Molecular Cytopathology
Part 1

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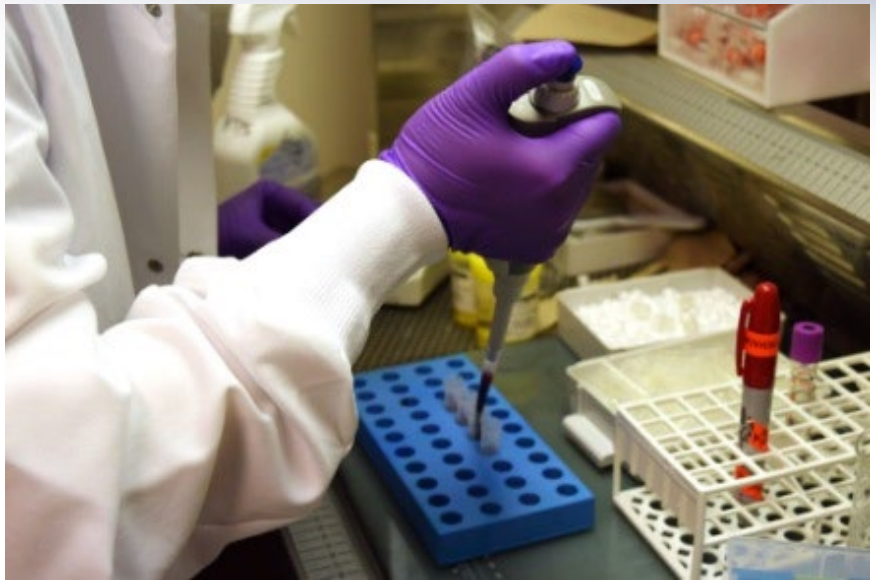
The Evolving Role of Pathology



Tissue is not only for diagnostic evaluation, but also for

clinically relevant molecular assays

Current Role of Molecular Diagnostics

Molecular testing helps with **diagnosis and classification**

DIAGNOSIS

GLIOBLASTOMA, IDH-WILDTYPE, WHO GRADE IV

IDH1 / *IDH2* status (PCR): NEGATIVE for mutation
MGMT status (methylation-specific PCR): NEGATIVE for promoter methylation

(SEE COMMENT)

Current Role of Molecular Diagnostics

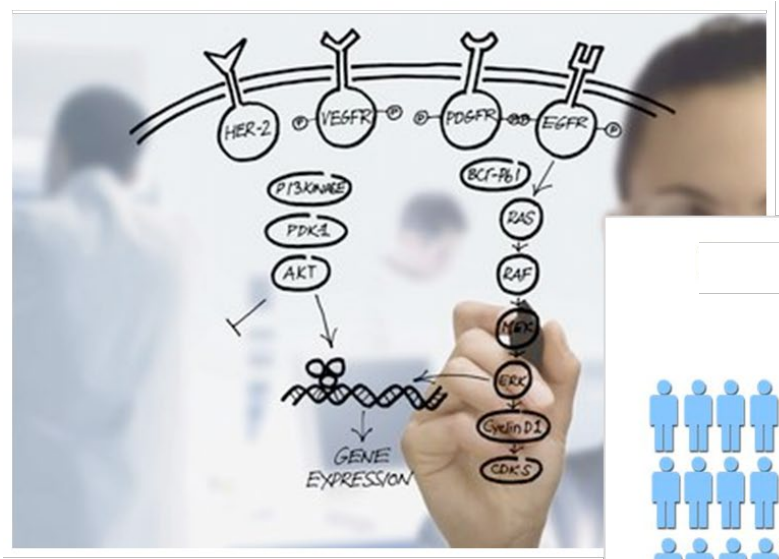
Molecular provides **predictive and prognostic** information

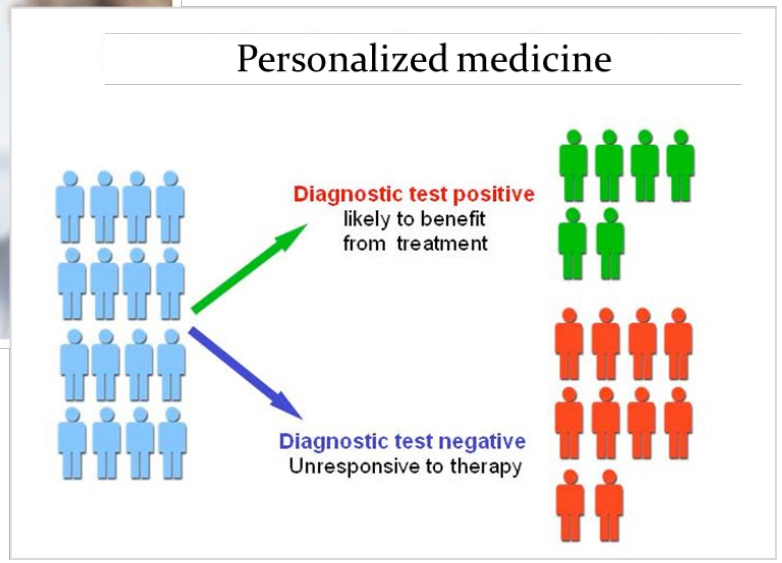
- ***Predictive*** marker indicates therapeutic efficacy (e.g. *EGFR, PD-L1*)
- ***Prognostic*** marker indicates patient survival independent of treatment received i.e. innate tumor aggressiveness (e.g. *TP53*)

Current Role of Molecular Diagnostics

Molecular profiling is now standard of care for various solid tumors:

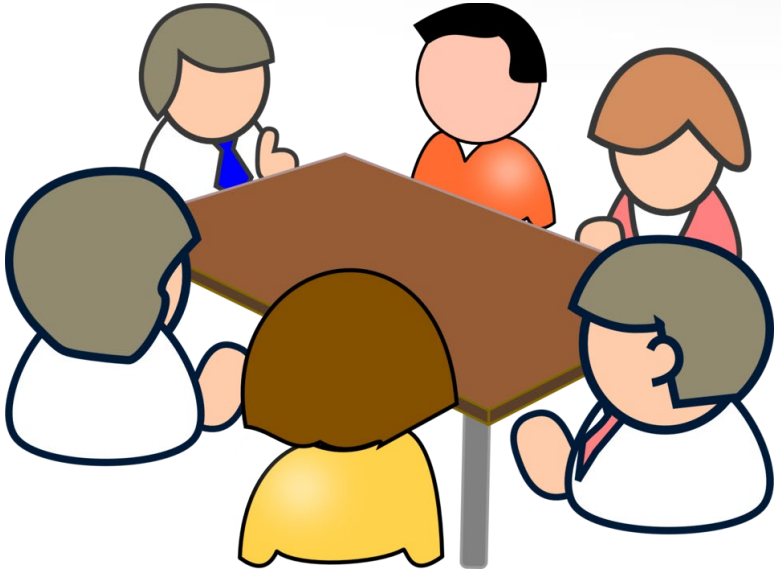
- Lung cancer, Colorectal Cancer, Melanoma, Thyroid etc.



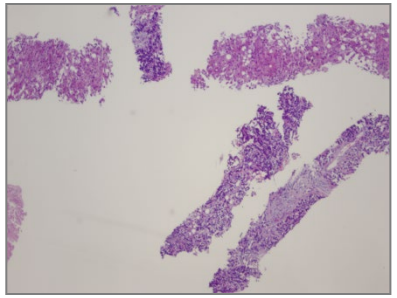


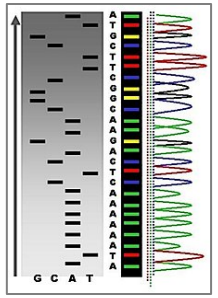
What is the role of the Pathologist?

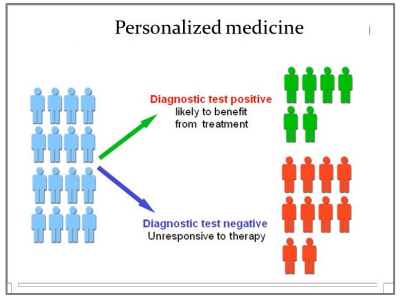
Pathologists Need to be "*Integrative Diagnosticians*"



Greg Fuller, MD PhD







Molecular Cytopathology

Demystifying Molecular Cytopathology

International Journal of Surgical Pathology
Supplement to 18(3) 213S–215S

DOI: 10.1177/1066896910370887
http://ijsp.sagepub.com
SAGE

Fernando C. Schmitt, MD, PhD, FIAC[1]

Molecular technology applied in the field of pathology is undoubtedly reshaping the practice of cytopathology worldwide. A recent multiinstitutional inquiry led to an interesting discussion concerning the introduction of these methodologies in order to optimize cytology procedures and solve old quandaries. Particular attention was devoted to the real utility of these new approaches and to the feasibility of introducing them in cytology laboratories. The conclusions were quite exciting because most of the cytopathologists now recognize the importance of molecular techniques as adjuncts to morphology for diagnosis. At present, the great challenge is deciding when to adopt a new molecular test and who should perform it and interpret it. Now and in the future, pathologists in general and cytopathologists in particular will play a vital role in the emerging world of molecular medicine.

Molecular cytopathology (MCP) can be defined as the application of molecular studies to any type of cytological specimen—namely, gynecological cytology, exfoliative nongynecological cytology, and fine needle aspirates. MCP has been applied to detect specific organisms or oncological changes at the molecular level. MCP techniques can be performed directly on the cytological specimens (eg, FISH [fluorescent in situ hybridization]) or on DNA/RNA extracted from smears, cell blocks, or cell suspensions.

The use of new technologies as applied to cervical samples collected with a liquid-based cytology medium has facilitated the identification of etiological agents and in to determine the optimal screening interval and algorithm. Other infectious agents can be identified in cytological material using molecular techniques. Recently, we demonstrated the feasibility of the polymerase chain reaction to detect *Mycobacterium ulcerans* in fine needle aspiration (FNA) material from lesions of Buruli ulcer.

The development of clinical cytogenetics has paralleled the emergence of clinical cytology as a major diagnostic specialty. Clinical applications of CISH (chromogenic in situ hybridization)/FISH have grown in the last decade. From a diagnostic point of view, a potential and emerging field is the use of ISH for the detection of recurrence of urothelial carcinomas in urine specimens and for the diagnosis in equivocal lung cytology. The use of a large combination of probes through the multiplex FISH technique will certainly improve the diagnostic capacity of cytological material. Moreover, better probes to detect specific translocations will be extremely useful in the characterization of soft-tissue tumors and malignant lymphomas in cytological material. New genetic information is coming from microarray technology, and specific probes can be generated and used to obtain diagnostic, prognostic, and predictive information for routine material.

Cytopathologists will also be expected to include specific prognostic and predictive information in their reports as well as to order ancillary tests and to contribute in clinical trials with their expertise. During the last decade, there have been

Reference: Schmitt FC. Int J Surg Pathol. 2010 Jun;18(3 Suppl):213S-215S.

Molecular Diagnostics is Used Commonly in Cytology Specimens

- Small specimens are *not necessarily* an obstacle
- It is becoming increasingly common for molecular testing to be performed on *cytology specimens*, including cell blocks, direct smears, and liquid-based preparations

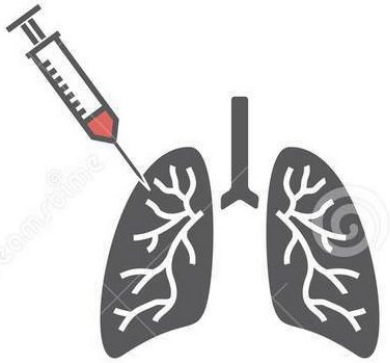
Key Objectives

- The variety and versatility of cytology specimen preparations offer several options for molecular testing
- A multitude of pre-analytical factors impact tissue quality and the success of molecular testing
- The pathologist plays a key role in triage and specimen handling that can improve the success of molecular testing

The Small Specimen:
A Closer Look at Cytology Specimens

Cytology Specimens Provide Multiple Options

Small Specimens for NSCLC Diagnosis



Histology

- Percutaneous transthoracic biopsy (CT guided)
- Trans/endobronchial biopsy (US guided)
- Biopsy of metastatic sites

Cytology

- Percutaneous transthoracic needle aspirate (CT guided)
- Transbronchial/endobronchial needle aspirate (US guided)
- FNA of metastatic sites
- Bronchial brushing
- Bronchial washing
- Bronchoalveolar lavage (BAL)
- Sputum
- Body cavity fluids/effusions

Cytology Specimens

Specimen Source:

- Aspiration
- Exfoliative

Advantages:

- Rapid on-site assessment (ROSE) and triaging
- Typically low proportion of interfering non-tumor cells
- Ideal for molecular testing in bony lesions (no decal)

Disadvantages:

- Small sample and may be inadequate for testing
- Historically underutilized for molecular testing

Cytology Specimens

Specimen Source:

- Aspiration
- Exfoliative

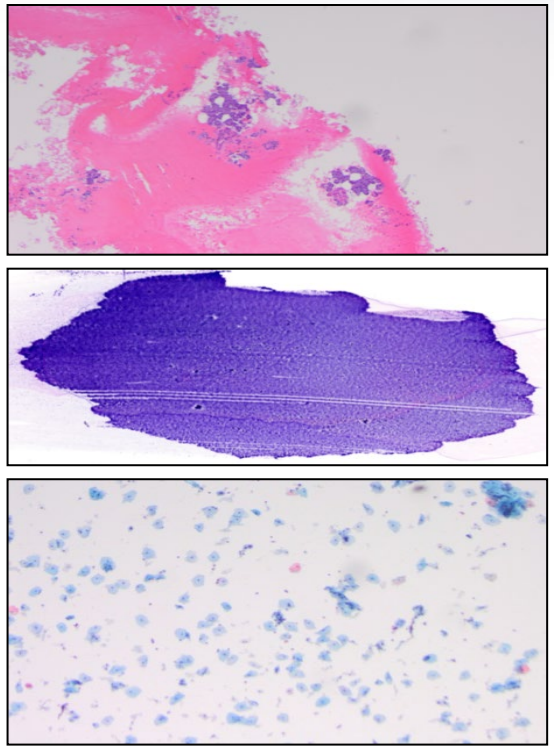
- **Advantages:**
  - May be more representative of metastatic tumor
  - May be large volume and amenable to repeat sampling
- **Disadvantages:**
  - Often has low tumor fraction with numerous non-tumor cells

Historically exfoliative samples used for molecular testing:
e.g. HPV testing of cervical GYN samples, FISH for urine samples etc.

Cytology Specimen Preparations

Specimen Preparation:

- Cell block
- Direct smear
- Liquid based cytology (LBC)
- Other*

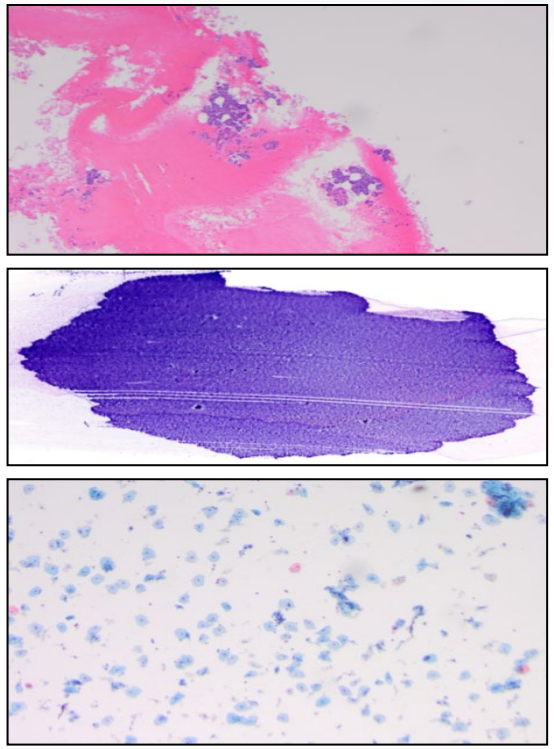


What is the most commonly used cytologic specimen preparation for molecular testing?

Cytology Specimen Preparations

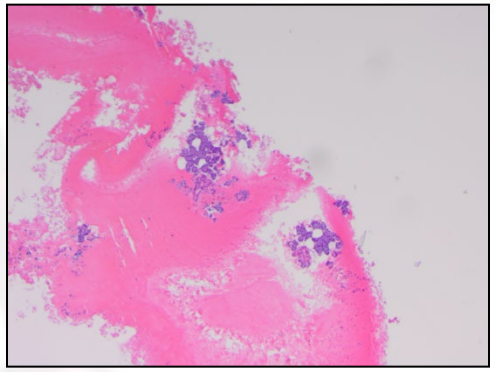
Specimen Type:

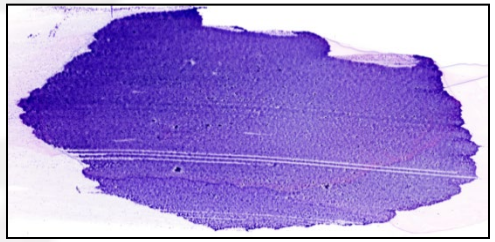
- Cell block
- Direct smear
- Liquid based cytology (LBC)
- Other*

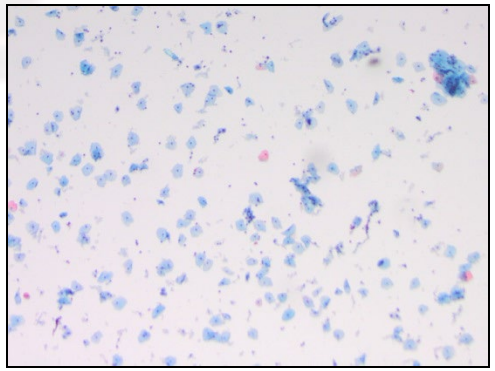


What is the ~~most commonly used~~ **best** cytologic specimen preparation for molecular testing?

Cytology Specimen Preparation Types







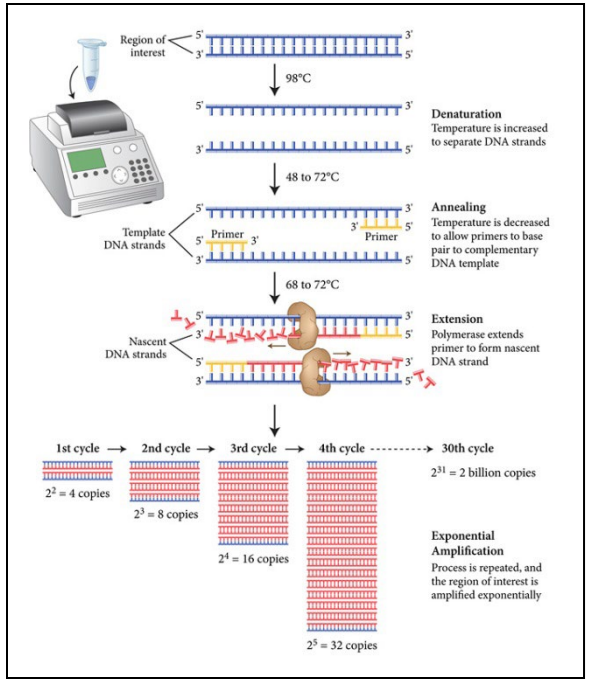
	Advantages	Disadvantages
Cell blocks (FFPE)	• Ease of acquisition • Ease of validation • Ease of getting serial sections	• Lack of immediate assessment • May have low cellularity • Formalin fixation artifact • Partial nuclei (4-5 µm sections)
Direct smears	• ROSE for tumor adequacy • High quality nucleic acid • Whole cells= whole nuclei (higher nucleic acid yield)	• Additional validation • Requires technical skill to prepare smears • Must sacrifice slide (medicolegal issues)
Liquid-based cytology	• Standardized processing with preservation of nucleic acids • Ease of use • Whole cells= whole nuclei (higher nucleic acid yield)	• Lack of immediate assessment • Inability to assess presence/ quantify tumor in tested sample • Additional validation

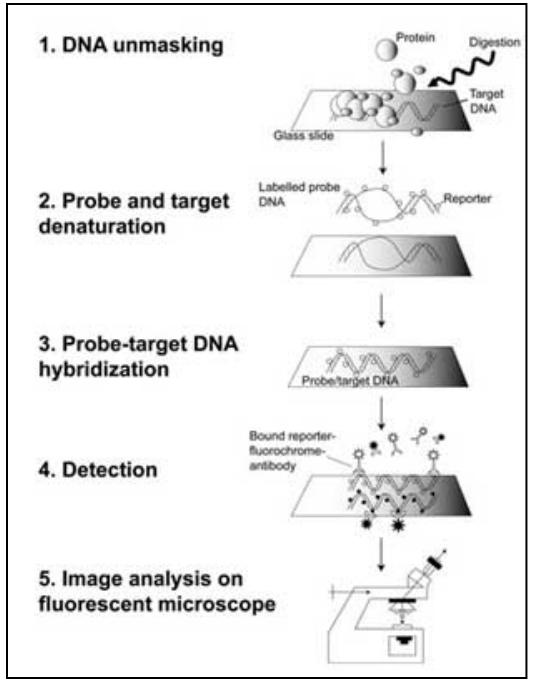
Preanalytical Factors Impact Molecular Diagnostics

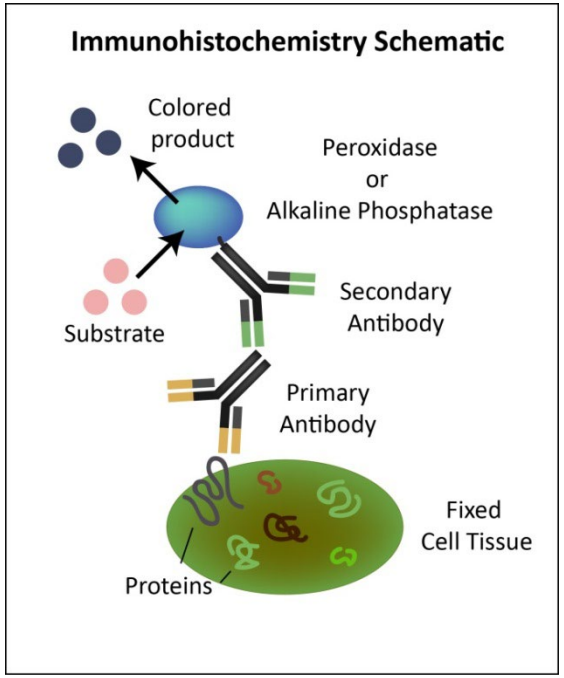
Molecular Diagnostics in the Clinical Setting

Nucleic acid (DNA or RNA)

Protein







Multiple Factors Impact Tissue Quality and Molecular Testing

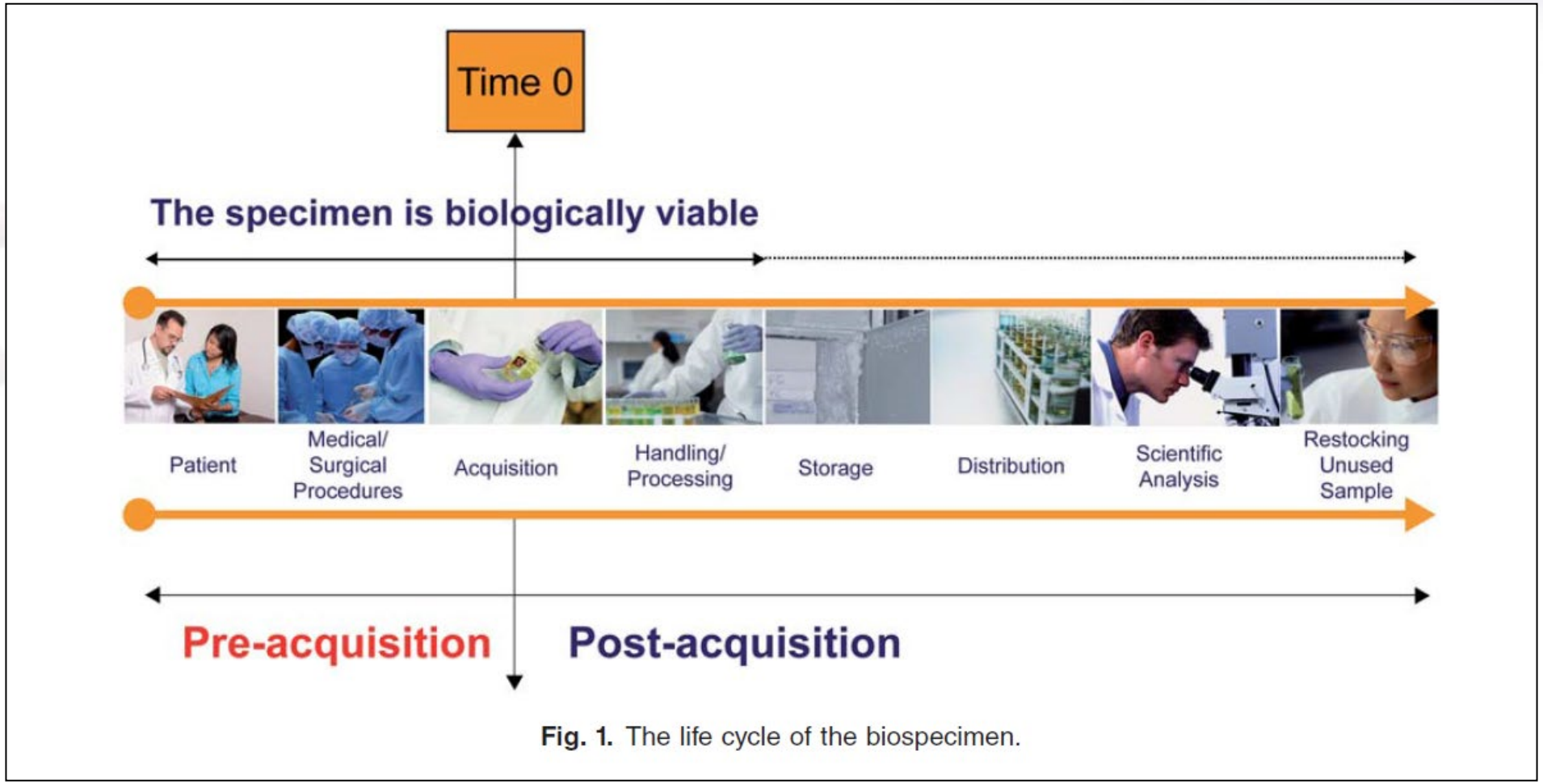
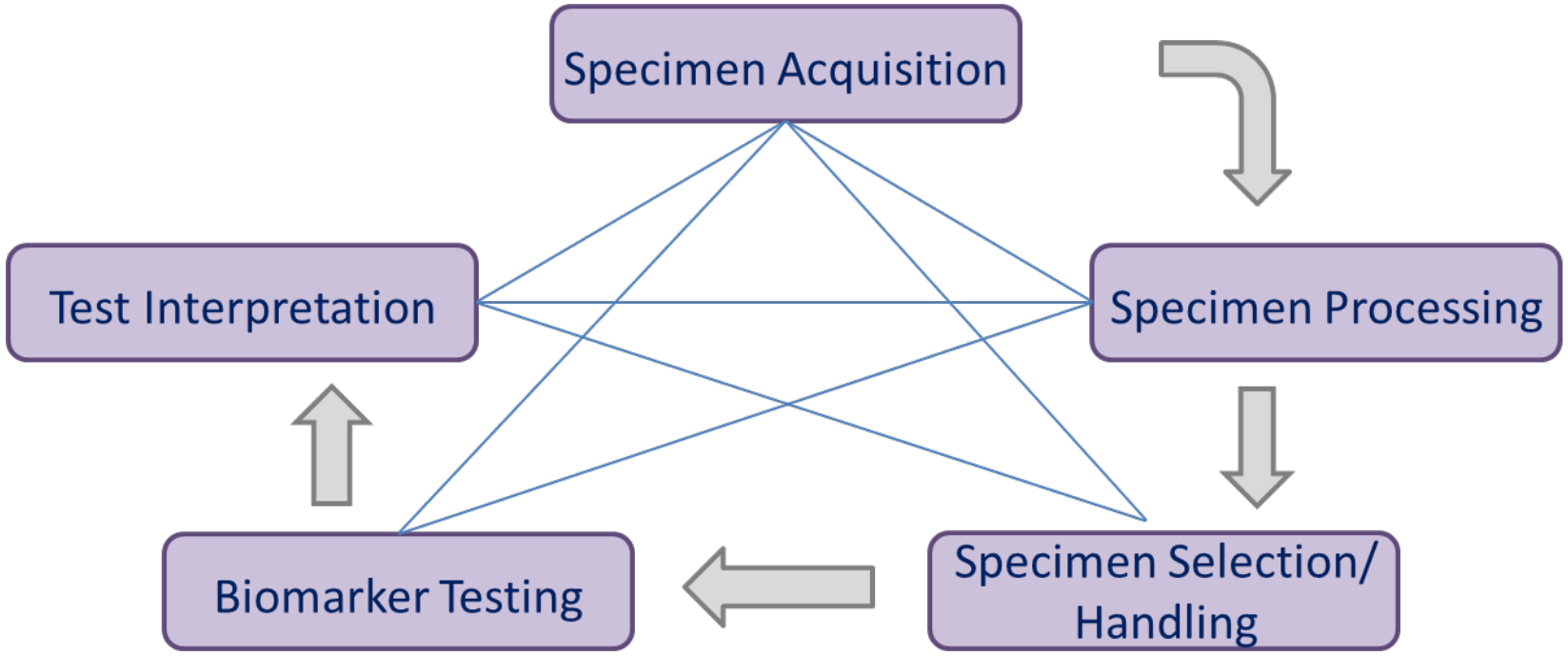


Fig. 1. The life cycle of the biospecimen.

Moore, H.M. Biotech Histochem. 2012 Jan;87(1):18-23. doi: 10.3109/10520295.2011.591833.

Steps to Ensuring Optimal Molecular Testing in Cytology Samples



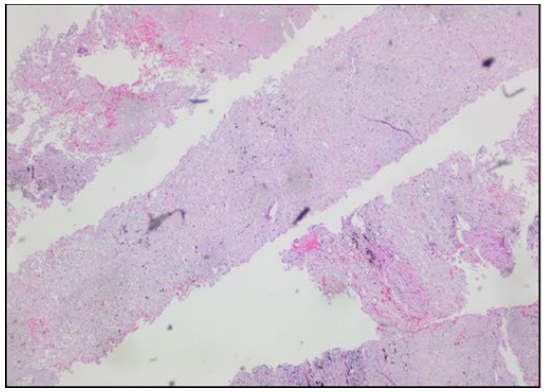
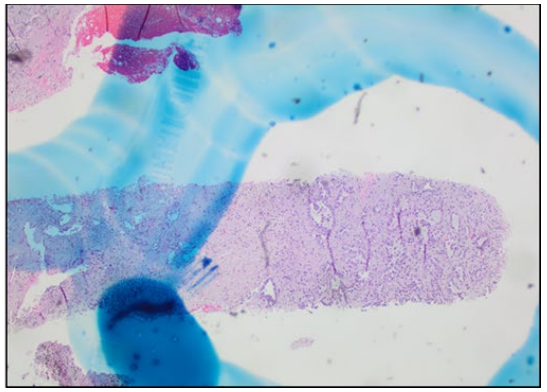
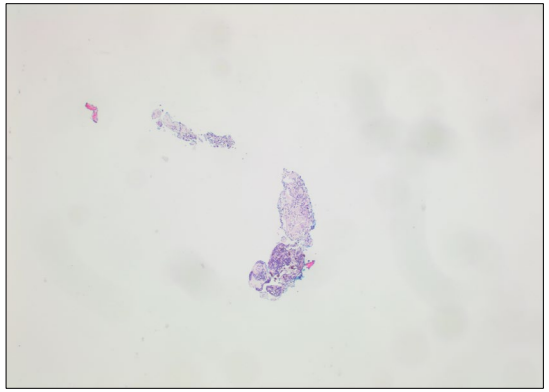
Specimen Acquisition

Step 1. Start with an Adequate Sample

Molecular testing works best when you have an adequate sample

Molecular Testing Case Scenario 1

- 72 y/o male with a lung mass
- Transthoracic biopsy performed

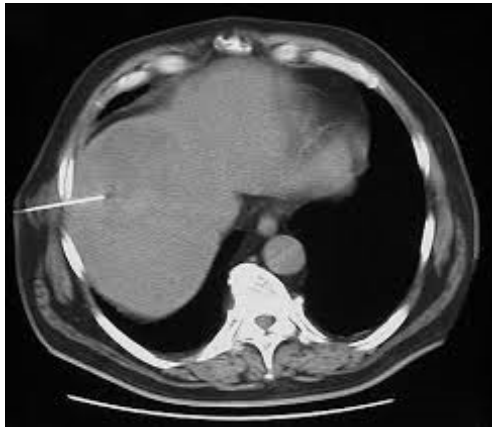




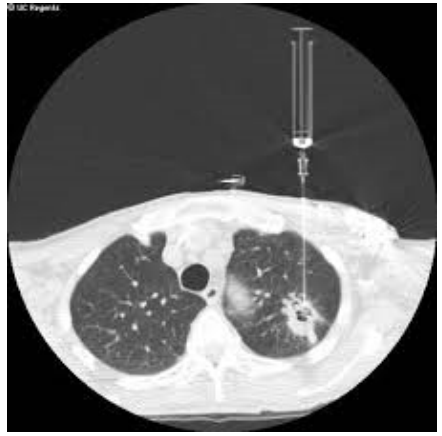
- Diagnosis: Lung adenocarcinoma
- Adequacy for Molecular Testing?

Step 1. Start with an Adequate Sample

How do you ensure an adequate sample?

- Using **image-guided procedure** for better diagnostic yield
- **Optimizing technique** for best diagnostic yield (e.g. needle gauge, number of passes, operator skill and training etc)

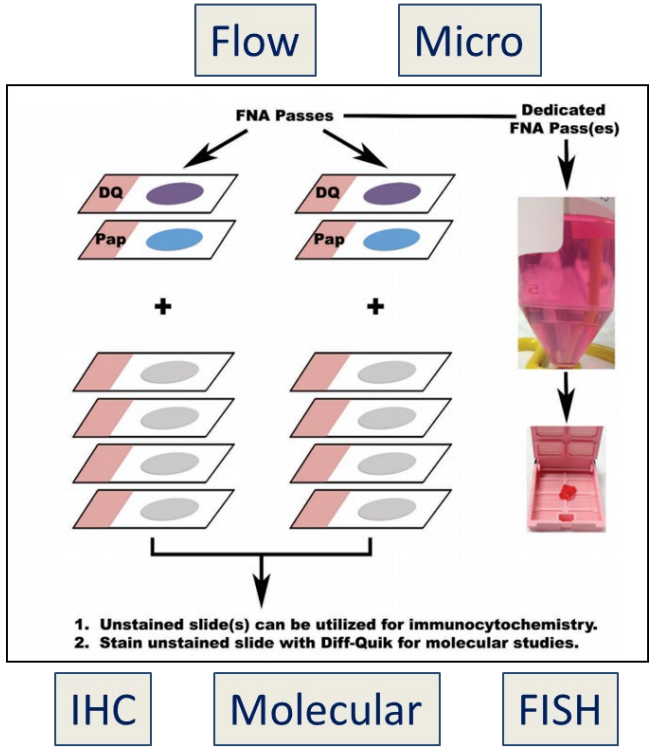




Step 1. Start with an Adequate Sample

How do you ensure an adequate sample?

- Utilizing **rapid on-site evaluation** (ROSE), if available, to guide adequacy assessment



Roh M. Arch Pathol Lab Med. 2013 Sep;137(9):1185-90.

Has ROSE been proven to increase diagnostic yield?
No

However, ROSE can

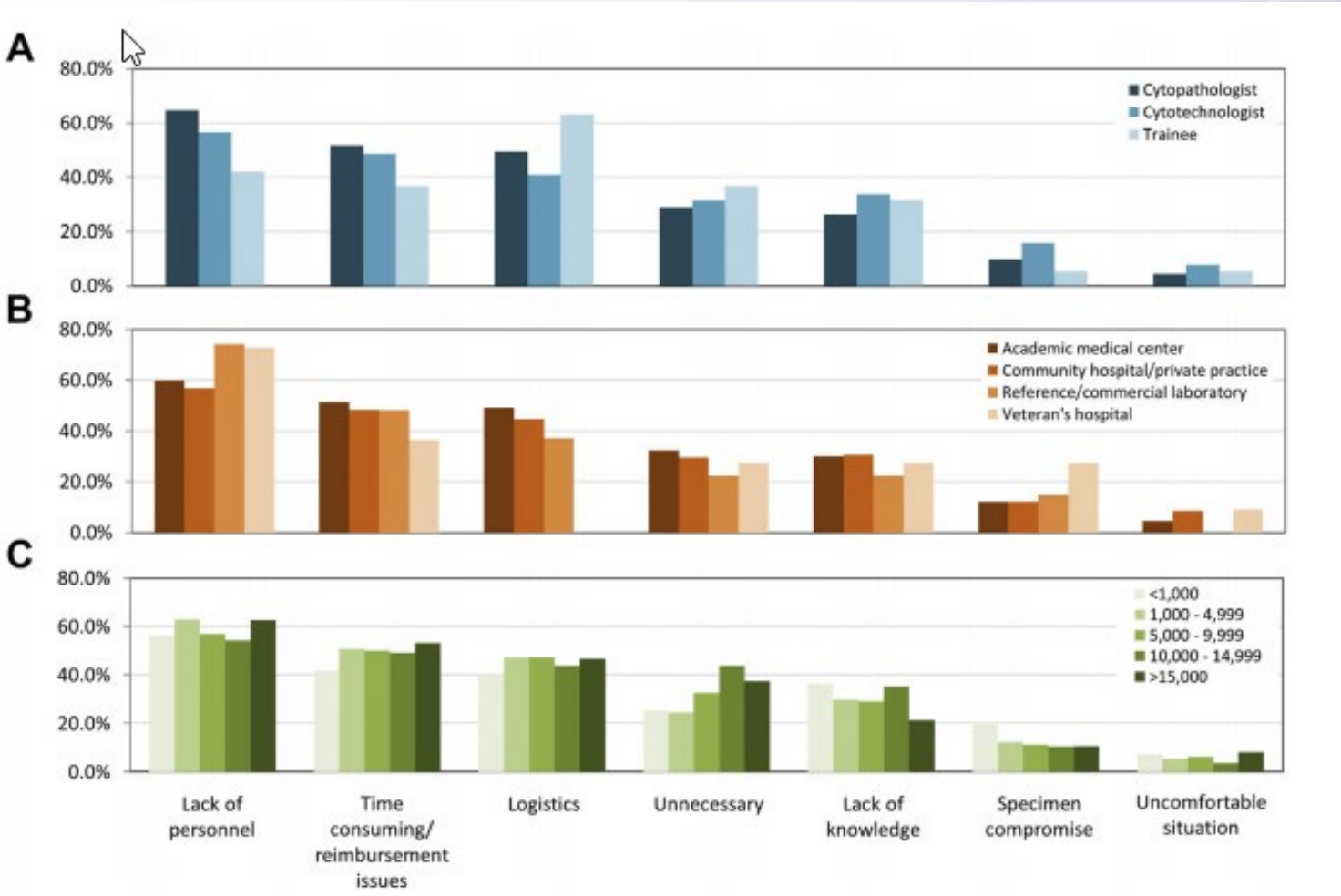
- Guide procedure
- Reduce number of passes
- Perform additional passes for ancillary studies
- Provide feedback
- Improve technique
- Reduce cost

Limitations for Wide-spread Adoption of ROSE

Results from the 2019 American Society of Cytopathology survey on rapid onsite evaluation (ROSE)—part 2: subjective views among the cytopathology community

Jennifer L. Sauter, MD[a,*], Yigu Chen, MPH, PMP[b], Deepu Alex, MD, PhD[c], Ronald Balassanian, MD[d], Jackie Cuda, BS, SCT(ASCP)[e], Melina B. Flanagan, MD, MSPH[f], Christopher C. Griffith, MD, PhD[g], Peter Illei, MD[h], Daniel N. Johnson, MD[i], Cindy M. McGrath, MD[j], Melissa L. Randolph, BS, SCT(ASCP)[k], Jordan P. Reynolds, MD[g], Amy J. Spiczka, MS, SCT, HTL, MB (ASCP)[l], Annemieke van Zante, MD, PhD[d], Paul A. VanderLaan, MD, PhD[b] on behalf of the American Society of Cytopathology Clinical Practice Committee

J. Am. Soc. Cytopathol. 2020 Jul 21;S2213-2945(20)30120-4.



ROSE Can Ensure Adequate Tissue Acquisition for Clinical Trials

Lessons learned from clinical trial queries on small biopsy collections: importance of rapid on-site evaluation

Jamie Voyten, BS[a], Matthew P. Holtzman, MD[b], Liron Pantanowitz, MD[c], Rajiv Dhir, MD[c], H. Scott Beasley, MD[d], Jackie Cuda, SCT[c], Sara E. Monaco, MD[c,*]

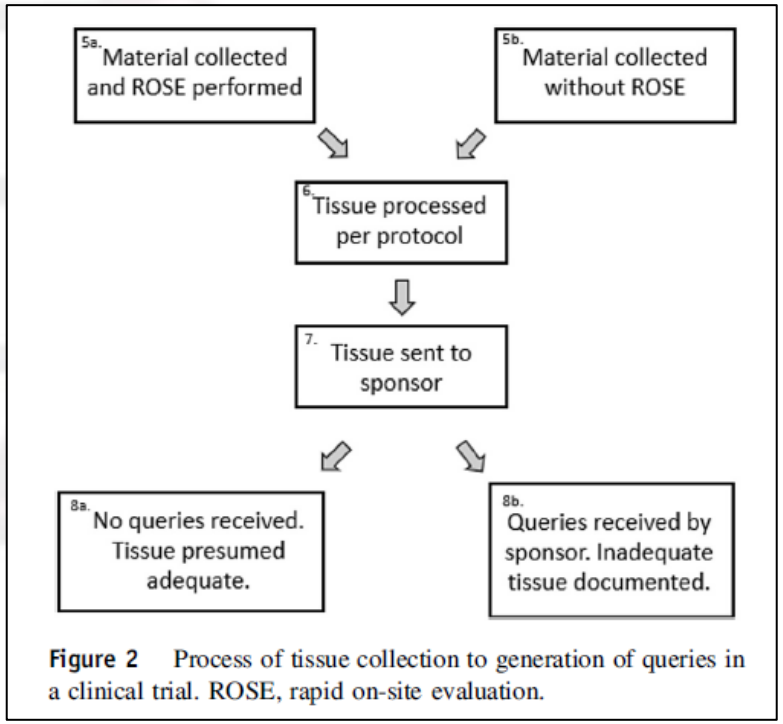


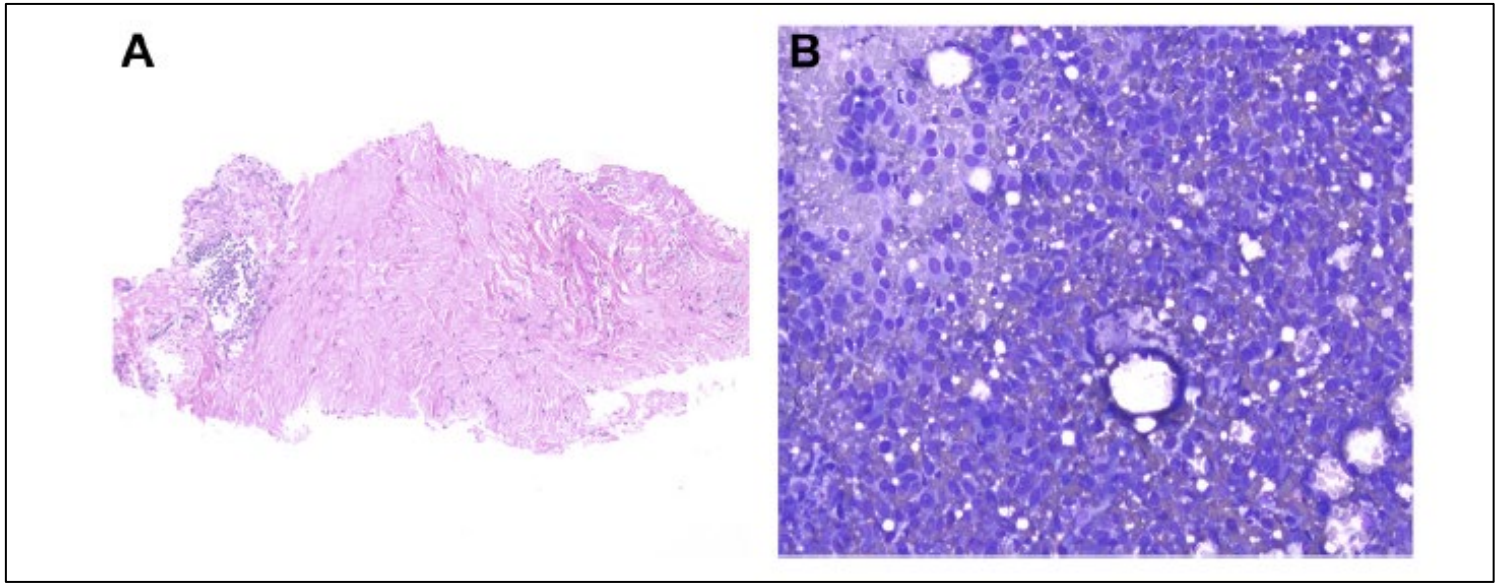
Figure 2 Process of tissue collection to generation of queries in a clinical trial. ROSE, rapid on-site evaluation.

Feedback from clinical trial sponsor	
Trials with feedback[b]	18 (18.9)
Trials without feedback[b]	77 (81.1)
Patient biopsies with feedback[a]	90 (12.9)
Patient biopsies with feedback that had ROSE[c]	52 (57.8)
Patient biopsies with feedback that did not have ROSE[c]	38 (42.2)
Sufficient biopsies on feedback[c]	63 (70.0)
Borderline biopsies on feedback[c]	17 (18.9)
Borderline biopsies without ROSE[e]	17 (100.0)
Borderline biopsies with ROSE[e]	0 (0.0)
Insufficient biopsies on feedback[c]	10 (11.1)
Insufficient biopsies without ROSE[d]	9 (90.0)
Insufficient biopsies with ROSE[d]	1 (10.0)

- With the use of ROSE, 93.4% of the biopsies were deemed adequate at ROSE, minimizing the number of inadequate biopsies

J. Am. Soc. Cytopathol. 2020 Jul 21;S2213-2945(20)30120-4.

Touch Imprint Preparations Can Be Used For Adequacy Assessment



Satturwar S et al. J Am Soc Cytopathol. 2020 Sep-Oct;9(5):322-331.

Concurrent FNA and Core Biopsies May Improve Chances of Molecular Testing Success

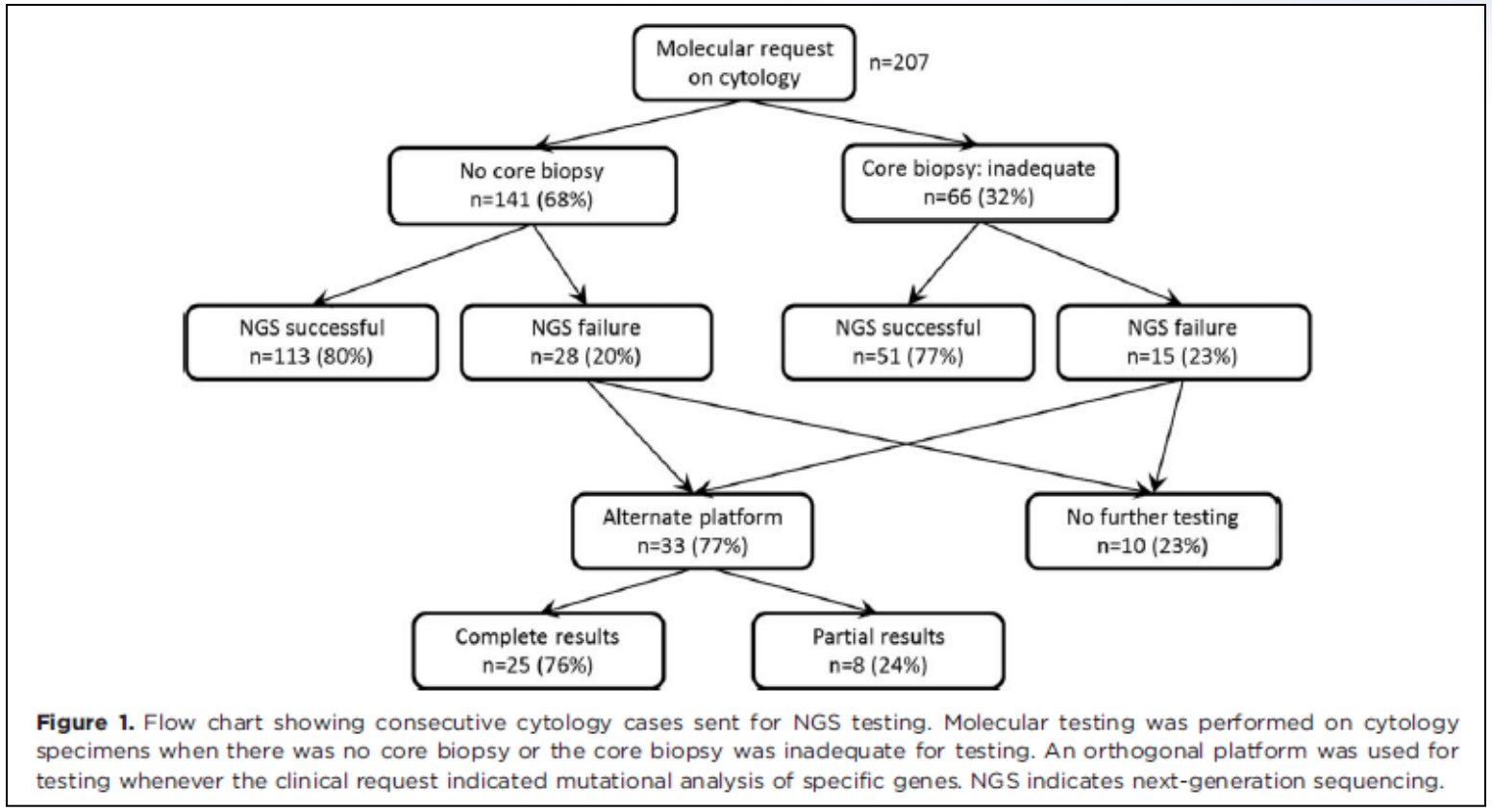
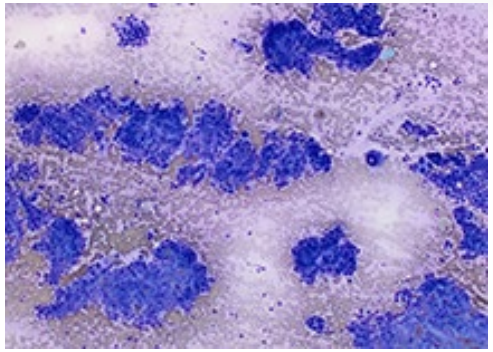
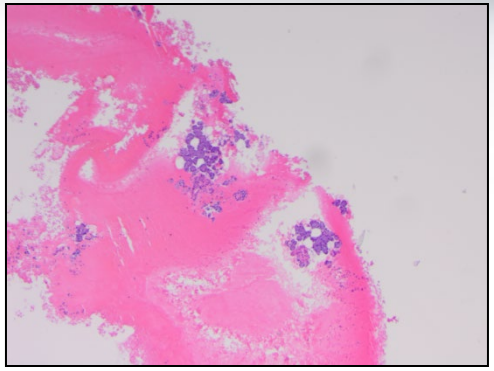
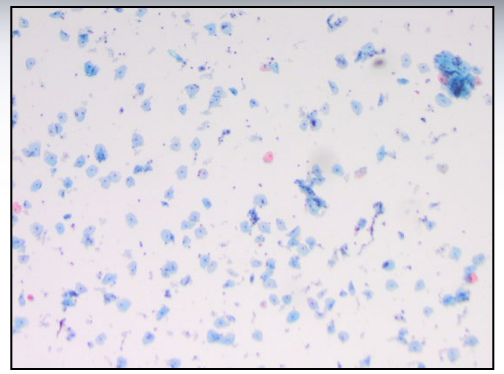


Figure 1. Flow chart showing consecutive cytology cases sent for NGS testing. Molecular testing was performed on cytology specimens when there was no core biopsy or the core biopsy was inadequate for testing. An orthogonal platform was used for testing whenever the clinical request indicated mutational analysis of specific genes. NGS indicates next-generation sequencing.

Roy-Chowdhuri, S. et. al. Cancer Cytopathol. 2015 Jul 31. doi: 10.1002/cncy.21597.

Specimen Processing and Handling

Step 2: Optimize Specimen Processing

Variety and versatility of cytology specimen preparations:

Collection Media, Preservatives, Fixatives, Stains, Glass slides, Mounting media, Extraction methods

- **Quantitative/qualitative differences** in nucleic acid and protein antigenicity due to varying processing techniques

FFPE cell blocks are the most common cytological substrate for molecular testing

- Are all FFPE blocks created the same?
- Are cytology cell blocks that are fixed in formalin after being collected in a different transport media/fixative the same as histologic FFPE?

A Review of Preanalytical Factors Affecting Molecular, Protein, and Morphological Analysis of Formalin-Fixed, Paraffin-Embedded (FFPE) Tissue

How Well Do You Know Your FFPE Specimen?

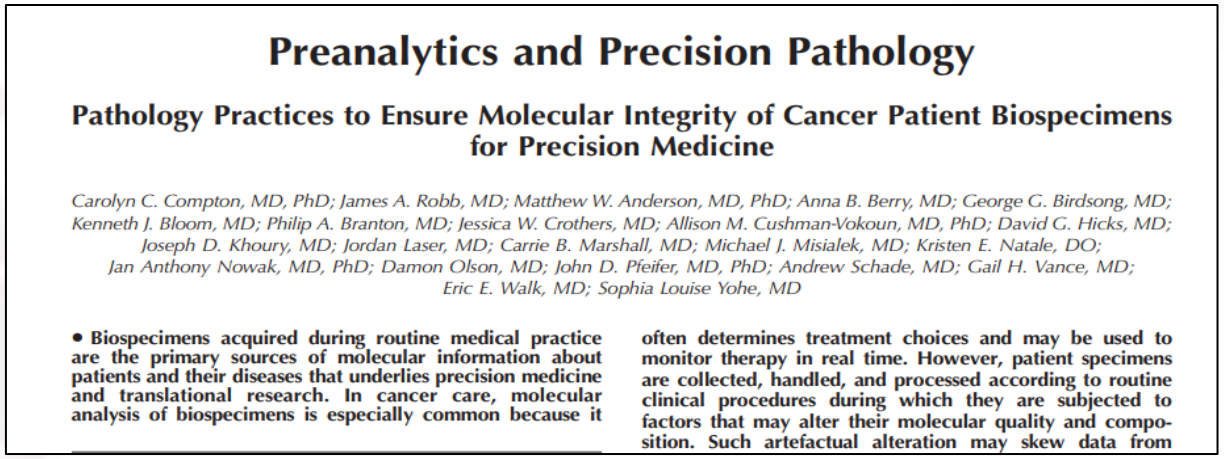
B. Paige Bass, PhD; Kelly B. Engel, PhD; Sarah R. Greytak, PhD; Helen M. Moore, PhD

Literature-based Recommendations for FFPE Tissue

	DNA	RNA	Protein
Cold ischemia time	<24 hrs for PCR <1 hr for FISH	<12 hrs	<12 hrs
Specimen size	3-10 mm^3	N/A	1.2-3.5mm^3
Fixative	NBF	NBF	NBF
Fixation time	<72 hrs	8-48 hrs	6-24 hrs
Embedding	Paraffin	N/A	N/A
Storage	<5-10 yr	<1 yr	<25 yr
Decalcification	EDTA	EDTA	Tissue/antigen specific

Based on data from Bass, B.P. Arch Pathol Lab Med. 2014 Nov;138(11):1520-30. doi: 10.5858/arpa.2013-0691-RA.

Preanalytical Factors Can Impact Molecular Testing



Preanalytics and Precision Pathology

Pathology Practices to Ensure Molecular Integrity of Cancer Patient Biospecimens for Precision Medicine

Carolyn C. Compton, MD, PhD; James A. Robb, MD; Matthew W. Anderson, MD, PhD; Anna B. Berry, MD; George G. Birdsong, MD; Kenneth J. Bloom, MD; Philip A. Branton, MD; Jessica W. Crothers, MD; Allison M. Cushman-Vokoun, MD, PhD; David G. Hicks, MD; Joseph D. Khoury, MD; Jordan Laser, MD; Carrie B. Marshall, MD; Michael J. Misialek, MD; Kristen E. Natale, DO; Jan Anthony Nowak, MD, PhD; Damon Olson, MD; John D. Pfeifer, MD, PhD; Andrew Schade, MD; Gail H. Vance, MD; Eric E. Walk, MD; Sophia Louise Yohe, MD

• Biospecimens acquired during routine medical practice are the primary sources of molecular information about patients and their diseases that underlies precision medicine and translational research. In cancer care, molecular analysis of biospecimens is especially common because it often determines treatment choices and may be used to monitor therapy in real time. However, patient specimens are collected, handled, and processed according to routine clinical procedures during which they are subjected to factors that may alter their molecular quality and composition. Such artefactual alteration may skew data from

Arch Pathol Lab Med. 2019 Nov;143(11):1346-1363. doi: 10.5858/arpa.2019-0009-SA.

Top 6 Preanalytical Factors for Tissue for the Maintenance of Nucleic Acid and Protein Quality and Integrity

Time to stabilization (cold ischemia time)
- 1 h or less

Method of stabilization
- Fixative: 10% phosphate-buffered formalin, pH 7.0
- Total time in formalin: at least 6 h, not more than 24–36 (tissue with high fat content may require 48 h)
- Acid decalcification, before or during stabilization, is contraindicated for nucleic acid analyses

Method of processing
- Specimen thickness not to exceed 4–5 mm
- Volume to mass ratio 4:1 at a minimum, preferably 10:1 with tissue completely submerged

Tissue processor variables
- Processor maintenance daily per manufacturer's recommendations
- Quality of processing fluids rigorously maintained
  - Maintenance of formalin purity and pH
  - Attention to water (ie, formalin) contamination of alcohol baths
- Type of paraffin
  - Low-melt paraffin (melts at <60°C)

Storage conditions
- Dry, pest-free conditions at room temperature (defined as 18°C–25°C)

Documentation data for the above factors and/or deviations from the recommendations

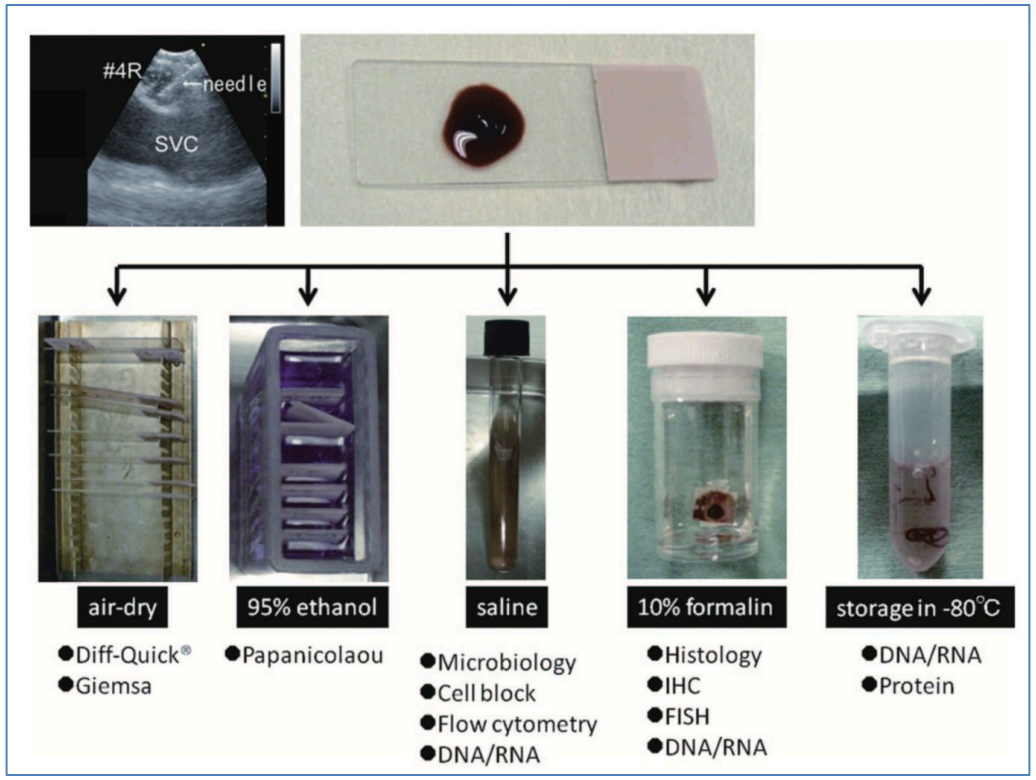
Note: Tissue specimens considered unacceptable for molecular testing include desiccated tissues or those known to have been improperly collected or stored

Preanalytical Factors Can Impact Molecular Testing

- The preanalytical variables for the vast majority of clinical specimens are largely uncontrolled, undocumented, and unknown
- An estimated 60% to 70% of laboratory-associated errors are due to preanalytical factors, involving mishandling during specimen collection, transport, processing, and storage

Arch Pathol Lab Med. 2019 Nov;143(11):1346-1363. doi: 10.5858/arpa.2019-0009-SA.

Preanalytical Factors in Cytology Specimens



J Thorac Oncol. 2011;6: 203–206

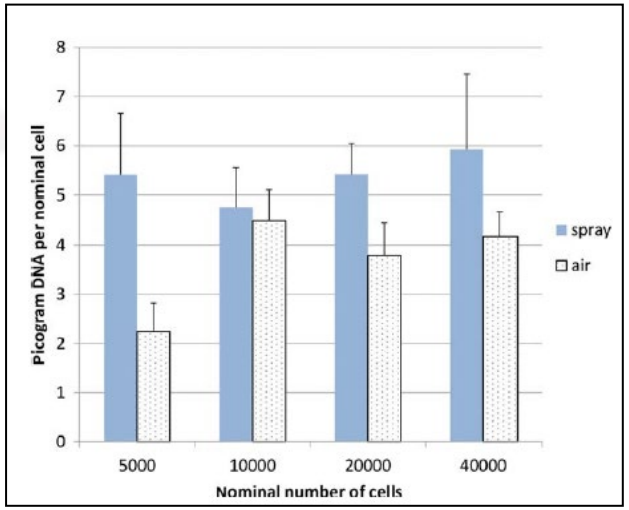
Is there a need to **standardize** cytology specimen collection and processing to **optimize** preanalytical variables for molecular testing?

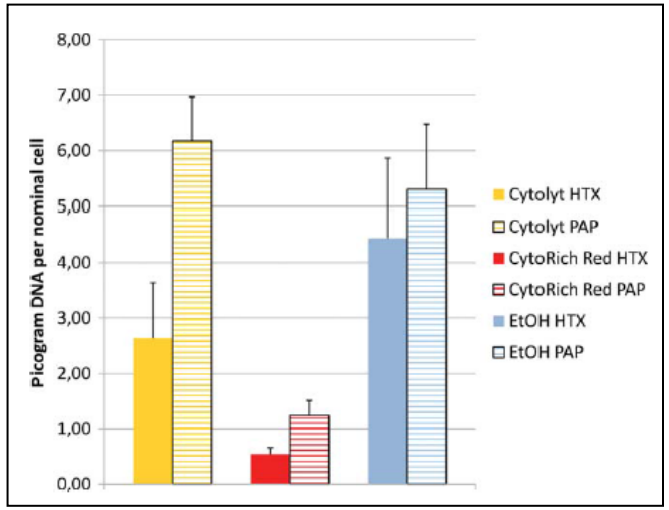
Specimen Processing Affects DNA Yield

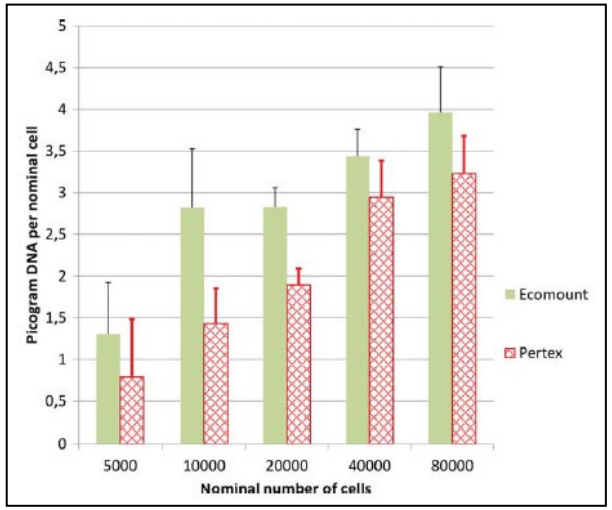
Preparation of DNA From Cytological Material

Effects of Fixation, Staining, and Mounting Medium on DNA Yield and Quality

Annika Dejmek, MD, PhD[1,2]; Nooreldin Zendehrokh, PhD[1];
Malgorzata Tomaszewska, MSc[3]; and Anders Edsjö, MD, PhD[1,2,4,5]







Dejmek A et. al. Cancer Cytopathol. 2013 Jul;121(7):344-53. doi: 10.1002/cncy.21276.

Specimen Processing Affects DNA Yield

Optimizing the DNA Yield for Molecular Analysis From Cytologic Preparations

Sinchita Roy-Chowdhuri, MD, PhD[1]; Chi-Wan Chow[2]; Mary K. Kane, CT (ASCP)[1]; Hui Yao, PhD[2,3]; Ignacio I. Wistuba, MD[2]; Savitri Krishnamurthy, MD[1]; John Stewart, MD, PhD[1]; and Gregg Staerkel, MD[1]

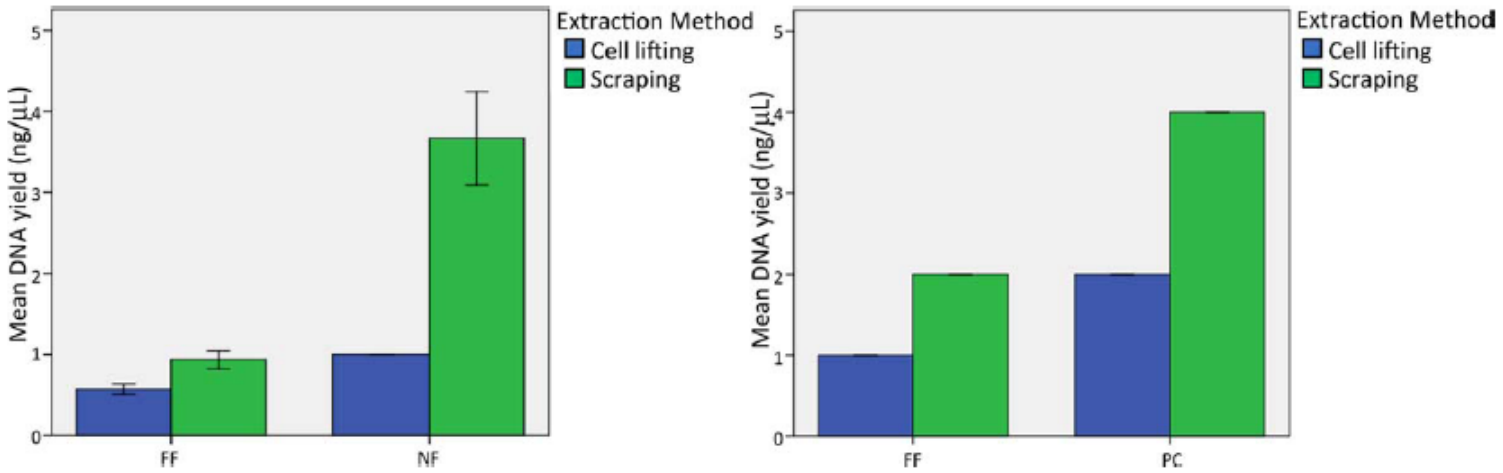


Figure 2. Comparing the DNA yields of cell-line cytospin preparations from FF and NF slides and from FF and PC slides with either scalpel-blade scraping or cell lifting as the tissue-extraction methodology. Error bars indicate ±1 standard deviation. FF indicates fully frosted; NF, nonfrosted; PC, positively charged.

Roy-Chowdhuri, S. et. al. Cancer Cytopathol. 2015 Dec 2. doi: 10.1002/cncy.21664.

Molecular Testing Success Depends on the Nucleic Acid Yield

TABLE 1. Tumor Fractions and DNA Yields of Cytology Cases That Were Successfully Sequenced With NGS or Failed

	Overall	NGS Success	NGS Failure
Cytology cases, No. (%)	207	164 (79)	43 (21)
Median tumor %	70	70	60
DNA yield, mean, ng/μL	4.7	5.2	2.5
DNA yield, median, ng/μL	1.8	2.5	0.2
DNA yield, range, ng/μL	0-32.5	0.07-32.5	0-25.2

Abbreviation: NGS, next-generation sequencing.

Cancer Cytopathol. 2015 Jul 31. doi: 10.1002/cncy.21597.

TABLE 3. Summary of the Factors Affecting RNA-Based Next-Generation Sequencing Testing Success

Variable	Testing Success	Testing Failure	*P*
Lesion size, Median (Range), cm	1.8 (0.7-12.7)	1.7 (0.8-7.4)	.45
No. of slides used for RNA extraction, Median (Range)	7 (1-27)	8 (1-25)	.85
CB sections	10 (1-27)	13 (8-25)	.10
Smear slides	2 (1-6)	2 (1-4)	.89
Tumor percentage, Median (Range)	60 (20-95)	60 (25-98)	.36
RNA yield, Median (Range), μg/μL	0.028 (0.002-0.654)	0.006 (0.002-0.224)	.03
Type of preparation, no.			
CB only, n = 78	72	6	.56
Smears: DQ or Pap, n = 56	50	6	

Abbreviations: CB, cell block; DQ, Diff-Quik stain; Pap, Papanicolaou stain.

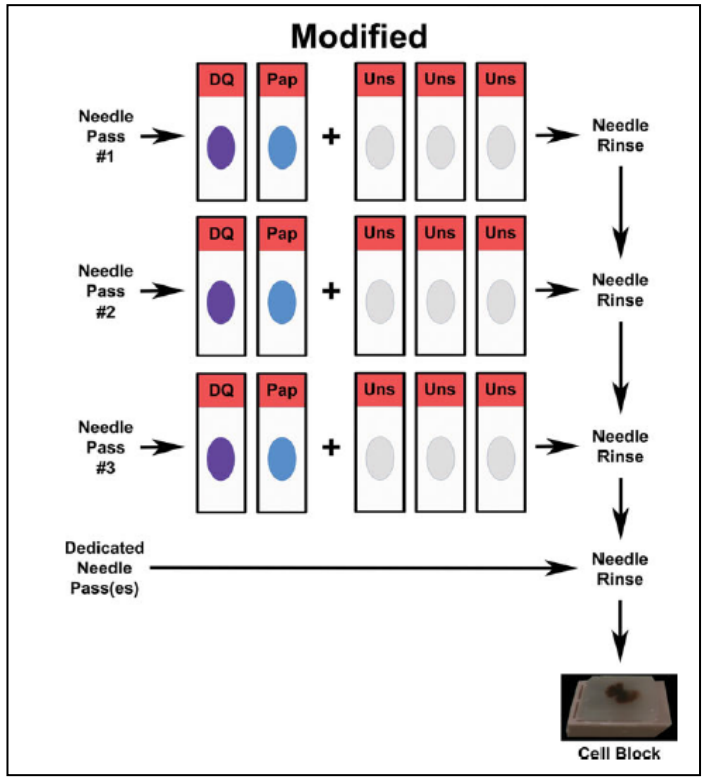
Cancer Cytopathol. 2021 May;129(5):374-382. doi: 10.1002/cncy.22381.

NGS success *positively correlates* with **DNA and RNA yield**

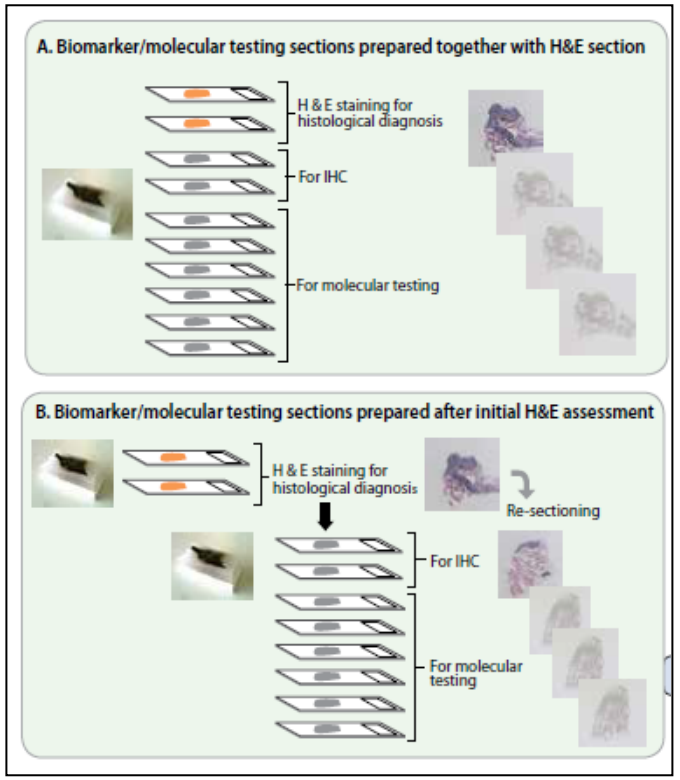
Specimen Processing and Handling

- Triaging material in anticipation of ancillary studies

  (e.g. preparing additional decoverslipped smears; cutting additional unstained cell-block sections)



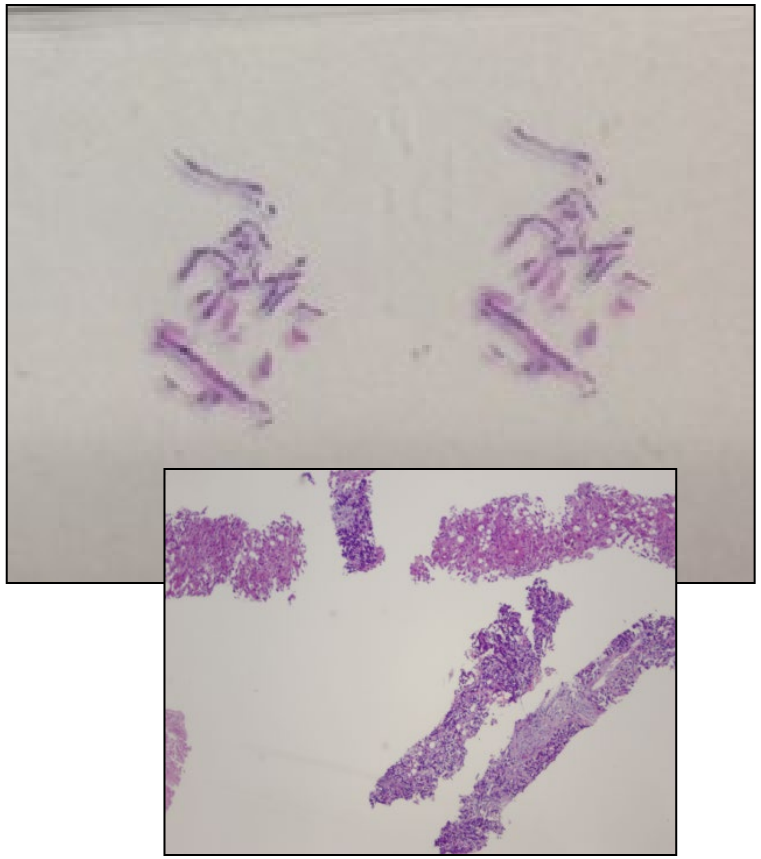
Cancer Cytopathol. 2013 Mar;121(3):120-8.

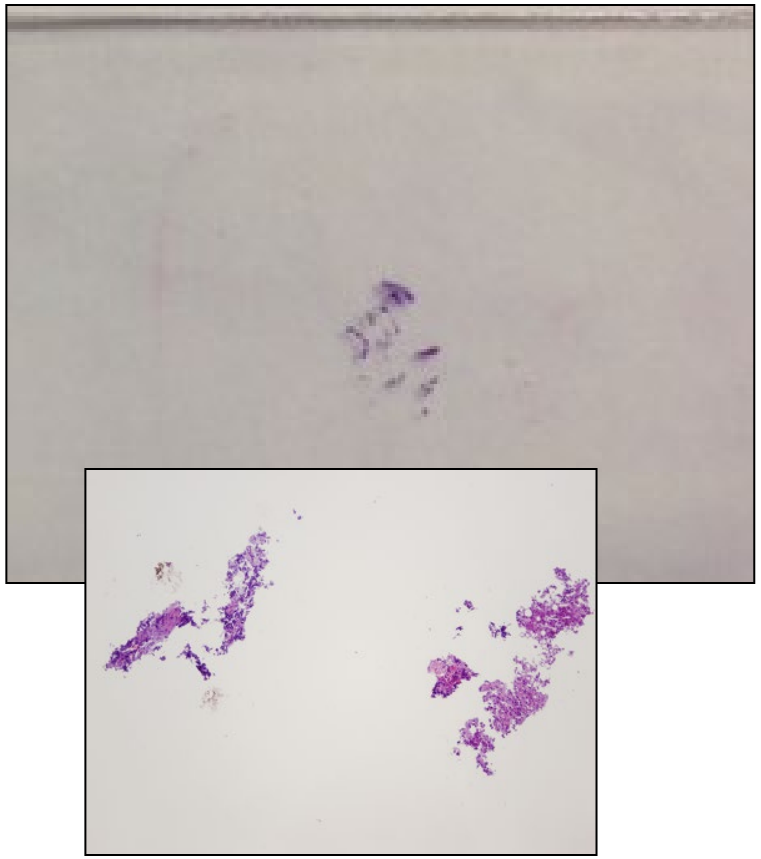


https://www.iaslc.org/sites/default/files/wysiwyg-assets/pd-l1_atlas_book_lo-res.pdf

Specimen Processing and Handling

- **Clear communication** between ordering clinician, proceduralist, laboratory technician/technologist, and pathologist





Specimen Selection

Step 3. Select the Best Specimen for Molecular Testing

- Knowledge about molecular adequacy criteria, nucleic acid-extraction techniques, and basic principles of molecular testing are needed to select the most appropriate material

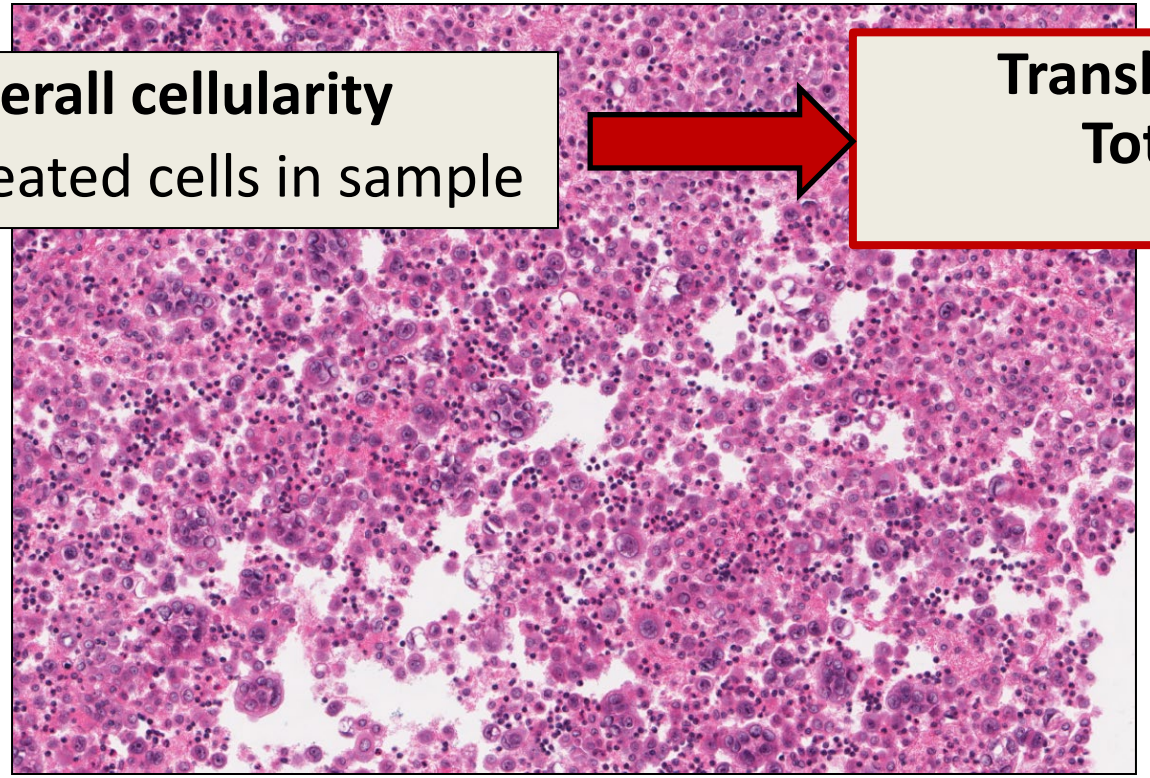
Cytology Specimen Assessment

Cellularity estimate

- Overall cellularity

Overall cellularity

All nucleated cells in sample

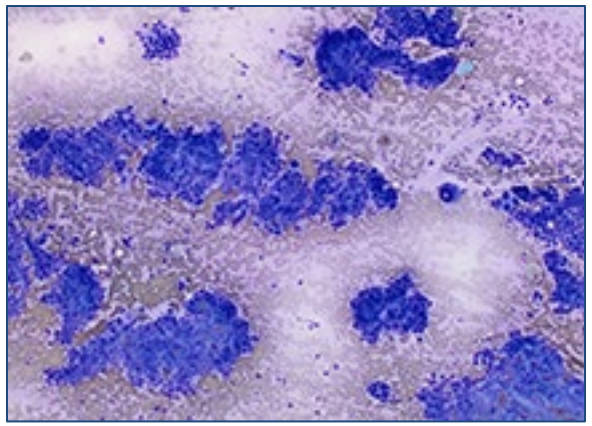


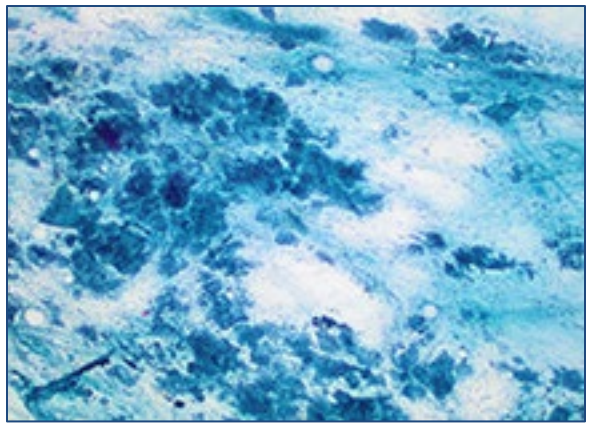
Translates to the Total yield

How many cells do you need?

- 1 cell ~7 pg of DNA
- Molecular assay requiring 1 ng of DNA input therefore needs ~166 intact cells
- NGS (Ion Torrent PGM)requires around 10 ng of DNA

  Therefore approximately 1660 *intact* cells

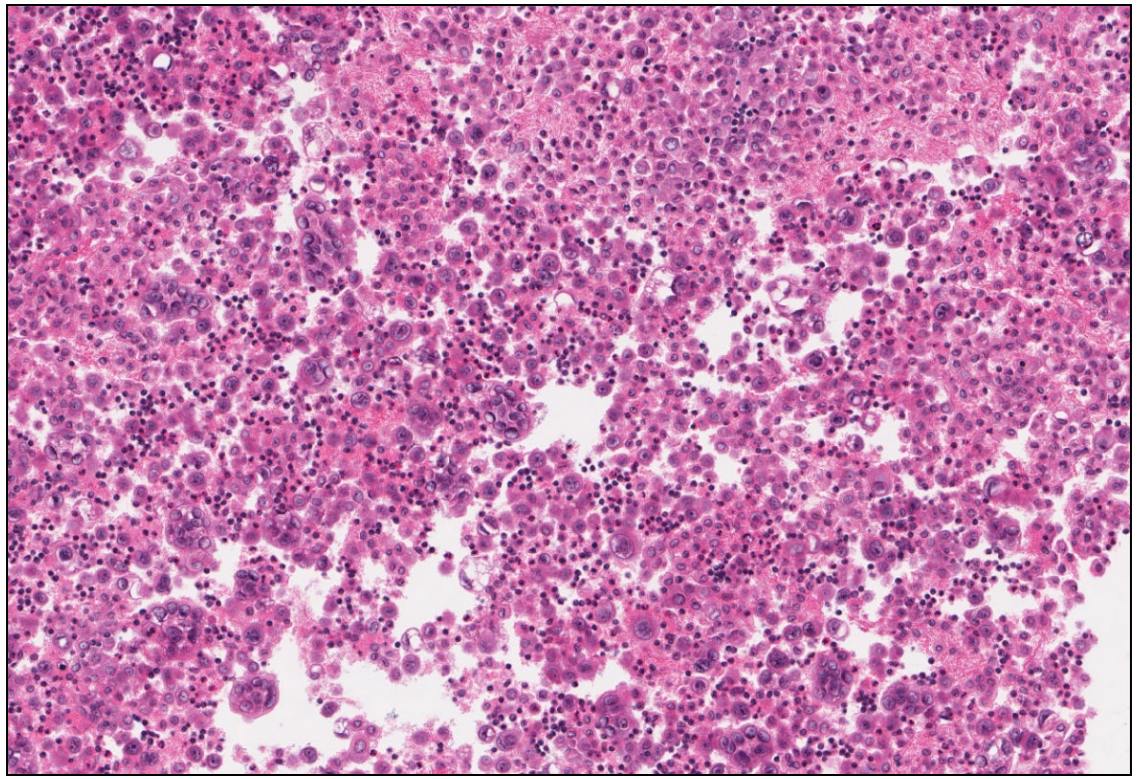




Cytology Specimen Assessment

Cellularity estimate

- Tumor cellularity/ tumor proportion/ tumor fraction/ tumor %



How do you Estimate Tumor Fraction?

A. Volume of tissue occupied by tumor cells/ total volume of tissue

B. Surface area occupied by tumor cells/ total volume of tissue

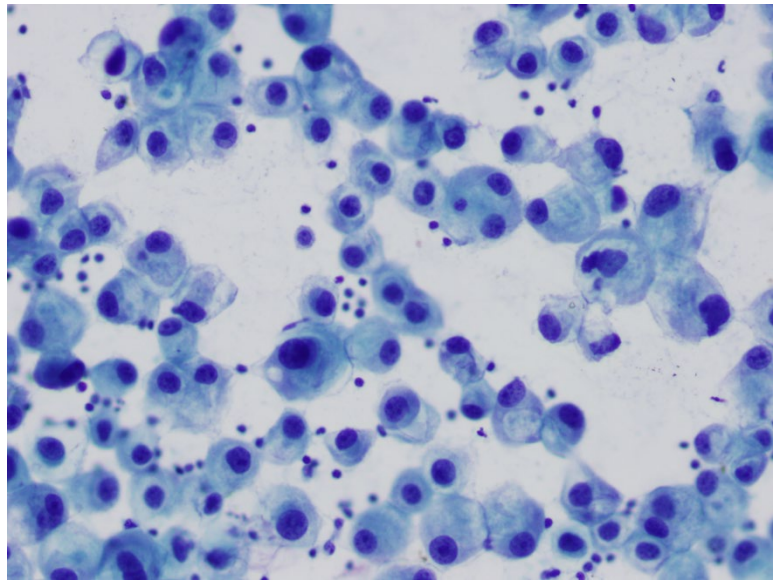
C. Number of viable tumor nuclei/total number of viable nucleated cells

How do you Estimate Tumor Fraction?

A. Do red blood cells count when estimating total number of cells?

B. Do necrotic tumor cells count in total number of tumor cells?

C. Does the size of the cell/nuclei matter when counting cells?



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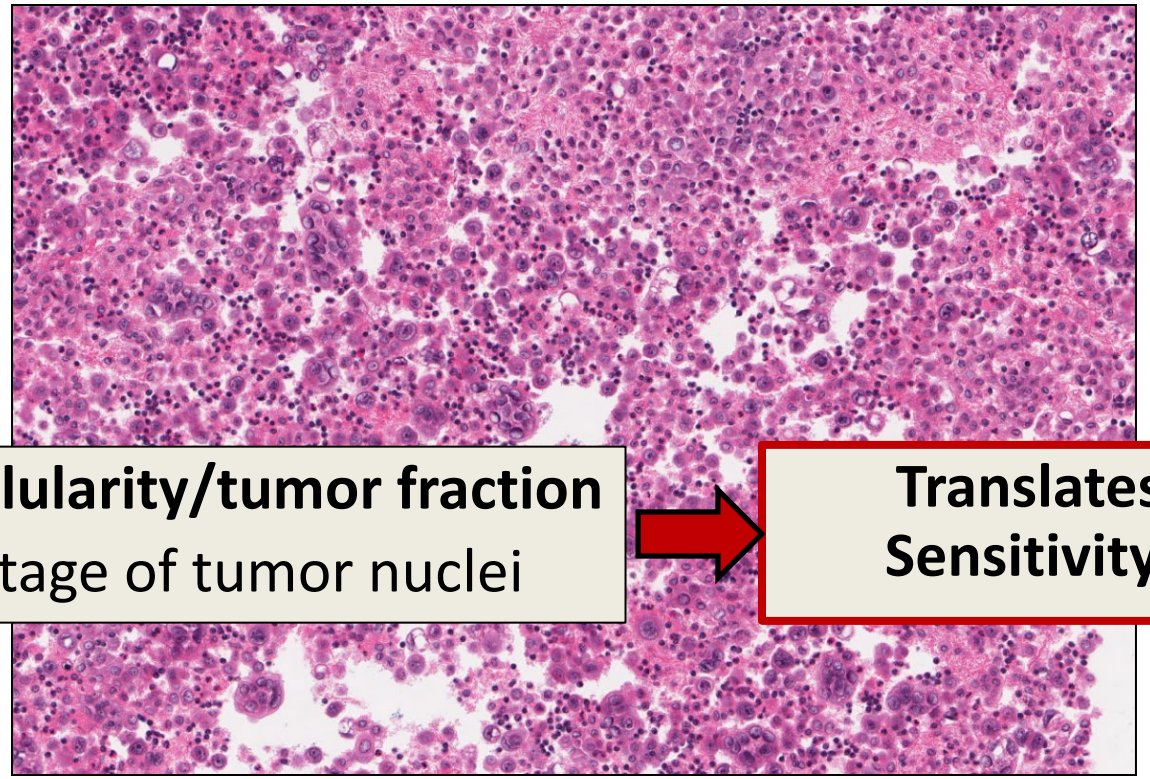
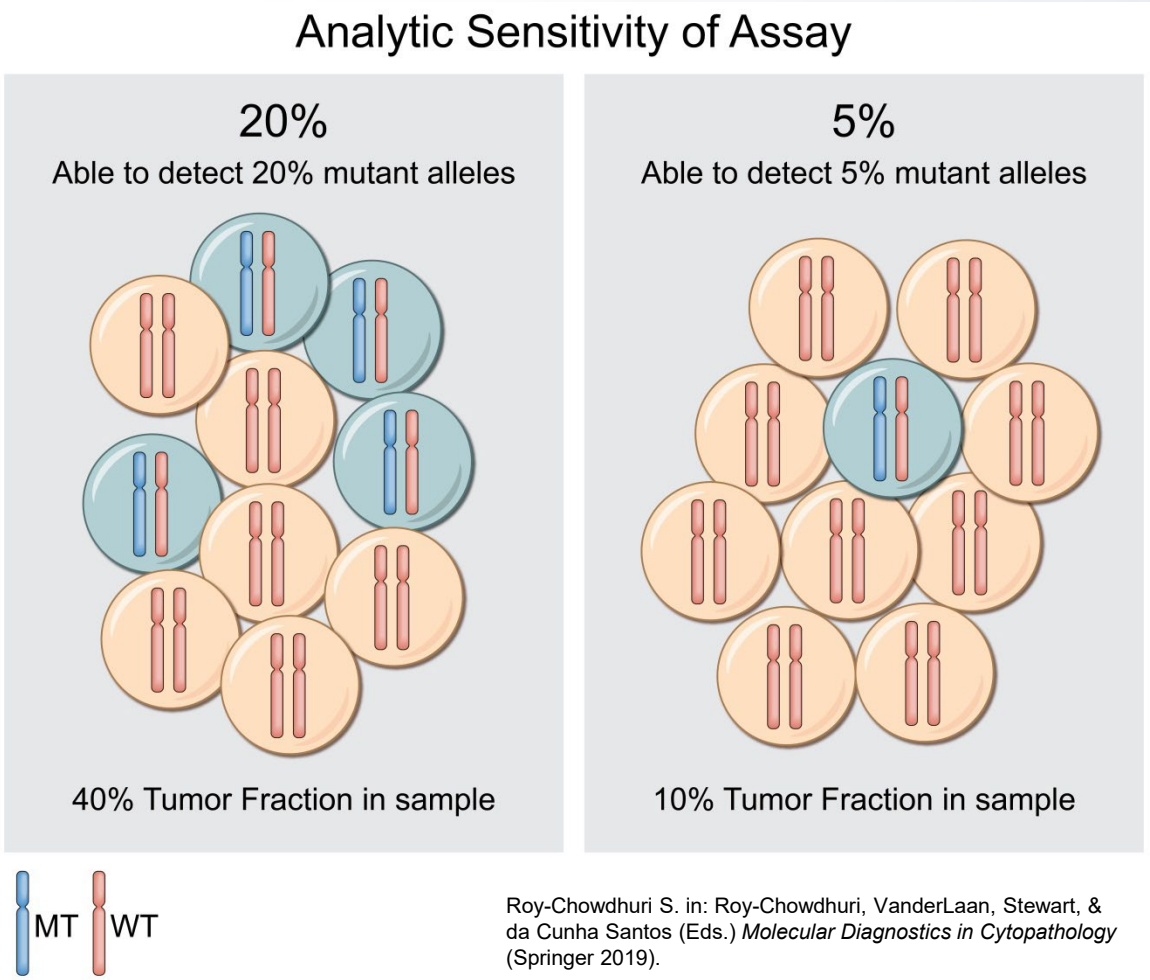


Tumor cellularity/tumor fraction

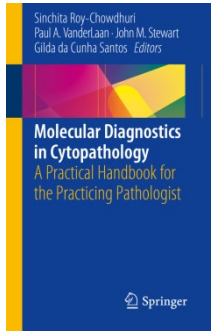
Percentage of tumor nuclei

Translates to Analytic Sensitivity of Platform

Analytic Sensitivity is the Lowest Limit of Detection



Roy-Chowdhuri S. in: Roy-Chowdhuri, VanderLaan, Stewart, & da Cunha Santos (Eds.) *Molecular Diagnostics in Cytopathology* (Springer 2019).



Analytic Sensitivity of Assay: Why do we need to care?

Problem Case

EGFR testing has been requested on this pleural fluid. The sample has a low tumor fraction on the smears and cell block. I tumor mapped several areas on two of the smears that show relative enrichment, but the absolute volume of material is relatively low. Because of the low tumor fraction there is a chance of a false negative, which should be evident since the patient has a known EGFR mutation. I assume this will be run by NGS, since the T% is 21% at best. This will be sent over tomorrow.

John

MOLECULAR RESULTS

EGFR mutation analysis:

No mutation detected in exons 18 to 21 of the *EGFR* gene.

METHODOLOGY: PCR-based DNA sequencing analysis was performed. The analysis was limited to exons 18 to 21 of the kinase domain of the epidermal growth factor receptor (*EGFR*) gene. The presence of mutations outside the tested exons

But patient has a known *EGFR* mutation

So, what went wrong?

Analytic Sensitivity of Assay: Why do we need to care?

Problem Case

EGFR testing has been requested on this pleural fluid. The sample has a low tumor fraction on the smears and cell block. I tumor mapped several areas on two of the smears that show relative enrichment, but the absolute volume of material is relatively low. Because of the low tumor fraction there is a chance of a false negative, which should be evident since the patient has a known EGFR mutation. I assume this will be run by NGS, since the T% is 21% at best. This will be sent over tomorrow.

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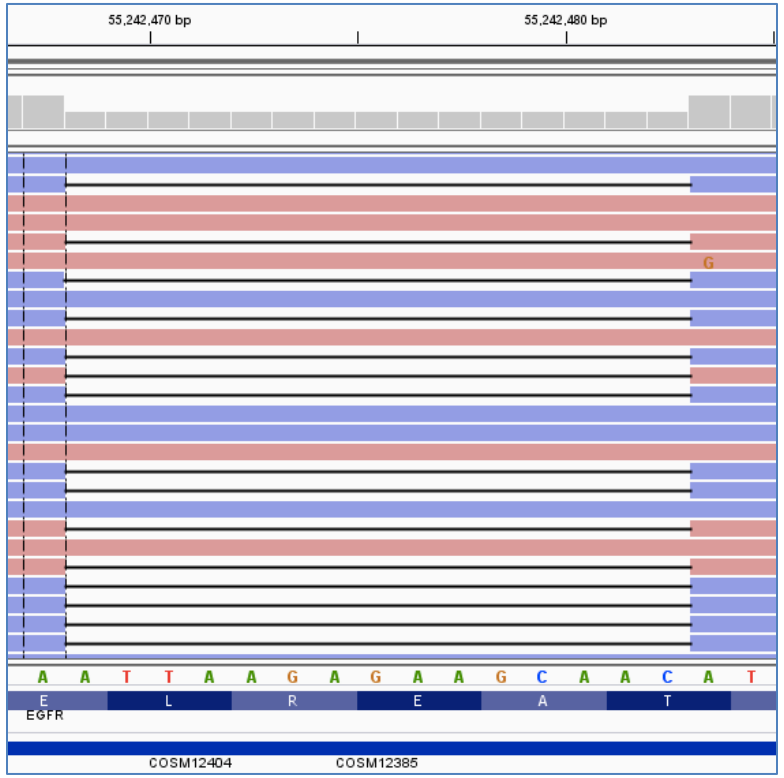
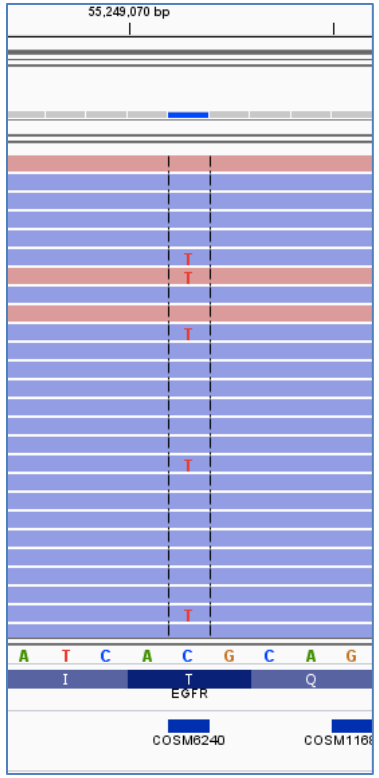
Low tumor fraction sample (approximately 20% tumor) tested by Sanger sequencing (low analytic sensitivity)

Analytic Sensitivity of Assay: Why do we need to care?

I,II. Somatic mutations						
Gene	Standardized Nomenclature (HGVS)	Location	DNA change	Protein change	dbSNP ID	COSMIC ID
EGFR	NM_005228.3(EGFR):c.2369C>T p.T790M	Exon 20	SNV	Missense	rs121434569	COSM6240
EGFR	NM_005228.3(EGFR):c.2240_2254del p.L747_T751del	Exon 19	Deletion	Deletion		COSM12369





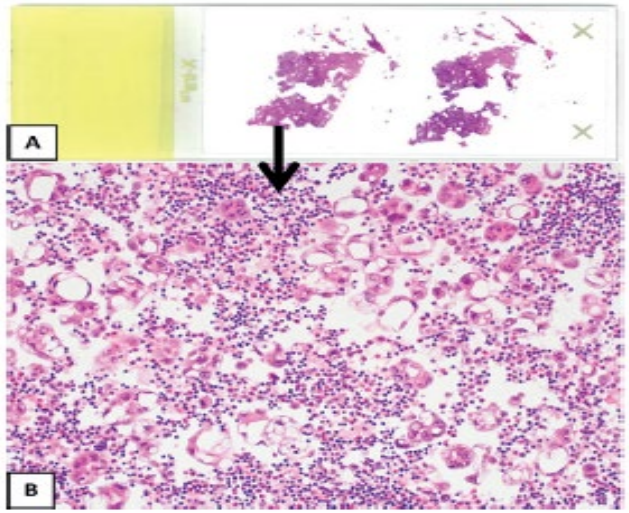
Key Element of Specimen Selection

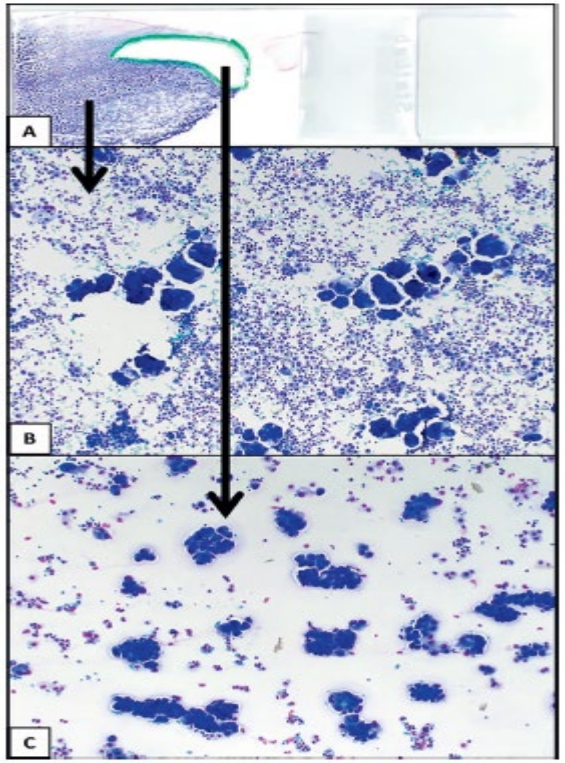
Role of the pathologist in **specimen selection** is finding the best fit (molecular assay) for the sample

- **Modulate the specimen** to fit the assay
- **Modulate the assay** to fit the specimen *(part 2)*

Modulate Specimen to Assay

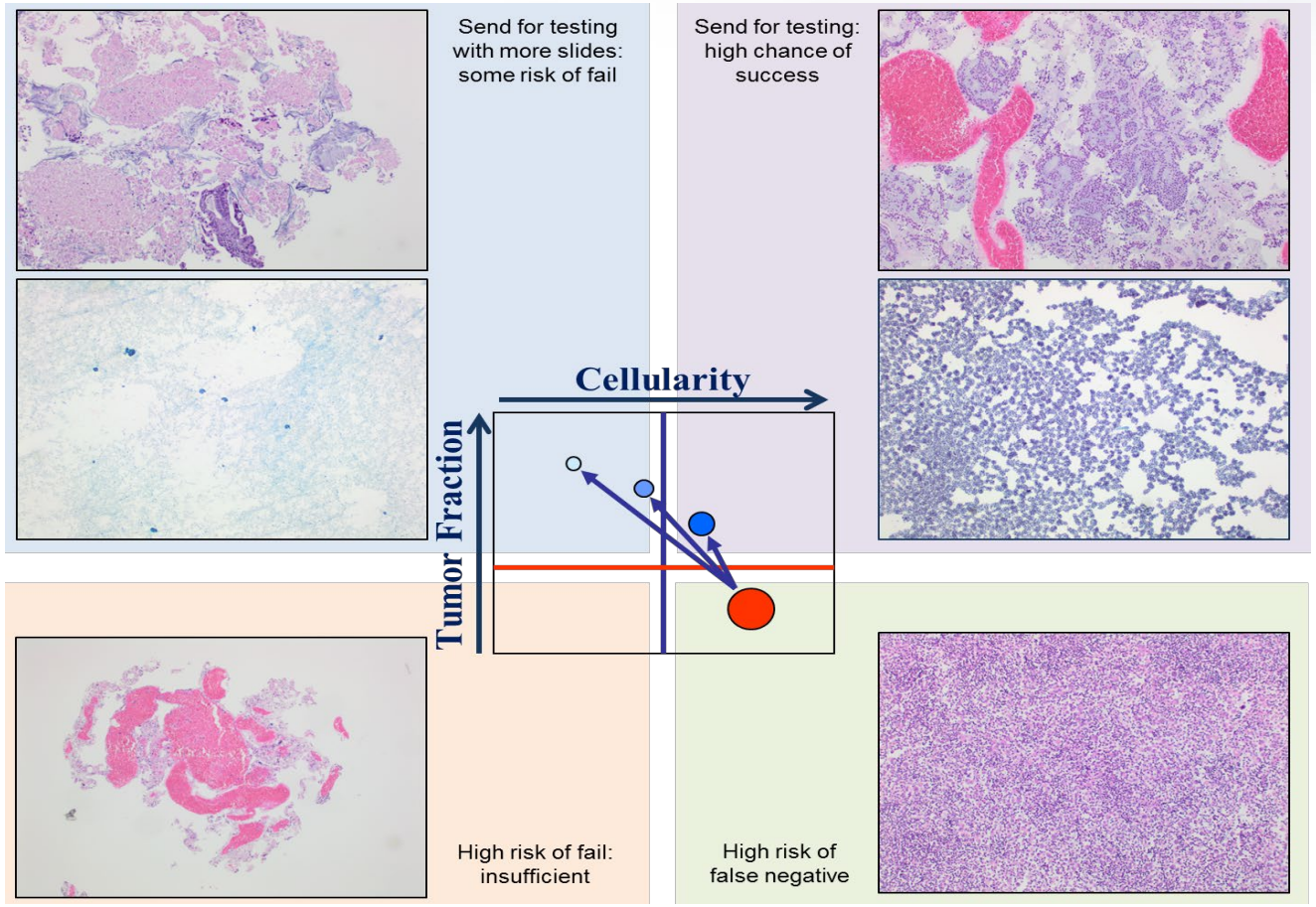
- Use **tumor enrichment** methods to increase the tumor fraction of the sample





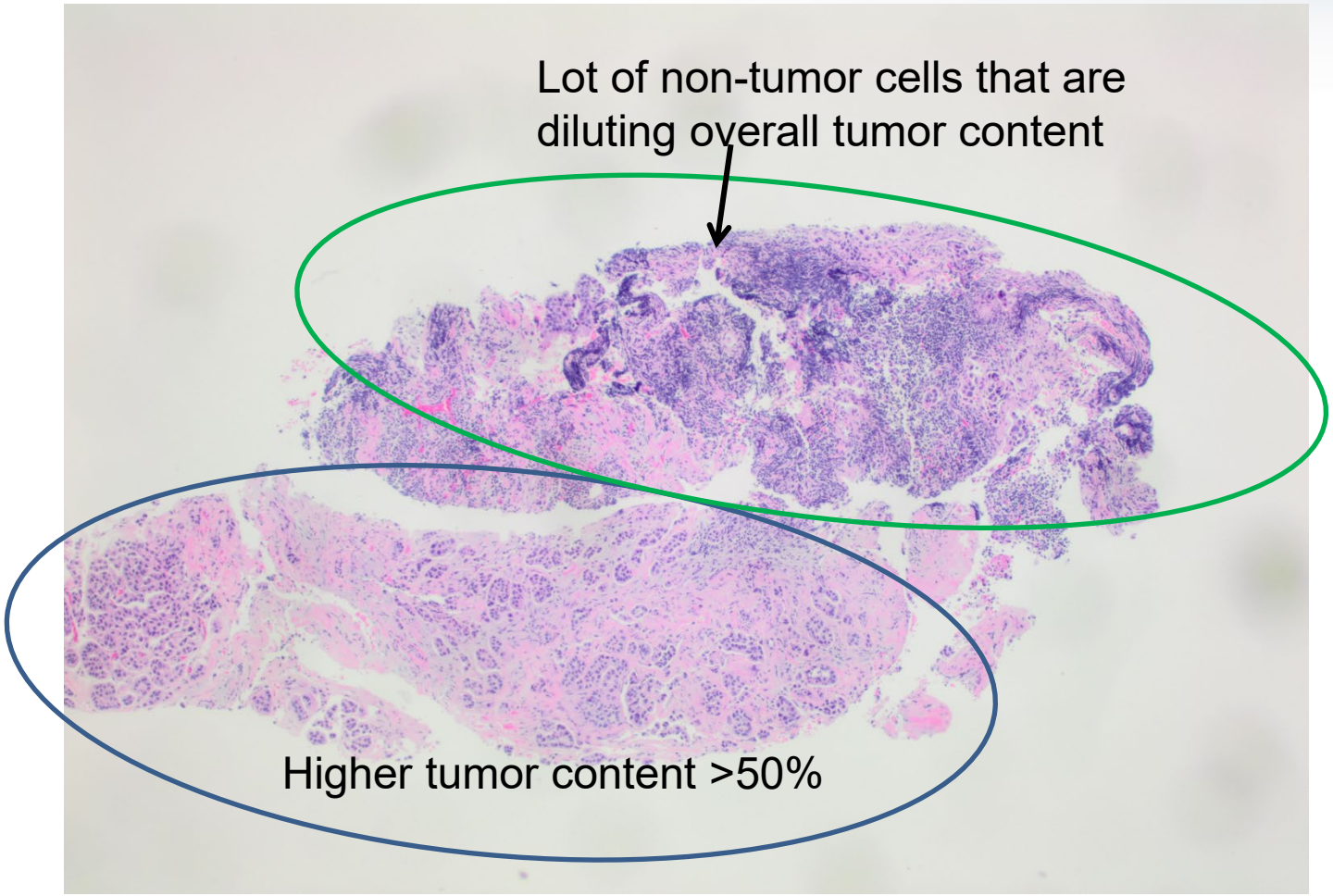
Roy-Chowdhuri, S and Stewart, J. Arch Pathol Lab Med. 2016 Jun 22.

Modulate Specimen to Assay



Roy-Chowdhuri, S and Stewart, J. Arch Pathol Lab Med. 2016 Jun 22.

Tumor Enrichment Reduces Risk of False Negative Results



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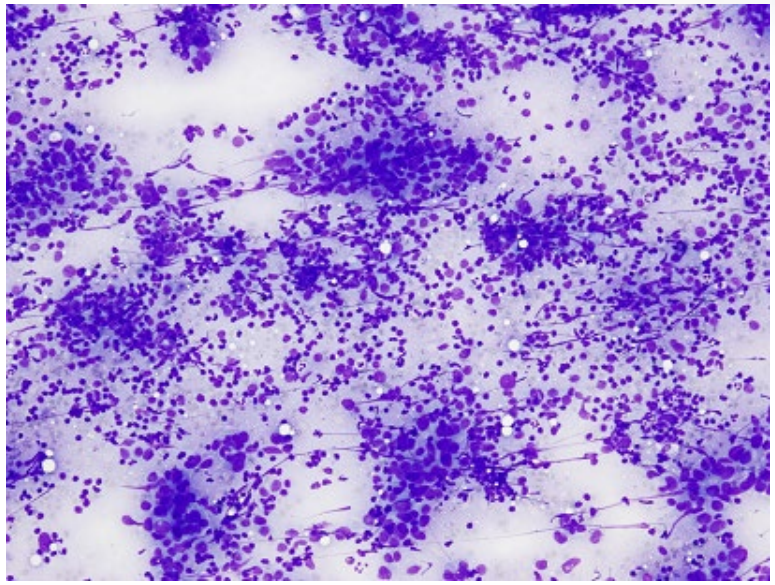
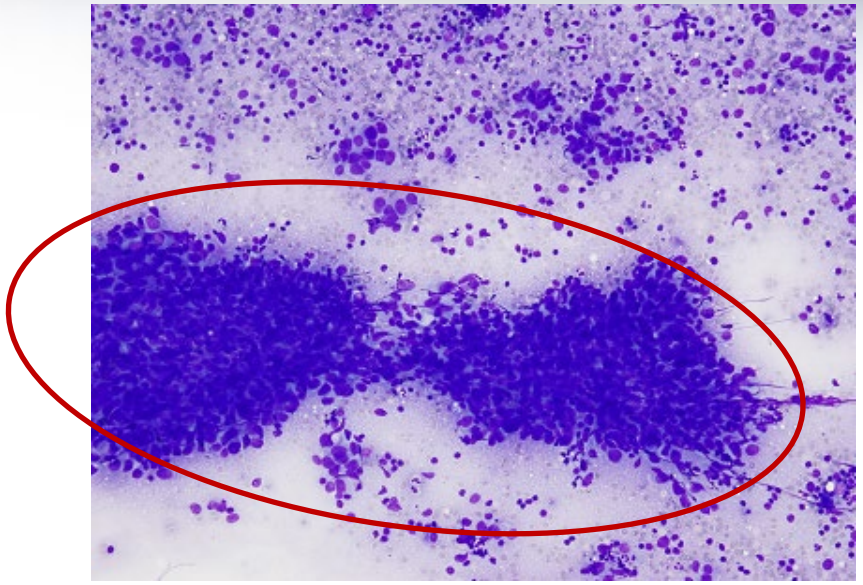
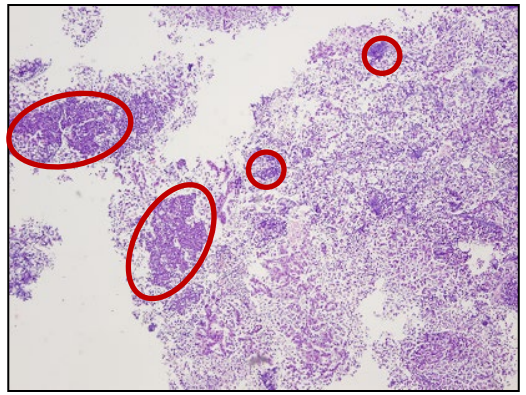
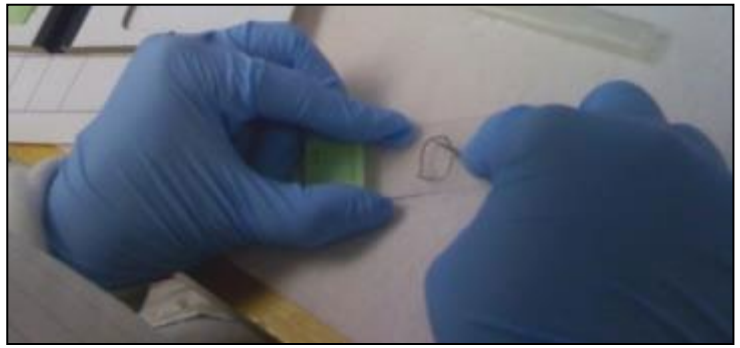
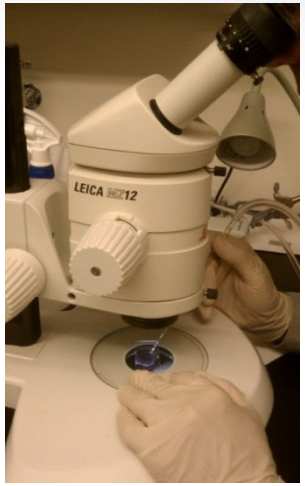






Tumor Enrichment: Techniques





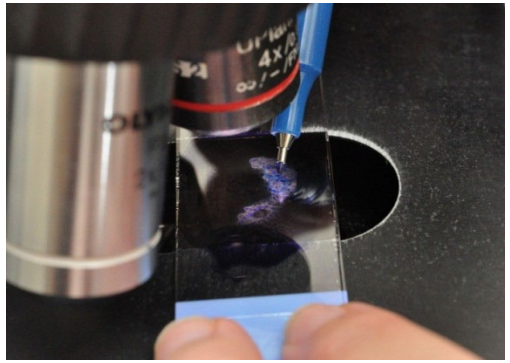
Tumor enrichment techniques:

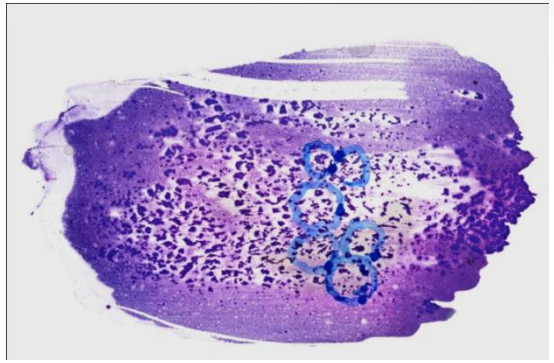
- Manual macrodissection
- Manual microdissection
- Laser capture microdissection

The majority of laboratories utilize macrodissection

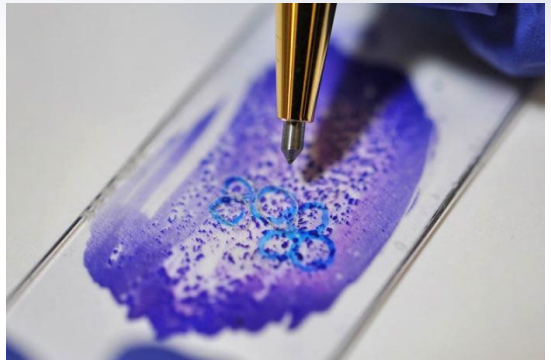
Pic courtesy: Dara L Aisner MD PhD, University of Colorado

Tumor Enrichment : Cytology Direct Smears

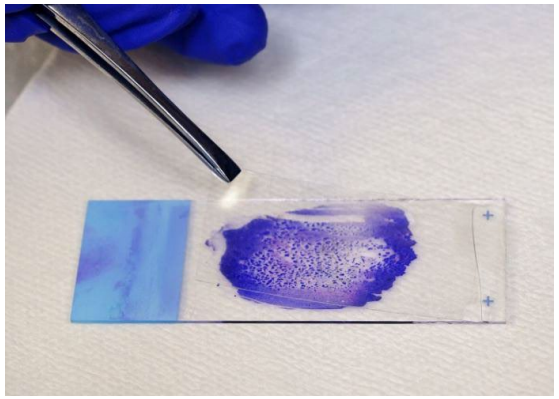




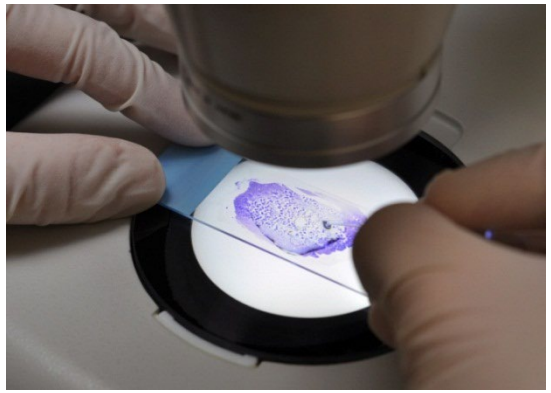
Slides are circled to enrich for tumor cells by a cytopathologist

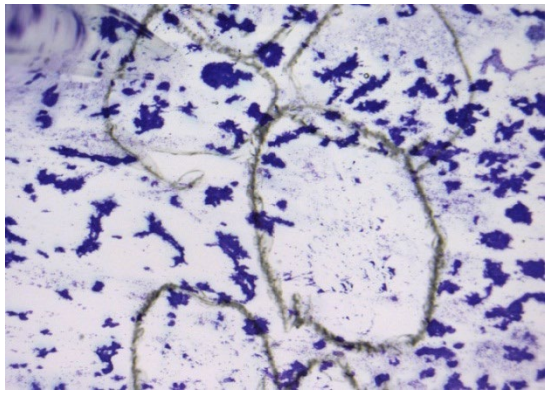


The circled areas are then etched on the bottom of the slide using a diamond-tip pen



Coverslip is removed by dipping the slide in xylene

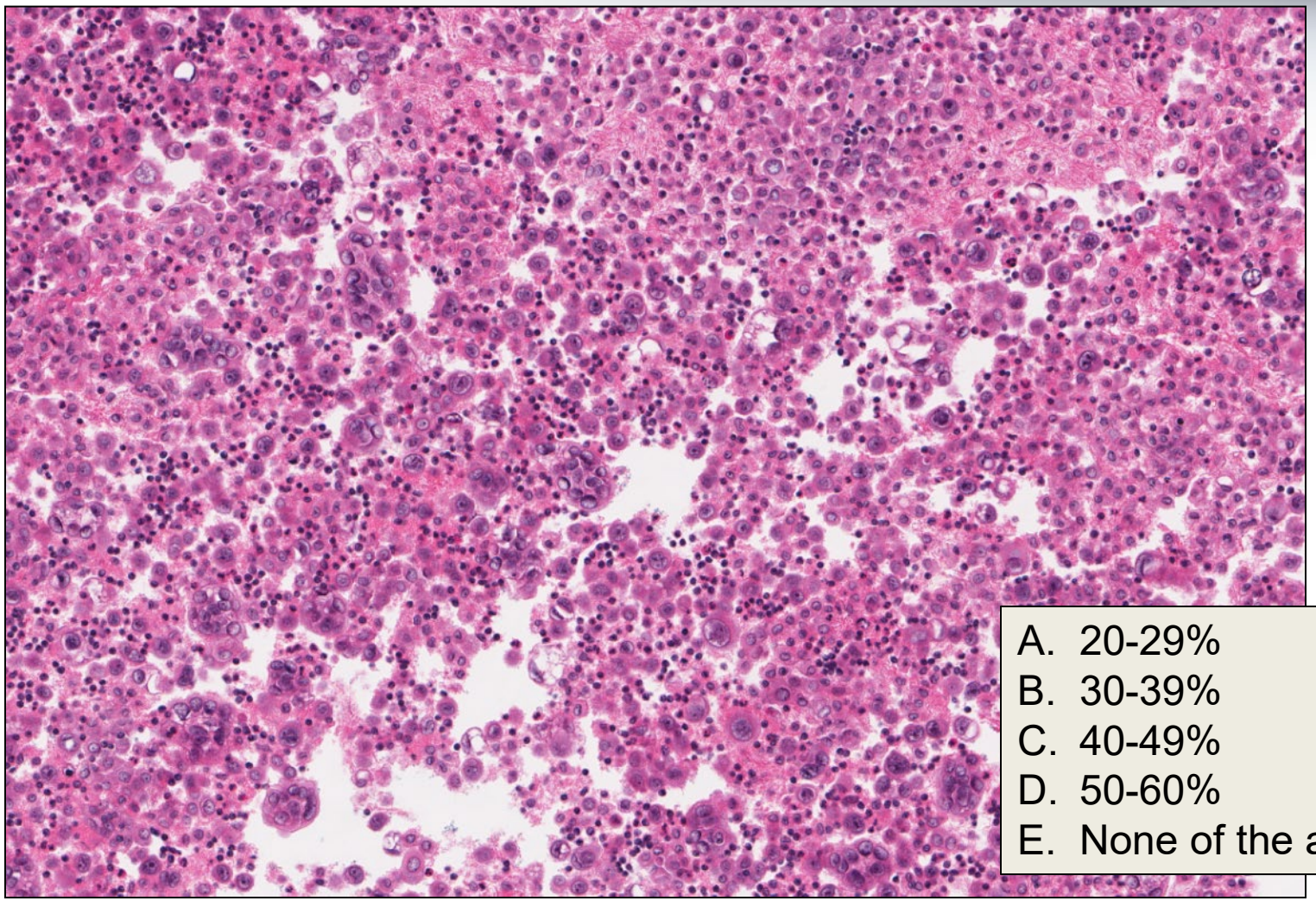




Circled areas are visualized under a microscope and cells are carefully scraped off the slide using a scalpel blade into a buffer for DNA extraction

Pic courtesy: Dara L Aisner MD PhD, University of Colorado

Assessing Tumor Fraction is an Imprecise Art



A. 20-29%
B. 30-39%
C. 40-49%
D. 50-60%
E. None of the above

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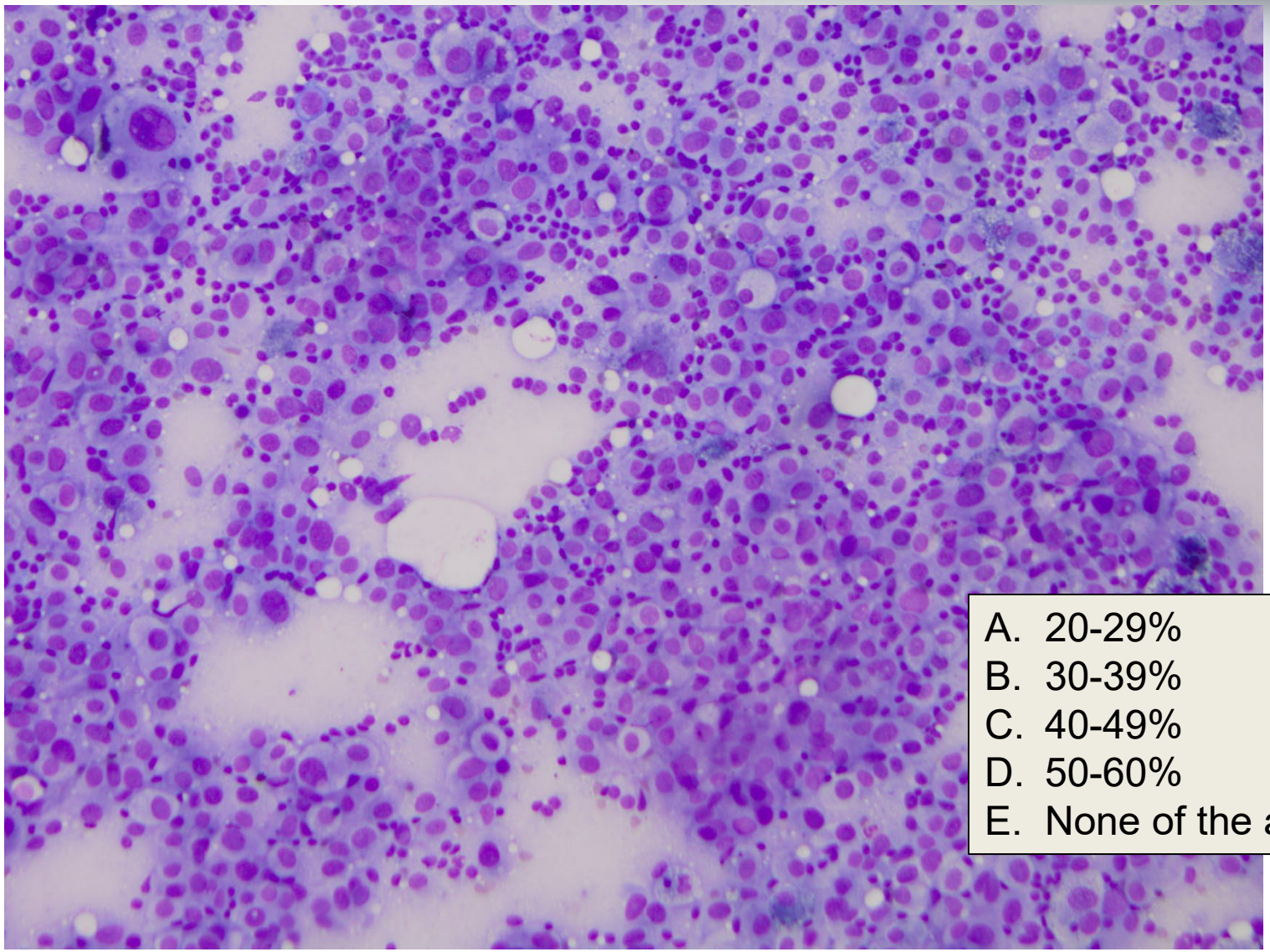

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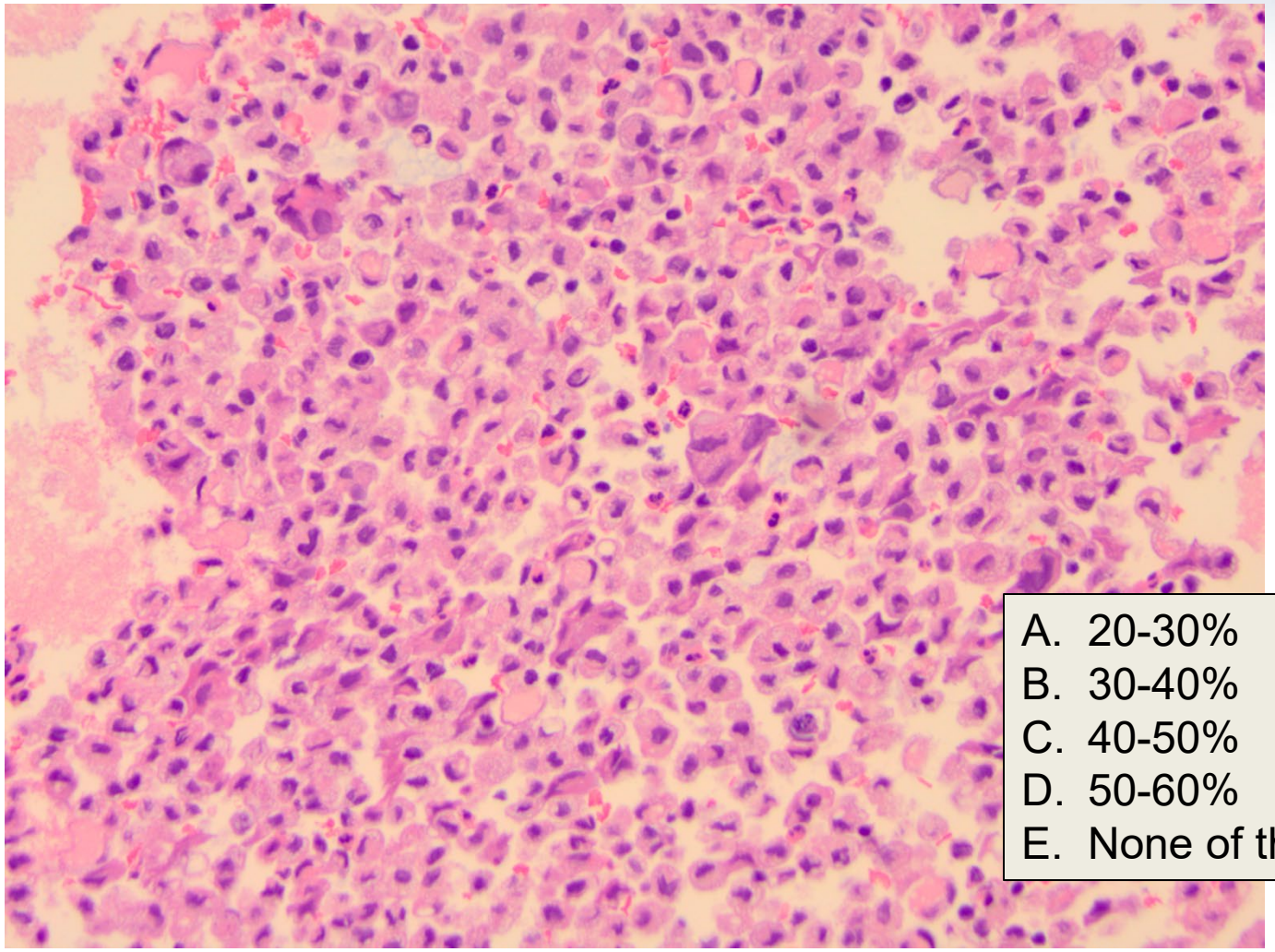


A. 20-30%
B. 30-40%
C. 40-50%
D. 50-60%
E. None of the above

Assessing Tumor Fraction is an Imprecise Art

Intra/interobserver variability

- Low inter-laboratory precision in tumor cellularity estimation
- Pathologists preferentially recognize atypical/ malignant cells

Assessing Tumor Fraction is an Imprecise Art

CAP Laboratory Improvement Programs

A Prospective, Multi-Institutional Diagnostic Trial to Determine Pathologist Accuracy in Estimation of Percentage of Malignant Cells

Hollis Viray, BS; Kevin Li; Thomas A. Long, MPH; Patricia Vasalos, BS; Julia A. Bridge, MD; Lawrence J. Jennings, MD; Kevin C. Halling, MD; Meera Hameed, MD; David L. Rimm, MD, PhD

• *Context.*—The fraction of malignant cells in tumor tissue submitted for tests of genetic alterations is a critical variable in testing accuracy. That fraction is currently determined by pathologist visual estimation of the percentage of malignant cells. Inaccuracy could lead to a false-negative test result.

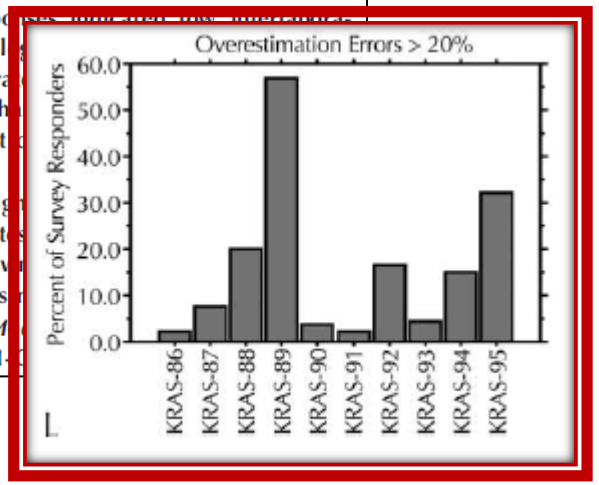
Objective.—To describe a prospective, multi-institutional study to determine pathologist estimation accuracy.

Design.—Ten ×20 magnification images of hematoxylin-eosin–stained colon tissue specimens were sent as an educational component of the College of American Pathologists KRAS-B 2011 Survey. Data from 194 labs were analyzed and compared to a criterion standard with comprehensive manual nuclear counts.

Results.—Survey resp
tory precision of pathol
mates were fairly accura
assessed showed more th
mating in a manner that
results.

Conclusions.—The sig
resulting in molecular t
for patient care is unknov
false-negative test results

(*Arch Pathol Lab M*
10.5858/arpa.2012-0561-



Assessing Tumor Fraction is an Imprecise Art

The estimation of tumor cell percentage for molecular testing by pathologists is not accurate

Alexander JJ Smits [1,2], J Alain Kummer[1], Peter C de Bruin[1], Mijke Bol[2], Jan G van den Tweel[2], Kees A Seldenrijk[1], Stefan M Willems[2], G Johan A Offerhaus[2], Roel A de Weger[2], Paul J van Diest[2] and Aryan Vink[2]

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Molecular pathology is becoming more and more important in present day pathology. A major challenge for any molecular test is its ability to reliably detect mutations in samples consisting of mixtures of tumor cells and normal cells, especially when the tumor content is low. The minimum percentage of tumor cells required to detect genetic abnormalities is a major variable. Information on tumor cell percentage is essential for a correct interpretation of the result. In daily practice, the percentage of tumor cells is estimated by pathologists on hematoxylin and eosin (H&E)-stained slides, the reliability of which has been questioned. This study aimed to determine the reliability of estimated tumor cell percentages in tissue samples by pathologists. On 47 H&E-stained slides of lung tumors a tumor area was marked. The percentage of tumor cells within this area was estimated independently by nine pathologists, using categories of 0–5%, 6–10%, 11–20%, 21–30%, and so on, until 91–100%. As gold standard, the percentage of tumor cells was counted manually. On average, the range between the lowest and the highest estimate per sample was 6.3 categories. In 33% of estimates, the deviation from the gold standard was at least three categories. The mean absolute deviation was 2.0 categories (range between observers 1.5–3.1 categories). There was a significant difference between the observers ($P<0.001$). If 20% of tumor cells were considered the lower limit to detect a mutation, samples with an insufficient tumor cell percentage (<20%) would have been estimated to contain enough tumor cells in 27/72 (38%) observations, possibly causing false negative results. In conclusion, estimates of tumor cell percentages on H&E-stained slides are not accurate, which could result in misinterpretation of test results. Reliability could possibly be improved by using a training set with feedback.

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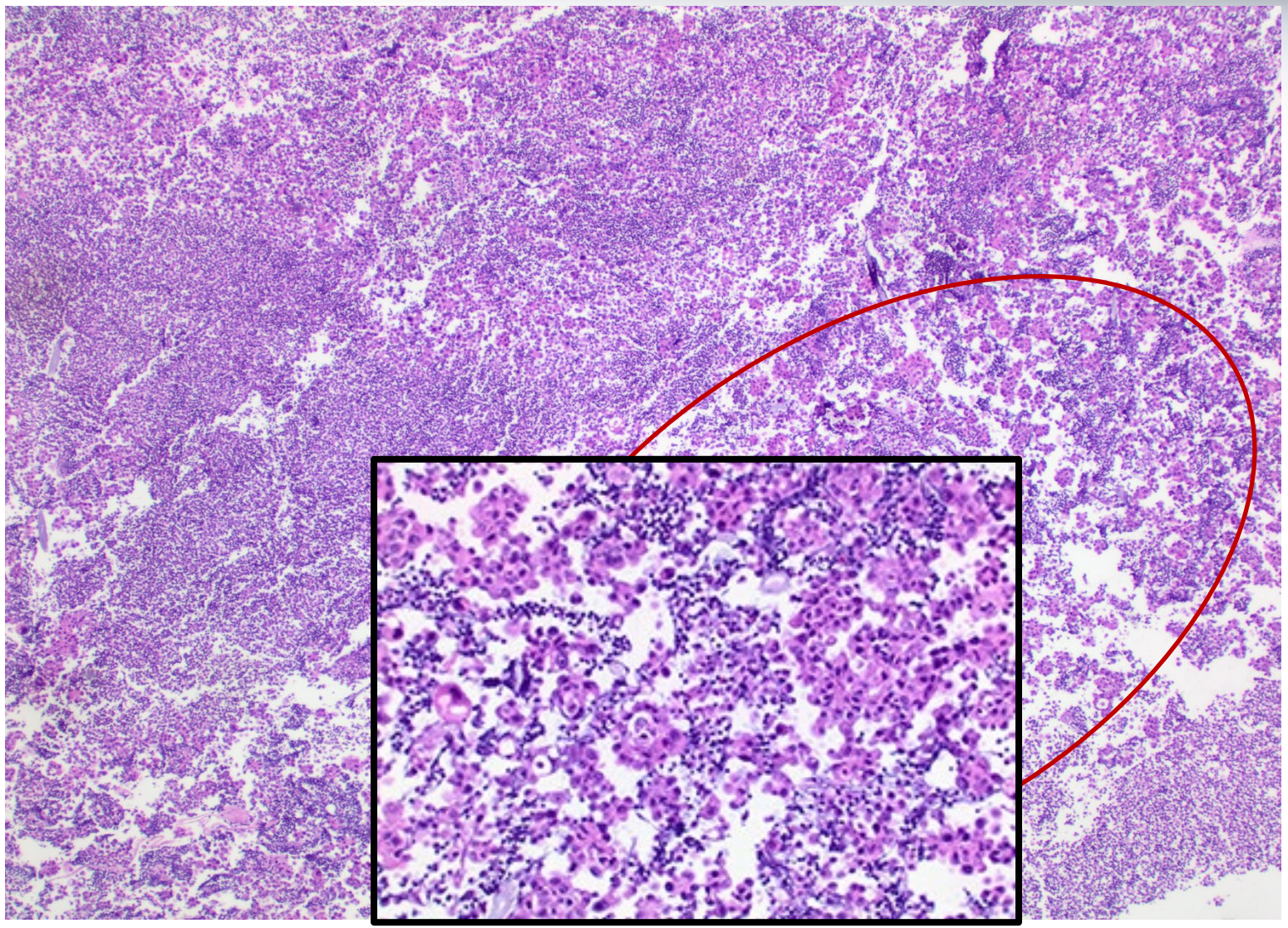
Assessing Tumor Cellularity is an Imprecise Art

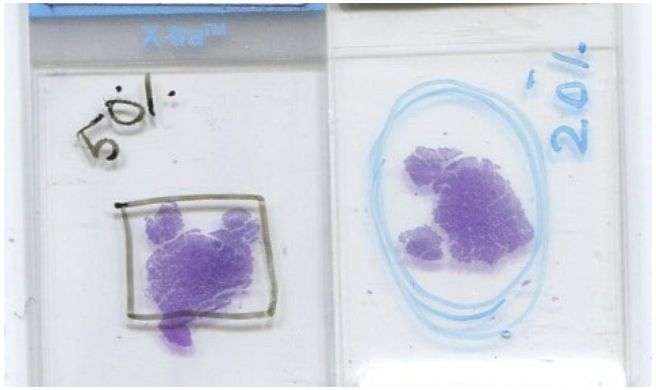
Problem Case



Assessing Tumor Cellularity is an Imprecise Art

Problem Case

- Cell block was evaluated (x2) and sent for molecular testing (x2) by 2 different pathologists.



NGS Success Rates is Impacted by Interobserver Variability

TABLE 4. Unadjusted and Adjusted NGS Success and Failure Rates of Cytopathologists Performing Molecular Adequacy Assessments

	Unadjusted Rates, %		Adjusted Rates, %				Preparation, %	
	NGS Success	NGS Failure	NGS Success	NGS Failure	Canceled Cases[a]	True Failure Rate[b]	Smear	Cell Block
Cytopathologist 1	71	29	57	23	20	43	67	33
Cytopathologist 2	90	10	82	9	9	18	55	45
Cytopathologist 3	85	15	76	14	10	24	67	33
Cytopathologist 4	77	23	65	19	15	35	50	50
Cytopathologist 5	58	42	54	39	7	46	83	17
Cytopathologist 6	70	30	68	29	3	32	50	50
Cytopathologist 7	83	17	53	10	37	47	42	58
Cytopathologist 8	83	17	75	15	10	25	28	72
Median	80	20	67	17	10	25	—	—

Abbreviation: NGS, next-generation sequencing.

[a] Canceled cases were deemed insufficient by pathologist review and were not sent for molecular testing.

[b] The true failure rate was the sum of the canceled case rate and the NGS failure rate.

Cancer Cytopathol. 2015 Jul 31. doi: 10.1002/cncy.21597.

Pathologists Need to be Trained for Specimen Adequacy Assessment

How can we reduce intra/interobserver variability?

- Appropriate training of pathologists in adequacy assessments to reduce variability

Pathologists Need to be Trained for Specimen Adequacy Assessment

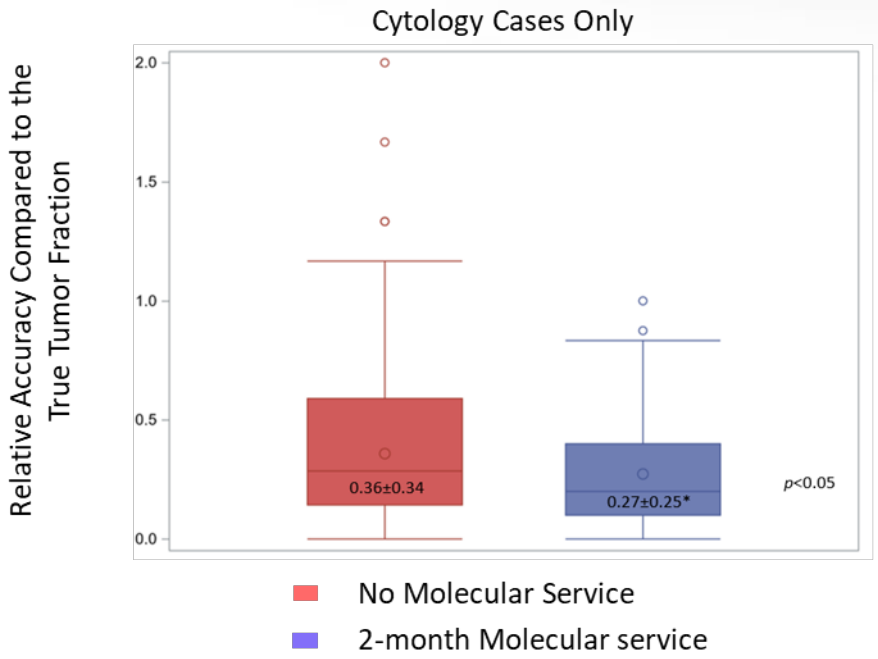


Image: Dr. Qiong (Jenny) Gan

Pathologists are the gatekeepers for ensuring the patient is matched to the appropriate treatment

Summary

- Cytology provides the versatility of specimen preparations that offer a variety of options for molecular testing
- A multitude of pre-analytic factors impact tissue quality and the success of molecular testing
- The pathologist plays a key role in triage and specimen handling that can improve the success of molecular testing

Thank You

Questions?

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