Supporting information

FEN1-assisted LAMP for specific and multiplex detection of pathogens associated with community-acquired pneumonia

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Experiment section

1. Reagents and devices

Bst DNA Polymerase (M0275), Thermostable FEN1 (M0645S), ThermoPol Reaction Buffer (B9004S) and Magnesium Sulfate Solution (B1003S) were purchased from New England Biolabs. The dNTP Mix was obtained from Shanghai Sangon Biotech. SYTO-9 (S34854) was purchased from Thermo Fisher Scientific. Plasmid sequences of *Mycoplasma pneumoniae*, *Streptococcus pneumoniae* and *Haemophilus influenzae* were obtained from GenBank database. The detailed plasmid information of three pathogens is listed in Table S1. Primers were designed by entering target sequence on the website of designing primer for loop-mediated isothermal amplification (https://primerexplorer.jp/e/index.html) containing FIP (consisted of F2 region and F1c

region), BIP (consisted of B2 region and B1c region), F3 region and B3 region. Sequences of each primer are shown in Table S2. Plasmids, primers and flap probes were synthesized by Shanghai Sangon Biotech. The amplification reaction and real-time fluorescence monitoring were performed on qTOWER^3 PCR device from Analytik Jena.

2. The design of flap probe

Each flap probe is composed of a binding region at 3' end and a flap region at 5' end, the latter of which can form a flap structure after the probes hybridize with target DNA. Additionally, all probes are labelled with a detectable fluorescent group at 5' end, such as FAM, ROX or HEX, and a quenching group was modified at the T base in the middle of each flap probe. The sequences of flap probe are demonstrated in Table S3.

3. FEN1-assisted LAMP assay

FEN1-assisted LAMP assay is with a total volume of 20 μL, which contains: 1 × ThermoPol reaction buffer, 8 mM $MgSO_4$, 1.4 mM dNTP Mix, 1.6 μM of FIP and BIP, 0.2 μM of F3 and B3 primers, 8 U Bst DNA polymerase, 1.2 U FEN1, 200 nM of flap probe, 2 μL of target sample and nuclease-free Water. The FEN1-assisted LAMP assay was carried out for 90 min at 65 °C. Threshold time (Tt) values were automatically determined by the system software. The conventional LAMP reaction did not contain FEN1 and flap probe, and SYTO-9 was added to monitor its fluorescence signal.

4. Clinical sample preparation and ethics approval

A total of 64 clinical serum samples (Table S4) were collected from the Fujian Provincial Hospital (Fuzhou, China). Genomic DNA in these serum samples were extracted using SUPRall super extraction kit and following the manufacturer's protocols provided by (Sansure Biotech Inc. China). The study was approved by the Ethics Committee of the Fujian Provincial Hospital (No. 2024-Ky-018). All clinical research was performed in accordance with relevant guidelines and regulations. All participants have provided informed consent.

Table S1. The plasmid sequence of *Mycoplasma pneumoniae*, *Streptococcus pneumoniae* and *Haemophilus influenzae*. Sequences marked with red, orange, blue and green represent F3, B3, FIP and BIP, respectively. The grey part among FIP or BIP is the binding region of flap probe.

Target	Sequence (5'-3')
Mycoplasma pneumoniae	GTTAAATACGGTAAGGAAAACGAGTTTGCTGCTAACGA GTACGAGCGCTTTAACCAGAAGTTAACGGTAGCTCCTA CCCAAGGAACAAACTGATCCCACTTCTCCCCCACGCTTT CCCGTTTCTCCACCGGGTTCAACCTTGGGATTTGGGGTC GGTGCTCGACCAGGTGTTGGATTATGTGCCCTGGATTGG GAATGGGTACAGGTATGGCAATAACCACCGGGGCGTGG ATGATATAACCGCGCCTCAAACCAGCGCGGGGTCGTCC AGCGGAATTAGTACGAACACAAGTGGTTCGCGTTCCTTT CTCCCGACGTTTTCCAACATCGGCGTCGGCCTCAAAGCG AATGTCCAAGCCACCCTCGGGGGCAGTCAGACGATGAT TACAGGCGGTTCGCCTCGAAGAACCCTCGACCAAGCCA ACCTCCAGCTCTGAACGGGGGCGGGGTGAAGGAATGAT AAGGCTTCAAGTGGACAAAGTGACGAAAACCACACCAA GTTCAC
Streptococcus pneumoniae	GGCGGTTGGAATGCTGAGACCTATGCAGCGGTTGAACT GATTGAAAGCCATTCAACTAAAGAAGAGTTCATGACGG ACTACCGCCTTTATATCGAACTCTTACGCAATCTAGCAG ATGAAGCAGGTTTGCCGAAAACGCTTGATACAGGGAGT TTAGCTGGAATTAAAACGCACGAGTATTGCACGAATAA CCAACCAAACAACCACTCAGACCATGTGGATCCATACC CTTACTTGGCAAAATGGGGCATTAGCCGTGAGCAGTTTA AGCATGATATTGAGAACGGCTTGACGATTGAAACAGGC TGGCAGAAGAATGACACTGGCTACTGGTACGTACATTC AGACGGCTCTTATCCAAAAGACAAGTTTGAGAAAATCA ATGGCACTTGGTACTACTTTGACAGTTCAG
Haemophilus influenzae	CGGAATTACTTCCACAAATGGGAATGGATTTGAAATTA GTTCAATTAATTGCTTATACAGATTGGAAAGAAACACA AGAAAAAGACCCAAAGGGTTATTGGGTAAACTATAATT ACGATTGGATGTTTAAACCTGGTGCAATGGCAGAAGTG GTTAAATATGCCGATGGTGTTGGCCCAGGTTGGTATATG TTAGTTAATAAAGAAGAATCCAAACCTGATAATATTGT GTACACTCCGTTGGTAAAAGAACTTGCACAATATAATGT GGAAGTGCATCCTTACACCGTGCGTAAAGATGCACTGC CCGAGTTTTTCACAGACGTAAATCAAATGTATGATGCCT TATTGAATAAATCAGGGGCAACAGGTGTATTTACTGATT TCCCAGATACTGGCGTGGAATTCTTAAAAGGAATAAAA

	TAA

Table S2. The sequences of primers used in detecting three pathogens.

Plasmids	primers	Sequence (5'-3')
Mycoplasma pneumoniae	F3	AACGAGTTTGCTGCTAACGA
	B3	GCGGTTATATCATCCACGCC
	FIP	TGGGGGAGAAGTGGGATCAGTTGTACGAGCG CTTTAACCAGA
	BIP	TCTCCACCGGGTTCAACCTTGCCTGTACCCAT TCCCAATCCFF
Streptococcus pneumoniae	F3	CTAGCAGATGAAGCAGGTT
	B3	ATCGTCAAGCCGTTCTCA
	FIP	GGTTATTCGTGCAATACTCGTGCCGAAAACGC TTGATACAGG
	BIP	ACCAAACAACCACTCAGACCATATCATGCTTA AACTGCTCAC
Haemophilus influenzae	F3	GGTTATTGGGTAAACTATAATTACG
	B3	TGAAAAACTCGGGCAGTG
	FIP	ATACCAACCTGGGCCAACACGATGTTTAAAC CTGGTGCAATG
	BIP	ACCTGATAATATTGTGTACACTCCGAGGATGC ACTTCCACATT

Table S3. The sequence of each flap probe.

Flap probe	Sequence (5'-3')
Mycoplasma pneumoniae	FAM-ACTATTAGCAGCGAGT/iTAMdT/AACGGTAGCTCCTACC CAAGGAACA
Streptococcus pneumoniae	ROX-CTCATTGTTAA/BHQ2T/GCCCCATTTTGCCAAGTAAGGG TATGGATCC
Haemophilus influenzae	HEX-CTCATTGTGGTGGCAGAAG/BHQ1T/GGTTAAATATGCCG ATG

Table S4. The detail information of clinical samples collected from Fujian Provincial Hospital.

Number	This method for MP	PCR result
1	positive	Mycoplasma pneumoniae
2	positive	Mycoplasma pneumoniae
3	positive	Mycoplasma pneumoniae
4	positive	Mycoplasma pneumoniae
5	positive	Mycoplasma pneumoniae
6	positive	Mycoplasma pneumoniae
7	positive	Mycoplasma pneumoniae
8	positive	Mycoplasma pneumoniae
9	positive	Mycoplasma pneumoniae
10	positive	Mycoplasma pneumoniae
11	positive	Mycoplasma pneumoniae
12	positive	Mycoplasma pneumoniae
13	positive	Mycoplasma pneumoniae
14	negative	Human rhinovirus
15	negative	Human rhinovirus
16	negative	Human rhinovirus
17	negative	Human rhinovirus
18	negative	Human rhinovirus
19	negative	Human rhinovirus
20	negative	Human rhinovirus
21	negative	Influenza A virus
22	negative	Influenza A virus
23	negative	Influenza A virus
24	negative	Influenza A virus
25	negative	Influenza A virus
26	negative	Influenza A virus
27	negative	Influenza A virus
28	negative	Human Adenovirus
29	negative	Human Adenovirus
30	negative	Human Adenovirus
31	negative	Human Adenovirus
32	negative	Human Adenovirus
33	negative	Influenza B virus
34	weak positive	SARS-COV-2

35	negative	SARS-COV-2
36	negative	SARS-COV-2
37	negative	negative
38	negative	negative
39	negative	negative
40	negative	negative
41	negative	negative
42	negative	negative
43	negative	negative
44	negative	negative
45	negative	negative
46	negative	negative
47	negative	negative
48	negative	negative
49	negative	negative
50	negative	negative
51	negative	negative
52	negative	negative
53	negative	negative
54	negative	negative
55	negative	negative
56	negative	negative
57	negative	negative
58	negative	negative
59	negative	negative
60	negative	negative
61	negative	negative
62	negative	negative
63	negative	negative
64	negative	negative