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Improved Detection of Mutated Human Cytomegalovirus UL97 by Pyrosequencing†

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Ganciclovir (GCV) resistance frequently occurs upon prolonged treatment of ongoing active human cytomegalovirus (HCMV) infection in individuals with immature or compromised immune functions (e.g., recipients of solid-organ and hematopoietic stem cell transplants). Using pyrosequencing (PSQ), we established fast and sensitive detection of GCV resistance-associated mutations occurring in the HCMV open reading frame UL97. These mutations have been repeatedly associated with clinical treatment failure. We designed four PSQ assays and evaluated them by analyzing mixtures of plasmids or bacterial artificial chromosome-derived viruses containing UL97 wild-type and mutant sequences. A minimum level of 6% mutant sequence variants could be detected in these mixtures. In order to further evaluate the novel PSQ assays, we tested clinical specimens from patients with active HCMV infections. The results were compared with those obtained by conventional dideoxy chain terminator sequencing. As the PSQ method was more sensitive in detecting minor HCMV mutant fractions in a wild-type population, it is suggested that pyrosequencing is a useful tool for the early detection of emerging GCV-resistant HCMV in GCV-treated patients.

Human cytomegalovirus (HCMV) is an important opportunistic pathogen for humans of all ages with immature or compromised immune functions (17). Drugs commonly used for the treatment of HCMV infections are ganciclovir (GCV), cidofovir, and foscarnet, which all target the viral DNA polymerase. However, all these compounds cause considerable adverse effects. Their prolonged application frequently results in the selection of drug-resistant HCMV mutants with eventual failure of therapy (reviewed in reference 6).

The first-line drug GCV is pharmacologically a prodrug that requires phosphorylation to the monophosphate by the HCMV UL97-encoded protein kinase to gain its antiviral activity. As a consequence, over 80% of GCV-resistant isolates carry mutations in the UL97 gene (4, 8). UL97 mutations known to confer GCV resistance occur predominantly at codons 460 and 520 and at several codons between 590 and 607, particularly at codon positions 592, 594, 595, and 603 (3). During the selection process of a spontaneously occurring UL97 mutation under drug pressure, mixed virus populations exist in the patient. At present, direct dideoxy chain termination sequencing (CTSQ) of PCR products spanning this part of the UL97 gene is the method of choice for the detection of mutant viruses (16). However, the sensitivity of the CTSQ method in detecting minor mutant fractions in samples with heterogeneous mutant-wild-type HCMV mixtures is only moderate. Approximately 20% of the total virus population has to be mutant virus in order to be solidly detected (2; B. Schindele and B. Ehlers, unpublished data). However, fast and early detection of emerging resistant virus is of particular importance, since mixed HCMV UL97 populations can influence GCV susceptibility, and detection may predict the outgrowth of resistant virus variants (5). Therefore, the early and sensitive detection of a resistance-associated HCMV mutant is highly desirable.

Pyrosequencing (PSQ) (18) has been successfully used to detect lamivudine-resistant variants of hepatitis B virus in heterogeneous virus populations (12) and drug-resistant influenza virus quasispecies (11). In the present study, we evaluated whether PSQ is also suitable for the fast detection of GCVresistant HCMV. The method was compared with CTSQ by analyzing a collection of samples containing mixed HCMV populations derived from patients with active HCMV infections. The results show that the PSQ method has considerable advantages over the currently used CTSQ method in detecting minor fractions of HCMV UL97 mutants in mixed virus populations.

Materials and Methods

UL97 PCR, plasmids, and viruses.
UL97 mutant and wild-type sequences (729 bp) were amplified by PCR with the primer pair UL97-1234-1PCR-P (5'-TCGACGTTTCCACACAGACATG)/UL97-1962-1PCR-M (5'-GTGGTAGAAGCGGCGAAAGG) (15, 16) from UL97-recombinant vaccinia viruses constructed earlier (1). UL97 sequences from patient samples were amplified by nested PCR (680 bp) using the primer pair described above in the first round and the primer pair UL97-1256-2PCR-P (5'-TTTCATCACGACCAGTGGAAG) and UL97-1935-2PCR-M (5'-GCGACACGAGGACATCTTGG) in the second PCR round (Fig. 1).

PCR was performed with 10 x AmpliTaq buffer, 2.5 mM MgCl2, 5% dimethyl sulfoxide (DMSO), deoxynucleoside triphosphates (dNTPs) at 10 mM each, 1.0 unit of AmpliTaq Gold polymerase, and 1 μM (each) sense and antisense primers under the following conditions: initial denaturation 95°C for 12 min; 45 cycles with denaturation at 95°C for 30 s, annealing at 61°C for 30 s, and elongation at 72°C for 30 s; and final elongation at 72°C for 15 min.

Amplification products were cloned in *Escherichia coli* using the Topo TA Cloning Kit pCR2.1TOPO (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. A plasmid with UL97 wild-type sequence and 10 plasmids with resistance-associated mutations were generated and named PL_WT, Pl_M460I, Pl_M460V, Pl_H520Q, Pl_C592G, Pl_A594G, Pl_A594V, Pl_L595F, Pl _L595S, Pl_C603W, and Pl_C607Y. The plasmid DNAs were used alone or as mutant–wild-type mixtures for PSQ evaluation.

Two HCMV strains, Titan wild type and Titan A594V carrying the UL97 mutation A594V, have been cloned as bacterial artificial chromosomes (14) and were used here for PSQ evaluation experiments.

HCMV-positive patient specimens and UL97 analysis by PSQ and CTSQ sequencing.
The applicability of the developed PSQ assays for virological diagnostics was tested with plasma, urine, spinal fluid, ascites fluid, throat swab, and breast milk samples (n = 27) from six individuals (designated A to F). Three of them (patients A, B, and C) had received GCV treatment and had shown clinical manifestations and laboratory parameters, i.e., increasing or continuously high HCMV genome copy numbers in real-time PCR (3.5×10^3 to 3.5×10^6 HCMV genome copies/ml), indicative of treatment failure. Six samples were available for patient A, seven for patient B, and eight for C. Two individuals (patients D and E) suffered from active HCMV infection as determined with real-time PCR but were therapy naïve (two samples each). From the sixth individual (F, the HCMVpositive, therapy-naïve mother of patient B), two milk samples were collected.

DNA was extracted from 200 μl of sample material using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The DNA was eluted in 50 μl double-distilled H2O (ddH2O) according to the manufacturer's recommendations. UL97 sequences were amplified by PCR and cloned in *E. coli* as described above. PCR products and recombinant plasmids were sequenced according to the method of Sanger with the Big Dye terminator cycle-sequencing kit (Applied Biosystems, Warrington, United Kingdom) in a 3130 DNA automated sequencer (Applied Biosystems) or pyrosequenced as described below.

For the development of pyrosequencing assays, all UL97 sequences available in GenBank were aligned using the software MacVector 10.6. Conserved regions flanking the codons of interest were identified and used for the design of PCR primers and pyrosequencing primers with the Pyrosequencing Assay Design software (Qiagen, Hilden, Germany). Four sets, each consisting of two PCR primers and one pyrosequencing primer, were selected to analyze UL97 codons (see Table S1 in

the supplemental material). Each PCR primer that did not bind to the same strand as the respective PSQ primer was biotinylated to allow immobilization of the PCR product on streptavidin-coated beads and preparation of single-stranded DNA for pyrosequencing with the PSQ primer.

PCR product preparation for PSQ and the PSQ reaction were performed essentially according to the manufacturer's recommendations (Qiagen, Hilden, Germany). Briefly, the biotinylated PCR product (20 µl) was bound to 200 µg of streptavidin-coated beads (GE Biosciences, Uppsala, Sweden) by shaking it at room temperature for 10 min. A single-stranded template was purified through a series of washes and hybridized with 200 µM of the appropriate PSQ primer (see Table S1 in the supplemental material). The pyrosequencing reaction was then performed using the PyroMark ID machine (Qiagen, Hilden, Germany).

A specific rather than a cyclic nucleotide dispensation strategy (order of nucleotide additions) was used to increase read length and quality. The dispensation orders used in pyrosequencing assays 1 to 4 were designed on the basis of the relevant UL97 sequence variants and are listed in Table 1.

Real-time PCR.
For quantification of HCMV genome copy numbers in patient samples, PCR products were generated in duplicate with the PSQ primer of PCR assay 2 as described above. For the real-time PCR assay, we used an MGB (minor groove binder) probe (FAM-CTGCTCATCTGCGACC MGB) that targets a conserved site within UL97. The reaction was performed in a Stratagene MX3000P-Cycler (Stratagene, La Jolla, CA). The detection limit was 10 copies/ reaction, and the assay was linear between 101 and 106 copies (data not shown)

Results

Optimization of the PCR for pyrosequencing.
Four PCR assays were set up for amplification of UL97 sequences comprising codon 460 (assay 1) or 520 (assay 2) or the region between codons 592 and 607 (assays 3 and 4) and optimized by using UL97 recombinant plasmids and DNA from HCMVpositive serum samples. After PCR with serially diluted UL97 recombinant plasmids (10^4 to 10^{-1} copies) and agarose gel electrophoresis, a minimum of 10 HCMV DNA copies/reaction were detected with each of the four PCR assays (data not shown). The PCR primers with their annealing temperatures are listed in Table S1 in the supplemental material, and the amplified UL97 regions are shown in Fig. 1.

Pyrosequencing of wild-type and mutant UL97.
PSQ primers were designed with binding sites immediately upstream of the target codons (see Table S1 in the supplemental material). Specific dispensation orders could be chosen (instead of cyclic dispensation) because the UL97 sequences that span the codons of interest and their flanking codons are sufficiently conserved. With these PSQ primers and the respective dispensation orders, we specifically addressed the resistance mutants M460V and M460I (PSQ assay 1); H520Q (PSQ assay 2); C592G, A594V, L595S, C603W, and C607Y (PSQ assay 3); and A594G and L595F (PSQ assay 4) (Table 1 and Fig. 1), allowing relative quantification of mutant and wild-type viruses in virus mixtures. In assay 3, polymorphisms without resistance association, frequently occurring in codons 598 and 605, were additionally incorporated into the dispensation order. Thereby, we ensured correct downstream sequencing and quantification of mutants by the pyrosequencing software (see Table 1 note *c*).

For each PSQ primer, the expected pyrogram patterns for wild-type and mutant sequences were theoretically designed. The patterns for the M460I and M460V (both assay 1) and H520Q (assay 2) mutants and the respective wild-type patterns are compared with experimentally created pyrograms in Fig. 2. Theoretically expected patterns and experimental pyrograms from assay 3 are shown in Fig. 3, demonstrating either the wild-type sequnce (Fig. 3A) or three different mutant sequences (C592G, C603W, and C607Y) (Fig. 3B to D). Those of the mutants A594V and L595S (assay 3) are shown in Fig. S1 in the supplemental material. Since assay 3 addresses several mutant positions and requires the dispensation of 55 consecutive nucleotides, the signal intensity was expected to decrease toward the end of the sequence. However, the experimental pyrogram patterns of assay 3 were clearly interpretable, including the final codon (592), with a potential mutation (Fig. 3). Theoretical pyrogram

patterns and experimental pyrograms for the wild-type sequence and mutant sequences A594G and L595F (assay 4) are shown in Fig. S2 in the supplemental material.

With assays 1 to 4, the important and frequent UL97 mutants were quantified. However, other, less frequent UL97 resistance mutants can also occur in patient samples. These mutants are also detectable in the experimental pyrogram patterns, but they cannot be quantified. An example is illustrated in Fig. S3 in the supplemental material. A plasmid containing a UL97 sequence with a deletion of codons 597 and 598 was analyzed and compared with the UL97 wild-type sequence. In the pyrogram of the deletion mutant, the mutant sequence could be unequivocally identified.

Detection of defined UL97 mutant–wild-type mixtures by pyrosequencing.
First, we tested the ability of PCR and pyrosequencing to detect 20, 50, and 80% UL97 mutant sequences in mixtures with the wild type. For this purpose, a plasmid containing a wild-type UL97 sequence (Pl_WT) was mixed with a plasmid containing a specific UL97 mutant sequence (either Pl_M460I, Pl_M460V, Pl_H520Q, Pl_A594G, Pl_A594V, Pl_L595S, or Pl_C607Y). Mixtures of plasmids (104 or 102 copies) were prepared at ratios of 80:20, 50:50, and 20:80 mutant and wild-type plasmids, respectively. In each of the mixtures, the presence of both mutant and wild-type sequences was detected with PSQ. With CTSQ, only the 50% and 80% (but not the 20%) fractions of mutant plasmids or the wild-type plasmid could be solidly detected (data not shown).

Next, mixtures of UL97 mutant and wild-type plasmids were prepared that contained only 0 to 10% mutant plasmid in increments of 1%. Each mixture was prepared 10 times and assayed. The PSQ data were analyzed by box-and-whisker plots. Examples are shown in Fig. 4, which illustrates the measurement of Pl_M460I/Pl_WT mixtures with assay 1, Pl_H520Q/Pl_WT mixtures with assay 2, and Pl_L595S/Pl_WT mixtures with assay 3. When 100% Pl_WT was assayed, falsepositive mutant signals were between 0% and 3%, with median values below 2% (Fig. 4). When mutant–wild-type mixtures were analyzed with assay 1, false-negative mutant signals were not observed. In assays 2 and 3, false negatives were measured when 1% (assay 2) or up to 4% (assay 3) mutant plasmids were present. In the presence of 5 to 10% mutant plasmid, mutant sequences were detected with linear increases in assays 1 to 3, and false negatives were not observed (Fig. 4). Mutant–wildtype mixtures using plasmid Pl_M460V, Pl_A594G, Pl_A594V, or Pl_C607Y gave similar results (not shown). For comparison, mixtures of 10% mutant plasmid with 90% wild-type plasmid were Sanger sequenced. None of the mutant plasmids in the mixtures could be detected (data not shown).

Two clonal HCMV strains, Titan wild type and Titan A594V, were then used for further evaluation of PSQ assay 3. Pure Titan wild type and mixtures of Titan A594V with 95% Titan wild type (either 5/95% or 10/90%) were assayed. The results were in line with the plasmid experiments (data not shown).

To further evaluate the performance of the PSQ assays, six samples from three HCMV-positive, therapy-naïve individuals (patients D and E) were analyzed. Most likely, these samples contained only wild-type sequences, since the patients had not been treated with GCV. Examination of 11 mutant positions with all PSQ assays revealed wild-type values of 95 to 100% and mutant signals of 0 to 5% (see Table S2 in the supplemental material). These mutant signals were taken as false positives, i.e., background noise.

Since the median values of all false-positive mutant signals, obtained either with wild-type plasmid (Fig. 4) or wild-type virus or with patients presumably infected with wild-type HCMV only (see Table S2 in the supplemental material), were between 0% and 3% and since individual false-positive measurements above 6% were never observed, we tentatively defined a mutant detection limit of 6% for all assays.

To further evaluate the assay reproducibility, the mutantpositive samples from GCV-treated patient A (see below) were analyzed in five independent PSQ runs. The resulting 90% confidence intervals amounted to a maximum of 88 to 112% of the mean and were slightly larger than those obtained with the plasmid mixtures (data not shown).

Detection of mutant–wild-type mixtures in patient samples by pyrosequencing.
Three patients who had received GCV treatment and had shown clinical manifestations and laboratory parameters indicative of treatment failure were analyzed with PSQ assays 1 to 4. The PCR for assay 2 was used for HCMV quantification by real-time PCR and then for PSQ. For comparison, a 680-bp UL97 product was amplified by nested PCR and sequenced using the conventional CTSQ method.

Patient A was a pediatric hematopoietic stem cell transplant recipient who had died after reactivation of a postnatal HCMV infection with bilateral retinitis and pneumonitis. Conventional sequencing of the HCMV UL97 region had revealed a compartment-specific H520Q mutation in urine that conferred GCV resistance (20). Limited amounts of DNA from some patient samples were still available and could be retrospectively analyzed with PSQ assays 2 and 3. An H520Q mutation was detected in urine by PSQ (37%) and by CTSQ (Table 2), thereby confirming the previously published results. In addition, H520Q was also detected in plasma as a minority of 10%. This could not be confirmed by conventional sequencing in the present study, and only a doubtful mutant peak had been seen in that sample in the previous study (20). Importantly, we additionally detected A594V (20%) and C603W (10%) mutations in the urine sample and C607Y mutants in plasma (13%) and urine (24%). These were not detected by CTSQ in either the previous or the present study. Also, C607Y was detected in leukocytes on days 90 and 171 after HSCT with PSQ only (14 and 13%, respectively) (Table 3).

Patient B was a preterm newborn with hydrops fetalis who was congenitally infected with HCMV. GCV therapy failed to control virus replication, and after 113 days, the infant died with anemia, ascites, and compromised lung function (S. E. Kampmann, B. Schindele, L. Apelt, C. Bührer, L. Garten, K. Weizsaecker, D. H. Krüger, B. Ehlers, and J. Hofmann, unpublished data). Seven samples were available for PSQ analysis. They had been collected on days 6 (throat swab), 8 (ascites fluid), 10 (cerebrospinal fluid [CSF]), 24 (urine), 30 and 99 (plasma), and 99 (ascites fluid) after birth. During the first 30 days, only small amounts of GCV resistance-associated mutants were detected in the CSF (M460V, 8%; A594G, 7%) on day 10 and urine (A594G, 7%) on day 24. On day 99, however, three or four GCV resistance-associated mutants (M460I, M460V, A594G, and C603W) were detected with PSQ in plasma and ascites fluid, which accounted for 44% and 60%, respectively, of the total HCMV population in these compartments
(Table 3). In addition, the D605E mutation, which is not associated with resistance but rather with resensitization to GCV (10a), was detected in all samples at almost 100%. Of these multiple mutations, only the C603W mutation (and the D605E mutation) was detected by conventional sequencing of 680-bp amplification products. To analyze whether individual viruses contained more than one resistance-associated mutation and to confirm the PSQ data,we cloned the 680-bp amplification products from plasma and ascites fluid (day 99) from patient B in *E. coli*. Clones were obtained from each product (24 from plasma and 22 from ascites fluid) and conventionally sequenced. All clones contained the D605E mutation. In addition, each of the GCV resistance-associated mutations, which had been identified by PSQ in these samples, was detected in individual clones, except the A594G mutation (Table 3). Furthermore, single clones with either an A594V or a C607Y mutation that had not been detected with PSQ were identified. This finding indicated the presence of HCMV with an A594V or a C607Y mutation at very low levels. No clone with more than one resistance-associated mutation was identified.

Finally, in patient C, an L595S mutation occurred with percentages of 26 to 50% and an A594V mutation with 8 to 14%, as measured by PSQ. While L595S was also observed by conventional sequencing, A594V was not (see Table S3 in the supplemental material).

Discussion

To our knowledge, this is the first report of a pyrosequencing method for the detection of GCV-resistant UL97 HCMV in clinical specimens. In comparison to conventional CTSQ, PSQ was shown to be considerably more sensitive in detecting minor UL97 mutant virus populations. In addition, 96 PCR products could be analyzed in 10 min (assays 1, 2, and 4) or 1 h (assay 3). Therefore, the analysis of suspected genotypically resistant HCMV by PSQ is also significantly faster than conventional sequencing. Although the limited readability of the PSQ sequence (to approximately 60 bp) is a drawback in the analysis of UL97 mutations because the most important mutations are located within a contiguous area spanning approximately 450 bp of the UL97 open reading frame, we have shown that clinically relevant mutations in that region can be detected with a limited number of PSQ assays.

To distinguish true mutations from background noise, we analyzed mutant–wild-type mixtures of plasmids and viruses. From these experiments, we defined a cutoff of 6% for the detection of mutant minorities. Compared to a cutoff of approximately 20% inherent in conventional CTSQ (2), PSQ allows much more sensitive and therefore earlier detection of emerging mutants in a GCV-treated patient. Rapid and sensitive detection of UL97 mutations in codons 460 and 520 using real-time PCR has been reported (9), and a real-time PCR method to detect mutations in codon 460 using molecular beacons has been published (21). Both studies claim a detection limit of 5% to confirm resistant virus. However, the assays do not offer the possibility of analyzing all clinically important UL97 mutations and therefore are of limited applicability. Ultradeep sequencing (UDPS) (19) has been reported to detect minor variants of hepatitis B virus with a sensitivity of 1 to 2% (13) and distinct genotypes of HCMV at an abundance of 0.1% to 1% (10). In addition, minor mutants of human immunodeficiency virus have been reliably detected by UDPS at a level of about 1% (reviewed in reference 7). However, the preprocessing of sample DNA, the run on the sequencer, and the processing of data require several days and expensive hardware. Therefore, UDPS is not suitable for cost-effective and fast routine screening of patient samples.

Importantly, during the clinical courses of two pediatric HCMV-infected patients with fatal outcomes and one HCMVinfected adult patient, we found multiple resistance-associated mutants (Tables 2 and 3; see Table S3 in the supplemental material). This emergence of multiple mutants was below the detection limit of conventional sequencing and was detected by PSQ only. PCR plus CTSQ sequence analysis, presently the method of choice for genotypic identification of GCV resistance, is able to detect multiple GCV-resistant HCMV mutants in a patient. However, from our findings with PSQ, it is tempting to speculate that this phenomenon might occur more often than is known today. In some of the samples analyzed here, the HCMV mutants made up more than 50% of the total HCMV population. Since identification of a GCV-resistant mutant in a patient with clinical GCV resistance is a prerequisite for a change of treatment, the high cutoff of Sanger sequencing for the detection of single mutants is not satisfactory. This is most clearly demonstrated by the results from the ascites fluid sample analysis (day 99) of patient B. Conventional sequencing revealed only one resistant mutant, which was close to the detection limit. PSQ revealed four resistant mutants with an abundance of 60% of the total virus population (Table 3). Therefore, we propose PCR plus PSQ as the method of choice for early detection of HCMV UL97 mutants.

In summary, we developed a novel genotypic assay on the basis of PSQ for the identification of GCV resistance-associated HCMV mutants in samples from patients infected with multiple HCMV strains. The method allows the detection of minor mutant fractions with high sensitivity and speed. It should therefore meet the requirements of clinical routine diagnostics.

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Tables and Figures

Table 1. PSQ target sequences and dispensation orders

PSQ assay	UL97 target codon(s)	Target sequence[a]	Dispensation order[b]
1	M460V/I	5′-A CCC A/gTG/c/t AAC G	(T)CGTCGATC(A)GT
2	H520Q	5′-CA C/g	(A)GC(A)TG
3[c]	C592G, A594V, L595S, C603W, C607Y	5′-T/gGC CGC GC/tG TT/cG GAG AAC G/aGC/t AAG CTC ACG CAC TGC/g TCC GAC/g GCC TG/aT C	(C)GACT(C)AGCG(A)TCGAGC(T)AGTGCGTGAGCTGAC T(C)GTCTCAGAC(T)GA(T)CGCGCA(T)
4	A594G, L595F	5′-C/gG TTG/t GA	TCG(C)TG(C)A

[a] Mutant bases are in lowercase letters; spaces in the sequence separate the codons.
[b] The DO must be reversed and complemented to allow comparison with the target sequence (except assay 4); control bases (automatically implemented by the software) are in parentheses.
[c] In assay 3, frequent mutations in codons 598 (GGt, synonymous; aGt and aGC, G598S) and 605 (Gag, D605E) are not resistance associated but were incorporated into the target sequence and the dispensation order to allow correct downstream operation of the sequencing. The codons are underlined.

Table 2. Detection of GCV-resistant HCMV mutants by pyrosequencing in patient A

No. of days after HSCT[a]	Specimen	HCMV-DNA (no. of copies/ml)	% Presence of UL97 mutant[b]						
			H520Q	C592G	A594V	L595S	C603W	D605E[c]	C607Y
38	Plasma	77,200	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**13** (−)
90	Leukocytes	14,700	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**14** (−)
109	Urine	7,510	**37** (+)	<6 (−)	**20** (−)	<6 (−)	**10** (−)	<6 (−)	**24** (−)
114	CSF	127,000	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)
150	Plasma	243,000	**10** (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)
171	Leukocytes	16,200	**15** (+)	<6 (−)	**6** (−)	<6 (−)	<6 (−)	<6 (−)	**13** (−)

[a] HSCT, hematopoietic stem cell transplantation.
[b] The mutant was detected (+) or not detected (-) by chain termination sequencing. Mutant percentages of <6% indicate that PSQ detected only the wild type; mutant signals were below the limit of detection (6%). Mutant percentages above the limit of detection are in boldface.
[c] The mutant D605E is not associated with resistance.

Table 3. Detection of GCV-resistant HCMV mutants by pyrosequencing in patient B

Day of life	Specimen	CMV DNA (no. of copies/ml)	% Presence of UL97 mutant[a]										
			M460I	M460V	H520Q	C592G	A594G	A594V	L595F	L595S	C603W	D605E[b]	C607Y
6	Throat swab	27.000	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**98** (+)	<6 (−)
8	Ascites	3.460	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**97** (+)	<6 (−)
10	CSF	54.600	<6 (−)	**8** (−)	<6 (−)	<6 (−)	**7** (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**94** (+)	<6 (−)
24	Urine	500.000	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**7** (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**97** (+)	<6 (−)
30	Plasma	306.000	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**97** (+)	<6 (−)
99	Plasma	3500.000	**11** (−)	**16** (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	<6 (−)	**16** (+)	**97** (+)	<6 (−)
E. coli clones[c]			**9 mut**/15 wt	**1 mut**/23 wt	0 mut/24 wt	0 mut/24 wt	0 mut/24 wt	**1 mut**/23 wt	0 mut/24 wt	0 mut/24 wt	**3 mut**/21 wt	**24 mut**/0 wt	**1 mut**/23 wt
99	Ascites	320.000	**14** (−)	**15** (−)	<6 (−)	<6 (−)	**13** (−)	<6 (−)	<6 (−)	<6 (−)	**17** (+)	**98** (+)	<6 (−)
E. coli clones[c]			**8 mut**/14 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	0 mut/22 wt	**22 mut**/0 wt	0 mut/22 wt

[a] The mutant was detected (+) or not detected (-) by chain termination sequencing. Mutant percentages of <6% indicate that PSQ detected only the wild type; mutant signals were below the limit of detection (6%). Mutant percentages (numbers for *E. coli* clones) above the limit of detection are in boldface.
[b] The mutant D605E is not associated with resistance.
[c] Recombinant plasmids from 24 (plasma) and 22 (ascites) *E. coli* clones were conventionally sequenced; *n* plasmids contained mutant (mut) and/or wildtype (wt) bases at the respective positions.

Figure 1. Amplification strategy. The thick black horizontal line represents UL97. Above, the resistance-associated codons are indicated. Below, the PCR targets of PSQ assays 1 to 4 are depicted. Open triangles represent the PCR primers, closed triangles the PSQ primers. The amplified sequences are shown as thin black lines. The PSQ PCR of assay 2 is depicted twice, with a biotinylated antisense primer for PSQ and with unmodified primers for real-time PCR. The MGB probe is indicated. At the bottom, nested PCR, which provides the basis for UL97 sequence analysis with CTSQ, is depicted.

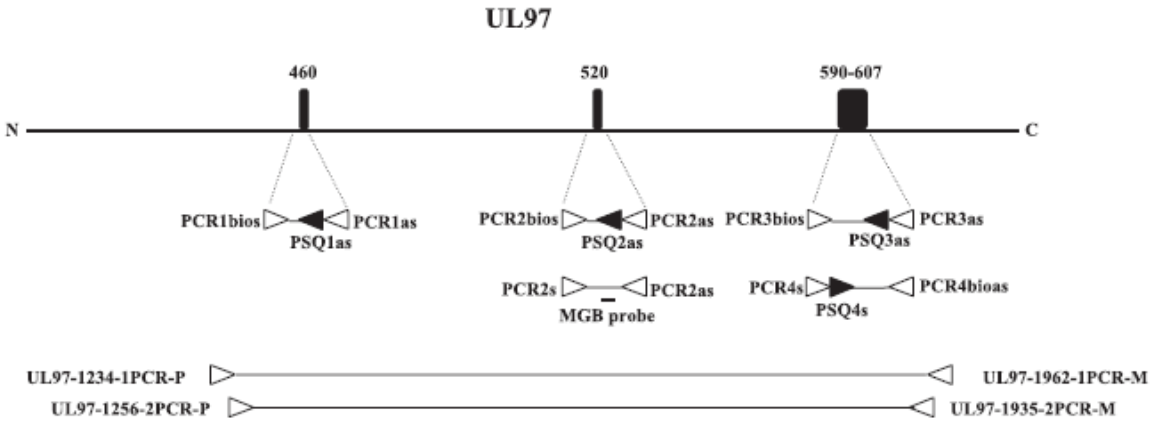

Figure 2. Theoretical pyrogram patterns and experimental pyrograms for analysis of UL97 codons 460 (assay 1) and 520 (assay 2). At the top of each panel, the amino acid sequence of the UL97 wild type, the number of the analyzed codon, and the amino acid sequences of the UL97 mutants are indicated. The corresponding nucleic acid sequence (in parentheses) is reversed. Below, theoretical pyrogram patterns and examples of PSQ reads are depicted. At the bottom of each panel, the dispensation order (Disp) and the determined sequence (Seq) are shown. The parts of the pyrograms that reveal changes upon the presence of a mutant are marked with brackets. Since the PSQ primer is a reverse primer, the determined sequence has to be reversed and complemented to be in line with the UL97 gene sequence. (A to C) Assay 1; (D and E) assay 2.

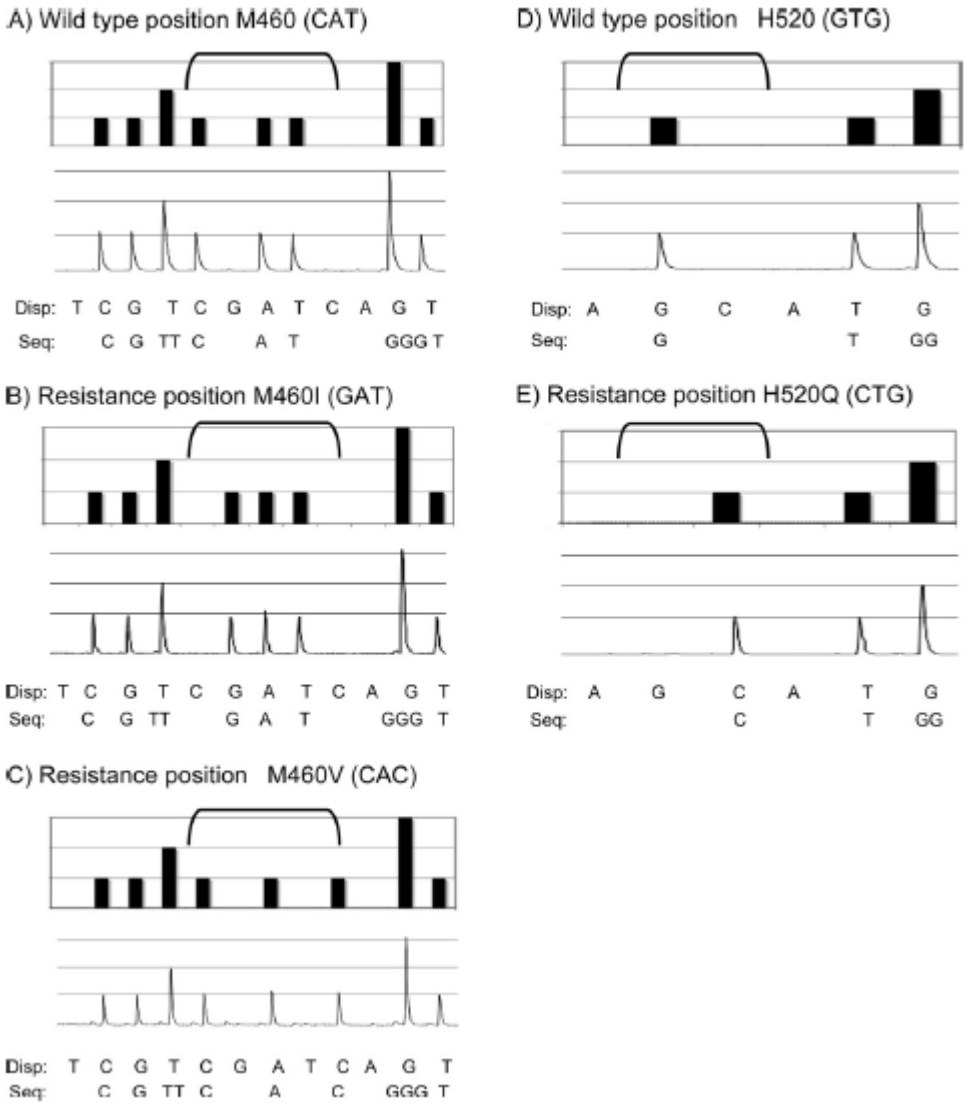

Figure 3. Theoretical pyrogram patterns and experimental pyrograms for analysis of the UL97 codons 592 to 607 (assay 3). At the top of each panel, the amino acid sequence of the UL97 wild type, the number of the analyzed codon, and the amino acid sequences of the UL97 mutants are indicated. (A) Theoretical pyrogram pattern of a UL97 wild-type sequence (codons 607 to 592) and an example of a PSQ read (below). (B to D) Theoretical pyrogram patterns of UL97 C592G (B), C603W (C), and C607Y (D) mutants and examples of PSQ reads. The parts of the pyrograms that reveal changes upon the presence of a mutant are marked with brackets, and the respective codon numbers are indicated above. At the bottom of each panel, the dispensation order (Disp) and the determined sequence (Seq) are shown. All determined sequences had to be reversed and complemented (see the legend to Fig. 2). In the last three positions of panel B, the experimentally determined sequence does not correspond to the theoretical PSQ pattern. This was caused by a silent mutation in codon 591 from GGC to TGC (underlined).

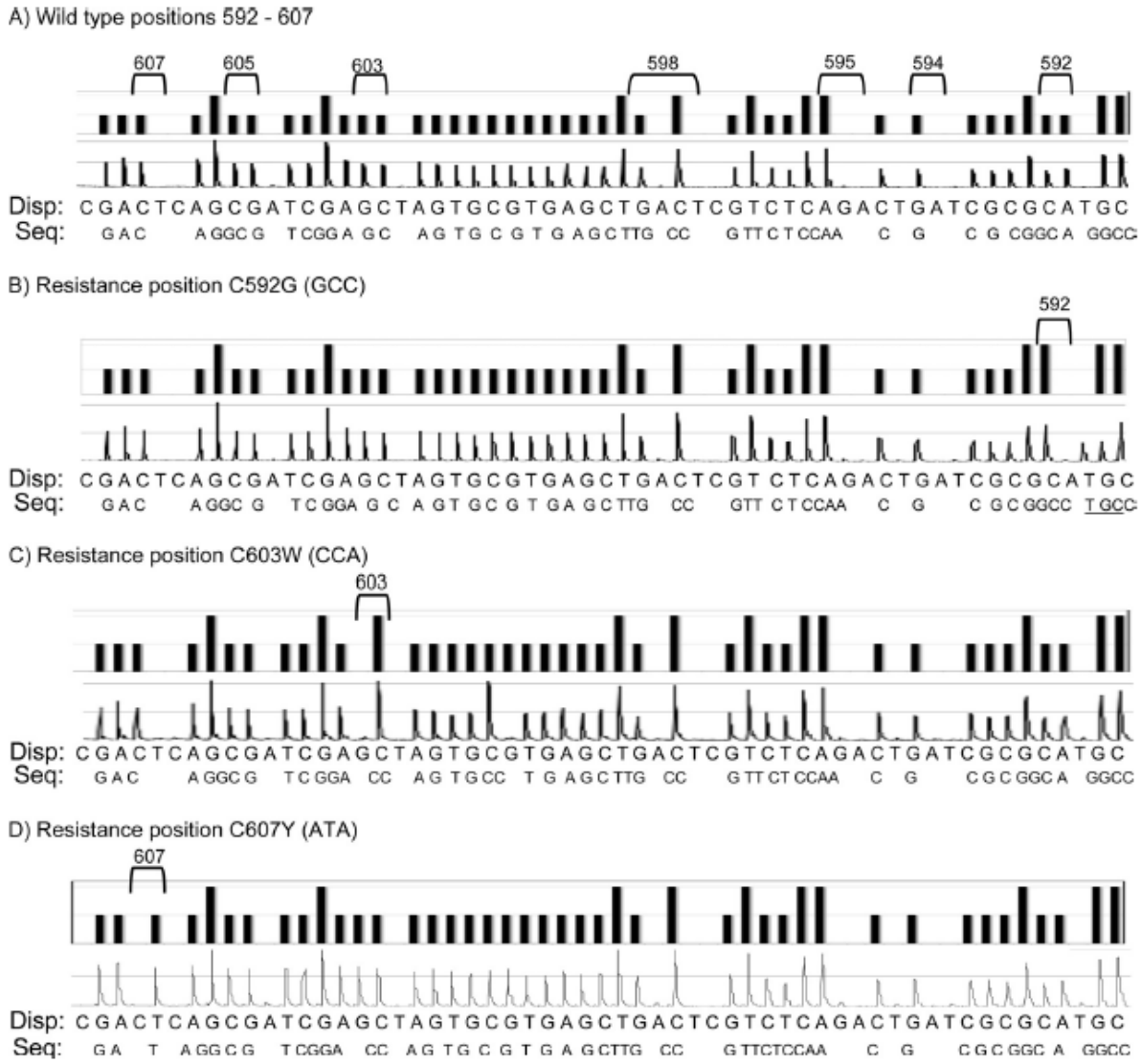

Figure 4. Detection of HCMV mutant minorities in the presence of the wild type. Plasmids (104 copies) containing either mutant or UL97 were mixed at ratios of 1:99 to 10:90 and analyzed with PSQ. (A) mutant plasmid M460I. (B) Mutant plasmid H520Q. (C) Mutant plasmid L595S. All mixtures were analyzed 10 times by PSQ, and the quantitative results were evaluated by box-whisker plotting. The areas containing 50% of the measured values are boxed. The medians are represented by black bars. Maxima and minima of the measured values are indicated by vertical lines above and below the boxes.