# Analytic Validation: NGS Tumor Genomic Profiling

Julia Elvin, MD, PhD
Sr. Associate Medical Director
Foundation Medicine
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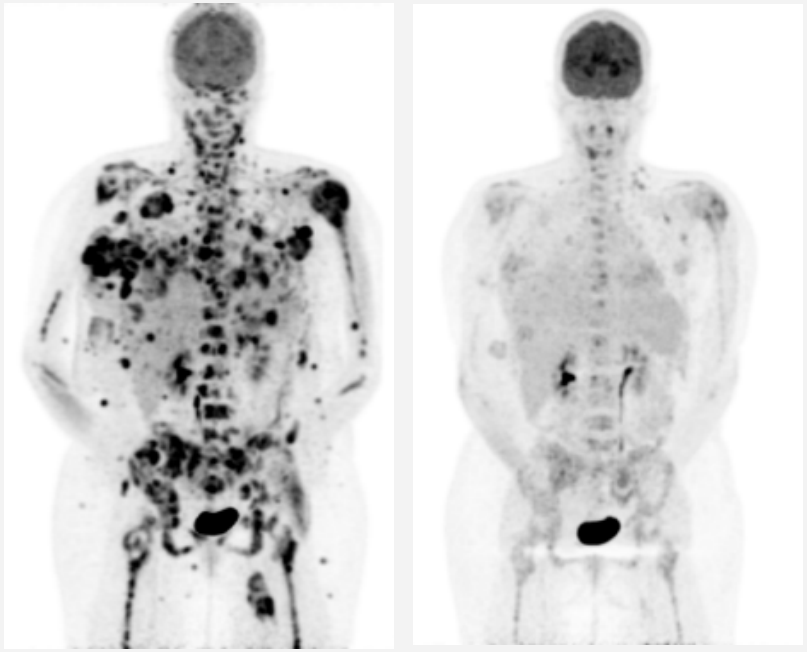
# Agenda

- Overview: the technical challenges of NGS tumor genomic profiling
- What is Analytic Validity?
- Who evaluates Analytic Validity?
- Why does Analytic Validity matter?
- Key Questions to Ask

# The Promise of Personalized Cancer Care

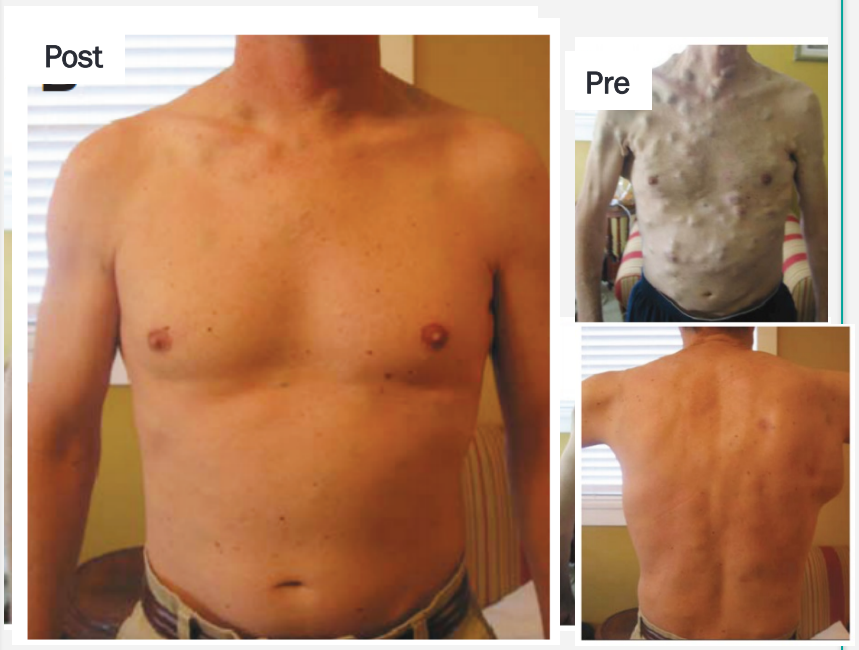
Case Example 1: Metastatic melanoma



After conventional treatment

15 days post Vemurafenib

Case Example 2: Metastatic melanoma




37 yo with metastatic melanoma after conventional treatment

23 weeks on Vemurafenib (targeted therapy matched to BRAF V600E mutation)

Tumor cells DEPEND on abnormal signaling for growth and survival.

- Step 1: *Identify* the genes of interest that have been mutated -> PROTEIN TARGETS
- Step 2: *Treat* with small molecules that INHIBIT ABNORMAL PATHWAYS = Hits "Achilles' heel" of the tumor.
- **GOAL** - Only tumor cells suffer, normal cells are spared from majority of treatment effects.

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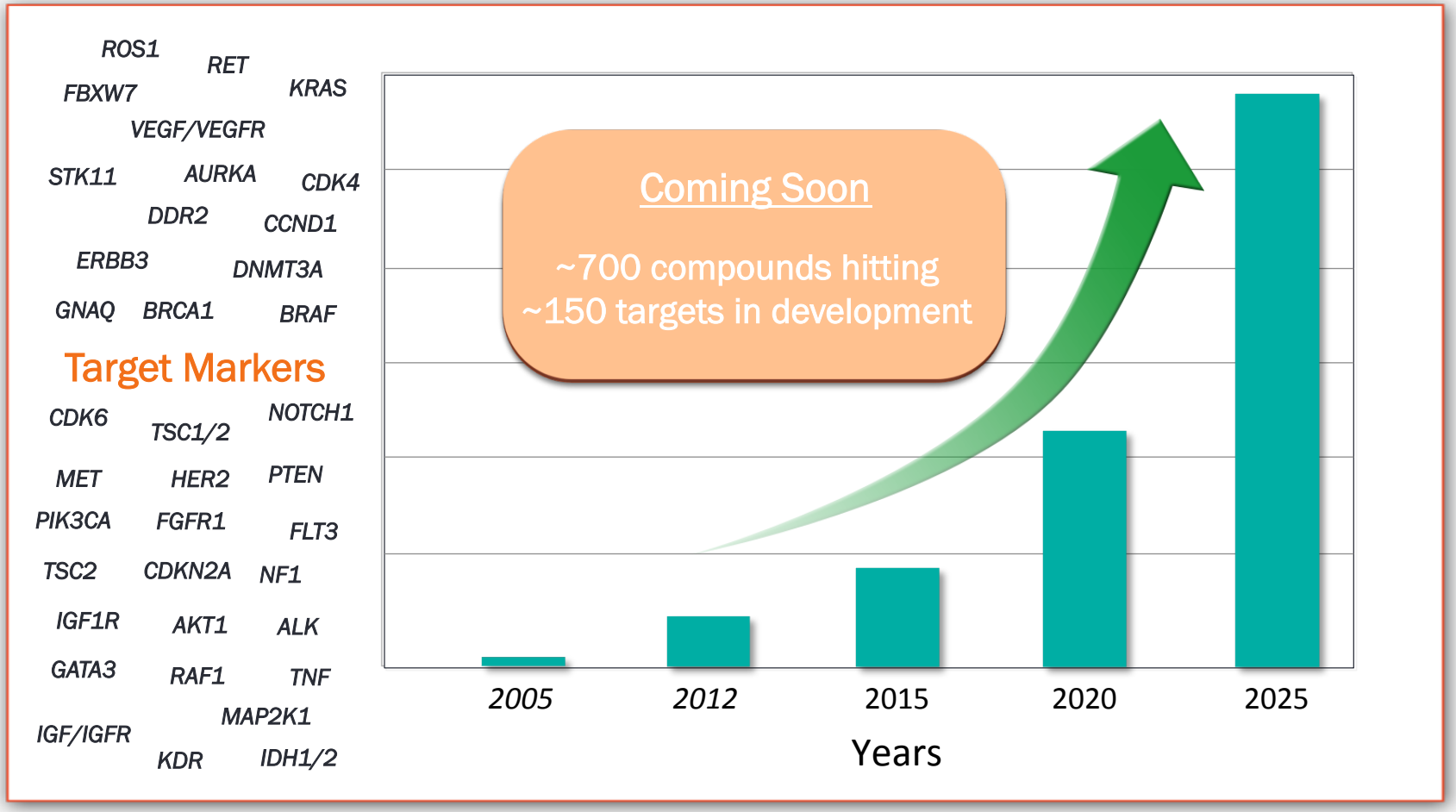
(Refs: Dr. Grant McArthur; Wagle et al., JCO 2011)

# Targeted Therapeutics Options Projected




*Knowing which tests to order for a tumor type increasingly challenging*

Extrapolated from BioCentury Online Intelligence Database

# Challenges to NGS in Oncology

- In oncology, molecular technologies are widely used to predict responsiveness to specific drugs
- Patients and physicians rely on these results for clinical decisions
- As we move to more multiplex technology to assess genomic drivers, the complexity of effectively dividing patients in the most relevant groups for clinical interventions is increasing
- Technical Variability must be minimized so that we can understand Biologic Variability

# NGS Assay Workflow Example

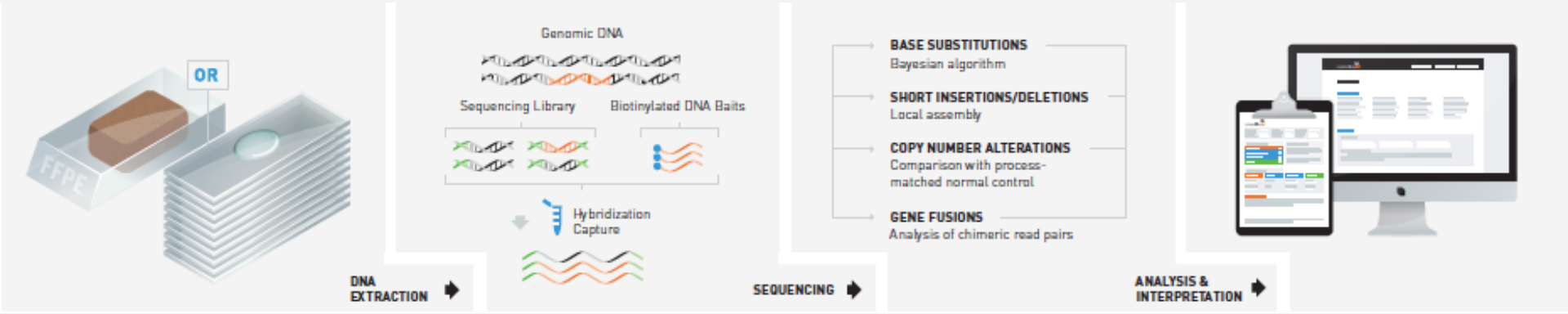
**Pre-analytic Variables Outside NGS Lab control (fixation, procedure, age, storage conditions) impact assay results & need evaluation.**

**1) DNA/RNA extraction:**
Extensive optimization

**2) LC, Hybrid Capture:**
Extensive optimization

**3) Analysis pipeline:**
Advanced computational biology

**4) Clinical report:**
Resource intensive




**Illumina HiSeq**

**Pre-Analytic Process (Pre-Sequencing)**

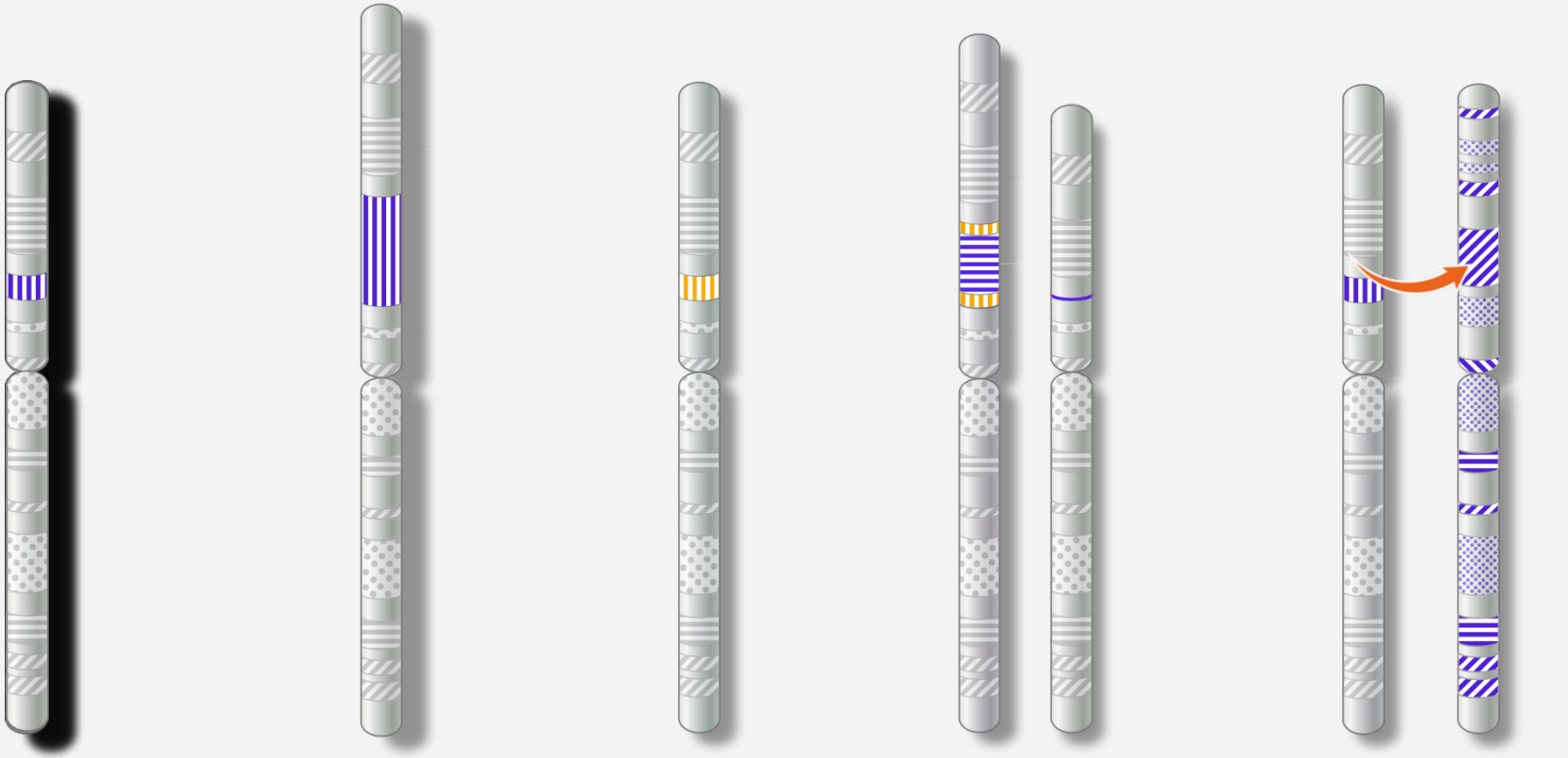
**Post-Analytic Process (Post-Sequencing)**

***Translating research grade NGS to a clinical cancer diagnostic assay requires extensive optimization and investment***

# Selected Examples of Cancer Genome Sequencing and Anti-Cancer Drug Selection

| Genetic Event | Disease | Drug |
|---|---|---|
| *KRAS* Mutation | CRC | Cetuximab/Panitumumab (contraindicated by KRAS mutation) |
| *BRAF* Mutation | Melanoma | Vemurafenib/Dabrafenib |
| *EGFR* Mutation | NSCLC | Gefitinib/Erlotinib/Afatinib |
| *EML4-ALK* Translocation | NSCLC | Crizotinib |
| *KIT* Mutation | GIST/melanoma | Imatinib/Sunitinib/Regorafenib/Pazopanib |
| *BCR-ABL* Translocation | CML | Imatinib/Dasatinib/Nilotinib/Bosutinib |
| *PML-RARA* Translocation t(15;17) | APL | ATRA |
| *HER2* Gene Amplification* | Breast and Upper GI Cancer | Trastuzumab/Lapatinib |
| *ROS1* Fusion | NSCLC | Cabozantinib (investigational) |
| *RET* Fusion | NSCLC | Cabozantinib (investigational) |

# Four types of ways genes can be altered



Normal

Copy number alterations

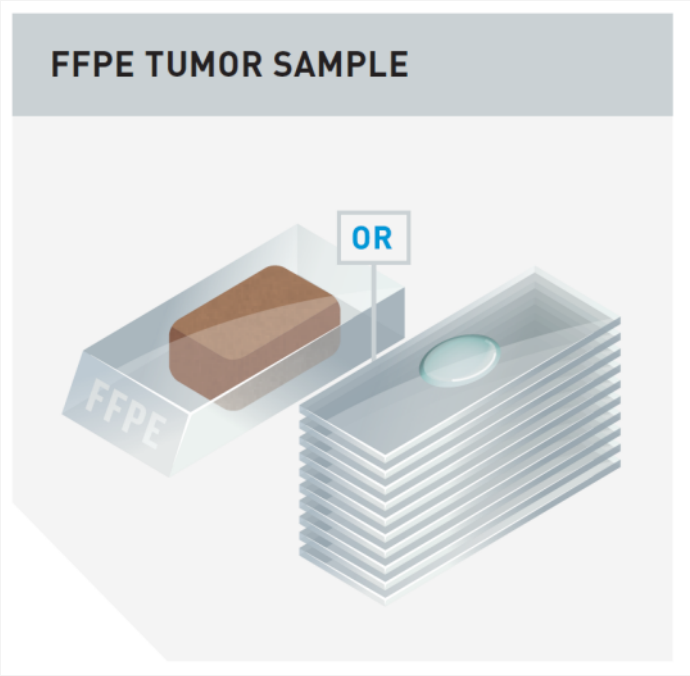
Substitutions

Insertions and deletions

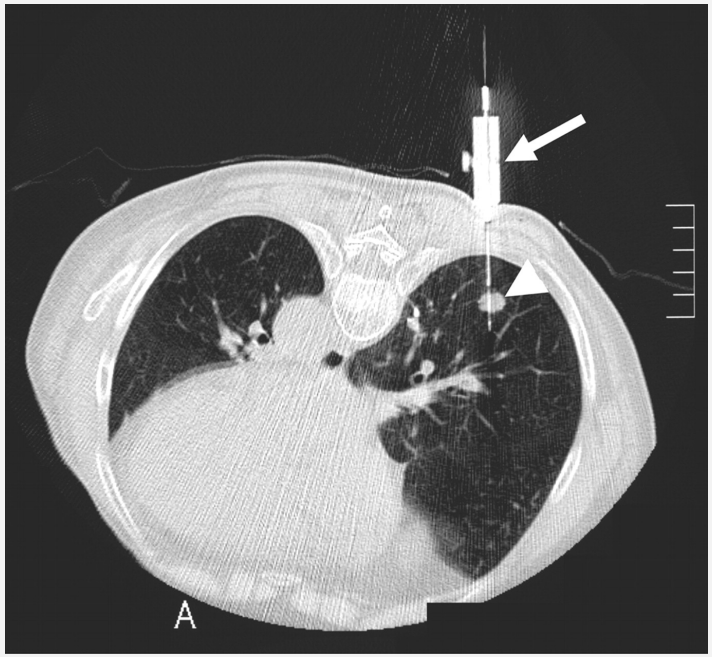
Rearrangements

# Diagnostic Challenge: Many Clinical Cancer Specimens are Small Needle Biopsies, FNAs, and Cell Blocks

**Formalin fixation and subsequent storage can damage nucleic acids**

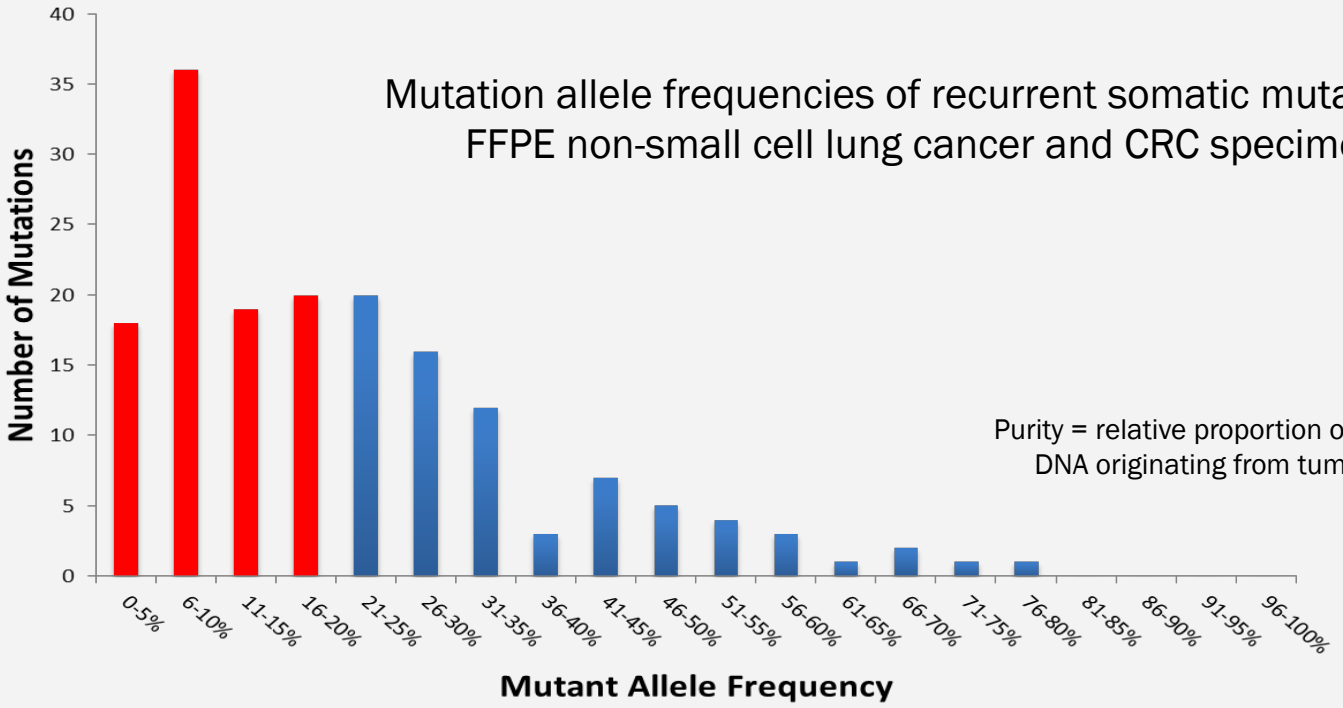



**Percutaneous needle biopsy of lung nodules under CT fluoroscopic guidance**




***Sample preparation needs be optimized to maximize accuracy and isolate sufficient material for diagnostic testing from tiny specimens***

# Diagnostic Challenge: Low Tumor Purity in Many Clinical Specimens Requires Diagnostic Tests with High Accuracy




| Fraction of mutations <5% | Fraction of mutations <10% | Fraction of mutations <20% | Fraction of mutations <25% | Fraction of mutations <50% | Fraction of mutations <100% |
|---|---|---|---|---|---|
| 11% | 32% | 55% | 67% | 93% | 100% |

***Capillary sequencing would have missed over half the mutations in this study as 20% allele frequency is the maximum limit of detection***

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# Challenges to NGS in Oncology

- With numerous different tests being used in different laboratories….
  - How different are they from one another?
    - Which genes are being analyzed?
    - How much of each gene is assessed (hot spots vs. all coding)?
    - What enrichment techniques are being employed (PCR vs. hybrid capture)?
    - Which instrumentation is being used (“the Box”)?
    - Which types of mutations can be detected and in which clinical contexts?
  - How do we know a various approach has value and can be trusted to provide accurate results?

# The Reality

"There is no assurance that every laboratory performing genetic tests for clinical purposes meets high standards."

*- Joint NIH-DOE Task Force on Genetic Testing*

National Human Genome Research Institute (1997). Retrieved July 2015 from: http://www.genome.gov/10001733.

# What is Analytic Validation?

- The process of determining whether an assay is able to discriminate the presence or absence of the event it was designed to detect.
- Measurements of Analytic Validation: Accuracy & Precision
  - Sensitivity: ability to correctly identify those patients with the disease (FNR).
  - Specificity: ability to correctly identify those patients without the disease (FPR)
  - Positive Predictive Value (PPV): 'How likely is it that this patient has the disease given that the test result is positive?' – Depends on prevalence
  - Negative Predictive Value (NPV): 'How likely is it that this patient does not have the disease given that the test result is negative?' – Depends on prevalence
  - Precision – When a test method is precise, the amount of random variation is small
    - Reproducibility - is the ability of an entire experiment or study to be duplicated
    - Repeatability - degree of agreement of tests or measurements on replicate specimens by the same observer in the same laboratory
  - Limits of Detection

# Why does Analytic Validation matter?

- 70% of medical decisions are based on diagnostic test results

- Ensuring the patient is stratified into the accurate subset and that the clinician has the information required to assess the reliability of the data they are receiving has critical implications

# Who Evaluates Analytic Validation?

- CLIA Certification
- CAP Accreditation
- NY State Department of Health
- MolDx Technical Assessment
- FDA

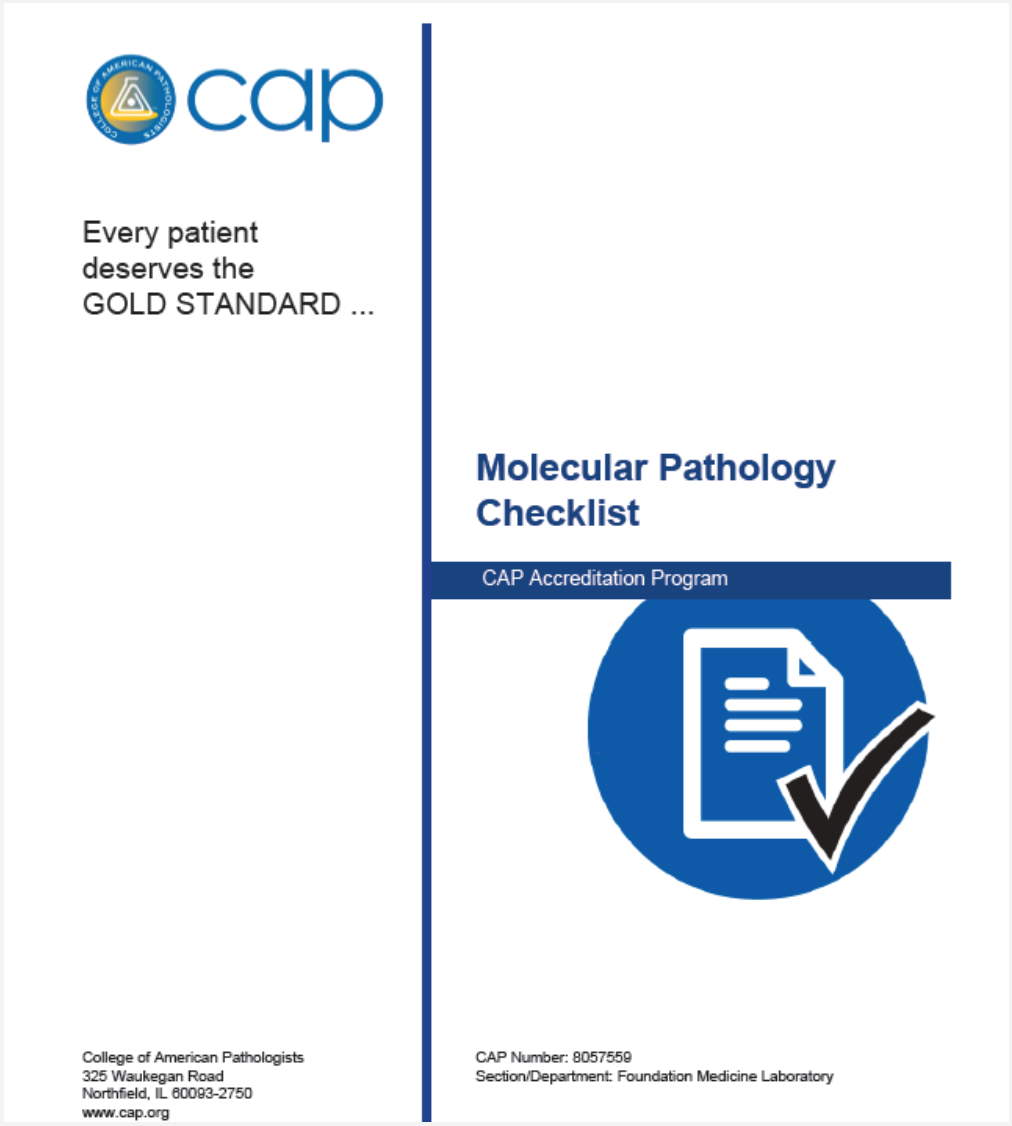
There is no single standard or guideline regarding analytic validation for NGS-based laboratory tests.

# Clinical Laboratories Improvement Act (CLIA)

- CLIA regulates laboratories performing clinical testing in order to ensure accurate and reliable test results
- Under CLIA, a laboratory performing a lab developed test (LDT; any test not FDA cleared/approved) may not release any test results prior to establishing certain performance characteristics relating to analytical validity for the use of that test system in the laboratory's own environment
- Under CLIA, a test's analytic validation is limited to the specific conditions, staff, equipment and patient population of the particular laboratory, so the findings of the laboratory-specific analytical validation are not meaningful outside of the laboratory that did the analysis – i.e. validations are not transferable.
- The laboratory's analytical validation of LDTs is reviewed during its routine every 2 year survey – after the laboratory has already started testing.
- **There are no minimum thresholds that must be met specific to NGS testing**

https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Downloads/LDT-and-CLIA_FAQs.pdf

# College of American Pathologists (CAP)




Laboratories can voluntarily pursue CAP-accreditation.

Inspections are performed every two years and evaluate:

- -Quality Management and Quality Control
- -Personnel
- -Laboratory Safety

There are NGS-specific checklists for validation and on-going QA/QC

# New York State License

- The state of New York requires that any laboratory performing clinical testing on samples from New York be certified by their Clinical Laboratory Evaluation Program (CLEP)
- Provides some minimum requirements for performance metrics
- Considered one of the most rigorous certifications a test can go through (outside of the FDA)

http://www.wadsworth.org/sites/default/files/WebDoc/1300145166/NextGenSeq_ONCO_Guidelines.pdf

# MolDx Technical Assessment

- Palmetto's MolDx program has established guidance for the components that should be evaluated in the Analytic Validation of NGS-based tests
- Components:
  - Sensitivity (e.g. Limit of Blank, Limit of Detection, Minimum tumor content)
  - Specificity (e.g. Primer and probe specificity, interfering substances)
  - Precision (e.g. Reproducibility, intra/inter-run repeatability)
- "Covered tests" (i.e. those that have passed the technical assessment) are listed on the MolDx website

http://www.palmettogba.com/palmetto/MolDX.nsf/DocsCat/MolDx%20Website~MolDx~Browse%20By%20Topic~Technical%20Assessment~8PKRZF3404?open

# FDA

- As part of the pre-market approval (PMA) process, the FDA does rigorously review the analytic validation of a test. HOWEVER, the FDA has practiced “enforcement discretion” with regards to laboratory developed tests (LDTs) for many years
- Most genetic and genomic tests currently available are NOT FDA-cleared or approved
- This is a keen area of interest for FDA
  - LDT DRAFT guidance issued-October, 2014
  - Optimizing FDA’s Regulatory Oversight of Next Generation Sequencing Diagnostic Tests Workshop-February 20, 2015

# Similarities between NYS, MolDx, and CAP Guidance

| Validation Guideline | Notes |
|---|---|
| Accuracy | -NYS specifies inclusion of 50 clinical specimens. |
| Analytical Sensitivity and Specificity | -MolDx requires limit of detection (LOD) to be established as minimum DNA input<br>-NYS requires mutant allele frequency (MAF) to be established (more pertinent to oncology challenges especially in low tumor purity samples and those with & subclonal alterations/resistance mutations) |
| Precision | -MolDx requires inclusion of reagent lot-to-lot reproducibility studies.<br>-This is not done as part of precision, however lot acceptance procedures are established as required by NYS, CAP and CLIA. |
| Reagent and Sample Stability | |
| Reference Intervals | |
| Quality Control | NYS specifies use of positive/sensitivity control, minor differences in recommended metrics however, substantially the same. |

# Differences Between NYS, MolDx, and CAP Guidance

| Authority | Guidance |
|---|---|
| NYS | Requires a single version of Pipeline |
| NYS, CAP | Establish key performance metrics for the entire process from extraction through data analysis. |
| MolDx (CTEP AVCV) | Establish LOD as defined as lowest DNA input for process |
| MolDx (CTEP AVCV) | Establish Lot-to-lot variability as part of reproducibility study. |
| NYS | Develop and include positive/sensitivity control |

# NGS Validation Example

# NGS Cancer Genomic Profiling Assay Specifications

- One comprehensive genomic profile for all solid tumors to simultaneously detect all clinically relevant classes of genomic alterations in a single assay
- Focused on 315 known clinically & biologically relevant cancer genes (all coding exons and selected introns)
- Validated high accuracy achieved by high, uniform coverage: >99.5% of exons covered >100X
- Permits testing small amounts of tissue from routine FFPE samples, including needle biopsies (≥50ng of DNA)
- Customized computational biology algorithms validated for high accuracy in clinical samples with high stromal contamination

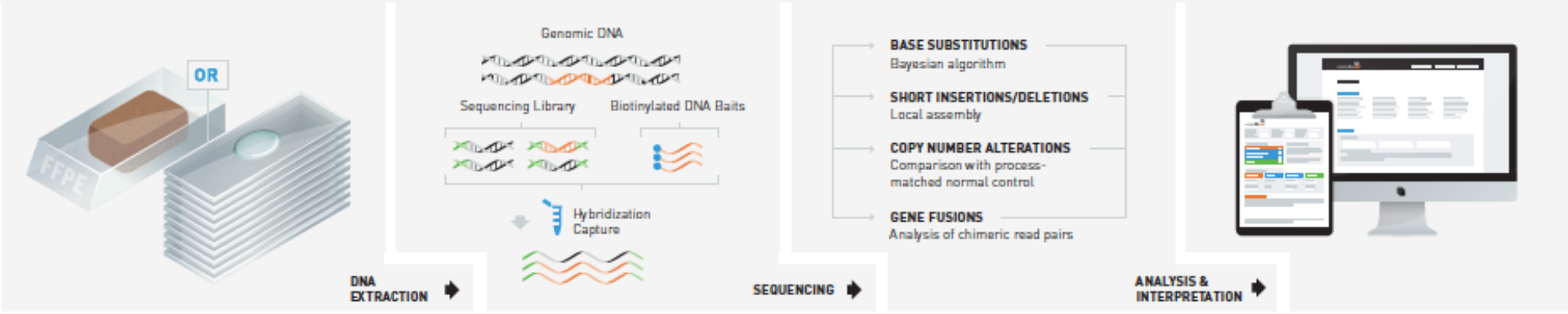
# NGS Assay Workflow (10-14 days)

**1) DNA/RNA extraction:**
Extensive optimization

**2) LC, Hybrid Capture:**
Extensive optimization

**3) Analysis pipeline:**
Advanced computational biology

**4) Clinical report:**
Resource intensive




**Illumina HiSeq**

**Pre-Analytic Process (Pre-Sequencing)**

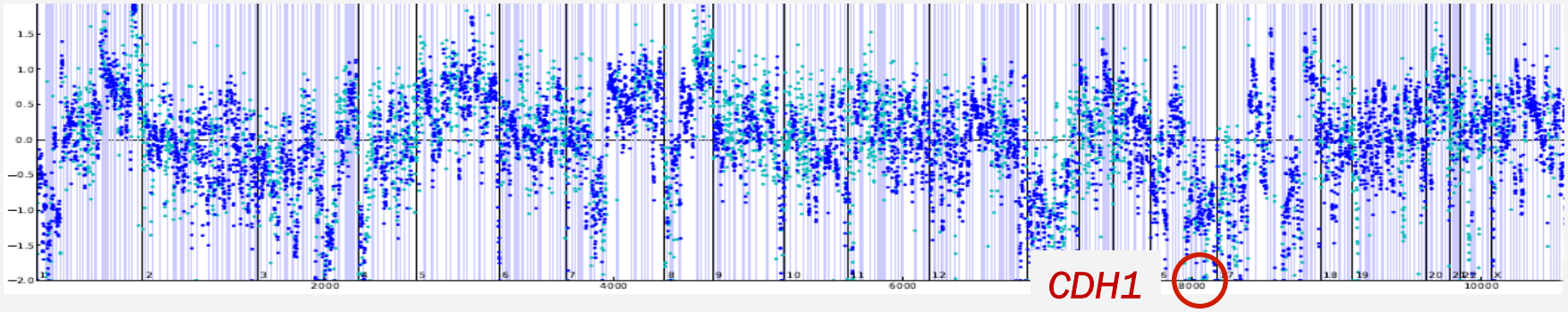
**Post-Analytic Process (Post-Sequencing)**

***Translating research grade NGS to a clinical cancer diagnostic assay requires extensive optimization and investment***

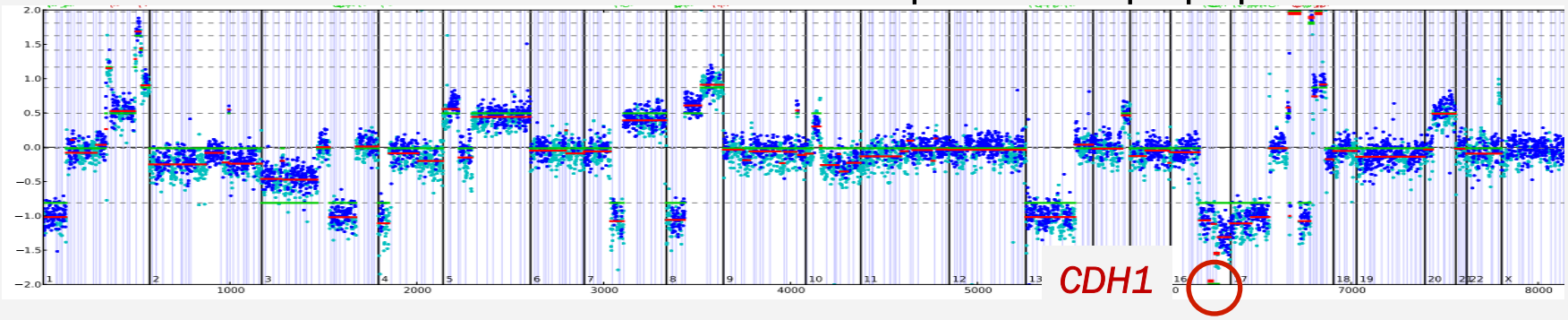
# Impact of DNA extraction optimization on Sequence Data

Copy Number

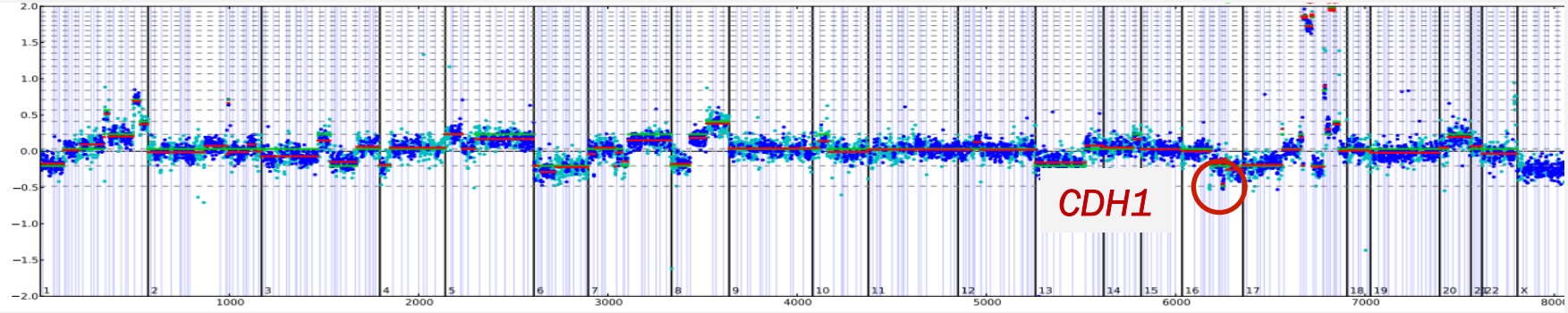
Breast cancer : 100% tumor "No optimization"




Breast cancer : 100% tumor "FMI optimized sample prep"



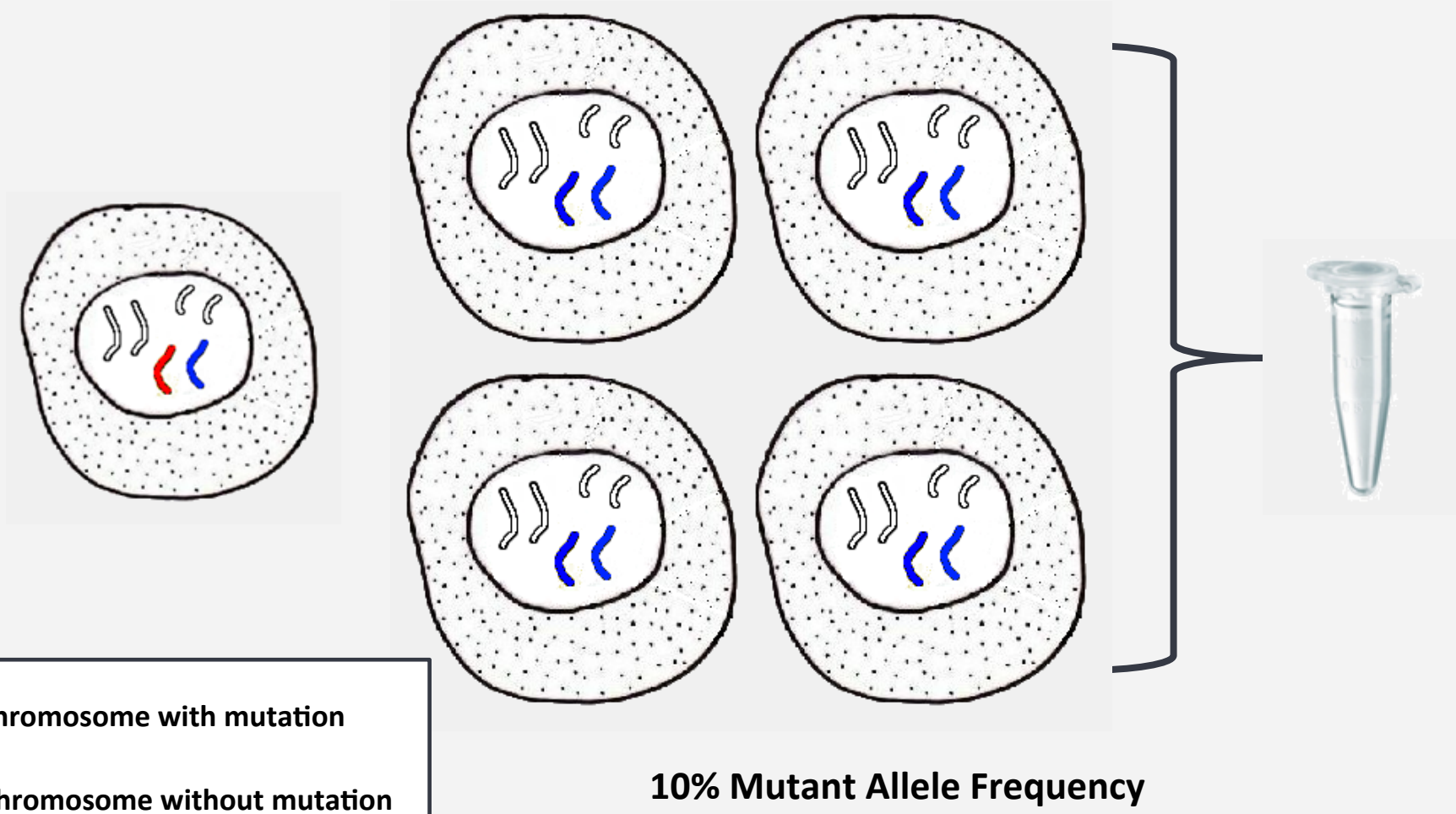

Breast cancer : 20% tumor 80% normal



# Challenges of Validating an NGS-based, Cancer Genomic Profiling Test

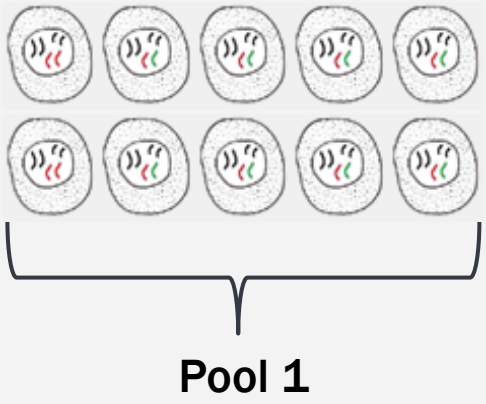
- Any Alteration Type
  - Substitutions
    - A>C, A>T, A>G, T>A, T>C, T>G, C>A, C>T, C>G, G>A, G>T, G>C
  - Short Insertions/deletions
    - A>AT, A>ATCG, ATCG>A, ATCGGCTA>TAGC, etc.
  - Gene amplifications or homozygous deletion
    - ERBB2, MYC, PTEN
- Any Position
  - Mutations can occur anywhere in the >1 Mb target region
- Any Mutant Allele Frequency
  - Mutations may be present at any mutant allele frequency
    - 1 – 100 %

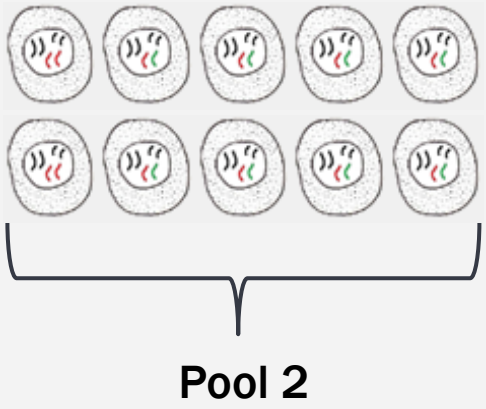
# Using Pools of Cell Line DNA to Model Somatic Mutations



# Base Substitution Detection Validation

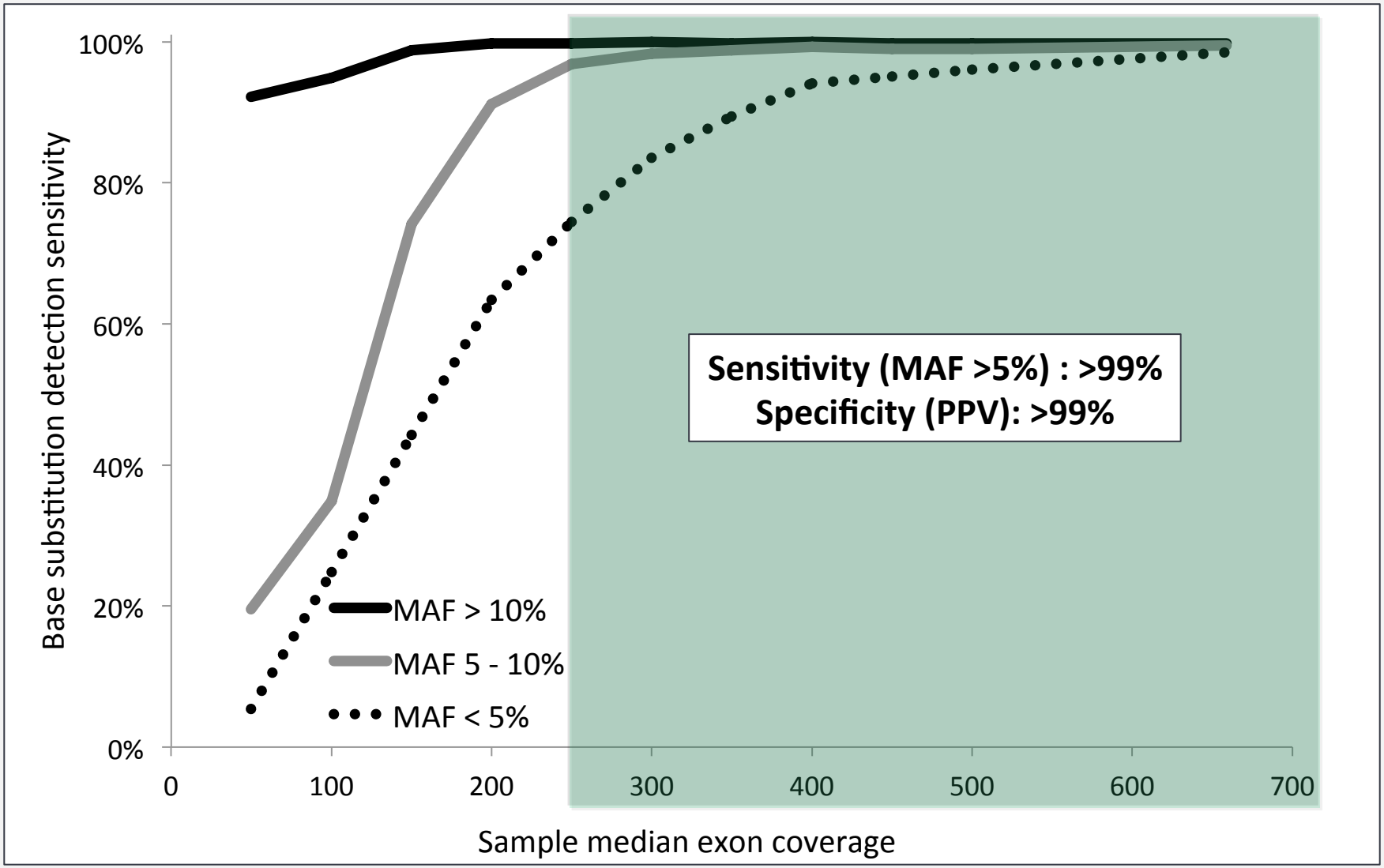
Pooling HapMap cell lines generates 2,056 base substitutions at a range of allele frequencies across the entire assay



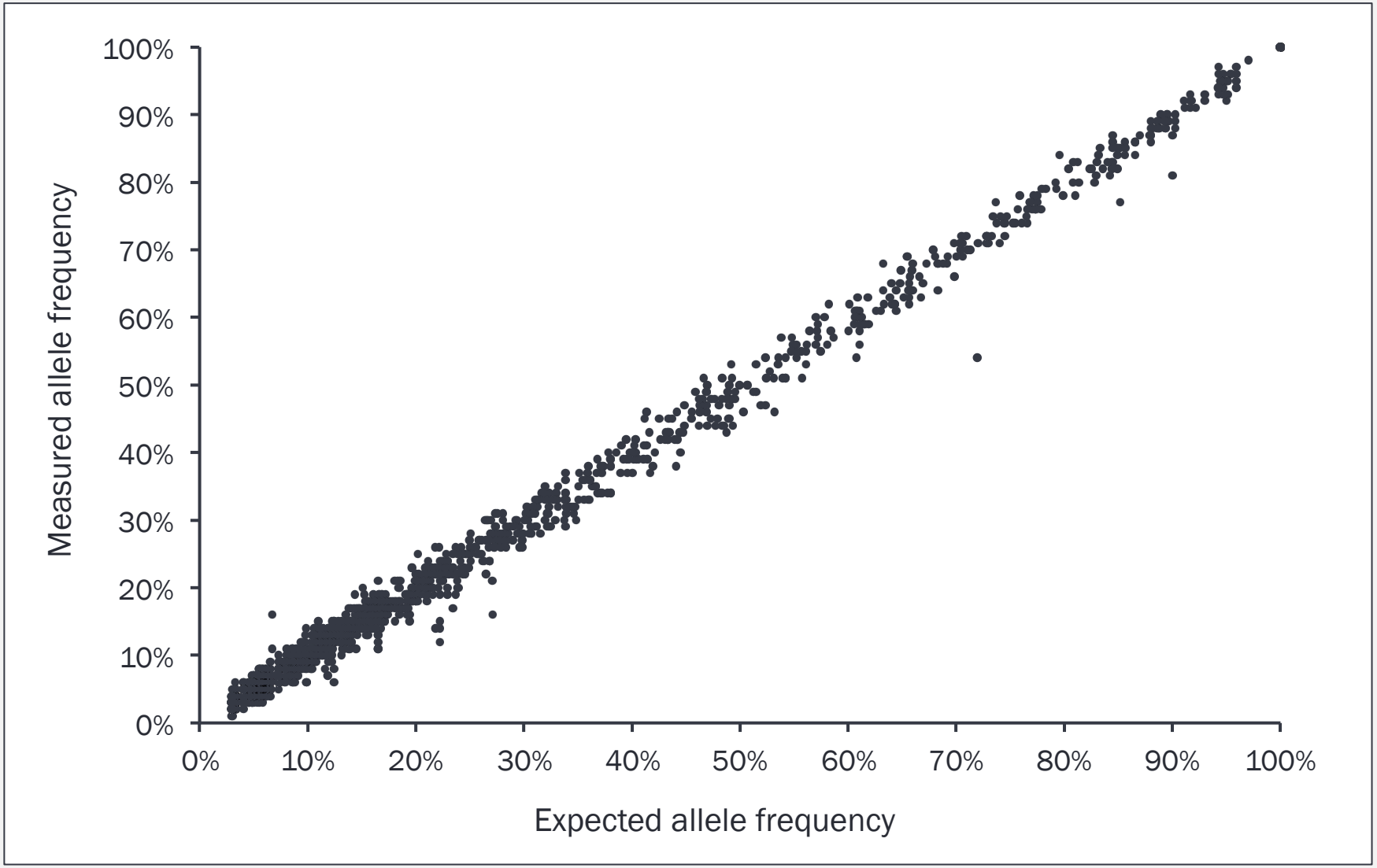




| Mutant Allele Frequency | Number of Subs | | |
|---|---|---|---|
| | Pool 1 | Pool 2 | Total |
| <5 % | 206 | 201 | 407 |
| 5 -10% | 314 | 300 | 614 |
| 10-15% | 130 | 103 | 233 |
| 15-20% | 75 | 78 | 153 |
| 20 - 100 % | 332 | 318 | 650 |
| Total | 1,057 | 1,000 | 2,057 |

# Base Substitution Detection Validation Results



# Base Substitution Detection Validation Results



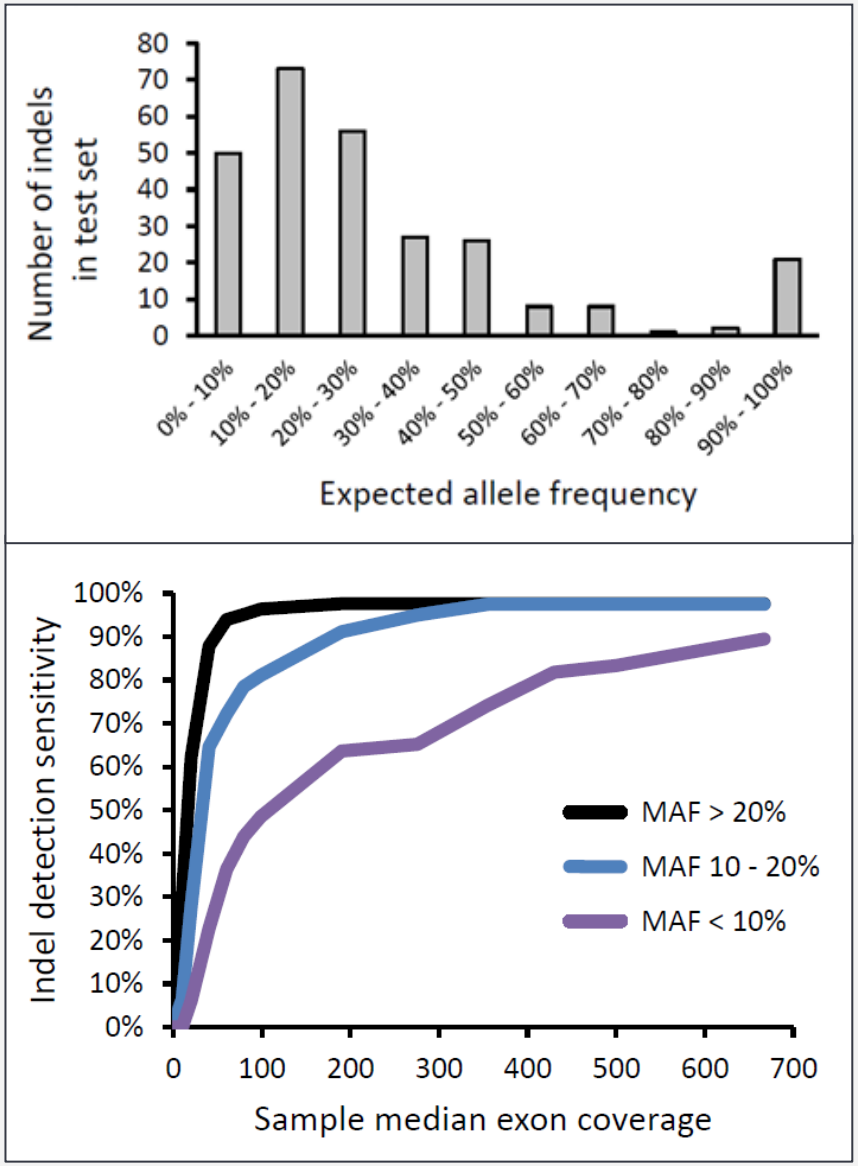
# Indel Detection Validation

28 cancer cell lines, containing 44 known InDels (1-40bp), were used to make 41 pools of 2 – 10 cell lines

*Results*

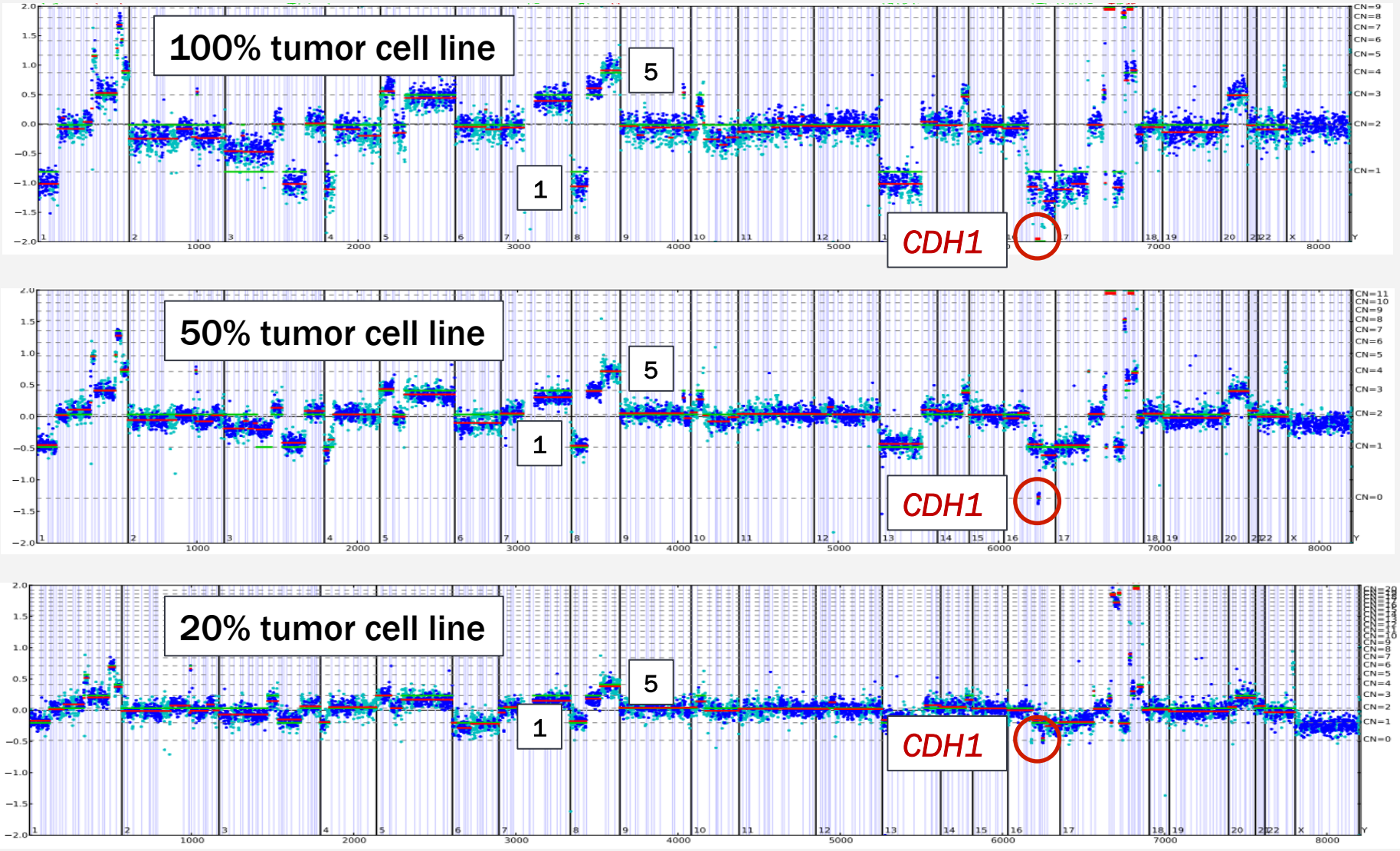
| Mutant Allele Frequency | **Sensitivity** | |
|---|---|---|
| <10% | 83% | 55 / 66 |
| 10 – 100% | **98%** | 157 / 161 |

| Mutant Allele Frequency | **Specificity (PPV)** | |
|---|---|---|
| All | **> 99%** | 872 / 875 |



# Detecting Copy Number Alterations: "CGH" From NGS, Corrected for Stromal Admixture

**Breast cancer cell line HCC2218 mixed with patient matched normal**



# Copy Number Alteration Validation Results

## Sensitivity

| Copy Number | Tumor fraction 20% | | Tumor fraction >= 30% | |
|---|---|---|---|---|
| Amplification CN ≥ 8 | 93% | 13/14 | 100% | 56/56 |
| Amplification CN ≥ 6 | 84% | 16/19 | 90% | 68/76 |
| Deletion | 89% | 8/9 | 97% | 35/36 |

## Specificity

| Copy Number | Positive Predictive Value | |
|---|---|---|
| Amplification | 100% | 84/84 |
| Deletion | 100% | 43/43 |

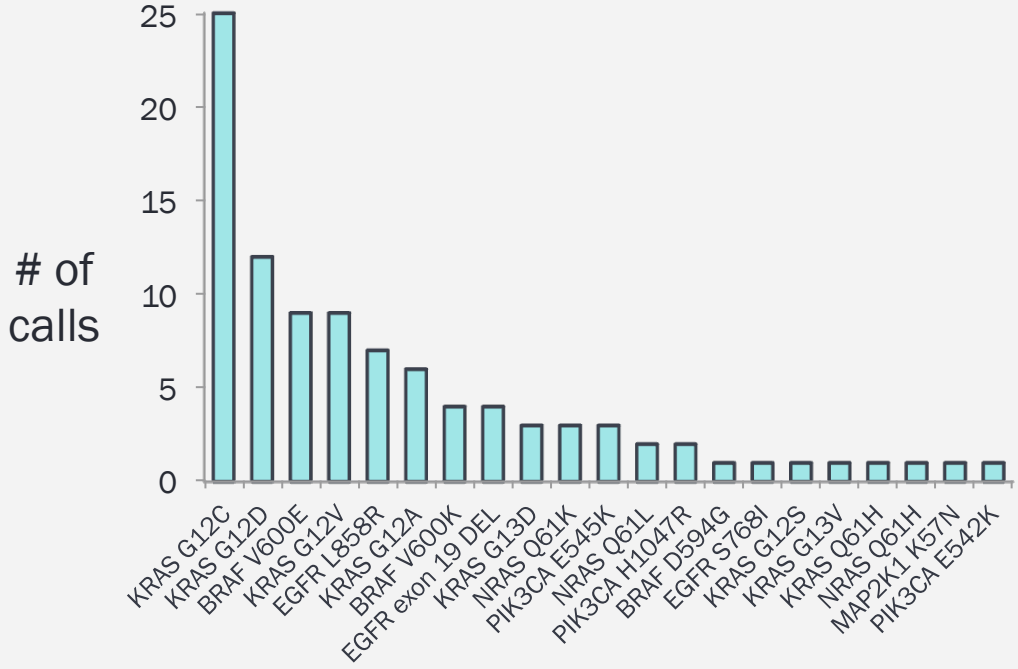
# Concordance With Other Testing Platforms: Sequenom

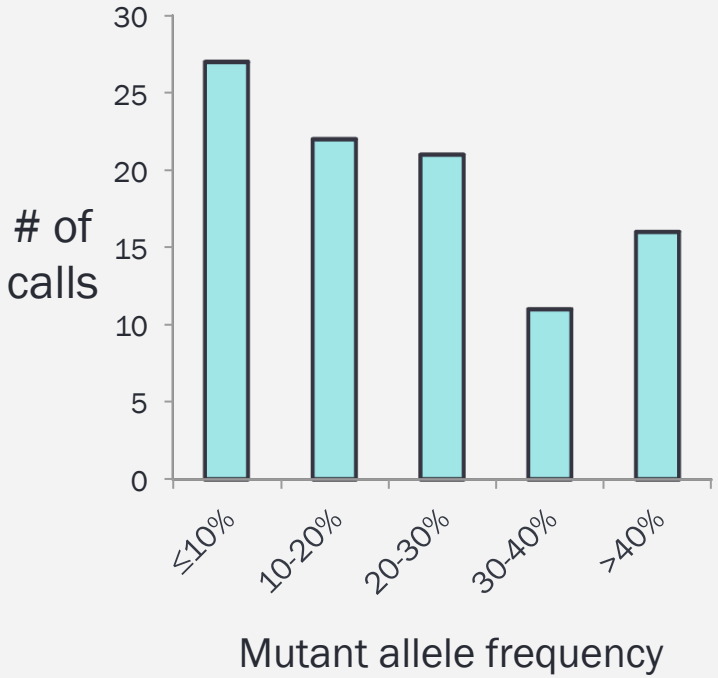
- Study samples: 118 FFPE specimens
  - 67 NSCLC, 31 colorectal cancer, 20 melanoma specimens
  - Originally tested for 91 oncogenic mutations in 8 oncogenes using Sequenom (mass-spec) in a CLIA-certified lab
- Assayed by comprehensive NGS genomic profiling test
  - DNA extracted from new 4x10µ unstained sections from original FFPE block used for Sequenom testing
  - Sequenced to average unique coverage >500x

# Concordance With Other Testing Platforms: Sequenom

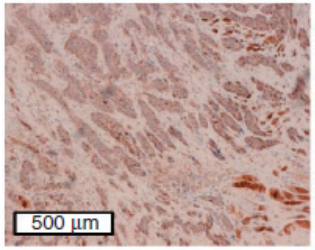
Sequenom:
101 mutations called


NGS:
104 mutations called

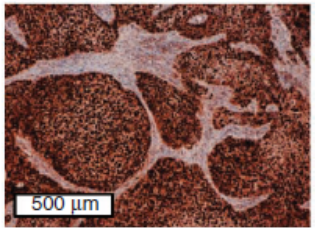


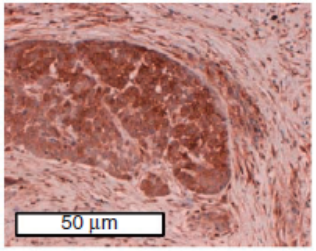
# Concordance With Other Testing Platforms: FISH/IHC



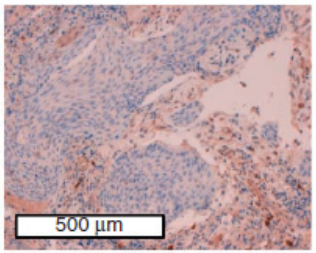

No *CCND1* amp by NGS




*CCND1* amp by NGS

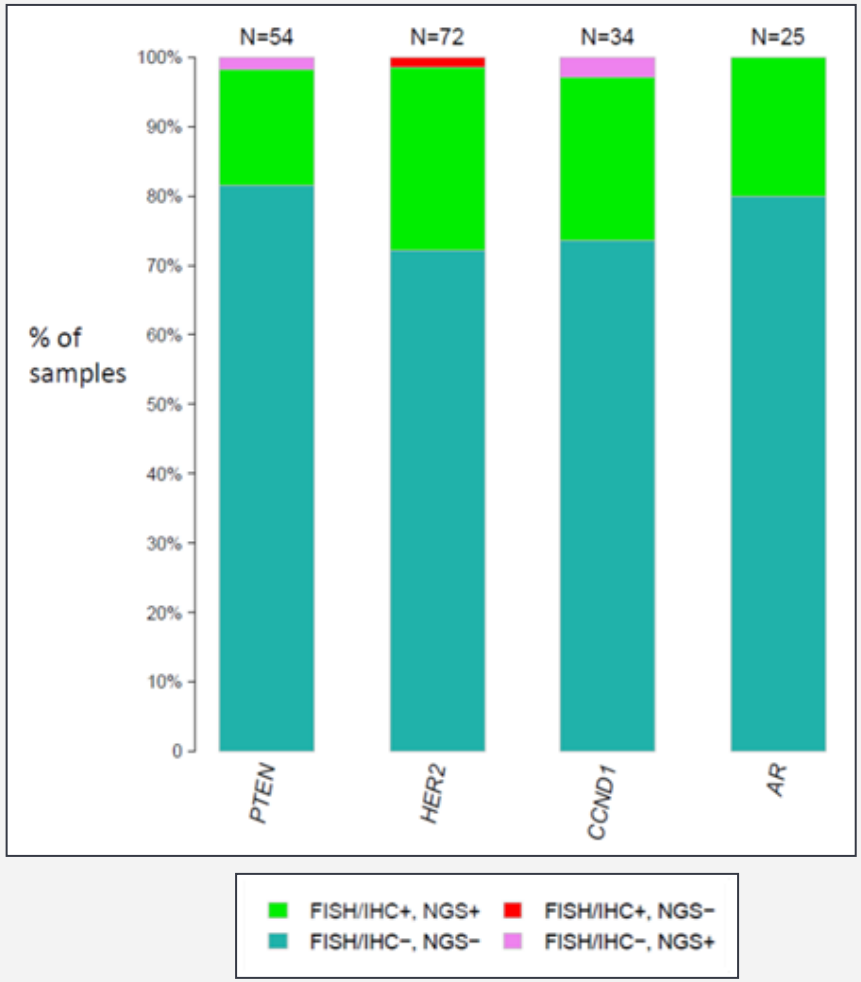



No *PTEN* loss by NGS




*PTEN* loss by NGS

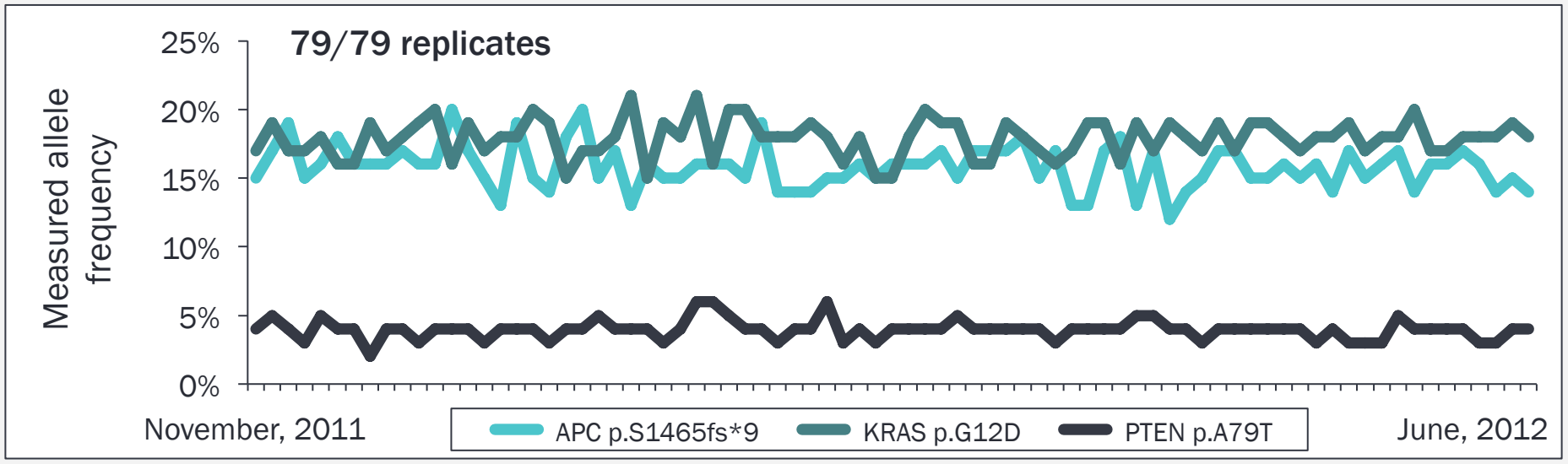
with UCL Cancer Institute, Weill Medical College, Yale School of Medicine, Albany Medical College

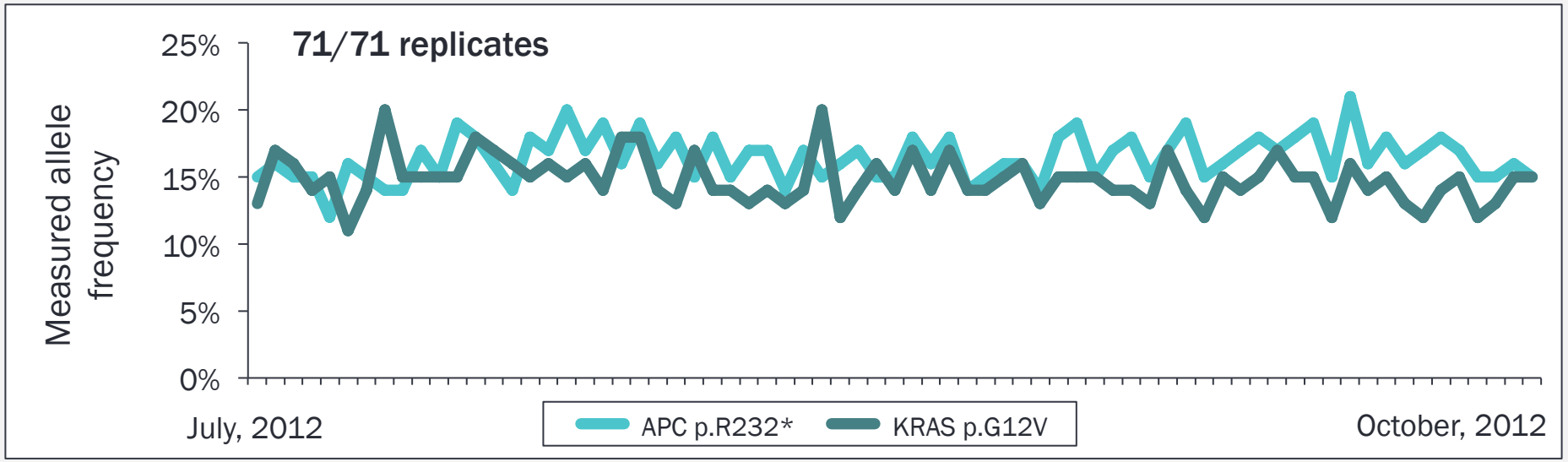


# Reproducibility

- 6 FFPE specimens, tumor content 20-60%
- 5 replicates of each specimen
- 3 inter- and 3 intra- batch comparisons
- 35 total variants detected
- 97% reproducibility across all variant calls

# Assay reproducibility



# Analytic validation study results

**Demonstration of high accuracy and reproducibility required for clinical use**

## Base Substitutions

(MAF 5-100%)

Sensitivity: >99% PPV: >99%

## Insertions/Deletions

(1-40bp, MAF 10-100%)

Sensitivity: >98% PPV: >99%

## Copy Number Alterations

(zero or ≥8 copies)

Sensitivity: >95% PPV: >99%

## Gene Fusions

Sensitivity: >95% (>99% for ALK fusion[1]) PPV: >99%

# Analytic Validation Publication

nature biotechnology

## Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing

Garrett M Frampton[1,9], Alex Fichtenholtz[1,9], Geoff A Otto[1], Kai Wang[1], Sean R Downing[1], Jie He[1], Michael Schnall-Levin[1], Jared White[1], Eric M Sanford[1], Peter An[1], James Sun[1], Frank Juhn[1], Kristina Brennan[1], Kiel Iwanik[1], Ashley Maillet[1], Jamie Buell[1], Emily White[1], Mandy Zhao[1], Sohail Balasubramanian[1], Selmira Terzic[1], Tina Richards[1], Vera Banning[1], Lazaro Garcia[1], Kristen Mahoney[1], Zac Zwirko[1], Amy Donahue[1], Himisha Beltran[2,3], Juan Miguel Mosquera[3,4], Mark A Rubin[3,4], Snjezana Dogan[5], Cyrus V Hedvat[5], Michael F Berger[5], Lajos Pusztai[6], Matthias Lechner[7], Chris Boshoff[7], Mirna Jarosz[1], Christine Vietz[1], Alex Parker[1], Vincent A Miller[1], Jeffrey S Ross[1,8], John Curran[1], Maureen T Cronin[1], Philip J Stephens[1], Doron Lipson[1] & Roman Yelensky[1]

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# Clinical Implications

## *Comprehensive Genomic Profiling Identifies Frequent Drug Sensitive EGFR Exon 19 Deletions in NSCLC Not Identified by Prior Molecular Testing*

Alexa B. Schrock, Siraj M. Ali, Garrett M. Frampton, Dana Herndon, Joel Greenbowe, Kai Wang, Doron Lipson, Roman Yelensky, Zachary Chalmers, Juliann Chmielecki, Julia A. Elvin, Mariane Wolner, Rodolfo Bordoni, Fadi Braiteh, Addie Dvir, Rachel Erlich, Mohamed Mohamed, Jeffrey S. Ross, Philip J. Stephens, Vincent A. Miller

400 NSCLC cases with EGFR exon 19 deletions identified by CGP

386 cases with Exon 19 del in classic range (743-754 aa)

14 cases with Exon 19 del in C-Helix (753-761 aa)

- 55 yo never smoker Asian M
- Negative prior EGFR testing
- 4th line: empiric erlotinib w/ 9 mo PR
- 6th line: CGP reveals exon 19 del & RET fusion not present in pretreatment specimen

(Ref Klempner et al 2015)

- 44 yo never smoker F
- T751_I759>N on CGP; neg on prior molecular testing
- Early PR to afatinib persists 8 mo
- F/u CGP on PD specimen: T790M and T751_I759>N
- Enrolls in 3rd gen EGFRi trial

17%

83%

| 12 cases with prior negative result for EGFR mutation | 59 cases with prior positive result for EGFR mutation | 5 cases with prior negative result for EGFR mutation | 1 case with prior positive result for EGFR mutation |
|---|---|---|---|

IASLC 40 YEARS

# *ALK* "negative" FISH in NSCLC

- *ALK* rearrangements identified by Comprehensive Genomic Profiling (CGP) compared to results from prior *ALK* FISH testing
- 32% of *ALK* rearranged cases identified by CGP previously tested negative by FISH

| | Positive Fish | Negative FISH | Unknown or not done |
|---|---|---|---|
| Cases | 19 | 9 | 19 |

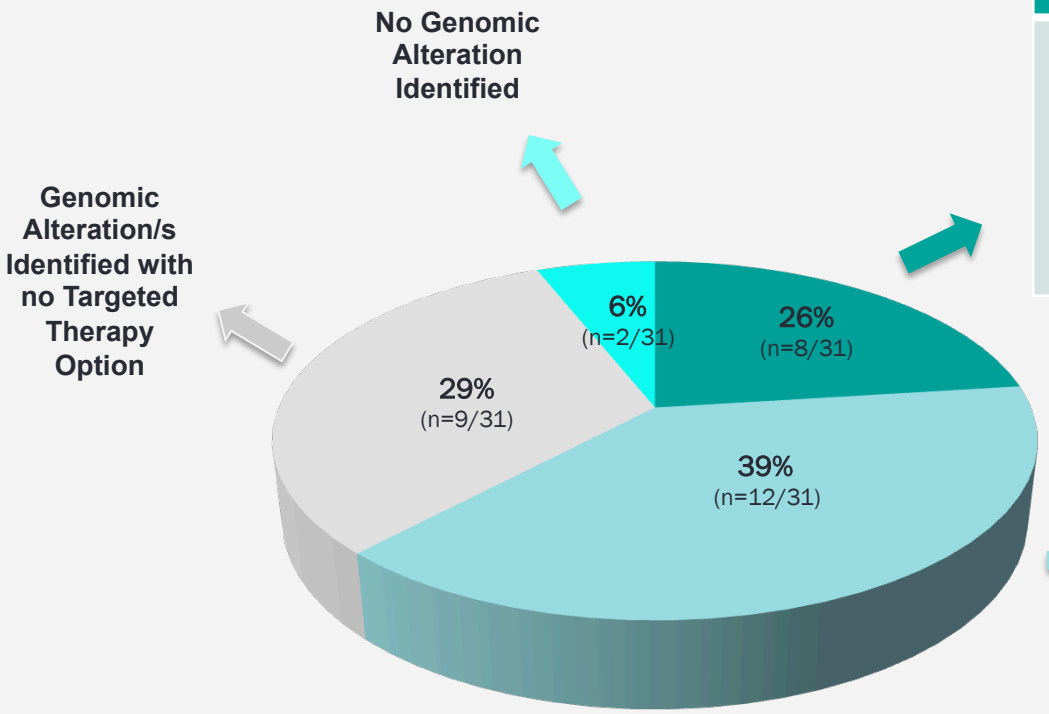
- 70% of FISH negative patients in this study responded to crizotinib

| ALK Fusion Detected on NGS | ≥Partial Response to Crizotinib | No Response to Crizotinib | Treatment History Unknown |
|---|---|---|---|
| FISH "+" Cases | 6 | 0 | 13 |
| FISH "-" Cases | 5 | 2 | 2 |

(ref: Ali AM, et al. Comprehensive Genomic Profiling Identifies a Subset of Crizotinib Responsive ALK-rearranged NSCLC Not Detected by FISH. **Accepted for publication, *The Oncologist*; March 7, 2016**)

# “Pan-Negative” NSCLC Study

- Living patients with adequate PS
- Prior testing was ***negative*** for EGFR, ERBB2, KRAS, NRAS, BRAF, MAP2K1, PIK3CA, and AKT1 by hotspot mutation testing and/or multiplex sizing; ALK, ROS1, RET by break apart FISH assays




| Genomic Alteration with Targeted Therapy in NCCN Guidelines | Patient’s Clinical Course |
|---|---|
| *EGFR* G719A | **Started erlotinib; eval pending** |
| *BRAF* V600E | Subsequently passed away |
| *SOCS5-ALK* | **Response to crizotinib** |
| *HIP1-ALK* | **Response to crizotinib** |
| *CD74-ROS1* | **Started crizotinib; eval pending** |
| *KIF5B-RET* | **Response to cabozantinib** |
| *KIF5B-RET* | **Response to cabozantinib** |
| *CCDC6-RET* | Stable on chemo; candidate for cabozantinib |

RESULTS: Highly validated, hybrid capture-based NGS genomic profiling assay identified clinically relevant genomic alterations in 65% of tumors deemed negative by earlier, extensive testing.

(**Ref: Drilon et al.**, Broad, hybrid capture-based next-generation sequencing identifies actionable genomic alterations in lung adenocarcinomas otherwise negativefor such alterations by other genomic testing approaches. *Clin Cancer Res (2015): volume 21: 3631–9.*

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# Summary

# Key Questions

| Question | Yes | No |
|---|---|---|
| Does the lab have a peer-reviewed, published analytic validation and/or have they successfully completed the MolDx Technical Assessment? | ✓ | |
| Does the lab provide the raw data for review? | ✓ | |
| Is the lab NY-State approved? | ✓ | |
| Were validation specimens representative of actual patient samples? | ✓ | |
| Were all types of alterations/variations represented in the validation specimens? | ✓ | |
| Were the sample sizes large enough and statistics appropriate to ensure narrow confidence intervals? | ✓ | |
| Was the entire process validated (including extraction) to ensure reproducibility and robustness? | ✓ | |
| Was a comparator method used, if available? | ✓ | |
| Did the precision studies include intra-assay as well as inter-assay, between operators and over multiple days? | ✓ | |

Questions?

# What does “99% accurate” mean?

- Could mean a variety of things
  - Is the patient assigned to the correct clinical category? 99 of 100 pts in the correct treatment group…..
  - Is each gene on the assay assigned to the correct functional category? 99 of 100 tests identify the function of this gene correctly…..
  - How often is each base pair of the control a match the reference sequence? Depending on the size of the assay 99% accuracy could be alarming. In a test which covers just 10kb there would be 100 errors of base pair calls…scale to 100kb to 1 Mb to 10 Mb……