

CMV DNA QUANTITATION (QT)

**Real -Time PCR
for the CMV genome
Quantitation**

- for "Research Use Only" -



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REF. CMVDNAQT.CE
25/50/100/150 Tests

CMV DNA

A. INTENDED USE

The **CMV DNA Quantitation (QT)** Real-Time PCR kit coded **CMVDNAQT.CE** is intended for the quantitative detection of Cytomegalovirus DNA in human sample (blood, plasma, amniotic fluid) with a simultaneous control of the extraction/amplification reaction through an **Internal Control (IC)**.

The kit has been adapted for its use on the Real-Time Thermocyclers ABI 7500 Sequence Detection System® (Applied Biosystems™*) or MiniOpticon® (Biorad™**) or MX3000P® (Stratagene™***).

* Applied Biosystems is a registered trademark and ABI PRISM® is a trademark of Applied Biosystems or its subsidiaries in the US and/or certain other countries.

** Biorad is a registered trademark.

***Stratagene is a registered trademark.

B. INTRODUCTION

Human cytomegalovirus (HCMV) is a ubiquitous member of the human herpesvirus family of viruses.

Clinical data indicate that HCMV infects various tissue and cell types and, hence, is responsible for a myriad of clinical complications. Depending on the tissue type and the host's immune state, HCMV engages in three different modes of infection: acute infections with highly productive growth, persistent infections with low levels of replication, and latent infections where no viral progeny are produced. The mechanism of reactivation is largely unknown but appears to be strongly related to impaired immune control of the virus. For this reason, CMV is one of the most common opportunistic pathogens complicating the care of transplant recipients; potentially it is a major cause of morbidity and mortality.

With a linear double-stranded DNA genome of >230 kb, HCMV is the largest member of the human herpesvirus family.

Within the family, HCMV is the prototype of the β -herpesvirus subgroup, which includes herpesviruses 6 and 7.

Treatment of CMV disease with specific antiviral drugs such as ganciclovir and foscarnet reduces disease severity and mortality in these patients. Prophylactic and preemptive antiviral strategies have been developed and aim at avoiding aggressive treatment of established end-organ disease.

The Quantification of CMV DNA viral load define specifically the progression of the disease. Real-time PCR-based assays are able to quantify viral DNA accurately and without a post-PCR sample handling, providing fast results and a really low risk of cross-contamination for the sample under evaluation.

C. PRINCIPLE OF THE TEST

The CMVDNAQT.CE Kit is based on a Real Time chemistry which uses specific Primers and Probes,

CMV DNA, recovered from the biological sample under investigation through an extraction step, is amplified using Real Time amplification system. The amplified product is detected and quantitated, against the standard curve using a fluorescent reporter dye probe specific for a CMV unique genomic sequence. An Heterologous Internal Control (IC) serves as an Extraction/Amplification control for each individually processed specimen aiming to the identification of reaction inhibitors.

A standard curve is supplied allowing the determination of the viral load.

D. COMPONENTS

The standard format of the product code CMVDNAQT.CE contains reagents for 50 tests.

Component	Contents	CMVDNAQT.CE 50 Reactions
A CODED: ALL/MM COLOR CODE: LIGHT BLUE	Master mix	N°2 vials / 0.350 ml
B CODED: CMV/CB COLOR CODE: YELLOW	Lyophilised Primers/Probes	N°2 vials (dissolve with 60 ul of ALL/C)
C CODED: ALL/C COLOR CODE: GRAY	MG Water	N°2 vials /1.5 ml
NTC CODED: ALL/NTC COLOR CODE: WHITE	Negative Control	N°1 vials /1.5 ml
STD Quantitation Standard (6x10 ⁶ copies/μl) CODED: CMV/STD COLOR CODE: RED	Lyophilised Quantitative Standard	N°2 vials (dissolve with 30 ul of ALL/C)
I.C. Internal Control CODED: ALL/IC COLOR CODE: GREEN	Lyophilised Internal Control	N°2 vials (dissolve with 300ul of ALL/C)
Package Insert	Instruction for Use	1

Important note: Upon request, Dia.Pro can supply reagents for 25, 100, 150 tests, as reported below :

1. Component A	n°1 vial/0.350	n°4 vials/0.350 ml	n°6 vials/0.350
2. Component B	n°1 vial	n°4 vials	n°6 vials
3. Component C	n°1 vial/1.5 ml	n°3 vial/1.5 ml	n°5 vial/1.5 ml
4. NTC	n°1 vial/1.5 ml	n°1 vial/1.5 ml	n°1 vial/1.5 ml
6. IC	n°1 vial	n°4 vials	n°6 vials
7. STD	n°1 vial	n°4 vials	n°6 vials
8.Pack. insert	n°1	n°1	n°1
Number of tests	25	100	150
Code	CMVDNAQT.CE.25	CMVDNAQT.CE.100	CMVDNAQT.CE.150

E. MATERIALS REQUIRED BUT NOT PROVIDED

1. Calibrated Micropipettes (0.5 μl < volume <1000 μl)
2. DNA extraction kit
3. MG EtOH
4. Thermal Block
5. Microcentrifuge
6. Tube racks
7. Sterile filtered tips with aerosol barrier
8. Nuclease-Free Microtubes
9. 0,2 ml Microtubes or Pcr Microplates recommended from the Real-Time PCR instruments manufacturers
10. Disposable gloves, powder-free
11. Real-Time PCR Thermalcycler (*)
12. Absorbent paper tissues.
13. Vortex or similar mixing tools.

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(*) **Attention:** A valid calibration of the pure dyes (Pure Spectra Component File) and of the background (Background Component File) must be done routinely.

F. WARNINGS AND PRECAUTIONS

1. The kit has to be used only by skilled and properly trained technical personnel, under the supervision of a medical doctor responsible for the laboratory.
2. The technical personnel must be deeply trained in the use of Real-Time thermal cyclers, in the manipulation of Molecular Biology reagents and in the Real-Time PCR amplification protocols.
3. The kit has to be used in a laboratory certified and qualified by the national authority in that field (Ministry of Health or similar entity) to carry out this type of analysis.
4. All the personnel involved in performing the assay have to wear protective laboratory clothes, powder-free gloves and glasses. The use of any sharp (needles) or cutting (blades) devices should be avoided. All the personnel involved should be trained in biosafety procedures, as recommended by the Center for Disease Control, Atlanta, U.S. and reported in the National Institute of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
5. All the personnel involved in sample handling should be vaccinated for HBV and HAV, for which vaccines are available, safe and effective.
6. The laboratory environment should be controlled to avoid contaminants such as dust or air-borne microbial agents.
7. Components A and B are light sensitive. Protect them from strong light exposition.
8. Avoid vibration of the bench surface where the test is undertaken.
9. Upon receipt, store the kit at 2..8°C into a temperature controlled refrigerator or cold room.
10. Do not interchange components between different lots of the kits. Moreover components of different kits coming from the same lot should not be interchanged.
11. Check that the reagents are clear and do not contain visible heavy particles or aggregates. If not, advise the laboratory supervisor to initiate the necessary procedures for kit replacement.
12. Avoid cross-contamination between samples by using disposable tips and changing them after each sample.
13. Avoid cross-contamination between kit reagents by using disposable tips and changing them between the use of each one.
14. Do not use the kit after the expiration date stated on the external container label.
15. Treat all specimens as potentially infective. All human blood/plasma/amniotic fluid specimens should be handled at Biosafety Level 2, as recommended by the Center for Disease Control, Atlanta, U.S. in compliance with what reported in the Institutes of Health's publication: "Biosafety in Microbiological and Biomedical Laboratories", ed. 1984.
16. Store and extract specimens separately from the other reagents and use a separate room for their handling
17. Dissolve the lyophilised reagents with the correct amount (stated in the labels) of Component C (Coded: ALL/C) supplied in the kit.
18. Carry on all the working operations as quickly as possible maintaining the components on ice or in a cooling block.
19. The laboratory Workflow must proceed in an unidirectional way, beginning in the Extraction Area and moving to the Amplification and Data Analysis Areas. Do not return samples, equipment and reagents to the area where the previous steps have been performed.
20. The use of disposable plastic-ware is recommended in the preparation of the liquid components or in the transferring of components into automated workstations, in order to avoid cross contamination.
21. Waste produced during the use of the kit has to be discarded in compliance with national directives and laws concerning laboratory waste of chemical and biological substances. In particular, liquid waste generated from sample extraction procedures, has to be treated as potentially infective

material and inactivated before waste. Do not put in contact with bleach the extraction waste.

22. Accidental spills from samples and operations have to be adsorbed with paper tissues soaked with household bleach and then with water. Tissues should then be discarded in proper containers designated for laboratory/hospital waste.

23. Other waste materials generated (example: tips used for samples) should be handled as potentially infective and disposed according to national directives and laws concerning laboratory wastes.

G. SPECIMEN: PREPARATION AND RECOMMENDATIONS

1. Blood has to be drawn aseptically by venepuncture and plasma has to be prepared using the standard procedures for clinical laboratory analysis.
2. Collection of amniotic fluid has to be done after 16 weeks from the beginning of gestation under continuous ultrasound control. Following the established and approved clinical guide lines.
3. No influence due to the preparation of the sample with citrate or EDTA has been observed
Attention: Heparin (≥ 10 IU/ml) affects the PCR reactions. Samples, which has been collected in tubes containing heparin as an anticoagulant should not be used. Also, samples of heparinised patients must not be used.
4. Avoid any addition of preservatives to samples.
5. Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.
6. Haemolysed (red) and visibly hyperlipemic ("milky") samples have to be discarded as they could generate false results. Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
7. Plasma and amniotic fluid, if not used immediately, must be stored at -20°.-80°C after collection. Samples can be stored frozen at -80°C for several months. Any frozen samples should not be frozen/thawed more than once as this may affect the test result.
8. The plasma samples for DNA extraction must be collected in EDTA according to the common laboratory procedures, transported and stored at +2 / +8 °C for a maximum period of 4 hours. The plasma samples can be stored frozen at - 20°C for a maximum period of 30 days or at -70 °C for longer periods.
9. The amniotic fluid samples must be centrifuged before DNA extraction and dissolved in PBS according to the common laboratory procedures. The amniotic fluid samples must be transported and stored at +2 / +8 °C for a maximum period of 4 hours. The amniotic fluid samples can be stored frozen at - 20°C for a maximum period of 30 days or at -70°C for longer periods.
10. We recommend you, for the optimal storage of samples, to split them in several aliquots (minimum volume 300 μ l) and store them frozen at -20°C for a maximum period of 30 day or -70°C for longer periods. Avoid repeated freezing / thawing cycles.
11. When using frozen samples, thaw the samples just before the extraction step in order to avoid the nucleic acid degradation.
12. The whole peripheral blood samples for DNA extraction must be collected in EDTA according to laboratory advices, transported and stored at +2 / +8 °C for a maximum period of 3 days. Do not freeze the whole peripheral blood samples to avoid cell lysis and viral titre loss.

H. PREPARATION OF COMPONENTS AND WARNINGS

Master Mix:

Component A. Ready to use. Mix well on vortex before use and centrifuge briefly to collect the whole volume.

WARNING: Component A is light sensitive. Protect it from strong light exposition.

Primers/Probes:

Component B.

- Centrifuge the vial for 1 min at 2000 rpm.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized Component B with the volume of Component C (Code: ALL/C) indicated on the vial label and in the Section D table.
- Keep it on the benchtop for at least 10 min at room temperature (15°C <RT< 25°C)

WARNING: Component B is light sensitive. Protect it from strong light exposition.

MG Water :

Component C. Ready to use.

Negative Control :

NTC. Ready to use.

Standard Curve:

STD.

- Centrifuge the vial for 1 min at 2000 rpm.
- Open carefully the vial cap avoiding powder dispersion.
- Dissolve homogenously the Lyophilized STD with the volume of Component C (Code: ALL/C) indicated on the vial label and in the Section D table of the IFU.
- Keep it on the bench top for at least 10 min at room temperature (15°C <RT< 25°C)
- Prepare the Nucleases-Free vials for the preparation of the Standard Curve
- Set up an STD 1:10 serial dilution in Component C (Code: ALL/C) to obtain the standard curve points as described in the table below:

Standard curve preparation		
STD	Calibrator 60000 copies/ µl	Add the Volume of Component C (Code: ALL/C) as written on the vial label
STD 1	6000 copies/ µl	10 µl (STD) + 90 µl Component C (Code: ALL/C)
STD 2	600 copies/ µl	10 µl (STD 1) + 90 µl Component C (Code: ALL/C)
STD 3	60 copies/ µl	10 µl (STD 2) + 90 µl Component C (Code: ALL/C)
STD 4	6 copies/ µl	10 µl (STD 3) + 90 µl Component C (Code: ALL/C)

Internal Control:

I.C.

Important Note: Dissolve with 300 ul of Component C ALL/C

- Centrifuge the vial for 1 min at 2000 rpm.

- Open carefully the vial cap avoiding the powder dispersion.
- Dissolve homogenously the Lyophilized I.C. with the volume of Component C (Code: ALL/C) indicated in the Section D table of the IFU
- Keep it on the bench top for at least 10 min at room temperature (15°C <RT< 25°C)

I. INSTRUMENTS AND TOOLS USED IN COMBINATION WITH THE KIT

1. **Micropipettes** have to be calibrated and must be submitted to regular decontamination (household alcohol, 10% solution of bleach, hospital grade disinfectants) of those parts that could accidentally come in contact with the sample. They should also be regularly maintained in order to show a precision of 1% and a trueness of +/-5%.
2. **Extraction Device:** The CMVDNAQT.CE Kit is intended for the use in combination only with QIAamp DNA Minikit Code.51306 (QIAGEN) and Nucleospin Blood kit Code: 740951. The end users must strictly follow the Instruction for use supplied by the manufacturers.
3. **Real-Time Thermocyclers.** The CMVDNAQT.CE Kit is intended for the use in combination only with the Real Time Thermal cyclers ABI PRISM 7500 (Applied Biosystems), and MX3000P (Stratagene) and MiniOpticon (Biorad) thermocyclers.
The end users must strictly follow the Instruments Instruction for use supplied by the manufacturers.

L. PRE ASSAY CONTROLS AND OPERATIONS

1. Check the expiration date of the kit printed on the external label of the kit box. Do not use it if expired.
2. Check that the liquid components are not contaminated by naked-eye visible particles or aggregates. Check that on the bottom of the Lyophilized component vials is present a well formed aggregate. Check that no breakage occurred in transportation and no spillage of liquid is present inside the box.
3. Dissolve the Lyophilized Components with the appropriate amount of Component C (Code: ALL/C) as described in the proper section (H).
4. Turn the Thermalcyclers on, check the settings and be sure to use the right assay protocol.
5. For the correct setting of the Real-Time Thermalcyclers follow strictly the Instrument Manual supplied by the manufacturers.
6. Check that the micropipettes are set on the required volumes.
7. Check that all the other equipment is available and ready to be used.
8. In case of problems, do not proceed further with the test and advise the supervisor.

M. ASSAY PROCEDURE

The assay has to be carried out according to what reported here below.

M.1 DNA extraction

The extraction step of the CMV genomic DNA has to be carried out exclusively in combination with the following kits:

Material	Description	Kit code	manufacturer
Plasma/Blood/ Amniotic fluid	Nucleospin Blood	740951	MN™
Plasma/Blood/ Amniotic fluid	QIAamp DNA mini kit®	51306	Qiagen™

NB: Before DNA isolation centrifuge the Amniotic fluid samples (10,000g, 5min), remove supernatant and dissolve the pellet in 200 ul of sterile PBS.

The DNA isolation must be carried out only according to the Instruction Manual supplied by the Manufacturer (QIAGEN™, MN™).

WARNING: The following volumes have to be strictly used in the extraction procedures for both the kits:

Sample Extraction volume : 200 µl
Elution Volume: 100 µl

The DNA extracted from the samples, not used in the run, has to be stored frozen (-20°C....-80°C).

Important note: The IC of the CMVDNAQT.CE Kit can be used in the isolation procedure as extraction control. The Internal Control Ct value for the negative samples is used to evaluate if the DNA extraction procedure has been performed correctly (see section P).

For this application add 5 µl of I.C. to the lysis buffer and sample mixture and proceed following the instruction manual supplied by the manufacturer of the Extraction Kit.

M.2 Setting up of the reaction

CMVDNAQT.CE kit is intended to be used exclusively in combination with ABI 7500 standard (Applied Biosystem) and MiniOpticon (Biorad) and MX3000P (Stratagene).

M.2.1 Preparing the PCR

Important: An example of dispensation scheme is reported in Section N. Please, refer to it before starting the operations described here below.

- Prepare the components as described in Section H;
- Prepare the required number of reaction tubes or a 96-well reaction plate for the samples under evaluation and for the Standard curve (prepared as described in section H).

Important note: Use only optical tubes or microplates suggested by the Real-Time thermalcyclers manufacturers.

- Consider that the samples, if possible, should be tested in duplicate;
- Include at least 1 tube for the NTC (negative control)
- Prepare the **Amplification Mix** for **Samples, NTC and standard curve** as table below:

Preparation of the Amplification Mix (I.C. as Amplification control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
I.C.	Internal Control	0,5 µl	6 µl
Tot vol.		15 µl	180 µl

Important note: If the Internal Control was added during the DNA isolation procedure, prepare the **Amplification Mix** for **Samples, NTC and standard curve**, as in table below:

Preparation of the Amplification Mix (I.C. as Extraction/Amplification control)

Number of Reactions		x1	x12
A	Master mix	12,5 µl	150 µl
B	Primers/probes	2 µl	24 µl
C	MG Water	0,5 µl	6 µl
Tot vol.		15 µl	180 µl

M.2.2 Amplification procedure

- Dispense 15 ul of the amplification mix in each reaction tube or microplate well
- Add 10 ul of the **Samples, NTC and standard curve** to the reaction tubes.
- Close firmly the reaction tubes
- Centrifuge briefly the reaction tubes at 2000 rpm
- Don't leave the reaction tubes at room temperature (RT) for more than 30 minute and at light exposure (cover the tubes).
- Load the tubes in the Real-Time Thermalcycler Thermoblock Holder.
- After the setting operations described in the Section M5 (Instrument Programming) start the Thermalcycler run.

Important note: The Lyophilized Components after dissolution in Component C (Code: ALL/C) are stable no more than 3 hours kept in ice or at 2°...8°C.

At the end of the working day discard adequately the material leftover of the STD Dilution Points.

The not used volume of Component B, STD and I.C. can be split in aliquots and kept frozen at -20°C. The aliquots must be used within seven (7) days from the freezing day.

M.3 Instrument programming

For programming the instrument refer to the Instrumentation Instruction Manual provided by the manufacturers.

Important Note: For Mx3000P set "Filter set gain settings" : ROX = x1, FAM = x8, HEX/JOE = x1. (see MxPro™ QPCR Software Instruction Manual, p.41)

M.3.1 Thermal Profile

The thermal profile is reported in the table below:

Step	Cycle	Temp.	Time
1	1	50°C	2 min
2	1	95°C	10 min
3	50	95°C	15 sec
		64°C (*)	1 min

IMPORTANT NOTE: (*) step for the real time data collection

WARNING: Keep attention to program the Real-Time Thermocycler with the correct Thermal Profile following the Instrument Manual supplied by the Instrument manufacturer.

M.3.2 Selection of the Detectors

Following the Instruction manuals of the Real-Time thermocyclers suggested (ABI 7500, Biorad MiniOpticon and MX300P Stratagene) select the Detectors reported in the table here below:

Detection	Reporter	Quencher
CMV	FAM	Non Fluorescent
Internal Control (I.C.)	JOE/HEX	Non Fluorescent
Passive Reference	ROX	Not Present

WARNING: Keep attention to program the Real-Time Thermocycler with the correct settings following the Instrument Manual supplied by the manufacturer.

N. ASSAY SCHEME

An example of dispensation scheme for the Quantitative Analysis is reported below:

Microplate or tubes

	1	2	3
A	STD 1 6000 copies/μl	Sample 4	
B	STD 2 600 copies/μl	Sample 5	
C	STD 3 60 copies/μl	Sample 6	
D	STD 4 6 copies/μl	Sample 7	
E	NTC	Sample 8	
F	Sample 1	Sample 9	
G	Sample 2	Sample 10	
H	Sample 3	Sample 11	

Legenda: NTC = Negative Control STD 1,2,3,4 = CMV DNA Standard Curve, Sample 1,2,3 = Samples under evaluation.

O. INTERNAL QUALITY CONTROL

O.1 Pre- Analysis setting

Before starting the interpretation of the data:

- Set the "Baseline" (the background fluorescence level) as reported in the table below:

"Baseline"	
ABI™PRISM® 7500 SDS	Auto Baseline
BIORAD™ MiniOpticon®	Average Over Cycle Range: Cycle Range from cycle n°3 to cycle n°15
STRATAGENE™ MX3000P®	Adaptive Baseline Important Note: <i>Do not use Mx4000 v1.00 to v3.00 algorithm</i>

- Set manually the FAM/JOE/HEX fluorescence "Threshold"

FAM fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	0.1
BIORAD™ MiniOpticon®	0.06
STRATAGENE™ MX3000P®	0.1

JOE/HEX fluorescence "Threshold"	
ABI™PRISM® 7500 SDS	0.1
BIORAD™ MiniOpticon®	0.02
STRATAGENE™ MX3000P®	0.02

O.2 Data Analysis

A check is carried out on the STD calibrators any time the kit is used in order to verify whether their Ct values are as expected and reported in the table below.

Check FAM	Requirements
STD 1	$23 \leq Ct \text{ (Threshold Cycle)} < 26$
STD 2	$26 \leq Ct \text{ (Threshold Cycle)} \leq 29$
STD 3	$30 \leq Ct \text{ (Threshold Cycle)} < 33$
STD 4	$33 \leq Ct \text{ (Threshold Cycle)} \leq 36$

Moreover the Slope and R2 values are checked in order to verify the quality of the run. The following requirements must be fulfilled.

Check FAM	Requirements
Slope	$-3.1 < \text{Slope} < -3.9$

Check FAM	Requirements
Efficiency	$R^2 > 0.98$

P. INTERPRETATION OF THE RESULTS AND TROUBLESHOOTING

For each samples FAM fluorescence (positive/negative Ct value) and Internal Control JOE fluorescence are assumed to validate CMV detection as described in the table below:

CMV FAM	Internal Control JOE/HEX	Assay Result
SAMPLE POSITIVE	+	CORRECT
	-	CORRECT*
SAMPLE NEGATIVE	Ct < 42	CORRECT
	Ct > 42 or undetermined	INVALID**

*High Initial concentration of CMV DNA in the sample (Positive FAM Signal) can lead to REDUCED or an ABSENT Fluorescent Signal for Internal Control I.C. due to the reagents Competition.

** Problems may be occurred during the amplification step (inefficient or absent amplification) or during the extraction step (presence of inhibitors or initial sample containing an insufficient number of cells) leading to an incorrect result. The test procedure must be repeated starting from the Extraction step using a fresh sample coming from the patient.

For each positive samples detected by kit code CMVDNAQT.CE a correct Quantitation of the viral load can be applied within the 6.0E+08 copies/ul and the 7.5E-01 copies/ul .
CMV viral load must be expressed as reported in the table below:

Sample CMV run data (copies/μl)	CMV viral load (copies/μl)
Quantity > 6.0E+08	CMV viral load >6.0E+08
7.5E-01 ≤ Quantity < 6.0E+08	QUANTITATION
Quantity < 7.5E-01	CMV viral load < 7.5E-01

IMPORTANT NOTE: For samples quantitation refer to [section Q](#)

The results obtained with the CMVDNA.CE Kit must be interpreted by the responsible of the laboratory keeping in consideration the clinical symptoms of the patients and the other laboratory markers of Infection.

The following results are possible:

Troubleshooting table

	FAM	JOE/HEX	Result	CHECK
SAMPLE unknown	+	+/-	CORRECT RESULT <u>Positive</u>	IMPORTANT: High Initial concentration of CMV DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of the Internal Control I.C. due to the reagents Competition.
SAMPLE unknown	-	-	ATTENTION ! POSSIBILITY OF: Inhibition, error in the procedure or malfunctioning of the Instruments	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the selected detection dyes are FAM for the CMV detection and JOE/HEX for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube 7. that the Extraction procedure have been executed correctly;
SAMPLE unknown	-	+	CORRECT RESULT <u>Negative</u>	
STD	+	+/-	CORRECT RESULT	IMPORTANT: High Initial concentration of CMV DNA (Positive FAM Signal) can lead to REDUCED or ABSENT Fluorescent Signal of the Internal Control I.C. due to the reagents Competition.
STD	-	-	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. that the selected dyes are FAM for the CMV detection and JOE/HEX for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly; 6. that no potential PCR inhibitors have been contaminated the tube

STD	-	+	ATTENTION ! POSSIBILITY OF: Error in the pipetting or in the procedure	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the selected detection dyes are FAM for the CMV detection and JOE/HEX for the I.C. detection; 4. that the Analysis has been run with the correct Instrument settings; 5. that the kit has been stored correctly;
NTC	-	+	CORRECT RESULT	
NTC	+	+/-	ATTENTION ! POSSIBILITY OF: Contamination	1. that the components have been prepared correctly 2. that no mistake has been done in the assay procedure; 3. That the work space and Instruments are decontaminated at regular intervals; 4.;that the kit has been stored correctly;

Important notes:

1. Interpretation of results has to be done under the supervision of the laboratory Responsible to reduce the risk of judgment errors and misinterpretations.
2. When the test results are transmitted from the laboratory to an informatics centre, attention has to be paid to avoid erroneous data transfer.

If the results of the test match to the CORRECT ASSAY RESULT requirements stated above, proceed to the next section.

If one of more of the problems described in the table above happen, after checking, report any residual problem to the supervisor for further actions.

Q. QUANTITATION

The STD calibrators are treated as patient samples and the same volume, 10μl, is used during the amplification step.
The STD calibrators concentration is expressed in copies/μl.
The **Viral Genome Concentration per mL** for each patient specimen is calculated applying the following formula:

$$\text{Results (copies/ml)} \equiv \frac{\text{copies/}\mu\text{l (run data)} \times \text{Elution sample volume (}\mu\text{l)}}{\text{Sample Extraction volume (ml)}}$$

Example:

$$\text{Results (copies/ml)} \equiv \frac{1500 \times 100}{0.2}$$

$$\text{Results (copies/ml)} \equiv 7.5 \text{ E}+05$$

R. PERFORMANCES

Evaluation of Performances has been conducted in accordance to what reported in the Internal Technical Specifications or ITS.
The performance evaluation was carried out in DiaPro's laboratories on materials supplied by the reference clinical labs.

R.1 ANALYTICAL SENSITIVITY

Analytical sensitivity may be expressed as **Limit of Detection** and as **Limit of Quantitation**.

Limit of detection (LOD): it is the lowest amount of target that can be detected by the system with a stated probability.

For the NAT tests it is expressed as the smallest concentration of the **analyte** that tested in multiple repetitions gives a positive result.

The **limit of detection (LOD)** is determined by testing serial dilutions containing known concentrations of the analyte.

The **LOD** is the lowest concentration of analyte that can be consistently detected (e.g. in $\geq 95\%$ of samples under routine laboratory conditions).

For the kit CMVDNAQT.CE the **LOD** has been determined by testing 1:5 and 1:2 serial dilutions (8 replicates for three different runs) of a plasmid carrying the viral target sequence.

The results were analyzed by a **Probit** analysis, to determine the detection limit at 95%.

The results of the PROBIT Analysis are the following:

LOD Limit of Detection ($p=0.05$)	
ABI™PRISM® 7500 SDS	0.45 copies/ μ l
BIORAD™ Miniopticon®	0.45 copies/ μ l
STRATAGENE™ MX3000P®	0.45 copies/ μ l

R1.1 Limit of quantitation

The **Limit of Quantitation** was determined by measuring the **linearity**, the **dynamic range** and the **reproducibility**.

The **Linearity** is the measure of the degree to which a curve approximates a straight line. It is expressed with the **SLOPE** value.

The **dynamic range** is the span of analyte concentrations for which the final output value (C_t threshold cycle) of the system is directly proportional to the analyte concentration, with acceptable trueness and precision.

The boundaries of the dynamic range are the lower and upper limits of quantitation (**Limit of quantitation**).

For the kit CMVDNAQT.CE a limiting dilution curve with defined copies/ μ l of a plasmid carrying the specific target viral sequence were prepared. The dilution points were tested in the analytical system and their C_t (threshold cycle) determined.

The upper **limit of quantitation** is $8.78 \log_{10}$ ($6.0E+08$ copies/ μ l) and the lower limit of quantitation is $-0.12 \log_{10}$ ($7.5E-01$ copies/ μ l).

R.2 ANALYTICAL SPECIFICITY

The Analytical specificity is the ability of the method to detect only the target DNA sequence.

The analytical specificity of CMV DNA assay has been studied as follow:

1. The primer/probe Set has been choose analysing the genome target sequence with an appropriate software (LionSoft v.1.0 supplied by Biotools and Primer Express v.3.0 supplied by Applied Biosystem Inc.).
2. The primer/probe Set and the target genome sequence has been controlled by the "BLAST" software, in order to check if any of the nucleotide sequences deposited in the worldwide genomic banks has any homology with CMV, and by the "ClustalX" software, in order to compare the genome target sequences of the different genotypes of CMV.
3. The specificity was improved through the selection of stringent reaction conditions.
4. Samples coming from patients suffering infections due to potential interfering organisms were obtained from a Reference Clinical Centre and tested.

The results are reported in the following table:

Organism	Result
VZV	negative
EBV	negative
HHV6	negative
HHV8	negative
HSV1	negative
HSV2	negative

R.3 DIAGNOSTIC SPECIFICITY AND SENSITIVITY

R.3.1 Diagnostic Specificity:

The Diagnostic specificity is the probability that the device gives a negative result in the absence of the target marker. So the **true negative** sample is a specimen known to be negative for the target marker and correctly classified by the device

This parameter was studied by examining 50 CMV DNA negative plasma samples:

TRUE NEGATIVES	50
FALSE POSITIVES	0
TOTAL SAMPLES	50
SPECIFICITY %	100

On the basis of the results obtained Diagnostic Specificity of the system has been calculated $\geq 99\%$.

R.3.2 Diagnostic Sensitivity

Diagnostic sensitivity is the probability that the device gives a positive result in the presence of the target marker. So the **true positive** sample is a specimen known to be positive for the target marker and correctly classified by the device.

For the kit code CMVDNAQT.CE the parameter was studied by examining 30 CMV DNA positive plasma samples.

The samples have been studied in duplicates in the same run and then it has been calculated the percentage (%) of positive samples.

CMV DNA Positive samples

TRUE POSITIVES	30
FALSE NEGATIVES	0
TOTAL SAMPLES	30
SENSITIVITY %	100

In addition, the QCMD 2002 Cytomegalovirus Panel was tested The Panel contains 10 positive samples (8 plasma and 2 amniotic fluid) and 2 negative samples (1 plasma and 1 amniotic fluid).

On the basis of the results obtained the Diagnostic Sensitivity of the system has been calculated in the 100%.

Diagnostic Sensitivity	100 %
Diagnostic Specificity	> 99.5 %

R.4 PRECISION

Precision shows the degree of the system's reliability. Every measurement procedure has an inherent random variation called "random error". Random error does not have a number value but it is determined by dispersion of measurement as standard deviation (DevST) and coefficient variation (CV%). Usually precision of an assay refers to the agreement between replicate measurements of the same material.

In the kit CMVDNAQT.CE, **precision** was expressed as intra-assay variability and inter-assay variability. 4 dilution points in 8 replicates were tested in the same run (intra-assay) and in three different runs (inter-assay).

On the basis of the results obtained Intra and inter-assay variability were then calculated.

In absence of established International parameters we have identified the following value of acceptability for the CMVDNAQT.CE Kit:

Intra-Assay Coefficient Variation (CV%) ≤ 10%.

Inter-Assay Coefficient Variation (CV%) ≤ 10%.

S. LIMITATIONS

The End user of the kit is advised to carefully read and understand this package insert. Strict adherence to the protocol is necessary in order to obtain reliable test results. In particular, accurate sample and reagent pipetting, application of a correct workflow along with careful programming of thermalcycling steps are essential for accurate and reproducible CMV DNA detection and quantitation.

The determination of the CMV DNA in a patient sample has extensive medical, social, Psychological and economic implications. It is recommended that confidentiality, appropriate counselling and medical evaluation be considered as an essential aspect of the testing sequence.







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5. Symbols

LEGENDA			
REF	Product code		Storage temperature
RUO	For Reseaech Use Only		See use instructions
LOT	Lot number		Manufacturer
	Expiry date		Number of tests
			Date of manufacturing

Produced by
Dia.Pro. Diagnostic Bioprobes Srl.
via Columella n°31 – Milano - Italy

RUO