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Electronic Supplementary Material

A Novel 2-dimensional Multiplex qPCR Assay for Single-Tube Detection of Nine Human Herpesviruses

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Table S1. Primers and probes used for the single-tube nonuple qPCR assay for eight human herpes viruses and one reference gene GAPDH.

Target	Primers (F/R)/Probes (P)	Sequence (5'-3')	Amplicon Size (bp)
HSV-1	P[a]	HEX-CCATACCGACCACACCGACGAATC-BHQ2	
	F[a]	CGGCCGTGTGACACTATCG	72
	R[a]	CTCGTAAAATGGCCCCTCC	
HSV-2	P[a]	TXR-CGCGGAGACATTCGAGTACCAGATCG-BHQ2	
	F[a]	CGCTCTCGTAAATGCTTCCCT	111
	R[a]	TCTACCCACAACAGACCCACG	
VZV	P[b]	HEX-TGTCTTTCACGGAGGCAAACACGT-BHQ2	
	F[b]	AACTTTTACATCCAGCCTGGCG	201
	R[b]	GAAAACCCAAACCGTTCTCGAG	
EBV	P[b]	CY5-TGTACACGCACGAGAAATGCGCC-BHQ3	
	F[b]	CGGAAGCCCTCTGGACTTC	90
	R[b]	CCCTGTTTATCCGATGGAATG	
HCMV	P[c]	VIC-CACCCTGCTTTCCGAC-MGB	
	F[c]	ACCGTCTGCGCGAATGTTA	67
	R[c]	TCGCAGATGAGCAGCTTCTG	
HHV-6	P[b]	CY5-AGCAGCTGGCGAAAAGTGCTGTGC-BHQ3	
	F[b]	GACAATCACATGCCTGGATAATG	176
	R[b]	TGTAAGCGTGTGGTAATGGACTAA	
HHV-7	P[b]	TXR-CTCGCAGATTGCTTGTTGGCCATG-BHQ2	
	F[b]	CGGAAGTCACTGGAGTAATGACAA	126
	R[b]	CCAATCCTTCCGAAACCGAT	
KSHV	P[d]	CY5- TCGTAACCCCCGTCTACTTTCCCCG -BHQ3	
	F[d]	TCGGTGGCGATGCTTTAGAC	97
	R[d]	TGAAGCAGACGATGCTTTGC	
GAPDH	P	TXR-TTGTTGCCATCAATGACCCCTTCATTG-BHQ2	
	F	CTGCTTTTAACTCTGGTAAAGTGG	145
	R	ATGACAAGCTTCCCGTTCTCA	

The final concentrations of primers and probes were 0.1 μmol/L and 0.04 μmol/L, respectively.

The actual Tm of each amplicon was determined by 10 replicates. F, forward primer; R, reverse primer; P, probe.

[a] Primers or probes were selected or modified from a previous study by Weidmann *et al.* (Weidmann *et al.*, 2008).

[b] Primers or probes were selected or modified from a previous study by Sugita *et al.* (Sugita *et al.*, 2008).

[c] Primers or probes were selected or modified from a previous study by Slavov *et al.* (Slavov *et al.*, 2016).

[d] Primers or probes were selected or modified from a previous study by Stamey *et al*. (Stamey *et al.*, 2001).

Table S2. Comparison of predicted and actual Tm values.

Fluorescence channel	Target	Theoretical Tm (℃)	Actual Tm of cloned target (℃)	Actual Tm of clinical samples (℃)
HEX	HSV-1	80.79 (80.55-81.31)	83.06 (83.01-83.13)	82.91 (82.51-83.23)
	VZV	86.33 (86.14-86.5)	86.23 (86.18-86.31)	86.12 (85.92-86.26)
	HCMV	84.48 (83.90-84.5)	83.99 (83.89-84.11)	84.13 (83.79-84.21)
CY5	EBV	82.71 (82.38-82.98)	82.76 (82.59-82.85)	82.67 (82.59-82.79)
	HHV-6A/B	80.19 (79.53-80.95)	78.96 (78.93-79.01)	-
	KSHV	85.24 (84.8-85.3)	84.72 (84.68-84.77)	84.83 (84.69-84.92)
Texas red	HSV-2	87.54 (87.29-87.8)	87.88 (87.82-87.97)	87.8 (87.74-87.88)
	HHV-7	81.14 (80.29-81.21)	79.71 (79.65-79.79)	79.78 (79.61-80.31)

Note: The mean Tm is shown for each HHV with the scope range in parenthesis. The theoretical Tm values were predicted using all available unique sequences in amplicon area. The Tm values of cloned target were obtained by ten replicates of the plasmid carrying HHV genomic segment.

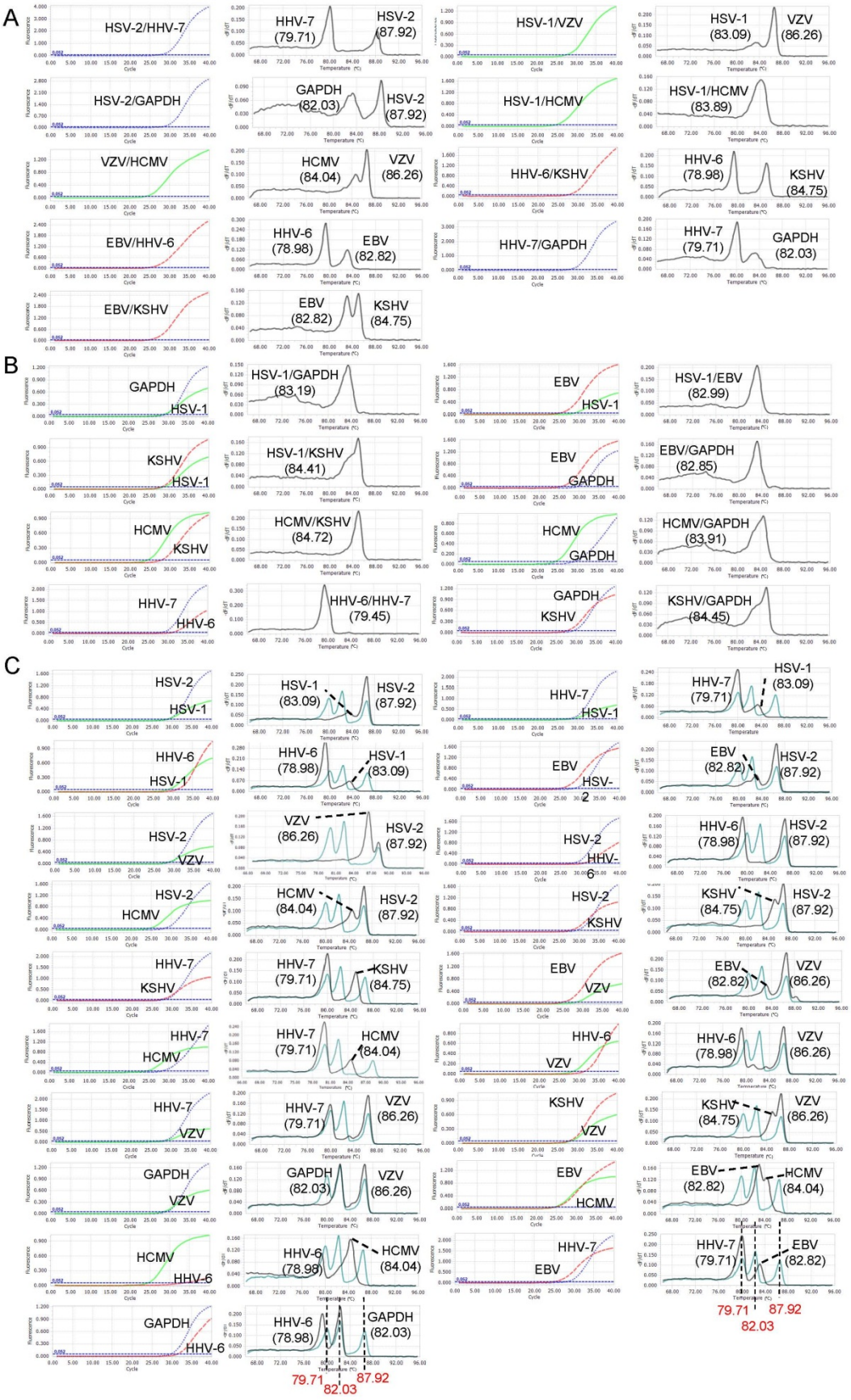
A
HSV-2/HHV-7
HHV-7 (79.71)
HSV-2 (87.92)
HSV-1/VZV
HSV-1 (83.09)
VZV (86.26)
HSV-2/GAPDH
GAPDH (82.03)
HSV-2 (87.92)
HSV-1/HCMV
HSV-1/HCMV (83.89)
VZV/HCMV
HCMV (84.04)
VZV (86.26)
HHV-6/KSHV
HHV-6 (78.98)
KSHV (84.75)
EBV/HHV-6
HHV-6 (78.98)
EBV (82.82)
HHV-7/GAPDH
HHV-7 (79.71)
GAPDH (82.03)
EBV/KSHV
EBV (82.82)
KSHV (84.75)
B
GAPDH
HSV-1
HSV-1/GAPDH (83.19)
EBV
HSV-1
HSV-1/EBV (82.99)
KSHV
HSV-1
HSV-1/KSHV (84.41)
EBV
GAPDH
EBV/GAPDH (82.85)
HCMV
KSHV
HCMV/KSHV (84.72)
HCMV
GAPDH
HCMV/GAPDH (83.91)
HHV-7
HHV-6
HHV-6/HHV-7 (79.45)
GAPDH
KSHV
KSHV/GAPDH (84.45)
C
HSV-2
HSV-1
HSV-1 (83.09)
HSV-2 (87.92)
HHV-7
HSV-1
HSV-1 (83.09)
HHV-7 (79.71)
HHV-6
HSV-1
HHV-6 (78.98)
HSV-1 (83.09)
EBV
HSV-2
EBV (82.82)
HSV-2 (87.92)
HSV-2
VZV
VZV (86.26)
HSV-2 (87.92)
HSV-2
HHV-6
HHV-6 (78.98)
HSV-2 (87.92)
HSV-2
HCMV
HCMV (84.04)
HSV-2 (87.92)
HSV-2
KSHV
KSHV (84.75)
HSV-2 (87.92)
HHV-7
KSHV
HHV-7 (79.71)
KSHV (84.75)
EBV
VZV
EBV (82.82)
VZV (86.26)
HHV-7
HCMV
HHV-7 (79.71)
HCMV (84.04)
HHV-6
VZV
HHV-6 (78.98)
VZV (86.26)
HHV-7
VZV
HHV-7 (79.71)
VZV (86.26)
KSHV
VZV
KSHV (84.75)
VZV (86.26)
GAPDH
VZV
GAPDH (82.03)
VZV (86.26)
EBV
HCMV
EBV (82.82)
HCMV (84.04)
HCMV
HHV-6
HHV-6 (78.98)
HCMV (84.04)
HHV-7
EBV
HHV-7 (79.71)
EBV (82.82)
GAPDH
HHV-6
HHV-6 (78.98)
GAPDH (82.03)
79.71
82.03
87.92
79.71
82.03
87.92

Fig. S1. Adaptability of the single-tube nonuple qPCR assay to co-existing HHVs and the internal reference (GAPDH). **A** Co-existing targets detected by the same color probe are distinguished by two different Tm peaks; **B** Co-existing targets with similar Tm values are detected by two different color amplification curves; **C** Co-existing targets are distinguished by different color probes and different Tm peaks. In panel (C), the melting curve of mixed template of HSV-2, HHV-7 and GAPDH was shown as the Tm reference (green line). The Tm of each target is shown in parenthesis.

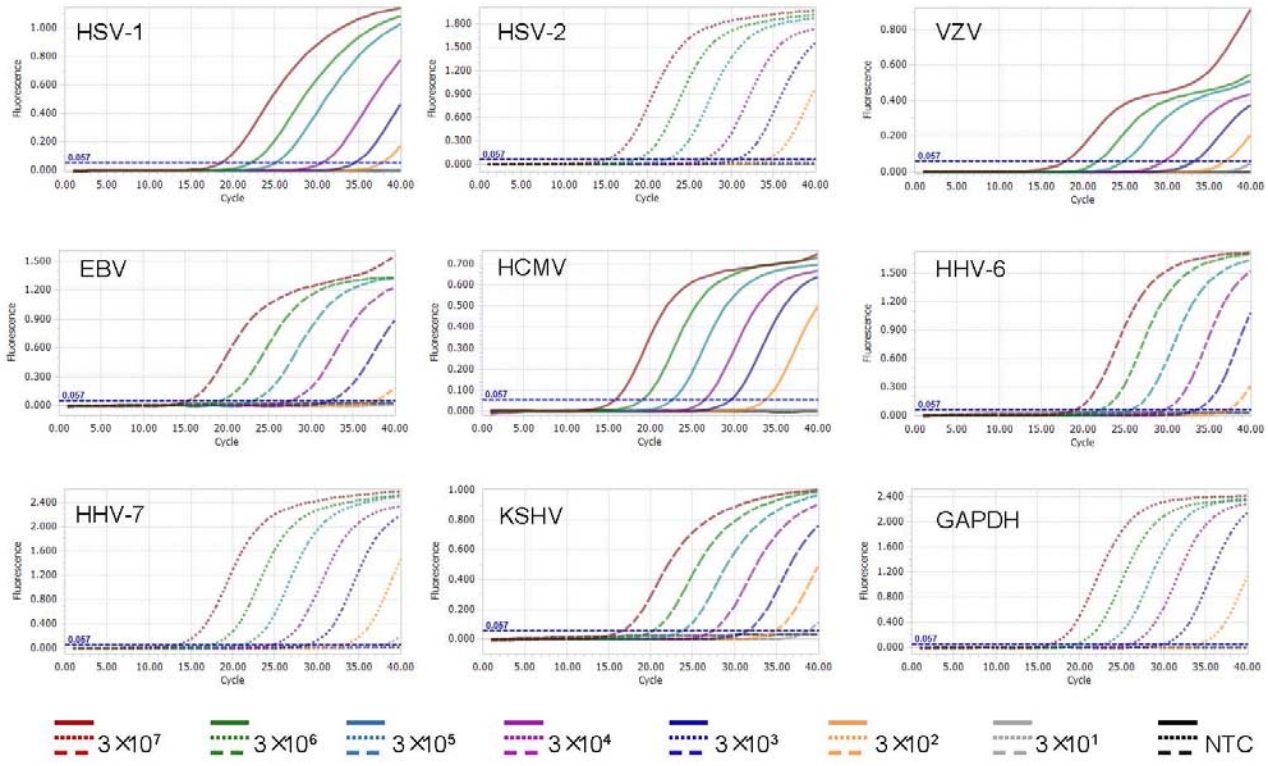

Fig. S2. Analytical sensitivity of the multiplex qPCR assay. NTC: non-template control.

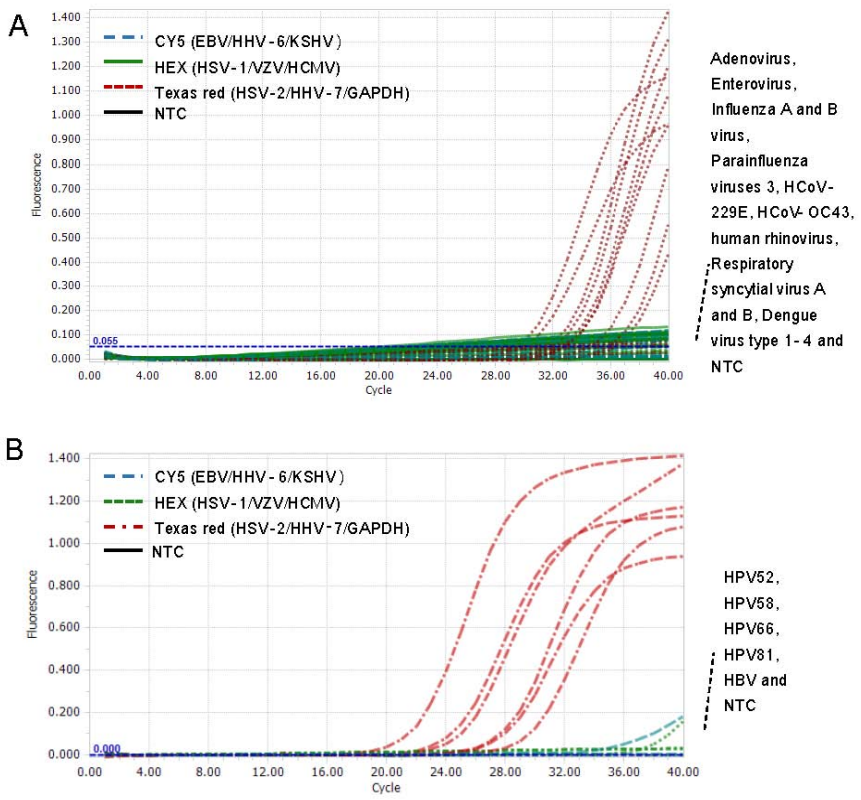

Fig. S3. Specificity of the multiplex qPCR assay. The common human viruses used in this assay include adenovirus, enterovirus; influenza A and B viruses, parainfluenza virus type 3, HCoV-229E, HCoV-OC43, human rhinovirus, respiratory syncytial virus A and B, dengue virus type 1-4, HPV52, HPV58, HPV66, HPV81, and HBV. NTC: non-template control.

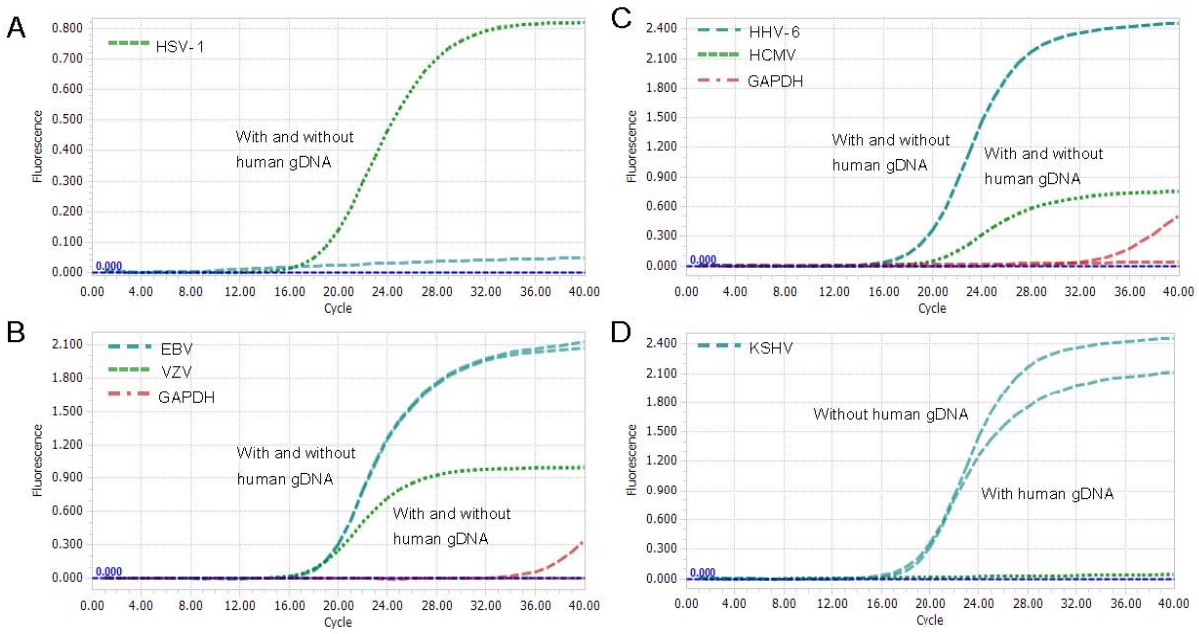

Fig. S4. The influence of human gDNA on the amplification of the multiplex qPCR assay. The amplification curves of HSV-1, VZV, EBV, HCMV, and HHV-6 were completely overlapped between the plasmid input with and without human gDNA. The results of HSV-2 and HHV-7 are not available since they shared same fluorescent channel with GAPDH. Each reaction used 3.56 ng of human genomic DNA.

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