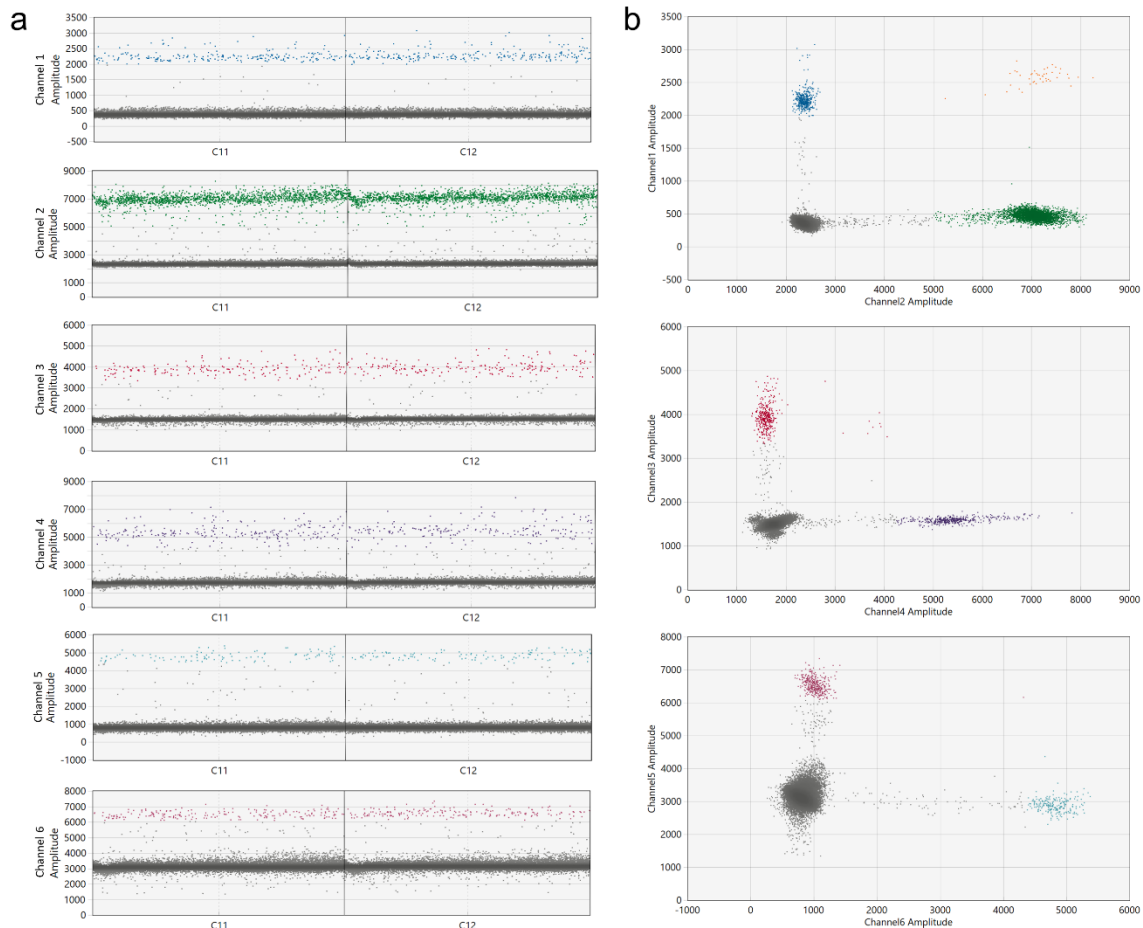


Development and validation of a methylation-specific droplet digital PCR multiplex for lung cancer detection

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Supplemental Figure S1. Initial test of 6-plex ddPCR on hypermethylated DNA. **a)** 1D and **b)** 2D fluorescence amplification plots from QXManager Software 2.0 showing duplicate analysis of Universal Methylated Human DNA Standard (Zymo Research) with a 6-plex ddPCR consisting of five cancer-specific markers (Channel 1: *HOXA9*, Channel 3: *OTX1*, Channel 4: *MCIDAS*, Channel 5: *SP9*, and Channel 6: *TFAP2B*) and one reference gene (Channel 2: *ALB*).

Supplemental Table 1 - Description of cohorts used for design and validation of the 6-plex ddPCR analysis. NSCLC: non-small cell lung cancer

Lung cancer patient cohort n = 109		NSCLC patient cohort n = 28	Healthy controls n = 60	
Age, median (range)		72 (41-91)	69.5 (57-79)	64 (25-88)
Sex				
	Male	56 (51%)	16 (57%)	29 (48%)
	Female	53 (49%)	12 (43%)	31 (52%)
Clinical TNM-stage				
	Noncancer			60 (100%)
	I	25 (23%)	3 (11%)	
	II	7 (6%)	2 (7%)	
	III	30 (28%)	5 (18%)	
	IV	47 (43%)	18 (64%)	
Pathology				
	Squamous cell carcinoma	26 (24%)	11 (39%)	
	Adenocarcinoma	65 (60%)	12 (43%)	
	Small cell lung cancer	9 (8%)		
	Large cell carcinoma		1 (4%)	
	Other*	9 (8%)	4 (14%)	

*Other include low differentiated carcinomas, carcinoid tumours, large cell neuroendocrine carcinomas, and sarcomatoid carcinomas

Supplemental Table 2 - List of identified differentially methylated CpGs using Illumina 450K methylation arrays.

Chromosome	Position	Islands Name	Relation to Island	Annotation	Detailed Annotation	Distance to Transcription Site [bp]	Nearest Gene	Lung Normal Tissue [mean beta-value]	Lung Tumour [mean beta-value]	Difference Mean Beta-value (tumour vs. normal)	Probe ID
chr11	627175	chr11:626728-628037	Island	promoter-TSS (NM_021920)	promoter-TSS (NM_021920)	-3	SCT	0.072900391	0.669151507	0.596251117	cg25774643
chr14	57278729	chr14:57278709-57279116	Island	Intergenic	CpG	-1171	OTX2-AS1	0.106328557	0.683261436	0.576932879	cg21039708
chr2	63283013	chr2:63282514-63283122	Island	exon (NM_001199770, exon 5 of 5)	exon (NM_001199770, exon 5 of 5)	5077	OTX1	0.08805333	0.664854105	0.576800775	cg07974511
chr2	63286049	chr2:63285949-63287097	Island	Intergenic	CpG	8113	OTX1	0.085542581	0.651651223	0.566108642	cg11536474
chr6	50791202	chr6:50791110-50791573	Island	exon (NM_003221, exon 2 of 7)	exon (NM_003221, exon 2 of 7)	4764	TFAP2B	0.12087363	0.685359055	0.564485424	cg27260772
chr2	208989248	chr2:208988834-208989269	Island	promoter-TSS (NM_006891)	promoter-TSS (NM_006891)	64	CRYGD	0.081847683	0.643283647	0.561435964	cg22399133
chr6	27647843	chr6:27647793-27648005	Island	Intergenic	CpG	-13970	LOC100507173	0.072536618	0.62182738	0.549290762	cg10903903
chr6	105400985	chr6:105400877-105401149	Island	Intergenic	CpG	-3937	LIN28B	0.198037533	0.746558005	0.548520473	cg02391713
chr1	63785946	chr1:63782394-63790471	Island	Intergenic	CpG-1195	-2783	FOXD3	0.08212014	0.630431916	0.548311776	cg06962177
chr2	172945058	chr2:172944988-172945384	Island	exon (NM_199227, exon 10 of 10)	exon (NM_199227, exon 10 of 10)	-5149	DLX1	0.209575047	0.753203424	0.543628377	cg16792302
chr6	105400993	chr6:105400877-105401149	Island	Intergenic	CpG	-3929	LIN28B	0.209230854	0.752227422	0.542996569	cg22620090
chr2	63283967	chr2:63283936-63284147	Island	3' UTR (NM_001199770, exon 5 of 5)	3' UTR (NM_001199770, exon 5 of 5)	6031	OTX1	0.045093758	0.588057711	0.542963952	cg21472506
chr11	627152	chr11:626728-628037	Island	promoter-TSS (NM_021920)	promoter-TSS (NM_021920)	20	SCT	0.051294897	0.590864478	0.539569581	cg00249511
chr1	110612044	chr1:110610265-110613303	Island	intron (NM_006492, intron 1 of 3)	CpG	1277	ALX3	0.098774656	0.631820532	0.533045876	cg06809252
chr2	63284132	chr2:63283936-63284147	Island	3' UTR (NM_001199770, exon 5 of 5)	3' UTR (NM_001199770, exon 5 of 5)	6196	OTX1	0.101370004	0.626194096	0.524824093	cg10122865
chr2	130971164	chr2:130970933-130971242	Island	Intergenic	CpG	-15131	TUBA3E	0.212424184	0.731706391	0.519282208	cg08566455
chr14	60973823	chr14:60973772-60974123	Island	Intergenic	CpG	-2114	SIX6	0.08735143	0.60649345	0.519142021	cg13539545
chr15	89952499	chr15:89952271-89953061	Island	Intergenic	CpG	31227	LOC254559	0.038202081	0.554060662	0.515858581	cg18698788
chr5	54516805	chr5:54516267-54516919	Island	intron (NM_001190787, intron 6 of 6)	CpG	6337	MCIDAS	0.054112251	0.569053786	0.514941535	cg18456523
chr2	172945274	chr2:172944988-172945384	Island	3' UTR (NM_199227, exon 10 of 10)	3' UTR (NM_199227, exon 10 of 10)	-4933	DLX1	0.083777253	0.595276161	0.511498909	cg08402365
chr12	6665330	chr12:6664425-6665336	Island	promoter-TSS (NM_001039670)	promoter-TSS (NM_001039670)	-82	IFFO1	0.129405187	0.639381514	0.509976327	cg08875705
chr2	63281139	chr2:63281034-63281347	Island	intron (NM_014562, intron 3 of 4)	CpG	3203	OTX1	0.034171611	0.543986162	0.509814552	cg25622366
chr2	66673316	chr2:66672431-66673636	Island	intron (NM_002398, intron 6 of 12)	CpG	10785	MEIS1	0.079556666	0.588032114	0.508475448	cg06833110
chr2	175208594	chr2:175208415-175209076	Island	Intergenic	CpG	8774	SP9	0.094550882	0.601734746	0.507183864	cg23064609
chr4	41882564	chr4:41882450-41882964	Island	Intergenic	CpG	-54572	TMEM33	0.068984096	0.572346272	0.503362176	cg17231999
chr16	31580989	chr16:31580559-31581023	Island	promoter-TSS (NR_027011)	promoter-TSS (NR_027011)	-145	YBX3P1	0.12192387	0.622185976	0.500262105	cg15811515

Supplemental Table 3 - Description of oligos used in quality control and methylation-specific ddPCR analyses.

Quality control multiplex

Assay	Gene	Assay component	Modifications	Sequence	Chromosome location	Strand	Manufacturer	Final concentration [nM]	Amplicon length	Reference
EMC7 250	<i>EMC7</i> (ER membrane protein complex subunit 7)	Forward primer Reverse primer Probe	5' 6-FAM/ZEN/IBFQ 3'	5'-AAGTACTACTGAGTATGATGTT-3' 5'-CTAGATTTTGCCAGATGATTTT-3' 5'-AGTTGCCTGATGTTTCTGAGTTCAT-3'	15	+	LGC Biosearch LGC Biosearch IDT	300 300 200	250 bp	Lefèvre, 2021
EMC7 65	<i>EMC7</i> (ER membrane protein complex subunit 7)	Forward primer Reverse primer Probe	5' HEX/IBFQ 3'	5'-CTTTCCCATGTTGCTTTAT-3' 5'-CTGACAACCTCTGATGTTTT-3' 5'-CAGAGCAAGATATGTGAATTACATCAA-3'	15	+	Biomers Biomers IDT	300 300 200	65 bp	Lefèvre, 2021
PBC	<i>IGVH3/IGJH2</i> (Immunoglobulin heavy locus)	Forward primer Reverse primer Probe	5' Cy5/TAO/IBRQ 3'	5'-ATCTGCAAATGAACAGYCTGAGA-3' 5'-CTTACCTGAGGAGACGGTGAC-3' 5'-CYGAGGACACRGCCTGTGATTACTGTGC-3'	14	+	Biomers Biomers IDT	900 900 400	90-125 bp	Pallisgaard, 2015*
CPP1	<i>CPP1</i>	Forward primer Reverse primer Probe	5' Cy5.5/IBRQ 3'	5'-CCATGGATGTATTGCGCAGTTAC-3' 5'-TAAATATTGTGCTTCACCTACTCTAGTG-3' 5'-TTGGCGTAGTTCTCCGCTTACCCCG-3'	-		LGC Biosearch LGC Biosearch IDT	300 300 200	191 bp	Pallisgaard, 2015*

Methylation-specific ddPCR multiplex assays

Assay	Gene	Assay component	Modifications	Sequence	Chromosome location	Strand	Manufacturer	Final concentration [nM]	Amplicon length	Reference
HOXA9	<i>HOXA9</i> (Homeobox A9)	Forward primer Reverse primer Probe	5' 6-FAM/ZEN/IBFQ 3'	5'-GAGTATTTTCGATTTTAGTTCGTGT-3' 5'-CGCGTACACTAAATCCAC-3' 5'-TTAGTTTAAGGCGACGGTGT-3'	7	-	LGC Biosearch LGC Biosearch IDT	200 200 200	78 bp	Wen, 2021
ALB	<i>ALB</i> (Albumin)	Forward primer Reverse primer Probe	5' VIC/MGBNFQ 3'	5'-GGGATGGAAAGAATTTATGTT-3' 5'-AAACAAACTAACCCCAAATTCT-3' 5'-AGGGTTTTTATAATTTA-3'	4	+	IDT IDT Applied Biosystems	400 400 200	76 bp	**
OTX1	<i>OTX1</i> (Orthodenticle homeobox 1)	Forward primer Reverse primer Probe	5' Cy5/TAO/IBRQ 3'	5'-TTTAGCGGAGTGCGTTGG-3' 5'-ACGAAACGAAATACGACTCTATACG-3' 5'-CTCCGACGAAACCTAACCCCTAACGC-3'	2	+	IDT IDT IDT	400 400 200	91 bp	**
MCIDAS	<i>MCIDAS</i> (Multiciliate Differentiation And DNA Synthesis Associated Cell Cycle Protein)	Forward primer Reverse primer Probe	5' Cy5.5/IBRQ 3'	5'-GATAGGGAAGAGCGTCGTTT-3' 5'-GTAATACGTAAACGCGCTAAATC-3' 5'-TTTCGGGTTCCGAGCGTGTA-3'	5	+	IDT IDT IDT	400 400 200	85 bp	**
SP9	<i>SP9</i> (Sp9 transcription factor)	Forward primer Reverse primer Probe	5' ROX/IBRQ 3'	5'-TTCGTTTAAGTTTGTGCGGTTTC-3' 5'-TTCGCTCGCTTTCCTAACTAC-3' 5'-TTTCGCGCGGGTCGGATTGTTT-3'	2	-	IDT IDT IDT	400 400 200	80 bp	**
TFAP2B	<i>TFAP2B</i> (Transcription factor AP-2β)	Forward primer Reverse primer Probe	5' ATTO 590/IBRQ 3'	5'-AGTTGGGTTTCGGTGTTT-3' 5'-AAATCCGACGACGAAATATAA-3' 5'-TTATTCCGAGCGTTTCGTGTTGTT-3'	6	+	Biomers Biomers IDT	400 400 200	70 bp	**

IDT: Integrated DNA Technologies, bp: base pair

*The assays have been modified to include Cy5 and Cy5.5 probes.

**All assays were designed for this study

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Supplemental Table 4. Description of markers in the 6-plex ddPCR above (+) or below (-) the limit of blank (LoB) in NSCLC patients undergoing immunotherapy. LoBs were based on Youden's J statistics, and samples were considered ctDNA positive (+) if they had ≥2 markers above the LoB. Empty fields represent missing samples.

Patient no.	ctDNA status (+/-)			Baseline					Before 2nd treatment cycle					Before 4th treatment cycle				
	Baseline	1st treatment	3rd treatment	HOXA9 (+/-)	MCIDAS (+/-)	OTX1 (+/-)	SP9 (+/-)	TFAP2B (+/-)	HOXA9 (+/-)	MCIDAS (+/-)	OTX1 (+/-)	SP9 (+/-)	TFAP2B (+/-)	HOXA9 (+/-)	MCIDAS (+/-)	OTX1 (+/-)	SP9 (+/-)	TFAP2B (+/-)
1	+	+		+	+	+	+	+	+	+	+	+	+					
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	-
8	+	-	-	+	+	+	+	+	-	-	-	-	-	+	-	-	-	-
9	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
10	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
11	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+
12	+	+		+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-
14	+	+	-	+	+	+	+	-	+	+	+	+	-	+	-	-	-	-
15	+	+		+	+	+	+	-	+	+	+	+	-					
16	+	+		+	+	+	+	-	+	+	+	+	-					
17	+	+	-	+	+	+	+	-	+	+	+	+	-	+	-	-	-	-
18	+	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	+	-
19	+	+	+	+	+	+	+	-	+	-	-	+	-	+	-	-	+	-
20	+	+		+	+	+	+	-	+	-	-	+	-					
21	+	+	+	+	-	-	+	+	+	-	-	+	+	-	-	-	+	+
22	+	-	+	+	+	-	+	-	-	-	-	-	-	+	+	+	+	-
23	+	-	+	+	-	+	+	-	-	-	-	+	-	+	+	+	+	-
24	+	+	+	+	-	+	+	-	+	+	+	+	-	+	-	-	+	-
25	+	-	-	+	-	+	+	-	-	-	-	-	-	-	-	-	-	-
26	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	+	-
27	-	-	+	+	-	-	-	-	-	-	-	-	-	+	+	-	+	-
28	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	-	+	-