Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

Supporting Information

Single-channel digital LAMP multiplexing using Amplification Curve Analysis

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Materials and Methods

1. LAMP primer sequences

Primer sequences for each of the targets are summarised in Table S1. A LAMP assay was designed for the detection of the M gene of the influenza A virus. Genomic sequences were retrieved from NCBI (http://www.ncbi.nlm.nih.gov/genbank/) and sequence alignment was performed using the MUSCLE algorithm. A conserved region of interest was selected, and the sequence was uploaded into Primer Explorer for the generation of several sets of LAMP assays. Further manual optimisation and design of loops primers were performed using GENEious Prime 2020.1.2 (https://www.geneious.com). Primer sequences specific to each of the targets were analyzed with IDT OligoAnalyzer software (https://eu.idtdna.com/pages/tools/oligoanalyzer) using the J. SantaLucia thermodynamic table for melting temperature (Tm) evaluation, hairpin, self-dimer, and cross-primer formation. Primers were purchased from IDT and rehydrated in TE (pH 8.00) at 500 µM. A 50x primer mix was prepared for each target and subsequently, the 10x 5plex-LAMP was obtained by mixing each specific primer mix at equitable volumes.

2. Multiplex real-time LAMP

Real-time LAMP reactions consisted of 6 µL final reaction volume including: 0.60 µL of 10x custom isothermal buffer, 0.30 µL of Mg SO4 (100 mM stock), 0.34 µL of dNTPs (25 mM stock), 0.36 µL of BSA (20 mg/mL), 0.48 µL of Betaine (5 M stock), 0.60 µL of 10x 5plex LAMP primer mix, 0.15 µL of NaOH (0.2 M stock), 0.03 µL of Bst 2.0 DNA polymerase (120 kU/µL stock), 0.30 µL of EvaGreen (20x stock), 1.8 µL of the target oligonucleotide and enough nuclease free water to have a final volume of 6 µL. Amplification reaction was performed at 63°C for 35 cycles of 60 s duration reading at the end of each cycle. Melting curve analysis was performed after the amplification reaction and consisted of 1 cycle at 95°C for 10 s, 65°C for 60 s, and gradual temperature change from 65°C to 97°C with a step of 2.2 °C/s reading every 0.2 °C. LAMP protocol was based and adapted from Rodriguez-Manzano et al.[1]

3. Multiplex real-time digital LAMP

Real-time digital LAMP reactions consisted of 6 µL final reaction volume including: 0.024 µL of ROX (50 µM stock), 0.60 µL of 20x GE Sample Loading Reagent (Fluidigm), 0.60 µL of 10x custom isothermal buffer, 0.30 µL of $MgSO_4$ (100 mM stock), 0.34 µL of dNTPs (25 mM stock), 0.36 µL of BSA (20 mg/mL), 0.48 µL of Betaine (5 M stock), 0.60 µL of 10x 5plex LAMP primer mix, 0.15 µL of NaOH (0.2 M stock), 0.03 µL of Bst 2.0 DNA polymerase (120,000 U/µL stock), 0.30 µL of EvaGreen (20x stock), 1.8 µL of the target oligonucleotide and enough nuclease free water to have a final volume of 6 µL. The qdPCR 37K integrated fluidic circuit (IFC) was used to perform the dLAMP experiments. Firstly, the 48.48 control lines fluid were injected into each accumulator of the qdPCR 37K IFC and primed in the IFC Controller MX. Secondly, reactions and 1x GE were loaded into the qdPCR 37K IFC following manufacturer's instructions and the qdPCR 37K IFC was loaded IFC Controller MX. Finally, the qdPCR 37K IFC was placed into the Fluidigm's Biomark HD system and the amplification reaction was performed at 63°C for 35 cycles of 1 min duration reading at the end of each cycle. Melting curve analysis was performed after the amplification reaction and consisted of 1 cycle at 95°C for 10 s, 65°C for 60 s, and gradual temperature change from 65°C to 97°C with a step of 2.2 °C/s reading every 0.2 °C. The qdPCR 37K IFC contains 48 inlets which correspond to 48 panels. Each of the panels contains 770 wells with a volume of 0.85 nL.

4. Synthetic oligonucleotides

Synthetic oligonucleotides (gBlock Gene Fragment) for each of the targets were purchased from IDT and resuspended at 5 ng/µL. Synthetic DNA sequences are included in Table S2.

5. Evaluation of the 5plex-LAMP assay

Performance of the 5plex-LAMP was evaluated by using 10-fold serial dilutions of synthetic DNA of each of the targets at concentrations ranging from 1.8×10^8 to 1.8×10^2 copies per reaction. A total of 8 replicates were performed per each concentration and target.

Specificity of the 5plex-LAMP assay was evaluated *in-silico* by testing the primers with the sequences of the target pathogens and experimentally by cross-testing each LAMP assay with all the other targets including non-template controls (NTC).

6. Machine learning methods for the detection of amplification events: ACA, MCA and FFI

Multiple standard packages and in-house scripts in Python (v3.7) were developed to analyse the data: (i) FFI values were extracted from each amplification curves, considering only the last values in the cycle time series. The FFI model consisted in a logistic regression classifier to distinguish different targets (please note that these assays are not optimised for improve FFI classification). (ii) A *k*-Nearest neighbour model was used to implement the ACA model using scikit-learn package with default parameters (for more information please see provided code and package documentation). The ACA classification accuracy (i.e., proportion of correctly identified events), sensitivity (i.e., true positive rate), and specificity (i.e., true negative rate) values in Tables 1 were computed for each binary classification subproblem in the one-vs-one multiclass classification scheme. (iii) The MCA classifier distinguished the melting peak temperature or peak T_m, using a supervised machine learning classifier, in this study we used a logistic regression. Performance of the models was evaluated based on out-of-sample classification accuracy, as determined by 10-fold cross-validation (using stratified splits). In order to assess the performance as a function of the volume of training data, shuffled stratified split was performed five times, with 5000 test samples. All data and code used in this study can be found at https://github.com/LMigliet/pyiACA.

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Table S1. Primer sequences of the LAMP assays used for the 5plex-LAMP.

Assay	Primer	Sequence (5' to 3')	Gene	LOD[a]	Author	Ref.
LAMP-FA1	F3	GGCTATGGAGCAAATGGCTG	M	180 copies/ reaction (1.8 µL sample in 6 µL reaction)	This study	
LAMP-FA1	B3	CACTTGAACCGTTGCATCTG				
LAMP-FA1	LF	CTGACTAGCAACCTCCATGG				
LAMP-FA1	LB	GCTGGTCTGAAAAATGATCTTCTTG				
LAMP-FA1	FIP	CGCTTGCACCATTTGCCTAGCGATCGAGTGAGCAAGCAGC				
LAMP-FA1	BIP	TGGGACTCATCCTAGCTCCAGTCACCCCCATTCGTTTCTGA				
LAMPcov	F3	ACCAATAGCAGTCCAGATGA	N	10 copies/ reaction (4 µL sample in 10 µL reaction)	*Rodriguez-Manzano et al.*	[1]
LAMPcov	B3	CACGATTGCAGCATTGTTAGC				
LAMPcov	LF	GGACTGAGATCTTTCATTTTACCGT				
LAMPcov	LB	ACTGAGGGAGCCTTGAATACA				
LAMPcov	FIP	TCTGGCCCAGTTCCTAGGTAGTCCAGACAAATTCGTGGTGG				
LAMPcov	BIP	GGACTTCCCTATGGTGCTAACAAACGGGTGCCAATGTGATCT				
LAMP-FB1	F3	AGGGACATGAACAACAAAGA	NS1	1 copy (5 µL sample in 25 µL reaction)	*Mahony et al.*	[2]
LAMP-FB1	B3	CAAGTTTAGCAACAAGCCT				
LAMP-FB1	LF	TCAAACGGAACTTCCCTTCTTTC				
LAMP-FB1	LB	GGATACAAGTCCTTATCAACTCTGC				
LAMP-FB1	FIP	TCAGGGACAATACATTACGCATATCGATAAAGGAGGAAGTAAACACTCA				
LAMP-FB1	BIP	TAAACGGAACATTCCTCAAACACCACTCTGGTCATAGGCATTC				
LAMP-HAdV	F3	GTGCGACAGGACCATGTG	HEXON	1.9×10^2 copies/ml of DNA	*Li et al.*	[3]
LAMP-HAdV	B3	GGTAGACGGCCTCGATGA				
LAMP-HAdV	LF	GGCCCCCCATGGACATGAA				
LAMP-HAdV	LB	CCACCCTGCTTTATCTTCTTTTCG				
LAMP-HAdV	FIP	AGCATGTTCTGTCCCAGGTCGGCATTCCCTTCTCCAGCAA				
LAMP-HAdV	BIP	GAGGTGGATCCCATGGATGAGCACTCTGACCACGTCGAARAC				
LAMP-KPn	F3	GGATATCTGACCAGTCGG	RCSA	10 copies /reaction (1 µL sample in 5 µL reaction)	*Dong et al.*	[4]
LAMP-KPn	B3	GGGTTTTGCGTAATGATCTG				
LAMP-KPn	LB	GAAGACTGTTTCGTGCATGATGA				
LAMP-KPn	FIP	CGACGTACAGTGTTTCTGCAGTTTTAAAAAACAGGAAATCGTTGAGG				
LAMP-KPn	BIP	CGGCGGTGGTGTTTCTGAATTTTGCGAATAATGCCATTACTTTC				

[a]Limit of detection (LOD).

Table S2. Synthetic DNA sequences (gBlocks) and accession numbers.

Target gene	Sequence (5' to 3')
Klebsiella Pneumoniae (RCSA) NC_012731.1	ATGTCAACGATGATTATGGATTTGTGCAGCTATACCCGGTTGGGATTGACGGGA TATCTGACCAGTCGGGGAATTAAAAAACAGGAAATCGTTGAGGTCAACAGTGCT GCGGATCTGCAGAAACACTGTACGTCGTGTTGCCCGGCGGTGGTGTTTCTGAAT GAAGACTGTTTCGTGCATGATGATGAAAGTAATGGCATTATTCGCCAGATCATT ACGCAAAACCCGGCGACGCTGTTTGTTATCTTTATGTCGCTGGCGAACATCCAT TTTGACCGCTATTTGCGGGTACGGAAGAATCTGCTAATCAGTTCAAAATCGATA ACCCCAAAAGACCTTGATGTTATTCTGGTTAATTATCTTAAATACAAAAACACC AGTGTAGGGCAGTTAACTTTACCGACATTGTCACTGAGTAAAACAGAATCAAAT ATGCTGCAAATGTGGATGGCCGGGCATGGTACTTCGCAAATCTCAACGCAAATG AACATCAAAGCGAAGACGGTATCGTCGCATAAAGGCAATATTAAAAAGAAAATA CAAACGCATAATAAGCAGGTGATTTATCATATCGTTCGGCTGACCGAAAACATC ACCTCCGGTATTCAGGTAAATATGCGCTGA
Human Adenovirus (HEXON) AF542104.1	AGGTCTCCATCATGTTTGACTCCTCAGTCAGCTGGCCTGGCAATGACAGGCTGT TGAGCCCAAATGAGTTTGAAATCAAGCGCACTGTGGACGGGGAAGGATACAACG TGGCACAATGCAACATGACCAAAGACTGGTTCCTAGTTCAGATGCTTGCCAACT ACAACATTGGCTACCAGGGCTTTTACATCCCTGAGGGATACAAGGATCGCATGT ACTCTTTTTTCAGAAACTTCCAGCCTATGAGCAGGCAGGTGGTTGATGAGGTTA ATTACACTGACTACAAAGCCGTCACCTTACCATACCAACACAACAACTCTGGCT TTGTAGGGTACCTTGCACCTACTATGAGACAAGGGGAACCTTACCCAGCCAATT ATCCATACCCGCTCATCGGAACTACTGCCGTTAAGAGTGTTACCCAGAAAAAGT TCCTGTGCGACAGGACCATGTGGCGCATTCCCTTCTCCAGCAACTTCATGTCCA TGGGGGCCCTTACCGACCTGGGACAGAACATGCTCTATGCCAACTCAGCCCATG CGCTGGACATGACTTTTGAGGTGGATCCCATGGATGAGCCCACCCTGCTTTATC TTCTTTTCGAAGTCTTCGACGTGGTCAGAGTGCACCAGCCACACCGCGGCGTCA TCGAGGCCGTCTACCTGCGCACACCGTTCTCGGCCGGCAACGCCACCACATAA
Influenza B (NS1) CY120033.1	TAATACGACTCACTATAGGGAGTAAAAATGATGAAAGTACTCCTATTTATGAATC CGTCTGCTGGAATTGAAGGGTTTGAGCCATACTGTATGAAAAGTTCCTCAAATAGC AACTGTCCGGAATACAATTGGACCGATTACCCTTCAACACCAGGGAGGTGCCTTGA TGACATAGAAGAAGAACCAGAGGATGTTGATGGCCCAACTGAAATAGTATTAAGGG ACATGAACAACAAAGATGCAAGGCAAAAGATAAAGGAGGAAGTAAACACTCAGAAA GAAGGGAAGTTCCGTTTGACAATAAAAAGGGATATGCGTAATGTATTGTCCTTGAG AGTGTTGGTAAACGGAACATTCCTCAAACACCCCAATGGATACAAGTCCTTATCAA CTCTGCATAGATTGAATGCATATGACCAGAGTGGAAGGCTTGTTGCTAAACTTGTT GCTACTGATGATCTTACAGTGGAGGATGAAGAAGATGGCCATCGGATCCTCAACTC ACTCTTCGAGCGTCTTAATGAAGGACATTCAAAGCCAATTCGAGCAGCTGAAACTG CGGTGGGAGTCTTATCCCAATTTGGTCAAGAGCACCGATTATCACCAGAAGAGGGA GACAATTAGACTGGTCACGGAAGAACTTTATCTTTTAAGTAAAAGAATTGATGATA ACATATTGTTCCACAAAACAGTAATAGCTAACAGCTCCATAATAGCTGCTTTAGTG AGGGTTAATT
Influenza A (M) NC_002016	TAATACGACTCACTATAGAGCGAAAGCAGGTAGATATTGAAAGATGAGTCTTCTAAC CGAGGTCGAAACGTACGTTCTCTCTATCATCCCGTCAGGCCCCCTCAAAGCCGAGAT CGCACAGAGACTTGAAGATGTCTTTGCAGGGAAGAACACCGATCTTGAGGTTCTCAT GGAATGGCTAAAGACAAGACCAATCCTGTCACCTCTGACTAAGGGGATTTTAGGATT TGTGTTCACGCTCACCGTGCCCAGTGAGCGAGGACTGCAGCGTAGACGCTTTGTCCA

	AAATGCCCTTAATGGGAACGGGGATCCAAATAACATGGACAAAGCAGTTAAACTGTA TAGGAAGCTCAAGAGGGAGATAACATTCCATGGGGCCAAAGAAATCTCACTCAGTTA TTCTGCTGGTGCACTTGCCAGTTGTATGGGCCTCATATACAACAGGATGGGGGCTGT GACCACTGAAGTGGCATTTGGCCTGGTATGTGCAACCTGTGAACAGATTGCTGACTC CCAGCATCGGTCTCATAGGCAAATGGTGACAACAACCAACCCACTAATCAGACATGA GAACAGAATGGTTTTAGCCAGCACTACAGCTAAGGCTATGGAGCAAATGGCTGGATC GAGTGAGCAAGCAGCAGAGGCCATGGAGGTTGCTAGTCAGGCTAGGCAAATGGTGCA AGCGATGAGAACCATTGGGACTCATCCTAGCTCCAGTGCTGGTCTGAAAAATGATCT TCTTGAAAATTTGCAGGCCTATCAGAAACGAATGGGGGTGCAGATGCAACGGTTCAA GTGATCCTCTCGCTATTGCCGCAAATATCATTGGGATCTTGCACTTGATATTGTGGA TTCTTGATCGTCTTTTTTTCAAATGCATTTACCGTCGCTTTAAATACGGACTGAAAG GAGGGCCTTCTACGGAAGGAGTGCCAAAGTCTATGAGGGAAGAATATCGAAAGGAAC AGCAGAGTGCTGTGGATGCTGACGATGGTCATTTTGTCAGCATAGAGCTGGAGTAAA AAACTACCTTGTTTCTACTCTTTAGTGAGGGTTAATT
SARS-CoV-2 (N)	IDT 2019-nCoV_N Positive Control

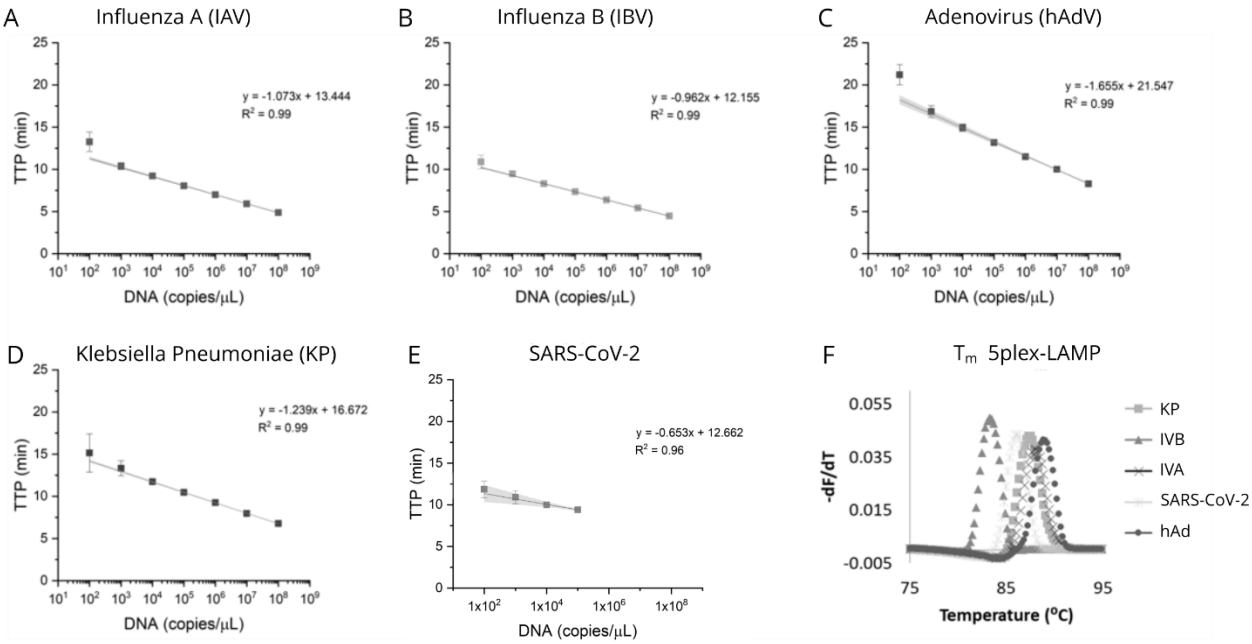

Figure S1. Standard and melting curves of the 5plex-LAMP in a real-time quantitative fluorescence-based instrument. (A) Standard curve for Influenza A virus. (B) Standard curve for Influenza B virus. (C) Standard curve for human adenovirus. (D) Standard curve of *Klebsiella Pneumoniae*. (E) Standard curve for severe acute respiratory syndrome coronavirus 2. (F) Specific melting curves of all the targets.

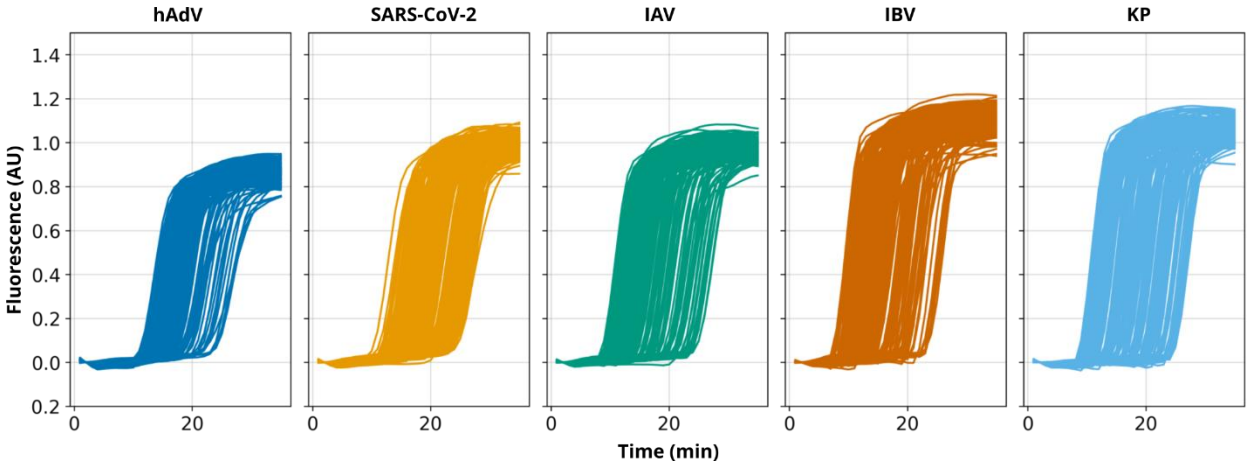

Figure S2. Amplification curves of the 5plex-LAMP in dLAMP using a single fluorescence channel.

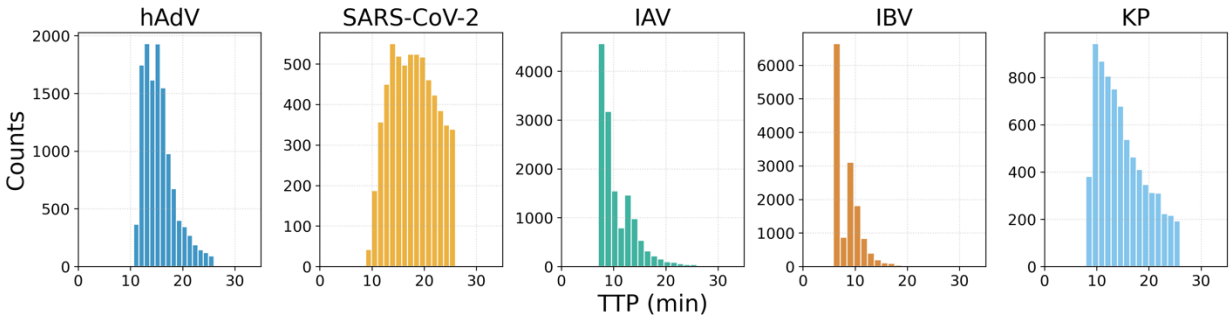

Figure S3. Histogram showing the distribution of Time-To-Positive (TTP) values of the 5plex-LAMP in dLAMP using a single fluorescence channel.

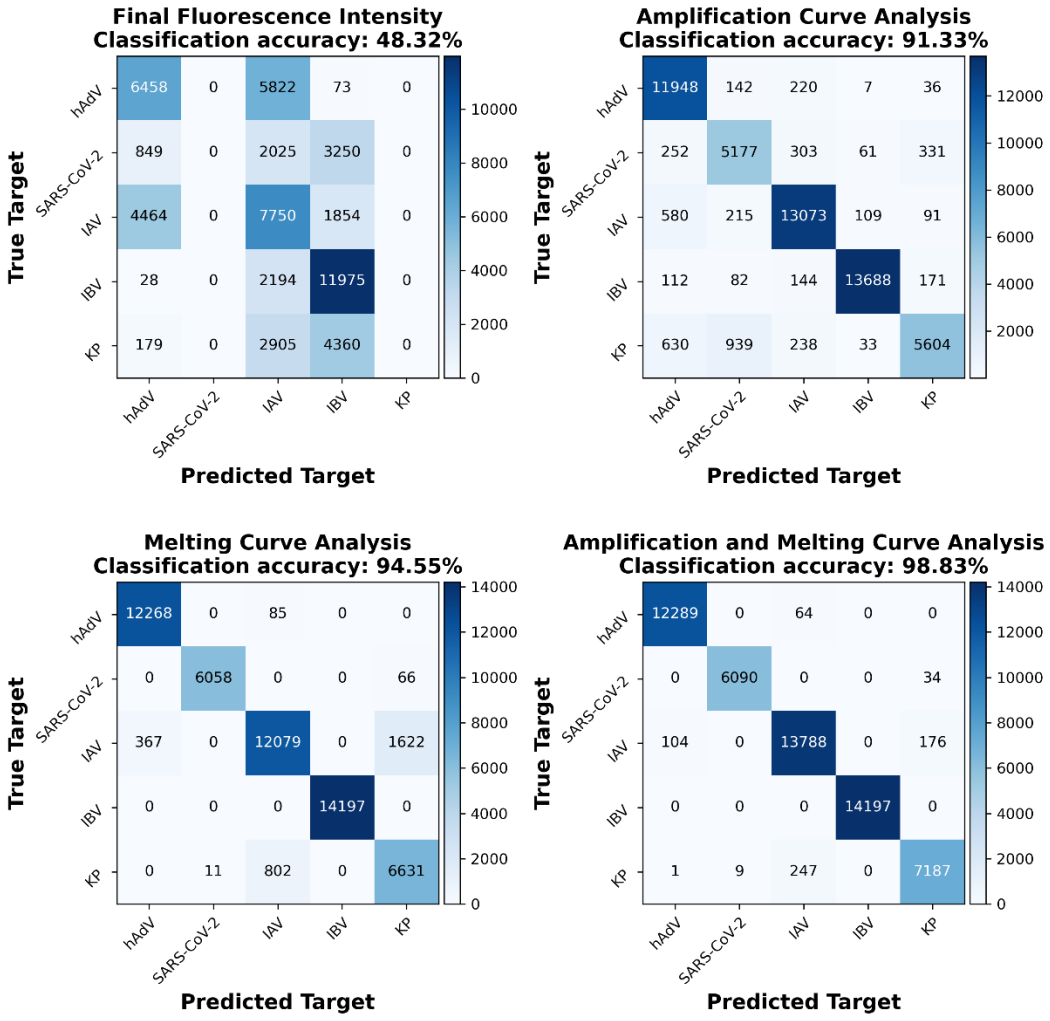

Figure S4. Confusion matrices showing the prediction performance of the four methods evaluated in this study: FFI, ACA, MCA and AMCA.

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