

# Cytomegalovirus Standard Reference Material<sup>®</sup> Development for Nucleic Acid Amplification Technologies

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### Abstract

Abostiractic 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 laboratorises. 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 antificial 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 *Escherchia coli*. Sequencing is being performed on the regions of interest, which were chosen to flank published quantitative PCR (qPCR) assays, are around the following genes: polymerase, glycorpotein B, UL83/pBS, major immediate early region, and US17. flank published quantitative PCR (qPCR) assays, are around the following genes: polymerase, glycoprotein B. U.B3/ppcB, 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. 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 assavs do not correlate with another, the assays will be re-examined until a reason for the discrepancy is found and corrected

### Fluidigm<sup>®</sup> 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



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 inhouse 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

#### 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 ℃ and 22 ℃, but signs of degradation appeared after 11 weeks at 37 ℃ •Homogeneity: 2 tubes (out of 240+) have been discovered with divergent concentrations



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

## 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

<ul> <li>save time and money in design &amp; testing</li> </ul>								
Example 1								
Та	anaka 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 negat	ive rate (21%) due to p	rimer and probe mismatch						
Isolate GenBack# AD169 X17403- Tome A7315197 PH AC3468005 Toledo AC146806 FIX AC346806 FIX AC346807 Mullio MC067272	gectaging particlapscass     gectaging takenyt     gectaging takenyt	<u>ttaktpanpro</u> r <u>ttaktpanpro</u> r <u>ttaktpanpror</u> <u>ttaktpanpror</u> <u>ttaktpanpror</u>						



#### Example 2 (Sanghavi 2008)

98%

		,
Threshold:	50 copies/mL	500 copies/mL
Sensitivity:	100%	92%



Number: 3422 anticoagulated peripheral blood samples Threshold of 500 copies/mL is clinically significant



Sensitivity and specificity are defined by the antigenemia test results.



"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

UL83										
and a second secon			Size based on AD169 (X17403)							
NOTE AD169	trains x17403. BK	000394, NC 001347, F35275	They perfect conservation in this region, therefore only Arabia only added will be displayed	Name	Size (bp)	Forward	Reverse	Probe	Aathor Date	Citation
				Polymerase						
					293	TEGCCETETTCEACTITEC	GAGCGCCATCTGTTCCTTG		Wirgart 1999 JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1998, p. 3662-3669	
				Pol innar	161	CAGCCTCTACCCTTCCATCA	GCACCGAGACGCGCACCGAA		Wigart 1999 JC	DURNAL OF CLINICAL MICROBIOLOGY, Dec. 1998, p. 3952-3959
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				CP1	72	GGCCGTTACTGTCTGCAGGA	GGCCTCGTAGTGAAAATTAATGGT	FAM CCGTATTGGTGCGCGATCTGTTCAA TAMRA	Sassenscheidt 2006 Jo	ournal of Virological Methods Volume 138 (2006) 40-48
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