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Title page

**Evaluation of four commercial extraction-
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load in whole blood.**

Adeline Baron, Albane Gicquel, Jean-Christophe Plantier, Marie Gueudin

Corresponding author: Jean-Christophe Plantier (jean-christophe.plantier@chu-rouen.fr)

Abstract : 246 words

Article : 2500 words

1 **1. Background**

2 Quantitative nucleic acid testing (QNAT) has become indispensable to measure viral load (VL) in the
3 management of cytomegalovirus (CMV) infection. Indications include CMV infection during
4 pregnancy and in newborns [1], viral surveillance of all immunocompromised patients [2] and follow-
5 up of treatment efficacy. The use of Epstein-Barr Virus (EBV) VLs is more recent and allows the
6 diagnosis, monitoring, and prevention of post-transplant lymphoproliferative disorders (PTLD) [3].

7 To respond to the need for QNAT, commercial assays have been developed on automated systems.

8 The first reagents for CMV and EBV VLs in whole blood were Abbott RealTime kits on the m2000
9 SP/RT system (Abbott Molecular Inc, Des plaines, USA) (M2000) [4, 5] and Artus QS-RGQ kits on the
10 QIASymphony RGQ system (Qiagen S.A.S., France) (QS/RGQ) [6]. Recently two new systems have
11 been commercialized: i) the eMAG/eSTREAM system (Biomerieux, Marcy-l'Etoile, France) (EMAG)
12 coupled with R-gene kits whose performance seems equivalent to the previous Nuclisens Easy Mag
13 system [7] and ii) the Versant kPCR system (Siemens Healthcare Diagnostics, Saint Denis, France)
14 with kPCR PLX kits (KPCR) [8]. A comparison of these systems is necessary to evaluate their
15 performances.

17 **2. Objectives**

18 The objective of this single center study was to compare the results obtained with our routine system
19 QS/RGQ and three other systems: M2000, EMAG and KPCR, to monitor CMV and EBV VL in whole
20 blood. We assessed analytical performances using World Health Organization International
21 Standards (WHO-IS) and clinical performances on samples with qualitative and quantitative analyses.

23 **3. Study design**

24 **3.1. Analytical assessment**

25 3.1.1. Specificity and Inter-sample contamination

26 Inter-sample contamination was assessed by testing 10 negative clinical samples in runs with positive
27 samples for each extraction system for both CMV and EBV.

28 3.1.2. Intra-assay reproducibility and accuracy

29 Intra-assay reproducibility was tested with an in-house Internal Quality Control (IQC) (corresponding
30 to a sample from a positive patient). For CMV, IQC was tested 9 times in the same run and for EBV 8
31 times.

32 Accuracy was tested with WHO-IS (code: 09/162 for CMV and 09/260 for EBV; NIBSC, Hertfordshire,
33 Great Britain). Four dilutions (range 500 – 5×10^5 IU/ml, 2.7 – 5.7 Log IU/ml) of each WHO-IS were
34 quantified in triplicate in the same run.

35

36 3.2. Clinical performances

37 Clinical performances were evaluated using blood samples collected on EDTA from hospitalized
38 patients. Whole blood samples were tested with the QS/RGQ system (our routine system to monitor
39 CMV or EBV VL) and frozen at -70°C . For analysis on the M2000, EMAG and KPCR systems, 56
40 samples (conserved less than 6 months) were selected from 24 patients for CMV and 45 samples
41 from 27 patients for EBV. Among these samples, we monitored the VL of 4 patients for CMV and of 3
42 patients for EBV.

43

44 3.3. Methods

45 All 4 assays were marked EC-IVD (European Conformity-In Vitro Diagnostic device) for whole blood
46 except EBV with KPCR. In each sample, an internal control was included allowing the detection of
47 potential inhibition. For QS/RGQ, M2000, and KPCR, the extraction automatons distributed the mix

48 and the eluate in the Polymerase Chain Reaction (PCR) plate. For EMAG this step was performed by
49 another automaton, the eSTREAM system. Results were calculated in IU/mL and Log₁₀ IU/mL.

50

51 3.3.1 QIASymphony RGQ system (Qiagen)

52 Whole blood samples (200µl) were dispensed onto the QIASymphony SP/AS automaton. This system
53 performs all steps of the purification procedure and preparation of 60µL of eluate with the extraction
54 kit QIASymphony DNA mini and the mix for DNA amplification (*artus* CMV QS-RGQ and *artus* EBV QS-
55 RGQ). Then, the thermocycler Rotorgene enabled amplification of 72 samples. The linearity was
56 between 2.9 and 7.6 Log IU/ml for CMV and between 2.2 and 6.8 Log IU/ml for EBV. The lower limit
57 of detection (LOD) was 123 IU/mL (2.1 Log IU/ml) for CMV and 40 IU/mL (1.6 Log IU/ml) for EBV
58 (Table 1).

59

60 3.3.2 m2000SP/RT system (Abbott)

61 Quantification was carried out on the m2000 system which includes the m2000SP instrument for
62 automated extraction of DNA from 500µl of sample using the *mSample* preparation system DNA kit
63 (eluate volume: 60µl). Amplification was performed on the thermocycler m2000RT with Realtime
64 CMV and EBV kits. The lower limit of quantification (LLQ) was 62.4 IU/mL (1.8 Log IU/ml) for CMV and
65 150 IU/mL (2.2 Log IU/ml) for EBV. The linearity extended from the LLQ to 8.2 Log IU/ml for CMV and
66 8.3 Log IU/ml for EBV.

67

68 3.3.3 eMAG/eSTREAM system (Biomerieux)

69 200µL of sample were used. After extraction executed with a DNA extraction kit onto the eMAG
70 system, 50µl of eluate were obtained. Distribution was performed by the eSTREAM automaton using
71 CMV R-gene and EBV R-gene quantification kits. Amplification was performed on an Abi Prism 7500
72 fast (Applied Biosystems, Foster City, USA). LOD was 535 IU/mL (2.7 Log IU/ml) for CMV and 328

73 IU/mL (2.5 Log IU/ml) for EBV. The linearity extended from the LLQ 2.8 to 9.4 Log IU/mL for CMV and
74 to 3.0 to 6.7 Log IU/ml for EBV.

75

76 3.3.4 Versant kPCR system (Siemens)

77 Extraction was performed with Versant Sample Preparation 1.2 Reagents kit using 400µL of sample.
78 Quantification was carried out on the Versant kPCR Molecular System which includes the SP module
79 for extraction and the AD module for real-time PCR. DNA amplification was performed with the CMV
80 and EBV kPCR PLX DNA Assays (Altona Diagnostics, Hamburg, Germany). LOD was 835 IU/mL (2.9 Log
81 IU/ml) for CMV in whole blood and 216.6 UI/mL (2.3 Log IU/ml) for EBV in plasma (not available
82 because not validated in whole blood). The linearity extended from LOD to 6 Log IU/ml for CMV in
83 whole blood and from 2.7 to 6 Log IU/ml for EBV in plasma.

84

85 3.4. Statistical analysis

86 Bland and Altman plots were used to represent the degree of agreement between the Log10-
87 transformed VLs obtained with the reference system and the three other systems using MedCalc
88 Statistical Software version 17.6 (MedCalc Software, Ostend, Belgium).

89

90 4. Results

91 4.1. Analytical performances

92 4.1.1. Specificity and inter-sample contamination

93 The 10 negative samples tested on each system for CMV and EBV were all undetected and no inter-
94 sample contamination was observed with the 4 systems.

95

96 4.1.2. Intra-assay reproducibility and accuracy

97 Standard deviations (SD) were: i) for CMV, QS/RGQ=0.04; M2000=0.07; EMAG=0.12 and KPCR=0.06
98 with a coefficient of variation from 1.09 to 2.65% and ii) for EBV, QS/RGQ=0.14; M2000=0.15;

99 EMAG=0.29 and KPCR=0.20 with a coefficient of variation from 4.13 to 8.50% (Table 2). SD were
100 below 0.25 Log IU/ml for all the assays except for EBV EMAG (SD=0.29).

101

102 **4.1.3. Performances on WHO international standard**

103 In contrast with the other assays, the KPCR system detected only 2 of the 3 replicates for CMV and 1
104 of the 3 for EBV for the lowest dilution (Table 3). Two points were answered “inhibited” by EMAG.
105 Due to a higher dispersion of the values mainly for KPCR and EMAG, this dilution was excluded from
106 the calculation of the mean. For an expected value of 4.70 Log IU/ml the mean values of WHO-IS
107 CMV were QS/RGQ=4.84, M2000=4.61, EMAG=4.33, KPCR=4.79 and of WHO-IS EBV were
108 QS/RGQ=4.70, M2000=4.61, EMAG=4.62, KPCR=4.57.

109

110 **4.2. Performances on clinical samples**

111 **4.2.1. A patient with discrepant results**

112 Ten of the 56 samples of whole blood tested with CMV assays were from Patient 1. This patient’s
113 samples were repetitively underquantified with QS/RGQ with a mean of 1.62 Log IU/ml for these 10
114 measures, whereas M2000=2.77, EMAG=2.59 and KPCR=3.33. These samples were excluded from
115 further analyses.

116

117 **4.2.2. Qualitative analysis**

118 Comparisons were performed using 46 CMV samples and 45 EBV samples (Table 4). For CMV,
119 quantifiable results, above the threshold announced by the manufacturers, were found for QS/RGQ
120 n=41, M2000 n=43, EMAG n=33 and KPCR n=24. Samples were undetectable for QS/RGQ n=1 (238
121 IU/ml with M2000, 404 IU/ml with EMAG), M2000 n=3 (24, 27 and 2412 IU/ml with QS/RGQ,
122 negative or invalid with the others), EMAG n=5 (VL maxi=505 IU/ml with QS/RGQ), and KPCR n=14

123 (VL maxi=2130 IU/ml with QS/RGQ). For EBV, quantifiable results were found for QS/RGQ n=43,
124 M2000 n=39, EMAG n=40 and KPCR n=32. Samples were undetectable for M2000 n=2 (77 and 759
125 IU/ml with QS/RGQ), EMAG n=4 (VL maxi=759 IU/ml), and KPCR n=13 (VL maxi=9438 IU/ml). Two
126 samples of 77 and 759 IU/ml were undetectable in all assays except QS/RGQ.

127

128 **4.2.3. Quantitative analysis**

129 The samples quantifiable by 2 assays were plotted for Bland and Altman representation (Figure 1).
130 One sample with very discordant results was excluded: CMV VL of 5.15 Log IU/ml with KPCR and
131 2.97, 3.19 and 3.89 Log IU/ml with QS/RGQ, M2000 and EMAG respectively. Between QS/RGQ and
132 M2000 the differences in mean were -0.06 Log IU/ml with SD=0.58 for CMV and 0.16 Log IU/ml with
133 SD=0.57 for EBV (Fig 1A, 1D). Three deviations greater than 0.5 Log IU/ml were observed for CMV
134 and 5 for EBV. Between QS/RGQ and EMAG the differences in mean were -0.27 Log IU/ml with
135 SD=0.57 for CMV and -0.27 Log IU/ml with SD=0.67 for EBV (Fig 1B, 1E). Seven deviations greater
136 than 0.5 Log IU/ml were observed for CMV and 10 for EBV. Between QS/RGQ and KPCR the
137 differences in mean were 0.01 Log IU/ml with SD=0.83 for CMV and 0.52 Log IU/ml with SD=1.45 for
138 EBV (Fig 1C, 1F). Six deviations greater than 0.5 Log IU/ml were observed for CMV and 22 for EBV.

139

140 **4.2.4. Comparison of the four assays for patient monitoring**

141 The clinical performances of the 4 assays were evaluated following the kinetics of VL in 4 patients for
142 CMV and 3 for EBV (Figure 2). The CMV VLs of patient 1 were always underquantified with QS/RGQ.
143 Patient 1 also replicated EBV but with QS/RGQ VLs similar to the other systems. In patients 2 and 4,
144 the first CMV VL was not detected with KPCR. Samples of patient 5 became undetectable for EBV
145 after 2 VLs with KPCR, after 3 VLs with EMAG whereas they stayed detected with M2000 and

146 QS/RGQ. Patient 6 was underquantified by KPCR with 3 positive EBV VLs with a mean of 2.4, whereas
147 the mean of the same VLs was 3.7 Log IU/ml with QS/RGQ.

148

149 **5. Discussion**

150 Technical features that matter

151 To evaluate automated systems it is important to take into account their simplicity, flexibility and
152 ergonomics. In the QS/RGQ system, Qiasymphony SP/AS automaton works in series from 1 to 24
153 samples but uses two different kits to extract DNA from whole blood and acellular samples. The
154 Rotorgene thermocycler uses small strips or tubes which require some dexterity to close and handle.
155 The Abbott system m2000 SP/RT is a robust and well-known system with a capacity of 96 tests/runs
156 also used in many laboratories to quantify HIV, HBV and HCV. M2000 can be used to extract DNA
157 from a variety of samples therefore without validation of Abbott. The main complaint of M2000 is
158 the duration of the extraction step which is the longest of the four systems (more than 3 hours). The
159 Biomerieux eMAG extractor has been validated for most types of samples using only one extraction
160 kit (serum, CSFs, BALs, urine, biopsies and amniotic fluids); it allows extraction of 1 to 48 samples in
161 consumables of eight wells but does not distribute, mix and extract in the PCR tubes (step ensured by
162 eSTREAM). The Siemens system, Versant kPCR, is probably the easiest and fastest system to use
163 despite a dilution step for all the blood samples before extraction. Particular attention should be paid
164 to the preparation of reagents to avoid mistakes with this system.

165 Analytical performances

166 No problem of specificity or contamination was observed with the four systems. The use of a dilution
167 of 500 IU/ml of WHO-IS allows evaluation of the announced sensitivity of the assays. KPCR assays are
168 less sensitive than the others particularly for EBV with two replicates out of three not detected. The
169 concordance of the results with the expected values of WHO-IS was very good for the four EBV
170 assays (maximum discrepancy=0.13 Log IU/ml) and for three of the four CMV assays with a slightly

171 low mean for EMAG (discrepancy of 0.37 Log IU/ml). These results confirm those obtained in other
172 studies [9, 10] and are very good news because in some pathologies there is a major difficulty in
173 determining a specific threshold value. For example, the ECIL-6 (Sixth European Conference on
174 Infections in Leukemia) cannot recommend a threshold of EBV DNAemia to give preemptive therapy
175 in patients with a high risk for EBV-PTLD because of the large variety of threshold values used by the
176 authors [11]. However, in order to establish these thresholds, it will be necessary to use data
177 obtained on the same matrix (plasma or whole blood) because differences between these two
178 matrices have been observed [12].
179 These results comparing four systems pave the way for future studies comparing results between
180 different laboratories and allowing the determination of universal thresholds for each type of
181 pathology to better manage CMV or EBV infections.

182

183 Clinical performance

184 CMV and EBV are known to have lower genetic variability than others viruses like HIV. The possibility
185 of underquantification because of mismatch of the primers is rare but has already been described for
186 CMV [13]. We found one patient repetitively underestimated by QS/RGQ CMV system. The major
187 clinical risk is not detecting a low CMV VL under antiviral treatment and stopping the treatment too
188 early. Indeed, many patients have recurrent CMV viremia and/or CMV disease following initial
189 therapy [2] and the consensus guidelines on the management of CMV in solid-organ transplantation
190 recommend two consecutive undetectable VLs to ensure viral clearance before stopping treatment
191 [14]. One option for dealing with this problem is the use of dual target PCRs but only one assay (the
192 M2000) amplifies two targets.

193 On clinical samples, the results were well correlated with the sensitivity announced by the
194 manufacturers of the assays with the greatest number of CMV samples quantifiable with M2000
195 (Threshold: 62.4 UI/ml) and the lowest obtained with KPCR (Threshold: 835 UI/ml). It should be
196 noted that this elevated threshold for KPCR can be problematic for the follow up of high risk patients

197 receiving T-cell depleted or umbilical cord blood grafts, indeed in these cases it is common to treat
198 when CMV viremia reaches 100 copies/mL to 1000 copies/mL [15]. For EBV the best sensitivity was
199 with the QS/RGQ system.

200 A good correlation between the QS/RGQ and M2000 systems was observed with a small number of
201 samples with more than 0.5 Log IU/ml of difference (7.3% of CMV and 12.8% of EBV). This
202 percentage was higher between QS/RGQ and EMAG and reached 71% between QS/RGQ and KPCR
203 for EBV.

204 Results of sequential samples confirm our previous results of sensitivity with no detection of the
205 majority of samples with KPCR. The sensitivity issues of the KPCR system are probably explained by
206 less efficient extraction in whole blood than on the other matrices. Indeed, during an evaluation
207 integrating different types of matrices Engelmann et al [8] found lower performance in whole blood
208 for CMV VL confirmed by our findings.

209 To conclude, each laboratory should select a system based on the importance given to the sensitivity
210 and the types of usable matrices. The four systems assessed here provide concordant results
211 compatible with routine monitoring of CMV DNA in whole blood. For EBV if we exclude KPCR which
212 was not validated in this matrix, the three other techniques were also concordant.

213

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217

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220

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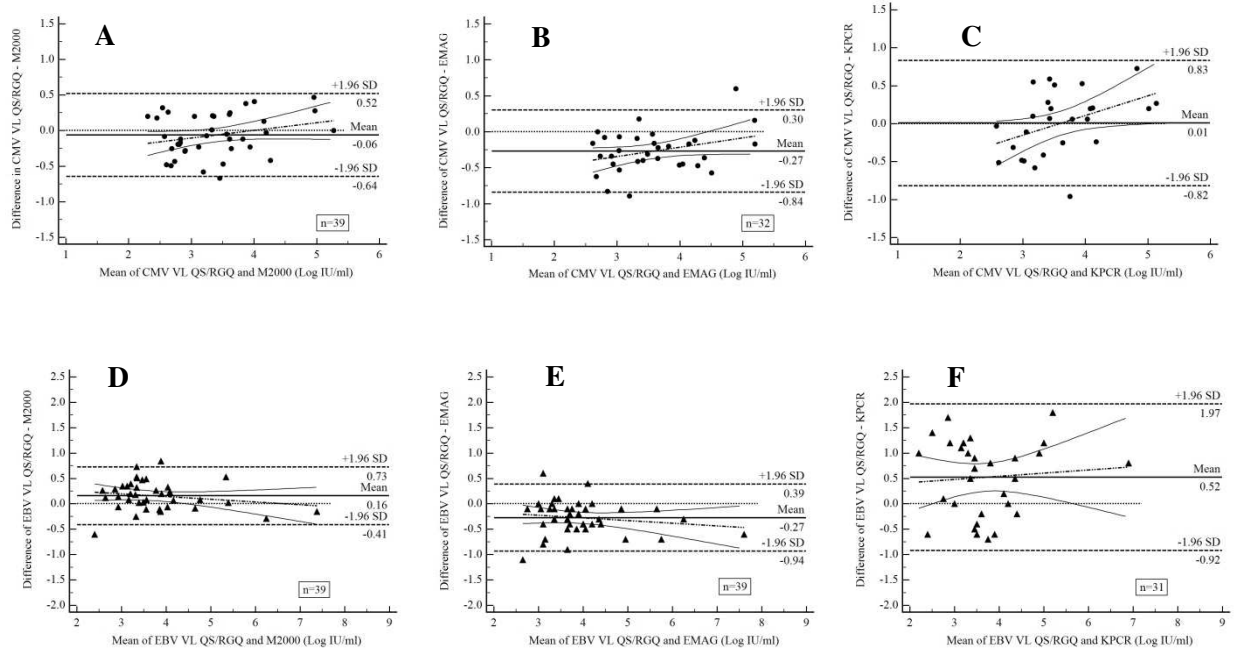


Fig 1: Degree of agreement in Log IU/ml between the different CMV VL (●) and EBV VL (▲) assays. (A) and (D) between QS/RGQ and M2000, (B) and (E) between QS/RGQ and EMAG, (C) and (F) between QS/RGQ and KPCR. For Bland and Altman curves, the mean values for each sample quantifiable by the 2 techniques are plotted on the x axis. The differences between the values obtained by the 2 techniques are plotted on the y axis. The solid lines show the differences in mean between the values, and the dotted lines show the differences in mean plus or minus 1.96 SD (95% limits of agreement).

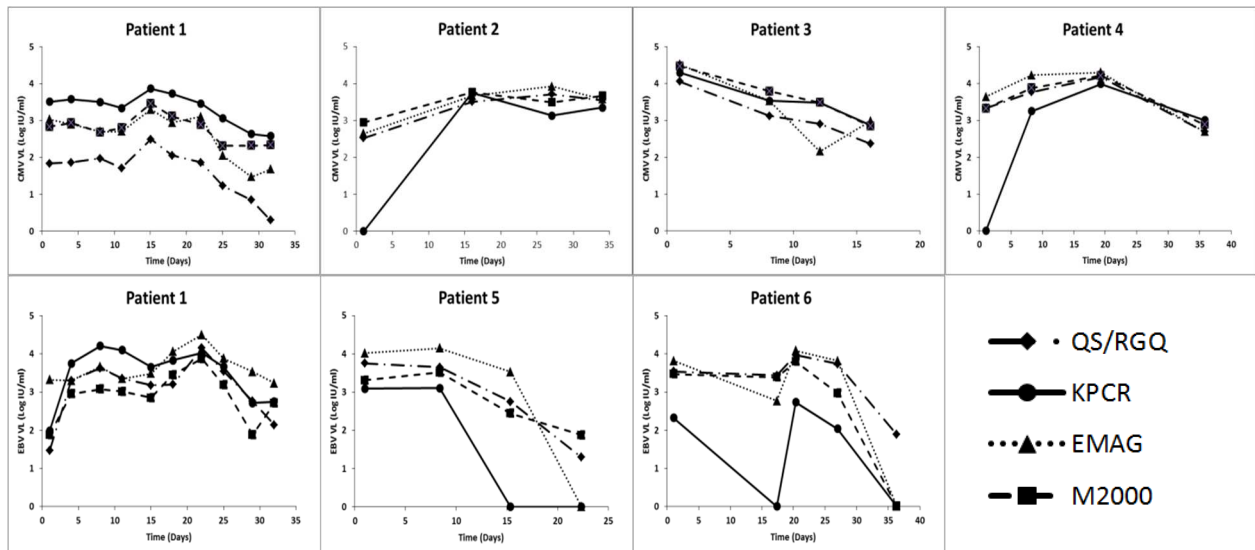


Fig 2: Follow-up of VL in patients with at least 4 measurements in the 4 assays (◆ QS/RGQ, ● KPCR, ▲ EMAG, ■ M2000). Patients 1 to 4 were followed for CMV and Patients 1, 5 and 6 for EBV.

	CMV				EBV			
	QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG	KPCR
LOD Log IU/ml	2.1	1.8	2.7	2.9	1.6	2.2	2.5	NA
LLQ Log IU/ml	2.9	1.8	2.8	2.9	2.2	2.2	3.0	NA
Target genes	ND	UL34 and UL80.5	ppUL83	ND	ND	BLLF1	BXLF1	ND

A: Not available ND : information kept confidential by the manufacturer

Table 1: Limit of detection (LOD), lower limit of quantification (LLQ) and target genes for each assay

	System	Mean concentration (IU/ml)	Mean concentration (Log IU/ml)	Standard deviation (Log IU/ml)	Coefficient of variation (%)
CMV	QS/RGQ	91203	4.96	0.04	1.09
	M2000	115267	5.06	0.07	1.38
	EMAG	35392	4.53	0.12	2.65
	KPCR	61593	4.79	0.06	1.18
EBV	QS/RGQ	2569	3.39	0.14	4.13
	M2000	1041	3.00	0.15	5.00
	EMAG	3002	3.41	0.29	8.50
	KPCR	1921	3.25	0.20	6.15

Table 2: Results of intra-assay reproducibility for CMV (N=9) and EBV (N=8)

Viral Load	Expected	CMV				EBV				
		Log IU/ml	Value	QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG
WHO IS										
			2.73	2.76	1.91	ND	3.02	3.02	3.03	2.46
STD 1	2.70	2.99	2.70	2.63	2.72	2.94	2.90	2.88	ND	
		2.56	2.80	1.64	2.91	3.02	3.09	IN	ND	
		3.82	3.65	3.55	3.71	3.73	3.88	3.67	3.26	
STD 2	3.70	3.77	3.64	3.33	3.77	3.76	3.88	3.89	3.41	
		3.79	3.62	3.37	3.82	3.73	3.86	3.64	3.46	
		4.82	4.63	4.31	4.74	4.70	4.87	4.42	4.76	
STD 3	4.70	4.85	4.59	4.32	4.77	4.68	4.89	4.44	4.74	
		4.82	4.59	IN	4.83	4.73	4.86	4.80	4.72	
		5.90	5.55	5.26	5.80	5.61	5.92	IN	5.68	
STD 4	5.70	5.89	5.58	5.25	5.84	5.64	5.91	5.23	5.50	
		5.90	5.60	5.24	5.84	5.67	5.94	5.70	5.61	
Mean	4.70	4.84	4.61	4.33	4.79	4.70	4.61	4.62	4.57	

ND = not detected IN = inhibited STD = Standard

Values in the gray zone have been excluded from the mean calculation. Values in bold deviate by more than 0.25 Log from the reference value.

Table 3: Values in triplicate obtained with the four systems on dilutions of WHO-IS CMV and EBV.

	CMV				EBV			
	QS/RGQ	M2000	EMAG	KPCR	QS/RGQ	M2000	EMAG	KPCR
LLQ	123 IU/ml	62.4 IU/ml	455 IU/ml	835 IU/ml	40 IU/ml	150 IU/ml	349 IU/ml	NA
Undetectable	1	3	5	14	0	2	4	13
Quantifiable	41	43	33	24	43	39	40	32
Detected	4	0	5	5	2	4	0	0
Invalid or inhibited	0	0	3	3	0	0	1	0

NA = not available

Table 4: Qualitative analysis of results obtained on 46 CMV samples and 45 EBV samples.