Report from the CCQM Nucleic Analysis Working Group for the period (April 2025 – 2026)

During this period the 20th and 21st CCQM NAWG meetings took place in Paris (April) and Washington DC (September) respectively with 21 institutes represented. Once again, the hybrid format of the meeting allowed more NAWG member to attend with >50 participants present at both meetings. The 21st Meeting hosted by NIST at the Institute for Bioscience and Biotechnology Research (IBBR) included the fifth joint meeting of the biological metrology working groups held on the 9th September 2025. This format is planned to be repeated in November 2026 with WG meetings hosted by NIMT.

The NAWG published its first Key Comparison dedicated to supporting claims associated with infectious disease molecular diagnosis (K181) focusing on the measurement of gene sequence copy number concentration from SARS-CoV-2 the causative agent of the COVID-19 pandemic. In addition two pilot studies were also published one dedicated to supporting epigenetic sequence analysis (P94.3) and the other assessing a rapid reference measurement response to potential situations where molecular testing (and therefor reference systems) are is required at pace (such as infectious disease outbreaks (P232)). This was the first pandemic fire drill performed as proposed by the CCQM pandemic task group. The NAWG is currently running run a range of KCs and pilot studies to support NMIs with nucleic acid analysis metrology needs to address an increasing range of measurements associated with nucleic acid sequence analysis.

1. Completed Key Comparisons and Pilot Studies

a. CCQM K181/P227 (NAWG) SARS-CoV-2 RNA copy number quantification

CCQM K181/P227 was a follow up study of CCQM P199 and P199.b and focuses on virus RNA sequence copy number concentration measurement to support calibration and measurement claims when measuring viral load, or copy number concentration of RNA molecules. The sequence analyte was derived from SARS-CoV-2 (the pathogenic cause of COVID-19) genes. 17 NMIs successfully participated in CCQM-K181 to support CMC claims associated with determining RNA copy number concentration in the range from 10^1 μL^{-1} to 10^6 μL^{-1} SARS-CoV-2 target sequences in a non-target RNA matrix or as a single RNA template in aqueous solution.

The CCQM-K181 report can be accessed at DOI 10.1088/0026-1394/62/1A/08016

b. CCQM P94.3 (NAWG) Quantitative analysis of DNA methylation of a defined human genomic DNA region

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. This study underscores the importance of validated methods to support low-level DNA methylation analysis and promotes the development of SI-traceable reference measurement systems for epigenetic biomarkers. Such advancements are particularly relevant in support of evaluation of the performance of diagnostic tests. The NAWG would like to thank Sema Tiryaki and colleagues at Tubitak UME and Inchul Yang and colleagues at KRISS for leading study which reflects an increasingly important aspect of nucleic acid analysis and VNIIM for assisting with the statistical analysis.

The final draft of CCQM P94.3 can be found here.

c. CCQM P232: Fire Drill Influenza RNA copy number quantification

The CCQM 'roadmap to metrology readiness for infectious disease pandemic response' recommends a series of 'fire drill' exercises to explore and develop the capacity within the NMI/DI community to meet the need for fast development of reference measurement procedure in response to rapid diagnostic deployment. A variety of analytes (including antigens, antibodies, nucleic acids) will be

planned for the future fire drill activities as they were targeted in the diagnostic response to COVID-19 and are analyte candidates for future outbreaks. CCQM P232 is led by NML at LGC and NIST represents the first of the CCQM fire drills and targets Highly Pathogenic Avian Influenza HPAI (H5N1) RNA sequences; which are the target analyte used by molecular diagnostic methods like PCR. Two different purified RNA sequence synthetic materials (in vitro transcribed gene fragments) were shared with 15 participating laboratories who had to select their in-house assays based on the sequence alone. The NAWG would like to thank Denise O'Sullivan and colleagues at NML at LGC and Megan Cleveland and colleagues at NIST for leading study which will contribute to demonstrating the potential future role for NMIs in supporting pandemic diagnostic response.

The final draft of CCQM P223 report can be found here

2. Key comparisons and pilot studies at report drafting stage

a. CCQM-K189/P242 Measurement of Single Nucleotide Variation (SNV) in Cancer Biomarker PIK3CA

The aim of K189 is to measure number concentration and fractional abundance of mixtures of the sequences variants to provide support for CMC claims when measuring variants 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 analytes are more challenging to measure than those evaluated than previous cancer markers evaluated in K176 as they must be distinguished from closely related (somatic) variant that predominate within the specimen. K189 draft B is due for sharing soon and it is anticipate this will be finalised in the coming months.

The NAWG would like to thank Lianhua Dong and colleagues at NIM China for leading this study with support from Alison Devonshire and colleagues at NML and Young-Kyung Bae and colleagues at KRISS.

b. CCQM P231: The specific meat composition determination of DNA extracted from meat

The study rationale is to support 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 or 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). CCQM P231 results were presented at the 19th NAWG meeting and the pilot report draft has been circulated for comment.

The NAWG would like to thank Burhanettin Yalcinkaya, Müslüm Akgöz and colleagues at TUBITAK UME and colleagues at NIMT for leading this study.

c. *CCQM K86.d/P133.5 (NAWG) Quantification (and fractional abundance) of genomic DNA extracted from a protein matrix*

CCQM K86.d is led by NIMT and Tubitak UME, with statistical support from VNIIM, and expands the K86 series scope by measuring nucleic acids sequences in a high protein meat matrix (a mix of pork and beef) relevant for processed food. While there have been some delays K86.d the draft B has been circulated and the NAWG is grateful to Phattaraporn Morris and colleagues at NIMT, TUBITAK UME and VNIIM for leading this study.

2. Ongoing Key Comparisons and pilot studies

a. CCQM NAWG P244: Lipid Nanoparticles with Encapsulated RNA

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 better reference measurement procedures and improved reference materials/standards. P244 is led by NIST, with support from NML, and is exploring measurements of mRNA lipid nanoparticles and also includes SAWG and IAWG participation. Materials were shared with NAWG members over the last reporting period and initial NAWG relevant results presented at the 21st NAWG meeting last September. NAWG members were able to explore and develop their capacity to assist the growing area of nucleic acid therapeutics. Analytical techniques explored by the NAWG included RNA number concentration and LNP encapsulation efficiency measurements:

1) RNA copy number concentration (Figure 1A) falls within existing capabilities through similar sequence measurement conducted in P199.b and K181 however this incorporates pre-examination extraction steps to purify the nucleic acids for analysis.

2) In addition P244 introduced the novel measurand of encapsulation efficiency (Figure 1B) not previously examined by NAWG members. Encapsulation efficiency is performed by stakeholders using a number of strategies however the common method, used by participants of P244, explored the 'RiboGreen Assay' which measures total RNA using fluorimetry with and without disruption of the LNPs. Increase in fluorescence with disruption is used as a surrogate for the RNA not measurable by fluorimetry due to encapsulation (when not disrupted).

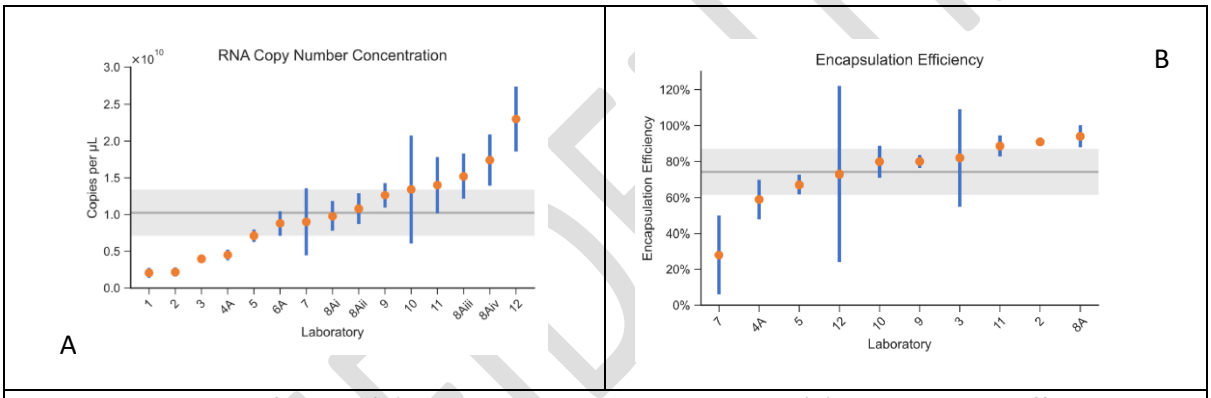


Figure 1. P244 result for RNA (A) copy number concentration and (B) encapsulation efficiency.

The results show a greater distribution of copy number concentration differences than in previous comparison which measured RNA sequence copy number concentration in buffered solution (e.g. K181, P199 and P199.b) suggesting the preprocessing (extraction) necessary to extract the RNA from the LNP added add additional sources of error. Additional ongoing activities (associated with CCQM K190) will allow us to determine whether this dispersion is a characteristic of the LNP material and preprocessing steps.

This pilot study is especially significant as it expands the NAWG topics from nucleic acid measurement associated with food and diagnostics to the first example of analyses linked to therapeutics associated with an especially exciting topic of RNA vaccines. The NAWG is grateful to Thomas Cleveland with support from colleagues and NIST and NML for championing this pilot study.

b. CCQM K198/P255 Cancer SNV measurement in cell-free DNA

K198 will build on learnings from the P184 and K189 to support CMCs when measuring genetic variants cell-free DNA (cfDNA) copy number which is an increasingly important metric in precision oncology. cfDNA containing single nucleotide variants (SNVs) can be used as a prognostic tool, whilst the ability to detect DNA fragments circulating in blood and plasma (liquid biopsy) provides a less invasive alternative to solid tissue biopsy. cfDNA provides additional challenges when compared to genomic DNA (the topic of focus for K189) as the cfDNA fragments are smaller and may vary in size. K184 material preparation has now been completed (Table 1) and it is anticipated materials will be sent in May 2026 with results being reported at the 23rd NAWG meeting in November 2026.

Table 1. Homogeneity results for K198 study materials for six different genetic targets

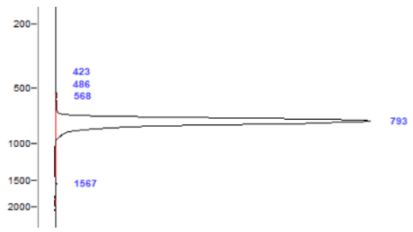
No.	Measurand	*Rel.* u_{bb}	
		SM1	SM2
1	EGFR L858R	10.48%	2.98%
2	EGFR L858R wild-type	0.91%	1.28%
3	VAF of EGFR L858R	10.48%	3.09%
4	TP53 R273H	5.21%	3.26%
5	TP53 R273H wild-type	1.01%	1.38%
6	VAF of TP53 R273H	5.21%	3.30%

c. *CCQM-K199a, CCQM-K199b and CCQM-P256 parallel pilot study traceable Mass fraction of (a) RNA and (b) corresponding DNA fragments.*

The Nucleic Acids Working Group (NAWG) has conducted numerous Key Comparisons and pilot studies comparing nucleic acid sequence copy number concentrations using 'molecular' methods such as digital PCR (dPCR) with alternative 'chemical' methods, primarily isotope dilution mass spectrometry (IDMS), used to provide a route to assess SI traceability (by using molecular weight to convert mass based measurements into copy number concentration estimations). Previous NAWG studies used orthogonal methods to evaluate the accuracy of dPCR (to measure DNA sequence copy number) and RT-dPCR (to measure RNA sequence copy number), focusing on how closely measurements using molecular methods align with SI traceable chemical methods. In CCQM-K181, IDMS results from three laboratories were used to establish a reference value against which RT-dPCR measurements where benchmarked. However, while sources of bias in RT-dPCR are known, possible inaccuracies in IDMS measurements (calibrator purity, incomplete digestion, chemical conversion issues and interfering substances) as well as molecular weight estimation are less clear.

Furthermore, while several NAWG members have capacity to use these alternative methods for nucleic acid analysis, the NAWG has not conducted any KCs to support them in claiming CMCs using SI traceable orthogonal methods. CCQM K199a and b represent the first in a series of KCs dedicated to supporting chemical analysis of nucleic acids. K199 aims to assess agreement between SI-traceable measurements of RNA and DNA and materials, characterised for fragment size (Figure 2), been distributed and results due to be discussed at the 22nd NAWG meeting in April 2023.

Figure 1 Capillary electropherogram of single stranded RNA fragment corresponding to the 732 nucleotide sequence given in the appendices. Purity estimate was 99.5%. The size estimate was within the specified error range for a 732 nucleotide fragment (~600 to ~850 nt).



K199 (a and b) represent the first in a series of studies to investigate the use of chemical methods for nucleic acid sequence measurement. For future studies within the CCQM-K199 series addressing factors that may include, but are not limited to, instrument bias, purity assessment and estimation

of molecular, the NAWG has requested future support of the BIPM to ensure its expertise can contribute to study design and planning and that its capabilities in small and large molecule primary reference material and calibrator characterisation facilities are made available to contribute to comparison coordination.

3. Plans for the near and medium future

The NAWG is planning to propose one Key Comparison and one Pilot study be initiated in the 2026-27 period. The KC will follow up on K189, which investigated measurement of single sequence base differences, to include insertions/deletions. The Pilot study will address bacterial drug resistance gene measurement and may include CAWG participation(to be confirmed during 22nd NAWG meeting on 13th April 2026)

Additional topics for potential activities in the near future include environmental DNA analysis as well as key comparisons to support CMCs in foods analysis for food pathogens and/or authentication (building on K86 series to potentially consider novel genetic techniques and precision bred organisms and cell cultivate products); many of these topics offer co activities with other working groups. Future topics exploring where chemical analysis (through K199 series) is used to estimate nucleic acid sequence number concentration will also be planned to consider additional factors (such as error from impurities that are difficult to determine but potentially present and estimation of the actual molecular weight). In addition to future key comparisons and pilot studies, the NAWG continues to explore routes to broaden stakeholder interaction both within and beyond the NMI community.

Jim Huggett and Maxim Vonsky (04-2026)