Digital PCR for lentivirus workflow

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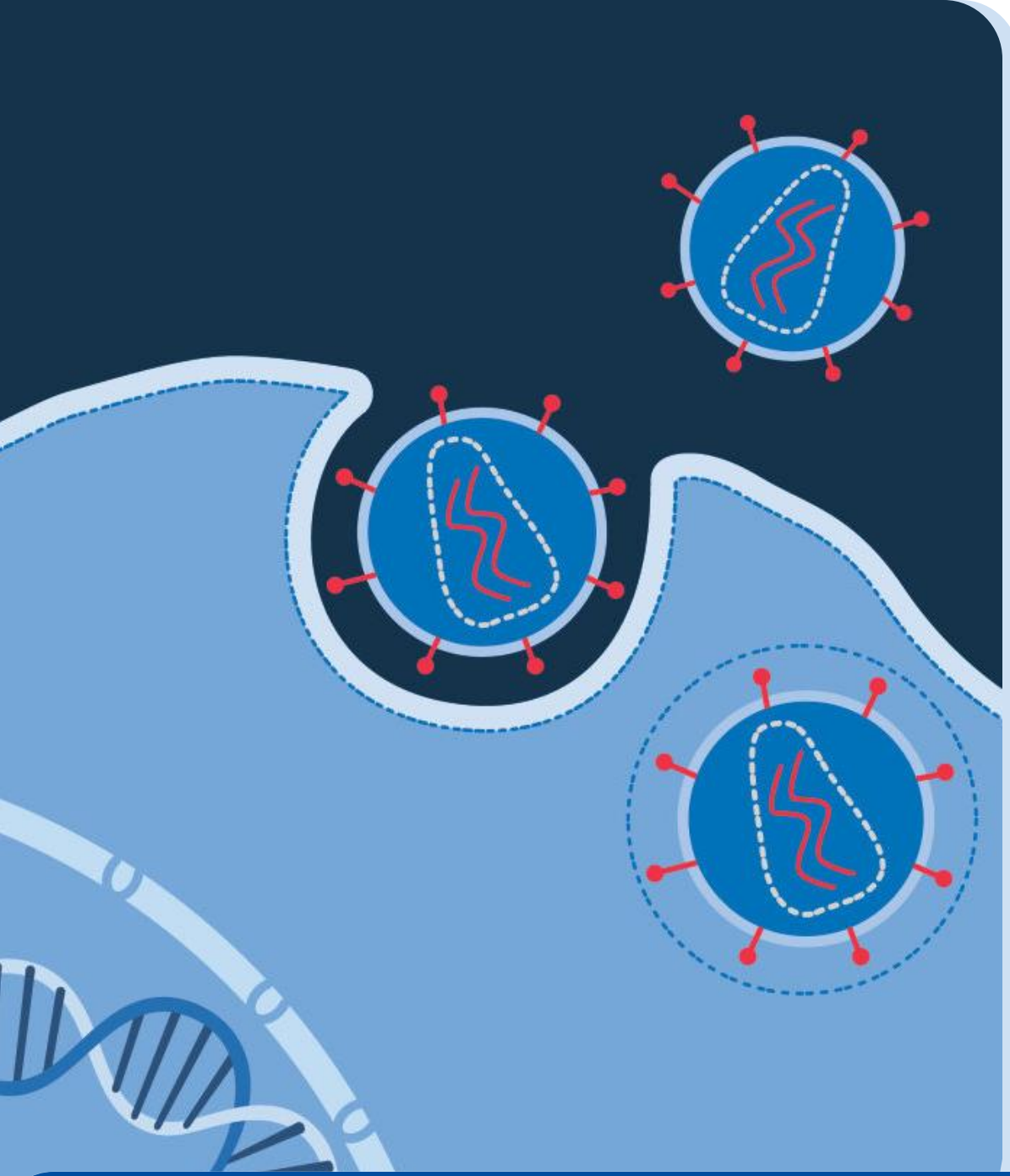
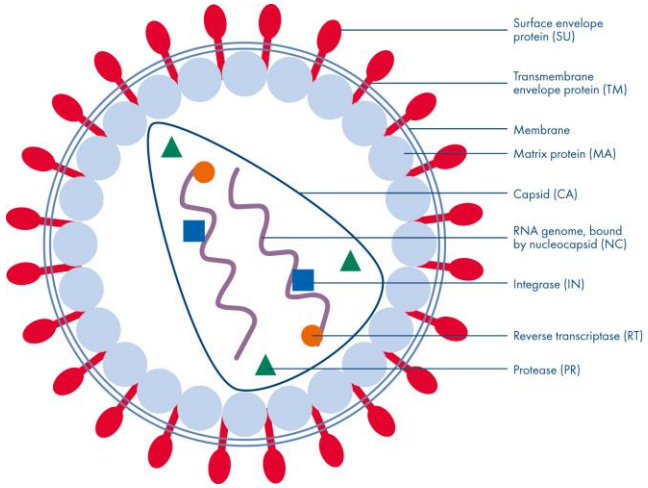
Agenda

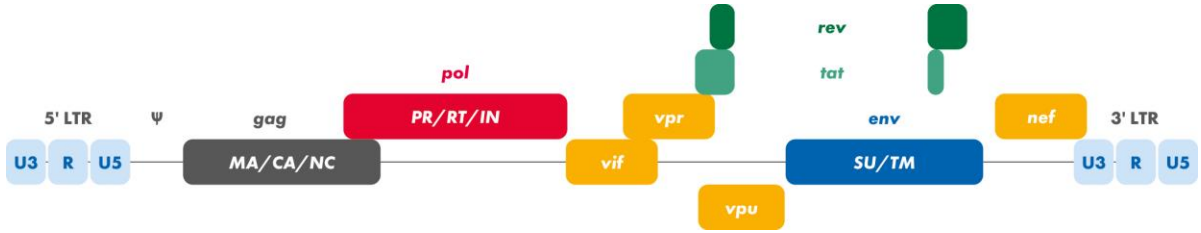


Lentivirus-based therapies

- Lentiviruses are one of the most commonly used viral vectors in research and cell & gene therapies (CGTs)
- Generation and purification of viral vectors require precise quality control to enable safe, effective and reliable therapies
- Analytical methods to extensively characterize and monitor multiple LV critical quality attributes (CQAs) are important to ensure product efficacy and safety
- Analytical methods such as qPCR and ELISA assess the identity, potency and purity of viral vectors, but accurate quantification requires well-characterized assays

Lentivirus (LV)





Profile

- Retroviruses family
- Single-stranded RNA viruses (diploid)
- Two sense-strand RNAs bound by nucleocapsid proteins
- Enveloped, ~100 nm in diameter
- Contains reverse transcriptase, integrase and protease
- Integrating into the host genome
- Stable long-term expression
- Transduction of dividing and non-dividing cells

Genomic structure (e.g., HIV genome)

- Common essential genes: 5'-*gag-pol-env*-3'
  - Flanked by 5′- and 3′ LTRs → viral genome replication
  - *gag* → capsid, matrix and nucleocapsid proteins (p24 = capsid)
  - *pol* → viral enzymes: RT, IN & PR
  - *env* → viral envelope glycoproteins
- Ψ (Psi) → encapsidation signal, required for packaging
- Additional genes: *tat, rev* → regulatory function
- Supplementary genes: *vif, vpr, vpu & nef* → elevate viral titer, infectivity and pathogenicity

Adeno-associated virus (AAV) and lentivirus (LV)

	AAV	LV
Size	~25 nm	~80–100 nm
Genome	ssDNA	ssRNA
Packaging capacity	~4.7 kb	8–9 kb
Transduction	Primarily non-dividing cells	Dividing and non-dividing cells
Transduction efficiency	Moderate	Moderate
Integration	Non-integrating/episomal	Integrating
Tissue tropism	Broad, multiple serotypes	Broad (e.g., through pseudotyping with VSV-G)
Expression	Transient/stable	Stable
Immunogenicity	Minimal	
Preferred for	In vivo	Ex vivo

Advantages of LVs:

- Higher packaging capacity delivers more complex or larger therapeutic genes
- Host cell integration allows sustained gene expression

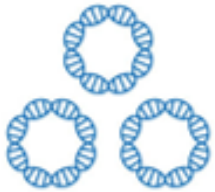
Main applications:

- Treatment of genetic disorders, cancer immunotherapy (e.g., CAR-T), infectious disease vaccines

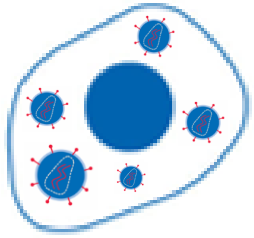
Risks of LVs:

- Replication-competent lentivirus (RCLs)
- Insertional mutagenesis – risk of disrupting oncogenes

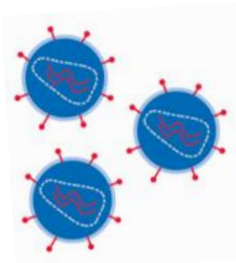
Characterization of LVs is key to safe and effective therapies



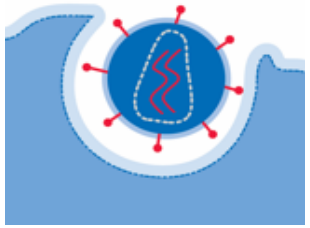
Plasmid development and production



LV production



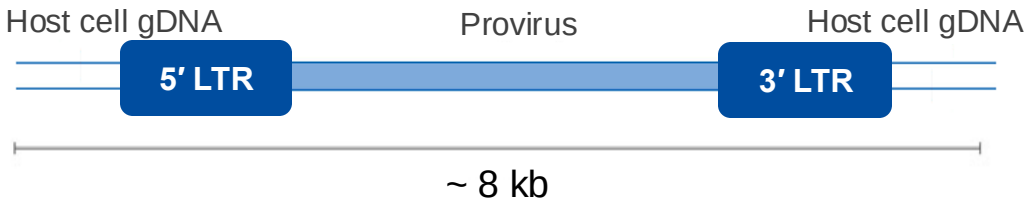
LV harvest (supernatant)



Delivery to target cell

Transfection

Transduction

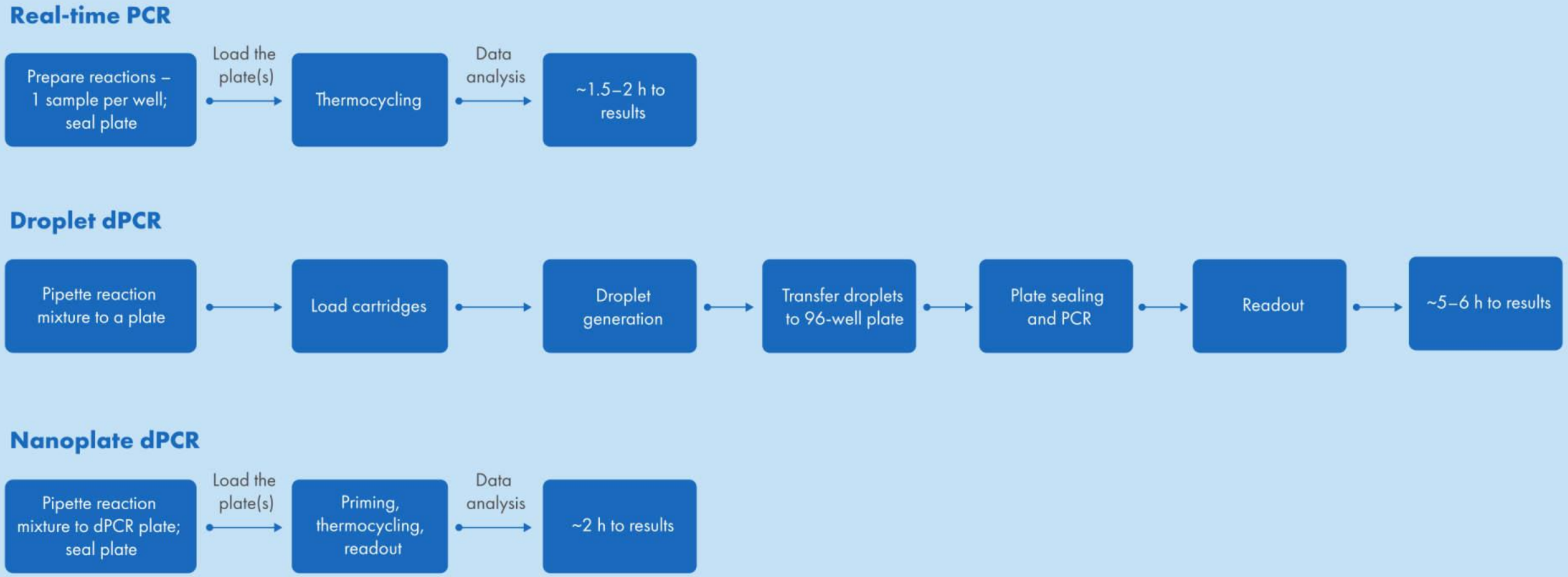


Digital PCR (dPCR) offers accurate and absolute quantification of CQAs

- Viral vector genome quantitation (physical virus titer)

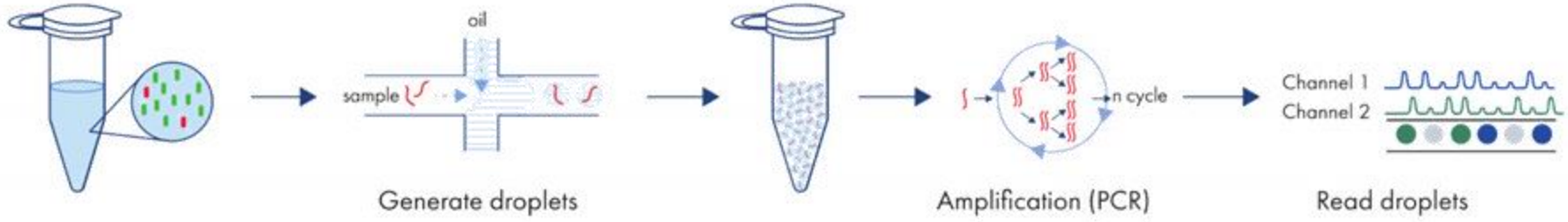
- VCN quantification
- RCL detection

Different PCR workflows for cell and gene therapy quality control

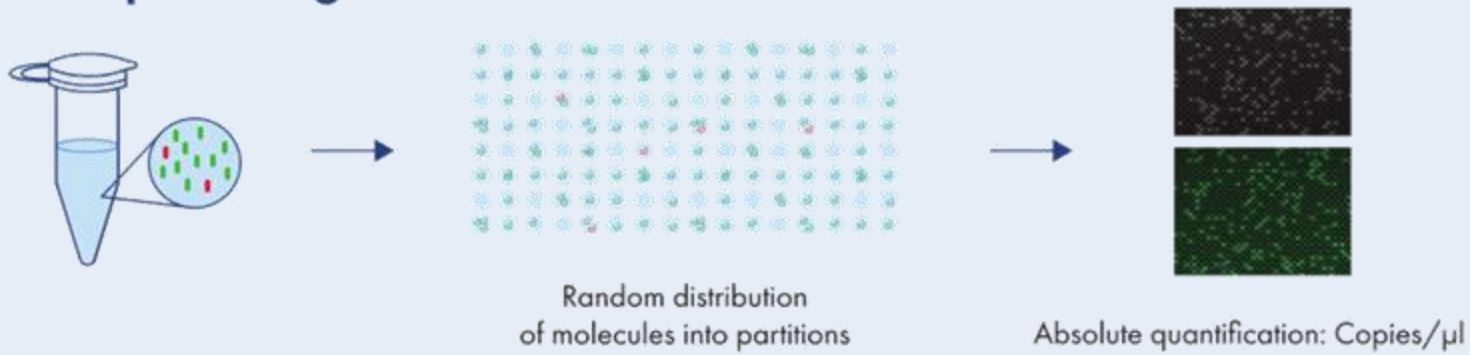


Select a platform that will easily transfer to a regulated environment

Droplet digital PCR



Nanoplate Digital PCR

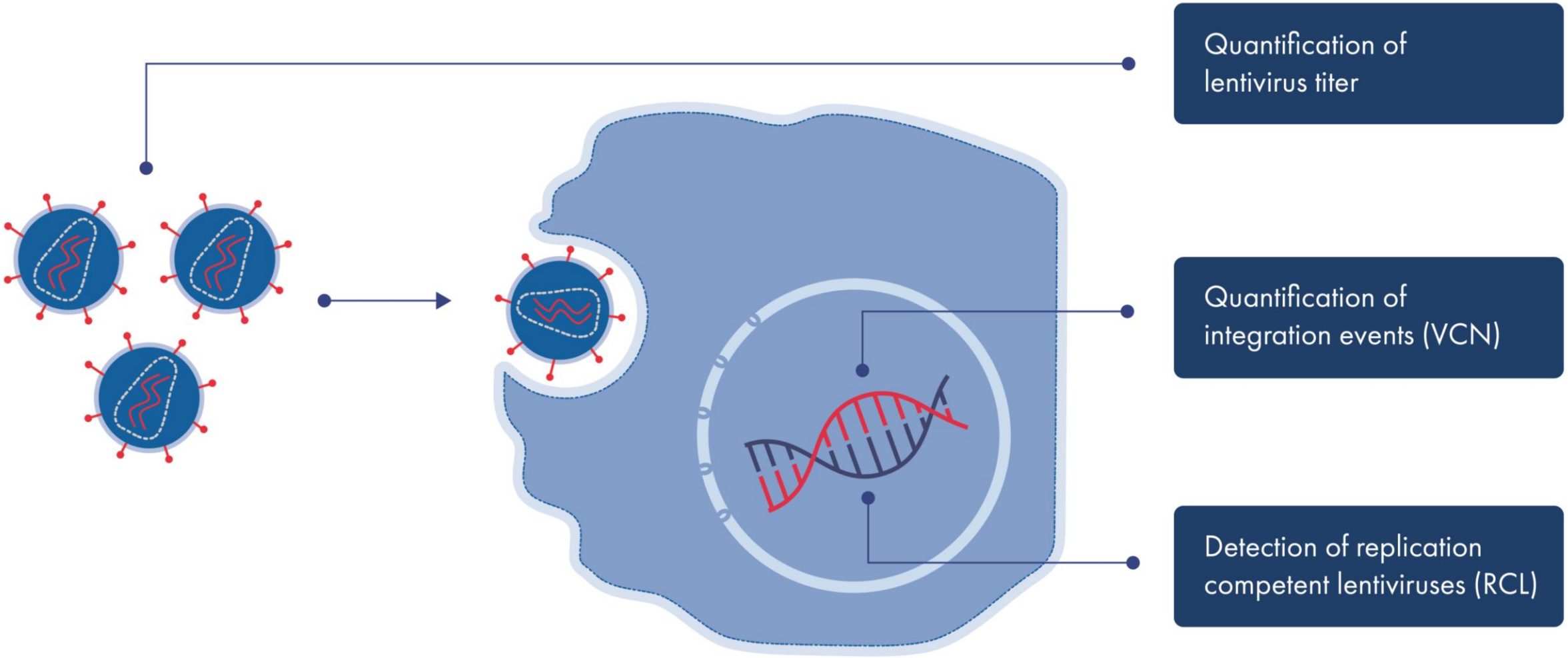


QIAcuity – just ONE unit

- ONE user requirement, ONE product maintenance, ONE qualification, ONE SOP
- Simple load and go
- Less user errors
- Chain of identity maintained
- Easier to train analysts
- Two-hour turnaround time (TAT)
- Unit is contained in user software that supports 21 CFR part 11
- Different user roles and individual authentication, audit trail tracking of changes
- One unit that is part of software
- Thermocycler part of the QIAcuity unit
- Analysis completed in the system software

(1) Nyaruaba R, et al. Developing multiplex ddPCR assays for SARS-CoV-2 detection based on probe mix and amplitude-based multiplexing. *Expert Rev Mol Diagn.* 2021;21(1):119-129.
(2) Nyaruaba R, et al. Digital PCR applications in the SARS-CoV-2/COVID-19 era: A roadmap for future outbreaks [published correction appears in *Clin Microbiol* Rev. 2023 Jun 21;36(2):e0005223]. *Clin Microbiol Rev.* 2022;35(3):e0016821.

Enhance your LV analytics with fast, reliable and resource-efficient solutions



Expansion of CGT dPCR Assays to LV workflow

Pack sizes:

CGT dPCR Assays (20x), 500 x 12 µL reactions

Different dye configurations:

FAM, HEX and Cy5

Quencher Zen and TAO IOWA Black

For singleplex, duplex and multiplex use

Ordering via QIAGEN website

www.qiagen.com/cgtdpcrassays

Kit components
CGT dPCR Assays (20X) (GOI)

Product	Description	Cat. No.
CGT dPCR Assays	One tube of target sequence dPCR Assay, 500 for 12 µL reaction in 8.5k Nanoplate	250230 – 250256 250300 – 250321 (New assays)
QIAcuity Probe PCR Kit (1 mL, 5 mL, 25 mL)	1 mL or 5 mL or 25 mL Probe MasterMix	250101, 250102, 250103
QIAcuity MasterMix (1 x 2 mL)	2 x 1 mL 4x concentrated MasterMix	1133251

QIAcuity RCL Quant Kit

Pack sizes:

96 x 40 µL reaction with positive and internal controls

Restriction enzyme compatibility:

P*vuII*

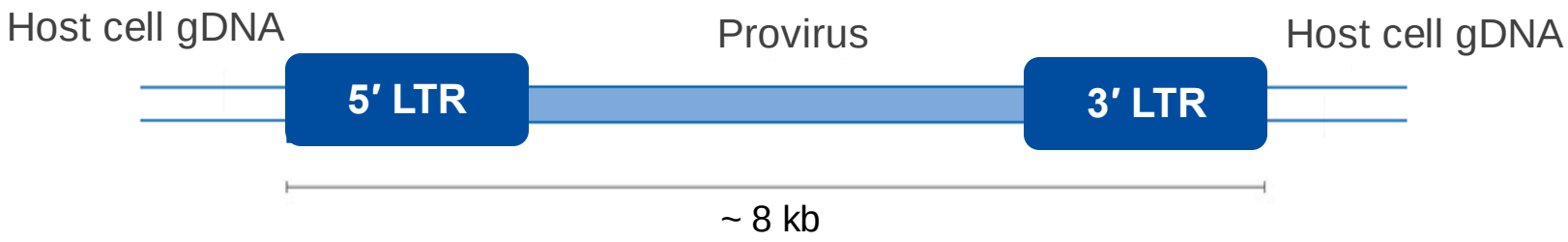
Ordering via QIAGEN website

www.qiagen.com/qiacurityrclkit

Product	Description	Cat. No.
QIAcuity RCL Quant Kit	Detection of the presence of RCL using VSV-G assay	250322

Kit components	Amount	Cap color
QIAcuity MasterMix (1/2)	1	Red
QN Internal Control DNA dPCR	1	Blue
QN IC Probe Assay 10x (200µl)	1	Orange
QIAcuity RCL (VSV-G) Assay	1	Violet
QIAcuity RCL (VSV-G) Assay Positive Control	1	Green
RNase-free Water (1.9 mL/2)	2	Transparent

Accurate and precise quantification requires well-designed assays



Design considerations

- GC content
- Probe system
- Primer and probe placement
- Length of primers and probes
- Melting temperatures

Validation process (examples)

- Specificity and reproducibility
- Optimal PCR cycling and imaging conditions
- Linearity
- Signal-to-noise ratio
- Ability to multiplex

Expansion of CGT dPCR Assays dedicated to LV workflow

2 **LV titer determination (vector backbone):** Direct lysis solution (Q2) and quantitation of genome-containing lentiviral particles

1 **VCN quantification:** Lentiviral targets in the gDNA of transduced cells in comparison to a reference housekeeping gene

3 **RCL:** Detection of replication-competent viruses

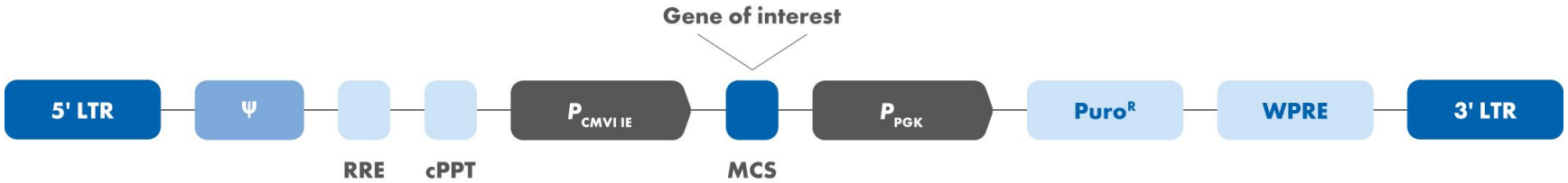
Reference genes
Albumin*
RPP30
RPL32

Reporter/resistance
GFP/eGFP
AmpR‡
PuroR§
KanR/NeoR

Promoter/enhancer
CMVp
CMVe
SV40p
WPRE*

Integration plasmid elements
Psi*
RRE*
5′ LTR*

Lentivirus replication (RCL)
VSV-G



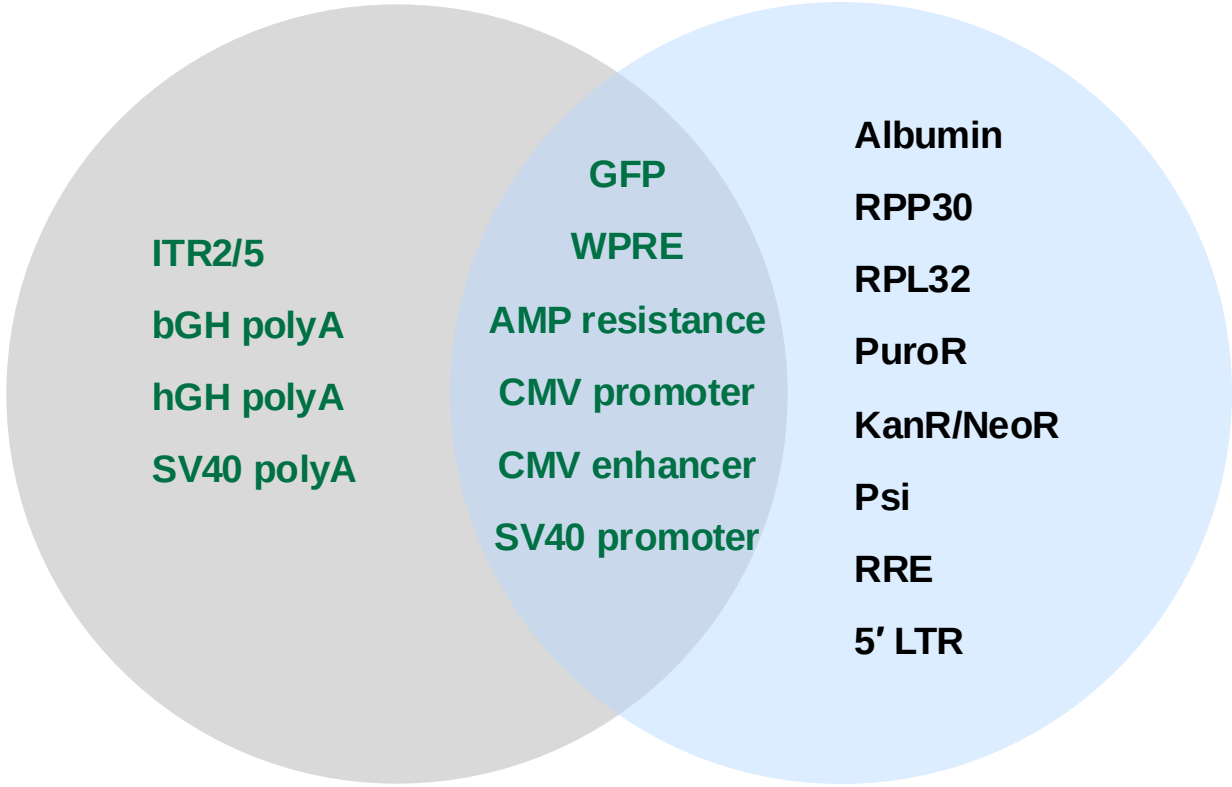
Eight new targets for lentiviral research and manufacturing process with up to 3 dyes (FAM, HEX, Cy5**).

* WHO recommendations I Assays already existing in CGT dPCR Assay portfolio for AAV workflow; ** Cy5 not available for PuroR and KanR/NeoR, ‡ AmpR only residual plasmid testing, § PuroR very high GC content – not intended for RNA applications

Expansion of CGT dPCR Assay portfolio – overview

Current AAV assays

- 10 targets
- QIAcuity Probe PCR Kit



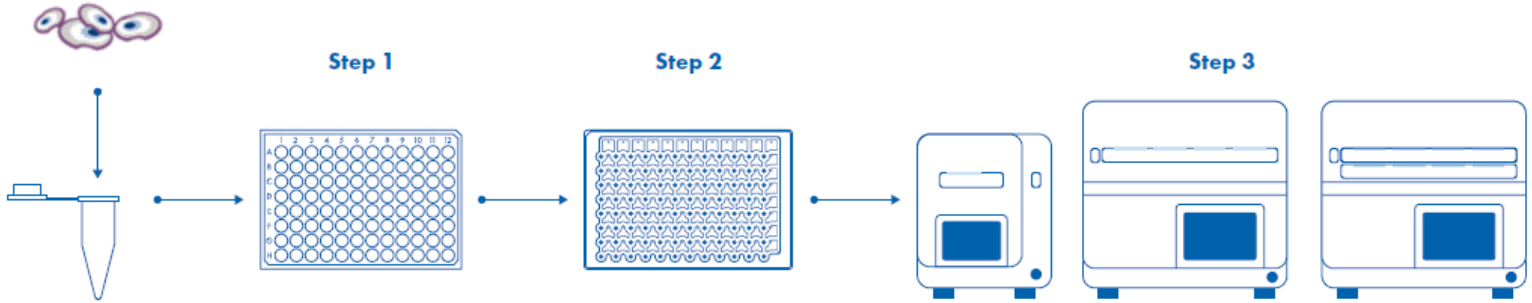
LV assays

- 8 new targets
- 6 existing assays
- QIAcuity MasterMix

Assays existing in CGT dPCR Assay portfolio for AAV workflow

A simple and fast protocol

Validated assays for LV workflows using digital PCR



Starting material* added to mastermix

Setup reaction mix in PCR pre-plate

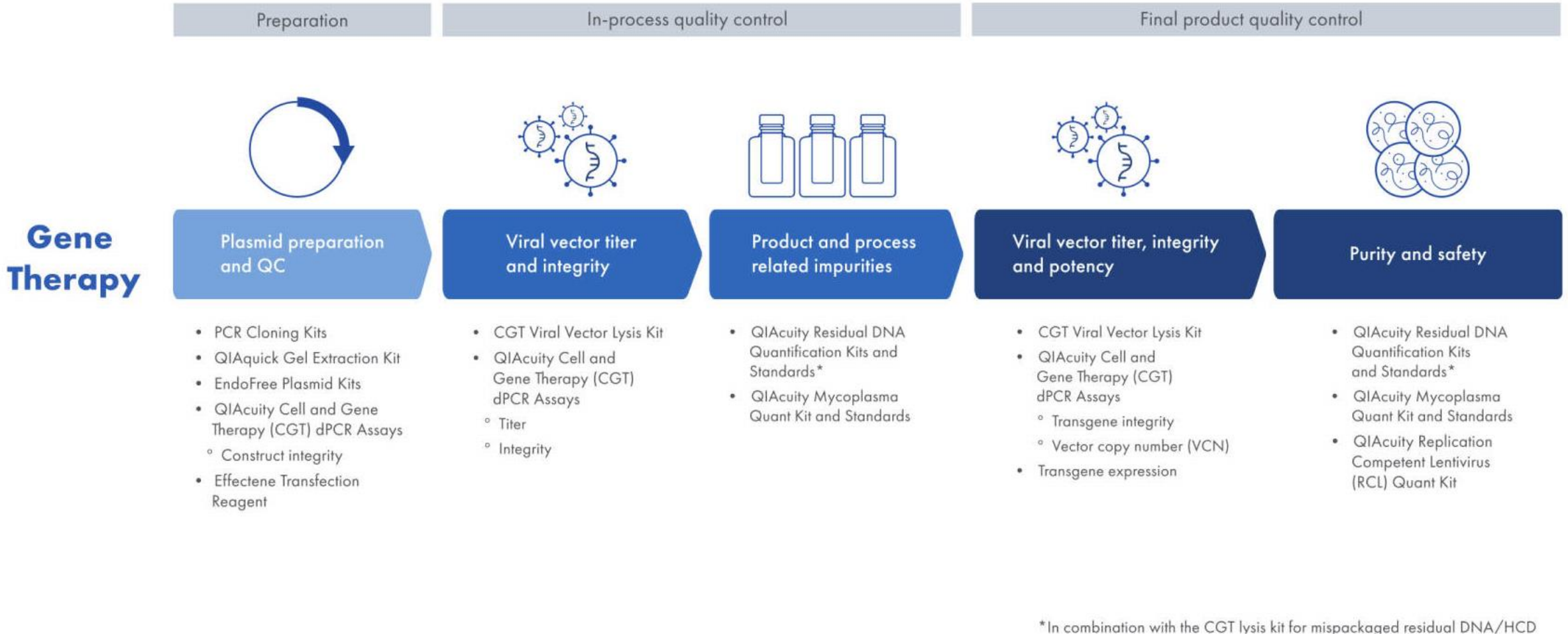
Pipet sample into a Nanoplate

Run and analyze results in the QIAcuity Digital PCR System

- 14 LVV targets available with different dye combinations for multiplexing
- 3-plex capacity (can be extended to 5-plex with customer-chosen GOIs)
- Complimentary, easy-to-use analysis in the QIAcuity Software Suite
- Used in combination with QIAcuity MasterMix and Nanoplates

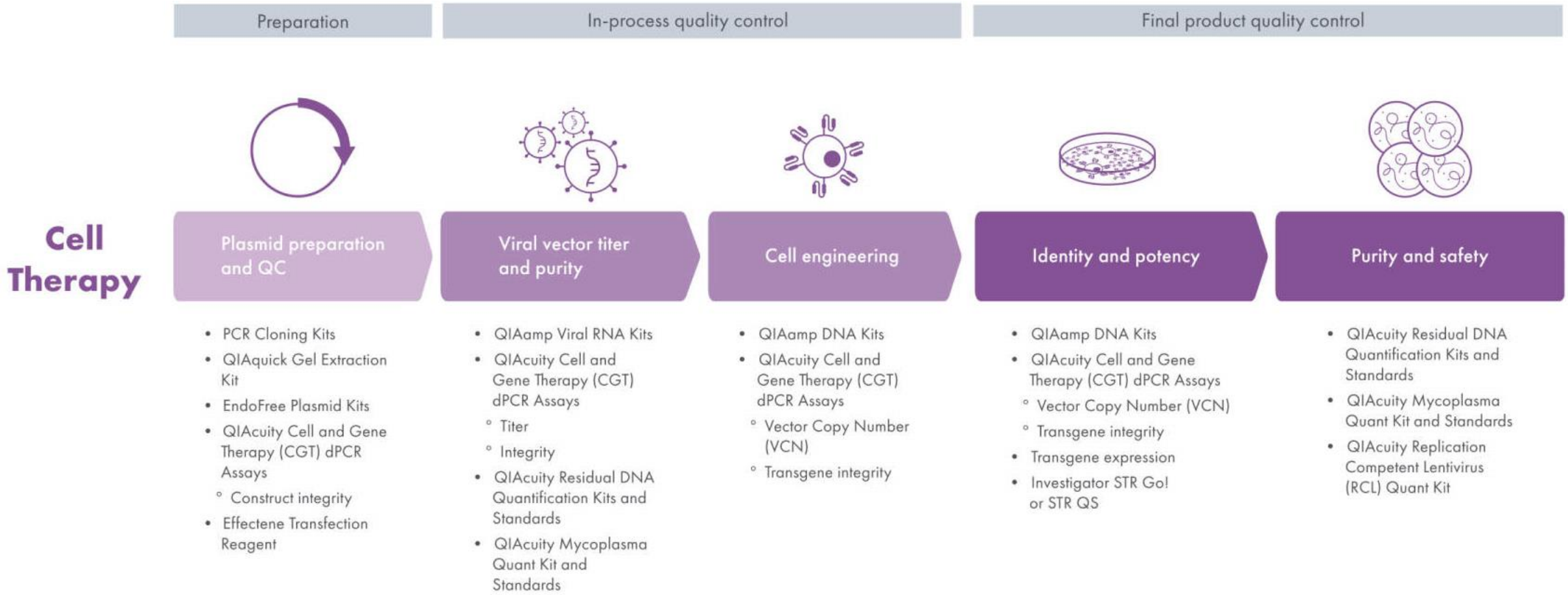
*Starting material differs depending on workflow: gDNA (VCN, RCL), LV genomic RNA (titer)

Growing dPCR portfolio for QC/QA of cell and gene therapies



Dedicated dPCR assays and kits for multiple viral vectors workflows.

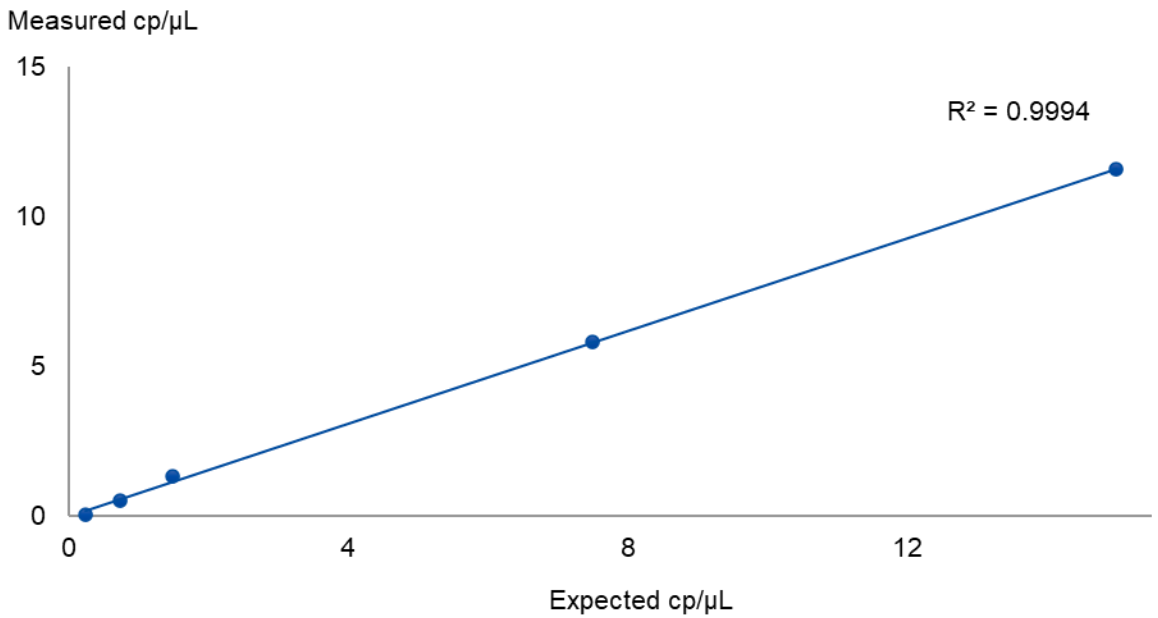
Growing dPCR portfolio for QC/QA of cell and gene therapies



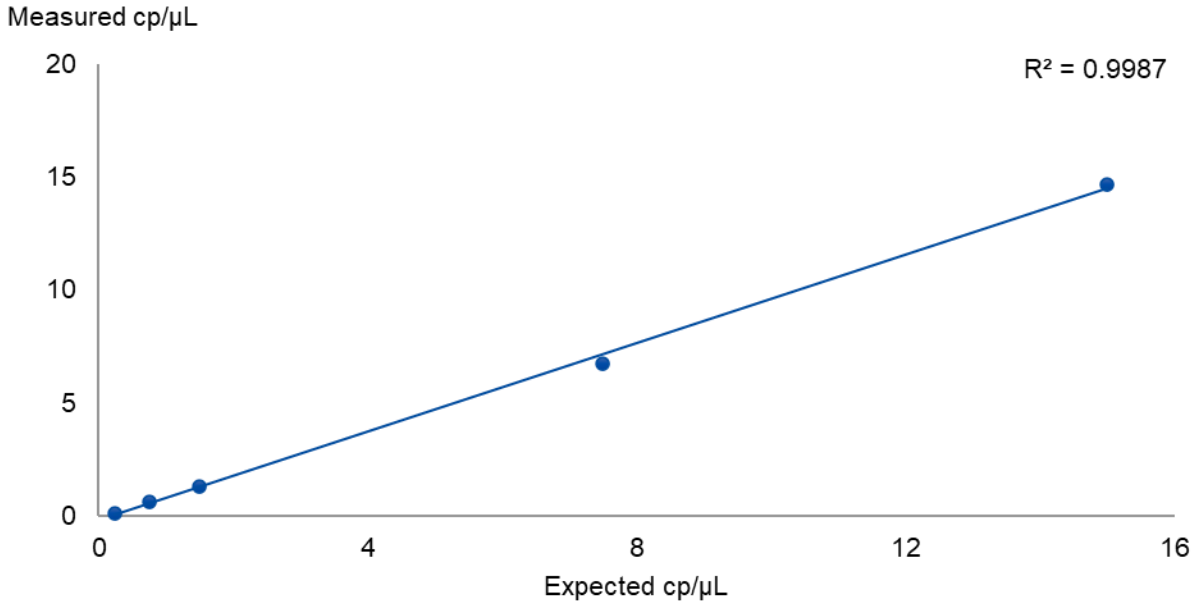
Dedicated dPCR assays and kits for multiple viral vectors workflows.

Linearity in quantification down to 0.25 copies/µL

RRE HEX: Lentivirus target



RPL32 FAM: Reference assay

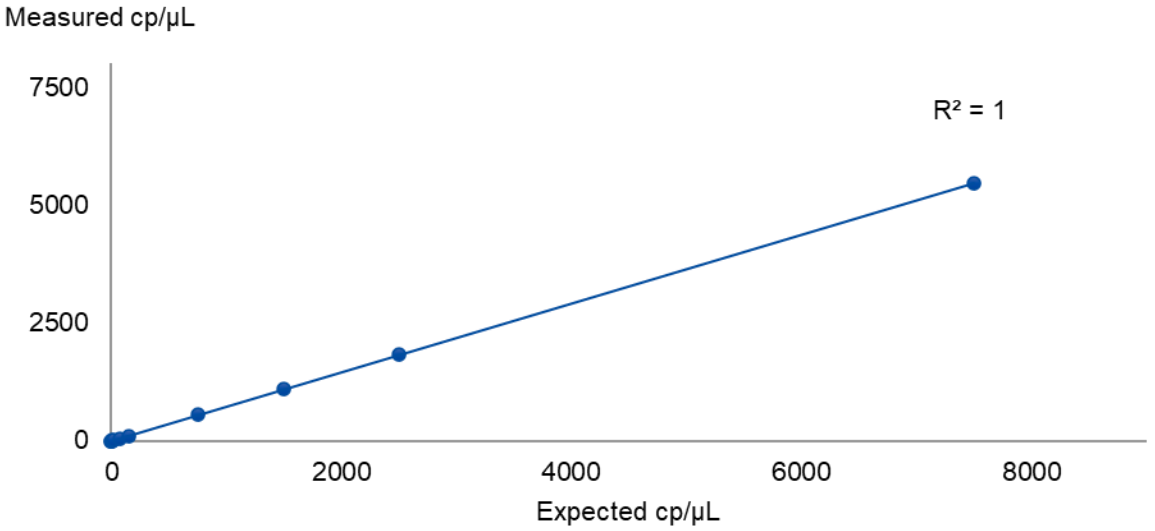


1 data point → 3 wells (8.5k Nanoplate)

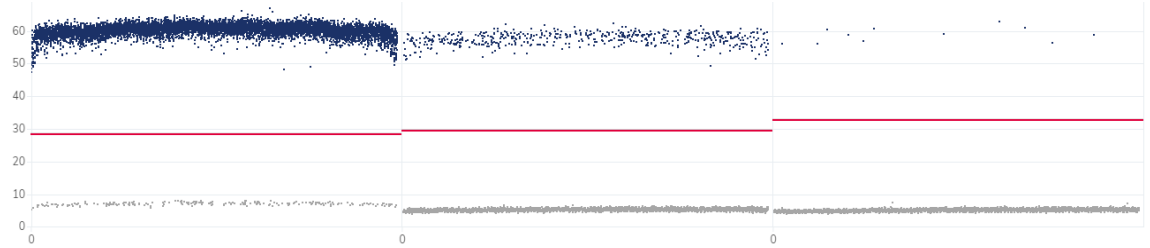
High linearity from 0.25–15 copies/µL on 8.5k Nanoplates (triplicates).

High precision over a broad dynamic range

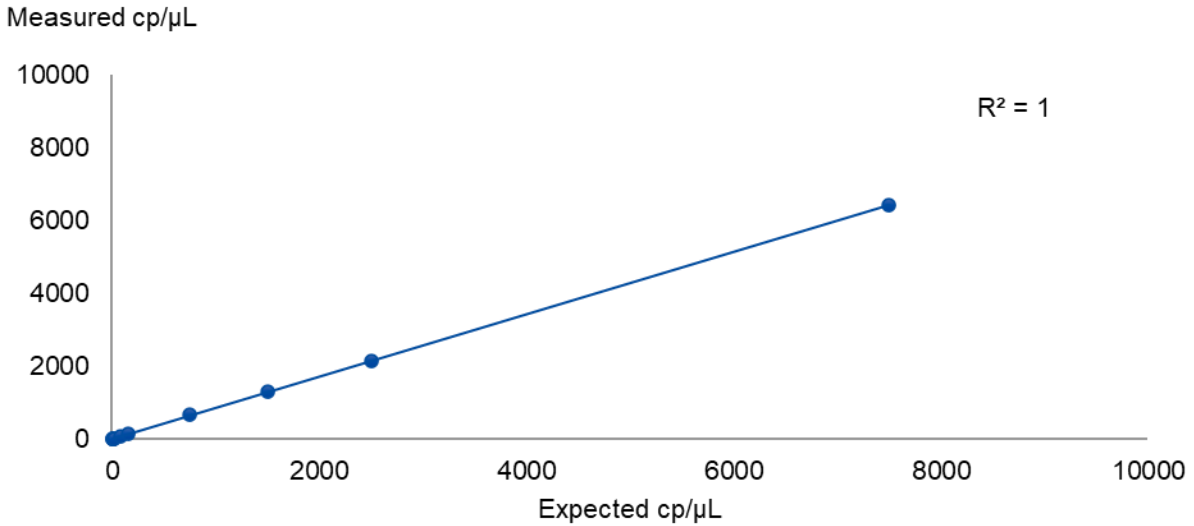
RRE HEX: Lentivirus target



7500 cp/µL **150 cp/µL** **1.5 cp/µL**



RPL32 FAM: Reference assay



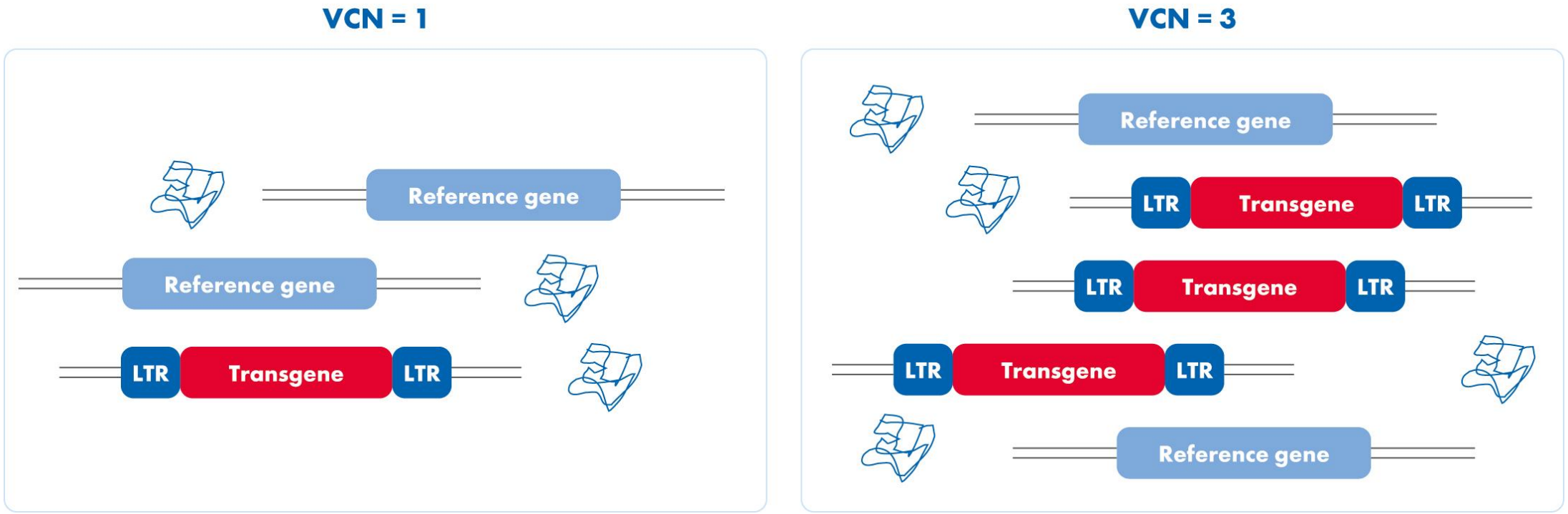
7500 cp/µL **150 cp/µL** **1.5 cp/µL**

High linearity from 0.25–7500 copies/µL on 8.5k Nanoplates.

Determination of VCN ratios

Formula for standard diploid genomes:

$$VCN = 2 \times \frac{vector\ target\ copies}{human\ reference\ target\ copies}$$

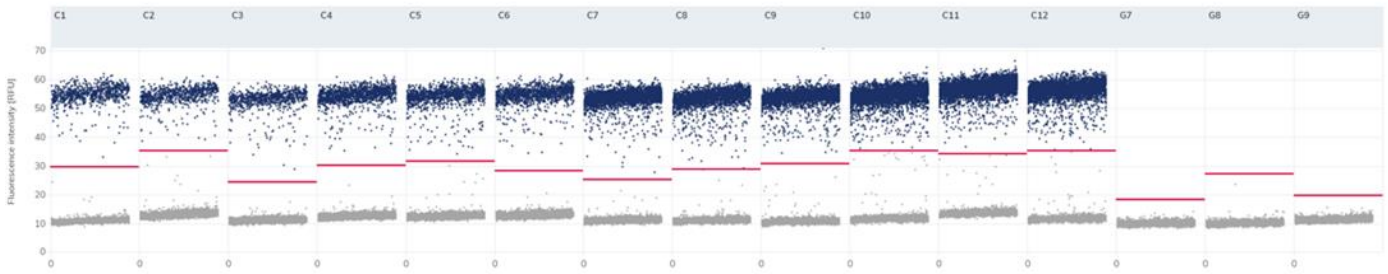


Accuracy for PuroR FAM + RPP30 HEX duplex reaction

Determination of VCN: gDNA with LV target spiked-in for VCN 1, VCN 2, VCN 5 and VCN 10

PuroR FAM

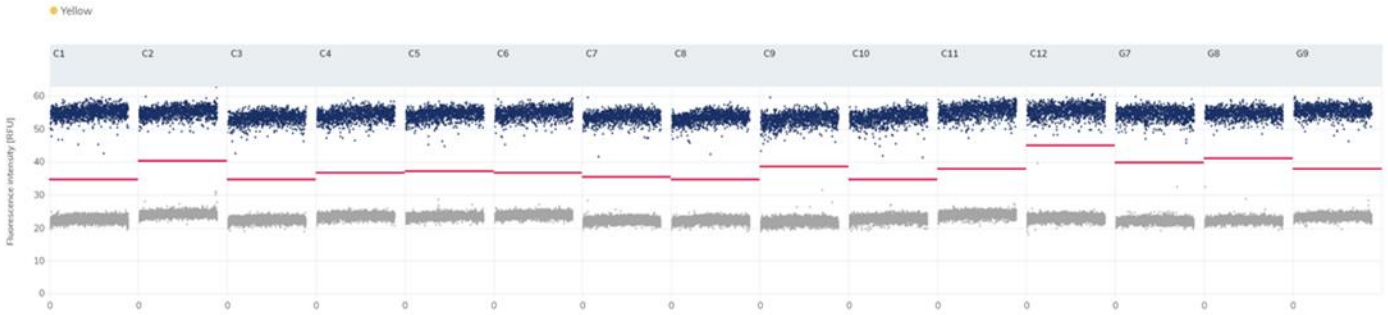
LV target



VCN 1 **VCN 2** **VCN 5** **VCN 10** **VCN 0**

RPP30 HEX

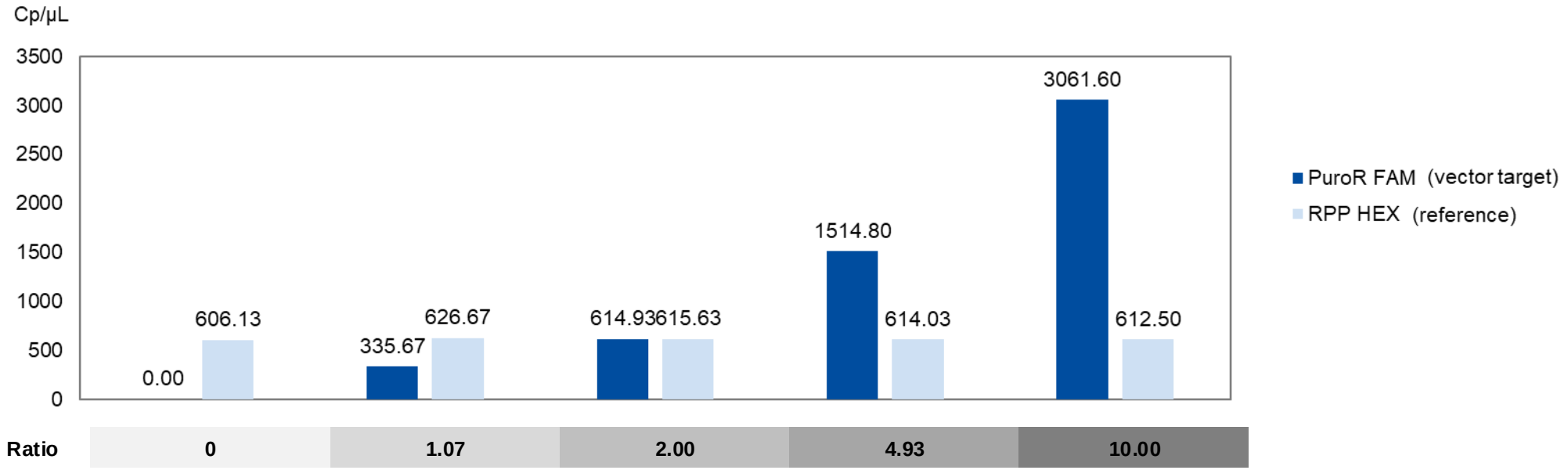
Reference target



Excellent signal to noise ratios over a broad range of LV target copy numbers.

Accuracy for PuroR FAM + RPP30 HEX duplex reaction

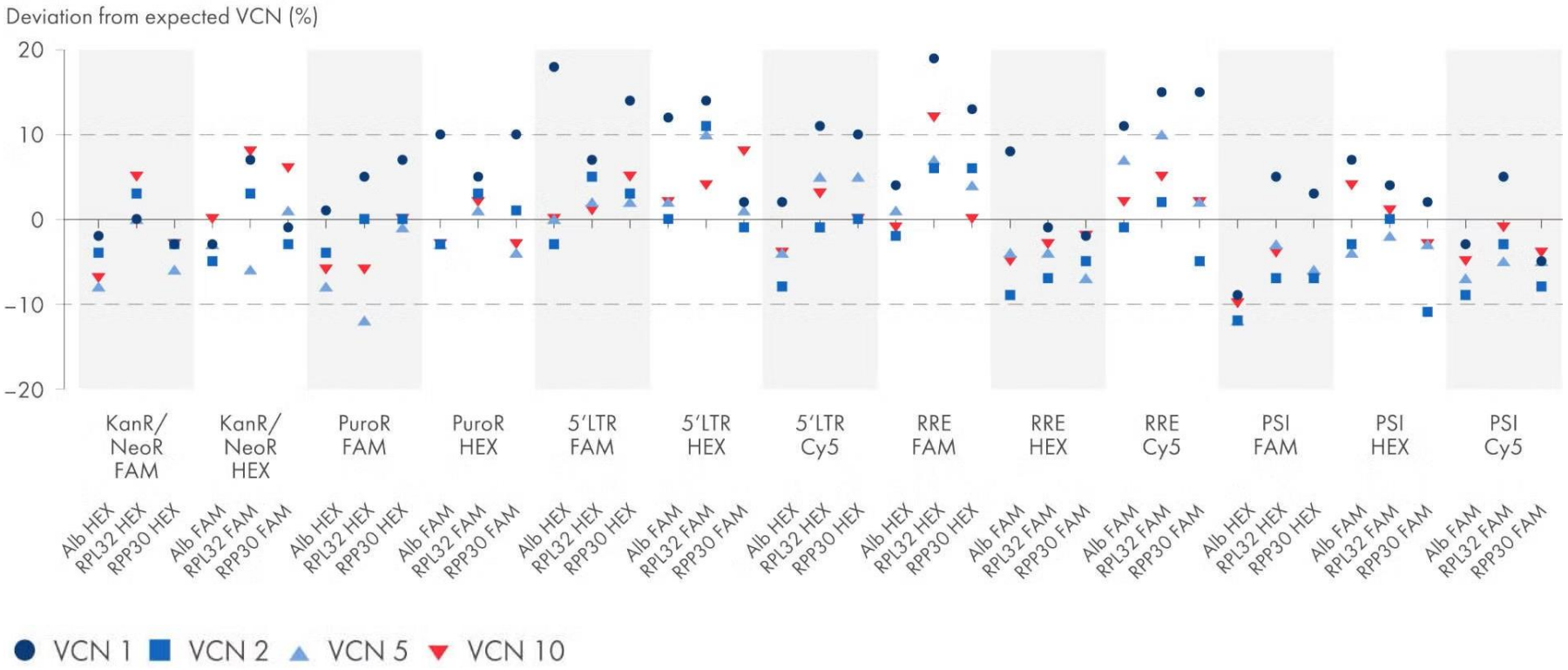
Determination of VCN: gDNA with LVV target spiked-in for VCN 1, VCN 2, VCN 5 and VCN 10



$$VCN = 2 \times \frac{vector\ target\ copies}{human\ reference\ target\ copies}$$

Very high accuracy for VCN quantification with PuroR FAM + RPP30 HEX duplex <5%.

High accuracy for VCN quantification



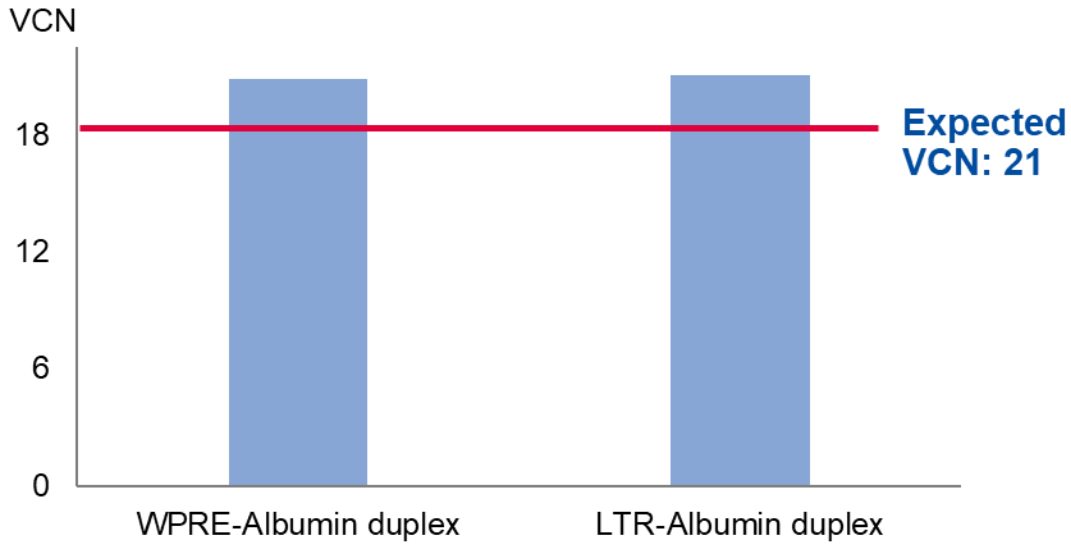
High accuracy for VCN quantification independent of fluorophores

- CGT dPCR vector backbone assays (KanR/NeoR, PuroR, 5' LTR, RRE, Psi) were run in duplex reactions with CGT dPCR genomic reference assays (albumin, RPL32, RPL30)
- Template was gDNA and 0/1/2/5/10-fold concentrations of gBlocks
- PCR was performed on the QIAcuity using a 96-well 8.5k Nanoplate
- All assays showed only less than 20% deviation from the expected VCN

For 24 out of 39 duplex combinations, deviation from expected VCN was less than 10%.

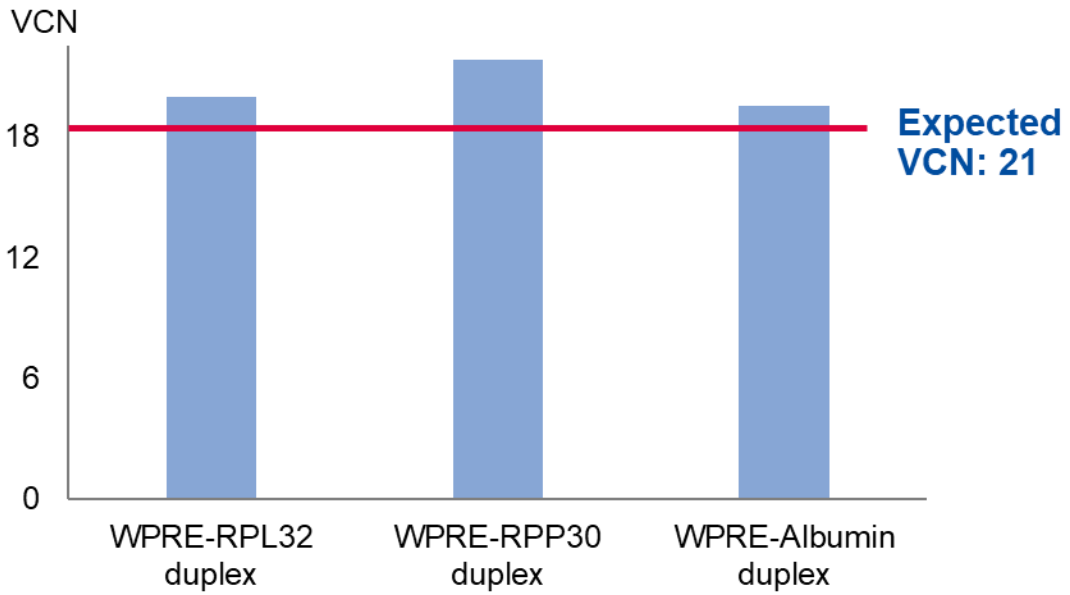
High-precision VCN determination

VCN of LV transduced control cell line



- VCN was determined for a control cell line using different LV targets
- Duplexes performed with albumin in FAM and LV targets in HEX

VCN of LV transduced control cell line

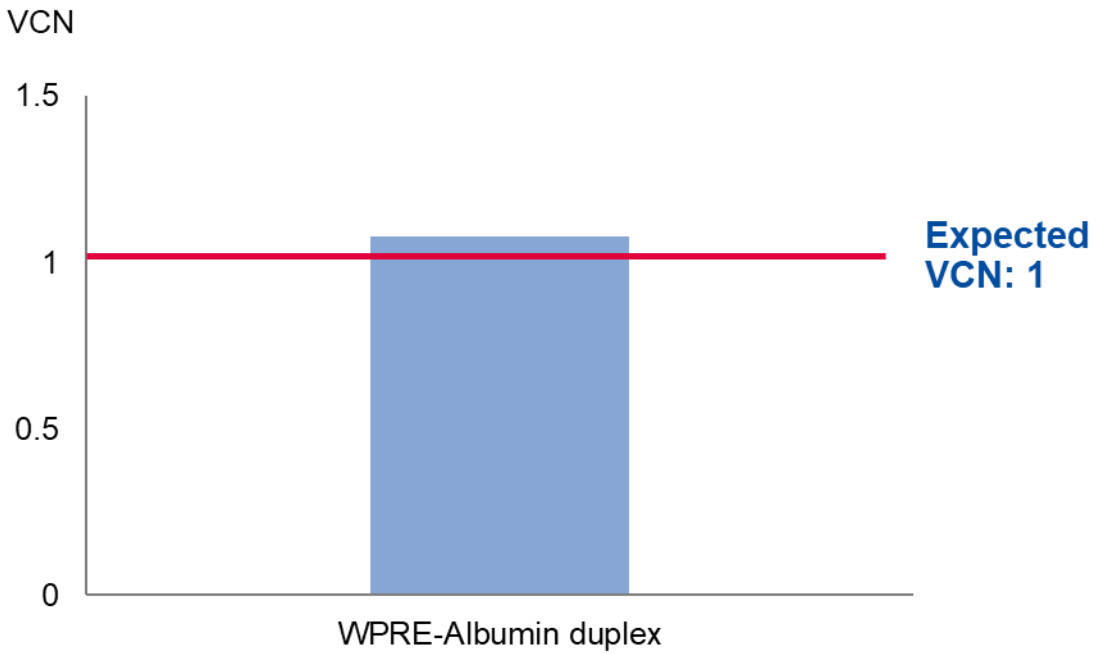


- VCN was determined for a control cell line using different reference assays
- Duplexes performed with references in HEX and WPRE in Cy5

Precise and comparable VCN determination with different LV targets and reference genes.

High-precision VCN determination

Duplex for VCN of control cell line



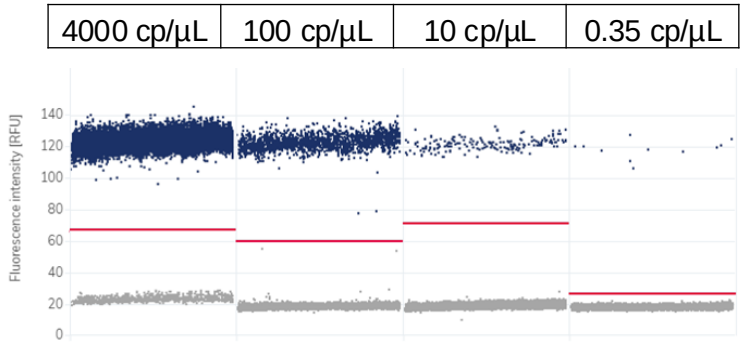
- VCN was determined for a control cell line using WPRE
- Duplexes performed with albumin in FAM and LV targets in HEX

Precise and comparable VCN determination from 0 to over 20 copies per genome.

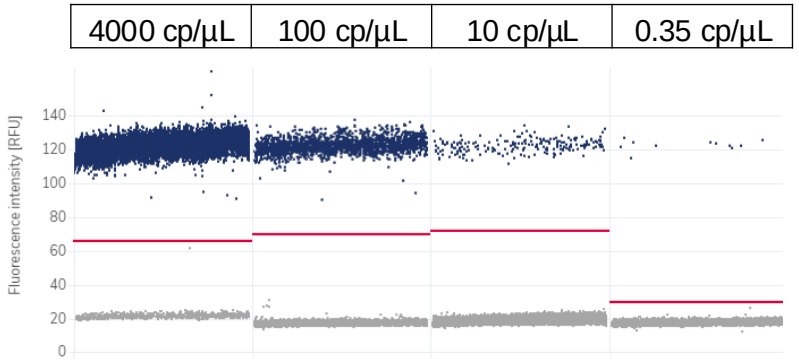
Robust RCLs detection using the QIAcuity RCL Quant Kit

RCL assay: Different concentrations of positive control spiked-in with increasing amounts of genomic background DNA

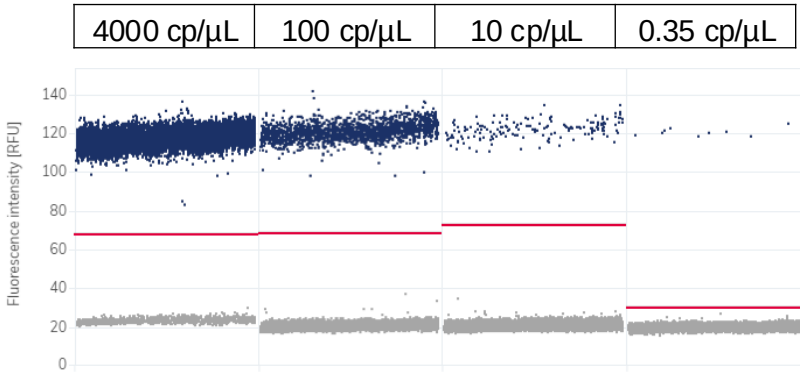
Background: 0 µg gDNA



1 µg gDNA

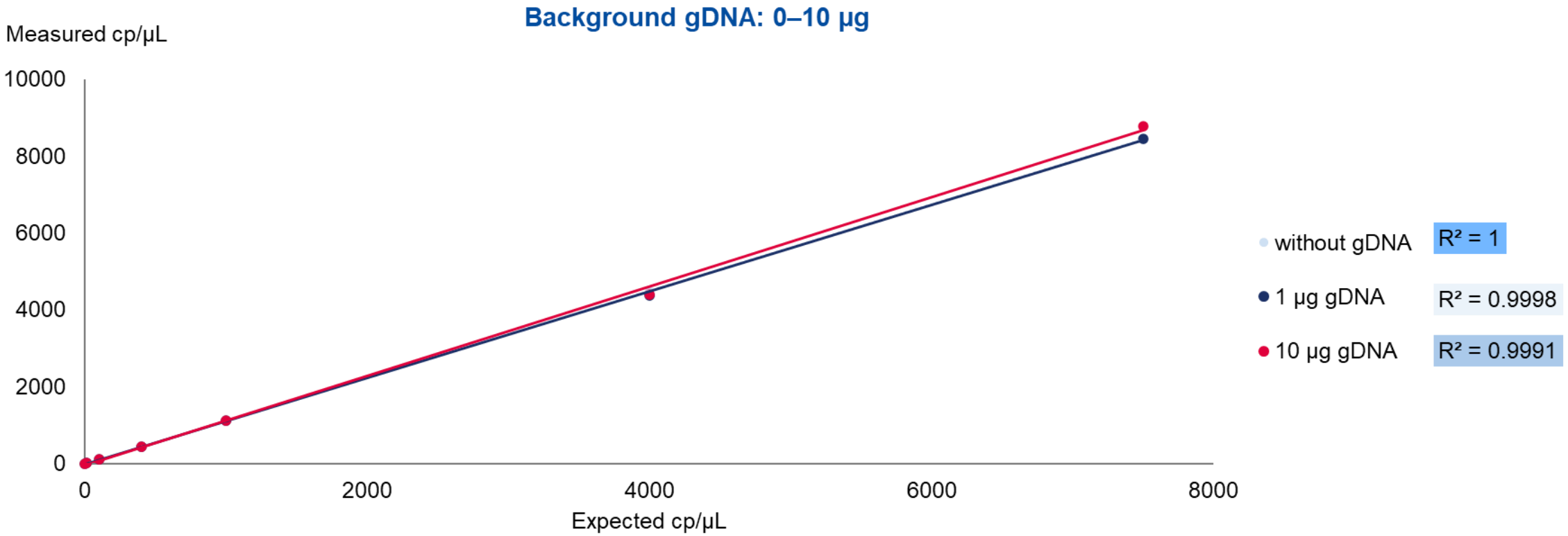


10 µg gDNA



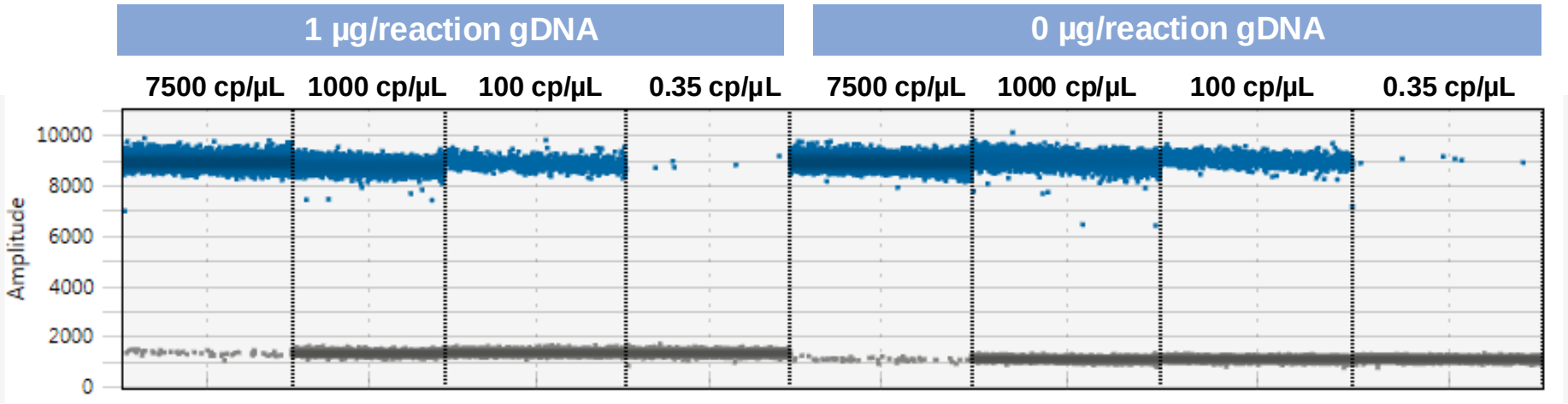
Up to 10 µg per reaction background for different concentrations of a positive control.

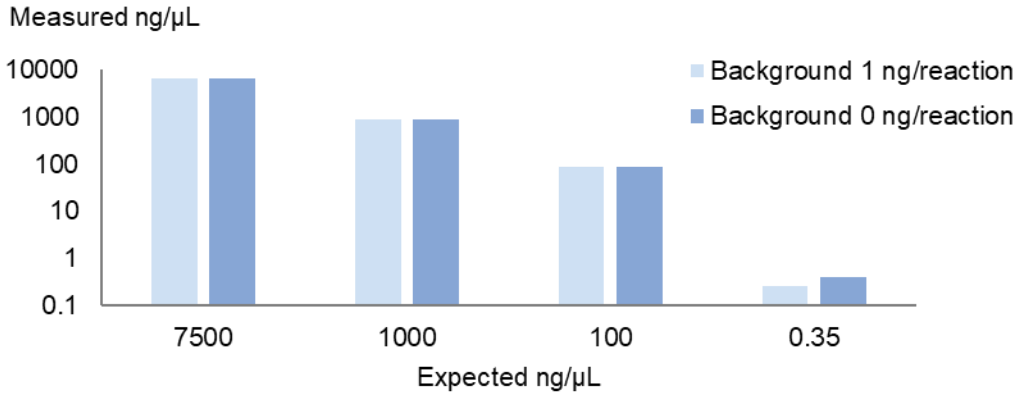
RCL Quant Kit with high gDNA background



Up to 10 µg per reaction background for different concentrations of a positive control.

RCL Quant Kit vs a kit from another supplier





- DNA input **limited to 1 µg/reaction**
- Quantification for VSV-G comparable between 0 and 1 µg/reaction

More than 1 µg of DNA not recommended according to Suppliers B handbook.

Data obtained through experiments conducted by QIAGEN R&D in Hilden, Germany.

Summary

Main features

- Mix and match approach using wet-lab tested **QIAcuity CGT dPCR Assays** for LV titer and VCN measurement
  - Assess LV titer using optimized assays for integration plasmid elements, regulatory elements or reporter/resistance genes
  - Use the same assays in combination with our genomic reference assays to easily assess the number of integrated vector copies
- A complete **QIAcuity RCL Quant Kit** for sensitive and robust detection of VSV-G absence in your cell material – including positive and internal controls
- QIAGEN sample prep solutions in combination with the dPCR assays offer a standardized workflow optimized for use on the QIAcuity

Benefits

- **Fast and reliable**: Experience a true qPCR-like workflow that eliminates the need for standard curves, reducing hands-on time, TAT and errors, while increasing accuracy and consistency
- **Standardized and wet-lab tested**: Use our wide range of pre-tested solutions for consistent, reproducible results across your workflows to save time and ensure efficiency
- **High throughput and scalability**: Meet the demands of both small research labs and large-scale screening and quality control facilities with a broad range of options

Thank you for your attention. Questions?