CURRENT AND FUTURE VIRAL DNA STANDARD REFERENCE MATERIALS: CYTOMEGALOVIRUS AS A MODEL

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Abstract

The first viral DNA Standard Reference Material® (SRM) produced at the National Institute of Standards and Technology (NIST) "SRM 2366 Cytomegalovirus for DNA Measurements" was released in 2011. The work preceding the release helped develop the model for future viral DNA SRMs; particularly, material production, packaging, and certification. The material that later became SRM 2366 was acquired as the entire Towne strain of CMV in a bacterial artificial chromosome (BAC) in *Escherichia coli*. At NIST the BAC was propagated and the DNA was isolated and purified. CMV DNA in buffer was packaged in perfluoroalkoxy fluoropolymer (PFA) (e.g. Teflon) tubes to minimize absorption of DNA onto tube walls. Digital PCR (dPCR) was used to certify the concentration of DNA. The use of dPCR allows the direct counting of molecules, so quantification can be achieved without the use of calibrants. In the past the number of replicate PCR reactions needed limited the accuracy and precision of digital PCR. However, new microfluidic technology makes the technique viable for measuring the concentration of DNA with reasonable measurement uncertainty. In addition to quantification, the DNA sequence of relevant regions of the CMV genome was verified by Sanger sequencing.

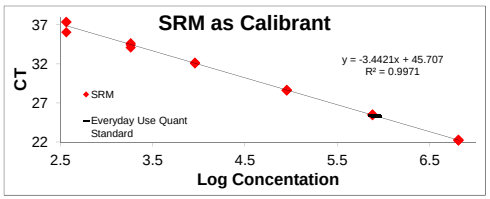
The second viral DNA SRM is currently in development for BK virus. This SRM will also be DNA in buffer, packaged in PFA, sequenced, and certified for concentration with digital PCR.

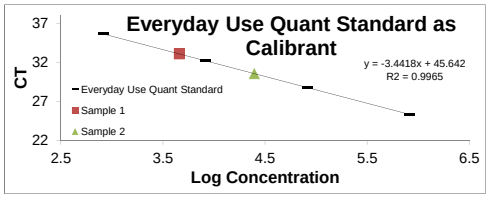
Certification of SRM 2366

- ***Materials***: DNA from Towne strain in a bacterial artificial chromosome (BAC) containing all of the viral genome except for regions IRS1, US1-15, and UL147; viral DNA to be provided in a buffer for dilution into a user's matrix of choice. BAC provided by Dr. Hua Zhu at the University of Medicine and Dentistry of New Jersey
- ***Certification***:
  - Concentration determined via digital PCR
- ***Information Values***:
  - DNA sequence of genes that are targets for qPCR
    - Covering published qPCR primers and commercial CMV detection assays

How to Use SRM 2366 to calibrate everyday-use-quant-standard

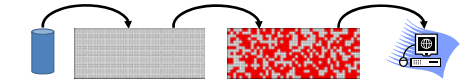
1. Make dilution of SRM and everyday-use-quant-standard
2. Optional: Process if necessary (e.g. if your everyday-use-quant-standard needs extraction, then extract both SRM and everyday-use-quant-standard)
3. Quant via qPCR, treating the SRM as the true value
4. Assign value to everyday-use-quant-standard (Note: this value is now traceable to a NIST Standard)
5. Use everyday-use-quant-standard with NIST-traceable value to quant patient samples





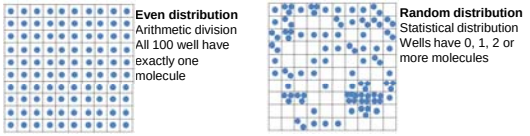
Digital PCR (dPCR) to determine Concentration of DNA

1. Create a PCR master mix as in qPCR with template DNA (some manufacturer specific reagents may be required)
2. Aliquot across hundreds or thousands of wells; either using microfluidics or microemulsions
3. Thermal cycle as in qPCR and count wells with amplification
4. Use Poisson statistics to determine the concentration of starting material



Random vs. Even distribution

- dPCR and Poisson statistics rely on random distribution
- Consider distributing 100 molecules over 100 wells



Poisson Statistics

1. Poisson equation for estimating total number of copies across all wells $N\lambda = N \ln(N/N-x)$; where λ = average copies per well, x = number of positive wells, and N = number of wells [9].
2. Divide estimated number of copies across all wells ($N\lambda$) by the total volume aliquoted; $N\lambda$/volume(µL) = copies/µL
3. Correct for dilution of DNA into mastermix and any other dilutions

Concentration of SRM Components

- Three components (A, B, & C) were certified for concentration using digital PCR.
- One TaqMan assay was used for the certification
- Six targets spread across the genome – three of which were overlapping varying by size of amplicon – were tested to verify concentration determination would not be skewed by any one target location or size of amplicon

Component	A	B	C
Value copies/µL	420	1702	19641
Standard uncertainty copies/µL	56	130	365
Relative uncertainty	13.30%	7.60%	1.80%
95 % confidence interval copies/µL	301 to 523	1446 to 1959	18924 to 20359

Sequencing

- Sanger sequence via Big Dye Terminator v3.1 (Applied Biosystems)
- Analyze by capillary electrophoresis using Applied Biosystems 3130xl with POP7 and an 80 cm capillary
- Towne strain sequence GenBank AY315197.2 used as reference
- Only sequences with multiple independent reads were reported in the certificate
- Two independent analysts confirmed sequence

Sequenced Regions

- Nucleotide numbering is based on the Towne sequence Genbank AY315197.2
- There is complete agreement between the NIST sequence and the Genbank AY315197.2 with one exception noted below
- Targets for commercial reagents: UL34, UL80, UL132
- Targets for published assays: UL54, UL55-56, UL83, UL122-126, US17
- Mutations in UL97 confer resistance to ganciclovir

Reference Sequences	Nucleotide range	# bases
UL34	43202 to 44971	1770
UL54 (except at 78651)*	77695 to 79992	2298
UL55 to 56	80848 to 82731	1884
UL80	114401 to 116793	2393
UL83	118890 to 119937	1048
UL97	140784 to 142090	1307
UL122 to 126	170525 to 173182	2658
UL132	176380 to 177192	813
US17	198929 to 199312	384

* The sequence at position 78651 is described as a Y (C/T) at NIST and a C in Genbank

BK Virus

- Polyomavirus closely related to JC virus and simian virus 40
- Genome is circular dsDNA approximately 5 kb
- Six common subtypes 1a, 1c, 3, 4, 5, & 6
- The Plan:
  1. Ideally the SRM would contain a panel of virus subtypes
  2. Sequence of all components would be given
  3. One concentration per subtype
  4. Concentrations would aim high so dilutions can be made to calibrate everyday use quantitative standard (see "How to Use SRM 2366 to calibrate everyday-use-quant-standard").
  5. Concentrations would be certified by dPCR

Additional Viruses Under Consideration

- Epstein Barr virus
- Adenovirus

Packaging

- Perfluoroalkoxy fluoropolymer (PFA) (e.g. Teflon) screw cap vials (0.5 mL) were used to package SRM 2366.
- PFA was chosen to minimize the absorption of DNA to the walls of the tube, which could change the concentration of the liquid.

Material Acquisition

- Obtaining material to create a SRM is a difficult part of the process
- If you have access to material and would be willing to sign a Material Transfer Agreement, please contact Ross Haynes Ross.Haynes@nist.gov 301-975-4469
- Any collaboration – intellectual or material – is welcome to help facilitate the best possible clinical SRMs

Clinical DNA Informational Resource

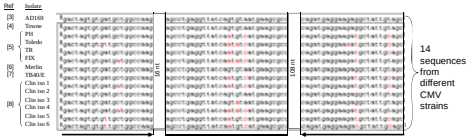
http://www.nist.gov/mml/biochemical/genetics/clinical_dna.cfm

Clinical DNA Informational Resource contains:

- Recent publications and presentations – including this one
- Information on DNA-based Standard Reference Materials (SRMs) and candidate SRMs
- Information to aid scientists in their research

Sequence alignments

The qPCR assay below [2] has a large number of mismatches that cause a false negative rate of 24%. A little more time in the library, probably would have lead this researcher to redesign the primer and probe binding sites.



Published Assays

- 73 unique qPCR assays have been collected, the primers and probes have been documented and put into one spreadsheet
- Errors have been corrected and traced to:
  1. Transcribing from paper to paper and
  2. Changes in GenBank sequences
- Each assay has a key to which strains of CMV [3] – [7] match that assay, which is a rough indicator of the sensitivity of the assay

References:
[1] Hemmerich 1998 www.deviselink.com/mpb/archive/98/07/002.html
[2] Tanaka 2000 Journal of Medical Virology 60:455–462
[3] X17403 "AD169" Chee et al. Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. Curr. Top. Microbiol. Immunol. 154, 125-169 (1990). Sequence revised Nov 14, 2006
[4] AY315197 "Towne" Dunn et al. Functional profiling of a human cytomegalovirus genome. Proc. Natl. Acad. Sci. U.S.A. 100 (24), 14223-14228 (2003). Sequence revised Jan 31, 2007
[5] AC146904 - AC146907 "PH-BAC", "Toledo BAC", "TR-BAC", "FIX-BAC" Murphy et al. Coding potential of laboratory and clinical strains of human cytomegalovirus. Proc. Natl. Acad. Sci. U.S.A. 100 (25), 14976-14981 (2003). Sequence added Dec 10, 2003
[6] NC_006273 Dolan et al. Genetic content of wild-type human cytomegalovirus. J. Gen. Virol. 85 (PT 5), 1301-1312 (2004). Sequence revised Aug 28, 2009
[7] EF999921 "TB40-BAC4" Sinzger et al. Cloning and sequencing of a highly productive, endotheliotropic virus strain derived from human cytomegalovirus TB40/E. J. Gen. Virol. 89 (PT 2), 359-368 (2008). Sequence added Jan 18, 2008
[8] M95634 - M95639 Chou. Effect of interstrain variation on diagnostic DNA amplification of the cytomegalovirus major immediate-early gene region. J. Clin. Microbiol. 30 (9), 2307-2310 (1992). Sequence added Aug 2, 1993
[9] Heyries et. al. Megapixel digital PCR. Nature Methods. 8 (8), 649-651 (2011).

Poster available for download from NIST Applied Genetics website: http://www.nist.gov/mml/biochemical/genetics/index.cfm