Analytical Validation of ctDNA Assays
General Principles

ctDNA in Clinical Cancer Research
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Analytical Validation Procedures

- Clinical laboratories routinely analytically validate assays prior to use in clinical applications
- Guidelines for analytical validation are available from several sources: CMS, CAP, manuscripts and other variety of other sources
- For those of you that don't live in the assay world:
  - It is important to understand how critical a well designed and executed analytical validation is for insuring confidence in results and result interpretation
  - Analytical performance testing permits understanding of the assay's
    - Performance characteristics
    - Strengths
    - Limits/weaknesses

Basic Parameters Tested in Analytical Validation
Assay Performance Testing

- Critical to test the assay "system" consistent with the intended use
- Assay system = all major processes that contribute to the assay results
  - Specimen collection, stabilization, shipment and processing?
  - Assay process (reagents, handling devices, instrumentation)
  - Data analysis (software)

Basic Parameters Tested in Analytical Validation
Assay Performance Testing

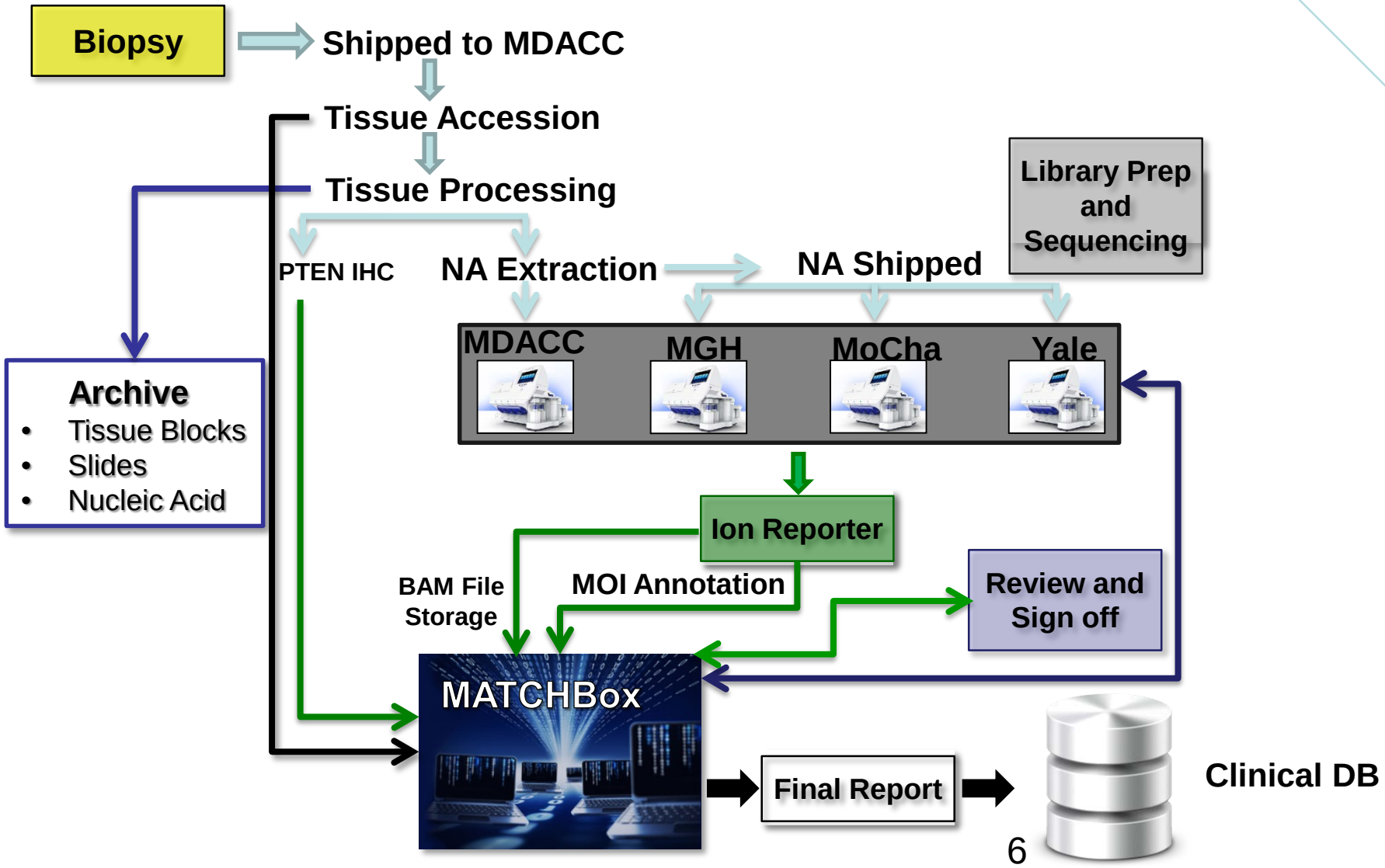
- Sensitivity* (testing for how many times the new assay accurately detects analytes known to be found in a specimen)
- Specificity*
- Accuracy*
- Reproducibility (day to day, operator to operator, instrument to instrument, reagent lot to lot)
- Limit of detection
- Full system testing
- Not all variants are created equal, therefore a multi-analyte test should minimally include a variety of snv's, indels, CNV (when appropriate)
- A representation of any known difficult to test analytes

* *Truth for a clinical ctDNA specimen often considered the matching solid tissue, but tumor heterogeneity may confound "truth"*

MPACT and MATCH Trial Assay Systems
Quality System, Design Control

1. "User Needs and Design Input:" What is the assay used for, assay claims, acceptable assay performance characteristics
   - ***a) Assay used for treatment selection for patients whose tumors were refractory to standard treatment (ctDNA, Dx, Rx selection, Rx response, resistance…the use will influence desired sensitivity/specificity)***
   - b) Assay is integral assay for trial (enrollment screening and treatment selection)
   - ***c) Assay requires new biopsy be obtained***
   - d) Assay system description (device description)
   - e) Assay system performance requirements defined in keeping with intended use and ***risk assessment***
2. "Design Verification:" Feasibility testing of assay performance using draft SOPs
3. Review processes and tweak as needed
4. Finalize assay parameters, lock SOPs, develop validation plan
5. Pre-submission discussion with FDA: assay intended use, risks and validation plan: Adjust Validation Plan reflecting discussion
6. Assay analytical performance testing and "validation"

NCI-MATCH Assay System & Work Flow



Feasibility Testing

- Non harmonized SOPs used by each lab
- IR v 4.2 used for data analysis
- 44 FFPE clinical samples tested within 4 laboratories
- 10 Cancer cell line genomes x4 labs
- 3 Hapmap genomes x3 replicates x 4 labs

Feasibility Data
Reproducibility

Variant allele frequencies are near identical across four lab replicates

Not detected

MATCH Assay - Oncomine Cancer Panel Gene List

Lots of Genes & Variants; Too Many Analytes to Validate Individually

SNV/Indel

Hotspot genes, n=73
(hotspot coverage)

ABL1	GNA11	MYD88
AKT1	GNAQ	NFE2L2
ALK	GNAS	NPM1
AR	HNF1A	NRAS
ARAF	HRAS	PAX5
BRAF	IDH1	PDGFRA
BTK	IDH2	PIK3CA
CBL	IFITM1	PPP2R1A
CDK4	IFITM3	PTPN11
CHEK2	JAK1	RAC1
CSF1R	JAK2	RAF1
CTNNB1	JAK3	RET
DDR2	KDR	RHEB
DNMT3A	KIT	RHOA
EGFR	KNSTRN	SF3B1
ERBB2	KRAS	SMO
ERBB3	MAGOH	SPOP
ERBB4	MAP2K1	SRC
ESR1	MAP2K2	STAT3
EZH2	MAPK1	U2AF1
FGFR1	MAX	XPO1
FGFR2	MED12	
FGFR3	MET	
FLT3	MLH1	
FOXL2	MPL	
GATA2	MTOR	

CNV

CDS, n=26
(full gene)

- APC
- ATM
- BAP1
- BRCA1
- BRCA2
- CDH1
- CDKN2A
- FBXW7
- GATA3
- MSH2
- NF1
- NF2
- NOTCH1
- PIK3R1
- PTCH1
- PTEN
- RB1
- SMAD4
- SMARCB1
- STK11
- TET2
- TP53
- TSC1
- TSC2
- VHL
- WT1

Copy gain,
n=49

ACVRL1	IGF1R
AKT1	IL6
APEX1	KIT
AR	KRAS
ATP11B	MCL1
BCL2L1	MDM2
BCL9	MDM4
BIRC2	MET
BIRC3	MYC
CCND1	MYCL
CCNE1	MYCN
CD274	MYO18A
CD44	NKX2-1
CDK4	NKX2-8
CDK6	PDCD1LG2
CSNK2A1	PDGFRA
DCUN1D1	PIK3CA
EGFR	PNP
ERBB2	PPARG
FGFR1	RPS6KB1
FGFR2	SOX2
FGFR3	TERT
FGFR4	TIAF1
FLT3	ZNF217
GAS6	

Gene Fusion

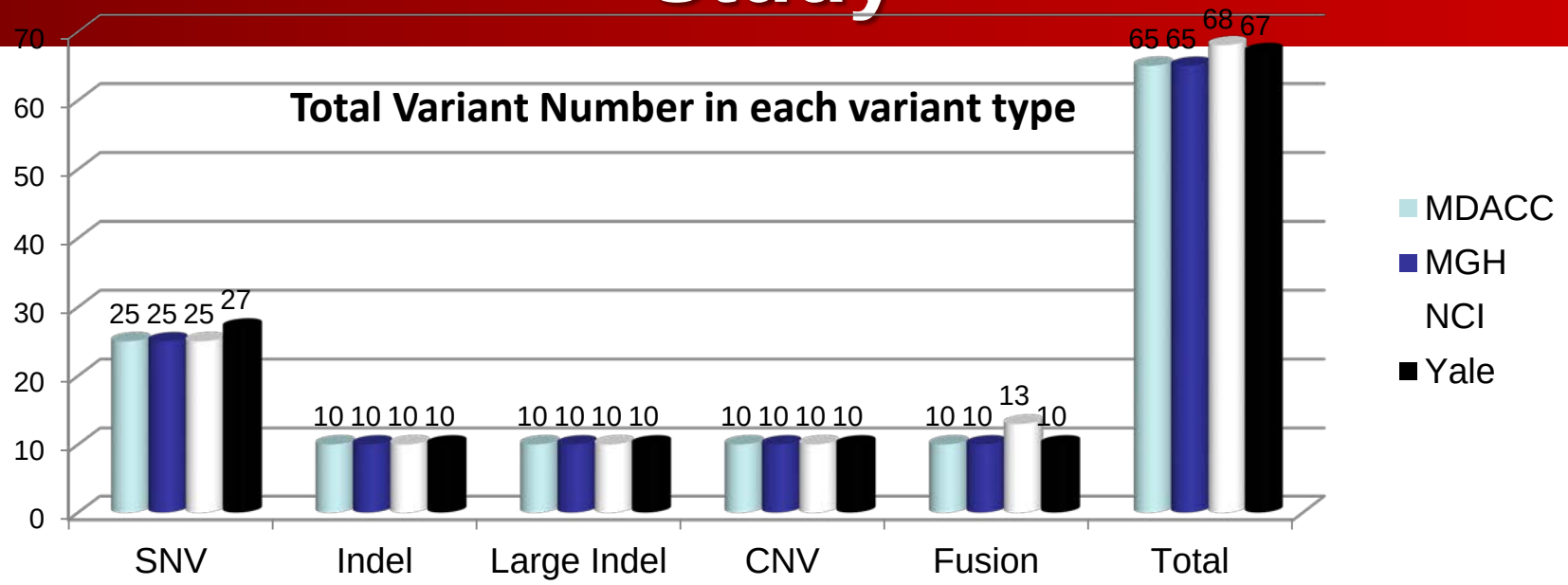
Fusion drivers,
n=22 (183 assays)

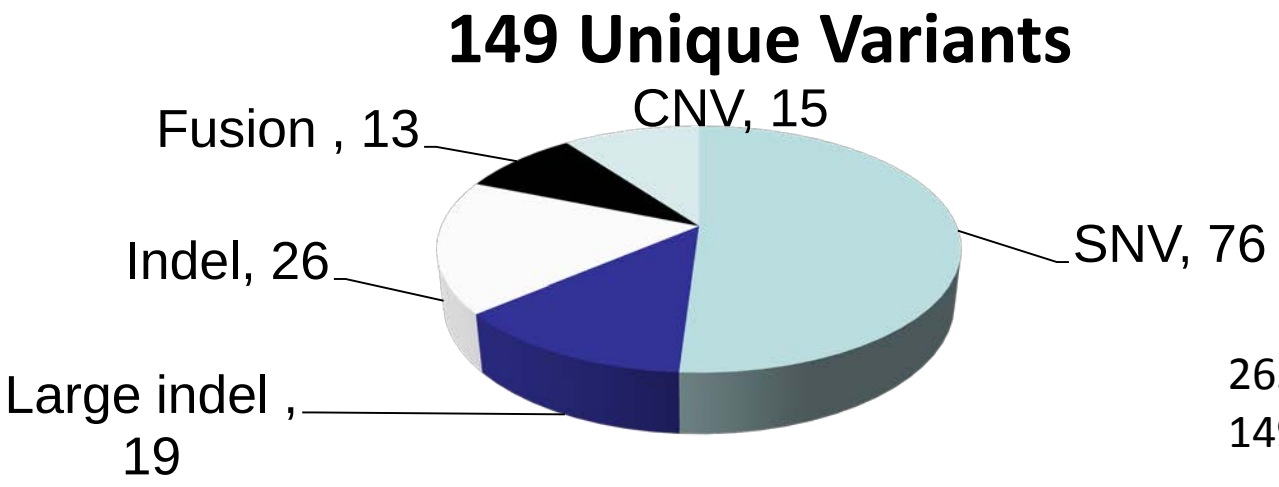
- ALK
- RET
- ROS1
- NTRK1
- ABL1
- AKT3
- AXL
- BRAF
- CDK4
- EGFR
- ERBB2
- ERG
- ETV1
- ETV4
- ETV5
- FGFR1
- FGFR2
- FGFR3
- NTRK3
- PDGFRA
- PPARG
- RAF1

143 Genes & >4,000 MOIs

Courtesy of Thermo Fisher

Variant Distribution in Sensitivity Study

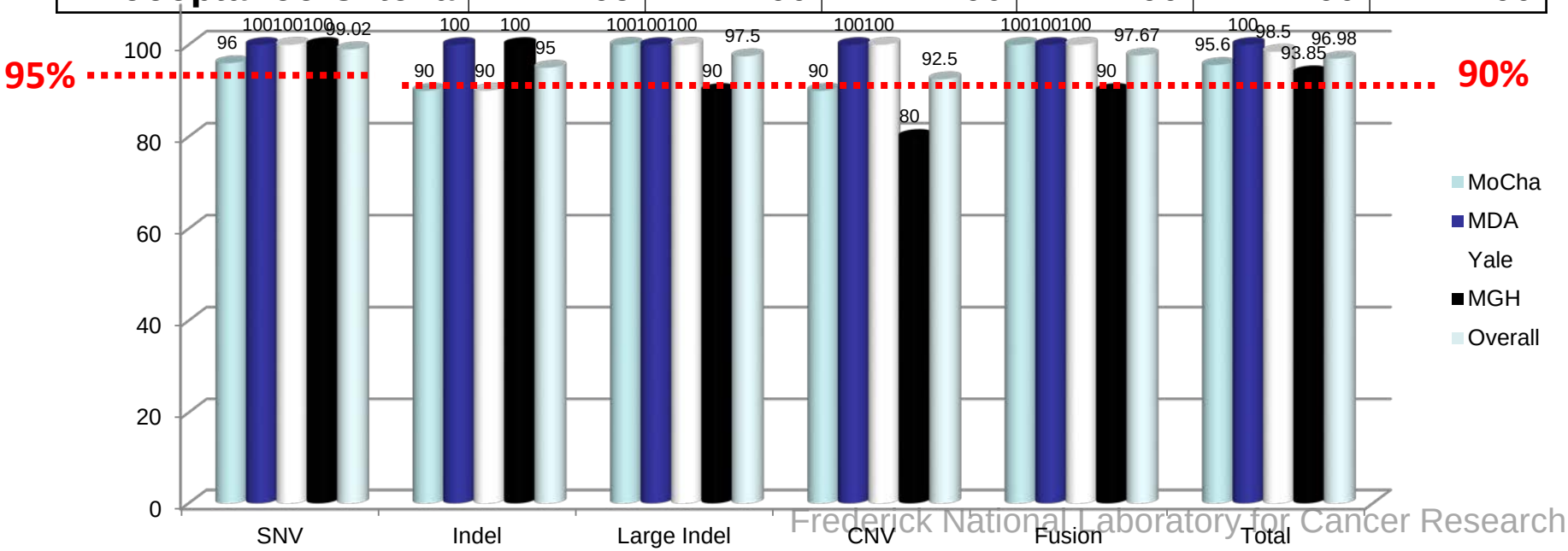




265 total variants
149 unique variants

Sensitivity

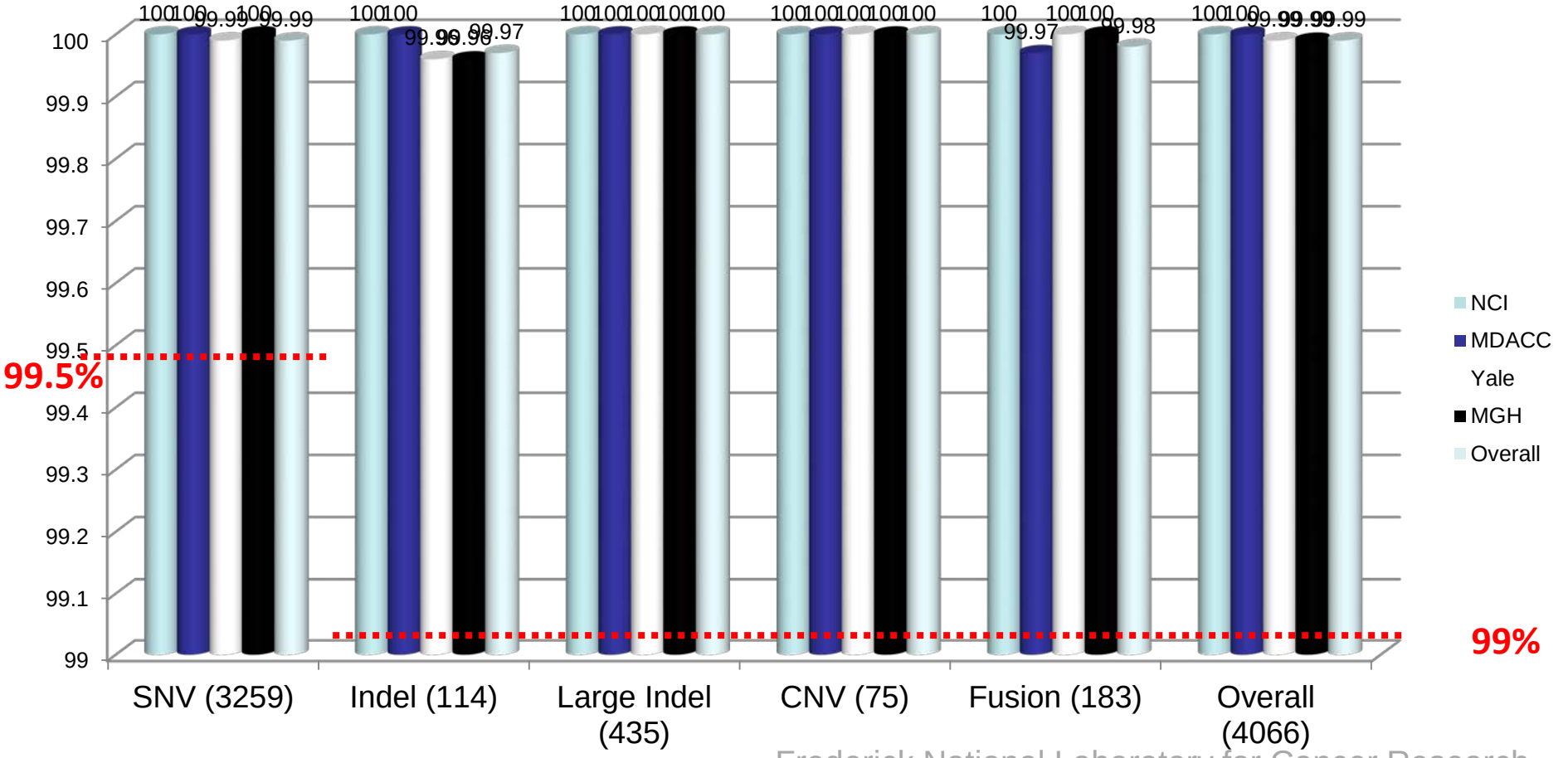
Sensitivity	SNV (102)	Indel (40)	Large Indel (40)	CNV (40)	Fusion (43)	Total (265)
MoCha	96	90	100	90	100	95.6
MDA	100	100	100	100	100	100
Yale	100	90	100	100	100	98.5
MGH	100	100	90	**80**	90	93.85
Overall	**99.02**	**95**	**97.5**	**92.5**	**97.67**	**96.98**
Acceptance Criteria	95	90	90	90	90	90



Specificity
Critical Parameter for Intended Use

True Negative / (True Negative + False Positive) over 4066 MOI loci

Acceptance criteria met by all laboratories

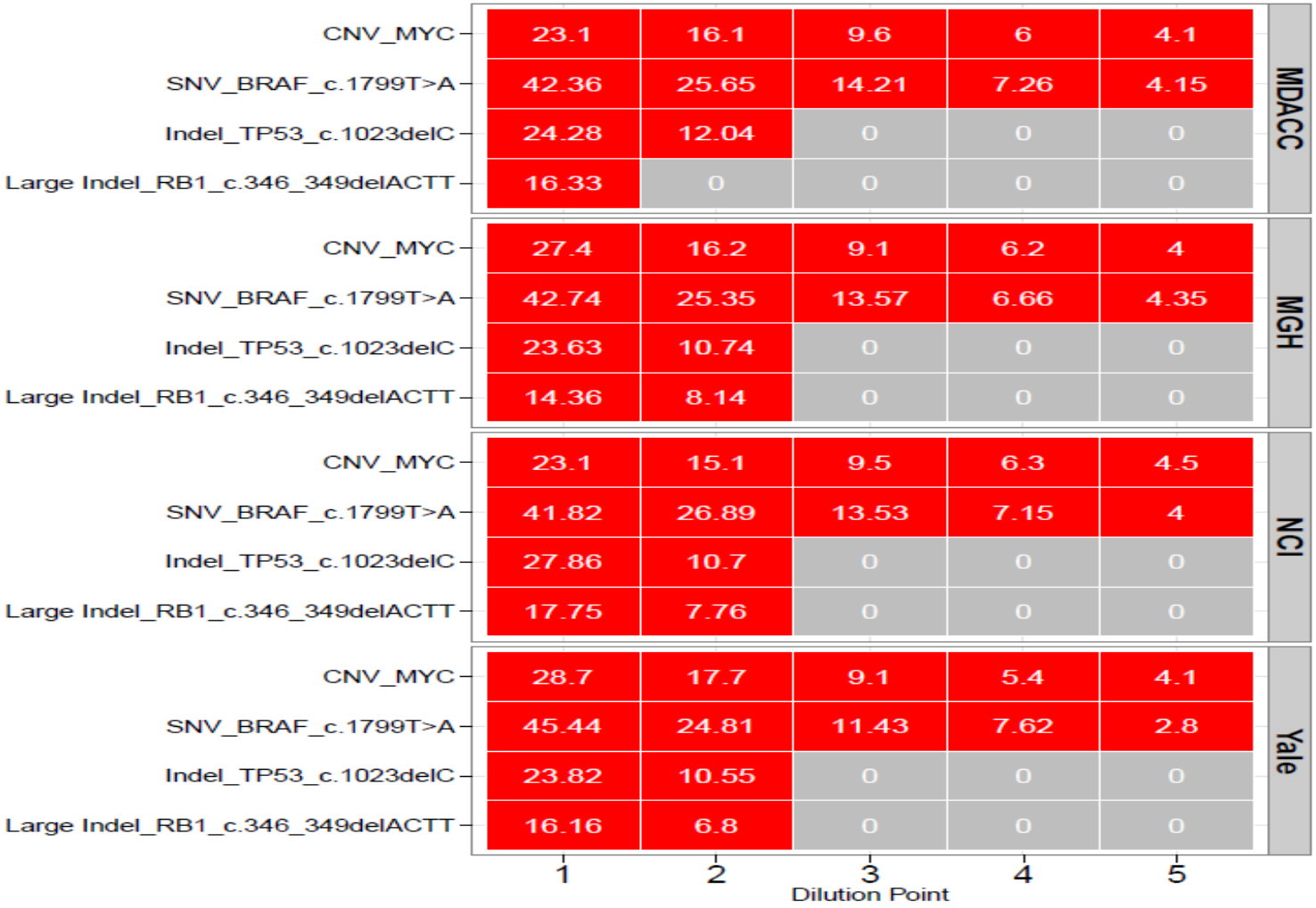


Reproducibility in Detected Variants

Acceptance criteria met by all laboratories

Reproducibility across 16 specimens	Nucleic Acid	Type	Number of Pairwise Concordances	Mean Concordance
Positive Concordance	DNA	Intra-Operator	72	96.20
	RNA	Intra-Operator	56	100
	DNA	Within lab Inter-Operator	144	96.20
	RNA	Within lab Inter-Operator	112	100
	DNA	Cross lab Inter-Operator	864	96.29
	RNA	Cross lab Inter-Operator	672	100
Overall Concordance	DNA	Intra-Operator	72	99.99
	RNA	Intra-Operator	56	100
	DNA	Within lab Inter-Operator	144	99.99
	RNA	Within lab Inter-Operator	112	100
	DNA	Cross lab Inter-Operator	864	99.99
	RNA	Cross lab Inter-Operator	672	100

Limit of Detection



NGS Assays and ctDNA

- NGS provides a powerful tool for massively parallel sequencing of patient tumors
- NGS ctDNA assays are being developed, applied and acted upon for patient management in oncology……"Everybody is doing it"
- I believe there is a need for:
  - Assay Standards *(eg. Genome in a Bottle, FNIH ctDNA)*
  - Agreement on clinical relevance of detected variants, levels of evidence
  - Public data sharing with assay details and clinical outcome may help drive clinical utility, adding knowledge of clinical impact and improved patient outcomes

Complexities Validating ctDNA Assays

- Tumor Specimen:
  - Can adequate numbers and amount of clinical specimens be obtained for performance testing? When and how should contrived specimens be used?
  - Is the specimen archival pre-treatment or recent post treatment (resistance mutations)
  - What is truth: Compare to solid tissue (heterogeneity of tumor) OR ctDNA data from orthogonal assay (is sample large enough to provide technical replicates?)
  - What tissue and stage of disease should be used? May impact amounts of ctDNA found
- Sequencing choices:
  - PCR based or probe capture
  - Platform and read depth
  - What is lower limit of detection ie. allele frequency reported

A Parting Question

- What is the best way to report result?:
  - Allele Fraction/% mutation
  - Genome equivalents per blood volume?
  - Absolute copy number per blood volume (requires a calibrator)

THANKS

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