Cytomegalovirus (CMV) detection & quantitation using SYBR Green format real-time qPCR.

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Cytomegalovirus humanbeta5 (CMV) is one of ten primate species (aotinebeta1, cebinebeta1, cercopithecinebeta5, macacinebeta3 and 8, mandrillinebeta1, paninebeta2, -3 & -4 and saimiriinebeta4) belonging to the *Betaherpesvirinae* subfamily *Orthoherpesviridae* family and *Herpesvirales* order. CMV has a double-stranded DNA (dsDNA) between 230–240 kbp in size, the largest of the human viruses, encoding more than 200 open reading frames (ORFs). Surface glycoprotein B (gB), encoded by the UL55 gene, is classified into four major: gB1, gB2, gB3, and gB4, and three non-prototypic: gB5, gB6, and gB7) variants.
Pediatric CMV infections can be congenital (transmitted in utero), perinatal (during delivery), postnatal (through breast milk, saliva, or other bodily fluids). CMV seroprevalence in the US has been estimated to be between 50% and 85% of adult women by 40 years of age. CMV does not usually cause disease in healthy individuals but establishes life long infections after primary infection which can reactivate during immunocompromises, especially after hematopoietic stem cell or solid organ transplantation and HIV infection where it can cause severe, sometimes fatal, diseases. CMV is the major cause of congenital infection occurring in 0.2% to 2.3% of all live births. Congenital CMV infections in the neonate range from asymptomatic infection to extreme prematurity, encephalitis, deafness, and even death. Urinary CMV PCR is a reliable and rapid method of screening infants for CMV infections and remains the standard of practice. Screening an infant for congenital CMV infection must be completed within the first 3 weeks of life. After 3 weeks of age, a positive result canot distinguish congential infections from perinatal or postnatal infections. This protocol allows for the detection of CMV gB glycoprotein genotypes 1, 2 and 3, other genotypes have not been tested.

Oligonucleotide primer features

Name	Sequence*	Bp	%GC	Tm[b]	Hair	HmD	HtD	Amplicon	Ref
CMVgb-F2	5´-ACT-gCA-CgT-ACg-AgC-TgT-TgR-3´	21	57	60	-0.45	-10.87	-8.25	91 pb	1
CMVgb-R2	5´- CTg-CgT-gAT-ATg-AAC-gTg-AAR-g -3´	22	55	56	-1.13	-6.3			

***** Reverse oligonucleotide primer sequences given in this table are the reverse-complement of the sequence present in the alignments and as they should be ordered for synthesis. Hairpin (Hair), homodimer (Hm) and heterodimer (Ht) ΔQ's are given in kcal/mol.

These primers were manually designed from the glycoprotein B UL55 gene sequence of genotypes gB1, gB2 and gB3 for use in the quantitative assessment of cytomegalovirus (CMV) infection based on those published by Guiver, Malcolm *et al* on Transplantation 71(11):p 1609-1615, June 15, 2001.

PCR components

Samples	1	8	16	24	32	40	48	56	64
dH2O	1.68	15.12	28.56	42	55.44	68.88	84	97.44	110.88
SybrMaster™ mastermix (2x)	5	45	85	125	165	205	250	290	330
ROX reference dye (50x)	0.02	0.18	0.34	0.5	0.66	0.82	1	1.16	1.32
CMVgb-Fwd (10 µM)	0.1	0.9	1.7	2.5	3.3	4.1	5	5.8	6.6
CMVgb-Rev (10 µM)	0.2	1.8	3.4	5	6.6	8.2	10	11.6	13.2
Template (50 to 200 ng/µL)	3	27	51	75	99	123	150	174	198
vf	10	90	170	250	330	410	500	580	660

Samples	72	80	88	96
dH2O	124.32	137.76	151.2	164.64
SybrMaster™ mastermix (2x	370	410	450	490
ROX reference dye (50x)	1.48	1.64	1.8	1.96
CMVgb-Fwd (10 µM)	7.4	8.2	9	9.8
CMVgb-Rev (10 µM)	14.8	16.4	18	19.6
Template (50 to 200 ng/µL)	222	246	270	294
vf	740	820	900	980

Volumes given in tables above consider using Jena Bioscience's qPCR SybrMaster (Cat. Num. PCR-372S or PCR-372L) which includes (red top tube) antibody-blocked hot start polymerase, dATP, dCTP, dGTP, dTTP, KCl, (NH4)2SO4, $MgCl_2$, SYBR® Green DNA intercalator dye, additives and stabilizers as well as Jena Bioscience 25 µM (50x concentration) ROX reference dye (Cat Num. PCR-351) λexc 576 nm, λem 601 nm.

PCR conditions

Use Applied Biosystems ABI7500 real-time cycler (total run time of 2 hr 10 min).

	Temp	Time	Cycles
qPCR	94 ºC	3 min	1
	94 ºC	15 sec	45
	62.8 ºC	60 sec*	
Dissociation curve	95 °C	15 sec	
	60 °C	20 sec	1
	Ramp	60 min*	
	95 ºC	15 sec	

Preparation of Standard Calibration Curve

All handling of artificial genes or positive template controls must be conducted exclusively in the Cell Biology section of the Viral & Human Genomics Laboratory.

1. Thaw one aliquot of CMV plasmid at $8.6x10^6$ cp/µL at room temperature.
2. Label a PCR tube strip with the corresponding serial dilutions to be prepared.
3. Add 90 µL of dH_2O to each tube using a p200 filter tip.
4. Take 10 µL from the CMV plasmid tube having $8.6x10^6$ cp/µL concentration and dispense into the first PCR tube of the strip (i.e., that one labeled with the number 5 corresponding to $8.6x10^5$ cp/µl).
5. Mix contents slowly by pipetting pipetting up and down 20 times without generating aerosols.
6. Cap the PCR tube, vortex briefly for 10 seconds, and spin contents down.
7. Using a new p10 filter tip, transfer 10 µL of this $8.6x10^5$ cp/µL dilution to the next PCR tube (i.e., that labeled with the number 4 corresponding to $8.6x10^4$ cp/µL).
8. Repeat steps 5 and 7 for the remaining dilutions.
9. Consistency and homogeneity is crucial for proper standard curve preparation.
10. Dispose of gloves used for standard curve preparation before leaving the cell biology area.

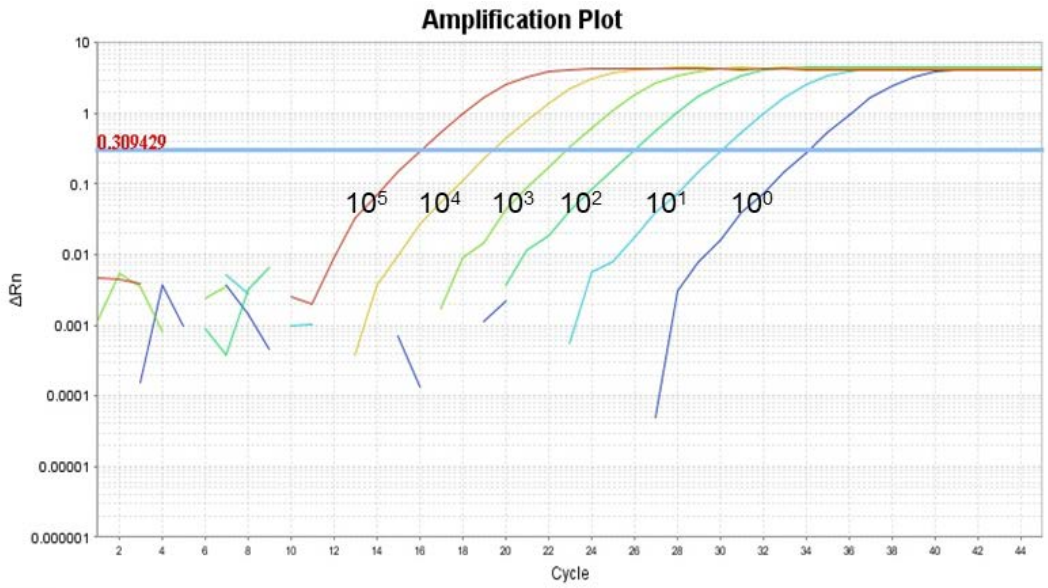
Realtime qPCR Assay performance

Comparison of SybrMaster qPCR results against those produced by the commercial standard real-Time PCR detection kit for CMV (HHV5) Cat. Num. Z-Path-CMV-STD (Genesig PrimerDesign, United Kingdom),

	Ct			Viral load (cp/µl)		Melt (ºC)
Sample	Local	Kit	ΔCt	Local	Kit	Local
CMV 01	31.4	34	2.6	11.72	1.5	84.3
CMV 02	32.3	35.0	2.6	14.3	12.6	84.3
CMV 03	32.4	34.4	2.0	14.0	18.7	84.6
CMV 04	12.9	15.3	2.4	8’389,865.0	8’630,000.0	84.8
CMV 05	24.5	27.7	3.3	3,052.5	1,760.0	84.8
CMV 06	15.3	18.0	2.6	1’609,674.3	1’420,000.0	85.0
CMV 07	26.1	28.8	2.7	1,031.0	871.5	84.8
CMV 08	22.4	24.7	2.3	12,429.0	13,000.0	84.8

The standard curve plots the log of the starting quantity of against the cycle threshold (CT) value and should show consistent Ct values across dilutions where the Ct values decrease in a consistent, predictable manner with each serial dilution. Typically, a 10-fold dilution should result in a Ct increase of approximately 3.3

cycles (see image below produced by us with this technique).



A good qPCR standard curve should exhibit the following key features (see image below produced by us with this technique):

1. High R^2 value (Correlation Coefficient), and as close to 1 (preferably > 0.99), indicating a strong linear relationship between the log of the starting quantity and the Ct values. This reflects the accuracy of the dilution series and the consistency of the qPCR amplification.

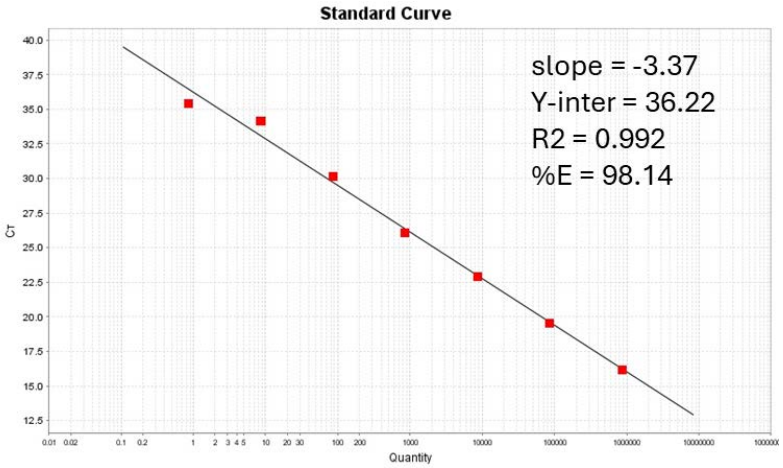
2. Slope (efficiency of the PCR reaction) within the optimal range, should ideally lie between -3.1 and -3.6. A slope of -3.32 indicates 100% amplification efficiency, meaning the DNA doubles with each cycle. A slope steeper than -3.6 suggests inefficient amplification, a flatter slope (greater than -3.1) indicates primer dimer formation or contamination.

3. PCR efficiency (90% to 110%) as calculated from the slope, should fall between 90% and 110%. Lower efficiencies (<90%) suggest poor amplification, while higher efficiencies (>110%) indicate the presence of inhibitors, contaminants, or non-specific amplification.

4. Noticeable separation between dilution points: The different dilutions should show clear and distinct separation in Ct values, providing sufficient dynamic range to assess the assay's linearity and detection limits.

5. Wide dynamic range covering several concentrations (at least 5-6 orders of magnitude), indicates the assay can detect both low and high quantities of the target DNA with accuracy.

6. Low variability between replicates, exhibiting minimal variation in Ct values (low standard deviation), indicating the precision and reproducibility of the assay.

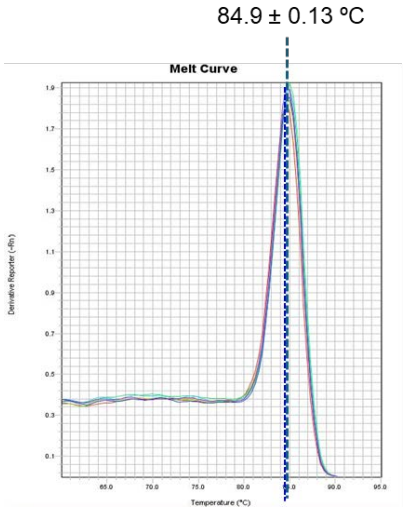


A good qPCR dissociation (or melting) curve plot should exhibit the following characteristics (see image below produced by us with this technique):

1. Single, sharp, well-defined peak at a specific melting temperature (Tm) corresponding to the specific amplicon. Primer-dimers appear as low-melting, broad peaks below 75 °C.

2. Consistent melting temperature (Tm) across all replicates and samples. Variability in the Tm between reactions can indicate inconsistencies in the reaction setup, reagent quality, or thermal cycling.

3. Gradual slope in pre- and post-melt regions: The pre- and post-melt regions should show a smooth, gradual slope, indicating a uniform melting transition and consistent DNA dissociation.



Viral load (cp/ul)	Ct	Tm
8.60E+05	16.1	84.6
8.60E+04	19.5	84.8
8.60E+03	22.9	84.8
8.60E+02	26.1	85.0
8.60E+01	30.1	85.0
8.60E+00	34.2	84.8
8.60E-01	35.4	84.8

Result interpretation

SYBR Green fluoresces upon intercalation into any double-stranded DNA, making it non-specific to individual reactions. Therefore, qPCR reactions using SYBR Green require an additional step: a melt curve or dissociation curve analysis. This step identifies a dissociation temperature specific to the composition and size of the target amplicon. Only samples with a dissociation temperature matching the positive control (84.9±0.2°C) are considered positive.

Notes

1. Clean work bench with 0.1% NaOCl and 70% ethanol before and after preparing PCR reactions.

2. Prepare PCR reactions on an ice tray or by using chilled tube racks to minimize static evaporative rate.

3. Vortex all reagents, except for the DNA, before preparing the master mix, vortex again after the master mix is prepared.

4. Use the most appropriate micropipette for each volume range to be dispensed.

5. Respect work-flow space segregation. The master mix should be prepared in a biological safety cabinet located in the Molecular Biology Area of our lab (run it for 5 minutes prior to preparing PCR but turn blower off while preparing PCR).

6. Do not exceed 500 ng DNA per reaction as final concentration.

References

1. Evaluation of CMV viral load using TaqMan CMV quantitative PCR and comparison with CMV antigenemia in heart and lung transplant recipients. Guiver M, Fox AJ, Mutton K, Mogulkoc N, Egan J. Transplantation. 2001 Jun 15;71(11):1609-15.

2. The ABCs of CMV. DeVries J. Adv Neonatal Care. 2007 Oct;7(5):248-55; quiz 256-7.

3. International Committee on Taxonomy of Viruses (ICTV): https://ictv.global/taxonomy/

Revision history

1. Original document.
2. Updated protocol format and validated technique against commercial CMV kit.
3. Introductory text incorporates new virus nomenclature and taxonomy, oligonucleotides modified to emsure genotype b3 detection.