GHENT UNIVERSITY

CRIG

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Can we trust RNA quantification? A case for meticulous standardization.

Jo Vandesompele

March 9, 2026

CCQM workshop

“Metrology for (multi)-omics measurements in clinical diagnostics”

The development path of a diagnostic test

1. analytical validation
   - Does the test measure the biomarker correctly and reliably?
     - accuracy: how close the measurement is to the true value
     - precision / reproducibility: whether repeated measurements give the same result
     - ‘sensitivity’ (LOD, LOQ): the lowest amount that can be detected, or quantified with precision
     - selectivity: whether the assay detects only the intended analyte
     - linearity and dynamic range
     - stability of the analyte during collection, storage, and processing
2. clinical validation
   - Does the biomarker correlate with a clinical condition?
     - sensitivity and specificity for detecting a disease
     - positive and negative predictive value
     - ROC curves and AUC
     - association with prognosis, response to therapy, or disease stage
3. clinical utility
   - Does using the test improve patient care?

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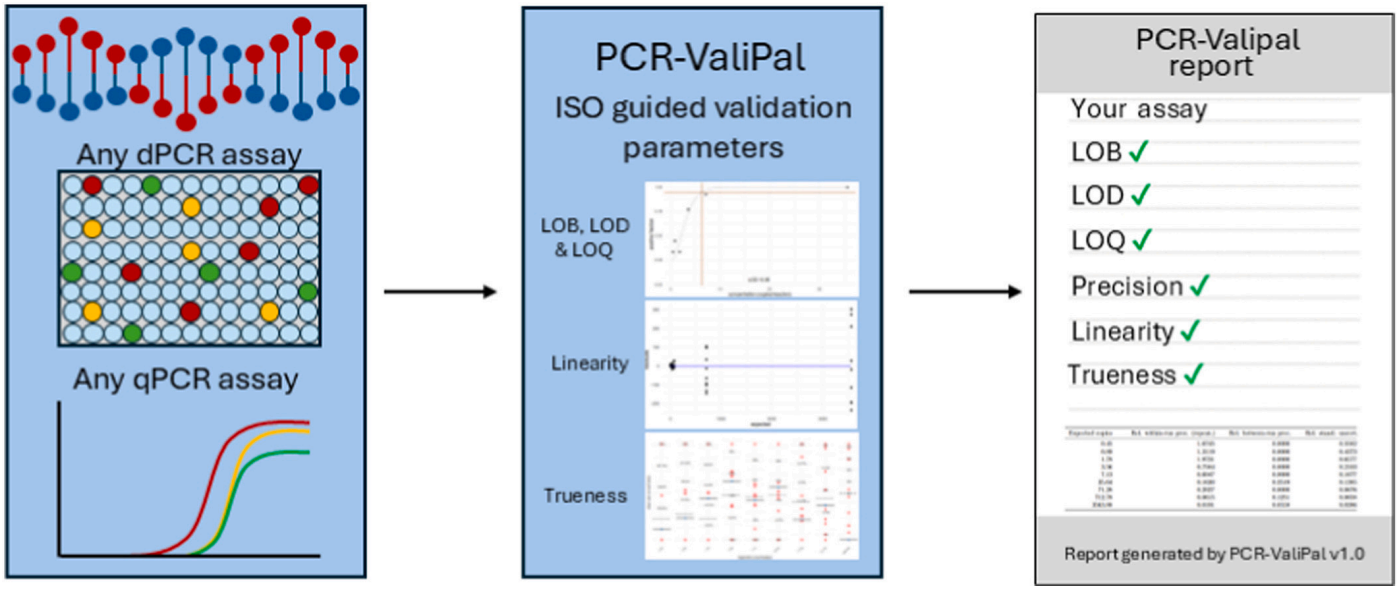
Analytical validation

- PCR-ValiPal web app - supports transparent, reproducible, and ISO-aligned validation of PCR-based assays



https://digpcr.shinyapps.io/valipal/
Gleerup, Vynck, *et al.*, Analytica Chimica Acta, 2026

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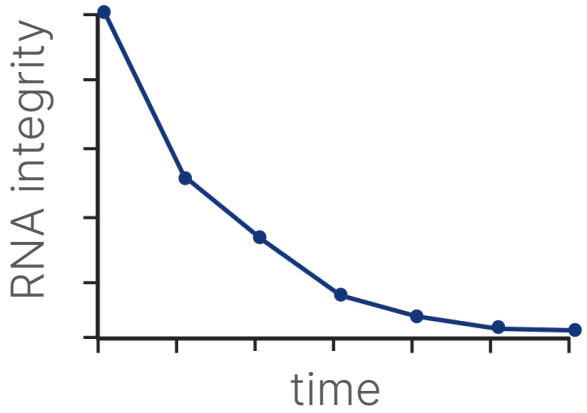
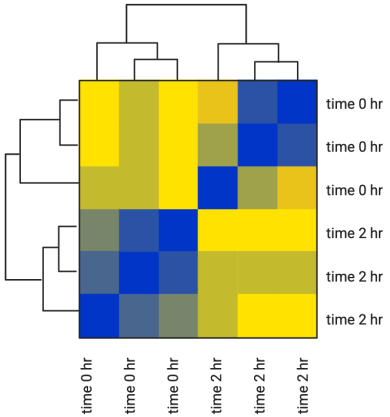
The pre-analytical phase is critical for RNA quantification

- RNA degrades rapidly after tissue or biofluid collection
- cells alter gene expression due to changing environment
- leads to poor RNA integrity and inaccurate (biased) molecular analyses





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Recommended use of RNA stabilizing sample collection buffers

- *CLSI MM13 guideline - Collection, transport, preparation, and storage of specimens for molecular methods, 2nd edition (2020)*

- blood and bone marrow
  - DRD Blood, PAXgene Blood RNA, ...
- tissue
  - RNA*wait*, RNA*later*, formalin-fixation, ...
- biofluids (swab, urine, saliva, ...)
  - DNA/RNA Defend, DNA/RNA Shield, ...

- prevent RNA degradation and transcriptional alterations
- accommodate freeze-thaw cycles
- some also render samples no-longer-infectious

~ISO 20658, ISO 20186-1

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RNA quantification is plagued by numerous issues

- no consensus on adequate RNA quality
- lack of reference materials
- reverse transcription black box
- need for normalization to correct technical variation
- relative measurement outcomes (Cq, blot) vs. count-based outcomes (digital PCR, UMI in MPS)
- pre-analytical factors impacting accuracy
- lack of transparent reporting

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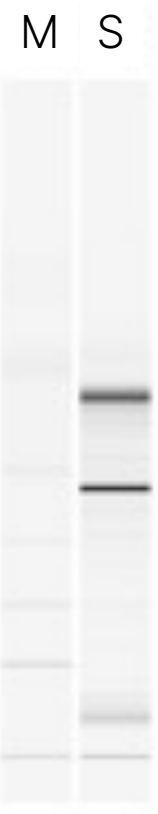
What is acceptable RNA integrity?



- *Don't get fooled by the RIN myth!*

- Depends on sample type (FFPE or fresh frozen tissue, biofluid, …), the RNA quantification method, target abundance, target sensitivity to degradation, # of targets in a biomarker test, target aggregation algorithm, sample classification algorithm, …
- Normalization does not completely rescue low RNA quality
- Reference gene stability depends on RNA quality
- Different methods appreciate RNA integrity in a different way
  - electrophoresis, 5'-3' assay, Alu repeat assay, normalization factor,

Vermeulen, De Preter *et al.*, Nucleic Acids Research, 2011

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Sample adequacy control

- new to ISO 20395
- definition
  - *Control included in a nucleic acid test to verify that the test sample contains sufficient and appropriate biological material and that the sample collection was performed correctly. The sample adequacy control is typically based on the detection of endogenous nucleic acid targets inherent to the sample matrix.*
    - sufficient quantity and quality (e.g., human endogenous control in swab to detect pathogen)
    - negative result is a true negative and not a false positive
- whole process control
  - *Control included in a nucleic acid test to verify the correct performance of all relevant analytical steps of the method, from sample preparation through nucleic acid extraction and amplification to detection. The whole process control is typically based on the detection of an exogenous control target introduced into the sample prior to sample processing.*

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Reference RNA materials

- few to no SI traceable reference materials
- each target (and method) may need its own reference material

- Universal Human Reference RNA (UHRR)
  - RT-qPCR data for 20,000 genes/assay; RNA-seq data in all its flavors
- Genome in a bottle (GiaB) RNA samples
  - immortalized B cells from well genetically characterized child-parent trios
- built-in-truth sample mixtures
  - samples A-B-C-D (MAQC, SEQC)
  - A and B are pure; C and D are 1:3 and 3:1 A:B mixtures
- IVT RNA from double-stranded synthetic DNA template

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MAQC – SEQC samples A/B/C/D

Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study

Pieter Mestdagh[1], Nicole Hartmann[2], Lukas Baeriswyl[2], Ditte Andreasen[3], Nathalie Bernard[4], Caifu Chen[4], David Cheo[5], Petula D'Andrade[6], Mike DeMayo[7], Lucas Dennis[8], Stefaan Derveaux[9], Yun Feng[5], Stephanie Fulmer-Smentek[6], Bernhard Gerstmayer[10], Julia Gouffon[7], Chris Grimley[8], Eric Lader[11], Kathy Y Lee[4], Shujun Luo[12], Peter Mouritzen[3], Aishwarya Narayanan[13], Sunali Patel[4], Sabine Peiffer[10], Silvia Rüberg[10], Gary Schroth[12], Dave Schuster[5], Jonathan M Shaffer[11], Elliot J Shelton[4], Scott Silveria[9], Umberto Ulmanella[4], Vamsi Veeramachaneni[13], Frank Staedtler[2], Thomas Peters[2], Toumy Guettouche[14], Linda Wong[4] & Jo Vandesompele[1]

- titration response
- trueness of fold changes
- selectivity
- linearity
- analytical sensitivity

A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the Sequencing Quality Control Consortium

SEQC/MAQC-III Consortium*

Mestdagh *et al.*, Nature Methods, 2014
SEQC/MAQC-III Consortium, Nature Biotechnology, 2014

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Reverse transcription black box

- RNA is no substrate for Taq polymerase
- reverse transcriptase is used to convert RNA into cDNA
  - enzyme, primer, input amount, target sequence/abundance, reaction volume, etc. dependent conversion efficiency
- solution 1: omit RT step
  - direct RNA sequencing
  - ligate two adjacent DNA oligonucleotide hybridized to the target
  - fluorescent *in situ* hybridization

  [all with their own biases]
- solution 2: calibrate the RT step
  - determine stochiometric relationship between RNA and cDNA
- solution 3: accept the limitation by quantifying ‘cDNA’

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Calibrate the RT step

- Representativeness of RNA reference material for complex biomaterial (varying purity, integrity, mass, sequence complexity, ...)
- RNA mass molarity conversion
- equimolar physically linked reference materials
  - proof-of-concept data in our lab
  - QC of material > circular problem

Vandenbroucke et al., Nucleic Acids Research, 2001

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Minimal information guidelines

- PCR expert community set of guidelines to design, execute, analyze, and report dPCR and qPCR experiments
- for users, authors, and reviewers
- standardize nomenclature, quality control, reporting

Clinical Chemistry 66:8
1012–1029 (2020)

Special Report

The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020

The dMIQE Group*

Clinical Chemistry 71:6
634–651 (2025)

Special Report

MIQE 2.0: Revision of the Minimum Information for Publication of Quantitative Real-Time PCR Experiments Guidelines

Stephen A. Bustin [a,*] Jan M. Ruijter,[b] Maurice J.B. van den Hoff,[b] Mikael Kubista,[c] Michael W. Pfaffl,[d] Gregory L. Shipley,[e] Nham Tran,[f] Stefan Rödiger,[g] Andreas Untergasser,[h] Reinhold Mueller,[i] Tania Nolan,[j] Mojca Milavec,[k] Malcolm J. Burns,[l] Jim F. Huggett [l] Jo Vandesompele [m] and Carl T. Wittwer[n]

dMIQE group, Clinical Chemistry, 2020
Bustin *et al.*, Clinical Chemistry, 2025

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Improving reproducibility across complex biomarker development workflows in molecular diagnostics

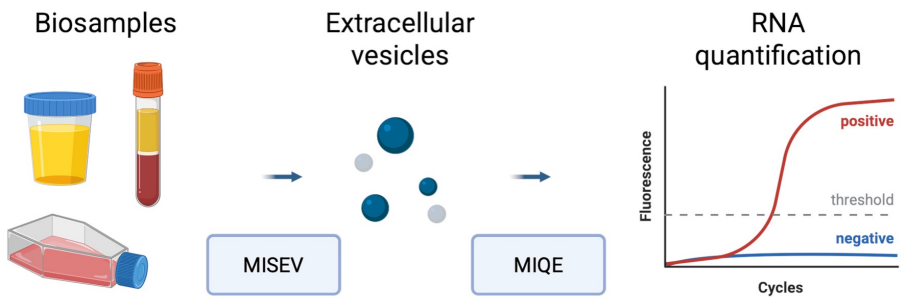
Commentary

Open Access

Check for updates

MISEV and MIQE: integrating domain-specific and general standards to strengthen extracellular vesicle biomarker research

Michael W. Pfaffl[1], Mikael Kubista[2], Jo Vandesompele[3], Stephen A. Bustin[4]



Pfaffl *et al.*, Extracellular vesicles and circulating nucleic acids, 2025

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ISO 20395

- part of the working group revising the standard (Dr. Carole Foy)
- more elaborate normalization strategies
  - spike-in controls
  - genomic DNA to normalize RNA
- extra controls, e.g., sample adequacy control

ISO 20395:2019

Biotechnology — Requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR

Published (Edition 1, 2019)

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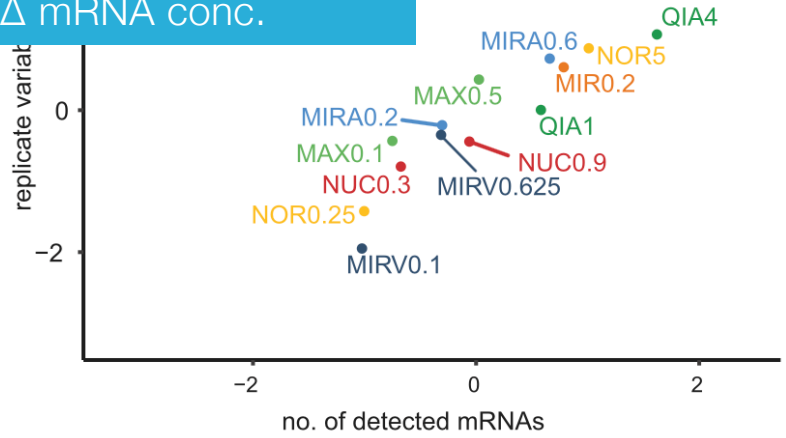
Impact of pre-analytical variables on blood plasma cfRNA

- large scale benchmarking
  - 456 exRNA transcriptomes
  - 10 blood collection tubes x 3 time intervals
  - 8 RNA purification kits
  - 11 performance metrics

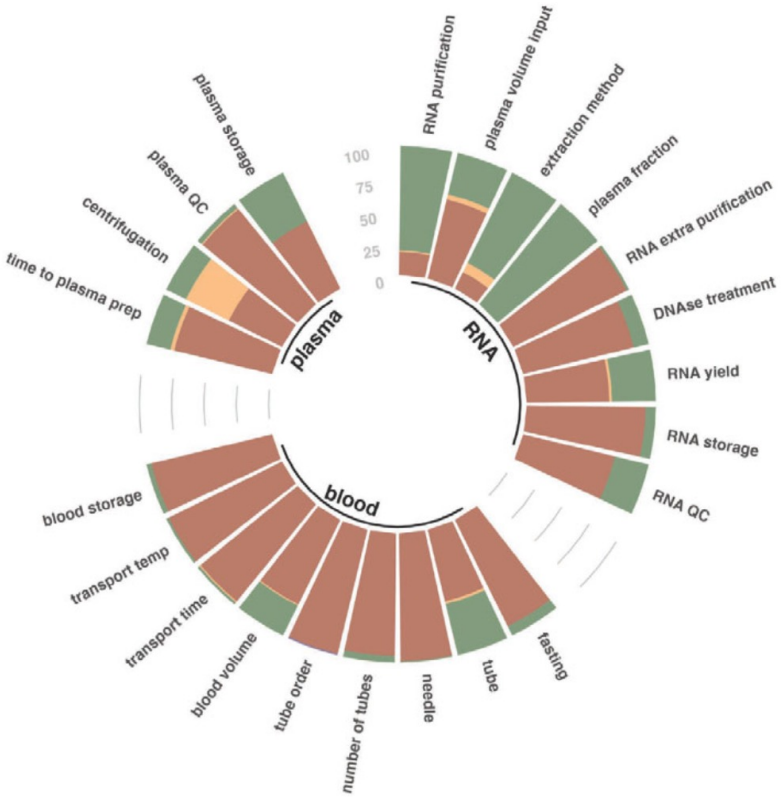
- recommendations for users and manufacturers
  - standardize workflow
  - maximize input in extraction
  - avoid ‘preservation’ tubes
    - EDTA/citrate processed within 4 h

- 11x Δ detected genes
- 30x Δ mRNA yield
- 76x Δ mRNA conc.



exRNAQC consortium, Nature Communications, 2025

Lack of reporting



Reported
Reported in insufficient detail
Not applicable
Unknown

- 200 peer-reviewed articles in 2018/2023 on ‘blood plasma + RNA’
- evaluated 22 pre-analytical parameters
  - median level of reporting is 7%
  - only 6/22 variables reported in >50% articles
- reporting checklist for exRNA-based studies > reliable interpretation & replication
- CEN/TS 17742:2022

Van Der Schueren, Molecular Oncology, 2024

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Normalization

- the goal is to remove or reduce technical variation
- best practices: geometric mean of at least 2 stably expressed reference genes
  - geNorm algorithm: +20K citations (monthly ~50 citations)
- despite multi-gene normalization with multiple stable reference genes, 15-30% CV remains
- When appropriate, avoid the use of reference genes

  “internally controlled measurements”
  - splice isoform quantification
  - ratio of up/down biomarker(s)
  - spike-in controls (e.g., normalize to volume of biofluid)
  - single cell applications
  - DNA normalization (bulk) > transcripts per cell

Vandesompele et al., Genome Biology, 2002

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Normalization

- ISO 20395 – normalization strategies
  - strategy d) exogenous spike-in material

d) Normalization to the amount of exogenous spike-in material added to a defined amount of the sample (e.g. volume of biofluid), either before nucleic acid extraction (preferred), or after extraction, or both (when using different spike-in nucleic acids). Spike-in nucleic acids shall have a different sequence compared to the organism under study. Multiple spike-in targets improve the normalization process. Apart from normalization, evaluating the ratio of pre- and post-extraction spikes enables the evaluation of extraction efficiency.

NOTE 2
As spike-in nucleic acids are fragile molecules and sensitive to nuclease-based degradation, pre-extraction spikes can be added to the lysis buffer or to the lysed sample.

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RNA normalization using DNA (1)

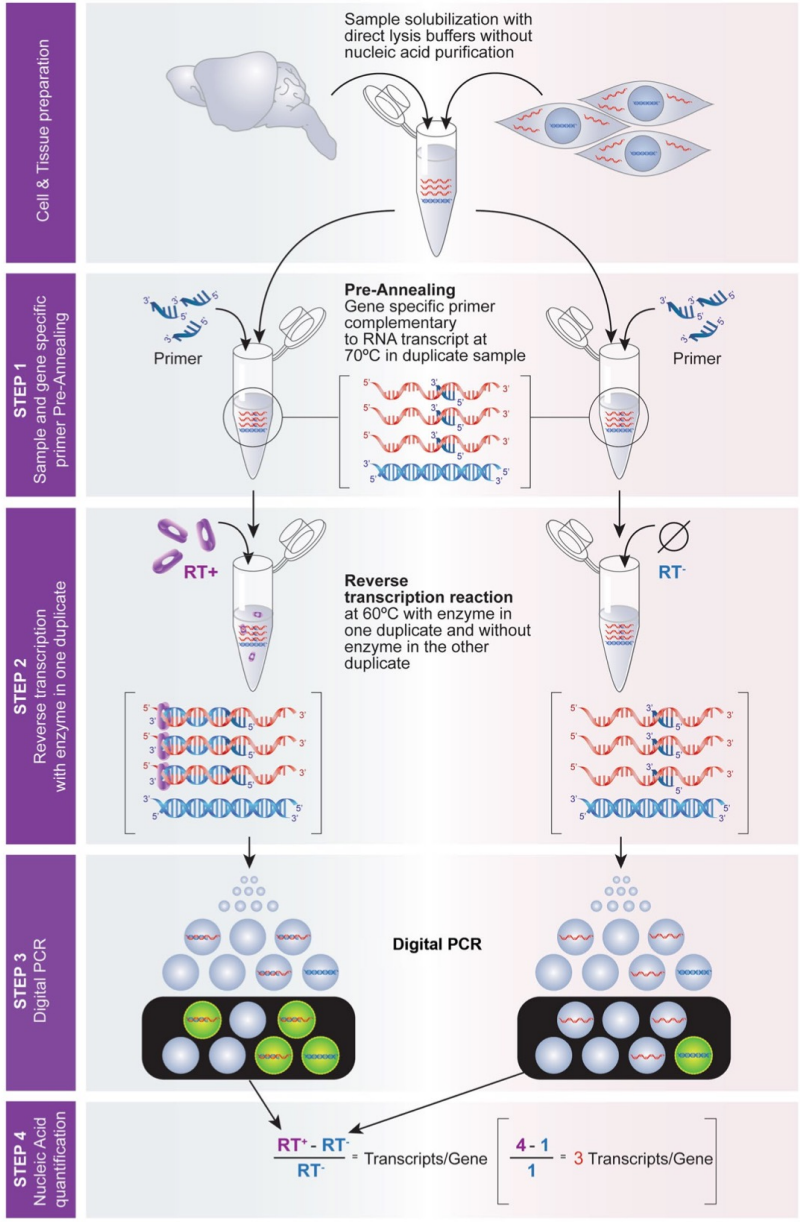
$$\frac{RT^{+} - RT^{-}}{RT^{-}} \cdot \text{ploidy} = \text{transcripts/cell}$$

SCIENTIFIC REPORTS
nature research

Sci Rep. 2017 Aug 21;7:8328. doi: 10.1038/s41598-017-08270-w

Absolute measurement of gene transcripts with Selfie-digital PCR

Petar Podlesniy [1,3], Ramon Trullas [1,2,3,4,✉]



RNA normalization using DNA (2)

- Selfie-digital PCR method does not work well for low abundant targets (close or lower than DNA gene copy number). For these scenarios, it's better to do a DNase treatment and quantify cDNA only.

$$\frac{RT^{+}_{DNase}}{RT^{-}} \cdot ploidy = \frac{cDNA}{DNA} \cdot ploidy = \text{transcripts/cell}$$

- instead of crude lysates (e.g. SingleShot, Cells-to-Ct, ...), co-purified DNA and RNA can be used
  - miRNeasy Advanced for biofluids
  - QIAamp ccfDNA/RNA Kit for biofluids
  - AllPrep DNA/RNA for tissue

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Co-purification of RNA and DNA from a precious liquid biopsy sample

- several commercial methods that co-purify cfDNA and exRNA
- evidence for increased mutation detection sensitivity (refs. 8-11)

RESEARCH Open Access

Digital PCR-based evaluation of nucleic acid extraction kit performance for the co-purification of cell-free DNA and RNA

Jill Deleu[1,2†], Kathleen Schoofs[1,2,3,4†], Anneleen Decock[1,2], Kimberly Verniers[1,2], Sofie Roelandt[2,3,4], Angie Denolf[2,3], Joke Verreth[1,2], Bram De Wilde[1,2,5], Tom Van Maerken[1,2,6], Katleen De Preter[2,3,4] and Jo Vandesompele[1,2*]

Deleu, Schoofs *et al.*, Human Genomics, 2022

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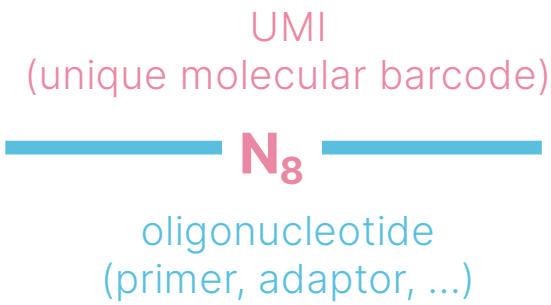
Relative vs absolute measurements

- Cq value (RT-qPCR) is a relative measure, linked to the experiment
  - threshold settings, accuracy calibration curve, instrument, ...
  - inherent limitation for commutability
  - likely explains few PCR tests in the clinic

- absolute quantification – counting molecules
  - digital PCR
  - UMI-based massively parallel sequencing
  - commutability inherently high



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Can we trust RNA quantification?

Yes, provided that ...

- good understanding of method dependencies
- use of validated standard operating procedures
  - pre-analytical phase
  - analytical phase
- RNA integrity preservation from sample to RNA eluate
- transcriptional processes are quenched (*in vivo* = *ex vivo* = *in vitro*)
- transparent reporting

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