A Guide to Start Liquid Biopsy Research

- Extracellular Vesicle, ctDNA and Circulating Tumor Cell

13 Apr 2018

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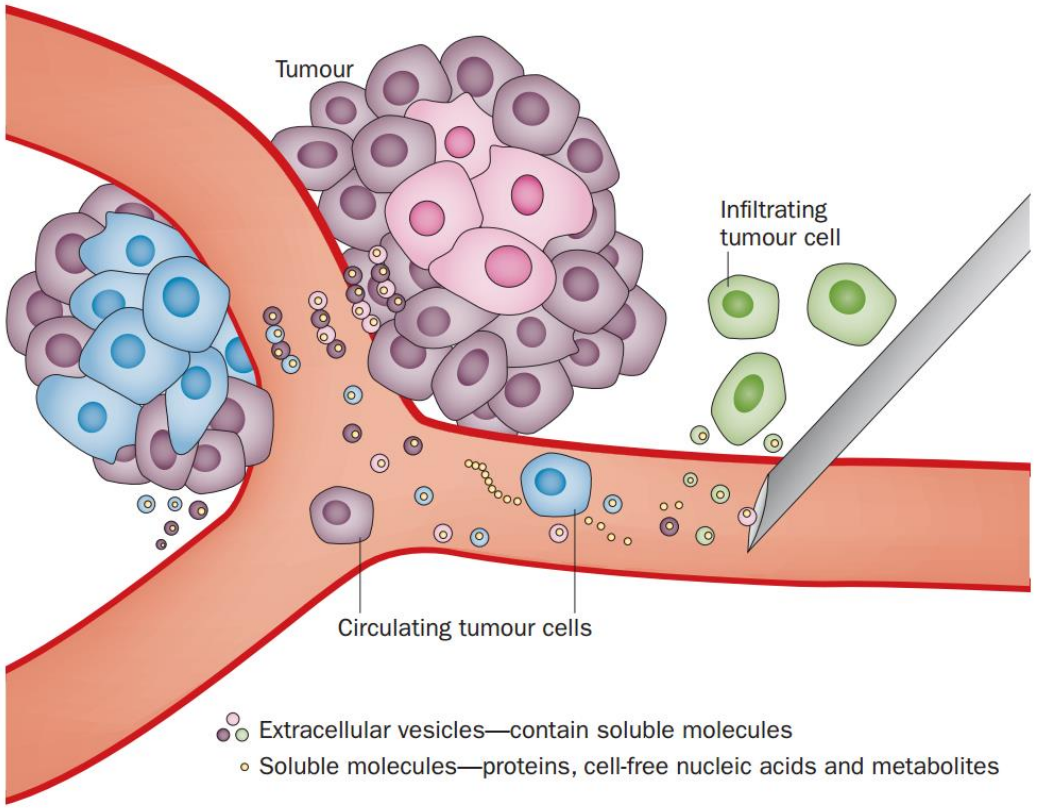
Blood-based tumor diagnostics
("Liquid Biopsy")

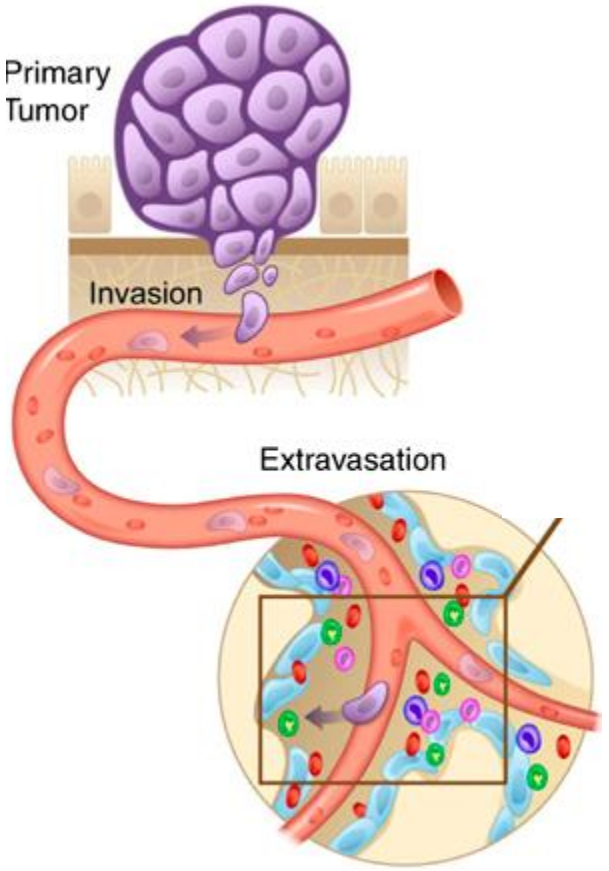
- Circulating tumor cells (CTC)
- Circulating cell-free nucleic acids
  - circulating tumor DNA (ctDNA)
  - circulating tumor RNA (ctRNA)
  - microRNA (miRNA)
- Nucleosome
- Exosome

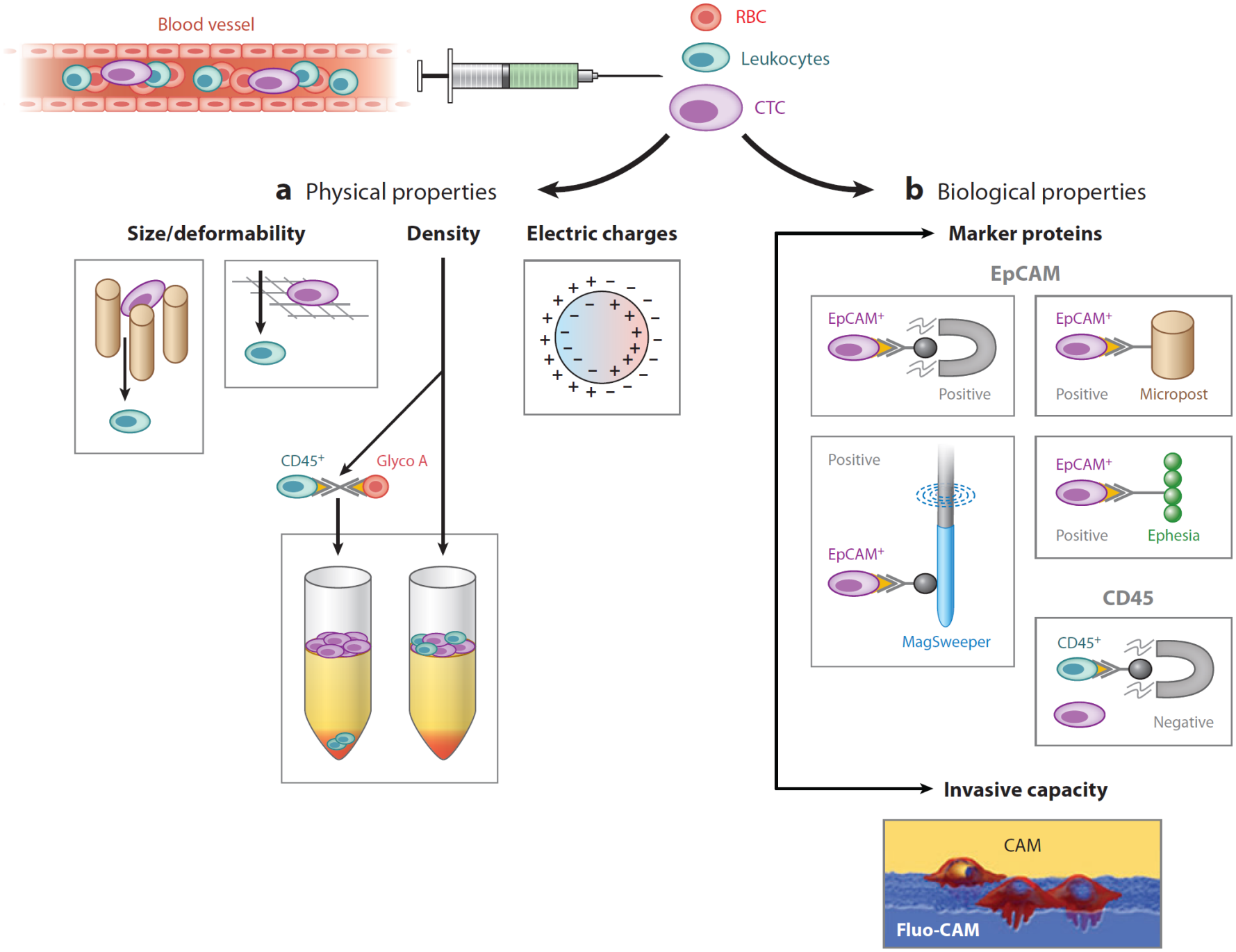


Circulating Tumor Cells (CTC)

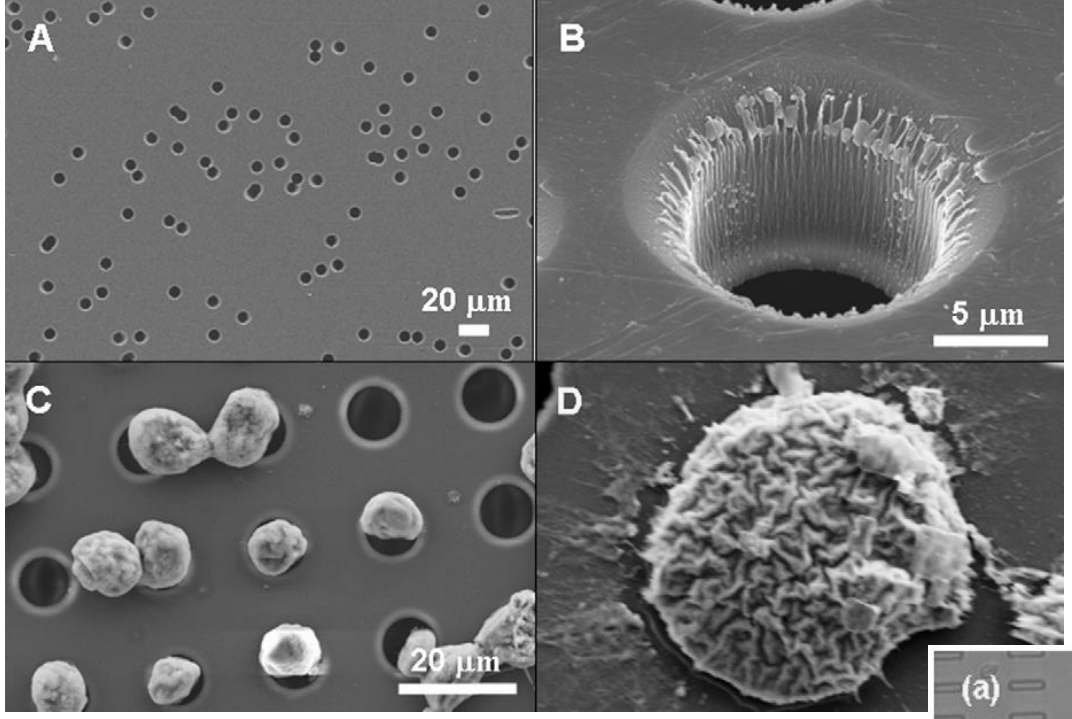
- Tumor cells shed into the blood stream
  - disseminated cancer cells
  - cancer stem cells
  - bystander cells

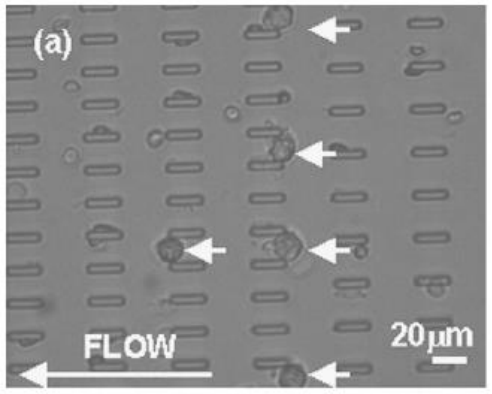
- 1 per 10^6~10^7 leukocytes
- Average 100 per 1 mL blood
  - Conventional methods may undercount the number of CTCs

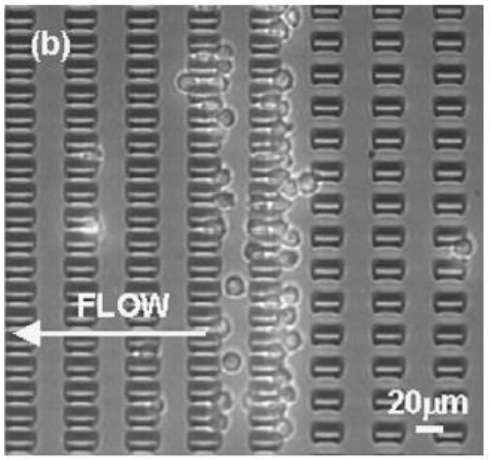


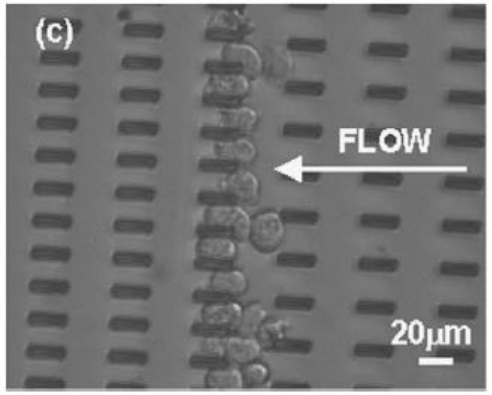

Blood vessel
RBC
Leukocytes
CTC
a Physical properties
b Biological properties
Size/deformability
Density
Electric charges
CD45+
Glyco A
Marker proteins
EpCAM
EpCAM+
Positive
EpCAM+
Positive
Micropost
Positive
EpCAM+
MagSweeper
EpCAM+
Positive
Ephesia
CD45
CD45+
Negative
Invasive capacity
CAM
Fluo-CAM

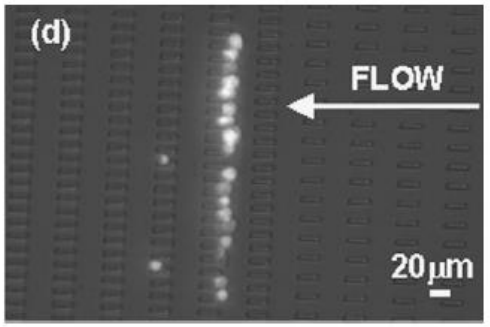
Size exclusion





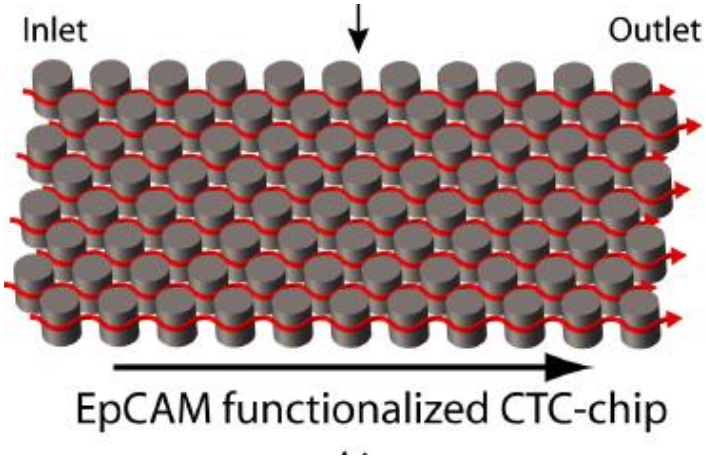


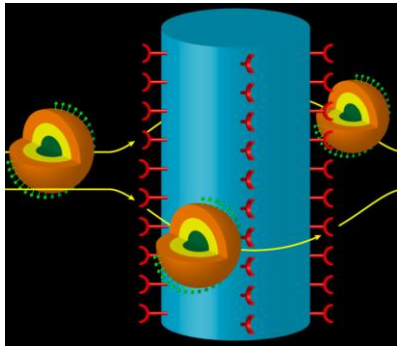


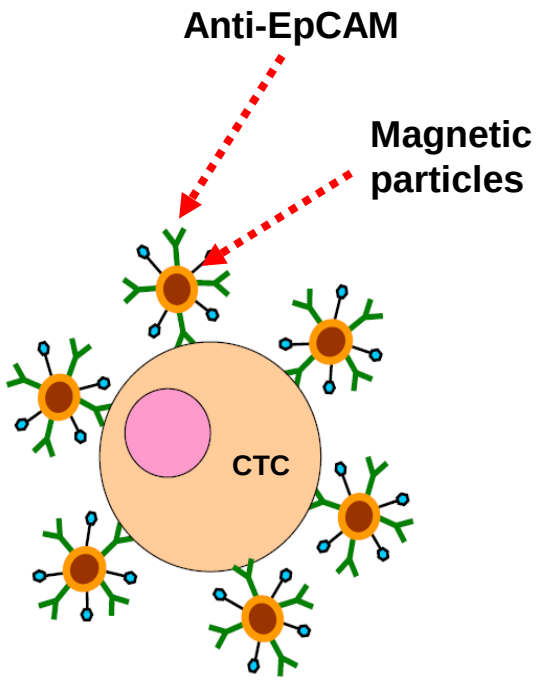


Antibody-mediated

- Cytokeratins (CK8, CK18, CK19)
- Epithelial membrane antigen (EMA)
- Epithelial cell adhesion molecule (EpCAM)

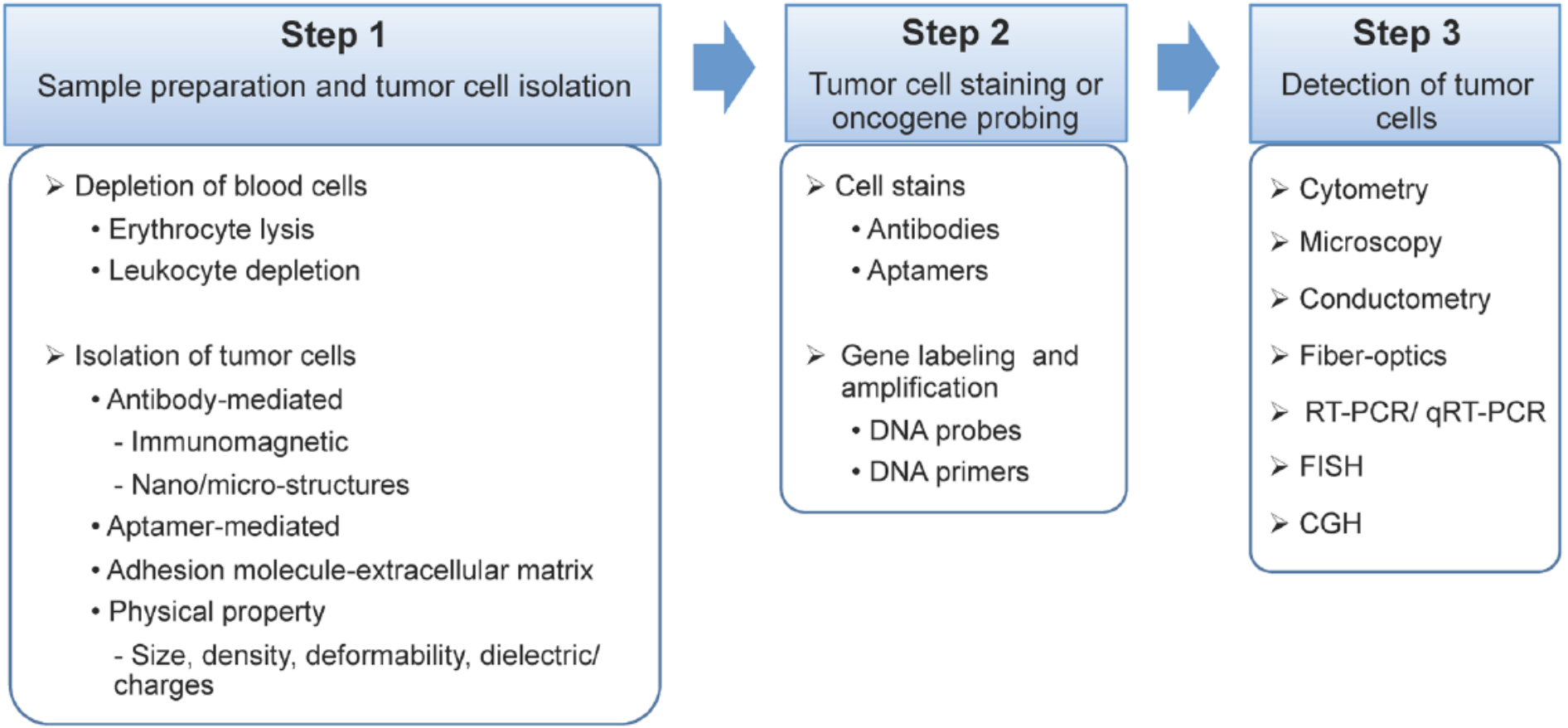






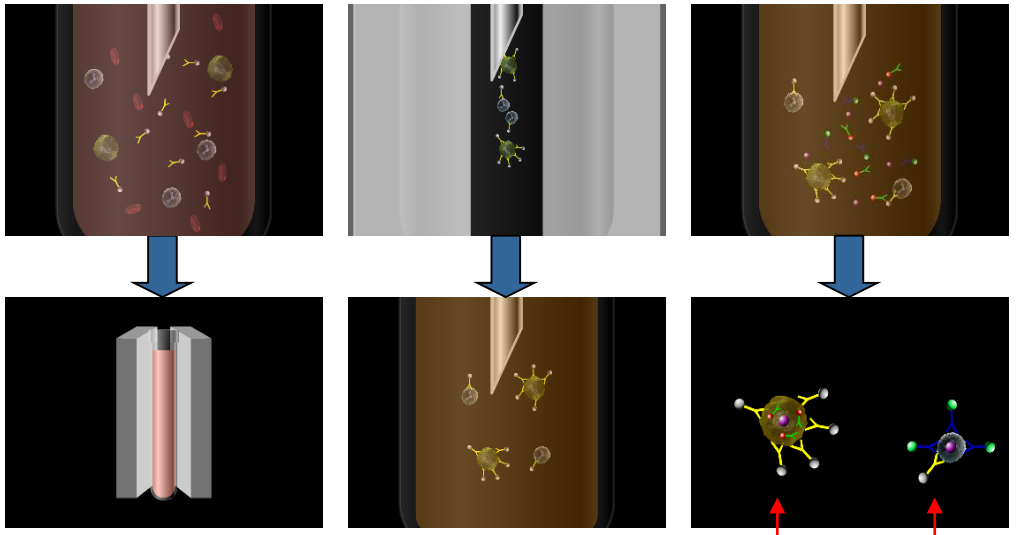
Method name	Developer (location)	Target cancer	Antibody against	Reference	Evidence level
AdnaTest	AdnaGen (Langenhagen, Germany)	Breast, prostate, and colon	EpCAM and MUC1	39	
autoMACS/MACS	MitenyiBiotec (BergischGladbach, Germany)		EpCAM, pan-CK, HER2/neu, or CD4	40	
Biofluidica	(Chapel Hill, NC)	Pancreatic, prostate, lung, breast, and colorectal	EpCAM	41	
CellSearch	Veridex, Johnson & Johnson	Metastatic breast, colon, prostate, lung, melanoma, and urothelial	EpCAM	42	FDA cleared, many clinical trials
ClearCell System	ClearbridgeBiomedics (Singapore)	Breast		Presented at ASCO 2014	
CTC iChip	Daniel Haber and Mehmet Toner, Dana-Farber Cancer Institute and Massachusetts General Hospital (Boston, MA)	Breast, colon, lung, prostate, and pancreas	EpCAM and CD45/ cytokeratin subtraction	43	FDA IDE
Dynabeads methods	Invitrogen (Carlsbad, CA, and Heidelberg, Germany)	Colorectal cancer	EpCAM and CD45 subtraction	44	
ISET	Metagenex (Paris, France)	Melanoma, mesothelioma, and NSCLC	Size (no antibody)	45	NCT01776385 for mesothelioma and NCT00818558 for NSCLC
IsoFlux Rare Cell Access System	Fluxion Biosciences (San Francisco, CA)	NSCLC and melanoma		46	
Lymphoprep (Ficoll-Isopaque)	Axis-Shield PoC (Oslo, Norway)	Prostate	EpCAM, PSA, and cytokeratin 7/8	47	
MagSweeper	Stephanie Jeffrey and Ronald W. Davis (Stanford University, Stanford, CA)	Breast and prostate	EpCAM	48	
Nanodetector	Gilupi (Potsdam, Germany)	Breast, lung, and prostate	EpCAM	49	
Negative enrichment QMS	Jeffrey Chalmers (Cleveland Clinic, Cleveland, OH)	Head and neck and breast	CD45 subtraction	50	
OncoQuick	Greiner Bio-One (Germany and Monroe, NC)	Breast, colorectal, melanoma, and pancreatic	Density (no antibody)	51	
RoboSep/EasySep	Stem Cell Technologies (Vancouver, BC, Canada)	Myeloma	CD33, CD66, and CD138	52	
ScreenCellCyto	ScreenCell Company (Sarcelles, France)	Lung and cell lines	Size (no antibody)	53	

Steps for CTC detection

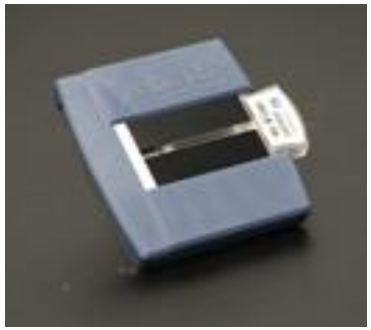


- Routinely 7.5 mL whole blood
- Larger volume can increase detection rate

CellSearch™



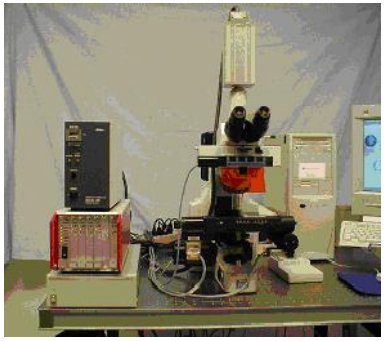
MagNest™



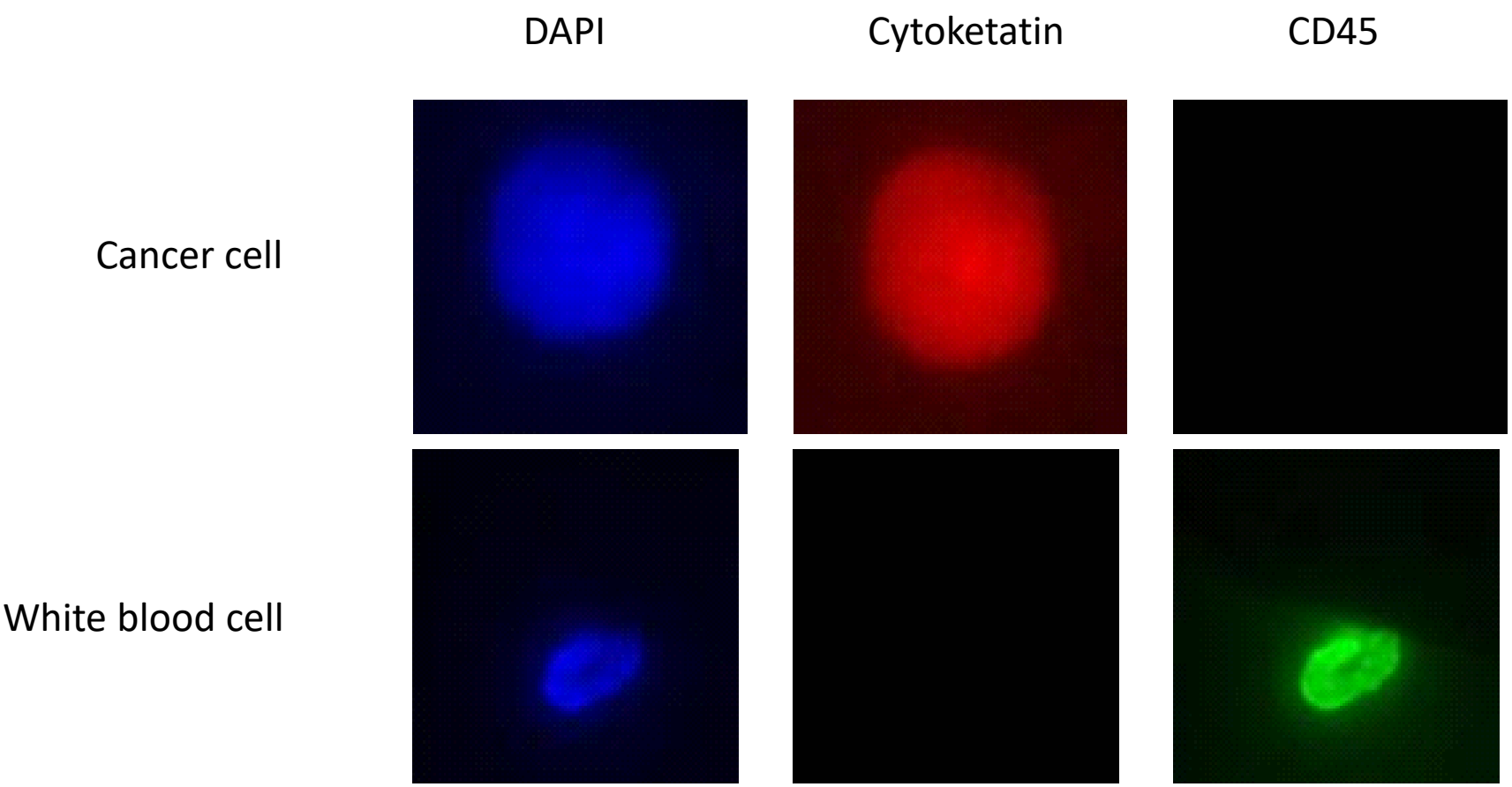
cellsave™ Preservative Tube

celltracks™ autoprep system

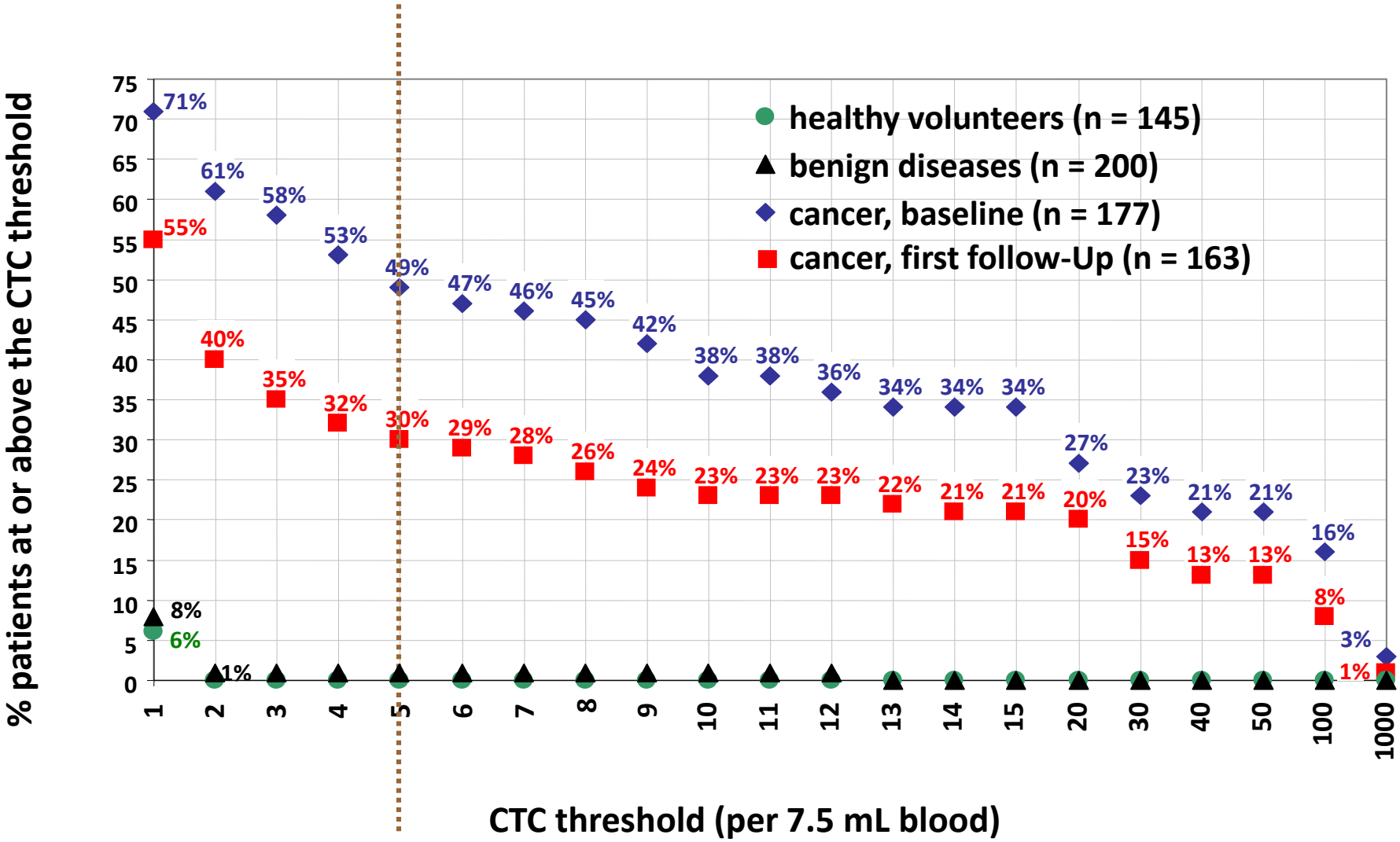
cellspotter™ analyzer



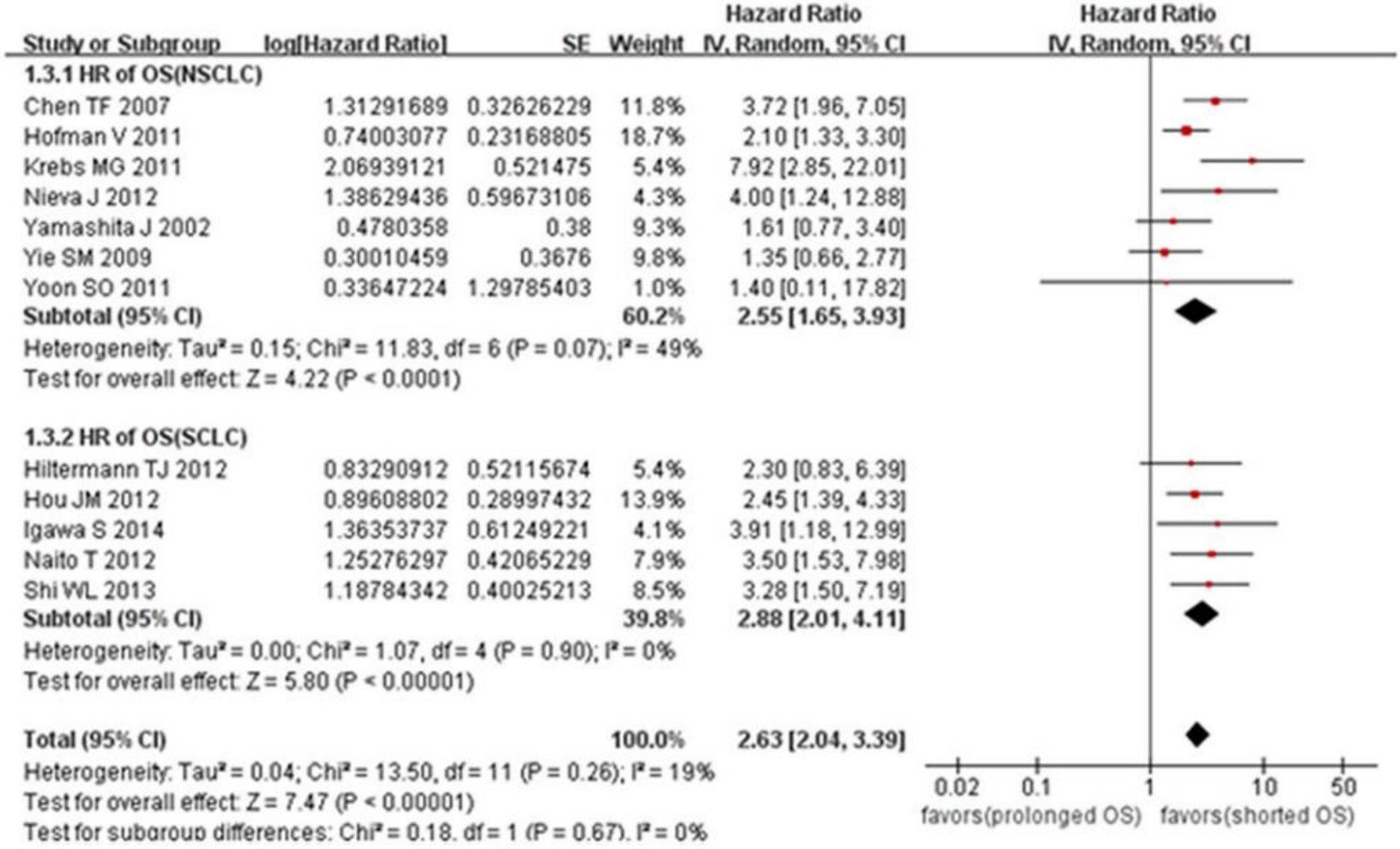
CellSearch™



Prevalence of CTCs



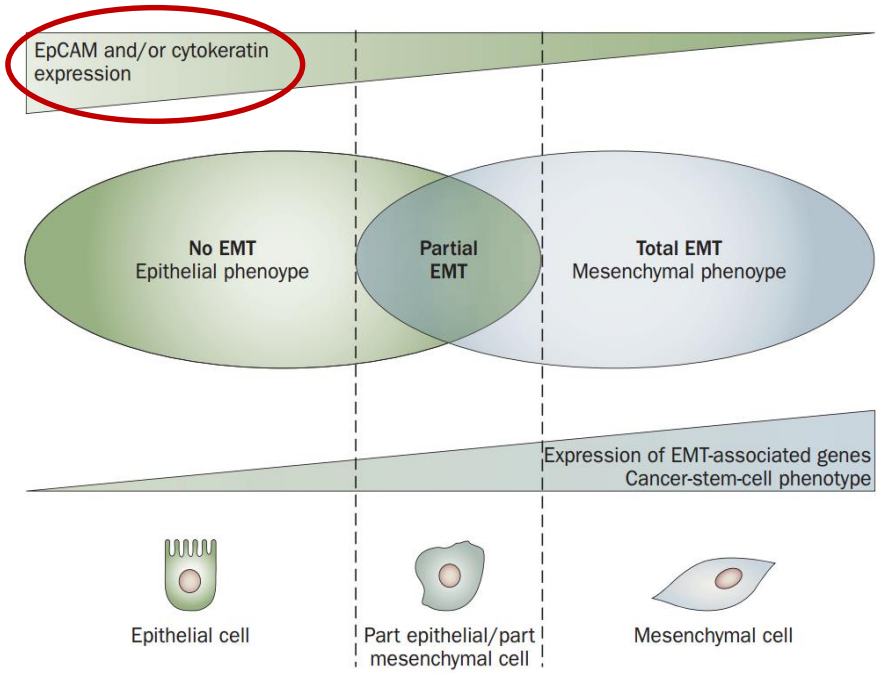
Detecting CTCs in lung cancers

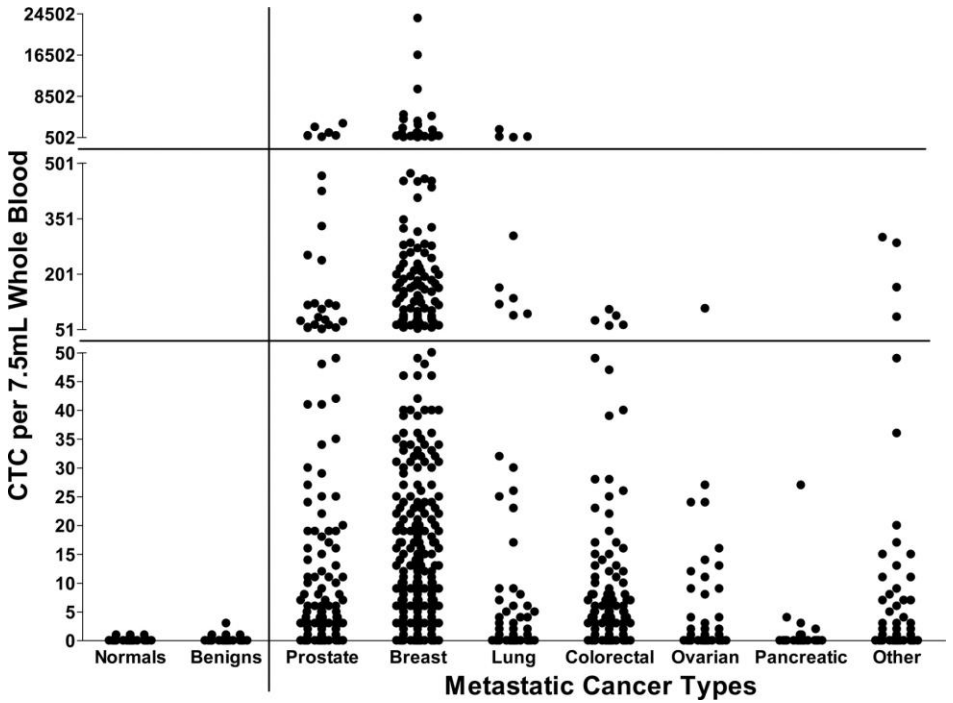


Xu et al. Oncotarget 2017

Limitations (1)

- Only a proportion of cases have detectable CTCs
- Epithelial–mesenchymal transition (EMT)
- Positive rates differ among different tumors





Allard et al. Clin Cancer Res. 2004

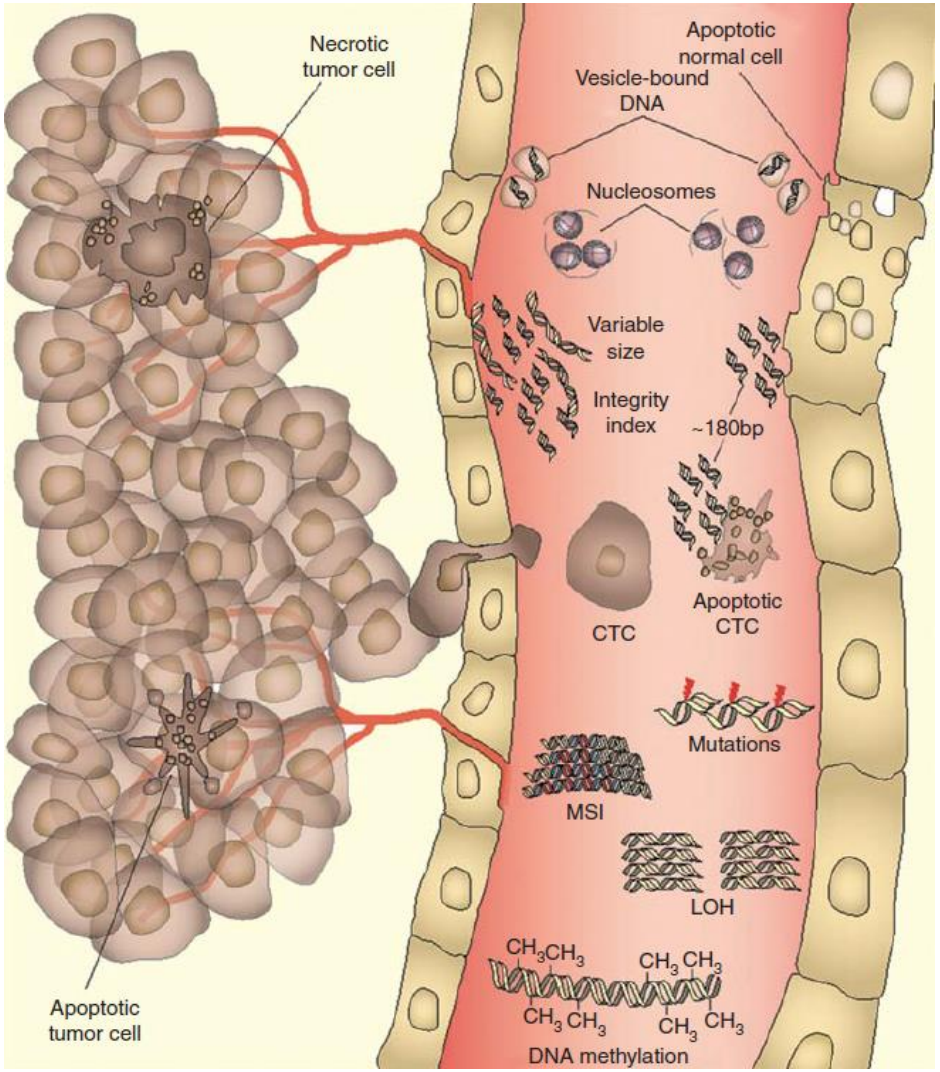
Limitations (2)

- No standards
- No guidelines
  - Not reccomended by ASCO, NACB, AACC, AJCC, BCTF

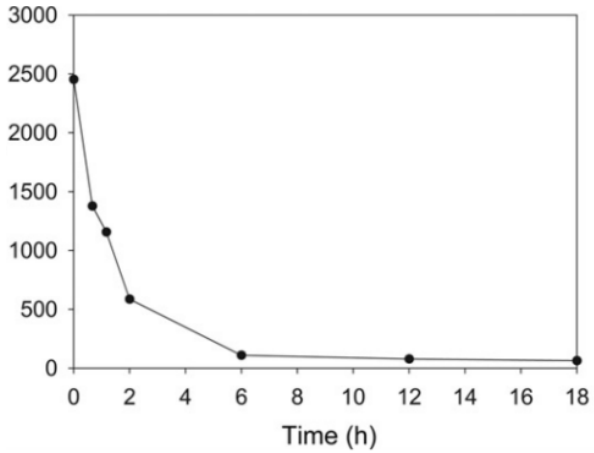
- Expensive, labor-intensive
- Long turnaround time

Circulating cell-free DNAs

- Various sources
  - Normal cells
  - Tumor necrosis
  - Tumor apoptosis
  - Circulating tumor cells
- Variable amount
  - <30 ng ~ > 1000 ng/5mL
- Variable fraction
  - ctDNA: <0.01% ~ >90%



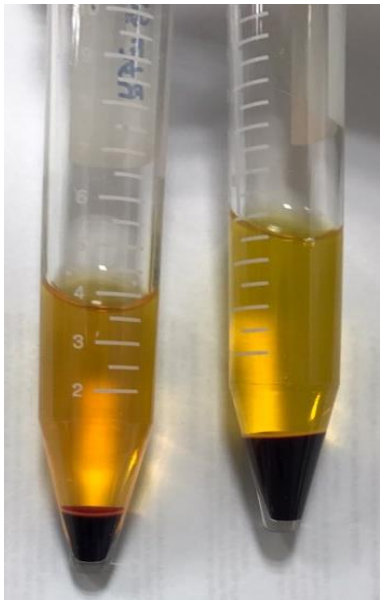
Collection of cell-free DNA



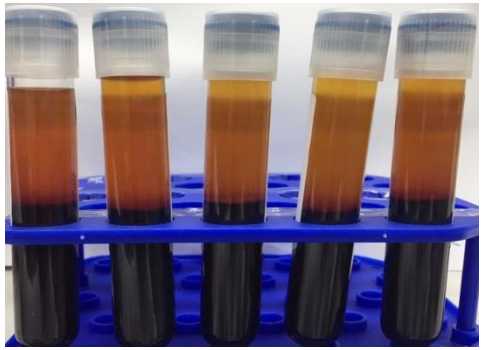
- Short half life (15 min ~ several hours) due to plasma DNases
- Blood can be sampled in ETDA tubes but the plasma has to isolated and stored at -80°C within one hour of collection
- Preservative tubes (e.g. Streck™ tube)
  - up to 4 days at room temperature
- Plasma vs. serum
  - DNA concentrations: serum > plasma
  - Serum contains large amount of leukocyte-derived DNAs
  - EDTA has some inhibitory effect on DNase

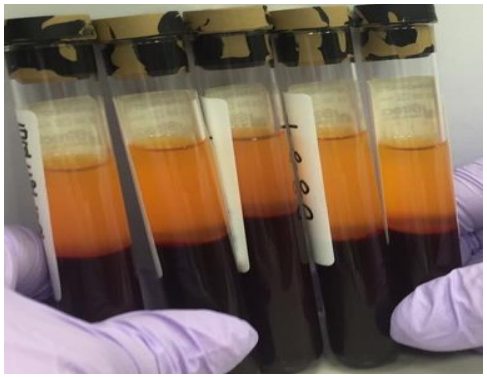
Evaluation of Preservation Tubes

0 day

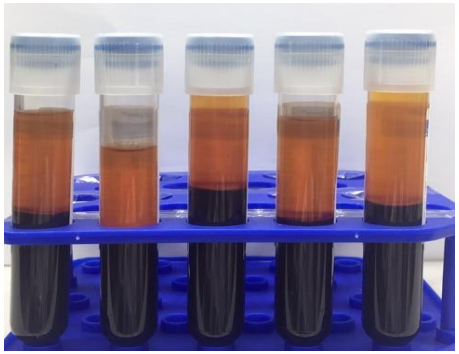


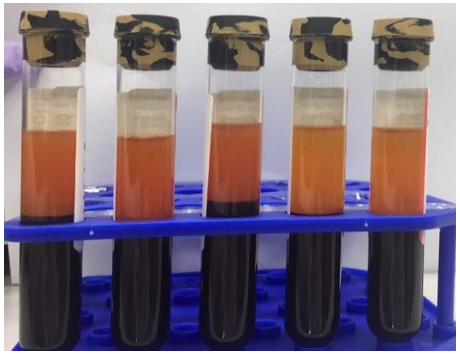
2nd day





5th day



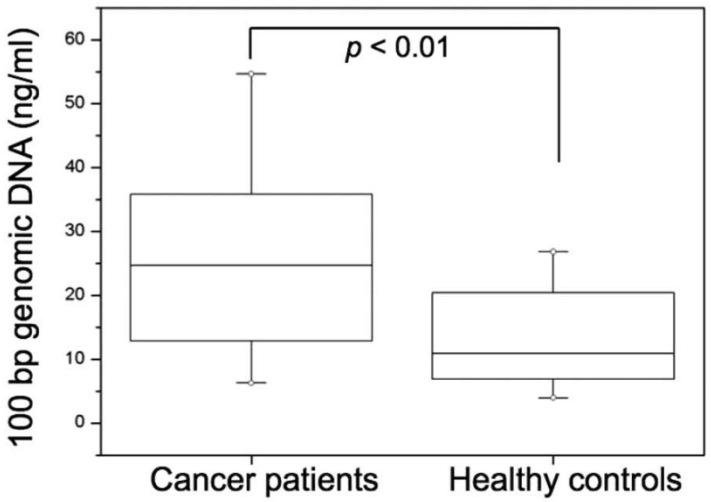


P tube

S tube

Genome equivalents

ng/assay	copies/uL	haploid genome equivalents (3.3 pg/haploid genome)
400	6060.6	121212.1
350	5303.0	106060.6
300	4545.5	90909.1
250	3787.9	75757.6
200	3030.3	60606.1
150	2272.7	45454.5
100	1515.2	30303.0
75	1136.4	22727.3
50.0	757.6	15151.5
40	606.1	12121.2
33	500.0	10000.0
25	378.8	7575.8
20	303.0	6060.6
15	227.3	4545.5
10	151.5	3030.3
5	75.8	1515.2
1	15.2	303.0
0.5	7.6	151.5
0.1	1.5	30.3
0.01	0.2	3.0



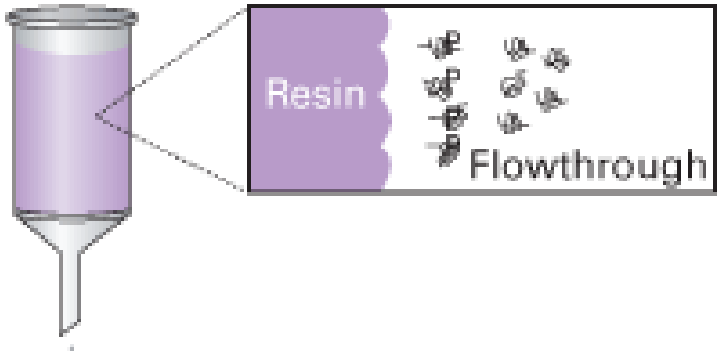
- cfDNA levels higher
  - Increased age
  - Underlying medical conditions
  - Pregnancy
  - Cancers

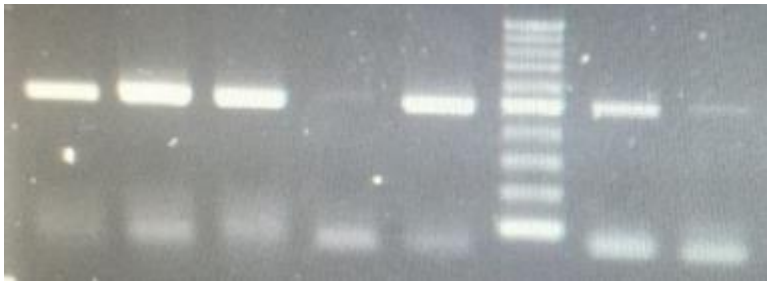
Quantitative estimates of plasma/serum of cell-free DNA

Material	Isolated method	Assay method	Patients		Controls		Progression free survival (PFS; m)	Median overall survival (OS; m)	References
			Number	DNA (ng/ml)	Number	DNA (ng/ml)			
Plasma	QIAamp DNA Mini kit	qPCR (hTERT)	104	270.0	205 (healthy controls)	122.7	NM	<20 ng/ml, 22.4 >20 ng/ml, 16.8	[6]
Plasma	QIAamp DNA Blood Mini Kit	qPCR (β-actin)	134	122.3 (11.5–2487.9)	NA	ND	6.35 (5.2–7.2) Low tertile ctDNA group, 6.61 Middle tertile ctDNA group, 5.89 High tertile ctDNA group, 5.62	23.3 (17.8–28.7) Low tertile ctDNA group, 28.6 Middle tertile ctDNA group, 26.0 High tertile ctDNA group, 16.0	[8]
Plasma	QIAamp DNA Blood Mini Kit	qPCR (hTERT)	446	49.8 (0.8–43735)	NA	ND	≦49.8 ng/ml, 6.3 >49.8 ng/ml, 4.9	≦49.8 ng/ml, 10.9 >49.8 ng/ml, 9.3	[18]
Plasma	NM	qPCR	50	8.02	50 (10 orthopedic patients and 40 healthy controls	2.27	High ctDNA concentrations were significantly associated with decreased survival in NSCLC patients		[10]
Serum	QIAamp DNA Mini kit	qPCR (GAPDH)	100	47.2 (0.7–251)	100 (healthy controls)	9.2 (2.2–184)	NM	NM	[5]
Plasma	QIAamp Blood Mini Kit	qPCR (GAPDH)	58	4.3 (0.82–49.8)	52 (healthy controls)	2.0 (0.03-26.9)	4.3 (2.8–5.8)	9.8 (5.7–13.9)	[19]
Plasma	QIAamp DNA Blood Mini Kits	qPCR (hTERT)	76	60.0	66 (smoker-matched controls)	5.0	High ctDNA concentrations were significantly associated with decreased disease free survival and overall survival in surgically treated NSCLC patients		[20]
Plasma	QIAamp DNA Blood Mini Kits	qPCR (β-actin)	102	22.6 (3.1–730.5)	105 (healthy controls)	10.4 (1.6–89.8)	NM	NM	[21]
Plasma	NM	qPCR	30	12.0 (1.5–50)	16	2.65 (0.9–7.0)	NM	NM	[22]
Plasma	QIAamp DNA Mini kit	qPCR (hTERT)	151	12.8	79 (healthy controls)	2.9	NM	NM	[23]
Plasma	BILATEST Viral DNA/RNA Kit	Duplex real-time PCR (β-actin)	88	66.5 (19.0–265.7)	200 (healthy controls)	22.4 (7.4–90.7)	High ctDNA concentrations were significantly associated with decreased survival after chemotherapy		[9]
Plasma	QIAamp DNA Blood Mini Kit	Fluorescence assay using PicoGreen dsDNA kit	42	95.1	100 (patients with benign diseases)	74.0	High ctDNA concentrations were significantly associated with decreased survival after chemotherapy	<93.8 ng/mL, 462.0 days (328.4–595.6) >93.8 ng/ml, 324.0 days (303.6–344.4)	[17]
Serum	NM	Fluorescence assay using PicoGreen dsDNA kit	19	12,414.4	28 (healthy controls)	7532	NM	NM	[24]

Nie et al. Tumor Biol. 2015

Extraction method



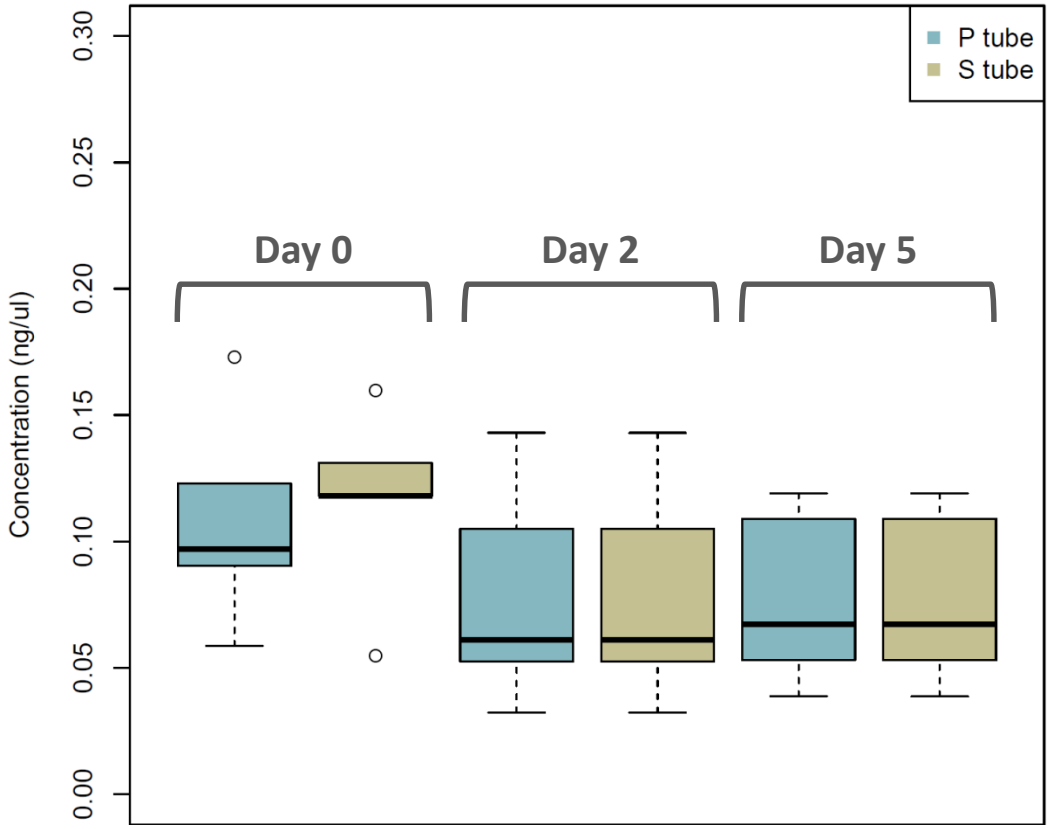


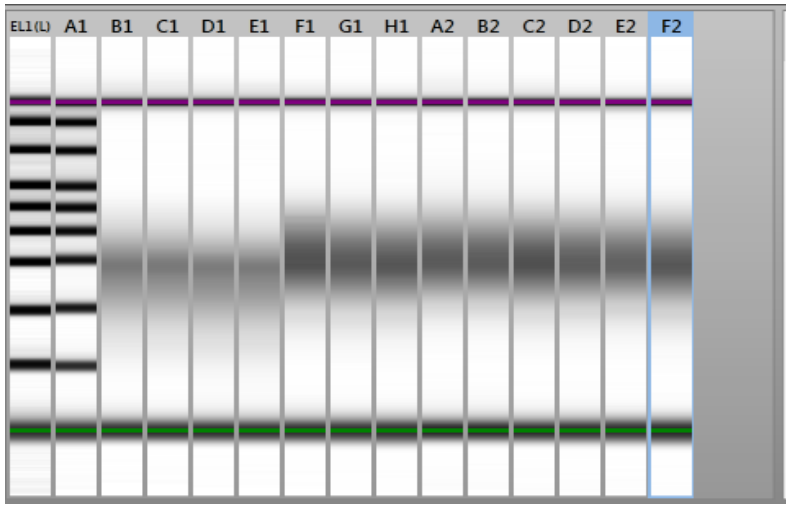
Carrier RNA

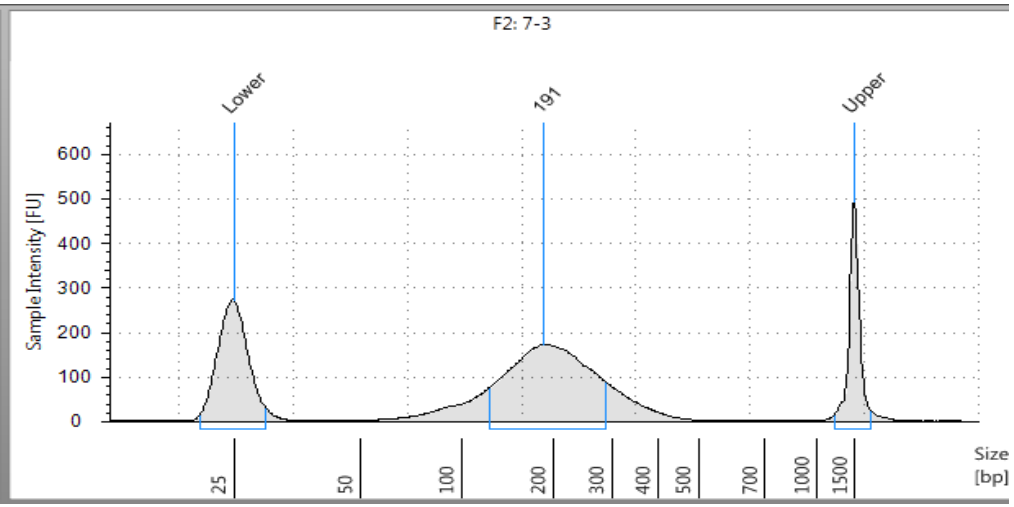
Extraction method	By fluorescent assay, ng	
	n	Median (SE)
PCI-glycogen[b]	12	367.958 (94.645)[c]
NaI method[e]	12	306.040 (61.228)[c]
Guanidine-resin method[g]	6	8.928 (0.364)
QIAamp DNA Blood Midi kit with carrier RNA[h]	12	228.915 (38.162)[c]
ChargeSwitch 1-mL serum kit[i]	12	83.165 (13.370)
ZR serum DNA kit[j]	6	15.363 (6.580)
Puregene DNA purification System Cell and Tissue Kit[k]	12	59.200 (11.652)

Fong et al. Clin Chem. 2009

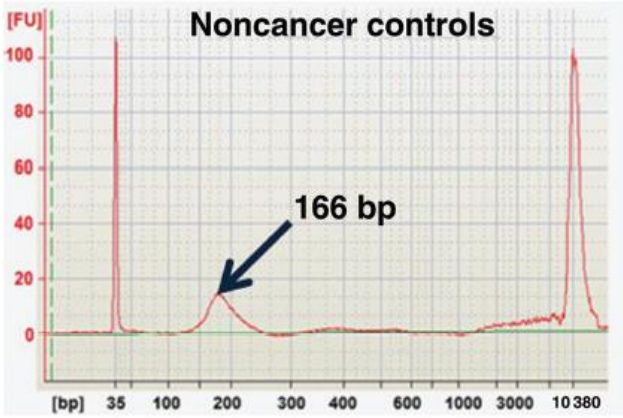
Preservation of DNAs

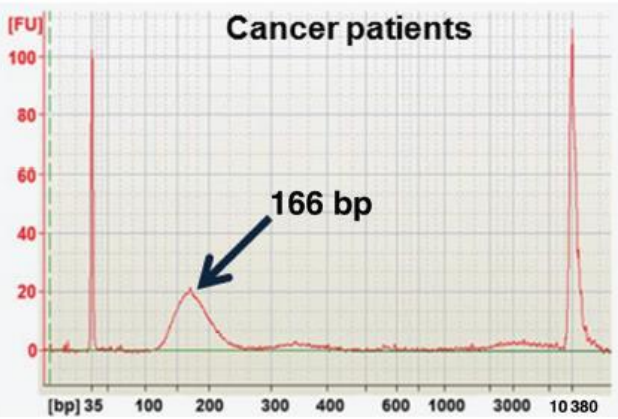


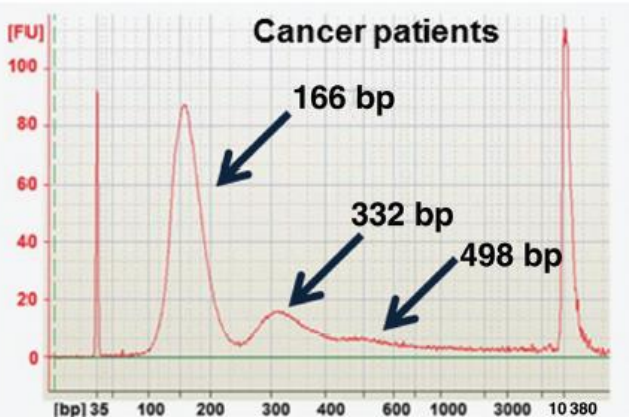


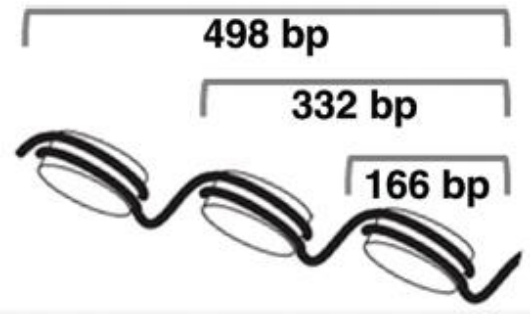


Size distribution of ctDNA



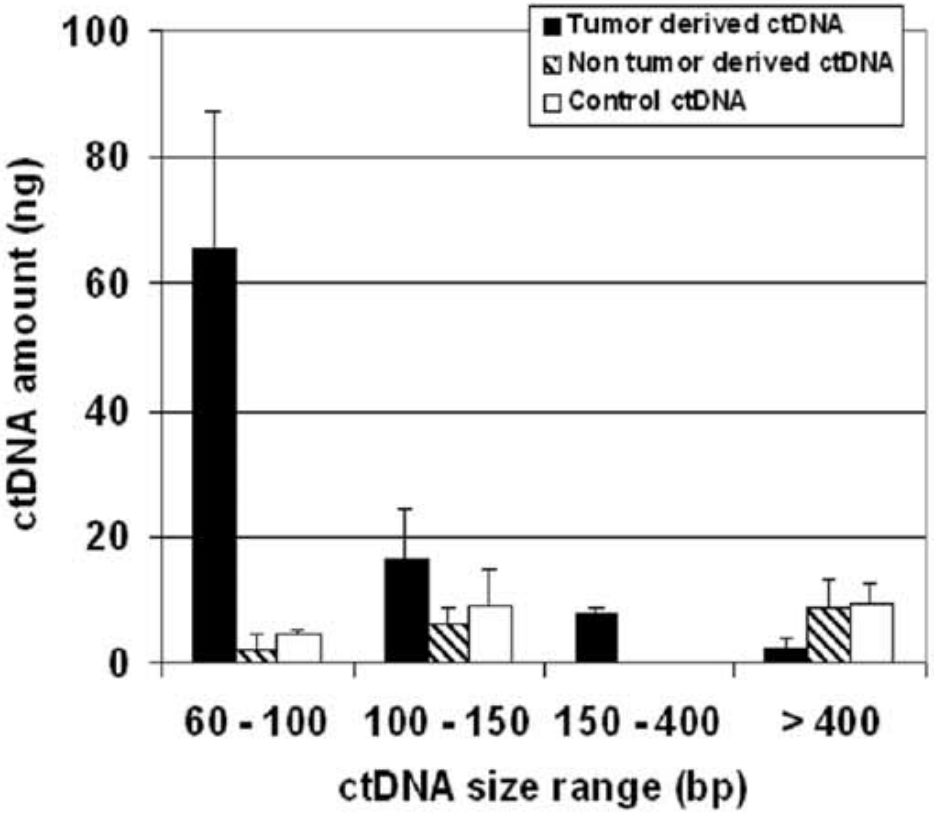


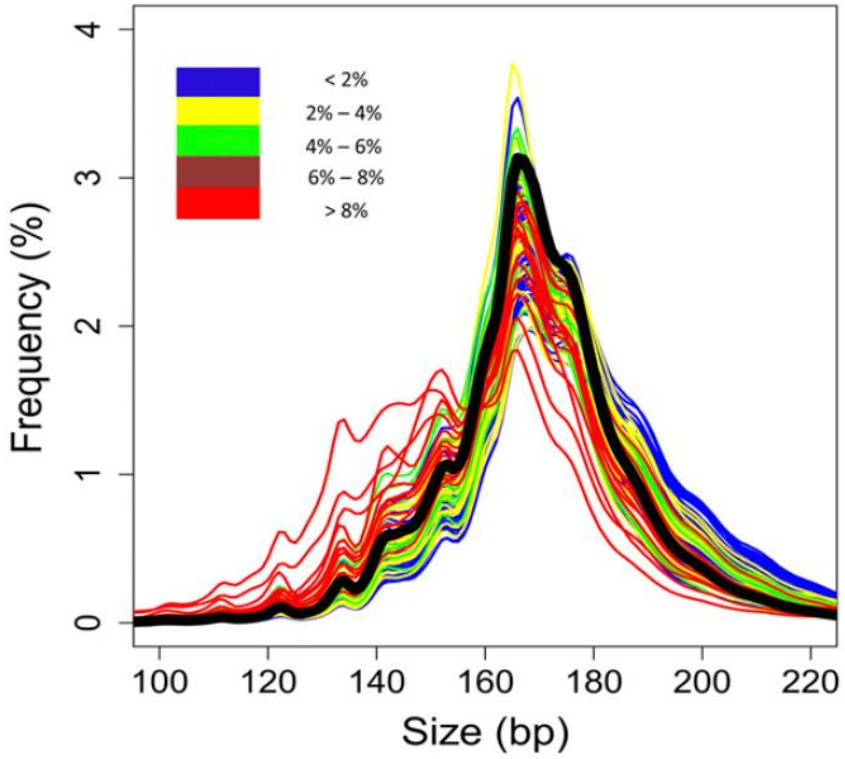




Heitzer et al. Clin Chem. 2015

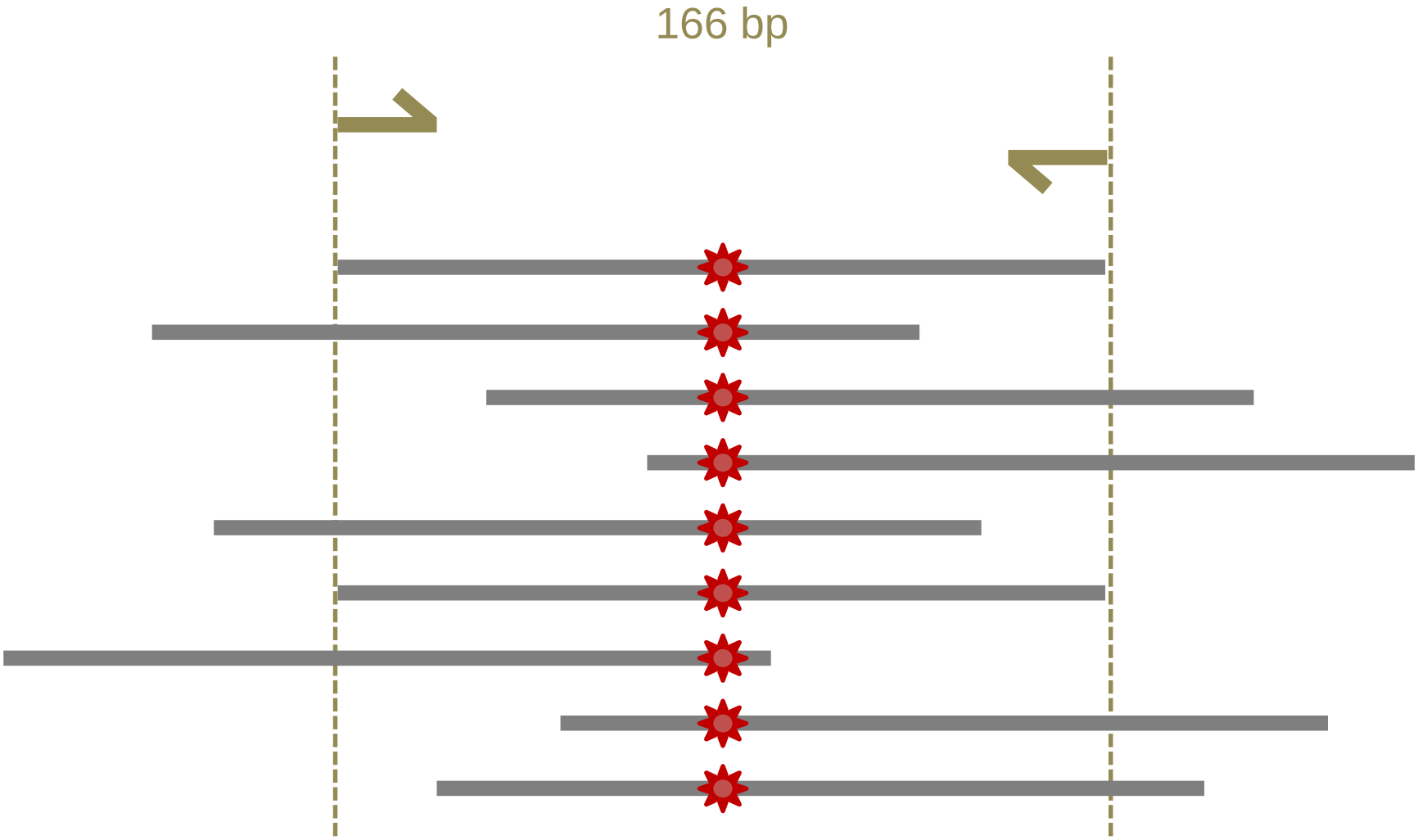
High fragmentation of ctDNA



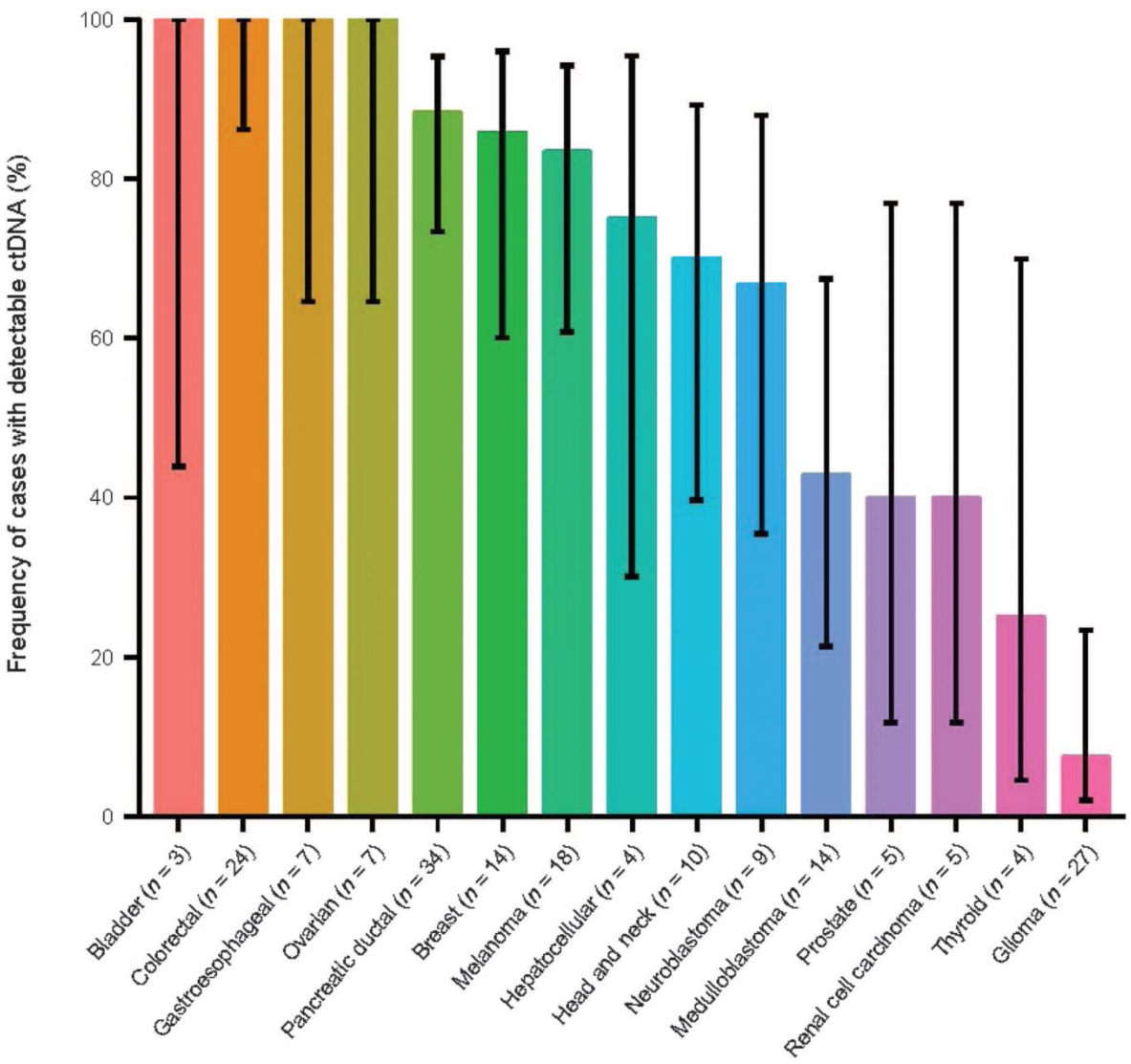


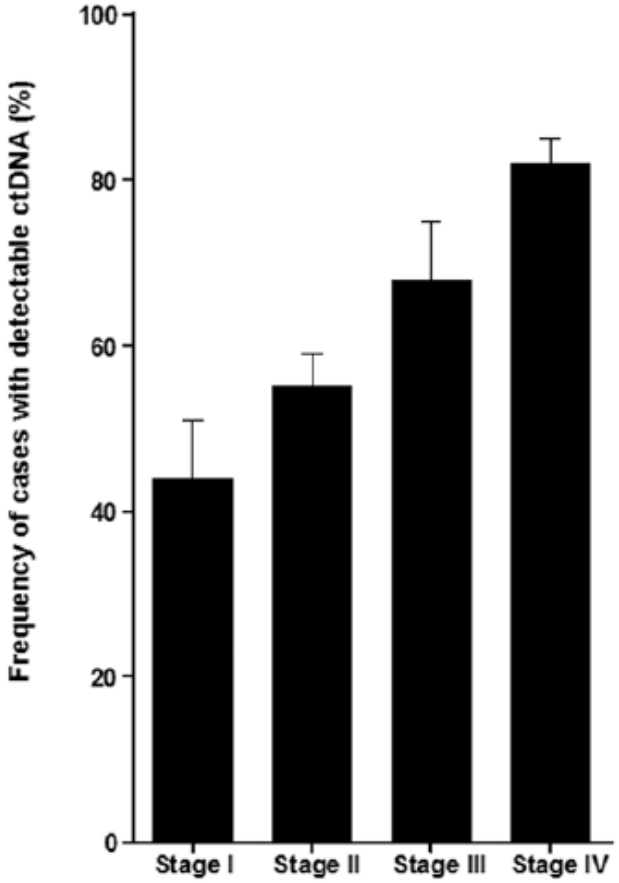
Mouliere et al. PLOS One 2011; Jiang et al. PNAS 2015

Location of target mutation



Detection rates of ctDNA





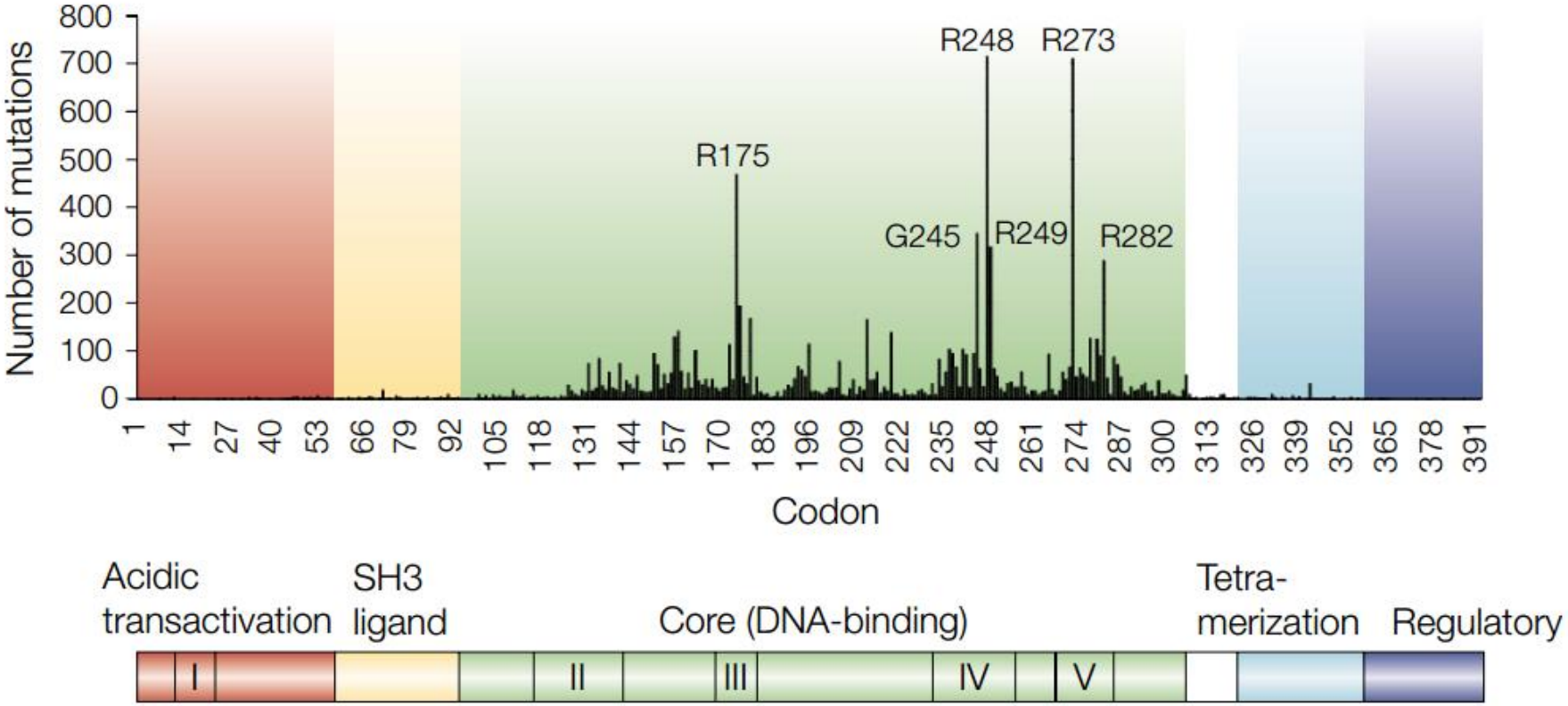
Bettegowda et al. Science Translational Med. 2014

Mutation frequencies in different cancers

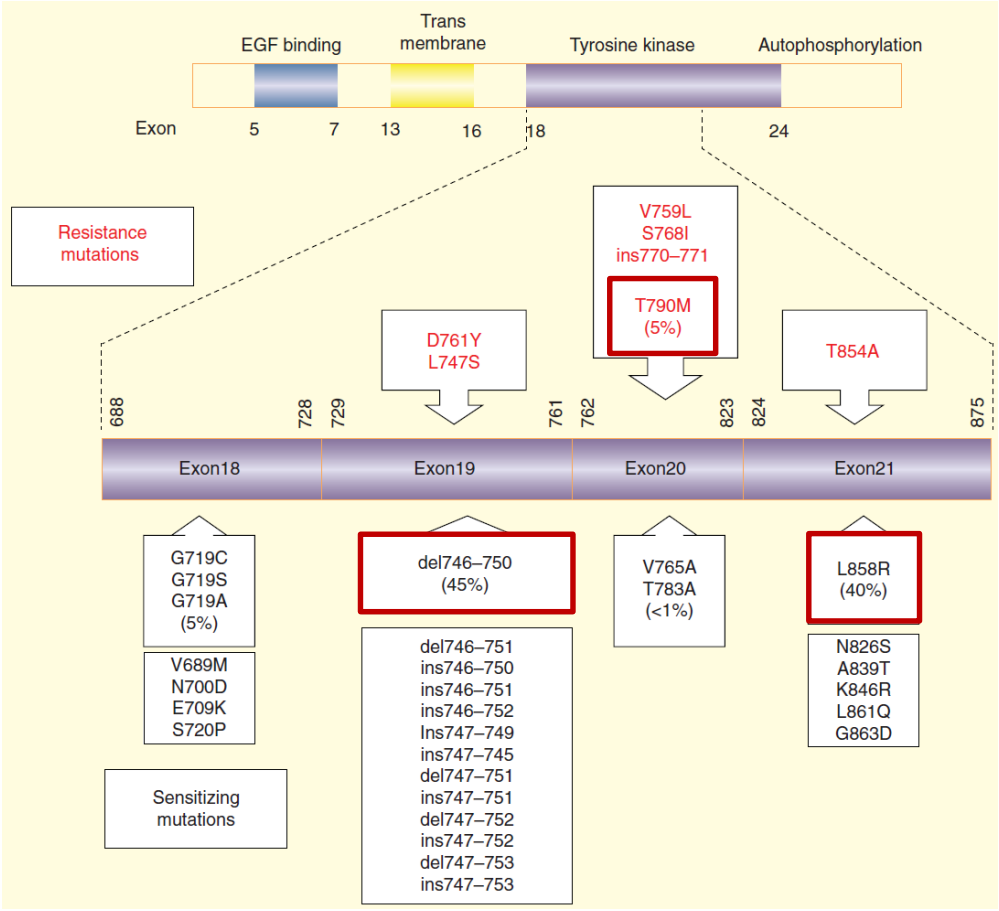
Gene	Breast	Colorectal	Head & Neck	Kidney clear cell	Lung Adeno	Lung Squamous	Ovarian	Melanoma	Thyroid	Uterus	Pan-cancer	Weighted pan-cancer
TP53	25.2	44.5	47.1	1.6	42.9	68.4	55.6	13.5	0.0	16.1	30.7	38.0
KRAS	0.3	44.1	0.0	0.5	30.0	0.8	0.0	1.1	2.7	21.7	15.2	13.7
PIK3CA	31.3	45.6	11.8	2.7	5.0	9.8	0.0	2.2	0.0	51.1	22.3	20.9
PTEN	2.4	35.7	2.9	2.2	1.7	7.5	0.0	6.7	0.0	57.8	15.0	11.2
VHL	0.0	14.7	0.0	41.1	0.0	0.8	0.0	1.1	0.0	1.1	7.7	4.5
FBXW7	0.3	28.7	5.9	0.5	2.9	6.8	0.0	3.4	0.0	18.3	8.7	8.4
CDH1	5.4	15.4	2.9	0.5	0.8	0.8	0.0	2.2	0.0	3.9	4.7	4.9
CDH10	0.3	9.6	16.2	1.1	17.1	17.3	0.0	14.6	0.0	11.1	8.8	10.1
SMAD4	0.0	28.7	1.5	0.0	3.3	2.3	0.0	0.0	0.0	2.2	6.0	6.3
DCAF4L2	1.0	7.4	5.9	0.0	8.3	4.5	0.0	4.5	0.0	4.4	4.2	4.7
CTNNB1	0.3	19.5	1.5	0.5	3.8	1.5	0.0	3.4	0.0	30.6	8.0	6.0
OR2M3	0.0	5.9	0.0	0.0	5.8	3.8	0.0	10.1	2.7	6.7	3.7	2.9
FAM47A	0.7	4.8	2.9	0.5	9.2	7.5	0.0	14.6	1.4	6.1	4.8	4.4
FAM5C	0.7	8.8	5.9	3.2	11.7	12.0	3.7	21.3	0.0	5.0	7.0	6.8
PIK3R1	0.7	9.6	1.5	0.5	1.3	1.5	0.0	4.5	0.0	38.3	6.9	4.3
ZNF676	0.0	3.3	5.9	0.5	9.2	11.3	0.0	20.2	0.0	3.3	4.8	4.9
APC	0.3	77.6	1.5	0.5	3.3	3.0	0.0	6.7	0.0	13.9	16.5	16.0
KLHL4	0.3	8.1	7.4	0.5	8.3	5.3	0.0	9.0	1.4	7.2	5.0	5.4
WBSCR17	1.4	10.3	0.0	0.5	7.5	3.8	0.0	11.2	0.0	6.1	4.9	4.2
PRDM9	0.3	8.8	4.4	0.5	12.5	6.8	0.0	16.9	4.1	9.4	6.6	6.1
TPTE	0.3	7.0	4.4	0.0	13.3	10.5	0.0	23.6	0.0	8.9	6.8	6.2
PCDH11X	0.7	7.7	5.9	2.2	15.8	15.0	3.7	7.9	2.7	7.2	7.2	7.2
MAGEC1	0.3	7.7	5.9	1.1	9.2	9.8	0.0	15.7	1.4	9.4	6.1	5.8
EGFR	0.3	15.1	4.4	0.5	10.4	2.3	0.0	6.7	0.0	2.2	5.4	5.8
FAM47C	2.0	5.9	2.9	1.1	12.5	3.8	3.7	12.4	0.0	7.2	5.5	5.1

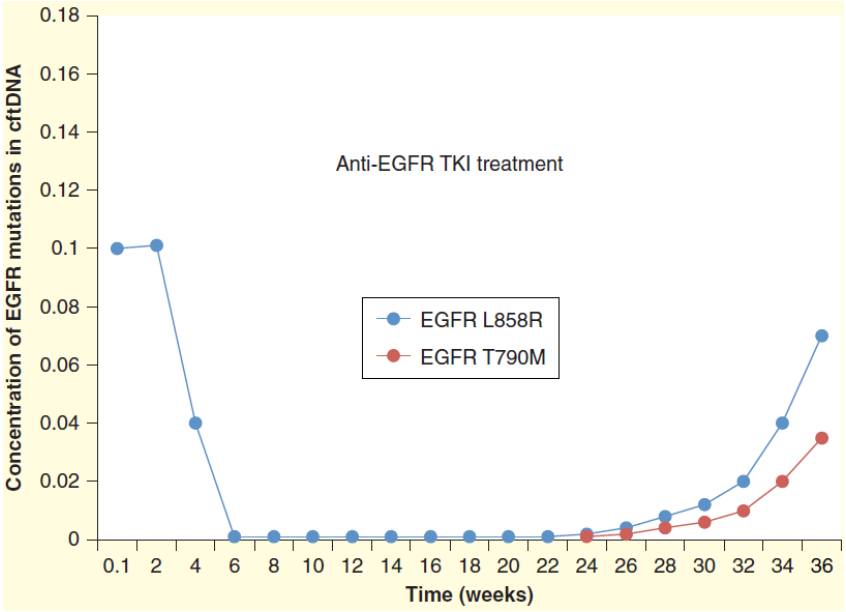
Martinez et al. Sci Reports. 2013

Distribution of *TP53* mutations

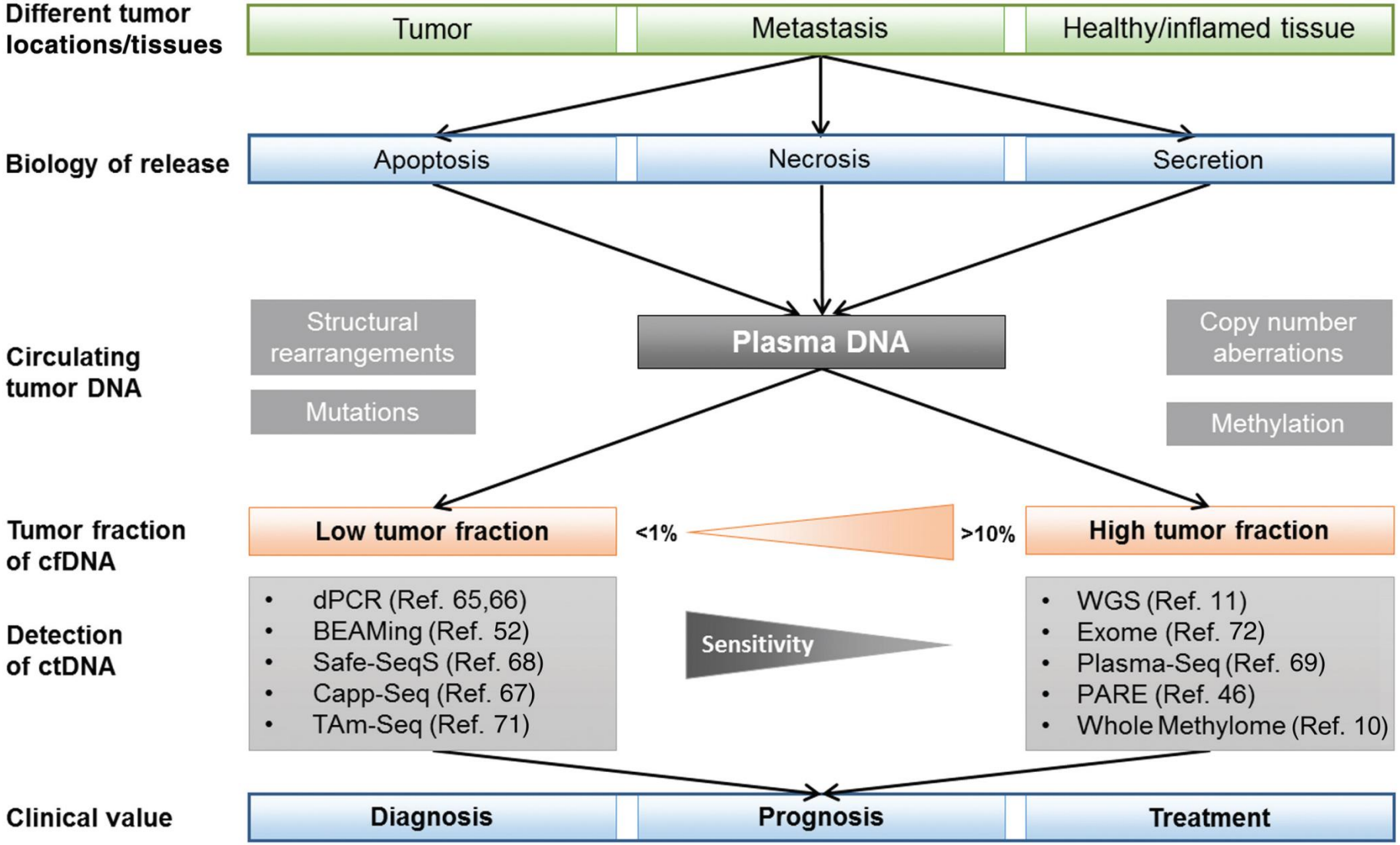


EGFR hotspot mutations in lung cancer



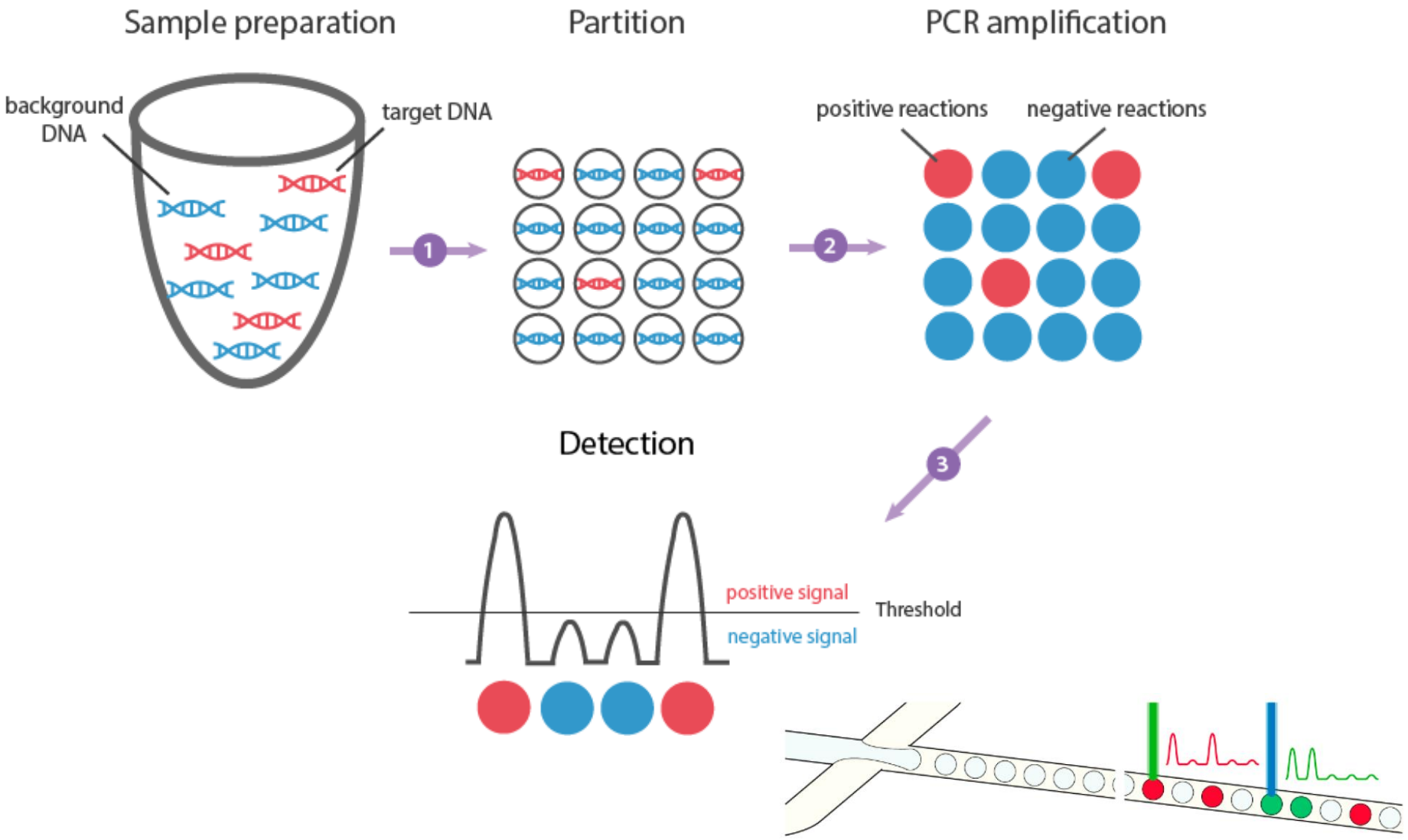


Re et al. Expert Rev Mol Diagn. 2014



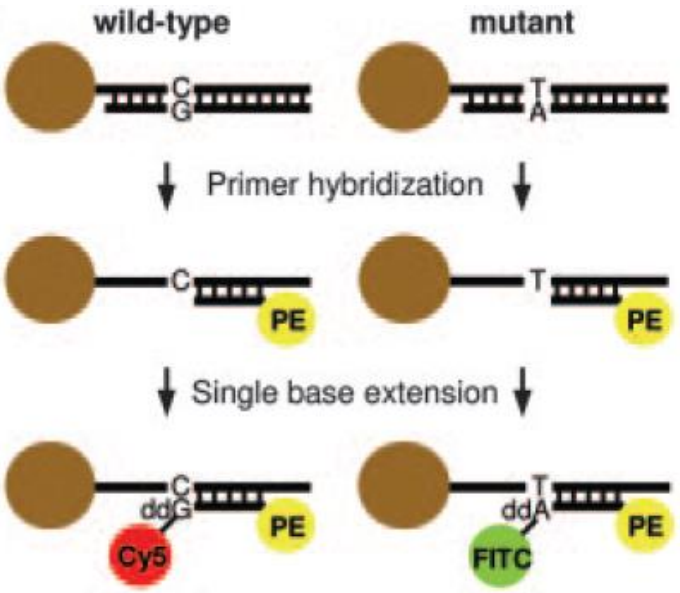
Heitzer et al. Clin Chem. 2015

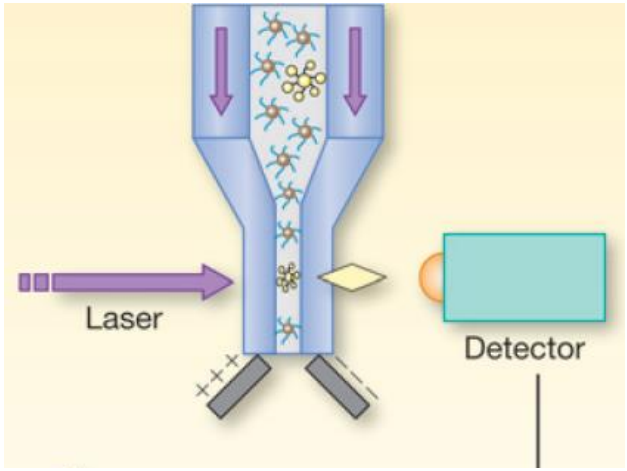
Digital droplet PCR

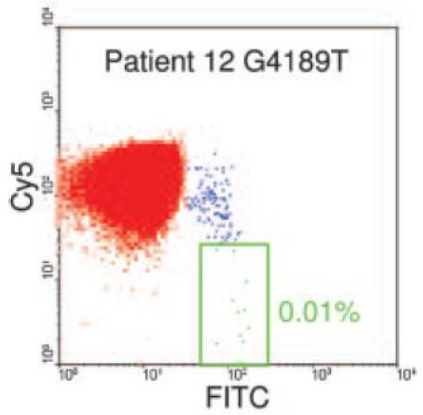


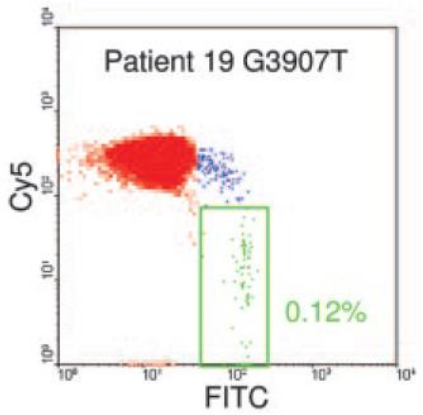
BEAMing method

(Beads, Emulsification, Amplification and Magnetics)



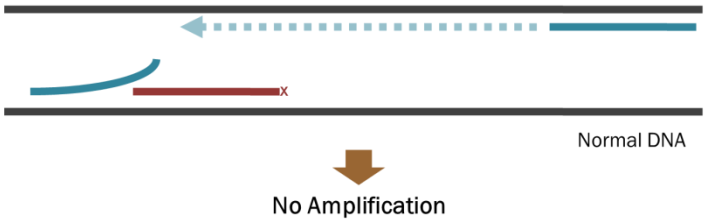


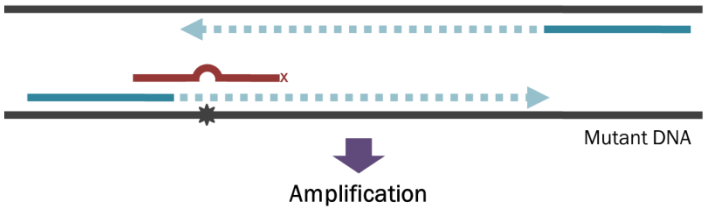




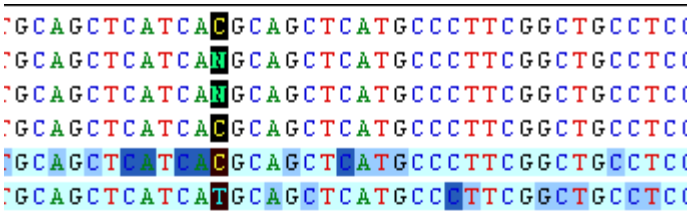
Mutant Enrichment with 3’-Modified Oligonucleotides (MEMO-PCR)

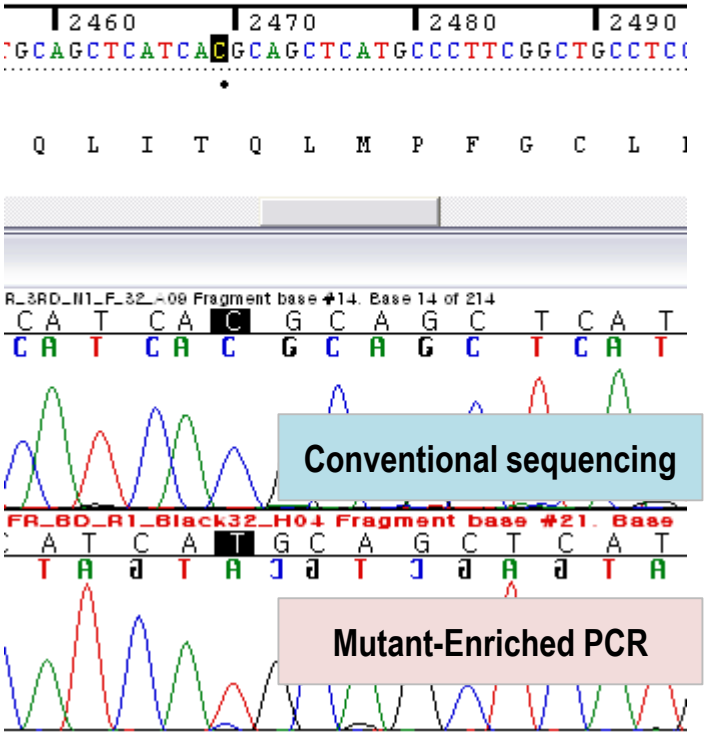
(*EGFR* T790M mutation in 40 lung cancer)





	After Chemotherapy		
Initial		Positive	Negative
	Positive	9	11
	Negative	3	17





MEMO-PCR

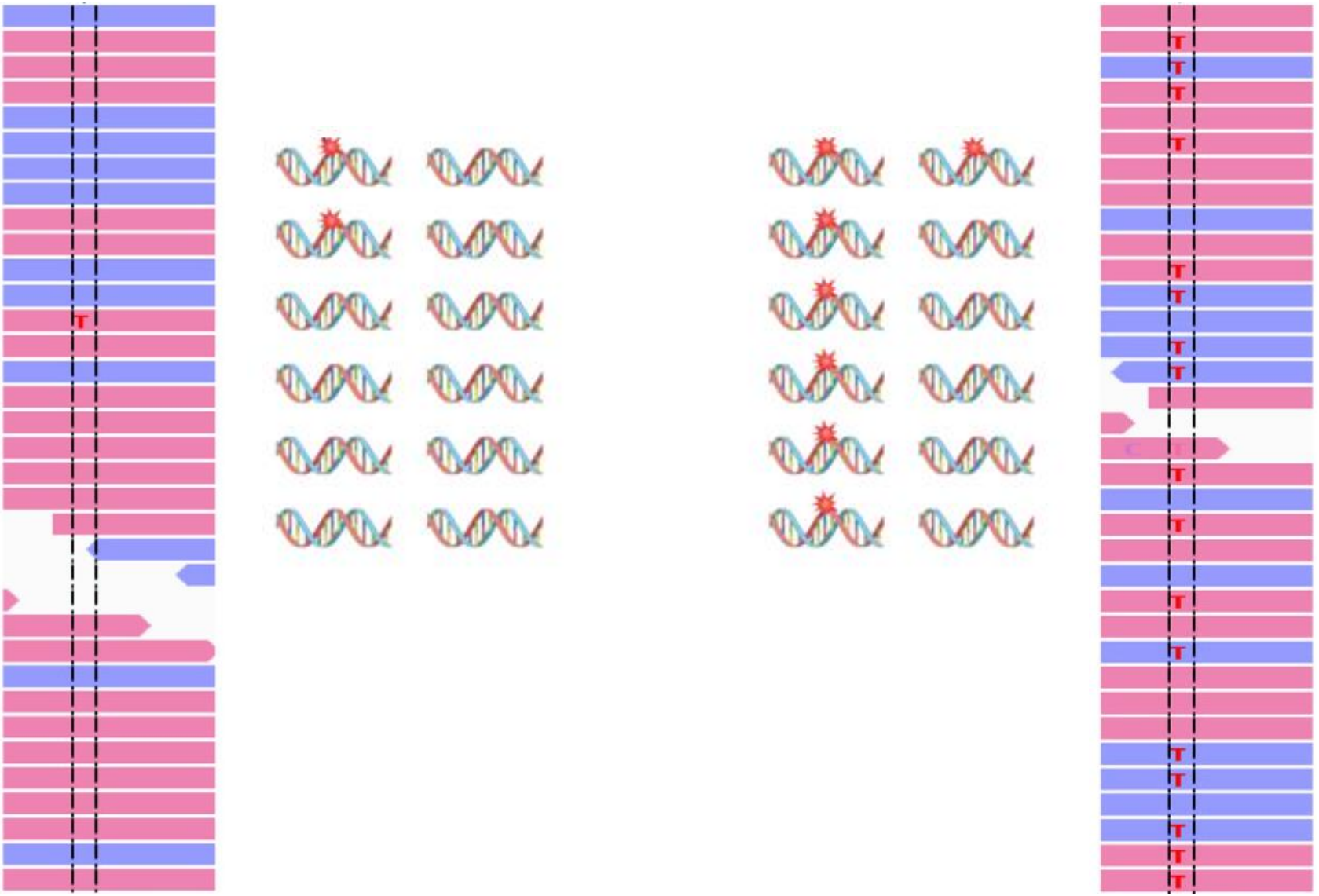
(*BRAF* V600E mutation in 52 thyroid cancers)

- 52 patients with papillary thyroid carcinoma
- 9.6% (5/52) positive in plasma

Pathologic diagnosis	Size	L/N metastasis	BRAF MEMO-PCR
Papillary carcinoma	2.2x1.7cm	Yes	Positive
Papillary carcinoma	1.0x0.9cm	Yes	Positive
Papillary carcinoma	1.5x1.2cm	Yes	Positive
Papillary carcinoma	4.3x3.5cm	No	Positive
Papillary carcinoma	1.6x1.0cm	Yes	Positive

Gene	Mutation	Specimen (cell line)	Best sensitivity*
EGFR	L858R	H1975	1.0×10^{-3}
	T790M	UC†	1.0×10^{-6}
	Exon 19 Del15	PC9	2.0×10^{-6}
BRAF	V600E	SNU-790	1.0×10^{-3}
TP53	R175H	CCRF-CEM	5.0×10^{-4}
	R248Q	Kasumi-1	1.0×10^{-3}
	R248W	MIA PaCa-2	5.0×10^{-5}
	R273H	H1975	2.0×10^{-4}
	R273C	SNU-1196	5.0×10^{-5}
KRAS	G12S	A549	5.0×10^{-4}
	G12C	MIA PaCa-2	2.0×10^{-4}
	G12D	CCRF-CEM	5.0×10^{-4}
	G12V	Capan-1	2.0×10^{-3}
	G12A	SW1116	2.0×10^{-3}
	G13D	DLD-1	2.0×10^{-4}
JAK2	V617F	HEL	2.0×10^{-5}
NPM1	Exon 12 Ins4	Patient sample	1.0×10^{-5}

Deep sequencing by NGS



Sequncing errors

Plasma EGFR testing

Sample	Exon 19 deletion		L858R		T790M		Exon 20 insertion	
	Result	SQI value	Result	SQI value	Result	SQI value	Result	SQI value
Level 1	Detected	15.2	Detected	10.09	Detected	11.62	Detected	4.69
Level 2	Detected	14.29	Detected	7.95	Detected	10.31	Detected	3.58
Level 3	Detected	11.69	Detected	6.47	Detected	5.97	Detected	1.95
Level 4	Detected	8.67	Not detected		Not detected		Not detected	
Level 5-1	Not detected		Not detected		Detected	11.4	Not detected	
Level 5-2	Not detected		Not detected		Detected	11.3	Not detected	
Level 5-3	Not detected		Not detected		Detected	11.42	Not detected	
Level 6-1	Not detected		Detected	9.55	Not detected		Not detected	
Level 6-2	Not detected		Detected	8.96	Not detected		Not detected	
Level 6-3	Not detected		Detected	9.69	Not detected		Not detected	
Level 7-1	Detected	14.99	Not detected		Not detected		Not detected	
Level 7-2	Detected	15.34	Not detected		Not detected		Not detected	
Level 7-3	Detected	15.35	Not detected		Not detected		Not detected	

Real-time PCR (Company R)

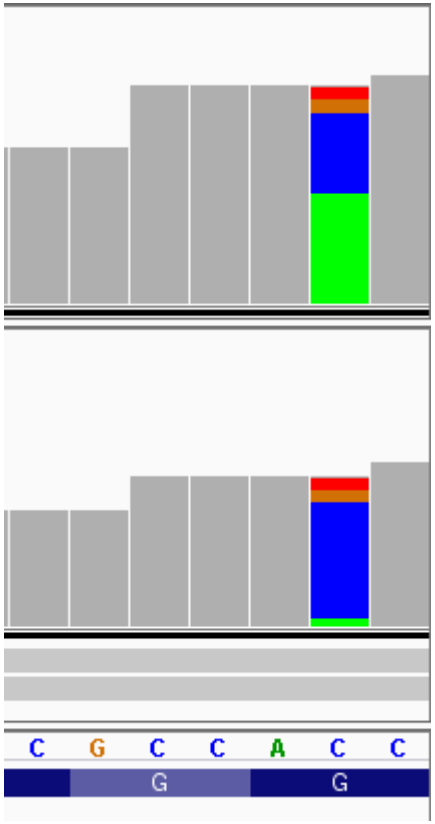
NGS analysis (Company Q)

Sample	Exon 19 deletion		L858R		T790M		Exon20 insertion	
	Result	Total read depth/variant read depth	Result	Total read depth/variant read depth	Result	Total read depth/variant read depth	Result	Total read depth/variant read depth
Level 1	Detected	5819/231	Detected	4594/171	Detected	1717/71	Detected	1091/30
Level 2	Detected	10001/130	Detected	4385/84	Detected	2572/87	Detected	2341/24
Level 3	Detected	9416/57	Detected	4681/52	Not detected		Not detected	
Level 4	Not detected		Not detected		Not detected		Not detected	
Level 5-1	Not detected		Not detected		Detected	2131/111	Not detected	
Level 5-2	Not detected		Not detected		Detected	2904/126	Not detected	
Level 5-3	Not detected		Not detected		Not detected		Not detected	
Level 6-1	Not detected		Detected	4067/148	Not detected		Not detected	
Level 6-2	Not detected		Detected	6335/344	Not detected		Not detected	
Level 6-3	Not detected		Detected	4638/139	Not detected		Not detected	
Level 7-1	Detected	9157/250	Not detected		Not detected		Not detected	
Level 7-2	Detected	8823/274	Not detected		Not detected		Not detected	
Level 7-3	Detected	14136/242	Not detected		Not detected		Not detected	

MEMO-PCR & deep sequencing using plasma

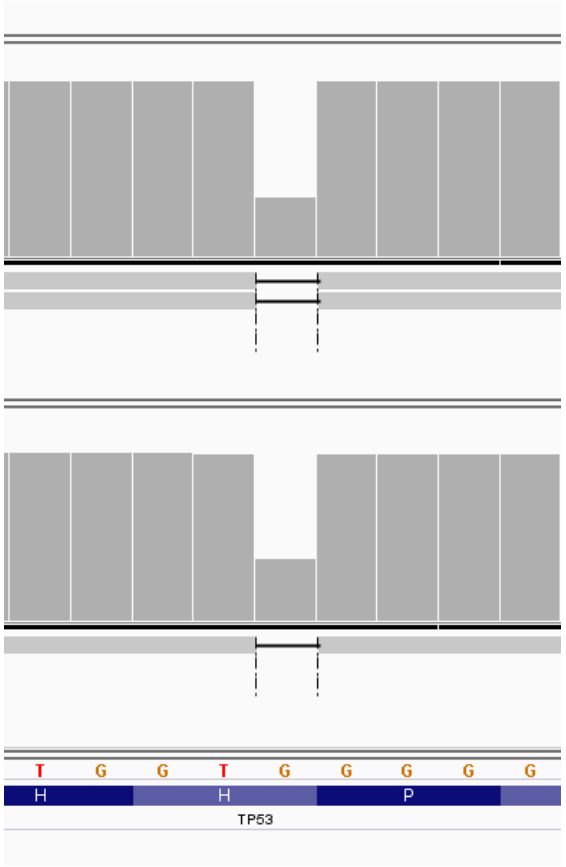
MEMO-PCR

Deep sequencing



KRAS p.G13D

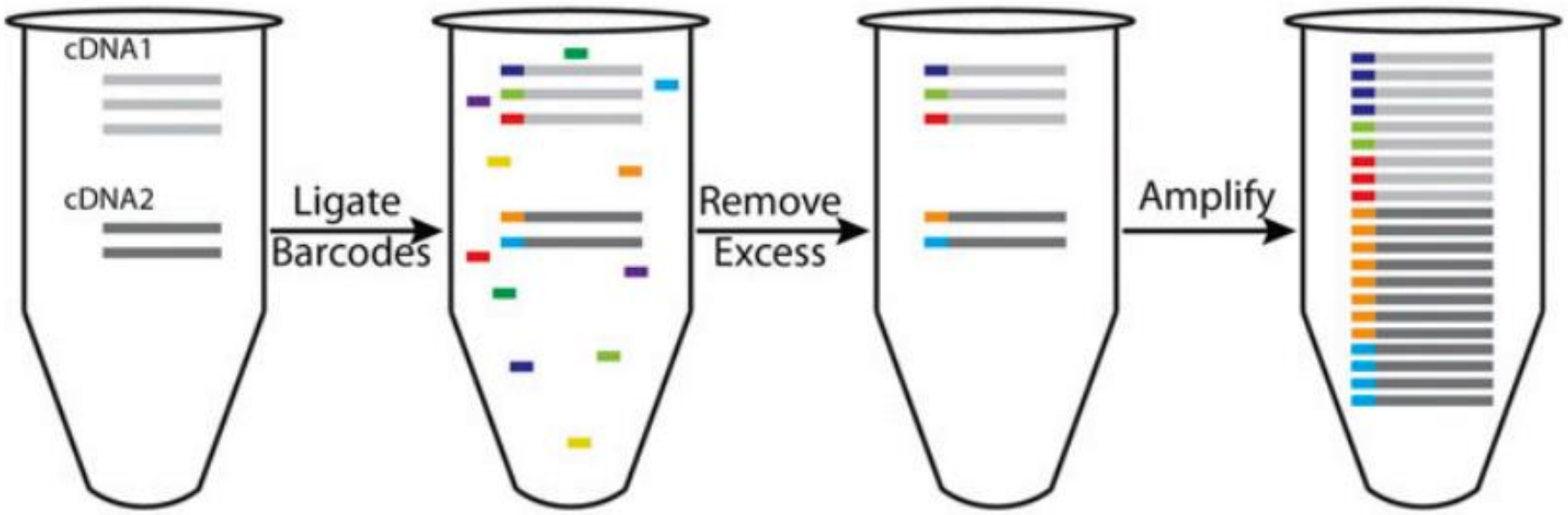
Lung adenocarcinoma
(Stage IIb)



TP53 c.532delC

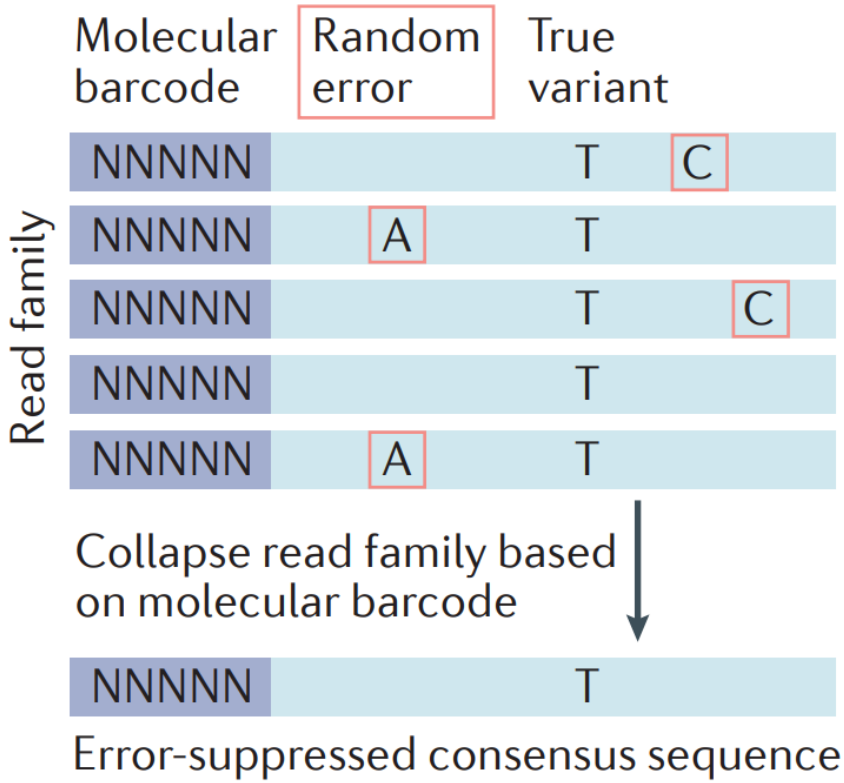
Colon adenocarcinoma
(Stage IV)

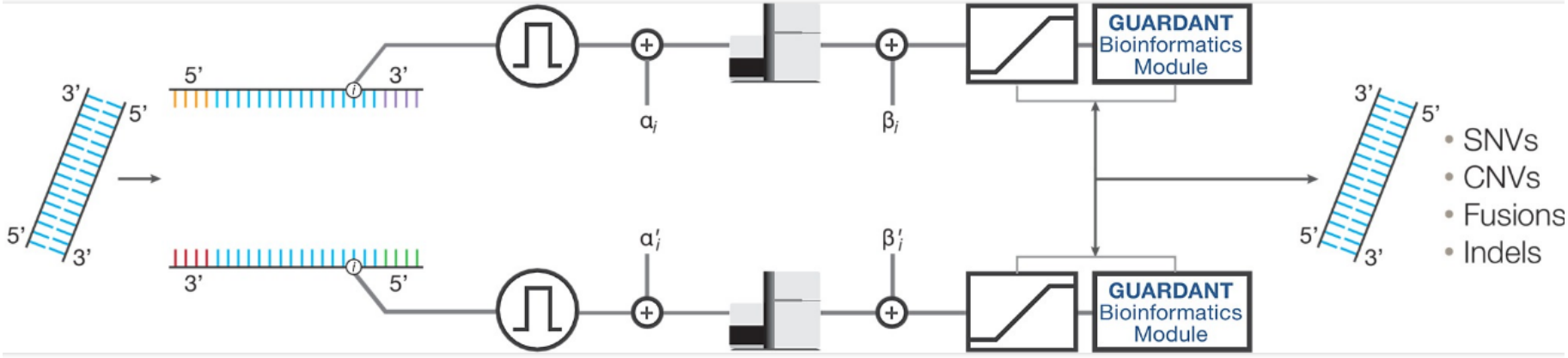
Molecular barcoding



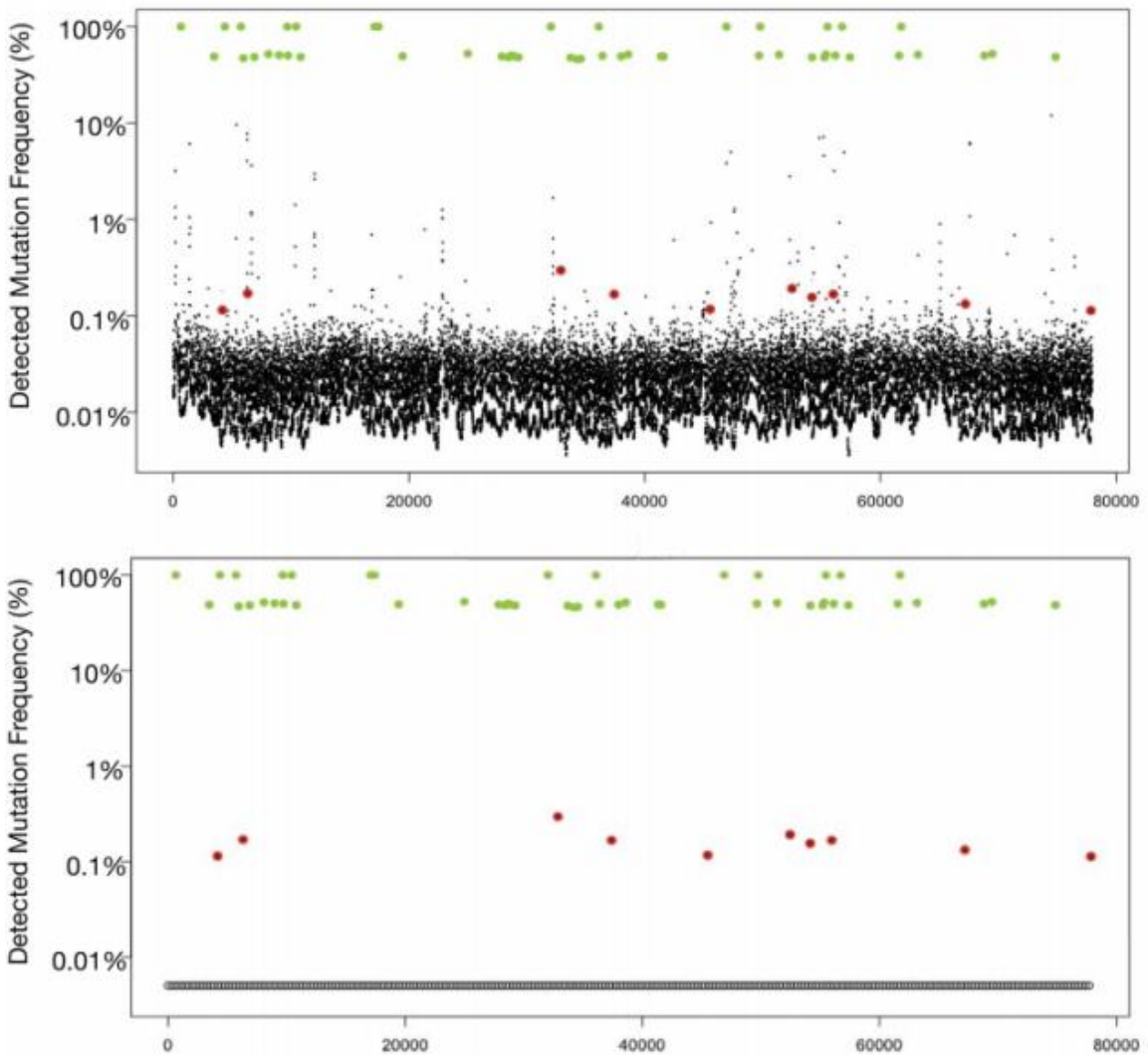
Barcode	
ATCGTAGG	DNA1
TGCGTGAT	DNA2
GTGCATGA	DNA3
CGTAGTTG	DNA4
AAGTCCTG	DNA5

Reducing background errors





Error reduction and increased sensitivity by digital sequencing



Lanman et al. PLOS One 2015

ctDNA *vs.* CTC

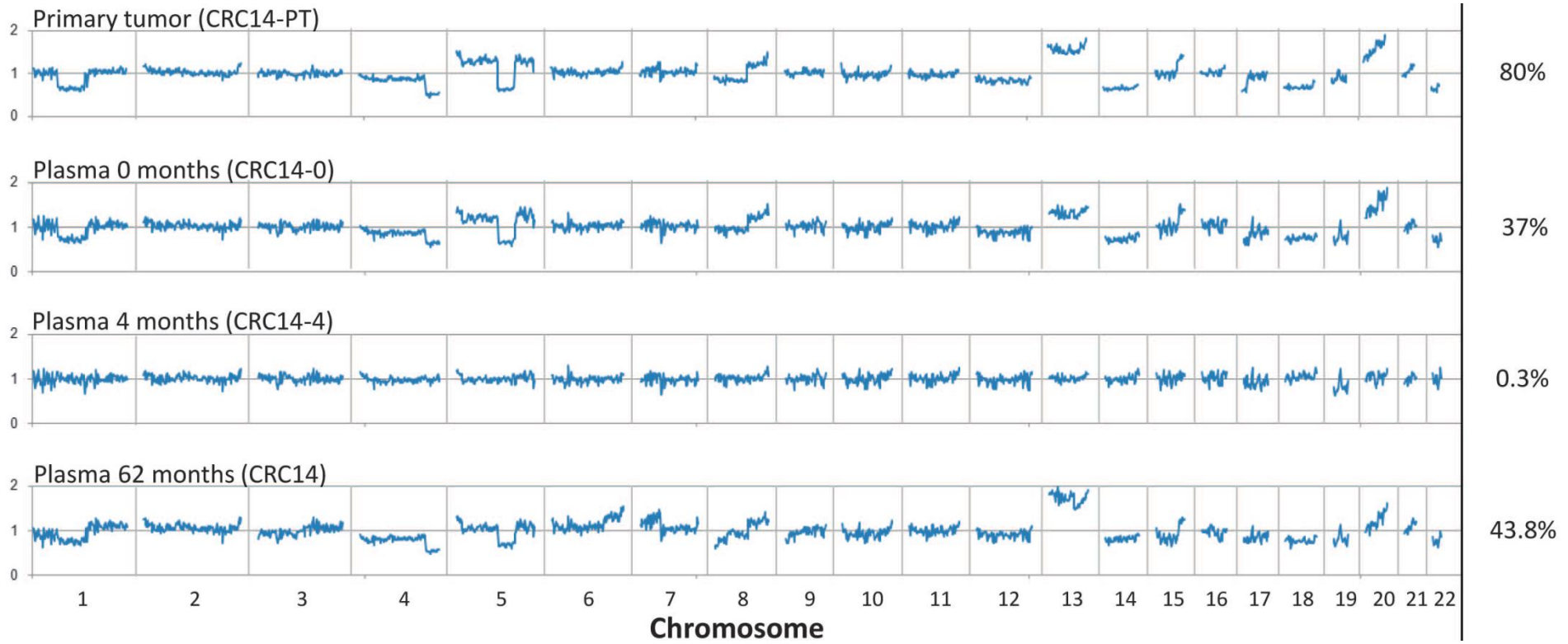
	Positive	Negative
ctDNA		
Positive	22	3
Negative	1	56
Sensitivity (95% CI)	0.957 (0.810–0.999)	
Specificity (95% CI)	0.949 (0.874–0.989)	
CTC		
Positive	12	7
Negative	11	52
Sensitivity (95% CI)	0.522 (0.335–0.732)	
Specificity (95% CI)	0.881 (0.789–0.951)	

Freidin et al. Clin Chem. 2015

Gene mutations detected in ctDNA of NSCLC patients

Material	Method	Genes analyzed	Patients		Median progression free survival (PFS)		median overall survival (OS)		Medicine	Sensitivity (%)	Specificity (%)	References
			Number	Positive (%)	Mutation type (m)	Wild type (m)	Mutation type (m)	Wild type (m)				
Plasma	qPCR	KRAS	308	8.8	5.77	5.43	9.07	10.03	Cisplatin and docetaxel	NM	NM	[25]
Plasma	qPCR+ARMS	KRAS	246	17.5	3.0	5.6	4.8	9.5	Carboplatin and vinorelbine	90.0	NM	[32]
Plasma	PCR-RFLP	KRAS	273	12.8	2.5	8.8	16.9	20.3	EGFR-TKI	76.7	95.1	[31]
Plasma	Scorpion+ARMS	KRAS	49	4.08	1.4	5.4	2.5	18.8	Docetaxel plus erlotinib	100	92.3	[44]
Plasma	Scorpion+ARMS	KRAS	24	20.8	KRAS mutation status in ctDNA was not associated with PFS.		NM	NM	Pertuzumab plus erlotinib	100	100	[45]
Plasma	qPCR+NGS	KRAS	30	20.0	NM	NM	NM	NM	Radiation, chemotherapy, targeted systemic therapy and/or surgery	100	100	[36]
Peripheral blood	Colorimetric membrane array	KARS	209	28.2	NM	NM	NM	NM	NM	83.1	96.4	[46]
Peripheral blood	Weighted chemiluminescent membrane array	KARS	209	31.6	NM	NM	NM	NM	NM	93.0	94.2	[46]
Plasma	qPCR	EGFR	56	23.2	11.2	2.7	21.8	5.8	Gefitinib	NM	NM	[26]
Plasma	Digital PCR	EGFR	35	43.0	NM	NM	NM	NM	EGFR TKIs	92	100	[35]
Serum	PCR+direct sequencing	EGFR	27	37.0	NM	NM	NM	NM	Gefitinib	75.0	71.4	[30]
Serum	nested PCR+ sequencing	EGFR	44	9.1	NM	NM	41.9	17.9	EGFR TKIs	NM	NM	[47]
Plasma	Scorpion+ARMS	EGFR	54	47	NM	NM	NM	NM	Gefitinib/erlotinib	70	85	[48]
Serum		EGFR	42	16.7	174 days	58 days	387 days	228 days	Gefitinib	75	97.1	[49]
Serum		EGFR	27	48.1	200 days	46 days	611 days	232 days	Gefitinib	50.0	85.7	[50]
Plasma		EGFR	49	20.4	Exon 19/21: 18.3 T790M: 3.9	4.0	Exon 19/21: 39.6 T790M: 24.2	17.8	Docetaxel plus erlotinib	66.7	100	[44]
Serum		EGFR	194	23.7	PFS was significantly longer with gefitinib than carboplatin/ paclitaxel in patients with EGFR mutation in ctDNA		EGFR mutation status in ctDNA was not associated with longer OS.		Gefitinib or carboplatin plus paclitaxel	43	100	[51]
Plasma		EGFR	24	16.7	EGFR mutation status in ctDNA was associated with longer PFS		NM	NM	Pertuzumab plus erlotinib	100	100	[45]
Plasma	DHPLC	EGFR	230	34.3	11.1 (8.7–16.8)	5.9 (2.1–9.7)	EGFR mutation status in ctDNA was not associated with longer OS		Gefitinib	81.8	89.5	[34]
Serum	Cycleave RT-PCR	EGFR	24	25.0	EGFR mutation status in ctDNA was not associated with longer PFS		EGFR mutation status in ctDNA was not associated with longer OS		NM	NM	NM	[52]
Plasma	Wild-inhibiting PCR Mutation-biased PCR	EGFR	39	Exon 19,: 44.7 L858R, 8.7	NM	NM	NM	NM	Operation or chemotherapy	NM	NM	[53]
Plasma	ME-PCR	EGFR	627	Exon19, 6.0	NM	NM	NM	NM	EGFR-TKI	Exon19, 100	Exon19, 50	[28]

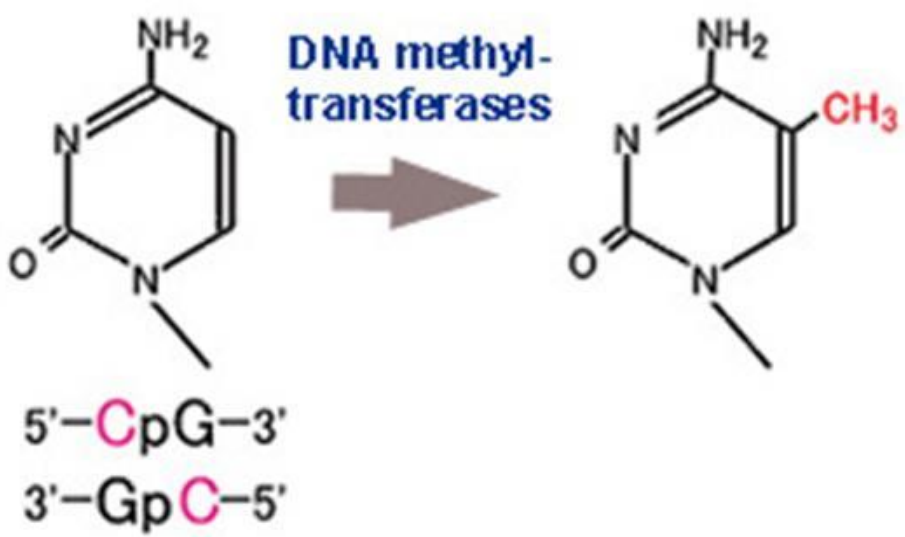
Copy number change in plasma

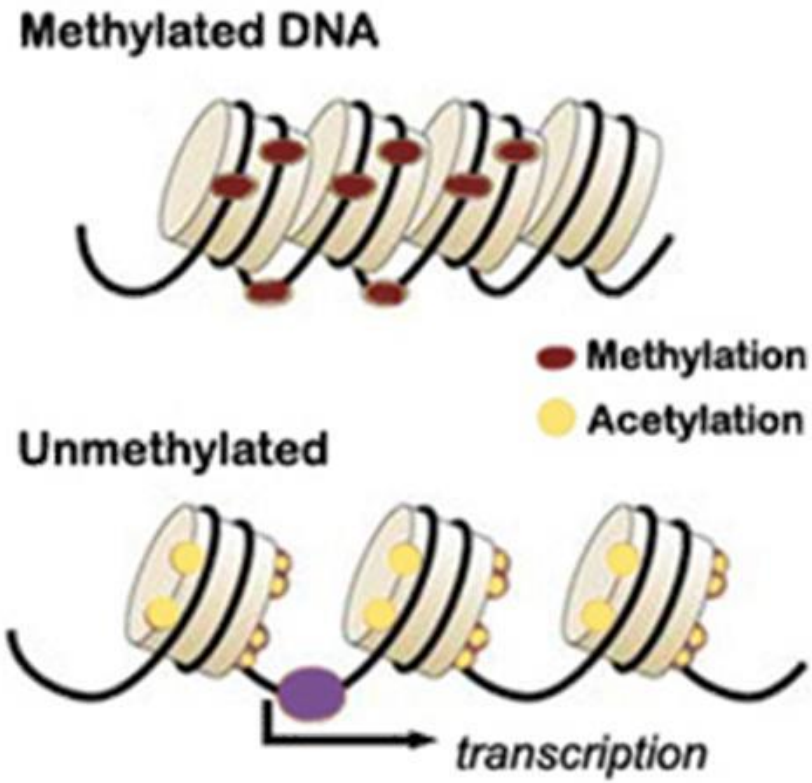


Leary et al. Science Translational Med. 2012

DNA methylation

- Methylation of cytosines within CpG dinucleotides
- 26.7 million CpG sites in the autosomal genome



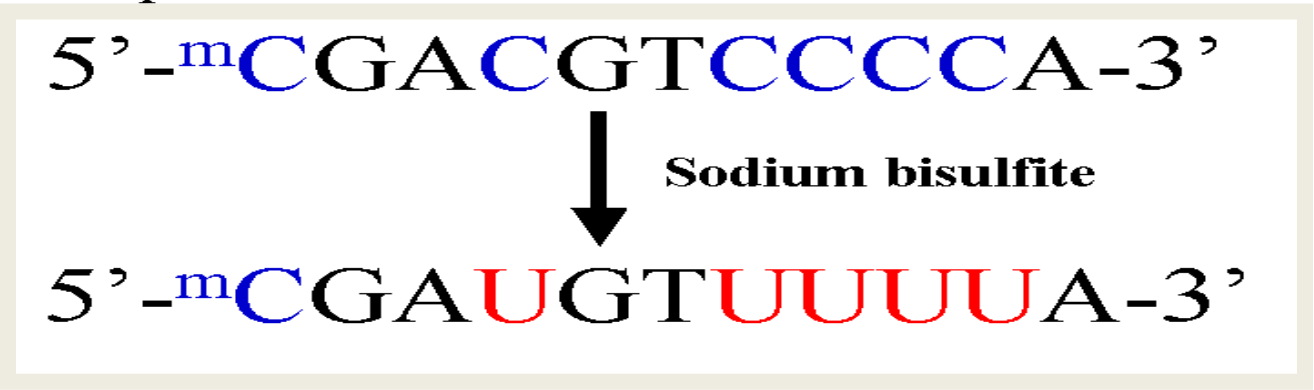


Assaying Methylation

- Multiple restriction enzyme assays
- Bisulfite conversion & methylation-specific PCR
- MeDIP (Methylated DNA immuno-precipitation)
  - Antibody to Methylated-C → ChIP
- Isoschizomer (HpaII/MspI) assays:
  - MIAMI (Microarray-based Integrated Analysis of Methylation by Isoschizomers)
  - HELP
- Bisulphite conversion & hybridize to array
- Bisulphite conversion & sequencing

Methylation-Specific PCR (MSP)

- Sodium bisulfite treatment of DNA
  - Methylated CpG: CpG → CpG
  - Unmethylated CpG: CpG → UpG
  - Cytosine except CpG: C→ U
- Example

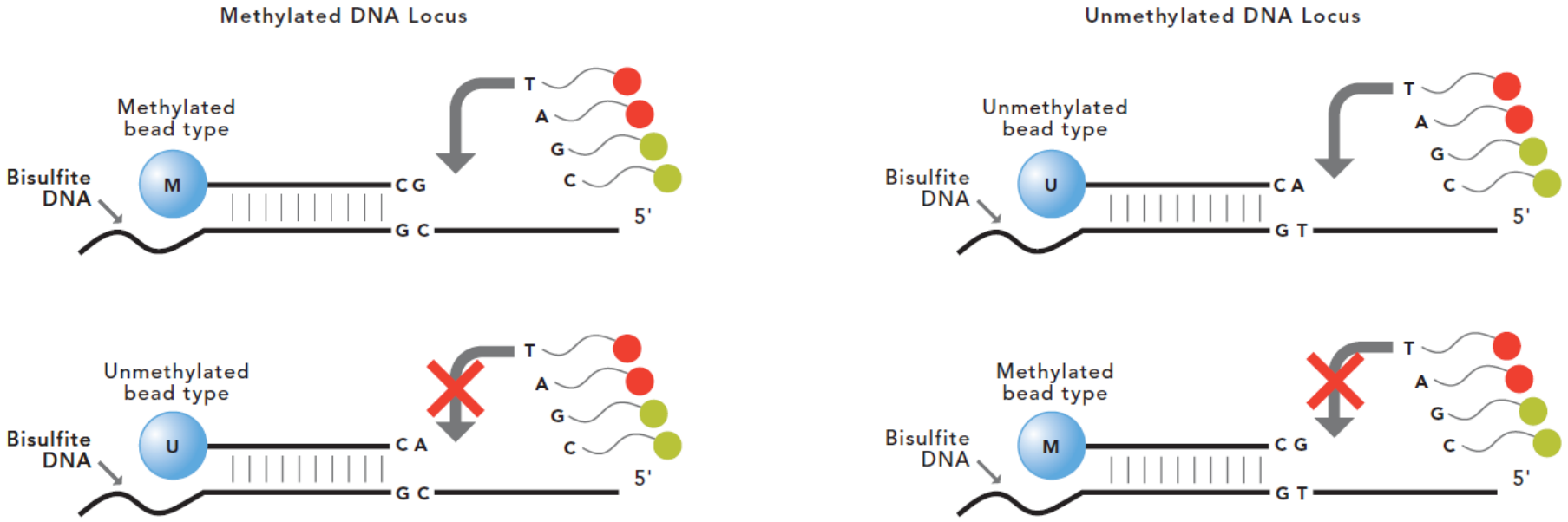


Genomic DNA is modified by treatment with sodium bisulfite.

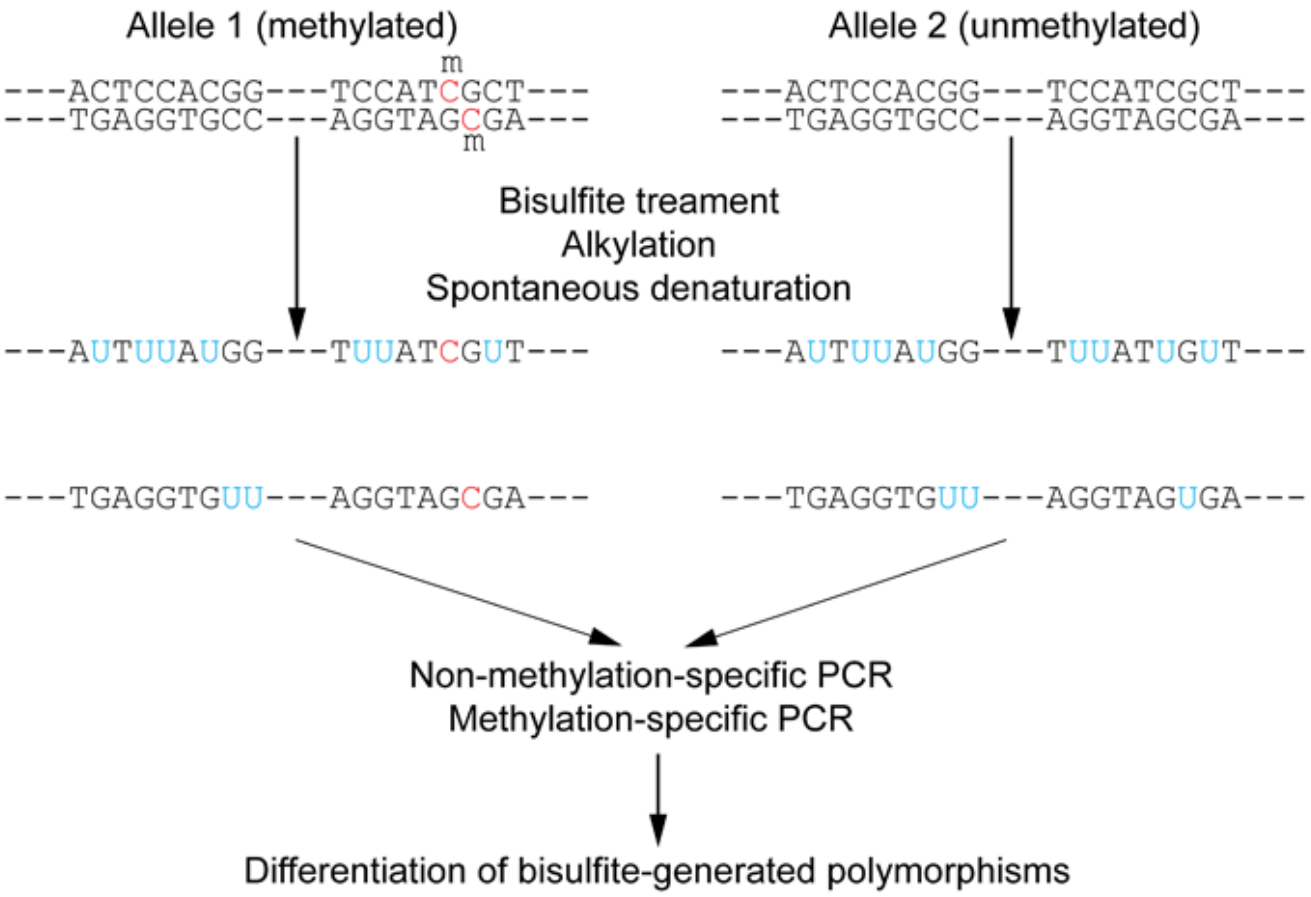
All unmethylated cytosines are converted to uracil, but methylated cytosines are resistant to this modification.

Illumina Infinium DNA methylation assay

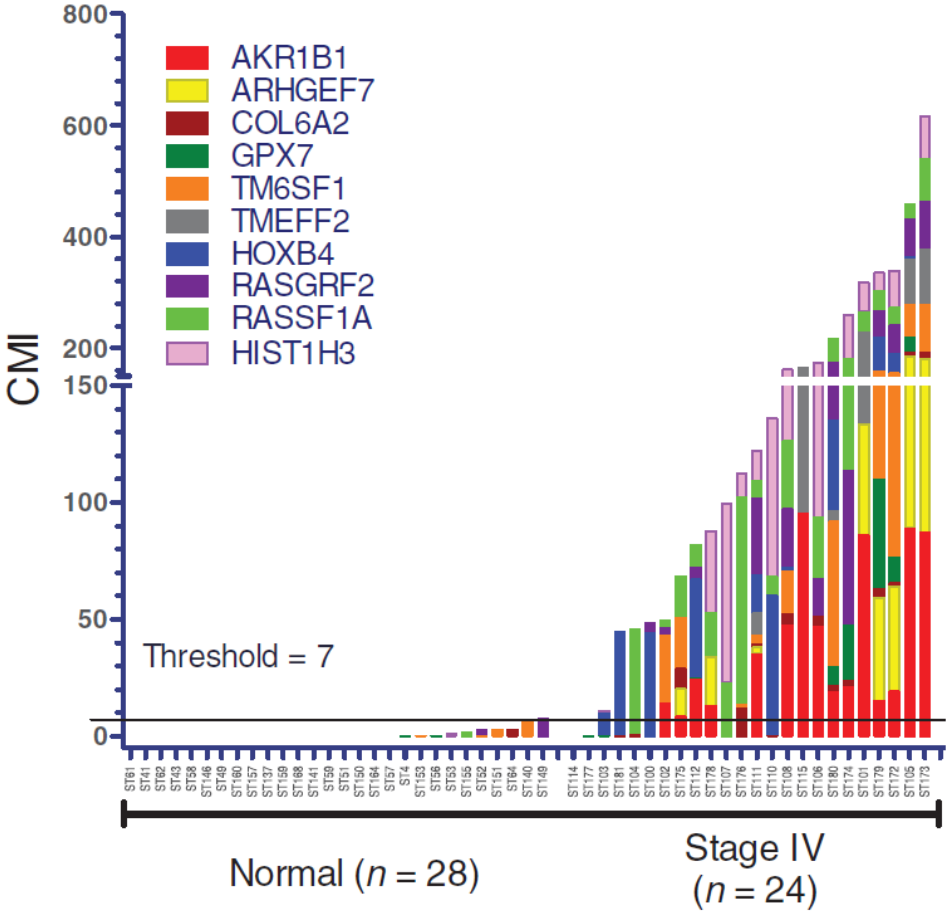
- Relative signal intensities of bisulfite-converted sequences
- 485,764 CpG sites for >32,000 transcripts
- Methylation levels are expressed as β-values (0~1.0; proportion of methylated DNAs)

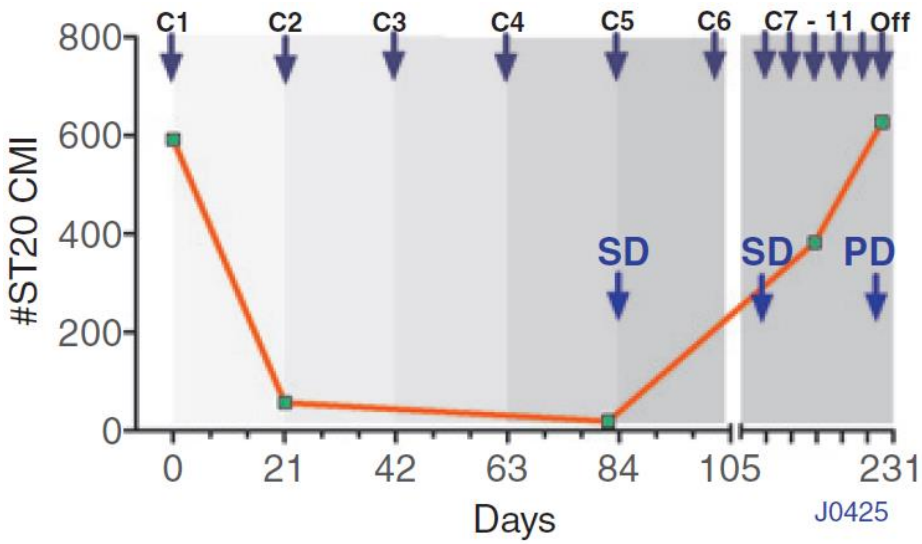


Bisulfite-sequencing



DNA methylation



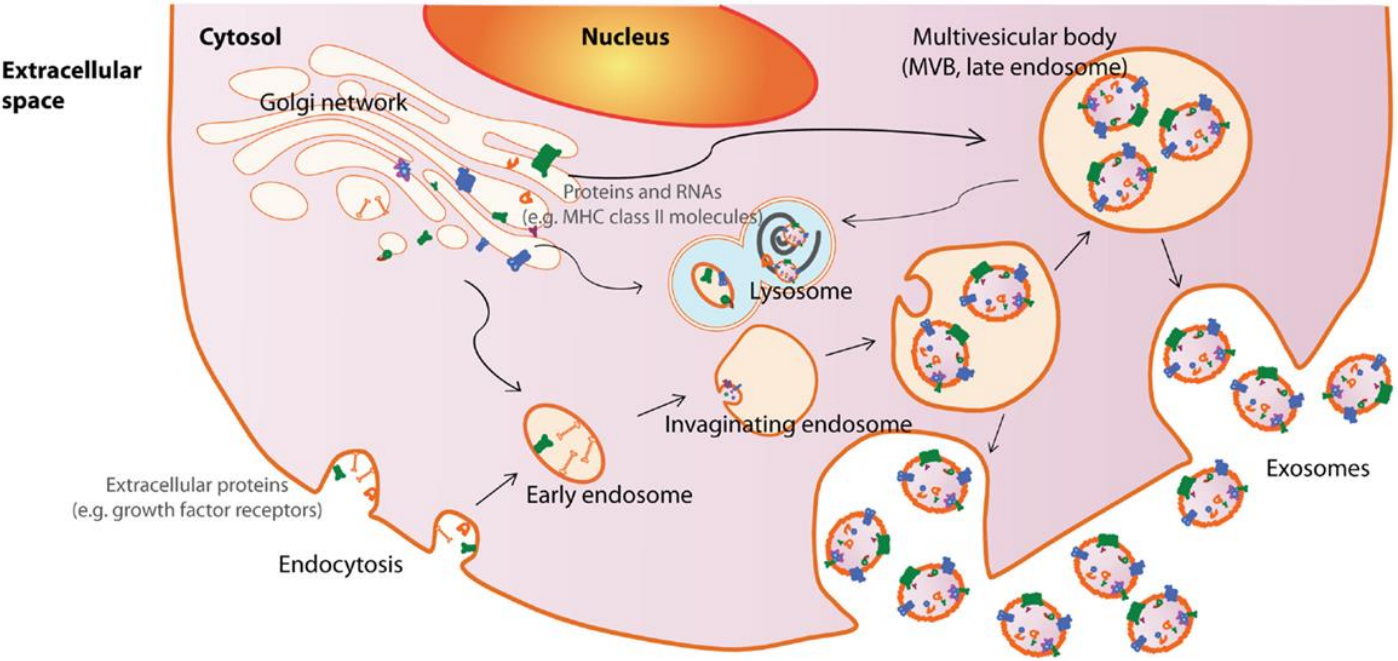


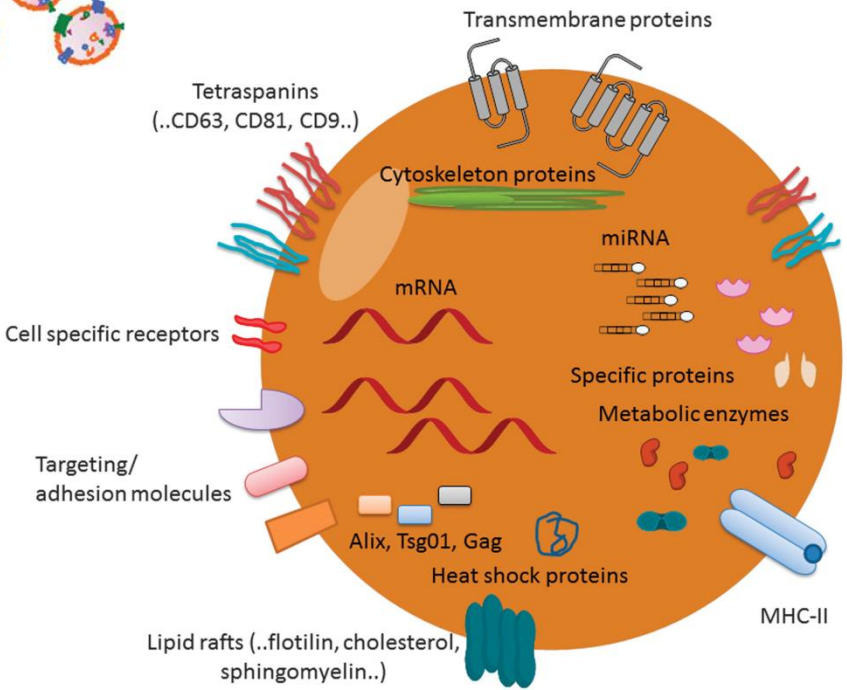
Fackler et al. Cancer Res. 2014

Material	Method	Genes analyzed	Patients		Controls		Median progression free survival		Median overall survival		Sensitivity (%)	Specificity (%)	References
			Number	Frequency (%)	Number	Frequency (%)	Methylated (m)	Unmethylated (m)	Methylated (m)	Unmethylated (m)			
Plasma	MSP	P16	67	17.9	NA	ND	31 weeks	24 weeks	53 weeks	43 weeks	NM	NM	[68]
Serum	Nested MSP	TMEFF2	316	9.2	50 (age-matched healthy controls)	0	NM	NM	NM	NM	9.2	100	[75]
Plasma	MSP	RARbeta2	52	51	26 (healthy controls)	18	NM	NM	NM	NM	70	63	[76]
Plasma	AQAMA	SHP1P2	38	92	52 (age- and sex-matched healthy controls)	38	<700 pg/ml, 5.2 >700 pg/ml, 2.6	NM	<700 pg/ml, 12.6 >700 pg/ml, 7.6	NM	89.5	90.4	[77]
Plasma	MSP	DLEC1	78	35.9	50 (healthy controls and patients with benign diseases)	2	NM	NM	NM	NM	84.4	97.8	[78]
Plasma	MSP	APC CDH13 KLK10 DLEC1 RASSF1A EFEMP1 SFRP1 RARbeta p16	110	47.27 33.64 29.09 25.45 36.36 21.82 23.64 20.0 22.73	50 (healthy controls and patients with benign diseases)	10.0 4.0 4.0 2.0 8.0 6.0 4.0 6.0 8.0	NM	NM	NM	NM	NM	NM	[73]
Plasma	MSP	SFRP1	78	28.2	50 (healthy controls and patients with benign diseases)	4.0	NM	NM	NM	NM	NM	NM	[79]
Serum	qMSP (b-Actin)	DAPK MGMT	76	68.4 7.9	NA	ND	NM	NM	NM	NM	NM	NM	[80]
Serum	nested MSP	APC1A DAPK FHIT p14(ARF) p16 RARbeta RASSF1A	92	30.8 26.1 47.3 30.4 25.9 44.6 33.3	14 (healthy controls)	0.0 0.0 0.0 0.0 0.0 14.3 0.0	NM	NM	8.6 11.6 9.7 12.9 8.2 12.0 12.0	11.8 10.5 11.3 9.0 11.2 8.8 10.5	NM	NM	[74]
Serum	MSP	CHFR	308	32.4	NA	ND	NM	NM	11.50 (chemotherapy) 14.12 (EGFR TKIs)	11.21 (chemotherapy) 21.36 (EGFR TKIs)	NM	NM	[47]
Plasma	MSP	KLK10	78	38.7	50 (healthy controls and patients with benign diseases)	4.0	NM	NM	NM	NM	80.6	97.6	[81]

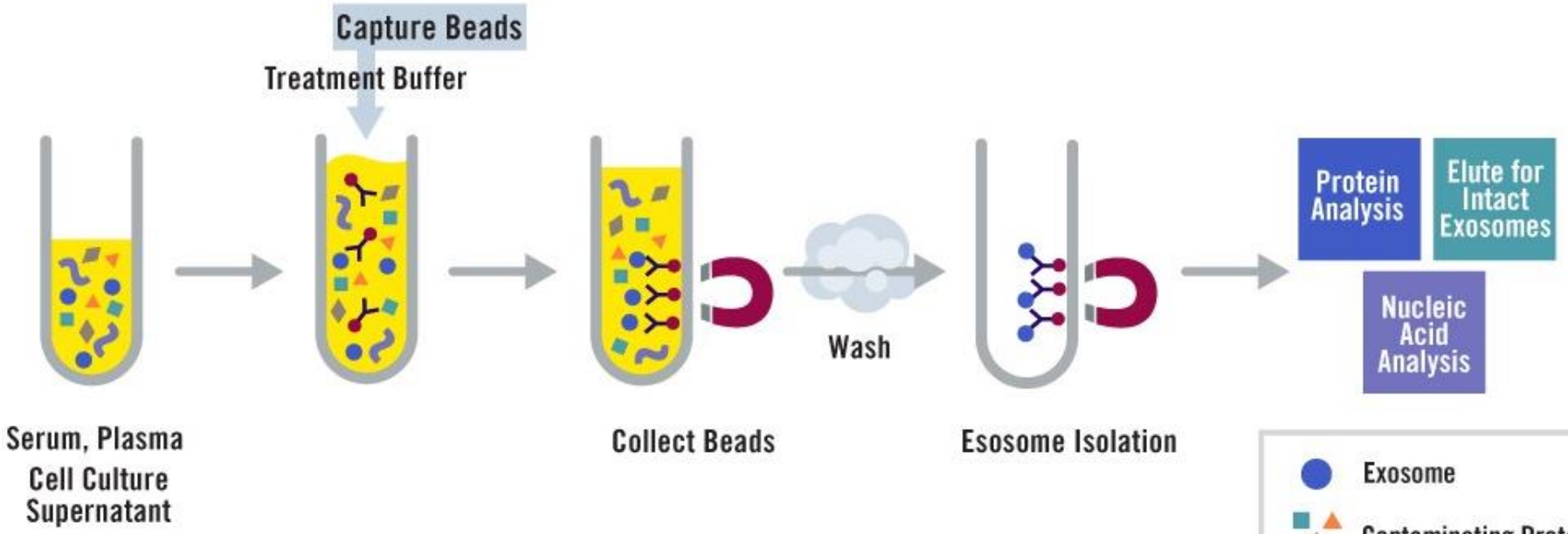
Nie et al. Tumor Biol. 2015

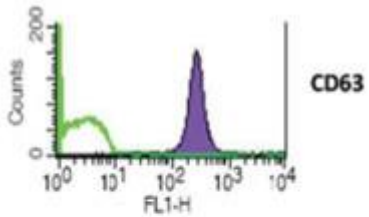
Exosomes, ctRNA, miRNA

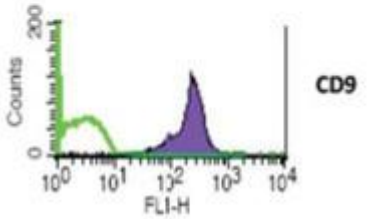


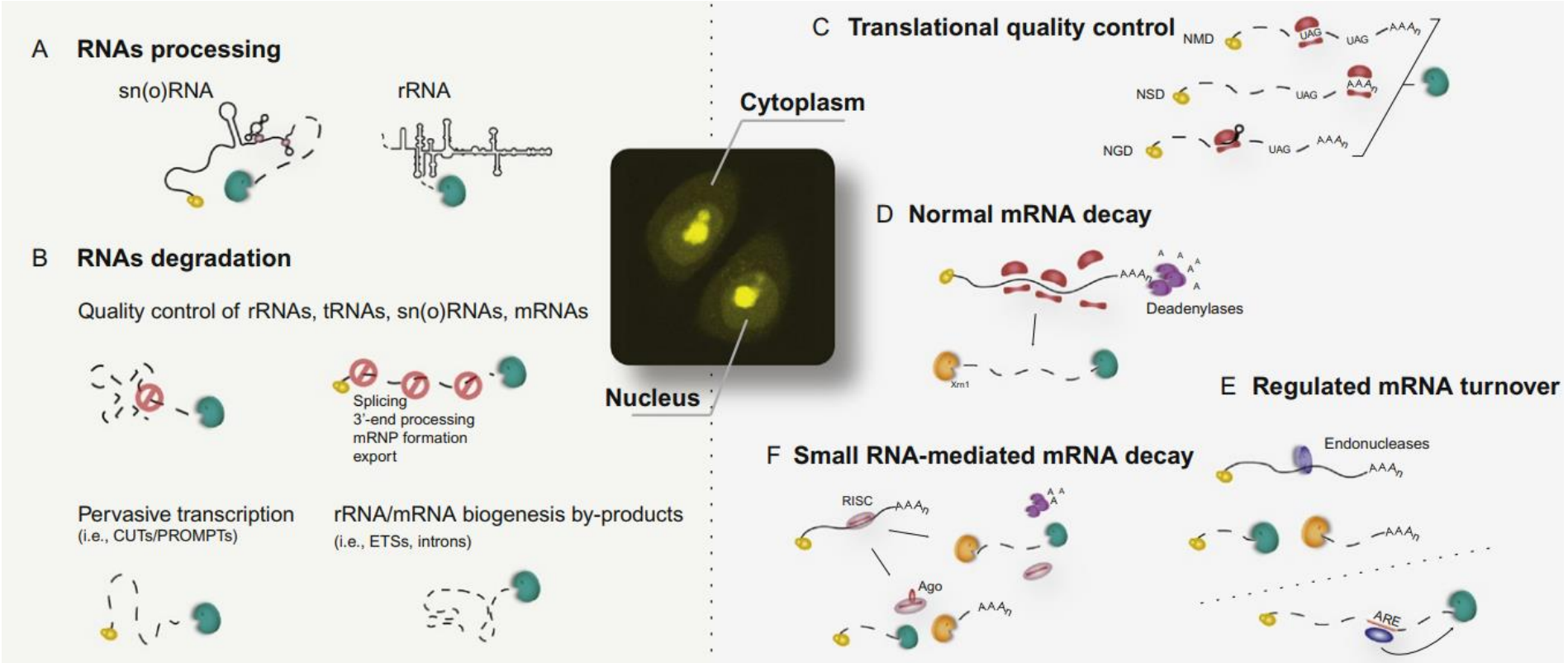


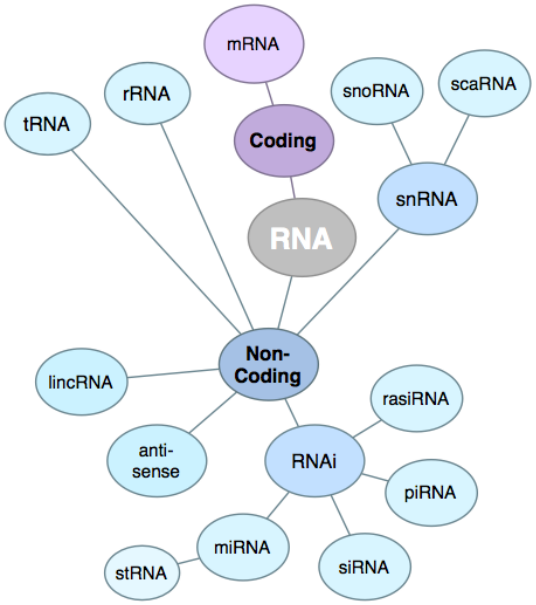
Isolation of Exosome





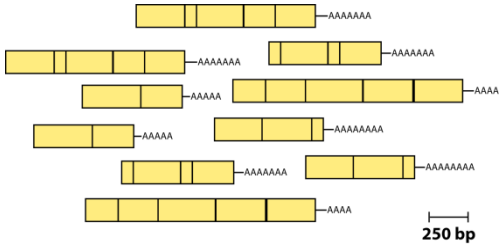



A RNAs processing
sn(o)RNA
rRNA
B RNAs degradation
Quality control of rRNAs, tRNAs, sn(o)RNAs, mRNAs
Splicing
3'-end processing
mRNP formation
export
Pervasive transcription
(i.e., CUTs/PROMPTs)
rRNA/mRNA biogenesis by-products
(i.e., ETSs, introns)
Cytoplasm
Nucleus
C Translational quality control
NMD
NSD
NGD
UAG
D Normal mRNA decay
Deadenylases
Xrn1
E Regulated mRNA turnover
Endonucleases
ARE
F Small RNA-mediated mRNA decay
RISC
Ago

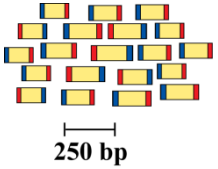

mRNA
rRNA
tRNA
Coding
snoRNA
scaRNA
snRNA
RNA
Non-
Coding
lincRNA
anti-
sense
RNAi
rasiRNA
piRNA
miRNA
siRNA
stRNA

Conceptual overview of RNA-sequencing (RNA-Seq)

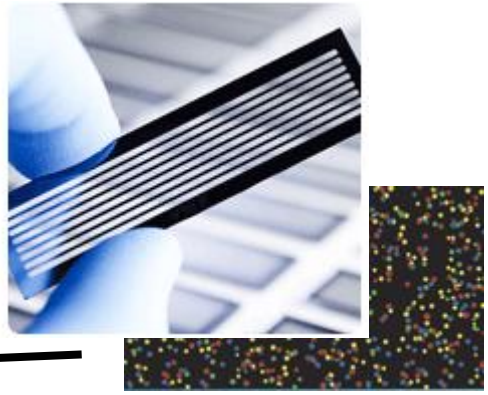
Isolate RNAs



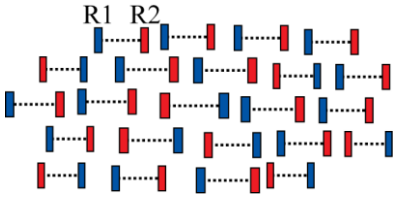
Generate cDNA, fragment, size select, add linkers



Sequence ends



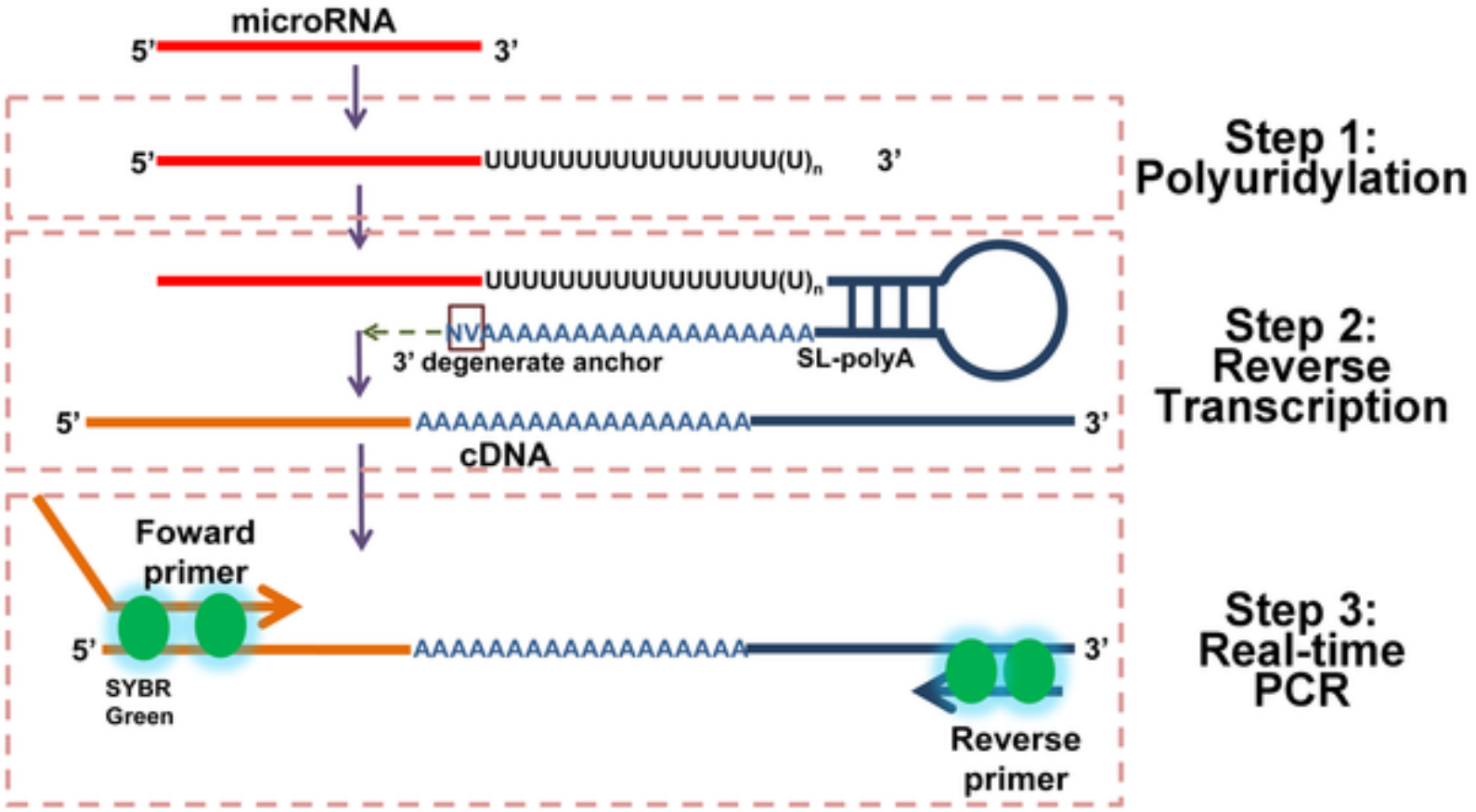
100s of millions of paired reads
10s of billions bases of sequence



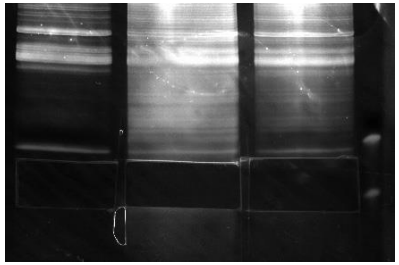
Map to genome, transcriptome, and predicted exon junctions

Downstream analysis

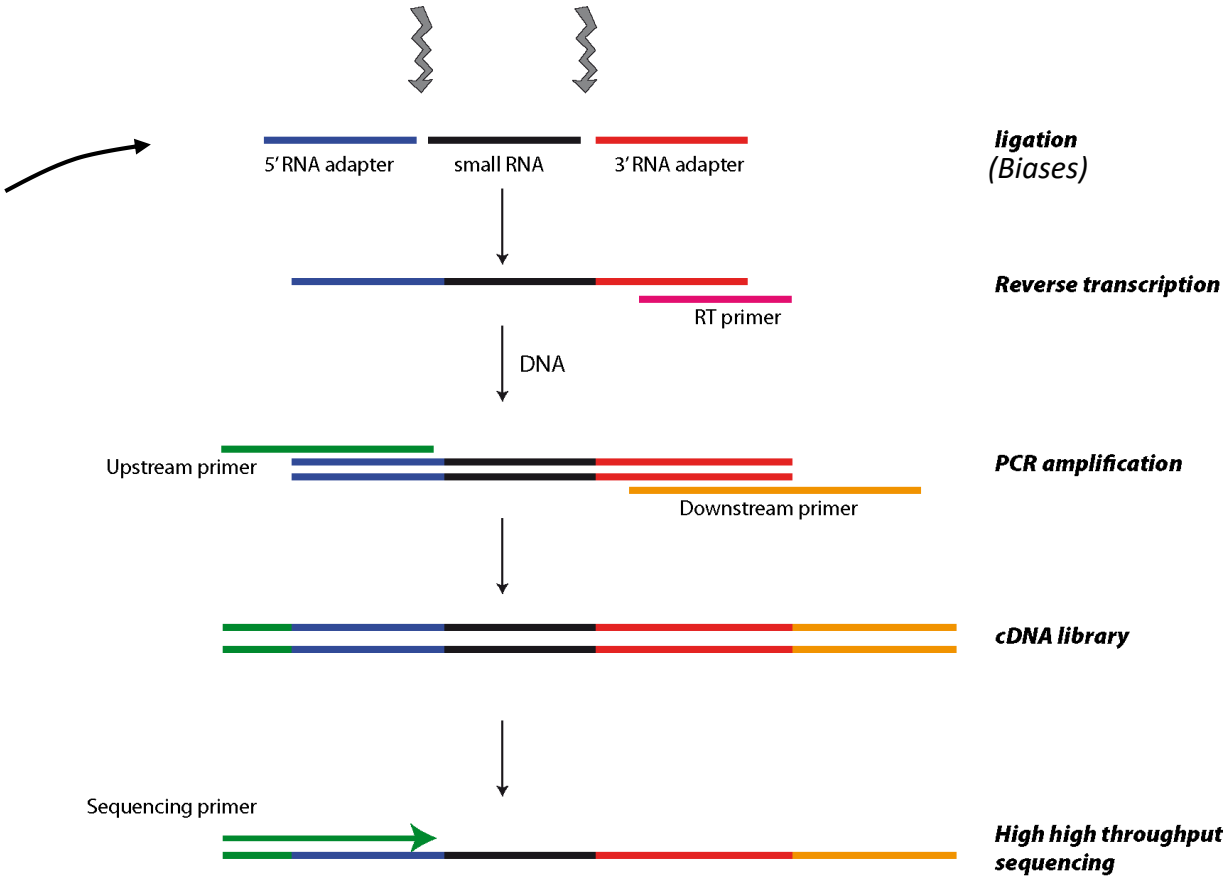
miRNA Real-time PCR



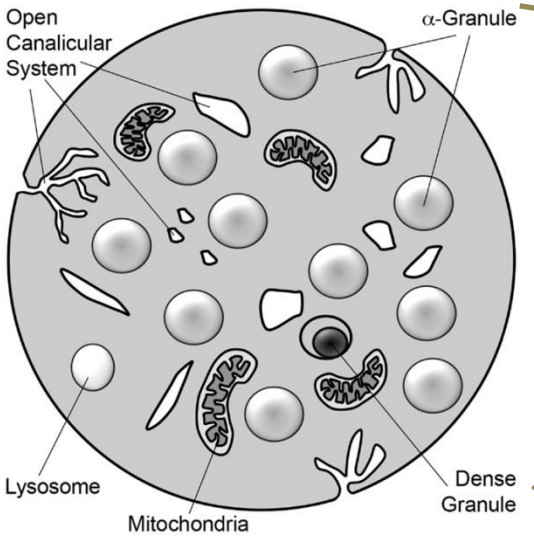
Small RNA sequencing



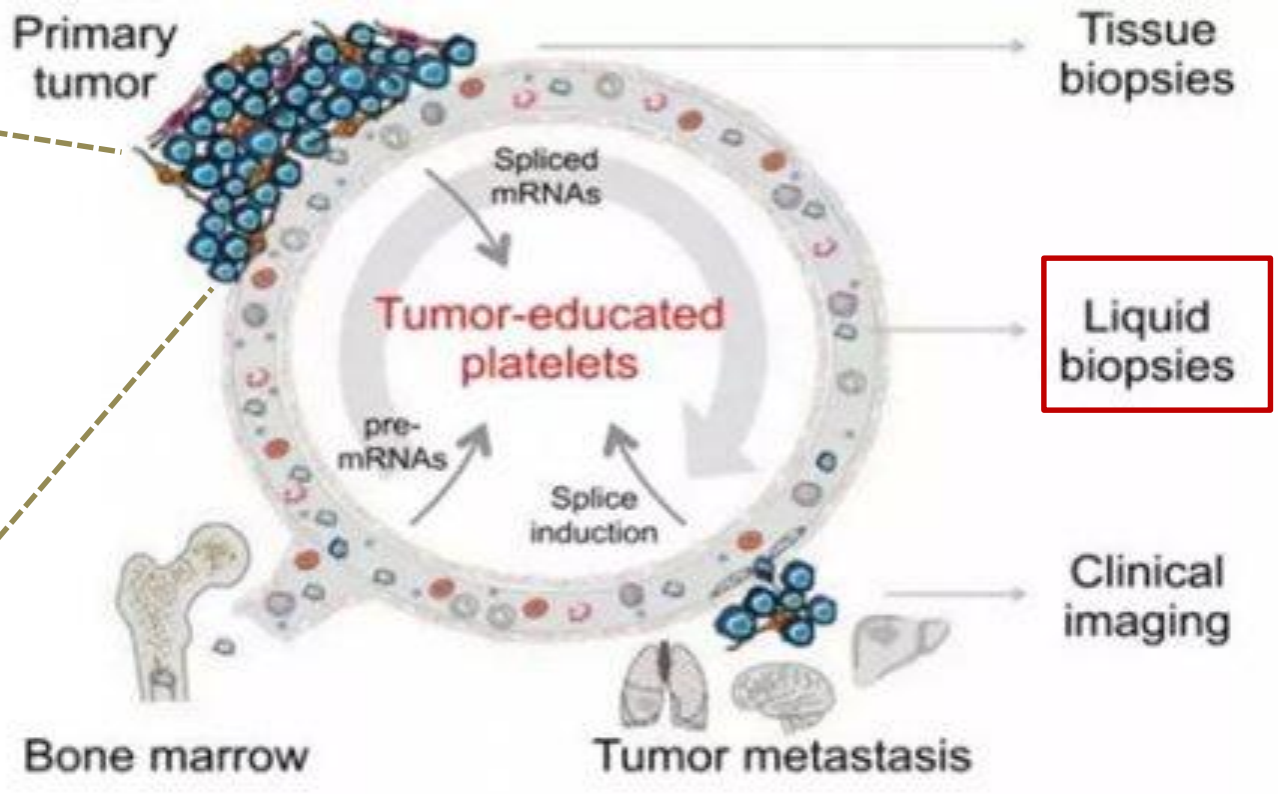
20-30nt RNA gel purification

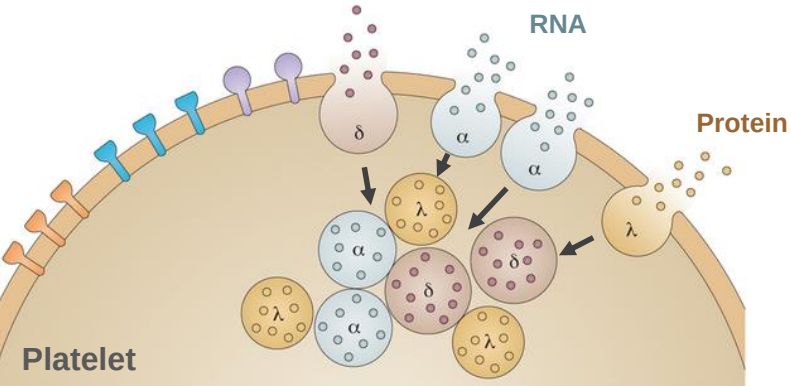


Tumor-educated platelets



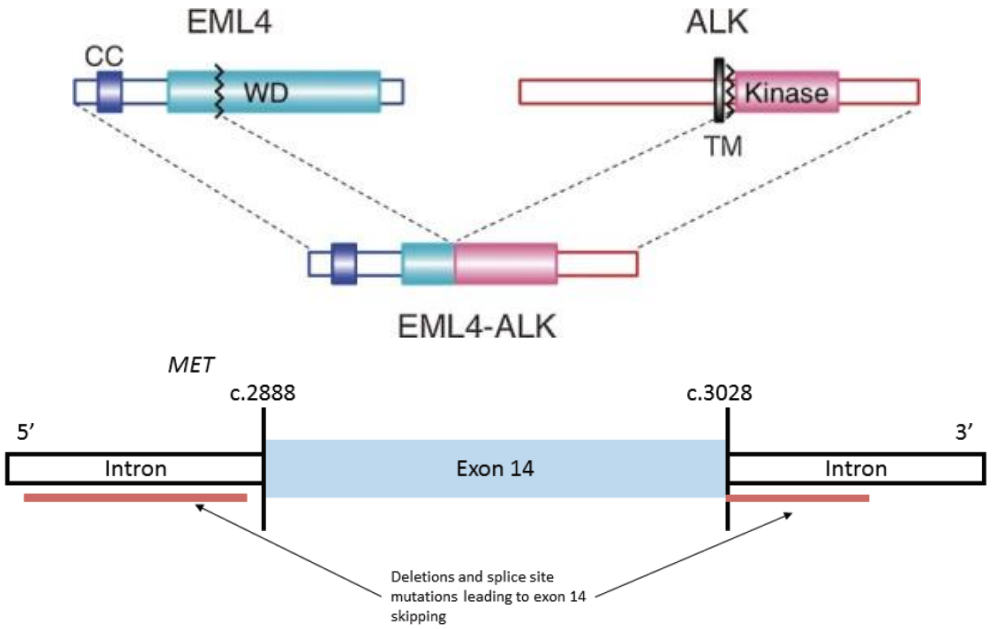
Platelet





Detecting RNA fusion in tumor-educated platelets

	EML4-ALK Rearrangement detected in Plasma	EML4-ALK Rearrangement detected in Platelets
NSCLC patients	*N* = 32*	*N* = 67*
EML4-ALK-rearranged tumor	3/14	22/34
EML4-ALK rearrangement not detected in tumor	0/18	0/33
Healthy controls	nd	0/21
Sensitivity	21%	65%
Specificity	100%	100%
Accuracy	66%	86%



Nilsson et al. Oncotarget. 2016

Summary

- Blood-based tumor diagnostics are evolving rapidly and recent studies show promising results.
- Blood has become an attractive specimen for diagnosis and therapeutic monitoring of various tumors.
- Technical optimization, further validation, standardization and practical application may be increase the utility of blood-based methods.