Preanalytic Variables in Cytology: Lessons Learned from Next Generation Sequencing

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Evolving Role of Cytopathology

Tissue is not only for diagnostic pathology evaluation, but also for molecular assays involving nucleic acids and proteins.

ASC/PSC Position Statement: Use of Molecular Testing on Cytologic Specimens

American Society of Cytopathology (ASC) and Papanicolaou Society of Cytopathology (PSC) Joint Position Statement on The Use of Molecular Testing on Cytologic Specimens


Molecular Testing of Cytologic Samples

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

ASC/PSC Position Statement: Use of Molecular Testing on Cytologic Specimens

• Cytology specimens provide an excellent platform for molecular testing.
• FNAs provide an excellent opportunity for rapid on-site evaluation (ROSE) that improves adequacy and helps triage the specimen.
• Cytotechnologists/cytopathologists should triage FNA material with reference to potential molecular testing.

• Small specimens are not necessarily an obstacle.
• It is becoming increasingly common for molecular testing to be performed on cytology specimens, including cell blocks, dried smears, and liquid-based preparations.
Cytologic Samples Provide a Variety of Substrates for Next Generation Sequencing

<table>
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<tr>
<th>Advantages</th>
<th>Disadvantages</th>
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| Cell blocks (FFPE) | • Ease of acquisition  
• Ease of validation  
• Easy to get serial sections |
| | • Lack of immediate assessment  
• May have low cellularity  
• Degradation of nucleic acid due to formalin fixation  
• Partial nuclei in standard 4-5 μm sections (lower DNA yield) |
| Direct smears | • Immediate assessment for tumor adequacy  
• High quality nucleic acid  
• Whole cells = whole nuclei (higher nucleic acid yield) |
| | • Difficult to validate  
• Requires technical support and skill to prepare smears  
• Must sacrifice slide (medicolegal issues) |
| Liquid-based prep | • Standardized processing with optimal 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  
• Variable preservative capacity of liquid preparations – requires validation for every type |

Challenges of Molecular Diagnostics

**Challenges:**

- Doing more with less
- Limited sample size
- Targeted therapy and evaluating multiple markers in tumor specimens
- Turn around time
- Timely and accurate reporting

Challenges:

- Next Generation Sequencing: A Novel Platform for Clinical Testing

**Advantages:**

- Highly multiplexed assay with high clinical sensitivity
- High analytical sensitivity (5-10%)
- Single platform to test multiple genomic alterations: SNVs, insertions and deletions, gene amplifications, gene rearrangements

**Disadvantages:**

- Cost and reimbursement
- Technical skill and expertise for validation and running assay
- Bioinformatics support for analysis and interpretation of variant calls
- Big data challenges

Next Generation Sequencing:

**Multiple Factors Impact Tissue Quality for Next Generation Sequencing**

- In the clinical setting, high-quality nucleic acids need to be obtained through the current practices of diagnostic pathology.
- However, there is significant variation in the quality of the tissue (analyte source) due to the diversity in specimen preparation and the lack of standardization across laboratories.

**Multiple Factors Impact Tissue Quality**
Preanalytic Factors Play a Major Role in Molecular Testing

Specimen Acquisition

- Clear communication among ordering clinician, radiologist, laboratory technician/technologist, and pathologist.
- Using image-guided procedure for better diagnostic yield.
- Optimizing technique for best diagnostic yield (e.g., needle gauge, number of passes, etc).
- Whenever clinically feasible, obtaining concurrent core biopsies and FNAs to maximize chances of sufficient material to perform ancillary studies.

Concurrently Acquired FNA and Core Biopsy Samples Can Improve NGS Success Rates

Specimen Acquisition

- Rapid on-site evaluation (ROSE) for specimen adequacy to ensure adequate sampling and proper triaging.
- Performing additional pass for smears or cell blocks in anticipation of ancillary studies.
- If cell block is the primary source of material for molecular testing, minimizing the amount of material expelled onto smears and maximizing the needle rinse material for preparation of cell block.

ROSE Can Ensure Appropriate Triage of Specimen
Specimen Processing

- Avoiding acidic and heavy metal fixatives like Bouin and Zenker solutions and harsh acid (nitric acid or hydrochloric acid) decalcification agents, which interfere with molecular testing.

Specimen Processing Affects DNA Yield

- Standardizing processing techniques for specimen preparations: glass slides, fixatives, stains, and preservative media.
- Understanding quantitative differences in DNA yield from different sources.


FF, Fully frosted slide
NF, Non-frosted slide
PC, Positively charged slide
SC, Silane coated slide
NGS Success Depends on the DNA Yield

NGS success positively correlates with DNA yield.

Specimen Processing

- Appropriate triaging of material for ancillary studies (e.g., preparing additional decoverslipped smears in anticipation of testing; cutting extra, unstained cell-block sections up front to avoid refacing block).

Specimen Selection

- Communication with molecular laboratory regarding criteria for adequacy, tissue-extraction strategies, and selecting the most appropriate material for next generation sequencing.

Specimen Adequacy Assessment

- Overall cellularity:
  - All nucleated cells in sample
  - Translates to the Total DNA yield
- Tumor cellularity/tumor fraction:
  - Percentage of tumor cells
  - Translates to Analytic Sensitivity
Low cellularity NOT EQUAL to low tumor fraction

How many cells do you need?
- 1 cell ~6-7 pg of DNA
- Molecular assay requiring 1 ng of DNA input therefore needs ~143-166 intact cells
- NGS (Ion Torrent PGM) requires around 10 ng of DNA
  Therefore approximately 1430-1660 intact cells

Specimen Handling for Molecular Testing

Specimen Selection
- Appropriate training of cytopatologists in adequacy assessments to improve interobserver variability.

NGS Success Rates Vary Among Pathologists
Molecular Processing

- Optimizing tissue-extraction strategies for maximal DNA yield (scraping versus cell lifting versus direct extraction from LBC samples or fresh cells.)

Tissue Extraction Method May Affect DNA Yield

Tissue extraction by scraping yields significantly higher DNA than by cell-lifting methodology (PinPoint Slide DNA Isolation system, Zymo Research).

Molecular Processing

- Establishing guidelines for tumor enrichment (e.g. macrodissection versus manual microdissection versus laser-capture microdissection).

Tumor Enrichment Reduces Risk of False Negative Results

Tumor Enrichment: Techniques

- Manual macrodissection
- Manual microdissection
- Laser capture microdissection
Tumor Enrichment: Cytology Cell Blocks (FFPE)

Dara Aisner, Uni. Of Colorado


Tumor Enrichment: Cytology Direct Smears

Slides are circled to enrich for tumor cells by a cytopathologist. Slides are circled on the bottom using a diamond-tip pen. Circled areas are then scraped with a scalpel blade into a buffer for DNA extraction, followed by coverslip removal.

Slides are etched on the bottom using a diamond-tip pen. 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.

Molecular Processing

• Validating next generation sequencing assay for a variety of cytologic substrates, establishing sensitivity, and input DNA threshold.

Input DNA Threshold Plays a Major Role in NGS Success

So, how do cytology samples compare to surgical samples?

Comparing FNA and CNB Samples for NGS

Comparing FNA and CNB Samples for NGS

- FNA samples provide significantly higher tumor fraction than the concurrently acquired CNB samples.

Mission Allelic Frequencies are higher in FNA samples than the concurrent CNB samples.
- Normalized average amplicon coverage is significantly higher in FNA samples than CNB samples.
- FNA samples had lower numbers of underperforming amplicons than the paired CNB samples.

Cytology: Underutilized Goldmine of Genomic Data

- General reluctance of molecular labs to validate a variety of cytologic specimen preparations (smears, cytospins, liquid-based cytology, fresh/frozen samples) for a multitude of molecular tests.
- Lack of standardization across cytology laboratories for specimen collection and processing for cytologic material.
- Overall reluctance of cytopathologists to sacrifice cytologic slides from the diagnostic archives.
- Lack of awareness among the oncology (and general pathology community) regarding utility of cytology specimens for molecular testing.