Optimizing DNA Methylation Detection for Early Lung Cancer

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Methylation Detection in Plasma and Sputum

Stage I Lung Cancers and Surgical Controls

Blood	Sensitivity	Specificity	PPV	NPV	AUC	95% CI
CDO1	**65%**	**74%**	**86%**	**46%**	**0.68**	**(0.58 - 0.77)**
TAC1	**76%**	**78%**	**90%**	**57%**	**0.78**	**(0.70 - 0.86)**
HOXA7	33%	94%	93%	36%	0.60	(0.51 - 0.69)
HOXA9	81%	52%	81%	52%	0.62	(0.52 - 0.73)
SOX17	**71%**	**86%**	**93%**	**54%**	**0.78**	**(0.70 - 0.86)**
ZFP42	81%	58%	83%	55%	0.66	(0.56 - 0.75)
CD01, TAC1, SOX17	**91%**	**64%**	**86%**	**74%**	**0.77**	**(0.68 - 0.86)**

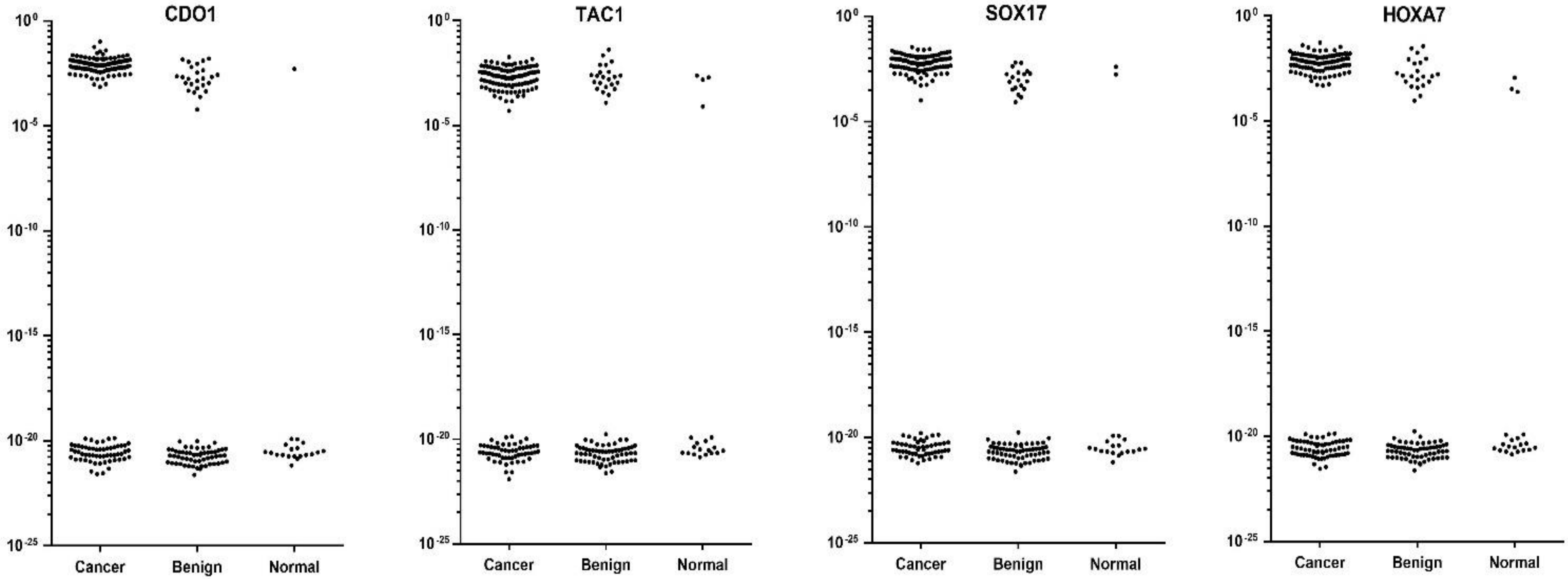
Sputum	Sensitivity	Specificity	PPV	NPV	AUC	95% CI
CDO1	78%	67%	90%	45%	0.70	(0.57 - 0.84)
TAC1	**84%**	**79%**	**94%**	**57%**	**0.84**	**(0.74 - 0.94)**
HOXA7	**63%**	**92%**	**97%**	**40%**	**0.77**	**(0.67 - 0.86)**
HOXA9	77%	42%	83%	32%	0.56	(0.41 - 0.69)
SOX17	**84%**	**88%**	**96%**	**59%**	**0.84**	**(0.75 -0.94)**
ZFP42	88%	62%	90%	58%	0.73	(0.60 - 0.87)
TAC1, HOXA7, SOX17	**93%**	**79%**	**94%**	**75%**	**0.89**	**(0.80 - 0.98)**

Hulbert A et al, *Clinical Cancer Research* 2017

Validation of Plasma DNA Methylation Detection

246 patients with screen detected pulmonary nodules referred to surgeon

Stage I: Nodules 3.0 cm or less, 163 cancer, 83 benign



Chen et al, Clinical Epigenetics, 2020

Performance of Plasma DNA Methylation Detection in Validation Cohort

Stage I NSCLCa T size ≤ 3.0 cm

Sensitivity, Specificity, PPV, and NPV at optimal cutoffs with AUC.

Gene	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*AUC*	*95% CI*
CDO1	63%	83%	88%	53%	0.78	0.71-0.83
TAC1	68%	70%	81%	52%	0.71	0.64-0.78
SOX17	68%	86%	90%	57%	0.82	0.76-0.87
HOXA7	55%	87%	89%	50%	0.73	0.67-0.80
HOXA9	64%	49%	71%	41%	0.56	0.48-0.64
GATA4	44%	58%	67%	35%	0.53	0.45-0.61
GATA5	43%	63%	70%	36%	0.52	0.44-0.60
PAX5B	41%	55%	64%	32%	0.54	0.45-0.62
CDO1, TAC1, SOX17	89%	61%	82%	74%	0.85	0.81-0.91
CDO1, SOX17, HOXA7	90%	71%	86%	78%	0.88	0.84-0.93

Specificity compared to benign nodules

Chen et al, Clinical Epigenetics, 2020

Performance of Plasma DNA Methylation Detection in Validation Cohort

Stage I NSCLCa T size ≤ 3.0 cm

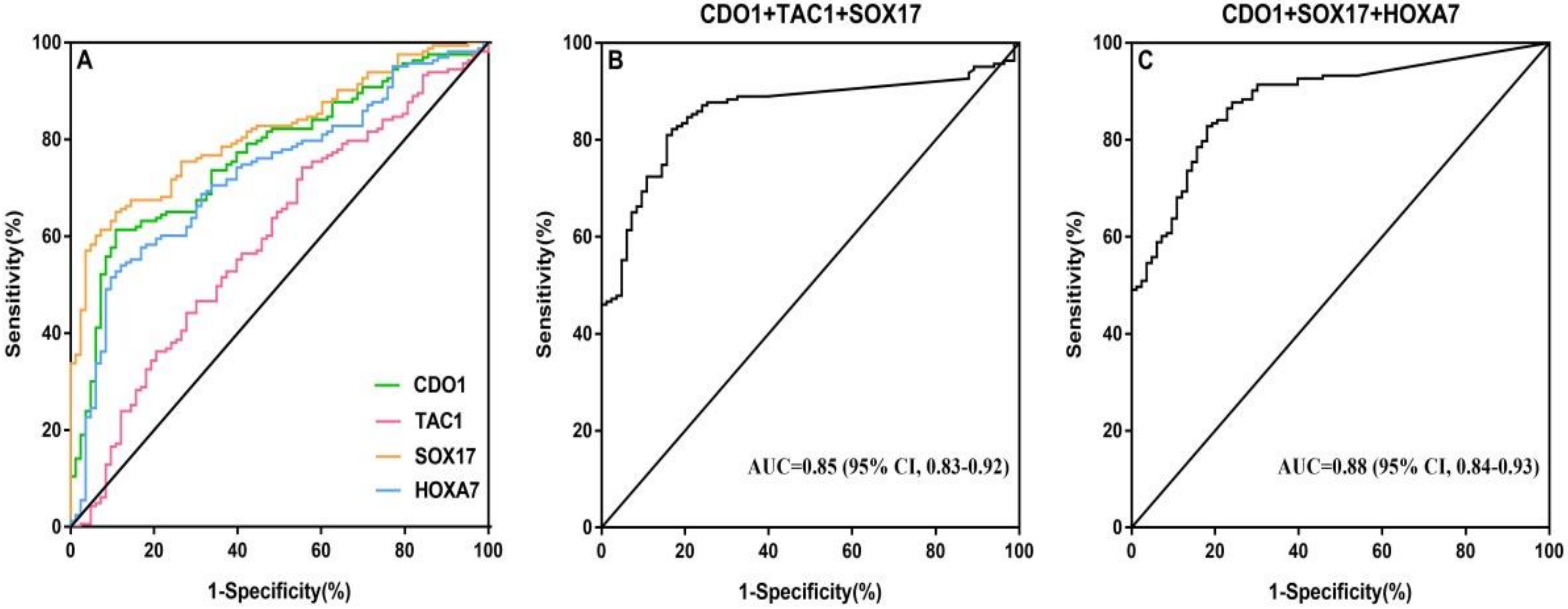
Sensitivity, Specificity, PPV, and NPV at optimal cutoffs with AUC.

Gene	*Sensitivity*	*Specificity*	*PPV*	*NPV*	*AUC*	*95% CI*
CDO1	*63%*	*83%*	*88%*	*53%*	*0.78*	*0.71-0.83*
TAC1	*68%*	*70%*	*81%*	*52%*	*0.71*	*0.64-0.78*
SOX17	*68%*	*86%*	*90%*	*57%*	*0.82*	*0.76-0.87*
HOXA7	*55%*	*87%*	*89%*	*50%*	*0.73*	*0.67-0.80*
HOXA9	*64%*	*49%*	*71%*	*41%*	*0.56*	*0.48-0.64*
GATA4	*44%*	*58%*	*67%*	*35%*	*0.53*	*0.45-0.61*
GATA5	*43%*	*63%*	*70%*	*36%*	*0.52*	*0.44-0.60*
PAX5B	*41%*	*55%*	*64%*	*32%*	*0.54*	*0.45-0.62*
CDO1, TAC1, SOX17	*89%*	*61%*	*82%*	*74%*	*0.85*	*0.81-0.91*
CDO1, SOX17, HOXA7	*90%*	*71%*	*86%*	*78%*	*0.88*	*0.84-0.93*

Specificity compared to benign nodules

Chen et al, Clinical Epigenetics, 2020

ROC curves for Methylation Detection in Plasma



Performance of Plasma DNA Methylation Detection

Stage I NSCLCa according to T size (all ≤ 3.0 cm)

Plasma	Sensitivity	Specificity	PPV	NPV	AUC	95% CI
CDO1	63%	83%	88%	53%	0.78	(0.71 - 0.83)
TAC1	68%	70%	81%	57%	0.71	(0.64 - 0.78)
SOX17	68%	86%	90%	57%	0.82	(0.76 - 0.87)
HOXA7	55%	87%	89%	50%	0.73	(0.67 - 0.80)
CD01, TAC1, SOX17	**89%**	**61%**	**82%**	**74%**	**0.85**	**(0.81 - 0.91)**
CD01, HOXA7, SOX17	**90%**	**71%**	**86%**	**78%**	**0.88**	**(0.84 - 0.93)**

CD01, HOXA7, SOX17	Sensitivity	Specificity	PPV	NPV	AUC	95% CI
T 2.1-3.0 cm	91%	90%	96%	81%	0.95	(0.57 - 0.84)
T 1.1-2.0 cm	74%	93%	90%	63%	0.92	(0.74 - 0.94)
T 0 - 1.0 cm	64%	82%	82%	64%	0.75	(0.62 - 0.89)
CDO1, SOX17, TAC1 **T 0 - 1.0 cm**	**71%**	**82%**	**83%**	**69%**	**0.81**	**(0.69 - 0.93)**

Comparison: stage 1 lung tumors (up to 5 cm included) CancerSEEK (Sensitivity of 43%)

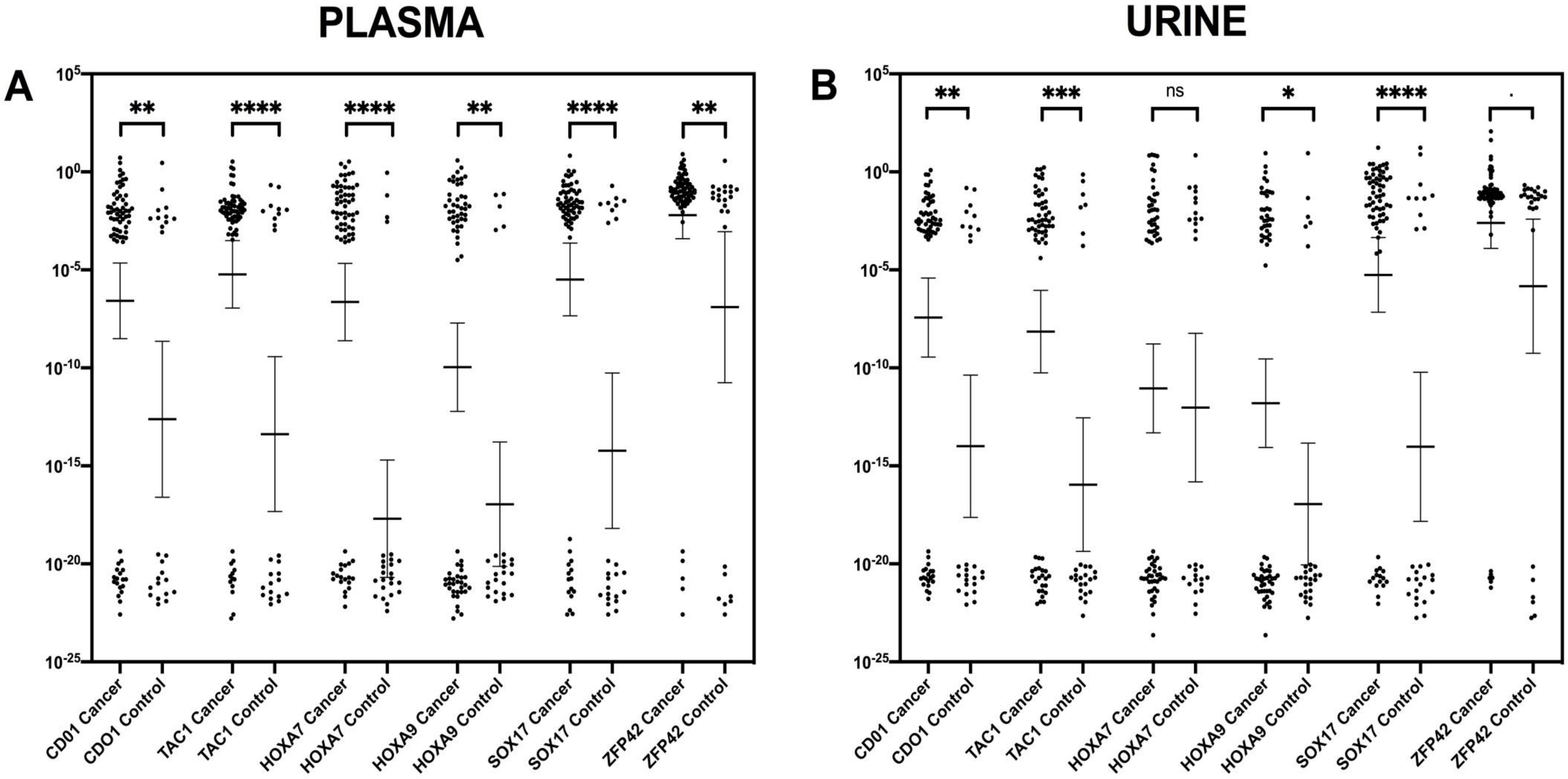
Can Urine be used for DNA Methylation Detection of Lung Cancer?

Rationale: ct DNA may be filtered by Kidney and be present in Urine
Oncogenic Driver mutations can be detected in Urine
Urine is easily collected, non-invasively

Approach: Utilize Established Lung Cancer Methylation Loci
MOB-qMSP Detection
Modify Assay for DNA Fragment Size in Urine vs Plasma
(~180bp Plasma, ~ or < 100 bp Urine)

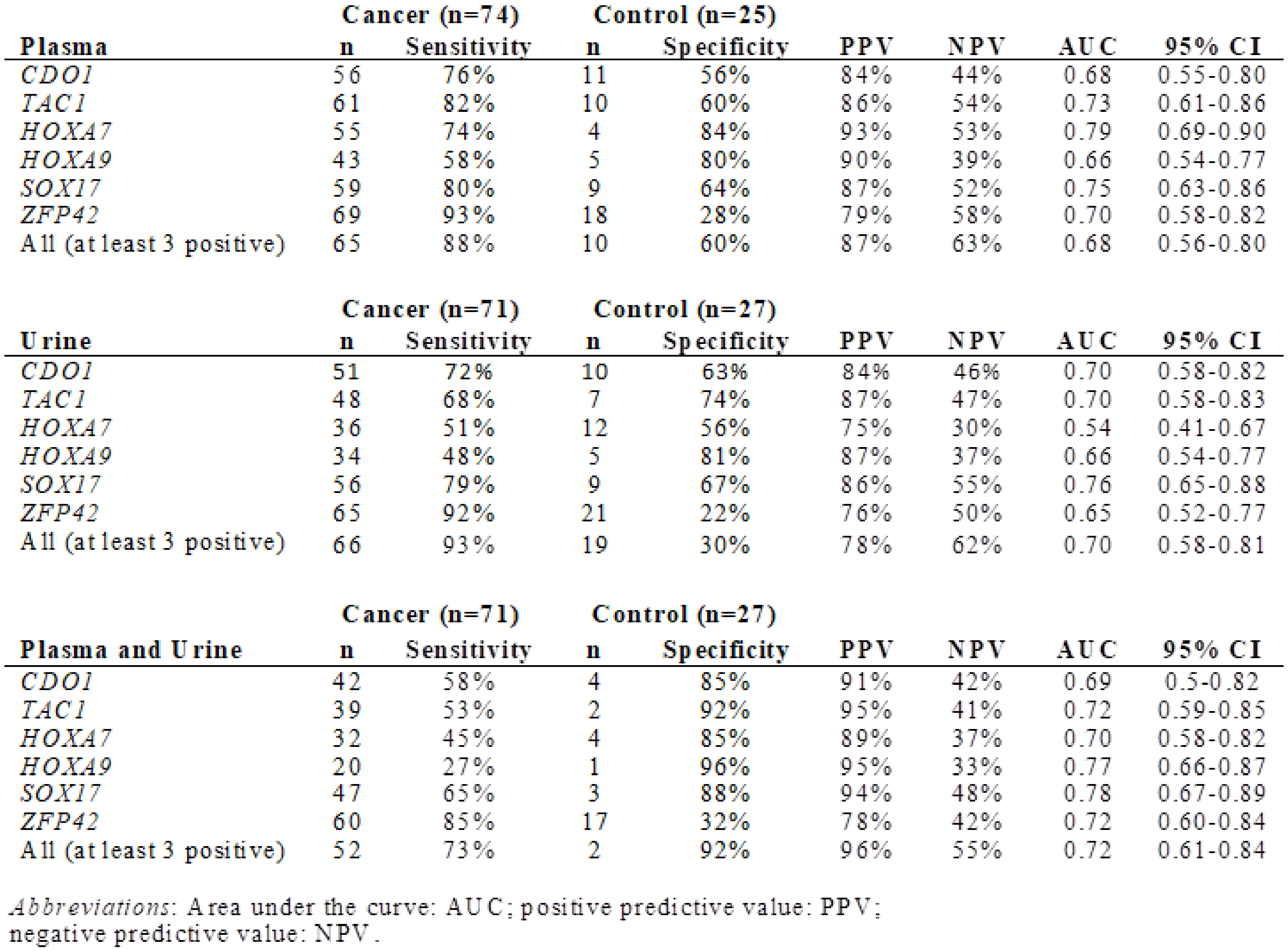
DNA Methylation Detection in Plasma and Urine

74 NSCLCa , 27 benign nodule controls



Liu B. … Herman, JG, Hulbert A. Clinical Cancer Research, 2020

DNA Methylation Detection in Plasma and Urine



	Cancer (n=74)		Control (n=25)					
Plasma	**n**	**Sensitivity**	**n**	**Specificity**	**PPV**	**NPV**	**AUC**	**95% CI**
CDO1	56	76%	11	56%	84%	44%	0.68	0.55-0.80
TAC1	61	82%	10	60%	86%	54%	0.73	0.61-0.86
HOXA7	55	74%	4	84%	93%	53%	0.79	0.69-0.90
HOXA9	43	58%	5	80%	90%	39%	0.66	0.54-0.77
SOX17	59	80%	9	64%	87%	52%	0.75	0.63-0.86
ZFP42	69	93%	18	28%	79%	58%	0.70	0.58-0.82
All (at least 3 positive)	65	88%	10	60%	87%	63%	0.68	0.56-0.80

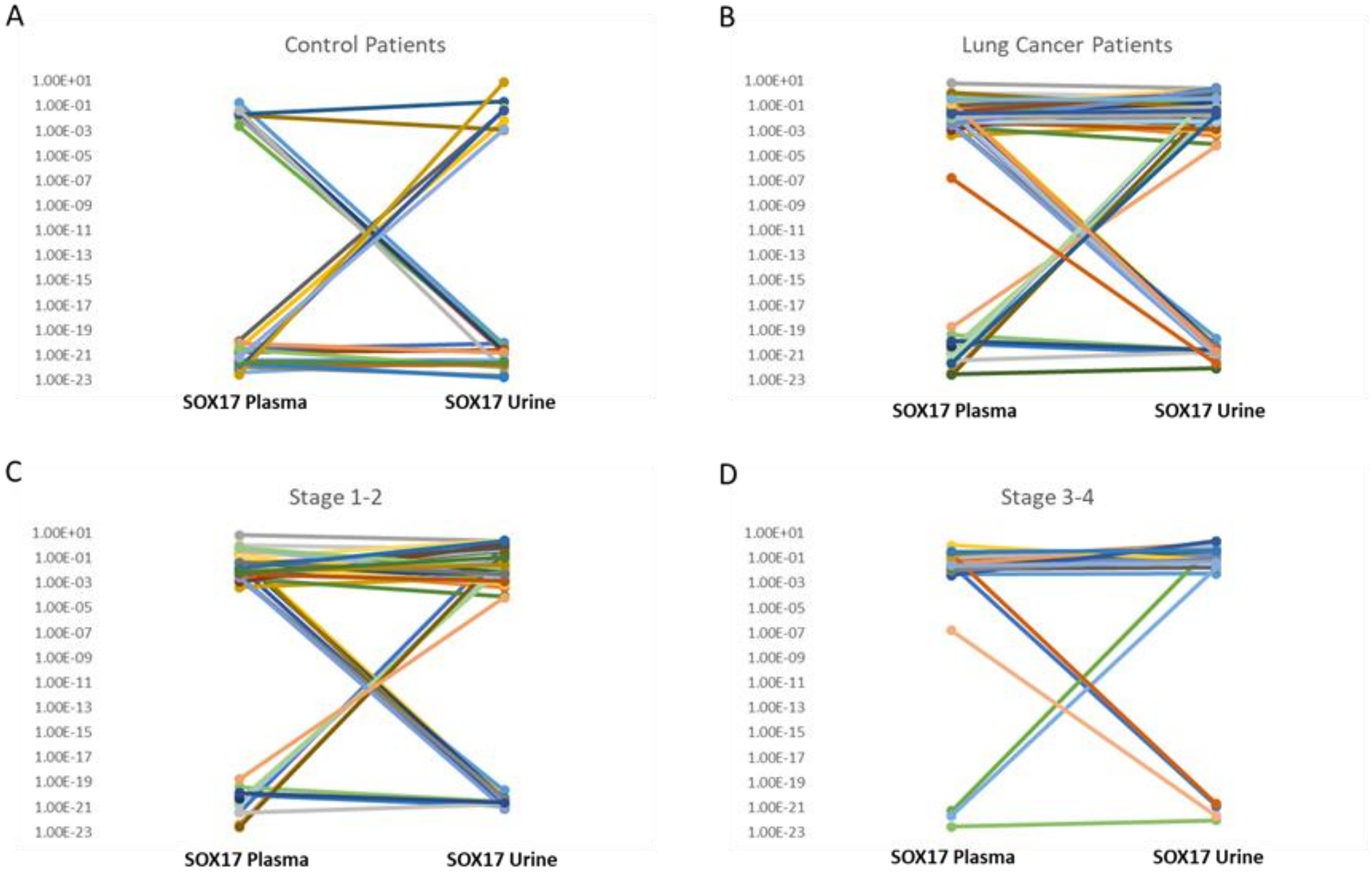
	Cancer (n=71)		Control (n=27)					
Urine	**n**	**Sensitivity**	**n**	**Specificity**	**PPV**	**NPV**	**AUC**	**95% CI**
CDO1	51	72%	10	63%	84%	46%	0.70	0.58-0.82
TAC1	48	68%	7	74%	87%	47%	0.70	0.58-0.83
HOXA7	36	51%	12	56%	75%	30%	0.54	0.41-0.67
HOXA9	34	48%	5	81%	87%	37%	0.66	0.54-0.77
SOX17	56	79%	9	67%	86%	55%	0.76	0.65-0.88
ZFP42	65	92%	21	22%	76%	50%	0.65	0.52-0.77
All (at least 3 positive)	66	93%	19	30%	78%	62%	0.70	0.58-0.81

	Cancer (n=71)		Control (n=27)					
Plasma and Urine	**n**	**Sensitivity**	**n**	**Specificity**	**PPV**	**NPV**	**AUC**	**95% CI**
CDO1	42	58%	4	85%	91%	42%	0.69	0.5-0.82
TAC1	39	53%	2	92%	95%	41%	0.72	0.59-0.85
HOXA7	32	45%	4	85%	89%	37%	0.70	0.58-0.82
HOXA9	20	27%	1	96%	95%	33%	0.77	0.66-0.87
SOX17	47	65%	3	88%	94%	48%	0.78	0.67-0.89
ZFP42	60	85%	17	32%	78%	42%	0.72	0.60-0.84
All (at least 3 positive)	52	73%	2	92%	96%	55%	0.72	0.61-0.84

Abbreviations: Area under the curve: AUC; positive predictive value: PPV; negative predictive value: NPV.

Concordance of DNA Methylation Detection

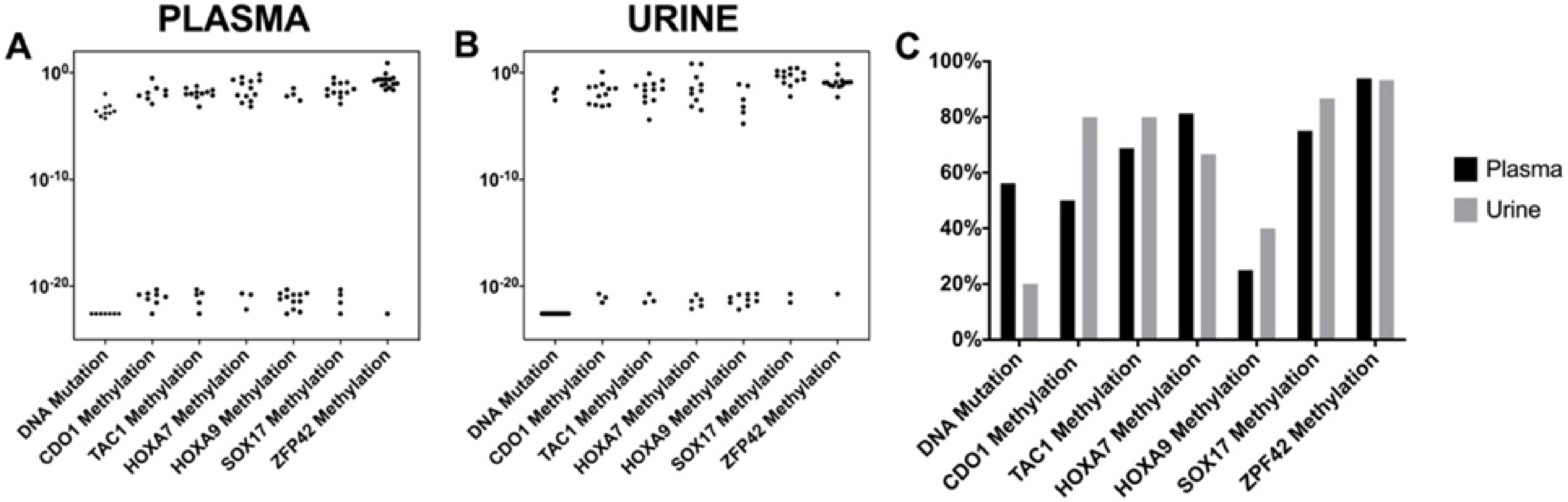
Plasma vs Urine



Comparison of DNA Methylation and Mutation Detection

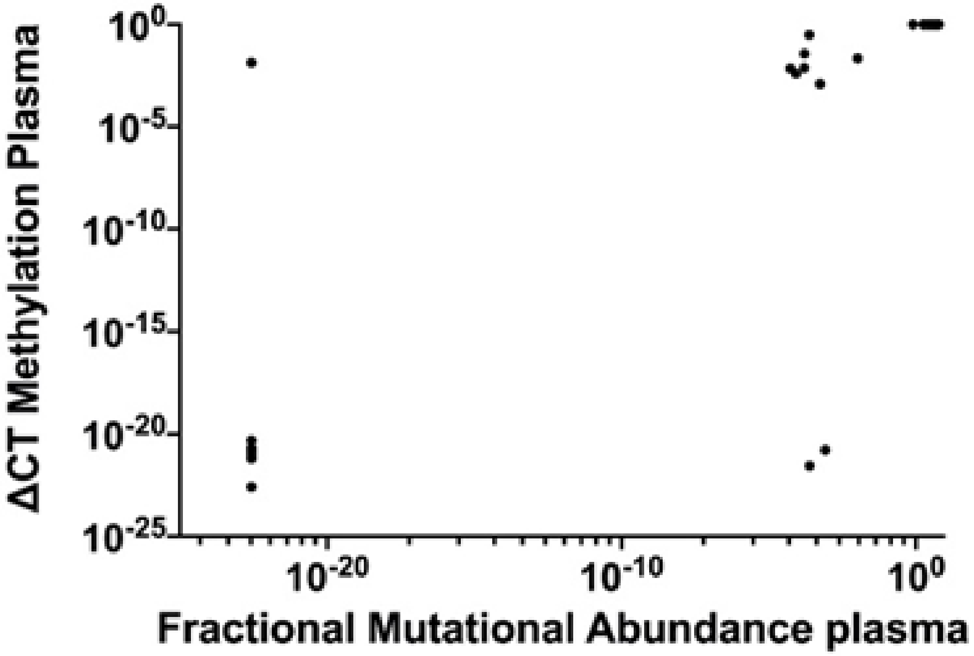
16 Patients with Oncogenic Driver Mutations (11 KRAS, 5 EGFR)

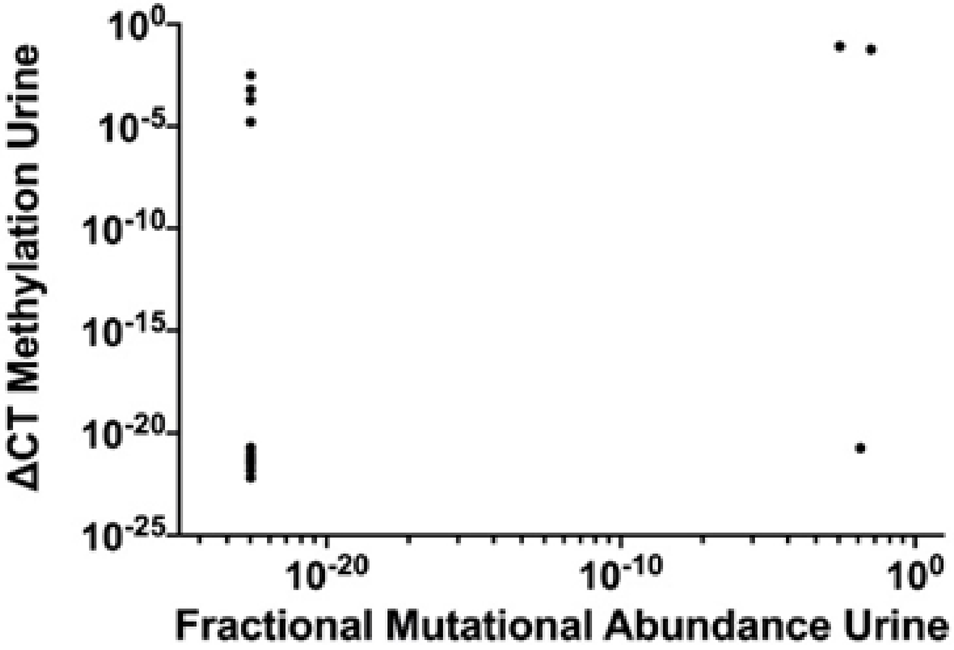
Droplet Digital PCR Mutation Detection



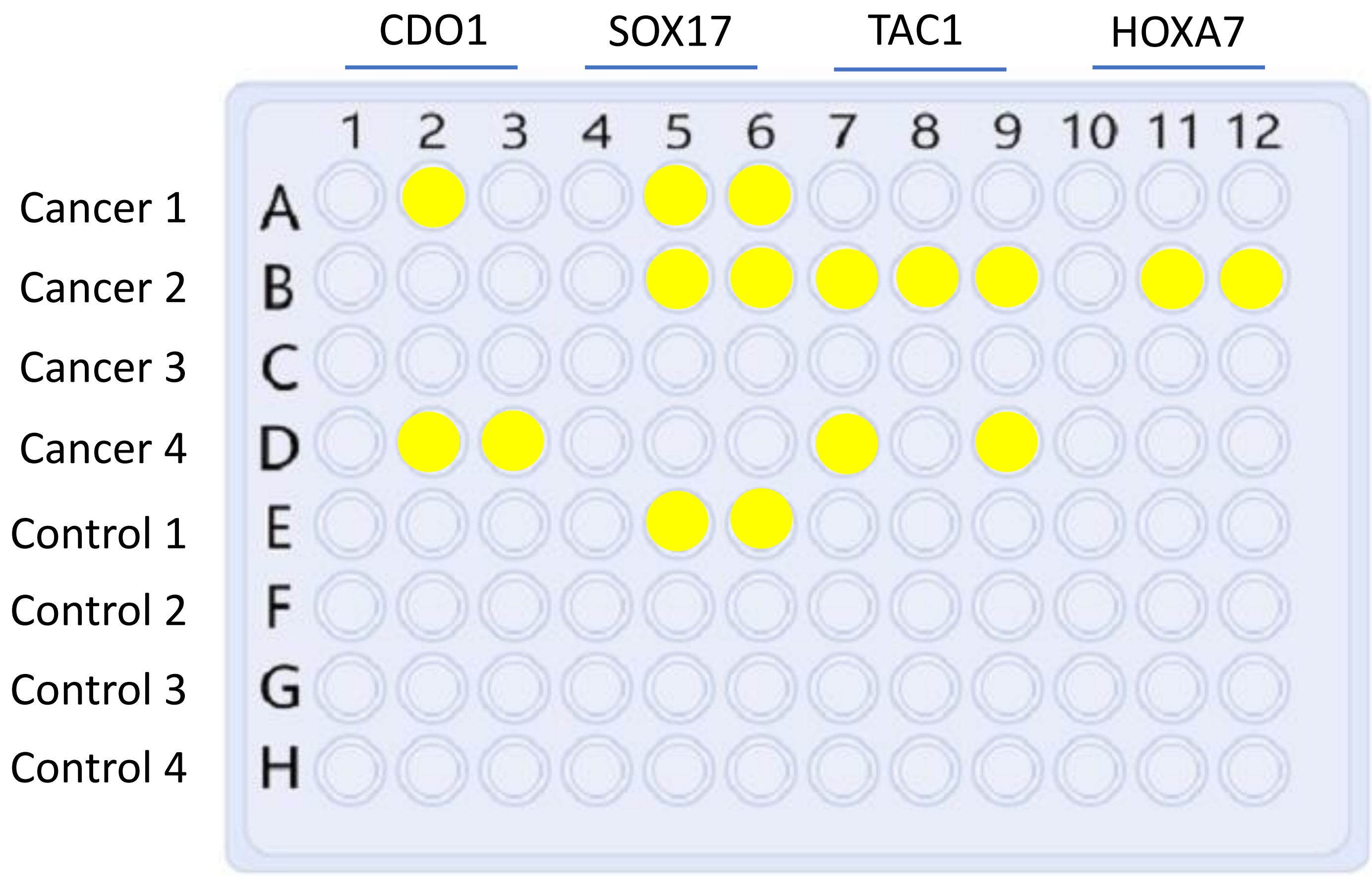
Concordance of DNA Methylation and Mutation Detection

16 Patients with Oncogenic Driver Mutations (11 KRAS, 5 EGFR)



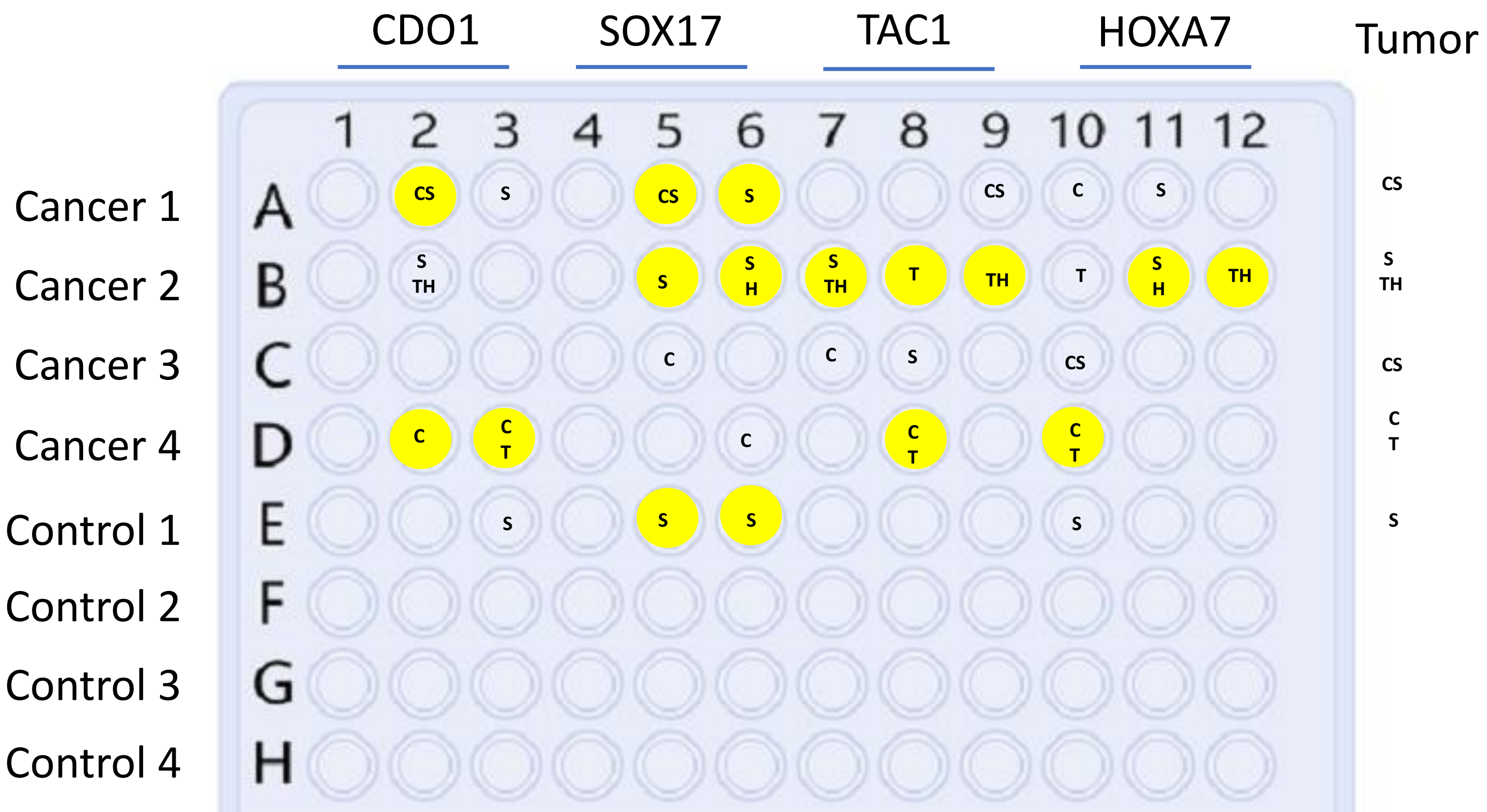


Multi-gene MOB-qMSP detection:
Addressing the challenge of stochastic sampling



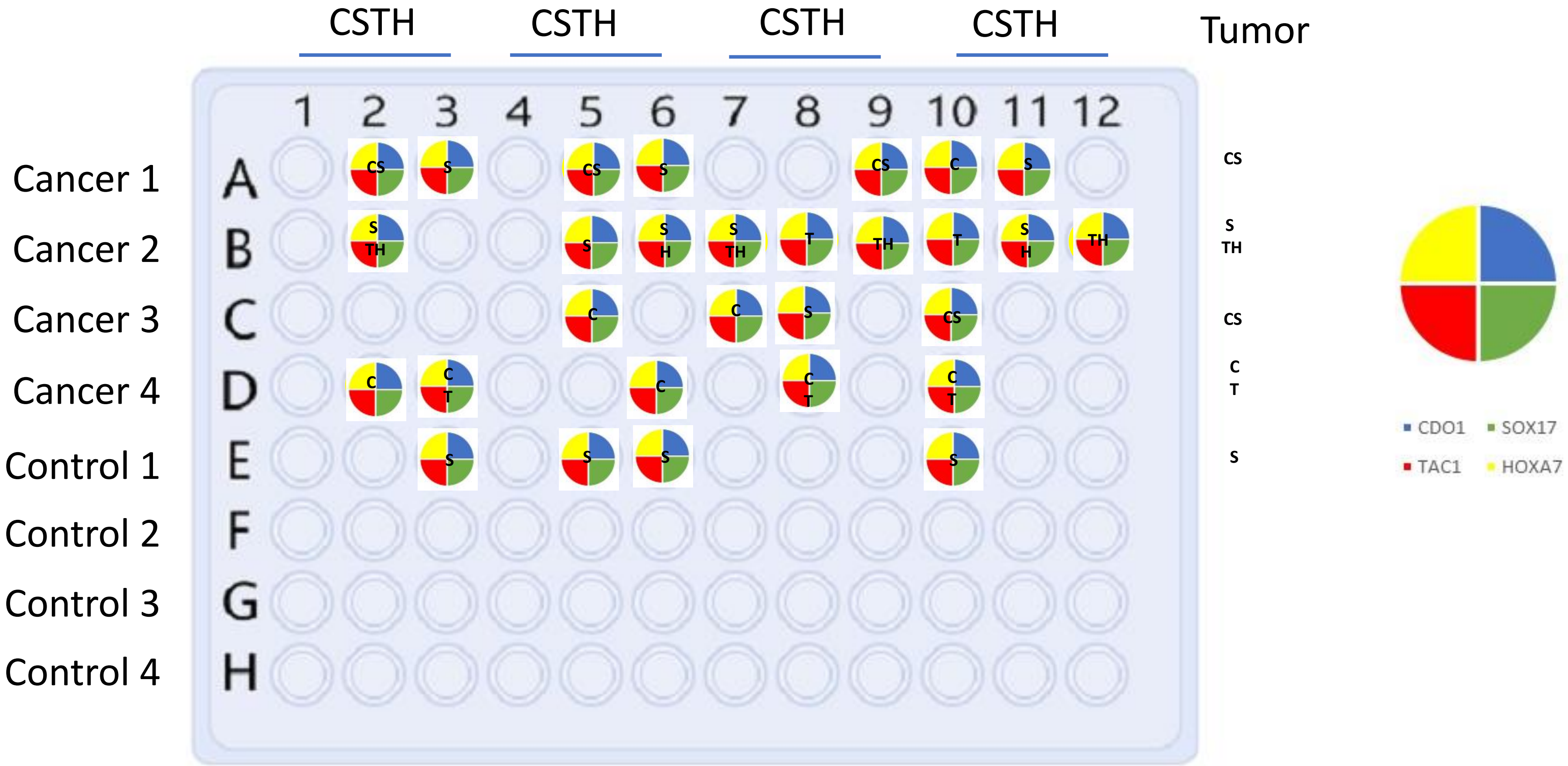
		CDO1			SOX17			TAC1			HOXA7		
		1	2	3	4	5	6	7	8	9	10	11	12
Cancer 1	A	○	●	○	○	●	●	○	○	○	○	○	○
Cancer 2	B	○	○	○	○	●	●	●	●	●	○	●	●
Cancer 3	C	○	○	○	○	○	○	○	○	○	○	○	○
Cancer 4	D	○	●	●	○	○	○	●	○	●	○	○	○
Control 1	E	○	○	○	○	●	●	○	○	○	○	○	○
Control 2	F	○	○	○	○	○	○	○	○	○	○	○	○
Control 3	G	○	○	○	○	○	○	○	○	○	○	○	○
Control 4	H	○	○	○	○	○	○	○	○	○	○	○	○

Multi-gene MOB-qMSP detection
Stochastic sampling leads to missed detection



		CDO1			SOX17			TAC1			HOXA7			Tumor
		1	2	3	4	5	6	7	8	9	10	11	12	
Cancer 1	A		● CS	S		● CS	● S			CS	C	S		CS
Cancer 2	B		S TH			● S	● S H	● S TH	● T	● TH	T	● S H	● TH	S TH
Cancer 3	C					C		C	S		CS			CS
Cancer 4	D		● C	● C T			C		● C T		● C T			C T
Control 1	E			S		● S	● S				S			S
Control 2	F													
Control 3	G													
Control 4	H													

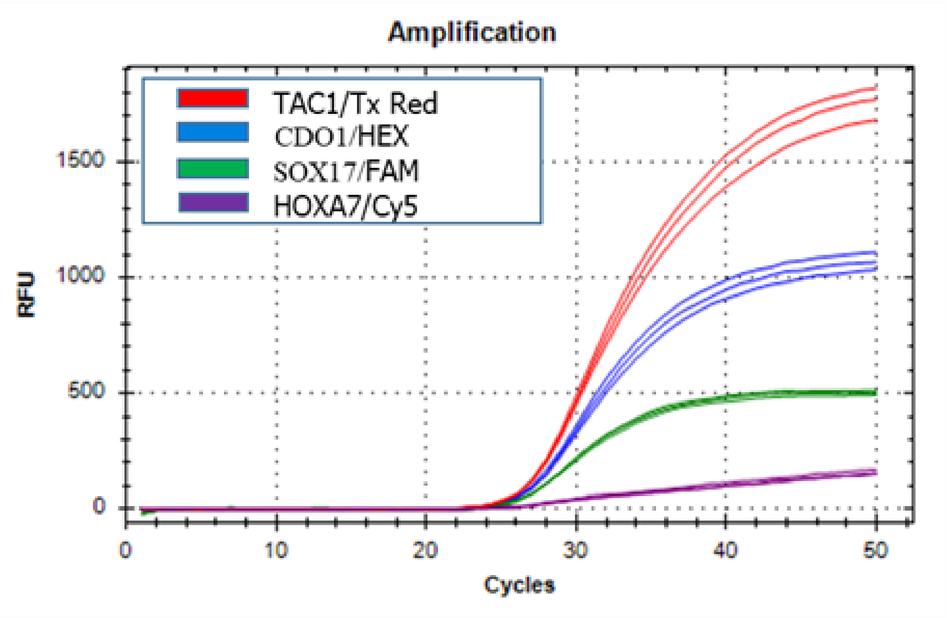
Multiplexing of Multi-gene MOB-qMSP detection
Elimination of Stochastic sampling improves detection



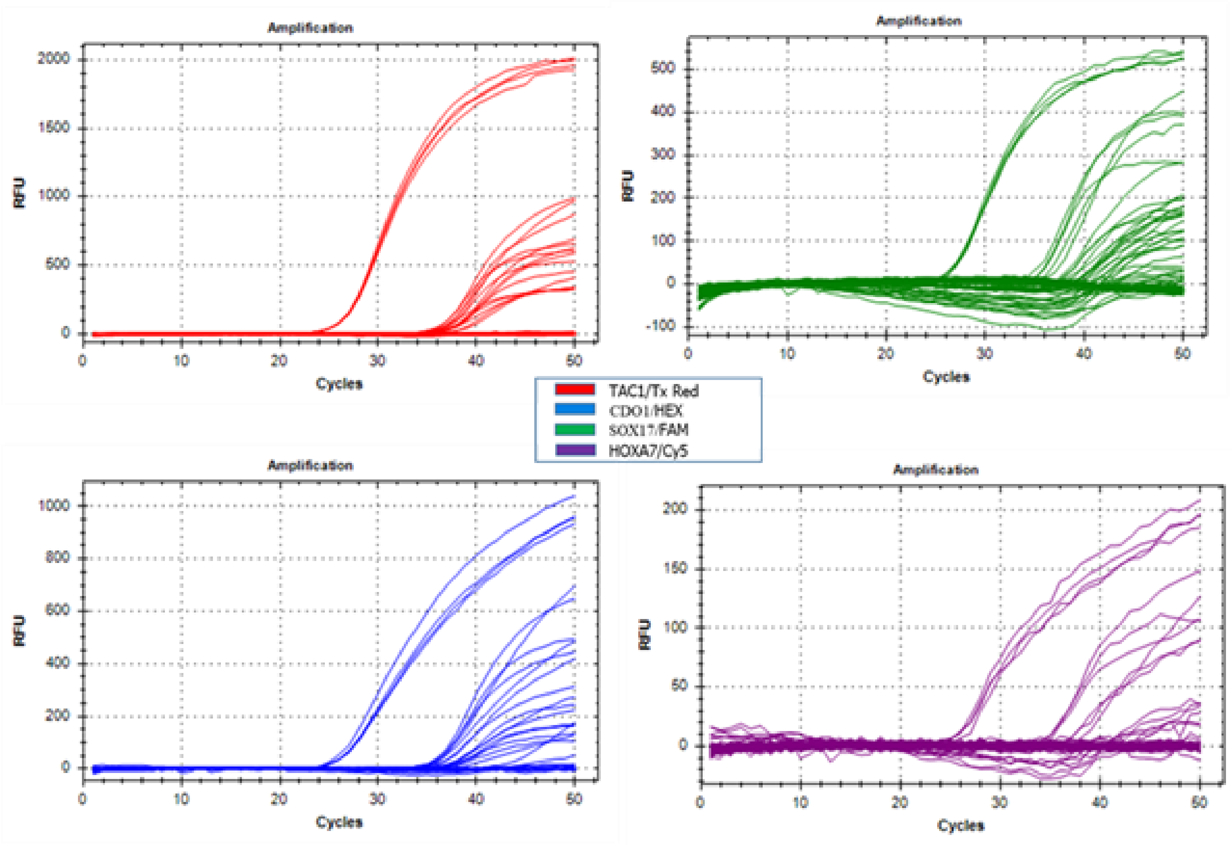
		1	2	3	4	5	6	7	8	9	10	11	12	Tumor
Cancer 1	A		CS	S		CS	S			CS	C	S		CS
Cancer 2	B		S TH			S	S H	S TH	T	TH	T	S H	TH	S TH
Cancer 3	C					C		C	S		CS			CS
Cancer 4	D		C	C T			C		C T		C T			C T
Control 1	E			S		S	S				S			S
Control 2	F													
Control 3	G													
Control 4	H													

Multiplexing for Multi-gene MOB-qMSP detection

A



B



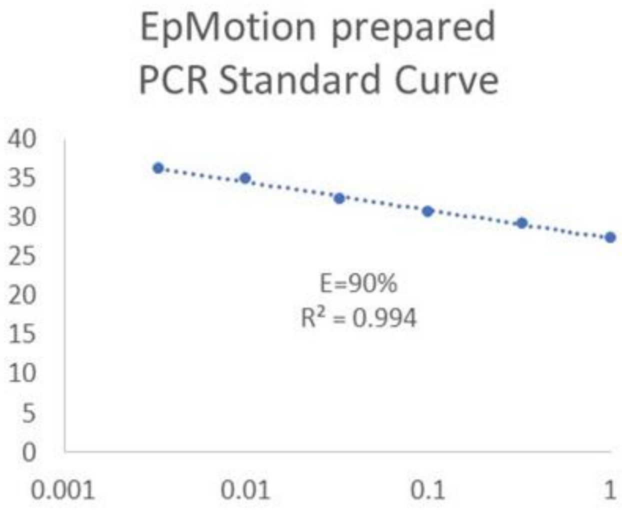
Eliminates stochastic issues for rare events
Reduces work (4 genes in single well)
Performance similar to individual loci detection

Robotics Processing: EpMotion Sample Prep

DNA Extraction		Bisulfite conversion					PCR Prep
Sample digestion	Washing	Sulphonation/ Deamination	Washing	Desulphonation	Washing	DNA Elution	PCR plate preparation

Objectives:

- Automated processing
- Enclosed environment
- High throughput

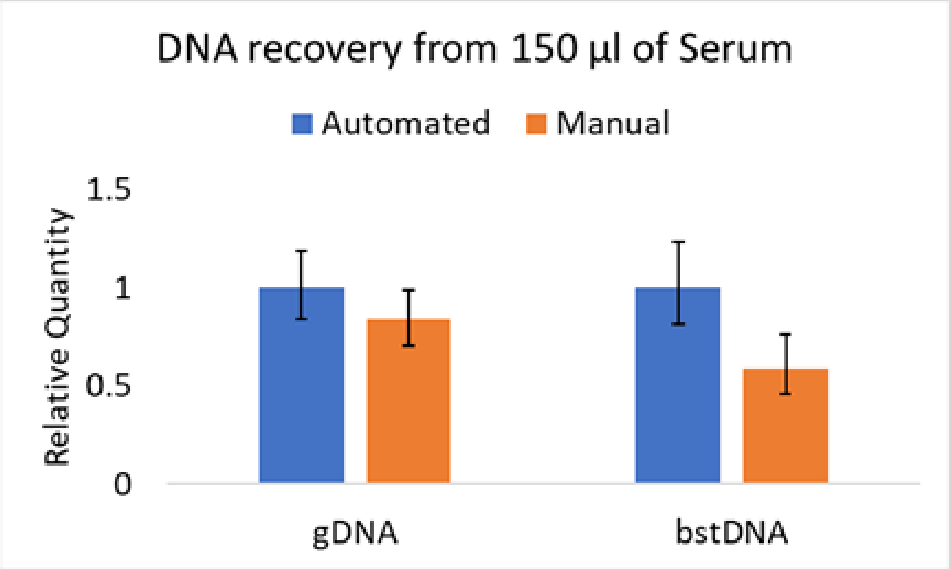


Alex Stark

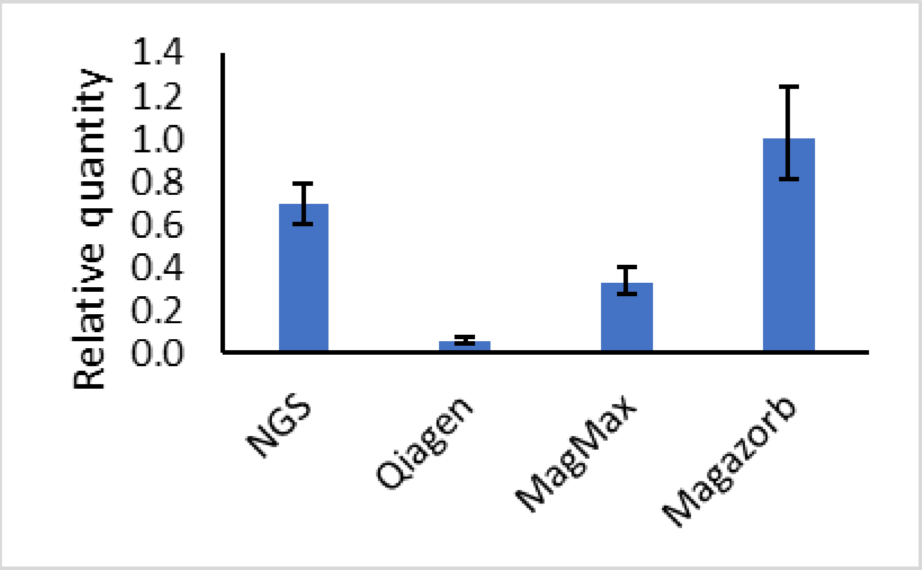
Robotics Processing: EpMotion Sample Prep

Improved Sample Quantity and Dependence on DNA extraction Reagents

Improvement in Automated vs Manual Processing



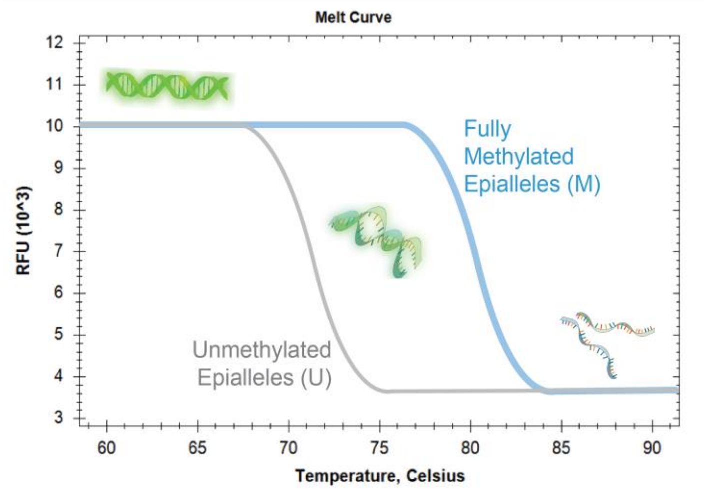
Efficiencies According to Extraction Reagents

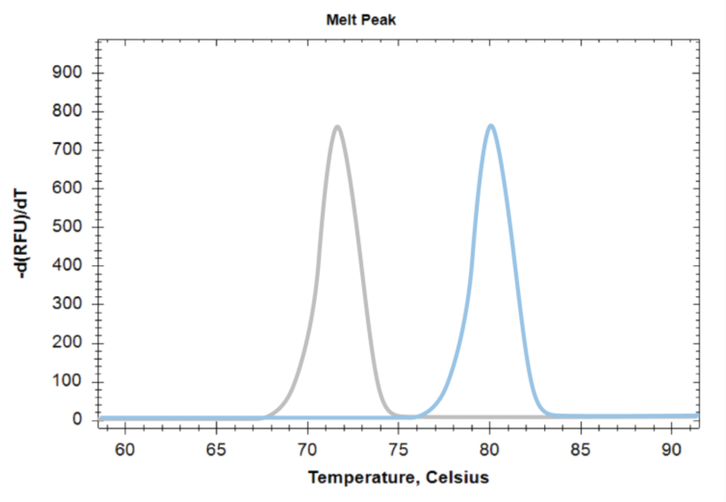


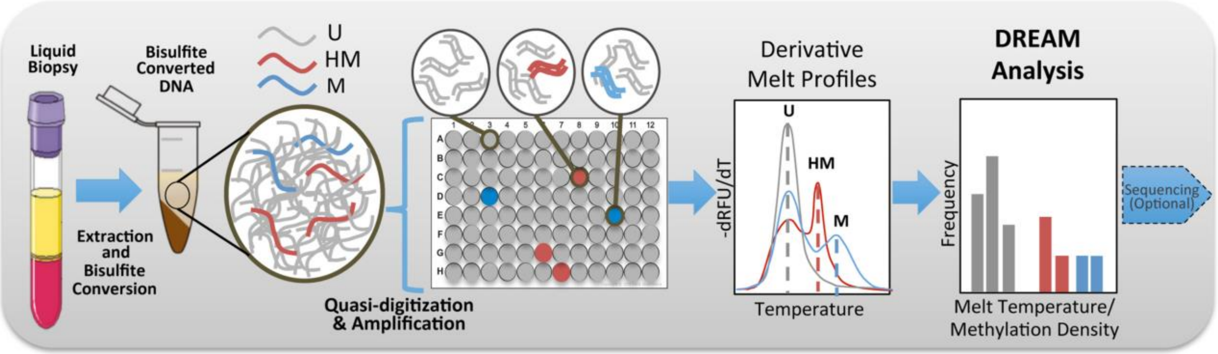
NeoGeneStar Circulating Cell Free DNA Kit (NGS)
Qiagen DNEasy Blood and Tissue Kit (Qiagen)
Thermo-Fisher MagMax kit
Promega Magazorb kit.

DREAMing

Discrimination of Rare EpiAlleles by Melt

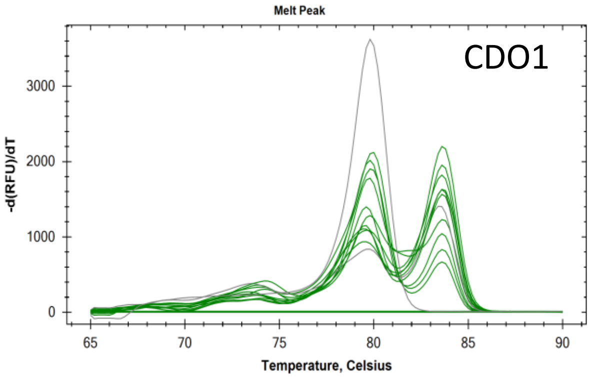


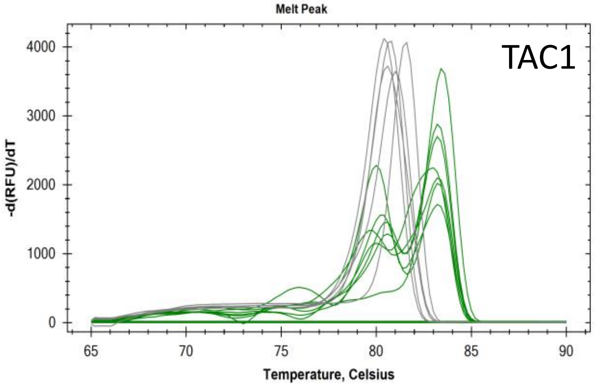


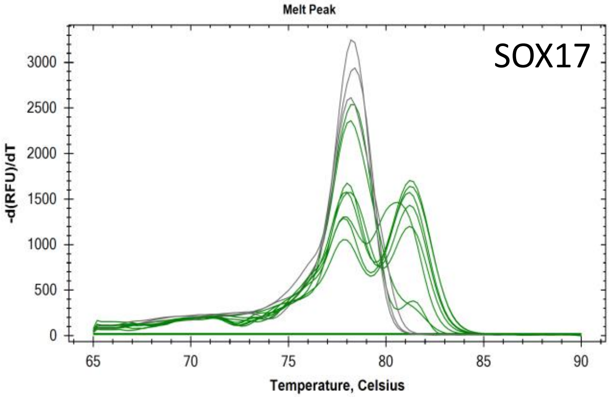


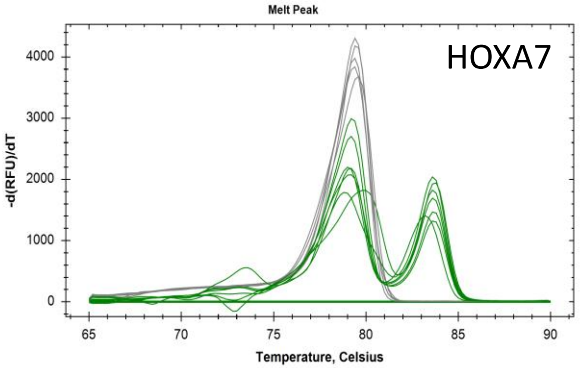
Pisanic II, et.al. *Nucl. Acids Res.* (2015)

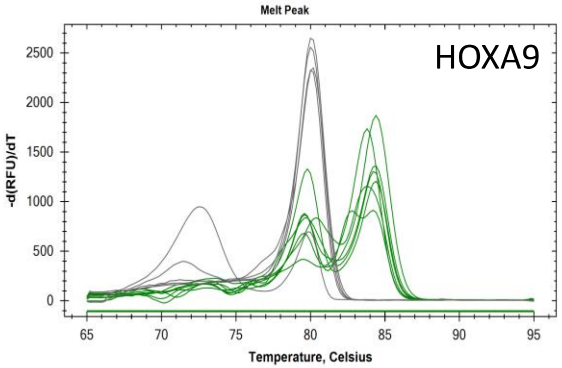
DREAMing
NSCLC Methylation Biomarkers







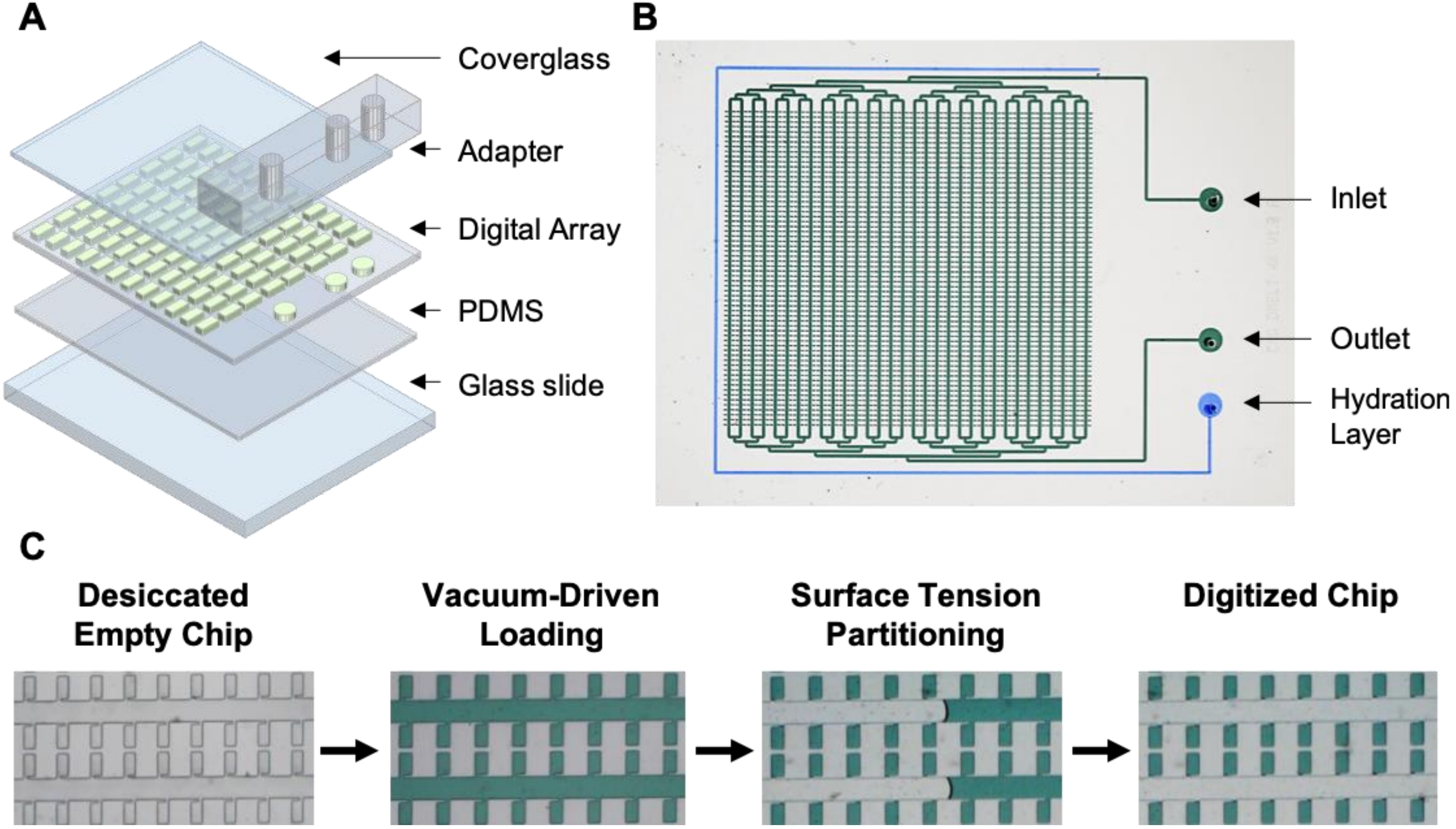




Increasing Detection of Rare events requires more wells

Microfluidic Digitization

Increasing Sensitivity using 4096-well array

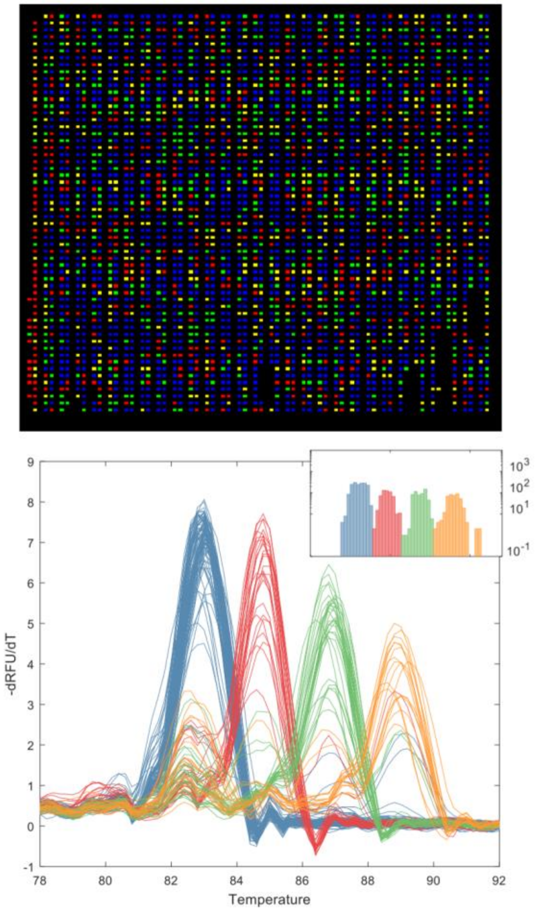


O’Keefe, et.al. *Sci. Advances* (2018)

Microfluidic DREAMing

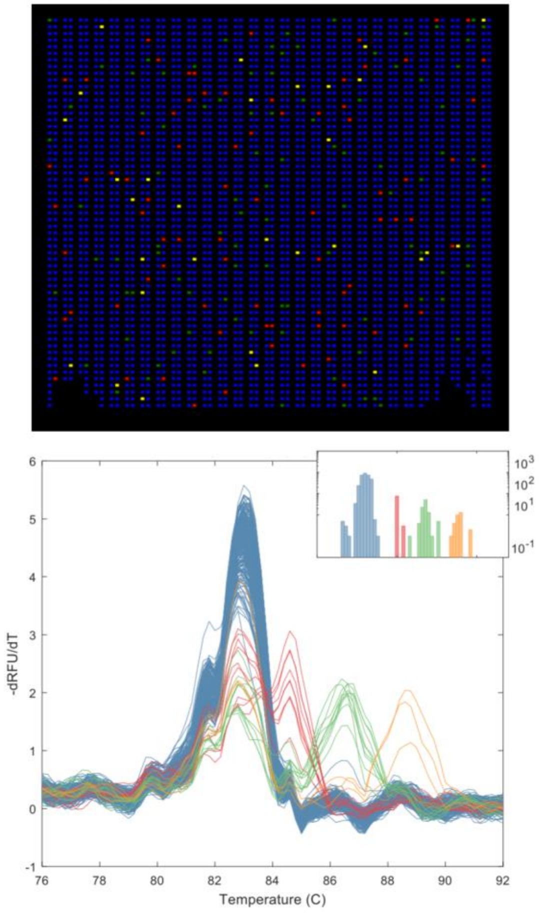
Analytical Validation

1000: 1000: 1000 : 2 million



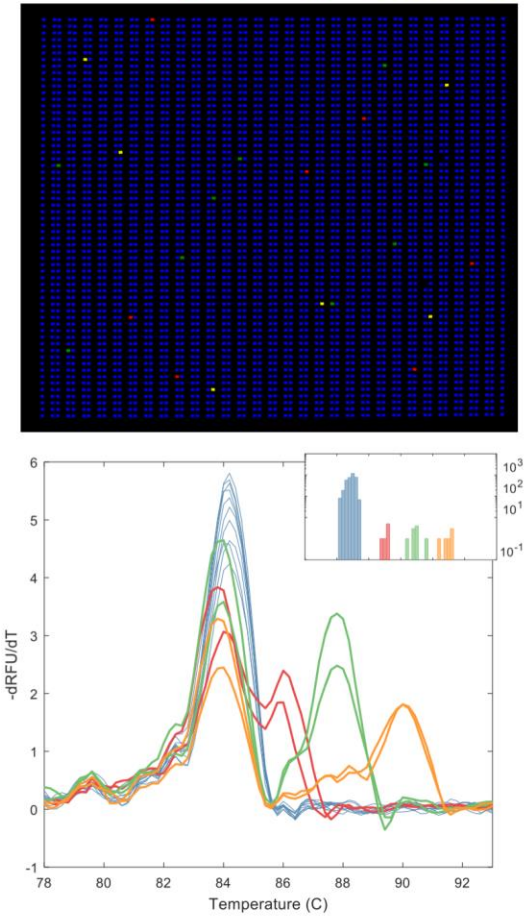
0.05%

100: 100: 100 : 2 million



0.005%

10: 10: 10 : 2 million

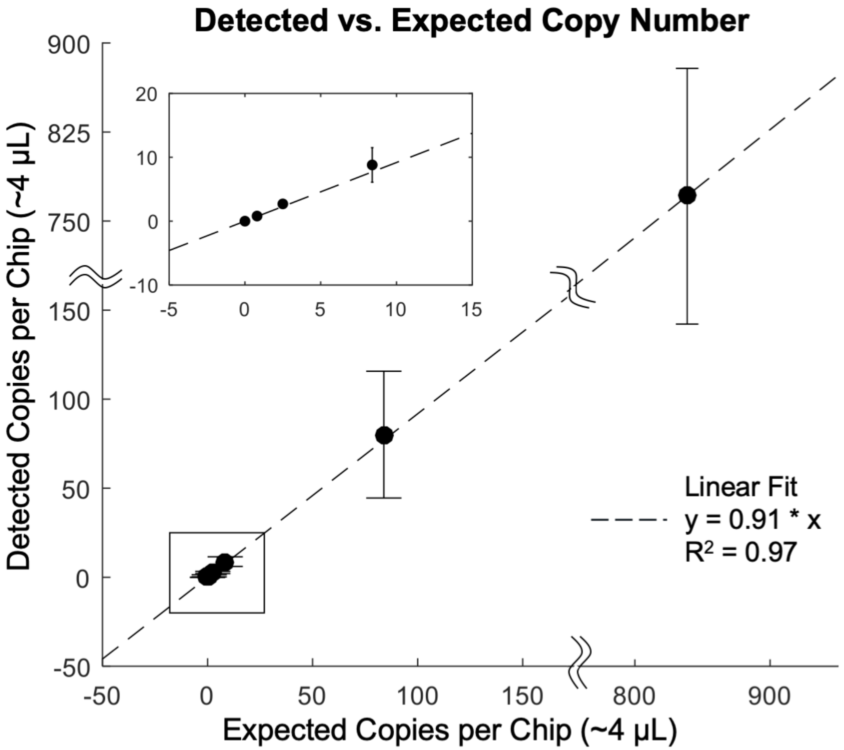


0.0005%

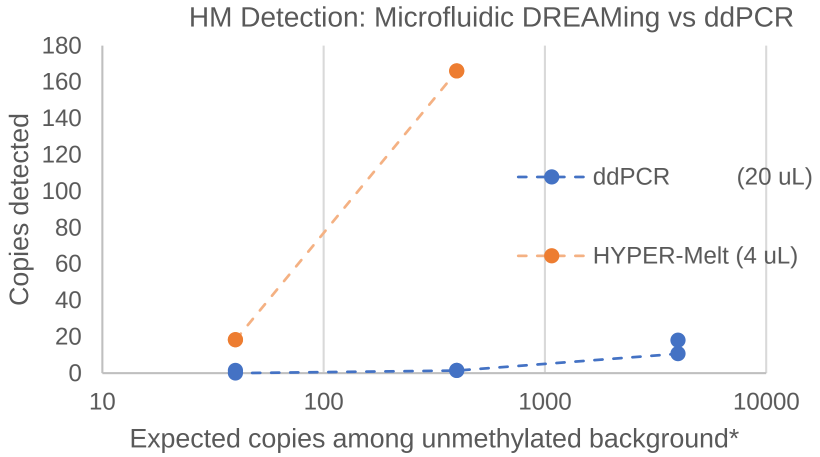
O’Keefe, et.al. *Sci. Advances* (2018)

Microfluidic DREAMing

Extreme Sensitivity and Analytical Validation



Expected Positive Copies	0	0.8	2.5	8.4	83.9	838.7
Detected Positive Copies	0 ± 0	0.7 ± 0.6	2.3 ± 0.6	7.7 ± 2.7	69.7 ± 35.6	669.3 ± 108.7



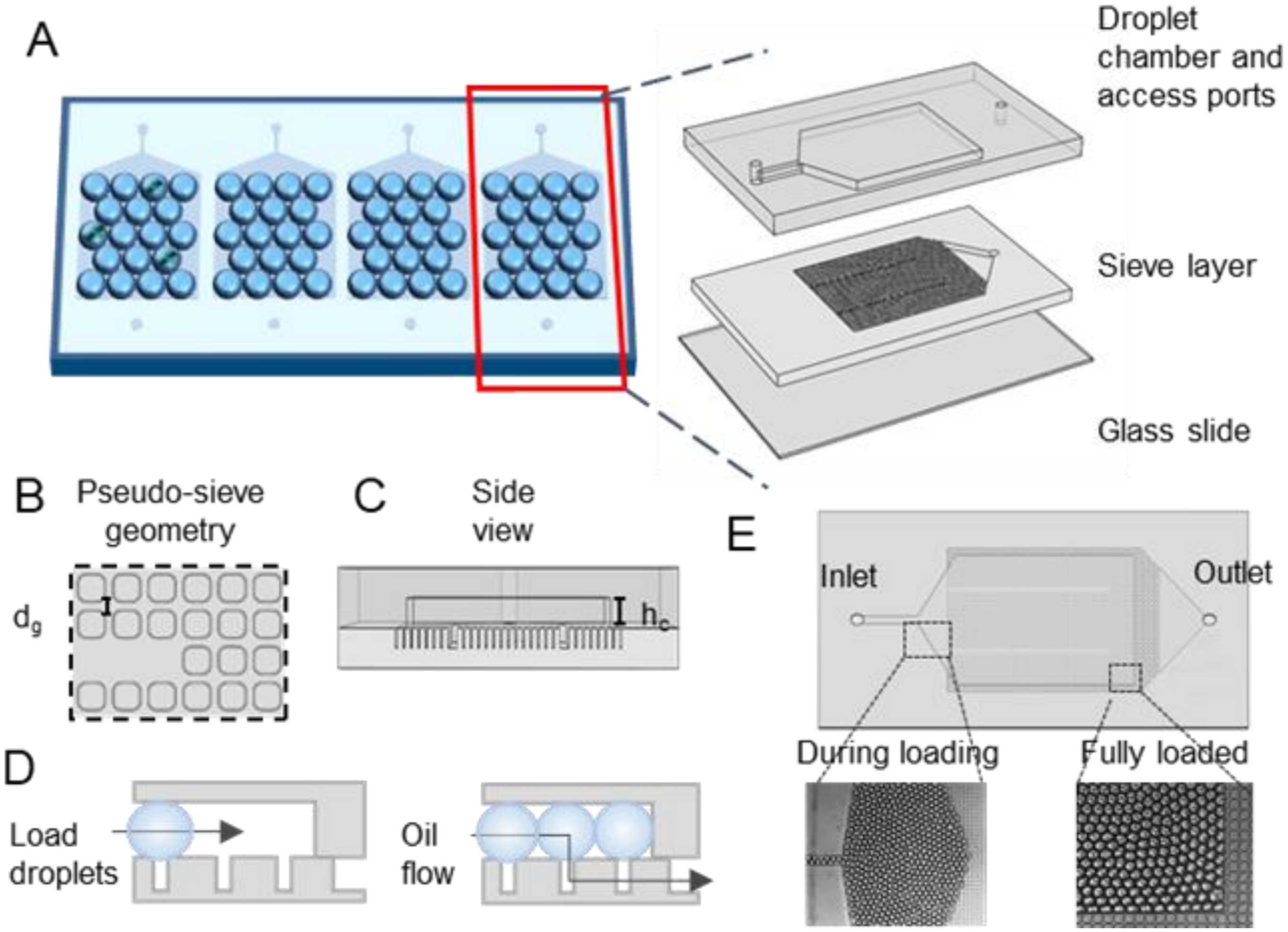
Input	DREAMing	ddPCR
40	**18.3**	0.7
400	**166.0**	1.4
4000	-	14.3

*Unmethylated background set to 2 million copies per reaction

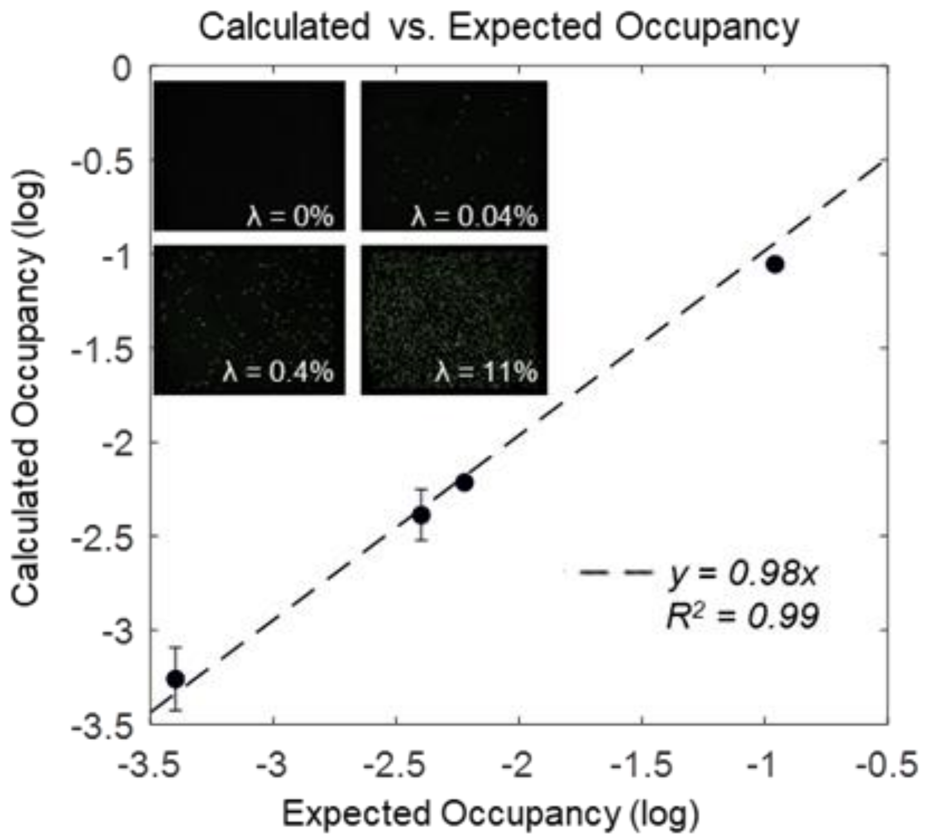
Drawback to this nanowell design: sample-reaction mixture could only be loaded into the device at ~20% efficiency, ultimately leading to an 80% loss in sample

Microfluidic Droplet Digital High-Resolution Melt

Near 100% loading efficiency



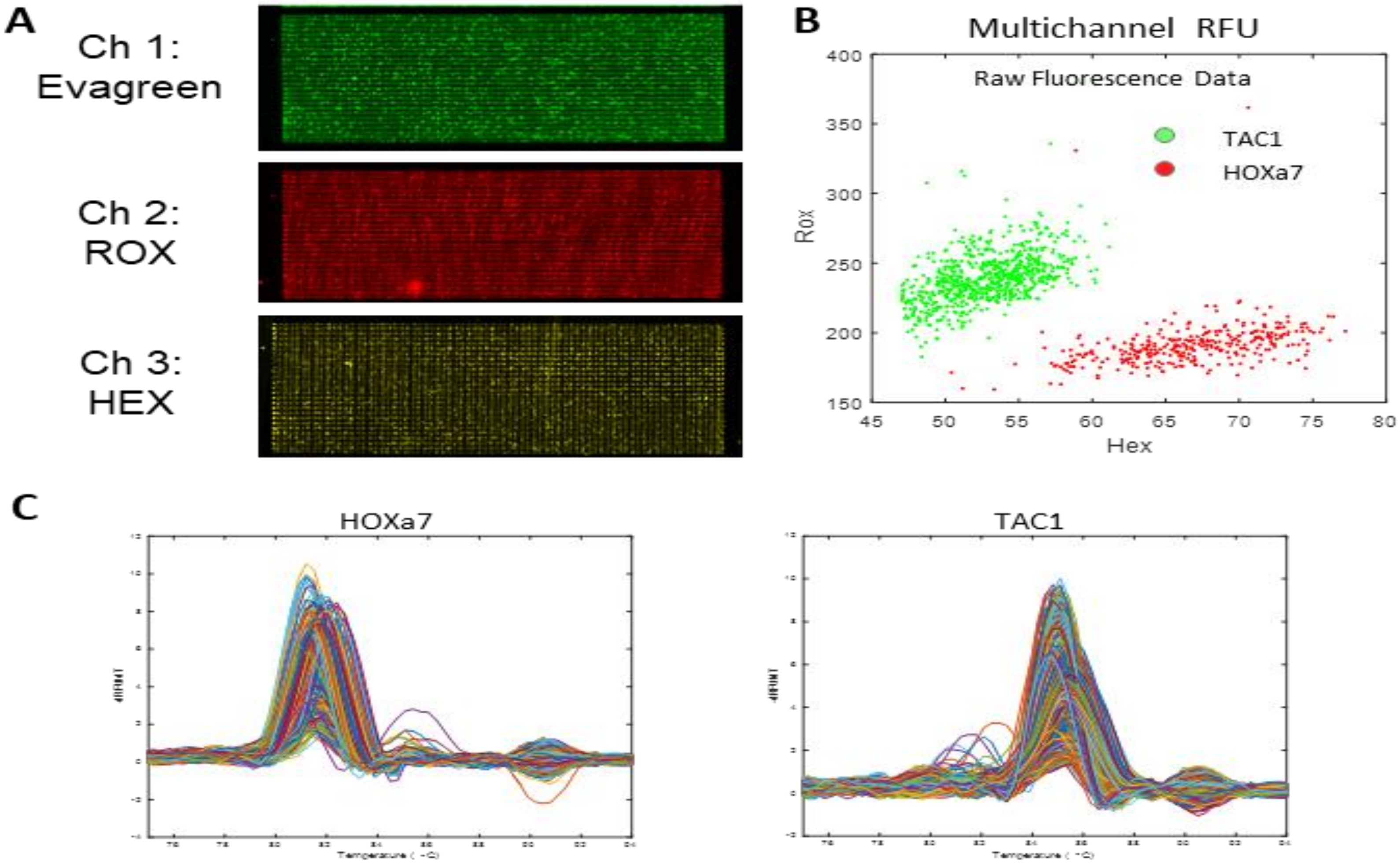
CDO1 methylation Detection



O’Keefe CM, et al, Anal Chem. 2019

Developing a Digital Microfluidic Multiplex DREAMing Assay

Solution for Sampling Issue



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SWOT for DNA Methylation Detection

Strengths

- Technical to Clinical team integration
- Extreme Sensitivity (Biologic and Analytic)
- Simplicity of data interpretation
- DNA methylation robustness—stable molecule
- Low cost of assay

Weakness

- Lack of Commercial Partner
- Complexity of Analyte Prep process
- Potential for detection of other malignancies
- Increased complexity of more sensitive approaches

Opportunities

- Robotics for throughput and standardization
- Extending approach to universal detection

Threats

- ctDNA competing approaches (sequencing)
  - If successful, competition
  - If fail, loss of interest in ctDNA