Methods comparison for molecular diagnosis of human herpesvirus 8 infections

Manon Corgiat, Vincent Calvez, Anne-Geneviève Marcelin, Aude Jary

TITLE: Methods comparison for molecular diagnosis of human herpesvirus 8 infections.

Manon Corgiat[a], Vincent Calvez[a], Anne-Geneviève Marcelin[a], Aude Jary[a]

[a]Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique (iPLESP), AP-HP, Pitié Salpêtrière Hospital, Department of virology, F-75013 Paris, France

Corresponding author: Aude Jary, 47-83 boulevard de l'Hôpital, 75013 Paris, +33 1 42 17 74 01, aude.jary@aphp.fr

Alternate corresponding author: Pr Anne-Geneviève Marcelin, 47-83 boulevard de l'Hopital, 75013 Paris, +33 1 42 17 74 01, anne-genevieve.marcelin@aphp.fr

Highlights

- HHV-8 DNA viral load in blood is an accurate marker for diagnosis and management of HHV-8 associated diseases.
- None of the commercial assays for HHV-8 diagnosis by real-time PCR were comparable with the in-house PCR.
- The HHV-8 R-gene™ assay overestimated by in median 0.46 $\log_{10}$ copies/10^6 cells the results obtained by the in-house PCR
- The HHV-8 Clonit® assay overestimated by in median 1.16 $\log_{10}$ copies/10^6 cells the results obtained by the in-house PCR

ABSTRACT

Background: Human herpesvirus 8 (HHV-8) virological diagnosis and monitoring relies mainly on real-time PCR.

Objectives: To evaluate two real-time PCR commercial kit (HHV-8 Premix R-gene™ and Clonit® HHV-8) and compare with in-house real-time PCR.

Study design. Twelve samples (3 undetectable and 9 detectable with viral load ranging from 10^1 to 10^5 per reaction) were tested for HHV-8 detection and quantification with the 3 methods. Methods comparison was supported with regression curve and diagram presenting difference or ratio between commercial and in-house PCR results and plotted against the in-house PCR results. Statistical analyses, specifically Student tests and Spearman correlation, were performed.

Results: In both cases, qualitative results obtained with commercial kit and in-house PCR were identical and HHV-8 quantitation results were significantly correlated (Clonit®, $R_s = 1$, $p < 0.001$ and R-gene™ $R_s = 0.98$, $p < 0.001$). However, Clonit® results were significantly higher compared to the in-house results with an overestimation in median [IQR] of 1.16 $\log_{10}$ copies/10^6 cells [1.12–1.18] whereas R-GeneTM results were not significantly higher, and an overestimation in median of 0.46 $\log_{10}$ copies/10^6 cells [0.37-0.52]. Otherwise, repeatability and reproducibility tests of undetectable sample failed with Clonit® technique contrary to the R-Gene™.

Conclusions: HHV-8 R-gene™ assay seems to be the most suitable since it showed consistent qualitative results with in-house HHV-8 PCR, a good quantitative correlation, an overestimation not significantly different and inferior to 0.50 $\log_{10}$ copies/10^6 cells and a good repeatability.

BACKGROUND

Human herpesvirus 8 (HHV-8) is involved in all forms of Kaposi's sarcoma (KS) and in two lymphoid malignancies: some forms of multicentric Castleman disease and primary effusion lymphoma [1,2].

As others human herpesviruses, HHV-8 establishes latency after primary infection and can provide symptomatic reactivations in immunocompromised patients [2]. Real-time Polymerase Chain Reaction (PCR) is an effective diagnostic tool for the detection and the quantification of HHV8-DNA and is based on the amplification of a conserved gene within different HHV-8 subtypes [3–6]. In the peripheral compartment, HHV8-DNA viral load (VL) monitoring was reported to be an accurate biomarker for assessment of the risk of further disease progression [7–9], but also to evaluate response to therapy [10,11] and to distinguish the three main HHV-8 pathologies at diagnosis [12]. Otherwise, whole blood seems to be the best sample to evaluate HHV8-DNA VL rate since it allows quantifying latent HHV-8 (intracellular), replicating HHV-8 (intracellular) and free viral particles (extracellular).

OJECTIVES

In our virology department, HHV8-DNA detection and quantification is performed with an in-house real time PCR amplifying ORF-73 [5]. This study aimed to evaluate two real time PCR commercial kits for the detection and quantification of HHV8-DNA by comparing them with the in-house PCR.

STUDY DESIGN

Samples

Twelve whole blood samples were selected [Supplementary Table 1]: 3 with undetectable HHV8-DNA VL and 9 with detectable HHV8-DNA VL ranging from 10^1 to 10^5 per reaction (or from 1.56 to 5.79 $\log_{10}$ copies/10^6 cells). Before extraction, DICO (Biomérieux®) internal control (IC) was added in each sample.

HHV-8 in-house real time PCR

HHV-8 in-house PCR was performed as previously reported [5]. Five quantitation standards (QS): QS1, QS2, QS3, QS4 and QS5 corresponding respectively to 10, 100, 1000, 10 000 and 100 000 copies/reaction were used and results expressed in copies/10^6 cells through quantification of human albumin gene [13]. Each run was validating by a negative (water RNAse-free) and two positive (QS2 and QS3) controls, and also by DICO IC amplified in the same well as the HHV-8.

Clonit HHV-8 PCR (Eurobio Ingen®)

This technique is based on the detection of a gene coding a minor capsid protein. The kit enabled 48 reactions and was composed as follows: R1 reagent amplification mix, R2 primers and probes, and R6, R5, R4 and R3 quantitation standard corresponding respectively to 500, 5000, 50 000 and 500 000 copies/reaction. IC corresponding to the human beta-globin gene, was amplified concurrently with HHV-8. The same controls as the in-house PCR were used.

HHV-8 Premix R-gene™ (Biomérieux®)

This test is based on the amplification of a 146 base pairs long of ORF26 and allows carrying out 20 reactions [14]. The quantification was performed with a calibration range (Quanti HHV-8 QS R-gene™) that included 4 QS: QS1, QS2, QS3 and QS4, corresponding

to 50, 500, 5000 and 50 000 copies/reaction respectively. The amplification of HHV-8 and DICO was done in two separate wells for each sample. The same controls as the in-house PCR were used.

Results of the two commercial kits were converted in copies/millions cells through quantification of human albumin gene performed once on each extracted-DNA.

Methods verification

We supported our method comparison using the following graphs: a regression curve presenting results of commercial kit compared to the in-house technique; a diagram presenting difference between commercial and in-house PCR results and plotted against the in-house PCR results; a diagram presenting ratio between commercial and in-house PCR results and plotted against the in-house PCR results. GraphPad software was used to perform Student's t-test (risk at 5%) and Spearman rank-order correlation. Repeatability and reproducibility were performed with 3 samples: each one was tested three times during the same experiment (repeatability) or four times in different experiments (reproducibility).

RESULTS

HHV-8 Clonit® and in-house PCR

Qualitative results obtained by the two techniques were identical; samples 1, 2 and 3 were undetectable whereas samples 4 to 12 were detectable [Figure 1A]. HHV-8 quantitation results between the two methods were significantly correlated (r_s=1, $p<0.001$). However, Student's t-test showed that the slope was not significantly different from 1, but the intercept was from 0, suggesting that Clonit® results were significantly higher compared to the in-

house results [Fig 1B]. This observation was further confirmed by difference and ratio analysis: respectively, Clonit® results were (i) in median [IQR] 1.16 log_{10} copies/10^6 cells [1.12–1.18] greater compared with the in-house PCR results [Fig 1C] and (ii) in median 1.34 [1.24-1.44] times higher than the in-house results [Fig 1D].

Repeatability and reproducibility tests were performed with an undetectable sample ($n^0$2), and two detectable samples ($n^0$5, Ct=32.87 and n^o10, Ct=24.23). For positive samples, both tests succeeded with a variation coefficient lower than the 5% accepted in our laboratory [Supplementary Table 2]. However, repeatability and reproducibility tests failed with sample 2 which was detectable once and twice, respectively. In order to exclude a contamination, we tested the same extracted-DNA and a new extracted-DNA from the same sample with the in-house PCR, and HHV8-DNA remained undetectable.

HHV-8 R-gene^{TM} and in-house PCR

Qualitative results obtained by the two techniques were identical; samples 1, 2 and 3 were undetectable whereas samples 4 to 12 were detectable [Figure 2A]. HHV-8 quantitation results between the two methods were significantly correlated (r_s=0.98, *p<0.001*). Student's t-test performed found that the slope was significantly different from 1 but the intercept was not from 0, suggesting that R-gene^{TM} results were not significantly higher compared to the in-house results [Fig 2B]. Commercial test values were in median 0.46 log_{10} copies/10^6 cells [0.37–0.52] greater compared with the in-house PCR results [Fig 2C], and thus less than 0.50 log_{10} usually accepted. The ratio chart showed that R-gene^{TM} results were in median 1.13 [1.12-1.14] times higher than the reference technique results [Fig 2D].

Repeatability test was performed with the same positive samples (no5 and 10) but with a different undetectable sample (n^o1). For all samples, repeatability tests succeeded with a

variation coefficient lower than 5% [Supplementary Table 2]. R-gene™ reproducibility could not be achieved due to a limited number of PCR-test.

DISCUSSION

In this study, we determined that none of the commercial assays were comparable with the in-house PCR. Indeed, although HHV-8 quantitation was correlated in both cases, there was an overestimation of the values. This could be explained by a better sensitivity, with more efficient amplifications and different molecular targets. However, this overestimation was less than 0.5 $\log_{10}$ with R-gene™, as opposed to Clonit® results which overestimated by more than 1 $\log_{10}$. Clonit® results overestimation may be explained by two parameters: a non-specific amplification as seen with reproducibility and repeatability tests failures of undetectable sample; the QS which ranged from 500 to 500 000 copies/reaction while the in-house PCR QS ranged from 10 to 10 000 copies/reaction. Moreover, Clonit® lower limit of quantification was high (45 000 copies/ml after correction of dilution factor) compared to the VL levels usually found in HHV-8 associated pathologies. Indeed particularly in KS, HHV8-DNA values were reported to be in median 2 $\log_{10}$ copies/10^6 cells [12].

Two limitations of this study are probably the number and the type of samples tested. However, although other specimens as effusion liquid and biopsies could be tested, blood sample remains the main specimen for diagnosis and management of HHV-8 associated diseases. Moreover, although few samples were used, wide range of VL rates including those found in the three main HHV-8 pathologies were tested.

In conclusion, R-gene™ technique seems to be the most suitable commercial kit since it showed consistent qualitative results with in-house HHV-8 PCR [14], a good quantitative

correlation, an overestimation not significantly different and inferior to 0.50 $\log_{10}$ copies/10^6 cells and a good repeatability.

Acknowledgement: This work was supported by Agence Nationale de Recherche sur le SIDA et les Hépatites virale (ANRS) AC43. We also thank Biomérieux® and Eurobio® for providing commercial kits for this study.

Declarations of interest: none

REFERENCES

[1] P.H. Goncalves, J. Ziegelbauer, T.S. Uldrick, R. Yarchoan, Kaposi sarcoma herpesvirus-associated cancers and related diseases, Curr. Opin. HIV AIDS. 12 (2017) 47–56. https://doi.org/10.1097/COH.0000000000000330.

[2] P.H. Gonçalves, T.S. Uldrick, R. Yarchoan, HIV-associated Kaposi sarcoma and related diseases, AIDS Lond. Engl. (2017). https://doi.org/10.1097/QAD.0000000000001567.

[3] D.J. Speicher, N.W. Johnson, Detection of human herpesvirus 8 by quantitative polymerase chain reaction: development and standardisation of methods, BMC Infect. Dis. 12 (2012) 210. https://doi.org/10.1186/1471-2334-12-210.

[4] J. Goudsmit, N. Renwick, N.H. Dukers, R.A. Coutinho, S. Heisterkamp, M. Bakker, T.F. Schulz, M. Cornelissen, G.J. Weverling, Human herpesvirus 8 infections in the Amsterdam Cohort Studies (1984-1997): analysis of seroconversions to ORF65 and ORF73, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 4838–4843.

[5] F. Lallemand, N. Desire, W. Rozenbaum, J.C. Nicolas, V. Marechal, Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay, J. Clin. Microbiol. 38 (2000) 1404–1408.

[6] I.E. White, T.B. Campbell, Quantitation of cell-free and cell-associated Kaposi's sarcoma-associated herpesvirus DNA by real-time PCR, J. Clin. Microbiol. 38 (2000) 1992–1995.

[7] A.S. Laney, M.J. Cannon, H.W. Jaffe, M.K. Offermann, C.-Y. Ou, K.W. Radford, M.M. Patel, T.J. Spira, C.J. Gunthel, P.E. Pellett, S.C. Dollard, Human herpesvirus 8 presence and viral load are associated with the progression of AIDS-associated Kaposi's sarcoma, AIDS Lond. Engl. 21 (2007) 1541–1545. https://doi.org/10.1097/QAD.0b013e3282202b7d.

[8] J. Stebbing, C. Adams, A. Sanitt, S. Mletzko, M. Nelson, B. Gazzard, T. Newsom-Davis, M. Bower, Plasma HHV8 DNA predicts relapse in individuals with HIV-associated multicentric Castleman disease, Blood. 118 (2011) 271–275. https://doi.org/10.1182/blood-2011-02-335620.

[9] F. Broccolo, C. Tassan Din, M.G. Viganò, T. Rutigliano, S. Esposito, P. Lusso, G. Tambussi, M.S. Malnati, HHV-8 DNA replication correlates with the clinical status in AIDS-related Kaposi's sarcoma, J. Clin. Virol. Off. Publ. Pan Am. Soc. Clin. Virol. 78 (2016) 47–52. https://doi.org/10.1016/j.jcv.2016.02.019.

[10] R. Tedeschi, M. Enbom, E. Bidoli, A. Linde, P. De Paoli, J. Dillner, Viral load of human herpesvirus 8 in peripheral blood of human immunodeficiency virus-infected patients with Kaposi's sarcoma, J. Clin. Microbiol. 39 (2001) 4269–4273. https://doi.org/10.1128/JCM.39.12.4269-4273.2001.

[11] A.-G. Marcelin, L. Aaron, C. Mateus, E. Gyan, I. Gorin, J.-P. Viard, V. Calvez, N. Dupin, Rituximab therapy for HIV-associated Castleman disease, Blood. 102 (2003) 2786–2788. https://doi.org/10.1182/blood-2003-03-0951.

[12] A. Jary, V. Leducq, R. Palich, A. Gothland, D. Descamps, V. Joly, S. Lambert-Niclot, C. Amiel, A. Canestri, A. Mirand, E. Gault, A. Vabret, M.-A. Valantin, C. Katlama, V. Calvez, N. Dupin, J.-P. Spano, A.-G. Marcelin, Usefulness of Kaposi's Sarcoma-Associated Herpesvirus (KSHV) DNA Viral Load in Whole Blood for Diagnosis and Monitoring of KSHV-Associated Diseases, J. Clin. Microbiol. 56 (2018). https://doi.org/10.1128/JCM.00569-18.

[13] I. Bièche, M. Olivi, M.H. Champème, D. Vidaud, R. Lidereau, M. Vidaud, Novel approach to quantitative polymerase chain reaction using real-time detection: application to the detection of gene amplification in breast cancer, Int. J. Cancer. 78 (1998) 661–666.

[14] C. Deback, F. Agbalika, C. Scieux, A.G. Marcelin, A. Gautheret-Dejean, J. Cherot, L. Hermet, O. Roger, H. Agut, Detection of human herpesviruses HHV-6, HHV-7 and HHV-8 in whole blood by real-time PCR using the new CMV, HHV-6, 7, 8 R-gene kit, J. Virol. Methods. 149 (2008) 285–291. https://doi.org/10.1016/j.jviromet.2008.01.026.

Figure 1: Results of methods comparison between HHV-8 Clonit® kit (Eurobio Ingen®) and HHV-8 in-house PCR. *HHV-8 DNA viral load levels obtained by the two techniques are listed in figure 1A. Correlation curve (y=0.9586x+1.3375) between results obtained by the two techniques is represented in figure 1B and theirs differences and ratio respectively in figure 1C and 1D.*

Figure 2: Results of methods comparison between HHV-8 R-gene™ kit (Biomérieux®) and HHV-8 in-house PCR. *HHV-8 DNA viral load levels obtained by the two techniques are listed in figure 2A. Correlation curve (y=1.0738+0.1928) between results obtained by the two techniques is represented in figure 2B and theirs differences and ratio respectively in figure 2C and 2D.*

Supplementary Table 1: **Characteristics of the samples selected for methods comparison**

Patient	Samples	HHV8-DNA viral load (log_{10} copies/10^6 cells)	HHV-8 serology	HIV status	Clinical context
1	Whole blood	<10	Negative	Negative	Liver transplantation No signs suggestive of HHV8-associated diseases
2	Whole blood	<10	Negative	Positive	HIV-infected patient with inflammatory syndrome No signs suggestive of HHV8-associated diseases
3	Whole blood	<10	Positive	Negative	Liver transplantation No signs suggestive of HHV8-associated diseases
4	Whole blood	1.56	NA	NA	Unknown
5	Whole blood	1.98	NA	Positive	Macrophage activation syndrome No signs suggestive of HHV8-associated diseases
6	Whole blood	2.48	Positive	Positive	Cutaneous, ganglionic and splenic KS in patient under effective ARVs.
7	Whole blood	3.20	NA	Positive	Multicentric Castleman disease
8	Whole blood	3.27	NA	NA	Unknown
9	Whole blood	3.87	NA	NA	Unknown
10	Whole blood	4.68	NA	Positive	Cutaneous KS and multicentric Castleman disease
11	Whole blood	4.97	Negative	Positive	Multicentric Castleman disease
12	Whole blood	5.79	NA	Positive	Multicentric Castleman disease

ARV: antiretroviral; KS: Kaposi's sarcoma; HHV-8: human herpesvirus 8; HIV: Human Immunodeficiency virus; NA: not available

Supplementary Table 2: Repeatability and reproducibility results of HHV-8 Clonit® and HHV-8 R-Gene™ PCR assay

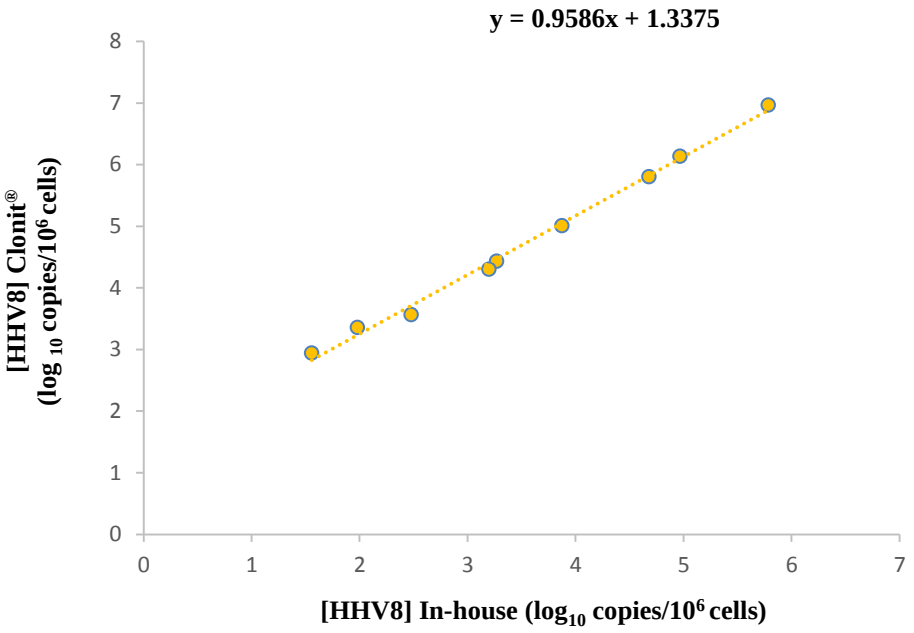
HHV-8 Clonit®				
	SAMPLES	**2**	**5**	**10**
Repeatability	Ct 1	Undetectable	32.87	24.23
	Ct 2	Undetectable	33	24.29
	Ct 3	Undetectable	32.78	24.19
	Ct 4	36.94	33.17	24.17
	Mean	NA	32.95	24.22
	Standard deviation	NA	0.17	0.053
	Variation coefficient (%)	NA	0.51	0.22
Reproducibility	Ct 1	Undetectable	33.54	24.51
	Ct 2	36.94	33.17	24.36
	Ct 3	37.03	33.42	24.63
	Ct 4	NR	32.88	24.25
	Mean	NA	33.25	24.43
	Standard deviation	NA	0.29	0.17
	Variation coefficient (%)	NA	0.88	0.68
HHV-8 R-Gene™				
	SAMPLES	**1**	**5**	**10**
Repeatability	Ct 1	Undetectable	33.39	23.92
	Ct 2	Undetectable	33.14	23.78
	Ct 3	Undetectable	33.03	24.03
	Mean	NA	33.18	23.91
	Standard deviation	NA	0.18	0.13
	Variation coefficient (%)	NA	0.56	0.52

NA: not applicable; NR: not realized

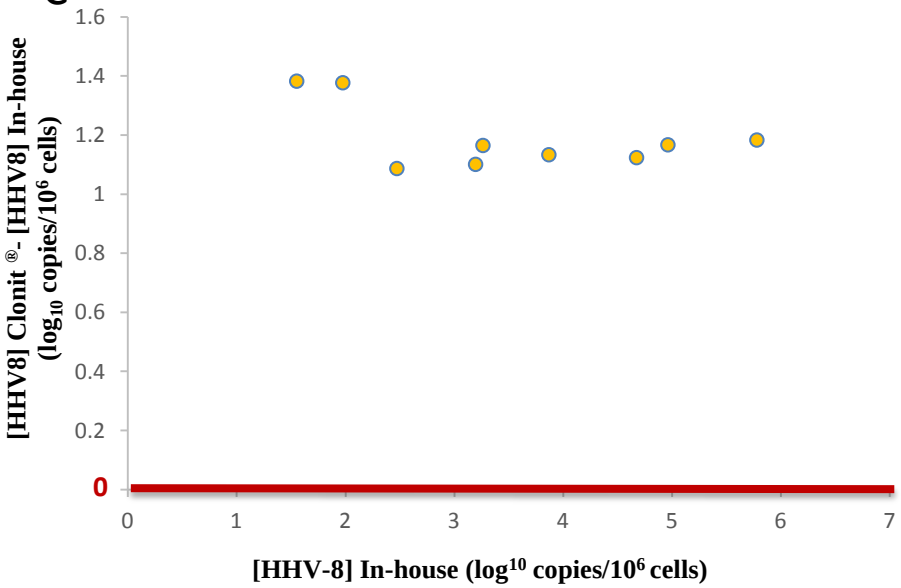
A

Patients	[HHV8] In-house (log_{10} copies/10^6 cells)	[HHV8] Clonit® (log_{10} copies/10^6 cells)
1	Undetectable	Undetectable
2	Undetectable	Undetectable
3	Undetectable	Undetectable
4	1.56	2.94
5	1.98	3.36
6	2.48	3.56
7	3.20	4.30
8	3.27	4.43
9	3.87	5.01
10	4.68	5.80
11	4.97	6.13
12	5.79	6.97

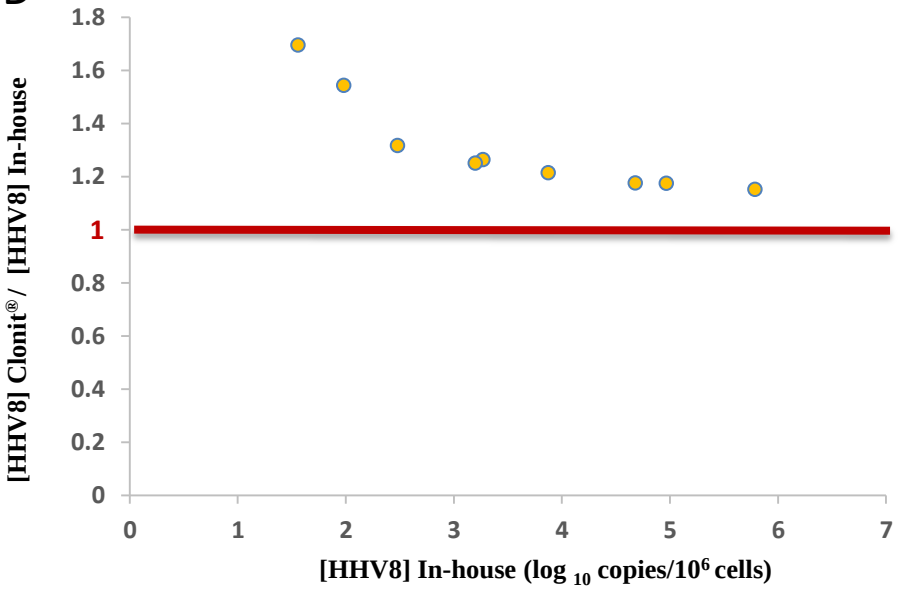
B



C



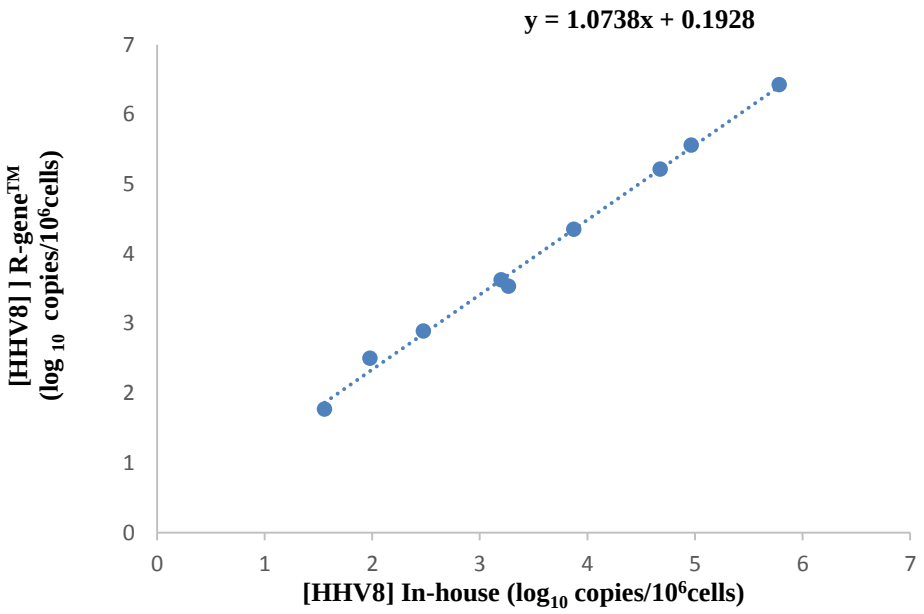
D



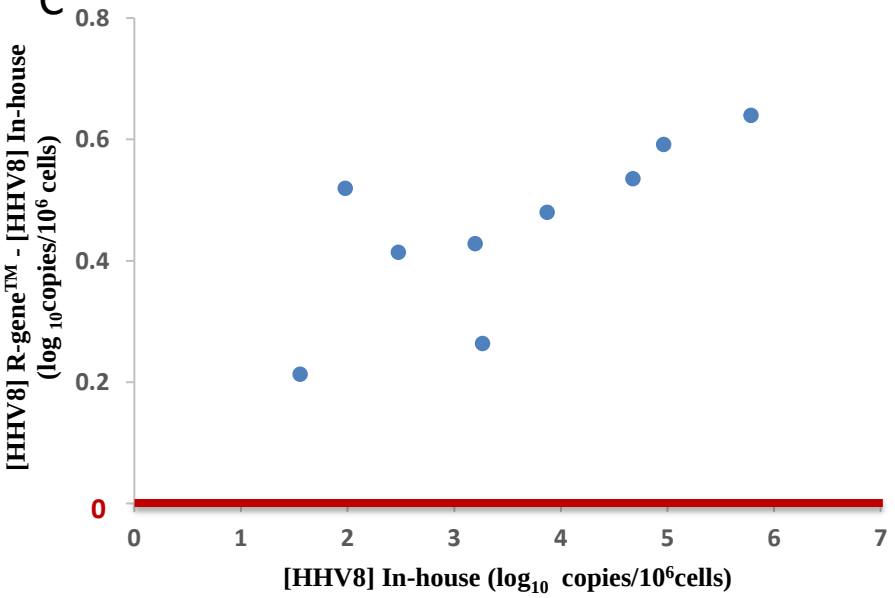
A

Patients	[HHV8] In-house (log_{10} copies/10^6 cells)	[HHV8] R-gene™ (log_{10} copies/10^6 cells)
1	Undetectable	Undetectable
2	Undetectable	Undetectable
3	Undetectable	Undetectable
4	1.56	1.77
5	1.98	2.50
6	2.48	2.89
7	3.20	3.63
8	3.27	3.53
9	3.87	4.35
10	4.68	5.21
11	4.97	5.56
12	5.79	6.42

B



C



D