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
- Personal consulting fees from Lilly

Agenda

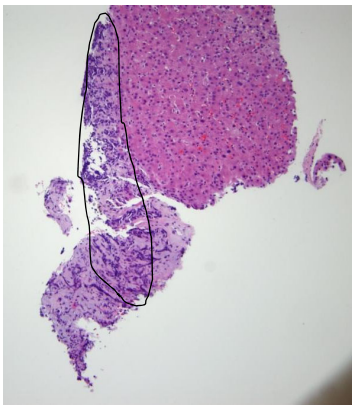
- Understand the basics of sample requirements for molecular testing
- Leveraging different types of cytology specimens/fluids for molecular biomarker testing
- Review the literature on best practices for cytology specimen handling for pathologists and proceduralists

Sample adequacy considerations

Quantity

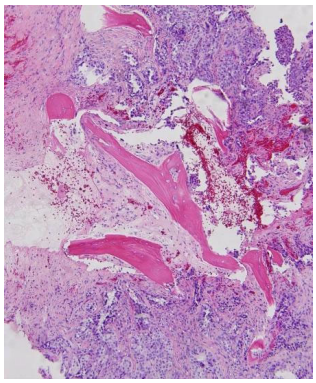
Tissue size = DNA content

Tumor content = mutant fraction



Quality

<u>Adverse factors:</u>
Delayed fixation
Inadequate fixation
Excessive fixation
Acid or heavy-metal fixatives (decalcification)



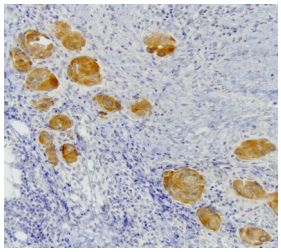
Defining "Specimen Adequacy":

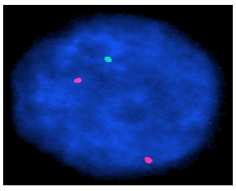
- No universal definition– this depends on the validated performance characteristics and limitations of the test being requested

- 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.

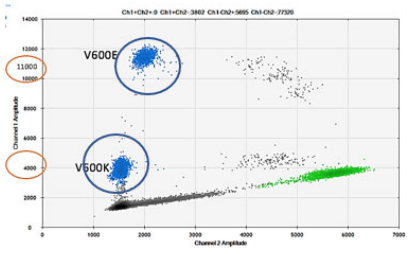
In situ assays





50-100 *cells*

High sensitivity single gene tests

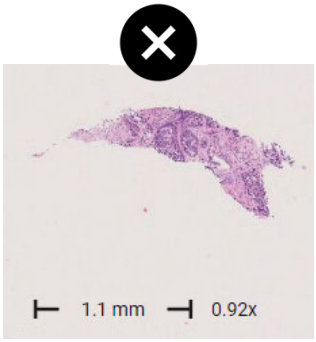


5-15 ng nucleic acids

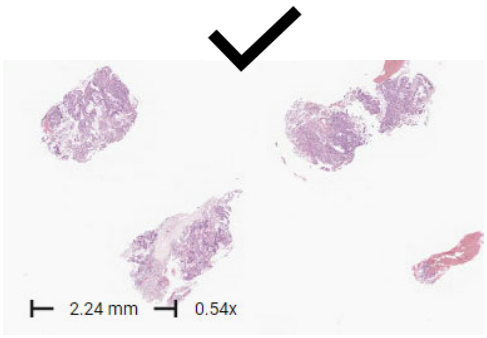
Panel NGS

50-100s ng nucleic acids

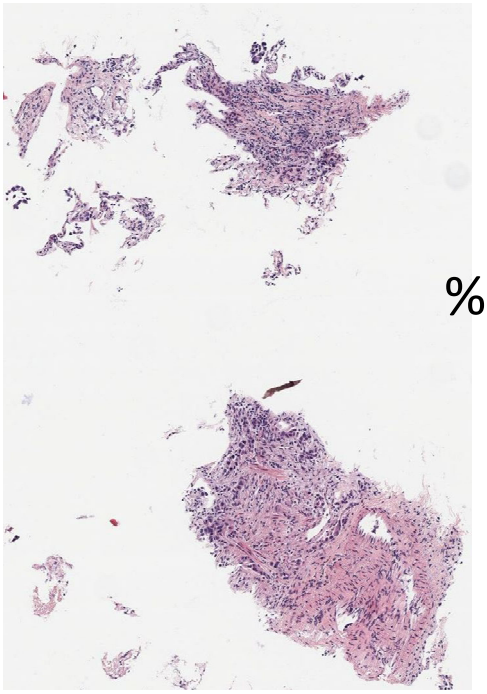
Sample size matters



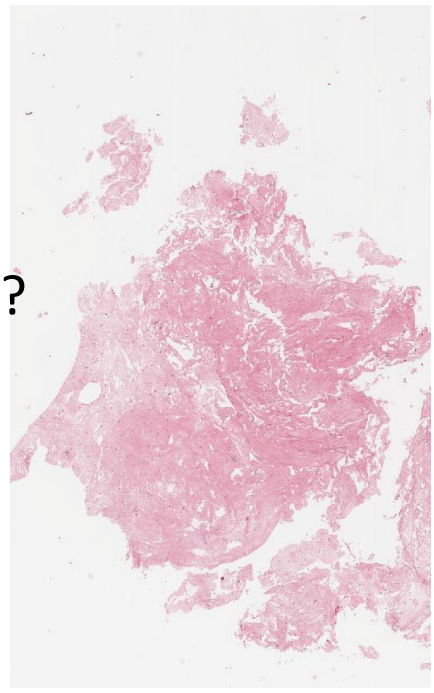
<2mm tumor → >80% failure rate

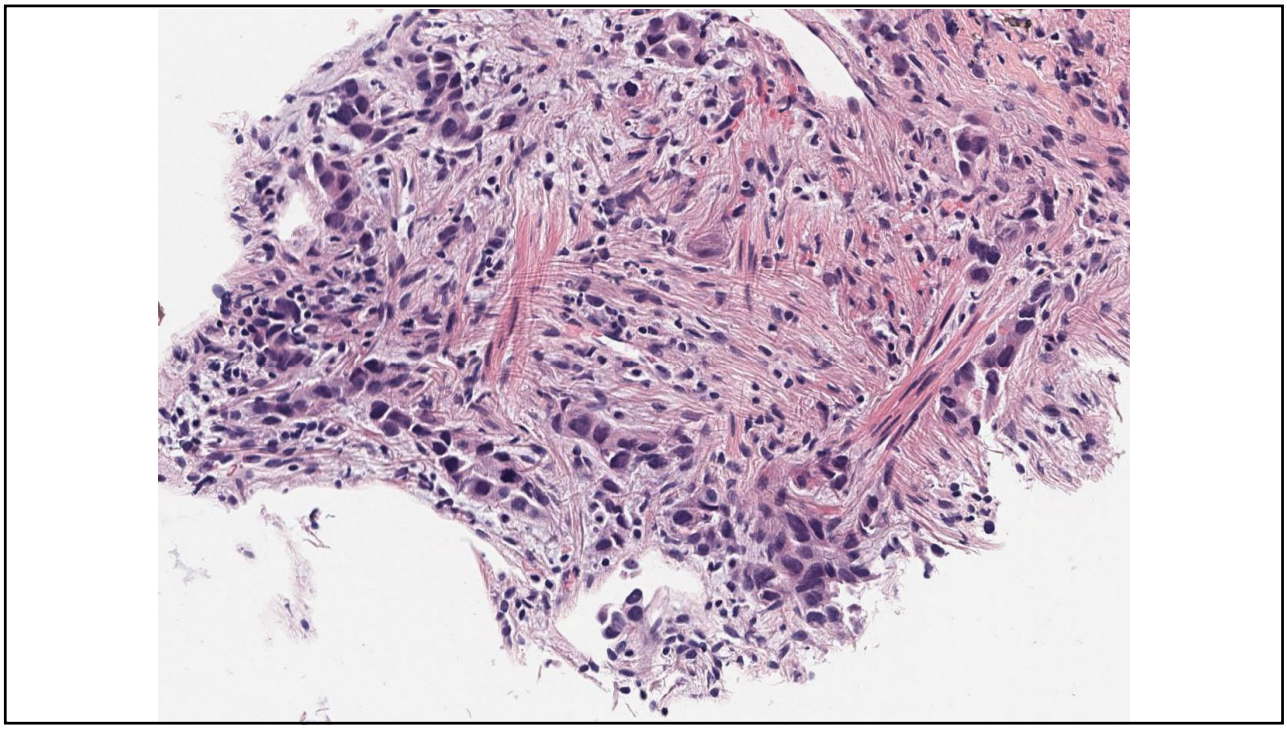


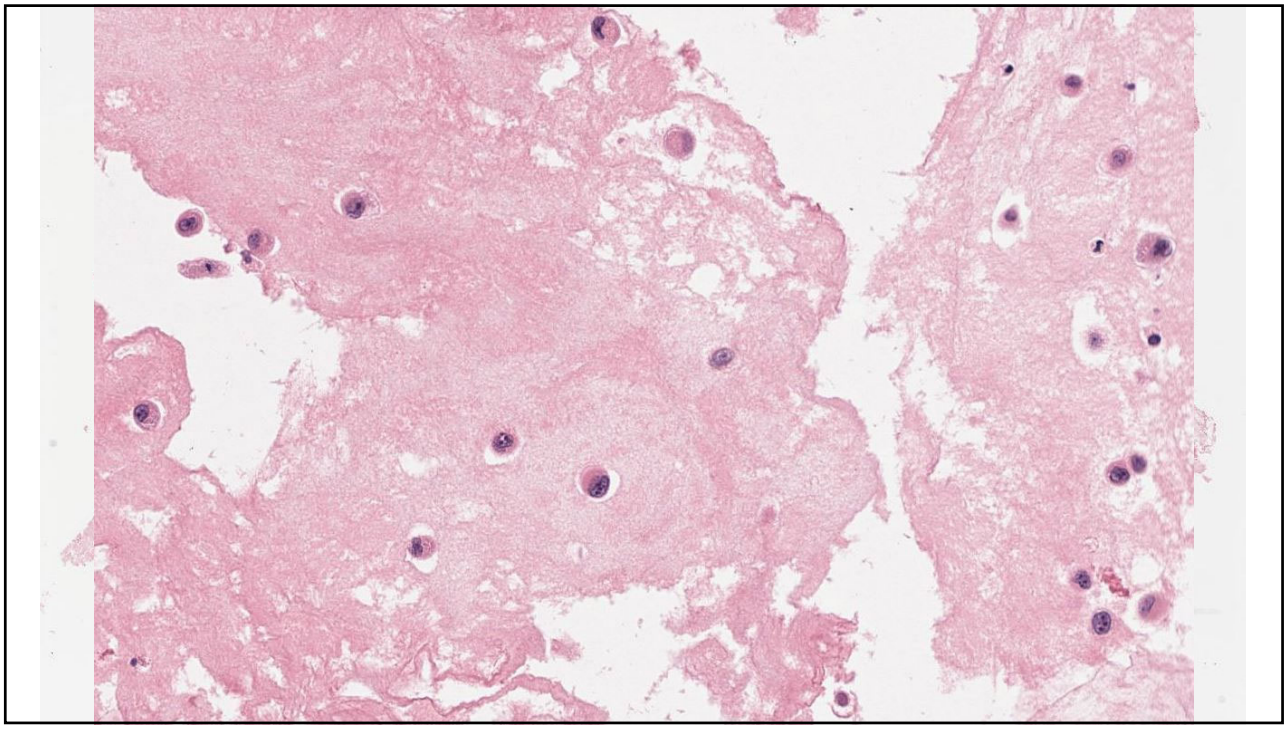
>3mm tumor → <20% failure rate

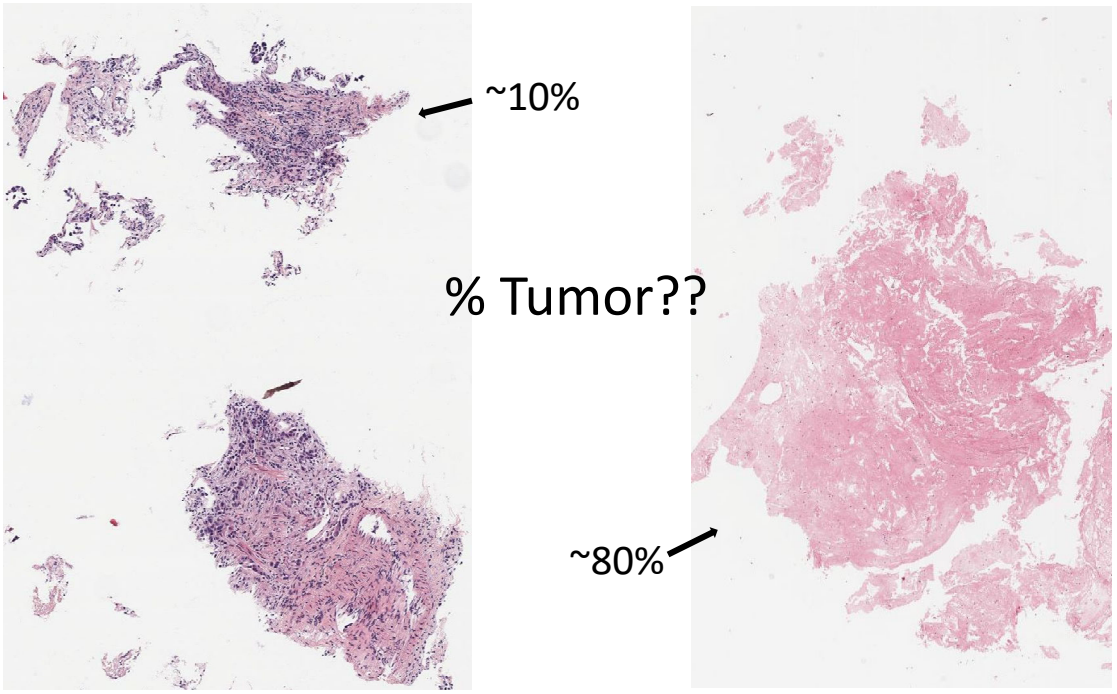


% Tumor??

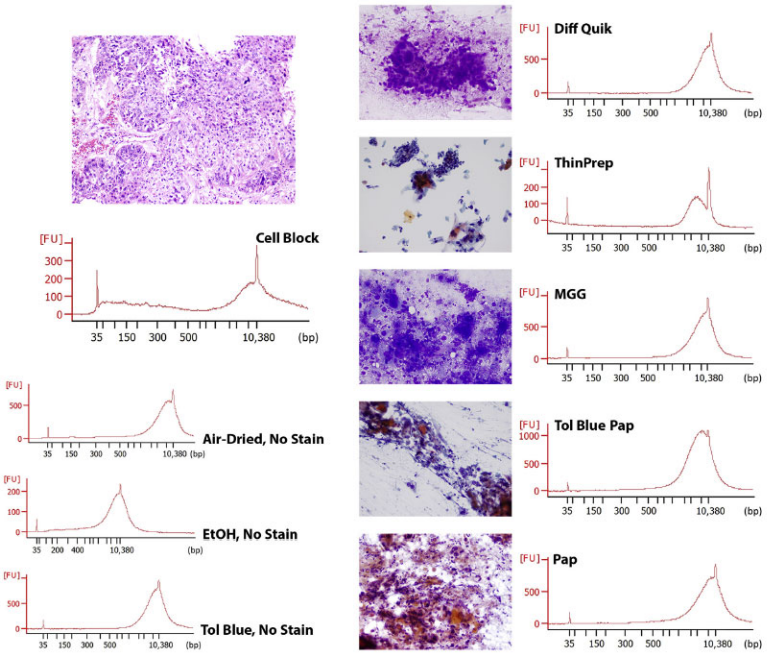






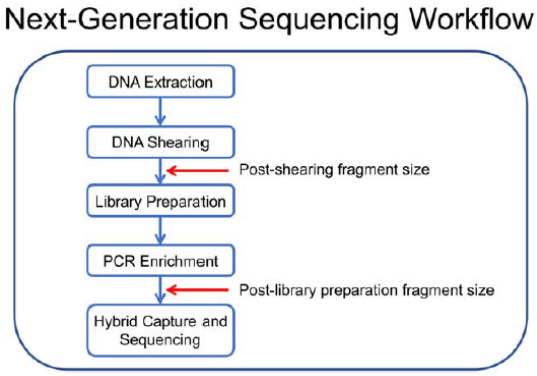


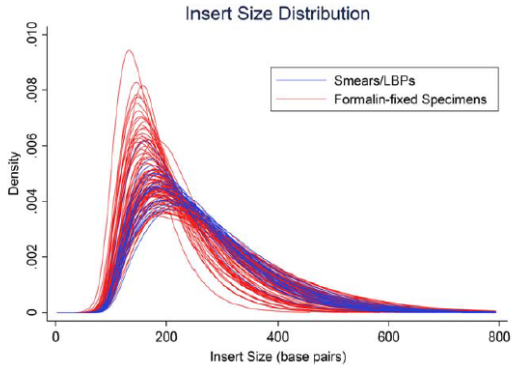
Embracing the non-FFPE sample



Balla A et al. *J Mol Diagn*. Vol. 20, No. 6, November 2018

Larger target DNA fragments from smears/liquid based cytology preps





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

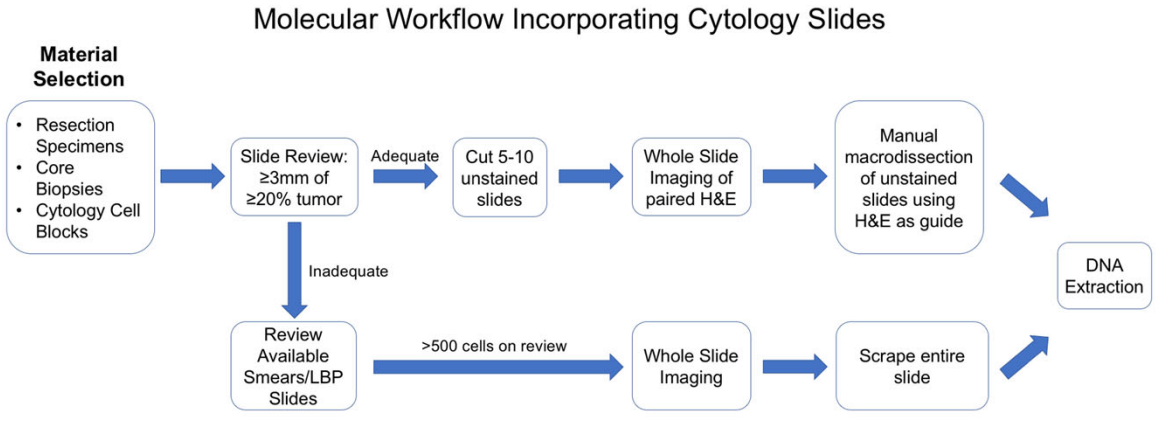
Superior sequencing quality metrics with smears/liquid based cytology preps

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

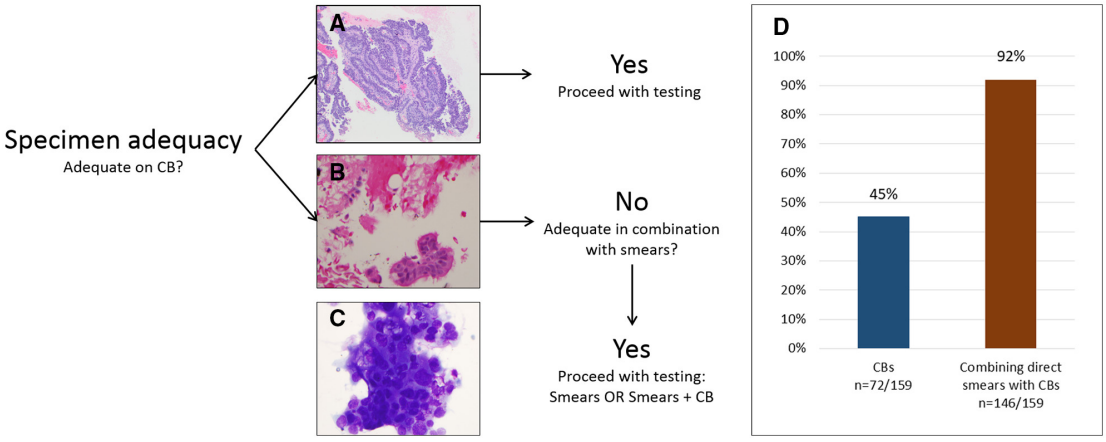
Abbreviations: bp, base pair; LBP, liquid-based preparation.
Median values are presented.
[a] Values within square brackets are values normalized by the flow cell average; *P* values are based on the normalized values.

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



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

Smear preps validated for RNAseq for fusion detection

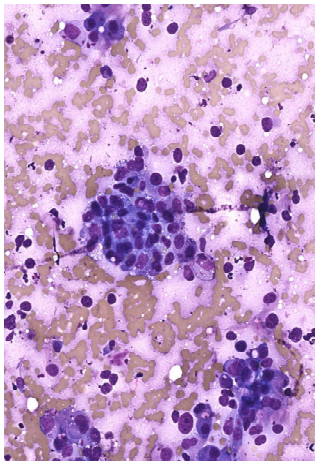


Ramani et al. Cancer Cytopathology. 2021 May;129(5):374-382.

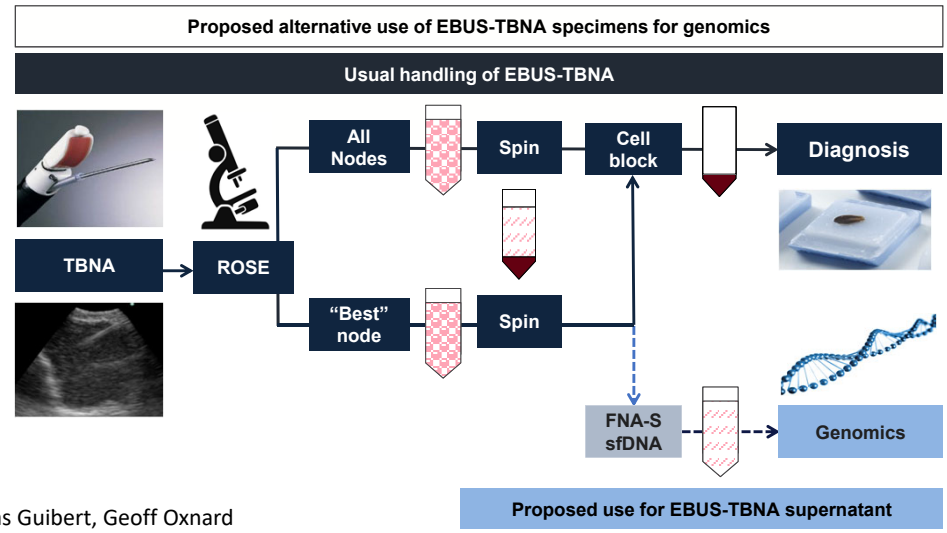
2018 AMP/CAP/IASLC guidelines:

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

- Sequencing quality metrics ≥ to FFPE samples
  - Hwang et al. *Cancer Cytopath* 2017
  - Roy-Chowdhuri et al. *Mod Pathol* 2017



Cytology supernatant – an overlooked genomic testing resource



Nicolas Guibert, Geoff Oxnard

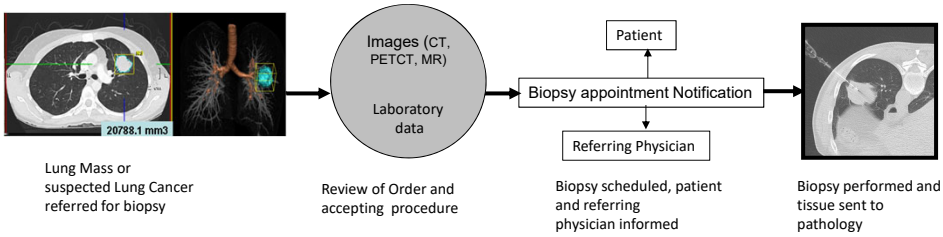
Mutation detection in cell free DNA from cytology supernatants

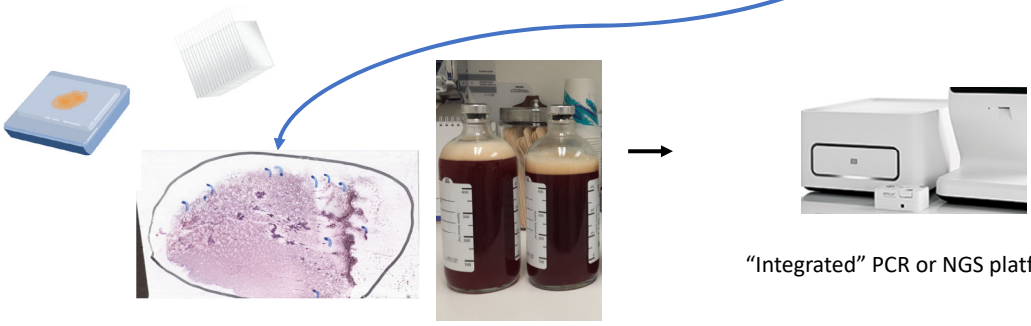
Reference	Supernatant source	n	Concordance with FFPE	PMID
Perrone et al. 2021	Body fluid or FNA rinse fluid	30	74%	34265180
Wu et al. 2020	CT-guided or EBUS FNA rinse fluid	214	97.2%	32286726
Hannigan et al. 2019	FNA rinse fluid	35	97%	30887015
Janaki et al. 2019	Endobronchial FNA rinse fluid	30	100%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

Biomarker Testing of Pleural Effusions

- In patients with an established diagnosis of NSCLC, targetable mutations can be detected in pleural effusion fluid even when it is cytologically negative.
- Supernatants superior to cell pellets for mutation detection.
- >85% concordance between pleural effusion and tumor tissue genotyping (sensitivity of pleural effusion testing is below 100%).
- Isolation of extracellular vesicle-derived DNA may enhance sensitivity of pleural effusion genotyping.

Zhengbo et al. *Lung Cancer.* 2019 Oct;136:23-29. Mahmood et al. *Chest*. 2023 Jul;164(1):252-61.
Wang et al. *Lung Cancer.* 2019 Sep;135:116-122. Xiang et al. *J Mol Diagn.* 2020 Apr;22(4):513-22.

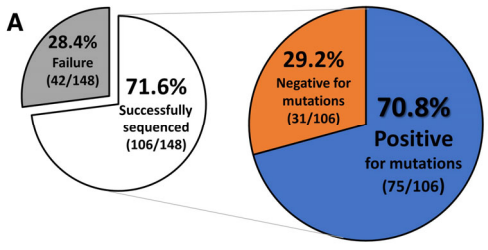




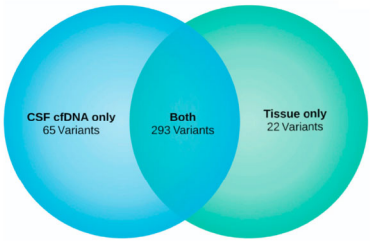
"Integrated" PCR or NGS platforms

CSF specimens – opportunities for molecular profiling

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.

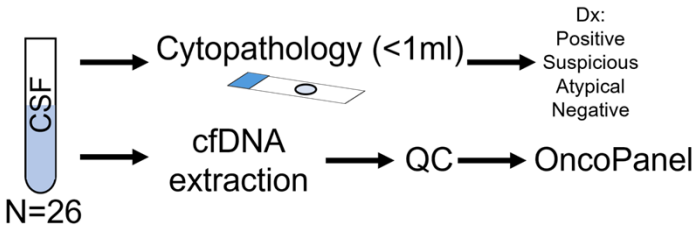
Received: 24 May 2023 | Revised: 28 July 2023 | Accepted: 21 August 2023
DOI: 10.1002/cncy.22768

ORIGINAL ARTICLE

Validation of targeted next-generation sequencing of cell-free DNA from archival cerebrospinal fluid specimens for the detection of somatic variants in cancer involving the leptomeninges: Cytopathologic and radiographic correlation

Alexander J. Neil MD, PhD[1] | Ugonma N. Chukwueke MD, MPH[2] | Nicholas Hoover CT (ASCP)[1] | Sean R. N. Marris BS[1] | Vanesa Rojas-Rudilla MS[1] | Danielle K. Manning PhD[1] | Jeffrey K. Mito MD, PhD[1] | Edmund S. Cibas MD[1] | Lynette M. Sholl MD[1]

B



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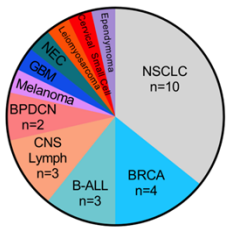
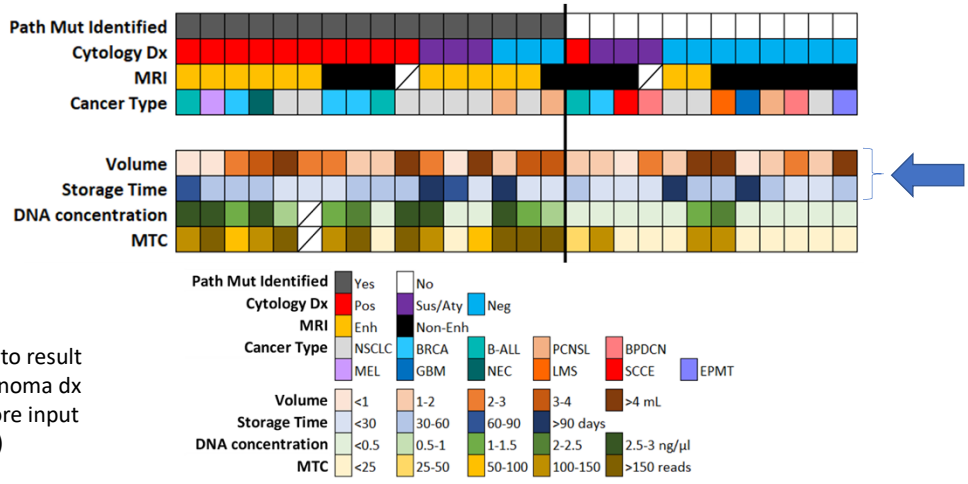


Patient Tumor Types

Factors influencing likelihood of mutation detection by NGS



Non-negative cyto result
Metastatic carcinoma dx
Higher MTC (more input DNA/more cells)

Neil A et al. Histopathology, April 2024.

Use of a dedicated cfDNA assay improves sensitivity of mutation detection for primary CNS tumors

- Most tissue NGS assays can reliably detect SNVs to ~5% VAF
- cfDNA assays may achieve 0.01-0.1% VAF detection

Journal of Neuro-Oncology (2024) 168:215–224
https://doi.org/10.1007/s11060-024-04645-y

RESEARCH

Detection of tumor-derived cell-free DNA in cerebrospinal fluid using a clinically validated targeted sequencing panel for pediatric brain tumors

Rebecca Ronsley[1,2,3,8] · Kristine A. Karvonen[1,2,3] · Bonnie Cole[4,5] · Vera Paulson[5,7] · Jeff Stevens[1,2] · Erin E. Crotty[1,2,3] · Jason Hauptman[6] · Amy Lee[6] · Shannon M. Stasi[4] · Christina M. Lockwood[5,7] · Sarah E. S. Leary[1,2,3]

1352 Neuro-Oncology
24(8), 1352–1363, 2022 | https://doi.org/10.1093/neuonc/noab299 | Advance Access date 4 January 2022

Liquid biopsy detection of genomic alterations in pediatric brain tumors from cell-free DNA in peripheral blood, CSF, and urine

Mélanie Pagès, Denisse Rotem, Gregory Gydush, Sarah Reed, Justin Rhoades, Gavin Ha, Christopher Lo, Mark Fleharty, Madeleine Duran, Robert Jones, Sarah Becker, Michaela Haller, Claire E. Sinai, Liliana Goumnerova, Todd R. Golub, J. Christopher Love, Keith L. Ligon, Karen D. Wright†, Viktor A. Adalsteinsson†, Rameen Beroukhim†, and Pratiti Bandopadhayay†

- But even these highly sensitive assays have limited ability to detect mutations in pediatric brain tumors (30-50% sensitivity)

Received: 14 September 2022 | Revised: 12 December 2022 | Accepted: 14 December 2022

DOI: 10.1002/cncr.34926

CONSENSUS STATEMENT

The American Cancer Society National Lung Cancer Roundtable strategic plan: Methods for improving turnaround time of comprehensive biomarker testing in non–small cell lung cancer

Sinchita Roy-Chowdhuri MD, PhD[1] | Haresh Mani MD[2] | Adam H. Fox MD[3] | Anne Tsao MD[4] | Lynette M. Sholl MD[5] | Farhood Farjah MD[6] | Bruce E. Johnson MD[7] | Raymond U. Osarogiagbon MBBS[8] | M. Patricia Rivera MD[9] | Gerard A. Silvestri MD[3] | Robert A. Smith PhD[10] | Ignacio I. Wistuba MD[1]

ACS NLCRT recommendations

- Endorses Rapid On-Site Evaluation (following CAP guidelines) for all EBUS, bronchoscopic, and transthoracic biopsiese
- Prioritize cytology samples for FFPE cell-block preparation, *especially where biomarker testing is performed from FFPE blocks only*
- Minimize diagnostic immunohistochemistry (TTF-1 & P40)
- Indicate the "best block" in the report or in the record

TUMOR CELL QUANTITY (BEST SMEAR, THINPREP, OR CYTOSPIN):
50-500 tumor cells

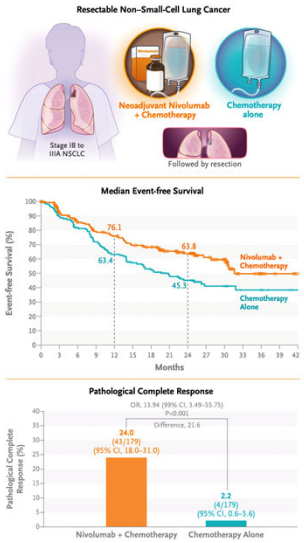
TUMOR CELL QUANTITY (CELL BLOCK):
50-500 tumor cells

TUMOR CELL PROPORTION (BEST SLIDE):
>50%

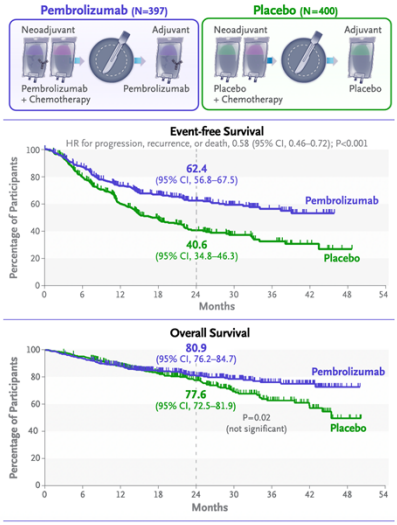
EBUS: supporting diagnosis, staging, *and biomarker testing*

Neoadjuvant therapy for NSCLC

Checkmate 816: Patients with *known* *EGFR* or *ALK* alterations excluded.



Forde et al. N Engl J Med 2022;386:1973-1985.



Keynote 671*: EGFR* and *ALK* were tested "at the discretion of the investigator"

Wakelee H et al. *N Engl J Med*. 2023;389(6):491-503.

NAUTIKA1: A Multicenter, Phase II, Neoadjuvant and Adjuvant Study of Multiple Therapies in Biomarker-Selected Patients with Resectable Stages IB-III Non-Small Cell Lung Cancer

Trial Description

This trial will evaluate the efficacy and safety of various therapies in patients with Stage IB, IIA, IIB, IIIA, or selected IIIB resectable and untreated non-small cell lung cancer (NSCLC) tumors that meet protocol-specified biomarker criteria

Eligibility Requirements

Inclusion Criteria for Neoadjuvant Therapy:

- Pathologically documented NSCLC:

- Newly diagnosed early-stage NSCLC stages IB, IIA, IIB, IIIA, or selected IIIB (T3N2 only) NSCLC of squamous or non-squamous histology. Staging should be based on the 8th edition of the American Joint Committee on Cancer (AJCC)/Union Internationale Contre le Cancer (UICC) NSCLC staging system.

- T4 primary NSCLC will be allowed only on the basis of size. Invasion of the diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, esophagus, vertebral body, carina, and separate tumor nodules in a different ipsilateral lobe is not permitted.

- All patients will undergo clinical staging using CT and PET scanning, as well as brain imaging using MRI. Invasive mediastinal staging by either mediastinoscopyor endo- bronchial ultrasonography is highly encouraged for patients with radiographically suspected mediastinal nodal disease (ie, N2) but not mandated if the CT or PET scans showed no evidence of N2 disease.

- Molecular testing results from CLIA-certified laboratories and showing at least one of the following abnormalities: ALK fusion, ROS1 fusion, NTRK1/2/3 fusion; BRAF V600 mutation (enrollment closed); RET fusion (enrollment closed), PD-L1, KRAS G12C expression in ≥ 1% tumor cells as determined by FDA-approved test.

ENROLLING

877-DF-TRIAL (877-338-7425)

Trial ID:

NCT04302025

View Complete trial on ClinicalTrials.gov

Protocol # :

20-098

Conditions

Non-Small Cell Lung Cancer

Phase

II

Disease Sites

Lung

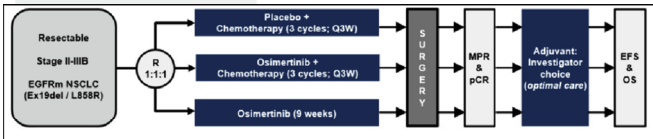
Principal Investigator

McNamee, Ciaran

Neoadjuvant targeted therapy trials

[NAUTIKA1: A Multicenter, Phase II, Neoadjuvant and Adjuvant Study of Multiple Therapies in Biomarker-Selected Patients with Resectable Stages IB-III Non-Small Cell Lung Cancer | Dana-Farber Cancer Institute]

[P03.02 Neoadjuvant Osimertinib with/without Chemotherapy vs Chemotherapy for EGFR Mutated Resectable NSCLC: NeoADAURA - Journal of Thoracic Oncology (jto.org)]



Ex19del, Exon 19 deletion; NSCLC, non-small cell lung cancer; EGFRm, epidermal growth factor receptor mutation-positive; R, randomisation, Q3W, every three weeks; MPR, major pathological response; pCR, complete pathological response; EFS, event-free survival; OS, overall survival.

How do we adjust the end-to-end practice to optimize molecular biomarker testing?

Practice Guideline > Chest. 2025 Mar;167(3):899-909. doi: 10.1016/j.chest.2024.08.056. Epub 2024 Sep 27.

Acquisition and Handling of Endobronchial Ultrasound Transbronchial Needle Samples: An American College of Chest Physicians Clinical Practice Guideline

Christopher R Gilbert [1], Claire Dust [2], A Christine Argento [3], David Feller-Kopman [4], Anne V Gonzalez [5], Felix Herth [6], Jonathan M Iaccarino [2], Peter Illei [7], Kevin O'Neil [8], Nicholas Pastis [9], M Patricia Rivera [10], Lynette Sholl [11], Gerard A Silvestri [12], Jeffrey Thiboutot [3], Momen M Wahidi [13], Kazuhiro Yasafuku [14], Lonny B Yarmus [3]

TABLE 1] PICO Questions

Question 1	In patients undergoing EBUS-TBNA, should alternative methods of specimen expulsion from the EBUS-TBNA needle be used compared to clinical practice?
Question 2	In patients being evaluated for malignancy undergoing EBUS-TBNA, should alternative collection media be used compared to clinical practice?
Question 3	In patients being evaluated for malignancy undergoing EBUS-TBNA, should rapid on-site evaluation be used?
Question 4	In patients being evaluated for malignancy undergoing EBUS-TBNA, should a larger or smaller needle be used?
Question 5	In patients being evaluated for malignancy undergoing EBUS-TBNA, should biopsy include four or more needle passes or three or less needle passes?
Question 6	In patients being evaluated for nonmalignant disease undergoing EBUS-TBNA, should alternative collection media be used compared to clinical practice?
Question 7	In patients being evaluated for nonmalignant disease undergoing EBUS-TBNA, should rapid on-site evaluation be used?
Question 8	In patients being evaluated for nonmalignant disease undergoing EBUS-TBNA, should a larger or smaller needle be used?
Question 9	In patients being evaluated for nonmalignant disease undergoing EBUS-TBNA, should biopsy include four or more needle passes or three or less needle passes?

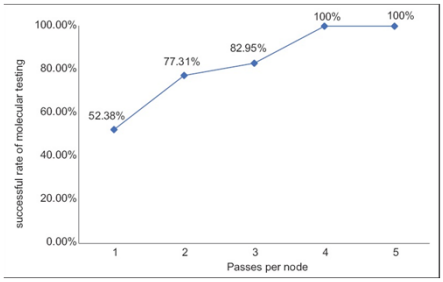
EBUS-TBNA = endobronchial ultrasound transbronchial needle aspiration; PICO = Population, Intervention, Comparator, and Outcome.

CHEST Recommendation: In patients with suspected malignant disease undergoing EBUS-TBNA, we suggest using ROSE over usual care (Conditional recommendation, very low certainty of evidence)

- Improves diagnostic yield
- Identification of ROSE-positive lymph nodes reduces need for biopsy of peripheral lung with associated risks
- May enhance molecular adequacy
- Controversial (resource intensive, logistically challenging)

CHEST Recommendation: In patients with suspected malignant disease undergoing EBUS-TBNA, we recommend using four or more needle passes over three or less (Strong recommendation, very low certainty of evidence)

- Several studies show increased passes → increased molecular yield
- 3-6 passes yield adequate specimens for NGS in 80-90% of samples
- Use of ROSE and discussion with cytopathology team may guide appropriate number of passes



Zhang Y, Xie F, Mao X, et al.. *Endosc Ultrasound*. 2019;8(6):404-411.

Take home points

- Understand the relevant molecular assays, including nucleic acid input requirements (tissue size, # of cells) and sensitivity (tumor %)
- Advocate for use of non-FFPE samples in your local lab, but anticipate barriers to use of these samples from commercial labs and plan accordingly
- Anticipate increased indications for molecular biomarker testing, including in earlier stages of disease (especially NSCLC)
- Explore use of ROSE in your institution to guide adequacy for diagnosis and biomarker testing
- Work with your proceduralists to ensure adequate passes to allow for diagnosis and biomarker testing