



# Recommendations for Validation of qPCR and dPCR Assays in Support of Cell and Gene Therapy Drug Development

Professor Mikael Kubista

[www.labgenexp.eu](http://www.labgenexp.eu)

[www.ibt.cas.cz/en/core-facilities/gene-core](http://www.ibt.cas.cz/en/core-facilities/gene-core)

[www.biocev.eu](http://www.biocev.eu)

[www.gu.se](http://www.gu.se)

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Too many of the findings that fill the academic ether are the result of shoddy experiments or poor analysis (see [article](#)). A rule of thumb among biotechnology venture-capitalists is that half of published research cannot be replicated. Even that may be optimistic. Last year researchers at one biotech firm, **Amgen, found they could reproduce just six of 53 “landmark” studies in cancer research.** Earlier, a group at Bayer, a drug company, managed to repeat just a quarter of 67 similarly important papers. A leading computer scientist frets that three-quarters of papers in his subfield are bunk. In 2000-10 roughly 80,000 patients took part in clinical trials based on research that was later retracted because of mistakes or improprieties.

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## NEW CEN/TS

and ISO Standards published!

[READ MORE](#)

SPIDIA-driven novel pre-analytical tissue technology enabled microbiome analysis as well as histological and parallel molecular evaluation, in two nota



### EVENTS

EVENT DATE: MAY 14-17, 2024

Save the date!

The Europe Biobank Week in 2024 will highlight state-of-the-art biobanking innovations and research. This action-packed congress will feature keynote presentations, panels and workshops organised by **ESBB** and former SPIDIA4P partner **BBMRI-ERIC**.



### NEWS

DECEMBER 14, 2023

Read the new paper! (preprint)

The recent publication from researchers from the Med. Univ. of Graz describes the benefits of ISO and CEN/TS standards for the pre-analytical quality of whole venous blood for circulation cell ...



### PARTNERS

SPIDIA4P-PARTNERS

Learn more about the highly experienced European project partners that collaborate to reach the goals of SPIDIA4P



### BACKGROUND

SPIDIA4P

Quality of samples matters – find out, why!

INTERNATIONAL  
STANDARDISO  
20395:2019

- **Molecular in-vitro diagnostic examinations - Specifications for preexamination processes for:**
  - Blood — Cellular RNA, gDNA, ccfDNA, ccfRNA
  - Blood – Exosomes / Evs
  - Blood Tumor Cells – DNA, RNA, staining
  - Tissue (FFPE) — DNA, RNA, Protein
  - Tissue (Frozen) – DNA, RNA, Proteins
  - Tissue (FFPE) – in situ staining
  - Fine Needle Aspirates – DNA, RNA, Proteins
  - Saliva – DNA
  - Urine & Body Fluids – cfDNA
  - Metabolomics – Urine, Serum, Plasma
  - Microbiome – Stool, Saliva etc.

Published CEN ⇒ progressing at ISO

Published ISO

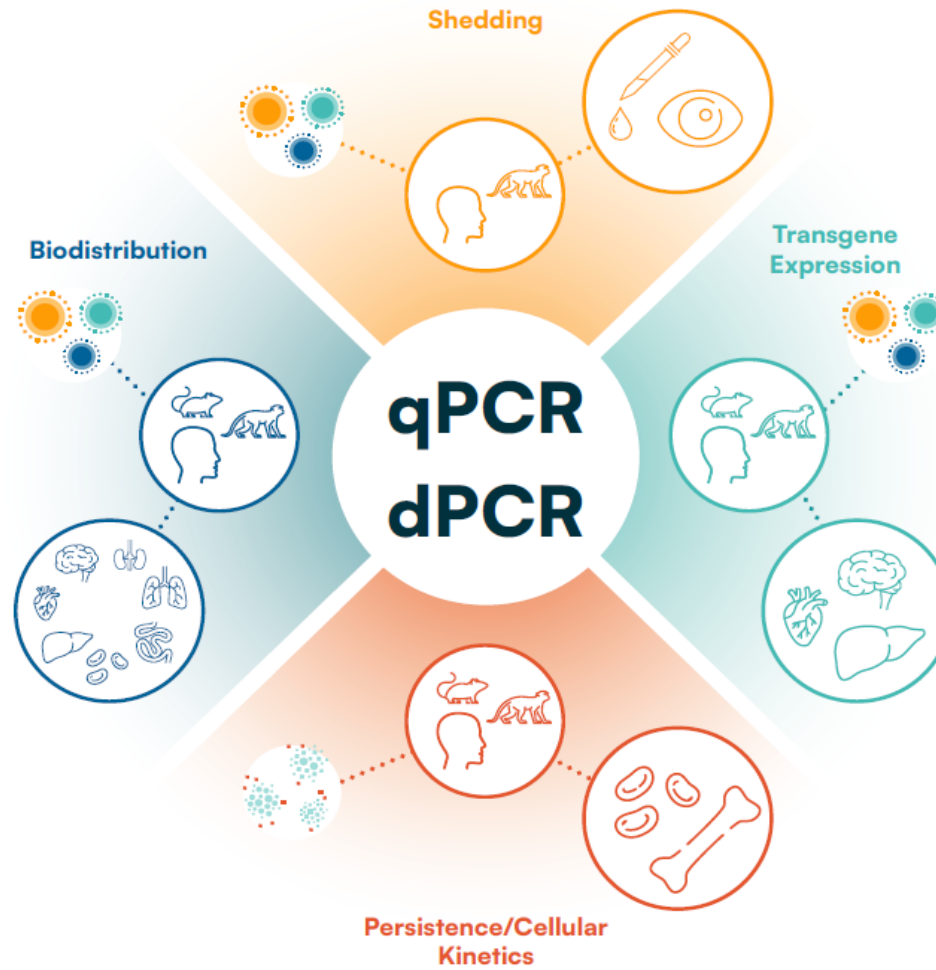
Total:22

Reference number  
ISO 20186-3:2019(E)

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# Application of qPCR and dPCR for C&GT



Recommendations from AAPS expert group



# Calibrators, Reference Material and Quality Controls

- Standard/calibrator curves should consist of at least **six data points** for each qPCR run.
- When possible, using the drug product and a **full process standard curve** is preferable, but choice of reference material should be guided by assay COU.
- In shedding studies, **encapsulated material** is preferred.
- For biodistribution studies, **plasmid DNA is acceptable** given that vector DNA is often no longer encapsidated and may appear in episomal or other forms following in vivo processing.
- For assay validation, it is recommended to use **drug product** or plasmids manufactured for drug product.
- For RT-PCR assays, **RNA template** should be used.



# Calibrators, Reference Material and Quality Controls cont.,

- In addition to the standard curve, **at least two QCs should** be tested (high and low) with a preference for three (high, medium, and low) on each sample analysis run.
- For studies and samples where confirming negative sample results is critical (i.e., vector shedding), a non-competing pre- or post-extraction **spike-in control** could be considered.
- **Extraction and assay NTCs** should be run for all applications (qPCR/dPCR) to confirm that no contamination has occurred.



# Precision & Accuracy – Validation criteria

- **Precision and Accuracy (P&A)** should be determined using **spikes of positive control template** in a background matrix of nucleic acid extracted from target tissues or biofluids from the species of interest.
- **Method accuracy** and precision are assessed in validation over **≥6 runs, ≥2 analysts, ≥2 days**.
- **ULOQ** (Upper Limit of Quantification), **HQC** (High Quality Control), **MQC** (Mid Quality Control), **LQC** (Low Quality Control), and **LLOQ** (Lower Limit of Quantitation) concentrations should be established **in method development** and their performance confirmed in method validation.
- **Three occasions/determinations** should be made **per QC/LOQ level per run**, with each QC/LOQ run in at least duplicate per determination.

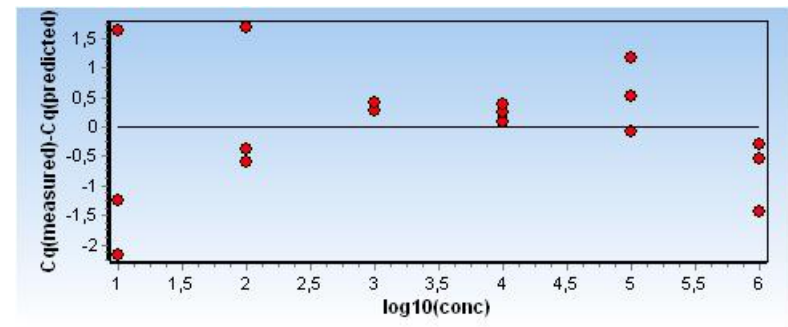
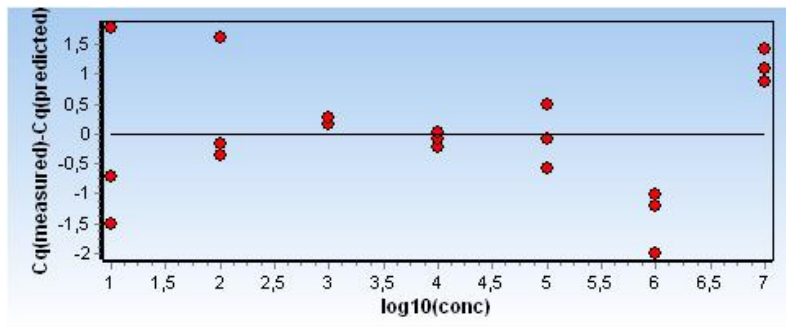
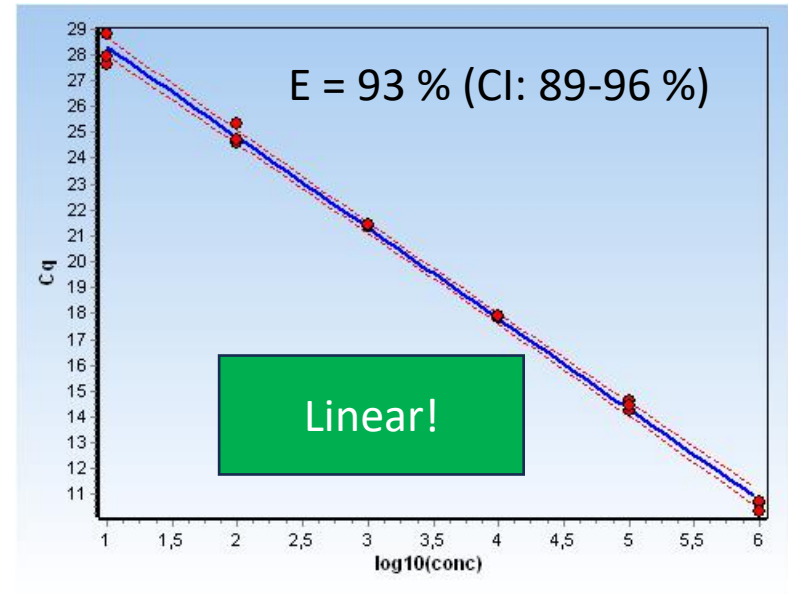
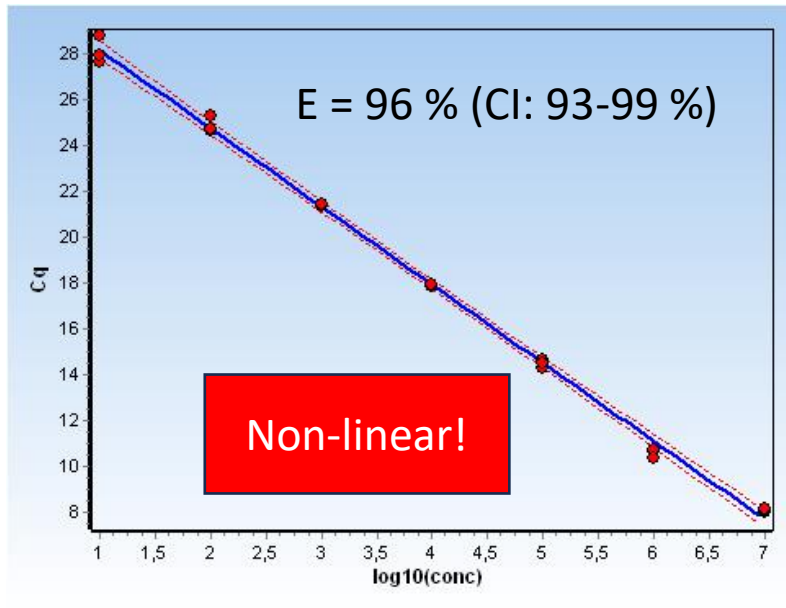


# PCR efficiency and linearity

- Intra and inter-assay precision  $\leq 30$  % **Coefficient of Variation (%CV)** for QCs and  $\leq 50$  %CV for LOQs.
- Intra and inter-assay **accuracy of -50 to 100 %RE** for qPCR
- For **dPCR**, the inter-assay accuracy for absolute copies measured should be **RE  $\leq 30$  %** for QCs ( $\leq 50$  % at LOQ).
- **PCR efficiency** should be **90 – 100 %** and **presented with CI**. ( $E > 100$  % can be accepted if 100 % is within CI)
- **Linear dynamic range** should be established (for qPCR and dPCR)
- The curves are assessed in  $\geq 6$  runs,  $\geq 1$  curve per run,  $\geq 2$  analysts,  $\geq 2$  days,  $\geq 2$  **PCR instruments**, when possible.



# Linearity



$$Y = a + b_1X$$

$$Y = a + b_1X + b_2X^2$$

$$Y = a + b_1X + b_2X^2 + b_3X^3$$

# Limit of Blank (LoB)

- LOB may be selected empirically at a 95% confidence (e.g., **19/20 replicate runs are negative**) independent of PCR platform
- In dPCR LOB can be derived assuming a Normal distribution of positive partitions from blank samples:

$$\text{LOB} = \text{Mean}_{\text{NTC}} + 1.645(\text{SD}_{\text{NTC}})$$

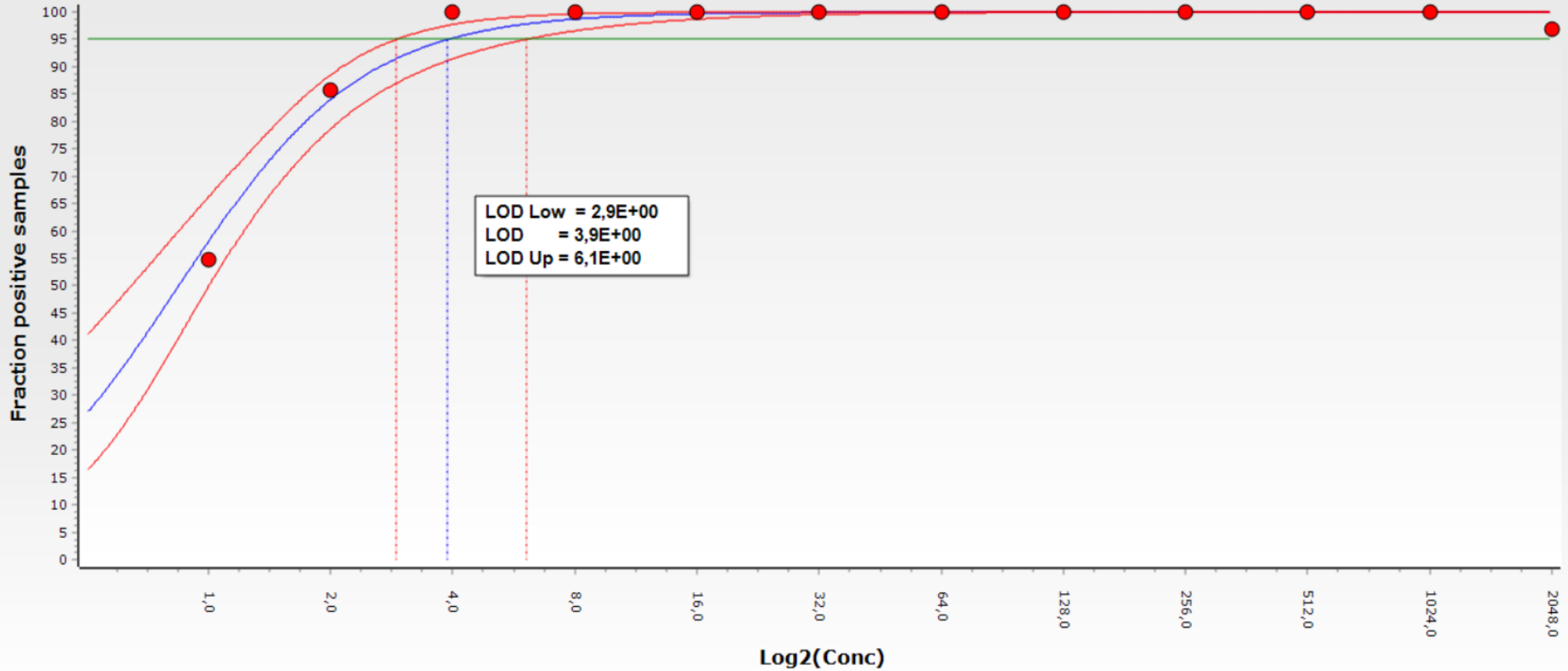
- The LOB is reported as determined.
- The LOB should be < LOD.

# Limit of detection (LoD)

LOD is the lowest analyte concentration that yields an assay response which can be reliably distinguished from the assay response in the absence of analyte (i.e., NTC) but is not required to meet any precision and accuracy criteria for quantification.

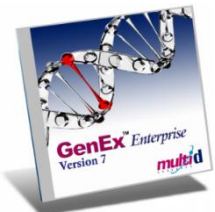


# Limit of Detection



Concentration when 95 % of replicates are positive

A. Frootan, R. Sjöback, J. Björkman, B. Sjögreen, L. Linz, M. Kubista. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification*. Vol 12, 1-6 (2017)



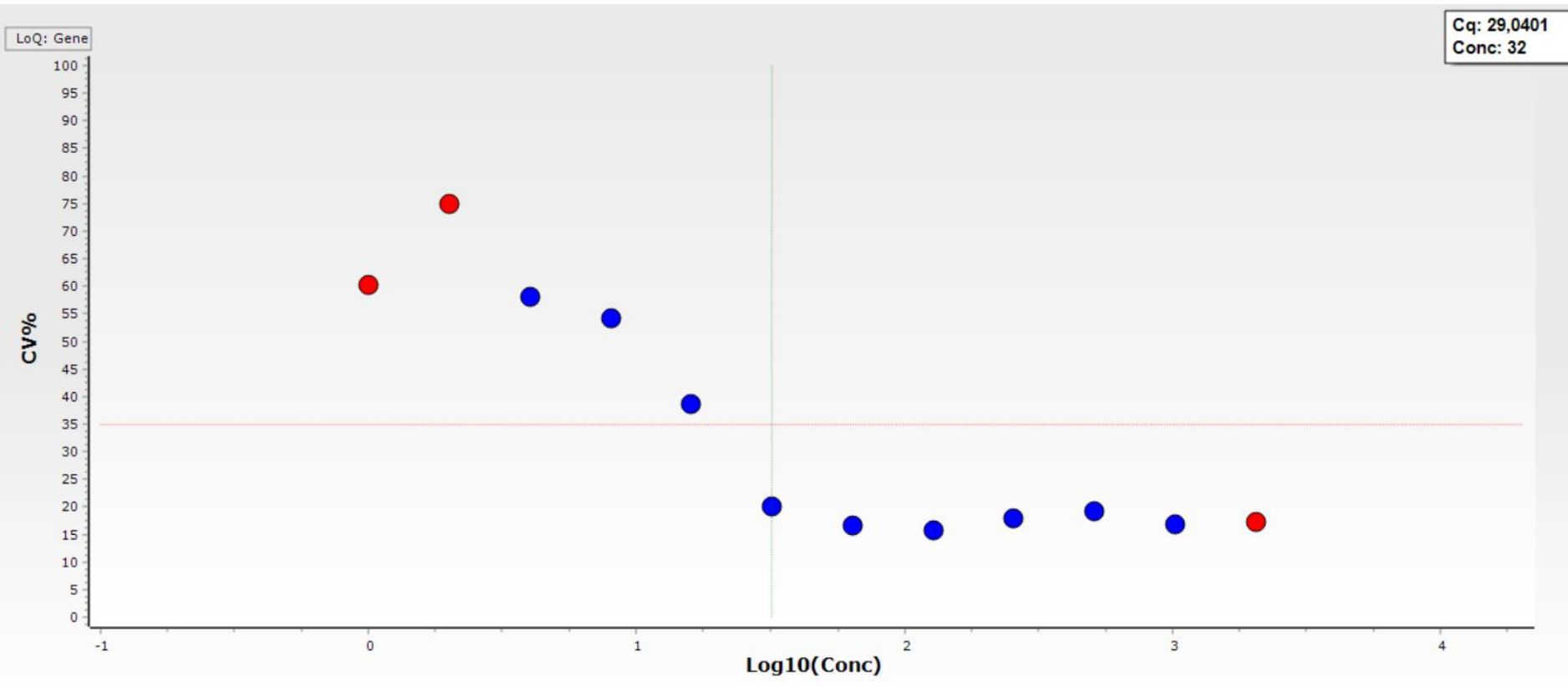


# Limit of quantification (LoQ)

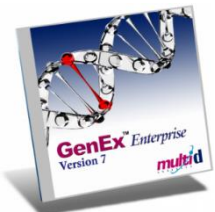
The LLOQ is the lowest concentration at which the target analyte can be quantified with demonstrated precision and accuracy within established acceptance criteria



# Limit of Quantification



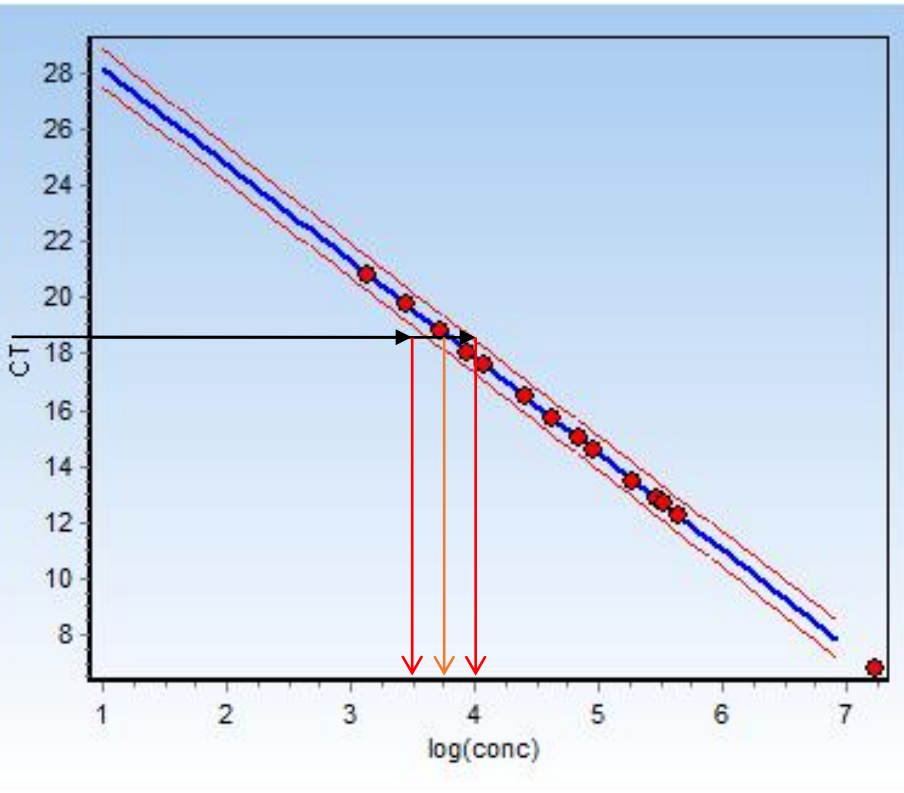
Lowest concentration when back calculated relative error in less than agreed specification, typically round 35 %



A. Frootan, R. Sjöback, J. Björkman, B. Sjögreen, L. Linz, M. Kubista. Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification*. Vol 12, 1-6 (2017)



# Prediction of unknowns



Logarithmic scale

CI(min)	Log10(Conc)	CI(max)
4.43918727...	4.59537671...	4.75156616...
3.79500779...	3.92024884...	4.04548989...
3.01480052...	3.13847172...	3.26214292...
4.23422830...	4.37826930...	4.52231031...
4.73327228...	4.90961112...	5.08594996...
3.31441614...	3.43366162...	3.55290710...
4.63641856...	4.80581855...	4.97521853...
5.24611234...	5.46285413...	5.67959592...
3.91572240...	4.04499038...	4.17425836...
5.02868575...	5.22765444...	5.42662312...
3.57977138...	3.70028476...	3.82079814...
5.20743361...	5.42095621...	5.63447881...
6.78429868...	7.14067544...	7.49705220...
5.36729201...	5.59426125...	5.82123049...

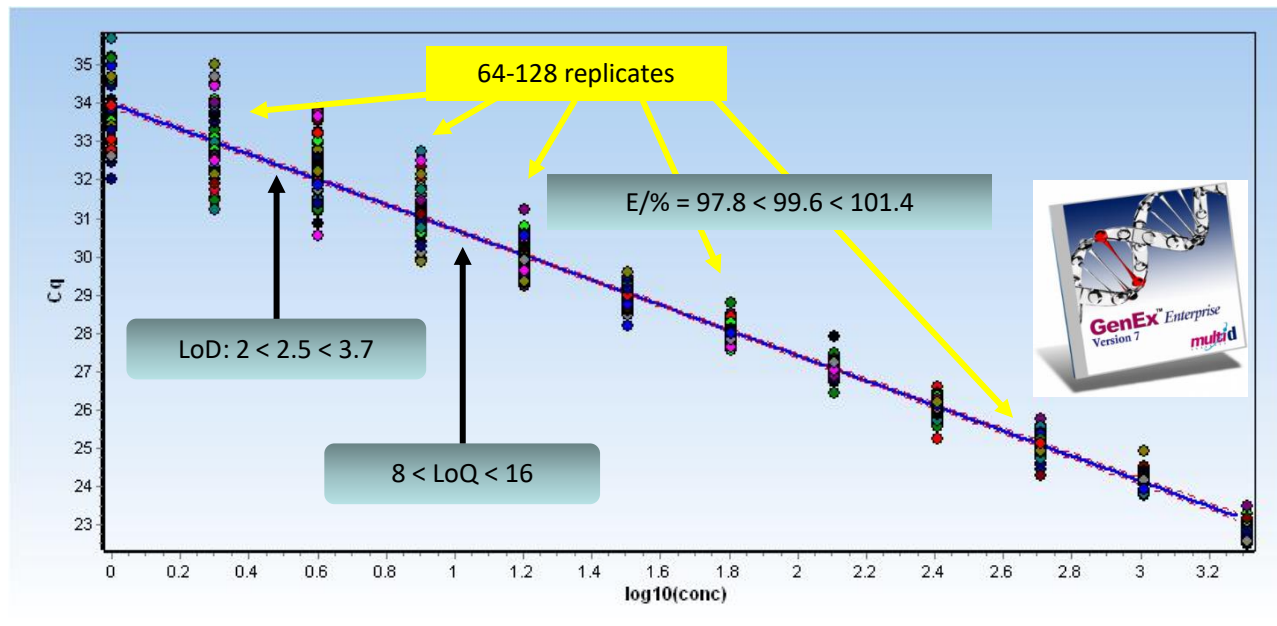
Linear scale

CI(min)	Conc.	CI(max)
27490,7935...	39389,1599...	56437,2912...
6237,46036...	8322,40503...	11104,2670...
1034,66682...	1375,53524...	1828,70192...
17148,5855...	23892,9243...	33289,7329...
54109,3465...	81210,3026...	121884,917...
2062,60537...	2714,32361...	3571,96425...
43293,0884...	63946,7609...	94453,6038...
176243,191...	290304,746...	478184,975...
8236,11509...	11091,5026...	14936,8275...
106828,162...	168909,642...	267068,782...
3799,89310...	5015,15962...	6619,08779...
161225,456...	263606,561...	431001,535...
6085533,86...	13825328,0...	31408862,3...
232965,717...	392881,206...	662568,054...

Symmetric error bars Asymmetric error bars

# ValidPrime standard assay

ValidPrime targets a unique non-transcribed conserved locus present in one copy per haploid human genome



[Nucleic Acids Research, Volume 40, Issue 7, 1 April 2012, Page e51 Correction of RT-qPCR data for genomic DNA-derived signals with ValidPrime. Henrik Laurell, Jason S. Iacovoni, Anne Abot, David Svec, Jean-José Maoret, Jean-François Arnal, Mikael Kubista](#)

[Methods to determine limit of detection and limit of quantification in quantitative real-time PCR \(qPCR\). Forootan, Sjöback, Björkman, Sjögreen, Linz, Kubista. Biomolecular Detection and Quantification 12 \(2017\) 1-62.](#)

## TECHNOLOGY FEATURE

Digital PCR on chips	542
Digital PCR in droplets	543
Thinking digitally	544

## Digital PCR hits its stride

Monya Baker



As the less familiar cousin of quantitative PCR moves mainstream, researchers have more options to choose from

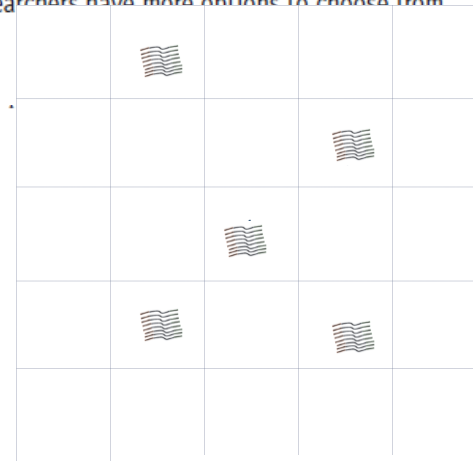


The most common applications of digital PCR at the TATAA Biocenter are standardizing qPCR assays, detecting copy number variations, detecting rare mutations and distinguishing differences between expression of nearly identical alleles, says Mikael Kubista.

ing blood<sup>4</sup>.

The concept behind digital PCR was first described in 1992 (ref. 5). A few years later, Bert Vogelstein and Ken Kinzler at Johns Hopkins University named the technique and showed that it could be used to quantify disease-associated mutations in stool from patients with colorectal cancer. But although the theory was simple, its implementation was not. Initial demonstrations were performed in commercially available 384-well plates with 5 microliters per partition, requiring volumes of reagents that would daunt most researchers<sup>6</sup>.

sensitivity and precision. In standard implementations, qPCR cannot distinguish gene expression differences or copy number variants smaller than about twofold. Identifying alleles with frequencies of less than about 1% is difficult because such tests would also detect highly abun-



Digital PCR works by diluting the number of partitions



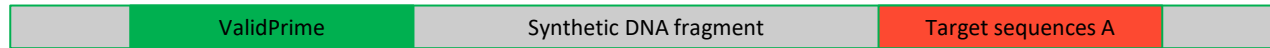
and counting up

TATAA Biocenter

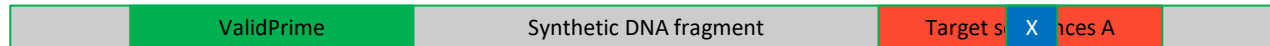
UNE 2012 | 541

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# ValidPrime for validation of new assays



Concentration determined with ValidPrime



- Synthetic dsDNA fragment (250 – 3000 bp) with ValidPrime and target sequences.
- Concentration of the fragment is measured by dPCR using the ValidPrime assay.
- Target assay sensitivity is validated using known amount of the fragment in dPCR.
- Multiple targets can be validated in a single fragment
- A second fragment with variant sequence can be designed to test assay specificity

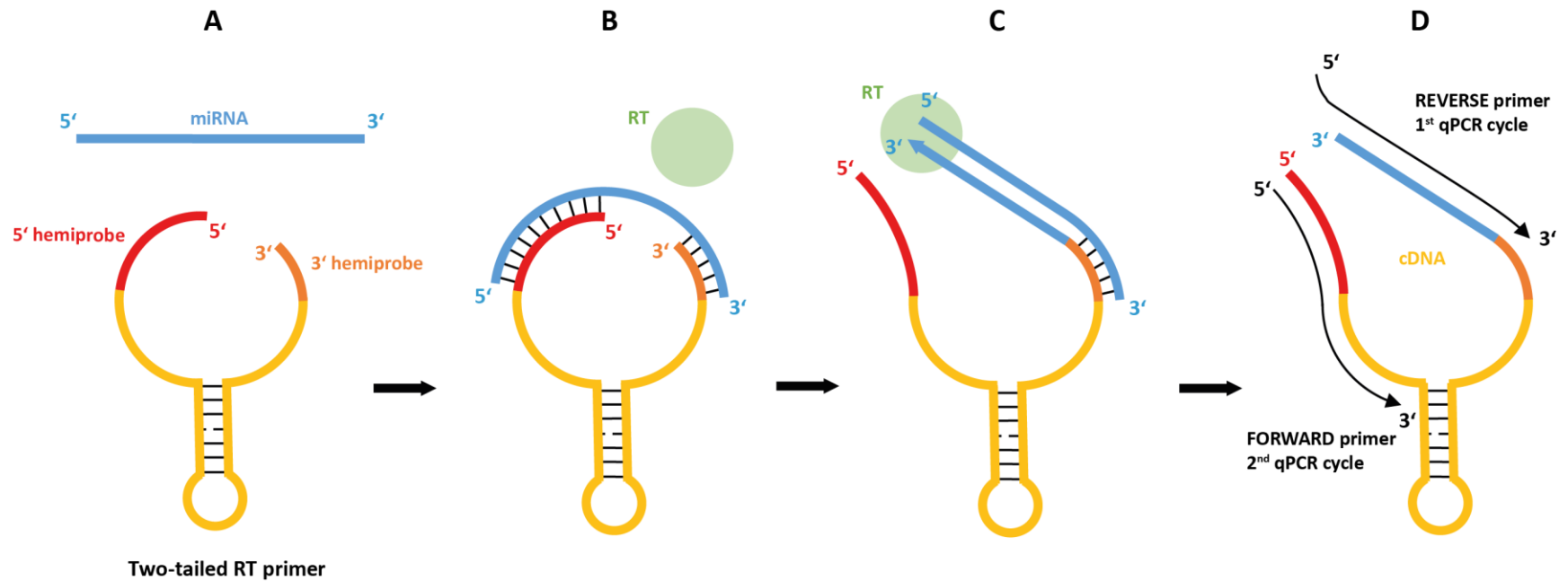
# Two-tailed PCR for short targets

**5'**  
Hemiprobe



**3'**  
Hemiprobe

# 2-Tailed PCR





# Two-tailed RT-qPCR: a novel method for highly accurate miRNA quantification

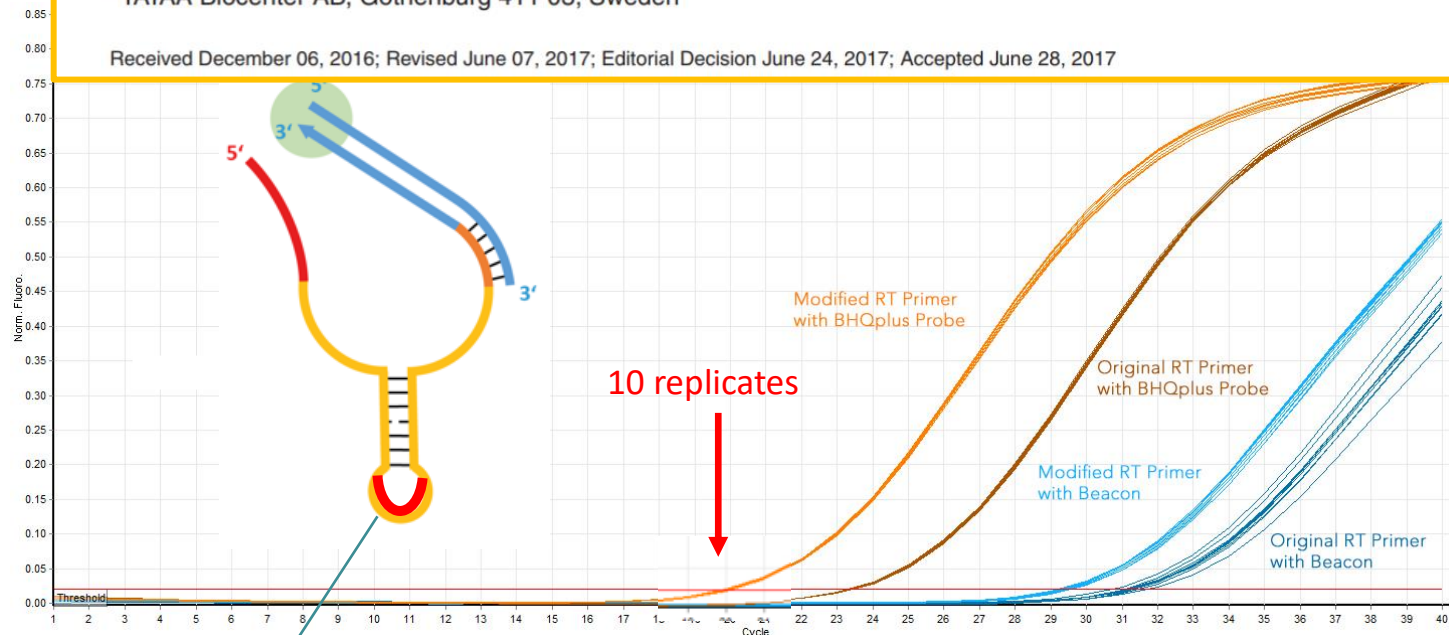
Peter Androvic<sup>1,2</sup>, Lukas Valihrach<sup>1</sup>, Julie Elling<sup>3</sup>, Robert Sjoback<sup>3</sup> and Mikael Kubista<sup>1,3,\*</sup>

<sup>1</sup>Laboratory of Gene Expression, Institute of Biotechnology CAS, Biocev, Vestec 252 50, Czech Republic,

<sup>2</sup>Laboratory of Growth Regulators, Faculty of Science, Palacky University, Olomouc 783 71, Czech Republic and

<sup>3</sup>TATAA Biocenter AB, Gothenburg 411 03, Sweden

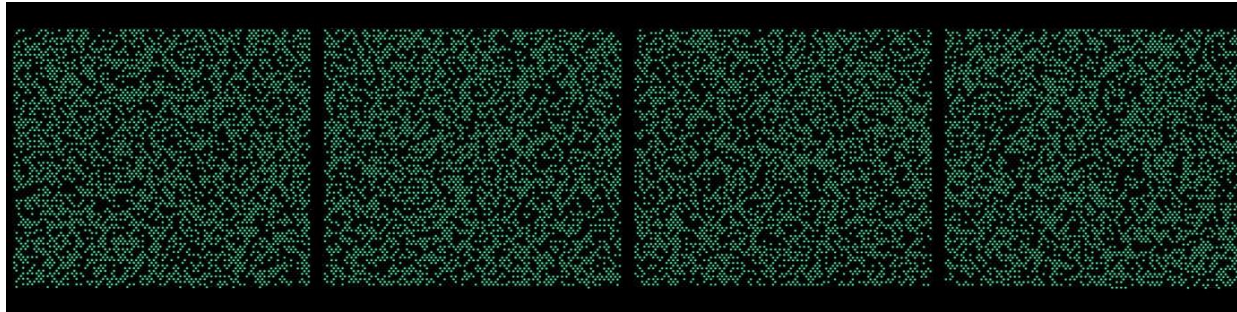
Received December 06, 2016; Revised June 07, 2017; Editorial Decision June 24, 2017; Accepted June 28, 2017



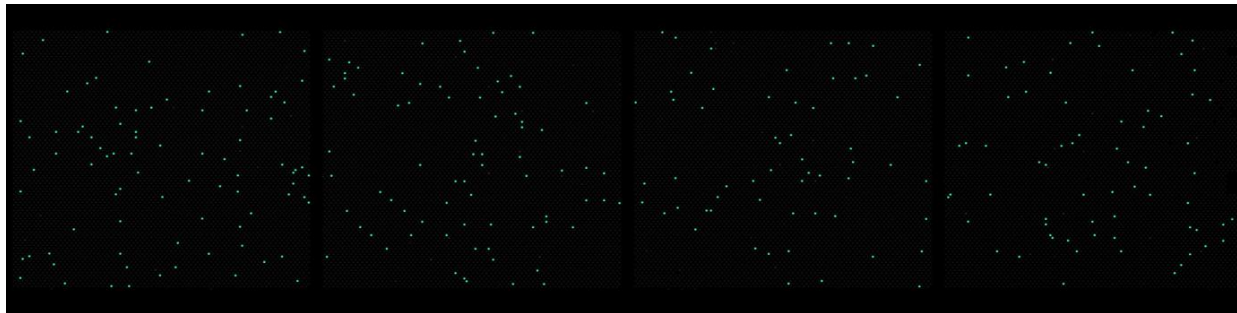
Generic probe



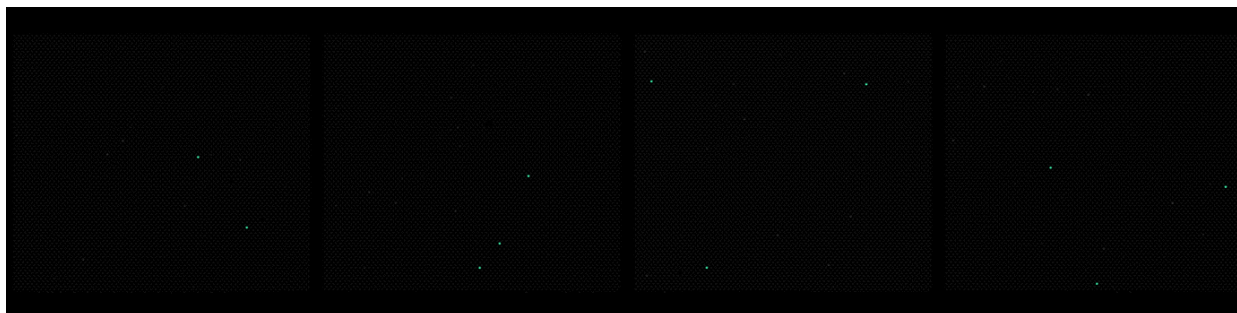
# 2T dPCR for KRAS mutations



50 %

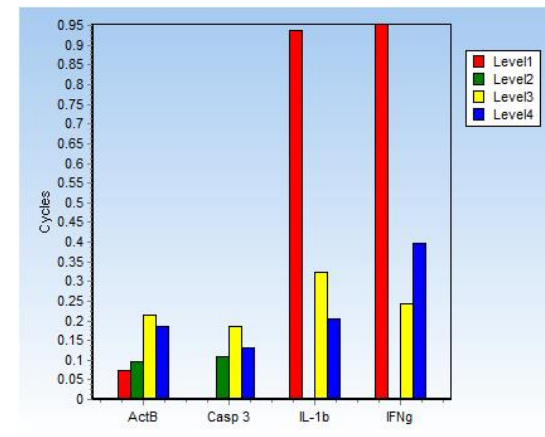
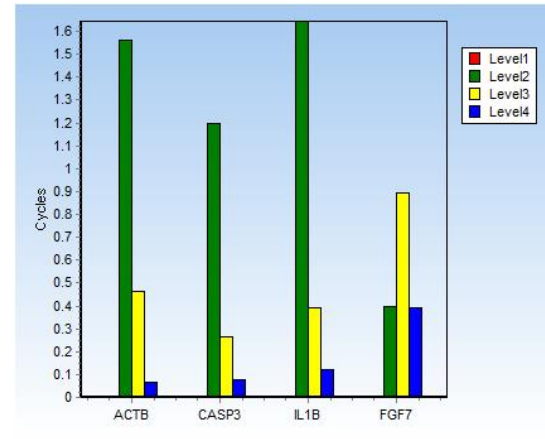
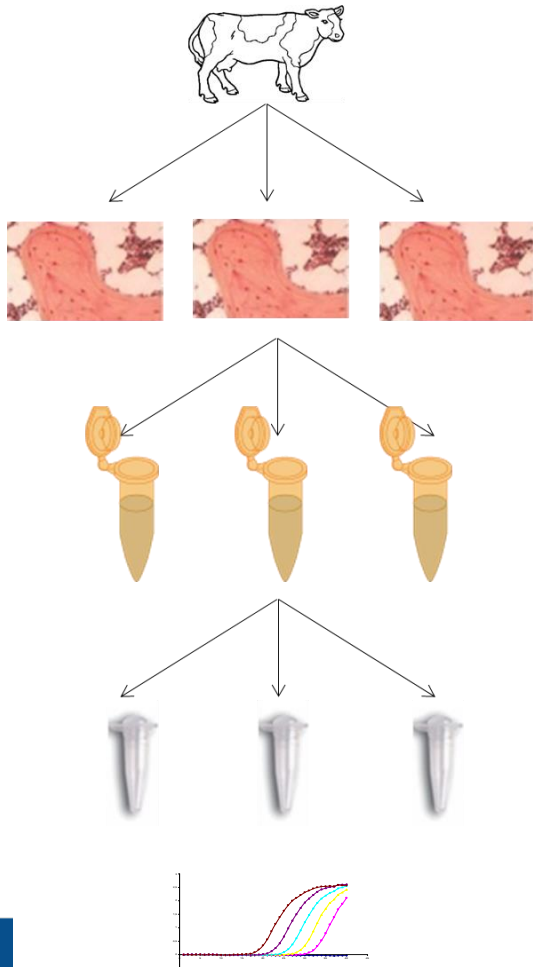


1 %

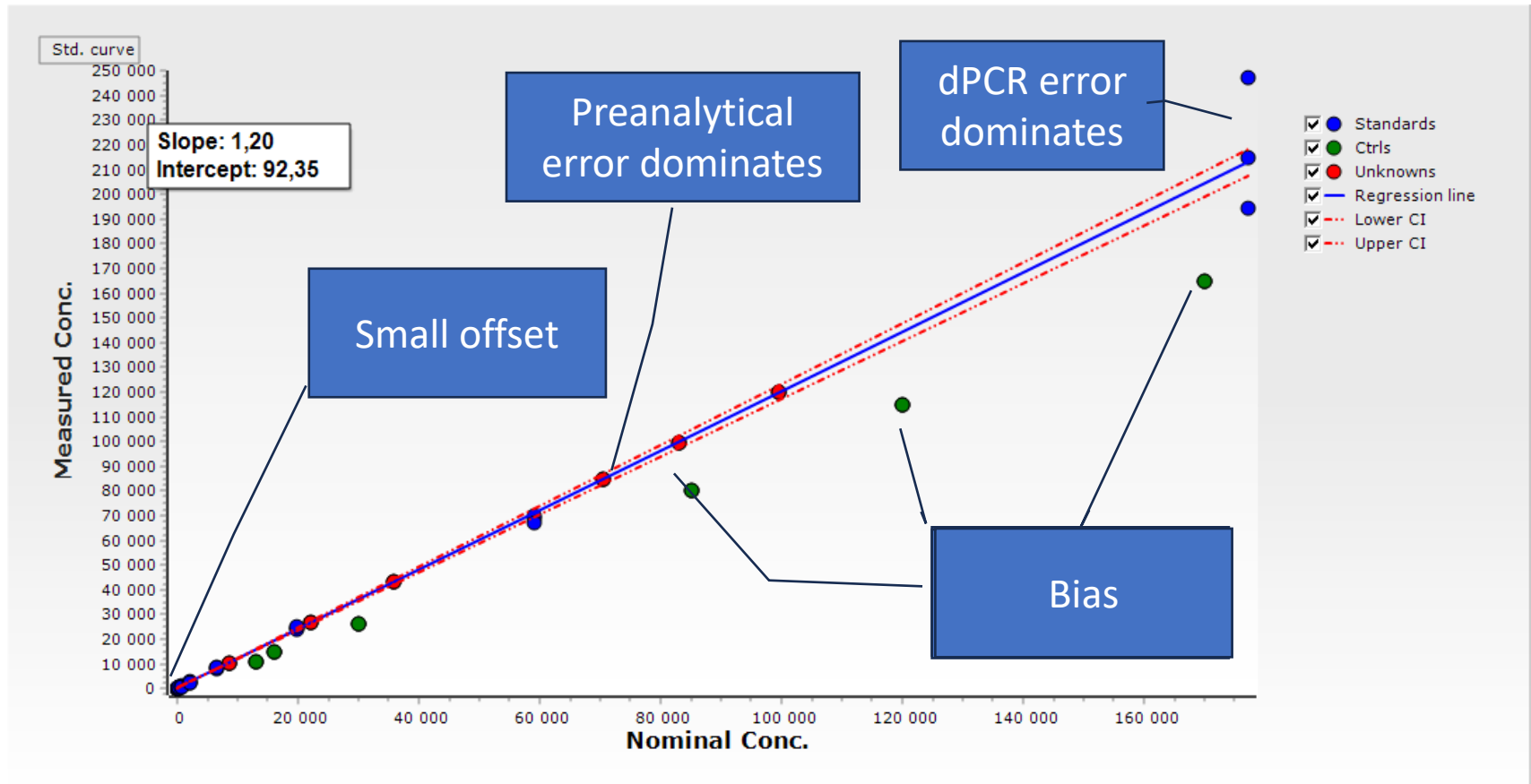


0.1 %

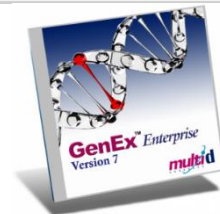
# Optimize the whole workflow



# Standard curve for complete dPCR workflow



Includes the preanalytical process!



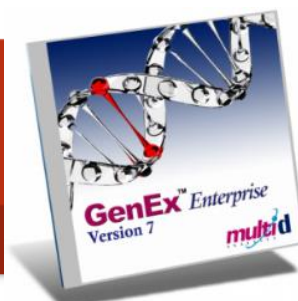
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Stephen A. Bustin,<sup>1\*</sup> Vladimir Benes,<sup>2</sup> Jeremy A. Garson,<sup>3,4</sup> Jan Helleman,<sup>5</sup> Jim Huggett,<sup>6</sup>  
Mikael Kubista,<sup>7,8</sup> Reinhold Mueller,<sup>9</sup> Tania Nolan,<sup>10</sup> Michael W. Pfaffl,<sup>11</sup> Gregory L. Shipley,<sup>12</sup>  
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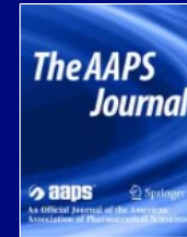
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