

Digital PCR for the Characterization of Reference Materials

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NIST National Institute of Standards and Technology U.S. Department of Commerce

MATERIAL MEASUREMENT LABORATORY

Digital PCR at NIST

Digital PCR has become our ‘go to’ method for the quantification of nucleic acid-based materials

Replacing UV spectroscopy (indirect method)

The typical downstream application of our ***reference materials*** is PCR or sequencing-based

We care about ***intact (and accessible) genomic targets***

Digital PCR

Droplet digital (ddPCR) used for copy number determination

Does not require an external calibrant

Bio-Rad QX200 instrument

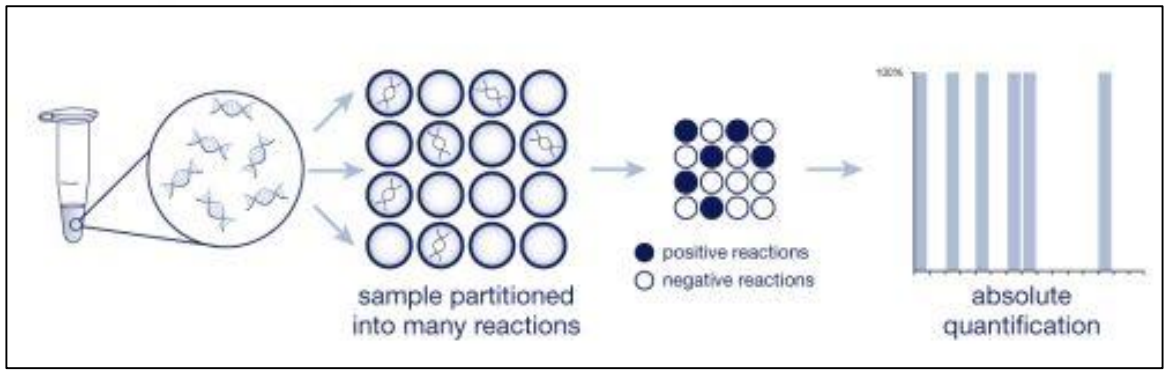


Image credit: http://digital-pcr.gene-quantification.info/

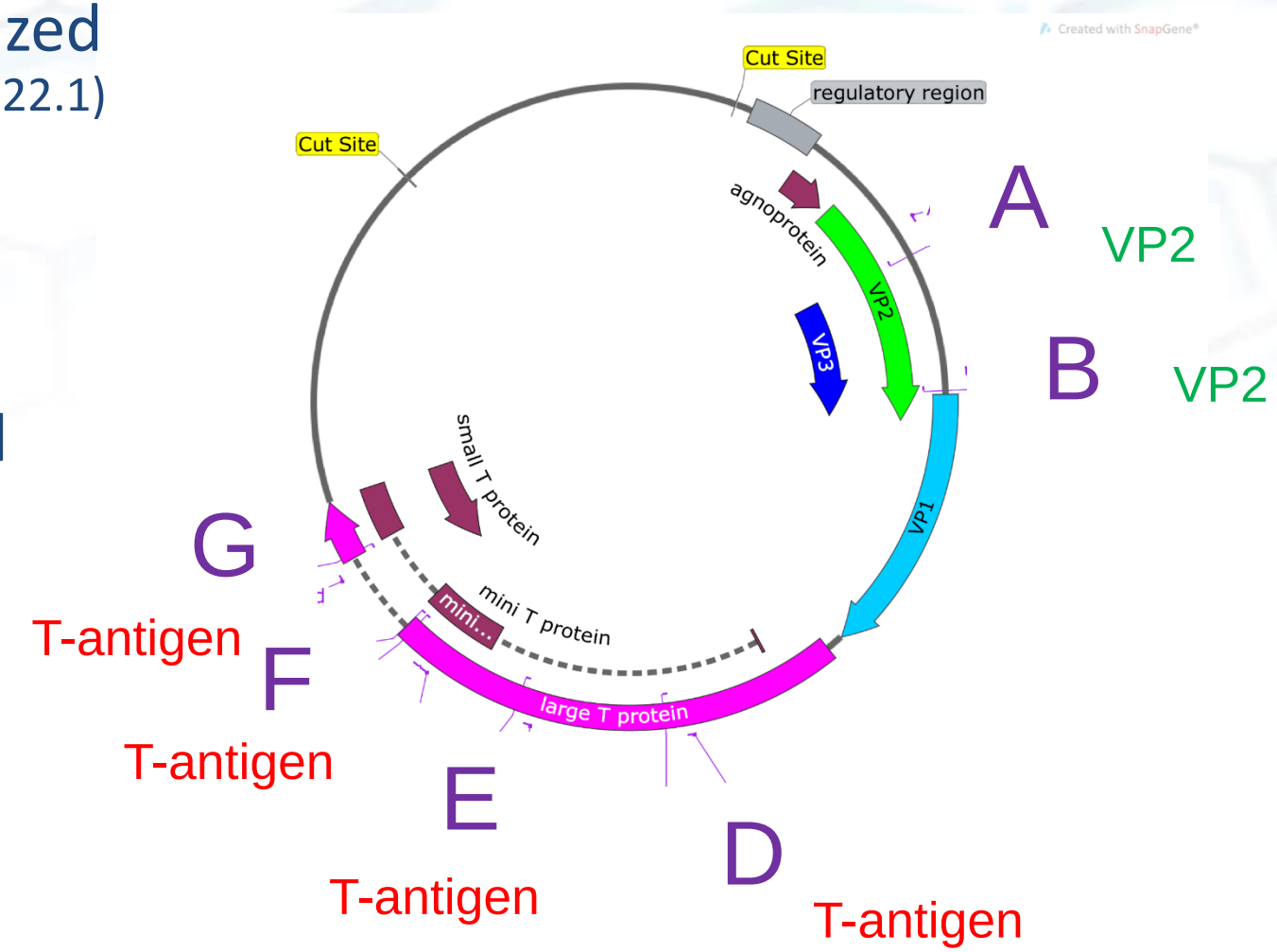
Image credit: http://bio-rad.com/

$$\lambda = -\ln(Fraction\ Negative)$$

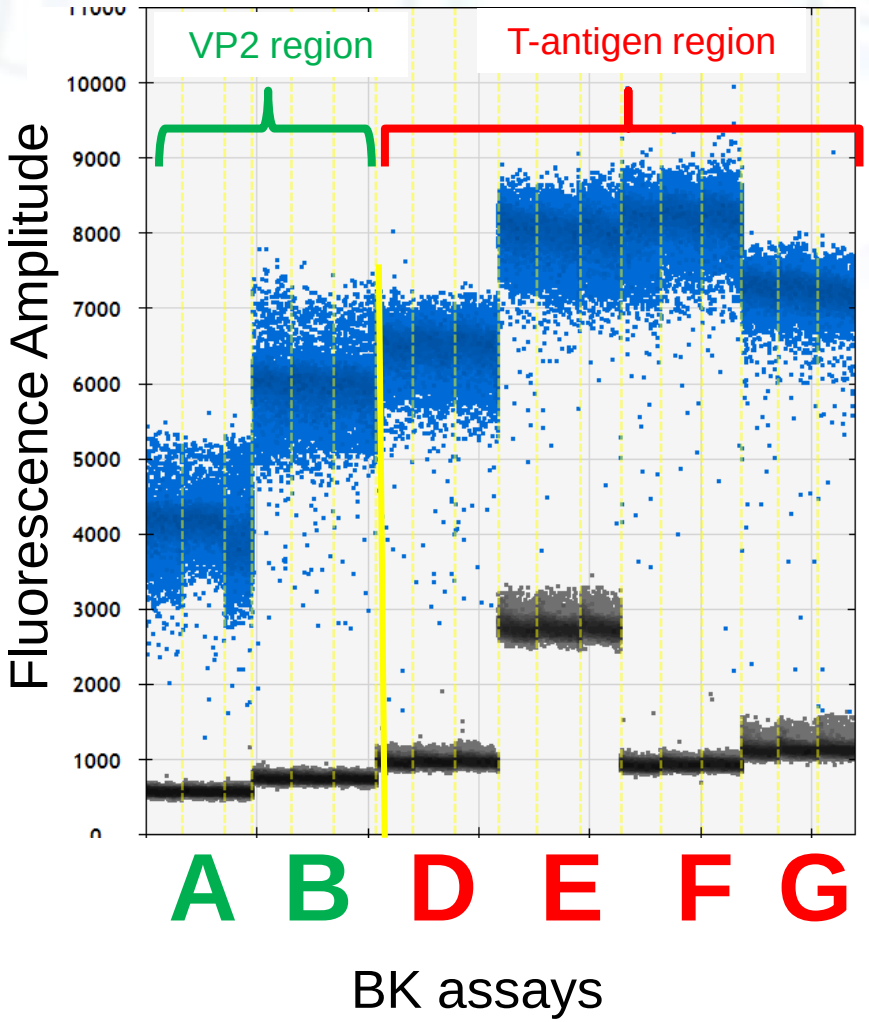
Copies/µL = λ / (Droplet Volume in µL)

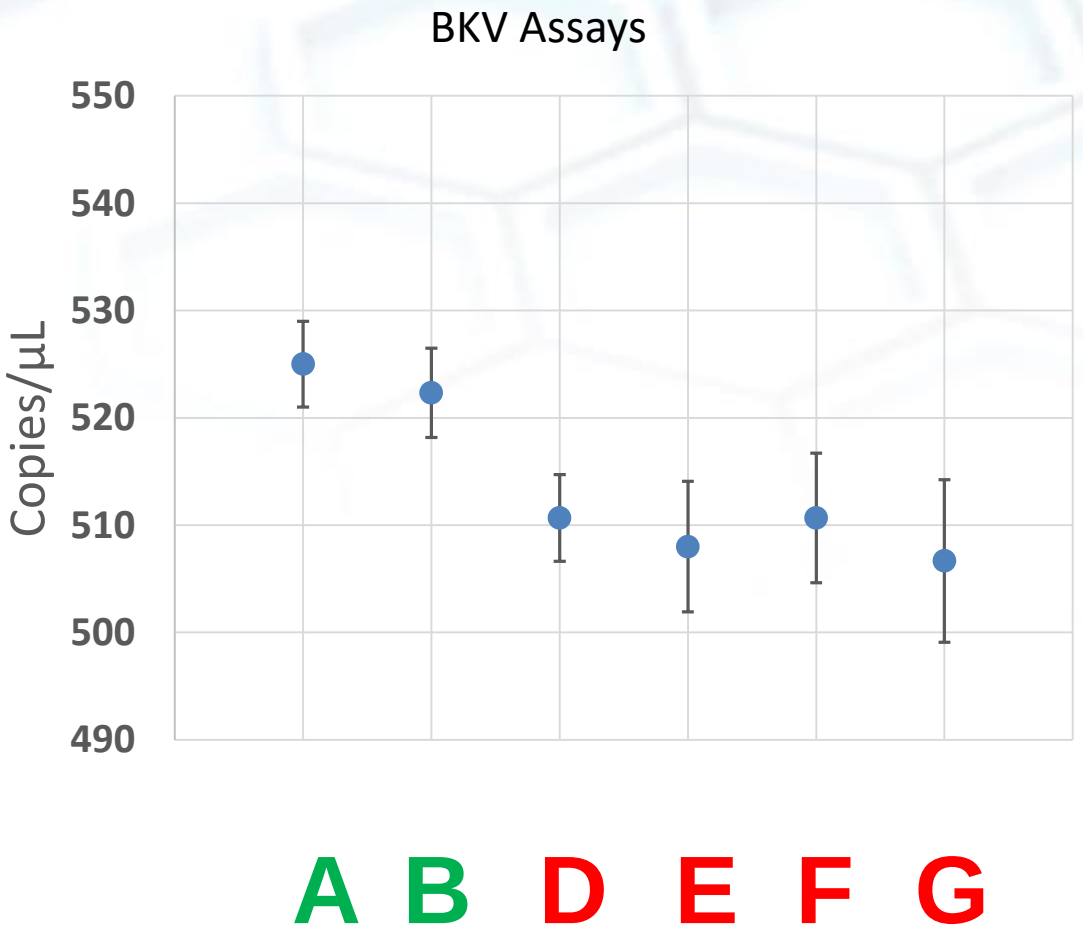
NIST Standards for BK Virus

- BKV genome was synthesized (Strain Ia – NCBI accession # JQ713822.1) and cloned into pUC57 plasmid
- Extracted DNA standard
- Linearized and in buffered solution at 4°C
- Concentration: 5.58 x 10^8 copies/mL
- tRNA added for stability



ddPCR assays for NIST BKV





SRM 2365 – BK Virus DNA Quantitative Standard

National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material® 2365

BK Virus DNA Quantitative Standard

This Standard Reference Material (SRM) is intended for use in the value assignment of BK virus deoxyribonucleic acid (DNA) quantitation materials, primarily those used for quantitative polymerase chain reaction (qPCR). SRM 2365 consists of a well-characterized, linearized plasmid, containing BK virus DNA solubilized in 10 mmol/L 2-amino-2-(hydroxymethyl)-1,3 propanediol hydrochloride (Tris HCl) and 1 mmol/L ethylenediaminetetraacetic acid disodium salt (disodium EDTA) pH 8.0 buffer (TE), with 50 ng/µL yeast tRNA added to ensure stability. A unit of the SRM consists of one 0.5 mL tube containing approximately 110 µL of DNA solution. The tube is labeled and is sealed with a screw cap.

Certified Values: Certified values are provided in Table 1. A NIST certified value is a value for which NIST has the highest confidence in that all known or suspected sources of bias have been accounted for. The copy number values are metrologically traceable to the natural units count 1 and ratio 1 and International System of Units (SI) derived units of volume.

Table 1. Certified Value for SRM 2365

Analyte	Certified Value (copies/µL)	95% Probability Uncertainty Interval (copies/µL)	Standard Uncertainty, $u(X)$ (copies/µL)	Effective Coefficient of Variation, $CV=100\times u(X)/X$
BK Virus DNA copy number	558,000	534,000 to 582,000	12,000	2.2%

WHO Standards / NIST Standards

	WHO Viral Standards	NIST Viral Standards
Contains	Viral particles	Extracted DNA
Intended Use	Extraction & Quantitation	Quantitation only
Concentration	International Units	Genome Copies per µL
How Concentration is Determined	Large, international multicenter effort	At NIST, multiple dPCR assays

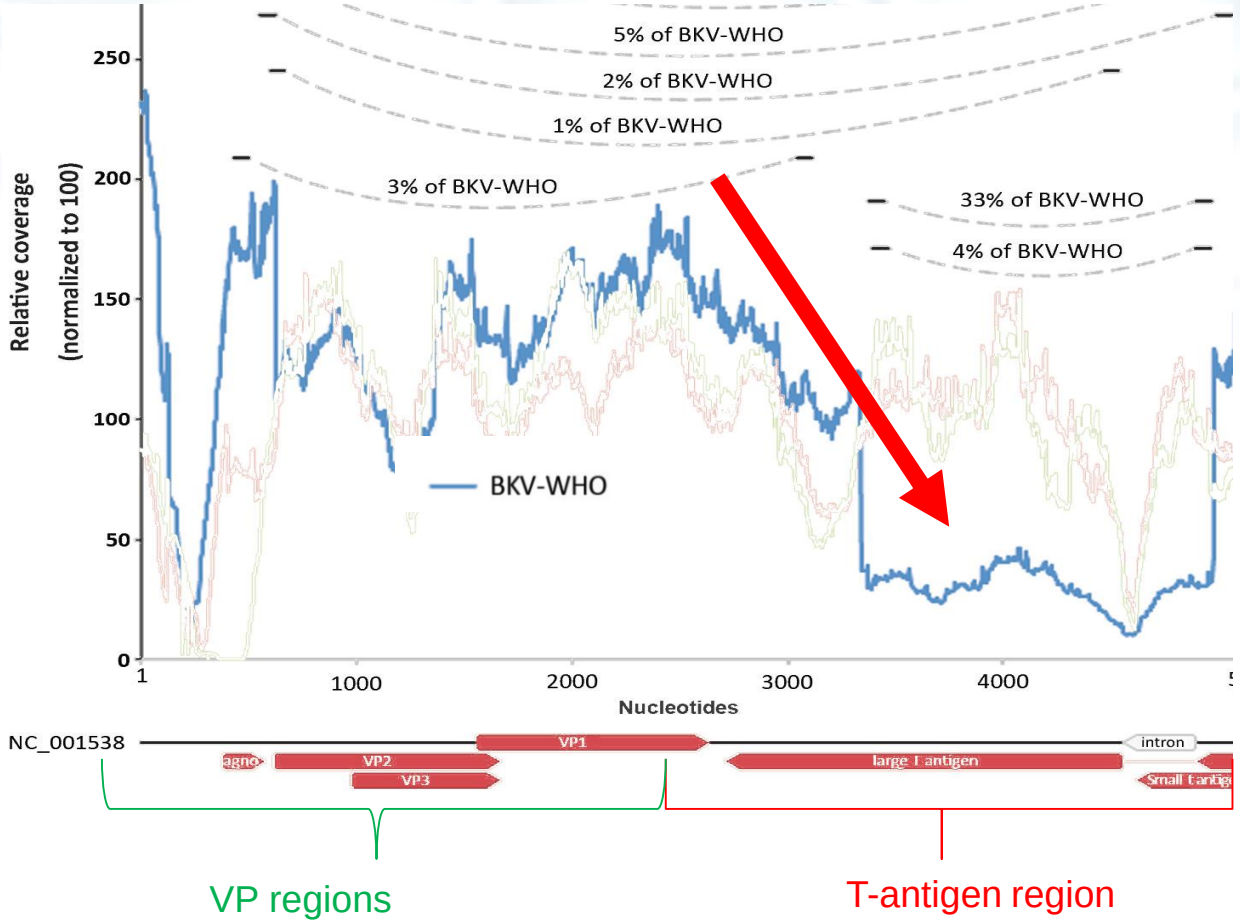
World Health Organization

NIBSC

NIST National Institute of Standards and Technology U.S. Department of Commerce

WHO International Standard for BKV

Deletion in the T-antigen region in subpopulations of the WHO BKV IS



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Molecular Diagnostics and Genetics

Quantification of BK Virus Standards by Quantitative Real-Time PCR and Droplet Digital PCR Is Confounded by Multiple Virus Populations in the WHO BKV International Standard

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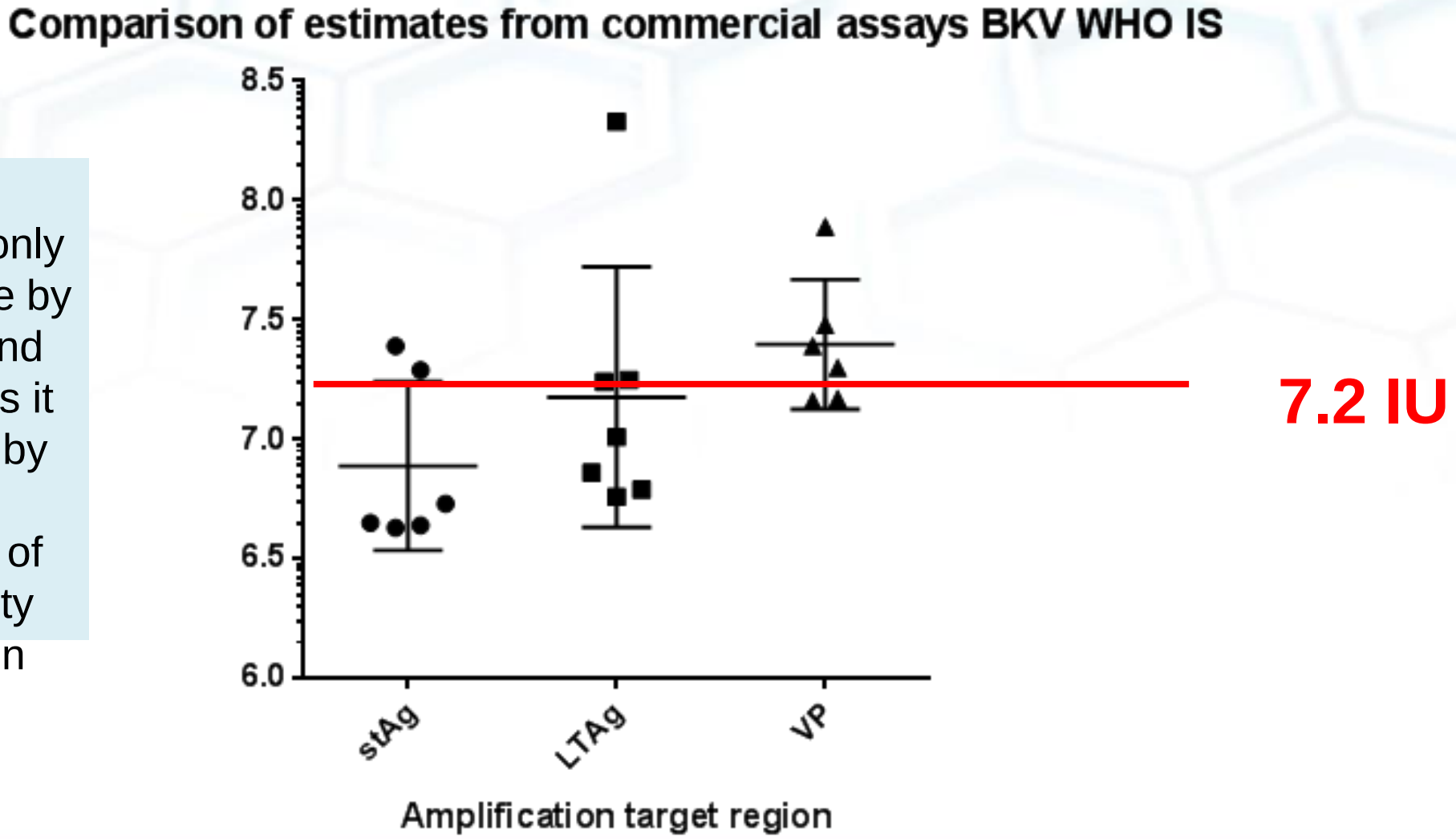
Collaborative Study

Is the deletion only detectable by dPCR and NGS or is it masked by other sources of variability between labs?



Experimental Outline

BKV 1st International Standard

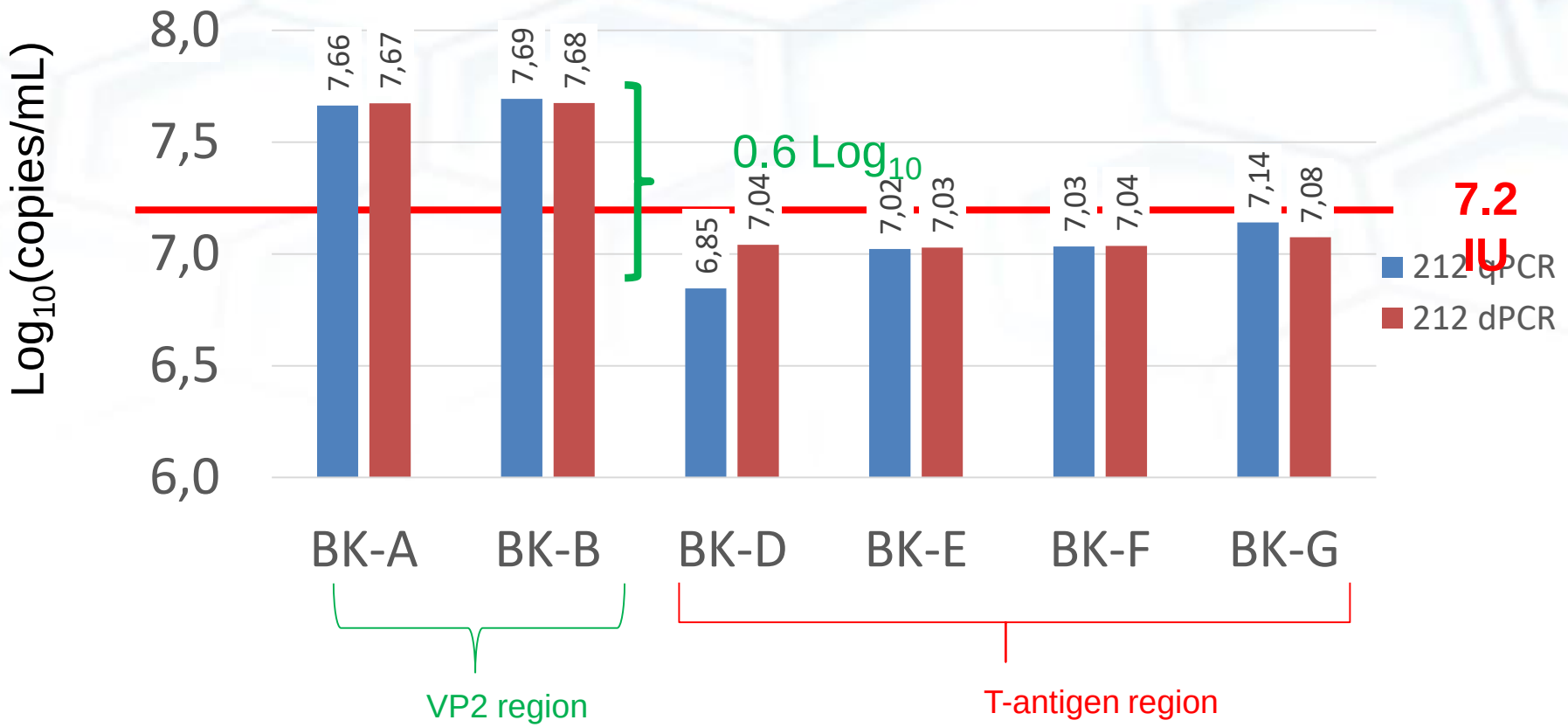
Extraction

Qiagen EZ1 Virus Mini 2.0

ddPCR

qPCR

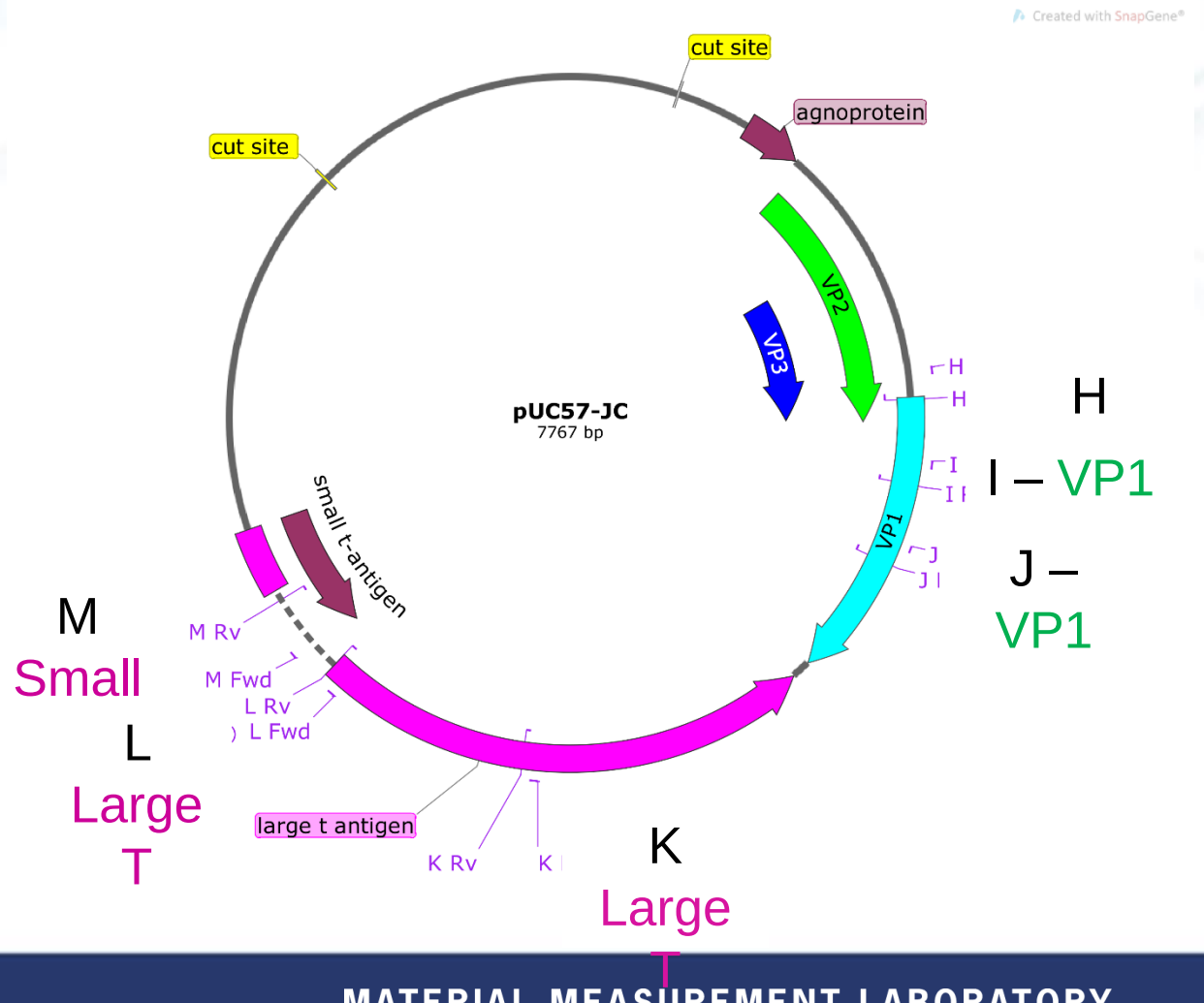
Evaluation of BK IS with dPCR and qPCR



Deletion is seen in both ddPCR and qPCR

NIST SRM for JC Virus

- Expected for release early 2020
- JCV genome was synthesized (Strain 1A – NCBI accession # J02226.1) and cloned into pUC57 plasmid
- Extracted DNA standard
- Linearized and in buffered solution at 4°C
- tRNA added for stability
- Concentration will be similar to BKV – 10^8 copies/mL



WHO IS for JC Virus

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Copy Number Heterogeneity of JC Virus Standards

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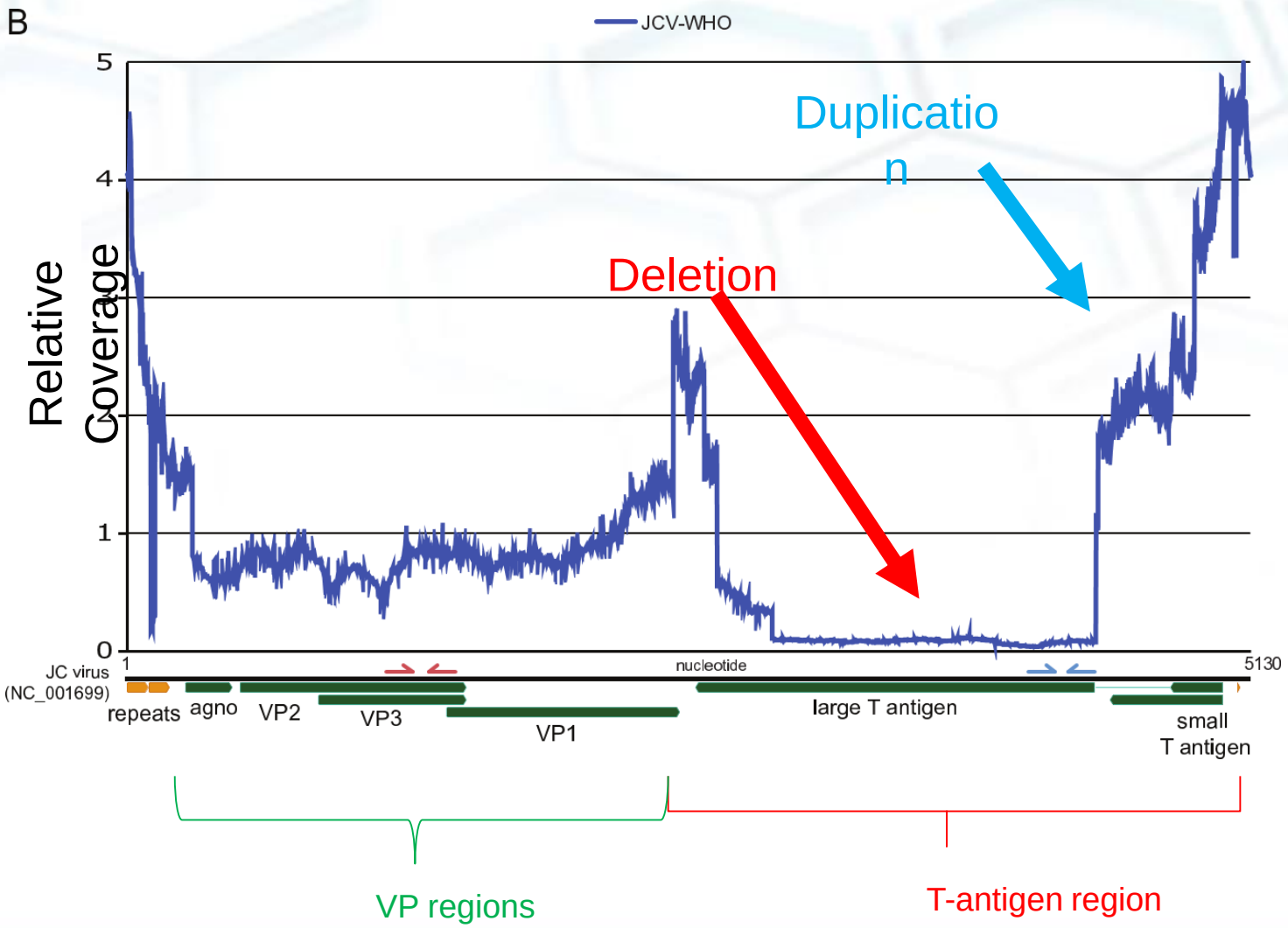
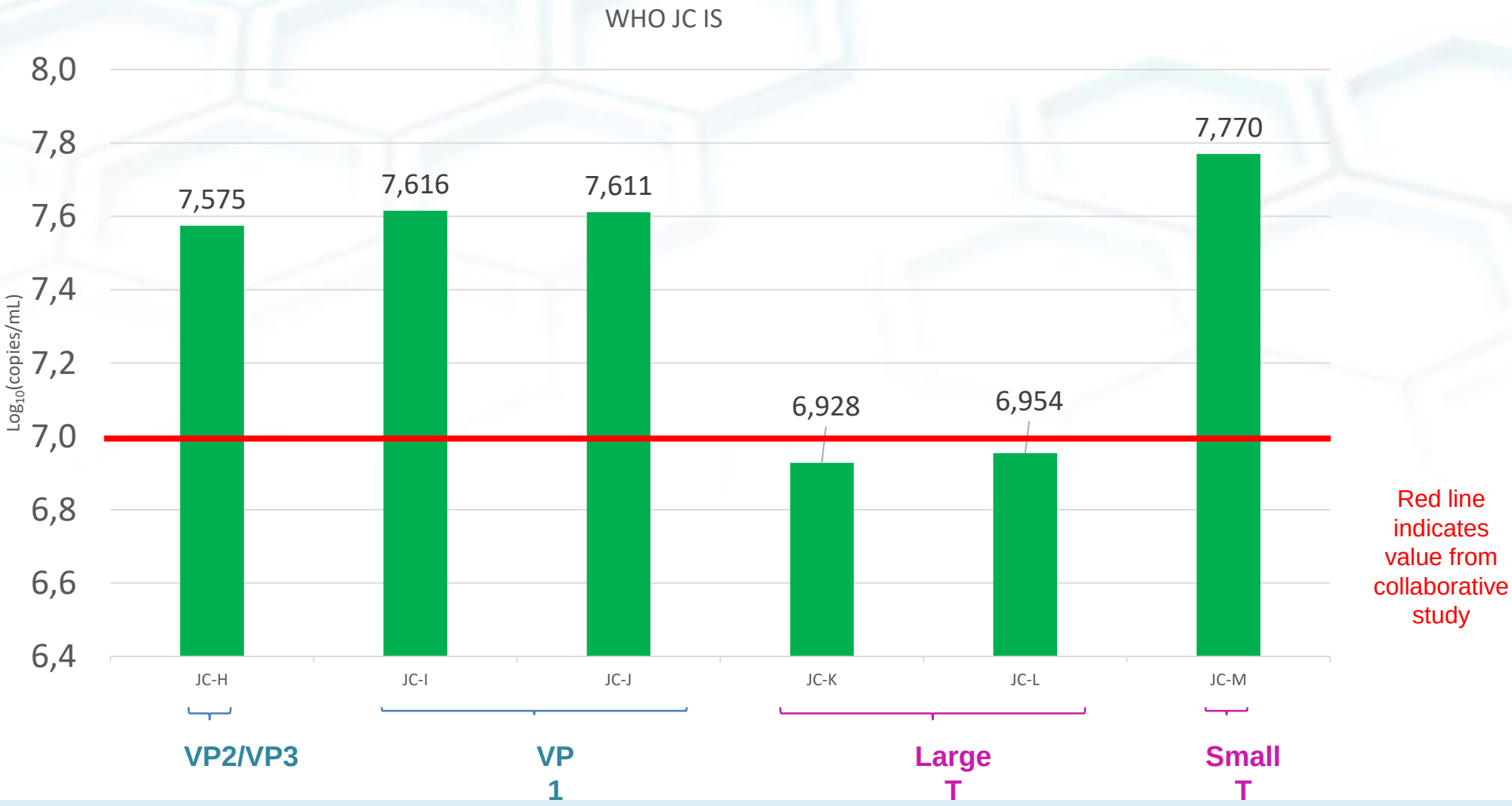


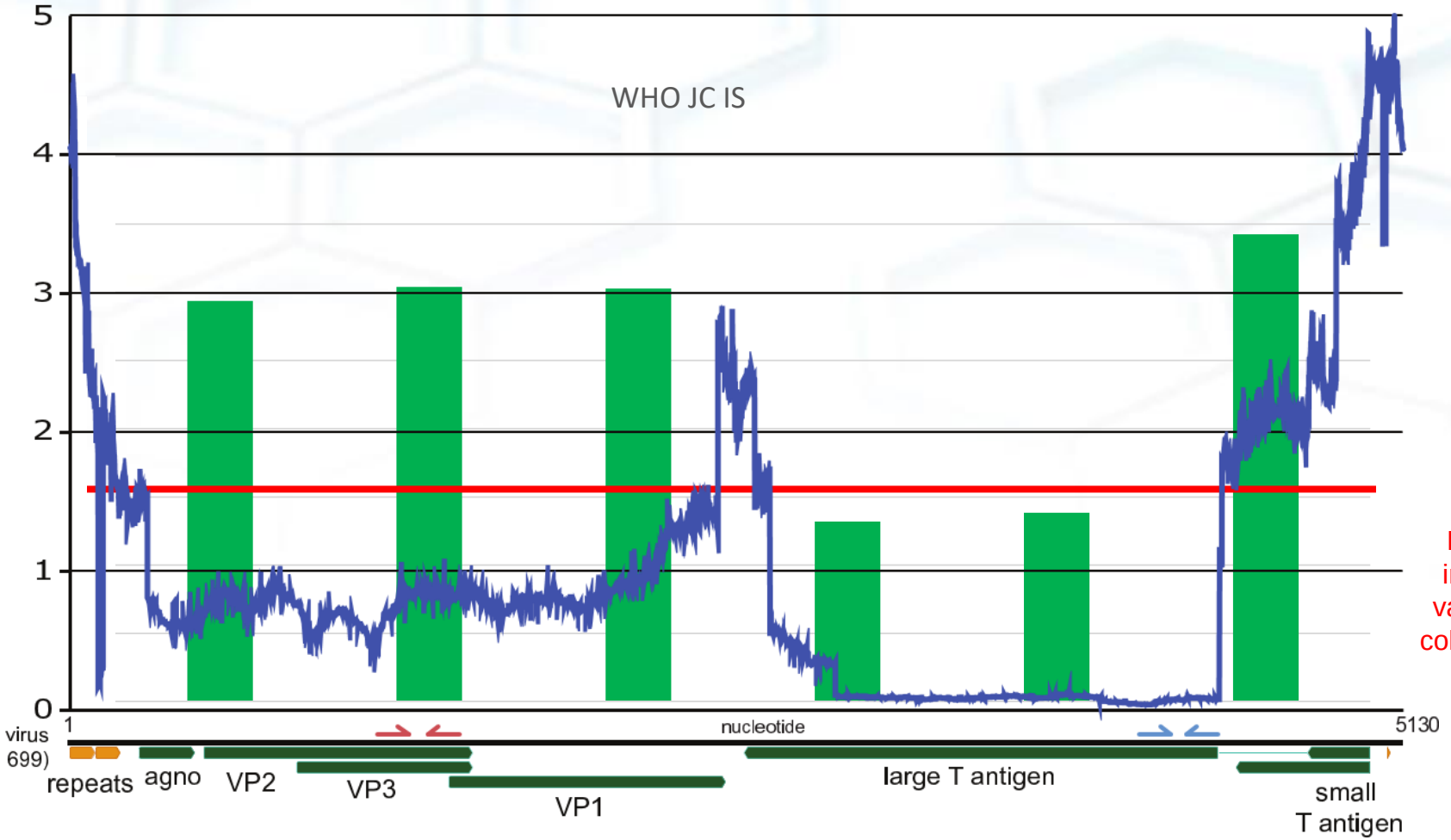
Characterization of JCV WHO IS with dPCR



Red line indicates value from collaborative study

80% of the viral population has a deletion in the Large T antigen region, 40% has a duplication in the Small T antigen region

Characterization of JCV WHO IS with dPCR



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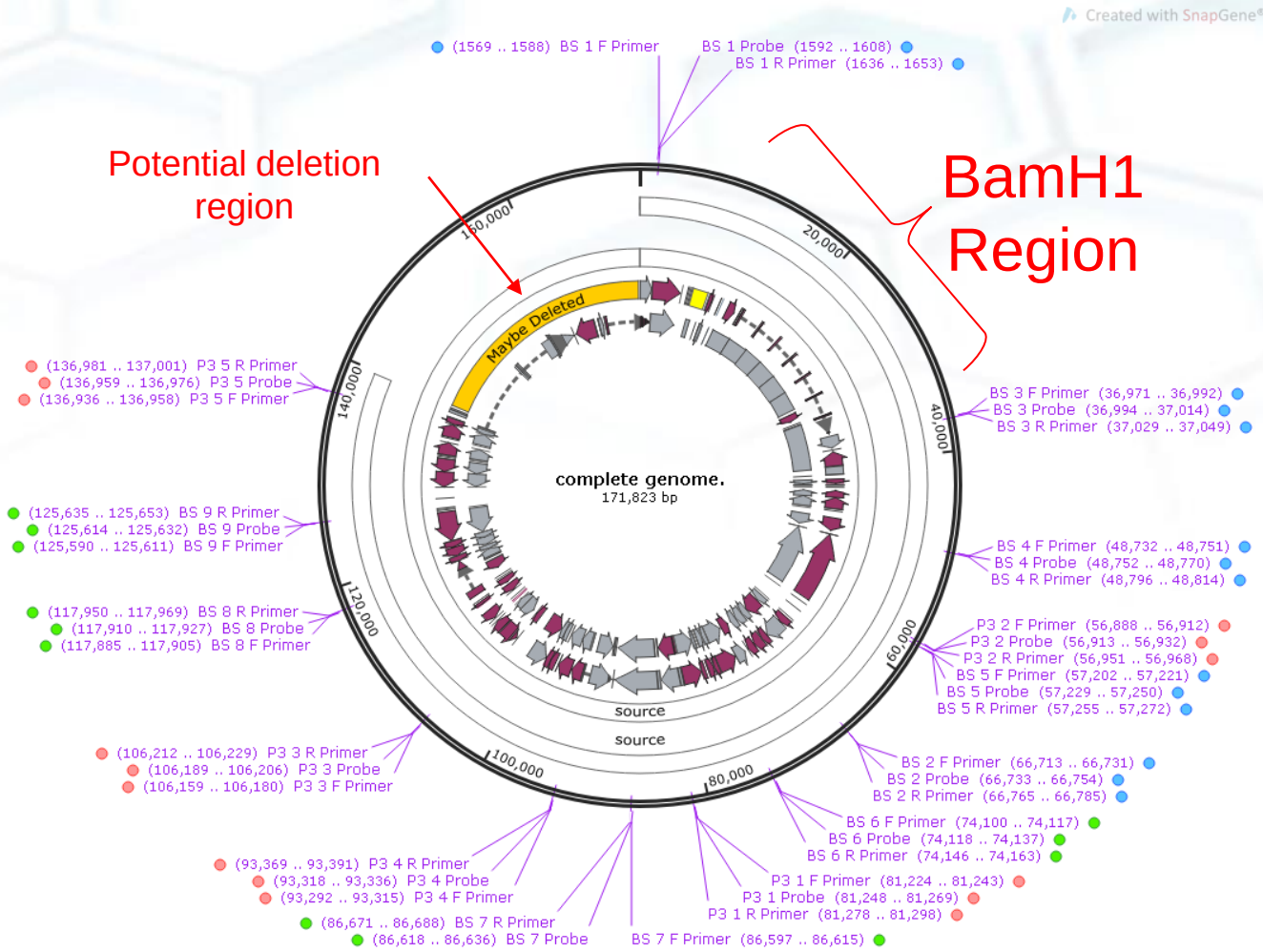
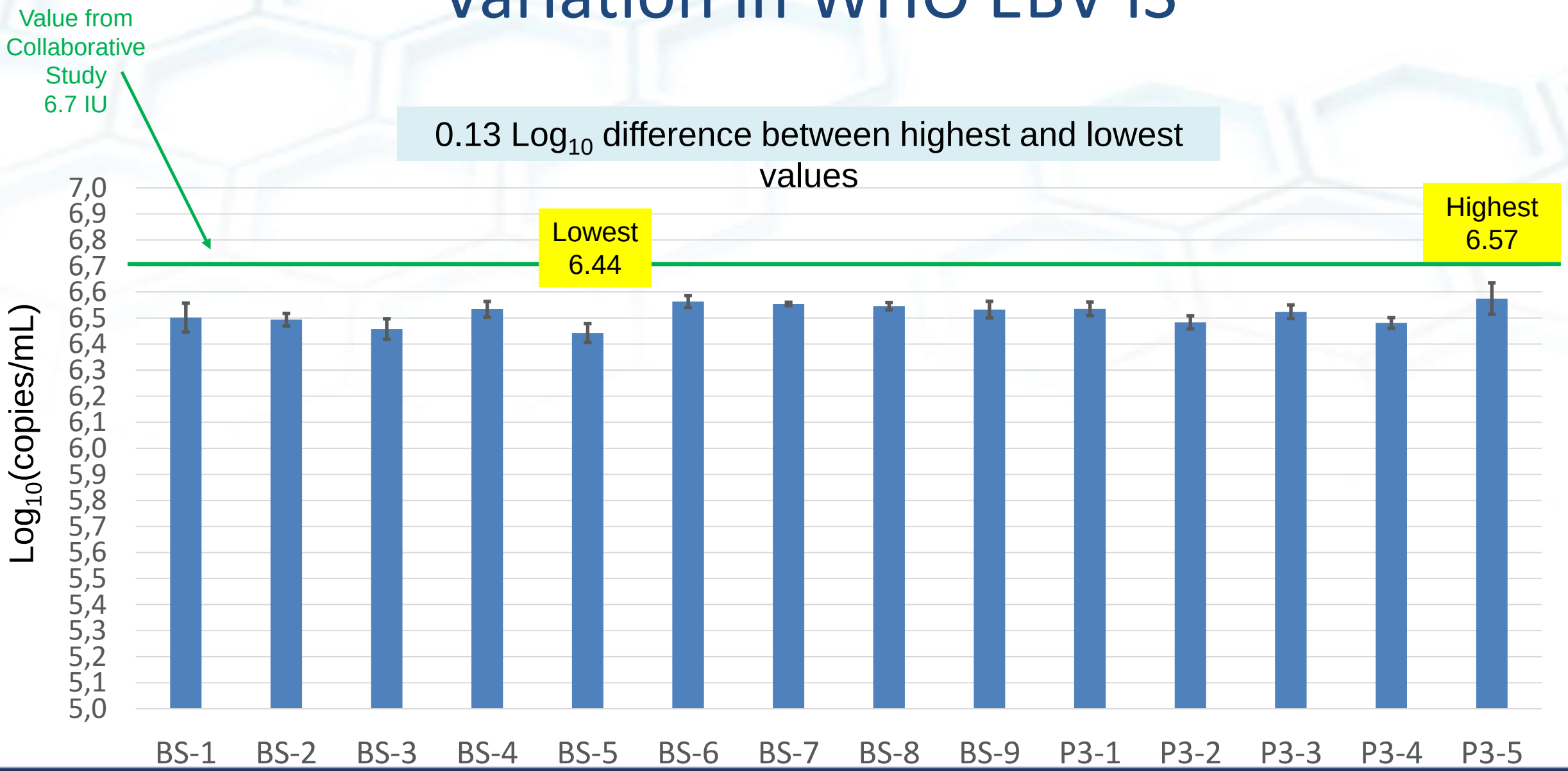
EBV Genome with Primers and Probes

- Primers and probes were evenly spaced around the genome, excluding the BamH1 region
- Total of 14 assays designed



Variation in WHO EBV IS



Conclusions

- dPCR can be used to assign values to DNA based reference materials
- dPCR is useful for characterizing reference materials
  - material purity issues (duplications, deletions)
- dPCR is useful for comparing different reference materials
- Multiple in-house assays strongly recommended
- Knowledge of the system is still required
- Single copy regions should be probed for accurate quantification of genome copy number by dPCR

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