Molecular Pathology Using Paraffin Embedded Tissue and Cytological Samples

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Rapid increase in demand for tissue-based molecular diagnostics
Molecular Diagnostic Testing

Specimen Processing → Nucleic Acid Analysis → Target Detection
Handling of All Clinical Samples

• Observe universal precautions for biohazards.
• Use protective gowns, gloves, face and eye shields.
• Decontaminate all spills and work areas with 10% bleach.
• Dispose of all waste in appropriate biologic waste containers
• Gloves on - your RNA depends on it!
SPECIMEN TYPES

- Whole blood
- Bone marrow
- PBSC (Phoresis Product)
- Serum/plasma
- Buccal cells
- Cultured cells
- Blood spots
- Liquid cytology
- FNA

• BODY FLUIDS
  - CSF
  - BRONCHIAL LAVAGE
  - AMNIOTIC
  - SEMEN
  - URINE

• TISSUE
  - FRESH/FROZEN
  - PARAFFIN-EMBEDDED

• HAIR (SHAFT/ROOT)
“This structure has novel features which are of considerable biological interest”

Watson and Crick, 1953
The Double Helix

The Structure of DNA

- One helical turn = 3.4 nm
- Sugar-phosphate backbone
- Base
- Hydrogen bonds
Whole Blood Used To Be The Most Common Specimen Type

- **EDTA**
  - LAVENDER TOP VACUTAINER
  - PREFERRED SPECIMEN

- **ACD**
  - YELLOW TOP VACUTAINER
  - REQUIRED FOR RNA ANALYSIS

- **HEPARIN**
  - GREEN TOP VACUTAINER
  - INHIBITS SEVERAL MOLECULAR ENZYMES
DNA Isolation Methods

Method #1: Liquid Phase Organic Extraction

- Phenol (50):chloroform(49): isoamyl alcohol (1)
- Lysed samples mixed with above; 2 layers form
- Proteins remain at interface
- DNA is removed with top aqueous layer
- DNA is precipitated with alcohol and rehydrated

- Disadvantages: slow, labor-intensive, toxic (phenol, chloroform), fume hood required, disposal issues
DNA Isolation Methods

Method #2: Liquid Phase Non-Organic Salt Precipitation

- Cell membranes are lysed and proteins are denatured by detergent (such as SDS)
- RNA is removed with RNase (optional)
- Proteins are precipitated with salt solution
- DNA is precipitated with alcohol and rehydrated
- Advantages: fast, easy, non-toxic, no hood required, high-quality
PUREGENE® Purification Process

Step 1) Lyse RBCs

Step 2) Lyse WBCs
(+Opt. RNA Digest)

Step 3) Precipitate Proteins

Step 4) Precipitate DNA

Step 5) Wash

Step 6) Rehydrate DNA
Precipitated DNA is visible in 100% Isopropanol.
DNA Isolation Methods

Method #3: Solid Phase Procedures

- Solid support columns
  Fibrous or silica matrices bind DNA

- Magnetic beads
  DNA binds to beads; separated with magnet

- Chelating resins

- Advantages: fast, easy, no precipitation
Qiagen Biorobot EZ1
Add samples
Insert protocol card
Easy nucleic acid purification

Sample

Lysis

DNA binds to magnetic particles

Magnet

Magnetic separation

Wash

Magnetic separation

Elute

Pure nucleic acids
Separation
Tissue-based Molecular Diagnostic Testing

Pre-analytic  Analytic  Post-analytic

Qualitative  Quantitative  Therapeutic Genotyping
Laboratory Analysis of Human Tissue

Gross Description

Surgical resection versus Cytology specimen?
Gross Examination

1. Label or identification
2. Correct tissue or diseased tissue
3. Cross contamination
Sectioning of Tissue

1. Label or identification
2. Correct tissue or diseased tissue
3. Pre-fixation issues
Tissue Processing

1. Types of fixatives
2. Time of fixation
Tissue Embedding

1. Label or identification
2. Correct positioning of tissue
Tissue Cutting

1. Label or identification
2. Cross contamination
   - Blade or floaters
Slide Staining and Coverslipping
Pathologist Review
Formalin Fixed, Paraffin Embedded Tissues (FFPE)

- Can be used for most molecular testing
- Various thickness sections
- 3-6 10 uM sections
- Mercury or heavy metal fixatives not acceptable
- Unstained slides can be used for in situ applications or for macro- and micro-dissections
Fluorescence In Situ Hybridization (FISH)

HER2 Gene Amplification

1. Tissue fixation
2. Morphology
3. Permeabilization
4. Hybridization

EGFR Gene Amplification

N-myc Gene Amplification
Nucleic Acid Extraction from FFPE

- Requires deparaffinization (chemical, heat, physical)
- Amplification technologies allow for smaller targets and smaller specimen size
- Tissue can be scraped off of stained slides
- FFPE rolls can be used
1. For FFPE Rolls:
   a. Cut two 10 \( \mu \text{m} \) tissue sections
   b. The collected tissue sections are placed in a nuclease free microcentrifuge tube labeled with the patient's name, surgical number, corresponding cassette identifier, and amount of tissue collected.

2. For Unstained Slides (resected tissue, FNA, core biopsy):
   a. Cut five 4 \( \mu \text{m} \) thick tissue sections using a microtome
   b. Float tissue sections in a protein free water bath at 50\( ^\circ \text{C} \)
   c. Mount tissues sections onto charged slides labeled with the patient's name, surgical number, corresponding cassette identifier.
   d. One slide is then stained with Hematoxylin and Eosin (H&E) for determination of tumor content
   e. The H&E stained slide is reviewed by the attending pathologist to determine the region of the tissue to be macro-dissected for DNA isolation
From the SOP: DNA Extraction

*Paraffin Extraction Day 1-For FFPE Tissue Rolls*

1. Add 1.0 mL of Xylene to the microcentrifuge tube containing tissue rolls
2. Place samples on the Nutator mixer for 5-10 minutes; Vortex vigorously
3. Centrifuge samples at maximum speed for 3 minutes
4. Completely remove supernatant without disturbing the tissue
5. Repeat Xylene wash steps 1-5 until all paraffin is removed from the sample
6. Add 1.0 mL of absolute ethanol to the sample tube; Vortex vigorously
7. Centrifuge samples at maximum speed for 3 minutes
8. Completely remove supernatant without disturbing the pelleted tissue
9. Repeat absolute ethanol wash steps 7-10
10. Add 1.0 mL of 95% ethanol to the sample tube; Vortex vigorously
11. Centrifuge samples at maximum speed for 3 minutes
12. Completely remove supernatant without disturbing the tissue
13. Add 1.0 mL of Phosphate Buffered Saline (PBS) to the sample
If small tissue specimens are being processed, it is best to forgo the wash with PBS and instead, allow tissue to completely dry prior to the addition of Cell Lysis Solution
14. Vortex vigorously; Centrifuge samples at maximum speed for 3 minutes
15. Completely remove supernatant without disturbing the tissue
16. Briefly centrifuge tubes and pipette residual buffer from bottom of tube
17. Add 300 μL of Cell Lysis Solution and 10 μL of Proteinase K to the sample (May need to scale up the volume of Cell Lysis Solution and Proteinase K)
18. Completely seal sample tubes (O-ring seal or parafilm)
19. Place in a 55°C incubation oven on a Nutator mixer overnight
Paraffin Extraction Day 1-For Unstained Slides (resected tissue, FNA, core biopsy)

1. Etch the region to be macro-dissected previously indicated by the attending pathologist on the back side of each of the unstained slides
2. Place the slides in an appropriate slide rack
3. Immerse slide rack in Xylene at room temperature for 5 minutes
4. Repeat Xylene incubation an additional two times using new Xylene
   ***All paraffin must be removed before continuing.
5. Immerse slides in 100% Ethanol for 5 minutes at room temperature
6. Repeat 100% Ethanol incubation an additional two times using new Ethanol
7. Immerse slides in nuclease-free water at room temperature for 5 minutes
8. Using a Size 11 disposable scalpel, scrape the scribed area of tissue
9. The collected tissue is placed in a nuclease free microcentrifuge tube labeled with the patients name, surgical number, corresponding cassette identifier
10. Add 300 μL of Cell Lysis Solution and 10 μL of Proteinase K to the sample
    Adjust the volume of Cell Lysis Solution and Proteinase K with respect to total tissue in the sample
11. Completely seal sample tubes (O-ring or parafilm)
12. Place in a 55ºC incubation oven on a Nutator mixer overnight

From the SOP: DNA Extraction
Paraffin Extraction Day 2-For FFPE or Unstained Slides

1. Evaluate samples to determine if all tissue particulates have dissolved
   ***If cell lysate is not homogeneous add additional proteinase K and incubate at 55ºC for additional time until all tissue particulates have dissolved
2. Add 1.5 µL of RNase A solution to the lysate
3. Mix by inverting 25 times; Incubate lysate at 37ºC for 30 minutes
4. Cool sample to room temperature on ice for approximately 5 minutes
5. Add 100 µL protein precipitation solution to the lysate; Vortex vigorously for 20 seconds
   ***The protein precipitation solution must be mixed uniformly with the cell lysate before continuing
6. Chill the sample on ice for 5 minutes; Vortex vigorously for 20 seconds
7. Centrifuge at 13,000-16,000 RPM for 3 minutes (The precipitate proteins should form a tight pellet)
8. Pipette the supernatant into a clean microcentrifuge tube containing 300 µL of 100% isopropanol and 1.0 µL of glycogen at a concentration of 20 mg/mL (Completely thaw glycogen before use).
9. Mix the sample by vortexing; Centrifuge at 13,000-16,000 RPM for 5 minutes
10. Pipette off and discard the ethanol; quickspin and remove the remaining ethanol
11. Allow to air dry for ≥5 minutes; There should be no visible evidence of alcohol before rehydrating
12. Add 20-50 µL of DNA hydration solution to the cell pellet (final concentration = 100-300 µg/mL)
13. Allow DNA to rehydrate overnight or heat the tube to 65 ºC for 1 hour.
14. Quantify each sample using the Nanodrop-1000
Troubleshooting Nucleic Acid Preparation Methods

• Problem: No or low nucleic acid yield.
  – Make sure that ample time was allowed for resuspension or rehydration of sample.
  – Repeat isolation from any remaining original sample (adjust procedure for possible low cell number or poorly handled starting material).
  – Concentrate dilute nucleic acid using ethanol precipitation.
Troubleshooting Nucleic Acid Preparation Methods

• Problem: Poor nucleic acid quality
  – If sample is degraded, repeat isolation from remaining original sample, if possible.
  – If sample is contaminated with proteins or other substances, clean it up by re-isolating (improvement depends on the extraction procedure used).
The Molecular Touch Prep
Getting An Upfront Specimen

The mid-’80s and PA Training

- Southern blots were going to revolutionize the industry
- Plasmids were a threat to human life
- Surgery and chemo weren’t curing anybody
- The first oncogenes and tumor suppressor genes were identified
- Tumors were gigantic

New imaging modalities and diagnostic procedures are detecting cancers earlier.
Questions we should be asking:

• Surgical vs. cytologic specimens?

• What is the state of “molecular cytopathology”?

• What are the impediments to using cytology specimens?

• Is there a future and utility of FNA specimens for molecular diagnostics?
Surgical vs. Cytologic specimens
Two most common modalities for collecting tissue samples

- Surgical specimens typically are fixed and thus compromise the quality of nucleic acids via formalin induced cross-linking and fragmentation.

- FNA procedures are used for rapid, cost-effective and accurate diagnosis with reduced patient morbidity.

- Cytologic preparations represent an average of 10-20% of archival hospital specimens.

- Two standard preparations of FNA materials: Air-dried Diff-Quik, Alcohol fixed Pap (no formalin fixation, alcohol fixation preserves DNA).
Impediments to using Cytology Specimens

• FNA samples under-utilized for molecular profiling

• Cell numbers required for analysis (20-30) are compatible with typical FNA yields, but because of the intra-tumoral heterogeneity, sampling artifacts may occur.

• To avoid false negative results, sequencing-based tests may require up to 50-70% tumor cells in a sample.

• Preanalytical recommendations in report from Molecular Assay in NSCLS Working Group of ASCO:
  “cytology smears are not acceptable for IHC and FISH”
  “at least 3 representative areas should be assessed per tumor section”
  “tissue block preferred”
  “FFPE is standard”

• Poor quality smears, poor quality FNA harvests

• Past and current concept of adequacy

• Maintaining records of cytomorphology
Matched Diff-Quik and Pap-stained FNAs show critical differences in DNA preservation and integrity

- Pap-specific degradation: nearly completely degraded to <400bp fragments
- DQ DNA preservation: migrates as a smear with over half in the mid-high molecular weight range

Unexpected finding: presumed alcohol fixation in Pap stain process would better preserve the DNA

Hypothesized that the hematoxylin may have had a comprising effect on DNA amplification

***more likely the HCl acid step in the Pap-staining process is responsible for the above finding
HSV 1-2 PCR Gel

Utility of FNA Specimens for Molecular Diagnostics

Reasons for FNA specimens to receive greater attention in future studies:

• Relative tumor purity in FNA specimens versus tissue extracts

• FNA samples contain copious high quality gDNA suitable for high-resolution genomic and epigenomic profiling

• Excellent potential source of patient materials for clinical molecular profiling, including:
  - retrospective genomic analysis
  - prospective collection for individual therapy or eligibility review for clinical trial enrollment

• Potential enhancement for biomarker detection

• Abundance of archival FNA smears
What if............
DHMC Molecular Pathology Laboratory and Translational Research Program

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