



Washington University in St. Louis

SCHOOL OF MEDICINE



ctDNA analysis

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Division of Cancer Biology

Department of Radiation Oncology

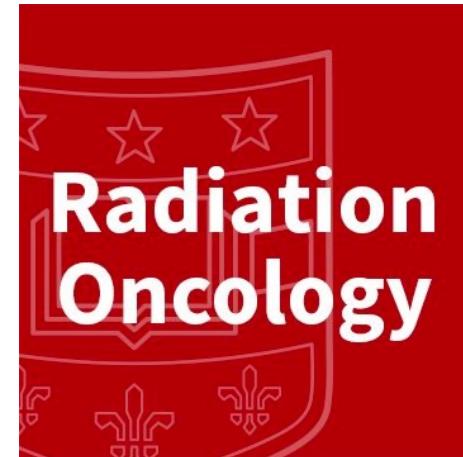
Department of Genetics

Dept of Computer Science & Engineering

WashU

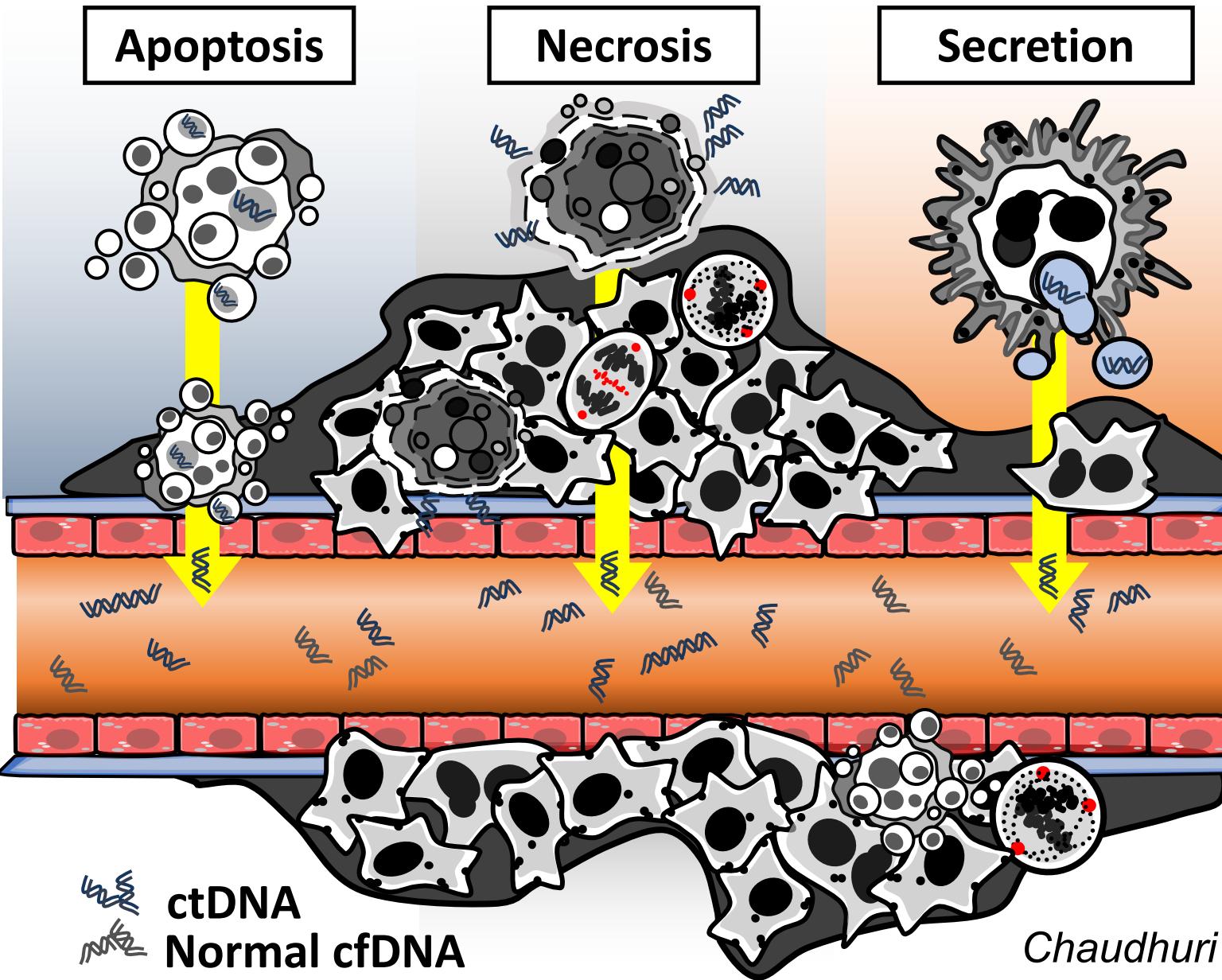
ENGINEERING

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Division of Cancer Biology

Cell-free circulating tumor DNA



ctDNA = circulating tumor DNA

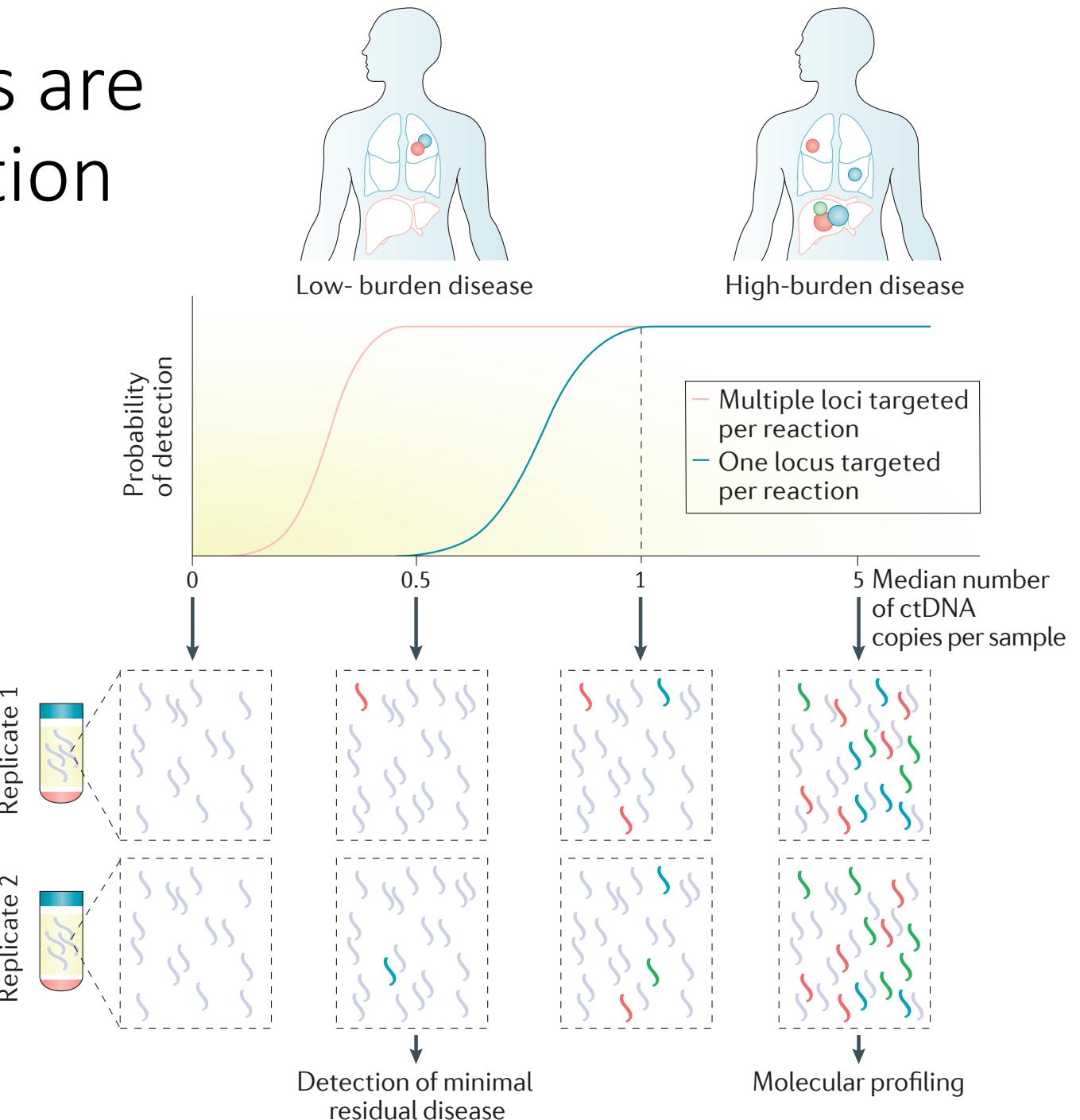
- At low levels in cell-free DNA
- A biomarker of tumor burden
- Biomarker of residual disease

MRD = Minimal Residual Disease

- Small volume of tumor cells after treatment in the absence of clinical evidence of disease

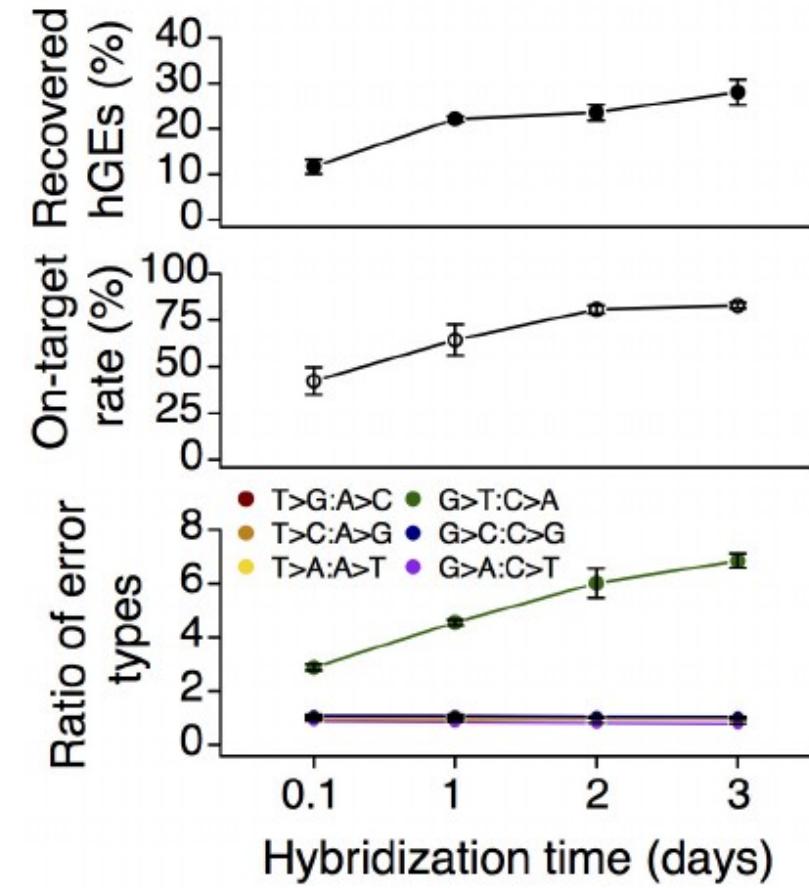
Technique	Example technologies	Scale of analysis	Method	Detection limit (% of cfDNA)	Cost	Assay personalization	Advantages	Limitations
AS-PCR	ARMS [112]	Single-locus or multiplexed assays	Preferentially amplifies rare mutant DNA molecules	~0.1–1	\$	Some required	Ease of use; ideal for detecting recurrent ‘hotspot’ point mutations	Can only query small number of variants concurrently; cannot detect mutations not known a priori
dPCR	dPCR [117] ddPCR [119, 125–127] BEAMing [128, 129]	Single-locus or multiplexed assays	Partitions target DNA into different reactions for massively parallel qPCR	~0.01	\$\$	Some required	High sensitivity	Can only query small number of variants concurrently; cannot detect mutations not known a priori
WGS	WGS [143, 144] Plasma-Seq [139, 140] PARE [145]	Genome-wide	NGS of whole genome	~10	\$\$–\$\$\$	Not required	Entire genome is interrogated	Low sensitivity; mostly limited to SCNA detection
Retrotransposon-based amplicon NGS	FAST-SeqS [141] mFAST-SeqS [152] WALDO [142]	Genome-wide retrotransposon sites	PCR amplification of retrotransposon insertion sites prior to NGS analysis	~5	\$\$	Not required	Rapid aneuploidy assessment with lower cost than WGS	Limited to aneuploidy detection
WES	WES [79]	Exome-wide	NGS of whole exome	~5	\$\$\$	Not required	Entire exome is interrogated	Low sensitivity
Multiplex PCR-based NGS	TAm-Seq [155] Enhanced TAm-Seq [156] Safe-SeqS [111] Natera® [64]	Targeted sequencing	PCR amplification enriches targets prior to NGS analysis	~0.01–2.0	\$\$	Some required	High sensitivity (modern methods)	Less comprehensive than other NGS methods; unable to detect SCNAs; unable to detect fusions without assay personalization
Hybrid Capture-based NGS	CAPP-Seq [70, 84] TEC-Seq [73] Guardant360® [161, 162] FoundationOne® Liquid [163]	Targeted sequencing	Subset of exome is hybridized to biotinylated probes and captured for NGS analysis	~0.001–0.5	\$\$	Not required	High sensitivity; detects multiple mutation types; broadly applicable without personalization	Less comprehensive than WGS or WES
Combination approaches	CAPP-Seq + GRP [149] CancerSEEK [169] UroSEEK [7]	Single-locus to genome-wide	Combines different ctDNA detection methods, sometimes including protein biomarkers	Variable	\$\$–\$\$\$	Variable	Improved detection compared to standard ctDNA analysis alone in certain settings	Potentially more time and resource intensive

Highly Sensitive Methods are Required for MRD detection



NGS Technical Artifacts

- Confound variant calling for SNVs
- Overall error rates 0.1-1%
- Types:
 - PCR polymerase errors
 - Oxidative damage (especially with long hybridization steps)
 - Errors in bridge amplification
 - Phasing

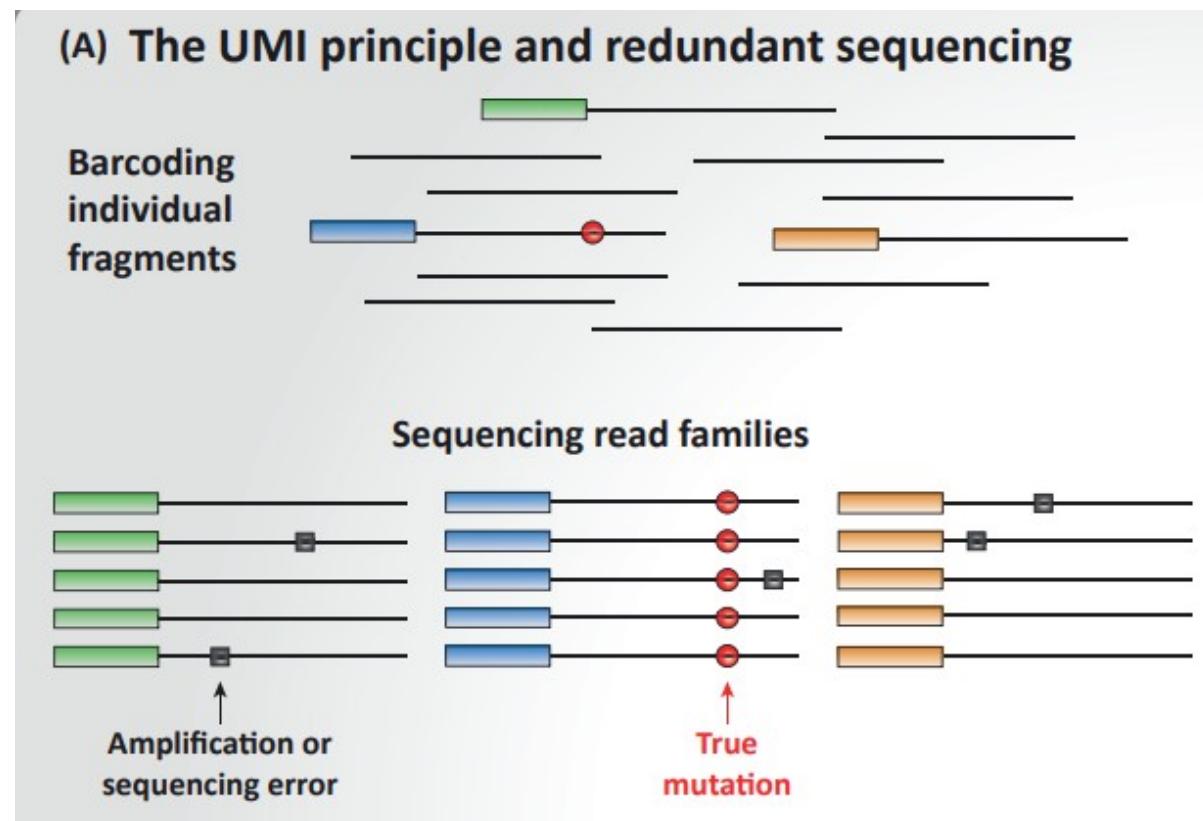


NGS Error Correction Strategies

- Unique molecular identifiers (UMIs)
- Duplexing
- Current state-of-art, post-duplexing
 - iDES
 - Singleton error correction

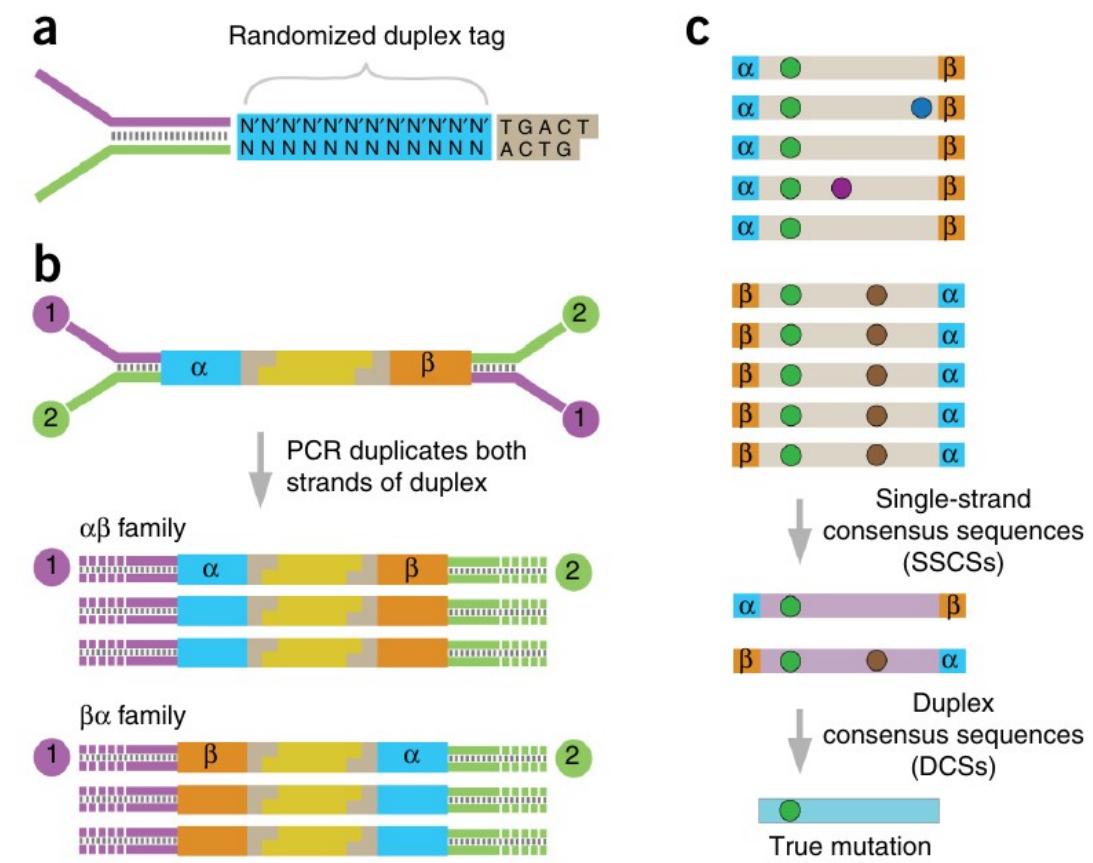
Unique Molecular Identifiers

- Nucleotide sequence that specifically identifies all library molecules derived from a single fragment present in the initial DNA sample
- UMIs are used to assemble read families and call single-stranded consensus sequence (SSCS)
- Strategy is dependent on high numbers of redundant reads (i.e. inefficient)



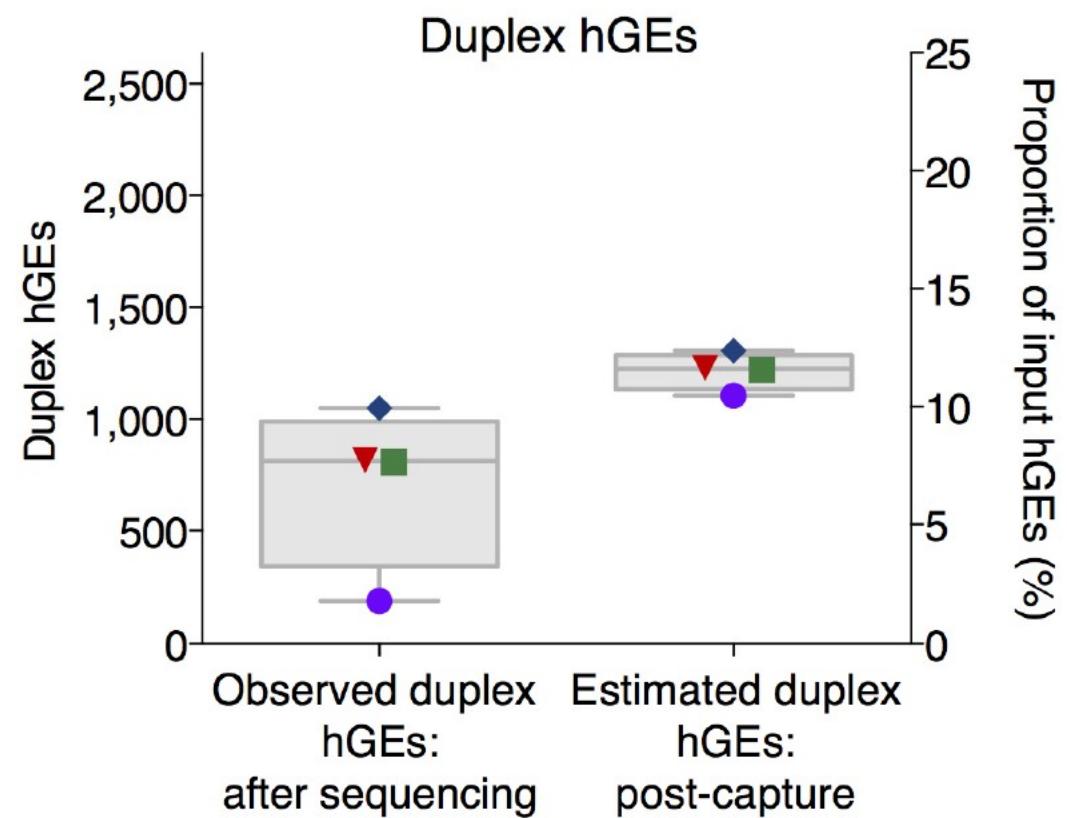
Duplex Sequencing

- Adapter design which can identify both strands of an original DNA molecule
- SSCS identified by adapter order (e.g. $\alpha\beta$ or $\beta\alpha$)
- True SNVs are present across duplex consensus sequences (DCS)
- Even more inefficient, requires
 - Duplexd hGEs
 - PCR duplication



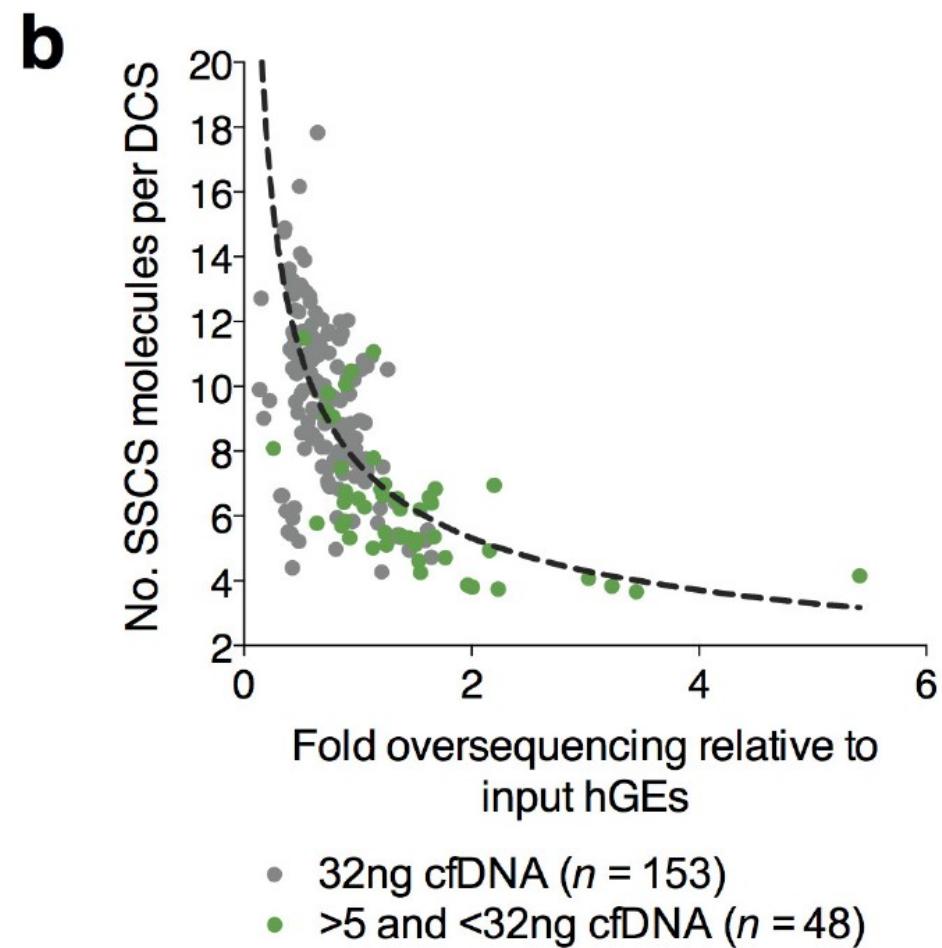
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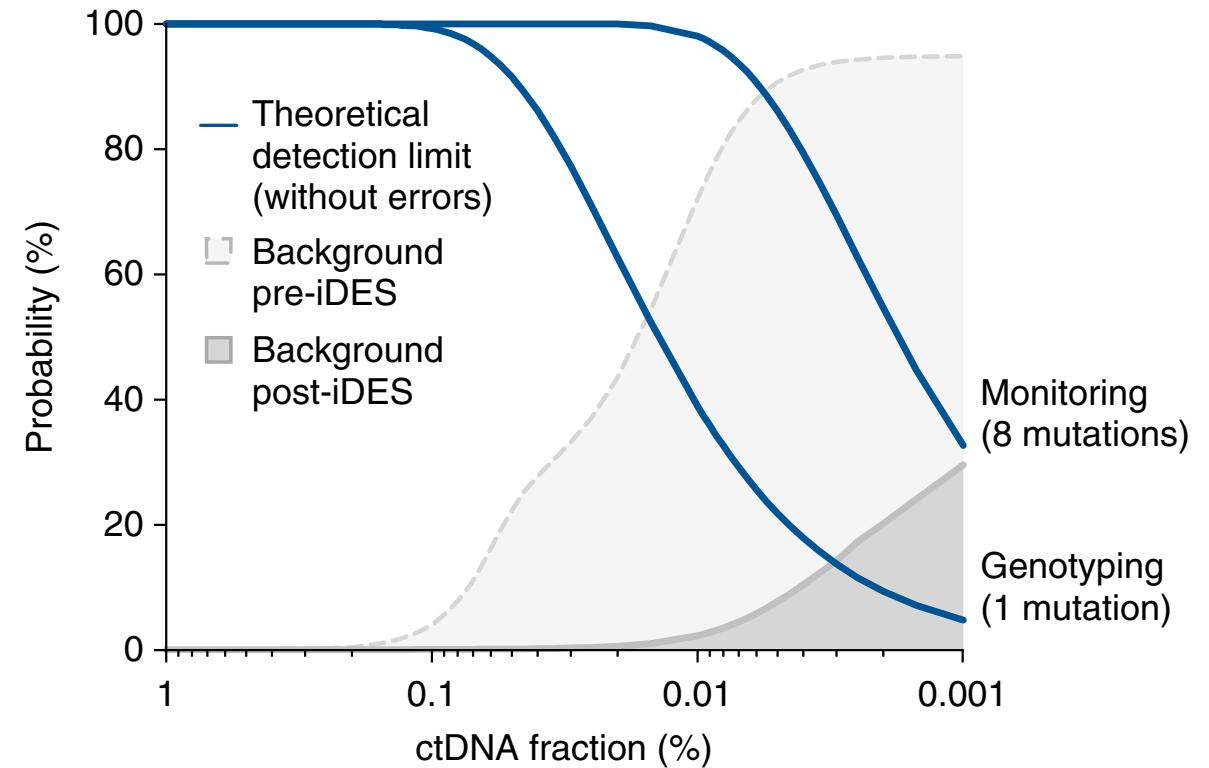
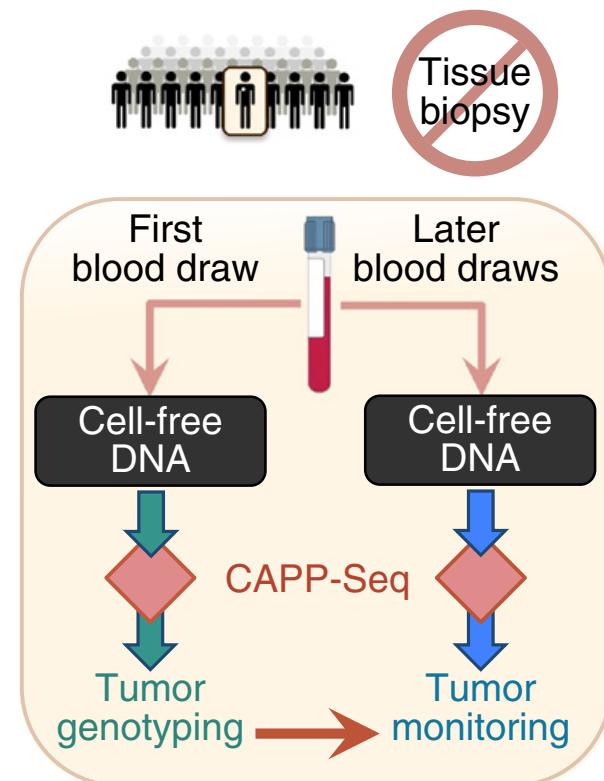
CAPP-Seq with integrated digital error suppression (iDES)

Ultra-low limit of detection and off-the-shelf workflow that doesn't require patient customization.

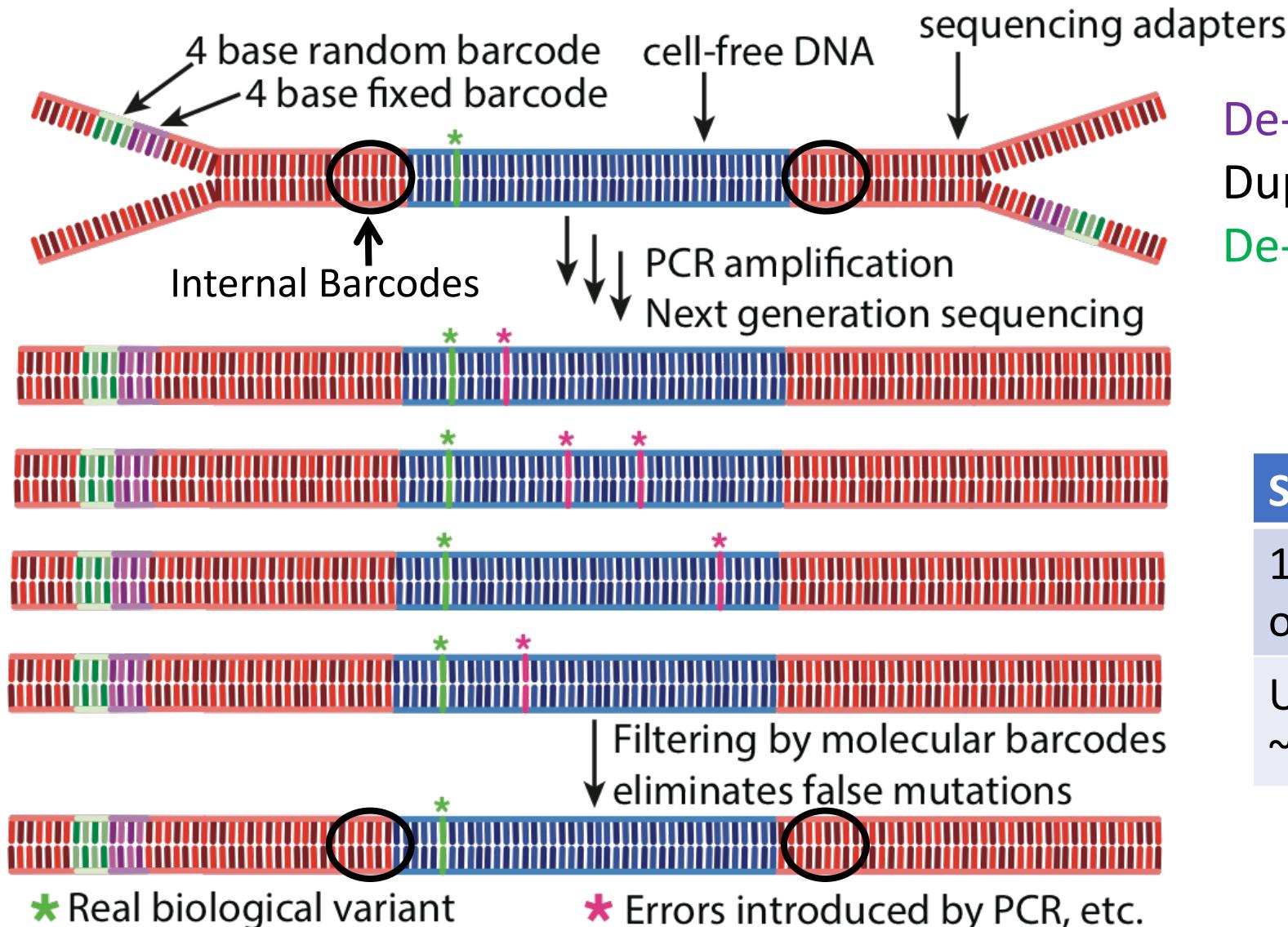
Noninvasive genotyping doesn't require tumor.

Integrated digital error suppression for improved detection of circulating tumor DNA

Aaron M Newman^{1,2,10}, Alexander F Lovejoy^{1,3,4,9,10}, Daniel M Klass^{1,2,4,9,10}, David M Kurtz^{1,2,5}, Jacob J Chabon¹, Florian Scherer², Henning Stehr⁴, Chih Long Liu^{1,2}, Scott V Bratman^{1,3}, Carmen Say³, Li Zhou⁴, Justin N Carter³, Robert B West⁶, George W Sledge, Jr^{2,4}, Joseph B Shrager⁷, Billy W Loo Jr³, Joel W Neal², Heather A Wakelee², Maximilian Diehn^{1-3,11} & Ash A Alizadeh^{1,2,4,8,11}



Molecular Barcoding Strategy – Reduces background error rate

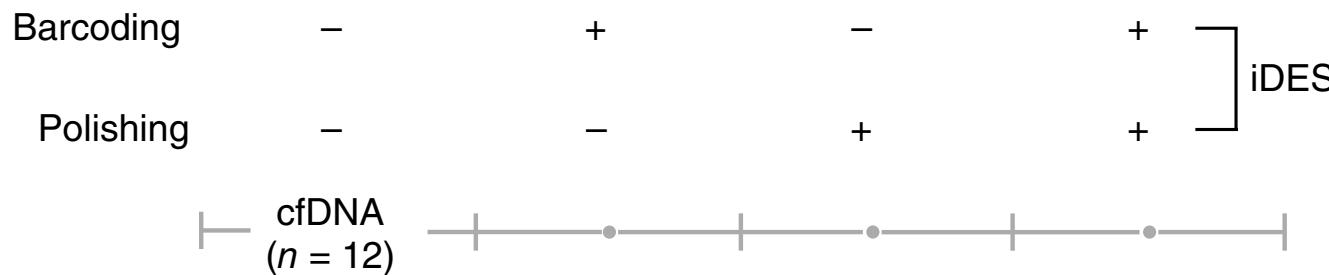


De-multiplex via fixed barcode
Duplex calling via internal barcode
De-duplicate via random barcode

Sample Sequencing

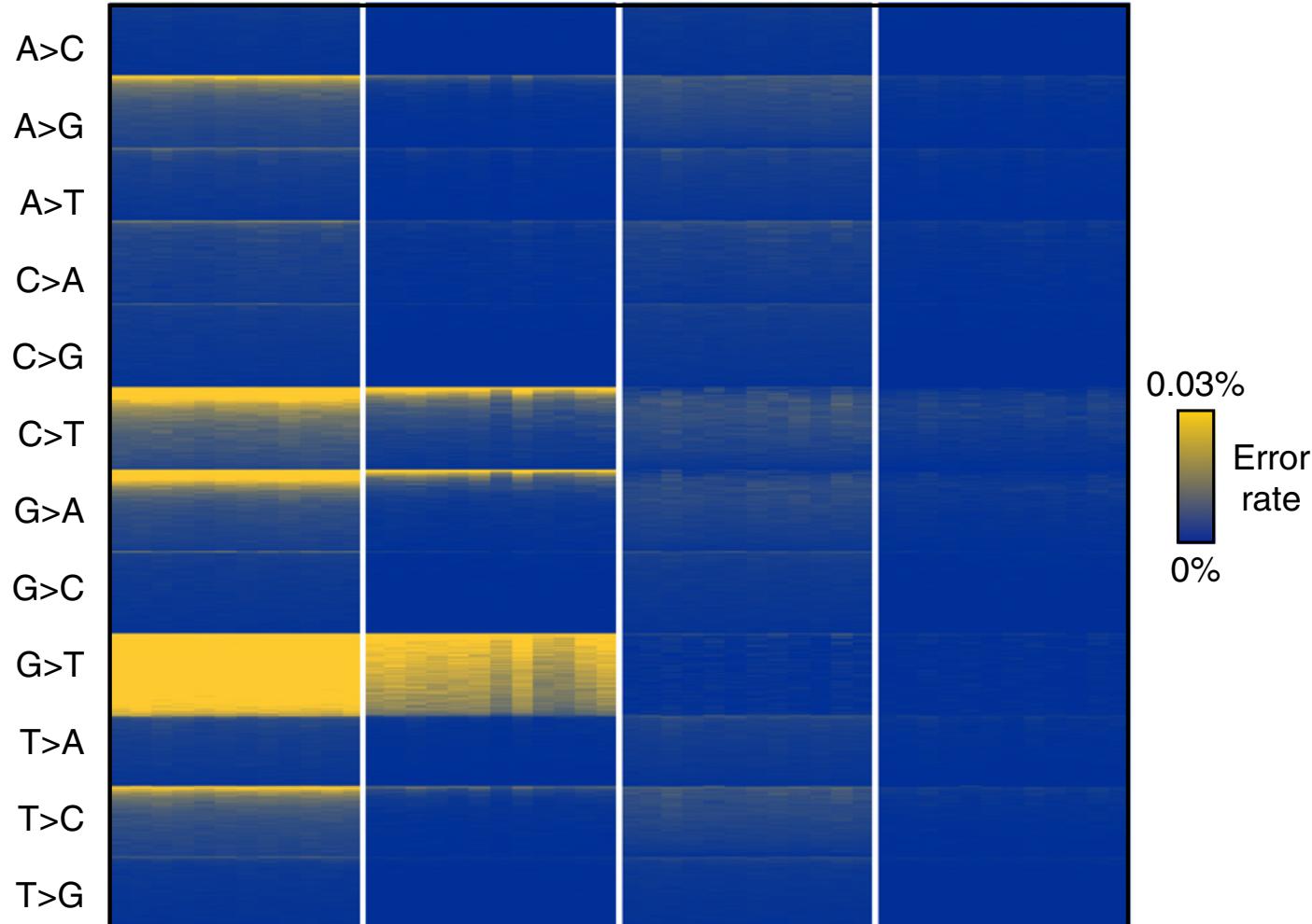
12-plex 2x150bp paired-end NGS
on HiSeq4000 lane

Unique depth: ~4000x across
~200kb NGS panel per sample

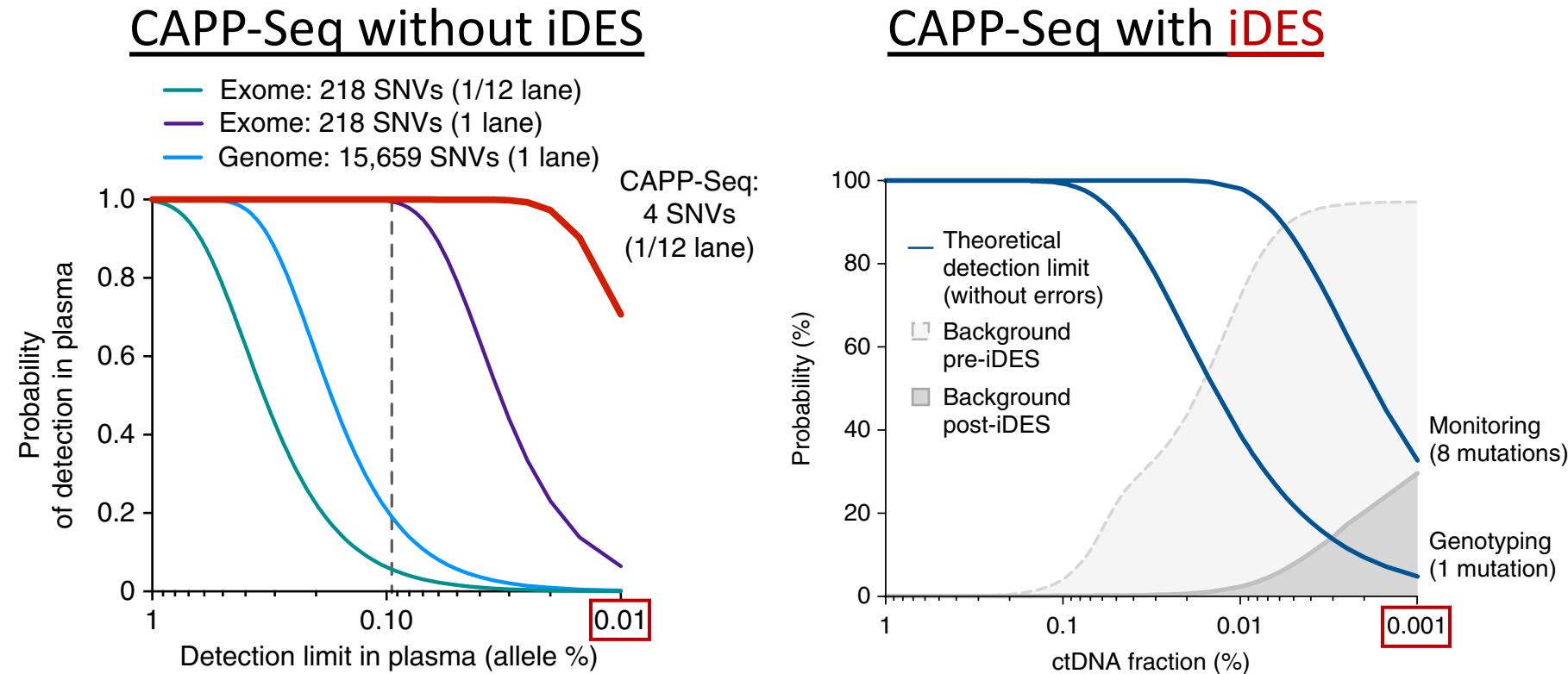


Background Polishing

Removes highly stereotypical background artifacts using CAPP-Seq data from healthy donors



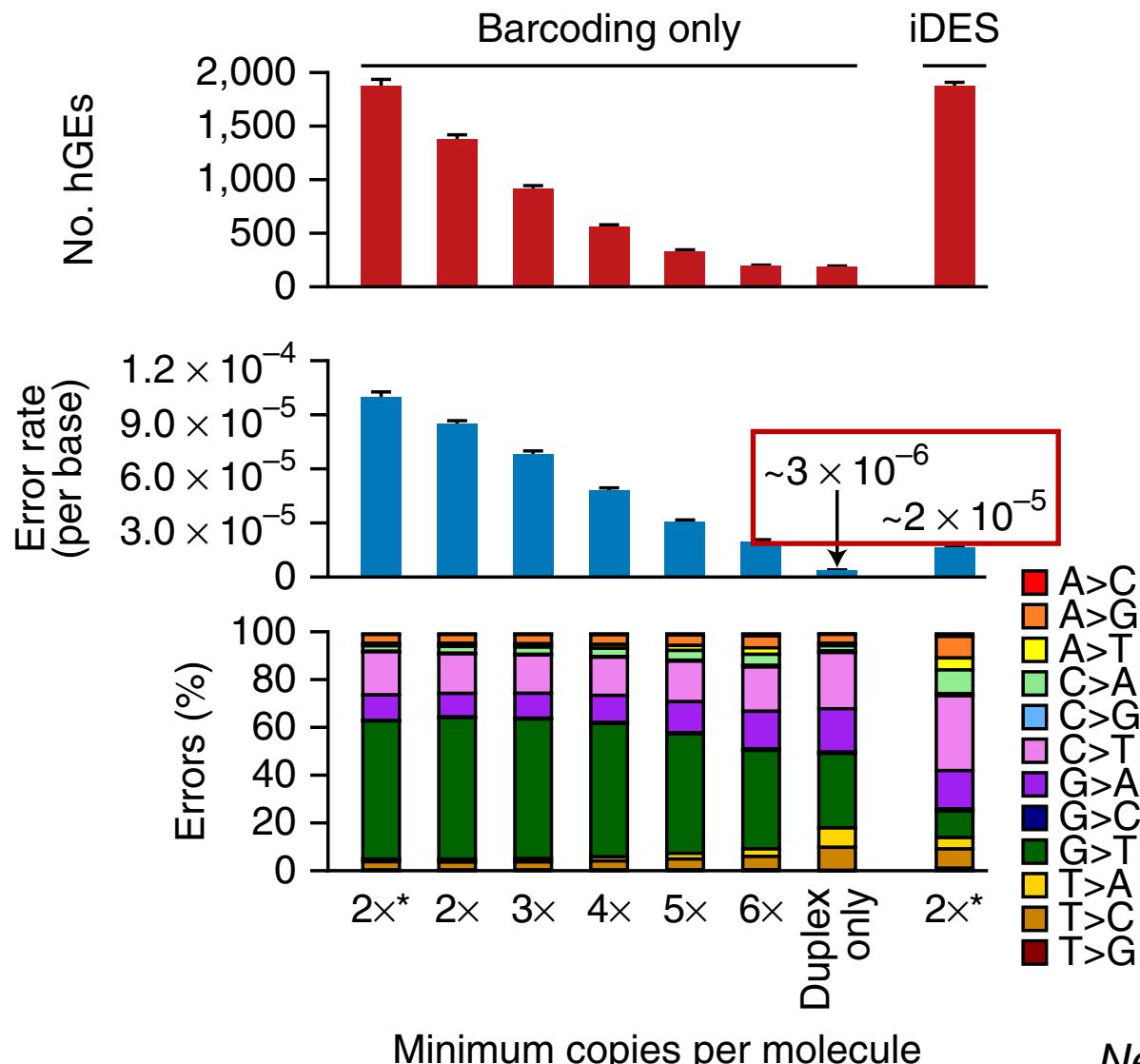
Integrated digital error suppression (iDES) lowers CAPP-Seq detection limit



Newman & Bratman et al, *Nature Medicine*, 2014

Newman, Lovejoy & Klass et al, *Nature Biotechnology*, 2016

Personalized selectors with duplex-only sequencing have potential for even lower detection limits



Duplex-only error rate is \sim 10-fold lower than iDES

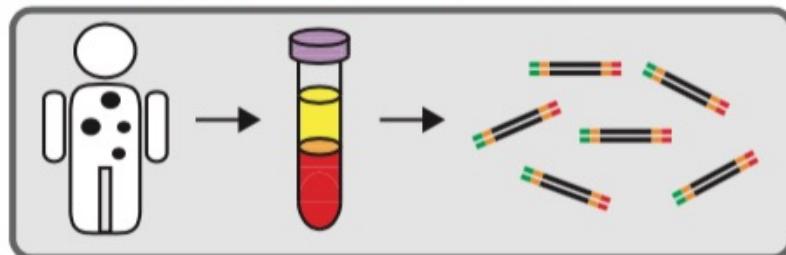
- Theoretical detection limit of \sim 3 parts per million
- Requires good recovery of duplex variants
 - Deeper sequencing
 - Personalized panels

Broad Institute - ichorCNA

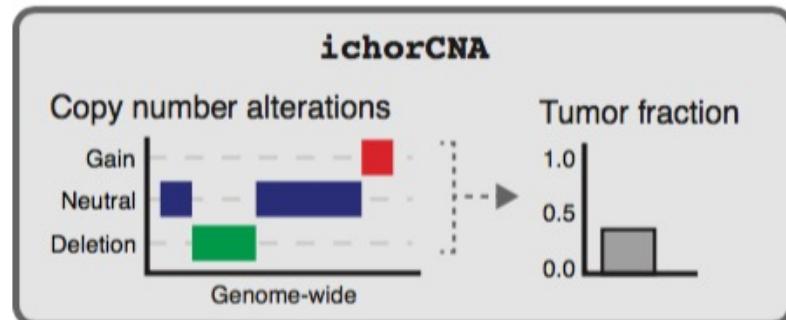
- ichorCNA is a tool for estimating the fraction of tumor in cell-free DNA from ultra-low-pass whole genome sequencing (ULP-WGS, 0.1x coverage)
- ichorCNA uses a probabilistic model, implemented as a hidden Markov model (HMM), to simultaneously segment the genome, predict large-scale copy number alterations, and estimate the tumor fraction of a ultra-low-pass whole genome sequencing sample (ULP-WGS). ichorCNA is optimized for low coverage (~0.1x) sequencing of samples and has been benchmarked using patient and healthy donor cfDNA samples.

Broad Institute - ichorCNA

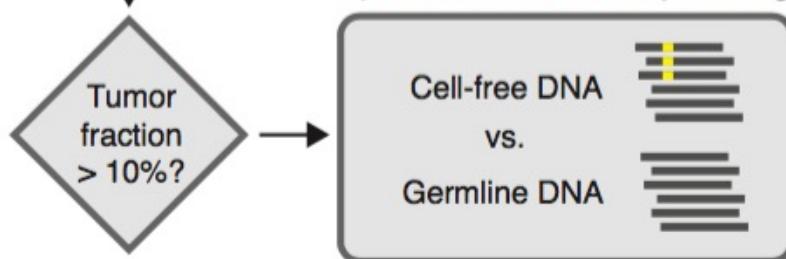
- ichorCNA can be used to inform the presence or absence of tumor-derived DNA and to guide the decision to perform whole exome or deeper whole genome sequencing. Furthermore, the quantitative estimate of tumor fraction can be used to calibrate the desired depth of sequencing to reach statistical power for identifying mutations in cell-free DNA. Finally, ichorCNA can be used to detect large-scale copy number alterations from large cohorts by taking advantage of the cost-effective approach of ultra-low-pass sequencing.

a
1) Cell-free DNA library construction

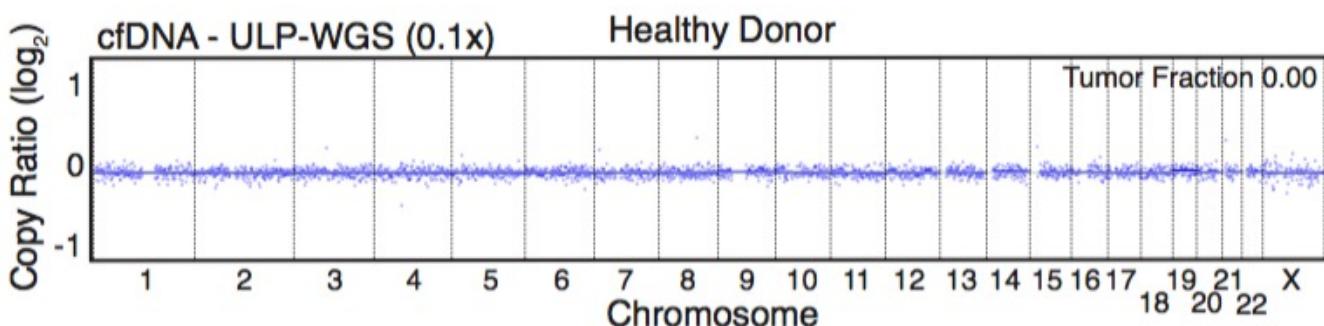
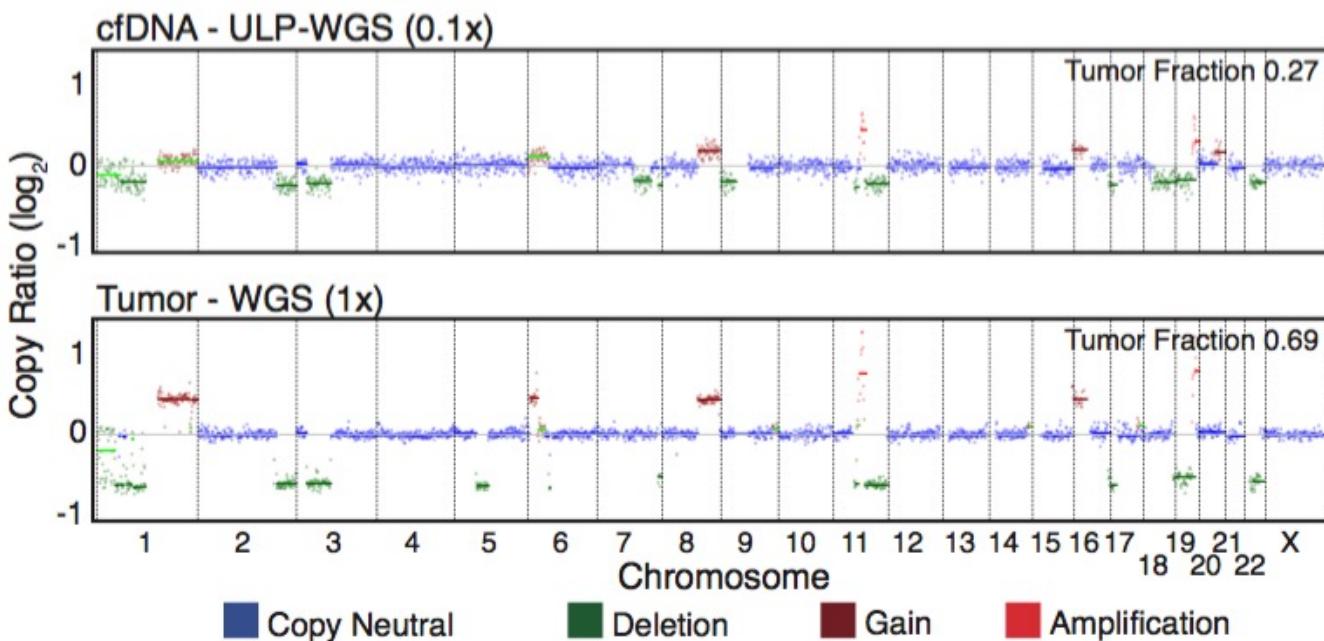
2) Ultra low-pass whole-genome sequencing (0.1x)



3) Whole-exome sequencing

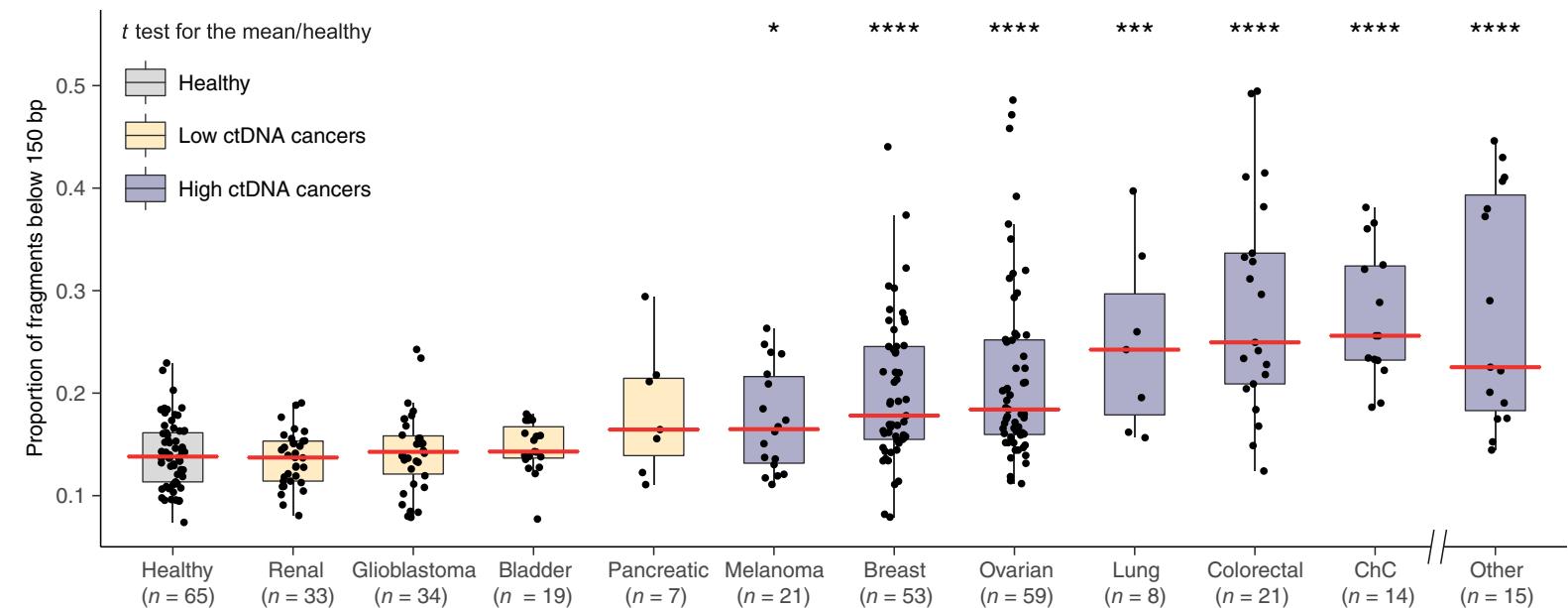
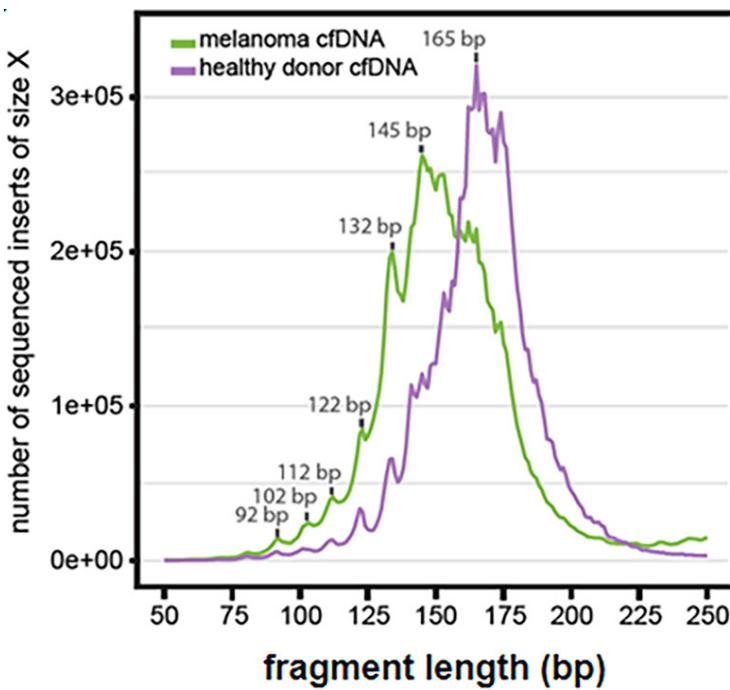


Application to large cohorts

**b****c**

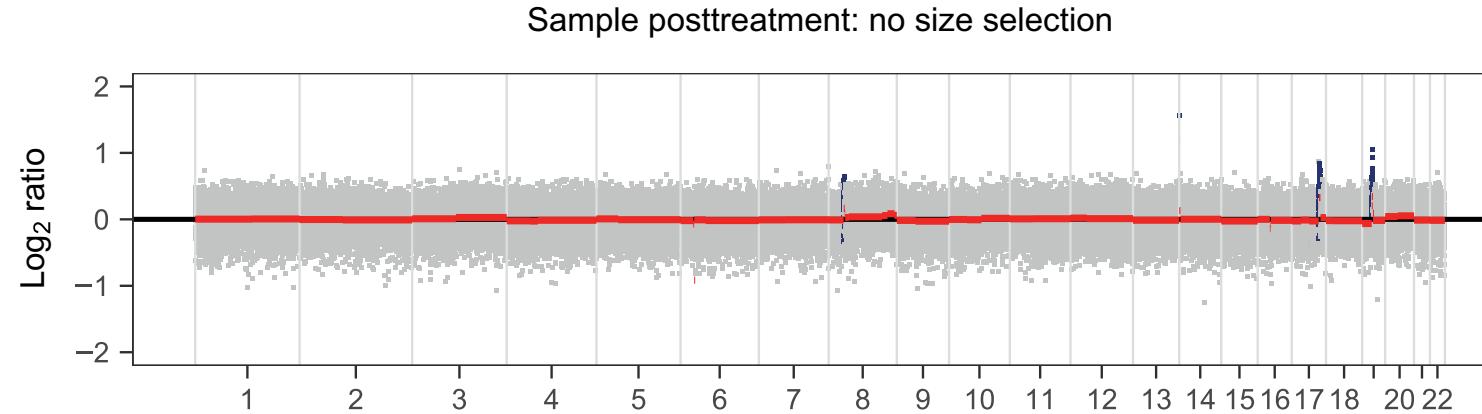
Ultra-low-pass WGS with Size Selection

ctDNA is shorter than healthy cfDNA



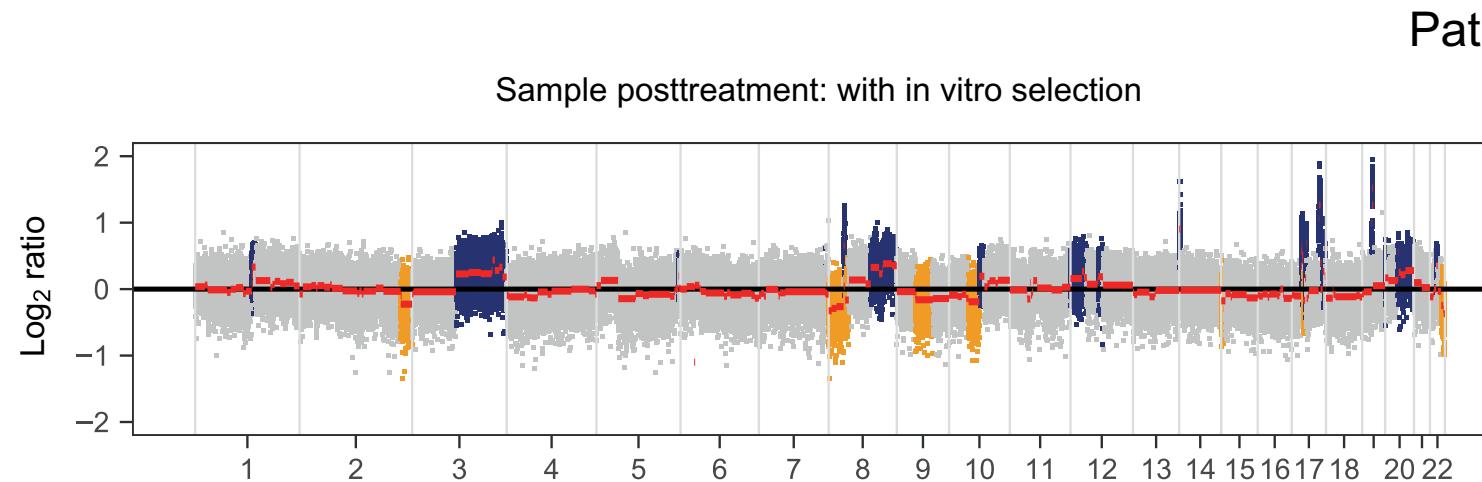
Underhill et al, PLoS Genetics, 2016
Mouliere et al, Science Translational Medicine, 2018

Size selection to select shorter fragments can significantly improve the sensitivity of ULP-WGS



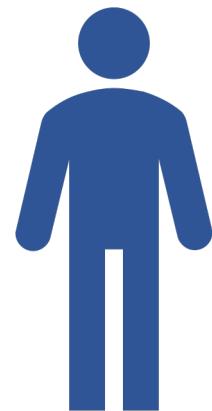
In vitro selection of short fragments between 90 and 150bp

Patient with Ovarian Cancer

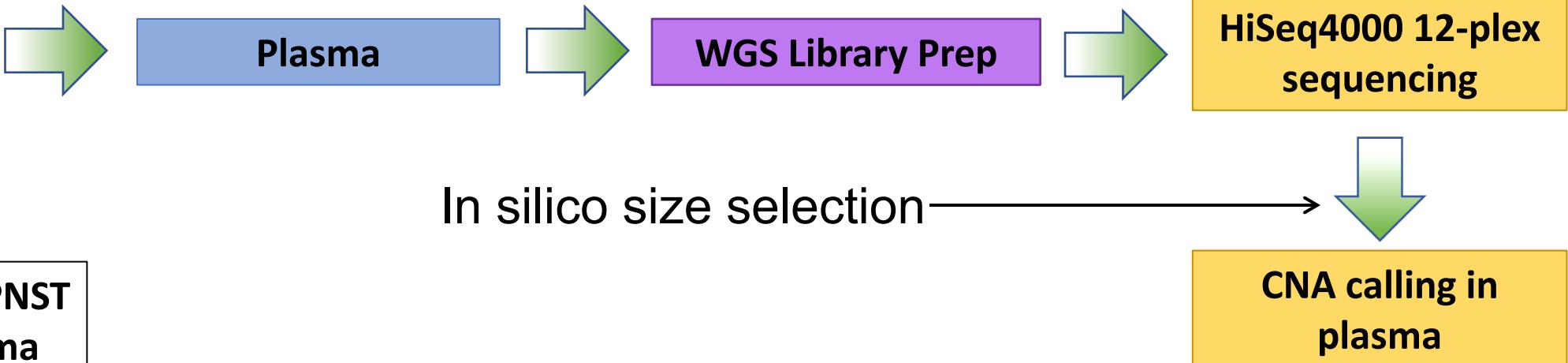


Cell-Free DNA Classification of MPNST vs. PN

Sample collection at
WashU and the NCI

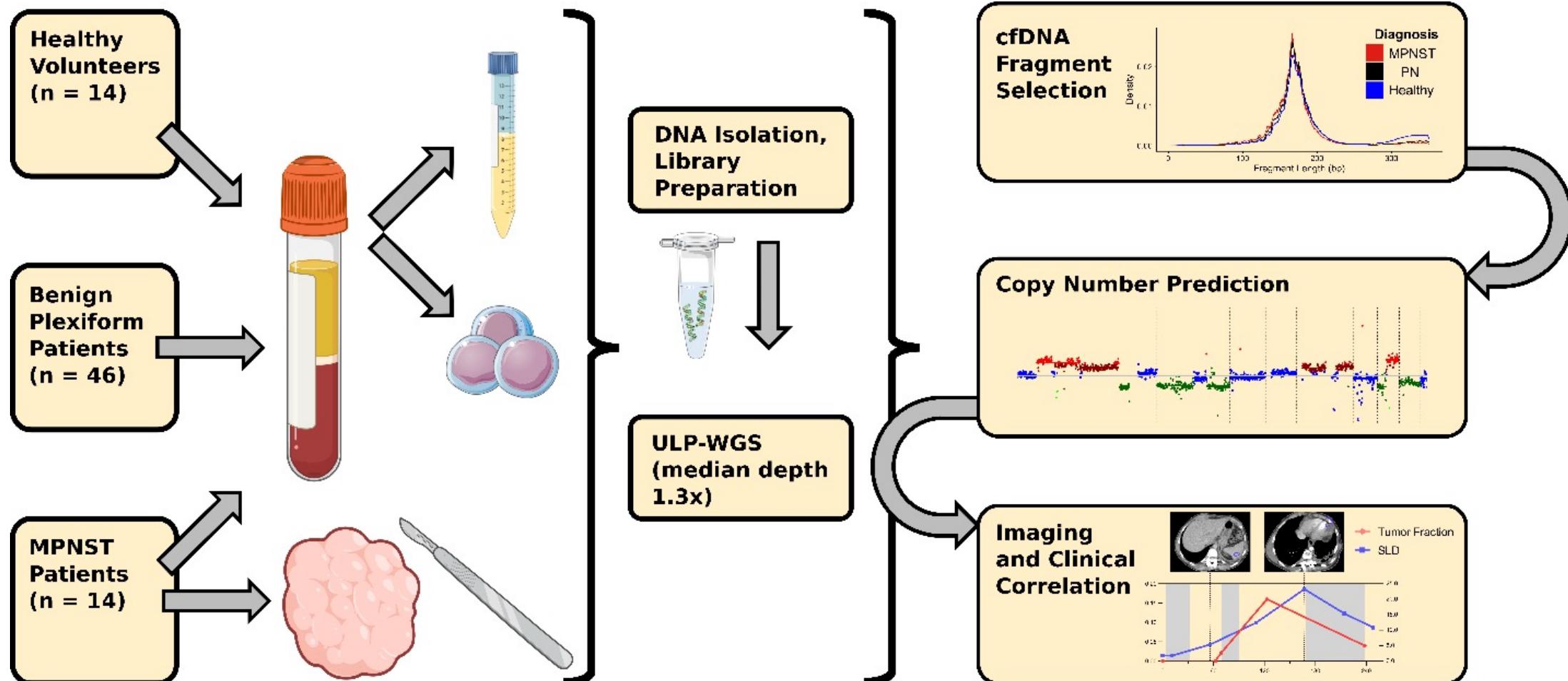


Patients with MPNST
vs. Neurofibroma

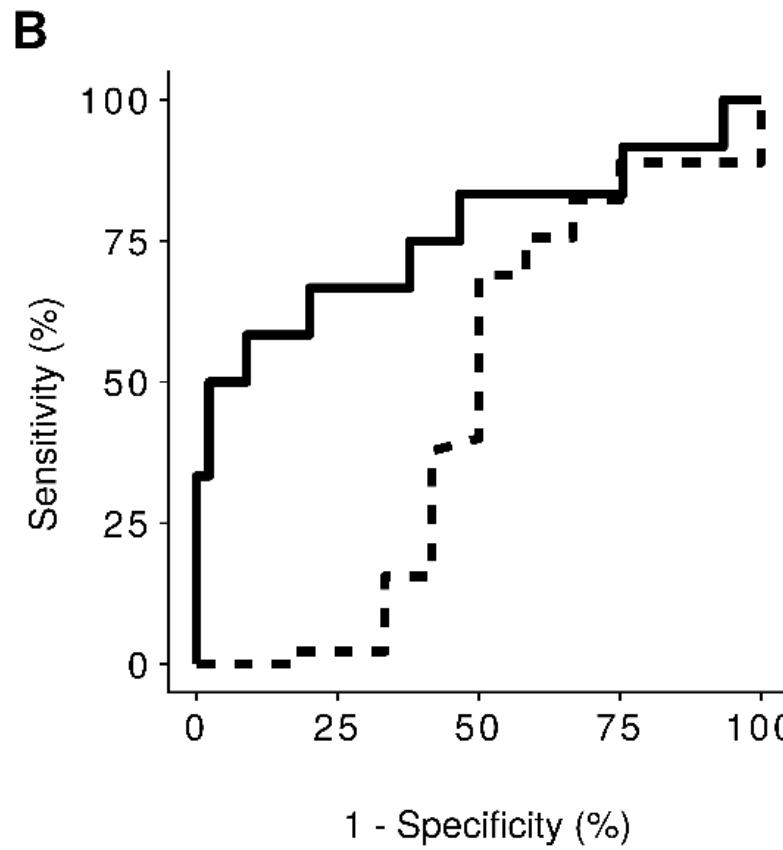
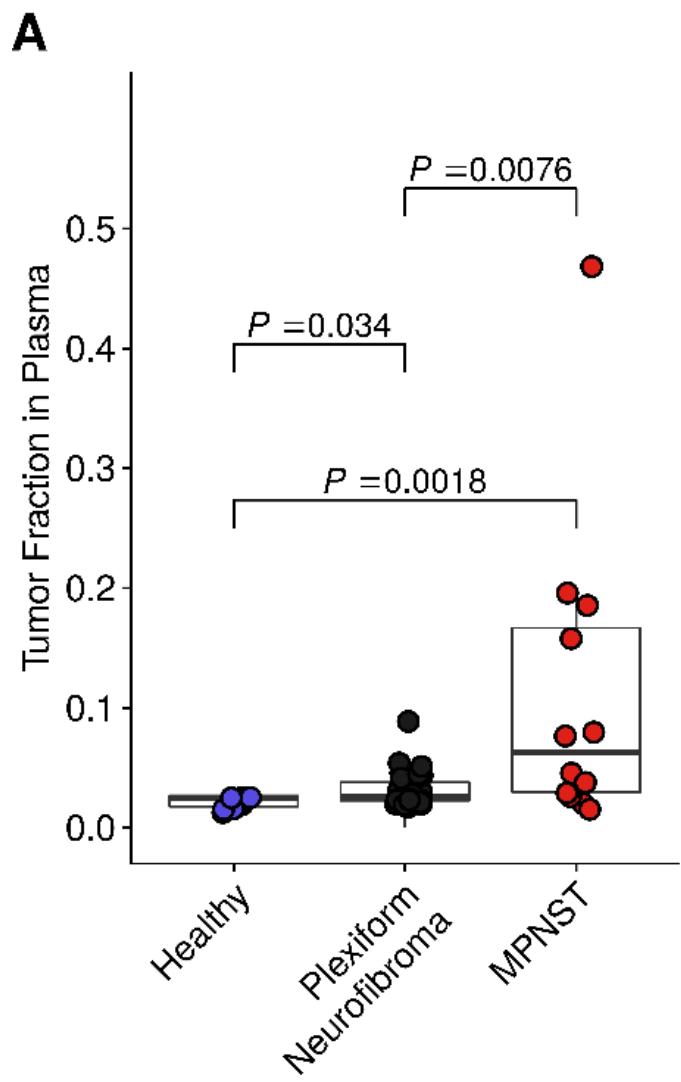


Labs of Chaudhuri, Hirbe & Shern

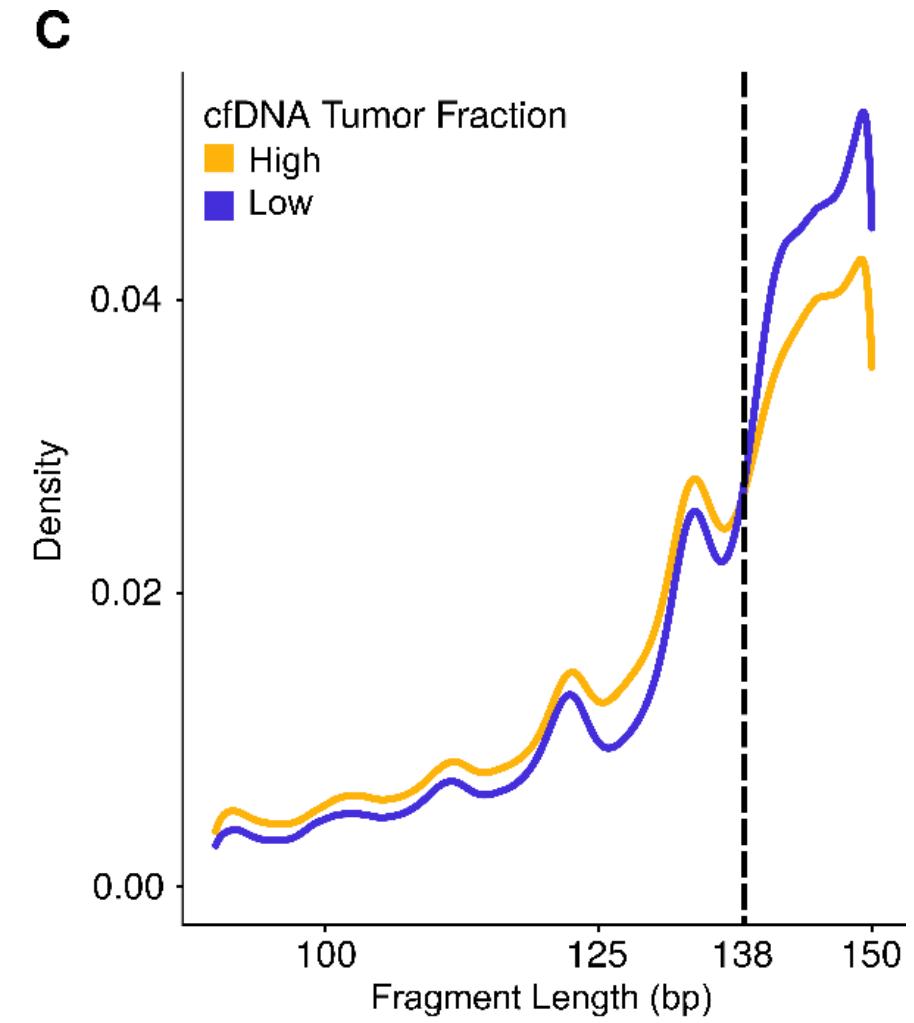
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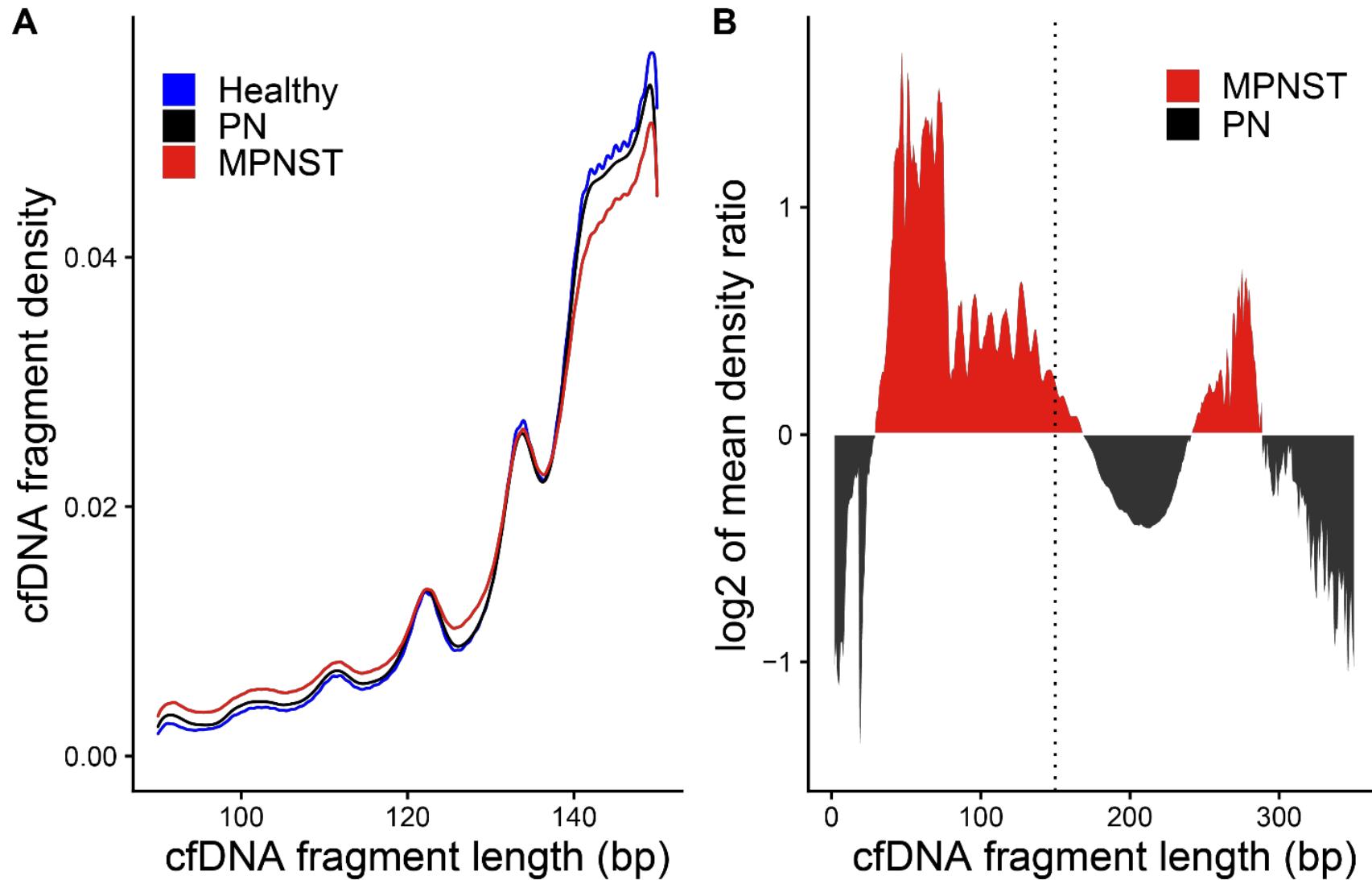
cfDNA analysis can discriminate MPNST from PN



	Sn	Sp	AUC
— 90–150 bp fragments	58	91	0.76
--- All fragment lengths	69	50	0.46

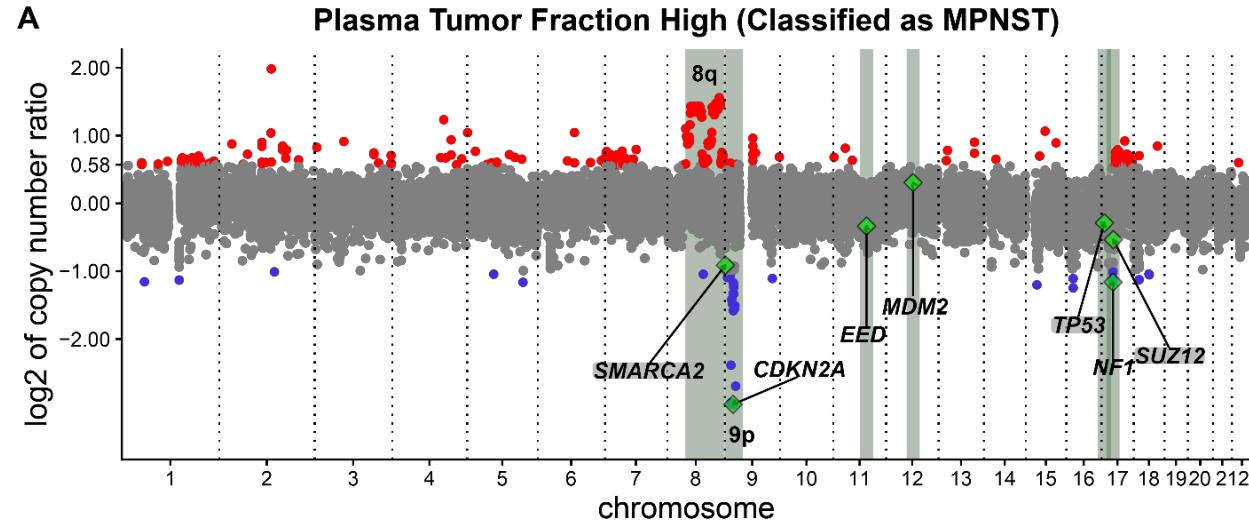


MPNST cell-free DNA fragments are shorter

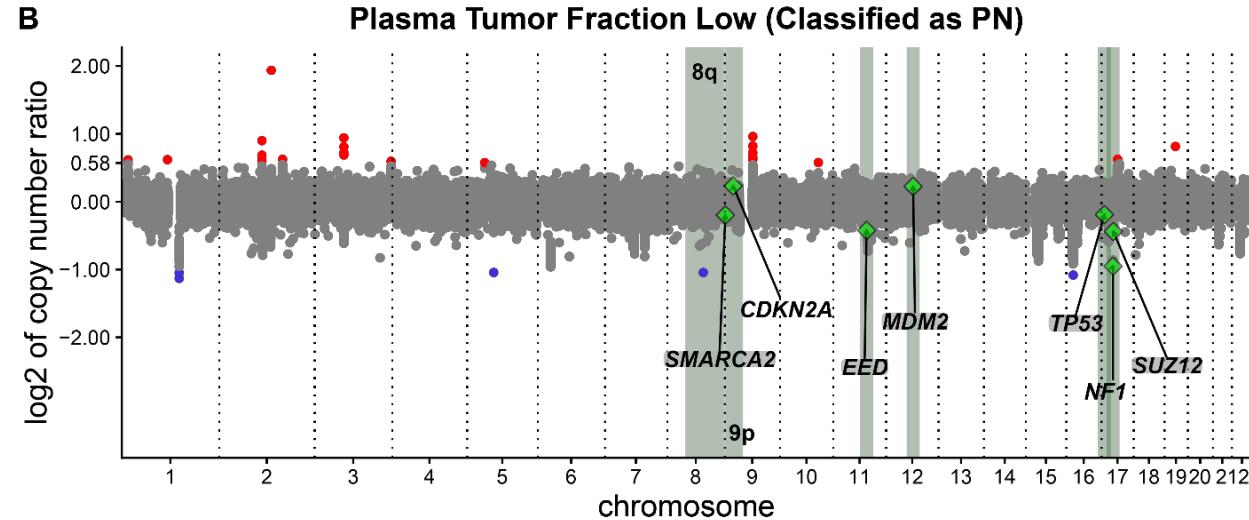


Copy number alterations are MPNST/NF1-specific

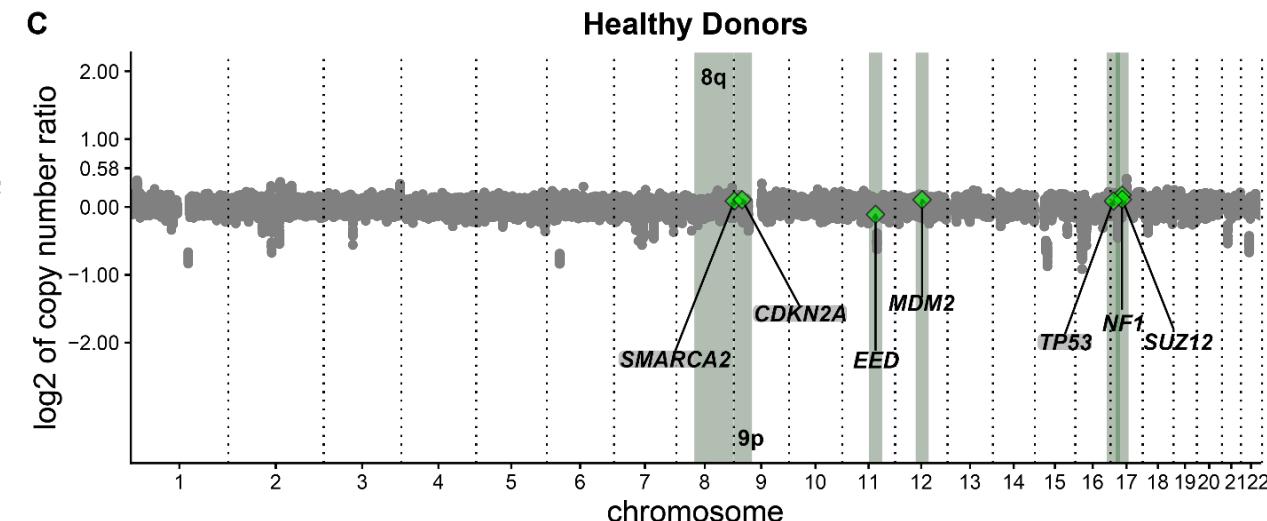
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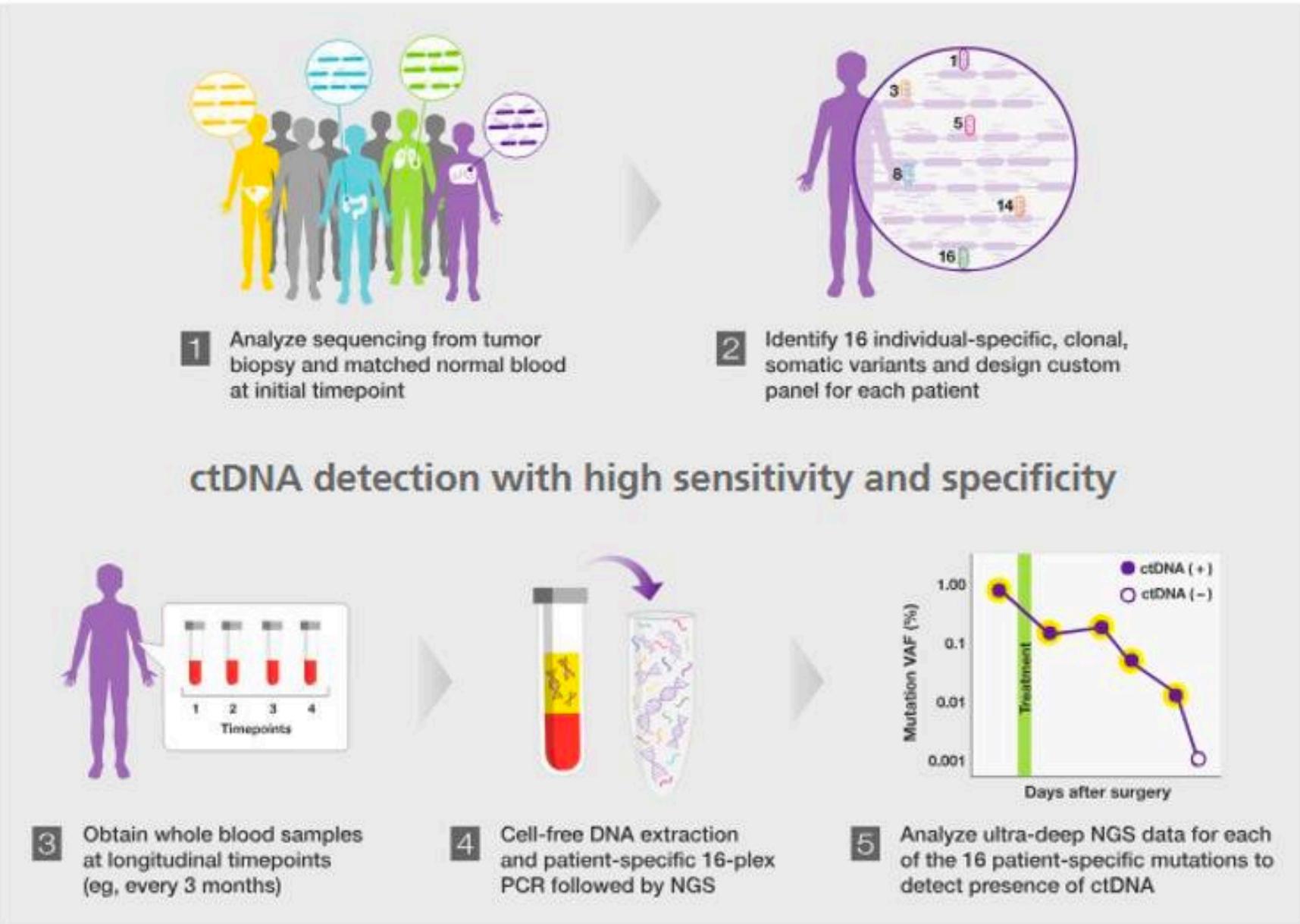
B



C



Natera Signatera ctDNA assay



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Patients & their families