Next generation sequencing: basic principles and cytological sample requirements

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Next generation sequencing: basic principles and cytological sample requirements
Cytology Specimens Provide Excellent Substrates for Next Generation Sequencing

- Small specimens are *not necessarily* an obstacle for next generation sequencing (NGS)
- Multiple pre-analytic factors impact tissue quality and the success of NGS
- The pathologist plays a key role in triage and specimen handling that can improve the success of NGS in cytology specimens
Evolving Role of Cytopathology

• Tissue is not only for diagnostic cytopathology evaluation, but also for clinical molecular assays

• Molecular profiling for key analytes is now standard of care for various solid tumors including lung cancer, colon cancer, melanoma, thyroid cancer etc.
Molecular Diagnostics is Used Commonly in Cytology Specimens

- Most patients diagnosed at an advanced stage are not surgical candidates
- Therefore, it is critical to be able to test on limited volume small samples including cytology
Small specimens are not necessarily an obstacle
Targeted Next-Generation Sequencing Using Fine-Needle Aspirates From Adenocarcinomas of the Lung

Hope E. Barnes, MD, PhD; Eric J. Duncavage, MD, PhD; and Cory T. Bernadt, MD, PhD

Young Investigator Challenge: Can the Ion AmpliSeq Cancer Hotspot Panel v2 be Used for Next-Generation Sequencing of Thyroid FNA Samples?

Claudio Bellevisc, MD, PhD; Roberta Sguarigilo, BS; Umberto Malapelle, PhD; Elena Vigilante, MD, PhD; Mariani Antonia Nacchio, BS; Giuseppe Ciaiania, MD, PhD; Markus Eslinger, PhD; Ralf Paschke, MD, and Giancarlo Troncone, MD, PhD

Next-Generation Sequencing of Lung Cancer EGFR Exons 18-21 Allows Effective Molecular Diagnosis of Small Routine Samples (Cytology and Biopsy)

Dario de Biase1, Michela Visani2, Umberto Malapelle1, Francesca Simonato1, Valentina Cesari1,2, Claudio Bellevisc3, Annalisa Pession3, Giancarlo Troncone1, Ambrogio Fassina4, Giovanni Tallini1

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Molecular Typing of Lung Adenocarcinoma on Cytological Samples Using a Multigene Next Generation Sequencing Panel

Aldo Scarpa1,2,9, Katarzyna Sikora1,9, Matteo Fassan1,2, Anna Maria Rachiaglio3, Rocco Cappelloso4, Davide Antonello5, Eliana Amato1, Andrea Mafficini1, Matilde Lambiase3, Claudia Esposito3, Emilio Bria8, Francesca Simonato1, Maria Scardoni1, Giona Turri1, Marco Chilosi2, Giampiero Tortora5, Ambrogio Fassina4, Nicola Normanno6

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Imaging, Diagnosis, Prognosis

Effective Assessment of egrf Mutation Status in Bronchoalveolar Lavage and Pleural Fluids by Next-Generation Sequencing

Fianna Butitta1,2, Ela Felcini1,2, Mela Del Graneretto1, Gaio Nullo1, Alessia Di Loiso1, Sara Malastesa1, Patrizia Volta1, Irene Centi1, Tommaso D’Antuono1, Roberta Zappacosta1, Sandra Rosati1, Franco Gucurruolo1, and Antonio Marchetti1

Methods in Pathology

Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics


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Multiple pre-analytic factors impact tissue quality and the success of NGS
Multiple Factors Impact Tissue Quality and Molecular Testing

Fig. 1. The life cycle of the biospecimen.

Specimen Acquisition
Specimen Acquisition is the First Step to Getting an Adequate Sample

• Clear communication between 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, operator skill and training etc)
ROSE Can Ensure Appropriate Triage of FNA Specimen

• Rapid on-site evaluation (ROSE) can ensure adequate sampling and triage
• Performing additional pass in anticipation of ancillary studies
• If molecular lab prioritizes cell blocks, minimize the amount of material expelled onto smears and maximize the needle rinse material for cell block
• If molecular lab prioritizes smears, minimize cell block material
Specimen Processing
The Cytology Specimen: A Closer Look

<table>
<thead>
<tr>
<th></th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
</table>
| Cell blocks              | • Ease of acquisition  
                          • Ease of validation  
                          • Easy to get serial sections | • Lack of immediate assessment  
                          • May have low cellularity  
                          • Degradation of nucleic acid (formalin fixation)  
                          • Partial nuclei in standard 4-5 μm sections |
| 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 cytology    | • Standardized processing with optimal preservation  
                          • 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 |
Specimen Processing in Cytology Lacks Standardization

- Quantitative/qualitative differences in nucleic acid and protein antigenicity due to varying processing techniques (glass slides, fixatives, stains, and preservative media)

And yes, that includes cell block preparations
Optimal Practices for Cell Block Processing

• 10% formalin (3.7% formaldehyde in water) most commonly used
• Neutral buffered formalin is the preferred choice
• Time of fixation (6-48 hr)
• Alternative fixatives containing acid or heavy metals are not recommended
• Decalcification using harsh acids should be avoided
Optimal Practices for Cell Block Processing

• Synthetic paraffin with low melting temperature is recommended
  Additives like beeswax should be avoided
• Block storage with appropriate temperature and humidity control
• Nucleic acid recovery may decrease with long periods of storage
• Fresh sections cut from the block are preferred over previously cut sections
## Optimal Practices for Cell Block Processing

### 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

<table>
<thead>
<tr>
<th></th>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cold ischemia time</td>
<td>&lt;24 hrs for PCR&lt;br&gt;</td>
<td>&lt;12 hrs</td>
<td>&lt;12 hrs</td>
</tr>
<tr>
<td>Specimen size</td>
<td>3-10 mm³</td>
<td>N/A</td>
<td>1.2-3.5mm³</td>
</tr>
<tr>
<td>Fixative</td>
<td>NBF</td>
<td>NBF</td>
<td>NBF</td>
</tr>
<tr>
<td>Fixation time</td>
<td>&lt;72 hrs</td>
<td>8-48 hrs</td>
<td>6-24 hrs</td>
</tr>
<tr>
<td>Embedding</td>
<td>Paraffin</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Storage</td>
<td>&lt;5-10 yr</td>
<td>&lt;1 yr</td>
<td>&lt;25 yr</td>
</tr>
<tr>
<td>Decalcification</td>
<td>EDTA</td>
<td>EDTA</td>
<td>Tissue/antigen specific</td>
</tr>
</tbody>
</table>

Specimen Processing in Cytology Lacks Standardization

Preparation of DNA From Cytological Material

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

Annika Dejmk, MD, PhD1,2; Nooreldin Zendehrokh, PhD1; Malgorzata Tomaszewska, MSc3; and Anders Edsjö, MD, PhD1,2,4,5

Specimen Processing in Cytology Lacks Standardization
Molecular Testing Success Often Depends on the DNA Yield

Factors Affecting the Success of Next-Generation Sequencing in Cytology Specimens

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Keyur P. Patel, MD, PhD; Mark J. Routbort, MD, PhD; Rajesh R. Singh, PhD;
Russell R. Broaddus, MD, PhD; Bedia A. Barkoh; Jawad Manekia; Hui Yao, PhD;
L. Jeffrey Medeiros, MD; Gregg Staerkel, MD; Rajyalakshmi Luthra, PhD; and John Stewart, MD, PhD

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

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

Abbreviation: NGS, next-generation sequencing.

NGS success positively correlates with DNA yield
Specimen Selection and Handling
Cytology Samples are Excellent Substrates for NGS

• Cytology specimens provide a variety and versatility of specimen preparations
  ➢ High quality nucleic acid (non-formalin fixed tissue)

• Ideal for testing metastatic tumors in bony lesions
  ➢ No decalcification
Cytology Samples are Excellent Substrates for NGS

Next-Generation Sequencing of Cytologic Preparations: An Analysis of Quality Metrics

David H. Hwang, MD; Elizabeth P. Garcia, PhD; Matthew D. Ducar, MS; Edmund S. Cibas, MD; and Lynette M. Sholl, MD

Smears/LBP show better quality metrics than FFPE

- higher mean insert size
- higher mean target coverage
- higher percent usable bases
- lower duplication rate
- lower post-shearing fragment size
Select the Best Sample for NGS

- FNA samples are inherently enriched in tumor cells
- FNA samples had lower numbers of underperforming amplicons
- Normalized average amplicon coverage is significantly higher in FNA samples

Specimen Selection/Handling is Key to NGS Success

Communication with molecular laboratory is critical

• Knowledge about molecular adequacy criteria, tissue-extraction strategies, and basic principles of NGS are needed to select the most appropriate material for testing
Step 1: Specimen Assessment

Overall cellularity
All nucleated cells in sample

Translates to the Total DNA yield
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 cells

• NGS (Ion Torrent PGM) requires around 10 ng of DNA

  Therefore approximately 1430-1660 intact cells
Step 2: Specimen Assessment

Tumor cellularity/tumor fraction
Percentage of tumor cells

Translates to Analytic Sensitivity of Platform
Tumor Fraction Estimation is an Imprecise Art

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; a false-negative test result is possible.

Objective.—To describe and utilize a comprehensive manual to determine pathologist accuracy in estimating the malignant cell percentage.

Design.—Ten 20 μm formalin-fixed, paraffin-embedded colon adenocarcinoma tissue sections were analyzed and compared with the comprehensive manual.

Results.—Survey responses indicated low interlaboratory precision of pathologist estimation, but mean estimates were fairly accurate. A total of 5 of the 10 cases were downstaged and 1 was overstaged.

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

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NGS Success Rates Vary Among Pathologists

- Appropriate training of pathologists is needed to reduce inter-observer variability
Modulate the Specimen to the Assay

Send for testing with more slides: some risk of fail
Send for testing: high chance of success

Cellularity
Tumor Fraction

High risk of fail: insufficient
High risk of false negative

Tumor mapping in high cellularity/low tumor fraction samples may reduce the risk of false-negative results, at the cost of decreased overall cellularity and DNA yield.
Tumor Enrichment: Techniques

Tumor enrichment techniques:
- Manual macrodissection
- Manual microdissection
- Laser capture microdissection

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

Tumor fraction at least 20%

- Tiny samples = 40ul (All slides)
- Small and small-medium samples = 40ul (up to 10 slides)
- Medium and Medium large samples = 40ul (up to 4 Slides)
- Large samples = 40ul (1 - 2 slides)
- Cytology smears = 40ul (All slides)
Input DNA Threshold Plays a Major Role in NGS Success

- NGS success *positively correlates* with input DNA \( p < 0.0001 \)

Preanalytic Factors Play a Major Role in Molecular Testing Success

<table>
<thead>
<tr>
<th>Strategies That Can be Used to Maximize Tissue From Cytologic Specimens for Molecular Testing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Steps</strong></td>
</tr>
</tbody>
</table>
| Specimen acquisition | • Clear communication among ordering clinician, proceduralist (eg, radiologist), laboratory technician/technologist, and pathologist for procuring adequate samples for appropriate testing  
  • Using image-guided procedure for better diagnostic yield  
  • Optimizing technique for best diagnostic yield (eg, needle gauge, number of passes, etc)  
  • Rapid, on-site assessment of specimen for adequacy, requesting additional passes to ensure adequate sampling and proper triaging  
  • Performing additional dedicated 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  
  • Whenever clinically feasible, obtaining concurrent core biopsies and FNAst to maximize chances of sufficient material to perform ancillary studies |
| Specimen processing | • Standardizing processing techniques for specimen preparations, glass slides, fixatives, stains, and preservative media; understanding quantitative differences in DNA yield from different sources  
  • Avoiding acidic and heavy metal fixatives like Bouin and Zenker solutions and harsh acid decalcification agents, which interfere with molecular testing  
  • Appropriate triaging of material for ancillary studies (eg, preparing additional decoverslipped smears in anticipation of testing; cutting extra, unstained cell-block sections up front to avoid refactoring block)  
  • Optimizing cell-block preparation method  
  • Limiting immunoperoxidase stains to conserve tissue (eg, dual stains, limited panels for diagnosis)  
  • Exploring the use of a variety of cytologic specimen preparations outside of paraffin-embedded cell-block preparation to maximize the use of limited samples  
  • Using tumor-enrichment techniques (macrodissection and microdissection) to enhance low tumor-cellularity samples  
  • For cell-block sections, providing a circled hematoxylin-eosin–stained slide to guide tissue extraction from unstained slides; for smears, decoverslipped slides that are appropriately etched on the bottom to demarcate tumor-rich areas for tissue extraction if macrodissection or microdissection is employed  
  • Communication with molecular laboratory regarding tissue-extraction strategies, testing platforms, criteria for adequacy, criteria for rejecting sample, and selecting the best material for the analytic platform  
  • Appropriate training of cytopathologists in adequacy assessments for visual estimation to improve interobserver and intraobserver variability |
| Specimen selection | • Optimizing tissue-extraction strategies for maximal DNA yield (scraping versus cell lifting versus direct extraction from LBC samples or fresh cells)  
  • Establishing guidelines for tumor enrichment (eg, macrodissection versus manual microdissection versus laser-capture microdissection)  
  • Selecting appropriate high analytic sensitivity platform to evaluate low tumor cellularity samples  
  • Establishing strict quality control and quality assurance procedures |

Abbreviations: FNA, fine-needle aspiration; LBC, liquid-based cytology.
Cytology Specimens Provide Excellent Substrates for Next Generation Sequencing

- Small specimens are *not necessarily* an obstacle for next generation sequencing (NGS)
- Multiple pre-analytic factors impact tissue quality and the success of NGS
- The pathologist plays a key role in triage and specimen handling that can improve the success of NGS in cytology specimens
Why are Cytology Samples Underutilized for Next Generation Sequencing?

• Lack of standardization across cytology laboratories for specimen collection and processing

• Reluctance of molecular labs to validate a variety of cytologic specimen preparations for a multitude of molecular tests

• Lack of awareness among the community regarding utility of cytology specimens for molecular testing

• Overall reluctance of cytopathologists to sacrifice irreplaceable cytologic smears from the diagnostic archives
Molecular Cytopathology: The Future is Bright
How to prepare cytological samples for molecular testing

Claudio Bellevicine, Umberto Malapelle, Elena Vigliar, Pasquale Pisapia, Giulia Vita, Giancarlo Troncone

Challenges and opportunities of next-generation sequencing: a cytopathologist’s perspective

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Challenges and opportunities of next-generation sequencing: a cytopathologist’s perspective

Abstract
Molecular cytopathology has gene sequencing as its core technology. Until recently, cytological samples were only tested by sequential single-gene mutational tests. Today, with the better understanding of the molecular events involved in malignancy and the mechanisms of pharmacotherapy, larger gene panels are more informative than a single biomarker. Next-generation sequencing (NGS), matched with the multiplex capture of targeted gene regions and analysed by sophisticated bioinformatics tools, enables the simultaneous detection of multiple mutations in multiple genes. With the development of miniaturised technology and benchtop sequencers, it is not unlikely that NGS will soon be adopted for routine molecular diagnostics, including cytological samples. This review addresses (1) the most relevant methodological and technical aspects of the NGS analysis workflow and the diverse platforms available; (2) the issues related to daily practice implementation, namely, the cytological sample requirement and the validation procedures; and (3) the opportunities that NGS offers in different fields of cytopathology, to increase mutation detection sensitivity in pan-cancer screens and to extend the analysis to a larger number of gene regions. Cytopathologists involvement and coordination in this rapidly evolving field is crucial for the effective implementation of NGS in the present and future cytopathological practice.

Keywords: Next-generation sequencing, targeted therapy, fine needle aspiration, cytology, 454 NGS, Illumina, Ion Torrent, Molecular Cytopathology
Thank You