



# Digital PCR for the Characterization of Reference Materials

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National Institute of Standards and Technology

# 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

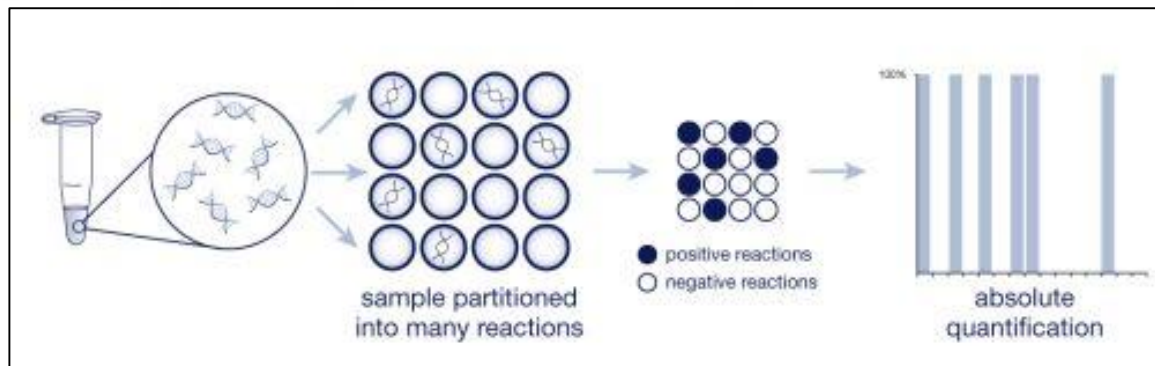


Image credit: <http://digital-pcr.gene-quantification.info/>



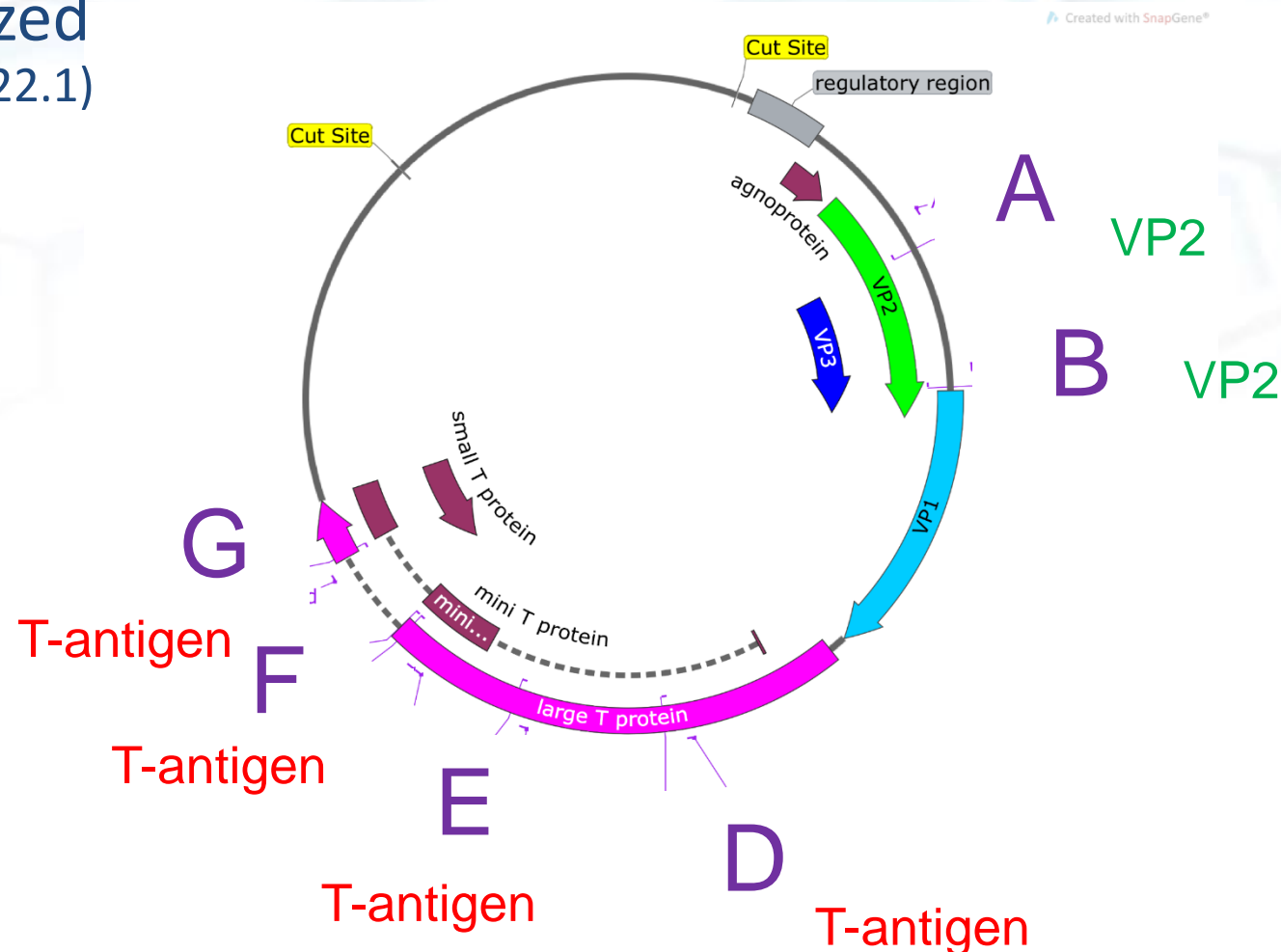
Image credit: <http://bio-rad.com/>

$$\lambda = -\ln(\textit{Fraction Negative})$$

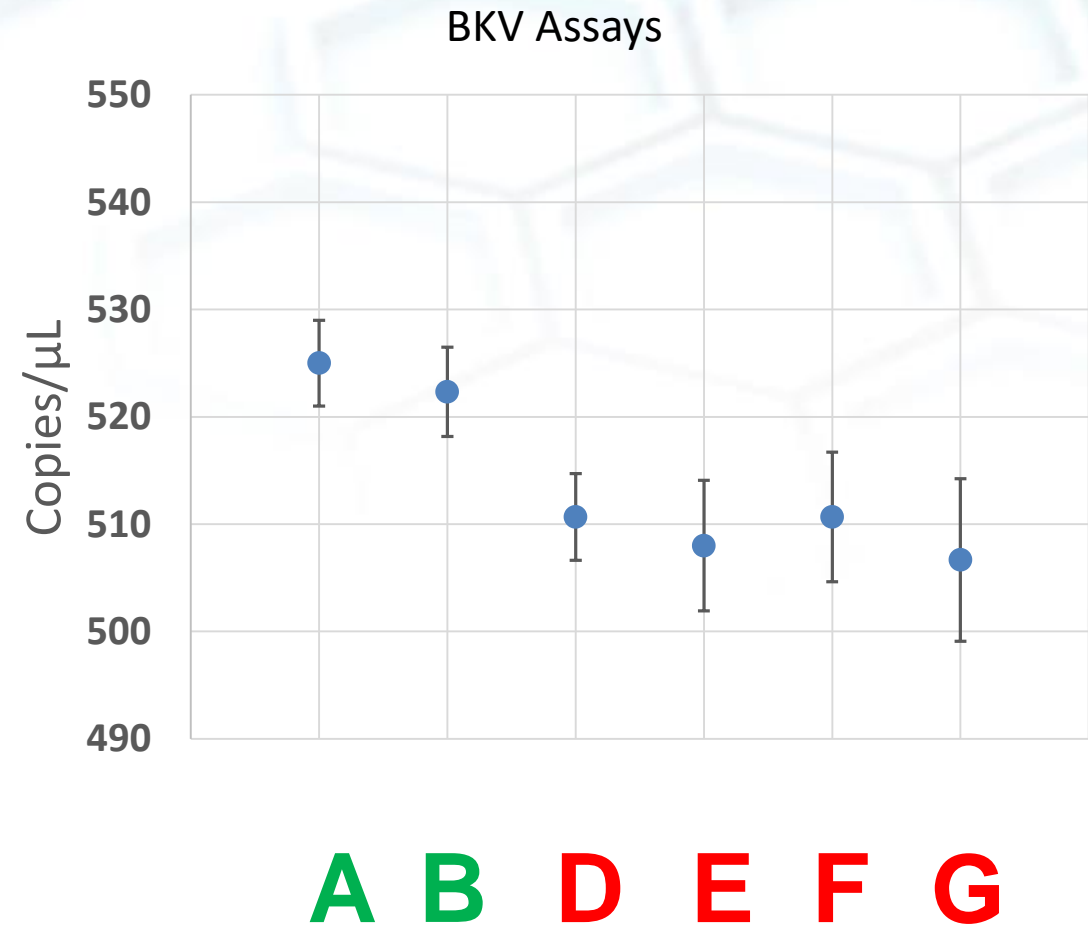
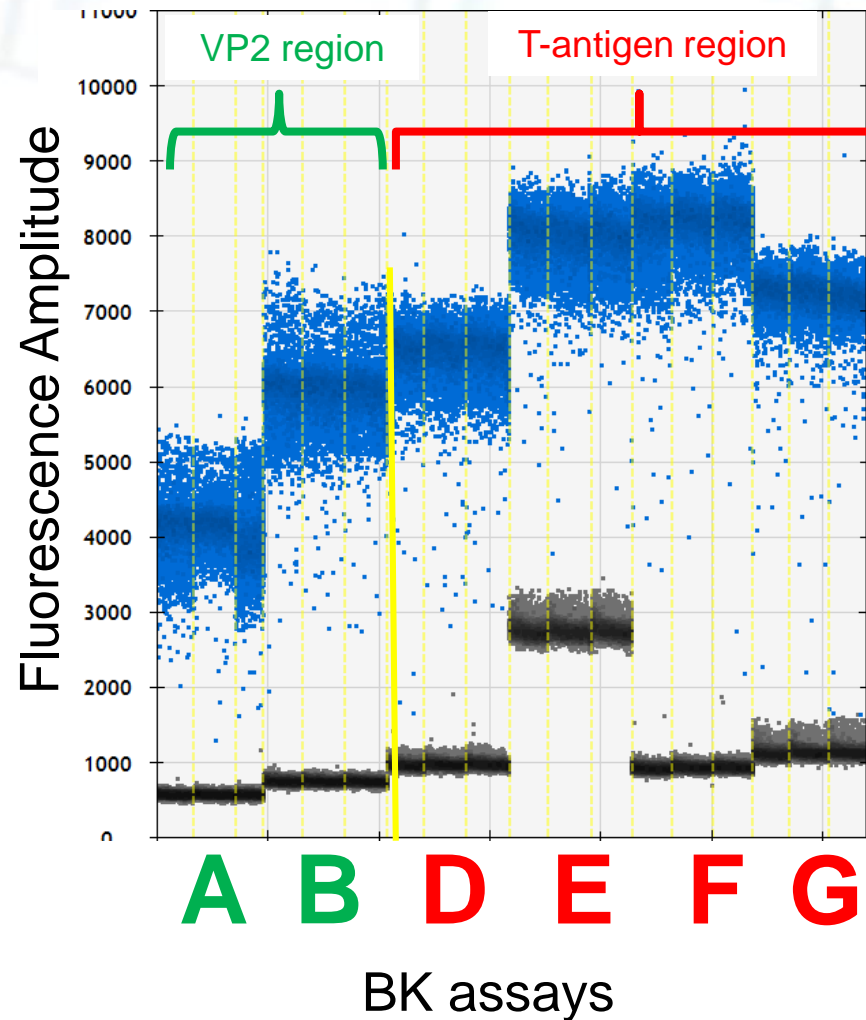
$$\text{Copies}/\mu\text{L} = \lambda / (\text{Droplet Volume in } \mu\text{L})$$

# 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:  $5.58 \times 10^8$  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

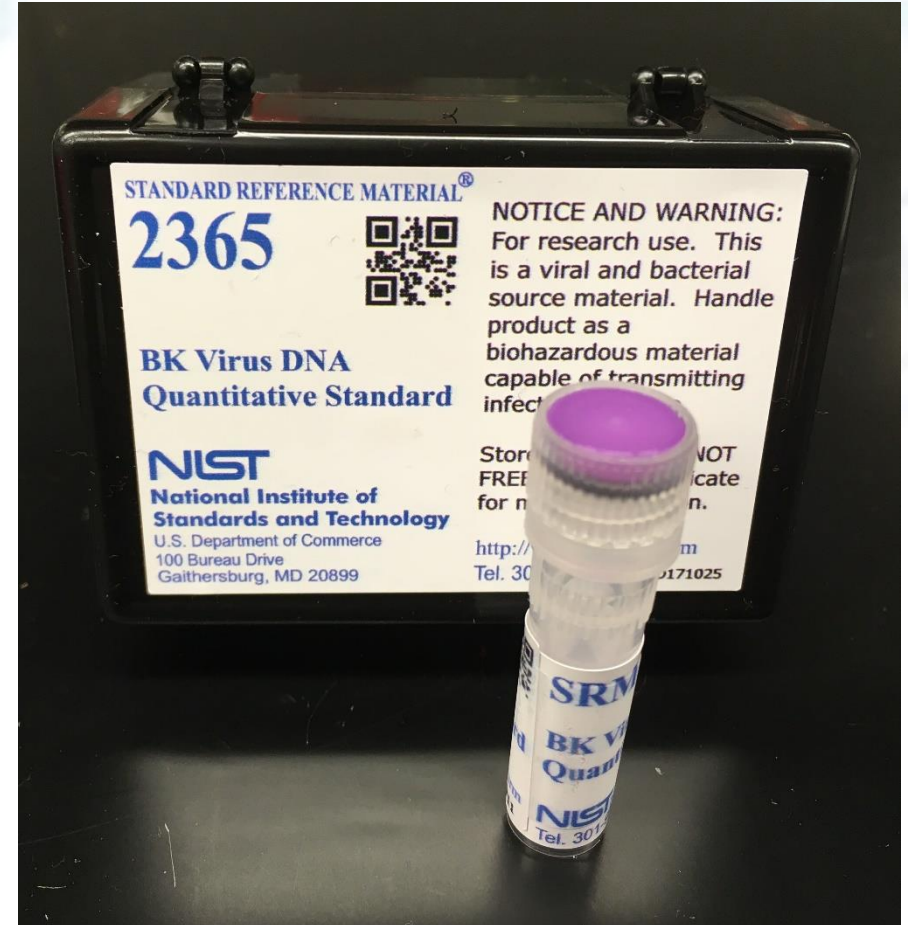
BK Virus DNA Quantitative Standard

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 $\mu\text{L}$
How Concentration is Determined	Large, international multicenter effort	At NIST, multiple dPCR assays



# WHO International Standard for BKV

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

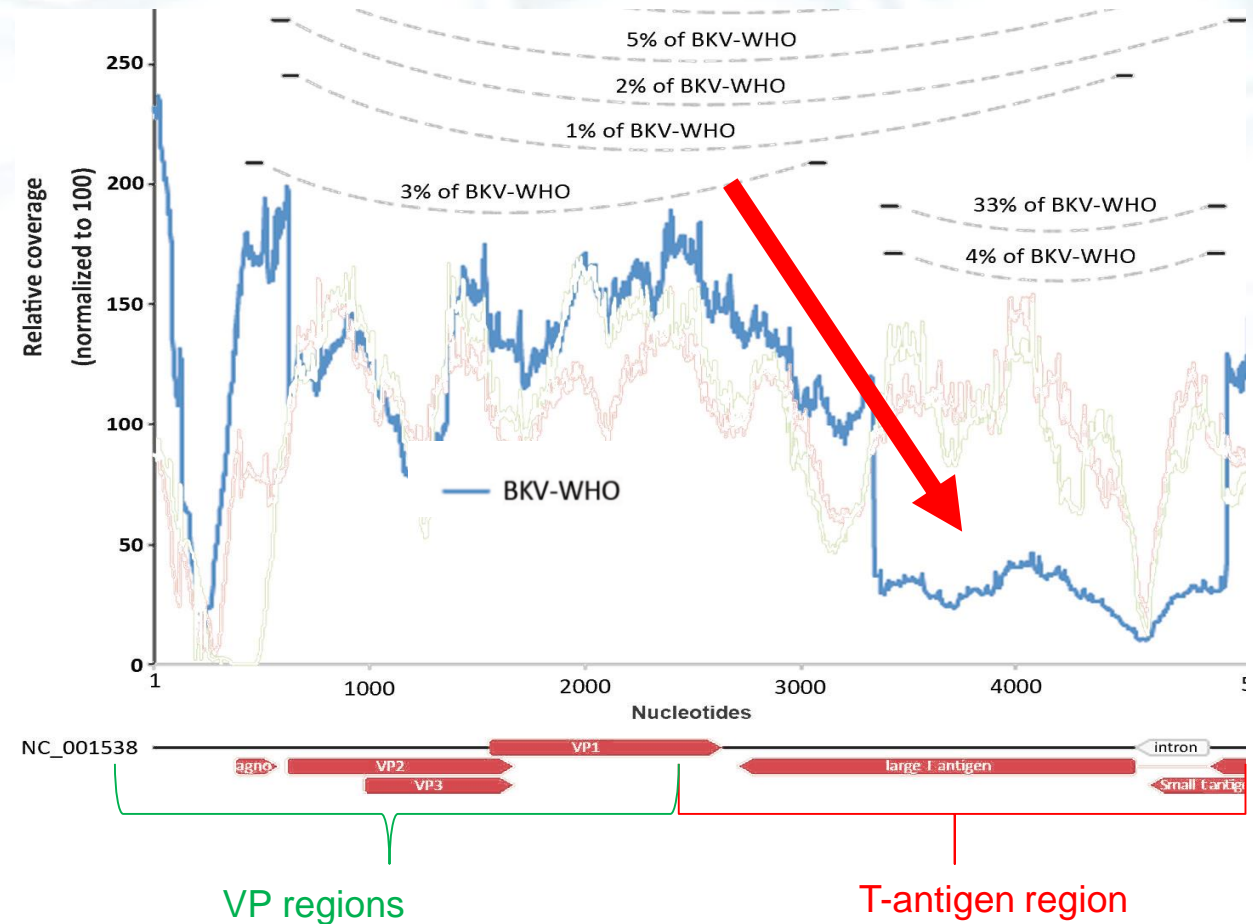


Clinical Chemistry 63:3  
761-769 (2017)

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

Allen C. Bateman,<sup>1\*</sup> Alexander L. Greninger,<sup>1</sup> Ederlyn E. Atienza,<sup>1</sup> Ajit P. Limaye,<sup>2</sup> Keith R. Jerome,<sup>1,3</sup> and Linda Cook<sup>1,3</sup>





### Comparison of estimates from commercial assays BKV WHO IS

Amplification target region

7.2 IU

Amplification target region	Mean (IU)	Min (IU)	Max (IU)
stAg	~6.9	~6.5	~7.4
LTA	~7.2	~6.6	~8.3
VP	~7.4	~7.1	~7.9

# Experimental Outline



BKV 1<sup>st</sup> International Standard

Extraction

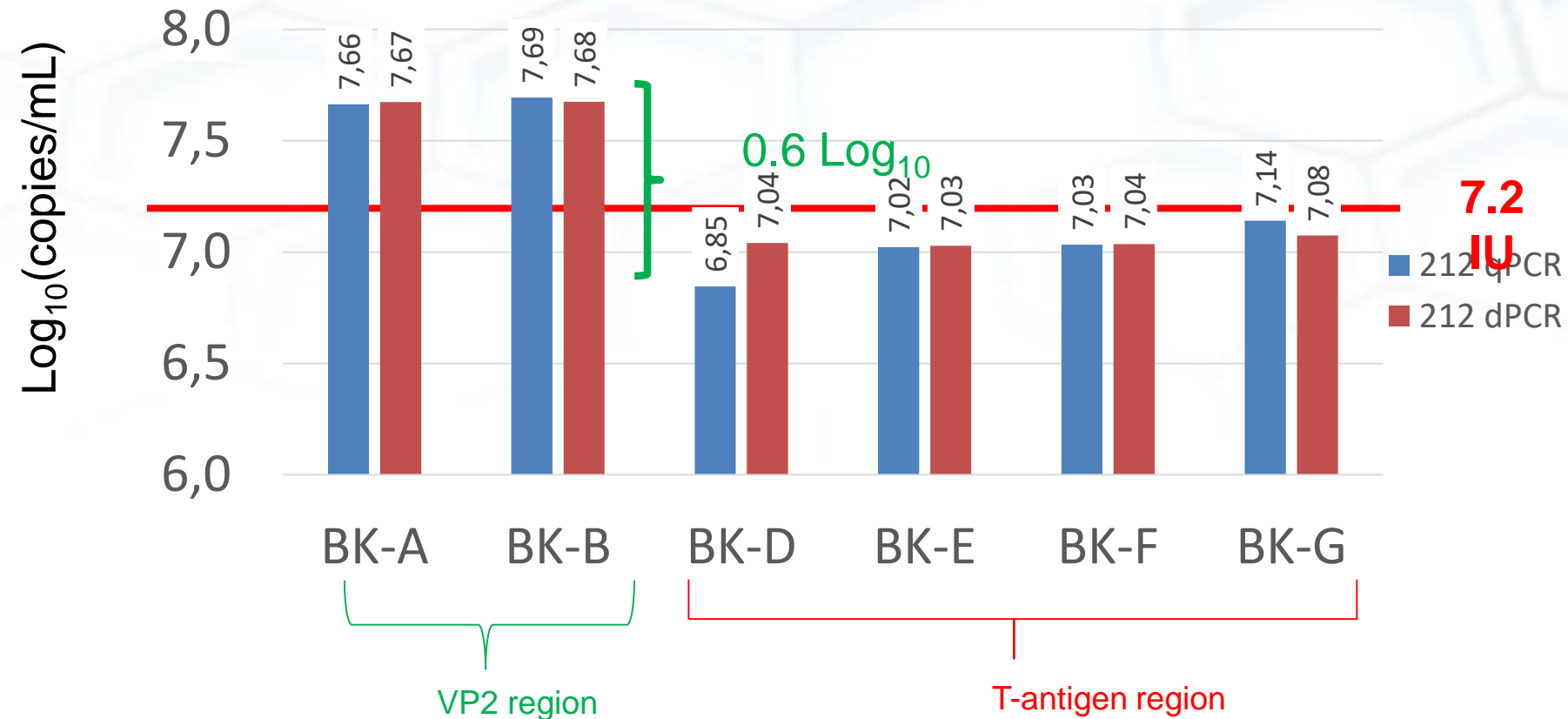
Qiagen EZ1 Virus Mini 2.0



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

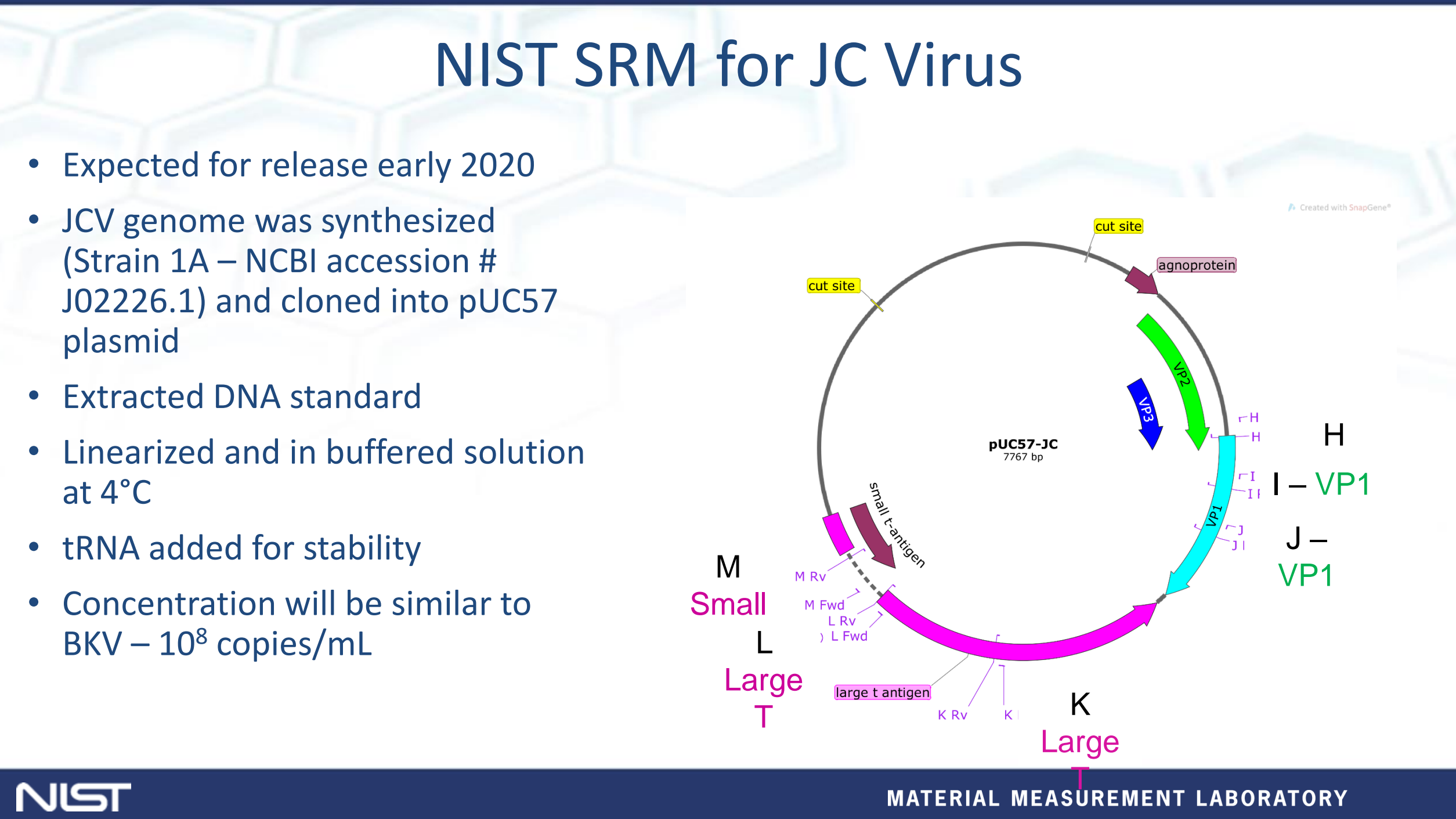
The diagram illustrates the circular plasmid pUC57-JC, which is 7767 bp in size. Key features include:

- Cut sites:** Indicated by yellow arrows labeled "cut site".
- agnoprotein:** A gene located near the top right.
- VP2:** A green arrow indicating the direction of transcription for the VP2 gene.
- VP3:** A blue arrow indicating the direction of transcription for the VP3 gene.
- VP1:** A cyan arrow indicating the direction of transcription for the VP1 gene.
- Small t-antigen:** A pink arrow indicating the direction of transcription for the small t-antigen gene.
- Large t antigen:** A pink arrow indicating the direction of transcription for the large t antigen gene.
- Genes and Regions:** Labeled regions include M, Small, L, Large, T, K, and H. Specific genes like M RV, M Fwd, L RV, L Fwd, K RV, and K are also indicated.

Created with SnapGene®

NIST MATERIAL MEASUREMENT LABORATORY

- # 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
- 
- The diagram illustrates the pUC57-JC plasmid and the JC virus genome structure. The plasmid is a circular DNA molecule with a size of 7767 bp. It contains two yellow 'cut site' markers. The JC virus genome is shown as a linear sequence of genes: VP1 (green), VP2 (blue), VP3 (blue), VP4 (blue), VP5 (blue), VP6 (blue), VP7 (blue), VP8 (blue), VP9 (blue), VP10 (blue), VP11 (blue), VP12 (blue), VP13 (blue), VP14 (blue), VP15 (blue), VP16 (blue), VP17 (blue), VP18 (blue), VP19 (blue), VP20 (blue), VP21 (blue), VP22 (blue), VP23 (blue), VP24 (blue), VP25 (blue), VP26 (blue), VP27 (blue), VP28 (blue), VP29 (blue), VP30 (blue), VP31 (blue), VP32 (blue), VP33 (blue), VP34 (blue), VP35 (blue), VP36 (blue), VP37 (blue), VP38 (blue), VP39 (blue), VP40 (blue), VP41 (blue), VP42 (blue), VP43 (blue), VP44 (blue), VP45 (blue), VP46 (blue), VP47 (blue), VP48 (blue), VP49 (blue), VP50 (blue), VP51 (blue), VP52 (blue), VP53 (blue), VP54 (blue), VP55 (blue), VP56 (blue), VP57 (blue), VP58 (blue), VP59 (blue), VP60 (blue), VP61 (blue), VP62 (blue), VP63 (blue), VP64 (blue), VP65 (blue), VP66 (blue), VP67 (blue), VP68 (blue), VP69 (blue), VP70 (blue), VP71 (blue), VP72 (blue), VP73 (blue), VP74 (blue), VP75 (blue), VP76 (blue), VP77 (blue), VP78 (blue), VP79 (blue), VP80 (blue), VP81 (blue), VP82 (blue), VP83 (blue), VP84 (blue), VP85 (blue), VP86 (blue), VP87 (blue), VP88 (blue), VP89 (blue), VP90 (blue), VP91 (blue), VP92 (blue), VP93 (blue), VP94 (blue), VP95 (blue), VP96 (blue), VP97 (blue), VP98 (blue), VP99 (blue), VP100 (blue). The genome is flanked by 'small t-antigen' and 'large t-antigen' regions. The plasmid is labeled 'pUC57-JC 7767 bp'. The diagram is created with SnapGene®.
- NIST**  
MATERIAL MEASUREMENT LABORATORY



# WHO IS for JC Virus



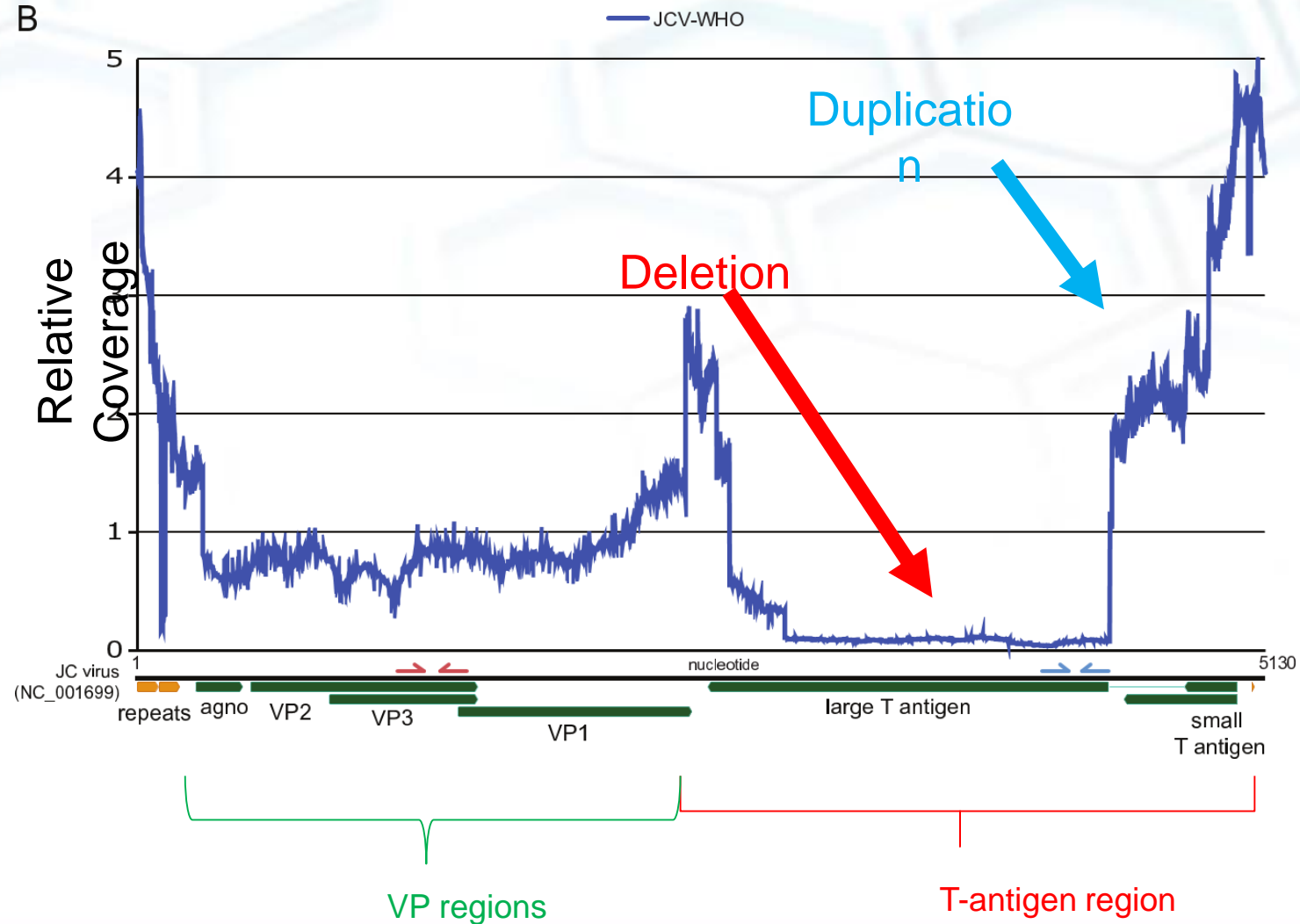
AMERICAN  
SOCIETY FOR  
MICROBIOLOGY

Journal of  
Clinical Microbiology®

## Copy Number Heterogeneity of JC Virus Standards

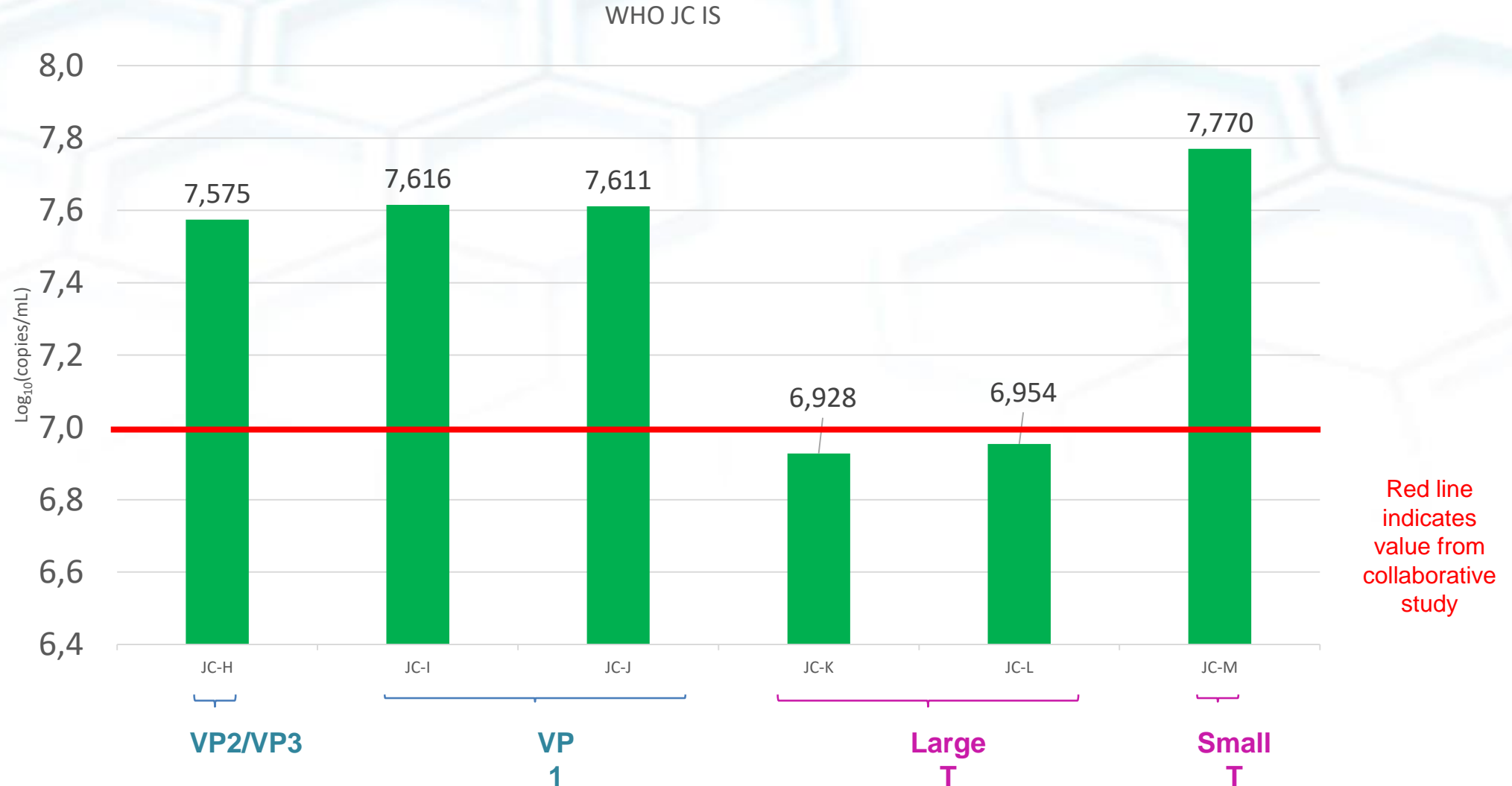
Alexander L. Greninger,<sup>a</sup> Allen C. Bateman,<sup>a</sup> Ederlyn E. Atienza,<sup>a</sup> Sharon Wendt,<sup>a</sup>  
Negar Makhsous,<sup>a</sup> Keith R. Jerome,<sup>a,b</sup> Linda Cook<sup>a</sup>

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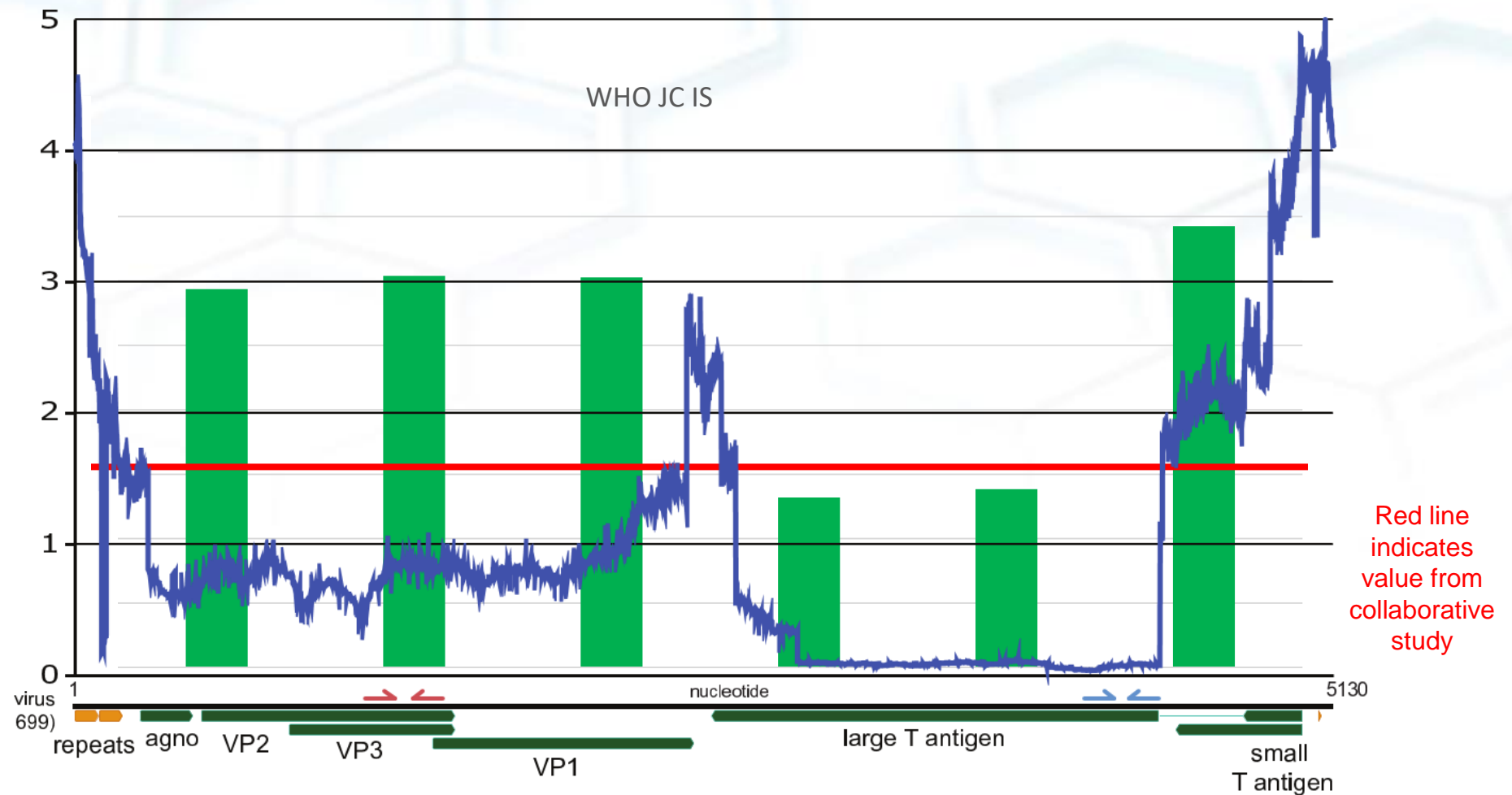


# Characterization of JCV WHO IS with dPCR



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

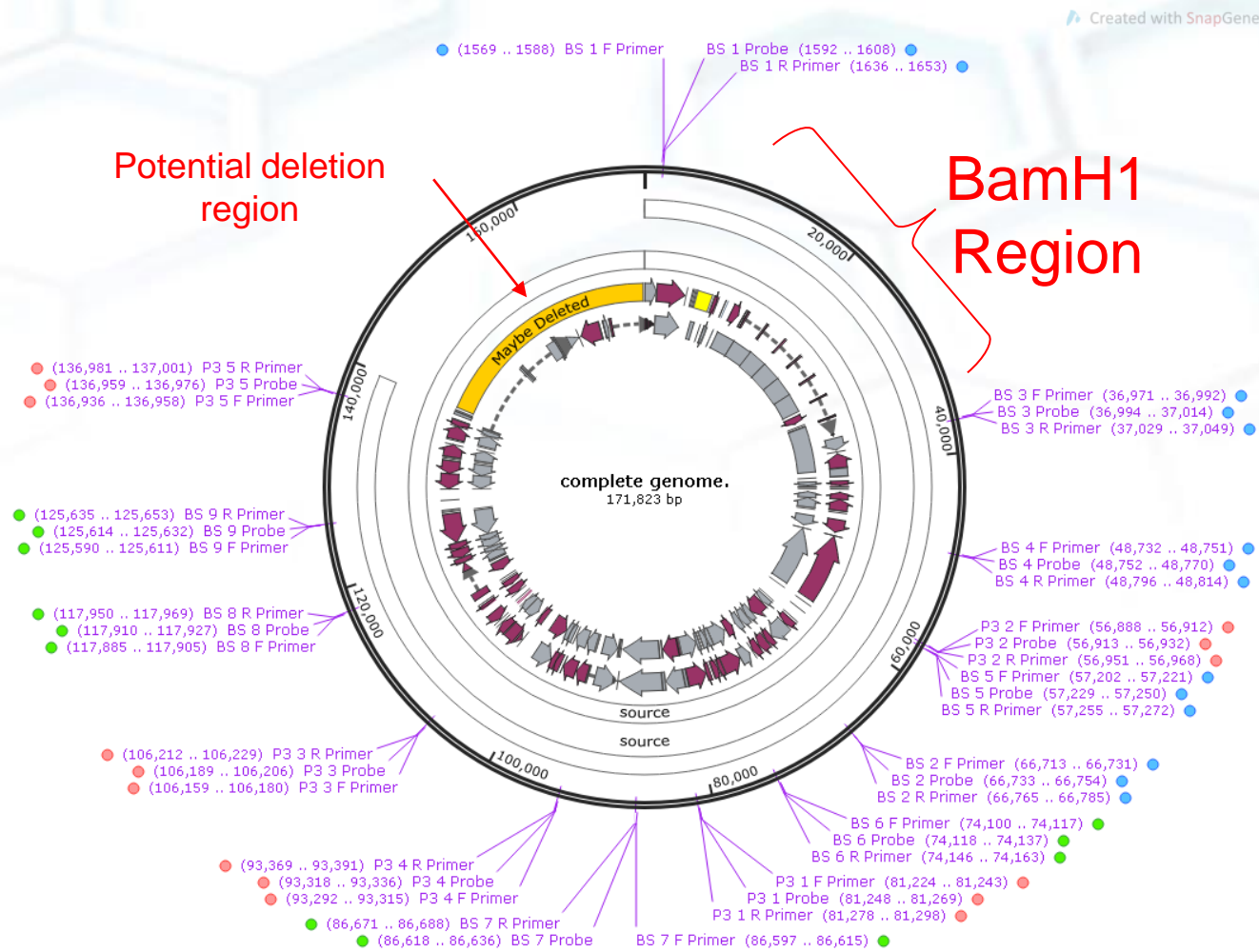
# Characterization of JCV WHO IS with dPCR



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# EBV Genome with Primers and Probes

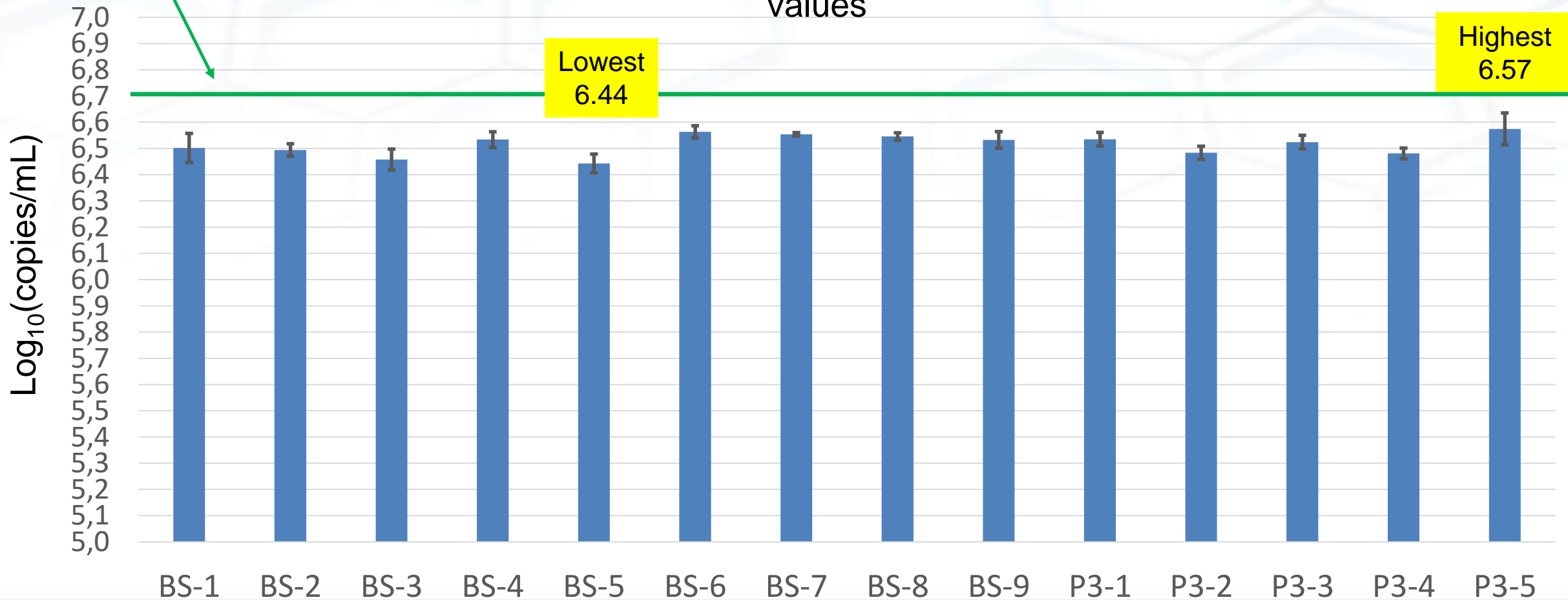
- Primers and probes were evenly spaced around the genome, excluding the BamH1 region
- Total of 14 assays designed



# Variation in WHO EBV IS

Value from  
Collaborative  
Study  
6.7 IU

0.13  $\text{Log}_{10}$  difference between highest and lowest values



# 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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Clare Morris

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