleic Acid Analysis Working group (NAWG)
Date Established: October 2015
Number of Members: 25 NMIs and DIs
Number of Participants at last meeting: 53
Periodicity between Meetings: 6 months
Date of last meeting: April 2025
CC WG Chair (Name, Institute, and years in post): Jim Huggett, National Measurement Laboratory, LGC UK, 6.5 years

Number of Nucleic Acid (NA) measurement KCs organized (from 1999 up to and including 2025): 10 (8 NAWG, 2 BAWG)
Number of Pilot studies organized (from 1999 up to and including 2025): 30 (17 NAWG, 13 BAWG)
Number of CMCs published in KCDB supported by CC body activities (up to and including 2025): 21 NIM (8), GLHK (4), CENAM (1), VNIIM (5), NIB (4), TUBITAK UME (4), NML at LGC (7), NIMT (2).

The agreed Terms of Reference (TOR) for the NAWG are:

To carry out Key Comparisons and pilot studies, to critically evaluate and benchmark NMI/DI claimed competences for measurement standards and capabilities for nucleic acid (NA) analysis. This includes, but is not limited to, the analysis of chromosomes, DNA, nucleotides, oligonucleotides, modified DNA (e.g. DNA methylation and other epigenetic modifications), mRNA, miRNA (and other non-coding RNAs) in a biological measurement context. The measurements in the studies include, but are not limited to, the identification and quantification of nucleic acids in complex matrices (such as those derived from plant, animal and microbial origins).

2. Case studies

I. Absolute SI Quantification of DNA

In plants, animals, bacteria and some viruses, DNA carries most of the basic inherited information that shapes the organism. Since the discovery of chemical and physical characteristics of DNA, the precise and accurate quantification of a specific DNA sequence has been intensely sought. Since the early 2000s, the CCQM NAWG (and BAWG prior to 2015) has worked to develop the SI-traceable methodologies for absolute DNA sequence quantification.

The CCQM P154 study demonstrated NAWG members' competency in calibration-free enumeration-based DNA quantification methods. The study was co-led by KRISS, NMIA, IRMM, and NML at LGC. There were 11 participating NMIs and DIs. Unlike previous studies which used relative quantification using quantitative polymerase chain reaction (qPCR), participating laboratories utilized enumeration-based quantification in order to achieve direct absolute quantification of DNA. This enables reliable value assignments of DNA reference materials, traceability of copy number counting quantity to International System of Units (SI) neutral element - unit one.

In this study, two enumeration-based methods, direct flow cytometric (FC) counting and digital polymerase chain reaction (dPCR), were used to quantify the pBR322 plasmid copy number concentration in a solution at a value of several thousand copies per microliter. Unlike DNA measurement methods based on chemical analysis such as mass spectrometry or capillary electrophoresis, direct flow cytometric (FC) counting offers simplicity once the system is set up and optimized. The counting of DNA molecules in a flow stream is independent of extra calibration or added internal standards. Double stranded DNA molecules are stained with a fluorescent intercalating dye and then counted by laser-induced fluorescence detection in capillary flow in a self-instrumented system [25]. Key achievements in this technology include electrohydrodynamic focusing of DNA molecules at the centre of the flow cross-section, optical adjustment for improving signal to noise ratio, self-controlled optical alignment, and exhaustive counting. This strategy applied with precise determination of sample volume allows calibration-free quantification of DNA copy number concentration. Using this optimized enumeration instrument, copy number concentration of target DNA molecules can be readily determined without any biochemical amplification steps.

Laboratories participating in the P154 study used a variety of methods [19] including FC-based direct counting, digital PCR, and two chemical-analysis methods which are based on nucleotide quantification: isotope-dilution mass spectrometry (IDMS) and capillary electrophoresis (CE). Nine dPCR results from eight laboratories showed some dispersion where relative standard deviation (RSD) was 11.8%. However the means from digital PCR results were comparable with those of the FC counting method and the chemical analysis methods, corrected for gravimetric dilution factors. When the average value of dPCR results was compared to the other methods, the RSD of all four methods was 1.8%. This strongly suggested these enumeration methods are valid in absolute quantification of DNA.

II. Towards SI traceable RNA measurement

Ribonucleic acid (RNA) is another analyte the NAWG is tasked with measuring. RNA encodes the genomic sequence for many viruses, is the intermediary molecule used to translate the genetic code into protein sequences, and also provides a range of other structural (ribosomal RNA), regulatory (micro RNAs) and even enzymatic functions (such as splicing and rybozymes). As with DNA, RNA molecules are comprised of a variation of the order of four monomers, allowing similar methods to be applied to different types of molecule. Challenges specifically associated with RNA include the fact that it is usually single stranded and can form secondary structures through complementary binding or where molecules like micro RNAs are very small making them difficult to measure using conventional molecular methods. RNA is also difficult to measure due to the fact that most of the methods used in molecular biology measure DNA. Consequently reverse transcription is required to convert RNA into complementary DNA (cDNA).

Over the last decade, the NAWG (and BAWG beforehand) has dedicated considerable effort to ensure the development of RNA measurement capability was also at the forefront of the strategic focus. CCQM P103 (Quantification of an RNA transcript) was the first pilot study to explore the measurement of RNA; it investigated the measurement of an RNA molecule (from the External RNA Controls Consortium [ERCC]). Two unknown concentrations of this RNA molecule were provided to laboratories, along with a calibrator to allow laboratories to perform reverse transcription quantitative PCR. The calibrator was value assigned using UV spectrophotometry with factors such as potential non-target fragments considered in the uncertainty. 11 NMIs/DIs participated in the pilot with majority using RT-qPCR and one applying RT-dPCR (corrected using the calibrator value). The pilot study concluded that most laboratories were able to accurately measure both concentrations of the RNA molecule in good agreement with the assigned value.

CCQM P103.1 (Measurement of multiple RNA transcripts) pilot study built upon the knowledge generated in P103 by including six ERCCs and three cellular RNA measurements using human RNA derived from cell lines [26]. The use of multiple ERCCs demonstrated that absolute RNA measurement could be widely applied and the inclusion of different concentrations of ERCCs and cellular RNA allowed for ratio-based measurements in the range typically measured by stakeholders. This study saw an increased number of participant laboratories to 14, the majority of which conducted RT-qPCR, although two laboratories submitted RT-dPCR results and one laboratory conducted massively parallel sequencing. The P103.1 study demonstrated that participating laboratories were able to measure target RNA copy number concentration and fractional abundance (ratios) in a complex (total RNA) background with good concordance. Additionally, the study highlighted the lack of a standardized approach for uncertainty calculations, especially when conducting fractional abundance quantification.

CCQM P155 (Multiple cancer cell biomarker measurement) expanded the earlier RNA pilot studies by incorporating whole cell materials for analysis. This necessitated a pre-processing step to purify the nucleic acid prior to molecular analysis and enabled participant laboratories to develop and compare their extraction capabilities. This also allowed the evaluation of the potential contribution of these initial steps to be considered when characterizing uncertainty both in terms of copy number concentration measurements as well as when measuring. CCQM-P155 demonstrated that participant laboratories were able to conduct measurements with good agreement when conducting both fractional abundance and absolute measurements, extending the findings of P103.1 to consider whole cell extracts.

The three preceding RNA pilot studies dealt with both sequence copy number fractional abundance and absolute measurements and saw a serial increase with time in the adoption and application of digital PCR technologies for RNA quantification. The measurement of RNA copy number concentration was supported with value assignment using spectrophotometry for the CCQM-P103 series and by RT dPCR for CCQM-P155. Neither approach, at the time, had been demonstrated to be suitable as an SI traceable method. Consequently an unmet need was identified to explore the potential for using higher accuracy SI traceable methods for RNA quantification. Following the success of CCQM P154, for the assessment of SI traceable measurement for DNA [19], the CCQM-P199 [22] (HIV-1 RNA copy number quantification) pilot study was proposed followed by CCQM-P199b [20] (SARS-CoV-2 copy number quantification) which was fast tracked in response to the COVID-19 pandemic. Both P199 studies demonstrated that NAWG members were capable of high accuracy measurement of RNA molecules in buffered solutions. The P199.b demonstrated SARS-CoV-2 quantification was possible with most laboratories submitting values with +/- 40% of the mean [27]. The five RNA studies open the possibility for the first CCQM key comparison to consider RNA analysis K181 which, like P199b copy number concentration measurement of SARS-CoV-2 RNA sequences

to support CMCs that allow NMIs to support stakeholders in viral RNA analysis [5]. K181 has been followed by K189 which expands on this capability to purification of viral RNA from whole virus.

III. Case study III: DNA measurements in Food

The NAWG (and the BAWG previously) has developed a series of studies to promote the development of technical capacities for measuring DNA in food matrices, to support members in nucleic acid RM development and calibration services. Plant (Genetic modified Crops - GM) and animal materials have been the selected matrices, covering high carbohydrate, high fat and high protein content.

Genetic Modified (GM) crops contributes to global feed, fiber, food, and fuel production; however, the safety of GM crops and their products are important issues. National legislation regulating GM crop cultivation and its products varies significantly in different jurisdictions. A global reference measurement system, including reference materials and reference methods, must be established to facilitate international trade and to provide information to consumers on GM ingredients content. In the other hand, for meat and meat products, it is also crucial to determine meat content for legislation purposes, ethical reasons, health concerns and religious matters, among others.

The measurement process includes DNA extraction from a complex biological matrix followed by measurement of two specific genomic DNA fragments by quantitative real-time PCR (qPCR) with an independent reference material as a calibrant or by digital PCR (dPCR), which is considered a direct SI-traceable or reference method for nucleic acid measurements.

In this sense, a series of key comparisons were performed to demonstrate and document the capacity of NMIs/DIs to determine the relative quantity of two specific genomic DNA fragments present in maize seed powder [2] (CCQM K86, 13 NMI/DI participants), rice seed powder [3] (K86.b, 9 NMI/DI participants), canola powder [4] (CCQM K86.c 12 NMI/DI participants) and pork and beef meat (K86.d, 12 NMI/DI participants) for which the draft B is soon to be completed.

Study participants had to perform extraction of genomic DNA followed by accurate detection and quantification of the relative amount of two defined DNA sequences in the extracted genomic DNA. The matrix in the previous CCQM K86 was a maize seed powder, whereas in CCQM K86.b, the matrix was a rice seed powder. Both these matrices are rich (between 75 and 80 g/100g) in polymeric carbohydrate (amylose and amylopectin), and poor in fat (<5 g/100g) requiring adapted DNA extraction methods to remove substances that can hinder the polymerase activity. In the CCQM K86.c study, the NAWG decided to challenge laboratories with a plant material with elevated oil/fat content, such as rapeseed, where study participants used a wide range of DNA extraction approaches, including phenol-chloroform extraction, CTAB extraction, and adsorption by silica and isolation with magnetic beads.

As the measurement space of the food matrix lacked measurements in high protein content matrix, CCQM-K86.d/P113.5 used a pork matrix in a beef matrix as a protein rich matrix model for genomic DNA quantification, focusing on samples with degraded genomic DNA to investigate the DNA extraction process for comparability of measurements for processed food samples. Two types of samples, protein rich matrix and DNA solution were used. In addition, in this comparison, both DNA copy number ratios and mass fractions were measured, setting the basis for delivering measurement services on mass fraction (obligatory in some jurisdictions) using a conversion factor.

Continuing with high protein matrices, the CCQM P231 study investigates the quantitative determination of species-specific meat composition using DNA analysis. The study involved the analysis of two types of materials: DNA from a single species of meat, or a mixture of DNA from more than one species of meat out of 6 different species which may be pork, beef, sheep, chicken, goat or horse. The DNA analysis was performed on total extracted DNA, including nuclear DNA, where each participant was required to sequence for the PCR assays to use.

To determine future applications, on september 2024, from the CCQM Task Group on food measurement (CCQM-TG-FOOD), a questionaire was sent to NAWG members; it covered topics related to CMC for supporting associated services, stakeholders, application areas, sequences and matrices of interest in order to identify gaps in measurement needs or unmet stakeholders engagement opportunities in the food and feed sector. Reference materials, proficiency test and reference measurements were the prinicpal services where organizations hold or plan to hold a CMC for next 5 years, in areas such as GMO, food safety, quality, adulteration / authentication, and with the advance of editing techniques, precision-bred organism. Current or potential stakeholders, include industry - IVD, official / control laboratories testing laboratories, PT providers, other NMI/DIs, academy and international organisations. With regard to sequences and matrices of interest, those associated with GMO biomarkers and specific sequences for identification and quantification were predominant followed by those associated with barcodes, InDels and in a lesser extent, Short Tandem Repeats (STR) and Single Nucleotide Variants (SNV); in matrices such as buffer solutions, plant and low- and highly-processed animal materials, plant- and animal-derived materials, sea food, and water, measured in absolute copies or relative abundance. The detection of editing sequences by molecular methods was a topic of interest for future interlaboratory studies and workshops or training activities.

Finally, a virtual Stakeholder Workshop on evolving needs in metrology for food and food safety, organized by the CCQM-TG-FOOD on February 2025, topics as the challenges with proper controls for sequencing techniques, reference databases for nucleic acids sequences as well as nucleic acid testing (NAT) for viable pathogen detection were highlighted as potential needs for the previously mentioned applications areas of authenticity, adulteration, labelling, identity and food safety for nucleic acid stakeholders.

3. REFERENCES

1. Milavec, M., et al., *Metrological framework to support accurate, reliable, and reproducible nucleic acid measurements.* Anal Bioanal Chem, 2022. **414**(2): p. 791–806.
2. Corbisier, P., et al., *CCQM-K86/P113.1: Relative quantification of genomic DNA fragments extracted from a biological tissue.* Metrologia, 2012. **49**(1A): p. 08002–08002.
3. Dong, L., et al., *Final report for CCQM-K86.b relative quantification of Bt63 in GM rice matrix sample.* Metrologia, 2018. **55**(1A): p. 08017–08017.
4. Mester, Z., et al., *Final report of CCQM-K86.c. Relative quantification of genomic DNA fragments extracted from a biological tissue.* Metrologia, 2020. **57**(1A): p. 08004–08004.
5. Dong, L., et al., *CCQM-K181 SARS-CoV-2 RNA copy number quantification.* Metrologia, 2025. **62**(1A): p. 08016.
6. Bhat, S., et al., *Comparison of methods for accurate quantification of DNA mass concentration with traceability to the international system of units.* Anal Chem, 2010. **82**(17): p. 7185–92.

7. Bhat, S., et al., *Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number.* Anal Bioanal Chem, 2009. **394**(2): p. 457–67.
8. Burns, M.J., et al., *Standardisation of data from real-time quantitative PCR methods - evaluation of outliers and comparison of calibration curves.* BMC Biotechnol, 2005. **5**: p. 31.
9. Corbisier, P., et al., *Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction.* Anal Bioanal Chem, 2010. **396**(6): p. 2143–50.
10. Dong, L., et al., *Comparison of four digital PCR platforms for accurate quantification of DNA copy number of a certified plasmid DNA reference material.* Sci Rep, 2015. **5**: p. 13174.
11. Haynes, R.J., et al., *Standard reference material 2366 for measurement of human cytomegalovirus DNA.* J Mol Diagn, 2013. **15**(2): p. 177–85.
12. Holden, M.J., et al., *Traceable Phosphorus Measurements by ICP-OES and HPLC for the Quantitation of DNA.* Analytical Chemistry, 2007. **79**(4): p. 1536–1541.
13. Pavsic, J., et al., *Inter-laboratory assessment of different digital PCR platforms for quantification of human cytomegalovirus DNA.* Anal Bioanal Chem, 2017. **409**(10): p. 2601–2614.
14. Redshaw, N., et al., *Quantification of epigenetic biomarkers: an evaluation of established and emerging methods for DNA methylation analysis.* BMC Genomics, 2014. **15**: p. 1174.
15. Sanders, R., et al., *Evaluation of digital PCR for absolute DNA quantification.* Anal Chem, 2011. **83**(17): p. 6474–84.
16. Sanders, R., et al., *Evaluation of digital PCR for absolute RNA quantification.* PLoS One, 2013. **8**(9): p. e75296.
17. Whale, A.S., et al., *Assessment of Digital PCR as a Primary Reference Measurement Procedure to Support Advances in Precision Medicine.* Clin Chem, 2018. **64**(9): p. 1296–1307.
18. Yoo, H.B., et al., *A candidate reference method for quantification of low concentrations of plasmid DNA by exhaustive counting of single DNA molecules in a flow stream.* Metrologia, 2014. **51**(5): p. 491–502.
19. Yoo, H.B., et al., *International Comparison of Enumeration-Based Quantification of DNA Copy-Concentration Using Flow Cytometric Counting and Digital Polymerase Chain Reaction.* Anal Chem, 2016. **88**(24): p. 12169–12176.
20. Devonshire, A.S., et al., *CCQM-P199b: Interlaboratory comparability study of SARS-CoV-2 RNA copy number quantification.* bioRxiv, 2024: p. 2024.03.27.584106.
21. CCQM, *CCQM Roadmap to Metrology Readiness for Infectious Disease Pandemic Response (2022)* https://www.bipm.org/documents/20126/74750777/Roadmap-to-Metrology-Readiness_050822_Final.pdf/c324192c-b145-1797-b29c-055b1b9044c1
22. Devonshire, A.S., et al., *CCQM-P199: Interlaboratory comparability study of HIV-1 RNA copy number quantification.* bioRxiv, 2024: p. 2024.04.12.589043.
23. Dong, L., et al., *CCQM-K176 breast cancer biomarker HER2 copy number variation (CNV) measurement.* Metrologia, 2024. **61**(1A): p. 08017.
24. Burke, D., et al., *Standardisation of cell-free DNA measurements: An International Study on Comparability of Low Concentration DNA Measurements using cancer variants.* bioRxiv, 2023: p. 2023.09.06.554514.
25. Lim, H.-M., et al., *Count-based quantitation of trace level macro-DNA molecules.* Metrologia, 2009. **46**(3): p. 375–387.
26. Devonshire, A.S., et al., *An international comparability study on quantification of mRNA gene expression ratios: CCQM-P103.1.* Biomol Detect Quantif, 2016. **8**: p. 15–28.
27. BIPM, *National Measurement Institutes demonstrate high accuracy reference measurement system for SARS-CoV-2 testing*. 2020.

4. DOCUMENT REVISION SCHEDULE

Document name; type of revisions; date