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

Professor Mikael Kubista

www.labgenexp.eu
www.ibt.cas.cz/en/core-facilities/gene-core
www.biocev.eu
www.gu.se
www.multid.se
https://goco.se/
https://strategiska.se/en/
https://www.aaps.org/

GoCo Health Innovation City

A health innovation city in Gothenburg, Sweden

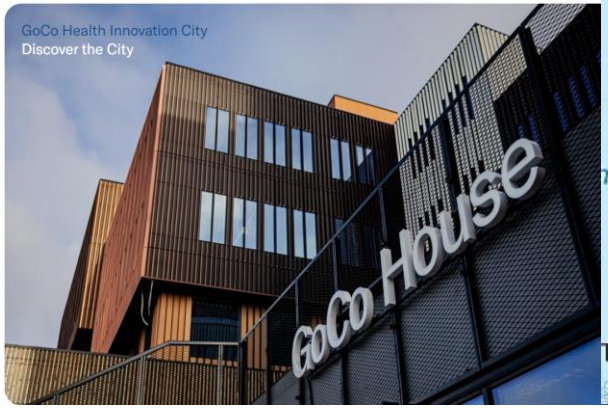
GoCo Health Innovation City is the new urban city center where over 200 000 square meters are being developed to attract researchers, entrepreneurs, and talents from around the world. The vision is to create a world-class innovation cluster for health, where collaboration is built into the core.

AstraZeneca

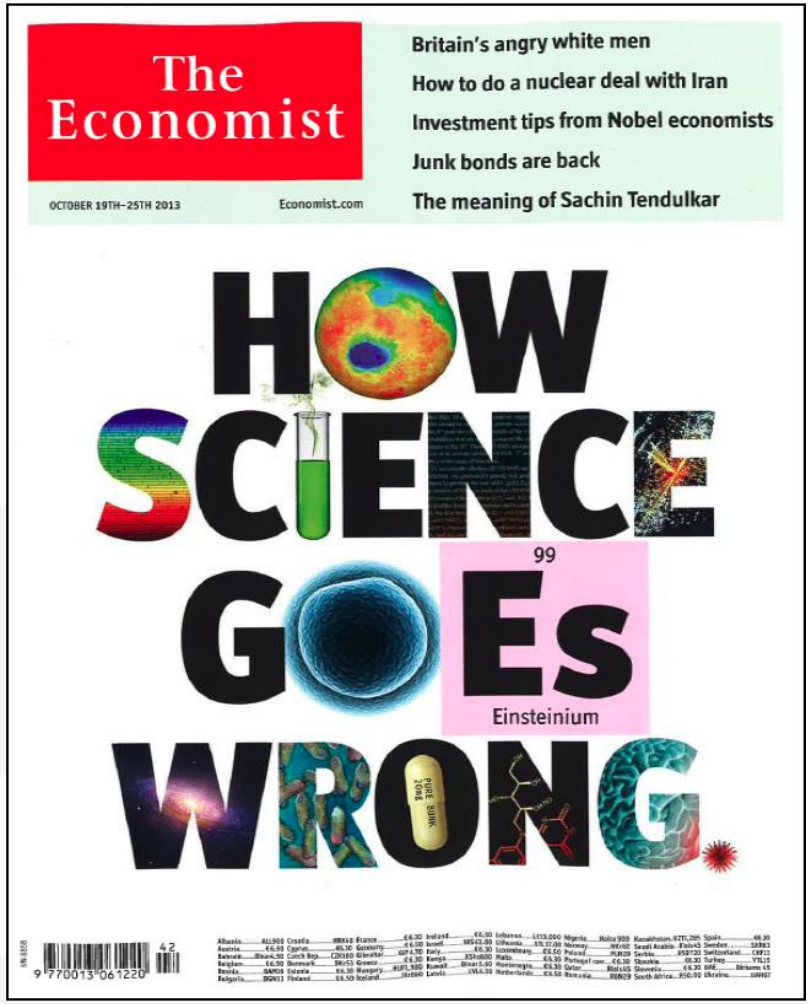
TaKaRa
Clontech TaKaRa cellartis

FUJIREBIO

+60 companies

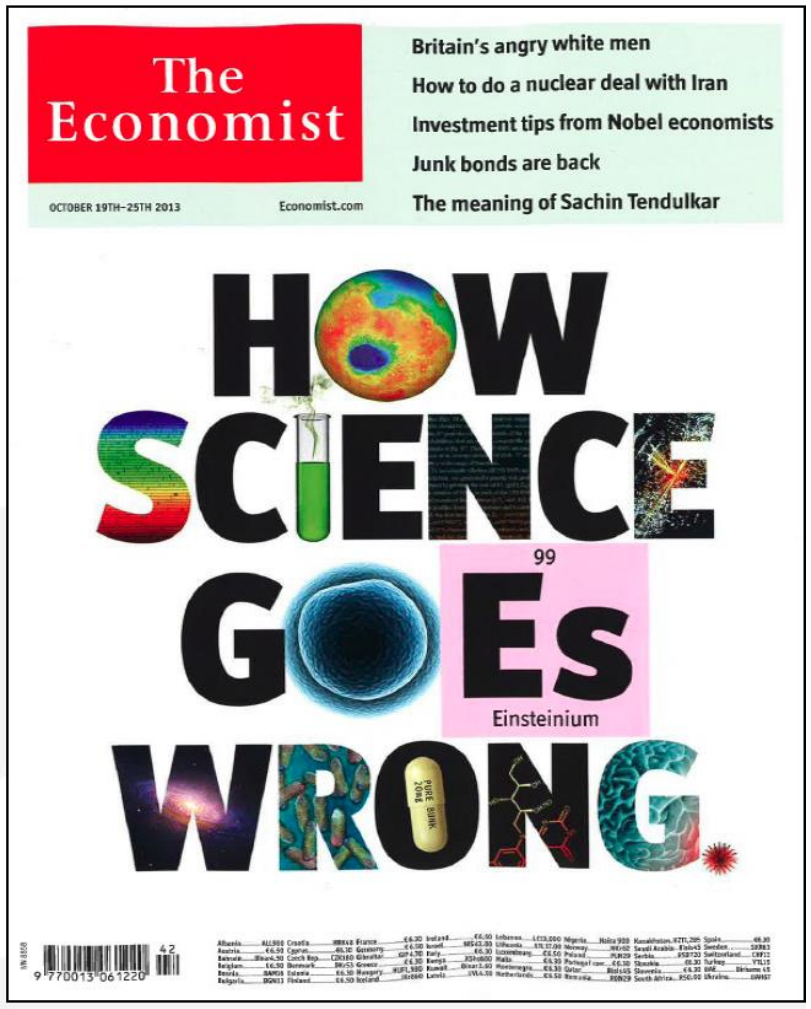


www.goco.se

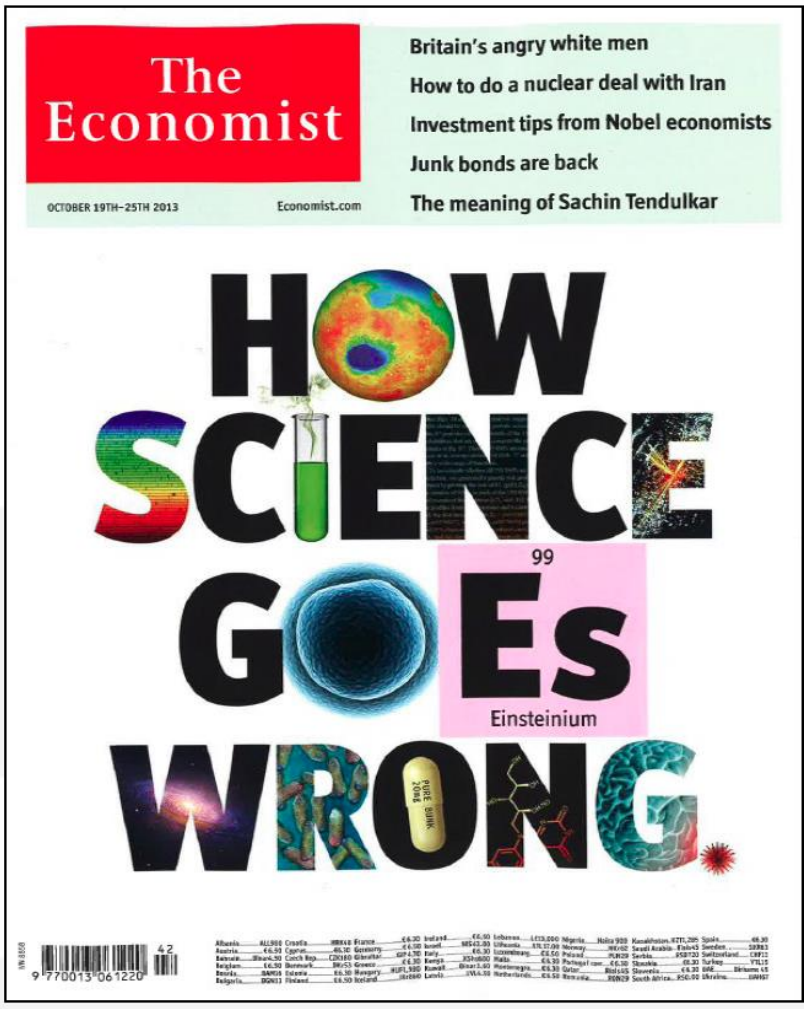


Too many of the findings that fill the academic ether are the result of shoddy experiments or poor analysis (see article). A rule of thumb among biotechnology venture-capitalists is that half of published research cannot be replicated. Even that may be optimistic. Last year researchers at one biotech 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.

multid ANALYSES



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.



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.

SPIDIA4P SPIDIA

ABOUT US PROJECTS NEWS & MEDIA EVENTS & TRAININGS PUBLICATIONS

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 form 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

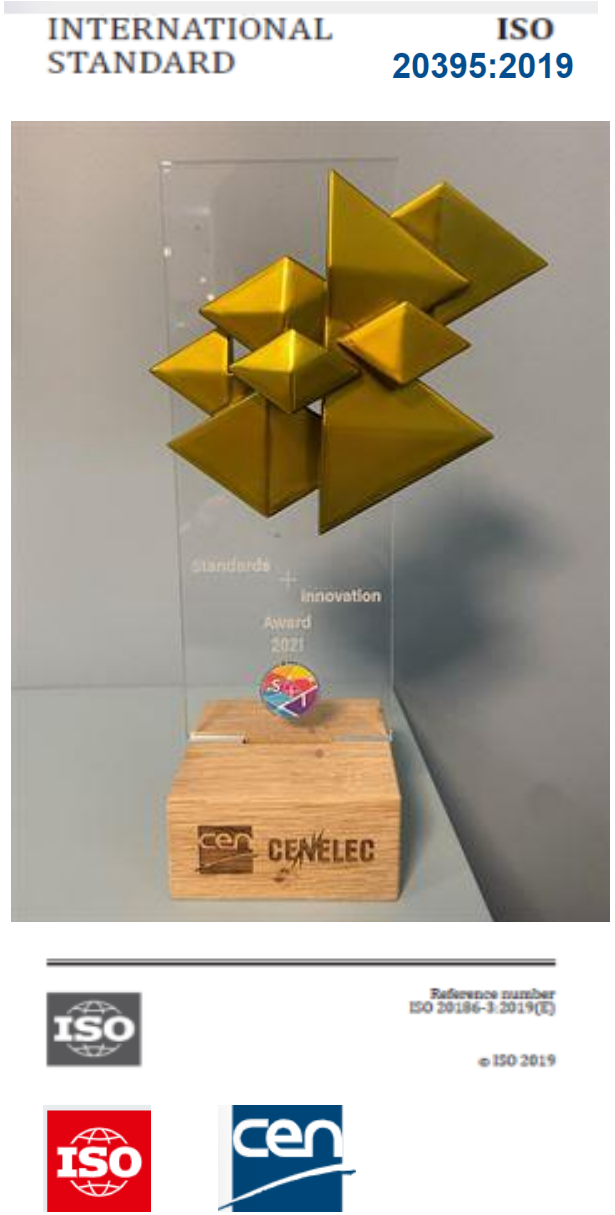
SPIDIA4P

Quality of samples matters – find out, why!

www.spidia.eu

QIAGEN

CEN & ISO Standard Documents and EQAs



- **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.

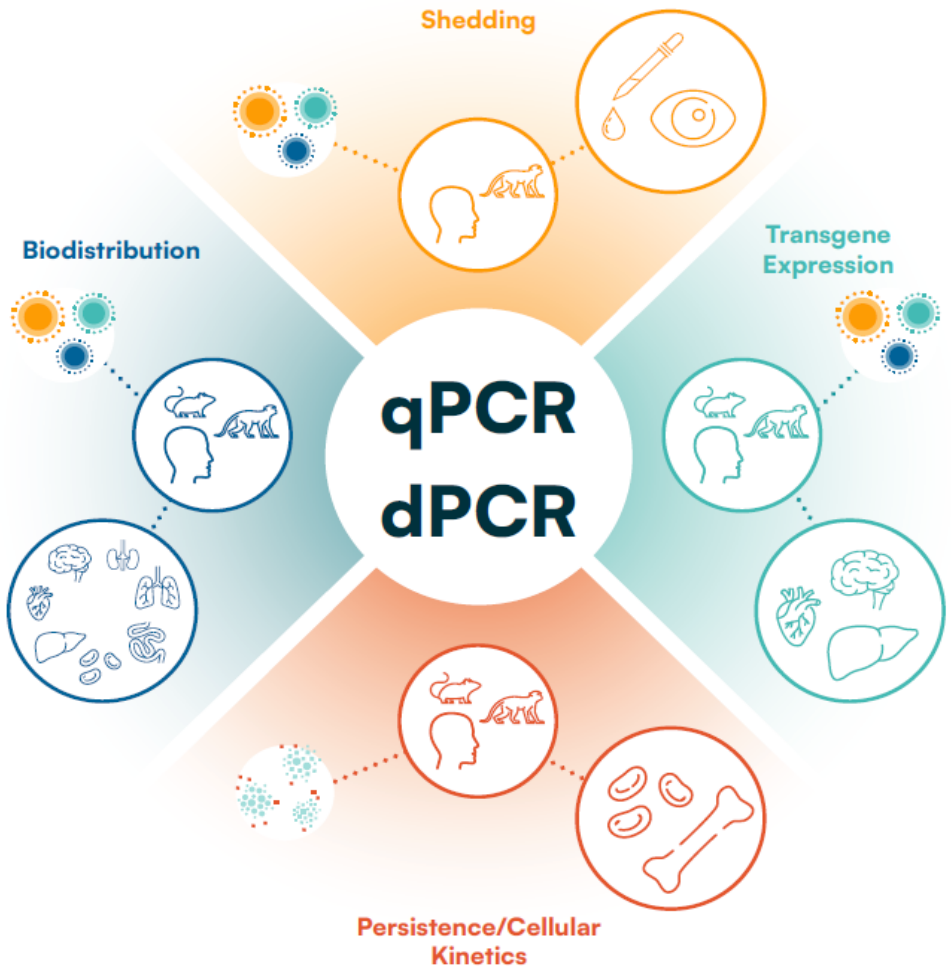
Published CEN ⇒ progressing at ISO

Published ISO

Total:22

QIAGEN

Application of qPCR and dPCR for C&GT



Recommendations from AAPS expert group

UNIVERSITY OF GOTHENBURG

lbt

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.

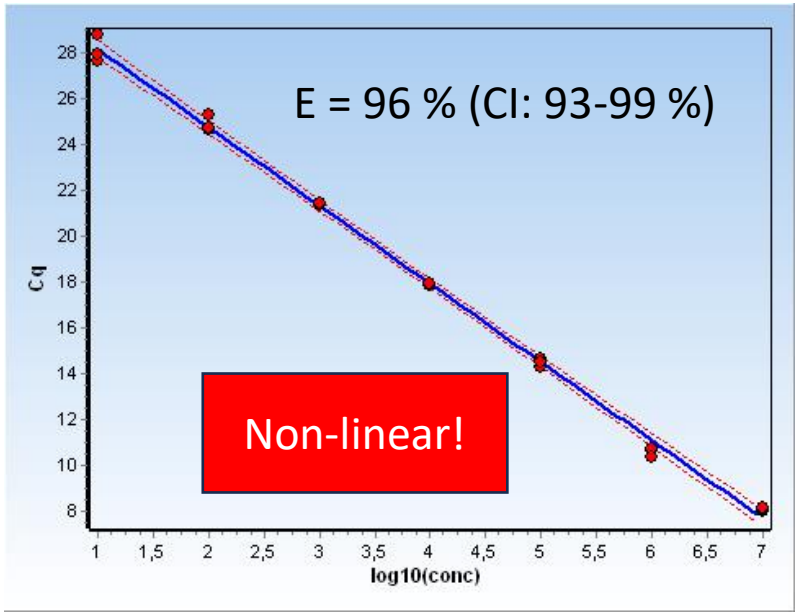
Precision & Accuracy – Validation criteria

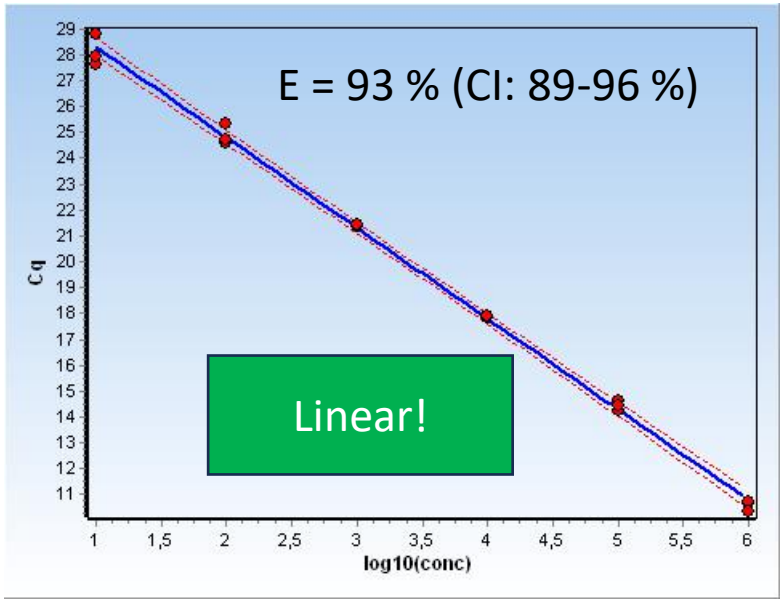
- **Precision and Accuracy** (P&A) should be determined using **spikes of positive control template** in a background matrix of nucleic acid extracted from target tissues or biofluids from the species of interest.
- **Method accuracy** and precision are assessed in validation over **≥6 runs, ≥2 analysts, ≥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.

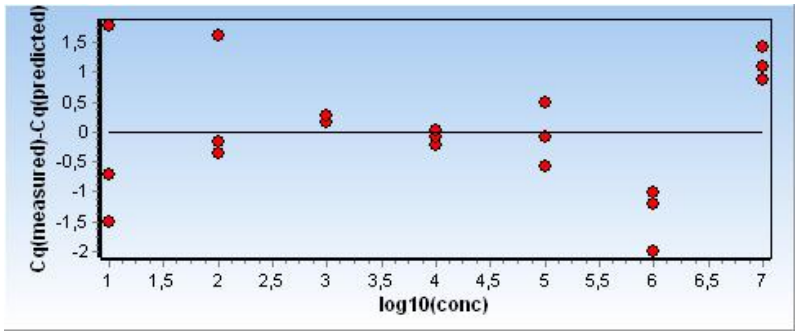
PCR efficiency and linearity

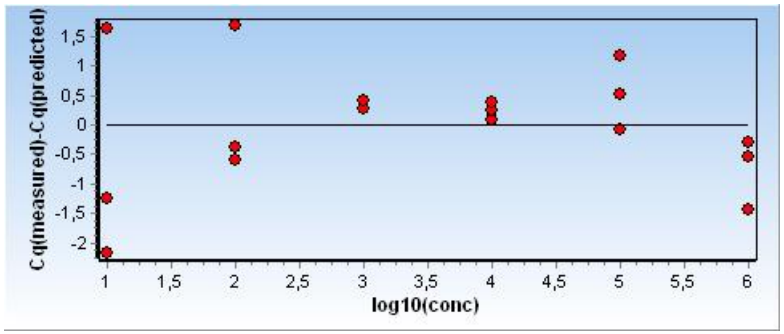
- Intra and inter-assay precision **≤30 % Coefficient of Variation** (%CV) for QCs and ≤50 %CV for LOQs.
- Intra and inter-assay **accuracy of -50 to 100 %RE** for qPCR
- For **dPCR**, the inter-assay accuracy for absolute copies measured should be **RE ≤ 30 %** for QCs (≤ 50 % at LOQ).
- **PCR efficiency** 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 **≥6 runs, ≥1 curve per run, ≥2 analysts, ≥2 days, ≥2 PCR instruments**, when possible.

Linearity









$Y = a + b_1X$

$Y = a + b_1X + b_2X^2$

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

UNIVERSITY OF GOTHENBURG

ibt

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 cam be derived assuming a Normal distribution of positive partitions from blank samples:

$$\mathbf{LOB = Mean_{NTC} + 1.645(SD_{NTC})}$$

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

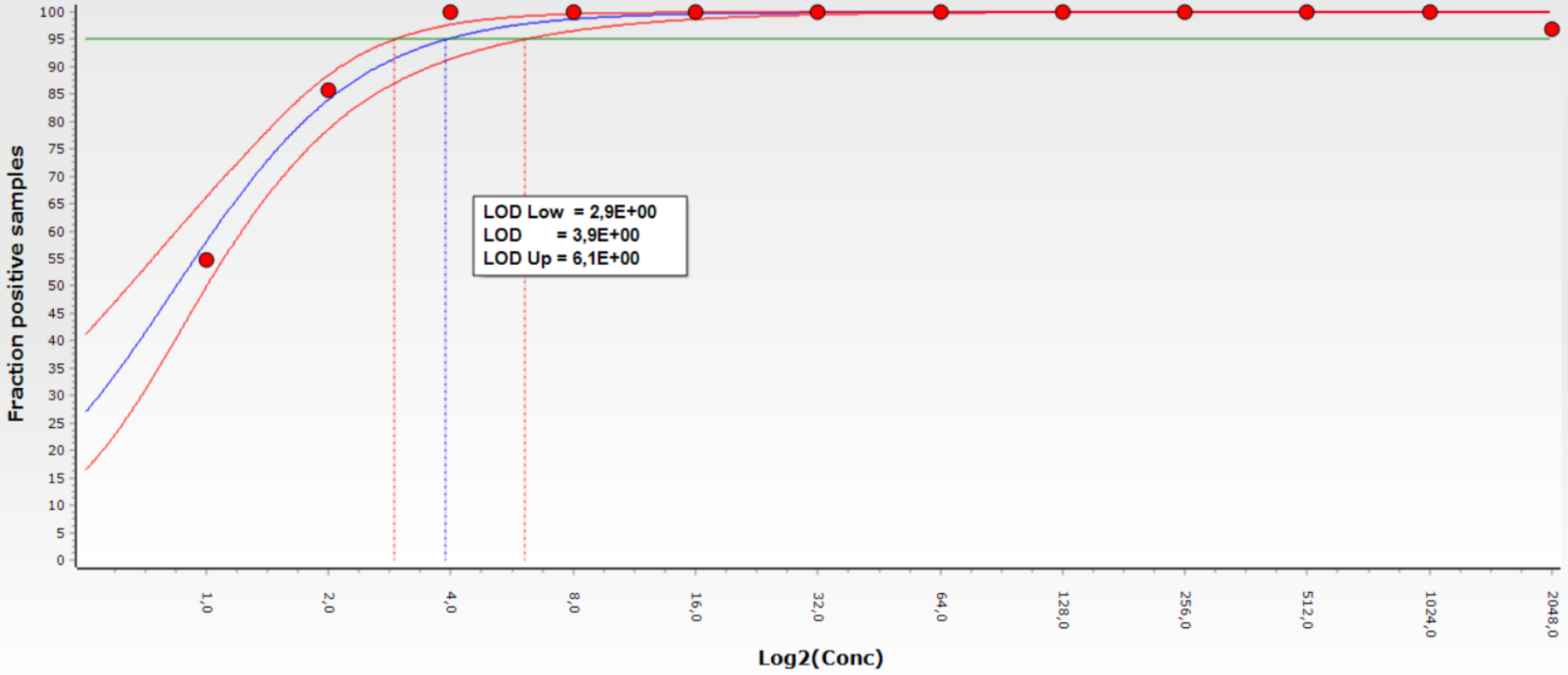
UNIVERSITY OF GOTHENBURG

ibt

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



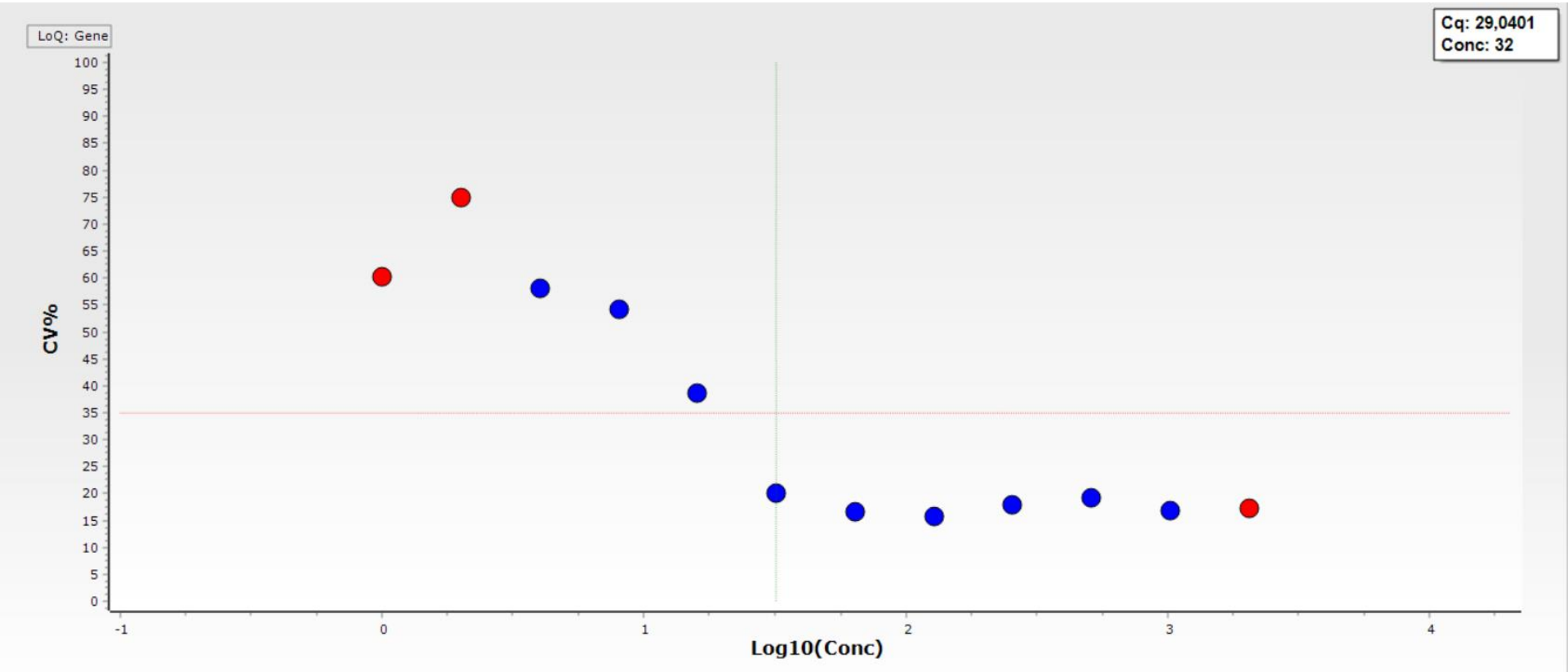
Concentration when 95 % of replicates are positive

A. Forootan, 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)

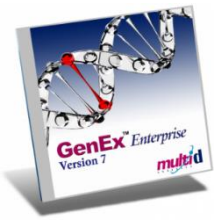
Limit of quantification (LoQ)

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

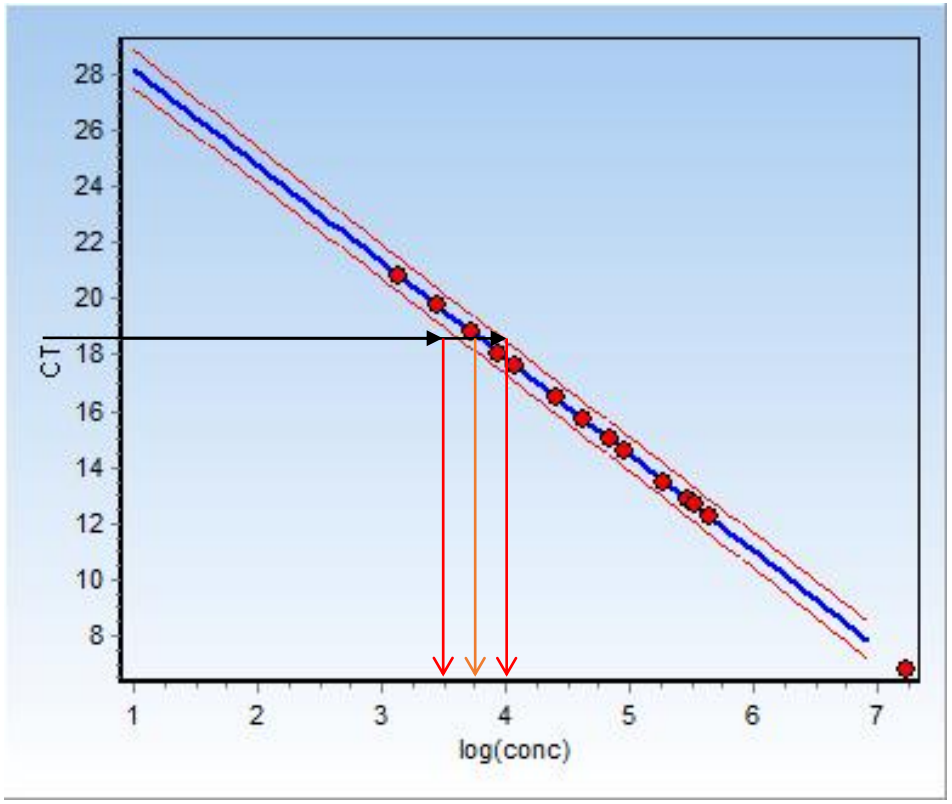


Lowest concentration when back calculated relative error in less than agreed specification, typically round 35 %



A. Forootan, 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...

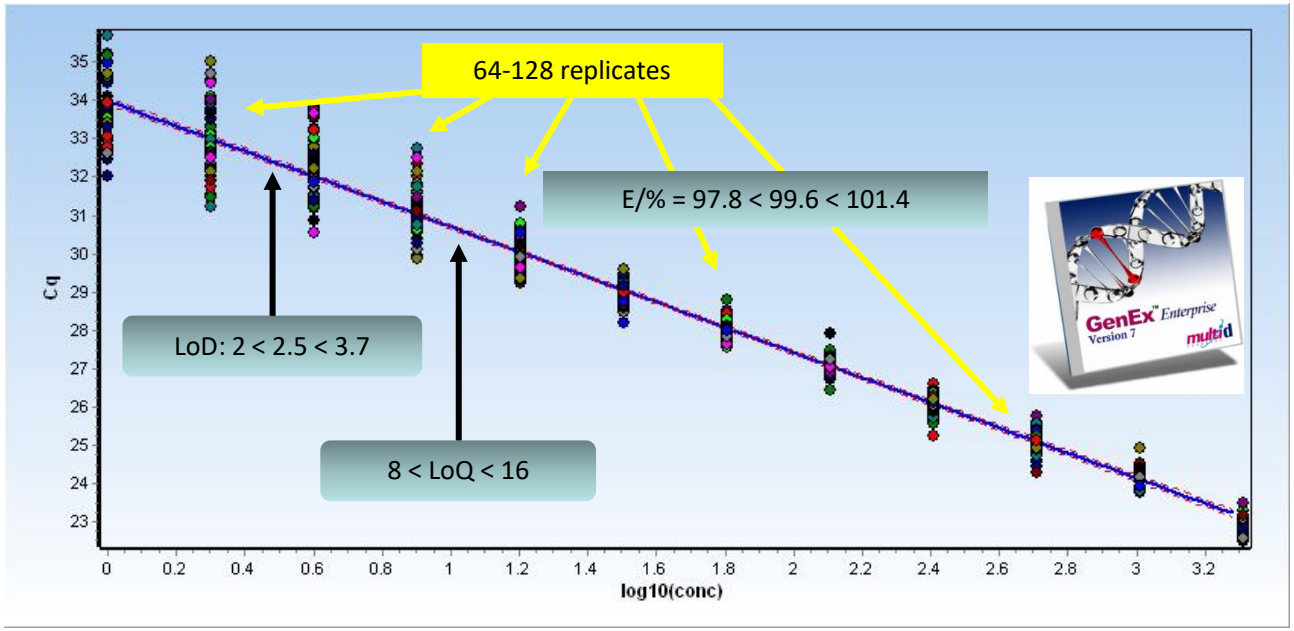
Linear scale

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.

UNIVERSITY OF GOTHENBURG

gibt

Digital PCR

Digital PCR hits its stride

Monya Baker

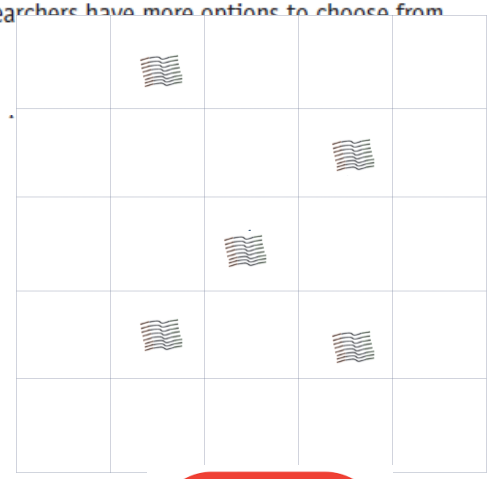
As the less familiar cousin of quantitative PCR moves mainstream, researchers have more options to choose from

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[4].

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[6].

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-



TATAA Biocenter

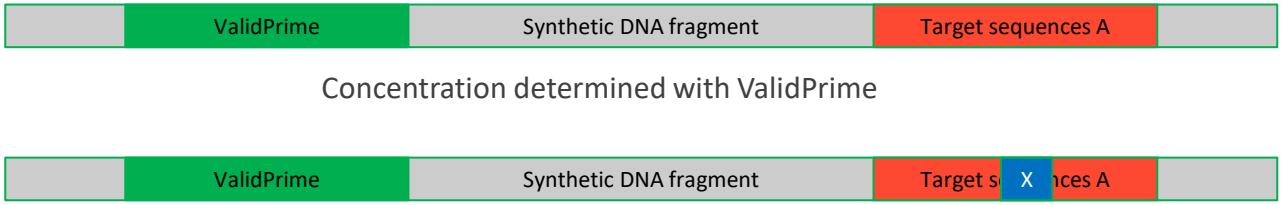
Digital PCR works by dilu … and counting up the number of partitions

5 PLUS

UNIVERSITY OF GOTHENBURG

ilbt

ValidPrime for validation of new assays

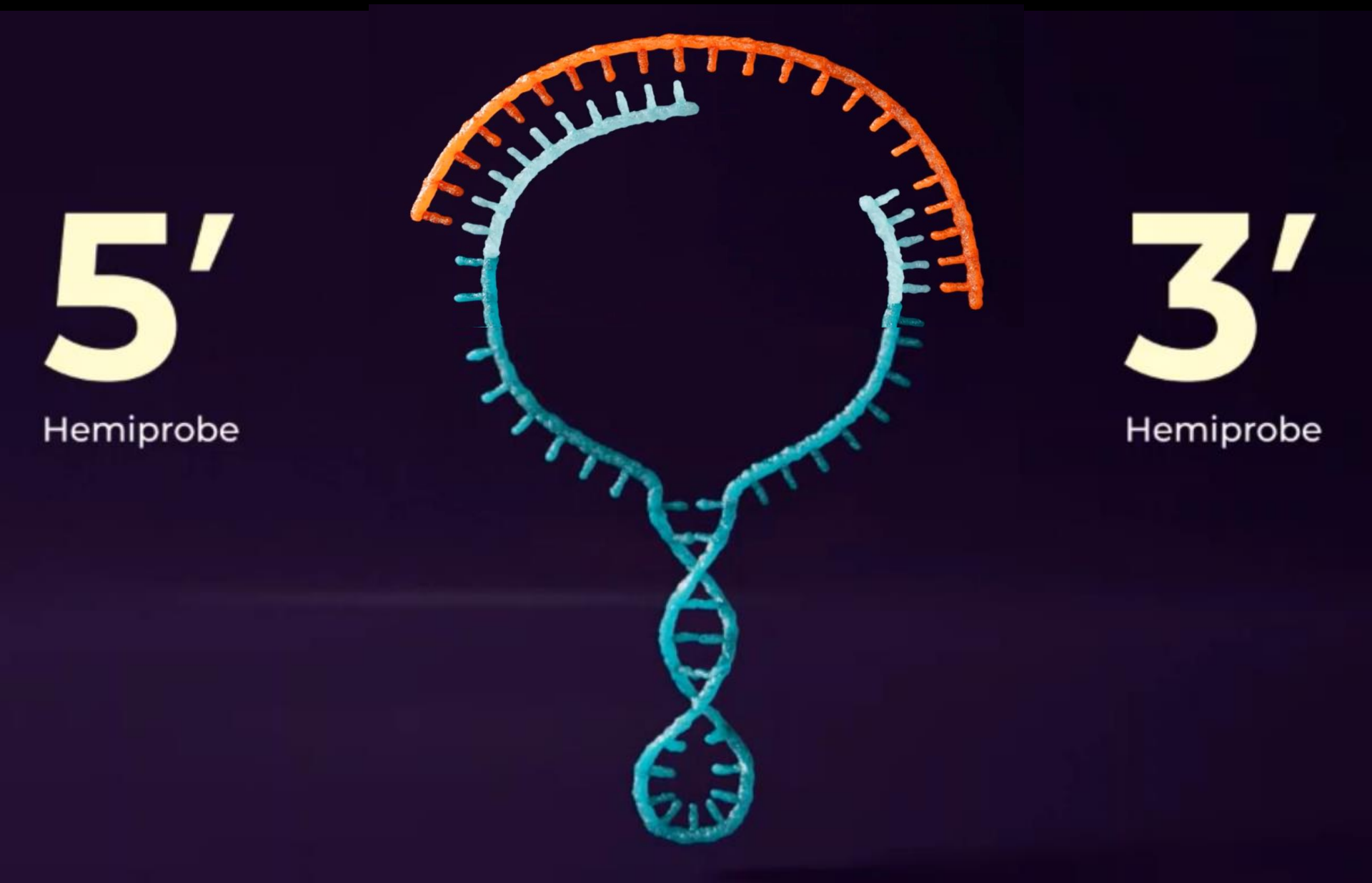


- Synthetic dsDNA fragment (250 – 3000 bp) with ValidPrime and target sequences.
- Concentration of the fragment is measured by dPCR using the ValidPrime assay.
- Target assay sensitivity is validated using known amount of the fragment in dPCR.
- Multiple targets can be validated in a single fragment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

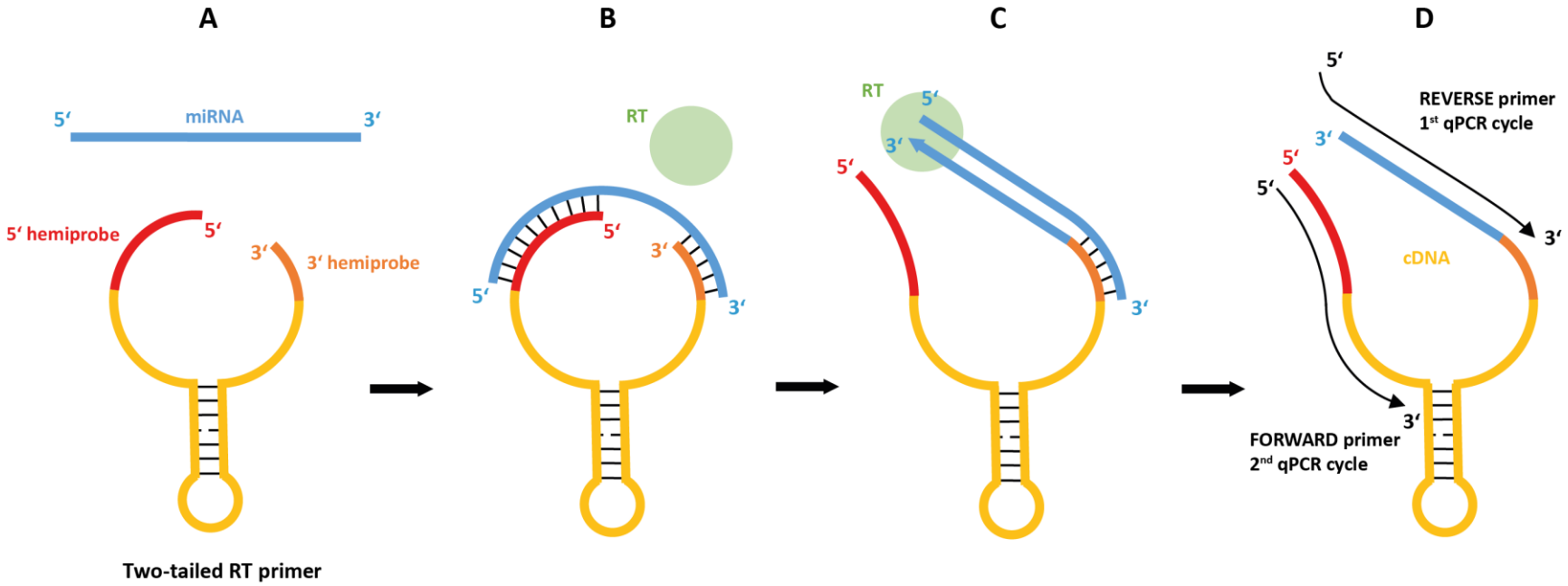
UNIVERSITY OF GOTHENBURG

lbt

Two-tailed PCR for short targets



2-Tailed PCR



UNIVERSITY OF GOTHENBURG

ibt

Multi

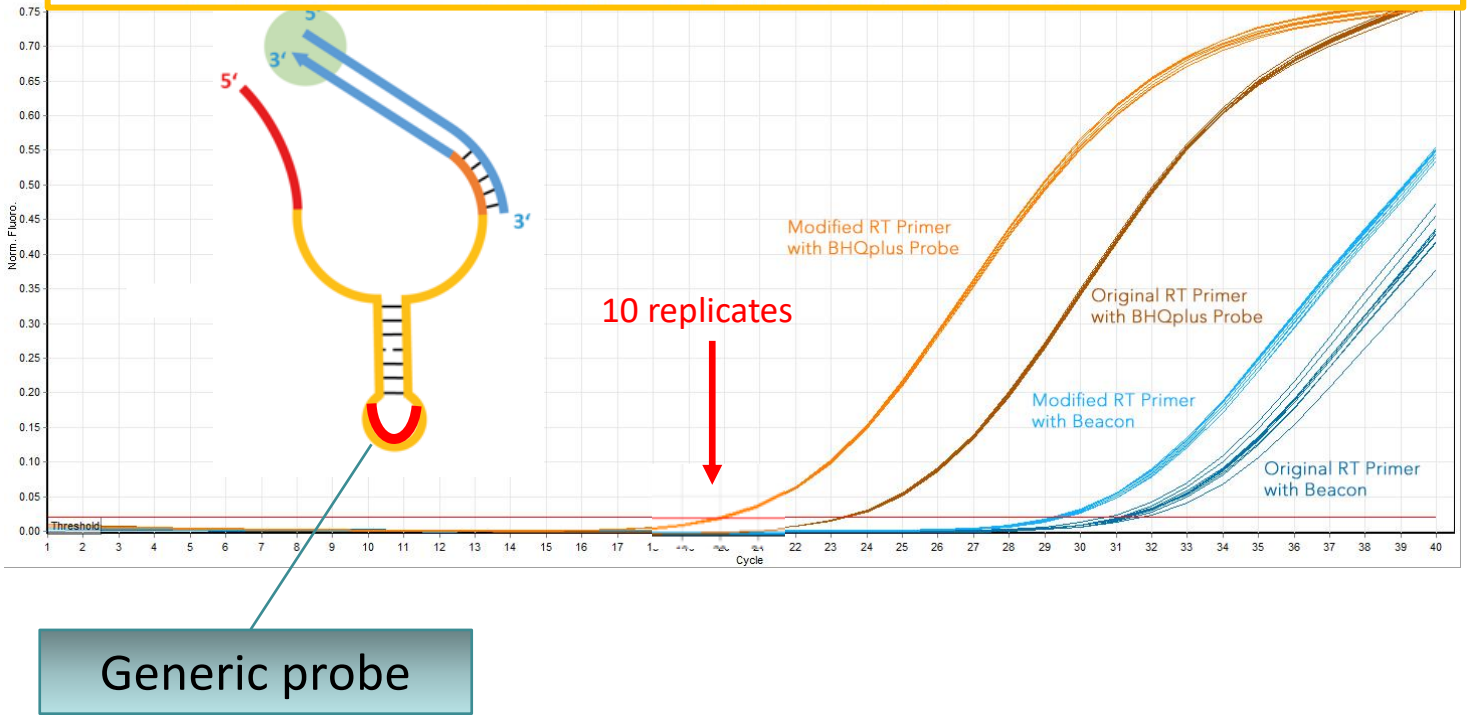
Nucleic Acids Research, 2017 **1**
doi: 10.1093/nar/gkx588

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

Peter Androvic[1,2], Lukas Valihrach[1], Julie Elling[3], Robert Sjoback[3] and Mikael Kubista[1,3,*]

[1]Laboratory of Gene Expression, Institute of Biotechnology CAS, Biocev, Vestec 252 50, Czech Republic,
[2]Laboratory of Growth Regulators, Faculty of Science, Palacky University, Olomouc 783 71, Czech Republic and
[3]TATAA Biocenter AB, Gothenburg 411 03, Sweden

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

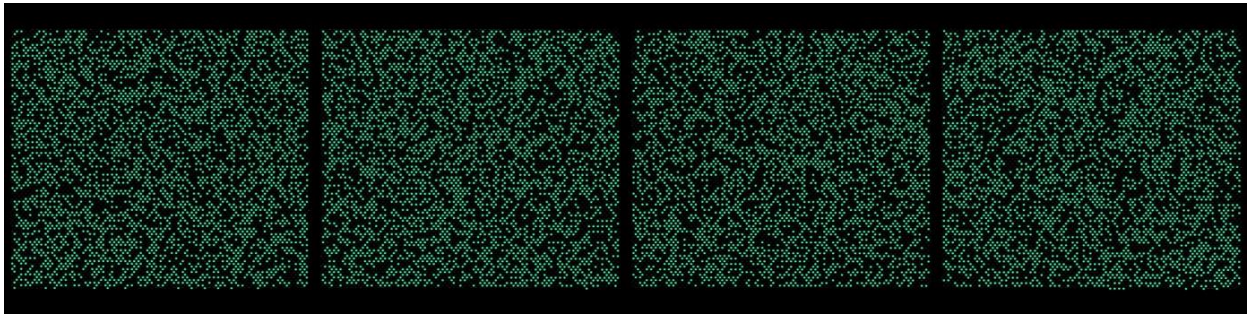


Generic probe

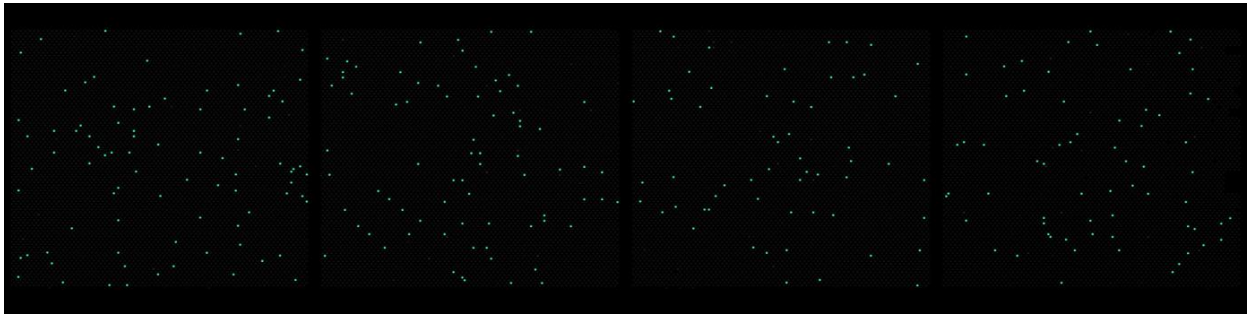
UNIVERSITY OF GOTHENBURG

ibt

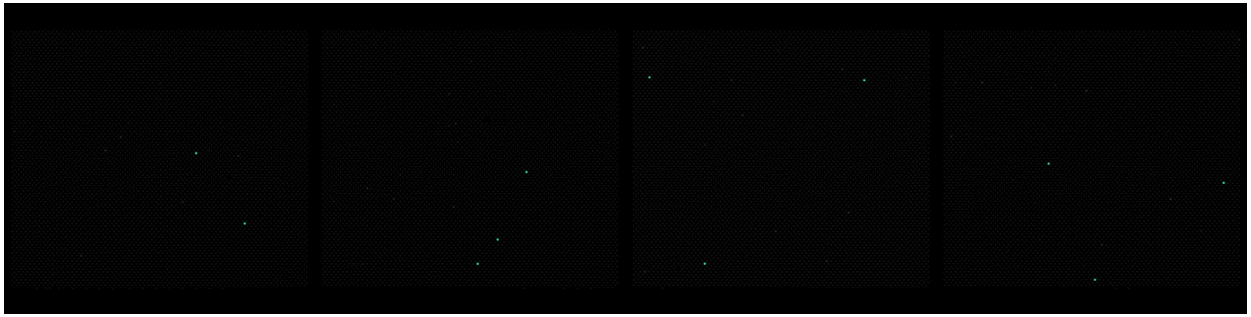
2T dPCR for KRAS mutations



50 %



1 %

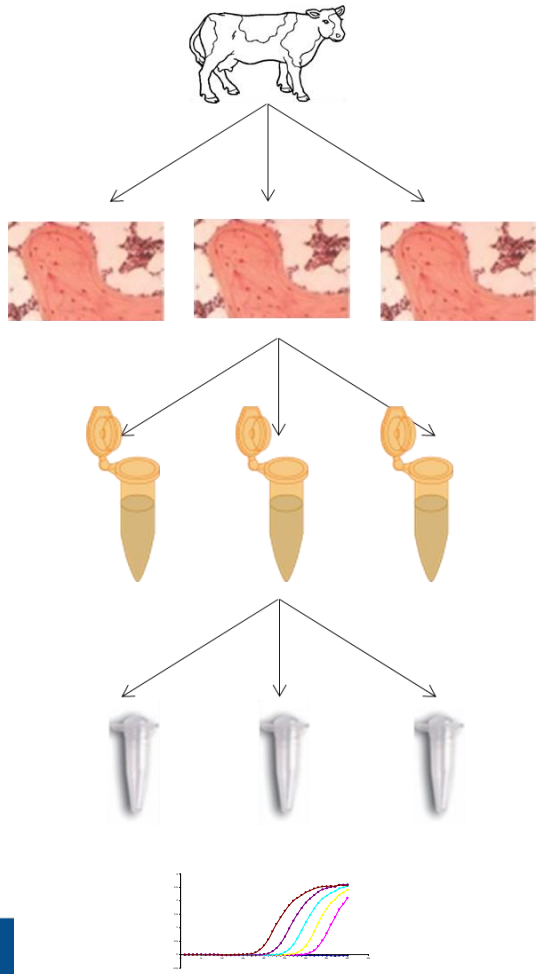


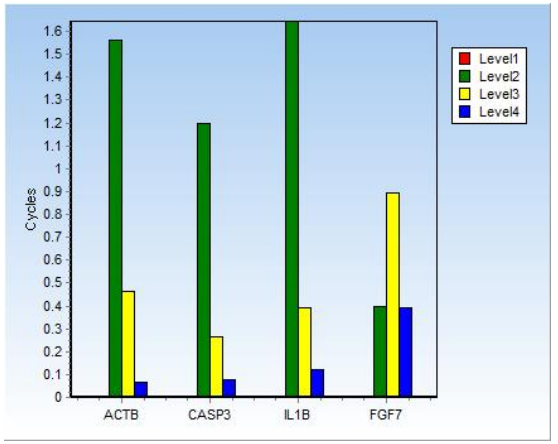
0.1 %

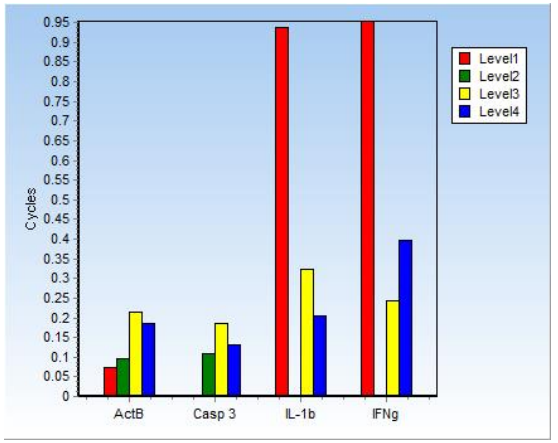
UNIVERSITY OF GOTHENBURG

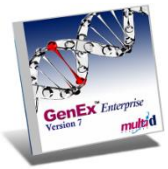
ibt

Optimize the whole workflow



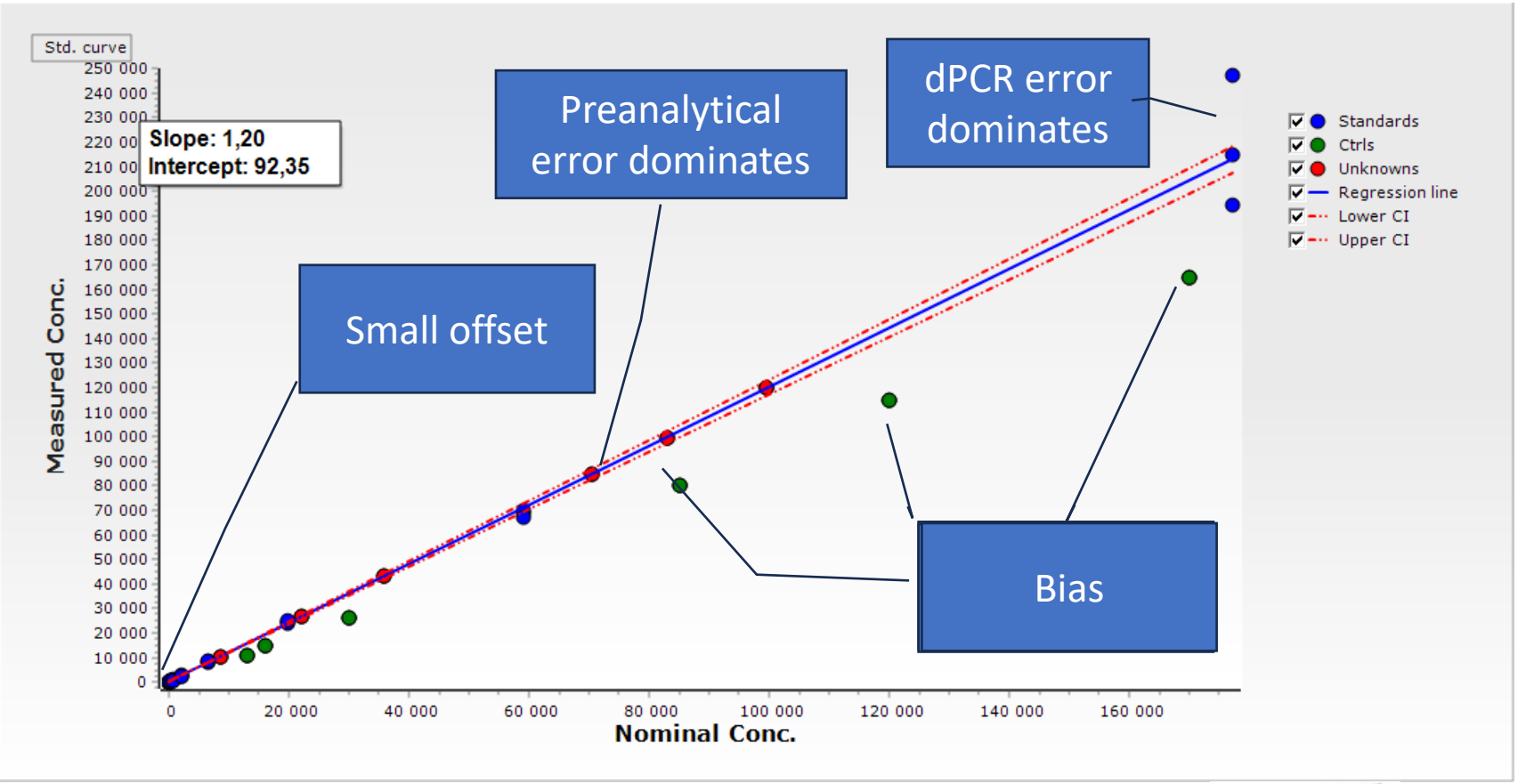




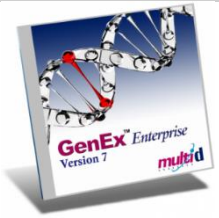


Ales Tichopad, Rob Kitchen, Irmgard Riedmaier, Christiane Becker, Anders Ståhlberg, and Mikael Kubista. Design and Optimization of Reverse-Transcription Quantitative PCR Experiments. Clin. Chem., Jul 2009; doi:10.1373/clinchem.2009.126201

Standard curve for complete dPCR workflow



Includes the preanalytical process!



UNIVERSITY OF GOTHENBURG

www.multid.se

ibt

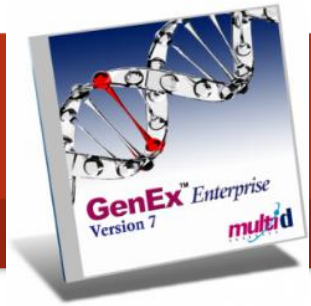
Clinical Chemistry 55:4
611–622 (2009)

15448 citations (3 per day)

Special Report

The MIQE Guidelines: *Minimum Information for Publication of Quantitative Real-Time PCR Experiments*

Stephen A. Bustin,[1*] Vladimir Benes,[2] Jeremy A. Garson,[3,4] Jan Hellemans,[5] Jim Huggett,[6] Mikael Kubista,[7,8] Reinhold Mueller,[9] Tania Nolan,[10] Michael W. Pfaffl,[11] Gregory L. Shipley,[12] Jo Vandesompele,[5] and Carl T. Wittwer[13,14]



Volume 59, Issue 6
1 June 2013

The Digital MIQE Guidelines: *Minimum Information for Publication of Quantitative Digital PCR Experiments* FREE

Jim F Huggett ✉, Carole A Foy, Vladimir Benes, Kerry Emslie, Jeremy A Garson, Ross Haynes, Jan Hellemans, Mikael Kubista, Reinhold D Mueller, Tania Nolan ... Show more

Clinical Chemistry, Volume 59, Issue 6, 1 June 2013, Pages 892–902,

AAPS recommendations

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

White Paper | Emerging Trends in Preclinical and Clinical Development of Cell and Gene Therapies | Open access
Published: 05 February 2024
Volume 26, article number 24, (2024) Cite this article

Amanda Hays, Mark Wissel, Kelly Colletti, Russell Soon, Mitra Azadeh, Justin Smith, Rajitha Doddareddy, Melanie Chalfant, Wendy Adamowicz, Swarna Suba Ramaswamy, Sanjay L. Dholakiya, Sebastian Guelman, Bryan Gullick, Jennifer Durham, Keith Rennier, Pruthvi Nagilla, Anamica Muruganandham, Manisha Diaz, Cassandra Tierney, Kaarthik John, Jenny Valentine, Timothy Lockman, Hsing-Yin Liu, Benjamin Moritz, ... Oskar Johansson Show authors

Part of a collection:
Emerging Trends in Preclinical and Clinical Development of Cell and Gene Therapies