Electronic Supplementary Material (ESI) for Analytical Methods.
This journal is © The Royal Society of Chemistry 2024

Title page

Rapid detection of monkeypox virus and differentiation of West African and Congo Basin strain using endonuclease restriction-mediated real-time PCR-based testing

Juan Zhou [1], Fei Xiao [1], Xiaolan Huang [1], Jin Fu [1], Nan Jia [1], Chunrong Sun [1], Min Chen [1], Zheng Xu [1], Hui Huang [2*] and Yi Wang [1*]

[1] Experimental research center, Capital Institute of pediatrics, Beijing, 100020, P.R. China

[2] Department of Infectious Diseases, Affiliated Children's Hospital, Capital Institute of Pediatrics, Beijing, 10020, P. R. China

*Correspondence: Yi Wang, wildwolf0101@163.com (Handing the Correspondence)
Hui Huang, huihui07@tom.com

Tables

Table S1 Primers used in this study

Primer	Sequences and modifications (5'-3')[a]	Length[b]	Genes
com-EF	FAM-TCGCG-CAACAAGA**T**(BHQ1)AGGGACACGCTTTC	28 mer	ATI (particle)
com-R	CAGTGCATCAACTTCTTGTGTGT	23 nt	
cen-EF	CY5-TCGCG-CAAC**T**(BHQ2)TGATATTGGCGGAGTAGACT	30 mer	D14L (particle)
cen-R	GTGCCTCAGGATTCCATACCAT	22 nt	
wes-EF	ROX-TCGCG-AGCA**T**(BHQ2)CTTCTGAGGAGGTAAATAGG	30 mer	ATI (particle)
wes-R	CCTGGCATTACCGAGTTCAGTTTTA	25 nt	

[a] BHQ1 and BHQ2 were labeled on the thymine presented in bold type.

[b] nt, nucleitide; mer, monomeric unit.

Table S2 Combinations of the three primer pairs for optimization of the reaction system of multiplex ET-PCR assay

Combinations	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
com (μl)	2	1	1	0.5	0.5	0.8	0.6	0.7	0.7	0.7	0.7	0.7
cen (μl)	2	1.5	1	1.5	1	1.5	1.5	1.5	1.6	1.8	2	2.2
wes (μl)	2	2	2	2	2	2	2	2	2	2	2	2

Figures

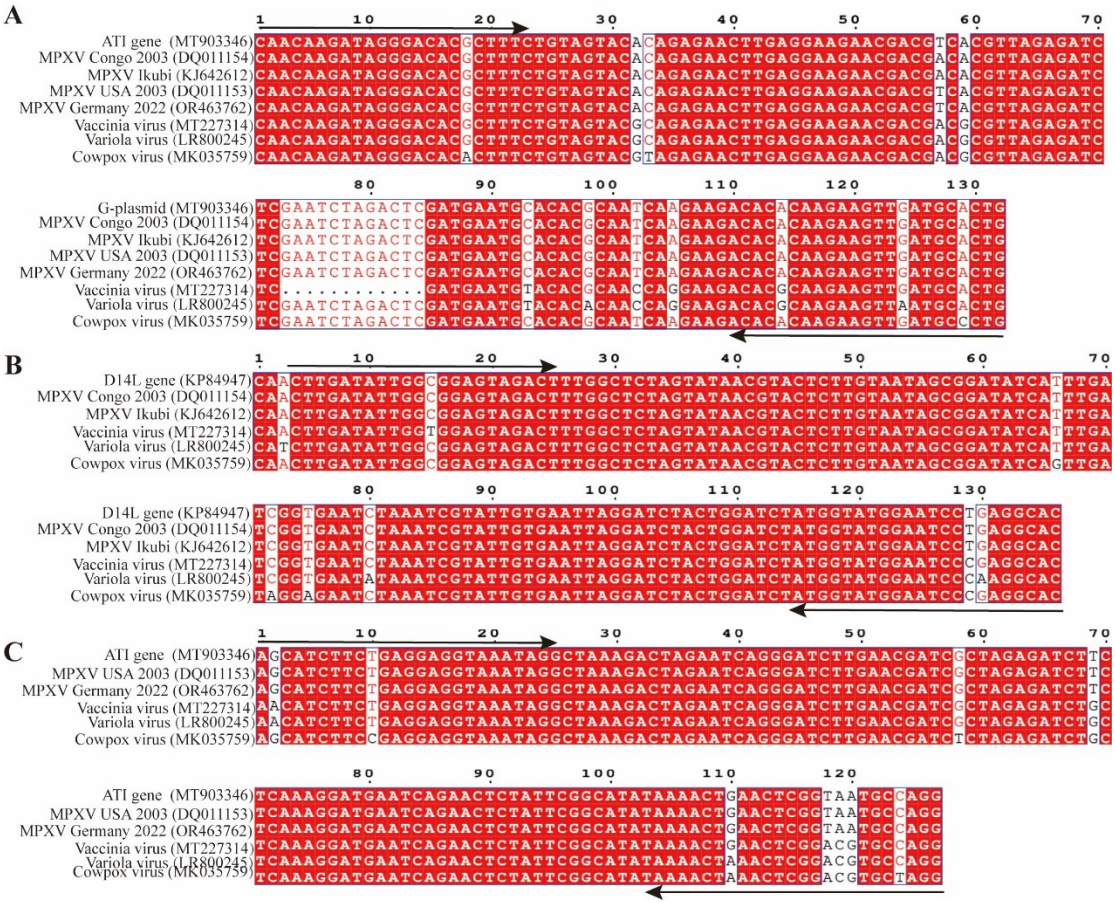

Figure S1. Sequence alignments of target regions for MPXV strains and other *Orthopoxviruses* species. Target sequences and primers of generic (**A**), clade I (**B**) and clade II (**C**) MPXV were aligned with genomic sequences of two Congo Basin clade strains (DQ011154, KJ642612), two West African clade strains (DQ011153, OR463762), and several *Orthopoxvirus* species (Vaccinia virus, MT227314; Variola virus, LR800245; Cowpox virus,MK035759). The arrows indicate the forward primers and reverse primers.

Figure S2. Sequences and locations of the three sets of ET-PCR primers. Location and sequence of conventional primers and modifications of the forward primers of ET-PCR (EF) for ATI gene (particle sequence), D14L gene (particle sequence) and another particle sequence of the ATI gene, which were specific for the generic (**A**), clade I (**B**) and clade II (**C**) MPXV strains, respectively, were shown. The locations of the primer sites are marked in red, right and left arrows indicate the sense and complementary sequences that are used. BstUI recognition site was shown in red bold type, and the reporter dye (FAM. Cy5, ROX) and quencher dye (BHQ1 and BHQ2) were shown in black bold type.

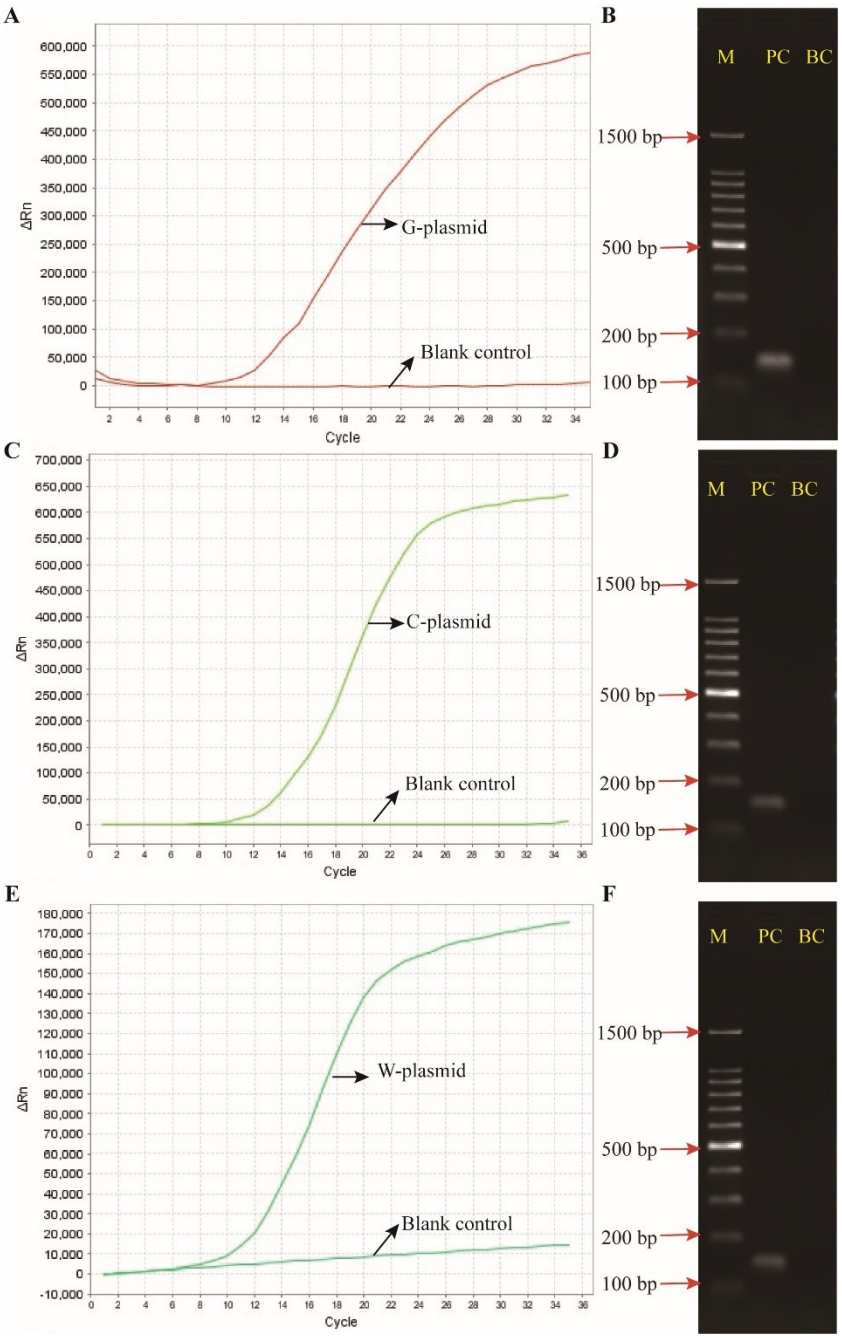

Figure S3. Confirmation and detection of ET-PCR products. A, **C** and **E** showed the results of ET-PCR products by real-time fluorescent format, **B**, **D** and **F** displayed the result by agarose gel electrophoresis format. The three real-time fluorescence images were obtained from FAM (labeled at com-EF of ATI), Cy5 (labeled at cen-EF of D14L), and ROX (labeled at wes-EF of ATI) channels, respectively. Ct value lower than 33 was considered as positive. PC, positive control; BC, blank control. M, 50 bp DNA marker.

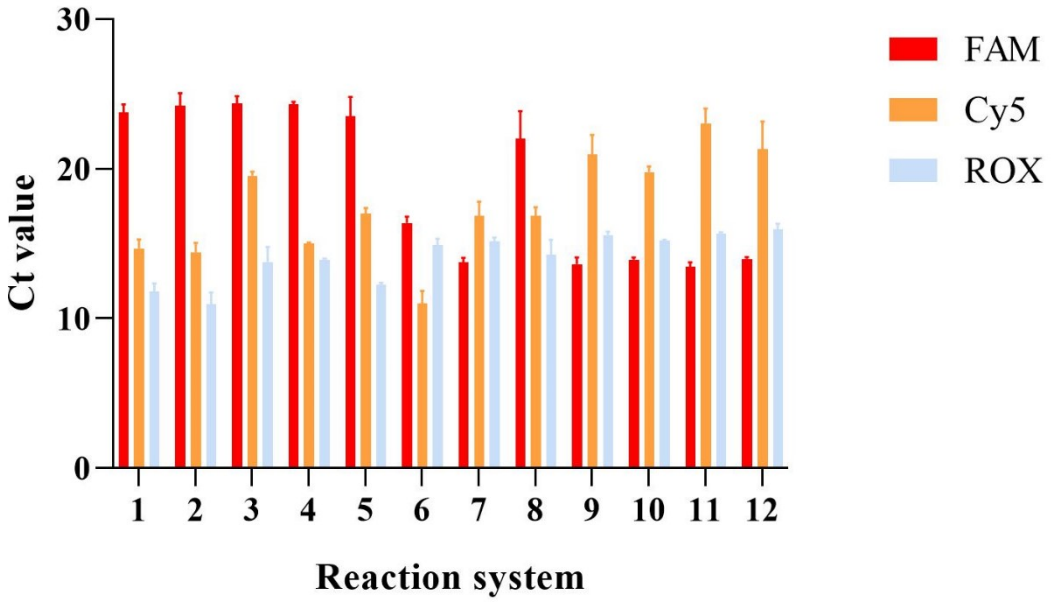

Figure S4. Optimization of the reaction system of multiplex ET-PCR assay. A total of 12 combinations (Table 3) of the three primer pairs were tested to choose the optimal one for the multiplex ET-PCR assay. The combination 6, including 0.8 μl of com-EF and com-R (10 μM), 1.5 μl of cen-EF and cen-R (10 μM) and 2 μl of wes-EF and wes-R (10 μM), was selected for the following detection.

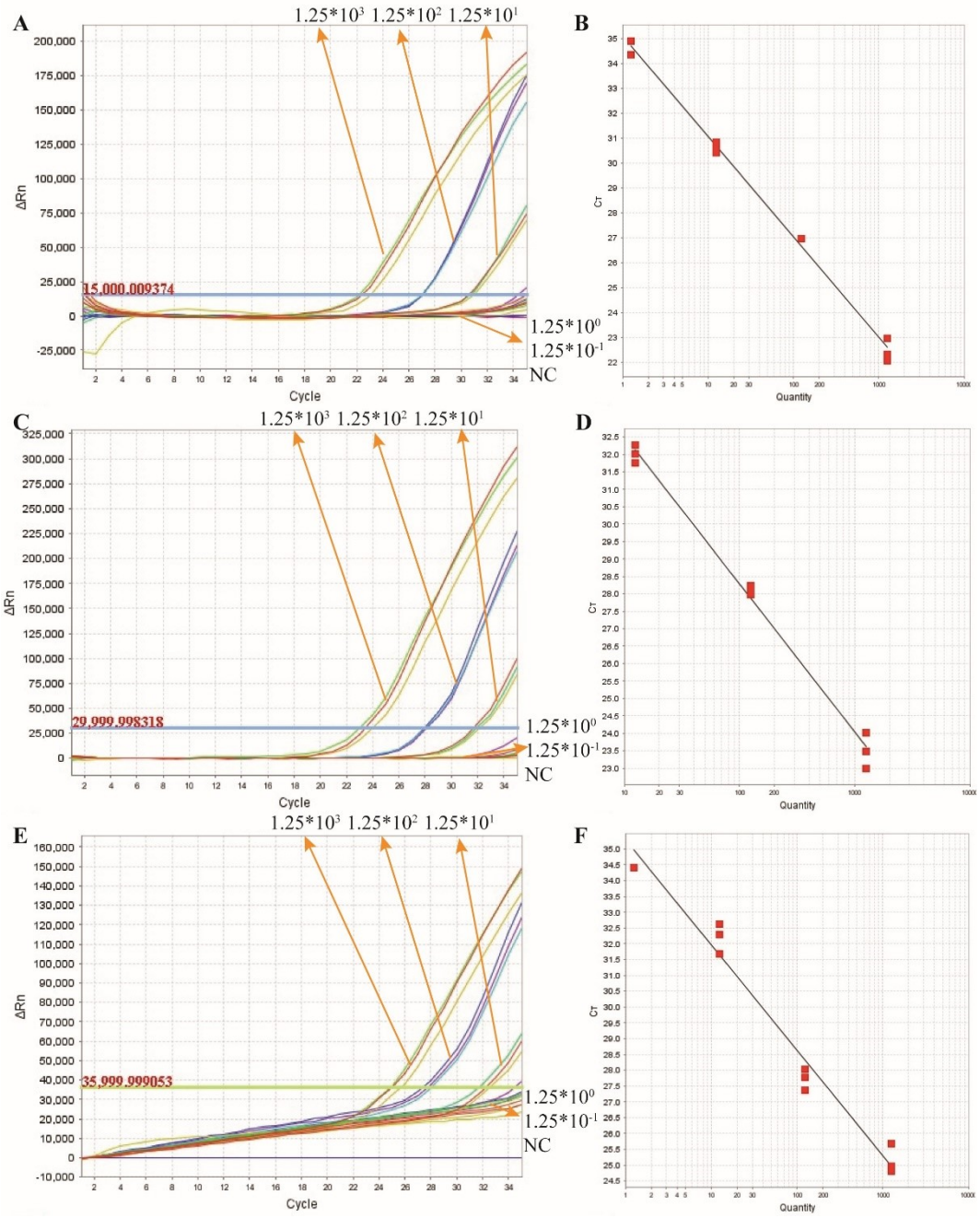

Figure S5 Analytical sensitivity of multiplex ET-PCR assay for detecting three targets simultaneously with pseudotyped virus. A, **C** and **E** displayed amplification curves of multiplex ET-PCR assay for detection of generic MPXV, clade I MPXV and clade II MPXV, respectively; **B, D and F** indicated the respective plots of different concentration against Ct value. Ct value lower than 33 was considered as positive.

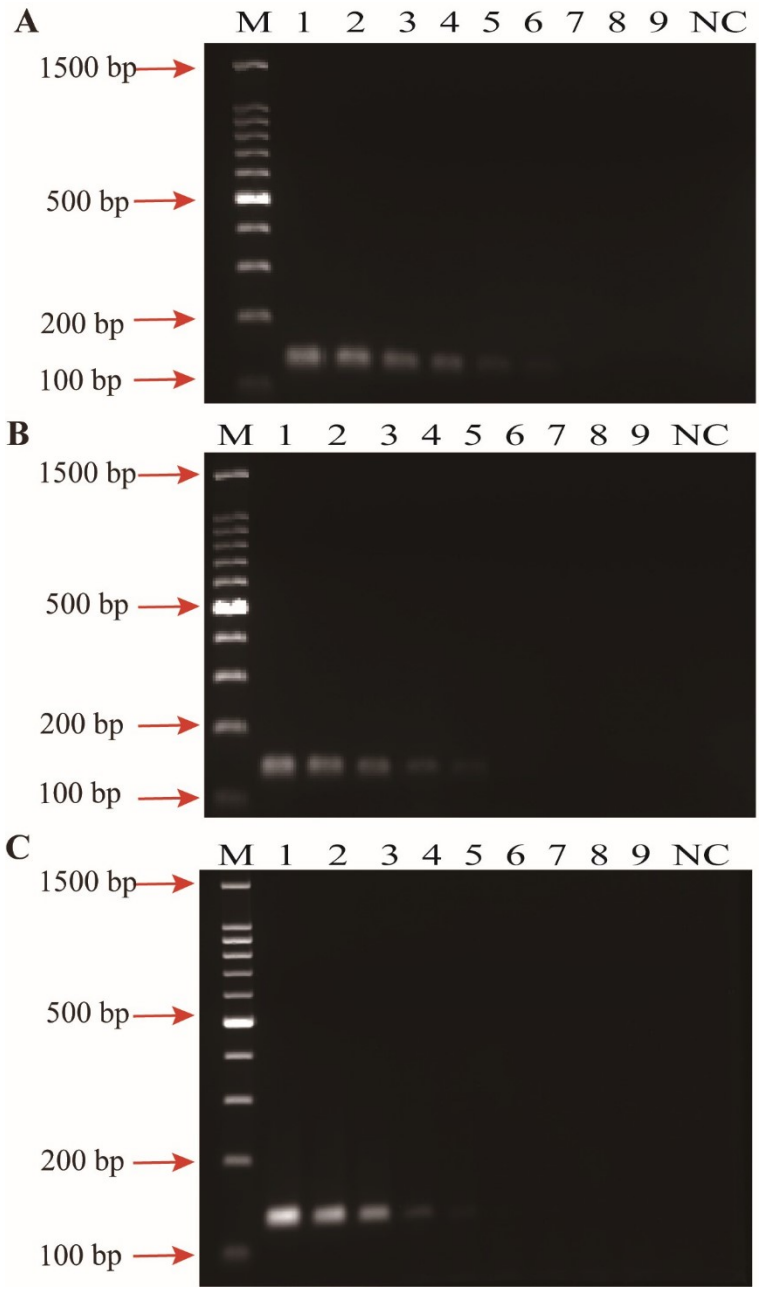

Figure S6. Analytical sensitivity of conventional PCR assay for detecting a single target of MPXV. A, **B** and **C** displayed the results of conventional PCR for detection of generic MPXV, clade I MPXV, and clade II MPXV, respectively, by agarose gel electrophoresis format. Lane A1-A8, B1-B8 and C1-C8 referred to 1.5×10^7 to 1.5×10^1 copies per microliter of G-, C- and W-plasmids, respectively; M, 50 bp DNA marker; NC, negative control (distilled water).

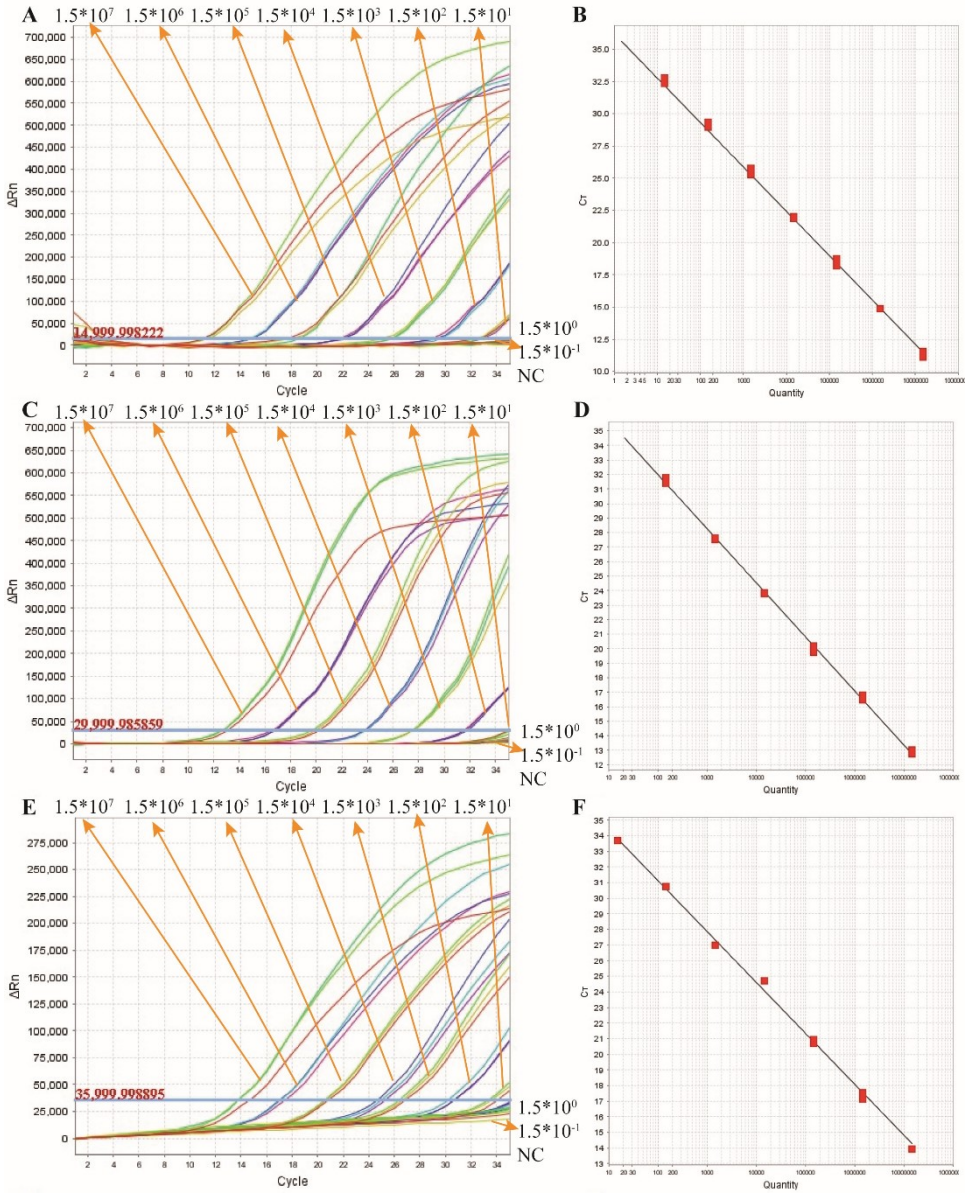

Figure S7. Analytical sensitivity of single ET-PCR assay. A, **C** and **E** displayed amplification curves of single ET-PCR assay for detection of generic MPXV, clade I MPXV and clade II MPXV, respectively; **B, D and F** indicated the respective plots of different concentration against Ct value. Ct value lower than 33 was considered as positive.

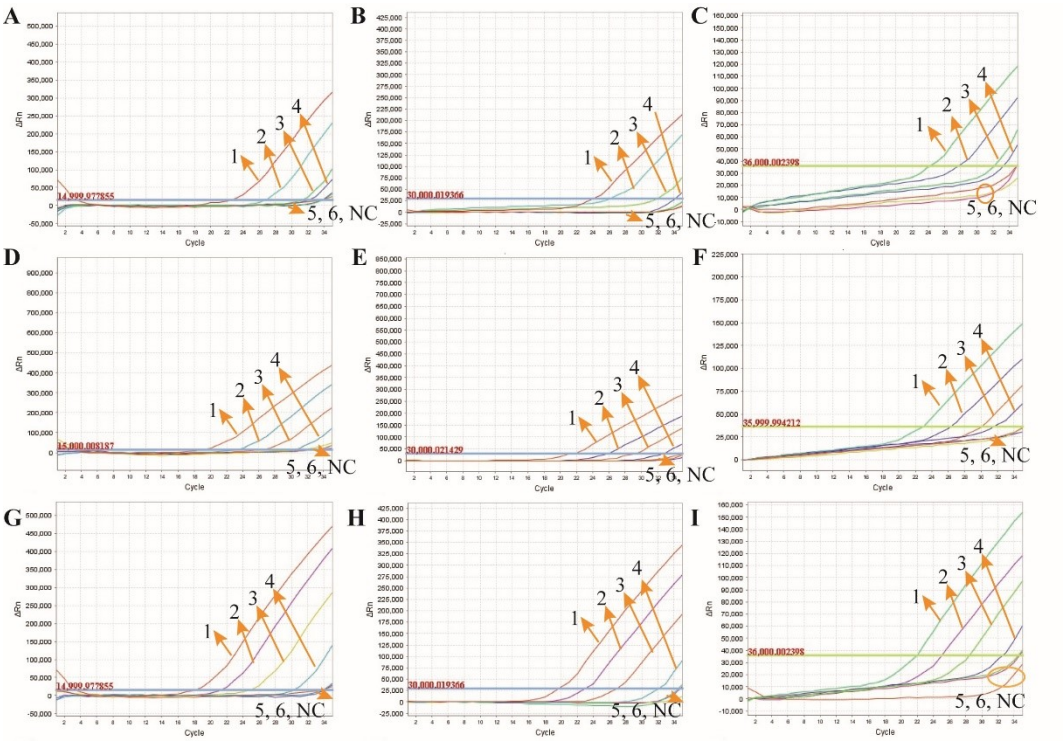

Figure S8. Clinical feasibility of multiplex ET-PCR assay in artificially simulated samples. A, B and **C** indicated the results of generic MPXV, clade I MPXV, and clade II MPXV with serially diluted artificially simulated plasma samples, **D, E** and **F** indicated the results with simulated nasopharyngeal swab samples, **G, H** and **I** indicated the results with simulated skin swab samples. Ct value lower than 33 was considered as positive. Signals A1-A6, B1-B6 and C1-C6 represented genomic DNA of simulated blood samples with pseudotyped virus from 1.25×10^{4} to 1.25×10^{-1} copies per microliter; signals D1-D6, E1-E6 and F1-F6, genomic DNA of simulated nasopharyngeal swab samples with pseudotyped virus from 1.25×10^{4} to 1.25×10^{-1} copies per microliter; signals G1-G6, H1-H6 and I1-I6, genomic DNA of simulated skin swab samples with pseudotyped virus from 1.25×10^{4} to 1.25×10^{-1} copies per microliter; NC, negative control (distilled water).