FUTURE STANDARD REFERENCE MATERIAL FOR BK VIRUS: ISSUES AND UPDATE

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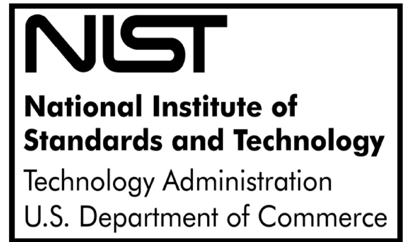

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Abstract

The National Institute of Standards and Technology (NIST) produced SRM 2366 Cytomegalovirus for DNA Measurements in 2011. SRM 2366 is composed of three concentrations of one bacterial artificial chromosome containing the entire Towne strain. Digital PCR was used to certify the DNA in buffer for concentration. About 17,000 bases of sequence were given as information value in the certificate so users could check for mutations under primer binding sites.

Currently NIST is working on a Standard Reference Material (SRM) for BK virus. This new SRM is currently slated to consist of three plasmids in buffer; two clinical strains cloned into plasmids and the Dunlop strain in pBKV(34-2) from American Type Culture Collection. BK virus DNA from genotypes 1a, 1c, 3, 4, 5, and 6 were obtained from University of Washington. Only genotypes 1a and 5 were successfully cloned into plasmids in E. coli. Cloning was only successful after omitting the non-coding regulatory region. There was an apparent E. coli toxicity preventing the entire BK virus genome to be cloned and which prevented four other subtypes to be successfully cloned.

Each plasmid in the SRM will be certified for concentration using two digital PCR systems using multiple qPCR assays spread across the genome. This will give greater confidence for results. Sequence information will be given for the entire BK virus genome in each plasmid. Whereas the CMV SRM was packaged in PFA (generic Teflon), to avoid a significant amount of DNA from binding to the tube walls, BK virus is known to have high virus concentrations; therefore, typical 0.5 mL tubes can be used without significant binding of DNA to tube walls.

Certification of Future BK Virus SRM

- ***Materials***: BK virus DNA was acquired from University of Washington and cloned at NIST into plasmid pACYC177
- Two clinical strains (1a and 5) were cloned and a plasmid containing the Dunlop strain was acquired from ATCC
- Viral DNA to be provided in a buffer at one high concentration

Certification:

- Concentration determined via digital PCR using two systems

Information Values:

- DNA sequence of BK virus portion of the plasmids

BK Virus

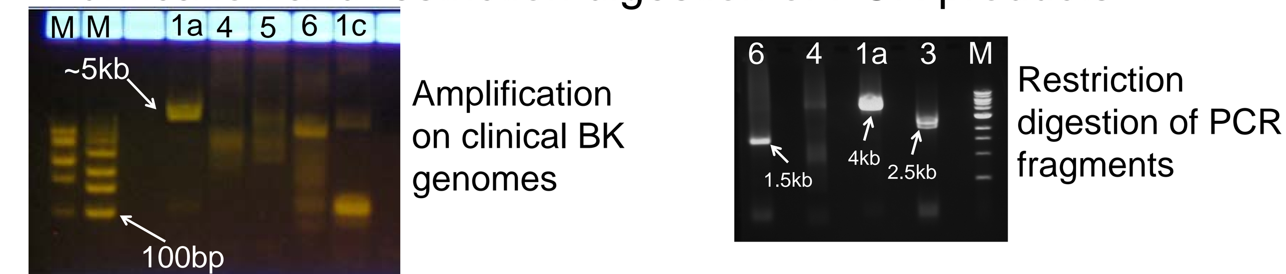
- Polyomavirus closely related to JC virus and simian-virus-40
- Genome is circular dsDNA approximately 5 kb
- Six common subtypes 1a, 1c, 3, 4, 5, & 6
- The Plan:
  1. Ideally the SRM would contain a panel of virus subtypes
  2. Sequence of all components would be given
  3. One concentration per subtype
  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

Packaging

- Two tube types were tested to determine if binding of DNA to the walls was a significant problem
- Sarstedt
- Perfluoroalkoxy fluoropolymer (PFA) (e.g. Teflon)
- Binding of DNA was not significant given the concentration used and Sarstedt tubes are being used
- SRM 2366 (CMV) was much less concentrated and PFA tubes were used

BK Cloning Challenges

1. Original plan was to design primers around the BamHI site and clone the entire BK genome – just as the Dunlop strain was cloned
   - Non-specific amplification
   - Clones only contained small insert
   - Purification and restriction digestion of PCR products



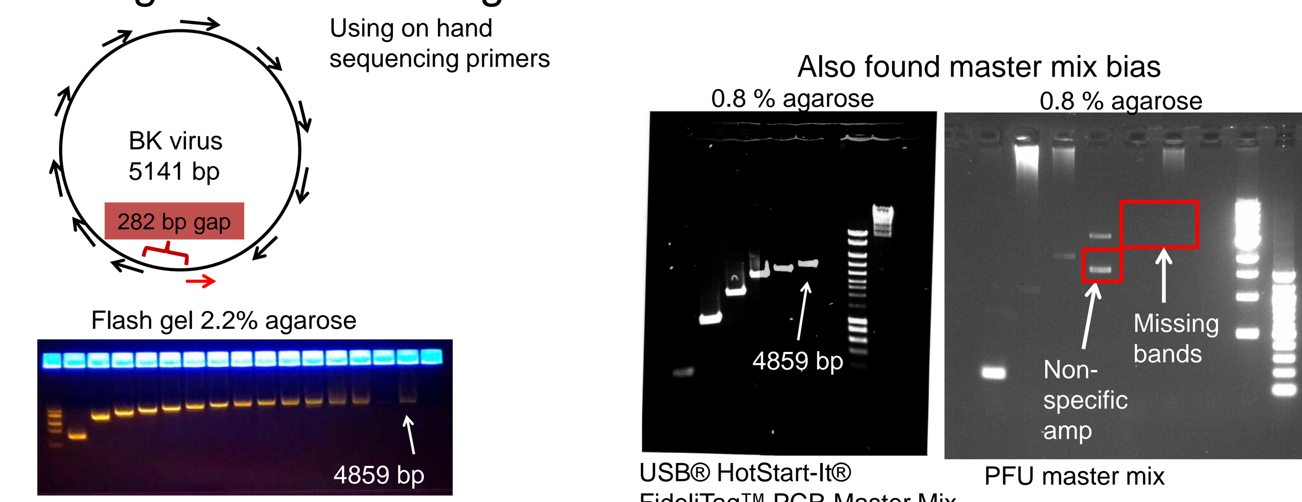
2. Rolling Circle Amplification, followed by restriction digestion
   - Restriction digestion failed to give a discrete band
   - Restriction enzymes
3. Designed primers around XhoI cut site
   - Nonspecific amplification
4. Sequenced clinical strains from U. of Washington to look for answers to our problems

Subtype	1a	1c	3	4	5	6
Ref GenBank #	V01108.1	AB211372.1	AB211386.1	AB211387.1	AB211370.1	AB211369.1
# of Diff.	103	5	68	48	55	13
SNPs	75	5	53	36	16	13
Insertions	8	0	6	12	39	0
Deletions	20	0	9	0	0	0
Largest In/Del	5	0	9	9	39	0
Bases in ref	5153	5142	5132	5142	5209	5141
Bases seq.	5161	4238	4750	5115	5110	5008
Gaps	0	904	382	27	99	133
% Difference	2.0%	0.1%	1.4%	0.9%	1.1%	0.3%

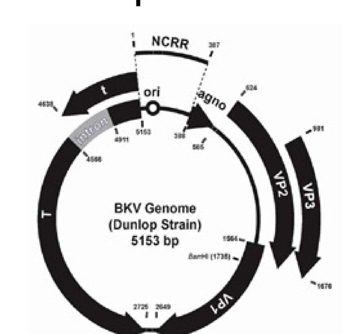
*Ref genomes from Hoffman J Clin Microbiol. 2008 Aug;46(8):2671-80. Dunlop is ref for 1a.

- Each genotype used a different reference
- Nonspecific primer binding sites were identified to explain some of the amplification issues

5. Fragmentation testing



6. Cloned individual genes – without problem
7. Removed Non-Coding Regulatory Region
   - Successfully cloned 1a and 5
8. Acquired Dunlop plasmid (pBKV 34-2) from ATCC



NCRR tends to mutate depending on the organ infected
Most quantitative PCR assays avoided the NCRR for that reason

3 genotypes for future BK virus SRM

- Two clinical genotypes (1a &5) from U. of Washington cloned into plasmids (sans NCRR)
- Dulop plasmid (pBKV 34-2) from ATCC

Current Cytomegalovirus SRM 2366

	SRM 2366 (CMV)	Future BK SRM
# components	3	3
Component makeup	dilutions of one genotype	3 genotypes (2 clinical, Dunlop)
Genome size	240 kb	5 kb
Sequenced	Regions of Interest (17 kb)	BK portions (5 kb x 3)
Concentration range	19,000 to 400 c/μL	~1,000,000 c/μL
Tube types	PFA	Sarstedt

Concentration of SRM 2366 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	1,702	19,641
Standard uncertainty copies/μL	56	130	365
Relative uncertainty	13.30%	7.60%	1.80%
95 % confidence interval copies/μL	301 to 523	1,446 to 1,959	18,924 to 20,359

1. Haynes RJ. "Standard reference material 2366 for measurement of human cytomegalovirus DNA." J Mol Diagn. 2013 Mar;15(2):177-85.
2. https://www-s.nist.gov/srmors/view_detail.cfm?srm=2366

Sequenced Regions of SRM 2366

- 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

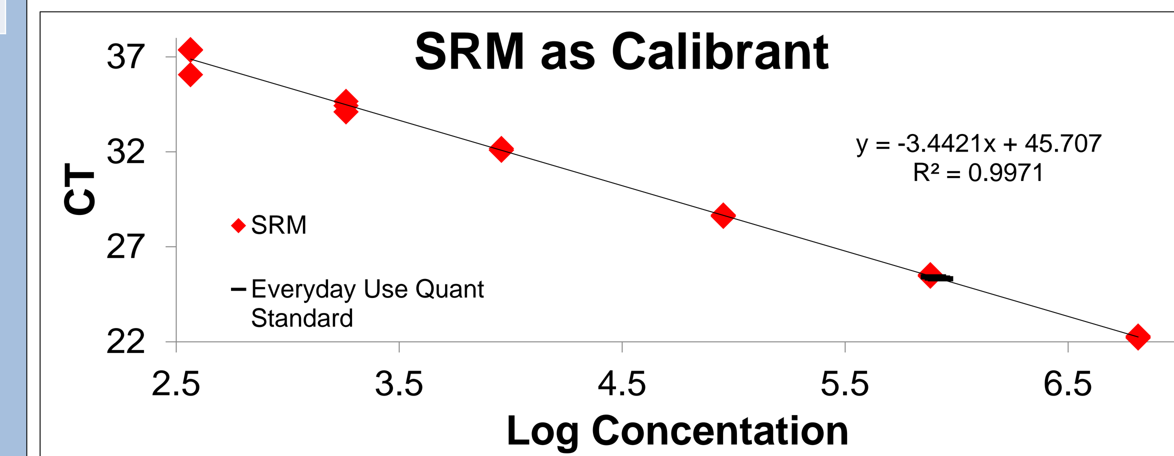
* The sequence at position 78651 is described as a Y (C/T) at NIST and a C in Genbank

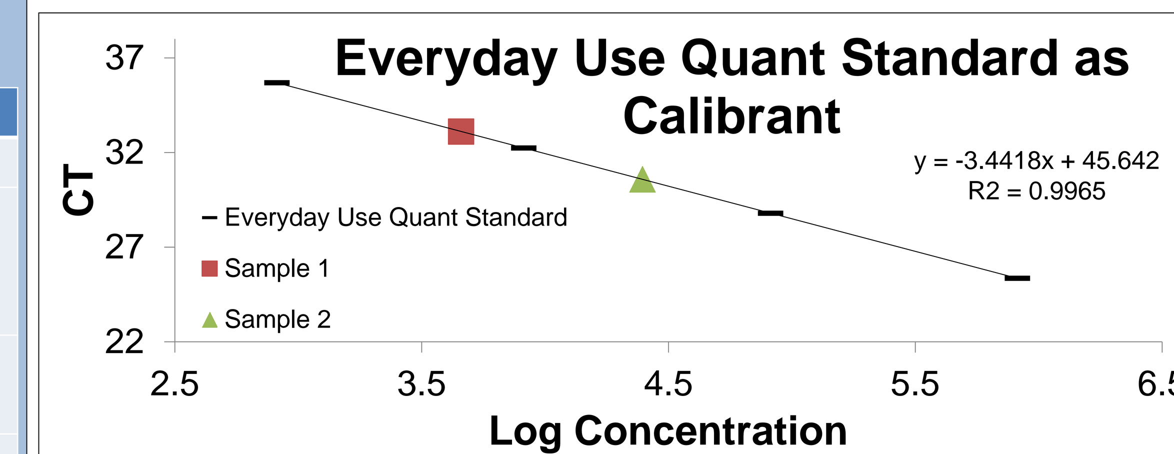
Disclaimer: Certain commercial equipment, instruments, or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

How to Use SRMs to calibrate everyday-use-quant-standard

NIST SRMs are higher-order standards that are meant to calibrate systems and are not intended for everyday usage

1. If necessary, process everyday-use-quant-standard
2. Quant via qPCR, treating the SRM as the true value
3. Assign value to everyday-use-quant-standard (Note: this value is now traceable to a NIST Standard)
4. Use everyday-use-quant-standard with NIST-traceable value to quant patient samples





Clinical DNA Information Resource

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

Clinical DNA Information Resource contains:

- Recent publications and presentations – including this one
- Information DNA-based Standard Reference Materials (SRMs) and candidate SRMs

Conclusions

- BK plasmids
  - Three components are anticipated; two clinical samples provided by University of Washington (1a & 5) and Dunlop plasmid (pBKV 34-2) from ATCC.
  - Clinical genomes were only cloned once the non-coding regulatory region was removed
  - Dunlop plasmid contains the entire BK virus genome
- Cytomegalovirus References

1. Haynes RJ. "Standard reference material 2366 for measurement of human cytomegalovirus DNA." J Mol Diagn. 2013 Mar;15(2):177-85.
2. https://www-s.nist.gov/srmors/view_detail.cfm?srm=2366

- 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
  - Both intellectual and material collaborations are welcome
- Additional Viruses Under Consideration
  - Epstein Barr virus
  - Adenovirus

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