

# Molecular Testing and Cytopathology

## Downsizing Precision Medicine but not Precision

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# Disclosures

- Medical Director of Molecular Oncology at ARUP Laboratories

# Outline

- Molecular Diagnostics in Cytopathology
- Cytology specimen types and molecular testing
- Performance of cytology specimens in molecular diagnostics
- Preanalytical factors and their influence on molecular testing
- Preanalytical optimization of cytology specimens
  - i.e. work with your molecular lab
- Summary and future directions

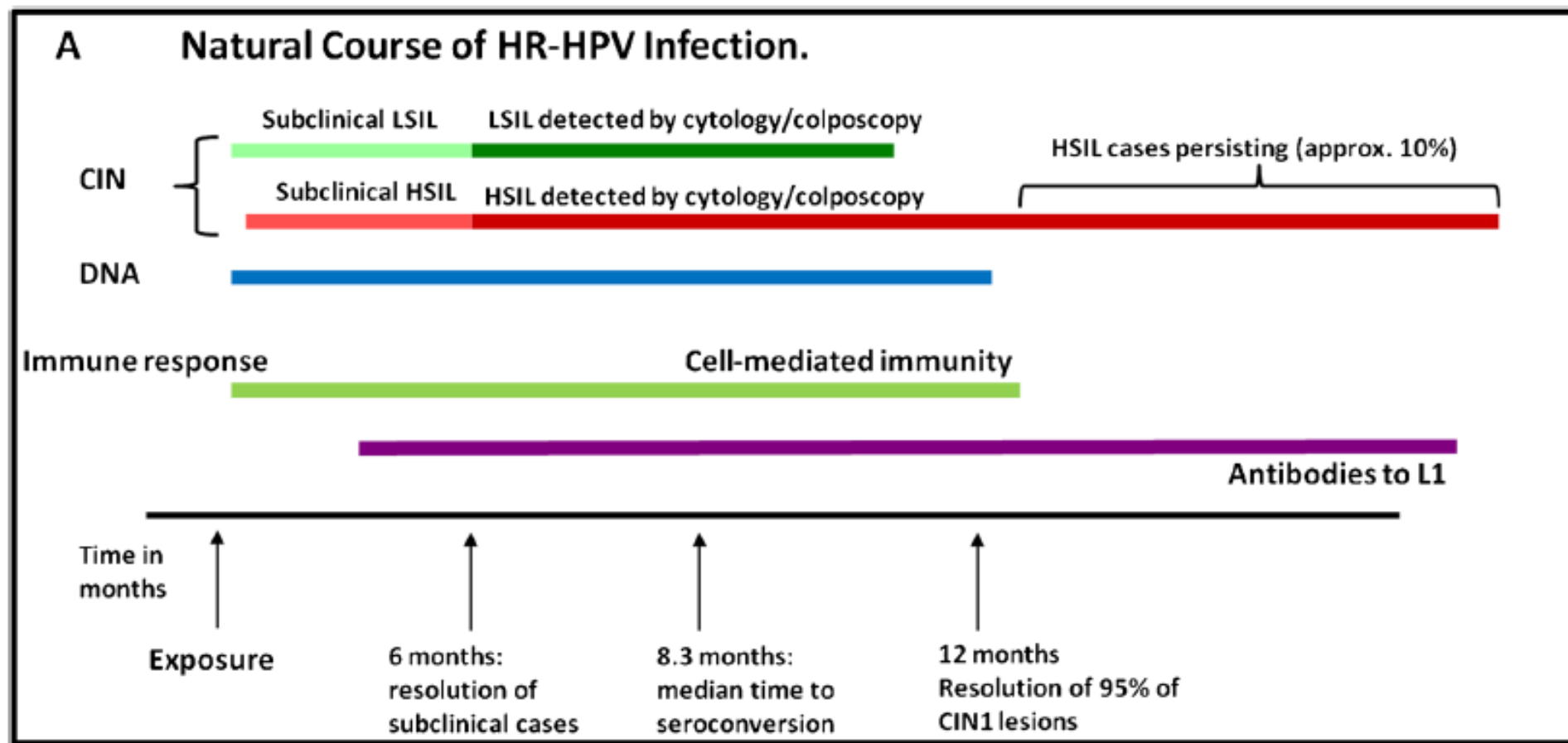
# Anatomic Pathology in the Era of Precision Medicine

- Molecular testing ordered routinely as standard of care for many different neoplasms
- More and more types of tumors tested for personalized treatment and patient stratification
- Expectations placed on AP to refer specimens for testing in an appropriate and timely manner
- Decision making on better use of the specimens
- Particularly important in Cytopathology due to scarcity of material

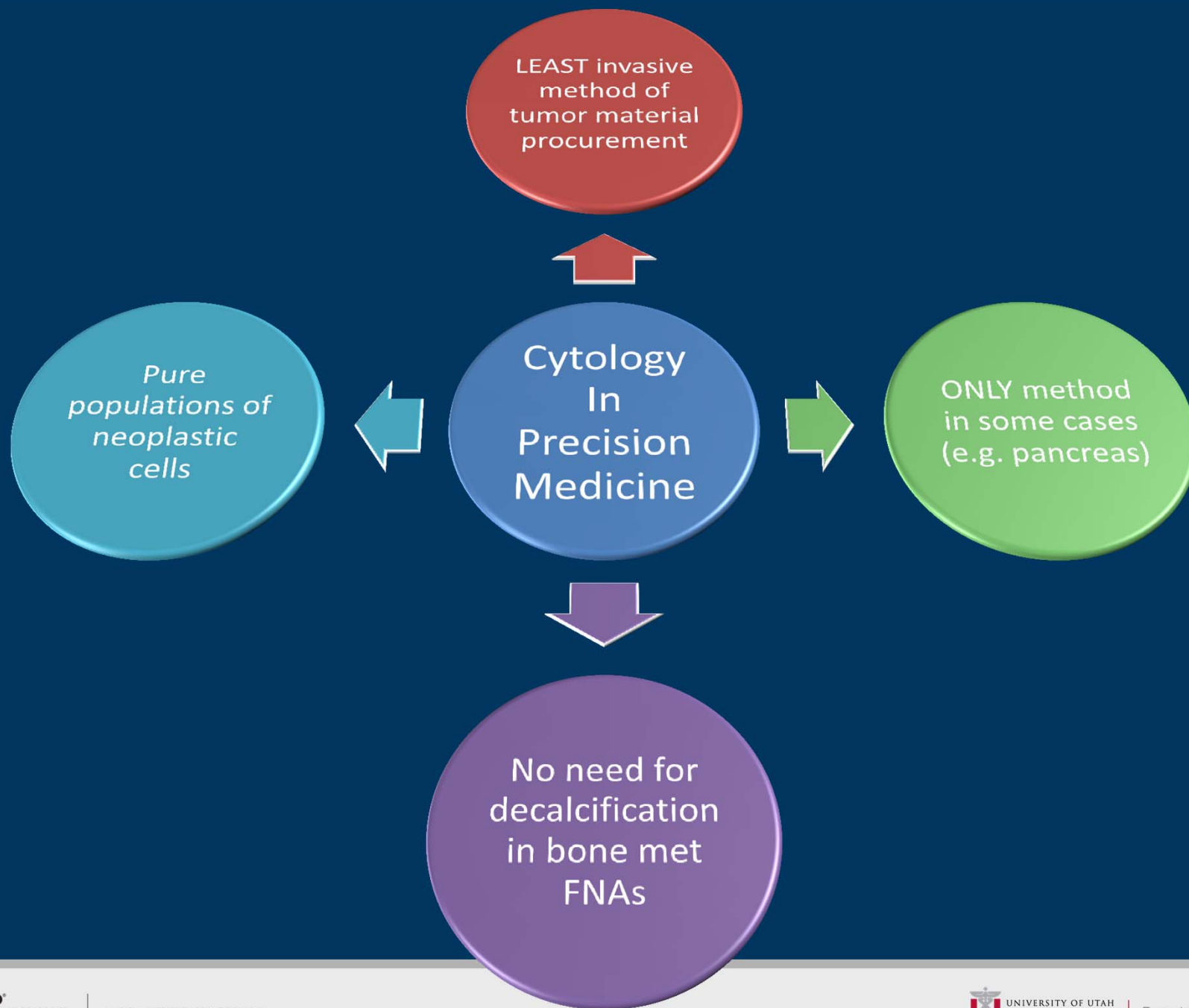
# Molecular Testing in Cytopathology

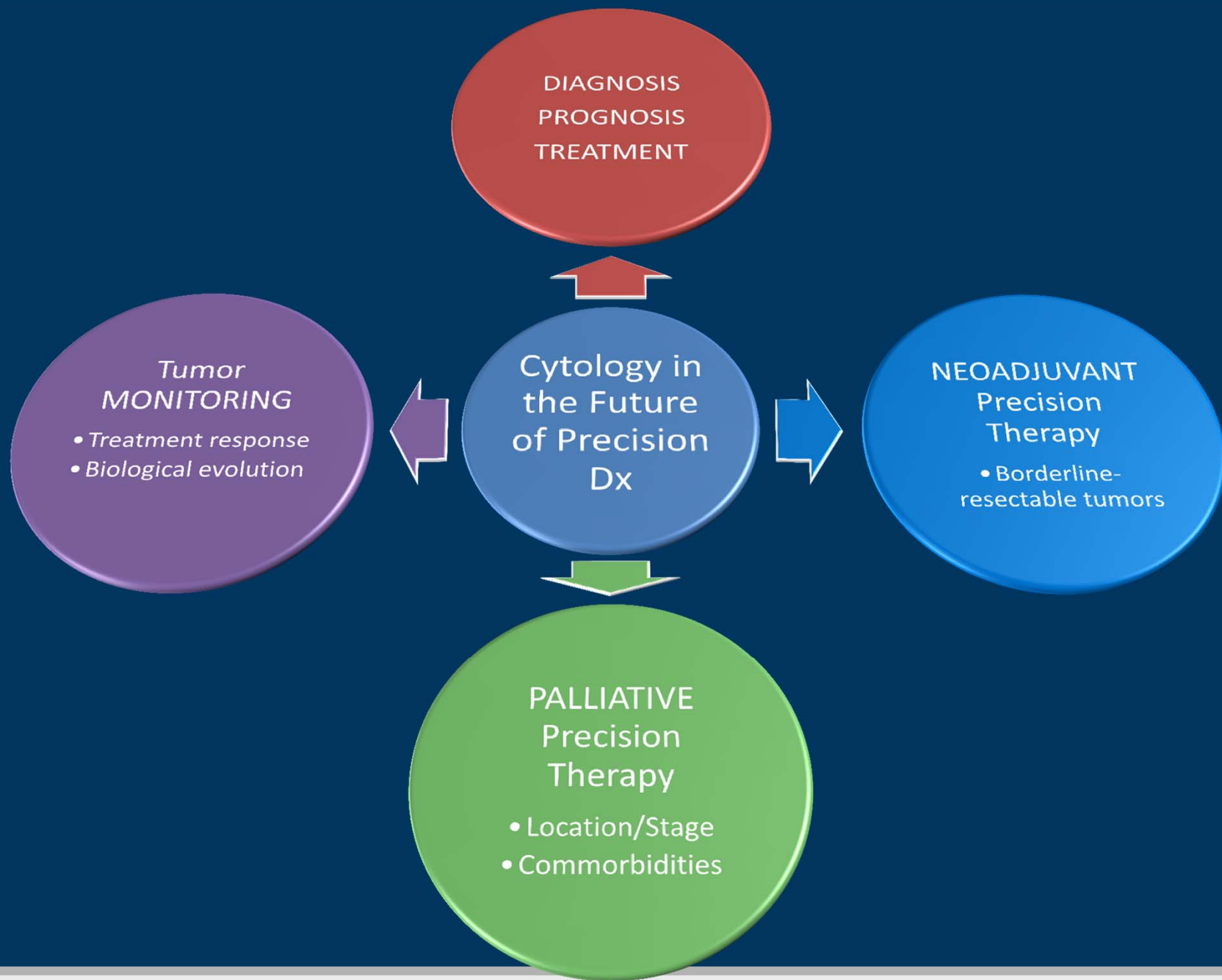
- Widespread testing for human papillomavirus (HPV) in liquid-based cervical cytology
- Increasing use in molecular oncology
- Small biopsies and cytology specimens acquired specifically for molecular testing
- Trend towards utilization of minimally invasive techniques to acquire diagnostic tissue
  - Demand for ancillary testing on smaller specimens, such as cytology specimens

# HPV Testing and Cervical Carcinoma Natural History



From: Deftereos G, Kiviat NB, 2014.

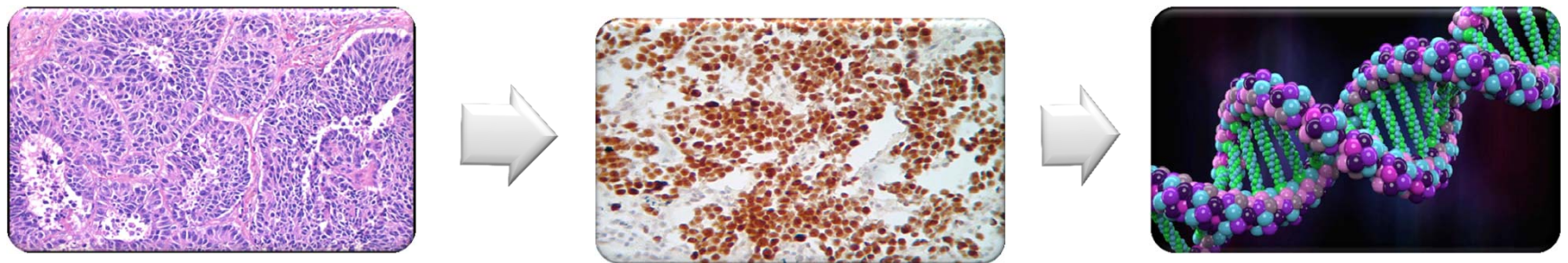






# Precision Medicine: The Evolution of “Biospecimen Information Extraction”

- Cytopathology: The art of doing more with less
- The biospecimen procurement process has somewhat evolved over the last decades
- However, the ability to extract information has changed dramatically



# Molecular Testing Guideline for Selection of Lung Cancer Patients for EGFR and ALK Tyrosine Kinase Inhibitors

Guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology

Neal I. Lindeman, MD; Philip T. Cagle, MD; Mary Beth Beasley, MD; Dhananjay Arun Chitale, MD; Sanja Dacic, MD, PhD; Giuseppe Giaccone, MD, PhD; Robert Brian Jenkins, MD, PhD; David J. Kwiatkowski, MD, PhD; Juan-Sebastian Saldivar, MD; Jeremy Squire, PhD; Erik Thunnissen, MD, PhD; Marc Ladanyi, MD

Arch Pathol Lab Med 2013;137:828-60.

## Question 1: Which Patients Should Be Tested for EGFR Mutations and ALK Rearrangements?

- 1.1a: Recommendation: *EGFR* molecular testing should be used to select patients for *EGFR*-targeted TKI therapy, and patients with lung adenocarcinoma should not be excluded from testing on the basis of clinical characteristics.
- 1.1b: Recommendation: *ALK* molecular testing should be used to select patients for *ALK*-targeted TKI therapy, and patients with lung adenocarcinoma should not be excluded from testing on the basis of clinical characteristics.
- 1.2: Recommendation: In the setting of lung cancer resection specimens, *EGFR* and *ALK* testing is recommended for adenocarcinomas and mixed lung cancers with an adenocarcinoma component, regardless of histologic grade. In the setting of fully excised lung cancer specimens, *EGFR* and *ALK* testing is not recommended in lung cancers that lack any adenocarcinoma component, such as pure squamous cell carcinomas, pure small cell carcinomas, or large cell carcinomas lacking any immunohistochemistry (IHC) evidence of adenocarcinoma differentiation.
- 1.3: Recommendation: In the setting of more limited lung cancer specimens (biopsies, cytology) where an adenocarcinoma component cannot be completely excluded, *EGFR* and *ALK* testing may be performed in cases showing squamous or small cell histology but clinical criteria (eg, young age, lack of smoking history) may be useful in selecting a subset of these samples for testing.
- 1.4: Recommendation: To determine *EGFR* and *ALK* status for initial treatment selection, primary tumors or metastatic lesions are equally suitable for testing.
- 1.5: Expert consensus opinion: For patients with multiple, apparently separate, primary lung adenocarcinomas, each tumor may be tested but testing of multiple different areas within a single tumor is not necessary.



## KEYTRUDA: START INFORMED WITH PD-L1 EXPRESSION IN METASTATIC NSCLC

# TEST

all patients for PD-L1 at diagnosis of  
squamous and nonsquamous NSCLC

### EDUCATION

PD-L1 IHC 22C3 pharmDx | Interpretation Manual



Agilent Pathology Solutions

## PD-L1 IHC 22C3 is FDA-approved

For In Vitro Diagnostic Use

» A minimum of 100 viable tumor cells must be present in the PD-L1 stained slide for the specimen to be considered adequate for PD-L1 evaluation.

Agilent/Dako, Santa Clara, CA

# Pulmonary Cytology Testing

- A small IHC panel is usually sufficient to distinguish between adenocarcinoma (positive TTF-1 and/or napsin-A) from squamous cell carcinoma (positive p63, p40 and/or CK5/6)
- Tumors are also routinely tested for EGFR mutations, ALK and ROS1 rearrangements, RAS mutations
- Oftentimes the tumor can be probed for less common driver mutations, BRAF, RET, PIK3CA etc.
  - NGS panels very often ordered

# Pulmonary Cytology Testing

- Relying on 1-off testing using these small biopsy or cytology specimens will inevitably reach an upper limit before depleting the cellular material
- Efforts by the cytopathologist to maximize cell-block cellularity and minimize material “loss” during the initial ROSE procedure or diagnostic workup
- Thus, multiplexed panel testing approaches are likely to become more widely used in the future

# Considerations on Rapid On-Site Evaluation (ROSE)

- Adequacy testing usually limited to assessment of material necessary for diagnosis and basic ancillary testing
- Necessity to consider all possible testing in the specimen's future
- Concept of “molecular adequacy”
- Grasp of mutation allelic frequency (MAF) concept and relation to tumor cell percentage (tumor burden)



## ■ RECOMMENDATION 15

(A) For nodules with AUS/FLUS cytology, after consideration of worrisome features and investigations such as ultrasound-guided FNA, used to supplement the diagnosis, proceeding directly to surgery is reasonable.

## ■ RECOMMENDATION 16

(A) Diagnostic surgical excision is the long-established standard of care for the management of FN/SFN cytology nodules. However, after consideration of clinical and

## ■ RECOMMENDATION 17

(A) If the cytology is reported as suspicious for papillary carcinoma (SUSP), surgical management should be similar to that of malignant cytology, depending on clinical risk factors, sonographic features, patient preference, and possibly results of mutational testing (if performed).

**(Strong recommendation, Low-quality evidence)**

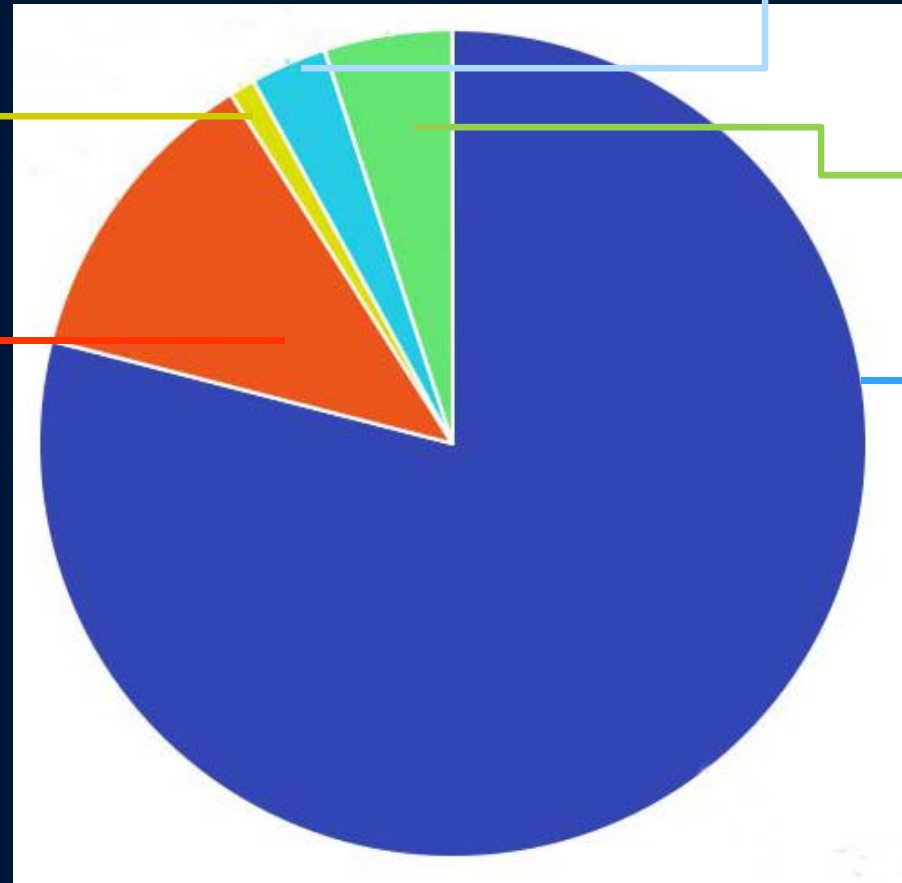
# Thyroid Cancer Genetic Alterations

## Poorly Differentiated

- RAS (25-30%)
- TP53 (20-30%)
- CTNNB1 (10-20%)
- BRAF (10-15%)

## Follicular

- Mutations in 70-75%
- RAS (40-50%; lower in Hurthle cell)
- PAX8/PPAR $\gamma$ (30-35%; lower in Hurthle cell)
- TP53 (21%)
- PTEN (8%)
- PIK3CA (7%)
- BRAF (2%)



## Anaplastic

## Medullary

## Papillary

- Mutations identified in  $\approx$ 70%
- BRAF (40-50%)
- RAS (7-20%)
- RET/PTC (clonal; 10-20%)
- EGFR (5%)
- NTRK (<5%)
- PIK3CA (2%)

Nikiforov et al. Arhc Pathol Lab Med 2011; COSMIC database.



# Oncogene Panels

- BRAF
  - Seen in PTC, including tall cell variant and reported to be in 51% of classic PTC, but only in 24% of follicular variant PTC
    - Can improve diagnostic accuracy of classic PTC
    - Rarely reported in FTC
  - Almost 100% PPV and associated with more aggressive behavior for PTC

# Oncogene Panels

- RAS (HRAS, KRAS, NRAS)
  - 40-50% of FTC and 15% of PTC (mostly follicular variant)
  - No clear role in tumor aggressiveness
    - Can be seen in aggressive carcinomas, but also in benign follicular nodules

# Oncogene Panels

- PAX8/PPAR $\gamma$ 
  - 20-40% of FTC
  - Lower prevalence in Hurthle cell carcinoma
  - 2-10% of follicular adenomas
  - Found occasionally in follicular variant PTC

# Oncogene Panels

- RET/PTC
  - 20% of thyroid carcinomas
  - 50-80% of radiation-induced thyroid carcinomas
  - 40-70% in children and young adults
  - RET/PTC1 associated with non-aggressive, classic PTC and low probability of progression to poorly differentiated/anaplastic forms
  - RET/PTC3 associated with more aggressive PTC forms

	Afirma®	ThyGenX®	ThyraMIR™	ThyroSeq®	Thyroid CA Mol. Panel
Provider	Veracyte, South San Francisco, CA	Interpace Diagnostics, Parsippany, NJ	Interpace Diagnostics, Parsippany, NJ	UPMC, Pittsburgh, PA	ARUP, Salt Lake City, UT
Methodology	mRNA gene expression	Multiplex PCR by sequence-specific probes. Limited panel, full sequencing	MicroRNA expression	Next generation sequencing, more extended panel, includes prognostic	PCR/Pyrosequencing, limited panel, hotspot analysis, RT-PCR for translocations
Strength	High NPV	High PPV	Good NPV and PPV when combined with ThyGenX	High NPV and PPV	High PPV
Limitation	Low PPV	Low NPV	Limited validation data	Limited validation data	Hotspot test – Low NPV
Specimen Collection	2 dedicated FNA passes	1 dedicated FNA pass with at least 50 ng of cellular material	1 dedicated FNA pass with at least 50 ng of cellular material	1-2 drops of 1 <sup>st</sup> pass if adequate cellularity on smear, otherwise add ½ of 2 <sup>nd</sup> pass, FFPE, frozen tissue	FNA slides (Diff-Quik or Pap), FNA passes in PreservCyt or CytoLyt, FFPE blocks/slides

# Melanoma

- It is not infrequent that metastatic melanoma is encountered by cytology personnel in the ROSE setting
- Over than 50% melanomas harbor activating BRAF gene mutations, with the vast majority of these being either V600E or V600K
  - Less commonly codon 601 mutations
- Tumors with a V600E BRAF mutation respond to TKIs like vemurafenib or dabrafenib.
- BRAF testing is frequently requested for metastatic melanoma on cytology specimens
- Melanomas arising in the setting of chronic sun exposure can harbor NRAS-activating mutations, generally rendering them insensitive to BRAF TKIs

# Melanoma

- Much less commonly, other major targetable mutations encountered in melanoma involve the KIT gene
- Most activating KIT mutations render these tumors sensitive to KIT TKIs, such as imatinib
- Resistance mutations possible
- Testing is generally PCR-based, as CD117/c-KIT IHC does not reliably correlate with mutation status
- Additional, potentially targetable mutations include MET, PTEN and ERBB2
  - Panel or multiplexed assay format (NGS)

# Hematological Malignancies

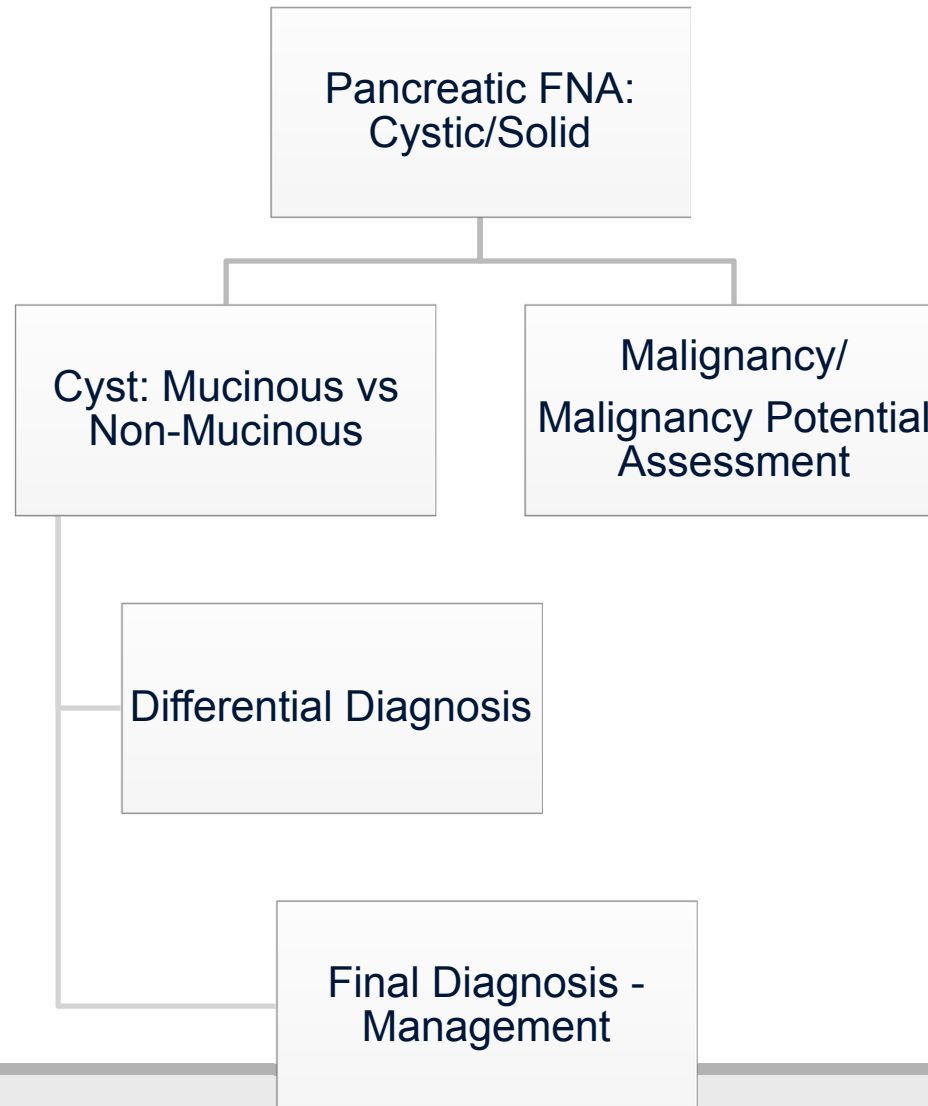
- When an atypical lymphoid population is seen on ROSE, effective triage of subsequent passes is crucial
- Material should be collected fresh into saline or cell culture media (e.g. RPMI or DMEM) and submitted for flow cytometry
  - Most flow cytometry platforms sensitive enough for cell block supernatant material (fresh, placed in RPMI)
- Needle passes for cell-block allocation or core-needle biopsy material should be obtained for
  - IHC
  - PCR (e.g. for Ig or TCR rearrangement)
  - Cytogenetic and/or FISH analysis
  - Molecular testing for patient stratification



# Head and Neck SCC

- HPV-associated squamous cell carcinomas of the oropharynx or base of tongue tend to have a better prognosis
- The identification of HPV not only can help localize the primary site of disease but also can direct subsequent therapy
  - p16 IHC not reliable for DDx
- HPV testing can take a variety of forms
  - p16 IHC
  - High-risk HPV DNA by ISH
  - Hybrid Capture II test (Qiagen, Gaithersburg, MD)
  - Roche COBAS HPV test (Roche Molecular Systems, Pleasanton, CA)
  - Cervista HPV HR test (Hologic, Inc., Bedford, MA)
  - APTIMA HPV Assay (Hologic/Gen-Probe Inc, San Diego, CA)
- Separate validation for cell blocks/FFPEs
  - Currently underway at ARUP (Salt Lake City, UT)
- CAP is currently developing a comprehensive, evidence-based guideline for HPV testing in head and neck squamous cell carcinoma

# Pancreatic FNA Molecular Testing



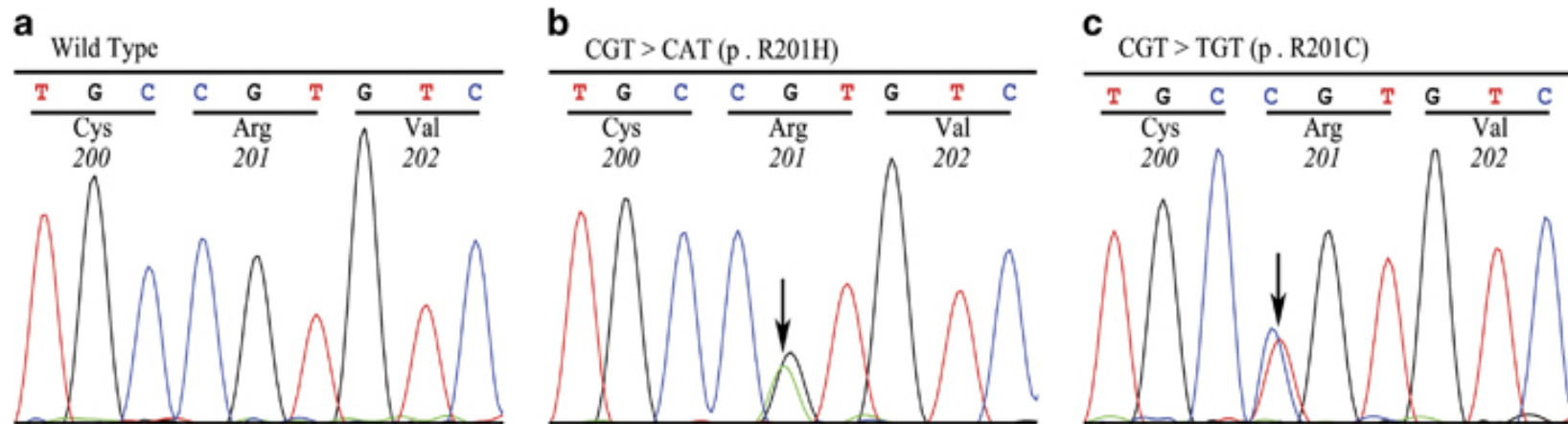
# CEA Testing

- A cystic fluid CEA concentration higher than 192 ng/ml strongly correlates with a mucinous cyst (Brugge et al. 2004)
  - Using this CEA threshold value for diagnosing a mucinous cyst, yields a sensitivity and specificity of 64% and 83%, respectively
  - No difference in CEA values is seen between mucinous premalignant and malignant cysts

# KRAS Mutation and CEA Analysis

- *KRAS* mutations (codon 12 or 13) are present in the fluid of about 30% of pancreatic cystic lesions
- *KRAS* testing of the cyst fluid is valuable, especially in those cases where the CEA level is low, as the presence of *KRAS* mutations supports the diagnosis of a mucinous cyst
- The added value of molecular testing can be small compared with the combination of cytology and CEA testing, at least in cases where the CEA levels are elevated
  - A negative *KRAS* test may be due to insufficient and possibly non-representative DNA and therefore does not exclude a neoplastic mucinous cyst.

# GNAS Mutation Analysis



- Guanine nucleotide protein, alpha stimulating (*GNAS*) mutations differentiate MCN from IPMN (Wu et al. 2011)
- Mutations at codon 201 of the *GNAS* gene were found in 66% of IPMNs
- Moreover, *GNAS* mutations were not found in other types of cystic neoplasms of the pancreas or in PDACs not associated with IPMNs

Image: Shi et al. Mod Pathol 2013;26:1023-31.

# KRAS/GNAS/VHL/RNF43/CTNNB1 Panel Analysis

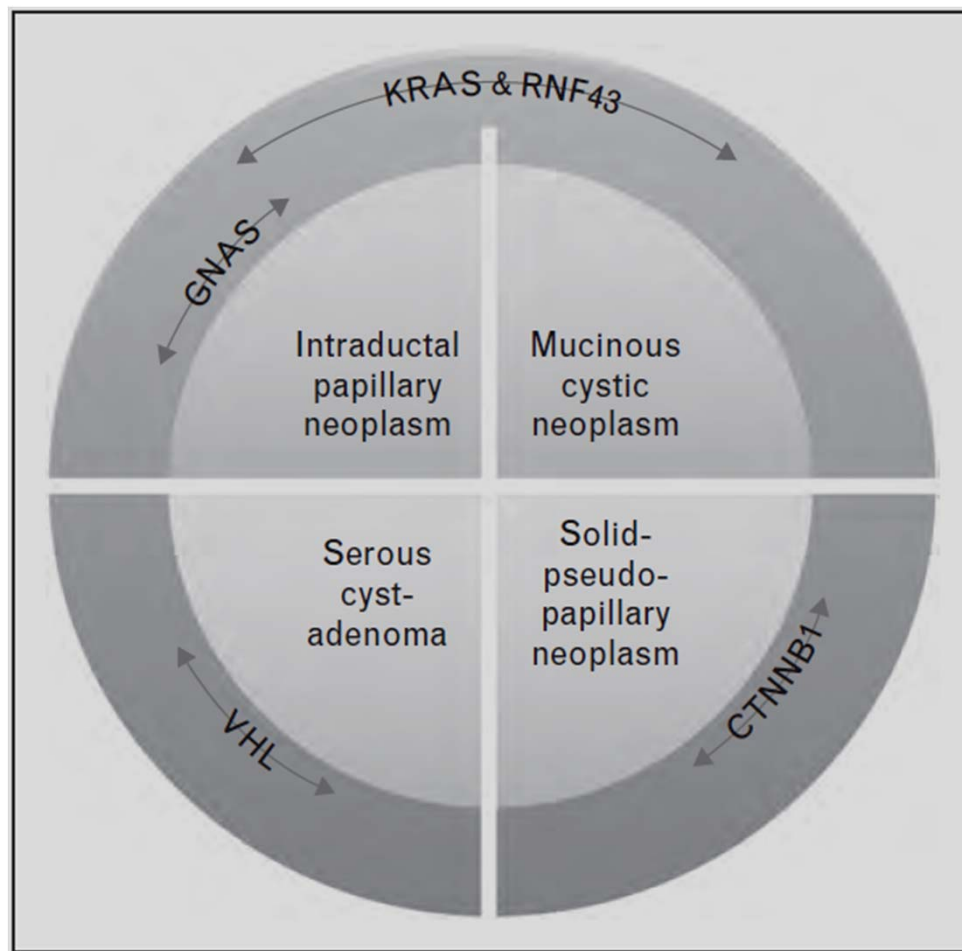


Image from: Law et al. Curr Opin Gastroenterol 2013;59:509-16.

# Main Platforms

- PancraGEN™, Interpace Diagnostics, Parsippany, NJ
  - Based on the PathFinderTG® offered by RedPath Integrated Pathology, Inc., Pittsburgh, PA
  - KRAS, GNAS sequencing
  - LOH mutations at the following loci: 1p, 3p, 5q, 9p, 10q, 17p, 17q, 18q, 21q, 22q
  - Integration with CEA, cytology findings radiology findings
- PancreaSeq®, University of Pittsburgh Medical Center, Pittsburgh, PA
  - AKT1, CTNNB1, GNAS, KRAS, PIK3CA, PTEN, TP53, VHL
  - NGS-based

Published in final edited form as:

*Diagn Cytopathol.* 2014 April ; 42(4): 351–362. doi:10.1002/dc.23093.

## Utilization of Ancillary Studies in the Cytologic Diagnosis of Biliary and Pancreatic Lesions:

The Papanicolaou Society of Cytopathology Guidelines for Pancreatobiliary Cytology

### Proposed Ancillary Tests for Solid Pancreatic Lesions

Marker	Purpose	Diagnostic finding	Utility
<i>KRAS</i> mutations	Identification of adenocarcinoma	Mutation present	Insufficient specificity for malignancy to warrant usage
<i>SMAD4</i>	Identification of adenocarcinoma	Mutation present [IHC shows loss of staining]	Supports the diagnosis of adenocarcinoma
FISH	Identification of adenocarcinoma	Presence of copy number abnormalities in CEP3, CEP7, CEP17 and abnormalities of band 9p21 favor malignancy	Most reliable test for confirming adenocarcinoma in conjunction with routine cytology
Mesothelin	Identification of malignancy	Overexpression of mesothelin by IHC	Supports the diagnosis of adenocarcinoma
Loss of heterozygosity	Identification of adenocarcinoma	Losses of chromosome arms 3p, 6Qp and 10pq along with gains of 5q, 12q, 18q, and 20q supports a diagnosis adenocarcinoma	Clinical importance to be determined
microRNAs	Identification of adenocarcinoma	Presence of miRNA including miR-21 and mi-155 supports a diagnosis of adenocarcinoma	Clinical utility to be determined



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Marker	Purpose	Diagnostic finding	Utility
Mucin (mucicarmine, alcian blue ph 2.5)	Identification of mucinous lesions	Positive stain	Diagnostically helpful
Cyst fluid amylase	Identification of pseudocysts and serous cystadenomas	Diagnosis of pseudocyst (level in 1000s, but not <250 U/L) and SCA (low levels, generally <1000 U/L); IPMNs have variable but elevated levels	Differential diagnosis of pancreatic cysts
Cyst fluid CEA	Identification of cystic mucinous lesions	CEA levels above 110 ng/mL support the diagnosis of a mucinous cyst	Distinction between mucinous and nonmucinous cysts
DNA analysis	Separation of benign from malignant cysts	Aneuploid and tetraploid results favor malignancy	Does not significantly improve diagnostic accuracy over routine cytology in majority of studies
<i>KRAS</i> mutations	Identification of mucinous cystic lesions	Presence of <i>KRAS</i> mutations supports diagnosis of a mucinous cyst	Distinguishes mucinous from nonmucinous cysts
CA 19-9	Separation of benign from malignant cysts	CA 19-9 level may be elevated in malignant cysts	Not generally useful in the diagnosis of pancreatic cysts
VHL gene mutation	Identification of SCA	Mutation present	Support the diagnosis of SCA
<i>CTNNB1</i> (beta-catenin) mutation	Identification of SPN	Mutation present	Supports the diagnosis of SPN
<i>GNAS</i> mutation	Identification of IPMN	Mutation present	Supports the diagnosis of IPMN
<i>RNF43</i> mutations	Identification of cystic mucinous lesions	Mutation present	Distinguishes mucinous from nonmucinous cysts

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## Utilization of Ancillary Studies in the Cytologic Diagnosis of Biliary and Pancreatic Lesions:

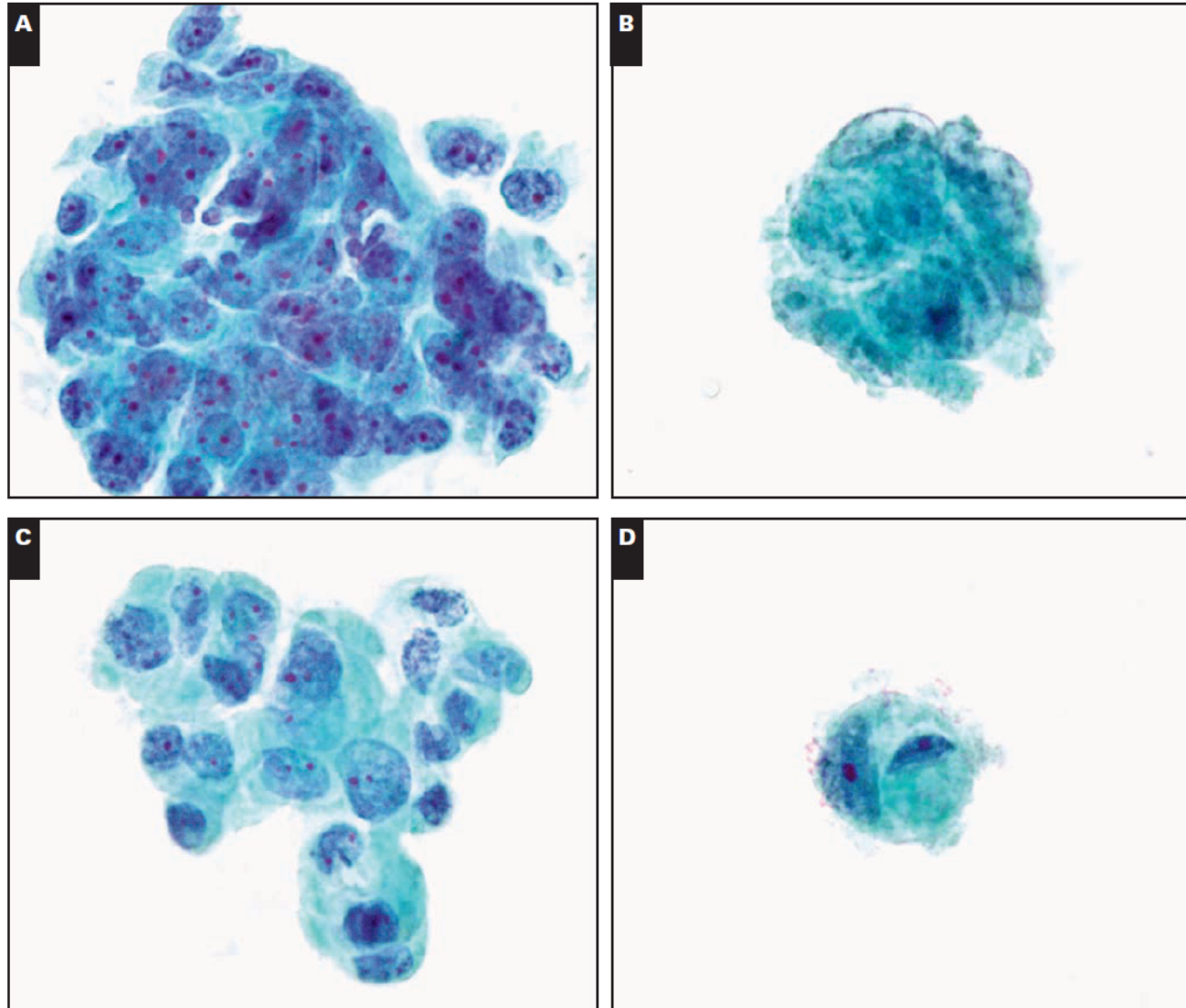
The Papanicolaou Society of Cytopathology Guidelines for Pancreatobiliary Cytology

Lester J. Layfield, M.D.<sup>1,\*</sup>, Hormoz Ehya, M.D.<sup>2</sup>, Armando C. Filie, M.D.<sup>3</sup>, Ralph H. Hruban, M.D.<sup>4</sup>, Nirag Jhala, M.D.<sup>5</sup>, Loren Joseph, M.D.<sup>6</sup>, Philippe Vielh, M.D., Ph.D.<sup>7</sup>, and Martha B. Pitman, M.D.<sup>8</sup>

### Proposed Ancillary Testing for Pancreatobiliary Strictures

Marker	Purpose	Diagnostic finding	Utility
Digital image analysis	Separation of benign from malignant strictures	Aneuploid and tetraploid results support malignancy	Does not improve diagnostic accuracy above that achievable by cytology alone
<i>KRAS</i>	Separation of benign from malignant strictures	Mutation present	Insufficient specificity for malignancy to warrant usage
Sequential mutational analysis	Separation of benign from malignant strictures	Loss of heterozygosity	Diagnostic utility to be determined by future studies
FISH	Separation of benign from malignant strictures	Presence of copy number abnormalities in CEP3, CEP7, CEP17, and abnormalities of 9p21 favor malignancy	Diagnostically useful. It is the preferred test to complement routine cytology

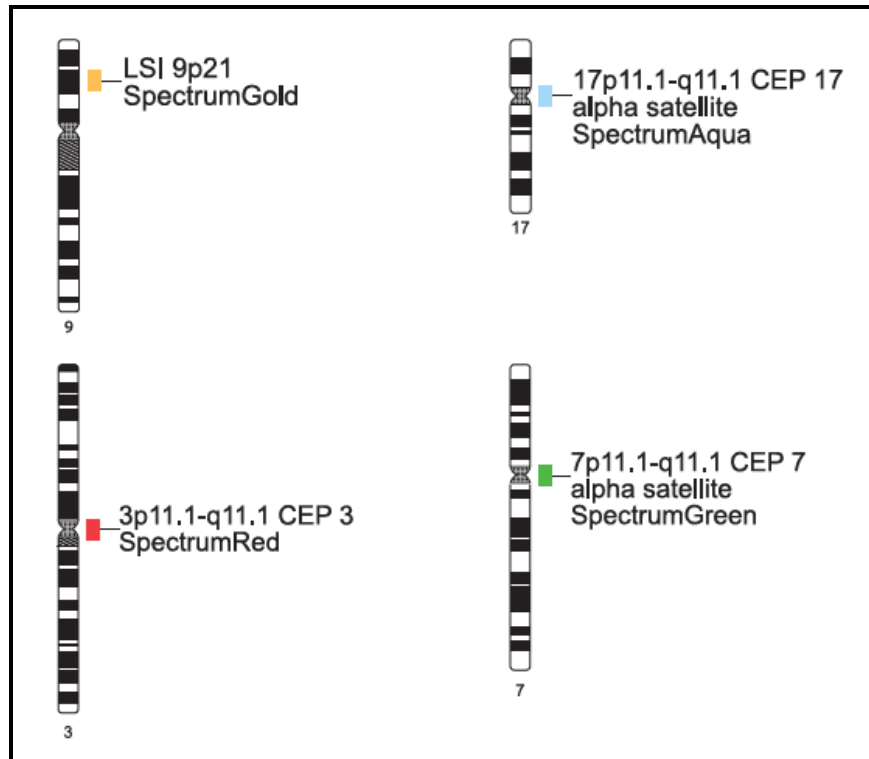
# Morphologic Criteria for Biliary Cytology



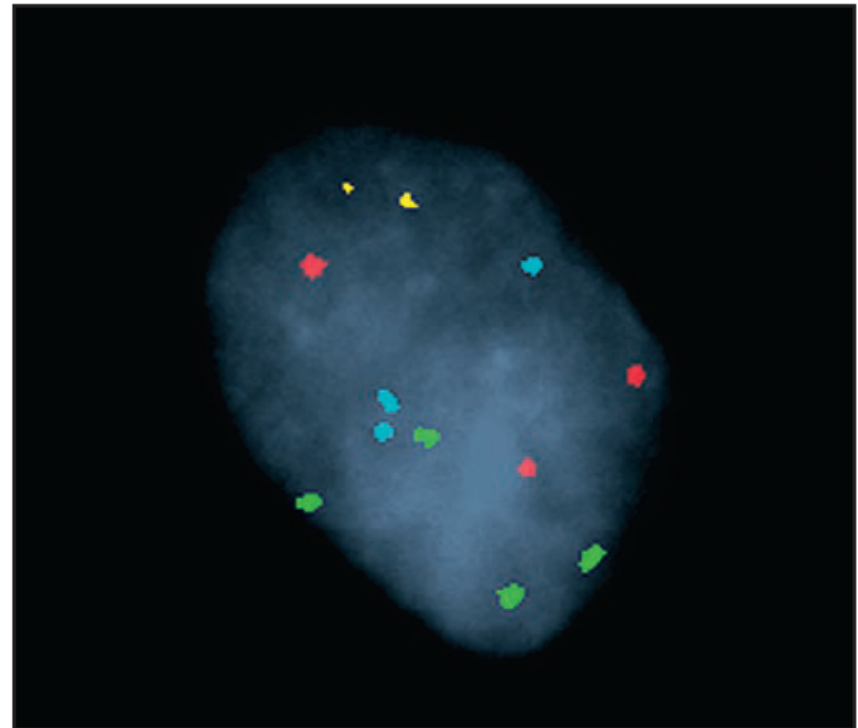
From: Fritcher et al., 2011.

# Urovysion

- Loss of 9p21 and chromosome 3, 7 and 17 aneuploidy correlates with urothelial carcinoma

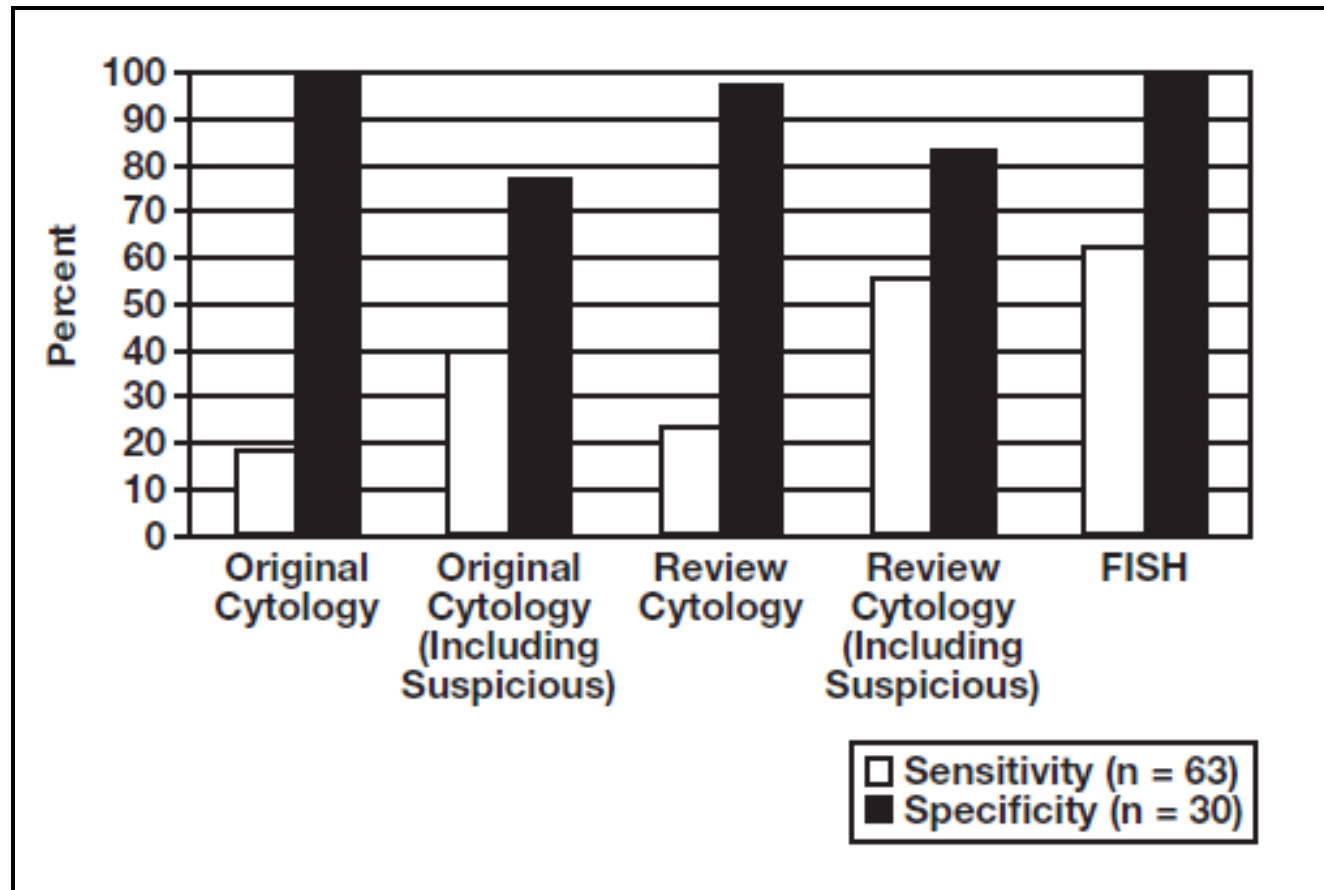


Abbott Molecular, Des Plaines, IL



From: Fritcher et al., 2011.

# Comparison of Morphology and FISH in CBD Cytology



From: Fritcher et al., 2011.

# Urovysion FISH in CBD Brushing Cytology

## Utilization of Ancillary Studies in the Cytologic Diagnosis of Biliary and Pancreatic Lesions:

### The Papanicolaou Society of Cytopathology Guidelines for Pancreatobiliary Cytology

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Diagn Cytopathol 2014;42(4):351-62

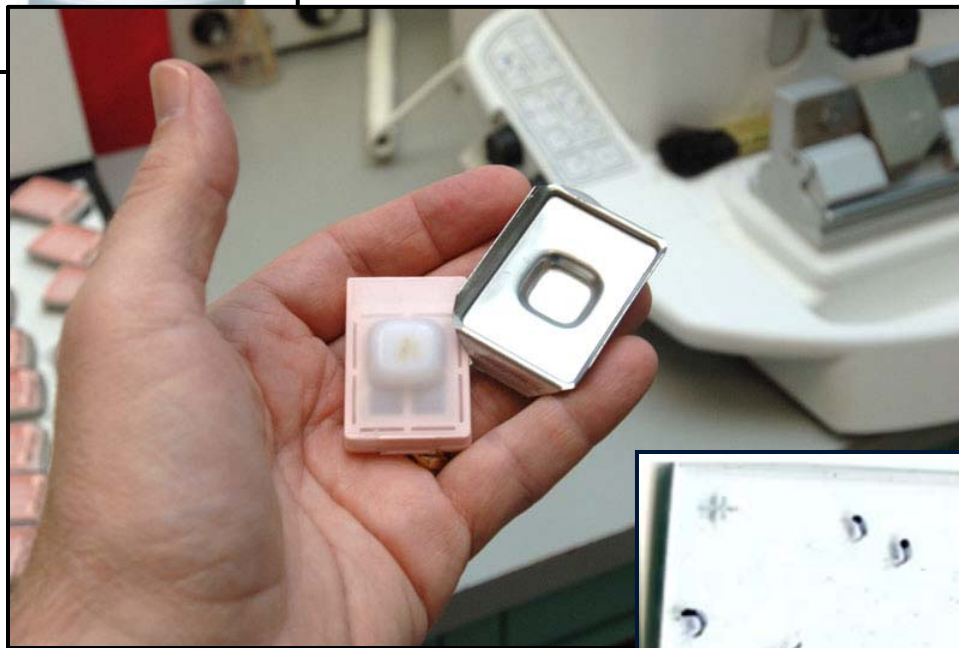
- “Most reliable test for confirming adenocarcinoma in conjunction with routine cytology.”
- Hard to convince giving up material from potential morphological evaluation

# Other Tumor Types

- Metastatic colorectal
- Metastatic Breast
- GIST
- Gastric and esophageal
- Bone and soft tissue



# CYTOLOGY SPECIMEN TYPES AND MOLECULAR TESTING



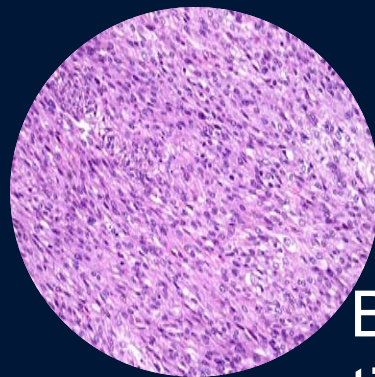
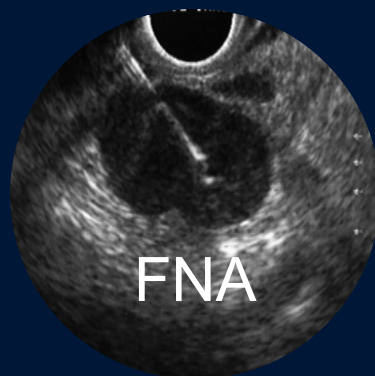
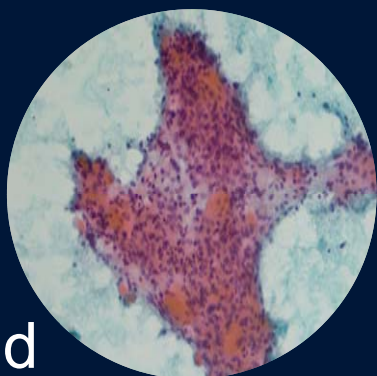


# Fine Needle Aspiration (FNA)

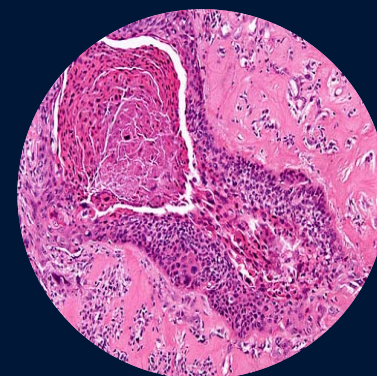
- Advantage of targeting a specific lesion and can be performed with minimal invasion
- Advantage of having a relatively pure population of lesional cells

# Molecular Diagnostics on FNAs

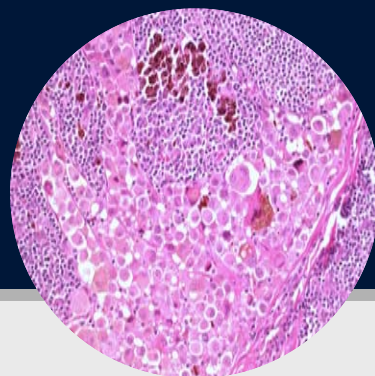
Thyroid  
undetermined  
diagnosis  
(AUS/FLUS/SFN)



Bone and soft  
tissue neoplasms



H&N Squamous  
cell carcinomas

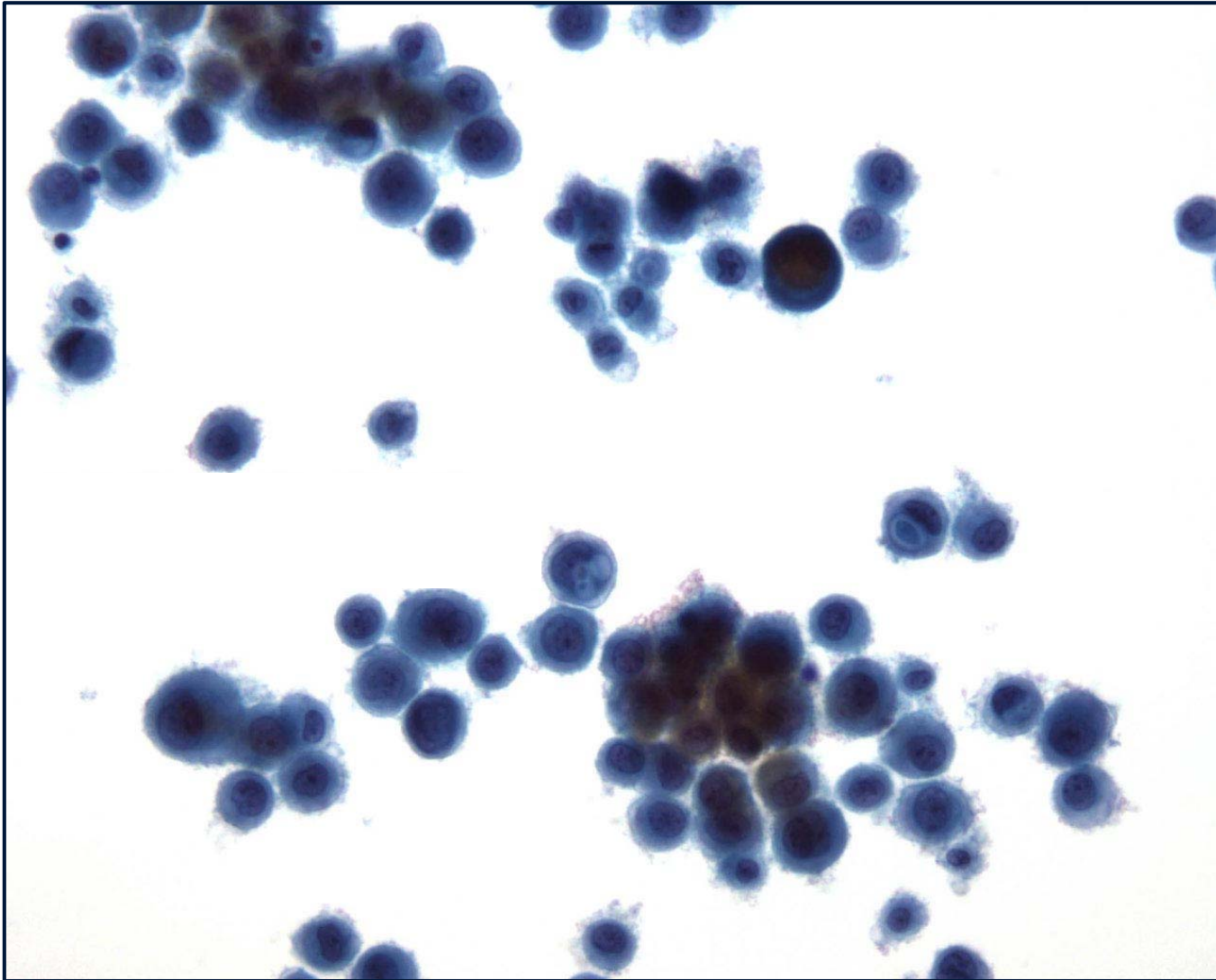


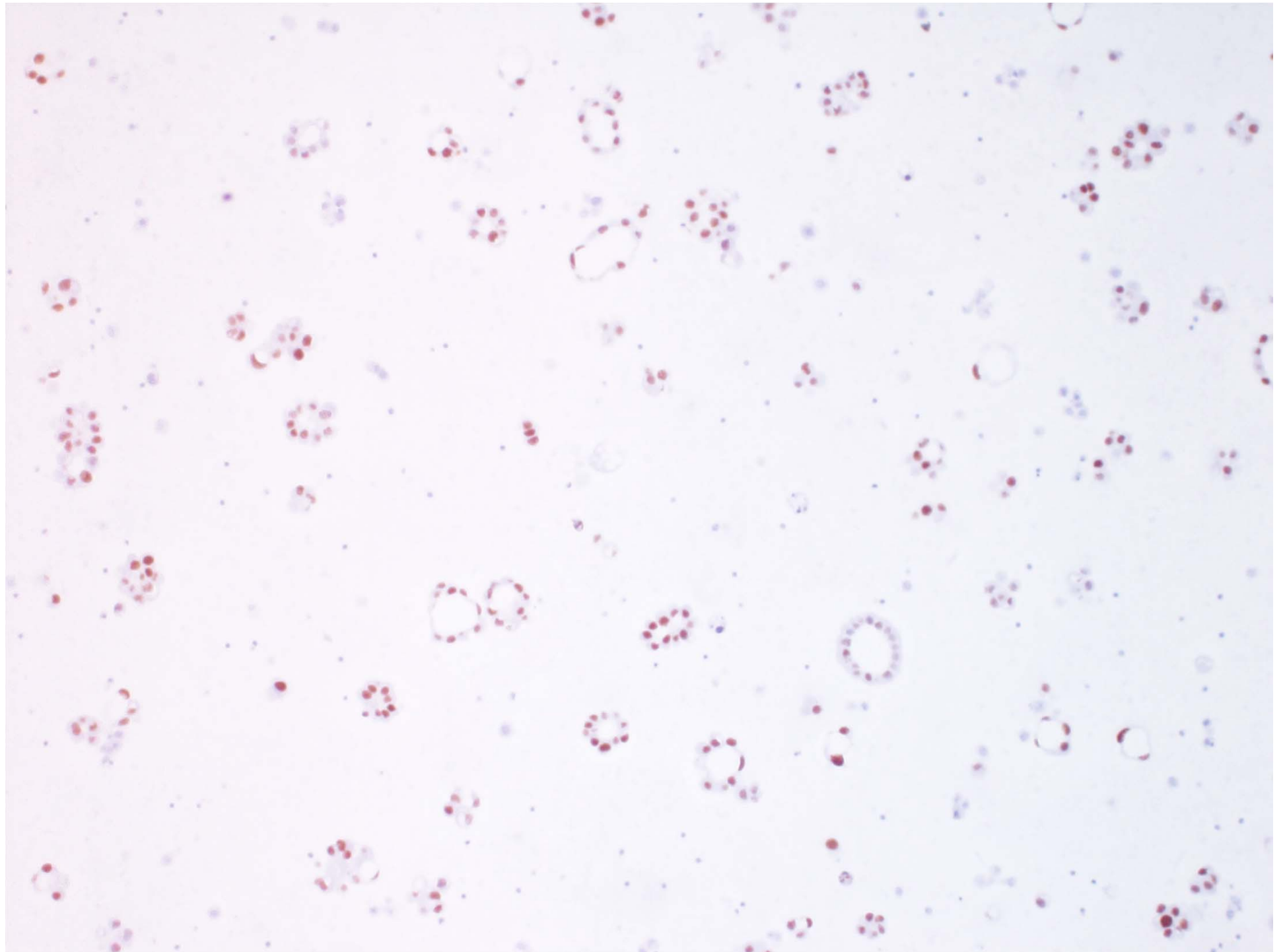
Metastatic  
melanomas

# Exfoliative Cytology

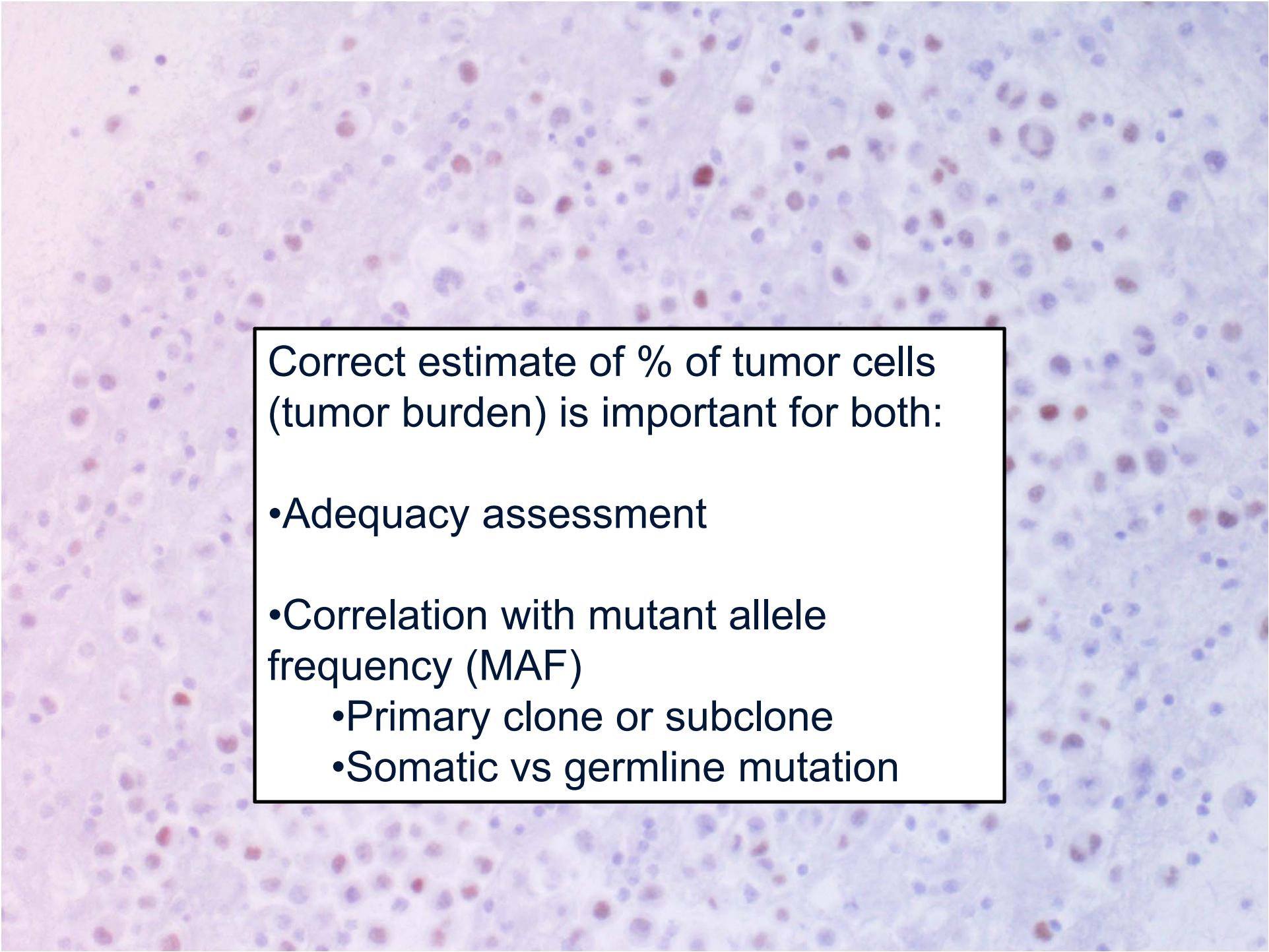
- Testing for high-risk HPV is standard of care in cervical screening and is used to clinically guide treatment
- Urovysion FISH for urine cytology specimens

# Effusion Cytology









Correct estimate of % of tumor cells  
(tumor burden) is important for both:

- Adequacy assessment
- Correlation with mutant allele frequency (MAF)
  - Primary clone or subclone
  - Somatic vs germline mutation



# SPECIMEN TYPES AND PREPARATION

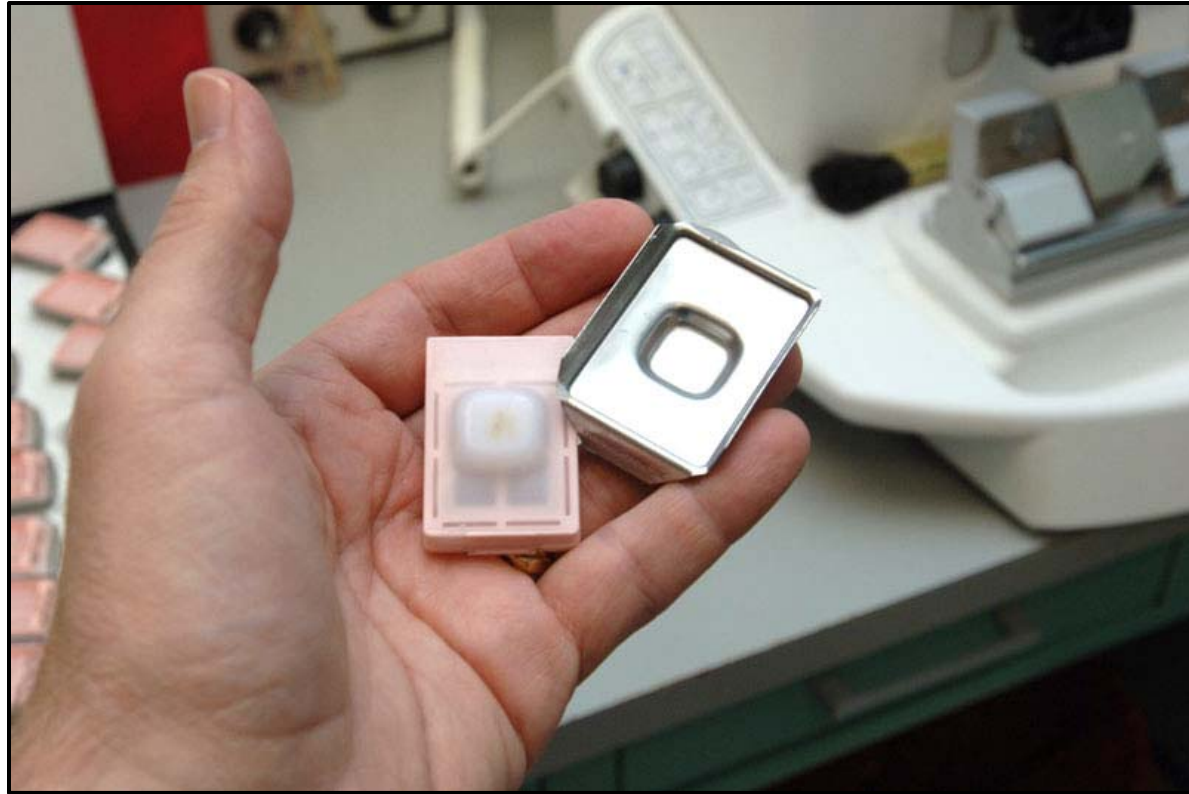
# Liquid-Based Collection



- Advantages:
  - Technical skills not necessary for slide preparation
  - Preservative solution designed for DNA(RNA) preservation
- Disadvantages:
  - Inability to perform immediate assessment
  - Potential solution the evaluation of 1 stained preparation from sample to be tested



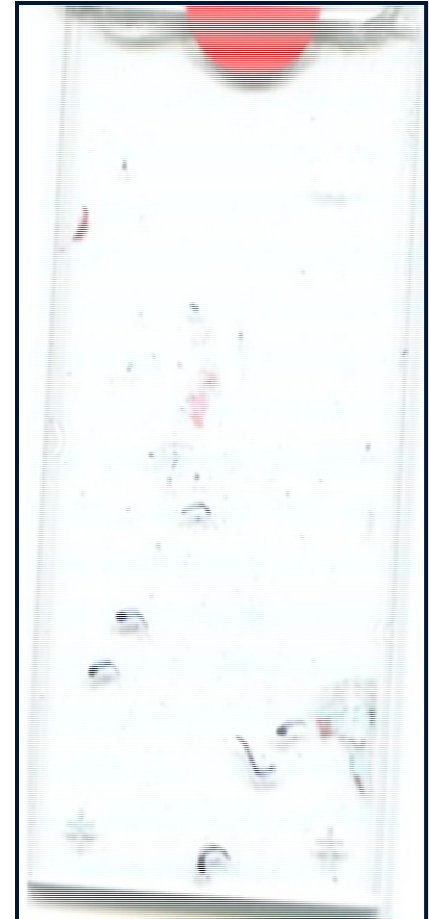
# Cell Blocks



- Best understood cytopathology specimen regarding extraction of DNA/in situ methods
- No need for separate validation from FFPE samples (in most cases)
- Applies to FNA, exfoliative and effusion cytology

# Direct Smears

- High quality of nucleic acids extracted with the common staining techniques, (Papanicolaou, Romanowsky/Diff-Quik)
- Great resource for thyroid FNAs
- Alcohol rather than formalin-based fixation
- Ease of immediate assessment
- Disadvantage:
  - The slide with lesional material must be sacrificed for molecular testing and is lost from the diagnostic archive
  - Slide scanning or photographic archive



# Harada et al. 2014

## Cytology Specimens for Molecular Testing/Harada et al

**TABLE 2.** QNS Rates With Different Types of Specimens

	Surgical Pathology		Cytology		Total
	Large Specimen	Small Biopsy	Cell Block	Cell Block + Smear	
Total	73	78	25	25	176
EGFR resulted, No. (%)	72 (98.6)	47 (64.1)	9 (36.0)	17 (68.0)	136 (77.3)
EGFR, limited results, No.		3			3
ALK resulted, No. (%)	72 (98.6)	70 (89.7)	16 (64.0)	16 (64.0)	158 (88.6)
QNS, No. (%)	1 (1.4)	28 (35.9) <sup>a</sup>	16 (64.0) <sup>b</sup>	8 (32.0) <sup>c</sup>	37 (21.0)

Abbreviations: ALK, anaplastic lymphoma kinase; EGFR, epidermal growth factor receptor; QNS, quantity not sufficient.

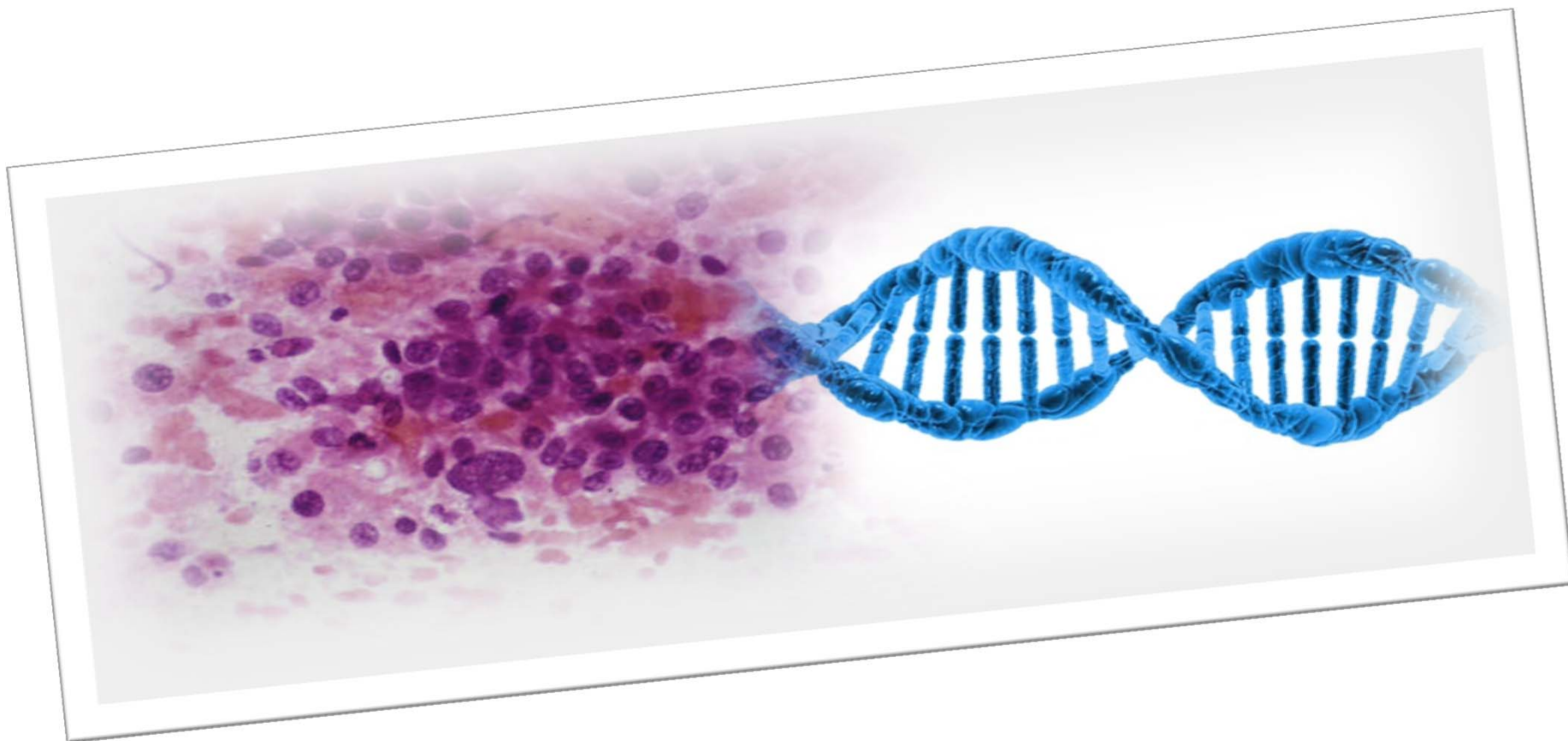
<sup>a</sup> $P < 0.01$  versus both large specimens and cell blocks.

<sup>b</sup> $P < 0.01$  versus both large specimens and small biopsies.

<sup>c</sup> $P = .024$  versus cell blocks.

# Role of the (Cyto)pathologist

- Cytopathologist evaluates for diagnostic adequacy of specimens
- Understanding of the indications for testing
- Appreciation of the methodology of molecular testing
- Appropriate referral of specimens for testing
- Institutions with “molecular adequacy” FNA assessment in place (e.g. MD Anderson)



# PERFORMANCE OF CYTOLOGY SPECIMENS IN MOLECULAR DIAGNOSTICS

# How do cytology specimens perform in molecular testing?

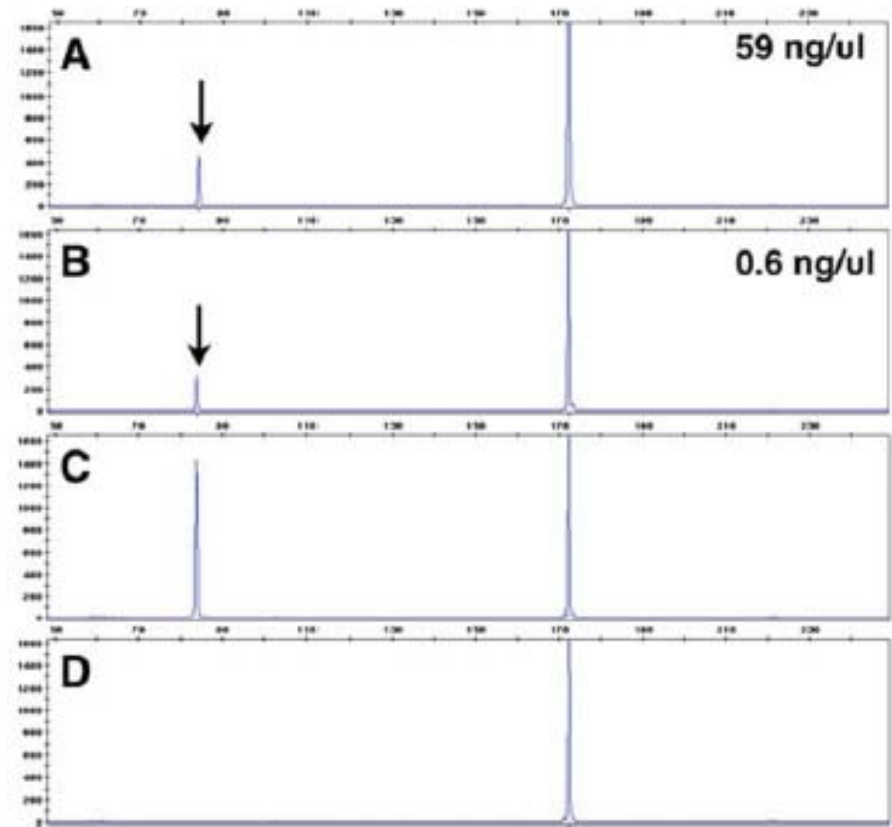
- Numerous studies available examining cytology specimens as sources of DNA for standard molecular testing and comparing them to standard biopsy specimens
  - E.g. EGFR/ALK/ROS-1 test in NSCLC

# Aisner et al., 2011

- Cytology cell block (CB) material was evaluated for EGFR exon 19 deletions and L858R mutations
- Retrospectively reviewed EGFR mutation analyses performed on 192 SP specimens and 42 CB specimens

**Table III.** Mutation Frequencies by Specimen Type

	<i>All Cases</i>	<i>Surgical cases</i>	<i>Cytology cases</i>
<i>N</i>	234	192	42
No. Positive (%)	42 (17.9)	31 (16.1)	11 (26.2)

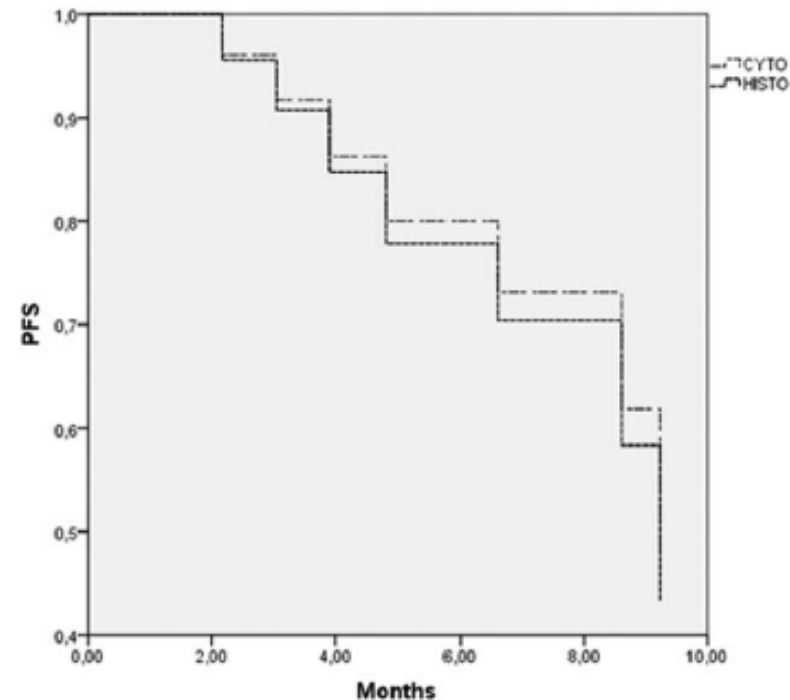


**Fig. 1.** Electropherograms of matched resection and cytology patient specimen for *EGFR* L858R mutation. Arrow indicates the peak generated from *Sau96I* digestion of mutant PCR product. A: Resection specimen; (B) cytology cell block; (C) positive control; (D) negative control.



# Malapelle et al. 2013

- 364 cytology samples and 318 histology samples
- EGFR exon 19 deletions and L858R point mutation in exon 21, detected by fragment analysis assay and TaqMan assay, respectively, were confirmed by direct sequencing
- The mutation rate was similar in histology samples (8.5%) and cytology samples (8.8%)



The disease control rate (responsive plus stable disease) was 92% in histologically selected patients and 100% in cytologically selected patients ( $p=0.88$ ).



# HOW DO PREANALYTICAL FACTORS INFLUENCE MOLECULAR TESTING ON CYTOLOGY SPECIMENS?



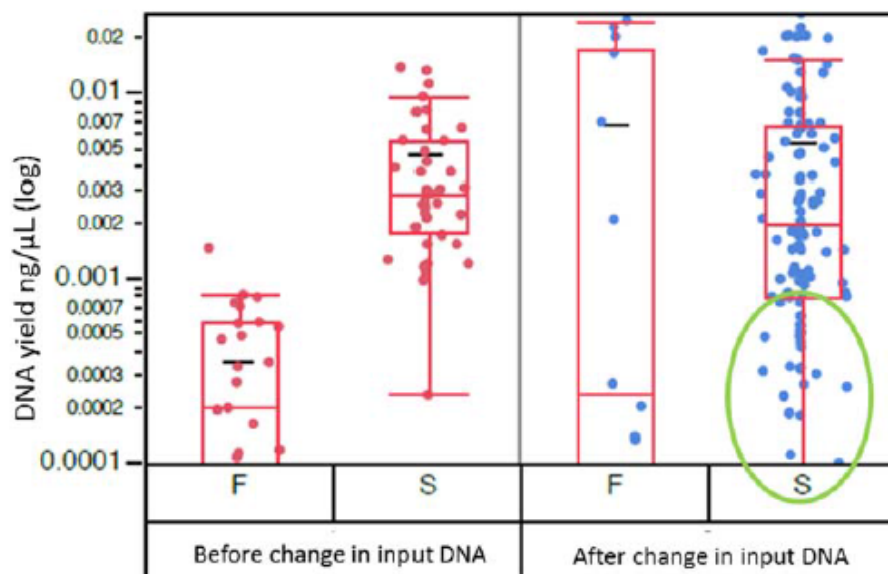
# Roy-Chowdury et al., 2015

- Reviewed variables associated with all 207 (116 smears, 91 CB) cytology analyzed by NGS with the Ion Torrent platform (IT AmpliSeq Cancer Hotspot v2 panel, ThermoFisher Scientific, Waltham, MA) during a 10-month interval
  - DNA input threshold
  - ~~– Specimen preparation~~
  - ~~– Slide type~~
  - Tumor fraction
  - DNA yield
  - Cytopathologist bias

# Roy-Chowdury et al., 2015

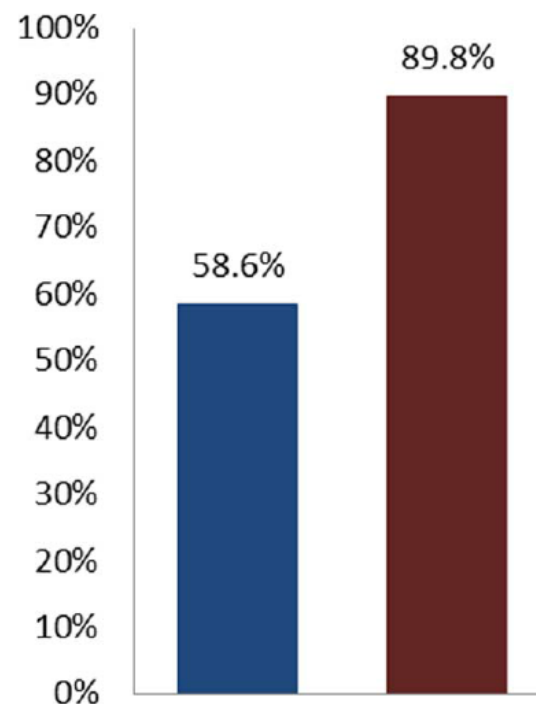
- MD Anderson has in place a molecular adequacy assessment by a cytopathologist
- $\geq 20\%$  tumor cells rule for adequacy
- 164/207 cases (79%) were successfully sequenced by NGS
- In comparing failed vs successful runs:
  - DNA yield correlated with success/failure
  - Tumor cell fraction not important successful NGS run

# Input DNA Threshold



10 ng

<10ng



Before  
n=41/70

After  
n=123/137

10 ng

<10ng

# Pathologist Bias and NGS Failure

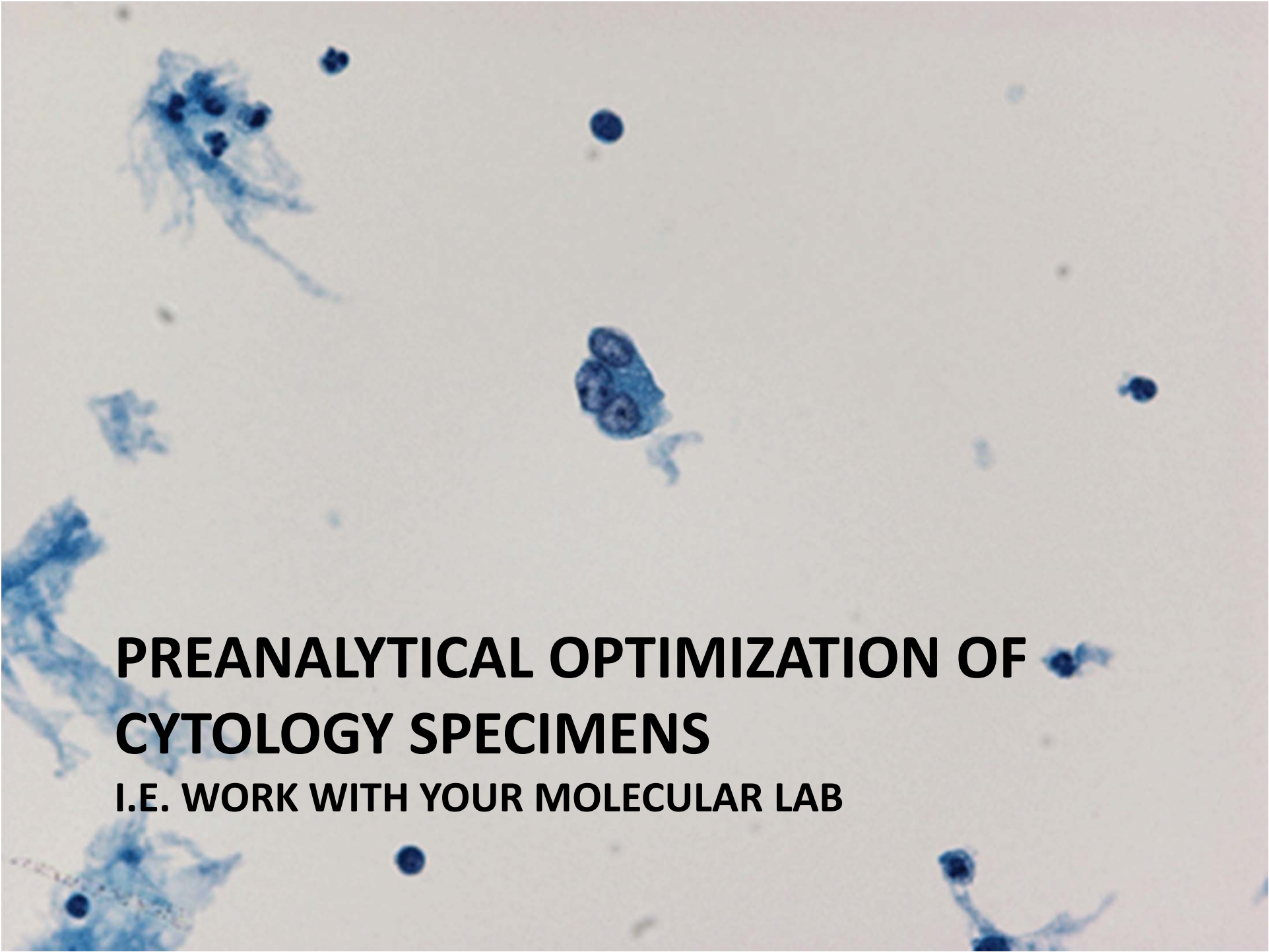
**TABLE 4.** Unadjusted and Adjusted NGS Success and Failure Rates of Cytopathologists  
Adequacy Assessments

	Unadjusted Rates, %		Adjusted Rates, %			
	NGS Success	NGS Failure	NGS Success	NGS Failure	Canceled Cases <sup>a</sup>	True Failure Rate <sup>b</sup>
Cytopathologist 1	71	29	57	23	20	43
Cytopathologist 2	90	10	82	9	9	18
Cytopathologist 3	85	15	76	14	10	24
Cytopathologist 4	77	23	65	19	15	35
Cytopathologist 5	58	42	54	39	7	46
Cytopathologist 6	70	30	68	29	3	32
Cytopathologist 7	83	17	53	10	37	47
Cytopathologist 8	83	17	75	15	10	25
Median	80	20	67	17	10	25

Abbreviation: NGS, next-generation sequencing.

<sup>a</sup> Canceled cases were deemed insufficient by pathologist review and were not sent for molecular testing.

<sup>b</sup> The true failure rate was the sum of the canceled case rate and the NGS failure rate.



# **PREANALYTICAL OPTIMIZATION OF CYTOLOGY SPECIMENS**

**I.E. WORK WITH YOUR MOLECULAR LAB**

# Scale of Sensitivities



## Analytical Sensitivity

- How sensitively can a test detect a rare change?
- Low AS can be overcome with enrichment (circling of tumor)
- FN related to allelic dilution (low tumor burden - % of tumor cells)

## Clinical Sensitivity

- How many of the possible changes are detected?
- Inherent in test design
- FN related to genetic alterations falling outside the range of testing



# Preanalytical Processing

- Assessment for adequacy:
  - Ratio of tumor to non-tumor nucleated cells in a specimen
  - An extremely small specimen with high tumor cellularity may be superior to an abundant specimen with low tumor cellularity
- Evaluation of specimen quantity is an important first step
  - Limiting material used for morphological diagnosis to necessary amount
- Thinking of ways of to better utilize the small cytology specimens



# Analytical Sensitivities of Different Sequencing Platforms

Platform	Limit of Detection – Mutant Allelic Frequency	Comments	Percentage of Tumor Cells for Testing (Tumor Burden)*
Sanger Sequencing	15-20%	Not a quantitative method	30-40%
Melt Curve Analysis	≈10%	Not a quantitative method	≈20%
Pyrosequencing	≈5%	Conservatively at 10%	10-20%
NGS	1-2%	May detect less than that	5-10%

1. Tsiatis et al. J Mol Diagn 2010;12:425-32.
2. Lin et al. Am J Clin Pathol 2014;141:856-66.

\*Assuming that tumor cells are heterozygous for the mutation

# Ways of improving utilization of specimens:

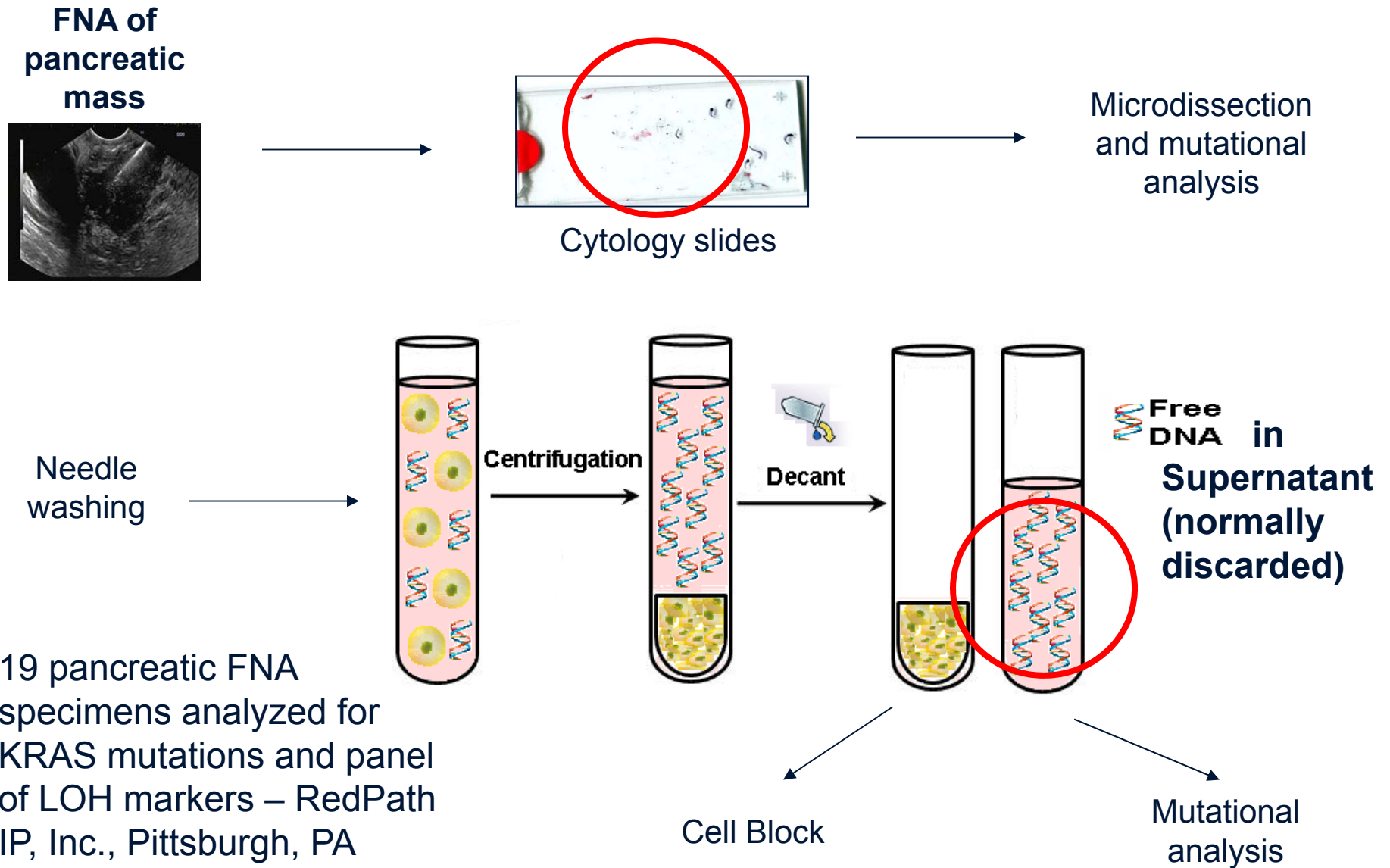
## Role of FNA Biopsy in the Diagnosis of Pancreatic Cancer

- FNA: approach of choice
- Core needle biopsy of the pancreas associated with high morbidity e.g. high risk of pancreatitis
- FNA associated with low morbidity
- Highly specific but has a negative predictive value (NPV) of around 65% (Hewitt et al., 2012)

# Molecular Testing of (Pancreatic) Cytological Samples

- The DNA analyzed often derives from microdissected cytology slides/cell blocks, or cyst fluid
- As many of the specimens are acellular or paucicellular
  - Low or no amount of DNA extracted
  - The use of microdissected cytology slides for DNA extraction further subtracts material from cytomorphological evaluation
- How about solid pancreatic tumors?
- Hypothesis
  - What if the FNA material contains cell-free tumor DNA, irrespective of the amount of tumor cells present in the smears/cell block?
  - Where does this material go?

# Deftereos et al., 2014.



# Summary of Results

- Mean paired DNA concentration supernatant:slides ratio: 6.76
- Mean paired Ct difference for KRAS amp.: 2.98
- Pancreatic Adenocarcinomas (5)
  - In all cases supernatant outperformed microdissected specimen
- Mucinous (3) and Endocrine (1) Neoplasms
  - Supernatant performed equally or better
- Pancreatitis (5) & Negative (5)
  - All negative results
- None of the KRAS mutations or LOHs found on the dissected slides missed by supernatants
- 3 cases had amplifiable DNA only in the supernatant
- Change in handling implemented – specimen validated

Deftereios et al., 2014

# Work with your molecular lab

- Consider including pertinent IHC slides along with slides/blocks sent for testing
- Consider including tumor burden estimate in the report comment
  - e.g. “The tumor cells represent approximately 30% of the entire cell population.”
- Consider including molecular adequacy information in the report comment
  - e.g. “The cell block H&E matches the smears in cellularity and may be used for ancillary testing.”
  - Or “The cell block material is scant; smears from passes 1 and 2 are the most cellular and may be used for ancillary testing.”

# Summary

- There are advantages in the development of molecular testing from cytology specimens
- Existing platforms can be adapted for low input specimens
- Novel techniques may be suitable for low input specimens
- Strategies can be developed to optimize the collection/adequacy assessment/usage of cytology specimens for molecular testing

# Future Directions

- Molecular laboratories increasingly recognize the need to optimize assays for use with small specimens
  - Validate cytology specimen types for testing
- Increasing number of clinically relevant analytes
  - Planning for material allocation becomes increasingly important
- Cytopathologists should anticipate an increasing demand to maximally preserve tissue for molecular testing



# Future Directions



**Precision Medicine begins at the bedside**

**Thank you!**



Department of Pathology

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