Cytomegalovirus Standard Reference Material® Development for Nucleic Acid Amplification Technologies

Ross J. Haynes, Marcia J. Holden, Margaret C. Kline, and John M. Butler

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 limiting the International System (SI) has resulted in difficulties in comparing results between laboratories. The National Institute of Standards and Technology (NIST) is developing a Standard Reference Material (SRM) for CMV that will provide certified sequence and copy number (number of copies per volume). Because CMV virus particles sometimes recycle 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 E. 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 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 calculate 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.

Cytomegalovirus Reference Material

• Disease: Cytomegalovirus (CMV) causes the 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 and microarray validation of BAC.
• Testing of various published PCR assays.
• Homogeneity and stability.

Results from Pilot Material #1:
• Digital PCR
  1) BAC DNA in buffer
  2) BAC DNA in buffer with poly-thymine carrier DNA
• Distributed into tubes for stability and homogeneity testing.

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 4.6 uL/partition = 4,460 uL.
• Software detects and counts positive partitions and calculates estimated count and upper and lower 95% confidence intervals.

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.

Table: Pilot Materials

<table>
<thead>
<tr>
<th>Example 1</th>
<th>Tanaka 2000 (org pub) Lengerova 2007 (review)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity: 76%</td>
<td>79%</td>
</tr>
<tr>
<td>Specificity: 97%</td>
<td>not reported</td>
</tr>
<tr>
<td>Number: 53</td>
<td>363</td>
</tr>
<tr>
<td>Solution: Use multiple assays. Don’t use this assay.</td>
<td></td>
</tr>
<tr>
<td>False negative rate (21%) due to primer and probe mismatch</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Example 2 (Sanghavi 2008)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Threshold: 50 copies/mL 500 copies/mL</td>
</tr>
<tr>
<td>Sensitivity: 100% 92%</td>
</tr>
<tr>
<td>Specificity: 94% 98%</td>
</tr>
<tr>
<td>Number: 3422 anticoagulated peripheral blood samples</td>
</tr>
<tr>
<td>Threshold of 500 copies/mL is clinically significant</td>
</tr>
</tbody>
</table>

Sensitivity and specificity are defined by the antigenemia test results.

Sequencing

Strategy
• Sequence via Big Dye Terminator v3.1 (Applied Biosystems)
• Analyze by capillary electrophoresis using Applied Biosystems 3130xD with POP7 and an 80cm capillary.
• Sequence via Big Dye Terminator v3.1 (Applied Biosystems)

Results
• BAC has the same sequence as Towne strain (AY315197) in regions already sequenced.

References:
Chou 1992 J of Clin Micro 30(9): 2307-2310
Sanghavi 2008 J of Clin Virol 42: 335-342

Some DNA-based SRMs:
• SRM2380 – commercial calibrants
• SRM2382 – Y-STRs
• SRM2372 – DNA quantitation
• SRM2384 – STRs (short tandem repeats)
• SRM2386 – Fragile X

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