

CAP Companion Society Meeting at USCAP 2009
Quality Assurance, Error Reduction, and
Patient Safety in Anatomic Pathology

Quality, Patient Safety, and Error Reduction in Molecular Diagnostics
Christopher A. Moskaluk, MD, PhD, FCAP
Associate Professor of Pathology, University of VA
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Learning objectives:

- Understand how procurement issues & target heterogeneity in tissue samples impacts the quality of molecular diagnostics
- Understand how protocol complexity & non-uniformity in molecular diagnostics can lead to errors
- Understand how application of quality assurance & quality control procedures in molecular diagnostics can reduce error

Syllabus

Introduction

The practice of Anatomic Pathology (AP) has for over 100 years been based primarily on the gross and histologic analysis of tissues. In the past few decades, the assessment of specific molecular constituents of tissue has been adopted into AP practices primarily through the histology-based *in situ* assays of immunohistochemistry and *in situ* hybridization. More recently it has become necessary to evaluate specific molecular constituents of tissue in solution or gel-based analytic techniques that require the extraction of biomolecules from tissue samples. It is this aspect of molecular diagnostics that this session will cover.

Pre-analytic variables in AP molecular diagnostics: Tissue and molecular target integrity

The traditional practice of anatomic pathology basically had to prevent the autolysis of tissue which precluded optimal histologic analysis. This allowed a relatively leisurely pace of tissue processing since autolysis typically does not become histologically evident for hours to days after excision. Moreover, the standard histologic practice of formalin fixation halts the process of autolysis and stabilizes the tissue for ambient temperature storage, and for histologic processing into paraffin blocks.

The analysis of specific molecular targets in tissue is much more problematic. Certain molecular constituents, particularly those involved in signal transduction, have a dynamic half-life that is significantly altered in the state of cellular stress incurred by devascularization that occurs as a result of surgical resection or biopsy. In particular, certain protein phosphorylation events have been shown to dramatically change levels within minutes of excision (1, 2). Formaldehyde fixation may preserve the original phosphorylation status, but only in areas of tissue that undergo rapid formalin penetration, making the assessment of protein phosphorylation in large organ resections problematic if rapid biopsy-type sampling is not undertaken (3). Other molecular constituents that are known to be particularly labile in devascularized tissue are certain proteins (particularly regulatory proteins) and RNA transcripts (4). One exception to this general rule is genomic DNA, which is generally resistant to marked degradation that would preclude molecular analysis. Since many of the initial techniques being employed in tissue diagnostics is the detection of specific gene mutations in genomic DNA, this is both a blessing and a curse. The blessing is that the majority of samples available in an AP laboratory will be amenable to analysis without altering customary practices and workflow. The curse is that as molecular assessment of

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more labile analytes becomes important, AP practices will not have been appropriately attuned to the necessary attention to rapid tissue procurement.

Some of the degradation of molecular targets is beyond the control of AP laboratories. In particular, the time of “warm ischemia” that occurs in an organ undergoing resection – the time between which the surgeon has severed the blood supply to a particular organ area and the time that the specimen is completely removed from the patient, cannot neither be controlled, nor often easily documented. However all steps after that, including the time of transport to the AP laboratories and the time of processing of the specimen (gross examination, inking, sectioning, etc.) need to be reviewed and reduced to the minimum time possible. It will become necessary to set up a triage system such as that now exists for specimens requiring intra-operative frozen section diagnosis, to identify cases needing or likely to need molecular diagnosis and have them processed and sampled in an expedient manner. Alternatively, changes to clinical practice, such as intra-operative biopsy of tissues for molecular diagnostics may need to be employed. Finally, techniques to rapidly stabilize samples that are complementary to the technique to be employed (e.g. snap freezing, emersion in “molecular friendly” fixatives) will have to be employed for the analysis of labile analytes.

Having made the general statement that speed is of the essence is obtaining tissue samples for molecular diagnostics, there is some data to suggest that a reasonable time period of 1-2 hours exists after the excision of tissue in which the global RNA transcript profile is relatively stable (5-7). However this not true of all transcripts, and the parameters for any individual analyte needs to be determined for appropriate specimen handling.

There is a substantial literature documenting the changes of biomolecules in tissue as a result of formalin fixation and paraffin embedding (8-12). Primarily, formaldehyde causes covalent cross links to occur within and between biomolecules. While the consequent stabilization of cellular and tissue structures is desirable for histology, this event precludes the complete solubilization of individual biomolecules that is required for many molecular analyses. In addition, the process of paraffin embedding causes additional denaturation and fragmentation of biomolecules, particularly nucleic acids. While this process does not preclude some molecular analyses, for many molecular technologies, formalin-fixation and paraffin-embedding (FFPE) precludes robust and/or accurate measurements.

Since it may not be possible to preclude that some tissue samples being subjected to a molecular diagnostic test do not have sufficient quality to yield an accurate result, there is a requirement to ascertain the molecular integrity of the sample. This may occur as either a separate pre-analytic assessment, or may be integrated by the addition of appropriate controls during the analysis. One of the most common of pre-analytic assessments is the determination of RNA quality. Generally, total RNA isolation is performed from tissue samples, yielding a mixture of processed messenger RNA, ribosomal RNA (rRNA) and heterogeneous nuclear RNA. The quality of the two major rRNA species (18s and 28s), as determined by their absolute and relative amount, has been shown to be generally reflective of the quality of the transcript population. Among the most common analytic methods to assess rRNA and global RNA quality is the use of microcapillary electrophoresis systems, and various computer algorithms have been developed to yield numerical scores of RNA quality that can be used as benchmarks or thresholds for subsequent analyses (13). Again, it is difficult to generalize for molecular diagnostics, and the minimum specimen quality required for any analyte and/or analytic platform needs to be determined on an assay by assay basis.

For those analyses not utilizing pre-analytic specimen integrity testing, generally an internal positive control is developed to ascertain sample quality. For instance, in a quantitative reverse transcriptase polymerase chain reaction assay (qRT-PCR) for a cancer-specific translocation gene product, an internal qRT-PCR for a “housekeeping” gene that is expected to be present in the sample will also be performed. If the concentration of this internal control gene

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does not reach a threshold level, then the sample will be rejected as being insufficient to confidently render a result.

Pre-analytic variables in AP molecular diagnostics: Tissue heterogeneity and the role of histology in molecular diagnostics

In general, molecular diagnostics concerns itself with the analysis of a specific analyte in a specific cell type (e.g. gene mutation in cancer cells). The inherent heterogeneity of tissue samples, precluding accurate assessment of cell type and cell viability of tissue specimens by gross assessment, and the vagaries of tissue sampling by biopsy methods requires some assessment of the type of constituent cells. At this juncture, histology remains the most universal and robust of methods to achieve this goal.

Two things are required to address this issue in AP molecular diagnostics:

- 1) During the development phase of the assay, a determination must be made as to minimum percentage of target cells is required in order to deliver an accurate assessment. This is typically done by “mixing experiments” using 2 populations of cell culture model systems, in which one cell line represents the target cell population and the other represents “non-target” cells.
- 2) There must either be a histologic assessment of the input tissue to determine if the target cell population meets the tolerances of the assay, or some sort of dissection technology needs to be employed to enrich the target cells to meet these tolerances.

Thus at a minimum, a matched FFPE tissue section needs to be examined to know what the input material is to a molecular assay. Alternatively, a histologic section is taken of the input sample (frozen or FFPE), and areas enriched for target cells are dissected. This may be performed as bulk or “macrodissection” in which the target areas are dissected from the block, or scraped from histologic sections (14). While this does not typically yield completely pure cell populations, for many samples >70% enrichment may be achieved.

For more pure cell populations, laser microdissection may be employed. This techniques may employ differing technologies, but what is in common is that a laser beam somehow captures discrete populations of cells from a histologic section by direct observer control through a microscope (15). Though capable of obtaining pure or near pure populations of cells, the drawbacks include that the amount of captured material is very small, too small for some types of analysis, and that the technique is relatively slow and cumbersome, taking up too much time of histopathology-trained personnel to be realistically deployed for the majority of molecular assessments in a clinical laboratory.

Patient safety issues: sample misidentification

Sample misidentification is a problem that is inherent to all laboratory testing. In addition to the standard problems of inaccurate pre-analytic and post-analytic identification of a sample or laboratory result, many molecular diagnostic methodologies suffer from numerous container transfers during both the biomolecule extraction phase and the analytic phase, increasing the odds of inappropriate specimen labeling. Often container labeling may be carried out manually, without computer generated labels or with bar-coding. These factors are often the result that many molecular diagnostic techniques are either emerging technology and/or are primarily research technique being newly applied to diagnostics. Such immature technologies often are not supported by robotic platforms requiring large amounts of manual specimen manipulation. A second problem is that some molecular tests may be carried out in hybrid research and clinical labs in which personnel are carrying out similar techniques using different standard operating procedures, leading to lapses in or confusion about the standards applied to clinical testing.

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Solutions include moving to robotic platforms whenever possible. While this may not be possible for many assay types, at least for biomolecule extraction, many platforms exist that support the multi-step isolation of DNA, RNA and/or proteins from tissue samples. Whenever possible, bar coding systems should be in place to track specimens in the pre-analytic and analytic process. And finally, appropriate personnel training and quality check procedures should be in place to maintain and verify the accuracy and reproducibility of specimen tracking through the process.

Quality Assurance and Quality Control procedures

While all of the preceding discussions have involved aspects of quality assurance (QA) and quality control (QC), this section will discuss the general implementation of these procedures in a molecular diagnostics laboratory. One definition of QA is: the policies, procedures and systematic actions established for the purpose of providing and maintaining a high quality of test integrity and accuracy. Quality control (QC) generally refers to the procedures in place to verify the accuracy of individual assay results (e.g. pre-analytic thresholds for specimen quality, positive and negative controls, etc.). QA/QC procedures involve steps to ensure all the steps involved in pre-analysis, analysis and post-analysis are being performed accurately and are being documented (see Table 1). Most of these procedure categories are not novel to molecular diagnostics; the standard QA/QC paradigm that governs the best practices of all clinical laboratories applies to molecular diagnostics (16, 17). The increased attention to tissue heterogeneity and integrity has already been covered. The specifics of appropriate quality control measures that pertain to the individual assays are unique, but the discussion of the controls used in the myriad types of molecular diagnostic assays is beyond the scope of this discussion.

Table 1: Areas that quality programs must address

Pre-analytical Variables

- Test requests/ordering
- Patient identification
- Specimen acquisition
- Specimen transport
- Specimen processing
- Presence/purity of target cells
- Specimen integrity
- Instrument maintenance

Analytical Variables

- Competency
- Controls
- Methodology/ procedure validation

Post-analytical Variables

- Result interpretation
- Result reporting
- Monitoring of equipment
- Monitoring of materials

Documentation

- Standard Operating Procedures (SOPs)
- Assay logs
- Equipment maintenance records
- Training records
- Results/diagnoses rendered

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A quality assurance program must create standard operating procedures (SOPs) for all aspects of specimen handling, analysis and report generation that all personnel must have access to and are familiar with. The program also devises, implements and documents training and retraining programs for all personnel involved in all of these aspects. Quality control measures are built into the procedures to ensure accuracy. Finally, a quality assurance program entails periodic quality monitoring (proficiency testing) of the system to ensure that quality measures are being adhered to and that accurate test results are being generated.

In order for a test to be considered valid, it must have at least first-line and second-line controls (18). First-line controls consist of the internal controls that ensure the accuracy of each assay (e.g. “no template” negative PCR control, a positive cell line DNA control for k-ras mutation assay, etc.). Second-line controls refer to local proficiency testing and quality monitoring. Examples of this would include giving a technician two aliquots of the same colon cancer to test for microsatellite instability to ensure the reproducibility of the assay, or supplying all technicians with the same cohort of known samples for k-ras mutation analysis to document the consistency of the entire laboratory.

It is also most desirable for the laboratory to participate in third-line controls. This refers to the analysis of externally validated samples. This typically takes place under the aspect of external proficiency testing under the auspices of an accrediting agency or organization (19). Unfortunately for many emerging molecular diagnostic tests, the clinical implementation of tests may outpace the availability of external proficiency testing from accrediting organizations. Such challenges have been faced in the past by clinical labs performing genetic testing, and which have been addressed by cooperative sample sharing between laboratories, or by quality assurance materials being validated and made available to members of regional cooperative groups. Similar arrangements may be made between cooperating molecular AP labs.

The issue of external proficiency testing is important in this area. As mentioned, molecular diagnostics is an emerging field. The result of this fact is that diagnostic tests for the same analyte are carried out using varying technologies, protocols and materials. This invariable will result in assays with varying sensitivity and specificity being deployed. The availability of external quality assurance materials being supplied in a cooperative, non-punitive manner is essential to determine for the field what technology and protocols yield the most accurate results, and to have these findings being disseminated widely.

To close with an evidence-based discussion of this topic, results from two reports of the experience of the Molecular Oncology Survey of the College of American Pathologists will be presented here. In the first study, the detection of the t(14;18)(q32;q21) translocation in samples of follicular lymphoma (FL) by PCR analysis of genomic DNA was studied (20). 24 well-characterized specimens were sent to participating laboratories in a proficiency testing program between 1997-2000 in which 25 to 61 laboratories participated annually. During the course of this program, 819 major breakpoint region and 323 minor cluster region determinations were performed, with an overall correct response rate of 91% and 94%, respectively. It was determined that no significant differences were detected in the detection rates between frozen and FFPE tissue samples, which confirms the previous assertion of DNA stability in this document. Interestingly, despite the overall good performance of the labs, in response to a survey of laboratory methods it was determined that no two laboratories in the survey carried out the analyses using exactly the same method, with the design of PCR primers showing the widest heterogeneity. This underscores the variability in technique inherent to emerging molecular diagnosis. The major deficiency that the survey uncovered was that most laboratories had not determined a sensitivity threshold for their assay. However, it was actually the laboratories that had determined the greatest level of sensitivity that reported false positive results, suggesting that “pushing” the sensitivity of PCR-based analysis may result in false positive results.

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The second report deals with the identification of immunoglobulin heavy chain (IGH) clonal gene rearrangement in the diagnosis of B cell lymphoproliferative disorders and malignancies (21). 39 well-characterized specimens were sent to participating laboratories in a proficiency testing program between 1998-2003 in which 161 laboratories participated. 944 results were reported using Southern blot technology, with a success rate of 95%. 2349 results were reported using PCR methodology, with a success rate of 72%. Since PCR methodology also varied widely in this survey, it could be determined that a specific grouping of genomic primers yielded a greater success rate (82%). It is of interest that though Southern blot technology appears to be a superior method, there appears to be wider spread adoption of PCR based methodologies. This may be due to the fact that PCR methodologies require less technician time and is a simpler procedure. PCR based methods also allow the analysis of FFPE material, which generally are precluded from Southern blot analysis. However when matched frozen and FFPE tissues were evaluated side by side by PCR analysis, the success rate for frozen material was 78% and for FFPE material was 66%. Participation in external proficiency testing such as this, with dissemination of results, is important for the diagnostic community to determine optimal protocols and techniques, and for diagnosticians and clinicians to understand the limitations of the assays when results are used in clinical decision-making.

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Patient Safety in Anatomic Pathology***

**Quality, Patient Safety, and Error
Reduction in Molecular Diagnostics**

Christopher A. Moskaluk MD, PhD, FCAP
University of Virginia



Education by the Experts

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Learning Objectives

- Understand how procurement issues & target heterogeneity in tissue samples impacts the quality of molecular diagnostics
- Understand how protocol complexity & non-uniformity in molecular diagnostics can lead to errors
- Understand how application of quality assurance & quality control procedures in molecular diagnostics can reduce error



Agenda

- Quality
 - Pre-analytic considerations for tissue samples
 - Tissue heterogeneity & target selection
- Patient Safety / Error reduction
 - Issues inherent to emerging technology that may contribute to sample misidentification
- Error reduction / Standardization of analytic techniques
 - Typical QA/QC must be applied to AP molecular diagnostics



Focus on “non-*in situ*” techniques

Specimen

Samples



FFPE

Fresh frozen

**Extract biomolecules
(DNA, RNA, protein)**

Histologic section



in situ assays

- immunohistochemistry
- in situ* hybridization
- in situ* PCR/RT-PCR

Solution or gel-based assays

- RT-PCR
- GeneChips™
- array CGH
- microsatellite PCR
- mass spectroscopy
- Western blots
- Northern blots
- Southern blots
- etc.

QuickTime™ and a decompressor are needed to see this picture.



Education by the Experts

Pre-analytic parameters: Target degradation

- Anatomic Pathologists are aware of tissue autolysis that degrades histologic analysis
 - Hours to days depending on tissue type and temperature
 - Formaldehyde-fixation stabilizes tissue architecture and cell morphology
 - Current AP clinical practice concerning tissue handling is based on this “leisurely” time table and on the almost universal use of formalin



Pre-analytic parameters: Target degradation in FFPE tissue

	Formalin-fixed paraffin-embedded	Fresh/Frozen
Nucleic acids	Cross-linked, sheared to ~200 base pair (bp)	Intact (thousands to millions of bp)
Proteins	Cross-linked, denatured	Intact
Histology	Excellent, best for clinical analysis based on morphology	Adequate for some but not all clinical assessments
Storage of archival samples	Cheap, common	Expensive, uncommon



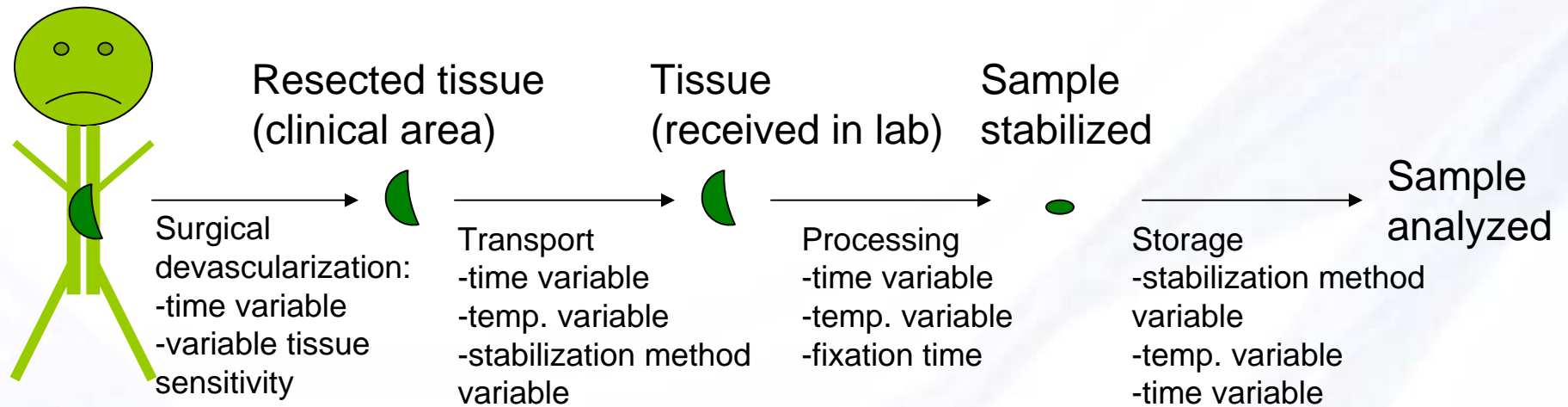
Pre-analytic parameters: Molecular target degradation

- Many targets of molecular assays are labile!
 - Post-translational protein modification (phosphorylation, etc.)
 - minutes-hours
 - Levels of regulatory protein
 - minutes-hours
 - Levels of RNA transcripts
 - minutes-hours
- DNA is an exception



Pre-analytic parameters: Myriad & some “uncontrollable”

Tissue in patient



Rate of degradation (generalities): Rapid increase immediately after devascularization. Rate stabilizes or decreases for a few hours, especially if specimen cools. Rate then rapidly increases as cell membranes breakdown.



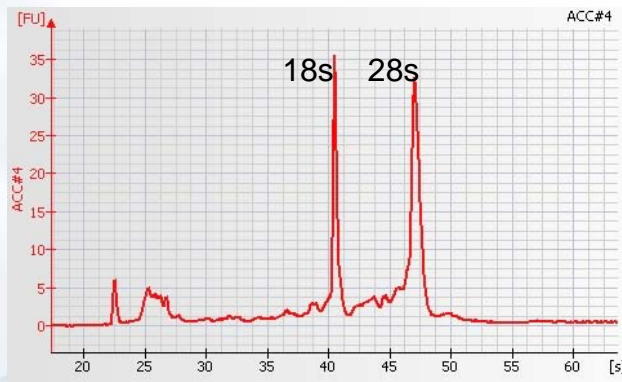
Pre-analytic parameters: Testing of specimen integrity

- Degradation in some samples will be unavoidable
- Assessment of overall molecular integrity or specific target integrity
 - Pre-analytic test
 - Integrity assessment may be incorporated into the analytical procedure
- Rejection of specimen/analytic results based on tissue/analyte quality

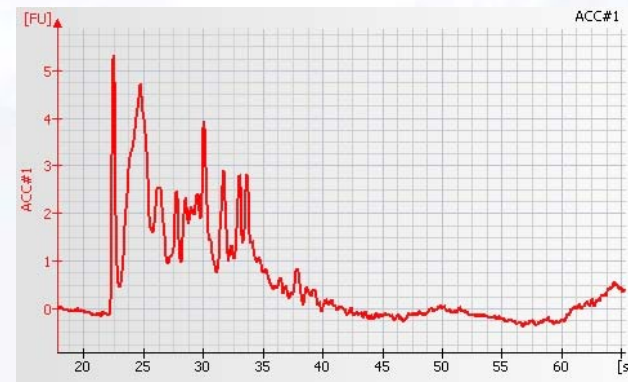


RNA integrity tests

- RNA integrity is one of the most common “pre-analytic” tests performed
- Integrity of 28s & 18s ribosomal RNA is commonly used as a global assessment
- Microcapillary electrophoresis



intact



degraded



Education by the Experts

Examples of intra-assay specimen integrity checks

- Threshold level for qRT-PCR of translocation gene product
 - Upper limit set for C_t of housekeeping gene
- PCR amplification of c-kit gene for sequencing
 - Minimum PCR product concentration after set number of PCR cycles



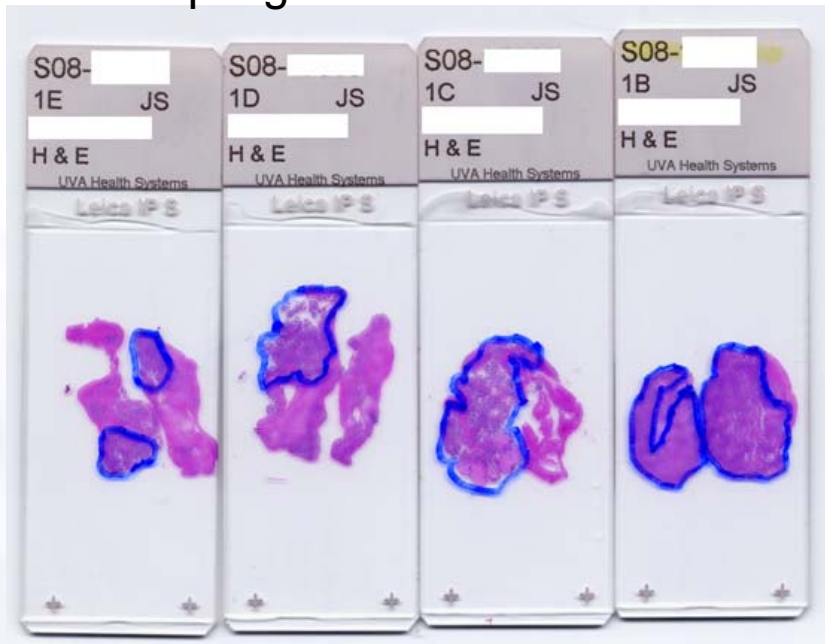
Pre-analytic parameters: Recommendations

- Review & streamline specimen transport & handling
 - May require increase in personnel & changes in work flow patterns
- Create triage system that identifies specimens requiring molecular analysis
- Integrate early specimen stabilization as much as possible
 - Freezing
 - LN₂ & -80°C ~equivalent, -20°C inappropriate
 - Stabilization solutions (probably not formalin!)



Pre-analytic parameters: Tissue heterogeneity

Sampling of same ovarian tumor

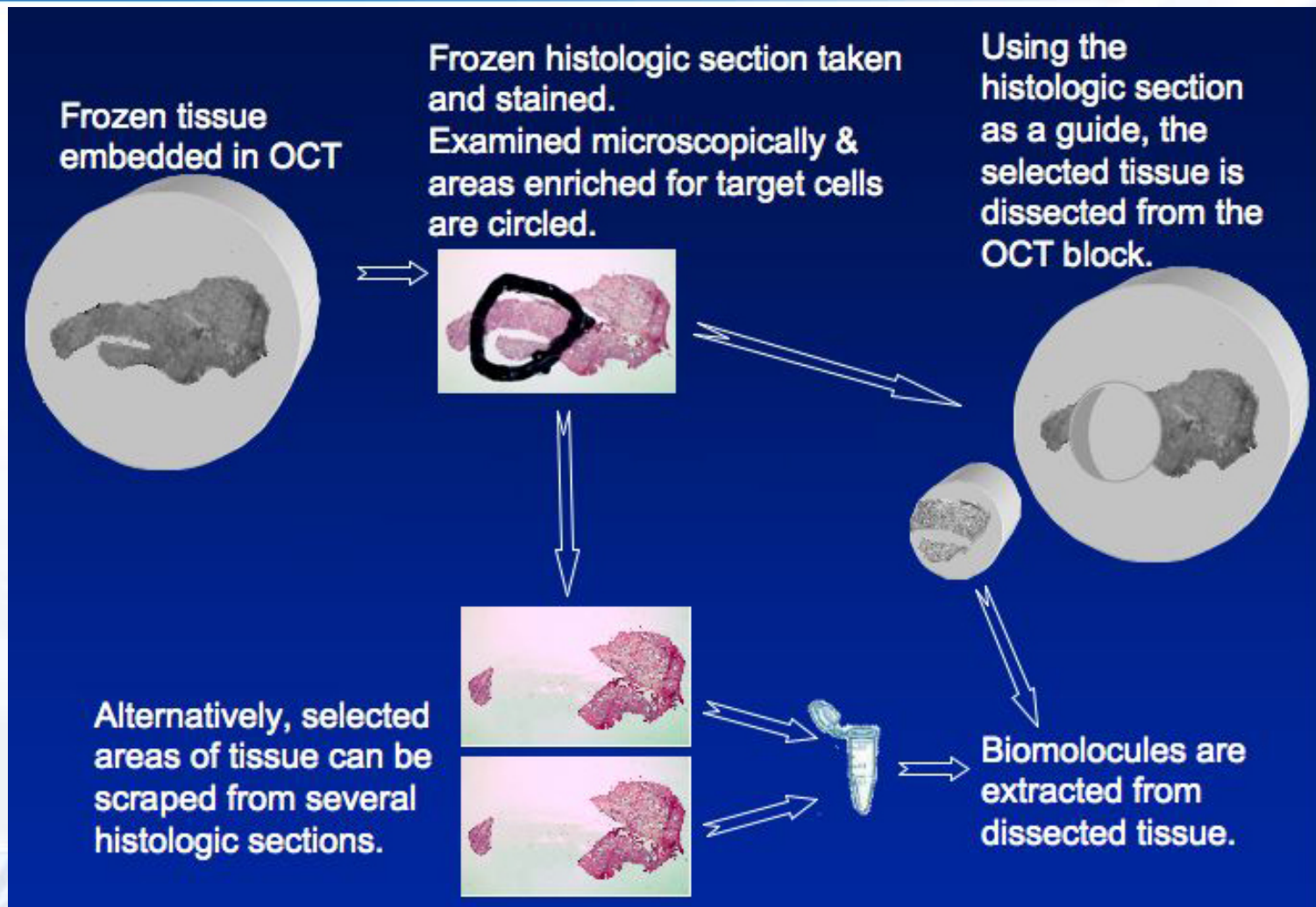


Areas of >70% tumor cellularity circled

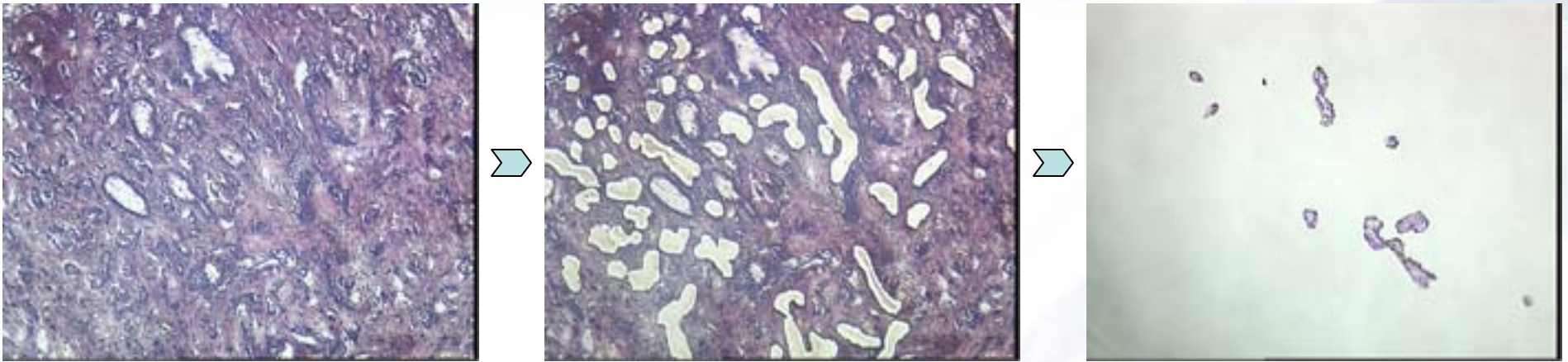
- Gross assessment does not accurately predict tumor cellularity and viability
- Major impediment to quantitative assays of molecular constituents



Target selection: Histology guided macrodissection



Target selection: Laser microdissection



- Several platforms & technologies
- Capture/propulsion/dissection of tissue from histologic section with direct microscopic visualization
 - Precise capture of target tissue
 - Very small sample size
- Laborious, cumbersome & finicky technique
 - Difficult to deploy in high throughput clinical setting with adequate cost-recovery



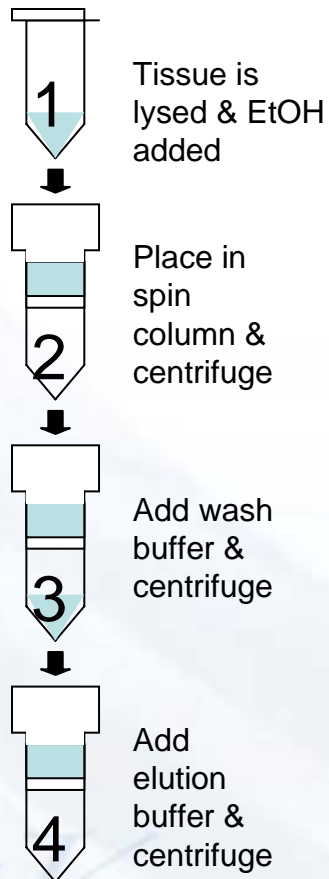
Pre-analytic parameters: Tissue heterogeneity

- Every tissue based molecular assay must in its development phase be tested for tolerances of “non-target” cells
 - Mixing experiments with tissue culture models
- Knowledge of these tolerances must be then applied in the sample input in the clinical testing phase



Patient Safety: Sample misidentification

Typical RNA isolation:
4 tube exchanges



- Many molecular techniques have attributes that can be sources of mislabeling error
 - Manually performed
 - Manual container labeling
 - Many container changes
 - Manual loading of gels/instruments
 - May be performed in hybrid research/clinical labs with varying standard operating procedures being performed in parallel



Education by the Experts

Patient Safety: Sample misidentification

- Remedies
 - Use robotic platforms whenever possible
 - Biomolecule extraction technology most robust at present time
 - Use computer-generated human-readable and bar coded labels whenever possible
 - Complexity of labeling software & hardware and variability in number and size of protocol containers are major obstacles
 - Quality assurance practices
 - Personnel training and retraining
 - Quality checks of protocol adherence



Quality issues in AP Molecular Diagnostics

- Many (most?) assays are:
 - newly emerging technologies
 - or are established research techniques being newly applied to anatomic pathology (emerging application)



Quality issues inherent to emerging technologies

- Protocols derived from research laboratories are focused on assay development
 - Manual
 - Often multi-step
 - Prone to being “operator-dependent”
 - Instrumentation tends to be “highly-tunable”, “tweakable”, hence complex
 - Emphasis is *not* on run-to-run reproducibility



Quality issues inherent to emerging technologies

- For the same analyte:
 - Different detection methodologies may be used by different laboratories
 - Even if the same basic detection method is used, protocols, reagents and instrumentation differ widely



Error elimination: QA/QC

- Quality Assurance (QA)
 - The policies, procedures and systematic actions established for the purpose of providing and maintaining a high quality of test integrity and accuracy.
 - Most standard QA procedures in place for clinical pathology laboratories apply to molecular diagnostics for anatomic pathology specimens
- Quality Control (QC)
 - Techniques that monitor performance parameters of individual assays
 - Are specific to the assays being performed



Typical QA/QC procedures

Pre-analytical Variables

- Test requests/ordering
- Patient identification
- Specimen acquisition
- Specimen transport
- Specimen processing
- Instrument maintenance

Documentation

- Standard Operating Procedures (SOPs)
- Assay logs
- Maintenance records
- Training records

Analytical Variables

- Competency
- Controls
- Methodology/ procedure validation
- Monitoring of equipment
- Monitoring of materials

Post-analytical Variables

- Result interpretation
- Result reporting



Error Reduction: QC/QA validation of analysis

- 1st-line (internal) controls
 - Check of results of each assay performed
 - e.g. “no template” negative PCR control
 - e.g. positive cell line DNA control for k-ras mutation assay
- 2nd-line controls (internal proficiency testing, quality monitoring)
 - Check on the spread of results produced in a lab
 - e.g. compare results of DNA isolation and microsatellite instability testing from adjacent sections of a colon cancer specimen
 - e.g. all technicians in a lab given known positive and negative samples of lung cancer for EGFR mutation analysis
 - Needs to be performed in validation-deployment phase of assay, and then periodically post-deployment



Error Reduction: QC/QA validation of analysis

- 3rd-line controls (external proficiency testing, quality monitoring)
 - essentially same as 2nd-line controls except performed with externally-validated materials
 - Often in context of accreditation
 - CAP is major source in U.S.A. for molecular genetic testing
 - Not available for all analytes



Error Reduction: Validation of analysis

- In the absence of validated reference materials from an accreditation agency:
 - Specimen sharing between collaborating laboratories
 - Development of external quality assessment (proficiency testing) materials by regional consortia groups



Summary

- Tissue handling procedures must be optimized for preservation of molecular targets
- Integrity of tissue specimens should be determined pre-analytically or concurrently
- Target selection or assessment by histologic analysis should be incorporated in most cases
- Protocol procedures should be optimized to prevent specimen misidentification
- A quality assurance program must be put in place to monitor assay performance and adherence to standard operating procedures



Pearls of Pathology

- Garbage in - garbage out
 - Molecular analysis of tissue *in the absence of histologic input* is fraught with problems of target cell validation
- Don't forget to take out the garbage
 - The rigorous practices of quality control and quality assurance that are well established in standard clinical pathology practice must be transferred to new molecular assessments of tissue specimens



Questions?

Relevant quotations from Yogi Berra:

If you ask me anything I don't know, I'm not going to answer.

There are some people who, if they don't already know, you can't tell 'em.

I never said most of the things I said.



It ain't over till it's over.
Yogi Berra

It's over!

Thank you for participating!

Please be sure to complete the course
evaluation online after the conference.



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