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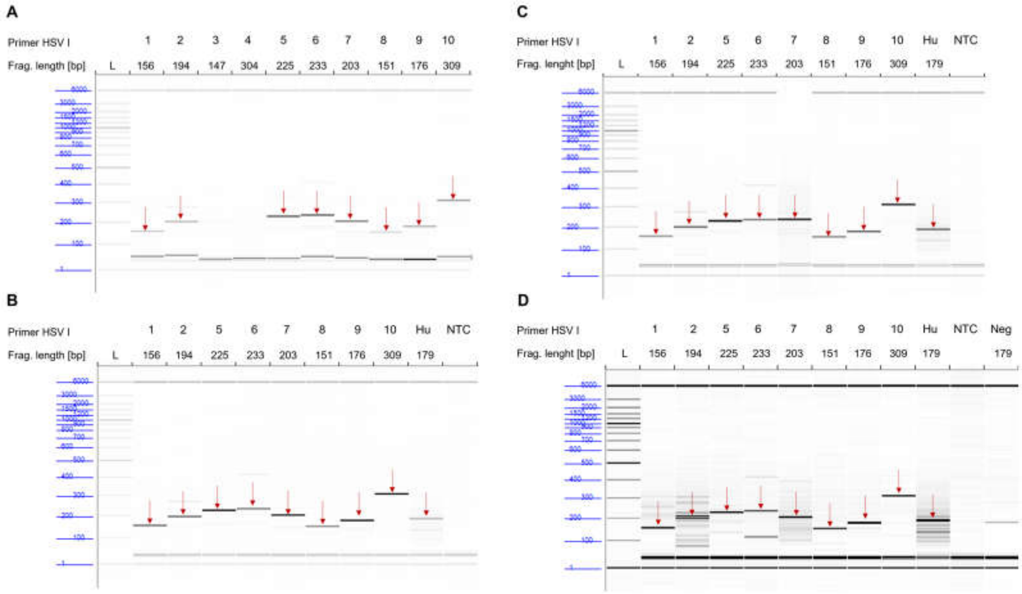
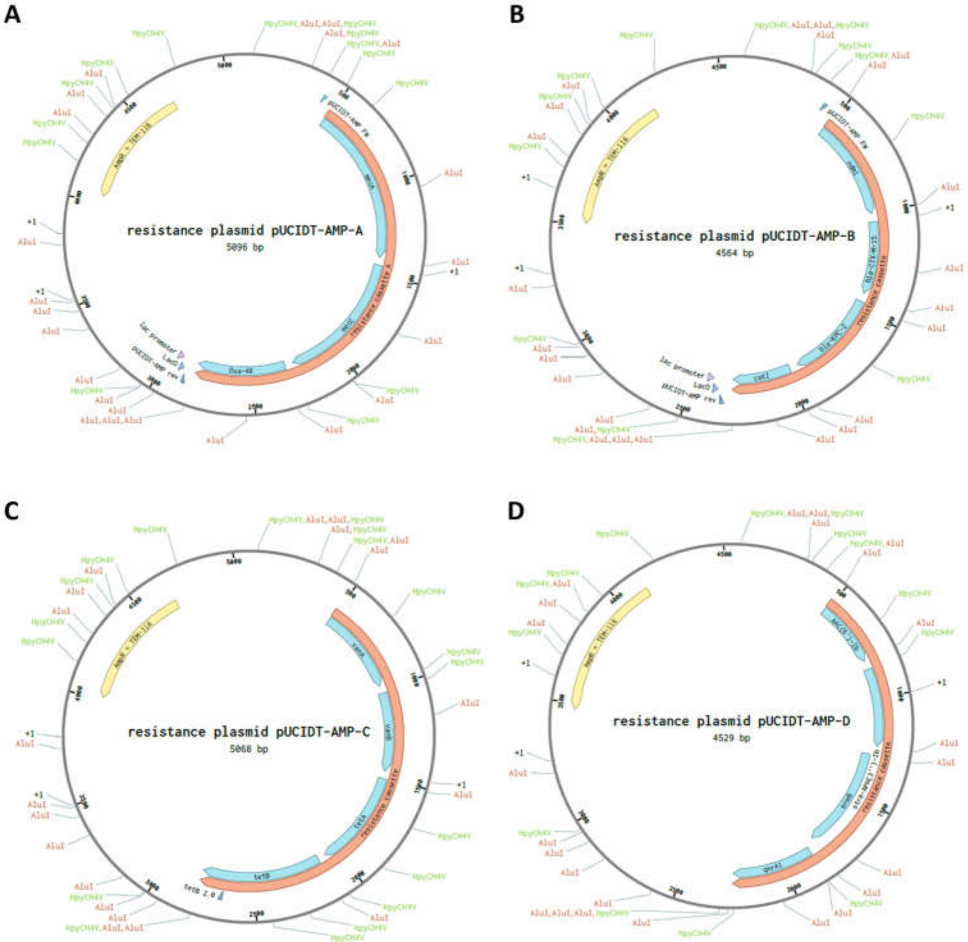
Article

Suppression PCR-based selective enrichment sequencing for pathogen and antimicrobial resistance detection on cell-free DNA in sepsis - A targeted, blood culture independent approach for rapid pathogen and resistance diagnostics in septic patients

Supplement



Supplement Figure S1. Identification of suitable HSV I primers. For the identification of most suitable SUPSETS target regions, bioinformatically identified primers were tested. Each lane represents a PCR product with the indicated HSV I primer. For all PCR experiments, the expected fragment length in bp is depicted and inside the gel image displayed with a red arrow. A: 40,000 GE of digested, adapter ligated HSV I DNA were tested in a one-step PCR as described in methods section. B: 40,000 GE digested, adapter ligated HSV I DNA were tested by the complete SUPSETS workflow. C: 40000 GE digested, adapter ligated HSV I DNA were spiked in 6,000 GE human digested, adapter ligated DNA and tested by the complete SUPSETS workflow. D: 4,000 GE digested, adapter ligated HSV I DNA were spiked in 6,000 GE human digested, adapter ligated DNA and tested by the complete SUPSETS workflow. NTC, non-template control; Hu, human only sample; Neg, HSV I DNA with human primer.



Supplement Figure S2. Antibiotic resistance plasmids. Antibiotic resistance plasmids. Resistance cassettes with relevant parts of AMR genes were cloned in the plasmid pUCIDT-AMP by Integrated DNA Technologies GmbH (IDT). A: Resistance plasmid A contains parts of the resistance genes mecA, mecC and Oxa-48. B: Resistance plasmid B contains parts of the resistance genes ndm-1, bla-CTX-M-15, bla-KPC-2 and catI. C: Resistance plasmid C contains parts of the resistance genes vanA, vanB, tetA and tetB. D: Resistance plasmid D contains parts of the resistance genes AAC(6')-Ib, strA-APH(3'')-Ib, ermB and qnrA1. All resistance cassettes are indicated in orange, while the resistance gene parts are depicted in blue. The selecting TEM116 ampicillin resistance is depicted in yellow. Furthermore, the used restriction enzymes AluI and HpyCH4V are shown. Images created in benchling.com.

Supplement table S1. Primer set used per experiment. Spike-In A: Spike-In *E. faecium*, *E. coli*, and HSV I. Spike-In B: Spike-In *B. fragilis*, *C. albicans*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*.

	Technical validation			**HSV I testing**		**Clinical samples**	
	Spike-In A	**Spike-In B**	**Multiplex**	**1 Step**	**SUPSETS**	**Flongle**	**Flow Cell**
B. fragilis	B. frag T1 & T2	B. frag T1 & T2	B. frag T1 & T2	-	-	B. frag T1 & T2	B. frag T1 & T2
C. albicans	C. alb T1 – T3	C. alb T1 – T3	C. alb T1 – T3	-	-	C. alb T1 & T3	C. alb T1 – T3
E. faecium	E. faec T1 & T2	E. faec T1 & T3	E. faec T1 & T2	-	-	E. faec T1 & T3	E. faec T1 & T2
E. coli	E. coli T1 & T2	-	E. coli T1 & T2	-	-	E. coli T1 & T2	E. coli T1 & T2

HSV I	HSV I T1 & T2	-	HSV I T1 & T2	HSV_01 HSV_02 HSV_03 HSV_04 HSV I T1 HSV_06 HSV_07 HSV_08 HSV I T2 HSV_10	HSV_01 HSV_02 HSV I T1 HSV_06 HSV_07 HSV_08 HSV I T2 HSV_10	-	HSV I T1 & T2
K. pneumoniae	K. pneum. T1 & T2	K. pneum. T1 & T2	K. pneum. T1 & T2	-	-	K. pneum. T1 & T2	K. pneum. T1 & T2
P. aeroginosa	P. aerug T1 & T2	P. aerug T1 & T2	P. aerug T1 & T2	-	-	-	P. aerug T1 & T2
S. aureus	S. aureus T1 & T2	S. aureus T1 & T2	S. aureus T1 & T2	-	-	-	S. aureus T1 & T2
16S	16S	16S	16S	-	-	-	16S
AMR	-	-	cat1_V2 ermB mecA_V2 mecC qnrA1 strA-APH(3'') tetA tetB vanA vanB AAC(6)-Ib TEM116 ndm1 OXA48 blaCTX-M-15_V2 blaKPC-2	-	-	cat1_V1 ermB mecA_V1 mecC qnrA1 strA-APH(3'') tetA tetB vanA vanB AAC(6)-Ib TEM116 OXA48 blaCTX-M-15_V1 blaKPC-2	cat1_V2 ermB mecA_V2 mecC qnrA1 tetA tetB vanA vanB AAC(6)-Ib TEM116 ndm1 OXA48 blaCTX-M-15_V2 blaKPC-2

Supplement table S2. Raw sequencing and mapping data for technical validation. Mapping on full target genome.

Spike-In	**Pathogen**		**Human**	
	Mapped reads	portion all reads [%]	Mapped reads	portion all reads [%]
S. aureus	144,613	7.05375591	1,786,390	87.1343449
P. aeruginosa	830,584	25.4745282	2,297,695	70.4717356
K. pneumoniae	613,937	37.2932865	1,031,292	62.6453008
HSV I	18,424	16.3754333	91,532	81.3545463
E. coli	23,888	32.4283232	48,803	66.2508145
E. faecium	120,240	60.1795778	78,440	39.2588663
C. albicans	1,550	0.07287955	1,989,341	93.5369478
B. fragilis	235,297	16.6421593	1,099,192	55.2896548

Supplement table S3. Characteristics of used clinical samples. Timepoint reflects days after ICU admission. BC, blood culture.

Sample	Septic status	Timepoint [days]	Age [years]	Sex	Localization	BC result	Clinical Metagenomics [21]
FL_01	sepsis	14	57	male	Abdominal (peritonitis)	Negative (VRE OP smear)	B. fragilis, E. faecium, E. coli, H. influenzae, S. epidermidis
FL_02	sepsis	2	74	male	Abdominal (peritonitis)	Negative (VRE abdominal smear)	E. faecium
FL_03	sepsis	0	41	male	Abdominal (peritonitis)	E. coli	E. coli
FL_04	sepsis	1	75	male	Abdominal (peritonitis)	negative	B. thetaiotaomicron, B. animalis, E. faecium, E. coli, K. pneumonaie, L. fermentum, V. parvula
FC_01	sepsis	7	78	female	Abdominal (peritonitis)	negative	E. faecium, Human herpesvirus 1
FC_02	sepsis	28	66	male	Abdominal	negative	K. pneumoniae, P. mendocina, S. aureus
FC_03	OP control	1	NA	NA	-	-	-
FC_04	Healthy	-	NA	NA	-	-	-
FC_05	OP control	2	NA	NA	-	-	-
FC_06	sepsis	28	57	male	Abdominal (peritonitis)	Negative (VRE wound swab)	B. fragilis, B. thetaiotaomicron, E. faecium, H. influenza, Human herpesvirus 1

Supplement table S4. Raw sequencing and mapping data for selected clinical samples on MinION Flow Cell. Reads considered with mapq >4, all human reads accumulated, pathogen reads per target amplicons accumulated. FC, Flow Cell.

FC samples	FC_01	FC_02	FC_03	FC_04	FC_05	FC_06
H. sapiens	127,496	9,795	150,899	7,877	68,041	113,028
B. fragilis	0	0	0	0	0	2,262
C. albicans	0	0	0	0	0	0
CARD	0	0	0	0	0	832
E. coli	0	0	0	0	0	662
E. faecium	18	0	0	0	0	1
HSV I	101	0	51	0	0	520
K. pneumoniae	0	0	0	0	0	823
P. aeruginosa	0	0	0	0	0	0
S. aureus	0	0	0	0	0	0

Supplement table S5. Raw sequencing and mapping data for selected clinical samples on MinION Flongle. Reads considered with mapq >4, all human reads accumulated, pathogen reads per target amplicons accumulated. Igκ reads resulted from mapping on full target genome. FL, Flongle.

FL samples	FL_01	FL_02	FL_03	FL_04	FL_04-single
Igκ	6,474	760	8,104	9,64	8,692
H. sapiens	42,572	23,975	98,992	113,712	54,278

B. fragilis	1,603	1	1	1	0
C. albicans	0	0	0	0	0
CARD	1,562	12	1,187	2	54
E. coli	1	0	708	114	219
E. faecium	1,048	51	0	42	90
K. pneumoniae	0	0	0	0	0

Supplement table S6. Approximate duration of SUPSETS workflow. h, hours; min, minutes.

Step	**Protocol**
cfDNA isolation	2 h
End-prep and adapter ligation	3 h
Template generation	50 min
Purification	20 min
Amplification	1 h 50 min
Library preparation	3 - 4 h
Sequencing	5 min - 24 h
Total	11 - 12 h 5 min - 24 h sequencing

Supplement table S7. Approximate costs and price per sample of SUPSETS. For multiplexing on a MinION Flow Cell, 10 samples per run are calculated. For MinION Flongle experiments, costs for one single sample per analysis are depicted. State of prices: 12/2023.

Reagent	**Selling price**	**Price per sample FC (10 samples)**	**Price per sample FL (1 sample)**
cfDNA isolation kit e.g. EZ1&2 ccfDNA Kit (48)	1000 € / 48 reactions	20.83 €	20.83 €
AmpliTaq Gold 360 Polymerase	1,304 € / 1,000 U	5.22 €	5.22 €
Primer / Adapter	-	~ 0.1 €	~ 0.1 €
AMPure XP Beads	1,200 € / 60 mL	~ 2 - 3 €	~ 2 - 3 €
Ligation Sequencing Kit V14 SQK-LSK114	570 € / 6 reactions	-	95 €
Native Barcoding Kit 24 V14 SQK-NBD114.24	665 € / 6 reactions	11 € (for 6 x 10 samples)	-
MinION Flongle R.10.4.1	797 € / 12 Flongles	-	66.42€
MinION Flow cell R.10.4.1	855 €	85 € (for 10 samples)	-
NEB Blunt/TA Ligase Master Mix	438 € / 250 reactions	1.75 €	1.75 €
dNTP Mix (10 mM)	100 € / mL	0.2 €	0.2 €
NEB Quick Blunting Kit	378 € / 100 reactions	3.78 €	3.78 €
NEBNext FFPE DNA Repair Mix	642 € / 96 reactions	6.69 €	6.69 €
NEBNext Ultra II End Repair/dA-Tailing Module	886 € / 96 reactions	9.23 €	9.23 €
NEBNext Quick Ligation Reaction Buffer (5x)	47 € / 2 mL	0.24 €	0.24 €
T4 Quick Ligase (400 U/µL)	288 € / 100,000 U	10 - 12 €	10 - 12 €
Total		~ 160 €	~ 225 €

Upfront costs:			
MinION Starter pack (1 Mk1B, 1 Flow cell, 1 sequencing kit, 1 washing kit)	950 €		
Optional: cfDNA isolation device e.g. EZ2 Connect	30,000 €		

Supplement table S8. Reference genoms and corresponding TaxIDs.

Reference	Corresponding ID
H. sapiens	GRCh38
B. fragilis	NZ_CP069563.1
C. albicans	C_albicans_SC5314
E. faecium	NZ_CP039729.1
E. coli	NC_000913
HSV I	NC_001806.2
K. pneumoniae	NC_016845.1
P. aeruginosa	NC_002516.2
S. aureus	NC_007795.1

Supplement table S9. List of primers and target sequence.

Primer name	Target Primer sequence (5′ – 3′)	Adressed Gen	Adressed genomic region
P. aerug T1	GATCGAGTCGTTCATCGG	PA2338	NC_002516.2:2,581,960-2,582,147
P. aerug T2	CAGGATATCGCTCAACCG	PA1813	NC_002516.2:1,971,919-1,972,054
S. aureus T1	GTAGGTACATCATCGTTAAGGC	SAOUHSC_00060	NC_007795.1:64,011-64,883
S. aureus T2	GCGATTATTGTTGCTGAAGG	SAOUHSC_02279	NC_007795.1:2,110,362-2,110,725
C.alb T1	GGATTGCCTTGTCTAACCC	FGR14	Ca21chrR_C_albicans_SC5314:1,129,506-1,131,023
C.alb T2	GGTATTTGGGTTGCTGGG	-	Ca21chr1_C_albicans_SC5314:173,093-173,226
C.alb T3	CTGCCTCCATACTTTGTAGGC	SFI 1 2.0	Ca21chr7_C_albicans_SC5314:645,263-645,549
K.pneu T1	GTGGTGGAAGTCTACACCG	KPHS_05240	NC_016845.1:562,800-563,175
K.pneu T2	GGCTATGGGATGATATTGCC	KPHS_18590	NC_016845.1:1,929,825-1,930,019
B.frag T1	TCGGAAGGAGAGAACATCC	I6J55_RS14525	NZ_CP069563.1:3,565,788-3,566,174
B.frag T2	TCTATAGATGGCAACACACGG	I6J55_RS00215	NZ_CP069563.1:44,925-45,117
E.faec T1	AAGACGCACTAGCGAAAGC	sufD	NZ_CP039729.1:568,370-568,596
E.faec T2	GCAATTGTAGAAGGCGTGGC	pslx	NZ_CP039729.1:101,372-101,564
E.faec T3	GTCAATCTGGTCTGCCTCA	RecG	NZ_CP039729.1:101,067-101,280
E.coli T1	CAGAACCGCGCTAATT	cynR	NC_000913:358,005-358,231
E.coli T2	CTCGCGCGATTATCC	yejB	NC_000913:2,275,142-2,275,233
HSV I T1	GGTCGAAAGCGTACTTGGC	UL12	NC_001806.2:25,762-25,934
HSV I T2	GCAACTCGTAGCAAATAGGC	UL16	NC_001806.2:30,224-30,342
HSV_01	CTGTTGGGTTAGAATTTCGG	UL41	NC_001806.2:91,653-91,672
HSV_02	AACGTCTCCATCCATGCC	US8	NC_001806.2:141,973-141,990
HSV_03	AGTGATGTAAATGGTGTCCG	UL4/5	NC_001806.2:12,022-12,041
HSV_04	GTGGTTCATGTGTGTGCC	-	NC_001806.2:94,673-94,690

HSV_06	AAAGCAGTAACCAGGTCCG	-	NC_001806.2:91,065-91,083
HSV_07	AAACCTAAGGACAGCGGC	UL15/17	NC_001806.2:31,426-31,443
HSV_08	CGATGTTAATGGTGAACGC	UL15/17	NC_001806.2:31,479-31,497
HSV_10	ACCACCAGGTGTTTAACCC	UL44	NC_001806.2:97,225-97,243
16S	CCATGAAGTCGGAATCGC	16S	-
Igκ	AGAGCAGGACAGCAAGGAC	Igκ constant chain	NC_000002.12:88,857,361-88,857,683
cat1_V1	AAGCACAAGTTTTATCCGGC	cat1	gb|KC243783.1|+|1-2007|mecA
cat1_V2	AAGATGTGGCGTGTTACGG	cat1	gb|KC243783.1|+|1-2007|mecA
blaCTX-M-15_V1	TTGTTAGGAAGTGTGCCGC	blaCTX-M-15	gb|AY044436.1|+|1436-2311|CTX-M-15
blaCTX-M-15_V2	GGAGACGAAACGTTCCG	blaCTX-M-15	gb|AY044436.1|+|1436-2311|CTX-M-15
ndm1	ACATCGCTTTTGGTGGC	ndm1	gb|FN396876.1|-|2407-3219|NDM-1
TEM116	TGAGGCACCTATCTCAGCG	TEM116	gb|U36911.1|+|1430-2290|TEM-116
mecA_V1	ACTACGGTAACATTGATCGC	mecA	gb|KC243783.1|+|1-2007|mecA
mecA_V2	AATCGATGGTAAAGGTTGGC	mecA	gb|KC243783.1|+|1-2007|mecA
mecC	CATCACCAGGTTCAACCC	mecC	gb|FR821779.1|-|35681-37678|mecC
strA-APH(3'')	GCATTCTGACTGGTTGCC	strA-APH(3'')	gb|AF313472.2|+|15594-16397|APH(3'')-Ib
ermB [46]	GAACATCTGTGGTATGGCG	ermB	gb|AF242872.1|+|2132-2878|ErmB
OXA48	GCACGTATGAGCAAGATGC	OXA48	gb|AY236073.2|+|2188-2985|OXA-48
blaKPC-2	TCTGACAACAGGCATGACG	blaKPC-2	gb|AY034847.1|+|6-887|KPC-2
vanA	CCAATACCGCACAACCGA	vanA	gb|M97297.1|+|6980-8010|vanA
vanB	GAAGTGGATCAAATCCGGC	vanB	gb|KF823969.1|+|5111-6139|vanB
qnr1	CAATGCCAATCTCAGCGG	qnr1	gb|DQ831141.1|+|8922-9578|QnrA1
tetA	AGGCAAGCAGGATGTAGC	tetA	gb|AF534183.1|+|2971-4245|tet(A)
tetB	CAAAGGCTTGGAATACTGAGTG	tetB	gb|AB089595.1|+|1-1206|tet(B)
AAC(6)-Ib	TCATATCGTCGAGTGGTGG	AAC(6)-Ib	gb|JQ808129.1|+|634-1188|AAC(6')-Ib