NIST Special Publication 260-191

Certification of Standard Reference Material® 2365

BK Virus DNA Quantitative Standard

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Abstract

Standard Reference Material (SRM) 2365 is intended for use value assigning the BK virus deoxyribonucleic acid (DNA) content of BK DNA controls and standards. A unit of 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 transfer RNA (tRNA) added to ensure stability. A unit of SRM 2365 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. This publication documents the production, analytical methods, and statistical evaluations involved in realizing this product.

Keywords

BK Virus (BKV);
Standard Reference Material (SRM)

Technical Information Contact for this SRM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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Purpose and Description

Standard Reference Material (SRM) 2365 is intended for use in value assigning the BK virus (BKV) deoxyribonucleic acid (DNA) content of BKV DNA controls and standards in units of copies per microliter solution (copies/µL).

A unit of SRM 2365 consists of a well-characterized, linearized plasmid, containing BKV 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 transfer ribonucleic acid (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 sealed with a screw cap.

Storage and Use

Until required for use, SRM 2365 should be stored in the dark between 2 °C and 8 °C.

The SRM 2365 component tube should be mixed briefly and centrifuged (without opening the tube cap) prior to removing sample aliquots for analysis. For the certified values to be applicable, materials should be withdrawn immediately after opening the tubes and processed without delay. Certified values do not apply to any material remaining in recapped tubes. The certification only applies to the initial use and the same results are not guaranteed if the remaining material is used later.

History and Background

BKV is a double-stranded DNA virus, with a genome size of approximately 5200 base pairs, from a family of viruses known as the *Polyomaviridae*. It was first isolated in 1971 from a renal transplant patient with the initials B.K. [1] Primary BKV infection is typically asymptomatic or extremely mild. More than 80 % of the adult population in the United States of America is seropositive for BK exposure. After the initial infection, the virus becomes latent in the kidney and brain tissue and can cause serious disease if the patient becomes immunocompromised. [2] The degree of immunosuppression is the greatest risk factor for BK viremia and BK nephropathy in transplant patients. Kidney transplant recipients should be monitored for BK viremia, and if BKV levels are increasing, the dose of immunosuppression should be decreased. [3]

Quantitative tests, such as quantitative real-time polymerase chain reaction (qPCR) for viral load are used to monitor patients. Results can vary markedly due to the variety of testing formats, lack of reference materials, and sequence heterogeneity between the different strains of BKV.[4]

Each unit of SRM 2365 consists of one vial containing 110 µL aqueous buffer solution of synthetic BKV DNA. SRM 2365 is certified for the number of BKV genome copies per µL of the solution. The value of this measurand was determined using six droplet digital polymerase chain reaction (ddPCR) assays that probe independent regions of the BKV genome.

The copy numbers of BKV DNA in the reaction mixture per partition, λ (lambda), were determined by ddPCR using six PCR assays optimized for use with our ddPCR platform (see Table 1).

The BKV DNA copy number in the component solutions, λ' (dilution-adjusted lambda), is calculated $\lambda' = \lambda/(VF)$ where V is the average droplet volume and F is the volume of component solution in the reaction mixture. SRM 2365 is certified for BKV DNA copy number per µL.

The manufacturer of the ddPCR platform used does not provide metrologically traceable droplet volume information. This lack of information prevented the metrologically traceable conversion of the number per partition measurements to the desired number per reaction volume units. In consequence, NIST staff developed a measurement method that provides metrologically traceable droplet volumes [5].

Experimental Methods

Digital Polymerase Chain Reaction Assays

Six PCR assays were developed for the characterization of SRM 2365. Primers were purchased from Eurofins Operon (Huntsville, AL). FAM-labeled Blackhole Quencher Plus probes were purchased from LGC Biosearch Technologies (Novato, CA). All assays were developed at NIST and have been optimized for ddPCR. Figure 1 displays exemplar droplet patterns for the assays. In Figure 1, blue dots represent "positive" droplets (those containing an amplifiable target) and black dots represent "negative" droplets (those without amplifiable targets).

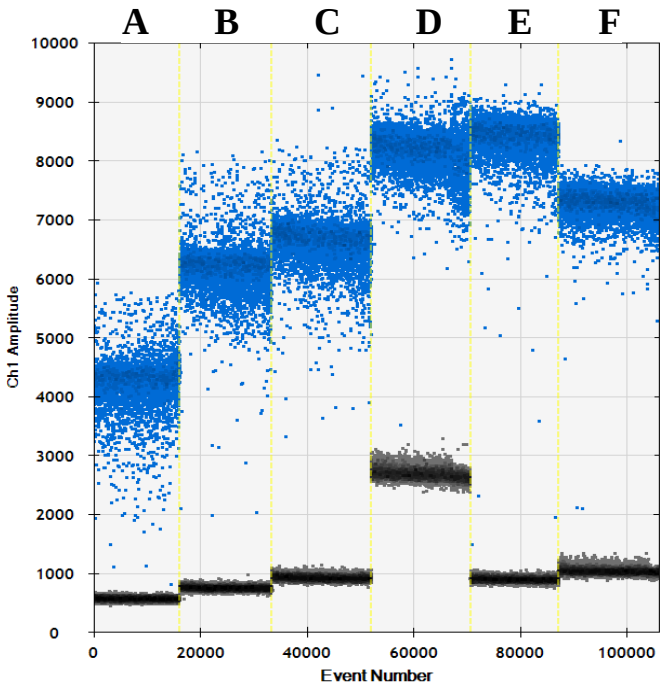

Figure 1: Exemplar Droplet Patterns for the six BKV Assays.

Table 1 details the six assays used to certify SRM 2365 for copy number. All primers and probes were diluted with TE buffer to a 10 µmol/L working solution.

Table 1: Primers and Probes for the PCR Assays Used

Assay Name	Region	Nucleotide Position[a]	Amplicon Size (in bp)	Primers and Probe[b]
A	VP2	812-940	129	F: tgctgggtttgctgctttaattc R: gccatgcctgattgctgatag P: ttgggatcacaaagtttcca
B	VP2	1422-1501	80	F: ccaggaggtgctaatcaaagaac R: gcaggtgttacagtcccgta P: ctcctcaatggatgttgcc
D	T-antigen	3290-3392	103	F: ccatgtcctgaaggcaaatcct R: ggctaacctttgagctaggtgta P: ttgattcagctcctgtcc
E	T-antigen	3910-3987	78	F: ctcacactttgtctctactgcatac R: aacccagaagagcctgaagaaac P: ttaatttccaagacacctgctt
F	T-antigen	4382-4495	114	F: ggtggtgttgagtgttgagaatc R: ggagtcctggtggagttccttt P: tgctgttgcttcttcatca
G	T-antigen	4812-4882	71	F: aggccattccttgcagtacag R: cctggagtagctcagaggtttg P: tctgggcaaagaggaaaatcagc

a Relative to the BKV Reference Genome JQ713822.1

b F: Forward primer, R: Reverse primer, P: Blackhole Plus quencher probe (FAM-labeled)

Droplet Digital Polymerase Chain Reaction Measurements

All BKV DNA ddPCR measurements used in this study followed the same measurement protocol:

- Droplets were generated on the Auto Droplet Generator (BioRad, Hercules, CA) using the ddPCR supermix for probes (BioRad, cat. # 186-3010, Control 64066349) and droplet generation oil for Probes (BioRad, cat # 186-4110).
- The mastermix setup described in Table 2 was used for all assays.
- Non-Template Controls (NTCs) with water instead of template DNA were included in every analysis for each assay.
- Droplets were thermal cycled on the ProFlex (Thermo Fisher, Waltham, MA) for 95 °C for 10 min followed by 40 cycles of 94 °C for 0.5 min and 60 °C for 1 min, then 98 °C for 10 min before a 4 °C hold until the plate was removed.

- Droplets were read on the QX200 Droplet Reader (BioRad, Hercules, CA) and analysis was performed using the QuantaSoft Analysis Software version 1.7.4.0917.
- The numbers of positive and negative droplets at the end of 40 cycles were determined and were exported into a spreadsheet for further analysis. Assay-specific intensity thresholds were determined by visual inspection [6] for each assay for each measurement session.

Table 2: Mastermix setup for NIST-Developed BKV Assays for ddPCR

Manufacturer	Part number	Reagent	µL/rxn
Bio-Rad	186-3010	ddPCR Supermix for probes	12.50
Thermo Fisher	AM9937	Water	8.125
Eurofins Operon	Salt-free oligos	Forward primer (10 µmol/L)	0.625
Eurofins Operon	Salt-free oligos	Reverse primer (10 µmol/L)	0.625
Biosearch Technologies	DLO-FBP-5 BHQplus	Probe (10 µmol/L)	0.625
		DNA (1→100 dilution)	2.50
		Total volume	25 µL/rxn

Sample Preparation

The BKV genome construct (NCBI accession # JQ713822.1) was synthesized and cloned into a pUC57 plasmid (GeneWiz, South Plainfield, NJ). Restriction sites (AhdI and BssHII) were added on either side of the BKV DNA, to allow the BK genome region to be isolated from the rest of the plasmid.

The plasmid was transformed into *E. coli* Sure2 cells (GeneWiz); a glycerol stock of this plasmid was delivered to NIST. At NIST, the plasmid was grown in a 2.5 L culture and purified using the Qiagen (Hilden, Germany) EndoFree Giga Kit (catalog #12391). The plasmid was linearized with restriction enzymes (AhdI and BssHII), by consecutive digests in 1 mL aliquots. The linearized material was pooled and then filtered to remove precipitate from bovine serum antigen (a component in the restriction digests). The material was then stored at a concentration of approximately 4.5×10^9 genome copies/µL in a perfluoroalkoxy fluoropolymer (PFA) container. The cleaning procedure for the PFA container is detailed in Appendix A.

As described below, a portion of the linearized DNA was diluted (volumetrically) to approximately 5.5×10^5 genome copies/µL, with 50 ng/µL yeast tRNA (catalog # AM7119, lot# 00426790, Thermo Fisher, Waltham, MA) added to help ensure stability. The quantities added are:

- 248.75 mL TE Buffer (Fisher Scientific, catalog # BP2473-100, lot # 142381)
- 1.25 mL yeast tRNA (10 mg/mL)
- 25 µL stock solution of BKV DNA (approximately 4.5×10^9 genome copies/µL)

This solution was stored in a 300 mL PFA container at 4 °C until packaging.

Packaging

The morning of packaging, the container holding the diluted BKV solution was removed from the refrigerated storage and placed on a slow orbital shaker for 2 h. The tubes selected for delivering the SRM (Sarstedt, catalog # 72.730.100, Newton, NC) were placed under a laminar flow hood in rows of 5 within 80-hole tube racks for a total of 30 tubes per rack. Tubes were placed in every third row of the racks to facilitate filling with an Eppendorf Repeater Xstream pipette (North America, Inc., Hauppauge, NY) fitted with a 10 mL positive displacement tip (Eppendorf, catalog # 0030089677) set to dispense 110 µL per tube. The solution container was removed from the shaker, a magnetic stir-bar was added, and the container was placed on a magnetic stir plate in the laminar flow hood and stirred gently. Filling proceeded until 2000 tubes were filled, with pipet tip replacement after filling every 180 tubes.

Packaging involved two individuals manually filling each tube within a biosafety cabinet (one pipetting, the other verifying that each tube was filled) and four individuals tightly closing the lids to each tube on a sterilized and covered bench. All individuals involved in the filling and capping process wore personal protective equipment (PPE) that included lab coats, hair covering, mouth and nose covering, and gloves.

When all 30 tubes in a rack were filled, the rack was moved out of the hood, the tubes checked for proper filling, their lids closed, and the tubes transferred to 100-unit pre-labeled storage boxes which were labeled 1 to 20 and filled in order. As the 100-unit boxes were filled, they were transferred to the labeling room where labels were applied by hand and purple inserts were added to the lids.

After all tubes were labeled, the 20 boxes were placed in a refrigerator. Filling the tubes took approximately 75 minutes. All units were transferred to the refrigerator within 4 hours of beginning the filling process. The material was equilibrated for a minimum of two weeks prior to beginning homogeneity analysis.

Component Homogeneity

One tube from the lower left corner in each box (1 to 20) was used to assess material homogeneity. Selected vials were labeled with a box number. These 20 vials were equilibrated to room temperature, then prepared for analysis using the ddPCR procedure described above (Table 2). The ddPCR system with the D and E assays was used for all homogeneity measurements. All tubes were assayed in triplicate on two 96-well plates. Figure 2 details the layout of the samples for the homogeneity measurements. One plate was run with Assay D and a second plate, with the same layout, was run with Assay E.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Box 1	Box 1	Box 1	Box 9	Box 9	Box 9	Box 17	Box 17	Box 17			
B	Box 2	Box 2	Box 2	Box 10	Box 10	Box 10	Box 18	Box 18	Box 18			
C	Box 3	Box 3	Box 3	Box 11	Box 11	Box 11	Box 19	Box 19	Box 19			
D	Box 4	Box 4	Box 4	Box 12	Box 12	Box 12	Box 20	Box 20	Box 20			
E	Box 5	Box 5	Box 5	Box 13	Box 13	Box 13	NTC	NTC	NTC			
F	Box 6	Box 6	Box 6	Box 14	Box 14	Box 14	NTC	NTC	NTC			
G	Box 7	Box 7	Box 7	Box 15	Box 15	Box 15	NTC	NTC	NTC			
H	Box 8	Box 8	Box 8	Box 16	Box 16	Box 16	NTC	NTC	NTC			

Figure 2: Plate Layout Design for Homogeneity Measurements

Homogeneity results were evaluated as copy number per droplet adjusted for the 1→100 dilution.

Component Stability

Six tubes for the stability study were chosen by random selection from boxes one through six. Two vials for each of three temperatures were used for stability testing: 4 °C, room temperature (≈21 °C) and 37 °C. Tubes at each temperature were opened, sampled, and resealed five times over the course of nine weeks.

Samples were prepared for analysis as described above shortly before making the measurements. Assays D and E were used for all stability measurements. All samples were assayed in triplicate. The data from the homogeneity study was used as timepoint 0 in the stability study, anchored to 4 °C by the storage recommendations. Initial stability measurements are referred to as "Week 1." Subsequent measurements were made on weeks 3, 5, 7 and 9. Figure 3 details the sample layout.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BK-D;Box 1; 4 C	BK-D;Box 1; 4 C	BK-D;Box 1; 4 C	BK-D; Box 4; 4 C	BK-D; Box 4; 4 C	BK-D; Box 4; 4 C						
B	BK-D;Box 2; 21 C	BK-D;Box 2; 21 C	BK-D;Box 2; 21 C	BK-D; Box 5; 21 C	BK-D; Box 5; 21 C	BK-D; Box 5; 21 C						
C	BK-D;Box 3; 37 C	BK-D;Box 3; 37 C	BK-D;Box 3; 37 C	BK-D; Box 6; 37 C	BK-D; Box 6; 37 C	BK-D; Box 6; 37 C						
D	BK-D;NTC	BK-D;NTC	BK-D;NTC	BK-D; NTC	BK-D; NTC	BK-D; NTC						
E	BK-E; Box 1; 4 C	BK-E; Box 1; 4 C	BK-E; Box 1; 4 C	BK-E; Box 4; 4 C	BK-E; Box 4; 4 C	BK-E; Box 4; 4 C						
F	BK-E; Box 2; 21 C	BK-E; Box 2; 21 C	BK-E; Box 2; 21 C	BK-E; Box 5; 21 C	BK-E; Box 5; 21 C	BK-E; Box 5; 21 C						
G	BK-E; Box 3; 37 C	BK-E; Box 3; 37 C	BK-E; Box 3; 37 C	BK-E; Box 6; 37 C	BK-E; Box 6; 37 C	BK-E; Box 6; 37 C						
H	BK-E; NTC	BK-E; NTC	BK-E; NTC	BK-E; NTC	BK-E; NTC	BK-E; NTC						

Figure 3: Plate Layout Design for Stability Measurements

Component Certification

After establishing homogeneity and stability, certification measurements were performed using randomly selected SRM tubes from the boxes listed in Table 3. The six BKV DNA assays described in Table 1 were run simultaneously on the same sample plate. Samples from each tube were prepared as 1→100 volumetric dilutions, followed by 1→10 volumetric dilution into the ddPCR mastermix and run in triplicate in two measurement campaigns. Figures 4 and 5 detail the plate layouts for the certification measurements.

Table 3: Tubes used for Certification Measurements

Box	Assay Day
1	Day 1
3	Day 2
8	Day 1
10	Day 2
13	Day 1
15	Day 2
19	Day 2
20	Day 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	Box 1; BK-A	Box 1; BK-A	Box 1; BK-A	Box 8; BK-A	Box 8; BK-A	Box 8; BK-A	Box 13; BK-A	Box 13; BK-A	Box 13; BK-A	Box 20; BK-A	Box 20; BK-A	Box 20; BK-A
B	Box 1; BK-B	Box 1; BK-B	Box 1; BK-B	Box 8; BK-B	Box 8; BK-B	Box 8; BK-B	Box 13; BK-B	Box 13; BK-B	Box 13; BK-B	Box 20; BK-B	Box 20; BK-B	Box 20; BK-B
C	Box 1; BK-D	Box 1; BK-D	Box 1; BK-D	Box 8; BK-D	Box 8; BK-D	Box 8; BK-D	Box 13; BK-D	Box 13; BK-D	Box 13; BK-D	Box 20; BK-D	Box 20; BK-D	Box 20; BK-D
D	Box 1; BK-E	Box 1; BK-E	Box 1; BK-E	Box 8; BK-E	Box 8; BK-E	Box 8; BK-E	Box 13; BK-E	Box 13; BK-E	Box 13; BK-E	Box 20; BK-E	Box 20; BK-E	Box 20; BK-E
E	Box 1; BK-F	Box 1; BK-F	Box 1; BK-F	Box 8; BK-F	Box 8; BK-F	Box 8; BK-F	Box 13; BK-F	Box 13; BK-F	Box 13; BK-F	Box 20; BK-F	Box 20; BK-F	Box 20; BK-F
F	Box 1; BK-G	Box 1; BK-G	Box 1; BK-G	Box 8; BK-G	Box 8; BK-G	Box 8; BK-G	Box 13; BK-G	Box 13; BK-G	Box 13; BK-G	Box 20; BK-G	Box 20; BK-G	Box 20; BK-G
G	NTC; BK-A	NTC; BK-A	NTC; BK-B	NTC; BK-B	NTC; BK-D	NTC; BK-D	NTC; BK-E	NTC; BK-E	NTC; BK-F	NTC; BK-F	NTC; BK-G	NTC; BK-G
H	NTC; BK-A	NTC; BK-A	NTC; BK-B	NTC; BK-B	NTC; BK-D	NTC; BK-D	NTC; BK-E	NTC; BK-E	NTC; BK-F	NTC; BK-F	NTC; BK-G	NTC; BK-G

Figure 4. Layout of certification plate 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Box 3; BK-A	Box 3; BK-A	Box 3; BK-A	Box 10; BK-A	Box 10; BK-A	Box 10; BK-A	Box 15; BK-A	Box 15; BK-A	Box 15; BK-A	Box 19; BK-A	Box 19; BK-A	Box 19; BK-A
B	Box 3; BK-B	Box 3; BK-B	Box 3; BK-B	Box 10; BK-B	Box 10; BK-B	Box 10; BK-B	Box 15; BK-B	Box 15; BK-B	Box 15; BK-B	Box 19; BK-B	Box 19; BK-B	Box 19; BK-B
C	Box 3; BK-D	Box 3; BK-D	Box 3; BK-D	Box 10; BK-D	Box 10; BK-D	Box 10; BK-D	Box 15; BK-D	Box 15; BK-D	Box 15; BK-D	Box 19; BK-D	Box 19; BK-D	Box 19; BK-D
D	Box 3; BK-E	Box 3; BK-E	Box 3; BK-E	Box 10; BK-E	Box 10; BK-E	Box 10; BK-E	Box 15; BK-E	Box 15; BK-E	Box 15; BK-E	Box 19; BK-E	Box 19; BK-E	Box 19; BK-E
E	Box 3; BK-F	Box 3; BK-F	Box 3; BK-F	Box 10; BK-F	Box 10; BK-F	Box 10; BK-F	Box 15; BK-F	Box 15; BK-F	Box 15; BK-F	Box 19; BK-F	Box 19; BK-F	Box 19; BK-F
F	Box 3; BK-G	Box 3; BK-G	Box 3; BK-G	Box 10; BK-G	Box 10; BK-G	Box 10; BK-G	Box 15; BK-G	Box 15; BK-G	Box 15; BK-G	Box 19; BK-G	Box 19; BK-G	Box 19; BK-G
G	NTC; BK-A	NTC; BK-A	NTC; BK-B	NTC; BK-B	NTC; BK-D	NTC; BK-D	NTC; BK-E	NTC; BK-E	NTC; BK-F	NTC; BK-F	NTC; BK-G	NTC; BK-G
H	NTC; BK-A	NTC; BK-A	NTC; BK-B	NTC; BK-B	NTC; BK-D	NTC; BK-D	NTC; BK-E	NTC; BK-E	NTC; BK-F	NTC; BK-F	NTC; BK-G	NTC; BK-G

Figure 5. Layout of certification plate 2.

Results/Discussion

Droplet Volume

Appendix B extracts the sections of the Special Test Method 11050S/-D Measurement Report that are pertinent to the ddPCR measurements used in the SRM 2365 certification process. These reports provide droplet volumes and diameters and make comparisons with previous measurements. Droplet volumes remain constant over long periods for given lots of supermix but vary between lots and different supermixes. Droplet volumes are not influenced by the presence or absence of DNA in the sample solution.

The volume for the batch of "Supermix for probes" (control number 64066349) was 0.7584 nL with a 95 % expanded relative uncertainty of 2.3 %. This lot of supermix was used for all homogeneity, stability and certification measurements.

Inconsistent droplet volume distributions may contribute to ddPCR measurement imprecision and bias[7], [8] but these effects become significant only with distributions wider than observed in Dagata et al[5] and at copy/droplet values larger than the ≤ 0.5 copy/droplet typically used in our measurements.

Homogeneity Results

Table 4 lists the λ results of the BKV DNA homogeneity measurements (adjusted for 1→100 sample dilution, but not 1→10 into the PCR reaction) from the homogeneity measurements. The data were analyzed using the two-level Gaussian hierarchical model described in Appendix C. Table 5 for assays D and E lists the values of the within- and between-tube variance components, expressed as percent relative standard deviations.

At the 95 % confidence level, the BKV units are homogeneous, except for materials in box 9, which will be excluded from sale. Homogeneity results were provided to the statistician for further analysis with the copy number per droplet adjusted for the 1 to 100 dilution factor. The average number of droplets for the homogeneity measurements was 16,808, with a standard deviation of 2,627. The lowest droplet count was 4,243 and the highest was 20,762.

Table 4: Homogeneity Data as λ, Copy Number per Droplet, Adjusted for 1→100 dilution

	Assay E			Assay D		
Box	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
1	39.58	43.41	40.87	39.62	TF	40.37
2	44.83	42.95	44.10	42.83	41.02	42.62
3	43.76	40.57	42.87	42.11	39.97	40.53
4	43.17	42.95	44.49	41.36	42.57	42.34
5	43.92	44.38	44.24	41.29	43.62	40.00
6	43.34	43.72	43.39	42.30	42.60	41.87
7	41.08	42.75	42.37	39.42	40.43	37.70
8	42.96	44.11	43.29	42.21	43.31	42.60
9	46.64	46.96	45.88	43.77	42.85	42.90
10	42.80	43.51	43.33	40.75	40.68	40.26
11	42.90	43.26	41.08	42.05	41.02	39.87
12	43.92	42.48	42.43	42.05	39.54	41.80
13	44.36	41.08	42.86	39.98	41.25	41.29
14	41.04	42.07	41.19	40.52	40.16	40.19
15	43.99	42.05	44.32	40.57	41.03	43.54
16	43.51	41.25	43.39	40.99	40.16	40.99
17	44.87	44.55	44.37	44.06	42.71	42.26
18	44.68	42.91	44.91	40.79	41.35	41.89
19	43.73	44.25	45.50	40.34	41.58	40.47
20	44.22	46.55	46.95	42.98	44.49	45.05

TF Technical Failure - Value not available due to a technical failure by either the droplet generator or droplet reader

Table 5: Posterior Means, γ_{ij}, and Standard Uncertainties, $u(\gamma_{ij})$ for Assays E and D.

	Assay "E"		Assay "D"	
Box	γ_{i1}	$u(\gamma_{i1})$	γ_{i2}	$u(\gamma_{i2})$
1	41.74	0.57	40.51	0.62
2	43.84	0.54	41.98	0.52
3	42.62	0.54	41.02	0.52
4	43.51	0.53	41.93	0.52
5	44.02	0.53	41.6	0.52
6	43.48	0.54	42.06	0.52
7	42.36	0.55	39.79	0.56
8	43.45	0.53	42.39	0.52
9	45.85	0.58	42.73	0.54
10	43.27	0.54	40.8	0.52
11	42.62	0.54	41.11	0.51
12	43.05	0.53	41.22	0.52
13	42.91	0.54	41.01	0.52
14	41.85	0.55	40.59	0.53
15	43.45	0.53	41.65	0.51
16	42.87	0.53	40.91	0.53
17	44.36	0.53	42.62	0.54
18	44.01	0.53	41.38	0.52
19	44.27	0.54	40.97	0.52
20	45.39	0.56	43.47	0.58

Stability Results

Table 6 lists the λ (adjusted for a 1→100 dilution) values from the stability measurements; Figure 6 summarizes these results. The results for the tubes held at 4 °C, room temperature (RT, ≈21 °C), and 37 °C are very similar. The absence of between temperature differences identifies the between-week differences as attributable to variation in the measurement processes rather than sample instability. These results indicate that the SRM 2365 BKV copy number is thermally stable from 4 °C to 37 °C over an extended period.

Table 6: Stability Data as Adjusted λ, Copy Number per Droplet

		Assay D				Assay E			
Week	Temp	Tube	Rep 1	Rep 2	Rep 3	Tube	Rep 1	Rep 2	Rep 3
1	4	1	42.34	41.56	41.99	1	41.98	41.05	40.73
		4	42.70	43.43	43.19	4	41.86	42.93	43.47
	21	2	42.63	43.44	41.80	2	40.84	41.98	40.76
		5	43.90	43.92	46.17	5	42.44	43.05	42.12
	37	3	43.01	44.31	42.89	3	42.12	41.52	43.46
		6	43.66	40.83	44.91	6	42.17	42.02	41.71
3	4	1	41.76	40.61	40.44	1	42.34	40.33	41.38
		4	42.10	42.66	42.14	4	42.33	44.20	42.59
	21	2	41.71	42.49	40.43	2	40.43	41.44	40.94
		5	43.74	41.18	42.91	5	42.26	42.61	43.46
	37	3	41.88	43.33	41.29	3	43.54	41.73	42.41
		6	41.88	41.13	40.20	6	40.20	41.88	41.97
5	4	1	39.52	43.63	40.98	1	42.05	41.87	42.03
		4	41.26	41.21	41.36	4	41.88	40.33	41.60
	21	2	42.04	41.68	a	2	41.69	40.88	40.93
		5	40.47	40.14	40.28	5	40.30	40.15	39.03
	37	3	40.19	39.18	41.59	3	39.63	38.76	39.44
		6	39.81	40.54	38.83	6	40.41	40.21	39.12
7	4	1	41.99	42.57	42.88	1	42.31	42.45	40.83
		4	43.23	41.99	41.13	4	44.09	42.94	42.81
	21	2	40.92	42.73	42.17	2	41.37	41.36	41.61
		5	41.61	40.72	40.68	5	40.41	42.21	41.65
	37	3	43.15	43.47	44.93	3	42.90	42.63	43.79
		6	39.62	40.92	40.94	6	41.23	42.66	41.80
9	4	1	39.59	41.88	40.60	1	42.59	42.62	42.46
		4	40.85	42.05	40.94	4	42.41	42.53	42.37
	21	2	41.41	40.73	40.51	2	41.71	42.20	42.64
		5	40.24	42.01	39.35	5	40.23	40.39	41.17
	37	3	42.69	43.09	41.61	3	42.39	43.36	42.55
		6	39.12	41.18	40.62	6	41.98	42.04	40.99

a Value not available due to a technical failure by either the droplet generator or droplet reader

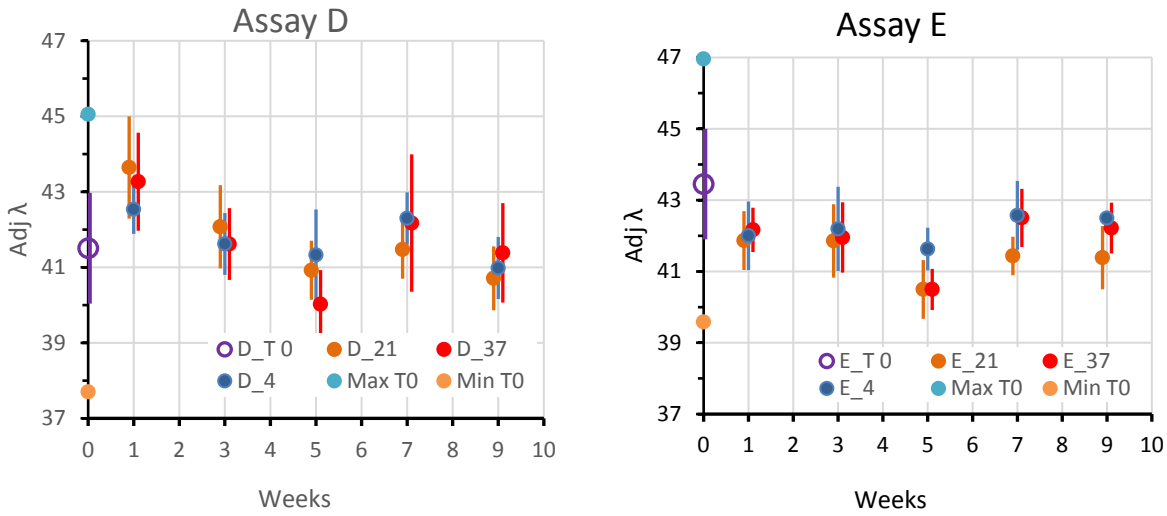

Figure 6: ddPCR Stability Measurements as Function of Time

The "D_T 0" and "E_T 0" symbol at Week 0 represent the mean and standard deviation of all the homogeneity measurements for assays D and E, respectively. The minimum and maximum values for the two assays are represented by the "Min T0" and "Max T0" symbols, also plotted at week 0. The "D_4", "D_21", "D_37", "E_4", "E_21", and "E_37" symbols represent the stability measurements made at 4 °C, 21 °C, and 37 °C. Error bars indicate one standard deviation.

Certification Results

Table 7 lists copy per µL values for all certification assay datasets. For the certification measurements, the average number of droplets was 18,015 with a standard deviation of 1,322. The lowest droplet count was 12,507 and the highest droplet count was 20,681.

The analysis detailed in Appendix C was used to calculate the uncertainty value. The derived uncertainty calculations are shown in Table 8.

The final certified values are 95% probability intervals for SRM 2365 are shown in Table 9.

Table 7: Certification Data as Copy Number per µL

Box3_10/11/17			Box10_10/11/17		
Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
534,852	538,601	530,618	562,983	558,521	565,206
548,708	545,971	547,294	564,930	553,871	558,753
535,170	538,308	536,546	554,992	572,672	556,854
552,091	534,058	530,540	555,285	562,783	558,935
540,671	540,923	536,888	558,959	572,960	550,797
533,746	528,368	540,005	558,105	551,164	554,462
Box15_10/11/17			Box19_10/11/17		
Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
523,174	499,837	533,144	542,962	564,129	546,803
524,687	512,263	531,017	543,401	562,536	557,708
521,301	502,803	532,651	544,474	553,160	538,705
524,910	515,603	529,041	549,552	564,655	547,620
531,759	526,512	532,683	551,067	557,967	548,064
533,518	510,900	523,419	546,362	547,137	537,142
Box1_10/12/17			Box8_10/12/17		
Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
535,404	563,292	527,299	593,177	584,420	589,032
565,107	553,751	549,235	584,362	590,104	582,089
550,124	559,293	541,736	574,658	566,911	575,540
535,479	586,059	540,863	564,629	565,927	577,114
534,432	557,921	538,426	566,079	572,074	579,705
543,990	578,599	528,767	558,040	571,945	573,465
Box13_10/12/17			Box20_10/12/17		
Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
575,495	604,728	554,293	601,675	607,096	593,840
582,714	596,330	562,011	602,994	603,868	619,142
588,022	568,942	543,309	587,454	607,383	594,790
591,011	577,122	566,913	618,619	591,867	592,905
559,525	575,859	549,716	589,101	603,390	589,897
559,040	577,089	545,416	591,843	582,847	596,474

Table 8: Components of Uncertainty for SRM 2365

Uncertainty Source	Relative Standard Uncertainty
Between boxes	1.60 %
Between assays in a box	0.03 %
Repeatability within assay and box	0.90 %
Droplet volume	1.15 %
Combined Uncertainty	2.20 %

Table 9: Certified Values for SRM 2365

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

These results are metrologically traceable to the International System of Units through 1) the counting unit one [9], 2) the validity of the Poisson endpoint transformation for digital PCR endpoint assays when applied to samples providing ≤ 0.5 copies per droplet and 3) calibrated mean droplet volume measurements made at NIST during sample dilution and mastermix preparation.

Conclusions and Recommendations

With high confidence, all units of SRM 2365 (excluding those in box 9) have the same copy number content within measurement repeatability.

With high confidence, the copy number content of SRM 2365 units is thermally stable from 4 °C to 37 °C over an extended period of time. The solution should not be shipped or stored below 4 °C.

The use of SRM 2365 as a calibrant for qPCR standard curves in screening for BKV may help limit the variability observed within the clinical community.

Certificate of Analysis

In accordance with ISO Guide 31: 2000, a NIST SRM certificate is a document containing the name, description, and intended purpose of the material, the logo of the U.S. Department of Commerce, the name of NIST as a certifying body, instructions for proper use and storage of the material, certified property value(s) with associated uncertainty(ies), method(s) used to obtain property values, the period of validity, if appropriate, and any other technical information deemed necessary for its proper use. A Certificate is issued for an SRM certified for one or more specific physical or engineering performance properties and may contain NIST reference, information, or both values in addition to certified values. A Certificate of Analysis is issued for an SRM certified for one or more specific chemical properties. Note: ISO Guide 31 is updated periodically; check with ISO for the latest version. [https://www.nist.gov/srm/srm-definitions]

For the most current version of the COA for NIST SRM 2365 BKV DNA Quantitative Standard, please visit: https://www-s.nist.gov/srmors/view_detail.cfm?srm=2365.

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Appendix A: Cleaning of Perfluoroalkoxy Fluoropolymer (PFA) Containers

1) Completely fill PFA container and the inverted lid of the PFA container with a 10 % to 20 % freshly made bleach solution.
2) Allow to stand for 20 minutes.
3) After 20 minutes, remove the bleach solution with a vacuum aspirator.
4) Flush the PFA container and lid with MilliQ water.
5) Allow to stand 5 to 10 minutes.
6) Remove water with a vacuum aspirator.
7) Repeat the water wash, steps 5 through 7 a total of five times.
8) Fill the entire container and inverted lid with ethanol.
9) Allow to stand 5 minutes.
10) Remove the ethanol from the container and lid by inverting
11) Place the open PFA container and inverted lid in a Biological Safety Cabinet Class II Type A/B3 (BSC) to air dry.

Appendix B: Droplet Measurement Report

The average volume for aqueous droplets dispersed in a propriety oil phase was determined according to Special Test Method 11050S/-D and is reported for the following protocols:

	Control #	Protocol	Average ± expanded uncertainty
S3	64066349	BK DNA, UNG MM	0.7584 nL ± 2.3% (*k*=2)
S4	64066349	No DNA, UNG MM	0.7576 nL ± 2.3% (*k*=2)

The detailed measurement results are shown in Table B-1. In addition to the average volume, the average diameter values as an equivalent spherical diameter obtained from the concentrated method are also included in Table B-2. Sufficient statistics was assured by preparing and measuring two samples for each protocol on different days with 3 replicate measurements per day. Standard deviations in the tables from left to right refer to measurement repeatability, within day reproducibility, and sample-to-sample variation. The expanded uncertainty of the volume measurements includes additional uncertainty components per Special Test Method 11050S/-D.

Panel A of Figure B-1 compares the results of the recent and previous studies. The measured droplet volumes for the UNG (Bio-Rad, ddPCR Supermix for Probes, cat# 186-3010) ddPCR Supermix for Probes No dUTP, cat# 186-3024) protocol are significantly lower than observed previously. Because of the somewhat unexpected results, droplet diameter was also determined by the dilute method as described in Special Test Method 11050S/-D. The results in Panel B of Figure B-1 confirm that the diameters of the UNG droplets are indeed lower than previously seen. Further investigation is suggested to determine whether it is due to formulation changes or lot-to-lot variation of the reagents.

Table B-1. Average Volumes from the Concentrated Method

			Measurement (nL)			Sample (nL)		Protocol (nL)	
Sample		Date	Replicate	Average	SD	Average	SD	Average	SD
S3		2/28/2017	1	0.7480	0.0086	0.7578	0.0089	0.7584	0.0009
			2	0.7655	0.0074				
			3	0.7598	0.0071				
		3/2/2017	1	0.7575	0.0067	0.7591	0.0059		
			2	0.7656	0.0061				
			3	0.7541	0.0094				
S4		2/28/2017	1	0.7477	0.0033			0.7576	0.0035
			2	0.7645	0.0046	0.7552	0.0086		
			3	0.7533	0.0067				
		3/2/2017	1	0.7559	0.0055				
			2	0.7653	0.0041	0.7601	0.0048		
			3	0.7591	0.0059				

Table B-2. Average Diameters from the Concentrated Method

		Measurement (nL)			Sample (nL)		Protocol (nL)	
Sample	Date	Replicate	Average	SD	Average	SD	Average	SD
S3	2/28/2017	1	113.38	0.44	113.87	0.45	113.90	0.04
		2	114.25	0.37				
		3	113.97	0.35				
	3/2/2017	1	113.85	0.34	113.93	0.30		
		2	114.26	0.30				
		3	113.68	0.47				
S4	2/28/2017	1	113.37	0.17	113.74			
		2	114.21	0.23		0.43	113.86	0.17
		3	113.65	0.34				
	3/2/2017	1	113.78	0.28	113.99			
		2	114.25	0.21		0.24		
		3	113.94	0.30				

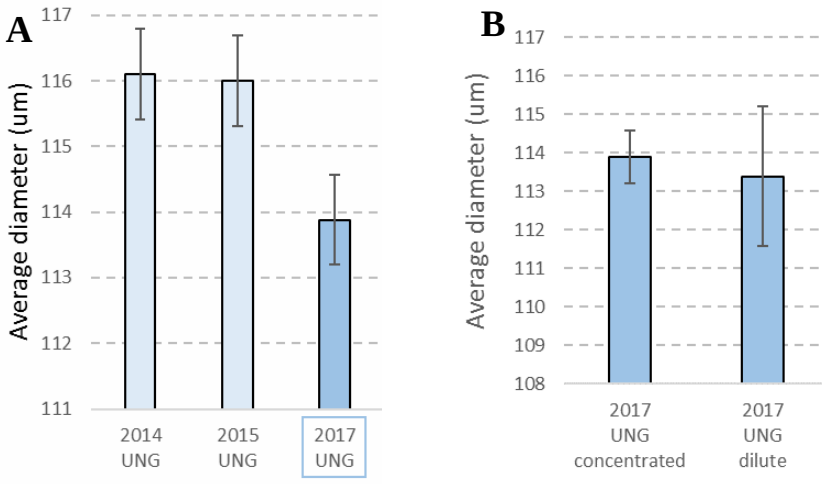

Figure B-1. Average Diameter Over Time and Test Method

Panel A: Results obtained by the concentrated method of the Special Test Method 11050S/-D compared to that from previous studies. Panel B: Results obtained by the concentrated and dilute methods.

Appendix C: SRM 2365 Statistical Analysis

Introduction

BK virus is a double-stranded DNA virus, with a genome size of approximately 5200 base pairs, from a family of viruses known as the Polyomaviridae. Quantitative tests, such as quantitative real-time polymerase chain reaction (qPCR) for viral load are used to monitor patients. Each unit of SRM 2365 consists of one vial containing 110 µL aqueous buffer solution of synthetic BK Virus DNA. SRM 2365 is certified for the number of BKV genome copies per µL solution. The value of this measurand was determined using six different droplet digital polymerase chain reaction (ddPCR) assays that probe different regions of the BKV genome.

Homogeneity assessment

Samples of the material were drawn from 20 different boxes. Two assays (D and E) were used to obtain three replicates of the measurements (lambda; copy number per droplet) for each sample and component. Table 4 lists the measurements.

The data were evaluated using a Gaussian hierarchical model with two levels, assay and box:

$$y_{ijk} \sim N(\gamma_{ij}, \sigma^2);\ \gamma_{ij} \sim N(\alpha_i, \sigma_i^2)$$

where i indexes box number (1 to 20); j indexes assay (j = 1 is assay E, j = 2 is assay D); k indexes replicate (1 to 3); γ_{ij} is the mean lambda value of box i, assay j; α_i is the mean lambda value of box i; σ_i^2 is the between assay variance; and σ^2 is the within box variance.

The analysis was accomplished using Bayesian MCMC [2] with noninformative priors using OpenBUGS [3]. The posterior means with standard uncertainties are listed in Table 5 and displayed in Figure C-1.

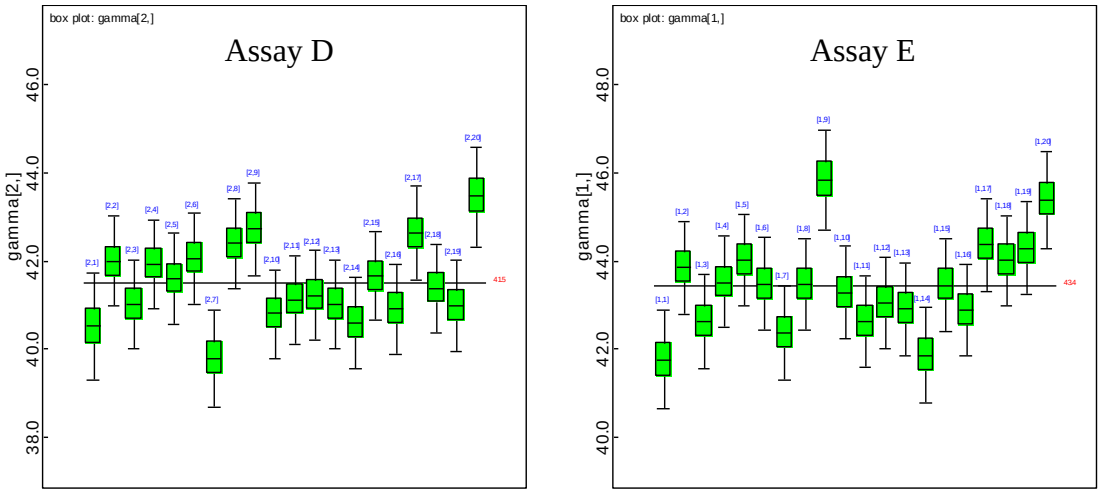

Figure C-1. Box plots of γ_{ij} for assays D and E

The following is the OpenBUGS code used to evaluate homogeneity.

```
# Model
ModelBegin{
sig~dgamma(1.0E-5, 1.0E-5); unc<-1/sqrt(sig)
for(j in 1:2){alpha[j]~dnorm(0,1.0E-5); sigg[j]~dgamma(1.0E-5, 1.0E-5); uncgg[j]<-
1/sqrt(sigg[j])}
for(i in 1:20){for(j in 1:2){gamma[j,i]~dnorm(alpha[j],sigg[j]);
pred[j,i]~dnorm(gamma[j,i],sig)}}
for(i in 1:20){for (j in 1:2){for(k in 1:3){measure[i,j,k]~dnorm(gamma[j,i],sig)}}}
}ModelEnd

# Initial values
list(sig=1 ,sigg=c(1,1))

# Data
list(measure=structure(.Data=c(
39.58,43.41,40.87,39.62,NA,   40.37,44.83,42.95,44.10,42.83,41.02,42.62,
43.76,40.57,42.87,42.11,39.97,40.53,43.17,42.95,44.49,41.36,42.57,42.34,
43.92,44.38,44.24,41.29,43.62,40.00,43.34,43.72,43.39,42.30,42.60,41.87,
41.08,42.75,42.37,39.42,40.43,37.70,42.96,44.11,43.29,42.21,43.31,42.60,
46.64,46.96,45.88,43.77,42.85,42.90,42.80,43.51,43.33,40.75,40.68,40.26,
42.90,43.26,41.08,42.05,41.02,39.87,43.92,42.48,42.43,42.05,39.54,41.80,
44.36,41.08,42.86,39.98,41.25,41.29,41.04,42.07,41.19,40.52,40.16,40.19,
43.99,42.05,44.32,40.57,41.03,43.54,43.51,41.25,43.39,40.99,40.16,40.99,
44.87,44.55,44.37,44.06,42.71,42.26,44.68,42.91,44.91,40.79,41.35,41.89,
43.73,44.25,45.50,40.34,41.58,40.47,44.22,46.55,46.95,42.98,44.49,45.05),
.Dim=c(20,2,3)))
```

Certification results

Six different assays were used to obtain triplicate measurements from eight different boxes. Table C-1 lists the measurements in units of λ, copy number per droplet, adjusted for the 1→100 dilution.

Table C-1. Certification Measurements, Dilution-Adjusted Copy Number Per Droplet

			Assay, Copy Number per Droplet					
Date	Box	Rep	A	B	C	D	E	F
10/11/2017	3	1	405.6	416.1	405.9	418.7	410.0	404.8
		2	408.5	414.1	408.3	405.0	410.2	400.7
		3	402.4	415.1	406.9	402.4	407.2	409.5
	10	1	427.0	428.4	420.9	421.1	423.9	423.3
		2	423.6	420.1	434.3	426.8	434.5	418.0
		3	428.7	423.8	422.3	423.9	417.7	420.5
	15	1	396.8	397.9	395.4	398.1	403.3	404.6
		2	379.1	388.5	381.3	391.0	399.3	387.5
		3	404.3	402.7	404.0	401.2	404.0	397.0
	19	1	411.8	412.1	412.9	416.8	417.9	414.4
		2	427.8	426.6	419.5	428.2	423.2	414.9
		3	414.7	423.0	408.6	415.3	415.7	407.4
10/12/2017	1	1	406.1	428.6	417.2	406.1	405.3	412.6
		2	427.2	420.0	424.2	444.5	423.1	438.8
		3	399.9	416.5	410.9	410.2	408.3	401.0
	8	1	449.9	443.2	435.8	428.2	429.3	423.2
		2	443.2	447.5	429.9	429.2	433.9	433.8
		3	446.7	441.5	436.5	437.7	439.6	434.9
	13	1	436.5	441.9	446.0	448.2	424.3	424.0
		2	458.6	452.3	431.5	437.7	436.7	437.7
		3	420.4	426.2	412.0	429.9	416.9	413.6
	20	1	456.3	457.3	445.5	469.2	446.8	448.9
		2	460.4	458.0	460.6	448.9	457.6	442.0
		3	450.4	469.6	451.1	449.7	447.4	452.4

Because the certified values are given in copies/µL, not copies/droplet, the measurements had to be converted using droplet volume. According to [1], the average droplet volume for BK DNA, 'UNG' MM is 0.7584 nL with standard relative uncertainty of 1.15%. The following statistical model was used to produce the certified values:

$$y_{ijk} \sim N(\gamma_{ij}, \sigma^2);\ \gamma_{ij} \sim N(\alpha_i, \sigma_i^2);\ \alpha_i \sim N(\mu, \sigma_{box}^2)$$

where: γ_{ij} is the mean lambda value of box i, assay j, α_i is the consensus mean lambda value of box i, µ is the overall consensus lambda value, σ^2 is the repeatability variance, σ_i^2 is the between assay variance within box i, and σ_{box}^2 is the between box variance.

Modelling the droplet volume of master mix BK DNA, 'UNG' MM, as

$$V \sim dnorm(0.7584, 0.0087^2),$$

the mean value of the measurand in copies per microliter is

$$copies/\mu L = \mu_{adj} = {}^{\mu}/_{V} .$$

The statistical model was analyzed using Bayesian MCMC, with non-informative Gaussian priors for the means and Gamma priors for the variance components.

The analysis detailed in Appendix C was used to calculate the uncertainty value. The derived uncertainty calculations are shown in Table 8.

The final certified values are 95% probability intervals for SRM 2365 are shown in Table 9.

Table 7 lists the volume-adjusted results. Table C-2 lists the posterior means for the eight boxes, α_i, and their standard uncertainties, $u(\alpha_i)$

Table C-2. Certification Results: Mean Values and Standard Uncertainties for Each Box

Values have units of dilution-adjusted copy number per droplet

Box	posterior mean of α_i	Standard uncertainty of α_i
1	408.5	2.2
2	424.3	2.1
3	397.0	2.1
4	417.5	2.2
5	416.8	2.1
6	436.7	2.4
7	432.8	2.5
8	453.6	2.2

In units of dilution-adjusted copies per droplet: the posterior median of the between box variability, σ_{box}, was 18; the posterior median of the between assay variability, σ_i , for all six assays was 0.3; and the posterior median of the repeatability, σ, was 10. The posterior mean and standard uncertainty of all measurements, $\mu \pm u(\mu)$, was (423.1 ± 7.5) dilution-adjusted copies per droplet.

The certified value, that is, the posterior mean of the droplet volume-adjusted value is 557,880 $copies/\mu L$ with standard uncertainty of 11,765. The 95% uncertainty interval in $copies/\mu L$ is (534,690 to 581,250). This uncertainty accounts for both between and within box repeatability, between assay variability, and the uncertainty in the droplet volume.

The following is the OpenBUGS code used to evaluate the certification data.

```
# Model
# Note: This model converges rather slowly.  Make sure that mu has reached a limiting value
# before defining the posterior distributions (approximately 60,000 iterations).
#
ModelBegin{
vol~dnorm(0.7584, 13179); mu~dnorm(0,1.0E-5)
sig~dgamma(1.0E-5,1.0E-5); sigalpha~dgamma(1.0E-5,1.0E-5)
for(i in 1:8){alpha[i]~dnorm(mu,sigalpha); xiNs[i]~dnorm(0, 0.0016)I(0.001,);
  chSqNs[i]~dgamma(0.5,0.5); siga[i]<-xiNs[i]/sqrt(chSqNs[i]); unca[i]<-1/sqrt(siga[i])}
for(i in 1:8){for( j in 1:6){gamma[i,j]~dnorm(alpha[i],siga[i])}}
for(i in 1:8){for(j in 1:6){for(k in 1:3){meas[i,j,k]~dnorm(gamma[i,j],sig)}}}
murep<-1000*(mu/vol)
}ModelEnd

# Initial values
list(sig=1,sigalpha=1)

# Data
list(meas=structure(.Data=c(
405.6,408.5,402.4,416.1,414.1,415.1,405.9,408.3,406.9,418.7,405.0,402.4,410.0,410.2,407.2,404.8,400.7,409.5,
427.0,423.6,428.7,428.4,420.1,423.8,420.9,434.3,422.3,421.1,426.8,423.9,423.9,434.5,417.7,423.3,418.0,420.5,
396.8,379.1,404.3,397.9,388.5,402.7,395.4,381.3,404.0,398.1,391.0,401.2,403.3,399.3,404.0,404.6,387.5,397.0,
411.8,427.8,414.7,412.1,426.6,423.0,412.9,419.5,408.6,416.8,428.2,415.3,417.9,423.2,415.7,414.4,414.9,407.4,
406.1,427.2,399.9,428.6,420.0,416.5,417.2,424.2,410.9,406.1,444.5,410.2,405.3,423.1,408.3,412.6,438.8,401.0,
449.9,443.2,446.7,443.2,447.5,441.5,435.8,429.9,436.5,428.2,429.2,437.7,429.3,433.9,439.6,423.2,433.8,434.9,
436.5,458.6,420.4,441.9,452.3,426.2,446.0,431.5,412.0,448.2,437.7,429.9,424.3,436.7,416.9,424.0,437.7,413.6,
456.3,460.4,450.4,457.3,458.0,469.6,445.5,460.6,451.1,469.2,448.9,449.7,446.8,457.6,447.4,448.9,442.0,452.4),
.Dim=c(8,6,3)))
```

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[2] Gelman A, Carlin J, Stern H, Dunson D, Vehtari A, Rubin D. Bayesian Data Analysis. Chapman & Hall, Boca Raton, 3rd edition, 2013.

[3] Lunn DJ, Spiegelhalter D, Thomas A, Best N. The BUGS project: Evolution, critique and future directions (with discussion), Statistics in Medicine 2009;28:3049–3082.