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## Methods comparison for molecular diagnosis of human herpesvirus 8 infections

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

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26 **Highlights**

- 27 - HHV-8 DNA viral load in blood is an accurate marker for diagnosis and management  
28 of HHV-8 associated diseases.
- 29 - None of the commercial assays for HHV-8 diagnosis by real-time PCR were  
30 comparable with the in-house PCR.
- 31 - The HHV-8 R-gene<sup>TM</sup> assay overestimated by in median 0.46 log<sub>10</sub> copies/10<sup>6</sup> cells  
32 the results obtained by the in-house PCR
- 33 - The HHV-8 Clonit® assay overestimated by in median 1.16 log<sub>10</sub> copies/10<sup>6</sup> cells the  
34 results obtained by the in-house PCR

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52 **ABSTRACT**

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

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

58 Study design. Twelve samples (3 undetectable and 9 detectable with viral load ranging from  
59  $10^1$  to  $10^5$  per reaction) were tested for HHV-8 detection and quantification with the 3  
60 methods. Methods comparison was supported with regression curve and diagram presenting  
61 difference or ratio between commercial and in-house PCR results and plotted against the in-  
62 house PCR results. Statistical analyses, specifically Student tests and Spearman correlation,  
63 were performed.

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

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

76

77 **BACKGROUND**

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

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

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93 **OJECTIVES**

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

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100 **STUDY DESIGN**

101 *Samples*

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

106

#### 107 ***HHV-8 in-house real time PCR***

108 HHV-8 in-house PCR was performed as previously reported [5]. Five quantitation  
109 standards (QS): QS1, QS2, QS3, QS4 and QS5 corresponding respectively to 10, 100, 1000,  
110 10 000 and 100 000 copies/reaction were used and results expressed in copies/ $10^6$  cells  
111 through quantification of human albumin gene [13]. Each run was validating by a negative  
112 (water RNase-free) and two positive (QS2 and QS3) controls, and also by DICO IC amplified  
113 in the same well as the HHV-8.

114

#### 115 ***Clonit HHV-8 PCR (Eurobio Ingen®)***

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

122

#### 123 ***HHV-8 Premix R-gene<sup>TM</sup> (Biomérieux®)***

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

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

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

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#### 134 ***Methods verification***

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

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## 145 **RESULTS**

### 146 ***HHV-8 Clonit® and in-house PCR***

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

152 house results [Fig 1B]. This observation was further confirmed by difference and ratio  
153 analysis: respectively, Clonit® results were (i) in median [IQR] 1.16 log<sub>10</sub> copies/10<sup>6</sup> cells  
154 [1.12–1.18] greater compared with the in-house PCR results [Fig 1C] and (ii) in median 1.34  
155 [1.24-1.44] times higher than the in-house results [Fig 1D].

156 Repeatability and reproducibility tests were performed with an undetectable sample (n<sup>0</sup>2), and  
157 two detectable samples (n<sup>0</sup>5, Ct=32.87 and n<sup>0</sup>10, Ct=24.23). For positive samples, both tests  
158 succeeded with a variation coefficient lower than the 5% accepted in our laboratory  
159 [Supplementary Table 2]. However, repeatability and reproducibility tests failed with sample  
160 2 which was detectable once and twice, respectively. In order to exclude a contamination, we  
161 tested the same extracted-DNA and a new extracted-DNA from the same sample with the in-  
162 house PCR, and HHV8-DNA remained undetectable.

163

#### 164 ***HHV-8 R-gene<sup>TM</sup> and in-house PCR***

165 Qualitative results obtained by the two techniques were identical; samples 1, 2 and 3 were  
166 undetectable whereas samples 4 to 12 were detectable [Figure 2A]. HHV-8 quantitation  
167 results between the two methods were significantly correlated ( $r_s=0.98$ ,  $p<0.001$ ). Student's t-  
168 test performed found that the slope was significantly different from 1 but the intercept was not  
169 from 0, suggesting that R-gene<sup>TM</sup> results were not significantly higher compared to the in-  
170 house results [Fig 2B]. Commercial test values were in median 0.46 log<sub>10</sub> copies/10<sup>6</sup> cells  
171 [0.37–0.52] greater compared with the in-house PCR results [Fig 2C], and thus less than 0.50  
172 log<sub>10</sub> usually accepted. The ratio chart showed that R-gene<sup>TM</sup> results were in median 1.13  
173 [1.12-1.14] times higher than the reference technique results [Fig 2D].

174 Repeatability test was performed with the same positive samples (no5 and 10) but with a  
175 different undetectable sample (n<sup>0</sup>1). For all samples, repeatability tests succeeded with a



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

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## 180 **DISCUSSION**

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

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

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

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

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227 providing commercial kits for this study.

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229 **Declarations of interest:** none

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

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351 **Figure 2: Results of methods comparison between HHV-8 R-gene™ kit (Biomérieux®)**  
352 **and HHV-8 in-house PCR. HHV-8 DNA viral load levels obtained by the two techniques are**  
353 *listed in figure 2A. Correlation curve ( $y=1.0738+0.1928$ ) between results obtained by the two*  
354 *techniques is represented in figure 2B and theirs differences and ratio respectively in figure*  
355 *2C and 2D.*

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

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



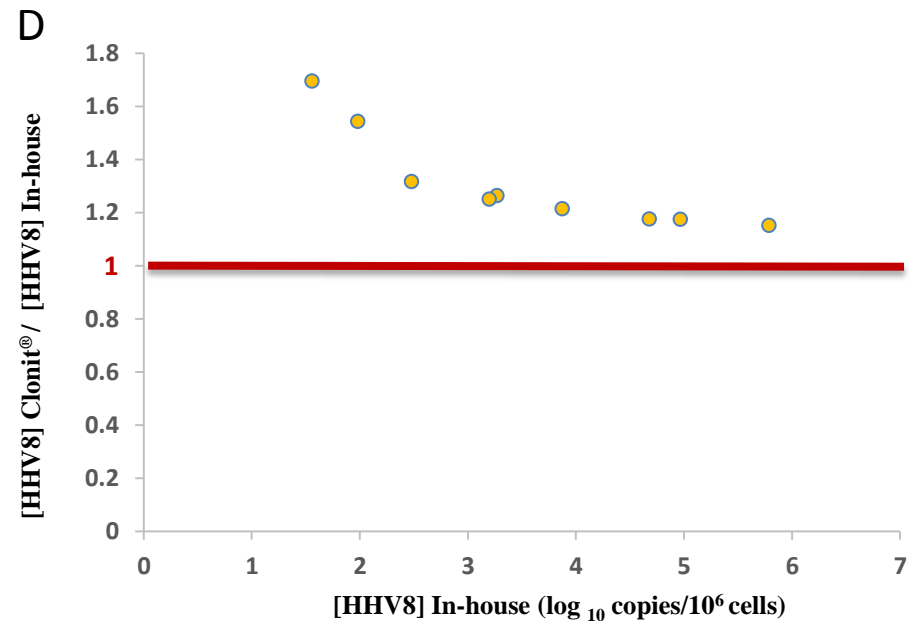
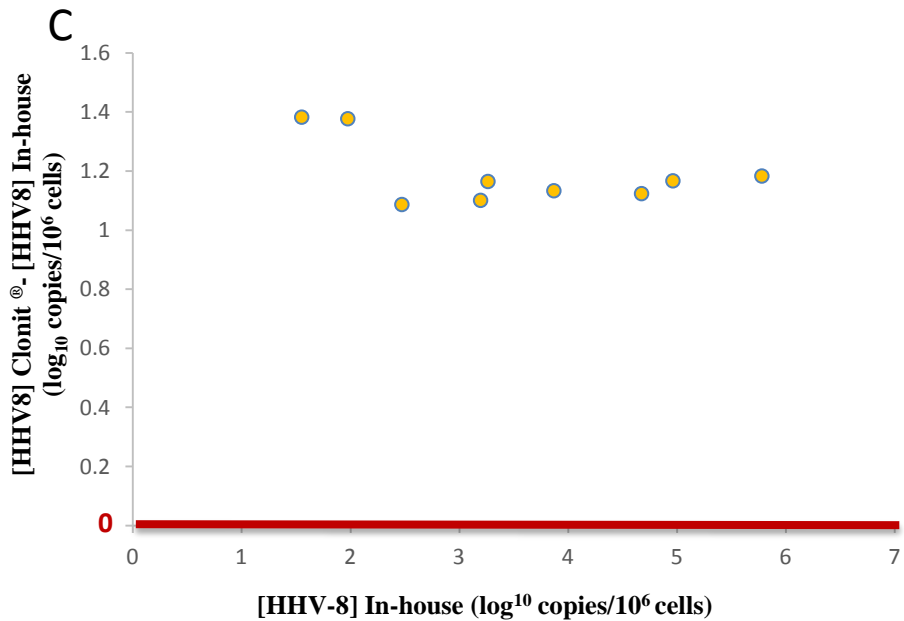
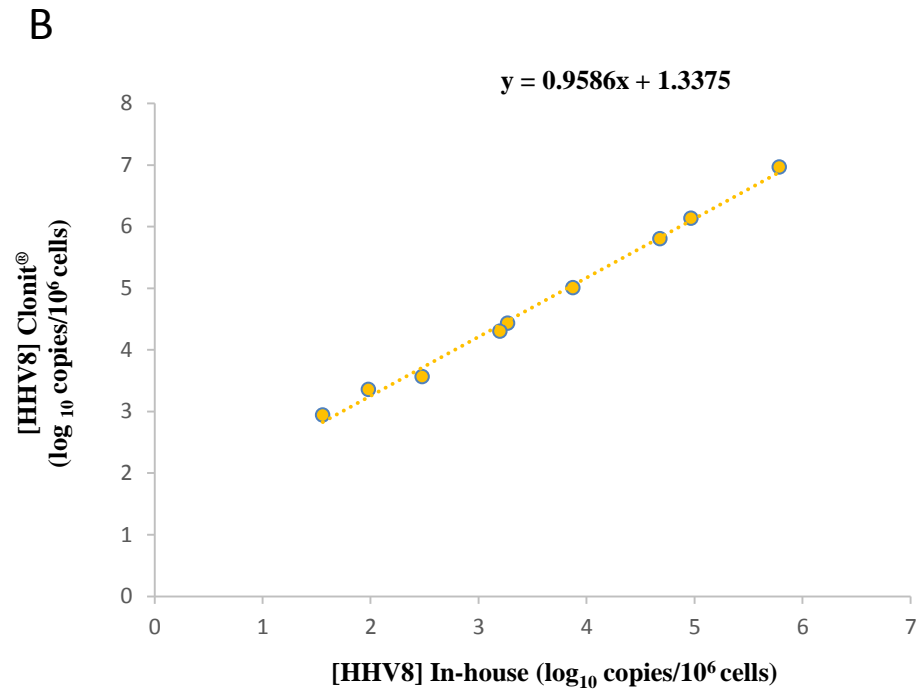
**Supplementary Table 2: Repeatability and reproducibility results of HHV-8 Clonit® and HHV-8 R-Gene™ PCR assay**

<b>HHV-8 Clonit®</b>				
	<b>SAMPLES</b>	<b>2</b>	<b>5</b>	<b>10</b>
<b>Repeatability</b>	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
<b>Reproducibility</b>	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
<b>HHV-8 R-Gene™</b>				
	<b>SAMPLES</b>	<b>1</b>	<b>5</b>	<b>10</b>
<b>Repeatability</b>	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 <sub>10</sub> copies/10 <sup>6</sup> cells)	[HHV8] Clonit® (log <sub>10</sub> copies/10 <sup>6</sup> 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



**A**

Patients	[HHV8] In-house (log <sub>10</sub> copies/10 <sup>6</sup> cells)	[HHV8] R-gene™ (log <sub>10</sub> copies/10 <sup>6</sup> 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

