Certification of Standard Reference Material® 2367
JC Virus DNA Quantitative Standard

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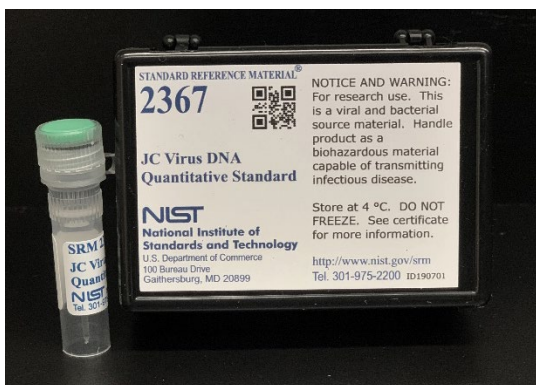# NIST Special Publication 260-216

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Abstract

Standard Reference Material (SRM) 2367 is intended for use in the value assignment of JC (John Cunningham) virus deoxyribonucleic acid (DNA) to JC virus DNA controls and standards. A unit of SRM 2367 consists of a well characterized, linearized plasmid, containing JC 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. This publication documents the production, analytical methods, and statistical evaluations involved in producing this SRM.

Keywords

JC Virus (JCV);
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) 2367 is intended for use in the value assignment (copies/µL) of JCV deoxyribonucleic acid (DNA) to JCV controls and standards.

A unit of SRM 2367 consists of a well characterized, linearized plasmid, containing JCV 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 sealed with a screw cap.

Storage and Use

Until required for use, SRM 2367 should be stored in the dark between 2 °C and 8 °C.

The SRM 2367 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

JCV is a double-stranded DNA virus from a family of viruses known as the Polyomaviridae. It was first isolated in 1971 from a patient with progressive multifocal leukoencephalopathy (PML)[1]. PML is caused by JCV reactivation in immunocompromised patients and results in progressive damage to the white matter in the brain. PML is a rare disorder but its incidence started to increase during the AIDS epidemic. Currently, PML can occur as a side effect of therapies that target immune system function, such as natalizumab, rituximab and infliximab. These drugs are used to treat autoimmune diseases such as multiple sclerosis, Crohn's disease, rheumatoid arthritis, and psoriasis[2]. Studies have shown that approximately (30 to 70) %[1, 3] of the population is seropositive for exposure to JCV, making nucleic acid tests important for measuring the viral load in patients.

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

Each unit of SRM 2367 consists of one vial containing 110 µL aqueous buffer solution of synthetic JCV DNA. SRM 2367 is certified for the number of JCV genome copies per µL solution. The value of this measurand was determined using six digital polymerase chain reaction (dPCR) assays that probe different regions of the JCV genome.

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

The copy numbers of JCV DNA in the component solutions, symbolized here as λ', is calculated as $\lambda' = \lambda/(VF)$ where V is the average droplet volume and F is the volume of component solution in the reaction mixture. SRM 2367 is certified for JCV DNA copy number per µL.

The manufacturer of the dPCR platform used does not provide metrologically traceable partition volume information. This lack of information prevented metrologically traceable conversion of the number per partition measurements to the desired number per reaction volume units. In consequence, the National Institute of Standards and Technology (NIST) staff developed a measurement method that provides metrologically traceable droplet volumes[4].

Experimental Methods

Digital Polymerase Chain Reaction Assays

Six digital PCR assays were developed for characterization of SRM 2367. FAM-labeled Blackhole Quencher Plus probes were purchased from LGC Biosearch Technologies[1] (Novato, CA); primers were purchased from Eurofins Genomics (Louisville, KY)

Table 1 details the six assays used to certify the JCV copy number of SRM 2367. Figure 1 displays exemplar droplet patterns for the assays. All assays were developed at NIST and have been optimized for dPCR.

All primers probes were diluted with TE buffer to a 10 µmol/L working solution.

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[1] Certain commercial entities, equipment, or materials may be identified in this document in order to describe an experimental procedure or concept adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the entities, materials, or equipment are necessarily the best available for the purpose.

Table 1: dPCR Assays Used

Assay Name	Locus	Nucleotide Position[a]	Amplicon Size	Primer and Probe Sequences (5' to 3')
H	VP2/VP3	1370-1475	106	F: caccaggaggtgcaaatcaaag
				R: gggccatcttcatatgcttcaagag
				P: tctgctcctcaatggatgttg
I	VP1	1693-1786	94	F: ggacatgcttccttgttacagtg
				R: cacagcctcccacatgagta
				P: ttccactacccaatctaaatgag
J	VP1	1990-2076	87	F: tgccacagtgcaatctcaag
				R: ggaacccaacattcaacaggata
				P: atgaacacagagcacaaggcgta
K	Large T Antigen	3597-3657	61	F: ctggtcatgtggatgctgtcaa
				R: gccagcaggctgttgatac
				P: ccctttgtttggctgctaca
L	Large T Antigen	4355-4427	73	F: caggtcttcatcccacttctcatta
				R: ggtgccaacctatggaacagat
				P: tattccaccaggattccca
M	Small T Antigen	4539-4731	193	F: cccagcaatgaagagcttcttg
				R: tgcaaggaatggcctaactg
				P: taagtcacacccaaaccattg

[a] Relative to the JCV Reference Genome J02226.1, F: Forward primer, R: Reverse primer, P: Blackhole Plus quencher probe (FAM labeled)
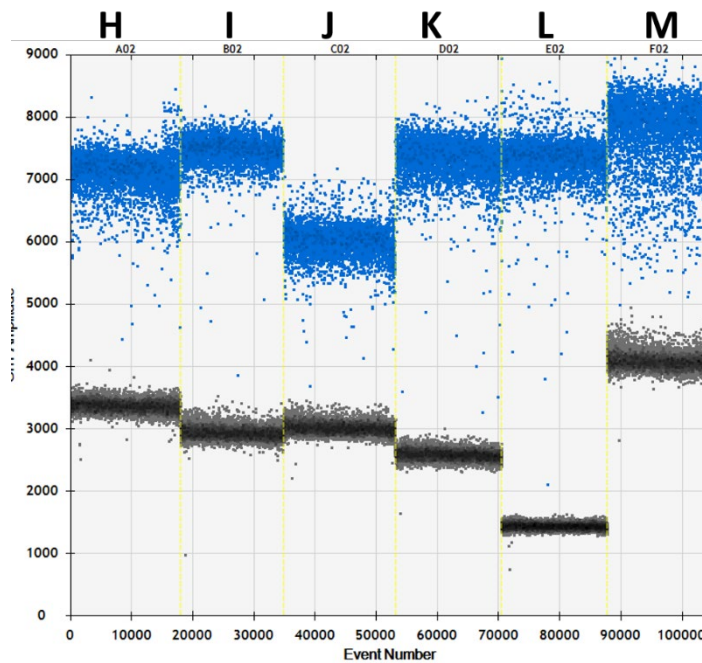
Figure 1. Exemplar Droplet Patterns for the six JCV Assays

Digital Polymerase Chain Reaction Measurements

All JCV DNA dPCR 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 64294601) 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 (ThermoFisher, 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 [5] for each assay for each measurement session.

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Table 2: Mastermix setup for NIST-Developed JCV Assays for dPCR

Manufacturer	Part number	Reagent	µL/rxn
Bio-Rad	186-3010	ddPCR Supermix for probes	12.50
Thermo Fisher	AM9937	Water	8.125
Eurofins Genomics	Salt free oligos	Forward primer (10 µmol/L)	0.625
Eurofins Genomics	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

<u>Sample Preparation</u>

The JCV genome construct (National Center for Biotechnology Information accession # J02226.1) was synthesized and cloned into a pUC57 plasmid (GeneWiz, South Plainfield, NJ). Restriction sites (AhdI and BssHII) were added on either side of the JCV DNA, to allow the JCV genome region to be isolated from the rest of the plasmid.
The plasmid was transformed into *Escherichia coli* Sure2 cells (GeneWiz) and 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 3.5×10^9 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 4.0×10^5 genome copies/µL, with 50 ng/µL yeast tRNA (catalog # AM7119, Thermo Fisher, Waltham, MA) added to help ensure stability.

The following components were added:

248.75 mL TE Buffer (Fisher Scientific, catalog # BP2473-100)
1.25 mL yeast tRNA (10 mg/mL)
25 µL stock solution of JCV DNA (approximately 3.5×10^9 genome copies/µL)

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

<u>Sample Production</u>

The morning of packaging, the container holding the diluted component was removed from the refrigerated storage and placed on a slow orbital shaker for 2 hours. The component tubes (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 component 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 component tubes were filled, with pipet tip replacement after filling every 180 tubes. The filling process consisted of two individuals manually filling each vial within a biosafety cabinet (one physically pipetting each sample, the other verifying each tube is filled) and four individuals tightly closing the lids to each tube on a sterilized and covered bench. All individuals performing the filling and capping process were required to wear 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 were 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 green inserts were added to the lids.

After all tubes for a given component were labeled, the 20 boxes were placed in a refrigerator. Filling of the component tubes took approximately 75 min. All units were transferred to the refrigerator within 4 hours of beginning the filling process. The material was equilibrated for four 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 box number. These 20 vials were equilibrated to room temperature, then prepared for analysis using the dPCR procedure described above (Table 2). The H and J dPCR assays were 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 H and a second plate, with the same layout, was run with Assay J.

	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 for Homogeneity Measurements

Homogeneity results were evaluated as copy number per droplet adjusted for the 1→100 pre-dPCR dilution and the 1→10 dPCR dilution factor.

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Component Stability

Typically, the homogeneity measurement values are used as timepoint 0 for the stability study and tubes from the homogeneity study are placed at 4 °C, 21 °C and 37 °C for a minimum of 8 weeks. Due to the COVID-19 pandemic, NIST moved to a "mandatory telework" mode shortly after the homogeneity measurements were completed. After the homogeneity measurements on March 6, 2020, SRM 2367 was not measured again until the preliminary certification measurements on August 12-13, 2020. Because of unusually high coefficient of variation values from the August 12, 2020 certification measurements, additional certification measurements were performed on April 29, 2021 and May 5, 2021. Additionally, 8 tubes were rechecked with the homogeneity assays (H and J) on August 30, 2021. The values from this 17 month period were used in lieu of the traditional stability assay setup.

Component Certification

For the certification measurements, all six dPCR assays (described above) were used. Samples were assayed in two measurement campaigns (over 4 days), using eight randomly selected SRM 2367 units from boxes 1, 3, 8, 10, 13, 15, 19 and 20. Measurements were made using the Bio-Rad QX200 ddPCR system. Three replicate measurements were made for each assay, with each tube. Table 3 lists the samples used and the dates of the measurement campaigns. All tubes were unopened prior to certification measurements. Figures 3 and 4 detail the plate layouts for the certification measurements.

Table 3: Tubes used for Certification Measurements

Box	Assay Date
1	August 12, 2020
8	August 12, 2020
13	August 12, 2020
20	August 12, 2020
3	August 13, 2020
10	August 13, 2020
15	August 13, 2020
19	August 13, 2020
1	April 29, 2021
8	April 29, 2021
13	April 29, 2021
20	April 29, 2021
3	May 5, 2021
10	May 5, 2021
15	May 5, 2021
19	May 5, 2021

	1	2	3	4	5	6	7	8	9	10	11	12
A	H Box 1	H Box 1	H Box 1	H Box 8	H Box 8	H Box 8	H Box 13	H Box 13	H Box 13	H Box 20	H Box 20	H Box 20
B	I Box 1	I Box 1	I Box 1	I Box 8	I Box 8	I Box 8	I Box 13	I Box 13	I Box 13	I Box 20	I Box 20	I Box 20
C	J Box 1	J Box 1	J Box 1	J Box 8	J Box 8	J Box 8	J Box 13	J Box 13	J Box 13	J Box 20	J Box 20	J Box 20
D	K Box 1	K Box 1	K Box 1	K Box 8	K Box 8	K Box 8	K Box 13	K Box 13	K Box 13	K Box 20	K Box 20	K Box 20
E	L Box 1	L Box 1	L Box 1	L Box 8	L Box 8	L Box 8	L Box 13	L Box 13	L Box 13	L Box 20	L Box 20	L Box 20
F	M Box 1	M Box 1	M Box 1	M Box 8	M Box 8	M Box 8	M Box 13	M Box 13	M Box 13	M Box 20	M Box 20	M Box 20
G	H NTC	H NTC	I NTC	I NTC	J NTC	J NTC	K NTC	K NTC	L NTC	L NTC	M NTC	M NTC
H	H NTC	H NTC	I NTC	I NTC	J NTC	J NTC	K NTC	K NTC	L NTC	L NTC	M NTC	M NTC

Figure 3. Layout of certification plates on August 12, 2020 and April 29, 2021

	1	2	3	4	5	6	7	8	9	10	11	12
A	H Box 3	H Box 3	H Box 3	H Box 10	H Box 10	H Box 10	H Box 15	H Box 15	H Box 15	H Box 19	H Box 19	H Box 19
B	I Box 3	I Box 3	I Box 3	I Box 10	I Box 10	I Box 10	I Box 15	I Box 15	I Box 15	I Box 19	I Box 19	I Box 19
C	J Box 3	J Box 3	J Box 3	J Box 10	J Box 10	J Box 10	J Box 15	J Box 15	J Box 15	J Box 19	J Box 19	J Box 19
D	K Box 3	K Box 3	K Box 3	K Box 10	K Box 10	K Box 10	K Box 15	K Box 15	K Box 15	K Box 19	K Box 19	K Box 19
E	L Box 3	L Box 3	L Box 3	L Box 10	L Box 10	L Box 10	L Box 15	L Box 15	L Box 15	L Box 19	L Box 19	L Box 19
F	M Box 3	M Box 3	M Box 3	M Box 10	M Box 10	M Box 10	M Box 15	M Box 15	M Box 15	M Box 19	M Box 19	M Box 19
G	H NTC	H NTC	I NTC	I NTC	J NTC	J NTC	K NTC	K NTC	L NTC	L NTC	M NTC	M NTC
H	H NTC	H NTC	I NTC	I NTC	J NTC	J NTC	K NTC	K NTC	L NTC	L NTC	M NTC	M NTC

Figure 4. Layout of certification plates on August 13, 2020 and May 5, 2021

Initially the certification measurements were performed in August 2020. When the data from the certification measurements was examined, the August 12, 2020 measurements had noticeably lower values compared to August 13, 2020 certification measurements and the homogeneity data. Therefore, it was decided that the certification should be redone. The second round of certification measurements were performed on April 29, 2021 and May 5, 2021, with the same plate layouts as shown above. The homogeneity measurements and the four days of certification measurements were examined. The August 12, 2020 certification measurements had a much higher coefficient of variation, relative to the homogeneity measurements and the other days of certification. Therefore, these measurements were excluded from the calculation of the certified value for SRM 2367.

Additionally, the original homogeneity measurements were slightly higher than the certification measurements, so eight additional tubes were pulled from the boxes and run with assays H and J. The values obtained for these eight tubes all fell within the uncertainty range for the certified value of SRM 2367.

Results/Discussion

Droplet Volume

Appendix B contains the sections of the Special Test Method 11050S/-D Measurement Report that are pertinent to the dPCR measurements used in the SRM 2367 certification process. These reports provide droplet volumes and diameters. 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 this batch of "Supermix for probes" (control number 64294601) was 0.7663 nL with a 95 % expanded relative uncertainty of 2.3 %. This lot was used for all homogeneity, stability and certification measurements.

Inconsistent droplet volume distributions may contribute to dPCR measurement imprecision and bias[6, 7] but these effects become significant only with distributions wider than observed in Dagata et al[4] 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 JCV DNA homogeneity measurements (adjusted for $1\rightarrow100$ sample dilution, and $1\rightarrow10$ into the dPCR reaction). The data was analyzed using the two-level Gaussian hierarchical model described in Appendix C. Tables 5 and 6 list the values of the within- and between-tube variance components, expressed as percent relative standard deviations for assays J and H. At the 95 % confidence level, all components are homogeneous. Homogeneity results were provided to the statistician for further analysis with the copy number per droplet (Lambda). Figures 5 and 6 show the effects plots of assays J and H.

Table 4: Homogeneity Data as λ, Copy Number per Droplet Adjusted for dilutions

Box	Assay "J"				Assay "H"		
	Rep1	Rep2	Rep3		Rep1	Rep2	Rep3
1	349.13	354.47	359.27		364.58	377.83	349.10
2	362.04	371.21	359.79		393.68	375.70	381.08
3	350.28	346.03	342.94		345.99	368.62	346.43
4	385.79	349.46	352.65		376.55	372.18	395.41
5	384.84	370.74	355.21		361.60	354.69	363.82
6	347.17	349.46	353.99		358.47	374.30	375.31
7	336.49	330.59	320.96		356.10	349.45	363.59
8	337.25	359.67	350.69		354.78	356.13	354.61
9	356.90	346.59	325.88		341.25	343.58	326.47
10	351.47	322.49	324.39		349.30	363.00	347.89
11	362.23	358.83	354.65		378.31	360.74	381.88
12	366.88	365.70	346.14		358.83	360.71	347.98
13	330.38	324.88	341.40		335.81	345.07	350.20
14	364.44	363.92	339.89		353.46	365.20	366.89
15	365.69	377.68	355.63		367.48	376.64	377.51
16	357.28	369.10	392.73		379.60	391.59	362.66
17	349.37	326.36	349.92		344.95	331.07	332.63
18	388.61	378.71	387.62		365.40	356.27	363.27
19	390.26	345.73	380.81		365.96	387.73	372.61
20	344.54	359.72	344.08		327.45	336.12	323.56

Table 5: Statistician Calculated Values for Assay J

Box	Posterior Mean	Posterior Standard Deviation
1	355	5.8
2	362	5.9
3	348	5.9
4	361	5.8
5	367	5.9
6	351	5.9
7	334	6.1
8	350	5.8
9	345	5.9
10	337	6.1
11	358	5.9
12	359	5.8
13	337	6
14	356	5.8
15	364	5.9
16	370	5.9
17	344	5.9
18	379	6.1
19	369	6
20	350	5.7
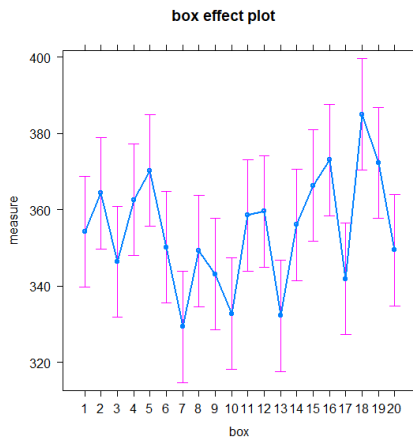
Figure 5. Effects plot for Assay J

Table 6: Statistician Calculated Values for Assay H

Box	Posterior Mean	Posterior Standard Deviation
1	363	5.9
2	379	6.1
3	355	5.9
4	378	6.1
5	360	5.9
6	368	5.9
7	357	5.9
8	356	5.8
9	341	6.1
10	355	5.9
11	371	5.9
12	357	5.9
13	348	6
14	361	5.9
15	371	5.9
16	375	6
17	341	6.1
18	361	5.9
19	373	6
20	335	6.3
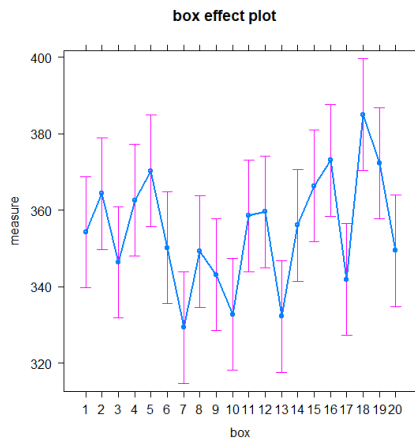
Figure 6. Effects plot for Assay H

Stability Results

Figure 7 displays the results of the homogeneity measurements, certification measurements, and homogeneity recheck measurements with all boxes and all assays. Although the original homogeneity measurements were slightly high compared to the certification measurements, the homogeneity recheck measurements confirm that the SRM 2367 maintained the same concentration at 4 °C for over 12 months. Additionally, SRM 2367 is nearly identical in manner of synthesis and storage conditions to SRM 2365, which has maintained its concentration for over 4 years [8]. These results indicate that SRM 2367 JCV copy number is stable at 4 °C over an extended period.
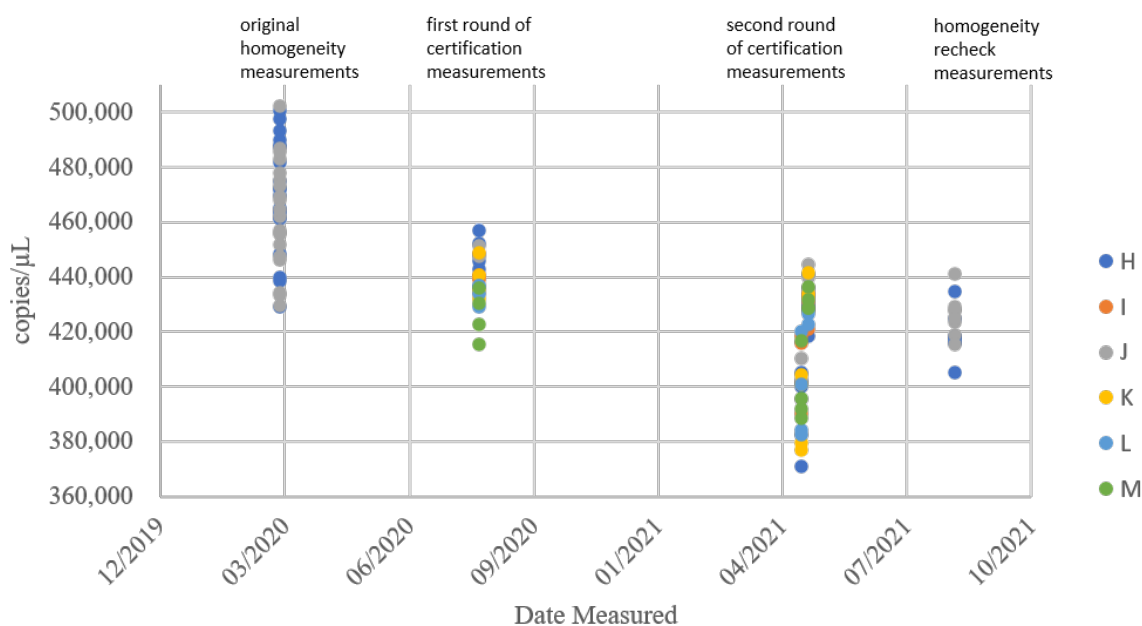
Figure 7. SRM 2367 Measurements over time.

Certification Results

Table 7 lists copy per µL values for all boxes and all assays. For the certification measurements, the average number of droplets was 18236 with standard deviation of 1965. The lowest droplet count was 12805 and the highest droplet count was 22835.

Table 7: Certification Data as Copy Number per µL

Box	Assay	Mean	Standard uncertainty	L95	U95
1	H	399961	11488		
1	I	401348	11465		
1	J	395368	11597		
1	K	379480	11602		
1	L	382647	11564		
1	M	391945	11395		
		390600	5460	382600	400900
8	H	371214	8290		
8	I	390034	8402		
8	J	382546	8441		
8	K	377213	8205		
8	L	384066	8418		
8	M	388618	8464		
		381700	4207	373300	389800
13	H	405338	8801		
13	I	415992	8786		
13	J	410420	8782		
13	K	400970	8773		
13	L	420267	8855		
13	M	416802	8787		
		410900	4291	402200	419200
20	H	401701	6224		
20	I	418271	6422		
20	J	402717	6250		
20	K	404341	6308		
20	L	400926	6244		
20	M	395669	6188		
		403500	3072	397500	409300

3	H	435476	7053		
3	I	436619	7102		
3	J	445720	7145		
3	K	434151	7044		
3	L	431669	7091		
3	M	429751	6997		
		435100	3202	428700	441400
10	H	443262	6348		
10	I	440734	6335		
10	J	446364	6411		
10	K	441108	6386		
10	L	430720	6244		
10	M	429562	6220		
		438100	3344	431500	444300
15	H	436102	6813		
15	I	430940	6850		
15	J	435126	6812		
15	K	430283	6824		
15	L	428717	6862		
15	M	422149	6795		
		430100	3225	423800	436400
19	H	443549	6992		
19	I	435466	7009		
19	J	441697	6969		
19	K	441,478	6983		
19	L	429683	6949		
19	M	433936	6939		
		437200	3351	430400	443500

The final certified values and 95 % probability intervals for SRM 2367 are shown in Table 8.

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Table 8: Certified Values for SRM 2367

Analyte	Certified Value	95 % Probability Uncertainty Interval	Standard Uncertainty, $u(X)$	Effective Coefficient of Variation, $CV=100 \times u(X)/X$	Units
JCV DNA copy number	415600	378000 to 442000	21320	5.1 %	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 tubes of SRM 2367 solution have the same copy number content within measurement repeatability.

With high confidence, the copy number content of the SRM 2367 solution is thermally stable at 2 °C to 8 °C over an extended period of time as defined by the most current Certificate of Analysis. The solution should not be shipped or stored below 2 °C.

The use of SRM 2367 as a calibrant for qPCR standard curves in measuring JCV 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 2367 JCV DNA Quantitative Standard, please visit: https://www-s.nist.gov/srmors/view_detail.cfm?srm=2367.

Acknowledgements

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Appendix A: Cleaning of perfluoroalkoxy fluoropolymer (PFA) containers

Cleaning procedure:
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 min.
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 min.
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 min.
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

Extracted from the Report of 3/25/2020
Natalia Farkas

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:

	Protocol	Average volume and expanded uncertainty
S1	DNA, 'UNG' MM	0.7663 nL ± 2.3 % (k=2)
S2	no DNA, 'UNG' MM	0.7686 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. Note that the diameter values are not corrected for the volume of oil phase remaining trapped at the vertices and edges of the Kelvin cells.

Sufficient statistics were assured by preparing and measuring the S1 and S2 samples on two 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 day-to-day variation. The expanded uncertainty of the volume measurements includes additional uncertainty components per Special Test Method 11050S/-D.

Table B-1. Average Volumes from the Concentrated Method

S1_3/11/2020	Average volume (nL)	St dev (nL)	Sample average (nL)	St dev (nL)	Protocol average (nL)	St dev (nL)
Measurement 1	0.7698	0.0058				
Measurement 2	0.7624	0.0112	0.7621	0.0078		
Measurement 3	0.7542	0.0055				
S1_3/13/2020	Average volume (nL)	St dev (nL)	Sample average (nL)	St dev (nL)	0.7663	0.0060
Measurement 1	0.7654	0.0043				
Measurement 2	0.7778	0.0050	0.7706	0.0065		
Measurement 3	0.7684	0.0052				
S2_3/11/2020	Average volume (nL)	St dev (nL)	Sample average (nL)	St dev (nL)	Protocol average (nL)	St dev (nL)
Measurement 1	0.7669	0.0061				
Measurement 2	0.7607	0.0079		0.0038	0.7686	
Measurement 3	0.7676	0.0052	0.7651			0.0050
S2_3/13/2020	Average volume (nL)	St dev (nL)	Sample average (nL)	St dev (nL)		
Measurement 1	0.7718	0.0081				
Measurement 2	0.7721	0.0081	0.77	0.0005		
Measurement 3	0.7728	0.0050				

Table B-2. Average Diameters from the Concentrated Method

S1_3/11/2020	Average diameter* (µm)	St dev (µm)	Sample average (µm)	St dev (µm)	Protocol average (µm)	St dev (µm)
Measurement 1	114.47	0.28				
Measurement 2	114.10	0.55	114.09	0.39		
Measurement 3	113.69	0.28				
S1_3/13/2020	Average diameter* (µm)	St dev (µm)	Sample average (µm)	St dev (µm)	114.30	0.30
Measurement 1	114.26	0.22				
Measurement 2	114.87	0.24	114.51	0.32		
Measurement 3	114.41	0.26				
S2_3/11/2020	Average diameter* (µm)	St dev (µm)	Sample average (µm)	St dev (µm)	Protocol average (µm)	St dev (µm)
Measurement 1	114.33	0.30				
Measurement 2	114.01	0.39	114.24	0.19		
Measurement 3	114.37	0.25				
S2_3/13/2020	Average diameter* (µm)	St dev (µm)	Sample average (µm)	St dev (µm)	114.41	0.25
Measurement 1	114.57	0.40				
Measurement 2	114.59	0.40	114.59	0.02		
Measurement 3	114.62	0.25				

uncorrected for the volume of the remaining oil phase

Appendix C: SRM 2367 Statistician's report

Extracted from the Report of 9/1/2021
Blaza Toman
SRM 2367 - JC Virus DNA Quantitative Standard Statistician's Report

1. Homogeneity study

Samples of the material were drawn from 20 different boxes, one tube per box. Two assays (H and J) were used to obtain three replicates of the measurements (lambda) for each sample. The following table shows the data.

	Assay "J"				Assay "H"		
	Rep1	Rep2	Rep3		Rep1	Rep2	Rep3
Box01	349.1295	354.4717	359.2685		364.5788	377.8256	349.1032
Box02	362.0424	371.2128	359.7888		393.6795	375.7021	381.075
Box03	350.2766	346.0322	342.9355		345.9935	368.6237	346.4293
Box04	385.7851	349.4573	352.6491		376.5487	372.1759	395.4086
Box05	384.8387	370.7363	355.2135		361.6004	354.6945	363.8166
Box06	347.1711	349.4562	353.986		358.474	374.3027	375.314
Box07	336.4859	330.5932	320.9569		356.1029	349.4531	363.5872
Box08	337.2496	359.673	350.6933		354.7846	356.1255	354.6148
Box09	356.8952	346.5926	325.8766		341.2485	343.5815	326.4674
Box10	351.4707	322.4876	324.3888		349.2982	362.9974	347.8946
Box11	362.2322	358.8326	354.6539		378.3131	360.7377	381.8834
Box12	366.8823	365.6959	346.1353		358.8257	360.7072	347.9814
Box13	330.3812	324.8769	341.3969		335.8051	345.0679	350.1978
Box14	364.4387	363.9205	339.8892		353.4579	365.2034	366.885
Box15	365.6896	377.6794	355.6287		367.4768	376.6364	377.5062
Box16	357.2811	369.0953	392.7339		379.6007	391.5867	362.6585
Box17	349.3687	326.3556	349.9187		344.9459	331.0708	332.6286
Box18	388.6083	378.7071	387.6163		365.3968	356.274	363.2676
Box19	390.2644	345.7329	380.8124		365.9552	387.73	372.6078
Box20	344.5386	359.7239	344.0785		327.4546	336.1188	323.558

Table C-1. Lambda homogeneity data.

Homogeneity was assessed using a Gaussian hierarchical model with two levels (assay and box):
$y_{ijk} \sim N(\gamma_{ij}, \sigma^2)$, i denotes box ($i = 1, ..., 20$), j denotes assay (1 ,…, 2 in the order as above),
$\gamma_{ij} \sim N(\alpha_i, \sigma_i^2)$,
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
σ^2 is the within box variance

The model parameters were analyzed using Bayesian MCMC [2] with noninformative priors using the OpenBUGS [3] code given in Appendix 1. The posterior means with standard uncertainties are given in Tables C-2 and C-3.

Box	Posterior mean	Posterior Standard deviation
1	355	5.8
2	362	5.9
3	348	5.9
4	361	5.8
5	367	5.9
6	351	5.9
7	334	6.1
8	350	5.8
9	345	5.9
10	337	6.1
11	358	5.9
12	359	5.8
13	337	6.0
14	356	5.8
15	364	5.9
16	370	5.9
17	344	5.9
18	379	6.1
19	369	6.0
20	350	5.7

Table C-2. Means and standard uncertainty for assay J.

The between box uncertainty is about 3.9 %, and the average repeatability uncertainty within a box is 3.3 %.

For assay H the following results were obtained:

Box	Posterior mean	Posterior standard deviation
1	363	5.9
2	379	6.1
3	355	5.9
4	378	6.1
5	360	5.9
6	368	5.9
7	357	5.9
8	356	5.8
9	341	6.1
10	355	5.9
11	371	5.9
12	357	5.9
13	348	6.0
14	361	5.9
15	371	5.9
16	375	6.0
17	341	6.1
18	361	5.9
19	373	6.0
20	335	6.3

Table C-3. Means and standard uncertainty for assay H.

The between box uncertainty for assay H is about 4.0 %, the average repeatability uncertainty within a box is 3.2 %. The following plots do not show any obvious outliers and illustrate the random heterogeneity between boxes.
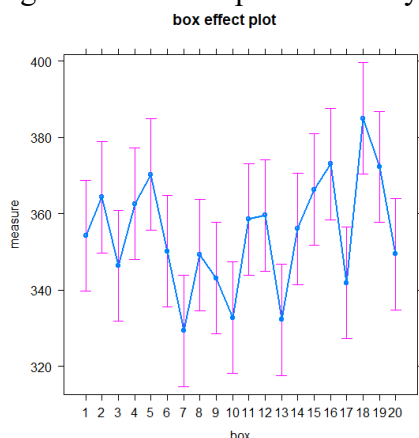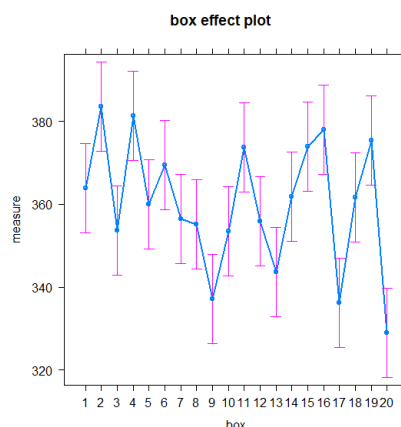
Figure C-1. Effect plots for assay J

Figure C-2. Effect plots for assay H

The random heterogeneity between the boxes will be accounted in the uncertainty of the certified values.

2. Certification
    2.1 The Data

Six different assays were used to obtain triplicate measurements from eight different boxes. The following table gives the results for lambda:

Box/Date:	Box3_08/13/20			Box10_08/13/20		
Assay:	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
H	354.9472	336.6405	348.312	339.1136	348.1473	337.5247
I	336.4969	328.9554	343.4656	351.8625	344.8092	334.729
J	341.9943	341.5115	354.1013	341.0374	338.9895	350.3296
K	335.1161	328.4124	338.088	333.3671	339.9001	339.4864
L	345.8318	324.9223	333.7285	339.5527	327.2739	330.2856
M	324.9493	323.3636	341.4774	316.7032	330.1028	325.5822

Box/Date:	Box15_08/13/20			Box19_08/13/20			Box1_04/29/21		
Assay:	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
H	332.0271	344.1375	342.2535	348.4821	343.8411	358.5932	301.171	309.2206	309.1542
I	337.7726	339.4304	336.3463	336.3253	337.3941	338.0111	289.6263	316.935	316.1174
J	335.4242	326.821	336.1443	348.1585	338.1762	342.9365	291.2945	311.4132	306.3519
K	326.1804	330.2845	337.0502	346.2018	333.8343	351.8073	278.956	282.7611	311.0352
L	323.9246	330.1804	332.8198	344.0356	334.6192	325.8419	289.9581	281.0599	308.9315
M	316.9753	314.6138	323.6987	336.2774	331.1128	334.9669	280.4934	307.2132	313.5646

Box/Date:	Box8_04/29/21			Box13_04/29/21			Box20_04/29/21		
Assay:	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
H	274.3804	277.4422	301.2357	315.7804	305.6069	310.5423	304.792	308.6921	310.1549
I	301.5821	299.7371	295.4989	324.2273	321.3827	311.023	316.4233	320.9631	324.2259
J	302.5376	288.5898	288.3196	313.4586	319.1218	310.9242	307.9575	312.1718	305.617
K	296.4404	286.1794	284.2149	319.2295	294.4628	308.5034	301.6426	316.9056	311.0486
L	297.5123	285.3576	300.3155	337.9842	319.7537	308.689	308.253	298.87	314.607
M	304.2301	284.5045	304.9226	325.8493	324.6264	307.8969	299.5428	301.1868	308.9492

Box/Date:	Box3_05/05/21			Box10_05/05/21			Box15_05/05/21		
Assay:	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
H	317.8743	326.6905	317.5936	335.8776	336.0723	341.4721	334.3194	329.1348	323.4492
I	332.6403	334.3196	331.761	328.8247	334.9044	331.3552	306.3285	322.9323	338.8292
J	337.6966	326.6556	347.2875	334.5019	341.6795	345.649	345.8092	338.964	317.4961
K	326.8879	328.6034	338.945	339.7496	331.5326	344.2568	337.0454	323.8995	324.0893
L	329.1115	333.7884	317.4376	316.6961	340.4217	326.1109	332.0738	334.6791	317.8131
M	333.2989	329.2273	323.6055	339.0656	336.5543	327.4276	334.1554	329.5435	322.3234

Box/Date:	Box19_05/05/21		
Assay:	Rep1	Rep2	Rep3
H	330.7697	322.3232	335.7336
I	327.3817	341.573	321.6225
J	337.275	332.8643	331.7896
K	325.9714	340.9066	330.7345
L	333.5462	322.0718	315.863
M	335.919	330.9443	326.1038

Table C-4. Lambda measurements for the certification runs

2.2 Assay means (in copies/µL) per box.

Because the certified values were to be given in copies/µL, not in lambda, the measurements in Table 4 had to be converted using droplet volume. According to [1], the average droplet volume for DNA, 'UNG' MM is 0.7663 nL with standard relative uncertainty of 1.15 %. The following statistical model was used to produce the certified values.
The response was represented as

$y_{ijk} \sim N(\gamma_{ij}, \sigma^2),$ i denotes box ($i = 1, ..., 8$),

         j denotes assay (1 ,…, 6 in the order as above),

         k denotes replicate (1 ,…, 3),

$\gamma_{ij} \sim N(\alpha_i, \sigma_i^2),$ γ_{ij} is the mean lambda value of box i, assay j

$V \sim dnorm(0.7663, 0.0088^2),$ represents droplet volume of master mix DNA, 'UNG' MM,

$$\mu_{adj} = \mu/V,$$ represents the measurand; mean $copies/\mu L$.

The variance components were $\sigma_i^2, i = 1, \ldots, 8$ which measured the between assay variability within box i, and σ^2, the repeatability variability of the eight boxes.

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 was implemented in OpenBUGS [3] and the code is given in Appendix 2. The results are given in Table 5 together with the box consensus means obtained using the NIST Consensus Builder (https://consensus.nist.gov/app/nicob), the Hierarchical Bayes (Gaussian) method [4]. The uncertainty of the consensus value includes the between assay uncertainty.

Box	Assay	Mean	Standard uncertainty	L95	U95
1	H	399961	11488		
1	I	401348	11465		
1	J	395368	11597		
1	K	379480	11602		
1	L	382647	11564		
1	M	391945	11395		
		390600	5460	382600	400900
8	H	371214	8290		
8	I	390034	8402		
8	J	382546	8441		
8	K	377213	8205		
8	L	384066	8418		
8	M	388618	8464		
		381700	4207	373300	389800
13	H	405338	8801		
13	I	415992	8786		
13	J	410420	8782		
13	K	400970	8773		
13	L	420267	8855		
13	M	416802	8787		
		410900	4291	402200	419200
20	H	401701	6224		
20	I	418271	6422		
20	J	402717	6250		
20	K	404341	6308		
20	L	400926	6244		
20	M	395669	6188		
		403500	3072	397500	409300
3	H	435476	7053		

3	I	436619	7102		
3	J	445720	7145		
3	K	434151	7044		
3	L	431669	7091		
3	M	429751	6997		
		435100	3202	428700	441400
10	H	443262	6348		
10	I	440734	6335		
10	J	446364	6411		
10	K	441108	6386		
10	L	430720	6244		
10	M	429562	6220		
		438100	3344	431500	444300
15	H	436102	6813		
15	I	430940	6850		
15	J	435126	6812		
15	K	430283	6824		
15	L	428717	6862		
15	M	422149	6795		
		430100	3225	423800	436400
19	H	443549	6992		
19	I	435466	7009		
19	J	441697	6969		
19	K	441478	6983		
19	L	429683	6949		
19	M	433936	6939		
		437200	3351	430400	443500

Table C-5. Assay means per box and consensus in copies/µL

The following plots (from the NICOB Hierarchical Bayes procedure) show the consensus mean with bands of ± 1 standard uncertainty, and the individual assay means with ±1 standard error uncertainty bands, for each box.
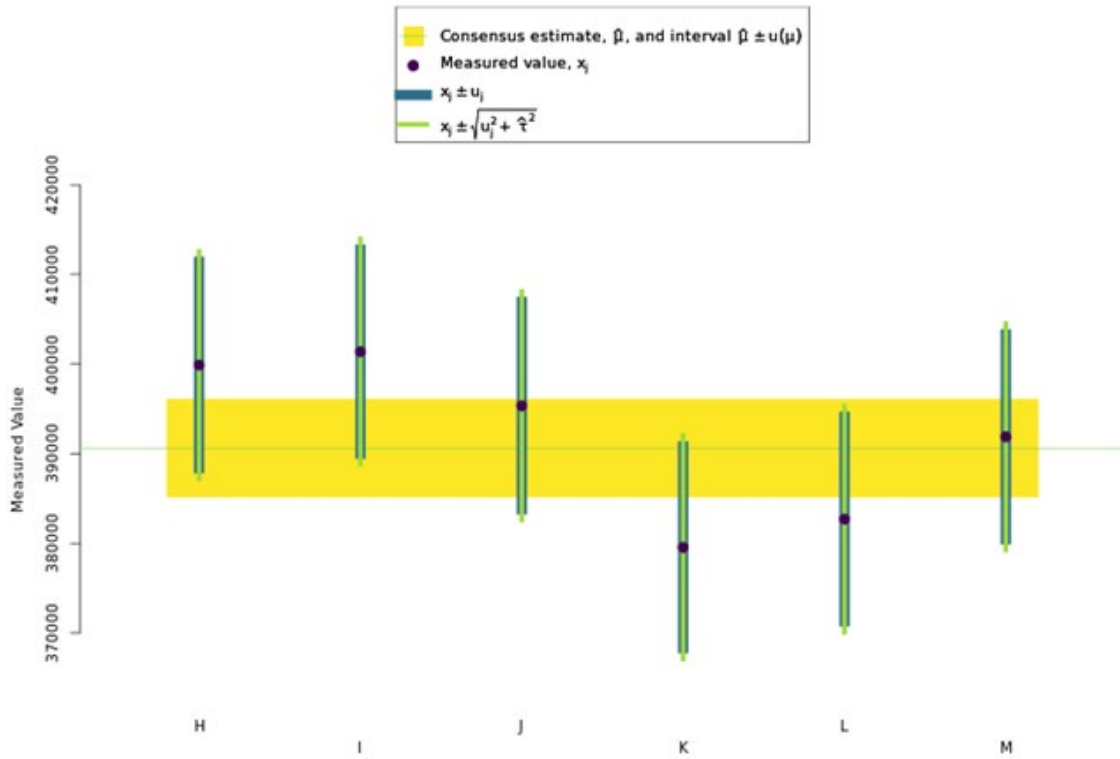
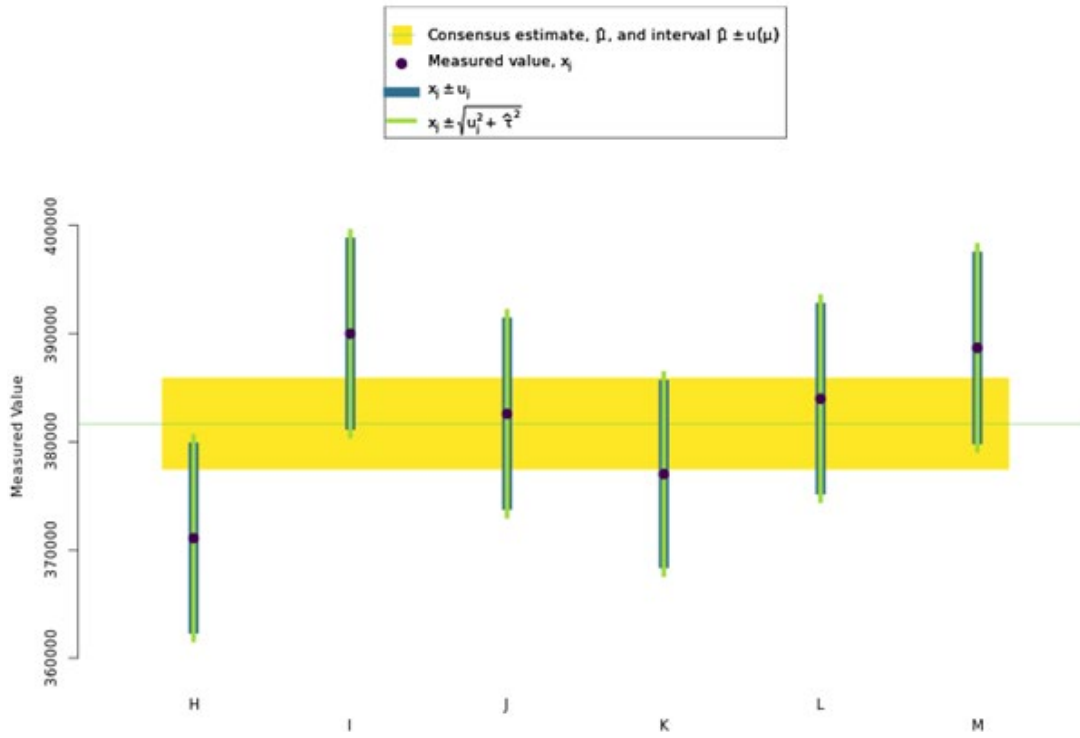Figure C-3. Consensus and individual assay means for Box 1.

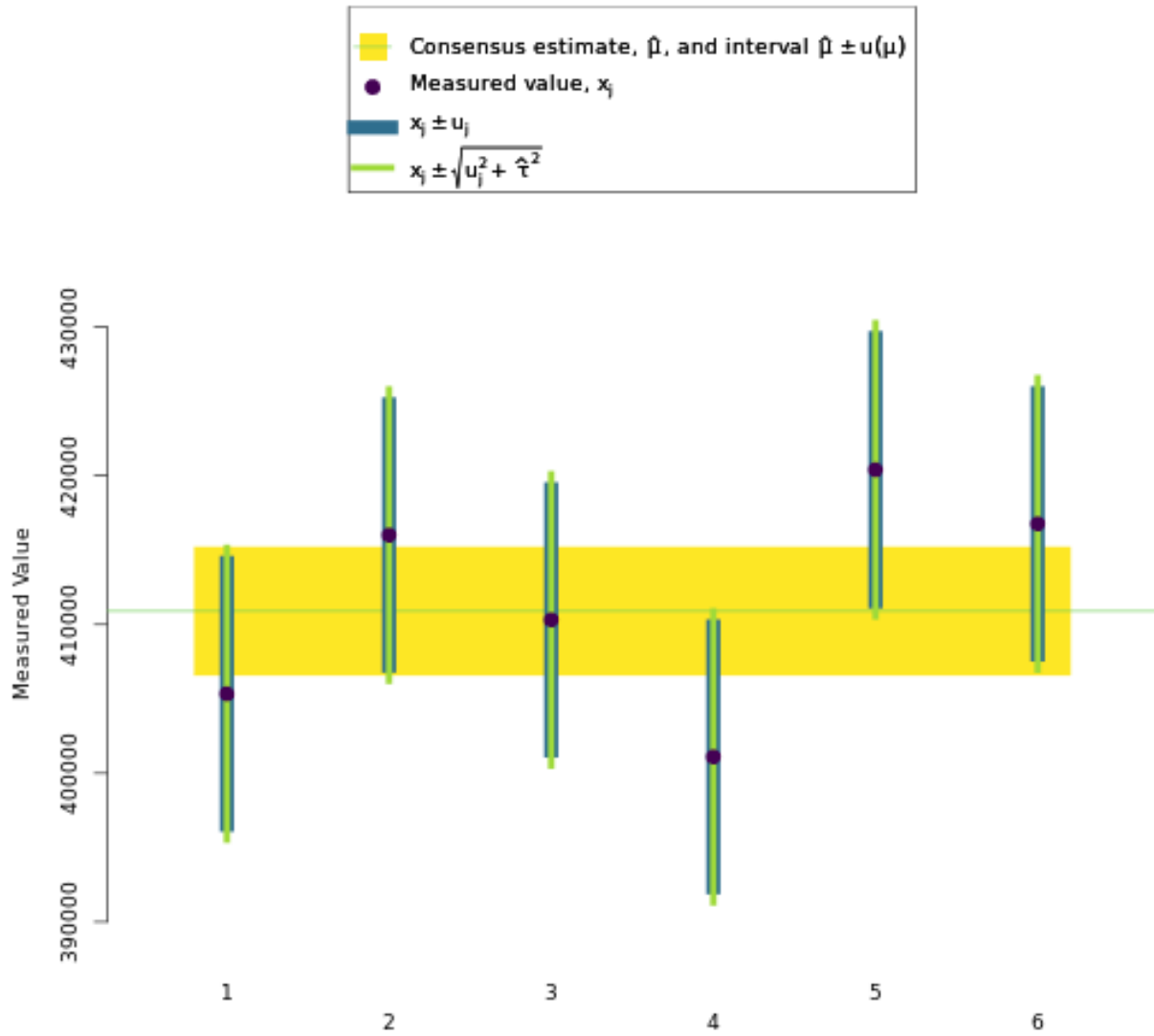Figure C-4. Consensus and individual assay means for Box 8.

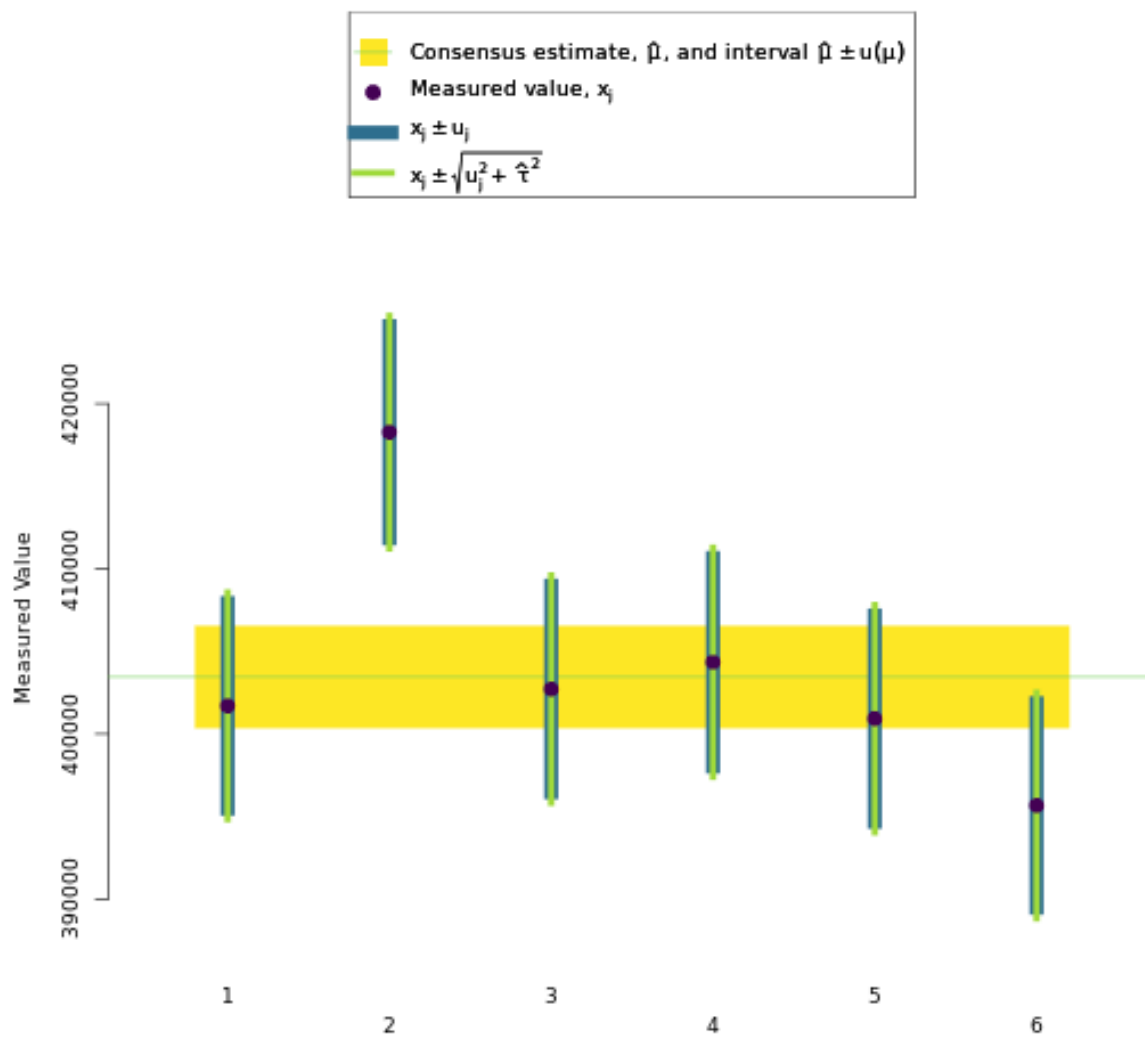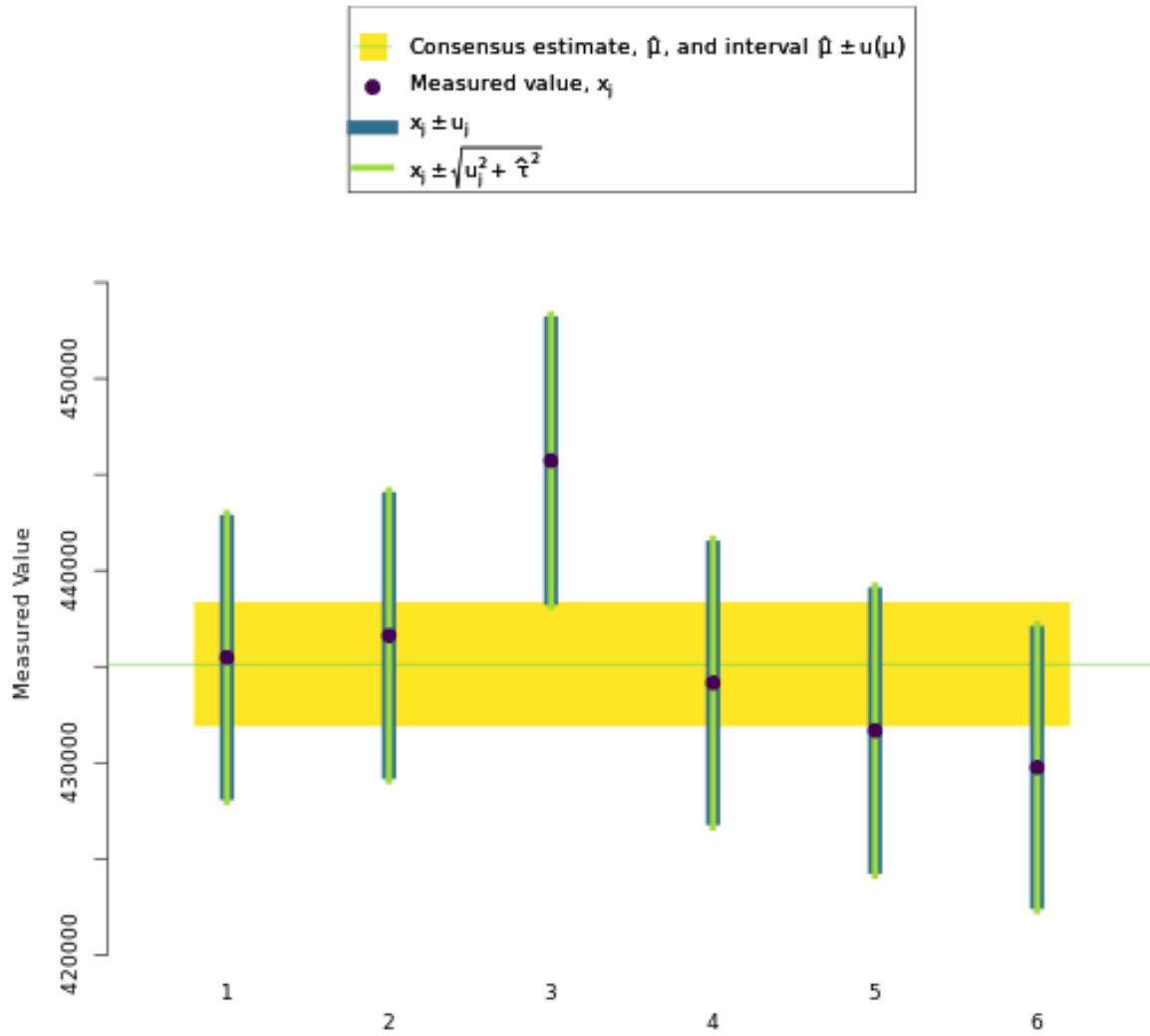Figure C-5. Consensus and individual assay means for Box 13.

Figure C-6. Consensus and individual assay means for Box 20.

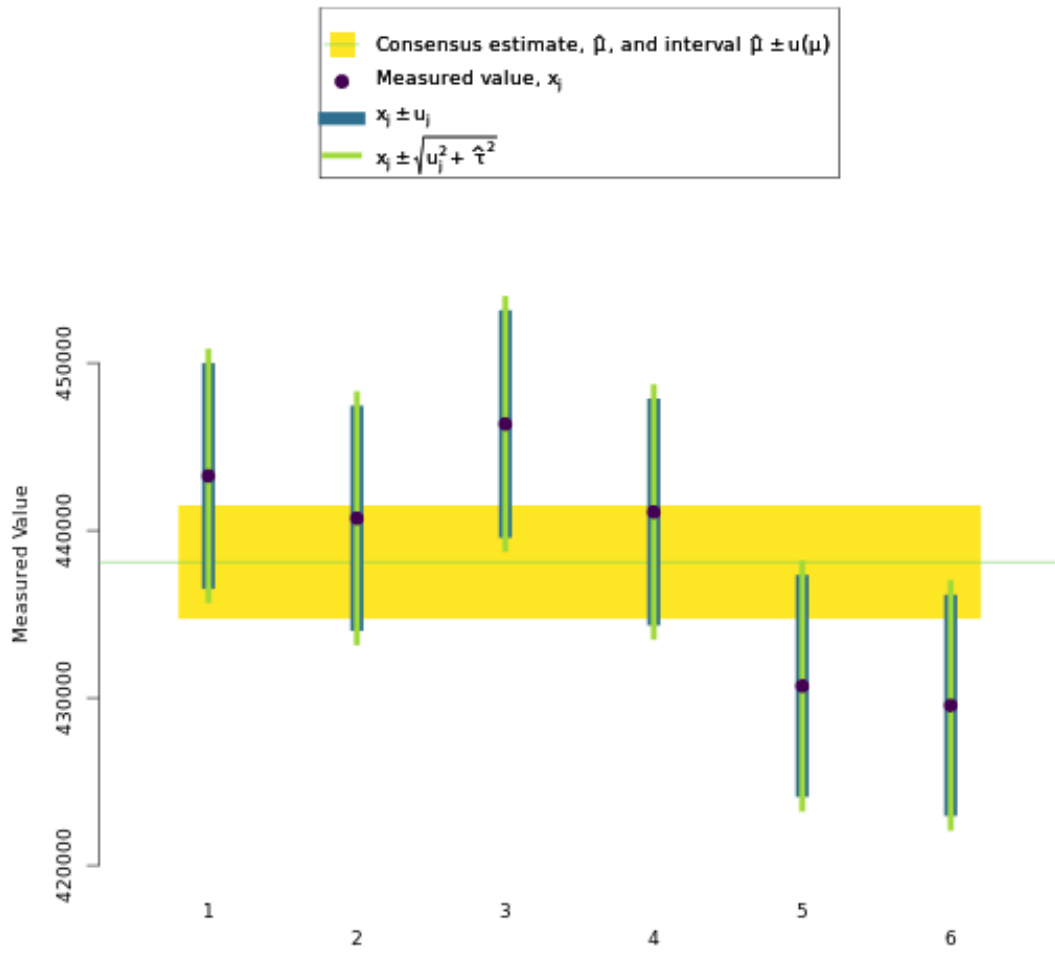Figure C-7. Consensus and individual assay means for Box 3.

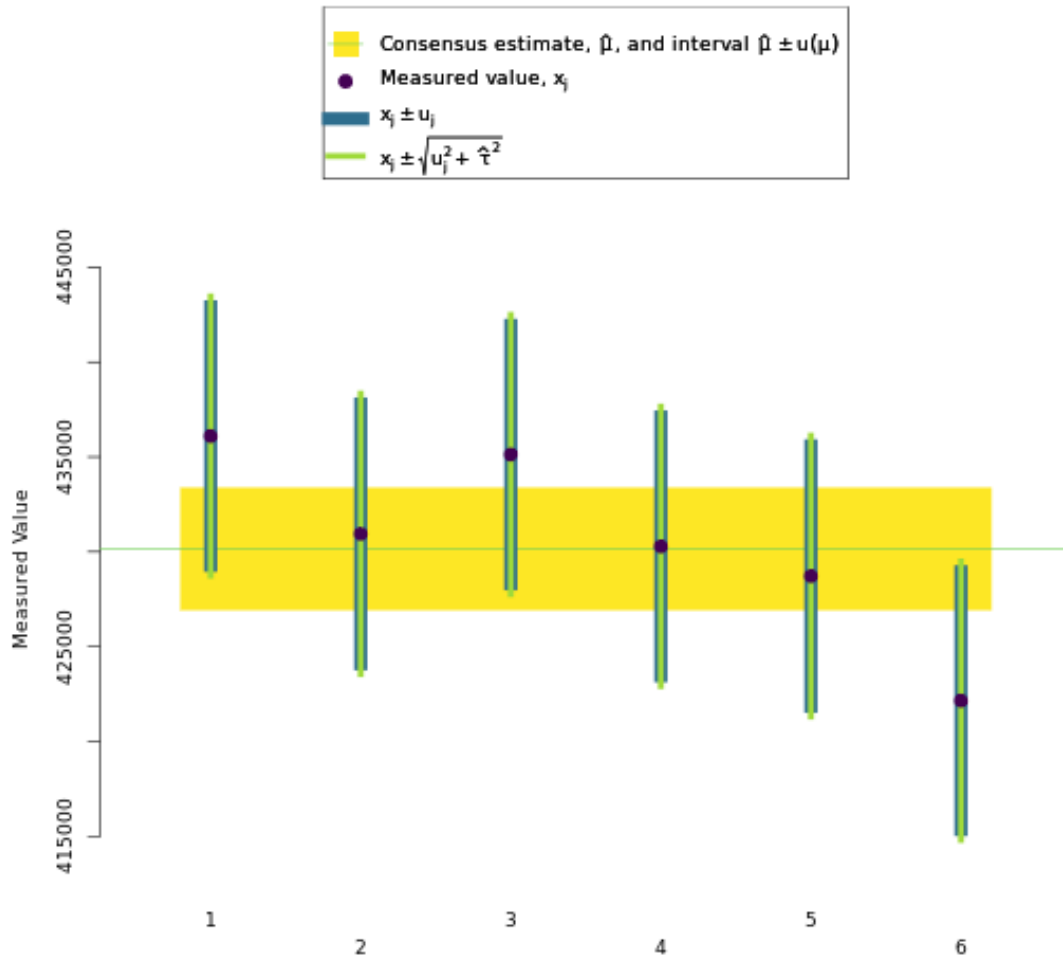Figure C-8. Consensus and individual assay means for Box 10.

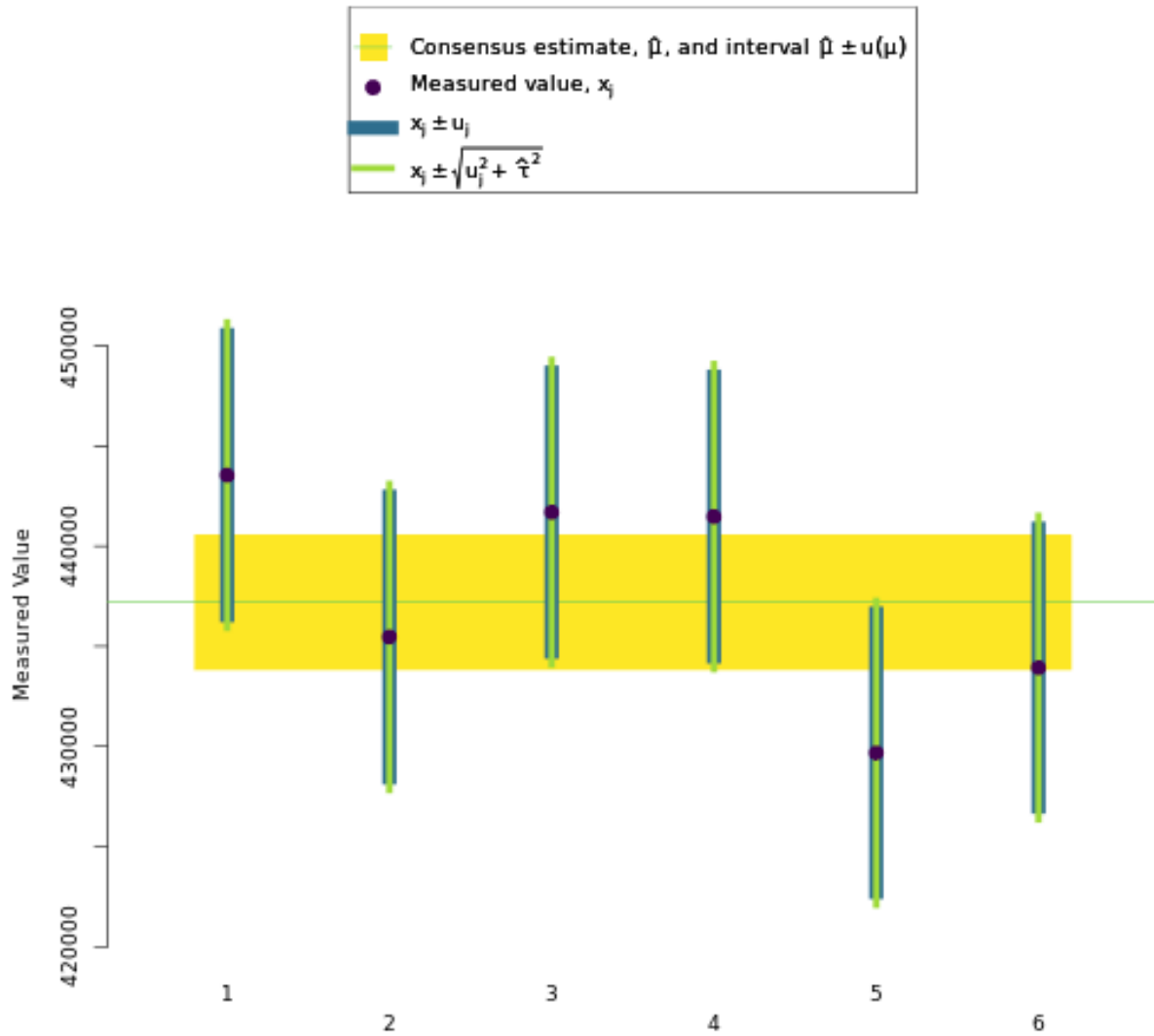Figure C-9. Consensus and individual assay means for Box 15.

Figure C-10. Consensus and individual assay means for Box 19.

2.3 The Certified value: combining across boxes:

Using the Linear pool method [4] of the NIST Consensus Builder to account for the between box variability, we obtain consensus mean of 415600 copies/µL, with standard uncertainty of 21320 copies/µL, and 95 % uncertainty band of 378000 copies/µL to 442000 copies/µL.

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OpenBUGS code for the homogeneity model

```
{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(k in 1:20){gamma[j,k]~dnorm(alpha[j],sigg[j])
pred[j,k]~dnorm(gamma[j,k],sig)}}
for(i in 1:119){measure[i]~dnorm(gamma[assay[i],box[i]],sig)
}

}
```

Initial values:
```
list(sig=1 ,sigg=c(1,1))
```

Appendix 2
OpenBUGS code for the certification data analysis.

```
{vol~dnorm(0.7663, 12877)
for( i in 1:8){
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(0,1.0E-6)
mup[i,j]<-gamma[i,j]/vol*1000}}
for(i in 1:216){
            meas[i]~dnorm(gamma[box[i],assay[i]],siga[box[i]])
            }

}
```