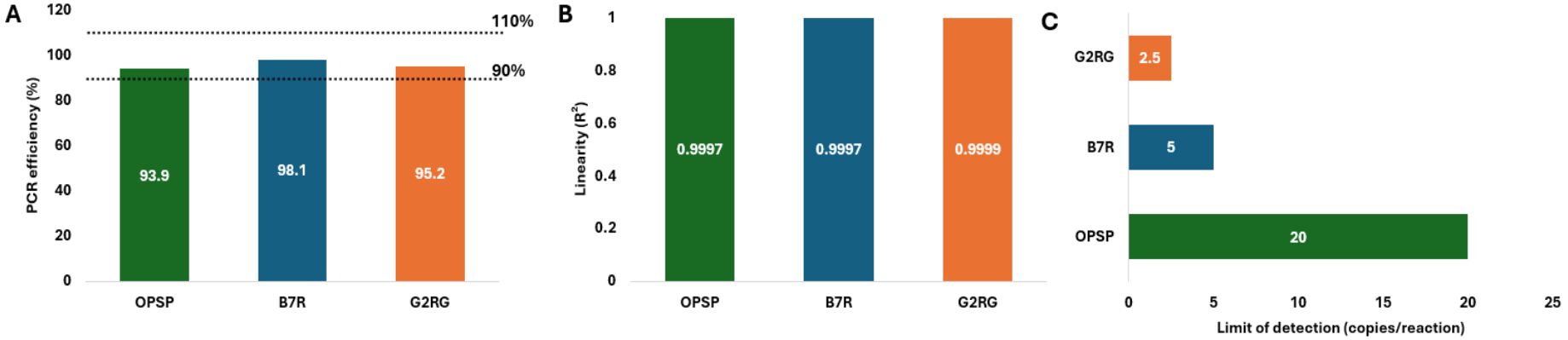
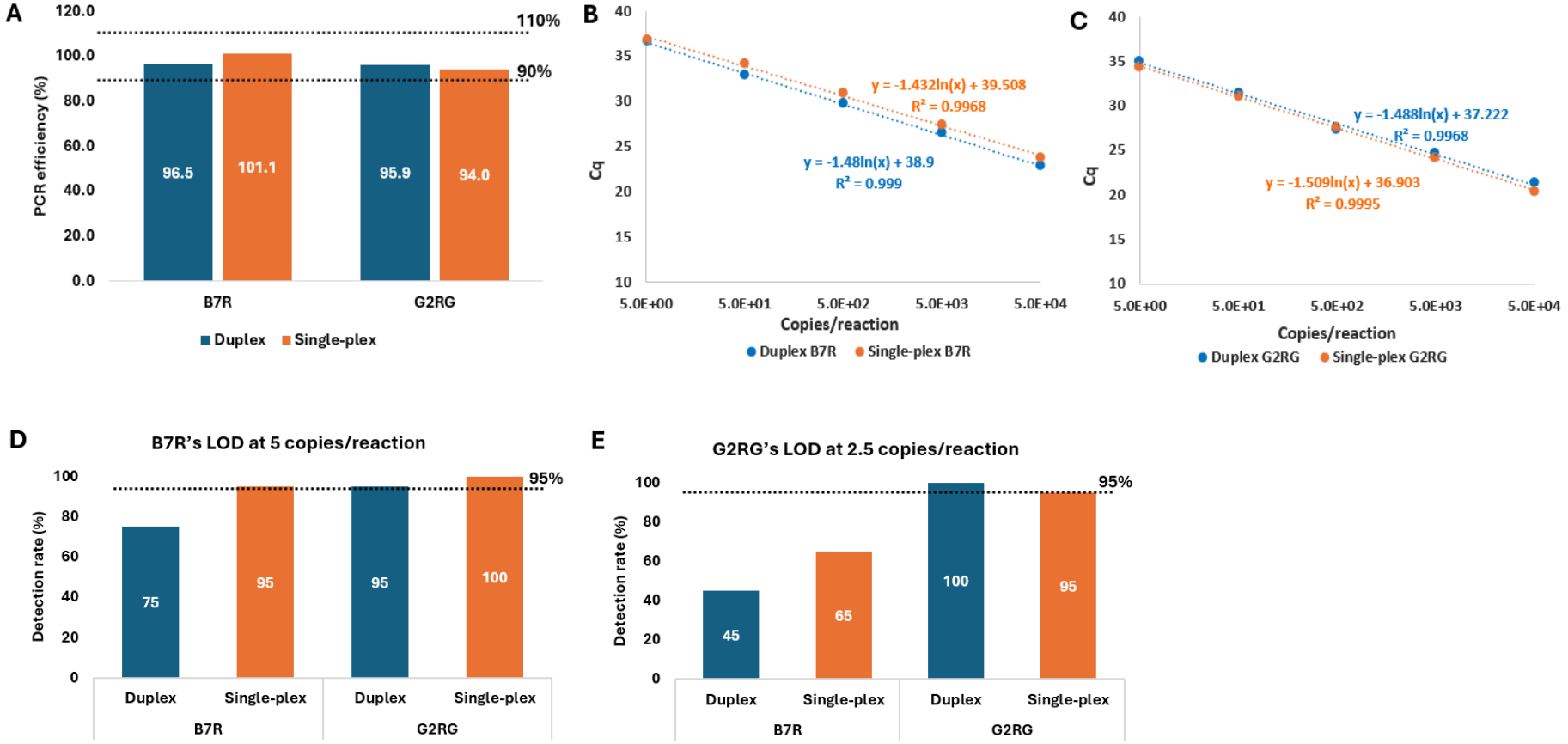
Supplementary Information

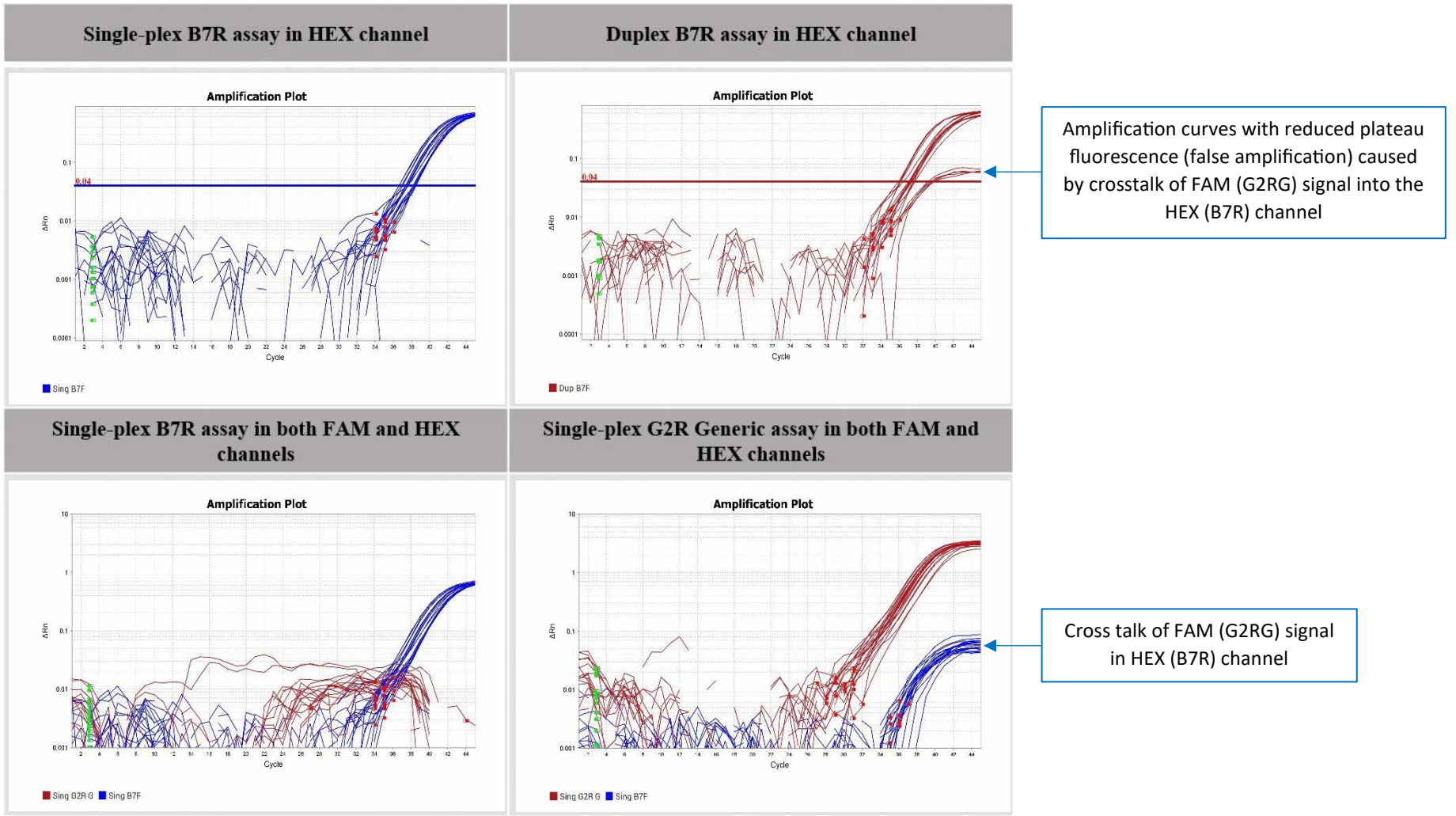
Additional File 1. Performance metrics of qPCR assays in a single-plex format evaluated using synthetic genomic DNA control (Clade II) spiked into molecular grade water. (A) PCR efficiency (acceptable range 90–110%). (B) Linearity (R^2). (C) Limit of detection: OPSP (20 copies/reaction), B7R (5 copies/reaction), G2RG (2.5 copies/reaction) assays.



Additional File 2: Performance comparison of B7R and G2RG assays in duplex and single-plex formats using single-quenched probes. (A) PCR efficiency. The acceptable efficiency range was between 90 to 110% (dotted lines). (B-C) Standard curves for B7R (B) and G2RG (C) assays. (B-C) Standard curves for B7R (B) and G2RG (C) assays. (D-E) Detection rate at B7R's LOD of 5 copies/reaction (D) and G2RG's LOD of 2.5 copies/reaction (E), with the 95% detection threshold indicated by the dashed line. B7R showed reduced sensitivity in duplex format at its LOD. Evaluations were conducted using the synthetic genomic DNA control (Clade II) spiked into molecular grade water. LOD, limit of detection. B7R and G2RG targets were detected using HEX and FAM dyes respectively.

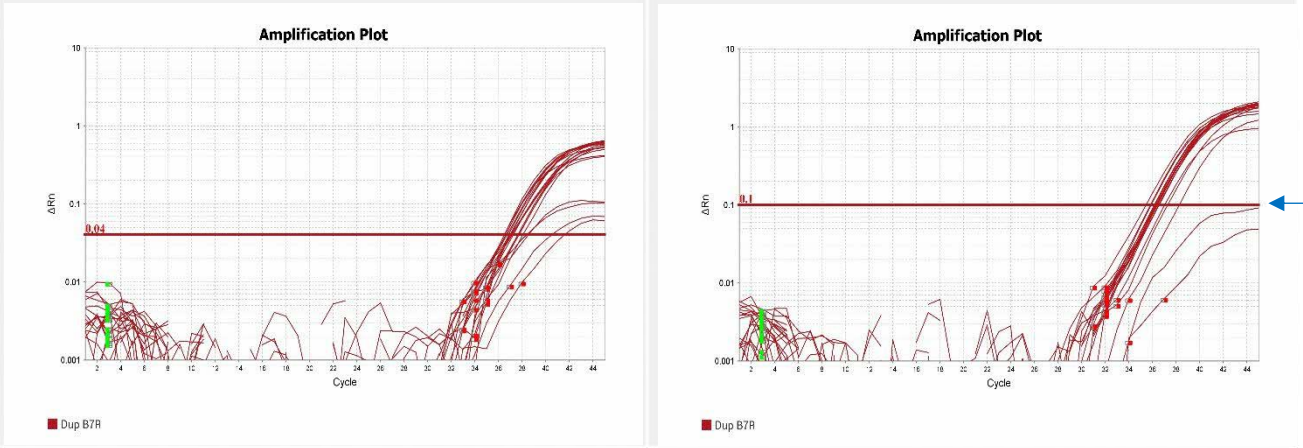


Additional File 3: Amplification curves of B7R (HEX) and G2RG (FAM) assays in single-plex and duplex formats to demonstrate crosstalk between fluorescent channels. Each target was tested using 5 genomic copies per reaction of synthetic genomic DNA control (Clade II) spiked into molecular grade water. In the duplex reaction (top-right plot), only the HEX channel (for the B7R assay) was monitored to demonstrate crosstalk, which is observed as amplification curves with reduced plateau fluorescence. This artefact is caused by bleed-through of the G2RG target (FAM) signal into the HEX (B7R) channel, which is further supported by the false amplification reported in the single-plex G2RG assay (shown in blue in the bottom-right plot) detected in the HEX (B7R) channel, where no signal should be detected due to the absence of the B7R target.

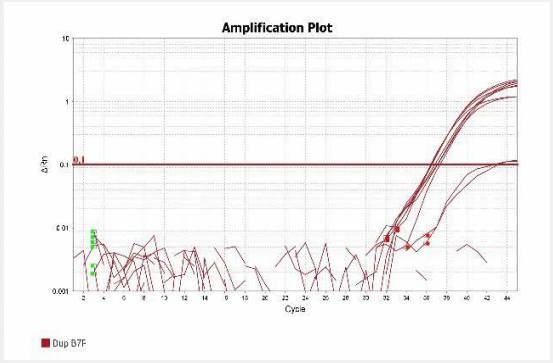
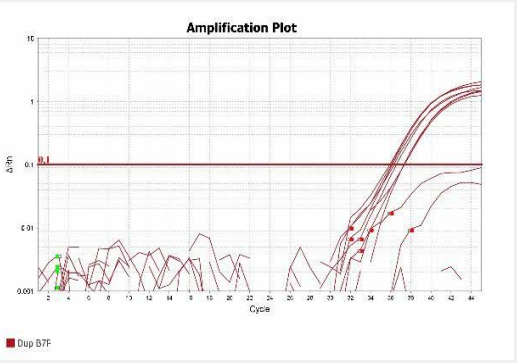
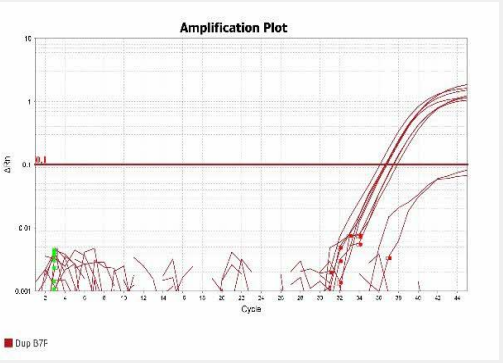


Additional File 4: Performance comparison of single-quenched and double-quenched probes in duplex assay format. Each target was tested in 20 replicates at 2.5 copies per reaction of synthetic genomic DNA control (Clade II) spiked into molecular grade water.

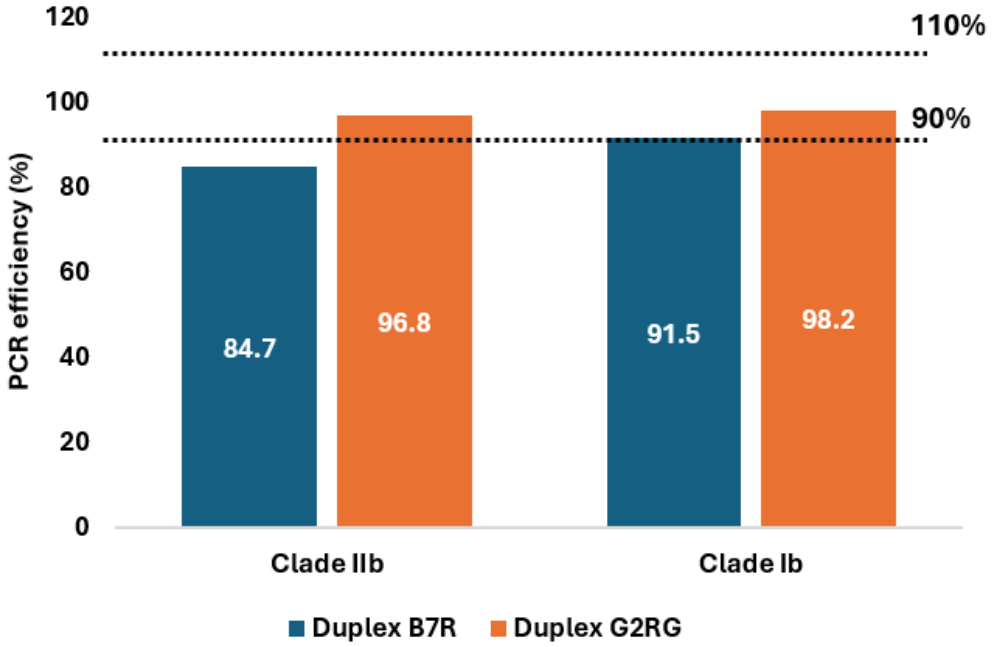
Duplex B7R assay with HEX labelled single-quenched probe	Duplex B7R assay with SUN labelled double-quenched probe
• 80% detection rate • Mean Cq = 37.08 • % Coefficient of Variation = 0.49	• 90% detection rate • Mean Cq = 36.49 • % Coefficient of Variation = 0.59



Additional File 5: Gradient PCR amplification of B7R target in duplex assay utilizing SUN-labelled double-quenched probe. Each target was tested in 10 replicates at 2.5 copies per reaction of synthetic genomic DNA control (Clade II) spiked into molecular grade water.

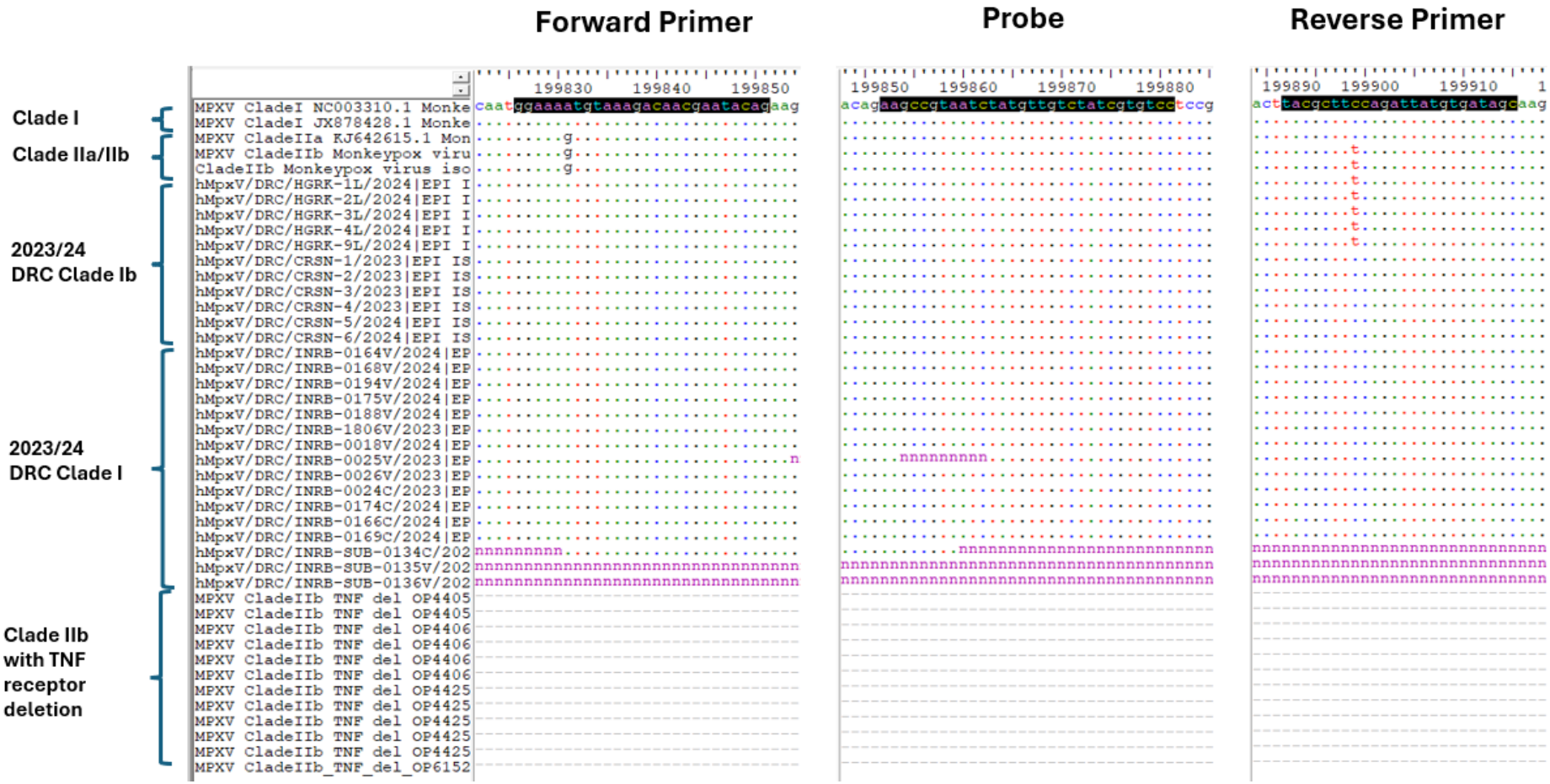
Ta = 56°C	Ta = 58°C	Ta = 60°C
• Mean Cq = 36.79 • % Coefficient of Variation = 0.99	• Mean Cq = 36.62 • % Coefficient of Variation = 1.70	• Mean Cq = 36.97 • % Coefficient of Variation = 1.55
		

Additional File 6: Comparison of amplification efficiency of duplex assay using 10-fold dilution series (prepared in molecular grade water) of genomic DNA extracted from culture supernatants of Clade IIb and Clade Ib. PCR efficiency was calculated based on the top six concentrations of template controls, each tested in triplicates.



Additional File 7: Alignment of oligonucleotide sequences of the B7R assay with the targeted sequence of DNA within reference genome sequences of several MPXV strains.

Additional File 8: Alignment of oligonucleotide sequences of the G2RG assay with the targeted sequence of DNA within reference genome sequences of several MPXV strains.

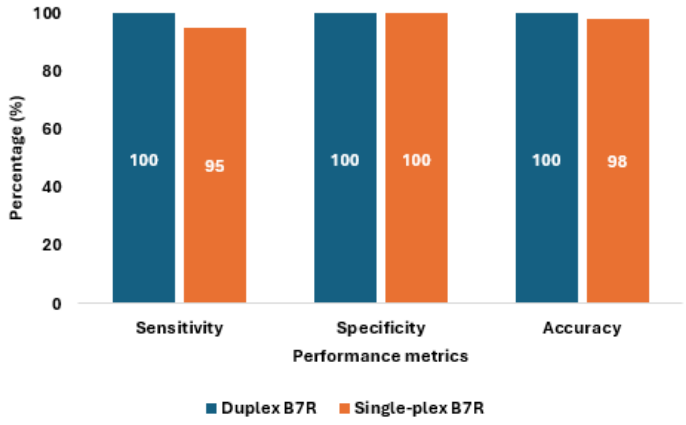


Additional File 9: Performance comparison of duplex and single-plex formats for MPXV detection using B7R and G2RG assays. Validation of B7R and G2RG assays in duplex and single-plex formats using 42 environmental samples comprising 21 MPXV-positive and 21 MPXV-negative samples. (A) Detailed classification results showing true positives, true negatives, false positives, and false negatives for both assays in each format. (B-C) Performance metrics (sensitivity, specificity, and accuracy) comparing duplex and single-plex formats of B7R (B) and G2RG (C) assays. Sensitivity = TP/(TP+FN) × 100; Specificity = TN/(TN+FP) × 100; Accuracy = (TP+TN)/(TP+TN+FP+FN) × 100, where TP, FN, TN, and FP denote true positives, false negatives, true negatives, and false positives, respectively.

A

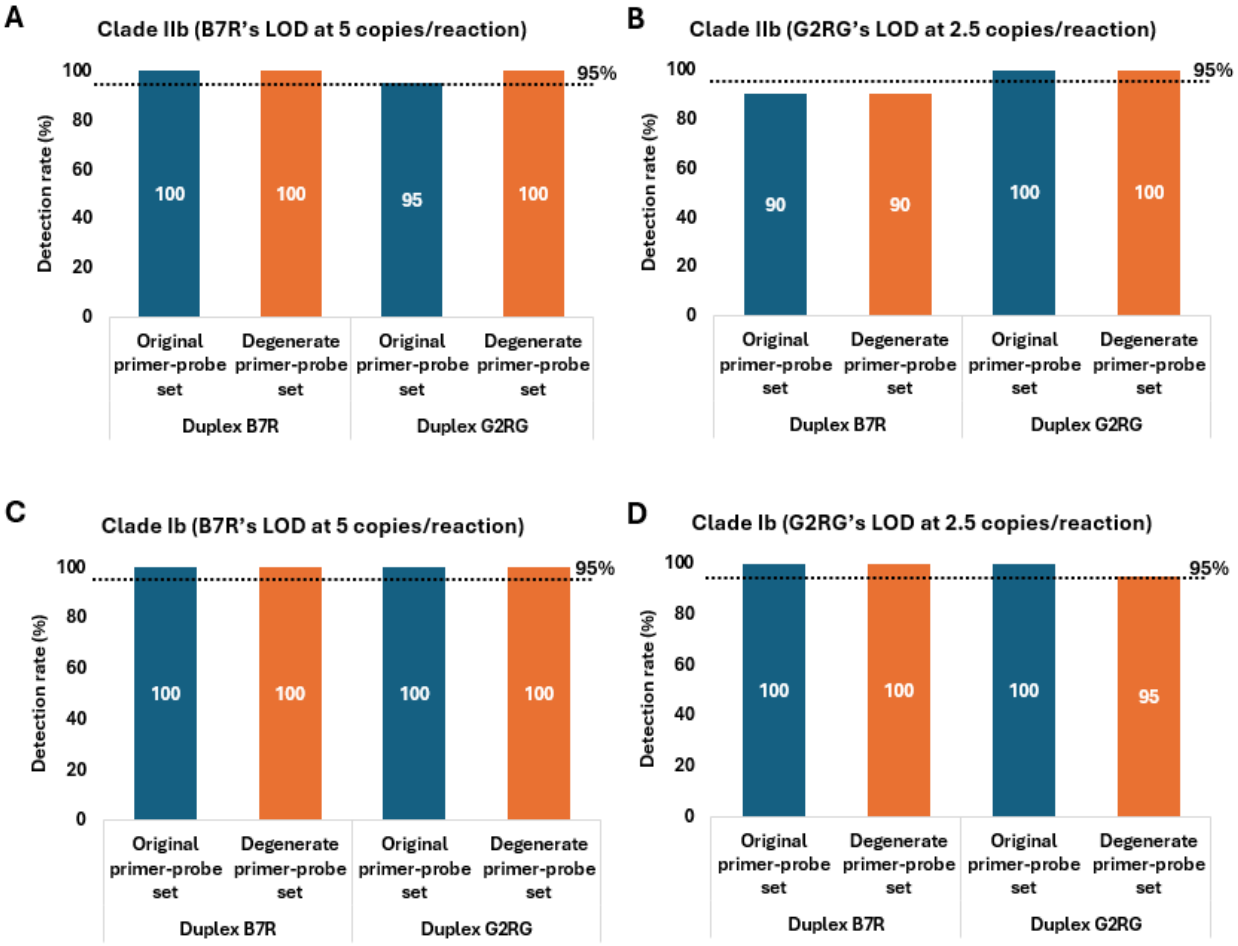
Assay	Assay Format	Status	MPXV-Positive (n=21)	MPXV-Negative (n=21)	Total
B7R	Duplex	Positive	21	0	21
		Negative	0	21	21
	Single-plex	Positive	20	0	20
		Negative	1	21	22
G2RG	Duplex	Positive	21	0	21
		Negative	0	21	21
	Single-plex	Positive	21	0	21
		Negative	0	21	21

B



C

Additional File 10: Performance comparison of original and degenerate primer-probe sets for MPXV detection. Detection rates (%) for duplex B7R and G2RG assays using original and degenerate primer-probe sets against MPXV Clade IIb (A and B) and Clade Ib (C and D) genomic DNA at predetermined LOD concentrations prepared in molecular grade water. Each condition was assessed in 20 replicates, with the 95% detection threshold indicated by the dashed line.

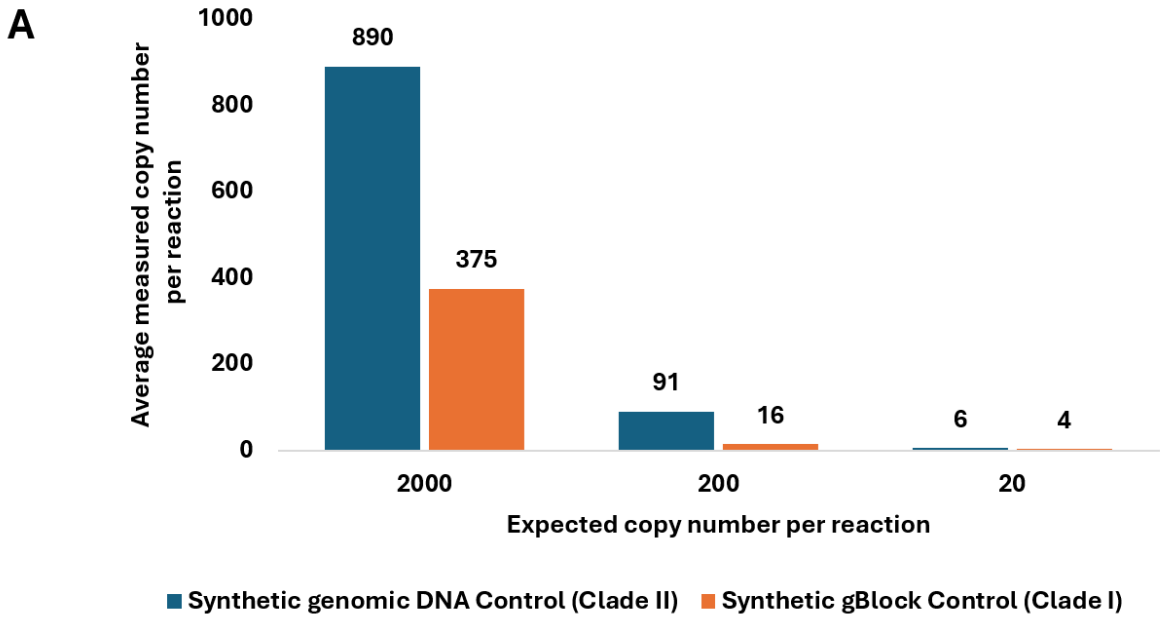


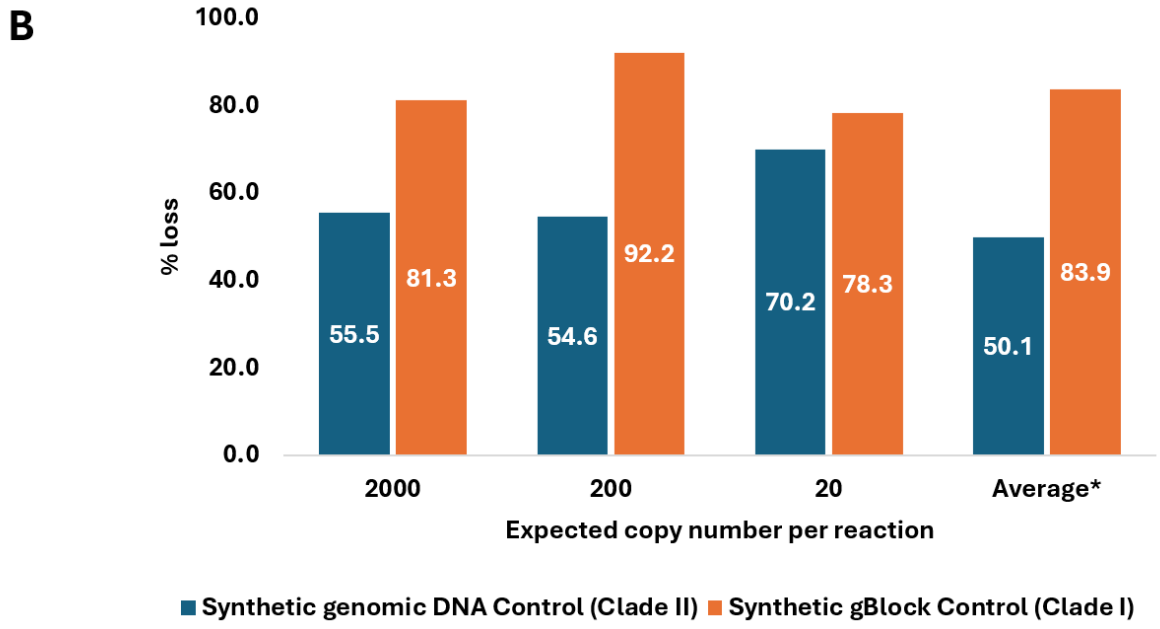
Additional File 11: Summary of qPCR assays for MPXV detection evaluated in the current study.

Assay	Gene target	Types of assays	Primer/ Probe name	Sequence	Final concentration in assay reaction (µM)	Annealing temperature (T_a) for single-plex assay	Annealing temperature (T_a) for duplex assay	Reference
***Pan*-Orthopox**								
OPSP	HA (J7R)	Single-plex	OPSP-F89	GATGATGCAACTCTATCATGTA	0.5	60°C	N.A.	Kulesh et al., 2003
			OPSP-R219	GTATAATTATCAAAATACAAGACGTC	0.5			
			OPSP-Probe	FAM-AGTGCTTGGTATAAGGAG-MGBNFQ	0.25			
Mpox-specific								
B7R	B7R	Single-plex	MPXV B7R forward	ACGTGTTAAACAATGGGTGATG	0.5	60°C	N.A.	Shchelkunov et al., 2001/2011
			MPXV B7R reverse	AACATTTCCATGAATCGTAGTCC	0.5			
			MPXV B7R probe	FAM-TGAATGAATGCGATACTGTATGTGTGGG-BHQ1	0.25			
		Single-plex/Duplex	MPXV B7R forward	ACGTGTTAAACAATGGGTGATG	0.5	56°C (HEX) 58°C (SUN)	56°C (HEX) 58°C (SUN)	
			MPXV B7R reverse	AACATTTCCATGAATCGTAGTCC	0.5			
			MPXV B7R probe	HEX-TGAATGAATGCGATACTGTATGTGTGGG-BHQ1* SUN-TGAATGAAT/ZEN/GCGATACTGTATGTGTGGG-3IABkFQ SUN-TGAATGAAT/ZEN/KCGATACTGTATGTGTGGG-3IABkFQ[#]	0.25			
G2R Generic	TNF receptor gene (or CrmB gene)	Single-plex/Duplex	G2R G-F	GGAAAATGTAAAGACAACGAATACAG GGAAARTGTAAAGACAACGAATACAG[#]	0.5	56°C** 58°C***	56°C** 58°C***	Li et al., 2010
			G2R G-R	GCTATCACATAATCTGGAAGCGTA GCTATCACATAATCTGRAAGCGTA[#]	0.5			
			G2R G-Probe	FAM-AAGCCGTAATCTATGTTGTCTATCGTGTCC-BHQ1	0.25			

* Probe used during the initial duplex assay development but provided less than ideal results; N.A. Not applicable; ** T_a initially used for optimization of duplex assay; *** Optimized T_a for both single-plex and duplex assays; [#] Degenerate primer-probe sequences.

Additional File 12: Digital PCR quantification of MPXV synthetic controls (Clade I and Clade II) spiked into molecular grade water. (A) Average measured copy number per reaction across the three dilution points (2000, 200, and 20 copies per reaction). (B) % loss, which was calculated by the formula (expected-measured copy numbers)/expected copy numbers × 100.





* Average % loss calculated from the three dilution points (2000, 200, and 20 copies per reaction).