Supplementary Material

Simultaneous Detection of Eleven Sexually Transmitted Agents Using Multiplexed PCR Coupled with MALDI-TOF Analysis

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Table S1 | Target gene and sequences of amplification primers and extension probes used in the STI-MS method

Target pathogen	Target	Oligonucleotide
Herpes simplex virus types 1	*gD*	Forward: ACGTTGGATGCTGCGGCTCGTGAAGATAAA Reverse: ACGTTGGATGCGTACTTACAGGAGCCCTTG Extension probe: ATAAACGACTGGACGG
	gG	Forward: ACGTTGGATGCGTGCCGTTGTTCCCATTAT Reverse: ACGTTGGATGTGGTGGAGGAGACGTTGGTG Extension probe: CGGTTCTTGTCGGTGTATCG
Herpes simplex virus types 2	*gD*	Forward: ACGTTGGATGCAGCTTAAAAATCGCCGGGT Reverse: ACGTTGGATGTTCCGGAACGAGTTCGGGT Extension probe: GACACCACCAACGC
	gG	Forward: ACGTTGGATGGTCGTCCGTCACGAGCCCC Reverse: ACGTTGGATGTTGCGTTGGGTGCGCCAAAT Extension probe: CGCTCGTTCCTCACGG
Chlamydia trachomatis	cryptic plasmid	Forward: ACGTTGGATGTTTCTTCAGCGCTACACACG Reverse: ACGTTGGATGTGACAAGCTTAGATCCGTTT Extension probe: TCACACGCTCAAATCATC
	omp1	Forward: ACGTTGGATGGTTTCGGCGGAGATCCTTG Reverse: ACGTTGGATGCACGGTCGAAAACAAAGTCA Extension probe: ACTTGGTGTGACGC
Treponema pallidum	*polA*	Forward: ACGTTGGATGACGTCCGGAACAATAAGAGG Reverse: ACGTTGGATGTGAAACGGATGGATTGCAT Extension probe: TGCTTCCTGAAAGCAGAT
	tpp47	Forward: ACGTTGGATGCAGGCTGACTTTGATTGCGA Reverse: ACGTTGGATGCAGCATCCATCAGAGTCTCC Extension probe: GCCTTCCCAAGTACGA
Neisseria gonorrhoeae	*opa*	Forward: ACGTTGGATGTATATCGGTGTGCGTGTCGG Reverse: ACGTTGGATGGCGGTAAGAGTATTTTTCGT Extension probe: TCAGACACGGTATCGAT
	porA	Forward: ACGTTGGATGCGTGGCGTTTGAAAATACCC Reverse: ACGTTGGATGGTTTGACGATGCCAGCAAAG Extension probe: TCGTTTGAAAATACCCAATTGC
Trichomonas vaginalis	*beta-tubulin*	Forward: ACGTTGGATGTTCCGTACACTCAAGCTCAC Reverse: ACGTTGGATGGCCGGACATAACCATGGAAA Extension probe: CCTCACAACACCAACATA
		Forward: ACGTTGGATGCACTCAAGGTCAAAGTGGCT

	repeated DNA target	Reverse: ACGTTGGATGCTTTGCGAACTGAGGGTAAG Extension probe: CCTTAGTAACCACATTAAAACCTAT
Mycoplasma hominis	*gap*	Forward: ACGTTGGATGCAGGCGCTTCATGTACTACT Reverse: ACGTTGGATGTGGTCTGCTGTATATGAGTG Extension probe: CCTGTTTAGCTCCTATTGC
	YidC	Forward: ACGTTGGATGTAGCTCTTGTCCAGAACCGA Reverse: ACGTTGGATGTATTATTTTTGGCTATCGG Extension probe: ATTTTACAGTAAATGATTGTACACAAC
Ureaplasma urealyticum	*ureA*	Forward: ACGTTGGATGTACCAACCATTGTATCTACA Reverse: ACGTTGGATGGACTTAATGCAATCTGCTCG Extension probe: CCATAACTTGATCAACACGTAA
	ureC	Forward: ACGTTGGATGAAAGGACGTACAATCCACGC Reverse: ACGTTGGATGTATCTGGAGCATGTCCACCA Extension probe: TACGCTTACCATACAGAAG
Mycoplasma genitalium	16S rRNA IR	Forward: ACGTTGGATGCCCAAATCAATGTTTGGTCTC Reverse: ACGTTGGATGGGAACTAGAAAGATTGTCGT Extension probe: CATTTGGTCAGTTTGTATCC
	mgpB	Forward: ACGTTGGATGCCTTAACCCCTTGGACTTGA Reverse: ACGTTGGATGGTTGTCATTTTGGCTTCTTAC Extension probe: GATTACTGGAGAGAACCCA
Ureaplasma parvum	*rpmA*	Forward: ACGTTGGATGCAGGGTAGATTTTATTGCCC Reverse: ACGTTGGATGGGTCGTGACTCAAATCCTAA Extension probe: CTTTGACCTGCTTTAGTAGATTGC
	ureD	Forward: ACGTTGGATGTTTACAATCATGGTTCCACA Reverse: ACGTTGGATGCAACCACGTAAAAATGATG Extension probe: GGGTTCCACAATAATGATAGCC
Haemophilus ducreyi	16S rRNA IR	Forward: ACGTTGGATGGTAGAAAGTCTGAGTAATC Reverse: ACGTTGGATGCGCGAGGCATATTGATATAC Extension probe: ATAATCTAAAATCTTAGCTGAACAAA
	recD	Forward: ACGTTGGATGCGACACTTTTACACGCGCTT Reverse: ACGTTGGATGTTTCTAGCGGAGTATAAGCA Extension probe: GCTGAATTAGATTATCAATTCGCT

Table S2 | Primers and probes of real-time PCR used in this study

Target pathogen	Target gene	Oligonucleotide	Reference
Herpes simplex virus types 1	*gD*	Forward: GGGCCGTGATTTTGTTTGTC Reverse: CCGCCAAGGCATATTTGC Probe: FAM–TAGTGGGCCTCCATGGG-MGB	This study
Herpes simplex virus types 2	*gD*	Forward: ACCTGACCATCGCCTGGTATC Reverse: CAGACCCCCAACGACTTGTT Probe: FAM–TTATGGAATACACCGAGTGC-MGB	This study
Chlamydia trachomatis	cryptic plasmid	Forward: TCCGGAGCGAGTTACGAAGA Reverse: AATCAATGCCCGGGATTGGT Probe: VIC–TGACTAATCTCCAAGCTTAA-MGB	Modified from [1]
Treponema pallidum	*polA*	Forward: AGGATCGCCCATATGTCCAA Reverse: GTGAGCGTCTCATCATTCCAAA Probe: FAM–ATGCACCAGCTTCGA-MGB	[2]
Neisseria gonorrhoeae	*porA*	Forward: CCGGAACTGGTTTCATCTGATT Reverse: GTTTCAGCGGCAGCATTCA Probe: FAM-CGTGAAAGTAGCAGGCGTATAGGCGGACTT-BHQ1	[3]
Mycoplasma hominis	*YidC*	Forward: CCTCAGTTTATTGCATTGCCAAT Reverse: AATACCCGGTTTAGTGAGTTTGCT Probe: VIC-AACAAGCAACCTGATATT-MGB	This study
Ureaplasma urealyticum	*ureD*	Forward: CATTGATGTTGCACAAGGAG Reverse: CGTGATTTTAATGTATCGGCTTTC Probe: VIC-TTGTCCGCCTTTACGAG-MGB	Modified from [4]
Mycoplasma genitalium	*mgpB*	Forward: GAGAAATACCTTGATGGTCAGCAA Reverse: GTTAATATCATATAAAGCTCTACCGTTGTTATC Probe: FAM-ACTTTGCAATCAGAAGGT-MGB	[5]
Ureaplasma parvum	*ureD*	Forward: CATTGATGTTGCACAAGGAG Reverse: CGTGATTTTAATGTATCGGCTTTC Probe: FAM-TTGACCACCCTTACGAG-MGB	[4]
Trichomonas vaginalis	*repeated DNA target*	Forward: CATTGACCACACGGACAAAAAG Reverse: CGAAGTGCTCGAATGCGA Probe: FAM-TCATTTCGGATGGTCAAGCAGCCA-TAMRA	[6]
Haemophilus ducreyi	*16S rRNA*	Forward: ACATCCATAGAAGAACTCAGAGATGA Reverse: TTGAGTTCCCATCAYTACATGCT Probe: FAM-GTGCCTTCGGGAACTATGTGACAGGT-BHQ1	[7]

Table S3 | Primers of nested PCR used in this study

Target Pathogen	Target gene	Direction	1st Primers	2nd Primers	Reference
Herpes simplex virus types 1	*gD*	Forward	TGCTCCTACAACAAGTC	ATCCGAACGCAGCCCCGCTG	[8]
		Reverse	CGGTGCTCCAGGATAAA	TCTCCGTCCAGTCGTTTATCTTC	
Herpes simplex virus types 2	*gG*	Forward	TCAGCCCATCCTCCTTCGGCAGTA	AGACGTGCGGGTCGTACACG	[9]
		Reverse	GATCTGGTACTCGAATGTCTCCG	CGCGCGGTCCCAGATCGGCA	
Chlamydia trachomatis	cryptic plasmid	Forward	GACGCGTCGGAAATTTGGTT	TGCGTCTGGGATTAACTTTCTTG	This study
		Reverse	TCTTTCAATGGAATAGCGGGT	CATGCCGCTCTAGCCTGTTT	
Neisseria gonorrhoeae	*porA*	Forward	AAATCATGTTGCGGGAAAGCA	GGGTAATTGGAGACTGATTGGGT	This study
		Reverse	AAGCCGATAAACGAGCCGAA	TGCGGCTTTTGGCCTTAGTA	
Mycoplasma hominis	*YidC*	Forward	TCACAGATTTGGCCTCAGTTT	GCCTCAGTTTATTGCATTGCC	This study
		Reverse	AGTCCGGCACTAAAGAATGCTC	TGGCATATATTGCGATAGTGCTT	
Ureaplasma urealyticum	*ureA*	Forward	GTAGTGGAAGGGGCAAGAGATG	GGAAGGGGCAAGAGATGGT	This study
		Reverse	GCCTTCAGCGAAGTTAATTGCTCC	TGGTACTAATTTACCTGGAGTGAA	
Mycoplasma genitalium	16S rRNA	Forward	TACATGCAAGTCGATCGGAAGTAGC	TTTAGAGGCGAACGGGTGAG	Modified from [10]
		Reverse	AAACTCCAGCCATTGCCTGCTAG	CCTTGGTAGGCCATTACCCT	

Table S4 | Discordant results verified by arbitration testing

Sample ID	STI-MS results [a]	Confirmed tests results	Arbitration testing results
M1	CT, MG, NG	MG, NG	CT, MG, NG
M2	CT	Negative	CT
M3	UU, CT	UU	UU, CT
M4	UU, CT, HSV1	UU	UU, CT, HSV1
M5	UU, CT	UU	UU, CT
M6	UU, CT, MH	UU, MH	UU, CT, MH
M7	UU, CT, MG, NG	UU, CT, NG	UU, CT, MG, NG
M8	MG	Negative	MG
M9	UU, MG	Negative	UU, MG
M10	MG	Negative	MG
M11	CT, MG, MH, NG	CT, MH, NG	CT, MG, MH, NG
M12	MG, HSV1, HSV2	HSV1, HSV2	MG, HSV1, HSV2
M13	UU, MH	UU	UU, MH
M14	MH	Negative	MH
M15	MH	Negative	MH
M16	NG	Negative	NG
M17	NG	Negative	NG
M18	UU, NG	UU	UU, NG
M19	UU, MG, HSV1	UU, MG	UU, MG, HSV1
M20	HSV1	Negative	HSV1
M21	UU, MH, HSV1	UU, MH	UU, MH, HSV1
M22	UU, MG, MH, HSV2	UU, MG, MH	UU, MG, MH, HSV2
M23	HSV2	Negative	HSV2
M24	HSV1, HSV2	HSV1	HSV1, HSV2
M25	NG, HSV2	NG	NG, HSV2
M26	HSV2	Negative	HSV2
M27	UU, CT, MH	UU, CT	UU, CT, MH
M28	UU	Negative	UU
M29	UU, CT, HSV1, HSV2	CT, HSV1, HSV2	UU, CT, HSV1, HSV2
M30	UU, CT, MH	UU, CT	UU, CT, MH
M31	UU	Negative	UU

[a] CT, *Chlamydia trachomatis*; MG, *Mycoplasma genitalium*; NG, *Neisseria gonorrhoeae*; UU, *Ureaplasma urealyticum*; HSV1, Herpes simplex virus types 1; MH, *Mycoplasma hominis*; HSV2, Herpes simplex virus types 2;

Table S5 | Multiple infections verified by confirmatory tests and arbitration testing

Infection type [a]	No. of confirmed samples
Dual infections (n=32)	
UU, MH	5
UU, MG	5
UU, CT	5
UU, NG	2
CT, MH	2
CT, NG	2
MG, MH	2
TP, MH	1
CT, HSV1	1
CT, HSV2	1
UP, MG	1
UP, MH	1
MG, NG	1
MH, NG	1
NG, HSV2	1
HSV1, HSV2	1
Triple infections (n=20)	
UU, MH, CT	6
UU, MG, MH	3
UU, CT, HSV1	2
UU, MH, HSV1	1
UU, MG, HSV1	1
CT, MG, NG	1
CT, MH, NG	1
MG, MH, NG	1
MG, NG, HSV1	1
MG, MH, HSV1	1
MG, HSV1, HSV2	1
NG, HSV1, HSV2	1
Quadruple infections (n=9)	
UU, CT, MH, NG	4
UU, CT, MG, NG	1
UU, MG, MH, HSV2	1
UU, MG, MH, NG	1
UU, NG, HSV1, HSV2	1
CT, MG, MH, NG	1
Quintuple infections (n=1)	
UU, CT, MH, HSV1, HSV2	1

[a] UU, *Ureaplasma urealyticum*; MH, *Mycoplasma hominis*; MG, *Mycoplasma genitalium*; CT, *Chlamydia trachomatis*; NG, *Neisseria gonorrhoeae*; TP, *Treponema pallidum*; HSV1, Herpes simplex virus types 1; HSV2, Herpes simplex virus types 2; UP, *Ureaplasma parvum*

Figure S1

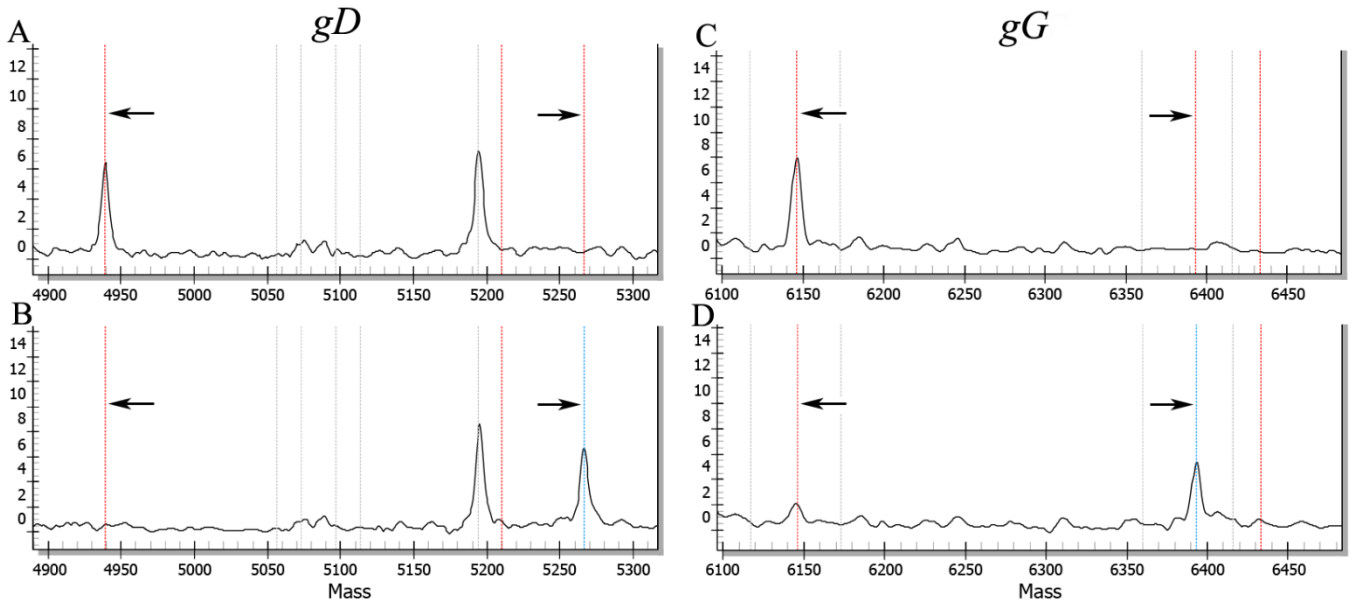

Figure S1 | Analytical specificity of Herpes simplex virus types 1 by using STI-MS assay. (A) Negative result of *gD* assay, (B) Positive result of *gD* assay, (C) Negative result of *gG* assay, (D) Positive result of *gG* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S2

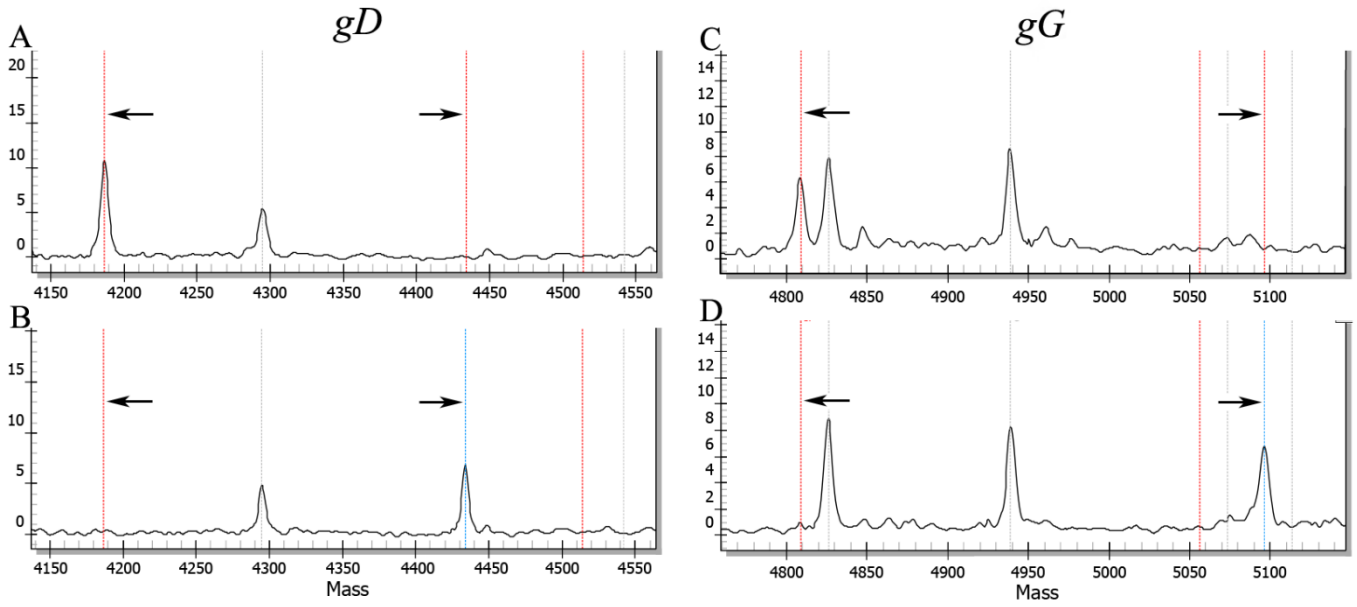

Figure S2 | Analytical specificity of Herpes simplex virus types 2 by using STI-MS assay. (A) Negative result of *gD* assay, (B) Positive result of *gD* assay, (C) Negative result of *gG* assay, (D) Positive result of *gG* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S3

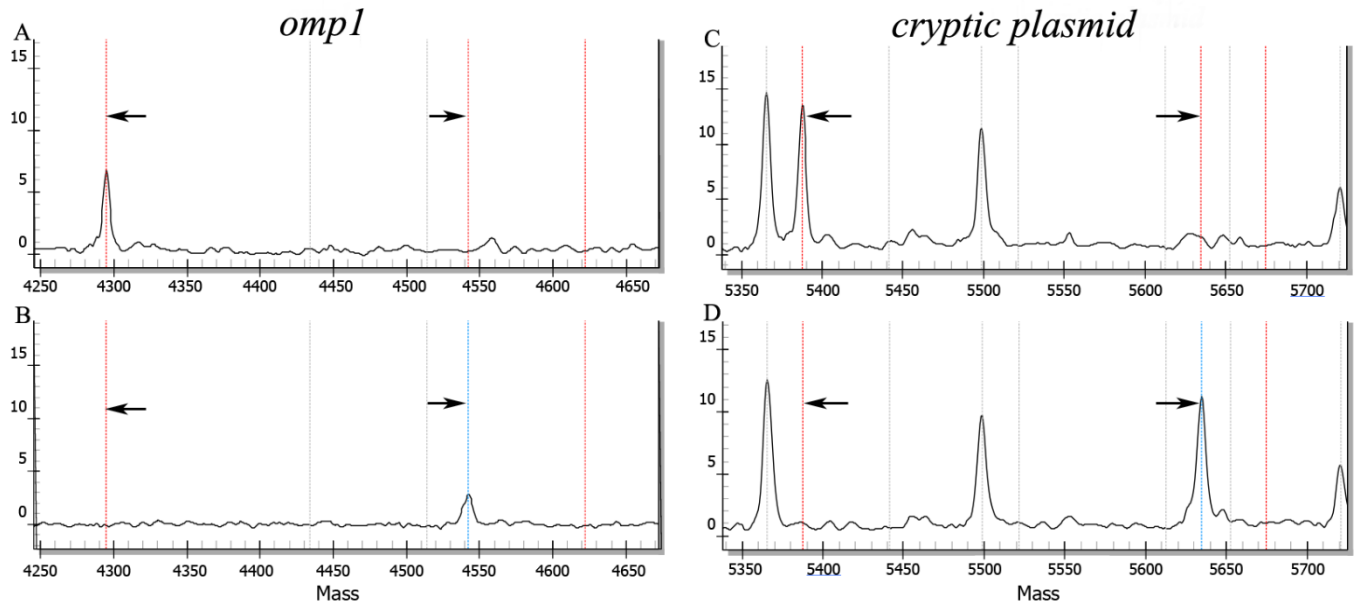

Figure S3 | Analytical specificity of *Chlamydia trachomatis* by using STI-MS assay. (A) Negative result of *omp1* assay, (B) Positive result of *omp1* assay, (C) Negative result of *cryptic plasmid* assay, (D) Positive result of *cryptic plasmid* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S4

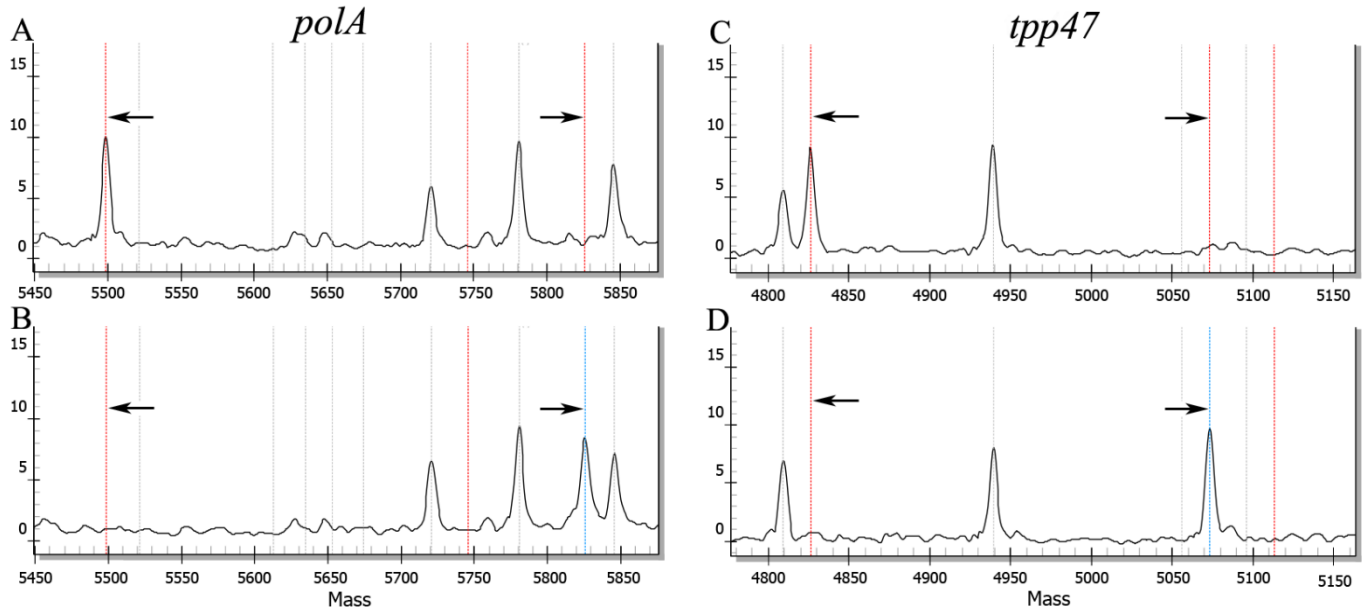

Figure S4 | Analytical specificity of *Treponema pallidum* by using STI-MS assay. (A) Negative result of *polA* assay, (B) Positive result of *polA* assay, (C) Negative result of *tpp47*assay, (D) Positive result of *tpp47* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S5

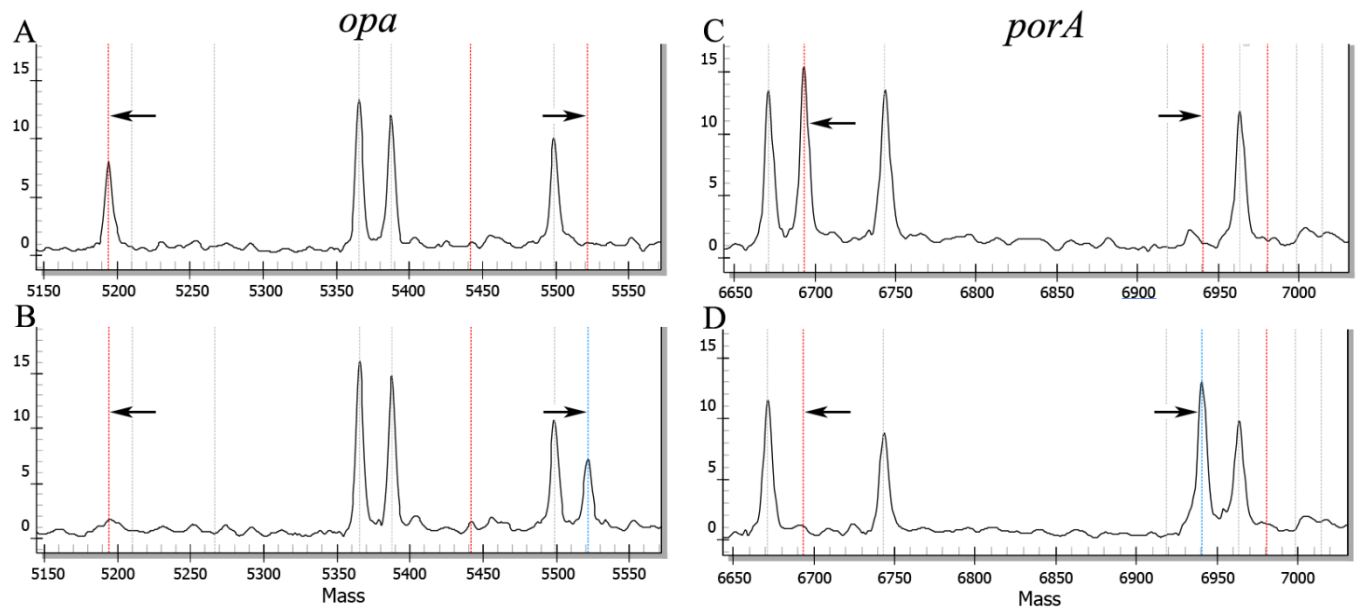

Figure S5 | Analytical specificity of *Neisseria gonorrhoeae* by using STI-MS assay. (A) Negative result of *opa* assay, (B) Positive result of *opa* assay, (C) Negative result of *porA* assay, (D) Positive result of *porA* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S6

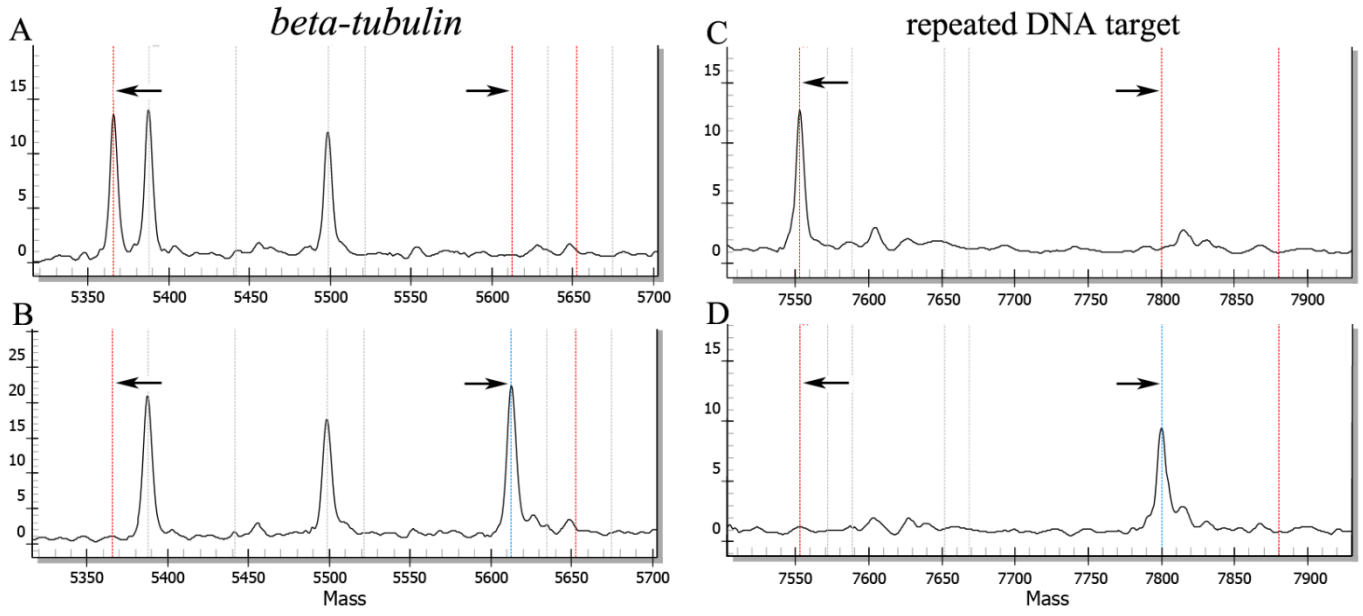

Figure S6 | Analytical specificity of *Trichomonas vaginalis* by using STI-MS assay. (A) Negative result of *beta-tubulin* assay, (B) Positive result of *beta-tubulin* assay, (C) Negative result of reprated DNA target assay, (D) Positive result of reprated DNA target assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S7

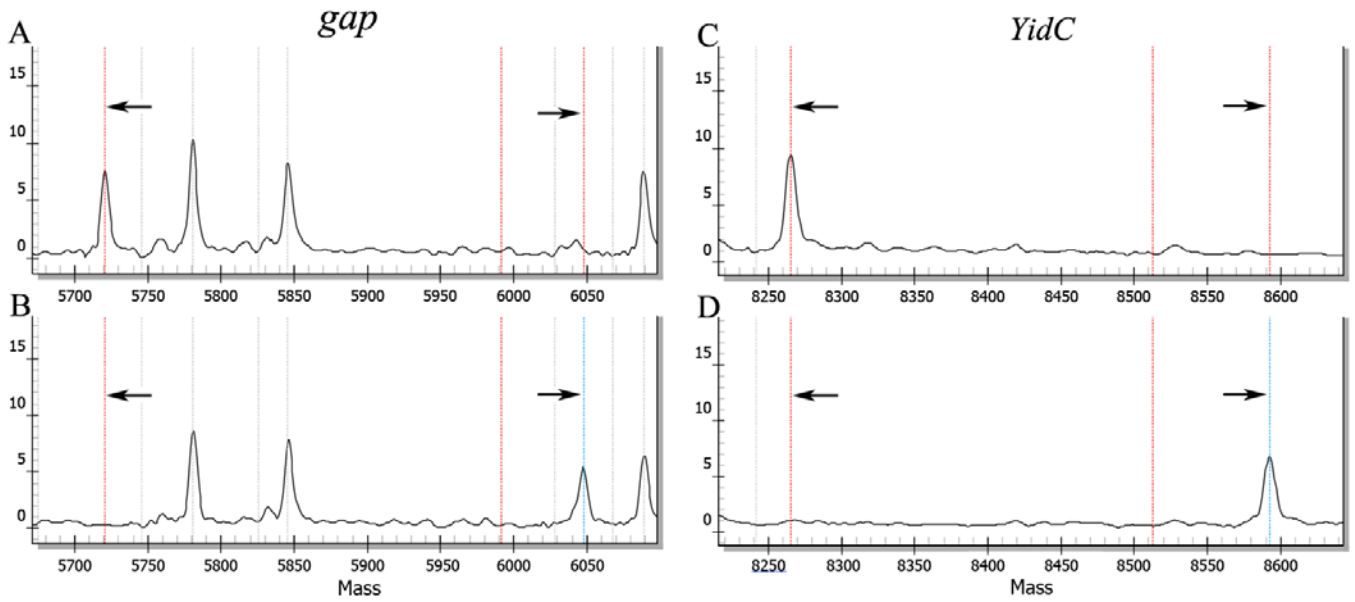

Figure S7 | Analytical specificity of *Mycoplasma hominis* by using STI-MS assay. (A) Negative result of *gap* assay, (B) Positive result of *gap* assay, (C) Negative result of *YidC* assay, (D) Positive result of *YidC* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S8

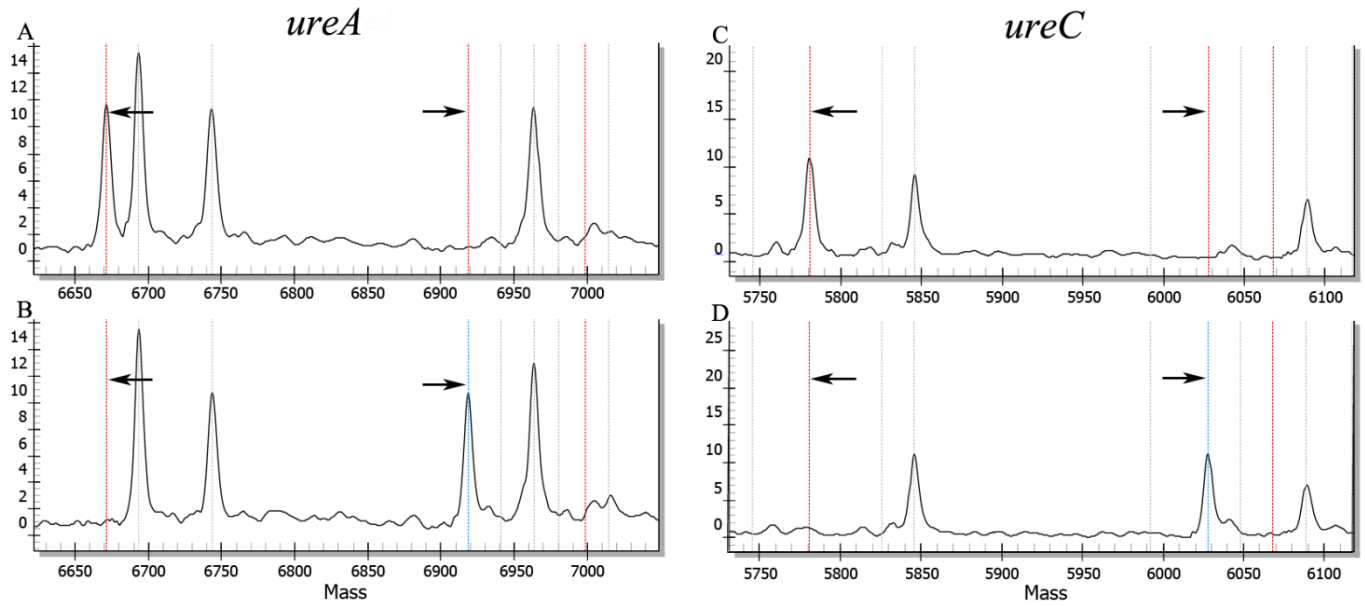

Figure S8 | Analytical specificity of *Ureaplasma urealyticum* by using STI-MS assay. (A) Negative result of *ureA* assay, (B) Positive result of *ureA* assay, (C) Negative result of *ureC* assay, (D) Positive result of *ureC* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S9

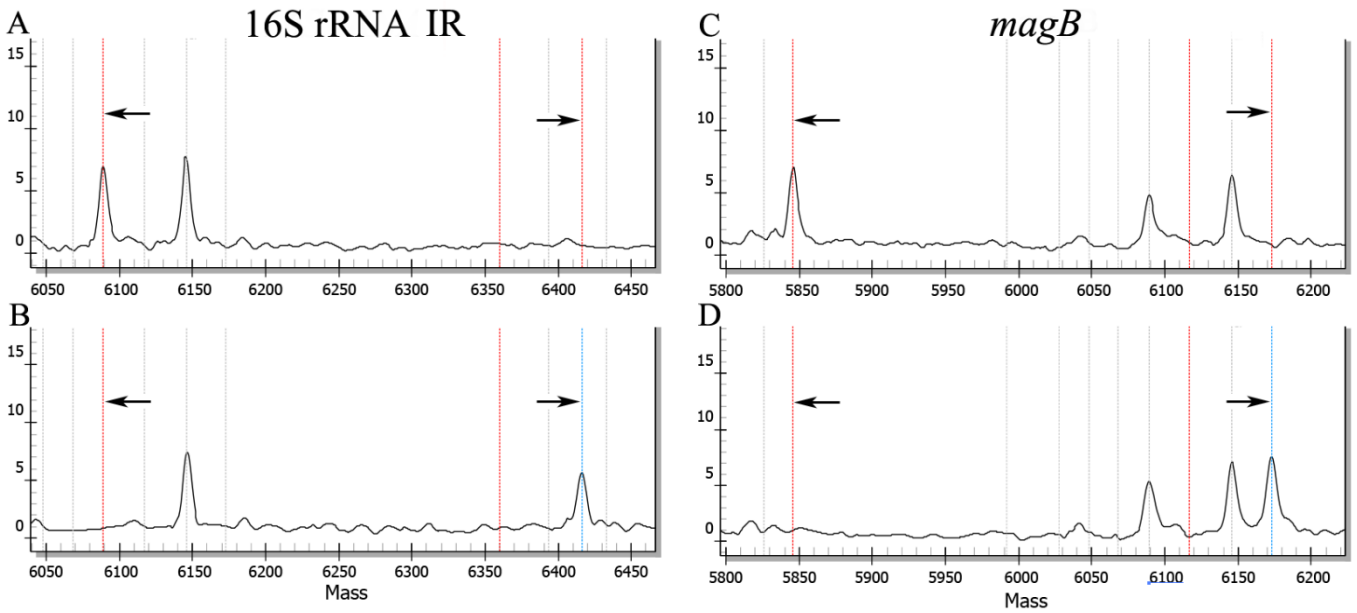

Figure S9 | Analytical specificity of *Mycoplasma genitalium* by using STI-MS assay. (A) Negative result of 16S rRNA IR assay, (B) Positive result of 16S rRNA IR assay, (C) Negative result of *magB* assay, (D) Positive result of *magB* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S10

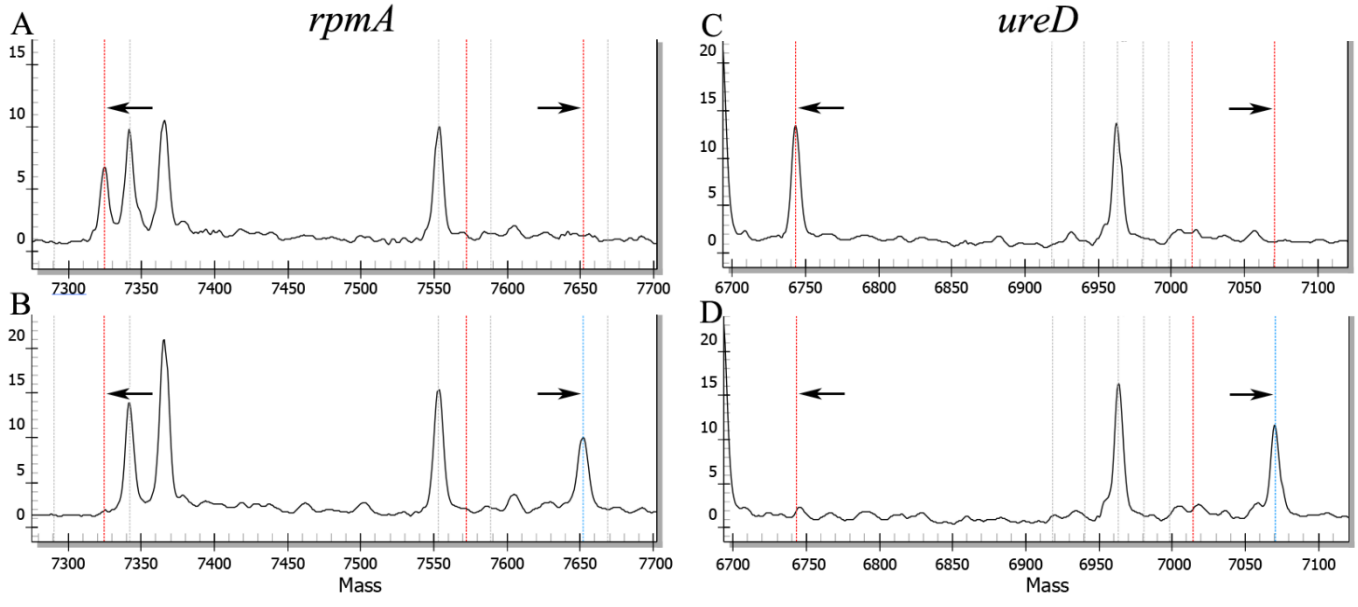

Figure S10 | Analytical specificity of *Ureaplasma parvum* by using STI-MS assay. (A) Negative result of *rpmA* assay, (B) Positive result of *rpmA* assay, (C) Negative result of *ureD* assay, (D) Positive result of *ureD* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S11

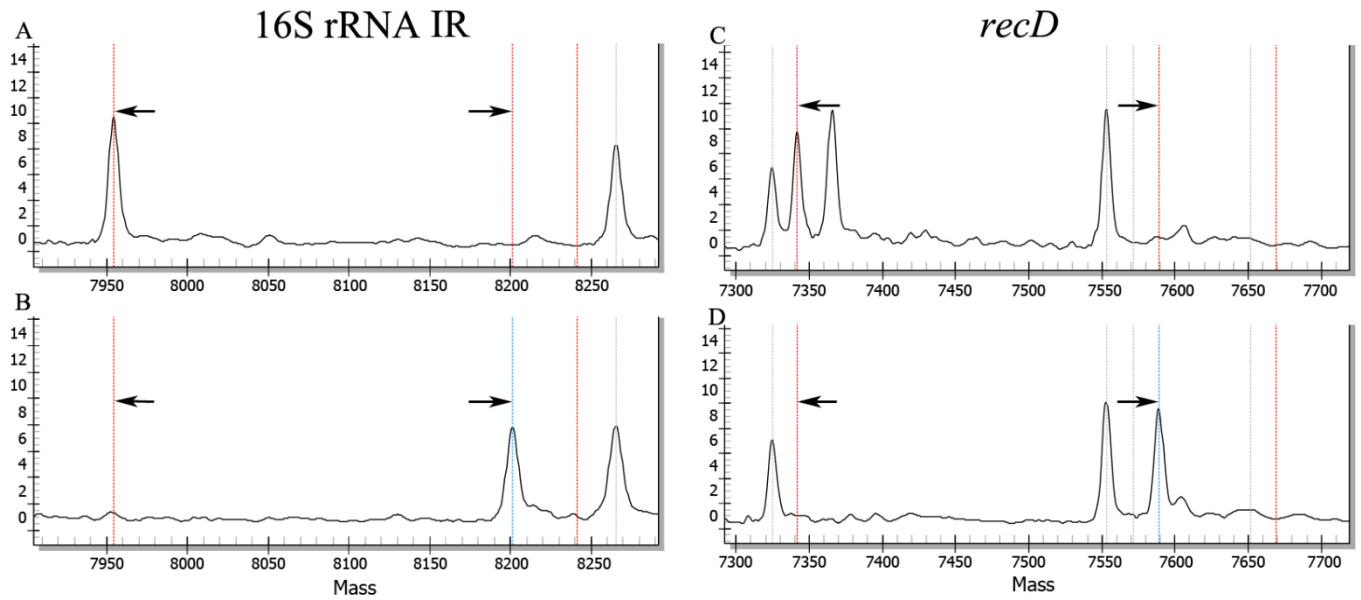

Figure S11 | Analytical specificity of *Haemophilus ducreyi* by using STI-MS assay. (A) Negative result of 16S rRNA IR assay, (B) Positive result of 16S rRNA IR assay, (C) Negative result of *recD* assay, (D) Positive result of *recD* assay. The left arrow and the right arrow represent the unextended probes and the extended probes of the assay, respectively. The x-axis represents the mass of extension probe and the y-axis represents the intensity.

Figure S12

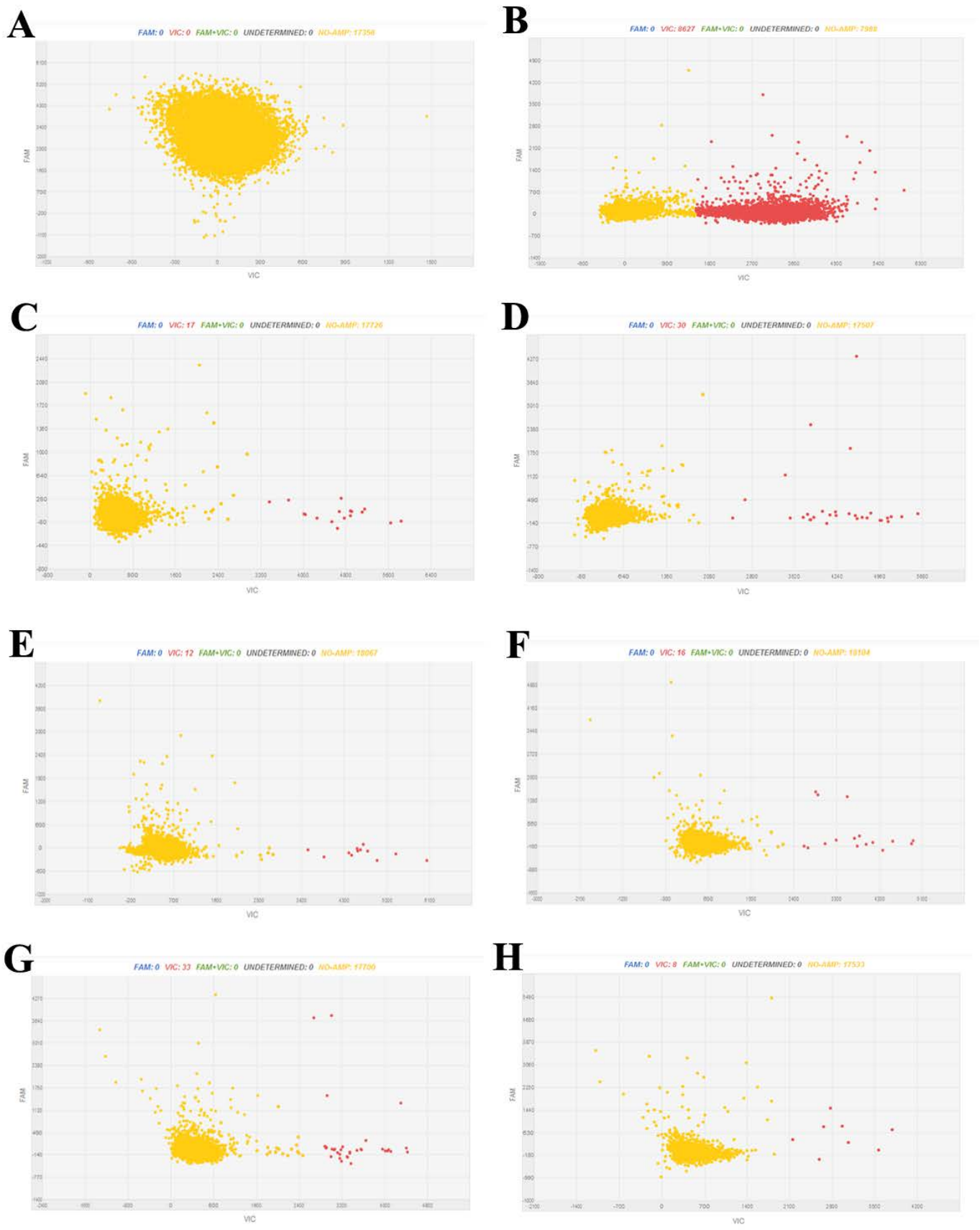

Figure S12 | Discrepant results of *Chlamydia trachomatis* verified by Digital PCR. Figure (A), (B), (C), (D), (E), (F), (G) and (H) represent the results of negative control, positive control, sample M1, sample M2, sample M3, sample M4, sample M5 and sample M6, respectively. The used probe was labeled by fluorescent VIC at the 5' end.

Figure S13

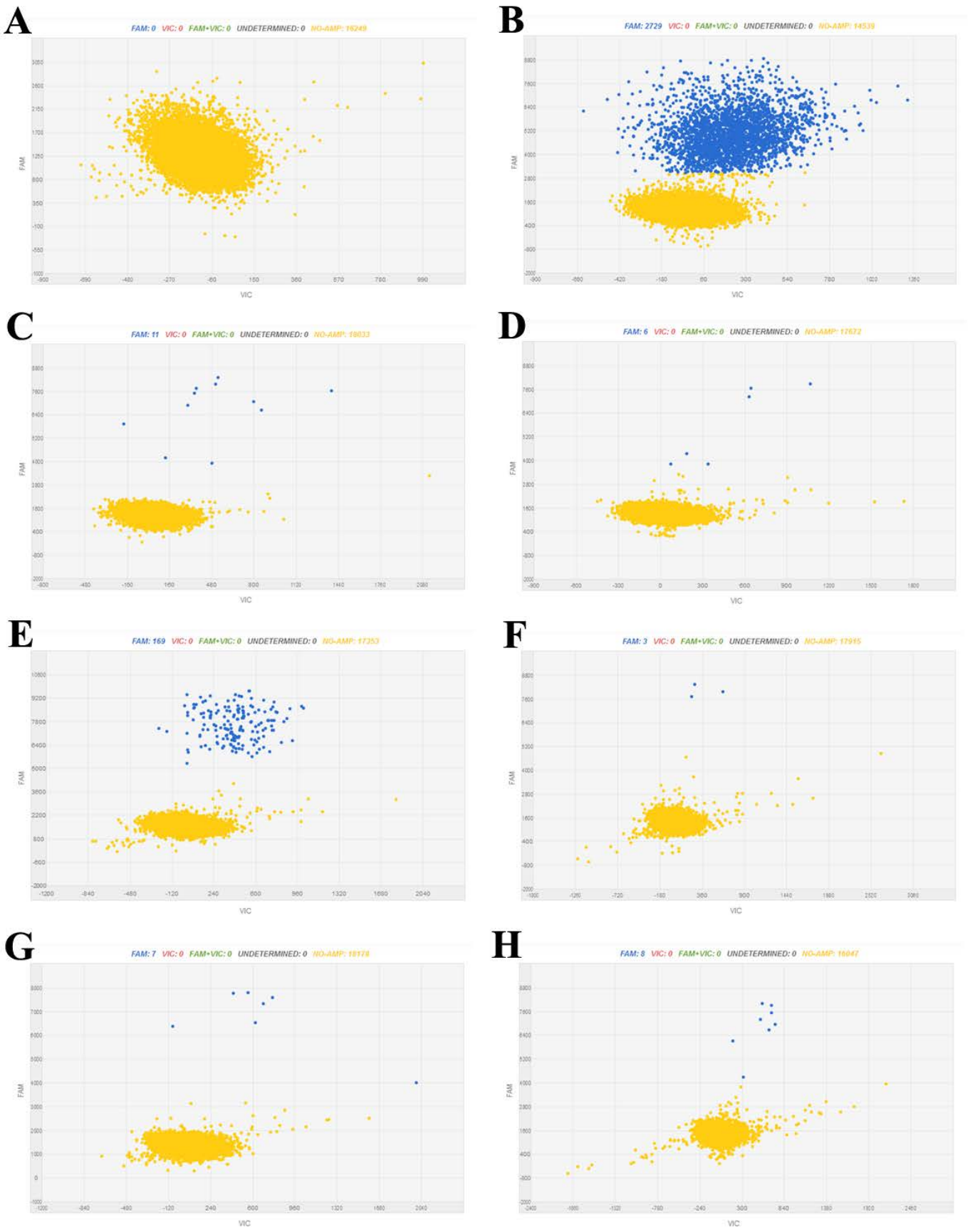

Figure S13 | Discrepant results of *Mycoplasma genitalium* verified by Digital PCR. Figure (A), (B), (C), (D), (E), (F), (G) and (H) represent the results of negative control, positive control, sample M7, sample M8, sample M9, sample M10, sample M11 and sample M12, respectively. The used probe was labeled by fluorescent FAM at the 5' end.

Figure S14

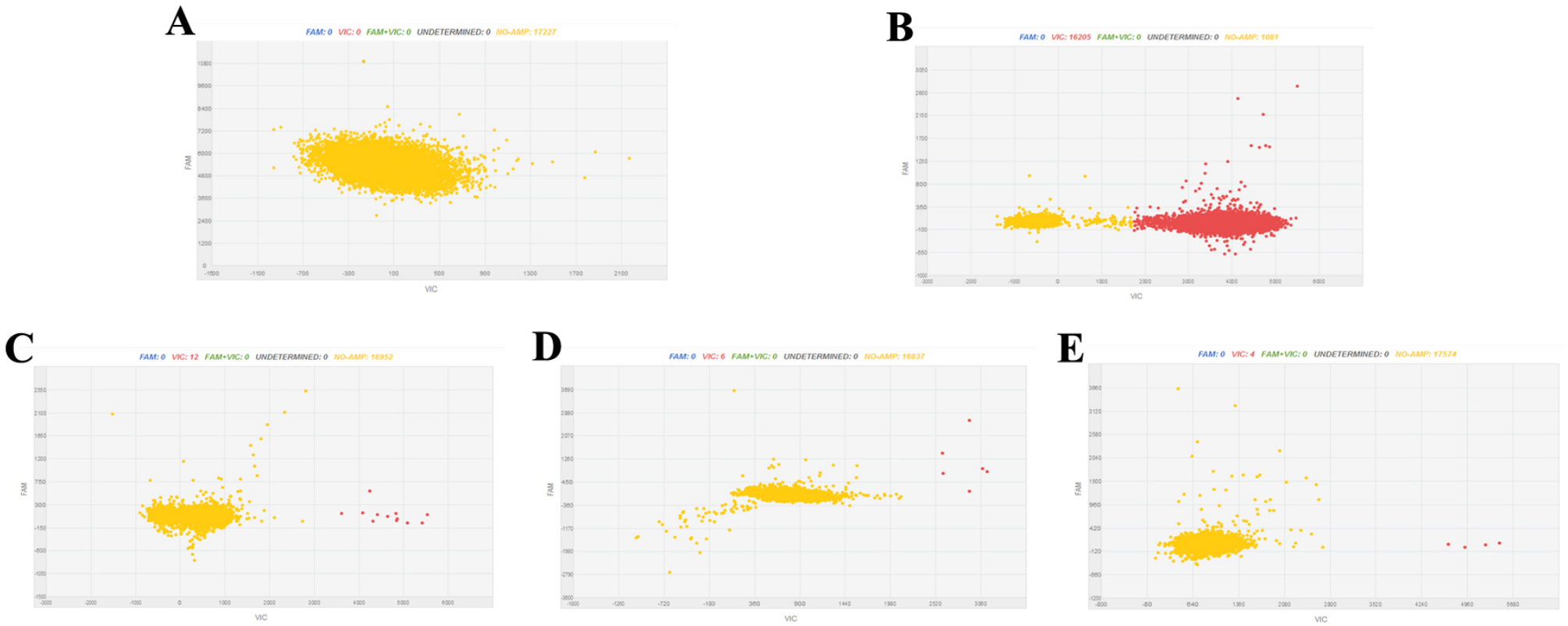

Figure S14 | Discrepant results of *Mycoplasma hominis* verified by Digital PCR. Figure (A), (B), (C), (D) and (E) represent the results of negative control, positive control, sample M13, sample M14, and sample M15, respectively. The used probe was labeled by fluorescent VIC at the 5’ end.

Figure S15

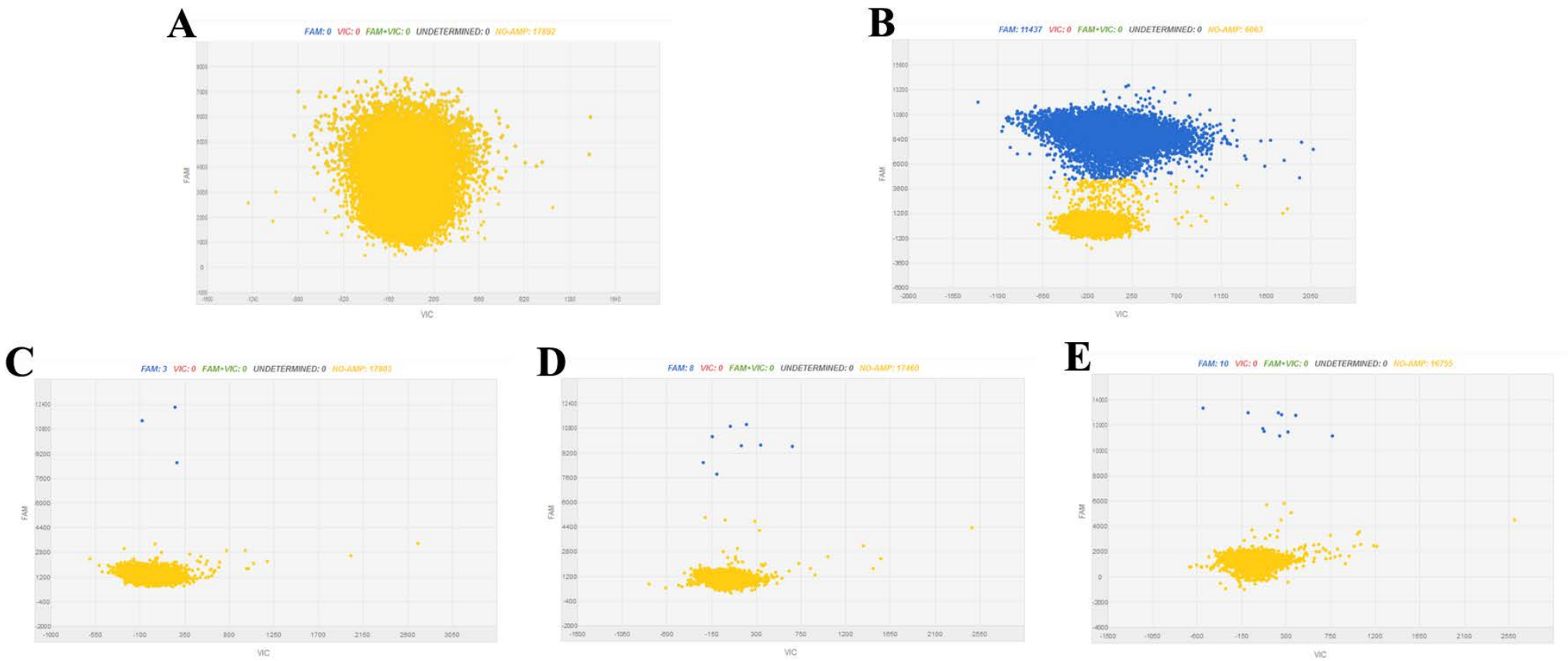

Figure S15 | Discrepant results of *Neisseria gonorrhoeae* verified by Digital PCR. Figure (A), (B), (C), (D) and (E) represent the results of negative control, positive control, sample M16, sample M17, and sample M18, respectively. The used probe was labeled by fluorescent FAM at the 5’ end.

Figure S16

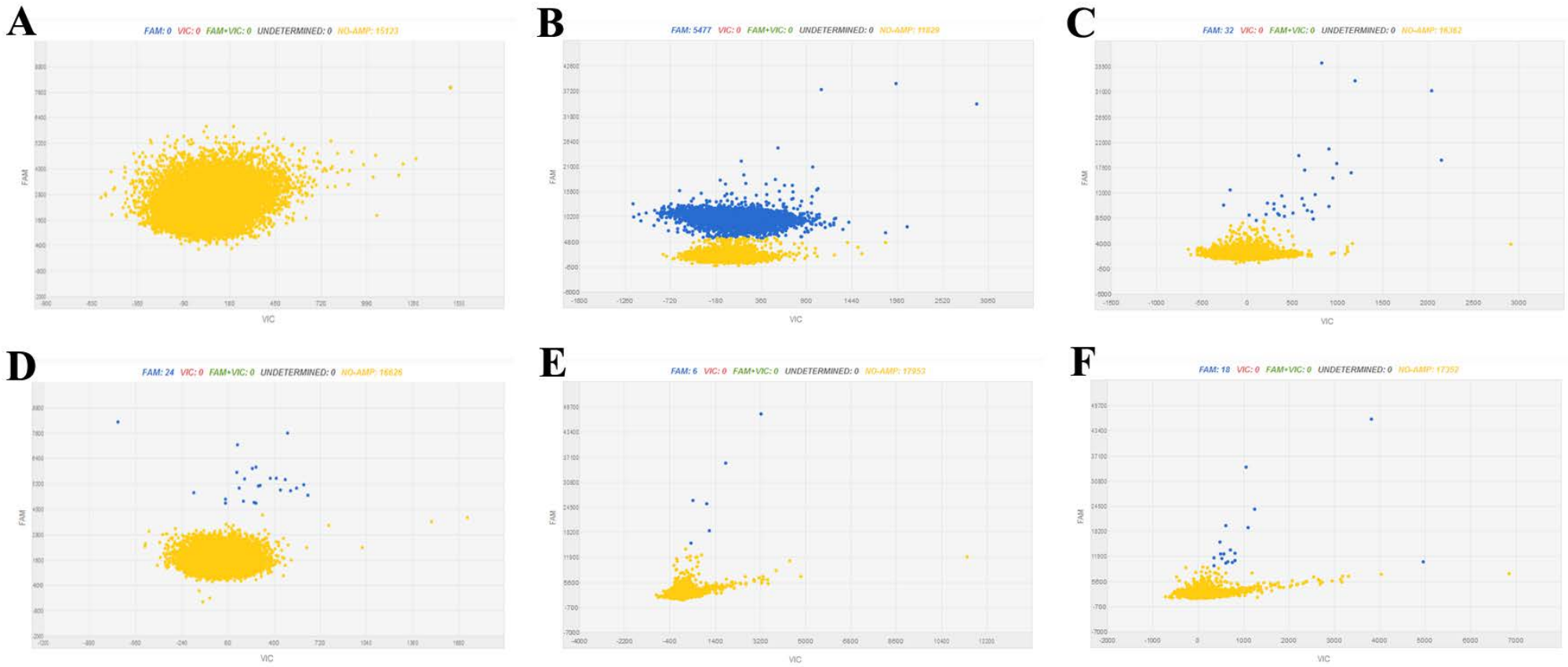

Figure S16 | Discrepant results of Herpes simplex virus types 1 verified by Digital PCR. Figure (A), (B), (C), (D), (E) and (F) represent the results of negative control, positive control, sample M4, sample M19, sample M20, and sample M21, respectively. The used probe was labeled by fluorescent FAM at the 5’ end.

Figure S17

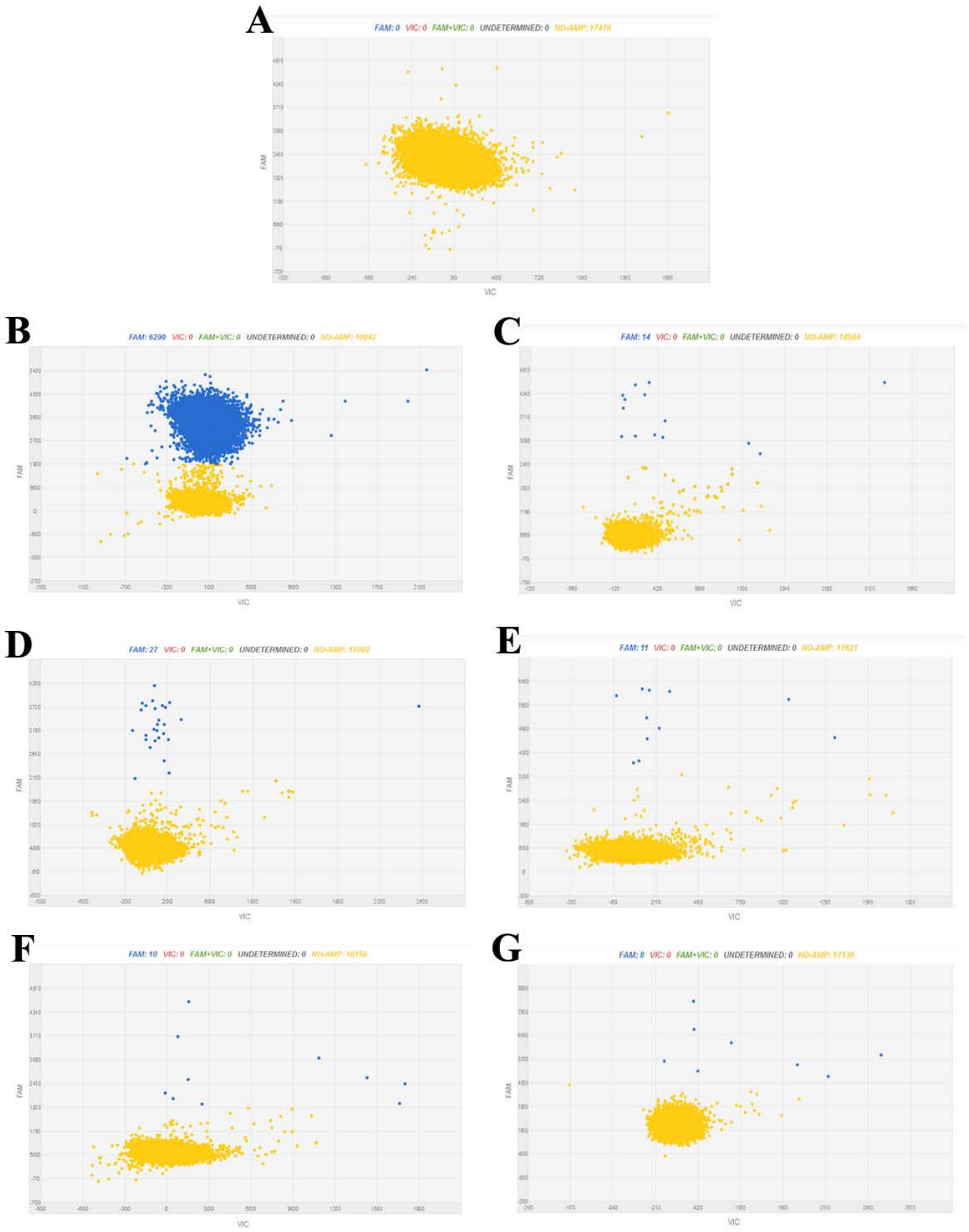

Figure S17 | Discrepant results of Herpes simplex virus types 2 verified by Digital PCR. Figure (A), (B), (C), (D), (E), (F) and (G) represent the results of negative control, positive control, sample M22, sample M23, sample M24, sample M25 and sample M26, respectively. The used probe was labeled by fluorescent FAM at the 5' end.

Figure S18

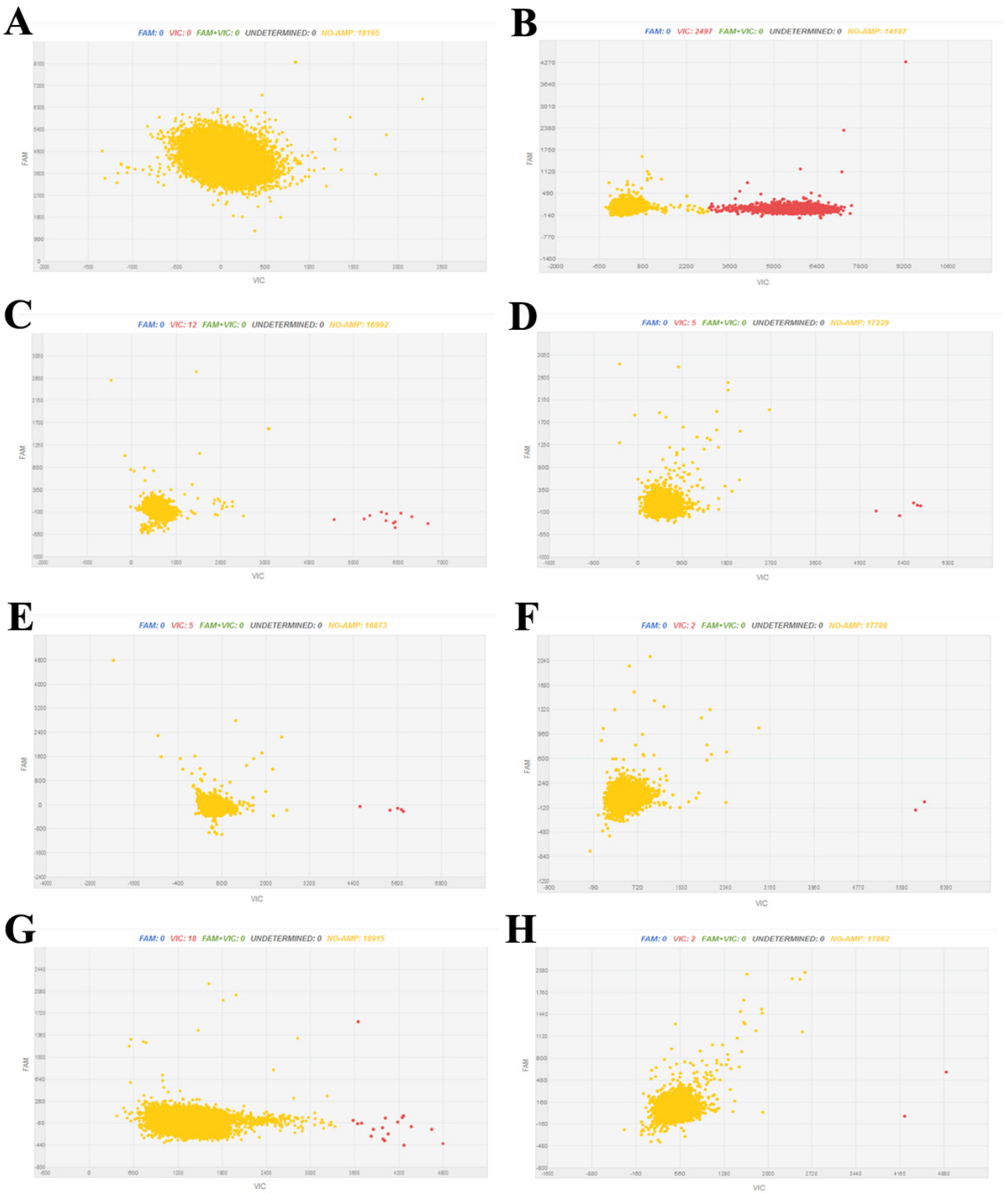

Figure S18 | Discrepant results of *Ureaplasma urealyticum* verified by Digital PCR. Figure (A), (B), (C), (D), (E), (F), (G) and (H) represent the results of negative control, positive control, sample M9, sample M27, sample M28, sample M29, sample M30 and sample M31, respectively. The used probe was labeled by fluorescent VIC at the 5' end.

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