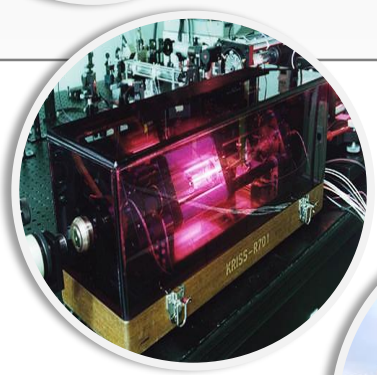

CCU/CCQM Workshop 2023

Quantification of Nucleic Acids by Counting

Inchul Yang (icy@kriss.re.kr)

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Contents

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Counting biological entities

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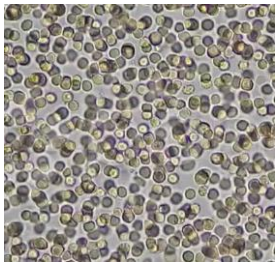
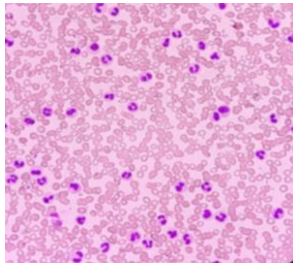
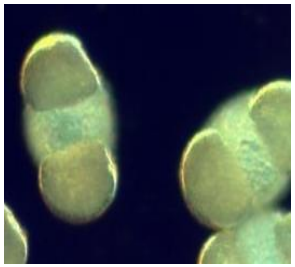
Naked Eye

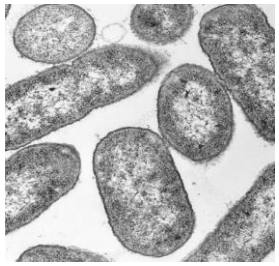
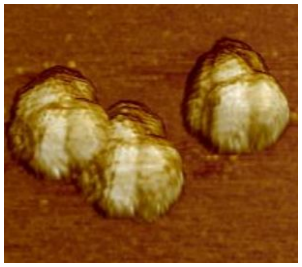
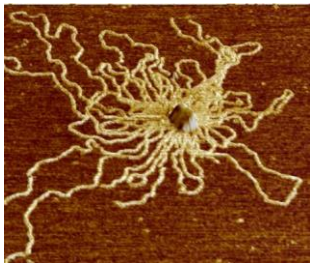
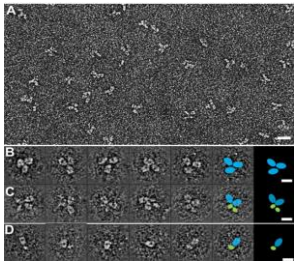



Optical Microscope

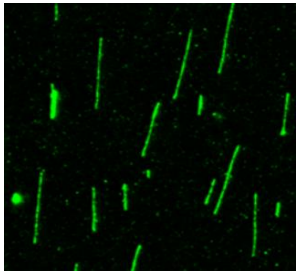
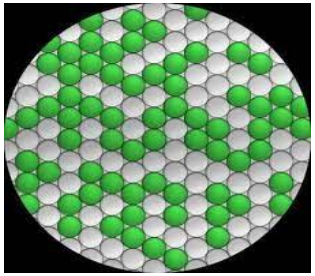
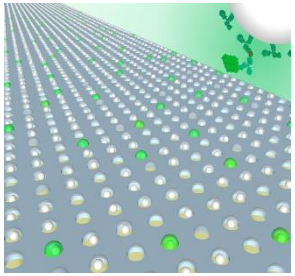





TEM/SEM/SPM

Amplification/colony Fluorescence

Bacterial colony

Fluorescence-DNA

Digital PCR

Single molecule Immuno-detection

Quantification by counting

KRISS

- Mole: fixed number of entities
- Quantification by counting directly realizes 'Mole'-traceable measurement
- Biological measurands are huge, complex and colloidal
  - limited applicability of chemical measurement methods
  - suitable for quantification by counting
  - count itself is more meaningful than 'kg' or 'mol'
    1 atto mol vs. 1 nano gram vs. 1 million (cell, DNA, protein)

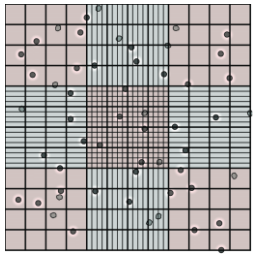
- Requires
- **separation/partitioning** of individual entities
- **making detectable/sensitization**
  magnification of size: growth, microscopy
  clonal multiplication: culture, PCR
  signal amplification: fluorescence, radio-isotope

- Hampered by
- sampling issues
- volume issues
- detection failure
- impurity and fragments

Separation-Sensitization-Counting

KRISS

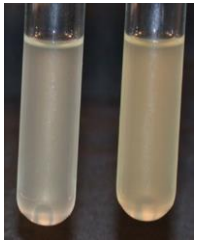
Dilution



Count/volume

Blood/cell

Counting chamber



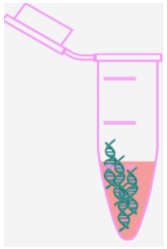
Dilution

Culture

Count/volume

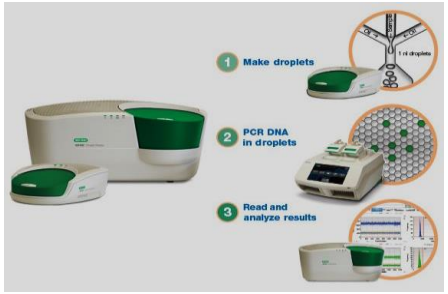
Bacteria

Colony counting



Dilution

PCR mixture



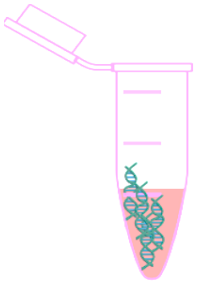
Count/volume

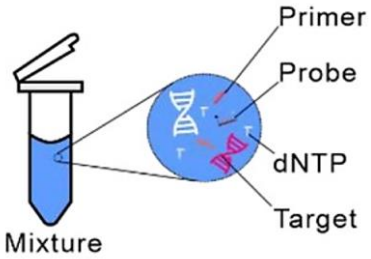
DNA/RNA

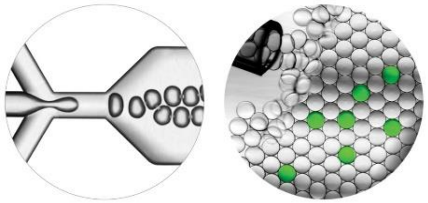
Droplet digital PCR

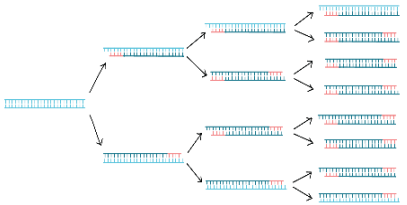
Digital PCR workflow

DNA → PCR mixture → Partitioning → PCR reaction

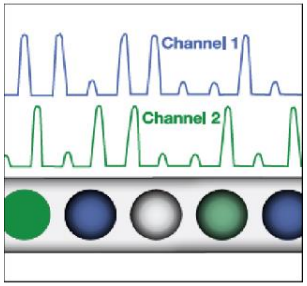


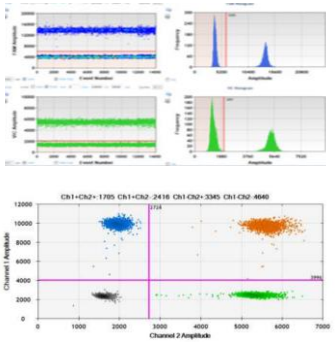






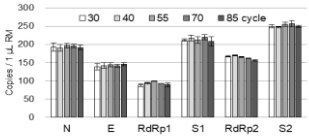
Fluorescence reading → Data analysis → Calculation → Interpretation

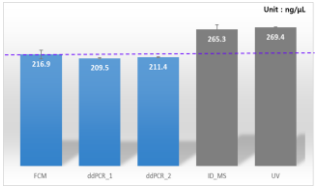




$$\lambda = -\ln(Fraction\ Negative)$$

Copies/μL = λ / (Droplet Volume in μL)





Strength of digital PCR

KRISS

Sensitivity and resolution
- single molecule sensitivity

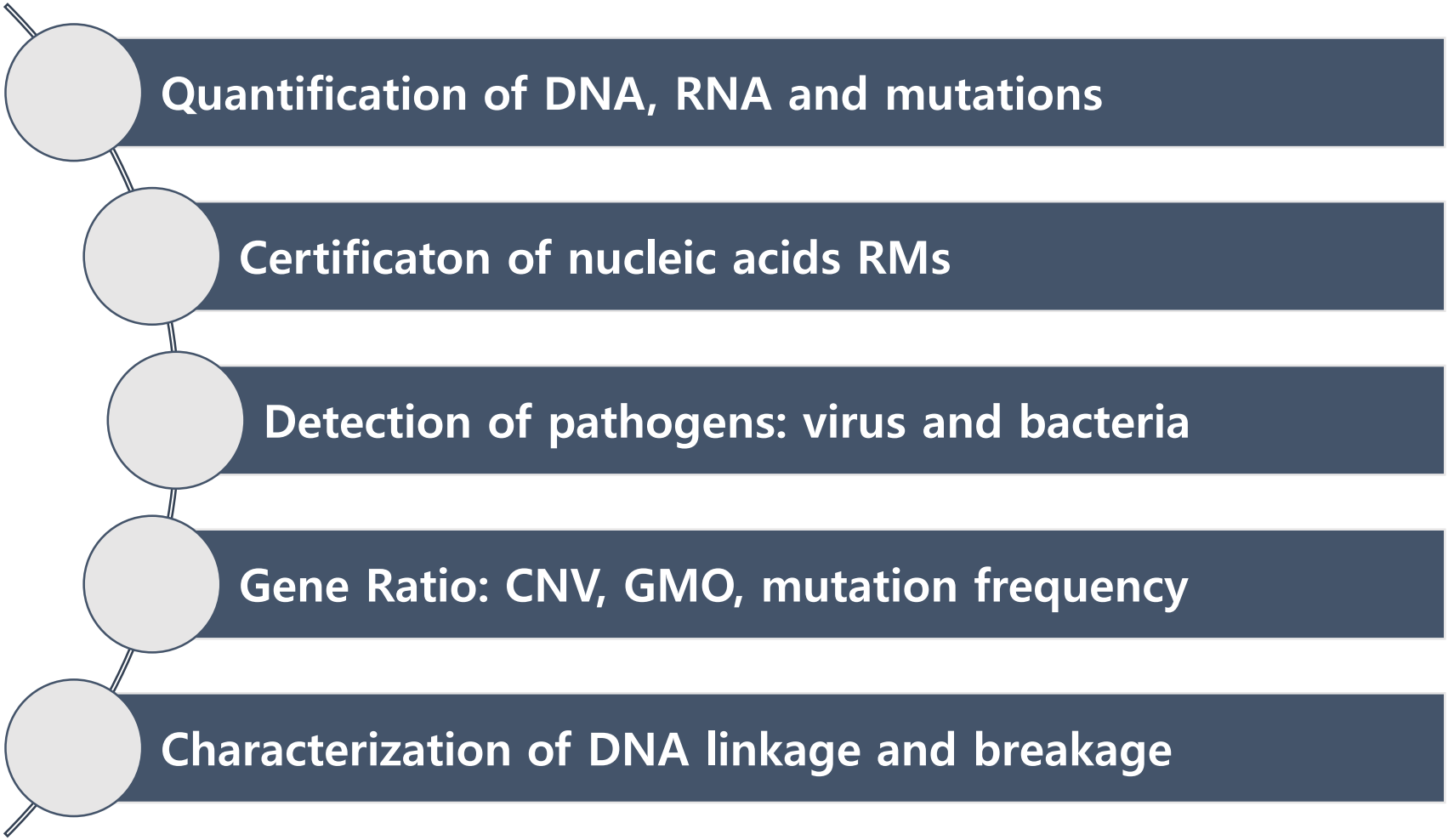
Precision
- higher precision and reproducibility
- higher tolerance to inhibitors and background nucleic acids

Absolute quantification
- no calibration needed under validated PCR conditions
- direct realization of 'mole'-traceable measurement

Throughput
- 96 samples in 5 hours including hand-on time less than 1 hour
- parallel measurement of different samples and controls

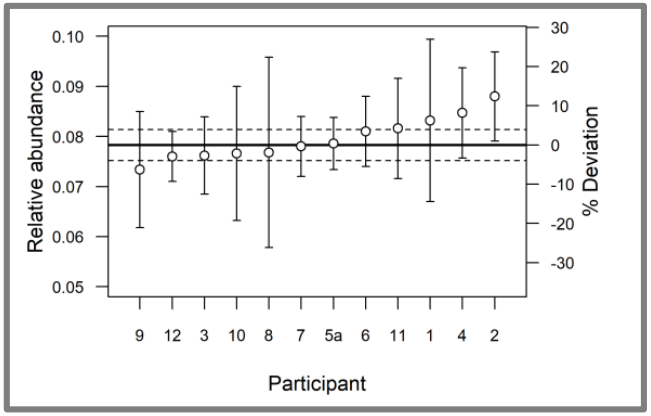
Multiplex PCR
- simultaneous quantification of multiple DNA/sequences in single tubes
- internal controls and standards can be used
- ratiometry, self-validation and linkage/breakage analysis

Applications of digital PCR

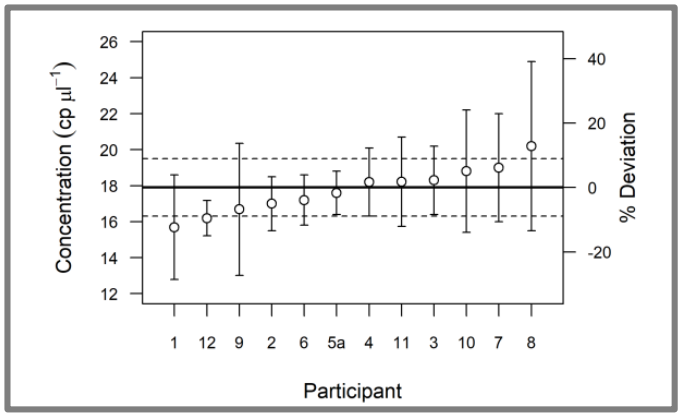


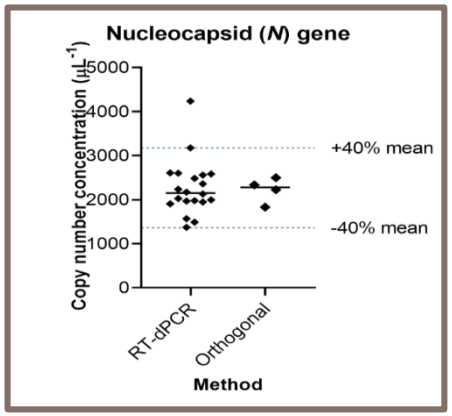
NAWG activities on dPCR

CCQM Comparison Studies	Description	Application area
P154.2	KRAS quantification and fractional abundance	Cancer
P184	EGFR and BRAF quantification and fractional abundance	Cancer
P199	HIV-1 RNA quantification	Infectious disease
P199b/K181	SARS-CoV-2 quantification	Infectious disease
K86c	GMO (canola seeds) quantification and fractional abundance	GMO, food
K86d	Meat authentication (pork and beef mixture)	Food
K176	HER2 Copy Number Variation	Cancer



CCQM P184





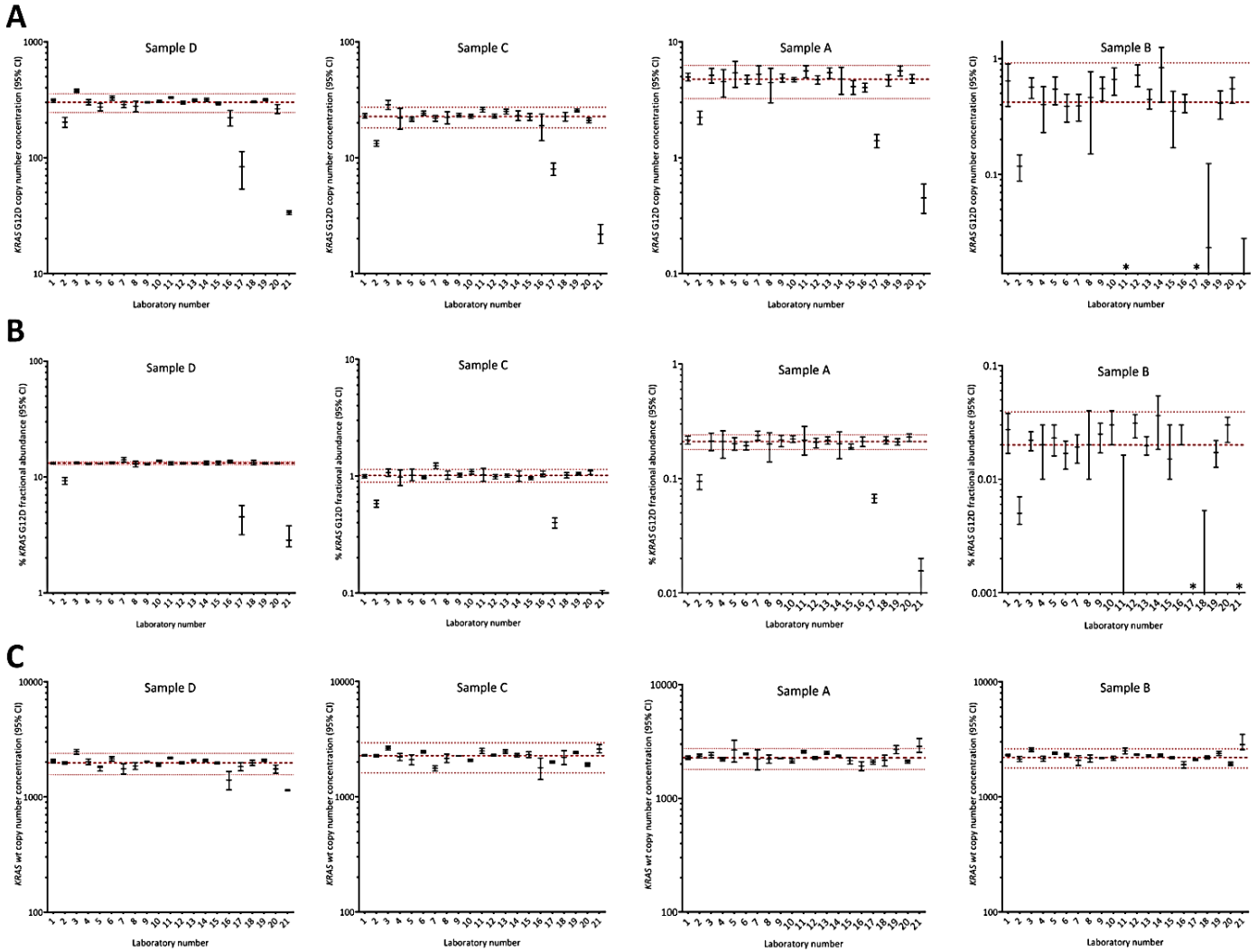
CCQM P199b

International comparison on 'absolute quantification & ratio of wild type and mutant KRAS, Coordinated by LGC

Mutant (copy/μL)

Ratio (%)

WT (copy/μL)



Reference materials by dPCR

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2365

BK Virus DNA Quantitative Standard

This Standard Reference Material (SRM) is intended for use in the value assignment of BK virus deoxyribonucleic acid (DNA) quantitation materials, primarily those used for quantitative polymerase chain reaction (qPCR). SRM 2365 consists of a well-characterized, linearized plasmid, containing BK virus DNA solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3 propanediol hydrochloride (Tris HCl) and 1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) pH 8.0 buffer (TE), with 50 ng/µL yeast tRNA added to ensure stability. A unit of the SRM consists of one 0.5 mL tube containing approximately 110 µL of DNA solution. The tube is labeled and is sealed with a screw cap.

Certified Values: Certified values are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in that all known or suspected sources of bias have been accounted for. The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume.

Table 1. Certified Value for SRM 2365

Analyte	Certified Value (copies/µL)	95% Probability Uncertainty Interval (copies/µL)	Standard Uncertainty, $u(X)$ (copies/µL)	Effective Coefficient of Variation, $CV=100\times u(X)/X$
BK Virus DNA copy number	558,000	534,000 to 582,000	12,000	2.2%

NIST

Australian Government
Department of Industry, Science, Energy and Resources

National Measurement Institute

CERTIFIED REFERENCE MATERIAL CERTIFICATE OF ANALYSIS

NMIA NA050 to NA055: SARS-CoV-2 Standard

Report ID: 210329 (supersedes ID 201221, revised expiry date) **Batch No.:** B200921

Certified concentration values of SARS-CoV-2 genome equivalents

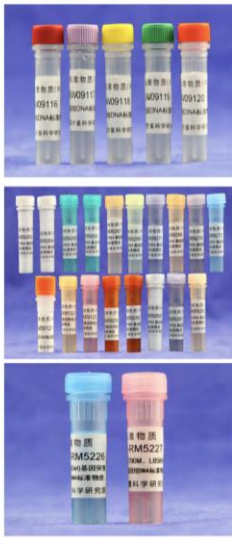
NMIA Code	Concentration (copies/mL)	Expanded Uncertainty	Expanded Uncertainty (%)
NA050	116,000	28,000	24
NA051	49,000	12,000	25
NA052	12,400	3,100	25
NA053	3,700	1,200	33
NA054	1,290	530	41
NA055	420	280	66

The uncertainty has been calculated according to the Guide to the expression of uncertainty in measurement [1] and ISO Guide 35 [2] and is stated at the 95% level of confidence.

NMIA

2021-2022 Newly Released CRM——Nucleic acid

Field	CRM number	CRM name
Nucleic acid	GBW09116-GBW09120	HER2 genomic DNA reference material
	GBW09121	GJB2 genomic DNA reference material
	GBW09122、GBW09123、GBW09258	GJB2 gene plasmid DNA reference material
	GBW09259-GBW09266	12S rRNA gene plasmid DNA reference material
	GBW09267-GBW09272	SLC26A4 gene plasmid DNA reference material
	GBW（E）091215	cfDNA containing EGFR T790M mutant reference material
	GBW（E）091216	cfDNA containing EGFR T790M wild type reference material

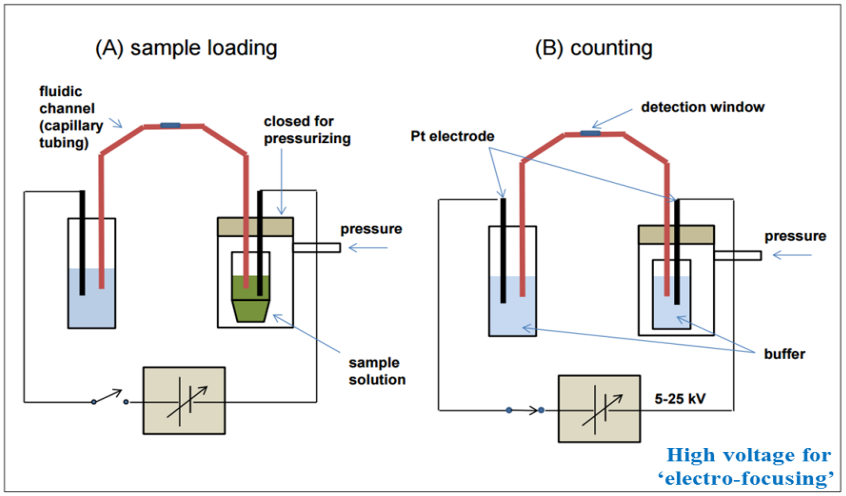


NIMC

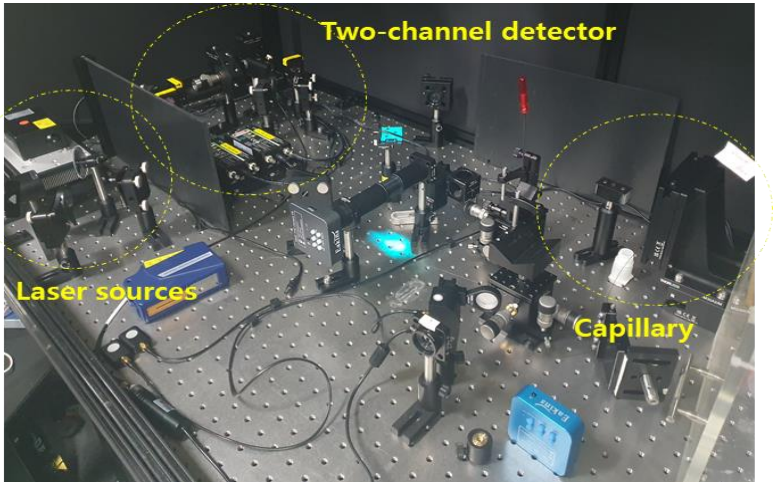
KRISS RM ID number	RM title	Method
111-10-506	SARS-CoV-2 RNA	dPCR (RM)
111-10-507	SARS-CoV-2 packaged RNA	
111-10-508	Severe fever with thrombocytopenia syndrome (SFTS) virus packaged RNA	
111-10-509	EGFR DNA for liquid biopsy	
111-10-510	Adenovirus Type 5 DNA (partial)	Single molecule counting & dPCR (CRM)
111-10-511	Adenovirus Type 5 E1 gene	
111-10-512	hAd5 E1-PSG4 DNA	
111-10-513	SARS-CoV-2 S gene	
111-10-514	Norovirus GI RNA	dPCR (RM)
111-10-515	Norovirus GI RNA	
111-10-526	Human Alphacoronavirus 229E RNA	
111-10-536	Human rhinovirus RNA	
111-10-537	RVFV lineage A RNA	
111-10-538	RVFV lineage D RNA	
111-10-539	RVFV lineage K RNA	
111-10-549	Severe fever with thrombocytopenia syndrome (SFTS) virus RNA	
111-10-551	SARS-CoV-2 Omicron variant RNA	

KRISS

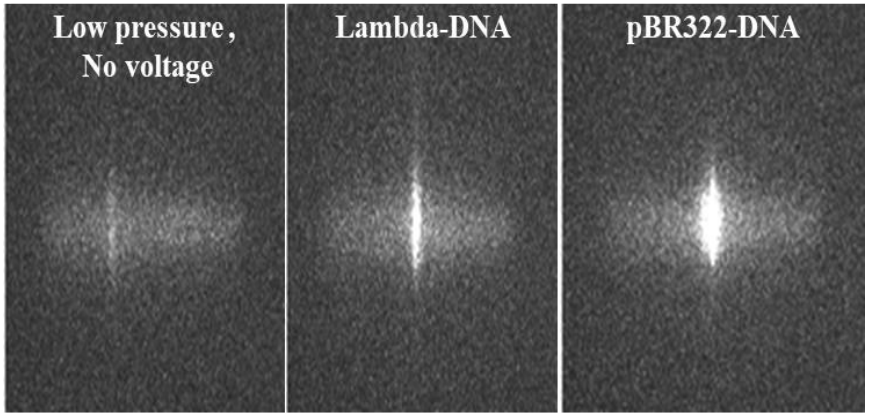
KRISS Single molecule counting instrument



Instrument design



Instrumentation (V2)



EMCCD images of DNA molecules

DNA and RNA count signals

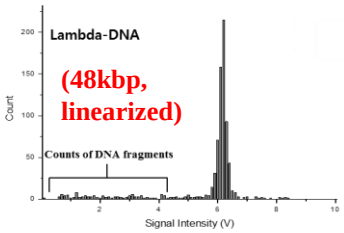
Nucleic acids quantification by direct counting

KRISS

Lambda DNA

Count-based quantitation of trace level macro-DNA molecules

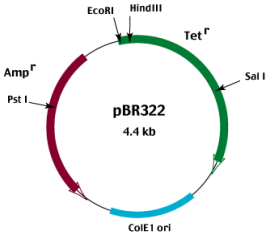
Hyuk-Min Lim, Hee-Bong Yoo, Nan-Sook Hong, Inchul Yang, Myung-Sub Han and Sang-Ryoul Park



Plasmid DNA

A candidate reference method for quantification of low concentrations of plasmid DNA by exhaustive counting of single DNA molecules in a flow stream

Hee-Bong Yoo, Donggeun Oh, Jae Yong Song, Mamoru Kawaharasaki, Jeeseong Hwang, In Chul Yang and Sang-Ryoul Park

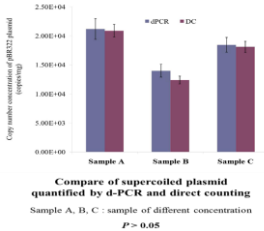


Bilateral comparison

SCIENTIFIC REPORTS

OPEN Accurate quantification of supercoiled DNA by digital PCR

Lianhua Dong, Hee-Bong Yoo, Jing Wang & Sang-Ryoul Park



Compare of supercoiled plasmid quantified by d-PCR and direct counting

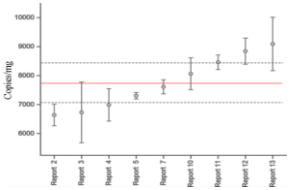
Sample A, B, C : sample of different concentration

$P > 0.05$

CCQM P154

analytical chemistry

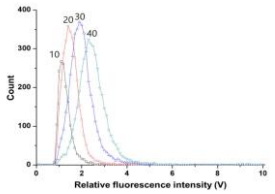
International Comparison of Enumeration-Based Quantification of DNA Copy-Concentration Using Flow Cytometric Counting and Digital Polymerase Chain Reaction



M13 DNA: Seq-specific

Quantification of single-strand DNA by sequence-specific counting in capillary flow cytometry

Hee-Bong Yoo, Chaeeun Lee, Kee-Suk Hong, Sang-Ryoul Park and Inchul Yang

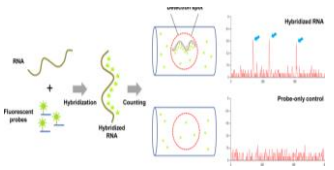


MS2 RNA: Seq-specific

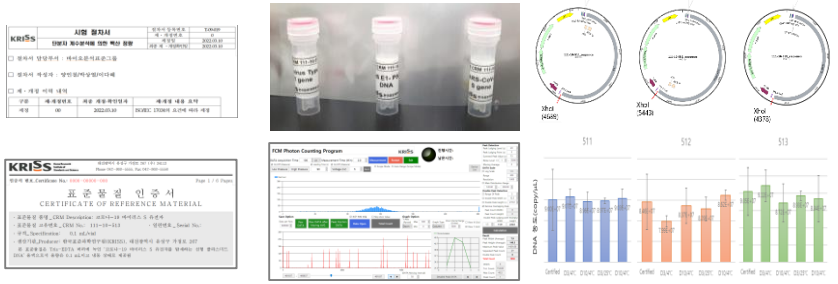
analytical chemistry

Precise RNA Quantification by Counting Individual RNA Molecules Using High-Sensitivity Capillary Flow Cytometry

Hee-Bong Yoo, Sang-Ryoul Park, Kee-Suk Hong, and Inchul Yang



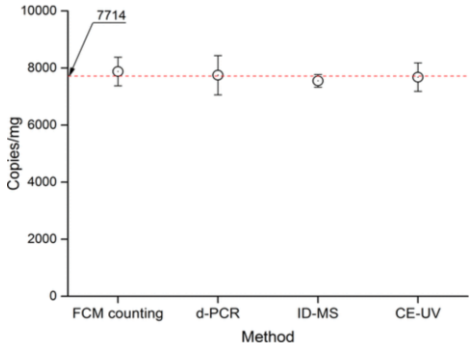
KRISS gene CRMs certified by counting



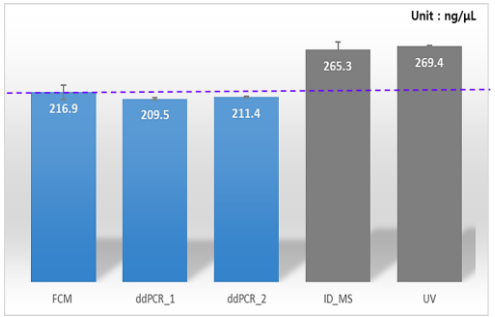
KRISS is now working on

- Cross-validation of different methods for nucleic acids measurements
- Quantification of virus particles by counting
- Two-channel optics, volumetric imaging and automation

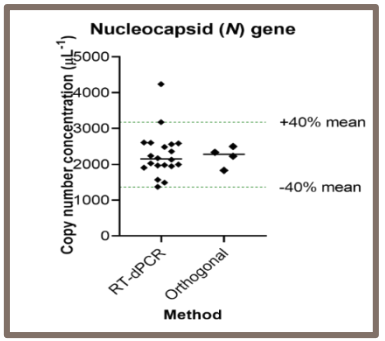
Comaparison of nucleic acids quantification methods



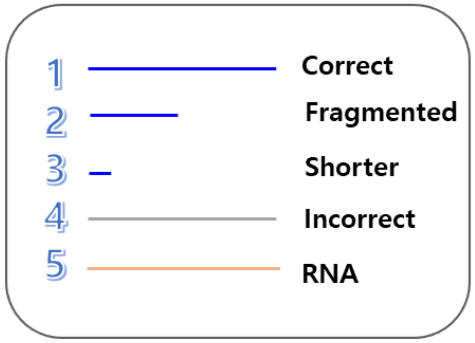
plasmid DNA



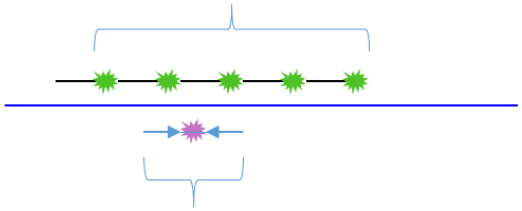
ss DNA



RNA



ss counting: ~ 1kb with correct sequence 1



PCR: ~ 200b with correct sequence 1+2

UV: Molecules with absorption at 260nm 1+2+3+4+5
Sequence-nonspecific
Size-independent

MS: Molecules producing deoxynucleotides 1+2+3+4
Sequence-nonspecific
Size-independent

Conclusion

- Counting is a simple and direct approach for realization of 'Mole'-traceable measurement of biological entities

- Biological entities(cells, virus, nucleic acids and proteins) are good targets for application of counting approaches

- Digital PCR is a popular and robust method for quantitative analysis of nucleic acids. It has a potential to be a primary method for mole-traceable quantification of nucleic acids owing to
  - extreme sensitivity and resolution
  - high precision, reproducibility and stability
  - throughput and multiplexing
  - varierty of applications

- KRISS has developed a method for direct counting of nucleic acids in capillary flowcytometry. We expect cross-validation of digital PCR, single molecule counting and chemical analysis will benefit one another in establishment of measurement standards of nucleic acids

Questions:

CCQM
NAWG

- What is the CCQM position on ‘use of non-SI and dimension-less descripts such as copy, cells and genomes’ when reporting results for such measurements in:
  - KC, pilot reports, etc?
  - Associated publications amongst the stakeholder communities?
- What recommendations may CCQM provide for expression of e.g. 1000 copies of biomolecular entities (cells, etc) per unit volume (mL) value:
  - 1000 copies/mL
  - 1000 1/mL
  - 1000 /mL
  - 1000 mL^{-1}
  - Other?

Bureau International des Poids et Mesures

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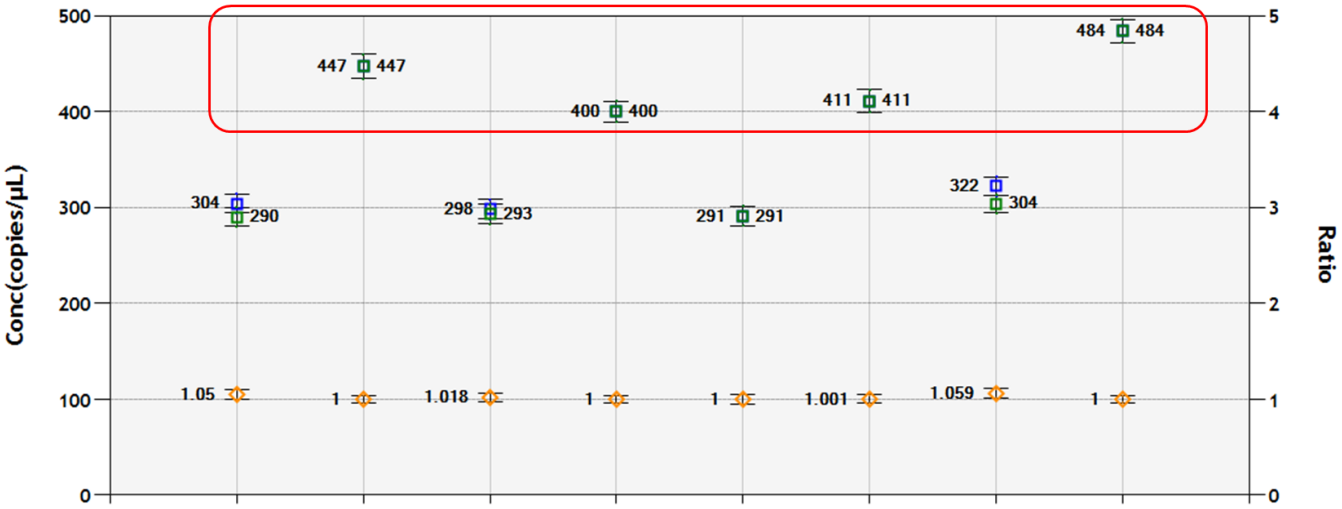
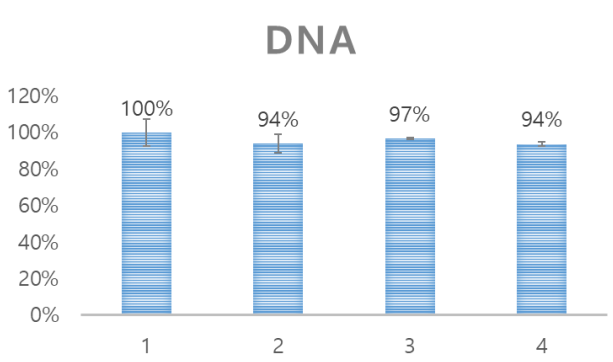
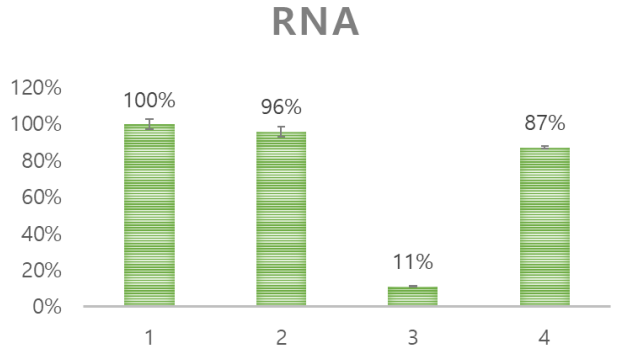
by Jim Hugget, NAWG chair


Conc(copies/μL)
Ratio
500
400
300
200
100
0
5
4
3
2
1
0
447 447
400 400
411 411
484 484
304 290
298 293
291 291
322 304
1.05
1
1.018
1
1
1.001
1.059
1


Norovirus
RNA 1.37 kb
1
2
3
4
Norovirus
DNA 1.37 kb


DNA
120%
100%
80%
60%
40%
20%
0%
100%
94%
97%
94%
1
2
3
4


RNA
120%
100%
80%
60%
40%
20%
0%
100%
96%
11%
87%
1
2
3
4