The potential of ddPCR for clinical research

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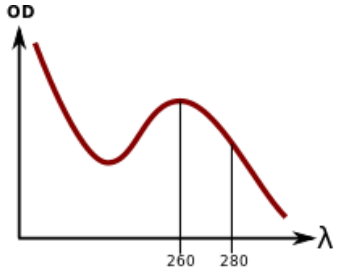
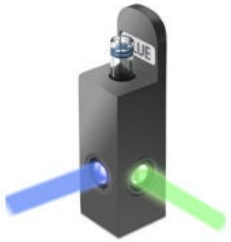
Different technologies for nucleic acid quantification

non-specific

- UV absorbance



- fluorescent dyes



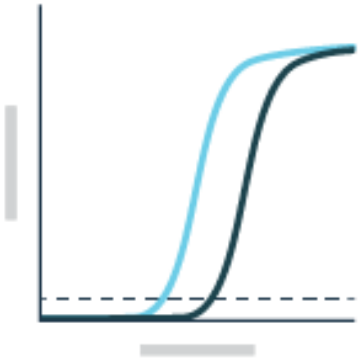
sequence specific

- PCR based [*few selected targets*]
  - qPCR
  - ddPCR
- probe based
  - e.g. microarrays
- sequencing
  - amplicon seq
  - gene panel / exome / genome seq
  - small RNA / polyA+ / total RNA seq

Principles

qPCR

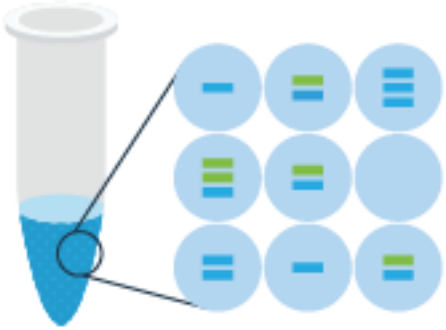
- real-time detection



- Cq values
  - relative quantities
  - + calibration curve → absolute quantities

dPCR

- end-point detection



- # pos partitions >*Poisson*> copies
  - copies / µl
  - ratio or fraction

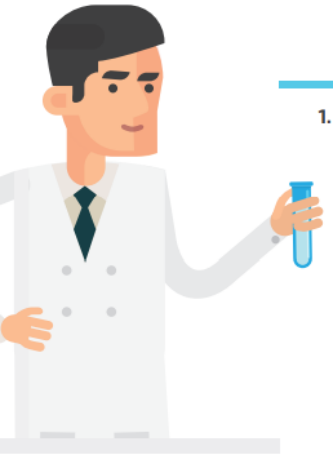
Comparison

qPCR

- price / measurement: **$**
- dynamic range **> 10^6**
- data points / day: **4 x 384**
- relative quantities (comparison between samples)
- absolute quantities **relying on calibration curve**
- variations in amplification efficiency (e.g. due to inhibitors) **affect results**

dPCR

- price / measurement: **$$**
- dynamic range **~10^4**
- data points / day: **2-3 x 96**
- ratios (comparison within a sample)
- absolute quantities (copies) **without a calibration curve**
- end point detection is **not affected** by changes in amplification efficiency



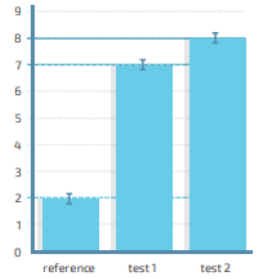
5 applications of digital PCR *on clinical samples*

1. rare allele detection, e.g. cancer biomarkers in liquid biopsies

intended sensitivity	ng cell-free DNA needed*
10%	0.46
1%	4.57
0.1%	45.71

* assuming at least 5 positive droplets are needed for confident calling, a perfectly discriminating assay between wild-type and mutant, 14,000 recovered droplets from 20,000 formed and 50% amplicon availability in cell-free DNA

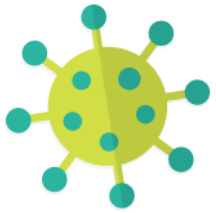
2.



gene copy number quantification

including transgenic animal characterization and oncogene amplification in cell-free DNA – digital PCR has higher accuracy and precision than qPCR

3.



quantification of pathogen load

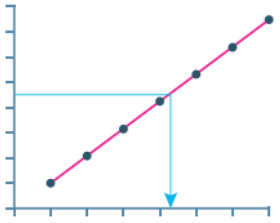
e.g. detecting human immunodeficiency virus (HIV) in clinical samples

4.

gene and microRNA expression analysis

ddPCR provides stand-alone absolute quantification of expression levels, especially low-abundance microRNAs with small differences, with high sensitivity and precision

5.



absolute quantification

of nucleic acid standards

5 benefits of digital PCR

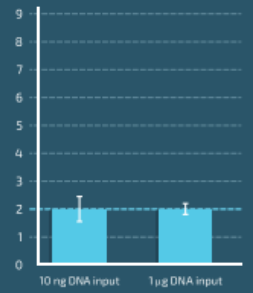
1. absolute quantification

no need to rely on references or standards

2. tolerant to inhibitors

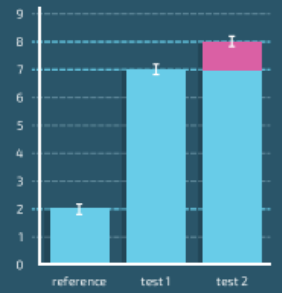
due to end point fluorescence measurement — up to 30% of dPCR reaction can be unpurified digested genomic DNA without inhibiting dPCR; up to 25% of dPCR reaction can be cDNA without inhibiting dPCR

3. scalable precision



precision can be improved by increasing number of dPCR replicates and by tuning input amount

4. unparalleled resolution



linear response to the number of input molecules allows for very small differences to be detected

5. Increased signal-to-noise ratio

high-copy templates and background are diluted, effectively enriching template concentration in target-positive partitions, allowing for the sensitive detection of rare targets and enabling increased precision in quantification

0.1%

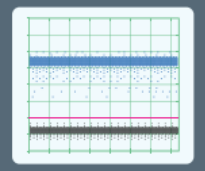
how it works

1. partitioning

the PCR reaction mixture is partitioned into 20,000 water-in-oil droplets with target and background DNA randomly distributed among the reactions

2. amplification

target DNA is amplified by PCR using standard thermal cycling with fluorescent dye or probe



3. detection

each reaction provides a fluorescent positive or negative signal indicating that target DNA was present or absent in partitioning

molecules = - ln (1 – p)

where p = **fraction of positive droplets**

4. calculation

the fraction of positive droplets is used to calculate the target DNA concentration using Poisson correction

services.biogazelle.com/solutions/digital-pcr

Rationale for Bio-Rad's QX200 platform

- right number of partitions
  - sufficient for good dynamic range and accuracy
  - variation in partition volume dominates inaccuracy at higher # of partitions
- good uncertainty (limited variation) on partition volume
- sufficient throughput (< 96 analysis / run would disqualify)
- well established & trusted instrument & reagent provider
- IVD version to provide GCLP compliancy

Guideline considerations

- General
  - ISO 17025
    general requirements for the competence of testing and calibration laboratories
  - GCLP

- Technology specific
  - digital MIQE guidelines *[Huggett et al., 2013 – update in preparation]*
  - ISO 20395 *[Aug 2019]*
    requirements for evaluating the performance of quantification methods for nucleic acid target sequences — qPCR and dPCR

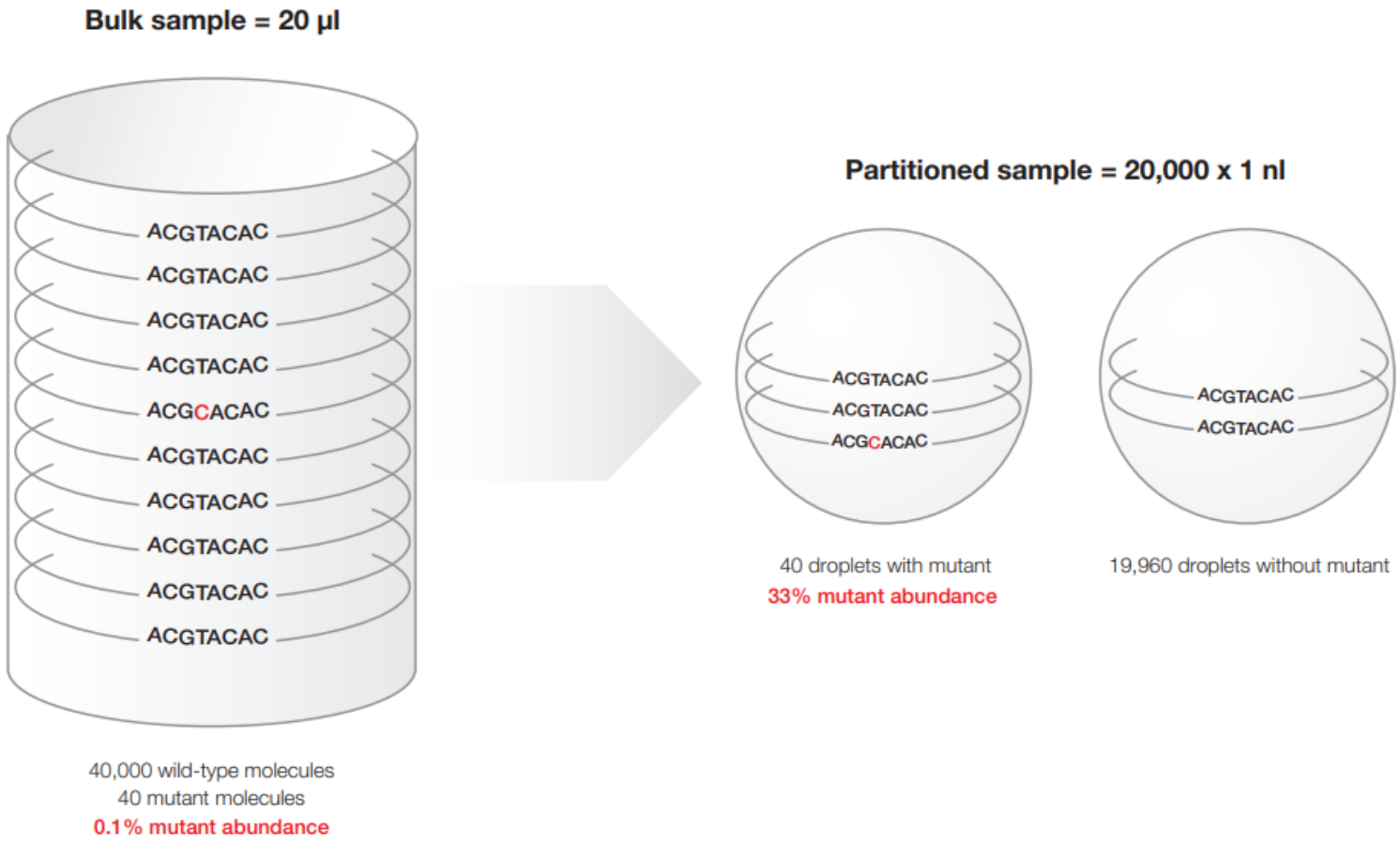
Case 1: rare variant detection

Background

- Context: phase 1 & 2 clinical trials for colorectal cancer treatment
- Goal: evaluate candidate response/resistance biomarkers with a required sensitivity <2%
- Set-up:
  - Custom assay design and validation of 30 ddPCR assays for rare variant analysis
  - Screening on plasma derived cfDNA of >200 colorectal cancer patients

Case 1: rare variant detection

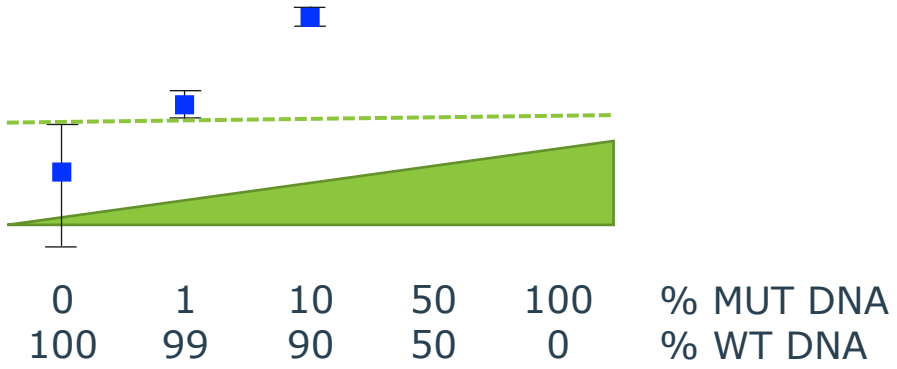
Rationale for ddPCR: partitioning increases the variant fraction



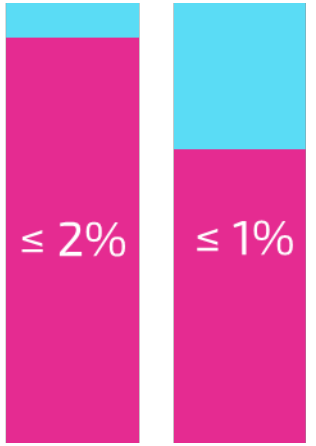
Case 1: rare variant detection

Data

- LOD: lowest mutant concentration that can be reliably distinguished from the mutation-negative control



- Results
  - 93% of reactions contained sufficient DNA to reach the target sensitivity of 2%
  - for 65% of reactions, detection sensitivity was ≤ 1%
  - fractional abundance varied from 0.4% to 63.5%



Case 2: small changes in RNA splicing

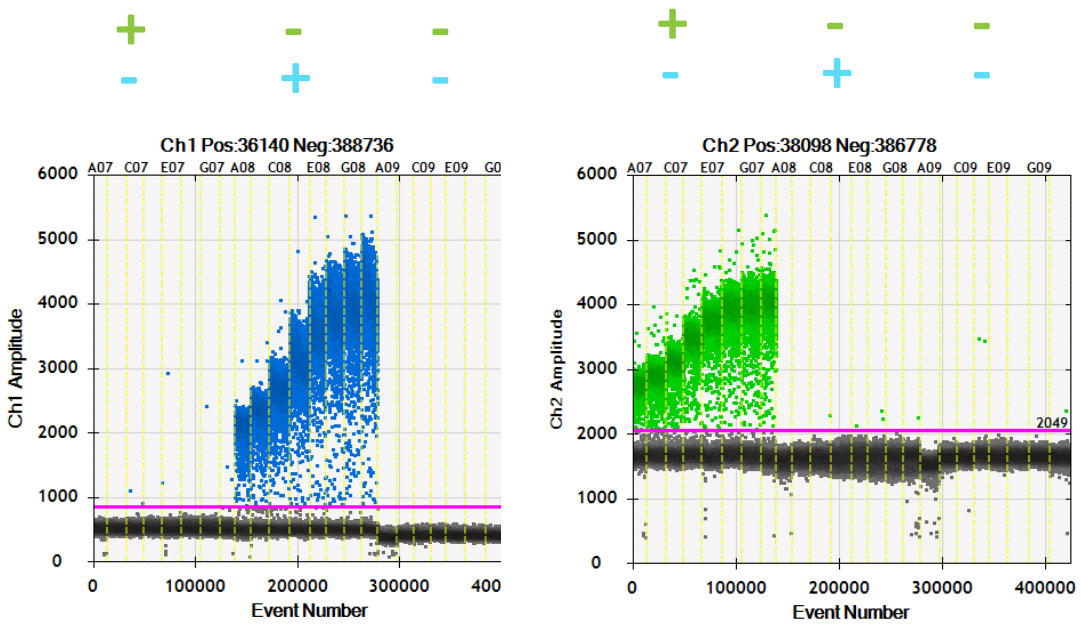
Background

- Context: first-in-human clinical trial for rare skin disorder
- Goal: quantify small changes in antisense induced alternative splicing
- Validation plan (key points):
  - identify optimal annealing temperature
  - assess linearity
  - assess robustness against variable RNA qualities
  - potential to call a 10% reduction
- Rationale for ddPCR: more accurate quantification of small differences
  (normalization uncertainty doesn't affect the results because of comparisons within a given sample)

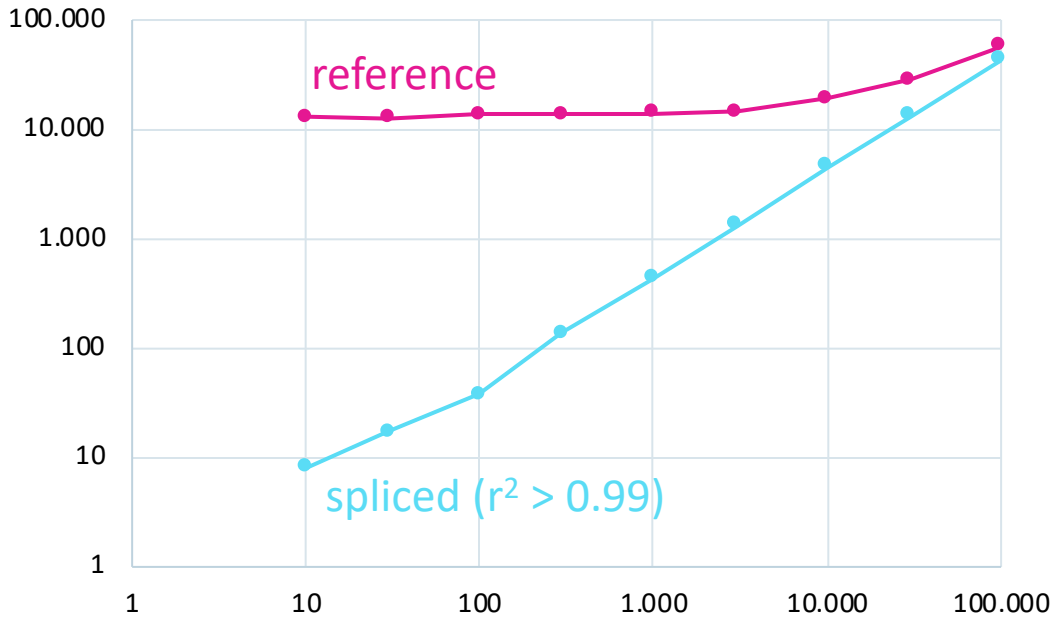
Case 2: small changes in RNA splicing

Data

optimal annealing temperature



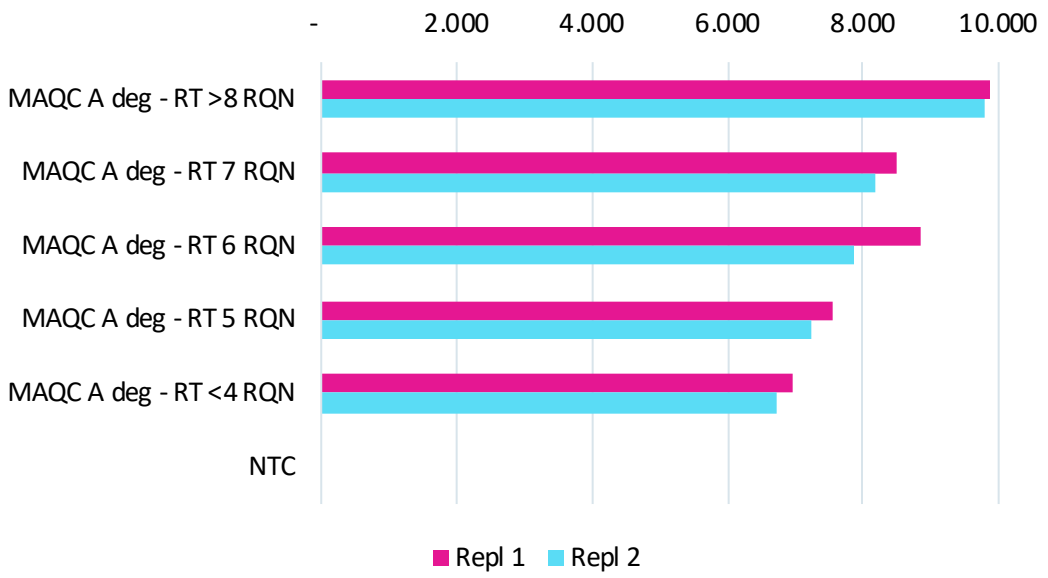
linearity



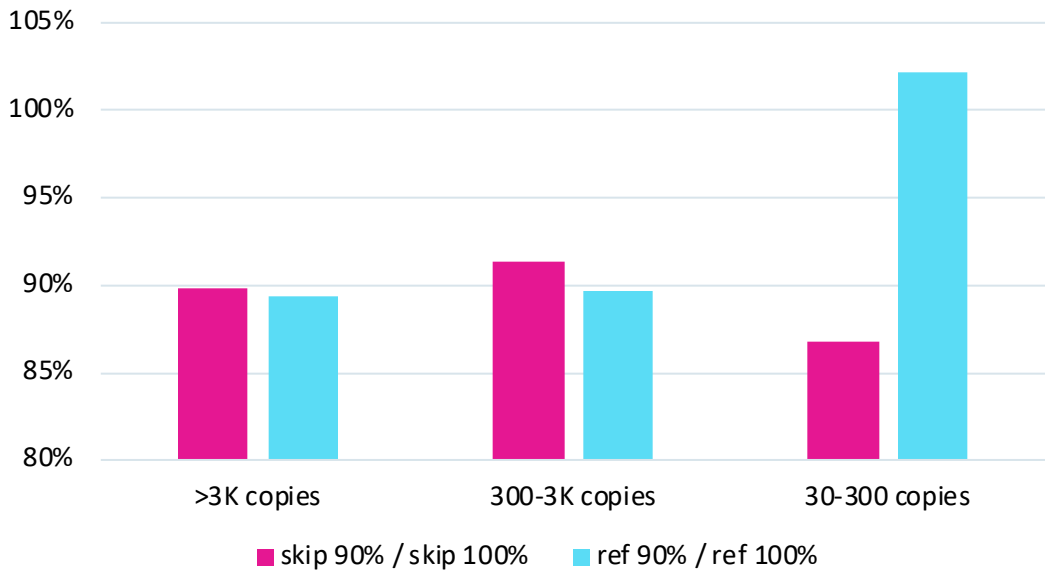
Case 2: small changes in RNA splicing

Data

impact of RNA degradation



call out a 10% reduction



→ normalize or analyze ratios

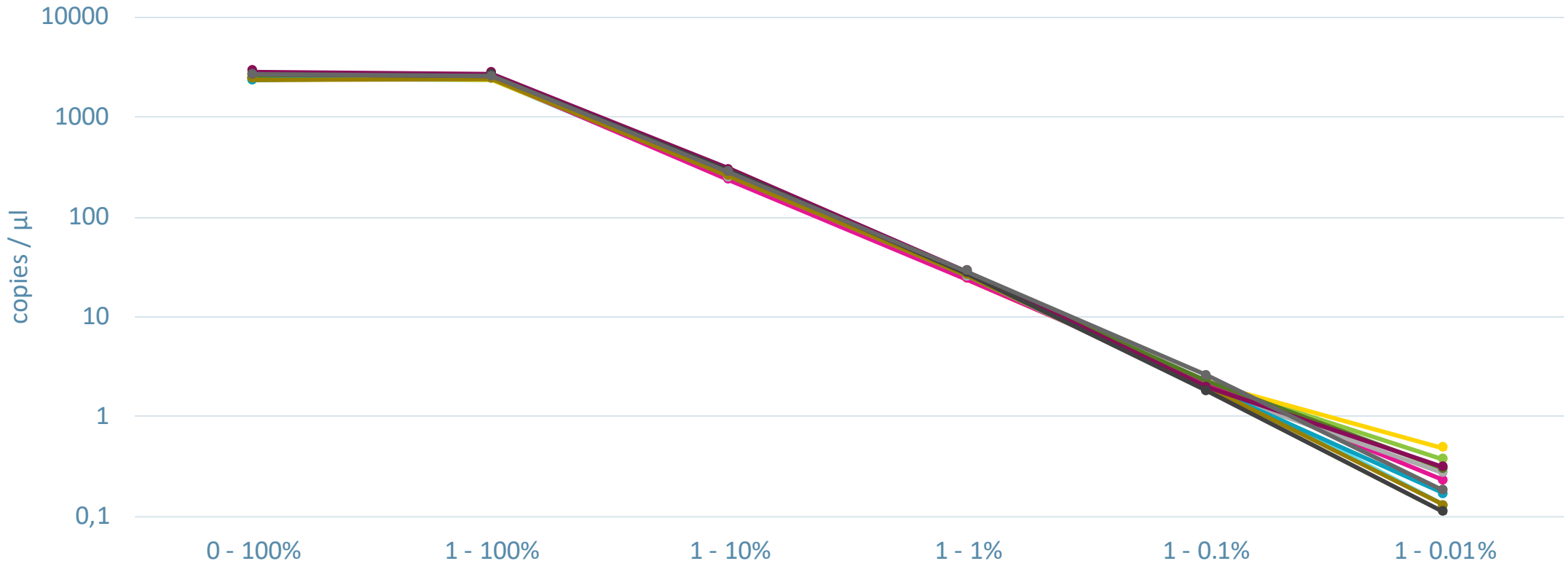
Case 3: CAR-T biodistribution

Background

- Context: pre-clinical study testing biodistribution of CAR-T cells *(chimeric antigen receptor T cells used in immune oncology)*
- Goal:
  - develop and validate assays to quantify mouse and engineered human cells
  - quantify the biodistribution of CAR-T cells across 8 different tissues (336 samples)
- rationale for ddPCR: absolute quantification at low copy numbers

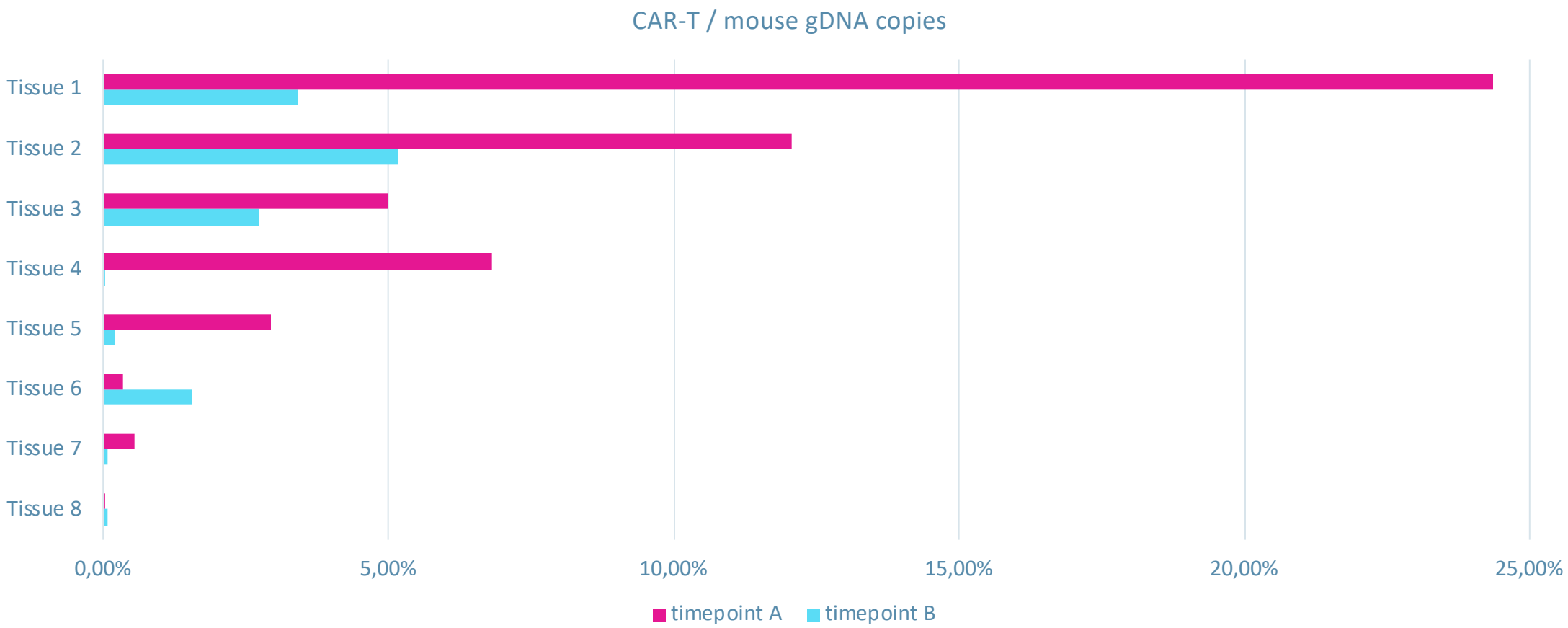
Case 3: CAR-T biodistribution

1 CAR-T cell in a background of 10,000 mouse cells can be detected, 10 cells can be quantified (different assays)



Case 3: CAR-T biodistribution

Strong tissue bias with abundances <1% in several samples



Case 4: VCN analysis in CAR-T therapy

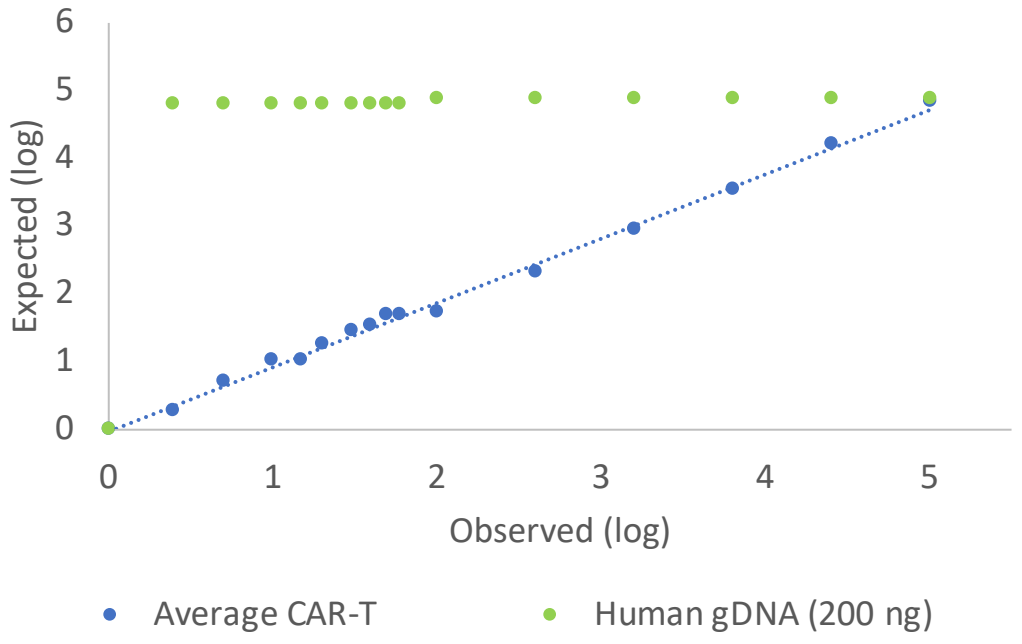
Background

- Context: monitor the persistence of CAR-T vector sequences in a phase I clinical trial
- Goal:
  - develop and validate ddPCR assays to quantify the CAR-T specific vector sequence
  - monitor DNA derived from whole blood & bone marrow samples until vector sequences can no longer be detected (safety evaluations required by the FDA)
- rationale for ddPCR: absolute quantification at low copy numbers

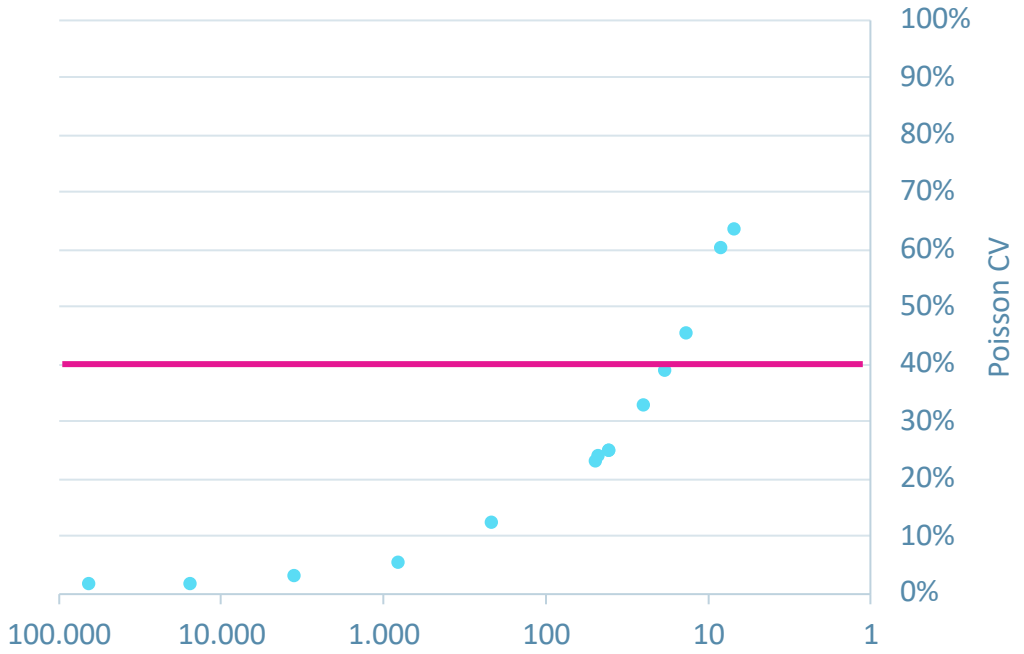
Case 4: VCN analysis in CAR-T therapy

Data

linearity ($r^2 > 0.99$)



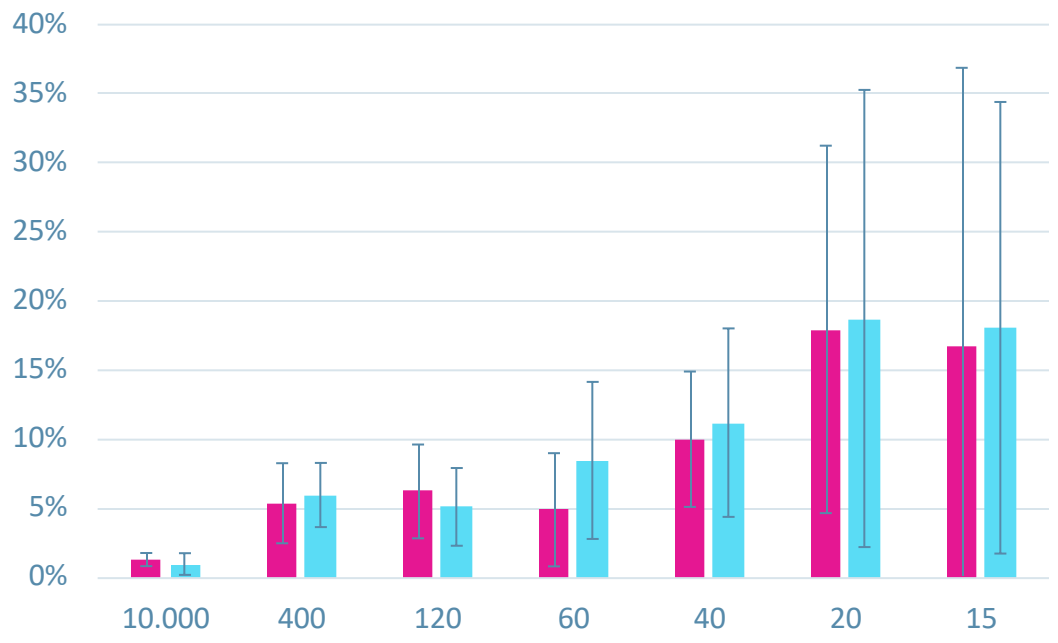
LOQ of 18 molecules



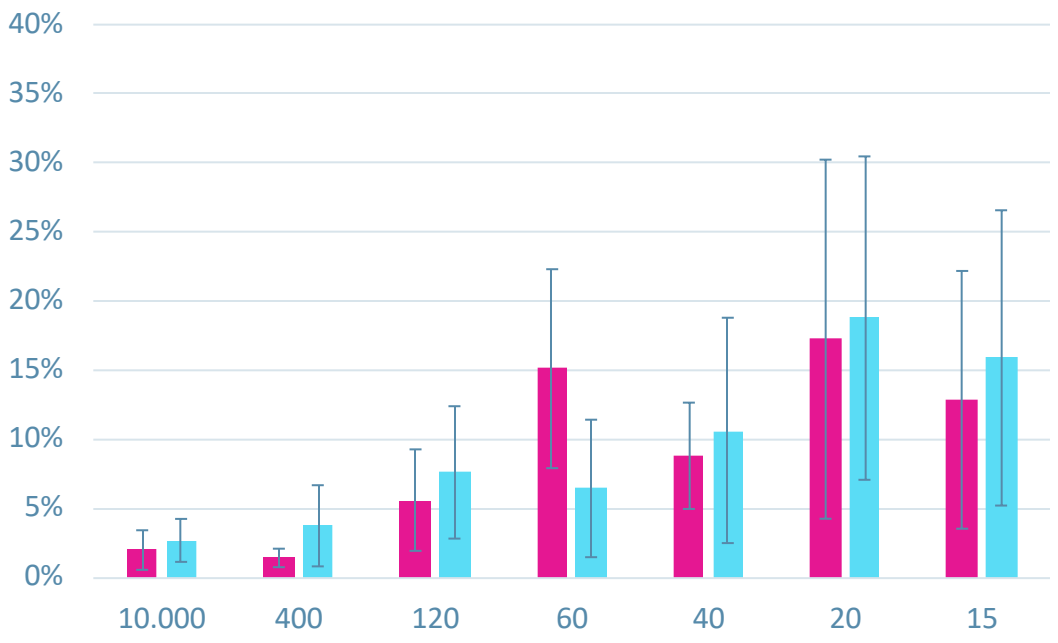
Case 4: VCN analysis in CAR-T therapy

Data

repeatability (%CV)



intermediate precision (%CV)



Conclusions

- For selected applications, ddPCR provides unique advantages over qPCR
  - rare variant detection (case 1)
  - detection of small differences (case 2)
  - sensitive absolute quantification without calibration curve (cases 3 & 4)
- By adhering to the relevant guidelines, ddPCR is well suited for clinical research & clinical trials
  - GCLP, ISO17025 & 20395, digital MIQE guidelines
- As a specialty service provider, Biogazelle offers ddPCR in a quality environment
- Bio-Rad's QX200 is a great platform to enable ddPCR quantification in a clinical research/trial setting

Back up

dPCR platform comparison

dPCR platform	BioMark	QX100	QuantStudio 12k	RainDrop
Partition number	765	13800 ± 464*	64	1695000 ± 24862*
λ (Mean copies/partition)	1.56	1.54	1.54	1.51
Measured pNIM-001 plasmid concentration	2.46E+ 08	2.34E +08	2.48E +08	2.49E +08
n (number of observation)	15	15	15	15
Relative standard uncertainty of all precision factors $\frac{u_M}{M}$ (%) (*M*, copies per panel)	2.9	1.6	2	1.5
Relative standard uncertainty of dilution factor $\frac{u_D}{D}$ (%) (*D*, dilution factor)	0.1	0.1	0.2	0.1
Relative standard uncertainty of a single droplet/partition volume $\frac{u_{V_P}}{V_P}$ (%) (V_p, partition volume)	0.7	0.8	2.3	2.9
Relative combined uncertainty *u* (%)	3.0	1.8	3.1	3.3
Relative expanded uncertainty U_{rel} ($k = 2$) (%)	6.0	3.6	6.1	6.5

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