

# Multiplex real-time NASBA for monitoring expression dynamics of human cytomegalovirus encoded IE1 and pp67 RNA

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## Abstract

**Background:** The monitoring for HCMV mRNA expression in whole blood provides an accurate and informative diagnostic approach. **Methods and materials:** A multiplex real-time NASBA with three molecular beacons (MR-NASBA) was developed for the simultaneous detection and quantification of HCMV-encoded immediate early-1 (IE1) and late pp67 mRNA. The assay was evaluated using RNA from in vitro HCMV-infected cells and sequential whole blood samples (100  $\mu$ l) of HCMV infected lung transplant recipients. **Results:** The MR-NASBA showed equal performance compared with standard NASBA assays (sensitivity of  $1-3 \times 10^3$  RNA molecules in 100  $\mu$ l blood and a linear range of  $10^3-10^6$  RNA molecules). The standard IE1 Q-RNA provides a reliable internal system control. No interference was observed between the individual beacon signals. The simultaneous 'one-tube' quantification of IE1-RNA levels combined with qualitative detection of pp67-RNA is feasible without loss of assay performance in clinical whole blood specimens. **Conclusion and comments:** MR-NASBA may be suitable for monitoring HCMV-activity in transplant recipients to aid in fine-tuning of antiviral intervention in high risk populations employing a traffic light diagnostic approach: no HCMV RNA signal (green light: safe) reflects absent or fully latent infection requiring no antiviral intervention; an 'IE1-RNA only' signal (yellow light: alert) indicates an emerging or subclinical active infection, opting for preemptive treatment in high risk populations; simultaneous 'IE1-RNA plus pp67-RNA' detection (red light:danger) indicates disseminating productive infection requiring immediate antiviral treatment. © 2002 Elsevier Science B.V. All rights reserved.

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## 1. Introduction

The presence of human cytomegalovirus (HCMV) can cause severe disease in immunocompromised host if left untreated (Britt and Alford,

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1996). In transplant recipients an accurate diagnosis can discriminate infection from rejection of the transplanted organ or graft-versus-host disease and prevent development of HCMV disease by antiviral treatment (Patel et al., 1996). Antigene-mia is the assay commonly used for determining the onset of HCMV disease (Bij et al., 1988; The et al., 1998). In addition, nucleic acid based diagnostic assays are emerging, enabling the detection and quantification of viral DNA and RNA using various techniques including PCR and NASBA. Qualitative PCR does not allow direct discrimination between latent infection and active virus replication, caused by the persistent presence of variable levels of HCMV in blood of healthy carriers (Caliendo et al., 2000; Patel et al., 1996). Quantification of HCMV genomes in plasma by PCR was shown to be relevant for determining the severity of infection and to predict the risk of developing HCMV disease (Caliendo et al., 2000; Spector et al., 1998). Detection of RNA is more directly reflecting viral activity and is associated with the progression of HCMV infection (Middel-dorp et al., 2000). The nucleic acid sequence-based amplification method (NASBA) detecting immediate early (IE1) and late pp67 RNA was evaluated for the monitoring of transplant recipients. IE1 RNA was shown to be a relevant early marker of lytic infection and pp67 RNA proved to be a marker for productive infection (Blok et al., 1999; Gerna et al., 2000; Oldenburg et al., 2000) comparable to the previous RT-PCR findings (Lam et al., 1998; Meyer-Köning et al., 1995). Recently, we described a quantitative NASBA for IE1 and pp67 RNA (Greijer et al., 2001a). Quantification of IE1 RNA may provide an early indication of progressive and clinically relevant viral activity. The quantification of pp67 RNA may not be essential, since presence of pp67 RNA alone reflects productive infection of HCMV in the circulation requiring immediate start of antiviral therapy (Gerna et al., 1999; Greijer et al., 2001a). However, most RNA detection methods are time consuming and involve multiple steps with obvious risk of cross-contamination. These disadvantages could be overcome by using a real-time detection method. Real-time automated quantification of DNA by PCR was

reported by Heid et al. This method measures PCR product accumulation by means of a dual-labeled fluorogenic probe (i.e. TaqMan probe), and it provides a very accurate and reproducible measure of gene copies. The use of real-time quantitative PCR assay to determine the HCMV viral DNA load (Tanaka et al., 2000) is described by using the US17 gene (Machida et al., 2000), immediate early gene (Nitsche et al., 2000) and a multiplex PCR using immediate early and glycoprotein B gene (Yun et al., 2000). However, due to the use of external calibration curves these real-time PCR approaches are semi-quantitative and do not correct for variation in PCR efficiency between samples.

For real-time detecting of HCMV RNA by the NASBA assay molecular beacons may be more convenient (Leone et al., 1998). Molecular beacons are single-stranded oligonucleotides having a stem-and-loop structure. The loop contains a probe sequence that is complementary to the target amplicon and the stem is formed by the annealing of short complementary sequences located on either side of the probe sequence. A fluorophore is linked covalently to the end of one arm and a quencher is linked covalently to the end of the other arm. When molecular beacons are in solution they will form a hairpin structure. The stem keeps the fluorophore in close proximity to the quencher, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the molecular beacon hybridizes to its target it undergoes a conformational change that separates the fluorophore and the quencher, allowing the fluorophore to emit fluorescent light upon excitation (Tyagi and Kramer, 1996). A comparison of hairpin probes with corresponding linear probes confirms that the presence of the hairpin stem in molecular beacons significantly enhances their specificity (Tyagi et al., 1998). In a multiplex assay a number of molecular beacons can be used simultaneously when different fluorophores are linked to each molecular beacons (Tyagi et al., 1998). The different fluorophores are measured simultaneously by the use of several defined excitation and emission filters. The use of four molecular beacons with differently colored fluorophores in patient samples was recently de-

scribed by Vet et al. using a multiplex qualitative RT-PCR assay for detection of HIV-1, HIV-2, HTLV types I and II (Vet et al., 1999).

The aim of this study was to develop a real-time NASBA assay that combines quantitative measurement of IE1 RNA with the qualitative detection of pp67 RNA. For this real-time approach the use of a multiplex NASBA was needed. Multiplex NASBA assay is described as being highly specific and sensitive (Deursen et al., 1999). The real-time multiplex NASBA assay is performed in a single tube, competitively co-amplifying and simultaneously detecting the wt IE1 RNA, the calibrator RNA of IE1, and pp67 RNA. The presence of a fixed amount of calibrator IE1 RNA spiked into the sample prior to isolation allows standardization irrespective of the clinical sample used.

## 2. Materials and methods

### 2.1. Cells and virus

Human lung fibroblasts (HLF) were infected with HCMV AD169 at an MOI of 5. After 1 h the virus suspension was removed and a 1:1 mixture of Ham's F12 and Dulbecco's modified Eagle medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah) was added. At different times after infection cells were harvested by trypsinization, washed with PBS, and lysed in NASBA lysis buffer (5 M guanidinium thiocyanate, 0.1 M Tris pH 6.4, 20 mM EDTA, 1.2% [wt./vol.] Triton-X100) at  $10^6$  cells per ml.

### 2.2. Clinical samples

Follow-up whole blood samples obtained from one primary infected lung transplant recipient were collected as described previously (Greijer et al., 2001a). One ml blood was mixed with 9 ml NASBA lysis buffer (5 M guanidine isothiocyanate, 0.1 M Tris pH 6.4, 20 mM EDTA, 1.2% [wt./vol.] Triton-X100) and was stored at  $-80$  °C. The patient was treated with antiviral therapy when the antigenemia assay became positive (Bij et al., 1988; Tanaka et al., 2000).

### 2.3. Primers and molecular beacons

The sequences of all primers used in this study are described elsewhere (Greijer et al., 2001a). Three molecular beacons were used to detect the NASBA amplicons. Each possessed a 6-nt stem sequence flanking 18 or 19 nt probe sequence. Each beacon was labeled with a different fluorophore: fluorescein (FAM) was used for detecting IE1 wt RNA, 6-carboxy-X-rhodamine (ROX) was used for detecting the IE1 Q RNA, and Cascade Blue (CB) was used for detecting pp67 wt RNA. All of the molecular beacons possessed the nonfluorescent quencher 4(4'-dimethylaminophenylazo) benzoid acid (DABCYL-200). The sequences of the molecular beacons were: IE1 wt/FAM: cgactcattgggctaactatgcagagagtcg, IE1 Q/ROX: gacctggtagctactagattgcacagtc, and pp67 wt/CB: cgactccaaaaagctagccgtcagagtcg, where underlines identify the probe sequences. All molecular beacons were synthesized by Eurogentec (Seraing, Belgium).

### 2.4. Nucleic acids isolation

The isolation of RNA was performed as described by Boom (Boom et al., 1990). RNA is isolated by lysis of 100  $\mu$ l of sample in 900  $\mu$ l NASBA lysis buffer (5 M guanidine isothiocyanate, 0.1 M Tris pH 6.4, 20 mM EDTA, 1.2% [wt./vol.] Triton X100). IE1 Q-RNA was spiked into the sample at a concentration of  $2 \times 10^4$  RNA copies. For quantification by the ECL detection method  $1 \times 10^4$  RNA copies was spiked into the sample. RNA was bound to silica and the silica was washed twice with wash buffer (5 M guanidine thiocyanate, 50 mM Tris pH 6.4), twice with 70% ethanol and once with acetone. The silica is dried at 56 °C for 10 min and RNA was eluted in 50  $\mu$ l elution buffer (1 mM Tris pH 8.5) for 10 min at 56 °C.

### 2.5. MR-NASBA

The MR-NASBA assay was performed with 5  $\mu$ l nucleic acid elute essentially performed as described by Kievits et al. (Kievits et al., 1991). A

premix was made by reconstitution of a basic kit NASBA reagent accu in 80  $\mu$ l accu diluent (Organon Teknika, Boxtel, The Netherlands) and adding 14  $\mu$ l 1.2 M KCL, 2.5  $\mu$ l of each 20  $\mu$ M primer of IE1 and pp67, 1.2  $\mu$ l of 20  $\mu$ M IE1 wt molecular beacon, 1.2  $\mu$ l of 20  $\mu$ M IE1 Q molecular beacon, and 2.4  $\mu$ l of 20  $\mu$ M pp67 wt beacon, where after the volume was brought to 120  $\mu$ l with H<sub>2</sub>O. 10  $\mu$ l of premix was added in a thin wall Eppendorf tube. After adding 5  $\mu$ l nucleic acid eluate the mix was incubated for 2 min at 65 °C and cooled down for 2 min at 41 °C. About 5  $\mu$ l enzyme mix (RNA polymerase [Pharmacia, Upsala, Sweden], RNase H [Pharmacia] and avian myeloblastosis virus-reverse transcriptase [Seikagaku, Rockville, MD]) was added. The NASBA reaction was measured in real-time using a Cytofluor (PerSeptive Biosystems, Foster City, CA USA) at 41 °C for 90 min using the filter combinations as presented in Table 1.

### 2.6. Quantification of IE1 RNA

The fluorescent increase ( $dA/dt$ ) of each sample for both wt and Q-RNA was determined by least squares regression analysis of all points within the lower and upper fluorescent cut-off. The average background plus five standard deviations from reading 3 to 6 determined the lower cut-off. The upper cut-off was defined as 50% of the maximum fluorescent increase. Both lower and upper cut-off values were automatically determined for each individual sample. For quantification of IE1 wt RNA, a standard curve ( $10^6$ ,  $10^4$  and  $10^3$  RNA copies in 100  $\mu$ l whole blood with six samples per concentration) was defined based upon the log input concentration over the  $\log(dA/dt)$  standard divided by  $dA/dt$  control).

Table 1  
The excitation and emission filters used in this study

Molecular beacon	Excitation $\lambda$ (nm)	Emission $\lambda$ (nm)
IE1 wt/FAM	485 (20)	530 (40)
IE1 Q/ROX	590 (20)	620 (25)
pp67/CB	360 (40)	460 (40)

### 2.7. ECL detection

The conventional quantitative NASBA for IE1 and pp67 is described previously (Greijer et al., 2001a). The amplification and detection is done separately for IE1 and pp67. For optimal ECL detection of IE1 and pp67 RNA, amplification products were diluted 76 and 21 times, respectively. Amplification products were detected by electrochemiluminescence, using capture probes coupled to magnetic beads and wt and Q specific ruthenium labeled oligonucleotide detection probes using the NASBA QR system (Organon Teknika) and quantified as described previously (Greijer et al., 2001a).

## 3. Results

### 3.1. Assay design

The MR-NASBA assay developed in this study was designed to quantify IE1 mRNA by competitive co-amplification of wt and calibrator RNA (Q-RNA) with simultaneous detection of late pp67 mRNA. Since this multiplex NASBA requires simultaneous co-amplification of three RNA targets, the primer concentration for each target was optimized. It appeared that for this assay an identical concentration of 2 nM of each primer was needed, similar as used for a single amplification (data not shown). For detecting three different amplicons, three different colored molecular beacons with unique complementary sequences in the loop were required. For accurate quantification, IE1 wt and IE1 Q fluorophores with identical fluorescent intensities and hybridization kinetics were used for detection (Table 2). The use of FAM and ROX as fluorescent labels fulfil this requirement as were described earlier (Greijer et al., 2001a; Tyagi et al., 1998). A third fluorophore was selected having a spectrum without overlap with the other two fluorophores. Two labels were evaluated, Alexa 350 and Cascade Blue. Characteristics of all fluorophores are shown in Table 2. All experiments described in this study for detecting pp67 RNA were performed with Cascade Blue as label, since this

Table 2  
The characteristics of the four fluorophores used in this study

	Excitation maximum (nm)	Emission maximum (nm)	EC( $\times 10^3$ ) <sup>a</sup>
Alexa-350	350	442	16
Cascade Blue	400	425	29
Rox	575	602	82
FAM	494	520	78

<sup>a</sup> EC: molecular extinction coefficient.

molecular beacon gave slightly better overall NASBA performance (data not shown).

### 3.2. Simultaneous detection of IE1 wt, IE1 Q, and pp67 wt RNA

The ability of the MR-NASBA assay to specifically detect the different RNA targets was evaluated. High input amounts of wt IE1 RNA ( $10^6$  RNA copies) were amplified in the absence of IE1 Q and pp67 wt RNA and the three beacons were simultaneously monitored in time. The concentration molecular beacons used for detecting IE wt and Q-RNA was 2 nM identical to the primer concentration, whereas the concentration of the molecular beacon detecting pp67 RNA was two times higher, since the fluorescent signal is lower. No signal was observed for the molecular beacons detecting IE1 Q and pp67 wt RNA, whereas the IE1 wt molecular beacon showed dynamic increase reaching a plateau within 30 min (Fig. 1A). All combinations were tested and only signals were observed from the molecular beacon of which the complementary amplicon was present (Fig. 1B and C). Consequently, the molecular beacons were specific and did not cross react with the different amplicons. In addition the emission spectra of the three fluorophores did not interfere with each other.

### 3.3. Sensitivity of the IE1 and pp67 real-time NASBA

In order to determine the sensitivity of the IE1 and pp67 real-time NASBA a serial dilution was made in whole blood ranging from  $10^3$  to  $10^6$  RNA copies per 100  $\mu$ l whole blood. The samples

were made and isolated in 6-fold. IE1 RNA was detected at 100% at concentrations above  $10^3$  copies per 100  $\mu$ l whole blood. The pp67 RNA amplification product was detected at 100% of the samples above  $3 \times 10^3$  RNA copies input (data not shown). These amounts are roughly similar to the RNA content of one in vitro infected cell equivalents (Greijer et al., 2001b).

### 3.4. Reproducibility of quantification of IE1 RNA levels

In a background of pp67 amplification the reproducibility of IE1 NASBA quantification was determined. RNA from samples randomly spiked with wt IE1 and wt pp67 RNA ranging from  $10^3$  till  $10^6$  RNA copies was isolated and amplified in 3-fold, and the amount of IE1 wt RNA copies was calculated. The results are presented in Fig. 2. Low concentrations of IE1 wt RNA around  $10^3$  copies per isolation were quantified with considerable variation as previously observed with ECL detection, whereas concentrations above  $10^4$  RNA copies yielded reproducible quantification results, showing a curve with a linear increase. The presence and amount of pp67 wt RNA did not influence the outcome of the quantification (data not shown) and was therefore not responsible for the fluctuations in the quantification at the lower RNA concentrations.

### 3.5. Real-time detection of IE1 and pp67 RNA in infected cells

To evaluate real-time NASBA for monitoring the dynamics of the expression of IE1 and pp67 RNA during the course of infection, HCMV in-

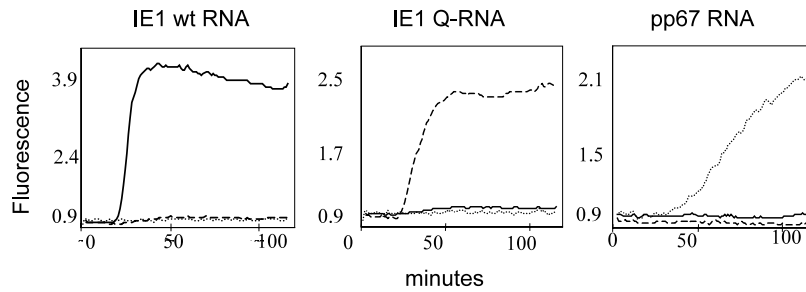


Fig. 1. Detection of specific RNA in the presence of IE1 wt \FAM, IE1 Q \ROX, and pp67 \CB molecular beacons, solid line: FAM (IE1 wt), discontinuous line: ROX (IE1 Q), dotted line: CB (pp67 wt).

ected HLF cells were harvested and analyzed for IE1 and pp67 RNA expression. The results of the conventional NASBA using separate ECL detection and the real-time NASBA were compared (Fig. 3). IE1 RNA levels were quantified at different time points in the course of HCMV infection. The ECL data of pp67 were of the quantitative NASBA and were compared with the results of the qualitative real-time NASBA. The results are shown in Fig. 3. The kinetics of IE1 RNA expression was similar as measured by both approaches. When analyzing pp67 RNA by ECL detection it appeared that ECL detection was more sensitive than the real-time measurement, which may be related to the suboptimal fluorescence emission intensity of the third molecular beacon. Despite the somewhat lower sensitivity of the real-time NASBA compared with the conventional NASBA, the quantification of IE1 simultaneously with the detection of the presence of pp67 RNA reproducible and accurate.

### 3.6. IE1 RNA quantification in a lung transplant recipient

The use of MR-NASBA for monitoring HCMV infected transplant recipients was analyzed in follow-up whole blood samples of a lung transplant recipient with a primary HCMV infection. The antigenemia assay is used to define active HCMV infection with consequently initiation of treatment with antiviral therapy as described before (Greijer et al., 2001a). The results are indicated in Fig. 4. Pp67 RNA was present at indicated time points as

determined by ECL (Fig. 4). The results of MR-NASBA and the conventional ECL NASBA have parallel courses, except for one time point at 22 weeks post transplantation. At this time the ECL detection quantified the IE1 RNA at the extraordinary high level of  $10^8$  RNA copies. The discrepancy between the assays may be explained by the unknown reliability of the quantification above  $10^6$  RNA copies. This experiment using whole blood samples of a lung transplant recipient with active HCMV infection demonstrates the feasibility of IE1 RNA quantification by MR-NASBA.

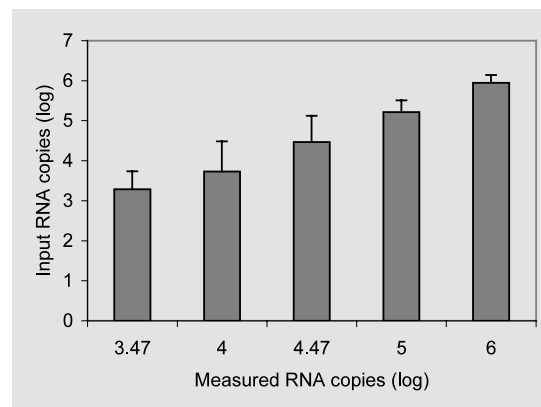


Fig. 2. Characterization of the quantification of IE1 RNA in the MR-NASBA assay using serial dilutions of in vitro IE1 RNA spiked in the presence of IE1 Q-RNA and different concentrations of pp67 RNA randomly spiked in 100  $\mu$ l blood of healthy individuals. Each concentration was analyzed in 6-fold.

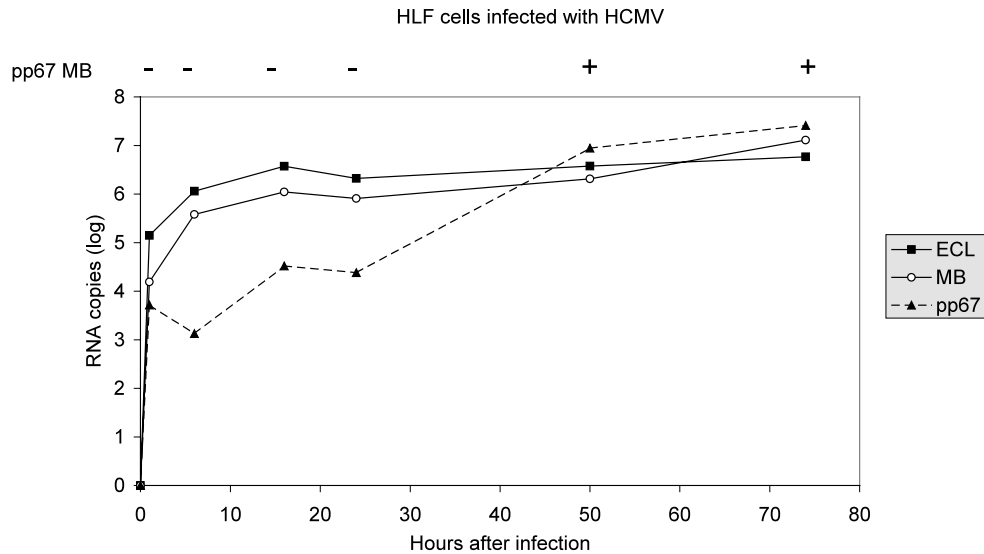


Fig. 3. The course of HCMV infection in HCMV infected HLF cells by the MR-NASBA and the ECL detection of IE1 and pp67 RNA by conventional NASBA assays.

#### 4. Discussion

HCMV IE1 mRNA expression in blood leukocytes is considered to be the earliest sign of active HCMV infection in the host, either during primary or secondary reactivating infection (Gerna et al., 2000; Greijer et al., 2001a). However, IE1 mRNA expression in blood may not always predict clinically relevant progression of infection and is poorly correlating with disease development. Monitoring the expression of an abundant late mRNA like UL65 encoded pp67 RNA is more closely related to viral dissemination and development of disease (Blok et al., 1999; Gerna et al., 1999; Oldenburg et al., 2000). Quantification of IE1 mRNA in blood was more suitable for monitoring progression of HCMV infection, since levels higher than  $10^4$  RNA copies per 100  $\mu$ l blood are related to episodes of high antigenemia and requirement of antiviral therapy. The combination of the clinical utility of IE1 quantification and late mRNA detection in a simple assay approach would further improve the diagnostic monitoring of HCMV infections in immunocompromised patients. In order to achieve this goal a multiplex real-time assay was developed detecting the dynamics of a very sensitive marker reflecting

early HCMV activity and detecting simultaneously the presence of a marker reflecting an active disseminating productive HCMV infection in a single assay. Three molecular beacons colored with three different labels were used to simultaneously quantify RNA levels of IE1 RNA and qualitatively detect pp67 RNA. An internal calibrator RNA enables accurate quantification of IE1 RNA, and simultaneously forms is an internal validation control for the assay. The quantification above  $10^4$  copies of IE1 RNA, which is a clinically relevant level (Greijer et al., 2001a), is reproducible. The reduced overall sensitivity for IE1 and the pp67 real-time NASBA compared with the conventional NASBA needs further optimization. Especially the molecular beacon used for detecting the pp67 amplicon may be improved by using a molecular beacon with an alternative fluorescent label, giving a stronger fluorescent signal. The decreased sensitivity of a real-time assay is also described for HCMV PCR assays of the US17 gene (Machida et al., 2000).

The MR-NASBA assay could be useful to accurately diagnose active HCMV infection and to fine-tune the period of antiviral therapy in transplant recipients. Previous studies with qualitative and quantitative IE1 and pp67 NASBA assays

showed that the presence of pp67 alone indicated direct requirement of antiviral therapy (Blok et al., 1998; Gerna et al., 1999). This is currently substantiated in prospective controlled clinical studies (Lunenbergh et al., 2000). Our previous study showed that the quantification of IE1 mRNA was predictive for the development of active disseminating HCMV infection and disease development in patients that did not receive pre-emptive therapy (Greijer et al., 2001a). These results can be extrapolated to the real-time NASBA assay. The results of MR-NASBA assay can be used as a traffic light for monitoring of HCMV activity and timely start of antiviral therapy. The absence of both IE1 and pp67 RNA means green light for the patient, since no HCMV activity can be detected (safe). Early increases in IE1 RNA reflect the onset of active HCMV infection and therefore indicate a yellow light, an alert for development of symptomatic HCMV infection. When increases of IE1 RNA levels are observed the patient may be more closely monitored in order to start the antiviral therapy on time. Decreasing levels of IE1 mRNA indicate that antiviral therapy may not be needed. The presence of pp67 RNA indicates the need of antiviral ther-

apy, meaning a red light. The presence of pp67 mRNA alone is reflecting active productive infection, which should be blocked at early stages by antiviral therapy. Quantification of pp67 RNA will not give additional information, as shown in a previous study (Greijer et al., 2001a).

The MR-NASBA assay has the advantage that the result of amplification is immediately available in contrast to the conventional ECL detection. This considerably reduces the assay time and more importantly, it lowers the hands on time and limits potential contamination between samples. Further studies to evaluate MR-NASBA in a clinical setting are needed, to validate its potential contribution for diagnostic monitoring and timing of antiviral treatment, thereby improving the overall management of HCMV infections in transplant recipients.

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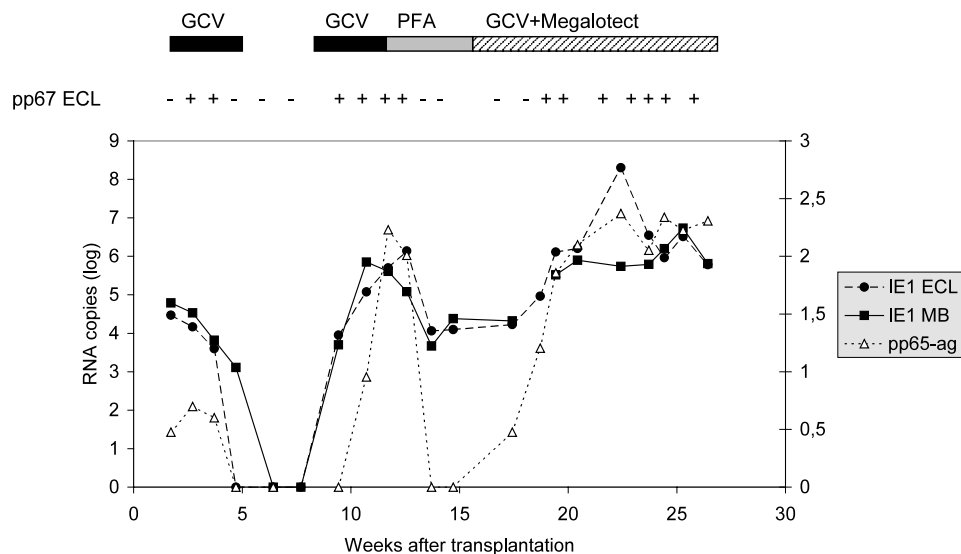


Fig. 4. The course of HCMV infection in a primary infected lung transplant recipient by antigenemia, IE1 MR-NASBA, and conventional ECL detection of IE1 and pp67 NASBA assays. The antiviral treatment is given in boxes; black: ganciclovir (GCV), gray: foscarnet (PFA), stripes: Megalotect.

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