Electronic Supplementary Material (ESI) for Analyst.
This journal is © The Royal Society of Chemistry 2022

Supporting Information

Multiplex Digital PCR with Digital Melting Curve Analysis on a Self-partitioning SlipChip

Yan Yu,[1] Ziqing Yu,[1] Xufeng Pan,[2] Lei Xu,[1] Rui Guo,[1] Xiaohua Qian,[1] Feng Shen[1,] *

1. School of Biomedical Engineering, Shanghai Jiao Tong University, 1954 Hua Shan Road, Shanghai, China
2. Department of Thoracic Surgery, Shanghai Chest Hospital, Shanghai Jiao Tong University, Shanghai, China

1. Single-plex real time qPCR protocol

Single-plex real-time qPCR and melting curve analysis were performed by the LightCycler 96 real-time PCR system (Roche Diagnostics, Germany). For real-time qPCR, each PCR amplification was performed in a total volume of 10 μL with 5 μL of Premix Taq, 0.5 μL of BSA, 0.5 μL of 20 × EvaGreen, 0.2 μL of forward primer (10 μM), 0.2 μL of reverse primer (10 μM), 2 μL of template and 1.6 μL of ddH_2O. The primers were previously characterized [1] and are listed in Table S1. The cycling conditions included preincubation at 95 °C for 180 s and the following amplification for a total of 35 cycles: denaturation at 95 °C for 10 s, annealing at 62 °C for 15 s, and extension at 72 °C for 30 s. Melting curve analysis was performed with one cycle at 95 °C for 60 s, 40 °C for 60 s and read from 65 °C to 97 °C with an increment of 0.07 °C. The concentrations were approximately 990 copies/μL, 4930 copies/μL, 1245 copies/μL, 885 copies/μL, 4348 copies/μL for the *S. aureus, A. baumannii, S. pneumoniae, H. influenzae,* and *K. pneumoniae* amplicons, respectively. Three replicates were performed to characterize the Tm.

Table S1. Sequences of the primers and property of amplicons

Primer	Primer sequence (5' - 3')	Amplicon length (bp)	Amplicon GC%
S. aureus-F	AGTCACGTCTCGATCGAACA	175	34%
S. aureus-R	GAAACTTGACCACGATCCGG		
A. baumannii-F	GGCTGGACATCATCAACTGC	193	38%
A. baumannii-R	GTCGGCCTGATCTCGTATGA		
S. pneumoniae-F	GCACACTCAACTGGGAATCC	110	47%
S. pneumoniae-R	ATGCAACCGTTCCCAACAAT		
H. infuenzae-F	CTGGTGTTGCGGCTAAAAGT	168	48%
H. infuenzae-R	TCATTAACTGGGGCTTCGGT		
K. pneumoniae-F	TACACAATCGCCCGTTGAAC	223	60%
K. pneumoniae-R	CCCGGTTAGATCCATGGTGA		

2. Experimental protocol for digital PCR of Stilla

Stilla digital PCR was used to quantify the concentration of the plasmids. The primers used in this study were the same as those used in real-time qPCR and digital melting curve analysis. Each amplification reaction consisted of a 25 μL final volume with 12.5 μL of 2 × PerfeCTa qPCR ToughMix, 2 μL of 20 × EvaGreen, 2.5 μL of forward primer (10 μM), 2.5 μL of reverse primer (10 μM), 1 μL of 25 × Alexa Fluor 647, 2 μL of template and 2.5 μL of ddH_2O. The PCR amplification protocol consisted of an initial denaturation step of 240 s at 95 °C followed by 35 cycles at 95 °C for 50 s, 62 °C for 30 s, and 72 °C for 60 s.

3. Optimization of the concentration of primers for multiplex digital melting curve analysis

The proportion of five pairs of primers was optimized on LC96. Five nucleic acid templates of *S. aureus*, *A. baumannii*, *S. pneumoniae*, *H. influenzae*, and *K. pneumoniae* were mixed at a 1:1:1:1:1 concentration ratio according to the quantification result of the Stilla digital PCR. Five pairs of primers were mixed at a 1:1:1:1:1 ratio at a concentration of 0.1 μM. The reagents, amplification

protocol and melting curve analysis protocol of 5-plex real-time qPCR were the same as those of single plex real-time qPCR. The melting curve of 5-plex qPCR is shown in Figure S1A. There was a large difference in the heights of five peaks. Then, the concentration ratios of five primer pairs of *S. aureus*, *A. baumannii*, *S. pneumoniae*, *H. influenzae*, and *K. pneumoniae* were adjusted and mixed at a 7:4:6:7:6 ratio. The melting curves are shown in Figure S1B. The heights of the melting peaks of the five plasmids were similar. Therefore, the concentration ratio of five pairs of primers for 5-plex digital PCR and digital melting curve analysis was identified as 7:4:6:7:6.

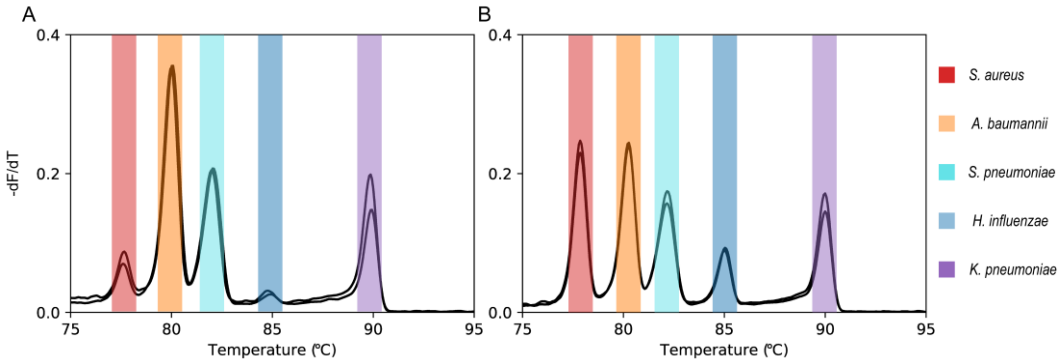

Figure S1. Comparison of the melting curve and melting temperature of different primer concentrations for multiplex melting curve analysis. A) Melting peaks with a primer ratio of 1:1:1:1:1 B) Melting peaks with a primer ratio of 7:4:6:7:6.

Supplementary tables and figures

Table S2. Concentrations of Combination 1

Species	Ratio	Expected concentration (copies/μL)	Calculated concentration (copies/μL)
S. aureus	1	1.5	2.1±0.6
A. baumannii	3	4.5	6.2±0.8
S. pneumoniae	9	13.5	23.4±2.3
H. influenzae	27	40.5	63.1±8.0
K. pneumoniae	81	121.5	140.8±5.2

Table S3. Concentrations of Combination 2

Species	Ratio	Expected concentration (copies/μL)	Calculated concentration (copies/μL)
S. aureus	3	4.5	6.2±0.8
A. baumannii	9	13.5	18.4±2.4
S. pneumoniae	27	40.5	54.8±2.4
H. influenzae	81	121.5	140.0±41.1
K. pneumoniae	1	1.5	1.5±0.5

Table S4. Concentrations of Combination 3

Species	Ratio	Expected concentration (copies/μL)	Calculated concentration (copies/μL)
S. aureus	9	13.5	20.1±1.8
A. baumannii	27	40.5	49.8±12.6
S. pneumoniae	81	121.5	153.3±20.4
H. influenzae	1	1.5	1.9±0.1
K. pneumoniae	3	4.5	3.9±0.5

Table S5. Concentrations of Combination 4

Species	Ratio	Expected concentration (copies/μL)	Calculated concentration (copies/μL)
S. aureus	27	40.5	64.6±1.9
A. baumannii	81	121.5	148.0±18.3
S. pneumoniae	1	1.5	1.7±0.5
H. influenzae	3	4.5	5.5±1.4
K. pneumoniae	9	13.5	15.4±3.5

Table S6. Concentrations of Combination 5

Species	Ratio	Expected concentration (copies/μL)	Calculated concentration (copies/μL)
S. aureus	81	121.5	162.4±23.0
A. baumannii	1	1.5	2.1±0.1
S. pneumoniae	3	4.5	5.3±1.4
H. influenzae	9	13.5	12.5±2.9
K. pneumoniae	27	40.5	36.4±4.2

Table S7. Expected and detected concentrations of target for the single-plex assay

	S. aureus	*A. baumannii*	*S. pneumoniae*	*H. influenzae*	*K. pneumoniae*
Expected concentration (copies/μL)	107	150	93.4	123	190
Calculated concentration (copies/μL)	177	200	95.0	197	289

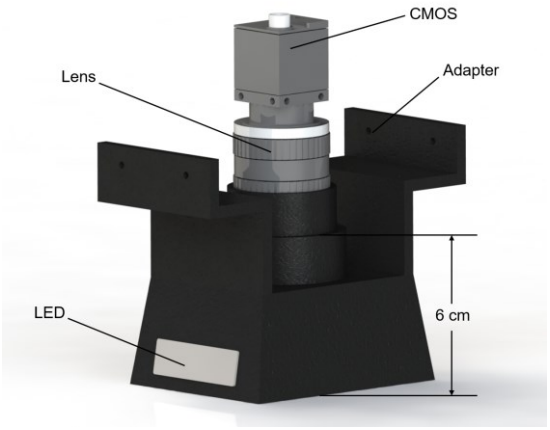

Figure S2. Schematic design of the fluorescence imaging module for digital melting curve analysis.

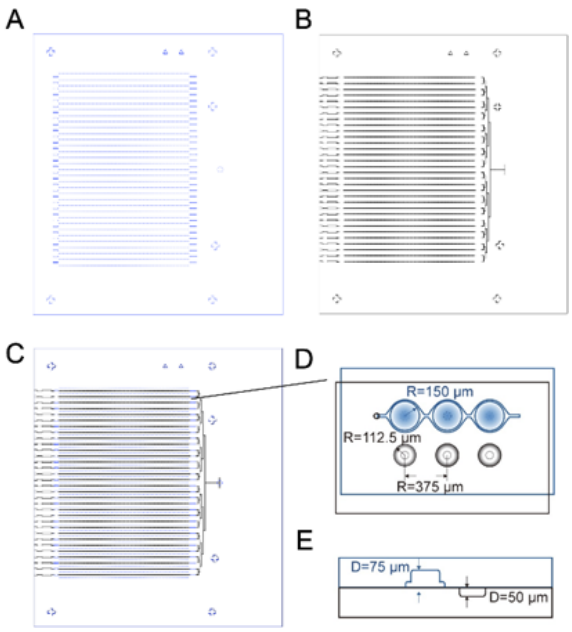

Figure S3. Schematic of the sp-SlipChip for digital melting curve analysis. A) Design of the bottom plate, which contains arrays of microwells. B) Design of the top plate, which contains "chain-of-pearl"-shaped microfluidic channels. C) Schematic of the assembled sp-SlipChip after slipping. D) Top view of the "chain-of-pearl" flow channel and microwells. E) Side view of the designed geometry of "pearls" and microwells.

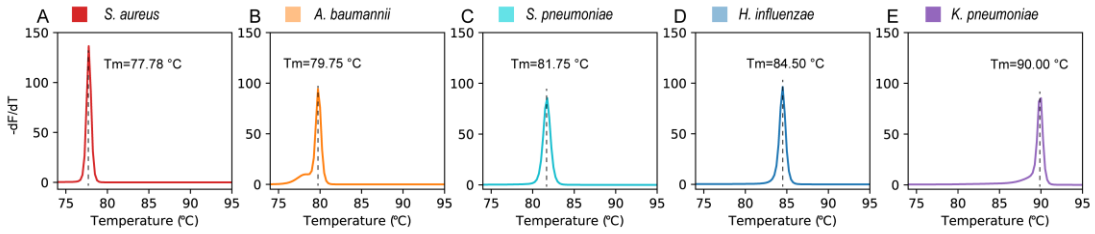

Figure S4. Graphs represent the predicted melting curves of target amplicons by uMelt. A) Predicted melting curve for amplicons of *S. aureus*. B) Predicted melting curve for amplicons of *A. baumannii*. C) Predicted melting curve for amplicons of *S. pneumoniae*. D) Predicted melting curve for amplicons of *H. influenzae*. E) Predicted melting curve for amplicons of *K. pneumoniae*.

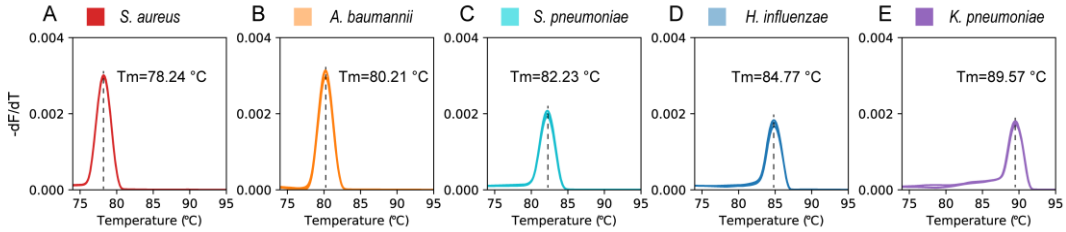

Figure S5. Graphs represent the experimental melting curves of target amplicons by the Roche LC96 real-time qPCR instrument. A) Experimental melting curve for amplicons of *S. aureus*. B) Experimental melting curve for amplicons of *A. baumannii*. C) Experimental melting curve for amplicons of *S. pneumoniae*. D) Experimental melting curve for amplicons of *H. influenzae*. E) Experimental melting curve for amplicons of *K. pneumoniae*.

Reference

[1] L. Hu, B. Han, Q. Tong, H. Xiao, D. Cao, Detection of Eight Respiratory Bacterial Pathogens Based on Multiplex Real-Time PCR with Fluorescence Melting Curve Analysis, Can. J. Infect. Dis. Med. Microbiol. 2020 (2020). https://doi.org/10.1155/2020/2697230.