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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

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The original version of "The Japanese Society of Pathology (JSP) Guidelines on the Handling of Pathological Tissue Samples for Genomic Research" appeared in Japanese as *Genome Kenkyu-yo Byori Soshiki Kentai Toriatsukai Kitei* from the JSP, Tokyo, in 2016 (http://pathology.or.jp/genome/). In addition to two parts that appeared in this special article, the Japanese version of "The JSP Guidelines on the Handling of Pathological Tissue Samples for Genomic Research" includes a part pertaining to "Appropriate Sites for Sample Collection in Surgically Resected Materials". Two parts of the Guidelines based on our empirical analyses, i.e., "Part 1: Appropriate methods for collection, storage, and transport of frozen tissue samples" and "Part 2: Appropriate methods for preparation and storage of formalin-fixed paraffin-embedded (FFPE) specimens", are here incorporated into this English-language article.

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Genome research using appropriately collected pathological tissue samples is expected to yield breakthroughs in the development of biomarkers and identification of therapeutic targets for diseases such as cancers. In this connection, the Japanese Society of Pathology (JSP) has developed "The JSP Guidelines on the Handling of Pathological Tissue Samples for Genomic Research" based on an abundance of data from empirical analyses of tissue samples collected and stored under various conditions. Tissue samples should be collected from appropriate sites within surgically resected specimens, without disturbing the features on which pathological diagnosis is based, while avoiding bleeding or necrotic foci. They should be collected as soon as possible after resection: at the latest within about 3 h of storage at 4°C. Preferably, snap-frozen samples should be stored in liquid nitrogen (about −180°C) until use. When intending to use genomic DNA extracted from formalin-fixed paraffin-embedded tissue, 10% neutral buffered formalin should be used. Insufficient fixation and overfixation must both be avoided. We hope that pathologists, clinicians, clinical laboratory technicians and biobank operators will come to master the handling of pathological tissue samples based on the standard operating procedures in these Guidelines to yield results that will assist in the realization of genomic medicine.

Key words: biobank, empirical analysis, genome research, pathological tissue sample, sample collection and storage

At the present time, which has seen rapid strides in the development of omics analysis technologies, such as genome analysis,[1,2] it is expected that data-driven studies based on analysis of clinical samples will help clarify the molecular mechanisms responsible for the occurrence, progression and treatment responsiveness of diseases, thereby leading to the development of new biomarkers for diagnosis and identification of drug discovery targets.[3] In particular, analysis of pathological tissue samples collected from the exact site of a disease, such as cancer, will be indispensable for the realization of genomic medicine.[4–12]

Besides detailed and accurate clinicopathological information about pathological tissue samples, the quality of the tissue samples itself holds the key to the success of such data-driven studies.[13,14] Appropriate collection and storage of pathological tissue samples enables reliable and high-quality analyses that can contribute to prevention and treatment of diseases.[15] To make appropriate pathological tissue samples available to a wide range of medical researchers, there have been active movements toward the development and management of biobanks at various institutions.[16] On the other hand, not uncommonly, researchers may be confounded by uninterpretable artifacts that are encountered during analysis of samples of varying quality because of inappropriate collection and storage methods,[17] and biobank operators do not have access to proper means of maintaining sample quality, despite a high level of motivation. In this connection, the Japanese Society of Pathology (JSP) has developed "The JSP Guidelines on the Handling of Pathological Tissue Samples for Genomic Research" (referred to hereafter as "the Guidelines") to facilitate the collection of adequate amounts of high-quality pathological tissue samples suitable for omics studies, including genome research.[18]

This paper includes two parts of the Guidelines, "Part 1: Appropriate methods for collection, storage, and transport of frozen tissue samples" and "Part 2: Appropriate methods for preparation and storage of formalin-fixed paraffin-embedded (FFPE) specimens", which have been compiled on the basis of abundant data obtained from empirical analyses of tissue samples actually collected and stored under various conditions. The authors, who belong to the "Editorial Committee on The Guidelines for Handling of Pathological Tissue Samples for Genomic Research" (Chair: Yae Kanai) of the JSP, and who are engaged in the development and management of biobanks at their own institutions, have performed empirical analyses in order to develop the two parts of the Guideline. Such analyses were approved by the Ethics Committees of the National Cancer Center, Tokyo, Keio University School of Medicine, Hokkaido University Graduate School of Medicine, Kanagawa Cancer Center Research Institute, Kyoto University Graduate School of Medicine, and Tokyo Medical and Dental University, and were performed in accordance with the Declaration of Helsinki. All of the patients concerned provided written informed consent prior to inclusion in the study.

In order to make the Guidelines widely applicable to genomic studies, they were reviewed and approved by the "Advisory Council for Developing the Guidelines on the Handling of Pathological Tissue Samples for Genomic Research" (Chair: Hitoshi Nakagama), consisting of representatives from BioBank Japan (BBJ),[19] the National Center Biobank Network (NCBN),[20] the National Hospital Organization (NHO), the Japan Clinical Oncology Group (JCOG),[21] the Japan Children's Cancer Group (JCCG), and the Japanese Cancer Association (JCA).[22]

The Guidelines provide practical descriptions of optimized procedures for the collection and storage of pathological tissue samples. We believe that, by reference to these empirical analysis data, readers will be able to choose the methods most suited to the actual situations at their respective institutions. We hope that pathologists, clinicians, clinical laboratory technicians and biobank operators will come to master the handling of pathological tissue samples. We also expect that researchers will come to understand the labor that goes into the collection of reliable pathological tissue samples and acquire good knowledge of the characteristic features of each sample before starting their analysis. It is our desire that the Guidelines are taken fully into account when conducting data-driven studies, in order to contribute to the realization of genomic medicine.

PART 1: APPROPRIATE METHODS FOR COLLECTION, STORAGE, AND TRANSPORT OF FROZEN TISSUE SAMPLES

The information in this section is intended to minimize the degeneration of DNA, RNA and proteins in frozen samples, to enable long-term storage and maintenance of tissue samples of high quality, and to maximize the versatility of such samples to allow their use in various analyses in the future. Explanatory notes are as follows:

- (E) means "issues that cannot be deemed essential because of excessive workload, although high quality better than (A) could be expected";
- (A) means "recommended issues";
- (B) means "recommended issues when (A) issues are not feasible"; and
- (N) "issues that should be avoided".

Collection of pathological tissue samples

1.1. Tissue samples to be used for genomic studies should be collected from appropriate sites within the surgical specimens, without disturbing the features on which pathological diagnosis

is based (without posing any disadvantage to the patient), while avoiding bleeding or necrotic foci where degeneration of nucleic acids and proteins can be expected (A).

Note: In cases of broad/comprehensive consent, it seems common policy for biopsy specimens not to be stored for biobanking after diagnosis and treatment. Therefore, the handling process for tissue samples collected from surgical specimens for genomic studies are first described in this section.

Note: In cases of individual consent, the use of biopsy specimens for genomic studies should conform to the rules in and after item 16 in Part 1, *"Snap freezing"*.

1.2. From patients with cancer, both cancerous and non-cancerous tissue (appropriate control tissue) should be collected (A).

Note: In general, cancerous tissue is heterogeneous in nature. Particularly, if macroscopic heterogeneity is evident, it is desirable to collect tissue specimens from multiple sites within the cancerous lesion (E).

1.3. A description of the site of collection, e.g., lesser curvature of the gastric body or segment X of the liver, should be given (A).

Note: It is also desirable to facilitate conveyance of information about the site of collection to the researchers by some means, including an indication of the approximate site of collection in a photo or schema, and recording of the block number prepared from an adjacent part for routine pathological diagnosis (E).

Note: When tissue is collected from multiple sites within a lesion, it is also desirable to describe the noteworthy macroscopic features in writing (E).

Person in charge of sample collection

1.4. In the case of surgical specimens, it is preferable for a board-certified pathologist to determine the propriety of tissue sample collection, the appropriate site of collection, and the appropriate amount of tissue to be collected (E). Alternatively, clinicians with relevant expertise who are familiar with the pathological diagnosis and possesses adequate ability for macroscopic pathological diagnosis may also make the judgment (A).

Note: About 2400 board-certified pathologists, who have passed qualification examinations consisting of a written examination, practical examination and an interview, were registered in JSP as of October, 2017. Before taking the qualification examination, each applicant has to obtain a Japanese doctor's license and complete two years of clinical training as well as three years of pathological training (histopathological diagnosis of more than 5000 cases, more than 30 autopsies, attendance at assigned seminars, and publication of papers focusing on human pathology).

1.5. Any pathology residents, clinical training physicians, clinical laboratory technicians or other biobank operators who are assigned responsibility for tissue collection should have appropriate training about suitable collection sites, as described in item 1.1 above, and should always collect samples under the supervision of a board-certified pathologist or clinician in the relevant specialty who is familiar with pathological diagnosis (B).

Time of collection

1.6. Tissue samples for use in genomic research should be collected from the surgical specimen as soon as possible after resection (A) (Figs. 1, 2 and Tables 1, 2).

Note: Keeping surgical specimens at room temperature for prolonged periods (more than 30 min) should be avoided (N) (Figs. 1, 2 and Tables 1, 2).

1.7. If the samples cannot be collected quickly, the surgical specimen should be kept at 4°C in a refrigerator. Tissue samples should be collected within about 3 h of storage at 4°C (B) (Figs. 1, 2 and Tables 1, 2).

Note: The quality of nucleic acids may depend on the techniques used by the researcher receiving the samples from the biobank rather than on the conditions of collection and storage at the biobank (Fig. 3).

1.8. When samples are provided from biobanks to researchers, the standard operating procedure (SOP), including the time and temperature at the institutions, should be disclosed (A). If there is deviation from the SOP at any institution, records of deviations in time, temperature and/or other items should be included in the sample-associated information (A).

Note: It is desirable to accurately record the time of sample collection or the time required for sampling after resection and the temperature of the specimen until sampling (E). However, as long as samples are collected according to the institutional SOP, the quality of the sample is predictable.

Note: The workload for recording the duration of intraoperative ischemia by reviewing the patient's medical records (E) is excessive, considering its relevance. However, it is desirable that a system for disclosing the duration of intraoperative ischemia be available upon request from researchers conducting studies whose results are influenced by the ischemia time (A).

Sample collection quantity

1.9. The quantity of the samples to be collected should be judged by a board-certified pathologist or clinician in the relevant specialty who is familiar with pathological diagnosis, in order to avoid any pitfalls in such diagnosis, as

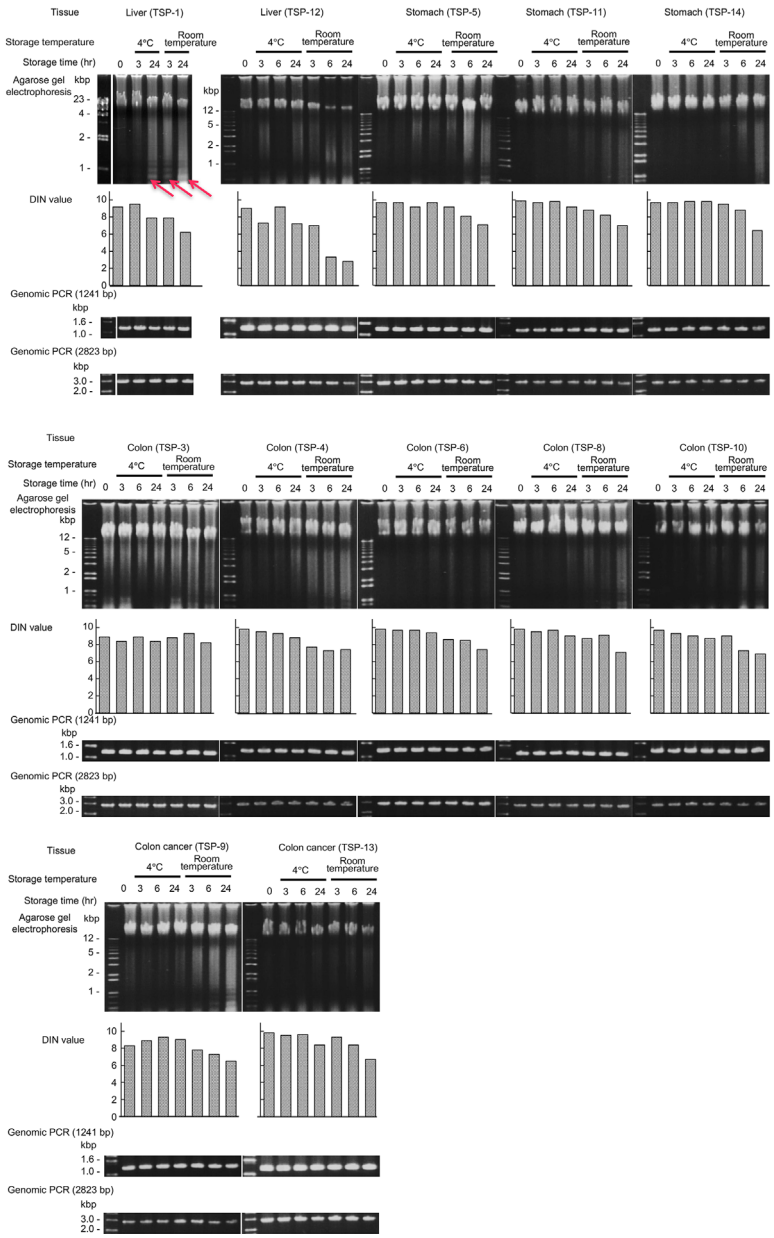

Figure 1 Effects of time until snap freezing and storage temperature on the quality of genomic DNA. Using tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous and cancerous parts) of a surgical specimen from the same patient, with varying times until snap freezing and storage temperatures, genomic DNA was extracted by the phenol-chloroform method to compare the quality of the genomic DNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen immediately after surgical resection; #2, Snap freezing in liquid nitrogen after 3 h of storage at 4°C; #3, Snap freezing in liquid nitrogen after 6 h of storage at 4°C; #4, Snap freezing in liquid nitrogen after 24 h of storage at 4°C; #5, Snap freezing in liquid nitrogen after 3 h of storage at room temperature; #6, Snap freezing in liquid nitrogen after 6 h of storage at room temperature; #7, Snap freezing in liquid nitrogen after 24 h of storage at room temperature. TSP, tissue sample for the Japanese Society of Pathology. The quality of the DNA was assessed by agarose gel electrophoresis, in terms of the DNA integrity number (DIN) measured in a 2200 TapeStation system (Agilent, Santa Clara, CA, USA), and by genomic PCR with amplification lengths of 1241 bp and 2823 bp. With any processing procedure, PCR amplification was possible for DNA fragments of 2823 bp, but degradation of genomic DNA was evident on agarose gel in cases of liver TSP-1 stored for many hours at 4°C or stored at room temperature (red arrows). There was a marked decrease in the DIN value in liver cases TSP-1 and TSP-12 with prolonged time until snap freezing and increased storage temperature. The effects of prolonged storage at 4°C and storage at room temperature on the quality of the DNA were generally milder in samples obtained from the gastrointestinal tract than in those from the liver. However, analysis of all the samples examined also showed a significant decrease in the DIN value following storage for 24 h at 4°C and storage at room temperature (Table 1). The specimens should be submitted for snap freezing as quickly as possible after surgical resection, and even if the specimen cannot be processed immediately, it is desirable that snap freezing be performed after storage for the shortest period of time at 4°C. The data in this figure suggest that storage for 6 h at 4°C is permissible. However, it is concluded that snap freezing should be conducted within about 3 h of storage at 4°C, because the quality of the RNA from some organs deteriorates after storage for 6 h at 4°C, as shown in Fig. 2.

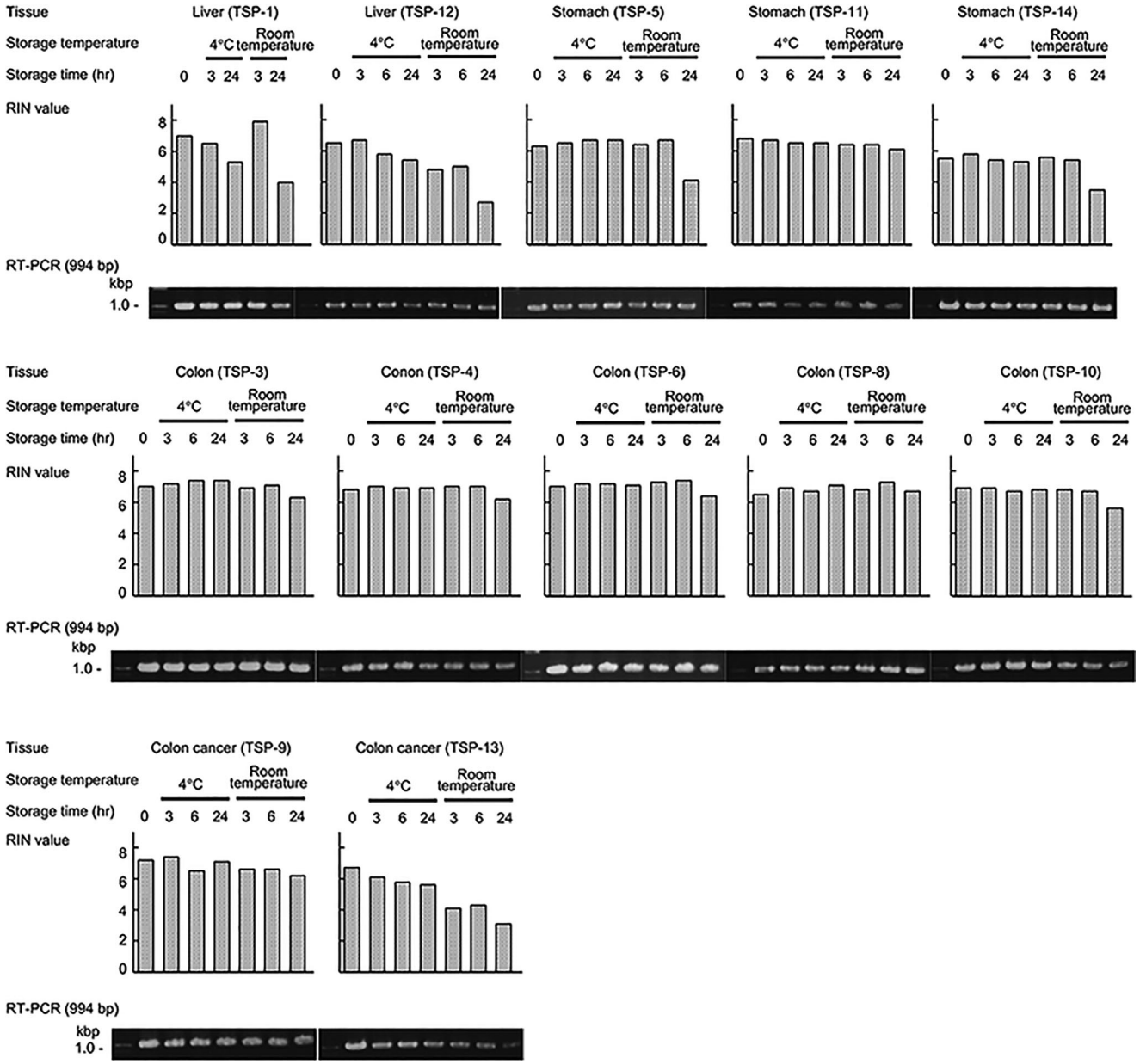

Figure 2 Effects of time until snap freezing and storage temperature on RNA quality. Using tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous and cancerous parts) of a surgical specimen from the same patient, with varying times until snap freezing and storage temperatures, total RNA was extracted using TRIzol (Thermo Fisher, Waltham, MA) to compare the quality of the RNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen immediately after surgical resection; #2, Snap freezing in liquid nitrogen after 3 h of storage at 4°C; #3, Snap freezing in liquid nitrogen after 6 h of storage at 4°C; #4, Snap freezing in liquid nitrogen after 24 h of storage at 4°C; #5, Snap freezing in liquid nitrogen after 3 h of storage at room temperature; #6, Snap freezing in liquid nitrogen after 6 h of storage at room temperature; #7, Snap freezing in liquid nitrogen after 24 h of storage at room temperature. RNA quality was assessed in terms of the RNA integrity number (RIN) measured in a 2100 Bioanalyzer system (Agilent), and reverse transcription (RT)-PCR with an amplification length of 994 bp. It was shown that there were variations in the effects of time until freezing and storage temperature on the quality of RNA among samples obtained from different organs. According to our analysis, the effects of prolonged storage at 4°C and storage at room temperature on RNA quality were generally milder in samples obtained from the gastrointestinal tract, particularly gastric mucosa, than in those obtained from the liver. Therefore, when all the samples examined were analyzed, the decrease in the RIN value was not statistically significant, unless the samples had been stored for many hours at room temperature (Table 2). However, for liver cases TSP-1 and TSP-12, there was a marked decrease in the RIN value with prolonged time until snap freezing and increased storage temperature. In the cases liver TSP-12 and colon TSP-9, etc., the RIN value began to decrease after 6 h of storage at 4°C. It is concluded that the processing should be conducted as quickly as possible, and even if the specimen cannot be processed immediately, snap freezing should be conducted within 3 h of storage at 4°C.

Table 1 Effects of time until snap freezing and storage temperature on the quality of genomic DNA

Procedure	Number of analyzed tissue specimens†	Yielded genomic DNA (μg)	A_{260}/A_{280}	DNA integrity number: Mean ± SD	DNA integrity number: *P*-value against #1 (Welch-*t* test)*
#1 Snap freezing in liquid nitrogen immediately after surgical resection	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#2 Snap freezing in liquid nitrogen after 3 h of storage at 4°C	12	206.5 ± 173.2	1.88 ± 0.04	9.2 ± 0.7	3.50×10^{-1}
#3 Snap freezing in liquid nitrogen after 6 h of storage at 4°C	11	196.0 ± 90.3	1.87 ± 0.03	9.4 ± 0.3	7.49×10^{-1}
#4 Snap freezing in liquid nitrogen after 24 h of storage at 4°C	12	197.1 ± 117.5	1.87 ± 0.03	8.8 ± 0.7	$\underline{1.62 \times 10^{-2}}$
#5 Snap freezing in liquid nitrogen after 3 h of storage at room temperature	12	195.2 ± 106.0	1.89 ± 0.02	8.5 ± 0.8	$\underline{1.65 \times 10^{-3}}$
#6 Snap freezing in liquid nitrogen after 6 h of storage at room temperature	11	164.8 ± 174.2	1.88 ± 0.05	7.8 ± 1.6	$\underline{2.77 \times 10^{-3}}$
#7 Snap freezing in liquid nitrogen after 24 h of storage at room temperature	12	192.8 ± 105.6	1.88 ± 0.03	6.6 ± 1.3	$\underline{6.00 \times 10^{-7}}$

*Cases with $P < 0.05$ are underlined. Also see Fig. 1.
†Detailed description of analyzed tissue specimens is shown in the legend for Fig. 1.

specified in item 1.4. However, if there is no particular difficulty with pathological diagnosis, and an appropriate site of collection is available, it is appropriate to collect tissue samples about half the size of a little fingertip (about 1 × 0.5 × 0.3 cm, 50–100 mg) (A).

Note: Even very small tissue samples may allow useful analysis. Therefore, there is no need to hesitate about collecting a sample simply because the amount of tissue involved is smaller than half a little fingertip in size (B).

Note: The weighing procedure has a disadvantage in that it prolongs the time until snap freezing. Weighing the whole collected tissue may not necessarily be useful for researchers, because cut tissue fragments are given to researchers, and weighing each of these would further prolong the time until snap freezing. If analysis that requires normalization by the wet weight of a tissue sample or other processing procedures that employ wet weight is highly likely, it is desirable to measure the wet weight of the tissue sample using an accurate measuring instrument (E).

Tissue processing

1.10. The tissue collected should be cut into pieces 2–3 mm square (A).

Note: It is indispensable to cut the tissue into pieces before snap freezing in order to prevent repeated freezing and thawing of any tissue other than that to be used, and to

Table 2 Effects of time until snap freezing and storage temperature on the quality of RNA

Procedure	Number of analyzed tissue specimens†	Yielded total RNA (μg)	A_{260}/A_{280}	RNA integrity number: Mean ± SD	RNA integrity number: *P*-value against #1 (Welch-*t* test)*
#1 Snap freezing in liquid nitrogen immediately after surgical resection	12	118.3 ± 53.0	2.10 ± 0.07	6.7 ± 0.5	–
#2 Snap freezing in liquid nitrogen after 3 h of storage at 4°C	12	98.4 ± 49.0	2.13 ± 0.14	6.7 ± 0.5	7.59×10^{-1}
#3 Snap freezing in liquid nitrogen after 6 h of storage at 4°C	11	91.5 ± 36.7	2.12 ± 0.06	6.5 ± 0.6	4.45×10^{-1}
#4 Snap freezing in liquid nitrogen after 24 h of storage at 4°C	12	101.1 ± 41.0	2.10 ± 0.07	6.4 ± 0.8	3.59×10^{-1}
#5 Snap freezing in liquid nitrogen after 3 h of storage at room temperature	12	102.6 ± 47.8	2.11 ± 0.06	6.2 ± 1.0	1.29×10^{-1}
#6 Snap freezing in liquid nitrogen after 6 h of storage at room temperature	11	85.3 ± 45.1	2.12 ± 0.14	6.4 ± 1.0	3.53×10^{-1}
#7 Snap freezing in liquid nitrogen after 24 h of storage at room temperature	12	81.1 ± 42.7	2.11 ± 0.08	5.1 ± 1.5	$\underline{3.12 \times 10^{-3}}$

*Cases with $P < 0.05$ are underlined. Also see Fig. 2.
†Detailed description of analyzed tissue specimens is shown in the legend for Fig. 2.

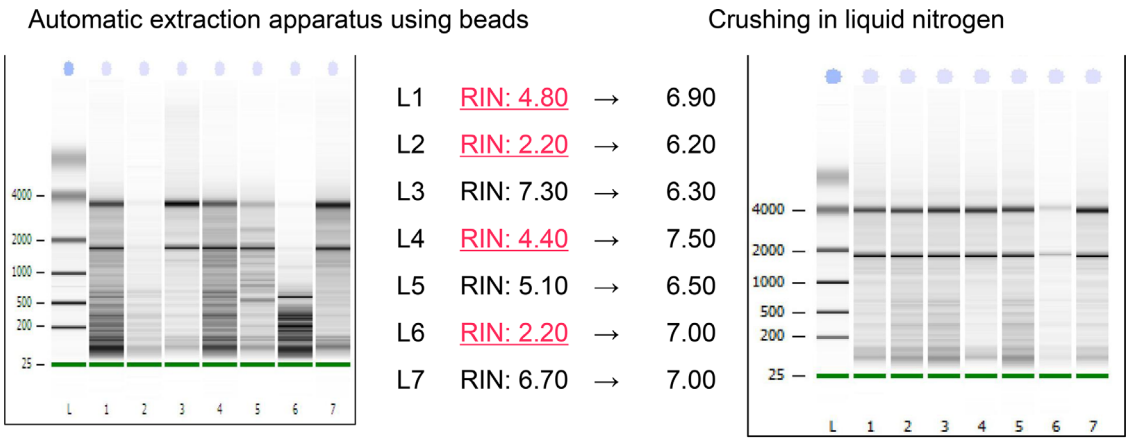

Figure 3 Effects of the extraction technique used on RNA quality. Using 7 groups of tissue specimens of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous part) of surgical specimens (various organs) from the same patient, RNA was extracted using different methods after storage under the same conditions (–80°C) to compare the quality of the RNA. The quality of RNA was assessed by RNA integrity number (RIN) measurement in a 2200 TapeStation system. Even for the same samples stored under the same conditions, the results varied greatly according to the method of extraction. Crushing of the sample in liquid nitrogen without thawing yielded higher-quality RNA, even in samples L1, L2, L4 and L6, in which low-quality RNA was provided by an automatic extraction apparatus using beads. The relevance of the quality of the RNA finally obtained may depend on the procedure that has been employed by the researcher, rather than the conditions of sample collection or storage at the biobank. If necessary, biobank operators should promote awareness about the proper choice of the procedure by the user. Biobanks that extract RNA by themselves for provision to researchers must also choose the most appropriate procedures.

rapidly conduct high-quality analysis while preventing activation of nucleases. (A).

1.11. Preferably, one cubical 2–3 mm tissue sample should be contained in one tube (E). When there is a limitation on the availability of storage containers, more than one tissue piece may be accommodated in one tube, while ensuring that the pieces are adherent to the inner wall of the tube and separate from one another (A).

Note: If the tissue pieces adhere to the inner wall of the tube separately from one another, the necessary number of individual pieces can be removed quickly with forceps, before the temperature of the tube containing the remaining tissue pieces increases.

Note: If there is more than one tissue piece per tube, it may be easier to use 1.5 mL to 2 mL volume tubes (A), but this is not the case when each tube contains only one piece of tissue.

1.12. Ultralow-temperature-resistant tubes should be used (A). Also, when writing or labeling the anonymized number or other marks on the tube, an ultralow-temperature-resistant marker or label should be used (A).

Note: It is desirable for tubes, markers and labels to be actually tested in advance for their ultralow temperature resistance at the storage facility in each institution (E).

Note: Depending on the management system at each institution, the use of tubes on which bar codes or other codes of anonymized numbers are printed is also recommended (E).

1.13. In order to prepare for unexpected temperature alterations (to prevent accidents due to expansion of liquid nitrogen), tubes with screw caps should be used (A).

1.14. Several tubes of samples described in item 1.11 should be prepared for each tissue collection site (for example cancerous and non-cancerous parts) (A).

Note: Even when more than one piece of tissue is contained in one tube, several tubes of samples should always be prepared, considering the convenience of storage at several places or provision to several researchers (A).

Note: If consensus is obtained from each institution, or particularly when storage of high-quality samples in a liquid nitrogen container is feasible, it is desirable to store some of the multiple sample tubes for a long period (e.g., more than 10 years) for future use in research using advanced analytical techniques (E).

1.15. A number of nucleic acid stabilization/preservation agents are commercially available, and tissue pieces are frequently immersed in a nucleic acid stabilization/preservation agent in tubes and frozen. However, at institutions where collection and snap freezing of tissue samples can be conducted as quickly as possible, it is also recommended not to use nucleic acid stabilization/preservation agents to ensure that the quality of the nucleic acids is maintained and the general versatility of the sample maximized.

Note: If tissue samples frozen in a nucleic acid stabilization/preservation agent are used for nucleic acid extraction, the quality of the DNA will generally not be affected (Fig. 4 and Table 3).

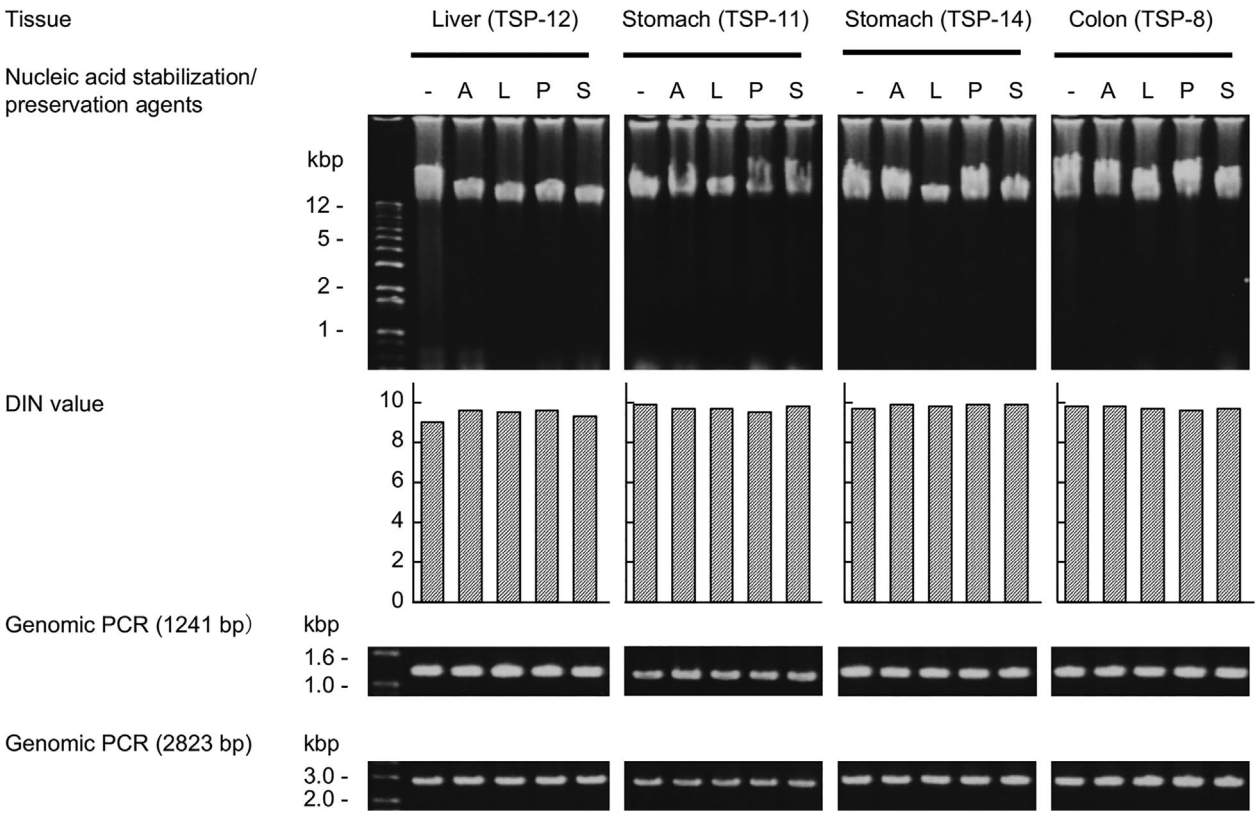

Figure 4 Effects of nucleic acid stabilization/preservation agents from various manufacturers on the quality of genomic DNA. Using tissue pieces of almost the same size (2 to 3 mm^3 cubes) collected from the same site (non-cancerous part) of a surgical specimen from the same patient, genomic DNA was extracted by the phenol-chloroform method after immersion in various nucleic acid stabilization/preservation agents and storage for a certain period of time in liquid nitrogen to compare the quality of the genomic DNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen without the use of any nucleic acid stabilization/preservation agent immediately after surgical resection; #8, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent A immediately after surgical resection; #9, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent L immediately after surgical resection; #10, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent P immediately after surgical resection; #11, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent S immediately after surgical resection. The quality of the DNA was assessed by agarose gel electrophoresis, in terms of the DNA integrity number (DIN) measured in an 2200 TapeStation system (also see Table 3), and by genomic PCR with amplification lengths of 1241 bp and 2823 bp. When tissue samples frozen in nucleic acid stabilization/ preservation agents were subjected to nucleic acid extraction, there was generally no influence on the quality of the DNA.

Table 3 Effects of nucleic acid stabilization/preservation agents from various manufacturers on the quality of genomic DNA

	Procedure	Number of analyzed tissue specimens[†]	Yielded genomic DNA (μg)	A_{260}/A_{280}	DNA integrity number: Mean ± SD	DNA integrity number: *P*-value against #1 (Welch-*t* test)
#1	Snap freezing in liquid nitrogen without the use of any nucleic acid stabilization/preservation agent immediately after surgical resection	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#8	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent A immediately after surgical resection	4	361.7 ± 186.6	1.90 ± 0.05	9.8 ± 0.1	9.58×10^{-2}
#9	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent L immediately after surgical resection	4	197.9 ± 112.7	1.93 ± 0.06	9.7 ± 0.1	2.08×10^{-1}
#10	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent P immediately after surgical resection	4	308.0 ± 70.2	1.90 ± 0.07	9.7 ± 0.2	4.94×10^{-1}
#11	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent S immediately after surgical resection	4	304.8 ± 103.7	1.93 ± 0.06	9.7 ± 0.3	4.47×10^{-1}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 4. Also see Fig. 4.

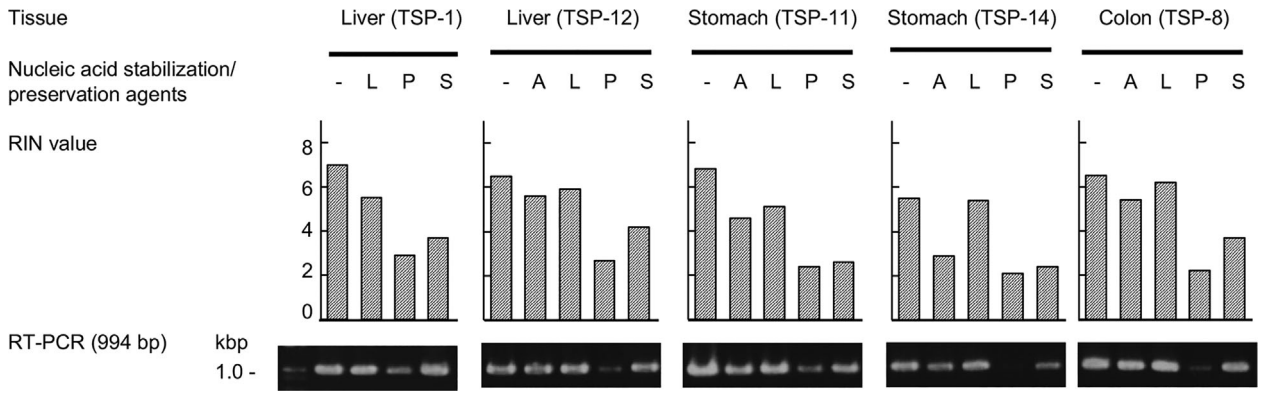

Figure 5 Effects of nucleic acid stabilization/preservation agents from various manufacturers on RNA quality. Using tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous part) of a surgical specimen from the same patient, total RNA was extracted using TRIzol after immersion in various nucleic acid stabilization/preservation agents and storage for a certain period of time in liquid nitrogen to compare the quality of the RNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen without the use of any nucleic acid stabilization/preservation agent immediately after surgical resection; #8, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent A immediately after surgical resection; #9, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent L immediately after surgical resection; #10, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent P immediately after surgical resection; #11, Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent S immediately after surgical resection. The quality of the RNA was assessed in terms of the RNA integrity number (RIN) measured in a 2100 Bioanalyzer system (also see Table 4), and by reverse transcription (RT)-PCR with an amplification length of 994 bp. There were considerable variations in the effect on RNA quality depending on the type of nucleic acid stabilization/preservation agent used. If tissue samples frozen in a nucleic acid stabilization/preservation agent are subjected to nucleic acid extraction, the quality of the RNA extracted may be decreased, e.g., because additional time is needed for thawing the nucleic acid stabilization/preservation agent, in comparison to extraction of nucleic acids from tissue samples frozen without the use of nucleic acid stabilization/preservation agents.

Note: On the other hand, the effects on RNA vary largely according to the type of nucleic acid stabilization/preservation agent used. If a nucleic acid stabilization/preservation agent is employed, the most appropriate one should be chosen based on quality that has been verified by actual extraction of nucleic acids at each institution (Fig. 5 and Table 4).

Note: If tissue samples frozen in a nucleic acid stabilization/preservation agent are used for nucleic acid extraction, the quality of the RNA extracted may be poorer because, for

Table 4 Effects of nucleic acid stabilization/preservation agents from various manufactures on the quality of RNA

	Procedure	Number of analyzed tissue specimens†	Yielded total RNA (μg)	A_{260}/A_{280}	RNA integrity number: Mean ± SD	RNA integrity number: *P*-value against #1 (Welch-*t* test)*
#1	Snap freezing in liquid nitrogen without the use of any nucleic acid stabilization/preservation agent immediately after surgical resection	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#8	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent A immediately after surgical resection	4	96.0 ± 44.0	2.08 ± 0.05	4.6 ± 1.2	$\underline{4.09 \times 10^{-2}}$
#9	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent L immediately after surgical resection	5	98.8 ± 41.3	2.08 ± 0.04	5.6 ± 0.4	$\underline{4.55 \times 10^{-4}}$
#10	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent P immediately after surgical resection	5	69.3 ± 45.6	2.08 ± 0.03	2.5 ± 0.3	$\underline{8.52 \times 10^{-12}}$
#11	Snap freezing in liquid nitrogen after immersion in nucleic acid stabilization/preservation agent S immediately after surgical resection	5	88.1 ± 37.5	2.07 ± 0.05	3.3 ± 0.8	$\underline{2.33 \times 10^{-4}}$

*Cases with $P < 0.05$ are underlined. Also see Fig. 5.

†Detailed description of analyzed tissue specimens is shown in the legend for Fig. 5.

example, additional time may be needed for thawing of the agent, as compared to that for extraction of nucleic acids from tissue samples that have been simply frozen without the use of nucleic acid stabilization/preservation agents (Fig. 5 and Table 4).

Snap freezing

1.16. Multiple sample tubes prepared according to item 1.14 should be rapidly frozen in liquid nitrogen (A).

Note: Tubes should be rapidly frozen by immersing them in corked Dewar bottles (double-wall insulated containers) containing liquid nitrogen (A).

Note: At institutions where samples are collected frequently (many each day), it is efficient to keep relatively small (about 30–50 L) liquid nitrogen storage containers for prompt snap freezing in surgical specimen processing rooms or other appropriate locations where fresh unfixed surgical specimens are handled, and to place the sample tubes prepared according to item 1.14 into these liquid nitrogen containers (E). This allows sufficiently frozen samples to be delivered regularly in bulk to long-term storage facilities.

Note: At institutions where liquid nitrogen is not readily available in surgical specimen processing rooms, the use of dry ice-acetone or other techniques may be useful alternatives for freezing (B) (Figs. 6, 7 and Tables 5, 6).

Note: At institutions where liquid nitrogen is not readily available in surgical specimen processing rooms, an ultra-deep freezer (–80°C) can be kept in the room, making it possible to promptly freeze any sample tubes (B) (Figs. 6, 7 and Tables 5, 6).

Long-term storage

1.17. It is preferable for snap-frozen sample tubes prepared according to item 1.16 to be stored in liquid nitrogen storage containers (about −180°C) until use for research purposes (A).

Note: Here, "long-term storage" means storage until use for research purposes. In general, equipment and standard procedures should be selected with a view to storage for 5–10 years or longer.

Note: Normally, gas-phase liquid nitrogen storage containers are used (A). It is desirable to use an isothermal-type liquid nitrogen storage container completely without liquid nitrogen in its inner tank, in order to avoid contamination by pathogenic microorganisms (E). However, from the viewpoint of cost-effectiveness, introduction of an

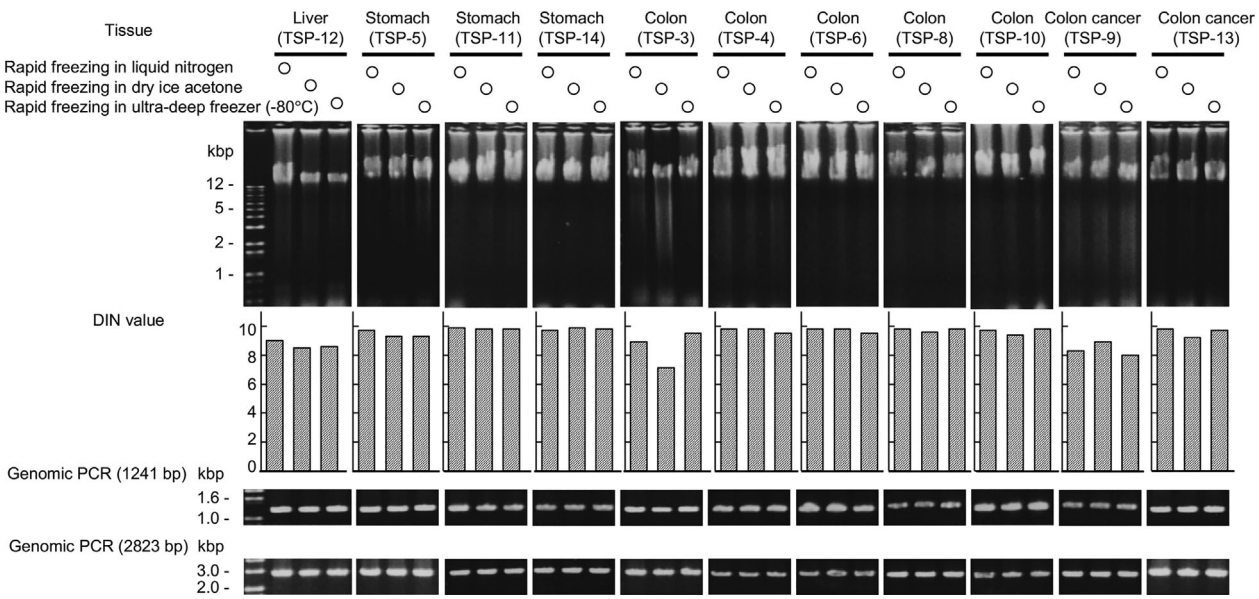

Figure 6 Effects of the freezing method on the quality of genomic DNA. Using tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous and cancerous parts) of a surgical specimen from the same patient, genomic DNA was extracted by the phenol-chloroform method after freezing by various methods and storage for a certain period of time, to compare the quality of the genomic DNA. The processing procedures for comparison were as follows: #1, The tubes containing the tissue samples were rapidly frozen in liquid nitrogen immediately after surgical resection; #12: The tubes containing the tissue samples were frozen in dry ice-acetone immediately after surgical resection, #13: The tubes containing the tissue samples were frozen and stored in an ultra-deep freezer (–80°C) immediately after surgical resection. The quality of the DNA was assessed by agarose gel electrophoresis, in terms of the DNA integrity number (DIN) measured in a 2200 TapeStation system, and by genomic PCR with amplification lengths of 1241 bp and 2823 bp. Even when the samples were frozen in dry ice-acetone or an ultra-deep freezer, instead of in liquid nitrogen, there was no marked influence on the quality of the DNA, as long as the storage period until analysis was short (also see Table 5).

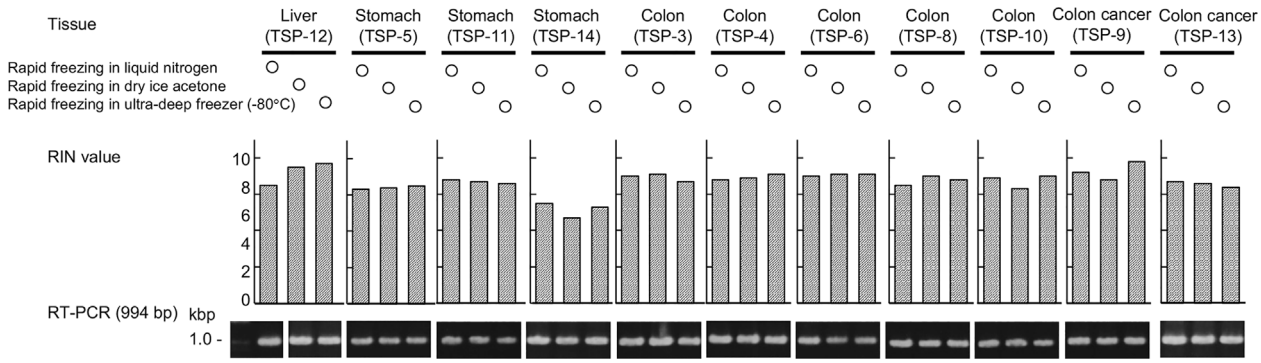

Figure 7 Effects of the freezing method on the quality of RNA. Using tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous and cancerous parts) of a surgical specimen from the same patient, total RNA was extracted using TRIzol after freezing by various methods and storage for a certain period of time to compare the quality of the RNA. The processing procedures for comparison were as follows: #1, The tubes containing the tissue samples were rapidly frozen in liquid nitrogen immediately after surgical resection; #12, The tubes containing the tissue samples were frozen in dry ice-acetone immediately after surgical resection; #13, The tubes containing the tissue samples were frozen and stored in an ultra-deep freezer (–80°C) immediately after surgical resection. The quality of the RNA was assessed in terms of the RNA integrity number (RIN) measured in a 2100 Bioanalyzer system, and by reverse transcription (RT)-PCR with an amplification length of 994 bp. Even when the samples were frozen in dry ice-acetone or an ultra-deep freezer, instead of in liquid nitrogen, there was no marked influence on the quality of the RNA as long as the storage period until analysis was short (also see Table 6).

Table 5 Effects of the freezing method on the quality of genomic DNA

	Procedure	Number of analyzed tissue specimens[†]	Yielded genomic DNA (μg)	A_{260}/A_{280}	DNA integrity number: Mean ± SD	DNA integrity number: *P*-value against #1 (Welch-*t* test)
#1	The tubes containing the tissue samples were rapidly frozen in liquid nitrogen immediately after surgical resection	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#12	The tubes containing the tissue samples were frozen in dry ice-acetone immediately after surgical resection	11	162.3 ± 79.3	1.87 ± 0.03	9.2 ± 0.8	3.70×10^{-1}
#13	The tubes containing the tissue samples were frozen in ultra-deep freezer (–80°C) immediately after surgical resection	11	158.1 ± 65.8	1.89 ± 0.04	9.4 ± 0.6	8.66×10^{-1}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 6. Also see Fig. 6.

isothermal-type container is left to the discretion of each individual institution.

Note: Liquid nitrogen-containing corked Dewar bottles (double-wall insulating containers) or other containers that have low thermal conductivity and adequate heat insulation capacity should be used for delivery to long-term storage facilities, even those within the institution.

Table 6 Effects of the method of freezing used on the quality of RNA

	Procedure	Number of analyzed tissue specimens[†]	Yielded total RNA (μg)	A_{260}/A_{280}	RNA integrity number: Mean ± SD	RNA integrity number: *P*-value against #1 (Welch-*t* test)
#1	The tubes containing the tissue samples were rapidly frozen in liquid nitrogen immediately after surgical resection	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#12	The tubes containing the tissue samples were frozen in dry ice-acetone immediately after surgical resection	11	77.9 ± 45.5	2.11 ± 0.07	6.6 ± 0.7	8.81×10^{-1}
#13	The tubes containing the tissue samples were frozen in ultra-deep freezer (–80°C) immediately after surgical resection	11	87.3 ± 53.0	2.10 ± 0.08	6.8 ± 0.7	5.77×10^{-1}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 7. Also see Fig. 7.

Note: Operations such as placement of sample tubes into a liquid nitrogen container for long-term storage are usually conducted manually, but as quickly as possible, to minimize any changes in sample temperature (A).

Note: From the viewpoint of quality preservation and labor-saving, it is also desirable to have a system at liquid nitrogen storage facilities to distinguish anonymized number bar codes or other types of codes printed on sample tubes, and a system for managing automatic robotic delivery in and out of the storage facility. A large outdoor liquid nitrogen storage tank and an automated system for supplying liquid nitrogen from it to each indoor container are also desirable (E). However, the long-term storage facility chosen at each institution should take cost-effectiveness into account.

1.18. When the sample tubes are placed in liquid nitrogen storage containers for long-term storage, sample-associated information including the place of storage of the sample should be registered in the appropriate management databases (A).

Note: To provide raw data for biobank operators to input into management databases, persons who collect the samples are required to generally specify the items summarized in Table 7.

1.19. It is possible to use an ultra-deep freezer (–80°C) for long-term storage instead of liquid nitrogen storage containers (B). In this case also, the sample tubes should preferably be immersed in liquid nitrogen for snap freezing according to item 1.16, and then placed in the ultra-deep freezer (A).

Note: The quality of DNA, RNA and proteins extracted from tissue samples after long-term storage in an ultra-deep freezer (–80°C) may be poorer than that of those extracted from tissue samples after long-term storage in liquid nitrogen storage containers. (In the case of DNA and RNA in particular, the quality may deteriorate after 10 or more years of storage.) (Figs. 8–10 and Table 8).

Note: Although long-term storage in liquid nitrogen storage containers is recommended to facilitate general versatility, DNA and RNA extracted from tissue samples after long-term storage at −80°C can be used for a number of analyses without any particular problems. Therefore, the temperature for long-term storage should be determined at the discretion of each institution, depending on feasibility (B). Researchers who use the samples should choose the analytical method appropriately according to the quality of the stored samples (A). For this purpose, see the empirical data in Figs. 8–10 and Table 8.

1.20. Use of any ordinary freezer (–20°C) for long-term storage should be avoided (N).

1.21. It is desirable for institutions such as biobanks providing samples to researchers to publish updated results of studies on the use of biobank samples (e.g., published papers) on their webpages, etc., in order to provide researchers with information on the properties and quality of the stored samples (A).

1.22. It is also desirable for institutions such as biobanks to regularly conduct demonstrative analysis of a few randomly selected samples, and to disclose the results (E). It is desirable for indices of nucleic acid integrity to be actually measured for all samples, and for the data to be disclosed when the samples are provided. However, from the viewpoint of feasibility or unnecessary consumption of nucleic acid samples, this may not be essential (E).

1.23. Considering the possibility of problems and accidents during long-term storage, it is recommended that a record of temperature management be provided, if possible (E).

1.24. Considering the possibility of problems and accidents during long-term storage, it is also desirable to disperse the samples and store them at separate facilities, if possible, as a backup archive (E).

Preparation of frozen tissue sections

1.25. When cutting tissue specimens into pieces according to item 1.10, it is recommended that 1 or 2 pieces measuring $0.5 \times 0.5 \times 0.2$ cm be taken separately, embedded in embedding medium for preparation of frozen tissue sections (so-called optimal cutting temperature [OCT] compound), and frozen in dry ice-acetone, etc., for storage in an ultra-deep freezer (–80°C) (E).

Note: It is recommended that, before they are frozen, tissue samples should be immersed once in OCT compound and then again in another volume of OCT compound to prevent the formation of ice crystals during the preparation of OCT-embedded specimens (E).

1.26. For long-term storage of OCT-embedded specimens in an ultra-deep freezer (–80°C), due caution should be exercised to prevent the samples from becoming desiccated, by means such as tightly wrapping the specimens with a material such as Parafilm (Bemis Flexible Packaging, Chicago, IL, USA), and then placing them in airtight containers.

Note: Usually, degraded nucleic acids alone can be extracted from desiccated OCT-embedded blocks (Fig. 11) (N).

Note: Although the quality of nucleic acids is generally maintained in OCT-embedded blocks stored for long periods under optimal conditions, verification of the quality of extracted nucleic acids should be done for each block before analysis (Fig. 11 and Table 9).

Note: Information on the percentage of cancer cells contained in the tissue to be provided to researchers can be obtained by microscopic examination of hematoxylin and

Table 7 Sample-associated information that should be deposited in the management databases

Item	Description
1. Date of surgery/collection	It is indispensable to note multiple episodes of surgery in the same patient.
2. Person who collects the sample	When a biobank contact person (pathologist in charge and clinicians in the relevant specialty, who will collaborate in the study for which the sample is to be used, and who will judge the propriety of providing of the sample from the biobank to researchers) has been assigned, the name of the person should be specified.
3. Name of the patient	In accordance with the catalogue database, the patient's name should be specified and not be abbreviated using initial letters or other similar abbreviations.
4. Patient ID	
5. Pathology ID	This item should be recorded to distinguish multiple episodes of surgery in the same patient with a single patient's ID.
6. Name of the clinical department	Because the name of a department can be a keyword in the catalogue search before research use, the official names of all clinical departments should be listed. It is also desirable to build a database into which the person collecting the sample can choose the official name of the department from a drop-down list in order to prevent typographical error.
7. Infection information	The specific diagnoses that must be confirmed for management and research use of the sample should be listed in advance, and the person collecting the sample should be requested to choose the precise diagnosis from the list, with free writing allowed in the “others“ section.
8. Gender	This item is not indispensable at institutions where a catalogue database of the biobank coupled with electronic medical records is available, where this item is automatically input from the patient ID.
9. Age at the time of surgery/collection	Entry of the patient age at the time of sample collection should be requested, because it is difficult, at the time when a sample is used for research, to automatically extract the patient age at the time of sample collection from the electronic medical records.
10. Clinical diagnosis	Free writing. This item is used for partial match retrieval in catalogue databases. In addition, to allow a search from the final diagnosis, it is necessary to couple the biobank catalogue database with the electronic medical records, in-hospital cancer registry, etc., after establishment of the pathological diagnosis, and to thereby enable a search based on the International Classification of Diseases (ICD) codes, etc.
11. Organ	If the name of the organ is used for classification in the management of samples, selection must be made from a drop-down list of organs, with no free writing option. The World Health Organization (WHO) tumor classification organ codes may be used.
12. Number of tubes	____ T tubes (tumor/lesion parts), ____ N tubes (non-tumor/control parts).
13. Sample-associated information	T1: Site of collection (e.g., lesser curvature of the gastric body, block number #; addition of photograph/schema of the organ with a mark of the site of sample collection should be allowed). Free writing of macroscopic findings, etc., should also be permitted. T2: Similarly, description is requested for the number of sample tubes. N1: Site of collection (e.g., lesser curvature of the gastric body, block number #; addition of photograph/schema of the organ with a mark of the site of sample collection should be allowed). Free description of macroscopic findings, etc., should also be permitted. N2: Similarly, description is requested for the number of sample tubes.
14. Deviation from the standard operating procedure (SOP) of the institution	“Present” or “absent” should be chosen. If present, an explanation of the situation should be given in free writing. However, at an institution where recording of the actual time between surgical resection and snap freezing of specimens and the temperature (e.g., room temperature, 4°C, etc.) at which the surgically resected materials are stored until freezing is always be requested, a description about the deviation from the SOP may not be needed.

The following may be specified if there is a consensus at the institution to allow such specification without any difficulty.

- Time of resection (24 h system, ___ h ___ min), time of snap freezing (24 h system, ___ h ___ min) or time between surgical resection and snap freezing (about ___ min)
- Duration of intraoperative ischemia (about ___ min)
- Wet weight (___ mg)

eosin-stained or immunostained thin sections prepared from OCT-embedded specimens. To calculate the cancer cell percentage from microscopic examination of tissue specimens, the following methods are available:

(a) Cancer cells in several fields of view are counted with a counter as accurately as possible, and the count is divided by the total cell count determined similarly (E), or

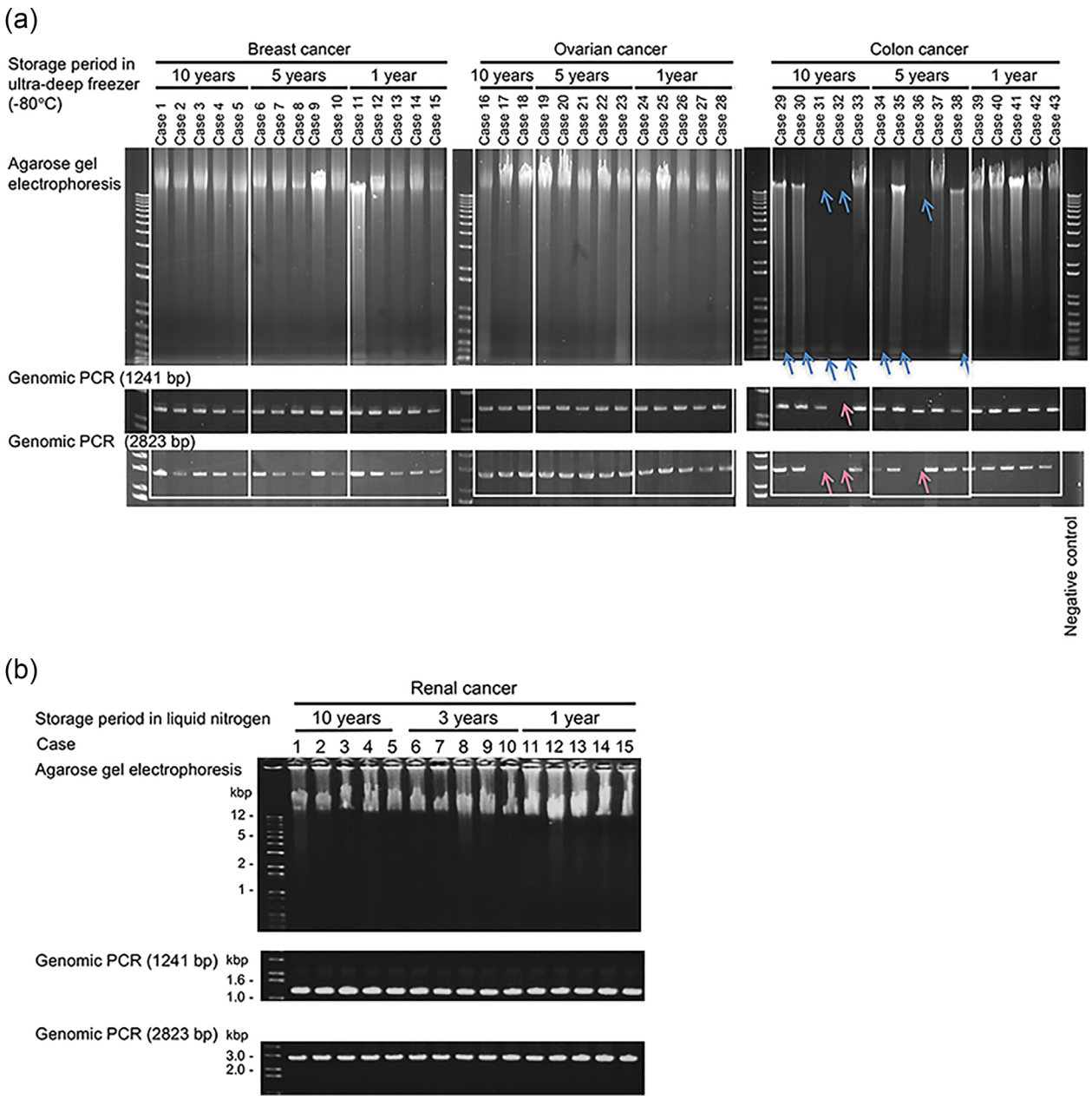

Figure 8 Effects of long-term storage temperature on genomic DNA. Using tissue samples stored for a long period of time in an ultra-deep freezer (–80°C) **(a)** and in a liquid nitrogen storage container (–180°C) **(b)**, genomic DNA was extracted by ZR-Duet^{TM} DNA/RNA MiniPrep (Zymo Reseaerch, Irvine, CA) and by the phenol-chloroform method, and the quality of the DNA was assessed by agarose gel electrophoresis and by genomic PCR with amplification lengths of 1241 bp and 2823 bp. **(a)** In some samples stored for long periods (5–10 years) in an ultra-deep freezer (–80°C), bands representing high-molecular-weight DNA disappeared, indicative of genomic DNA degradation (blue arrows), and there was no amplification by PCR at an amplification length of 1241 bp or 2823 bp (red arrows). **(b)** In contrast, in samples stored for long periods in liquid nitrogen, such degradation and PCR failure were rare. When using samples stored for particularly long periods, it is desirable to thoroughly verify the quality of the nucleic acids before performing analysis, because the quality of DNA may deteriorate with storage at −80°C.

(b) a rough classification, e.g., less than 10%, 10–20%, and in incremental 10% classes, can be obtained at a glance by low-power microscopic examination (E).

Useful information can be obtained even using method (b) (Table 10). Method (a) or (b) should be chosen at each institution according to feasibility.

Note: Microdissection can be conducted after observation of histologic features in hematoxylin and eosin-stained thin sections prepared from OCT-embedded specimens, or nucleic acids can be extracted without microdissection for various analyses. Thin sectioning, i.e., destruction of cell and/or nuclear membranes, may be advantageous for some

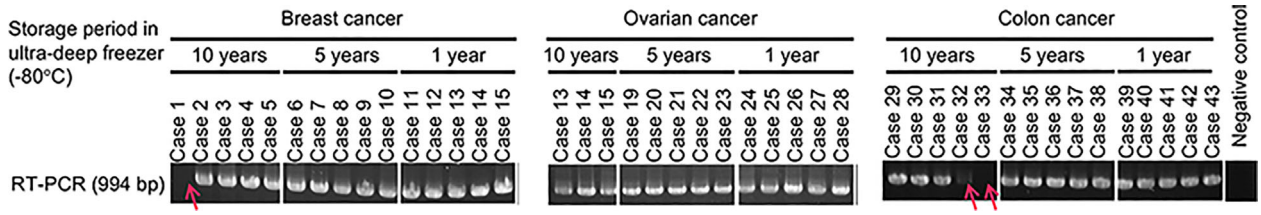

Figure 9 Effects of long-term storage temperature on RNA quality. Using tissue samples stored for long periods of time in an ultra-deep freezer (–80°C), total RNA was extracted by ZR-Duet DNA/RNA MiniPrep and TRIzol, and the quality of the RNA obtained was assessed by measurement of the RIN values in a 2100 Bioanalyzer system and by reverse transcription (RT)-PCR with an amplification length of 994 bp. In some samples stored for long periods (10 years) in an ultra-deep freezer (–80°C), no (RT)-PCR amplification was observed at an amplification length of 994 bp (red arrows). In addition, the average RNA integrity number tended to be lower in samples stored for long periods (10 years) in an ultra-deep freezer (–80°C) (See Table 8). In contrast, such deterioration was rare in samples stored for long periods in liquid nitrogen (Table 8). When using samples stored for a particularly long period, it is desirable to thoroughly verify the quality of the nucleic acids before performing analysis, because the quality of RNA may deteriorate with storage at −80°C.

analyses. On the other hand, contamination by the embedding agent may interfere with the reaction. In particular, OCT compound may interfere with column purification of nucleic acids and protein analysis.

Note: Preparation of OCT-embedded specimens has the merit of allowing histologic features to be confirmed, thus allowing various analyses. However, management of these samples requires extreme care, including prevention of desiccation, and this substantially increases the banking workload, including assessment of the cancer cell percentage. Therefore, OCT-embedded specimens should be prepared taking feasibility into account, and only when there is adequate consensus.

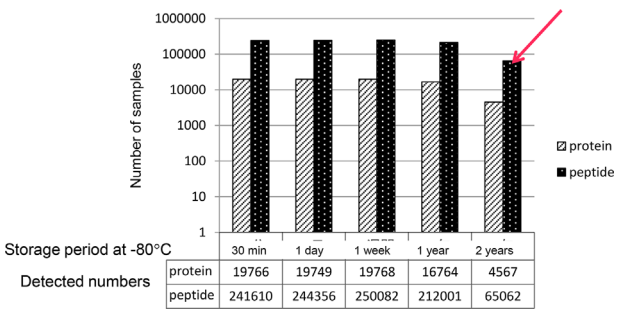

Figure 10 Effects of storage in an ultra-deep freezer (–80°C) on the quality of protein. Liver tissue stored for up to 2 years in an ultra-deep freezer (–80°C) was dispersed and crushed in protease inhibitor-containing radio-immunoprecipitation assay (RIPA) buffer. The samples were subjected to gelation, washing, dehydration, reduction treatment, and trypsin digestion by the usual methods,[29] and proteome analysis was conducted by liquid chromatography-electrospray ionization-quadrupole-time of flight mass spectrometry (LC/ESI-QTOF MS). It became apparent that storage for 2 years or more in an ultra-deep freezer (–80°C) was associated with a decrease in the number of proteins and peptides detected. In contrast, in the samples stored for long periods of time in liquid nitrogen, such proteome analysis failure was rare. When using samples stored for a particularly long period, it is desirable to thoroughly verify the quality of the proteins before performing analysis, because protein quality may deteriorate following storage at −80°C.

Research use

1.27. Operations to remove samples from the liquid nitrogen storage container for research purposes are normally conducted manually, but as quickly as possible to minimize any effects of exposure on other samples (A).

Note: High sample quality should be maintained as far as possible until provision to researchers or the beginning of transport by placing the tube containing the tissue specimens on dry ice after removal from the container (A).

1.28. Information about the provision of samples and their intended use should be registered in an appropriate management application (A).

Note: At biobanks that require the return of extracted nucleic acids, results of analysis, and remaining pieces of tissue, the history of usage (researchers, subjects of study, etc.) should be recorded in precise detail in the management databases (E).

Transport

1.29. For samples obtained at institutions that lack facilities for long-term storage, the tubes containing the tissue samples should be frozen sufficiently in liquid nitrogen or dry ice-acetone in Dewar bottles and then transported to institutions that do have liquid nitrogen storage facilities. Appropriate means of transport should also be used when supplying samples to researchers in remote locations.

1.30. Usually, non-airtight containers that allow maintenance of temperature (polystyrene containers are a simple example) filled with dry ice should be used, and samples in a frozen state should be entrusted to a forwarding agency (A).

Table 8 Effects of long-term storage temperature on the quality of RNA

Storage temperature	Organ	Storage period	Number of analyzed tissue specimens	RNA integrity number	
				Mean ± SD	*P*-value against 1-year storage (Welch-*t* test)*
–80°C	Breast	1 year	5	8.0 ± 0.5	
		5 years	5	7.9 ± 0.4	7.93×10^{-1}
		10 years	5	5.0 ± 2.9	8.75×10^{-2}
	Ovary	1 year	5	7.3 ± 0.6	
		5 years	5	6.8 ± 2.2	6.20×10^{-1}
		10 years	3	5.4 ± 3.4	4.19×10^{-1}
	Colon	1 year	5	7.9 ± 1.0	
		5 years	5	6.2 ± 2.0	1.27×10^{-1}
		10 years	5	4.4 ± 2.3	$\underline{2.53 \times 10^{-2}}$
–180°C	Kidney	1 year	11	7.4 ± 1.9	
		5 years	2	7.1	8.60×10^{-1}
		10 years	12	6.8 ± 0.8	4.44×10^{-1}

*Cases with $P < 0.05$ are underlined. Also see Fig. 9.

Note: Some forwarding agencies specializing in the delivery of biological samples guarantee temperature maintenance (e.g., at −80°C) and use transport containers with a built-in temperature recording chip (E). However, for samples that are transported in a frozen state using polystyrene containers filled with dry ice, there is no clear indication of any deterioration in the quality of extracted nucleic acids within a few days, even if such specialist forwarding agencies are not employed (Figs. 12, 13 and Tables 11, 12).

Biosafety

1.32. If tissue banking operations are likely to pose a risk of exposure to infectious agents, even in cases where only a

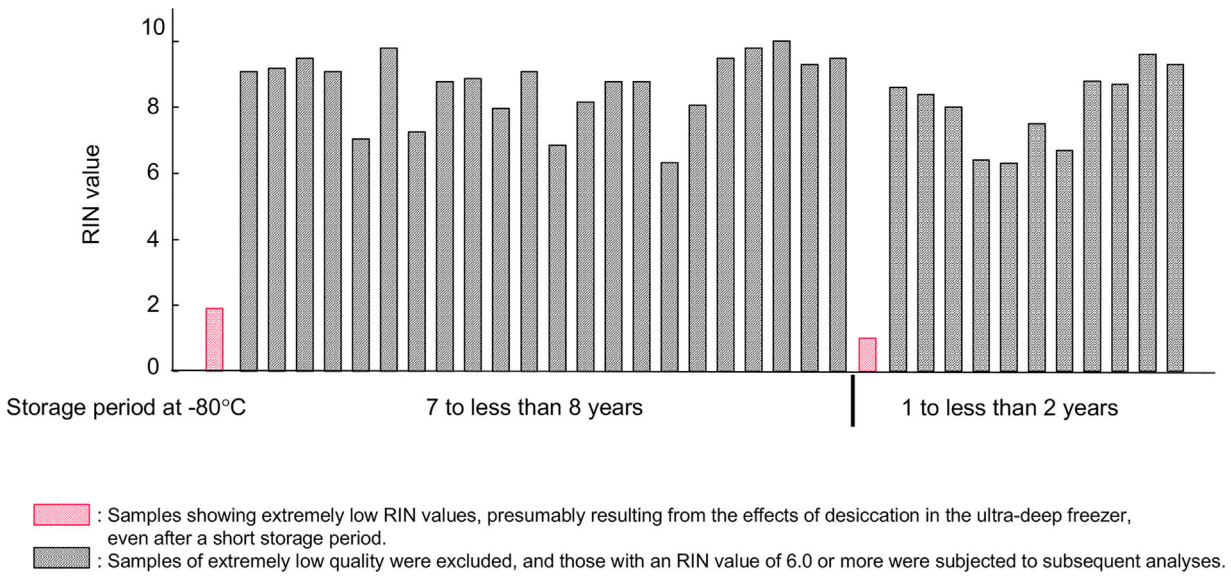

Figure 11 The quality of RNA extracted from specimens embedded in optimal cutting temperature (OCT) compound. OCT-embedded specimens were prepared from surgical specimens of consecutive cases of biliary tract cancer, stored for a long period in an ultra-deep freezer (–80°C), and cut into thin sections to extract total RNA. RNA integrity numbers (RINs) were then determined using a 2100 Bioanalyzer system. Notwithstanding the same period of storage, there were some samples that showed extremely low RIN values (red), presumably resulting from the effects of desiccation in the ultra-deep freezer. It was considered important to protect OCT-embedded specimens from desiccation by some means, such as tightly packing them in Parafilm, etc., and putting the packed specimens in airtight containers. After excluding the dried specimens, 215 samples with RIN values of 6.0 or more were subjected to subsequent analyses (Table 9). In the 215 samples analyzed, the relationship between the storage period and the quality of the RNA was described in Table 9. When samples with extremely low RIN values resulting from desiccation were excluded, it was possible to extract RNA of good quality, indicating that long-term storage of OCT-embedded specimens in an ultra-deep freezer (–80°C) does not cause any marked deterioration in the quality of the RNA.

Table 9 The quality of RNA extracted from specimens embedded in optimal cutting temperature (OCT) compound

Storage period	Number of analyzed tissue samples[†]	A_{260}/A_{280}	RNA integrity number	
			Mean ± SD	*P*-value against storage for less than 1 year (Welch-*t* test)
Less than 1 year	18	2.05 ± 0.06	8.1 ± 0.5	–
1 year to less than 2 years	11	2.03 ± 0.16	8.0 ± 1.2	7.89×10^{-1}
2 years to less than 3 years	20	2.11 ± 0.13	8.5 ± 0.8	8.48×10^{-2}
3 years to less than 4 years	21	2.04 ± 0.11	8.4 ± 0.9	2.91×10^{-1}
4 years to less than 5 years	13	2.05 ± 0.22	8.6 ± 0.9	7.52×10^{-2}
5 years to less than 6 years	27	2.05 ± 0.08	8.0 ± 1.1	5.34×10^{-1}
6 years to less than 7 years	26	2.04 ± 0.08	8.5 ± 0.9	9.57×10^{-2}
7 years to less than 8 years	22	2.08 ± 0.07	8.5 ± 1.0	1.48×10^{-1}
8 years to less than 9 years	21	2.06 ± 0.07	7.9 ± 0.9	3.96×10^{-1}
9 years to less than 10 years	30	2.07 ± 0.07	8.0 ± 1.0	5.07×10^{-1}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 11. Also see Fig. 11.

portion of the specimen contains infectious agents, all samples should be handled as biohazards.

1.33. Biobank managers should refer to appropriate documents, such as "National Cancer Institute (NCI) Best Practices for Biospecimen Resources" (first released in 2007 and updated in both 2011 and 2016),[23] "World Health Organization (WHO): Biorisk Management; Laboratory Biosecurity Guidance (2006)"[24] and "Organisation for Economic Co-operation and Development (OECD): Best Practice Guidelines on Biosecurity for Biological Resource Centres (2007)",[25] to formulate safety handling manuals, and to ensure all operators clearly understand the contents of the manuals.

1.34. Operators must read safety handling manuals with care, and perform their activities according to the methods specified therein. In addition, managers must provide the operators with appropriate training.

Table 10 Representative cases revealing the significance of at-a-glance assessment of the proportion (percentage) of cancer cells for interpretation of mutant allele frequency

Amplicon sequencing			
Read depth	Mutant allele frequency (%)	Proportion of tumor cells as assessed by the pathologist at a glance (%)	Interpretation of the results
45 989	48.76	90	Proportion of cancer cells × 0.5 = mutant allele frequency → Mutation in unilateral allele?
3560	50.22	80	Proportion of cancer cells × 0.5 = mutant allele frequency → Mutation in unilateral allele?
2321	59.11	80	Proportion of cancer cells × 0.5 = mutant allele frequency → Mutation in unilateral allele?
17 684	53.15	90	Proportion of cancer cells × 0.5 = mutant allele frequency → Mutation in unilateral allele?
6309	3.53	10	Proportion of cancer cells × 0.5 = mutant allele frequency → Mutation in unilateral allele?
5113	54.57	30	Generally 50%, unlike the mutant allele frequency predicted from the percentage of cancer cells → Heterozygous or germline mutation
133	11.28	80	Mutant allele frequency inconsistent with the proportion of cancer cells → Read depth is also shallow, indicating artifact or heterogeneity
5279	83.16	80	Proportion of cancer cells = mutant allele frequency → Mutation in both alleles?
8335	51.22	20	Generally 50%, unlike the mutant allele frequency predicted from the proportion of cancer cells → Heterozygous or germline mutation
14 506	9.27	80	Mutant allele frequency inconsistent with the proportion of cancer cells → Heterogeneity?

In 10 hematoxylin and eosin-stained samples of non-small cell lung cancer, a pathologist assessed the proportion (percentage) of cancer cells at a glance under low magnification and classified them in 10% groups, such as <10%, 10% − <20%, etc. Cases in which the proportion of cancer cells assessed at a glance was useful for interpretation of the read depth and mutant allele frequency obtained using the TruSeq Cancer Panel (Illumina, San Diego, CA, USA) using genomic DNA extracted from the same specimen are shown in this table. Useful findings may be obtained by at-a-glance assessment of the proportion of cancer cells under low magnification, even without actual determination of the cancer cell count and the total cell count using a counter.

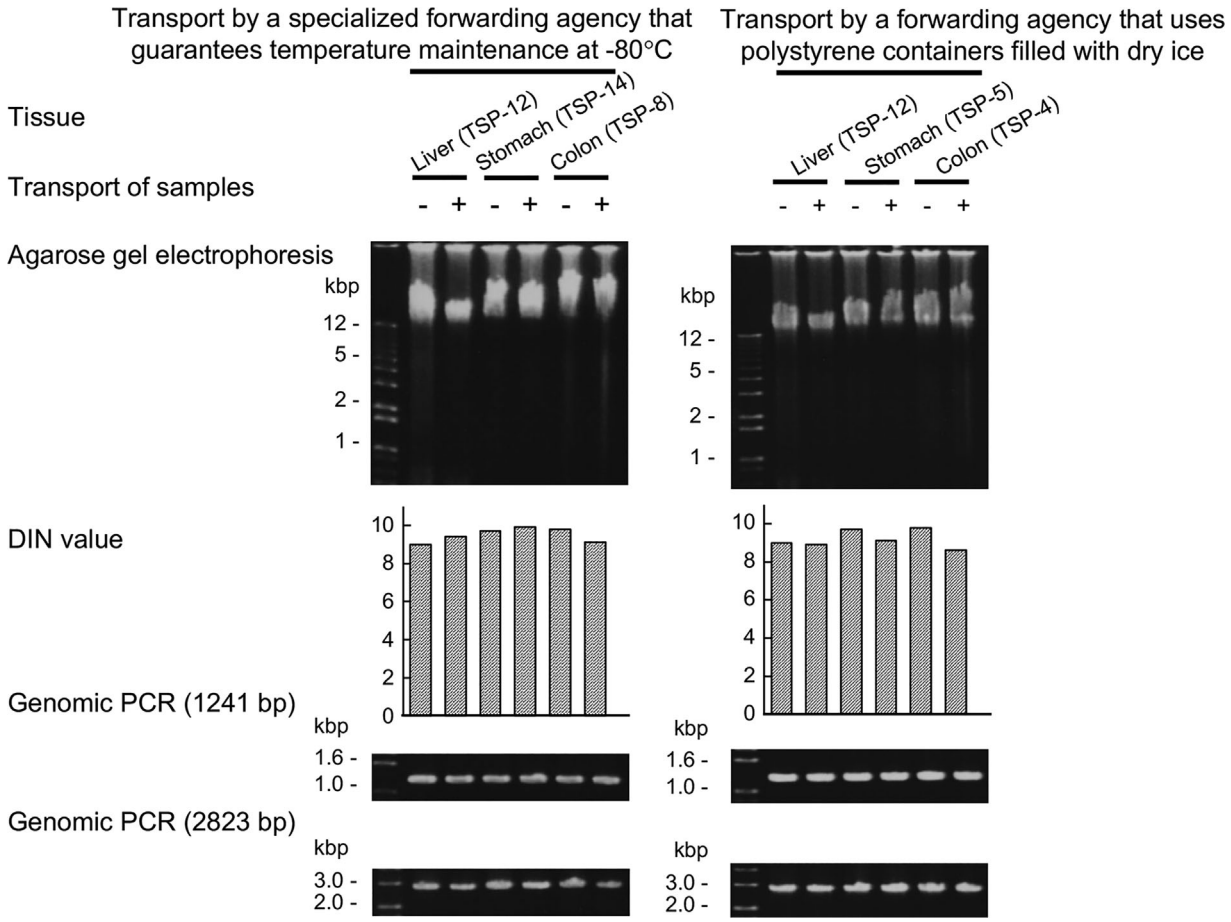

Figure 12 Effects of the method of transport of samples on the quality of genomic DNA. Tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous part) of the surgical specimen from the same patient were transported by various methods after they were immediately subjected to snap freezing and storage for a certain period in liquid nitrogen, and genomic DNA was extracted by the phenol-chloroform method to compare the quality of DNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen immediately after surgical resection, and no transport; #14, Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a forwarding agency specialized in the delivery of biologic samples that guarantees temperature maintenance at −80°C, and uses transport containers with a built-in temperature recording chip; #15, Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a forwarding agency that uses polystyrene containers filled with dry ice. Extraction and assessment of the quality of genomic DNA were performed by the same operator in the same laboratory. The quality of DNA was assessed by agarose gel electrophoresis, by DNA integrity number (DIN) measurement in a 2200 TapeStation system (Table 11), and by genomic PCR with amplification lengths of 1241 bp and 2823 bp. As shown in this figure and Table 11, if the methods of collection and of storage prior to the transport are appropriate, the method used for transport of the samples appears to exert no marked effects on the quality of the genomic DNA.

Note: Specifically, preventive measures similar to those in clinical practice must be adopted in addition to the general safety control measures used in the laboratory. Namely, operators should wear gloves and face shields and be vigilant about hand washing.

Note: In particular, due caution should be exercised to prevent tuberculosis infection, which pathologists and clinical laboratory technicians involved in pathology are at a high risk of contracting.[26]

1.35. If there is a possibility of exposure to droplets and aerosols, the containers of the samples should be opened in a safety cabinet in accordance with Biosafety Level 2 prescribed in the "WHO: Laboratory Biosafety Manual, 3rd edn. (2004)".[27]

1.36. All operators in biobanks should be vaccinated against hepatitis virus B, etc., and all exposed operators must receive post-exposure examinations, and be followed up as appropriate.

Disposal of samples

1.37. When disposal of frozen pathological tissue is necessary because of withdrawal of consent by the

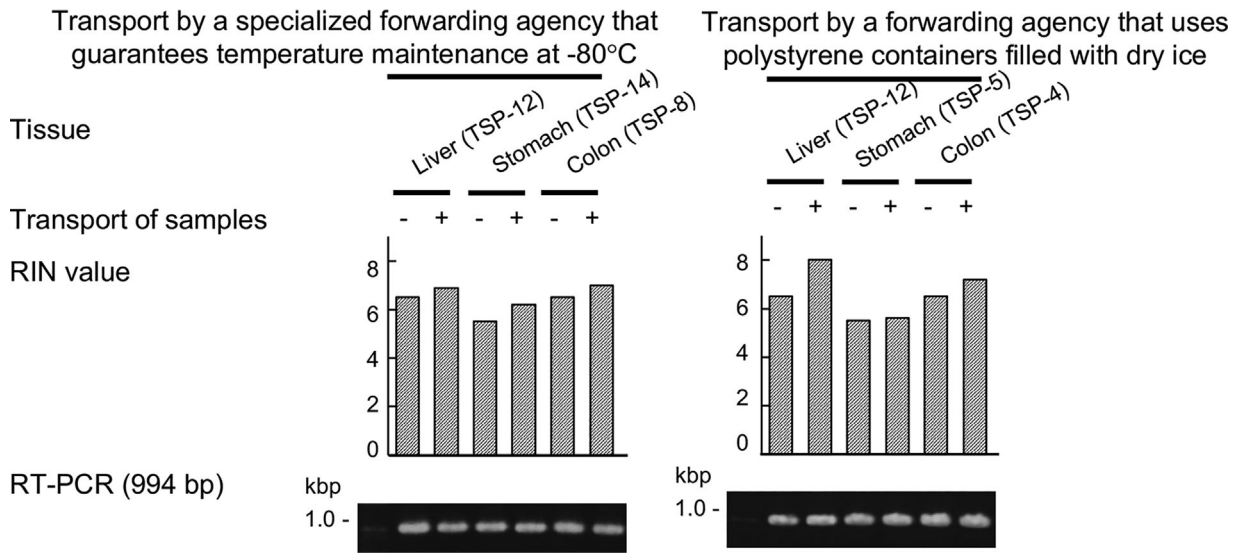

Figure 13 Effects of sample transport method on RNA quality. Tissue pieces of almost the same size (2 to 3 mm^3) collected from the same site (non-cancerous part) of the surgical specimen from the same patient were transported by various methods after they had been immediately subjected to snap freezing and storage for a certain period in liquid nitrogen, and total RNA was extracted using TRIzol to compare the quality of the RNA. The processing procedures for comparison were as follows: #1, Snap freezing in liquid nitrogen immediately after surgical resection, and no transport; #14, Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a forwarding agency specializing in the delivery of biologic samples that guarantees temperature maintenance at −80°C and uses transport containers with a built-in temperature-recording chip; #15, Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a forwarding agency that uses polystyrene containers filled with dry ice. Extraction and assessment of the quality of all the RNA were performed by the same operator in the same laboratory. The quality of RNA was assessed by measurement of the RNA integrity number (RIN) in a 2100 Bioanalyzer system (Table 12) and by reverse transcription (RT)-PCR with an amplification length of 994 bp. As shown in this figure and Table 12, if the methods of sample collection and storage prior to transport are appropriate, the method used for transport of the samples appears to have no marked effects on the quality of the RNA.

Table 11 Effects of transport method on the quality of genomic DNA

	Procedure	Number of analyzed tissue specimens[†]	Yielded genomic DNA (μg)	A_{260}/A_{280}	DNA integrity number: Mean ± SD	DNA integrity number: *P*-value against #1 (Welch-*t* test)
#1	Snap freezing in liquid nitrogen immediately after surgical resection, and no transport	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#14	Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a specialized forwarding agency at −80°C	3	260.7 ± 119.2	1.89 ± 0.03	9.5 ± 0.4	1.00
#15	Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a forwarding agency using polystyrene containers filled with dry ice	3	269.7 ± 157.5	1.92 ± 0.02	8.9 ± 0.3	7.05×10^{-2}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 12. Also see Fig. 12.

Table 12 Effects of the method of transport of the samples on the quality of RNA

	Procedure	Number of analyzed tissue specimens[†]	Yielded total RNA (μg)	A_{260}/A_{280}	RNA integrity numebr: Mean ± SD	RNA integrity numebr: *P*-value against #1 (Welch-*t* test)
#1	Snap freezing in liquid nitrogen immediately after surgical resection, and no transport	12	190.0 ± 104.0	1.87 ± 0.03	9.5 ± 0.5	–
#14	Snap freezing in liquid nitrogen immediately after surgical resection, and 2-day transport by a specialized forwarding agency at −80°C	3	123.9 ± 18.1	2.10 ± 0.01	6.7 ± 0.4	9.55×10^{-1}
#15	Snap freezing in liquid nitrogen immediately after extraction, and 2-day transport by a forwarding agency using polystyrene containers filled with dry ice	3	36.6 ± 38.8	2.07 ± 0.03	6.9 ± 1.2	7.59×10^{-1}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 13. Also see Fig. 13.

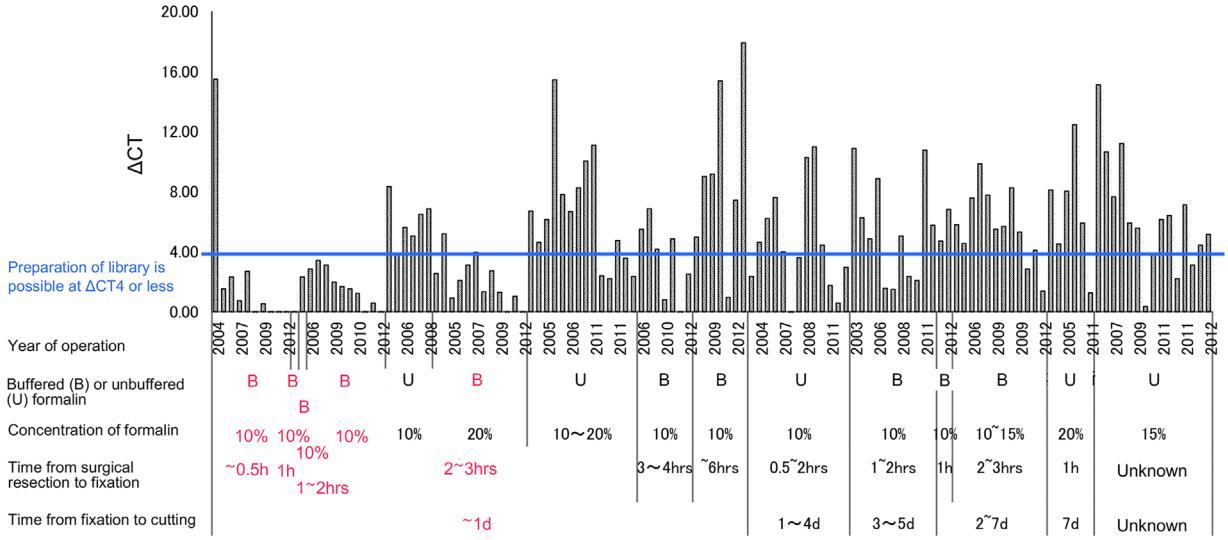

Figure 14 Effects of the time until fixation, the fixation time, and the type and concentration of formalin on the quality of DNA. At 15 institutions participating in a multicenter study, a verbal survey was conducted concerning the average time until fixation of surgical specimens, the fixation time, and the type and concentration of formalin used in the routine procedure for preparation of pathological tissue samples. Using formalin-fixed paraffin-embedded specimens prepared at the 15 institutions, the ΔCT values against control DNA were determined by real-time PCR to verify the quality of DNA by TruSight Tumor (Illumina). Preparation of a library for a search of cancer-related gene mutations by next-generation sequencing is expected to be possible for cases with ΔCT <4 (blue line).[30] As shown in red, fixation in 10% buffered formalin soon after surgical resection (within about 3 h) and cutting of the specimen on the day after surgery were considered desirable.

sample donor for use in research, or for other reasons, the sample should be treated as pathological waste (resected organs and tissues, etc.), and the date and reason for disposal should be recorded in the management database.

PART 2: APPROPRIATE METHODS FOR PREPARATION AND STORAGE OF FFPE SPECIMENS

In order to minimize any degeneration of DNA, RNA and proteins, etc., maintain high sample quality during long-term

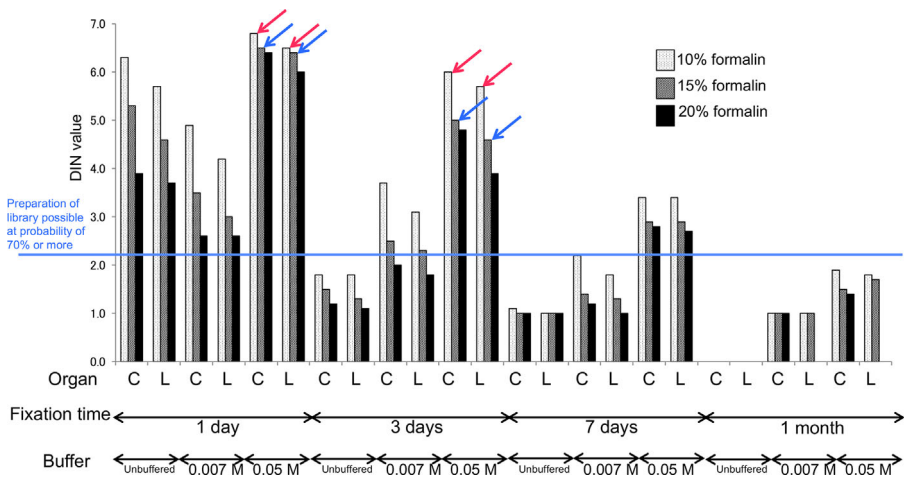

Figure 15 Effects of fixation time and the type and concentration of formalin on the quality of DNA. Colonic mucosa (C) and liver (L) specimens obtained at autopsy within 3 h of death were analyzed at a single institution. Genomic DNA was extracted using the QIAamp DNA FFPE Tissue kit (Qiagen, GmbH, Hilden, Germany) and the DNA integrity numbers (DINs) were determined using TapeStation-genomic DNA. Here, preparation of a library allowing amplicon sequencing was considered possible at a probability of 70% or more for samples with DIN values of 2.3 or higher (blue line) (H. Nishihara, unpubl. data, 2015). Fixation in highly buffered (0.05 M) 10% formalin within 1–3 days is desirable (red arrows). It is possible to increase the concentration of highly buffered formalin for fixation within 1–3 days, as long as attention is paid to conservation of the morphology (blue arrows). At institutions where the fixation time cannot be reduced to 3 days or less, it is indispensable to use highly buffered formalin. It is considered that analysis is possible using specimens fixed for less than a day if unbuffered formalin is used.

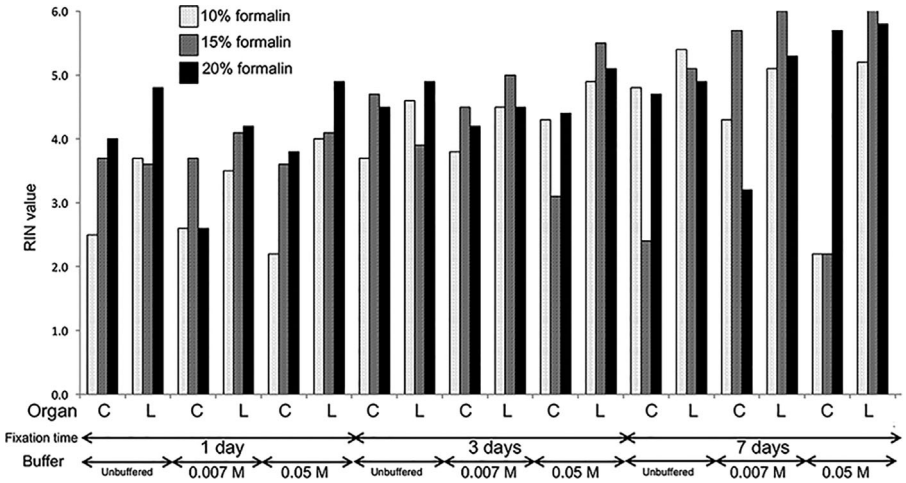

Figure 16 Effects of fixation time and the type and concentration of formalin on the quality of RNA. At a single institution, colonic mucosa (C) and liver (L) specimens obtained at autopsy within 3 h of death were analyzed. Total RNA was extracted using the RNeasy FFPE Kit (Qiagen) and the RNA integrity numbers (RINs) were determined using TapeStation. Unlike the effects on the quality of DNA described in Fig. 15, use of 15% or 20% formalin tended to provide RNA of higher quality than the use of 10% formalin. The quality of RNA also tended to be better with fixation for 3–7 days than with fixation for 1 day. Namely, in cases of RNA analysis, more thorough fixation relative to the optimal conditions for DNA analysis may yield better results, for a variety of possible reasons, including complete inactivation of RNase. It is considered that the conditions of fixation should be chosen according to the purpose for which the samples are used.

storage, and maximize the versatility of samples for use in a variety of analyses in the future, appropriate methods for preparation of FFPE specimens that are applicable to genomic studies as well as to routine pathological diagnosis are specified in this section. Explanatory notes, (E), (A), (B) and (N), are the same as those in Part 1.

FFPE specimens are prepared from surgical or biopsy specimens mainly for routine pathological diagnosis. Even at institutions where paraffin-embedded specimens are mostly prepared for research use, the processes of fixation and cutting of surgical specimens are inseparable from those used in routine handling of specimens. Therefore, it is important that the present recommended rules are incorporated as far as possible at each institution, while respecting each institution's conventional method(s) for preparing FFPE specimens for pathological diagnosis.

Timing of fixation

2.1. Tissue specimens should be immersed in fixative as soon as possible after resection (A).

2.2. At institutions where immediate fixation is not feasible, it is desirable for surgically resected material to be kept refrigerated (4°C) until it is subjected to fixation within about 3 h (Fig. 14) (B).

Note: Leaving surgically resected material at room temperature for more than 30 min should be avoided as far as possible (N).

2.3. Insufficient fixation must be avoided (N).

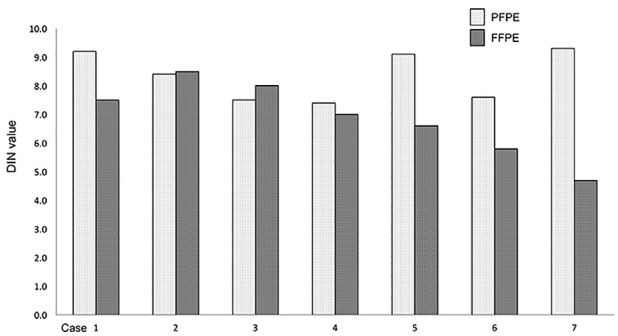

Figure 17 Effects of formalin-free fixative on the quality of genomic DNA. Using surgical specimens obtained from the same patients with ovarian, gastric or lung cancers, formalin-fixed paraffin-embedded (FFPE) specimens fixed for 3–5 days in 10% neutral buffered formalin and PAXgene[31]-fixed paraffin-embedded (PFPE) specimens fixed for 4-6 h in PAXgene Tissue container (Qiagen) were prepared. Genomic DNA was extracted from 2 to 4 sections 8 μm thick by QIAsymphony (Qiagen). The quality of genomic DNA was generally equivalent in both types of specimens, although it tended to be better in some PFPE specimens; there were no statistically significant differences between the two types of specimens in this experiment. Subsequently, mutations of cancer-related genes were searched for in the same genomic DNA samples using the Comprehensive Cancer Panel (Qiagen), which revealed no distinct differences in the detected mutations among samples 2, 3 and 4, for which no evident differences in the DNA integrity numbers (DINs) were observed between the FFPE and PFPE specimens (also see Table 13). In contrast, for samples 1, 5, 6 and 7, for which the FFPE specimens showed lower DIN values, the threshold for the number of reads representative of significant mutations had to be set up appropriately because of the high frequency of C/T substitution including artifacts based on the formalin fixation-related deamination reaction of cytosine.[23] Whereas highly reliable analysis can be carried out using genomic DNA extracted from PFPE specimens, genomic analyses such as mutation analysis are also possible with FFPE specimens, when appropriate conditions are set.

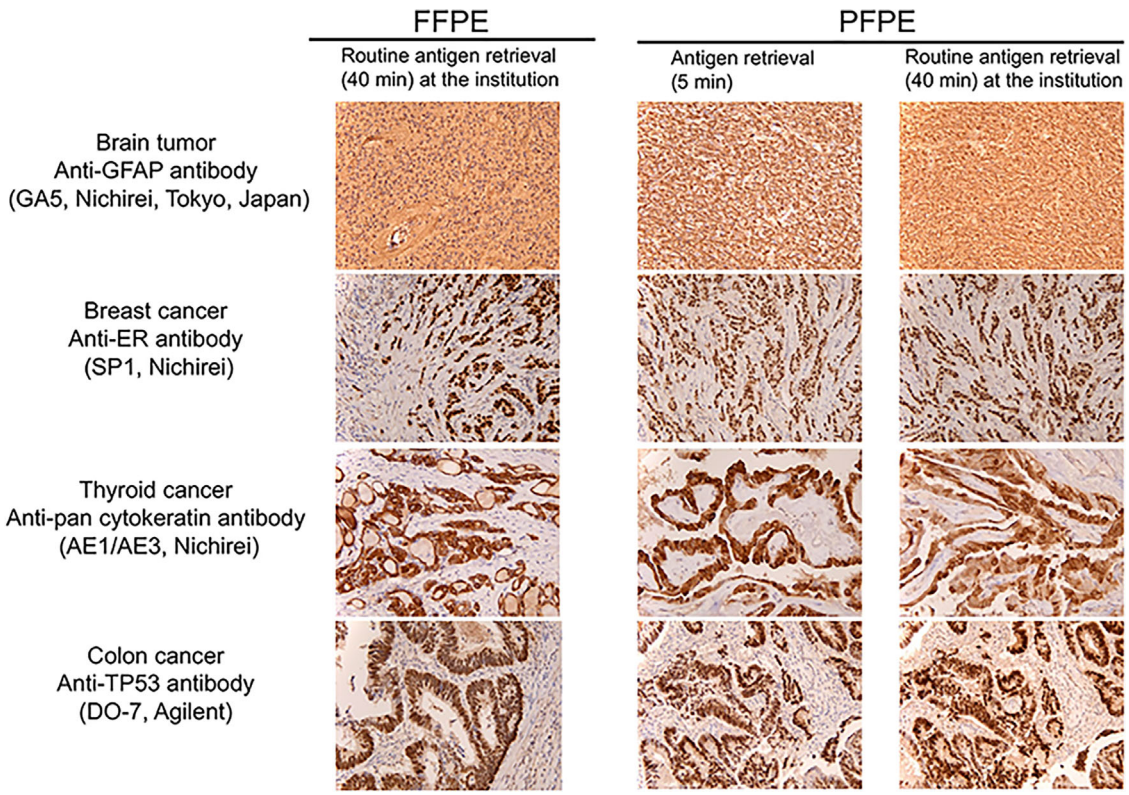

Figure 18 Effects of formalin-free fixative on immunohistochemistry. Formalin-fixed paraffin-embedded (FFPE) specimens and PAXgene[31]-fixed paraffin-embedded (PFPE) specimens were prepared from surgical specimens obtained from the same patients with brain tumor and thyroid, breast or colon cancers (specimens of equal size were obtained from the opposite side to each other in the cases of brain tumor, thyroid and breast cancers, whereas specimens were obtained from a different site in the case of colon cancer), and the results of immunohistochemistry using various antibodies were assessed. In this experiment, although non-specific stainability was slightly higher in the PFPE specimens when 40 min antigen retrieval was used, reduction of the antigen retrieval time to 5 min yielded favorable results. Thus, PAXgene fixation is considered to be suitable for immunohistochemical studies.

Note: Insufficient fixation causes a marked decrease in the quality of DNA, RNA, and protein.

Note: It is necessary to secure a sufficient fixation time, considering that the infiltration rate of formalin, the most common fixative, is about 1 mm/h. Before proceeding to fixation, the tissue specimen should be sliced at an appropriate thickness that allows sufficient fixation prior to cutting out (A).

Concentration and type of fixative

2.4. It is desirable to use neutral buffered formalin solution, not an unbuffered (acidic) solution, for fixation (Figs. 14, 15) (A).

2.5. When intending to use the extracted DNA for analysis, it is desirable to use 10% formalin (3.5% formaldehyde) rather than 20% formalin (7% formaldehyde) for fixation (Fig. 15) (A).

Table 13 Effects of formalin-free fixative on the quality of genomic DNA

				DNA integrity number	
Tissue specimens[†]	Number of analyzed samples	Yielded genomic DNA (μg)	A_{260}/A_{280}	Mean ± SD	*P*-value against #1 (Welch-*t* test)
Formalin-fixed paraffin-embedded specimens	7	4.01 ± 1.98	1.93 ± 0.03	6.87 ± 1.31	–
PaxGene-fixed paraffin-embedded specimens	7	7.90 ± 3.90	1.97 ± 0.24	8.36 ± 0.85	6.70×10^{-2}

[†]Detailed description of analyzed tissue specimens is shown in the legend for Fig. 17. Also see Fig. 17.

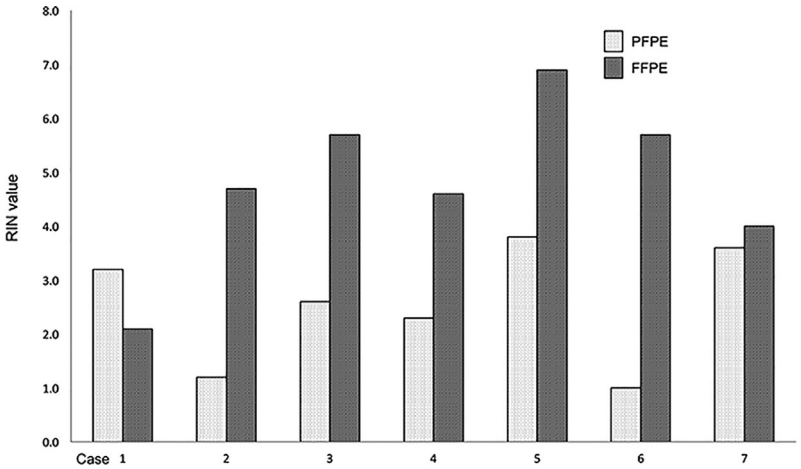

Figure 19 Effects of formalin-free fixative on the quality of RNA. Using surgical specimens obtained from the same patients with ovarian, gastric or lung cancers, formalin-fixed paraffin-embedded (FFPE) specimens fixed for 3–5 days in 10% neutral buffered formalin and PAXgene[31]-fixed paraffin-embedded (PFPE) specimens fixed for 4–6 h in PAXgene Tissue container were prepared. Total RNA was extracted from 2 to 4 sections 8 μm thick by QIAsymphony (Qiagen). In this experiment, the RNA integrity number (RIN) was generally equivalent in both types of specimens, tending to sometimes be worse in the PFPE specimens. It was presumed that fixation in PAXgene for 4–6 h resulted in insufficient fixation. It appeared that the results of RNA analysis are more susceptible to the influence of insufficient fixation than those of DNA analysis. For analysis of RNA, in particular, PAXgene fixation should be conducted for 12–24 h.

Note: However, for analysis using RNA, more thorough fixation using 20% formalin (7% formaldehyde) relative to the optimal conditions of analysis for DNA may yield better results, because RNase becomes completely inactivated (Fig. 16). Therefore, fixation conditions should be chosen according to the purpose for which the study samples are to be used/applied.

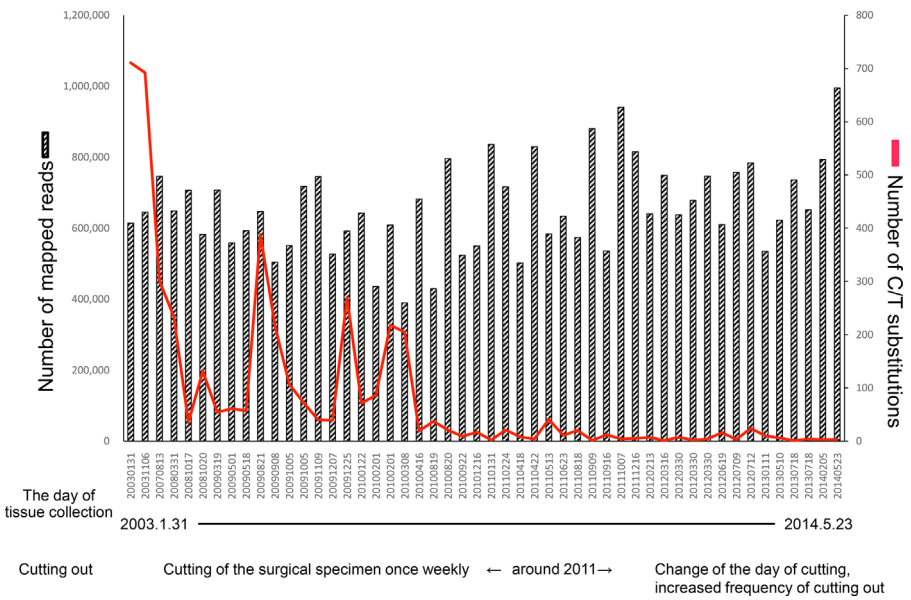

Figure 20 Effects of overfixation on the quality of genomic DNA. Using formalin-fixed paraffin-embedded surgical specimens from a total of 53 patients with colon and uterine corpus cancers, genomic DNA was extracted and its quality was assessed by the comparative Ct method.[33] Using 10 ng of genomic DNA that satisfied the quality criteria, cancer-related gene mutations were searched for by means of amplicon sequencing with Ion AmpliSeqTM Cancer Hotspot Panel v2 (Thermo Fisher), using the semiconductor sequencer Ion PGM (Thermo Fisher). The TaqMan RNaseP Detection Reagent Kit (Thermo Fisher) and TaqMan MGB gene expression Kit (Thermo Fisher) were used. The relative value ratio (quality control [QC] value) of the Ct value for short amplicon (86 bp)/long amplicon (256 bp) was 0.82 on average, and the QC value of 0.2 recommended by Thermo Fisher was met in 94% of the specimens (50 cases). Genomic DNA that satisfied the QC value criterion allowed synthesis of a sufficiently sized library for base sequence analysis. In the 50 cases for which the QC values met the criteria, a mean map read number of 638 661, map rate of 93%, and mean read depth of 2759 were obtained, and base sequence analysis by amplicon sequencing was conducted successfully in all cases. The frequency of C/T substitution including artifacts based on the formalin fixation-related deamination reaction of cytosine[32] was higher in the samples collected before 2011, but markedly decreased in the samples collected in or after 2011 (red line). At this institution, the fixation time for each surgically resected specimen has been reduced since around 2011, by adjusting the cutting day. Blocks were cut twice every week after the end of March 2012, and every day after April, 2013, reducing the time until cutting to 2–3 days. The frequency of C/T substitution is considered to be significantly affected by fixation time as well as time-related deterioration. To ensure accurate analysis, overfixation exceeding 1 week should be avoided, as a rule.

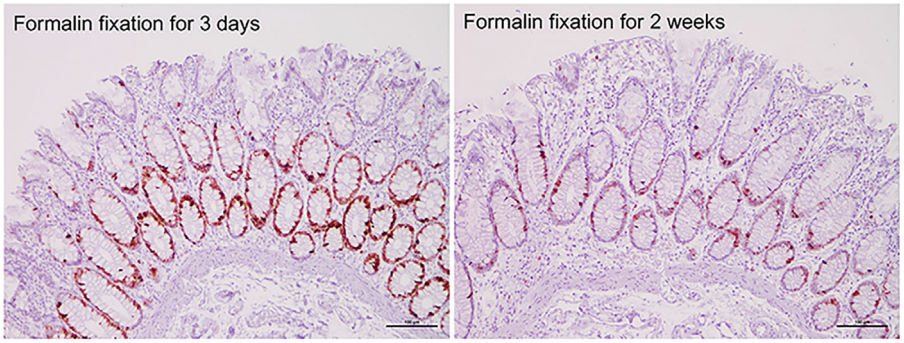

Figure 21 Effects of overfixation on immunohistochemistry. Paraffin-embedded specimens were prepared from colonic mucosa of the same patient using various periods of formalin fixation, and subjected to immunohistochemistry with anti-Ki-67 antibody (MIB-1, Agilent). The results indicated that overfixation would interfere with accurate assessment of the MIB-1-labeling index; therefore, overfixation should be avoided.

Note: Several tissue fixatives not containing formalin have been developed and are currently available commercially. Some of them have been demonstrated to be of sufficient quality for histological observation, and superior for preservation of nucleic acids, proteins, etc. (Figs. 17, 18 and Table 13).

Note: When conducting analysis of RNA using tissue fixatives not containing formalin, more thorough fixation relative to the optimal conditions for DNA analysis may yield favorable results (Fig. 19).

Note: At institutions possessing apparatuses that can be used for preparation of specimens in the absence of formalin, and where there is consensus among the staff involved, paraffin-embedded specimens that have undergone non-formalin fixation are recommended for research purposes, in parallel with routine FFPE specimens (E).

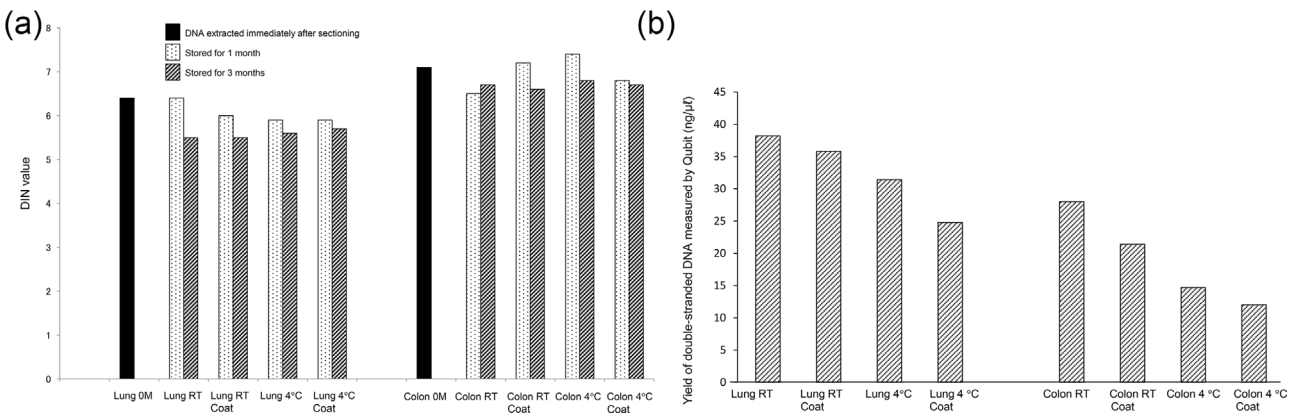

Figure 22 Effects of the state of preservation of unstained slides on genomic DNA. **(a)** Effects of the state of preservation of unstained slides on the quality of genomic DNA. Using unstained 5 μm-thick sections prepared from surgical specimens of the large intestine and lung fixed in formalin for 3 days and embedded in paraffin, the following storage conditions were compared: (i) Storage at room temperature without paraffin coating, (ii) Storage at room temperature with paraffin coating, (iii) Storage at 4°C without paraffin coating, and (iv) Storage at 4°C with paraffin coating. These samples were stored for 1 or 3 months, deparaffinized with xylene, and subjected to extraction of genomic DNA with the QIAamp DNA FFPE Tissue kit. The yield of double-stranded DNA was assessed by the Qubit system (Thermo Fisher), and the quality of DNA was assessed by the DNA integrity numbers (DINs) determined using the TapeStation system. Although the DIN values were slightly lower in the samples stored for 3 months than in those stored for 1 month, it is considered that unstained slides stored for 3 months can be used favorably for analysis of the DNA. Whether the samples were stored at room temperature or 4°C, and whether unstained slides were paraffin-coated or not appeared to exert no marked influence on the quality of the DNA. RT; room temperature, Coat; paraffin coating. **(b)** Effects of paraffin coating of unstained specimens on the yield of genomic DNA. A comparison of the yields of genomic DNA from specimens after storage for 3 months showed that the yield from paraffin-coated unstained slides tended to decrease, perhaps depending on the thoroughness of the deparaffinization process. RT, room temperature; Coat, paraffin coating.

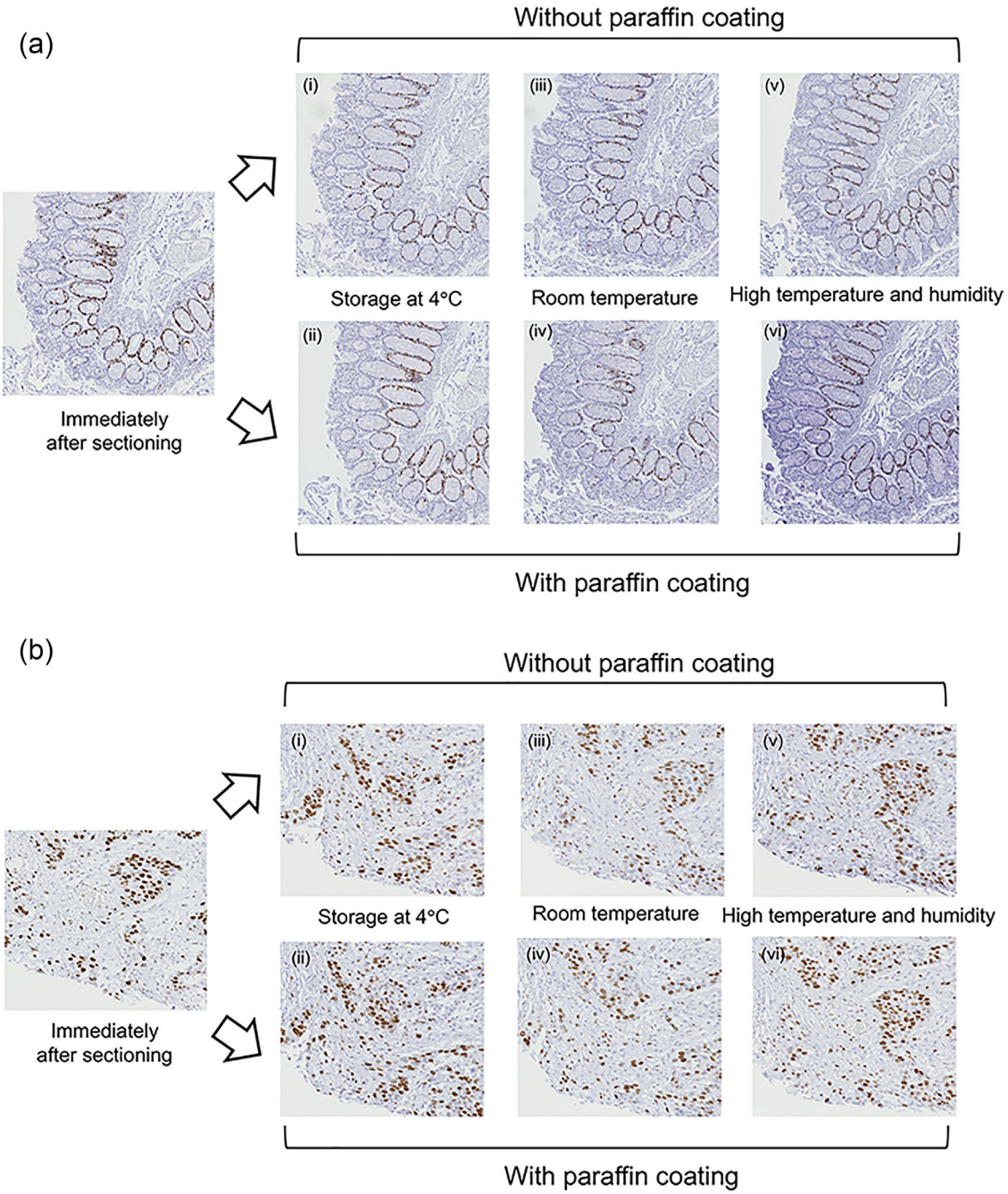

Figure 23 Effects of the state of preservation of unstained slides on immunohistochemistry. Unstained 5 μm-thick sections prepared from surgical specimens of the **(a)** colon and **(b)** lung fixed in formalin for 3 days and embedded in paraffin were stored for 2 months under the following storage conditions, and then subjected to immunohistochemical studies using anti-Ki-67 antibody (MIB-1, Agilent). The following storage conditions were compared: (i) Storage at 4°C without paraffin coating, (ii) Storage at 4°C with paraffin coating, (iii) Storage at room temperature without paraffin coating, (iv) Storage at room temperature with paraffin coating, (v) Storage in a high-temperature and humid environment without paraffin coating, and (vi) Storage in a high-temperature and humid environment with paraffin coating. Although the stainability was slight in comparison with that just after sectioning, no interference with the results of the MIB-1 labeling index was observed for any sample, and there was also no marked difference of stainability in relation to paraffin coating.

Fixation time

2.6. It is desirable to avoid overfixation and to cut blocks on an adequate number of occasions (A). Although it is most desirable to conduct cutting the day after surgery (within 24 h) (E), reasonably favorable preservation of nucleic acids and other elements can be expected when cutting is conducted within 3 days of surgery (Figs. 14, 15, 20, 21) (A).

Note: It is desirable to avoid fixation in formalin for more than 1 week (Figs., 21 20) (N).

Handling of unstained slides

2.7. In general, it is recommended to conduct extraction of nucleic acids from unstained slides as soon as possible after thin sectioning, and it is generally considered that unstained slides should be kept at 4°C if they need to be stored for a certain period of time for research reasons. However, there are generally no effects on the quality of nucleic acids even when the specimens are kept at room temperature, provided that the storage period is not too long (Fig. 22a).

2.8. Many institutions use paraffin coating for unstained slides. Paraffin coating of unstained slides, which reduces dust and physical damage, generally has no obvious influence on the quality of nucleic acids (Fig. 22a).

Note: When unstained slides are coated with paraffin, it is necessary to perform thorough deparaffinization before analysis (Fig. 22b).

Note: Lack of paraffin coating of unstained slides does not cause any particular problems in immunohistochemical studies (Fig. 23).

2.9. When unstained slides are to be stored for a certain period of time for research reasons, extremely bad conditions such as exposure to direct sunlight should be avoided (N).

Decalcification

2.10. If there is a possibility that samples containing hard tissues may be subjected to genomic studies, decalcification with ethylenediaminetetraacetic acid should be performed (A), while rapid decalcification (Plank-Rychlo method)[28] should be avoided (Fig. 24) (N).

PERSPECTIVES

Even though the Guidelines issued so far have focused on procedures for use of pathological tissue samples for research purposes, the fruits of genome research have been rapidly introduced into clinical practice over the last few years. Genome research should provide continuous support to clinical practice to facilitate precise and personalized medicine based on clinical sequencing of genomic material from pathological tissue samples. In this context, the bioresource banking system is becoming more pervasive among various institutions. In view of the constant innovations being made in wet analytical techniques and improvements in the dry bioinformatics pipelines, such as the application of artificial intelligence to deal with integrated

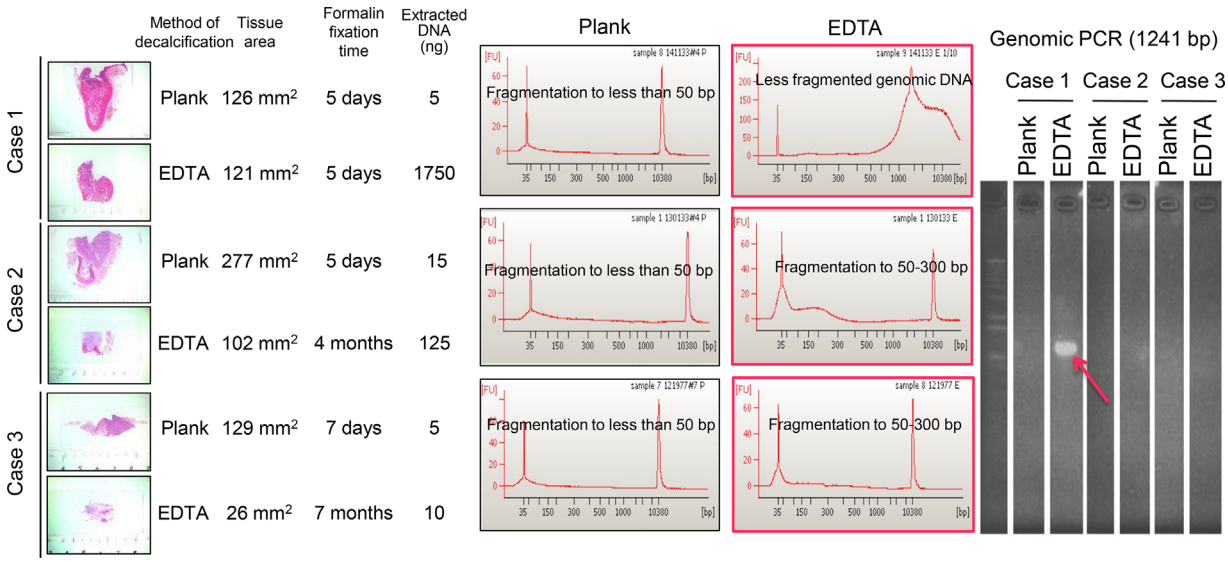

Figure 24 Effects of decalcification method on the quality of genomic DNA. Surgical specimens from a patient who underwent mandibulectomy for gingival squamous cell carcinoma were fixed in formalin and processed into paraffin-embedded (FFPE) blocks. Genomic DNA was extracted from thin sections with a total thickness of at least 20 μm using the QIAamp DNA FFPE Tissue Kit to compare the quality of the DNA. Samples decalcified rapidly by the Plank-Rycholo method and paraffin embedded for routine histopathological diagnosis (Plank) and samples decalcified with 10% ethylenediaminetetraacetic acid and paraffin embedded for research use (EDTA) were compared. Less fragmented DNA was occasionally extracted from the FFPE blocks processed by formalin fixation for a few days and decalcification in EDTA (red frames and red arrow). Fragmentation of genomic DNA (about 50-300 bp) was often observed when the duration of formalin fixation before decalcification with EDTA was prolonged. Extraction of genomic DNA of suitable quality for genomic analysis is difficult after decalcification by the Plank-Rychlo method carried out for routine histopathological diagnosis.

multi omics data, appropriate handling of pathological tissue samples is now a key issue essential to the success of genome research. The JSP intends to update its guidelines regularly to ensure that they incorporate the most up-to-date analytical techniques.

On the other hand, as next-generation sequencing using targeted gene panels is now being performed widely in a clinical setting, appropriate procedures for the handling of pathological tissue samples for both research and clinical purposes are basically becoming standardized. However, specific procedures for clinical purposes focusing more on next-generation sequencing will need to be developed using the research-oriented guidelines as a basis.

In the near future, the fruits of both genome research and genome medicine based on pathological tissue samples will be reapplied to histopathological research and diagnostic pathology in practice: The histological classification of malignant tumors derived from various organs will be reorganized so that it better reflects the somatic mutation spectrum and multiomics data profiles. In this way, genome research and histopathological research will become an inseparable combination for realization of precise and personalized medicine.

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DISCLOSURE STATEMENT

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