Cytomegalovirus Standard Reference Material® Development for Nucleic Acid Amplification Technologies

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Abstract

A variety of nucleic acid amplification technologies (NAT) based diagnostic tests have been developed both commercially and in-house for the surveillance of cytomegalovirus (CMV) infection. Lack of traceable standards utilizing the International System (SI) has resulted in difficulty in comparing results between laboratories. The National Institute of Standards and Technology (NIST) is developing a Standard Reference Material (SRM) for CMV that will be certified for sequence and copy number (number of copies per volume).
Because CMV virus particles sometimes truncate a portion of the genome during packaging, we are using a bacterial artificial chromosome (BAC) which contains the entire CMV genome from the Towne strain minus a small segment of the genome known as UL147. Our BAC is also non-infectious and propagated in *Escherichia coli*.
Sequencing is being performed on the regions of interest via dye-terminator sequencing and analyzed by capillary electrophoresis. Our regions of interest, which were chosen to flank published quantitative PCR (qPCR) assays, are around the following genes: polymerase, glycoprotein B, UL83/pp65, major immediate early region, and US17.
Our strategy for measuring copy number is to use digital PCR (DPCR) using multiple validated qPCR assays spread throughout our regions of interest. DPCR is a primary method of counting copy number. DPCR works by running multiple replicates so that some, but not all, wells have target DNA, then based on probability equations the number of wells with target DNA translates into number of copies of target per volume.
In addition to the CMV SRM, NIST is building a free database for CMV relevant information including, published primer and probe sequences overlaid on sequence alignments and errors in published primer and probe sequences. This information should be useful for scientists using or designing qPCR assays for diagnostic testing.

Digital PCR

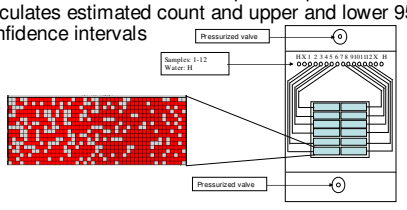
- qPCR assay (ie TaqMan assay)
- Run multiple replicates so that some, but not all, wells have target DNA
- Based on probability: number of wells with target translates to number of copies of target per volume.

Strategy

- Multiple qPCR assays – spread throughout the genome – will be used to perform digital PCR on the SRM
- Ideally the different assays will all render the same concentration, which can then be used to generalize about the concentration of the BAC
- If the results from the assays do not correlate with another, the assays will be re-examined until a reason for the discrepancy is found and corrected

Fluidigm® Technology

- Integrated fluidic circuit – automatically partitions sample into 765 reactions.
- Each chip has 12 panels = 12 samples
- Each panel uses 4.6 uL of master mix
  - 765 partitions x 6 nL/partition = 4.6 uL
- Software detects and counts positive partitions and calculates estimated count and upper and lower 95% confidence intervals



Results from Pilot Material #1

- Digital PCR was performed using two qPCR assays, IE and CP1, which target the major immediate early region and the polymerase gene respectively
- Slight between group difference is explained by slight difference in amplification efficiency which should be corrected by increasing the number of amplification cycles

Molecules/uL	
IE	CP1
4394	3452
3631	3537
4192	3600
3972	3653
3833	3308
4031	4232
$\bar{X}$	3820
SD	189
RSD	5%
U_{95}	10%

Anova: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
IE	6	24055	4009	71496
CP1	6	21781	3630	101496

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	430658	1	430658	4.97891	0.04973	4.965
Within Groups	864965	10	86496			
Total	1295623	11				

Cytomegalovirus Reference Material

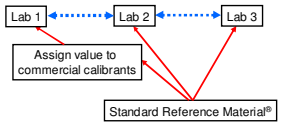
- **Disease:** Cytomegalovirus (CMV) causes life-threatening infections in immunocompromised patients and in congenital transmission to infants, though commonly found and usually latent in the general population
- **Standard needs:** Calibration and quality control of quantitative real-time PCR assays of blood and other body fluids
- **Customers:** Producers of secondary standards or clinical laboratory using in-house assays for CMV detection
- **Current Plans:**
  - ***Materials***: Pure DNA from Towne strain in a bacterial artificial chromosome 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
  - ***Certification***:
    1. DNA sequence of genes that are targets for qPCR
    2. Copy number via digital PCR measurements of multiple CMV specific qPCR targets
  - ***Additional Information***:
    - Restriction digest and microarray validation of BAC
    - Testing of various published PCR assays
    - Homogeneity and stability

Standard Reference Materials®

http://www.nist.gov/srm

Traceable standards to ensure accurate and comparable measurements between laboratories

Some DNA-based SRMs:
- SRM 2391b – autosomal STRs
- SRM 2392 &-I – mtDNA sequencing
- SRM 2395 – Y-STRs
- SRM 2372 – DNA quantitation
- SRM 2394 – mtDNA heteroplasmy
- SRM 2399 – Fragile X



Calibration with SRMs enables confidence in comparisons of results between laboratories

Helps meet ISO 17025 needs for traceability to a National Metrology Institute

Pilot Materials

1) BAC DNA in buffer
2) BAC DNA in buffer with poly-thymine carrier DNA

- Distributed into tubes for stability and homogeneity testing
- Concentration determined with digital PCR
- Any difference in the two materials will allow us to determine if carrier DNA is necessary to ensure the concentration of CMV DNA is stable

Thus far:

- Stability: materials are stable at 4 °C and 22 °C, but signs of degradation appeared after 11 weeks at 37 °C
- Homogeneity: 2 tubes (out of 240+) have been discovered with divergent concentrations

Sequencing

Strategy

- Sequence via Big Dye Terminator v3.1 (Applied Biosystems)
- Analyze by capillary electrophoresis using Applied Biosystems 3130xl with POP7 and an 80cm capillary
- Sequence will be checked against the Towne strain

Results

- BAC has the same sequence as Towne strain (AY315197) in regions already sequenced

Database

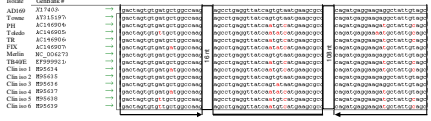
"Although primer mismatching does not necessarily prevent PCR amplification, use of primers having a known high degree of sequence conservation…should reduce assay variability in clinical practice" (Chou 1992).

- Primer size and location of mismatch affect amplification efficiency which can effect clinical sensitivity.
- Database of sequence alignments will:
  - aid scientists designing and using primers and probes
  - save time and money in design & testing

Example 1

	Tanaka 2000 (org pub)	Lengerova 2007 (review)
Sensitivity:	76%	79%
Specificity:	97%	not reported
Number:	53	363
Solution:	Use multiple assays	Don't use this assay.

False negative rate (21%) due to primer and probe mismatch



Example 2 (Sanghavi 2008)

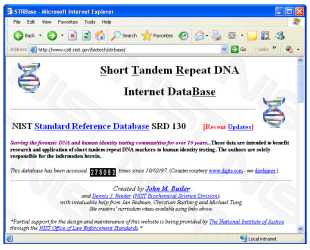
Threshold:	50 copies/mL	500 copies/mL
Sensitivity:	100%	92%
Specificity:	94%	98%

Number: 3422 anticoagulated peripheral blood samples

Threshold of 500 copies/mL is clinically significant

Sensitivity and specificity are defined by the antigenemia test results.

STRBase: a Community Resource for Forensic DNA Applications of STRs…



A similar standard information resource is being developed for clinical diagnostics

"More-uniform detection sensitivity was achieved with primers of conserved sequence" (Chou 1992).

References:
Chou 1992 J of Clin Micro 30(9): 2307-2310
Tanaka 2000 J of Med Virol 60: 455–462
Lengerova 2007 J of Clin Micro 45(3): 1042–1044
Sanghavia 2008 J of Clin Virol 42: 335-342