Diploma Thesis

Extraction of CMV DNA out of EDTA whole blood samples: comparison of automated platforms

Submitted by

Barbara Klara Maier

Mat.No.: 0433232

For receiving the academic degree

Medical Doctor, MD
(Dr. med. univ.)

at the

Medical University of Graz

Institute of Hygiene, Microbiology and Environmental Medicine

First supervisor: Prof. Dr. Harald H. Kessler

Graz, Juli 2011

Declaration of Originality

The undersigned, Barbara Klara Maier, certifies that, to the best of her knowledge, the following diploma thesis has been written only by the undersigned and without any assistance from third parties.
Furthermore, I confirm that no sources has been used in the preparation of this thesis other than those indicated in the thesis itself.

Graz, the signature

Acknowledgements

First of all, I would like to give a special thank to Prof. Dr. Harald H. Kessler for agreeing to be my supervisor and for his time, patience and understanding.
He supported me whenever I needed his advice and supplied ideas and suggestions throughout the writing process.

I would like to thank the wonderful team at the Molecular Diagnostics Lab at the Medical University of Graz, Bettina, Evelyn, Margit, Michael and Petra, for their good company and support. It was a pleasure to work with you.

I truely appreciate the help of Chris Lunde, thank you for supporting me with your ideas and knowledge.

I would like to thank all my friends who were there for me when I needed a break or an ear to listen.

Finally, I owe thanks to my parents who made it possible for me to study medicine.
A special thanks goes to my little brother Alexander Maier who supported and believed in me the entire time.

Table of Contents

1 Abstract 5
1.1 English 5
1.2 German 6
2 Introduction 7
2.1 Structure and Morphology of CMV 7
2.2 Genome 8
2.3 Life cycle 9
2.4 Tropism 10
2.5 Epidemiology 11
2.6 Transmission 12
2.7 Laboratory Diagnostics 12
2.7.1 Indirect diagnostics (serology) 12
2.7.2 Direct (molecular) diagnostics 13
2.8 Clinical manifestations 18
2.9 Treatment 18
2.10 Goals of the study 20
3 Materials and methods 21
3.1 Study design 21
3.2 Clinical specimens 22
3.3 Nucleic acids extraction 23
3.3.1 Extraction of CMV DNA on the NucliSens easyMAG 23
3.3.2 Extraction of CMV DNA on the QIAsymphony SP 25
3.3.3 Extraction of CMV DNA on the MagNA Pure LC 2.0 26
3.3.4 Extraction of CMV DNA on the VERSANT kPCR SP 27
3.4 Amplification and detection 29
3.4.1 The qPCR kit used in this study 29
3.4.2 qPCR on the LightCycler 480 II 31
3.4.3 qPCR on the VERSANT kPCR AD 32
3.5 Statistical methods 32

4 Results 33
4.1 Comparison of automated nucleic acids extraction systems..... 33
4.2 Comparison of CMV viral loads obtained with different extraction systems 35
4.3 Comparison of viral concentrations when amplifying with the VERSANT kPCR AD and the LightCycler 480 II..... 39
4.4 Comparison of times-to-result..... 40
5 Discussion 41
6 Conclusion..... 42
7 References..... 43
8 Figures and Tables..... 47

1 Abstract

1.1 English

Background: Detection and quantification of cytomegalovirus (CMV) DNA by real-time PCR (qPCR) is the standard method to detect early CMV infection. Because the qPCR technology is unlikely to be further improved, the current focus is on optimization of nucleic acid extraction.

Objectives: To evaluate and compare automated platforms useful for extraction of CMV DNA from different sample types including EDTA whole blood, plasma, urine, and bronchoalveolar lavage. To compare results obtained from different real-time PCR systems. To compare time-to-result when employing different assays.

Materials & Methods: Twenty clinical samples obtained from patients with CMV infection including 5 EDTA whole blood, 5 plasma, 5 urine, and 5 bronchoalveolar lavage samples were analyzed on 4 different automated extraction platforms (easyMAG, QIAsymphony, MagNA Pure LC 2.0, VERSANT kPCR SP). Nucleic acid extracts were amplified and detected on 2 different qPCR instruments, the LightCycler 480 II and the VERSANT kPCR AD.

Results: When the VERSANT kPCR SP instrument in conjunction with the VERSANT kPCR AD was compared to the alternative extraction systems, significantly higher CMV DNA concentrations were obtained for ETDA whole blood and plasma samples ($p<0.05$). When comparing the two amplification systems, the LightCycler 480 II gave significantly higher values for the majority of samples. The VERSANT kPCR SP has the longest total time-to-result.

Conclusion: With each assay and sample type, consistent results could be generated within an acceptable timeframe. It should be considered that a statistically significantly higher CMV DNA concentration does not necessarily imply clinical significance.

1.2 German

Hintergrund: Der quantitative Nachweis von Cytomegalievirus (CMV) DNA mittels real-time PCR (qPCR) stellt die Standardmethode zur Frühdiagnose einer CMV-Infektion dar. Da eine weitere Verbesserung der qPCR-Technologie in naher Zukunft nicht zu erwarten ist, liegt der Schwerpunkt derzeit auf einer Optimierung der Nukleinsäurenextraktion.

Ziele: Evaluierung und Vergleich von Nukleinsäurenextraktionsautomaten unter Verwendung von verschiedenen Probenmaterialien (EDTA-Vollblut, Plasma, Urin, und bronchoalveoläre Lavage). Vergleich von den mit verschiedenen qPCR-Instrumenten erhaltenen Resultaten. Vergleich der gesamten Analysenzeit der verschiedenen Assaykombinationen.

Methoden: Es wurden 20 klinische Proben (jeweils 5mal EDTA-Vollblut, Plasma, Urin und bronchoalveoläre Lavage) von PatientInnen mit CMV-Infektion unter Verwendung von 4 verschiedenen automatisierten Extractionssystemen (easyMAG, QIAsymphony, MagNA Pure LC 2.0, VERSANT kPCR SP) analysiert. Die extrahierten Nukleinsäuren wurden mit 2 verschiedenen qPCR-Instrumenten, dem LightCycler 480 II und dem VERSANT kPCR AD, amplifiziert und detektiert.

Ergebnisse: Im Vergleich zu anderen Extraktionssystemen wurde mit dem VERSANT kPCR SP in Kombination mit dem VERSANT kPCR AD signifikant höhere Werte bei ETDA-Vollblut und Plasmaproben beobachtet ($p<0.05$). Der Vergleich der beiden Amplifikationssysteme ergab für den LightCycler 480 II signifikant höhere Werte bei der Mehrzahl der Proben. Die längste Analysenzeit wurde bei Verwendung des VERSANT kPCR SP beobachet.

Schlussfolgerung: Alle untersuchten Testkombinationen ergaben für die verwendeten Testmaterialien gute Ergebnisse innerhalb einer akzeptablen Analysenzeit. Es sollte immer beachtet werden, dass statistisch signifikante Ergebnisse nicht unbedingt klinisch relevant sein müssen.

2 Introduction

The human cytomegalovirus (CMV) is a ubiquitous beta human herpesvirus type 5 that causes a lifelong subclinical infection in healthy adults. The virus may lead to morbidity and mortality in neonates and immunocompromised individuals. Its tropism for different cell types is responsible for CMV-associated diseases including mental retardation, retinitis, and vascular disorders (Dunn et al. 2003).
Congenital CMV infection is a major public health concern. The virus causes serious neurodevelopmental sequelae including cerebral palsy, mental retardation, and sensorineural hearing loss. Despite antiviral therapy, these injuries are often irreversible. The pathogenesis of injury to the developing fetal central nervous system (CNS) is unknown. Multiple mechanisms are proposed to play a potential role in CNS injury including CMV acting as a “teratogen” disrupting normal cellular differentiation and morphogenesis pathways and the impact on apoptosis and antiapoptotic mechanisms. Additional factors include the role of neural stem cells, the critical developmental windows of susceptibility, the role of the inflammatory processes in potentiating CNS injury, and the potential pathogenic impact of CMV on the endovascular system. Insights into the pathogenesis of CNS injury caused by CMV have been obtained from studies in primate, mouse, and guinea pig models (Cheeran et al. 2009).

2.1 Structure and Morphology of CMV

The CMV is an enveloped DNA-virus. The lipid bilayer envelope contains at least 20 viral glycoproteins that are involved in cell attachment and penetration (Mocarski et al. 2007). Viral glycoproteins include glycoprotein B (gB), gH, gL, gM, gN, and gO (Varnum et al. 2004). Mature virions range in diameter from 200 to 300 nm (Mocarski et al. 2007) (**Fig. 2.1**).
The viral tegument lines the space between the envelope and nucleocapsid. The tegument contains proteins that aid in viral DNA replication and evasion of the immune response (Chen et al. 1999). The function of the tegument proteins can

be separated into two classes: (i) proteins that play a structural role and (ii) proteins that modulate the host cell response to infection.
The icosahedral nucleocapsid encloses the viral DNA (Davison et al. 2003).

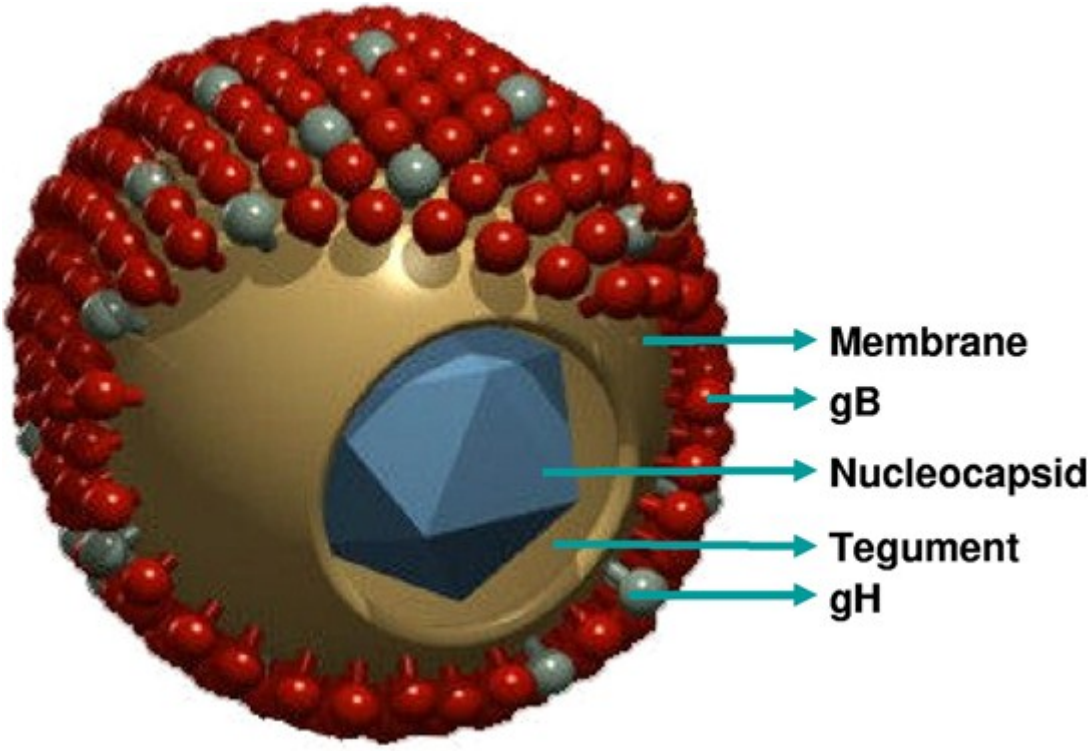

Fig. 2.1.: Three-dimensional model of CMV showing the different components of the virus (Reschke and Eickmann 1994).

2.2 Genome

CMV is a double-stranded positive linear DNA virus. Compared to other human herpesviruses, the CMV genome is the largest, with a genome size of approximately 235 kb encoding approximately 165 genes (Davison et al. 2003). The CMV genome consists of two regions of unique sequences, the unique long sequence (UL) and the unique short sequence (US), flanked by two sets of inverted repeats (TRL/IRL) and (IRS/TRS), respectively (Kotenko et al. 2000) (**Fig. 2.2**).
The different sections of the UL encodes several important proteins including the UL 83 section encoding the lower matrix phosphoprotein 65 (pp65), the UL82

section encoding the virion transactivator pp71, and the UL32 section encoding the maturation protein pp150 (Varnum et al. 2004).

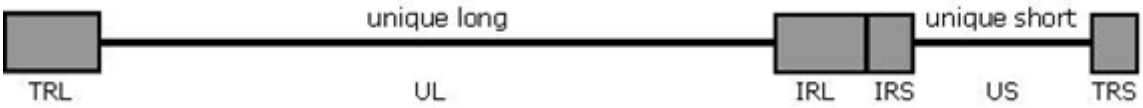

Fig. 2.2: Genomic structure of CMV. UL, unique long sequence; US, unique short sequence; TRL, terminal repeat long sequence; IRL, internal repeat long sequence; TRS, terminal repeat short sequence; IRS, internal repeat short sequence.

2.3 Life cycle

CMV enters human cells either through direct fusion or through the endocytic pathway. The virus attaches to the cell via interactions between viral glycoproteins (e.g., gB and gH) and one or more specific surface receptors (e.g., platelet-derived growth factor alpha), followed by the fusion of the envelope with the cellular membrane to release nucleocapsids into the cytoplasm (**Fig. 2.3**). These nucleocapsids are translocated into the nucleus, where viral DNA is released. This initiates the expression of IE-1/IE-2 genes. Furthermore, replicated viral DNA is encapsulated in the capsid. Secondary envelopment occurs at the endoplasmic reticulum (ER)-Golgi intermediate compartment. This is followed by a complex two-stage final envelopment and egress process that leads to virion release by exocytosis at the plasma membrane (Crough and Khanna 2009).

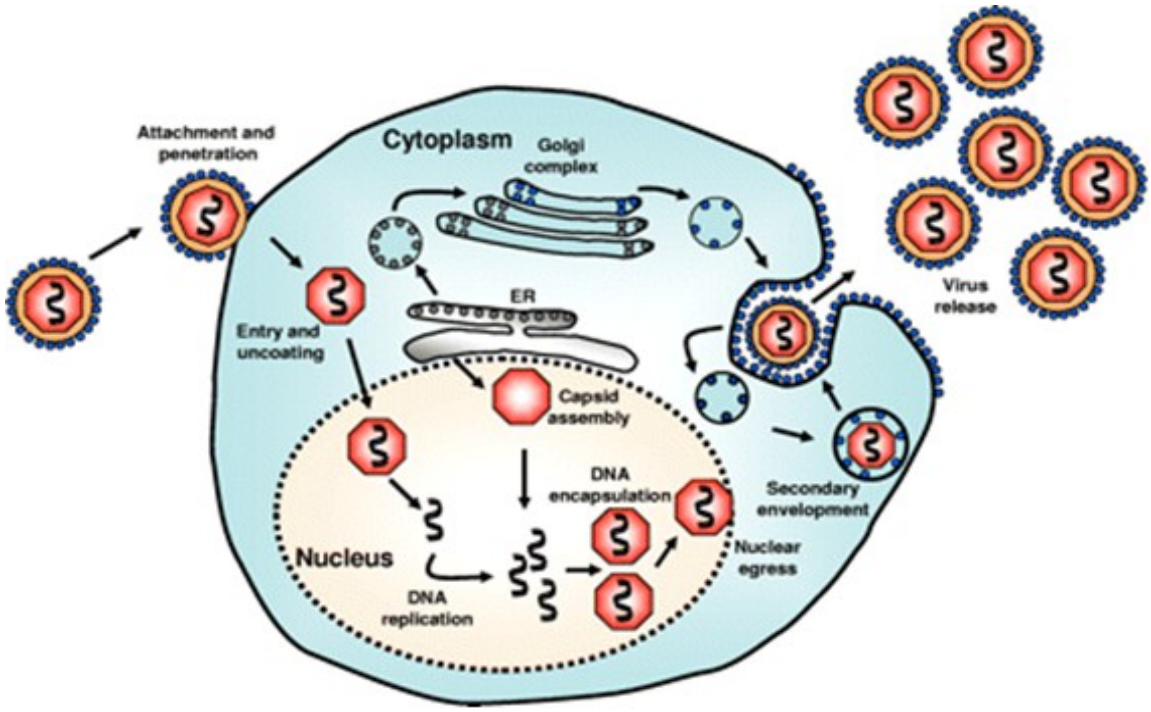

Fig. 2.3: Life cycle of CMV in a human cell (Crough and Khanna 2009).

2.4 Tropism

During primary infection, the first cells to be infected are epithelial cells of the rhinopharynx, if the route of transmission is oral, or epithelia of the genital tract, if transmission occurs by sexual contact. If infection is through blood transfusions, endothelial cells of the vascular tree are the first sites to be infected. Leukocytes are recruited at the sites of primary infection, take up the virus and viral products initiating hematogenous dissemination. Following viral spread through the blood stream, CMV localizes mostly in the salivary glands, kidneys, liver, and mammary glands. Consequently, virus can be isolated or detected in secretion products from these organs such as saliva, urine, or milk. Once the acute phase of infection is controlled through the immune response, CMV undergoes latency (Revello and Gerna 2010).

Studies on CMV cell tropism *in vivo* revealed three characteristics. These include: (1) ubiquitously distributed cell types such as epithelial cells, endothelial cells, and fibroblasts are the major targets of CMV infection; (2) leukocytes circulating in the peripheral blood are susceptible to the virus; (3) specialized parenchymal cells such as smooth muscle cells in the gastrointestinal tract and hepatocytes may also

be infected (Sinzger and Jahn 1996). Among leukocyte subpopulations, polymorphonuclear leukocytes were shown to be the major reservoir of viral products. However, monocytes and macrophages were also found to contribute to viral dissemination at lower rates. Additionally, epithelial cells are the cell type most involved in disseminated CMV infection *in vivo*. Alveolar epithelial cells were shown to be the main CMV target in lung tissues. Epithelial cells are also the predominantly infected cell population of the gastrointestinal tract, secretory glands, and kidneys. Detachment of CMV-infected epithelial cells from the basal membrane is the source of infectivity in several body fluids such as bronchoalveolar lavage fluid, saliva, urine, and stool (Revello and Gerna 2010).

2.5 Epidemiology

The prevalence of past CMV infection in a surveyed population, indicated by seropositivity rate, varies with the socioeconomic status of that population. Adults of high socioeconomic status in industrialized societies may have an antibody seroprevalence as low as 40%, while nearly 100% of persons in lower socioeconomic groups may have evidence of past infection with CMV. In Sweden, approximately 50% of newborns show CMV antibodies; as maternal, placentally acquired antibodies disappear, seropositivity rates decrease to 13% among infants four to six months of age. Thereafter, the rate of acquisition of CMV infection increases with age, with the greatest increase among infants six months to two years of age. The proportion of newborns with maternally acquired CMV antibodies varies according to the mother's socioeconomic status. Since women of low socioeconomic status acquire CMV infection early in life, 70% of those of childbearing age show CMV antibodies. In higher socioeconomic groups, infection is acquired later; only 45% of women of childbearing age are seropositive. The role of sexual contact in the transmission of CMV is not clear. Homosexual males, particularly those engaged in passive anal-genital intercourse, are known to have a higher prevalence of antibody to CMV (95%) than heterosexual males (50%) (Onorato et al. 1985).

2.6 Transmission

CMV shares with other herpesviruses the ability to remain latent in tissues after acute infection. Primary infection occurs by direct close personal contact via exposure to body fluids such as saliva, tears, urine, stool, semen, and breast milk. Infants may acquire CMV transplacentally because of maternal viremia or perinatally via breast milk. In later childhood, close physical contact facilitates transmission. Notably, infection in children is usually asymptomatic. Children in day care centers transmit the virus to other children as well as to susceptible adults including transplant recipients. In large day care centers, approximately 50% of the children experience CMV infection and 10 to 15% of uninfected children become infected each year. The highest rates of active viral transmission and excretion are found in children 13 to 24 months of age (Dobbins et al. 1994).
Although CMV is difficult to transmit, the household appears to be a favorable setting for transmission. Adler et al. (1991) reported that 30% of mothers of children infected in day care centers became infected within a year, compared to only 3% of mothers of uninfected children. CMV can be transmitted heterosexually and homosexually; seroprevalence is high among patients showing up at sexually transmitted disease clinics. Approximately 50% of transplant patients excrete CMV in body secretions (e.g., saliva and urine) at some stage after organ transplantation (Sia and Patel 2000).

2.7 Laboratory Diagnostics

Because of the considerable impact of CMV infection in transplantation, rapid and accurate diagnostics is of paramount importance. Today, diagnostic tools allow not only precise serologic determination of past exposure to CMV infection but also the detection of CMV in blood during viremia and in infected organs (Sia and Patel 2000).

2.7.1 Indirect diagnostics (serology)

Serological diagnosis of CMV infection is based on the detection of IgG and IgM antibodies, mainly by enzyme-linked immunosorbent assay (ELISA) (Grangeot-

Keros 2001). In organ transplantation, serologic studies are an accurate and sensitive means of determining a history of CMV infection in donors and allograft recipients (Sia and Patel 2000). CMV serology may also be useful for screening blood donors. However, serology is an insensitive marker of active CMV infection in the transplant population and is therefore of limited diagnostic usefulness (Schmidt et al. 1995).

Serology has a short time to result, is safe, and can be performed fully automated. The most widely used technology is the ELISA with several commercially available kits on the market.

2.7.2 Direct (molecular) diagnostics

Tissue biopsy is an important tool for diagnosing the causes of allograft dysfunction and can often differentiate inflammatory response provoked by CMV from cellular rejection. The direct diagnosis of CMV infection with histopathology has traditionally been based on the histologic recognition of cytomegalic inclusion bodies that show the characteristic intranuclear owl-eye appearance in hematoxylin and eosin-stained tissue specimens. *In situ* hybridization with CMV-specific complementary DNA probes applied to cellular material has facilitated the histopathological identification of infected cells in tissue. Hybridization has conventionally been performed with probes labeled with radioactive isotopes, which then allows the detection of sequence specific nucleic acid following autoradiographic analysis (Sia and Patel 2000).

Productive infection leads to the coordinated synthesis of proteins (antigens) in three overlapping phases based on the time of synthesis after infection, namely, immediate-early (IE) (0 to 2 h), delayed-early (<24 h), and late (>24 h) viral proteins (Stinski 1978). For detection of CMV antigenemia, the pp65 assay has been a major advance in the diagnosis of CMV infection transplantation. The presence of CMV antigenemia in blood leukocytes provides an early marker of active CMV infection (van den Berg et al. 1991).

In recent years, detection and quantitation of CMV DNA has largely replaced pp65-antigenemia testing in the routine diagnostic laboratory. Usually, blood samples (EDTA whole blood or plasma) are used for detection of CMV DNA. Further specimen types for detection of CMV DNA in the immunocompromised patient include cerebrospinal fluid, bronchoalveolar lavage, aqueous humor, and bone marrow (**Table 2.1**). For prenatal issues, amniotic fluid may be an additional specimen type (Ciotti and Kessler 2010).

Table 2.1: Choice of adequate sample material for molecular diagnostics of CMV infection (modified from Rabenau et al. 2010).

Clinical presentation	Sample material
Pneumonia	Bronchoalveolar lavage (BAL), EDTA whole blood
Encephalitis	Cerebrospinal fluid (CSF), EDTA whole blood
Disseminated CMV infection	BAL, CSF, EDTA whole blood
Hepatitis	EDTA whole blood
Colitis	EDTA whole blood, biopsy
Retinitis	Aqueous humor
Pre-emptive monitoring under immunosuppression (BMT, solid organ transplantation)	EDTA whole blood, bone marrow
Suspected CMV infection/reactivation under Immunosuppression	EDTA whole blood, bone marrow, BAL, CSF
Resistance testing	EDTA whole blood
Prenatal infection	Amniotic fluid, fetal EDTA whole blood
Perinatal infection	EDTA whole blood, breast milk, urine

In the routine diagnostic laboratory, extraction of CMV DNA is usually performed on an automated nucleic acid extraction platform based on the magnetic glass (also called silica) particle technology. This technology consists of four major steps: lysis, capture, purification, and elution (**Fig. 2.4**). In the first step, a certain

amount of lysis buffer is added to the sample. The especially composed lysis buffer is designed to break up the pathogen, followed by the release but simultaneous stabilization of total nucleic acids (at high salt concentration and neutral pH value). Furthermore, it degrades inhibitory proteins and RNases through protease digestion and inactivation of nucleases through chaotropic salt. In the following step, released total nucleic acids are captured through binding to the silica surface of added magnetic glass particles in the presence of chaotropic salt (at high salt concentration and neutral pH value). Today, magnetic glass particles with a significantly increased silica surface have been brought to the market. After nucleic acids capture, purification is performed through repeat washing steps. The wash buffer employed removes unbound substances and impurities such as denatured proteins and cellular debris which may act as potential PCR inhibitors (at low salt concentration and low pH value). Finally, the purified total nucleic acids are released at elevated temperature (low salt concentration and high pH value) and recovered in a small amount of elution buffer. This buffer keeps the nucleic acids in proper condition to secure them from degradation. As smaller elution buffer volumes are chosen, nucleic acid detection increases (Ciotti and Kessler 2010).

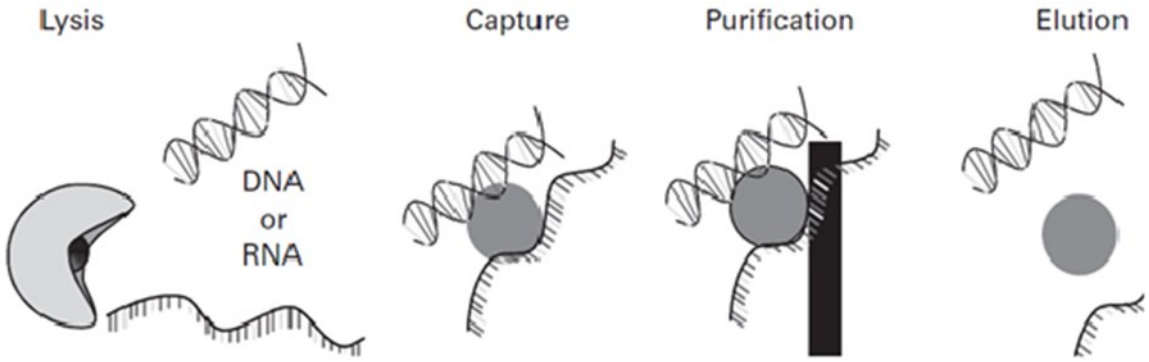

Fig. 2.4: The four major steps of the magnetic glass (also called silica) particle technology (Ciotti and Kessler 2010).

Following extraction of CMV DNA, amplification and detection is preferably performed by real-time PCR. With this technology, CMV DNA can be easily quantified. Quantitative results are usually reported as copies/ml. Several commercial assays have been developed for the quantitation of CMV DNA (**Table 2.2**).

The majority of those assays employ the real-time PCR method while the COBAS Amplicor CMV Monitor Test is based on conventional PCR technology thus being impaired by a limited range of linearity. To improve the limit of detection, this assay may be performed in an ultrasensitive version by addition of a high-speed precentrifugation step to the standard procedure. However, in comparison to molecular assays based on real-time PCR, the COBAS Amplicor CMV Monitor Test seems to underestimate the viral DNA concentration in plasma samples (Ciotti and Kessler 2010).

Table 2.2.: Comparison of currently frequently used commercially available assays for the quantitation of cytomegalovirus (CMV) DNA.

Characteristics	**Manufacturer and details**				
	Argene	Nanogen Advanced Diagnostics	Qiagen GmbH	Roche Molecular Diagnostics	Roche Molecular Diagnostics
Kit name	CMV R-gene	CMV Real Time Complete Kit	artus CMV PCR Kit	COBAS Amplicor CMV Monitor Test	LightCycler CMV Quant Kit
Target sequence	UL83 (lower matrix Phosphoprotein 65)	UL123 (major immediate early protein)	UL122 (immediate early protein)	UL54 (DNA polymerase)	UL54 (DNA polymerase)
Amplification method	Real-time PCR	Real-time PCR	Real-time PCR	Conventional PCR	Real-time PCR
Detection method	Fluorescence	Fluorescence	Fluorescence	EIA	Fluorescence
Internal control	Heterologous	Heterologous	Heterologous	Homologous	Heterologous
Standards	Four EQS	Four EQS	Four EQS	One IQS	Four EQS
Range of linearity	5.0×10^2–2.5×10^6 copies/ml	3.2×10^2–1.3×10^7 genome equivalents/ml	3.2×10^2–1.0×10^7 copies/ml	6.0×10^2–1.0×10^5 copies/ml	1.0×10^3–2.0×10^7 copies/ml

EQS: external quantitation standards; IQS: internal quantitation standard.

2.8 Clinical manifestations

Primary infection is usually asymptomatic or mild in immunocompetent individuals; however, CMV has the ability to establish lifelong latent infection following primary exposure. Major sites of latency are white blood cells and endothelial cells (Forbes et al. 2007). Re-activation is usually asymptomatic but in the immunocompromised it is potentially life threatening because of pneumonia, encephalitis, retinitis, colitis and/or hepatitis.

CMV is thus the most important opportunistic viral pathogen for immunocompromised patients, with CMV seronegative transplant recipients at highest risk of developing CMV disease and its complications. In transplant recipients, acute/chronic graft rejection may be produced, together with bacterial and fungal super-infections leading to acceleration of underlying viral infections.

Congenital CMV infection occurs in 0.6-0.7% of all newborns and is the most prevalent infection-related cause of congenital neurological handicap. Vertical transmission occurs in around 30% of cases, but the fetus is not always affected. Symptomatic newborns at birth have a much higher risk of suffering severe neurological sequelae. Systematical testing of pregnant women for CMV has been discussed controversially. It should be restricted to pregnancies where a primary infection is suspected or among pregnant women at high risk (Coll et al. 2009).

2.9 Treatment

Anti-CMV treatment options remain very limited. There is not a single anti-CMV drug, which is potent and safe enough to be given to all individuals infected with this virus, on the market.

The nucleosides Acyclovir (ACV) and Ganciclovir (GCV) have been used in anti-CMV treatment for over 20 years. Both drugs show a similar chemical structure and are anabolized by a common cellular pathway (Fig. 2.x). After activation, they are competitive inhibitors of CMV encoded DNA polymerase. In cells infected with CMV, the first stage of phosphorylation is achieved by the UL97 protein kinase.

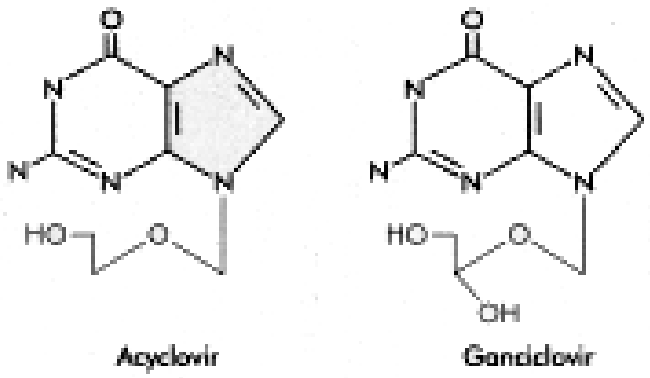

Fig. 2.5: Chemical structure of Acyclovir and Ganciclovir.

ACV can be activated by UL97. ACV triphosphate is a potent inhibitor of CMV DNA polymerase (Talarico et al., 1999; Mar et al., 1985). It is an obligate chain terminator and, additionally, a suicide inhibitor of herpesvirus DNA polymerase (Furman et al., 1984). In combination, these characteristics potently inhibit CMV replication. However, the intracellular half-life of ACV triphosphate is significantly shorter than that of GCV triphosphate so that high drug levels and frequent dosing are needed for ACV to control CMV replication *in vivo* (Lowance et al., 1999). Selectivity of ACV triphosphate for virus-infected cells is achieved by both, UL97 activation and superior inhibition of CMV-encoded DNA polymerase in comparison to cellular DNA polymerase.

GCV is not an obligate chain terminator, although chain termination usually occurs after incorporation of one or more molecules. The ability to allow chain elongation is theoretically undesirable because it might occur in uninfected cells leading to a mutagenic event in cellular DNA. Once ACV and GCV are mono-phosphorylated within the virus-infected cell, they are charged and thus unable to diffuse out of the cell. A concentration gradient is thereby formed across the plasma membrane, aiding diffusion of additional GCV into the infected cell. Cellular enzymes convert GCV monophosphate to the triphosphate. GCV triphosphate is a potent inhibitor of CMV DNA polymerase and has a long intracellular half-life. The oral absorption of GCV is poor while that of ACV is better but also variable between individuals. Oral bioavailability of these compounds has been improved via esters which are absorbed and then cleaved in the intestinal wall and/or liver to release free compound. Valganciclovir is the valine ester of GCV; Valaciclovir is the valine ester of ACV.

Nucleotides used in anti-CMV treatment include Cidofovir (CDV) and Foscarnet (FSC). These compounds are phosphonates, structurally equivalent to the nucleoside monophosphate but without the charge which would prevent the molecule crossing the plasma membrane.

CDV bypasses the UL97 step with subsequent conversion to the diphosphate by cellular enzymes. The selectivity of CDV resides in the preferential inhibition of CMV DNA polymerase rather than cellular DNA polymerase by CDV diphosphate.

FSC is structurally analogous to pyrophosphate and inhibits CMV DNA polymerase by binding to the enzymatic site for pyrophosphate. Because pyrophosphate is one of the products of DNA enzyme activity, foscarnet is a product inhibitor, not a substrate inhibitor, and thus does not compete with the natural nucleotides.

Recently, antisense molecules have been studied regarding anti-CMV treatment. Molecules complementary (antisense) to mRNA bind mRNA to prevent expression of the encoded gene. For CMV, the antisense molecule Fomivirsen binds to the major immediate-early transactivator gene. The half-life of this compound is long because it contains modified nucleosides such as phosphorothioates (substitution of sulfur into the phosphodiester background) and/or modified sugar residues, which are not readily degraded by host cell enzymes. In contrast to the other compounds mentioned above, Fomivirsen is administered intravitreally only to the end organ involved with CMV retinitis and thus does not affect systemic CMV (Griffiths and Boeckh, 2007).

2.10 Goals of the study

In this study, automated platforms useful for extraction of CMV DNA from different sample types including EDTA whole blood, plasma, urine, and bronchoalveolar lavage were evaluated and compared. In addition, results obtained from different real-time PCR systems were compared. Finally, time-to-result when employing different assays was compared.

3 Materials and methods

3.1 Study design

The study design is shown in **Fig. 3.1**. Clinical specimens were extracted on 4 different automated extraction platforms. Nucleic acid extracts were amplified and detected on 2 different qPCR instruments.

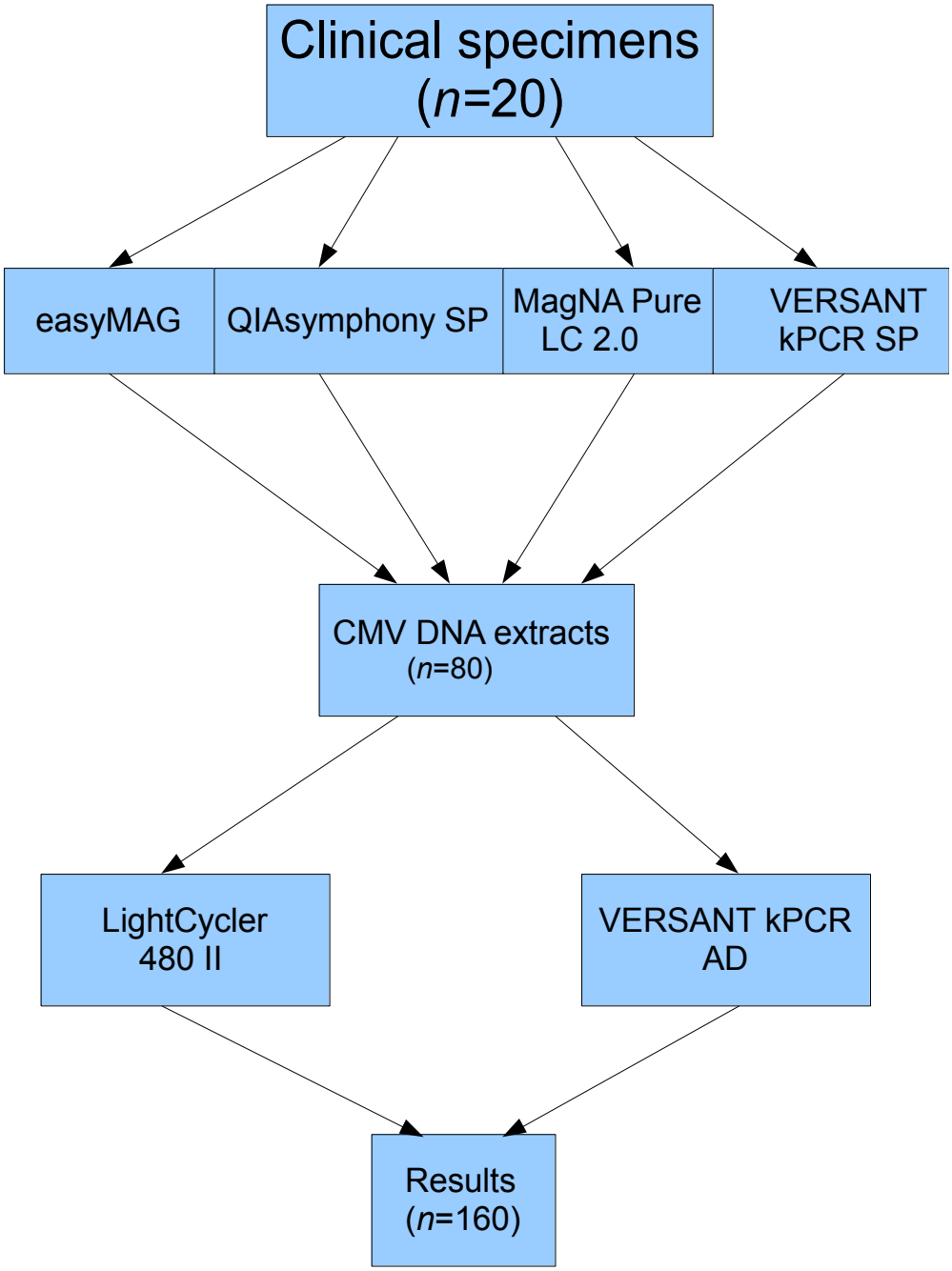

Fig. 3.1: The study design

3.2 Clinical specimens

Clinical specimens were collected from transplant recipients treated at different departments of the Graz University Hospital. Collection vessels are shown in **Fig. 3.2**.

Fig. 3.2: Vessels for collection of clinical specimens used in this study. 1a and 1b, EDTA whole blood tubes from different manufacturers; 2, urine collection vessel; 3, BAL collection vessel (see text).

Blood was collected in 3.5 ml-EDTA whole blood tubes (Vacuette®, Greiner Bio-One, Kremsmuenster, Austria). Immediately after receipt in the International Standards Organization (ISO9001:2008)-certified routine diagnostic laboratory, the Molecular Diagnostics Laboratory, Medical University of Graz, tubes were either frozen at ms 70°C or centrifuged with 2000 x *g* for 10 min in order to prepare EDTA plasma. Immediately after centrifugation, plasma samples were frozen at ms 70°C until analysis.

Bronchoalveolar lavages (BALs) and urine specimens were collected in sterile screw-cap collection vessels (Polycon®,mwe, Corsham Wiltshire, England). Immediately after receipt, vessels were frozen at ms 70°C until analysis.

3.3 Nucleic acids extraction

Frozen specimens were thawed and analyzed within 3 hours. Commercially available sample preparation kits designed for DNA extraction from different matrices on automated platforms were employed.

3.3.1 Extraction of CMV DNA on the NucliSens easyMAG

The NucliSens easyMAG (bioMérieux, Boxtel, The Netherlands) is a nucleic acid extraction system utilizing silica-based extraction technology (Boom et al., 1990) (**Fig. 3.3, Table 3.1**). For this study, the NucliSens easyMAG accessory products/ Generic2.0 Protocol were employed (**Table 3.2**). The extraction method is universal and can be applied to a broad range of different sample types including blood, sputum, serum, and throat swabs (Tang et al. 2005).

Table 3.1: Basic features of extraction systems used in this study.

System	Manufacturer	CE label	Run size
easyMAG	Biomerieux	YES	24
QIAsymphony SP	Qiagen	YES	24
MagNAPure LC 2.0	Roche	NO	32
VERSANT kPCR SP	Siemens	YES	96

Fig. 3.3: The NucliSens easyMAG instrument.

3.3.2 Extraction of CMV DNA on the QIAsymphony SP

The QIAsymphony SP (Qiagen, Hilden, Germany) has been recently introduced for fully automated extraction of nucleic acids (**Fig. 3.4, Table 3.1**). It utilizes the magnetic silica particle technology to capture nucleic acids. For this study, the DSP Virus/Pathogen Mini Kit / Cellfree 200 Protocol for EDTA WB/plasma; Complex 200 Protocol for urine and BALs were employed (**Table 3.2**). This extraction method enables extraction of DNA and RNA from a wide range of matrices.

Fig. 3.4: The QIAsymphony SP instrument.

3.3.3 Extraction of CMV DNA on the MagNA Pure LC 2.0

The MagNA Pure LC 2.0 (Roche Diagnostics, Rotkreuz, Switzerland) is based on the well-established magnetic bead technology offering DNA and RNA isolation from various matrices including blood, cells, plasma/serum, and tissue (**Fig. 3.5**, **Table 3.1**). For this study, the MagNA Pure LC Total Nucleic Acid Isolation Kit / High Performance Protocol were employed (**Table 3.2**). The MagNA Pure LC 2.0 is capable of performing fully automated pipetting of subsequent reactions such as the setup of qPCRs or reverse transcriptase-qPCRs (Kirchgesser et al. 2008).

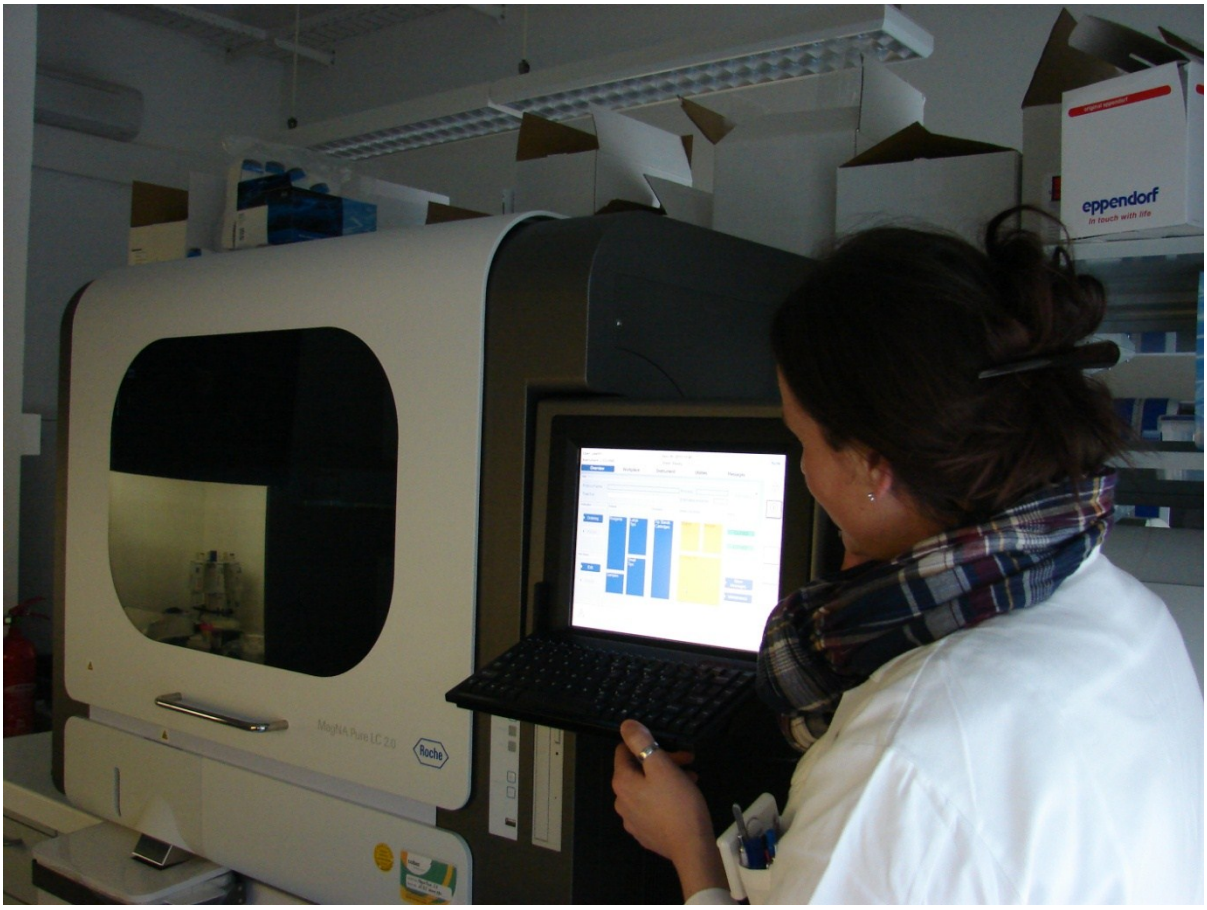

Fig. 3.5: The MagNA Pure LC 2.0 instrument.

3.3.4 Extraction of CMV DNA on the VERSANT kPCR SP

The VERSANT kPCR SP (Siemens, Tarrytown, NJ, USA) is capable to isolate and purify both DNA and RNA from diverse clinical samples including plasma, serum, urine, urogenital swabs, dried blood spots, stool, nasopharyngeal swabs, and breast milk (**Fig. 3.6**, **Table 3.1**). It utilizes a single set of reagents. The extraction technology is based on magnetic particles with silica coating for efficient isolation of nucleic acids. For this study, the SP 1.2 Kit / WBS 10B Protocol were employed (**Table 3.2**).

Fig. 3.6: The VERSANT kPCR SP instrument.

Table 3.2: Kits and protocols for extraction of CMV DNA used in this study.

Manufacture	Kit name	Protocol	Loading vol (µl)	Processed vol (µl)	Elution vol (µl)	IC / Sample
Biomerieux	NucliSens easyMAG accessory products	Generic2.0 Protocol	200	200	55	10 µl into sample manually
Qiagen	DSP Virus/Pathogen Mini Kit	Cellfree 200 Protocol for EDTA WB/plasma; Complex 200 Protocol for urine and BALs	300	200	Delivers 60 of 90	15 µl into sample manually
Roche	MagNA Pure LC Total Nucleic Acid Isolation Kit	High Performance Protocol	200	200	55	10 µl into sample manually
Siemens	SP 1.2	WBS 10B Protocol	400	250	Delivers 50 of 70	Preload into DWP(deep well plates) 10 µl manually

3.4 Amplification and detection

Extracted CMV DNAs were amplified and detected by the CMV HHV6,7,8 R-gene™ kit (Argene SA, Varilhes, France; **Fig. 3.7**), a commercially available, CE/IVD-labeled molecular assay based on the qPCR technology. Two different qPCR platforms were employed.

Fig. 3.7: The CMV HHV6,7,8 R-gene™ kit.

3.4.1 The qPCR kit used in this study

The IVD/CE-labeled CMV HHV6,7,8 R-gene™ kit is designed to measure the viral load of CMV and HHV-6 and to detect HHV-7 and HHV-8 by qPCR using the 5'nuclease TaqMan® technology. Several types of specimens including EDTA whole blood, plasma, serum, urine, BAL, CSF, amniotic fluid, samples derived from Guthrie cards, and biopsies, nucleic acids extraction systems (automated and manual), and commonly available qPCR platforms have been validated with this kit. The CMV HHV6,7,8 R-gene™ kit includes external (positive and negative) run controls and a

heterologous internal control (enveloped DNA particle). All controls are added prior to the lysis step thus subjected to the entire molecular test system. The ready-to-use PCR mix includes: primers, dNTPs, amplification buffer, Hot StarTaq® Polymerase (Qiagen), primers for specific amplification of a 283-base pair genome section encoding the CMV ppUL83, primers for the specific amplification of the internal control, and probes for the detection of the CMV-specific and the internal control-specific amplification products. The CMV HHV 6,7,8 R-gene quantification kit was performed according to the manufacturer`s instructions.

For real-time PCR, 15 µl of the PCR mix and 10 µl of the eluate were pipetted into each well of the microwell plate which was then placed on the qPCR instrument (either VERSANT kPCR AD or LightCycler 480 II).

According to the manufacturer's package insert, the limit of detection (LOD) is 150 cop/ml. The categories of results reported by the quantitative CMV test are "over upper limit of linearity", a numeric value (copies/ml) within the range of linearity, "positive under LOD", or "not detectable".

3.4.2 qPCR on the LightCycler 480 II

The LightCycler 480 II (Roche Diagnostics) is a fully integrated 96-well plate based qPCR instrument for qualitative and quantitative detection of nucleic acids (**Fig. 3.8**). The LightCycler 480 II consists of the following components: the LightCycle 480II instrument, the LightCycler 480 II software, the LightCycler 480 II disposables, and the LightCycle 480II reagents.

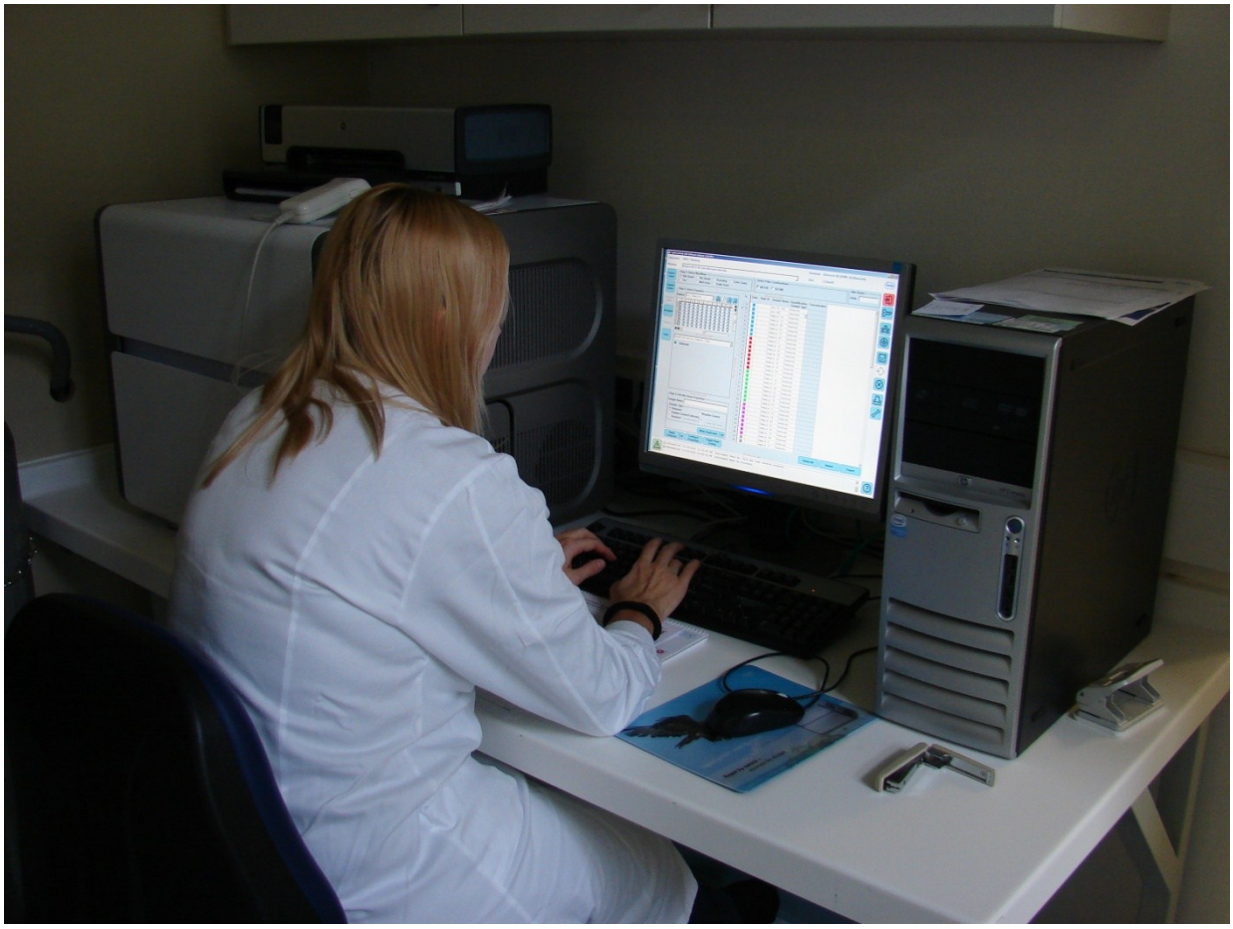

Fig. 3.6: The LightCycler 480 II instrument.

3.4.3 qPCR on the VERSANT kPCR AD

The VERSANT kPCR AD (Siemens) is a fully integrated 96-well plate based qPCR instrument for qualitative and quantitative detection of nucleic acids (**Fig. 3.9**). Similar to the LightCycler 480 II, this instrument automatically performs the reverse-transcription, amplification and detection steps, and the AD software provides a report with viral load quantitations.

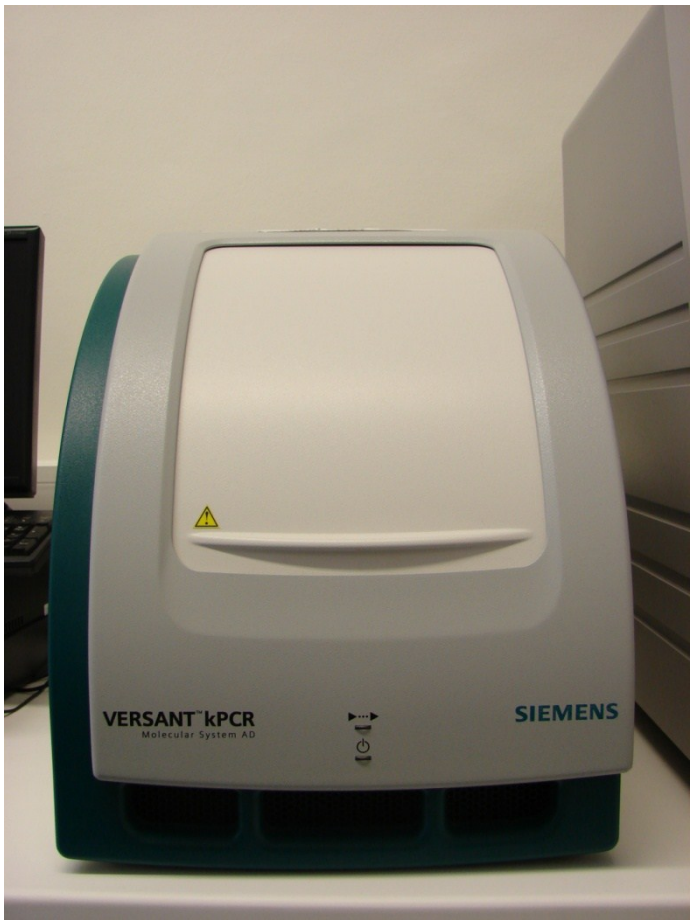

Fig. 3.6: The VERSANT kPCR AD instrument.

3.5 Statistical methods

Comparisons of viral loads were carried out using the Student's paired t-test. The results were expressed as the mean log quantity, and $p<0.05$ was considered significant.

4 Results

Twenty clinical samples obtained from patients with CMV infection including 5 EDTA whole blood, 5 plasma, 5 urine, and 5 BAL samples were analyzed.

4.1 Comparison of automated nucleic acids extraction systems

When the VERSANT kPCR SP instrument in conjunction with the VERSANT kPCR AD was compared to the alternative extraction systems, significantly higher values were obtained for ETDA whole blood and plasma samples (p<0.05) (**Table 4.1**). Similar results, although not reaching the significance level (except for one value), were found with urine and BAL samples.

When the VERSANT kPCR SP instrument in conjunction with the LightCycler 480 II was compared to the alternative extraction systems, significantly higher values were obtained for ETDA whole blood (p<0.05) (**Table 4.2**). For plasma, urine, and BAL samples, results were comparable.

Table.4.1: Comparison of CMV DNA concentrations obtained from extraction using automated nucleic acids extraction systems in conjunction with real-time PCR on the VERSANT kPCR AD.

Specimen	easyMAG	MagNAPure LC2.0	QIAsymphony	VERSANT SP	easyMAG vs VERSANT SP	MagNAPureLC2.0 vs VERSANT SP	QIAsymphony vs VERSANT SP
	Mean log quantity				*p*-value*		
EDTA WB	2.68	2.96	3.20	3.86	0.001*	0.011*	0.019*
Plasma	3.28	3.56	3.59	3.78	0.004*	0.006*	0.040*
Urine	2.83	2.66	2.41	3.02	0.250	0.427	0.486
BAL	3.97	3.90	3.75	4.10	0.138	0.178	0.016*

**p-value less than 0.05 (highlighted in blue) indicates that the VERSANT kPCR 1.2 yielded significantly higher quantitations than the alternative protocol.*

Table.4.2: Comparison of CMV DNA concentrations obtained from extraction using automated nucleic acids extraction systems in conjunction with real-time PCR on the LightCycler 480 II.

Specimen	easyMAG	MagNaPure LC2.0	QIAsymphony	VERSANT SP	easyMAG vs VERSANT SP	MagNaPure LC2.0 vs VERSANT SP	QIAsymphony vs VERSANTSP
	Mean log quantity				*p*-value*		
EDTA WB	3.26	3.63	3.46	4.11	0.015*	0.015*	0.038*
Plasma	3.63	3.92	3.97	3.88	0.001*	0.346	0.185
Urine	3.29	3.25	3.35	3.27	0.152	0.399	0.213
BAL	4.53	4.35	4.41	4.28	0.068	0.317	0.130

**p-value less than 0.05 (highlighted in blue) indicates that the VERSANT kPCR 1.2 yielded significantly higher quantitations than the alternative protocol.*

4.2 Comparison of CMV viral loads obtained with different extraction systems

CMV DNA concentrations in 5 different samples of EDTA whole blood, plasma, urine, and BAL obtained when using 4 different extraction systems in conjunction with real-time PCR on the VERSANT kPCR AD instrument are shown in **Fig. 4.1a-d**.

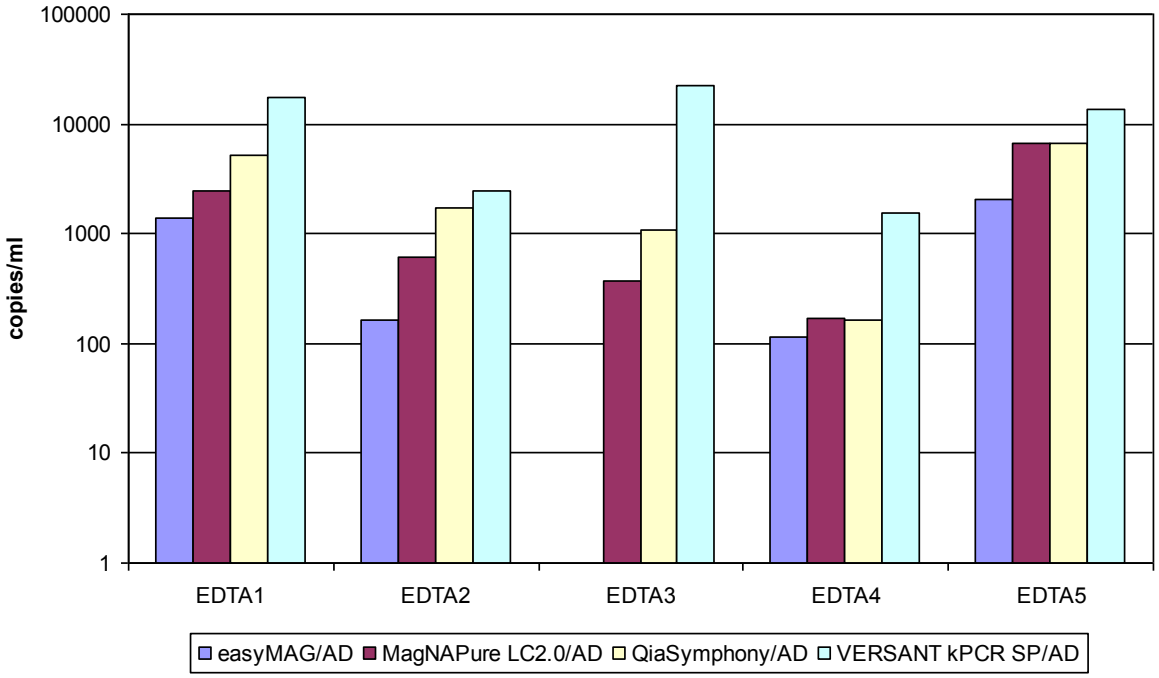

Fig. 4.1a: CMV DNA concentrations in EDTA whole blood obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

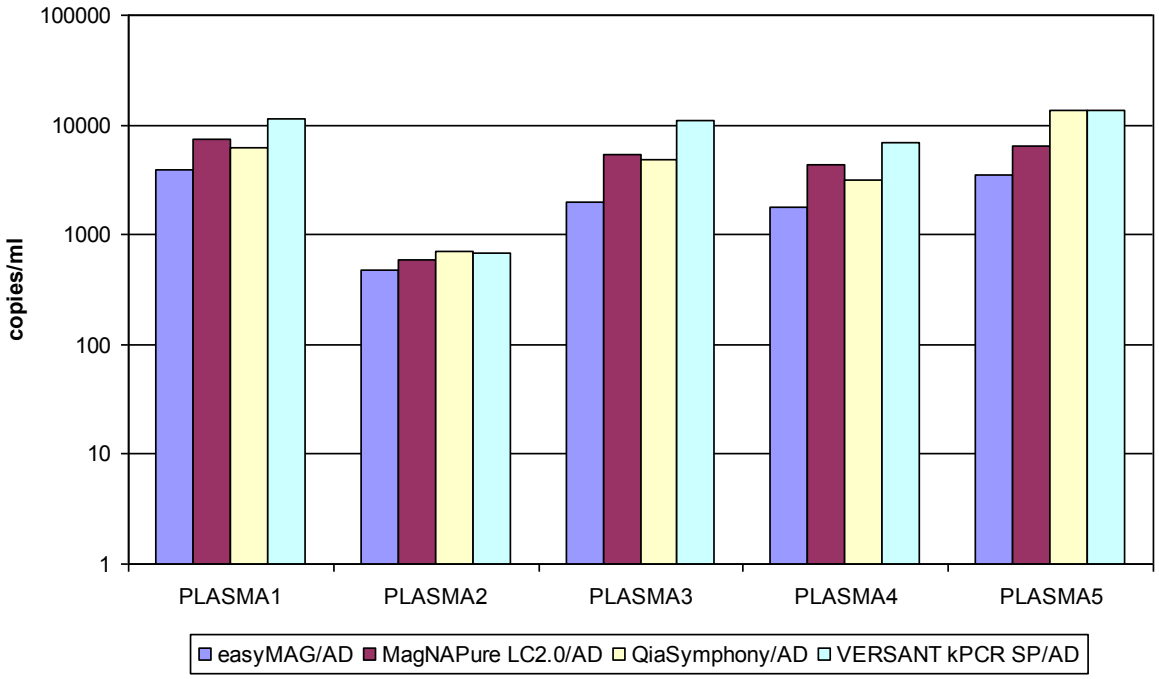

Fig. 4.1b: CMV levels in EDTA plasma obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

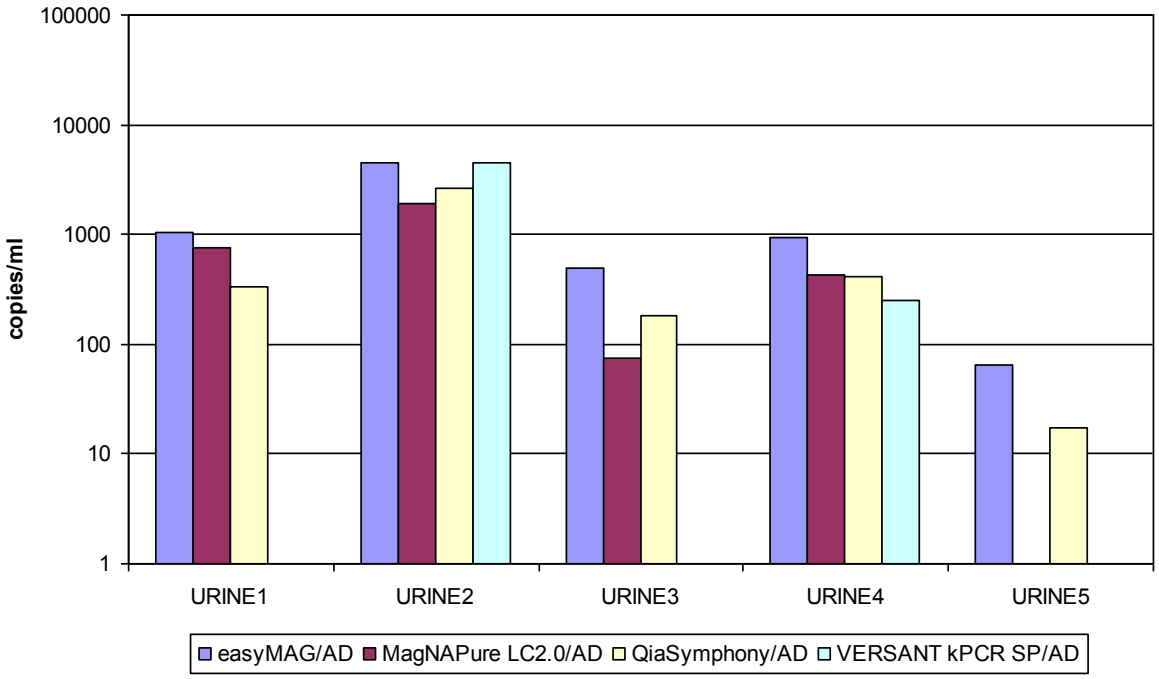

Fig. 4.1c: CMV levels in urine obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

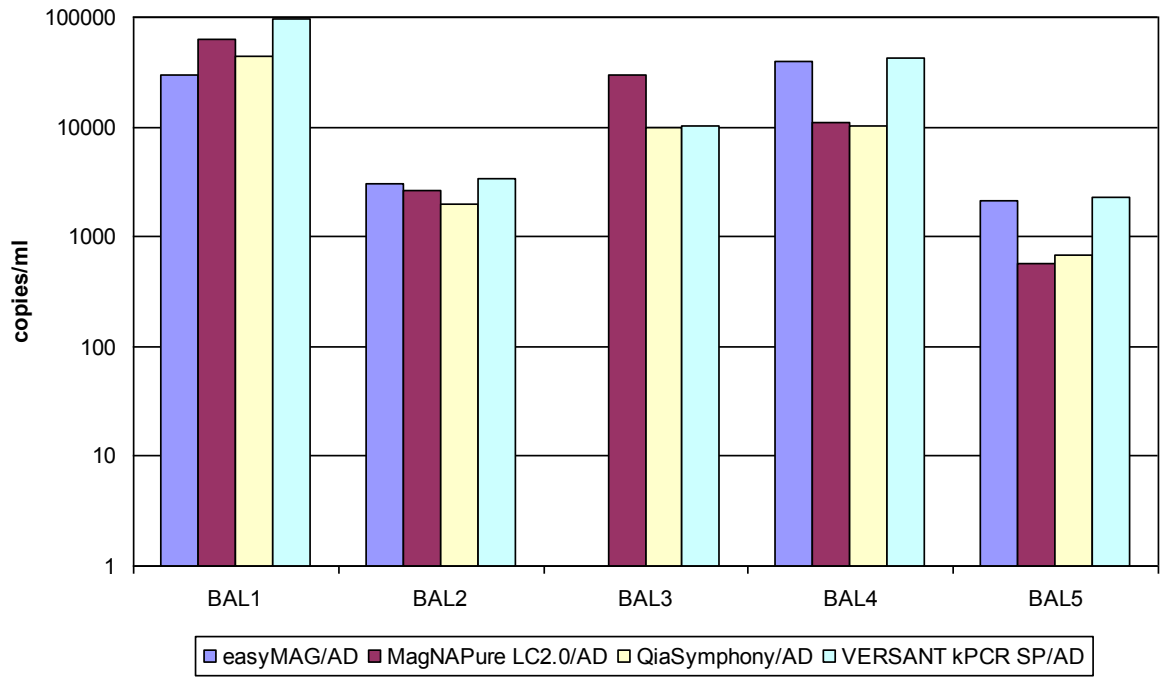

Fig. 4.1d: CMV levels in BALs obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

The viral loads of 5 different samples of EDTA whole blood, plasma, urine, and BAL obtained when using 4 different extraction systems in conjunction with amplification on the LightCycler 480 II instrument are shown in **Fig. 4.2a-d**.

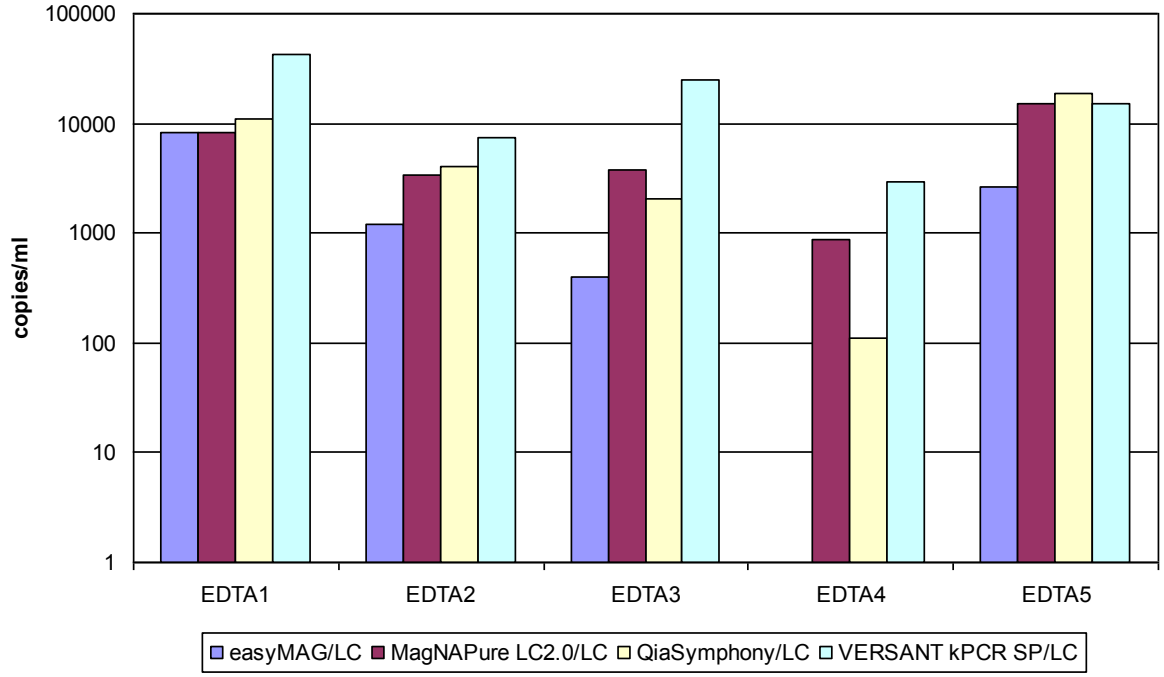

Fig. 4.2a: CMV levels in EDTA whole blood obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

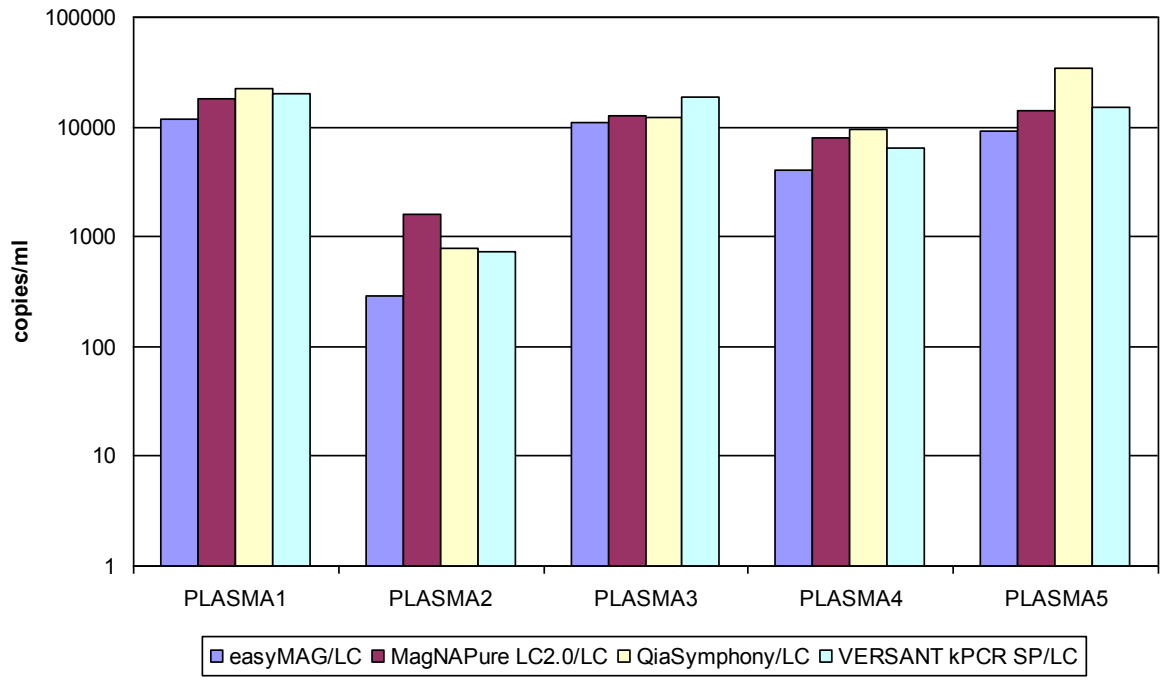

Fig. 4.2b: CMV levels in EDTA plasma obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

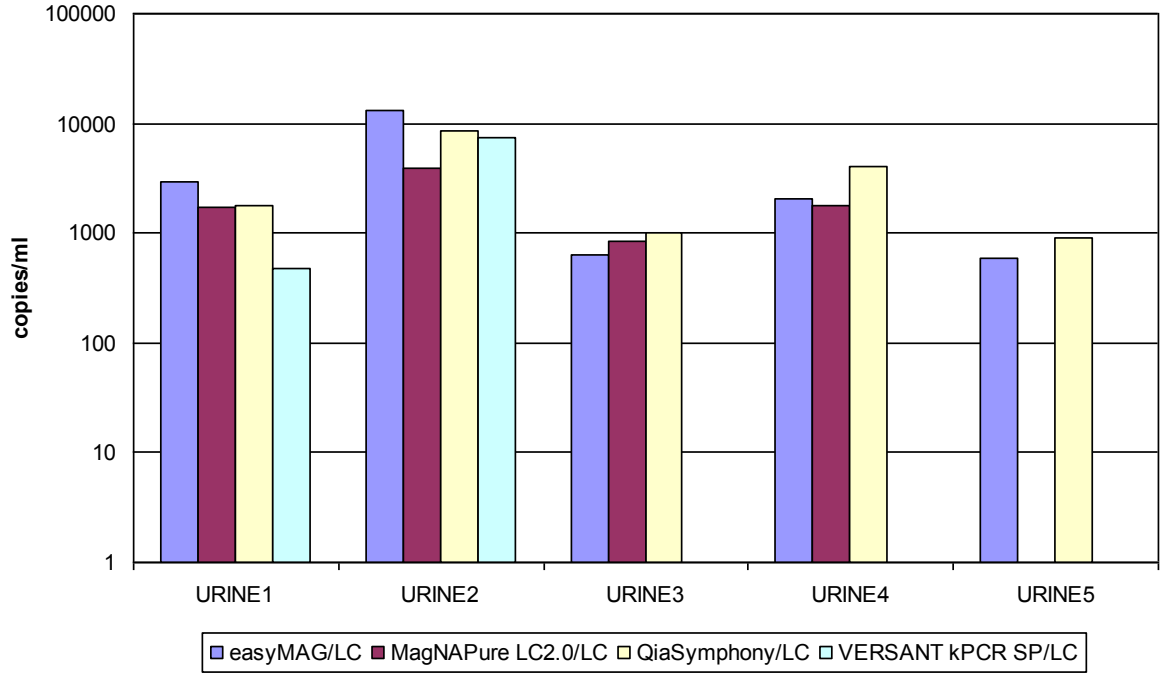

Fig. 4.2c: CMV levels in urine obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

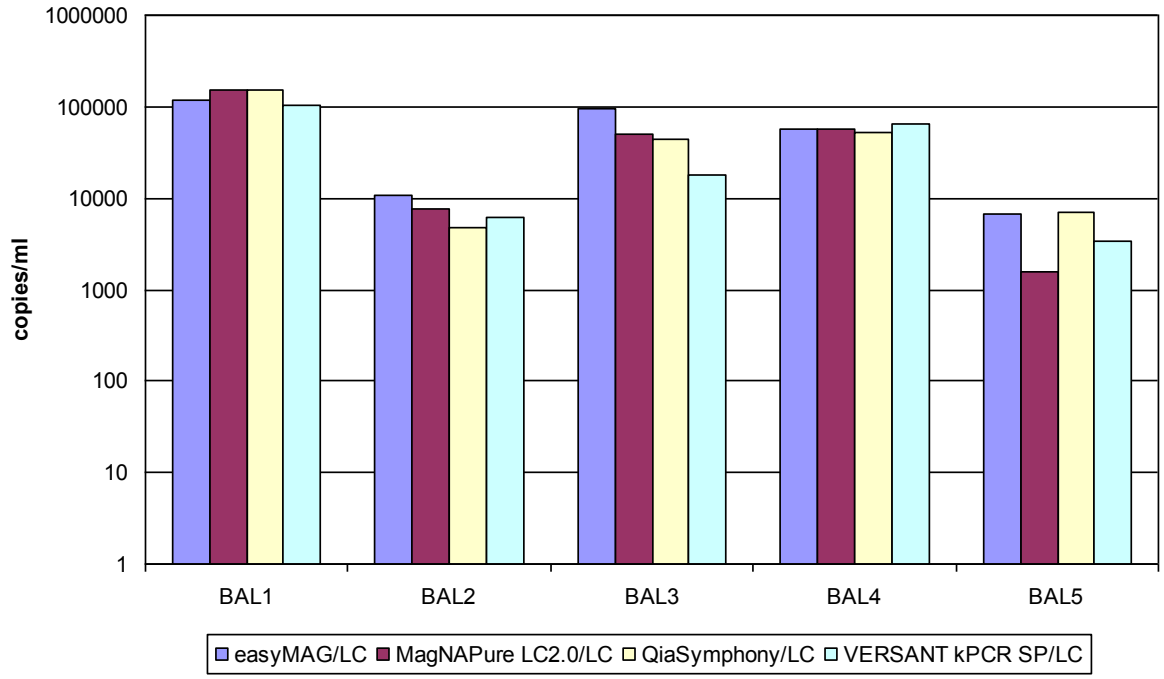

Fig. 4.2d: CMV levels in BALs obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

4.3 Comparison of viral concentrations when amplifying with the VERSANT kPCR AD and the LightCycler 480 II

When comparing the two amplification systems with the *Student's paired t-test*, the LightCycler 480 II gave significantly higher values (**Table 4.3**)

Table 4.3: Comparison of CMV DNA concentrations when amplifying with the VERSANT kPCR AD and the LightCycler 480 II.

Specimen	**Mean log quantity (copies/ml)**		***p*-value***
	easyMAG/AD	**easyMAG/LC**	**AD vs LC**
EDT	2.68	3.26	0.066
Plasma	3.28	3.63	0.047
Urine	2.83	3.29	0.015
BAL	3.97	4.53	0.010
	MagNaPureLC2.0/AD	**MagNaPureLC2.0/LC**	**AD vs LC**
EDT	2.96	3.63	0.002
Plasma	3.56	3.92	<0.001
Urine	2.66	3.25	0.020
BAL	3.90	4.35	0.002
	QIAsymphony/AD	**QIAsymphony/LC**	**AD vs LC**
EDTA	3.20	3.46	0.042
Plasma	3.59	3.97	0.006
Urine	2.41	3.35	0.005
BAL	3.75	4.41	0.002
	VERSANT kPCR SP/AD	**VERSANT kPCR SP/LC**	**AD vs LC**
EDTA	3.86	4.11	0.022
Plasma	3.78	3.88	0.071
Urine	3.02	3.27	NA**
BAL	4.10	4.28	0.005

**p*-value less than 0.05 (highlight in blue) means that VERSANT kPCR AD yielded significantly higher quantitations than the LightCycler 480 II. The *Student's paired t-test* was used.
**Not applicable for the AD vs LC comparison due to only 2 positive results.

4.4 Comparison of times-to-result

The hands-on-times, automated times, and total times were compared (**Table 4.4**).

Table 4.4: Comparison of times-to-result (min) when extracting 24 samples in parallel.

	easyMAG	**QIAsymphony**	**MagNA Pure LC 2.0***	**VERSANT kPCR SP**
Hands-on time	15	15	15	25
Automated	60	90	90	90
Total	75	105	105	115

*Time-to-result when extracting 32 samples in parallel.

5 Discussion

Human cytomegalovirus (CMV), one of the eight herpesviruses that commonly infect humans, is best known for its ability to cause disease in immunocompromised patients, especially transplant recipients, patients with advanced AIDS, and congenitally infected newborns. Advances in molecular detection and treatment options have vastly improved our understanding and ability to manage CMV infections (Boeckh and Geballe, 2011). Because real-time PCR is unlikely to be further improved, the current focus is on optimization of nucleic acid extraction.

When the VERSANT kPCR SP instrument in conjunction with the VERSANT kPCR AD was compared to the alternative extraction systems, higher CMV DNA concentrations were obtained for all types of samples tested in this study. Similar results were observed for the VERSANT kPCR SP instrument in conjunction with the LightCycler amplification system. These results indicate that the VERSANT kPCR SP instrument may be superior in comparison to alternative extraction systems. This effect is even more notable because the EDTA whole blood protocol optimized for extraction of nucleic acids from EDTA whole blood was used for all sample types tested in this study.

When single samples were compared, the easyMAG, QIAsymphony, and VERSANT kPCR SP systems failed to detect some of those samples containing a low concentration of CMV DNA. In conjunction with the VERSANT kPCR AD, the easyMAG failed to detect CMV DNA in a BAL sample containing a relatively high concentration of CMV DNA. This failure might have been caused by air bubbles in the pipette tips operated automatically by the machine.

When comparing the two amplification systems, the VERSANT kPCR SP instrument in conjunction with the LightCylcer 480 II gave higher values than the VERSANT kPCR SP in conjunction with the VERSANT kPCR AD. This effect was observed with all sample types. These results might indicate that the two VERSANT systems are not yet optimized with each other.

Another important factor to consider when selecting a molecular assay for use in the routine diagnostic laboratory is time-to-result. When comparing the hands-on-time of the 4 different extraction systems, the VERSANT kPCR SP has the longest hands-on time due to extra steps for dilution of the reagent in the cartridge and for labeling the samples before starting the automated procedure. Theoretically, the hands-on-time might be shortened when using the QIAsymphony system which allows for the use of primary sample tubes.

6 Conclusion

In this study, detection and quantitation of CMV DNA on 4 different automated nucleic acid extraction systems in conjunction with qPCR was evaluated with different sample types including EDTA whole blood, plasma, urine, and BAL. With each assay and sample type, consistent results could be generated within an acceptable timeframe. The VERSANT kPCR SP instrument in conjunction with the VERSANT kPCR AD was found to provide statistically significantly higher CMV DNA concentrations when using EDTA whole blood and plasma. However, it should be considered that a statistically significantly higher CMV DNA concentration does not necessarily imply clinical significance. When using the VERSANT kPCR SP instrument, the time-to-result was longer than with the other extraction systems compared.

7 References

Adler SP. Cytomegalovirus and child day care: risk factors for maternal infection. Pediatr Infect Dis. 1991;10:590-594

Atmar RL, Estes MK Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses, Clin Microbiol Rev. 2001 Jan;14(1):15-37.

Atmar RL. Noroviruses – State of the Art. Food and Environmental Virology 2010;2(3):117–126.

Boeckh, M. & Geballe, A.P., 2011. Cytomegalovirus: pathogen, paradigm, and puzzle. Journal of Clinical Investigation, 121(5), 1673-1680.

Cheeran, M.C., Lokensgard, J.R. & Schleiss, M.R., 2009. Neuropathogenesis of Congenital Cytomegalovirus Infection: Disease Mechanisms and Prospects for Intervention. Clinical Microbiology Reviews, 22(1), 99-126.

Chen, D. H., H. Jiang, M. Lee, F. Liu, and Z. H. Zhou. 1999. Three-dimensional visualization of tegument/capsid interactions in the intact human cytomegalovirus. Virology 260:10-16.

Ciotti Marco; Kessler HH (2010) Pathogens relevant in transplantation medicine *In*: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York.

Coll O et al. Guidelines on CMV congenital infection. Journal of Perinatal Medicine 2009;37(5):433-45.

Crough Tania and Khanna Rajiv, Immunobiology of Human Cytomegalovirus: from Bench to Bedside, Clinical Microbiology Reviews, January 2009, p. 76-98, Vol. 22, No. 1

Davison, A. J., A. Dolan, P. Akter, C. Addison, D. J. Dargan, D. J. Alcendor, D. J. McGeoch, and G. S. Hayward. 2003. The human cytomegalovirus genome revisited: comparison with the chimpanzee cytomegalovirus genome. J. Gen. Virol. 84:17-28.

Dobbins, J. G., S. P. Adler, R. F. Pass, J. F. Bale, Jr., L. Grillner, and J. A. Stewart. 1994. The risks and benefits of cytomegalovirus transmission in child day care. Pediatrics 94:1016-1018

Dunn, W., 2003. Functional profiling of a human cytomegalovirus genome. Proceedings of the National Academy of Sciences, 100(24), 14223-14228.

Forbes, B.A., Sahm, D. F., & Weissfeld, A.S., Bailey and Scott's Diagnostic Microbiology, 12th Edition., Elsevier Mosby, 2007

Graham DY et al. Norwalk virus infection of volunteers: New insights based on improved assays. Journal of Infectious Diseases 1994;170(1):34–43.

Grangeot-Keros, L., 2001. Diagnosis and prognostic markers of HCMV infection. Journal of Clinical Virology, 21(3), 213-221.

Griffiths, Paul D, and Michael Boeckh, Michael. In Human Herpesviruses: Biology, Therapy, and Immunoprophylaxis. Chapter 66Antiviral therapy for human cytomegalovirus Arvin A, Campadelli-Fiume G, Mocarski E, et al., editors.
Cambridge: Cambridge University Press; 2007.

Hirai K. Nippon Rinsho. 1998 Jan; 56(1):36-43, The mechanisms of human cytomegalovirus DNA replication

Kessler HH (2010) Extraction of nucleic acids. *In*: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York.

Kirchgesser M et al. The new MagNA Pure LC 2.0 system: new design and improved performance combined with a proven nucleic acid isolation technology. Biochemica 2008;(3):20-22.

Le Guyader FS et al. Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. J Clin Microbiol. 2008 Dec;46(12):4011-7. Epub 2008 Oct 8

Mar, E.C. u. a., 1985. Inhibition of cellular DNA polymerase alpha and human cytomegalovirus-induced DNA polymerase by the triphosphates of 9-(2-hydroxyethoxymethyl)guanine and 9-(1,3-dihydroxy-2-propoxymethyl)guanine. J. Virol., 53(3), 776-780.

Mattner F, Sohr D, Heim A, Gastmeier P, Vennema H, Koopmans M. Risk groups for clinical comoplications of norovirus infections: An outbreak investigation. Clinical Microbiology and Infection 2006;12(1):69–74.

Mocarski, E. S., Jr., T. Shank, and R. F. Pass. 2007. Cytomegaloviruses, p. 2701-2772. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), Fields virology, 5th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.

Onorato Ida M., Morens David M.,Martone William J., Stansfieldt Sally K. 1985: Epidemiology of Cytomegaloviral Infections: Recommendations for
Prevention and Control. Reviews of Infectious Deseases Vol 7, No.4.

Rabenau H, Raggam RB, Salzer HJF (2010) Choice of adequate sample material, *In*: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York.

Revello, M.G. & Gerna, G., 2010. Human cytomegalovirus tropism for endothelial/epithelial cells: scientific background and clinical implications. Reviews in Medical Virology, 20(3), 136-155.

Schmidt, C. A., H. Oettle, R. Peng, P. Neuhaus, G. Blumhardt, R. Lohmann,
F. Wilborn, K. Osthoff, J. Oertel, H. Timm, et al. 1995. Comparison of polymerase chain reaction from plasma and buffy coat with antigen detection and occurrence of

immunoglobulin M for the demonstration of cytomegalovirus infection after liver transplantation. Transplantation 59: 1133–1138

Sergei V. Kotenko,* Simona Saccani, Lara S. Izotova, Olga V. Mirochnitchenko, and Sidney Pestka, 2000. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc Natl Acad Sci U S A. 2000 February 15; 97(4): 1695–1700.
Sia, I. G., and R. Patel. 2000. New strategies for prevention and therapy of cytomegalovirus infection and disease in solid-organ transplant recipients. Clin. Microbiol. Rev. 13:83-121.

Sinzger C, Jahn G. 1996. Human cytomegalovirus cell tropism and pathogenesis. Intervirology. 39(5-6):302-19.

Stinski, M. F. 1978. Sequence of protein synthesis in cells infected by human cytomegalovirus: early and late virus-induced polypeptides. J. Virol. 26:686-701.

Talarico, C.L. u. a., 1999. Acyclovir Is Phosphorylated by the Human Cytomegalovirus UL97 Protein. Antimicrobial Agents and Chemotherapy, 43(8), 1941-1946.

van den Berg, A. P., I. J. Klimpmaker, E. B. Haagsma, A. Scholten-Sampson, C. M. A. Bijleveld, J. Schirm, M. van der Giessen, M. J. H. Slooff, and T. H. The. 1991. Antigenemia in the diagnosis and monitoring of active cytomegalovirus infection after liver transplantation. J. Infect. Dis. 164:265–270.

Varnum, S. M., D. N. Streblow, M. E. Monroe, P. Smith, K. J. Auberry, L. Pasa-Tolic, D. Wang, D. G. Camp II, K. Rodland, S. Wiley, W. Britt, T. Shenk, R. D. Smith, and J. A. Nelson. 2004. Identification of proteins in human cytomegalovirus (HCMV) particles: the HCMV proteome. J. Virol. 78:10960-10966.

Yee EL et al. Widespread outbreak of norovirus gastroenteritis among evacuees of Hurricane Katrina residing in a large "megashelter" in Houston, Texas: lessons learned for prevention. Clin Infect Dis. 2007 Apr 15;44(8):1032-9. Epub 2007 Mar 2.

8 Figures and Tables

Fig 2.1: Three-dimensional model of CMV showing the different components of the virus; Reschke M. and Eickmann M. 1994-2005, Institute of Virology, Germany http://www.biografix.de/

Fig. 2.2: Genomic structure of CMV; Kotenko S.V., Saccani S., Izotova L.S., Mirochnitchenko O.V., and Pestka S., 2000. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc Natl Acad Sci U S A. 2000 February 15; 97(4): 1695–1700.

Fig. 2.3: Life cycle of CMV in a human cell; Crough Tania and Khanna Rajiv, Immunobiology of Human Cytomegalovirus: from Bench to Bedside, Clinical Microbiology Reviews, January 2009, p. 76-98, Vol. 22, No. 1

Fig. 2.4: The four major steps of the magnetic glass (also called silica) particle technology; Ciotti Marco; Kessler HH (2010) Pathogens relevant in transplantation medicine In: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York

Fig.: 2.5: Chemical structure of Acyclovir and Ganciclovir available in http://pathmicro.med.sc.edu/lecture/images/acvgan.gif

Fig. 3.1: The study design

Fig. 3.2: Vessels for collection of clinical specimens used in this study; © 2011 by Barbara Klara Maier

Fig. 3.3: The NucliSens easyMAG instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 3.4: The QIAsymphony SP instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 3.5: The MagNA Pure LC 2.0 instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 3.6: The VERSANT kPCR SP instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 3.7: The CMV HHV6,7,8 R-gene™ kit; © 2011 by Barbara Klara Maier

Fig. 3.6: The LightCycler 480 II instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 3.6: The VERSANT kPCR AD instrument; © 2011 by Prof. Dr. Harald H. Kessler

Fig. 4.1a: CMV DNA concentrations in EDTA whole blood obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

Fig. 4.1b: CMV levels in EDTA plasma obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

Fig. 4.1c: CMV levels in urine obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

Fig. 4.1d: CMV levels in BALs obtained from different extraction systems in conjunction with the VERSANT kPCR AD.

Fig. 4.2a: CMV levels in EDTA whole blood obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

Fig. 4.2b: CMV levels in EDTA plasma obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

Fig. 4.2c: CMV levels in urine obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

Fig. 4.2d: CMV levels in BALs obtained from different extraction systems in conjunction with the LightCycler 480 II instrument.

Table 2.1: Choice of adequate sample material for molecular diagnostics of CMV infection; Rabenau H, Raggam RB, Salzer HJF (2010) Choice of adequate sample material, In: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York.

Table 2.2.: Comparison of currently frequently used commercially available assays for the quantitation of cytomegalovirus (CMV) DNA; Ciotti Marco; Kessler HH (2010) Pathogens relevant in transplantation medicine In: Kessler HH (Ed.): Molecular Diagnostics of Infectious Diseases. De Gruyter, Berlin, New York.

Table 3.1: Basic features of extraction systems used in this study.

Table 3.2: Kits and protocols for extraction of CMV DNA used in this study.

Table.4.1: Comparison of CMV DNA concentrations obtained from extraction using automated nucleic acids extraction systems in conjunction with real-time PCR on the VERSANT kPCR AD.

Table.4.2: Comparison of CMV DNA concentrations obtained from extraction using automated nucleic acids extraction systems in conjunction with real-time PCR on the LightCycler 480 II.

Table 4.3: Comparison of CMV DNA concentrations when amplifying with the VERSANT kPCR AD and the LightCycler 480 II.

Table 4.4: Comparison of times-to-result (min) when extracting 24 samples in parallel.