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Towards High-quality Clinical Trials and
Implementation of Genomic Medicine

ATLAS Training Program

Course: Cancer Genome-based Medicine Course
Lecture Title : Sample Handling
Speaker : Taiki Hashimoto

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TAIKI HASHIMOTO

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■ Education

- 2016–2019 Juntendo University, graduate student
- 2004–2010 Kyoto University, medical student

■ Work Experience

- 2013–present National Cancer Center Hospital
- 2011–2013 Kyoto University Hospital
- 2010–2011 Kitano Hospital

■ Specialty and Research Field of Interest

- Diagnostic Pathology
- Molecular pathology of GI tract

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SAMPLE HANDLING

Guidelines on the Handling of Pathological Tissue Samples

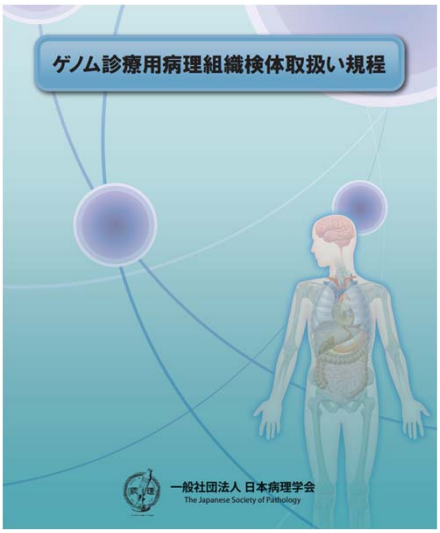
Pathology International

Pathology International 2018; **68:** 63–90 doi:10.1111/pin.12631

Special Article

The Japanese Society of Pathology Guidelines on the handling of pathological tissue samples for genomic research: Standard operating procedures based on empirical analyses

Kanai Y. *Pathol Int*. 2018



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Why is it important to manage sample handling?

- In genomic cancer medicine, NGS analysis is conducted using FFPE samples collected during routine medical practice.
- NGS analysis depends on the quality of the FFPE samples.
- Samples beyond a certain quality threshold are needed for molecular diagnosis.
- Sample quality is affected by various factors at the pre-analytical and analytical phases.

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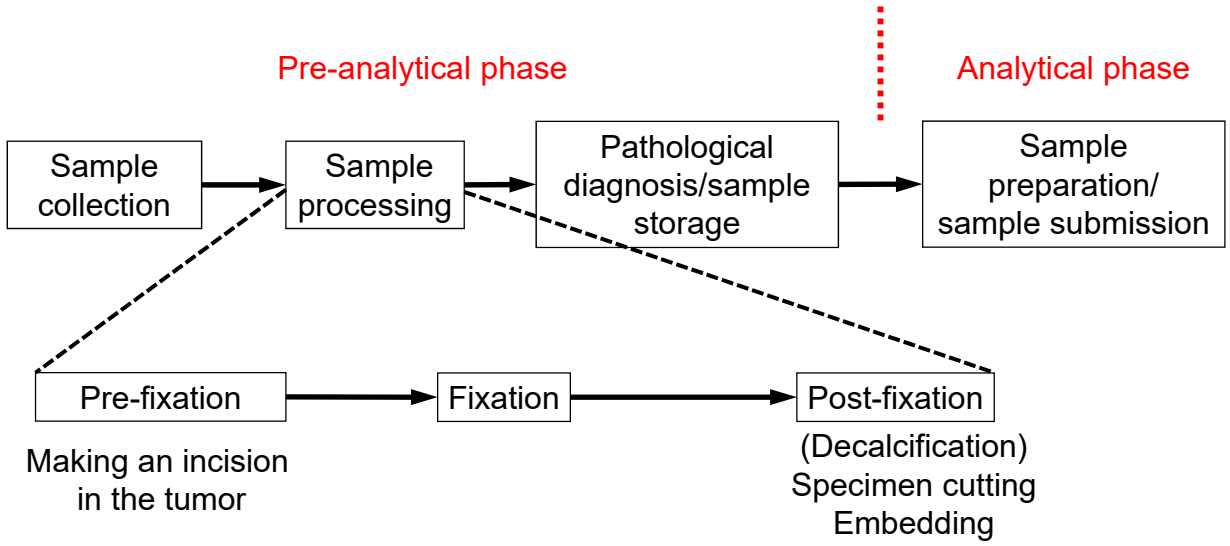
Sample Handling Process in Cancer Genomic Medicine



Pre-analytical Phase：Pre-fixation processing

1. Surgical specimens should be immediately stored in a refrigerator (4° C) and fixed within 1–3 hours.
2. Biopsy specimens should be fixed immediately.

The quicker, the better!!

Sample Storage Refrigerator (4° C)

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Pre-analytical Phase：Pre-fixation Processing

- Warm ischemia time
  Time from the interruption of the blood supply to the excision
- Cold ischemia time
  Time from excision to fixation
  - ➢ Both of these times affect sample quality.
  - ➢ The laboratory should make efforts to reduce the cold ischemia time.
  - ➢ As clinicians often perform the processing steps from sample excision to fixation, cooperation between the laboratory and clinician is important.

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Who fixes surgical samples?
Pathologists or surgeons?

Shorten the processing time according to the hospital system

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Pre-analytical Phase：Fixation process

1. 10% neutral-buffered formalin should be used as the formalin fixative.
2. The fixation time should be 6 to 48 hours
3. Under-fixation and over-fixation should be avoided
4. A 10-fold amount of fixative with respect to the amount of tissue should be used
5. Formalin fixation should be performed at room temperature

10% Neutral-Buffered Formalin (NBF) Solution

- NBF is prepared by adding sodium phosphate to formalin to adjust the pH to approximately 7.4.
- When formalin is stored for a long time, it degrades and produces formic acid.
- Addition of buffer prevents the acid from affecting the tissue.
- Fixatives with higher formalin concentrations improve tissue fixation and are suitable for morphological diagnosis, but 10% is preferable from the perspective of preserving DNA.

Use of 10% Neutral-Buffered Formalin is preferred when preparing samples for routine histological diagnosis.
This enables the samples to be used in genomic medicine as well.

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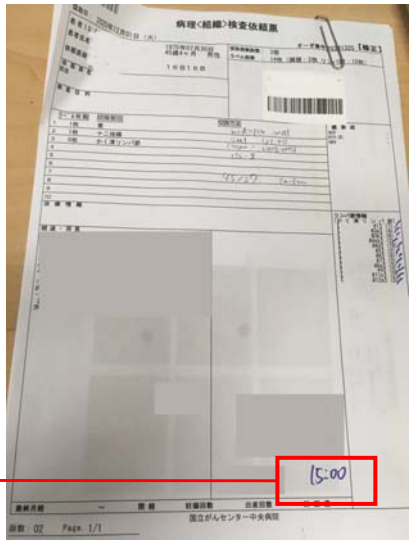
Fixation Time should be 6 to 48 Hours

- Both under-fixation and over-fixation reduce nucleic acid quality.
- Longer formalin fixation times induce G>T mutations (Williams C. *Am J Pathol*. 1999).
- Record the fixation start time if possible.



Starting fixation from 15:00

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Approaches For Maintaining Appropriate Fixation Times

Weekdays

Sample collection day	Next day
Sample collection and fixation 10:00–18:00	Specimen cutting and embedding from 10:00

16–24 hours

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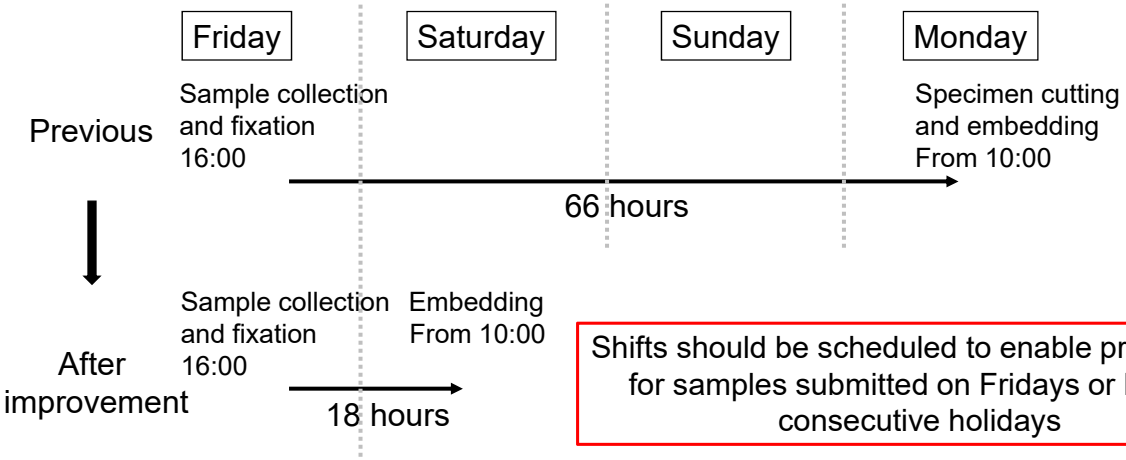
Approaches For Maintaining Appropriate Fixation Times

When procedures are performed on weekends or consecutive holidays



Shifts should be scheduled to enable processing for samples submitted on Fridays or before consecutive holidays

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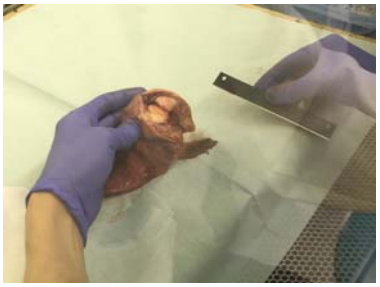
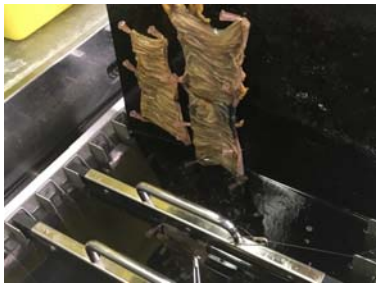
Appropriate Fixation Method

- Formalin penetration rate (1 mm/1 hour) (Srinivasan M. *Am J Pathol*. 2002).
- If necessary, make incisions in surgical samples before fixation.
- Fixation with formalin injection is also preferable.
- Use a sufficient amount of fixative (10-fold the amount of the tissue).





Pre-analytical Phase: Post-fixation Process

1. Close attention should be paid to contamination during specimen cutting and embedding.
2. Decalcification of samples containing hard tissue should be avoided; instead, EDTA decalcification should be performed.
3. FFPE blocks may be stored at room temperature but it is advisable to avoid high humidity and store the blocks in a cool, dark place.

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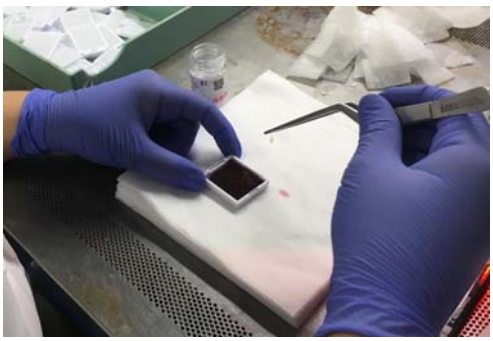
Cutting and Embedding While Avoiding Contamination

- Small sections of tissue that have adhered to the knife, scissors, tweezers, or cutting board may result in cross-contamination of patient samples.
- Wash and replace the instruments used for each patient specimen.



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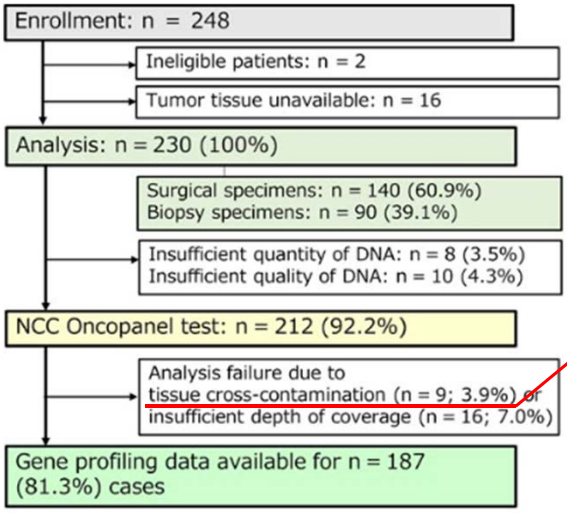
Contamination Adversely Affects NGS Analysis



DNA contamination from other individuals was detected in 3.9% of samples in the TOPGEAR project

Sunami K. *Cancer Sci*. 2019

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Acid Decalcification and EDTA Decalcification

- Acid decalcification results in nucleic acid fragmentation.
- EDTA decalcification takes time but does not significantly affect nucleic acid quality.
- For samples containing hard tissue, it may be possible to prepare sections without decalcification by collecting the tumor part separately.

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FFPE Block Storage

- Whether the storage temperature of FFPE blocks affects sample quality is controversial.
- Currently, it is thought that there are no major issues if the blocks are stored in a cool and dark place. (Avoid high temperature and high humidity)

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Analytical Phase

1. The pathologist should select a suitable FFPE block (tumor sample evaluation).
2. If multiple samples have been resected and collected at different times from the same patient, the most recent samples must be used.
3. Precautions must be taken to prevent cross-contamination of samples, such as by replacing the microtome blade for each sample during tissue sectioning.
4. HE slides and unstained slides should be prepared at the same time, the tumor volume and tumor ratio should be evaluated, and, if necessary, the region occupied by the tumor should be marked in the slide.

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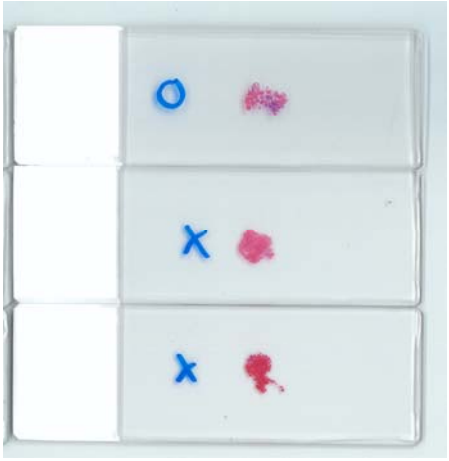
Tumor Sample Evaluation

- Tumor area ranges for reporting
  (<4 mm^2/4–16 mm^2/16–25 mm^2/≥25 mm^2)
- Tumor cell content
  (If macrodissection is performed, the amount after dissection)
- Incorrect tumor evaluation may result in untestable or false-negative results



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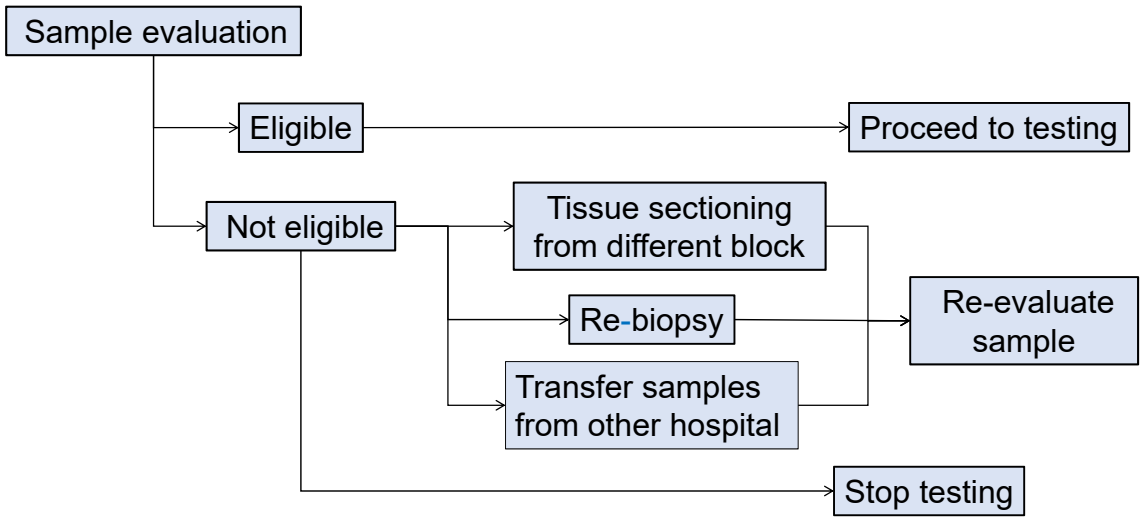
Tumor Sample Evaluation



Appropriate Block Selection

- Be cognizant of the sample requirements for the NGS panel tests.
- The best samples are as new as possible, contain numerous tumor cells, and have a high tumor cell content.

Example: Sample requirements for NGS panel test

	OncoGuide™ NCC Oncopanel System	FoundationOne®CDx Cancer Genomic Profile
Sample volume	5 μm × 10 slides (≥16 mm^2 is recommended, but ≥4 mm^2 is possible)	4–5 μm × 10 slides (generally ≥25 mm^2)
Tumor cell content	≥20%	≥30% is recommended (at least ≥20%)

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Appropriate FFPE Block Selection

- Formalin-induced DNA fragmentation continues after FFPE block preparation.
- The longer the block is stored, the lower the success rate of the test.
- The guidelines of Japanese Society of Pathology recommend the use of blocks within 3 years of preparation.
- Clinician should be consulted or re-biopsy should be considered if only old blocks remain.

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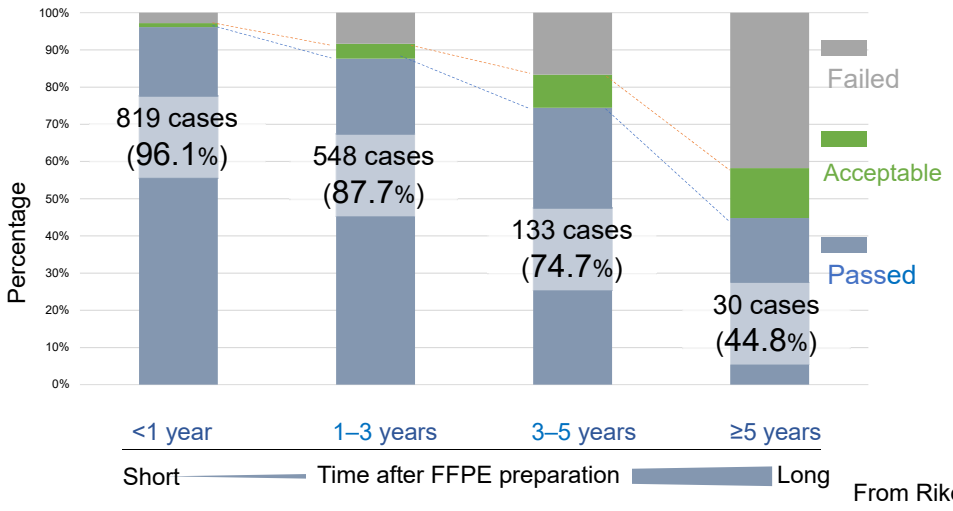
Time After FFPE Block Preparation and DNA Quality Pass Rate (NCC Oncopanel)



From Riken Genesis

Determining the Number of Tissue Sections

- 5 μm × 10 slides or 10 μm × 5 slides is common.
- If the number of tumor cells is insufficient, the number of tissue section slides should be added.
- The amount of DNA per one cell is approximately 6 pg.
- Generally, 10–500 ng of DNA is required for NGS panel testing.
- Approximately 2000 cells are needed to obtain 10 ng of DNA.

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Tissue Sectioning For Genomic Testing

- Wear a mask and gloves
- Replace the microtome blade for each sample
- Replace the water in the water bath for each case



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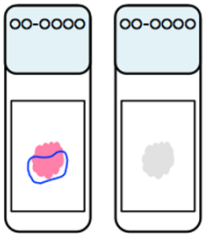
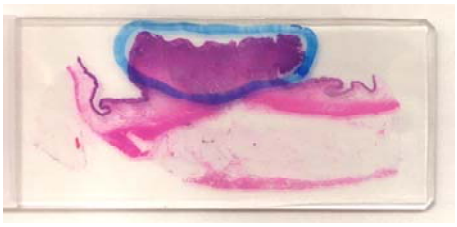
Checking the Slides and Marking the Tumor Area

- Prepare HE slides and unstained slides at the same time.
- Check that a sufficient number of tumor cells and tumor cell content can be assured.
- If necessary, mark the tumor area to ensure a sufficient tumor cell content (marking is not required for FoundationOne).





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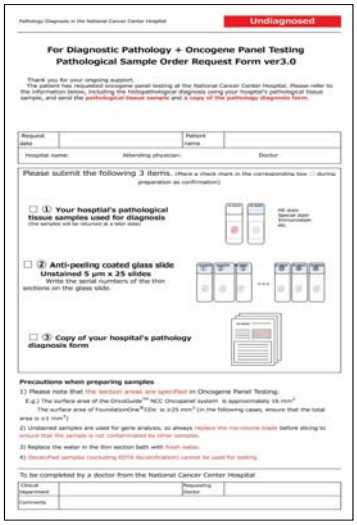
Transfer Samples from Other Hospitals

- It is necessary to request another hospital to select the appropriate block and make sections for genomic medicine.
- Create an easy-to-understand explanation sheet.


Undiagnosed

For Diagnostic Pathology + Oncogene Panel Testing
Pathological Sample Order Request Form ver3.0

Please submit the following 3 items.

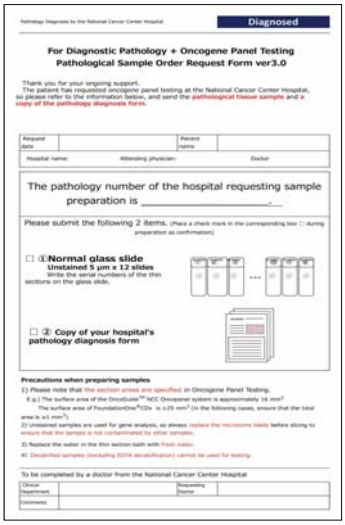
① Your hospital's pathological tissue samples used for diagnosis

② Anti-peeling coated glass slide
Unstained 5 μm × 25 slides

③ Copy of your hospital's pathology diagnosis form

Precautions when preparing samples

To be completed by a doctor from the National Cancer Center Hospital


Diagnosed

For Diagnostic Pathology + Oncogene Panel Testing
Pathological Sample Order Request Form ver3.0

The pathology number of the hospital requesting sample preparation is _____.

Please submit the following 2 items.

①Normal glass slide
Unstained 5 μm x 12 slides

② Copy of your hospital's pathology diagnosis form

Precautions when preparing samples

To be completed by a doctor from the National Cancer Center Hospital

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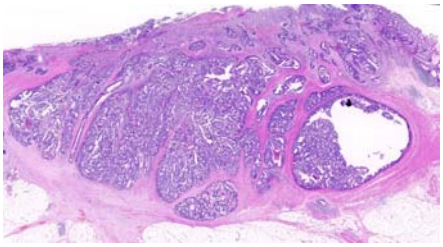
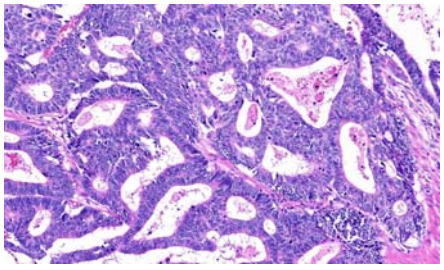
Examples of Tumor Evaluation #1





Surgical specimen of Colorectal Cancer

Diagnosis : Adenocarcinoma

Tumor cell content : 50% (after macrodissection)

Number of sections: 5 μm × 10 slides

DNA yield : 18.655 μg

ΔΔCq value : 0.36

- *APC* R216* (27.8%)
- *APC* Q1338* (25.6%)
- *TP53* R278H (46.9%)

Ensure that the dissection range of the surgical sample includes as little non-tumor tissue as possible, including necrosis, abscess, and lymphoid follicles.

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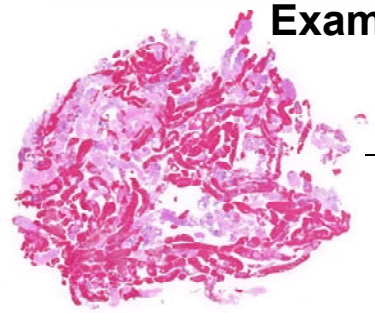
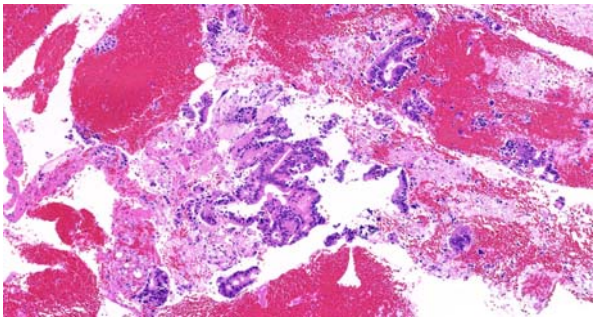
Examples of Tumor Evaluation #2





EUS−FNA sample of Pancreatic Cancer

Diagnosis : Adenocarcinoma

Tumor cell content : 30%

Number of sections: 5 μm × 15 slides

DNA yield : 1.41μg

ΔΔCq value : 0.00

- *RET* R67H (46.9%)
- *KRAS* G12V (31.7%)
- *BRCA2* N986fs*2 (13.8%)
- *TP53* C238fs*2 (41.4%)
- *SMAD4* Q245* (15.4%)

Small samples can be dealt with by increasing the number of tissue sections.

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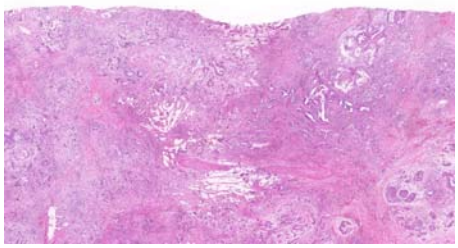
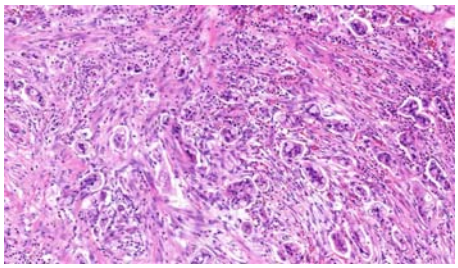
Examples of Tumor Evaluation #3





Surgical sample of Pancreatic Cancer

Diagnosis : Adenocarcinoma

Tumor cell content: < 20% (after macrodissection)

Sufficient tumor cell content could not be secured even after macrodissection because of prominent fibrosis and inflammatory cell infiltration.
Testing was discontinued.

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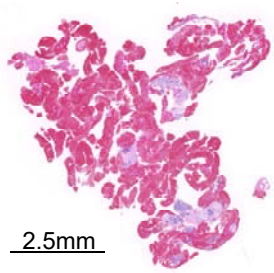
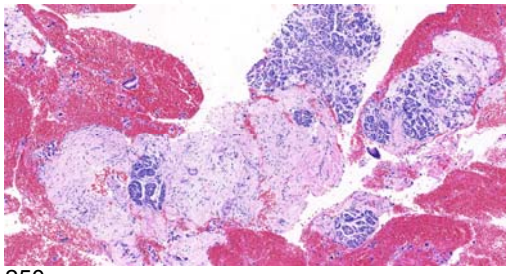
Examples of Tumor Evaluation #4





EUS-FNA sample of Pancreatic Cancer

Diagnosis : Adenocarcinoma

The sample was small, and the tumor cell content was low. Testing was discontinued.

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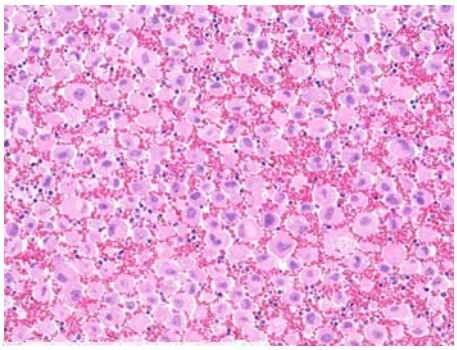
Examples of Tumor Evaluation #5



Lung Adenocarcinoma Pleural fluid Cell Block

Diagnosis	:	Adenocarcinoma
Tumor cell content	:	60%
Number of sections	:	5 μm × 15 slides
DNA yield	:	0.84 μg
ΔΔCq value	:	–0.41

- *EGFR* E746_A750delELREA (22.5%)
- *TP53* M237K (43.6%)

If the specimen is large and has sufficient tumor cell content, even body cavity fluid cell block samples can be used for testing.

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Tumor Sample Evaluation (Tumor Cell Content)

- Tumor cell content tends to be overestimated by pathologists who are unfamiliar with evaluation *Smith AJ. Mod Pathol. 2014*
- NCC Oncopanel will yield negative results if the variant allele frequency (VAF) is less than 5%.
- Overestimation produces “false-negative results”.
- The correct evaluation of the tumor cell content is one of the most important jobs of pathologists in genomic medicine.
- This ability can be improved by using training sets.

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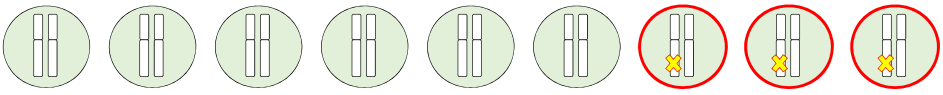
Checking Tumor Cell Content Evaluation

- The true value of tumor cell content can be estimated based on the VAF results of panel testing.

Example

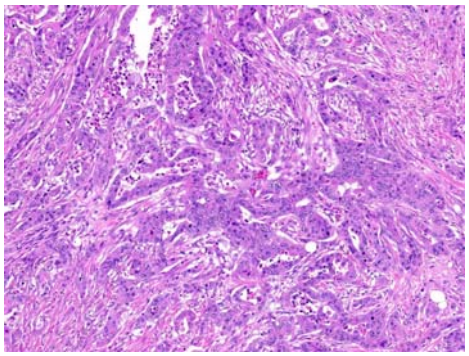
Tumor cell content: 30%



Variant allele frequency = 15%

- If the mutation is activating type and is present in only one allele, the VAF will be half the tumor cell content.
- Calculations are difficult with samples containing gene amplification such as *EGFR* and some tumor suppressor genes.

Example of Tumor Cell Content Evaluation



Surgical sample of Pancreatic Cancer

Diagnosis : Adenocarcinoma
Tumor cell content : 20%

NGS analysis results

- *KRAS* c.35G>A (12.4%)
- *TP53* c.375+1G>A (16.2%)

KRAS (c.35G>A) is an activated driver mutation

The true tumor cell content is 24.8%, as predicted from the *KRAS* variant allele frequency of 12.4%

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Summary

- It is essential to perform routine operations while considering that the samples may be used not only for histological diagnosis, but also for genomic medicine.
- Each individual point is a small point to note, but it is important to perform all tasks properly and to continue these practices.
- Do not deprive patients of treatment opportunities due to inappropriate sample handling.

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References

1. Kanai Y, Nishihara H, Miyagi Y, et al. The Japanese Society of Pathology Guidelines on The Handling of Pathological Tissue Samples For Genomic Research: Standard Operating Procedures Based on Empirical Analyses. *Pathol Int.* 2018;68:63-90.

2. Williams C, Pontén F, Moberg C, et al. A High Frequency of Sequence Alterations is Due To Formalin Fixation of Archival Specimens. *American J Pathol.* 1999;155:1467-1471.

3. Srinivasan M, Sedmak D, Jewell S. Effect of Fixatives And Tissue Processing on The Content And Integrity of Nucleic Acids. *Am J Pathol.* 2002;161:1961-1971.

4. Sunami K, Ichikawa H, Kubo T, et al. Feasibility And Utility of A Panel Testing For 114 Cancer-associated Genes in A Clinical Setting: A Hospital-based Study. *Cancer Sci.* 2019;110:1480-1490.

5. Smits AJ, Kummer JA, de Bruin PC, et al. The Estimation of Tumor Cell Percentage For Molecular Testing By Pathologists is Not Accurate. *Mod Pathol.* 2014;27:168-174.