

Molecular Testing in Cytology Specimens

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Disclosures

- Research funding to my institution from Genentech, Bristol Myers Squibb
- Consulting income to my institution from Genentech, Lilly
- Personal consulting fees from AstraZeneca

Agenda

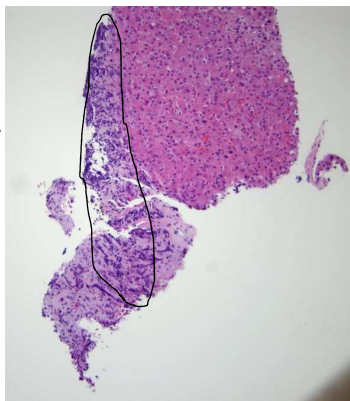
- Basics of sample requirements for molecular testing
- Leveraging cytology specimens for molecular biomarker testing
- Effects of sample types on sequencing quality metrics
- Direct testing of diagnostic fluids
- Validation of cytology samples

Sample adequacy considerations

Quantity

Tissue size = DNA content

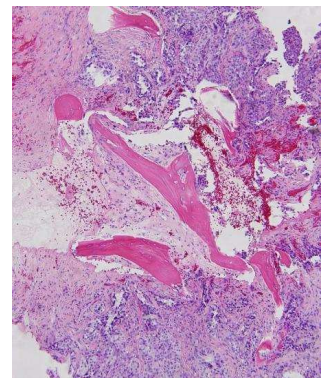
Tumor content = mutant fraction



Quality

Adverse factors:

Delayed fixation
Inadequate fixation
Excessive fixation
Acid or heavy-metal fixatives
(decalcification)

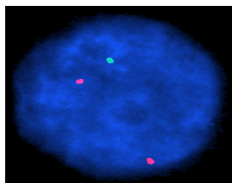
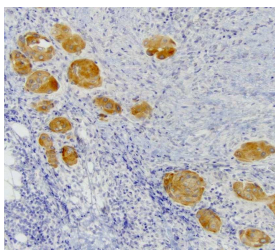


Defining “Specimen Adequacy”:

- No universal definition– this depends on the validated performance characteristics and limitations of the test being requested
- Interplay between nucleic acid quantity and quality
 - Lower input quantity may be acceptable if quality is high
 - Higher input quantity may be required if quality is low

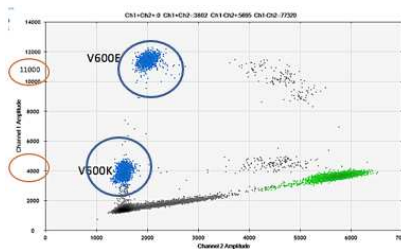
Hadd AG et al. *J Mol Diagn.* 2013, 15:234-247.

In situ assays



50-100 *cells*

High sensitivity single gene tests



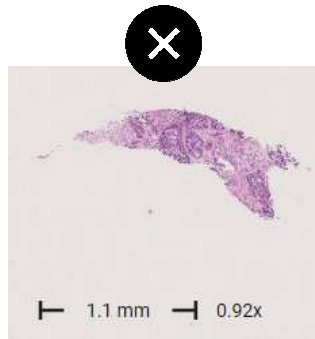
5-15 ng
nucleic acids

Panel NGS

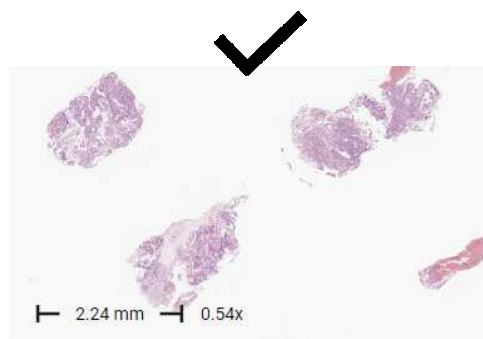


50-100s ng
nucleic acids

Sample size matters

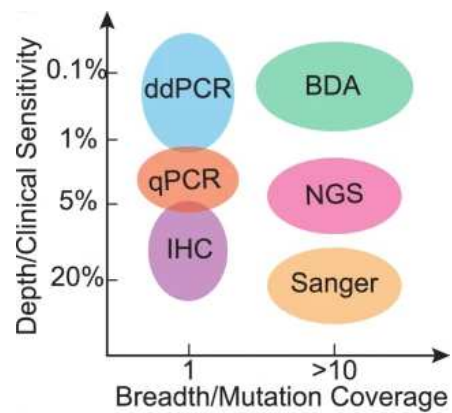


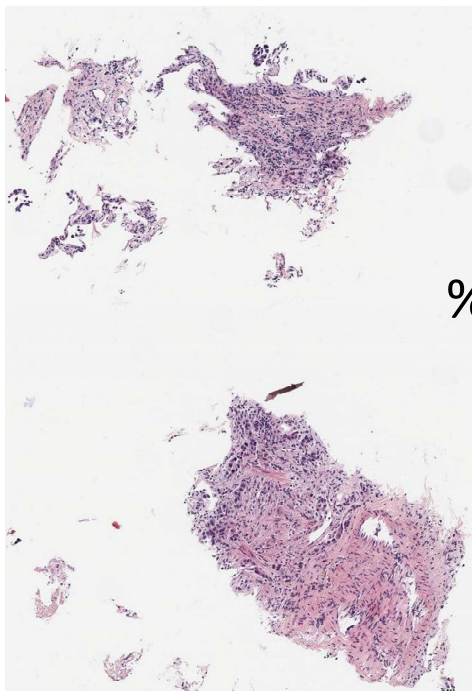
<2mm tumor → >80% failure rate



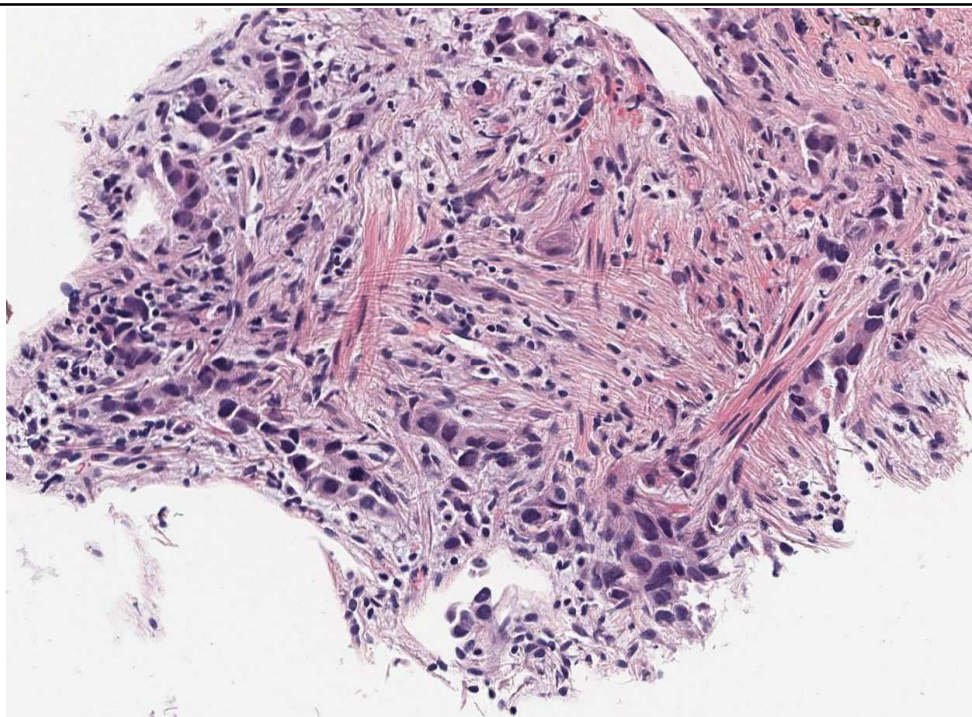
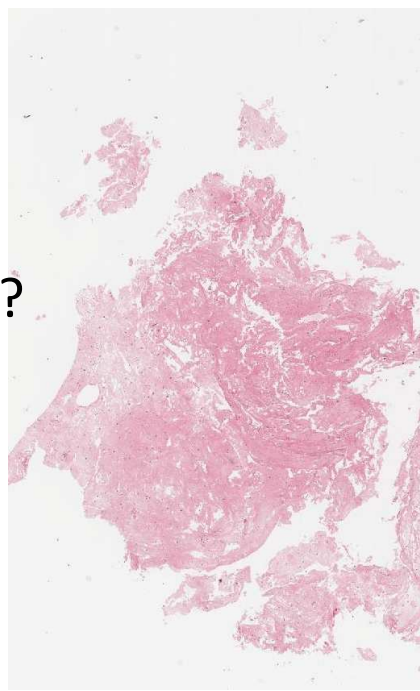
>3mm tumor → <20% failure rate

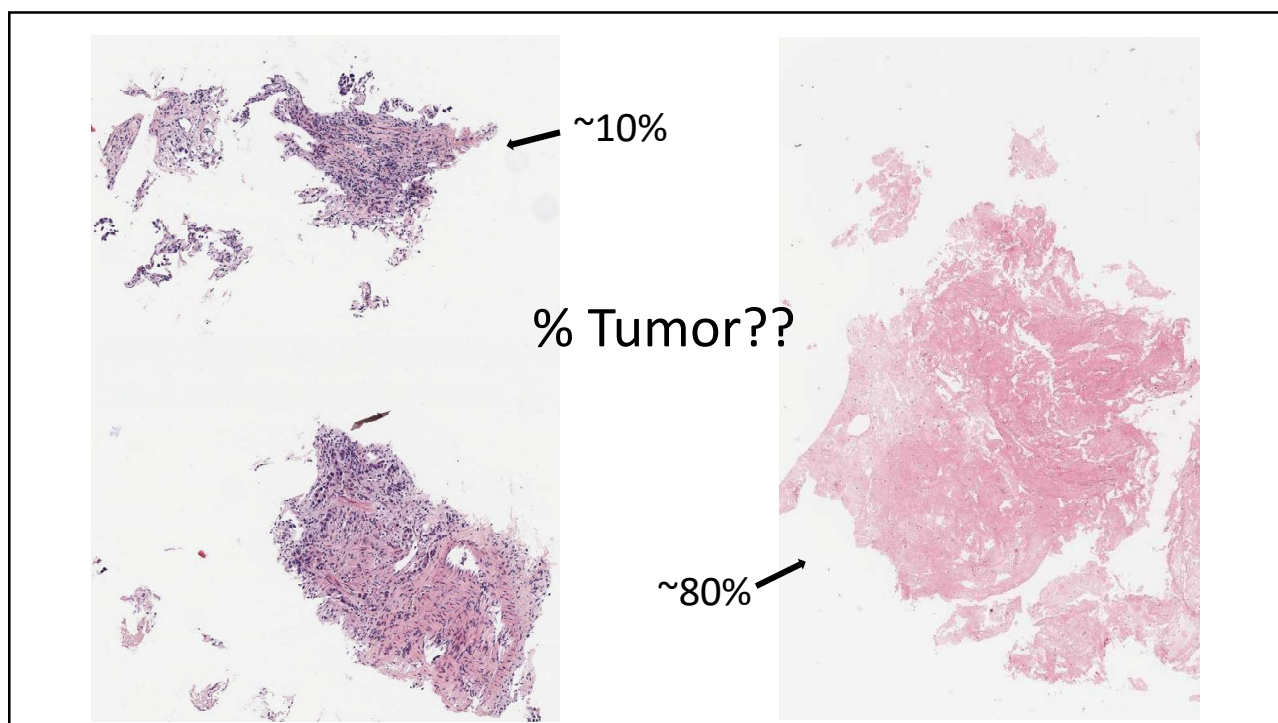
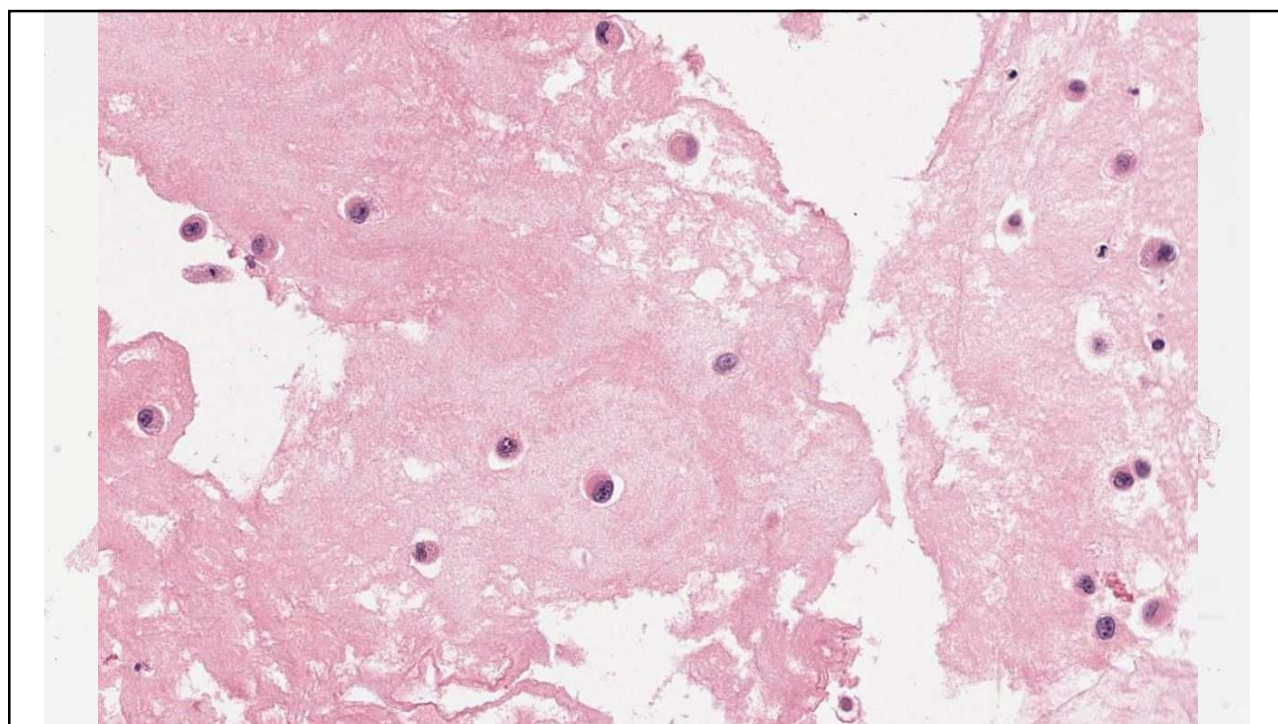
Quantity: % tumor content



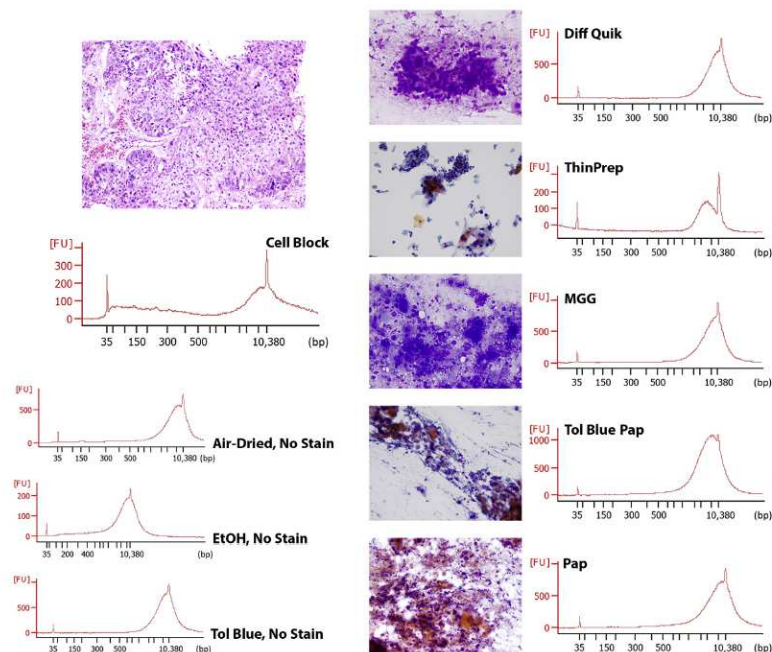


% Tumor??





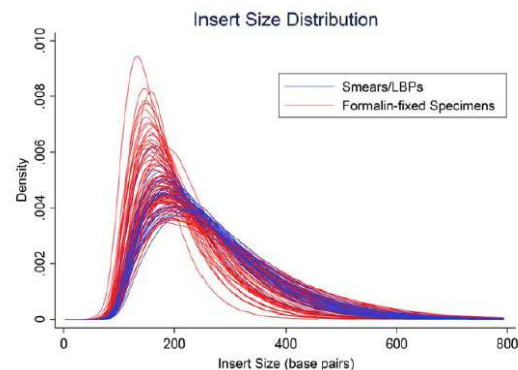
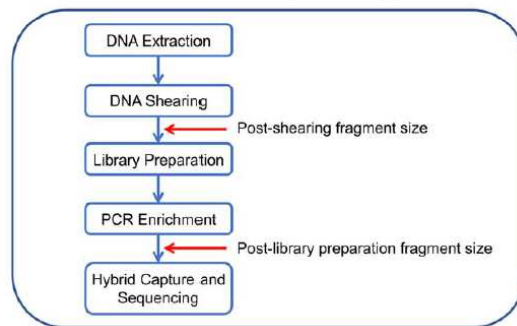
Embracing the non-FFPE sample



Larger target DNA fragments from smears/liquid based cytology preps



Next-Generation Sequencing Workflow



Hwang et al. Cancer Cytopathol. 2017 Oct;125(10):786-794.

Superior sequencing quality metrics with smears/liquid based cytology preps

TABLE 3. Comparison of Quality Metrics

| Quality Metric | Smears/LBPs | Core Biopsies | Cell Blocks | P |
|--|--------------------------------|--------------------------------|--------------------------------|-------|
| Adequacy rate, n/N (%) | 23/26 (88) | 77/87 (89) | 29/30 (97) | .41 |
| Initial DNA concentration, ng/μL | 6.84 | 7.70 | 10.45 | .70 |
| Postshearing fragment size, bp | 317.2 | 411.7 | 385.8 | <.001 |
| Post-library preparation fragment size, bp | 356.3 | 336.3 | 355.6 | .21 |
| Fragment size difference, bp | 52.5 | -72.3 | -47.6 | <.001 |
| Insert size, bp | 191 | 177 | 179 | <.001 |
| Total reads ^a | 2.79 × 10 ⁷ [1.085] | 2.48 × 10 ⁷ [0.983] | 2.50 × 10 ⁷ [1.002] | .29 |
| Passing-filter reads aligned ^a | 2.59 × 10 ⁷ [1.085] | 2.30 × 10 ⁷ [0.982] | 2.29 × 10 ⁷ [1.003] | .33 |
| Percent passing-filter unique reads aligned ^a | 96.3% [1.001] | 94.3% [1.001] | 94.1% [1.000] | .70 |
| Mean target coverage ^a | 400.3% [1.181] | 156.0% [0.989] | 147.8% [1.006] | .04 |
| Percentage of loci with >100× coverage ^a | 97.2% [1.013] | 76.2% [0.988] | 77.0% [1.003] | .24 |
| Percent duplication ^a | 32.0% [0.929] | 70.5% [1.001] | 70.5% [0.996] | <.001 |
| Percent selected bases ^a | 49.5% [1.019] | 49.0% [1.010] | 48.7% [1.003] | .14 |
| Percent usable bases on bait ^a | 26.7% [1.049] | 11.1% [1.002] | 10.7% [0.999] | .03 |

Abbreviations: bp, base pair; LBP, liquid-based preparation.

Median values are presented.

^aValues within square brackets are values normalized by the flow cell average; P values are based on the normalized values.

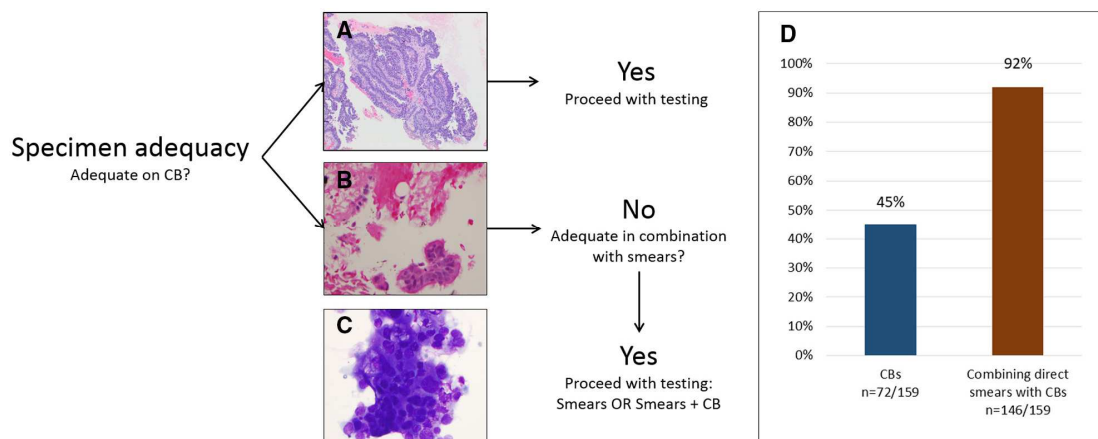
Hwang et al. Cancer Cytopathol. 2017 Oct;125(10):786-794.

Molecular Workflow Incorporating Cytology Slides



Hwang et al. Cancer Cytopathol. 2017 Oct;125(10):786-794.

Smear preps validated for RNAseq for fusion detection



Ramani et al. Cancer Cytopathology. 2021 May;129(5):374-382.

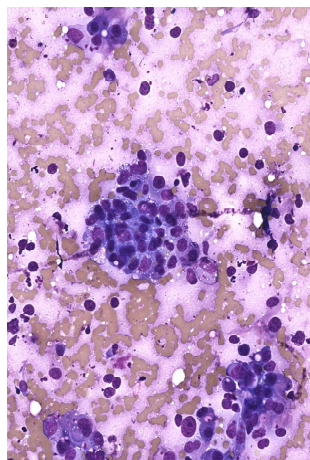
2018 AMP/CAP/IASLC guidelines:

ANY cytology sample with adequate cellularity is ok for testing,
including smear preps:

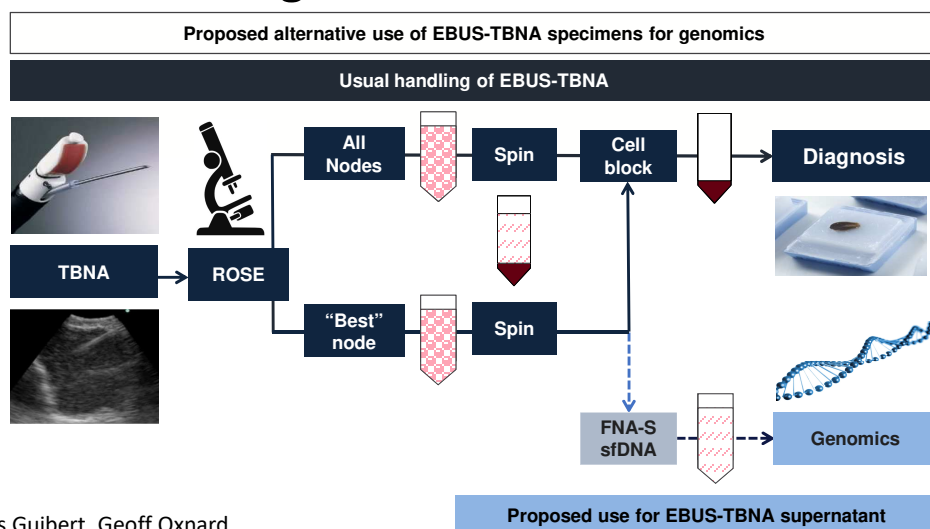
Sequencing quality metrics \geq to FFPE samples

Hwang et al. *Cancer Cytopath* 2017

Roy-Chowdhuri et al. *Mod Pathol* 2017



Cytology supernatant – an overlooked genomic testing resource

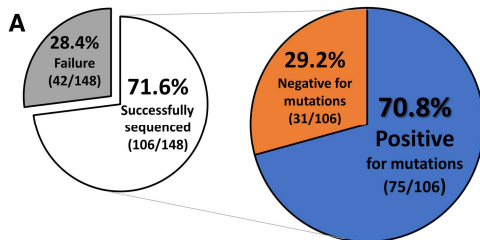


Mutation detection in cell free DNA from cytology supernatants

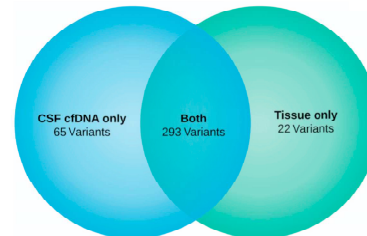
| Reference | Supernatant source | n | Concordance with FFPE | PMID |
|---------------------------|-----------------------------------|-----|-----------------------|----------|
| Perrone et al. 2021 | Body fluid or FNA rinse fluid | 30 | 74% | 34265180 |
| Wu et al. 2020 | CT-guided or EBUS FNA rinse fluid | 214 | 97.2% | 32286726 |
| Hannigan et al. 2019 | FNA rinse fluid | 35 | 97% | 30887015 |
| Janaki et al. 2019 | Endobronchial FNA rinse fluid | 30 | 100% | 30933438 |
| Roy-Chowdhuri et al. 2018 | FNA rinse fluid | 35 | 100% | 29463880 |

Cell free DNA testing from discarded CSF specimens

Cell free DNA from cerebral spinal fluid in patients with leptomeningeal metastases



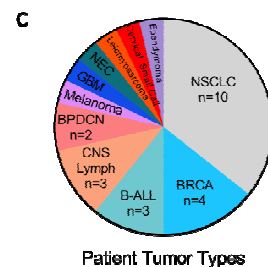
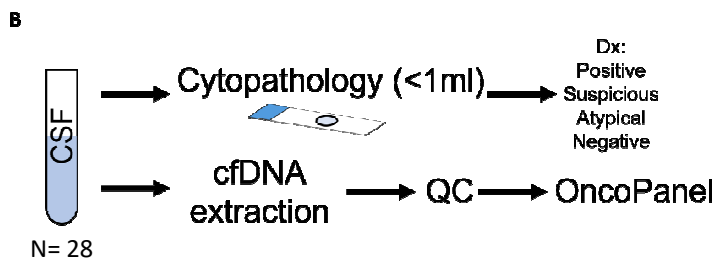
High sequencing success rates for cfDNA isolated from CSF in patients with leptomeningeal spread, including those with negative cytology.



Comparison of CSF and tissue sequencing reveals tumoral heterogeneity.

Bale et al. J Mol Diagn. 2021 Jun;23(6):742-752.

Validation of CSF cfDNA for tumor variant detection from “discarded” cytology specimens

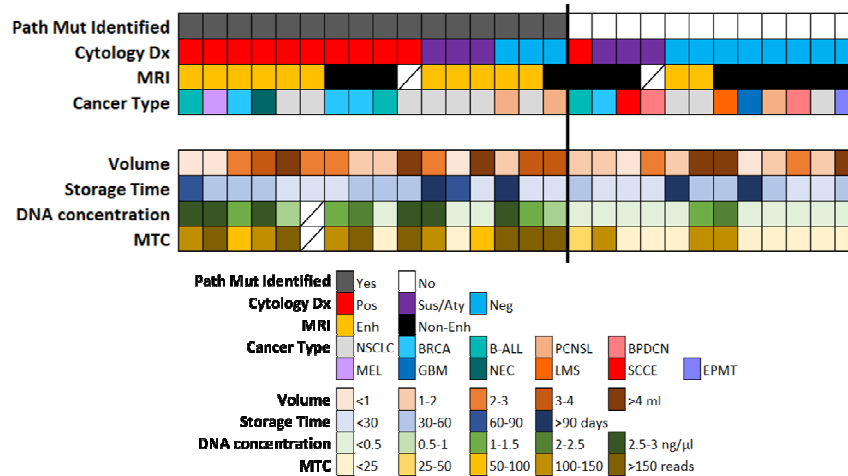


Performance of NGS from CSF cfDNA relative to tumor tissue

| | PPA | PPV |
|--|-----|------|
| Truncal pathogenic variant in POS, SUS, ATY, NEG | 56% | 100% |
| Truncal pathogenic variant in POS, SUS, ATY | 79% | 100% |

Neil A et al. Manuscript under review.

Factors influencing likelihood of mutation detection by NGS



Neil A et al. Manuscript under review.

Validation principles

Verification/Validation

UNMODIFIED FDA-Approved or Cleared Tests – Requirements

For UNMODIFIED FDA-approved or FDA-cleared tests, laboratories must verify that test(s) perform(s) as expected by obtaining data on:

- Accuracy
- Precision
- Reportable range
- Linear range (for quantitative assays)
- Reference intervals (normal values) for laboratory patient population

These performance characteristics are published in the manufacturer's package insert.

MODIFIED FDA-Approved or non-FDA Cleared Tests – Requirements

For MODIFIED FDA-Approved tests or for non-FDA cleared tests (e.g., Laboratory Developed Procedures (LDP), previously LDTs) laboratories must **establish** the following performance characteristics:

- Accuracy
- Precision
- Reportable range
- Linear range (for quantitative assays)
- Reference intervals (normal values) for laboratory patient population
- Analytic sensitivity
- Analytic specificity

For some tests there may be additional parameters which are necessary to assess:

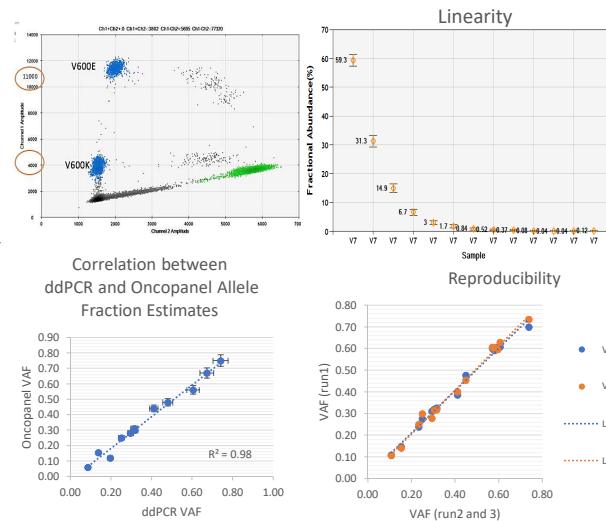
- Frequency or call rate for genotyping assays
- Specimen stability
- Carryover (e.g., well-to-well cross-contamination for automated nucleic acid extraction)

<https://www.amp.org/AMP/assets/File/resources/201503032014AssayValidationWhitePaper.pdf?pass=38>

Lab developed procedure validation – BWH CAMD BRAF V600E/K ddPCR

N=55 Samples
(FFPE/cytology
preps/fresh frozen)

- Accuracy
- Precision
- Linear Range
- Reportable Range
- Limit of Detection/Blank
- Analytic sensitivity
- Analytic specificity



- Establish reporting parameters
- Acceptable sample types
- Qualitative/quantitative requirements

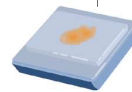
Validation of a “new” sample type



| Cytology Case | Cytology Diagnosis | Surgical Case | Surgical Diagnosis | BRAF mutation status |
|---------------|------------------------------|---------------|--|----------------------|
| BC1 | AUS | BS1 | Nodular hyperplasia | WT |
| BC2 | Malignant (PTC) | BS2 | Papillary thyroid carcinoma | V600E |
| BC3 | Malignant (PTC) | BS3 | Papillary thyroid carcinoma | V600E |
| BC4 | Malignant (PTC) | BS4 | Papillary thyroid carcinoma | WT |
| BC5 | Metastatic thyroid carcinoma | BS5 | Metastatic poorly differentiated thyroid carcinoma | WT |
| BC6 | AUS | BS6 | Hurthle cell adenoma | WT |
| BC7 | AUS | BS7 | Follicular adenoma | WT |
| BC8 | FOL | BS8 | NIFTP | WT |
| BC9 | Suspicious for PTC | BS9 | Papillary thyroid carcinoma | V600E |
| BC10 | Malignant (PTC) | BS10 | Papillary thyroid carcinoma | V600E |



VS



Kristine Wong, BWB

ThinPrep fluid vs Tissue (gold standard): Concordance and reproducibility analysis

| Cytology Case | Volume Used (ml) | Pellet Size | Pellet Concentration (ng/ul) | Supernatant Concentration (ng/ul) | Surgical Specimen BRAF %VAF | ThinPrep Specimen BRAF %VAF |
|---------------|------------------|-----------------|------------------------------|-----------------------------------|-----------------------------|-----------------------------|
| BC1 | 4 | Moderate | 9.2 | 0.20 | 0.0% | 0.0% |
| BC2 | 3 | None | 1.8 | 0.35 | 36.2% | 34.3% |
| BC3 | 4 | Very Small | 0.2 | 0.26 | 16.6% | 8.0% |
| BC4 | 3 | Small | 17.4 | 0.23 | 0.0% | 0.0% |
| BC5 | 4 | None/Very Small | 5.9 | 0.30 | 0.0% | 0.0% |
| BC6 | 3 | None | 0.3 | 0.29 | 0.0% | 0.0% |
| BC7 | 4 | Small | 2.1 | 0.22 | 0.0% | 0.0% |
| BC8 | 4 | Small | 6.3 | 0.26 | 0.0% | 0.0% |
| BC9 | 2.5 | None | 0.79 | 0.24 | 36.2% | 15.8% |
| BC10 | 4 | Large Pellet | 2.0 | 0.25 | 36.7% | 22.2% |

| Case | Replicate 1 VAF | Replicate 2 VAF |
|------|-----------------|-----------------|
| BC1 | 0.00 | 0.00 |
| BC2 | 0.34 | 0.34 |
| BC3 | 0.07 | 0.09 |
| BC4 | 0.00 | 0.00 |
| BC5 | 0.00 | 0.00 |
| BC6 | 0.00 | 0.00 |
| BC7 | 0.00 | 0.00 |
| BC8 | 0.00 | 0.00 |
| BC9 | 0.17 | 0.15 |
| BC10 | 0.23 | 0.22 |

| | | ThinPrep pellet | |
|-------------------|------------|-----------------|---|
| Surgical specimen | BRAF V600E | 4 | 0 |
| | BRAF WT | 0 | 6 |
| | | | |

Concordance 100%
Reproducibility 100%

Take home points

- Understand your assays, including nucleic acid input requirements and sensitivity
- Advocate for use of non-FFPE samples in your local lab
- Consider all your options, as the steward of the specimens throughout their lifecycle
- Work with your molecular pathologist to validate “nonconventional” sample types
- Validating a new sample type for use on an existing local assay can be straightforward