



quany CMV (IEA region)

REF: RT-12

Detection and quantification of the Cytomegalovirus genome with Real Time PCR

INTRODUCTION AND PURPOSE OF USE

The quany CMV system is a quantitative test that allows the DNA amplification and quantification, by means of Real Time PCR, of IEA region of CMV genome.

The Procedure allows the detection of the DNA target by means a genomic amplification reaction. The analysis of the results is made by an instrument of Real Time PCR, composed by a thermal cycler with a system of fluorescence detection.

CONTENT

The kit contains reagents enough to perform 48 amplification tests

Quantity	Description
R1 3 x 200 µl	Amplification mMix dNTPs, Tris-HCl, KCl, MgCl ₂ , Taq Polymerase, Nuclease-free water (Blue plug)
R2 3 x 120 µl	CMV probes Mix Upstream primer, downstream primer, Target probe (FAM), Internal control (β-globina) Probe (VIC) Nuclease-free water (Green plug)
R3 3 x 35 µl	Cloned DNA corresponding to the IEA region at the concentration of 10 ⁵ copies/µl
R4 3 x 35 µl	Cloned DNA corresponding to the IEA region at the concentration of 10 ⁴ copies/µl
R5 3 x 35 µl	Cloned DNA corresponding to the IEA region at the concentration of 10 ³ copies/µl
R6 3 x 35 µl	Cloned DNA corresponding to the IEA region at the concentration of 10 ² copies/µl
R7 1 x 30 µl	Negative Control (Yellow plug)

MATERIALS AND STRUMENTATION REQUIRED BUT NOT SUPPLIED

Disposable latex powder-free gloves or similar material;
Bench microcentrifuge (12,000 - 14,000 rpm);
Micropipettes and Sterile tips with aerosol filter;
Vortex;
Plastic materials (microplate and optica adesive cover);
Real Time PCR Instrumentation.

ACCESSORY PRODUCTS

EX05 – DNA Extraction from Blood, Uretral, Vaginal and Cervical Swab

The kit allows the DNA extraction from Human samples. The kit contains reagents enough to perform the DNA extraction for 50 samples.

SAMPLES AND STORAGE

The quany CMV system must be used with extracted DNA from the following biological samples: Blood and plasma EDTA. Collected samples must be shipped and stored at +2 - +8°C and used within 3 days from the collected data.

Store the sample at -20°C if it is used after 3 days.

PRECAUTIONS USE

This kit is for in vitro diagnostic (IVD), for professional use only and not for in vivo use.

After reconstitution of the amplification master mix, it must be used in once (16 reactions). Do not re-frost already used material.

At all times follow Good Laboratory Practice (GLP) guidelines.

Wear protective clothing such as laboratory coats and disposable gloves while assaying samples.

Avoid any contact between hands and eyes or nose during specimens collection and testing.

Handle and dispose all used materials into appropriate bio-hazard waste containers. It should be discarded according to local law.

Keep separated the extraction and the reagents preparation.

Never pipette solutions by mouth.

Avoid the air bubbles during the master mix dispensing. Eliminate them before starting amplification.

Wash hands carefully after handling samples and reagents.

Do not mix reagents from different lots.

It is not infectious and hazardous for the health (see Material Safety data Sheet – MSDS).

Do not eat, drink or smoke in the area where specimens and kit reagents are handled.

Read carefully the instructions notice before using this test.

Do not use beyond the expiration date which appears on the package label.

Do not use a test from a damaged protective wrapper.

LIMITS OF THE METHOD

The extreme sensitivity of gene amplification may cause false positives due to cross-contamination between samples and controls. Therefore, you should:

- physically separate all the products and reagents used for amplification reactions from those used

for other reactions, as well as from post-amplification products;

- use tips with filters to prevent cross-contamination between samples;
- use disposable gloves and often change them;
- carefully open test tubes to prevent aerosol formation;
- close every test tube before opening another one.

The proper functioning of the amplification mix depends on the correct collection, correct transportation, correct storage and correct preparation of a biological sample.

As with any diagnostic device, the results obtained with this product must be interpreted in consideration of all the clinical data and other laboratory tests done on the patient.

As with any diagnostic device, there is a residual risk of obtaining invalid results, false positives and false negatives with this product.

STORAGE AND STABILITY

Store the product quany CMV (IEA region) at -20°C.

An intact and well stored product has a stability of 24 months from the date of production.

Do not use beyond the expiration date which appears on the package label.

ANALYTICAL PROCEDURE

ABI 7500 Instrument

Software setting:

Turn the instrument and the computer on and open the control software. Click on “Advance Setup”: by default the software will show the page “experiment properties”. Write in the “experiment name” the file name, choose the type of instrument (ABI7500 o ABI7500fast), the type of reaction (quantitation standard curve), the type of used reagent (Taqman•Reagents) and the reaction time of analysis (Standard ~ 2 hours to complete a run).

Open the page named “page setup” (sheet Define Target and Samples).

In the window Define Targets set:

CMV probe:

Reporter = FAM; Quencher = TAMRA

IC (β-globina) probe:

Reporter = VIC; Quencher = TAMRA

Set the samples’ name in the window “Define Samples”.

In the same page “plate setup” select the sheet “Assign Target and Samples”. On the screen you will see the microplate draft.

Select an area of the plate where the controls will be placed: select wells of the plate and set both targets (CMV and b-globin). Select “Assign target to selected wells” in the blank, the “task Standard (S)” for CMV target and set the controls’ concentration.

Choose an area in the plate where negative control will be placed: select “Assign target to selected wells” in the blank, the “task Negative (N)” for the CMV target.

Select an area of the plate where samples will be placed: select the wells and set both targets (CMV and β-globine). Link every well to a sample, through the window “Assign samples to selected wells”.

For each sample, select in the blank “Assign targets to selected wells” the “task UnKnown (U)” for the CMV target.

S1		C1		C9					
S2		C2		C10					
S3		C3		C11					
S4		C4							
		C5							
		C6							
		C7							
N		C8							

Set ROX as passive reference, using as normalizer of detecting fluorescence.

Open “Run Method” (sheet Graphic View) and set the right thermal cycling:

cycles	denaturation	annealing/extension
1	50° C 2 min	
1	95° C 10 min	
45	95° C 15 sec	60° C 1 min

In the window “Reaction volume plate per well” set a volume of 25 µl.

After making the plate, and correctly inserting it in the instrument, press the button “Start Run”.

Preparation of the reactions:

Defrost a tube of Amplification mMix;

Defrost a tube of CMV probes Mix;

Mix carefully by vortex 200 µl of Amplification mMix and 120 µl of CMV probes Mix (the mix as produced is enough to prepare 16 reactions of amplification: 4 positive controls, 1 negative control and 11 samples).

Distribute, in the amplification plate, 20 µl of just reconstituted mix in chosen positions and already settled on the instrument software.

Distribute, in the negative control position, 5 µl of solution taken by the negative control vial.

Distribute, in chosen position for each sample, 5 µl of corresponding sample.

Distribute, in chosen positions for the positive controls, 5 µl of 10² copies/µl solution, 10³ copies/µl solution, 10⁴ copies/µl solution and 10⁵ copies/µl solution.

Seal up accurately the plate using an optical adhesive film and verify that there aren't air bubbles in the mix, because of amplification interferences.

Transfer the plate in the instrument and push the button "Start Run".

INTERPRETATION OF RESULTS

The use of positive and negative control in each amplification session allows to verify the correct functioning of the amplification mix and the absence of any contamination.

The instrument software is able to analyze the fluorescences that are emitted by the specific probe for CMV (FAM) and by the specific probe for the positive internal control (β-globina VIC). A proper results' analysis needs a correct settings of the instrumentation. For this aim, set:

Baseline fluorescence level from cycle 6;

Threshold for FAM detector: 0.2

Threshold for VIC detector: 0.09

The software uses Ct values obtained from 4 controls with known concentration for calculating the calibration curve and for interpolating unknown samples on it.

A proper functioning of the amplification mix can be verified analyzing these parameters:

Parameter	Reference
RTS conc. 10 ⁵ copies (FAM)	Ct ≤ 25
Correlation coefficient	0.990 ≤ r ² ≤ 1
Slope	-3,6 ≤ Slope ≤ -3.2
Efficiency di PCR	90 ≤ Efficiency ≤ 100

If the result of the amplification reaction of RTS at 10⁵ copies concentration produces a Ct > 25 or undetermined or if the correlation coefficient value (r²), the slope and then the reaction efficiency don't enter in the table indicated limits, the session can't be considered valid and so it must be cancelled.

Be sure that there isn't any specific fluorescence increasing for examining target in negative control (FAM).

In the amplification reaction of each sample, the Ct values for the internal control (β-globina) specific probe are used for validating the analysis session. Beginning from extraction process until detection stage. Be sure that emitted fluorescence from internal control amplification hasn't got a Ct > 35 or undetermined. If a sample presents an undetermined CMV DNA and internal control Ct > 35 means that some problems happened in the extraction stage or in the amplification stage; therefore the sample could be a false negative. **Repeat the sample.** We can evaluate as valid the samples with a Ct > 35, if we consider internal control, and a high concentration of CMV DNA. In this case, the competitive nature of PCR reaction can hide or disadvantage the internal control amplification.

FAM Detector	VIC Detector	Assay	Sample
undetermined Ct	Ct > 35 or undetermined	Not valid	Repeat
undetermined Ct	Ct < 35	Valid	Negative
positive Ct	Ct < 35	Valid	Positive
low Ct	Ct > 35 or undetermined	Valid	High Positive

QUANTITATIVE ANALYSIS




Through Real Time PCR reaction it is possible to give the DNA quantification of CMV DNA, with the correct settings of positive controls values, that compose the calibration curve. This setting has to consider all the dilutions and the passages that the sample has to be undergone during extraction and amplification stages.

The system can take over from 1.000.000 to about 10 copies of DNA a reaction.

When the extraction system is used as suggested in technical sheet, it is possible to set on software these parameters and to obtain the results in terms of copies/ml directly:

	RTS 1	5.000.000 copies
	RTS 2	500.000 copies
	RTS 3	50.000 copies
	RTS 4	5.000 copies

When alternative systems are used it is suggested to use these concentrations:

	RTS 1	100.000 copie
	RTS 2	10.000 copies
	RTS 3	1.000 copies
	RTS 4	100 copies

In this case, sample concentration expressed in copies/ml will be found using this formula:

$$copie/ml = \frac{1000}{V_e} \times \frac{E_v}{E_a} \times C_{react}$$

where:

- **Ve:** extracted sample Volume
- **Ev:** eluted sample Volume during extraction stage
- **Ea:** extracted sample Volume used for amplification
- **C_{react}:** copies provided from instrument.

PERFORMANCES

Analytical sensitivity:

Limit of sensitivity

For the purposes of this evaluation is considered as analytical sensitivity the highest serum dilution (title) to which a positive sample can be subjected without the system losing the ability to detect it as positive. The analytical sensitivity of the system was assessed by analyzing plasmid DNA (pCM5018), quantified by spectrophotometric analysis, containing the genomic regions of interest (IEA region) of the virus in serial dilutions from 100.000 copies to 1 copy of DNA in 5µl of extracted material added in the amplification reaction.

Concentration	1 copy	10 copies	100.000 copies
N° of Tests	15	15	15
N° of Positive	3	15	15
N° of Negative	12	0	0

Analytical sensitivity enables to detect 10 copies of CMV DNA in 5µl of extracted material added in the amplification reaction.

Clinical sensitivity:

For the purposes of this evaluation is considered as clinical sensitivity the skill of determining true positives in the totality of positive screened samples. The analysis is made on CMV positive samples and the test is performed following the method advices. Positive samples are confirmed with another disposable method.

Samples	N	Positive	Negative
Donors EDTA Blood	5	5	0

Obtained results show a clinical sensitivity of 100%.

Linearity/Proportionality

System linearity is valued analyzing plasmidic DNA (pCM5018), quantified by spectrophotometric analysis, containing the genomic regions of interest (IEA region) of the virus in serial dilutions (1:10) from 100.000 copies to 1 copy of DNA in 5µl of extracted material added in the amplification reaction. The evaluation is performed analyzing 10 calibration curves, that showed these parameters:

RTS conc. 10 ⁵ copies (FAM)	Ct ≤ 25	medium Ct 23.45
Correlation Coefficient	0.990 ≤ r ² ≤ 1	medium r ² 0.9986
Slope	-3,6 ≤ Slope ≤ -3.2	medium slope - 3.479
PCR	90 ≤ Efficiency ≤ 100	medium Eff. 94%

Reproducibility and Repeatability:

The reproducibility and repeatability of the system are valued analyzing 3 dilutions of plasmidic DNA containing the IEA region of interest for CMV e quantified by spectrophotometric analysis (pCM5018) and 1 negative control (negative DNA). For each session 5 replicates are made for 3 different sessions, made by different workers, with 3 different lots.

Hypothetical value	Lot	N° repetitions	Med. Reveal. Conc.	Inaccuracy %
100.000 copies	L1	5	110944	11 %
100.000 copies	L2	5	106796	7 %
100.000 copies	L3	5	104803	5 %

Hypothetical value	Lot	N° repetitions	Med. Reveal. Conc.	Inaccuracy %
1.000 copies	L1	5	1083	8 %
1.000 copies	L2	5	1036	4 %
1.000 copies	L3	5	980	2 %

Hypothetical value	Lot	N° repetitions	Med. Reveal. Conc.	Inaccuracy %
10 copies	L1	5	13.9	39 %
10 copies	L2	5	12.5	25 %
10 copies	L3	5	12.6	27 %

The medium inaccuracy % is on 14%.

Diagnostic Specificity:

For the purposes of this evaluation is considered as diagnostic specificity the skill of the method of determining real negative samples. The diagnostic specificity of the system is valued analyzing human genomic samples tested and confirmed as negative with another disposable system.

Samples	N	Positive	Negative
Donors EDTA blood	20	18	2

Diagnostic specificity is 90% for material extracted from EDTA blood.

Analytical Specificity:

Test's specificity is guaranteed by the use of specific primers for determining CMV. The alignment of the choose regions for specific primers' hybridization for CMV with available sequences of the IEA region present in database, demonstrated: their conservation, the absence of significant mutations and the complete specificity for the analyzed target.

Cross-Reactivity:

The alignment of the choose regions for specific primers' hybridization for CMV with available sequences of the IEA region present in database, demonstrated: their conservation, the absence of significant mutations and the complete specificity for the analyzed target. Furthermore positive samples analysis is performed for other herpetic viruses and the test is made following the method advices.

Sample	Sample positive for	Result
1	HSV-1	< 10 copies
2	HSV-2	< 10 copies
3	VZV	< 10 copies
4	EBV	< 10 copies
5	HHV-6	< 10 copies
6	HHV-8	< 10 copies

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1	HSV-1	< 10 copies
2	HSV-2	< 10 copies
3	VZV	< 10 copies
4	EBV	< 10 copies
5	HHV-6	< 10 copies
6	HHV-8	< 10 copies

Sample	Sample positive for	Result
1	HSV-1	< 10 copies
2	HSV-2	< 10 copies
3	VZV	< 10 copies
4	EBV	< 10 copies
5	HHV-6	< 10 copies
6	HHV-8	< 10 copies

INTERFERENCES:

Verify that in DNA extracted from the sample there aren't nucleoproteins and haemoglobin, in way of exclude possible inhibition of PCR reactions. The interference due to contaminants can be highlighted through the spectrophotometric analysis and obtained data report at 260 nm (maximum absorbtion of Nucleic Acids) and 280 nm (maximum absorption of Proteins). A pure DNA might have a rate of approximately 1.8.

QUALITY CONTROL

It is therefore recommended to insert as quality control of every extraction session, amplification and detection of a negative sample and of a positive sample which have already tested before or referential material with known concentration.

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TROUBLESHOOTING

Presence, in the negative control, of the specific fluorescence for examining Target:









Possible causes	Solutions
Error displacing the positive sample or the positive control.	<ul style="list-style-type: none"> Use only micropipettes and Sterile tips with aerosol filter Always change tip between a sample and another Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use During analytical stage, distribute positive control for last During analytical stage, before distribute negative control
Contamination of the amplification mixes	<ul style="list-style-type: none"> Don't dispense the amplification mix with the same pipettes used in the extraction or detection area Use only micropipettes and Sterile tips with aerosol filter Clean surfaces and instruments with aqueous detergents, wash lab coats, replace test tubes and tips in use Close the amplification mix and return it at -20°C before open the other sample tubes
Badly sealed microplate	<ul style="list-style-type: none"> Seal very carefully the microplate
Mistake during the instrument setup of the plate	<ul style="list-style-type: none"> Check the correct correspondence between setting on the instrument and the real positions on the microplate (for samples, negative controls and standard)
Contamination of the preparation area for amplification reaction	<ul style="list-style-type: none"> Clean surfaces and instruments with aqueous detergents that can degrade DNA, wash lab coats, replace test tubes and tips in use

Failure or incorrect amplification of positive control:

Possible causes	Solutions
Possible mistakes in the dispensation of the positive control	<ul style="list-style-type: none"> Dispense carefully the positive control Check the calibration of micropipettes used Verify that the amount of positive control used corresponds to the method described in the Analytical Procedure (S_{MI})
Positive Control Degradation	<ul style="list-style-type: none"> Test the mixture through a new lot of positive control
Wrong heat protocol	<ul style="list-style-type: none"> Check the thermal cycler setting
The reaction mix is maintained at room temperature for too long	<ul style="list-style-type: none"> Keep the amplification mix on ice during the use and store it at -20 ° C immediately after finishing the preparation of test samples

Failure or incorrect amplification of internal control in testing samples:

Possible causes	Solutions
Presence of possible interferences	<ul style="list-style-type: none"> Carefully repeat sample extraction stage
Insufficient extracted DNA quantity	<ul style="list-style-type: none"> Carefully repeat sample extraction stage Accurately samples storage

	In vitro diagnostic device
	Read the instruction's manual
	Range of temperature
	Use within (dd/mm/yyyy: year-month)
	Lot (xxxx)
	Code
	Manufacturer
	Contains sufficient for <n> tests

EDMA code: 1504024000
CND: W0105040205



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