HERQ-9 Is a New Multiplex PCR for Differentiation and Quantification of All Nine Human Herpesviruses

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Abstract: Infections with the nine human herpesviruses (HHVs) are globally prevalent and characterized by lifelong persistence. Reactivations can potentially manifest as life-threatening conditions for which the demonstration of viral DNA is essential. In the present study, we developed HERQ-9, a pan-HHV quantitative PCR designed in triplex reactions to differentiate and quantify each of the HHV-DNAs: (i) herpes simplex viruses 1 and 2 and varicella-zoster virus; (ii) Epstein-Barr virus, human cytomegalovirus, and Kaposi’s sarcoma-associated herpesvirus; and (iii) HHV-6A, -6B, and -7. The method was validated with prequantified reference standards as well as with mucocutaneous swabs and cerebrospinal fluid, plasma, and tonsillar tissue samples. Our findings highlight the value of multiplexing in the diagnosis of many unsuspected, yet clinically relevant, herpesviruses. In addition, we report here frequent HHV-DNA co-occurrences in clinical samples, including some previously unknown. HERQ-9 exhibited high specificity and sensitivity (LOD95s of 10 to 17 copies/reaction), with a dynamic range of 10^1 to 10^6 copies/l. Moreover, it performed accurately in the coamplification of both high- and low-abundance targets in the same reaction. In conclusion, we demonstrated that HERQ-9 is suitable for the diagnosis of a plethora of herpesvirus-related diseases. Besides its significance to clinical management, the method is valuable for the assessment of hitherto-unexplored synergistic effects of herpesvirus coinfections. Furthermore, its high sensitivity enables studies on the human virome, often dealing with minute quantities of persisting HHVs.

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HERQ-9 a New Multiplex PCR for Differentiation and Quantification of All Nine Human Herpesviruses

Lari Pyöriä¹, Maija Jokinen¹², Mari Toppinen¹, Henri Salminen¹, Tytti Vuorinen³⁴, Veijo Hukkanen³, Constanze Schmotz¹, Endrit Elbasani⁵, Päivi M. Ojala⁵⁶, Klaus Hedman¹⁷, Hannamari Välimaa¹⁸*, Maria F. Perdomo¹*

*These authors contributed equally to this work

1. Department of Virology, University of Helsinki, Helsinki, Finland.
2. Department of Evolutionary Biology and Environmental Studies, University of Zürich, Switzerland
3. Institute of Biomedicine, University of Turku, Turku, Finland
4. Clinical Microbiology, Turku University Hospital, Turku, Finland
5. Translational Cancer Medicine Research Program, Faculty of Medicine, University of Helsinki, Helsinki, Finland
6. Department of Infectious Diseases, Imperial College London, London, UK
7. Helsinki University Hospital, HUSLAB, Helsinki, Finland.
8. Department of Oral and Maxillofacial Surgery, Helsinki University Hospital, Helsinki, Finland

Corresponding authors

Maria F. Perdomo maria.perdomo@helsinki.fi
Hannamari Välimaa hannamari.valimaa@helsinki.fi
Abstract

Infections with the nine human herpesviruses (HHVs) are globally prevalent and characterized by life-long persistence. Reactivations can potentially manifest as life-threatening conditions for which the demonstration of viral DNA is essential.

In the present study, we developed *HERQ-9*, a pan-HHV qPCR designed in triplex reactions to differentiate and quantify each of the HHV-DNAs: 1) HSV-1&2, VZV; 2) EBV, HCMV, KSHV; and 3) HHV-6A/B-7. The method was validated with pre-quantified reference standards as well as with mucocutaneous swabs, cerebrospinal fluid, plasma and tonsilar tissue samples.

Our findings highlight the value of multiplexing in the diagnosis of many unsuspected, yet clinically relevant, herpesviruses. In addition, we report here frequent HHV-DNA co-occurrences in clinical samples, including some previously unknown.

*HERQ-9* exhibited high specificity and sensitivity (LOD\textsubscript{95} of ~10 - ~17 copies/reaction) with a dynamic range of $10^1$ to $10^6$ copies/μl. Moreover, it performed accurately in the co-amplification of both high and low abundance targets in the same reaction.

We demonstrated that *HERQ-9* is suitable for the diagnosis of a plethora of herpesvirus-related diseases. Besides its significance to clinical management, the method is valuable for the assessment of hitherto unexplored synergistic effects of herpesvirus co-infections. Furthermore, its high sensitivity enables studies on the human virome, often dealing with minute quantities of persisting HHVs.
The importance

By adulthood, almost all humans become infected by at least one herpesvirus (HHV). The maladies inflicted by these microbes extend beyond the initial infection as they remain inside our cells for life and can reactivate, causing severe diseases. The diagnosis of active infection by these ubiquitous pathogens includes the detection of DNA with sensitive and specific assays. We developed the first quantitative polymerase chain reaction assay (HERQ-9) designed to identify and quantify each of the nine human herpesviruses. The simultaneous detection of HHVs in the same sample is important since they may act together to induce life-threatening conditions. Moreover, the high sensitivity of our method is of extreme value for assessment of the effects of these viruses persisting in our body and their long-term consequences on our health.
Introduction

The nine human herpesviruses (HHVs) are ubiquitous pathogens that persist life-long after primary infection. HHVs cause many disorders ranging from mild mucocutaneous diseases to severe central nervous conditions, birth defects and cancer. Their ability to reactivate poses significant risks, particularly to immunosuppressed patients, such as transplant recipients, in whom Epstein-Barr virus (EBV), human cytomegalovirus (HCMV), HHV-6B and Kaposi’s sarcoma-associated herpesvirus (KSHV) can induce life-threatening conditions (1–8).

The investigation of active HHV infections includes, among other markers, the detection of viral nucleic acids, typically by quantitative polymerase chain reaction (qPCR). In addition, the simultaneous detection of these pathogens has been shown to be beneficial as their recognition may be difficult based on the clinical presentation alone (9–15).

While several multiplex qPCRs have been introduced for detection of HHVs (16–19), none are designed to quantify them all. In addition, only few of the existing protocols distinguish between the closely related HHV-6A and HHV-6B, a distinction that may be crucial as the former still lacks clear association to disease (20–23).

In the present study, we developed a pan-herpes multiplex assay, HERQ-9, that quantifies and discriminates each of the HHVs using three separate triplex-qPCR reactions: the first amplifies herpes simplex virus types 1&2 (HSV-1&2) and varicella-zoster virus (VZV), the second EBV, HCMV and KSHV, and the third HHV-6A, 6B and 7. We validated our assay using pre-quantified reference materials and evaluated its performance with various clinical samples as well as solid tissue material.

HERQ-9 simplifies diagnosis and improves the clinical management and risk assessment of highly susceptible populations (1, 5, 8, 24–26). Moreover, its high sensitivity is of significant value for studies on the impact of HHV persistence on health (27, 28) and disease (29, 30).
Results

In silico evaluation of amplicons, primers and probes

The designed primers and probes were queried against all available sequences (full or partial genomes) in the NCBI database. The oligonucleotides showed perfect match for the different strains except for four sequences of HHV-6A (KY316054.1, KT3555575.1, KY316056.1, KY316047.1) and two of HCMV (KY490070.1, KP745685.1) for which one to two mismatches were observed far from the 3’-end.

We found no unspecific binding to other viruses or human DNA except for the primers and probe of HSV-2 that had also complete homology to chimpanzee alpha-1 herpesvirus (JQ360576.1).

In silico analysis of amplicons, primers and probes revealed no relevant secondary structures, primer-dimers or cross-dimers (see Fig. S1 and S2 in the supplemental material).

Analytical sensitivities and specificities

We evaluated the sensitivities using eight replicates of the respective plasmids in seven dilutions ranging from 50 to 1 copies per reaction. Based on probit link function, the limits of detection (LOD\textsubscript{95}) of HERQ-9 for HSV-1, HSV-2, VZV, EBV, HCMV, KSHV, HHV-6A, HHV-6B and HHV-7 were respectively 12, 13, 13, 10, 16, 17, 11, 11 and 11 copies per reaction. The values were similar in singleplex format (see Fig. S3 in the supplemental material).

The multiplex assay detected all HHVs correctly from infected cell lines without cross-amplification of other HHVs, human DNA or near-full/full-length genomes of B19V, polyomaviruses BKV, JCV and MCV. All the no-template water controls remained negative throughout the PCR analyses.

Repeatability and reproducibility
We tested the intra- and interassay variations in three separate qPCR runs using five replicates of the individual HHV plasmids (10⁶ – 10¹ copies /µl) and plasmid mixes pMIXI (HSV-1,2; VZV), pMIXII (EBV, HCMV, KSHV) and pMIXIII (HHV-6A,B; 7). The assay showed excellent short-term repeatability and long-term reproducibility in both singleplex and multiplex formats as well as with the pMIXI-III. The highest standard deviations in C_q values (intraassay) and coefficients of variation between runs (interassay) were seen at the lowest template copies (Tables 1 & 2).

The method was linear in the range of 10¹ to 10⁶ copies per µl and the qPCR efficiencies were between 95.9% and 103.8% in all the experiments.

The HHV plasmid dilutions spiked with 500ng of human DNA (HaCaT cells) showed equal linearity to pure HHV plasmids (Fig. 1, see also Fig. S4 in the supplemental material).

Comparison to pre-quantified reference samples.

We correlated the quantification of HERQ-9 to several pre-quantified reference materials. The results are summarized in Table 3. Each of the two strains of HSV-1 and HSV-2 DNA had spectrophotometrically estimated quantities of 4.3E9 and 6.1E9 copies/µl and 1.9E9 and 3.8E9 copies/µl, respectively. The calculated loads by our multiplex qPCR were 4.2E9 and 6.1E9 copies/µl for HSV-1, and 9.1E8 and 1.9E9 copies/µl for HSV-2. The conversion factors were hence 0.99 (HSV-1) and 0.49 (HSV-2).

A VZV-DNA extract (ATCC) containing 5.6E5 copies/µl was calculated to have 1.8E6 copies/µl by HERQ-9 with a conversion factor of 3.2.

EBV, HCMV and HHV-6B WHO international standards contained 1.0E4, 1.0E4 and 1.1E5 IU/µl, respectively. The calculated copies by HERQ-9 were 1.0E4 copies/µl for EBV, 3.2E4 copies/µl for HCMV and 1.8E5 copies/µl for HHV-6B. Hence, the conversion factors (copies/IU) were 1.0, 3.2 and 1.6, respectively.
Spectrophotometrically estimated copies of KSHV DNA were 2.2E9 copies/µl and calculated to be 5.0E9 copies/µl with our assay, yielding a conversion factor of 2.3.

HERQ-9 showed good correlation with HHV-6A, HHV-6B and HHV-7 spiked in sera (Fig. 2). The conversion factors were 1.1, 1.3 and 6.8, respectively.

Analysis of clinical samples
We tested several types of clinical samples and compared the positive and negative agreements against reference methods. A summary of the results is presented in Table 4.

Plasma
We tested 60 plasma samples, previously studied for EBV and/or HCMV at Turku University Hospital. Altogether 13/13 and 16/16 plasma samples positive for EBV and HCMV by a reference qPCR, respectively, were positive by HERQ-9 with good correlation in viral loads (Fig. 3). Seven out of nine samples reported as borderline for EBV by the clinical laboratory (50-200 copies/ml of plasma) were positive in the new assay, as were four of five HCMV-borderline samples (50-200 copies/ml of plasma). Furthermore, the new qPCR found additional samples positive for EBV (n=3) and HCMV (n=5) that in the hospital laboratory had been tested only for either of the virus. These samples were re-analyzed with a reference qPCR confirming one EBV and three HCMV positivities.

Furthermore, among the 60 plasma samples studied with HERQ-9, six were positive for HHV-6B (median 2.7E2 copies/ml of plasma, range 1.7E2-5.2E3); one for HHV-6A (6.3E2 copies/ml of plasma); and five for HSV-1 (median 1.2E4 copies/ml of plasma, range 8.0E2 - 5.1E4). The co-occurrence of HHVs in plasma was seen in 14 patients of whom three had EBV/HCMVHSV-1; one EBV/HCMV/HHV-6B; one EBV/HHV-6BHSV-1; five EBV/HCMV; two EBV/HHV-6B; one HCMV/HHV-6B; and one EBVHSV-1 (Fig. 4A).

Mucocutaneous swabs
We tested 114 mucocutaneous swab samples previously investigated for HSV-1&2 or VZV at Turku University Hospital.
HERQ-9 identified correctly all the mucocutaneous swab samples that had tested positive by rapid viral culture for HSV-1 (n=35, median 5.6E7, range 1.4E5 to 8.2E9 copies/ml of collection media) and HSV-2 (n=30, median 3.3E7, range 2.9E5 to 3.5E8 copies/ml of collection media). In contrast, the 15 culture-negative controls showed no amplification for HSV-1 or HSV-2. However, two of these negative samples were positive instead for VZV, at 2.0E7 and 3.2E4 copies/ml of collection media and were confirmed to be VZV-DNA positive with a control PCR (4, 31). In addition, 5/5 HSV-1 and 4/4 HSV-2 positive DNA extracts previously tested by a reference PCR (4, 31) were also positive with HERQ-9.

All the VZV samples positive (n=15) by enzyme immunoassay (EIA) were positive with the new assay, at median quantities of 4.5E7 copies/ml of collection media (range 8.0E6 to 2.7E9). On the other hand, among 10 VZV antigen-negative samples, two contained VZV DNA at 3.6E6 and 4.7E3 copies/ml of collection media. Of these, the former was confirmed to be VZV DNA positive by the reference PCR. Incidentally, among the remaining eight samples negative for the VZV antigen, three showed positivity for HSV-1 instead, at loads of 7.7E7, 7.2E7 and 6.3E1 copies/ml of collection media. Only the two samples with the highest copy numbers were confirmed to be positive for HSV-1 DNA by the control PCR.

Moreover, we co-detected other HHVs in these mucocutaneous swabs (Fig. 4C and 4D). Of the HSV-1 positive samples, 20.9% were also positive for EBV-DNA, 4.7% for HCMV-DNA, 9.3% for HHV-7 DNA and 2.3% for HHV-6B DNA. Among the HSV-2 positive swabs, 5.3% were also positive for EBV, 8.8% for HCMV, and 2.9% for HHV-7. From 19 VZV positive swabs, one was positive for EBV-DNA (5.3%). Of all the 114 swabs, two were quadruple positive (HSV-1/EBV/HCMV/HHV-7 and HSV-2/EBV/HCMV/HHV-7) and four were triple positive (HSV-1/EBV/HCMV, HSV-1/HHV-6B/HHV-7 and two HSV-2/EBV/HCMV). The copy numbers of the other co-detected HHVs (generally log2-log3 copies/ml of collection media) were always lower than those for HSV-1, HSV-2 or VZV. However, a few samples had log5-log6 copies/ml of EBV DNA. Of all the mucocutaneous swab samples negative for HSV-1, HSV-2 and VZV (n=18), one (5.6%) tested positive for EBV DNA.

*Cerebrospinal fluid (CSF)*
Eight CSF samples were analyzed with the pan-herpes multiplex assay. Two were positive for VZV (3.23E3 and 1.64E5 copies/ml of CSF) in concordance with the hospital laboratory reference PCR (4, 31).

HHV prevalences in tonsillar tissue.

The HHV DNA prevalences in 35 tonsillar tissue samples were 80% for HHV-7, 71% for EBV, 66% for HHV-6B, 3% for HSV-1 and 3% for HCMV. No HSV-2, VZV or KSHV were found (Fig. 5). From these samples, two were positive for four HHVs, 17 for three, 10 for two, while four tonsils were negative for all the HHVs (Fig. 4B). The median viral loads (copies/million cells) were highest for EBV (2.1E2, IQR 9.6E2), followed by HHV-7 (3.6E1, IQR 1.3E2) and HHV-6B (1.1E1, IQR 2.2E1).

Co-quantification of mixed high- and low-abundance targets

We tested uneven copies of whole HHV genomes in the same reaction (range 4.5E0 to 1.1E6 copies/µl) and found that all the viruses were correctly differentiated and accurately quantified by HERQ-9 (Pearson correlation coefficient r = 0.996, p<0.01). Higher coefficients of variation were seen at lower viral copies (Table 5).

Discussion

Our newly developed pan-herpes multiplex-qPCR assay, HERQ-9, outstands for its ability to differentiate and quantify the genomes of all nine human herpesviruses.

HERQ-9 was designed on three distinct triplex-qPCR reactions to meet, on the one hand, the clinical needs and, on the other, the technical constraints inherent to PCR multiplexing. Indeed, the capacity to co-detect several targets is restricted by the spectral overlap of different fluorophores as well as the number of channels in the qPCR instrument (maximum of six) (32). Moreover, a greater number of targets can increase cross-reactions between primers and probes, hampering assay performance.
The new multiplex assay performed remarkably well on cell, plasma, CSF and mucocutaneous swabs as well as on palatine tonsils. HHV genoprevalences in this lymphoid organ have been reported to be highest for EBV (20.4 – 88.8%), followed by HHV-7 (71.4%), HHV-6B (50.7%), HCMV (0-5.4%) and HSV-1 (1.8-6.3%) (33–35), in line with our results. In addition, we frequently co-detected several HHVs in the same tonsil, a phenomenon only previously reported by Berger et al. in young children (36).

HERQ-9 had good agreement with reference materials. The observed dissimilarities were likely to be related to the types and sensitivities of different methodologies (e.g., viral culture, EIA, spectrophotometry), sample processing (e.g., DNA extraction methods), as well as the design of the PCR methods compared. Regarding the latter, the primer and probe design, amplicon size, target gene and its polymorphisms, reagents and standards can all account for disagreements between qPCRs (37–39). In fact, these discrepancies have urged the introduction of WHO international standards for EBV, HCMV and HHV-6B to increase the commutability between assays (39–42). However, this has had only relative value since, even after standardization, the inter-laboratory variabilities continue to be high (up to 1.5 log_{10} IU/ml on average) (39, 42).

Our findings emphasize the importance of multiplexing for comprehensive diagnosis and clinical management. Indeed, we identified additional HHVs in clinical samples that had been tested only for a single pathogen, encountering several unforeseen HSV-1 or VZV findings in mucocutaneous swabs, as well as EBV-HCMV co-reactivations in plasma of immunodeficient patients. In addition, we co-detected other HHVs in plasma in several combinations (EBV/HSV-1, EBV/HHV-6B, HCMV/HHV-6B, EBV/HCMV/HSV-1, EBV/HCMV/HHV-6B and EBV/HHV-6B/HSV-1). These coincidental discoveries, also noted by others (4, 19, 43), may have a significant impact on risk assessment and prognosis. Indeed, HHVs are thought to individually or synergistically contribute to viral syndromes (5, 8, 44), organ rejection (19) or the development of cancer (45, 46).

Moreover, we found other HHVs besides HSV-1, HSV-2 or VZV in mucocutaneous lesions (up to four in the same sample). The most common were EBV, HCMV and HHV-7, whose
low viral loads were likely to represent skin virome (47) or latency in mobilized leukocytes (48). Yet, in a few samples, EBV-DNA levels approached those of HSV-1 or HSV-2, suggestive of in-situ co-reactivation or -infection. Our detection of both EBV and HSV-1 in mucocutaneous lesions and plasma supports an interplay between these two viruses, as has been shown in vitro by Wu et al. (49). To the best of our knowledge, we are the first to report on HHV co-occurrences in classical herpetic lesions.

In conclusion, we demonstrated that HERQ-9 is suitable for the diagnosis of a plethora of herpesvirus-related diseases. Besides its significance for clinical management, the high sensitivity and specificity of this method will be of particular value for studies of the human virome generally dealing with minute quantities of persisting HHVs.

Materials and Methods

Plasmids

Sequences of the reference strain (90-336 bp), including the corresponding qPCR amplicon, were inserted into a pIDTSmart backbone (Integrated DNA technologies). The plasmids (pHSV-1, pHSV-2 and pVZV; pHHV-6A, pHHV-6B, pHHV-7, pEBV, pHCMV and pKSHV) were transformed into E.coli, extracted (see DNA extraction) and confirmed with restriction analysis to contain the correct insert (for whole insert sequences, see Text S1 in the supplemental material). The concentrations were measured spectrophotometrically, and the plasmids (diluted serially from $10^6$ to $10^1$ copies/µl in 10mM TE-buffer) were stored at -80°C. In addition to single-plasmid analysis, triple-plasmid combinations were made: pHSV-1, pHSV-2, pVZV (pMIXI); pEBV, pHCMV, pKSHV (pMIXII); pHHV-6A, pHHV-6B, pHHV-7 (pMIXIII).
Plasmids containing full/near-full length genome of parvovirus B19 genotype 1 (50) and polyomaviruses BKV (NC_001538), JCV (NC_001699) (generous gifts from Dr. Eeva Auvinen) and MCV (inserted in vector backbone pJ241, a gift from Patrick Moore (51); Addgene plasmid # 32059) were used to test unspecific amplification.

Infected cell cultures

Primers and probes were initially tested using viral DNA extracted from virus-infected cell cultures. HSV-1 (strain F), HSV-2 (strain G) and VZV (strain Ellen) were propagated in HaCat, human foreskin fibroblast and Vero cells; EBV in Raji cells; HCMV (strain AD169) in human lung fibroblast MRC-5, HHV6A (strain GS) in HSB-2 cells, HHV6B (strain Z29) in MOLT-3 cells, HHV7 (strain JI) in SupT-1 cells and KSHV (strain rKSHV.219) in latent and lytic iSLK.219 cells. (52)

Uninfected HaCaT cells were used for human DNA spiking experiments.

Pre-quantified reference material

All the reference materials are presented in Tables 3 & 4.

Cell-free viral nucleocapsids

HSV-1 and HSV-2 nucleocapsids were isolated from pseudonymized dermal or mucosal lesion samples at the virus diagnostic unit of Turku University Hospital. The viruses were initially typed by a rapid viral culture immunoperoxidase assay (53) and confirmed by HSV type-specific gD (US6) gene-based PCR (54). For viral nucleocapsid DNA preparations, low-passage stocks were generated in Vero cells (African green monkey kidney; ATCC), and the viral genomic DNA was prepared as described (55, 56) (see Text S2 in the supplemental material for more detailed description). Two strains of HSV-1 (HSV-H1211 and HSV-H1215) and HSV-2 (HSV2-H12211 and HSV2-H1526) were prepared and the viral copies determined spectrophotometrically to be used as reference standards in dilutions 1:10 000 and 1:100 000.
WHO international standards

WHO international standards for EBV, HCMV and HHV-6B (NIBSC codes 09/260, 09/162 and 15/266, respectively) (57–59) were tested undiluted and in dilutions 1:10 and 1:100 with HERQ-9, to obtain conversion factors (viral DNA copies/International units).

KSHV genome in bacterial artificial chromosome (BAC)

KSHV-BAC16 DNA (a generous gift by prof. Carolina Arias, UCSB, CA, USA), derived from the KSHV strain of JSC-1 primary effusion lymphoma (PEL) cell line, was purified from E. coli (60). The viral copy numbers were estimated spectrophotometrically. This reference was analyzed in dilutions 1:10 000, 1:100 000 and 1:1000 000 with the multiplex assay.

Spiked sera

Serum samples spiked with HHV-6A (n=15) or HHV-6B (n=15) (4 x 10⁶ to 4 x 10² copies/ml of sera), and HHV-7 (n=12) were obtained from the HHV-6 Foundation (Santa Barbara, California, USA). Reference copy numbers given by the providing institute were used for HHV-6A and HHV-6B (59), while the HHV-7 DNA was quantified with a commercial HHV-7 qPCR kit (PCRmax) in our laboratory.

ATCC standard

Quantitative Genomic DNA of VZV (ATCC® VR1367DQ ™) was analyzed with HERQ-9 undiluted and at 1:10 and 1:100 dilutions.

Clinical samples

All the clinical HHV samples were collected at the virus diagnostic unit of Turku University Hospital, and are presented in Table 5. These included: 80 mucocutaneous swab samples of which 35 were positive for HSV-1 and 30 for HSV-2 by rapid viral culture immunoperoxidase assay (53); 25 mucocutaneous swab samples of which 15 were positive for VZV by antigen enzyme immunoassay (61); five HSV-1 and four HSV-2 PCR positive DNA extracts from mucocutaneous swab samples tested by a reference PCR (4, 31); and eight CSF samples of which two were VZV positive by a control PCR (4, 31).
In addition, 60 plasma samples were investigated of which 17 had been studied only for EBV (GeneProof Epstein-Barr Virus (EBV) PCR Kit), 17 only for HCMV (GeneProof Cytomegalovirus (CMV) PCR Kit) and 26 for both. From these 13 and 16 samples were reported as EBV and HCMV positive (>200 copies/ml of plasma), respectively, while nine and five had borderline copy numbers (50-200 copies/ml of plasma).

Tonsillar tissues

Altogether 35 mechanically homogenized tonsillar tissues were screened for the nine HHVs. The patients were 2 to 69 years of age (mean, 26), with eight <12 years (48). The viral loads were normalized per 10^6 cells, determined with the human single-copy gene RNase P-qPCR (48).

Herpesvirus DNA mixes

Mixtures of three viral genomes extracted from the previously mentioned infected cell lysates [i) HSV-1, HSV-2 and VZV; ii) EBV, HCMV and KSHV] or spiked serum [iii) HHV-6A, 6B and 7] were tested at uneven quantities, ranging from 4.5E0 to 1.1E6 copies/μl of DNA extract.

Primers and hydrolysis probes

Primers and probes were designed for all HHVs, except for EBV (62). For each virus, several primer pairs were constructed in silico, in conserved genes (16, 18, 63, 64). Degenerate primers were designed for HCMV to cover polymorphisms in the target area. Moreover, for HHV-6A, two probes were custom-designed to contain six locked nucleic acids (LNA) each (for shorter probe length), and a single-nucleotide difference for specific binding to different strains (Table 6).

The tendency of primers and probes to form secondary structures, primer dimers and cross-dimers was evaluated with Multiple Primer Analyzer (Thermo Fisher Scientific), while the propensity of the amplicons to form secondary structures was checked with Mfold Web Server. A BLAST search (65) was performed to confirm primer binding to each of the virus strains in the nucleotide collection database (NCBI).
Primer candidates designed in silico were tested at 200 nM concentration with plasmid dilutions (see Plasmids), human DNA (HaCaT 500 ng/reaction) and nuclease-free water in a SYBR green format (Maxima SYBR Green qPCR master mix, Thermo Fisher Scientific) followed by melting curve analysis. Primer pairs showing the highest efficiency and sensitivity with no primer dimer formation were chosen for further testing with the hydrolysis probes. Concentrations of the primer pairs were optimized empirically with a matrix of reactions ranging from 100 nM to 600 nM. The probes were tested in a 100 nM to 400 nM range. The final primer and probe concentrations are presented in Table 6.

Primers and hydrolysis probes were purchased as HPLC purified except for two degenerative primers (HCMV), which were cartridge purified (Sigma Aldrich). For the triplex reactions, the probes were labeled with 6-FAM-BHQ-1, JOE-BHQ-1 and TxRd-BHQ-2.

Quantitative PCR protocol

Four commercial master mixes were pre-tested with HSV-1, HSV-2 and VZV plasmids and viral genomes with special consideration given to the performance in the presence of human DNA and the co-amplification of markedly low and high-abundance targets. Consequently, TaqPath ProAmp Multiplex Master Mix (Thermo Fisher Scientific) was chosen for the multiplex assay.

The qPCR thermal profile comprised initial denaturation at 95 °C for 10 min followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C. The qPCR reactions contained 5 μl of template, 2X TaqPath ProAmp Multiplex Master Mix, primers and probes (Table 6), and nuclease-free water in a final volume of 20 μl. Water was used as negative control in all the qPCR runs. The samples were run in duplicate in AriaMx Real-time PCR System (Agilent) and analyzed with the Aria Real-Time PCR Software (v. 1.3) provided by the manufacturer. The adaptive fluorescence baseline, efficiency, slope, $R^2$-values and intercept were calculated by the software. Background-based threshold was set for cycles 5 to 9 for the FAM and TexasRed dyes, and 8 to 11 for the JOE dye.
Pre-testing of primers in SYBR green format consisted of the above-mentioned thermal profile, followed by a melting curve analysis at 95 °C for 10 min, 45 °C for 30 s and 95 °C for 30 s. The melting curve analysis was performed with a resolution of 0.5 °C and soak time of 5 s.

DNA extraction

DNA from plasma, mucocutaneous swabs, CSF and WHO international standards were extracted from 200 µl of starting material with the QIAamp DNA Blood Mini Kit (Qiagen); DNA from cells and virus-infected cell lines with the QIAamp DNA mini kit (Qiagen); DNA from KSHV BAC with NucleoBond® Xtra Midi EF kit (Macherey-Nagel); and transformed plasmids with GeneJET Plasmid Miniprep kit (Thermo Fisher Scientific), according to manufacturer instructions. The final elution volumes were 100 µl (with the exception of 50 µl for plasma and 60 µl for CSF samples). In every extraction, at least two negative controls (phosphate-buffered saline) were included.

Analytical sensitivity and specificity

The analytical sensitivities were determined in singleplex and multiplex formats using eight replicates of each HHV plasmid template in 50, 25, 15, 10, 5, 3 and 1 copies per reaction. The proportion of positive results was fit into a generalized linear model using probit link function (MATLAB v.R2018b) to approximate the limit of detection (LOD\textsubscript{95}) for a given target.

The analytical specificities were evaluated by cross-testing: a) 10\textsuperscript{7} copies of viral genomic DNA extracted from infected cell lysates and plasmid constructs of each HHV; b) Plasmids containing near full/full-length genomes of polyomaviruses BKV, JCV and MCV; and parvovirus B19 genotype 1. In addition, 1000 ng of cellular DNA extracted from HaCaT cells and 500 ng from human foreskin fibroblasts were tested for unspecific amplification of human DNA.
Repeatability and reproducibility

The intra- and interassay variations were calculated using three separate qPCR runs using five replicates of i) HHV plasmids \((10^6 - 10^1 \text{ copies /µl})\) in singleplex and multiplex formats and ii) plasmid mixes pMIXI, pMIXII, pMIXIII \((10^6 - 10^1 \text{ copies /µl})\) in multiplex format. Two of the replicates were used to generate a standard curve and three were marked as unknowns. The standard deviations of the C\(_q\) values of the five replicates were used as a measure of intraassay variation. A coefficient of variation calculated from the copy numbers of unknown replicates from three separate runs was used to estimate the interassay variation.

Statistical analysis

Boxplots were built with Rstudio (v.1.2.5001) and Excel 2016 (v.16.0.4964.1000) was used to create scatterplot graphs. Pearson correlation coefficient between estimated and quantified copies in virus mixes was calculated with SPSS (v.25). Venn diagrams were made with InteractiVenn (66).

Ethics statement

The Ethics Committee of the Helsinki and Uusimaa Hospital District approved the collection of tonsils. Informed consent was obtained from all the donors or their parents prior to the surgery.

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The authors state no conflict of interests

Author´s contributions
L.P., M.T., K.H, H.V. and M.F.P. contributed to the design of this study. C.S., and H.V. planned the target gene areas. L.P., M.J., and H.S. executed the development of the qPCR and optimization experiments. L.P. carried out experiments with clinical and reference samples and analyzed the data. H.V., M.F.P., V.H., E.E., and P.M.O. participated in the collection of reference materials. T.V., and H.V. coordinated the collection of clinical samples. L.P., and M.F.P. drafted the manuscript. K.H., M.T., M.J., H.S., P.M.O., E.E., V.H., and H.V. participated in writing of the manuscript.

All authors read and approved the final manuscript.
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Figure Legends

Figure 1. A) Amplification and standard curves of HSV-1 plasmid dilutions from $10^6$ to $10^1$ copies/µl in singleplex (Red), multiplex (Green) and together with HSV-2 and VZV plasmids (pMIXI) in the same mix (Blue). (B) pMIXI dilution series spiked with 500 ng/reaction of human DNA (HaCaT cells). The y-axis represents baseline-corrected fluorescence signal (amplification curve) or cycles (standard curve). The x-axis represents cycle number (amplification curve) or plasmid copies in reaction($\log_{10}$) (standard curve). Analogous illustrations for other herpesviruses can be found in Fig. S4.

Figure 2. Reference copy numbers of (A) HHV-6A (B) HHV-6B (C) HHV-7 spiked sera plotted against copy numbers quantified by HERQ-9. Viral DNA copy numbers are presented per ml of serum ($\log_{10}$ transformed). $R^2$ indicates the coefficient of determination between the copy numbers.

Figure 3. Comparison of (A) EBV and (B) HCMV quantities (copies/ml of plasma in $\log_{10}$) as determined by HERQ-9 and GeneProof EBV and CMV PCR kit –reference assays.

Figure 4. A Venn diagram representing HHV co-occurrence in (A) 60 plasma samples, (B) 35 palatine tonsils, (C) 43 HSV-1 positive mucocutaneous swab samples and (D) 34 HSV-2 positive mucocutaneous swab samples; n = number of positive cases.

Figure 5. HHV-6B, HHV-7, EBV, HSV-1 and HCMV copies per million cells. Notches represent IQR (interquartile range) of the samples and whiskers range +/- 1.5 IQRs from the upper and lower quartiles.

Figure S1. Primer dimers, cross-dimers and secondary structures analysis of the primers and probes with Multiple Primer Analyzer (Thermo Fisher Scientific). The sensitivity of three was used with a primer concentration of 0.5 µM and a salt concentration of 50 mM.
Figure S2. Amplicon secondary structure analyzed with Mfold Web Server. The reaction temperature was set to 60°C, [Na+] to 50mM, [Mg++] to 3.0 mM and other settings left as default. The green nucleotides represent probe binding areas.

Figure S3. Probit link function estimating LOD\textsubscript{95} of qPCRs in singleplex and multiplex formats. The proportion of positives from eight plasmid replicates at 50, 25, 15, 10, 5, 3 and 1 copies/reaction was fit into the model to approximate the assay sensitivity.

Figure S4. Amplification and standard curves in 10\textsuperscript{6} to 10\textsuperscript{1} copies/\mu{l} of plasmid dilutions of HSV-2 (A) and (B), VZV (C) and (D), EBV (E) and (F), HCMV (G) and (H), KSHV (I) and (J), HHV-6A (K) and (L), HHV-6B (M) and (N), HHV-7 (O) and (P). On the left are represented plasmids in singleplex (Red), multiplex (Green) and pMIXI-III (Blue). On the right, pMIXI-III dilution series spiked with 500 ng/reaction of human DNA (HaCaT cells). The \textit{y}-axis represents baseline-corrected fluorescence signal (amplification curve) or cycles (standard curve). The \textit{x}-axis represents cycle number (amplification curve) or plasmid copies in reaction(\textit{log}_{10}) (standard curve).

Text S1. Sequences of reference strains in the plasmids

Text S2. Preparation protocol of HSV-1 and HSV-2 nuclecapsids
Table 1. Intraassay variation

| copies | Mean Cq values ±SD | | | | | | |
| /µl | | | | | | |
| | Mean Cq values ±SD | | | | | | |
| | HSV-1 | HSV-2 | VZV | HSV-1 | HSV-2 | VZV | HSV-1 | HSV-2 | VZV |
| | singleplex | multiplex | pMIXI | singleplex | multiplex | pMIXI | singleplex | multiplex | pMIXI |
| 10 | 32.4 ± 0.7 | 32.4 ± 0.6 | 32.9 ± 0.6 | 32 ± 0.4 | 32.5 ± 0.3 | 32.1 ± 0.4 | 32 ± 0.4 | 32.5 ± 0.3 | 32.1 ± 0.4 |
| 10 | 29.2 ± 0.2 | 29.1 ± 0.2 | 29.4 ± 0.3 | 28.9 ± 0.2 | 28.8 ± 0.2 | 28.7 ± 0.2 | 28.9 ± 0.2 | 28.8 ± 0.2 | 28.7 ± 0.2 |
| 10 | 26.1 ± 0.3 | 25.8 ± 0.1 | 25.9 ± 0.1 | 25.7 ± 0.2 | 25.5 ± 0.1 | 25 ± 0.3 | 25.7 ± 0.2 | 25.5 ± 0.1 | 25 ± 0.3 |
| 10 | 22.6 ± 0.2 | 22.6 ± 0.2 | 22.5 ± 0.2 | 22.1 ± 0.2 | 22.1 ± 0.2 | 22 ± 0.1 | 22.1 ± 0.2 | 22.1 ± 0.2 | 22 ± 0.1 |
| 10 | 19.4 ± 0.4 | 19.2 ± 0.1 | 19.2 ± 0.1 | 18.8 ± 0.1 | 18.9 ± 0.4 | 18.8 ± 0.1 | 18.8 ± 0.1 | 18.9 ± 0.4 | 18.8 ± 0.1 |
| 10 | 16.3 ± 0.2 | 16.1 ± 0.1 | 16 ± 0.2 | 15.8 ± 0.1 | 15.8 ± 0.2 | 15.6 ± 0.2 | 15.8 ± 0.1 | 15.8 ± 0.2 | 15.6 ± 0.2 |
| Eff. % | 100.1 | 101.7 | 100.3 | 101.0 | 101.7 | 100.5 | 103.2 | 100.9 | 100.7 |
| R² | 0.998 | 0.997 | 0.997 | 0.994 | 0.997 | 0.998 | 0.997 | 0.997 | 0.997 |
| Slope | -3.32 | -3.28 | -3.32 | -3.3 | -3.28 | -3.31 | -3.25 | -3.3 | -3.31 |
| Interc. | 39.1 | 38.9 | 39.1 | 38.3 | 38.9 | 38.2 | 37.5 | 37.8 | 37.6 |

| copies | EBV | HCMV | KSHV |
| /µl | | | |
| | singleplex | multiplex | pMIXII | singleplex | multiplex | pMIXII | singleplex | multiplex | pMIXII |
| 10 | 33.6 ± 0.8 | 32.6 ± 0.8 | 32.9 ± 0.7 | 33.6 ± 0.8 | 32.6 ± 0.8 | 32.9 ± 0.7 | 33.6 ± 0.8 | 32.6 ± 0.8 | 32.9 ± 0.7 |
| 10 | 29.4 ± 0.4 | 29.3 ± 0.5 | 28.9 ± 0.4 | 29.4 ± 0.4 | 29.3 ± 0.5 | 28.9 ± 0.4 | 29.4 ± 0.4 | 29.3 ± 0.5 | 28.9 ± 0.4 |
| 10 | 25.8 ± 0.1 | 26.1 ± 0.2 | 25.7 ± 0.2 | 25.8 ± 0.1 | 26.1 ± 0.2 | 25.7 ± 0.2 | 25.8 ± 0.1 | 26.1 ± 0.2 | 25.7 ± 0.2 |
| 10 | 22.6 ± 0.2 | 22.6 ± 0.2 | 22.4 ± 0.1 | 22.6 ± 0.2 | 22.6 ± 0.2 | 22.4 ± 0.1 | 22.6 ± 0.2 | 22.6 ± 0.2 | 22.4 ± 0.1 |
| 10 | 19.3 ± 0.1 | 19.1 ± 0.1 | 19.0 ± 0.2 | 19.3 ± 0.1 | 19.1 ± 0.1 | 19.0 ± 0.2 | 19.3 ± 0.1 | 19.1 ± 0.1 | 19.0 ± 0.2 |
| 10 | 16.1 ± 0.1 | 16 ± 0.1 | 15.5 ± 0.2 | 16.1 ± 0.1 | 16 ± 0.1 | 15.5 ± 0.2 | 16.1 ± 0.1 | 16 ± 0.1 | 15.5 ± 0.2 |
| Eff. % | 97.8 | 99.5 | 98.6 | 98.2 | 98.4 | 99.7 | 95.9 | 98.9 | 97.2 |
| R² | 0.998 | 0.998 | 0.999 | 0.997 | 0.994 | 0.996 | 0.996 | 0.992 | 0.996 |
| Slope | -3.37 | -3.33 | -3.36 | -3.37 | -3.36 | -3.33 | -3.42 | -3.35 | -3.39 |
| copies | HHV-6A | HHV6-B | HHV-7 |
|-------|--------|--------|--------|
|       | singleplex | multiplex | pMIXIII | singleplex | multiplex | pMIXIII | singleplex | multiplex | pMIXIII |
| $10^1$ | 32.4 ± 0.6 | 33.0 ± 0.4 | 33.3 ± 0.6 | 31.6 ± 0.9 | 31.8 ± 0.5 | 31.5 ± 0.5 | 31.5 ± 0.7 | 31.6 ± 0.7 | 32.2 ± 0.7 |
| $10^2$ | 29.5 ± 0.3 | 29.5 ± 0.4 | 29.6 ± 0.3 | 28.7 ± 0.4 | 28.6 ± 0.2 | 29.1 ± 0.3 | 28.1 ± 0.4 | 28.2 ± 0.4 | 28.3 ± 0.1 |
| $10^3$ | 26.1 ± 0.2 | 25.8 ± 0.1 | 26.1 ± 0.3 | 25.3 ± 0.1 | 25.3 ± 0.1 | 25.6 ± 0.2 | 25 ± 0.1 | 24.9 ± 0 | 25.0 ± 0.1 |
| $10^4$ | 22.7 ± 0.1 | 22.5 ± 0.1 | 22.9 ± 0.2 | 21.8 ± 0.2 | 22.0 ± 0.2 | 22.2 ± 0.1 | 21.6 ± 0.2 | 21.5 ± 0.1 | 21.5 ± 0.2 |
| $10^5$ | 19.2 ± 0.1 | 19.4 ± 0.2 | 19.6 ± 0.2 | 18.6 ± 0.1 | 18.8 ± 0.2 | 19 ± 0.2 | 18.1 ± 0.1 | 18.2 ± 0.1 | 18.4 ± 0.1 |
| $10^6$ | 16.3 ± 0.1 | 16.6 ± 0.1 | 16.1 ± 0.1 | 15.6 ± 0.1 | 15.7 ± 0.1 | 15.8 ± 0.1 | 15.0 ± 0.2 | 15.2 ± 0.1 | 15.1 ± 0.1 |
| Eff. % | 100.6 | 100.2 | 98.2 | 100.3 | 103.8 | 101.9 | 100.7 | 102.7 | 98.2 |
| R² | 0.995 | 0.995 | 0.995 | 0.992 | 0.998 | 0.998 | 0.995 | 0.993 | 0.994 |
| Slope | -3.31 | -3.32 | -3.37 | -3.32 | -3.23 | -3.28 | -3.31 | -3.26 | -3.37 |
| Interc. | 38.3 | 38.4 | 38.7 | 37.6 | 37.4 | 37.7 | 37.01 | 36.9 | 37.5 |
Table 2. Interassay variation

| copies/µl | HSV-1 (singleplex) | HSV-1 (multiplex) | HSV-1 (pMIXI) | HSV-2 (singleplex) | HSV-2 (multiplex) | HSV-2 (pMIXI) | VZV (singleplex) | VZV (multiplex) | VZV (pMIXI) |
|---------|-------------------|-------------------|---------------|-------------------|-------------------|---------------|----------------|----------------|-------------|
|         |                   |                   |               |                   |                   |               |                 |                 |             |
| 10^1    | 12                | 16                | 7             | 23                | 27                | 33            | 14             | 27             | 13          |
| 10^2    | 21                | 20                | 12            | 12                | 10                | 8             | 6              | 9              | 16          |
| 10^3    | 8                 | 15                | 7             | 11                | 3                 | 7             | 11             | 6              | 13          |
| 10^4    | 4                 | 7                 | 4             | 9                 | 2                 | 9             | 4              | 8              | 2           |
| 10^5    | 6                 | 3                 | 1             | 4                 | 9                 | 5             | 2              | 2              | 8           |
| 10^6    | 7                 | 4                 | 10            | 9                 | 6                 | 3             | 5              | 9              | 7           |

| copies/µl | EBV (singleplex) | EBV (multiplex) | EBV (pMIXII) | HCMV (singleplex) | HCMV (multiplex) | HCMV (pMIXII) | KSHV (singleplex) | KSHV (multiplex) | KSHV (pMIXII) |
|-----------|-----------------|-----------------|-------------|-------------------|------------------|----------------|------------------|------------------|----------------|
| 10^1      | 24              | 23              | 20          | 15                | 14               | 16            | 15              | 14              | 16            |
| 10^2      | 11              | 4               | 7           | 13                | 9                | 8             | 13              | 9               | 8             |
| 10^3      | 11              | 10              | 4           | 9                 | 6                | 5             | 9               | 6               | 5             |
| 10^4      | 12              | 1               | 12          | 11                | 2                | 7             | 11              | 2               | 7             |
| 10^5      | 11              | 2               | 4           | 18                | 3                | 12            | 18              | 3               | 12            |
| 10^6      | 7               | 4               | 14          | 10                | 6                | 3             | 10              | 6               | 3             |

| copies/µl | HHV-6A (singleplex) | HHV-6A (multiplex) | HHV-6A (pMIXIII) | HHV6-B (singleplex) | HHV6-B (multiplex) | HHV6-B (pMIXIII) | HHV-7 (singleplex) | HHV-7 (multiplex) | HHV-7 (pMIXIII) |
|-----------|---------------------|--------------------|------------------|---------------------|--------------------|------------------|------------------|--------------------|------------------|
| 10^1      | 15                  | 4                  | 20               | 27                  | 28                 | 19               | 19               | 25                 | 31               |
| 10^2      | 7                   | 19                 | 7                | 22                  | 13                 | 5                | 10               | 4                  | 14               |
| 10^3      | 14                  | 9                  | 7                | 13                  | 3                  | 6                | 11               | 9                  | 12               |
| 10^4      | 9                   | 3                  | 6                | 4                   | 12                 | 10               | 9                | 10                 | 3                |
| 10^5      | 3                   | 1                  | 2                | 3                   | 3                  | 6                | 8                | 2                  | 4                |
| 10^6      | 12                  | 15                 | 0                | 6                   | 11                 | 4                | 3                | 8                  | 14               |
| Virus   | Sample type                          | Quantification method | Strain(s)                     | Genbank accession number | Reference (copies/µl) | HERQ-9 (copies/µl) | Conversion factor | Difference between quantifications (log₁₀) |
|---------|--------------------------------------|-----------------------|--------------------------------|----------------------------|-----------------------|---------------------|-------------------|--------------------------------------------|
| HSV-1   | DNA from purified nucleocapsids      | Spectrophotometer     | HSV-H1211, HSV-H1215          | MH999843, MH999846         | 4.3E9, 6.1E9          | 4.2E9, 6.1E9        | 0.99              | <0.02                                      |
| HSV-2   | DNA from purified nucleocapsids      | Spectrophotometer     | HSV2-H12211, HSV2-H1526       | KY922725, KY922724         | 1.9E9, 3.8E9          | 9.1E9, 1.9E9        | 0.49              | 0.31                                       |
| VZV     | Quantified DNA, ATCC                 | ddPCR,                | Ellen                         | JQ972913.1                 | 5.6E5                 | 1.8E6               | 3.2               | 0.51                                       |
| EBV     | WHO international standard          | qPCR, (NIBSC code 09/260), (57) | BS5-8                         | NC_007605                 | 1.0E4                 | 1.0E4               | 1.0               | <0.02                                      |
| HCMV    | WHO international standard          | qPCR, (NIBSC code 09/162) (58) | Merlin                        | GU179001.1                 | 1.0E4                 | 3.2E4               | 3.2               | 0.51                                       |
| KSHV    | Genome in BAC                        | Spectrophotometer     | BAC16 JSC-1                   | MK208323.1                 | 2.2E9                 | 5.0E9               | 2.3               | 0.36                                       |
| HHV-6A  | Spiked serum, HHV-6 Foundation       | qPCR (59)             | GS                            | KC465951.1                 | 1.6E4-1.6E0           | 2.1E4-1.1E0         | 1.1               | 0.03                                       |
| HHV-6B  | WHO international standard          | qPCR, (NIBSC code 15/266), (59) | Z29                          | AF157706.1                 | 1.1E5                 | 1.8E5               | 1.6               | 0.20                                       |
| HHV-7   | Spiked serum, HHV-6 Foundation       | HHV-7 qPCR kit (PCRmax) | Ji                            | U43400.1                   | 1.7E2-2.4E-1          | 1.1E3-2.4E0         | 6.8               | 0.83                                       |
Table 4. Clinical samples and other sample material

| Virus   | Sample type | n = | reference method(s)                                      | Positive agreement | Negative agreement | type          | Strain(s) | Genbank accession number |
|---------|-------------|-----|---------------------------------------------------------|--------------------|--------------------|---------------|------------|--------------------------|
| HSV-1   | mucocutaneous swab | 92  | rapid viral culture immunoperoxidase, qPCR (4, 31, 53)  | 42/42 (100%)       | 49/50 (98%)       | infected cell cultures | strain F   | KM222724.1               |
| HSV-2   | mucocutaneous swab | 34  | rapid viral culture immunoperoxidase, qPCR(4, 31, 53)   | 34/34 (100%)       | 58/58 (100%)      | infected cell cultures | strain G   | KP143740.1               |
| VZV     | mucocutaneous swab | 27  | EIA, qPCR(4, 31, 61)                                    | 20/20 (100%)       | 14/15 (93.3%)     | infected cell cultures | Ellen      | JQ972913.1               |
| EBV     | Plasma       | 46  | EBV (GeneProof Epstein-Barr Virus (EBV) PCR Kit)        | 14/14 (100%)       | 21/23 (91.3%)     | Raji-cells    | Raji       | KF717093.1               |
| HCMV    | Plasma       | 48  | GeneProof Cytomegalovirus (CMV) PCR Kit                 | 19/19 (100%)       | 22/24 (91.7%)     | Infected cell cultures | AD169      | FJ527563.1               |
| KSHV    | -            | -   | -                                                       |                    | -                  | infected cell cultures | JSC-1 clone BAC16 | GQ994935.1               |
| HHV-6A  | -            | -   | -                                                       |                    | -                  | Infected cell cultures | GS         | KC465951.1               |
| HHV-6B  | -            | -   | -                                                       |                    | -                  | Spiked serum, Infected cell cultures | Z29       | AF157706.1               |
| HHV-7   | -            | -   | -                                                       |                    | -                  | Infected cell cultures | JI         | U43400.1                 |

*Positive agreement with samples reported borderline with reference assay
Table 5. Three viral genomes intermixed into the same reaction.

| Virus mix | Estimated viral genome copies/µl | Measured viral genome copies/µl |
|-----------|---------------------------------|--------------------------------|
|           | HSV-1   | HSV-2  | VZV   | HSV-1   | HSV-2  | VZV   |
| 1         | 1.1E+06 | 5.6E+03 | 2.8E+02 | 1.2E+06 (6) | 1.0E+04 (42) | 1.9E+02 (28) |
| 2         | 1.3E+04 | 5.9E+04 | 3.4E+01 | 1.3E+04 (1) | 6.3E+04 (4) | 1.4E+01 (57) |
| 3         | 1.2E+03 | 6.2E+03 | 4.2E+04 | 1.1E+03 (9) | 5.1E+03 (13) | 2.7E+04 (29) |
| 4         | 1.2E+02 | 4.5E+01 | 4.2E+03 | 1.1E+02 (9) | 7.2E+01 (33) | 3.3E+03 (15) |
| 5         | 1.1E+05 | 4.6E+01 | 3.6E+04 | 1.1E+05 (2) | 8.9E+01 (45) | 3.0E+04 (13) |
| 6         | 1.2E+02 | 5.9E+04 | 4.2E+04 | 1.6E+02 (18) | 4.9E+04 (13) | 4.2E+04 (1) |
| 7         | 1.1E+05 | 1.5E+03 | 5.4E+03 | 1.2E+05 (5) | 2.6E+03 (37) | 6.7E+03 (15) |
| 8         | 1.1E+04 | 1.7E+05 | 4.9E+05 | 1.3E+04 (12) | 1.8E+05 (4) | 6.4E+05 (20) |
| 9         | 1.2E+03 | 1.8E+04 | 5.7E+04 | 1.4E+03 (10) | 1.5E+04 (11) | 6.9E+04 (14) |
| 10        | 7.1E+01 | 1.3E+02 | 6.0E+05 | 1.1E+02 (31) | 1.9E+02 (26) | 6.2E+05 (2) |
| 11        | 1.1E+05 | 1.7E+05 | 4.1E+02 | 1.2E+05 (6) | 2.0E+05 (12) | 9.7E+02 (57) |
| 12        | 9.6E+01 | 1.8E+04 | 5.7E+04 | 1.3E+02 (22) | 1.5E+04 (14) | 5.4E+04 (3) |
| 13        | 1.9E+03 | 6.2E+03 | 1.0E+02 | 1.7E+03 (9) | 7.1E+03 (9) | 1.3E+02 (24) |
| 14        | 1.9E+03 | 6.1E+02 | 1.0E+02 | 1.7E+03 (10) | 4.6E+02 (18) | 1.1E+02 (10) |
| 15        | 9.3E+01 | 6.1E+03 | 5.2E+01 | 1.2E+02 (21) | 5.6E+03 (6) | 3.6E+01 (25) |
| 16        | 1.3E+03 | 6.1E+01 | 5.2E+01 | 1.4E+03 (9) | 1.2E+02 (47) | 3.6E+01 (26) |
| 17        | 4.5E+00 | 7.9E+01 | 1.0E+02 | 1.1E+01 (58) | 5.2E+01 (30) | 1.1E+02 (4) |
| 18        | 1.3E+03 | 6.1E+03 | 5.2E+01 | 1.9E+03 (27) | 1.1E+04 (40) | 4.9E+01 (4) |

On the left are the estimated genome copies per dilution and on the right the copy numbers/µl of DNA extract quantified by HERQ-9. In parentheses are the coefficient of variations (CV %) between the estimated and measured copy numbers. Estimated and measured viral genome copies showed significant correlation (pearson’s r = 0.996, p<0.01).
| Virus | Oligo name | Concentration | Sequence 5’ – 3’ | Position of the amplicon in the genome (length) | Target gene | Reference Sequence (GenBank) |
|-------|------------|---------------|------------------|-----------------------------------------------|-------------|-----------------------------|
| HSV-1 | HSV-1 FWDLP1 | 300 nM | GTTAGAAGCTAGCGAGCGCA | 93580-93683 (124 bp) | UL42 | X14112.1 |
| HSV-1 | HSV-1 REVLP1 | 300 nM | CTATATAAGCTAGCGAGCGCA | | | |
| HSV-1 | HSV-1 probeLP1 | 250 nM | [FAM]CGCGAAGCTAGCGAGCGCA | | | |
| HSV-2 | HSV-2 FWD-2 | 400 nM | GTTAGAAGCTAGCGAGCGCA | 46783-46872 (90 bp) | UL23 | Z86099.2 |
| HSV-2 | HSV-2 REVLP1 | 400 nM | CTATATAAGCTAGCGAGCGCA | | | |
| HSV-2 | HSV-2 probeLP1 | 200 nM | [FAM]CGCGAAGCTAGCGAGCGCA | | | |
| VZV | VZV FWDLP1 | 200 nM | GGCGAAGCTATTTAGAGC | 48283-48145 (139 bp) | ORF28 | KUS29566.1 |
| VZV | VZV REVLP1 | 200 nM | ACATGCGAGAAATCCTGGG | | | |
| VZV | VZV probeLP1 | 150 nM | [JOE]CGCGAAGCTATTTAGAGC | | | |
| EBV | EBV FWD | 200 nM | GTTAGAAGCTAGCGAGCGCA | 153036-152947 (90 bp) | BALF5 | (62) |
| EBV | EBV REV | 300 nM | CTATATAAGCTAGCGAGCGCA | | | |
| EBV | EBV Probe | 300 nM | [FAM]CGCGAAGCTAGCGAGCGCA | | | |
| HCMV | H5 FWD211 | 400 nM | GTGGCTCCTAGCTGTTTAC | 80396-80329 (68 bp) | UL54 | AB329634.1 |
| HCMV | H5 rev 211 | 500 nM | ACATGCGAGAAATCCTGGG | | | |
| HCMV | H5 Probe 20 | 300 nM | [FAM]CGCGAAGCTAGCGAGCGCA | | | |
| KSHV | HHV8 fwd 3.1 | 200 nM | ATATACCGCGACAGCACGCTC | 13603-13761 (159 bp) | ORF9 | AP017458.1 |
| KSHV | HHV8 REV 10 | 200 nM | GAGCGAGAAGGACTGTTGAG | | | |
| KSHV | H8 Probe 300 | 200 nM | [JOE]CGCGAAGCTAGCGAGCGCA | | | |
| HHV-6A | HHV6A FWD1-3 | 500 nM | GTGCTGTTGCTGTTGCTGTC | 133969-133894 (76 bp) | U90 | KP257584.1 |
| HHV-6A | HHV6A REV 10 | 500 nM | ATATACCGCGACAGCACGCTC | | | |
| HHV-6A | HHV6A LNA Probe A1 | 100 nM | GAGCGAGAAGGACTGTTGAG | | | |
| HHV-6A | HHV6A LNA Probe A2 | 100 nM | [JOE]CGCGAAGCTAGCGAGCGCA | | | |
| HHV-6B | H6B FOTY1 | 300 nM | TTTGACAGGAGTTGCTGAG | 136176-136258 (83 bp) | U90 | AB021506.1 |
| HHV-6B | H6B ROTY 1 | 300 nM | GAGCGAGAAGGACTGTTGAG | | | |
| HHV-6B | H6B PROBE MVP | 200 nM | [JOE]CGCGAAGCTAGCGAGCGCA | | | |
| HHV-7 | HHV7 1. FWD | 400 nM | CTCGCAGAGTGCCTTGAG | 88332-88490 (159 bp) | U57 | AF037218.1 |
| HHV-7 | HHV7 1. REV | 400 nM | GATACCGAGAAGGACTGTTGAG | | | |
| HHV-7 | H7 MOP PROBE | 300 nM | [FAM]CGCGAAGCTAGCGAGCGCA | | | |

* Nucleotides in [] refer to locked nucleic acids.
$R^2 = 0.98$
$R^2 = 0.99$
\[ R^2 = 0.99 \]
$R^2 = 0.85$
$R^2 = 0.83$
