A Real Time PCR Assay for the Detection of CMV Using Eclipse Hybridization Probes

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ABSTRACT

We developed a qualitative real time PCR assay to detect CMV in patient samples, using a novel hybridization probe termed the Eclipse probe (Epoch Biosciences). These are single probes with a fluorescent molecule attached to the 3' end and a dark quencher on the 5' end. Also conjugated to the 5' end of the probe is a minor groove binding moiety that stabilizes probe binding and allows much shorter probe sequences to be used. The assay is run on the HT7900 Sequence Detection System (Applied Biosystems) in a 96-well plate. We incorporated a heterologous internal control in the assay to monitor the extraction process and detect PCR inhibition. The internal control target is a plasmid, containing a fusion of GFP and *C. elegans* DNA. We validated ten sample types (spinal fluid, plasma, serum, amniotic fluid, ocular fluid, buffy coat, tissue, urine, bone marrow and bronchioalveolar lavage), and demonstrated the dependence that the limit of detection has on sample type. Since this assay went on-line last year, we have identified a number of patient samples that generated discrepant results between our qualitative test and the Roche CMV Amplicor Quantitative assay. Here we present the validation data for our real time assay, and results of the discrepant analysis.

MATERIALS and METHODS

Specimens:

During the clinical validation of this assay we tested 453 samples (amniotic fluids, spinal fluids, plasma, serum, buffy coats, bone marrows, urine, bronchioalveolar lavage, ocular fluids, swab samples and tissues). Due to the infrequent number of positive samples obtained for serum, buffy coats, ocular fluids, bone marrows, BALs, and tissues, some or most of the positive samples for these sample types were spiked with virus. In these cases, positive control material was doped into the appropriate CMV negative sample type, at a ratio of 1:1. We obtained all un-doped positive samples from the clinical lab. These samples had previously been identified as CMV positive using the current nested PCR assay (gel detection). Positive control material consists of cell culture material (obtained from the ARUP Virology Lab) diluted to have a crossing threshold of approximately 35. A negative control of extracted water is included in each run.

Extractions:

With the exception of the bone marrows and tissue samples that were extracted with the Puregene Tissue Extraction Kit, all samples were extracted using the Qiagen 96-well Blood Kit, with a few minor modifications. Protease (25 µL), patient sample (200 µL) and pre-warmed lysis buffer AVL (200 µL) were added to each well of the round well block in that order. For quality control of the extraction, internal control was added to the lysis buffer at a concentration of 50,000 copies per mL (10,000 copies per extraction). The block was then shaken for 15 seconds and spun down briefly at 3000 rpm. The block was incubated at 70°C for 10 minutes and spun down as before. 200 µL of ethanol was added to each extraction, the block shaken for 15 seconds and spun down again briefly at 3000 rpm. Samples were transferred to the filter plate, covered with airpore tape and spun for 4 minutes at 6000 rpm to apply the samples to the filter. The samples were washed with 500 µL of buffer AW1 (spun 2 minutes at 6000 rpm) and 500 µL buffer AW2 (spun 3 minutes at 6000 rpm). The filter block was incubated for 10 minutes at 70°C. The filter plate was placed over the collection tubes and 50 µL of prewarmed elution buffer (buffer AE, 70°C) was added. The plate was incubated at 70°C for 5 minutes and spun for 4 minutes at 6000 rpm. An additional 50 µL of elution buffer was added to each sample well and the plate spun another 4 minutes

Amplification and detection:

Primer and probe sequences for the Eclipse CMV reaction are from the major immediate early protein gene (gi:330620). The probe and primers were purchased from Epoch Biosciences. The probe is labeled at the 5' end with a non-fluorescent quencher (NFQ) and a minor groove binding molecule (MGB). The 3' end of the probe is labeled with FAM. Real time primer sequences are in red, probe sequence is in blue. Primer sequences used in the discrepant analysis are underlined.

- CMV upstream primer: 5' - CGAGACACCCGTGACCAAGGC- 3'
- CMV downstream primer: 5' - TCTGCCAGGACATCTTTCTC- 3'
- CMV probe: 5' - NFQ-MGB- AAGTGACCGAGGATTGC- FAM - 3'

5' CGAGACACCCGTGACCAAGGCCACGACGTTCCTGCAGACTATGTTGAGGAAGGAGGTTAACAGTCAGCTGAGTCTGGGAGACCCGCTGT

TTCCAGAGTTGGCCGAAGAATCCCTCAAAACTTTTGAACAAGTGACCGAGGATTGCAACGAGAACCCCGAGAAAGATGTCCTGGCAGA 3'

Primer and probe sequences for the Eclipse internal control reaction are from a plasmid containing a fusion of *C. elegans* and GFP sequences. The probe is purchased from Epoch Biosciences, and is labeled at the 5' end with non-fluorescent quencher (NFQ) and a minor groove binding molecule (MGB). The 3' end of the probe is labeled with TET. Primers are synthesized in-house. Primer sequences are in red, probe sequence is in blue.

- IC upstream primer: 5' - CTGCACGGACCAGTTACTTTACG- 3'
- IC downstream primer: 5' - CTCATTTTTTCTACCGGAGATCTTGT- 3'
- IC probe: 5' - NFQ-MGB - TGCGGTACGTGGTC - TET- 3'

5' CTGCACGGACCAGTTACTTTACGGACCACGTACCGCATTGGTACAAGATCTCCGGTAGAAAAAATGAG 3'

Figure 1a

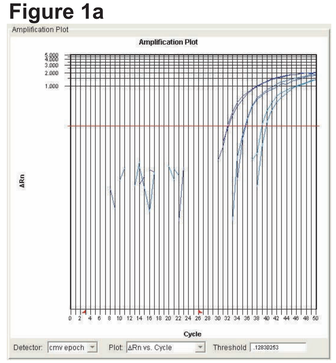


Figure 1b

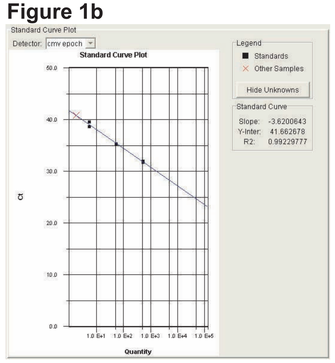


Figure 1c

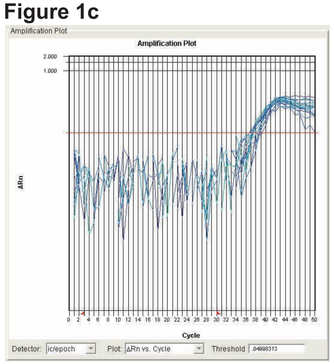


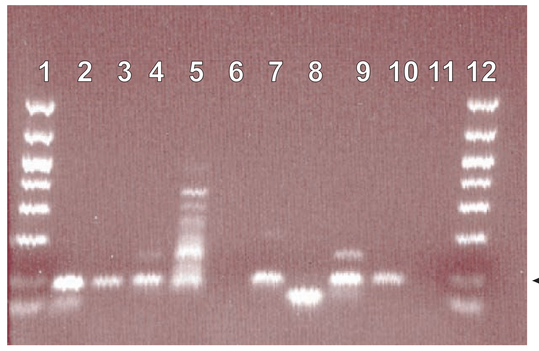
Table 1

Sample Type	Positives	% Positives Spiked	Negatives
amniotic fluid	22/23 (96%)	0% (0/23)	21/22 (95%)
BAL	21/23 (91%)	52% (12/23)	18/23 (78%)
bone marrow	22/23 (96%)	96% (22/23)	19/22 (86%)
buffy coat	20/20 (100%)	60 %(12/20)	20/23 (87%)
CSF	21/21 (100%)	0% (0/21)	32/33 (97%)
ocular fluid	23/23 (100%)	70% (16/23)	6/7 (86%)
plasma	20/21 (95%)	0% (0/21)	36/38 (95%)
serum	19/20 (95%)	60% (12/20)	19/21 (90%)
tissue	31/31 (100%)	77% (24/31)	9/15 (60%)
urine	21/21 (100%)	0% (0/21)	21/23 (91%)
totals	220/226 (97%)	43% (98/226)	201/227 (89%)

Table 2

Sample Type	Sensitivity
amniotic fluid	1250 copies/mL
bal	250 copies/mL
buffy coat	650 copies/mL
csf	400 copies/mL
ocular	800 copies/mL
plasma	3800 copies/mL
serum	3800 copies/mL
urine	300 copies/mL

Figure 2



lane 1 1 kb ladder
lane 2 positive control
lane 3 patient A, extract 1
lane 4 patient A, extract 2
lane 5 patient B, extract 1
lane 6 patient B, extract 2
lane 7 patient B, extract 3
lane 8 patient C, extract 1
lane 9 patient C, extract 2
lane 10 patient C, extract 3
lane 11 negative control
lane 12 1 kb ladder

MATERIALS and METHODS, cont'd

We perform the assay using 10µl of extracted DNA in a 50µL total reaction volume. In addition to the target DNA, we include the following reagents:

CMV upstream primer	1000 nM
CMV downstream primer	1000 nM
CMV probe	200 nM
Internal control upstream primer	300 nM
Internal control downstream primer	300 nM
Internal control probe	50 nM
UNG	0.5 U
$MgCl_2$	4 mM
10X Roche FastStart Master Hybridization Probes Mix	1X
10X PCR Enhancer	1X
ROX passive reference	60 nM

The cycling conditions for amplification are as follows:

1)	50°C	2 minutes	
2)	95°C	8 minutes	
3)	95°C	15 seconds	50 cycles
4)	58°C	30 seconds	
5)	76°C	30 seconds	
6)	95°C	15 seconds	
7)	45°C	15 seconds	
8)	95°C	15 seconds	

All ramp rates are set to 100% except for the final temperature ramp between steps 7 and 8 which is set to 5%.
Data is collected at step 4 (annealing) and during the temperature ramp between steps 7 and 8 (dissociation analysis).

RESULTS

To evaluate the analytical sensitivity of this real time CMV assay, we amplified serial log dilutions of a plasmid containing the cloned CMV amplicon, ranging from 500 copies per reaction down to 5 copies per reaction. In this experiment, the internal control plasmid was added to the master mix and co-amplified in the reaction. The results of the CMV amplification are shown in Figure 1a and the standard curve in Figure 1b. The slope of the standard curve corresponds to a PCR efficiency of 89%. Figure 1c shows the amplification plot for the internal controls, which amplified in all reactions.

The validation was performed by comparing this real time PCR assay with the gel assay (nested PCR) that was being performed in the clinical lab at ARUP. Accuracy, precision, sensitivity and specificity of the new assay were all evaluated. For measuring accuracy, at least twenty positive and twenty negative CMV samples were run by each method, on each of the sample types to be validated (except ocular fluids). For serum, ocular fluid, tissue, bone marrow, buffy coats and bronchioalveolar lavage, an insufficient number of positive samples were available. In these cases, positive CMV control (CMV positive culture) was spiked into these sample types (previously collected; CMV negative) at a concentration of approximately 5000 copies/mL (100 copies per reaction). Validation data is shown in Table 1

Sensitivity of the assay was measured by running serial 2-fold dilutions of quantitated positive control material, doped into each of the validated sample types (sensitivity was not performed for tissue extracts or bone marrows). Our experience suggests that the differences seen in sensitivity between sample types is likely a function of the extraction. When target DNA is spiked into extracted patient sample post-extraction, the differences between sample type are minimal (data not shown).

In the course of performing routine CMV testing in the clinical lab, we identified a small number of patient samples that gave discrepant results between the real time qualitative assay described here and our quantitative assay. Quantitation of CMV is done on plasma, using the Roche CMV Amplicor test. In most cases, samples are not run on both assays. Occasionally however, when a qualitative test is positive, the ordering physician adds a request for quantitation of the CMV viral load. In 3 cases, the qualitative assay was positive, but a negative result (<600) was generated on the quantitative assay. These 3 samples displayed late crossing thresholds (>40) on the qualitative assay. To resolve these 3 discrepants, we amplified the extracted DNA with a new primer pair (see Materials and Methods), designed to amplify a region within the original qualitative amplicon. These PCR reactions were run on a gel to identify the products. All three patient samples showed amplification products of the correct size (91bp), in a majority of the reactions (Figure 2), indicating that these patient samples are likely true CMV positives.

DISCUSSION

We designed and validated a qualitative real time PCR assay for detecting CMV that displays a similar sensitivity to the nested PCR assay with gel detection formerly run in our clinical lab. Addition of an internal control that is co-extracted and co-amplified with each patient sample provides confidence in our ability to call a true negative. All positive results are re-extracted and re-amplified, minimizing the likelihood of reporting a false positive result. Since real time PCR is inherently a quantitative platform, this assay has the potential to be easily converted to a quantitative format, possibly replacing our current quantitative assay.

While the limit of detection provided for the Roche CMV AMPLICOR assay (600 copies per mL) is greater than what we measured for the real time CMV assay described here (3800 copies per mL in plasma), the results of our discrepant analysis suggest otherwise. In reality, our conclusion is that both these assays have similar limits of detection. The crossing thresholds we observed for these discrepant samples indicate that the CMV viral loads are likely very low, nearing our limit of detection. These results highlight the difficulty in being able to consistently detect a DNA target in samples with low viral loads. Even in the discrepant analysis presented here (Figure 2), CMV was not detected in 100% of the sample extracts. Furthermore, these results point out the difficulty in determining an accurate value for assay sensitivity. This figure can vary based on a number of factors, including the accuracy of sample quantitation and the criteria used to define the limit of detection.